Mustansiriyah University
College of Science
Department of Chemistry

Practical biochemistry for third class students

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Preface

This practical lab booklet has been prepared and written to suit the main requirements of the third class students - Department of Chemistry - College of Science at Al-Mustansiriyah University.
It contains four chapters, each chapter deals with the most important practical experiments used by the third class students to detect and diagnose the biological molecules in human daily life, which include Carbohydrates, Lipids, Amino acids and Proteins.
Finally, I want to thank Dr. Falah AL-Fartusie who supplied me with an Arabic lab booklet copy and helped me in translating and revising, and Dr. Salwa Hameed Naser who reviewed the drafts and encouraged me to write this lab booklet.

The editor

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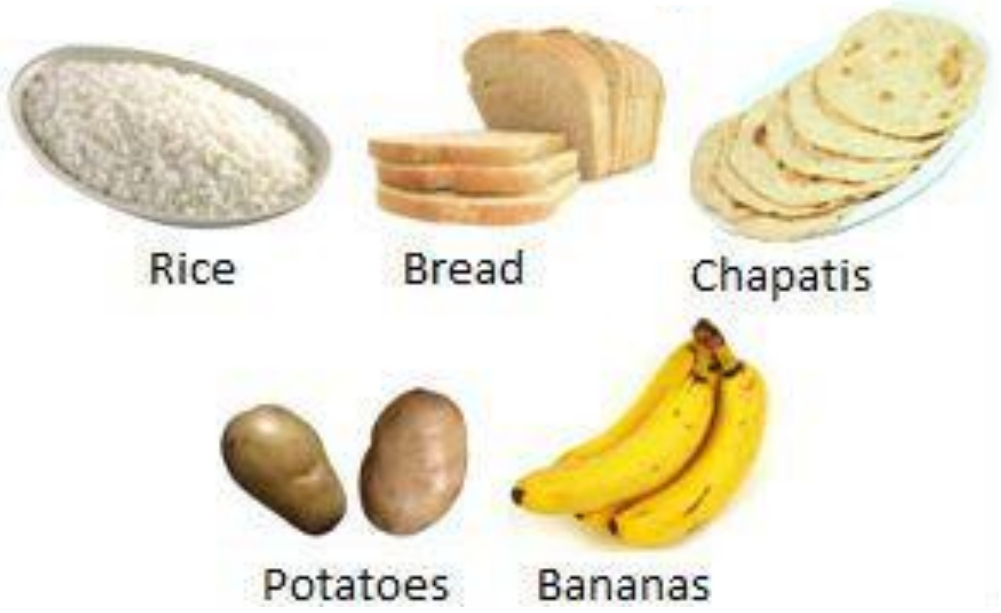
Qualitative test of carbohydrates

Foods high in carbohydrates:



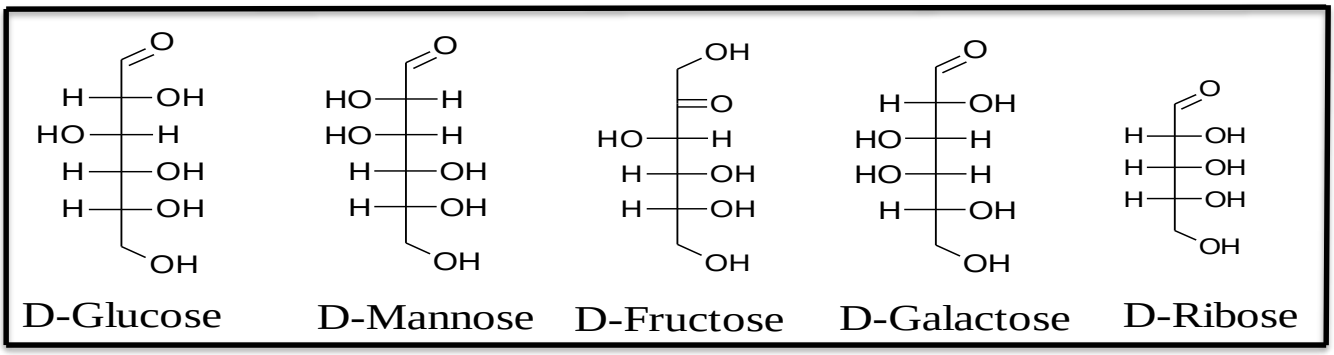
Carbohydrates

Carbohydrates:

They are polyhydroxy aldehydes or ketones compounds. The general formula is $(CH_2O)n$, when (n) ranges from three to several thousand. The structure involves only two functional groups (ketone or aldehyde carbonyls and alcohol hydroxyl groups). Carbohydrates are classified into;

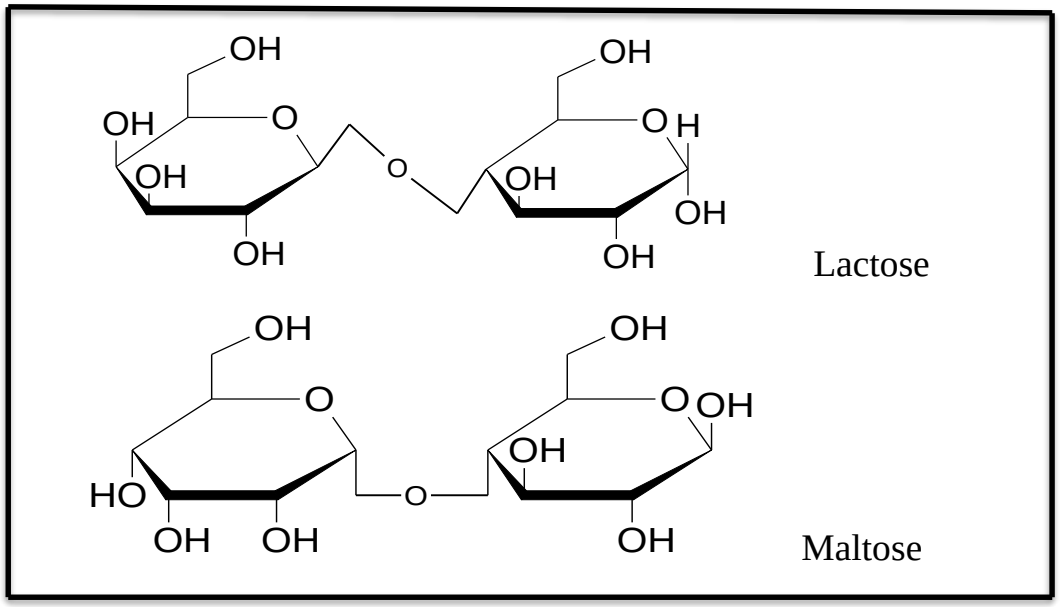
1- Monosaccharaides :

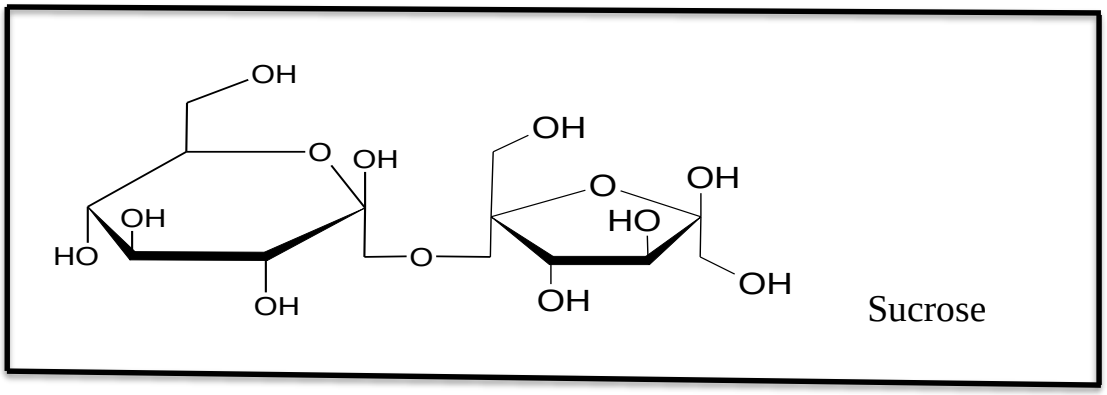
Monosaccharides are the simplest carbohydrates, they cannot be hydrolyzed to simpler chemical compounds. They classified as trioses, tetroses, pentoses, hexoses and heptoses (depending on the number of carbon atom) and classified as aldoses or ketoses depending upon whether they have an aldehyde or ketone group. The most important are:



2- Disaccharides :

Disaccharides are sugars containing two of monosaccharides joined by glycosidic bond (C-O-C) and these are the simplest polysaccharides. Three common examples are:

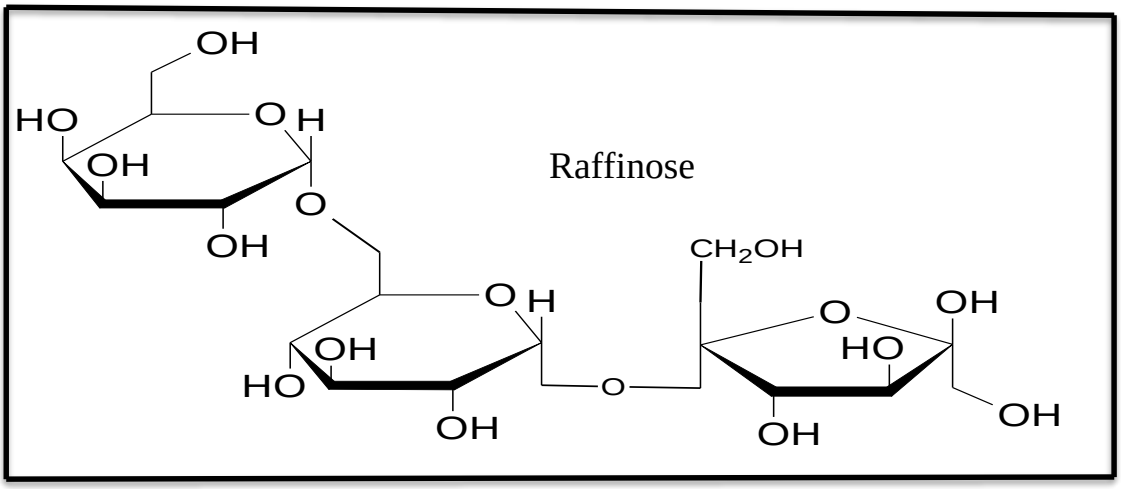




Sucrose

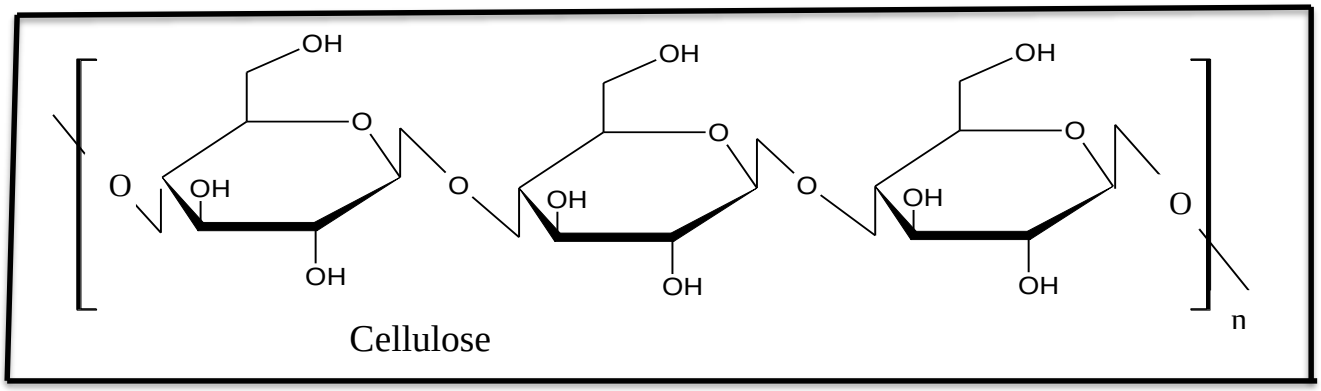
3- Oligosaccharides :

They are sugars containing three to ten monosaccharide joined by glycosidic bond.



4- Polysaccharides :

They are sugars of high molecular weight, such as polymers of monosaccharaides. Examples include storage polysaccharides such as starch and glycogen, and structural polysaccharides such as cellulose and chitin. All polysaccharides are not reduced because of their large molecular weight, although they contain OH group.

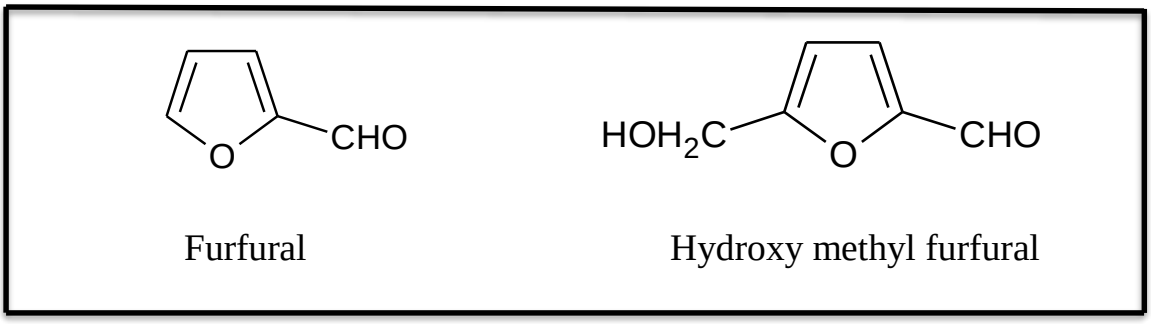


Cellulose

Qualitative tests of Carbohydrates

1- **Molicsh`s test :**

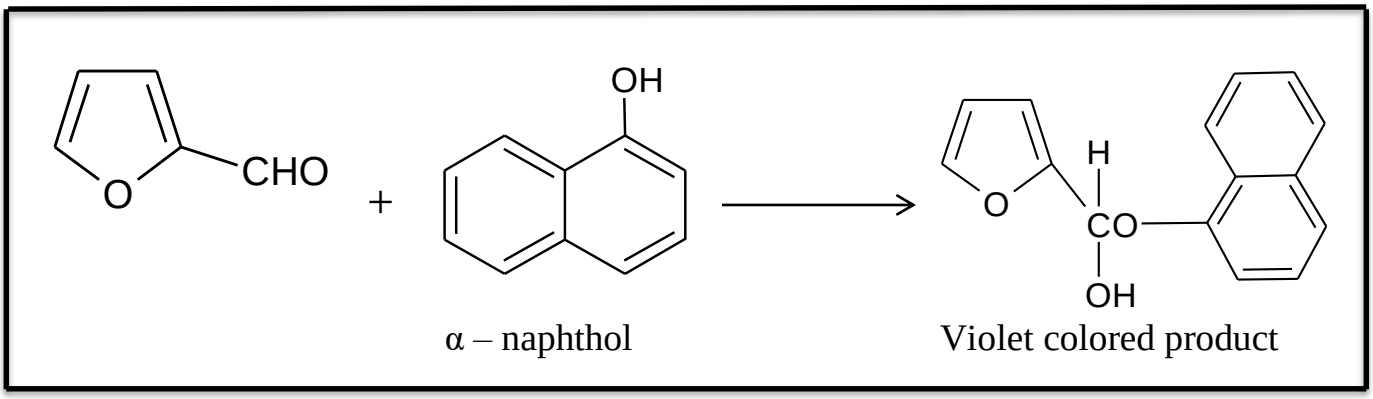
It is a general test of carbohydrate based on the dehydration of carbohydrate by a using strong acid such as sulfuric acid H_2SO_4. Pentoses are dehydrated to furfural, while hexoses are dehydrated to hydroxy methyl furfural, then furfural reacts with α-naphthol to give violet colored product (purple ring).



Furfural Hydroxy methyl furfural

Method :

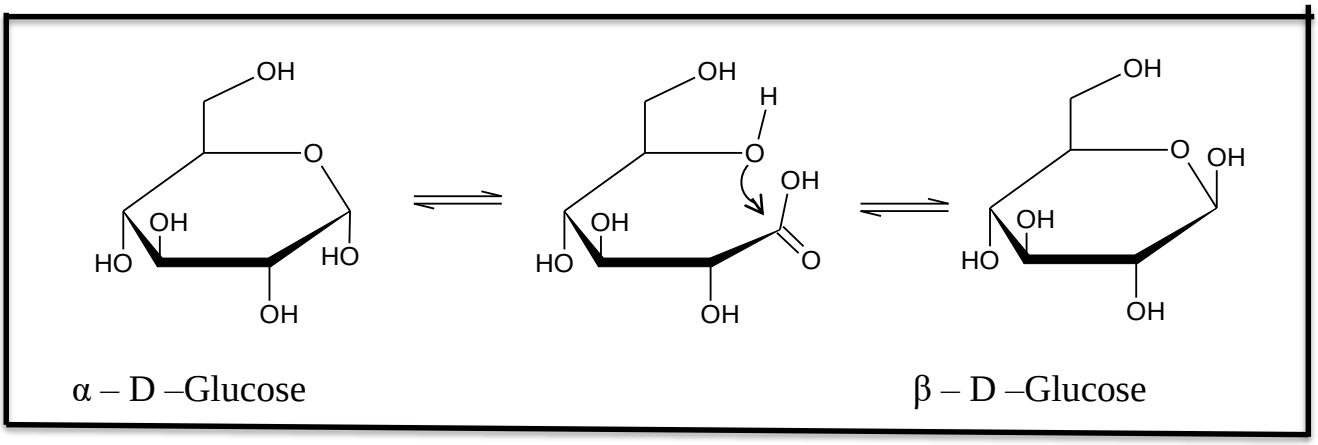
1 – In a clean and dry test tube, 2 drops of α-naphthol are added to 0.5 ml of sugar solution and mixed well.
2 – A small amount of concentrated sulfuric acid is slowly added down the sides of the sloping test tube without mixing.
3 – The violet colored product (purple ring) will appear between two layers (water and organic layers).



α – naphthol Violet colored product

Detecting the reduction properties:

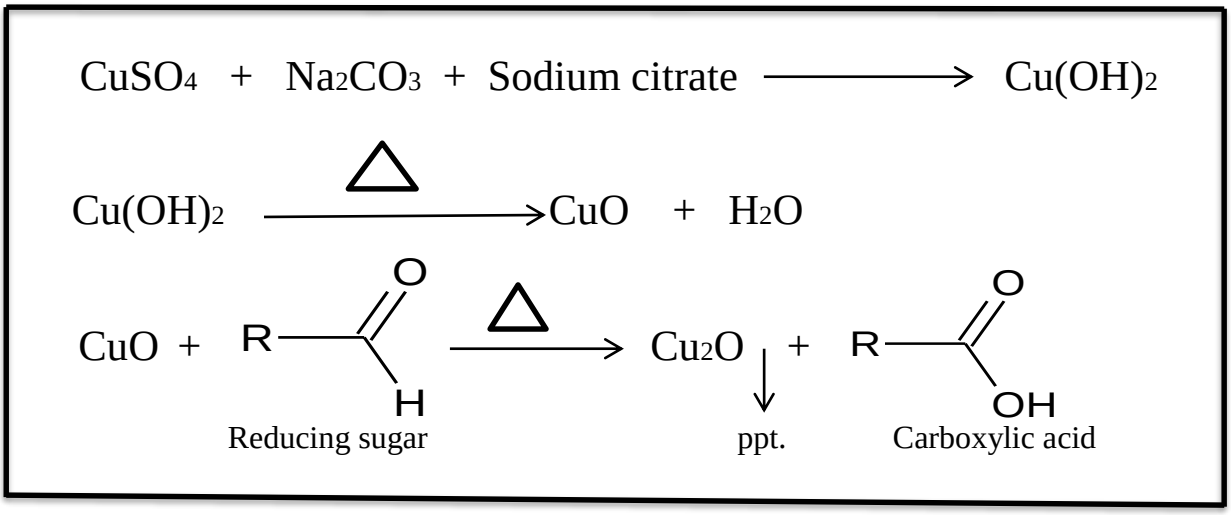
Sugars generally have reduction properties such as aldehydes and ketones, which contain free carbonyl group. The reduction properties depend on the number of monosaccharaide units, for example disaccharides have less reduction than monosaccharide, and so for polysaccharides (is not considered to be a reduction in the laboratory) although they contains free carbonyl group because they have large size of the molecule. The open aldehyde or ketone form in the sugar solution (where the oxidation process is performed) is 1% only and the rest 99% will be cycle.



α – D –Glucose β – D –Glucose

2-Bnedict`s test :

It is a general test of all reducing sugars (this include monosaccharaides and disaccharides). The cupric ions (Cu^{+2}) are reduced in a weak alkali medium to the cuprous ions (Cu^{+}) which get precipitated as insoluble red copper (I) oxide. Benedict`s reagent can be used to test for the presence of glucose in urine (glucosuria) and can be indicative of diabetes mellitus.



Method :

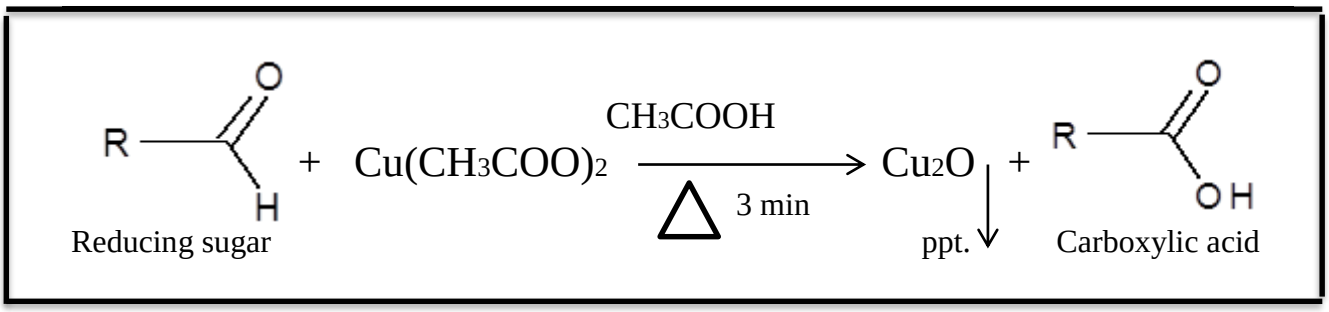
1- Benedict`s reagent is prepared by adding 173 gm of sodium citrate and 100 gm of sodium carbonate anhydrous to 800 ml of distilled water, the solution is filtered, then 17.3 gm of copper (II) sulfate pentahydrate is added and the volume of the solution is completed to the 1000 ml by using distilled water.
2- In a clean and dry test tube, 1 ml of Benedict`s reagent is added to 1ml of sugar solution and mixed well.
3- The solution is heated in a boiling water bath for 3-5 min.
4- The change in the color of the solution is noticed (red precipitate is present).

Caution:

All sugars give a positive reaction only sucrose, because it has not the free reduction group.

3-Barfoed`s test :

It is a chemical test used to detect the presence of monosaccharides. In this test, the cupric ions (Cu^{+2}) are reduced in a weak acidic medium to the cuprous ions (Cu^{+}) which get precipitated as insoluble red copper (I) oxide after cooling. Only monosaccharide can reduce the cupric ions in the weak acidic medium (hardly), so we can distinguish between monosaccharides and disaccharides. The heating time has an important role in determining the positive detection (increasing in time, disaccharide will be able to gives a positive result because disaccharide will be hydrolyzed in weak acidic medium to a smaller units (monosaccharide).

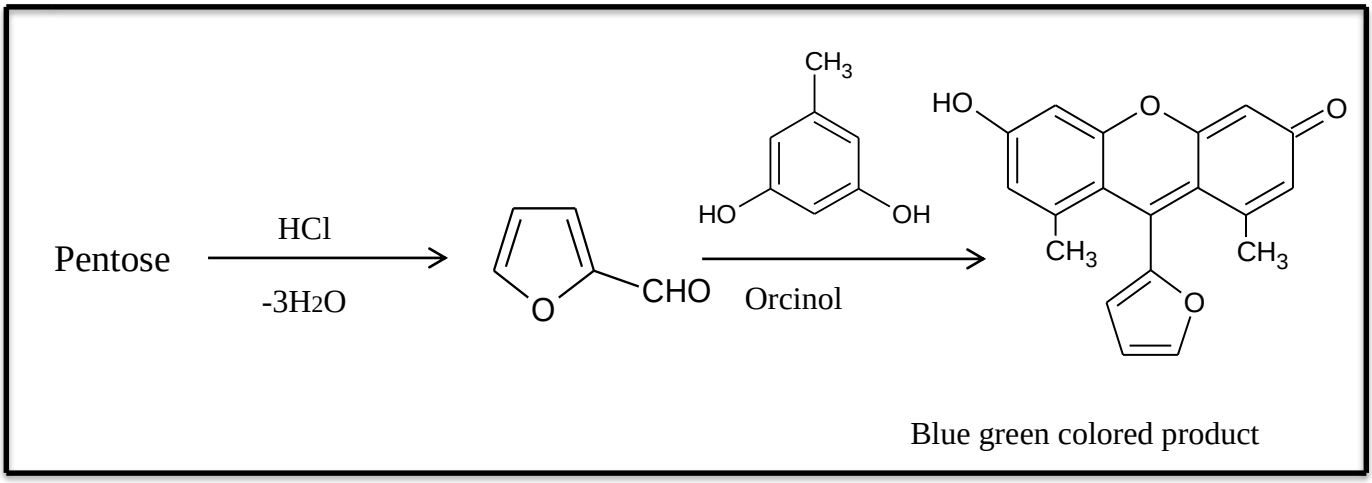


Method :

1- Barfoed`s reagent is prepared by adding 13.3 gm of copper acetate in 200 ml distilled water, the solution is filtered, then 1.8 ml of glacial acetic acid are added to the solution.
2- In a clean and dry test tube, 1 ml of Barfoed`s reagent is added to 1ml of sugar solution.
3- The solution is heated in a boiling water bath for 3 min.
4- The change in the color of the solution is noticed (red precipitate is present).

4-Bial`s test:

It is a special test of pentose like ribose and xylose. Pentose interact with concentrated hydrochloric acid to get furfural, then furfural reacts with orcinol to get a blue green colored solution. The hexose interacts with concentrated hydrochloric acid to get a hydroxyl methyl furfural which react with orcinol to get a brown colored solution.



Method :

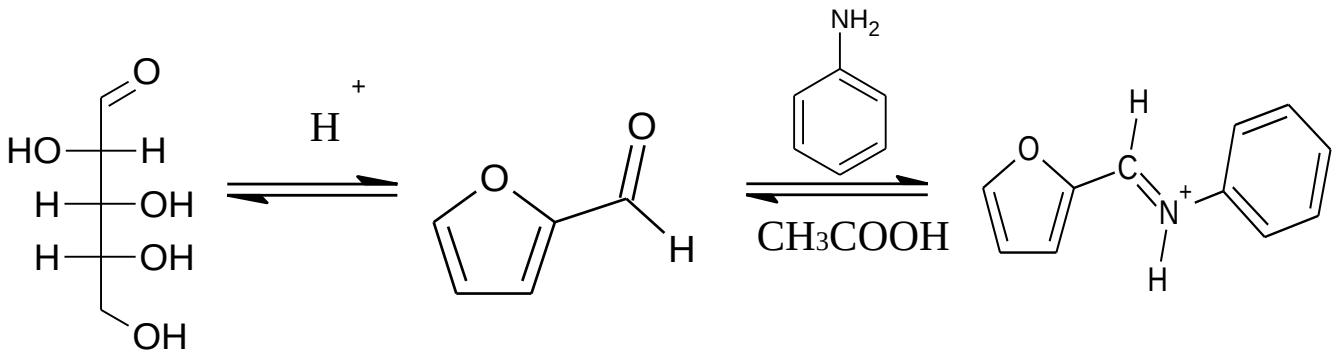
1- Bial`s reagent is prepared by adding 1.5 gm of orcinol in 500 ml concentrated hydrochloric acid, then 1 ml of 10 % ferric chloride is added to the solution.
2- In a clean and dry test tube, 1 ml of Bial`s reagent is added to 1ml of sugar solution.
3- The solution is heated in a boiling water bath for 3-5 min.
4- The change in the color of the solution is noticed (Blue green colored solution is present).

Caution :

In the case of the uses large amount of pentose, we will get a blue-purple colored product.

5-Aniline acetate test :

It is another test to detect pentose. Pentose interact with concentrated hydrochloric acid to get furfural, then furfural react with aniline reagent to get a bright pink colored solution but hexoses is not produce a pink colored solution.



Arabinose furfural Pink colored product

Method :

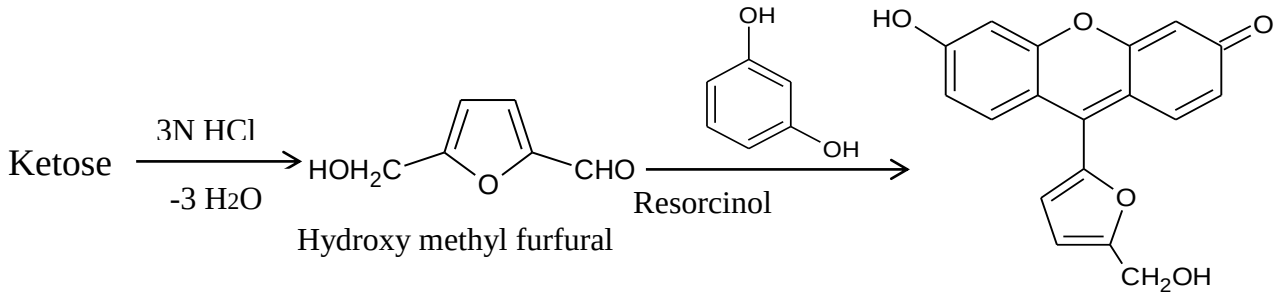
1- Aniline reagent is prepared by adding 5 ml of aniline in 5 ml distilled water, then 5 ml of glacial acetic acid is added and mixed well.
2- In a clean and dry test tube, 1 ml of Bial`s reagent is added to 1ml of sugar solution. The change in the color of the solution is noticed (pink colored solution is present).

6- Seliwanoff`s test :

It is a special test of ketose, where they interact with concentrated hydrochloric acid 3N HCl to get hydroxyl methyl furfural, then hydroxyl methyl furfural react with resorcinol to get a pink red colored solution with attention of heating time.

Method :

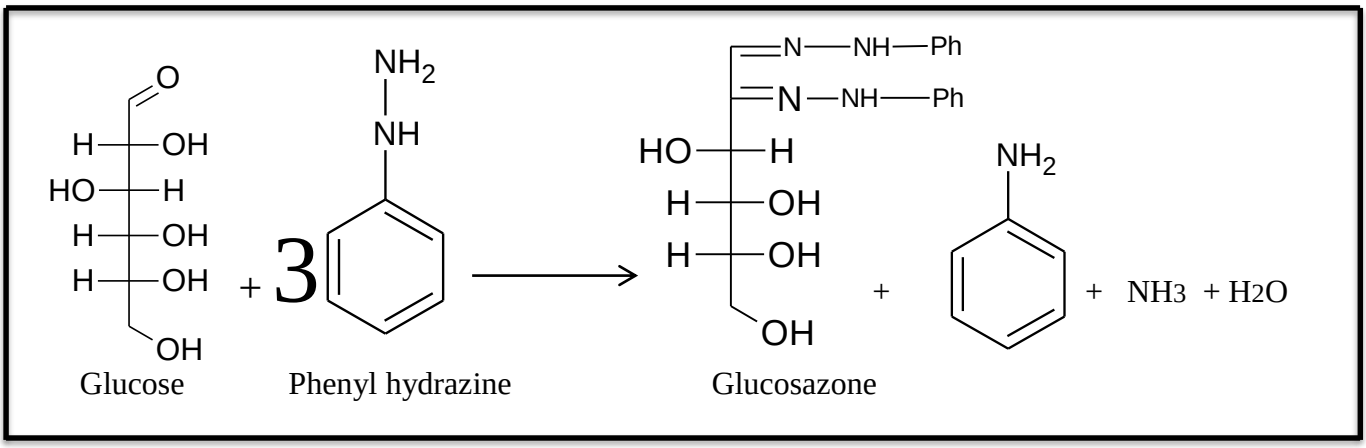
1- Seliwanoff`s reagent is prepared by adding 0.05 gm of resorcinol in 500 ml concentrated hydrochloric acid 3N HCl.
2- In a clean and dry test tube, 1 ml of Seliwanoff`s reagent is added to 1ml of sugar solution.
3- The solution is heated in a boiling water bath for 3-5 min. The change in the color of the solution is noticed (pink colored solution is present).



Pink colored product

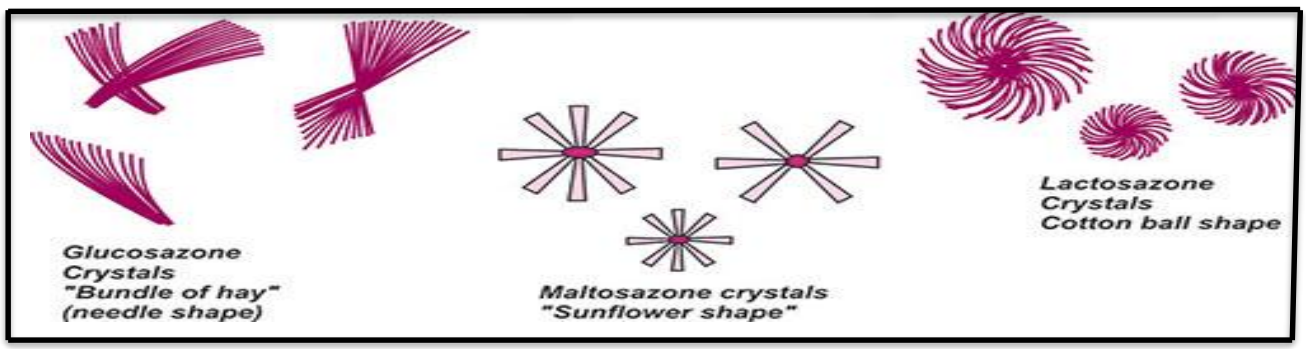
7-Osazone test :

The compounds that contain free aldehyde group or free ketone group (free reduction groups) react with phenyl hydrazine to get a yellow crystals with different geometric shapes and certain melting points that can be easily detected under a light microscope. Glucose and galactose can be distinguished by this test only. Sucrose is the only sugar that does not give a positive reaction because it does not contain free reduction group. The osazone crystals for monosaccharide, other than galactosazone, are not dissolved in hot solution, but maltosazone and lactosazone crystals are dissolved in hot water which get precipitated when leaving the tubes to be cooled.



Method :

1- In a clean and dry test tube, 2 ml of phenyl hydrazine reagent (3 gm of sodium acetate + 3 gm of phenyl hydrazine) are added to 2ml of sugar solution. The solution is heated in a boiling water bath with shaking for 30 min.
2- The osazone crystals for monosaccharide will appear and separated into the hot solution after ten minutes.
3- If the crystals do not appear after 30 minutes (at the end of the period without separation of crystals), there is a possibility that disaccharide is presence. The solution is cooled and the osazone crystals will appear.
4- The crystals are placed at a microscope slide and the shape of crystals is noticed under a light microscope.



Polysaccharide:

They are tasteless and odorless compounds contain a large number of monosaccharide chains and these chains are either branched like a glycogen, or straight like cellulose.

8-Iodine test :

Iodine test is used to detect the presence of starch or dextrin. A triiodide (I_3^-) solution formed by mixing iodine and iodide (usually from potassium iodide KI). Starch contains an alpha – amylose (a helical saccharide polymer) and amylopectin. Iodine forms a coordinate complex between the helically chain and iodine depending on the occurrence of the adsorption process and gives a dark blue color for starch and violet color for dextrin. This process occurs at room temperature because the high temperature lead to increase the motion of the particles and that will be not help the process of adsorption to occur, so when we increase the temperature, the color of the test will disappear .This test is influenced by:

1- **The temperature** ; when the temperature increased, the color of the test will disappear.

2- **pH of the solution**; This reaction occurs in the acidic or neutral medium and it does not occur in the alkali medium because iodine will react with NaOH , the reaction will give iodides and iodates salts according to the following reaction:

$$3I_2 + 6NaOH \longrightarrow 5NaI + NaIO_3 + 3H_2O$$

But when HCl is added, iodine will be returned again and the color will be back again according to the following reactions:

$$NaIO_3 + HCl \longrightarrow HIO_3 + NaCl$$

$$5\,NaI + 5\,HCl \longrightarrow 5HI + 5\,NaCl$$

$$HIO_3 + 5HI \longrightarrow 3\,I_2 + 3\,H_2O$$

Method ;

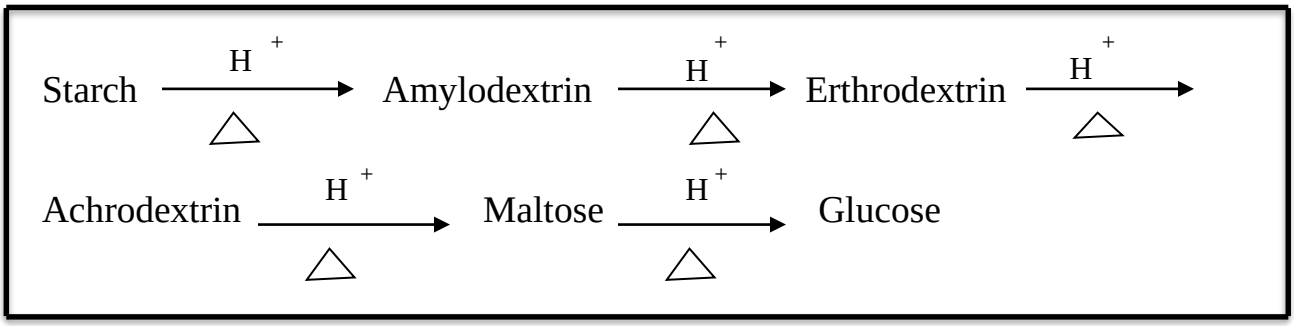
1- In a clean and dry test tube, 2-3 drops of Iodine solution are added to 1 ml of starch solution, the dark blue color will appear.
2- After that, the tube is heated in the water bath and then cooled it. The different between heating and cooling is noticed when you continue the process of heating and cooling, and we will reach a stage where the color will disappear and not return after cooling because iodine will evaporate as a whole.

Caution ;

The I_2/KI solution is toxic, corrosive, and an irritant. If you spill the solution on yourself or on the bench, immediately notify the instructor of the laboratory.

Hydrolysis of starch by acid;

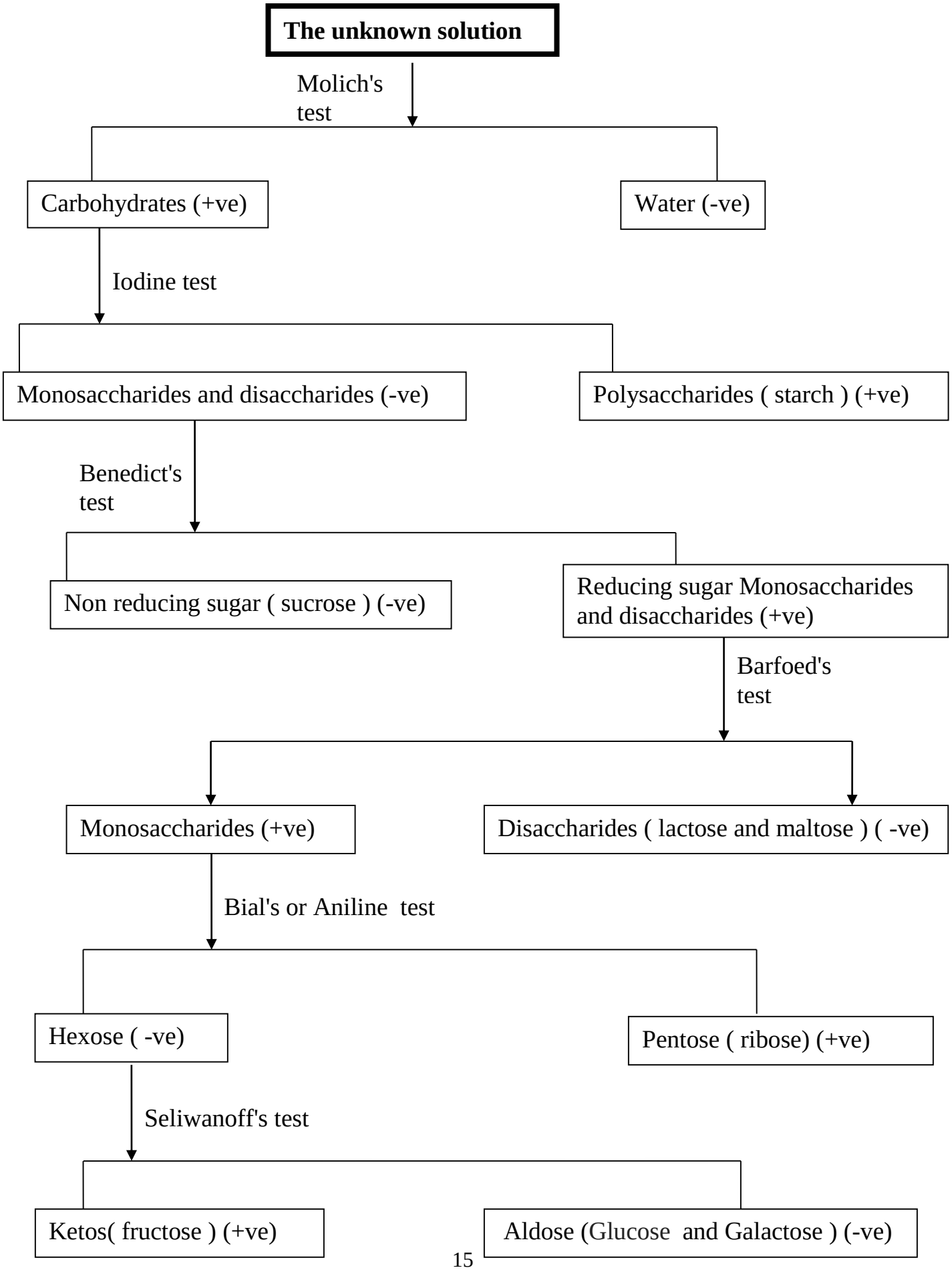
The process of hydrolysis of starch occurs with HCl and it occurs sequentially and thermally stimulated according to the following reaction:



Method ;

1- In a clean and dry test tube, 3 ml 3N HCl are added to 10 ml of starch solution with shaking.
2- The solution is heated in the boiling water bath.
3- After every three minutes, 1 ml of the solution during times 0, 3, 6, 9, 12, 15 min is taken and divided it into two parts, the first part is used to iodine test, and the second to the benedict's test.

Time	Sugar	Benedicts test	Iodine test
0	Starch	(-) Not reduced	(+) Dark blue color
3	Amylodextrin	(-) Not reduced	(+) Violet color
6	Erthrodextrin	(-) Not reduced	(+) Red color
9	Achrodextrin	(-) Not reduced	(+)Yellow–brown color
12	Maltose	(+) Reduced	(-) Yellow color
15	Glucose	(+) Reduced	(-) Yellow color


The unknown solution
Molich's test
Carbohydrates (+ve)
Water (-ve)
Iodine test
Monosaccharides and disaccharides (-ve)
Polysaccharides (starch) (+ve)
Benedict's test
Non reducing sugar (sucrose) (-ve)
Reducing sugar Monosaccharides and disaccharides (+ve)
Barfoed's test
Monosaccharides (+ve)
Disaccharides (lactose and maltose) (-ve)
Bial's or Aniline test
Hexose (-ve)
Pentose (ribose) (+ve)
Seliwanoff's test
Ketos(fructose) (+ve)
Aldose (Glucose and Galactose) (-ve)

Qualitative test of Lipids

Foods high in lipids:







Lipids

Lipids are heterogeneous organic substances that are soluble in non-polar organic solvents such as benzene, ether, chloroform, but they are insoluble in water. The functions of lipids are:

1- Storing energy (A form of energy stock) .
2- Acting as structural components of cell membranes .

There are different types of lipids contain non-polar structures similar to hydrocarbons, which are given an oily or waxy state insoluble in water.

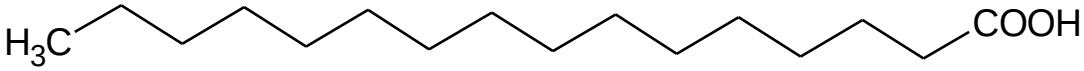
Lipids are divided by chemical properties into types:

1- **Simple lipids**: contains fats, oils, and waxes.
2- **Compound lipids**: contains phospholipids, glycolipids, sphingolipids.
3- **Derivative lipids:** contains cholesterol(Steroids) and fat-soluble vitamins.

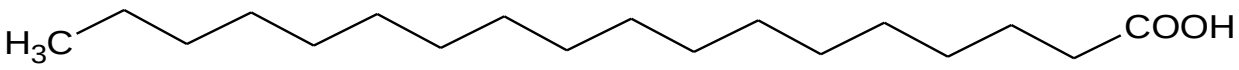
fatty acids :

A fatty acid is a carboxylic acid with a long aliphatic chain, which is either saturated or unsaturated. They are principal building blocks of simple and complex lipids. Fatty acids that have carbon–carbon double bonds are known as unsaturated, while fatty acids without double bonds are known as saturated. Most naturally occurring fatty acids have an unbranched chain of an even number of carbon atoms from 4 to 28.

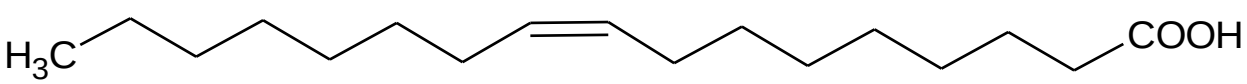
16:0 Palmitic acid



18:0 Stearic acid



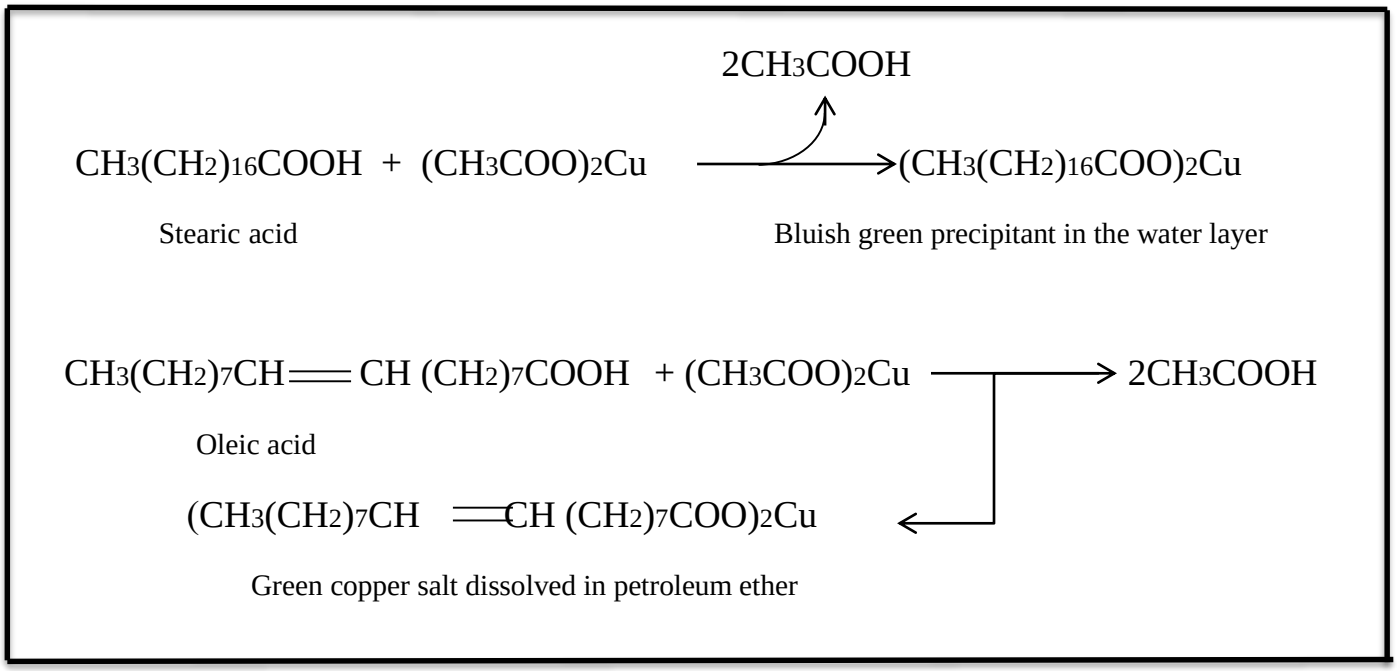
18:1 cis Δ^9 Oleic acid



Qualitative tests of lipid :

1-Copper acetate test for detecting fatty acids:

This test is used to distinguish between neutral fat and fatty acids (saturated and unsaturated). In this test, Triglyceride does not react with copper acetate solution, while fatty acids react with copper acetate to form copper salt. Saturated fatty acids react with copper acetate solution and give (bluish green precipitant in the water layer), while the unsaturated fatty acids give (green copper salt dissolved in petroleum ether (organic layer)).



Method :

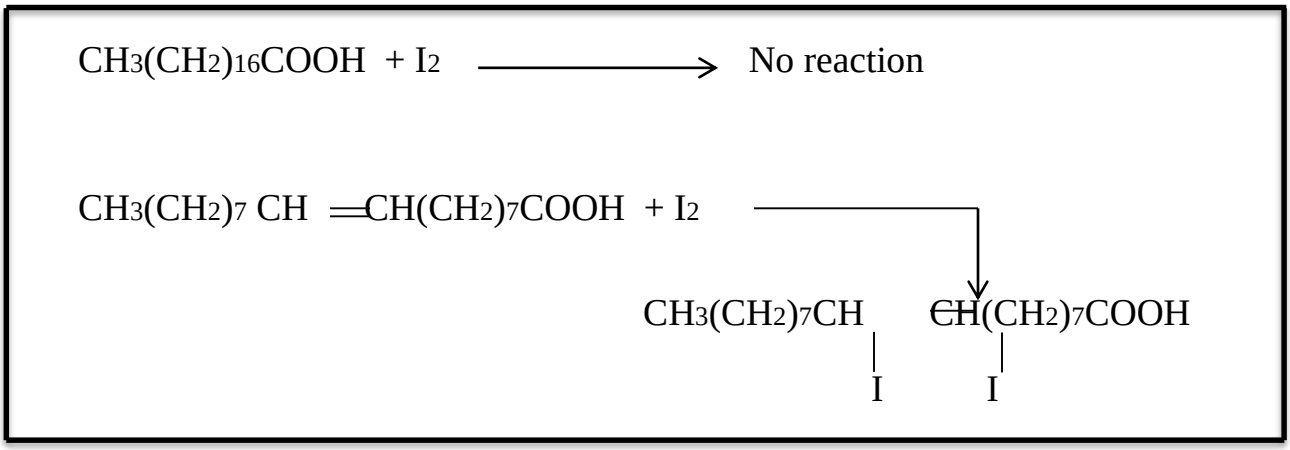
1- In a clean and dry test tube, 10 drops of 10% copper acetate are added without shaking to 1ml of unsaturated fatty acid (oleic acid that dissolved in petroleum ether), then 1ml of saturated fatty acid (stearic acid dissolved in petroleum ether) is added in the other test tube and the 10 drops of 10% copper acetate are added in the same tube (without shaking).

2- The different between two tubes is noted that the first reaction is given a green copper salt dissolved in petroleum ether and the second reaction is given a bluish green precipitant in the water layer.

Caution :
Do not shake the test tube because it will give a heavy emulsion.

1- Iodine test :

Fatty acids found in animal fats are usually fully saturated while those in plant oils (or vegetable oils) are generally unsaturated (contain one or more of double bonds). When iodine solution is added to the unsaturated fatty acid, the iodine will reacts with the double bonds and as a result the pink color will disappear (decolorization). On the other hand, the iodine color does not change when it added to the saturated fatty acids. Iodine test is used for distinguish between saturated and unsaturated fatty acids as well as between oils and fats.



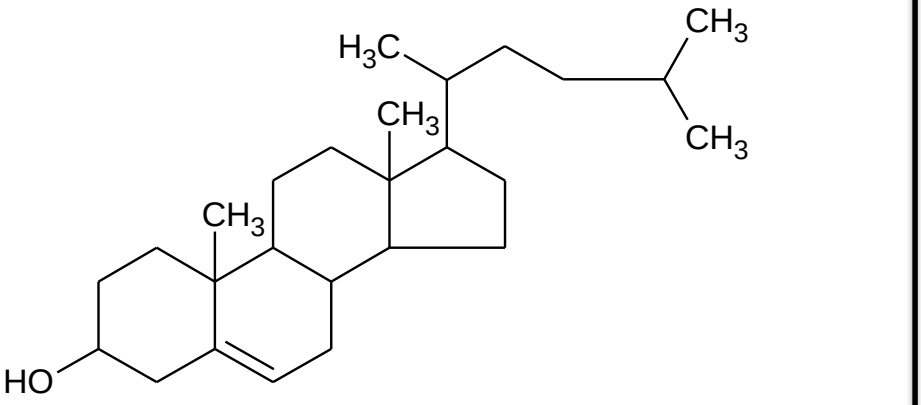
Method :

1- In a clean and dry test tube, Iodine solution is added drop by drop to the two test tubes, the first one contain saturated fatty acid and the other one contain unsaturated fatty acid.
2- The tubes are shaken after addition of iodine solution until.
3- The disappearance of iodine pink color is an indication of the presence of unsaturated fatty acids

3- Qualitative tests of cholesterol:

Cholesterol is a steroid lipid built from four linked hydrocarbon rings. It is an amphipathic molecule, with a polar head group (the hydroxyl group at C-3) and

nonpolar hydrocarbons body (the steroid nucleus and the hydrocarbon side chain at C-17), about as long as a 16- carbon fatty acid in its extended form.



Cholesterol $C_{27}H_{46}O$

When steroids that contain unsaturated bonds are treated in non-aqueous conditions with strong acids, they interact yield distinct color outputs depending on the conditions of the experiment, the resulting colors show significant differences from one compound to another.
There are the tests used to detect cholesterol:

1- *Salkowaki test* :

Salkowski test is used to detect cholesterol in a solution. It is an important test used to detect cholesterol depending on the colors (distinct and clear colors) that yield from the reaction of cholesterol with concentrated sulfuric acid.

Method:

1- One milliliter of cholesterol is added to a test tube and then the same volume of H_2SO_4 is added and shaken well.

2- The tube is allowed to stand until the mixture separates sharply into two layers; the top layer is red and the bottom layer is green.

Caution: In order for this test to be successful, the tubes must be dry and the solutions should be non-aqueous.

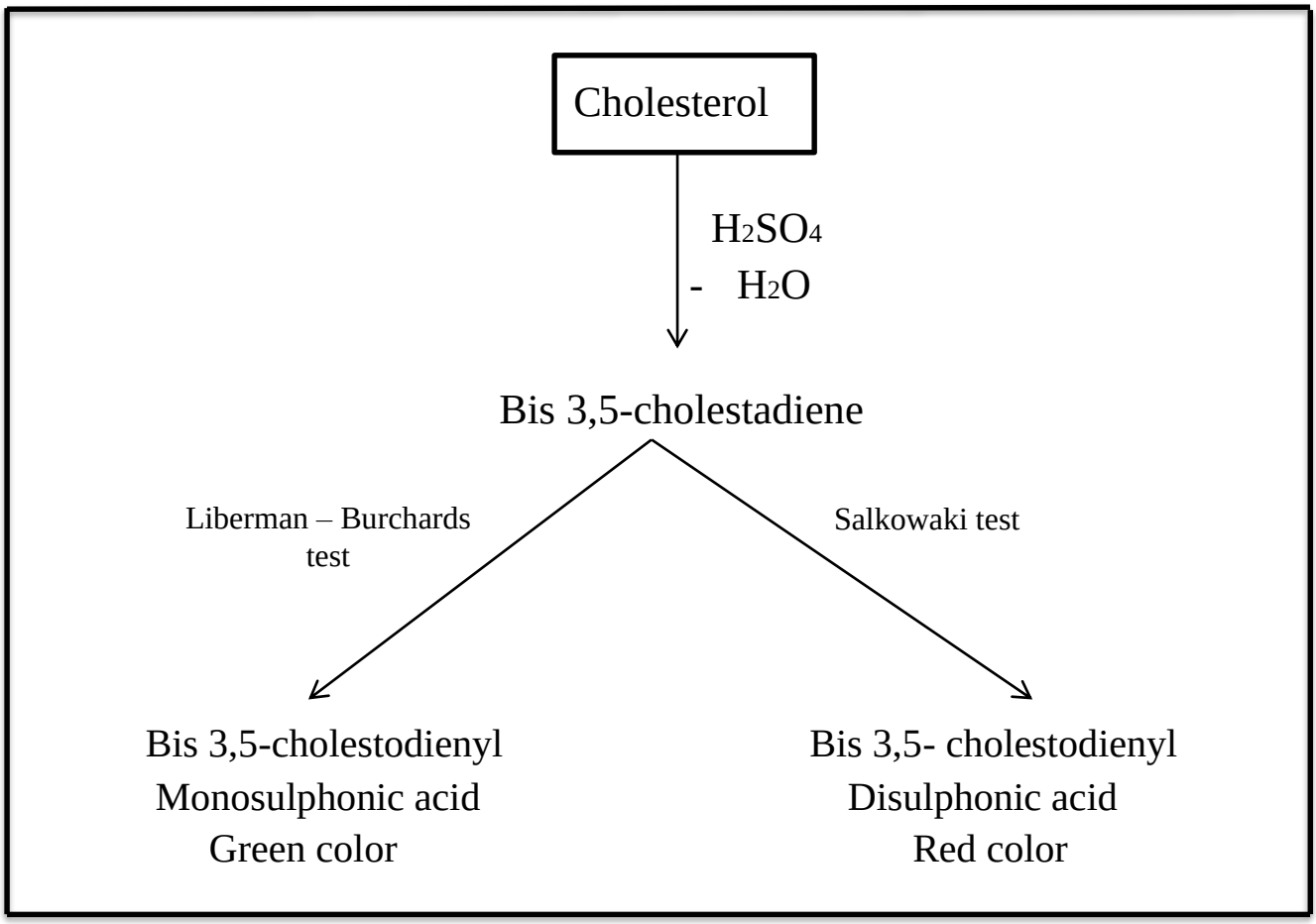
2 - *Liberman – Burchards test :*

Liebermann – Burchard test is a chemical estimation of cholesterol. The cholesterol is react as a typical alcohol with a strong concentrated acids to give colored substances. In this test acetic anhydride are used as solvent and dehydrating agents, and the sulfuric acid is used as dehydrating and oxidizing agent. After adding these acids to the cholesterol solution a result is observed when the solution becomes red or pink, then purple, blue and finally bluish – green colour.

Method :

1- In a clean and dry test tube, 1 ml of 5% cholesterol dissolved in chloroform is added to 1 ml of acetic anhydride., then 2 drops of H_2SO_4 are added to the same tube and shaken well.

2- The color change is noticed (Observe the appearance of pink color which gradually turns into deep green).

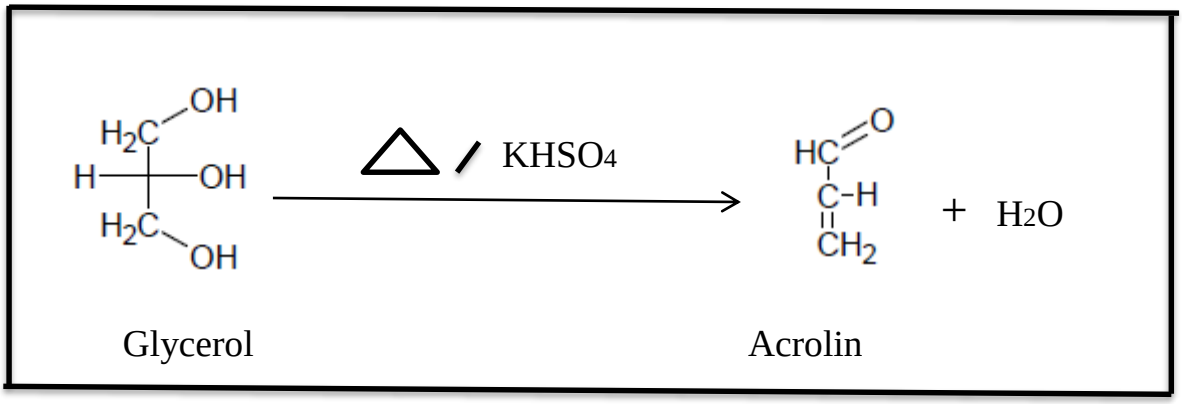


4-Acrolin test:

Acrolin test is a general test for the presence of glycerin or fats. It is an important test for glycerol where in the presence of a dehydrating agent potassium bisulfate ($KHSO_4$); the glycerol part of the molecule is dehydrated and formed a volatile substance with a smell that is similar to the smell of burned fat (unsaturated aldehyde) that called Acrolin. This test is distinctive for glycerol whether it is free or combined with fatty acids.

Method :

1- In a clean and dry test tube, 0.5 ml of glycerol is added with 0.5ml of $KHSO_4$.
2- The tube is heated and the change of the solution in the tube is noticed.



Glycerol Acrolin

5-Rancidity of lipids:

Rancidity is a complete or incomplete oxidation or hydrolysis of fats and oils process which is accompanied by the formation of undesired odor and taste, as a result of the action of moisture, air (O_2) and enzymes. This process occurs to fat because it contains volatile fatty acids (VFA), where there is a change in its physical and chemical properties. To guard against rancidity it should protect fats from moisture and direct light as well as its storage must be in a cold place to deactivate lipase. There are two types of rancidity:

1- Hydrolytic rancidity:

It is a process occurs by enzymes or microorganisms that causing the release of volatile short chain fatty acids (VFA) with undesirable smell (as it happens

for butter). The two factors that accelerate this type of rancidity are (heat and humidity).

2- Oxidative rancidity:

Oxidative rancidity occurs especially in oils containing unsaturated fatty acids where these acids are oxidized and converted into compounds with a short chain (ketones, aldehydes and volatile fatty acids). The factors that accelerate this type of rancidity are (oxygen, light and heat). Oxidative rancidity can be prevented by light-proof packaging, oxygen-free air-tight containers and by the addition of antioxidants.

Method :

1- A fresh reagent is prepared by adding 2 drops of 1% phenolphthalein to (0.5%) NaOH.
2- A sample of old and new fat is putting in two separate test tubes, then the previous reagent is added to each tube (drop by drop).
3- The colors formed in both tubes are noticed.

6- Acid value of lipids*:*

Acid value is a common parameter in the specification of lipids. Lipid is become rancid as a result of storage. As a result of rancidity process, the amount of fatty acids found in fat gives a large indicator about the age and quality of lipid.

Acid value: It is the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in one gram of lipid.

Method:

1- In a clean conical flask 2 ml of 12% of olive oil are added, and then 2 drops of Phenolphthalein are added.
2- The solution in the conical flask is titrated with 0.1N KOH until the pink color is appeared (equivalence point).

Calculation :

1- Calculation of weight of oil :

12	**100**
X	**2**

X = 0.42 g (weight of oil in the sample).

2- Calculation the volume of base :

$$\mathbf{N} = \frac{\text{wt.}}{\text{M.wt}} \times \frac{1000}{V_{ml}} \qquad 0.1 = \frac{\text{wt.}_{KOH}}{56} \times \frac{1000}{V_{ml}}$$

Multiply (M x 1000) to convert it into milligrams.

M is represents the number of milligrams KOH that needed to equalize free fatty acids found in (0.24) gram of lipid.

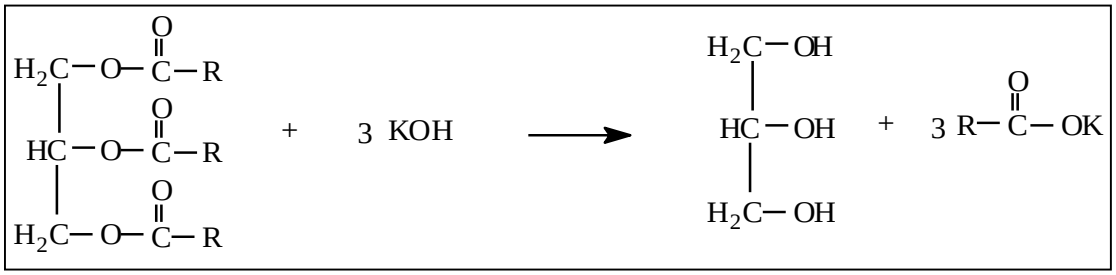
M	0.24
X	1

X = ? mg

X is represents the number of milligrams (KOH) that needed to equalize free fatty acids found in 1 gram of lipid.

7-The saponification value:

Saponification is a process that involves conversion of fat or oil into soap and alcohol in the presence of aqueous alkali. Oils can be saponified with KOH to form soap and glycerol as follows:



Saponification number:

is the number of milligrams of potassium hydroxide (KOH) required to neutralize free fatty acids that produced by the complete decomposition of one gram of lipid. At the condensation process, the triglyceride is treated with a strong base, which cleaves the ester bond, releasing glycerol and potassium salts of fatty acids (soap). The saponification value gives important information about the nature of fatty acids found in lipid.

Method:

1- One gram of lipid is weighted and added into a conical flask and dissolved in a suitable solvent.

2- Twenty five ml of potassium hydroxide KOH (0.5 mole/L) is added.

3- At the same time, another conical flask containing all the components of the first conical flask is prepared without adding lipid (the blank flask).

4- The process of condensation for two conical flasks is done for a 30 minutes, then the conical flasks are cooled by leaving them at room temperature.

5- The contents of the two conical flasks are titrated against hydrochloric acid (0.5 mole/L) in the presence of phenolphthalein as an indicator.

Calculation :

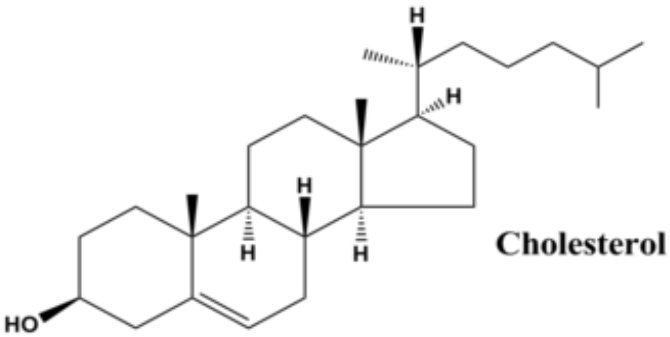
The difference in volumes between two conical flasks gives the number of millimeters of KOH required for the saponification of 1 gm of lipid.

M.Wt of KOH = 56 gm/mol.

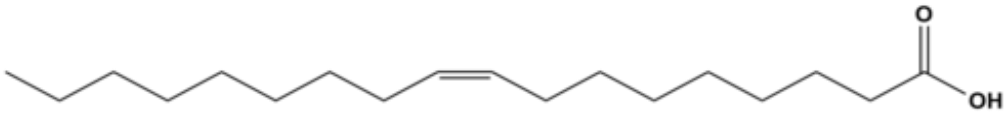
As the three molecules of fatty acid are removed from the triglyceride, so:

$$\text{Saponification value (S)} = \frac{3 \times 56 \times 1000}{\text{Average molecular weight of fat}}$$

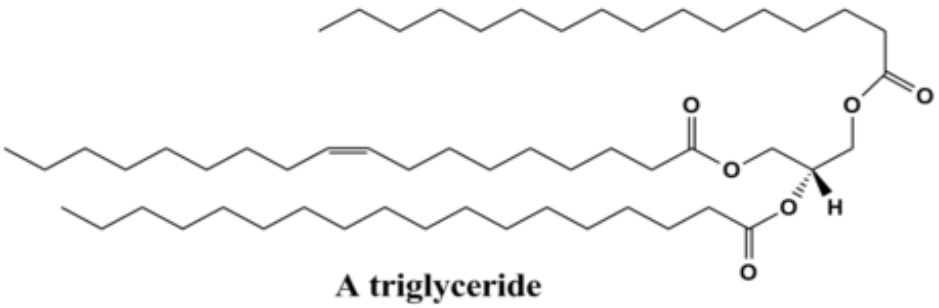
$$\text{Average molecular weight of fat} = 3 \times 56 \times 1000 / S$$



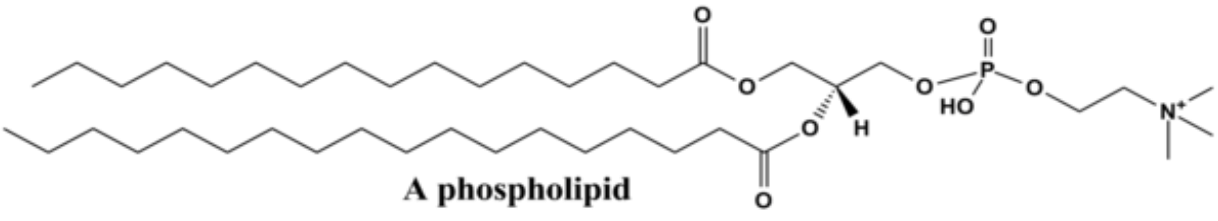
Cholesterol



A free fatty acid

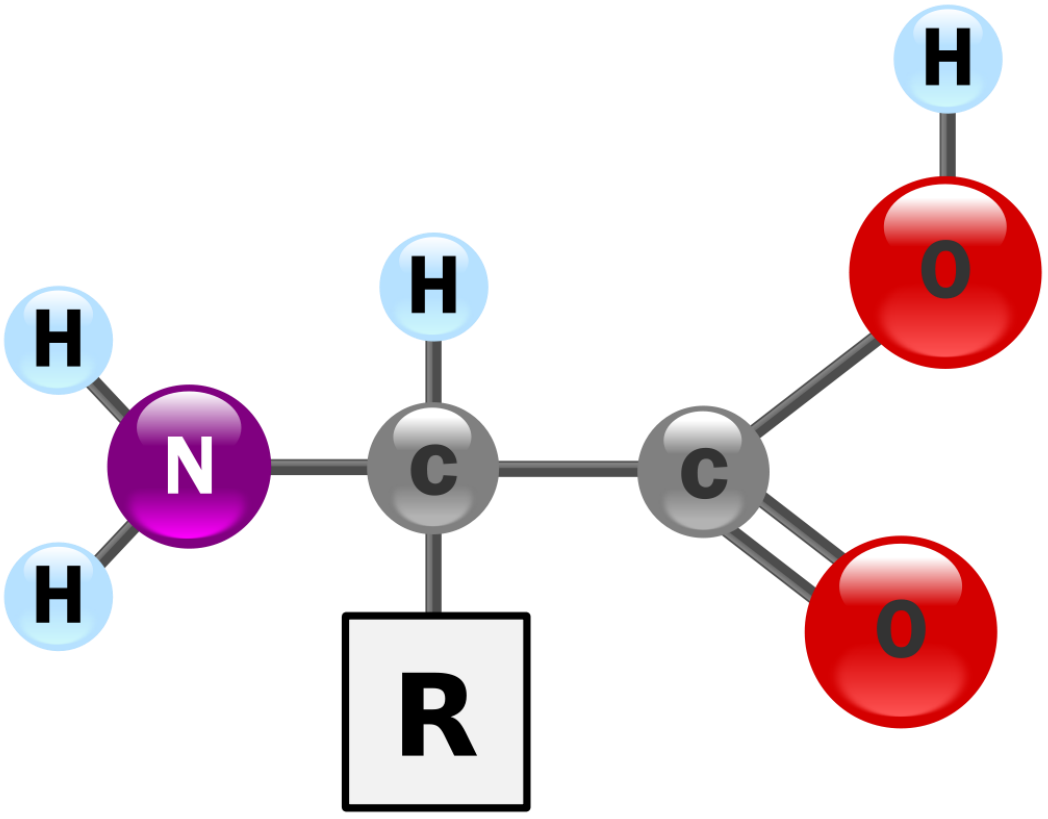


A triglyceride



A phospholipid

Qualitative test of amino acids



Amino acids

They are organic compounds that have two or more active groups. All amino acids contain an acidic group (carboxyl group COOH) and an alkaline group (amino group NH_2), because of that, amino acids have an amphoteric behavior (weak acid and weak base behavior).

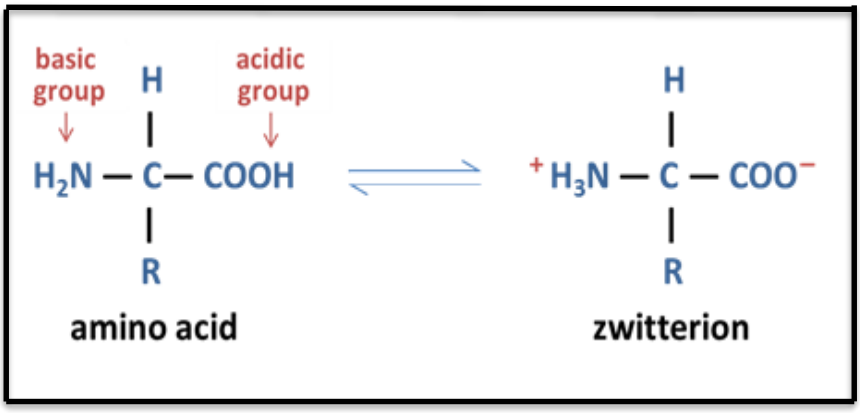
There are 20 amino acids which act as building blocks of protein molecules found in living tissues, including eight (in adult person), the body cannot manufacture them sufficiently, that is why it should be got from food or through medication.

They are crystalline materials, which are different from each other in taste such as:

1- Alanine, Serine, Glycine and Proline have sweet test.

2- Tryptophan has no test.

3- Arginine has bitter test.



All amino acids have high melting point, they are dissolved in polar solvents such as water and ethanol but not dissolved in non-polar solvents such as benzene, hexane and ether. They are optically active because they contain an asymmetric carbon atom except Glycine. Amino acids are always in the form of a Zwitter ion when **pH** equals to **pI.**

(pI) Isoelectric point: It is a certain pH value at which the sum of the negative charges equal to the sum of the positive charges (the molecule carries no net electrical charge).

Qualitative test of Amino acids

1- Ninhydrin test :

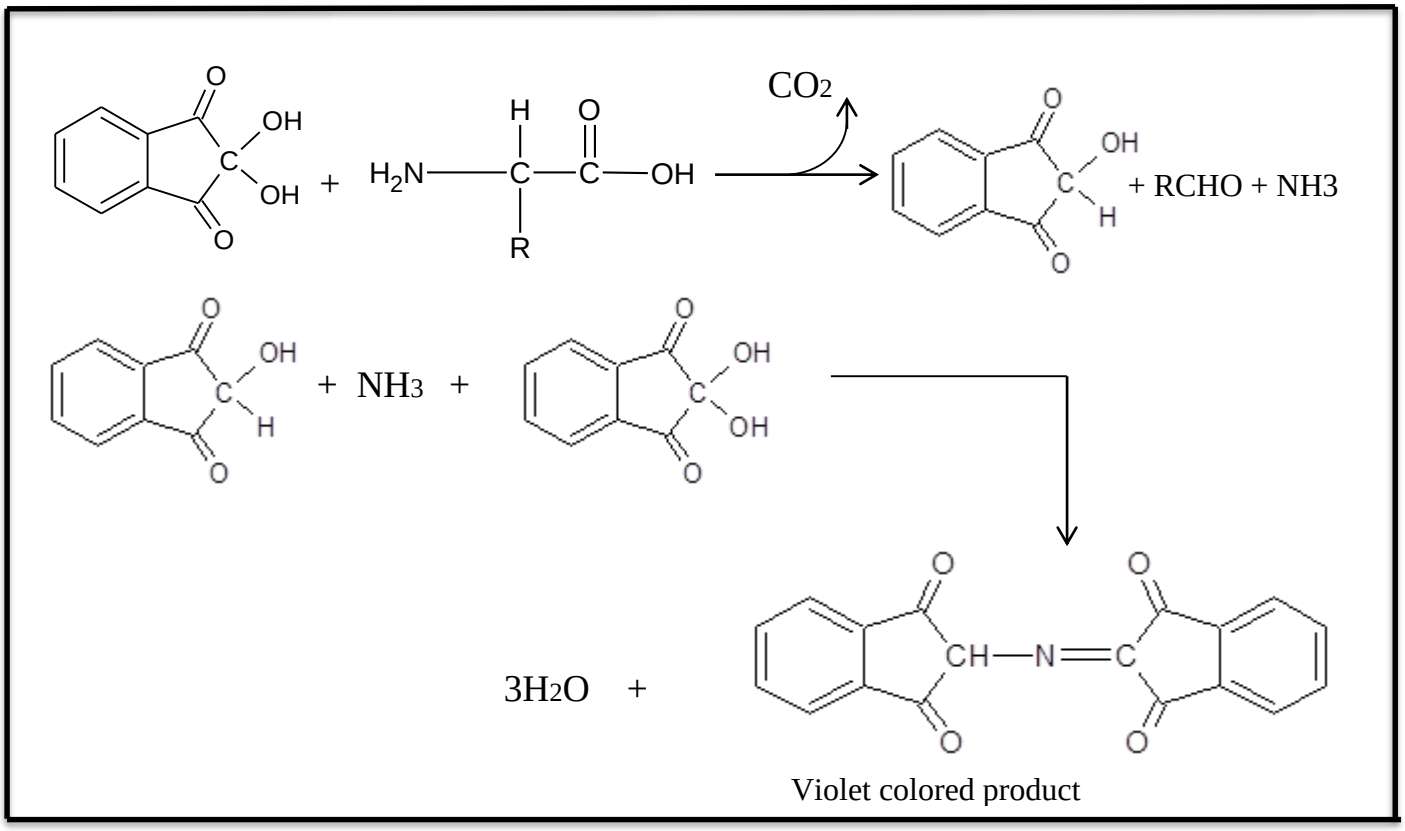
It is a general test of all amino acids and proteins. Amines and ammonia also give a positive ninhydrin test, but without releasing CO_2. The reaction is based on the presence of amino and carboxylic groups. Ninhydrin solution is very strong oxidizing agent that reacts with amino acids and yield aldehyde RCHO , CO_2 , NH3 , and hydrindantin which reacts with ammonia and another ninhydrin molecule to yield violet colored solution (but it gives a yellow colored solution with Proline). We can use this test in quantitative estimation of amino acids (the amount of free CO_2 can be used to estimate the amount of amino acid).

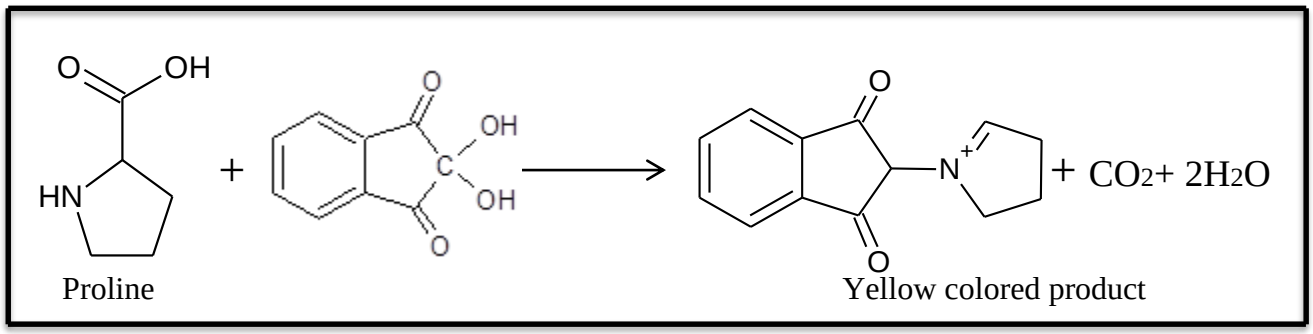
Method :

1 –In a clean and dry test tube, 10 drops 0.2% of ninhydrin solution are added to 1ml of amino acid solution and mixed well.

2 -The solution is heated in a boiling water bath for 5 min.

3 – A violet color will develop.





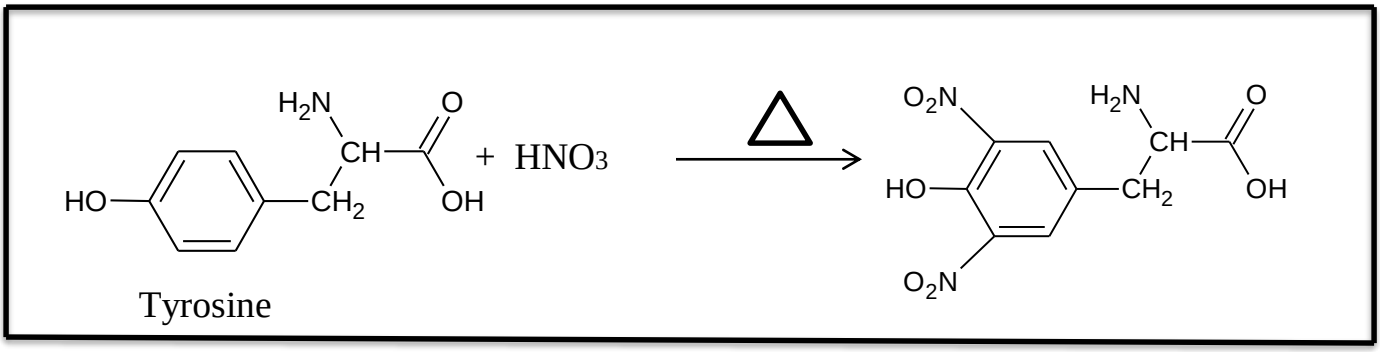
Proline

Yellow colored product

2-Xanthoprotic test :

This test depends on the presence of the aromatic ring in the structure of amino acid, therefore it is possible to distinguish between the aliphatic amino acids and aromatic amino acids.

In this test, concentrated nitric acid reacts with the aromatic amino acid to yield a yellow color and it turns to orange upon the addition of a strong base (ammonia or sodium hydroxide).



Tyrosine

Method :

1- In a clean and dry test tube, 1 ml of concentrated nitric acid is added to 2 ml of amino acid solution and mixed well.

2-The solution is heated in a boiling water bath for 3 min .

3-The change in the color of the solution is noticed.

4-After cooling the solution, 4 ml 10N of NaOH are added and the change in the color of the solution is noticed.

3-Millon`s test:

It is a special test for amino acid that contains an aromatic hydroxyl group (phenol group), therefore, it is carried out only to distinguish Tyrosine. The interaction of

Tyrosine with mercuric nitrate dissolved in nitric acid $Hg(NO_3)_2$ yields a red color solution or red precipitate.

Method :

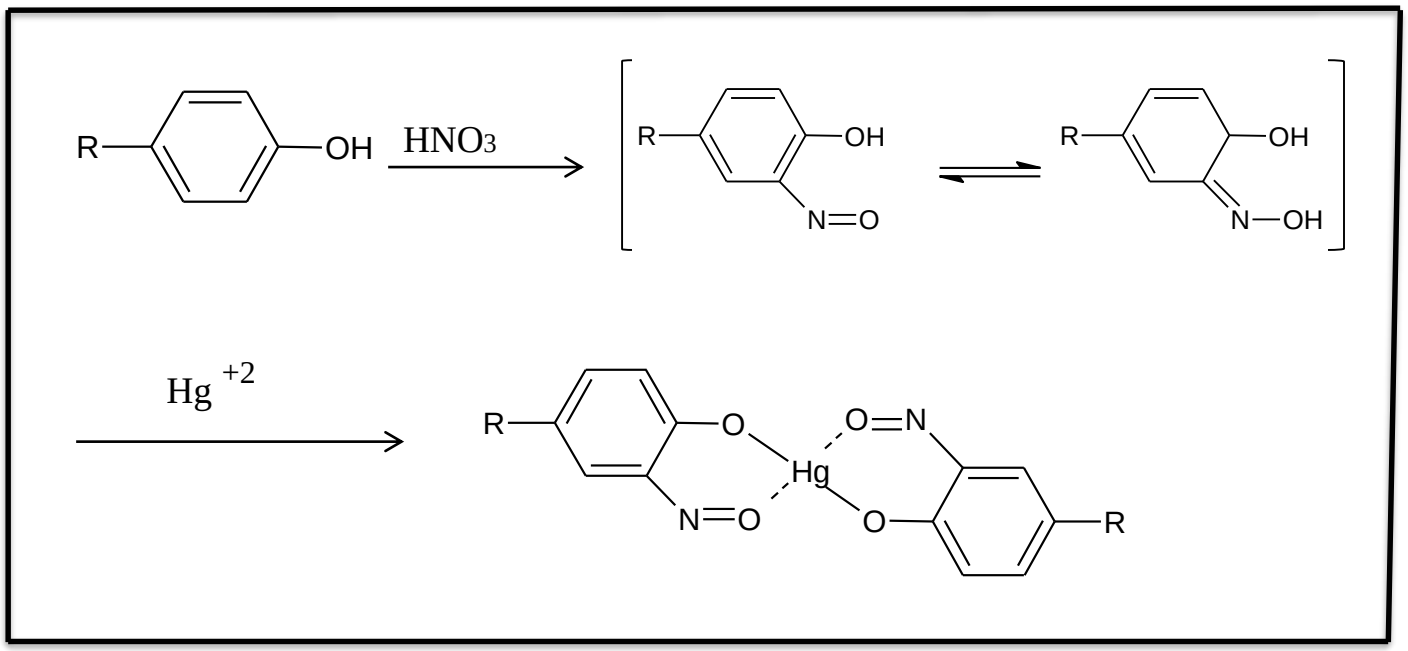
1- In a clean and dry test tube, 1 ml of Millon`s solution is added to 1 ml of amino acid solution and mixed well.

2- The solution is heated in a boiling water bath for 3 min .

2- The change in the color of the solution is noticed (a red color solution or red precipitate).

Preparing Millon`s solution :

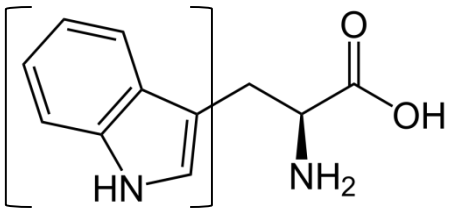
15% of mercuric nitrate is dissolved in 15% v/v nitric acid.



4-Hopkins – cole test :

This test is specific for Tryptophan because it contains an indole group which interacts with glyoxylic acid in the presence of concentrated sulfuric acid to yield a violet ring between two layers. Glyoxylic acid is prepared by exposing glacial acetic acid to light.

Indole group



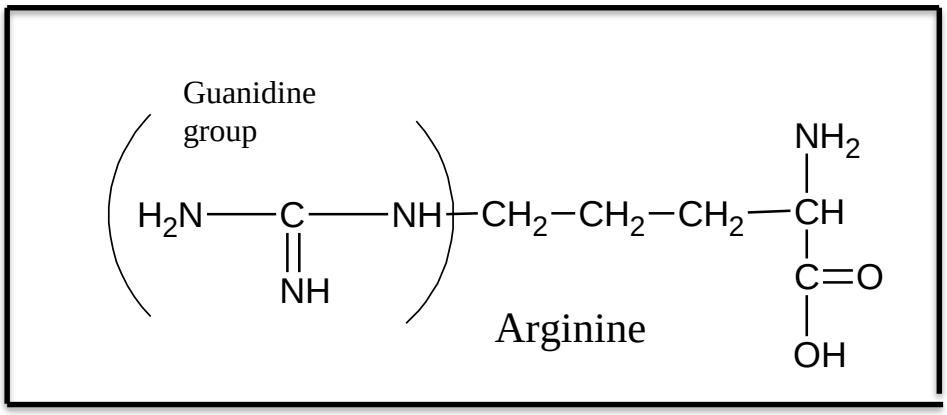
Glacial acetic acid $\xrightarrow{\text{Light}}$ Glyoxylic acid

Method :

1- In a clean and dry test tube, 1 ml of glyoxylic acid is added to 2 ml of amino acid solution and mixed well.
2- A small amount of concentrated sulfuric acid is slowly added down the sides of the sloping test-tube, without mixing, the violet ring will appear between two layers.

5-Sakakoguchi test :

This test is specific for Arginine, because it contains guanidine group which interacts with α-naphthol and sodium hypochlorite (bleach) to yield a red colored solution.

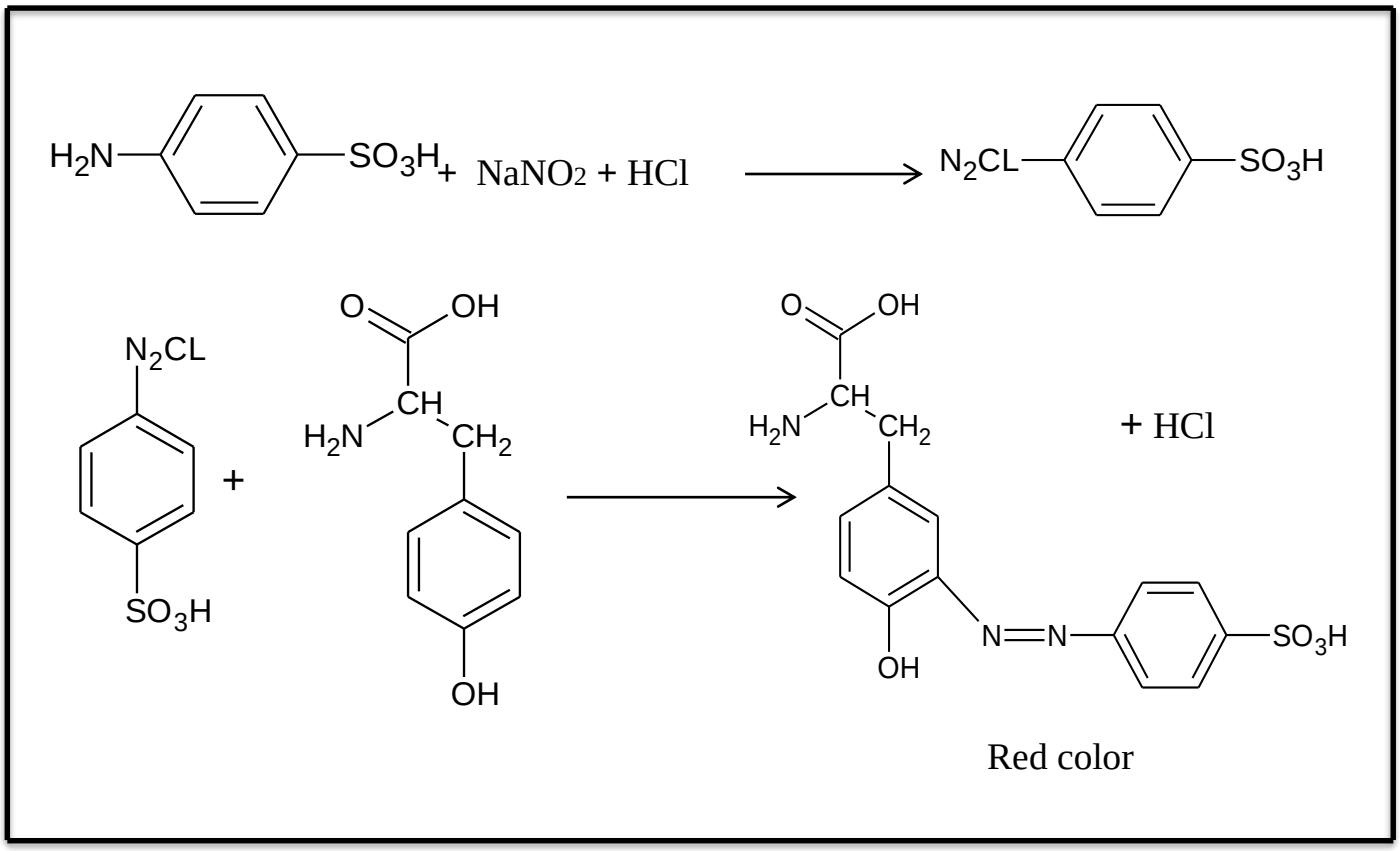


Method :

1- In a clean and dry test tube, 10 drops of 40% NaOH are added to 1 ml of amino acid solution.
2- After mixing well, 2 drops of α-naphthol are added in the same test tube and mixed well, then 3 drops of sodium hypochlorite (bleach) are added and the change of the color is noticed (red colored solution).

6-Pauly`s test:

This test is used to detect Histidine and Tyrosine, which have the ability to bind with Diazonium salts to form Azo compounds with strong and distinct colors and they only react in cold conditions.



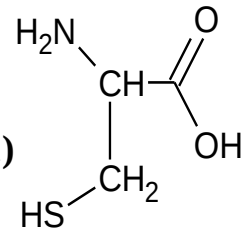
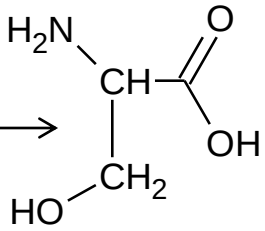
Method :

1- In a clean and dry test tube, 1 ml of Sulphanilic acid is added to 2 ml of amino acid solution with mixing well.

2- The tube is cooled in an ice bath for 3 min , then 1 ml of sodium nitrite $NaNO_2$ is added with 2 ml of 1% sodium carbonate and the change of the color is noticed (red color).

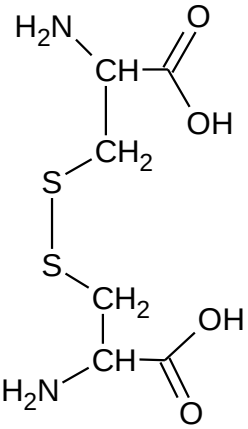
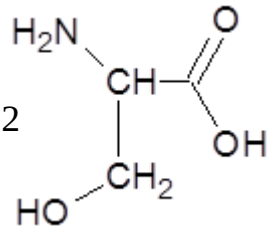
7-Lead sulfide test:

When Cysteine and Cystine are treated with strong base like NaOH, sodium sulfide will be formed, in turn, it will react with the lead acetate to yield a black precipitate of lead sulfide.

1)  + 2 NaOH →  + Na_2S + H_2O

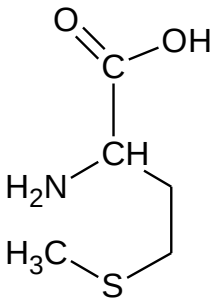
Na_2S + $(CH_3COO)_2Pb$ → PbS ↓ + 2 CH_3COONa

Black ppt.

2)  + 4 NaOH → 2  + Na_2S + H_2O

Na_2S + $(CH_3COO)_2Pb$ → PbS ↓ + 2 CH_3COONa

Black ppt.

3)  + NaOH → No reaction

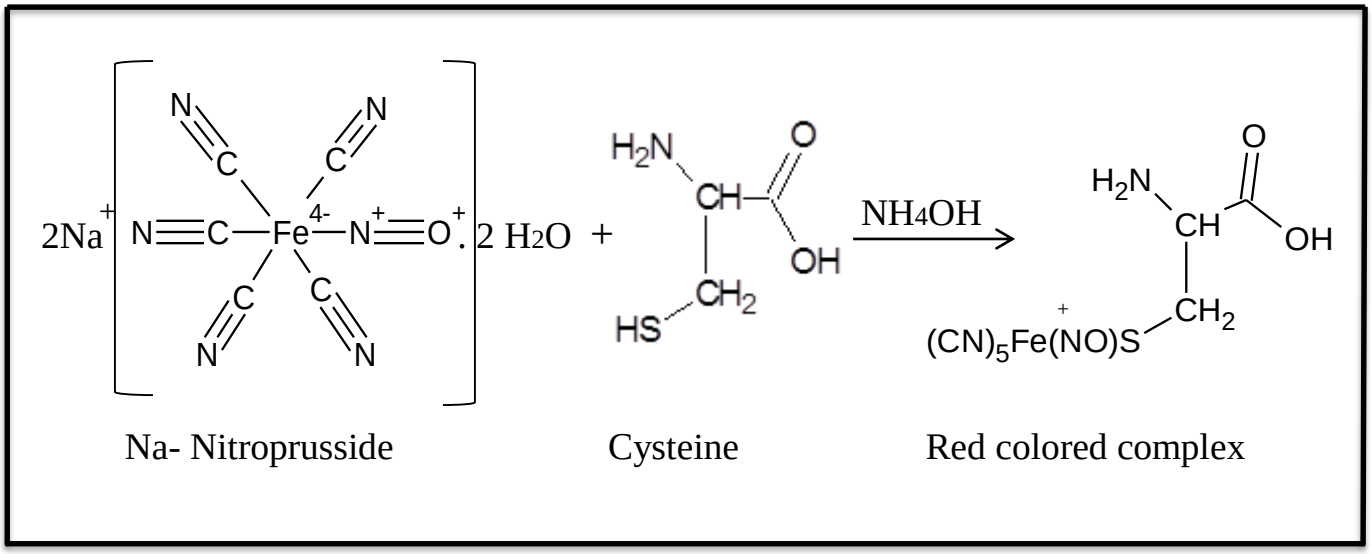
Method :

1-In a clean and dry test tube, 1 ml of sodium hydroxide is added to 1 ml of amino acid solution with mixing well.

2- The tube is heated in a boiling water bath for 2 min, then 5 drops of lead acetate are added and the change of the color is noticed (black precipitate).

8-Nitroprusside test:

The thiol group SH in the Cysteine reacts with sodium nitroprusside $Na_2(CN)_5Fe(NO).2H_2O$ in the presence of ammonia to give a red color solution. This test distinguishes Cysteine from Cystine.

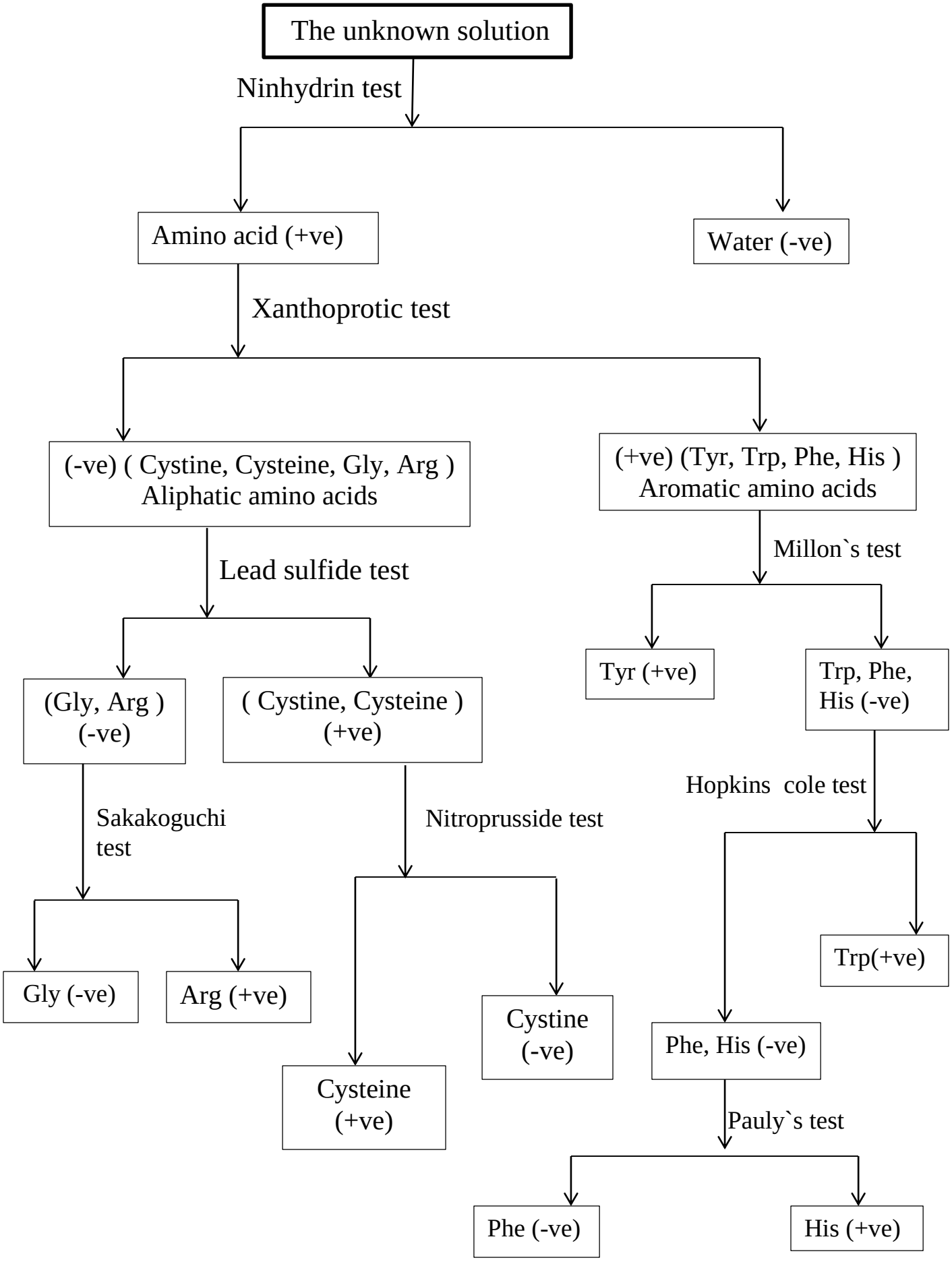


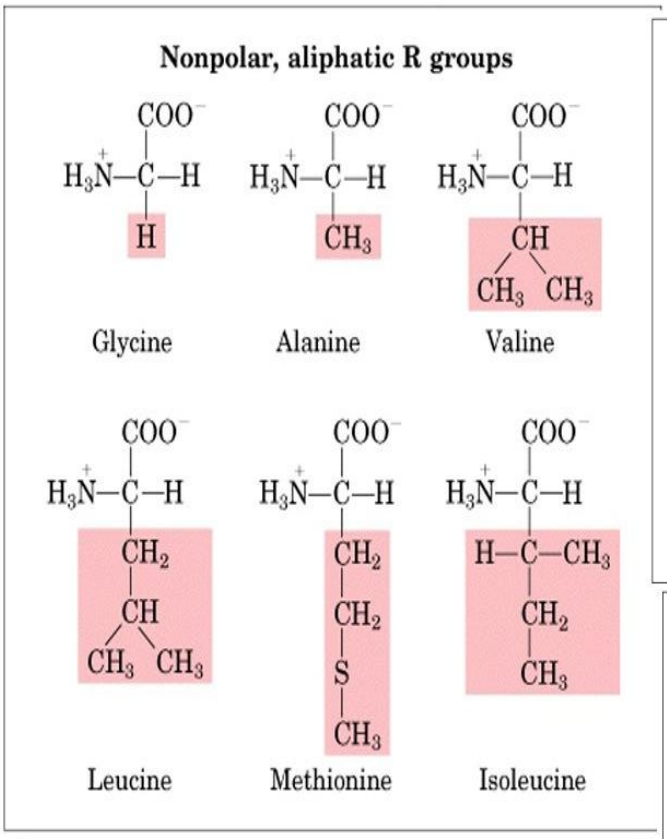
Na- Nitroprusside Cysteine Red colored complex

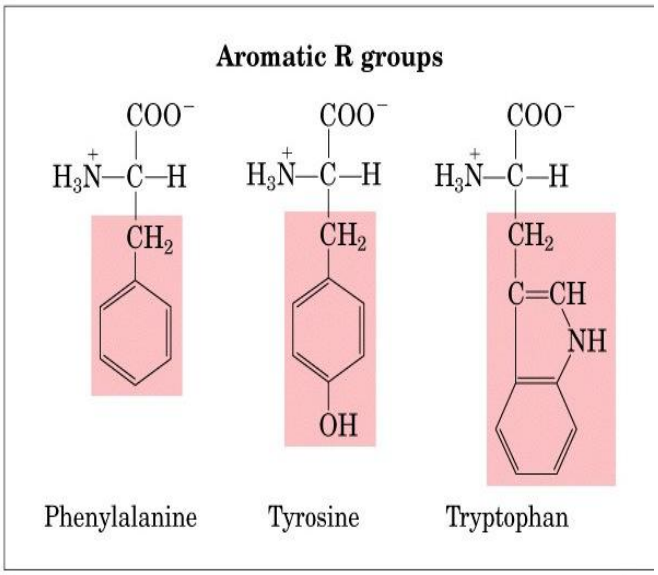
Method:

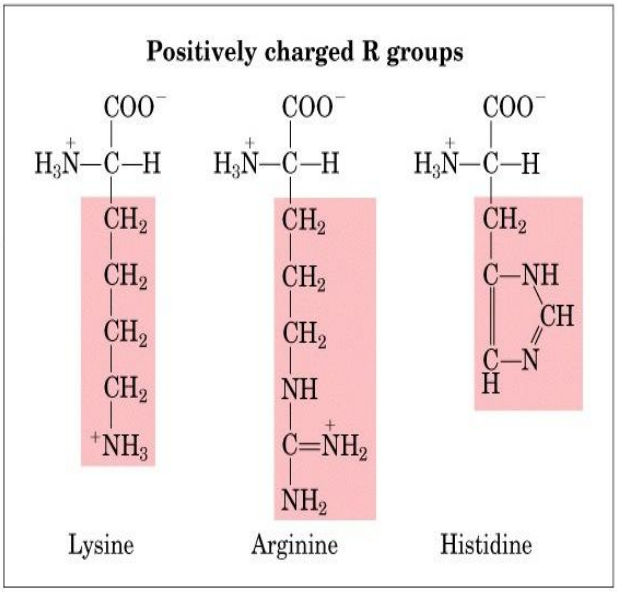
1-In a clean and dry test tube, 0.5 ml of sodium nitroprusside is added to 2 ml of amino acid solution .

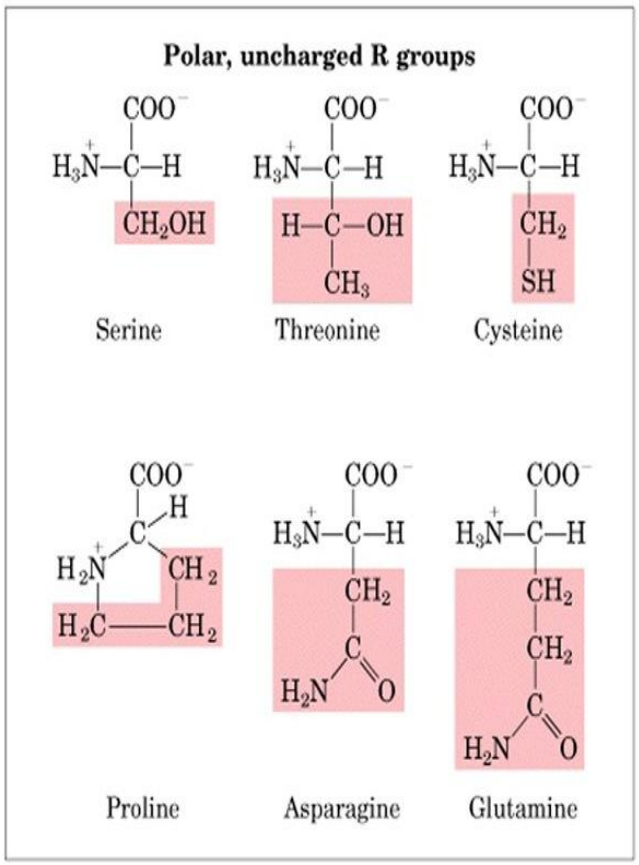
2- After mixing well, 0.5 ml of NH_4OH is added, then the change in the color of the solution is noticed (red colored complex).

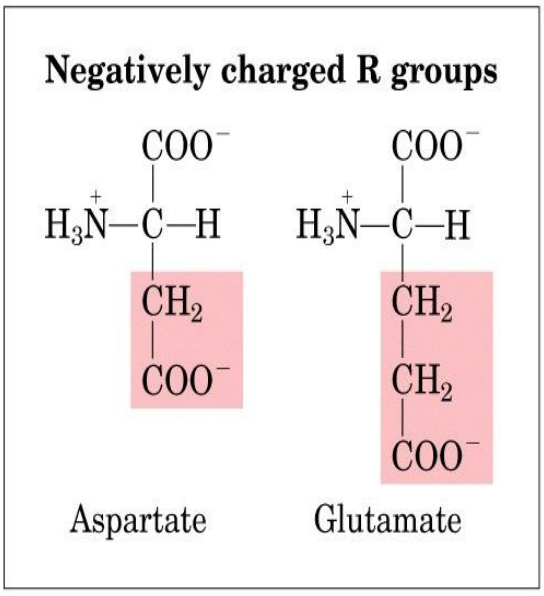

The unknown solution
Ninhydrin test
Amino acid (+ve)
Water (-ve)
Xanthoprotic test
(-ve) (Cystine, Cysteine, Gly, Arg)
Aliphatic amino acids
(+ve) (Tyr, Trp, Phe, His)
Aromatic amino acids
Millon`s test
Lead sulfide test
Tyr (+ve)
Trp, Phe,
His (-ve)
(Gly, Arg)
(-ve)
(Cystine, Cysteine)
(+ve)
Hopkins cole test
Sakakoguchi
test
Nitroprusside test
Trp(+ve)
Gly (-ve)
Arg (+ve)
Cystine
(-ve)
Phe, His (-ve)
Cysteine
(+ve)
Pauly`s test
Phe (-ve)
His (+ve)


Nonpolar, aliphatic R groups
Glycine
Alanine
Valine
Leucine
Methionine
Isoleucine

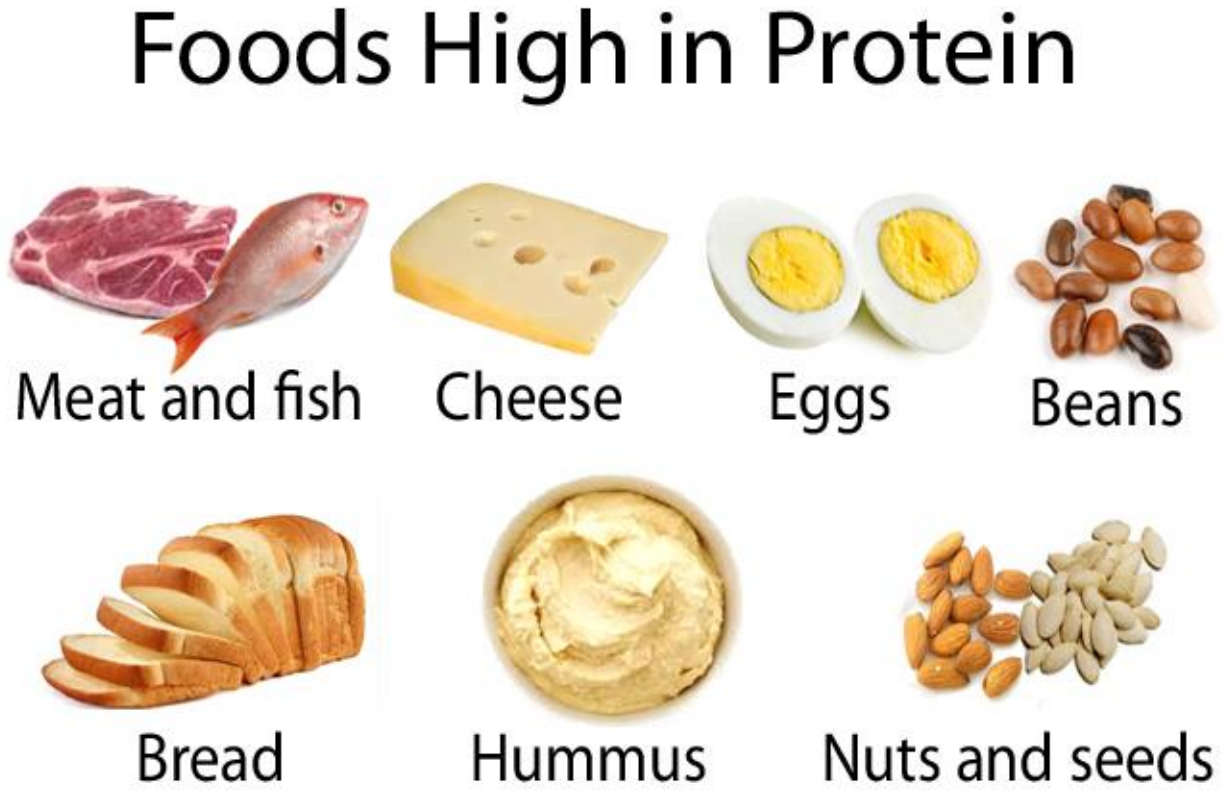

Aromatic R groups
Phenylalanine
Tyrosine
Tryptophan


Positively charged R groups
Lysine
Arginine
Histidine


Polar, uncharged R groups
Serine
Threonine
Cysteine
Proline
Asparagine
Glutamine


Negatively charged R groups
Aspartate
Glutamate

Qualitative test of protein

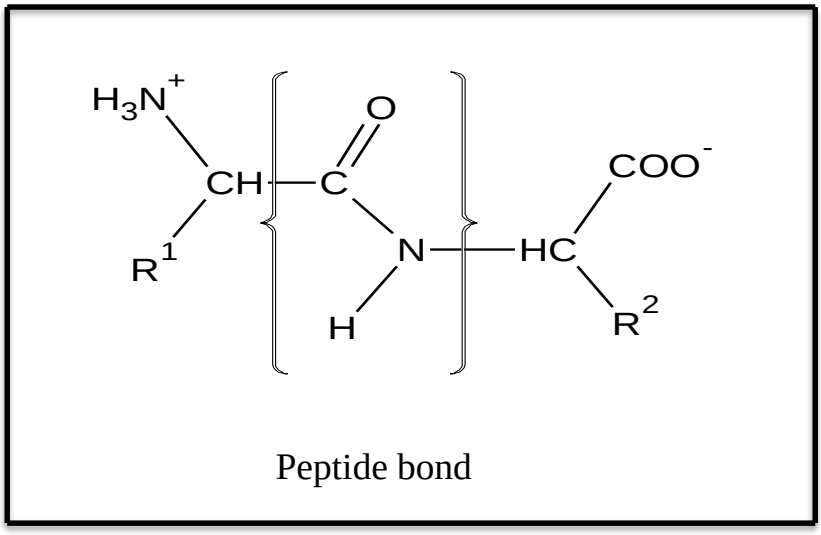


Proteins

They are complex organic compounds with high molecular weights consisting of a large number of amino acids, which are linked to each other by peptide bonds. Proteins consist of essential elements such as carbon, nitrogen, oxygen, hydrogen and other elements such as sulfur, phosphorus and iron.

Proteins are the main components of animal and plant tissues, they stimulate the chemical reactions of life such as enzymes, these reactions are organized such as hormones.

They are found within the components of cell walls, as well as parts of cells such as the (nucleus and mitochondria ……..). All proteins do not contain equal amounts of amino acids and do not contain all 20 amino acids.



Peptide bond

Proteins are classified according to their chemical composition or solubility properties into :

1- **Simple protein.**
2- **Associated proteins.**
3- **Dissociative proteins.**

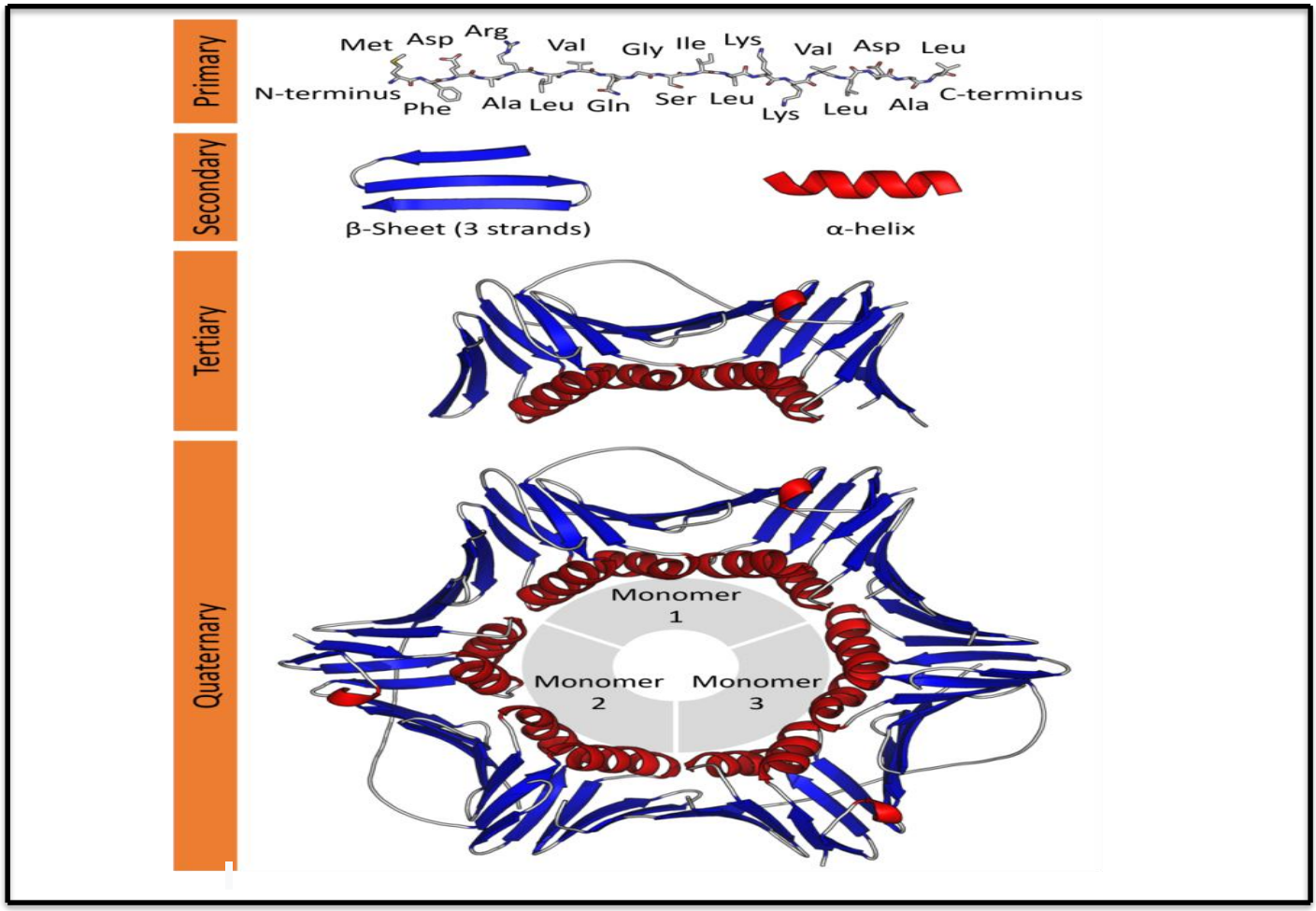
Four levels of structure can be noticed in proteins :

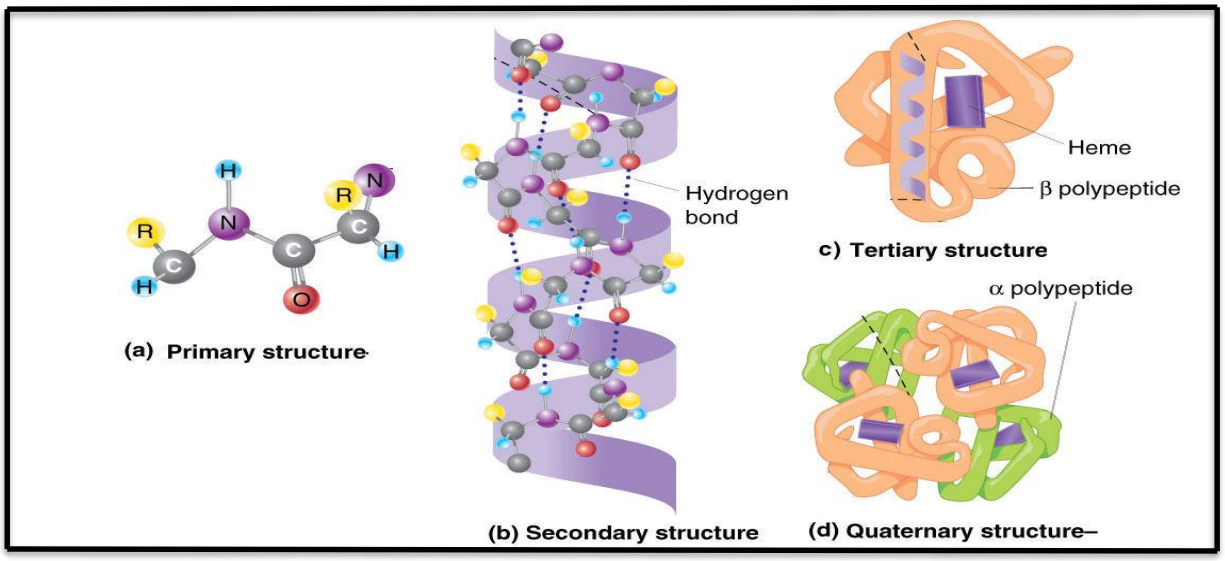
1- **Primary structure :**
It represents the number and sequence of amino acids in the peptide chain and only the peptide bonds are responsible for this level of structure.

2- **Secondary structure :**
It is the 3-D arrangement of the right-handed alpha helix, or alternative structures such as the β-sheets.

3- **Tertiary structure :**
This is the 3-D folding of the alpha helix and β-sheets, shaped by structures such as proline corners, disulfide bridges between cysteine residues, and electrostatic bonds.

4- **Quaternary structure** :
Where more than one protein chain contributes to form on protein, the quaternary structure is the arrangement of these subunits. In hemoglobin, the quaternary structure comprises two alpha and two beta polypeptides, held together by electrostatic bonds.





The most important bonds involved in stabilizing the protein is:

1- Peptide bonds.
2- Hydrogen bonds.
3- Disulfide bonds.
4- Hydrophobic interactions.
5- Electrostatic forces (or attractions).
6- Van der Waals force.

Solubility of proteins :

Proteins are differ in solubility in solutions, they generally are low soluble in water and polar solvents (there are some proteins easy to dissolve in water, such as albumin and others do not dissolve in water such as creatine).

The solubility of proteins depends on four factors:

1- **pH**:

The degree of solubility is affected by pH value, because of the amphoteric behavior, where the solubility rate is at its lowest point at the point of pI and is increased whenever it changes either by increase the acidity or increase of the basicity and it forms of negative or positive ions. This property is used in the separation of proteins because they have different pI values.

2- Temperature:

It leads to change in the quaternary structure and tertiary structure of proteins, causing loss of its vital effectiveness and this is called Denaturation of protein , where protein precipitates from its solution when exposed to high temperature.

Denaturation of protein :

It is a process of changing the tertiary and quaternary structure of proteins that leads to loss of their basic functions. This process occurs due to several factors including heating, using a strong acid and using a strong base because they destroy the weak bounds and affect the tertiary and secondary structure, but not the primary structure .

3- Ion concentration:

When a neutral solution is added with a low concentration of table salt NaCl 0.9%, the solubility of a low-soluble protein will increase and this effect is called salting – in, but when high concentrations of neutral salts is added, proteins are precipitated from their water solutions and that effect is called salting – out.

4- The solvents:

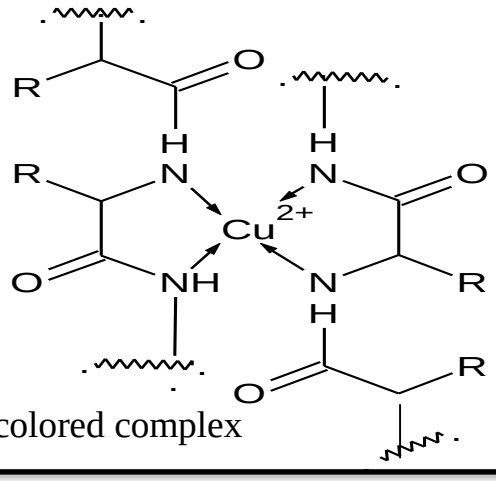
Proteins in their aqueous solutions are precipitated by adding of polar solvents and mixing such as alcohol and acetone, where the process of protein denaturation occurs and leads to precipitate it.

Qualitative tests of proteins

1- Biuret test :

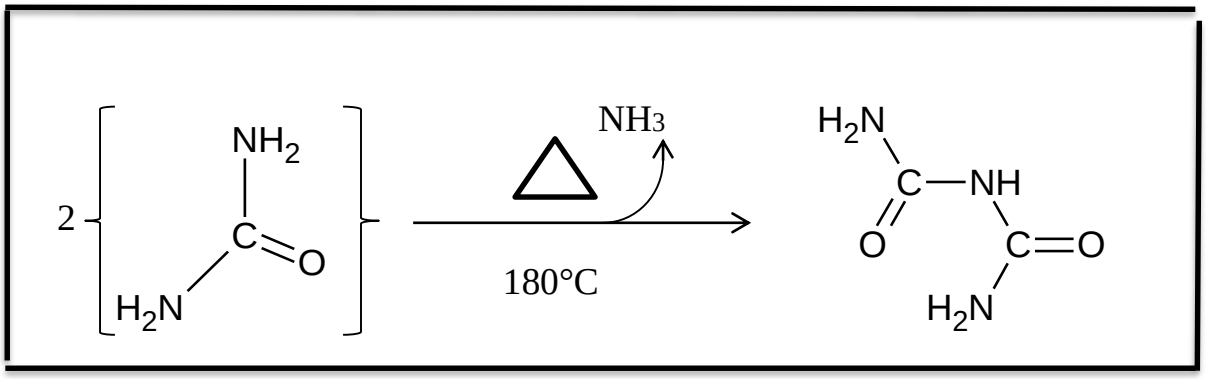
It is a general test for all proteins and peptides that are composed of three amino acids or more (contains two or more peptide bonds), where a coordination complex is formed between the copper ions and the nitrogen atoms present in the peptide bonds of the protein.

Protein + $CuSO_4$ + NaOH ⟶



Violet colored complex

The test name was derived from the Biuret compound, which can be obtained from heating urea . This compound has the ability to form a complex with copper ions (violet colored complex).



Method :

1-The Biuret solution is prepared by adding 3 gm of copper (II) sulfate pentahydrate and 9 gm of sodium – potassium tartarat to 500 ml of NaOH 0.2N, then 5 gm of potassium iodide are added and the volume of the solution is completed to the 1000 ml by using 0.2N NaOH.

2- In a clean and dry test tube, 0.5 ml of Biuret solution are added to 1ml of protein solution and mixed well.

3- The change in the color of the solution is noticed (violet colored complex is present).

2-Precipitation by using heat:

Proteins can be precipitated by heating as it gets a denaturation process. Proteins cannot be dissolved again by cooling as the denaturation process is irreversible.

Method:

1- In a water bath, 2 ml of protein solution such as albumin or egg globulin are boiled.

2- The change in the solution is noticed.

3- Precipitation by using concentrated acids:

A- By using Heler`s test (nitric acid):

In a dry and clean test tube, nitric acid is added drop by drop to 1 ml of protein solution until the precipitate appears. The precipitation process is irreversible because it leads to change in the tertiary structure of proteins.

B- By using H_2SO_4 and HCl :

In a dry and clean test tube, H_2SO_4 and HCl is added drop by drop to 1ml of protein solution until the precipitate appears. The precipitation process is reversible.

4- Precipitation by using heavy metals (positive charge) :

In general, proteins negative charged at pH= 7 or more, the process of adding metals that carry positive charge (heavy metals) leads to the equivalence of these charges and thus bring the proteins to the pI and precipitate it.

This method occurs in cases of poisoning by one of these salts (eggs or milk can act as an antidote) where the metal will precipitate without absorbed by the human body.

Method:

1- In a dry and clean test tube, a few drops of 0.1 M of copper sulfate, ferrous chloride or lead acetate solution are added to 2ml of protein solution until the precipitate appears.

2- The increasing of the drops (heavy metal solution) leads to disappear of precipitate. (why?)

5- Precipitation by using alkaloids reagents (complex acids):

Talic acid, picric acid, tungstic acid and sulfosalicylic acid solutions are the most alkaloid reagents which cause protein to precipitation in their solutions.These acids carry negative charges that have the ability to equalize positive charges of proteins and form insoluble salts.

Method:

In a dry and clean test tube, a few drops of 20% of the alkaloid reagents are added to 2ml of protein solution until the precipitate appears.

6- Precipitation by using alcohol :

In a dry and clean test tube, a few drops of ethanol are added to 2ml of protein solution until the precipitate appears. The precipitation process is irreversible.

7- Precipitation by using neutral salts (salting – out):

By using high concentrations of neutral salts, a denaturation of the protein occurs. This type of precipitation depends on salt type, salt concentration, type of protein.

This method is used in the separation of proteins in biological applications.

Method:

In a dry and clean test tube, 1 ml of the ammonium sulfate, sodium chloride or magnesium sulfate is added to 1ml of protein solution and the change in the solution is noticed.

Reference :

1- فلاح سموم دغل. "ملزمة الكيمياء الحياتية العملي". الجامعة المستنصرية (2004).

2- Seager, Spencer L.; Slabaugh, Michael R.; Hansen, Maren S. "Safety Scale Laboratory Experiments". Cengage Learning.2016. p. 358.

3- Peter J. Russell". iGenetics A Molecular Approach". 3rd Edition, 2010 Pearson Education. p.105-106.

4- Baldwin, E. and Bell, D.J.," Cole's Practical Physiological Chemistry", published by Heffer, Cambridge, 1955, p. 189.

5- Katoch, Rajan . "Analytical Techniques in Biochemistry and Molecular Biology". Department of Crop Improvement, SCKHPKV.India. 2011. p. 71.

6- Abramoff, Peter; Thomson, Robert." An experimental approach to biology". WH Freeman & Company, San Francisco.1966. p. 47.

7- Walsh, Edward O'Farrell." An Introduction to Biochemistry". London: The English Universities Press Ltd.1961. p. 406–407.