Chapter 7

Qualitative Analysis of Lipids, Proteins, Carbohydrates

1. Lipids

A lipid is characterized as a water-insoluble biomolecule which has a high solvency in nonpolar natural solvents, for example, chloroform. The simplest lipids are the fats, which are triesters comprised of one glycerol and three unsaturated fats. The term fats is additionally utilized as a general equivalent word for lipids, so the more exact terms triacylglycerols or triglycerides are best for the most straightforward lipids. Triacylglycerols are utilized basically for vitality stockpiling as a part of creatures. More unpredictable lipids, the phospholipids, glycolipids and cholesterol, are the significant constituents of natural cell membrane.

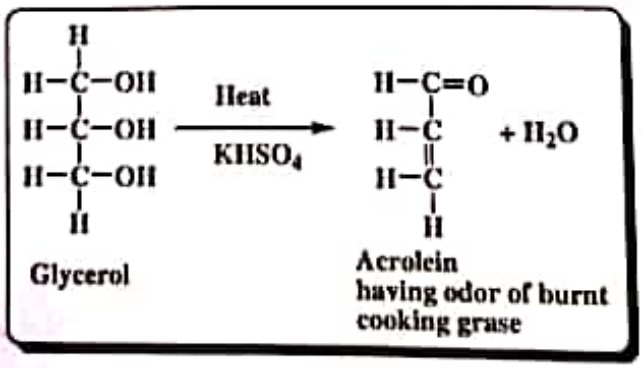
Qualitative Tests for Lipids

1. Solubility Test

Triacylglycerol having little chain unsaturated fats are soluble in water to some extent however those containing nonpolar long chain unsaturated fats are insoluble and they form emulsions in water. All triacylglycerols are soulble in diethyl ether, chloroform and benzene. They are somewhat soluble in cold methanol, ethanol and acetone yet their solubility increases on warming.

1.Acrolein Test

When a fat is heated strongly in the presence of a dehydrating agent such as potassium bisulfate ($KHSO_4$). The glycerol portion of the molecule is dehydrated to form the unsaturated aldehyde, acrolein (CH_2=CH–CHO), which has the odor peculiar to burnt cooking grease.



Material and Reagents

1. Lipid sample (butter, olive oil, stearic acid, and glycerol).
2. Anhydrous potassium hydrogen sulphate.

Procedure

1. Take approximately 1.5g potassium hydrogen sulphate in a test tube and add 5 drop of liquid test sample or an approximately equivalent weight of the test sample if it is solid. Cover the test sample completely by adding more of the solid potassium hydrogen sulphate on top of it.

2. Heat the test tube slowly on burner and note the odor of the fame evolved from the tube.

2. Sudan IV Test.

Sudan IV is a non-polar stain that readily stains lipids red-orange but does not stain polar compounds or aqueous solutions

3. Copper Acetate Test

The copper acetate solution does not react with the oils, while saturated and unsaturated fatty acids react with copper acetate to form copper salt. Unsaturated fatty acids can only be extracted by petroleum ether.

- In the case of olive oil notice that petroleum ether upper layer containing the dissolved oil and appears colorless, aqueous solution remains blue in the bottom.
- In the case of oleic acid the upper layer of petroleum ether becomes green as a result of copper oleate. The lower layer becomes less blue.
- In the case of stearic acid notice that the petroleum ether upper layer remains colorless, while consists of pale green precipitate of copper stearate at the bottom

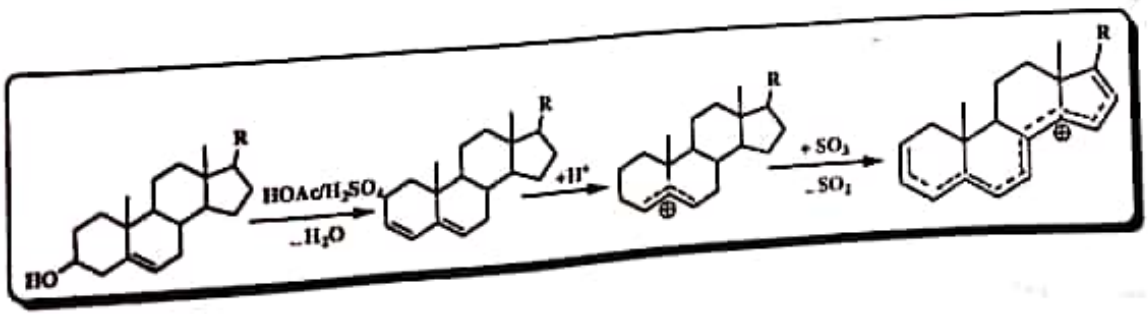
Method: Take two test tubes put 1 / 2 g of each sample and then added 3 ml of petroleum ether and an equal volume of a solution of copper acetate.

General Procedure for Qualitative Analysis of Lipids

No	Experiments	Observations	Inference
1.	**Solubility:** Sample + Water	Insoluble in water	May be fat or oil
2.	**Acrolein Test:** Sample + $KHSO_4$ and heated	Odor of burnt cooking grease observed	Lipids indicated
3.	**Copper Acetate Test:** 0.5 g Sample + 3 ml of petroleum ether + 3 mL aq. Copper acaettae solution and shake well. Allowed to stand.	(i) Upper layer of petroleum ether, containing the dissolved oil, appears colorless, aqueous solution remains blue in the bottom (ii) Upper layer of petroleum ether, containing the dissolved oil, becomes green; the lower layer becomes less blue.	Oil such as olive oil indicated. oleic acid indicated
4	5 mL Aq. NaOH + 1 drop of phenolphthalein solution + Add lipid sample drop wise and shake **Test for Unsaturation:** 5 mL Test solution + Bromine water dropwise	Pink color of phenolphthalein solution disappears Colour of bromine solution discharged	Presence of free fatty acid indicated Unsaturated lipids indicated.

4.Qualitative Estimation of Cholesterol by Liebermann - Burchard Test

The cholesterol reacts as a typical alcohol with a strong concentrated acids and the product are colored substances. Acetic anhydride is used as solvent and dehydrating agents, and the sulfuric acid is used as dehydrating and oxidizing agent. A positive result is observed when the solution becomes ... (red → blue, and finally → bluish –green color).

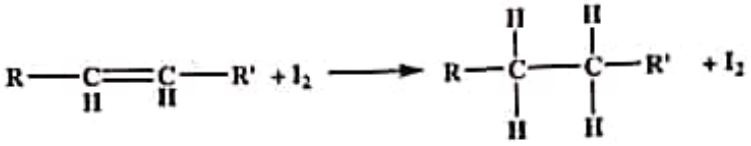


No	Experiments	Observations	Inference
	Dissolve a few crystals of cholesterol in 2 mL of chloroform in a dry test tube and add 10 drops of acetic anhydride. Add to this solution few mL conc. H_2SO_4 with shaking	A colour change is observed: red → blue, and finally → bluish –green color	Cholesterol indicated

5. Unsaturation Test

This test is used to indicate the amount of presence of double bonds in the lipid sample.

All neutral fats contain glycerides of some unsaturated fatty acids. These unsaturated fatty acids become saturated by taking up iodine. If the fat contains more unsaturated fatty acids, it will take up more iodine.



Method:

Add 10 mL of chloroform equally into 4 flasks and then 10 drops of Hubl's iodine reagent, the chloroform shows pink color due to presence of iodine.

- To one test flask add the oil sample drop by drop shaking the tube vigorously for about 30 seconds after addition of each until the pink color is discharged and count the number of drops.
- The pink color is discharged owing to the taking up of iodine by the unsaturated fatty acids of the oil.
- Compare unsaturation, it should be remembered that more the number of drops required to discharge the pink color, the less is the unsaturation.

2. Qualitative Tests for Carbohydrates

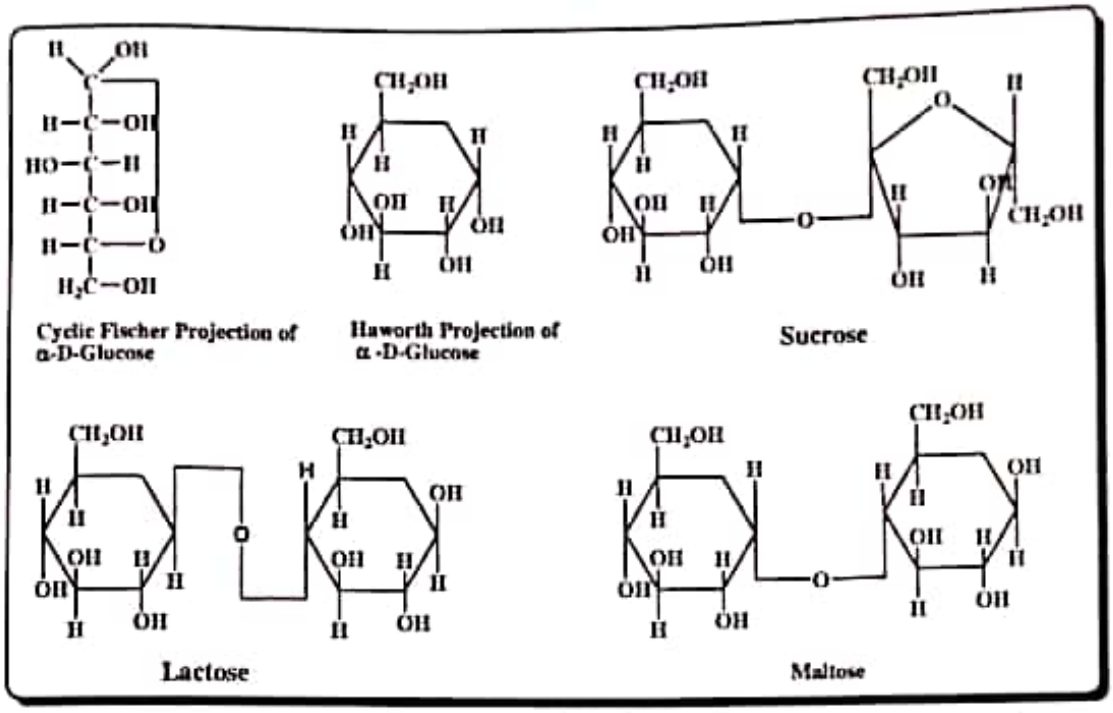
Introduction:

Sugars can be defined as polyhydroxy aldehydes or ketones. Hence the simplest sugars contain at least three carbons. The most common are the aldo- and keto-trioses, tetroses, pentoses, and hexoses. The simplest 3C sugars are glyceraldehye and dihydroxyacetone

Carbohydrates can be classified as either monosaccharides, oligosaccharides or polysaccharides. Anywhere from two to ten monosaccharide units, linked by glycosidic bonds, make up an oligosaccharide. Polysaccharides are much larger, containing hundreds of monosaccharide units. The presence of the hydroxyl groups allows carbohydrates to interact with the aqueous environment and to participate in hydrogen bonding, both within and between chains. Derivatives of the carbohydrates may contain nitrogens, phosphates and sulfur compounds. Carbohydrates also can combine with lipid to form glycolipids or with protein to form glycoproteins.

Monosaccharides

The monosaccharides commonly found in humans are classified according to the number of carbons they contain in their backbone structures. The major monosaccharides contain four to six carbon atoms.



Structures of Common sugars

Disaccharides

Covalent bonds between the anomeric hydroxyl of a cyclic sugar and the hydroxyl of a second sugar are termed glycosidic bonds, and the resultant molecules are glycosides. The linkage of two monosaccharides to form disaccharides involves a glycosidic bond. Physiogically important disaccharides are sucrose, lactose and maltose.

- **Sucrose:** Sucrose, commonly named table sugar or sugar, is cane and beet sugar. Saccharose is an obsolete name for sugars in general, especially sucrose. The molecule is a disaccharide combination of the monosaccharides glucose and fructose with the formula $C_{12}H_{22}O_{11}$. In sucrose, the components glucose and fructose are linked via an ether bond between C1 on the glucosyl subunit and C_2 on the fructosyl unit. The bond is called a glycosidic linkage.
- **Maltose:** Maltose also known as maltoblose or malt sugar, is a disaccharide formed from two units of glucose joined with an α(1→4) bond, formed from a condensation reaction. The isomer isomaltose has two glucose molecules linked through an α(1→6) bond. Maltose is the second member of an important biochemical series of glucose chains. Maltose is the disaccharide produced when amylase breaks down starch. It is found in germinating seeds such as barley as they break down their starch stores to use for food. It is also produced when glucose is caramelized.

Polysaccharides:

Most of the carbohydrates found in nature occur in the form of high molecular weight polymers called polysaccharides. The monomeric building blocks used to generate polysaccharides can be varied; in all cases, however, the predominant monosaccharide found in polysaccharides is D-glucose. When polysaccharides are composed of a single monosaccharide building block, they are termed homopolysaccharides. While the Polysaccharides composed of more than one type of monosaccharide are termed heteropolysaccharides.

Preparation of Reagents for Carbohydrate Analysis

1. **Molisch's reagent:** 5% α naphthal in alcohol, i.e., 5g of αnaphthal dissolved in 100ml of ethanol.
2. **Fehling's soln. A:** 35g of $cuso_4.5H_20$ + water and make the volume to 500mL.

   Fehling soln. B :120g of KOH + 173g Na-K tartrate (Rochelle salt) in water to make volume up to 500 mL.

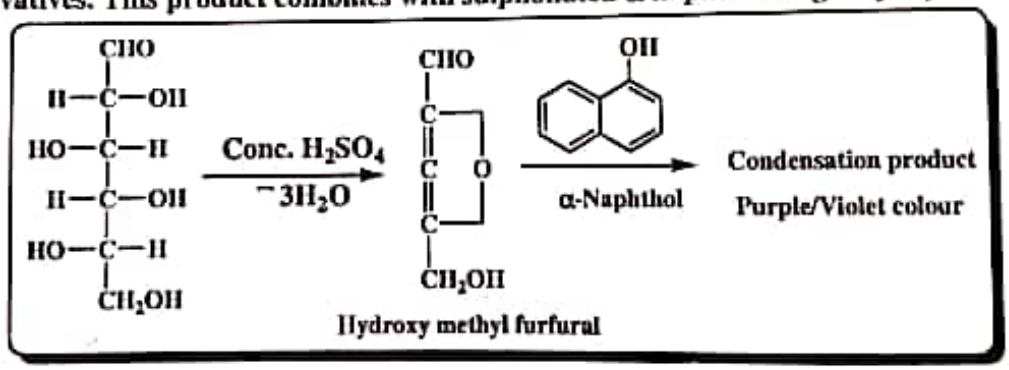
Fehling's reagent: Mix equal volumes of Fehling's soln. A & B. This soln. must be mixed immediately prior to use.

3. **Anthrone:** Anthrone (2g/L in conc H_2SO_4).
4. **Iodine solution:** 0.005% in 3% KI, i.e., 3g of KI dissolved in 100ml water and then 5mg of Iodine is dissolved.
5. **Benedict's solution:** 17.3g of sodium citrate and 10g of sodium carbonate are dissolved in 75ml of water. 1.73g of $CuSO_4.5H_2O$ is dissolved in 20ml of water. Mix the $CuSO_4$ solution with alkaline citrate with constant stirring, finally the whole volume is made up to 100ml with water.
6. **Barfoed's reagent:** 13.3g of copper acetate in 200ml of water and add 2ml of glacial acetic acid.
7. **Fearons test:** (methylamine Hydrochloride (MH) 5% in H_2O + NaOH (20%).
8. **Bial's reagent:** Dissolve 300mg of orcinol in 100mL of concentrated HCl.
9. **Seliwanoff's reagent:** Dissolve 50g of resorcinol in 100mL of con.HCl in the ratio of 1:2.
10. Concentrated HCl
11. Concentrated H_2SO_4
12. Osazone Reagent
    - Phenyl hydrazine hydrochloride
    - Sodium acetate
    - Acetic acid

Mechanistic Principles of Qualitative Identification of Carbohydrates

1. Molisch's Test:

It is a common test for all carbohydrates. As conc. H_2SO_4 hydrolyses glyscosidic bonds, used to produce monosaccharides which in the presence of an acid get dehydrated to form furfural and its derivatives. This product combines with sulphonated α naphthal to give purple colour.



2. Anthrone Test:

The same principle outlined above, except that the furfural reacts with anthrone (10-keto-9,10-dihydroanthracene) to give a blue-green complex.

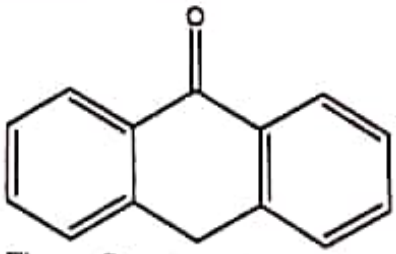


Figure: Structure of anthrone

3. Iodine Test:

Iodine forms colored adsorption complexes with polysaccharides.
Starch → Blue color,
Glycogen → Reddish brown colour complex. Iodine will form a polysaccharide inclusion complex.

4. Benedict's Test

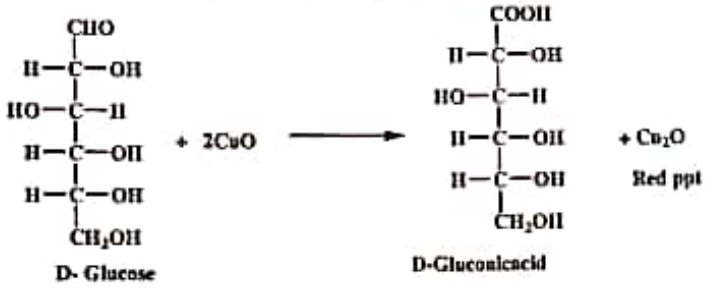
Carbohydrates with a potential aldehyde or ketone group have reducing property when placed in an alkaline solution. Cupric ions present in the solution will be reduced to cuprous ion. This will give a red coloured precipitate. Moreover, this test is more specific for reducing sugars.

5. Barfoed' Test:

It is an important for distinction of reducing disaccharides from monosaccharides. Reducing disaccharides react in about 7-12 min while monosaccharides take 1-2 min to get hydrolysed, react with reagent. Brick red color is appeared in this reaction due to formation of cuprous oxide. If the saccharide is a reducing sugar it will reduce Cu (11) ions to Cu(I) oxide

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

$$Cu(OH)_2 + 2H_2O \longrightarrow CuO + H_2O$$

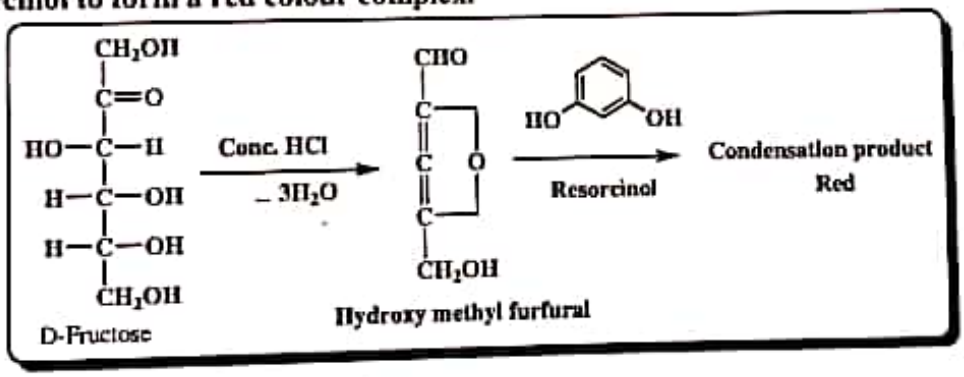


6. Bial's Test:

When pentose is heated with conc.HCl, furfural, which condenses with orcinol in the presence of ferric ion to give a blue green colour.

7. Seliwanoff's Test:

Ketoses are dehydrated more rapidly than aldose to give furfural derivatives, which then condenses with resorcinol to form a red colour complex.



8. Fehling's test

This forms the reduction test of carbohydrates. Fehling's solution contains blue alkaline cupric hydroxide solution, heated with reducing sugars gets reduced to yellow or red cuprous oxide and is precipitated. Hence, formation of the yellow or brownish-red colored precipitate helps in the detection of reducing sugars in the test solution.

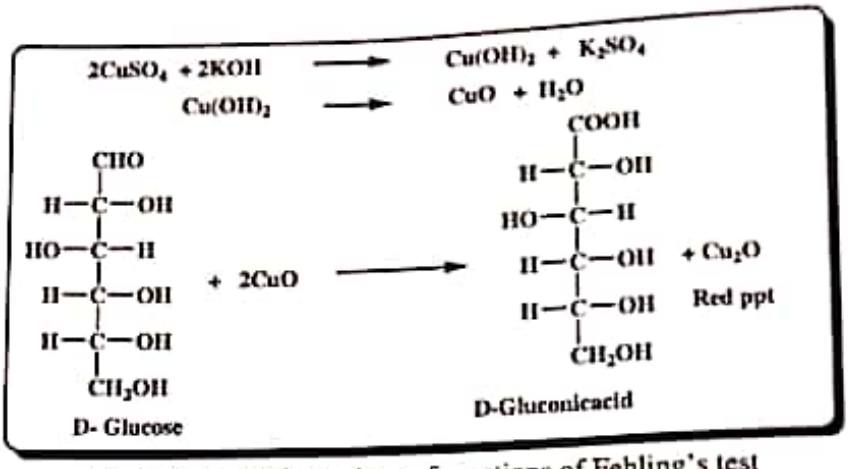


Figure: Chemical reactions of reactions of Fehling's test

9. Osazone Test:

Compounds containing aldehyde and keto groups form crystalline osazone with phenyl hydrazine hydrochloride. Osazone crystals have characteristic shape and melting point which helps in the identification of reducing sugar.

General Procedure for Qualitative Analysis of Carbohydrates

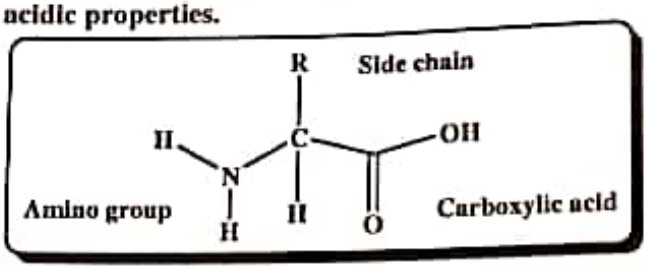
No.	*Experiment*	*Observation*	*Inference*
1.	**Molisch's test** 1mL test solution + 2 drops of Molisch's reagent. Then add conc. H_2SO_4 carefully along the sides of the test tube.	Violet coloured ring is formed at the junction of the 2 layers.	Carbohydrate is present
2.	**Anthrone test** 5 drops of sugar solution +2 mL *Anthrone* reagent	Blue-green color complex is formed	Carbohydrate present
3.	**Iodine test** 1ml test solution + 2 drops of Iodine solution	(i) Deep blue colour is obtained. (ii) Dark brown colour is obtained. (iii) No characteristic colour change.	Polysaccharide indicated (Starch) Polysaccharide (Glycogen) Polysaccharide is absent
4.	**Benedict's test** 5mLof Benedict's reagent was mixed with 1mLof test solution and the contents are boiled for a few minutes.	(i) Orange red precipitate is obtained. (ii) No characteristic colour change.	Reducing sugar indicated Reducing sugar is absent
5.	**Barfoed's test** To 2mL of test solution, 2mL of Barfoed's reagent was added and boiled for 3 minutes and the colour change was noted.	(i) Brick red precipitate is obtained (ii) No characteristic colour change.	Reducing monosaccharide indicated Reducing monosaccharide absent
6.	**Fearon's Test** 1mL of the test solution, 2mL of Fearons reagent is added and the	(i) Red coloration appeared	Reducing disaccharide indicated

	content is heated. Then NaOH was added to the cold mixture.	(ii) No color change.	Reducing disaccharide absent
7.	**Bial's Test** To 1ml of the test solution, 5mL of Bial's reagent was added. The contents are boiled and cooled.	(i) Blue green colour is obtained. (ii) No characteristic colour change.	Presence of pentose sugar. Absence of pentose sugar.
8.	**Seliwanoff's Test** To 1mL of the test solution, 3mL of Seliwanoff's reagent is added and the contents are boiled	(i) Cherry red colour is obtained. (ii) No characteristic colour change	Presence of fructose. Absence of fructose.
9.	**Osazone Test** To 1mL of the test solution, , add 2-3 drops of glacial acetic acid, followed by the addition of a pinch of phenyl hydrazine hydrochloride and twice the amount of sodium acetate and the contents are boiled	(i)Yellow colour crystals are formed in 5 minutes, as observed through a microscope. Haystack structure form of fructosazone. (ii) Yellow colour crystals are formed in 5-10 minutes, as observed through a microscope. Haystack structure form of glucosazone (iii) Yellow colour crystals are formed in 10 minutes. Broad fan structure crystals of galactosazone are observed through microscope. (iv) Yellow colour crystals are formed in 20-25 minutes, Sunflower shaped crystals of maltosazone are observed through the microscope. (v)Yellow colour crystals are formed in 5-10 minutes, scattered needles shaped crystals of Xylosazone are observed through the microscope. (vi) Yellow colour crystals are formed in 20-30 minutes, Powderpuff shaped crystals of Lactosazone are observed through the microscope.	Fructose is confirmed Glucose is confirmed. Galactose is confirmed. Maltose is confirmed. Xylose is confirmed. Lactose is confirmed.

Qualitative Analysis of Proteins and Amino acids

The proteins are essential components of living organisms, including the human body. Proteins are polymers of amino acid. 22 different amino acids are commonly known except proline and hydroxyproline which are Imino acids.

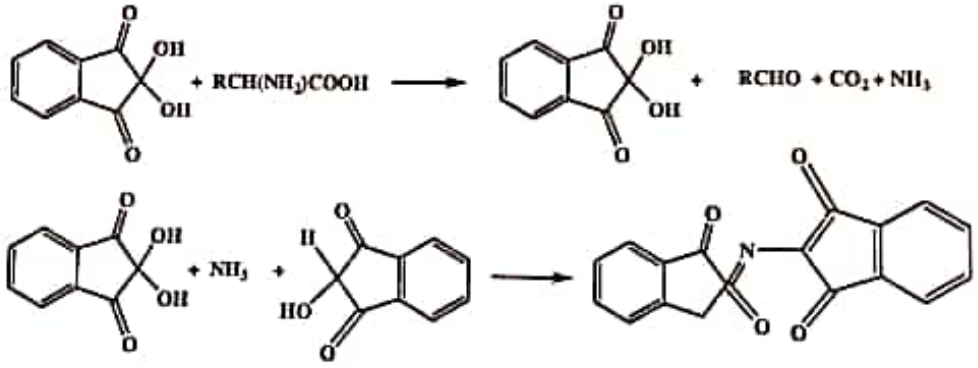
The structure of an amino acid contains an amino group, a carboxyl group, and a R group which is usually carbon based and gives the amino acid's specific properties. As amino acids contain carboxylic & amino groups, so have both basic & acidic properties.



Ninhydrin Test

This test is widely used for detection of proteins. Although compounds other than proteins and amino acids also give positive reactions, standard procedures used in analysis can make the reaction a positive test for amino acids and proteins. Amino acids contain free carboxyl & amino group which condense with ninhydrin to yield colored products. As ninhydrin is a good oxidizing agent so when it reacts with α-amino group, it produce a purple colored product (diketohydrin) known as Rhuemann's purple.

All primary amines and ammonia react in similar way but without the liberation of carbon dioxide. Other complex structures like proteins, peptides & peptones also give this reaction.



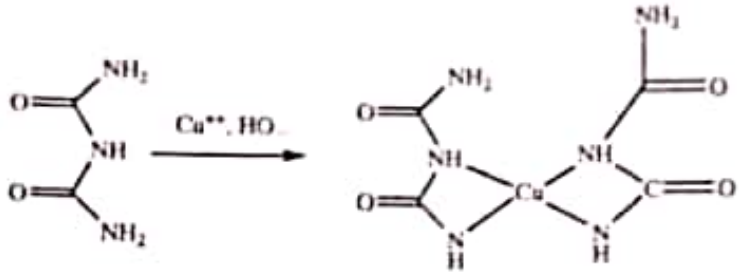
Procedure

- Dissolve the contents of the vial marked "albumin" in 100 mL water. Heat and shake gently.

Put 2.0 mL (34 drops) of ninhydrin reagent. Mix the solutions and boil in a water bath for 2 minutes. In this way, test albumin, gelatin, the unknown compound, and at least two of the ammo acids. Pink, purple, or violet-blue color development normally. But Imino acids like proline and hydroxyproline give a complex of yellow color.

Biuret Test for Protein

It is general test for detecting the presence of peptide bonds. If the peptide bonds are present, a copper (II) ion gives violet-colored coordination complexes in an alkaline solution. Compounds containing 2or more peptide bond, when condensed with alkaline copper sulphate develop a pinkish or violet colored product, which is due to coordination complex formation of cupric ion with unshared electron pairs of oxygen of water and peptide nitrogen.



Procedure

1) Add 0.5 ml NaOH into 1 mL of test soln.
2) Mix it very well.
3) Add 2-5 drops of copper sulphate.

Development of pink or violet color indicates the presence of peptide or protein in the sample.
In this way, test albumin, gelatin, the unknown, and two amino acids.

Test for Cysteine and Cystine

The sulfur group of cysteine and cystine is liberated by heating with strong alkali. If lead ions are present lead sulfide is formed as a dark precipitate. This reaction distinguishes between these two amino acids and methionine. Treatment with alkali does not liberate sulphur from methionine.

Procedure

Dissolve the cysteine in aqueous NaOH solution. Add lead acetate solution to the alkaline solution of cysteine. Formation of dark precipitates indicates the presence of cysteine.

Sakaguchi's Test for Arginine

Ammonia and ammonium ions give positive reactions in this test. For this and other reasons the reagents always give some color in the reaction. Therefore, a blank or control containing reagents only should be prepared for comparison.

Procedure

Prepare a 4% solution of NaOH by adding 20 mL 40% NaOH made up for experiment 4 to 180 mL water. Set up a number of test tubes as follows: one with 2.0 mL water, one with 2.0 mL arginine solution, one with 2.0 mL unknown compound, one with 2.0 mL glutamic acid solution, and one with 2.0 mL albumin solution. To each of these add 1.0 mL 4% NaOH solution, and then add 2 drops of alpha-naphthol reagent. Add 1.0 mL of sodium hypochlorite reagent to each solution and mix. Observe any color change which occurs over the next 5 minutes. What color is positive for arginine? Does albumin contain arginine?

Systematic Analysis of Proteins and Amino acids

No	Experiments	Observations	Inference
1	Ninhydrin Test: Dissolve the sample in 100 mL water. Heat and shake gently. Put 2.0 mL (34 drops) of ninhydrin reagent	A purple colored product was noted	Amino acid or protein indicated
2	Biuret Test: Sample + NaOH solution + 2-5 drops of copper sulphate	Pink or violet color	Presence of protein indicateed
3	Dissolve the sample in aqueous NaOH solution. Add lead acetate solution	Dark ppt. noted	Cystein confirmed