Indira Gandhi National
Open University
School of Sciences

MOLECULES OF LIFE: LAB

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MOLECULES OF LIFE: LAB (BCHEL-150)

You have performed several experiments in your core chemistry courses and have familiarized yourself with the techniques involved. You are aware that all those lab courses were integrated to the relevant theory courses. We are sure that the lab work must have been beneficial in a number of ways including the verification of various aspects of a particular theme. This laboratory course has been designed to understand the facts and concepts dealt in the related theory course i.e., BCHET-149 by performing some experiments. You would be using most of the techniques that are already used in the previous labs and involve both conventional and instrumental techniques. You know that the theory course dealt with the nature, structure, function, classification and properties of the major biomolecules that included carbohydrates, proteins, lipids, enzymes and nucleic acids. Their metabolism and bioenergetics were also described. However, in this lab course we have restricted to the qualitative and quantitative analysis of only the biomolecules.

The Molecules of Life lab course, BCHEL-150 consists of a total of ten experiments. The first three experiments are based on the properties of amino acids which are the building blocks of proteins, the important biomolecules. The experiments deal with the separation, determination of concentration and pK_a values of amino acids. The techniques used here are chromatography, titrimetry and pH metry. pH metry is the instrumental method of determining the pK_a and isoelectric point in amino acids.

Next two experiments are based on the catalytic property of enzyme and the structural property of starch, the polysaccharide. You would be able to observe the action of amylase on starch and the effect of temperature on the catalytic reaction.

You are familiar with the structures of oils and fats. These are either saturated or unsaturated in nature. The unsaturated might contain one two or more double bonds that can be observed with the help of chemical reactions. Two experiments are devoted to the determination of saponification number and iodine value of oils and fats.

You have read in Unit 2 of the theory course, BCHET-149 about the differences between reducing and nonreducing sugars as indicated by some of their reactions. In this course you will perform an experiment to observe the differences in the behaviour of the two types. The tests would be performed using some reagents leading to colour changes.

You have studied the structure of DNA and its significance in the heredity of living beings. Here, in one of the experiments you will learn and isolate DNA from an onion peel. This is relatively an advanced level of experiment which in a very simple way gives you the feel of an important molecule called the gene.

You very well know the importance of drugs and medicines and have studied the action of drugs in a human system in Unit 9 of the theory course. Therefore, one experiment is based on the type of organic molecules belonging to this category. You would be able to synthesise a common drug and compare its purity with the one available in the market using thin layer chromatographic technique.

Expected Learning Outcomes

After studying and performing the experiments given in this laboratory course you should be able to:

- explain the principle of paper chromatography and separate a mixture of amino acids by paper chromatography,

- explain the principle of titrimetry and determine the concentration of glycine by formylation method,
- explain the principle of titration curves in amino acids and determine the pK_a and pI of glycine using pH metry,
- describe the reaction between enzyme amylase and starch and perform the experiment to show the changes that take place due to the reaction,
- interpret the effect of temperature on the action of amylase on starch,
- define saponification and iodine value of oils and fats and calculate the values of the given samples,
- differentiate between reducing and nonreducing sugars and perform the tests justifying it,
- explain the acetylation reaction, prepare aspirin using this reaction and compare the product with the commercial tablet using thin layer chromatography.
- extract DNA from an onion peel by the prescribed procedure, and

GUIDELINES FOR DOING EXPERIMENTS

This laboratory course contains experiments based on the concepts covered in the BCHEL-149 course on 'Molecules of Life'. It is desirable that you go through the contents of the theory course. However, the relevant concepts have been suitably recalled, wherever necessary, for the sake of essential understanding. Thus like other lab courses, this course is also a standalone course. The course is worth **2 credits** which as per IGNOU norms amount to **60 hours** of lab work. This includes reading the contents, performing the experiments, participating in the interaction with the peers and the counsellor, preparing the laboratory notebook, etc. It is strongly advised that you read the details of the experiments before performing these. This experimental work will be conducted at the study centre allocated to you for the purpose.

The duration of the lab would be of **7 days**; each day having **2 sessions** of **4 hours** each. Thus, there are **14 sessions** in total. The first session is meant for the introduction where your counsellor will give you an overview of the entire experimental work and any useful instructions to be followed in the course of the laboratory work. The last two sessions are meant for the term-end evaluation. In the rest of the sessions you would be performing the experiments, detailed in the block, under the guidance of your counsellor. These experiments are termed as **'Guided Experiments'** and would be evaluated on a continuous basis. These guided experiments carry a weightage of **70%.** You are required to maintain a laboratory notebook wherein you would record the experiments performed and get them corrected by the counsellor regularly. On the last day you would be required to perform one of the experiments, allocated by your counsellor, on your own. This is termed as '**Unguided Experiment**' and has a weightage of **30%.** It is necessary for you to get pass marks **(at least 35%)** in both of these components of evaluation.

We hope that you would find the contents of the course and your laboratory experience stimulating and useful. In case you find difficulty in understanding any concept, you may get it clarified in the counselling sessions or feel free to mail your queries to us at:

lalitaskumar@ignou.ac.in / bifozdar@ignou.ac.in / jafarooqi@ignou.ac.in

Wish you Good Luck……..

IMPORTANT

- **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 **5 days** duration:
  - **4 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

SEPARATION OF THE COMPONENTS OF A GIVEN MIXTURE OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

Structure

1.1 INTRODUCTION

In chemistry, it is necessary to separate, isolate, purify and identify components of complex mixtures. Simple separation techniques such as distillation, crystallisation, extraction are quite useful when the components of mixture are chemically and physically different and are few in number. For example, the isolation of pure benzoic acid from its diethyl ether solution requires the use of distillation to remove the more volatile diethyl ether. The chemical structures, intermolecular forces and hence boiling points of these two substances differ considerably, allowing the use of such a technique of separation. However, where the components of a mixture resemble each other very closely in structure, simple separation techniques are of little value. For example, amino acids are closely related chemically and physically, none of the techniques mentioned above, can be used for the separation of such types of mixtures.

Similarly when, we carry out reactions in the laboratory, many a times the resulting product is not a single compound but a mixture having many components which are structurally related. Thus techniques are needed which could be used to separate such closely related substances. There are techniques, known as **chromatographic techniques** available for separation of such mixtures. In this experiment and in the next experiment, you will be studying about these techniques. You will also perform two experiments: Experiment 7 and Experiment 8 based on paper chromatography, which is one of the simplest chromatographic technique**.**

Expected Learning Outcomes

After performing the experiment, you should be able to:

- explain the meaning of the terms chromatography, stationary phase and mobile phase;
- explain the mechanisms of partition as applied to chromatography;
- calculate R_f values for different organic compounds;
- set up experimental arrangement for paper chromatography; and
- separate and identify amino acids from the mixture of two amino acids by paper chromatography.

1.2 THE ORIGINS OF CHROMATOGRAPHY

The word **chromatography** is Greek in origin (*chroma* - colour; *graph* - writing). The technique of chromatography was first applied to the separation of coloured pigments and dyestuffs, as evident from the pioneering work of Tswett who in 1906 separated the pigments from leaves into coloured bands by passing a solution of the pigments down a glass tube packed with powdered chalk. The separation of pigments that Tswett achieved was due to the process called **'adsorption'**. The most adsorbed pigment remains close to the top of the column and the least adsorbed pigment is 'washed through' to form the lowest band in the column.

The term *chromatography* is applied to the process which involves the distribution of a sample between a stationary phase and a mobile phase.

In 1941 Martin and Synge used liquid-impregnated columns of absorbent to obtain good separation of components in complex mixtures. The mechanism of separation was in this case a **partitioning** or **distribution phenomenon** involving each component in the mixture. We will consider the nature of these mechanisms of separation in more detail in the experiment part.

A. J. P. Martin and R. L. M. Synge were jointly awarded the Nobel Prize in 1952 for their work on partition chromatography.

Apart from column chromatography, many other chromatographic techniques were developed in last few decades. These can be listed as:

(1) Paper chromatography (PC)

(2) Thin-layer chromatography (TLC)

(3) Column chromatography (CC)

(4) Gas chromatography (GC)

(5) Gas-liquid chromatography (GLC)

(6) High resolution gas chromatography (HRGC)

(7) High performance liquid chromatography (HPLC).

The last four methods are useful in achieving a high degree of separation or resolution and often involve use of elaborate and expensive apparatus.

Chromatography is used to isolate, purify the components of complex mixtures in the laboratory both for analytical and preparative purposes. Chromatography is used in labs right from the simple techniques such as paper chromatography to the sophisticated ones such as Gas chromatography and HPLC which find applications in research labs and industries.

In this experiment, you will be using the simplest laboratory chromatographic method, paper chromatography "which is relatively cheap." Nevertheless, the basic principles remain the same and with skillful usage surprisingly good separations can be achieved. You will also do some experiments based on this chromatography during practical sessions. Let us now study the language of these techniques. But before that answer the following SAQ.

SAQ 1

Which of the following characteristics would you say must be present for a mixture to be satisfactorily separated by column chromatography? (Tick the correct choice).

a) Be coloured ☐

b) Be volatile ☐

c) Be related by similar chemical structure ☐

d) Show a differential adsorption to the column. ☐

1.3 TERMINOLOGY OF CHROMATOGRAPHY

Various terms are frequently used and they form the language of chromatographic techniques. These are explained below:

(i) Chromatography

The historical origins of the word chromatography have already been discussed earlier. All types of chromatography have certain basic features, although from the design of the apparatus, this may not be apparent. The basic methodology involved is as follows:

The mixture to be separated is added to a moving 'solvent' which may be liquid or gas. This moving stream, now containing components, is passed over or through a fixed medium, which is specially designed to separate the individual constituents in the original mixture.

However, there are many variations and hence in many cases, it is difficult to see the common link between the various chromatographic methods.

(ii) Mobile Phase and Stationary Phase

The moving component in the chromatographic technique is called the **mobile phase**, which is normally a liquid or a mixture of liquids, except in gas chromatography where a gas is employed. The fixed medium is called the **stationary phase**. Thus if the sample consisting of a mixture of components (usually referred to as solutes) is added to the mobile phase, then the components by a variety of physical processes will be carried along in the moving stream to varying degrees.

Table 1.1 classifies some of the chromatographic methods. However, the nature of the process whereby separation takes place is rarely confined to one physical mechanism, and although the table indicates the nature of the distribution process in each case, this is merely the most important factor.

Table 1.1: Classification of chromatographic methods

Nature of the Separation Process	Mobile Phase	Stationary Phase	Kind of Chromatography
Partition	Liquid	Liquid	Partition chromatography Paper chromatography Column chromatography
Partition	Gas	Liquid	Gas-liquid chromatography
Adsorption	Liquid	Solid	Adsorption chromatography Thin-layer chromatography Column chromatography Ion-exchange chromatography
Adsorption	Gas	Solid	Gas-solid chromatography

Now work through the following SAQ.

SAQ 2

For the examples given below, indicate what are (i) the mobile phase and (ii) the stationary phase?

a) Paper chromatography

(i)…… (ii)……

b) Thin layer chromatography

(i)…… (ii)……

c) Partition column chromatography

(i)…… (ii)……

d) Ion-exchange chromatography.

(i) …… (ii) ……....

Let us now understand the basic ideas involved in partition chromatography by performing the experiment given in the next Section.

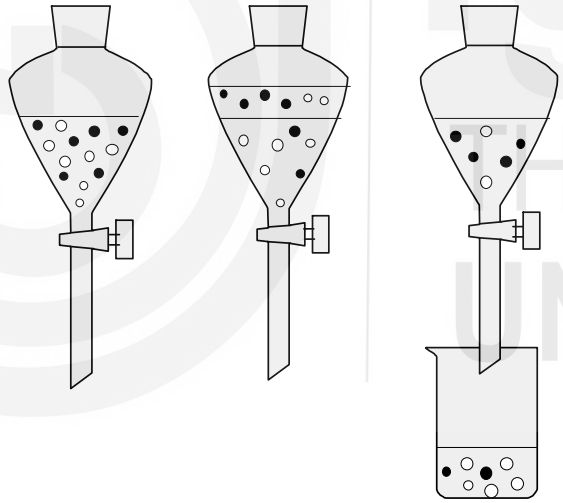
1.4 EXPERIMENT: SEPARATION AND IDENTIFICATION OF AMINO ACIDS IN A MIXTURE

In this experiment you are going to separate and identify amino acids using paper chromatographic technique. This chromatographic technique is the simplest among all the chromatographic methods. It does not require much complicated apparatus and surprisingly good separation can be achieved. Let us now study the basic principle and mechanism of separation of this technique.

1.4.1 Principle of Paper Chromatography

Paper chromatography is based on partition principle. The underlying principle governing the separation of components in paper chromatography (liquid-liquid) chromatography can be demonstrated using a static partitioning model.

Suppose a solvent, such as water, containing two solutes is added to a separating funnel as shown in Fig. 1.1 (a).



(a) (b) (c)

Fig. 1.1: Partial separation of solutes using two immiscible solvents

In Fig.1.1, the two solutes are represented by black and white circles. If a second solvent immiscible with the first is added and the two solvents are shaken together, then some of the solute molecules will transfer to the second solvent forming the upper layer.

As you can see in Fig. 1.1 (b), the black molecules are more soluble in the second solvent. If the stopper is removed and the first solvent is run off via the tap, then a partial separation will occur, with white molecules predominantly in the first solvent and black molecules predominantly in the second solvent. This is shown in Fig. 1.1 (c).

You can show this phenomenon readily in your own laboratory as follows:

(1) Dissolve a small crystal of iodine in 5 cm^3 of aqueous potassium iodide solution in a test-tube. Note the brown coloration that forms.

(2) Then carefully add 5 cm^3 of tetra chloromethane (carbon tetrachloride), using a dropping pipette and shake the test-tube gently. Notice how the tetra chloromethane layer, which was clear, is now purple.

(3) Cork the test-tube and shake vigorously, with care. The purple colour in the organic layer intensifies while the aqueous layer becomes paler.

The colour changes that you observed in this experiment reflect the fact that the iodine, which started off in the aqueous layer, has transferred mainly to the organic layer. No matter how much the mixture is shaken, the ratio of concentrations of iodine in the two layers will remain constant, irrespective of the actual volumes of both solvents, provided only that the temperature remains constant.

Although solid iodine is deep purple in colour, its colour in KI solution is brown while in the organic layer it reverts to purple colour again.

When equilibrium is reached, i.e. there are no more tendencies for the iodine to pass into the organic layer; we can define mathematically this partition or distribution process by the following expression:

$$\frac{\text{Concentration of solute in organic layer}}{\text{Concentration of solute in aqueous layer}} = K_D \qquad \ldots.(7.1)$$

Where K_D is called the **partition** or **distribution coefficient**.

This value of K_D is independent of the amount of solute taken or a volume of solvents used but depends on temperature because solubility is temperature dependent.

Although it was originally believed that the paper chromatography is based on partition principle. Therefore it was originally named as paper partition chromatography. It is now generally recognised that the paper more commonly acts by a combination of partition, adsorption, and ion exchange mechanisms.

The explanation just given relates to static systems, where a component, on the basis of solubility consideration, partitions itself between two immiscible solvents. But in partition chromatography, one of the immiscible solvents is *mobile*.

Let us now understand the mechanism of separation of components of solute in paper chromatography.

1.4.2 Mechanism of Separation

In paper chromatography, the stationary phase is water held on paper in cellulose fibers and the mobile phase is some solvent which runs up or down the paper and the solutes are partitioned between it and the water. The solutes separate out according to their partition coefficients; the one which favours the mobile phase being carried along the paper more quickly. The components of solute are separated completely or partially in distinct colour zones if sample components are coloured, otherwise they are located by the application of different reagents.

To understand how the separation takes place, we need to look more closely at the structure of the filter paper. We know that filter paper consists of numerous cellulose fibers which attract a certain amount of water from the atmosphere. Each fiber can be considered to be made up of a number of cells and each cell consists of a fibrous part with its associated water. The

To demonstrate the water content of a filter paper, weigh a filter paper circle on an analytical or top-loading balance (accurate to ± 0.01 g). Dry the circle in an air oven set at 105 °C for one hour. Then cool it in a desiccator and reweigh it. You can then calculate the percentage of absorbed water.

separation is achieved by partitioning the components of a mixture between the moisture in the cells and the moving solvent flowing over these cells. (Remember the static model which you have read earlier.) It is the relative solubility of each component in the stationary phase (water) and the mobile phase (solvent) which decides the rate at which the component is transferred to the moving phase. Those components which are more soluble in the mobile phase move further; and the components which are more soluble in water will tend to remain longer in the water adhering to the fibers.

A pictorial representation of the partitioning process is given in Fig. 1.2.

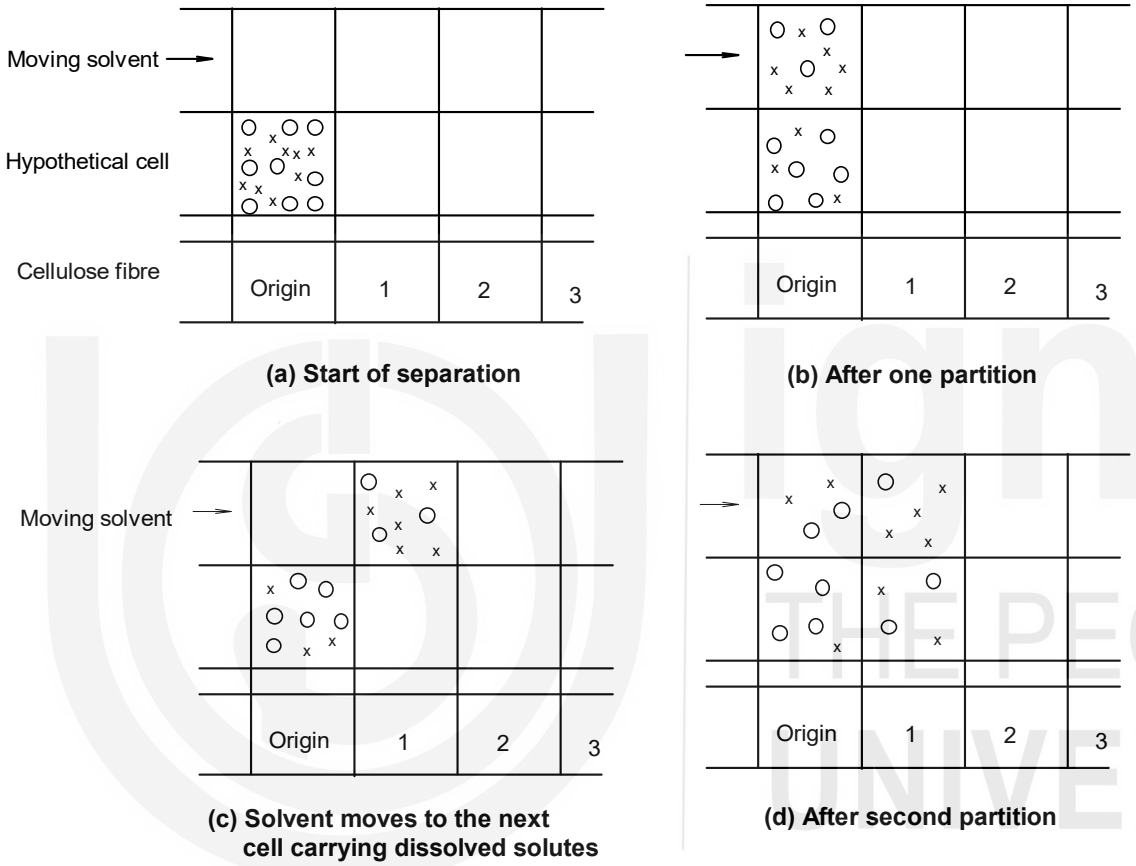


Fig. 1.2: A diagrammatic representation of the partitioning process in paper chromatography

In this figure we have shown how the separation of two components has taken place. Component A (represented by the circles) is *twice* as soluble in the stationary phase, i.e. its partition coefficient is 0.5. Component B (represented by the crosses) is *twice* as soluble in the mobile phase, i.e. its partition coefficient = 2. Fig. 1.2 (a) represents the start of the process, where equal amounts of A and B (equal numbers of crosses and circles) are dissolved in the water cell. In Fig. 1.2 (b), an equal volume of moving solvent is in contact with the first hypothetical water cell, and partitioning (i.e. dividing of the components between the two liquids according to their solubility) will take place so that twice the number of molecules of B will be found in the mobile phase. Remember that B is twice as soluble in the moving solvent. Pictorially, then, there are twice as many crosses as circles. The situation is reversed in the static water cell where twice as many molecules of A are to be found.

Chromatography is a *dynamic* partition process so that a mobile solvent moves on to a situation represented by Fig. 1.2 (c). A second partition will take place to give situation represented by Fig. 1.2 (d), where two adjustments will take place - one at the origin and one at cell 1. At the origin, components A and B will again partition into the mobile solvent in the ratio of 2:1 in favour of B. Thus there are now two crosses in the moving phase and only one in the water cell. A second readjustment will also occur between the mobile and stationary phases in cell 1. In this case, however, as A is more soluble in the water, there will be twice as many molecules of A in this phase (two circles as opposed to one in the mobile phase).

By an extension of this process, it is possible to see why B is carried along in the mobile phase to a greater extent than A. In practice, this process is repeated countless times, eventually giving a sharp separation of the two components in the form of two spots on a paper chromatogram (see Fig. 1.3). The rate at which a component has moved is then determined by its **Retardation factor**, (R_f) or **Retention factor**, which is defined as follows:

$$R_f = \frac{\text{Distance moved by the centre of the solute spot}}{\text{Distance moved by solvent front}}$$

Thus, R_f values for component A will be as shown in Fig. 1.3.

$$R_{f(A)} = \frac{\text{Distance moved by the centre of spot of component A}}{\text{Distance moved by solvent front}} = \frac{x_A}{y}$$

Similarly, retardation factor for component B,

$$R_{f(B)} = \frac{\text{Distance moved by the centre of spot of component B}}{\text{Distance moved by solvent front}} = \frac{x_B}{y}$$

For good separation, the components should have different R_f.

Where, x_A and x_B are linear distances of centre of the spots measured from the line of origin where mixture of solute is applied and y is the distance travelled by solvent front from the starting point as illustrated in Fig. 7.3. R_f values range from 0.00 to 1.00. Small R_f values indicate little tendency to move with the solvent and thus reflect low solubility of solute in mobile phase. Large R_f values conversely indicate a high solubility of solute in mobile phase.

So as long as the correct solvent and type of chromatography paper are used, a component can be identified from its Retardation factor.

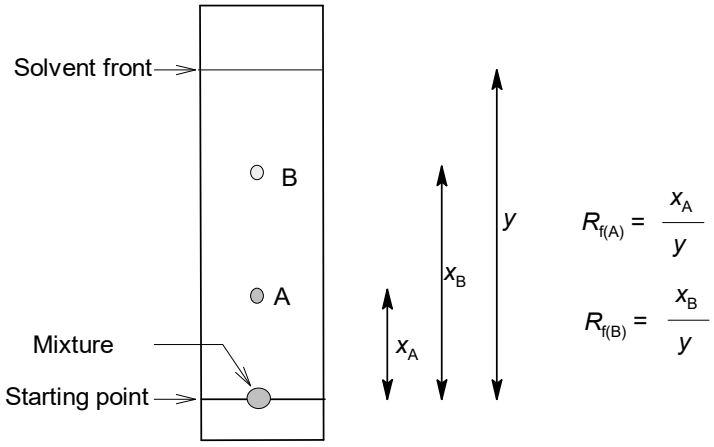
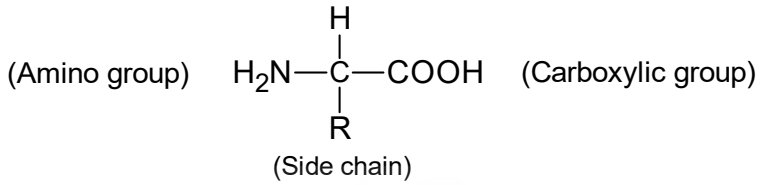


Fig. 1.3: Procedure for calculation of retardation factor (ratio), R_f

Solvent System (Mobile Phase) for Amino Acid Separation:

As mentioned above, the separation of the components mainly depends on the relative solubility of the components of solute (sample) in mobile phase. Therefore, the nature of solvent system plays important role in the development of paper chromatogram. Various solvent systems have been tried out for the separation of amino acids. While selecting a solvent system for the separation of amino acids, some basic understanding of the structure of amino acids is necessary.

As the name suggests, each amino acid contains an amino group, $– NH_2$, and a carboxylic acid group, – COOH. The molecular structure of a generic amino acid is provided below:



Amino acids with nonpolar R	Amino acids with polar R	Amino acids with polar charged R
R = $-CH_3$, Alanine	R = $-CH_2OH$, Serine	R = $-CH_2COOH$, Aspartic acid
R = $-CH(CH_3)CH_3$, Valine	R = $-CH_2SH$, Cysteine	R = $-CH_2CH_2COOH$, Glutamic acid
R = $-CH_2CH(CH_3)_2$, Leucine	R = $-CH_2(OH)CH_3$, Threonine	R = $-(CH_2)_4 - NH_2$, Lysine
R = $-CH_2Ph$, Phenylalanine		R = $-(CH_2)_3 - NH(NH) - NH_2$, Arginine

There are 20 different amino acids that make up our proteins, and they closely resemble in structure except the structure of the side chain R. In glycine, the simplest amino acid, R is a hydrogen atom. Amino acids can be categorised into three main types, on the basis of nature of their side chains. These are amino acids with nonpolar, uncharged polar and charged polar side chains, respectively. The nonpolar groups have generally an aliphatic or an aromatic side chain. For examples, alanine, valine, phenylalanine, etc., have nonpolar hydrocarbon side chains, therefore, are less soluble in water. The uncharged polar types have side chains containing hydroxyl, amide or sulphydryl groups (thiols). These amino acids like serine, cysteine and have polar but neutral R groups thus tend to promote water solubility. The charged polar groups may have carboxylic group (aspartic acid, glutamic acid) or a basic group (lysine, arginine) in their side chain. Both acidic and basic R groups tend to promote water solubility. Therefore, because of the differences in interaction of polar and nonpolar side chains with water, amino acids will show different partition coefficients i.e. relative solubility with solvent system of mobile phase and water of stationary phase. Thus, one which favours the mobile phase being carried along the paper more quickly and one which favours the stationary phase remain near the base line.

There is no universal solvent system which is capable of separating all the usual amino acids. We can try different solvent systems such as acidic (phenol-water, 1-butanol-acetic acid-water), basic (pyridine-water, 1-propanol- aqueous ammonia) or neutral (1-butanol, methyl ethyl ketone) solvent systems, which can provide us the best separation.

The paper chromatogram can be developed in many ways such as ascending: here development of paper occurs due to the solvent movement in upward direction on the paper; descending: here the development of paper occurs due to solvent moves downwards on the paper; horizontal or radial mode: here the solvent travels from centre towards periphery of circular chromatographic paper; two dimensional: here the chromatogram development occurs in two directions at right angle to each other. In our present experiment we will be using ascending mode to develop our chromatogram.

For our present experiment, we will be following an easy ascending paper chromatography technique and 1-butanol-glacial acetic acid-water in the ratio 12:3:5 as solvent system. This experimental set up does not require special apparatus.

SAQ 3

In paper chromatography, the rate at which the components move in the mobile phase is decided by which of the following factors:

a) The number of components in the mixture that is spotted on the paper

b) The relative solubility of the components in the mobile and the stationary phases

c) The time for which the chromatogram is allowed to run

The basic principles discussed above now can be easily demonstrated using the simplest of apparatus and chemicals. You can try the following experiment at your Study Centre's Chemistry Lab. The exercise will take up very little time.

1.4.3 Requirements

Apparatus		Chemicals
Chromatographic tank	1	1-Butanol
Boiling tubes	4	Glacial acetic acid
Measuring cylinder (100 cm^3)	1	Any three amino acids from the following: [L-alanine, L-leucine, L-lysine L-aspartic acid, L-methionine]
Test tubes	5	
Spotting capillaries	5	
Separatory funnel	1	Ninhydrin
Whatman No. 1 filter paper sheet		
Spraying bottle	1	

Solutions provided

1. **Sample solutions:** Provide solution of any three amino acids as mentioned above. Make one unknown sample solution by mixing any two of these three amino acids.

Their solutions can be prepared by dissolving 15 mg of each amino acid separately in 1 cm^3 of distilled water. Warm if a particular amino acid is not soluble in cold.

2. **Detector:** Ninhydrin reagent (0.2%): Take 100 cm^3 of 1-butanol and 100 cm^3 of water in a separating funnel. Shake gently and allow it to form the layers. Remove the lower aqueous layer. Transfer the upper organic layer to a spraying bottle and to this add 0.2 g of ninhydrin, shake well and use as the detector for amino acids.

1.4.4 Procedure

Proceed according to the following steps:

1. Preparation of Developer (solvent system): Prepare the developer as per your requirement by mixing 1-butanol-glacial acetic acid-water in the ratio 12:3:5 by measuring the required volumes.

2. Place the developer in the chromatographic tank so that the height of the developer is less than 1 cm. Place the lid and allow the tank to be saturated with the solvent vapours.

3. Cut the chromatographic paper strip of the required size (usually 10 cm x 10-30 cm for four spot and breadth may be changed as per the number of spot(s). On the strip draw a line with pencil at about 1 cm from one end. This will be the bottom of the chromatogram. Mark off equally spaced points along this line. (They should be separated by about 2 cm). Your samples will be applied to these spots.

4. Apply the solutions of three known amino acids and solution of unknown mixture of the amino acids to the point of application separately on the marked strips using spotting capillaries or tooth picks. Use a fresh capillary or tooth pick for each solution. Remember the position of the known amino acids on the strip. You can label the paper at the top with the name of each of the known amino acid and label the last as unknown.

5. After spotting, dry the spots by allowing the solvent to evaporate. You can use a hot air dryer to dry the paper as illustrated in Fig. 1.4. Repeat spotting process again.

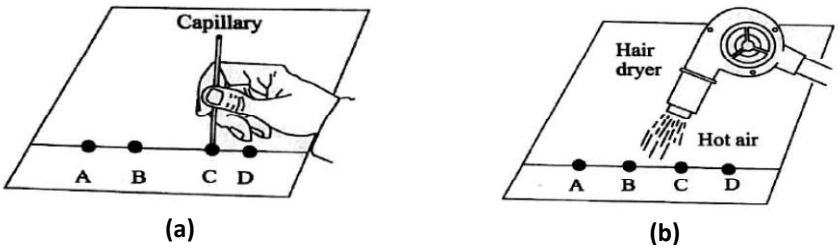


Fig. 1.4: Illustration of (a) spotting of solute sample using glass capillary tube and (b) drying process using hair dryer

6. Suspend the spotted and dried paper strip in the chromatographic tank with the help of a supporting glass road or hanger so that the lower end touching the developer. Care should be taken to see that this is done gently and the strip is vertical. The spots should always be above the developer level (see Fig. 1.5). Cover the chromatographic jar with its lid.

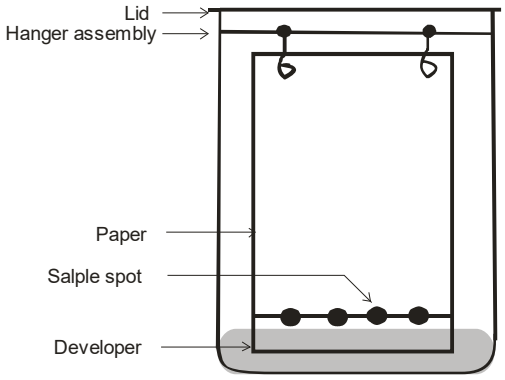
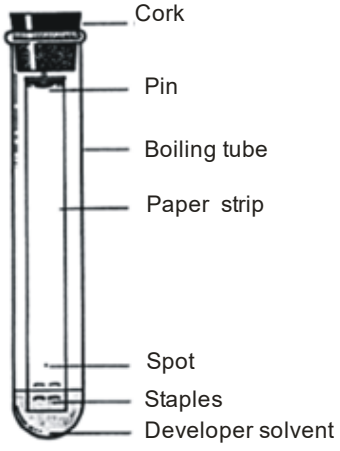


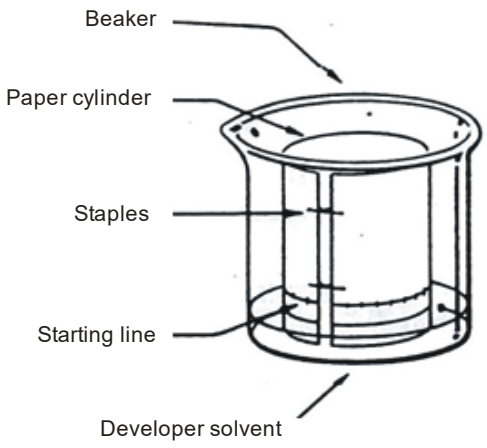
Fig. 1.5: Some typical experimental arrangement for developing paper chromatogram

7. Allow the developer to rise along the paper and wait till the developer (solvent front) reaches near the upper end of the paper.
8. Remove the paper strip from the tank and mark the solvent front with the help of a pencil.
9. Dry the strip until the acetic acid odour from the strip is no more present.
10. After the chromatogram has dried, spray the paper with the ninhyrin reagent solution using a spraying bottle.
11. Heat the strip at 105 ^{o}C either in an oven or a hot plate or use hot air dryer until the coloured zones of amino acids are seen.
12. Encircle the coloured zones and mark the centre of each zone.
13. Measure the distance of each spot centre from the starting line and also the distance by which the solvent front has moved.
14. Calculate the R_f value for each spot on the strip. From the comparison of R_f values, you can determine which amino acids are present in your unknown sample. Report your results in the Observation Table I.

Alternatively you can run a paper chromatogram in 500 ml beaker by making a paper cylinder as shown in Fig. 1.6. Common boiling tubes can also be used to run paper chromatograms (see Fig. 1.6).



Boiling tube for developing



Running a chromatogram by making paper cylinder
Beaker can be covered by aluminium foil

Fig. 1.6: An alternate method for developing paper chromatogram

1.4.5 Observations and Calculations

Observe the colour of the spots of various amino acids on paper chromatogram. Measure the distance (*x*), to which the centre of spot for each amino acid has moved from the original point of application and the distance (*y*), to which the solvent front has moved from the original line on the paper.

Calculate the R_f values by the relation: R_f=x/y, for each known amino acid and for the unknown. Present the data in an Observation Table below.

Observation Table I

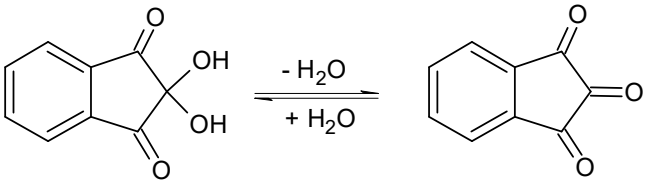
Amino acid	***x***	***y***	$R_f = \frac{x}{y}$
1. --------			
2. --------			
3.---------			
Given Mixture Unknown A Unknown B			
Unknown A's R_f resembles with ..... Unknown A's R_f resembles with			

1.4.6 Result and Discussion

The given mixture sample contains:

1. A.....
2. B.....

The mobile phase rises up along the paper by capillary action, rapidly at first, and then more slowly as the solvent front rises. The movement of an amino acid along the paper depends on its solubility in mobile phase, and a number of other factors. Therefore, the different amino acids move along the paper at different rates and may have different R_f values. The significant differences in R_f values of certain amino acids result into a clean separation. The most widely used reagent for detecting amino acids is ninhydrin. Ninhydrin is the 2-hydrate of indane-1,2,3 trione (or triketohydrindene hydrate) with the following formula.

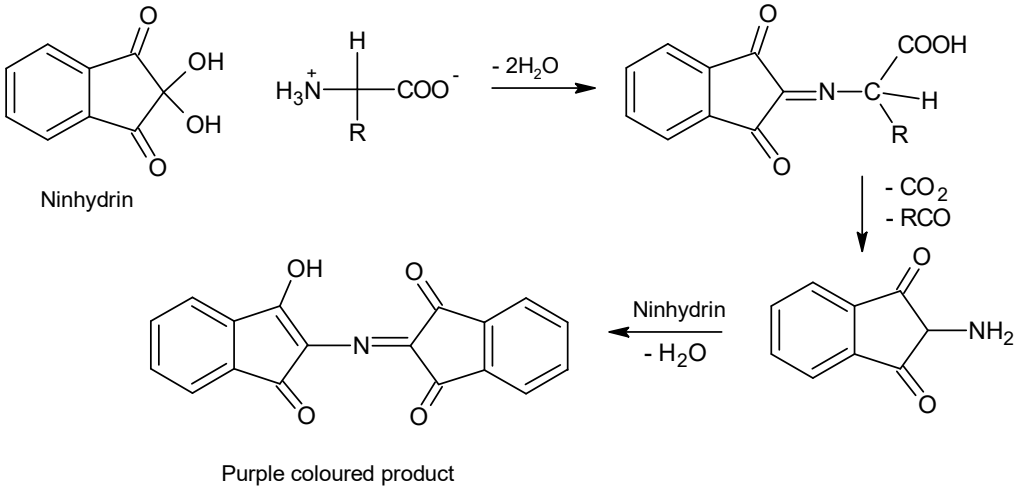


Hydrated form of Ninhydrin Unhydrated form of Ninhydrin

It reacts with amino acids to yield highly coloured products.

$$\text{Amino acid} + \text{Ninhydrin} \xrightarrow{\text{Heat}} \text{coloured product}$$

The different steps in colour formation between ninhyrine and amino acids are shown below:



The formation of visible colour with ninhydrin has limits of detection that may vary from 0.01-0.5 μg depending on the particular amino acid.

1.5 ANSWERS

Self-Assessment Questions

1. You should have answered (d) as this is the chief mechanism of separation on a column. It usually happens that there is a structural similarity between components separated in this way though this is not an essential characteristic.

2. a) (i) liquid – (ii) liquid b) (i) liquid – (ii) solid c) (i) liquid – (ii) liquid d) (i) liquid – (ii) solid

3. (b) is the correct answer. Paper chromatography separations are mainly decided by the differential solubility of the components between the two liquid phases.

EXPERIMENT 2

Determination of the Concentration of Glycine Solution by Formylation Method

Structure

2.1 INTRODUCTION

In Courses BCHCL132 and BCHCL 134, you were introduced to the various techniques for quantitative and qualitative analysis. We have described different methods which are used in quantitative analysis i.e., titrimetric methods refer to measurements of a volume, and physiochemical (instrumental) methods are based on the measurement of some physical or chemical property. In this experiment, we will use titrimetric methods for quantitative analysis of amino acid i.e. glycine. In this experiment you will use formylation methods for the determination of the concentration of glycine solution.

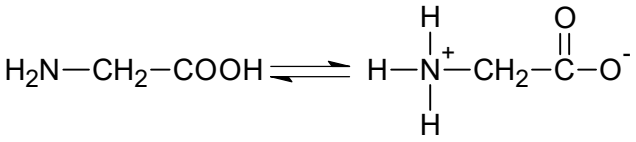
Expected Learning Outcomes

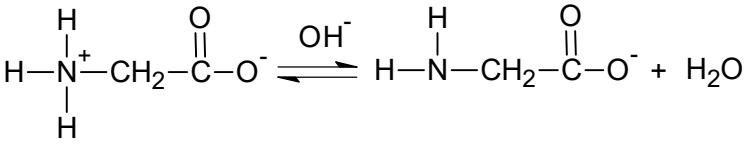
After performing the experiment given, you should be able to:

- describe the significance of quantitative analysis;
- determine the amount of glycine in the given sample;
- describe formylation reaction; and
- perform acid-base titration using standard alkali.

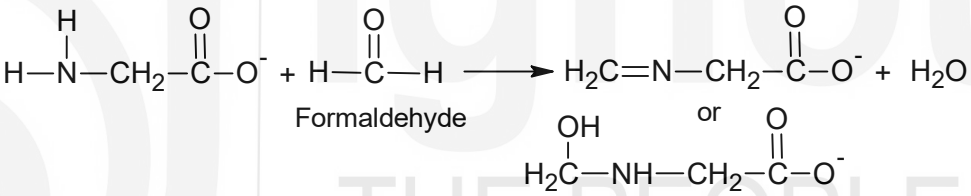
2.2 PRINCIPLE

Amino acids like glycine, alanine, etc. contain one amino group and one carboxylic group as part of their structures. These groups being of opposite nature neutralise one another intramolecularly and form internal salts called zwitter ions or dipolar ions. These ions are held together by electrostatic attraction. They are neutral but in presence of alkali the dissociation favours formation of acid ion.





The free amino group then undergoes condensation with formaldehyde to form mono and dimethyl derivatives. Thus, the formation of these condensation products greatly reduces the basic character of amino group and the carboxylic group can readily be titrated with standard alkali.



2.3 REQUIREMENTS

Burette (50 cm^3)	1	Sodium hydroxide
Pipette (25 cm^3)	1	Formalin solution
Vol. flask (250 cm^3)	1	Phenolphthalein indicator
Conical flask (250 cm^3)	1	
Weighing bottle	1	
Wash-bottle for distilled water	1	
Funnel (small)	1	
Test-tube	1	
Burette stand	1	

Solution Provided

i) Sodium hydroxide solution (0.l *M*): Dissolve 2 g of sodium hydroxide in a 250 cm^3 volumetric flask and make up to the mark with distilled water.

ii) Neutral 40% formalin solution: Take 50 cm^3 of 40% formalin solution in a 250 cm^3 conical flask and add 8-10 drops of phenolphthalein indicator. To

it add carefully from a burette a dilute solution of sodium hydroxide (0.1 *M*), till the solution is just faintly pink.

iii) Phenolphthalein indicator: Dissolve 1.0 g of phenolphthalein in 100 cm^3 of ethanol and then dilute with 100 cm^3 of water.

2.4 PROCEDURE

i) Preparation or Standard solution of glycine: Weigh accurately 0.8 g of glycine and transfer to a 100 cm^3 volumetric flask and make up to the mark with distilled water.

ii) Titration with standard solution: Take 20 cm^3 of standard glycine solution in a 250 cm^3 conical flask and add 3-4 drops of phenolphthalein indicator. Add dilute sodium hydroxide solution (0.1 *M*) taken in burette drop by drop to it until a pink colour is just obtained. Now add 10 cm^3 of neutral formalin solution to it. The pink colour of the solution immediately disappears. Continue adding sodium hydroxide slowly till pink colour is restored. Note the volume of sodium hydroxide used and repeat the experiment until two concordant readings are obtained. Record the observations in the Observation Table I.

iii) Titration with unknown glycine solution: Perform the titration as described above for 20 cm^3 unknown glycine solution and note the volume of sodium hydroxide used in this titration. Record the observations in Observation Table II.

2.5 OBSERVATIONS

Mass of the weighing bottle = m_1 = …….g

Mass of the bottle + glycine = m_2 = …….g

Mass of the bottle (after transferring the compound) = m_3 = …….g

Mass of glycine transferred $m_2 - m_3 = m$ = …… g

Observation Table I
Standard Glycine Solution vs. Sodium Hydroxide Solution

Sl. No.	Volume of glycine solution in cm^3	Burette reading		Volume of sodium hydroxide solution in cm^3 (Final - Initial)
		Initial	Final	
1	20			
2	20			
3	20			

Observation Table II
Unknown Glycine solution vs. Sodium hydroxide

Sl. No.	Volume or glycine solution in cm^3	Burette reading		Volume of sodium hydroxide solution in cm^3 (Final - Initial)
		Initial	Final	
1	20			
2	20			
3	20			

2.6 CALCULATIONS

Mass of glycine dissolved in 100 cm^3 = m g

The volume of sodium hydroxide solution used for 20 cm^3 of standard glycine solution = V_1 cm^3

The volume of sodium hydroxide solution used for 20 cm^3 of unknown glycine = V_2 cm^3

Volume of glycine solution taken for titration = 20 cm^3

V_1 cm^3 of sodium hydroxide solution = 20 cm^3 of standard glycine solution

$$= \frac{m}{5} \text{ g of glycine}$$

V_2 cm^3 of sodium hydroxide solution = 20 cm^3 of unknown glycine solution

$$= \frac{m}{5} \times \frac{V_2}{V_1} \text{ g of glycine}$$

Thus strength the strength of the unknown glycine solution

$$= \frac{m}{5} \times \frac{V_2}{V_1} \times \frac{1000}{20} \text{ g dm}^{-3}$$

$$= \frac{m \times V_2 \times 10}{V_1} \text{ ……g dm}^{-3}$$

2.7 RESULT

The amount of glycine in the given solution = …….g.

The strength of the unknown glycine solution = ……..g dm^{-3}.

EXPERIMENT 3

DETERMINATION OF pK_a OF GLYCINE BY pH METRY

Structure

3.1 INTRODUCTION

In the previous two experiments you have learnt to separate a mixture of amino acids and to determine the concentration of amino acid, glycine in the given solution. These involved techniques of chromatography (paper) and titrimetry respectively. You have used these techniques earlier in the core chemistry elective lab courses in the previous semesters. In the current experiment you would learn about and perform the determination of isoelectric point (pI) of the amino acid glycine. The difference from earlier techniques is that you will use an instrumental technique, viz. pH metry about which you learnt in detail in the core lab course BCHCL-134.

You would recall that amino acids contain at least one carboxyl group and one amino group. Both groups are capable of ionisation in an aqueous solution. Therefore, amino acids can act as both acids and bases in water and are known as **amphoteric** molecules. At or around neutral pH, an amino acid forms a **dipolar ion** or a **zwitterion.** The pH at which amino acids carry no net charge is called its **isoelectric point (pI).**

In this experiment you will determine the pK_a values of the ionisable groups of glycine by performing a titration with a standard solution of NaOH using pH meter and drawing a titration curve. You will find out the values of pK_1 (corresponding to ionisation of COOH group) and pK_2 (corresponding to the ionisation of NH_3^+ group) from the curve and calculate the isoelectric point (pI) of the amino acid.

In the next experiment you would perform a reaction showing the effect of an enzyme on starch, a polysaccharide.

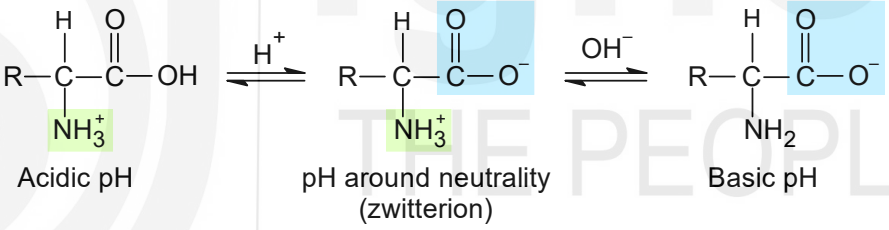
Expected Learning Outcomes

After performing the given experiment, you should be able to:

- ❖ explain the principle of determination of pI value of glycine by pH metry;
- ❖ calibrate the pH meter to be used for the experiment;
- ❖ perform pH metric titration of acidified glycine with sodium hydroxide and draw the titration curve; and
- ❖ determine the pK_1, pK_2 and isoelectric point of glycine.

3.2 PRINCIPLE

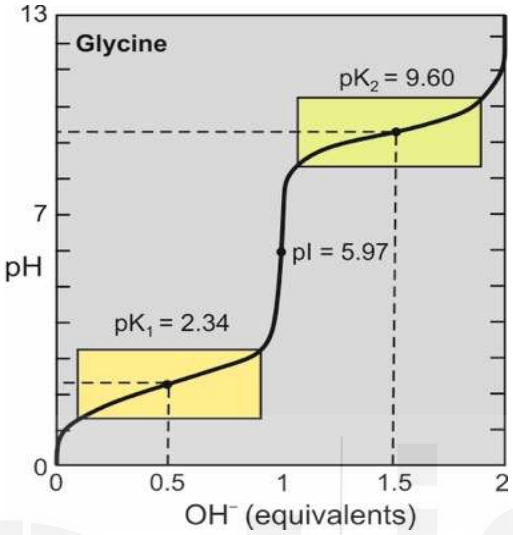
As stated above, the amino acids are amphoteric molecules and in aqueous solutions these can act as an acid or a base. In acidic solutions these are protonated and in basic solutions these are de-protonated whereas around the neutral pH i.e., at pH equal to their isoelectric point (pI) these are zwitterionic and carry no net charge.



A solution of an amino acid (say glycine) in acidic medium would be monocationic. Quantitative amounts of this species can be obtained by adding a calculated amount (1 mole equivalent) of standard HCl solution to the glycine solution. This species would have two ionisable protons: one on carboxylic acid group and the other on NH_3^+ group; the former being more acidic. These can be successively titrated with a standard solution of an alkali like NaOH. The addition of a standard solution of alkali would cause the neutralisation of these groups; initial addition of NaOH would cause the neutralisation of the COOH group. The pH of the solution is determined by the ratio of the concentration of ionised and unionised forms as per the Henderson-Hasselbalch equation about which you have learnt in BCHCT-133 course.

Addition of more and more of NaOH would alter the ratio and the pH would increase gradually. At the stage of half neutralisation i.e., when the COOH group in 50% of the glycine molecules is ionised, the concentrations of the ionised and unionised forms would be equal, and the pH of the solution would be equal to the **pKa** of the COOH group. With continued addition of solution of NaOH the ionisation of COOH group would continue. At the stage of complete neutralisation there will be a sharp increase in pH. The **inflection point** gives

the **pI** value. Further addition of the base would cause the neutralisation of NH_3^+ group. Once again, the pH of the solution is determined by Henderson-Hasselbalch equation and there would be a gradual change in the pH. Once the neutralisation of NH_3^+ group is complete the pH would again increase sharply. A schematic titration curve of glycine with sodium hydroxide is given below.



Schematic titration curve of glycine (0.1M) at 25°C

The titration curve can be used to determine the pKa values corresponding to the ionisation of COOH and NH_3^+group by determining the pH corresponding to their half neutralisation as shown in the titration curve. The pI value can be obtained as the arithmetic mean of the two pKa values.

3.3 REQUIREMENTS

Apparatus		Chemicals
Volumetric flask (100 cm^3)	1	Glycine
Burette (50 cm^3)	1	Oxalic acid
Pipette (10 cm^3)	1	Hydrochloric acid
Weighing bottle	1	Sodium hydroxide
Burette stand with clamp	1	Buffer solutions / tablets for pH meter calibration
Conical flasks	2	
Funnel	1	
pH meter with combination electrode	1	
Magnetic stirrer with stirring bar	1	

Solutions Provided

1. **Sodium hydroxide solution** (**~ 0.2 M**): It is prepared by dissolving about 8 g of sodium hydroxide in about 150-200 cm^3 of distilled water and diluting it to 1 litre.

2. **Hydrochloric acid solution (~ 0.1 M)**: It is prepared by adding about 10 cm^3 of concentrated hydrochloric acid to about 100 cm^3 of distilled water and diluting it to 1 litre.

3. **Glycine solution (0.1 M)**: It is prepared by dissolving 7.5 g of glycine powder in 1000 cm^3 distilled water.

4. **Phenolphthalein indicator**: It is prepared by dissolving 1 g of the reagent in 100 cm^3 of ethanol and adding 100 cm^3 of water to it. If a precipitate is formed, it is filtered.

3.4 PROCEDURE

The procedure consists of the following steps.

a) Preparation of a standard solution of oxalic acid

b) Standardisation of sodium hydroxide solution by titrating against standard solution of oxalic acid.

c) Standardisation of given HCl solution by titrating against standardised solution of sodium hydroxide

d) Calibrating the pH meter

e) Acidifying the 0.1 M solution of glycine with HCl and Performing pH metric titration with standardised solution of sodium hydroxide

f) Drawing the titration curve and the determination of the pK_1 and pK_2 values of glycine.

g) Calculation of pI value of glycine

Follow the procedure given below in a step wise manner to determine the pI value of glycine.

a) Preparation of 100 cm^3 of standard solution of 0.05 M oxalic acid

You know that to prepare a standard solution we need to first calculate the amount of the substance required. The amount of oxalic acid (M_m = 126 g mol^{-1}) required to prepare 100 cm^3 of 0.05 M solution of oxalic acid would be:

$$\text{mass(in g)} = \frac{M\,V\,M_m}{1000} = \frac{0.05\,\text{mol}\,\text{dm}^{-3} \times 100\ \text{cm}^3 \times 126\,\text{g}\,\text{mol}^{-1}}{1000\,\text{cm}^3\,\text{dm}^{-3}} = 0.63\,\text{g}$$

i) Weigh about 0.65 g of oxalic acid on a rough weighing balance and transfer it to a clean dry weighing bottle and accurately weigh the weighing bottle with oxalic acid.

ii) Transfer the oxalic acid through a funnel to a clean volumetric flask of 100 cm^3 capacity.

iii) Weigh the weighing bottle (may contain some oxalic acid) again and find the exact mass of oxalic acid transferred by subtracting this mass from the mass of the weighing bottle plus oxalic acid.

iv) Dissolve oxalic acid in about 30-40 cm^3 of distilled water taken in the volumetric flask. Once dissolved, make up the volume up to the mark with distilled water.

b) Standardisation of sodium hydroxide solution by titrating against standard oxalic acid solution

i) Fill the burette with the given solution of sodium hydroxide using a funnel and mount it on the burette stand. Note the reading on the burette and record it in the Observation Table 3.1 under the initial reading column.

ii) Carefully pipette out 10 cm^3 of the standard solution of oxalic acid and transfer it to a clean 100 cm^3 conical flask. Add two to three drops of phenolphthalein indicator to it.

iii) Titrate the colourless solution with NaOH using constant swirling till a persistent pink colour is obtained. The colour should not fade on shaking the solution. Record the reading in the Observation Table 3.1 under the final reading column.

iv) Repeat the titration to get at least two concordant readings and record the same in the Observation Table 3.1

c) Standardisation of given HCl solution by titrating against standardised sodium hydroxide solution

i) Fill the burette with the given standardised solution of sodium hydroxide using a funnel and mount it on the burette stand. Note the reading on the burette and record it in the Observation Table 3.2 under the initial reading column.

ii) Carefully pipette out 10 cm^3 of the given solution of HCl and transfer it to a clean 100 cm^3 conical flask. Add two to three drops of phenolphthalein indicator to it.

iii) Titrate the colourless solution with NaOH using constant swirling till a persistent pink colour is obtained. The colour should not fade on shaking the solution. Record the reading in the Observation Table 3.2 under the final reading column.

iv) Repeat the titration to get at least two concordant readings and record the same in the Observation Table 3.2.

d) Calibrating the pH meter

The general procedure for the calibration of pH meter is given below. You may need to consult the operational manual of the pH meter being used. Follow the instructions from the operation manual accompanying the instrument and calibrate the instrument. (*Your counsellor will help you in this step*)

i) Connect the combination glass electrode to the pH meter; switch on the pH meter and allow it to stabilize for about 15 minutes.

ii) Take the standard buffer solution of pH=7.0 in a clean beaker and place the combination glass electrode connected to the pH meter in this solution. Gently

While measuring pH ensure that the bulb of the glass / combination electrode is completely dipped into the solution. If not fully dipped add some more solution.

stir the solution and note the pH as described in the operational manual of the pH meter.

iii) It is expected to be 7.0. If it is not seven adjust it to 7.0 with the calibration knob on the pH meter.

iv) Wash the electrode with distilled water and wipe off the water adhering to it by using a tissue paper. Repeat step (ii) with the standard buffer of pH 4.0.

v) Repeat steps (ii) to (iv) till you get consistent values with the standard buffer solutions of pH 4.0 and 7.0. You may take the help of your counsellor or the laboratory assistant for this.

After calibration, the pH meter is ready for the measurement.

e) Acidifying the 0.1 M solution of glycine with HCl and performing pH metric titration with standardised solution of sodium hydroxide

i) Before starting the titration, you should determine the **drop volume** of your burette. For this adjust the burette (containing NaOH solution) at a certain value (say 0) and note it. Then carefully drain out 100 drops of the solution into a beaker and note the burette reading again. Divide the difference of the two readings by 100 to get the drop volume of the burette.

ii) Pipette out 10.0 cm^3 of the given solution of 0.1 M glycine in a 50 cm^3 beaker; dilute with about 10.0 cm^3 of distilled water and to this add 1 mole equivalent of standardised HCl solution. The volume of HCl solution required can be calculated as follows

Volume of HCl required = (10 cm^3 x 0.1 M) / (molarity of HCl)

iii) Drop a magnetic stirrer bar in the beaker containing test solution and keep the beaker on a magnetic stirrer and dip the combination glass electrode into it. Make sure that it does not touch the walls or the bottom of the beaker. In case the bulb of the electrode does not dip completely into the solution add a little more of distilled water.

iv) Fill the burette with the given solution of NaOH and mount the burette on an iron stand with a clamp.

v) Add a few, say 4-5 drops of NaOH solution to the glycine solution, stir and allow it to become still and measure the pH as per the operation manual provided with the instrument. Record your observation in Table 3.3.

vi) Keep repeating the above step and record your observation in Table 3.3. You would observe gradual change in the pH value. When you observe larger increase in the value of pH, decrease the volume of NaOH being added to 2-3 drops and continue recording your data.

vii) You may once again observe much smaller increase in the pH, you may increase the volume of NaOH being added to 4-5 drops or more and continue recording your data in Table 3.3. Continue the titration till the solution pH becomes 12 or a bit more.

f) Drawing the titration curve and the determination of the pK_1 and pK_2 values of glycine

i) Use the data from Table 3.3 to draw the titration curve. Mark x-axis in terms of number of drops of NaOH and y-axis as the pH of the solution.

ii) Note down the number of drops corresponding to the steepest increase in the pH. Let it be N. This would be equivalent to the volume of NaOH required for complete neutralisation of COOH group.

iii) Note the pH corresponding to N/2 drops of NaOH from the titration curve. This would give the value of pK_1.

iv) Similarly, note the pH corresponding to 3N/2 drops of NaOH from the titration curve. This would give the value of pK_2.

g) Calculation of pI value of glycine

i) Calculate the value of pI by taking average of pK_1 and pK_2.

ii) Report the results.

3.5 OBSERVATIONS

a) Preparation of 100 cm^3 of standard solution of 0.05 M oxalic acid

Mass of weighing bottle + oxalic acid = m_1 g = ...g

Mass of weighing bottle (after transferring oxalic acid) = m_2 g = ... g

b) Standardisation of the given solution of sodium hydroxide by titrating against standard solution of oxalic acid.

Volume of standard oxalic acid solution taken in conical flask, V_O = …cm^3

Solution in the burette: Sodium hydroxide

Indicator used: Phenolphthalein

Observation Table 3.1: Standardisation of sodium hydroxide

S.No.	Volume of oxalic acid (cm^3)	Burette reading		Titre value (cm^3) (Final-initial reading)
		Initial	Final	
1				
2				
3				
			Concordant reading	

c) Standardisation of the given solution of HCl by titrating against standardised solution of sodium hydroxide.

Volume of HCl solution taken in conical flask, V_H = …cm^3

Solution in the burette: Sodium hydroxide

Indicator used: Phenolphthalein

Observation Table 3.2: Standardisation of HCl solution

S.No.	Volume of HCl (cm^3)	Burette reading		Titre value (cm^3) (Final-initial reading)
		Initial	Final	
1				
2				
3				
			Concordant reading	

d) pH metric titration of glycine with sodium hydroxide solution

Record the following readings for the calculation of drop volume of burette

Initial reading of the burette: R_1=…….

Burette reading after draining 100 drops of solution, R_2=…….

Drop volume $= \frac{(R_2 - R_1)}{100} = ...cm^3$

Volume of glycine solution taken in beaker V_H = …cm^3

Solution in the burette: Sodium hydroxide

Molarity of sodium hydroxide = M_N=….M

Observation Table 3.3: pH metric titration of 0.1 M glycine with NaOH

S.No.	Total number of drops of NaOH	pH	S.No.	Total number of drops of NaOH	pH
1			21		
2			22		
3			23		
4			24		
5			25		
6			26		
7			27		
8			28		
9			29		
10			30		
11			31		
12			32		

13			33		
14			34		
15			35		
16			36		
17			37		
18			38		
19			39		
20			40		

3.6 CALCULATIONS

Perform the calculations based on the observations recorded above. You need to perform the calculations stepwise as following.

a) Preparation of 100 cm^3 of standard solution of 0.05 M oxalic acid

Molarity of the prepared standard solution of oxalic acid can be calculated as follows:

Mass of weighing bottle + oxalic acid = m_1 g = ...g

Mass of weighing bottle (after transferring oxalic acid) = m_2 g = ... g

Amount of oxalic acid transferred = $m_1 - m_2 = m$ g = ... g

Molar mass (M_m) of oxalic acid = 126 g mol^{-1}

Volume of solution of oxalic acid prepared = 100 cm^3

Molarity of oxalic acid solution = $M_O = \frac{m \times 1000}{126 \times 100} = \frac{10m}{126} = ...M$

b) Standardisation of the given solution of sodium hydroxide by titrating against standard solution of oxalic acid

Volume of standard solution of oxalic acid taken in conical flask, V_O = …cm^3

Volume of solution of NaOH used (concordant value from Observation Table 3.1) = V_N = ...cm^3

The concentration of the given solution of sodium hydroxide can be determined as follows:

The reaction involved in the titration:

$$2NaOH + (COOH)_2 \rightarrow (COONa)_2 + H_2O$$

Molarity equation: $M_N V_N = 2M_O V_O$

The molarity of sodium hydroxide = $M_N = \frac{2M_O V_O}{V_N} = ...M$

c) Standardisation of the given solution of HCl by titrating against standardised solution of NaOH.

Volume of solution of HCl taken in conical flask, V_H = …cm^3

Volume of solution of NaOH used (concordant value from Observation Table 3.2) $= V_N$ = ...cm^3

The concentration of the given solution of HCl can be determined as follows:

The reaction involved in the titration:

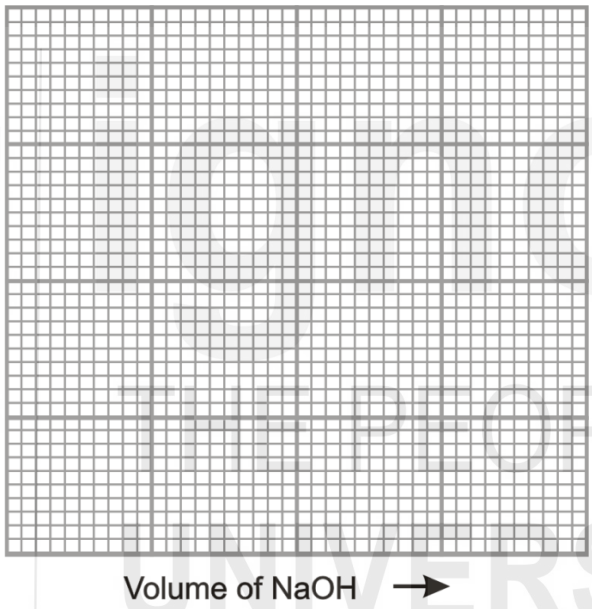
$NaOH + HCl \rightarrow NaCl + H_2O$

Molarity equation: $M_N V_N = M_H V_H$

The molarity of HCl = $M_H = (M_N V_N) / V_H$

F. Drawing the titration curve and the determination of the pK_1 and pK_2 values of glycine

i) Plot a graph between the measured pH (on y-axis) and the volume of NaOH (in number of drops; on x-axis) using the data from Observation Table 3.3.



Titration curve for Glycine versus NaOH titration

ii) Note the number of drops of NaOH used for complete neutralisation of COOH group as explained above i.e., corresponding to steepest increase in pH.

iii) Determine and report p*K* values by determining the pH values corresponding to N and 3N/2 drops of NaOH.

iv) Calculate and report the pI value calculated as

pI = (pK_1 + pK_2) /2

3.7 RESULTS

The pK_1 of glycine = …….

The pK_2 of glycine = …….

The p*I* of glycine = …….

EXPERIMENT 4

ACTION OF SALIVARY AMYLASE ON STARCH AND EFFECT OF TEMPERATURE ON THE ACTION OF SALIVARY AMYLASE ON STARCH

Structure

4.1 INTRODUCTION

In Block 2 of BCHET-149, we have discussed the chemistry of proteins. We have also mentioned that enzymes are protein catalysts and they act as catalysts for biological reactions. Enzymes also, like other catalysts, increase the rate of reactions without being used up themselves. They do this by lowering the activation energy of a reaction. As enzymes are proteins, they can be denatured on variation of temperature and pH. Most of enzymes have optimum activity at a static pH and at body temperature.

In this experiment we will take up the salivary amylase enzyme to study the effect of temperature. This enzyme is responsible for hydrolysis of starch.

The major enzyme found on human saliva is α-amylase. Healthy people produce about 1.5 dm^3 of saliva per day.

Expected Learning Outcomes

After performing the experiment given, you should be able to:

- explain various factors which influence enzyme activity;
- describe the reaction of amylase enzyme with starch;

- describe how temperature affects the hydrolysis reaction of starch and amylase, and;
- demonstrate the effect of temperature on the activity of salivary amylase using indicator method.

4.2 PRINCIPLE

The ability of enzymes to function as catalyst depends on its shape of the protein. Recall that all proteins have a specific shape and they have many different types of active binding sites. Because of these characteristics, they are very specific – They only act on one substrate or one class of related substrates. A typical enzyme catalyzed reaction can be represented as

$$\text{Enzyme} + \text{Substrate(s)} \rightleftharpoons \underset{\text{complex}}{\text{Enzyme} - \text{Substrate(s)}} \rightleftharpoons \underset{\text{complex}}{\text{Enzyme Product}} \rightleftharpoons \text{Enzyme} + \text{Product(s)}$$

Factors Influencing Enzyme Activity

Enzymes are among the fastest catalysts known. The ability of an enzyme to convert substrate into product is called enzyme activity.

Enzyme activity is not necessarily constant – there can be a number of factors that influence their activity. Here, we consider some common factors that can influence enzyme activity.

1. Effect of Enzyme Concentration

In enzyme catalysed reaction each enzyme molecule has one more active binding site for conversion of substrate molecule to product molecules. Thus the more enzyme is available, the quickly substrate can be converted into product. If we consider that all these factors are constant, in general as enzyme concentration increases, there is a proportional increases in reaction rate (see Fig. 13.1).

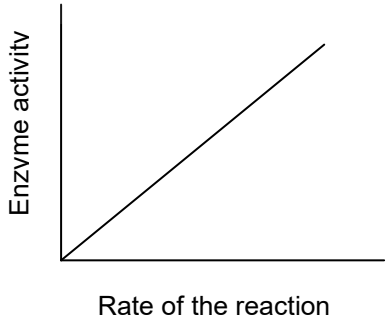


Fig. 13.1: Plot showing the mathematical relationship between enzyme concentration and enzyme activity.

2. Effect of pH

Each enzyme has an optimum pH. Above or below an enzyme optimum pH, its activity is lower. Mostly in human body function optimally at pH between 7-8, some enzymes, such as those of our digestive system, may have optimal pH at very acidic (pH 2) or very alkaline (pH < 8). This

effect is actually due to the change in protein structure of the enzyme at different pH values similar to colour indicators in acid base titrations. In other words, enzymes become denatured when exposed to different acidic condition than its optimum pH conditions. The variation in the rate of enzyme – catalysed reaction with pH is shown Fig. 4.2.

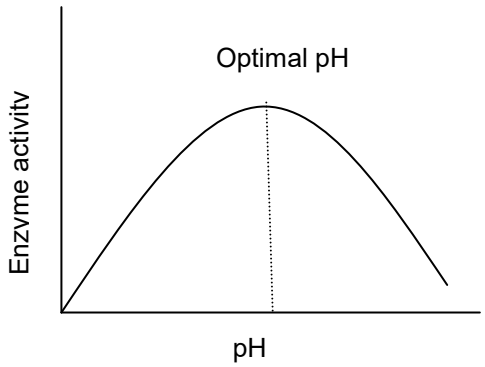


Fig. 4.2: Plot showing variation in the rate of enzyme – catalysed reaction with pH.

The secondary, tertiary, and quaternary structures of proteins all rely on relatively weak non-covalent bonds, for example hydrogen bonds, van der Wall forces, and ionic bonds to link different regions of the protein together. Increased temperature causes random movement in different regions of the protein, thus destabilising these weak bonds and causing a charge in the shape of the protein(denaturisation). If large numbers of these weak bonds are broken, the shape of the active site will begin to distort and the enzyme will lose it ability to bind the substrate and catalysed the reaction.

Effect of Temperature

In general, rate of chemical reaction both catalysed and non-catalysed reactions, tend to increase as temperature increases. This is because at higher temperature, in reaction mixture molecules tend to be moving more quickly than at lower temperature and thus, there is more tendency of collision with one another with greater energy. However, enzyme-catalysed reactions become slower or stop if the temperature becomes too high (above 70°C), because enzymes become denatured at high temperature. Fig. 4.3 illustrates the effect of temperature on enzymatic activity.

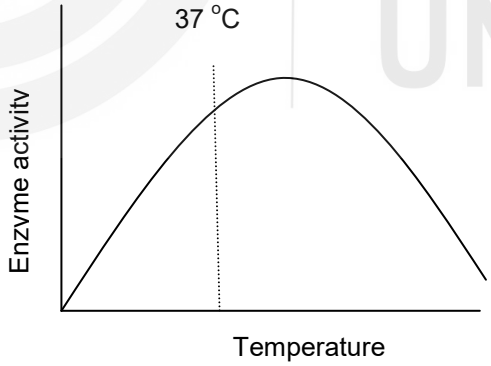


Fig. 4.3: Plot showing the relationship between the rate of enzyme catalysed reaction and temperature.

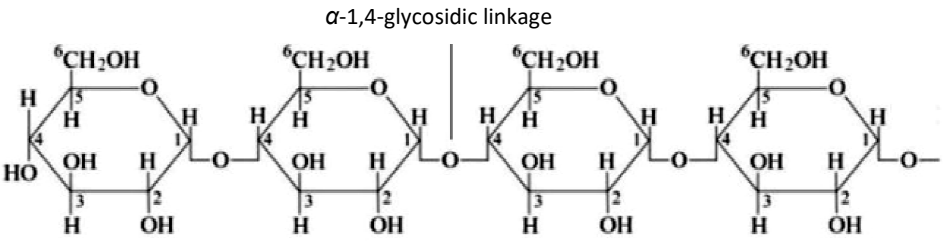
There are few more other factors which also influence the enzyme activity such as effect of substrate concentration, effect of co-factor/coenzyme concentration and inhibitors etc., At this stage, we are not going in details of these factors.

Reaction between starch and salivary amylase

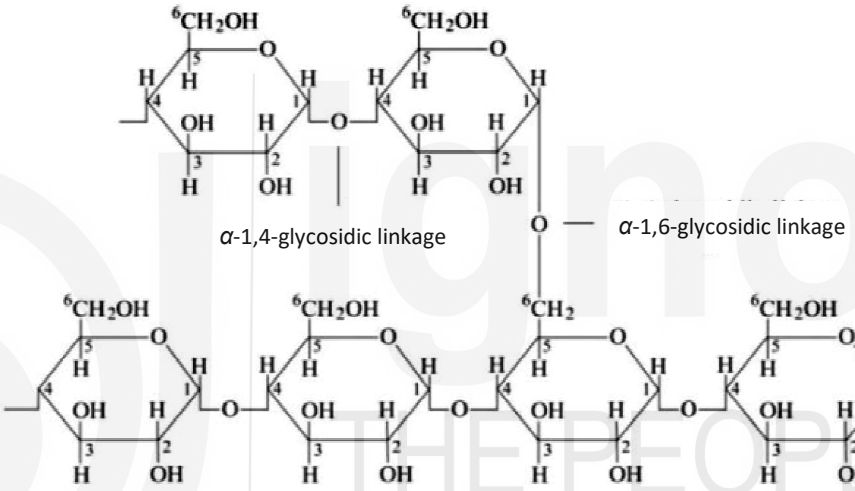
Our saliva contains an enzyme called salivary amylase. This reacts with the starch component of our food and hydrolyses it mainly into smaller saccharide

units such as maltose. Complete hyrolysis of starch into glucose occurs in the small intestine in the presence of a very active pancreatic enzyme.

Starch is a polysaccharide made of two compounds, amylose and amylopectin. These two polymers consist of glucose monomers that are joined by glycosidic bonds. The enzyme amylase catalysed the breakdown of starch into disaccharides and finally to glucose molecules.

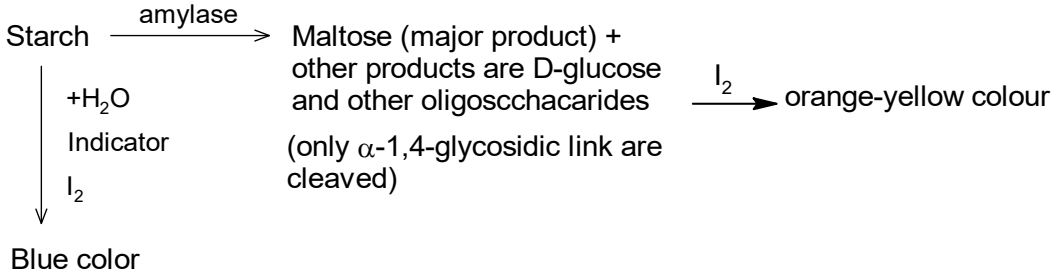


Amylose



Amylopectin

An easy way to track the rate of reaction catalysed by enzyme is by means of an indicator. For this reaction under study, iodine solution is used as a starch indicator and can be used to track the digestion of starch by amylase. Iodine solution is typically orange-yellow in color, and in the presence of starch it becomes blue-black. Iodine molecules in aqueous solution are arranged in random order. However, when starch is added to the iodine solution, it binds to iodine and organises it in non-random arrangement. Iodine in this non-random arrangement imposed by the starch chain is blue. When starch gets broken down into maltose or glucose, it can no longer arrange iodine non-randomly. So the solution turns yellowish again. The reaction of I_2 and starch with amylase enzyme can be represented as follows:



4.3 REQUIREMENTS

1.	Stop watch	-	1
2.	Test tubes (or 6 test tubes + 3 spot plates)	-	20
3.	Droppers	-	3
4.	Constant temperature water bath	-	2
5.	Thermometers	-	3
6.	Beakers	-	3

Solution Provided

1. 1% Starch solution:

   i) Place 90 cm^3 of distilled water in a 250 cm^3 beaker and bring to boiling on a hot plate.

   ii) Make a smooth paste with 1 g of soluble starch in 10 cm^3 of distilled water.

   iii) Once the water is boiling, carefully remove the beaker containing the boiling water from the hot plate. Pour the starch paste into the boiling water and stir until all of the starch is dissolved. The resulting solution may be somewhat cloudy.

   iv) Allow the starch solution to cool to room temperature before use.

2. 1% NaCl Solution: 1 g of NaCl in 100 cm^3 of distilled water.

3. Iodine solution: Dissolve 1 g of iodine crystals and 2 g of potassium iodide in 100 cm^3 of distilled water.

4.4 PROCEDURE

1. If your laboratory has two multi holes water baths, set one of them at 37°C and other can be set close to 100°C (boiling temperature), and every one of you will use these water baths turn by turn. You will also need one more low temperature water bath. You can make your own using a 250 cm^3 beaker or copper water bath; fill it with some water add some ice to the water and maintain temperature between 0-5°C. If you Lab does not have large water baths for higher temperatures, then you have to make own water bath using 250 cm^3 beaker or copper bath and regulate their temperature using Bunsen burner/hot plate.

2. Take 15 cm^3 1% starch solution and 3 cm^3 NaCl solution in a beaker and also take about 50 cm^3 of iodine solution in another beaker.

3. You can prepare your own fresh amylase solution by collecting about 1-2 cm^3 of your own saliva in a small beaker (You will need to spit politely into the beaker). Take 1 cm^3 of saliva, add 19 cm^3 of water to saliva and

mix well. If a commercial amylase solution is available, you may use it instead of your own saliva.

4. Take three sets of test tubes (6 test tubes in each set) in three separate test tube stands. Add 1 cm^3 of iodine solution in each test tube. Mark each test tube stands as A (for 5 ^oC), B (for 37 ^oC), C (for 100 ^oC) and also mark test tubes of each set as 0, 1, 2, 3….

5. Take another three test tubes and mark them as Experiment Tube A, Experiment Tube B and Experiment Tube C and add 5 cm^3 mixture of starch solution + NaCl solution to each test tube.

6. Now transfer Experiment Tube A into water bath/beaker containing ice maintained at 5°C. Transfer the second Experiment Tube B into water bath set at 37°C and the third Experiment Tube C into boiling water bath maintained at 100°C.

   Keep the tubes in their baths for 10 minutes to allow them to reach the temperature of their baths.

7. Read and record the temperature of all water baths. Add 1 cm^3 of saliva solution into each test tube marked as Experiment Tube A, B and C.

8. Immediately, using a dropper, take few drops from tube A and transfer them into tube 0 of A series of test tubes having iodine solution.

9. Similarly, using fresh droppers do the same procedure for Experiment Tube B and Experiment Tube C and transfer the few drops of solution into B and C series of test tubes having iodine solution. Note the first addition of reaction mixture to iodine as zero minute reading.

10. At intervals of every 2 minutes keep on repeating the above step and note the change in colour of iodine solution. Continue this till the colour of iodine does not change.

11. Note the time taken for different experimental tubes till they do not give any colour with iodine.

4.5 OBSERVATIONS

Table

Time/ minutes	Reaction with Iodine from Experiment Tube A	Reaction with Iodine from Experiment Tube B	Reaction with Iodine from Experiment Tube C
0			
2			
4			
6			
8			

4.6 Result

Result: To digest completely 5 cm^3 of 1% starch solution, 1 cm^3 of enzyme takes …….. minutes at 5°C …… minutes at 37°C and …….. minutes at 70°C.

EXPERIMENT 5

DETERMINATION OF SAPONIFICATION VALUE OF AN OIL OR FAT

Structure

5.1 INTRODUCTION

Oils and fats are triglycerides with three long chains of fatty acid group randomly esterified with glycerol. The difference between oils and fats is that, oils are liquid whereas fats are solid at ordinary temperature.

Fats and oils mainly come from plant seed. In animals they are present under the skin, in tissues and muscles. Some of the fatty acids like linoleic, linolenic and arachidonic acids are very essential for our body. Fats and oils are widely distributed in food and are of great nutritional value. They are also used in manufacture of soaps, detergents, glycerine, candles, printing ink etc.

The industrial value of a particular oil or fat depends upon its physical and chemical characteristics, e.g. melting point, specific gravity, refractive index, viscosity, saponification value, iodine value, acid value, acetyl value, etc. In this experiment and Experiment 6 we will determine two of these parameters, that are saponification value and iodine value.

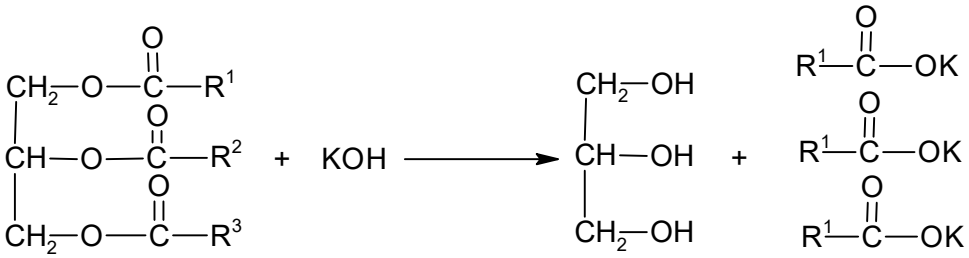
Expected Learning Outcomes

After performing the given experiments you should be able to:

- define the term saponification value; and
- determine saponification value.

5.2 PRINCIPLE

You have already studied chemistry of oil and fats in Unit 10 of BCHET 149. Please revisit the unit to recall it. Esters are hydrolyzed either by aqueous base or by aqueous acid to yield components acid and alcohol fragments. Hydrolysis of an ester in alkaline medium is called saponification.



Saponification number or saponification value is an arbitrary unit that is defined as "number of mg of potassium hydroxide required to saponify 1 gm of oil or fat" i.e. to neutralise completely the fatty acids resulting from complete hydrolysis of 1 gm fat or oil.

The saponification value gives an idea about the molecular weight of fat or oil. The smaller the saponification value, the higher the molecular weight. As the average molecular weight of oil or fat depends on the average length of carbon chain of the fatty acid components, the saponification value also gives an indication of the average length of the carbon chain in the oils or fats. The saponification value for each oil or fat has its own characteristic value.

In this experiment first we will boiling of the sample under reflux condenser with ethanolic KOH solution, and then titration of excess potassium hydroxide with standard HCl in presence of an indicator.

5.3 REQUIREMENTS

Apparatus

Reflux condenser -1

Conical flask (250 cm^3) -1

Dropper -1

Burette– 1

Volumetric flask (250 cm^3) – 4

Pipette (20 cm^3) – 1

Measuring cylinder (10 cm^3) -1

Water bath

Chemicals

Standard HCl (*M*/2)

Alcohol

Potassium hydroxide

Phenolphthalein(indicator)

Solutions Provided:

1. **2.5 *M* KOH Solution:** It can be prepared by dissolving 24.2g KOH in 100 cm3 water.

2. ***M*/2 HCl:** Standard solution

5.4 PROCEDURE

1. Weigh accurately about 1 g of oil in 250 cm^3 conical flask,
2. Dissolve the ester in 25 cm^3 alcohol and then add 25 cm3 of 2.5 M KOH solution with the help of a pipette,
3. Attach a reflux water condenser to the flask and add some boiling chips,
4. Heat the flask in water bath for about 1 hour with occasional shaking,
5. After 1 hour, stop heating. Add to the hot solution 0.5 to 1.0 cm^3 of phenolphthalein.
6. Titrate the excess alkali with standard *M*/2 HCl until the colour of the indicator changes. Record the volume of HCl used in Observation Table-II.
7. Carry out determination with the same prepared sample again to get at least two concordant readings.
8. Carry out a blank test upon the same quantity of KOH solution at the same time under the same conditions. Record the volume of HCl used in Observation Table-I

5.5 OBSERVATIONS

Mass of the conical flask = m_1 =…….g

Mass of conical flask + oil = m_2 =…….g

Mass of oil = $m_2 - m_1 = m$ =…….g

=……g

Observation Table I

(Blank Experlment)
KOH Solution vs. HCl

Sl. No.	Volume of KOH Solution solution (cm^3)	Burette reading		Volume ofHCl solution (cm^3) (Final - Initial)
		Initial	Final	
1.				
2				
3				

Observation Table II

(Original Experiment)
Oil Sample + KOH Solution vs. HCl

Sl. No.	Volume of oil Sample + KOH Solution solution (cm^3)	Burette reading		Volume ofHCl solution (cm^3) (Final - Initial)
		Initial	Final	
1.				
2				
3				

5.6 CALCULATIONS AND RESULT

The saponification value (S.V.) is given by the formula:

$$\text{S.V.} = \frac{56.1 \times M_m \times (V_0 - V_1)}{m}$$

where: V_0 = Volume of HCl used in the blank titration cm^3

V_1 = Volume of HCl used in the original titration cm^3

M_m = Molarity of HCl

m = mass of the oil in g.

S.V. of given sample of oil = ……

EXPERIMENT 6

DETERMINATION OF IODINE VALUE OF AN OIL OR FAT

Structure

6.1 INTRODUCTION

Iodine value or iodine number of fats or oil is a measure of its degree of unsaturation and gives an idea of its drying character. Iodine value depends on the number of double bond present in the molecule. Low iodine value means that the carbon chain of triglyceride contains very few carbon-carbon double bonds, while a higher iodine number indicates a large number of double bonds present in the molecule. If the triglycerides do not contain any double bond then the iodine value will be zero.

Iodine value can be defined as "the number of grams of halogen (Iodine) absorbed by 100 grams of the oils fat, and expressed· as the weight of iodine.

Expected Learning Outcomes

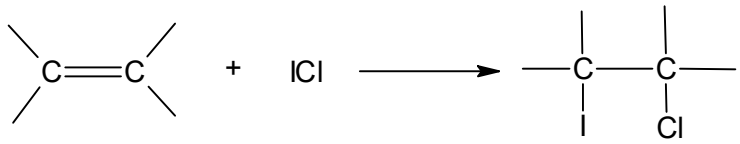
After performing the given experiments you should be able to:

- define the term iodine value; and
- determine iodine value.

6.2 PRINCIPLE

A know weight of oil is treated with ICI solution in CCl_4 or $CHCl_3$ and then the excess of iodine monochloride is treated with potassium iodide. The amount of liberated iodine is estimated by titration with sodium thiosulphate solution. By comparing volumes of thiosulphate used in this process and with bank

experiment the amount of iodine consumed can be determined. In this process following reaction occurs:



After the completion of the reaction, KI solution is added which is oxidised to I_2 by unreacted ICl :

$$ICl + KI \longrightarrow I_2 + KCl$$

The liberated iodine is titrated with standard NaS_20_3 solution using starch solution as indicator.

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

6.3 REQUIREMENTS

Apparatus

Reflux condenser -1

Conical flask (250 cm^3) -1

Dropper -1

Burette– 1

Pipette (20 cm^3) – 1

Water bath

Chemicals

Glacial acetic acid

Carbon tetrachloride/Chloroform

Potassium iodide

Starch solution (indicator)

Iodine trichloride

Iodine, pure, resublimed

Sodium thiosulphate

Solutions Provided:

1. **Potassium iodide:** It can be prepared by dissolving 100 g potassium iodide in 1 dm^3 water.

2. **Wijs Solution (ICl):** This can be prepared by dissolving 9 g of iodine monochloride in a mixture of 700 cm^3 glacial acetic acid (pure) and 300 cm^3 carbon tetrachloride.

3. **Starch solution:** Mix 5 g of starch and 0.01 g of the mercuric iodide with 30 ml of cold water and slowly pour it with stirring into one dm^3 of boiling water. Boil for three minutes. Allow to cool and decant off the supernatant clear liquid.

4. ***M*/10 sodium thiosulphate solution:** Prepare a 0.1 *M* thiosulphate solution by dissolving approximately 25 g of sodium thiosulphate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$; FW 248.19) in the sodium carbonate solution. Transfer the solution to a clean storage bottle, and store the solution in the dark. For more accurate results it necessary to

standardized sodium thiosulphate solution with potassium dichromate before use.

6.4 PROCEDURE

The amount of oil to be taken varies according to its expected iodine value and is given in the following table:

Iodine Value expected	Weight to be taken (g)
0-4	3.0
5-20	1.0
21-50	0.40
51-100	0.20
101-150	0.15
151-200	0.10

1. Dissolve the appropriate quantity of oil, in 250 cm^3 conical flask in 15 cm^3 carbon tetrachloride.
2. Add 25 cm^3 of Wijs solution, from the burette, to the mixture.
3. Close the flask using cork, shake gently and allow it to stand in dark at about 20°C.
4. For oils having iodine value below 150, leave the flask in dark for 1 hour for those with iodine value above 150 leave the flask for 2 hours.
5. After that add 25 cm^3 of potassium iodide solution and 150 cm^3 of water.
7. Titrate the liberated iodine with standard *M*/10 sodium thiosulphate solution, using starch solution as indicator, continue the titration until the blue colour just disappears. Record the value of $Na_2S_2O_3$ used in Observation Table-II.
8. Carry out experiment again to get at least two correspondent readings,
9. Carry out a blank test without oil under the same conditions. Record the volume of $Na_2S_2O_3$ used in Observation Table-I

6.5 OBSERVATIONS

Mass of the conical flask = m_1 =…….g

Mass of conical flask + oil = m_2 =…….g

Mass of oil = $m_2 - m_1 = m$ =…….g

=……g

Observation Table I

(Blank Experlment)
ICl Solution vs. M/10 Sodium Thiosulphate Solution

Sl. No.	Volume of ICl solution in cm^3	Burette reading		Volume of Sodium thiosulphate solution (cm^3) (Final - Initial)
		Initial	Final	
1. 2 3				

Observation Table II

(Original Experiment)
Oil Sample + ICl Solution vs. M/10 Sodium Thiosulphate Solution

Sl. No.	Volume of oil sample + ICl solution in cm^3	Burette reading		Volume of sodium thiosulphate solution in cm^3 (Final - Initial)
		Initial	Final	
1. 2 3				

6.6 CALCULATIONS AND RESULT

The iodine value (I.V.) is given by the formula:

$$\text{I.V.} = \frac{12.69 \times M_m \times (V_0 - V_1)}{m}$$

where: V_0 = Volume of sodium thiosulphate solution used in the blank titration cm^3

V_1 = Volume of sodium thiosulphate solution used in the original titration cm^3

M_m = Molarity of sodium thiosulphate solution

m = mass of the oil in g.

I.V. of given sample of oil = ……

The iodine value of commonly used edible oils is given as below.

Type of Oil	Iodine Value	Type of Oil	Iodine Value
Coconut oil	7.5-10.5	Groundnut oil	87-98
Safflower oil	138-146	Soybean oil	125-140
Cottonseed oil	98-115	Mustard oil	98-110
Sunflower oil	100-140	Rice bran oil	90-105
Sesame oil	103-115	Palm oil	44-58

EXPERIMENT 7

DETERMINATION OF REDUCING SUGARS AND NON-REDUCING SUGARS

Structure

7.1 INTRODUCTION

You have already studied the chemistry theory of carbohydrate in the BCHCT-135 Course. Carbohydrate, you should know by now, are compound of carbon, hydrogen and oxygen having empirical formula such as $C_n(H_2O)_n$ or $C_n(H_2O)_{n-1}$. There formulas suggest that they are hydrates of carbon and that is why early chemist gave the general name carbohydrates. We commonly call carbohydrates sugars and they also known as saccharides. Having studied the chemistry of carbohydrates in your theory course, now in the experiment we will perform some tests to distribution reducing and non conducting sugars.

Expected Learning Outcomes

After performing the experiment given, you should be able to:

- define monosaccharides, disaccharides and polysaccharides;
- explain the chemistry of various qualitative tests used to distinguish reducing and now reducing sugar;
- perform different tests such as Fehling's Test, Benedict's Test and Barfoed's Test to distinguish reducing and non-reducing sugars.

7.2 PRINCIPLE

Before going in further details of this experiment let us recapitulate some main points related to the chemistry of carbohydrates. Carbohydrates are actually polyhyroxy aldehydes and polyhyroxy ketones, therefore they are also referred

as aldoses and ketoses, respectively. As you have studied in your theory course, carbohydrates are divided into three general classes, depending on the number of carbohydrate molecular units they contain: Monosaccharides are carbohydrates that cannot be broken into smaller units upon hydrolysis. They may contain between 3 and 7 carbons. Common examples of monosaccharides are glucose and fructose. Oligosaccharides upon hydrolysis yield between 2-6 monosaccharide units and, depending upon the number of monosaccharide units so obtained, are known as disaccharides, trisaccharides, etc. Common examples of this class are sucrose and lactose. Polysaccharides produce a large number of monosaccharide units (more than six) upon hydrolysis. They may contain as many as 3000 monosaccharide units. Starch and cellulose are common examples of polysaccharides

Six carbon monosaccharides, such as glucose undergoes intramolecular reaction between a hydroxyl group and the carbonyl group to form hemiacetal form. We refer to the carbonyl carbon involved in the reaction as the **anomeric carbon**. At his carbon atom there are two stereo chemical configuration possible, alpha-(α) and beta (β). The two molecules contain these configuration are referred **anomers**. The α- and β-forms may be inter converted by a process called mutarotation, which proceeds via the acyclic aldehyde (see Fig. 8.1). In aqueous solution, an equilibrium mixture contains 36% α-anomer and 64% β-anomer. The two forms undergo inter conversion through the open form of glucose (0.02%). A similar intramolecular reaction occurs between a hydroxyl group and the ketone group of fructose to form a hemiketal.

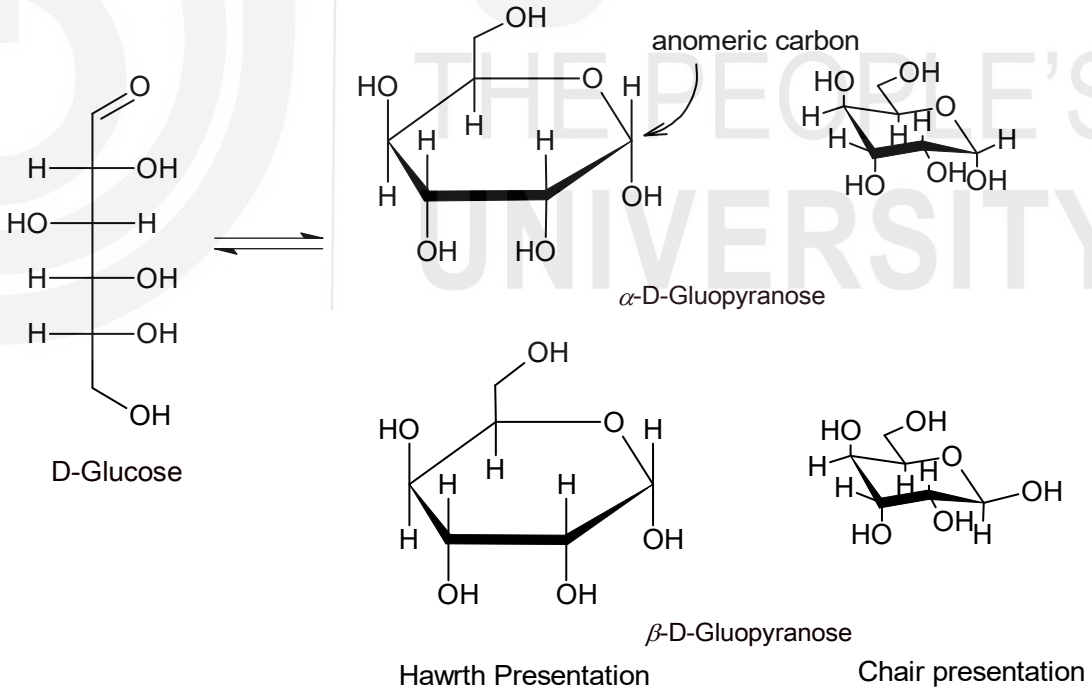


Fig. 8.1: Anomers of Glucose.

Carbohydrate can qualitatively characterized on the basis of their reactions with dehydrating acid (H_2SO_4) and condensation reagents such as phenols. Molisch's test, Seliwanoff test and Bial test come in this category. These tests are used to distinguishing between carbohydrates and non-carbohydrates; adose and ketose sugars; and pentose and hexose sugars. Second class of reagents are oxidizing agents such as solution of Cu(II) ions. In this

experiment we will focus on the oxidation reactions of carbohydrates with Copper(II) ions solutions. Three common reagents of this class are Fehling's solution, Benedict's solution and Barfoed's reagent. These reagent are mild oxidizing agents and consist of solutions contain Copper(II) ions which oxidize contain carbohydrates.

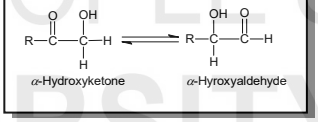
Alkaline Cu(II) ions solution readily oxidize many mono- and disaccharides with the oxidation of the sugar molecules. In this procee, there is a simultaneously reduction of the Coper(II) ions [Cu^{2+}] to a copper(I) ions [Cu^{+}]. This change in reaction can be identified easily by the colour change from a deep blue to a red or reddish brown.

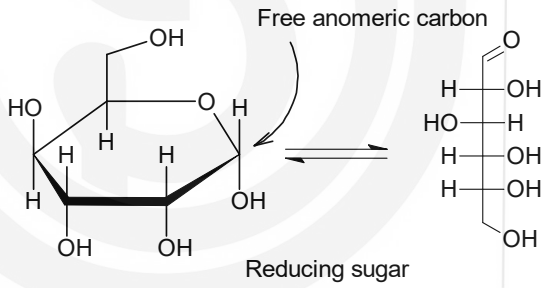
$$\text{Sugar} + Cu^{2+} \longrightarrow \text{Oxidized Sugar} + Cu^{+}$$

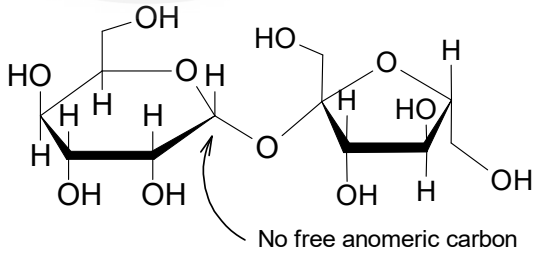
Reducing and Non-reducing Sugar

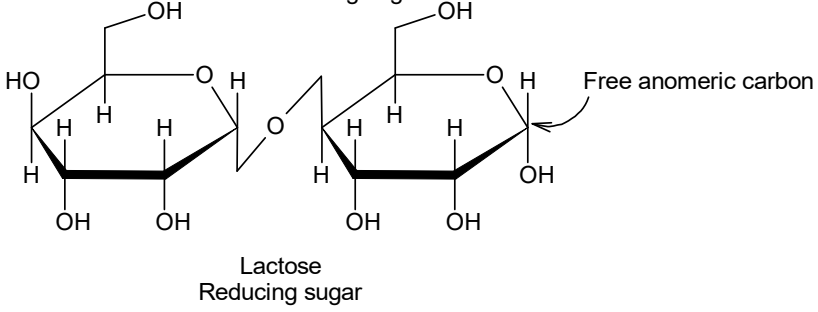
Any Sugar which is capable of being oxidized and causes the reduction of oxidizing agents such a Copper(II) ions (Cu^{2+}) to copper(I) ions (Cu^{+}) is known as reducing sugar, but those which are unable to be oxidized and do not reduce oxidizing agents are known as non-reducing sugars. All monosaccharide are reducing sugar, they all have a free reactive carbonyl group in the terms of aldehyde or cyclic hemiacetal. We can also distinguish between reducing sugar and non-reducing sugars on the basis of presence of anomaric carbon. If the oxygen on the anomeric carbon of a sugar is not attached to any other structure, that sugar can easily change to aldose and can act as a reducing agent, which is termed a reducing sugar.

Both aldose and ketose sugars can be reducing sugars. Under reaction conditions, ketose sugars undergo base-catalyzed rearrangement reaction to aldose sugars, which subsequently react with oxidizing agent such as Fehling's, Benedict's or Barfoed's reagents.









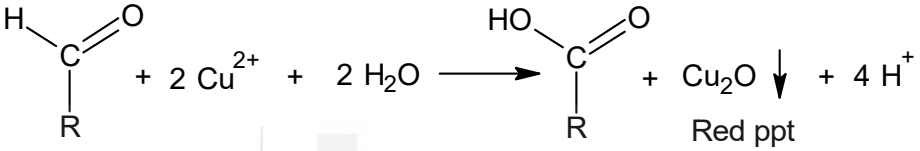
In the case of reducing sugars, the presence of alkali causes enolization especially at higher pH and temperature. This leads to a higher susceptibility to oxidation reactions that at neutral or acidic pH. These sugars, therefore, become potential agents capable of reducing certain mild oxidants such as copper(II) ions and Ag+ ions. Most commonly used reagents of this category for differentiating between reducing and non-reducing sugars are Fehling's test, and Benedict's test. In Barfoed's test mild acidic conditions are used (copper (II) acetate and acetic

Some disaccharides have free anomeric carbon and also reducing sugars like lactose. While other disaccharides such as sucrose are non-reducing sugars and will not react with copper(II) solution.

Large polymer of glucose such as starch, are not reducing sugars, since the concentration of hemiacetal group is very low. With this brief discussion on reducing and non-reducing sugars, now we take up the chemistry of the commonly used reagents for distinguishing them.

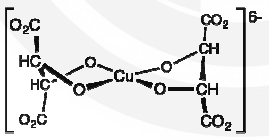
Fehling's and Benedict's Test

Both Fehling and Benedict's test are used to differentiate between reducing and non-reducing sugars. In these tests, Cu^{2+} is reduced to Cu^{+} and the reducing sugar is oxidized to a carboxylic acid:



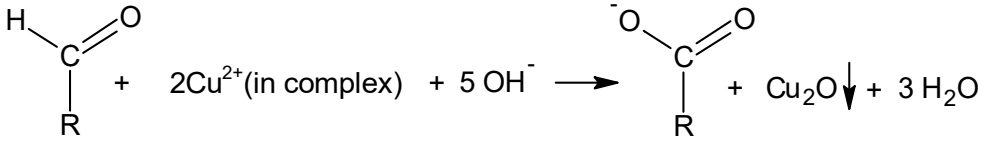
The appearance of copper(I) oxide, which is brick red, confirms the sugar to be a reducing sugar. Non-reducing sugars do not react. In both the reagent alkaline medium is used, therefore, to improve the solubility of Copper(II) ions and to prevent precipitation of copper ion as copper hydroxide, complexing agents are used. For Fehling's solution we use tartrate ions and for Benedict's solution we use citrate ions.

Fehling's solution is always prepared fresh in the laboratory. It is made from 2 separate solutions viz., Fehling's A and Fehling's B. The former is a blue colour solution of copper sulphate pentahydrate ($CuSO_4.5H_2O$), while the latter is a clear and colorless solution of aqueous potassium sodium tartrate (Rochelle salt) and a strong alkali (NaOH). In the final mixture, aqueous tartrate ions chelate to Cu^{2+} ions as bidentate ligand giving the bistartratocuprate(II) complex as shown in figure in margin. The tartrate ions, by complexing with copper prevent the formation of $Cu(OH)_2$ from the reaction of $CuSO_4.5H_2O$ and NaOH present in the solution.



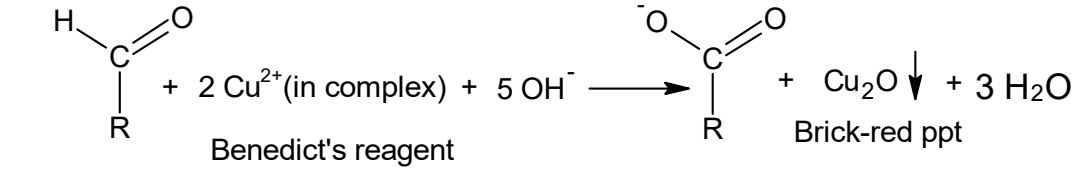
Bistartratocuprate (II)complex

The net reaction between an aldose sugar and the copper(II) ions of Fehling's solution may be written as:



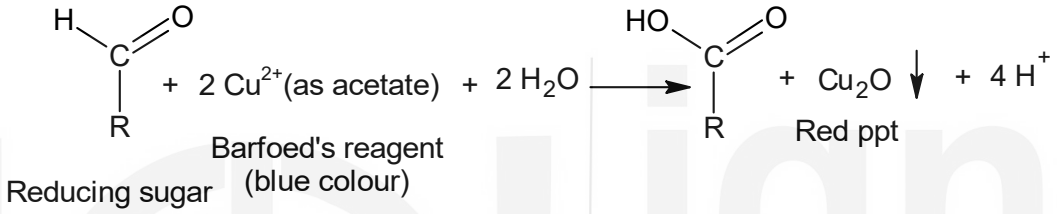
Reducing sugar Fehling's solution (blue colour) Brick-red ppt

Benedict's solution is a bright blue solution prepared by mixing copper sulphate $CuSO_4.5H_2O$, sodium citrate ($Na_3C_6H_5O_7$), and sodium carbonate (Na_2CO_3) in distilled water. Citrate provided better stability for copper(II) ions. Net reaction may be written as



Barfoed's Test

Barfoed's reagent, a mixture of ethanoic (acetic) acid and copper(II) acetate. This also differentiates between reducing and non-reducing sugars, but because it is more sensitive than the Benedict's test, it can be used to differentiate between reducing monosaccharides and reducing disaccharides. The reduction of Cu^{2+} to copper(I) oxide occurs more rapidly for monosaccharides than for disaccharides. The rate of appearance of the red precipitate can distinguish reducing monosaccharides from reducing disaccharides. Net reaction may be written as:



7.3 REQUIREMENTS

Apparatus		Chemicals
Test tubs	6	Fehling's solution A and B
Water bath Or 500 cm^3 Beaker	1 1	Benedict's solution
Test tube stand	1	Barfoed's solution
Test tube holder	1	Sugars: glucose, fructose, lactose, maltose, sucrose, starch.

Solution Provided

Fehling's:

Fehlin's Solution A: Dissolve 7 g of $Cu_2SO_4.7H_2O$ in water and make up to 100 cm^3.

Fehling's Solution B: 35 gm of sodium pottassium tartarate and 10 gm of NaOH Dissolved in 100 cm^3 of distilled water.

Benedict's solution:

100 cm^3 Benedict's solution can be prepared from 10 g of anhydrous sodium carbonate, 17 g of sodium citrate and 1.7 g of copper(II) sulphate pentahydrate.

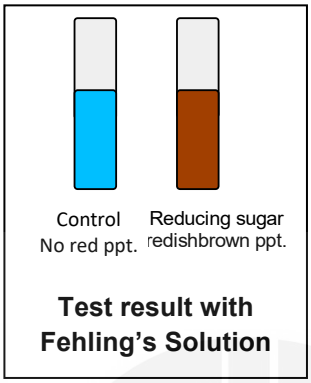
Barfoed's solution:

Dissolve 13.3 g of copper acetate in 200 ml of distilled water and add 1.8 ml of glacial acetic acid to it.

5 % Sugar solution of each glucose, fructose, lactose, maltose, sucrose, starch, unknown sugar solution:

Dissolve 5 g of each sugar in 100 cm^3 distilled water.

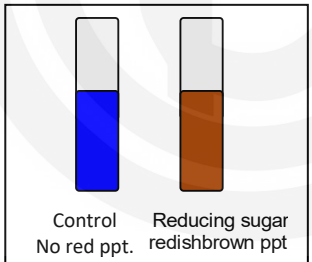
7.4 PROCEDURE



Test result with Fehling's Solution

Procedure of Fehling's test:

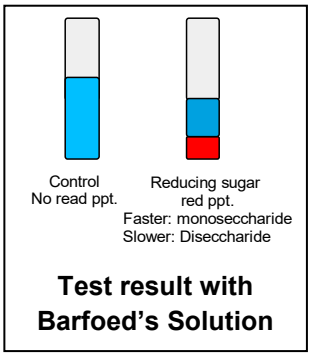
1. Take 1 cm^3 of test sample in dry test tube.
2. Take 1 cm^3 of distilled water in another test tube as control.
3. Take 2 cm^3 each Fehling's solution A and B in a test tube and add 2 cm^3 of this mixture to the sugar sample and distilled water.
4. Keep both sample solution and control test tubes in boiling water bath for 3-5 minutes.
5. Observe for the development of radish brown/brick red precipitate.
6. Record the observations in the Observation Table I.
7. Repeat the experiments for other sugar samples and unknown sugar solution.



Test result with Benedict's Solution

Procedure of Benedict's test:

1. Take 1 cm^3 of test sample in dry test tube.
2. Take 2 cm^3 of distilled water in another tube as control.
3. Add 2 cm^3 of Barfoed's reagent to both the tubes.
4. Keep both the test tubes in boiling water bath for 3-5 minutes.
5. Observe for colour change in the solution of test tubes or precipitation formation for the development of radish brown/brick red precipitate..
6. Record the observations in the Observation Table II.
7. Repeat the experiments for other sugar samples and unknown.



Test result with Barfoed's Solution

Procedure of Barfoed's test:

1. Take 1 cm^3 of test sample in dry test tube.
2. Take 1 cm^3 of distilled water in another tube as control.
3. Add 3-4 cm^3 of Barfoed's reagent to both the tubes.
4. Keep both the test tubes in boiling water bath for some time.
5. Look for the development of brick red precipitate.

6. To check whether the red precipitates or red color are formed or not, remove the test tube regularly from the bath. Put the test tube again and again in the bath. Note the time taken to develop the color.

7. Record the observations in the Observation Table III.

8. Repeat the experiments for other sugar samples and unknown.

7.5 OBSERVATION

Observation Table I
Test with Fehling's Solution

Material		Colour of mixture	Reducing/non-reducing sugar
1	Control	Blue	--
2	Glucose		
3	Fructose		
4	Maltose		
5	Sucrose		
6	Lactose		
7	Starch solution		
8	Unknown		

As compare to the original sugar solution, Barfoed's reagent should be added in more quantity. This ensures the good reaction and a positive result will be more observable.

It is very necessary to keep track of the time. Make sure that you have a digital stop watch beside you while you are performing the test. Timing is everything in this test and you might lose your experiment if not taken care of the time.

Observation Table II
Test with Benedict's Solution

Material		Colour of mixture	Reducing/non-reducing sugar
1	Control	Blue	
2	Glucose		
3	Fructose		
4	Maltose		
5	Sucrose		
6	Lactose		
7	Starch solution		
8	Unknown		

As compare to the original sugar solution, Barfoed's reagent should be added in more quantity. This ensures the good reaction and a positive result will be detected easily.

It is very necessary to keep track of the time. Make sure that you have a continually watch time while you are performing the test. Timing is important criteria in this experiment.

Observation Table III
Test with Berfoed's Solution

Material		Colour of mixture	Reducing/ nonreducing sugar	Monosaccharide/ Diseccharide
1	Control	Blue		
2	Glucose			
3	Fructose			
4	Maltose			
5	Sucrose			
6	Lactose			
7	Unknown			

7.6 RESULT

Identification of Unknown carbohydrate:

Test reagent

		Observation	Conclusion
1.	Fehling's solution:	_____	_____
2.	Benedict's solution:	_____	_____
3.	Berfoed's Solution:	_____	_____

EXPERIMENT 8

SYNTHESIS OF ASPIRIN AND ITS COMPARISION WITH AN ASPIRIN TABLET BY TLC

Structure

8.1 INTRODUCTION

In the previous eight experiments you performed experiments based on quantitative analysis (mainly titrimetry), paper chromatography and pH metric titration. Now you will learn some techniques involved in organic synthesis like, dissolution, recrystallisation, filtration, etc. and its identification by thin layer chromatography. In this experiment you will synthesise an organic compound which is used in our day-to-day life as a common drug, aspirin.

Aspirin is an antipyretic, an analgesic, and an anti-inflammatory drug. It is probably the most extensively used analgesic drug. As an anti-inflammatory agent aspirin is used extensively in the treatment of arthritis. It is also universally being recommended as a medicine which may prevent heart attack by checking blood clotting in the arteries. It is attributed to the fact that it effects platelets which are important for clotting of blood. On the negative side in excess doses, it causes gastric problems like irritation of mucous membrane. It is also said to be responsible for brain disorder (Reye's syndrome) in people below the age of 18 years.

In this experiment you will learn and perform the synthesis of aspirin from salicylic acid and compare this product with a commercial sample of aspirin by thin layer chromatographic technique which is a separation and purification technique. Thus, the experiment has two parts: one for the synthesis and other for the chromatography. However, the principle and procedure etc. for

the two parts are given separately. In the preparation part (Section 9.2) you will learn how to perform " acetylation", an important organic reaction. As you will see later, acetylation reaction can be carried out in a number of ways. We are giving procedures for two methods. Depending upon the convenience of time and availability of chemicals, your counsellor can choose any of these. The chromatography part (Section 9.3) may be carried out with any of the available nonbuffered commercial tablet of aspirin and doing its comparison with the lab based product. In the next experiment you will learn and perform extraction technique and extract DNA from a fruit like, strawberry or a vegetable like, onion.

Expected Learning Outcomes

After performing the given experiments you should be able to:

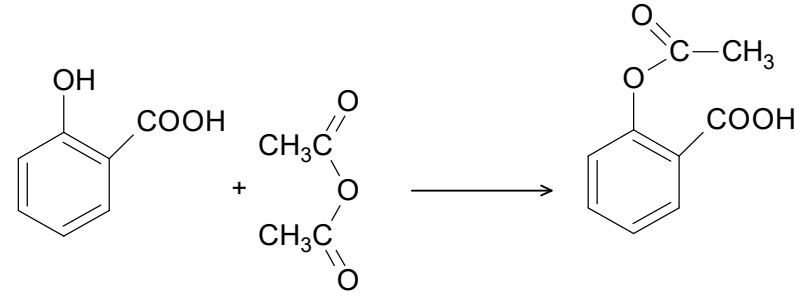
- explain the principle involved in the synthesis of aspirin,
- prepare aspirin from salicylic acid by acetylation method;
- explain the mechanism of acetylation of salicylic acid;
- measure the yield and the melting point of aspirin;
- explain the principle of thin layer chromatography; and
- compare the R_f of the synthesised aspirin with a commercial sample of aspirin by thin layer chromatography.

8.2 PREPARATION OF ASPIRIN

As said above, this experiment has two parts. In the first part, related to synthesis of aspirin, you will learn about the mechanism of acetylation of salicylic acid and the procedure to be followed for the synthesis. The second part will be described in Section 9.3.

8.2.1 Principle

Aspirin is chemically named as **acetyl salicylate.** It is the acetyl derivative of salicylic acid and is prepared by acetylation reaction of salicylic acid. You know that salicylic acid is *ortho* – hydroxybenzoic acid. The reaction between salicylic acid and acetic anhydride to form acetyl salicylic acid is given as follows.



Salicylic acid Acetic anhydride Acetysalicylic acid

Generally, in an acetylation reaction the reactive hydrogen of hydroxyl (alcohol or phenols) or amino (primary and secondary amines) functional group is replaced by $-COCH_3$ group. This reaction can be written as follows.

$$\begin{matrix} R-OH \\ \text{or} \\ RNH_2 \end{matrix} \xrightarrow{\text{acetylation}} \begin{matrix} R-OCOCH_3 \\ \text{or} \\ RNHCOCH_3 \end{matrix}$$

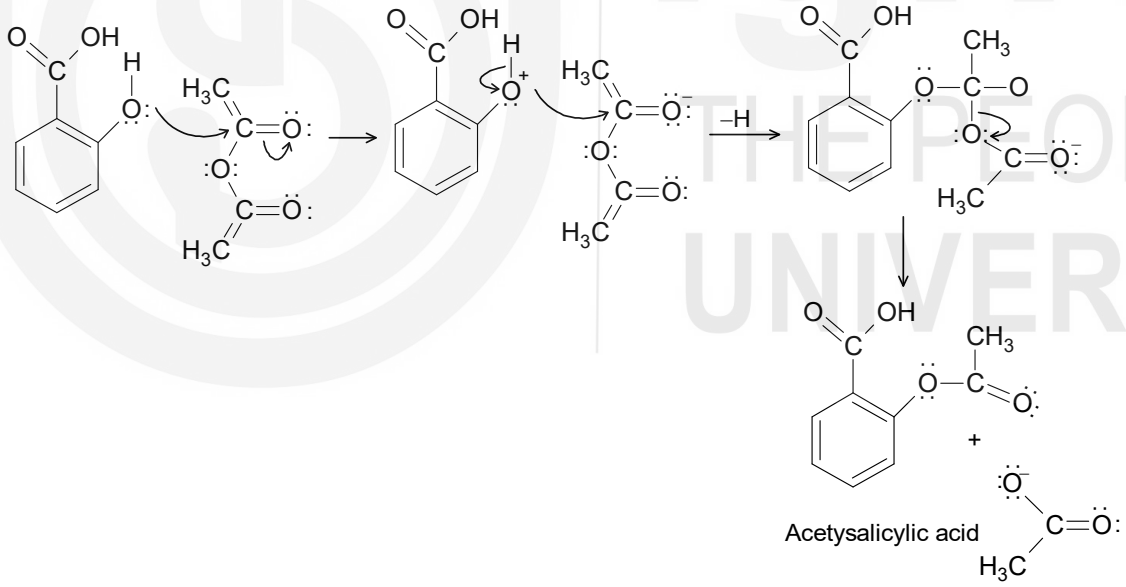
The acetylation of $-OH$ group is equivalent to the esterification of acetic acid. It is so because the product obtained, $R—OCOCH_3$ is essentially an alkyl/aryl ester of acetic acid depending on whether R is alkyl or aryl group. Acetylation reaction can be accomplished in a number of ways. These are:

i) with acetic anhydride in presence of a catalyst

ii) with acetyl chloride in presence of a base, like pyridine

iii) with a mixture of acetic acid and acetic anhydride

Commercially, aspirin is prepared by first method i.e., with acetic anhydride in presence of a catalyst. The procedures for both first and second method are given here, you may use any method as said above. The mechanism for acid catalysed acetylation of salicylic acid is represented as follows.



8.2.2 Requirements

Apparatus

Conical flask (100 cm^3)
Water bath
Beakers (100 cm^3)
Glass rod

Chemicals

Salicylic acid
Acetic anhydride
Sulphuric acid
Acetyl chloride
Pyridine
Alcohol

8.2.3 Procedure

Method I: This makes use of acetic anhydride as the acetylating agent. You have to proceed following the steps given below.

1. Take 2.75 g (0.02 mole) of salicylic acid in a 100 cm^3 conical flask and to this add about 6 cm^3 of acetic anhydride (0.06 mol) and a few drops of conc. sulphuric acid (acts as a catalyst).

   In this method acetic anhydride is taken in excess. It acts as acetylating agent as well as the solvent.

2. Swirl this flask in a water bath (temp = 50 – 60^{O}) for a few minutes till the solid material dissolves.

3. Leave the flask in water bath for about 10 minutes with occasional swirling.

4. Allow the solution to come to room temperature and then add about 50 cm^3 of ice cold water to it. You may even add crushed ice.

   Water is added to destroy the excess acetic anhydride, which gets converted to acetic acid.

5. Scratch the sides of flask with glass rod to induce crystallisation and filter the solid, so obtained.

6. Take about 10 -- 15 mg (a speck) of the crude aspirin in a test tube and dissolve it in about 1cm^3 of alcohol. Add a drop of 1% ferric chloride solution to it and observe the colour. Formation of intense colour indicates the presence of unreacted salicylic acid.

7. **Recrystallise** about half of the crude sample by ethanol/water solvent system. For this dissolve aspirin in minimum quantity of hot alcohol and to this add warm (50 - 60) water with constant swirling of the solution till a turbidity persists. If some crystals do form at this stage, dissolve these by gently heating the solution.

8. Allow the solution to cool till the crystallisation is complete. Collect the crystals by vacuum filter and wash these with cold water and dry the crystal in the folds of filter paper and measure the weight.

9. Determine the melting point of the recrystallised sample and report it in the result.

10. Report the yield of the product and calculate the % yield.

Method II: This makes use of acetyl chloride as the acetylating agent. You have to proceed following the steps given below.

1. Dissolve 2.75 g of salicylic acid to about 2 cm^3 of dry pyridine taken in a 100 cm^3 conical flask.

2. Quickly add about 2.5 ml of acetyl chloride to the above solution in small lots with constant swirling/shaking.

Caution: This reaction is highly exothermic and the temperature of reacting mixture rises rapidly. Don't let the temperature go beyond about 60°C (unbearable to touch). You may cool the flask occasionally in cold water/under the tap.

3. Heat the mixture on a water bath for about 5 minutes.

4. Proceed exactly as in method I from step 4 onwards and get the recrystallized product. Take the melting point of this product and report in the result.

5. Report the yield and the % yield as done in the previous method.

8.3 COMPARISON OF SYNTHESIZED ASPIRIN WITH THE ASPIRIN TABLET BY THIN LAYER CHROMATOGRAPHY

As mentioned before this is the second part of Experiment 9 in which you will compare the **Retardation factor** or the **Retention factor** (R_f) values of the synthesized aspirin and the tablet aspirin taken from the market. It is suggested that you brush up the concepts of chromatographic separation technique as discussed in your core courses and also what has been recapitulated in Experiment 1 of this course. In Experiment 1 you have again studied the concept of chromatography in detail. You learnt to use paper chromatography for the separation of a mixture of amino acids in that experiment. For the present experiment you will make use of thin layer chromatography (TLC). Thin layer chromatography is an efficient method of separating complex mixtures. It is a sensitive, fast, simple and inexpensive analytical technique in carrying out small scale experiments and also it is an excellent technique for small laboratories which do not have access to more sophisticated chromatographic equipment. You can carry out an easy variation of stationary phase in this technique.

Retention factor is defined as:

Distance moved by the centre of the solute spot / Distance moved by the solvent front

Beside separation, chromatographic techniques are identification techniques too, which you are going to perform here. The latter is an important application of TLC that helps in identifying the impurity if present in a sample. Thus, in this experiment you will be able to observe the purity of the compound synthesized by you in the lab and see if the commercial aspirin has any impurity or not.

8.3.1 Principle

Thin layer chromatography belongs to the adsorption type of chromatography. It is similar to paper chromatography in that the sample is spotted near one end of a plate of glass or plastic coated with a thin layer of an adsorbent. The TLC plate is place in a covered jar containing a shallow layer of developer. The developer rises up by capillary action and the solute is distributed between the stationary (absorbent) phase and the mobile phase. A solute which is more strongly adsorbed onto the stationary phase, will spend less time in the mobile phase, and hence it will migrate more slowly up the TLC plate. The sample is subsequently separated by development (elution). Treatment with a detector forms the coloured zones of the solutes. The

components of a mixture are identified by the calculation and comparison of R_f values. **Retention factor** is defined as:

Distance moved by the centre of the solute spot (**ds**) / Distance moved by the solvent front (**dm**)

Where **s** stands for the stationary phase and **m** stands for the mobile phase and **d** for the distance measured

8.3.2 Requirements

Apparatus	Chemicals
TLC jar (An alternative is beaker covered by watch glass or aluminum foil)	Salicylic acid
Spotting capillaries	Acetic acid
Measuring cylinder 100 cm^3	Ethanol/Methanol
TLC plates (prepared/procured) / sheets	Ethyl acetate
Spraying bottle	Hexane
Funnel	Iodine crystals for iodine chamber
Fitration/Vacuum filtration apparatus	Silica gel G
Slides/plates for TLC	Aspirin tablet (commercial)
TLC applicator	Propanol
Test tubes	Conc. Ammonia solution
	Chloroform

Preparation of TLC Plates

Thin layer chromatography can be performed on a sheet of an inert substance such as glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminum oxide (alumina), or cellulose. The aluminum foil sheets are the most common now-a-days due to the convenience in handling and use. The glass plates were very commonly used earlier. In case the plastic or aluminum foil sheets are not available in the study centre, you may use the glass plates. The plates are to be coated with an adsorbent for which the procedures are given here. In all probability, your counsellor would provide you the prepared plates.

TLC plates can be prepared by one of the following methods:

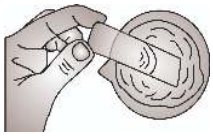
Screw cap jar

A. Dipping Method

- Take 33 cm^3 of methanol and 67 cm^3 of chloroform in a 125 cm^3 **screw-cap jar** (figure in the margin).
- Add 35 g silica gel G to this jar and shake the capped jar vigorously for about a minute.
- **Stack** two clean microscope slides back-to-back, holding these together at the top.

- Without delay **dip the stacked slides** into the slurry for about 2 seconds. Touch the bottom of the stacked slides to the jar to drain off the excess slurry (Figure in the margin).
- Let these air dry a minute or so to evaporate the solvent, separate the two slides and wipe the excess adsorbent off the edges with a tissue paper.
- **Activate** the slides by heating these in oven at 110°C for 15 minutes, or by placing these in covered beaker heated to that temperature.

Dipping method of slide preparation



B. Spreading Method

- Mix about 10 g of silica gel G with 20 cm^3 of water (stirring and shaking well to avoid formation of any lumps) in a beaker or any other container and get a free flowing paste.
- Take a glass plate or slide and pour the paste prepared in the first step on the glass plate.
- Spread it out with the help of a TLC applicator (shown in the Fig. 1 below). Let the plate air dry for ten minute and put the plate at 110°C in an oven for at least 5 minutes to activate the adsorbent.
- Alternately the slurry can be poured on the slide or the plate carefully from the beaker **directly** or a lab made applicator can be used for this purpose as shown in Fig. 2 below. The plate should be left for drying and then oven drying as in the previous case.

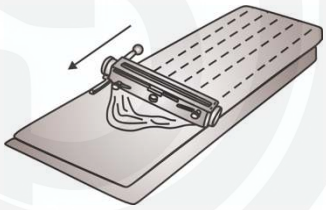


Fig. 1: Commercially available applicator

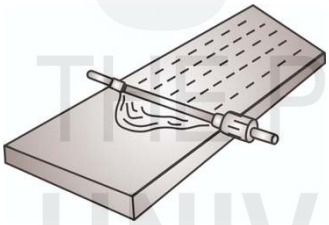


Fig. 2: A lab made applicator

Solutions provided

1. Sample Solution: Make a solution of the aspirin synthesized by you in ethanol/methanol in a test tube. The solution of the tablet can also be prepared in a similar way.

2. Visualisation reagent: You would be provided with an iodine chamber or a jar which has iodine crystals inside and the jar is covered with a lid so that the iodine vapours do not escape. Iodine binds with organic compounds to give these a yellow or orange colour spot.

8.3.3 Procedure

Proceed according to the following steps given:

1. Preparation of **Developer**: Prepare the developer by mixing hexane, ethyl acetate and acetic acid in the proportion of 65:35:5 respectively by measuring the required volumes with the help of a measuring cylinder.

2. Take the developer solution in a TLC jar and cover the jar with its lid to avoid any evaporation.
3. Take 4 silica get coated TLC plates from your counsellor and dry the plates in an oven for 30 minutes at 100°C, so that these are activated for adsorption chromatography.
4. Take a plate and make a light pencil line across it, 1 cm above the bottom of the plate and put three equidistant marks at the marked line where the compounds will be spotted.
5. Label the plate at the top end to indicate the spotted compounds i.e., acetyl salicylic acid, synthesized aspirin and the commercial aspirin.
6. Hold the plate in the left hand cautiously, so that the fingers do not touch the adsorbent layer. Take a capillary and place in the solution of acetyl salicylic acid to be spotted, let the solution rise into the capillary, take out the capillary from the solution and gently touch the capillary to the layer side of the TLC plate at the marked left hand side corner. Allow to flow the solution on the plate for a short duration so that a spot of the solution is formed but not larger than 2 mm in diameter.

   (**Note: The counsellor would demonstrate this step in the lab**).
7. Allow the spot to dry. You can blow in order to make the evaporation quick. Apply more solution at the sample place (if required). The aim is to apply to small but visible and built up spot.
8. Apply the synthesized aspirin solution spot on the TLC plate at the centre mark in a similar manner.
9. Apply the commercial aspirin tablet solution spot on the TLC plate at the marked right hand side corner in a similar manner.
9. After spotting all the three solutions, insert the plate into the developing jar (TLC jar).
10. Pour the mobile phase solution (prepared as per Step 1) into the jar with the help of a pipette till the developer level reaches nearly at 1 cm height of the lower edge of the adsorbent layer on the plate (**Remember that the spot should be above this level**).
11. Cover the jar and allow the developer to ascend along the plate. The position of the solvent front can be seen visually as the damp portion of the plate appears darker than the dry portion.
12. When the developer ascends to a required height on the plate, remove the plate from the developing chamber, mark the solvent front and dry the plate at 110°C for about 1-2 minutes.
13. After the plate is dried you will have to identify the product, called the **visualisation of the spots.**
14. The visualisation of the spots is done after heating the developed plate at ~100°C, either in an oven or on a hot plate, for 1-2 minutes.

Remove a thin strip of the layer from the edge of the TLC plate by means of a thumb nail or a spatula.

While marking the plates with pencil, do not press so hard on the pencil that you remove the silica gel.

The spot applied should be kept as small as possible. Application of too much of the solute should be avoided, as this will result in an elongated zone and will affect the correct calculation of R_f values.

15. In case of organic compounds, the developed, dried and hot TLC plate is put inside an **iodine chamber/jar**. The iodine jar is made by adding a few fresh crystals of iodine in a jar which is covered by a lid.

16. Observe the development of a colour on the plate. You would observe yellow or light orange colour spots.

17. Take out the plate after ~15 minutes or after the colour of all the three spots have developed properly and keep the plate on a flat surface.

18. Measure the distance of each spot (coloured) from its spot on the starting line and also the distance to which the solvent front has moved. Calculate the R_f values obtained using the formula given earlier.

17. From the comparison of R_f values of both the aspirin samples you can determine whether there is any impurity in any of these. You might recall that **a pure sample gives a single spot** in TLC. In case the reaction has not completed, the starting spot would also appear in the product spot. In such cases one should recrystallize the synthesized c

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Visualisation under a UV Lamp: In case your study centre has a UV lamp you can request your counsellor to allow you to put your developed and dried TLC plate under UV light and observe. You would be able to see the florescent spots of the compounds spotted. In this case also a pure compound will show only a single spot.

Remember that UV lamp visualization should be done prior to putting the plate in the iodine jar.

8.3.4 Observations and Calculations

Observe the colour of the spots of the three compounds on TLC plates. Measure the distance to which the three compounds have moved from the original (ds), and the distance which the solvent front has moved from the point of application (dm) on the TLC plate.

Calculate the R_f values by the relation: $\boldsymbol{R_f = ds/dm}$

The R_f of all the three compounds along with the remarks may be entered in the table given below.

Compound	ds	dm	Remark (no. of spots)
1. Acetyl salicylic acid			
2. Aspirin (prepared)			
3. Commercial Aspirin			

8.3.5 Results

1. The yield of the aspirin synthesized from 2,75 gm of acetyl salicylic acid = …….. gm

Theoretical yield = 3.6 g

$$\%\,\text{yield} = \frac{\text{yield obtained} \times 100}{\text{theoretical yield}}$$

= ……..

2. The melting point of aspirin was found to be = …….°C

3. The R_f by TLC of aspirin synthesized in the lab = ……

4. The R_f by TLC of commercial aspirin tablet = …….

EXPERIMENT 9

EXTRACTION OF DNA FROM MASHED ONION

Structure

9.1 INTRODUCTION

In the earlier experiments of this course, you have performed some chemical tests and experiments based on techniques like titrimetry, chromatography, pH metry, etc. Also, you carried out the chemical synthesis of a common drug, aspirin, in the previous experiment. All the experiments were based on the nature, structure, classification, and properties of the biomolecules found in living beings. In this last experiment of this lab course will give a different insight into the experimental work. It concerns the extraction of deoxyribonucleic acid (DNA), an important biomolecule and involves basic processes like, dissolution, filtration, decantation, etc.

Here you would recall from Unit 12 of this course that DNA is the genetic material, which is the blueprint for everything that happen in living cells. You may also remember that structurally DNA is a double helix in which two polynucleotide chains wind around each other. In this experiment you will learn to extract crude DNA from onion and can see it by naked eye. Onions are easily available, soft, and easy to pulverize. Further, onions have a low starch content, which allows the DNA to be seen clearly with naked eye. You can use banana as an alternate material to extract DNA.

Expected Learning Outcomes

After learning about and performing this experiment, you should be able to:

- explain the principle of extraction of DNA from onion (or banana);
- prepare the extraction liquid for the extraction of DNA,

- ❖ extract crude DNA from mashed onion; and
- ❖ observe coiled structure of crude DNA through naked eye.

9.2 PRINCIPLE

You know that DNA, the genetic material, is present mostly in the nucleus of the cell. The nucleus is surrounded by a nuclear membrane. It is difficult to extract out DNA from the nucleus. Therefore, to isolate DNA we need to disrupt or break the nuclear membrane to release an intact DNA into solution. Here, we make use of the fact that the proteins and lipids constituting the membranes can get denatured by the interaction with a detergent present in the salt solution. A sample of mashed onion when treated with the extraction solution that consists of a solution of detergent and salt in water releases the DNA in the solution. Here, the detergent causes the cell membrane to break down by dissolving the lipids and proteins of the cell. This solution is filtered and followed by precipitation of DNA by alcohol. The salt shields the negatively charged phosphate groups on the DNA backbone and allows it to precipitate out of a cold solution of alcohol.

9.3 REQUIREMENTS

Apparatus		**Chemicals**
Measuring cylinder (100 cm^3)	1	Absolute alcohol
Test tubes	2	NaCl (Salt)
Beaker (100 cm^3)	2	Distilled water
Funnel	1	Dishwashing liquid or any liquid detergent
Mortar and Pestle	1	Onion
Petri dish	1	
Rough balance and weight box	1	

9.4 PROCEDURE

- Prepare **extraction liquid** by taking 5 g of NaCl in 50 cm^3 of distilled water in a 100 cm^3 beaker and adding 9 cm^3 of liquid detergent (dishwashing liquid) to it. Dissolve the salt by stirring slowly to avoid foaming and set the mixture aside.
- Take about 50 cm^3 of absolute alcohol (95% ethanol) in a 100 cm^3 beaker and keep the beaker in the freezer as cooled alcohol is necessary for extraction.
- Take a small onion, peel, and cut it into small pieces. Place the chopped onion into the mortar and thoroughly grind it with the pestle.

- Cover the mashed onion with the extraction liquid and mix it well with a glass rod for about 5 minutes. Filter the contents of the mortar into a test tube.
- Transfer the filtrate to a petri dish and carefully add the chilled alcohol to it. You may observe some whitened strings or filament like structures in the dish. These are clusters of DNA molecules.
- You may try to spool the DNA with the glass rod.

9.5 OBSERVATION

White solid mass precipitated out on addition of chilled alcohol. A closer look revealed the filament like structure.

10.6 RESULT

The DNA sample from mashed onions is extracted as white coiled material.