# CLS 232 Manual

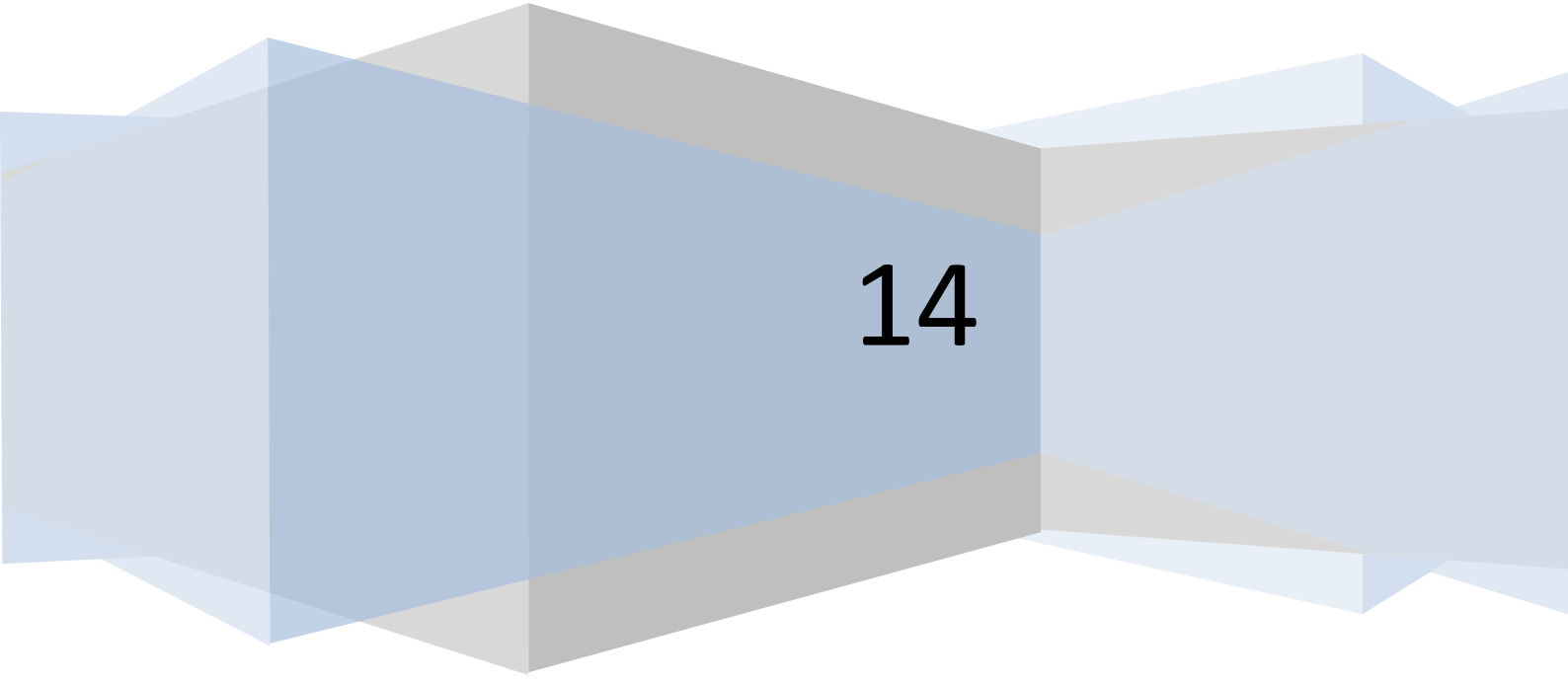
**Done by: Najla Al-Khumatium**

**Reviewed and edited by:**

**Sahar AL-Subaie**

**Alhanouf Aleydan**

**Supervision: Dr. Nora Al- Jameil**



**Experiment: 1**

# Color Tests for Proteins and Amino Acids

## A)General Proteins Color Tests

### 1/Biuret Test:

*It is a general test used for detecting the presence of proteins and peptides.

*Protein sample treated with Copper Sulphate ($CUSO_4$) in an alkaline solution (NaOH) formed a pink-violet colored complex.

*This color is due to a reaction between Copper ions (CU++) and peptide bonds (CO-NH) in alkaline solution (at least two peptide bonds are required for a positive test).

*Biuret ($H_2N$-CO-NH-CO-$NH_2$) react with $CUSO_4$ in an alkaline solution and give the same color like protein, that is why the test is called Biuret test.

### Procedure:

1-Take six separate test tubes. Add 1 ml of : water, 1% egg albumin, 1% alanine, 1% sucrose, 1% glucose , 1% casein respectively.

2-Add 1 ml of 10% NaOH to each tube.

3-Add 5 drops of 0.1% $CUSO_4$ to each tube.

4-Mix, describe any color change that occurred.

| $H_2O$ | 1%egg albumin | 1%alanine | 1%sucrose | 1%glucose | 1%casein |
|---|---|---|---|---|---|
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| 10% NaOH | | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| 0.1% CUSO4 | | | | | |
| 5 drops | 5 drops | 5 drops | 5 drops | 5 drops | 5 drops |

Mix, describe any color change that occurred

Question

1.why is an excess of copper to be avoided?

2. why do ammonium salts interfere?

3. name two substances other than proteins which give a positive biuret test.

4. of what value is the biuret in studying the hydrolysis of proteins?

5. indicate whether the following substances would give + or – biuret test and give a reson for your choice.

a. Tyrosylphenylalanine

b. Insulin

c.Alanylglycine

## 2/Ninhydrin Test:

*a.a. (that have α-amino group) react with ninhydrin to form blue colored complex.

*This color is due to librate $NH_3$ with ninhydrin.

*Ninhydrin is used to locate the α-amino acid in paper chromatography as a blue to purple spots.

*Also, permits the quantitative estimation of α-amino acid and peptides in column chromatography.

*Proline give yellow color due to lack of α-amino group.

## Procedure:

1-Take seven test tubes. Add 1 ml of: water, 1% egg albumin, 1%alanine, 1% proline, 1% sucrose, 1% casein respectively, and to the seventh tubes add 5 drops diluted ammonia.

2-Add 1 ml of 0.1% aqueous ninhydrin to each tube.

3-Mix, incubate in boiling water bath for 4 minutes, and observe the colors after standing a few minutes.

Describe the color changes that occur in each test tube.

| $H_2O$ | 1%egg albumin | 1%alanine | 1%proline | 1%sucrose | 1%casein | Dilute ammonia |
|---|---|---|---|---|---|---|
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 2 drops |
| 0.1% aqueous ninhydrin | | | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |

Mix, incubate in boiling water bath for 4 minutes, cool.

Describe the color changes that occur in each test tube.

Questions:

1. What color was produced on cooling?
2. What structure in the protein molecule is indicated by the ninhydrin test?
3. Write the formula of alanine and the products obtained from it when it is oxidized by ninhydrin. Encircle the product that reacts with the ninhydrin molecules to form a coloured substance.
4. Which amino acids do not produce a purple colour with ninhydrin? Why?
5. Why would ninhydrin test be more useful and reliable for showing the absence rather than the presence of proteins?

## 3/Xanthoproteic Test:

*Nitration of the aromatic rings in Tyrosine and Tryptophan, with concentrated $HNO_3$, produce a yellow color.

Tyrosine or Tryptophan + con.$HNO_3$ $\xrightarrow{\text{heat}}$ Yellow color

*Phenylalanine does not produce the color because the benzene ring is not activated for nitration.

## Procedure:

1-Take seven test tubes. Add 1 ml of: water, 1% egg albumin, 1% alanine, 1% phenylalanine, 1% sucrose, 1% phenol respectively and 2 ml of 0.02% tryptophan to the seventh tube.

2-Add 1 ml of concentrated HNO3 to each tube.

3-Incubate all the tubes in boiling water bath for 2 mins. Cool. Describe the result in each test tubes, then, add carefully 10% NaOH and see the deepens of yellow color to orange.

| H2O | 1%egg albumin | 1%alanine | 1%phenylalanine | 1%sucrose | 1%phenol | 0.02%tryptophan |
|---|---|---|---|---|---|---|
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 2 ml |
| Con. HNO3 | | | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |

Incubate in boiling water bath for 2 mins. Cool. Describe the change in color in each test tube. Then, add 10% NaOH and see the deepens of yellow color to orange.

Questions:

1.what happened when $HNO_3$ was added to egg albumin solution?

2.what was the color of test solutions before and after neutralizing with NaOH?

3.for what chemical group is Xanthoproteic reaction a test

4. write the formulas and names of the amino acids that contain these groups.

5.do most proteins give a positive Xanthoproteic test?

**Experiment:2**

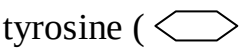
# Color Tests for Proteins and Amino Acids

## B)Color Test for Specific Amino Acids

### 1/Millon's Test:

*Used for detecting the presence of monohydroxybenzene derivatives (e.g: Tyrosine, Tyrosine derivatives, Phenol).

*Millon's reagent is a solution of mercuric and mercurous ions in nitric and nitrous acids.

*Phenolic group of tyrosine (  OH ) reacts with Millon's reagent and give a red color, which is due to a mercury salt of nitrated tyrosine.

### Procedure:

1-Take seven separate test tubes. Add 2 ml of: water, 1% egg albumin, 0.02% salicylic acid, 0.02% tyrosine, 1% gelatin, 0.02% phenylalanine and 0.02% phenol, respectively.

2-Add 3 drops of Millon's reagent to each tube.

3-Incubate in boiling water bath for 2 minutes. Note the color formed.

| H2O | 1% egg albumin | 0.02% salicylic acid | 0.02% tyrosine | 1% gelatin | 0.02% phenylalanine | 0.02% phenol |
|---|---|---|---|---|---|---|
| 2 ml | 2 ml | 2 ml | 2 ml | 2 ml | 2 ml | 2 ml |
| Millon's Reagent | | | | | | |
| 3 drops | 3 drops | 3 drops | 3 drops | 3 drops | 3 drops | 3 drops |

Incubate in boiling water bath for 2 min. Not the color formed

Questions:

1. Why does albumin solution coagulate?
2. Which of the protein solutions give a weak or negative test?why?
3. What is the composition of Millon's reagent?
4. What happened when Millon's reagent was added to egg albumin solution?
5. For what chemical group is Millon's reagent a test?
6. Write the formula and name of the amino acid that contains this group?
7. Is this amino acid present in gelatin? What is the evidence for your answer?

## 2/Hopkins-Cole Test:

*This test for Tryptophan.

Tryptophan + Hopkins-Cole reagent ⟶ purple ring

*Hopkins-Cole reagent contain magnesium glyoxylate (Magnesium powder and Oxalic acid).

*Purple ring is formed by the reaction of glyoxylic acid ( CHO-COOH ) with the indole ring of tryptophan in presence of $H_2SO_4$.

## Procedure:

1-Take six separate test tubes. Add 2 ml of: water, 1% egg albumin, 1% gelatin, 1% casein, 0.02% tryptophan and 0.02% tyrosine, respectively.

2-Add 3 ml of Hopkins-Cole reagent to each tube and mix.

3-Carefully add 5 ml of con. $H_2SO_4$ from buret by touching the tip of the stopcock to the inside wall of the test tube and allowing the acid to slowly drain down the tube so that the two liquids form separate layers. Add 5 ml of con. $H_2SO_4$ to each tube. Observe the color at the zone of contact of the two fluids. If no color appears, swirl the tube gently, but do not mix.

| $H_2O$ | 1%egg albumin | 1% gelatin | 1% casein | 0.02% tryptophan | 0.02% tyrosine |
|---|---|---|---|---|---|
| 2 ml | 2 ml | 2 ml | 2 ml | 2 ml | 2 ml |
| Hopkins-Cole Reagent | | | | | |
| 3 ml | 3 ml | 3 ml | 3 ml | 3 ml | 3 ml |
| Concentrated $H_2SO_4$ | | | | | |
| 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml |

Observe the color formed at the junction of the two liquids.

If necessary, gently rotate the tube to develop the colored ring.

Questions:

1. What protein fails to give this test ? why?
2. What is the composition of Hopkins-Cole reagent?
3. What is the active constituent of this reagent?
4. Write the formula and name of amino acid for which this reaction is a test>
5. Is this amino acid present in gelatin?

## 3/Sakaguchi Test:

*This test is specific for the guanidine group of arginine ($H_2N$-C-NH), but a non-amino acid like creatine which contain this group will also answer this test.

*This test positive for all proteins containing arginine and for arginine itself.

*When arginine reacts with α-Naphthol and Sodium hypobromite (NaOBr) a red color result.

*Red color is due to a reaction between the hypobromite and the –NH2 group of the guanidino part of arginine.

## Procedure:

1-Take five separate test tubes. Add 5 ml of: water, 0.1% gelatin, 0.02% arginine, 0.02% creatine and 0.02% urea, respectively.

2-Add 1 ml of 10% NaOH to each tube.

3-Add 1 ml of 0.02% α-Naphthol to each tube.

4-Incubate all the tubes at room temperature for 3 minutes.

5-Add 4 drops of NaOBr to each tube. Note the color formed.

| $H_2O$ | 0.1% gelatin | 0.02% arginine | 0.02% creatine | 0.02% urea |
|---|---|---|---|---|
| 5 ml | 5 ml | 5 ml | 5 ml | 5 ml |
| 10% NaOH | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| 0.02% α-Naphthol | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| Mix, incubate at room temperature for 3 minutes | | | | |
| NaOBr | | | | |
| 4 drops | 4 drops | 4 drops | 4 drops | 4 drops |

Note the color formed

Questions:

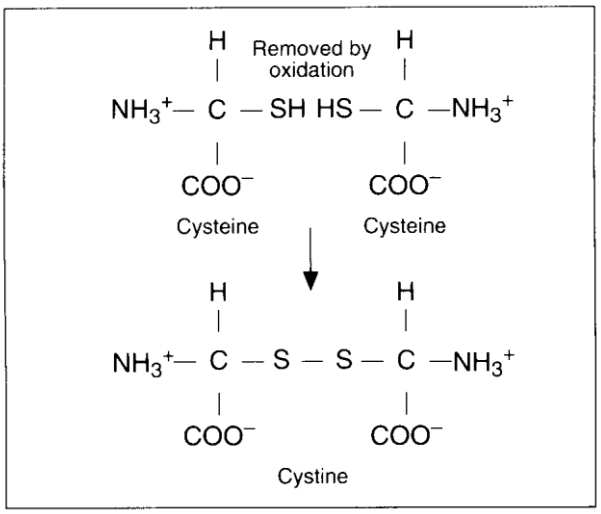
1.write the formula and name of the amino acid for which this reaction is a test.
2. for what chemical group is this test?
3. what protein fails to give this test? Why?
4.what causes the red color to fade in the Sakaguchi test for arginine, and how could this fading be prevented?

## 4/Lead Acetate Test:

*Used for detecting the presence of Sulfur in Cysteine , Cystine and methionine .

*Proteins or peptides containing these amino acid heated with NaOH to split the sulfide group from amino acids.

*Then, lead acetate react with free sulfide ions resulting in the formation of lead sulfide ( pbs ) a brown to black color.




## Procedure:

1-Take four separate test tubes. Add 2 ml of: water, 1% egg albumin, 0.02% cystine and 0.02% methionine, respectively.

2-Add 5 ml of 5% NaOH to each test tube.

3-Add few crystals of lead acetate. Incubate all the tubes in boiling water bath for 10 minutes, with occasional mixing of the contents of the tube.

| $H_2O$ | 1% egg albumin | 0.02% cystine | 0.02% methionine |
|---|---|---|---|
| 2 ml | 2 ml | 2 ml | 2 ml |
| 5% NaOH | | | |
| 5 ml | 5 ml | 5 ml | 5 ml |
| Lead Acetate | | | |
| Few crystals | Few crystals | Few crystals | Few crystals |

Incubate in boiling water bath for 10 minutes with occasional mixing.

Describe the color changes in each test tubes.

Questions:

1.what happens when the protein is boiled with NaoH?

2. What are the formulas and names of amino acids for which this

reaction is a test?

3.Why does silverware turn black when used with eggs?

## 5/Folin's Test:

*This test for free cystine, therefore protein or peptide must be first hydrolyzed by sodium carbonate and sodium sulfite.

*Then, Folin's uric acid reagent (phosphotungstic acid) is added which is reduced to tungsten blue.

*This can be used for the quantitative determination of cystine.

## Procedure:

1-Take four separate test tubes. Add 1 ml of: water, 0.1% cystine, 1%egg albumin and 0.1% methionine, respectively.

2-Add 7 ml of saturated sodium carbonate to each tube.

3-Add 3 ml of 20% sodium sulfite to each tube.

4-Incubate at room temperature for 5 minutes.

5-Add 1 ml of uric acid reagent to each tube. Note the color formed.

| $H_2O$ | 0.1% cystine | 1% egg albumin | 0.1% methionine |
|---|---|---|---|
| 1 ml | 1 ml | 1 ml | 1 ml |
| Sodium Carbonate (saturated) | | | |
| 7 ml | 7 ml | 7 ml | 7 ml |
| 20% Sodium Sulfite | | | |
| 3 ml | 3 ml | 3 ml | 3 ml |
| Incubate at R.T for 5 min. | | | |
| Uric acid reagent | | | |
| 1 ml | 1 ml | 1 ml | 1 ml |

Note the color formed

**Experiment:3**

# Precipitation of Protein

## Introduction:

*When the structures of native proteins are altered by chemical or physical means, the protein molecules tend to agglomerate and precipitate and the protein becomes denaturated.

*Denaturation can be either irreversible (excessive heating or extreme PH changes) or reversible (treatment with ammonium sulfate).

*Denaturation accompanied by loss of protein biological activity.

*Protein precipitation is due to a disruption of hydrogen bonds, ionic bonds and sometimes the stronger covalent disulfide bonds.

## 1/Influence of Strong Mineral Acids

*Proteins are amphiprotic substance (can accept or give protons).

*When strong acids are added to a neutral protein solution, the carboxylate groups become undissociated carboxyl groups, and the nitrogen atoms become protonated, resulting a positively charged protein molecule.

*The protein will precipitate because the ionic bonds and hydrogen bonds are disrupted.

## Procedure:

1- To three separate test tubes, add 3 ml of clear 1% egg albumin to each tube.

2- To one, add 2 ml concentrated HCL, to another 2 ml concentrated $HNO_3$ and to the third 2 ml concentrated $H_2SO_4$.

Note and describe the changes that occur.

| Test Tube No. | 1 | 2 | 3 |
|---|---|---|---|
| 1% egg albumin | 3 ml | 3 ml | 3 ml |
| Con. HCL | 2 ml | x | x |
| Con. HNO3 | x | 2 ml | x |
| Con. H2SO4 | x | x | 2 ml |

Note and describe the changes that occure.

## 2/Precipitation by Alkaloidal Reagent

*Alkaloidal reagents are acids that can combine with alkaloids (Alkaloids are organic bases from plants).

*Certain acidic reagents (alkaloidal reagents) e.g : Trichloroacetic acid, Tannic acid, Phosphotungstic acid, Picric acid, Sulfosalicylic acid combine with protein to form insoluble protein salts (e.g Protein tannate).

## Procedure:

| Test Tube No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| 1%egg albumin | 3 ml | 3 ml | 3 ml | 3 ml | x | x |
| 1% urea | x | x | x | x | 3 ml | x |
| 1%alanine | x | x | x | x | x | 3 ml |
| 10%Trichloroacetic acid | 5 drops | x | x | x | 5 drops | 5 drops |
| 5%aqueous tannic acid | x | 5 drops | x | x | x | x |
| 20%phosphotungstic acid | x | x | 5 drops | x | x | x |
| 20%sulfosalicylic acid | x | x | x | 5 drops | x | x |

## 3/Precipitation by Metallic Salts

When heavy metal cations (e.g: Pb, Cu, Hg, Ag) are added to protein solution, the metal ions combine with the negatively charged groups to form insoluble metal ion proteinate.

## Procedure:

| Test Tube No. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1%egg albumin | 2 ml | 2 ml | 2 ml | x |
| 1%urea | x | x | x | 2 ml |
| 0.2M copper sulfate | 5 drops | x | x | 5 drops |
| 0.2M lead acetate | x | 5 drops | x | x |
| 0.2M mercuric chloride | x | x | 5 drops | x |

Mix well, and describe the result

**Experiment:4**

# Estimation of Proteins

## Biuret Method:

*All proteins contain a large number of peptide bonds.

*When a solution of protein is treated with an alkaline solution of dilute Copper Sulfate, a colored complex is formed between the cupric ion (Cu+2) and the carbonyl (-C=O) and imine (=N-H) groups of the peptide bonds.

*An analogous reaction takes place between the Cu+2 ion and the organic compound biuret, therefore the reaction is called biuret reaction.

*The reaction takes place between the cupric ion and any compound containing at least two $NH_2CO$-, $NH_2CH_2$-, $NH_2CS$.

*Amino acids and dipeptides cannot give the reaction, but tri- and polypeptides and proteins react to give pink to violet products.

*In the biuret reaction one copper ion is linked between 4 and 6 nearby peptide linkages by coordinate bonds, the more protein present, the more peptide bonds available for reaction.

*The intensity of the color produced is proportional to the number of peptide bonds undergoing reaction. Thus, the biuret reaction can be used as the basis for a simple and rapid colorimetric method for determining protein.

*Biuret reagent composed of: Copper Sulfate, Rochelle salt (sodium potassium tartrate) used as complexing agent to keep the copper in solution (not precipitate), NaOH and Potassium iodide to prevent autoreduction of copper.

## Procedure:

| Blank (H2O) | Std. 1 | Std. 2 | Std. 3 | Std. 4 | Std. 5 | Unknown |
|---|---|---|---|---|---|---|
| Protein Standard 10 mg / ml | | | | | | |
| x | 0.20 ml | 0.40 ml | 0.60 ml | 0.80 ml | 1.0 ml | x |
| 1% NaCL | | | | | | |
| 1.0 ml | 0.80 ml | 0.60 ml | 0.40 ml | 0.20 ml | x | x |
| Unknown | | | | | | |
| x | x | x | x | x | x | 1 ml |
| Biuret reagent | | | | | | |
| 4 ml | 4 ml | 4 ml | 4 ml | 4 ml | 4 ml | 4 ml |

Mix the tubes and incubate all the tubes at R.T for 30 minutes. Read absorbance of standards and unknown at wavelength 545 nm using the spectrophotometer. Plot the standard curve and from the standard curve find out the concentration of protein in the unknown.

**Experiment:5**

# General Color Tests for Carbohydrates

## 1/Molisch Test:

*It is a general test for carbohydrate.

*It is effective for any compound which can be dehydrated to furfural or a substituted furfural (such as hydroxymethyl furfural) by concentrated sulfuric acid.

*If the carbohydrate is an oligosaccharide (e.g. disaccharide, trisaccharide …etc) or a polysaccharide, the hydrolysis of the carbohydrate acetal linkage occurs simultaneously with the dehydration reaction (in polysaccharide the color develops slower).

*The purple ring color is due to condensation products of furfural or it's derivatives with α-Naphthol.

Carbohydrate $\longrightarrow$ Furfural or it's derivatives.

Furfural or it's derivatives + α-Naphthol $\longrightarrow$ Purple ring.

*A negative result by this reaction is a very good evidence of the absence of carbohydrates, but a positive test is indication to the probable presence of carbohydrate.

*Thymol may be used as a reagent instead of α-Naphthol.

*Thymol is more stable than α-Naphthol, and can be applied to insoluble carbohydrates like cellulose or wood.

**Procedure:**

1-To three separate test tubes, add 10 drops of 0.5% solutions of glucose, sucrose and starch respectively.

2-Dilute each sugar solution with 2 ml of water.

3-Add 2 drops of α-Naphthol solution to each tube and mix.

4-Incline the test tube slowly and carefully add 3ml of concentrated sulfuric acid down the side of the tube to form a layer below the sugar solution.

| | **0.5% glucose** | **0.5% sucrose** | **0.5% starch** |
|---|---|---|---|
| | 10 drops | 10 drops | 10 drops |
| **Water** | 2 ml | 2 ml | 2 ml |
| **α-Naphthol** | 2 drops | 2 drops | 2 drops |
| **Mix** | | | |
| **Con. H2SO4** | 3 ml | 3 ml | 3 ml |

A purple ring at the interface is indicative of acarbohydrate.

Questions:

1. Explain the production of the colour.
2. What is the composition of Molisch reagent?
3. Why do many proteins give the Molisch test?
4. To what other reactions of carbohydrates is this test similar?

## 2/ Anthrone Test:

*It is another general test for carbohydrates.

*Based on similar reactions in which anthrone is used instead of α-Naphthol in Molisch test to form the color product (green to blue green color).

Furfural or furfural derivatives + Anthrone ⟶ colored compound
green to blue green color

*It is very sensitive, it will give a positive reaction with filter paper (cellulose).

*It can be used for quantitative determination of glycogen, inulin and sugar of blood.

*It can be used as qualitative test, since different sugars dehydrate at different rates and produce a variety of colors.

*Furfural give green color but can be differentiated by the fact that the test is rapidly obscured by brown precipitate when the sample is diluted with 50% $H_2SO_4$ or glacial acetic acid.

## Procedure:

1-To five separate test tubes, add 1 drop of : water, 0.5% glucose, 0.5% sucrose and 0.5% starch, respectively, and to the fifth test tube, add some filter paper.

2-Dilute each one with 1ml of water.

3-Add 3 ml of Anthrone reagent to each test tube.

4-Mix thoroughly by swirling, heat in a boiling water bath for 3 mins. Observe the color formed.

| Blank | 0.5%glucose | 0.5%sucrose | 0.5%starch | Filter paper |
|---|---|---|---|---|
| 1 drop | 1 drop | 1 drop | 1 drop | Small piece |
| **Water** | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| **Anthrone reagent** | | | | |
| 3 ml | 3 ml | 3 ml | 3 ml | 3 ml |

Heat for 3 mins in boiling water bath.

Green to blue green = positive.

Questions:

1. Is there a change in intensity of the colour upon standing?
2. If the test solution contains other organic compounds besides carbohydrates, brown colours may be produced, why?
3. Explain the chemical reactions involved in this test, giving equations where possible.

# Reducing Properties:

*A reducing sugar is sugar that has free or potentially free aldehydic or ketonic group.

*When blue cupric hydroxide in alkaline liquid is heated, it's converted into insoluble black cupric oxide, but, if a reducing agent like certain sugars is present, the cupric hydroxide is reduced to insoluble yellow or red cuprous oxide.

$Cu(OH)_2$ (cupric hydroxide) $\longrightarrow$ CuO (insoluble black sugar) + $H_2O$

2 $Cu(OH)_2$ + Reducing sugar $\longrightarrow$ $Cu_2O$ (insoluble yellow to red sugar) + $2H_2O$ + $O_2$

## 1/Benedict's Test:

*Carbohydrate with a free or potentially free aldehyde or ketone group have reducing properties in alkaline solution.

*In addition monosaccharides act as a reducing agent in weakly acid solution.

*Benedict modified the original Fehling's test to produce a single solution which is more convenient for tests, as well as being more stable than Fehling's reagent.

*Benedict's test is a rapid and general test for reducing sugar.

*Benedict's reagent composed of copper sulphate and sodium citrate, made alkaline with sodium carbonate.

*Citrate in Benedict's reagent act as a complexing agent to form deep, blue, stable, soluble complex ions with $Cu^{++}$, this is done to prevent the precipitation of $CuCO_3$.

*The alkalinity of Benedict's solution (PH = 10.5) is due to the hydrolysis of sodium carbonate.

Reducing sugar + Benedict's reagent ⟶ yellow to red ppt. + oxidation product

*Sucrose does not reduce Benedict's solution, because it has no free aldehyde or ketone group.

**Procedure:**

a) 1-To seven separate test tubes, add 1 ml of: water, 1% glucose, 1% xylose, 1% fructose, 1% sucrose, 1% lactose and 1% starch respectively.

2-Add 5 ml of Benedict's reagent to each tube and mix.

3-Incubate all the tubes in boiling water bath for 5 mins. Allow to cool and observe changes in the color of solutions and color of any precipitates.

b)For sucrose and starch, do acid hydrolysis, then run again with Benedict's reagent.

a)

| Blank | 1%Glucose | 1%Xylose | 1%Fructose | 1%Sucrose | 1%Lactose | 1%Starch |
|---|---|---|---|---|---|---|
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| Benedict's reagent | | | | | | |
| 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml |

Incubate in boiling water bath for 5 mins.

Observe changes in the color of solutions and formation of any precipitate.

**b)Hydrolysis step**

| | 1% Sucrose | 1% Starch |
|---|---|---|
| | 5 ml | 5 ml |
| **HCL (3M )** | 10 drops | 10 drops |
| Incubate for 5 mins. In boiling water bath | | |
| Take 1 ml | | |
| **Benedict's Reagent** | 5 ml | 5 ml |

Incubate in boiling water bath for 5 mins. Compare the results with those obtained without acid treatment.

Questions:

1. What is the colour of the precipitate?
2. Discuss the chemistry of the test?
3. What compounds other than those of copper maybe used?
4. What is the function of the Sodium Citrate?
5. How does Benedict's reagent differ from Fehling's reagent?
6. Give the name and structure of the functional group in aldoses and ketoses responsible for a positive Benedict's test.
7. What is meant by the term reducing sugar?

## 2/Barfoed's Test:

*Barfoed's reagent composed of copper acetate in acetic acid.

*By use of the Barfoed's reagent we can distinguish monosaccharides from disaccharides by controlling such conditions as PH and time of heating.

*Monosaccharides are stronger reducing agents towards Cu++ than the disaccharide.

*Under the acidic conditions of the Barfoed's test, the cuprous ion precipitates to the red cuprous oxide which settles to the bottom of the tube. (The solution still remains dark blue).

*Aldose and ketose sugar reduce this reagent, but hexoses act more rapidly and more vigorously than reducing disaccharide.

*Sucrose very easily hydrolyzed by the dilute acid reagent and the librated fructose is exceedingly reactive in this test.

*This test is positive with solutions of all monosaccharides of con. 0.1% and above.

*Disaccharide do not produce any reduction unless they are present in very high con.

## Procedure:

a)

| Water | 1%Glucose | 1%Xylose | 1%Fructose | 1%Sucrose | 1%Maltose | 1%Starch |
|---|---|---|---|---|---|---|
| 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml |
| Barfoed's Reagent | | | | | | |
| 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml |
| Mix, incubate all the tubes in boiling water bath for 3.5 mins<br>Note any change in color or clarity of the solution. | | | | | | |

Red Precipitate = positive

## b)Hydrolysis step

| | 1% Sucrose | 1% Starch |
|---|---|---|
| | 2 ml | 2 ml |
| **Barfoed's Reagent** | 5 ml | 5 ml |
| Incubate in boiling water bath for 3.5 mins<br>Note any change in color formed or clarity of solution | | |

Red Precipitate = positive

Questions:

Which of the sugars are oxidized?

What is the objection to boiling the solution for too long time ?

How does Barfoed's Reagent differ from Bendict's reagent?

Can the Barfoed's test be used in place of the Bendict's test for the detection of sugar in the urine?

What are the two fundamental chemical reasons why lactose and glucose reduce Barfoed's solution at different rates?

**Experiment: 6**

# Color Test for Ketoses and Pentoses

## 1/Seliwanoff's Resorcinol Test:

*This test is used to distinguish between aldoses and ketoses.

*Ketohexoses form considerably more furfural derivatives (about 20 to 25 times) and at a faster rate than aldohexoses.

*Resorcinol, condenses with the higher concentration of the furfural derivatives formed from the ketohexoses but not with the lower concentration formed from the aldohexoses. Consequently, the resorcinol test can be used to identify ketohexoses.

*At very high concentrations the aldohexoses will give positive test.

*Also, upon continued boiling, aldoses will give a red color with resorcinol reagent because of their gradual conversion to ketoses by the HCL.

*This test can be used to detect the presence of fructose (example of hexose) in either free or combined state. Sucrose give a positive test. Pentoses react in this test to give a blue to green product.

*Due to the interference of glucose, it must not be present in amounts greater than 2%. Concentration of HCL must not be more than 12%.

*The reaction (red color) and the precipitate must be observed after not more than 26-30 seconds boiling.

Ketose + resorcinol $\longrightarrow$ red complex

Aldose + resorcinol $\longrightarrow$ light yellow to faintly pink color

Pentose + resorcinol $\longrightarrow$ blue to green color

## Procedure:

| Water | 1%Fructose | 1%Glucose | 1%Sucrose | 1%Sorbose | 1%xylose |
|---|---|---|---|---|---|
| 2 drops | 2 drops | 2 drops | 2 drops | 2 drops | 2 drops |
| Resorcinol Reagent | | | | | |
| 5 ml | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml |
| Incubate in boiling water bath for 1 min.<br>See the color and continue incubation to 4 mins. See change in color. | | | | | |

Questions:

1. Which solution gives the test in the shortest time?
2. Could the test be used to distinguish sucrose from fructose?
3. When glucose or maltose solutions containing the Seliwanoff's reagent are heated for a long time , a color will also be produced , what is the reason for this ?
4. Write equations for the reactions involved in this test>
5. What conditions must be controlled if this test is to be used to distinguish lactose from ketose?
6. Ketohexoses are monosaccharides. Why will sucrose (a disaccharide) raffinose ( trisaccharide), and inulin ( a (fructosan polysaccharide) ngive a positive Seliwanoff's test?
7. Why is it necessary to use such small amounts of sugar in this test?

## 2/Bial's Orcinol Test:

*It is a simple, rapid qualitative test for pentoses.

*Bial's  gives a green color with the furfural from dehydrated pentoses.

*Bial's reagent consist of: orcinol, HCL and ferric chloride. (Ferric chloride increase the sensitivity of the test).

*Compounds containing pentoses and uronic acid, give a blue-green product when heated with a strong non-oxidizing acid in the presence of orcinol and FeCL3.

*It can be used for quantitative assay of pentoses (e.g:Ribonuclic acid) in the absence of interfering substances.

Pentose + heat & HCL ⟶ furfural

Furfural + orcinol ⟶ blue-green color

## Procedure:

| water | 1%xylose | 1%glucose | 1%fructose | 1%lactose | 1%starch |
|---|---|---|---|---|---|
| 1 drop | 1 drop | 1 drop | 1 drop | 1 drop | 1 drop |
| Bial's Reagent | | | | | |
| 3 ml | 3 ml | 3 ml | 3 ml | 3 ml | 3 ml |
| Incubate in boiling water bath for 3-5 mins.<br>Observe the change in color formed | | | | | |

Blue-Green color product = positive

Questions:

1. Both furfural from pentoses and hydroxymethyl furfural from hexoses will give a red color with aniline acetate. Why do hexoses not interfere in the aniline acetate test when properly done?
2. Shredded wheat contains mainly starch, a hexosan. Why does it give a good positive test with aniline acetate?
3. Would the following substances give a positive aniline acetate test? Explain each case.
    A. Ribonucleic Aicds (RNA)
    B. Refined wheat flour
    C. Wood pulp

## Iodine Test for Polysaccharide:

*Iodine forms colored adsorption complexes with polysaccharides.

*Starch gives a blue color with iodine, while glycogen gives red-brown colors.

*This iodine color is due to coordination complex between the helically coiled polysaccharide chains and the iodine centrally located within the helix.

*The iodine can be removed by extraction with ethanol or by reduction with sodium thiosulphate. So, the iodine is very loosely bound in the complex and that is still in the oxidized state.

Starch + Iodine ⟶ blue to black color

Dextrin + Iodine ⟶ red to violet color

Glycogen + Iodine ⟶ red to brown color

*Agar and xylan suspended particles will absorb the iodine to give blue to purple colors.

## Procedure:

| **water** | **1%starch** | **1%glycogen** | **1%dextrin** | **1%cellulose** |
|---|---|---|---|---|
| 2 ml | 2 ml | 2 ml | 2 ml | 2 ml |
| **Iodine solution** | | | | |
| 2 drops | 2 drops | 2 drops | 2 drops | 2 drops |
| Note the color developed | | | | |

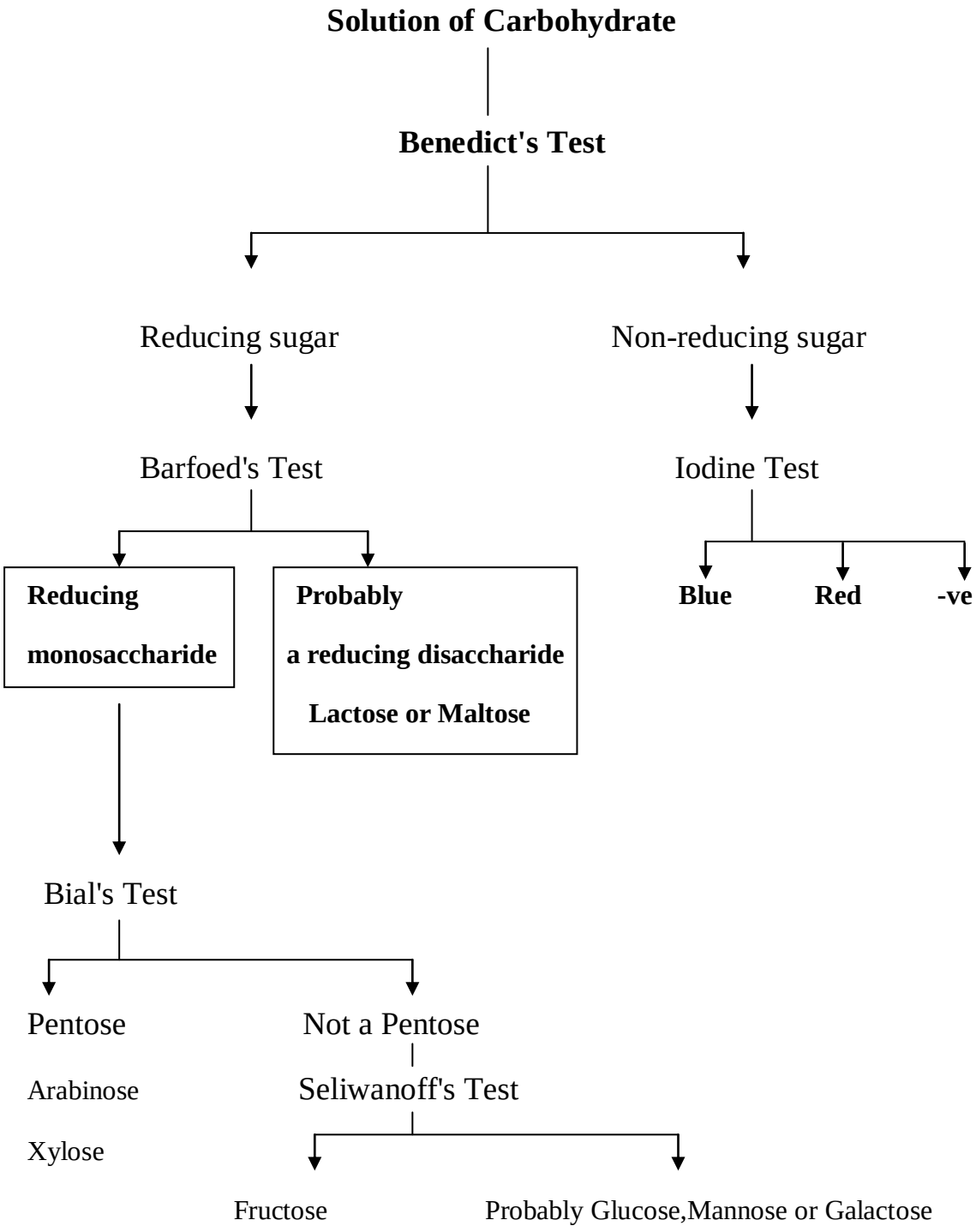
Questions:

1.what substances other than starch give a colour with iodine?

2.how would you compare the sensitivity of this test with that of the anthrone test?

**Experiment:7**

# Scheme for the Identification of an Unknown Carbohydrate



**Experiment:8**

# Copper Reduction Method (Somogyi-Nelson Method)

-In hot alkaline solution, glucose reduces cupric ion to cuprous ion with formation of cuprous oxide.

Cu +2 + glucose $\longrightarrow$ $Cu_2O$ + oxidation products of glucose

-The reaction depends on the alkalinity, the time and temperature of heating, and the concentration of reagents.

-Reoxidation of cuprous ion by oxygen from the air is prevented by adding Sodium Sulphate in the reagent to decrease the solubility of oxygen.

-Added Phosphomolybdic (or Arsenomolybdic) acid (MO+6) is reduced by the cuprous ion to form compounds with lower oxidation states of molybdenum, which have a blue color and suitable for photometric measurement.

-Due to the interference of proteins in this method, the proteins are precipitated by the addition of Barium hydroxide and Zinc sulphate.

-Protein is removed as Zinc proteinate, Sulphydryl compounds as Zinc salts and the remaining zinc and barium ions as Zinc hydroxide and Barium sulphate.

$ZnSO_4 + Ba(OH)_2 \longrightarrow Zn(OH)_2 + BaSO_4$

-Recently, rapid colorimetric procedures using O-toluidine or enzymes (such as glucose oxidase and peroxidase) have replaced the Somogyi-Nelson method.

**Procedure:**

| **Blank** | **St. 1** | **St. 2** | **St. 3** | **St. 4** | **St. 5** | **unknown** |
|---|---|---|---|---|---|---|
| Distalled water | | | | | | |
| 1 ml | 0.8 ml | 0.7 ml | 0.5 ml | 0.3 ml | x | x |
| Diluted Glucose standard (0.2 mg/ml) | | | | | | |
| x | 0.2 ml | 0.3 ml | 0.5 ml | 0.7 ml | 1.0 ml | x |
| Protein-free filtrate | | | | | | |
| x | x | x | x | x | x | 1 ml |
| Copper reagent | | | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| Incubate in boiling water for 20 min., then cool. | | | | | | |
| Arsenomolybdate Reagent | | | | | | |
| 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| Let it stand for 1 min. | | | | | | |
| Distalled water | | | | | | |
| 7 ml | 7 ml | 7 ml | 7 ml | 7 ml | 7 ml | 7 ml |

-Mix , read glucose standard and the unknown .

-With spectrophotometer, at 520 nm, against blank, plot standard curve, find out the concentration of your unknown.

**Experiment: 9**

# The Determination of the Acid Value of a Fat

## Introduction:

*During storage, fats may become rancid as a result of peroxide formation at the double bonds by atmospheric oxygen and hydrolysis by micro-organisms with the libration of free acid.

*The amount of free acid present therefore gives an indication of the age and quality of the fat.

*The acid value is the number of milligrams of KOH required to neutralize the free fatty acid present in 1 g of fat.

## Procedure:

1-Weigh about 5 ml of fat in a flask.

2-Add 25 ml of fat solvent to the flask.

3-Add 1 ml of phenolphthaleine solution, mix well.

4-Titrate with 0.01N KOH. End point is when the faint pink color persist for 30 seconds.

5-Note the volume (V) for KOH required. Calculate the acid value

## Calculation:

Molecular weight of KOH is 56

1 liter N KOH contains 56 g.

1 ml of N KOH contains 56 mg.

1 ml of 0.01N KOH contanis 0.56 mg

Acid value = (V X 0.56) / weight of fat used

Questions:

1.Define the acid number.

2. what is the significance of the acid value in an oil analysis?

# The Iodine Number of a Fat

*Each carbon-to-carbon double bond of an unsaturated fat under suitable conditions readily combines with two atoms of halogen (iodine, bromine and chloride).

*The number of grams of iodine absorbed by 100 g of fat, called the "iodine number", therefore, is a measure of the degree of unsaturation.

*Two methods are generally used for iodine number determination:

1)The Wijs method, which uses iodine chloride (ICL).

2)The Hanus method, which uses iodine bromide (IBr).

*The Hanus reagent is more stable, but the Wijs method gives results 2 to 5 percent higher and the iodine numbers are closer to the theoretical values.

*To determine the iodine number, a weighed sample of fat is allowed to react with excess iodine and titrating the librated iodine with standard sodium thiosulfate solution.

$I_2 + Br_2 \longrightarrow 2\ IBr$ (Iodine Bromide)

Fat + Excess IBr $\longrightarrow$ Halogenated Fat + unreacted IBr

$KI + IBr \longrightarrow I_2 + KBr$

$I_2 + 2\ Na_2S_2O_3 \longrightarrow 2\ NaI + Na_2S_4O_6$

**Procedure:**

Each student will analyse two fat samples dissolved in chloroform. Note the concentration of each sample.

1-Pipette 10 ml of each fat sample provided in separate flasks. Label each flask.

2-Add exactly 25 ml of Hanus iodine solution from a burette to each flask.

3-Set up a blank (separate flask) by adding 10 ml of chloroform to 25 ml of Hanus iodine solution. Only one blank flask is enough for all the students.

4-Close the flasks with glass stoppers, mix well by swiriling and allow to stand at room temperature for 30 mins. In a dark cabinet with occasional swirling.

5-Add 10 ml of 15% potassium iodide solution to each flask and mix.

6-Add about 50 ml of water, washing down any iodine solution that may be found on the wall of the flask and the stopper.

7-Titrate the iodine with 0.1N sodium thiosulphate from 50 ml burette until the color of the solution is pale yellow.

8-Add 2 ml of 1% starch solution as indicator. The solution in the flask turns blue.

9-Continue the titration until the blue color disappears, mixing well during the final stages of titration.

10-To ensure complete removal of the iodine, stopper the flask and shake vigorously. If the blue color returns, continue the titration.

11-Record the volume used for your sample and blank.

**Calculation:**

*Milli equivalent of iodine absorbed by sample:

$A = (B - S) \times N$

Where B = ml of Na2 S2 O3 used for blank titration.

S = ml of Na2 S2 O3 used for sample titration.

N = normality of Na2 S2 O3 used.

*Grams of iodine absorbed by the sample (C)

C = mEq iodine x 0.1269 g

Where 0.1269 is the milli equivalent weight of iodine.

*Grams of fat absorb (D)

D = 10 ml x (A g / 100 ml)

Where 10 ml = volume of sample fat used.

A g / 100 ml = concentration of sample fat used.

*Iodine number is equal to grams of iodine absorbed by 100 g of fat.

100 g of fat absorb (C / D) X 100

Iodine Number = (C / D) X 100

Questions:

1. Define the iodine number?
2. What is the significance of the iodine number in an oil analysis?
3. What is the composition of Hanus iodine solution?

4. Why is KI added before titrating with sodium thiosulfate?
5. How does $NaS_2O_3$ react with iodine .write the equation?
6. Why is the addition of the starch indicator delayed until most of the iodine has been titrated?
7. Explain the relationship between the iodine number and the fatty acid composition of most fats.
8. How do you account for the following :
    a) The low iodine number (8-10) of coconut oil?
    b) The high iodine number (175-205) of linseed oil?

9.a)what is meant by a "polyunsaturated fat"?

b)can you tell from the iodine number if a fat is a polyunsaturated? Explain.

10.calculte the iodine number of linoleopalmitolinolenin whose molecular weight is 881?

11. A 0.250 gm sample of butter was dissolved in $CHCl_3$ and treated with 25.00 ml of Hanus iodine solution . After 30 min. KI was added and the liberated iodine required 40.15 ml of 0.014 N $Na_2S_2O_3$. The blank titration for 25.00 ml of Hanus iodine was 46.50 ml of the standard $Na_2S_2O_3$. Calculate the iodine number of the butter.