Practical Manual

Biochemistry-1 (Bio202)

Department of Biotechnology

Virtual University of Pakistan, Lahore

Pakistan

Practical Manual

Biochemistry I (Bio202)

Safety Rules for Biochemistry Laboratory

1. Wear lab coat during the practical classes and wear closed toe shoes.

2. Wash your hands thoroughly when exiting and entering the lab. Biochemical experiments are very sensitive to contamination & the chemicals can harm you without any symptoms being apparent.

3. Keep your desk and apparatus neat and clean. At the end of the laboratory period, clean and put away all apparatus, and wash and dry the desktop.

4. Leave any food, beverages outside the lab. Never drink water or eat anything in the laboratory.

5. Properly label and store all chemicals.

6. Do not smell or taste chemicals

7. Do not keep a vessel with the base or acid near your eyes.

8. Never use mouth suction for pipetting or starting a siphon

9. Do not discard any chemicals down the sink unless instructed to do so.

10. Never leave spilled chemicals or water on a desktop or floor. Clean up immediately.

11. Read the label carefully before using any chemical.

12. Never return unused chemicals to the stock bottles. This may lead to contamination of the entire supply of reagent.

13. Be careful not to mix stoppers or caps of reagent bottles.

14. Never pour water into conc. acid. Pour the acid slowly into the water with stirring. (In a fume hood).

15. Know the properties (i.e., flammability, toxicity, and reactivity) of the chemicals in use. All hazardous chemicals must be handled in the fume hood.

16. Consult the teacher before using any unfamiliar equipment.

17. Report any injury, no matter how slight, to your instructor.

Practical Manual

Biochemistry I (Bio202)

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Biochemistry I (Bio202)

Experiment No. 1
Preparation of laboratory solutions and pH determination.
Apparatus Required
Weighing balance, volumetric flask, Cylinder, beaker, stirrer, pH meter
Chemicals required
Sodium chloride, Hydrochloric acid, sulfuric acid
Theory
Solution:
"A solution is a homogenous mixture of atoms, ions or molecules of two or more substances."
A homogenous mixture is that which has uniform composition throughout its body.

Solvent and Solute
The solvent is the component of a solution that is visualized as dissolving another component
called a solute. Usually the component present in the larger quantity is called the solvent, and the
component present in the smaller quantity is called the solute.

Types of solution

Unsaturated solution
A solution that is capable of dissolving more solute at a given temperature than it already
contains, is known as unsaturated solution.
Saturated solution
A saturated solution is the solution which can dissolve no more amount of the solute, at a
given temperature.
Supersaturated solution
A solution that contains more dissolved solute than a saturated solution is called super saturated
solution.
Concentration and its units
Concentration means the relative amounts of the components of a solution. It tells the ratio of the
quantity of one component to the quantity of the other or to the total quantity of solution. It has
many units. Some common units are discussed below.

Mass Percentage
The ratio of the mass of the solute to the mass of the solution multiplied by 100 is called mass
percentage.
Mass Percentage of a solute = Mass of the solute x 100
                                                    Mass of solution
For liquid-liquid solutions, it is sometimes more convenient to express the concentration in the
units of percentage by volume:

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Volume Percentage of a liquid = $\dfrac{\text{Volume of the liquid} \times 100}{\text{Total volume}}$

Parts per Million (ppm)

This is used to express very dilute concentrations of a substance. One ppm is equal to 1 mg of solute dissolved per litre of solvent or 1 mg of solute dissolved per kg of solvent.

Molarity (M)

Molarity or the molar concentration is the number of moles of solute dissolved per dm^3 of solution. (1 dm^3 is equal to 1 Liter)

$$\text{Molarity} = \frac{\text{Number of moles of solute}}{\text{Volume of solution in liters}}$$

Molality (m)

Molality is the number of moles of solute dissolved per kilogram of solvent.

$$\text{Molality} = \frac{\text{Number of moles of solute}}{\text{Kg of solvent}}$$

Normality (N)

Normality is the number of equivalents dissolved per liter of solution.

$$\text{Normality} = \frac{\text{No. of equivalents}}{\text{Volume of solution in litres}}$$

pH

The pH is defined as follows:

"The pH of a solution is the negative logarithm to the base 10 of the hydrogen ion (or hydronium ion) concentration".

$$pH = -\log_{10} [H^+]$$

$$pH = -\log_{10} [H3O^+]$$

The pH of a neutral solution is 7, the pH of acids is less than 7 while that of bases is higher than 7.

Procedure

i. Preparation of 5% NaCl solution

1. Weigh 5 grams of sodium chloride.

2. Dissolve the sodium chloride (NaCl) in 25 ml of distilled water in a beaker with the help of stirrer.

3. Transfer the contents into a volumetric flask. Add some more water (10-20 ml) to the beaker and rinse the walls of beaker and add that to the same flask.

4. Raise the volume to 100 ml in the volumetric flask to prepare 5% NaCl solution.

5. Check the pH of the solution with pH meter.

ii. Preparation of 10% hydrochloric acid solution (100 ml) from 37% HCl

1. Take 50 ml of distilled water in a volumetric flask.

2. Calculate the volume of HCl required by using the formula.

$$C_1V_1 = C_2V_2$$
$$37 \times V_1 = 10 \times 100$$
$$V_1 = 1000/37$$
$$= 27.02 \text{ ml}$$

3. Add 27.02 ml of HCl to the volumetric flask slowly and raise the volume to 100 ml with distilled water to prepare 100 ml of 10% HCl solution.

4. Record the pH of the solution using pH meter.

Experiment No. 2
Qualitative and quantitative tests for carbohydrates.

Apparatus Required
Test tubes, test tube holder, dropper, beaker, spirit lamp

Theory
"Carbohydrates are polyhydroxy aldehydes or ketones, or substances that yield such compounds on hydrolysis". Many of the carbohydrates have the empirical formula $(CH_2O)n$. Some carbohydrates also contain phosphorus, nitrogen or sulfur.

- Carbohydrates are classified into **four** major groups:
  - ➢ Monosaccharides
  - ➢ Disaccharides
  - ➢ Oligosaccharides
  - ➢ Polysaccharides

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Monosaccharides (simple sugars) are those which cannot be hydrolyzed further into simpler forms. The backbones of monosaccharides are unbranched carbon chains in which all the carbon atoms are connected by single bonds. For example glucose, fructose

Disaccharides are those sugars which yield two molecules of the same or different monosaccharides on hydrolysis. For example, maltose, lactose.

Oligosaccharides are small chains of monosaccharide units (3-10) linked by distinctive linkages called glycosidic linkage.

Polysaccharides (Glycans) are those which yield more than 10 molecules of monosaccharides on hydrolysis. Some have hundreds or thousands of units. For example cellulose, glycogen

1. Molisch's test

It is a sensitive group test that is used regularly for the detection for all carbohydrates present either in free or combined form. Monosaccharides react rapidly whereas disaccharides and polysaccharides react slowly.

Principle: This test makes use of concentrated H_2SO_4 to catalyze the dehydration of sugars to form hydroxymethyl furfural (from hexoses) or furfural (from pentoses). The furfurals then condense with α-naphthol to give a violet or purple colored product.
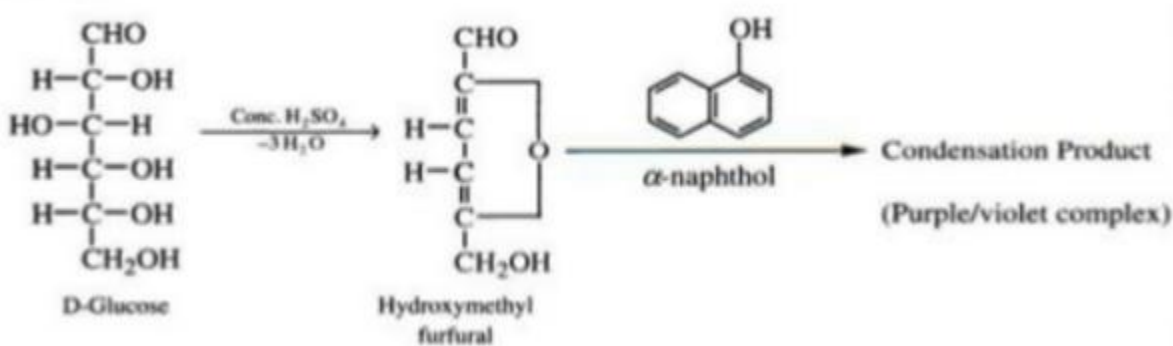
Reagents Required
i. Conc. sulfuric acid
ii. Molisch's Reagent: 5% α-naphthol solution in 95% ethanol
iii. Carbohydrates (glucose, maltose)

Biochemistry I (Bio202)

Procedure

1. Add 1-2 ml of unknown solution in a test tube.

2. Add 2-3 drops of Molisch's reagent ad mix well.

3. Hold the test tube in an inclined position and add carefully 1-2 ml of Conc. Sulfuric acid down the side of the tube to make a layer of it at the bottom of the tube.

4. Formation of purple product/ring at the border of two layers indicates a positive test.

2. Benedict's Test

Benedict's test is used for the detection of reducing sugars (sugars having free reactive carbonyl group).

Principle

This test utilizes a mixture of sodium citrate, copper (II) sulfate and sodium carbonate in a slightly basic solution. Reducing sugars reduce the copper (II) ions to copper (I) oxide (Red ppt).

$$\textbf{R-CHO} + \textbf{2Cu}^{2+} + \textbf{5OH}^- \longrightarrow \textbf{R-CO}_2^- + \textbf{Cu}_2\textbf{O (red ppt)} + \textbf{3H}_2\textbf{O}$$

R-CHO = Reducing carbohydrates

$R-CO^{2-}$ = Carbohydrate ion

Reagents Required

i. Benedict's Reagent**:** Dissolve Sodium Citrate (173 g) and anhydrous Sodium Carbonate (100 g) in about 700 ml of distilled water by gently heating the contents. Dissolve Copper Sulfate (17.3 g) in about 100 mL of distilled water in a separate beaker. Transfer this solution gradually into the Carbonate-Citrate mixture with constant stirring and raise the volume to 1 L with distilled water.

ii. Carbohydrates (glucose, starch/sucrose)

Procedure

1. Add 1 ml of sample in a test tube.

2. Then add 2 ml of Benedict's reagent.

3. Heat the solution in a boiling water bath for 3 minutes.

4. Formation of red precipitate indicates positive test.

3. Iodine test

This test is used to differentiate polysaccharides from monosaccharides and disaccharides.

Principle

Different carbohydrates e.g. starch form colored adsorption complexes with iodine due to the adsorption of iodine on the polysaccharide chains. The intensity of the color is dependent on the

length of the unbranched or linear chain accessible for the complex formation. Iodine forms complex with starch to give deep blue-black color. Glycogen (highly branched polysaccharide) gives red color with iodine.

Reagents Required

1. Iodine solution: Dissolve Iodine (1.0 g) and potassium iodide (2.0 g) in 300 ml of distilled water.
2. Starch solution (1%)

Procedure

1. Add 2 ml of sample in a test tube.
2. Add 1-2 drops of iodine solution.
3. Formation of blue black color indicates the presence of starch.

Experiment No. 3
Qualitative and quantitative tests for proteins.

Apparatus

Test tubes, test tube holder, dropper, beaker, spirit lamp

Theory

Amino acids have an amino group and a carboxyl group bonded to the same carbon atom (the α carbon) and a side chain that varies for different amino acids.

$$H_3\overset{+}{N}-\underset{\underset{R}{|}}{\overset{\overset{COO^-}{|}}{C}}-H$$
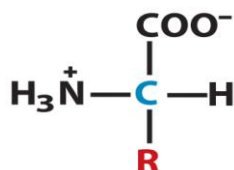
"Proteins are polymers of amino acids, with each amino acid residue joined to its neighbor by a specific type of covalent bond termed the peptide bond."

$$H_3\overset{+}{N}-\underset{\overset{|}{R^1}}{\overset{\overset{R^1}{|}}{C}H}-\underset{\overset{||}{O}}{C}-OH \;+\; H-\underset{\overset{|}{R^2}}{\overset{\overset{H}{|}}{N}}-CH-COO^-$$

$$H_2O \Updownarrow H_2O$$

$$H_3\overset{+}{N}-\underset{\overset{|}{R^1}}{\overset{\overset{R^1}{|}}{C}H}-\underset{\overset{||}{O}}{C}-\underset{}{N}-\underset{\overset{|}{R^2}}{\overset{\overset{H}{|}}{C}H}-COO^-$$
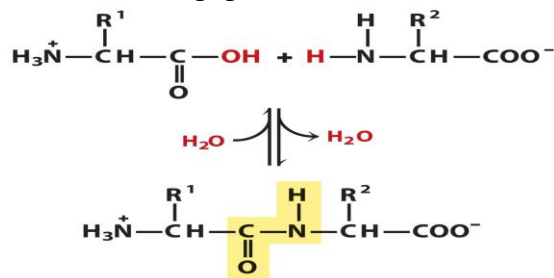
Biochemistry I (Bio202)

1. Ninhydrin Test

It is general test used for the detection of proteins, peptides and amino acids.

Principle

Ninhydrin reacts with α-amino acids ($-NH_2$) to give a purple colored complex. Proteins also have a free amino group on the α carbon that can react with ninhydrin to give a blue purple product.

| amino acid | ninhydrin | purple colored complex |
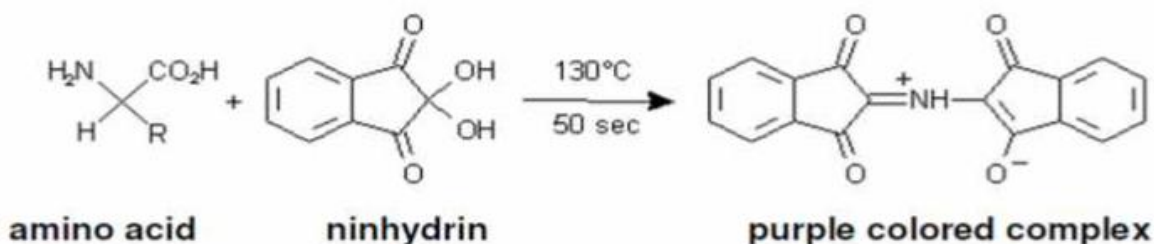
Amino acids such as Proline which has a secondary amino group react with ninhydrin to form a yellow reaction product.

| ninhydrin | proline | yellow product |
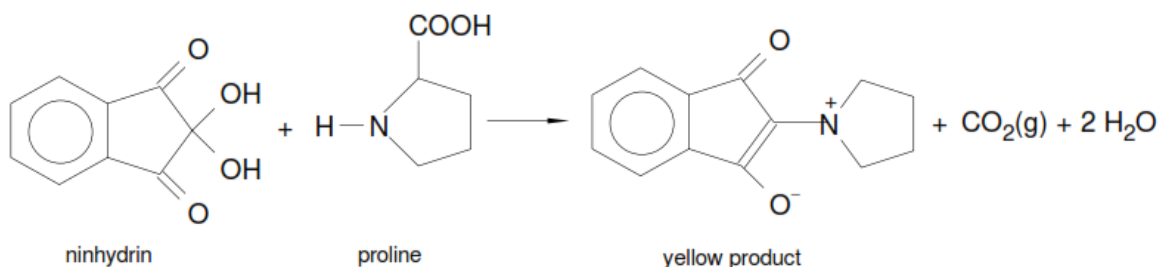
Reagents Required

1. Ninhydrin solution: Add 0.2 g of ninhydrin in 50 ml of acetone and raise the volume to 100 ml with acetone.
2. Amino acid solution (Lysine/alanine/proline etc..)

Procedure

1. Add 1 ml of amino acid solution in a test tube.
2. Add 5 drops of 0.2% ninhydrin solution.
3. Place the test tubes in boiling water for 5 min.
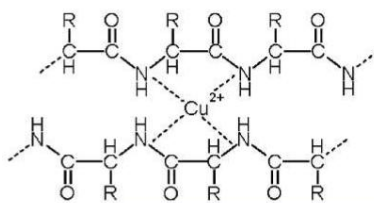4. Allow it to cool and observe the purple color formed.

Biuret Test

This test is used for the detection of proteins containing at least two peptide bonds.

Principle

The reaction incorporates the violet complex formation of the proteins with Cu^{2+} ions in an intensely alkaline solution.

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Biuret complex

Reagents Required
1. 10% sodium hydroxide solution
2. 1% copper sufate solution
3. Protein solution (casein, gelatin, albumin)

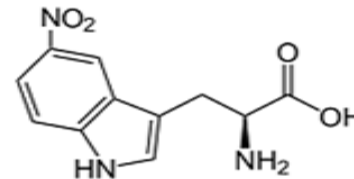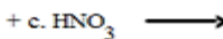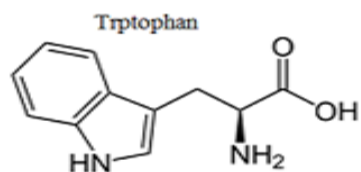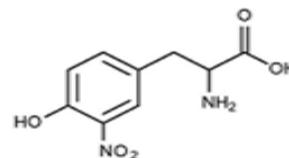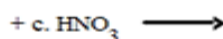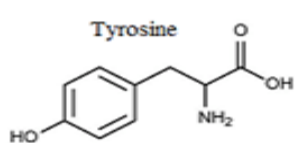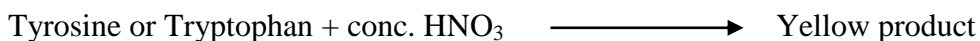## Procedure
1. Add 1 ml of protein solution in a test tube.
2. Add 1 ml of 10% sodium hydroxide solution and 2-3 drops of 1% copper sulfate solution.
3. Mix well and observe the formation of violet color.

Xanthoproteic Test
It is a general test used for the detection of tyrosine, tryptophan and proteins containing these two amino acids.

Principle
Some amino acids have aromatic groups that are derivatives of benzene. The amino acids tryptophan and tyrosine contain activated benzene rings that readily undergo nitration and form yellow product. The intensity of yellow color increases when the reaction is carried out in basic solution. Phenylalanine also carries a benzene ring but the ring is not activated and hence does not undergo nitration readily.

Tyrosine or Tryptophan + conc. HNO_3 $\xrightarrow{\text{Heat}}$ Yellow product

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Reagents Required
i. Conc. Nitric acid
ii. 40% sodium hydroxide solution
iii. Amino acid solution (Tyrosine/Tryptophan)

Procedure
i. Add 2 ml of amino acid solution in a test tube.
ii. Add equal volume of conc. HNO_3.
iii. Heat over the flame for two minutes and observe the color. Positive test is indicated by the formation of yellow color.
iv. Cool the test tube thoroughly under the tap water and add sufficient 40% NaOH solution to make the solution alkaline.
v. Observe the color (orange) of the nitro derivative of aromatic nucleus.

Experiment No. 4
Qualitative and quantitative tests for lipids.

Apparatus
Test tubes, dropper, filter paper, spirit lamp

Materials required
Oil/Ghee, Chloroform/alcohol, 20% alcoholic KOH

Theory
The lipids are a heterogeneous group of compounds that contain hydrocarbons and include fats, oils, steroids, waxes, their derivatives and precursors. They are insoluble in water but are soluble in nonpolar solvents such as ether and chloroform.

1. Spot Test

i. Take a piece of filter paper.
ii. Add a drop of sample on it.
iii. A greasy spot penetrating the paper indicates lipids. The spot is also translucent in nature.

2. Solubility Test
i. Take a small amount of sample in two test tubes.
ii. To one test tube, add 5 ml of water and add 5 ml of chloroform in the second test tube.
iii. Shake well.
iv. Miscibility of sample in chloroform and immiscibility of sample in water indicates lipids.

3. Saponification Test
Principle

Saponification is the process that produces soap from fats. In this process, oil or fat is hydrolyzed with alkali that results in the formation of glycerol and salt of fatty acids (soap).

Triacylglycerol   Glycerol   Soap
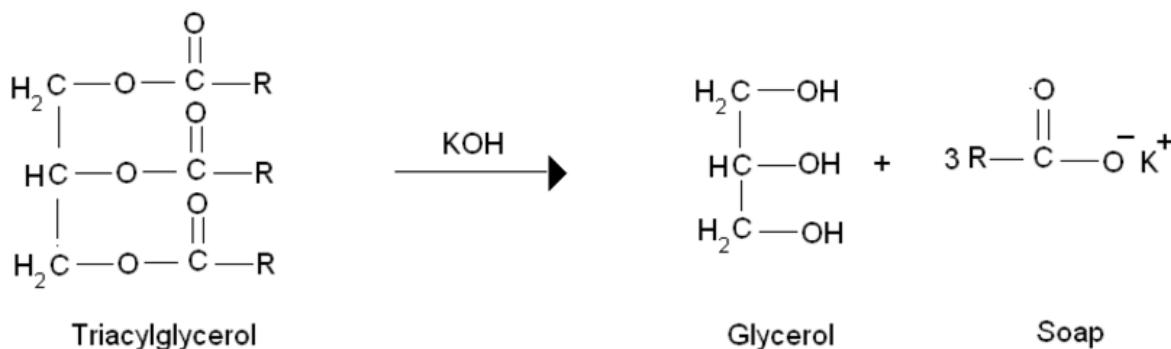
Procedure
i. Take sample and alcoholic potassium hydroxide in 1:3 ratio.
ii. Boil for 10-20 minutes.
iii. After heating, a semi solid layer develops (soap).
iv. Separate the upper semi solid layer.
v. Add little water to upper separated layer and shake well. Formation of bubbles indicates soap formation.

Experiment No.5
(a) Enzyme assays and the effect of pH on enzyme activity.

Apparatus Required
Test tube, stirrer, beaker, water bath, spectrophotometer
Materials Required
Salivary amylase/commercial amylase, soluble starch, Iodine, potassium iodide,
Preparation of reagents
1. Enzyme solution:
Add one ml of saliva in a beaker and dilute it by adding 9 ml of water.
2. Starch solution (2%)
Prepare stock starch solution by dissolving 2 g of starch in 70 ml of distilled water by heating on hot plate and then raise the volume to 100 ml with distilled water.

3. Stock Iodine Solution:
Add Iodine (5 g) and KI (50 g) to 500 ml of distilled water and raise the volume to I Litre with distilled water .

4. Dilute Iodine Solution: Take 1 ml stock iodine solution and raise the volume to 100 ml with distilled water.

5. Phosphate buffer
a) 11.876 g of $Na_2HPO_4.2 H_2O$ was dissolved in 500 ml of distilled water and the volume was raised to 1.0 L.
b) 9.08 g of KH_2PO_4 was dissolved in 500 ml of distilled water and the volume was raised to 1.0 L. Desired pH was obtained by adding enough solution (b) to make a total volume of 100 ml.

pH	Solution (a) ml
5	1
5.5	4.2
6	12.1
6.5	32.5
7	61.2
7.5	80.4
8	94

6. 0.1 N HCl

Theory
Enzyme assays are the methods used in laboratory for measuring the enzyme activity. Enzyme activity is a measure of quantity of active enzyme present. Different factors that affect enzyme activity include pH, temperature and substrate concentration. Enzymes are active within a certain range of pH and temperature. Within the range, there is a pH value at which the enzyme shows maximum activity. This point is known as optimum pH for enzyme. Amylase is an enzyme that catalyzes the hydrolysis of starch into simpler carbohydrates such as maltose. It is present naturally in human saliva. Amylase activity can be determined by the use of iodine solution. Iodine reacts with starch to form a blue black complex.
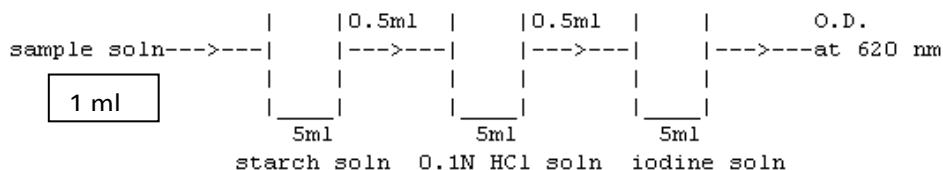Addition of amylase to starch solution results in the hydrolysis of starch to maltose and the intensity of blue/black color fades away. If complete hydrolysis of starch is carried out by amylase, then the reaction mixtures gives a yellowish color upon addition of iodine.

Biochemistry I (Bio202)

Procedure
Effect of pH
i. Add 5 ml of buffer solutions of different pH (5.0-8.0) to 5.0 mL of the 2% starch solution. This will result in 1% starch solution of different pH. Take 5 ml of each starch solution in test tubes.

ii. Add 1 ml of amylase solution to each test tube.

iii. Place the test tubes in water bath for 10 minutes at 37 ^{o}C.

iv. Add 0.5 mL of the reaction mixture to 5 mL of the 0.1 N HCl stopping solution.

v. Add 0.5 mL of the above mixture to 5 mL iodine solution to develop color. Shake and mix. (Deep blue coloration = residual starch present; brown-red coloration= partially degraded starch, clear/yellow= totally degraded starch)

vii. Measure the absorbance with a spectrophotometer at 620 nm setting the spectrophotometer to zero with distilled water.

```
                    |   |0.5ml  |     |0.5ml  |    |          O.D.
    sample soln--->---|   |--->---|     |--->---|    |--->---at 620 nm
                    |   |       |     |       |    |
    ┌───────┐       |___|       |___|       |___|
    │ 1 ml  │        5ml         5ml         5ml
    └───────┘    starch soln  0.1N HCl soln  iodine soln
```

viii. Plot a graph between pH and Absorbance by taking pH along x-axis and absorbance along y-axis.

b. Effect of temperature on enzyme activity.
Procedure
i. Add 5 ml of 1% starch solution (pH: 7.0) in 7 test tubes and place at different temperature (30-90°C) for equilibration.

ii. Add 1 ml of amylase solution to each test tube.

iii. Keep the test tubes in water bath for 10 minutes at different temperature (30-70°C with increments of 10°C).

iv. Add 0.5 mL of the reaction mixture to 5 mL of the 0.1 N HCl stopping solution.

v. Add 0.5 mL of the above mixture to 5 mL iodine solution to develop color. Shake and mix.

vii. Measure the absorbance with a spectrophotometer at 620 nm setting the spectrophotometer to zero with distilled water.

vii. Plot a graph between temperature and Absorbance by taking temperature along x-axis and absorbance along y-axis.

c. Effect of substrate concentration on enzyme activity.
i. Prepare starch solution (pH: 7.0) of different concentration (0.1-1%).

ii. Add 5 ml of starch solution of different concentration in test tubes.

iii. Add 1 ml of amylase solution to each test tube.

iv. Place the test tubes in water bath for 10 minutes at different temperature (37°C).

v. Add 0.5 mL of the reaction mixture to 5 mL of the 0.1 N HCl stopping solution.

vi. Add 0.5 mL of the above mixture to 5 mL iodine solution to develop color. Shake and mix.

vii. Measure the absorbance with a spectrophotometer at 620 nm setting the spectrophotometer to zero with distilled water.

viii. Plot a graph between substrate concentration and Absorbance by taking substrate concentration along x-axis and absorbance along y-axis.

Experiment No. 6
Paper chromatography of amino acids.

Materials Required

Whatmann filter paper (12x22 cm), Butan-1-ol, Acetic acid, ninhydrin spray (2% solution of ninhydrin in ethanol), Capillary tube, beaker (500 ml), oven.

Theory

Chromatography is an analytical method used for separation of different biomolecule on the basis of their chemical properties. In paper chromatography, a biomolecule (or mixture of biomolecules) is spotted on a piece of filter paper and is placed in an organic solvent. The hydrophobic organic solvent moves up the paper by capillary action. As the solvent reaches the biomolecule, the biomolecule starts to move up the paper. The degree of movement of biomolecule up the paper is associated to its relative affinity for paper (hydrophilic in nature) and the solvent (hydrophobic in nature).

Paper chromatography is very valuable method for characterizing amino acids. Different amino acids migrate at different rates on the paper because of difference in their R groups. The rate of movement of a biomolecule during paper chromatography is known as its relative mobility (R_f). R_f is also known as retardation factor. R_f is simply "the distance the biomolecule moved through the filter paper divided by the distance the moved by solvent through the paper".

$$R_f = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by solvent}}$$

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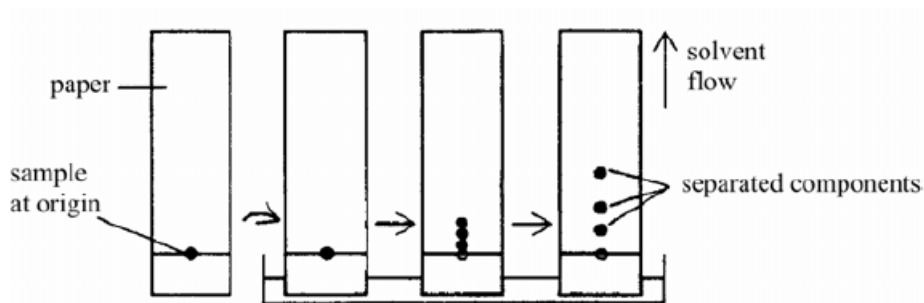
Procedure

i. Place enough chromatography solvent [Butan-1-ol : Acetic acid : Water in 60 : 15 : 25 ratio] in tank or beaker to achieve a depth of about ½ inch. Cover tank and allow solvent to saturate the atmosphere in the tank for at least 30 minutes.

ii. Draw a line across the bottom of the chromatography sheet about 2.5 cm from the bottom edge of the chromatography paper with the aid of lead pencil.

iii. Take a small volume of amino acid into the capillary and deposit it onto the paper by touching the capillary to the line drawn. The spot should not be larger than ¼ inch in diameter and the two spots should be separated by 2 cm. Allow the spots to dry.

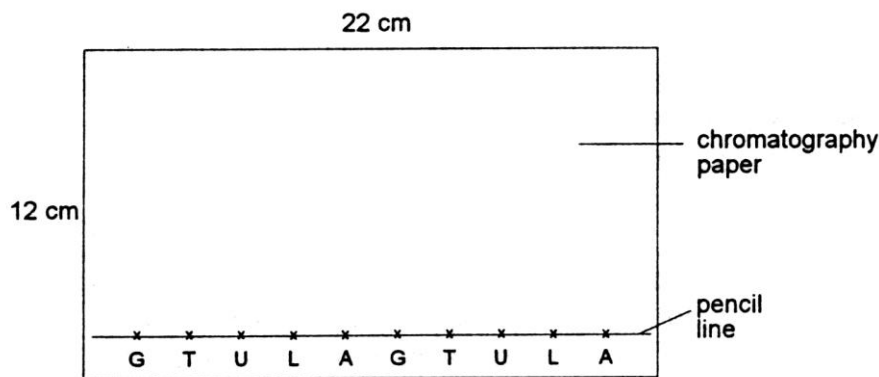iv. After drying, roll the paper into a cylinder and staple so that the ends do not touch.
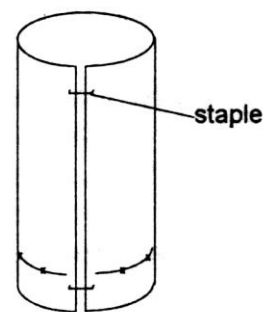

Figure 1

Figure 2

Development of the Chromatogram

i. Place the cylinder (chromatography paper) in a chromatography chamber/beaker (under the hood). Cover the chamber and allow the solvent to migrate up the paper for 60-90 minutes or till the solvent line is about an inch from the top of the sheet.

ii. Remove the chromatography paper from the tank and mark the solvent front line with pencil. Allow it to dry.

iii. Spray the paper with Ninhydrin solution (used to detect the location of amino acids).

iv. Dry the chromatography paper in a drying oven for about $100^{\circ}C$ for 3-4 minutes to allow the color to develop. Amino acids give blue/purple color when they react with ninhydrin.

v. Measure the distance the solvent migrated and the distance each of the amino acids migrated. Calculate the relative mobility (Rf)/retardation factor for each amino acid.

$$Rf = \frac{\text{distance the amino acid migrated}}{\text{distance the solvent migrated}}$$

Experiment No. 7
**Estimation of saponification value of fats/oils.**

Apparatus
Test tube, conical flask, Beaker, weighing balance, Dropper, Boiling water bath, Glass pipette, Burette, reflux condenser

Materials Required
Oil/Fat, 0.5 N alcoholic KOH, 0.5 N HCl, Phenolphthalein, Fat solvent (equal volumes of 95% ethanol and ether)

Principle
Saponification is the hydrolysis of fats or oils under basic conditions to give glycerol and the salt of the corresponding fatty acid. Saponification literally means "soap making". The saponification number is "the number of milligrams of potassium hydroxide required to neutralize the fatty acids resulting from the complete hydrolysis of 1g of fat". The long chain fatty acids found in fats have low saponification value because they have a relatively fewer number of carboxylic functional groups per unit mass of the fat and therefore high molecular weight.

Triacylglycerol   Glycerol   Soap
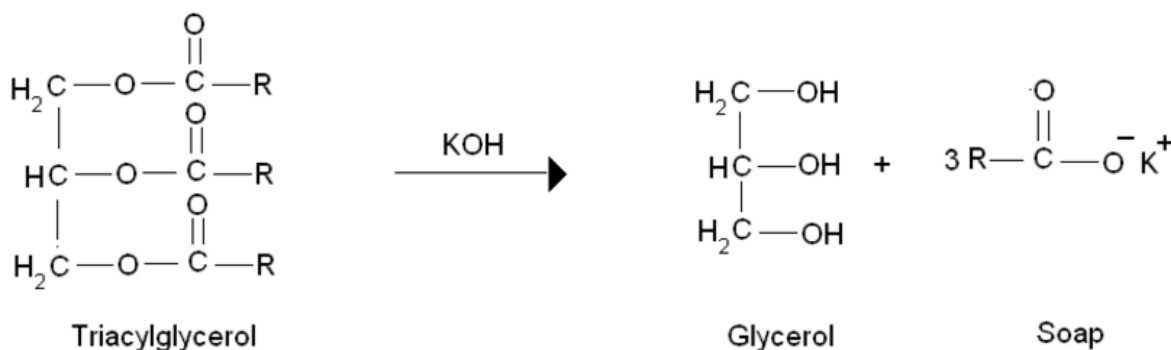
Procedure

i. Weigh 1 g of fat in a small beaker and dissolve it in about 3 ml of the fat solvent. Quantitatively transfer the contents of the beaker to a 250 ml conical flask by rinsing the beaker three times with a further milliliters of solvent.

ii. Add 25 ml of 0.5 N alcoholic KOH solution to the flask and attach it to a reflux condenser.

iii. For blank, follow the same procedure but do not add fat.

iv. Place both flasks in a boiling water bath for 30 minutes.

v. Cool the flasks to room temperature.

vi. Add 2-3 drops of phenolphthalein indicator in both the flasks and titrate using 0.5 N HCl. with phenolphthalein indicator.

vii. The disappearance of pink indicates the end point.

viii. Note down the end point of blank and test.

ix.   The difference between the blank and test reading gives the number of millilitres of 0.5N KOH required to saponify 1g of fat.

x. Calculate the saponification value using the formula

$$\text{Saponification Value (mg/g)} = \frac{(\text{ml blank} - \text{ml sample}) \times \text{N of KOH} \times \text{Equivalent wt of KOH}}{\text{Weight of sample in grams}}$$

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References

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