LAB MANUAL

BIOCHEMISTRY LAB

CODE: NBT-652

IMS ENGINEERING COLLEGE

A NAAC Accredited & ISO 9001:2008 Certified Institution

(Approved by AICTE and affiliated to APJ Abdul Kalam Technical University, Lucknow)

NH-24, Delhi-Hapur by Pass Road, Adhyatmik Nagar, Ghaziabad-201 009 (U.P.)

PRACTICAL - 1 **Date:**

AIM: Preparation of Solutions

The concentration of the compound present within the sample can be expressed as:

1. **Molar Solution:** It contains gm molecular weight of the solute present per liter of the solution. It can be calculated by using following formula.

$$\text{Molarity of Solution} = \frac{\text{Numbers of moles of solute}}{\text{Dissolve in 1 liter of solution}}$$

Example:
1M solution of NaOH: Dissolve 40gm of NaOH in 500 ml and make up the final volume 1 liter with D/W. (Molecular weight (M.W.) of the NaOH = 40)

2. **Normal Solution:** It contains gm equivalent weight of the solute in 1 liter of the solution.

$$\text{Normality of Solution} = \frac{\text{Numbers of gm equivalent weight}}{\text{Dissolve in 1 liter of solution}}$$

Example:
1N solution of HCl: Dissolve 49.04gm of H_2SO_4 in 500 ml and make up the final volume 1 liter with D/W. (M.W. of the H_2SO_4= 98.08 The gram equivalent weight is calculated by calculating the dissociable H^+/OH^- ions in the solutions. H_2SO_4 consist $2H^+$ dissociable ions, hence 98.08/2 = 49.04).
Similarly, 1 mole of HCl, 0.5 mole of H_2SO_4 and 0.333 mole of H_3PO_4 dissolved in 1000ml are 1N solution.

3. **% Solution (w/v):** Dissolve required gm of the solute in 100 ml of solution.

Example:
1% NaCl: Dissolve 1g of NaCl in 100ml of water.

4. **% Solution (v/v):** Dissolve required gm of the solute in 100 ml of solution, by considering the density of the liquid solute.

Example:
1% Acetic acid: 1ml Acetic acid = 1.05- 1.15 gm of Acetic acid
Dissolve 1.1 g of Acetic acid in 98.9ml of water.

5. **Expression of the result**: Express your result for concentrations of the bimolecules or metabolite always as mg% (mg/dl) or g% (g/dl).

ORIENTATION TO PREPARATION OF BUFFER

INTRODUCTION

A buffer system is one that resists a change in pH on addition of acid or alkali. It constitutes of conjugate acid-base pair. Most commonly, the buffer solution consists of a mixture of a weak acid and its conjugate base.

Buffers are extensively used in biochemical studies since these aids in maintaining a near constant pH of the media while performing various laboratory operations such as during extraction, isolation and purification of various biomolecules. Selection of an appropriate buffer with optimal pH is important as it may have a profound influence on extractability, stability, and even biological functioning of cell constituents. All the biochemical reactions in a cell are catalyzed by enzymes whose stability as well as activity is highly dependent on pH of the system.

Measurement of pH

By using pH indicators an approximate value of pH of a solution can be obtained. The pH papers coated with indicators of different pH range are routinely used. However, accurate pH can be measured using pH meter which measures e.m.f. of a concentration cell developed from a reference electrode, test solution and a glass electrode sensitive to H^+ ions. The combined electrode constituting of glass and reference electrode is dipped into the test solution for accurate measurement of pH.

Buffers can be made in stock solutions and these are diluted before use. Always check the pH of solution before use.

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PRACTICAL - 2 **Date:**

AIM: Preparation of buffer

PRINCIPLE

By using Henderson-Hasselbalch equation, pH of the solutions of various concentration ratios of a conjugate acid-base pair of known pK' can be calculated and the ratio of conjugate acid-base of unknown pK' to obtain a buffer of desired pH can be found out.

$$pH = pK' + \log \frac{[\text{conjugate base}]}{[\text{conjugate acid}]}$$

REQUIREMENT:

Reagents

1. **Stock solutions :**
   - Sol. A 0.2 M Monobasic Sodium phosphate solution: Add 2.78g of NaH_2PO_4 in 100ml D/W.
   - Sol. B 0.2 M Dibasic Sodium phosphate solution: Add 5.36g of $Na_2HPO_4.7H_2O$ in 100ml D/W.
2. 1 N HCl
3. 1 N NaOH

Equipments: Balance, pH meter or pH strips
Glassware: Beakers, Glass Pipettes

PROCEDURE:

Calibrate the pH meter by standard buffer (pH 7 and PH 4/ pH9). The buffer of desired pH can be prepared by using following table.

Solution A (ml)	Solution B (ml)	pH
68.5	31.5	6.5
56.5	43.5	6.7
39.0	61.0	7.0
16.0	84.0	7.5

1. Add 16 ml of solution A and 84 ml of solution B.
2. Dilute to a total volume of 200ml.
3. Check the pH of the 0.1M buffer using pH meter.

RESULT:

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ORIENTATION TO QUALITATIVE AND QUANTITATIVE ANALYSIS OF CARBOHYDRATES

INTRODUCTION

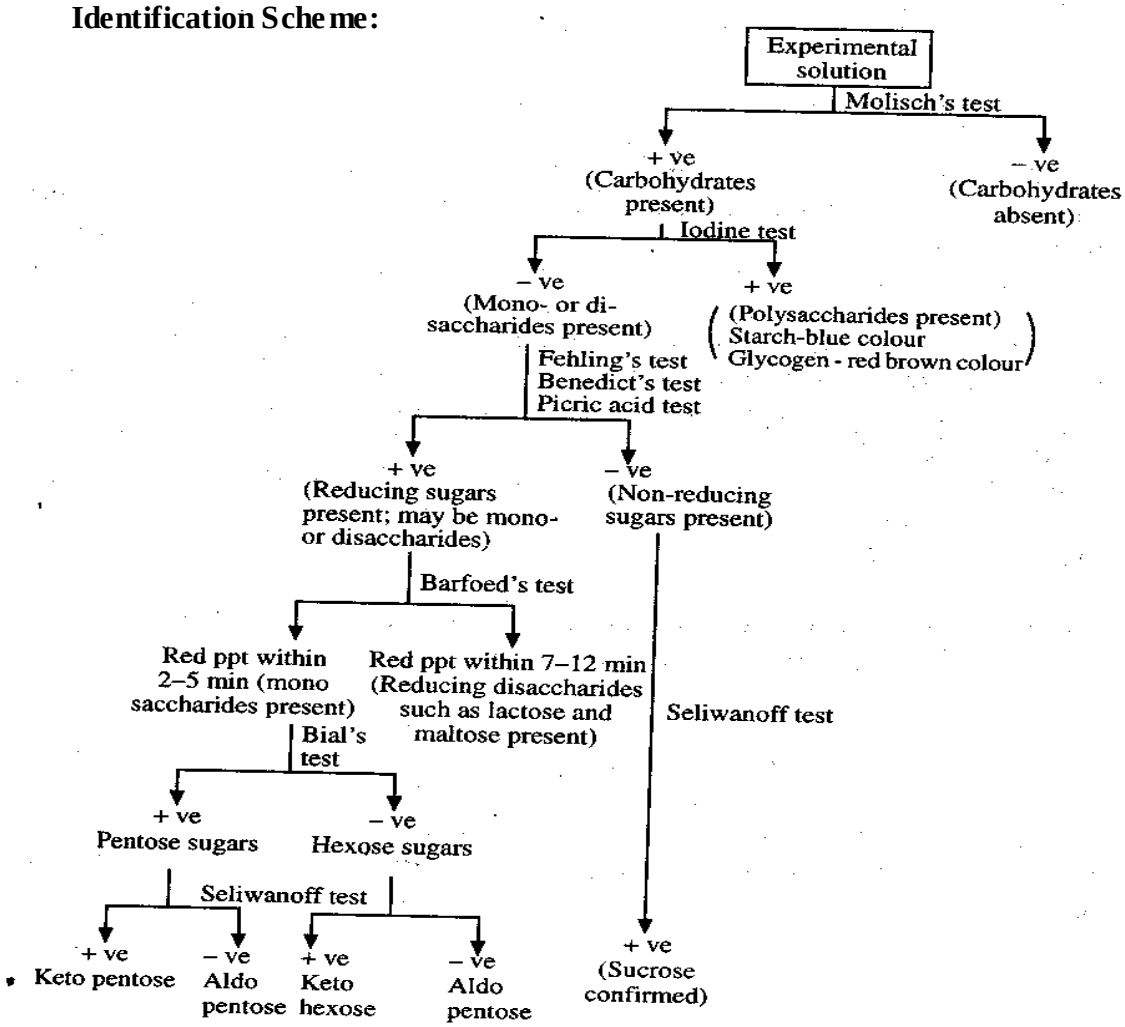
The majority of the most important metabolic and structural roles in nature are filled by carbohydrates. Carbohydrates are organic biomolecules and defined chemically as polyhydroxy aldehydes or ketones with the empirical formula $Cn(H_2O)n$ (where n=numbers of atoms). Depending on the numbers of carbons are present they are broadly classified as monosaccharide, oligosaccharides and polysaccharides. Along with this basic formula sometimes they have also consist nitrogen (N), sulfur (S) and phosphorus (P). Depending on their free aldehyde or ketone group they are classified as reducing and rest as non-reducing.

The qualitative test used for the carbohydrates are broadly categorized into three groups.

1. Based on their reducing properties
2. Oxidation and reduction properties
3. Formation of derivatives after reaction with certain chemical compounds.

Depending on the types and reaction carried out by the individual or groups of carbohydrates following test and scheme can be applicable for the identification of the carbohydrates present in the sample.

Identification Scheme:

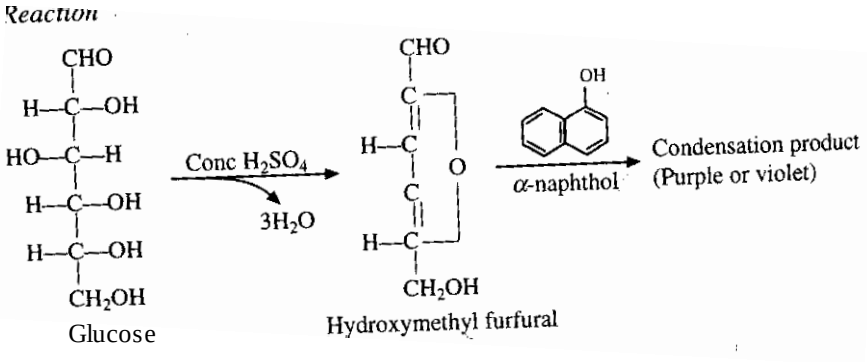


PRACTICAL -3 **Date:**

AIM: Identify the carbohydrate present in the given sample by qualitative method.

A. <u>Molisch's Test</u> (for the presence of carbohydrates)

PRINCIPLE:
Reactions of sugar with concentrated acids produce dehydration of sugars and forming furfural and their derivatives. These derivatives react with α-naphthol to produce purple colored complex.



REQUIREMENTS:

1. **Molisch's reagent:** Dissolve 0.5g α-naphthol in10ml of 95% ethanol. Store the reagent in brown bottle and preserve at room temperature.
2. Concentrated H_2SO_4
3. Test sample

PROCEDURE:

i) Take 2 ml of test solution and add two drops of Molisch's reagent in a test tube.
ii) After mixing, tilt the tube and carefully add without mixing, add 1ml of conc. H_2SO_4 from the side of the tube.

OBSERVATION: A red-violet layer at the interface between the acid (bottom) and aqueous (upper) layers is a positive test for carbohydrates.

RESULT:

B. <u>Benedict's Test</u> (for reducing sugars)

PRINCIPLE:
Under alkaline conditions free aldehyde or ketone group present in the mono, di (except sucrose) or oligosaccharides reacts with $Cu(OH)_2$ and reduced to red colored Cu_2O compound.

Reaction

$$Na_2CO_3 + 2H_2O \rightarrow 2NaOH + H_2CO_3$$
$$2NaOH + CuSO_4 \rightarrow Cu(OH)_2 + Na_2SO_4$$
$$Cu(OH)_2 \rightarrow CuO + H_2O$$
$$\text{D-Glucose} + 2\ CuO \rightarrow \text{D-gluconic acid} + Cu_2O\ (\text{Red ppt})$$

REQUIREMENTS:

1. **Solution A:** Dissolve 1.73g trisodium citrate (dihydrate) and 1g sodium carbonate (anhydrous) in 8m of warm D/W.
2. **Solution B:** Dissolve 1.73 g copper sulfate (pentahydrate) in 20ml of D/W.
3. **Benedict's reagent:** Mix 0.8ml of Solution A with 0.2ml of Solution B, before use. Prepare always fresh.
4. Test sample

PROCEDURE:

i) Take 1ml of test solution and add 1ml of Benedict's reagent in test tube.
ii) After mixing heat in a boiling water bath for 5 minutes. Record your observation for change in color.

OBSERVATION: A brick-red precipitate indicates a positive test for reducing sugars

RESULT:

C. <u>Iodine Test</u> (for non reducing sugars)

PRINCIPLE:

Under mild acidic conditions non-reducing sugar such as starch forms blue color complex when reacts with iodine.

REQUIREMENTS:

1. **Iodine solution:** Prepare 0.005N iodine solution in 3% potassium iodide (KI). Store in brown bottle and preserve at room temperature.
2. **0.1N HCl**
3. **Test sample**

PROCEDURE:

i) Take 1 ml of test solution and add few drops of iodine reagent in test tube and add few drops of 0.1N HCl
ii) Mix well and record the color change.

OBSERVATION: A blue color indicates presence of starch and brown color for glycogen.

RESULT:

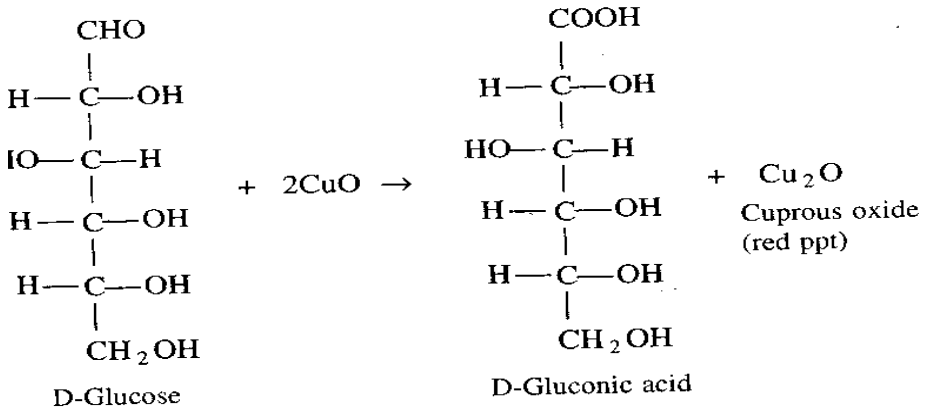
D. <u>Barfoed's Test</u> (for reducing monosaccharide)

PRINCIPLE:
Under acidic conditions free aldehyde or ketone group present in the mono- or reducing disaccharides reacts with $Cu(OH)_2$ and reduced to red coloured Cu_2O compound.

$$(CH_3COO)_2Cu + 2H_2O \rightarrow 2CH_3COOH + Cu(OH)_2$$

Cupric acetate — Cupric hydroxide

$$Cu(OH)_2 \rightarrow CuO + H_2O$$



REQUIREMENTS:

1. **Barfoed's reagent:** Dissolve 0.66g cupric acetate (monohydrate) and 0.18ml glacial acetic acid in 10ml of D/W.
2. Test sample

PROCEDURE:

i) Take 1ml of test solution and add 2mlof Benedict's reagent in test tube.
ii) After mixing heat in a boiling water bath for 1min.
iii) Cool the mixture to room temperature. Record your observation for change in color.

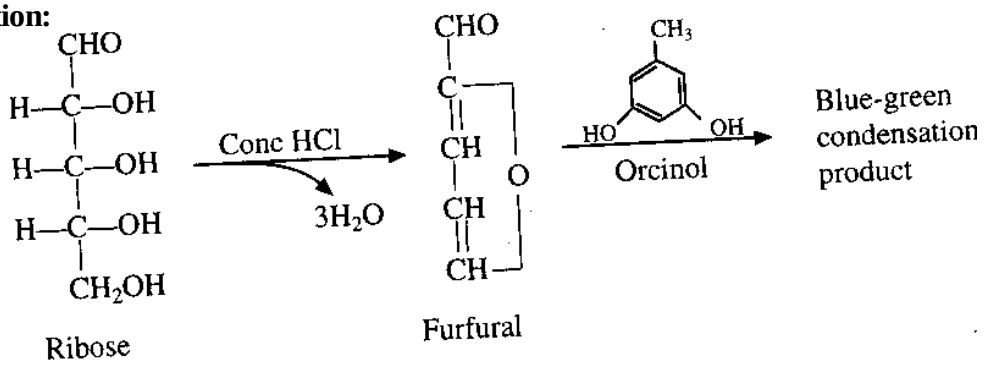
OBSERVATION: A copious amount of brick-red precipitate indicates a reducing monosaccharide. Some hydrolysis of disaccharides may lead to trace precipitates.

RESULT:

E. Bial's Test

PRINCIPLE:
Under strong acidic conditions pentoses converted to the furfural or their derivatives.

Reaction:



REQUIREMENTS:

1. **Bial's reagent:** Dissolve 0.3g Orcinol and 0.05g ferric chloride in 100ml of concentrated (12 M) HCl. Store the reagent in brown bottle.
2. Test sample

PROCEDURE:

i) Take 2 ml of test solution and add 5ml of Bial's reagent in a test tube
ii) After mixing, heat the solution in a boiling water bath for 2 minutes.
4. Observe your result for the color change within 2 minutes.

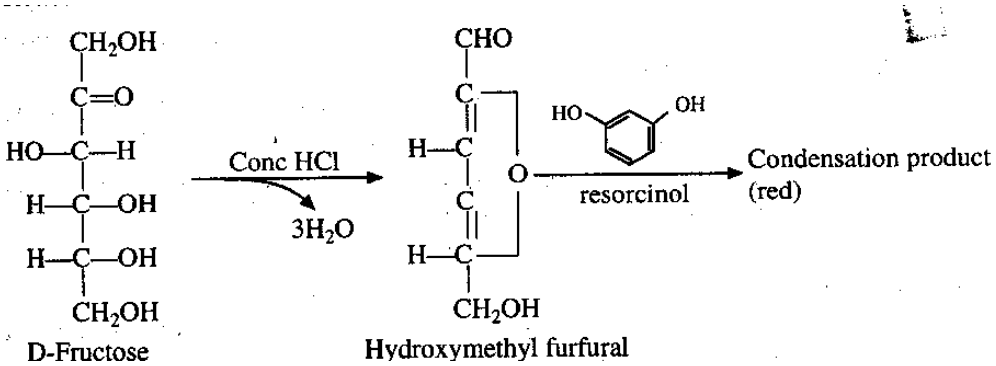
OBSERVATION: A blue-green color indicates presence of pentoses or nucleotides. A yellow-green color indicates hexoses, where as, disaccharides gives in color

RESULTS:

F. Seliwanoff's Test (for ketohexoses)

PRINCIPLE

Under strong acidic conditions ketoses reacts with resorcinol and converted to corresponding furfural and their derivatives buy forming deep red colored complex.



REQUIREMENTS:

1. **Seliwanoff's reagent:** Dissolving 50mg resorcinol in 100ml of 3M HCl. Store protected from light.
2. Test sample

PROCEDURE:

i) Take 1 ml of test solution and add 5ml of Seliwanoff's reagent in a test tube
ii) After mixing, heat the solution in a boiling water bath for 5 minutes.
iii) Observe your result for the color change.

OBSERVATION: A deep red colored precipitate within 5min. indicates ketohexoses. Sucrose also gives a positive test because of partial hydrolysis to glucose and fructose. Other sugars give a red color upon prolonged heating.

RESULTS:

INTERPRETATION:

CONCLUSION:

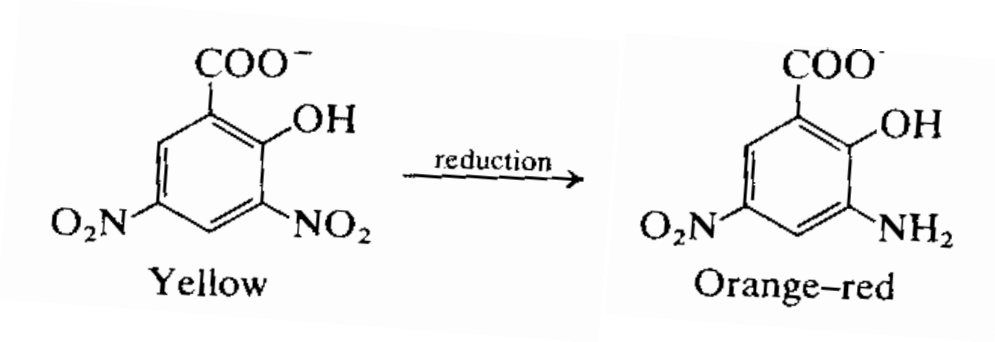
[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 4 **Date:**

AIM: Estimate the concentrations of reducing sugar present in given sample by DNSA method.

PRINCIPLE:

Under alkaline condition reducing sugar is converted to furfural, which reduces one of the nitro group (-NO_2) of Dinitro Salicylic Acid (DNSA) to amino group (-NH_2) to produce orange-brown colored 3-amino-5-nitrosalicylic acid. The orange-brown colored compound can be measured spectrophotometrically at 540 nm. Since the chemistry of the reaction is complicated standard curves do not always go through the origin and different sugars give different color. The method is therefore not suitable for the determination of complex mixture of reducing sugars.



3, 5 Dinitrosalicylic acid **3-amino-5-nitrosalicylic acid**

REQUIREMENTS

Reagents:

1. **Standard Sugar solution** (maltose 1mg/ml): Dissolve 100mg of maltose and make up the final volume 100 ml with D/W.
2. **DNSA reagent:** Dissolve 10g DNSA powder in 250 ml of 2N sodium hydroxide. Allow it to cool. Add 300 g sodium potassium tartarate, and make final volume 1000ml with D/W. Stored in brown bottle.

Equipments: Balance, Water bath, Spectrophotometer,
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 100-1000 µg/ml
Working concentration: 1mg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with maltose (std) with 100, 200, 300 up to 1000 µg and test solution sequentially as per the protocol table.
3. Make up the final volume 1ml with D/W along with the blank tube.
4. Add 1ml DNSA reagent in all tubes. Mix all the reagents properly and incubate the mixture in BWB as mentioned in the protocol table.
5. After cooling under running tap water, add 8.0ml D/W in all tubes.
6. Measure the color complex spectrophotometrically at 540 nm.
7. Draw the standard curve of Concentration of maltose vs Optical Density (O.D.)

8. Put the O.D. of the unknown solution and calculate out the concentration of the maltose present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \textbf{100}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

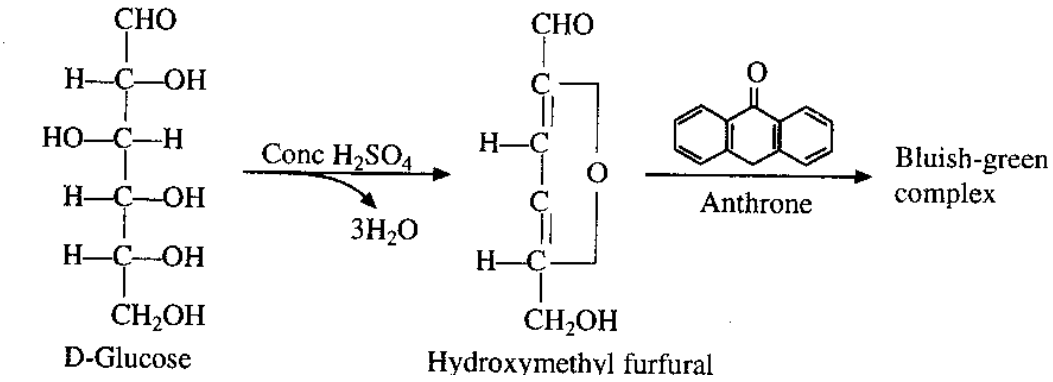
PRACTICAL - 5 **Date:**

AIM: Estimate the concentrations of sugar present in given sample by Anthrone method.

PRINCIPLE:

Carbohydrates dehydrated under strong acidic condition to produce furfural. Which finally condensed to anthrone and forms green-blue colored complex can be measured spectrophotometrically at 620 nm.

Reaction



REQUIREMENTS

Reagents:

3. **Standard Sugar solution** (Glucose 100µg/ml): Dissolve 10mg Glucose and make up the final volume 100ml with D/W.
4. **Anthrone reagent:** Dissolve 0.2g anthrone powder in 100ml concentrated H_2SO_4. Stored in brown bottle.

Equipments: Balance, Water bath, Spectrophotometer,
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 10-100 µg/ml
Working concentration: 100µg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with glucose (std) solution with 10, 20, 40 up to 100 µg and test solution sequentially as per the protocol table.
3. Make up the final volume 1ml with D/W along with the blank tubes
4. Add 4ml Anthrone reagent in all tubes with the help of burette only.
5. Mix all the reagents properly but with care and incubate the mixture in BWB as per the protocol table.
6. Allow to cool and measure the color complex spectrophotometrically at 620 nm.
7. Draw the standard curve for Concentration of glucose vs Optical Density (O.D.)
8. Put the O.D. of the unknown solution and calculate out the concentration of the Glucose present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Aliquot (test)}} \times \frac{100}{1000}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

ORIENTATION TO QUALITATIVE AND QUANTITATIVE ANALYSIS OF PROTEINS

INTRODUCTION

Amino acids are organic compounds that contain amino and carboxyl groups and therefore possess both basic and acidic properties. Along with the basic structure the 20 standard amino acids are classified chemically on the basis of their side chain designated as "R groups". Proteins are polymers of amino acids linked together with the peptide bond (-CO-NH) formed between the amino groups ($-NH_3^+$) of the second amino acid to the carboxyl group (COO^-) of the first amino acids.

Qualitative determination of amino acids and proteins

There are various qualitative tests for detection of amino acids and these are largely based on the nature of the R group. Some of these tests are general and are given by all the amino acids present in the proteins for example Ninhydrine test, whereas, others are specific for a particular amino acid and hence can be used for identification of a particular amino acid. Many of these tests give positive response irrespective of the fact whether the concerned amino acid exists in free form in a solution or happens to be constituent of a protein molecule. Hence, these tests can also be employed for the purpose of detection of proteins in a sample.

Depending on the concentration of the protein present in the given sample various quantitative methods can be used for protein estimation such as,

i) Biuret Method (1-20 mg/ml)
ii) Folin-Lowry Method (50-250 µg/ml)
iii) Bradford's Method (4-20 µg/ml)
iv) Eosin Y and Eosin B dye binding Method (2-2000 ng/ml)

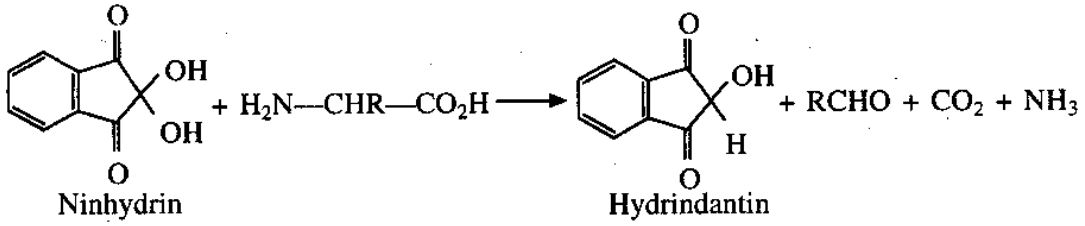
PRACTICAL - 6 **Date:**

AIM: Identify the amino acid present in the given sample by qualitative method

A. Ninhydrin Test (for amino acid)

PRINCIPLE:
This test is due to reaction between α-amino group of free amino acid and ninhydrin (triketohydrindene hydrate). Ninhydrin is a powerful oxidizing agent and in its presence, amino acids undergo oxidative deamination liberating ammonia, carbon dioxide, a corresponding aldehyde and reduced form of ninhydrin. The ammonia formed from α-amino group reacts with ninhydrin and its reduced product (hydrindantin) to give a blue substance diketohydrin (Ruhemann's purple). However, in case of imino acids like proline, a different product having a bright yellow colour is formed. Asparagine which has a free amide group reacts to give a brown coloured product. This test is also given by proteins and peptides.

Reaction



REQUIREMENTS
Reagents:
1. Test solution: prepared solution containing 50µg/ml of individual amino acids.
2. **Ninhydrin Reagent** : 0.2% Ninhydrin prepared in acetone

Equipments: Balance, Water bath
Glassware: Test tubes, beaker, glass pipette etc……

PROCEDURE: Add 2-5 drops of ninhydrin solution to 1ml of test solution or sample preparation. Mix and keep for 5 min in BWB.

OBSERVATION: Appearance of purple colour indicates the presence of amino acids. However proline and hydroxyproline give yellow colour.

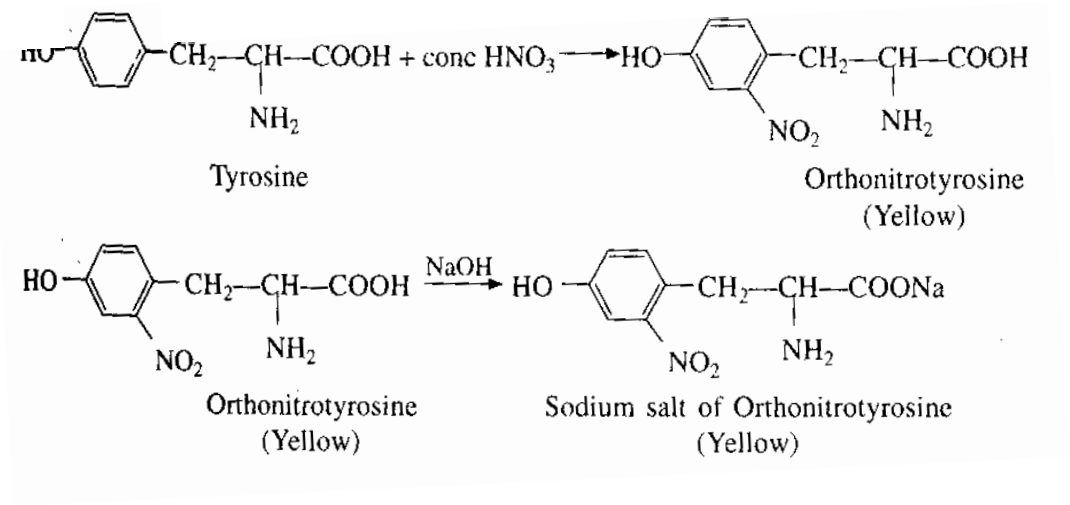
RESULT:

B. <u>Xanthoproteic Test</u> (for aromatic amino acid)

PRINCIPLE

Aromatic amino acids (tyrosine, tryptophan, and phenylalanine) form yellow nitro derivatives on heating with conc. HNO_3. The salt of these derivatives are orange in colour. Proteins containing these amino acids also give a positive response of this test.

Reaction:



REQUIREMENTS:

Reagents:

1. conc. HNO_3.
2. NaOH solution (40%, w/v): Dissolve 40gm of NaOH in water and make the final volume to 100ml.
3. Test solution: prepare separate solutions containing 50µg/ml of amino acids such as tyrosine, glycine, tryptophan, phenylalanine, lysine, etc.

Equipments: Balance, Water bath

Glassware: Test tubes, beaker, glass pipette etc……

PROCEDURE:

1. Add 1ml conc. HNO_3 into 1 ml of test solution.
2. Mix the contents and keep in BWB for 1 min. and allow to cool.
3. Slowly pipette 40% NaOH till the solution becomes alkaline.
4. Note down the change in the color.

OBSERVATION: Development of orange red colour indicates positive test for aromatic amino acids.

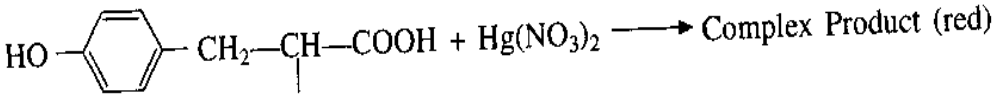
RESULT:

C. Millon's Test (for tyrosine)

PRINCIPLE:
Amino acids are compounds containing hydroxybenzene radical. It react with Millon's reagent which constitute of mercurous and mercuric nitrates containing HNO_3 to form a red complex thus this test is specific for tyrosine.

Reaction:

$$Hg + 4HNO_3 \rightarrow Hg(NO_3)_2 + 2NO_2 + 2H_2O$$



Tyrosine

RQUIREMENTS
Reagents:

1. **Millon's reagent**: Dissolve 10g of mercury (Hg) metal in 20ml of concentrated HNO_3, dilute to 100 ml with water after cessation of evolution of the brown fumes.
2. Test solution of different amino acids made as in the preceding test. Use a solution of phenol also as a test sample.

Equipments: Balance, Water bath
Glassware: Test tubes, beaker, glass pipette etc……

PROCEDURE:

1. Add 2-5 drops of Millon's reagent into 1ml of test solution.
2. Mix well and place the test tubes in boiling water bath for 5 min.

The same experiment may be carried out with a modified Millon's reagent which contains 10% mercuric sulphate in 10%H_2SO_4. To 1ml of test solution, add 1 ml of modified millon's reagent and boil gently for 1min. cool under running tap water and then add one or two drops of 1% $NaNO_2$ solution and heat slightly.

OBSERVATION: Appearance of red colour denotes presence of tyrosine. The advantage of using modified Millon's test lies in its being less susceptible to interference from inorganic salts.

RESULT:

CONCLUSION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 7 **Date:**

AIM: Estimate the concentration of amino acids in given sample by Ninhydrin method

PRINCIPLE

Ninhydrin is a powerful oxidizing agent and in its presence, amino acids undergo oxidative deamination liberating ammonia, carbon dioxide, a corresponding aldehyde and reduced form of ninhydrin in between the pH 4-8. . The ammonia formed from α-amino group reacts with ninhydrin and its reduced product (hydrindantin) to give a blue substance diketohydrin (Ruhemann's purple). However, in case of imino acids like proline, a different product having a bright yellow colour is formed. Asparagine which has a free amide group reacts to give a brown coloured product. This test is also given by proteins and peptides.

REQUIREMENTS

Reagents:

1. **Standard amino acid solution:** (Leucine/ aspartic acids/ arginine/ proline: 0.1 mmol/l): Prepare 0.1mM amino acid solution by dissolving gram molecular weight into liter of D/W.
2. **Ninhydrin reagent:** Dissolve 0.8g of ninhyrine in 25ml acetone and add 25 ml of 0.2 M acetate buffer (pH 5.5). Store in brown bottle to protect from light.
3. **0.2 M acetate buffer (pH 5.5):** Prepare 0.2M Sodium acetate and make the pH 5.5 with glacial acetic acid. Make the final volume 1 liter with D/W.
4. 70% ethanol
5. Test sample

Equipments: Balance, pH meter, water bath, spectrophotometer
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 10-100 µM
Working concentration: 0.1 mmol/l

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with amino acids (std) solution with 10, 20, 30 up to 100 µM and test solution sequentially as per the protocol table.
3. Make up the final volume 4ml with D/W along with the blank tubes
4. Add 1ml Ninhydrine reagent in all tubes. Mix all the reagents properly and incubate the mixture in BWB as per the protocol table.
5. After cooling add 1ml of 70% ethanol in all tubes.
6. Mix it properly and measure the color complex spectrophotometrically at 550nm.
7. Draw the standard curve for Concentration of amino acids vs Optical Density (O.D.)
8. Put the O.D. of the unknown solution and calculate out the concentration of the amino acids present in given sample from standard graph and formula (given below) and express your result in µM of amino acids present in 100ml.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \textbf{100}$$

RESULT:

CONCLUSION/INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

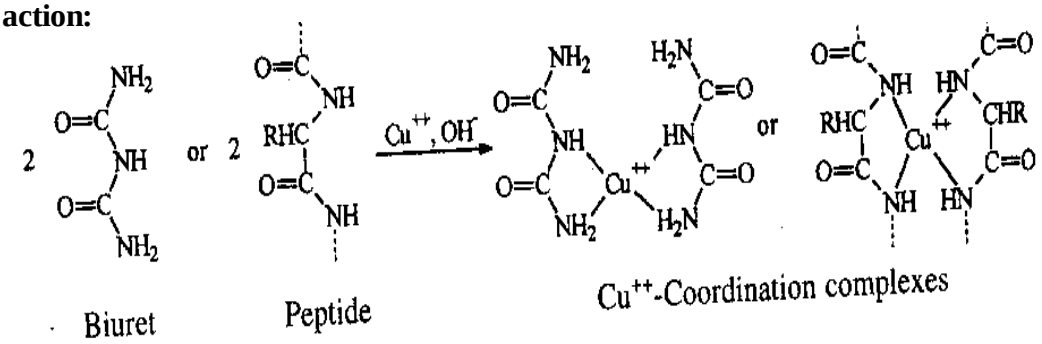
PRACTICAL - 8 **Date:**

AIM: Estimate the concentration of protein in given sample by Biuret method

PRINCIPLE

This is the most commonly used method for the estimation of proteins. Under alkaline conditions copper ions present in Biuret reagent interact with –CO-NH- groups present in adjacent di-peptide and form a purple colored complex. This can be measure spectrophotometrically at 540 nm. The method is fairly specific and there is little interference with other compound containing -CO-NH- groups like urea and other like reducing sugar can interact with cupric ions.

Reaction:



REQUIREMENTS

Reagents:

1. **Standard Protein solution** (Bovine Serum Albumin (BSA) 5mg/ml): Dissolve 500mg of BSA and make up the final volume 100 ml with D/W.
2. **Biuret reagent:** Dissolve 3g of $CuSO_4$ and 9gm sodium potassium tartarte in 500ml of 0.2 M NaOH. Add 5gm of KI and make up the final volume one liter with 0.2 M NaOH.
3. **D/W**
4. Test sample

Equipments: Balance, water bath, spectrophotometer
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 2-20 mg
Working concentration: 5mg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with protein (std) solution with 1, 2, 3 up to 5mg and test solution sequentially as per the protocol table.
3. Make up the final volume 2ml with D/W along with the blank tubes
4. Add 3ml Biuret reagent in all tubes. Mix all the reagents properly and incubate the mixture as per the protocol table.
5. Measure the color complex spectrophotometrically at 520 nm.
6. Draw the standard curve of Concentration of BSA vs Optical Density (O.D.)

7. Put the O.D. of the unknown solution and calculate out the concentration of the protein present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \textbf{100}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 9 **Date:**

AIM: Estimate the concentration of protein in given sample by Folin-Lowry's method

PRINCIPLE:

The aromatic amino acids (tyrosine and tryptophan) present in proteins react with phosphomolybdic acid and tungstate present in Folin – Ciocalteau reagent and form a blue colored complex which can be measure spectrophotometrically at 660 nm. This method is 10 times more sensitive than Biuret method.

REQUIREMENTS

Reagents:

1. **Standard protein solution** (Bovine Serum Albumin (BSA) 250µg/ml): Prepare stock solution (1mg/ml) and dilute with D/W to make final concentration 250µg/ml.
2. **Folin–Ciocalteau reagent:** 2N Folin–Ciocalteau reagent is available commercially. Dilute it to 1:1 proportion for use. Prepare fresh and keep in brown bottle.
3. **Reagent A:** 2% Sodium carbonate in 0.1N NaOH
4. **Reagent B:** 0.5% $CuSO_4$ and 1% Sodium potassium tartarate in 100ml D/W.
5. **Reagent C:** Mix 50ml Reagent A with 1ml Reagent B. Prepare always fresh.
6. **D/W.**
7. Test sample

Equipments: Balance, spectrophotometer

Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 50-250 µg.

Working concentration: 250µg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with protein (std) solution with 50, 100, 150 up to 250µg and test solution sequentially as per the protocol table.
3. Make up the final volume 4ml with D/W along with the blank tubes
4. Add 5.5ml alkaline reagent (Reagent C) in all tubes. Mix all the reagents properly and incubate the mixture as per the protocol table.
5. Add 0.2 ml Folin–Ciocalteau reagent in all tubes. Mix well and incubate the mixture as per the protocol table
6. Measure the color complex spectrophotometrically at 660 nm.
7. Draw the standard curve for Concentration of BSA vs Optical Density (O.D.)
8. Put the O.D. of the unknown solution and calculate out the concentration of the protein present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Aliquot (test)}} \times \frac{100}{1000}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

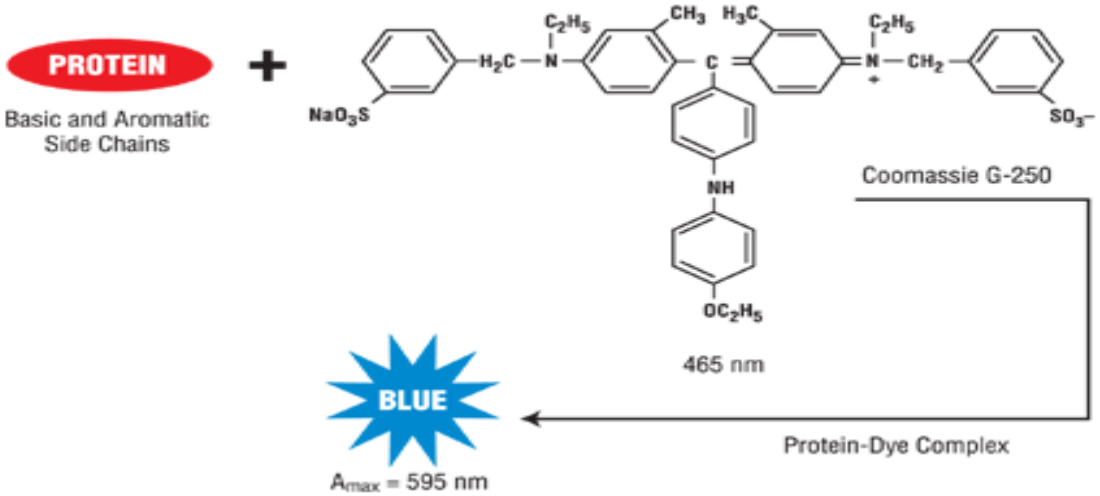
PRACTICAL –10 **Date:**

AIM: Estimate the concentration of protein in given sample by Bradford's method.

PRINCIPLE :

This mechanism is based on the protein-dye binding method. The binding of Commassie Brilliant Blue (CBB)G-250 to protein in acidic conditions shift the λmax of dye from 465nm to 595 nm. Absorption of the blue colored protein-dye complex at 595nm is directly related to concentration of protein present in the sample. The method is more sensitive than the Biuret and Folin-Lowry's method. But, the formed colored complex is unstable with time interval.

Reaction



REQUIREMENTS

Reagents:

1. **Standard Protein solution** (BSA) 20µg/ml: Prepare stock solution (1mg/ml) and dilute with D/W to make concentration 20µg/ml.
2. **Bradford reagent:** Dissolve 100mg of CBB-G250 in 50ml of ethanol. Cover it tightly and incubate for an hour. Slowly add 100ml of 85% Ortho-Phosphoric acid and make the volume to 1 liter with D/W. Filter the reagent through Whatman filter paper no.1 and Store in brown bottle. Reagent is stable only for 2-3 weeks.
3. **0.2M Phosphate Buffer (pH 7.5):** Prepare 0.2M monobasic sodium phosphate and 0.2 M dibasic sodium phosphate and mix until the pH becomes 7.5.

Equipments: Balance, pH meter, Spectrophotometer

Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 4-20µg

Working concentration: 20µg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with protein (std) solution with 4, 6, 8 upto 20µg and test solution sequentially as per the protocol table.

3. Make up the final volume 2.0 ml with phosphate buffer and add 2ml Bradford reagent in each tube.
4. Mix all the reagents properly and incubate the mixture as per the protocol table.
5. Measure the color complex spectrophotometrically at 595nm.
6. Draw the standard curve for Concentration of BSA vs Optical Density (O.D.)
7. Put the O.D. of the unknown solution and calculate out the concentration of the protein present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \frac{\textbf{100}}{\textbf{1000}}$$

RESULTS:

CONCLUSION / INTERPRETATION:

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[Signature of Student] **[Signature of Instructor]**

PRACTICAL -11 **Date:**

AIM: Estimate the amylase activity in the given sample by calculating units (U) of enzyme.

PRINCIPLE:

Amylases hydrolyse starch and glycogen into sugars. There are two types of amylases: α-amylase and β-amylase. α-amylase is an endohydrolase which hydrolyses α-1,4-linkage within the chain and breaks these polymers into small fragments. In contrast β-amylase is an exohydrolase and it starts acting from non-reducing end removing two glucose molecules at a time. The hydrolytic action of β-amylase results in increasing reducing power of the starch solution due to the release of a large number of molecules of the reducing sugar (maltose).The reducing sugars can be determined quantitatively by their reaction with DNSA reagent which undergoes reduction to a red-brown coloured product with absorption maxima at 540nm.

REQUIREMENTS:

Material and Reagents:

1. **Experimental material**: use barley seeds which have been kept for germination for 2-3 days at 25^{o}C.
2. **DNSA Reagent**
3. **Sodium Potassium Tartarate (40%):** Take 40g of sodium potassium tartarate and dissolve it in small amount of water and make the final volume to 100 ml with D/W.
4. **Substrate (1% Starch, w/v):** Weigh 1g of starch and add 100ml of boiling water to it. Continue boiling for 2-3 min. Let the solution cool down and then filter it. Use the filtrate as the substrate solution.
5. **Sodium phosphate buffer (0.05M, pH 7.0)** containing 0.5M NaCl.
6. **Standard maltose solution (**1 mg/ml**):** Dissolve 100 mg of maltose in 100ml of D/W.

Equipments: Balance, water bath, spectrophotometer.
Glassware: test tubes, beakers, measuring cylinders, glass pipettes etc.

a) Preparation of Standard curve for maltose

1. Take different aliquot of standard maltose solution in range of 0.2-1mg/ml.
2. Make up final volume to 1ml with D/W.
3. Add 1ml DNSA reagent in each tube and heat all the tubes on boiling water bath for 10 min.
4. Allow to cool mixture under running tape water and add 8ml of D/W to each tube.
5. Measure the absorbance at 540nm against reagent blank.
6. Plot a standard curve of A_{540} Vs Concentration of maltose (mg/ml).

Protocol Table:

Volume of maltose sol. (ml)	Conc. of maltose sol. (mg/ml)	Volume of water added (ml)	Total volume (ml)	Absorbance (540nm)
0.2		0.8	1.0	
0.4		0.6	1.0	
0.6		0.4	1.0	
0.8		0.2	1.0	
1.0		-	1.0	
-		1.0 (blank)	1.0	

b) Determination of Enzyme activity

PROCEDURE:

1. Extraction of the enzyme should be done in cold at 0-4^{0}C. Macerate 500 mg of washed germinating seeds in a chilled pestle and mortar with 5ml of 0.05 M phosphate buffer (pH.7.0) containing 0.5 M NaCl.
2. Centrifuge the homogenate at 10,000 X g for 15 min in a refrigerated centrifuge at 4^{0}C. Decant the supernatant and use it as the enzyme preparation.
3. Pipette 1 ml of starch solution and 1 ml of an appropriately diluted enzyme preparation into two tubes (control and experimental).
4. In one of the tubes, which serve as a zero min control, terminate the reaction immediately by adding 2ml of dinitrosalicylic acid (DNSA) reagent.
5. Incubate the other test tube at 37^{0}C for 15 min and then stop the reaction by adding 2 ml dinitrosalicylic acid reagent.
6. Keep both the tubes in a boiling water bath for 5 min and then add 1 ml of sodium potassium tartarate to each of the tubes.
7. Cool the tubes under running tap water. Make the volume to 10 ml with water.
8. Set the colorimeter to zero absorbance at 540nm with the zero min. control and then record the absorbance of the second tube.

Protocol Table:

Test tube	**Enzyme (ml)**	**Substrate (ml)**	**Buffer (ml)**
Enzyme Blank	-	1.0	1.0
Substrate Blank	1.0	-	1.0
Test	1.0	1.0	-

CALCULATIONS:

$$\textbf{Unit (U)} = \frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \frac{\textbf{1}}{\textbf{Time}}$$

= _____mg maltose /ml/min

= _____μ moles of maltose /ml/min

RESULTS:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL -12 **Date:**

AIM: Investigate the effect of temperature on amylase activity and find out the temperature optima for amylase.

PRINCIPLE: All the enzymes have narrow temperature range for their efficient functioning. To determine enzyme activity in the reaction mixture, assay should be carried out at optimum temperature, where enzyme and substrate binding are more feasible compare to rest of the temperature.

REQUIREMENTS: Same as in the Practical 11

PROCEDURE:

1. Carried out the similar steps 1-4 as mentioned in the Practical 11
2. Incubate the different reaction mixture tubes at different temperature such as, 20^0C, 25^0C, 30^0C, 37^0C and 45^0C for 15 min.
3. Stop the reaction by adding 2 ml DNSA reagent.
4. After that the process is remain the same as mentioned in the Practical 11.
5. Plot graph with Enzyme activity vs temperature and determine temperature optima.

RESULT:

CONCLUSION / INTERPRETATION:

ORIENTATION TO ANALYSIS OF LIPID

INTRODUCTION:

Lipids are heterogeneous group of compound with non-plolar nature and soluble in non-polar solvents such as, hexane, benzene, chloroform is utilized for extracting lipids from various tissues. In biological materials the lipids are generally bound to proteins and they are therefore extracted either with a mixture of ethanol and diethyl ether or a mixture of ethanol and methanol, which helps in breaking the bonds between the lipids and proteins.

They are broadly classified into simple, compound and derived lipids. Where, so many side groups along with saturated or unsaturated fatty acids chain are attached to it such as, phosphorus, sulfur, nitrogen etc. The existence of side groups and specific solubility of the lipid is mainly used for the quantitative estimation of lipids.

PRACTICAL -13 **Date:**

AIM: **Extraction and Gravimetric estimation of lipid from ground nut by Soxhlet's method.**

PRINCIPLE:

Lipids are soluble in some organic solvents. This property of specific solubility in non-polar solvents such as hexane, benzene, chloroform is utilized for extracting lipids from various tissues. In biological materials the lipids are generally bound to proteins and they are therefore extracted either with a mixture of ethanol and diethyl ether or a mixture of ethanol and methanol, which helps in breaking the bonds between the lipids and proteins.

REQUIREMENTS

Reagents:

1. **Ground nut powder:** Crush the ground nut homogenously to make fine powders (5gm)
2. **Non polar solvents**: Hexane, Chloroform, Methanol and benzene
3. **Thimble Preparation:** Pack 5gm of fine crushed ground nut powder in filter paper and tight it with the strings. Put the thimble in extractor of Soxhlet apparatus.

Equipments: Balance, Mortar and Pestle, Soxhlet apparatus

PROCEDURE:

1. Perform the soxhlet extraction till all the oil has been extracted (Color of the RBF solvent turn to yellowish black or greenish black).
2. Distill the solvent by removing the thimble and repeat the round of soxhlet extraction.

CALCULATION:

A = Weight of RBF (Preweight)

B = Weight of Dry RBF + Oil contain

So weight of sample B-A = _____.

$$\% \text{ of oil in Ground nut} = 100 \times \frac{\text{Weight of substance}}{\text{g of substance in thimble}}$$

RESULTS:

CONCLUSION / INTERPRETATION

[Signature of Student] **[Signature of Instructor]**

PRACTICAL – 14 **Date:**

AIM: Estimate the lipid present in given sample by Sulfo-phosphovanilin method.

PRINCIPLE:

Lipid reacts with vanillin in presence of concentrated H_2SO_4 and phosphoric acid to form pink colored complex which can be measure spectrophotometrically at 456nm.

REQUIREMENTS

Reagents:

1. **Standard lipid solution** (100µg/ml): Dissolve 1gm ground nut oil in 100 ml of chloroform
2. **Color reagent:** Dissolve 2.1gm Vanillin in 350ml D/W. and slowly add 600ml O-phosphoric acid with continuous stirring. Make the final volume 1 liter with D/W.
3. Concentrated H_2SO_4.
4. **Unknown solution:** Prepare homogenous mixture of 5gm fine crushed ground nut powder with 20ml of hexane, crush it well and add hexane if needed. Filter the mixture (till the clear solution is obtained) and make up the final volume to 100 ml with hexane. Use this solution for the estimation of lipid.

Equipments: Balance, Mortar and Pestle, water bath Spectrophotometer
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 20-100 µg.
Working concentration: 100µg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with lipid (std) solution with 20, 40, 60 up to 100µg and test solution sequentially as per the protocol table.
3. Incubate all the tubes in BWB for the complete evaporation of solvent.
4. Carefully add 2ml concentrated H_2SO_4 with the help of burette in all the tubes mix well and again incubate in BWB as per the protocol table.
5. After cooling measure the color complex spectrophotometrically at 456nm.
6. Draw the standard curve for Concentration of lipid vs Optical Density (O.D.)
7. Put the O.D. of the unknown solution and calculate out the concentration of the lipid present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\text{OD (test)}}{\text{OD (std)}} \times \frac{\text{Conc (std)}}{\text{Aliquot (test)}} \times \frac{100}{1000}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL- 15 **Date:**

AIM: Estimate concentration of inorganic phosphorus present in the given sample by Fiske & Subbarrow's method.

PRINCIPLE:

Digestion of the sample containing organic phosphorus with perchloric acid liberates inorganic phosphorus. The released inorganic phosphorus in the sample under the treatment with Amino Naptho Sulphonic Acid (ANSA) reacts with molybdate to form a phosphomolybdate and develops blue colored product, which is measured spectrophotometrically at 640nm.

REQUIREMENTS

Reagents:

1. **Standard inorganic phosphorus (Pi) solution (KH_2PO_4):** Dissolve 439mg KH_2PO_4 in 10N H_2SO_4 and make the final volume to 1liter with D/W. Solution contain 0.439mg KH_2PO_4/ml. so stock concentration is 0.1mg/ml)

   31 gm Pi is present in the 136gm (MW) KH_2PO_4
   31 mg Pi is present in the 136mg KH_2PO_4
   So, 100mg Pi is present in the 438.7mg KH_2PO_4)
2. **Ammonium Molybdate Reagent**: Dissolve 25gm of ammonium molybdate in 400 ml of DD/W. Add slowly 500ml of 10N H_2SO_4 and make up the volume to 1liter with DD/W.
3. **ANSA Reagent:** Prepare triturate powder in mortar pestle by mixing 200mg ANSA, 1.2gm Sodium sulphate and 1.2gm Sodium bisulphate. Dissolve 400mg of above mixture in 10ml D/W for preparation of ANSA Reagent.
4. **Preparation of unknown sample:**
   1 gm chicken liver + 5ml H_2SO_4 → Digest it in microkjeldhal tube by heating for 5-6 hours, at the time of heating add perchloric acid 3-5 drops up to the point when the clear solution is obtained and neutralized by $NaHCO_3$. Than make up the final volume 100 ml with D/W. Use this as a unknown sample.

Equipments: Balance, spectrophotometer
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 10-100 µg.
Working concentration: 100µg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with Pi (std) solution with 10, 20, 30 up to 100 µg and test solution sequentially as per the protocol table.
3. Make up the final volume 3ml with D/DW along with the blank tubes
4. Add 0.4ml molybdate reagent and 0.2 ml ANSA in all tubes.
5. Mix all the reagents properly and incubate the mixture as per the protocol table.
6. Measure the color complex spectrophotometrically at 640 nm.
7. Draw the standard curve for Concentration of Pi vs Optical Density (O.D.)

8. Put the O.D. of the unknown solution and calculate out the concentration of the Pi present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \frac{\textbf{100}}{\textbf{1000}}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

ORIENTATION TO ANALYSIS OF NUCLEIC ACIDS

INTRODUCTION

It will be redundant to mention that Deoxyribonucleic acid (DNA) is the genetic material of the cell, with few exception where Ribonucleic acid RNA (viruses) and Priones (Proteins). Chemically nucleic acids are polymers of nucleotides joined together with phosphodiester bonds. Nucleotides are phosphorylated form of nitrogen base such as Purine (Adenine, Guanine) and pyrimidine (Cytosine, Thiamine and Uracil) attached to the pentose sugars (Ribose or deoxyribose). Reaction of pentose sugars under strong acidic condition leads to dehyradation of the ring structure, which is one of the properties used for the estimation of the nucleic acids.

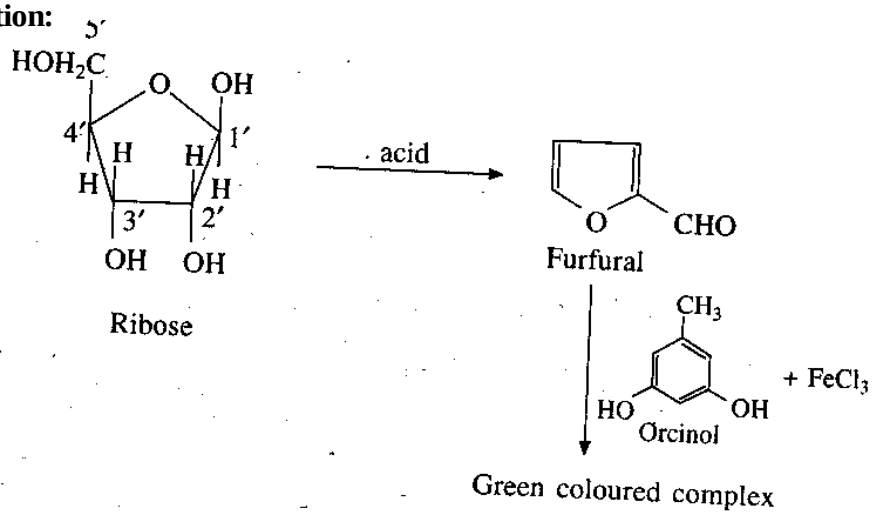
PRACTICAL - 16 **Date:**

AIM: Estimate the concentration of RNA present in given sample by orcinol method.

PRINCIPLE

The pentose (ribose) in the presence of concentrated acid converted to furfural, which then reacts with orcinol in presence of ferric chloride ($FeCl_3$) to yield a green color complex which can be measure spectrophotometrically at 660nm. Formation of color complex largely depends on the concentration of HCl, and Orcinol. Only purine nucleotides give specific color reaction.

Reaction:



REQUIREMENTS

Reagents:

1. **Standard RNA solution:** (100µg/ml): Prepare stock solution of RNA in buffer saline and dilute with buffer saline to make final concentration 100µg/ml
2. **Orcinol reagent:**
   Solution A: Dissolve 300mg orcinol in 5ml ethanol
   Solution B: 0.1 % $FeCl_3$: Dissolve 100mg of FeCl3 in 100ml of Concentrated H_2SO_4
   Orcinol reagent: Take 3.5 ml of solution A and add to the 100ml solution B just prior to use.
3. **Buffer Saline:** Dissolve 0.15M NaCl (2.2gm) in 0.015M Sodium Citrate (1.1gm) (pH 7.0) in 250 ml D/W.
4. Test solution

Equipments: Balance, Water bath, Spectrophotometer
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 20-200 µg.
Working concentration: 100µg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.

2. Take all the reagents along with RNA (std) solution with 20, 40, 60 up to 200µg and test sequentially as per the protocol table.
3. Make the final volume 2ml with buffer saline in all tubes along with blank.
4. Add 3ml of orcinol reagent with care in all the tubes.
5. Mix all the reagents properly and incubate the mixture in BWB as per the protocol table.
6. Measure the color complex spectrophotometrically at 660nm.
7. Draw the standard curve for Concentration of RNA vs Optical Density (O.D.)
8. Put the O.D. of the unknown solution and calculate out the concentration of the RNA present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \frac{\textbf{100}}{\textbf{1000}}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

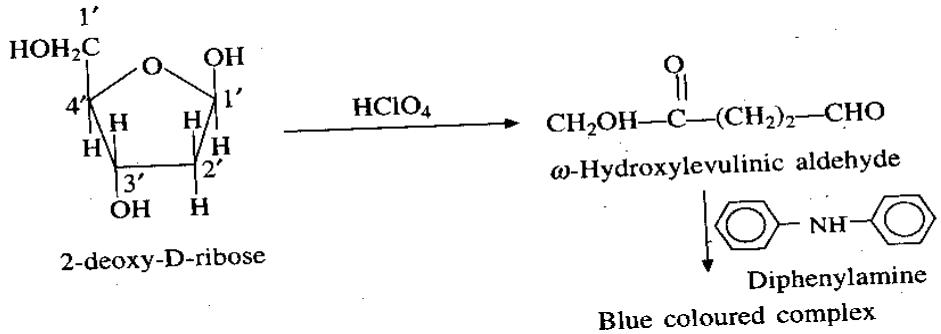
PRACTICAL - 17 **Date:**

AIM: Estimate the concentration of DNA present in given sample by Diphenylamine (DPA) method.

PRINCIPLE:

Under extreme acid conditions deoxypentose of DNA is initially converted to β-hydroxylevulinyaldehyde, which reacts with Diphenylamine (DPA) to produce deep blue color compound with the absorption maximum at 595nm. In DNA only deoxyribose purine nucleotides reacts, so the value obtained represents half of the total deoxyribose present.

Reaction:



REQUIREMENTS

Reagents:

1. **Standard DNA solution:** (100µg/ml): Prepare stock solution of DNA in buffer saline and dilute with buffer saline to make final concentration 100µg/ml
2. **DPA reagent:** Dissolve 10gm DPA in 1 liter glacial acetic acid and add 25ml concentrated H_2SO_4. Always prepare fresh.
3. **Buffer Saline:** Dissolve 0.15M NaCl (2.2gm) in 0.015M Sodium Citrate (1.1gm) (pH 7.0) in 250 ml D/W.
4. Test solution

Equipments: Balance, Water bath, Spectrophotometer
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 100-200 µg.
Working concentration: 100µg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with DNA (std) solution with 100, 120, 140 up to 200µg and test solution sequentially as per the protocol table.
3. Make the final volume 3ml with buffer saline in all tubes along with blank.
4. Add 3ml of DPA reagent with care in all the tubes.
5. Mix all the reagents properly and incubate the mixture in BWB as per the protocol table.
6. Measure the color complex spectrophotometrically at 595nm.

7. Draw the standard curve for Concentration of DNA vs Optical Density (O.D.)
8. Put the O.D. of the unknown solution and calculate out the concentration of the DNA present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \frac{\textbf{100}}{\textbf{1000}}$$

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

ORIENTATION TO CHROMATOGRAPHY

INTRODUCTION

The term "chromatography" was coined by a Russian botanist Tswett in 1906. It includes all those techniques in which various components in a mixture get separated during their passage through a porous media due to the differences in their migration rates. In chromatography, the sample is applied at once end of a porous support which hold the stationary phase which may be a solid or a liquid and the mobile phase a liquid or a gas sample is then made to flow over it. The various constituents in a simple get separated essentially due to differences in their partition and distribution behaviour between the stationary phase and the mobile phase. The partition or distribution coefficient (K_d), is defined as the ratio of concentration of compound in two phase (A & B) at equilibrium.

$$K_d = \frac{\text{Concentration of the compound in solvent A}}{\text{Concentration of the compound in solvent B}}$$

Different compound vary in their distribution coefficients in a given biphasic system and this constitutes the underlying principle for their separation in the different type of chromatographic techniques. In simple terms, movement of those substances in the sample which have greater affinity for stationary phase is related since they tend to spend more time in this phase. On the other hand, the compound with relatively higher affinity for mobile phase travel at a much faster rate as there are carried along with the flowing mobile phase. This eventually results in the separation of various components in the mixture.

CLASSIFICATION OF CHROMATOGRAPHIC TECHNIQUES

Chromatographic techniques may broadly be classified on the basis of nature of support used for holding the stationary phase, these include:

1. Paper chromatography: in this system a filter paper sheet is used as a support for stationary phase.
2. Thin layer chromatography (TLC): in this technique a glass plate, plastic sheet or a piece of metal foil serves as a support for the stationary phase which is applied in the form of a thin layer on these materials.
3. Column chromatography: here stationary phase is packed into a tubular glass, polypropylene or metal columns.

PRACTICAL - 18 **Date:**

AIM: Separation and identification of amino acids by ascending paper chromatography.

PRINCIPLE:

Amino acids in a given mixture are separated on the basis of differences in their solubilities and hence differential partitioning coefficients in a binary solvent system. The amino acid with higher solubilities in stationary phase moves slowly compared to those with higher solubilities in the mobile phase. The separated amino acids are detected by spraying the air dried chromatogram with ninhydrin reagent. All amino acids give purple or bluish purple color on reaction with ninhydrin except proline and hydroxyproline which give a yellow colored product.

REQUIREMENTS:

Reagents:

1. **Developing solvent:** Take butanol, acetic acid and water in the ratio of 4:1:5 in a separating funnel and mix it thoroughly. Allow the phase to separate out completely. Used the lower aqueous phase for saturating the chamber. The upper organic phase is used as mobile phase.
2. **Ninhydrin reagent**: prepare fresh by dissolving 0.2g ninhydrin in 100 ml acetone.
3. **Amino acids solutions:** prepare solutions of authentic samples of amino acids such as glycine, proline etc.(1mg/ml of amino acid in 10% iso-propanol)
4. Test sample containing amino acid.

Material and Equipments:

1. Whatman no.1 filter paper sheet.
2. Micropipette/ micro syringe/Capillaries
3. Hair drier.
4. Sprayer
5. Oven set at 105^0 C.
6. Chromatographic chamber saturated with vapours

PROCEDURE:

1. Take What man no.1 filter paper and lay it on a rough filter paper
2. Fold the Whatman no1 filter paper about 2.0-2.5 cm from one edge reverse the paper and again fold it 2cm further down from the first fold.
3. Draw a line across the filter paper with a lead pencil at a distance of about 2 cm from the second fold. Put circular marks along this line as a distance of 2.5 cm from each other.
4. With the help of a micropipette or micro syringe apply 20µl of solution of each standard amino acid on separate mark. Also apply a spot of the sample or mixture to be analyzed, preferably on the mark at centre of this base line. The size of the spot should be as small as possible so that the developed spots are compact and do not overlap. If necessary, the wet sample spot should be dried with hair drier before applying additional aliquot.

5. Pour sufficient volume of the mobile phase into the chromatographic tank/chamber to saturate the tank
6. Gently place the filter paper upright in the chamber ensuring that it does not touch the sides of the chamber and at the same time taking care that the base line where the spot have been applied does not dip into the solvent.
7. Close the tank with an airtight lid or a glass plate to which sufficient amount of silicon grease has been applied.
8. Leave the set up undisturbed and allow the solvent move up till it reaches about 5 cm from the upper edge.
9. Remove the chromatogram from the chamber and mark the solvent front with lead pencil and let it dry at room temperature.
10. Spray the paper with ninhydrin reagent and let it dry again at room temperature prior to transferring it to an oven at 105^0 C for 5-10 minutes.
11. Blue or purple colored spot with appear on the paper. Mark the boundary of each spot with lead pencil.
12. Measure the distance between the centre of the spot and also the distance of the solvent front from the base line.
13. Calculate the R_f value of standard amino acids as well as in the given sample as follows :

$$R_F = \frac{\text{DISTANCE TRAVELLED BY UNKNOWN AMINO ACID}}{\text{DISTANCE TRAVELLED BY THE SOLVENT SYSTEM}}$$

14. Identify the amino acid in sample by comparing their R_f values with those of the reference standard.

OBSERVATIONS:

Distance traveled by the solvent front from base line	=	cm
Distance traveled by glycine from base line	=	cm
Distance traveled by proline from base line	=	cm
Distance traveled by spot no.1 in a sample from base line	=	cm

CALCULATIONS:

R_f value of glycine =
R_f value of proline =
R_f value of spot no.1 =

RESULT AND CONCLUSION:

The amino acid present in the given test sample is =

PRECAUTIONS:

1. Do not touch the paper with naked hands because sweat on hands contains significant amount of amino acid.
2. The spot of the applied sample should be as compact as possible. Larger the spot poorer will be resolution.

3. At the time of fixing paper in chromatography chamber, it should be ensured that the base line on which the sample has been applied does not dip in to the solvent otherwise the sample might get washed away in the solvent.
4. Solvent front should advance in a straight line. It should not be zig-zag or sloping but should be parallel to the base line.
5. Dry the paper thoroughly before spraying with the detection reagent. Wet paper may interfere with the appearance of evenly shaped compact spots.
6. The chromatography should be carried out in temperature controlled room because any fluctuation in the temperature would cause the uneven flow of the solvent and may alter the R_f value.

[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 19 **Date:**

AIM: **Separation and identification of sugars by adsorption thin layer chromatography (TLC).**

PRINCIPLE:

Sugars get separated on the basis of differential adsorption on silica gel. The sugars which have higher affinity for stationary phase are adsorbed more strongly and hence they migrate slowly when mobile phase moves over them. On the other hand, those having lower affinity for stationary phase are weakly adsorbed and are move easily carried by the mobile phase. The separated sugars are then located as colored zones by spraying TLC plates with aniline di phenyl amine reagent.

REQUIREMENT

Reagents:

1. **Solvent system**: prepare a mixture of ethyl acetate: iso-propanol: water: pyridine (26:14:7:2 v/v).
2. **Standard sugars solution**: prepare 1% solution of standard sugars such as glucose, maltose etc. in 10% iso-propanol (v/v).
3. A sample solution containing unknown sugar.
4. **Aniline diphenylamine reagent**: mix 5 volumes of 1% aniline and 5 volumes of 1% diphenylamine in acetone with 1 volume of 85% phosphoric acid.

Material and Equipments:

1. TLC chromatographic chamber
2. Glass plates (20 x 20 cm).
3. Spreader
4. Micropipettes / micro syringes
5. Oven set at 105^0 C.
6. Hair drier
7. Sprayer

PROCEDURE:

1. Place thoroughly cleaned and dried glass plates (20 x 20cm) on a flat plastic tray side by side with no gap between the two adjacent plates.
2. Prepare the slurry of the stationary phase (silica gel G) free of clumps in water or in an appropriate buffer.
3. Spread the uniform layer of 250 µm thickness with the help of a spreader or applicator by moving it from one end of the tray to its other end.
4. Activate the plates by keeping them at 105^0 C for 30 min. Allow the plates to cool in a desiccator before use.
5. Gently put marks in a straight line with the help of a pin at a distance of about 2 cm from of one edge of the plates. The adjacent marks should be about 1.5-2.0 cm apart from each other. Extreme care should be taken that silica does not get scratched off while putting these marks.
6. Carefully apply the solution of individual standard sugars and the mixture on the separated marks spots.
7. Gently put marks or draw a line 1 cm from the opposite edge.

8. Place the plate in chromatographic tank which has already been equilibrated with the solvent (mobile phase) taking care that base line on which samples have been applied does not dip into the solvent.
9. Close the chromatographic tank with airtight lid and allow the solvent to ascend along the plate by capillary action till the solvent reaches the mark line on the upper side of the plate. This may take about 90 min.
10. Remove the plate from the chromatographic tank and let it dry at room temperature.
11. For determining the location of sugars on the TLC plates, spray it with freshly prepared aniline-diphenylamine reagent ensuring that silica gel is not remove or blown off while spraying.
12. Place the plate in hot air oven at 100^0 C for 10 min. Appearance of bluish spot on the white background indicates presence of sugars at that region of the plate.
13. Measure the distance from the base line to the center of the colored spot and calculate the R_F value of each sugar.
14. Identify the sugars in the given mixture or sample by comparing their R_F values with those sugars standard.

OBSERVATIONS:

Distance traveled by the solvent front from base line	=	cm
Distance traveled by glucose from base line	=	cm
Distance traveled by maltose from base line	=	cm
Distance traveled by spot no.1 in a sample from base line	=	cm

CALCULATIONS:

R_f value of glucose =
R_f value of maltose =
R_f value of spot no.1 =

RESULT & CONCLUSION:

The sugar present in the given test sample is =

PRECAUTIONS:

1. Thoroughly cleaned glass plates free of any greasy spot or finger marks should be used.
2. Thickness of the layer should be uniform throughout the length of the plate.
3. The slurry of the chromatographic media should be free of any clumps. This can be ensured by vigorously shaking it in an Erlenmeyer flask or by gently preparing the slurry in pestle and mortar to ensure uniform mixing.
4. The TLC plates should be activated at recommended temperature and duration. Poor resolution of components occurs on over or under activated plates.
5. The layer of chromatographic media should not get scraped off at the time of putting marks of application of sample.
6. Size of the applied spot should be as small as possible. If large volume of the sample has to be spotted, then it should be done in small aliquots with an intermittent drying. Overloading of the sample should be avoided.
7. The chromatographic tank should be airtight and chromatography should be performed under temperature controlled conditions.

PRACTICAL - 20 **Date:**

AIM: Separation of pigments from leaves or flowers by ascending paper chromatography.

REQUIREMENT

Reagent:

1. **Solvent system** : benzene: methanol (2:1)
2. solid anhydrous $Na_2 SO_4$
3. **Spraying reagent**: 80 % acetone solution.

Material and Equipments:

1. Leaves or flower
2. What man no. 1 filter paper
3. Micropipette/ micro syringe.
4. Hair drier.
5. Sprayer
6. Oven set at 105^0 C.
7. Chromatographic chamber saturated with water vapours.
8. Pestle and mortar

PROCEDURE:

(a) Preparation of extract:

1. Homogenize the leaves or flower (5 g weight) in a pestle and mortar, using sand as an abrasive in 20 ml of benzene: methanol (2:1) adding a small amount of this extract at a time. Filter the extract through whatman no.1 filter paper and transfer the filterate to a separating funnel.
2. Add 10 ml of water to the filtrate and after shaking the contents and allowing the phase to separate out, drain out the lower aqueous methanol layer. Repeat this step. Avoid very vigorous shaking.
3. Collect the benzene layer in a beaker and add small amount of solid anhydrous $Na_2 SO_4$ to remove the traces of moisture.
4. Decent the clear benzene layer to another beaker and concentrate the extract by evaporating the solvent on a boiling water bath.

(b) Sample application:

Same as Experiment no. 18

OBSERVATIONS:

Distance traveled by the solvent front from base line	=	cm
Distance traveled by spot no.1 from base line	=	cm
Distance traveled by spot no.2 from base line	=	cm
Distance traveled by spot no.3 in a sample from base line	=	cm

Note: the change in the colors of the collected fractions. In case of the leaf extract, the initial fractions are colorless followed by yellow colored and then by the green colored ones. The colorless frictions do not contain any pigments but it is quite possible that these fractions may contain some UV absorbing

RESULT & CONCLUSION:

[Signature of Student] **[Signature of Instructor]**