Indira Gandhi National
Open University
School of Sciences

BBCCL-110 METABOLISM OF CARBOHYDRATES AND LIPIDS (PRACTICAL)

METABOLISM OF CARBOHYDRATES AND LIPIDS

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BBCCL-110: METABOLISM OF CARBOHYDRATES AND LIPIDS

Dear learners welcome to the practical sessions of the course "Metabolism of carbohydrates and lipids". The lab exercises and experiments provided in this manual are based on the syllabus that you have studied in BBCCT-109. You would be able to observe effect of some of the metabolic reactions you studied in theory course while performing these experiments in the lab.

This lab course is worth 2 Credits and consists of four laboratory experiments. The experiments are designed in such a way that you are able to experience and connect the theoretical concepts explained so far. Basic principle on which these experiments are based has also been explained in each exercise.

Expected learning outcomes

The broad objective of this lab course is to enable you to:

- Estimate blood glucose levels;
- Demonstrate and interpret the effects of microbial fermentation;
- Extract total lipids from egg yolk;
- Separate different types of lipids using thin layer chromatography and visualize them; and
- Estimate different types of lipids by biochemical assay.

Study Guide

We advise you to go through respective blocks of BBCCT-109 before you come to attend the practical sessions. This will enable you to easily understand the purpose of doing experiments and their applications. You should also read the principle of each experiment of this course along with procedure before you start performing the experiment. It is always good to prepare all the reagents freshly and store them under appropriate storage conditions. Adhere to all the safety measures and follow the safety instructions while handling the reagents. One of the good laboratory practices is to maintain your log books up-to-date i.e., enter the observations made while performing the experiments. Carry this laboratory manual and your log book during lab sessions.

Like all other IGNOU laboratory courses, this is an intensive residential exercise requiring one week for completing 2 credits. Everyday there will be two laboratory sessions of 4 hours each. So there will be a total of 14 sessions. The first session will be introductory and the remaining 2^{nd} to 12^{th} sessions will be based on the exercises given in the course. A schedule for laboratory exercises will be given to you in the first session. Sessions 1 to 12 will have guided exercises under the supervision of the academic counsellor. The last two sessions i.e., 13^{th} and 14^{th} will be unguided sessions and that would pertain to evaluation for the term end examination. In each session you will perform exercises for 3 hours and in the remaining 1 hour you are advised to complete your practical note book. The laboratory notebook must be submitted to the counsellor for corrections and grading. 70% marks have been assigned for doing the experiments and recording them properly.

You should be aware that due to time constraint as you will have limited access to laboratory work; therefore, you are required not to miss any of the laboratory sessions.

Performance during the practical sessions will be graded and you will have to appear for the *viva-voce* at the end of the practical session. At the end of the laboratory session you would be asked to perform the assigned experiment, which will be graded. Final assessment will be made based on the continuous performance during the laboratory sessions, maintenance of log books and records followed by *viva-voce*. 30% marks are reserved for the assigned experiments.

For the better understanding of how to use laboratory apparatus or perform experiments, few video links may be provided where ever available. There might be a slight difference in the steps or procedure being explained in the video when compared to the procedure provided in this self-instructional material. However, the principles and reagents remain same. Hence, there is no need to worry about slight modifications adopted in the procedure.

We wish you best in this endeavour!!

IMPORTANT INFORMATION

- **Attendance** is compulsory in the Laboratory Course work held generally at the Study Centre.
- The Laboratory Course is worth **2 credits** to be completed over **7 days** duration.
  6 days of Guided Laboratory work
  1 day for the **Unguided** Laboratory work
- To successfully complete the laboratory course you will have to pass (at least **35% marks**) in the Guided and Unguided components separately.

EXPERIMENT 1

ESTIMATION OF BLOOD GLUCOSE BY COUPLED ENZYME ASSAY

Structure

1.1 INTRODUCTION

Estimation of blood glucose is generally carried for diagnosis of diabetes mellitus. This disease is characterized by abnormally high concentration of glucose in blood (hyperglycemia). High blood glucose levels also occur in thyroid or pituitary dysfunction, renal failure and liver diseases. Low blood glucose level (hypoglycemia) is associated with insulinoma (insulin secreting tumours), hypopituitarism, adrenal cortical insufficiency and insulin induced hypoglycemia.

A number of methods have been developed for glucose estimation. The earlier methods such as Nelson-Somogyi and Folin-Wu were **reduction based** that suffered from a number of limitations. These methods were non-specific; less sensitive, non stoichiometric, cumbersome and required rigid control of experimental conditions. Currently, enzymatic oxidation based assays have taken over the above methods. They are specific for glucose and single step rapid procedures. The test is not influenced by uric acid, creatinine, vitamin C, anticoagulants and bilirubin, etc. Glucose can be estimated with very small volumes of blood and serum samples. Two popular enzyme based **coupled assays** are glucose oxidase –peroxidase method (GOD-POD) and hexokinase-glucose-6 phosphate dehydrogenase. In this exercise we shall use GOD-POD method.

Expected Learning Outcomes

After studying this unit, you should be able to:

- separate serum or plasma from the blood;

- ❖ perform glucose estimation in blood/serum sample(s); and
- ❖ explain the clinical significance of blood glucose estimation.

1.2 PRINCIPLE

The test uses two enzymes; glucose oxidase (GOD) and peroxidase (POD). GOD oxidises glucose to glucuronic acid with the reduction of the flavin prosthetic group, FAD to $FADH_2$. The reaction requires oxygen to reoxidise $FADH_2$ to FAD and H_2O_2. The enzyme is specific for the β- anomer of glucose. The two anomers (α; β) are interchangeable via the open chain form in solution therefore, total glucose in the sample can be estimated. The liberated H_2O_2 is acted upon by peroxidase which transfers oxygen to the chromogenic system. It results in oxidation of phenol that combines with 4-aminoantipyrine (APP) to form a pink coloured quinoneimine dye with absorbance maxima (λ_{max}) at 510 nm. The intensity of pink / red colour dye is directly proportional to the concentration of glucose in the sample (Fig. 1.1).

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{POD}} \text{Red quinone} + 4\,H_2O$$

Fig. 1.1: The reactions catalysed by GOD (glucose oxidase) and POD (peroxidase).

1.3 MATERIALS REQUIRED

1. Human blood (2-3 ml)
2. Fluoride-EDTA vial or fluoride vial
3. Phosphate buffer, pH of 7.5
4. Enzymes: (a) Glucose oxidase (30 U / ml)
   (b) Peroxidase (1.0 U / ml)
5. Phenol (0.1 % by weight)
6. 4-aminoantipyrine (0.1 % by weight)
7. Glucose standard (100 mg /dL)
7. Centrifuge
8. Colorimeter or spectrophotometer
9. Distilled water.

1.4 PROTOCOL

(a) Separation of plasma or serum from blood

Separation of plasma

(1) Collect the blood into fluoride-EDTA vials and mix gently two to three times.

(2) Now transfer the blood to an Eppendorf tube (2ml) and centrifuge at 2000 g (4° C) for 15 minutes.

(3) After centrifugation, you would see upper light yellow colour liquid (plasma) separated from blood cells. Plasma is the liquid cell free part of blood that has been treated with anticoagulants; it contains clotting factors.

(4) Transfer it carefully using a clean pipette into 2.0 ml Eppendorf tube. Take care not to disturb the other layers of blood cells.

(5) Freeze the plasma immediately in a –80° C degree freezer and discard the pellet.

Separation of Serum

(1) Collect the blood into fluoride vials and mix gently two to three times.

(2) Now leave it undisturbed at room temperature (37°C) for 30 minutes to allow clot formation. With the help of a glass rod try to release the clot sticking to the vial.

(3) Transfer the clotted blood to Eppendorf tubes (2ml) and centrifuge at 1000 g (4° C) for 10 minutes.

(4) After centrifugation, the upper light clear yellow colour fluid (serum) is separated by using clean pipette into 2.0 ml Eppendorf tube. Serum lacks clotting factors.

(5) Freeze the serum immediately in–80° C degree freezer (Fig.1.2).

Note: The plasma / serum can be directly used for analysis. The glucose level remains stable for 24 hr at 2-8°C if serum /plasma is prepared within 30 min after collection. If analysis is delayed, it must be stored at –80° C.

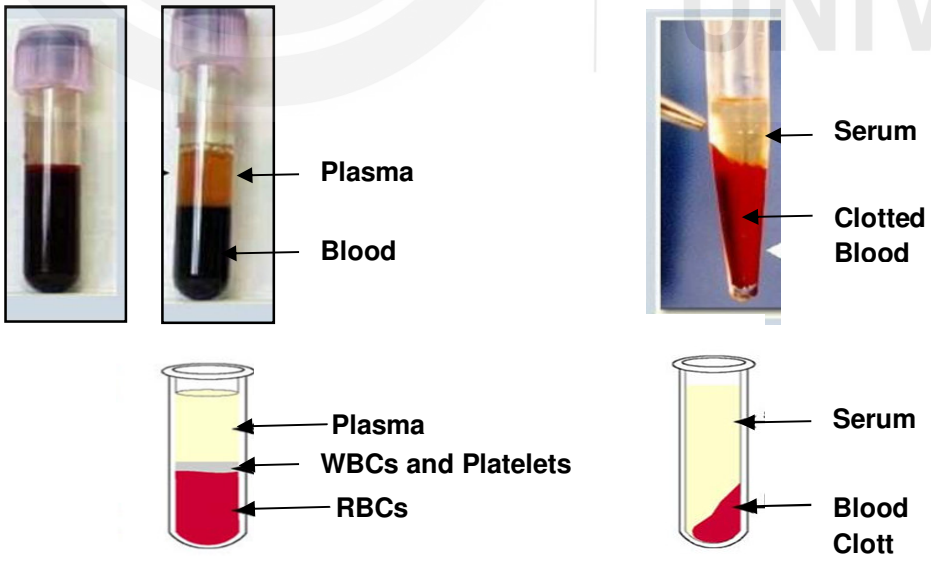

Fig. 1.2: Separation of serum and plasma from blood. Lower panel shows their constituents.

(b) Preparation of reagents

Before you begin the estimation, you need to prepare the solutions

(1) Phosphate buffer (pH of 7.5): Prepare 1M dipotassium hydrogen phosphate (K_2HPO_4) and 1M potassium dihydrogen phosphate (KH_2PO_4) stock solutions. Then mix 9.4 ml of 1M KH_2PO_4 with 40.6 ml of 1M K_2HPO_4 for preparing 50 ml of 1M buffer (pH 7.5). Check the pH of the solution after mixing.

(2) Dilute the standard glucose oxidase (GOD) and peroxidase (POD) solutions with phosphate buffer to prepare working reagents.

(3) Standard glucose (100mg %) solution: Dissolve 100 mg glucose in 100ml phosphate buffer.

(4) Phenol (0.1% by weight): Weigh 100 mg phenol and dissolve it in 100 ml phosphate buffer**.**

(5) 4-aminoantipyrine (0.1% by weight): Dissolve 100mg of 4-aminoantipyrine in phosphate buffer and make up the volume to 100 ml.

(c) Estimation of glucose

(1) Take three test tubes and mark these as Blank (B), Standard (S) and Test (T).

(2) Pipette 980µl of phenol and 980µ of 4-aminoantipyrine in each tube.

(3) Then add 20µl of GOD and 20 µl of POD in all the three tubes.

(4) Mix gently all the contents.

(5) Pipette 20 µl of plasma or serum sample in the tube marked (T). Make sure that the frozen sample is thawed completely before performing the estimation.

(6) Add 20µl of standard in tube marked (S).

(7) Make up the volume to 3ml in all the test tubes by adding distilled water (DW). Mix the contents and incubate the tubes at 37℃ for 10 minutes.

(8) Record absorbance at 510 nm in the spectrophotometer.

Nowadays readymade kits are also available for glucose estimation.

1.5 OBSERVATION AND RESULTS

Record the observations in the Table 1.1

The absorbance is directly proportional to the concentration of glucose in the blood. This is calculated by using the following formula:

Glucose (mg/dL) = (Absorbance of Test/Absorbance of Standard) X100

The biological reference range:

Glucose fasting is 60-110 mg/dL.

Glucose PP (postprandial) is 90-140 mg/dL.

Glucose Random is 60-130 mg/dL.

Table 1.1: Observation table

S.No.	Volume of serum / plasma (µl)	Volume of standard glucose (µl)	Volume of GOD (µl)	Volume of POD (µl)	Volume of 4-APP (µl)	Volume of Phenol (µl)	Volume of DW (µl)		Absorbance at 510 nm
1. Blank	0.0	0.0	20	20	980	980	2000	**Mixed and incubated at 37°C for 10 minutes**	
2.Standard	0.0	20	20	20	980	980	1980		
3. Test	20	0.0	20	20	980	980	1980		

Values beyond these references may indicate abnormalities in sugar metabolism and are used for diagnosing diabetes. While lower values point towards hypoglycemia, higher values may be indicative of diabetes. The test is repeated to confirm the diagnosis. In addition, **Glucose tolerance test** (GTT) is performed to determine response of your body towards sugar. The patient is made to drink sugar solution containing 100 g of glucose and the glucose levels are monitored by taking samples at regular intervals to determine how long does it take for glucose levels to come down. After two hours:

(i) If blood glucose level is lower than 140 mg/dL - normal

(ii) If blood glucose level between 140 and 199 mg/dL - impaired glucose tolerance, or prediabetic which indicates risk of developing type 2 diabetes.

(iii) If blood glucose level are 200 mg/dL or higher- may indicate diabetes.

1.6 PRECAUTIONS

(1) The reagent is light sensitive. It must be protected from direct light (store in a dark bottle). It is stable for 2 weeks at 2-8°C.

(2) Mixing of blood in fluoride or fluoride-EDTA vials should be gently performed to minimise blood haemolysis.

(3) The absorbance should be preferably recorded within 1 to 1.5 hrs.

(4) If the glucose concentration of the test sample exceeds 500mg%, then dilute the sample appropriately and repeat the estimation.

EXPERIMENT 2

SUGAR FERMENTATION BY MICROORGANISMS

Structure

2.1 INTRODUCTION

The fermentation of sugars (monosaccharides and disaccharides) by microorganisms produces a variety of end products. Among the common products of carbohydrate breakdown are organic acids, for example, acetic acid, lactic acid and propionic acid and gases like carbon dioxide and hydrogen. The type(s) and proportion of these end products are dependent on the organism and the carbohydrate fermented. Some microorganisms produce both acid and gas; some only acid while others produce neither acid nor gas.

In this lab exercise, you will use simple procedures to detect acid and gas formation by fermentation of sugars (carbohydrate) in the given bacterial cultures.

Expected Learning Outcomes

After studying this unit, you should be able to:

- perform fermentation of carbohydrates;
- determine their ability to produce acid and gas by simple methods; and
- learn the fermentation pathways of bacteria responsible for acid and gas production.

2.2 PRINCIPLE

Microorganisms are extremely versatile in their ability to degrade and ferment carbohydrates. They possess an assorted combination of enzymes to process carbohydrates and other biomolecules to a variety of end products. Some can cleave glycosidic bonds and can begin with disaccharides such as lactose, sucrose and maltose or even more complex carbohydrates. Most of these

monosaccharides are converted into pyruvic acid by Embden- Meyerhof pathway (Glycolytic pathway). They diversify from pyruvate to yield a variety of end products. Even a given bacterial species can produce multiple products as in mixed acid fermentation. An overview of this capability can be had from Fig. 2.1.

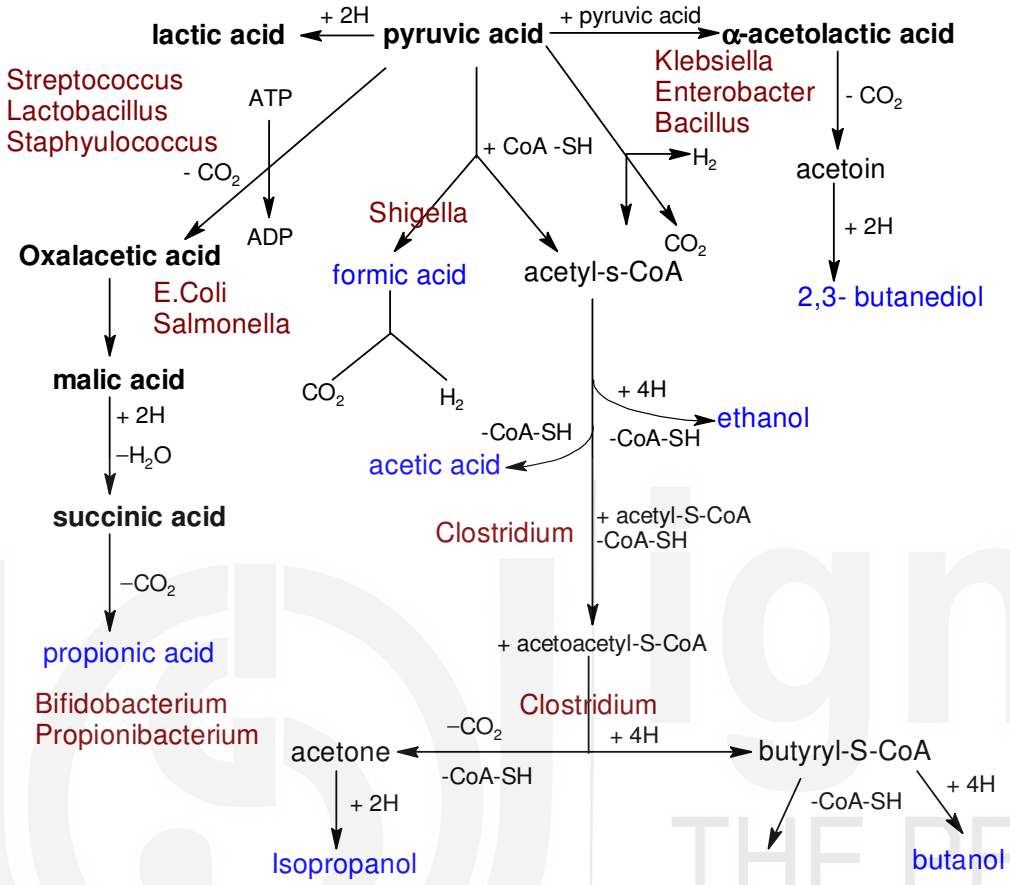

Fig. 2.1: Sugar fermentation pattern of different bacterial species.

The degradation of carbohydrates under anaerobic conditions is carried out in a fermentation broth containing a Durham tube (an inverted inner vial) for the detection of gas production which is visible as a bubble in the inverted vial and pH indicator (phenol red) to detect change in pH upon acid production.

2.3 MATERIALS REQUIRED

1. Broth Media: You would need the following to prepare 1L of medium.

   Sodium Chloride 1 gm

   Proteose peptone number 3 10 gm

   Sugar (Glucose or sucrose or maltose or lactose) 10 gm

   Beef extract: 1 gm

   Phenol red indicator: 0.018 gm (7.2 ml of 0.25% phenol red solution)

Note: Phenol red indicator, which is red in neutral or basic solution and yellow in acid solution. You can also use bromocresol / bromocresol purple (BCP), bromothymol/ bromothymol blue (BTB).

2. Test tubes (13 x 100 mm size)
3. Durham tubes (These are inverted vials, which are used to trap any gas produced)
4. Distilled water.

2.4 PROTOCOL

(a) Preparation of the broth media

(1) Prepare the Broth media by mixing all ingredients (refer to section 2.3) in distilled water. Make up the volume to 1000ml. Heat the contents gently to dissolve.

(2) In order to determine the fermentative capabilities of an organism, a single sugar source is used for the preparation of each broth media. The broth media is named based on the added sugar. So you are going to have four different broth media, namely:

(a) Phenol Red Glucose Broth,

(b) Phenol Red Lactose Broth,

(c) Phenol Red Maltose Broth,

(d) Phenol Red Sucrose Broth

(3) For each bacterial culture, take four test tubes with 5ml of different broth medium. Make sure to label them appropriately with name of media and the organism you are going to add.

(4) Insert Durham tube in the test tube.

(5) Finally, close the test tube with a rubber lid.

(6) Sterilize the broth media in tubes by autoclaving at 121℃ for 15 minutes. You would see that the media has light red color upon cooling.

(b) Inoculation and Incubation

(1) With the sterile loop, uncap the culture tube/plate and aseptically pick a loopful of bacteria from it and replace the culture tube/plate in the rack.

(2) Take the sugar broth tube, remove the lid, and sterlize the neck by rapidly passing through the flame few times.

(3) Transfer bacteria from the loop below the broth surface.

(4) Replace the lid, and return the tube to its rack.

(5) Flame the loop.

(6) Repeat the inoculation process for the remaining sugar broth tubes (Fig. 2.2).

(7) Incubate the tubes for 24 to 48 hours at 37℃. A set of uninoculated control tubes should be included.

(8) Observe the tubes for acid and gas production. Record your observations.

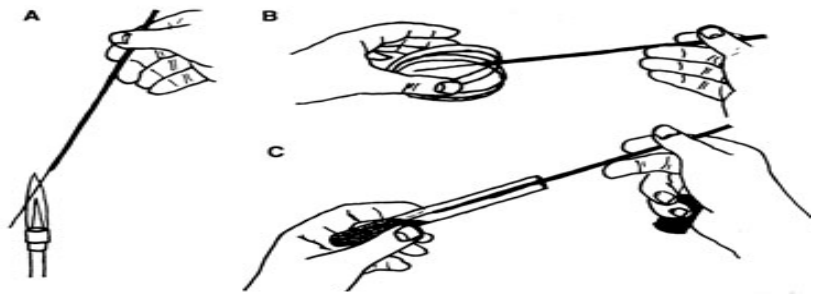

Fig. 2.2: Inoculation of bacteria in sugar broth; A. Sterilize the inoculating needle by heating to redness in a flame and then cooling. B. Streaking of bacterial culture or C. Place the inoculating needle / loop containing bacteria below the broth surface while shaking it lightly.

2.5 OBSERVATIONS AND RESULTS

Examine the Durham tube in each test tube for the presence of gas bubble and change in broth color from red to yellow for acid production (Fig. 2.3).

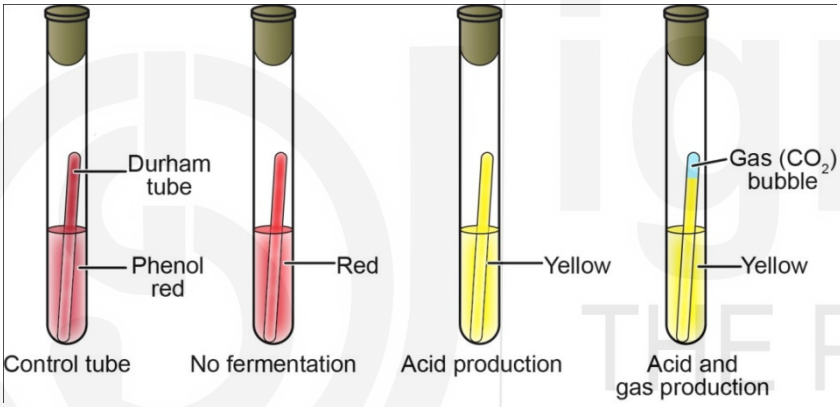

Fig. 2.3: A control tube and three experimental tubes showing the possible results of the sugar fermentation by bacteria.

Record the results of sugar fermentation test in the Table 2.1.

Table 2.1: Observation table

Name of the bacteria → / Broth medium ↓	Sugar fermented			Acid Production			Gas Production		
	A	B	C	A	B	C	A	B	C
Glucose									
Lactose									
Maltose									
Sucrose									

A- Bacterial culture 1; B- Bacterial culture 2; C- Bacterial culture 3

Find the names of the bacterial cultures from your instructor.

2.6 PRECAUTIONS

i) The nutrient broth ingredients must support the growth of all microorganisms investigated.

ii) The inoculating needle or loop must be sterilized after each transfer by holding it in the hottest portion (light blue cone) of the flame until the entire wire turns red hot.

iii) Allow it to cool before picking bacterial cells from the culture.

iv) Do not put the loop down or wave it around.

v) Always have a control tube (uninoculated) for each sugar tested.

vi) During inoculation do not shake the inoculation tube as it may force a bubble of air into the inverted glass vial, giving a false positive.

vii) (Label each tube to be inoculated with the name of the organism and sugar source.

viii) Observe the cultures within 48 hours. Refrigerate the tubes if there is a delay.

2.7 SUMMARY

Organism: ---------- ---------- ---------- ----------

Sugar : ---------- ---------- ---------- ----------

Test results: ---------- ---------- ---------- ----------

EXPERIMENT 3

DEMONSTRATION OF STARCH DIGESTION BY SALIVARY AMYLASE

Structure

3.1 INTRODUCTION

Starch is storage polysaccharide which is obtained from plant sources such as wheat, rice, potato etc. It is a homopolymer of glucose that polymerises to form a linear polymer, amylose and a branched polymer, amylopectin. The proportion of the two polymers varies from one plant source to another. When food containing starch is ingested, its digestion begins in the mouth due to the activity of salivary amylase. This enzyme plays an important role in the digestion of starch. In this lab exercise, you will qualitatively monitor starch digestion by salivary amylase using iodine.

Expected Learning Outcomes

After studying this unit, you should be able to:

- prepare a crude extract of salivary amylase;
- demonstrate the digestion of starch by salivary amylase; and
- explain the physiological digestion of starch in mouth.

3.2 PRINCIPLE

Iodine is commonly used to detect presence of starch. Linear amylose structure of starch forms a helical chain in which iodine gets trapped giving blue color Fig. 3.1.

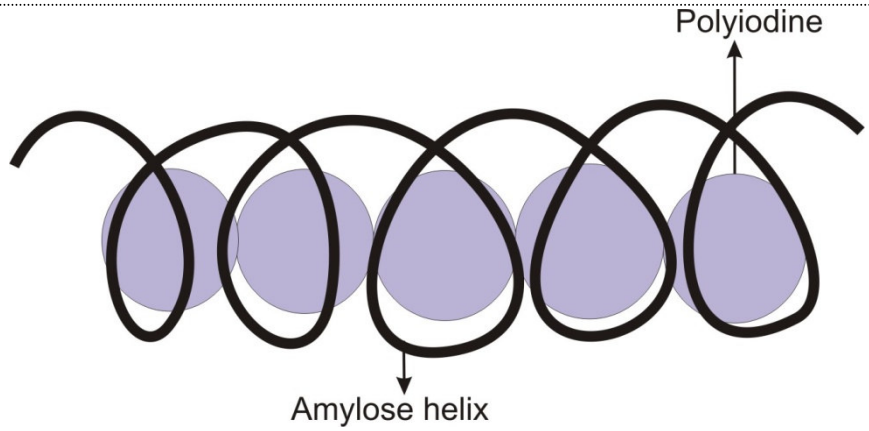

Fig. 3.1: Formation of starch iodine complex.

You will see that while preparing iodine solution, KI is added. It is because molecular iodine (I_2) is not very soluble in water; addition of KI increases its solubility. Further it results in formation of charge transfer (CT) complex which gives dark brown color to iodine. I_2/KI together form polyiodide ions such as I_3^-, I_5^- or I_7^-. These charged ions donate charge to neutral I_2 forming CT complexes which when excited with light give different colors leading to overall brownish color. When amylose is added, it forms complex which absorbs at a wavelength different form polyiodide, resulting in blue color.

3.3 MATERIALS REQUIRED

1. Starch solution (1 %; 1 g starch is dissolved in 100 ml distilled water).
2. Distilled water
3. Iodine / KI (I_3K) solution

   Iodine 2.0 g

   Potassium iodide 4.0g

   Distilled water 100ml

   Grind iodine and KI in a mortar; transfer it to a 100ml conical flask. Dissolve by slowly adding water and mixing. Make up the volume to 100ml. Store in a dark bottle protected from direct sunlight.
4. Saliva solution (source of salivary amylase)
5. Muslin cloth
6. Test tubes
7. Dropper
8. Ice cubes.
9. Stop watch.
10. Thermometer.
11. Water bath.

3.4 PROTOCOL

(a) Preparation of crude extract of salivary amylase

1. Rinse your mouth thoroughly with distilled water. This is to ensure that the saliva does not contain any food particles.
2. Take 5 ml of distilled water in the mouth and gargle for about 3 minutes. In this way, saliva is properly mixed with distilled water.
3. Now, spit it into a beaker.
4. Filter the saliva solution through a muslin cloth to get rid of any suspended particles.
5. Keep the filtered saliva solution on ice and use it as a crude source of salivary amylase.

(b) Preparation of starch solution

1. Weigh 1 g of starch and place it in a 250 ml beaker.
2. Measure 100 ml of distilled water.
3. Add only 2-5 ml of distilled water to starch to begin with and mix to make a smooth paste.
4. After this, add the rest of the water and mix well.
5. Now, boil this solution for 5 minutes and allow it to cool at room temperature.

(c) Digestion of starch

1. Pipette out 5 ml of 0.1% starch solution in a test tube.
2. Add 2 ml of saliva solution.
3. Mix the two solutions properly by the gently shaking the tube for 10-20 seconds.
4. Place the experimental tube in a water bath maintained at 37^{o}C.
5. Now, start the stop watch.
6. After completion of 1 minute, pipette out 1-2 drops of solution with a dropper.
7. Add it to 1ml of iodine solution containing test tube. Observe the colour produced, if any.
8. Repeat steps 6 and 7 after every one minute until the blue-black colour of iodine with starch disappears.
9. Disappearance of the blue-black colour indicates that the digestion of starch is completed Fig. 3.2.

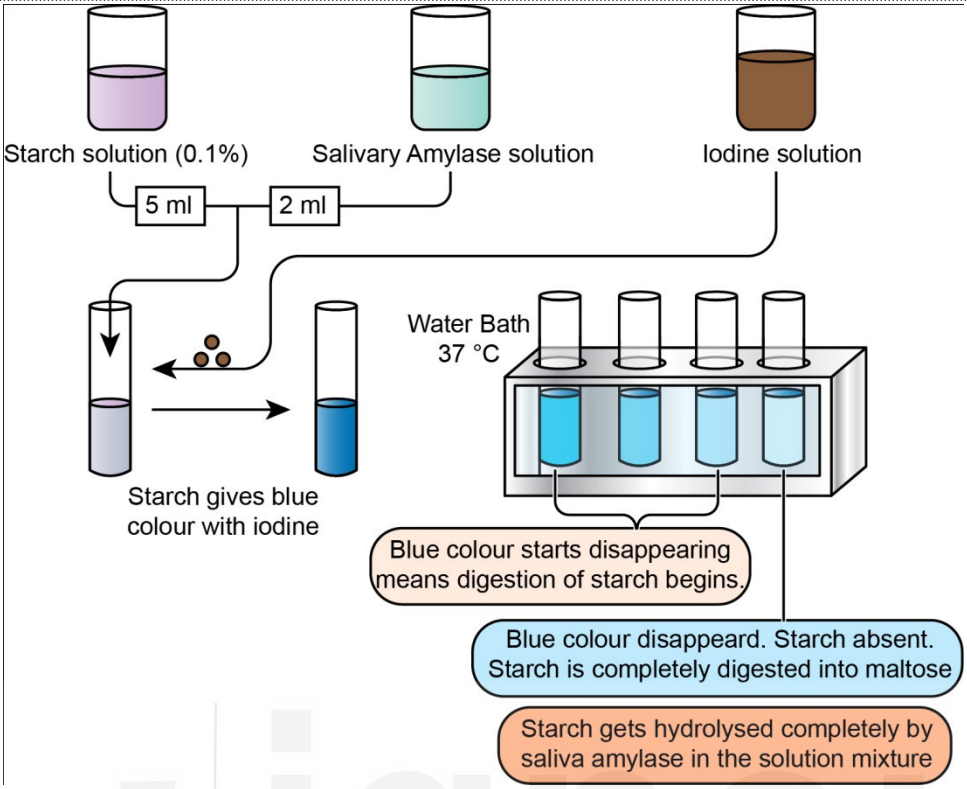

Fig. 3.2: Digestion of starch by salivary amylase monitored with addition of iodine.

3.5 OBSERVATIONS AND RESULTS

The change in colour intensity is recorded at one minute intervals by pipetting 1-2 drops of starch solution undergoing enzymatic digestion, until the completion of digestion. Present your results in the Table 3.1 by grading the change in colour intensity from deep blue to blue to light blue to colorless. You may continue for few more minutes if digestion is incomplete.

Table 3.1: Observation table of starch hydrolysis

Time	1 min.	2 min.	3 min.	4 min.	5 min.
Colour intensity (colour of solution)					

3.6 PRECAUTIONS

1. Rinse your mouth thoroughly before collecting saliva. The solution must be filtered.
2. Keep the crude enzyme extract on ice to prevent loss of activity.
3. Never do mouth pipetting.
4. Carefully remove aliquots at 1min interval.

EXPERIMENT 4

ISOLATION AND FRACTIONATION OF EGG LIPIDS BY TLC AND THEIR ESTIMATION

Structure

4.1 INTRODUCTION

Lipids represent a chemically heterogeneous group of biomolecules that share the property of relatively insolubility in water but soluble in organic solvents such as alcohols, ether, chloroform and hydrocarbons. They can are classified based of their structure (glycerolipids sphingolipids, cholesterol, waxes, etc) or function (membrane lipids, storage lipids, signaling molecules, lipid anchors for proteins, carriers). Lipids are also subdivided into saponifiable (phosphoglycerides; triglycerides) or non-saponifiable (cholesterol) depending on their reaction with strong alkali. In this lab exercise, you are going to learn about the isolation and fractionation of chicken egg lipids by thin layer chromatography (TLC), and their estimation (Fig. 4.1).

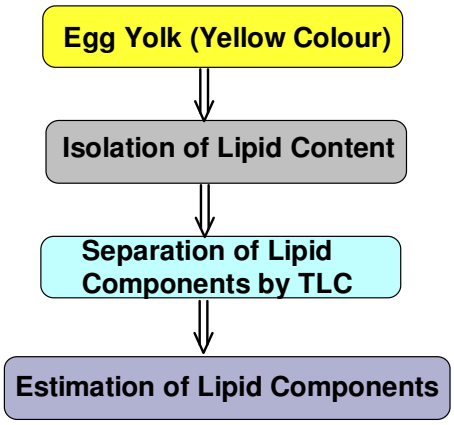

Fig. 4.1: Summary of the experimental procedure.

The egg yolk (yellow colour) is the chief source of lipids (31 %) in chicken egg. It has 46% of triglycerides, 20% phospholipids and 3% cholesterol. The lipids are rich in unsaturated fatty acids [oleic acid (47%), linoleic acid (16%), palmitoleic acid (5%) and linolenic acid (2%)] and saturated fatty acids [palmitic acid (23%), stearic acid (4%) and myristic acid (1%)].

Expected Learning Outcomes

After studying this unit, you should be able to:

- extract lipids from egg yolk;
- separate lipids by thin layer chromatography (TLC);
- explain the principle of separation of different lipids; and
- estimate the concentration of lipids in the separated fractions.

4.2 PRINCIPLE

Lipids are non polar class of biomolecules that are readily extracted from biological sources using non polar solvents. Total lipids from egg yolk can be isolated using sodium chloride, isopropyl alcohol and petroleum ether or any other combination of two or more solvents of different polarities. The extracted lipids may be separated by adsorption chromatography on columns or thin layers. In this exercise we shall separate lipids by thin layer chromatography (TLC).

The earliest reported use of chromatographic adsorbent in the form of thin layer on an inert support is by two Russian chemists, **M.A. Ismailov and M.S. Schreiber (1938).** In the 1950s the technique of thin layer chromatography became more widely used essentially due the improvements in plate preparation, use of binder and the variety of separations that can be carried out. It is performed on a glass or metallic sheet coated with silica gel or any material that can be finely ground, made into slurry and spread into thin layers. The layer of adsorbent is known as the stationary phase. Minimum volume of sample dissolved in a volatile solvent is applied on the coated plate. The plate is kept in a chamber saturated with vapours of the mobile phase (solvent / mixture of solvents) which moves up the plate by capillary action. The mobile phase is chosen according to the properties of the components in the mixture.

In case of lipid fractionation the coated silica gel is activated by removing water. Separation is predominantly on the basis of adsorption and to some extent liquid-liquid partitioning. Molecules are separated if they have different adsorption isotherm. The less polar lipids move farthest as they bind relatively less to polar silicic acid (higher Rf value; (Fig. 4.2). In general, neutral lipids are separated with non-polar solvents and polar lipids with relatively polar solvents.

The separated lipids can be visualized using different methods. These methods are either destructive (50% sulphuric acid followed by heating) or non destructive (iodine vapors; dichlorofluorescein); some reagents help to detect specific lipids. If non destructive methods are used lipids can be recovered by

scraping the adsorbent from the plate and processed for estimation. However, in this exercise we will do the estimation in the total lipid extracted from the egg yolk using standard colorimetric methods.

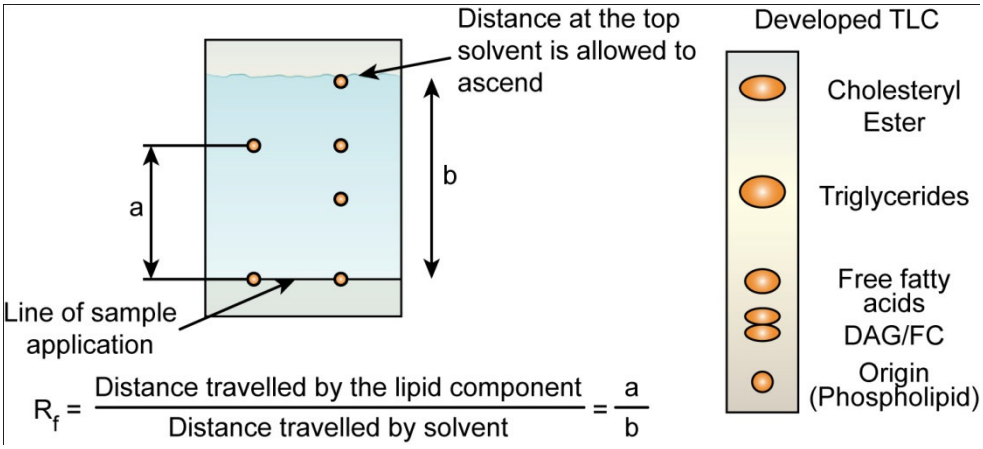

Fig. 4.2: Separation of lipid components by thin layer chromatography.

4.3 MATERIALS REQUIRED

(a) Isolation of lipids from egg yolk

1. Chicken egg (1-2),
2. Sodium chloride (IM),
3. Isopropyl alcohol,
4. Petroleum ether,
5. Glass tubes and test tubes,
6. Separating flasks.

(b) Fractionation of isolated lipids by TLC

1. Thin layer plates coated with silica gel G,
2. Separation chambers,
3. Solvent system (petroleum ether: diethyl ether: glacial acetic acid; 80:20:1),
4. Hydrocarbons (n-hexadecane and n-octadecane),
5. Standard lipids:
   (a) Triacylglycerols (glycerol trioleate, glycerol tristearate and glycerol tripalmitate),
   (b) Cholesterol esters (cholesterol acetate, cholesterol oleate and cholesterol stearate),
   (c) Free fatty acids (oleic acid, palmitic acid and stearic acid),
6. 2', 7'-Dichlorofluorescein (2 g/litre in 95 % v/v ethanol),

7. Sulphuric acid (50% v/v),
8. Oven (set at 110 °C),
9. Ultraviolet lamp.

(c) Estimation of fractionated lipids

(i) Estimation of cholesterol

1. Ferric chloride - uranyl acetate reagent

   To prepare this solution, dissolve 500 mg of ferric chloride in 10 ml of water and then add 3.0 ml of concentrated ammonia. Mix properly and centrifuge at 2000 rpm for 5 minutes. After centrifugation, decant the supernatant and wash the precipitate 4-5 times with distilled water and dissolve it in 1 litre of glacial acetic acid. Finally, add 100 mg uranyl acetate to the solution, mix thoroughly and leave over night.

   Note: The reagent is stable for six months.

2. Sulphuric acid - Ferrous sulphate reagent

   Dissolve 100 mg of ferrous sulphate in 100 ml glacial acetic acid. You would notice generation of heat during solubilization. Once the solution cools down, make up the volume to one liter with concentrated sulphuric acid.

   Note: The reagent can be used for six months.

3. Cholesterol standard

   Dissolve 200 mg of cholesterol in 100 ml of acetic acid. The working standard (200 µg / ml) is made by diluting 1 ml of the stock solution to 100 with acetic acid.

(ii) Estimation of triacylglycerol

1. Chloroform and methanol mixture (ratio 2:1).
2. Activated silicic acid

   Wash silicic acid with 4 N or 2 N HCl. Remove the traces of acid by washing repeatedly with water. Dry the washed silicic acid by addition of sufficient amount of ether and continuous stirring. Discard the supernatant, dry silicic acid at 60°C and activate by heating at 100°C over night prior to use.

3. H_2S0_4 (0.4N)

   Measure 25ml of DW, transfer it to beaker and add carefully 1.11 ml of conc. sulphuric acid; mix and make up the volume to 100 ml with distilled water. Normality of commercially available sulphuric acid is 36 N. You may calculate the required volume of acid by using the formula, $N_1V_1=N_2V_2$; where N_1 and N_2 are normality; V_1 and V_2 are volume of conc. H_2S0_4 and diluted acid, respectively.

4. Saponification reagent

   Weigh 5g of KOH and dissolve in 60 ml distilled water. After complete solubilization, 40 ml of isopropanol is added to it.

5. Sodium metaperiodate reagent

   Weigh 77 g anhydrous ammonium acetate and dissolve it in 700 ml distilled water. After complete solubilization, add 60 ml acetic acid and 650 mg of sodium metaperiodate. Before use the reagent is diluted 11 times with distilled water.

6. Acetyl acetone reagent

   Mix 0.75 ml acetyl acetone properly with 20 ml of isopropanol. Then add 80 ml of distilled water and mix again.

7. Tripalmitin standard

   Dissolve tripalmitin in chloroform to make 100 pg /ml

(iii) Estimation of phospholipids

1. Perchloric acid (70%).
2. Ammonium molybdate (3%; 3g / 100ml distilled water)
3. Ascorbic acid (3%)
4. Standard phosphorous solution (80 pg / ml)

   Dissolve KH_2P0_4 (35.1 mg) in100ml distilled water.

(iv) Estimation of free fatty acids (FFA)

1. Chloroform-heptane-methanol mixture (CHM mixture) in the ratio of 200:150: 7.
2. Activated silicic acid: Prepare as described in preparation for estimation of triacyl glycerol
3. Copper nitrate - triethanolamine solution

   Measure 9 ml of 1M aqueous triethanolamine, 1 ml of 1 N acetic acid, 10 ml of $Cu(NO_3).23H_2O$ (6.45%) and weigh 33g of sodium chloride. Mix properly and adjust the pH to 8.1.

4. Diethyl dithiocarbamate (0.1%)

   Dissolve 100 mg diethyl dithiocarbamate in 100 ml n-butanol

5. Standard palmitic acid (200mg/100 ml) is prepared in CHM mixture. The working solution is diluted 1: 10 (200 µg /ml).

4.4 PROTOCOL

(a) Isolation of lipid from egg yolk

1. Separate egg yolk from chicken egg (Fig.4.2) and measure its volume.

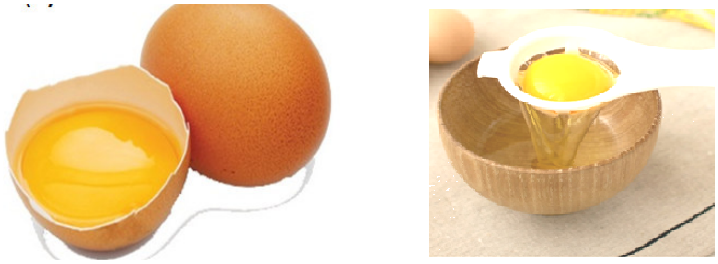

Fig. 4.2: (A) Egg yolk with egg white and (B) separation of egg yolk from egg white.

2. Add the 1M sodium chloride solution to the egg yolk in the ratio 1:5 v/v.
3. Take 2 ml of diluted egg yolk in a test tube and mix it with 3 ml isopropyl alcohol.
4. Next add 2 ml volume of petroleum ether to it.
5. Transfer the above mixture to a clean flask and covered it with rubber stopper.
6. Mix well for 3-5 minutes.
7. Allow the mixture to stand for 10 minutes to get two distinct layers.
8. Carefully pick the lower layer, transfer to a clean test tube and cover it.

(b) Fractionation of isolated lipids by TLC

You can either use readymade TLC sheets after cutting to the desired size or prepare plates manually, although the former is commonly used in most laboratories. To prepare TLC plates follow the following steps:

1. Clean the glass plates well with soap and water and let them dry.
2. Wipe the glass plates with ethanol to remove any greasy material.
3. Weigh 4gm of silica gel G.
4. Prepare silica gel slurry by adding 8ml of water to it (1:2 w/v).
5. Mix it thoroughly and immediately pour it into a clean glass plate.
6. Spread it manually to prepare a plate of uniform thickness (250 µm), taking care to remove all air bubbles. You can also use a TLC maker.
7. Leave the plates to air dry.
8. Now activate them by heating at 110℃ for one hour in an oven.
9. Allow the plates to cool down before spotting.
10. Next, prepare the solvent system by mixing petroleum ether: diethyl ether: glacial acetic acid in the ratio of 80:20:1. Prepare the required volume depending on the size of the solvent tank. After you add the solvent system in the solvent tank, leave it covered for 2hr for saturation with the solvent vapors.

11. Now you are ready for spotting. For standard lipids approximately 1 % w/v solution of each lipid is prepared; of which 10µl is spotted. Similarly 10-15 µl of the isolated lipids are spotted. However, actual volume may vary depending upon the concentration of the sample. Do not spot the entire amount in one go while spotting,. Instead, first spot 5 µl of the lipid at the designated place on the plate, let it dry and then spot another 5 µl on top of previous spot, again let it dry. Repeat till entire amount is spotted. Similarly spot the other lipids also. After spotting is complete, place the plate in the saturated solvent tank and allow it to run till 2-3 cm from top is left (Fig. 4.3). Mark the position o the solvent immediately after taking out the plate and let it dry. Also note the time taken for the run.

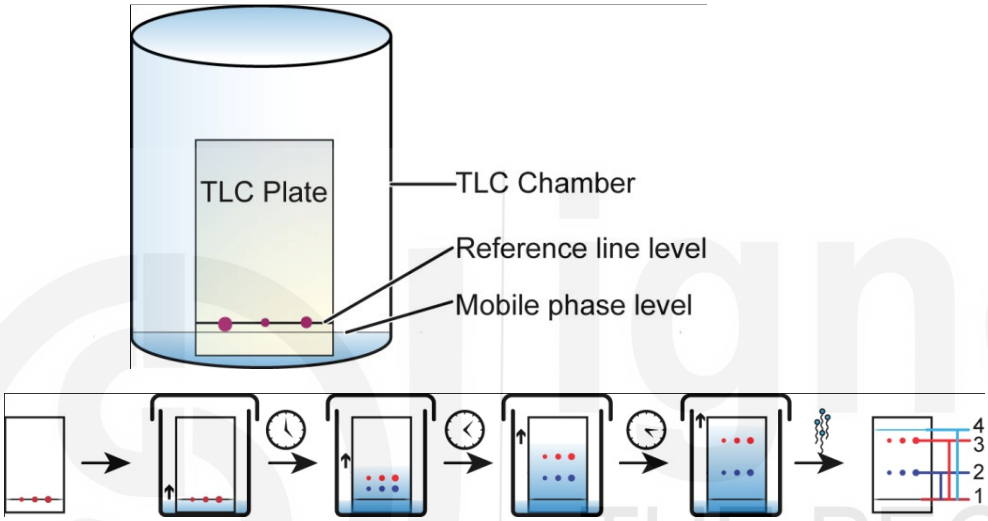

Fig. 4.3: Fractionation of lipids by TLC. The reference line and mobile phase level; TLC plate orientation and TLC chamber are marked in the figure.

12. Locate the fractionated lipids by spraying with the dichlorofluorescein solution and place it under ultraviolet light at 270 nm for visualization.

13. In the case of unavailability of UV spectroscopy, the spots can also be visualized by spraying the plates with 50% v/v sulphuric acid. After spraying, heat the plates at 110°C for 10 minutes and allow them to cool down to complete the process of development of spots.

14. After development of the spots, mark them with pencil and take their picture.

(c) Estimation of lipid components of egg yolk

Estimation of cholesterol (Parekh and Jung method; 1970)

1. Take 0.1 ml of total lipid extract, evaporate it to dryness, and add 3.0 ml of ferric chloride uranyl acetate reagent.

2. Standard cholesterol solution containing 20-60 µg of standard is evaporated to dryness and made up to 3.0 ml with ferric chloride uranyl acetate reagent.

3. The blank has only 3 ml of ferric chloride uranyl acetate reagent.
4. Add 2.0 ml of the sulphuric - ferrous sulphate reagent and transfer all three tubes to an ice bath for 20 min for color development.
5. The purple color is read at 540 nm with the help of spectrophotometer.

Estimation of triacylglycerol

1. Take 0.1 ml of total lipid extract and evaporate it to dryness; then add 40 ml isopropanol and 400 mg of silicic acid.
2. Mix properly in a mechanical shaker for 20 minutes.
3. Centrifuge the solution at 1000 g for 15 minutes and collect the supernatant.
4. To 2.0 ml of the supernatant, add 0.6 ml saponification reagent and incubate at 60-70°C for 15 min.
5. After cooling added 1.0 ml of sodium metaperiodate and mix well. Then add 0.5 ml of acetyl acetone reagent and mix again.
6. Incubate the tubes at 50°C for 30 minutes and allow them to cool for 10 minutes. The solution turns yellow.
7. Record absorbance at 405 nm in a spectrophotometer.
8. Repeat the above steps with standard tripalmitin (20-100 pg).

Estimation of phospholipids

1. Take 0.1 ml of total lipid extract, evaporate to dryness.
2. Add 1.0 ml of perchloric acid and digest it on a sand bath. The solution will become colourless after digestion is complete.
3. Make up the volume of digested lipid to 5.0 ml with water.
4. Add 0.5 ml of ammonium molybdate followed by 0.5 ml of ascorbic acid solution. Mix well.
5. Keep the tubes in a boiling water bath for 6 min. You would see development of blue color. Take OD at 700nm and record the readings in the observation table.

Estimation of free fatty acids

1. Take 0.1 ml of total lipid extract, evaporate to dryness and mix well with 6.0 ml of chloroform-heptane-methanol mixture.
2. Now add 200 mg of activated silicic acid and mix again.
3. Centrifuge the solution at 2000 rpm for five minutes and transfer the supernatant to another clean tube.
4. Add 2.0 ml of copper nitrate - TEA solution and mix it on a mechanical shaker for 20 min.
5. Again centrifuge at 2000 rpm for 5 minutes. Two distinct phases can be seen.

6. Transfer 2.0 ml of the upper phase to another tube and add 1.0 ml of the coloring reagent, mix well.

7. Read the blue color developed at 430 nm and record in the observation table.

4.5 OBSERVATIONS AND RESULTS

Table 4.1: Cholesterol estimation

Test tube	Lipid extract (ml)	Standard cholesterol (µl)	Ferric chloride uranyl acetate reagent (ml)	H_2SO_4-Ferrous sulphate reagent (ml)		Absorbance at 540 nm
Blank	0.0	0.0	3.0	2.0	Mixed and incubated in ice bath for 15 minutes	
Standard	0.0	10	3.0	2.0		
Test	0.1	0.0	3.0	2.0		

$$\text{Concentration of cholesterol in egg yolk} = \frac{\text{Absorbance of extracted lipid} \times \text{concentration of standard cholesterol}}{\text{Absorbance of standard cholesterol}}$$

Table 4.2: Triacylglycerol estimation

S. No.	Lipid extract (ml)	Standard TAG (µl)	Vol. of isopropanol (ml)	Silicic acid (mg)		Supernatant (ml)	Saponification reagent (ml)		Sodium etaperiodate (ml)	Acetyl acetone (ml)		Absorbance at 405 nm
1 Blank	0.0	0.0	40	400	Mixing in a mechanical shaker; centrifuged	2.0	0.6	Incubated at 60-70° C for 15 min. Kept for cooling	1.0	0.5	Incubated at 50° C for 30 min, cooled	
2 Std.	0.0	10	40	400		2.0	0.6		1.0	0.5		
3 Test	0.1	0.0	40	400		2.0	0.6		1.0	0.5		

$$\text{Concentration of triglycerides in egg yolk} = \frac{\text{Absorbance of extracted lipid} \times \text{concentration of standard triglycerides}}{\text{Absorbance of standard triglycerides}}$$

Table 4.3 Phospholipids estimation

S. No.	Lipid extract (ml)	PCA (ml)	Standard Phosphate (µl)	Digestion on sand bath colourless	Distilled Water (ml)	Ammonium molybdate (ml)	Ascorbic acid (ml)	Heat the tubes on boiling water bath for 6 min; cool	O.D 700 nm
1. Blank	0.0	1.0	0.0		5.0	0.5	0.5		
2. Standard	0.0	1.0	250		5.0	0.5	0.5		
3. Test	0.1	1.0	0.0		5.0	0.5	0.5		

$$\text{Concentration of phospholipids in egg yolk} = \frac{\text{Absorbance of extracted lipid} \times \text{concentration of standard phosphates}}{\text{Absorbance of standard phosphates}}$$

Table 4.4 Free fatty acid estimation:

S. No.	Lipid extract (ml)	CHM mixture (ml)	Standard (ml)	Activated silicic acid (mg)	Mix well; centrifuge and collect supernatant	Copper nitrate-triethanolamine solution (ml)	Mix on a mechanical shaker for 20 min followed by centrifugation. Two phase separate. Transfer the upper phase to another tube.	Diethyl dithiocarbamate in n-butanol (ml)	Mixed and shaken well	A 440 nm
1. Blank	0.0	6.0	0.0	200		2.0				
2. Standard	0.0	6.0	1.0	200		2.0				
3. Test	0.2	6.0	0.0	200		2.0				

$$\text{Concentration of free fatty acids in egg yolk} = \frac{\text{Absorbance of extracted lipid} \times \text{concentration of standard fatty acids}}{\text{Absorbance of standard fatty acids}}$$

4.6 PRECAUTIONS

1. The silica gel slurry must be spread as quickly as possible otherwise the binder will start setting.
2. In order to obtain reproducible results, the activation time must be kept constant. Overheating should be avoided.
3. The sample should be dissolved in a volatile solvent of low polarity.
4. Keep the chromatographic jar covered so that evaporation does not change the composition of the solvent system.
5. In non preparative thin layer plates load minimum samples volumes (5-15 µl). Overloading will lead to merging of spots. Do not load the entire sample in one go. Load 5µl, let it dry and then load another 5µl till whole amount is loaded on the plate.
6. Take care not to remove the coated gel while loading.

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