**About the authors**

Dr. Sattanathan, Assistant Professor in Life Science, at MASS College of Arts and Science, Kumbakonam, Thanjavur Dt. Tamilnadu, India, has teaching Biotechnology for the last 5 years. He has also worked in other institutes and he has publihsed a number of scientific papers in national and international journals.

Dr. Padmapriya has working in Dharmapuram Gnanambigai Govt. Arts College for Women, Mayiladuthurai. She has been in the Fish Immunology field for the last 10 years.

Dr. B. Balamuralikrishnan, Ph.D. is an Assistant Professor in Department of Food Science and Biotechnology, Sejong University, Seoul, South Korea. His area of research focuses multidisciplinary in biological science on Molecular Genetics, Food Microbiology, Animal Science, Nutrition Science and Food Biotechnology, especially Food Resources and Microbiological Science. His interests include synthesis of nanomaterials/bioactive compounds from natural-by products and their applications nutraceutical; isolation, and characterization of probiotic for food/feed supplementation and biological field; interested in Nutrition in Aquaculture. To, his credit, he has participated in various International/symposia/conferences in USA, Canada, Japan, Austria, Italy, Czech Republic, Thailand, South Korea and has published more than seventy five research papers in international journal of repute. He has been serving as Academic Editor in Plos One, Guest Editor in Animals [MDPI] and act as potential reviewer in many high reputed journals. He has been acted Life Member in various scientific societies such as The Korean Society of Food Science and Technology, Poultry Science Association, Korean Society of Animal Science and Technology, Animal Nutrition Society of India (ANSI). Dr. B. Balamuralikrishnan, worked as Post-doctoral researcher in Department of Animal Science, Dankook University, South Korea and Research Assistant in Department of Zoology, Bharathiar University, India.

Practical Manual of Biochemistry

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Email:skyfox@skyfox.org.in

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Dr. G. Sattanathan, Ph.D.,
Dr. S.S. Padmapriya, Ph.D.,
Dr. B. Balamuralikrishnan, Ph.D.,

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**Dr. S.S. Padmapriya, Ph.D.,**
**Dr. B. Balamuralikrishnan, Ph.D.,**

## AUTHOR INFORMATIONS

**Dr Sattanathan Govindharajan Ph.D.,**
Assistant Professor
Department of Life Science
MASS College of Arts and Science, Kumbakonam,
Taminadu, India

**Dr Swaminathan Padmapriya Ph.D.,**
Guest Lecturer
Department Of Zoology
Dharmapuram Gnanambigai Government Arts College For Women,
Mayiladuthurai, Tamil Nadu, India.

**Dr Balasubramanian Balamuralikrishnan Ph.D.,**
Assistant Professor
Department of Food Science and Biotechnology,
College of Life Science, Sejong University,
Seoul 05006, South Korea

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Website: www.skyfox.co

# Preface

Biochemistry, a fascinating subject dealing with all the body's functions and reactions. Clinical biochemistry has a tremendous effect on patients' diagnosis and treatment. Medical students should be aware of the procedures, the parameters of diagnosis and their estimations. They should acquire sound knowledge of the diagnostic reports and their consequences that contribute to disease diagnosis and prognosis.

The most rapidly growing subject is biochemistry, which is extensively applicable to molecular understanding of the disease. Estiamations of various biochemical parameters definitely provide an insight into the normal metabolism and its aberrations, which form the basis for medicine, leading to diseases. In relation to health and disease, biochemistry shpuld is encouraged that will make the topic more interesting and fascinating for the students. We hope that this practical biochemistry book will help medical students to think about the different facts found in the body's reactions.

Dear students, let us admit that, given the fact that it is non-clinical, extensive, volatile and there is little in it to arouse any amount of interest in medical graduates, biochemistry is seldom a favorite of medical graduates. We have realized that practice is where they have shown some enthusiasm for biochemistry in our biochemistry teaching to undergraduate medical students.

Our main objective is to make this book as simple and attractive as possible for undergraduate biochemistry, allied biochemistry, medical, non-medical students, which is evident from the book's title. Each practical session has been reorganized to achieve this objective in such a way that it is easy to understand. Wherever possible, the subject is presented in tabular format so that it becomes very concise, and all test results are given color so that one realizes the practice of the simple task of reading the book itself. The basic concepts and values behind each experiment are explained in a simple manner.

Our only real concern is to help you understand the subject in an easy and organized way so that not only in your examinations, but also in your future medical career, this little knowledge comes as a big help.

To make this book a better one, we will be happy to accept constructive criticism and fruitful suggestions.

**Dr. K. Saravanan**
Principal,
MASS College of Arts and Science,
Kumabkonam.

# ACKNOWLEDGMENTS

I would like to thank God for enabling me to do this work. I thank my parents, teachers for molding me to reach this level. I extend my gratitude to my colleagues for their support.

## CONTENTS

# 1. RULES AND PROCEDURES OF GENERAL SAFETY

1. Prior to attending that laboratory session, the laboratory procedures must be read.
2. Laboratory smoking, eating and drinking are absolutely prohibited at any time in the laboratory.
3. Only closed-toe shoes should be worn in the lab. Due to the constant risk of cuts and infections from broken glass found on the laboratory floors and the possibility of chemical spills, sandals or open-toed or canvas shoes are not allowed.
4. Keep your face, nose, eyes, ears and mouth away from your hands and other objects. In the laboratory, the use of cosmetics in the laboratory is prohibited.
5. Before and after use, work areas or surfaces must be disinfected.
6. While in the laboratory, laboratory coats must be worn and buttoned. Outside of the laboratory, laboratory coats should not be worn.
7. When conducting any exercise or procedure in the laboratory, protective eyewear must be worn.
8. To minimize the fire hazard or contamination of experiments, long hair should be secured behind your head.
9. Prior to leaving the laboratory, hands must be washed.
10. Coats, books and other paraphernalia, such as purses, briefcases, etc., should be placed in specified locations when entering the laboratory and never on bench tops (except for your lab manual).
11. Never mouth-pipet anything (including water). Always use appliances for pipetting.
12. Label all materials with your name, date and any other information applicable (e.g., media,organism, etc.).
13. Waste disposal in its proper containers (see Biohazard Waste Disposal below).
14. Note the hazard code on the bottle when handling chemicals and take the appropriate precautions indicated.
15. Do not pour down the sink with chemicals.
16. Return to their appropriate places all chemicals, reagents, cultures, and glassware.
17. Do not pour fluids that are biohazardous down the sink.

18. It is necessary to wash the glassware with soap and water, then rinse it with distilled water.
19. Flame transfer loops, wires, or needles for transferring biological material before and immediately after use.
20. Do not walk around the laboratory with infectious matter containing transfer loops, wires, needles, or pipettes.
21. Around Bunsen burners, be careful. It is not always possible to see flames.
22. Turn off the incinerators before the laboratory leaves.
23. Report any broken equipment, report any broken glass, in particular those containing infectious materials immediately.
24. Contact your course instructor or TA immediately if you are injured in the laboratory.
25. In the event of further treatment being required, spills, cuts and other accidents should be reported to the instructor or TA.
26. Familiarize yourself with safety equipment and emergency escape routes in the laboratory.
27. Before putting it away, always wipe and clean your microscope's lenses. To this end, use the relevant tissue paper and cleaning solution.
28. With all biological fluids, apply appropriate universal precautions.
29. Without the written permission of the course instructor or TA, do not remove any materials from the laboratory.

# 2. BUFFER SOLUTION PREPARATION AND PH MEASUREMENT

**Principal**

A buffer's main purpose is to control the solution's pH. Buffers can also play secondary roles in a system, such as controlling ionic strength or species solving, perhaps even affecting the structure or activity of protein or nucleic acid. Nucleic acids, nucleic acid-protein complexes, proteins, and biochemical reactions are stabilized by buffers (whose products might be used in subsequent biochemical reactions). Complex buffer systems in electrophoretic systems are used to control the pH and to establish the pH gradient. Weak acids and bases are made up of buffer solutions that make them comparatively resistant to pH change. Theoretically, buffers offer a ready source of both acid and base to either supply additional H+ if the process consumes H+ or if a reaction produces acid, combine it with excess H+.

**Reagents:**

a. Acetic acid 0.2 M: glacial acetic acid 1.5 ml is made up to 100 ml by using distilled water.

b. Citric acid: citric acid 2.10 gm in 100 ml distilled water.

c. Dibasic sodium phosphate: 5.3 gm of disodium hydrogen phosphate in 100 ml distilled water.

e. Monobasic sodium phosphate: 2.78 gm sodium dihydrogen phosphate in 100 ml distilled water.

f. Sodium acetate solution: 0.64 gm of sodium acetate in 100 ml distilled water.

g. Sodium bicarbonate solution: sodium bicarbonate 1.68 gm in 100 ml distilled water.

h. Sodium carbonate solution 0.2 M:2.12 gm anhydrous sodium carbonate in 100 ml distilled water.

i. Sodium citrate solution 0.1 M: sodium citrate 2.94 gm in 100 ml distilled water.

**Procedure:**

**a. Acetic acid-sodium acetate buffer**

Take a 100 ml flask and use a pipette to add 36.2 ml of sodium acetate solution, and then add 14.8 ml of glacial acetic acid to it. Using

distilled water to produce a total volume of 100 ml. The resulting acetic acid-sodium acetate buffer is 0.2 M. With the help of a pH meter, the pH is measured. With distilled water, the electrode is washed, and then dipped in the prepared buffer solution. The resultant pH is 4.6.

**b. Barbitone buffer**

In distilled water, mix 2.85 gm of diethyl barbituric acid and 14.2 gm of sodium diethyl barbititrate and then produce 1000 ml of final volume. That's the buffer of barbitone. With the help of the pH meter, the pH is measured, and the final pH comes out as 6.8.

**c. Citrate buffer**

Using distilled water, mix 46.5 ml of citric acid with 3.5 ml of sodium citrate solution and add 100 ml to the final volume. This is a citrate buffer of 0.1 M. With the help of a pH meter, the pH of this buffer is measured and the pH is 2.5.

**d. Carbonate-Bicarbonate buffer**

Put 27.5 ml of sodium carbonate solution in a flask and add 22.5 ml of sodium bicarbonate solution to it. Then make 100 ml of the total volume with the aid of distilled water. This is a buffer of 0.2 M carbonate-bicarbonate. The pH meter is standardized, and the pH of the buffer solution that is prepared is measured. The pH would be 10.2.

**e. Phosphate buffer**

Dihydrogen sodium phosphate (39 ml) is mixed with disodium hydrogen phosphate (61 ml), and 200 ml of distilled water is added to the final volume. This solution results in the phosphate buffer being 0.2 M. Using a pH meter, the pH of the phosphate buffer is measured and 6.8 is obtained.

**Preparation of solutions (Molar, Normal) and Dilution**

**a. Solution Preparation**

A solution is a homogeneous mixture produced in a solvent by dissolving one or more solutes. The smaller chemical, the solute, is soluble in the solvent (the chemical present in a larger amount). As standard (stock)

solutions, solutions with precisely known concentrations can be referred to. These solutions are purchased directly from the manufacturer or formed by dissolving the desired amount of solute into a specific volume volumetric flask. Stock solutions are often diluted into lower concentration solutions for experimental use in the laboratory.

**b. Preparing a Standard Solution from a Solid**

By two similar methods, a solution of known concentration can be prepared from solids. Although there are inherent errors with each of the methods, either for making solutions in the General Chemistry Laboratory will be sufficient with careful technique.

In the first method, the solid solvent is weighed on paper or in a small container and then transferred directly to a volumetric flask (commonly called a "vol flask"). In transferring the solid into the slim neck of the vol flask, a funnel could be helpful. In the vol flask, a small amount of solvent is then added and the contents are gently swirled until the substance is completely dissolved.

More solvent is added until the liquid meniscus reaches the calibration mark (a process called diluting to volume) on the neck of the volume flask. Until the contents are mixed and completely dissolved, the vol flask is then capped and reversed several times. The drawback of this method is that the original container, weighing paper, or funnel can adhere to some of the weighed solid. Also, when it is transferred into the slim neck of the vol flask, solid can be spilled.

In the second method, in a small beaker, the solid is weighed out first. A small amount of solvent is added to the beaker and until the solid is dissolved, the solution is stirred. The solution is then moved to the flask of vol. Again a funnel may need to be inserted into the flask's slender neck.

The beaker, stirring rod, and funnel must be carefully rinsed before adding additional solvent to the flask, and the washes added to the vol flask to ensure that all remaining traces of the solution have been transferred. The vol flask is finally diluted to volume (additional solvent is added to the

flask until the liquid level reaches the calibration mark). As before the flask is capped and inverted until the contents are mixed thoroughly. The downside to this technique is that if not thoroughly washed, some of the solution may stick to the beaker, stirring rod, or funnel. Also, if they have not been washed carefully, there is a chance of contamination from the beaker, rod, or funnel.

**c. Molar Solution**

It consists of one mole of solvent in a solution equal to one liter.
Molar solution = Molecular weight in the solution in grams / liters.
Example: Sodium chloride molar solution I (NaCl).
Sodium atomic weight = 23
Chloride atomic weight = 35.5
Total molecular weight = 58.5 gram / mol
Now dissolve 58.5 grams of NaCl in distilled water and make the solution to one liter.

**d. Normal Solution**

The normal solution is defined as the solution's gram equivalent weight per liter.

Normal solution = gram equivalent solvent weight/solution liter.
These alternatives are expressed as N.
Gram equivalent weight = weight/valence of molecules
Example
1 N sodium chloride solution to make
• The NaCl molecular weight is 58.55.
• Gram equivalent NaCl weight = molecular weight / 1 (valency)
Dissolve 58.5 grams of NaCl in one liter of distilled water and make-up.

**e. Percent Solution**

1. This is per hundred of the total solution component.
2. A percentage solution has three possibilities.
Weight/weight is a solvent percentage equal to solute + solvent in 100 grams of final solution.
1. Weight/weight is a solvent percentage equal to solute + solvent in 100 grams of final solution.

1. For example, dissolving 5 grams of NaCl in 95 grams of water, which is around 95 mL.

2. The weight/volume of for example 5 grams of NaCl dissolved in water and the volume of 100 ml is called a 5 percent NaCl solution.

3. Two solutions consist of volume/volume. For example, if we take 5 mL of acid and dilute it to 100 mL of water, that acid is a 5 percent solution.

**f. The Dilution**

1. This procedure is very common for the preparation of serum dilution where, if it is above 300 mg/dL, there is a high concentration of chemicals such as urea in the blood.

2. If we manufacture a serum dilution like this:

    1. Serum = 1 millilitre
    2. Diluting 4 mL of fluid
    3. This will be a dilution of 1:5 (1+4 =5).

# 3. INTRODUCTION OF CARBOHYDRATES

This experiment is intended to introduce you to one of the three major classes of macronutrients found in food, carbohydrates. You will also learn a variety of ways to categorize carbohydrates and several tests used in the analysis of carbohydrates.

The experiment is composed of four parts that should be completed in the order listed below (background, prelab, experiment, postlab).

You may proceed directly to the section on carbohydrate tests if you are already familiar with carbohydrates.

$$nCO2 + n\,H2O + \text{energy} \longrightarrow CnH2nOn + nO2$$

It is possible to classify carbohydrates as simple or complex:

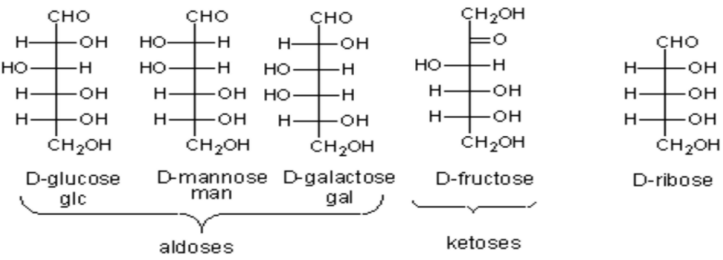
1- Simple carbohydrates, often referred to as simple sugars or monosaccharides, contain one unit of saccharide and cannot be broken down into smaller carbohydrates.

2- Those containing more than one group of saccharides are complex carbohydrates.

- Disaccharides contain two units of monosaccharide.
- Oligosaccharides have 3-6 units of monosaccharides.
- Over 7 or more monosaccharide units may be contained in polysaccharides. Through a process known as hydrolysis, complex carbohydrates can be broken down into smaller carbohydrate units.

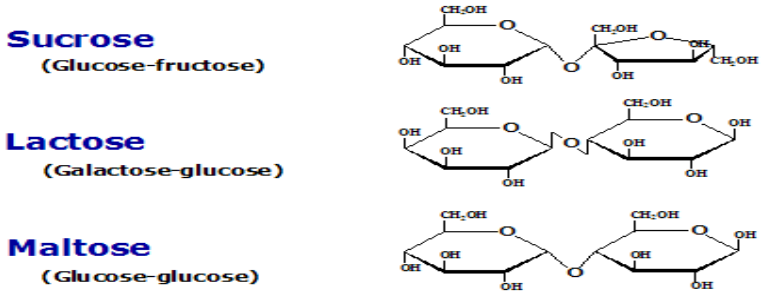
It is possible to classify monosaccharides in a number of ways. They can be categorized by the number of atoms of carbon they contain. While there are others, the most common are pentose (5 carbons) and hexose (6 carbons).

Ketoses or aldoses may also be classified as monosaccharides. In addition to having one or more hydroxyl groups, ketose contains a carbonyl group. Besides the hydroxyl groups, an aldose contains an aldehyde group. For an aldose or "aldohexose" for ketose, which is also a hexose, the two descriptors are usually combined into a single term like "ketohexose". See several examples of monosaccharides below.



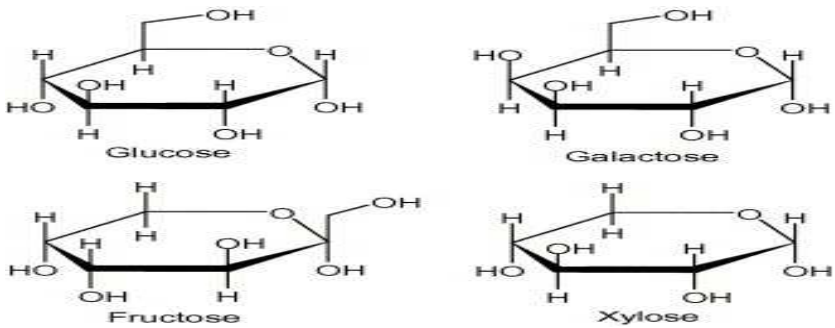

Monosaccharides

Hydrolysis can break disaccharides into two monosaccharide units and lose one molecule of water.




FDi-saccharides

Oligosaccharides can be broken by hydrolysis into 3-6 monosaccharide units and lose one or more water molecules, see the example of oligosaccharides below.




Poly-saccharides

Polysaccharides can be broken into 7 or more units of monosaccharides by hydrolysis and one or more molecules of water can be lost.

# 4. QUALITATIVE ANALYSIS OF CARBOHYDRATES

**1. Solubility**

Due to the polar hydroxyl groups that form H-bonds with water, monosaccharides and oligosaccharides are readily soluble in water. However, due to their large molecular weight, the polysaccharides make translucent colloidal solutions.

**2. Qualitative tests for carbohydrates:**

There are several difficulties in their qualitative as well as quantitative analysis when analyzing a sample containing a mixture of carbohydrates, particularly sugar. These problems are attributed to their structural and chemical resemblance, as well as their stereoisomerism. It is therefore necessary to determine, during biochemical investigations, whether or not a given sample contains carbohydrates. In order to establish the presence or absence of a sugar or a carbohydrate in a sample, several rapid tests are available. Such tests are based on specific typical color reactions for their group. It is advisable to carry out these tests with the individual in the laboratory rather than with a sugar mixture. The sensitivity of these tests can be confirmed by using different concentrations of sugar solutions (0.1- 1 percent ).

**A. General tests for carbohydrates:**

In order to detect the presence of carbohydrates in a solution, the most common tests used are:

For all carbohydrates, whether free or in combined form, it is a group test. It is routinely used to detect the presence of carbohydrates, despite its limitations.

**a. Molisch's Test**

**Principle**

The response is based on the fact that H2SO4 concentrated catalyzes the dehydration of sugars to form furfural (from pentoses) or hydroxymethyl furfural (from hexoses). These furfurals then condense to give a purple or violet colored product with sulfonated alpha-naphthol. A+ve reaction is also provided by polysaccharides and glycoproteins. The

acid first hydrolyses it into monosaccharides, which are then dehydrated to form furfural or its derivatives, in the case of the carbohydrate being a poly- or disaccharide.

**Reagents**

(i) Conc.H2SO4

(ii) Molisch's reagent: 5 percent (w/v) of alpha-naphthol in 95% ethanol.

**Procedure**

Take 2 mL of the unknown solution and add the contents to the 2-mixture. Incline the tube and pour 1-2 mL of conc.H2SO4 carefully down the side of the tube. Tube so that a layer beneath the aqueous solution forms the acid. The formation at the junction of two layers of a purple or violet ring or zone indicates the presence of carbohydrates.

**Precautions:**

(i) The solution of alpha-naphthol is unstable and should be made fresh.

(ii) Conc.H2SO4 should be carefully added along the sides of the test tube, causing the contents of the tube to be minimally disturbed.

**Limitations:**

This test is also performed in addition to carbohydrates, furfurals as such, certain organic acids, aldehydes and ketones. Secondly, due to the charring action of acid, a concentrated sugar solution can give a red colour instead of purple.

**b) Anthrone test**

**Principle**

Another general test for carbohydrates is anthrone reaction. Its principle is the same as that of Molisch's, except that furfurals and hydroxyl-methyl furfurals offer bluish green condensation products with anthrone.

**Reagents**

Anthrone reagent: in conc.H2SO4, 0.2% (w/v) solution.

**Procedure**

In a test tube, add about 2 mL of Anthrone reagent to approximately 0.5-1mL of the test solution and mix thoroughly. Watch if the colour changes to bluish green. If not, after keeping them in a boiling water bath for ten minutes, re-examine the tubes. A positive test indicates a

blue-green color.

**B. Specific tests for carbohydrates:**

**a) Polysaccharide iodine test**

This test is performed to differentiate polysaccharides from mono- and disaccharides.

**Principle**

With various polysaccharides, iodine forms coloured adsorption complexes. Because of iodine adsorption on the polysaccharide chains, these complexes are formed. The colour intensity depends on the length of the available unbranched or linear chain for the complex formation. Therefore, amylose gives a deep blue color and amylopectin, the unbranched helical component of starch, the branched component gives red color because the chains do not effectively coil. Glycogen, which is also highly branched, adds iodine to the red colour. This experiment is performed in acidic or neutral solutions.

**Reagents**

(i) Iodine solution: prepare a 2% (w/v) KI solution in water to which a few iodine crystals are added until the solution is deep yellow.

(ii) Starch solution: dissolve 1g of starch in about 10-20mL of boiling water and add saturated sodium chloride solution to a volume of 100mL.

**Procedure**

In a test tube, take 3 mL of the test solution and add 1-2 drops of dil.HCl.

Mix and then add 1-2 drops of the solution of iodine. Mix and observe the change in colour. Heat the tube and again observe the colour. When heated, the blue colour disappears and reappears when cooled.

**(b) Tests based on the reduction of carbohydrate properties**

Sugars with a free or potentially free group of aldehydes or ketones act as reducing agents, and this becomes the basis for the tests carried out to distinguish them from non-reducing sugars. These sugars have the ability to easily reduce alkaline metal solutions such as copper, bismuth, mercury, iron and silver. Aldo sugars are oxidized into the corresponding aldonic acids, whereas shorter chain acids are produced by keto sugars. If in the

absence of sugar reduction, the alkaline copper solution is heated, the black precipitate of cupric oxide forms:

$$Cu(OH)_2 \xrightarrow{\text{Heat}} 2OH$$

However the alkaline solution of copper is reduced to insoluble yellow or red cuprous oxide in the presence of a sugar reducer:

$$\text{Sugar} + 2\,Cu(OH)2 \xrightarrow{\text{Heat}} 2O + 2\,H2O$$

**A) Fehling's test**

In this reaction, Rochelle salt acts as a chelating agent:

$$CuSO4 + 2KOH \text{ ---------- } 2 + K2SO4$$

$$2Cu(OH)2 + \text{Reducing Sugar ---------- } 2O + \text{Aldonic acid}$$

**Reagents**

**i) Fehling solution A:**

Dissolve 69.38 g of copper sulfate in distilled water and make 1 L of copper sulfate.

**ii) Fehling's solution B:**

Dissolve 250 g of NaOH in DW, add 346 g of potassium sodium tartrate and add up to 1 L of volume.

Just before use, mix equivalent volumes of A & B solutions because mixing causes deterioration with time.

**Procedure**

1 mL of Fehlings reagent is added to the remaining 1 mL solution. Mix the test tubes thoroughly and place them in a boiling water bath. The formation of yellow or red Cuprous Oxide precipitates indicates the presence of sugar reduction.

**Note**

i) Leave the solution to stand for 10-15 minutes in the case of a mild reduction, then decant the supernatant. It is then possible to see a small amount of red or yellow precipitates adhering to the inner side of the tube.

ii) The Fehling test is only carried out with an alkaline solution

iii) Cuprous oxide is ammonia-dissolved. Small amounts of sugar reducers

can therefore not be detected in fluids saturated with ammonium salts, such as urine.

**B) Benedict's Test**

Benedict modified the Fehling solution to produce a fairly stable, enhanced single reagent. As a chelating agent, Sodium Citrate works. It is very sensitive and sufficient precipitates are produced by even small amounts of sugar reduction (0.1 percent).

**Reaction**

$$Na_2CO_3 + 2H_2O \rightarrow 2NaOH + H_2CO_3$$

$$2NaOH + CuSO_4 \rightarrow Cu(OH)_2 + Na_2SO_4$$

$$Cu(OH)_2 \rightarrow CuO + H_2O$$

$$\text{D-Glucose} + 2\ CuO \rightarrow \text{D-gluconic acid} + Cu_2O \quad \text{(Red ppt)}$$

**Reagents:**

**Qualitative reaction of Benedict:**

Dissolve 173g of Sodium Citrate and 100g of Anhydrous Sod. In about 800mL of water, carbonate by gently heating the contents. Then, dissolve 17.3g of copper sulfate in about 100mL DW in a separate beaker. Slowly pour this solution into the Carbonate-Citrate mixture with constant stirring, and make up to 1 L with DW.

**Procedure:**

To add about 2 mL of Benedict's reagent, add 0.5-1 mL of the test solution. Keep the test tubes in a bath of boiling water. .Note that green, orange, yellow or red precipitates are formed, indicating the presence of sugar reduction in the solution.

**Note:**

i) This test is particularly suitable for urinary sugar reduction detection because it is more specific than the Fehling test, which is also positive for non-reducing substances such as urates present in urine.

ii) This is a semi-quantitative test.

**C) Barfoed's Test:**

This test is performed to differentiate between mono- and disaccharide reduction. Monosaccharides are more reactive reducing agents than disaccharides and thus react in about 1-2 minutes, while it takes 7-12

minutes for the reducing disaccharides to get hydrolysed and then react in the acidic solution. Therefore it is possible to detect the difference in property reductions.

**Reagents:**

**Barfoed's reagent:**

66.5 g of Cupric acetate dissolved in approximately 900 mL DW. Add 9 mL of Glacial Acetic Acid and boil. Cool and use DW to cool the volume to 1 L and filter if necessary.

**Procedure:**

Take 2- solution: Keep the test tubes for only 1-2 min in a boiling water bath. Then allow a while for the tubes to cool down. Thin red precipitates indicate the presence of a reduction of monosaccharide at the bottom or sides of the tube.

**Note:**

i) The boiling should not be prolonged beyond 1-2min, otherwise the disaccharide reduction will respond to this test as well.

ii) This test is not effective in detecting urine sugar reduction due to the presence of chloride ions.

**D) Picric Acid Test:**

This is another test for sugar reduction detection. To form a red colored Picramic Acid, the decreasing sugars react with Picric Acid.

**Reagents:**

i) Picric acid saturation:

Dissolve 13 g of picric acid in 100 mL of DW, boil and cool.

(ii) Sodium Carbonate (10% solution).

**Procedure**:

Procedure: Add 1 mL of the above-mentioned reagent to 1 mL of the test solution, followed by 0,5 mL of the 10% solution of sodium carbonate. In a boiling water bath, heat the test tube. The presence of sugar reduction in the solution indicates the appearance of a red color.

**E) Seliwanoff's Test**

**Principle:**

This test is a keto hexose-specific timed colour reaction. It is therefore used for the differentiation of aldoses from ketoses. Dehydration

is carried out in the presence of HCl ketohexoses to yield 4-hydroxy methyl furfural more rapidly than aldohexoses. In addition, these furfural derivatives condense to form a red coloured complex with resorcinol.

**Reagents:**

**Seliwanoff's reagent:**

Dissolved in 100 mL dilute HCl (1:2)

**Procedure**:

Add 1 mL of the test solution to about 2 mL of the Seliwanoff reagent and warm it in a boiling water bath for 1 min. The presence of ketohexose indicates the appearance of a red colour (fructose).

**Note:**

i) Aldohexoses, e.g. glucose, also react if the boiling is prolonged because the catalytic action of the acid is converted into fructose.

ii) Sucrose and inulin are also tested because they are hydrolysed by fructose-giving acids.

Bial's Test for Pentoses

Principle:

This test is specific to pentoses and to pentose-containing compounds and is therefore useful for pentose sugar determination. The reaction is due to the formation of furfural in the acid medium which in the presence of ferric ions, condenses with orcinol to give a blue-green complex.

**Bial's reagent:**

Dissolve 1.5g of orcinol in 100 mL of Conc HCL and add 20 drops of 10% solution of Ferric Chloride. Make yourself fresh.

**Procedure:**

Add 4 to 5 drops of the test solution to about 2 mL of Bial's reagent. Heat until bubbles of gas rise to the surface in a boiling water bath. The presence of pentose sugar indicates the formation of the green solution and the precipitate.

**E) Test for sucrose:**

This test is performed only if the Barfoed test does not contain any precipitation.

**Principle:**

In an unknown solution, the sucrose present is hydrolyzed to glucose and fructose by acid. Then Seliwanoff's reagent forms the resulting fructose in the solution.

**Reagents:**

i) Conc. of HCL
ii) Seliwanoff's reagent
iii) Sodium carbonate

**Procedure:**

Add 1-2 drops of conc HCl to approximately 2-3mL of test solution and boil for about 8-minutes in a water bath. The appearance of the red color indicates the presence of the hydrolytic sucrose product fructose.

Note:

Benedict's reagent can be tested for sugar reduction by acid hydrolyzed sample after cooling and then neutralizing with sodium carbonate.

**f) Mucic acid test for galactose**

**Principle:**

This test is highly specific to galactose, which is either present in solutions independently or obtained by lactose hydrolysis. On heating with HNO3, Galactose is converted to Saccharic acid (a strong oxidizing agent). Mucic acid (galactaric acid) formed from galactose due to the aldehyde & primary alcoholic group oxidation at C1&C6. It is the only saccharic acid that is insoluble in cold water and thus assists in galactose identification.

**Reagents:**

i) Conc. of HNO3

**Procedure:**

Take 50 mg of galactose and 50 mg of glucose into the test tubes separately. Add to each tube 1mL DW and 1mL conc HNO3. Heat the tubes for about 1hr in a boiling water bath. Add 5mL of DW and slowly let the tubes stand and cool. The presence of galactose will be indicated by colorless needles such as crystals.

**Note**:

This test will also be administered with lactose.

**(g) Osazone test / Phenylhydrazine test**

This test is used to distinguish between lactose and maltose.

**Principle:**

The organic compound phenylhydrazine reacts to form osazones with the carbonyl carbon of sugar. The shapes and melting point, time of formation and solubility of these osazone crystals have yellow color characteristics. In the following table, the characteristic characteristics of osazone are given:

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

**Procedure:**

In a test tube, take 7-8 ml of carbohydrate solution and add a pinch of phenylhydrazine to that and double the amount of sodium acetate and 10 drops of acetic acid. Dissolve and allow slow cooling by shaking. Observe the shape of the crystal under low microscope power (10x).

**Observations and conclusion:**

Lactose forms crystals in the form of powder puffs, maltose forms sunflower-shaped or star-shaped crystals, while glucose and fructose form identical crystals in the form of needles.

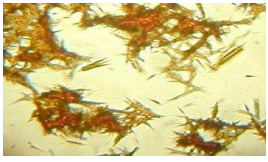



**Table: 5.1 Colour reactions of Carbohdyrates**

| S.No | Test | Observation | Inference |
|---|---|---|---|
| 1 | **Molisch Test (Ring test)** Aqueous or alcoholic solution of substances _ 10% alcoholic solution of α-napthol shake + concentrated sulphuric acid along the side of the tube | Violet ring at the junction o two liquids | Carbohydrate present |
| 2 | **Solubility** Compound+water | Soluble<br><br>Insoluble | Mono & Disaccharides<br>Polysaccharides |
| 3 | **Fehling's test** 2 ml of Fehling's A solution + 2 ml of Fehling's B solution + 2 ml of sugar solution. Boil | Yellow or brick red precipitate | Reducing sugars present |
| 4 | **Benedict's test** 5 ml of benedict's reagent + 8 drops of sugar solution. Boil for 2 min. Cool | Green, yellow or red precipitate | Reducing sugars present |
| 5 | Tommer's test 2 ml of tommer's reagent + 3 ml of sugar solution. Boil for 2 minutes | Yellow or red precipitate | Reducing sugars present |
| 6 | Barfoed's test 2 ml of sugar solution + add 2 ml of Barfoed's test reagent. Boil on water bath. | Brick red precipitate at the bottom of the test tube. | Monosaccharides present |
| 7 | Rapid furfural test 1 to 2 ml of sugar solution + add 1 ml of α-napthol solution (1% in alcohol) + add 5 ml of conc. HCl and Boil. | Deep purple colour | Ketoses like Fructose, Sucrose present |

| | | | |
|---|---|---|---|
| 8 | Osazone test<br>0.2 gram of sugar + add 0.4 gram of phenyl hydrazine hydrochloride + 0.6 gram of sodium acetate + 4 ml of distilled water. Add 6-8 drops of glacial acetic acid. Heat on a water bath for 20 minutes. Cool and allow crystallization. Observe crystals under microscope | a) Greenish yellow needle shaped crystals arranged in fan-shape<br>b) Thin small needle shaped crystals appear luke ball of prickles<br>c) Sunflower like crystals | Glucosazone is glucose present<br><br>Lactose present<br><br>Maltose present |
| 9 | Iodine test<br>Suspension or sugar solution of polysaccharides + add 1-2 drops of iodine solution | i) Blue violet colour develops<br>ii)Brown wine colour develops | Starch present<br><br>Glycogen present |

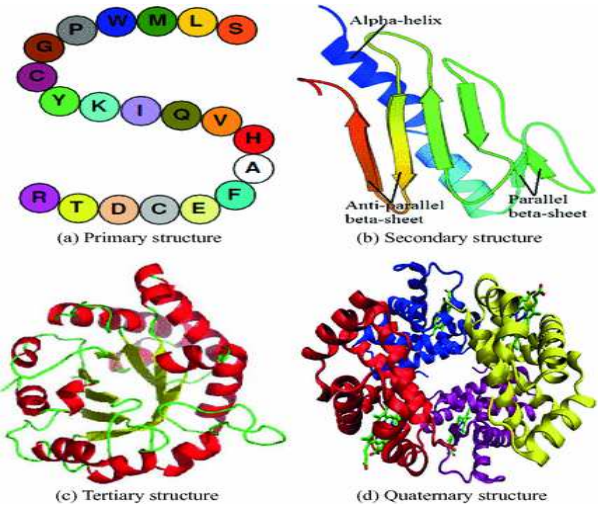
# 5. INTRODUCTION OF PROTEINS

Protein is an important macronutrient essential for survival. They are constituent of calls and hence are present in all living bodies. 10-35% of calories should come from protein. Protein is found in meats, poultry, fish, meat substitutes, cheeses, milk etc.

Proteins are large biological molecules of alpha-amino acids (amino acids in which the amino group is attached to alpha-carbon, which are crystalline in nature and exist as zwitter ions). They contain carbon, hydrogen, oxygen, nitrogen, phosphorus and sulphur in some cases.

Molecules containing both the amino (NH2) and carboxylic (COOH) groups are amino acids. To form a specific type of linkage known as peptide linkage, amino acid molecules undergo a condensation reaction.

The products formed are classified as; depending on the number of amino acid molecules involved in the condensation reaction.



(a) Primary structure (b) Secondary structure

(c) Tertiary structure (d) Quaternary structure

**Dipeptide**

They are the products formed by the condensation of two molecules of alpha-amino acid.

**Tripeptide**

They are formed by the condensation of three molecules of al-amino acid.

If a large number of molecules of amino acids combine, the formed product is called a polypeptide. A protein is called a polypeptide that has a molecular mass greater than 10000. Proteins differ primarily from each other in their amino acid sequence. There are about 20+ amino acids here. Some amino acids are not produced by the body and are delivered by diet. They are called amino acids, which are essential.

## 6. TESTS FOR PROTEINS

**Solubility Tests**

The solubility of amino acids and proteins is largely dependent on the pH of the solution. The structural changes that occur at different pH values in an amino acid or protein change the molecule's relative solubility. Amino and carboxylic groups are both protonated in acidic solutions. Both groups are deprotonated in basic solutions.

In water, amino acids are essentially soluble. Depending on the structure of its side chains, their solubility in water, dilute alkali and dilute acid varies from one compound to another. Glycine, tyrosine, glutamic acid and cysteine will be used for this test.

**Procedure:**

The solubility of amino acids in water and alcohol should be noted by placing a small quantity in the test tube, adding a few mL of solvent and if necessary, warming up.

Use the litmus paper to determine whether the amino acid solution is acidic or basic, while testing the solubility in water.

Using dilute HCl and dilute NaOH, repeat the solubility test.

WARNING: Avoid spilling ninhydrin solutions on your skin because it is hard to remove the resulting stains.

**Stability to Alkali**

Amino acids do not develop NH3 or alkaline vapor when boiled with alkali, unlike amides and volatile amines. In order to differentiate amino acids from amines and amides, this method can be used. Apply this test to the amine or amide provided and to glycine as well.

**Procedure:**

Pipette 1 mL 1% glycine and the amide or amine solution into separate test tubes.

Add 1 mL dilute NaOH to each test tube and boil.

Test the vapor from each boiling tube with wet litmus paper.

**Specific Reactions for Individual Amino Acids:**

**a) Xanthoproteic Test:**

There are aromatic groups that are derivatives of benzene in some amino acids. Reactions that are characteristics of benzene and benzene derivatives can be experienced by these aromatic groups. The nitration of a

benzene ring with nitric acid is one such reaction. The amino acids that have the benzene ring activated can easily undergo nitration. This nitration reaction forms a yellow product in the presence of the activated benzene ring. Apply this test to tyrosine, phenylalanine, tryptophan, and glutamic acid.

**Procedure:**

To 2 mL amino acid solution in a boiling test tube, add equal volume of concentrated HNO3.

Heat over a flame for 2 min and observe the color.

Now COOL THOROUGHLY under the tap and CAUTIOSLY run in sufficient 40% NaOH to make the solution strongly alkaline.

**Observe the color of the nitro derivativative of aromatic nucleus.**

**a) Millon's Test:**

The test by Millon is specific to phenol that contains structures (tyrosine is the only common phenolic amino acid). Millon's reagent is a concentrated HNO3 reagent that dissolves mercury. A red precipitate or a red solution is regarded as a positive test as a consequence of the reaction. A yellow HgO precipitate is NOT a positive response, but it usually shows that the solution is too alkaline. This test can be applied to tyrosine, phenylalanine.

**Procedure:**

To 2 mL amino acid solution in a test tube, add 1-2 drops of Millon2s reagent.

Warm the tube in a boiling water bath for 10 min.

A brick red color is a positive reaction.

Note that this is a test for phenols, and the ninhydrin test should also be positive if it is to be concluded that the substance is a phenolic amino acid.

**b) Hopkin's Cole Test:**

In the presence of concentrated H2SO4, the indole tryptophan group reacts with glyoxylic acid (glacial acetic acid, which has been exposed to light, always contains CHOCOOH glyoxylic acid as an impurity) to give a purple color. Apply this test to glycine, tyrosine and tryptophan.

**Procedure:**

To a few mL of glacial acetic acid containing glyoxylic acid, add 1-2 drops of the amino acid solution.

Pour 1-2 mL $H_2SO_4$ down the side of the sloping test tube to form a layer underneath the acetic acid.

The development of a purple color at the interface proves a positive reaction.

**c) Lead-Sulfide Test:**

When cystine is boiled with 40 percent NaOH, sodium sulfide is covered with some sulfur in its structure ($Na_2S$). Using a sodium plumbate solution that causes the precipitation of PbS from an alkaline solution, $Na_2S$ can be detected. In order to carry out this test, the sodium plumbate solution must be prepared first. This test is applied to cysteine and cystine.

**Procedure:**

Sodium Plumbate Solution Preparation:

Add 5 mL dilute NaOH to 2 mL dilute lead acetate.

A white precipitate of lead hydroxide forms.

Boil until the precipitate dissolves with the formation of sodium plumbate.

Boil 2 mL amino acid solution with a few drops of 40% NaOH for 2 min.

Cool and add a few drops of the sodium plumbate solution.

A brown color or precipitate is a positive test for sulfides.

**d) Ehrlich Test:**

Aromatic amines and many organic compounds (indole and urea) provide this test with a color complex. This test is applied to tryptophan, urea and glycine.

**Procedure:**

Put 0.5 mL of the amino acid solution to a test tube.

Add 2 mL Ehrlich reagent and observe the color changes.

Repeat the test with urea solution.

**e) Sakaguchi Test:**

To test for a certain amino acid and proteins, the Sakaguchi reagent is used. The amino acid that this test detects is arginine. As arginine has a group of guanidine in its side chain, in the presence of an oxidizing agent

such as bromine solution, it gives a red color with alpha-naphthol. Apply Arginine to this test.

**Procedure:**

1 mL NaOH and 3 mL arginine solution is mixed and 2 drops of α-naphthol is added.

Mix thoroughly and add 4-5 drops of bromine solution UNDER THE HOOD!!

Observe the color change.

**f) Nitroprusside Test:**

The nitroprusside test is specific to cysteine, the only sulfhydryl group amino acid containing cysteine (-SH). In the presence of excess ammonia, that group reacts with nitroprusside. This test is used for cysteine, cystine and methionine.

**Procedure:**

Put 2 mL amino acid solution into the test tube.

Add 0.5 mL nitroprusside solution and shake thoroughly.

Add 0.5 mL ammonium hydroxide.

Observe the color change.

**g) Biuret Test:**

The Biuret Test recognizes positively the presence of proteins (not less than two peptides). The reaction in this test involves the complex formation in a strongly alkaline solution of the proteins with Cu2+ ions. This test is applied to gelatin, casein and albumin.

Procedure:

To 2 mL protein solution, add 5-6 drops of dilute CuSO4 (Fehling's solution A diluted 1/10 with water)

Add 3 mL 40% NaOH solution.

Observe the color change.

If the protein tested is insoluble in water, then apply the procedure given below:

Measure 3 mL acetone and 1.5 mL water into a test tube.

Add 1 drop of dilute NaOH and a little piece of protein to be tested.

Boil continuously over a small flame for 2 min and cool.

Add 0.5 mL 40% NaOH and 2 drops of a 1/10 diluted Fehling's solution A.

Observe the color change.

**h) Ninhydrin Test:**

This test is given by only amino acids and proteins which contain free –NH2 groups in their structure. Apply this test for all the proteins provided.

**Precipitation of Proteins:**

The precipitation of a protein occurs in a stepwise process. The addition of a precipitating agent and steady mixing destabilizes the protein solution. Mixing causes the precipitant and the target product to collide. Enough mixing time is required for molecules to diffuse accross the fluid.

**By Neutral Salts:**

The precipitation of a protein by neutral salt is commonly known as salting-out method. Addition of a neutral salt, such as ammonium sulfate, compresses the solvation layer and increases the protein-protein interaction. As the salt concentration of a solution is increased, more of the bulk water becomes associated with the ions. As a result, less water is available to take part in the solvation layer around the protein, which exposes hydrophobic parts on the protein surface. Therefore, proteins can aggregate and form precipitates from the solution. The amount of neutral salt required to cause protein precipitation varies with the nature of the protein and the pH of the solution. Apply this test to all the proteins provided.

**Procedure:**

Add solid ammonium sulfate to about 5 mL of protein solution in a test tube (the salt should be added in quantities of approximately 1 g at a time)

Agitate the solution gently after each addition to dissolve the ammonium sulfate.

**By salts of Heavy Metals:**

Heavy metal salts usually contain Hg2+, Pb2+, Ag1+, Tl1+, Cd2+ and other metals with high atomic weights. Since salts are ionic, they disrupt

salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt. Apply this test to all the proteins provided.

**Procedure:**

Treat 3 mL of the protein solution provided with a few drops of mercuric nitrate.

A white precipitate formation should be observed.

**By Acid Reagents:**

The precipitation of a protein in the presence of acid reagents is probably due to the formation of insoluble salts between the acid anions and the positively charged protein particles. These precipitants are only effective in acid solutions. Apply this test to all the proteins provided.

**Procedure:**

Treat 3 mL of protein solution provided with a few drops of trichloroacetic acid solution.

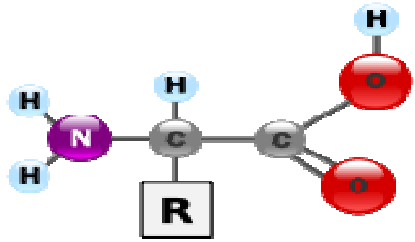
Note the protein precipitate formed.

**Table: 6.1 Colour reactions of Proteins**

| S. no | Test | Observation | Inference |
|---|---|---|---|
| 1 | **Biuret test**<br>Take 1 ml of albumin solution and add 1 ml of 5% sodium hydroxide then add 1-2 drops copper sulphate solution and mixed well. | Violet colour is formed | Indicates albumin is protein |
| 2 | **Ninhydrin test**<br>Take 1 ml of albumin solution and add 5-10 drops of ninhydrin reagent. Heat to boiling. | Ruhemann's purple colour formed | Alpha aminoacids are present |
| 3 | **Heat coagulation test**<br>Take 5 ml of albumin solution and heat flame slanting position, add 3 drops of 1% acetic acid glacial. | A white cloudy precipitate is formed | Albumin present |
| 4 | Half saturation test<br>Take 3 ml of test sample and add an equal vol. of saturated ammonium sulphate solution to make it half saturated. Mix well and wait for 3 minutes then filter the sample | No white precipitate is seen | Albumin is not precipitated by half saturation as it is a low Molecular weight protein. hence, the biuret test is positive with the filtrate. |
| 5. | **Biuret test with filtrate**<br>Take 2 ml of the filtrate of the half-saturation test and add an equal volume of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution. Mix well | Violet colour is formed | Albumin is not precipitated by half saturation as it is a low Molecular weight protein. hence, the biuret test is positive with the filtrate. |

# 7. INTRODUCTION OF AMINOACIDS

Amino acids are molecules containing an amine group, a group of carboxylic acids, and a side chain that varies from one amino acid to another. In some reactions, amino acids of the general formula RCH(NH2)COOH are amphoteric, acting as amines and in others as carboxylic acids. An amino acid has no overall charge at a certain pH, known as the isoelectric point, as the number of protonated ammonium groups (positive charges) and deprotonated carboxylate groups (negative charges) is equal.




For life, amino acids are critical. They have important functions in particular, such as being the building blocks of proteins and the intermediates in metabolism.

Amino acids are usually classified into four groups according to the properties of their side chain. The side chain can make a weak acid or a weak base of an amino acid, and if the side chain is polar, a hydrophobe or if it is nonpolar, a hydrophile.

Proteins (also known as polypeptides) are organic compounds arranged in a linear chain made of amino acids. The peptide bonds between the carboxyl and amino groups of adjacent amino acid residues are joined together by the amino acids in a polymer.

Proteins are essential parts of organisms, like other biological macromolecules such as polysaccharides and nucleic acids, and participate in virtually every process within cells. In: Proteins are significant in:

-catalyzing biochemical reactions (enzymes)
-structural and mechanical functions (actin and myosin)
-cell signaling
-immune responses
-cell adhesion
-cell cycle

# 8. TESTS ON AMINO ACIDS

**Solubility Tests**

The solubility of amino acids and proteins is largely dependent on the pH of the solution. The structural changes that occur at different pH values in an amino acid or protein change the molecule's relative solubility. Amino and carboxylic groups are both protonated in acidic solutions. Both groups are deprotonated in basic solutions.

In water, amino acids are essentially soluble. Depending on the structure of its side chains, their solubility in water, dilute alkali and dilute acid varies from one compound to another. Glycine, tyrosine, glutamic acid and cysteine will be used for this test.

**Procedure:**

The solubility of amino acids in water and alcohol should be noted by placing a small quantity in the test tube, adding a few mL of solvent and if necessary, warming up.

Use the litmus paper to determine whether the amino acid solution is acidic or basic, while testing the solubility in water.

Using dilute HCl and dilute NaOH, repeat the solubility test.

WARNING: Avoid spilling ninhydrin solutions on your skin because it is hard to remove the resulting stains.

**a) Millon's Test:**

The test by Millon is specific to phenol that contains structures (tyrosine is the only common phenolic amino acid). Millon's reagent is a concentrated HNO3 reagent that dissolves mercury. A red precipitate or a red solution is regarded as a positive test as a consequence of the reaction. A yellow HgO precipitate is NOT a positive response, but it usually shows that the solution is too alkaline. This test can be applied to tyrosine, phenylalanine.

**Procedure:**

To 2 mL amino acid solution in a test tube, add 1-2 drops of Millon2s reagent.

Warm the tube in a boiling water bath for 10 min.

A brick red color is a positive reaction.

Note that this is a test for phenols, and the ninhydrin test should also be

positive if it is to be concluded that the substance is a phenolic amino acid.

**b) Hopkin's Cole Test:**

In the presence of concentrated H2SO4, the indole tryptophan group reacts with glyoxylic acid (glacial acetic acid, which has been exposed to light, always contains CHOCOOH glyoxylic acid as an impurity) to give a purple color. Apply this test to glycine, tyrosine and tryptophan.

**Procedure:**

To a few mL of glacial acetic acid containing glyoxylic acid, add 1-2 drops of the amino acid solution.

Pour 1-2 mL H2SO4 down the side of the sloping test tube to form a layer underneath the acetic acid.

The development of a purple color at the interface proves a positive reaction.

**c) Lead-Sulfide Test:**

When cystine is boiled with 40 percent NaOH, sodium sulfide is covered with some sulfur in its structure (Na2S). Using a sodium plumbate solution that causes the precipitation of PbS from an alkaline solution, Na2S can be detected. In order to carry out this test, the sodium plumbate solution must be prepared first. This test is applied to cysteine and cystine.

**Procedure:**

Sodium Plumbate Solution Preparation:

Add 5 mL dilute NaOH to 2 mL dilute lead acetate.

A white precipitate of lead hydroxide forms.

Boil until the precipitate dissolves with the formation of sodium plumbate.

Boil 2 mL amino acid solution with a few drops of 40% NaOH for 2 min.

Cool and add a few drops of the sodium plumbate solution.

A brown color or precipitate is a positive test for sulfides.

**d) Ehrlich Test:**

Aromatic amines and many organic compounds (indole and urea) provide this test with a color complex. This test is applied to tryptophan,

urea and glycine.

**Procedure:**

Put 0.5 mL of the amino acid solution to a test tube.

Add 2 mL Ehrlich reagent and observe the color changes.

Repeat the test with urea solution.

**e) Sakaguchi Test:**

To test for a certain amino acid and proteins, the Sakaguchi reagent is used. The amino acid that this test detects is arginine. As arginine has a group of guanidine in its side chain, in the presence of an oxidizing agent such as bromine solution, it gives a red color with alpha-naphthol. Apply Arginine to this test.

**Procedure:**

1 mL NaOH and 3 mL arginine solution is mixed and 2 drops of α-naphthol is added.

Mix thoroughly and add 4-5 drops of bromine solution UNDER THE HOOD!!

Observe the color change.

**f) Nitroprusside Test:**

The nitroprusside test is specific to cysteine, the only sulfhydryl group amino acid containing cysteine (-SH). In the presence of excess ammonia, that group reacts with nitroprusside. This test is used for cysteine, cystine and methionine.

**Procedure:**

Put 2 mL amino acid solution into the test tube.

Add 0.5 mL nitroprusside solution and shake thoroughly.

Add 0.5 mL ammonium hydroxide.

Observe the color change.

**Table 9.1 Colour test for aminoacids and proteins**

| Test | Reactions | Responsible Amino acid | Colour formation |
|---|---|---|---|
| Biuret | Alkaline Copper sulphate | Peptide proteins | Purple colour formation |
| Ninhydrin | Ninhydrin | All aminoacid, peptide proteins | Purple |
| Millon's test | Silver nitrate with nitrous acid | Tyrosine | Brick red colour |
| Xanthoproteic test | Boiling with Coc. Nitric acid | Tyrosine, Tryptophane, phenualanine | Yellow in acid which turns orange yellow |
| Pauly's test | Daizotized sulphanilic acid in alkaline solution | Tyrosine, Tryptophan, Histidine | Red colour |
| Hopkins cole test | Glycoxylic acid in Conc. Sulphuric acid | Tryptophan | Violet ring at interface |
| Sakaguchi test | Alpha-napthol and sodium hypochlorite | Arginine | Red |
| Lead sulphide test | Lead acetate and NaOH | Cysteine | Black or Black Colour |

# 9. QUALITATIVE TESTS FOR LIPIDS

A large number of heterogenous compounds are referred to as lipids including fats, steroids, waxes, and related compounds, which are related more by their physical than their chemical properties.

**They have the common property of being:**

(1) Relatively insoluble in water

(2) Soluble in polar solvents such as ether and chloroform.

Fatty acids are aliphatic carboxylic acids. If the aliphatic chain contains no double bond then it is called saturated and if it contains one or more double bond it is called unsaturated. Most naturally occurring unsaturated fatty acids have cis-double bonds. Some of the most common fatty acids are palmitic acid and stearic acid. Palmitic has 16 carbon atoms and stearic has 18 carbon atoms.

As it is clear from the formulae, both are saturated fatty acids. Some fatty acids like oleic acid may be unsaturated. Naturally occurring animal fats consist largely of mixed glyceride of oleic, palmitic and stearic acids. They are usually mixture of individual fats. Fats have more saturated fatty acids whereas oils have more of unsaturated ones.

Lipids are simple, complex or derived. Simple lipids are esters of fatty acids with various alcohols, e.g., fats (esters of fatty acids with glycerol) and waxes (esters of fatty acids with higher molecular weight of monohydric alcohols). Complex lipids are esters of fatty acids containing groups in addition to an alcohol and a fatty acid, e.g., phospholipids or glycolipids etc. Derived lipids include fatty acids, glycerol, steroids, other alcohols, fatty aldehydes, and ketone bodies, lipid soluble vitamins, and hormones.

Phospholipids yield in addition to alcohol and fatty acids, phosphate and a nitrogenous base like choline, ethanolamine, etc. Lecithin's and cephalous are representatives of the phospholipids. Similarly glycolipids contain carbohydrates, and sulpholipids contain sulphate. Lipoproteins are combinations of lipids with proteins.

**Qualitative Tests:**

**I. Physical Test:**

**1. Grease spot test:**

Take a small amount of oil on a piece of paper, a greasy spot

penetrating the paper will be formed. This happens because lipid does not wet paper unlike water.

**2. Test for free fatty acids:**

Take a few drops of phenolphthalein solution in a test tube and add to it one or two drops of very dilute alkali solution, just sufficient to give the solution a pink colour. Now add a few drops of the oil and shake. The colour will disappear as the alkali is neutralized by the free fatty acids present in the oil.

**3. Emulsification:**

Oil or liquid fat becomes finely divided and is dispersed in water when shaken with water to form emulsification. Emulsification is permanent and complete in the presence of emulsifying agent. The important emulsifying agents are bile salts, proteins, soaps, mono- and diglycerides. Emulsification is important in the processes of fat digestion in the intestine. Emulsifying agents lower surface tension of the liquid.

Take 2 clean and dry test tubes, in one test tube added 2 ml water and in other 2ml dilute bile salt solution. Now to each tube added 2 drops of mustard oil and shaken vigorously for about one minute. Allow the tubes to stands for two minutes and note that the water, oil is broken in small pieces and floats on the surface; where as in the bile salt solution, the oil can be seen in minute droplets suspended in the liquid (permanent emulsification).

**4. Saponification test:**

Esters can be hydrolysed by alkali to yield the parent alcohol and salt. When the fatty acid possesses a long chain the salt formed is a soap which we commonly use. This process is called saponification. Oils and fats usually contain long chain fatty acids and are, therefore, the starting materials for the preparation of soap.

Take 1 ml of the oil in a test tube and add an equal amount of alcoholic KOH solution, mix them thoroughly and keep the mixture during the course of warming and shake up gently with a little distilled water. Appearance of some oil drops will indicate the incomplete saponification. After complete saponification no oil drops will appear.

**5. Tests for unsaturation of fatty acids:**

Unsaturated fatty acids like oleic acid can react with halogens like bromine and iodine due to presence of double bonds as shown below.

$CH_3 (CH_2)_7CH = CH (CH_2)_7COOH + Br_2 \rightarrow CH_3 (CH_2)_7CHBr\text{-}CHBr$

$(CH_2)_7COOH$

The amount of $Br_2$ or $I_2$ taken up will indicate the amount of unsaturation present in a particular acid. Approximate idea about the unsaturation in a different oils and fats can be obtained by the following test. Set up four clean and dry test tubes each containing 5 ml of $CCl_4$.

To the first, add one drop of shark liver oil, to the second, one drop of coconut oil, to the third, a drop of vegetable ghee and add nothing to the fourth tube. Now test for the unsaturation of the added oil by adding bromine water drop by drop to each tube followed by shaking.

Record the number of drops required to obtain a permanent yellowish red colour in each tube and infer the relative unsaturation in the three samples used. It may be mentioned here, vegetable ghee is prepared by hydrogenating vegetable oil. Hydrogenation means saturation of unsaturated fatty acid by hydrogen.

**6. Isolation of free fatty acids from soap:**

Take a few ml of 20% $H_2SO_4$ in a test tube and gradually add 5 ml of some soap solution. The fatty acids will separate out in a distinct layer due to the hydrolysis of the soap.

$RCOONa + H_2O \rightarrow RCOOH + NaOH$

Cool the solution which will become hot and skim off the surface layer and wash it several times with water till free from $H_2SO_4$. Then dissolve it in some water and add alkaline phenolphthalein solution and shake. The pink colour will be discharged indicating the presence of free fatty acids.

**Calcium soap formation:**

To a small amount of the soap solution in a test tube add $CaCl_2$ solution. A white precipitate will be formed. The white precipitate is due to insoluble calcium salt of fatty acid. This is referred to as calcium soap.

**Lead soap formation:**

To a small amount of the soap solution in a test tube add lead acetate solution, a white precipitate will appear. The white ppt is due to insoluble lead salt of fatty acids. This is referred to as lead soap.

**7. Tests for Glycerol:**

**I. Acrolein test:**

Take pure glycerol in a dry test tube; add to it a few crystals of

potassium hydrogen sulphate. Warm gently to mix and then heat strongly. A very pungent odour of acrolein is produced. Acrolein is formed due to removal of water from glycerol by potassium hydrogen sulphate.

**II. Dichromate Test:**

Take in a dry test tube 3 or 4 ml of glycerol solution, to it add a few drops of 5% potassium dichromate solution and 5 ml of conc. $HNO_3$, mix well and note that the brown colour is changed to blue. This test is given by the substances containing primary and secondary alcohol groups. The chromic ions oxidize the glycerol and in this process they are reduced to chromous ions which give the blue colour. This test is also given by reducing sugars, so before confirming glycerol be sure that the reducing sugars are not present.

**Quantitative Tests:**

**1. Determination of Iodine Number:**

The iodine number of a fat is the amount in gm. of iodine taken up by 100 gm. of fat. Not only iodine but also equivalent amounts of other halogens will add at double bonds; so bromine is often used instead of iodine because it is more reactive. 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 determining 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 0.1 (N) $Na_2S_2O_3$ (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.

**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.

**Qualitative Test of Cholesterol:**

Cholesterol is a lipid with a structure quite different from that of phospholipids. It is a steroid, built from four linked hydrocarbon rings. A hydrocarbon tail is linked to the steroid at one end, and a hydroxyl group is attached at the other end. In membranes, the molecule is oriented parallel to the fatty acid chains of the phospholipids, and the hydroxyl group interacts with the nearby phospholipid head groups.

Cholesterol is absent from prokaryotes but is found to varying degrees in virtually all animal membranes. It constitutes almost 25% of the membrane lipids in certain nerve cells but is essentially absent from some intracellular membranes.

The main test for cholesterol is known as Liberman-Burchard test. This is carried in the following way. In a dry test tube take a small amount of solution of cholesterol in chloroform. Add 1 ml of acetic anhydride and 1 drop of conc. $H_2SO_4$. Mix and observe that a purple colour is formed which soon changes to green. It may take 15-30 min for full development and it is advisable to put the tube in dark during this time.

**Enzymatic Methods:**

Assays have been developed in which cholesterol oxidase obtained from the bacterium Nocardia erythropolis is used to convert cholesterol into cholest-4-en-3-one with the formation of Hydrogen peroxide. The

cholest-4en-3-one formed has been measured by reading at 240 nm after extracting into isopropanol. Alternatively, hydrogen peroxide has been quantified by formation of chelate complex with quadrivalent titanium and xylenol orange.

**Other Tests for Cholesterol:**

**1. Salkowski's Test ($H_2SO_4$ Test):**

Dissolve cholesterol in 2 ml of chloroform in dry test tube. Add equal amount of con. $H_2SO_4$. Shake gently. The upper layer turns red and the sulphuric acid layer shows a yellow colour with a green fluorescence.

**2. Formaldehyde-$H_2SO_4$ Test:**

Add 2 ml of formaldehyde-sulphuric acid solution (1 part of 40% formaldehyde to 50 parts of the acid) to 2 ml of chloroform solution in a dry test tube. The cherry colour is developed in the chloroform. Pour off the chloroform in another test tube and add 2-3 drops of acid anhydride. The blue colour develops.

# 10. SAMPLE COLLCETION

Samples may be taken from animals or their environment for the purpose of disease diagnosis, health monitoring or vaccine response monitoring. It is imperative on the part of a clinician to collect the most appropriate material from live or dead animals in order to get a timely and correct diagnosis of a suspected infectious disease. There may be a great variety of different combinations of samples and animal species. In order to collect the most suitable specimen, knowledge of infectious disease pathogenesis is the single most important factor. It is better to collect specimens from new cases of the disease in the face of an outbreak where animals may be seen in different stages of the clinical disease. In all cases in order to provide a statistically valid result, the samples need to be appropriate for the purpose required and adequate in number and amount.

To avoid undue stress or damage to the animal or danger to the operator, samples must be taken with care. For instance, it is not necessary to open a carcass suspected of being infected with Bacillus anthracis, but a drop of blood should be obtained from a superficial vein. Aseptic techniques are generally important to adopt, and care must be taken to avoid cross-contamination between samples. A number of intestinal bacteria may invade the host tissues just prior to death and shortly thereafter.

It must be carefully packaged, labeled and transmitted to the laboratory using the fastest practicable method after the appropriate material has been obtained. Relevant shipping laws must be followed. This laboratory must be consulted in advance to ensure that it is willing to receive the material if the material is sent to a laboratory in another country. All samples must be accompanied by a written note indicating the origin of the material, the historical background and the required tests.

**A. Equipment required for collection of samples**

(1). Sterile forceps, scissors, and scalpels.

(2). Sterile swabs

(3). Vials for containing transport medium for collection of samples for isolation or identification

(4). Bottles for collection of faeces, blood, and other samples that do not

require transport medium
(5). Bottles containing formalin saline for tissues to be examined histologically.
(6). Blood collection equipment- without additive for serum, and with anticoagulant for isolation
(7). Notebook and equipment for labeling specimens
(8). Swabs and transport medium for bacteriological investigation (9). Cool box (Thermos flask)
(10). Heavy duty plastic bags for postmortem material.

## B. COLLECTION OF SAMPLES

### 1. Tissues (in general):

For the post-mortem examination of the species of animals with which they work, animal health personnel should be trained in the correct procedures. The required equipment will depend on the size and species of the animal, but for the opening of the intestines, a knife, saw and cleaver will be required, as well as scalpels, forceps and scissors, including scissors with a rounded tip on one blade.

There must be an abundant supply of containers appropriate to the nature of the sample required, as well as labels and reporting forms. For transporting samples from the field, special media may be required. Protective clothing should be worn by the operator: overalls, rubber gloves and rubber boots. It is common to detach the animal's head if rabies is suspected, and the operator should wear a face mask and goggles, gloves, and a plastic apron.

Tissues may be collected for culture or histopathology and occasionally in serological tests for use as an antigen. In the post-mortem technique, the person removing the tissues should be experienced and have sufficient pathology knowledge to select the right organs and the most promising lesions for sampling.

Using ordinary instruments, the skin of the dead animal may be removed, but the body cavities should be opened with sterile instruments, and a fresh set of sterile instruments should be used to collect the required pieces of the various organs. Each piece of tissue should be placed in a separate plastic bag or sterile screw-capped jar, fully labeled with the date, tissue and identification of the animal. It is necessary to take care not to

contaminate one tissue with another. Instruments can be heated with portable packs of liquid gas on a burner or by lighting a fire using local fuel. To be sampled for bacterial culture or virus isolation, disinfectants must not be used on or near tissues.

**2. Blood**

Blood samples can be taken for haematology or culture purposes and/or for direct examination of bacteria, viruses, or protozoa, in which case anti-coagulants such as heparin are added to the blood. They may also be taken for serology, which requires a clotted sample. A blood sample is taken through venepuncture, as cleanly as possible.

The jugular vein or caudal vein is chosen in most large mammals, but brachial veins and mammary veins are also used. A wing vein (brachial vein) is generally selected in birds. Blood can be taken through a syringe and needle or through a needle and vacuum tube (not easy in delicate veins but convenient in strong veins). Ideally, the skin should be shaved (plucked) and swabbed with 70 percent ethyl alcohol at the venepuncture site first and allowed to dry.

In order to reduce bacterial growth, whole blood samples can have antibiotics added, taking care that the antibiotics are chosen to avoid interference with the growth of the pathogens concerned. Thorough mixing is required for samples with anti-coagulant and/or antibiotics as soon as the sample has been taken. A smear of fresh blood on the microscope slide may also be required.

A smear of fresh blood on the microscope slide may also be required. Blood should be left to stand at room temperature for serum samples (but protected from excessive heat) until the clot begins to contract. With a rod, the clot can then be ringed round and the bottles then placed at 4o C in a refrigerator. Later, after centrifugation, the serum can be decanted or removed.

The use of chemical preservatives such as boric acid or merthiolate in sera in virus neutralization tests should be avoided. An alternative method is to transport on a filter paper disk a drop of dried blood that contains sufficient material for sensitive antibody assay systems.

**3. Faeces:**

It is necessary to select freshly voided faeces and send them with or

without a means of transport. Taking swabs from the rectum (or cloaca), taking care to swab the mucosal surface, is an alternate and sometimes preferable method. It is also possible to transport swabs either dry or in transport media. In order to reduce air and prevent hatching of parasite eggs, Faeces for Parasitology should fill the container.

**4. Skin**

Samples are taken from the lesions themselves in diseases which produce vesicular rashes or where the lesions are exclusively in the skin. Scrapings of the lesion can be taken, and where unruptured vesicles are present, the vesicular fluid should also be sampled.

**5. Genital tract:**

Samples may be taken by washing them vaginally or prepucially, or by using appropriate swabs. The cervix or urethra is also sometimes sampled through swabbing.

**6. Nasal discharge (saliva, tears):**

Samples may be taken by soaking cotton swabs that are wetted with transport medium and sent to the laboratory at 4º C.

**8. Milk:**

Samples of milk should be taken after cleansing the tip of the teat. The initial stream of milk is discarded and a tube filled with the next stream(s). In severe mastitis, there may be little fluid present.

**9. Environment:**

Samples may be taken to monitor hygiene or as part of a disease inquiry, for example, from litter, ventilation ducts, feed troughs, drains, soil, hatcheries and slaughter houses.

**10. Serum:**

Serum samples are the most commonly collected specimens from live animals for conducting various serological tests. Generally serum samples early in the course of disease (acute, within 1-4 days) and during convalescence (convalescent, around 21 days) are collected. Such samples

are called paired serum samples and are used to demonstrate the rising antibody titre. In comparing the antibody titres of acute and convalescent phase sera, a minimum four fold rise is considered significant.

**C. SELECTION OF SAMPLES**

Considerable skill and care are required to decide on the correct samples to be sent to the laboratory. Frequently a combination of blood samples for serology and tissues from dead or culled animals for microbiological culture will be required. Also, it is usually important to collect tissues for fixation for histology.

**D. SAMPLE SIZE**

There are some general statistical rules which should be borne in mind, particularly when sampling herds or flocks for a health surveillance scheme. It is possible to calculate how many animals must be sampled from a herd/flock of a certain size, to achieve a 95% probability infection assumed to be present in a certain percentage of the animals.

**E. INFORMATION TO BE SENT WITH SAMPLES**

Information and case history should always accompany the samples to the laboratory, and ideally should be placed in a plastic envelope. The information should include the following points:

1. Name and address of owner/occupier where disease occurred, with telephone and fax numbers,
2. Disease suspected,
3. Samples submitted and tests required (transport medium used),
4. Different species on the farm and number, age and sex of each affected animal,
5. Length of time on the farm; if recent arrival, where from,
6. Date of first cases and of subsequent cases or losses,
7. Description of the spread of infection in the herd or flock,
8. Number of animals dead, the number showing clinical signs, and their age, sex and breed,
9. The clinical signs and their duration including the condition of mouth, eyes and feet, and milk or egg production data,
10. Type and standard of husbandry, including the type of feed available,

possible contact with poison or poisonous plants,
11. A list of description of the samples submitted for examination, and post-mortem findings,
12. Any medication already applied to the animals, and when given,
13. Any vaccination already given, and when given,
14. Name and address of sender, with telephone and fax number, and date of submission.

## F. TRANSPORT OF SAMPLES

Samples must be carefully packed, to avoid any possibility of leakage or cross-contamination. They should be delivered within 48 hours and must be kept cool during transit. Some samples should not be frozen. Screw-capped bottles should be used and should be additionally sealed with adhesive tape or paraffin wax. Samples in individually identified containers should be placed in larger strong, outer containers and packed with enough absorbent material to protect from damage. Official shipping regulations must be consulted. It is advisable to contact the laboratory in advance in the case of unusual requests. It is essential to do so, where material is sent to a laboratory in another country. Many countries require a special import license to be obtained in advance for any biological material, especially for tissues which could contain animal pathogens. This should accompany the package and be attached in an envelope to the outside of the parcel.

## G. PRESERVATION OF SPECIMENS

Various preservatives are used for different specimens, e.g. phosphate buffered glycerin for tissues; EDTA, sodium citrate, heparin or OCG mixture for whole blood and transport media (TPB) for swabs. The preserved specimens are most frequently transported on ice in a thermos flask or other suitable containers.

### a. Phosphate buffered glycerin (PBG):

It is prepared by mixing equal parts of phosphate buffered saline and neutral glycerin (pH 7.0).

| | |
|---|---|
| Phosphate buffered saline | (PBS) |
| Sodium chloride (Nacl) | 8.00 g |
| Pot. chloride(Kcl) | 0.20 g |

| | |
|---|---|
| Di-Sod. hydrogen phosphate (Na2 HPO4) | 1.15 g |
| Pot. dihydrogen orthophosphate (KH2 PO4) | 0.20 g |
| Glass Dist. water | 1000 ml. |

The final pH of PBG sol. is adjusted between 7.2 to 7.4, before autoclaving.

**b. Oxalate-Carbolic acid-Glycerin (OCG) Mixture:**

| | |
|---|---|
| Potassium oxalate | 5.0 g |
| Phenol (Carbolic acid) | 5.0 g |
| Glycerin | 500 ml |
| Dist. water | 500 ml |

The pH is adjusted to 7.2 before autoclaving.

**c. Tryptose phosphate broth (TPB):**

It is used as a transport medium for nasal, eye, rectal swabs etc.

| | |
|---|---|
| Tryptose (Difco) | 20.0 g |
| Dextrose | 2.0 g |
| Sod. chloride | 5.0 g |
| Di-sod. hydrogen phosphate | 2.5 g |
| Dist. water | 1000 ml. |

The pH is adjusted to 7.2-7.4 before autoclaving. Antibiotics (Penicillin, Streptomycin, Mycostatin) are added before collection of swabs to check bacterial contamination.

# 11. ANTICOAGULANTS

Anticoagulants are medicines that prevent the blood from clotting as quickly or as effectively as normal. Some people call anticoagulants blood thinners. However, the blood is not actually made any thinner - it just does not clot so easily whilst you take an anticoagulant.

Anticoagulants are used to treat and prevent blood clots that may occur in your blood vessels. Blood clots can block blood vessels (an artery or a vein). A blocked artery stops blood and oxygen from getting to a part of your body (for example, to a part of the heart, brain or lungs). The tissue supplied by a blocked artery becomes damaged or dies, and this results in serious problems such as a stroke or heart attack. A blood clot in a large vein, such as a clot in a leg vein - a deep vein thrombosis (DVT), can lead to serious problems. For example, it can lead to a clot that travels from a leg vein to the lungs (a pulmonary embolism). Anticoagulants are used to prevent blood clots as well - the most common condition for this is atrial fibrillation (AF).

**EDTA**

Salt of ethylene diamine tetraacetic acid. Dipotassium (K2), tripotassium (K3) (41) and disodium (Na2) salts are used; concentrations: 1.2 to 2.0 mg/mL blood (4.1 to 6.8 mmol/L blood) based on anhydrous EDTA

**Citrate**

Trisodium citrate with 0.100 to 0.136 mol/L citric acid. Buffered citrate with pH 5.5 to 5.6: 84 mmol/L tris odium citrate with 21 mmol/L citric acid. Differences were noticed between 3.2% and 3.8% (v/v) citrate when reporting results in INR (1, 145, 192, 210). WHO and NCCLS recommend 0.109 mol/L (3.2%) citric acid. The International Society for Thrombosis and Haemostasis (ISTH) recommends the use of Hepes buffered citrate for all investigations of haemostastic functions.

a. A mixture of one part citrate with nine parts blood is recommended for coagulation tests.

b. One part citrate mixed with four parts blood is recommended to

determine the erythrocyte sedimentation rate.

**Heparinates**

12 to 30 IU/mL of unfractionated sodium, lithium or ammonium salt of heparin with a molecular mass of 3 to 30 kD is recommended to obtain standardized heparinized plasma.

Calcium-titrated heparin at a concentration of 40 to 60 IU/mL blood (dry heparinisation) and 8 to 12 IU/mL blood (liquid heparinisation) is recommended for the determination of ionized calc ium

**Hirudin**

Hirudin is an antithrombin extracted from leeches or prepared by a genetic engineering process. Hirudin inhibits thrombin by forming a 1:1 hirudin-thrombin complex. Hirudin is used at a concentration of 10 mg/L

. The colour codes of anticoagulants described in ISO/DIS 6710 are:

EDTA = lavender/red; citrate 9 + 1 = light blue/green;

citrate 4 + 1 = black/mauve;

heparinate = green/orange;

no additives (for serum) = red/white (86).

**Sodium Citrate**

Citrate is used as trisodium citrate salt.

It is a white hygroscopic crystalline powder.

**Purpose**:

Sodium citrate is widely used for coagulation studies.

For PT and PTT.

The sample can be used for ESR by the Westergren method.

**Mechanism of action:**

it is used in solution form.

This will chelate calcium. Inactivates Ca++ ions.

This will prevent the rapid deterioration of labile coagulation factors like factor V and factor VII.

Sodium citrate mechanism as an anticoagulant

**Solution preparation and uses:**

Trisodium citrate= 3.2 to 3.8 g/dL (3.2% solution).

Mix well Trisodium citrate 3.8 grams in distle water.

This can be used as 0.109 mg/mL.
In blood, its ratio is 1:9 where 9 parts are blood and 1 part is sodium citrate.
PT and PTT= Blood: Sodium citrate = 9: 1 part (blood 9 parts: sodium citrate 1 part)
ESR = Blood: Sodium citrate = 4:1 (1.6 mL of blood: o.4 mL Sodium citrate).

**Potassium Oxalate**

This may be sodium, potassium, ammonium, or lithium oxalic acid salt used as an anticoagulant.
This form insoluble complex with calcium ions (precipitate with calcium as a salt).
Potassium oxalate mechanism as an anticoagulant

This is the most popular oxalate salt used as an anticoagulant in powder form.
Solution:

Potassium oxalate at a concentration of 1 to 2 mg/mL of blood is used.

Mix 30 grams/dL in distal watr.

Now add a few drops in the test tube side and dry it in the oven below 100 °C.

The combination of ammonium/potassium oxalate does not lead to shrinkage of the RBCs.

While other oxalates cause shrinkage.

**Sodium Fluoride**

This is a weak anticoagulant but used an antiglycolytic agent to preserve the glucose.

This inhibits the system involved in glycolysis and preserve the glucose.
This can be used as a dry additive.

Mechanism of action: It acts in two ways:
As an anticoagulant by binding the calcium.
As an enzyme inhibitor which prevents the glycolytic enzyme to destroy the glucose.

Sodium fluoride mechanism as an anticoagulant

Solution:

This is effective at a concentration of 2 mg/mL of blood along with another anticoagulant like potassium oxalate.

When used alone then more concentration than 2 mg/mL is needed.

This can be used in combination with oxalate as a fluoride-oxalate mixture.

Most specimens are preserved at 25 °C for 24 hours and at 4 °C for 48 hours.

Sodium fluoride is poorly soluble so mix blood thoroughly before effective anti-glycolysis occurs. This is mostly used for glucose estimation.

| Order of Draw | Tube Stopper Color | Additive | Dept. | Tests | Liquid Part post - centrifugation |
|---|---|---|---|---|---|
| 1 | Yellow  | Sodium polyethanol sulfonate (SPS) | Microbiology | Blood Culture | Plasma |
| 2 | Light Blue  | Sodium Citrate | Coagulation | PT, PTT | Plasma |
| 3 | Red (plain)  | No additive | Tube Blood Bank | Type, RH, antibody screen, type & crossmatch | Serum |
| 4 | Red & Grey or Gold  | Clot Activator | Routine Chemistry | All STAT tests + Iron, folate | Serum |
| 5 | Green  | Heparin | STAT Chemistry | BMP, CMP, Glucose, K, Troponin, Bilirubin | Plasma |
| 6 | Lavender  | K2EDTA | Hematology | CBC, ESR | Plasma |
| 7 | Pink  | EDTA | Gel Blood Bank | Type, RH, antibody screen, type & crossmatch | Plasma |
| 8 | Gray  | Sodium Flouride (inhibits glycolysis) | Chemistry | Lactic Acid, Gluc (not run right away) | Plasma |

**Sodium Iodoacetate**

This is also an antiglycolytic agent at a concentration of 2 g/L.

This may be substituted for sodium fluoride.

This has no effect on urease.

Drawback:

It inhibits creatine kinase.

Adverse effects of the additives:

The additive may contain the substance to be tested like Na+oxalate for the

estimation of Na+.

The additive may remove the component to be tested like in oxalate, removes the calcium.

The additive may affect enzymes like Na+flouride. This may destroy many enzymes.

A small amount of the anticoagulant gives rise to microclots and this will interfere with cell count.

The additive may distort the cells like oxalate will change the cell morphology like RBCs and these will become crenated. While WBCs show vacuoles. Lymphocytes and monocytes will have distorted shapes.

If the excess quantity is used that will dilute the substance to be tested.

# 12. TYPE OF COLLECTION PROCEDURES

**Capillary blood (skin puncture)**

This is good for a small quantity of blood. Warm the finger taking the blood sample.

In the newborn, under 3 months the heel is the best site to get a small quantity of blood.

The depth should not be >2.4 mm on the heel.

Avoid the central portion and back of the heel.

**Venous blood (venipuncture):**

For larger quantities, venous blood will be taken.

The blood sample is taken from the forearm, wrist, or ankle veins.

A forearm site is preferred. Blood is taken directly from the vein, called phlebotomy.

The median cubital vein is usually preferred.

Mostly venous blood is drawn in the fasting state.

Blood collected after the meal is called a postprandial sample.

There are biological variables in the blood collection like:

Patient lying in the bed or standing up.

After the exercise.

Diurnal variations.

Recent food intake.

Recent intake of Tea/coffee (caffeine), smoking (nicotine), alcohol ingestion, and administration of the drugs.

**Various Blood Samples:**

**Whole Blood**

This blood sample obtained in the test tube containing an anticoagulant.

This sample will contain cells (white blood cells, platelets, RBCs, proteins) and plasma.

**Plasma**

This is a pale yellow liquid which contains RBCs, white cells, and platelets. Plasma forms with the help of anticoagulants which will prevent the clotting.

There is the presence of fibrinogen in the plasma.

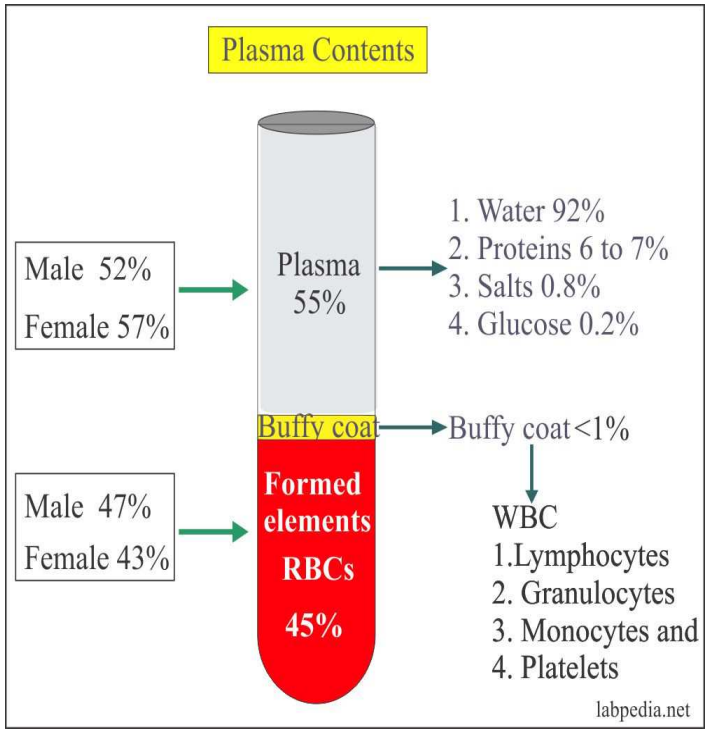
**Serum**

This is a clear fluid that is separated from the clotted blood. There are no RBCs, white cells, or platelets. There is no need for anticoagulants.

Clotted blood is kept at 37 C for at least 20 minutes and then centrifuged.

The upper portion is called serum.

There is no fibrinogen. serum and plasma difference



## 13. PROXIMATE ANALYSIS

**Moisture**

One gram sample of samples were taken in a Petri dish. The weight of petridish with sample was taken. Then it was placed in the oven at 60ºC for 12 hours or until it is dried. The dried samples were transferred to desiccators for minutes and weighted. The samples were again kept in oven for one to two hours until constant weight (W2) was obtained. The loss in weight was recorded as moisture.

$$\text{Moisture (\%)} = \frac{W1-W2}{W3} \times 100$$

Where,

W1 = weight of Petri dish + sample before drying;

W2 = weight of Petri dish + sample after drying;

W3 = weight of the sample.

**Total ash**

The total ash was determined by burning 2g of feed in a pre-weighed China dish and then samples were placed in a muffle furnace for ignition at 550 – 600°C till residue was obtained after 4 – 5 hours. Then the sample residue were placed in desiccators to cool and weight was recorded. Percentage of ash was obtained by using the following formula:

$$\text{Total ash (\%)} = \frac{\text{Wt. of ash}}{\text{Wt. of feed}} \times 100$$

**Dry matter**

Dry matter content was determined by difference between fresh weight of sample and moisture content. The crude protein, crude lipid, crude fibre, nitrogen free extract (NFE), ash contents were calculated on % dry matter basis. Dry matter percentage was calculated by the following.

Dry matter (%) = 100 - moisture (%)

**Nitrogen free Extract**

The nitrogen free extract

NFE = 100-(% crude protein + % of crude lipid + % of moisture + % ash).

# 14. ESTIMATION OF REDUCING SUGAR BY BENEDICT'S METHOD

Principle

Benedict's quantitative reagent is a modification of qualitative. It contains copper sulphate, sodium acetate and sodium corbonate. It also contains potassium thio cyanate and small amount of potassium ferricyanide. The inclusion of acetate prevents the precipitation of copper carbonate by chelating $Cu^{3+}$ion. The thiocyanate causes the precipitation of white cuprous thio cyanate rather than red cupric oxide. On reduction of $Cu^{3+}$ ions which enables the end point of the titration ie., the transition from blue to white to be readily observable. Methylene blue will be used as an additional indicator. The small amount of potassium ferricyanide prevents the re-oxidation of copper. A non-stoicheometric reaction is on which does not follow a defined pathway and cannot be described by an equation either quantitatively or qualitatively. The reduction of $Cu^{3+}$ ions by sugar is a non-stoicheometric equation and is only constant over a small range of sugar concentration. To obtain accurate results the volume of sugar added must be with in 6- 12 ml for 10 ml of benedict's reagent. If the preliminary titre value Falls outside this range the sugar solution must be titrations are repeated.

Reagents Required:

**Standard Glucose Solution:**

200 mg of glucose was weighed accurately and made upto 100 ml with distilled water (concentration: 2 mg / ml)

**Benedict's Quantitative Reagent**

100 ml of solution acetate, 37.5 g of sodium carbonate and 62.5 g of potassium thiocyanate were dissolved in 300 ml of distilled water by warming gently and filtered. 9 g of copper sulphate is dissolved in 50 ml of water, added with continuous stirring. 2.5 ml of potassium ferricyanide is added and the volume is made upto 500 ml with water.

**Anhydrous Sodium Carbonate**

**Procedure**

100 ml of benedict's reagent was pipetted out into a clean conical flask. About 600 mg of anhydrous sodium carbonate was added to

provide the required allcaling with A few porcelain bits and heated to boiling over a moderate flame.

The standard glucose solution is taken in the burette when the benedict's solution Boils, glucose solution is added drop by drop (one drop per second) till the last trace of blue colour disappears. The volume of glucose rundown is noted and the titrations are repeated for concordant value.

The given unknown sugar solution was made upto 100 ml in a standard flask with distilled water. Then the burette was filled with unknown sugar solution and the benedict's reagent was titrated as before. The volume of sugar solution rundown was noted and titrations are repeated for concordant values.

Estimation Of Reducing Sugar By Benedict's Method

Titration 1

Standardisation Of Benedict's Reagent Benedict's Reagent Vs Standard Glucose Solution

| S.No | Volume of Benedict's reagents(ml) | Burette Readings | | Volume of standard glucose(ml) | Indicator |
|---|---|---|---|---|---|
| | | Intial ml | Final ml | | |
| | | | | | Self |

TITRATION 2:

Estimation Of Glucose Standardised Benedict's Reagent Vs Unknown Glucose

| S.No | Volume of Benedict's reagents(ml) | Burette Readings | | Volume of unknown glucose(ml) | Indicator |
|---|---|---|---|---|---|
| | | Intial ml | Final ml | | |
| | | | | | Self |

**Calculation:**

The standard glucose solution 2 mg / ml
5 ml of Benedict's solution react with……..ml of the standard glucose solution.

…….ml of standard glucose solution which contains x 2 = mg
5 ml of Benedict's solution reacts with……..
mg of unknown glucose 100 ml of unknown glucose contains is
100 x……..

**Result:**

The amount of glucose present in 100 ml of given unknown solution is……..

## 15. ESTIMATION OF PROTEIN BY LOWREY'S METHOD

**Principle:**

Protein in the given solution when treated with alkaline copper sulphate and Folin's phenol reagent produces a blue colored complex. The intensity of the colour is directly proportional to the concentration of protein present in the given sample solution.

**Reagents Required**

Stock Solution:

Bovine serum albumin of 100mg is weighed accurately and dissolved in 100ml of distilled water in a standard flask. (Concentration:1mg/ml)

Working Standard:

The stock solution of 10ml is diluted to 100ml with distilled water in a standard flask. (Concentration:100mg.ml)

Folin;s Phenol Reagent:

Folin's phenol reagent is mixed with distilled water in a the ratio 1:2

Alkaline CuS04 Reagent: Solution A:

Sodium carbonate of 2% in 0.1N sodium hydroxide.

Solution B: Sodium Potassium tartrate of 1%

Solution C: Copper sulphate of 0.5%

Solutions A, B, C are mixed in the proportion of 50:1:0.5

Unknown Preparation:

The given protein is made upto 100ml with distilled water.

**Procedure:**

Working standard of 0.2 to 1.0ml is pipetted out into clean test tubes labelled as S1, to S5 . Test solution of 0.2 and 0.4 ml is taken in test tubes labelled as T1 and T2. The volume is made upto 1.0ml with distilled water. Distilled water of 1.0ml serves as blank. To all the test tubes 4.5 ml of alkaline copper sulphate reagent is added and it is incubated at room temperature for 10 minutes. To all the test tubes 0.5ml of Folin's Phenol reagent is added. The contents are mixed well and the blue colour developed is read at 640nm after 15 minutes. From the standard graph the amount of protein in the given unknown solution is calculated.

## Estimation of Protein by Lowrey's Method

| S.No | Reagents | B | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $T_1$ | $T_2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Volume of working standard (ml) | - | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | - | - |
| 2 | Concentration of working standard (mg) | - | 20 | 40 | 60 | 80 | 100 | - | - |
| 3 | Volume of unknown solution (ml) | - | - | - | - | - | - | 0.2 | 0.4 |
| 4 | Volume of distilled water (ml) | 1.0 | 0.8 | 0.6 | 0.4 | 0.2 | - | 0.8 | 0.6 |
| 5 | Volume of alkaline copper reagent (ml) | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
| 6 | Volume of Folin's phenol reagent | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| The contents are mixed well and kept at room temperature for 10 minutes. The blue colour developed is read at 640nm | | | | | | | | | |
| 7 | Optical density 640nm | | | | | | | | |

**Calculation:**

……of unknown solution corresponds to……..xOD

OD corresponds of …….. mg of protein

i.e. 0.2ml of unknown solution contains …….of protein 100ml of unknown solution contains

100 x ----

**Result:**

The amount of protein present in the given solution is……

## 16. ESTIMATION OF CHOLESTEROL BY ZAK'S METHOD

To estimate the amount of cholesterol in an unknown food sample.

**Principle:**

Cholesterol in glacial acetic acid gives a red colour with ferric chloride and apolar sulphuric acid. This reaction has been employed by ZAK'S to estimate the cholesterol in an unknown food sample.

**Reagents Required:**

Stock Standard Solution :

About 100 mg of cholesterol was dissolved and made up to 100 ml with glacial acetic acid (concentration 1 mg / ml).

Working Standard :

About 4 ml of stock solution was made up to 100 ml with ferric chloride acetic acid reagent (concentration in 40 mg / ml).

Ferric chloride of 0.05% in acetic acid.

Apolar sulphuric acid.

Glacial acetic acid.

**Preparation of unknown sample:**

20 ml of sample and 40 ml of chloroform was added and centrifuged. The supernatant was used for estimation.

**Procedure:**

0.5 ml to 2.5 ml of working standard were Pipetted out into a clean test tubes. About of 0.5 ml and 1 ml of unknown food sample supernatant was taken in a test tubes. The volume was made upto 5.0 ml with ferric chloride and 3.0 ml of concentrated sulphuric acid were added. The test tubes were kept at room temperature for 15 minutes. The pinkish red colour formed was measured at 540 nm. Standard graph was drawn for the values obtained. From the standard graph the amount of cholesterol present in the food sample can be calculated.

**Estimation of Cholesterol by Zak's Method**

| S.No | Reagents | B | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $T_1$ | $T_2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Volume of standard chloresterol (ml) | _ | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | _ | _ |
| 2. | Concentration of cholesterol (mg) | _ | 20 | 40 | 60 | 80 | 100 | _ | _ |
| 3. | Volume of sample supernatant (ml) | _ | _ | _ | _ | _ | _ | 1 | 1 |
| 4. | Volume of 0.05% ferric chloride acetic acid reagent (ml) | 5 | 4.5 | 4 | 3.5 | 3 | 2.5 | _ | _ |
| 5. | Volume of cone sulphuric acid (ml) | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | | Incubated the tubes for 15 minutes at room temperature | | | | | | | |
| 6. | O.D at 540 nm | | | | | | | | |

**Calculations**

0.5 ml of standard corresponds to O.D 0.03 O.D corresponds to……..mg.
0.5 ml of unknown corresponds 100 ml of unknown corresponds to.....
100x……..

**Result:**

The amount of cholesterol present in unknown food sample was found to be…….

# 17. ESTIMATION OF DNA BY DIPHENYLAMINE METHOD

**Principle:**

The deoxyribose of DNA in the presence of acid forms hydroxyl levolinic aldehyde which reacts with diphenylamine to give a blue colour. But only the deoxy ribose of purine nucleotide react.

**Reagents required:**

Stock standard solution: Weighed 100 mg of DNA and dissolved in 100 ml of distilled water.

Working standard solution: 10 ml of stock was diluted to 100 ml using distilled water.

Diphenyl amine reagent:

It should be prepared freshly by dissolving 1 gm of diphenyl amine in 100 ml of glacial acetic acid and by adding 2.5 ml of concentrated sulphuric acid.

**Procedure:**

Pipetted out 0.2 ml – 1.0 ml of DNA solution into a series of test tubes and made up the volume to 3.0 ml with distilled water. 0.2 ml and 0.4 ml of unknown is taken and made upto 3.0 ml with water. Added 5.0 ml of disphenylamine reagent, mixed well and is heated in a boiling water bath for 10 minutes. Cooled and the colour developed is read at 595 nm.

**Calculations**

0.2 ml of unknown corresponds to 0.02 O.D

---O.D corresponds to ----

0.2 ml of unknown corresponds to ----

100 ml of unknown corresponds to = 100x……..

**Results:**

The amount of DNA present in 100 ml of given unknown solution is……

## Estimation of Dna by Diphenylamine Method

| S.No | Contents | B | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $U_1$ | $U_2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Volume of working standard in (m1) | - | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | - | - |
| 2. | Concentration in □ g | - | 20 | 40 | 60 | 80 | 100 | - | - |
| 3. | Volume of unknown in (m1) | - | - | - | - | - | - | 0.2 | 0.4 |
| 4. | Volume of distilled water in (ml) | 3 | 2.8 | 2.6 | 2.4 | 2.2 | 2.0 | 2.8 | 2.6 |
| 5. | Volume of diphenyl amine reagent in (ml) | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | | Heated in a boiling water bath for 10 minutes | | | | | | | |
| 6. | Optical density at 595 mm | | | | | | | | |

# 18. SEPARATION OF AMINO ACIDS BY THIN LAYER CHROMATOGRAPHY

**Principle:**

Chromatography is a method by which a mixture of substances in smaller quantities can be separated both qualitatively and quantitatively. In chromatography there are twophases-the stationary phase and other mobile phase. When the mobile phase moves along stationary phase, separation of substances takes place. In thin layer chromatography, the thin layer of gel functions as an inert supporting material. When the mobile phase moves along the gel solvent, as the partition coefficient differ for different sugars, the rate of flow differs and therefore separation occurs.

**Materials Required:**

Silica gel G 2.Microscopic slides 3.n-butanol

Acetic acid

Spraying reagent (0.3% solution of Ninhydrin in butanol containing 3 ml acetic acid)

Amino acids

**Procedure:**

A slurry of silicagel G is prepared in 0.02M sodium acetate buffer. Taken the microscopic slides and keeping them flat, pipetted out about 1-2 ml of the slurry into them. By tilting the slides spread the slurry evenly on the surface. Lining the edges with Vaseline will be of help. Allowed the slides to dry completely leaving them flat. 5□l samples of amino acid (or mixture) are spotted and the slide is then dipped in a trough containing n-butanol-acetic acid-water in the ration 8:2:2. The slide must be handled with care not to break the surface. After development, that is, when the solvent has reached the top, the slide is dried and sprayed with the developing reagent. The slide is then heated in an oven at 1100 C for 10 minutes and Rf values of the spots are measured.

$$Rf = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

**Result:**

The given sample Rf value is ……

# 19. SEPARATION OF SUGARS BY PAPER CHROMATOGRAPHY

**Principle:**

Chromatography is a method by which a mixture of substances in smaller quantities can be separated both qualitatively and quantitatively. In chromatography there are two pahses the stationary phase and other mobile phase. When the mobile phase moves along stationary phase, separation of substances takes place. In paper chromatography the paper functions as an inert supporting material. When the mobile phase moves along the paper solvent, As the partition coefficient differ for different sugars the rate of flow differs and therfore separation occurs.

**Materials Required:**

Whatmann No :1 filter paper 2.N-butanol

Glacial acetic acid 4.Sugars

Spraying reagent-Alkaline permanganate spraying agent 0.1% KmnO4 in 2% sodium carbonate

**Procedure:**

3 strips of whatman No 1 filter paper of size 12□ 2 cm are cut. A line is drawn at a distance of 2.5 cm from the base and a pencil mark made at the mid point. Sugar solutions of glucose and fructose at a concentration of 100 mg/10ml is prepared. The chromatography paper is placed on a box having a slit with lighting arrangment underneath the slit. Spotting is done on the paper using a micropipette. Care is taken to see that the spot does not spread beyond a particular diameter. 10ml each glucose and fructose are spotted on a paper A and B. To the strips a mixture is applied. The three strips are placed in three different boiling tubes each containing 5ml of n-butanol acetic acid, water in the ration 4:2:1. The boiling tubes are plugged with cotton, The paper are kept in a a pre-saturated chromatographic chamber and the solvent is allowed to rise. When the solvent front has reached three fours of the paper the strips are removed and air dried. It is then sprayed with the spraying agent and dried in hot air oven at 1000C.

The sugars appeared as yellow spots in a purple background. The distances travelled by the solvent are mesured. The distance from the base line to the centre of each spot are measured, Rf values is then calculated as

follows.

$$Rf = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

**Result:**

The given sample Rf value is ……

# 20. ESTIMATION OF CSF SUGAR BY TRINDERS METHOD (GLUCOSE OXIDASE PEROXIDASE METHOD)

**Principle**

Glucose in the presence of atmospheric oxygen is completely oxidised by enzyme glucose –oxidase to gluconic acid & hydrogen peroxide. The hydrogen peroxide formed is broken down by peroxidase enzyme to water & oxygen. The latter oxidizes phenol which combines with amino -4-antipyrine to give a red coloured complex quinonimine The intensity of red colour is proportional to the concentration of glucose in the sample and the intensity of colour is measured colorimetrically at 520 nm using green filter

**Enzyme reagent**

Consisted of amino 4 antipyrine (0.125 m mol /L) glucose (30000 m/l ) Peroxidase (10000 m/1) & phosphate butter (100 m mol /L)
Phenol (16 m mol /l)
Standard glucose (5.55 m mol /L )
Preparation of working enzyme reagent

The enzyme reagent was prepared in 500 ml distilled water to which 5 ml of phenol reagent was added. It was mixed gently & used after 3 hours.

**Procedure**

Three clean dry test tubes were marked as T for test S for standard & B for blank & following solutions were added in ml in given order.

| Reagent | Test (ml) | Standard (ml) | Blank (ml) |
|---|---|---|---|
| CSF Reagent | 0.02 | - | - |
| DIW | - | 0.02 | - |
| Working Reagent | 3.0 | 3.0 | 3.0 |

All the contents of the test tubes were mixed & were incubated for 15 minutes at 37 0C. After incubation 2 ml DW was added, mixed well & O.D of test & standard were measured against blank using green filter (520nm).

**Result**:

The total CSF sugar is present in the given sample ……

## 21. ESTIMATION OF RNA BY ORCINOL REACTION

**Principle:**

This is a general reaction for pentoses and depends on the formation of furfural when the pentose is heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour, which can be measured at 665 nm.

**Requirements**:

Standard RNA solution- 200µg/ml in 1 N perchloric acid/buffered saline.

Orcinol Reagent- Dissolve 0.1g of ferric chloride in 100 ml of concentrated HCl and add 3.5 ml of 6% w/v orcinol in alcohol.

Buffered Saline- 0.5 mol/litre NaCl; 0.015 mol/litre sodium citrate, pH 7.

**Procedure:**

Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled test tubes.

Pipette out 1 ml of the given sample in another test tube.

Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.

Now add 2 ml of orcinol reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.

Mix the contents of the tubes by vortexing / shaking the tubes and heat on a boiling water bath for 20 min.

Then cool the contents and record the absorbance at 665 nm against blank.

Then plot the standard curve by taking concentration of RNA along X-axis and absorbance at 665 nm along Y-axis.

Then from this standard curve calculate the concentration of RNA in the given sample.

**Result:**

The given unknown sample contains ----------µg RNA/ml.

| S.No | Volume of Standard (200 µg/ml) RNA | Volume of distilled water | Conc. of RNA (µg) | Vol. of Orcinol Reagent (ml) | | OD 665 nm |
|---|---|---|---|---|---|---|
| 1. | 0.0 | 1.0 | 0 | 2 | | |
| 2. | 0.2 | 0.8 | 40 | 2 | | |
| 3. | 0.4 | 0.6 | 80 | 2 | Incuate in boiling water bath for 20 min & Cool | |
| 4. | 0.6 | 0.4 | 120 | 2 | | |
| 5. | 0.8 | 0.2 | 160 | 2 | | |
| 6 | 1.0 | 0.0 | 200 | 2 | | |
| 7 | Test 1.0 ml | 0.0 | To be estimated | 2 | | |

# 22. DETERMINATION OF REDUCING SUGARS BY NELSON-SOMOGYI METHOD

**Principle**

The reducing sugars when heated with alkaline copper tartrate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue color developed is compared with a set of standards in a colorimeter at 620nm.

**Materials**

Alkaline Copper tartrate

(A) Dissolve 2.54g anhydrous sodium carbonate, 2g sodium bicarbonate, 2.5g potassium sodium tartrate and 20g anhydrous sodium sulphate in 80mL water and make up to 100mL.

(B) Dissolve 15g copper sulphate in a small volume of distilled water. Add one drop of sulphuric acid and make up to 100mL. Mix 4mL of B and 96mL of solution A befire use.

Arsenomolybdate Reagent*:* Dissolve 2.5g ammonium molybdate in 45mL water. Add 2.5mL sulphuric acid and mix well. Then add 0.3g disodium hydrogen arsenate dissolved in 25mL water. Mix well and incubate at 37°C for 24 to 48 hours.

Standard Glucose Solution: *Stock:* 100mg in 100mL distilled water.

Working Standard: 10mL of stock diluted to 100mL with distilled water [100mg/mL].

**Procedure**

Weigh 100mg of the sample and extract the sugars with the hot 80% ethanol twice (5mL each time)

Collect the supernatant and evaporate it by keeping it on a water bath at 80°C.

Add 10mL water and dissolve the sugars.

Pipette out aliquots of 0.1 or 0.2mL to separate test tubes.

Pipette out 0.2, 0.4, 0.6, 0.8 and 1mL of the working standard solution into a series of test tubes.

Make up the volume in both standard and samp-le tubes to 2mL with distilled water.

Pipette out 2mL distilled water in a separate tuibe to set a blank.

Add 1mL of alkaline copper tartrate reagent to each tube.

Place the tubes in a boiling waterfor 10 minutes.

Cool the tubes and add 1mL of arsenomolybolic acid reagent to all the tubes.

**Calculate**

Absorbance corresponds to 0.1mL of test = 'x' mg of glucose

10mL contains = ('x' ÷ 0.1) × 10mg of glucose = % of reducing sugars

## 23. EFFECT OF DIFFERENT TEMPERATURES ON THE ACTIVITY OF SALIVARY AMYLASE ON STARCH

Principle

All enzymes are proteinaceous in nature. At a lower temperature, the enzyme salivary amylase is deactivated and at the higher temperature, the enzyme is denaturated. Therefore, more time will be taken by an enzyme to digest the starch at lower and higher temperatures. Optimum temperature for the enzymatic activity of salivary amylase ranges from 32 °C to 37 °C. The optimum temperature means that the temperature at which the enzyme shows the maximum activity. At this optimum temperature, the enzyme is most active and hence, takes less time to digest the starch.

Materials Required

Three series of test tubes having iodine solution in each, test tubes, ice cubes, water,

15 ml 1% starch solution + 3 ml 1% NaCl,

saliva solution, droppers, thermometer, Bunsen burner and wire gauze.

**Procedure**

Take beaker containing 15 ml of 1% starch solution + 3 ml of 1% NaCl solution.

Divide and pour this solution into three test tubes and mark them as A, B and C.

Maintain the temperature of the beaker containing ice cubes at 5°C.

Take beaker containing ice cubes and keep it on the table.

Take another two beakers containing water and heat over the Bunsen burner.

Now transfer experimental tube A into a beaker containing ice.

Transfer the second experimental tube B into water bath set at 37°C and third experimental tube C into the beaker maintained at 50°C.

Using a dropper, take 1 ml saliva solution and transfer the solution into test tube A.

Similarly, add 1 ml saliva solution into test tube B and test tube C.

Immediately, using a dropper, take few drops from experimental tube A and transfer this into first series of test tubes having iodine solution.

Similarly, using fresh droppers, do the same procedure for test tube B and test tube C and transfer the solution into second and third series of test tubes having iodine solution.

Note this time as zero minute reading.

After an interval of 2 minutes, again take a few drops from each tube and add to the iodine tubes and note the change in colour of iodine.

Keep on repeating the experiment at an interval of every 2 minutes till colour of iodine does not change.

**Results**

It takes less time to reach achromic point at 37°C, as the enzyme is maximum active at this temperature, while at higher and lower temperatures more time is taken to reach the achromic point.

# 24. EFFECT OF DIFFERENT PH ON THE ACTIVITY OF SALIVARY AMYLASE ON STARCH

**Principle**

The optimum pH for the enzymatic activity of salivary amylase ranges from 6 to 7. Above and below this range, the reaction rate reduces as enzymes get denaturated. The enzyme salivary amylase is most active at pH 6.8. Our stomach has high level of acidity which causes the salivary amylase to denature and change its shape. So the salivary amylase does not function once it enters the stomach.

**Materials Required**

Three series of test tubes having iodine solution
test tubes, pH tablets of 5, 6.8 and 8,
15 ml 1% starch solution + 3 ml 1% NaCl,
saliva solution, droppers, Bunsen burner and wire gauze.

**Procedure**

Take a beaker containing 15 ml of 1% starch solution + 3 ml of 1% NaCl solution.

Divide and pour this solution into three test tubes and mark them as A, B and C.

Add pH tablet 5 into test tube A, pH tablet 6.8 into test tube B and pH tablet 8 into test tube C.

Now transfer experimental tube A, B and C into a beaker containing water and a thermometer for recording temperature. Temperature of this beaker is to be maintained at 37°C.

Using a dropper, take 3 ml saliva solution and add 1 ml of solution to each of the three test tubes.

Immediately using a dropper, take few drops from experimental tube A and transfer this into the first series of test tubes having iodine solution.

Similarly, do the same procedure for test tube B and test tube C and transfer the solution into second and third series of test tubes having iodine solution.

Note this time as zero minute reading.

After an interval of 2 minutes, again take a drop from each tube and add to the iodine tubes and note the change in colour of iodine.

Keep on repeating the experiment at an interval of every 2 minutes till colour of iodine does not change.

**Results**

pH 5 is acidic and pH 8 is alkaline, therefore salivary amylase did not act in these tubes. Whereas, the enzyme acted in the tube with pH 6.8 (i.e., slightly acidic) and digested the starch.

## 25. ESTIMATION OF HAEMOGLOBIN BY SAHLI'S/ACID HEMATIN METHOD

**Principle**:

Blood is mixed with N/10 HCl resulting in the conversion of Hb to acid hematin which is brown in color. The solution is diluted till it's color matches with the brown colored glass of the comparator box. The concentration of Hb is read directly.

Equipment required

Hemocytometer which consists of z comparator box which has brown colored glass on either side

Hb pipette which is marked upto 20mm3(0.02ml blood) z Tube with markings of Hb on one side z glass rod

Dropper

**Reagents required**

N/10 HCl Distilled water Sample:

Venous blood collected in EDTA

**Procedure**

1. Add N/10 HCl into the tube upto mark 2g%
2. Mix the EDTA sample by gentle inversion and fill the pipette with 0.02ml blood. Wipe the external surface of the pipette to remove any excess blood.
3. Add the blood into the tube containing HCl. Wash out the contents of the pipette by drawing in and blowing out the acid two to three times. Mix the blood with the acid thoroughly.
4. Allow to stand undisturbed for 10min.
5. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop stirring with the glass rod till it's color matches with that of the comparator glass. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.
6. Remove the stirrer and take the reading directly by noting the height of the diluted acid hematin and express in g%.

# 26. ESTIMATION OF HAEMOGLOBIN BY CYANMETHEMOGLOBIN METHOD

**Principle:**

Blood is diluted in a solution containing potassium cyanide and potassium ferricyanide. The latter converts Hb to methemoglobin which is converted to cyanmethemoglobin (HiCN) by potassium cyanide. The absorbance of the solution is then measured in a spectrophotometer at a wavelength of 540nm or in a colorimeter using a yellow green filter.

**Equipment required**

Hb pipette
Spectrophotometer

**Reagents required**

Drabkin's solution pH7.0-7.4 which contains
Potassium cyanide 50 mg Potassium ferricyanide 200 mg
Potassium dihydrogen phosphate 140 mg
Nonionic detergent 1 ml
Distilled water 1 L

The solution should be clear and pale yellow in color. When measured against water as a blank in a spectrometer at a wavelength of 540 nm, the absorbance must be zero. The solution is unstable if exposed to light and can be stored at room temperature in brown borosilicate bottles for several months. However, if the room temperature is higher than 30°C, the solution should be stored in a refrigerator but brought to room temperature before use. The solution must never be frozen. The pH of the solution must be checked every month. Discard the solution if found to be turbid/if pH is outside range/ it's absorbance is not zero at 540 nm. Do not pipette Drabkin's solution by mouth. Sample: Venous blood collected in EDTA.

**Procedure**

1. Take 5ml of Drabkin's solution in a test tube.

2. Mix the blood sample by gentle inversion and draw 0.02ml of blood into the Hb pipette. Wipe the outer surface of the pipette to remove excess blood.

3. Place the pipette into the tube containing Drabkin's solution and slowly expel the blood into the solution. Mix well and let it stand undisturbed for 5min.

4. Measure the absorbance of this solution at 540nm in a spectrophotometer after adjusting the OD at 0 by using Drabkin's solution as blank.

5. Calculate the hemoglobin concentration using a standard curve.

# 27. ISOLATION OF CHLOROPLAST DNA

**Principle**

The DNA of plant cells is found in three distinct genomes. First, there is nuclear DNA familiar as the DNA that makes up the chromosomes, but mitochondria and chloroplasts each have DNAs of their own. These genomes are closed circular DNA molecules encoding many of enzymes necessary for the function of the organelles.

**Materials**

Extraction buffer (EB):
50 mM Tris pH 8.0
1% CTAB; 50 mM EDTA
1 mM 1,10 *o*-phenanthroline
0.7 M NaCl
0.1% β-mercaptoethanol
chloroform; isopropyl alcohol
80% ethanol
15 mM ammonium acetate pH 7.5.
TE buffer:
10 mM Tris pH 8.0
1 mM EDTA,
centrifuge tubes, water bath (65°C), centrifuge.

**Protocol**

Start with the frozen chloroplast preparation. This sample will have a volume of several mL. For each mL of chloroplasts, add 4 mL EB. If necessary, transfer the mixture to a capped centrifuge tube of at least twice the volume of the chloroplasts and EB.

Incubate the mixture at 65°C for 1 h on a water bath.

Remove the tube from the water bath and allow cooling on the bench for several minutes to proceed further.

Add an approximately equal volume of chloroform to the tube, recap, and mix by inversion.

Centrifuge at >3500*g* for 10 min.

The tube contents will be separated into two distinct layers. Transfer the upper (aqueous) layer into a fresh centrifuge tube with the help of an

ultra pipette. This tube should be of the same size as that used in the first step. The lower (organic) layer is hazardous water, dispose as per safety guidelines.

Add 0.6 mL of isopropanol for each mL of DNA containing extract in the centrifuge tube and mix by inversion.

Centrifuge at >10,000*g* for 20 min.

Decant liquid in the tube away from the DNA containing pellet. The tube's inside can be wiped carefully to remove liquid, but take care not to dislodge the DNA pellet.

**Result**

The yield of chloroplast DNA is expected to be low and will depend on the quality of the chloroplast preparation produced.

# 28. ISOLATION OF MITOCHONDRIA

**Principle**

Roots of pea seedlings are used to isolate mitochondria by differential centrifugation and assay the activity of a mitochondrial enzyme to assess the success of the isolation protocol. Differential centrifugation separates cell components based on differences in the rate at which they sediment in a centrifugal field. The first centrifugation step will remove whole cells, cell wall fragment, nuclei, starch, etc. Mitochondria, because of their small size, will not be sedimented by this step but will remain suspended in the supernatant. A subsequent spin at a higher speed will then be used to pellet the mitochondria to the bottom of the centrifuge tube.

**Materials**

Pea seedlings, cheesecloth/miracloth, centrifuge tubes,
Pasteur pipettes, small paint brush, ice bucket, blender
centrifuges
UV–Vis spectrophotometer.

Harvest pea root tissue, prepare extract, isolate mitochondria by differential centrifugation.

**Homogenization buffer:**

70 mM sucrose, 220 mM mannitol, 0.5 g/L BSA, 1 mM HEPES pH 7.4 homogenization buffer.

**Solutions**:

0.1 M potassium phosphate buffer, pH 7.4,
0.8 M ascorbic acid,
4% Triton X-100,
5 mg/mL cytochrome,
sodium dithionate crystals.

**Isolation of Mitochondria**

**Procedure**

All solutions and samples should be kept on ice while working.

Harvest 5 g of 7-day-old pea roots, shake off vermiculite in which they are growing, and rinse in distilled water in a beaker.

Chop roots into small pieces with a razor blade or scissors and put

into a chilled blender with 20 mL ice-cold homogenization buffer.

Homogenize the tissue with five 2–3 s bursts of the blender at high speed.

Filter the homogenate through four layers of cheesecloth plus one layer of miracloth. It may be necessary to squeeze the filtrate through the cloth. Wear gloves.

Pour the filtrate into a centrifuge tube balance against a tube of water and centrifuge at 4°C, 700*g* 10 min (2500 rpm in a Sorvall SS34 rotor).

Decant the supernatant into a clean centrifuge tube and centrifuge at 4°C, 10,000*g* 10 min (9500 rpm in a Sorvall SS34 rotor).

Decant the supernatant from the tube into a beaker and save it on ice. Use it for the next experiment. The pellet at the bottom of the centrifuge tube should contain isolated mitochondria.

Wash them gently by resuspending in 20 mL of fresh homogenization buffer. This is most easily done by pipetting 1–2 mL of the buffer into the tube and using a small paintbrush to break up the pellet.

Once the pellet is resuspended in this small volume, it can be diluted with the remaining 18–19 mL of buffer.

Recentrifuge the washed mitochondria as in Step 6 earlier.

Discard the supernatant from this spin and resuspend the mitochondrial pellet in 5 mL of homogenization buffer.

# 29. IDENTIFICATION OF LIPIDS BY THIN LAYER CHROMATOGRAPHY

**Principle**

In biological materials, lipids are found as lipoprotein complexes and these have to be extracted. Lipids being soluble in nonpolar organic solvents and proteins being soluble in polar aqueous solvents, the efficient lipid extraction can be achieved only with an aqueous solvent like chloroform and diethyl ether. This would help in breaking the lipoprotein complexes. Extracted lipid components can be separated on TLC based on their differential mobility along the porous stationary phase such as silica gel, and these can be located by spraying the plates with either 2',7'-dichlorofluorescein or 50% sulfuric acid.

**Materials**

TLC tank; glass plates (20 × 20 cm) for TLC; 2',7'-dichlorofluorescein (prepare 0.2% solution of 2',7'3-dichlorofluorescein in 95% v/v ethanol).

glass plates; spreader;

silica gel G;

oven set at 110°C;

UV lamp; solvent system—petroleum ether (b.p. 60°C–70°C).

**Procedure**

**Extraction of lipids from sample**

Grind 1 g of the tissue in the extraction solvent (either diethyl ether/ethanol, 3:1, or chloroform/methanol, 2:1) in a pestle and mortar. Transfer the homogenate to a separating funnel. Shake the contents vigorously, and allow it to stand till the two phases have completely separated. Drain out the lower organic layer that contains the lipids. Evaporate the solvent under vacuum, and keep the concentrated lipid extract protected from light under N2 atmosphere.

Prepare the TLC plates using silica gel G as described in the experiment for sugars.

Activate the TLC plates at 110°C for 30 min, cool them in a desiccator, and spot the lipid samples, standards, as well as unknown.

Develop the plates in the solvent system consisting of petroleum ether (b.p. 60°C–70°C) or hexane/diethyl ether/glacial acetic acid (80:20:1) till the solvent has traveled up to 1 cm from the opposite side of the plate.

Remove the plate and allow it to air-dry.

Locate the lipid spots by either of the following methods:

a. Spray the plate with 2',7'-dichlorofluorescein and examine it under UV light. Lipids show up as green fluorescent regions against the dark background.

b. Spray the plate carefully with 50% H2SO4 and heat it in an oven at 110°C for 10 min. Areas containing lipids get charred and appear as black spots.

**Observation and Calculations**

Calculate the *Rf* value of the lipid components in the sample and identify them by comparing their *Rf* values with lipid standards.

# 30. DETERMINATION OF STARCH IN PLANT TISSUES

**Principle**

Sugars are first extracted by treating the finely powdered dried grain or leaf sample repeatedly with hot 80% (v/v) alcohol. The residue is then treated with cold perchloric acid to solubilize starch. This derivative is then condensed with anthrone to give a blue-colored complex that is determined quantitatively by anthrone sulfuric acid procedure.

**Materials**

Dry leaf powder, test tubes, water bath, refrigerated centrifuge, polypropylene tubes, colorimeter, cuvettes, anthrone, sulfuric acid, ethyl alcohol, perchloric acid, glucose.

Anthrone–sulfuric acid reagent:

Dissolve 2 g of anthrone in cold 95% conc. $H_2SO_4$, store at 4°C. Use freshly prepared reagent.

80% absolute alcohol: Eighty milliliters of ethyl alcohol make up to 100 mL with distilled water.

52% perchloric acid: Add 270 mL of 72% perchloric acid to 100 mL of water.

Glucose standard:

Dissolve 0.1 g of anhydrous glucose in 100 mL of water containing 0.001% benzoic acid as preservative. The standard solution contains 1 mg glucose per mL.

**Procedure**

For extraction of sugars and starch, take 0.2 g of finely ground sample in a 50 mL centrifuge tube, and add 20 mL of hot 80% alcohol. Shake the tubes for 5–10 min, and after centrifuging at 3000 rpm for 10 min, decant the supernatant.

Repeat this extraction with 80% hot ethanol five to six times till the supernatant is free of sugars as judged by a negative test with anthrone reagent.

Cool the residue in ice water and add 6.5 mL of 52% perchloric acid while stirring the contents with a glass rod.

Let it stand for 15 min with occasional stirring and then centrifuge

at 4°C. Collect the supernatant and repeat this extraction Step four to five times. Combine the supernatant fractions and make up the volume to 100 mL with water.

Dilute the previous extract so that it contains 5–20 μg of glucose per mL. Take 5 mL aliquot of this diluted extract and place the tubes in a cold water bath.

Add 10 mL freshly prepared anthrone reagent. Mix properly and transfer the tubes to boiling water bath for 5–7 min.

After cooling the tubes under running tap water, note the absorbance of these solutions at 630 nm.

Prepare a standard curve using 0–100 μg glucose and anthrone reagent (Step 3). Calculate the amount of glucose in the sample aliquot.

**Results**

From the standard curve, determine the amount of glucose in 5 mL of the diluted aliquot of sample extract.

# 31. ISOLATION OF CASEIN FROM MILK

**Principle:**

Casein is the main protein found in milk and is present at a concentration of about 35 g/L. It is actually a heterogeneous mixture of phosphorus-containing proteins and not a single compound. Most proteins show minimum solubility at their isoelectric point, and this principle is used to isolate the casein by adjusting the pH of milk to 4.8, its isoelectric point. Casein is also insoluble in ethanol, and this property is used to remove unwanted fat from the preparation.

**Materials:**

Milk, sodium acetate buffer (0.2 mol/L, pH 4.6)
Ethanol (95% v/v), ether

**Procedure:**

Place 100 mL of milk in a 500 mL beaker and warm to 40°C; also warm 100 mL of the acetate buffer and add slowly with stirring.

The final pH of the mixture should be about 4.8, and this can be checked with a pH meter. Cool the suspension to room temperature and then leave to stand for a further 5 min before filtering through muslin.

Wash the precipitate several times with a small volume of water, and then suspend it in about 30 mL of ethanol.

Filter the suspension on a Buchner funnel and wash the precipitate a second time with a mixture of equal volumes of ethanol and ether.

Finally, wash the precipitate on the filter paper with 50 mL of ether, and suck dry. Remove the powder and spread out on a watch glass to allow evaporation of the ether.

Weigh the casein and calculate the percentage yield of the protein.

Result:

The total casein is present in the given sample is ……

# 32. DETERMINATION OF THE ACID VALUE OF A FAT

**Principle**

During storage, fats may become rancid as a result of peroxide formation at the double bonds by atmospheric oxygen and hydrolysis by microorganisms with the liberation of free acid. The amount of free acid present, therefore, gives an indication of age and quality of the fat. The acid value is the number of milligrams of KOH required to neutralize the free fatty acid present in 1 g of fat.

**Materials**

Olive oil, butter and margarine,

fat solvent (equal volumes of 95% v/v alcohol and ether neutralized to phenolphthalein),

Phenolphthalein (10 g/L in alcohol),

KOH (0.1 mol/L)

**Protocol**

Accurately weigh out 10 g of the test compound and suspend the melted fat in about 50 mL of fat solvent.

Add 1 mL of phenolphthalein solution, mix thoroughly, and titrate with 0.1 mol/L KOH until the faint pink color persists for 20–30 s.

Note the number of milliliters of standard alkali required and calculate the acid value of the fat.

**Results**

The acid value present in the given fat sample is ……g/mL.

# 33. SAPONIFICATION VALUE OF FAT

**Principle**

On refluxing with alkali, glyceryl esters are hydrolyzed to give glycerol and the potassium salts of the fatty acids (soaps). The saponification value is the number of milligrams of KOH required to neutralize the fatty acids resulting from the complete hydrolysis of 1 g of fat. The saponification value gives an indication of the nature of the fatty acids in the fat since the longer the carbon chain, the less acid is liberated per gram of fat hydrolyzed.

**Materials**

Fats and oils (tristearin, coconut oil, corn oil, and butter),
Fat solvent (equal volumes of 95% ethanol and ether),
Alcoholic KOH (0.5 mol/L),
Phenolphthalein (10 g/L in alcohol),
HCl (0.5 mol/L)

**Protocol**

Weigh 1 g of fat in a tarred beaker and dissolve in about 3 mL of the fat solvent.

Quantitatively transfer the contents of the beaker to a 250 mL conical flask by rinsing the beaker three times with a further addition of solvent; add 25 mL of 0.5 mol/L alcoholic KOH and attach to a reflux condenser.

Set up another reflux condenser as blank with everything present except the fat, and heat both flasks on a boiling water bath for 30 min.

Leave to cool to room temperature and titrate with 0.5 mol/L HCl and phenolphthalein indicator.

The difference between the blank and test reading gives the number of milliliters of 0.5 mol/L KOH required to saponify 1 g of fat.

**Results**

The molecular weight of KOH is 56, and since three molecules of fatty acid are released from a triglyceride

# 34. ESTIMATION OF BLOOD CHOLESTEROL

**Principle**

Acetic anhydride reacts with cholesterol in a chloroform solution to produce a characteristic blue-green color. Blood or serum is extracted with an alcohol–acetone mixture that removes cholesterol and other lipids and precipitates protein. The organic solvent is removed by evaporation on a boiling water bath and the dry residue dissolved in chloroform. The cholesterol is then determined colorimetrically using the Liebermann–Burchard reaction. Free cholesterol is equally distributed between the cells and plasma, while the esterifies form occurs only in plasma. It is essential to use absolutely dry glassware for this estimation.

**Materials**

Serum or blood, alcohol–acetone mixture (1:1)

Chloroform, acetic anhydride–sulfuric acid mixture (30:1)

Stock cholesterol solution (2 mg/mL in chloroform)

Working cholesterol solution (dilute the previous solution in 1:5 ratios in chloroform to give a strength of 0.4 mg/mL).

**Protocol**

Place 10 mL of the alcohol–acetone solvent in a centrifuge tube, and add 0.2 mL of serum or blood.

Immerse the tube in a boiling water bath with shaking until the solvent begins to boil. Remove the tube and continue shaking the mixture for a further 5 min. Cool to room temperature and centrifuge.

Decant the supernatant fluid into a test tube and evaporate to dryness on a boiling water bath.

Cool and dissolve the residue in 2 mL of chloroform. At the same time, set up a series of standard tubes containing cholesterol and a blank with 2 mL of chloroform.

Add 2 mL of acetic anhydride–sulfuric acid mixture to all tubes and thoroughly mix. Leave the tubes in the dark at room temperature and read the extinction at 680 nm.

**Results**

The normal serum cholesterol lies within the range of 100–250 mg/100 mL.

## 35. ISOLATION OF RNA FROM YEAST

**Principle**

Total yeast RNA is obtained by extracting a whole-cell homogenate with phenol. The concentrated solution of phenol disrupts hydrogen bonding in the macromolecules, causing denaturation of the protein. The turbid suspension is centrifuged, and two phases appear: the lower phenol phase contains DNA, and the upper aqueous phase contains carbohydrate and RNA. Denatured protein, which is present in both phases, is removed by centrifugation. The RNA is then precipitated with alcohol. The product obtained is free of DNA but usually contaminated with polysaccharide. Further purification can be made by treating the preparation with amylase.

**Materials**

Dried yeast, phenol solution (90%),
Potassium acetate (20%, pH 5),
Absolute ethanol, diethyl ether

**Protocol**

Suspend 30 g of dried yeast in 120 mL of water previously heated to 37°C. Leave for 15 min at this temperature and add 160 mL of concentrated phenol solution.

Stir the suspension mechanically for 30 min at room temperature, and then centrifuge at 3000*g* for 15 min in the cold to break the emulsion. Carefully remove the upper aqueous layer with a Pasteur pipette, and centrifuge at 10,000*g* for 5 min in a refrigerated centrifuge to sediment denatured protein.

Add potassium acetate to the supernatant to a final concentration of 20 g/L, and precipitate the RNA by adding two volumes of ethanol. Cool the solution in ice and leave to stand for 1 h.

Collect the precipitate by centrifuging at 2000*g* for 5 min in the cold. Wash the RNA with ethanol–water (3:1), ethanol, and, finally, ether; air-dry and weigh. (*Note*: Yeast contains about 4% RNA by dry weight.) Compare with a commercial preparation by measuring the pentose, phosphorus, and DNA content and by determining the absorption spectrum. Keep your preparation for use in later experiments.

# 36. ESTIMATION OF CHLOROPHYLL CONCENTRATION IN THE CHLOROPLAST SUSPENSION

## Principle

Pigments absorb light to different extents depending on the exposure of light of varying wavelength of the light spectrum. The ideal wavelength to select is the peak of the pigment's absorption spectrum for quantification of a pigment. For example, for chlorophyll a, 663 nm is generally used, but for chlorophyll b, 645 nm is a more appropriate wavelength to choose. The greater the concentration of a pigment in solution, the larger the proportion of light absorbed by the sample at that wavelength.

## Procedure

Take 1 mL aliquot of the chloroplast suspension into a 10 mL graduated cylinder, and dilute to 10 mL with 80% acetone. Cover the cylinder with parafilm and mix by inverting.

Prepare the spectrophotometer to read the absorbance of the diluted chlorophyll extract.

Adjust the wavelength to read 652 nm (why is this wavelength chosen?). Without a cuvette in the machine, adjust to 0% transmittance (left-hand knob).

Blank the spectrophotometer with the reagent blank (80% acetone) to read 0 absorbance (right-hand knob). Transfer some of diluted chlorophyll extract to a cuvette and read the absorbance. Record the absorbance value.

Chlorophyll absorbance ($A652$) = OD

Calculate the chlorophyll content in the diluted sample using the following equation. Record the chlorophyll concentration of the diluted sample.

$A = ECd$ where $A$ is the observed absorbance, $E$ is a proportionality constant (extinction coefficient) (=36 mL/cm), $C$ is the chlorophyll concentration (mg/mL), and $d$ is the distance of the light path (=1 cm).

Calculate the chlorophyll concentration in the original chloroplast

suspension (undiluted) by adjusting for the dilution factor. In order to determine the concentration of chlorophyll in the original suspension, you must multiply the chlorophyll concentration in the diluted sample by the dilution factor.

Knowing the chlorophyll content of the undiluted chloroplasts, prepare 10 mL of chloroplast suspension containing approximately 0.02 mg/mL chlorophyll by diluting an appropriate aliquot of original chloroplast suspension with cold 0.5 M sucrose. *Keep this on ice.*

# 37. DETERMINATION OF TOTAL ERYTHROCYTE COUNT

For RBC count, a method devised by Yokayama (1974) and later modified by Christensen et al., (1978) was followed. Hayems diluting fluid, which had the following composition, was used for RBC count.

**Chemicals**:

Mercuric chloride : 0.5gm
Sodium chloride : 1.0gm
Sodium sulphate : 5.0 gm
Distilled water : 200ml

**Procedure**

An improved Neubauer,s counting chamber was used for counting RBC (Baker and Silverton, 1982).

Using RBC pipette, the blood was drawn upto 0.5 mark and the diluting fluid to the mark 101.

Although fluid is drawn to the mark 101 but the real dilution is 0.5:100 or 1:200 because the fluid in the capillary tube is discarded before the count.

**Calculations**

The number of RBC's per sq mm was calculated as follows:

Area of a small square : 1/400 sq mm
Depth of the counting chamber : 1/10mm
The volume of Small Squareis : 1/4000cumm
The dilution of the blood is : 1/200

Total RBC = cu mm

N = No of cells in 80 small squares

Result

The total RBC present in the given sample is …….

## 38. DETERMINATION OF TOTAL LEUCOCYTE COUNT

A white cell count (TLC) estimates the total number of white cells in a cubic millimetre of blood. WBC diluting fluid or Turk fluid contains a weak acid to lyse the red blood cells and Gentian violet stain for staining the nucleus of white blood cells.

**Chemicals**:

The Turks fluid with following composition was used for TLC:
Glacial acetic acid :1.5ml
1% aqueous solution of Gentian violet :1 ml
Distilled water :100ml

**Procedure**

Neubauer,s hemocytometer (Baker and Silverton, 1982) was used for counting of leucocytes.

Using white cell pipette, the blood was drawn upto 0.5 mark and the diluting fluid to 11 mark, thus the dilution was 1:20.

**Calculations**

The number of RBC's per sq mm was calculated as follows:

Area of a small square : 4 sq mm
Depth of the counting chamber : 1/10mm
The dilution of the blood is : 1/20

$$\text{Total WBC} = \frac{N \times 20/10}{4} \text{ cu mm}$$

**Result**

The total WBC present in the given sample is …….

# 39. DETERMINATION OF PACKED CELL VOLUME (PCV) OR HAEMATOCRIT

PCV was determined by micro haematocrit method of Schalm et al., (1975).

**Chemicals**:

EDTA blood

PCV tube

**Procedure**

The heparinised blood was filled upto the mark 100 of the haematocrit tube with the help of Pasteur pipette and centrifuged at 3000 rpm for 30minutes.

The relative volume of the height of the RBC's packed at the bottom of the haematocrit tube was recorded as (PCV) in terms of percentage of total blood column taken in the haematocrit tube.

**Result**

The PCV or Haematocrit present in the given sample is …….

## 40. DETERMINATION OF MEAN CORPUSCULAR VOLUME (MCV)

MCV indicates the average size of the RBC in a given sample of blood.

MCV was calculated by the following formula and represented in cubic microns.

$$\text{MCV (fl)} = \frac{\text{PCV} \times 10}{\text{RBC Count}}$$

**Result**

The MVC present in the given sample is ……..

# 41. DETERMINATION OF MEAN CORPUSCULAR HEMOGLOBIN (MCH)

MCH represents the average weight of the hemoglobin contained in each RBC in a given volume of the blood.

MCH is influenced by the size of the cell and concentration of the hemoglobin.

MCH was calculated by the following formula and expressed in pictograms (pg).

$$\text{MCH (pg)} = \frac{\text{Hemoglobin(g / dL)} \times 10}{\text{RBC Count}}$$

**Result**

The MCH present in the given sample is …….

## 42. MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (MCHC)

MCHC reflects the average concentration of the hemoglobin in the RBC in a given volume of the blood.

MCHC was obtained by the following formula and expressed in terms of percentage.

$$\text{MCHC (\%)} = \frac{\text{Hemoglobin(g / dL)}}{\text{PCV}} \times 100$$

**Result**

The MCHC present in the given sample is …….

# 43. DIFFERENTIAL LEUKOCYTE COUNT

Differential Leucocyte Count in blood was determined by the method Ghai, (1993). A dried blood film stained with Leishman's stain, was examined under oil a immersion objective and the different type of white blood cells were identified. The percentage of distribution these cells was then determined.

**Reagents**

Leishman's stain (Eosin and methylene blue dyes dissolved in acetone-free absolute methyl alcohol).

**Procedure**

A blood film was prepared, dried and placed on the staining rack and was covered with Leishman's stain, allowed to stand for 2 minutes.

Then equal volume of distilled water was added and mixed by the slide first one way and then other.

Allowed to stand it for 6 minutes. Drained off diluted stain in a stream of distilled water from a wash bottle for about 20 seconds and allowed the slide to remain on the staining rack for 1-2 minutes with the last wash covering it.

Then kept the slide against a support in an inclined position, stained smear facing down and allowed it to dry.

Then the stained slides were studied under low and high power objectives for differential leucocyte count by placing two drops of glycerol on the stained smear and using oil-immersion lens.

**Calculations**

Differential leucocyte was expressed as percentage.

$$\text{DLC (\%)} = \frac{\text{Number of type cells}}{\text{Total number of leukocytes}} \times 100$$

## 44. DETERMINATION OF SERUM AMYLASE

**Principle:**

Serum is incubated with starch substrate. The amylase in the serum hydrolyses the starch to simpler units with a resulting increase in reducing groups. In the method presented here iodine is added which reacts with the starch molecules not hydrolysed by the amylase. The iodine-starch complex is blue in colour and is measured in the spectrophotometer. The degree of loss in colour is proportional to the amount of starch hydrolysed and hence to the activity of the amylase in the serum. A substrate control is carried though the procedure to give a reference value for the amount of starch substrate present before hydrolysis.

Procedure

1. Pipette 5 ml of substrate into two 50 ml volumetric flasks.
2. Place the 'test' flask into a 34oC water bath for 5 minute to warm the contents.
3. Using a pipette that deviloers between two marks add 0.1 ml of serum to the 'test' flask and mix. Do not use blow out pipettes as the smallest amount of saliva can give a large error.
4. Time the addition of serum using a stop watch.
5. After exactly 7.5 minutes add 5 ml or working iodine solution, mix and immediately remove from the water bath.
6. Similarly add 5 ml of the working iodine solution to the flask containing the 'substrate control', which has not been incubated.
7. Dilute the contents of both flasks to the 50 ml mark with distilled water and mix the flasks well.
8. Read the absorbance of both against water using the large (19 mm) cuvettes at 660 nm.

Calculation

Absorbance of substrate control – absorbance of test x 800 Absorbance of control

units of amylase activity per 100 ml of serum.

## 45. ESTIMATION OF SERUM URIC ACID

**Principle:**

Phosphotungstic acid in alkaline medium oxidizes uric acid to allantion and itself gets reduced to tungsten blue which is estimated colorimetrically at 700mm.

**Reagents :**

Sodium tungstate 10%.

2/3 N Sulphuric acid.

Tungstic acid: Add 50ml of 10% sodium tungstate 50ml 2/3 N $H_2SO_4$ and a drop of phosphoric acid with mixing to 800ml water. Discard when cloudy. Store in brown bottle.

Phosphotungstic acid: Stock-Dissolve 50g sodium tungstate in about 400ml of water. Add 40ml 85% phosphoric acid and reflux gently for 2 hours, cool, make volume to 500m. store in brown bottle. Dilute 1 to 1 for use.

$Na_3CO_3$ 10%.

Standard uric acid solution stock-100mg%.

Working uric acid solution-1mg%.

**Procedure :**

In a centrifuge tube pipette 0.6ml serum and add 5.4ml. tungstic acid while shaking. Centrifuge and process as follows.

| | **B** | **T** | **S** | **$S_2$** | **$S_3$** |
|---|---|---|---|---|---|
| 1. Standard uric acid (1mg%) ml. | - | - | 1.0 | 2.0 | 3.0 |
| 2. Supernatant (ml) | - | 3.0 | - | - | - |
| 3. D. Water (ml) | 3.0 | - | 2.0 | 1.0 | - |
| 4. $Na_3CO_3$ (ml) | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| 5. Phosphotungstate (ml) | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |

Mix well stand at room temperature for 30 min. Read absorbance at 700 nm or using a red filter plot a standard curve between concentration of standard and absorbance and calculate the uric acid conc, in test.

$$\text{Serum Uric acid} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \frac{\text{Conc. of Std.}}{\text{Vol. of Serum}} \times 100$$

# 46. PHYTOCHEMICAL ANALYSIS

The chemicals produced by plants are referred to as plant chemicals. These are generated by the primary and secondary metabolism of the plant. These phytochemicals are essential for other plants, animals, insects and microbial pests and pathogens to prosper or thwart them. Plants are also assisted and protected from disease and harm caused by environmental hazards such as pollution, UV, stress and draught. They are used as conventional medicine and as ancient poisons.

Phytochemicals are not the essential nutrients they are, rather than the essential nutrients because there is no evidence that they are not yet established to cause any possible health effects in humans. They are known to have a role in the protection of human health. More than 4,000 phytochemicals are classified by protective function, physical characteristics and chemical characteristics and have been catalogued. Phytochemicals are usually classified into the following types; carotenoids and polyphenols, including phenolic acids, stilbenes/lignans, are included. In addition, flavones, anthocyanins, isoflavones and flavanols are further categorized into classifications such as flavonoids.

**Steps in Preparation of Plant Sample**

**Drying**

The plant materials are dried to remove the water content and thus to store them after the water is removed. As soon as the plants are collected, this process should be done immediately so that spoilage is prevented. In drying the plants, there are two techniques,

**Natural process**

This process include sun-drying. In this the plants are kept in the shades and are air dried in sheds. This process takes few weeks for complete drying of the moisture. This time depends on the temperature and humidity.

**Artificial drying**

Using the help of artificial driers, artificial drying is done. The time consumed is reduced to a few hours or minutes by this process. Warm-air drying is the common method used for the drying of medicinal plants. The

hot air furnace on which hot air is blown is used to do this. For drying succulent parts of plants and fragile flowers, this method is applicable. To avoid disintegration of the thermolabile compounds, the drying must be done at a lower temperature.

**Grinding of plant materials**

The plant samples are to be powdered for further analysis following complete drying of moisture. There are various forms of powdering, including the following,

1. Grinding can be done by grinding in an electric grinder or a spice mill or in a mortar or pestle as well.
2. Due to the increased surface area of the plants, grinding increases the effectiveness of extraction. The reduction in the area of the surface can lead to dense packing of the material.
3. It is always ideal to mill the plants into a fine powder, but if they are too fine, this impacts the flow of the solvent and also generates more heat that could degrade some thermolabile compounds.

Choice of Solvent

For the determination of biologically active phytochemicals from plants, the solvent that is used for the extraction process is very important. These solvents must be less toxic, easy to evaporate in less heat, preserve and not dissociate the compounds in them. For extraction, the different solvents commonly used include:

1. Water:

It is a universal solvent; plant extracts are usually extracted with water for anti-microbial activity. But when compared to water, the organic solvents give consistent results in anti-microbial activities. No significant results can be obtained from water soluble compounds in the extract.

2. Alcohol:

Due to the presence of higher amounts of polyphenols, these alcoholic extracts from plants show more activity than aqueous extracts. This is due to the higher alcohol degradation of the cell wall and seed, which releases the polyphenols that will be degraded if aqueous extracts are extracted. Ethanol, however is microbicidal rather than water. 70 percent ethanol is used to extract more bioactive compounds than pure ethanol. Intracellular ingredients from plant materials are also found to be easier to

extract from ethanol. For the extraction of phenolic compounds, polar solvents like methanol, ethanol and their aqueous mixtures are used. The addition of water to alcohol will enhance the extraction rate. Because of its cytotoxic nature, methanol is more polar but is unsuitable for extraction.

3. Acetone:

Acetone dissolves many of the plants' hydrophilic and lipophilic compounds and is water-mixable. It is low in toxicity and volatile and is used to extract antimicrobial activity. Tannins and other phenolic compounds are extracted using acetone. In addition, they are used to extract saponins.

4. Chloroform:

Terpenoid lactones are obtained from barks by extraction with chloroform. Tannins and Terpenoids are treated with less polar solvents.

5. Ether: They are used for the extraction of coumarins and fatty acids.

**Methods of Extraction:**

**Homogenization**

One of the most commonly used methods for extraction is this technique. This is done using either the dried or wet method of extraction. The dried plant samples are finely powdered in this dried extraction method and added to the solvent blended for a few minutes and kept for about 24 hours in an orbital shaker. The parts of the plants are cut into small pieces during the wet extraction process, ground in a mortar and pestle and added to a solvent and shaken for 24 hours in an orbital shaker and then filtered. The filtrate can be used for the further analysis.

**Serial Exhaustive Extraction**

It is done to extract a wide range of polarities of compounds with a variety of solvents from a non-polar solvent such as hexane to more polar solvent such as methanol. The drawback is that due to the high heat leading to the degradation, thermolabile compounds can not be extracted.

**Soxhlet Extraction**

It is used when the compound in the solvent is less soluble and the impurities in the solvent are soluble. The impurities can be eliminated by

simple filtration if the desired compound is highly soluble in the solvent. The benefit is that the solvent is recycled in this method and therefore the solvent is less wasted. Thermolabile compounds cannot be extracted in this method, similar to the above method.

**Maceration In this method,**

With frequent agitation, the entire plant or the powder can be kept in the solvent for a certain period until the soluble compounds are dissolved. For thermolabile compounds, this method is the most appropriate method.

**Decoction**

Heat-stable and water-soluble compounds are extracted in this process. The plant materials extracted are cooked in the water for about 15 minutes and cooled, filtered and used for further analysis. 4.6. Infusion

By diluting the compounds in the solvents, it is done. It is prepared by macerating the compounds in cold or boiling water for a brief period.

**Digestion**

This is a process where the extraction is done as maceration with a gentle heat applied. It is used when the elevated temperature do not interfere the solvent efficiency or the compounds.

**Percolation**

For this process an instrument called percolator is used which is a narrow, cone shaped Vessel with open ends. The ingredients are moistened with an appropriate amount of the specified menstrum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstrum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstrum is added as required, until the percolate measures about three quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

**Sonication**

In this method the ultrasound with higher frequencies of 20 kHz – 2000 kHz are used which will disrupt the cells and releases the constituents. Although the process is useful in some cases, like extraction of rauwolfi a root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

**Qualitative Analysis of Primary Metabolites:**

**Test for carbohydrates**

Benedict's test:

About 0.5 ml of the filtrate was taken to which 0.5 ml of Benedict's reagent is added. This mixture was heated for about 2 minutes in a boiling water bath. The appearance of red precipitate indicates the presence of sugars

Molisch's test:

To about 2ml of the sample, 2 drops of alcoholic solution of α-napthol was added and to the mixture after being shaken well. Few drops of conc.H2SO4 were added along the sides of the test tube. A violet ring indicates the presence of sugars

Test for Starch

To about 5 ml of distilled water, 0.01g of iodine and 0.075 g of potassium iodide were added and this solution was added to about 2-3 ml of the extract. Formation of blue colour indicates the presence of starch.

**Test for proteins**

Biuret test:

2ml of filtrate was taken to which 1 drop of 2% copper sulphate solution was added; 1ml of 95% ethanol was added. Then it was followed by excess addition of KOH. The appearance of pink colour indicates the presence of protein.

2ml of extract was mixed with 2ml of water and about 0.5% of conc. HNO3 was added. The appearance of yellow colour indicates the presence of proteins.

To about 2 ml of the extract, 2ml of miller's reagent was added white precipitate which turns red on heating will confirm the presence of proteins.

**Test for amino acids**

To 1ml of the extract, few drops of ninhydrin reagent (10mg of ninhydrin in 200ml of acetone) were added. The appearance of purple colour indicates the presence of amino acids.

To 2ml of extract few drops of nitic acid were added along the sides of the tube the appearance of yellow colour indicates the presence of protein and free amino acids.

**Test for fatty acids**

1 ml of the extract was mixed with 5 ml of ether. There extracts were allowed to evaporate on a filter paper and the filter paper was dried. The appearance of transparency indicates the presence of fatty oils

**Miscellaneous compounds**

**Test of resins**

1. Precipitation test: about 0.2 g of extract was extracted with 15ml of 95% ethanol. The alcoholic extract was then poured into a beaker containing about 20ml of distilled water.
2. 1ml of extract was taken and to this few ml of acetic anhydride was added to this 1ml of conc.H2SO4 was added. The appearance of orange to yellow colour indicates the presence of resins

**Test of fixed oils and fats**

1. Spot test: small quantity of the extract was taken and pressed between 2 filter papers. The appearance of spots indicates presence of oils
2. Saponification test: To the extract, few drops of 0.5N alcoholic KOH and few drops of phenolphthalein were added. This mixture was heated for about 2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils or fats

Gums and mucilage

To 1ml of extract, distilled water, 2ml of absolute ethanol was added with constant stirring white or cloudy precipitate indicates the presence of gums or mucilage

**Carboxylic acids**

1. To 1ml of extract a pinch of sodium bicarbonate is added. The production of effervescence indicates the presence of carboxylic acids

2. 2ml of alcoholic extract was taken in warm water and filtered. The filtrate was then tested with litmus paper and methyl orange. The appearance of blue colour.

**Qualitative Analysis of Secondary Metabolites**

Test for anthraquinones

To 5ml of extract, few ml of conc.H2SO4 was added and 1ml of diluted ammonia was added to it. The appearance of rose pink confirms the presence of anthraquinones

**Test for quinones**

To 1ml of extract, alcoholic KOH is added the presence of red to blue colour indicates the presence of quinones

**Test for alkaloids**

1. Mayer's test: to a few ml of filtrate, 2 drops Mayer's reagent was added a creamy or white precipitate shows a positive result for alkaloids.

2. Wagner's test (iodine – potassium iodine reagent): To about an ml of extract few drops of Wagner's reagent were added. Reddish – brown precipitate indicates presence of alkaloids.

3. To 5ml of extract 2ml of HCl was added. Then 1 ml of Dragendroff"s reagent was added an orange or red precipitate shows a positive result for alkaloids.

**Test for glycosides**

1. Borntrager's test: to 2ml of filtrate, 3ml of chloroform is added and shaken. The chloroform layer is separated and 10% ammonia solution was added. The pink colour indicates the presence of glycosides

2. 5ml of extract was hydrolysed with 5ml of conc. HCl boiled for few hours in a boiling water bath, small amount of alcoholic extract was dissolved in 2ml of water and 10% of aqueous 10% NaOH was added the presence of yellow colour was a positive result for the glycosides.

3. 2ml of extract is mixed with about 0.4 ml of glacial acetic acid containing traces of ferric chloride and 0.5 of conc. H2SO4 was added the production of blue colour is positive for glycosides.

**Test for cardiac glycosides (Keller-Killani test)**

1. 5ml of solvent extract was mixed with 2ml of glacial acetic acid and a drop of ferric chloride solution was added followed by the addition of 1ml of conc. H2SO4. A brown ring in the interface indicates the presence of deoxy sugars of cardenoloides. A violet ring may appear beneath the brown ring while acetic acid layer a green ring may also form just gradually towards the layer.

**Test for phenol**

1. Gelatine test:

To 5ml of extract 2ml of 1% solution of gelatine containing 10% of NaCl is added. Appearance of white precipitate indicates the presence of phenol

2. Lead acetate test: To 5 ml of extract 3ml of 10%lead acetate solution was added and mixed gently. The production of bulky white precipitate is positive for phenols. Test for polyphenols 1. To the 3ml of extracts 10ml of ethanol was added and were warmed in a water bath for 15 minutes. To this few drops of ferric cyanide (freshly prepared) was added. The formation of blue – green colour indicates presence of polyphenols.

2. To 1ml of extract few drops of 5% solution of lead acetate was added. The appearance of yellow precipitate indicates the positive results for polyphenols

3. To the 5ml of ethanolic extract 3ml of 0.1% gelatine solution was added. The formation of precipitate was positive for polyphenols

**Test for tannins**

1. To 5ml of extract few drops of neutral 5% ferric chloride solution was added, the production of dark green colour indicates the presence of tannins

**Test for Flavonoids**

1. To the aqueous solution of the extracts 10% ammonia solution is added

and is heated. The production of fluorescence yellow is positive for flavonoids.
2. 1ml of extract was taken and 10% of lead acetate was added. The yellow precipitate is positive inference for the flavonoids
3. The extract is treated with concentrated H2SO4 resulting in the formation of orange colour indicates the positive result for flavonoids.
4. To 5ml of dilute ammonia the plant extract is added and shaken well. The aqueous portion is separated and concentrated H2SO4 is added. The yellow colour indicates the presence of flavonoids.

**Test for phytosterols**

1. The extract is dissolved in 2ml of acetic anhydrite and to which 1 or 2 drops of concentrated H2SO4 is added along the sides an array of colour change indicates the presence of phytosterols.
2. The extract was refluxed with alcoholic KOH and saponification takes place. The solution was diluted with ether and the layer was evaporated and the residue was tested for phytosterols. It was dissolved in diluted acetic acid and few drops of concentrated H2SO4 are added. The presence of bluish green colour indicates the presence of phytosterols.

**Test for phlobatannins**

1. Aqueous extract was boiled with diluted HCl leading to the deposition of reddish precipitate indicates the presence of phlobatannins Test for saponins 1. 0.5 mg of extract was vigorously shaken with few ml of distilled water. The formation of frothing is positive for saponins
2. The froth from the above reaction is taken and few drops of olive oil is added and shaken vigorously and observed for the formation of emulsion. Test for steroids 2ml of extract with 2ml of chloroform and 2ml of concentrated H2SO4 are added, the appearance of red colour and yellowish green fluorescence indicates the presence of steroids

**Test for xanthoproteins**

1ml of extract is taken and to this few drops of nitric acid and ammonia are added. Reddish brown precipitate indicates the presence of xanthoproteins Test for chalcones 2ml of ammonium hydroxide is added to 0.5 g of extract. The appearance of red colour indicates the presence of

chalcones

Test for Terpenoids (Salkowski test) 3ml of the extract was taken and 1ml of chloroform and 1.5 ml of concentrated $H_2SO_4$ are added along the sides of the tube. The reddish brown colour in the interface is considered positive for the presence of terpenoids

**Test for triterpenoids**

To 10 mg of extract 1ml of chloroform is added and is mixed to dissolve it. 2ml of concentrated $H_2SO_4$ is added followed by 1ml of acetic anhydride. Formation of reddish violet colour is positive for the presence of triterpenoids.

**Test for anthocyanins**

2ml of aqueous extract was taken to which 2N HCl was added and it was followed by the addition of ammonia, the conversion of pink-red turns blue-violet indicates the presence of anthocyanins.

**Test for Leucoanthocyanins**

To 5ml of extract dissolved in water, 5ml of Isoamyl alcohol is added. The red appearance of the upper layer indicates the presence of Leucoanthocyanins

**Test for Coumarins**

To 2 ml of the extract, 3 ml of 10% aqueous solution of NaOH is added. The production of yellow colour indicates the presence of coumarins

**Test for emodins**

To 5ml of extract, 2ml of $NH_3OH$ and 3ml of benzene are added. The production of red colour indicates the presence of emodins

**Qualitative Analysis of Vitamins**

Test for Vitamin – A

In 5 ml of chloroform, 250mg of the powdered sample is dissolved and it is filtered, to the filtrate, 5ml of antimony trichloride solution is added. The appearance of transient blue colour indicates presence of vitamin-A

Test for vitamin – C

In 5ml of distilled water, 1ml of the sample was diluted and a drop of 5% sodium nitroprusside and 2ml of NaOH is added. Few drops of HCl are added dropwise, the yellow colour turns blue. This indicates the presence of vitamin- C

Test for vitamin – D

In 10 ml of chloroform, 500mg of powdered extract is dissolved and filtered. 10ml of antimony trichloride is added, the appearance of pinkish-red colour indicates the presence of vitamin – D

Test for vitamin – E

Ethanoic extract of the sample was made and filtered (500mg in 10ml), few drops of 0.1% ferric chloride were added and 1ml of 0.25% of 2'- 2'dipyridyl was added to 1ml of the filtrate. Bright-red colour was formed with a white background.

**Qualitative and Quantitative**

Analysis Qualitative and quantitative analysis of phytochemicals can be done using Gas Chromatography Mass Spectroscopy (GCMS). GCMS can be applied to solid, liquid and gaseous samples. First the samples are converted into gaseous state then analysis is carried out on the basis of mass to charge ratio. High Performance Liquid Chromatography is applicable for compounds soluble in solvents. High performance thin layer chromatography is applicable for the separation, detection, qualitative and quantitative analysis of phytochemicals.

Gas Chromatography

Volatile compounds are analysed using gas chromatography. In this method, there is a gas and a liquid phase. The liquid phase is stationary where the gas phase is a mobile phase. These compounds to be analysed are also in the mobile phase with a carrier gas which is usually helium, hydrogen or argon. The chemicals are separated depending on the migration rate into the liquid phase. Higher percentage of the chemical will lead to faster migration in the liquid phase. This is widely used in qualitative and

quantitative phytochemical analysis.

High Performance Liquid Chromatography: (HPLC)

HPLC is also known as High- Pressure Liquid Chromatography. This method involves the interaction of liquid solvent in the tightly packed solid column or a liquid column. These acts as the stationary phase while the liquid (solvent) acts as the mobile phase, high pressure enables the compounds to pass to the detector. As HPLC compounds are analysed after vaporisation, thermolabile compounds cannot be analysed with this technique.

High Performance Thin Layer Chromatography: (HPTLC)

This method is modified form of thin layer chromatography. It is a type of planer chromatography where the separation is done by high performance layers with detection and the sample components are acquisition using an advanced work- station. The reduction of the thickness of the layer will increase the efficiency of the separation and hence HPTLC is more advanced method for qualitative, quantitative and micro-preparative chromatography.

Optimum Performance Laminar Chromatography:

(OPLC) OPLC combines the advantages of TLC and HPLC. The system separates about 10-15 mg samples, with simultaneous processing of up to 4 or 8 samples at a time depending on the model. In OPLC a pump is used to force a liquid mobile phase through a stationary phase, such as silica or a bonded-phase medium.

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