

PRACTICAL MANUAL OF

SSAC-354
BIOCHEMISTRY

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PRACTICAL MANUAL

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EXERCISE NO. - 1

QUALITATIVE TESTS FOR CARBOHYDRATES

Carbohydrates are defined as "aldehyde or ketone derivatives of polyhydroxy alcohols". Hence each carbohydrate contains either aldehyde ($-\text{CHO}$) or ketone ($\text{C}=\text{O}$) group in its molecular structure. These functional groups may be free or involved in the formation of glycosidic linkages. Most of the chemical properties of the carbohydrates are mainly due to these groups. On the basis of number of sugar units involved in the structure, and their behavior on hydrolysis, carbohydrates, are classified as under:

1. **Monosaccharides** : Simple sugars that cannot be split further by hydrolysis
 - a) **Aldoses** : Monosaccharides containing $-\text{CHO}$ group e.g. glucose, galactose, mannose.
 - b) **Ketoses** : Monosaccharides containing $\text{C}=\text{O}$ group e.g. fructose.

All Monosaccharides are reducing sugars.

2. **Oligosaccharides** :
Sugars that yield two to ten molecules of Monosaccharides on hydrolysis.
 - A) **Disaccharides** : Sugars made up of two Monosaccharides, e.g. sucrose, lactose, maltose.
 - i) **Reducing sugar** : Sugars having free or potentially free $-\text{CHO}$ or $\text{C}=\text{O}$ groups, e.g. lactose, maltose
 - ii) **Non-reducing sugar** : Sugars having no free or potentially free $-\text{CHO}$ or $\text{C}=\text{O}$ groups e.g. sucrose
 - B) **Trisaccharides** : Sugars made up of three Monosaccharides e.g. Raffinose
 - C) **Tetrasaccharides** : Sugars made up to four monosaccharides e.g. stachyose
3. **Polysaccharides** : Carbohydrates made up of more than ten molecules of monosaccharides, e.g. starch, cellulose, glycogen.

The tests for detection of carbohydrates in the sample are usually based on the chemical reaction with functional groups. Following tests are carried out for qualitative detection of carbohydrates in the sample. The qualitative tests only indicate the presence of compound in the sample.

TEST 1: Anthrone reaction

- **Principle** : The polysaccharids and Oligosaccharides are hydrolyzed to simple sugars by conc. H_2SO_4 . All these simple sugars are further dehydrated and cyclized to furfural by conc. H_2SO_4 . The furfural so formed reacts with anthrone to produce blue colour complex.
- **Reagents** : **Anthrone solution** (0.2% in conc. H_2SO_4): Dissolve 0.2g anthrone in 100 ml conc. H_2SO_4 . Keep the solution in coloured bottle or in dark. Always prepare fresh anthrone reagents for test.
- **Procedure** : Add 5 drops of sample solution to 2 ml of anthrone reagent, mix, heat in water bath for 5 min. and cool under running tap water. Occurrence of blue colour indicates presence of carbohydrates.

TEST 2: Fehling's test

- **Principle** : The reducing sugars under alkaline conditions reduce cupric copper (blue) to cuprous oxide (brick red). The test is useful only for reducing sugars.
- **Reagents** :
 1. **Fehling solution A**: Dissolve 35 g of $CuSO_4 \cdot 5H_2O$ in water and make up to 500 ml.
 2. **Fehling solution B**: Dissolve 120 g of KOH and 173g Na-K-tartrate (Rochelle salt) in water and make up to 500 ml.
- **Procedure** : Mix equal volumes (2.5 ml each) of Fehling solution A and B. Add 5 ml of the test solution and boil for 5 min. Occurrence of brick red precipitate indicates the presence of reducing sugars.

TEST 3: Benedict's test

- **Principle** : Sugars reduce the cupric copper to green, yellow or red precipitate, depending on the concentration of the reducing sugars. Benedict's quantitative reagent (commercially available) contains potassium thiocyanate and potassium ferrocyanide. The reduced copper is, therefore, precipitated as white cuprous thiocyanate instead of the red cuprous oxide.
- **Reagents** : Benedict's solution: Dissolve 173 g of sodium citrate and 100 g of sodium carbonate in about 820 ml water. Filter and make up the volume to 850 ml. Dissolve separately 17.3 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in water and make up to 150 ml. Mix both solutions slowly with stirring.
- **Procedure** : Mix 5 ml of Benedict's solution with 5 ml of sample solution and boil in waterbath for 5 minutes. The ~~white~~ ^{greenish red} precipitate is formed indicates the presence of reducing sugars. .

blue-green-red

TEST 4 : Seliwanoff's Test

- **Principle** : This test is applied for ketose sugars. Ketoses are dehydrated more rapidly than Aldoses to give furfural derivatives, which then condense with resorcinol to form a red colour complex.
- **Reagents** : Seliwanoff's reagent: 0.05% resorcinol in 3N HCl.
- **Procedure** : Add 0.5 ml of sample solution to 2 ml of Seliwanoff's reagent and warm in boiling water bath for 1 minute, red colour occurs.

TEST 5: Iodine test

- **Principle** : Iodine forms coloured adsorption complexes with polysaccharides. Starch gives a blue colour with iodine, while glycogen and amylopectin form red-brown colour.
- **Reagents** : 1) **Iodine solution** : 0.005 N iodine in 3% potassium iodide.
2) 1% solutions of cellulose, glycogen, starch.
- **Procedure** : Acidify the test solution with dilute HCl, then add two drops of iodine and compare the colours obtained with that of water and iodine (Blue colour indication).

TEST 6 : Inversion test of sucrose

- **Principle** : Sucrose is a non-reducing sugar made up of glucose and fructose. The aldehyde group of glucose and ketone group of fructose are involved in the formation of glycosidic linkage. Upon acidification the glycosidic bond is broken and equal amount of glucose and fructose are liberated. The optical rotation is changed from $+66.5^\circ$ (sucrose) to $+52.5^\circ$ (glucose) and -92° (fructose). The result is change in rotation from dextro (+ve) to levo (-ve).
- **Procedure** : Add 5 drops of conc. HCl to 5 ml of sucrose solution. Heat for 5 minutes on a boiling water bath, cool and add carefully solid Na_2CO_3 to give a neutral or slightly alkaline solution. Perform the reduction test (Fehling's test) on the hydrolysed, solution. (Brick red colour is obtained).

Sr. No.	Test	1% solution of					
		Glucose	Fructose	Lactose	Sucrose	Starch	Hydrolysed starch
1.	Anthrone Test						
2.	Fehling's Test						
3.	Benedicts Test						
4.	Seliwanoff's test						
5.	Iodine Test						

Questions:

- 1) What is inversion?
- 2) What is Fehling's solution?
- 3) Name the common reducing sugars?
- 4) Name the common non-reducing sugars.
- 5) Why does starch form blue colour with iodine?
- 6) What are polysaccharides?
- 7) What are the storage polysaccharides?
- 8) What are the structural polysaccharides?

EXERCISE NO. 2

QUALITATIVE TESTS FOR DETECTION OF AMINO ACIDS AND PROTEINS

Proteins are the high molecular weight organic compounds having complex structures which upon hydrolysis yield either amino acids only or amino acid plus non-protein portion (prosthetic group). Amino acids are organic acids which contain in their molecular structure at last one – COOH group and one – NH₂ groups attached to the carbon chain. There are 20 different amino acids are found in most of the naturally occurring proteins.

TEST 1: The Ninhydrin (Triketohydrindene hydrate) reaction

- **Principle** : Ninhydrin reacts with all α - amino acids between pH 4 and 8 to give a purple coloured compound. The amino acids (proline and hydroxyproline) react with Ninhydrin to produce yellow colour.
- **Reagents** :
 - 1) **Amino acids**: 1% solution of glycine, proline, lysine..
 - 2) **Ninhydrin**: 0.2% in ethanol/methanol.
- **Procedure** : Place 1 ml amino acid solution in a test tube and adjust the pH to about neutrality, add 5 drops of Ninhydrin solution and boil for 2 minutes. Note the colour for each amino acids.

TEST 2: The Xanthoproteic test

- **Principle** : Amino acids (tryptophan, tyrosine, phenylalanine) which contain an aromatic ring and forms yellow nitro-derivatives on heating with conc. nitric acid.
- **Reagents** :
 - 1) 1% solutions of above amino acids.
 - 2) Conc. nitric acid
 - 3) 40% NaOH
- **Procedure** : Mix 1 ml each of nitric acid and amino acid solution, heat, cool and observe the colour. Add sufficient 40% NaOH to make the solution strongly alkaline. A yellow colour in acid solution which turns bright orange with alkali indicates a positive test.

TEST 3: The Nitroprusside test

- **Principle** : Thiol groups in amino acids react with sodium nitroprusside in the presence of excess ammonia to give a red colour.
- **Reagents** :
 - 1) 0.5% solutions of sulphur containing amino acids (methionine, cystine, cysteine).
 - 2) 2% freshly prepared sodium nitroprusside solution.
 - 3) Ammonium hydroxide.
- **Procedure** : Mix 0.5 ml of a fresh solution of sodium nitroprusside with 2 ml of the test solution and add 0.5 ml of ammonium hydroxide to form red colour.

TEST 4: The Biuret test

- **Principle** : Alkaline copper sulphate reacts with compounds containing two or more peptide bonds to give a violet blue coloured complex. It is not given by free amino acids.
- **Reagents** :
 - 1) 1% copper sulphate.
 - 2) 40% sodium hydroxide
 - 3) 0.5% solution of bovine serum albumin, egg albumin, casein.
- **Procedure** : Add 5 drops of copper sulphate solution to 2 ml of the test solution followed by 2 ml of NaOH, mix thoroughly and note the colour produced (violet).

TEST 5: Acid precipitation of proteins

- **Principle** : The positive charges on the protein are neutralized by acid to form an insoluble salt of protein.
- **Reagents** :
 - 1) 1% solution of protein (casein, albumin)
 - 2) Acid reagent (20% w/v Trichloroacetic acid)
- **Procedure** : Mix 5 ml of protein solution with 2 ml of Trichloroacetic acid and stir. Observe the precipitate.

TEST 6: Salting out test

- **Principle** : Salts at high concentration compete with proteins for solvent which results in rendering proteins insoluble and precipitate (white).
- **Reagents** :
 - 1) 2% solution of albumin
 - 2) Ammonium sulphate
- **Procedure** : To 10 ml of protein solution add increasing amounts of ammonium sulphate with stirring, till the proteins are precipitated.

Questions:

- 1) Define proteins.
- 2) Write the chemical reaction of biuret test.
- 3) What is denaturation of proteins?
- 4) Why do proteins get precipitated on addition of acid.

EXERCISE NO. 3

QUALITATIVE TESTS FOR LIPIDS/OIL

Lipids are characterized by their limited solubility in water considerable solubility in organic solvents (benzene, acetone, ether). It is a heterogeneous class of compounds and traditionally classified as simple lipids (oils, fats and waxes) and compound lipids (phospholipids, sphingolipids, sulfolipids, glycolipids, sterols, carotenoids and fat soluble vitamins).

Test 1 – Lipid's solubility

Principle: "Like dissolves like" A major portion of lipids being nonpolar, they are soluble only in nonpolar solvents. These solvents are also called "organic" or "fat solvents".

Reagents: (1) Vegetable oil samples

(2) Organic solvents : Acetone, Ether and Benzene..

Procedure: Mix 1 ml of oil in water and other solvents. Observe the solubility. Place 1 drop of solution from each group on a filter paper and leave it to dry. Observe the formation of a clear greasy spot in solutions containing oil after drying of filter paper..

Test 2 - Unsaturation test

Principle : The vegetable oils contain relatively higher proportion of unsaturated fatty acids. The unsaturated fatty acids contain one or more double bonds. Halogens such as iodine, chlorine or bromine can be easily added at these double bonds. This process is called "**Halogenation**". Halogenation results in the decolourization of a solution of bromine or iodine by a lipid which indicates the presence of double bond.

Reagents : 1. Vegetable oil

2. Bromine water.

Procedure: Slowly add bromine water to the test solution drop by drop, shaking after each addition, until the bromine just fails to be decolorized. Compare the ability of test compounds to decolorize the solutions of iodine and bromine.

QUESTIONS

1. What are lipids?
2. What is the difference between fats, oils and waxes?
3. What is halogenation?
4. Give examples of monoethenoid acids, diethenoid acid, triethenoid acid and tetraethenoid acids.
5. Enlist different fat solvents.

EXERCISE NO. 4

ESTIMATION OF CRUDE FAT/ OIL BY SOXHLET'S METHOD

Principle: Lipids in sample is dissolved in organic non polar solvents like petroleum spirit, benzene, hexane etc. Lipids/ Fat dissolved in solvent can be extracted by heating and cooling simultaneously in a condenser.

Material: 1. Soxhlet apparatus : It consists of three parts fitted into one another. These three parts are extraction flask, extraction thimble and water condensor.

2. Petroleum spirit B.P. 40-60° C.
3. Whatman No. 1 filter paper sheet..

PROCEDURE:

- 1) Weigh 2 to 5 gm of 60 mesh oil seed sample (previously ground and dried at 105 °C for 24 hours for removing moisture) or 10 gm of other sample.
- 2) Prepare a small packet of sample with Whatman No.1 filter paper.
- 3) Take weight of empty dry extraction flask.
- 4) Plug the bottom of thimble by putting cotton or glass wool to avoid the possibility of passing out the sample particles in extraction flask.
- 5) Connect the rubber tube, water tap to condenser. See that water supply to the condenser is constantly flowing.
- 6) Put the packet of sample in thimble and pour organic solvent to 2/3 capacity of thimble. Take extraction flask containing 2/3 organic solvent.
- 7) Connect these extraction flask and thimble to the condenser unit with heating coil.
- 8) Put the apparatus on heating mantle and start water supply to the condenser. Regulate the rate of heating to allow continuous volatilization of solvent, its simultaneous condensation.

- 9) Continue heating slowly till 6-8 siphoning collected in extraction flask. And stop heating.
- 10) Take out extraction flask from the extraction unit. Which contains crude fat with little ether.
- 11) Evaporate excess ether on water bath OR in open air.
- 12) Keep the flask in the oven at 105 °C for 1 hour and evaporate remaining spirit.
- 13) Cool to the room temperature and weigh it accurately to know the quantity of crude fat / oil extracted.

Observations:

- i) Wt. of sample taken = 5 gm (X)
- ii) Wt. of empty flask = g (W₁)
- iii) Wt. of flask + oil =g (W₂)

Calculation:

$$\% \text{ Crude fat / oil} = \frac{(W_2 - W_1)}{X} \times 100$$

Results: Given sample contains.....% of oil.

Questions :

1. What is meant by crude fat ?
2. State the different parts of Soxhlet apparatus.
3. Explain the principle of extraction of crude fat by Soxhlet method.
4. Name the solvent for extraction of fat.

EXERCISE NO. 5

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

Principle:

Protein reacts with the folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein as in the biuret test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Materials:

1. Alkaline sodium carbonate solution.
(2% Na_2CO_3 in 0.1 N NaOH)
2. Copper sulphate – sodium potassium tartarate solution.
(0.5% CuSO_4 in 1% Na & tartarate)
3. 'Alkaline solution' – prepare on day of use by mixing 50 ml of 1st solution and 1 ml of 2nd solution.
4. Folin – Ciocalteau reagent – Dilute the commercial reagent with an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid.
5. Standard protein – Albumin solution 0.2 mg/ml.

Method:

Add 5 ml of the 'alkaline solution' to 1 ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10 minutes or longer. Add 0.5 ml of diluted Folin – Ciocalteau reagent rapidly with immediate mixing. After 30 minutes read the extinction against the appropriate blank at 750 nm.

Estimate the protein concentration of an unknown solution after preparing a standard curve.

EXERCISE NO. 6

DETERMINATION OF ACID VALUE OF OIL/FAT

Definition:

Number of milligrams of potassium hydroxide (KOH) required to neutralize the free fatty acids present in one gram of fat is known as acid value of fat.

Principle:

Fats contain small quantity free fatty acids. During storage, the free fatty acid contents increases. These are determined by titration of fats with standard potassium hydroxide solution.

Reagents:

- 1) **Alcohol-Ether mixture:** Mix alcohol (95%) and ether in equal proportions by volume: 25 ml of each are enough for one experiment.
- 2) **Phenolphthalein indicator** (1% solution in alcohol).
- 3) **KOH solution (0.1 N):** Dissolve 5.6g KOH in water and make up the volume to 1 litre with water. Standardize with potassium hydrogen phthalate.
- 4) **Fat or oil sample.**

Procedure:

- 1) Weigh out accurately by difference 10 g oil or fat into a well dried 250 ml conical flask.
- 2) Add 50 ml neutralized mixture of alcohol and ether.
- 3) Add 1 ml of phenolphthalein indicator and shake the flask.
- 4) Titrate against 0.1 N KOH solution till pink colour forms and stays for 30 seconds.

Observations:

- 1) Weight of oil or fat = \dots ⁵~~10~~ gm
- 2) Vol. Of std. KOH solution = \dots ml
required for neutralization.
- 3) Indicator used – Phenolphthalein.

Calculations:

1 ml of 0.1 N KOH = 5.6 mg KOH

$$\text{Acid value} = \frac{5.6 \times \text{Vol. of KOH required for titration } 0.7}{\text{Weight of sample (g) } 5 \text{ gm}}$$

- Ques

Questions:

- 1) Define acid value of fat.
- 2) How are fatty acids developed in fat and oil?
- 3) What is the object of acid value determination?
- 4) How the factor 5.6 is derived?

for cotton seed oil

wt. of oil = 10 gm

vol of KOH soln = 0.7 ml

$$\text{acid value} = \frac{5.6 \times \text{volume KOH required}}{\text{weight of sample}}$$

$$= \frac{5.6 \times 0.7}{10}$$

$$= 0.392 \text{ mg/g}$$

the given sample of determination
of acid value content

EXERCISE NO. 7

DETERMINATION OF SAPONIFICATION VALUE OF FAT/OIL

Definition:

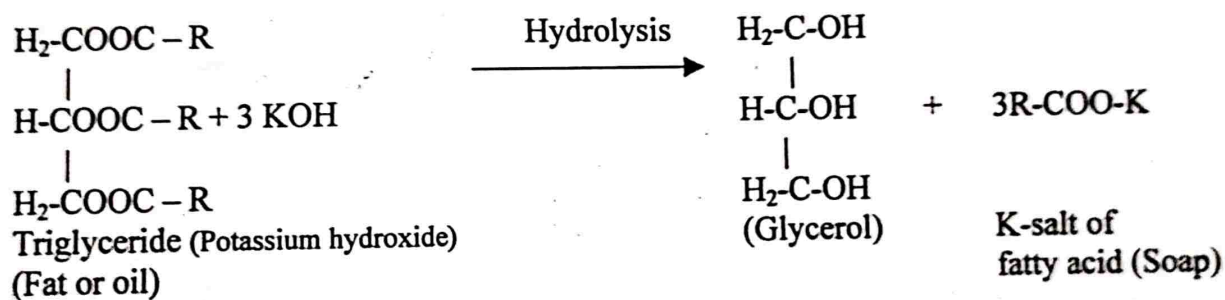
The number of milligrams of KOH required to neutralize the fatty acids resulting from the complete hydrolysis of one gram of fat or oil is called its specification value.

It is an indication of the nature of the fatty acids in the fat or oil, since longer the carbon chain less acid is liberated per gram of the fat or oil hydrolysed.

Principle:

When a fat or oil is heated with excess of alcoholic KOH fat/oil is hydrolysed to glycerol and the potassium salts of fatty acids (soaps).

Reaction:



Reagents:

- 1) **Alcohol – Ether mixture (1:1), (v/v):** Mix alcohol (95 %) and ether in equal proportions by volume.
- 2) **Alcoholic KOH :** (0.5 mol/L or 0.5 N): Dissolve 7g KOH in 250 ml of alcohol.
- 3) **Hydrochloric acid :** (0.5 mol/L or 0.5 N): Measure 45 ml conc. hydrochloric acid into a 250 ml flask. Dilute with water to 1000 ml.
- 4) **Phenolphthalein indicator :** Dissolve 1g of phenolphthalein into 100 ml of alcohol.

Procedure:

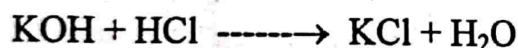
- 1) Weight out by difference about 1g of oil or fat into a 250 ml conical flask.
- 2) Add 10 ml of alcoholic KOH and 25 ml alcohol – ether mixture to the flask.
- 3) Reflux with air condenser in boiling water bath for 30 minutes.
- 4) Cool the contents of the flask.

- 5) Simultaneously process another flask using all the reagents but without fat or oil (blank).
- 6) Titrate the contents of both the flasks with 0.5 N HCl using phenolphthalein indicator.
- 7) The titre value of the flask containing fat or oil will be less than that for blank experiment.

Observations:

- 1) Weight of fat or oil = 1 g
- 2) Volume of 0.5 N alcoholic KOH used = 10 ml
- 3) Volume of 0.5 N HCl required to titrate the blank experiment flask (x). =ml
- 4) Volume of 0.5 N HCl required to titrate the flask containing the oil (y) =ml
- 5) Indicator used = Phenolphthalein

Calculations:



- Hence, 1 mole of HCL = 1 mole of KOH
- 1000 ml 1 N HCl = 56 g KOH
- 1000 ml 0.5 N HCl = 28 g KOH
- 1 ml of 0.5 N HCl = 0.028 g KOH (28 mg)

$$\text{Specification value} = \frac{(X-Y) \times 28}{\text{Weight of sample (g)}}$$

Questions:

- 1) Define saponification.
- 2) What is saponification? Explain with chemical reaction.
- 3) What is relationship between saponification value and chain length of fatty acids in fat/oil.
- 4) What is the purpose of using reflux condenser in this experiment?
- 5) How the factor 28 is derived?
- 6) Give the saponification values of important fats and oils.

EXERCISE NO. 8

DETERMINATION OF IODINE VALUE OF FAT/OIL

Definition:

The number of grams of iodine absorbed by 100 g of fat or oil is called its iodine value.

Principle:

The degree of unsaturation of a fat is determined by allowing a known amount of fat/oil to react with standard solution of iodine chloride (Wig's solution) or iodine bromide (Hanus solution). The iodine adds to the double bonds of the unsaturated fatty acids of the triglyceride molecules. Each double bond absorbs two atoms of iodine.

Reagents:

- 1) **Iodine bromide solution (Hanus):** Weigh out 13.2 g iodine crystals into a beaker and add 400 ml of glacial acetic acid to it. Stir well. Add carefully 3 ml of liquid bromine to this solution, drop wise and with constant stirring. Transfer the mixture to one litre measuring flask and make up the volume to litre by glacialacetic acid. Shake and filter if necessary through a glass wool plug.
- 2) **Sodium thiosulphate solution (0.1 N):** Dissolve 24.8 g of sodium thiosulphate crystals in enough water and make up the volume to 1000 ml in a measuring flask. Standardize against N/20 $K_2Cr_2O_7$ (2.4525 g/l) as primary standard.
- 3) **Potassium iodide solution:** 10% in water
- 4) **Starch solution:** Stir 1 gram of starch powder in 20 ml water. Boil 100 ml water in a breaker and the suspension of starch to it with stirring. Boil for two minutes and cool. The starch solution is used as indicator.
- 5) **Carbon tetrachloride (CCl_4) or chloroform.**

Procedure:

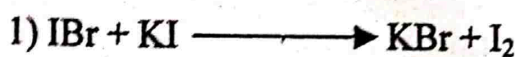
- 1) Weigh out accurately about 1 g of fat or oil by difference into a 250 ml clean and dry glass stoppered iodine flask.

- 2) Add 10 ml of carbon tetrachloride.
- 3) Add 25 ml of iodine bromide solution and stopper the flask.
- 4) Shake the flask to mix the content well.
- 5) Allow the flask to stand in dark for 30 minutes with occasional shaking.
- 6) At the same time, prepare a similar flask containing the same quantity of reagents but without fat/oil as a blank and place for 30 minutes.
- 7) After 30 minutes take out the flask and add 15 ml of 10% KI solution and 50 ml of water in each flask.
- 8) Titrate both the flasks with the standard sodium thiosulphate solution until pale straw colour appears.
- 9) At this stage add 1 ml of starch indicator solution.
- 10) Continue the titration by dropwise addition of sodium thiosulphate solution until blue colour disappears.
- 11) Record the readings obtained for both flasks.

Observations:

1. Weight of the fat/oil taken = 1 g
2. Volume of std. Sodium thiosulphate solution required for the blank titration = ml
3. Volume of std. Sodium thiosulphate solution required for titration when fat/oil used. = ml
4. Normality of sodium thiosulphate solution = 0.1 N
5. Indicator used = Starch

Reaction:



From reaction,

1 mole of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ = 1 mole of iodine

1000 ml 1 N $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ = 127 g iodine

1 ml of 1 N $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ = 0.127 g iodine (Volumetric factor)

1 ml of 1 N $\text{Na}_2\text{S}_2\text{O}_3 - 5\text{H}_2\text{O}$ = 0.0127 g iodine

Calculations:

$$\text{Iodine value} = (\text{Blank} - \text{Test}) \times \text{Normality of thiosulphate solution} \times 0.0127 \times \frac{100}{\text{Weight of sample (g)}}$$

Questions:

- 1) What is the importance of estimation of iodine value of an oil?
- 2) Define Iodine value.
- 3) Explain the principle involved in the estimation of iodine value.
- 4) What are the reactions involved in the estimation?
- 5) Can the iodine value be zero? If yes why?
- 6) How the factor 0.0127 is derived?

ESTIMATION OF REDUCING SUGARS BY BENEDICT'S METHOD

Principle : The cupric copper in alkaline solution is reduced by glucose, fructose, lactose and maltose. The cuprous oxide formed, combines with potassium sulphocyanide(KCNS) in the solution to form a bulky white cuprous thiocyanate. This prevents the formation of red or yellow precipitate. On complete reduction, whole of CuSO_4 disappears and the solution shows no blue colour.

Reagents : 1. benedict's quantitative reagent : Dissolve 200 g of sodium citrate, 75 g of anhydrous sodium carbonate and 125 g of potassium thiocyanate in about 600 ml of water gentle heating. Filter and cool.

Dissolve 18 g of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in about 100 ml of water. Add this to the potassium thiocyanate solution with stirring. Add 5 ml of 5% potassium ferrocyanide solution and make up the volume to 1 litre with distilled water. If the solution is not clear, filter. (The reagent is also available commercially).

2. Anhydrous sodium carbonate.

3. Standard solution of glucose: Weigh out accurately 0.5 g of reagent grade glucose, dissolve in water and make up to 100 ml (5gm/ml).

4. Solution of glucose or any of the reducing sugars: Fructose, lactose or maltose of unknown concentration.

Procedure :

1. **Standardization of benedict's quantitative reagent :** Pipette 25 ml of Benedict's reagent in 100 ml. flask with a long narrow neck. Add 2 to 3 g of anhydrous sodium carbonate and a few pieces of porcelain. Heat the flask on a burner. Keep the contents of the flask boiling through out the titration period. Take the standard glucose solution (0.5g/100 ml) in a burette and slowly run this solution into the boiling reagent. A bulky white precipitate of cuprous thioyanate is former. Add the glucose solution drop wise until the last trace of the blue colour due to CuSO_4 disappears.

Allow the titration mixture to cool . The white precipitate settles down. Supernatant liquid exhibits light green colour. if the fluid shows a bluish colour, boil the contents and add more glucose solution until the end point is reached. If the supernatant liquid show green tinge then excess glucose has been added. Then titrate until constant readings are obtained.

2. Determination of reducing sugar :

Take the test solution into the burette and repeat the titration as in the standardization experiment. End point of the titration is reached when the supernatant liquid becomes greenish. Repeat the titration to obtain constant reading. Record the readings in a table.

1. Standard glucose- Benedict's reagent

Sr. No.	Volume of Benedict's Reagent	Burette Reading		Volume of Standard glucose	Constant Reading
		Initial	Final		
1.	25	5	6.1	1.1	1.4
2.	25	6.1	7.5	1.4	1.4
3.	25	7.5	9.1	1.6	1.4

Therefore, 25 ml Benedict's solution = X ml standard glucose

2. Unknown glucose (or reducing sugar) with benedict's reagent

Sr. No.	Volume of Benedict's Reagent	Burette Reading		Volume of unknown glucose solution	Constant Reading
		Initial	Final		
1.					
2.					
3.					

Therefore, 25 ml Benedict's solution = Y ml unknown glucose solution

Calculations :

The standard glucose solution contains 5 mg glucose / ml

- 25 ml Benedict's solution = X ml standard glucose solution
- 25 ml Benedict's solution = Y ml sample solution

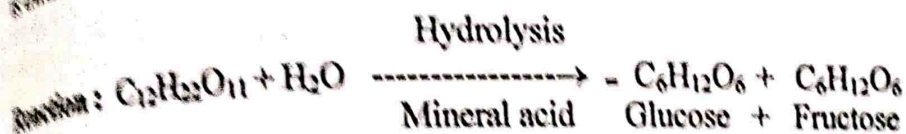
$$\text{Reducing sugar per 100 ml (\%)} = \frac{5 \times X \times 100}{Y \times 1000} = \frac{5 \times X}{10 \times Y} = \frac{X}{2Y}$$

Questions :

- Define reducing sugars. Give examples of reducing sugars?
- How would you prepare Benedict's reagent?
- Write down the principle involved in the estimation of reducing sugars by the Benedict's method?
- Give the formula for determination of per cent reducing sugars?
- Why the titration is carried out under boiling condition?

ESTIMATION OF NON-REDUCING SUGARS BY BENEDICT'S METHOD

Principle : The sucrose is inverted by boiling with mineral acid to obtain invert sugar solution. It is titrated against Benedict's quantitative reagent as in the estimation of glucose.



Reagents :

1. Benedict's quantitative reagent.
2. Anhydrous sodium carbonate
3. Hydrochloric acid (1N)
4. Sodium hydroxide (1 N)
5. Sucrose solution about 2.5%

Procedure : Pipette 25 ml. of the sucrose solution in a beaker, add 12 ml. of 1N HCL. Stir well and heat on flame. Heat to boiling. Allow the content to boil for two minutes. Cool under running tap water. Add with stirring 12 ml of 1 N NaOH (alkali equivalent to acid). Confirm the neutralization of acid by testing a small drop of solution with red litmus. It should become blue. Transfer the contents of the beaker to a 250 ml volumetric flask. Make up the volume to 250 ml. Mix thoroughly by inverting the flask several times. The hydrolyzed solution of sucrose, thus formed, is called 'Invert sugar solution'. Fill this solution in the burette and titrate it with 25 ml. Benedict's quantitative reagent as described in the determination of glucose. The end point is obtained in the same manner as described for glucose estimation. Repeat the titration until constant reading is obtained.

Observations :

Sr. No.	Volume of Benedict's Reagent	Burette Reading		Volume of Invert Solution	Constant Reading
		Initial	Final		
1.					
2.					
3.					

25 ml Benedict's solution = Y ml invert solution

1. The standard glucose solution contains 5 mg glucose/ml.
2. 25 ml of Benedict's solution = $X \times 5$ mg glucose (Earlier Expt. No. 9)
3. Hence Y ml of Invert sugar solution = $X \times 5$ mg glucose (Earlier Expt. No. 9)

$$4. \text{ Invert sugar (g/100 ml)} = \frac{X \times 5 \times 100}{Y \times 1000} \times \frac{250}{25}$$

$$= \frac{5 \times X}{Y}$$

$$= Z \text{ gms}$$

$$5. \text{ Non-reducing sugar g/100 ml sucrose} = \text{glucose} + \text{fructose}$$

$$\text{MW } 342 = 180 + 180$$

$$= 360$$

$$\text{If } 360 \text{ g of invert sugar} = 342 \text{ g of non-reducing sugar}$$

$$\text{Hence, for } Z \text{ g} = \frac{342}{360} \times Z$$

$$= 0.95 \times Z$$

Questions :

1. What do you mean by hydrolysis.
2. How will you carry out the hydrolysis reaction ?
3. Give the reaction of hydrolysis of sucrose.
4. Give the structural formulae of sucrose.
5. Why the sucrose is called a non reducing sugar ?

Result -: In the sample solⁿ present 3.8 gm
sugars per. 100 ml of glucose solⁿ total amount
of sugars is there.

ESTIMATION OF ASCORBIC ACID IN CITRUS JUICE

Principle :

Ascorbic acid (Vit. C) present in the sample extract is oxidized by 2, 6 dichlorophenol- Indophenol (Dye) to dehydroascorbic acid in acid medium. From volume of dye required for oxidation, the amount of Vit. C is calculated.

Reagents:

- 1) **3% Metaphosphoric acid (HPO_3)**: Prepare by dissolving the sticks or pellets of HPO_3 in glass distilled water.
- 2) **Standard Ascorbic acid solution**: Weigh accurately 100 mg of L-ascorbic acid and make up to 100 ml with 3% HPO_3 . Dilute 10 ml to 100 ml with 3% HPO_3 . (1 ml = 0.1 mg of ascorbic acid).
- 3) **Dye solution**: Dissolve 50 mg of the sodium salt of 2, 6 - dichlorophenol indophenol in approximately 150 ml of hot distilled water containing 42 mg of sodium bicarbonate. Cool and dilute with distilled water to 200 ml.

Procedure:

Take 5 ml of standard ascorbic acid solution and add 5 ml of HPO_3 . Fill a burette with the dye. Titrate with the dye solution to a pink colour which should persist for 15 seconds. Determine the dye factor, i.e. mg of ascorbic acid per ml of the dye using the formula.

$$\text{Dye Factor} = \frac{0.5}{\text{Titre}}$$

Preparation of sample:

Fruit juices: Take 10 to 20 ml of sample and make up to 100 ml with 3% HPO_3 . Filter or centrifuge.

Solid or semi-solid food: Take 10 g of sample, blend with 3% HPO_3 and make up to 100 ml with HPO_3 . Filter or centrifuge.

Assay of Extract:

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Take an aliquot (2-10 ml) of the HPO_3 extract of the sample and titrate with the standard dye to a pink end-point which should persist for at least 15 Sec. Titrate rapidly. The aliquot of sample taken should be such that the titre should not exceed 3 to 5 ml.

Calculation:

Calculate the ascorbic acid content of the sample from the following formula:

$$\begin{array}{l} \text{mg of Ascorbic acid} \\ \text{Per 100 g or ml} \end{array} = \frac{\text{Titre} \times \text{Dye factor} \times \text{Vol. made up} \times 100}{\begin{array}{l} \text{Aliquot of extract} \\ \text{Taken for estimation} \end{array} \times \begin{array}{l} \text{wt. or volume of sample taken for} \\ \text{estimation} \end{array}}$$

EXERCISE NO. 12

ESTIMATION OF TOTAL CHLOROPHYLL FROM PLANT TISSUES

Principle:

The Chlorophyll is extracted by treating fresh plant tissues with 85% acetone. The absorbance of acetone extract is measured at 642.5 and 600 nm wave length. From the absorbance values at these wave lengths, the total chlorophyll content is calculated.

Reagent:

- 1) Acetone 85%

Procedure:

- 1) Weigh 1 to 5 g fresh plant tissue sample.
- 2) Macerate tissue in mortar and pestle with small amount of acid washed quartz sand.
- 3) Add 85% acetone and mix well.
- 4) Filter through whatman No. 1 filter paper and collect filtrate in 150 ml volumetric flask.
- 5) Repeat the process of maceration and extraction with acetone and filtration till filtrate is colorless.
- 6) Make up the volume with 85% acetone.
- 7) Measure the colour intensity at 642.5 and 660 nm on Spectronic 20 colorimeter.

Observations:

- 1) Weight of fresh plant tissue
- 2) Volume made with acetone
- 3) Absorbance at 642.5 nm
- 4) Absorbance at 660 nm

= (W) g
 = 50 ml (V)
 = A
 = B

Calculations:

Total chlorophyll = $7.12 \times B + 16.8 \times A = Z \text{ mg/1000 ml}$

$$\text{Total chlorophyll (mg/100g)} = \frac{Z \times \text{Vol. of extract} \times 100}{1000 \times \text{weight of plant sample in g}}$$

Questions:

- 1) Write functions of chlorophyll.
- 2) Write the components of chlorophyll.
- 3) Mention extractant of chlorophyll.

Reference:

A.O.A.C. (1974). Methods of Analysis, pp. 50-51.

EXERCISE NO. 13

SEPERATION AD IDENTIFICATION OF AMINO ACIDS BY
PAPER CHROMATOGRAPHY (TWO DIMENTIONAL)**Principle:**

The partition principle used in one dimensional chromatography has been refined and used for separating mixture of amino acids. The mixture is chromatographed in one direction using n-butanol-acetic acid-water. Then the paper is dried and subjected to chromatography with a different solvent-phenol-water at right angles to the first direction (by rutning 90^0) for achieving considerable separation of amino acids over the entire sheet. The paper after drying again at 105^0C is sprayed with Ninhydrin solution for locating spots and thereafter identification room their R_f values is done.

 R_f value:

It is the relation of distance moved/traveled by a compound to that traveled/moved by the solvent is known as R_f values and is more or less constant for a particular compound, solvent system and paper under carefully controlled conditions of solute conc. temperature and pH etc.

$$R_f \text{ value} = \frac{\text{The distance moved by the compound}}{\text{The distance moved by the solvent}}$$

Materials and Reagents:

- 1) Chromatocap: An airtight rectangular chamber on wooden frame with side glasses, supporting rod and through for filling solvent.
- 2) Whatman No. 1 : Chromatographic paper sheets of 20 x 20 cm size.
- 3) An electric oven with thermostatic control (optional)
- 4) Solvent 1 : (n-butanol-glacial acetic acid-water in 12:3:5 ratio v/v)

- 5) Solvent 2: (Phenol-water in 4:1 ratio, add 125 ml H_2O to 500g bottle of phenol, replace stopper and allow it to stand overnight, add few drops of ammonia liquor 0.88 sp. Gravity to the solvent and mix well.
- 6) Ninhydrin location solution: (Dissolve 0.2g in 100 ml acetone just before use).
- 7) Standard amino acids: (Prepare small volumes of 10g/L solutions in 10% v/v isopropanol, sometimes a drop of acid or alkali is needed to bring the compound in the solution.

Procedure:

Rule faint straight line across one side of chromatography paper sheet at 5 cm from the edge and another at right angle from the another edge lightly by a pencil. Mark the point of intersection.

Apply the mixture on a spot along on any one line with 1 cm away from the point of intersection i.e. 6 cm from the edge of a sheet.

Spot 10 to 20 ml of the mixture with the help of a micropipette or moicrosyringe and dry the spot on a current of air. A hair dryer may be used to hasten the evaporation of the solvent and the paper be kept clean and handled as little as possible. The diameter of the spot should not be more than 0.5 cm.

Pour solvent 1 in glass though to saturate air of the Chromatocap and hand the upper end of the chromatography paper sheet fastened to the support with paper clips over the trough for sometimes by closing the chamber. Place the edge of the sheet adjacent to the test spot slightly immersed (0.5 cm deep) in the solvent- 1 kept the glass through and allow the solvent to rise till it reaches almost to the upper edge (To run for most of the day). Mark the position of the front immediately.

Remove the paper sheet and dry it in a current of cooled air to allow the evaporation of solvent.

Turn the sheet through 90° after drying and carry out the separation preferably overnight in the second solvent – Phenol + Water + ammonia by arranging the immersion of second edge adjacent to spot. Remove the paper when the solvent front reaches the top

edge. Mark the position. Dry the papers previously and spray it with Ninhydrin solution to reveal the position of zones (or alternately rapidly deep the sheet through the Ninhydrin reagent followed by hanging it overnight to evaporate acetone and heating at 105°C for 2 to 3 minutes to develop colour.

Describe the resulting chromatogram by characterizing zones from R_f values by measuring the distance from starting line to the solvent front and the centre of zone.

Run the amino acid standard chromatograms on paper sheet identical to one described above containing one application (10 to 20 ml) of each of amino acids along the 5 cm line at about 2 to 2.5 cm interval either 7.0 to 7.5 cm from the edge.

Examine the table showing R_f values of amino acids and mark

AA	R_f		AA	R_f	
	Butanol	Phenol solvent		Butanol solvent	Phenol solvent
Histidine	0.87	0.65	Proline	0.30	0.91
Serine	0.18	0.36	Tyrosine	0.32	0.64
Lysine	0.18	0.48	Methionine	0.40	0.80
Arginine	0.11	0.59	Valine	0.47	0.77
Aspartic acid	0.13	0.15	Tryptophan	0.47	0.83
Glutamic acid	0.14	0.25	Isoleucine	0.55	0.86
Glycine	0.17	0.48	Phenylalanine	0.58	0.89
Alanine	0.22	0.54	Leucine	0.60	0.85
Threonine	0.22	0.50	-	-	-

(Source: Chaykin, 5(1966). Biochemistry Lab Techniques John Wiley N.Y.)

Plot positions on a square sheet of graph paper by selecting appropriate scale for R_f value of AAs with butanol/acetic acid/water solvent on "y" co-ordinate and that for phenol/water/ammonia on 'X' co-ordinate, compare this graph to check your experimental findings.

Note: Paper chromatographic methods are microanalytical in character and can not be usually applied with more than about 100 ug of sample. In the mixture to be assayed only 5 to 15 ug of each AA needs to be included in 2 to 5 ul of total solution (Vogel, 1961).

EXERCISE NO. 14

SEPARATION OF PLANT PIGMENTS BY THIN LAYER CHROMATOGRAPHY (TLC)

Principle:

The separation of compounds in TLC is based on differential adsorption as well as partitioning of the analytes between the liquid stationary phase and mobile solvent phase. This technique is rapid as compared to paper chromatography. Molecules get separated between the hydrated stationary phase and non-polar mobile phase. Hydrophilic analytes have more affinity to the polar stationary matrix, while less hydrophilic molecules tend to have more affinity towards mobile phase, resulting in its faster movement and separation. The separated analytes are identified by comparing their R_f values to that of reference standards. The R_f value of an analyte depends upon the (i) solvent system (ii) degree of saturation of the mobile phase in the chromatographic chamber (iii) particle size of the adsorbent (iv) type of adsorbent (v) temperature and humidity. Thus, the R_f value for an analyte is constant for a given set of experimental conditions.

$$R_f = \frac{\text{Distance traveled by the analyte}}{\text{Distance traveled by the mobile phase (solvent)}}$$

Commonly used stationary matrix for TLC include, silica gel – G, silica gel H, micro-porous cellulose, alumina, florisil, polyamide and octadecylsilane. Normally, glass, aluminium or polyester supports (size 20 x 20 cm) are used for coating stationary matrixes.

Material: (i) Glass plates (20 x 20 cm, thickness 3 mm) (ii) Chromatographic glass jar with vacuum greased lid (iii) TLC applicator with plastic plate support and TLC rack (iv) TLC-spotting guide (v) Calibrated capillary tubes (SPL/10 uL capacity) or micro-syringe (10 uL). Silica gel-G, TLC grade.

Sample: *Spirulina* powder (commercially available in drug stores/medical shops as capsules).

Solvent system: Petroleum ether: Acetone (7:3 v/v).

Procedure:

- i) Arrange 5 clean glass plates on the plastic support unit along with silica gel applicator. Weight 40 g of silica gel-G into a 250 ml iodine flask. Add 100 ml of distilled water and thoroughly shake the contents. Immediately, pour the slurry into the applicator and coat the gel along the glass plate, by rapidly moving the applicator. The coating of the adsorbent should be of uniform thickness (25-75 μm). Air dry the plates (placed in TLC rack) at room temperature for 5 hours. Activate the coated TLC plates in a hot air oven at 110°C for 1 hour. Later, the plates are removed and allowed to cool at room temperature in a desiccated chamber.
- ii) Prepare an acetone extract of the Spirulina powder (500 mg), by transferring the contents of one capsule into a 20 ml glass test tube containing 5 ml of acetone. Vortex and allow the contents to stand at room temperature for 30 minutes. Collect the acetone extract by filtering through glass wool. The clear filtrate is concentrated by evaporation to a small volume (500 μL volume).
- iii) Spot equidistantly 5, 10, 15 and 20 μL aliquots of the acetone extract onto the activated silica gel plate, about 3 cm away from the edge of the plate by using a TLC guide and capillary tube. The area of the spot should be kept to minimum (1-2 mm dia), which can be achieved by repeated spotting of the sample volume followed by air drying at the same spot. The chromatogram is developed by placing the TLC plate vertically in a TLC chamber, saturated with the mobile phase, in such a manner that the spotted edge dips into the solvent system. Run the chromatogram until the solvent front reaches the top edge of the plate. Remove and mark the solvent front as soon as the plate is removed from the chamber. Air dry the plate at room temperature. Outline the coloured pigment spots using a pencil and calculate the R_f values of the pigments.

Caution: Do not touch or damage the coated area and the edges of the plate. For the purpose of identification, following are the reference R_f values (Table 8.1) of the pigments in the petroleum ether: acetone solvent system.

Table 8.1 R_f values of plant pigments

Pigment	R_f value
Chlorophyll a	0.68
Chlorophyll b	0.54
Chlorophyll c	0.03
β - carotene	0.94
Fucoxanthin	0.51
Lutein	0.43
Violaxanthin	0.22

Workout: Analyse the pigments present in Spinach leaves, by TLC and compare the profile with Spirulina.

Note: The separated pigment spots on the TLC plate should be scored immediately, as the pigment undergoes destruction/bleaching in the presence of light.