KOMAR UNIVERSITY OF SCIENCE AND TECHNOLOGY

KOMAR UNIVERSITY

OF SCIENCE AND TECHNOLOGY

(KUST)

DEPARTMENT OF MEDICAL LABORATORY SCIENCE

BIOCHEMISTRY LAB MANUAL

(CHM2415C)

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Introduction

The aim of the following set of experiments is to acquaint students with some basics in understanding Biochemistry lab and Biochemical. This Biochemistry laboratory seeks to introduce undergraduate students techniques used in biochemistry.

A collection of eleven experiments has been presented that teach students how to detect, estimate different biomolecules with simple equipment. Each experiment set introduces a theoretical principle and the needed equipment and chemicals used in each experiment.

Course objectives

-This course regarded as an introduction to basic biochemistry and will be useful for students who want to study clinical chemistry.

-The course uses simple protocols and available materials and instruments to understand Biochemical substances.

-Some experiments were put to teach students how to work independently in the any Lab.

- Modern lab researchers should know the principles of the biochemical methods of analysis and to learn the main theoretical statements. For it, medical Lab Science students have to get the minimum of manual skills during a research of biochemistry, eg. measuring out solutions and biological liquids, centrifugation, colorimetry of colored solutions, determination of pH, peculiarities of the technique of enzyme investigations etc. The given manual contains the Descriptions of the biochemical methods of analysis which all the skills are required in.

Table of Contents

Introduction to the Biochemistry lab (Rules for laboratory experiments, laboratory Safety Signs/Labels and laboratory reports).

1. Safety Rules:

What are the Safety Do's and Don'ts for Students?

Life threatening injuries can happen in the laboratory. For that reason, students need to be informed of the correct way to act and things to do in the laboratory. The following is a safety checklist that can be used as a handout to students to acquaint them with the safety do's and don'ts in the laboratory.

Conduct

- Do not engage in practical jokes or boisterous conduct in the laboratory.
- Never run in the laboratory.
- The use of personal audio or video equipment is prohibited in the laboratory.
- The performance of unauthorized experiments is strictly forbidden.
- Do not sit on laboratory benches.

General Work Procedure

- Know emergency procedures.
- Never work in the laboratory without the supervision of a teacher.
- Always perform the experiments or work precisely as directed by the teacher.
- Immediately report any spills, accidents, or injuries to a teacher.
- Never leave experiments while in progress.
- Be careful when handling hot glassware and apparatus in the laboratory. Hot glassware looks just like cold glassware.
- Never point the open end of a test tube containing a substance at yourself or others.
- Never fill a pipette using mouth suction. Always use a pipetting device.
- Make sure no flammable solvents are in the surrounding area when lighting a flame.
- Do not leave lit Bunsen burners unattended.
- Turn off all heating apparatus, gas valves, and water faucets when not in use.
- Do not remove any equipment or chemicals from the laboratory.
- Coats, bags, and other personal items must be stored in designated areas, not on the bench tops or in the aisle ways.
- Notify your teacher of any sensitivities that you may have to particular chemicals if known.
- Keep the floor clear of all objects (e.g., ice, small objects, and spilled liquids).

Housekeeping

- Keep work area neat and free of any unnecessary objects.
- Thoroughly clean your laboratory work space at the end of the laboratory session.
- Do not block the sink drains with debris.
- Never block access to exits or emergency equipment.
- Inspect all equipment for damage (cracks, defects, etc.) prior to use; do not use damaged equipment.
- Never pour chemical waste into the sink drains or wastebaskets.
- Place chemical waste in appropriately labeled waste containers.
- Properly dispose of broken glassware and other sharp objects (e.g., syringe needles) immediately in designated containers.
- Properly dispose of weigh boats, gloves, filter paper, and paper towels in the laboratory.

Apparel in the Laboratory

- Always wear appropriate eye protection (i.e., chemical splash goggles) in the laboratory.
- Wear disposable gloves, as provided in the laboratory, when handling hazardous materials. Remove the gloves before exiting the laboratory.
- Wear a full-length, long-sleeved laboratory coat or chemical-resistant apron.
- Wear shoes that adequately cover the whole foot; low-heeled shoes with non-slip soles are preferable. Do not wear sandals, open-toed shoes, open-backed shoes, or high-heeled shoes in the laboratory.
- Avoid wearing shirts exposing the torso, shorts, or short skirts; long pants that completely cover the legs are preferable.
- Secure long hair and loose clothing (especially loose long sleeves, neck ties, or scarves).
- Remove jewelry (especially dangling jewelry).
- Synthetic finger nails are not recommended in the laboratory; they are made of extremely flammable polymers which can burn to completion and are not easily extinguished.

Hygiene Practices

- Keep your hands away from your face, eyes, mouth, and body while using chemicals.
- Food and drink, open or closed, should never be brought into the laboratory or chemical storage area.
- Never use laboratory glassware for eating or drinking purposes.
- Do not apply cosmetics while in the laboratory or storage area.
- Wash hands after removing gloves, and before leaving the laboratory.
- Remove any protective equipment (i.e., gloves, lab coat or apron, chemical splash goggles) before leaving the laboratory.

Emergency Procedure

- Know the location of all the exits in the laboratory and building.
- Know the location of the emergency phone.
- Know the location of and know how to operate the following:
- Fire extinguishers
- Alarm systems with pull stations
- Fire blankets
- Eye washes
- First-aid kits
- Deluge safety showers
- In case of an emergency or accident, follow the established emergency plan as explained by the teacher and evacuate the building via the nearest exit.

Chemical Handling

- Check the label to verify it is the correct substance before using it.
- Wear appropriate chemical resistant gloves before handling chemicals. Gloves are not universally protective against all chemicals.
- If you transfer chemicals from their original containers, label chemical containers as to the contents, concentration, hazard, date, and your initials.
- Always use a spatula or scoopula to remove a solid reagent from a container.
- Do not directly touch any chemical with your hands.
- Never use a metal spatula when working with peroxides. Metals will decompose explosively with peroxides.
- Hold containers away from the body when transferring a chemical or solution from one container to another.
- Use a hot water bath to heat flammable liquids. Never heat directly with a flame.
- Add concentrated acid to water slowly. Never add water to a concentrated acid.
- Weigh out or remove only the amount of chemical you will need. Do not return the excess to its original container, but properly dispose of it in the appropriate waste container.
- Never touch, taste, or smell any reagents.
- Never place the container directly under your nose and inhale the vapors.
- Never mix or use chemicals not called for in the laboratory exercise.
- Clean up all spills properly and promptly as instructed by the teacher.
- Dispose of chemicals as instructed by the teacher.
- When transporting chemicals (especially 250 mL or more), place the immediate container in a secondary container or bucket (rubber, metal or plastic) designed to be carried and large enough to hold the entire contents of the chemical.
- Never handle bottles that are wet or too heavy for you.
- Use equipment (glassware, Bunsen burner, etc.) in the correct way, as indicated by the teacher.

Chemical Waste

- All containers used for chemical waste should be labeled with:

  "WASTE" or "HAZARDOUS WASTE"

  Chemical name (as it appears on the MSDS)

  Accumulation start date

  Hazard(s) associated with the chemical waste

Storage Don'ts

- Do not place heavy materials, liquid chemicals, and large containers on high shelves.
- Do not store chemicals on tops of cabinets.
- Do not store chemicals on the floor, even temporarily.
- Do not store items on bench tops and in laboratory chemical hoods, except when in use.
- Do not store chemicals on shelves above eye level.
- Do not store chemicals with food and drink.
- Do not store chemicals in personal staff refrigerators, even temporarily.
- Do not expose stored chemicals to direct heat or sunlight, or highly variable temperatures.

Proper Use of Chemical Storage Containers

- Never use food containers for chemical storage.
- Make sure all containers are properly closed.
- After each use, carefully wipe down the outside of the container with a paper towel before returning it to the storage area. Properly dispose of the paper towel after use.

Appendix A. Common Safety Symbols

Flammable

Poison

Explosive

Radioactive

Corrosive

Compressed Gas

The above safety symbols may be replaced by the following symbols that are internationally accepted*:

Flammable

Oxidizer

Explosive

Low Level Hazard

Corrosive

Severe Chronic Hazard

Poison

Environmental Hazard

2. Lab Reports:

Lab reports should be filled using the following format or style:

Lab Report Form

Course name

Course number-Lab section

The title of the experiment

Date

Student name (registration no.)

Lab partners

Instructor name

Lab no.

All these should be written in the first page.

The second page should be filled as follows:

Mark
_____

Title: ----------

ABSTRACT:

INTRODUCTION:

MATERIALS:

METHODS:

RESULTS:

DISCUSSION:

CONCLUSION(s):

REFERNCES:

Lab 1: Carbohydrate Qualitative tests

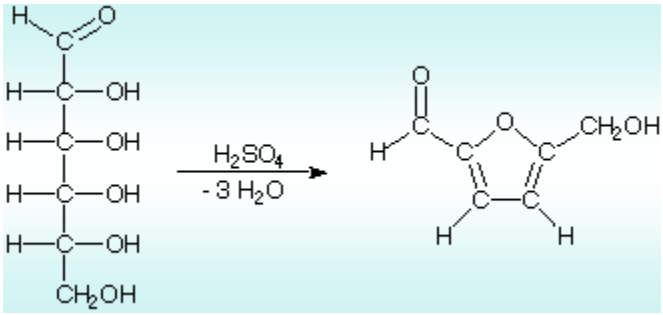
1. Molisch's Test:

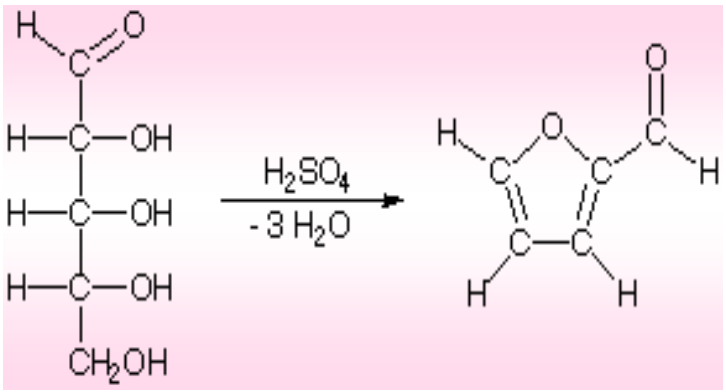
It is the general test for all carbohydrates. Monosaccharides give a rapid positive test. Disaccharides and polysaccharides react slower. WHY?

Principle:

The reaction is due to the formation of Furfural and it's derivatives by the dehydrating action of acid on sugar in the event of Sugar being (poly saccharide or disaccharide) the acid first hydrolyzes it into monosaccharides and then acts as a dehydrating agent.

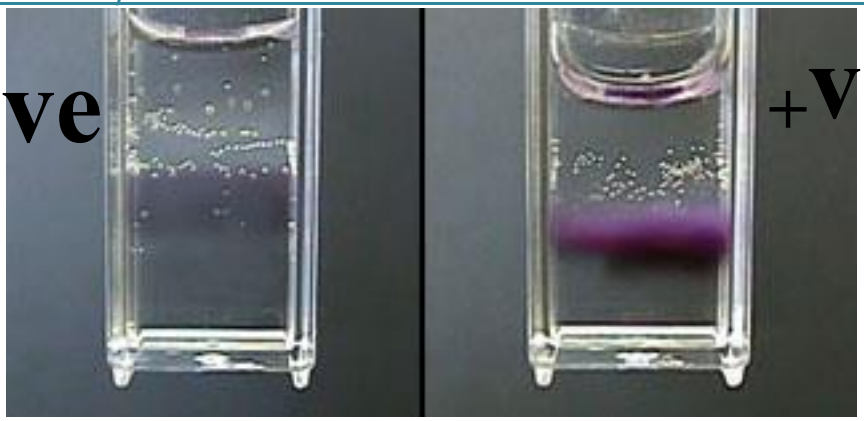
The Molisch reagent dehydrates pentoses to form furfural. It dehydrates hexoses to form 5-hydroxymethyl furfural. The furfurals further react with α-naphthol present in the test reagent to produce a purple product.

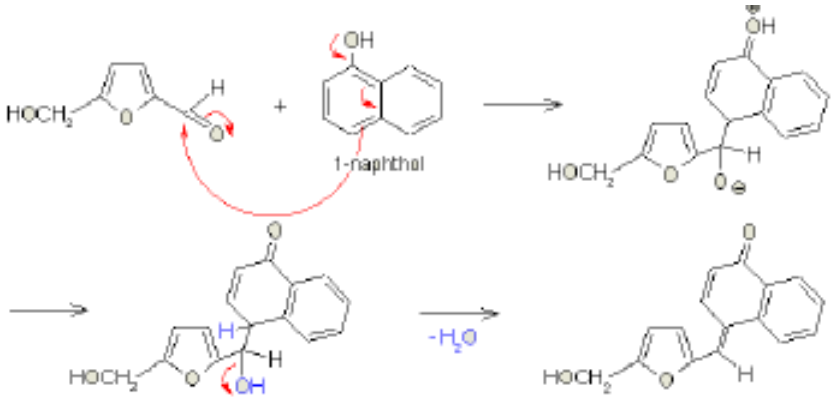




■ Method:

- 1ml test solution + 2 drops of α-naphthol
- mix well
- Add conc. H_2SO_4 down the side of the tube to form the ring at the interface of the two layers.





Molisch's Reaction

Lab results: Glucose, maltose, arabinose and starch will all display the purple ring compound at the interface of the acid and solution.
Limitations:
-Not specific to carbohydrates
-Generalized test that cannot distinguish carbohydrates and further testing must be undertaken to identify the carbohydrate.

2. Benedict's Test:

All reducing sugars give positive Benedict's Test
Principle: reducing sugars have free aldehyde or keton group, it under go converting in to enediol forms under hot alkaline condition. The enediol are strong reducing agents converting cupric ions (Cu+2) of the Benedict's solution into Cuprose ion which altimately oxide as a red precipitate red copper(I) oxide by aldehydes.

- Benedict's reagent contains blue copper(II) sulfate ($CuSO_4$) · 5H2O which is reduced to red copper(I) oxide by aldehydes, thus oxidizing the aldehydes to carboxylic acids.
- The copper oxide is insoluble in water and so precipitates. The color of the final solution ranges from green to brick red depending on how many of the copper (II) ions are present.
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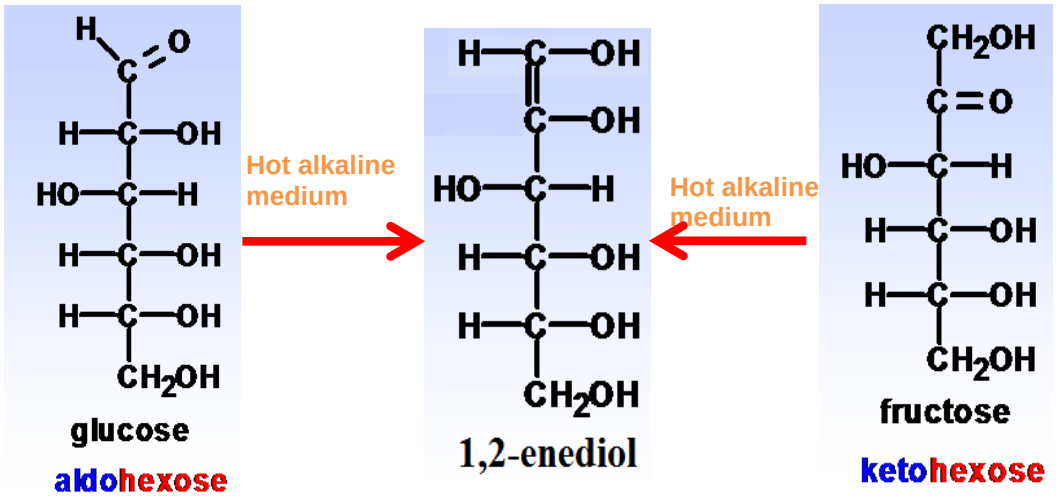
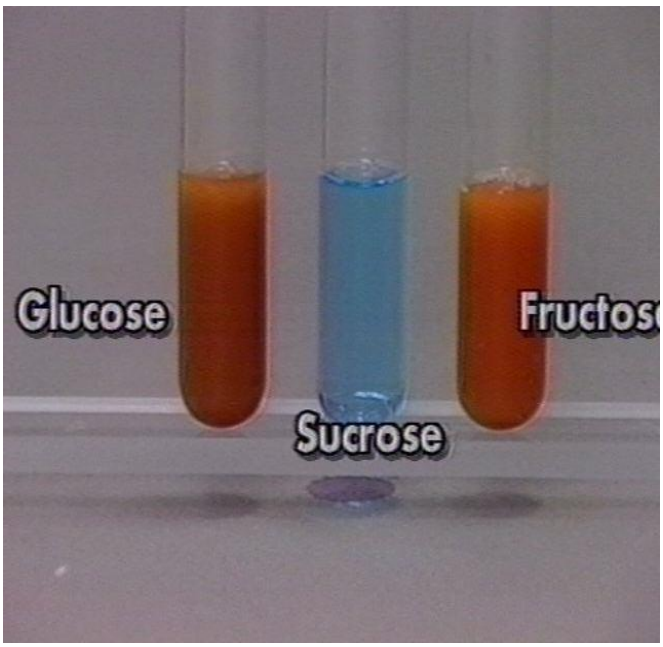
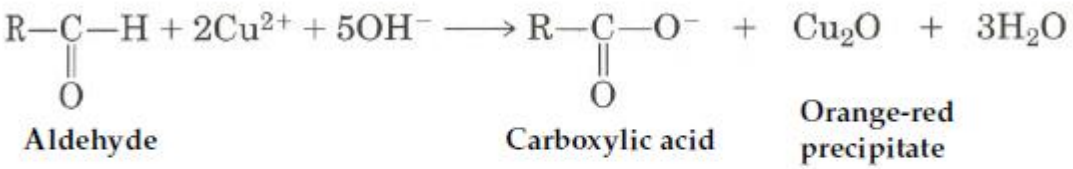


■ Method:

- 1ml test solution + 1ml Benedict's reagent
- heat the mixture in Boilingf Water Bath for (5min)
- Reddish brown ppt.





Benedict's Reaction

3. Barfoid's Test:

The test is used to distinguish between monosaccharides and reduced disaccharides

- This reaction will detect reducing monosaccharides in the presence of disaccharides. Reagent uses copper ions to detect reducing sugars in an acidic solution. Barfoed's reagent is copper acetate in dilute acetic acid (pH 4.6)

- Reducing monosaccharides are oxidized by the copper ions in a weak acidic medium to form a carboxylic acid and a reddish ppt of Cu_2O (cuprous oxide).

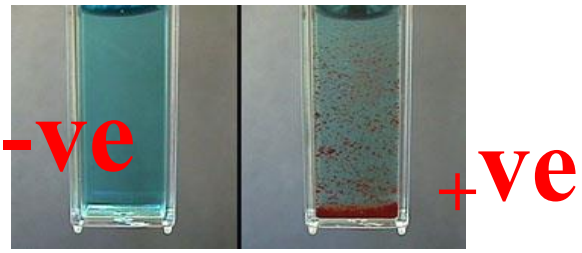
- Reducing disaccharides (lactose but not sucrose) undergo the same reaction but at slower rate. Therefore, boiling time is critical and positive test within (2min.) indicate the monosaccharides in case of disaccharides they are first converted into monosaccharide and then colored precipitate are formed.

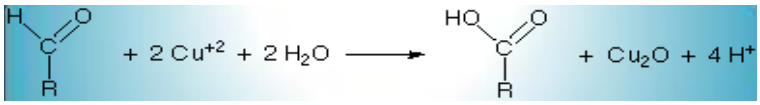
- So the porolonged boiling may hydrolyses disaccharides to give a false positive test.

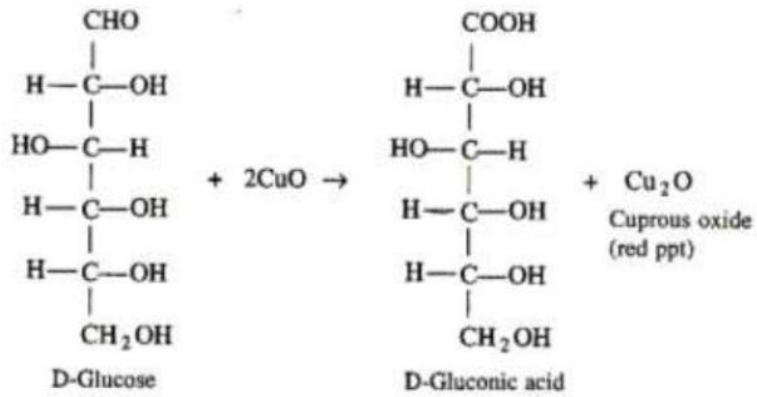
- **Method:**

- 1 ml of the solution to be tested + 2 ml of freshly prepared Barfoed's reagent.

- Place test tubes into a boiling water bath and heat for 2 minutes.
- Remove the tubes from the bath and allow to cool.
- Formation of a green, red, or yellow precipitate is a positive test for reducing monosaccharides.
- Do not heat the tubes longer than 3 minutes, as a positive test can be obtained with disaccharides if they are heated long enough.

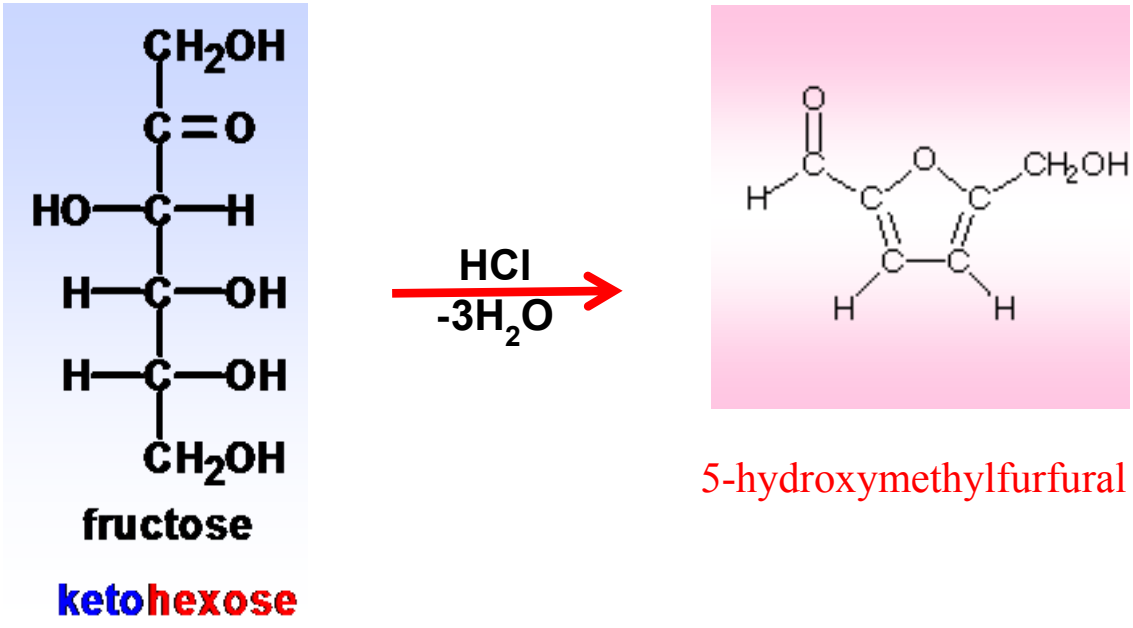






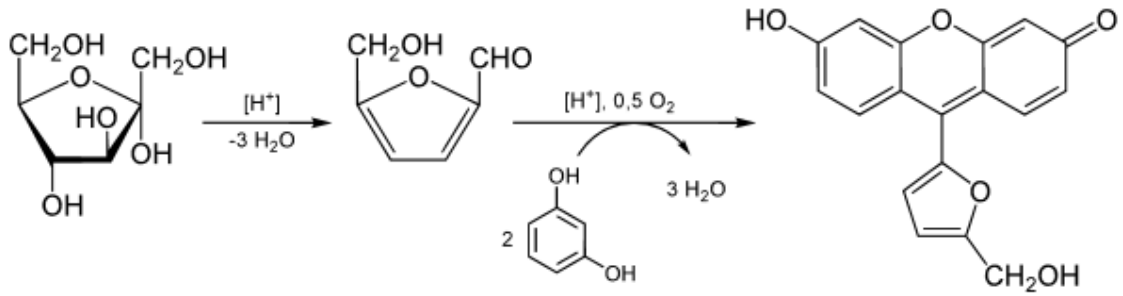
4. Seliwanoff's Test:

The test reagent dehydrates ketohexoses to form 5-hydroxymethylfurfural



5-hydroxymethylfurfural

5-hydroxymethylfurfural further reacts with resorcinol present in the test reagent to produce a red product within two minutes.



Resorcinol red product

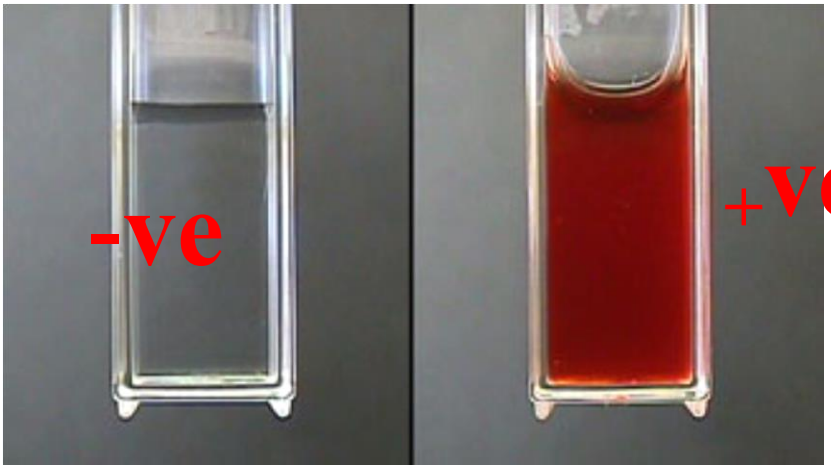
Note:

1. Aldohexoses react to form the same product, but do so more slowly.

2. In case of sucrose, avoid over-boiling because sucrose may be hydrolyzed to its component (glucose and fructose) and gives false positive result.

■ Method:

- 1/2 ml of a sample + 2ml of Seliwanoff's reagent (a solution of resorcinol and HCl) is added.
- The solution is then heated in a boiling water bath for two minutes.
- A positive test is indicated by the formation of a red product.



Lab result: Fructose is a ketose sugar that will react with the reagent to form the cherry-red complex after 1 minute. The Glucose solution will also form a pink to red complex but not after 1 minute. It will form the complex after a longer period.

Limitations:

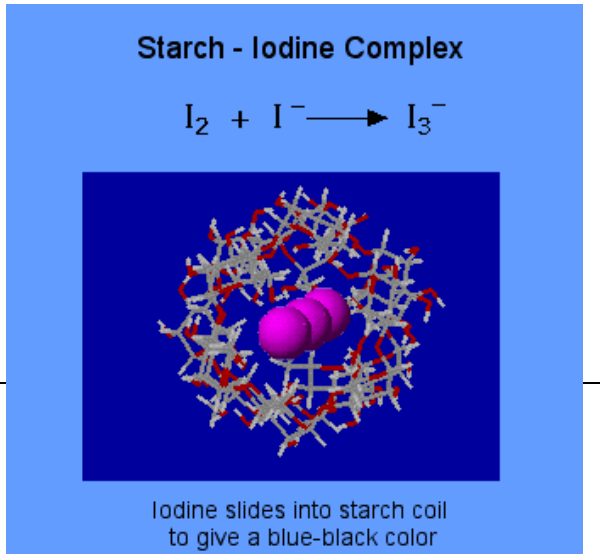
Although this test is able to adequately distinguish a ketose sugar from an aldose sugar, it is not very specific since an aldose may also form the complex. The difference is the time taken to do so.

This test also is a generalized test that does not differentiate the specific ketose present but rather illustrates that a ketose sugar is present. Specific ketose sugar identification must be performed by further testing.

5. Iodine's Test:

(Test for Polysaccharides)
Poly Saccharides adsorb I_2
And form colour complex Starch gives blue colour while glycogen gives reddish-brown colour.

Note: On heating (poly saccharide-Iodine) complexes losses blue colour because of dissociating the complex



while on cooling the blue colour again appears due to re-association of complex

a- Starch:

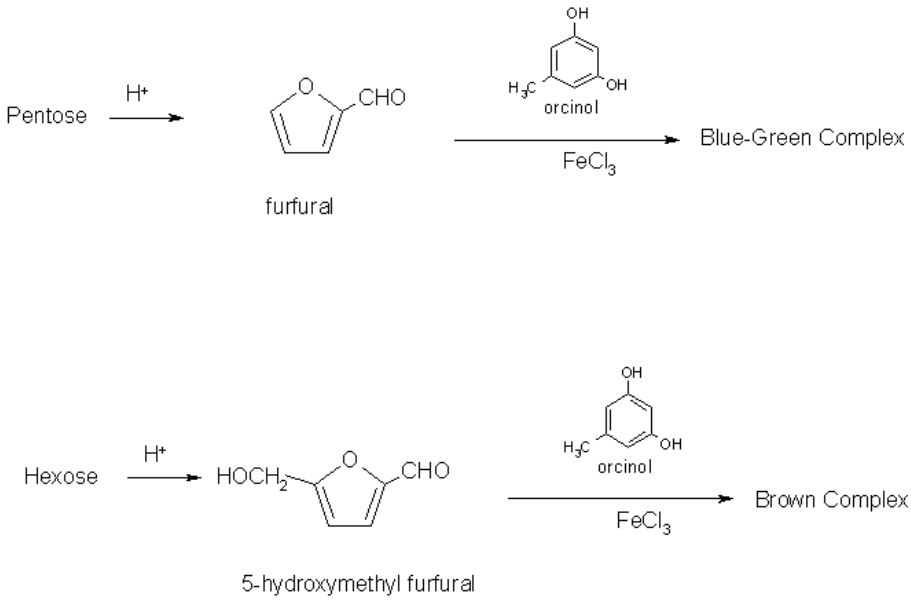
- 1/2 mL of the fresh starch solution + 1 drop of the iodine solution.
- A dark blue color indicates a positive test for starch.
- If the yellow color of the iodine reagent simply becomes diluted, no starch is present.
- Record the observation as positive (blue) or negative (yellow).

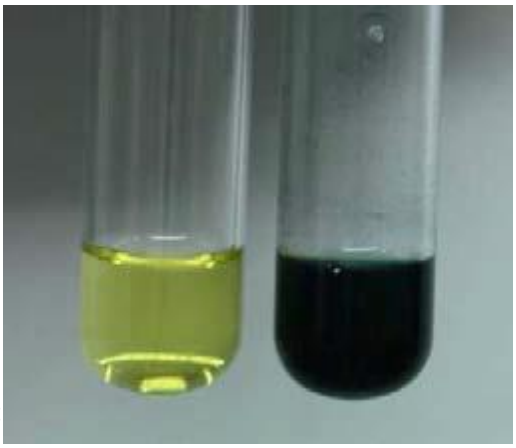
b- Dextrin:

- 1/2 mL of the fresh dextrin solution + 1 drop of the iodine solution.
- A violet color indicates a positive test for dextrin.
- If the yellow color of the iodine reagent simply becomes diluted, no dextrin is present.
- Record the observation as positive (violet) or negative (yellow).

6. Bial's Test:

This test is used to distinguish pentose sugars.
The reaction: Bial reagent contains HCl, orcinol and ferric chloride. The pentose sugars are hydrolysed by the HCl to form a furfural derivative. This derivative then reacts with the orcinol to form a green-yellow complex in the presence of ferric ions via a condensation reaction. Polysaccharides made up of pentose units are hydrolysed to break the glycosidic bonds and then undergo the same reaction to form the complex. Hexoses are also hydrolysed and react with the orcinol but form a red to brown complex rather than a green-yellow colouration.



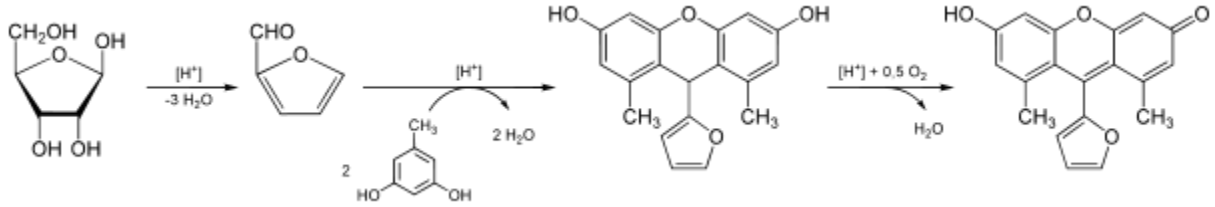


Procedure: **right=+ve**
Left:-ve
1. Take 1 ml of Standard and Test sugar solutions in four test tubes
2. Add 2.5 ml of Bial's reagent to each tube and heat in a boiling water bath for 1 minute and allow the tubes to cool down to room temperature. Look for the formation of blue-green colour.

Lab results: Arabinose is a pentose sugar that will yield the green yellow colour change. Gum Arabic is a polymer of arabinose, rhamnose and galactose and therefore when hydrolysed, will react similar to arabinose and give the green-yellow colour change. However, glucose is a hexose sugar and therefore will not react similar to arabinose and will therefore not yield a green colour change. Rather it may produce a red to brown colour change or no change at all.

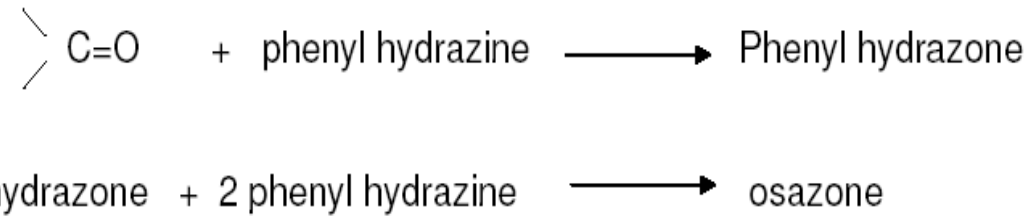
Limitations:

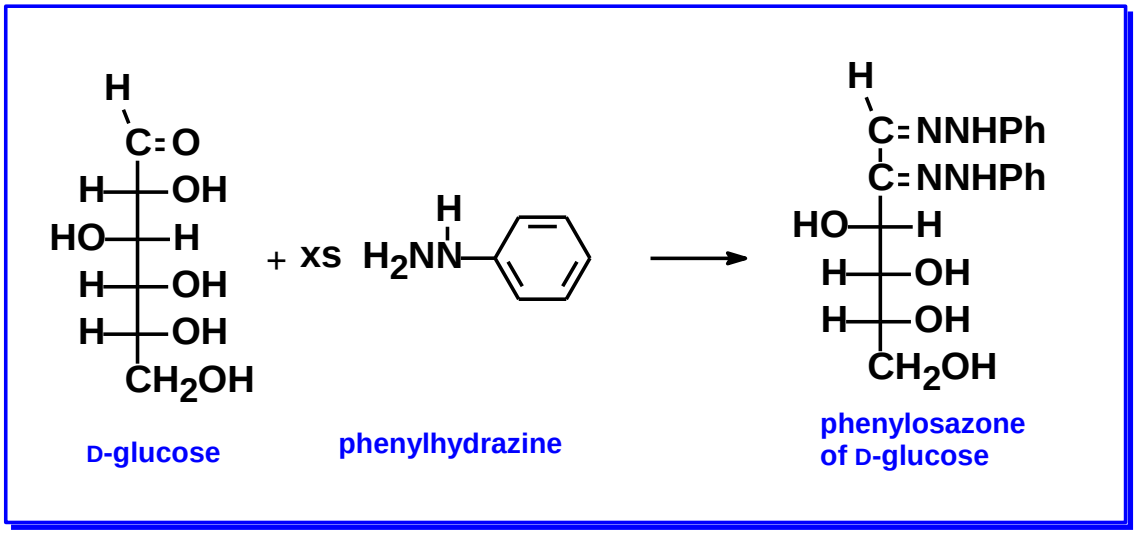
It is not specific to which pentose is present and further tests must be conducted to identify specific pentose sugar.



7. Phenylhydrazene's Test (Osazone Test)

- Osazones are formed when the sugars (monosaccharides) react with a compound known as phenylhydrazine. These sugars are reducing ones which have either a free aldehyde or a ketone group to react with the phenylhydrazine. This reaction is complete in 3 step and consume 3 moles of phenylhydrazine
- During reaction with monosaccharides, additional phenyl hydrazine is consumed in oxidizing the adjacent OH-group to carbonyl group which then forms a second phenyl hydrazone.
- Such bisphenyl hydrazones are called osazones.





Mannose, a pentahydroxyaldehyde that differs in configuration from glucose only at C-2, gives an osazone with exactly the same structure as that of the osazone of glucose

How can these reactions help us determine whether we have glucose or mannose as an unknown? The answer is by timing the reactions. The

osazone from glucose forms in approximately five minutes; whereas, the osazone from mannose forms in less than one minute. Thus, if we allow our unknown glucose or mannose to start forming an osazone at exactly the same time we start osazone formation with authentic samples of glucose and mannose, our unknown will form its osazone at the same time as one of the known compounds but not the other.

- Osazone is a crystalline compound with a sharp melting point will be obtained
- Since only C1 & C2 of a saccharide are involved in osazones, sugars with the same configuration at the remaining carbon atom gives the same osazone.
- D-fructose and D-mannose give the same osazone as D-glucose
- seldom used for identification; we now use HPLC or mass spectrometry
- Depending on the time required to form the insoluble yellow osazone, different sugars can be classified into the following:

Mannose: 1-5 min

Fructose: 2 min

Glucose:5 min

Xylose: 7 min

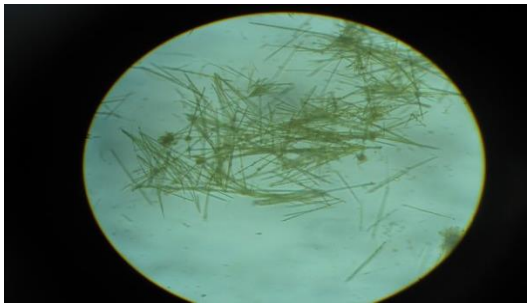
Arabinose: 10 min

Galactose: 20 min

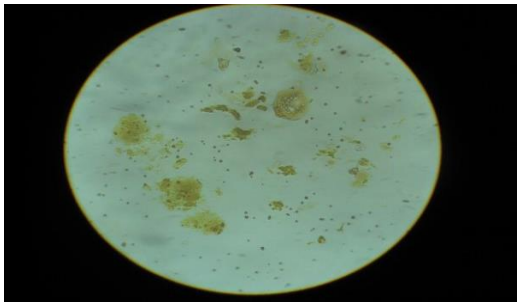
Maltose osazone soluble in hot water

Reagents:

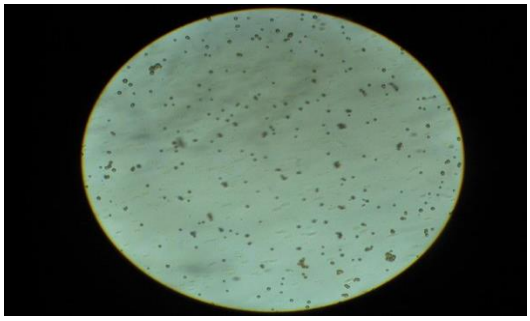
- 1% solutions of glucose, fructose, maltose, mannose, and xylose
- Phenyl hydrazine mixture (2 parts phenyl hydrazine hydrochloride are mixed with 3 parts sodium acetate).
- To 300 mg of phenyl hydrazine mixture add 5 ml of the tested solution,
- Shake well, and heat on a boiling water bath for 30 – 45 min.
- Allow the tubes to cool slowly (not under tap) and examine the crystals microscopically, draw the shapes of the crystals



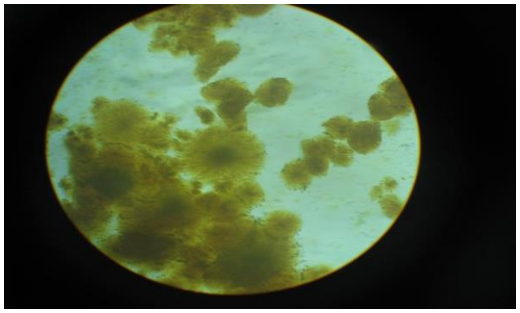
Glucosazone



fructosazone



Xylosazone



Galactosazone

Carbohydrate (Osazone)	Time of formation (Minutes)	Solubility in b water	Crystalline structure
Fructosazone	2	Insoluble	Needle shape
Glucosazone	5	Insoluble	Needle shape
Galactosazone	20	Insoluble	Thorny ball shape
Maltosazone	30-45	soluble	Sunflower/Star shape
Lactosazone	30-45	soluble	Cotton ball/Powder puff shape

8. Hydrolysis of Starch using Saliva or mineral acids:

Outline. The experiment illustrates the conversion of starch to a reducing sugar by the action of hydrochloric acid at boiling point. The longer the starch is exposed to the acid the further hydrolysis proceeds. The experiment is intended to show the contrast with enzymes, which do not need high temperatures and prolonged exposure to reagents and give a quick reaction.

Objective

It demonstrates the hydrolysis of starch into glucose.

- Polymers are broken down by hydrolysis which is essentially the reverse of condensation.
- An –OH group from water attaches to one monometer and a H attaches to the other.
- This is a hydrolysis reaction because water (hydro) is used to break (lyse) a bond.
- When a bond is broken, energy is released.
- Polysaccharides such as starch, dextrin and glycogen, give positive iodine test.
- Starch is a non reducing polysaccharide therefore it does not give positive result with Benedict's, Fehling's and Barfoed's reagents, nor does it form any Osazone.
- However after hydrolysis into monosaccharide by the actions of strong acid , its components (glucose molecules) give all the test positive.

PROCEDURE

- Take test tubes and label them as 'T' (for test) ,Take 15ml of starch sol in test tube
- Add 10 drops of conc. HCl in test tube 'T'
- Put test tube in boiling water bath and start performing iodine test after every min in an indicator dish by taking 1 drop of iodine and 1 drop of starch sol till the iodine test becomes –ve in test tube 'T'
- Heat test tubes for 2 min more
- Take out test tube from boiling water bath, then cool them at room temperature
- Add very small quantity of solid sodium carbonate in test tube

- Continue to add sodium carbonate till the effervescence stops in test tube 'T'
- Now from test tube take small quantities in separate test tubes to perform Benedict's ,Selivanoff's and Osazone tests.

INTERPRETATION

In test tube 'C' Benedict's, Selivanoff's, Osazone test remain –ve because due to absence of conc. HCl in this test tube, the starch is not hydrolyzed. In this test tube iodine test will also remain +ve.

In test tube 'T' Benedict's, Selivanoff's , Osazone test become +ve because due to presence of conc. HCl in this test tube the starch is hydrolyzed into glucose, which is a strongly reducing monosaccharide.

Although acid completely hydrolyze starch to give glucose but this process occurs through various stages.Before complete hydrolysis it gives various products which react with iodine and produce different colors.

Stage of hydrolysis	Color
Starch(insoluble)	Blue
Starch(soluble)	Blue
Amylodextrin	Blue purple
Erythrodextrin	Red
Achrodextrin	No color
Maltose	No color
Glucose	No color

PRINCIPLE

- Heating of starch in the presence of conc. HCl causes its hydrolysis into glucose. because glucose have free Aldehyde group, therefore it is a strongly reducing monosaccharide, and hence Benedict's, Selivanoff's and Osazone tests become positive.
- Sodium carbonate is added to neutralize excessive HCl, because the reducing ability of reducing sugars is high in alkaline medium, and hence gives good results of Benedicts ,Selivanoff's and Osazone tests.

- Erythrodextrin give red colour.its further hydrolysis produces achrodextrins, which gives negative iodine, test. When the iodine test becomes negative, we heat test tubes for two minutes more.The reason being is to provide time to complete hydrolysis of achrodextrin into maltose and maltose into glucose.

Lab2: Carbohydrate quantitative tests

Test: Determination of Glucose using Benedict's Solutions

1. Estimation of Glucose by Benedict's Method:

During qualitative analysis of sugars we have already learnt that glucose reduces copper sulphate in Benedicts reagent under alkaline conditions and a red precipitate is formed. This qualitative method has been exploited for its use in quantitative analysis.

The Benedicts quantitative reagent contains the following ingredients, copper sulphate, sodium carbonate, sodium or potassium citrate, potassium thiocyanate and potassium ferrocyanide. Of these, copper sulphate has to be very accurately measured as the amount of copper sulphate reduced will correspond to the amount of glucose present in solution.

Due to presence of potassium thiocyanate in Benedict's reagent a white precipitate of cuprous thiocyanate instead of red precipitate of cuprous oxide will be formed when copper sulphate is reduced. As the precipitate formed is white it is very easy to determine the end point. Blue tint of Benedict's reagent disappears completely at this point.

The small amount of potassium ferrocyanide added helps to prevent the oxidation of cuprous oxide. Sodium or potassium citrate added does not allow the formation of copper carbonate. The alkaline condition is produced by sodium carbonate which is a mild alkali in comparison with NaOH and is, therefore, less destructive for the sugar. The Bennedict's reagent prepared as follows is stable for long periods of time.

To prepare quantitative Benedict's reagent 18.0 gm. of crystalline copper sulphate is dissolved in 100 ml of water (solution A). Further, 100 gm. of sodium carbonate, 200 gm. of anhydrous sodium citrate and 125 gm. of potassium thiocyanate are dissolved in 800 ml of water with heating (solution B). If solution B is not clear it should be filtered. Solution A is added slowly to solution B with stirring. Then 5 ml of potassium ferrocyanide solution is added and the volume is finally made up to 1 litre after cooling.

The reaction of $CuSO_4$ with glucose is quite complicated and a number of molecules of $CuS0_4$ are reduced by one molecule of glucose. Therefore, it is not possible to write the stoichiometric equation for reaction between

$CuSO_4$ and glucose. But it has been found that 25 ml of the above mentioned quantitative reagent corresponds to 50 mg glucose. Determination of the unknown amount of glucose will be based on this.

Procedure:

Pipette out in a conical flask 25 ml of the Benedict's quantitative reagent. Add about 5 to 10 gm. of Na_2CO_3 and a few porcelain chips to the flask to prevent bumping. Heat the contents of conical flask to boiling and then run in the glucose solution from a burette at first rapidly and then slowly until the blue colour becomes fade.

Allow it to boil for 2-3 minutes more and add glucose solution drop by drop till the solution becomes colourless. Note down the volume of the glucose solution used and calculate the percentage of glucose in solution as described below. Sometimes the solution in the flask becomes too much concentrated due to evaporation of water. To avoid it more water may be added.

Suppose 20 ml of the glucose solution is required to titrate 25 ml of Benedict's quantitative reagent. As 25 ml of the Benedict's quantitative regent is equivalent to 50 mg of glucose, hence 20 ml of the solution contains 50 mg of glucose. Therefore, 100 ml of the glucose contains 50×100/ 20 =250 mg of glucose and the strength of the solution 250 mg per cent.

Estimation of Lactose by Benedict's Quantitative Regent:

Principle is same as for glucose, only difference being 25 ml of Benedict's quantitative regent is equivalent to 67 mg of lactose.

Even sucrose after acid hydrolysis can be estimated by this method.

2. Glucose Oxidase Method for Estimation of Glucose:

In this method, the aldehyde group of β-D-Glucose is oxidized by glucose oxidase to give gluconic acid and hydrogen peroxide.

β-D-Glucose + H_2O + O_2 → gluconic acid + H_2O_2

The hydrogen peroxide may be broken down to water and oxygen by a peroxidase and if an oxygen acceptor is present, it will convert to a coloured compound which can be measured. The reagent usually used is oxidation product of phenol condensed with 4-aminophenazone to give a coloured product as in determination of alkaline phosphatase.

Lab 3: Amino acids and protein qualitative tests

1- Elements of protein

The main elements of proteins are carbon (50%), hydrogen (7%), oxygen (23%), nitrogen (16%), and sulfur (0-3%).

Protein powder is heated in dry test tube then; nitrogen and sulfur are converted to NH_3 and H_2S gases respectively.

Oxygen and hydrogen are appearing as moisture on the wall of the test tube.

Some of carbon content is liberated as CO_2 gas the other is converted to black residue in the bottom of the tube.

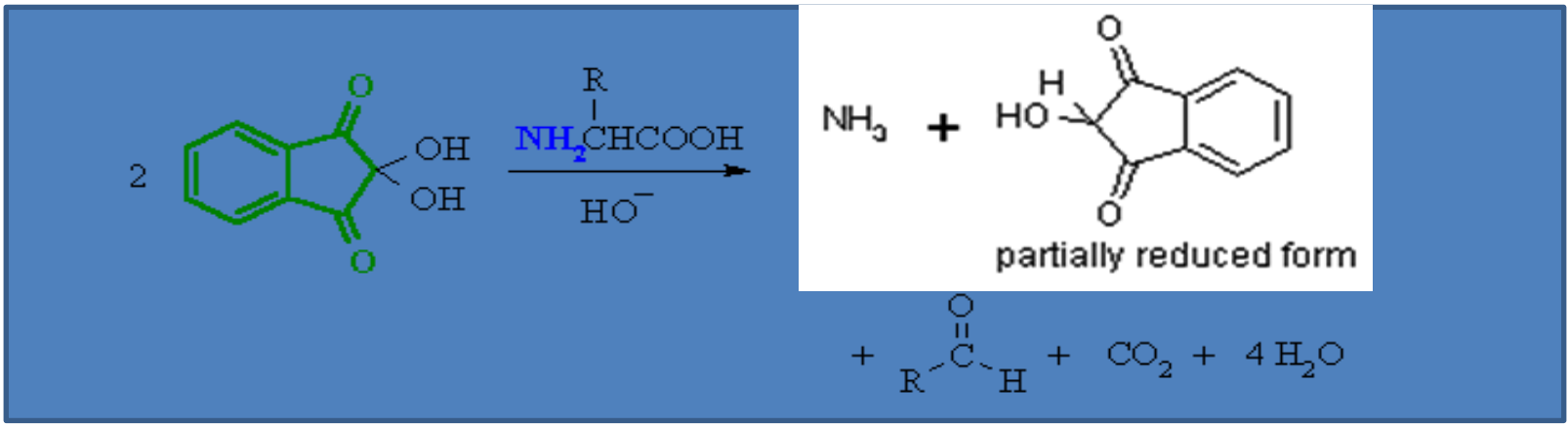
$$\textbf{Protein} + \textbf{O}_2 \xrightarrow{\text{heat}} \textbf{C} + \textbf{CO}_2\uparrow + \textbf{H}_2\textbf{O}\uparrow + NH_3\uparrow + H_2S\uparrow$$

Elements	Detection
Sulfur	**By lead acetate paper/ appearance of black ppt. on the paper**
Nitrogen	**By litmus paper /color change from red to blue**
Hydrogen and oxygen	**Appearance of the moisture on the wall of the test tube confirm the presence of Hydrogen and Oxygen in the sample**
Carbon	**Remaining black residue in the bottom of the tube confirm the presence of Carbon in the sample**

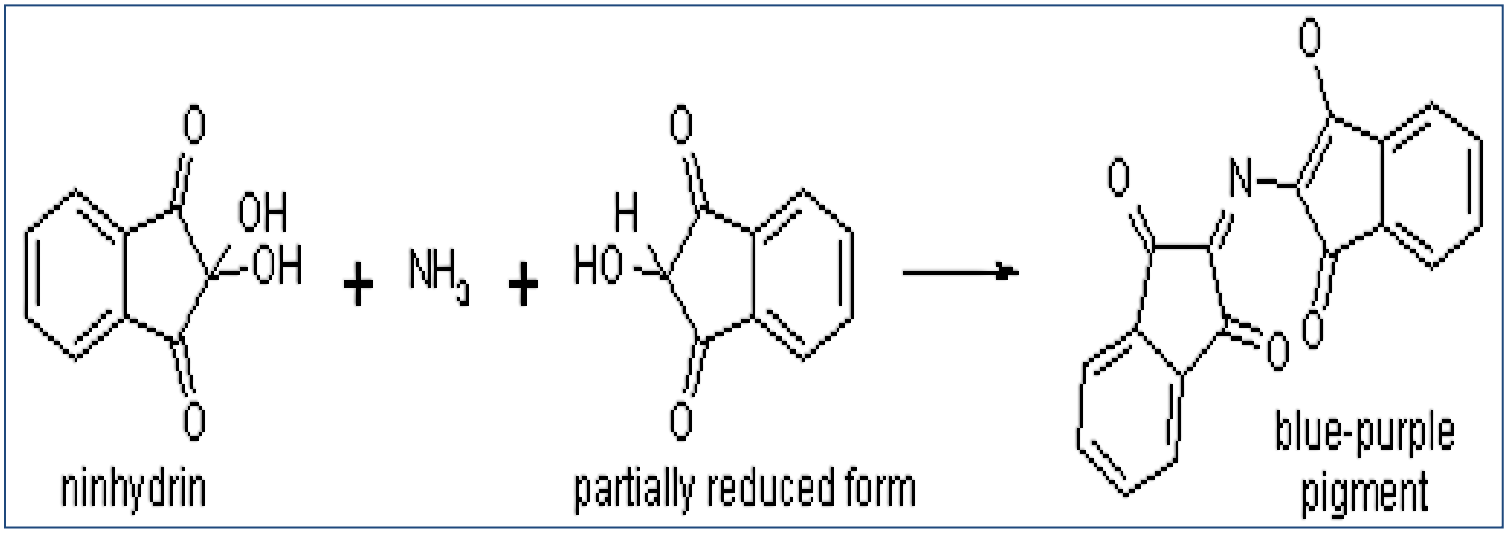
2-Ninhydrin test:

Ninhydrin (triketohydrindene hydrate), a powerful oxidizing agent, react with all α-amino acids to give blue or purple-colored compound. The reaction is also given by primary, secondary amines, amino containing compounds (proteins, peptides) and ammonia without the liberation of CO_2. The imino acids, proline and hydroxyproline, also react with ninhydrin, but in this case a yellow color is formed.

Ninhydrin degrades amino acids into aldehyde, ammonia, and CO_2 through a series of reactions; the net result is ninhydrin in a partially reduced form hydrindantin:



Ninhydrin then condenses with ammonia and hydrindantin to produce an intensely blue or purple pigment, sometimes called **Ruhemann's purple**:



Procedure

1. Add 5 drops of ninhydrin soln. to 1ml of the sample
2. Shake it and put in a boiling water bath for 2 min.

Note: A blue – purple colour indicates that we have protein or aminoacid**.**

3-Biuret test (General test for proteins)

copper(II) ion forms a violet-coloured complex in an alkaline solution when mixed with a compound containing tripeptides and larger polypeptides or proteins

Note that Single amino acids (free) and dipeptides do not give the biuret reaction.

The purple colour is formed when copper (II) ions in the Biuret reagent react with the lone pair of electrons on the N in the peptide bonds to form a complex. Cu2+ forms a "tetra dentate" coordination complex through the four nitrogen donor atoms.

The test produces the light blue to violet complex that absorbs light at 540nm.

The intensity of the colour produced is proportional to the number of peptide bonds participating in the reaction. Thus, the biuret reaction is the basis of colorimetric method used to quantitatively to determine total protein concentration.

In spite of its name, the reagent does not in fact contain "biuret" molecule .The test is so named because it also gives a positive reaction with the "biuret molecule"

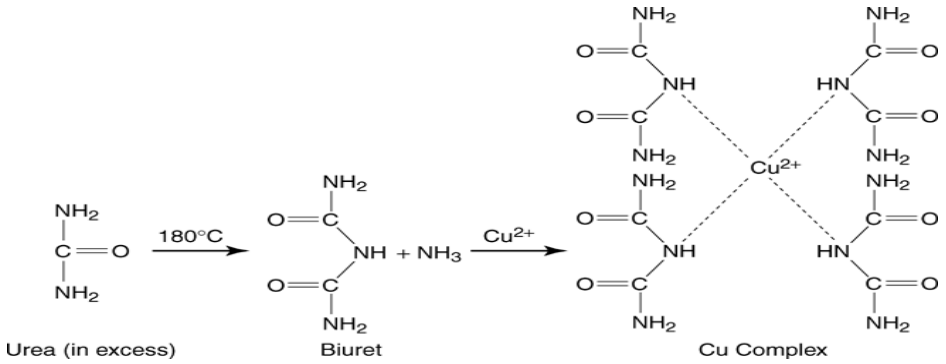
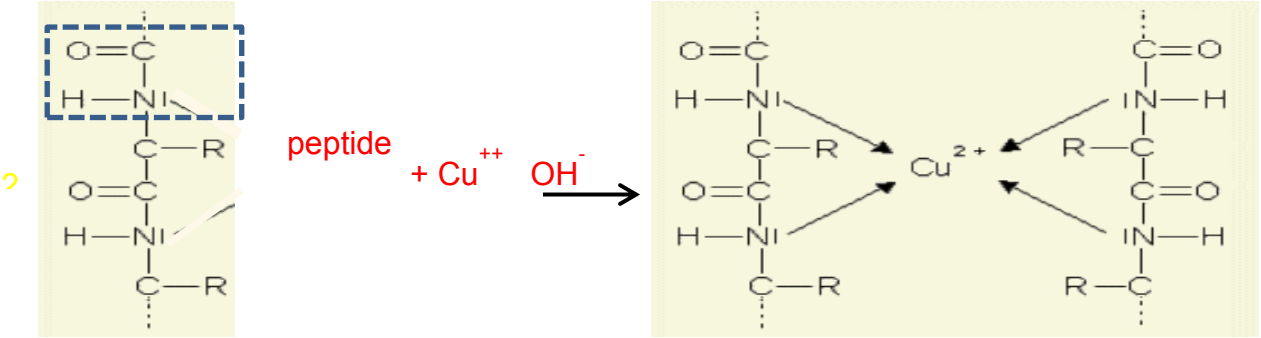


Figure: Biuret is formed by condensation of two molecules of urea, when heated at 180 °C , note biuret forming a complex with Cu^{2+}.

Because polypeptides have a structure similar to Biuret molecule, they are able to complex with copper by the biuret reaction.

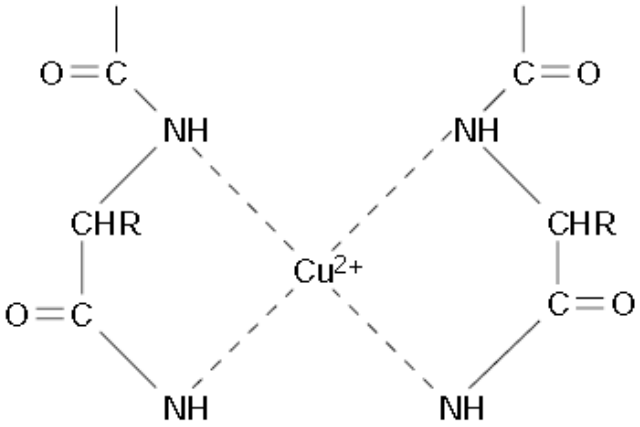


Polypeptide

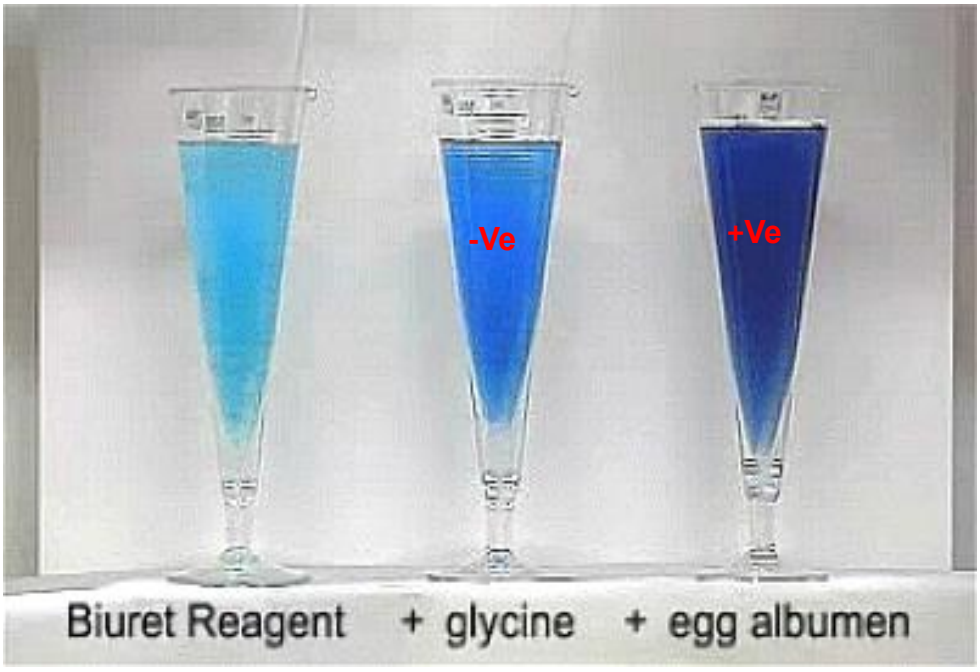
Note: other non-protein compounds such as oxamide and "Biuret" give a positive test.

Procedure:

1. Label 4 clean, test tubes with the names of the following solutions: 1% tyrosine, 2%albumin, , 2% gelatine and phenylalanine.
2. Place 15 drops of each in the corresponding test tube.
3. Add 5 drops of 3M sodium hydroxide and 2 drops of 0.1M copper (II) sulfate solution to each test tube.
4. Mix the contents. Record your observations on Data Sheet.



Protein–copper (II) ion complex, also called the biuret complex.

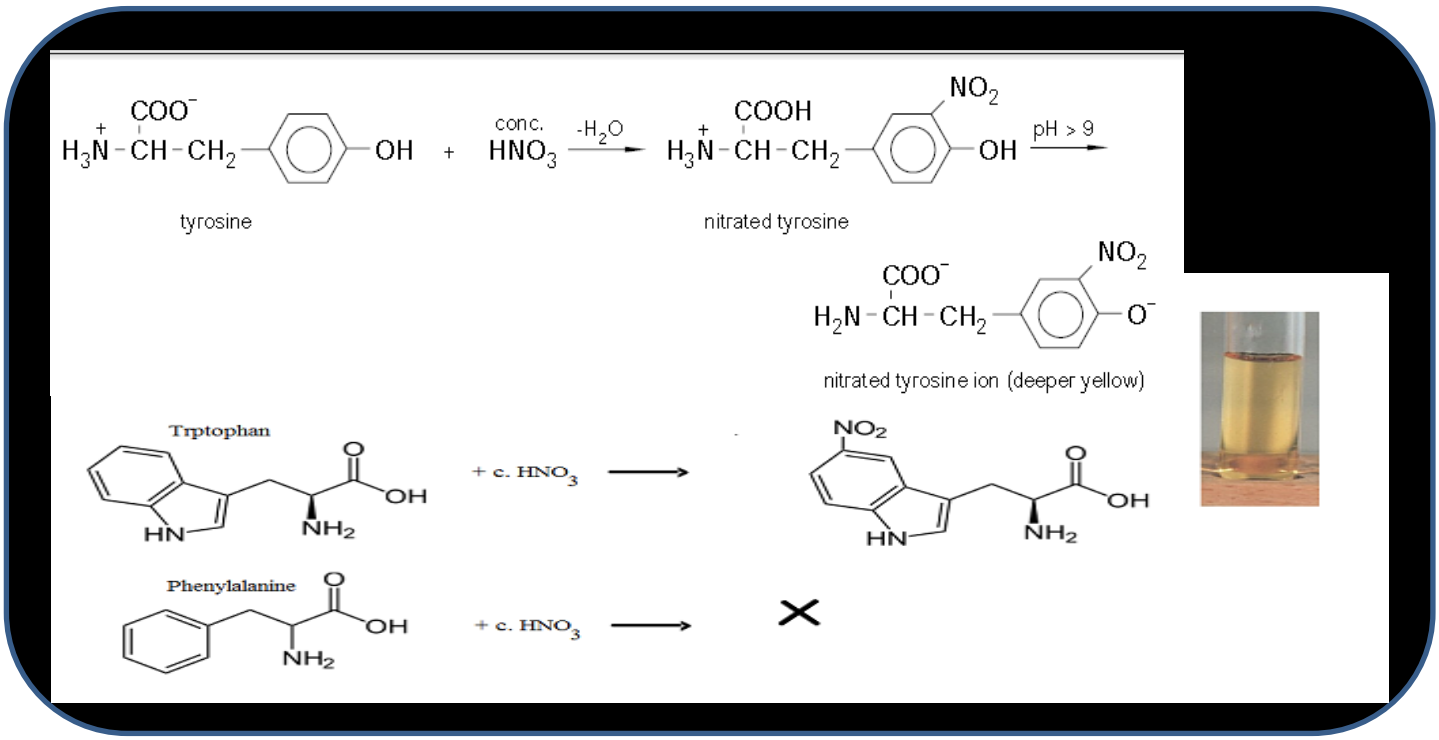


Exp4: Xanthoproteic Test

general test for those amino acids and proteins that contain aromatic groups.

Some amino acids contain aromatic groups that are derivatives of benzene. These aromatic groups can undergo some reactions. One of these reactions is the nitration of a benzene ring with nitric acid. The amino acids tyrosine and tryptophan contain activated benzene rings and readily undergo nitration.

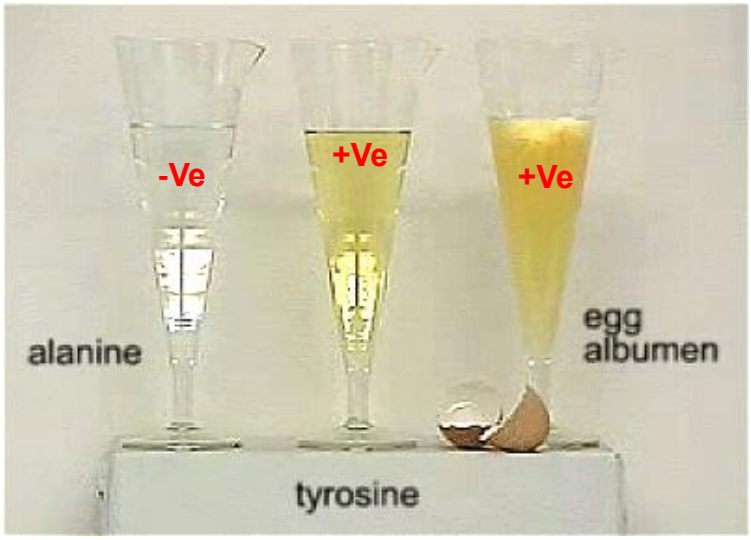
The amino acid phenylalanine also contains a benzene ring, but the ring is not activated and therefore does not readily undergo nitration why???. This nitration reaction, when used to identify the presence of an activated benzene ring, is commonly known as the **xanthoproteic test, because the product** is yellow. Xanthoproteic comes from the Greek word xanthos, which means "yellow." The intensity of the yellow colour deepens when the reaction occurs in basic solution. The xanthoproteic test for tyrosine is shown in this Equation bellow. This reaction is one of the reactions that occurs if you spill a concentrated solution of nitric acid onto your skin. The proteins in skin contain tyrosine and tryptophan, which become nitrated and turn yellow.



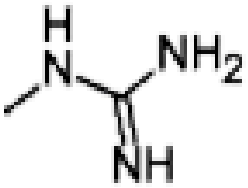
Note: concentrated nitric acid—toxic, corrosive, and strong oxidant.

Procedure:

1. Label 4 clean, test tubes with the names of the following solutions: 1% tyrosine, 2%albumin, 2% gelatine and phenylalanine.
2. Place 15 drops of each in the corresponding test tube. Working under a **fume hood,**
3. **Add 10** drops of concentrated nitric acid to the 1% tyrosine test tube. Using a test tube holder.
4. Place the test tube into the boiling-water bath. the contents gently for 1–2 min.
5. Remove the test tube from the boiling-water bath. Place it into a test tube rack and allow it to cool.
6. Record your observations on Data Sheet 2.

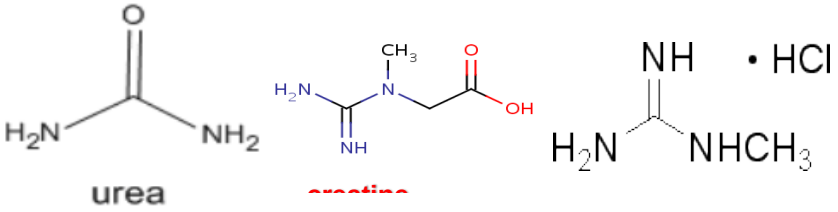


5. **Sakaguchi Test:** this test is specific for guanido group the only amino acid containing guanide group is arginine.



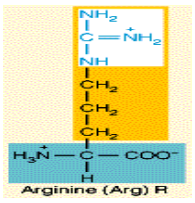
guanido group

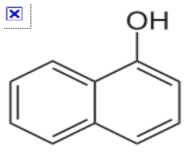
Principle: Free arginine or arginyl residue in proteins react with α- naphthol and an oxidizing agent like (sodium hypo chlorite or sodium hypo bromite (NaOCl or NaOBr)) in alkaline medium (NaOH) to give a red coloured product the other compounds that contains guanidine group can interfere with sakaguchi test with α- naphthol and oxidizing agent in alkaline medium to give red compound for example:



urea

Methyl guanidine





NaOCl
NaOBr

Red complex

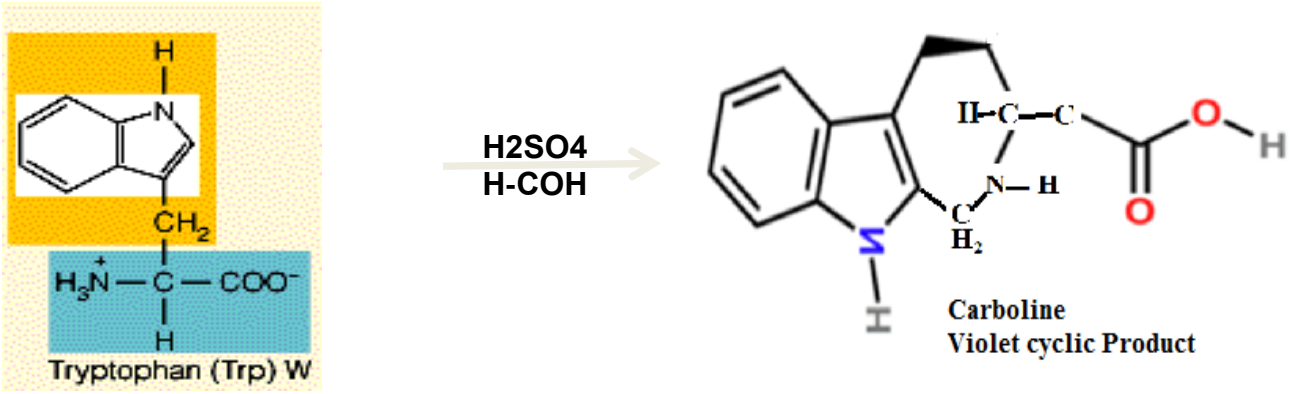
Procedure:

1. add 5 drop of NaOH to 3 ml of test solution
2. Mix and add 2 drops of molische's reagent α- naphthol

3. Mix and add 4-5 drops of NaOCl
   Red colour indicate of presence of arginine

6. **RosenheimTest:** this test is specific for indole group the only amino acid which containing indole group is tryptophan, so this test is specific for tryptophan.

Principle: The indole rings of tryptophan react with formaldehyde in the presence of strong acid (rosenheim reagent) to form violet cyclic product named carboline.



The rosenheim reagent only react with protein that contain tryptophan, the protein is hydrolysed by concentrated sulphuric acid and the tryptophan to be free, so it react with formaldehyde to form violet product.

Protein + H_2SO_4 → tryptophan + other A.A

Tryptophan + Formaldehyde H_2SO_4 → Violet product

Procedure:

1. Add 0.5 ml of the test solution to a clean test tube
2. Add 2-3 drop of formaldehyde shaking more quickly and add 2 drops of $FeCl_3$
3. Add Concentrated H_2SO_4 drop by drop to produce violet colour.

7. Lead Acetate Reaction:

Principle: This reaction is specifically given by sulphur group as in cysteine and cystine, such group can be removed by treatment with alkali as sodium sulphide and if lead ions are present, lead sulphide will precipitate.

Note: strong alkali does not liberate sulphur group from methionine thus giving negative result.

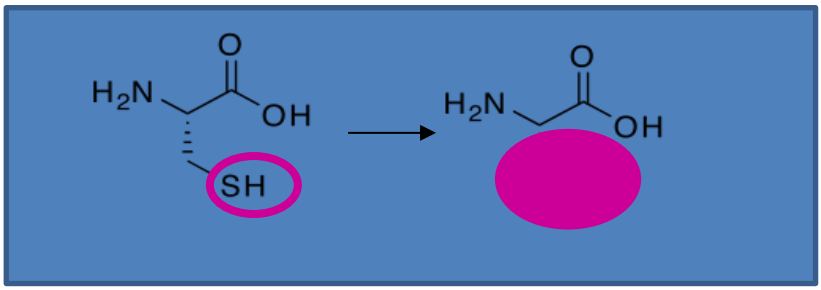
$$R\text{-}SH + NaOH \xrightarrow{\Delta} Na_2S + ROH + H_2O$$

$$(CH_3COO)_2Pb + Na_2S \xrightarrow{\Delta} PbS\downarrow + 2CH_3COONa$$

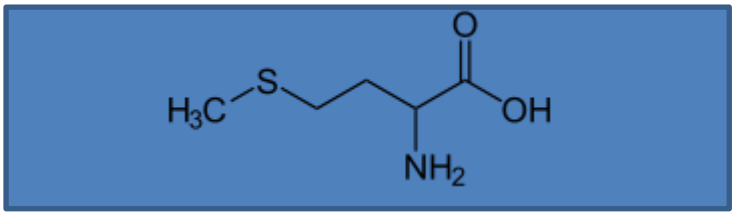
Black ppt

Lead acetate reaction:

- Liberates sulfur content to detect (cys)
- Sulfur group of cysteine is liberated through heating with strong alkali.
-

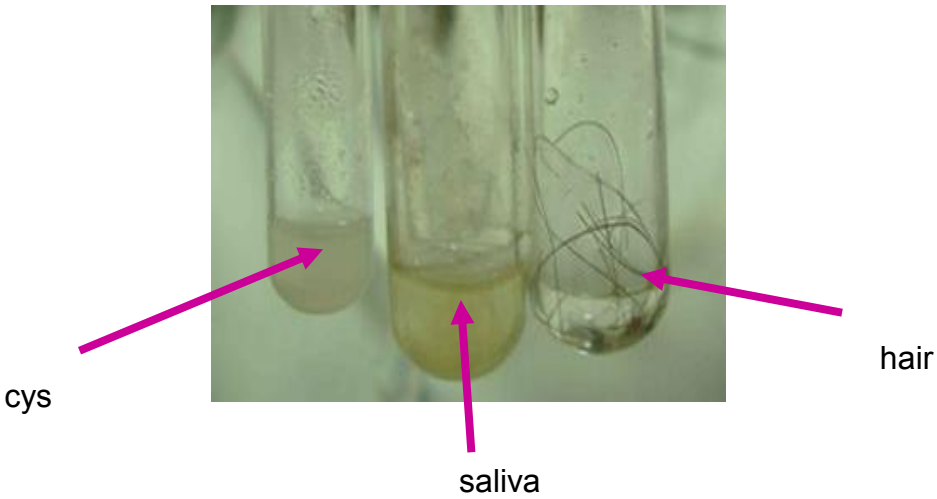


NOTE: Treatment with alkali does not liberate sulfur from methionine



Procedure:

1. To 2 ml of test solution, add 2ml of 40% NaOH.
2. Heat the solution for 1 min. in a boiling water bath.
3. Add 3-5 drops of 10% lead acetate. gray, brown or black indicates cysteine (cys)



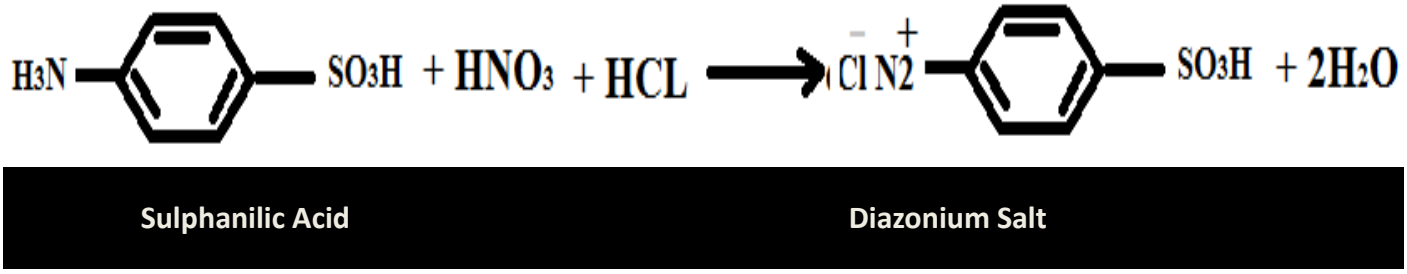
8. **Pauly's test:** This test answers for tyrosine, tryptophan and histidine residues.

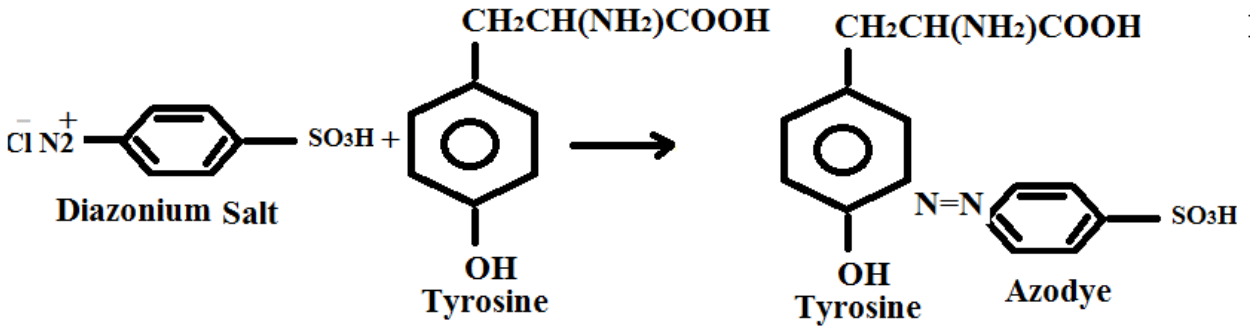
Principle: Diazotized sulphanilic acid couple with amines, phenols (Tyrosine) and imidazole (Histidine) to form highly colored compounds. The diazonium compound is only formed in the cold, so all solution must be cooled in ice before diazotization

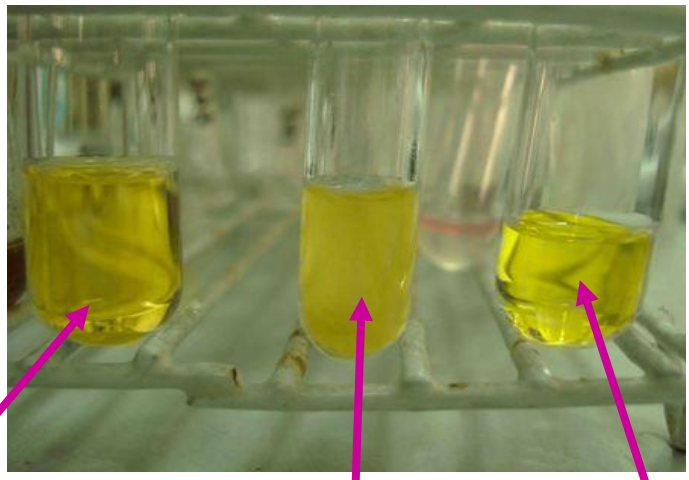
Procedure:

1. Mix 1ml of sulphanilic acid with 2ml of test solution
2. Cooled in ice and add 1ml of sodium nitrite solution and leave in a cold for 3 min.
3. Make the solution alkaline by addition of 1ml of sodiumcarbonate solution

- POSITIVE TEST : dark yellow or orange
  : histidine, tryptophan and tyrosine residues



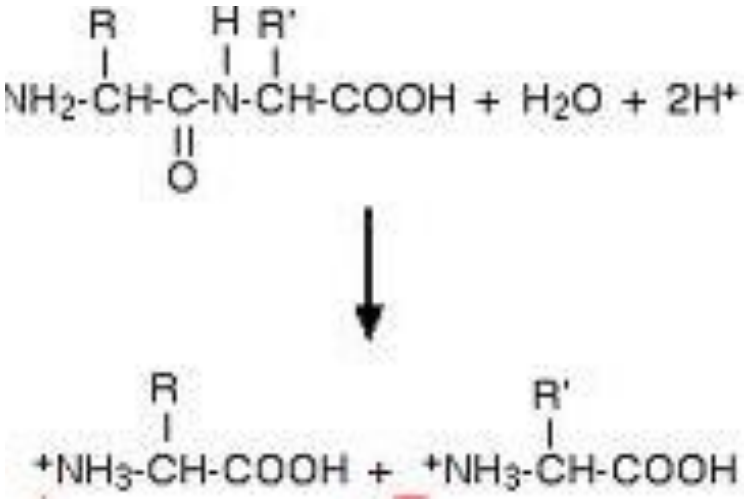




Chemical properties of protein

1. **Hydrolysis**: protein hydrolysis is the reverse of peptide bond formation and the peptides are broken down to amino acids. Digestion is an example of protein hydrolysis.

   Note: In the laboratory the protein can be hyrolyzes by heating with hydrochloric acid.



2. **Denaturation:** denaturation of proteins refers to the unfolded and rearrangement of the secondary, tertiary, and quaternary without breaking the peptide bonds (primary structure will remain) . A protein that is denatured loses its biological activity**.**

3. Renaturation: is the process of returning a denatured protein structure to its original structure and normal level of biological activity

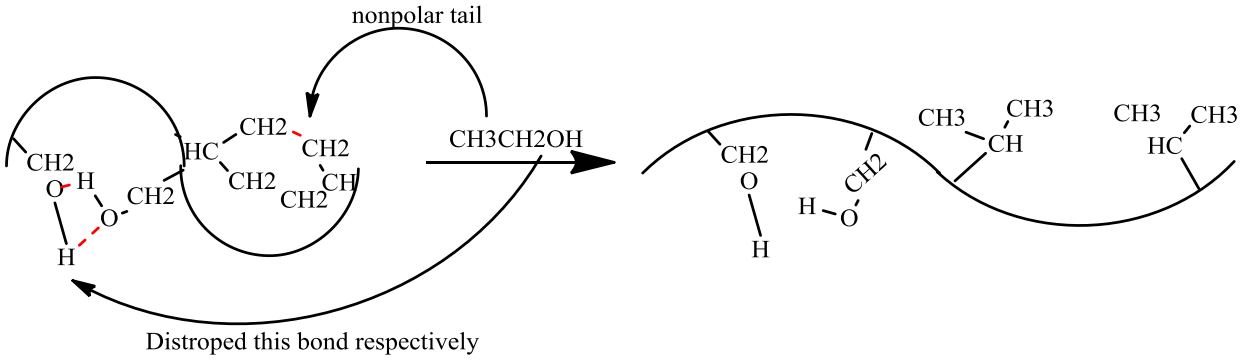
1. Protein denaturation by organic solvent

Organic solvent (such as alcohol) denature protein by disrupting the side chain intermolecular hydrogen bonding and hydrophobic interaction. New hydrogen bonding and hydrophobic interaction are produced between protein side chains and polar head and hydrophobic tail of alcohol respectively.

Addition of organic solvent such as alcohol and acetone decrease the concentration of water in the solution. This effect tends to decrease solubility of protein.

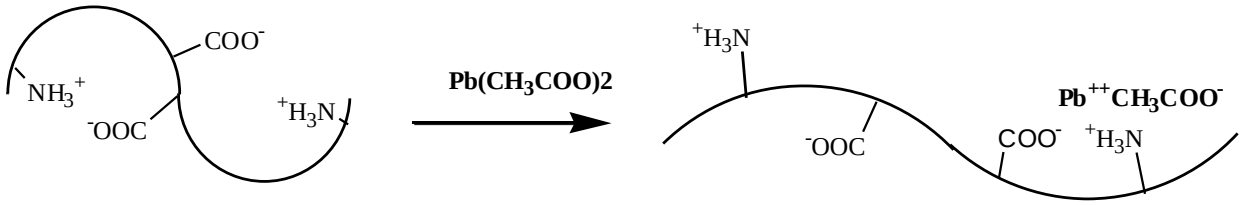
A 70% alcohol solution is used as a disinfectant on skin. This concentration of alcohol is able to penetrate the bacterial cell wall and denature the proteins and enzymes inside of the cell.

95% alcohol solutions coagulate the protein on the outside of the cell wall and prevent any alcohol from entering the cell.



2. **Protein denaturation by heavy metal ions**

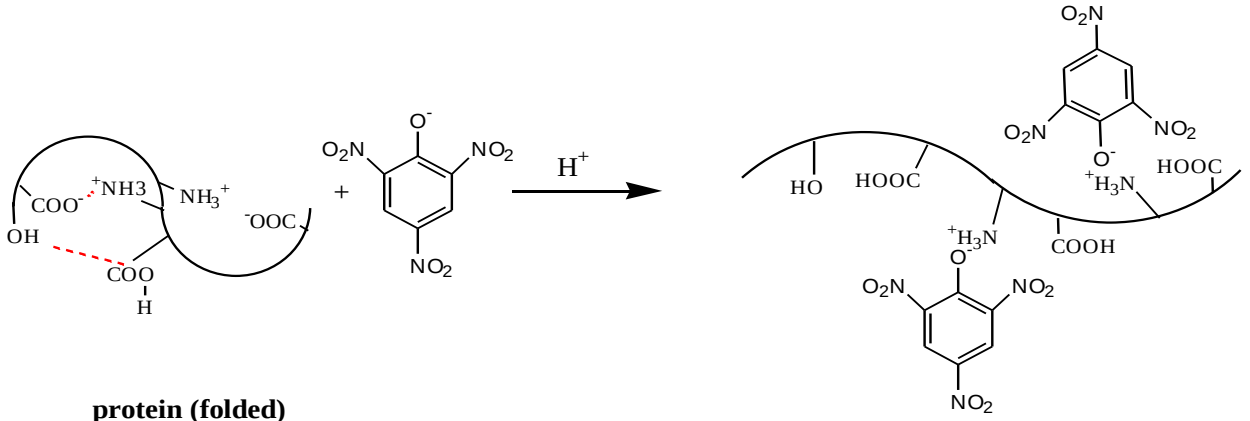
Heavy metal act to denature protein by disrupting salt bridges in protein molecule. Heavy metal salts usually contain Hg^{2+}, Pb^{2+}, Ag^{1+}, Ti^{1+}, Cd^{2+} and other metals with high atomic weight. these cations react with negatively charged groups on the proteins (**carboxylate ions of the acidic amino acids**) **. Thus, they disrupt both disulfide bridges and salt linkages and cause the protein to precipitate out of solution as an insoluble metal-protein salt.**



This reaction is used for its disinfectant properties in external applications. This reaction is used in reverse in cases of acute heavy metal poisoning. In such a situation, a person may have swallowed a significant quantity of a heavy metal salt. As an antidote, a protein such as milk or egg whites may be administered to precipitate the poisonous salt. Then an emetic is given to induce vomiting so that the precipitated metal protein is discharged from the body.

3. **Protein denaturation by acidic reagent**

These compounds are acid and carry a large negative charge which neutralizes positively charged groups on protein to form insoluble salt. The acidic reagents are, therefore most effective at acid pH values where proteins are positively charged.

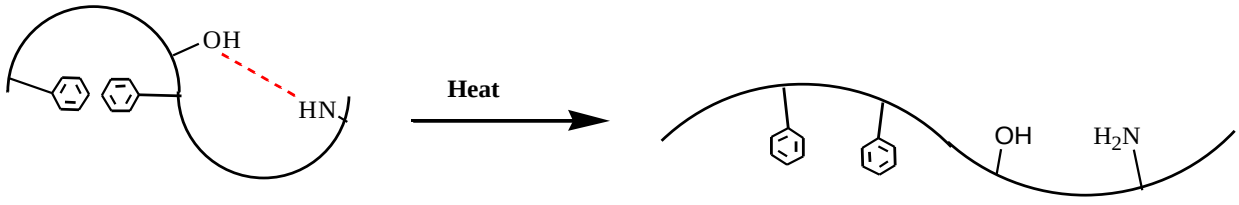


protein (folded)

Denaturated protein (unfolded)

4. **Protein denaturation by heat**

Heat can be used to disrupt hydrogen bonding and non polar hydrophobic interaction. This occurs because heat increases the kinetic energy and causes the molecules to vibrate so rapidly and the bonds are disrupt.



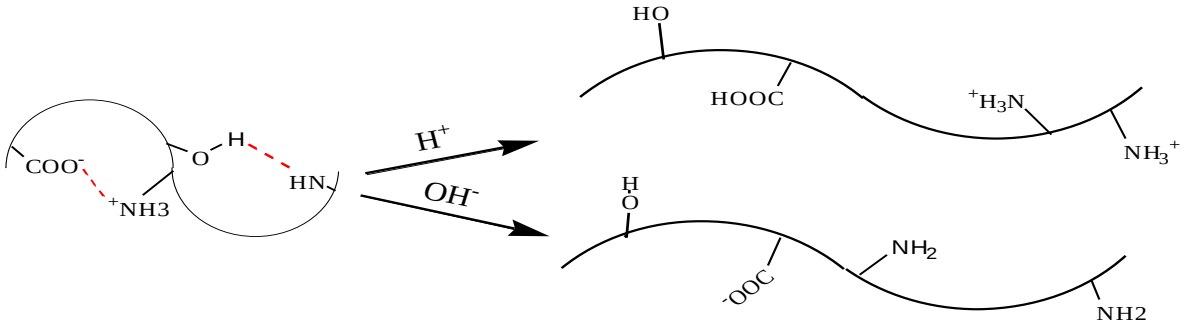
Protein (folded) Protein (unfolded)

The protein in eggs denature and coagulate during cooking, other foods are cooked to denature the protein to make it easier for enzymes digest them.

Medical supplies and instrument are sterilized by heating to denature proteins in bacteria and thus destroy the bacteria.

5. **Protein denaturation by extreme pH**

Addition of strong acid or base result in protonation or deprotonation of ionizable R –groups. As a consequence hydrogen bonds and ionic bonds are disrupted.



Note: - precipitation of protein by concentrated nitric acid is called Heller's test

6. **Precipitation of protein by salts**

Solubility of protein

Proteins are large molecule with a definite size, shape and charge. Solubility of protein depends on the proportion and distribution of polar hydrophilic groups and non-polar hydrophobic groups in the molecule.

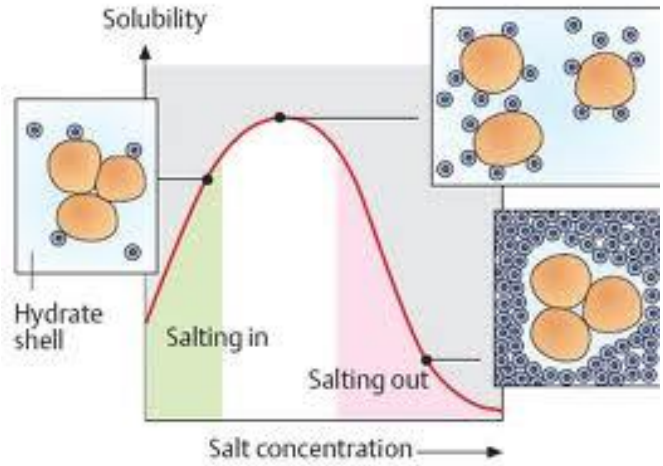
Salting in

Addition of small amount of inorganic salt increases the solubility of protein. The cations and anions of salts interact with polar groups on proteins, tending to increase solubility. The process is called salting in.

Salting out

Generally, the high molecular weight substances can be precipitated from their solution by addition of salt. When an inorganic salt like ammonium sulfate or sodium sulfate is added to a protein solution, the effective concentration of water available for the protein is decreased and the protein is precipitated .The process is known as salting out.

The higher is the molecular weight of protein, lesser is hydrated, lower is the concentration of salt required for precipitation.



Lab 4: Quantitative determination of proteins by biuret reagent

Spectrophotometric Determination of Total Protein-Biuret Method

Background: Proteins are essential to human life. They are macromolecules which are assembled on an as needed basis. They regulate may bodily functions and can function as enzymes.

Biological/Clinical Significance: Proteins are macromolecules with molar masses ranging upwards of 6000 Daltons. They are formed from colvalently bonded amino acids units. The simplest amino acid is glycine whose structure is: $^{+}NH_3CH_2COO^{-}$

Proteins are formed by the linkage of these amino acid groups via an amine group-carboxylic acid group covalent bond which also produces water. Hundreds to thousands of amino acids are present in proteins. There are 20 standard amino acid groups which vary only in what is called the "side chain". That is, the group which is attached to the central carbon (in glycine it is Hydrogen, in others it may be a methyl group or a phenyl group or something else). It is the combination of these 20 amino acids and how they are ordered which dictates the function of a protein.

Proteins form between 50 and 70 % of a cells dry weight and are found in all cells, secretions, fluids and excretions of the body. The concentration of proteins in the body ranges from 6.0 g/dL to 8.3 g/dL. The most abundant protein is albumin which can make up 60% of the total protein concentration.

Emphasis and Technique: The experimental procedure teaches the concepts of spectrophotometric analysis. The Lambert-Beer Law relationship is introduced as are rudimentary analytical spectroscopy considerations. Standard (or calibration) curves are generated and the goodness of fit to a linear relationship emphasized.

Spectrophotometric analysis relies on the interaction of electromagnetic radiation (light) with the matter of interest. Strictly speaking, every compound has a distinct absorption spectrum which allows its identification, in many cases, in the presence of other compounds. In addition to the identification of a compound, it is also possible to determine quantitatively the concentration of that compound. The relationship between absorbance and concentration is given by the Lambert-Beer Law and is written mathematically as:

$A = \varepsilon bC$

Where A is the absorbance, a unit-less quantity;

ε is the molar absorptivity constant (a constant of the compound having units of $L*mol^{-1}*cm^{-1}$); and b is the path length over which the light interacts with the sample in cm.

In a more practical sense, the absorbance is defined as the negative logarithm of the transmittance This is given mathematically as:

$$A = -\log T = -\log \frac{I}{I_0}$$

In analytical spectrophotometry, the molar absorptivity of the unknown compound may not be known. It is therefore a common practice to generate a calibration or standard curve. A standard curve is generated by measuring the absorbance of a series of samples for which the concentration is known. Then, since the Lambert -Beer Law shows a linear relationship between absorbance and concentration, a linear least squares fit of the standard curve will yield a mathematical relationship between the absorbance and concentration. This relationship in turn can them be used to calculate the concentration of the unknown sample.

The Biuret Method, which is the most widely used method for total protein determination, relies on the complexation of Cu^{2+} by the function groups involved with the peptide bond. A minimum of two peptide bonds is needed for the complexation to occur.

Upon complexation, a violet color is observed. The absorbance of the Cu^{2+}-protein complex is measured at 540 nm and compared to a standard curve.

Reagents

1) NaOH, 6.0 M. Dissolve 60g of NaOH in distilled water and dilute to 250 mL. Store in a tightly closed polyethylene bottle at room temperature.
2) Biuret reagent. Dissolve 1.50 g of copper (II) sulfate in 250 mL of distilled water. Add 4.5g of sodium potassium tartrate and 2.5 g of potassium iodide. After solids have dissolved, add 50 mL of 6.0 M NaOH and dilute to a total volume of 500 mL with distilled water. Store in a tightly capped ploye thylene bottle at room temperature. The reagent is stable for approximately six months.
3) Biuret reagent blank. Prepare exactly as in step 2 but omit the copper (II) sulfate.
4) Sodium Azide solution 1.5 mM. Add 0.05 g of sodium azide to 250mL of distilled water. Dilute to a total volume of 500 mL.
5) Protein standard. Dissolve 1.0 g of 7 mL of sodium azide solution. Dilute to 10 mL total volume with sodium azide solution.
6) Standard solutions. Dilute the Protein standard to 20, 40, 60, 80, and 100 g/L by adding the appropriate amount of water. The total volume of each should be 10 mL.

Procedure

1) Pipette 5.00mL of the biuret reagent into a each of 7 test tubes.
2) Pipette 5.00mL of the biuret blank reagent into a each of 7 test tubes.
3) Prepare a reagent-series by adding 100 μL of each of the protein standards to five separate test tubes filled with the biuret reagent. Prepare a reagent blank by adding 100 μL of water to a sixth test tube different test tube filled with biuret reagent. Prepare the serum unknown by adding 100 μL of serum to a seventh test tube filled with biuret reagent. Mix each tube by placing a piece of a parafilm on the top and inverting several times.
4) Prepare a blank-series by adding 100 μL of each of the protein standards to five separate test tubes filled with the biuret blank reagent. Prepare a reagent blank by adding 100 μL of water to a sixth test tube different test tube filled with biuret blank reagent. Prepare the serum unknown by adding 100 μL of serum to a seventh test tube filled with biuret blank reagent. Mix each tube by placing a piece of a parafilm on the top and inverting several times.
5) Allow the cuvettes to stand at room temperature for 30 minutes.
6) Using the reagent-series blank, zero the Spec 20 at 540 nm and measure the absorbance of the reagent series including the serum unknown. Be sure to mix by inversion before making this measurement.
7) Using the blank-series blank, re-zero the Spec 20 and measure the absorbance of the blank series including the serum unknown. Be sure to mix by inversion before making this measurement.
8) Conduct a blank subtraction by subtracting the absorbance of the blank-series from its reagent series counterpart. Plot the new absorbance vs concentration, perform a least squares fit of the standard curve and determine the concentration of the unknown.

Lab 5: Lipids Qualitative tests:

Lipids are classified as simple or complex

1. **Simple lipid**
   a. Fatty acids
   b. neutral fats (monoglyceride, diglycerde, and triglyceride)
   c. waxes
2. **Complex lipids**
   a. Phospholipids
   b. Glycolipids
   c. Lipoproteins

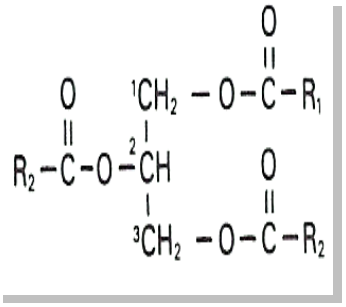


Figure 1 : Triglyceride

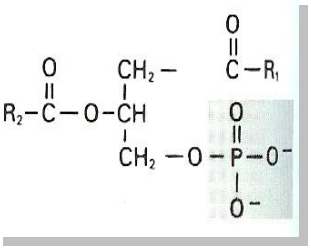


Figure 2 : Phosphatidic acid

Grease-spot Test

This simple test for lipids has been used for centuries. Lipids that are derived from glycerol and sphingosine, a long-chain base that is the backbone of sphingolipids, will produce translucent "spots" or "stains" on fabrics. If the lipid is not a derivative of glycerol or sphingosine, it will not produce a translucent spot on the fabric. The grease-spot test requires that the lipid be in liquid form.

Note: Semi-solid lipids, because of the higher degree of saturation in the fatty acid chains, have melting points higher than room temperature and therefore need to be mildly heated before testing.

Procedure

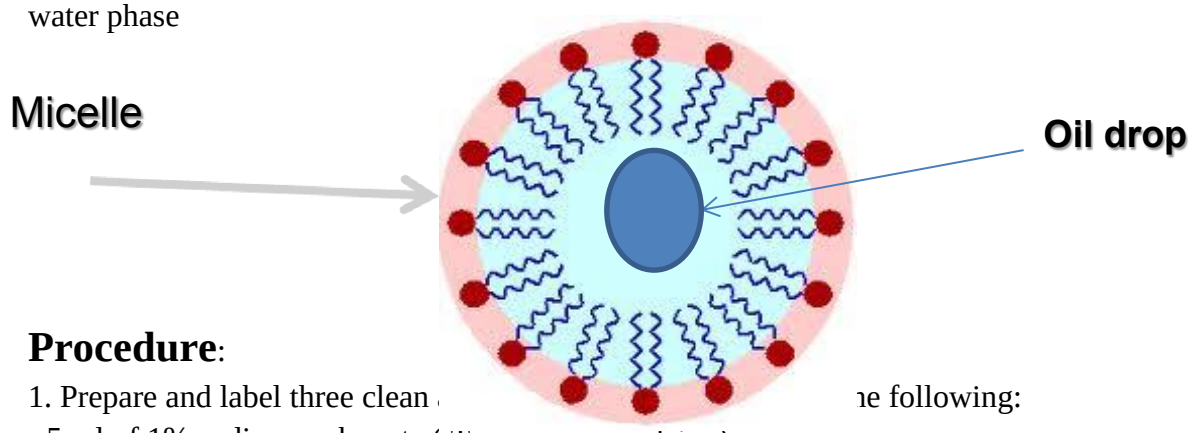
1. Dissolve small amount of oil in ether
2. Put one drop of the ether soluble oil on a piece of filter paper.
3. Heat the spot on the paper until the solvent evaporates.
4. Grease spot can be observed on filter paper and it will be transparency.
5. Record your observations.

Emulsification Test

- As mentioned in the solubility test the lipids are insoluble in water and when mixing lipids with water temporarily mixed and after a few minutes the water and lipids are separate as a two layers but by a process known as emulsification we able to stabilize this mixing and changing the temporarily mixing the (water and lipid) to permanent mixing .
- Emulsification: is the stabilizing of colloidal dispersion of lipids by an emulsifying agent.
- It is the process of diffusion a small droplet between the molecules of water , sometime leaving the mixture (oil + water) it is turn back and large of drops on the surface of water can temporarily emulsification.
- But it can be converted to permanent emulsification when we using emulsifing agent , the best emulsifing agent is bile salt and lecithin.
- The emulsifing agent possess both (hydrophilic) and non-polar (hydrophobic) groups

The hydrophobic (non-polar) tail of the emulsifying agents directed to the oil drop while hydrophilic (polar) head directed to water and forming a micelles molecules.

This micelles composed of the oil drop in the core of it, and the hydrophobic part of the emulsifying agent directed to oil drop, while the surface of the micelle molecules is hydrophilic and directed to the water phase



Procedure:

1. Prepare and label three clean ıe following:
- 5 ml of 1% sodium carbonate (Temporary emulsion)
- 5 ml of 1% soap solution (Permanent emulsion)
- 5 ml of Albumin (Permanent emulsion)
2. Add 3 drops of oil to each tube
3. Shake for 10 minutes and then let to stand.
4. Record your observations

Test for unsaturation

Lipids can be of two types:

Saturated: which are solid at room temperature

unsaturated: which are liquid at room temperature such as oil , higher is the degree of unsaturation, lower is the temperature required to liquefy it.

Unsaturation fatty acid can react with halogens like bromine or iodine due to the presence of double bonds.

$CH_3(CH_2)_7\ CH{=}CH\ (CH_2)_7COOH + Br_2 \longrightarrow CH_3(CH_2)_7\ CH\ Br\text{-}\ CHBr\ (CH_2)_7COOH + 2HBr$

Oleic acid Di bromostearic acid

Bromine goes into the solution forming a dibromide. It adds to the double bonds. In other words bromine solution is decolorized but when all bonds are saturated, bromine solution imparts its own colour.

Procedure:

1. Take 2 test tubes and add 2 to 3 ml of alcohol to each tube.
2. To one tube add a pinch of palmitic acid (saturated) to the second tube add oleic acid (unsaturated)
3. To both tubes add 5% bromine solution
4. Observe that for first tube only one or two drops of bromine needed to produce end point. While for the unsaturation more than two drops require.

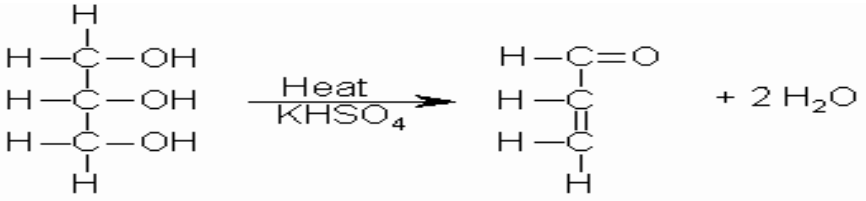
Acrolein test:

Qualitative test for glycerol.

- The principle behind the acrolein test is a specific chemical reaction. This reaction is utilized to determine the presence of glycerine in a fat. By heating the fat sample in the presence of potassium bisulfate ($KHSO_4$), which acts as a dehydrating agent, acrolein (C_3H_4O, or CH_2=CH-CHO) is formed and can easily be detected by its odour. Whenever fat is heated in the presence of a dehydrating agent, the fat molecule will shed its glycerol in the form of the unsaturated aldehyde - acrolein.
- Acrolein smells like burned grease, and this toxic chemical was used in the first World War as a chemical weapon. Even in small concentrations, exposure irritates the mucous membranes and causes the eyes to tear up. It can incapacitate individuals very quickly at levels of only a few parts per million.

Procedure:

- Take pure glycerol in a dry test tube. Add to it a few crystals of potassium hydrogen sulphate (KHSO4)
- warm gently mix and then heat strongly
- A very pungent odour of a acroline is produced acroline is formed due to removal of water From glycerol by potassium hydrogen sulphate.



Saponification

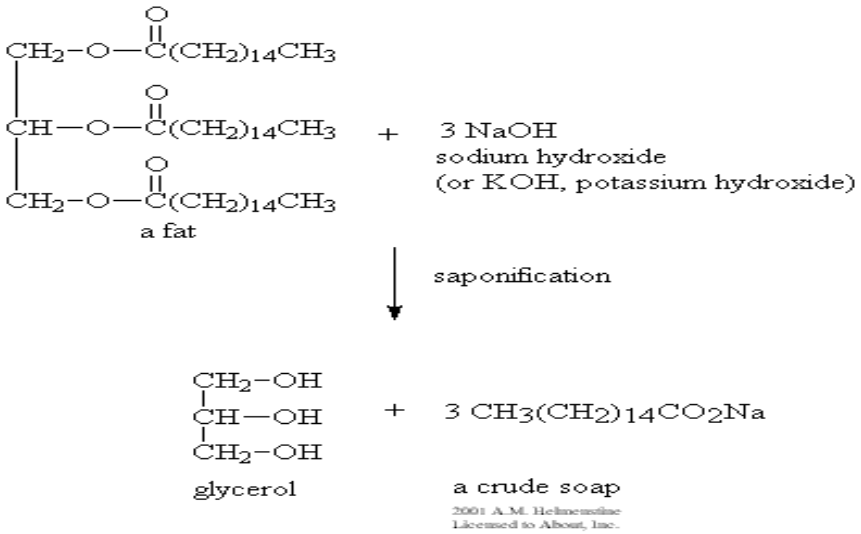
Is a chemical reaction that occurs when a vegetable oil or animal fat (triglycerides, ester) mixed with a strong alkali (NaOH, KOH).

The products of the reaction are soap (fatty acid salt) and glycerol (free alcohol).

The soap produced can be precipitated by adding concentrated sodium chloride solution, if necessary

Sodium hydroxide (NaOH) gives "hard soap", whereas, when

Potassium hydroxide (KOH) is used, a soft soap or liquid soap because of its greater solubility.



Structure of Soap:

Tail		***Head***
Non-Polar		***Polar***
Hydrophobic		***Hydrophilic***
Insoluble in water	***dissolve oil***	***Soluble in water***

Oxidation (Rancidty)

- This toxic reaction of triglycerides leads to unpleasant odour or taste of oils and fats developing after oxidation by oxygen of air, bacteria, or moisture.
- Also this is the base of the drying oils after exposure to atmospheric oxygen.
  Example is linseed oil, which is used in paints and varnishes manufacturing**.**

Rancidity

Definition:

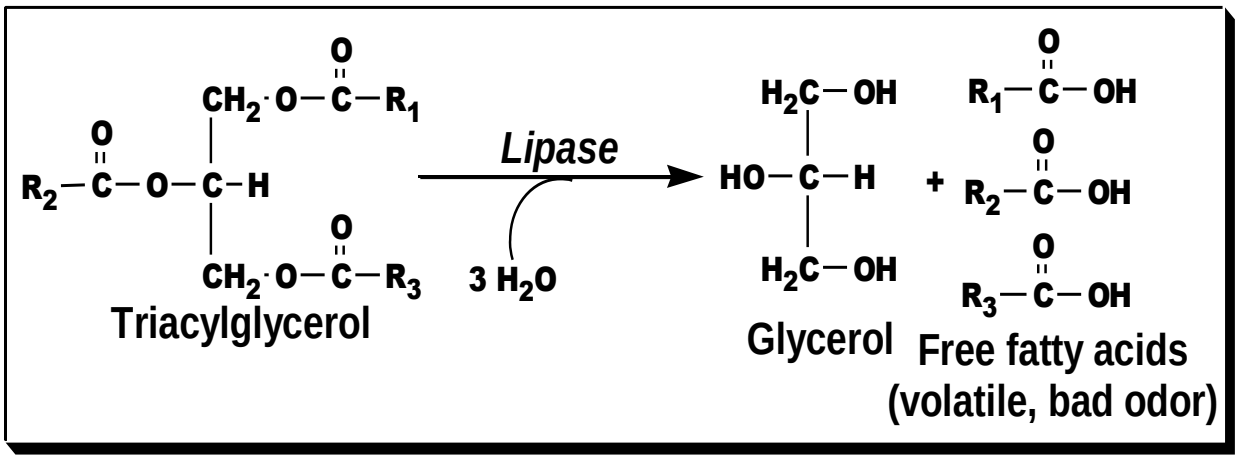
- It is a physico-chemical change in the natural properties of the fat leading to the development of unpleasant odor or taste or abnormal color particularly after exposure to atmospheric oxygen, light, moisture, bacterial or fungal contamination and/or heat.
- Saturated fats resist rancidity more than unsaturated fats that have unsaturated double bonds.

Types and causes of Rancidity:

1. Hydrolytic rancidity
2. Oxidative rancidity
3. Ketonic rancidity

1-Hydrolytic rancidity:

- It results from slight hydrolysis of the fat by lipase from bacterial contamination leading to the liberation of free fatty acids and glycerol at high temperature and moisture.
- Volatile short-chain fatty acids have unpleasant odor.



2-Oxidative Rancidity:

- It is oxidation of fat or oil catalyzed by exposure to oxygen, light and/or heat producing peroxide derivatives which on decomposition give substances, e.g., peroxides, aldehydes, ketones and dicarboxylic acids that are toxic and have bad odor.
- This occurs due to oxidative addition of oxygen at the unsaturated double bond of unsaturated fatty acid of oils.

3-Ketonic Rancidity:

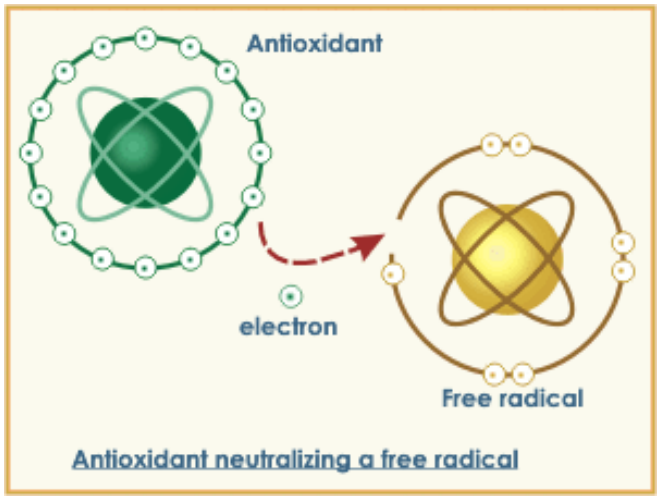
- It is due to the contamination with certain fungi such as Asperigillus Niger on fats such as coconut oil.
- Ketones, fatty aldehydes, short chain fatty acids and fatty alcohols are formed.
- Moisture accelerates ketonic rancidity.

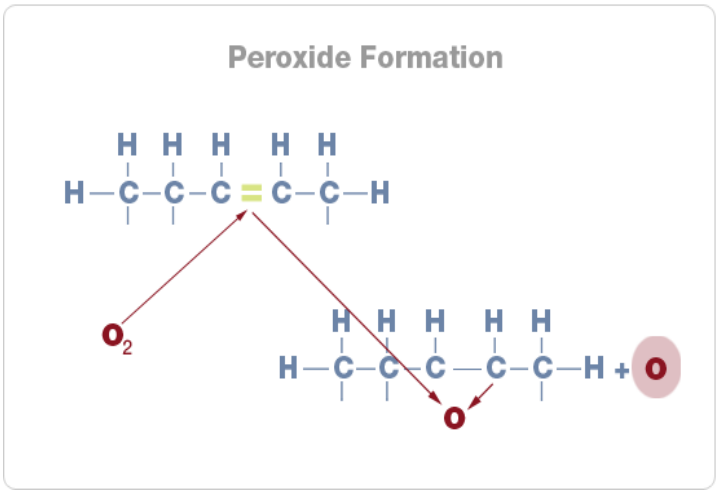
Prevention of rancidity is achieved by:

- Avoidance of the causes (exposure to light, oxygen, moisture, high temperature and bacteria or fungal contamination). By keeping fats or oils in well-closed containers in cold, dark and dry place (i.e., good storage conditions).
- Removal of catalysts such as lead and copper that catalyze rancidity.
- Addition of anti-oxidants to prevent peroxidation in fat (i.e., rancidity). They include phenols, naphthols, tannins and hydroquinones. The most common natural antioxidant is vitamin E that is important *in vitro* and *in vivo*.
-

Hazards of Rancid Fats:

1. The products of rancidity are toxic, i.e., causes food poisoning and cancer.
2. Rancidity destroys the fat-soluble vitamins (vitamins A, D, K and E).
3. Rancidity destroys the polyunsaturated essential fatty acids.
4. Rancidity causes economical loss because rancid fat is inedible.





Procedure:

1. Weigh 1g of oil or fat into a clean dry boiling tube and add 1g of powdered potassium iodide and 20mL of solvent mixture.
2. Place the tube in boiling water so that the liquid boils within 30 seconds and allow to boil vigorously for not more than 30 seconds.
3. Transfer the contents quickly to a conical flask containing 20mL of 5% potassium iodide solution.
4. Wash the tube twice with 25mL water each time and collect into the conical flask.
5. Titrate against N= 0.1 sodium thiosulphate solution until yellow color is almost disappeared.
6. Add 0.5mL of starch, shake vigorously and titrate carefully till the blue color just disappears.
7. A blank should also be set at the same time.

Calculation

$$\text{Peroxide value (milliequivalent peroxide/kg sample)} = \frac{S \times N \times 1000}{\text{Weight of sample (g)}}$$

where,
S = mL $Na_2S_2O_3$ (Test-Blank) and
N = normality of $Na_2S_2O_3$

Qualitative test for Cholesterol

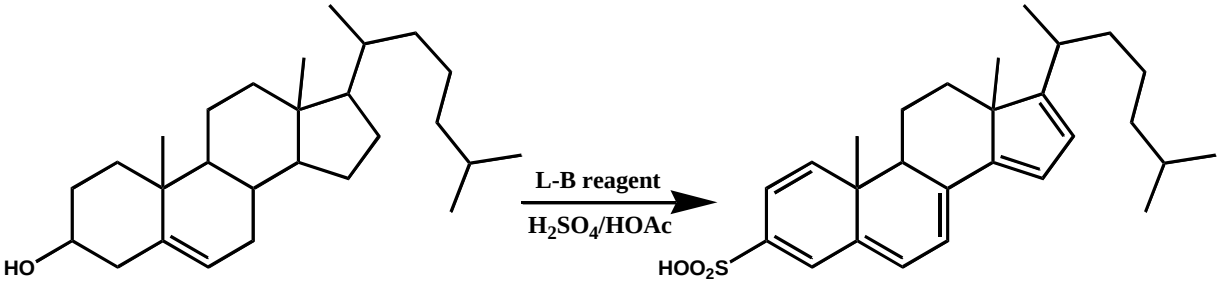
The presence of a double bond in one of cholesterol rings is responsible for its ability to form colour products in the presence of concentrated inorganic acids. The action of concentrated sulphuric acid results in dehydration of cholesterol molecule (Salkowski test). With a formation of a red bicholestadien

disulphonate, which in the presence of acetate anhydride forms a green colour bicholestadien monosulphonate (Lieberman-Burchard test)? Traces of water make this reaction impossible.

Qualitative test for Cholesterol

Lieberman reaction:

It is a test for unsaturated steroids (as cholesterol) and triteness based on the formation of a series of colors (as pink to blue to green) with acetic an-hydride in the presence of concentrated sulfuric acid called also Liebermann-Burchard test



Cholesterol **Cholestahexaene sulfonic acid**

λ_{max} = 620 nm

1. To 2ml of chloroform solution of cholesterol add 10 drops of acetic anhydride and 2 drops of concentrated H2SO4 along the side of the test tube.

2. Solution turns rosy red , blue and finally deep green colour

3. Indicates the presence of cholesterol the changing of colours in this reaction is due to dehydration of cholesterol by sulphuric acid and acetic acid this reaction is used in the colorimetric estimation of cholesterol in blood.

Note: Results are quite sensitive to the presence of water, so keep everything dry.

Salkowiski's reaction:

Salkowsk's test is a test for cholesterol; when concentrated sulfuric acid is added to a chloroform solution of cholesterol, the chloroform layer shows a red to blue colour and the acid layer shows a green fluorescence.

. To 2ml of chloroform solution of cholesterol add 2ml of concentrated H2SO4 slowly along the side of the test tube. Mix gently and allow to stand observe the colour at the junction of two layers

2. a red colour is seen in the upper chloroform layer , a yelloe colour within green fluorescence is seen in the lower acid layer

3. Indicates the presence of cholesterol

Lab 6: Vitamins, Qualitative and Quantitative tests

Vitamins are complex organic molecules required in small amounts by the body in order to maintain health and well-being. Vitamin C is important for the human body because it helps the body to absorb iron, helps wounds to heal, helps red blood cell formation and helps to fight infections. For example, a lack of vitamin C can cause a disease called scurvy, iron deficiency and poor wound healing.

OBJECTIVE

The objectives of the study is to determine the concentration of vitamin C in different commercial fruit juices, (apple, orange and lychee) of same brand by using DCPIP titration.

Titration Method

In this experiment, titration method is used to determine the concentration of Vitamin C in freshly prepared and commercial fruit juice samples. Titration or called as volumetric analysis is a common laboratory method of quantitative analysis that can be used to determine the concentration of a known analyte. A titrant of known concentration is used to react with a solution of the analyte of unknown concentration. Using a calibrated burette, it is possible to determine the exact amount of titrant that has been consumed when the endpoint is reached. The endpoint is the point at which the titration is complete, as determined by the colour change of an indicator.

Procedure

Part A: Preparation of Standard Ascorbic Acid Solution

1. 0.2 g of ascorbic acid is weighted out and makes up to 1 L of distilled water.
2. The concentration of the ascorbic acid solution is calculated by using the formula below:

$$\text{Concentration of ascorbic acid} = \frac{\text{Mole}}{\text{Volume}} = \frac{\frac{\text{Mass}}{\text{Molar mass}}}{\text{Volume}}$$

PART B Preparation of DCPIP Solution

1. Approximately 0.24 g DCPIP is weighted out and make up to 1 L of distilled water.
2. The concentration of DCPIP solution is calculated by using the formula below:

$$\text{Concentration of DCPIP solution} = \frac{\text{Mole}}{\text{Volume}} = \frac{\frac{\text{Mass}}{\text{Molar mass}}}{\text{Volume}}$$

PART C Standardization of DCPIP Solution

1. 25 ml of 0.5% oxalic acid is measured and transferred into a 250 ml conical flask.

2. 10 ml of standard ascorbic acid solution is added into the conical flask which contains oxalic acid by using a pipetman.
3. A trial run of titration is carried out with a titration set. The ascorbic acid solution is titrated rapidly with the DCPIP solution. The DCPIP solution is added through the burette and the solution is vortex well. Colour change of DCPIP solution to pink is observed when the solution contacts with the ascorbic acid solution and then becomes colourless after shaking well.
4. After the trial run, another three actual titrations to the ascorbic acid standard solution is conducted and the results are being average. Then, DCPIP solution is added drop by drop carefully when the volume of DCPIP solution used is close to the end point volume.
5. The volume of DCPIP solution used is recorded.
6. The concentration of the DCPIP solution is calculated by using the formula below:

$$CV\ (\text{Ascorbic acid}) = CV\ (\text{DCPIP})$$

* C refer to concentration
* V refer to volume

Part D Determination of the Vitamin C Concentration in Fruit Juice

1. A fruit is cut in half with knife and the juice is squeeze out.
2. Fresh fruit juice is collected with the aid of a Buchner funnel and filter paper, the flesh and seed is separated from the juice.
3. 10 ml of the fruit juice is pipetted into a 250 ml conical flask, which contains 25ml of 0.5% oxalic acid, and 10 ml of distilled water is added.
4. The fruit juice solution is titrated with the DCPIP solution in the burette to a pink end point.
5. The test is triplicated and average the results are being averaged.
6. The vitamin C concentration in the fruit juice is calculated by using the following formula.

$$\text{Mole (Vitamin C)} = CV\ (\text{DCPIP solution})$$

$$\frac{\text{Mass}}{\text{Molar mass}} = CV$$

$$\text{Mass} = Mr\ (\text{Vitamin C}) \times C\ (\text{DCPIP}) \times V\ (\text{DCPIP})$$

* Mr refer to molar mass
* C refer to concentration
* V refer to volume

3.0 OBSERVATION AND RESULTS

3.1 PART A : Standardization of DCPIP solution

Table 3.1 : Titration of ascorbic acid with DCPIP Solution (Standard solution)

Sample Number	Initial Reading (ml)	Final Reading (ml)	Volume of DCPIP used to titrate 1 ml of Ascorbic Acid Standard Solution (ml)
Trial 1			
Trial 2			
Trial 3			
Average			

Concentration of Ascorbic Acid Solution (Standard)

Molecular formula of ascorbic acid is $C_6H_8O_6$.

Molar mass of ascorbic acid = 176.12

Mass of ascorbic acid is 0.2 g.

$$\text{Concentration of ascorbic acid} = \frac{\text{Mole}}{\text{Volume}}$$

$$= \frac{\frac{\text{Mass}}{\text{Molar mass}}}{\text{Volume}}$$

$$= \frac{\frac{0.20}{176.12}}{1\text{L}}$$

$$= 0.00114 \text{ mol/L}$$

Concentration of DCPIP Solution

Molecular formula of DCPIP is $C_{12}H_7NCl_2O_2$.

Molecular formula of DCPIP = 268.10

Mass of DCPIP is 0.24 g.

$$\text{Concentration of DCPIP solution} = \frac{\text{Mole}}{\text{Volume}}$$

$$= \frac{\frac{\text{Mass}}{\text{Molar mass}}}{\text{Volume}}$$

$$= \frac{\frac{0.24}{268.10}}{1\text{L}}$$

$$= 0.000895 \text{ mol/L}$$

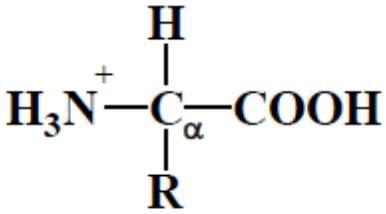
Lab 7: Amino acids titration curves

Objectives: A) To determine the titration curve for an amino acid and B) to use this curve to estimate the pKa values of the ionizable groups of the amino acid and the amino acid's pI.

Introduction: A titration curve of an amino acid is a plot of the pH of a weak acid against the degree of neutralization of the acid by standard (strong) base. Consider the ionization of a weak organic acid such as acetic acid by NaOH.

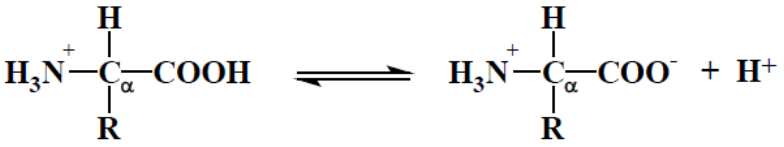
As more of the strong base (titrant) is added to the aqueous solution, more of the weak acid is converted to its conjugate base. During this process, a buffer system forms and the pH of the system will follow the Henderson-Hasselbalch relationship.

Based on the number of plateaus on a titration curve, one can determine the number of dissociable protons in a molecule. The one plateau observed when acetic acid is titrated indicates that it is a **monoprotic** acid (i.e., has only one dissociable H^+). Many organic acids are **polyprotic** (have > one dissociable H^+). The protein building blocks, amino acids, are polyprotic and have the general structure.

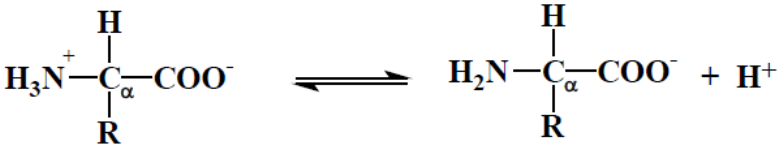


The majority of the standard amino acids are diprotic molecules since they have two dissociable protons: one on the alpha amino group and other on the alpha carboxy group. There is no dissociable proton in the R group. This type of amino acid is called a "simple amino acid". A simple amino acid is electrically neutral under physiological conditions. **NOTE: Under this definition it is possible to have a simple amino acid which is triprotic. Which of the 20 common or standard amino acids are simple & triprotic?** Ionization of a diprotic amino acid will proceed as follows:

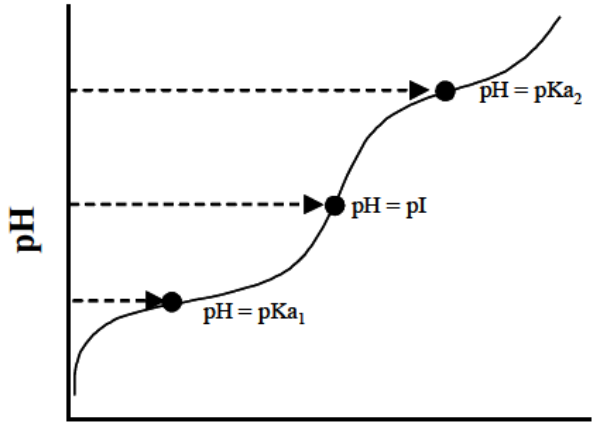
Dissociation 1:



Dissociation 2:



The order of proton dissociation depends on the acidity of the proton: that which is most acidic (lower pKa) will dissociate first. Consequently, the H^+ on the α-COOH group (pKa_1) will dissociate before that on the α-NH_3 group (pKa_2). The titration curve for this process looks similar to the following:



This curve reveals, in addition to the same information observed with a monoprotic acid, an additional characteristic of polyprotic acids and that is the pH at which the net charge on the molecule is zero. This pH defines the **isoelectric point** (**pI**) of the molecule, a useful constant in characterizing and purifying molecules. Using a titration curve, the pI can be empirically determined as the inflection point between the pKa of the anionic and cationic forms. Mathematically, the pI can be determined by taking the average of the pKa for the anionic and cationic forms. The ionic form of the molecule having a net charge of zero is called the zwitterion.

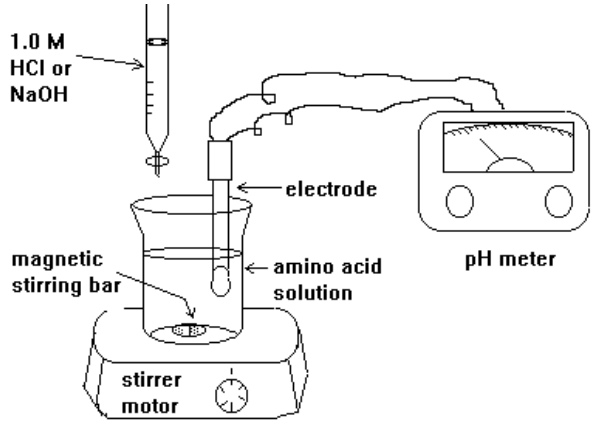
A few amino acids are classified as triprotic. This is because, in addition to the ionizable protons of the α-COOH and α-NH_3 groups, they also have a dissociable proton in their R group. Although triprotic amino acids

can exist as zwitterions, under physiological conditions these amino acids will be charged. If the net charge under physiological conditions is **negative**, the amino acid is classified as an **acidic** amino acid because the R group has a proton that dissociates at a pH significantly below pH 7. The remaining triprotic amino acids are classified as **basic** amino acids due to a) their having a net **positive** charge under physiological conditions and b) an R group dissociable proton with a pKa near or greater than pH 7. Titration curves for triprotic amino acids generate the same information as those for the diprotic amino acids. The pI for a triprotic amino acid can be determined graphically, although this is somewhat more challenging. Graphical determination, as was the case with the diprotic acids, requires one to know the ionic forms of the amino acid and finding the inflection point between the cationic and anionic forms. Mathematically, the pI for an acidic amino acid is the average of pKa_1 and pKa_R (the pKa of the dissociable proton in the R group); for a basic amino acid, it is the average of pKa_2 and pKa_R.

Procedures:

A) Determine the titration curve for an amino acid

1. Using a 25-mL graduated cylinder or serological pipet, transfer 25 mL of a 0.2 M amino acid solution to a 150 - 250 mL beaker. Set up the apparatus as shown below:



2. Titrate the amino acid with 1.0 M HCl (**titrant**)

a. Determine the pH of the amino acid solution before the addition of titrant.

b. Initially add approximately **0.5 mL** of the titrant to the amino acid at a time. Record the data **IN YOUR NOTEBOOK** as indicated below.

mL 1.0 M HCl	pH
0.0	
0.5	
1.0	
etc.	

Note: In the beginning, the pH will change very dramatically with each addition of titrant. As you get closer to the pKa of the ionizable group, the pH will change much more slowly. When this phenomenon occurs, add **1 mL** of titrant at a time.

c. After the addition of each volume of HCl, stir the solution briefly.

d. Turn the stirrer off and measure the pH using the pH meter.

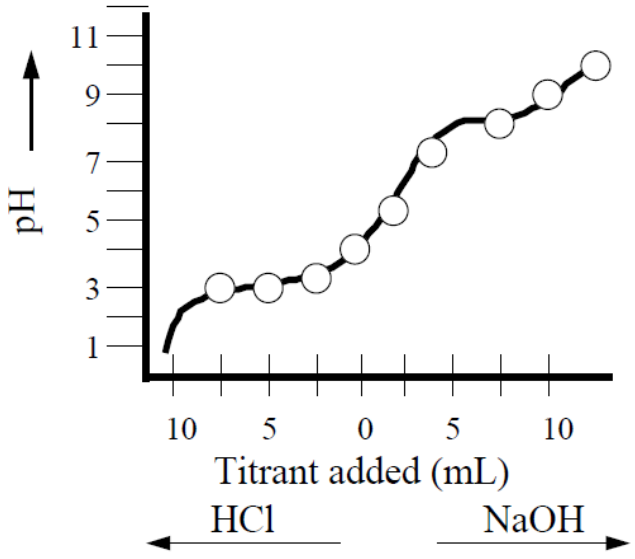
e. Continue with the titration until the pH ~1.5.

3. Repeat steps 1 and 2 above, this time using 1.0 N NaOH as the titrant for **a fresh 25-mL sample** of the same amino acid. Record the data **IN YOUR NOTEBOOK** until you get to pH ~13.

mL 1.0 M NaOH	pH
0.0	
0.5	
1.0	
etc.	

B) Estimate the amino acid's pKa values of the ionizable groups and its pI.

4. Using Microsoft Excel (or some similar program), construct your titration curve plotting **pH versus mL of acid and base** added to the amino acid solution as indicated below.



5. On your curve, designate the buffer region(s), pKa(s), and the amino acid's pI.

6. From your graph, estimate the pKa values of the ionizing groups and the pI of the amino acid. Compare your experimental values with those found in the literature. You can, for example, use either the Handbook of Biochemistry or your textbook. Cite some reasons why your values might differ from those found in the literature.

In your report, you must categorize your amino acid as diprotic or triprotic. Based on the pKa values in the lecture Textbook state which amino acids are possibilities.

Record data in your notebook:

Lab 8: RNA preparation and Qualitative tests

Nucleic acids are large biological molecules essential for all known forms of life. They include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). Together with proteins, nucleic acids are the most important biological macromolecules. One DNA or RNA molecule differs from another primarily in the sequence of nucleotides. Ribonucleic acid (RNA) functions in converting genetic information from genes into the amino acid sequences of proteins. In this experiment, RNA was isolated from yeast (*Saccharomyces cerevisiae*) by heating the active dry yeast with alkaline NaOH. This method of RNA extraction involved the disruption of the cell membrane and subcellular nucleus to break open and is charge the nucleic acids. RNA was extracted from associated proteins with HCl extraction and was treated with ethanol and ether to remove lipids.

MATERIALS AND METHODS

In a beaker, 5mL of 1% NaOH solution and 25mL of water were diluted and was added with 5.0g of dry yeast. The mixture was set to heat in a water bath for 15 minutes with occasional stirring. After heating, the mixture was strained with cheesecloth. The filtrate was centrifuged and the supernate was transferred to a different test tube and was added glacial acetic acid dropwise until faintly acidic. It was observed that the supernate was turbid; it was centrifuged and decanted after. Twenty milliliters of 95% ethanol containing 0.2 mL of conc. HCl was poured to the supernate while it was stirred vigorously.

All residues were decanted, centrifuged, and transferred in one big test tube and was washed twice with 2mL of 95% ethanol. The decantation and centrifugation processes were repeated every after washing. It was washed again twice with 2mL ether with the same process as the previous; it was centrifuged and decanted every after washing. After the washing, the residue was divided into two portions in two separate test tubes and cover. The test tubes were refrigerated to be used in the following experiment in the next laboratory meeting.

Do the following tests:

a. Solubility
b. Biuret
c. Molisch and Bial’s test
d. Test for P and Purines.

Lab 9: Horizontal and Vertical Electrophoresis

Theoretical clarification of both types of electrophoresis will be prepared later.

Lab 10: Test for a solid unknown

1. Physical characteristics of the unknown as color, odor, morphology.
2. Effect of dry heating on the substance.
   a. Carbohydrates charred and give burnt sugar odor.
   b. Proteins charred and give burnt feather odor.
   c. Lipids melt and refreeze on cooling.
   d. Urea melts and solidifies immediately with the release of ammonia odor.
3. Solubility
4. Do the tests for carbohydrates, lipids and proteins.

Lab11: Test for a liquid unknown

The same experiments as for solid unknowns can be performed.