Practical Manual

**[For Elementary Plant Biochemistry (ABB-159) &
Plant Biochemistry (FBS-143) students]**

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Syllabus:

Elementary Plant Biochemistry Practical: Preparation of standard solutions and reagents; Carbohydrates: Qualitative reactions; Estimation of starch; Estimation of reducing and non-reducing sugars from fruits; Amino acids: Reactions of amino acids; Proteins: Estimation of proteins by Lowry's method; Fatty acids: Estimation of free fatty acids; Determination of iodine number of vegetable oils; Vitamins: Estimation of Ascorbic acid; Techniques: Paper chromatography, Thin layer chromatography; Electrophoresis of pigments extracted from flowers, Extraction of oil from oil seeds; Enzymes: Enzyme assay, Enzyme Immobilization.

Plant Biochemistry Practical: Qualitative tests for carbohydrates, Quantitative estimation of reducing sugars by DNS method, Quantitative test for total carbohydrates by Anthronere agent, Qualitative tests for lipids, Determination of Saponification number of oils/fats, Determination of Iodine number of fatty acids, Qualitative tests for proteins/ amino acids, Estimation of protein by Lowry's method, Determination of Michaelis constant of enzymes, Estimation of RNA.

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CONTENTS

Exercise	Particulars	Page No
Exercise No.1	To study material safety data sheet and hazard pictograms of different laboratory chemicals.	3
Exercise No.2	To identify important laboratory glassware and equipments.	4
Exercise No.3	To prepare 0.1 N HCl solution and to determine its strength by titration against 0.1N Na_2CO_3.	7
Exercise No.4	To determine pH of HCl, NaOH and some buffer solutions by using indicators.	9
Exercise No.5	To determine the pH of a given solution by using pH meter.	11
Exercise No.6	To perform qualitative tests on carbohydrates.	12
Exercise No.7	To perform qualitative tests on amino acids and proteins.	14
Exercise No.8	To perform qualitative test on lipids.	16
Exercise No.9	To identify the unknown sample for sugar, amino acid, protein or lipid by performing qualitative tests.	17
Exercise No.10	To estimate reducing sugars by Nelson-Somogyi method.	18
Exercise No.11	To estimate the lysine content in Cereal grains.	19
Exercise No.12	To estimate the Methionine content in cereal grains.	21
Exercise No.13	To estimate the tryptophan content in cereal grains.	23
Exercise No.14	To estimate the strength of Glycine by formol titration.	24
Exercise No.15	To estimate total free amino acids by ninhydrin method.	25
Exercise No.16	Quantitative estimation of protein by Lowry's method.	26
Exercise No.17	Quantitative estimation of protein by Biuret method.	27
Exercise No.18	Quantitative estimation of protein by Micro-Kjeldahl method.	28
Exercise No.19	Quantitative estimation of oil content by Soxhlet method	29
Exercise No.20	Determination of ascorbic acid using 2,6-dichlorophenolindophenol dye.	30
Appendix	Introduction to laboratory procedures	31
	Preparation of Solutions	32
	Estimation of pH	34
	Preparation of Buffer solution	36
	Colloidal Solutions	37
	Quantitative Estimations of Biomolecules	38

Laboratory Exercise No. 1

Objective: To study material safety data sheet and hazard pictograms of different laboratory chemicals

Introduction: A material safety data sheet (MSDS) contains data regarding the properties of a particular chemical. Its main aim is to provide guidelines for using or working with a particular chemical in laboratory in a safe manner. It provides the information about the chemicals which include physical data (melting point, boiling point, flash point, etc.) toxicity, health effects, first aid, reactivity, storage, disposal, protective equipment, and spill handling procedures. The exact format of an MSDS can vary from source to source within a country depending on how specific is the national requirement. However typical MSDS will contain the chemical name of the compound and its CAS Number (Chemical Abstracts Service Number) perhaps some other identifying number, a product code and the name of the supplier. If the material is a mixture, its composition will be given. Suppliers of the chemicals are responsible for providing accurate information on the safety aspects through MSDS sheets which are supplied along with chemicals. MSDS also provides the information about the potential hazards associated with a particular chemical. Different hazard symbols or hazard pictograms with different colours, borders etc. are used to signify the hazards associated with a particular chemical substance. A pictogram is used to denote an object, concept or activity with the help of illustration/diagram. These are easily recognizable symbols which warn about the nature/property of hazardous material.

Common chemical hazard labels: Internationally agreed chemical hazard symbols are given in Fig.

GHS pictogram	Code and meaning	GHS pictogram	Code and meaning	GHS pictogram	Code and meaning
	C Corrosive		N Nature polluting		Xn Harmful
	E Explosive		O Oxidising		Non-flammable gas
	F Flammable		T Toxic		Hazardous to health

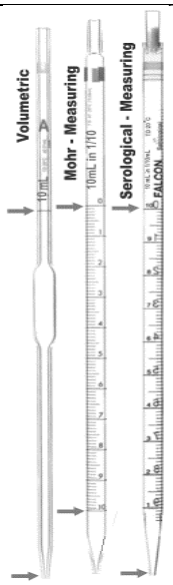
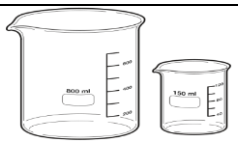
Grades of chemicals: Depending upon the application and purity level, chemicals can be divided into following grades:

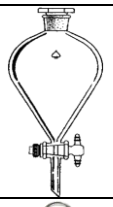
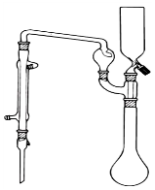
1. **Technical grade:** Such chemicals are used for general washing and cleaning in the laboratory and their minimum purity level is 96%.
2. **Analytical grade reagents (AR):** These reagents are used in specific applications like analysis of various chemicals and calibration, etc. Since quality of the product is important in such applications, hence such AR grade chemicals have a purity level of 99%.
3. **Guaranteed reagent (GR):** These are basically AR chemicals where batch to batch variation is specially controlled so as to have reproducible results in various analytical research works.
4. **Molecular biology (MB) grade:** These chemicals are 99% pure and free from nucleases and proteases, which may degrade nucleic acids and proteins. Hence such chemicals are specially used for Molecular Biology work.
5. **Laboratory grade (LR):** These chemicals are of midrange quality and are used in those applications where quality of the product is more important in the lab. Their purity level is 98% which falls in between AR and technical grade.

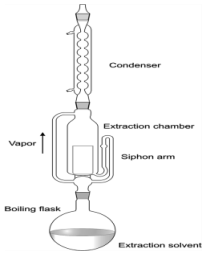
6. **Pharmacopoeia standard:** Such chemicals are of very high quality and used in pharmaceutical research work.

Laboratory Exercise No. 2

Objective: To identify important laboratory glassware and equipments.

1.	**Pipettes:** Laboratory instrument/ glassware used to transport a measured volume of liquid. Three types of glass pipets used in the laboratory ▪ **Volumetric pipets** Designed to transfer a fixed amount of liquid when filled to the mark, e.g. 10 mL and only 10 mL. There is generally only one "fill-line" on a volumetric pipet. ▪ **Serological pipets** are TD = to deliver. To accurately dispense the measured volume the last bit must be blown out. ▪ **Mohr pipets** are TC = to contain. These pipets are designed to dispense the correctly measured volume, so there will be a minute amount of liquid left in the tip.	
2.	**Burette:** Burette also spelled Burette, laboratory apparatus used in quantitative chemical analysis to measure the volume of a liquid or a gas. It consists of a graduated glass tube with a stopcock (turning plug, or spigot) at one end.	
3.	**Beaker:** Used for transferring liquid to another container or to transfer a small amount of reagent for use in procedures. Volume is not accurate, just an estimate.	
4.	**Erlenmeyer Flask or Conical flask :** Features a conical base, a cylindrical neck and a flat bottom. They are marked on the side (*graduated*) to indicate the approximate volume of their contents.	
5.	**Graduated Cylinder or Measuring cylinder** For rapid measurement of liquid volume. They are generally more accurate and precise for this purpose than flasks.	
6.	**Volumetric Flask:** A *volumetric flask* is used to measure very precisely one specific volume of liquid. This flask is used to prepare a solution of known concentration.	
7.	**Funnel:** A funnel is a pipe with a wide (often conical) mouth and a narrow stem. It is used to channel liquid or fine-grained substances into containers with a small opening.	

8.	**Separatory Funnel** A separatory funnel, also known as separation funnel or separating funnel, is a laboratory glassware used in liquid-liquid extractions to separate (partition) the components of a mixture into two immiscible solvent phases of different densities.	
9.	**Desiccator** Desiccators are sealable enclosures containing desiccants used for preserving moisture-sensitive items. A common use for desiccators is to protect chemicals which are hygroscopic or which react with water from humidity.	
10.	**Test tubes:** Test tubes are used by chemists to handle chemicals, especially for qualitative experiments and assays. Their spherical bottom and vertical sides reduce mass loss when pouring, make them easier to wash out, & allow convenient monitoring of the contents.	
11.	**Centrifuge tubes** Centrifuge tubes are used in laboratory centrifuges, machines that spin samples in order to separate solids out of liquid chemical solutions. The centrifuge tubes can be made of glass or plastic, and resemble miniature test tubes with tapered tips	
12.	**Kjeldahl flask** Kjeldahl flask is a round bottom flask with a long wide neck that is used in the determination of nitrogen by Kjeldahl's method.	
13.	**Reagent bottles**, also known as media **bottles** or graduated **bottles**, are containers made of glass, plastic, borosilicate or related substances, and topped by special caps or stoppers and are intended to contain chemicals in liquid or powder form for laboratories.	
14.	**Wash bottle** A **wash bottle** is a squeeze bottle with a nozzle, used to rinse various pieces of laboratory glassware, such as test tubes and round bottom flasks. Wash bottles are sealed with a screw-top lid. When hand pressure is applied to the bottle, the liquid inside becomes pressurized and is forced out of the nozzle into a narrow stream of liquid.	
15.	**Dropper or Pasteur pipette** Pasteur pipettes, also known as droppers are used to transfer small quantities of liquids. They are usually glass tubes tapered to a narrow point, and fitted with a rubber bulb at the top.	
16.	**Nitrogen distillation unit** The distillation unit is used to perform nitrogen analysis and protein determination according to Kjeldahl method in food & feed industry and other applications in environmental and chemical industries after having digested the sample accurately.	

17.	**Soxhlet extraction** A Soxhlet extractor is a kind of laboratory equipment. It is made of glass. Franz vonSoxhlet invented it in 1879. It has a flask, an extraction chamber, and a condenser. It can be used for solid-liquid extractions.	
18.	**Heating mantle** A heating mantle, or isomantle, is a piece of laboratory equipment used to apply heat to containers, as an alternative to other forms of heated bath.	
19.	**Centrifuge** A centrifuge is a laboratory device that is used for the separation of fluids or liquid, based on density. Separation is achieved by spinning a vessel containing material at high speed; the centrifugal force pushes heavier materials to the outside of the vessel.	
20.	**Hot air Oven** Hot air ovens are electrical devices which use dry heat to sterilize. They were originally developed by Pasteur. Generally, they can be operated from 50 to 300 °C, using a thermostat to control the temperature.	
21.	**pH meter** pH meter, electric device used to measure hydrogen-ion activity (acidity or alkalinity) in solution. Fundamentally, a pH meter consists of a voltmeter attached to a pH-responsive electrode and a reference (unvarying) electrode.	
22.	**Water bath** A water bath is laboratory equipment made from a container filled with heated water. It is used to incubate samples in water at a constant temperature over a long period of time.	
23.	**Analytical balances** The Analytical balances in the General Chemistry labs are very sensitive instruments used for weighing substances to the milligram (0.001 g) level.	

Laboratory Exercise No. 3

Objective: To prepare 0.1 N HCl solution and to determine its strength by titration against 0.1N Na_2CO_3.

Requirements:

1. Sodium carbonate (AR grade)	2. Hydrochloric acid (35%) specific gravity 1.18
3. Distilled water	4. Methyl orange indicator
5. Volumetric flask 1000 ml & 250 ml	6. Beaker 500 ml
7. Burette	8. Pipette 10 ml
9. Hot Air oven	10. Desiccators
11. Analytical balance	

A. Preparation of 0.1N solution of Na_2CO_3 :

- A.R. Grade Sodium Carbonate (Na_2CO_3) is dried in an electric oven at 260-270^{0}C for 30 minutes and then cooled in a desiccators before weighing.
- Since Standard Solution of Na_2CO_3 is only used to standardize secondary standard solutions, only 250 ml of this solution is sufficient.

$$\text{Quantity of } Na_2CO_3 \text{ for 250 ml 0.1N solution} = \frac{53}{10} \times \frac{1}{4}$$
$$= 1.325\text{g}$$

- Hence, weigh out accurately on chemical balance 1.325g of dehydrated Na_2CO_3 and dissolve it in distilled water in 250 ml volumetric flask, makeup the volume with distilled water. It will give 0.1N Na_2CO_3 Solution.

B. Preparation of 0.1N solution of HCl :

- To prepare 0.1 N HCl, 3.65g of 100% pure HCl should be present in one litre of solution. The purity of common HCl (LR) is 35% by weight with specific gravity of 1.18.
- The equivalent mass of 35% HCl for 3.65g pure HCl will be = $\frac{100}{35} \times \frac{3.65}{1}$
$$= 10.428\text{g}$$
- This mass can be converted to corresponding volume of HCl as be = $\frac{10.428}{1.18}$ ml
$$V = 8.84 \text{ ml}$$

For convenience, it is always advisable to prepare secondary standard solution of HCl slightly stronger than the required and then dilute it to the required volume after titration. So measure out by measuring cylinder or burette 9.00 ml of 35% HCl and pour it into one litre volumetric flask or measuring cylinder containing about 500ml of distilled water. Mix and makeup to the mark with distilled water. Mix again thoroughly by shaking. It will give approximately 0.1N HCl solution.

Procedure:

- Fill the burette to zero mark with HCl
- Pipette 10 ml 0.1 N Na_2CO_3 in a beaker, add 1-2 drops of Methyl orange indicator
- Titrate Na_2CO_3 against HCl until appearance of a orange or faint pink
- Note the reading. Repeat titration to get two constant readings.
- Calculate the strength of the HCl solution by using following formula. $N_1V_1=N_2V_2$

Where,

N_1 is the strength of Na_2CO_3 =0.1N

V_1 is the volume of Na_2CO_3 solution = 10 ml

N_2 is the strength of HCl solution to be determine

V_2 is the volume of HCl solution that is noted from the burette after titration

Hence,

$$N_2 = \frac{N_1V_1}{V_2}$$

Observations: Volume of HCl required to neutralize 10 ml 0.1N Na_2CO_3

Reading	Initial reading	Last reading	Final reading
1.			
2.			
3.			
4.			
5.			

Calculations:

$N_1V_1=N_2V_2$

Result:

Strength of the HCl Solution is ……..N

Laboratory Exercise No. 4

Objective: To determine pH of HCl, NaOH and some buffer solutions by using indicators.

Requirements:

1. 0.1M HCl	2. 0.1M NaOH
3. Indicator solutions: Thymol blue, Bromophenol blue, Chlorophenol red, Bromothymol blue, Phenol red, Methyl red, Methyl orange, Phenolphthalein, Bromocresol green.	4. Buffer solutions of different pH values
5. Test tubes	

Procedure:

1. Take nine test tubes. In each take nearly 1 mL 0.1M HCl solution.
2. Add 2-3 drops of indicator in each tube (separate indicator in different tubes).
3. Mix and note the colour.
4. Note the pH range for each indicator and fill information in a tabular form as given under observations.
5. Finally find out the narrowest possible pH range for the test solution. Repeat all the steps with NaOH and given buffer solutions.

Observations: For 0.1M HCl

Indicators	Colour	pH<	pH>
Bromocresol green			
Bromophenol blue			
Bromothymol blue			
Chlorophenol red			
Methyl orange			
Methyl red			
Phenol red			
Phenolphthalein			
Thymol blue			

Resultant pH range =

For 0.1M NaOH

Indicators	Colour	pH<	pH>
Bromocresol green			
Bromophenol blue			
Bromothymol blue			
Chlorophenol red			
Methyl orange			
Methyl red			
Phenol red			
Phenolphthalein			
Thymol blue			

Resultant pH range =

For Buffer 1

Indicators	Colour	pH<	pH>
Bromocresol green			
Bromophenol blue			

Bromothymol blue			
Chlorophenol red			
Methyl orange			
Methyl red			
Phenol red			
Phenolphthalein			
Thymol blue			

Resultant pH range =

For Buffer 2

Indicators	Colour	pH<	pH>
Bromocresol green			
Bromophenol blue			
Bromothymol blue			
Chlorophenol red			
Methyl orange			
Methyl red			
Phenol red			
Phenolphthalein			
Thymol blue			

Resultant pH range =

For Buffer 3

Indicators	Colour	pH<	pH>
Bromocresol green			
Bromophenol blue			
Bromothymol blue			
Chlorophenol red			
Methyl orange			
Methyl red			
Phenol red			
Phenolphthalein			
Thymol blue			

Resultant pH range =

Result:

The pH of the given solutions is as under:

1. 0.1M HCl……..
2. 0.1M NaOH…….
3. Buffer 1……..
4. Buffer 2……
5. Buffer 3……

Laboratory Exercise No. 5

Objective: To determine the pH of a given solution by using pH meter.

Requirements: pH Meter, solutions of HCl (0.1M), NaOH (0.1 M), three buffer solutions of different pH. Standard buffer solutions of pH 7.0. Tissue paper roll, 100 mL beakers, wash bottle, waste container.

Procedure:

1. Switch on the pH meter, allow it to stabilize for few minutes.
2. Transfer nearly 20 mL of standard buffer solution of pH 7.0, check the pH by turning the knob to 0-7. The meter should indicate pH 7.0. If it varies, bring it to 7.0 by turning the set pH knob.
3. After each pH measurement turn the knob to stand position.
4. Before taking a fresh solution to measure pH, wash the beaker and electrode bulb thoroughly with distilled water.
5. Now step by step take nearly 20 mL test solutions and measure their pH without disturbing the calibration.
6. Compare the pH values of these solutions with pH values determined by using indicators.

Observations and Result:

Test solution	pH measured by pH meter	pH determined by indicators
0.1M HCl		
0.1M NaOH		
Buffer 1		
Buffer 2		
Buffer 3		

Discussion:

Laboratory Exercise No. 6

Objective: To perform qualitative tests on carbohydrates.

Theory: Carbohydrates are defined as polyhydroxy aldehydes or ketones, hence, called aldoses or ketoses. The basic unit of carbohydrate is monosaccharide, which cannot be hydrolyzed again. Most of the tests for carbohydrates are based on two facts:

(i) Reducing reaction due to the presence of free reducing group, which may be aldehyde or ketone in nature. Due to this property carbohydrates give some tests like Fehling test, Benedict's test, Barfoed's test etc.

(ii) The conversion of sugar into furfural or its derivatives in presence of strong acids. These derivatives condense with phenol (α-napthol, resorcinol, orcinol) to give coloured derivatives. The relative rate of dehydration of different sugars also depends on the nature and strength of the acidity. Due to this fact they give some tests e.g., Molisch test, Seliwanoff's test and Bial's test.

1. Molisch Test

Materials: Conc. H_2SO_4, α-naphthol, alcohol and test solution.

Principle: Concentrated H_2SO_4 hydrolyses glycosidic bond to give the monosaccharides, the latter on further reaction with acid dehydrates to form furfural or fufural derivatives. These compounds combine with sulfonated α-naphthol to give a Purple coloured complex. All carbohydrates give this fest.

Method: Add 2 drops of Molisch reagent to two ml of test solution. Then carefully pour about 1ml. concentrated H_2SO_4 down the side of the tube so as to form two layers. A reddish violet zone or ring is formed at the junction of two liquids.

2. Benedict's Test

Material: Benedict reagent, Test solution

Principle: Carbohydrates with a free aldehyde or ketone group have reducing property in alkaline solution. Alkaline copper reagents when react with a reducing sugar give rise cuprous oxide, which depending on concentration imparts green, yellow, orange or red colour.

Methods: Add 5 drops of the test solution to 2 ml of Benedict's reagent and heat to boiling. Cool the tube. If a reducing sugar is present a colour will form which may be red, orange, yellow or green depending upon the amount of sugar present. This test is positive for all reducing sugars.

3. Seliwanoff's Test

Materials: Seliwanoff's reagent, Test solution

Principle: Ketoses are dehydrated more rapidly than the aldoses, to give furfural derivatives, which then condense with orcinol to form a red complex. Prolonged heating must be avoided.

Method: Add 2 drops of test solution to 2ml of Seliwanoff's reagent. Heat to boiling for 1 min. If a ketose is present a red colour will appear as in fructose. Sucrose also gives as positive test due to hydrolysis into glucose and fructose.

4. Bial's Test

Material: Bial's reagent

Principle: When pentoses are heated with concentrated HCl, furfural is formed which condenses with orcinol in presence of ferric ions to give a blue green colour. The colour is specific for pentoses, glucuronate and their polymers. Prolonged heating of some hexoses also yield hydroxy methyl furfural, which also reacts with orcinol to givecoloured complex.

Method: Add about 1ml of test solution to 2.5 ml of the reagent in a test tube and heat to boiling. A blue green colour on cooling indicates the presence of pentose sugar.

5. Barfoed's Test

Material: Barfoed's reagent, test solution

Principle: Barfoed's reagent is weakly acidic and is only reduced by monosaccharides. Prolonged heating may also hydrolyze disaccharides to give a positive result. The precipitation of cuprous oxide is less dense than with the Benedict's solution and is best to leave tube to stand to allow the precipitate to settle. The colour of the cuprous oxide is also different being a more brick red rather than orange brown as obtained in Benedict's test

Method: Add 4 ml. of reagent to 1ml. of the test solution. Put the tube in water bath and note the time for the formation of red spot at the bottom of the tube. Monosaccharides take lesser time than the disaccharides.

6. Iodine Test

Principle: Iodine test is performed to distinguish polysaccharide from mono and disaccharides. Iodine forms coloured adsorption complexes with polysaccharides. Adsorption is a surface phenomenon and it decreases with temperature and vice versa. That's why polysaccharide-iodine complexes lose and gain colour on heating and cooling respectively.

Method: Take 3 ml of test solution in a test tube and add single drop of dil HCI. Mix and add one or two drops of Iodine solution. Mix again and observe the colour change. A blue colour indicates the presence of starch, a reddish blue or purple for dextrin and wine red colour for glycogen.

Observations: Write + or - for positive or negative test, respectively in the following table. Also note time in case of Barfoed's test.

Sugar	Molisch Test	Benedict Test	Barfoed Test	Bial's test	Seliwanoff's Test
Fructose					
Glucose					
Lactose					
Sucrose					
Xylose					

Laboratory Exercise No. 7

Object: To perform qualitative tests on amino acids and proteins.

Amino acids are organic compounds that have amino and carboxylic groups due to which they have both acidic and basic properties. Amino acids are the building blocks of proteins and are joined together in the protein molecule by peptide bond. Hence, tests performed by the amino acids are also performed by the proteins but proteins impart some additional reactions due to peptide bonds.

1. Ninhydrin Test

Materials: Test solution, Ninhydrin (0.2%) in acetone

Principle: Ninhydrin is a powerful oxidizing agent, which reacts with all α-amino acids to give a purple coloured complex. The reaction is also given by primary amines and ammonia, but there is no liberation of CO_2. Proline and hydroxy proline react with ninhydrin but in this case yellow colour is obtained instead of purple colour.

Method: Place 1ml of test solution in a test tube and add 5 drops of ninhydrin solution and boil for 2 minutes. A purple colour indicates the presence of amino acids. This test is given by all α- amino acids and proteins. Many primary amines may also give a positive ninhydrin test.

Note: Sometimes only glycine gives purple complex and other amino acids such as tyrosine, tryptophan, arginine etc. gives yellow colour, which is due to their less solubility in water due to which their solution contains a little amount of NaOH which is responsible for this colour.

2. Hopkins-Cole test

Material: Test solution, glacial acetic acid, which has been exposed, to light and sulphuric acid.

Principle: This test is given by those amino acids, which contain indole group, such as tryptophan. The indole group of amino acid reacts with glyoxylic acid in the presence of concentrated H_2SO_4 to give a purple colour (glacial acetic acid, which has been exposed to light, contains glyoxylic acid).

Method: Add 2ml of glacial acetic acid to 2ml of the test solution then pour about 2ml of concentrated H_2SO_4 carefully down the side of a sloping test tube so as to form two layers. A violet ring is formed at the junction of two liquids, which indicates the presence of indole group.

3. Pauly's Test

Material: Test solution, Diazotized sulphanilic acid, Sodium nitrite (5%), Sodium carbonate (1%)

Principle: Diazotized sulphanilic acid couples with amines, phenols and imidazoles to form highly coloured azo compounds. The diazonium compound is only formed in cold, so all solution are cooled in ice before diazotization.

Method: Mix 1ml of sulphanilic acid with 2ml of test solution cooled in ice. Now add 1ml of sodium nitrite solution and leave in cold for 3 minutes. Make it alkaline by the addition of 2ml of sodium carbonate solution. Orange-red colour indicates the presence of tyrosine or histidine because they contain phenolic and imidazoles groups, respectively.

4. Sakaguchi Test

Materials: Test solution, sodium hydroxie 40%, α-naphthol, bromine water

Principle: This test is specific for only those compounds, which contain guanidine group, such as arginine. Guanidine reacts with α- naphthol in presence of an oxidizing agent, e.g., bromine water to give a red colour.

Method: Mix 1 ml of 40% strong alkali with 2 ml of amino acid solution and add 1-2 drops of α-naphthol. Mix thoroughly and add 5 drops of bromine water. A red colour indicates the presence of arginine.

5. Xanthoproteic test

Materials: Test solution, conc. HNO_3, 40% NaOH solution

Principle: Amino acids containing aromatic nucleus when react with concentrated HNO_3 form yellow coloured nitro derivatives. The salts of these derivatives are orange in alkaline condition. Phenols also impart a positive test.

Method: Take 0.5 ml test solution in a test tube and add equal amount of concentrated HNO_3. On cooling yellow colour forms. Now to this solution add 40% NaOH solution to make it alkaline, a bright orange colour confirms the amino acid containing aromatic ring.

6. Millon's Test

Materials: Millon's reagent, Test solution

Principle: Compounds containing the hydroxybenzene radical react with Millon's reagent to form the red complexes. The only phenolic amino acid is tyrosine and its derivatives and only these amino acids give a positive test. The original millon's reagent was a solution of mercuric nitrate in 50% nitric acid.

Method:

a. 2ml of test solution add 1ml of Millions reagent. If white ppt. comes which turns red on heating. It indicates the presence of proteins.

b. Take 2ml of test solution and add 10 drops of Millon's reagent then heat in boiling water bath for 10min. Cool to room temperature. Add 10 drops of sodium nitrate solution. A red colour indicates the presence of tyrosine.

7. Biuret Test

Materials: $CuSO_4$ solution (1%), NaOH solution (40%), Proteins (0.5% albumin, casein, gelatin)

Principle: The test is positive for proteins only. Alkaline copper sulphate reacts with compounds containing two or more peptide bonds to give a violet coloured complex. The name of the test comes from the compound biuret (NH_2-CO-NH-CO-NH_2), which gives typical positive reaction

Method: Add 5 drops of copper sulphate solution to 2 ml of the test solution. Now add 2 ml of 40% sodium hydroxide solution, mix thoroughly. A purple violet colour indicates the presence of protein. (Egg albumin)

Observations: Write + or - for positive or negative test, respectively in the following table.

	1	2	3	4	5	6	7
Arginine							
Glycine							
Tryptophan							
Tyrosine							
Egg albumin							

Laboratory Exercise No. 8

Objective: To perform qualitative test on lipids.

Lipids are defined as the group of compounds that are soluble in organic (non-polar) solvents and insoluble or sparingly soluble in aquecus solvents (water). Most lipids are fatty acid esters of glycerol, where the fatty acids may have a saturated or unsaturated (with one or more double bonds) hydrocarbon chains. Following tests are routinely performed on lipids.

Requirements:

Ghee, Soybean or mustard oil, Lecithin, Stearic acid, Ethyl alcohol, Chloroform, carbon-tetra chloride, Ether, Paper, Potassium bi-sulphite

1. Solubility test

Take about half a gram of sample (1 ml if in liquid form) in each of the 5 test tubes and add to separate tubes water, alcohol, chloroform, ether and carbon-tetra chloride. Examine the solubility of lipids in these solvents.

2. Translucency test

Place a drop of lipid (oil) on a paper and leave to dry. Semi-transparent, appearance on paper at the point of contact with lipid is a positive test.

3. Acrolein test

Place about 2 g of potassium bisulphate in a clean dry test tube and add 10 drops of oil and heat the test tube slowly. Note the characteristic pungent odour of acrolein. This test is for glycerol, hence, all fats and oils impart a positive acrolein test.

4. Test for unsaturation

Double bonds in fatty acids absorb iodine or bromine, hence iodine or bromine solution when added to a fat or oil decolourizes.

Add bromine water slowly to the test solution drop by drop and shake it. Note decolourization of bromine water.

Observations:

Test/Compound	Ghee	Oil	Lecithin	Fatty acid
Solubility				
Translucency test				
Acrolein test				
Decolorization of Bromine				

Laboratory Exercise No. 9

Objective: To identify the unknown sample for sugar, amino acid, protein or lipid by performing qualitative tests.

Observations:

S. No.	Test	Observation	Inference

Result and discussion:

Laboratory Exercise No. 10

Objective: To estimate reducing sugars by Nelson-Somogyi method.

Principle: Some sugars have reducing property and can reduce reagents like Fehling solution, Benedict's solution' Barfoed's solution etc. These are called reducing sugars, e.g., glucose, fructose etc. Reducing property of sugar is due to the presence of free aldehydic or ketonic group, which can reduce metal ions under alkaline conditions. The reducing sugars when heated with alkaline copper tartarate, reduce the copper from the cupric (Cu^{++}) to cuprous (Cu^{+}) state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue colour developed is compared with a set of standards in a colorimeter at 520 nm.

Precaution: Proteins are major interfering agents in estimation of reducing sugars by this method, the deproteinization of sample should be performed before estimation.

Deproteinization of sample for sugar estimation

Add 0.2ml of 0.3N Ba $(OH)_2$ to 1 ml of sample extract and mix well. Then add 0.2 ml $ZnSO_4$ (5%), shake thoroughly and after 10 min filter it through waterman No 1 filter paper. During this step, proteins are precipitated by Zn (OH_2) to give a protein free sample extract.

Requirements: Alkaline copper tartarate, Arsenomolybdate reagent, Standard glucose solution (1mg/ml), working standard (100µg/ml), Unknown sugar solution

Procedure:

Preparation of Sample:

1. Prepare a set of eight test tubes as detailed below:

S. No	Standard glucose (ml)	Distilled water (ml)	µg glucose (to be calculated)	Alk. Copper tartarate (ml)	Keep the tubes in boiling water bath for 10min	Arsenomo-lybdate reagent	Distilled water (ml)	Absorbance at 520nm
1.	0.2	1.8	20	1.0		1.0	6.0	
2.	0.4	1.6	40	1.0		1.0	6.0	
3.	0.6	1.4	60	1.0		1.0	6.0	
4.	0.8	1.2	80	1.0		1.0	6.0	
5.	1.0	1.0	100	1.0		1.0	6.0	
6.	0.0	2.0	0	1.0		1.0	6.0	
7.	0.4	1.6	40	1.0		1.0	6.0	
8.	0.6	1.4	60	1.0		1.0	6.0	

2. Record absorbance for all the tubes by setting zero with control tube at 520 nm.

3. Draw the standard curve on graph paper using absorbance (on Y axis) versus µg glucose (on X axis) and draw a straight line best fitting the plot. Find out the amount of glucose in the unknown solution by extrapolation of its absorbance.

Calculations:

Result: Report your result as µg glucose/100ml sample.

Laboratory Exercise No. 11

Objective: To estimate the lysine content in Cereal grains.

Principle: The defatted biological sample (protein) is hydrolyzed by proteolytic enzyme (papain) to free amino acids. The α-amino group of the free amino acids are blocked by complexing with copper while the ε-amino group the of lysine is then converted to α-dinitropyridyl derivative of lysine by the reaction of 2 chloro-3,5-dinitropyridine and its excess is removed by extraction reaction mixture with ethyl acetate, leaving an aqueous solution of ε-dinitropyridyl derivative of lysine. The colour intensity of this solution is then measured at 390nm.

Materials:

1. **Solution A:** Dissolve 2.89 g of $CuCl_2.2H_2O$ in distilled water and make upto 100ml.
2. **Solution B:** Dissolve 13.6g of $Na_3PO_4.12H_20$ in distilled water and make up to 200ml.
3. **Sodium Borate Buffer:** 0.05 M pH-9.0
4. **Copper Phosphate Reagent:** Pour solution A into B with swirling. Centrifuge it and collect the precipitate. Then precipitate is washed three times with 15 ml of borate buffer followed by centrifugation. After last washing again suspend the precipitate in 80ml of borate buffer. This suspension may be used for a week.
5. 3% solution of 2-chloro-3,5-dinitropyridine in methyl alcohol. Always prepare this solution prior to use.
6. 0.05M sodium carbonate buffer (pH 9.0)
7. **Amino Acid mixture:** Mix by grinding in a pestle and mortar 30 mg alanine, 300mg glutamic acid, 60mg aspartic acid, 20mg cystine, 40 mg phenylalanine, 20 mg methionine, 40 mg valine, 30 mg histidine, 50mg arginine, 50mg serine, 30 mg Isoleucine, 30mg threonine, 30mg tyrosine, 80mg leucine, 40mg glycine and 80mg proline. Weigh 100mg of this mixture and dissolved in 10 ml of sodium carbonate buffer (0.05M-pH 9.0)
8. **Standard solution of papain:** Dissolve 400mg of technical grade papain in 100ml of 0.1M sodium acetate buffer (pH 7.0)
9. 1.2 N HCl.
10. Ethyl acetate.

Procedure:

1. Weight 100mg, finally powdered defatted sample in a glass vial and add 5 ml of papain solution. Shake it carefully so that the sample is totally wet. Incubate the samples over night at 65°C. Cool to room temperature.
2. Centrifuge at 3000x g for 5 min.
3. Take 1 ml of supernatant fraction in a centrifuge tube and add 0.5ml of carbonate buffer and then 0.5ml of copper phosphate suspension. Shake the mixture for 5 minutes and centrifuge to separate the excess copper phosphate.
4. Pipette out 1ml of supernatant in a test tube, add 0.1ml 2-chloro-3, 5-dinitropyridine solution and shake well.
5. Allow the reaction to proceed for two hours at room temperature.
6. Acidify this is reaction mixture by adding 5 ml of 1.2N HCl and mix well.
7. Add 5 ml of ethyl acetate and mix well by inverting the stoppered tube several times.
8. Discard the upper layer. This step must be repeated at least 3 times.
9. Read the absorbance of aqueous phase containing ε-dinitropyridyl lysine at 390nm. Carry out a blank with only 5 ml of standard papain solution and repeat all the above steps.

Preparation of standard curve:

1. Dissolve 62.5 mg lysine monohydrochloride in 50ml of carbonate buffer (1mg lysine/ml).
2. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml and make up to 1 ml with carbonate buffer.
3. Add 4 ml papain to each tube and mix.
4. Now pipette out 1ml from each tube and add 0.5 ml of amino acid mix and 0.5ml of copper phosphate suspension.
5. Carry out further steps as above. The absorbance values of these will represent 40, 80, 120, 160 and 200µg lysine.

Observations:

Standard Solution (ml)	Carbonate buffer (ml)	Papain (ml)	Amino acid mix (ml)	Copper phosphate (ml)	µg lysine (to be calculated)	Absorbance at 390 nm
0.2	0.8	4.0	0.5	0.5	40	
0.4	0.6	4.0	0.5	0.5	80	
0.6	0.4	4.0	0.5	0.5	120	
0.8	0.2	4.0	0.5	0.5	160	
1.0	0.0	4.0	0.5	0.5	200	

Calculation:

Prepare a standard curve from the above readings of standard lysine. After subtracting the absorbance of the blank from the samples, calculate the lysine content in the aliquot from the graph.

$$\text{Lysine content of the sample} = \frac{\text{Lysine value from graph in µg} \times 0.16}{\text{\% N of sample}}$$

$$= \text{g/16gN}$$

Result: Lysine content in the given sample=

Laboratory Exercise No. 12

Objective: To estimate the Methionine content in cereal grains.

Methionine is one of the essential, sulphur-containing amino acids. Although it is present in many food proteins, methionine is the limiting amino acid in most of the grain legumes.

Principle: The protein in the grain is first hydrolyzed under mild acidic condition. The liberated methionine gives a yellow colour with nitroprusside solution under alkaline condition and turns red on acidification. Glycine is added to the reaction mixture to inhibit colour formation with amino acids. The intensity of colour is measured at 520nm.

Materials:

1. 2 N Hydrochloric acid
2. 10 N NaOH (40%)
3. NaOH (10%)
4. 10% Sodium nitroprusside
5. 3% Glycine
6. Orthophosphoric acid (Sp.gr. 1.75)
7. Standard methionine: Dissolve 100 mg of DL-Methionine in 4 mL of 20% HCl and dilute with water to 100 ml.

Procedure:

1. Weigh 0.5 g of defatted sample into 50 ml conical flask. Add 6 ml of 2N HCl and autoclave at 15 lb pressure for 1 h.
2. Add a pinch of activated charcoal to the hydrolyzed (autoclaved sample) and heat to boil. Filter when hot and wash the charcoal with hot water.
3. Neutralize the filtrate with 10 N NaOH to pH 6.5. Make up the volume to 50 ml with water after cooling to ambient temperature.
4. Transfer 25 ml of the made up solution into a 100 ml conical flask.
5. Add 3 ml of 10% NaOH followed by 0.15 ml sodium nitroprusside.
6. After 10 min add 1 ml of glycine solution.
7. After another 10 min add 2 ml ortho-phosphoric acid and shake vigorously.
8. Read the intensity of red colour after 10 min at 520 nm against a blank prepared in the same way but without nitroprusside.

Preparation of standard curve:

1. Pipette out 0,1,2,3,4, and 5ml of standard methionine solution and make up to 25 ml with distilled water.
2. Follow steps with the addition of 3 ml of 10% NaOH onwards as above to develop colour in standard.
3. The zero (O) level of standard serves as blank.

Observations:

Standard methionine (ml)	Distilled water (ml)	mg methionine (to be calculated)	Absorbance at 520 nm
0	25	0	
1	24	1	
2	23	2	
3	22	3	
4	21	4	
5	20	5	

Calculation:

Draw a standard curve and calculate the methionine content from the graph.

Methionine Content from the graph x 4 = mg/g

Methionine is usually expressed as percent in protein or in grams/16gN.

$$\text{Methionine content of the sample} = \frac{\text{Methionine content from graph x 6.4}}{\text{\% N of sample}}$$

or= g/16gN

Result: Methionine content in the given sample=

Laboratory Exercise No. 13

Objective: To estimate the tryptophan content in cereal grains.

Principle: In strongly acidic conditions, tryptophan yields a blue coloured derivative with p-dinethyl-amino benzaldehyde in the presence of sodium nitrite. The colour intensity of derivative tryptophan is measured at 545mm.

Materials:

1. 19N sulphuric acid.
2. p-dimethyl aminobenzaldehyde
3. 0.45% sodium nitrite
4. Standard Tryptophan: Dissolve 10mg tryptophan in 100ml-distilled water (0.1mg/ml). Add few drops of sulphuric acid to dissolve tryptophan to get clear solution, if necessary.

Procedure:

1. Weigh out 100mg of air-dried, finely powdered defatted biological sample (grain etc.)
2. Transfer it to a 50 ml stoppered conical flask.
3. Add 30mg of p-dimethylamino benzaldehyde and 10 ml of 19N H_2SO_4 solution and shake well.
4. Incubate the reaction mixture at room temperature for 12 hrs. in dark.
5. Centrifuge the reaction mixture at 5000 rpm for 15 min. and collect the supernatant in separate 50ml conical flask.
6. Then add 0.1ml of 0.45% sodium nitrite solution and shake properly.
7. After 30 min. measure the blue colour at 545 nm.
8. Set a blank without sample and develop colour as above.

Standard curve:

1. Pipette out 0, 0.2, 0.4, 0.6, 0.8 and 1.0ml of standard tryptophan solution and make up to 1ml with distilled water.
2. Develop the colour as above and measure colour intensity at 545nm and draw a standard curve.

Observations:

Standard methionine (ml)	Distilled water (ml)	mg methionine (to be calculated)	Absorbance at 520 nm
0	25	0	
1	24	1	
2	23	2	
3	22	3	
4	21	4	
5	20	5	

Calculation: Calculate the percentage of tryptophan in the sample from the graph.

$$\text{Tryptophan (g/100g protein)} = \frac{\text{Tryptophan \% in sample}}{\text{Protein \% in sample}} \text{ X100}$$

Result: Tryptophan content in the given sample=

Laboratory Exercise No. 14

Objective: To estimate the strength of Glycine by formol titration.

The titration in which formaldehyde is used is called formol titration. At neutral pH an amino acid in solution remains in zwitter ion form, in which NH_3 groups is capable of donating protons hence behaves as bronsted acid, when titrated against standard NaOH, this group is only partially saturated because of its high pKa value and phenolphthalein changing its colour at a pH 8.3 or above. Formaldehyde reacts with the amino group and converts the primary amino $(H_3N^+)^-$ group to tertiary amino group, which is monoacidic and has a lower pKa value.

The tertiary amino group is almost completely titrated with NaOH.

Materials:

1. Glycine 0.1N
2. NaOH 0.1N
3. Oxalic acid 0.1N
4. Phenolphthalein indicator
5. Formaldehyde solution (Neutralized)

Method:

1. Fill burette with NaOH solution to zero mark.
2. Pipet: 10mL of 0.1 N oxalic acid in a conical flask and add 1 or 2 drops of indicator
3. Titrate oxalic acid against NaOH and note the end point
4. Calculate strength of NaOH solution using the formula $N_1V_1 = N_2V_2$
5. Fill the burette again with NaOH upto zero mark
6. Pipette out 10ml. of glycine solution in conical flask and add 1drop of indicator. Titrate with NaOH till the appearance of faint pink colour. Note the volume of NaOH used. Now add 1.0 ml of HCHO solution (pink colour disappears), continue titration till the reappearance of faint pink colour, note the final reading. Repeat titration until two constant readings is obtained. Calculate the total volume of NaOH used in glycine titration calculate the concentration of glycine solution in the form of Normality.

Observation: Table for NaOH normality

S. No.	Volume of oxalic acid (ml)	Volume of NaOH used in ml			
		Initial reading (ml)	Final reading (ml)	Total volume of NaOH used (ml)	Mean value (ml)
	10				
	10				
	10				

S. No.	Volume of Glycine (ml)	Volume of NaOH used in ml			
		Initial reading (ml)	Final reading (ml)	Total volume of NaOH used (ml)	Mean value (ml)
1.	10				
2.	10				
3.	10				

Calculation:

Normality of NaOH=

$N_1V_1=N_2V_2$

Result:

Laboratory Exercise No. 15

Objective: To estimate total free amino acids by ninhydrin method.

Principle: Ninhydrin, a powerful oxidizing agent, reacts with α-amino acids between pH 4 and 8 to give a purple coloured compound which is measured colorimetrically at 570 nm. Proline and hydroxyl proline give a yellow colour that absorbs at 540 nm.

Reagent:

1. 80% ethanol
2. 0.2M citrate buffer (pH 5.0)
3. Ninhydrin reagent (Dissolve 0.8 g of stannous chloride in 500 mL of 0.2 M citrate buffer, pH 5. Add this solution to 20 g ninhydrin in 500 mL of methyl cellosolve), prepare fresh and store in brown bottle.
4. Standard leucine solution (10mg/100ml water). Standard graph is different for different amino acids. So it is advisable to prepare a composite mixture of alanine, aspartic acid, tryptophan, proline and cysteine in equal amounts.
5. Diluent solvent 50% ethanol or 50% propanol

Preparation of sample:

Grind a known quantity (500mg) of sample in a pestle and mortar with small quantity of acid-washed sand. Add 5-10 ml of 80% ethanol (Boiling 80% ethanol may also be used if the tissue is tough). Filter or centrifuge. Repeat the extraction and pool the supernatant. The volume of the sample can be reduced by evaporation. This extract is used for estimation of total amino acids.

Procedure:

1. Pipette out 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of aliquot from the standard solution in different test tubes.
2. Make up the volume to 1.0 ml with water. Add 1 ml of Ninhydrin reagent and mix
3. Heat in a boiling water bath for 20 minutes
4. Add 5ml of the diluent while still on the water bath and mix.
5. Keep the tube for 10 minutes and read the O.D. at 570nm.
6. In the same way, the unknown sample can be processed.
7. Draw a standard curve and determine the concentration of unknown solution using standard curve.
8. Calculate the concentration of amino acid as mg/100 ml

Observations:

Standard Solution (ml)	Distilled water (ml)	Quantity of amino acid (to be calculated)	Ninhydrin reagent (ml)	Heat for 20 min boiling water bath	Diluent (ml)	Absorbance at 570nm
0	1		1.0		5.0	
0.1	0.9		1.0		5.0	
0.2	0.8		1.0		5.0	
0.3	0.7		1.0		5.0	
1.4	0.6		1.0		5.0	
0.5	0.5		1.0		5.0	
Unknown 0.2	0.8		1.0		5.0	

Calculations:

Result:

Laboratory Exercise No. 16

Objective: Quantitative estimation of protein by Lowry's method.

Principle: Proteins react with the Folin-Ciocalteau reagent to give a blue coloured complex. The colour so formed is due to the reaction of the alkaline copper sultate with the protein and the reduction of phosphotungstomolybdate (major component of Folin Ciocalteu reagent) by tyrosine and tryptophan present in the protein. The intensity of colour depends on the amount of these aromatic amino acids present and will thus vary for different proteins.

Materials:

1. 2% Sodium carbonate in 0.1N NaOH (Reagent A)
2. 0.5% Copper sulphate ($CuSO_4.5H_2O$) in 1% Potassium sodium tartarate (Reagent B)
3. 49 ml. of reagent 'A' + 1 ml. of reagent 'B' (Reagent C)
4. Folin-Ciocalteau reagent (Reagent D), Dilute commercial reagent with equal volume of water
5. Protein solution (Stock standard) 1 mg/ml
6. Working standard 200 µg/ml.

Procedure:

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml. of the working standard into a series of test tubes. Also pipette out 0.4 & 0.6 ml of the unknown protein. Make-up the volume to 1 ml. in all the test tubes. A test tube with 1 ml of water serves as the blank (control).
2. Add 5ml of reagent C to each tube including the blank, mix well and allow to stand for 10 min.
3. Then add 0.5ml. of 'reagent D, mix vigorously and incubate, at room temperature in dark for 30 min. Blue colour develops.
4. Record absorbance at-660 nm.

Observation:

Standard Solution (ml)	Distilled water (ml)	Quantity of protein (to be calculated)	Alkaline reagent (Regent C)	Keep 10 min for incubation at room temperature	Arsenomolybdate reagent	Keep 30 min for incubation at room temperature in darl	Absorbance at 520nm
0.2	0.8	20	5.0		0.5		
0.4	0.6	40	5.0		0.5		
0.6	0.4	60	5.0		0.5		
0.8	0.2	80	5.0		0.5		
1.0	0.0	100	5.0		0.5		
0.0	1.0	0	5.0		0.5		
0.4	0.6	40	5.0		0.5		
0.6	0.4	60	5.0		0.5		

Draw the standard curve between absorbance and quantity of protein and calculate the amount of unknown protein using standard curve.

Calculations:

Result:

Laboratory Exercise No. 17

Objective: Quantitative estimation of protein by Biuret method.

Principle: When powdered samples of biological material is treated with the biuret reagent composed of alkaline sodium potassium tartarate and copper sulphate solution, the formation of blue-violet colour takes place due to formation of copper protein complex. The intensity of colour which largely depends on the peptide bonds (-CONH) in protein is proportional to the amount of protein present in sample and can be measured at 575nm.

Materials:

1. **Solution A :** 4% $CuSo_4$
2. **Solution B :** 10N NaOH or KOH solution
3. **Solution C :** Weigh 2.5g of sodium potassium tartarate, transfer it to 1000ml volumetric flask and dissolve in about 500ml of distilled water. Add then 15 ml of solution B to the volumetric flask and mix well, now add to it 30ml of solution A, mix well and make up to the mark. This solution known as biuret regent.

Procedure:

1. Weigh out 100mg of finely powdered biological sample in to boiling test tube.
2. Add 2-3 carbon tetrachloride to moist the sample and then add accurately 10 ml of solution C (Biuret reagent) and mix well.
3. Incubate this for 1 hr. at room temperature.
4. Filter this coloured solution and collect the clear filtrate.
5. Read the intensity of the coloured filtrate at 575nm.
6. Solution C is used as a blank.
7. Standard protein sample is also run side by side.

Observation:

S. No.	Weight of sample	Sample reading	Standard protein sample reading	Blank reading
1.	100mg			
2.	100mg			
3.	100mg			

Calculation:

$$\text{Protein \%} = \frac{\text{(Reading of unknown sample-Blank) X (Protein \% of known sample)}}{\text{(Reading of known sample-Blank)}}$$

Result: Protein % of given sample is =

Laboratory Exercise No. 18

Objective: Quantitative estimation of protein by Micro-Kjeldahl method.

Theory: For the determination of crude protein in biological sample, first the nitrogen content of the biological sample is determined by Conventional Kjeldahl methods and then it is multiplied with a suitable factor which vary from material to material to get the value of crude protein. In most biological proteins, the nitrogen constitutes 16% of the total make up and hence the nitrogen content of sample is multiplied by 6.25 to get the value of crude protein.

Principle:

1. **Digestion:** The biological sample is digested in concentrated H_2S0_4 to convert the nitrogen in protein to ammonium sulphate during digestion.
   $$\text{Organic N} + H_2SO_4 \rightarrow CO_2 + H_20 + (NH_4)2SO_4$$
2. **Distillation:** On steam distillation, ammonium sulphate liberates ammonia, which is collected in boric acid solution
   $$(NH_4)2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$
   $$NH_3 + H_3BO_3 \text{ (boric acid)} \rightarrow NH_4^+ + H_2BO_3^- \text{ (borate ion)}$$
3. **Titration:** The nitrogen content is then estimated by titration of the ammonium borate formed with standard hydrochloric acid, using a suitable indicator to determine the end-point of the reaction.
   $$H_2BO_3^- + H^+ \rightarrow H_3BO_3$$
   The results can be expressed in % N, % NH_3 or protein (%N x factor).

Materials:

1. Concentrated sulphuric acid (sp. gr. 1.84)	**2.** Digestion Mixture (Potassium sulfate + Copper sulfate) 5:1
3. 40% NaOH solution	**4.** 4% Boric acid solution
5. 0.1N HCL	**6.** Mixed Indicator (Methyl red & methylene blue)

Procedure:

1. Weigh out 100mg of the given sample into a 30ml digestion flask.
2. Add 2g digestion mixture followed by 2 ml conc. H_2SO_4. Then add some boiling chips and digest the sample till the solution becomes colourless.
3. After cooling the digested sample, transfer it to distillation apparatus with the help of washing with ammonia free distilled water.
4. Place a 100ml conical flask containing 5-10 ml boric acid solution with a few drops of mixed indicator with the tip of condenser dipping in boric acid solution.
5. Add 10 ml of sodium hydroxide solution in the test solution in the distillation flask. Distil and collect ammonia in boric acid solution.
6. About 15-20 ml of distillate is sufficient Rinse the tip of condenser with ammonia free distilled water and titrate it against standard solution of an acid until the end point (violet colour) is obtained.
7. Also carry out a reagent blank using all reagents and distilled water as above except sample and subtract its titre volume from that of sample titre volume.

Observation:

S. No.	Weight of sample	Volume of 0.1N HCL in sample (ml)	Volume of 0.1N HCL in blank (ml)
1.	100mg		
2.	100mg		
3.	100mg		

Calculation: Calculate the nitrogen percentage as follows

$$\%N = \frac{\text{Volume of 0.1N HCl sample (ml) - Volume of 0.1N HCl blank (ml) X 1.4mg}}{\text{Weight of sample (mg)}} \text{ X } 100$$

% Protein = % N X 6.25 factor or for a specific crop

Result: Protein % of given sample is =

Laboratory Exercise No. 19

Objective: Quantitative estimation of oil content by Soxhlet method.

Lipids are heterogeneous group of compounds including fats, oils, steroids, waxes and related compounds. These are the organic substances synthesized in plants which are relatively insoluble in water but soluble in organic solvents (alcohol, ether etc.). Lipids occur in plants and animals as storage and structural components. In plants, they are preferably found in seeds, fruits and nuts while in animals they are present in the form of meat. Fat supplies twice energy per unit weight as compared to protein or carbohydrate. Lipids have lower specific gravities than water.

Principle: The oil and fats are completely miscible with non-polar organic solvents such as ether, petroleum ether and hexane. This property of lipids is used to extract them from tissues. It involves repeated extraction of the material with the solvent in the Soxhlet apparatus. The solvent is then distilled off completely. Oil (%) is calculated gravimetrically.

Materials:

1. Soxhlet apparatus
2. Thimble
3. Petroleum ether/hexane
4. Pestle and mortar

Procedure:

1. Take about 2g of dried seed sample.
2. Grind the seed sample in a pestle mortar and pour it in thimble.
3. Place the thimble having sample in extractor of the Soxhlet apparatus.
4. Weigh the flask and add organic solvents, two and half times the capacity of the flask.
5. Extract oil for at least a period of 6-8 hrs. till the solvent in the extractor becomes colourless.

Observation:

S. No.	Weight of sample	Weight of the defatted meal
1.	2g	
2.	2g	
3.	2g	

Calculation:

Weight of the seed = W g
Weight of the empty flask = W_1g
Weight of flask and lipid = W_2 g
Weight of lipid = (W_2-W_1)g

Calculate the oil percentage as follows

$$\text{Oil content (\%) in seed} = \frac{(W_2-W_1)}{W} X100$$

Result: Oil content (%) in given seed sample is =

Laboratory Exercise No. 20

Objective: Determination of ascorbic acid using 2,6-dichlorophenolindophenol dye.

Principle: Ascorbic acid is oxidized by the coloured dye 2,6-dichlorophenolindophenol to dehydroascorbic acid. At the same time the dye is reduced to a colourless compound so that the end point of the reaction can be easily determined. The end point is appearance of pink colour. The dye is pink coloured in acid medium. Oxalic acid is used as the titrating medium. Other compounds as well as ascorbic acid decolorize the dye, but the specificity can be increased to some extent by carrying out the reaction in an acid solution where interfering substances react only slowly.

Materials:

1. Oxalic acid 4%
2. **Dye solution:** Weigh 42mg sodium bicarbonate into a small volume of distilled water. Dissolve 52mg 2,6-dichlorophenolindophenol in it and make up to 200ml with distilled water.
3. **Stock standard solution:** Dissolve 100mg ascorbic acid in 100ml of 4% oxalic acid solution in a standard flask (1mg/ml)
4. **Working standard:** Dilute 10ml of the stock solution to 100ml with 4% oxalic acid. The concentration of working standard is 100µg/ml.

Procedure:

1. Fill the burette to zero mark with the dye solution.
2. Pipette out 5ml of the working standard solution into a 100ml conical flask.
3. Add 10 ml of 4% oxalic acid and titrate against the dye and note the volume (V_1ml) reading. End point is the appearance of pink colour, which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid.
4. Extract the sample (0.5-5gm depending on the sample) in 4% oxalic acid and make up to a known volume (100ml) and centrifuge.
5. Pipettes out 5ml of this supernatant, add 10ml of 4% oxalic acid and titrate against the dye (V_2ml)
6. For coloured solution, 1 ml of chloroform is added to the reaction mixture (because it is difficult to see the end point when test solution is coloured). End point is obtained when pink colour is seen is organic phase. Standard solution is also treated in the same way.

Observation: For known (standard) ascorbic acid

S. No.	Volume of ascorbic acid (ml)	Volume of dye			
		Initial reading (ml)	Final reading (ml)	Volume of dye used (ml)	Mean value (ml)
1.	5.0				
2.	5.0				
3.	5.0				

For unknown (test solution)

S. No.	Volume of ascorbic acid (ml)	Volume of dye			
		Initial reading (ml)	Final reading (ml)	Volume of dye used (ml)	Mean value (ml)

1.	5.0				
2.	5.0				
3.	5.0				

Calculation:

$$\text{Amount of ascorbic acid (mg/100ml)} = \frac{\text{Test solution - Blank}}{\text{Standard - Blank}} \times \text{conc. of standard(mg/100ml)} \times \text{Dil. factor}$$

Results:

APPENDIX

INTRODUCTION TO LABORATORY PROCEDURES

For the individual safety and convenience, following rules and reputations must be followed in the biochemistry laboratory.

General instructions

1. Before each laboratory period, read over the exercises to be done and plan your work carefully, know how each exercise is to be done and what basic principles it is intended to convey.
2. Each laboratory meeting will begin with a short discussion and instruction period. Don't begin work until you have received your instructions. Ask questions whenever you do not understand the method and purpose of any experiment. Good laboratory technique depends primarily on knowing what you have to do.
3. Properly record all observations in practical file at the time they are made. An index page containing the exercise and page number should be attached in the beginning of observation note book or practical file. Each laboratory exercise should include the objective of exercise, principle, materials required, procedure, result/observation in the form of tables/graphs and discussion of the observations/findings.
4. Laboratory examinations will cover both the information given by your laboratory instructor and that contained in the manual, as well as you own observations and inferences.

Laboratory Regulations

1. Wear a coat, smock, or apron to protect your clothing
2. Sponge off the top of your laboratory desk with germicide solution in the beginning and the end of each laboratory period.
3. Keep your desk free of non-essential materials during the lab time and leave it free of all materials and equipment at the end of the period.
4. Place all solid waste material in the waste cans and all dirty glassware in the trays. Do not spill any chemical on the desk. Your laboratory grade will depend to some extent on your knowledge of techniques, orderliness, and cleanliness.
5. Label everything you use or prepare with your initial, date and name of the reagent, buffer or medium.
6. Report immediately all accidents such as cuts, burns to your instructor, take all precautions to avoid such accidents.
7. Make yourself familiar with the laboratory equipments such as incubators, centrifuges and microscopes etc. If you have any problem in their operation, approach the lab technician or course instructors.
8. There should not be any eating, drinking or smoking in the lab.
9. There should not be any storage of food in the lab.
10. Since many chemicals used in the lab may be hazardous, always refer to MSDS (material safety data sheet) before using a new chemical. MSDS sheet provides information about safe handling of the chemical. Wear gloves for handling certain chemicals like phenol concentrated acids, acrylamide, etc.
11. Avoid exposure to UV light which is a potentially mutagenic radiation and severely damages the eyes.

Cleaning of glassware

It is extremely important to use clean glassware for experimental purpose. The following procedure ensures clean for flasks, tubes, petri dishes and other glassware.

1. Immerse all glassware in 2-5% detergent immediately following use for 20-24 hr. Heating considerably accelerates the cleaning process.

2. To remove resistant dirt soak the glassware in chromic acid for 3-4 hours. Chromic acid is a solution of 140 g of potassium dichromate in 100 ml of distilled water and 2:1 of concentrated sulfuric acid. Note that the preparation of this solution is hazardous and should be done in fume hood.
3. Rinse thoroughly in tap water.
4. Rinse in hot water.
5. Soak in distilled water for at least 3 hr.
6. Rinse in distilled water and dry in a hot air oven.

PREPARATION OF SOLUTIONS

Solution: The homogeneous mixture of two or more substances is called as solution. When a solid is dissolved in a liquid, the solid is called as the solute and the liquid is called as the solvent and the resultant mixture is known as solution. The concentration of a solution may be defined as the amount of the solute present in the given quantity of the solvent.

The following types of solution are prepared in the laboratory:

1. Saturated solution: A Saturated solution is one, which holds as much solute it can.

2. Standard Solution: The solution whose concentration (strength) is known is called as standard solution.

The standard solution may be expressed as:

(i) Percent solution: A percent solution is one, which contains a known weight of the solute in 100ml of the solution. The percentage of a solution can be expressed in three ways:

- **Weight per unit weight (w/w):** A 1% w/w solution has 1g of solute and 99g of solvent to make 100g of solution
- **Weight per unit volume (w/v):** A 1% of w/v solution contains 1g of solute in 100ml of solution.
- **Volume by volume (v/v):** A 1% of v/v solution contains 1 ml of solute in 100ml of solution.

(ii) Molar solution: The solution that contains one mole (molecular weight in g) of a substance (solute) per 1000ml of solvent is known as molar solution.

Relationship of molarity and moles:
Molarity is the most useful concentration for chemical reaction in solution because it directly relates moles of solute to volume of solution. It is defined as the number of moles of a solute dissolved per 1000ml of the solution.
One molar solution = 1mole/liter = 1 millimole/ml = 1 micromole/µl
Molarity = Number of moles /litre

(iii) Molal solution: The solution which contains one mole (molecular weight in g) of a substance (solute) per 1000g of solvent is termed as molal solution.

(iv) Parts per million (ppm): 1g solute dissolved in 1000 liters solvent or 1mg solute in 1 liter solvent is called 1ppm solution.

(v) Normal solution: The solution which contains 1g equivalent weight of a substance (solute) per 1000 ml of solvent is called as normal solution.

The equivalent weight of a substance is defined as the amount that reacts with 1 part of hydrogen, 8 part of oxygen or 35.5 parts of chlorine.

Equivalent weight of an acid: An acid has ionisable or replaceable hydrogen ion (H^+). If an acid has one replaceable H^+ it is called a monobasic acid, if 2 it is called a dibasic acid, and 3 it is tribasic acid. A monobasic acid forms one type of salt, dibasic two types and tribasic acid forms three types of salts on treating with base.

$$\text{Equivalent weight of an acid} = \frac{\text{Molecular weight}}{\text{No. of replaceable } H^+}$$

e.g., Equivalent weight of HCl=36.5/1=36.5
Equivalent weight of H2SO4 = 98/2=49

Equivalent weight of base: A base has replaceable hydroxyl ions, hence to calculate the equivalent weight of a base, its molecular weight is divided by the number of replaceable hydroxyl ions, e.g.. NaOH has one replaceable OH ion. Thus the equivalent weight of NaOH = Molecular weight of NaOH/1, i.e. 40/1= 40.

Equivalent weight of salts: The number of electrons, which are given or taken up during reaction, determines the equivalent weight of a salt, such as $AgNO_3$, $KMnO_4$ and $K_2Cr_2O_7$.

For example, $AgNO_3$, gives 1 electron, therefore its equivalent weight is its molecular weight divided by 1. While potassium permanganate takes 5 electrons, its equivalent weight is its molecular weight divided by 5. Same way equivalent weight of $K_2Cr_2O_7$ is molecular weight divides by 3

A. Primary standard substances: The Chemicals, which are analytically pure and can be used directly on the basis of their equivalent weight or molecular weight for the preparation of standard solution of different normality or molarity, are called primary standard substances. A few of them are given below:

S. No	Name of Chemical	Formula	Equivalent wt. (g)
1	Potassium hydrogen phthalate	$HK(C_8H_4O_4)$	204.22
2	Oxalic acid	$(COOH)_2.2H_2O$	63.034
3	Sodium carbonate	Na_2CO_3	53.00
4	Sodium oxalate	$Na_2C_2O_4$	67.01

B. Secondary standard substances: The Chemicals which have impurities or hygroscopic nature and hence cannot be used according to their equivalent or molecular weight directly for preparation of standard solution are called as secondary standard substances. These chemicals are used to prepare their approximate solutions which are then standardized against a primary standard solution. Details of a few such chemicals are given below:

S. No	Name of Chemical	Formula	Purity	Equivalent wt. (g)
1	Hydrochloric acid	HCl	35%, sp.gr. 1.18	36.5
2	Sodium hydroxide	NaOH	Hygroscopic	40.0
3	Sulphuric acid	H_2SO_4	98%, sp.gr. 1.84	49.0

ESTIMATION OF pH

Each aqueous solution whether acidic, alkaline or neutral, contains both H^+ and OH^- ions. The product of their concentration is always constant i.e. $1x10^{-14}$ at 25^0C. The nature of a solution whether it is acidic or alkaline depends upon which of the two ions are present in greater concentration than the other. But for convenience acidity or alkalinity of a solution can be expressed in terms of hydrogen ions (H^+) concentration only. Since after knowing the concentration of one of these two ions, concentrations of other ion can be calculated. The hydrogen ion concentration can vary within wide range, usually from 1 mole/litre (as in HCl) to about 10^{-14} moles/litre (as in 1M NaOH).

Danish Biochemist Soren Soreson used a logarithmic scale for expressing the hydrogen ion concentration. The scale was called as pH, where P stands for power and H for hydrogen ion concentration. According to him, "The pH of a solution is the negative logarithm of concentration (in mole/litre) of hydrogen ions which it contains". Thus,

$$pH = -\log [H^+]$$

$$\text{or} \quad = \log \frac{1}{[H^+]}$$

pH gives us an idea about the acidity or alkalinity of a solution. The ionization product of water a neutral molecule) makes the basis of pH scale. Pure water, after dissociation, produces equal amounts of H^+ and OH^-.

$$H_2O \rightleftharpoons H^+ + OH^-$$

At 25^0C, only one out of about 10^7 molecules is ionized at any given time. The equilibrium constant for the above ionization reaction can be written

$$K' = \frac{[H^+][OH^-]}{[H_2O]} \text{ ----------(1)}$$

The concentration of water molecule is = 1000/18 or 55.5 while the value of K' as measured by electrical conductivity of water which comes to 1.8×10^{-16} at 25^0C. After replacing these values in equation (1) we get.

$55.5 \times 1.8 \times 10^{-16} = [H^+] [OH^-]$
$99.9 \times 10^{-16} = [H^+] [OH^-]$
$10^2 \times 10^{-16} = [H^+] [OH^-]$
$10^{-14} = [H^+] [OH^-]$
$1x10^{-14} = [H^+] [OH^-]$
or $Kw = 1 \times 10^{-14} = [H^+] [OH^-]$

Kw is called the ionization product of water which always remains constant i.e. $1x10^{-14}$ at 25°C. This means that when concentration of $[H^+]$ is exactly equal to the concentration of [OH), the solution is said to be neutral such as distilled water under these conditions. Concentration of $[H^+]$ or $[OH^-)$ can be calculated as

$$Kw = [H^+] [OH^-] = 1 \times 10^{-14} \text{ ----------(2)}$$

Expressing only in terms of $[H^+]$

$$Kw = [H^+] [OH^-] = [H^+]^2 \quad \text{or}$$
$$[H^+]^2 = Kw = 1 \times 10^{-14}$$
$$\text{or } [H^+] = \sqrt{Kw}$$
$$= \sqrt{1\text{-}10^{-14}}$$
$$= 1x10^{-7}$$
$$[H^+] \text{ or } [OH^-] = 1 \times 10^{-7}M. \text{ ----------(3)}$$

The equations (2) and (3) also refer that whenever the concentration of $H^+ > 1x10^{-7}$ M, the concentration of OH^- must be $< 1 x10^{-7}$ M and vice-versa.

The pH of pure water or a neutral solution where hydrogen ion concentration is $1x10^{-7}$ M, will be 7 as follows:

$$pH = \log \frac{1}{[H^+]}$$

$$= \log \frac{1}{1x10^{-7}}$$

$$= \log (1x10^{-7})$$

$$= \log 1 + \log 10^{-7}$$

$$= 0 + 7 \text{ or } 7$$

Determination of pH: pH of a solution can be determined by two methods

1. Colorimetric method
2. Electrometric method

1. Colorimetric method:

In this method dyes are used as pH indicators. Certain dyes change colour with change in pH; such dyes are called indicators. By using these indicators, one can know the pH of the unknown solution. Some dyes and their colours in acidic and basic media are given in Table.

Table: pH range of indicators

Indicators	pH range	Acidic	Basic
Thymol blue	1.2-2.8	Red	Yellow
Bromophenol blue	3.0-4.6	Yellow	Blue
Chlorophenol red	5.0-6.6	Yellow	Red
Bromothymol blue	6.0-7.6	Yellow	blue
Phenol red	6.7-8.3	Yellow	Red
Methyl red	4.2-6.3	Red	Yellow
Methyl orange	3.1-4.4	Red	Yellow
Phenolphthalein	8.3-10.0	Colorless	Pink
Bromocresol green	3.8-5.4	Yellow	Blue

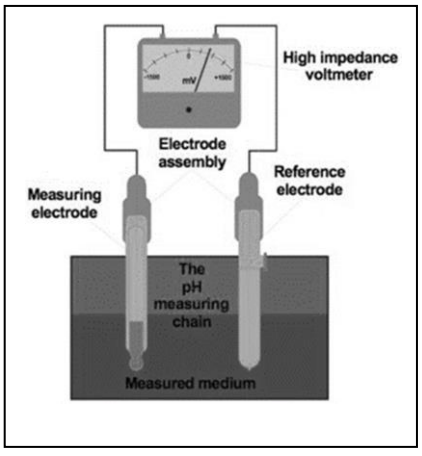
Using a combination of these dyes approximate pH of a solution can be determined.

2. Electrometric method (Accurate measurement of pH)

In this method, pH-meter is used for measuring pH, which measures the e.m.f. of concentration cell formed from a reference electrode, the test solution and a glass electrode sensitive to hydrogen ions.

Glass electrode: The glass electrode consists of a very thin bull about 0.1 mm thick blown onto a hard glass tube of high resistance. Inside the bulb is a solution of HCI acid (0.1 mol/l) connected to a pt-wire via silver-silver chloride electrode which is reversible to H^+. The glass electrode rapidly responds to H^+ concentration. Salts, protein or oxidizing and reducing agents do not readily affect this potential, so the electrode can be used in a wide range of media.

The glass electrode in the test solution constitutes a half-cell and a reference electrode completes the measuring circuit, which is sensitive to hydrogen ions.



The voltaic cell formed during the measurement of pH

Preparation of Buffer solution: The resistance of a solution to change its hydrogen ion concentration upon the addition of small amounts of acid or alkali is called as buffering system. A solution, which tends to maintain its pH after addition of small amounts of an acid or base, is known as buffer solution. Buffer solution contains a H^+ donor and a H^+ acceptor form of weak acids and weak bases. A buffer system is most

effective when the concentration of H^+ donor and H^+ acceptor is equal.

In blood plasma, carbonic acid-bicarbonate is a very common buffering system. The weak carbonic acid readily dissociates into H^+ and HCO_3^- as follows.

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

When small amount of HCl is added to this buffer solution, H^+ produced from HCl combine with HCO_3 to form H_2CO_3. Now, If small amount of NaOH is added, the OH^- produced, reacts with H^+ dissociated from H_2CO_3 to form H_2O. Thus, this buffering system soaks the H^+ and OH^-produced from strong acid or base and tend to maintain the original pH of the solution. Similarly weak bases and their salts also work as buffer system.

Buffer system in the organisms help in carrying out most of the biochemical reactions in a narrow range of pH between 6 and 8. For example, the blood maintains its constant pH of about 7.4 in spite of carrying a large number and variety of chemicals. Apart from this, buffer system provide protection to cells and tissues against sudden change in their pH.

Preparation of Acetate Buffer:

A. Prepare 0.2M solution of acetic acid by diluting 11.5ml of acetic acid to 100ml with distilled water.

B. Prepare 0.2N solution of sodium acetate by dissolving 14.4g of C_2H_2 Na or 27.2g of $C_2H_30_2$ Na $3H_2O$ in water and then diluting it to 1000 ml.

Now mix the above stock solution and dilute to 100ml as given below to get buffer situations of different pH.

A (ml)	B (ml)	pH
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0

Preparation of Carbonate-Bicarbonate Buffer:

A. Prepare 0.2M solution of anhydrous sodium carbonate by dissolving 21.2g of it in distilled water and then diluting to 100ml.

B. Prepare 0.2N solution of sodium bicarbonate by dissolving 16.8g of it in distilled water and then diluted to 1000ml.

Mix the stock solutions as given in the following table and finally dilute to 100ml get buffer solution of desired pH.

A (ml)	B (ml)	pH
16.0	34.0	9.6
19.5	30.5	9.7
22.0	28.0	9.8
25.0	25.0	9.9
27.5	22.5	10.0
30.0	20.0	10.1
33.0	170	10.2
35.5	14.5	10.3
38.5	11.5	10.4
40.5	9.5	10.5
42.5	7.5	10.6
45.0	5.0	10.7

Preparation of Citrate Buffer:

A. Prepare 0.2M solution of citric acid by dissolving 21.01g of it in distilled water and diluting to 1000ml.

B. Prepare 0.1N solution of sodium citrate by dissolving 29.41g of it ($C_6H_5O_7Na.2H_2O$) in distilled water and diluting to 1000ml.

Mix above stock solutions as given below and finally dilute to 100ml to get the buffer solution of different pH.

A (ml)	B (ml)	pH
35.0	15.0	3.8
33.0	17.0	4.0
31.5	18.5	4.2
28.0	22.0	4.4
25.5	24.5	4.6
23.0	27.0	4.8
20.5	29.5	5.0
18.0	32.0	5.2
16.0	34.0	5.4
13.7	36.3	5.6
11.8	38.2	5.8
9.5	40.5	6.0
7.2	42.8	6.2

COLLOIDAL SOLUTIONS

Thomas Graham (1961) classified soluble substances according to their power of diffusion across vegetable or animal membranes into the following two groups.

(A) Crystalloids: The substances, which are readily obtained in the crystalline form and diffuse rapidly across the membrane, are called crystalloids. Such as urea, sugar, salt etc.

(B) Colloids: The amorphous substances, which exhibit little or no tendency to diffuse across the membrane, are known as colloids such as gelatin, starch, gum. etc. (Colloids-Greek; Kolla = glue and eidos = like).

According to Graham, NaCl is a crystalloid but it has been obtained in the colloidal state when dissolved in benzene. Similarly, soap behaves as colloid in water but as crystalloid in alcohol. Hence, all the substances can be obtained in colloidal condition by using appropriate method. Therefore, we may call the colloidal state of matter as we speak of the liquid, solid and gaseous state of matter. "The solution of organic substances like gelatin, gum, albumin etc. whose particle size is between 0.2×10^{-4} cm and visible only under the ultra-microscope is called as colloidal solution."

Nature of Colloidal Solutions: If the solute particles of a solution are of or below of size 1×10^{-7} cm and not visible under the ultra-microscope, the solution will be called as true solution while the solution having solute particles of size 0.2 x 10 cm which are visible under microscope and even by naked eyes is termed as suspension but the solution whose solute particle size is between 0.2×10^{-4} to 1 x 10-7 cm and visible only under the ultra-microscope is called as colloidal solution.

Type of Colloidal System: A true solution is a homogenous mixture of solute and solvent and make a single phase solution. The colloidal solution, on the other hand, is a diphasic system in which a solid as dispersed phase and a liquid as a depression medium. In diphasic system, each one of the two phases may either be a gas, liquid or solid as given below:

Colloidal System	Name	Examples
Gas dispersed in liquid	Foam	Foam of soap solution
Gas dispersed in solid	Solid foam	Minerals with gaseous inclusions & Pumice stone
Liquid dispersed in gas	Mist. Fog. Cloud	Rain, Mist, cloud
Liquid dispersed in liquid	Emulsion	Milk, water in benzene, oil in water
Liquid dispersed in solid	Gel or jelly	Silicic acid gel
Solid dispersed in gas	Aerosol	Coal smoke from chimney, Ammonium chloride fumes.
Solid dispersed in liquid	Sole Colloidal solution	Gold or silver in water, nickel in benzene
Solid dispersed in solid	Solid solution	Coloured gems and glasses

QUANTITATIVE ESTIMATIONS OF BIOMOLECULES

Amount of a biomolecule in a tissue of body fluid can be determined by any of the following procedures:

1. **Gravimeteric method:** In this method, the biomolecule is precipitated, dehydrated and then weighed. Since procedures to precipitate all types of biomolecules are not available as well as complete removal of water from a biomolecule is also not possible, gravimeteric methods are limited to only few biomolecules like proteins.
2. **Volumetric method:** These methods involve titration of the solution of a biomolecule against a suitable standard solution in presence of a suitable indicator. It is less sensitive, time consuming, less accurate and need more amount of sample. Many biomolecules are present in very low amounts, hence this method is also limited to only few types of biomolecules such as amino acid, ascorbic acid etc.
3. **Spectrophotometric methods:** These methods depend on the amount of light absorbed by a chromophore, which is either the compound itself or its derivative formed after a suitable chemical reaction. The Beer-Lambert Law relates the light absorbed with concentration of the chromophore:

   $$E = k.c.1$$

   If the Beer-Lambert's law is obeyed and l' is kept lcm, then a plot of absorbance against concentration 'c' gives a straight line passing through the origin.

For Rough Work

For Rough Work