PRACTICAL

MANUAL

OF

BIOCHEMISTRY

Practical manual

For B. Sc (Agriculture 1st year students)

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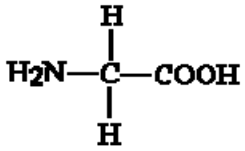
Contents

Atomic models of amino acids :

To understand the molecular logic of life, one needs to see the relationship between a molecular structure and the function it carries out in a cell. The structure plays a major role in emphasizing the function. If the structure is altered then the function is lost. One has to see the structure in relation to various atoms. Physical models play an important role to over come this problem to some extent. Let us see how to build the atomic models of amino acids.

Amino acids*:* Amino acids are alpha amino carboxylic acids. In amino acids, there are two functional groups, an amino group and a carboxylic group. Both these groups are attached to the α carbon atom only. The carbon atom which is tetrahedral in shape where the various groups attached to it are placed in different positions. Since the valence of the carbon atom is four, four groups can be attached to the carbon atom. Based on the groups attached to the carbon atom it may be of two types.

1. Symmetric carbon atom: When the valence of the carbon is satisfied by more than one similar atoms/ groups then the particular carbon atom is called as symmetric carbon atom. Eg : Glycine

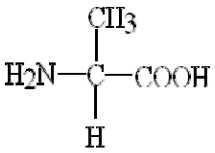


glycine

Compounds containing symmetric carbon atoms are optically inactive since they cannot rotate the plane of polarized light.

2. Asymmetric carbon atom: When the valence of the carbon is satisfied by four different groups, then that particular carbon atom is called as asymmetric carbon atom.

Eg: Alanine



alanine

In amino acids, to α carbon atom, an amino group, a carboxylic group and a hydrogen atom are attached and the fourth group is the R group which varies for each amino acid. All amino acids except glycine have at least one asymmetric carbon atom, hence they are optically active.

The structure of proteins can be easily understood by molecular models like ball and stick models. This was further modified by Cochranes to make it a sufficiently accurate. In this concept the atoms are represented by plastic spheres with holes in it pertaining to its valency. The atoms are joined by plastic sticks to show the bond formation. To make it more accurate each atom is represented by different colour.

Let us prepare the models of amino acids

First prepare molecular model of amino acids and then go on substituting the R groups to see the structure of each amino acid.

S.No	R groups	Structure	Name of the amino acids
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			

S. No	R groups	Structure	Name of the amino acids
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			

S. No	R groups	Structure	Name of the amino acids
17.			
18.			
19.			
20.			

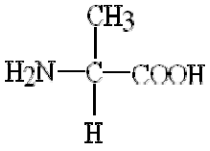
R.S Configuration :

All the amino acids except glycine have at least one asymmetric (chiral) carbon atom, to which four different atoms groups are attached. Hence they exhibit steroisomerism and such pair of molecules are called steroisomers. One member of the steroisomer pair solution rotates the plane of polarized light either to the right (dextrorotatory) and the other to the left (levorotatory). The amino acid stereoisomer can be classified as d or l based on the rotation. But a more precise and clear way of notation is R and S configuration, which was given by Cahn-Ingold and Prolong and it is named after them as Cahn-Ingold-Prolong convention.

In this convention, the groups that are attached to the α -carbon are taken into consideration and they are arranged according to the atomic number when sequence of groups arranged in descending priority which will decide the configurations either R or S.

Let us take one example of the simplest amino acid alanine to explain the phenomenon

The structure of alanine



alanine

The four groups that are attached to the α carbon are, NH_2 COOH,CH_3,H

These groups are given priorities based on their atomic number. Let us first consider the atoms that are directly attached to the α carbon. They are N,C,C,H. Now arrange them according to their decreasing atomic numbers ie: N-C-H. If two similar atoms are attached, then one has to take into account the atoms attached to these carbon in COOH and CH_3. The atoms attached to carbon are O& H. Hence when priority to the groups is as follows NH_2 – COOH-CH_3 –H

1. Prepare the model of alanine amino acid
2. Now orient the model in such a way that the atom which has the least priority ie:- Hydrogen will be away from you. And all the three groups are projected towards you.
3. Now arrange the groups according to these descending order of their atomic atomic number from greatest to least.

If the descending priority is in clock wise direction around the α carbon, then it is said to be R (rectum or right handed)

If the descending priority is in anti clock wise direction around the α carbon then it is said to be S(sinister, left handed)

Fill in the blanks

1. If the descending priority is in clock wise then it is _____
2. if the descending priority is in anti clock wise then it is_____
3. R and S convection is given by_____
4. R and S convection is based on_____
5. The atom with least priority will be faced toward _____
6. The scientists who gave R & S configuration are_____

Reactions of amino acids:

The chemical reactions of amino acids are those characteristic of their functional groups. All amino acids have an amino group and carboxyl group.

Ninhydrin test :

Ninhydrin test is the general test given by all the amino acids. One can identify the amino acids by performing this test. The amino group of amino acids reacts with the ninhydrin an oxidizing agent and the aminoacid gets itself oxidatively decarboxylated and deaminated to an aldehyde. Ninhydrin reacts with amino acids in two steps and two ninhydrin molecules react with one amino acid. In the first step ninhydrine reacts with amino acids it itself gets reduced and the aminoacid forms the corresponding aldehyde and ammonia and carbon di oxide are released, in the next step the reduced ninhydrine and a fresh ninhydrin react with the ammonia and forms a colored complex is formed. Alpha amino acids react fast when compared to beta and gama. Imino amino acids give a different color.

Materials :

1. Test solutions 0.1 % of the solutions prepared in 10% isopropanol
2. Filtered paper whatman No 1 paper, the size should be in accordance to fit in your manual.
3. Ninhydrin solution 0.1% in acetone (ninhydrin is carcinogenic handle with care)
4. Capillary tubes
5. Petri dishes
6. Reagent bottles
7. Measuring cylinder
8. Distil water
9. Hot air oven

Method :

1) Take the filter paper as required and make small circles each a part so that they do not overlap each other.
2) Against each spot write the name of different test solutions as given in manual with the pencil
3) Now carefully spot the test solutions in each circle with the help of capillary tubes
4) Dry the paper than apply excess ninhydrin slowly without over lapping each other
5) Heat the paper in oven at 105^{oc} for 4 min
6) Note down the color immediately

Group	Name	Filter paper Attachment	Color observed
α- amino	Glycine		
β- amino	B-alanine		
Imino	Proline		
Peptide	Asparetame		
Protein	Albumin		
Unknown	Sweat drop		

Individual test for amino acids :

Xanthoproteic test (Mulder's test):
This test gives positive result with aromatic amino acids containing phenyl groups in their structures (phenyl alanine, tyrosine & tryptophan)

Principle :
This test is based on the ability of aromatic amino acids containing substituted phenyl groups to react with concentrated nitric acid to give dinitro derivates. These are further converted to quinoid structures by reacting with strong alkali.

Materials :

1. Test solution 1% in 10% iso proponal
2. Concentrated nitric acid
3. 40% sodium hydroxide
4. Test tubes
5. Dropper
6. Test tube stands

Method :

Take 3 drops of test solution in a test tube and add 1 drop of concentrated nitric acid. Slowly mix and observe the change, now cool the test tube for 2-3 min by keeping under the tap. Now slowly add 2to 3 drops of 40% sodium hydroxide drop by drop and mixing thoroughly. The colour intensities will change in the alakine medium. Note down the changes.

S.No.	Test Solution	Colour obsevered in Acidic medium	Colour obsevered in Alkaline medium
1	Tyrosine		
2	Phenol		
3	Glycine		

Millons test :
It is generally Known as millon- Nasse's test.
This test gives a +ve result with tyrosine amino acid it gives a +ve result both in free state as well as being present as constitutive of protein.

Priniciple : phenyl /radical or hydroxy benzene radical present in tyrosine reacts with millons reagent to yield a purple red nitro-hydroxy phenyl mercurial, on heating with mercuric sulfate and sodium nitrite. This reaction is also gieven by phenolic compound. In amino acids only tyrosine and its derestatives gives the +ve result.

Materials :

1. Amino acids solution (1gm/lit)
   glycine, tyrosine, phenylalanine
2. Millon's reagent Commerically available
3. Phenol (1gm/lit)
4. sodim nitrite (10/lit)
5. boiling water bath

Proceure : Take 10 drop of the solution in a test tube and add 5 drops of millon's reagent to mix and boil for 2 min then cool the test tube by keeping under the tap and than add one drop of sodium nitrite slolution and observe the colour change note down the observation
Be cautious while heating phenol.

S.No	Name of the test solution	Observation	Inference
1.	Glycine		
2.	Phenol		
3.	Tyrosine		

Glyoxylic acid test (Hoplin-cole' test):

This test is given by those amino acids which have the indole groups.

Principle: The indole groups present in the tryptophan molecules react with glyoxylic acid in presence of con.H_2SO_4 forms a condensation product which is reddish violet, the sulfuric acid acts as dehydrationg agent, eliminating a molecule of water.

Tryptophan glyoxylic condensation product (reddish violet)

Materials

1. Amino acids solution 1g/lit
glycine, tyrosine, tryptophan
2.Glacial acetic acid which has been exposed to the light
3.Con. sulphuric acid.

Procedure:

Take 1ml of the test solution in a test tube and then add 1 ml of gly oxylic mix the solution than care-fully add around 2ml of con.sulphuric acid drop by drop by inclining the test tube so that the acid moves through the walls of the test tube and carefully see the colour at the junction where the two solutions meet. Care should be taken while using H_2SO_4. Record the observations.

S.No	Name of test solution	Observation	Inference
1	Glycine		
2	Tryptophan		

Electrophoresis

This technique was first introduced by the Swedish Biochemist Tiselius in 1937 and called moving boundary electrophoresis at that time. The zone electrophoresis, an improvement over the moving boundary method and was devised by Ionsden, Gardon and Martin. Paper electrophoresis, where Whatman No1 fillter paper was used as a support. It was one of its simplest and earliest version. This technique has become so much versatile that a number of support media eg:- Cellulose acetate strips, polyacrylamide gel, agar gel, sodiaum dodecyl sulfate gel are being used.

Any biological molecules possessing or carrying charge can be separated by electrophoresis. The magnitude of the charge depends upon the ionizable groups present on the molecule as also on the pH and compositiionof the suspending medium.

The electrophoretic mobility of a molecule depends upon a number of factors like size shape and charge of the molecule. However, the separation of closely related macromolecules such as proteins or simple molecules like amino acids depends mainly on the ioizable groups present on the molecule. In addition to this, the magnitude of the charge carried by the ionizing groups, vary according to the ionic strength and pH of the suspending medium and choice of the ionic strength of the medium plays a crucial role.

Let us take the example of an amino acid. Generally amino acid has ionisable groups ie:- COOH group which has a negative charge and NH3 group which has a positive charge. But when we take glutamic acid or Aspartic acid, which are dicarboxylic acids. They have two negative and one positive charge. Hence the net charge is –ve. Thus they drift towards the positive electrode anode(+) under the influence of an electric field. On the other hand, let us consider basic amino acids like lysine. It has two NH2 groups (two +ve charges) and one COOH group (one negative charge). Hence the net charge is +ve. It drifts towards negative electrode cathode, under the influence of applied electric field.

When an amino acid possess equal number of opposite charges ie:-the number of negative charges equals to the number of positive charges, the resultant net charge is zero then the amino acid will neither drift towards anode nor towards cathode in an applied electric field and remains wherever it is present .

Each amino acid has net charge of zero at some pH. This pH is called as isoelectric pH or isoelectric point (PI) where it does not move to either electrode when some external electric field is supplied.

When pHis more than PI then the amino acid bears a negative total charge and drift towards anode and exists as anion. When pH is less than PI then the amino acid bears a positive total charge and drifts towards cathode and exists as cation.

In the present experiment we are going to separate plant pigments and amino acids by paper electrophoresis. The two amino acids are aspartic acid and lysine and plant pigments which are colored derivatives of amino acids are

betalains and anthocyanidins. In addition to this, two synthetic pigments are taken as reference components, an anion eosin and a cation, methylene.

Principle :

Under the influence of an electric field, charged species / particles in colloidal solution move towards the oppositely charged electrodes. Electrophoresis is the migration of electrically charged particles under influence of applied electric field. Charged species are applied on to a support which is saturated with an conducting solution. Different species move with different velocities.

Material :

1. Electrophoresis equipment along with power pack
2. Whatman filter paper with suitable size to fit in the equipment.
3. Capillary tubes.
4. Phosphate buffer,pH6.3(7.2g KH_2PO_4+ Na_2HPO_4 1,liter distilled water.
5. Acidified methanol (2ml of con Hcl in 100ml of methanol)
6. Aspartic acid 0.1%
7. Lysine 0.1%
8. Mortar and pestle
9. Petri dishes
10. Muslin clothe
11. Eosin (2'2'5'7'tetra bromo fluorescein)
12. Methylene blue
13. Hibiscus flower
14. Bougainvilla flowers.

Preparation of plant extract :

a) To extract anthocyanidin hibiscus flowers are used
   1. Take 2 or 3 hibiscus flowers and separate the petals and grind them into a fine paste in motar and pestle.
   2. Then add 8ml of acidified methanol. Now filter the contents into a peridish using muslin cloth. The filtrate is the source for anthocyanidin. The hibiscus flower extract is stickly and thick, do not press the cloth tightly.

b) To extract betalains bougainville flowers are used
   1. Take 10 to 15 bougainville flowers and separate the petals of bougainville flowers which are dark in color and then grind them into a fine paste in motar and pestle.
   2. Then add 5ml of acidified methanol. Now filter the contents into a peridish using muslin cloth. The filtrate is the source for betalins.

Method :

The procedure or these experiments can be divided into three steps

1) Applying the sample
2) Development
3) Visualising the result

Applying the sample:

a) Take the Whatman No-3 filter paper of required size. The size varies depending upon the instrument used. Then fold the paper in the centre

length wise. Measure the distance and divide it into six equal parts with the help of scale and put the marks with pencil.

b) Now apply the sample by using a capillary tube in the form of thin streak between two points leaving a gap after each sample so that the spots do not interact ie:- Aspartic acid, Lysine, Betalins and Anthocynidins.

c) Now apply the synthetic dyes on either side of the paper ie:-eosin and methylene blue.

Development *:*

a) Pour the phosphate buffer into the anodic and cathodic cups of the chamber of electrophoresis to a height of 1-2cm phosphate buffer acts as electrolyte.

b) Now place the What man No.3. filter paper on which the samples were loaded into the electrophoretic chamber dipping both the ends, one in cathodic cup and other in anodic cup.

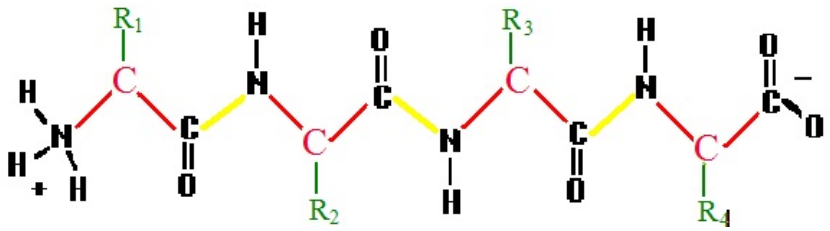
c) Due to capillary action, there is a rise of the electrolyte on the paper. When electrolyte from both the cups is about to meet then switch electric power supply which supplies current at 250 volts for 45 minutes. Closely watch for the drift of the colored components from the centre. If sufficient movement is observed switch off the power. If not continue for 15 more minutes. Then remove the paper and dry it in oven for 5 minutes.

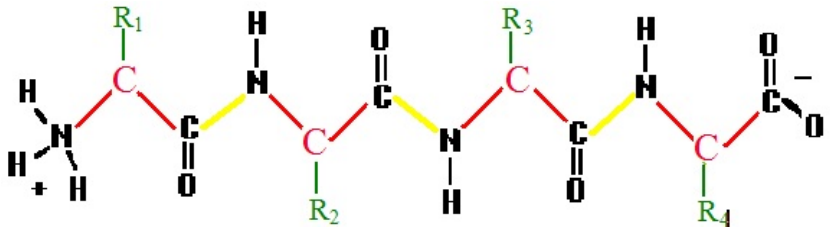
Visualization:

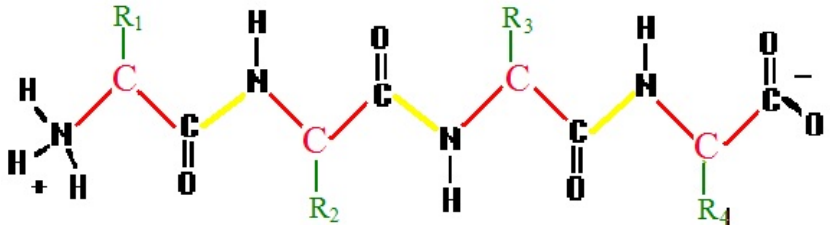
The amino acids are white in color. They have to be visualized by applying visualizing agent ie:- Ninhydrin. Apply it along the length of the filter paper where amino acids applied carefully and keep the filter paper in the oven at 104^{o} for 5 minutes. With the development of spots visualize the Results.

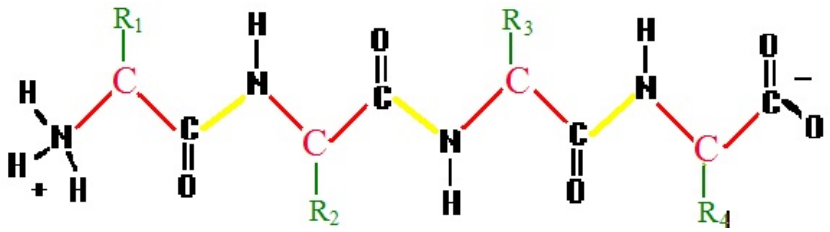
Components	charge possessed	move towards electrode
Asparatic acid		
Lysine		
Anthocyanidin		
Betalins		
Eosin		
Methylene blue		

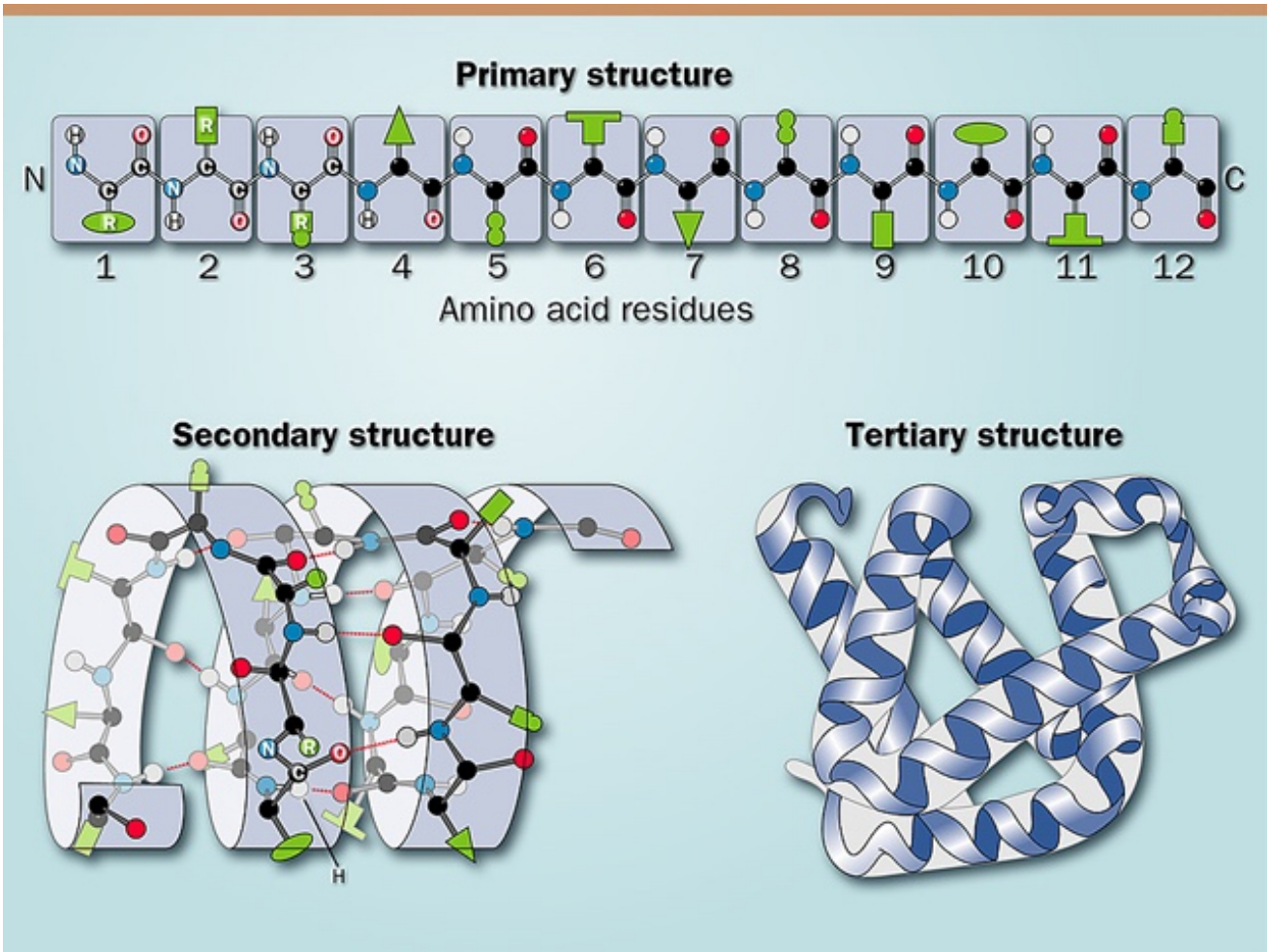
Paper model of proteins











Alpha helix : Take the polypeptide present in the above figure and identify the peptide bonds and encircle the amino acids and fold them in the form of helix by giving small slits where ever necessary. Show the hydrogen bonds in the helix

Beta pleated sheets : Your are provided with four polypeptide chains to build the model, identify the peptide bonds and encircle the amino acids in the form of triangles. Now identify the N and C terminal and paste antiparallel beta pleated sheet with one N & C terminal on either side show the hydrogen bonds between the two polypeptide chains. To build the parallel beta pleated sheets paste both C terminals on the same side and N terminals on the other side.

Denaturation of proteins

The secondary and the tertiary structures of the proteins are stabilized by hydrogen, ionic and covalent bonds. These bonds are broken by heat, which increases the random thermal motion. Extremes changes in pH will alter the charges on the side chains of amino acid residues of a protein, which may result in internal electronic repulsion, or loss of electrostatic attraction. Hydrogen bonds are broken. This leads to opening up of the molecules, loss of original shape and often activity.

Subunits of a protein may separate by application of urea, detergents like sodium dodecyl sulphate, guanidine and freezing. In some proteins urea weakens the hydrophobic bonds, which makes them loose and more viscous.

Materials :

1. Protein, (egg white)
2. Hydrochloric acid
3. Sodium hydroxide pellets
4. Test tubes

Method:

Protein are subjected to various types of processes as given below

1. Heat:- Take few drops of protein solution in a test tube and heat over a flame for few seconds. Observe the change.
2. Acid:- Take few drops of the protein solution in a test tube and add a drop of concentrated hydrochloric acid. Observe the change.
3. Alkali:- Take few drop of test solution in a test tube and add one or two pellets of Sodium hydroxide and observe the change.

Denaturating agent	Change observed
Heat	
Acid	
Alkali	

Preciptation with Heavy metals: Free side chains on the proteins have both positive and negative charges. By neutralizing these charges the protein becomes least soluble and may precipitate out. This can be brought by .

1. adjusting the pH to isoelectric point.
2. by neutralizing the negative charges (at alkaline pH) with heavy metal cations.
3. by neutralizing the positive charges (at acidic pH) with acidic reagents which have excess negative charges. The latter include trichloracetic acid and tannic acids..

Heavy metal ions react with S-S bonds to from solids.

Materials :
Protein source: gelatin solution 0.5% w/v in water.
Lead acetate solution 45% w/v in water.
Test tube
Method : Add one drop of lead acetate solution to two drops to test solution and observe the change
Inference :

Precipitation by acidic reagent : acidic reagents like trichloro acetic acid and phospotungstic acid can precipitate proteins. Acid induce precipitation is often used for concentrating proteins.

Materials :

Trichoroacetic acid solution 10% w/v in water
Protein gelatin solution 0.5%w.v in water.

Method : To five drops of protein solution in a test tube, add a drop of the TCA solution . Observe the change.
Inference :

Precipitation with organic solvents: The solubility of a protein depends on dielectric constant. When organic solvents are added they decrease the dielectric constant of the medium (if it is water) there by decreasing the solubility.

Material:

Protein solution egg white
Acetone/ Benzene
Test tube

Method : Take two drops of protein solution in a test tube and add a drop of acetone and note the change.
Inference :

Protein estimation by Lowry method

Protein can be estimated by different methods as described by Lowry and also by estimating the total nitrogen content. No method is 100% sensitive. Hydrolyzing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry et al is sensitive enough to give a moderately constant value and hence largely followed. Protein content of enzyme extraction is usually determined by this method.

Priniciple

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in Lowry's method.

Materials:

1. 2% Sodium Carbonate in 0.1N Sodium Hydroxide (Reagent A)
2. 0.5% Copper Sulphate (CuSO4. 5H20) in 1% Potassium sodium tartrate (Reagent B).
3. Alkaline Copper solution: Mix 50mL of A and 1mL of B prior to use (Regant C).
4. Folin –Ciocalteau Reagent (reagent D)Commercial reagent. Dilute 1:1 With distil water
5. Protien solution (stock standard)

Weigh accurately 50 mg. of bovine serum albumin (Fraction V) and dissolve in distilled water and make up to 50mL in a standard flask. This is stock solution, dilute it to prepare the working standard.

Working standard.

Dilute 10mL of the stock solution to 50mL with distilled water in a standard flask. 1mL of this solution contains 200ug protein.

Procedure

1. Pipette out 0.2,0.4,0.6,0.8 and 1mL of the working standard into a series of test tubes.
2. Pipette out 0.1mL and 0.2mL of the sample extract in two other test tubes.
3. Make up the volume to 1mL in all the test tubes. A tube with 1mL of water serves as the blank.
4. Add 5mL of reagent C to each tube including the blank. Mix well and allow to stand for 10 min.
5. Then add 0. 5mL of reagent D, mix well and incubate at room temperature in the dark for 30 min. Blue color is developed.
6. Take the readings at 660nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

Calculation :

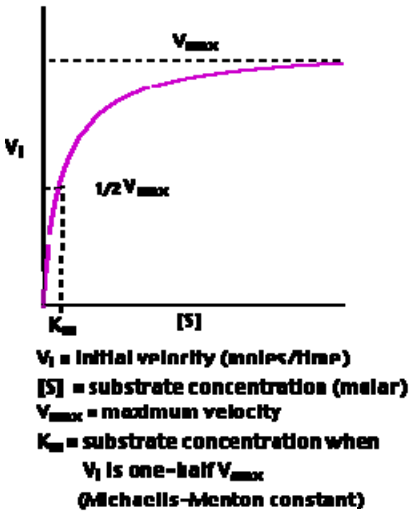
From the graph calculate the amount of protein present in the given sample. The OD obtained for the sample should be taken and on y axis and draw a straight line intercepting the standard graph. From there draw a tangent on to x axis, which will give the information regarding the concentration of the sample

S.No	Ml of protein	Ml of wate	Concentration of protein in mg	Reagent C	Reagent D	O.D at 660 nm
1.	0	1	0	5	0.5	
2.	0.2	0.8	0.2 x 0.2 = 0.04	5	0.5	
3.	0.4	0.6	0.2 x 0.4 = 0.08	5	0.5	
4.	0.6	0.4	0.2 x 0.6 = 0.12	5	0.5	
5.	0.8	0.2	0.2 x 0.8 = 0.16	5	0.5	
6.	1	0	0.2 x 1 = 0.2	5	0.5	
7.	sample		?	5	0.5	

Result :

Enzyme kinetics (graphical representation of data)

Some experimental data is supplied in the tables below. Use graph sheet to plot the michaeli's menton graph from the date provided. From the graph show vmax and find Km value.



S.NO	Substrate Concentration umoles	Rate of the reaction m/mg
1.	5	0.1
2.	10	0.177
3.	15	0.26
4.	20	0.31
5.	30	0.38
6.	40	0.43
7.	50	0.46
8.	60	0.48
9.	70	0.48

S.NO	Substrate Concentration umoles	Rate of the reaction m/mg
1.	5	0.02
2.	15	0.06
3.	30	0.11
4.	40	0.13
5.	50	0.14
6.	60	0.15
7.	70	0.15

Competitive inhibition

Competitive inhibitors due the structural similarity compete with the regular substrate for the catalytic site on the enzyme. For example malonate resembles succinate and competes with in the reaction of sucinic dehydrogenase.

Three important features of competive inhibition.
Inhibitor binds at the same active site as substrate.
Substrate and inhibitor mutually exclusive
Inhibition can be overcome by increasing substrate concentration.

E + S --- ES --- E+P
E+I---EI but it cannot form products.

The reaction between succinate and succinic dehydrogenase can be easily monitored by passing the electrons and protons to a dye.

Enzyme activity will be measured by determining the extent of color loss of the dye 2,6- dichorophenol indophenol(DCPIP). This dye intercepts the flow of electrons in the electron transport chain. When it accept electrons it becomes reduced and changes color, from blue (oxidized from) to colorless (reduced form). The amount of color lost is proportional to the number of electrons activated due to enzyme activity. Longer the time to vanish color, slower the reaction. Methylene blue can also be used instead of DCPIP.

Materials :

Sodium succinate 0.1M,Dissolve 16.2g in one liter water.
Sodium malonate 0.1M dissolve 14.8g in one liter water.
Phosphate buffer pH6.8
2,6-dichlorophenol indophenol solution 1% w/v in water (100mg/10ml)

Enzyme succinate dehydrogenase crude preparation : soak green gram seeds or Kabuli channa and allow them to germinate. Take 25 seedlings of green gram or 10 seedlings of Kabuli channa of Remove the seed coats from germinated seeds and grind in 25ml ice cold water in cold mortar. Filter with muslin cloth. Use filtrate as enzyme source. Store it at low temperature until use. Take Three test tubes.

Method

To three test tubes add the following, mix and stopper them immediately

Test tube number	Volume of buffer ml	Volume of DCPIP ml	Volume of succinate Substrate ml	Volume of malonate Inhibitor ml	Volume of enzyme ml	Time taken for decolration
1	2	0.5	1	--	1	
2	2	0.5	1	--	1	
3	1	0.5	1	1	1	

Immobilization of enzymes

Enzymes can be immobilized by an insoluble matrix (calcium alginate, polyacrylamide, gelatine) by physical adsorption method, encapsulation, entrapment and chemical covalent bonding means, while still retaining their catalytic activity. Matrix entrapment is the focus of this experiment it is based on the physical occlusion of enzyme molecules within a changed gel structure such that the diffusion of enzyme molecules to the surrounding medium severely limited. A highly cross- linked gel has a fine wire mesh structure and can more effectively hold smaller enzymes in its cages. Advantages over soluble enzymes are.
Reduced costs as they can be repeatedly used
Greater stability of the enzyme
Reduced inhibition effects on the enzyme.

Of late this technique has assumed greater importance as a tool biotechnology. In all entrapment protocols, enzymes are well mixed with monomers/ polymer and cross-linking agents in a solution. The solution is then exposed to polymerization promoters to start the process of gel formation. Commercially, it is common to force the unpolymerized solution through a set of nozzles to from spherical beads, whose size can be controlled by adjusting the back pressure the resulting beads may be further hardened to enhance structural integrity. Or alternatively, the solution is poured into mold to achieve the desired shapes. A gel block may be cut into smaller cubes to increase the surface area.

We will use calcium alginate as our matrix. Polymer molecules are cross-linked by calcium ions. Because of this calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. If needed, the trapped enzymes or even the trapped microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

Material :

Enzyme invertase crude preparation. Soak 10g dried yeast in 30ml 0,1m sodium bicarbonate for 24hrs at 40^{o}c.
Centrifuge.
Decant the supernatant which contains the enzyme invertase,
This stock is stable and can be stored at 5^{o}c for several months
Dilute 1: 25 with water just before use.
Sodium alginate soln.1.5%w/v in water
Calcium chloride 0.1M
Teat pipette
Sucrose solution 1.8M in water
DNS reagent: mix 100ml of 3,5-dintrosalicyulic acid 5% w/v in 2M sodium hydroxide with 250ml sodium potassium titrate 60% w/v in water.
Buffer sodium acetate 0.05M, PH 4.7

Method:

Immobilization

1. Mix 1ml of diluted invertase solution with 5ml of alginate solution
2. Stir the calcium chloride solution by placing the beaker on a magnetic stirrer.
3. Add invertase + alginate mixture drop wise from teat pipet intothe stirred calcium solution.

Testing the immobilized enzyme

1. Add 0.5ml sucrose solution, 1.5ml water and 1.0ml buffer to each of the three test tubes.
2. Equilibrate them at 40^{o}c
3. Add a gel – enzyme bead to the first
4. Shake continuosly.
5. Exactly after 10min, decant the contents of the first tube into another into another tube containing 2ml DNS reagent.
6. Add the second batch of equilibrated buffered substrate to the bead-containing original tube.
7. Incubate for another ten min.
8. Decant the contents like before retaining the bead.
9. Repeat the process for the third time.

DNS Assay

1. Place the three tube containing DNS reagent and reacted mixture in water bath at 40^{o}c for 5min.
2. Cool. Note the color intensity.

Enzyme induction

Introduction:

Enzyme induction is a process in which a molecule (*e.g.* a drug/ substrate) induces (*i.e.* initiates or enhances) the expression of an enzyme. In the late 1950s and early 1960s, the French molecular biologists François Jacob and Jacques Monod became the first to explain enzyme induction, in the context of the lac operon of Escherichia coli. In the absence of lactose, the constitutively expressed lac repressor protein binds to the operator region of the DNA and prevents the transcription of the operon genes. When present, lactose binds to the lac repressor, causing it to separate from the DNA and thereby enabling transcription to occur. Nitrate reductase and invertase are induced by their corresponding substrates.

Materials :
Green gram seeds 50 soaked
Surose solution 5% in water 5g in 100ml
Diastrix reagent strips
Beakers 2 of 25ml

Method :
1. Pour 10ml 0f sucrose solution in a 25ml beaker and add 25 soaked seeds of green gram
2. In another beaker pour 10ml water and add 25 soaked seeds of green gram
3. Incubate both the beakers at room temperature.
4. After one hour use the reagent strips and test for the amount of glucose present in the beakers
5. Note the intensity of the color and match with the color on the reagent strip bottle and quantify the amount of glucose present in both the beakers.
6. Record the color after each hour.

Time in hours	Sucrose beaker		Water beaker	
	Color observed	Glucose present in mg	Color observed	Glucose present in mg
0				
1				
2				
3				

Extraction of DNA

Introduction:
Nucleic acid is macromolecule composed of chains of monomeric nucleotides. The most common nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Nucleic acids were first discovered by Friedrich Miescher.

The term “nucleic acid” is the generic name from a family of biopolymer, named for their role in the cell nucleus. The monomers from which nucleic acids are constructed are called nucleotides. Each nucleotide consists of three components: a nitrogenous heterocyclic base, which is either a purine or a pyimidine; a pentose sugar; and a phosphate group. Nucleic acid types differ in the structure of the sugar in their nucleotides. DNA contains 2-deoxyriboses while RNA contains ribose (where the only difference is the presence of a oxygen). Also, the nitrogenous bases found in the two nucleic acid types are different: adenine cytosine, and guanine are found in both RNA and DNA, while thymine only occurs in DNA and uracil only occurs in RNA.

Nucleic acids are usually either single-stranded or double-stranded. A double-stranded nucleic acid consists of two single- stranded nucleic acids held together by hydrogen bonds, such as in the DNA double helix. In contrast, RNA is usually single-stranded, but any given strand may fold back upon itself to form secondary structure as in tRNA and rRNA. Within cell DNA is usually double-stranded, though some viruses have single-stranded DNA as their genome. Retroviruses have single-stranded RNA as their genome.

The sugars and phosphates in nucleic acids are connected to each other in an alternating chain, linked by shared oxygens, forming a phosphodiester bond. In conventional nomenclature, the carbons to which the phosphate groups attach are the 3’ end and the 5’ end carbons of the sugar. This gives nucleic acids polarity. The bases are joined through N-1 of pyrimidines and N-9 of purines to 1’ carbon of ribose through N-B glycosyl bond.

During extraction of biomolecule one should be aware of some facts. For any extraction processes the source is very important. One should choose source where the quantity of the biomolecule to be extracted should be present in abundance. Secondly one should be keen during the extraction process that the molecule should not be cleaved by hydrolysis or by any other processes, so one should use the extraction agents in such a way they do not hydrolysis the nucleic acids. Since nucleic acids are made three components one should see that all the three components are in tact.

Materials:

Fresh Onions
Test tube
Blender
Beaker
Ethanol 95% ice cold
SDS

Papain
Detergent salt solution is prepared by taking 20ml of 10% SDS and 20g of Nacl and 180ml of distil water.
Protease solution : 5 grams of papain in 95ml distilled water.

Method :

1. Take and onion and cut it along the four sides so as to prepare a square retaining the centre core of the onion.
2. Now chop it into very small pieces.
3. Transfer the contents into a mortar and pestle or blender and grind into a very fine paste.
4. Now add 80ml of the above detergent solution to the onion paste and mix gently.
5. Filter the above solution by using a cheese cloth.
6. Transfer the filtrate into a beaker and add 25ml protease solution. Mix it and keep it a side for 10 minutes so that the enzyme acts on proteins present in the onion extract.
7. Now measure 6ml of this solution with the help of measuring cylinder and transfer into a boiling tube.
8. Now add ice cool ethanol to the solution slowly by keeping the pipette in slanting position.
9. Keep the boiling tube aside and don't disturb as the bubbles are coming out.
10. Now carefully watch at the junction of water and ethanol. One can clearly see, DNA threads floating in ethanol.
11. Now transfer the small quantity of DNA extract from boiling tube into a test tube. Perform the conformation test.

Test of DNA : Nucelic acids are characterized on the basis of the three components present nucleotide. Nucleotide is made up of a nitrogen base, a pyramidine or purine base. A sugar molecule (deoxy ribose) and a phosphate molecule.

Method : Take small quantity of DNA Extracted from onion in a test tube add twice the quantity of diphenylamine. Keep the solution for 10min in a water bath at 100^{o}c note the change in color

Observation :

Separation of nucleotides by column chromatography:

Introduction : In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column and the mobile phase, a liquid, is added to the top and flows down through the column (by either gravity or external pressure). Column chromatography is generally used as a purification technique, it isolates desired compounds from a mixture.

The mixture to be analyzed by column chromatrography is applied to the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of air pressure. An equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutents, are collected and identified. Nucleotides can be separated from each other with ion exchange chromatography. In the present experiment the stationary phase (ion exchanger is packed into a glass column and the mobile phase (water) sample is applied on the ion exchanger is elutant and distributes between the two phases.

Stationary phase: Amberlite IR 120 resin is used an ion exchanger which is water insoluble polymer to which charged groups like coo- and NH3+ have been covalently linked eg: styre- SO3 cation exchanging. Cellulose an anion exchange. Hydrophobic polystyrene is used for separation of small molecules, while hydrophilic cellulose and agarose are used for nucleic acids and proteins.

Mobile phase. eluent is generally a buffer, whose composition is either kept same or variable with regard to pH and ionic strength during development of the column.

As attraction between two opposite charges forms the main basis for separation, the method can be used for separation of only charged molecules. The ion exchanger is placed in a glass column, equilibrated with the eluant and the mixture to be resolved is filtered through the column. Oppositely charged molecules are bound to the polymer, thus separating from others.

Neutral and like – charged molecules do not bind and are eluted quickly without the contaminating oppositely charged molecules. Like-charged ions are eluted sequentially from the column. Change of solvent composition helps in better resolution. A simple ion exchange column chromatographic analysis of RNA nucleotides follows.

Material :

1. Column: glass tubing 0.7 – 1 cm diameter, 8 cm long with a flow regulator at the bottom. Discarded or broken burettes can be salvaged for this purpose.
2. Stand to hold column
3. Fraction collector: Test tubes on a stand. Positions should be serially numbered.

4. Sample to be analysed: RNA hydrolysate is prepared by taking 50mg of RNA which is hydrolysed by alkali NaoH 0.2M. Incubate the RNA in alkali for 20hours and than adjust the PH to neutral by using HCL

Eluant : distil water

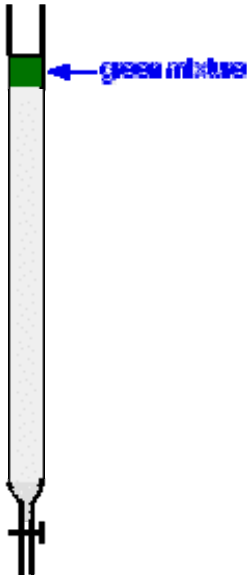
Ion exchange resin: Dowex -50/Amberlite IR120 / Zeocarb 225/ Biorad AG50 (strongly acidic, cation exchanging). Wash 2-3 times with water before use .

Visualizing agent : 2', 7'-Dichlorofluorescein 0.2% w/v in methanol. Store in amberlite bottle. Ultraviolet viewing chamber.

Method :

Filling the column :

1. Plug the bottom of the tube by pushing glass wool with a rod.
2. Make a slurry of the with water and pour it into the tube.
3. Allow it to settle. Drain it upto the surface. Fill the bed to a height of 5 cm
4. Wash the bed with 5ml M HCL and then with distilled water to neutrality.

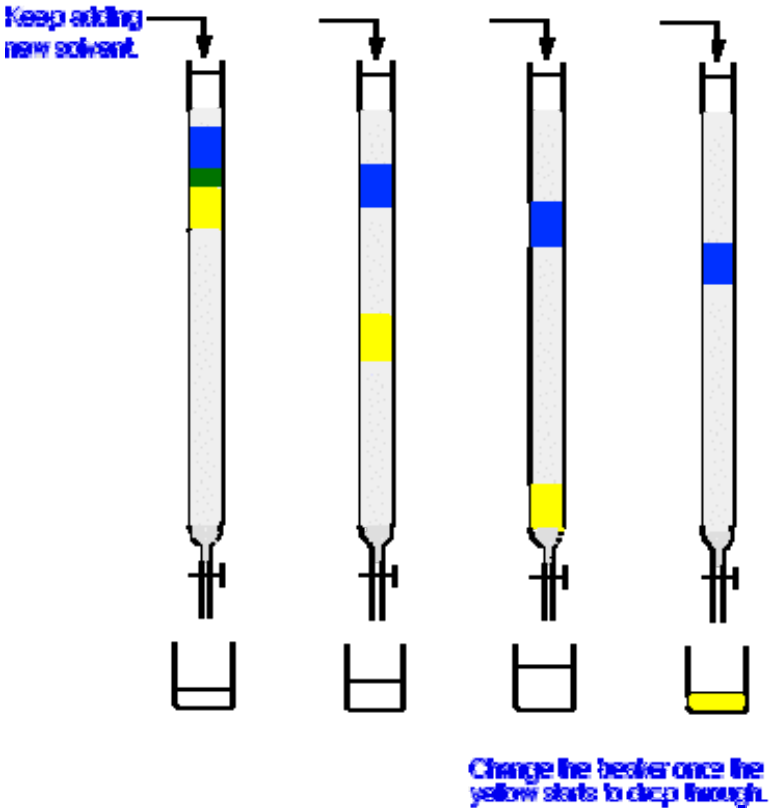


Applying the sample

1. Allow the water to run down until the fluid surface touches the top of the settled resin bed. Close the regulator
2. Apply the sample about 0.2ml carefully without disturbing its surface.
3. Allow the sample to run into bed just to expose the surface
4. Never allow the bed to dry throughout the experiment.

Elution

1. Carefully add eluant to the column continuously.
2. keeping a flow rate of 0.5ml/ min, collect the eluted fractions in test tubes @ 10 drops each.



Identification of fractions :

Fractions into which nucleotides are eluted can be identified by the sugar, phosphate and nitrogenous base present in the nucleotides. Take a whattman No 2 filter paper and draw a straight line on a paper and mark 1-24 circles on it serially 1 cm apart. Apply a streak of dichlorofluorescein solution over the entire line. Spot the effluent fractions with capillary on the corresponding numbers. Wash the capillaries after application to avoid contamination of the adjacent fraction. View the strip in ultraviolet light. UV absorbing spots indicate presence of nucleotides

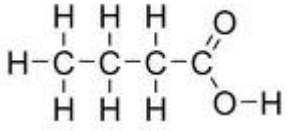
Results :

Interpretation :

Atomic model of fatty acid

Fatty acid consist of the elements carbon © , Hydrogen (H) and oxygen (O) arranged as a carbon chain skeleton with a carboxyl group (COOH) at one end. Saturated fatty acids (SFAs) have all the hydrogen that the carbon atoms can hold, and therefore, have no double bonds between the carbons. Mono unsaturated fatty acids (MUFs) have only one double bond, polyunsaturated fatty acids (PUFs) have more than one double bond.

Butyric acid (butanoic acid) is one of the saturated short-chain fatty acids responsible for the characteristic flavor of butter.



The number present at the beginning of the scientific names indicate the location of the double bonds. By convention, the carbon of the carboxyl group is carbon number one. Number of carbons present in a fatty acid are mentioned by symbol eg 18:0 is used to emphasie stearic acid. Which contain 18 carbons and zero double bonds. “9,12-octadecadienoic acid” indicates that there is an 18-carbons chain (octa deca) with two double bonds (di en) located at carbons 9 and 12, with carbon 1 constituting carboxyl group (oic acid). The structural formula corresponds to:

CH_3 CH_2CH_2 CH_2CH_2 $CH{=}CHCH_2{=}CH_2CH_2CH_2CH_2CH_2CH_2COOH$

9,12- octadecadieonic adid (Linoleic Acid)

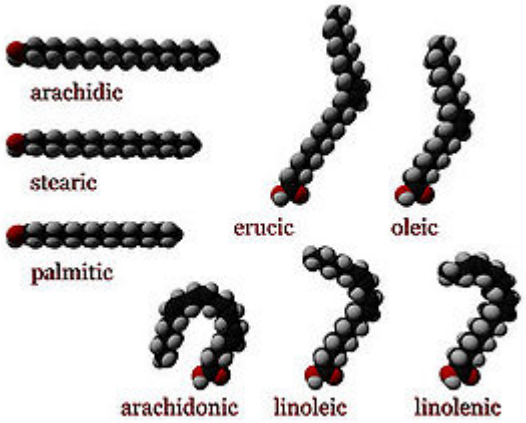
which would be abbreviated as:

$CH_3(CH2)4$ $CH{=}CHCH_2CH{=}(CH_2)_7COOH$

Fatty acids are frequently represented by a notation such as C18:2 that indicates that the fatty acid consists of an 18-carbons chain and 2 bonds.

Fatty Acid Configurations: Double bonds bind carbon atoms tightly and prevent rotation of the carbon atoms along the bond axis. When ever a double bond is introduced into a fatty acid there is a bend in the structure of the corresponding fatty acid. Double bonds present in the fatty acid are either arranged in cis configuration or trans configuration.

Atomic models of fatty acids



Cis / trans arrangement

Let us build the model of fattyacid

Prepare a saturated fatty acid model and introduce the formation of cis double bonds and notice the change is structure.

Trivial name	Systemic name	Structure	Abbreviation
Pamitic acid			
Stearic acid			
Oleic acid			
Lionoleic acid			
Linolenic acid			

Characterization of lipids by thin layer chromatography

Lipids which can be characterized by physical methods like melting point. For this large quantity of the sample is used and it gives us the picture of over all lipids present in the samples. But chromatography has largely replaced these classical methods.

Principle : Naturally occurring lipids have wide range or polarity. Neutral lipids and their hydrolytic products can be separated into classes according to polarity by adsorption on silica gel. Saturated hydrocarbons are absorbed only slightly, hence they migrate very fast. On the other hand unsaturated fatty acids are absorbed to a high extent and the degree of adsorption increases. Hence they migrate slowly.

Thin layer chromatography works on the basic phenomena of chromatography which requires a support system on which the sample is loaded and the solute the solvent and visualizing agent. This experiment is divided into four phases.

1.Preparation of the plate
2.Applying the sample
3.Development
4.Detection and calculation

Material:
Glass plate 10x20cm
Silica gel G
Layer applicator
Oven at 100^{o}C with see through glass window
Hot air blower
Development chamber
Solvent mixture: Petroleum ether: diethyl ether: acetic acid in the ratio of 70:30:2
Visualizing agent: 2'7'-dichlorofluorescein 0.2% w/v in methanol

Standards: The standards if they are solids, they have to be dissolved in their respective solvents ie

1. Hydrocarbons eg: Grease/ Vaseline (petroleum ether)
2. Triacyl glycerol eg : Triolein
3. Fatty acid eg: Plamitic acid (carbon tetra chloride of chloroform)
4. sterols: Cholesterol (acetone)
5. Sample

Method:

1. **Preparation of the plate:** weigh around 10g of silica gel and add 20ml of distil water to it in a beaker and stir once it has become uniform apply on a thin glass plate uniformly with the help of glass rod role over the plate for uniform thin layer of silica gel on the plate and allow it to air dry. Just before using the plate, activate the plate by keeping at 100 degrees for 10 min.
2. **Applying the sample or spotting:** Use a transparent applicator provided along the instrument. Leave one and half cm from the

bottom of the base of the plate, and one cm on either side of the plate, use a needle to mark them. Now measure the distance present between the two spots and divide it into four equal parts and mark them with the needle. Over the five points apply the sample with the help of capillary tubes as a small circles and even the four standards and make a note of the order of the standards applied,

3. **Developing:** Pour the solvent to a height of about 2cm in the development chamber and cover it. After some time the vapors will saturate the chamber. Now insert the plate with the spotted side downwards. Allow the solvent to rise up to 85% of the plate. Remove the plate and draw the solvent front movement with the help of needle and dry the plate.
4. **Detection:** spray the visualizing agent 2'7'dichlorofluorescein observe in U.V light and encircle the spots and calculate the R.F value for each spot and then identify the sample
5. **calculation:**

R.F = Distance traveled by solute
Distance traveled by the solvent

Name of the standard/Sample	Distance from the origin to the spot				Ratio of fronts
	Upper edge of solute	Lower edge of solute	Average value of solute	Solvent front	

Result

ESTIMATION OF OIL IN OILSEEDS

Fats are fatty acid esters of glycerol. Fat as liquid is called oil. Seeds like gingely, groundnut, castor, sunflower, coconut sesame etc. contain oil as reserve food material for the embryo.

Principle :

Oil from a known quantity of the seed is extracted with petroleum ether. Then the whole fraction is collected from distilled flask, ether and other components are evaporated completely and then the oil collected is weighed and the % oil is calculated.

Materials :

Petroleum ether (40^0-$60\ ^0$C)

Whatman No 2. Filter Paper

Absorbant cotton

Soxhlet Apparatus

Procedure :

1. Fold a piece of filter paper in such a way to hold the seed meal. Wrap around a second filter paper which is left open at the top like a thimble. A piece of cotton wool is placed at the top to evenly distribute the solvent as it drops on the sample during extraction.
2. Weigh 4-5g of ground sample into the thimble.
3. Place the sample packet in the butt tubes of the Soxhlet extraction apparatus.
4. Extract with petroleum ether (150 drops/min) for 6hours without interruption by gentle heating. (For castor beans use hexane)
5. Allow to cool and dismantle the extraction flask.
6. Transfer the contents of the extraction flask into a clean, dry, previously weighed 250ml beaker with repeated washings with petroleum ether.
7. Evaporate the ether on a hot sand bath until no odor of ether remains.
8. Dry the beaker in an oven at 105^0 C for 30 min to evaporate the traces of ether and moisture, if any.

9. Cool the beaker in a desiccator and weigh it with contents.

Calculation

W1 weight of the thimble
W2 weight of the sample + thimble
W3 weight of the beaker
W4 weight of the beaker + oil

$$\% \text{ Oil in the sample} = \frac{\text{Weight of oil (g)}}{\text{Weight of sample (g)}} \times 100$$

$$= \frac{w4 - w3}{w2 - w1} \times 100$$

Gas chromatography

Introduction

Gas chromatography is an instrumental method for the separation and identification of chemical compounds. A sample is introduced into a heated injector, carried through a separating column by an inert gas, and detected as a series of peaks on a recorder when components leave the column. The first commercial gas chromatographs appeared on the market in 1956. Since that time gas chromatography has undergone considerable development and there are at present about 50 firms engaged in the manufacture of gas chromatographs

It is a common type of chromatography used in analytic chemistry for separating and analysing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations; GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture

In gas chromatography, the *mobile phase* (or "moving phase") is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (a homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a *gas chromatograph* (or "aerograph", "gas separator").

The gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time, known as the *retention time* of the compound. The comparison of retention times is what gives GC its analytical usefulness.

The process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, (Hence the full name of the procedure is "Gas-liquid chromatography", referring to the mobile and stationary phases, respectively.). The column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography (typically) has no such temperature control. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas.

Gas chromatography is also sometimes known as vapor-phase chromatography (VPC), or gas-liquid partition chromatography (GLPC). These alternative names, as well as their respective abbreviations, are frequently found in scientific literature. Strictly speaking, GLPC is the most correct terminology, and is thus preferred by many authors.

CARRIER GAS SYSTEM

The carrier gas ensures the migration of the components of the sample to be separated. The nature of the carrier gas in principle is any gas which does not interfere either with the stationary phase or the components of the sample.

The earlier gases that the widely used in gas chromatography are,

i. Nitrogen-this is easily available ad handled. ii. Argon, iii. Helium, iv. Neon and v. carbon dioxide.

The constant value of flow rate of the carrier gas is of paramount importance in gas chromatographic analysis. The- simplest way to keep the flow rate at a constant value is to produce such a large pressure drop outside the column that compared to it the resistance change within the column is negligible. In recent years the method of programmed carrier gas flow has been evolved.

Sample injector :

The task of sample injector systems is to introduce the gaseous, liquid or solid sample rapidly and in a reproducible manner in to the column. The sample injector system should be in such a way that,

i. the sample shall reach the column like a plug in the shortest possible time

ii. there shall be no change in flow and thermal condition of the system during the injection of the sample and

iii. the quantity of the sample and manner of injection i.e., the process in time shall be reproducible.

Syringes : The syringes manufactured by Hamilton Ltd., are the most widely used, they are marketed with 1 to 500 Jll volume. Syringes of 1, 10 and 50 Jll volumes are the most common in gas chromatography with 1Jll syringe a sample of 0.01 ml can be directly measured. To ensure reproducibility the syringes may be fibled with a micrometer (Chaney) adaptor whicl;1 provides for the preliminary adjustment of sample quantity.

Columns

With respect to their dimensions and arrangement of two basic types of columns, the packed and the open tubular (capillary) column are rather different, though the latest type (packed capillary columns) represents a certain transition between the two.

Material of the column :

The first packed columns were made of glass and later of metal, such as aluminium, copper and stainless steel. For handling ad assembly the metal columns are more convenient, especially at higher temperatures and pressures. In the case of certain samples the aluminium and copper columns may be reactive or have a catalytic effect, so that the stainless steel colul1\!l can be considered to be the most satisfactory. For the analysis of high molecular weight, easily decomposed substances (Steriods, aminoacids etc.) glass columns are suggested.

For capillary columns aluminium, copper stainless steel, glass or plastics (polyamide) are used. At high temperatures hereagain stainless steel is the best material.

Dimensions:

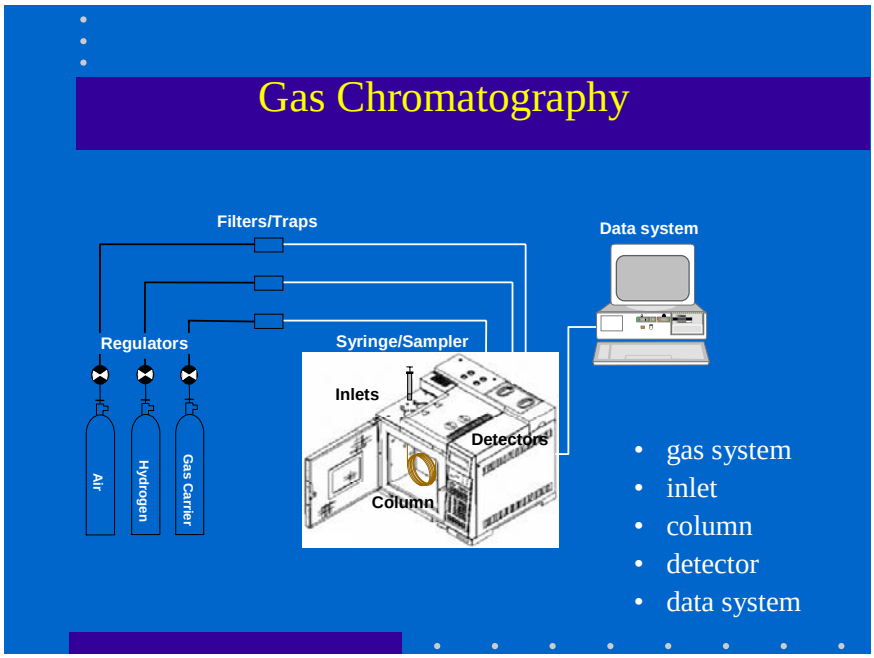
In the 1950's the internal diameter of packed columns was 6-8 mm. To

increase the efficiency the diameter of the later columns was 3-4mm, and to-day columns of 1-2mm internal diameter are also used. The length of packed columns is usually 1-6m. For most tasks 2-3 columns usually suffice, while in certain cases a column length of 10-15 m may be necessary, though the trend in recent analytical methods is to apply shorter columns and to improve separation by some other way.

Packed columns may be straight tubes, U, W, spiral or flat spiral shaped. The shape of the column should occupy only a small space and packing should be uniform and compact which is best realised with straight and U shaped columns.

Detectors :

Detectors have the task to sense continually, rapidly and with high sensitivity the components which appear in the carrier gas as it emerges from the column. The detector senses the changes in certain physical or chemical property of the effluent gas stream on the appearance of the components. With the help of an appropriate

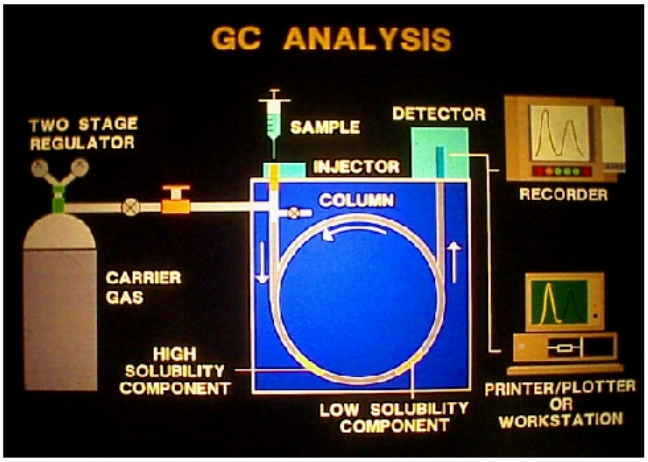


Procedure

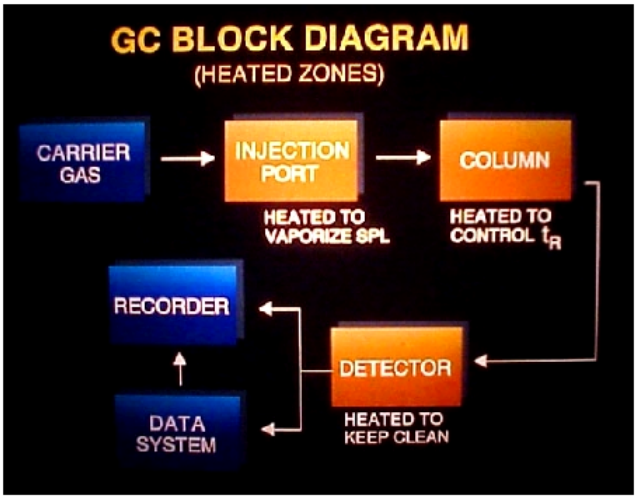
1. Add the sample to be injected to the syringe. Only 2-4 µL of sample is injected onto the column.
2. Inject the sample into the injector port. Push the needle of the syringe through the injection port and immediately press the plunger to inject the sample, then immediately press the start button on the recorder. You will feel a bit of resistance from the rubber septum in the injection port; this is to be expected and you should be prepared to apply some pressure to the syringe as you force the needle into the instrument all the way to the base of the needle.
3. Sit back and wait. Observe the recorder. Within several minutes, it should record several peaks.
4. End the GC run. When you have seen all of the peaks which you suspect are in the mixture, or when the recorder has shown a flat baseline for a few minutes or so, press stop on the recorder. When you

press stop, the recorder will print out the peaks, the retention times, and the areas under the peaks. When it is done printing, Carefully tear the paper off the recorder.

Slide 1



Slide 7



Advantages of gas chromatography

i. Gas chromatographs can be used for the solution of widely varying analytical tasks from the analysis of permanent gases to that of high boiling liquids or volatile solids.

ii. Preparation applications: For this pure substances or narrow fractions as standards or for further detailed investigations.

iii. Continuous monitoring and automatic process control.

iv. Study of structure of chemical compounds.

v. Study of the mechanism and kinetics of chemical reactions.

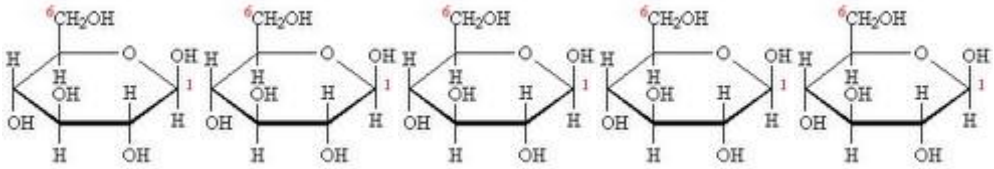
vi. Physico chemical measurements such as determination of isotherms, heat of solution etc.

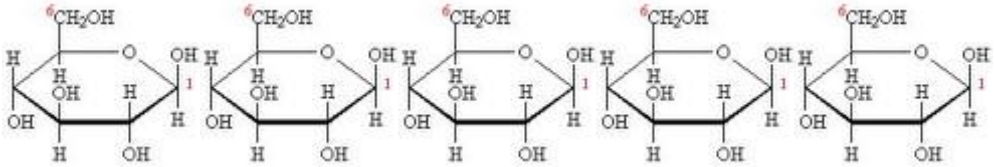
vii. Determination of specific surface of solids, plotting of distillation curves and elementary analysis of organic substances.

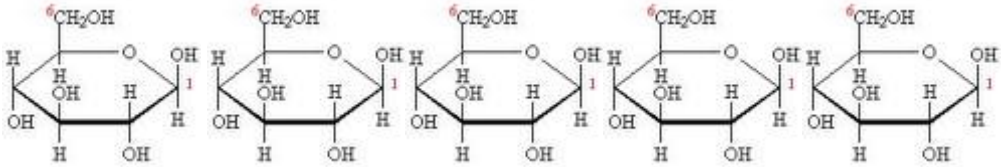
From the point of view of the construction and operation of the apparatus there is no difference between the two types of gas chromatography namely, gas-solid and gas-liquid chromatography. The difference between the two is in the method of separation partition between the two phases is based on adsorption in the first case and in second case on dissolution in the liquid.

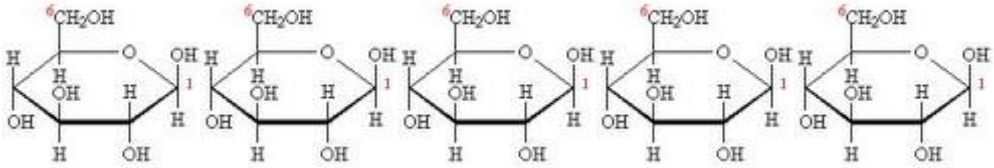
Atomic models of sugars

Paper model of starch









Separation of sugars and amino acids by Paper Chromatography

Chromatography is a powerful separation technique, which has revolutionized the scientific research. Difference in adsorption, electrical charge, solubility or size exploited for the separation. Paper chromatography is largely based on partition of the solute in two solvent, one immobile (water in cellulose) and another mobile. It is like the running competition based on various aspects one of the participate wins. In the present experiment it is based on solubility the movement of solutes varies.

Principle: It is the partition between the two solvent which will decide the distance to which the solute moves. More the solubility in mobile solvents, faster will be the movement of that solute and vice versa.

Material:

1. Support- What man Chromatography paper
2. Developing chamber- Tall beaker with watch glass
3. Sample- Any source of our interest
4. Standards

Amino Acids	Sugar
a. Phenylalanine	a. Glucose
b. Proline	b. Fructose
c. Alanine	c. Sucrose
d. Histidine	d, Ribose

5. Solvent mixture;
   n-Propanol: Ethyl Acetate: Water in the ratio 7:1:2 (For sugars)
   n-Butanol: Acetic acid: water in the ratio 12:3:5 (For amino acids)
6. Visualizing agents:
   Ninhydrin (for amino acid)
   Aniline diphenylamine phosphate (for sugar)

Method: Chromatography is divided into three steps.
1. Applying the sample
2. Development
3. Visualizing

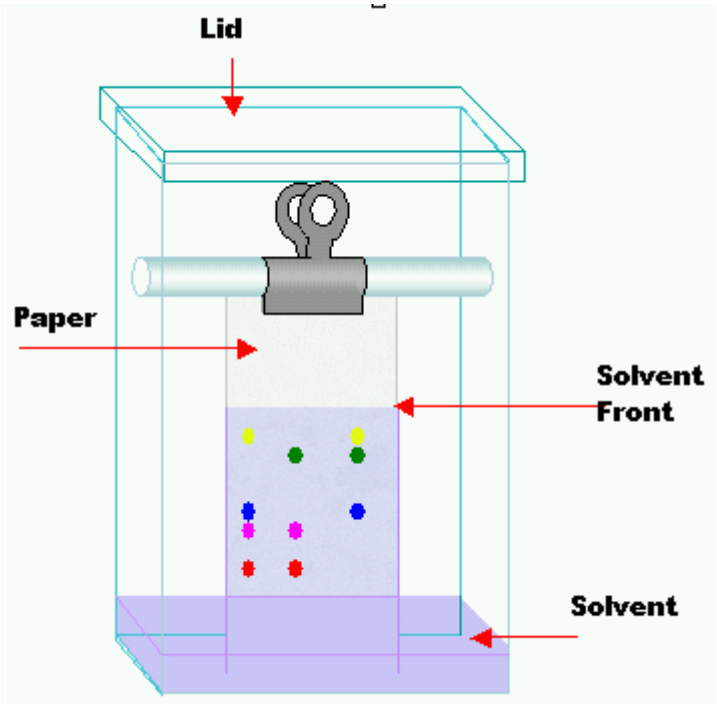
Applying the sample:

1.Take the whatman No1 chromatography paper of appropriate size
2. Place it on a rough paper and with the help of pencil and scale draw a line leaving $1^1/_2$ cm from the bottom.
3.Now on the line mark two spots leaving $1^1/_2$ cm on either side of the edges.
4. Now measure the distance between the spots and divide it into four equal parts and mark three more points, in total we will get five points.
5. Carefully draw five small circles touching the line, below the line under each circle write the name of the standard.
6. In the centre will be the sample. With the help of capillary tube apply standard and the sample give a feather touch and see that the solute do not spread below the line.

7. Now fold the paper in the form of a cylinder and staple at three different positions with the help of stapler. While stapling it, be careful and check that the two ends of the paper are equal and the spots are present outside the circle and there is a gap between the two edges.

Development:

1. Pour enough solvent mixture about 15ml in the beaker after shaking.
2. Now carefully place the cylinder in the beaker with the spots below.
3. Cover the beaker with the help of watch glass.
4. Due to the capillary action the solvent in the beaker rises.
5. While the solvent is moving above, along with the solvent the solute particles are also carried away.
6. When the solvent front reaches nearly to one cm to the top of the paper
7. Remove the paper, draw the solvent front with the pencil and dry the paper.



Visualizing:

On the chromatogram apply the visualizing agents mentioned above and dry the papers. The visualizing agents react with the solutes and the spots will be developed. Calculate the R.F values for each spot and identify the sample.

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

Calculations for Amino acids :

Name of the standard/Sample	Distance from the origin to the spot				Ratio of fronts
	Upper edge of solute	Lower edge of solute	Average value of solute	Solvent front	

Result :

Calculations for Sugars :

Name of the standard / Sample	Distance from the origin to the spot				Ratio of fronts
	Upper edge of solute	Lower edge of solute	Average value of solute	Solvent front	

Result :

Quantitative estimation of sugars after removing interfering substances

1. Removal of interfering substances:

For quantitative analysis of sugars from natural compounds, interfering substances like proteins are removed by complexing with heavy metals (lead acetate). Excess lead, which interact with cupric oxide of the assay, is removed by adding di sodium hydrogen phosphate (deleading agent). Two major glycosidic bond hydrolyzing agents are removed during this process.

a. Acids: generally fruits are acidic in nature. This acidity is removed by adjusting the PH to the neutral state by using dilute NaoH.
b. Enzymes: since enzymes are proteins. They are removed by adding lead acetate.

Material

Fruit juice 25ml or 25gms of pulp
Lead acetate 45% w/v in water
Di sodium phosphate solution saturated.
Red litmus paper
Sodium hydroxide solution 1M.

Method:

1.Take the weight of the fruit.
2. Remove the epicarp then take the weight of the eatable portion of the fruit.
3. Now extract the juice and measure the total volume of the juice.
4. Take 25ml if it is plain juice if not weight the pulp and than take 25gms of the pulp.
5. Make up the volume to 100ml mix well.
6. Check the pH and neutralized it with the help of NaOH.
7. Now add 2ml of lead acetate solution mix well
8. After 5 min centrifuge the juice and transfer the supernatant into a beaker.
9. Add 1 ml of saturated solution of di sodium hydrogen phosphate to remove excess lead.
10. Filter or centrifuge it and than transfer the supernatant into 250ml volumetric flask and make up the volume to 250ml with distil water. This is the clarified juice.

2. Estimation of amount of reducing sugars with Benedict's reagent.

For quantitative estimation of reducing sugar, every care has to be taken to prevent reoxidation of cuprous ions by the atmospheric oxygen. This is done with the help of a steam barrier, which is formed when the titration is carried on while Benedict's solution is boiling. It can be further assured by generating carbon dioxide in the flask with the addition of sodium carbonate.

Principle: Benedict's quantitative reagent forms white cuprous thiocynate instead of red cuprous oxide. Discoloration is the end point.

Material:

1. Clarified juice
2. Benedict's quantitative reagent

3. Anhydrous sodium carbonate
4. Conical flask 50ml
5. Burette 25ml

Method:

1. Fill the burette with the clarified juice.
2. Now pipette out 5ml of benedict's quantitative reagent into conical flask
3. Add two spatulas of anhydrous sodium carbonate.
4. Add 15ml distil water to it and keep the conical flask for boiling.
5. When the solution is vigorously boiling titrate with clarified juice.
6. The end point is indicated by means of discoloration of the benedict's solution.

Calculations:

Weight of the fruit
Weight of the pulp / volume of the juice
Percentage of pulp / juice
Volume of the juice taken
Volume of the clarified juice
Titer value
Result

$$\frac{10 \times 250}{X} \times \frac{\text{Total volume of the juice}}{25} \times \frac{100}{\text{weight of the fruit}} \times \frac{1}{1000}$$

When clarified juice is given directly use this formula for calculation
5ml of Benedict's quantitative reagent can reduced 10mg of glucose
So 5ml of Benedict's quantitative reagent = 10mg glucose
X ml reduced 5ml of Benedict's quantitative reagent
So x ml = 10mg of glucose (where x is the titer value)
100ml =?

$$\frac{100 \times 10\text{mg}}{X} = y\text{ mg} = \frac{y\text{ mg}}{1000} = \text{in grams or \%}$$

Estimation of Total Phenols

Phenols, the aromatic compounds with hydroxyl groups are widespread in plant kingdom. They occur in all parts of the plants. Phenols are said to offer resistance to diseases and pests in plants. Grains containing high amount of polyphenols are resistant to bird attack. Phenols include an array of compounds like tannins, flavonols etc. Total phenol estimation can be carried out with the Folin-Ciocalteau reagent.

Principle : Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Materials :

80% Ethonal
Folin-ciocalteau reagent
Na_2Co_3 20%
Standard (50mg Catechol in 100mL water)
Dilute 10 times for preparing working standard solution.

Procedure :

1. Weigh exactly 0.5 g of the sample and grind it in a mortar and pestle. Than add 5 ml of 80%ethanol, mix it and transfer into a centrifuge tube.

2. Centrifuge the homogenate at 10,000rpm for 20min.Save the supernatant. Re-extract the residue with 3 ml 80% ethanol, centrifuge and pool the supernatants.

3. Evaporate the supernatant to dryness.

4. Dissolve the residue in 5 ml of distilled water.

5. Prepare the stock solution using catechol, whose concenteration is 0.5mg / ml. ie 50mg in 100ml of distil water

6. Pipette 10ml of the above solution and make up the volume to 100ml in a volumetric flask.

7. Pipette out different aliquots (0 .1, 0.2, 0.3, 0.4, 0.5.ml) and blank into test tubes.

8. Make up the volume in each tube to 3mL with water.

9. Simultaneously pipette out .1 ml and .2ml of the sample and make up the volume to 3ml with distil water

10. Add 0.5mL of Folin Ciocalteau reagent. And wait for 3 minutes.

11. After 3minutes, add 2mL of 20%Na2co3 solution to each tube.

12. Mix thoroughly. Place the tubes in a boiling water for exactly one min, cool and measure the absorbance at 650nm against a reagent blank.

13. Prepare a standard curve using different concentrations of catechol. And calculate total phenol concentration

Calculation

From the standard curve find out the concentration of phenols in the test sample and express as mg phenols/ 100g material.

Stock solution concentration is 0.5mg/ml
Working standard concentration is 10ml ----------→ 100ml
10 times dilution 1/10 = .05mg/ml

S.N	Ml of catechol	Ml of water	Concentration of catechol in mg	Folin Reagent ml	20% Na2Co3 ml	O.D at 650 nm
1.	0	3	0 x 0.05 =0	0.5	2	
2.	0.1	2.9	0.1 x 0.05 = 0.005	0.5	2	
3.	0.2	2.8	0.2 x 0.05 = 0.010	0.5	2	
4.	0.3	2.7	0.3 x 0.05 = 0.015	0.5	2	
5.	0.4	2.6	0.4 x 0.05 = 0.020	0.5	2	
6.	0.5	2.5	0.5 x 0.05 = 0.025	0.5	2	
7.	Sample I			0.5	2	
8	Sample 2			0.5	2	

$$\frac{S1+S2/2}{2} = Y \text{ mg is present in } 0.1\text{ml.}$$

In 0.1ml ----------→ Y mg
In 5ml ----------→Y mg x 5 = Z
Z is present in 5ml and also in .5grams
In 0.5g ----------→ Z mg
In 100gms----------→ ?

$$\frac{100 \times Z\text{mg}}{0.5}$$