SOPs: BIOCHEMISTRY PRACTICAL EXERCISES FOR UNDERGRADUATE STUDENTS

(DBT Life Sciences Star College Scheme, Ministry of Science & Technology, Govt. of India)

Reaccredited with 'A' grade by NAAC

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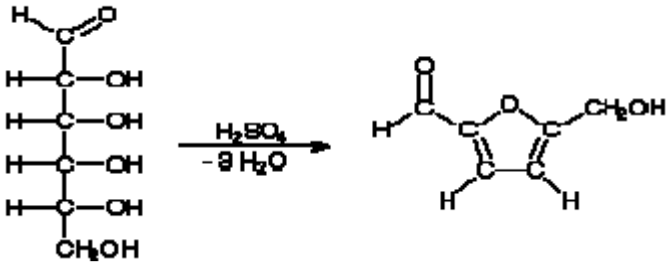
Experiment No.1 : Estimation of Sugar by Dubois Method

Background: Phenol Sulphuric Acid Method is the most easiest and reliable method amongst the quantitative assays for carbohydrate estimation. It is mostly used in measuring neutral sugar content in oligosaccharides, proteoglycans, glycoproteins and glycolipids.In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This forms a yellow-brown coloured product with phenol and has absorption maximum at 490 nm. This is one of the best methods to estimate total carbohydrate.



Material and Reagent requirements: H_2SO_4 (95.5%)**,** Phenol (5%)**,** Standard stock glucose solution: 100mg in 100ml distilled water, Working standard: Dilute 10ml of stock solution to 100ml with distilled water. Weighing balance, spectrophotometer, test tubes, beakers, micropipette and hot plate.

Methodology:

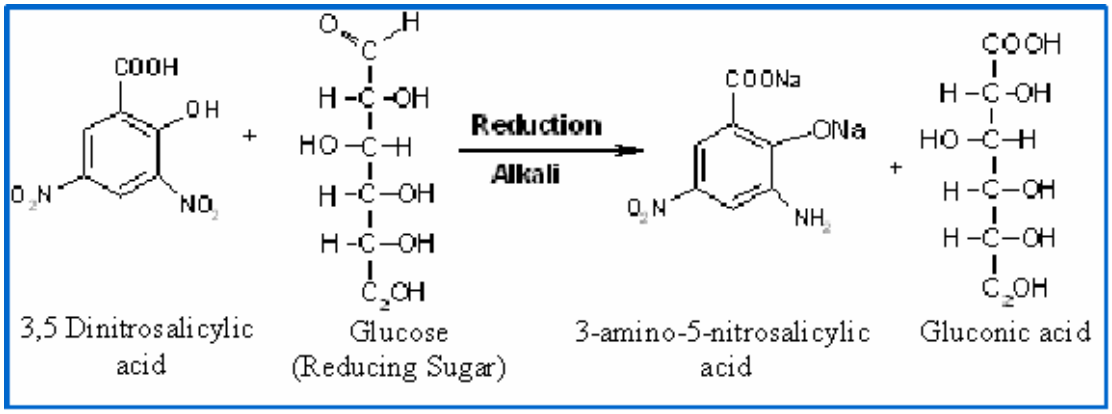
1. 0.2 – 1.0 ml of standard glucose solution was taken in different test tubes and volume was made up to 1 ml with distilled water.
2. To each test tube 1 ml of 5 per cent phenol was added.
3. The test tubes were placed in ice cold water and 5 ml of 95.5 per cent H_2SO_4 was added swiftly. The contents were mixed well and boil in water bath for 5 minutes.
4. Cool the test tubes.
5. Absorbance of the color developed was read at 490 nm. The standard curve was plotted using standard glucose concentrations versus absorbance at 490 nm recorded on spectrophotometer.

Precautions:

1. Always wear lab coat and gloves when you are in the lab.
2. Care should be taken while handling reagents.
3. After completing the experiment, clean the glassware and wipe the lab bench.
4. Reading should be taken accurately.

Experiment No. 2 : Determination of Reducing Sugars by DNS Method

Background: A reducing sugar is one that in a basic solution forms an aldehyde or ketone. Several reagents have been employed which assay sugars by using their reducing properties. This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions. The formation of 3-amino-5-nitrosalicylic acid results in a change in the amount of light absorbed, at wavelength 510 nm. The absorbance measured using a spectrophotometer is directly proportional to the amount of reducing sugar.



Material and reagent requirements:

1. Dinitrosalicylic Acid Reagent (DNS Reagent): Dissolve by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 mL 1% NaOH. Store at 4°C. Since the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite may be added at the time of use.
2. 40% Rochelle salt solution (Potassium sodium tartrate).
3. Standard stock glucose solution: 100mg in 100ml distilled water.
4. Working standard: Dilute 10ml of stock solution to 100ml with distilled water.

5. Weighing balance, spectrophotometer, test tubes, beakers, micropipette and hot plate.

Methodology:

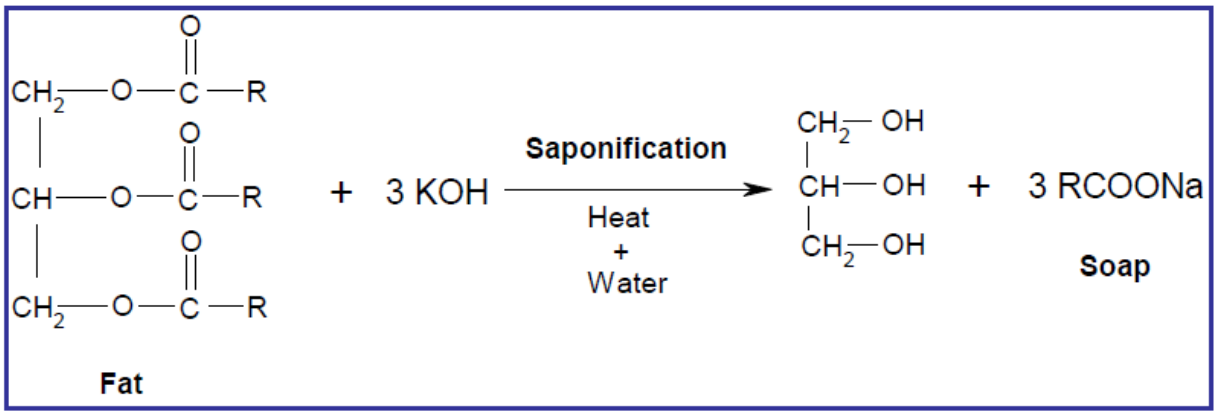
1. Pipette out 0.5 to 3 mL of the glucose in test tubes and equalize the volume to 3 mL with distilled water in all the tubes.
2. 3 mL of DNS reagent add in test tubes.
3. The contents were heated in a boiling water bath for 5 min.
4. When the contents of the tubes were still warm, add 1 mL of 40% Rochelle salt solution.
5. The test tubes were cooled to room temperature.
6. Switched on the spectrophotometer and selected the wavelength of 510 nm. First took the absorbance (OD) of Blank and made it zero.
7. Run a series of standards using glucose and plot a graph.

Precautions:

1. Always wear lab coat and gloves when you are in the lab.
2. Care should be taken while handling reagents.
3. After completing the experiment, clean the glassware and wipe the lab bench.

Experiment No.3 : Estimation of saponification value of fat

Background: On refluxing with alkali, triacylglycerols (fatty acid esters) are hydrolyzed to give glycerol and potassium salts of fatty acids (soap). Such process is known as, *Saponification* . The saponification value is the number of milligrams of KOH required to neutralize the fatty acids resulting from the complete hydrolysis of 1g of fat.



The saponification value gives an indication of the nature of the fatty acids constituent of fat and thus, depends on the the average molecular weight of the fatty acids constituent of fat. The greater the molecular weight (the longer the carbon chain), the smaller the number of fatty acids is liberated per gram of fat hydrolyzed and therefore, the smaller the saponification number and vice versa.

Material and reagent requirements:

1 Fats and oils (olive oil, coconut oil, sesame oil, and butter)

2 Fat solvent (equal volumes of 95% ethanol and ether)

3 Alcholic KOH (0.5 mol/liter)

4 Reflux condenser.

5 Boiling water bath.

6 Phenolphethalein.

7 Hydrochloric acid (0.5 mol/liter)

8 Burettes (10 ml and 25 ml)

Methodology :

1 Accurately weighed 1g of fat in a small beaker and dissolved it in about 3ml of the fat solvent.

2 Quantitatively transfered the contents of the beaker to a 250 ml conical flask by rinsing the beaker three times with a further milliliters of solvent.

3 Add 25ml of alcoholic KOH and attached to a reflux condenser .

4 Set another reflux condenser as blank with everything present except the fat.

5 Heat both flasks on a boiling water bath for 30 min.

6 Leave to cool to room temperature and titrate with 0.5 mol/liter HCl and use phenolphthalein as indicator. Until the pink color disappeared.

7 Recorded your readings as T ml for test and B ml for blank.

Calculations:

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

To calculate the saponification value:

1ml (0.5 N HCl) = 28.05 mg KOH

saponification value (S) = $\frac{(B-T) \times 28.05}{}$ = mg KOH/1g Wt. of fat (1g)

Precautions:

1. Always wear lab coat and gloves when you are in the lab.
2. Care should be taken while handling reagents.
3. After completing the experiment, clean the glassware and wipe the lab bench.
4. Reading should be taken accurately.

Experiment No. 4 : Determination of Total Carbohydrate by Anthrone Method

Background:

The anthrone reaction is the basis of a rapid and convenient method for the determination of hexoses, aldopentoses and hexuronic acid either free or present in polysaccharides. Carbohydrates are dehydrated by conc. sulphuric acid to form furfural. Furfural condenses with anthrone to form green coloured product with an absorption maximum at 630 nm.

Material and reagent requirements:

1. Anthrone reagent: Dissolve 200 mg anthrone in 100 mL of ice-cold 95% H_2SO_4. Prepare fresh before use.
2. Standard glucose: Stock—Dissolve 100 mg in 100 mL water. Working standard—10 mL of stock diluted to 100 mL with distilled water.
3. Weighing balance, spectrophotometer, test tubes, beakers, micropipette and hot plate.

Methodology:

1. Prepared the standards by taking 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL of the working standard. '0' serves as blank.
2. Made up the volume to 1 mL in all the tubes including the sample tubes by adding distilled water.
3. Then add 4 mL of anthrone reagent.
4. Heat for eight minutes in a boiling water bath.
5. Cooled rapidly and read the green to dark green colour at 630 nm.
6. Drawn a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.

Precautions:

1. Always wear lab coat and gloves when you are in the lab.
2. Care should be taken while handling reagents.
3. After completing the experiment, clean the glassware and wipe the lab bench.

Experiment No.5 : Protein estimation by bradford method

Background:

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Material and Reagent requirements:

1. Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman 1 paper just before use.
2. 1 M NaOH (to be used if samples are not readily soluble in the color reagent).
3. Weighing balance, spectrophotometer, test tubes, beakers, micropipette.

Methodology:

1. Prepared the standards by taking 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL of the working standard. ‘0’ serves as blank.
2. Made up the volume to 1 mL in all the tubes including the sample tubes by adding distilled water.
3. 0.1 ml aliquot of the each sample was taken in the test tube and made up the volume of 1 ml with distilled water.
4. If desirred, add an equal volume of 1 M NaOH to each sample and vortex.
5. Warm up the spectrophotometer before use.
6. Add 5 ml dye reagent and incubate 5 min.
7. Measured the absorbance at 595 nm.

Calculations: Prepare a standard curve of absorbance versus micrograms protein and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

Experiment No. 6 : Protein estimation by Lowry's method.

Background:

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin- Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5.

Material and reagent requirements :

1. Reagent A: 2% Sodium carbonate in 0.1N sodium hydroxide
2. Reagent B: 0.5% Copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartrate
3. Reagent C: Alkaline copper solution: Mix 50 ml of A and 1ml of B prior to use
4. 1 N Folin-Ciocalteau Reagent
5. Standard bovine serum albumin solution was used as the standard for total soluble protein estimations. 10 mg of bovine serum albumin was dissolved in 40 ml distilled water.
6. Weighing balance, spectrophotometer, test tubes, beakers, micropipette and hot plate.

Methodology:

1. Prepared the standards by taking 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL of the working standard. ‘0’ serves as blank.
2. Made up the volume to 1 mL in all the tubes including the sample tubes by adding distilled water.
3. 0.1 ml aliquot of the each sample was taken in the test tube and made up the volume of 1 ml with distilled water.
4. Then add 5 ml of reagent C was added in each tube and mixed immediately.

5. 0.5 ml of 1N Folin's reagent was added after 10 min and the test tube was shaken vigorously to ensure proper mixing.
6. After 30 min, the intensity of color was measured at 660 nm against a reagent blank.
7. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.

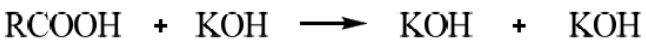
Precautions:

1. Always wear lab coat and gloves when you are in the lab.
2. Care should be taken while handling reagents.
3. After completing the experiment, clean the glassware and wipe the lab bench.
4. Incubate the test tubes should be in dark condition.

Experiment No. 7 : Determination of acid value of a fat.

Background:

The acid value (*AV) is* the number that expresses, in milligrams the quantity of potassium hydroxide required to neutralize the free acids present in 1 g of the substance. The acid value may be overestimated if other acid components are present in the system, *e.g.* amino acids or acid phosphates. The acid value is often a good measure of the break down of the triacylglycrols into free fatty acids, which has an adverse effect on the quality of many lipids.



Material and reagent requirements:

1. Fat or Oil
2. Absolute ethanol alcohol
3. Phenolphthalein
4. 0.1 N KOH

Methodology:

1. Placed 5.0 g of fat or oil in a dried conical flask.
2. Add 25 ml of absolute ethanol alcohol and add (2-3) drops of phenolphthalein
3. Heat with shaking in water bath (65%) for 10 minutes ,then cool it.
4. Titrate the solution against 0.1 N KOH until pink color appears (end point).
5. Record your observations.
6. Calculate the acid value (AV).

Calculations: ml of KOH x N x 56

AV= ---------- = mg of KOH

Weight of Sample where N = Normality of KOH

Precautions:

1. Always wear lab coat and gloves when you are in the lab.
2. After completing the experiment, clean the glassware and wipe the lab bench.
3. Reading should be taken accurately.

Experiment No. 8 : The absorbance curve of two dyes.

Background:

The method described in this protocol to determine the maximum absorbance of two different dyes. Colored solutions are colored because they absorb certain wavelengths of light while allowing other wavelengths of light to pass through. By measuring the amount of light absorbed, we can find the concentration of solutions. The process involves recording the absorbance over the range of 350 nm to 650 nm, usually in intervals of 25 nm. The data can be graphed to visualize the highest absorbance.

Material and Reagent requirements:

Bromophenol blue, methyl red, distilled water, beakers, rod, weighing balance, cuvet and spectrophotometer.

Methodology:

1. Prepared 0.15 M solution of bromophenol blue and methyl red and dissolved it properly.
2. Hold cuvet by the rough sides and fill it with distilled water to be used as a blank.
3. Rinsed the cuvet with a small amount of distilled water and then fill with the colored solution of bromophenol blue and methyl red, respectively.
4. Then observed the absorbance of both solution from wavelength 375 nm to 650 nm.
5. The wavelength of maximum absorbance of both dyes can be determined by creating a graph of the data - placing wavelength on the x-axis and absorption on the y-axis.

Precautions:

1. Always wear goggles and an apron in the lab.
2. Gloves may be preferred to keep coloring from staining hands.

Experiment No. 9: Casting of vertical gels for electrophoresis.

Background:

Generally the sample is run in a support matrix such as paper, cellulose acetate, starch gel, agarose or polyacrylamide gel. Which provide a means of separating molecules by size, in that they are porous gels. A porous gel may act as a sieve by retarding, or in some cases completely obstructing, the movement of large macromolecules while allowing smaller molecules to migrate freely. Because dilute agarose gels are generally more rigid and easy to handle than polyacrylamide of the same concentration, agarose is used to separate larger macromolecules such as nucleic acids, large proteins and protein complexes. Polyacrylamide, which is easy to handle and to make at higher concentrations, is used to separate most proteins and small oligonucleotides that require a small gel pore size for retardation.

Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders).

Material and reagent requirements:

(1) Acrylamide stock solution

Acrylamide 30.0 g

Bisacrylamide 0.8 g

Dissolved in water and the final volume was made 100 ml. Filtered the solution through whatman no. 1 filter paper and stored in brown bottle at $4^{o}C$.

(2) Stacking gel buffer stock (Tris-HCl, pH 6.8)

Tris 0.45 g

0.5 M HCl 7.8 g

Adjusted pH to 6.8 and final volume was made to 100 ml with distilled water. Filtered through whatman no. 1 filter paper and stored at 4 ^{0}C.

(3) Resolving gel buffer stock (Tris-HCl, pH 8.8)

Tris 15.39 g

1.5 M HCl 3.69 g

Adjusted pH to 8.8 and the final volume was made 100 ml. It was filtered through whatman no. 1 filter paper and stored at 4 ^{o}C.

(4) Polymerising Agents

Ammonium persulphate (1%) 0.1 g/10ml, prepared freshly before use

TEMED Fresh from refrigerator

(**5**) Spacer, glass plates, gel casting unit, beakers, rods, measuring cylinder, weighing balance .

Methodology:

1. Assembled the glass plates and spacers in the casting frame and tested for water leaks.
2. The comb was placed into gel cassette for marking. The glass plate was marked 1 cm below comb teeth and this was the level up to which the resolving gel poured. The comb was then removed.
3. Resolving gel 12 % prepared (table 1) by combining all reagents except APS and TEMED. Degassed solution under vacuum for at least 15 minutes. The APS and TEMED were added to degassed solution. Gently swirl solution before pouring into gel mold and gel was allowed to polymerize for 45 minutes to 1 hr.
4. After polymerization, rinsed the gel surface completely with distilled water.

5. Five per cent stacking gel also prepared by combining all reagents given in table 1. Before pouring the stacking solution, water was allowed to drain from resolving gel by tilting the gel assembly.

6. Gently the comb was inserted between spacers after pouring and allowed it to polymerize for 30 minutes. After polymerization, comb was removed. The gel assembly was removed from casting frame after polymerization.

Table 1 Composition of gels for SDS-PAGE

Chemicals	Stacking gel for 5 per cent gel	Resolving gel for 12 per cent
Stock acrylamide solution	0.83 ml	8.3 ml
Tris-HCl (pH 8.8)	-	10 ml
Tris-HCl (pH 6.8)	0.63 ml	-
Water	3.4 ml	5.0 ml
Ammonium persulphate solution	0.05 ml	0.2 ml
10% SDS	0.05 ml	0.2 ml
TEMED	5 µl	8 µl

Precautions:

1. Acrylamide is a potent neurotoxin and should be handled with care.
2. Wear disposable gloves when handling solutions of acrylamide and a mask when weighing out powder.
3. Polyacrylamide is considered to be non-toxic, but polyacrylamide gels should also be handled with gloves due to the possible presence of free acrylamide.

Experiment No. 10: Separation of bio-molecules by vertical gel electrophoresis.

Background:

Proteins are amphoteric compounds, their net charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a net negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. The net charge carried by a protein is in addition independent of its size - ie: the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecule differs from protein to protein. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone. SDS confers a negative charge to the polypeptide in proportion to its length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size. This is done with 2- mercaptoethanol.

Material and reagent requirements: protein sample (egg oe gram seed), tris, glycine, SDS, coomassie brilliant blue R-250, methanol, acetic acid, bromophenol blue, mercaptoethanol, distilled water, sodium phosphate monobasic, sodium phosphate diabasic, polyvinyl pyrolidone, ready to use protein marker, glycerol, pestle mortar, beakers, test tubes, micropipette, tips, centrifuge, weighing balance, power supply, staining and destaining chamber and gel rocker.

Methodology:

1. **Protein Extraction**

   Extraction of seed protein was carried out with 0.1 M sodium phosphate buffer (pH 7.2). 0.5 g of sample was ground well with pestle and mortar in 10 ml of 0.1 M sodium phosphate buffer containing 0.5 ml of β- mercaptoethanol and 12 mg of PVP. The contents were transferred to centrifuge tube(s) and centrifuged at 18,000 rpm for 10 minutes. The supernatant was collected and used for protein profiling.

2. **Preparation of protein sample for SDS- PAGE**

   The sample was prepared for SDS- PAGE in a sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10 per cent SDS, 0.5 per cent bromophenol blue,

denoised water) and concentration of the protein in sample was adjusted to 1.0 µg / µl. The sample and buffer was taken in 1:1 ratio in eppendorf tube and boiled for 3-5 min(s).

3. Separation of protein sample through SDS-PAGE

1. First of all gel was prepared by using gel preparation method. The gel assembly was removed from casting frame after polymerization and inserted into the electrode assembly.
2. The running buffer was poured in the buffer tank. The 10 µl sample(s) was loaded into the wells with pipette using gel loading tips along with 10 µl protein marker.
3. The rest of the running buffer was poured into outer chamber of electrode assembly. The power was applied at 90 V for running gel.
4. When the dye front was near bottom of gel (about 1 cm from bottom), turns off the power supply and disconnects the electric leads.
5. Removed the gel sandwich and disassembled the glass plates.
6. The gel was placed in small tray containing water. Rinsed the gel for about 5 minutes with water.
7. Immersed the gel in the Coomassie dye staining solution until it is uniformly blue.
8. The gel was transferred into destaining solution. The successive changes of destaining solution were given till the Coomassie dye was soaked out of the gel and only the protein bands remain blue.
9. Molecular weight of different protein bands were determined by the help of protein marker.

Experiment No. 11: To study separation of bio-molecules by paper chromatography.

Background:

The basic procedure in this experiment consists of applying a small drop of the solution containing the substances to be separated near one end of a strip of absorbent paper. This end of the paper is then placed into a developing solvent, which flows upward along the paper by capillary action. The degree of solubility of the components of the mixture in the solvent, as well as the degree of attraction of these components to the wet cellulose molecules in the paper fibers, will determine the distance that the solvent will carry each substance along the paper during a given time interval. Those components that are quite soluble in the developing solvent, or that have a low affinity for cellulose, will be carried the greatest distance from the origin.

Material and reagent requirements:

Whatmann filter paper-1, 2 % individual amino acid solution (tryptophan, proline, methionine), solvent mixture (butanol: acetic acid: distilled water) in 12:3:5 volume , isopropanol, ninhydrin reagent, acetone, capillary tubes, weighing balance, chromatographic chamber, sprayer, pencil, beakers, test tubes, measuring cylinder.

Methodology:

1. Take sheet of filter paper and draw a faint pencil line about 1 to 2 cm from one of the long edge.
2. Load the sample of different amino acids on filter paper above the marking with the help of capillary tube.
3. Dried the sample with the help of dryer.
4. Pour the solvent mixture of (butanol: aceic acid: water) into the chromatography chamber (jar) just to cover the bottom.
5. Put the strip of the chromatography paper with sample(s) in the chromatography chamber, so that bottom of the strip touches the solvent.
6. Solvent started to move upper side, strip of filter paper was removed when solvent reached top.

7. Mark where the solvent front. Dried the strip and sprayed with ninhydrin solution.
8. Then placed the strip in hot air oven. Colored spots were developed.

Calculations:

Distances traveled by spots of various compounds determined by Rf value.

$$Rf = \frac{\text{distance the spot travels}}{\text{distance the solvent travels}}$$

Precautions:

1. Wear gloves when using ninhydrin solvent.

2. Use different capillary tube for each amino acid and unknowns so that cross contamination can be avoided.

3. Dispose of any left over developing solvent (butanol/acetic acid/water) in reclaim container provided. No solvent should be allowed to go down the drain.

4. Butanol is a flammable liquid. Make sure there are no open flames and heat near the developing solvent.