NATIONAL OPEN UNIVERSITY OF NIGERIA

FACULTY OF HEALTH SCIENCES

DEPARTMENT OF ENVIRONMENTAL HEALTH SCIENCES

COURSE CODE: EHS 209

COURSE TITLE: GENERAL BIOCHEMISTRY PRACTICAL FOR ENVIRONMENTAL HEALTH

COURSE GUIDE

EHS 209 GENERAL BIOCHEMISTRY PRACTICAL

Course Developers /Writers:

Dr. (Mrs.) Oluremi A. Saliu
Department of Environmental Health Science
Faculty of Health Sciences
National Open University of Nigeria, Abuja

Dr. Oluwafemi A. Idowu
Department of Biochemistry
Faculty of Life Sciences
University of Ilorin, Kwara State

Course Editor:

Professor (Mrs.) Adenike T. Oladiji
Department of Biochemistry
Faculty of Life Sciences
University of Ilorin, Kwara State

Course Coordinator:

Professor Grace C. Okoli
Department of Environmental Health Science
Faculty of Health Sciences
National Open University of Nigeria, Abuja

Programme Coordinator:

Professor Grace C. Okoli
Department of Environmental Health Science
Faculty of Health Sciences
National Open University of Nigeria

National Open University of Nigeria
Headquarters
91, Cadastral Zone
Nnamdi Azikiwe Expressway,
Jabi Abuja
Nigeria
Abuja Annex
245 Samuel AdesujoAdemulegun Street
Central Business District
Opposite Arewa Suites, Abuja
E-mail: **centralinfo@noun.edu.ng**
URL: **www.nou.edu.ng**

First Printed 2019

ISBN:

Printed by……

For

National Open University of Nigeria
Headquarters
91, Cadastral Zone
Nnamdi Azikiwe Expressway,
Jabi, Abuja
Nigeria

E-mail: centrainfo@nou.edu.ng
Url/:www.niu.edu.g

CONTENTS **PAGE**

Introduction……....5
What you will Learn in this Course……....5
The Course Aim……..5
Working through this Course…….. ……...5
The Course Materials……..5
Study Units…….....6
Presentation Schedule…….....6
Assessment…….6
Sources of Information……...7
Appendix……...73

INTRODUCTION

This course guide informs you about the course, the materials available and how to work with them to take full advantage of learning through effective time management.

General Biochemistry Practical is a second semester, two-credit unit course for Environmental Health Science students. It is a practical course addressing the practical aspects necessary for students offering General Biochemistry in the Department of Environmental Health Science of Faculty of Health Sciences. Being a practical based course, you are expected to carry out the experiments in your laboratory.

WHAT YOU WILL LEARN IN THIS COURSE

In this course you will learn how to determine the pH and pKa value of solutions, how to carryout qualitative and quantitative test for carbohydrates, amino acids, proteins, lipids and vitamins. Finally, you will learn how to purify and analyze compounds using chromatographic separation techniques such as paper and thin layer chromatography.

THE COURSE AIMS

Generally, the course is aimed at encouraging you to have a practical knowledge of the important aspects of biochemistry as a course.

WORKING THROUGH THIS COURSE

Each unit has specific learning laboratory experiment with specific objectives. Endeavour to achieve these objectives when you go through these experiments. Attend the practical classes and make sure you participate fully. Again, go through the objectives after completing each unit to see whether you have understood the concepts treated in the unit.

Read textbooks and other materials which may be provided by the National Open University of Nigeria. Make sure you do not miss the practical classes.

THE COURSE MATERIALS

The main components of the course are:
1. The Course Guide
2. Study Units
3. Laboratory Experiments
4. Tutor-Marked Assignments
5. References/Further Reading

STUDY UNITS

The following are the units contained in this course:

Module 1 pH Measurement and Qualitative Test

Unit 1 Measurement of pH
Unit 2 Qualitative Test for Carbohydrates
Unit 3 Qualitative Test for Amino Acid
Unit 4 Qualitative Test for Lipid

Module 2 Quantitative Analysis and Chromatography

Unit 1 Quantitative Estimation of Carbohydrates
Unit 2 Quantitative Estimation of Proteins
Unit 3 Quantitative Estimation of Lipids
Unit 4 Quantitative Estimation of Vitamins
Unit 5 Paper and Thin Layer Chromatography

The first module has 4 units. Unit 1 focuses on the measurement of pH of solutions. Units 2, 3, 4 and 5 focus on the qualitative test for carbohydrates, amino acids and lipids respectively.

The second module has 5 units. Units 1, 2, 3 and 4 focuses on the quantitative estimation of carbohydrates, protein, and vitamins respectively while Unit 5 which is the final unit; focus on paper and thin chromatography separation techniques.

PRESENTATION SCHEDULE

As you must have read earlier, this course is practical. It is important you attend the practical classes that will be organized by your study centers and participate. Submit your report on time. You should guard against falling behind in your work.

SOURCES OF INFORMATION

Folin, O. and Ciocalteu, V. (1927). On tyrosine and tryptophan determination in proteins. J. Biol. Chem. 73, 627-650.

Lowry, O.H; Rosebrough, N.J; Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Murphy, J.B. and Kies, M.W. (1960). Note on spectrophotometric determination of in dilute solutions. Biochem. Biophys. Acta 45, 382-384.

www.vlab.amirita.edu/?sub=3&brch=63&sim=10948int=1

https://biologyreader.com/qualitative-analysis-of-lipids.html

https://www.onlinebiologynotes.com/millons-test-objective-principle-reagents-procedure-and-result/

https://vlab.amrita.edu/?sub=3&brch=63&sim=1094&cnt=1

https://www.onlinebiologynotes.com/sakaguchi-test-objective-principle-reagents-procedure-and-result/

https://www.onlinebiologynotes.com/lead-sulfide-test-detection-of-amino-acid-containing-sulfhydral-group-sh/

https://www.onlinebiologynotes.com/ninhydrin-test-principle-requirements-procedure-and-result/

http://www.biologydiscussion.com/carbohydrates/test/qualitative-and-quantitative-tests-for-carbohydrates/13042

http://www.biologydiscussion.com/lipids/tests/qualitative-and-quantitative-tests-for-lipids/13050

http://www.chem.wisc.edu/courses/342/Fall2004/TLC.pdf

http://www.bc.edu/schools/cas/chemistry/undergrad/org/fall/TLC.pdf

http://orgchem.colorado.edu/Technique/Procedures/TLC/TLC.html

http://www.google.com.ng/search?q=Experiment4%3AThin Layer Chromatography&ie=utf-8&oe=utf-8&aq=t&rls=org.mozilla:en-US:official&client=firefoxa&source=hp&channel=np

www.academiya.org/sites/default/files/Chromatography%20MCQs.doc

MAIN COURSE

CONTENTS **PAGE**

Module 1	**pH Measurement and Qualitative Test**	**9**
Unit 1	Measurement of pH Solutions	9
Unit 2	Qualitative Test for Carbohydrates	13
Unit 3	Qualitative Test for Amino Acid	19
Unit 4	Qualitative Test for Lipid	25
Module 2	**Quantitative Analysis and Chromatography**	**39**
Unit 1	Quantitative Estimation of Carbohydrates	40
Unit 2	Quantitative Estimation of Proteins	44
Unit 3	Quantitative Estimation of Lipids	52
Unit 4	Quantitative Estimation of Vitamins	61
Unit 5	Paper and Thin Layer Chromatography	66

UNIT 1 Measurement of pH of solutions

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
    3.1 The pH scale
    3.2 Measuring pH of Solutions with pH Paper and pH Meter
    3.2 Experiments on pH Measurement using pH Indicators
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

The pH of a solution is the hydrogen ions concentration [H^+] of the solution. In a simple term, it is the value that gives the amount of hydrogen ions present in a solution or a media. The pH of a solution may be measured approximately with indicators. These pH indicators are chemical substances which may be in the form of paper (e.g. litmus and pH paper indicator) or prepared in solution (e.g. as listed in Table 1) that changes colour when they come in contact with acids or bases.

For example, litmus and pH paper are indicator which turns red in strong acids and blue in strong bases. It is possible to estimate the pH of a solution from the colours given by various indicators and because only a few pH indicators measure pH over a wide range of pH values, you will need to find out the pH range of the indicator used. Typically, there is colour chart kit provided for each pH indicator that shows the pH range of indicators. This however, only provides an approximate measure of the pH value, or the strength of the acid or base. The pH of a solution is more accurately measured with a pH meter which uses a glass electrode and a calomel reference electrode.

Indicators are used to provide information about the degree of acidity of a substance or the state of some chemical reactions within a solution being tested or analysed.

2.0 OBJECTIVES

At the end of this unit, you should be able to:

- carryout experiments on pH measurement
- measure the pH of some substances and solutions using various pH indicators

3.0 MAIN CONTENT

3.1 The pH Scale

The pH scale is used to rank solutions as acidic or basic (alkaline). The pH scale ranges from 0 to 14. A pH below 7.0 is acidic, above 7.0 is alkaline, or basic while pH of 7 is considered neutral.

3.2 Measuring pH with pH Paper and pH Meter

When measuring pH of a mixture with pH paper, dip the end of a strip of pH paper into each mixture you want to test. After about two seconds, remove the paper, and immediately compare the colour at the wet end of the paper with the colour chart provided with that pH indicator. Always use a clean, unused strip of pH paper for each mixture that you test. However, when measuring pH of mixtures with pH meter, dip the end of electrode of pH meter into the mixture you want to test and observe the pH value on the meter. Write down the pH value of each mixture.

Materials Needed

pH paper indicator and colour chart (pH range 3 to 12) or pH meter, distilled water, indicators (phenolphthalein, methyl red and thymol blue).

Table 1: List of Indicators and their pH range

Indicators	pH range	Acid Colour Change	Basic Colour Change
Thymol blue	1.2 – 2.8	Red	Yellow
Bromophenol blue	2.8 – 4.6	Yellow	Violet
Congo red	3.0 – 5.0	violet	Orange
Bromocresol green	3.7 – 5.4	Yellow	Blue
Alizarin yellow	10.1 – 12.0	Yellow	Red
Methyl red	4.4 – 6.0	red	Yellow
Methyl orange	3.1 – 4.1	Red	Yellow
Phenolphthalein	8.2 – 10.2	Colourless	Pink

3.3 Experiments on pH measurement using pH indicators

In this experiment, you will determine the pH of some solutions and classified them as acidic or basic. A practical way to evaluate the relative acidity or basicity of solutions is to compare their effect on indicators.

Procedure

1. Dip an unused strip of pH paper into the solutions provided by your facilitator (vinegar, distilled water, caustic soda and ammonia). Leave until wet (about 2 seconds). Immediately compare with the color chart provided and write down the approximate pH value of the solution.

2. If you're using a pH meter, dip the end of the electrode of pH meter into the solution, and observe the value on the meter. Write down the pH value and compare with what you have in step 1 above.

3. Two solutions of A and B of unknown pH are provided. To 5 ml of each solution add 2 drops of phenolphthalein. Repeat this process using methyl red and thymol blue; observe the colours obtained and draw conclusions about the approximate pH of the solutions.

4.0 CONCLUSION

The pH is used to measure the amount of hydrogen ion in a solution or media. It is used to determine if a solution is acidic or basic in nature. The pH scale ranges from 0 to 14. A pH of less than 7 is acidic, above 7 is basic while at pH value of 7 is neutral.

5.0 SUMMARY

In this unit, you have been:

• exposed to using pH paper to measure pH of a solution
• exposed to different indicators and their pH range
•exposed to measuring the pH value of common substances using pH meter.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is pH?
2. How can you measure pH of a solution in the laboratory?
3. Which indicator appears colourless in acidic medium and pink in basic medium?

7.0 REFERENCES/FURTHER READING

http://swc2.hccs.edu/pahlavan/intro_labs/Exp_20_pH_of_Common_Substances.pdf

http://www.sciencegeek.net/Chemistry/taters/Unit8pH.htm

Working Sheet

Observation:

Conclusion:

UNIT 2 QUALITATIVE TEST FOR CARBOHYDRATES

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
    3.1 Classes of Carbohydrates
    3.2 Experimental Tests for Carbohydrates
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

Carbohydrates are compounds containing carbon, hydrogen and oxygen and are widely distributed in plants and animals. They have the general formula of $C_nH_{2n}O_n$. Carbohydrates are more abundant in nature than all other organic compounds of biological importance. The most abundant carbohydrate is cellulose which is found in woody structures and fibers of plants. Starches are also abundant in grains, tubers and roots where they serve as food reserves for plants.

2.0 OBJECTIVES

At the end of this unit, you should be able to:

- carryout the different experimental tests for carbohydrate sugars
- know how to test for starch

3.0 MAIN CONTENT

3.1 Classification of Carbohydrates

1. Monosaccharides	(i) Hexoses: glucose, fructose, galactose (ii) Pentoses: ribose, ribulose, xylose
2. Disaccharides	(i) Sucrose (glucose + fructose) (ii) Maltose (glucose + glucose) (iii) Lactose (glucose + galactose)
3. Polysaccharides	Starch, cellulose, glycogen, inulin etc

3.2 Experimental Test for Carbohydrates

Materials Needed

Benedict's reagent, Barfoed's reagent, Fehling's solution A and B, iodine, Molisch reagent (α-naphthol, 5% (w/v) in 95% ethanol) distilled water, Conc.H_2SO_4, glucose, fructose, sucrose, starch, unknown source of carbohydrate, water bath. See Appendix for the preparation of the reagents

Carbohydrates can be tested using any of the tests explained below:

1. **Molisch's Test:** It is a group test for all carbohydrates, whether free or in combined form. The reaction depends on the condensation of α-naphthol with the furfural formed by the action of tetra-oxo-sulphate (VI) acids (H_2SO_4) giving a purple or violet coloured product. Polysaccharides also give a positive reaction. In the event of the carbohydrate being a poly- or di-saccharide, the acid first hydrolyses it into component monosaccharides, which then get dehydrated to form furfural or its derivatives.

Procedure

To 1 ml of test solution (carbohydrate source), add 3 drops of 1% α-naphthol in ethanol. Mix and pour 1 ml of conc. H_2SO_4 slowly down the side of the tube to give a separate lower layer. A violet colour at the junction of the two layers indicates the presence of a carbohydrate. All carbohydrates except amino sugars (e.g. glucosamine, galactosamine) give a positive reaction.

2. **Fehling's Test:** Fehling's reagent contains copper II or cupric (Cu^{2+}) ions in an aqueous basic solution. In the presence of a reducing agent, the Cu^{2+} is reduced to (copper I or cuprous) Cu^{+} ion which forms a red precipitate of copper I oxide or cuprous oxide (Cu_2O). Therefore, if Fehling's solution is added to a solution containing a reducing sugar, a red precipitate is formed. Sometimes the reaction mixture must be heated in order for the precipitate to form. The color of the precipitate can vary from red to orange to green (the green color is actually a mixture of an orange and a blue precipitate).

Procedure

In this part of the experiment, you will test for glucose, fructose, lactose, sucrose, starch, and an unknown solution which will be provided. Add 6 drops of the solution to be tested to each of the 6 labeled test tubes containing the samples above respectively. In a larger test tube, mix 6 ml of Fehling's solution A with 6 ml of Fehling's solution B. Add 2 ml of this mixture to each of the 6 test tubes, and mix

each tube thoroughly by shaking the tube well. Place these tubes in a boiling water bath for 5 minutes. After 5 minutes, remove the tubes from the water bath and record your observations. The formation of a red precipitate indicates a positive reaction (i.e. presence of a reducing sugar).

3. **Barfoed's Test:**Barfoed's test is similar to Fehling's test, except that in Barfoed's test, different types of sugars react at different rates. Barfoed's reagent is much milder than Fehling's reagent. Reducing monosaccharides react quickly with Barfoed's reagent, but reducing disaccharides react very slowly or not at all. Therefore, it is possible to distinguish between a reducing monosaccharide and a reducing disaccharide using Barfoed's reagent.

Procedure

You will again test glucose, fructose, lactose, sucrose, starch, and your unknown sample. Add 1 ml of the solution to be tested to each of the 6 labeled test tubes. Add 3 ml of Barfoed's reagent to each of the 6 test tubes, and mix each tube thoroughly by shaking the tube. Place these tubes in a boiling water bath for 5 minutes. After 5 minutes, remove the tubes from the water bath, let them cool, and then cool them further by running cold water over the outside of each test tube. Record your observations. The formation of a red precipitate indicates a positive reaction which is an evidence of a reducing monosaccharide. The precipitate formed in this test is less dense than that produced with Benedict's solution.

4. **Seliwanoff's test**: In Seliwanoff's test a dehydration reaction is involved. Seliwanoff's reagent contains a non-oxidizing acid, hydrogen chloride (HCl) and resorcinol. When a ketose sugar (a sugar with ketone group e.g. fructose, ribulose etc.) is reacted with this reagent, it becomes dehydrated and a cherry-red complex is formed (not a precipitate). Aldoses (sugars with aldehyde and hydroxyl group e.g. glyceraldehyde, glucose, mannose, galactose etc.) also react with this reagent, but much more slowly than ketoses. When Seliwanoff's reagent is reacted with a disaccharide or a polysaccharide, the acid in the solution will first hydrolyze them into monosaccharides, and the resulting monosaccharides can then be dehydrated. Disaccharides and polysaccharides will therefore react slowly with Seliwanoff's reagent. When you carry out this test, it is important to note the time required for a reaction to occur.

Procedure

For this part, you will test glucose, fructose, lactose, water, and your unknown. Add 10 drops of the solution to be tested to each of 5 labeled test tubes. Add 4 ml of Seliwanoff's reagent to each of the 5 test tubes, and mix each tube thoroughly by shaking the tube. Place these tubes in a boiling water bath and note the time it take for

any color change to occur. After 10 minutes, stop heating the tubes. Record your observations. The appearance of a deep red colour indicates the presence of a ketose group in the sugar.

5. **Benedict's test:** Benedict test often used in place of Fehling's solution to detect the presence of reducing sugars in a sample. The principle of Benedict's test is that when reducing sugars are heated in the presence of an alkali or a base, they reduce the cupric ion (Cu^{2+}) present in the Benedict's reagent to cuprous ion (Cu^{+}) to form an insoluble precipitate of copper(I) oxide or cuprous oxide (Cu_2O).

Procedure

Add 5 drops of the test solution (glucose, fructose, lactose, water, and your unknown) to 2 ml of Benedict's reagent. Heat the mixture in a boiling water bath for 5 minutes. If the test solution contains a reducing sugar (i.e. all monosaccharides, maltose and lactose but not sucrose), a yellow or brick-red precipitate of cupric oxide will be observed. If the blue colour of the Benedict's reagent persists, add more drops of the test solution and boil the mixture.

6. **Test for Starch:** Iodineforms a blue, black, or gray complex with starch and is used as an experimental test for the presence of starch. The colour of the complex formed depends on the structure of the polysaccharide and the strength and age of the iodine solution. Starch such as amylase forms a blue complex precipitate upon contact with iodine while other starch like amylopectin, glycogen and cellulose forms different colour complex (red, brown or purple) with iodine. Iodine does not form a complex with simpler carbohydrates (i.e. monosaccharides and disaccharides).

Procedure

You will test glucose, fructose, lactose, sucrose, starch (yam cassava, cocoyam potato etc.), water. Add 1 ml of the solution to be tested to each of 7 labeled test tubes. Add
3 drops of iodine solution to each of the 7 test tubes, and mix each tube. Compare the colours and record your observations. Formation of blue, black, or gray complex shows positive to starch.

Note: The purpose of using water is to prove water contains no sugar and act as control for negative test.

4.0 CONCLUSION

Carbohydrates are classified into monosaccharides, disaccharides and polysaccharides. In the laboratory, several rapid tests are available to establish the presence or absence of a sugar or a

carbohydrate in a sample and these tests are based on specific colour reactions typical for their group.

5.0 SUMMARY

In this unit, you have learnt:

- The classes of carbohydrates
- The laboratory tests for carbohydrates

6.0 TUTOR-MARKED ASSIGNMENT

i. What are the classes of carbohydrates?
ii. How do you distinguish a disaccharide from a monosaccharide in the laboratory?
iii. What test would you use to distinguish a polysaccharide from other sugars?

7.0 REFERENCES / FURTHER READINGS

www.vlab.amirita.edu/?sub=3&brch=63&sim=10948int=1

Working Sheet

Observation:

Conclusion:

UNIT 3 QUALITATIVE TEST FOR AMINO ACIDS

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
    3.1 Classes of Amino Acids
    3.2 Experimental Test for Amino Acids
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

Amino acids are building blocks of all proteins, and are linked in series by peptide bond (-CONH-) to form the primary structure of a protein. Amino acids possess an amine group, a carboxylic acid group and a side chain that differs between different amino acids.
There are 20 naturally occurring amino acids, which vary from one another with respect to their side chains. Amino acids respond to all typical chemical reactions associated with compounds that contain carboxylic acid and amino groups, usually under conditions where the zwitter ions (positive and negative ions) form is present in only small quantities.

2.0 OBJECTIVES

At the end of this unit, you should be able to:

- carry out the experiment to identify the presence of amino acid from a given unknown sample

3.0 MAIN CONTENT

3.1 Classes of Amino Acids

(i). Acidic amino acids: e.g. aspartate, glutamate
(ii) Basic amino acids: e.g. lysine, arginine, histidine
(iii) Neutral amino acids: e.g. asparagine, leucine valine
(iv) Aromatic amino acids e.g. histidine, phenylalanine, tryptophan and tyrosine
(v) Amino acids with sulphur atom: e.g. cysteine, methionine

1.1 Experimental Test for Amino Acids

1. **Ninhydrin Test:** This test is a general test for all amino acids. This test is due to a reaction between an amino group of free amino acid and ninhydrin. Ninhydrin is a powerful oxidizing agent and in its presence, amino acids undergo oxidative deamination liberating ammonia (NH_3), Carbondioxide (CO_2), a corresponding aldehyde and reduced form of ninhydrin (hydrindatin). The NH_3 formed from an amino group reacts with another molecule of ninhydrin and its reduced product, hydrindatin to give a blue substance diketohydrin (Ruhemanns complex). However, in case of imino acid like proline and hydroxyproline, a different product having a bright yellow color is formed. Asparagine, which has a free amide group, reacts to give a brown colored product.

Materials Needed

Test solution (1% alanine, 1% proline, 1% asparagine, egg albumin or soy bean), distilled water, 2% ninhydrin in acetone or ethanol, water bath, dry test tubes and pipettes.

Procedure

Take 1 ml test solution (1% alanine, 1% proline, 1% asparagines, egg albumin or soy bean) in dry test tube and 1 ml distilled water in another tube as a control, pour few drops of 2% ninhydrin in both the test tubes and place the test tubes in water bath for 5 minutes. Look for the development of blue or violet color which is a positive test.

Note: In the case of proline and hydroxyproline, yellow color will develop instead of blue color. Similarly, asparagine will give brown color.

2. **Xanthoproteic acid test**: Aromatic amino acids, such as phenylalanine, tyrosine and tryptophan, respond to this test. In the presence of concentrated nitric acid, the aromatic phenyl ring is nitrated to give yellow colored nitro-derivatives. At alkaline pH, the color changes to orange due to the ionization of the phenolic group.

Materials Needed

Conc. Nitric acid, water bath, 40% NaOH

Procedure

Add 1 ml of conc. nitric acid to 1 ml of the sample (protein source). Warm the mixture in boiling water bath and allow cooling. Observe the colours and formation of precipitates. Add sufficient amount of 40% NaOH to make the solution strongly alkaline in nature. A yellow colour in acid solution which turns bright orange in alkali indicates the presence of one or more of the aromatic amino acids tryptophan, phenylalanine or tyrosine.

3. **Million's test:** Amino acids such as Tyrosine and its derivatives respond to this test. Million's reagent is a solution of mercuric sulphate in sulphuric acid. This test is specific for the amino acid tyrosine and the protein containing this amino acid. Tyrosine when reacted with acidified mercuric sulphate solution gives yellow precipitate of mercury-amino acid complex. On addition of sodium nitrate solution and heating, the yellow complex of mercury-amino acid complex converts to mercury phenolate which is in red colour.

Materials Needed

Test solution, Million's reagent (Acidified mercuric sulphate), distilled water, test tubes, 1% tyrosine and 1 % sodium nitrite. See Appendix for preparation of Million's reagent

Procedure

To 1 ml of the unknown test solution (protein source e.g. egg albumin, soy bean etc) and distilled water in separate test tubes, add 5 drops of millions reagent and mix well. Boil the mixture gently for 2 minutes and cool under tap water add 5 drops of 1% sodium nitrate. Heat the solution lightly and look for the development of brick red precipitate which indicates the presence of tyrosine or its derivatives and phenols. Distilled water and tyrosine are used as the negative and positive control respectively. Distilled water will not form brick red precipitate but tyrosine will.

4. **Sagakuchi test:** This is used to test for the presence of arginine in a sample. The arginine reacts with α – napththol and an oxidizing agent such as bromine water or sodium hypochlorite/sodium hypobromite to give a red colored product. The other guanidinium containing compounds other than amino acid also give this reaction.

Materials Needed

Test solution (1 % arginine, 5 % egg white (albumin) or soy bean), 1 % α naphthol in alcohol, sodium hypochlorite, 1 % urea solution, Dry test tubes and pipettes

Procedure

Take 1ml each of test solution and distilled water into separate dry test tubes, add 2 drops of α-naphthol and mix well. Add 2 ml sodium hypochlorite or sodium hypobromate to all test tubes and immediately add 1ml of urea solution. Note the colour change that appears. The formation of red coloured complex establishes the presence of arginine.

Note: Arginine serves as the positive control while distilled water as the negative control

5. **Lead sulphide test:** This test is used to test for sulphur containing amino acids such as cysteine and methionine in a sample. When these amino acids are heated with strong alkali like NaOH, the sulphur (organic) is converted to inorganic sodium sulphide (Na_2S) which can be detected by precipitation as lead sulphide (PbS) from the alkaline solution.

Materials Needed

Test solution (1 % cysteine, distilled water, 5% egg white (albumin) or soy bean), Bunsen burner and Foli's reagent: Lead acetate ($Pb(CH_3COO)_2$), 40 % NaOH.

Procedure

Take 1 ml of the test solution and 1 ml of glycine into separate dry test tubes, add 2 ml of 40 % NaOH and mix well. Add 1 ml Foli's reagent (lead acetate) to all test tubes and heat over the flame of Bunsen burner. Record your observation. The development of a black precipitate indicates the presence of cysteine. Glycine serves as the negative control while cysteine as the positive control which will yield a black precipitate.

6. **Biuret test:** The Biuret method is basically used to test for proteins in foods and biological fluids e.g. plasma, serum and organ homogenates both qualitatively and quantitatively. However, it can also be used to test for amino acids but it is only histidine that is positive to biuret test when testing for amino acids.

Materials Needed

Biuret reagent: Sodium, hydroxide, Copper sulphate and sodium potassium tartarate.

Procedure

To 1 ml of the test solution and to another 1 ml of distilled water in dry test tubes separately, add to each of the solution 1 ml of biuret reagent and mix thoroughly with the distilled water serving as the control. Observe what happens, the development of violet colour is an indication of a positive test.

4.0 CONCLUSION

Amino acids are the building blocks of protein. Amino acids are made up of different functional groups that chemical reacts with many colours producing agents (dyes) with characteristically coloured products**.** The colour intensity of the product formed by a

particular functional group varies in proportion to the number of reacting functional or free groups present and their accessibility to the reagent.

5.0 SUMMARY

In this unit, you have learnt about:

- amino acids and their classes
- how to test for amino acids in a sample using different reagent test methods

6.0 TUTOR-MARKED ASSIGNMENT

i. What are the classes of carbohydrates?
ii. What test would you use to identify the presence of aromatic amino acids in a sample and mention two reagents that will be required for the test?

7.0 REFERENCES / FURTHER READINGS

https://www.onlinebiologynotes.com/millons-test-objective-principle-reagents-procedure-and-result/

https://vlab.amrita.edu/?sub=3&brch=63&sim=1094&cnt=1

https://www.onlinebiologynotes.com/sakaguchi-test-objective-principle-reagents-procedure-and-result/

https://www.onlinebiologynotes.com/lead-sulfide-test-detection-of-amino-acid-containing-sulfhydral-group-sh/

https://www.onlinebiologynotes.com/ninhydrin-test-principle-requirements-procedure-and-result/

Working Sheet

Observation:

Conclusion:

UNIT 4 QUALITATIVE TEST FOR LIPIDS

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
    3.1 Classes of Lipids
    3.2 Methods for Qualitative Analysis of Lipids
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

The qualitative analysis of lipid is used to detect the presence or absence of lipid on the basis of colour change. Lipids are the organic biomolecules which are soluble in non-polar solvents like chloroform, ether, benzene, and acetone etc. but insoluble in water. In nature, there are different classes of lipids which show structural diversity among each other. Some common lipids are fatty acids, fats, oils, waxes, phospholipids etc.

2.0 OBJECTIVES

At the end of this unit, you should have learnt about:

- the classification of lipids
- the various qualitative test for lipids

3.0 MAIN CONTENT

3.1 Classification of Lipids

Lipids are classified on the basis of their chemical composition into the following groups:

(i). **Fatty acids:** All organic acids are usually classified as fatty acids. Fatty acids may be saturated or unsaturated.
(ii). **Neutral fats:** These groups are the triacylglycerol (also called triacylglyceride) and are the most abundant lipids in animal tissues.
(iii). **Phospholipids:** This group includes phosphatidic acid, phosphatidylethanolamine (cephalin), phosphatidylcholine (lecithin), diphosphatidylglycerol (cardiolipin).

(iv). **Sphingolipids:** This group includes sphingomyelins, cerebrosides and gangliosides.

(v). **Steroids:** Steroids include sterol and steroid hormones

(vi). Waxes

3.2 Methods for Qualitative Analysis of Lipids

There are several methods which are used for the qualitative analysis of lipids and its components.

1. **Experimental Test on Solubility**

Solubility test is the preliminary test which detects the presence of all lipids. This test detects the solubility of lipid in various solvents to check whether it is miscible or immiscible in polar or non-polar organic solvents.

Principle: Solubility test is based on the property of lipid to dissolve in different solvents. Lipids are readily miscible in non-polar solvents like chloroform, partially soluble in a polar solvent like ethanol and immiscible in a polar solvent like water.

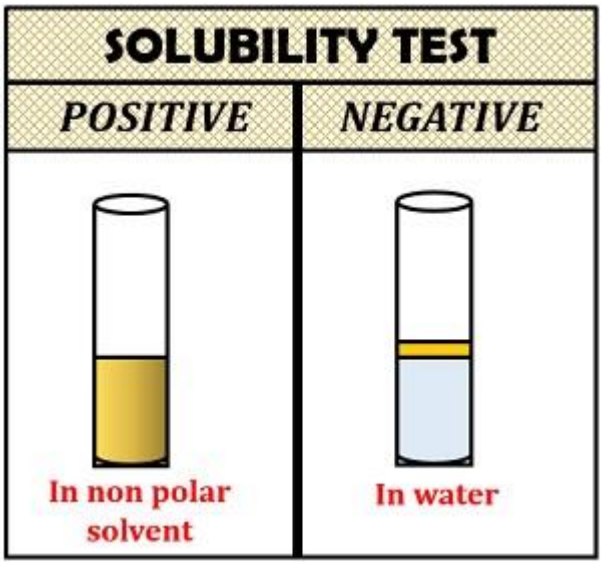
Materials Needed

Olive oil, groundnut oil, palm oil, coconut oil, margarine, glycerol, palmitic acid, cholesterol, ethanol, diethyl ether, chloroform and test tubes

Procedure:

Take each of the samples of lipid into separate test tubes and label them, then add two drops of water. Shake the tubes and allow standing for 1 minute. Repeat the experiment for each sample using ethanol, diethyl ether and chloroform. Record your observation for the solubility of each sample in each solvent.

Interpretation of Result



Positive result: Lipids are soluble in non-polar solvent i.e. chloroform and partially soluble in ethanol which can solubilize upon heating.

Negative result: Lipids are insoluble in a polar solvent i.e. water.

2. **Translucent Spot Experimental Test of Lipid**

A translucent spot test is also a preliminary test for the lipids which can be detected by the appearance of a translucent and greasy spot.

Principle: The lipid will not wet the filter paper, unlike water. Lipids will form a greasy spot because they are having a greasy texture that will penetrate into the filter paper. In contrast to lipid, the spot of water will disappear from the paper whereas the spot of lipid appears as the "Translucent spot".

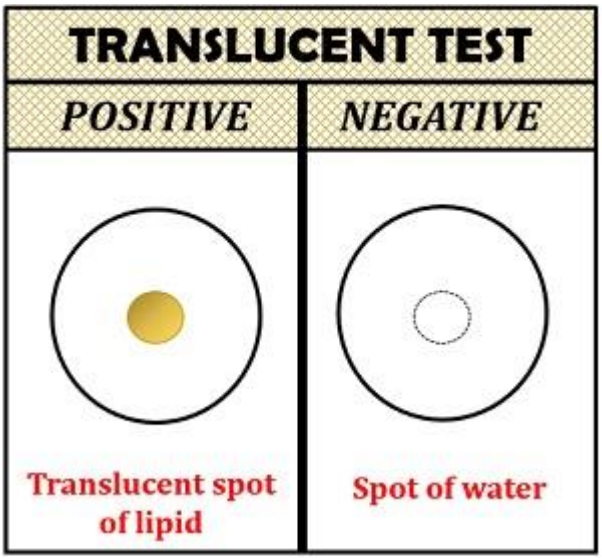
Materials Needed

Lipid source, distilled water and filter paper.

Procedure

Take two filter papers separately, to one add one drop of water on one and a drop of oil or the lipid source on the paper and observe the appearance on the filter paper.

Interpretation of result:



Positive result: Translucent (grease) spot will appear on the filter paper containing the lipid.

Negative result: Translucent (grease) spot will not appear on the filter paper containing water.

3. Experimental Test on Emulsification of Lipid

Emulsification test is used to detect the presence of lipids.

Principle: Emulsification is the process which stabilizes the water and oil emulsion by the help of emulsifying agents. The lipid or oil in water appears on the top of the water because of the high surface tension of water which gets together to form a separate layer. On the addition of emulsifying agents like bile salts, soap etc. emulsifying agents emulsify the lipid by which the lipid appears as the tiny droplets suspended in the solution.

Materials Needed

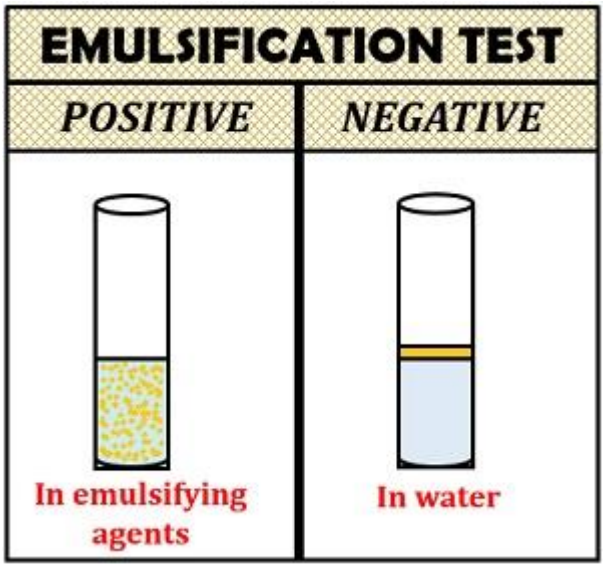
Lipid source, emulsifying agents (soap or bile salt)

Procedure

Take two test tubes and label them as test tube A and test tube B. Add two drops of oil to each of the test tubes, shake the test tubes and allow to stand for about two minutes.

Then add detergent or bile salt into test tube A only, allow to stand for few minutes and record your observation.

Interpretation of result



Positive result: It gives a permanent or stable emulsion of lipid and water.

Negative result: Oil in water emulsion will form at the top, due to the high surface tension of water.

4. **Experimental Test on Saponification of Lipid**

Principle: It is based on the "Saponification reaction", where the triglycerides of lipid react with an alkali NaOH and produce soap and glycerol in the presence of ethanol. This reaction also refers to as "Alkaline hydrolysis of esters".

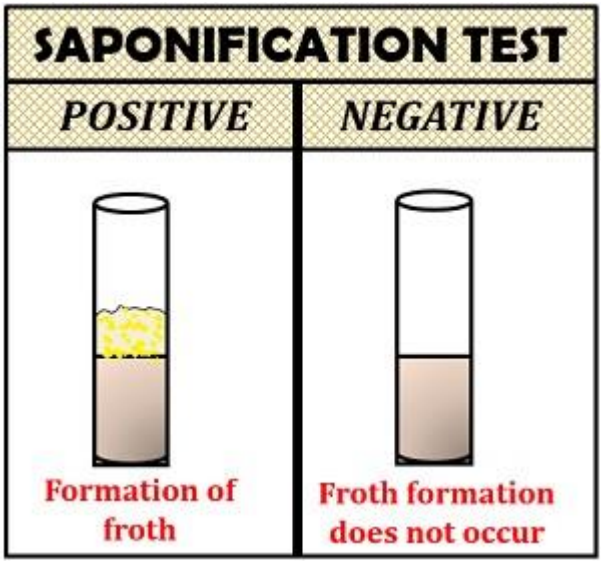
Materials Needed

Lipid source, sodium hydroxide, ethanol, test tubes and water bath

Procedure

Take a sample of the lipid into a test tube, and add strong alkali sodium hydroxide (NaOH). Boil the solution in a water bath for 5 minutes. At the end of the boiling add ethanol and record your observation.

Interpretation of result



Positive result: Froth appears in the test tube.

Negative result: Froth does not appear in the test tube.

5. **Sudan IV Test on Lipid**

Sudan IV test is used to detect the presence of lipid in a solution.

Principle: This test is based upon the principle of binding and solubility of lipid to non-polar compounds. As Sudan IV is a non-polar stain, therefore the lipid will bind with it and retain the colour of the stain and gives a red-orange colour. Sudan IV does not stain or binds to the polar compounds.

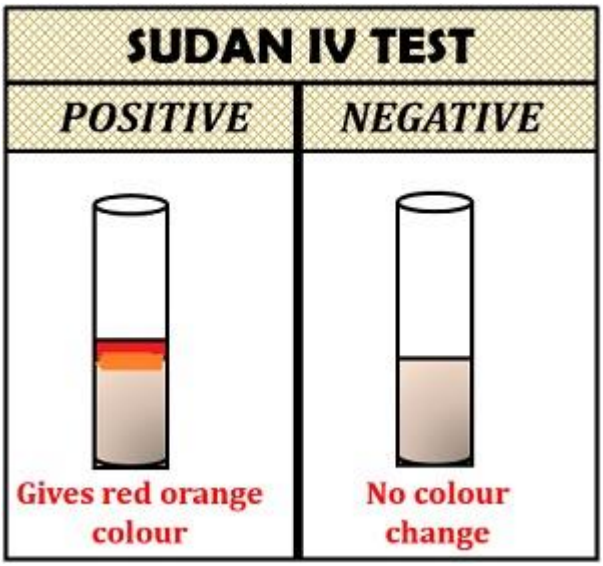
Materials Needed

Lipid source, test tube and Sudan IV solution

Procedure

Take 1 ml of the lipid sample into a test tube and add 1-2 drops of Sudan IV to the solution. Observe the tube for the appearance of red-orange colour to the solution.

Interpretation of result



Positive result: Gives red-orange colour to the solution.

Negative result: The solution to the colour will remain unchanged.

6. Acrolein Test on Lipid

Acrolein test is used to detect the presence of glycerol and fat.

Principle: This test is based on the "Dehydration reaction", where the water molecules removed from glycerol by the addition of potassium hydrogen sulphate reagent. The reaction between glycerol and potassium hydrogen sulphate results in the formation of "Acrolein" that is characterized physically by the release of the pungent smell.

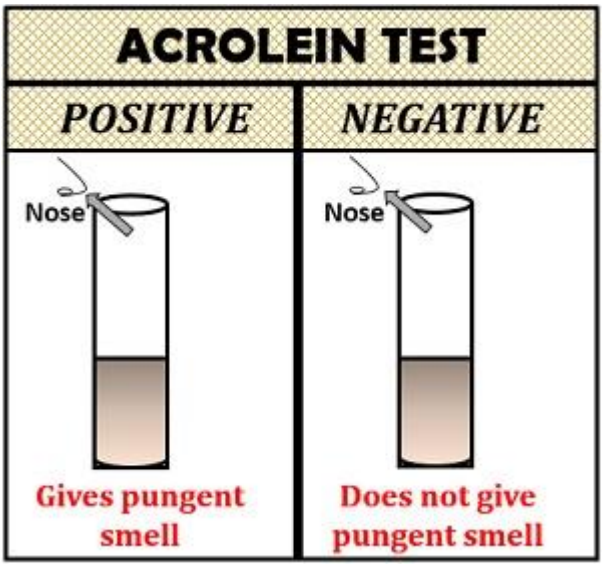
Materials Needed

Test tube, crystals of potassium hydrogen sulphate

Procedure

Take 1 ml of the lipid sample into a test tube and add crystals of potassium hydrogen sulphate. Heat the solution for a few minutes and observe the test tube for the pungentsmell.

Interpretation of result



Positive result: If glycerol present in the sample it will give a pungent smell.

Negative result: If glycerol is absent in a sample, then it will not produce a pungent smell.

7. Dichromate Test on Lipid

Dichromate test is also used to detect the presence of glycerol.

Principle: It is based on the principle of "oxidation reaction". In this, glycerol and dichromate ions react to give a brown colour to the solution. Then the chromic ions oxidize the glycerol and are reduced to chromous ions in the presence of nitric acid giving a blue colour to the solution.

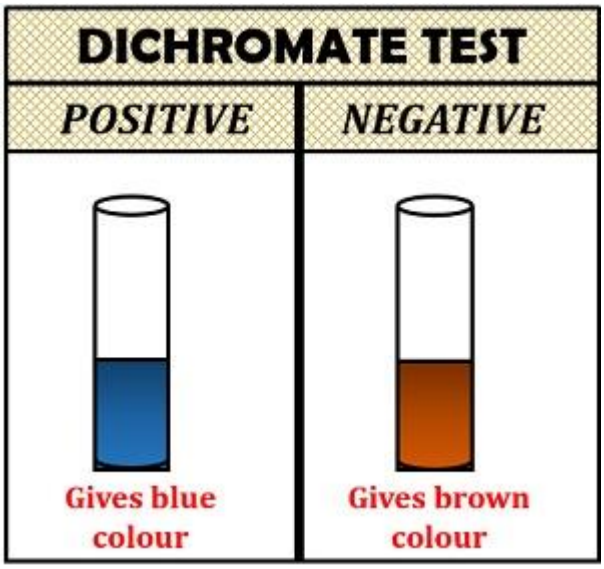
Materials Needed

5% potassium dichromate, distilled water and concentrated nitric acid.

Procedure

Take 2-3 ml of the sample of lipid into a test tube and add a few drops of 5% potassium dichromate solution this should be followed by the addition of 5 ml of concentrated nitric acid. Record your observation on the colour formed in the test tube.

Interpretation of result



Positive result: If the colour of the solution changes from brown to blue, then it indicates the presence of glycerol.

Negative result: The brown colour will not change to blue.

8. Neutralization Test on Lipid

This test is used to detect the presence of fatty acids in a sample.

Principle: This is based on the "Neutralization reaction" where an alkali is neutralized by the addition of free fatty acids in the lipids.

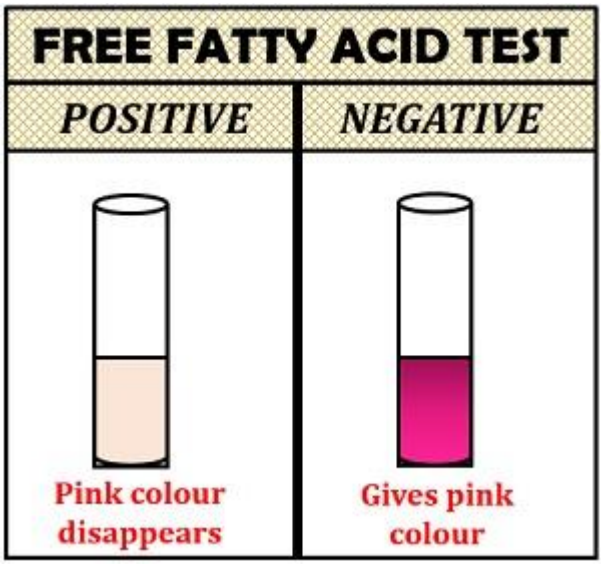
Materials Needed

Phenolphthalein, dilute sodium hydroxide, test tubes and lipid sample

Procedure

Add 2 drops of phenolphthalein solution into a test tube and then add few drops of dilute alkali (e.g. NaOH) to the above solution which will give a pink colour. To this mixture, add 2 ml of the lipid sample and observe the colour change as you add the lipid sample to the mixture.

Interpretation of result



Positive result: If the pink colour disappears by the addition of the lipid sample, then it indicates the presence of free fatty acids in the sample.

Negative test: if the pink colour does not disappear, it means the lipid sample contain no fatty acids.

9. **Unsaturation Test on Lipid**

Unsaturation test is used to detect the presence of unsaturated fatty acids or the amount of double bond in a lipid sample.

Principle: All the neutral fat contains glycerides of fatty acids. The double bond found in the structure of unsaturated fatty acids becomes saturated by taking up or decolorizing either bromine or iodine when it comes in contact with them. If the lipid contains more unsaturated fatty acids or more double bonds that means, it will take more iodine (i.e. high degree of unsaturation by producing a more pronounced discoloration of bromine or iodine).

Materials Needed

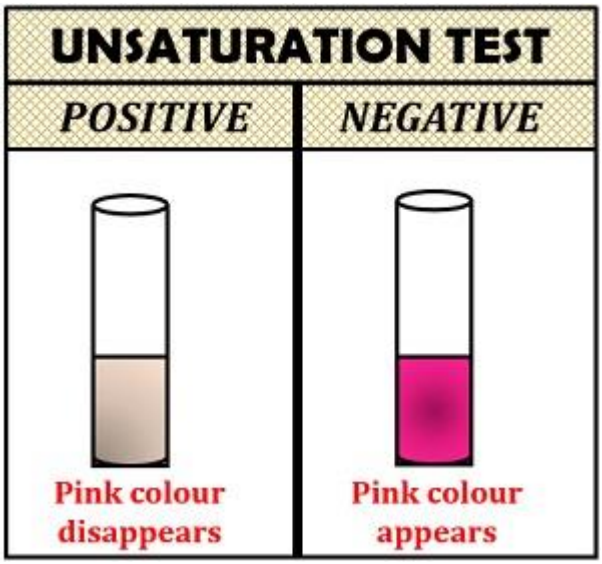
Chloroform, Hubel's iodine, beaker, lipid sample

Procedure

Take 5 ml of chloroform and 5 ml of Hubel's iodine reagent into a beaker which will give pink colour to the solution. Add the lipid sample drop by drop and shake vigorously, until the pink colour disappears.

Observation: Count the number of drops added to the solution of chloroform and Hubel's iodine solution till the pink colour disappears. The number of drops determines the taking up of iodine by the unsaturated fatty acid of lipids.

Interpretation of result



Positive result: Pink colour will disappear by the addition of unsaturated fatty acids.

Negative result: Pink colour will not disappear.

10. Burchard Test

Burchard test was first given by the scientist "Liebmann" to detect the presence of cholesterol. The test is also called Liebmann-Burchard test.

Principle: Cholesterol reacts with the strong concentrated acid i.e. sulphuric acid and acetic anhydride. Sulphuric acid and acetic anhydride act as a dehydrating and oxidizing agent.

Materials Needed

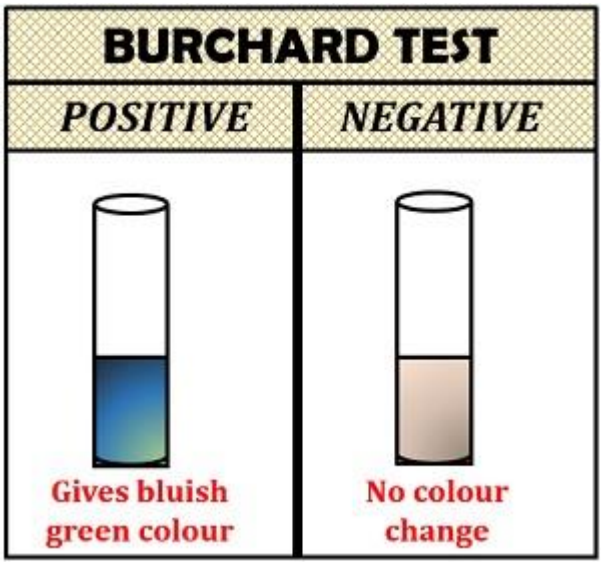
Acetic anhydride, Conc. H_2SO_4, lipid source, chloroform

Procedure

Carefully add 1 ml of acetic anhydride to 1 ml of a chloroform solution of cholesterol (10 mg/l), followed by the addition of 2-3 drops of concentrated sulphuric acidH_2SO_4.

Note the change in colour. Acetic anhydride reacts with cholesterol solution to produce a characteristic blue-green colour.

Interpretation of result



Positive result: It indicates the presence of cholesterol in a sample by giving bluish-green colour to the solution.

Negative result: It is indicated by no colour change.

4.0 CONCLUSION

The qualitative analysis of lipid is the preliminary test to detect the presence or absence of lipids and to classify the different groups of lipids based on their chemical reactivity with the chemical reagent. The presence of lipids in the qualitative analysis is measured by the colour change.

5.0 SUMMARY

In this unit you have learnt about:

- Classes of lipids
- qualitative test for various lipid

6.0 TUTOR-MARKED ASSIGNMENT

(i). Describe one qualitative test that may be employed to detect the presence of cholesterol in a sample

(ii). It was observed on the addition of bromine to a solution of lipid in a test tube, the bromine colour decolorizes. State what type of lipid that is responsible for the discoloration of bromine.

7.0 REFERENCES / FURTHER READINGS

https://biologyreader.com/qualitative-analysis-of-lipids.html

Working Sheet

Observation:

Conclusion:

MODULE 2 QUANTITATIVE ANALYSIS

Unit 1 Quantitative Estimation of Carbohydrates

Unit 2 Quantitative Estimation of Proteins

Unit 3 Quantitative Estimation of Lipids

Unit 4 Quantitative Determination of Vitamins

Unit 5 Paper and Thin Layer Chromatography

UNIT 1 QUANTITATIVE ESTIMATION OF CARBOHYDRATE

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

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

2.0 OBJECTIVES

At the end of this unit you would have learnt about:

- the quantitative estimation of glucose and lactose

3.0 MAIN CONTENT

1. **Estimation of Glucose by Benedict's Method:**

Principle: 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 white precipitate is formed, it is very easy to determine the end point as the blue tint of Benedict's reagent disappears completely at the end point.

Materials Needed

Copper sulphate, sodium carbonate, anhydrous sodium citrate, potassium thiocyanate, potassium ferrocyanide, glass bead, distilled water, glucose source, conical flask, and burette

Preparation of Benedict's reagent for the quantitative analysis

- To prepare quantitative Benedict's reagent 18 g of crystalline copper sulphate is dissolved in 100 ml of water (solution A). Further, 100 g of sodium carbonate, 200 g of anhydrous sodium citrate and 125 g 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 Benedict's reagent prepared as described is stable for long periods of time.

- 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.

Procedure

Pipette out in a conical flask 25 ml of the Benedict's quantitative reagent. Add about 5 to 10 mg. of Na_2CO_3 and a few porcelain chips (glass bead) to the flask to prevent bumping (splashing of the reactants in the conical flask). Heat the contents of conical flask to boiling and then run in the glucose solution (e.g. urine of a diabetic patient or other sample containing glucose) 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 the volume of the glucose solution used and calculate the percentage of glucose in solution as described below:

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.

Note: Sometimes the solution in the flask becomes too much concentrated due to evaporation of water. To avoid it more water may be added. The reaction of $CuSO_4$ with glucose is quite complicated and a number of molecules of $CuSO_4$ 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 therefore be based on this.

2. **Estimation of Lactose by Benedict's Quantitative Test:**

The Principle and procedure is the same as that of glucose stated in the step above, the only difference being 25 ml of Benedict's quantitative regent is equivalent to 67 mg of lactose when interpreting the result.
Note: Sucrose after acid hydrolysis can be estimated by this method.

4.0 CONCLUSION

The amount or concentration of carbohydrates varies in sample and quantitative analysis is adopted for estimating the amount of carbohydrate sugar in a sample.

5.0 SUMMARY

In this unit you have learnt how to:

- estimate glucose and lactose which is the principal form of fuel in the living system using Benedict's quantitative test.

6.0 TUTOR-MARKED ASSESSMENT

i. what are the components of Benedict's reagent used to estimate glucose?

ii. What is the purpose of using a glass bead during Benedict's quantitative estimation of glucose?

7.0 REFERENCES / FURTHER READINGS

http://www.biologydiscussion.com/carbohydrates/test/qualitative-and-quantitative-tests-for-carbohydrates/13042

Working Sheet

Observation:

Conclusion:

UNIT 2 QUANTITATIVE ESTIMATION OF PROTEIN

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

The concentration of proteins in various biological fluids is an important indicator often used for the diagnosis of a number of diseases, particularly those due to pronounced metabolism disorders. In clinical diagnostics, this indicator is usually designated “total protein. This term “total protein” unites a large amount of proteins present in biological fluids, differing in their structure, functions, physical and chemical properties.

2.0 OBJECTIVES

In this unit, you will learn about:

- some methods that can be adopted to determine the concentration of proteins in solution and biological fluids

3.0 MAIN CONTENT

1. Spectrophotometric estimation of the protein content of dilute solutions

Protein concentrations of dilute solutions in the region of 10 to 200 µg/ml can be determined by measuring the difference in absorbance at 215 and 225 nm according to the procedure of Murphy and Kies (1960). All proteins absorb strongly below 230 nm due to the presence of peptide bonds. A standard curve can be prepared by plotting a graph of the difference in absorbance (ΔA) against various concentrations of a standard protein (e.g. bovine serum albumin).

Materials Needed

Spectrophotometer, bovine serum albumin (BSA), protein source, 5 mM NaOH

Procedure

Prepare a standard curve by dissolving various amounts of a standard protein (e.g. bovine serum albumin) in 5 mM NaOH as shown in the table below. Measure the absorbance of the solutions at 215 nm and 225 nm. Estimate the concentrations of the two protein samples A and B provided from your standard graph.

Tube No	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Bovine Serum Albumin (1 mg/ml) (µl)	0	10	20	50	80	100	150	200
5 mM NaOH (µl)	1000	990	980	950	920	900	850	800
Absorbance at 215nm								
Absorbance at 225nm								
Δ A								

Δ A = change in absorbance ($A_{215nm} - A_{225nm}$)

As a first approximation, the protein concentration can be calculated from the following formula:

$$\text{Concentration (µg/ml)} = 144\ (A_{215nm} - A_{225nm})$$

2. Biuret Method of Protein Determination

The Biuret Test is often used to determine the presence of peptide bonds in protein. The method can be used for the qualitative test of protein in solution and also employed to quantitatively measure the concentration of total protein in biological samples such as in serum and tissue homogenates using the spectrophotometer.

Principle

When compounds containing peptide bonds are treated with alkaline copper sulphate, a purple coloured complex is obtained. The intensity of the colour is proportional to the number of peptide bonds in solution.

Materials Needed

Biuret reagent, 3% Sodium deoxycholate, distilled water, bovine serum albumin (BSA), protein sources (e.g. serum or tissue homogenates) and spectrophotometer

Procedure

You are provided with two protein samples A and B, e.g. serum (or plasma) and rat liver homogenate or any other protein sample. Place the following into various test tubes as stated below:

	Test (ml)	**Blank (ml)**
Test Sample	0.1	-
3% Sodium deoxycholate	0.4	0.4
Distilled water	0.5	0.6
Biuret reagent	4.0	4.0
Total Volume	5.0	5.0

Mix the contents of the test tubes by gentle shaking and allow to stand for 20 minutes for colour development. Then read the absorbance of each test solution against the blank at 540 nm. You are also provided with a standard solution of albumin (bovine serum albumin) with a concentration of 10 mg/ml. prepare a standard curve using various concentrations of the albumin. This can be done by replacing the test tube sample in the table above with various amounts, i.e. 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml, of the albumin. Adjust the amount of distilled water accordingly in order to keep the total volume constant at 5 ml. Read the absorbance of the solution in each tube after 20 minutes and plot a graph of absorbance against concentration of albumin. Estimate the concentration of the protein samples provided from your graph.

3. **Determination of Protein Content by Lowry's Method**

The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution, biological cell fractions, chromatography fractions, enzyme preparations, and so on. The amount of protein found in most biological membranes is usually very low and is best estimated using the procedure of Lowey *et al.* (1951). The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. It is named after the biochemist Oliver H. Lowry who developed the reagent in the 1940s.

Principle

The Lowry method is based on the reaction of divalent copper Cu^{2+} (cupric ion) with peptide bonds of protein (especially aromatic residues mainly tryptophan, also tyrosine and cysteine) in an alkaline copper medium which produces monovalent copper ion (Cuprous ion) Cu+. The Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction) to produce an unstable intense blue molecule known as heteropolymolybdenum Blue (molybdenum/tungsten blue).

Reagents needed and their preparation

i. *Reagent A* (2% Na_2CO_3 in 0.1M NaOH)

Dissolve 20 g of anhydrous sodium carbonate (Na_2CO_3) and 4g of sodium hydroxide pellets in a little quantity of distilled water in a 1 litre standard volumetric flask and make up to the mark with distilled water.

ii. *Reagent B* (2% Sodium Potassium Tartarate)

Dissolve 20 g of sodium potassium tartarate ($NaKC_4O6.4H_2O$), in distilled water in a litre standard volumetric flask and it up to the mark.

iii. *Reagent C* (1 % Copper Sulphate)

Dissolve 10 g of copper sulphate ($CUSO_4.5H_2O$) in distilled water in a 1 litliter standard volumetric flask and make it up to mark.

iv. *Reagent D* (Alkaline Copper Solution)

This should be prepared fresh, just before use by mixing 50 ml of reagent A with 0.5 ml of reagent B and 0.5 ml reagent C. Add the tartarate (reagent B) first to prevent the solution from growing cloudy.

v. *Reagent E* (Folin-Ciocalteu Reagent)

Reagent E is commercially available in 2M solutions and contains phosphomolybdictungstic complex, lithium sulphate and bromine water. It should be diluted to 1M concentration on the day of the assay. If the commercial preparation cannot be procured, the instructions for preparing the reagent from its active ingredients can be found in the Appendix.

Procedure

Place the following reagents into various test tubes

	Test	**Blank**
Test Sample	10 µl	-
Distilled Water	590 µl 590 µl	6000 µl
Reagent D	3.0 ml	3.0 ml

Mix well and wait for 10 minutes

Reagent E	0.3 ml	0.3 ml

Mix rapidly and read absorbance at 750 nm after 30 minutes.
The rapid mixing after the addition of reagent E is very important since reagent E is unstable at alkaline pH and can be destroyed before it reacts with the protein copper complex.

You will also be provided with a standard solution of albumin (bovine serum albumin) with concentration of 10 mg/ml (i.e. 1 mg of BSA in 9 ml of distilled). Prepare a standard curve using various concentrations of the albumin. This can be done by placing the test sample with various amounts of the albumin as shown below

Tube No	1	2	3	4	5	6	7
Bovine Serum Albumin (1 mg/ml) (µl)	0	25	50	75	100	150	200
Distilled water	1000	975	950	925	900	850	800
Reagent D (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0

Mix and wait for 10 minutes

Reagent E	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml

Mix rapidly and take absorbance at 750 nm for 30 minutes.

4.0 CONCLUSION

The quantitative analysis is used to test the total amount or concentration of protein in cell fractions, chromatographic fractions, solutions etc. Various quantitative tests such as Spectrophotometric technique, Biuret method Lowry method may be adopted for quantifying proteins however each method has its own specificity and advantage.

5.0 SUMMARY

In this unit you have learnt about:

- spectrophotometric estimation of protein content in dilute solutions
- biuret method of protein determination
- Lowry method of protein determination

6.0 TUTOR-MARKED ASSIGNMENT

i. What absorbance is taken in Lowry's method of protein determination?
ii. What is the principle behind Lowry method of protein determination?

8.0 REFERENCES / FURTHER READINGS

Folin, O. and Ciocalteu, V. (1927). On tyrosine and tryptophan determination in proteins. J. Biol. Chem. 73, 627-650.

Lowry, O.H; Rosebrough, N.J; Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Murphy, J.B. and Kies, M.W. (1960). Note on spectrophotometric determination of in dilute solutions. Biochem. Biophys. Acta 45, 382-384.

Working Sheet

Observation:

Conclusion:

UNIT 3 QUANTITATIVE ESTIMATION OF LIPIDS

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

The quantitative test for lipids involves analyses to estimate the amount of lipids in various forms present in food samples or biological system. Some quantitative analysis such as iodine value, Saponification value, acid value and total cholesterol can be estimated in lipid samples and are explained in detail in this unit.

2.0 OBJECTIVES

In this unit you will learn about the following:

- Iodine value of lipids
- Saponification value of lipids
- Acid value of lipids
- Estimation of Total cholesterol

3.0 MAIN CONTENT

1. **Iodine Value:** The iodine number is defined as the number of grams of iodine which can be absorbed by 100 grams of the lipid. Not only iodine but also equivalent amounts of other halogens will add at double bonds in fatty acids; so bromine is often used instead of iodine because it is more reactive with double bonds of fatty acids. Therefore, iodine value is a measure of the degree of unsaturation.

   The iodine equivalent of the bromine used in the reaction is then obtained by adding potassium iodide to the mixture and titrating with a standard solution of potassium.

Materials Needed

Lipid source (olive oil, groundnut oil, coconut oil, margarine and glycerol), 0.5% chloroform, Dam's iodine, 10% potassium iodide, distilled water, 1% starch, 0.1M thiosulphate, conical flask, burette,

Procedure

Prepare a blank to determine the iodine equivalent of the blank by placing 5 ml of 0.05% chloroform solution into a conical flask. Add 5 ml of Dam's iodine from a burette. Cork and shake thoroughly, then place the flask in a dark cupboard for 10 minutes. Add 5 ml of 10% potassium iodide solution followed by 20 ml of distilled water. Mix thoroughly and titrate the liberated iodine with the standard thiosulphate provided. Towards the end of the titration, i.e. when the solution has a pale straw colour, add 1 ml of a 1% starch solution and continue the titration until the blue colour disappears. The conical flask must be shaken thoroughly throughout the titration to ensure that all the iodine is removed from the chloroform layer.

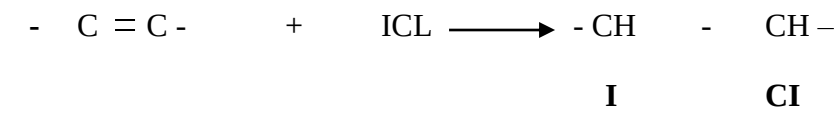
Repeat the entire procedure using in place of chloroform, 5 ml of a 0.5% chloroform solution of (a) olive oil (b) groundnut oil (c) coconut oil (d) margarine and (e) glycerol and calculate the iodine values of the various lipids.

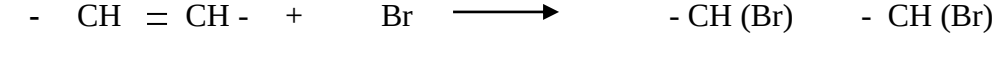
The difference between the blank and test readings (BI – T) gives the number of ml of 0.1M thiosulphate needed to react with the equivalent volume of iodine. The amount of lipid taken is 0.025 g and 1 litre of 0.1 M iodine contains 12.7 g of iodine. Therefore, the iodine number can be calculated as follows:

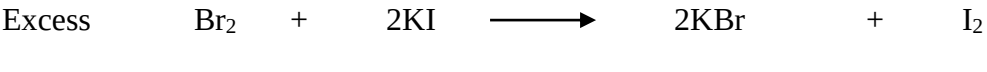
Iodine number = (BI – T) x 12.7 / 1000 x 100 / 0.025

= (BI – T) x 50.8

Calculations







$2S_2O_3^{2-} \longrightarrow S_4O_6^{2-} + 2e^-$

$I_2 + 2e^- \longrightarrow S_4O_6^{2-} + 2I^-$

Let (BI – T) = y ml

2 moles of thiosulphate ($S_2O_3^{2-}$) is equivalent to 1 mole of iodine (I_2)

1 litre of 0.1M $S_2O_3^{2-}$ contains 0.1 moles.

y ml of 0.1M $S_2O_3^{2-}$ will contain 0.1y / 1000 moles.

2 moles of $S_2O_3^{2-} \longrightarrow$ 1 mole of I_2

1 mole of $S_2O_3^{2-} \longrightarrow$ 1/2 mole of I_2

Therefore 0.1y /1000 moles of $S_2O_3^{2-} \longrightarrow$ 1/2 x 0.1y / 1000 moles of I_2

= 1/2 x 0.1y / 1000 x 254g of I_2

1 mole of lipid used = 5 ml of 0.5% solution

i.e. 100 ml of lipid contains 0.5 g and

5 ml of lipid contains 0.5 / 100 x 5 g = 0.025 g

0.025g of lipid absorbs ½ x 0.1y / 1000 x 254 g of I_2

Therefore, 100g of lipid will absorb ½ x 0.1y /1000 x 254 x 1000 / 0.025

= (1/2 x 10 x 254y) / 25

= 50.8 y

Alternatively

Suppose the halogenating reagent used in this method is pyridine sulphate di-bromide. This reagent can be prepared by adding carefully 8.1 ml pyridine in 20 ml glacial acetic acid and making the volume up to 1 litre with glacial acetic acid.

Weigh the bottle containing sample of oil plus a medicine dropper and then transfer about 0.1 to 0.3 gm. of oil to a flask. Reweigh the bottle containing oil and dropper to find out the exact quantity of the sample transferred. Add 10 ml of chloroform and then 25 ml of the pyridine sulphate di-bromide reagent.

Shake thoroughly; allow standing for 5 minutes and then determine the residual bromine. To do this, add 10 ml of 10% KI and titrate the equivalent amount of iodine liberated by the residual bromine with the help of 0.1N $Na_2S_2O_3$ (sodium thiosulphate). The titration can be done by adding sodium thiosulphate solution through a burette to the flask. When the colour of the solution in flask becomes light yellow add 1 ml of starch solution. It will become blue. Slowly add the thiosulphate solution again till it becomes colourless. Note the total volume of thiosulphate used.

The total amount of bromine originally added is found by titrating 25 ml of the pyridine sulphate di-bromide reagent with thiosulphate after adding KI as in the previous case. The amount of bromine taken up by the fat sample can be determined by the difference between the two titers and then the iodine number can be calculated as presented below

Suppose with a sample of 0.2 gm. oil the data obtained are as follows:

0.1 (N) $Na_2S_2O_3$ used for titration of blank = 47.0 ml

0.1 (N) $Na_2S_2O_3$ used for titration of sample = 27.0 ml

0.1 (N) $Na_2S_2O_3$ equivalent to iodine absorbed by the sample = 20.0 ml

As 1 ml 0.1 (N) $Na_2S_2O_3$ = 1.0 ml of 0.1 (N) Bromine = 1 ml of 0.1 (N) Iodine

Hence, 20 ml of 0.1 (N) $Na_2S_2O_3$ = 20 ml of 0.1 (N) Iodine = 20×12.7/1000 gm Iodine =

0.254 gm Iodine

Thus 0.2 gm of oil can take up 0.254 gm of iodine.

Therefore, iodine number of oil used = 127.

2. **Acid Value of Lipids:** Acid value is the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in 1 gram of the lipid.

   Freshly prepared animal and vegetable oils contain very little free fatty acids (FFA). However, during storage the oils may become rancid due to peroxide formation at the double bonds as a result of oxidation by atmospheric oxygen and hydrolysis by microorganisms. This results in the liberation of free fatty acids. With low grade

vegetable oils, as much as 30% of the oil may be degraded in a very short time. The amount of FFA present is, therefore, an indication of the age and quality of the oil.

Materials Needed

Lipid sample, 50 ml chloroform, 0.1M KOH (potassium hydroxide), burette, conical flask, phenolphthalein indicator.

Procedure

Weigh out exactly 10 g of the lipid sample and add 50 ml of chloroform. Add 1 ml of phenolphthalein solution, mix thoroughly and titrate with 0.1M KOH from a burette until the faint pink colour persists for 20 to 30 seconds. Note the volume of alkali used and calculate the acid value of the lipid provided using the expression:

Acid value = (56.1 PV/ m) milliliters of potassium hydroxide; *P* = exact molarity of potassium hydroxide; and *m* = mass (g) of the test portion.

3. **Saponification Value:** Saponification value is the number of milligrams of potassium hydroxide (KOH) required to neutralize the fatty acids resulting from the complete hydrolysis of one gram (1 g) of the lipid. The Saponification value gives an indication of the nature of the fatty acids in the lipid since the longer the carbon chain the less acid is liberated per gram of the lipid hydrolyzed.

Principle

When glyceryl esters are refluxed with alkali they give glycerol and the potassium salts of the fatty acids (soaps).

Materials Needed

Lipid source, potassium hydroxide, reflux condenser, 0.5M HCl, phenolphthalein indicator, ethanol, ether, conical flask.

Procedure

Weigh 1 g of the lipid in a beaker and dissolve it in 3 ml of a mixture of ethanol and ether (1:1 v/v). Quantitatively transfer the contents of the beaker to a 250 ml conical flask by rinsing the beaker three times with more of the solvent then add 25 ml of 0.5M alcoholic KOH and attach to a reflux condenser. Set up another reflux condenser as blank with everything present except the lipid and heat both flasks on a boiling water bath for 30 minutes. Leave to cool to room temperature and titrate with 0.5M HCl using phenolphthalein as indicator. The difference between the blank and test reading gives the number of mls of 0.5M KOH required to saponify 1 g of the lipid.

4. **Estimation of Cholesterol:** The estimation of cholesterol is divided into two parts the first part examines cholesterol estimation in organic food samples like margarine, olive oil, coconut oil etc while the second part gives an account on the determination of cholesterol in plasma.

To estimate cholesterol content from an organic food source e.g. olive oil, groundnut oil, coconut oil, margarine etc. the procedure below can be adopted. In the case where the lipid source, is fat e.g. margarine it is advisable to dissolve small amount of the fat with small amount of non-polar organic solvent in a test tube.

Materials Needed

Glacial acetic acid, test tubes, cholesterol source, acetic anhydride sulphuric acid reagent, spectrophotometer

Procedure

All volumes for test tubes 1 to 5 and B are in ml						
	B	1	2	3	4	5
Glacial acetic acid	2.0	1.7	1.4	1.1	0.8	0.2
Cholesterol solution (1 mg/ml)	–	0.3	0.6	0.9	1.2	1.8
Acetic anhydride sulphuric acid reagent	4.0	4.0	4.0	4.0	4.0	4.0

Following the mixture in the table above, shake the tubes well and keep them at room temperature for 30 minutes. Blue colour will develop in all the tubes except blank tube. Measure the absorbance at 625nm against the blank tube and plot these against the amount of cholesterol.

Note: Acetic anhydride-sulphuric acid reagent.

This reagent has to be freshly prepared before use. Acetic anhydride (20 ml) is taken in a glass stoppered flask which is then chilled in ice water. When cold, add 1 ml of conc. H_2SO_4 to it drop by drop. The contents are mixed and cooled during the addition. After completion of the addition the flask is stoppered and shaken vigorously for a few minutes. The solution has to be kept cold in ice and should be used within an hour.

(b). Determination of Plasma Cholesterol: Cholesterol is widely distributed in animal tissues. Its concentration in the blood cells remains constant, even in disease. However, the plasma concentration of cholesterol is sensitive to changes in diet, state of health etc.

Principle

The determination of plasma cholesterol is based on the Lieberman-Buchard reaction. Plasma proteins are first precipitated by a mixture of ether and ethanol which also removes cholesterol and other lipids at the same time. The organic solvent is removed by evaporation and the dry residue is dissolved in chloroform.

Materials Needed

Centrifuge machine, pipette, graduated glass cylinder, chloroform and beaker.

Procedure

Pipette 0.3 ml of plasma into a centrifuge tube containing 2 ml of ether and 8 ml of absolute ethanol. Cork the test tube with a dry stopper and shake vigorously for two minutes. Then let the mixture stand on the bench for 30 minutes. Centrifuge the test tube with its content in a bench centrifuge and carefully decant the clear supernatant into a hot (50 ml) beaker and allow the solvent to evaporate. Take up the residue in three separate washings using 1 ml of chloroform each time. Combine these in a stopper, graduated glass cylinder and make it up to 5 ml with chloroform.

In another set of graduated glass cylinder, set up a series of cholesterol standards and a blank containing 5 ml of chloroform. Add 2 ml of acetic anhydride to each tube, followed by 3 drops of conc. sulphuric acid. Mix the contents by inverting the cylinders several times, then stand them in a dark cupboard for 10 minutes. Compare the test and standard solution by reading the absorbance in a spectrophotometer at 680 nm.

Note: It is essential to use dry glassware in carrying out all the tests.

4.0 CONCLUSION

Some quantitative test used for lipid analysis includes iodine value, saponification value, acid value and total cholesterol. This analysis could help to determine the degree of saturation and rancidity of lipids

5.0 SUMMARY

In this unit you have learnt about:

- iodine value, Saponification value and Acid value of lipids and their estimation.

- how to estimate cholesterol in plasma protein and organic food source.

6.0 TUTOR-MARKED ASSIGNMENT

(i). Define is Iodine value and Saponification value of lipid?
(ii). Mention the reagents that you will need to carry out analysis on iodine value of lipid?

7.0 REFERENCE / FURTHER READINGS

http://www.biologydiscussion.com/lipids/tests/qualitative-and-quantitative-tests-for-lipids/13050

Working Sheet

Observation:

Conclusion:

UNIT 4 QUANTITATIVE ESTIMATION OF VITAMINS

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

Vitamins are essential constituents of the diet which are required for the maintenance of the body's normal metabolism. Although they are present in minute amounts in foodstuffs, deficiency and any of the vitamins leads to corresponding disease. There are two classes of vitamins: the fat-soluble vitamins (A, D, E, and K) and water-soluble vitamins (B and C). There are methods for determining the amounts of vitamins occurring in diet. Firstly, the bioassay methods which involve growth effect on bacterial cultures and, secondly, colorimetric methods which require the use of a variety of chromogenic reagents.

2.0 OBJECTIVES

At the end of this unit you would have learnt about:

- how to test for vitamin A
- the quantitative estimation of ascorbic acid (vitamin C)

3.0 MAIN CONTENT

1. **Test for Vitamin A**

Materials Needed

Test tube, antimony trichloride, vitamin source (cod liver oil), and pipette

Procedure

Place 2 ml of saturated solution of antimony trichloride in chloroform in a test tube. With a clean, dry, pipette add 0.2 ml of a 20% solution of cod liver oil in chloroform. Mix and observe the development of a blue colour.

Note: Antimony trichloride is poisonous, and care should be taken in handling it.

3. **Quantitative Estimation of Ascorbic acid (Vitamin C) in Tablet**

Principle

Vitamin C (ascorbic acid) exhibits strong reducing properties in solution. It decolorizes potassium iodate by rapid redox reaction to form a blue-black complex.

Materials Needed

0.025 M KIO_3 (Ptassium iodate), 1 M HCl, 1 M NaI, Magnetic stirrer, beaker, 100 ml volumetric flask, conical flask, pipette, burette, fruit juice, high and low dose vitamin C tablets.

- For Low-dose Tablet (< 250 mg)

Procedure

Weigh 1 tablet of vitamin C, crush the tablet and dissolve with 50 ml of distilled water in a conical flask. Add 2 ml of 1 M NaI, 2 ml of 1 M HCl, 1 ml of 2% starch and stir till it dissolve. Titrate the solution with 0.025 M KIO_3 in a burette until blue-black complex is formed, which marks the end point. Record the volume and repeat the produce for a duplicate titration and then calculate the ascorbic acid content in mg/tablet.

Calculation

$$\frac{[IO_3^-]}{1} = \frac{[I_2]}{3} = \frac{[C_6H_8O_6]}{3}\ (\textbf{ascorbic acid})$$

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_20 \ldots\ldots\ldots\ldots(1)$$

$$C_6H_8O_6 + I_2 \longrightarrow 2I^- + 2H^+$$

Therefore ascorbic acid content = ΔVml x 3 x 0.025 M x 3 x 176 (mg/tablet)

Where 176 = molecular mass of ascorbic acid

- For High Dose Tablet (> 250 mg)

Procedure

Weigh 1 tablet of vitamin C, crush the tablet and dissolve with 50 ml of distilled water in a beaker. Stir the mixture in a magnetic stirrer and then transfer the content to 100 ml volumetric flask and fill up to the mark level with distilled water. Pipette 25 ml of the ascorbic acid solution into a conical flask and then add 2 ml of 1 M NaI, 2 ml of 1 M HCl, 1 ml of 2% starch then titrate with 0.025 M KIO_3 in a burette until a blue-black complex is formed, which marks the end point and record the volume. Take another 25 ml portion of Vitamin C and carryout a duplicate titration by repeating the procedure and then calculate the ascorbic acid content in mg/tablet for the high dose tablet using the expression.

$$\textbf{Ascorbic acid content} = \frac{\Delta V\text{ml} \times 3 \times 0.025\text{ M} \times 3 \times 176 \times 100\text{ ml}}{25\text{ ml}}\ (\text{mg/tablet})$$

3. **Quantitative Estimation of Ascorbic acid (Vitamin C) Content in Fruit Juice**

Procedure

Pipette 50 ml of fruit juice containing vitamin C and dissolve with 50 ml of distilled water in a conical flask. Stir the mixture thoroughly, then add 2 ml of 1 M NaI, 2 ml of 1 M HCl, 1 ml of 2% starch and titrate with 0.025 M KIO_3 in a burette until a blue-black complex is formed, which marks the end point and record the volume. Take another 50 ml portion of the fruit juice and carryout a duplicate titration by repeating the procedure and then calculate the ascorbic acid content in fruit juice in mg/100 ml using the expression.

$$\textbf{Ascorbic acid content (mg/100 ml)} = \frac{\Delta V\text{ml} \times 3 \times 0.025\text{ M} \times 3 \times 176 \times 100\text{ ml}}{50\text{ ml}}$$

4.0 CONCLUSION

Vitamins are organic constituents of food required for proper functioning of the body. Vitamins in food samples can be determined both qualitatively and quantitatively.

5.0 SUMMARY

In this unit, you learnt about:

- the different classes of vitamins
- how to estimate vitamin

6.0 TUTOR-MARKED QUESTIONS

(i). what vitamins are classified as fat soluble vitamins?

(ii). what colour appearance is used to mark the presence of vitamin C in ascorbic acid when titrating with KIO_3?

7.0 REFERENCE / FURTHER READINGS

https://www.onlinebiologynotes.com

Working Sheet

Observation:

Conclusion:

UNIT 5 PAPER AND THIN LAYER CHROMATOGRAPHY

CONTENTS

1.0 Introduction
2.0 Objectives
3.0 Main Content
4.0 Conclusion
5.0 Summary
6.0 Tutor-Marked Assignment
7.0 References/Further Reading

1.0 INTRODUCTION

Chromatography is a separation and analytical technique widely used in chemistry and the biological sciences for separating substance. Most things that occur in nature are a mixture of substances which can only be separated or analyzed using any of the techniques known.

2.0 OBJECTIVES

At the end of this unit, you should be able to:

- explain the principle on which chromatography as a separation technique is based
- explain paper and thin layer chromatography
- carryout the procedures for paper and thin layer chromatography.

3.0 MAIN CONTENT

Chromatography works on the principle of redistribution of the substances between two or more phases. One of these phases is held stationary by adsorption on a suitable support, while the other liquid phase moves continuously over it. The various phases which could be used in chromatographic procedures include:
(i). two immiscible liquids such as in partition chromatography
(ii). a liquid and the surface of a solid adsorbent such as chalk or aluminum oxide as in adsorption chromatography.
(iii). A gas and an adsorbent such as oil or grease in gas chromatography.

3.1 Types of Chromatography

Two of the most widely used chromatographic techniques are paper and thin layer chromatography. The separation of mixtures of amino acids, sugars and many other

biological substances by the process of paper chromatography has been found to be very useful. When a strip of filter paper is suspended vertically with a lower end dipping into a mixture of water and an organic solvent (e.g. n-butanol, n-propanol, butanoic acid) the mixture moves up the filter paper. The water in the mixture is strongly held by the filter paper and represents the mobile phase.

In thin layer chromatography, an adsorbent in the form of a slurry is spread in thin layer on glass plate.A wide range of adsorbents are used for thin layer chromatography. These include silica gel, alumina, diatomaceous earth, cellulose powder, polyacrylamide, magnesium phosphate and calcium sulphate.

3.2 Experimental Procedure

In this experiment, you will separate some amino acids and sugars using paper and thin layer chromatography.

1. **Separation of Amino acids by Paper Chromatography**

Materials Needed

13 x 18.5 cm Whatman No. 1 chromatography paper, glycine, alanine, glutamate, tyrosine and phenylalanine, butanol, glacial acetic acid, distilled water, 0.2% ninhydrin reagent, acetone

Procedure

Obtain a 13 x 18.5 cm Whatman No. 1 chromatography paper. When you handle this paper, ensure you hold it only on one of the long (18.5 cm) sides, which will be considered the “top” of the chromatographic paper. The amino acids from your fingers will contaminate the paper and lead to erroneous results if it is touched on the “bottom”. Lay the chromatography paper on a piece of notebook paper, and draw a line in pencil, not pen, 1.5 cm above the bottom. Make small marks along the line using the dimensions given by your supervisor. Write labels at the top. Spot 10 µl of 1% solution of each of amino acids (e.g. glycine, alanine, glutamate, tyrosine and phenylalanine) and the “unknown” sample that contains one or more of these four amino acids to the filter paper all at intervals of 4 cm along the line you have drawn. Allow the spot to dry, and then reapply the solution at the exact same place, again touching the paper quickly and lightly. Allow the spot to dry, and repeat one more time. After all six solutions have been applied to the paper in this manner; allow the spots to dry for five minutes in the sun. Roll the paper into a cylinder with the spots on the outside, and then staple it so that the edges do not overlap or touch. Pour 50 ml of the amino acid developing solution into a 1000 ml beaker. The developing solvent is comprised of a four-to-one mixture of 1-butanol and glacial acetic acid that has been saturated with water i.e. (butanol/glacial

acetic acid/water solvent in the ratio of 12:3:5 v/v). Run the chromatogram in this developing solution i.e. position the cylinder inside the beaker with the bottom edge immersed in the solvent. Make sure the paper does not touch the glass. Place a piece of aluminum foil over the mouth of the beaker. Allow the chromatogram to develop undisturbed for 60 to 75 minutes. Do not move the beaker while the chromatogram is developing. When you remove the paper from the beaker, mark the solvent front with a pencil. Set the cylinder on notebook paper, and allow it to dry. When the chromatogram is completely dry, remove the staples, and hang it from the clips in the fume hood and visualize the spots by spraying the paper with freshly prepared 0.2% ninhydrin reagent in butanol or acetone and do not allow the paper to become dripping wet. Place the chromatogram in an oven set at 80°C for about 5 minutes. Circle the spots with a pencil. Measure the distance from the origin to the center of each spot and the distance from the origin to the solvent front. Identify the amino acids in the unknown sample by comparing their rate of movement with those of the standards i.e. Rf values.

2. **Separation of Sugars by Paper chromatography**

Materials Needed

Sugar source: glucose, fructose, xylose, arabinose and sucrose, silica gel, isopropanol, aniline-diphenylamine

A mixture of sugars (e.g. glucose, fructose, xylose, arabinose and sucrose) can also be separated by paper chromatography. The same procedure of paper chromatography separation of amino acids explained above (1) is applied except that developing solvent comprised of isopropanol/water solvent (4:1 v/v) and the spot is visualise by spraying the filter paper with freshly prepared aniline-diphenylamine in the fume cupboard.

3. **Analyzing a Non-prescription Medicine Tablet with TLC**

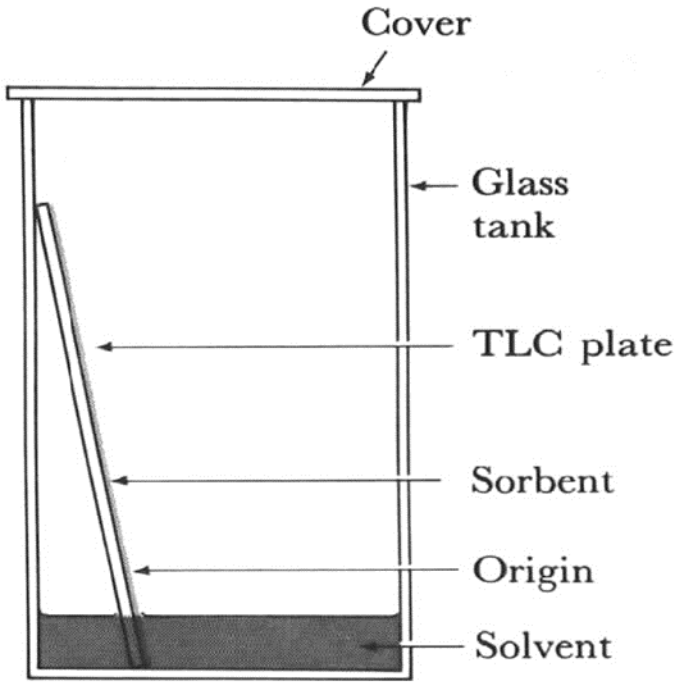
Materials Needed

TLC plate, with the dimensions of 6 cm x 6.7 cm, acetylsalicylic acid, acetaminophen and caffeine

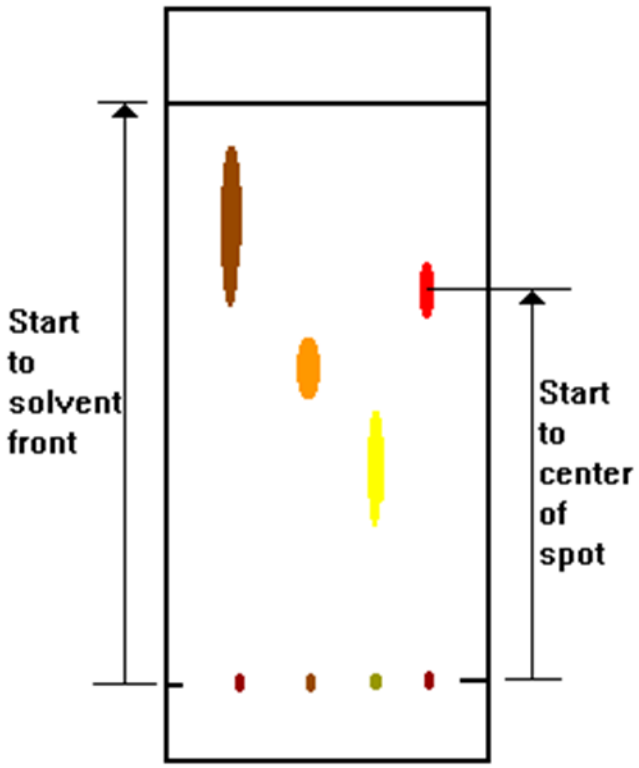
Procedure

Obtain a silica gel TLC plate with the dimensions of 6 cm x 6.7 cm. Draw a line in pencil 1 cm from the bottom along the short (6 cm) side of the plate. Be careful not to disturb the silica gel as you draw the line! Use the small capillary tube provided to spot 3 spots, one of each active ingredient solution (acetylsalicylic acid, acetaminophen and caffeine), along the line. Leave room for a fourth spot. Your supervisor will assign a non-prescription medicine tablet for you to analyse. Spot your TLC plate with the supernatant from a mixture of this crushed tablet and methanol. When applying these solutions to the plate, touch the capillary

to the surface of the silica gel quickly and lightly so the spot is very small. In each case, reapply the spot, allowing it to dry in between applications, two more times. Place a small amount of the ethyl acetate developing solvent in a 400 ml beaker. The liquid should cover the bottom of the beaker to a depth of about 0.5 cm. Line the beaker with a piece of filter paper to saturate the atmosphere within. Fit a piece of aluminum foil over the mouth of the beaker. Place the plate that you have spotted in the beaker, cover it with the foil, and allow the solvent front to move up the plate until it is approximately 1 cm from the top. Do not disturb the beaker while the chromatogram is developing. In this case, the solvent will travel up the silica gel plate very quickly and will reach the top in two to three minutes. Remove the plate and mark the solvent front with a pencil. Allow the plate to dry for a few minutes, then observe it under short-wave ultra-violet light. With a pencil, circle any spots that are illuminated. Write your initials in a corner of the plate, and place it in an iodine chamber. Position the plate so that the silica gel surface is completely exposed to the iodine vapors and is not covered by other plates in the chamber. Leave it there for 5-10 minutes. After removing the plate from the chamber, record in your notebook whether or not any coloured spots appeared as a result of exposure to the iodine vapors. If new spots appear, circle them with a pencil. Sketch a diagram of the chromatogram in your notebook. Measure the distance from the origin to the center of each spot and the distance from the origin to the solvent front.



Method used for developing TLC plates



TLC plate spotted with four coloured food dyes

4.0 CONCLUSION

Various forms of chromatography are well known analytical and separation techniques used in the laboratory by chemists, biologists and other natural scientists to determine the quality and quantity of particular substances in different mixtures. TLC is a simple, quick, and inexpensive procedure that gives a quick answer as to how many components are in a mixture. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action.

5.0 SUMMARY

In this unit, attempts have been made to:

- explain chromatography
- describe the basic technique method of paper and thin layer chromatography
- describe an experimental procedure of paper chromatography.

6.0 TUTOR-MARKED ASSIGNMENT

(i). What is the main advantage of thin layer chromatography overpaper chromatography?

(ii). Explain how your observations of the paper chromatogram led to the identification of the components of the unknown amino acid mixture.

7.0 REFERENCES / FURTHER READINGS

http://www.chem.wisc.edu/courses/342/Fall2004/TLC.pdf

http://www.bc.edu/schools/cas/chemistry/undergrad/org/fall/TLC.pdf

http://orgchem.colorado.edu/Technique/Procedures/TLC/TLC.html

http://www.google.com.ng/search?q=Experiment4%3AThin Layer Chromatography&ie=utf-8&oe=utf-8&aq=t&rls=org.mozilla:en-US:official&client=firefoxa&source=hp&channel=np

www.academiya.org/sites/default/files/Chromatography%20MCQs.doc

Working Sheet

Observation:

Conclusion:

APPENDIX

PREPARATION OF REAGENTS AND SOLUTIONS

Barfoed's Reagent

Dissolve 66 g of copper acetate and 10 ml of glacial acetic acid in about 800 ml of boiling distilled water. Make the solution up to 1 litre. Allow to stand overnight in a cylinder and decant off the supernatant from the precipitate which forms.

Benedict's reagent (Qualitative)

Heat 100 g of anhydrous sodium carbonate and 175 g of sodium citrate in about 600 ml of distilled water until they dissolve. Filter (if necessary) into a measuring cylinder. Dissolve 17.5 g of copper sulphate in 100 ml of distilled water and add it slowly, with constant stirring, to the carbonate-citrate solution. Make the solution up to 1 litre with distilled water.

Benedict's Reagent (Quantitative)

Dissolve 100 g of anhydrous sodium carbonate in 200 ml of cold distilled water. Dissolve 100 g of sodium citrate and 125 g of potassium thiocyanate in 600 ml of oiling distilled water. Mix the two solutions. Dissolve 18 g of copper sulphate in 100 ml of distilled water and add it slowly, with constant stirring, to the above solution. Then add 5 ml of 5% potassium ferrocyanide and make the solution up to 1 litre.

Biuret Reagent

Dissolve 1.5 g of copper sulphate and 6 g of potassium sodium tartarate in 500 ml of distilled water in a litre standard volumetric flask. With constant shaking, add 1 g of potassium iodide and 300 ml of 10% sodium hydroxide. Make the solution up to 1 litre with distilled water.

Dam's Iodine

Dissolve 8.2 ml of pyridine and 6 ml of conc. H_2SO_4 in 20 ml of cold glacial acetic acid. Add this to a solution of 2.6 ml bromine in 20 ml glacial acetic acid. Dilute the mixture to litre with glacial acetic acid and keep in the dark.

Fehling's Solution A

Dissolve 69.28 g of copper sulphate in distilled water and make up to 1 litre.

Fehling's Solution B

Dissolve 350 g potassium sodium tartarate and 100 g of sodium hydroxide in distilled water and make up to 1 litre.

Folin & Coicalteu Phenol Reagent

Sodium tungstate (100 g), Sodium molybdate (25 g), 85% Phosphoric acid (50 ml), Conc. HCl (100 ml), Lithium sulphate (150 g).

Dissolve 100 g of sodium tungstate and 25 g of sodium molybdate in about 700 ml of distilled water. Add 50 ml of 85% phosphoric acid and 100 ml of conc. HCl. Reflux for 10 hours using an all glass apparatus, then add 150 g of lithium sulphate, 50 ml of distilled water and a few drops of bromine.Boil the mixture without a condenser for 15 minutes to remove excess bromine. Allow to cool, then make the solution up to 1 litre with distilled water and filter with Whatman No. 1).

Iodine Solution (0.1M)

Dissolve 12.685 g of iodine in 150 ml of 12% potassium iodide (KI) solution in a 1 litre standard volumetric flask. Shake until the iodine dissolves completely, then make the solution up to 1 litre with distilled water.

Million's Reagent

Dissolve 15 ml of mercury in 285 ml of conc. HNO_3. Dilute the resulting solution with twice its volume of distilled water. (Millions reagent is a mixture of mercuric nitrate and nitrite.

Ninhydrin Reagent

Dissolve 2 g of ninhydrin in 100 ml of absolute ethanol.

Seliwanoff's Reagent

Dissolve 0.5 g of resorcinol in 100 ml of 50% hydrochloric acid.