KARPAGAM ACADEMY OF HIGHER EDUCATION

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DEPARTMENT OF BIOCHEMISTRY

Subject	**:**	**Practical I- QUANTITATIVE ESTIMATION AND SEPARATION TECHNIQUES**	**Semester**	**:**	**I**
Subject code	**:**	**17BCP111**	**Class**	**:**	**I M.Sc Biochemistry**

Lecture Plan

S.NO	NAME OF THE EXPERIMENT	SUPPORT MATERIALS
Colorimetry		
1.	Isolation and estimation of starch from potato, Isolation and estimation of glycogen from liver	R1: 11-12
2.	Estimation of total carotenoids, Estimation of fructose in fruits	R1: 15-16
3.	Estimation of ascorbic acid, Estimation of vitamin-E	R1: 184-185
Fluorimetry		
4.	Estimation of thiamine from cereals or fruits, Estimation of riboflavin	R1: 179-182
Titrimetry		
5.	Estimation of lactose in milk, Estimation of calcium in milk	W1
Separation techniques		
6.	Separation of amino acids by paper chromatography-circular, ascending & descending, Separation of plant pigments by TLC	R1: 220-225
7.	Separation of plant pigments by column chromatography, Estimation of quercetin using HPLC (Demo)	R1: 230-233
Cell biology		
8.	Preparation of standard buffer and determination of pH of buffers,	R1: 246-250

	Subcellular fractionation by differential centrifugation and purity assessment with marker enzymes (Group experiment)	R1: 235-236
9.	Salting out of proteins using ammonium sulphate precipitation	R2: 130-135
10.	Model exam	

REFERENCES

R1: Sadasivam S and Manickam A. 2009. Biochemical methods, New age International publishers, New delhi.

R2: Jayaraman, J., (2011). Laboratory Manual in Biochemistry, New Age International Publishers, New Delhi.

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Experiment No: 1

ESTIMATION OF STARCH BY ANTHRONE REAGENT

Starch is an important polysaccharide. It is the storage form of carbohydrate in plants abundantly found in roots, tubers, stems, fruits and cereals. Starch, which is composed of several glucose molecules, is a mixture of two types of components namely amylose and amylopectin. Starch is hydrolysed into simple sugards by dilute acids and the quantity of simple sugars is measured colorimetrically.

Principle

The sample is treated with 80% alcohol to remove sugars and then starch is extracted with perchloric acid. In hot acidic medium starch is hydrolysed to glucose and dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone.

Materials

1. Anthrone: Dissolve 200mg anthrone in 100mL of ice-cold 95% sulphuric acid
2. 80% ethanol
3. 52% perchloric acid
4. Standard Glucose: Stock – 100mg in 100mL water.
5. Working Standard – 10ml of stock diluted to 100mL with water.

Procedure

1. Homogenize 0.1 to 0.5g of the sample in hot 80% ethanol to remove sugars. Centrifuge and retain the residue. Wash the residue repeatedly with hot 80% ethanol

till the washing do not give color with anthrone reagent. Dry the residue well over a water bath.

2. To the residue add 5.0mL of water and 6.5mL of 52% perchloric acid.

3. Extract at 0°C for 20min. Centrifuge and save the supernatant.

4. Repeat the extraction using fresh perchloric acid. Centrifuge and pool the supernatant and make up to 100mL.

5. Pipette out 0.1 or 0.2mL of the supernatant and make up the volume to 1mL with water.

6. Prepare the standards by taking 0.2, 0.4, 0.6, 0.8 and 1mL in each tube with water.

7. Add 4mL of anthrone reagent to each tube.

8. Heat for eight minutes in a boiling water bath.

9. Cool rapidly and read the intensity of green to dark green color at 630nm.

Result

Amount of starch present in 1g of sample is =

Amount of glucose present in 100ml of given unknown solution =

Calculation

Find out the glucose content in the sample using the standard graph. Multiply the value by a factor 0.9 to arrive at the starch content.

Readings

S.No	Solution	Volume of solution (ml)	Concentration (µg)	Volume of water(ml)	Volume of Anthrone (ml)	Heated in a boiling water bath for 8 minutes	Optical Density at 630 nm
1	Blank	-	-	1.0	4.0		
2	Standard						
	S1	0.2	20	0.8			
	S2	0.4	40	0.6			
	S3	0.6	60	0.4			
	S4	0.8	80	0.2			
	S5	1.0	100	-			
3	Sample	0.1	-	0.9			
		0.1	-	0.9			

4	**Unknown**	**1.0**	**-**	**-**			
		1.0	**-**	**0.5**			

Experiment No. 2

ESTIMATION OF GLYCOGEN IN LIVER

Glycogen is a storage form of glucose in animal and acts as an important food reserve which is stored in the liver.

Principle

From the deproteinised homogenate of liver, glycogen was precipitated by ethanol. Glycogen was treated with 45% alcohol to extract glucose. Glucose is dehydrated by sulphuric acid to furfural derivative which then complexes with anthrone to give a green coloured complex, which was read at 620nm.

Reagents

1. Glucose Standard: 100 mg of glucose was dissolved in 100 ml of water in a standard flask.10 ml of the stock was diluted to 100 ml with water. 1ml of this solution contains 100µg of glucose.
2. Anthrone reagent: 0.2 % anthrone in concentrated sulphuric acid (freshly prepared)
3. 45% Ethanol
4. 5% TCA

Sample extraction

Excised liver was washed in ice cold saline to remove any blood by quickly blotting between folds of the paper. Weighed 10g of liver tissue mixed thoroughly in a beaker surrounded by ice. The mixed tissue was then homogenized in a homogeniser or blender for 2 minutes, after adding 5% TCA (2.3ml /g). This homogenate was centrifuged at 3000rpm for 10 minutes in cold. Rehomogenised the sediment with half the volume of 5% TCA , then centrifuged in cold.

To the combined supernatant added twice the volume of 45 % ethanol, mix well and left in a refrigerator overnight. This precipitate glycogen. This precipitate was collected by centrifugation and dissolved in minimal volume of water. Then repeated as before by adding twice the volume of ethanol. The precipitate obtained was again washed with ethanol once and then with ethyl ether and was made up to 25 ml with water.

Procedure

Pipetted out 0.2 to 1 ml of working standard solution into a series of test tubes corresponding to µg values 20-100. 1.0 ml of extracted sample was pipetted out. The volume was made up to 1 ml in all the tubes with distilled water. Set up a blank along with the working standard. Added 4 ml of anthrone reagent to all the tubes and heated in a boiling water bath for 8 min. Cooled the tubes, read the colour developed at 620 nm in a spectrophotometer.

A standard graph was drawn by plotting concentration of glucose on X-axis and optical density on Y- axis. From the graph, concentration of glucose present in sample and unknown solution was calculated.

The amount of Glycogen present in liver is expressed as mg of glucose/g tissue.

The amount of glycogen in the aliquotused was then calculated using the following equation: **µg of glycogen in aliquot = 100 U/1.11S**

U is the optical denisty of the unknown solution.

S is the optical density of the 100 µg glucose

1.11 is the factor determined by Morris standard (Morris, 1948) for the conversion of the glucose to the glycogen.

Readings

Solution		Volume of water (ml)	Volume of anthrone reagent (ml)	Condition	Optical density (620nm)
Volume (ml)	**Concentration (µg)**				
Blank Standard 0.2 0.4 0.6 0.8 1.0 unknown 0.5 Sample 1.0					

Experiment No. 3

ESTIMATION OF TOTAL CAROTENOIDS

AIM:

To estimate the amount of carotenoid present in the given sample.

PRINCIPLE:

The carotenoids in the samples were saponified in alcoholic potassium hydroxide and extracted with petroleum ether. The extracted carotenoids are then estimated in a UV visible spectrophotometer at 450nm.

REAGENTS:

1. 12% alcoholic potassium hydroxide(2g/100ml of ethanol)
2. Petroleum ether
3. Calcium carbonate
4. Anhydrous Sodium sulphate

PROCEDURE:

To a weighed 2g amount of the carrot added a small amount of 12% alcoholic potassium hydroxide and saponified for an half an hour in a shaking water bath at 37°C. The alkaline mixture was transferred to a separating funnel containing petroleum ether (60°-80°C). Calcium carbonate was added to remove non carotenoids present if any. The upper petroleum ether layer was collected discarding the lower aqueous layer and the extraction was continued until no colour was observed in the petroleum ether layer. The petroleum ether extracts were collected in a volumetric flask, filtered through glass wool and anhydrous sodium sulphate was used to remove turbidity. The final volume of the petroleum ether extract was noted down and the optical density of the sample was measured at 450nm. Then the total carotenoid content of the sample was found out by using the formula

$$\text{Total carotenoids} = \frac{P*4*V*100}{W} \text{ mg}$$

Where,

P = Optical density of the sample

V = Volume of the sample

W = Weight of the sample

RESULT:

The amount of carotenoids in 100mg of carrot = ----------mg.

Experiment No. 4

ESTIMATION OF FRUCTOSE BY RESORCINOL METHOD

Fructose, a keto-hexose (called as fruit sugar), is usually accompanied by sucrose in fruits like apple. Honey is a rich source of fructose.

Principle

The hydroxymethyl furfural formed from fructose in acid medium reacts with resorcinol to give a red color product.

Materials

1. Resorcinol reagent: Dissolve 1g resorcinol and 0.25g thiourea in 100mL glacial acetic acid. This solution indefinitely stable in the dark.

2. Dilute HCl: Mix five parts of conc. HCl with one part of distilled water.

3. Standard fructose solution: Dissolve 50mg of fructose in 50mL water. Dilute 5mL of this stock to 50mL for a working standard.

Procedure

1. To 2mL of the solution containing 20 to 80□g of fructose add 1mL of resorcinol reagent.
2. Then add 7mL of dilute hydrochloric acid.
3. Pipette out 0.2, 0.4, 0.6, 0.8 and 1mL of the working standard and make up the volume to 2mL with water. Add 1mL of resorcinol reagent and 7mL of dilute HCl as above.
4. Set a blank along with the working standard.
5. Heat all the tubes in a water bath at 80°C for exactly 10min.
6. Remove and cool the tubes by immersing in tap water for 5min.
7. Read the color at 520nm within 30min.
8. Draw the standard graph and calculate the amount of fructose present in the sample using the standard graph.

Solution		Volume of distilled water (ml)	Volume of Resorcinol reagent (ml)	Volume of dilute HCL (ml)	Condition	Optical density (520nm)
Volume (ml)	Concentration (µg)					
Blank						
Standard						
0.4						
0.8						
1.2						
1.6						
2.0						
unknown						
1.0						
1.0						
sample1						
0.1						
sample2						
0.1						

Experiment No. 5

ESTIMATION OF ASCORBIC ACID (VITAMIN C)

Principle

Ascorbic acid is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbate then reacts with 2, 4-dinitrophenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophotometrically at 540 nm.

Reagents

1. 4% TCA
2. 10% Thiourea
3. 2% DNPH (2g-2,4 Dinitro phenyl hydrazine in 100ml of 0.5N sulphuric acid)
4. 85% Sulphuric acid

5. Standard Ascorbic acid (20-100 µg /ml)-

100 mg of ascorbic acid was dissolved in water and was made up to 100 ml with water. Convert 10mL of stock ascorbic acid solution into dehydro form by bromination. This contains 100µg uric acid /ml.

6. Extraction:

1g of leaves were ground and homogenized in 4% TCA and made up to 10 ml with the same and centrifuged at 2000 rpm for 10 minutes. The supernatant was treated with a pinch of activated charcoal residue, shaken well and kept for 10 minutes. Once again centrifugation was carried out to remove the charcoal residue. The volume of the clear supernatant obtained was noted. Transfer an aliquot (10mL) to a conical flask . and add bromine water drop wise with constant mixing. The enolic hydrogen atoms in ascorbic acid are removed by bromine. When the extract turns orange yellow due to excess bromine, expel it by blowing in air. Make up to a known volume (25 or 50mL) with 4% oxalic acid solution.

Procedure

1. Pipette out 10-100µg standard dehydroascorbic solution into a series of tubes.
2. Similarly pipette out different aliquots (0.1mL-2mL) of brominated sample extract.
3. Make up the volume in each tube to 3mL by adding distilled water.
4. Add one mL of DNPH reagent followed by 1-2 drops of thiourea to each tube.
5. Set a blank as above but with water in place of ascorbic acid solution.
6. Mix the contents of the tubes thoroughly and incubate at 37°C for 3h.
7. After incubation dissolve the orange-red osazone crystals formed by adding 7mL of 80% sulphuric acid.
8. Measure absorbance at 540nm.
9. Plot a graph ascorbic acid concentration versus absorbance and calculate the ascorbic acid content in the sample and expresse as the amount of ascorbic acid in mg/g or 100 g sample.

TABLE

S.No	Solution	Volume of solution (ml)	Concentration (µg)	Volume of water (ml)	Volume of DNPH (ml)	Volume of Thiourea (ml)	Incubate at 37 ° C for 3 hours	Volume of H_2So_4 (ml)	Allow to standard for 30 minute at room temperature	Optical Density at 630 nm
1	Blank	-	-	3.0	1.0	1-2 drops		7.0		
2	Standard									
	S1	0.2	20	2.8						
	S2	0.4	40	2.6						
	S3	0.6	60	2.4						
	S4	0.8	80	2.2						
	S5	1.0	100	2.0						
3	Sample	0.5	-	2.5						
		0.5	-	2.5						
4	Unknown	1.0	-	-						
		1.0	-	2.5						

Experiment No. 6

ESTIMATION OF VITAMIN-E

Aim

To estimate the amount of vitamin-E present in the given unknown sample.

Principle

Vitamin E can be estimated using reaction of ferric to ferrous ions by tocopherols, which forms a red colour with 2,2'-dipyridyl tocopherols and carotenes were first extracted with xylene and read at 460nm to measure carotenes. A correlation is made for these after adding ferric chloride and read at 520nm.

Reagents

i) 2-2' dipyridyl: 1.2g/L of n-Propanol

ii) Ferric chloride: 1.2g of $FeCl_3$ or 720mg of anhydrous ferric chloride in 1 L of ethanol.

iii) Standard Tocopherol: 10mg/L in absolute ethanol and 1ml of α-tocopherol is equivalent to 100mg of tocopherol acetate.

iv) Absolute ethanol

v) Xylene

Sample preparation

The plant sample (2.5g) was homogenized in 500ml of 0.1N H_2SO_4 and allowed to stand for overnight. The contents of the flask were shaken vigorously and filtered through filter paper. Aliquot of the filtrate were used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank) pipetted out 1.5 ml of each tissue extract, 1.5 ml of the standard and 1.5 ml of water respectively. To the test and blank added 1.5 ml ethanol and to the standard added 1.5 ml of water. Added 1.5 ml of xylene to all the tubes, stoppered, mixed well and centrifuged. Transfererred 1 ml of xylene layer into another stoppered tube, taking care not to include any ethanol or protein. Added 1.0 ml of 2, 2' dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixtures into spectrophotometer cuvettes and read the absorbance of test and standard against the blank at 460 nm. Then in turn beginning with the blank, add 0.33 ml of ferric chloride solution. Mixed well and after exactly 1.5 minutes read test and standard against the blank at 520 nm.The amount of vitamin E can be calculated using the formula

$$\text{Vitamin E } (\mu g/g) = \frac{(\Delta A520nm - \Delta A450nm \times \text{conc [s]} \times 0.29) \times \text{Total volume}}{(\Delta A520nm \times \text{Vol for experiment} \times \text{wt of sample})}$$

Experiment No. 7

ESTIMATION OF LACTOSE

Aim

To estimate the amount of lactose present in the milk.

Principle

Lactose is reduce in disaccharide when it is treated with appear containing reagent like fehlings reagent. The cupric ion present in the reagent and converted into cuprous ions by reducing the activity of lactose. This property is mainly used in titration.

Reagent

1. Fehling's solution A:

   Dissolve 34.64 gm of pure cuprous sulphate crystals in distilled water in a 500ml measuring flask and made up to 500ml.

2. Fehling's solution B:

   Dissolve 173gm roucheller (sodium potassium hydroxide). In distilled water and dissolved 5gm at pure sodium hydride in 5ml of distilled water and added to the former potassium tartarate and made the solution to 500ml with distilled water.

3. Methylene blue indicator:

   Dissolve 1gm of methylene blue powder and made up to 100ml.

4. Lactose solution:

   Small amount of lactose was taken and dried in a vaccum desicator over concentrated sulphuric acid for six hours. Dissolve 5gm of dried lactose crystal in 100ml with distilled water and boiled, cooled in dissolved water.

Procedure

Standardizing fehling's solution as follows.

10ml of fehling's solution A and B are pipetted separately into 250ml conical flask with 25ml of lactose was added into a flask containing Fehlings solution and mixed and boiled gently for 2 minutes. Added 2-3 drops of methylene blue indicator while boiling.

The addition of lactose was continued with 2 drops at 10 seconds by holding the burette in hands stop for the further addition of lactose as soon as the blue colour of the methylene blue is disappeared. The titration should be heated with 10 ml portion of Fehling's solution. Thus the value of lactose is determined. The percentage of lactose in milk is estimated as follows. 10 ml of milk is estimated as measuring flask and made up to 100 ml by adding distilled water. 25 ml of dilute milk was taken in conical flask containing 10 ml of fehling's solution A and B. Boil for 2 minute and added 3-5 drops of lactose solution at a

time intervals of 10 seconds until the end point reached. The titration is repeated two or more proportion of 25ml distilled milk to determine the exact quantitiy of lactose.

Assuming that Fehling's solution along required with 25ml of dilute milk. Since 1ml of lactose solution contain 0.005gm of lactose and the amount of lactose present in 25ml of dilute milk is obtained.

Result

The amount of lactose present in 100ml of undiluted milk is _____.

Standadization

Volume of Fehling's solution (ml)	Volume of milk (ml)	Burette reading		Volume of standardized lactose (ml)	Indicator
		Initial (ml)	Final (ml)		
10ml of fehling's A + 10ml of fehling's B	25	0.0 0.0			Methylene blue

Estimation

Volume of Fehling's solution (ml)	Volume of milk (ml)	Burette reading		Volume of standardized lactose (ml)	Indicator
		Initial (ml)	Final (ml)		

10ml of fehling's A + 10ml of fehling's B	25	0.0 0.0			Methylene blue

Calculation

Sample diluted A =

Blank value B =

Difference (A-B) =

1ml of lactose contains _____ gm of lactose.

25ml of diluted milk contain (A-B) * 0.005 = _____

100ml of diluted milk contain = (_____ /25)*100

100ml of undiluted milk contain = (_____*100)/10

Experiment No. 8

ESTIMATION OF CALCIUM IN MILK- PERMANGANATE METHOD

Aim

To estimate the amount of calcium in milk sample.

Principle

Calcium in milk is precipitated as calcium oxalate with ammonium oxalate(Magnesium is not precipitated as the conditions are selected to increase the solubility of magnesium oxalate).

The precipitate was washed with dilute ammonia to remove the excess ammonium oxalate and then dissolved in normal sulphuric acid. The oxalic acid formed was titrated with permanganate solution. The end point of titration was indicated by the formation of pink colour stable for atleast 30 seconds.

Reagents

1. 4% Ammonium oxalate solution
2. 2% Ammonium (v/v)

   Dilute 2.0 ml of ammonia (specific gravity 0.08) to 100 ml water.
3. 0.01N potassium permanganate solution

   It is prepared freshly before use by diluting the stock potassium permanganate (0.01N) solution
4. approximately weighed normal sulphuric acid.

Procedure

Into each of the two centrifuge tubes pipetted out 2.0 ml of distilled water. 20 ml of milk and 1.0 ml of ammonium oxalate, mixed the tubes and allowed to stand overnight at room temperature.

Centrifuge for 10 minute of 2000 rpm and carefully drained the supernatant. Inverted the tubes and allowed to drain on a pad of filter paper for 3 minutes. Added 3.0 mnl 2% ammolnia drain the sides of the solution. This process was repeated until the precipitate was washed completely and supernatant gave no precipitate with calcium chloride.

Pipetted out 2.0 ml of normal sulphuric acid rotating the tubes to wash down, then placed the tubes in water bath for 5 mins at 70-80°c to dissolve the precipitate. Removed centrifuged and titrated the contents with 0.01N potassium permanganate solutipon taen in a

burette till a pale pink was got. Which persist for about a minute. Repeat the titration with duplicate tubes.

Performed a blank titration with 2.0 ml of normal sulphuric acid. Kept in water bath for 5 mins and titrated with potassium permanganate to a pink colours the difference between the two titre values gives the volume of 0.1N kmno4 required to titrate calcium oxalate.

Result

The amount of calcium present in 100 ml milk was formed to be _____.

Calculation

Volume of solution (ml)	**Burette reading**		**Volume of $KmnO_4$ (ml)**	**Indicator**
	Initial (ml)	**Final (ml)**		
Blank 2.0 2.0 sample 2.0 2.0	 0.0 0.0 0.0 0.0			H_2So_4

0.1ml of 0.01N $KMno_4$ of calcium

Volume of 0.01N $KMno_4$ required by 2ml of blank =_____

Volume of 0.01N $KMno_4$ required by =_____

2ml of milk contains =

100ml of milk contains = (_____*_____)/20

Experiment No: 9

Separation of amino acids by paper chromatography

Paper Chromatography

Chromatography is the most powerful technique to separate chemically closely related substances into the individual components on the basis of their physicochemical properties. The compounds are separated on the basis of their partition coefficients between two immiscible phases. The static phase may be a solid or liquid while the mobile phase may be a solid, liquid or gas. Depending upon the static and mobile phases, a variety of chromatographic techniques are available. These include chromatography on paper, thin layer gel, ion-exchange resin etc. Although modern instrument facilities such as High Performance Liquid Chromatography (HPLC) are available for the separation of chemical substances, the classical techniques - paper chromatography and thin layer chromatography are still easy, can be set up even in an ordinary laboratory without much expenditure. It may be recalled that Calvin and his associates used paper chromatography to elucidate the pathway of carbon dioxide fixation in photosynthesis. The separation, identification and (semi) quantification of amino acids using paper chromatography is described below. The same methodology can be used to separate other smaller molecules such as sugars, organic acids etc. by changing the mobile phase and detection (spray) agents.

Principle

The separation of the solutes (amino acids) is based on the liquid-liquid partitioning of amino acids in paper chromatography. The partitioning takes place between the water molecule (static phase) adsorbed to the cellulosic matter of the paper and the organic (mobile) phase.

Materials

1. Whatman No.1 filter paper
2. Chromatography chamber
3. Hair-dryer or spot-lamp
4. Atomizer
5. Microsyringe or micropipette

Mobile Phase (Solvent System)

Mix n-butanol, glacial acetic acid and water in the ratio 4 : 1 : 5 in a separating funnel and stand to equilibrate for 30 min. Drain off the lower aqueous phase into a beaker and place it

inside to saturate the chromatography chamber. Save the upper organic phase and use it for developing the chromatogram.

Dissolve different individual amino acids in distilled water at a concentration of 1mg/mL. Use very dilute (0.05N) HCl to dissolve the free amino acids tyrosine and phenylalanine. Dissolve tryptophan in very dilute (0.05N) NaOH.

Extraction of Sample

Grind a known quantity of the sample material (dry/wet) in a pestle and mortar with 10-fold volume of 70% ethanol. Shake the contents at 55°C for 30 min. Centrifuge the contents at 10,000rpm for 10 min. Collect the supernatant. Repeat the extraction of the pellet at 55°C at least twice. Pool the supernatants (for leaf extracts, treat with equal volume of petroleum ether 40-60°C) and shake vigorously. Discard the petroleum ether layer containing chlorophyll. Evaporate the alcohol fraction to dryness under vacuum using either a water-pump or rotary evaporator at 40-45°C. Dissolve the residue in a known volume of absolute ethanol or water for analysis.

Ninhydrin Reagent

Dissolve 100mg ninhydrin in 100mL acetone.

Elution Mixture

Prepare 1% copper sulphate solution. Mix ethanol and copper sulphate solution in the ratio 80:20 (v/v).

Procedure

1. Cut the chromatography sheet carefully to a convenient size (40 x 24cm). Draw a line with pencil across the sheet about 5cm away from one end. Mark a number of points at intervals of 3cm.
2. Apply a small volume (say, 25 L)of each amino acid as a separate small spot using a microsyringe. A stream of hot air from a hair-dryer facilitates fast drying of spot. The spot should be as small as possible for better resolution.
3. Similarly spot different known aliquots of sample extract.
4. After spotting, place the sheet in a stainless steel trough in the chromatography chamber, firmly hold it by placing a long steel rod over the sheet. The spot-end of the sheet should be in the trough (descending chromatography). Otherwise, the sheet may be rolled as a cylinder, tied together with fine thread and placed upright with the spots as the bottom in a large Petridish for upward movement of solvent (ascending chromatography).what mann no:1 filter paper was act into a circle of diameter 20 cm.

pencil line was drawn 4 cm away from the centre. The circle was divided into four parts. The three amino acids and the unknown mixture was spotted along the pencil line at specific spots. As hole was made in the centre of the paper which was introduced into it. The paper was kept in the petridish containing 5 ml of solvent. The solvent was sucked by the wider which transfer solvent to the chromatographic chamber (Circular chromatoghraphy).

5. Add the organic (phase) solvent to the trough/petri dish and close the chamber airtight. Develop the chromatogram, preferably overnight or longer, until the solvent moves almost to the other end.
6. Note the solvent front and dry the chromatogram free of solvent in a fume chamber.
7. Spray the chromatogram with the ninhydrin reagent using an atomizer. Dry the paper for about 5 min at room temp followed by at 100°C in an oven for 2-3 min. Amino acids appear as purple spots; hydroxyproline and proline give yellow colored spots.
8. Mark all the spots and calculate their R_f values by the formula.

$$R_f = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

9. The amino acids present in the sample are then identified by comparing the *Rf* values with that of the authentic amino acids, co-chromatographed.
10. For quantitative estimation, cut each spot into several small bits and transfer to the bottom of the test tube.
11. Add 3mL of elution mixture. Shake the tubes vigorously for 15 min. Decant the liquid and elute the pieces with another 2mL of elution mixture. Repeat the elution with small aliquots until the bits are colorless. Combine and clear the eluate by centrifuging at 10,000rpm for 10 min. Read the intensity of purple color at 570nm in a colorimeter. Use the spot of leucine *(50□g)* run as standard for comparison.

Notes

1. Handle the chromatography sheet very carefully until developed as otherwise amino acids from fingers will contaminate. Hold the chromatographic paper between a fold of filter paper piece.
2. After developing the chromatogram with the first solvent system, it may be developed with second solvent system such as phenol: H_2O (80 : 20). This is then called

bidimensional chromatography. In such case, the sample alone is applied at near one corner of the paper. Bidimensional chromatography is carried out for fine separation.

3. As the *Rf* value varies from run to run, due to solvent system, paper, room temperature, size of the chromatography chamber etc., it is advisable to co-chromatograph the standards every time.
4. Similarly, analysis of sugars, organic acid, phenolic compounds can also be done by paper chromatography (For solvent systems, spraying agents etc., see under thin layer chromatography).

Result:

Experiment No: 10

Separation of amino acids by thin layer chromatography

The separation and identification of organic compounds is a routine work in many service laboratories. Thin layer chromatography (tlc) is an easy technique to adopt for the said purpose. It is highly useful in research laboratories to separate, identify and characterize unknown compounds. A variety of small molecules like amino acids, sugars, organic acids, lipids etc. are separated by tic technique. The greater advantage of tic is the speed at which separation is achieved. When volatile solvents are used the time required to effect separation is only about 30 min and with nonvolatile solvents it is seldom longer than 90 min.

Principle

The general Principle involved in tlc is similar to that of column chromatography i.e. adsorption chromatography. In the adsorption process, the solute competes with the solvent for the surface sites of the adsorbent. Depending on the distribution coefficients, the compounds are distributed on the surface of the adsorbent. Of course, in tic the partition effect in the separation is also not ruled out. The adsorbent normally used contains a binding agent such as calcium sulphate which facilitates the holding of the adsorbent to the glass plate.

Materials

- Glass Plate (20 x 20 cm or 20 x 10 cm)

- Glass Tank with Lid
- Spreader
- Developing Solvents
- Adsorbent Silica GelG/Alumina
- Sample (should be extracted following the procedures indicated for each group of compounds. For e.g., extraction with 80% alcohol for amino acids and sugars)
- Standards Spraying Agent (This also differs as for the group of compounds of interest).

Procedure

Preparation of Plates

1. Place dry, clean glass plates (5 nos, 20 x 20cm) on the plastic base plate over a plane surface.
2. 1. Prepare a slurry of the adsorbent in water (sometimes buffer) in the ratio 1:2 (w/v).
3. Stir the slurry thoroughly for 1-2 min and pour into the applicator positioned on the head glass plate.
4. Coat the slurry over the glass plates at a thickness of 0.25mm for qualitative analysis by moving the applicator at a uniform speed from one end to the other. (One has to gain some experience by practicing to prepare uniformly coated plates.) Leave the plates to dry at room temperature for 15-30 min.
5. Heat the plates in an oven at 100-120°C for l-2h to remove the moisture and to activate the adsorbent on the plate. The dried plates in a rack can be stored in a desiccator over silica gel to prevent moisture absorption.

Sample Application

1. Leave 2.5cm from one end of the glass plate and at least an equal distance from the edges.
2. Apply the sample and standards by means of a micropipette or syringe as small spots. All spots should be placed at equal distance from one end of the plate. See that the adsorbent does not flake off at the sample application point. (Measured volumes are applied for quantitative analysis.)

3. Allow the sample to dry so that spotting can be done repeatedly for a more concentrated sample spot.

Developing Chromatogram

1. Pour the developing solvent into the tank to a depth of 1.5cm. Allow it to stand for at least an hour with a cover plate over the top of the tank to ensure that the atmosphere within the tank becomes saturated with solvent vapor. This is called equilibration.
2. After equilibration, remove the cover plate, and place the thin layer plate (sample applied) vertically in the and so that it stands in the solvent with the spotted end dipping in the solvent.
3. Replace the cover plate. The separation of the compounds occurs as the solvent moves upward. Develop the chromatogram at constant temperature in order to avoid anomalous solvent-running effects.
4. Once the solvent reaches the top of the plate, remove it from the tank, dry and proceed for the identification of the separated compounds.

Result:

Experiment No: 11

COLUMN CHROMATOGRAPHY

On several occasions, a researcher requires a specific group of macromolecules separated from a biological extract in order to understand the molecule or the process indepth. Chromatography is one of the techniques to separate biological molecules. There are many types of chromatography based on the physicochemical properties of the molecules.

These different types of chromatography include gel filtration - ion-exchange - adsorption - affinity chromatography and so on, based on the molecular size and shape, ionic nature, molecular topography and biological specificity of the molecule.

Essentially, any chromatography consists of two phases: one is stationary phase which may be a solid, liquid or a solid/liquid mixture and is immobilized while the other, mobile phase, is a fluid which flows through the stationary phase.

Chromatographic separations in practice may take any one of three modes-paper, thin layer or column chromatography.

In column chromatography, the stationary phase is packed in a cylindrical column made of plastic or glass.

The concept of gel filtration chromatography is the different molecules are separated based on the molecular size and shape where the stationary gel matrix serves as a sieve.

The principle underlying the ion-exchange chromatography is the attraction between the biological compounds and the stationary phase, each with opposite electrical charges, thus attracting each other. The ionic nature of chemical compounds is hence exploited.

Affinity chromatography is based on the attraction of a partially compound specifically to combine with the molecule of our interest. For instance, the inhibitor of an enzyme serves as affinity compound to separate that particular enzyme. However, the inhibitor has to be initially combined with an inert matrix to serve as stationary phase.

Majority of the chromatography is routinely carried out Using the column mode. The apparatus and general techniques used for gel exclusion, ion-exchange, adsorption and affinity chromatography have much in common. Gas-liquid chromatography and high performance liquid chromatography each have their own special apparatus, materials and protocols. The column chromatography nowadays been made sophisticated, easier and faster by combining together pumps, detectors, recorders etc. to the columns.

As a model, column chromatographic separation of proteins based on their molecular size by gel filtration is described below:

Principle

The basis of any form of chromatography is the partition or distribution coefficient (Kd) which describes the way in which a compound distributes itself between two immisible phases, such as solid/liquid or gas/liquid.

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for the solute to achieve complete equilibrium between the mobile and stationary phases. Each zone is called a theoretical plate and its length in the column is called the plate height. The more efficient the column is the greater the number of theoretical plates involved.

Materials

Ä Chromatographic column of suitable dimension made up of transparent plastic or glass: Generally, gel filtration is carried out in longer columns (up to 1 m) depending upon the type of gel filtration medium used and the size of the protein to be purified from the bulk

Ä Stationary phase: (ex) Sephadex G 100

There are different types and grades of gel filtration media available. It is to be chosen on the basis of the size of the protein under study

Ä Elution buffer

Ä Fraction collector

Ä Peristaltic pump

Ä Marker proteins (a set of highly pure proteins with known molecular weight (e.g.) cytochrome C, 12,400; carbonic anhydrase, bovine 1,50,000; b-amylase, potato 2,00,000; blue dextran 2,00,000 daltons.

Procedure

A. Packing the column

1. Suspend the gel (for instance, Sephadex G 100) in a large volume of water or preferably in elution buffer until the gel is fully swollen. The swelling can be done by overnight suspension or by heating in a water bath for 2-4 hr (Follow the manufacturer's instructions for this purpose, carefully).

2. Plug the bottom of column tube with glasswool or sintered filter and stand upright the column.

B. Packing the column

3. Make a good slurry of the gel (stationary phase) in a suitable buffer after proper swelling of the gel.

4. Pour a small volume of buffer into the column to avoid trapping of any air bubbles in the plug immediately followed by the slurry to the full of column. The top portion may be carefully, gently stirred prior to pouring additional slurry to the growing column, if necessary. Wait until the gel settle down to the desired height by gravitational force.

5. Place a suitable filter circle on top of the gel bed.

6. Equilibrate the column thoroughly by passing through the column buffer.

7. Apply the sample in column buffer onto the top of bed. The sample volume should preferably limited to 1-3% of the total bed volume. The sample can be applied to the top by careful pipetting or conveniently through the buffer pipeline.

8. Now connect the bufferline to the elution buffer to develop the chromatogram.

9. Protein molecules pass through the gel space while small molecules distribute between the solvent inside and outside the gel and then pass through the column at a slower rate.

10. The effluent emerging out of the column can be routed through a suitable spectrophotometer to monitor the absorbance at a particular wavelength (for proteins either 280, 230 or 210 nm) and the, data recorded. The effluent is then collected using a fraction collector. The effluent is manually collected in the absence of a collector in a fixed time-or volume intervals in tubes and measured subsequently.

11. The volume of mobile phase required to elute a particular solute is known as the elution volume while the corresponding time for elution of the solute at a given flow rate is known as the retention time.

12. The elution is continued (usually 2-3 times bed volume of buffer) until the absorbance monitored reached baseline value.

13. Thereafter the column is extensively reequilibrated with the column buffer for subsequent run.

14. The reference proteins are loaded onto the bed and the chromatography is carried out as above.

15. Plot the logarithms of molecular weight of marker proteins against their respective ratios of elution volume to column void volume (Ve/Vo), the column volume being the elution volume of a very large molecule such as blue dextran.

16. Compute the elution volume of protein of interest and deduce its molecular weight from the above linear graph.

Notes

1. The column chromatography is, nowadays, very much modernized/sophisticated and automated. A family of equipment such as pumps, cooling devices, detectors, collector and data processor put together make the technique much interesting and easier.

2. The column chromatography experiment is an art. Every step in the experiment needs to be carefully done for good results.

3. The experimenter should choose the right stationary and mobile phases, column etc. for optimal results. It is mostly trial and error when there is no details available on the molecule.

4. No single column chromatography procedure will result in the complete purification of a molecule except the affinity chromatography procedure. Therefore, chromatography exploiting two or more physicochemical traits of the molecule has to be employed for purification.

5. Column development using a single solvent is known as isocratic separation. However, in many cases by continuously changing the pH, ionic strength or polarity of the eluent, the resolving power of the eluent could be increased. This kind of elution is called as gradient elution.

Experiment No: 12

ESTIMATION OF QUERCETIN USING HPLC (DEMO)

MATERIALS AND METHODS

Instrumentation:

The output signal was monitored and processed using LC Solutions Version 1.21 SP1 software on a

Pentium computer (Hewlett Packard). Preparation of Standard Solution The standard was prepared by accurately weighing about 10 mg Quercetin in 10 ml of methanol in a volumetric flask. It was then sonicated for 10-15 minutes .The contents of flask were filtered through Whatman no.41 paper (Merck, Mumbai, India) to attain the stock solution of 1 mg/ml.

Preparation of Sample Solution: 500 mg Sample (Neem bark extract) is accurately weighed and dissolved in 50 ml of methanol in a volumetric flask. It was then sonicated for 10-15 minutes then the contents of the flask were filtered through Whatman No. 41 paper (Merck, Mumbai, India) to attain the stock solution of 10 mg/ml.

Methodology : High-performance liquid chromatography method development.The separation was performed by using column C18 ODS thermo 250 × 4.6 mm, 5 µm on a Shimadzu class LC(California ,USA), which consisted of a FCV-10 ACVP pump, DGU-14A degasser, a thermo stated CTO-10AVP column oven compartment, an auto sampler, and a SPD-M10AVP diode array detector and Rheodyne 7725i injector with 20 µl loop volume and a flow rate of 1.0 ml/min. The output signal was monitored and processed using SPI Software LC Solutions, Version 1.21 on a Pentium computer (Hewlett Packard). All analyses were performed at a ambient temperature of 37ºC, with a mobile phase of orthophosporic acid

/Acetonitrile, The analysis was performed using a high-performance liquid chromatographic system an injection volume of 20 µl, and a flow rate of 1.0 ml/min. The UV absorbance of the eluent was measured at 350 nm.

Chromatography

HPLC Column C18 ODS thermo (250 x 4.6 mm) 5µm; Wavelength 350 nm

Injection volume 20 µl; Flow rate 1.0ml/min.

Mobile phase Solvent A-0.1 % orthophosphoric acid

Mobile phase ratio Solvent B-Acetonitrile 80:20v/v

HPLC ANALYSIS

Calculation of Assay of Quercetin

(Std Wt / Sample Wt) x (Sample Area / STD Area)x Assay of Standard = Assay of Sample.

Example:

Calculation of assay of Quercetin in Acacia catechu Bark (Ethanolic)

Extract Sample Weight taken : 10.02mg

Area of sample : 410112

Standard Weight taken : 1 mg

Area of Standard : 5754965

Assay of Quercetin : 100%

Assay of Quercetin : (1.0 /10.02) x (410112 /5754965) x 100 =0.070% w/w.

Experiment No: 13

PREPARATION OF STANDARD BUFFER AND DETERMINATION OF PH OF BUFFERS.

Preparation of standard buffer

pH standard solutions are used as standards of pH. Use the water, directed below, to prepare the pH standard solutions.

Distill purified water, boil the distillate for more than 15 minutes to expel carbon dioxide, and cool in a container fitted with a carbon dioxide-absorbing tube (soda lime). Store the pH standard solutions in hard glass or polyethylene bottles. As the pH value may change during storage for a long period, usually use acidic standard solutions within 3 months, and use basic

standard solutions within 1 month under storage in containers fitted with a carbon dioxide-absorbing tube (soda lime).

Oxalate pH Standard Solution: Reduce potassium tetraoxalate for pH determination to a fine powder, and dry in a desiccator. Weigh 12.71 g exactly, and dissolve in water to make exactly 1,000 ml.

Phthalate pH Standard Solution: Reduce potassium hydrogen phthalate for pH determination to a fine powder, and dry at 110ˆto constant weight. Weigh 10.21 g exactly, and dissolve in water to make exactly 1,000 ml.

Phosphate pH Standard Solution: Reduce monopotassium phosphate for pH determination and anhydrous disodium phosphate for pH determination to fine powders, and dry at 110ˆto constant weight. Weigh 3.40 g (0.025 mol) of monopotassium phosphate and 3.55 g of disodium phosphate exactly, and dissolve in water to make exactly 1,000 ml.

Borate pH Standard Solution: Place sodium borate for pH determination in a desiccator (sodium bromide moistened with water), and allow to stand to constant weight. Weigh 3.81 g exactly, and dissolve in water to make exactly 1,000 ml.

Carbonate pH Standard Solution : Dry sodium hydrogen carbonate for pH determination in a desiccator to constantweight, and weigh 2.10 g exactly. Dry sodium carbonate for pH determination at 300 to 500ˆto constant weight, and weigh 2.65 g exactly. Mix both, and dissolve in water to make exactly 1,000 ml.

Calcium Hydroxide pH Standard Solution: Reduce calcium hydroxide for pH determination to a fine powder, transfer 5 g into a flask, add 1,000 ml of water, shake well, maintain the temperature at 23ˆ27ˆ, saturate thoroughly, and filter the supernatant at the same temperature. Use the clear filtrate (about 0.02 mol/l).

pH Meter: A pH meter generally comprises a detecting unit consisting of a glass electrode and a reference electrode, and an indicating unit for indicating the pH value corresponding to the electromotive force detected. The indicating unit usually has dials for zero point adjustment and temperature compensation, and some have a dial for sensitivity adjustment.

When the pH value is tested five times by the procedure below for one of the above pH standard solutions, the reproducibility of the meter should be within ±0.05. Wash well the detecting unit with water at each measurement

DETERMINATION OF PH

Phosphate Buffer; pH range 5.8 to 8.0

(a) 0.1 M Sodium phosphate monobasic; 13.8 g/l (monohydrate, M.W. 138.0)

(b) 0.1 M Sodium phosphate dibasic; 26.8 g/l (heptahydrate, M.W. 268.0)

Mix Sodium phosphate monobasic and dibasic solutions in the proportions indicated and adjust the final volume to 200 ml with deionized water. Adjust the final pH using a sensitive pH meter

ml of Sodium phosphate, Monobasic	92.0	81.5	73.5	62.5	51.0	39.0	28.0	19.0	13.0	8.5	5.3
ml of Sodium phosphate, Dibasic	8.0	18.5	26.5	37.5	49.0	61.0	72.0	81.0	87.0	91.5	94.7
pH	5.8	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0

Procedure for pH Measurement

1. A pH meter may require a warm up time of several minutes. When a pH meter is routinely used in the laboratory, it is better to leave it "ON" with the function switch at "standby."

2. Set the temperature control knob to the temperature of your buffer solution. Always warm or cool your buffer to the desired temperature before checking final pH.

3. Before you begin make sure the electrode is well rinsed with deionized water and wiped off with a clean absorbent paper.

4. Always rinse and wipe the electrode when switching from one solution to another.

5. Calibrate your pH meter by using at least two standard buffer solutions.

6. Do not allow the electrode to touch the sides or bottom of your container. When using a magnetic bar to stir the solution make sure the electrode tip is high enough to prevent any damage.

7. Do not stir the solution while taking the reading.

8. Inspect your electrode periodically. The liquid level should be maintained as per the specification provided with the instrument .

9. Glass electrodes should not be left immersed in solution any longer than necessary. This is important especially when using a solution containing proteins. After several pH measurements of solutions containing proteins, rinse the electrode in a mild alkali solution and then wash several times with deionized water.

10. Water used for preparation of buffers should be of the highest possible purity. Water obtained by a method combining deionzation and distillation is highly recommended.

11. To avoid any contamination do not store water for longer than necessary. Store water in tightly sealed containers to minimize the amount of dissolved gases.

12. One may sterile-filter the buffer solution to prevent any bacterial or fungal growth. This is important when large quantities of buffers are prepared and stored over a long period of time.

Experiment No: 14

CELL FRACTIONATION OF SUBCELLULAR ORGANELLES

Aim

To separate the sub cellular organelle by differential centrifugation and to assess its purity by marker enzymes.

Prinicples

Cell contains different structural entities like nucleus, mitochondria, ribosome's and cell wall membrane each of which has its unique function. They can be separated from one another by differential centrifugation. In this teqniques, fractions varying their density are sedimented at different centrifugal forces in a centrifuge. The speed to be used varies from about 3000g for nuclear fraction to about 10430g for sediment microsomal fractions.

Instrument

The primer requirement is refrigerated centrifuge that prevent temperature rise and consequent denaturation and also generated speed up to 15000 rpm which is sufficient to pellet down cell wall membrane and mitochondria.

Reagents

1. homogenizing medium :

the ideal homogenizing medium is 0.25M sucrose which is isotonic

2. 50mM Tris HCL buffer pH7.5 – neutralize acidic vacuolar content
3. 10mM Mg2+ - keep ribosome intact
4. 1mM ca2+ - prevent clumping of nuclei

Procedure

Preparation of cell free homogenate

Freshly excised rat liver (5g) was washed in ice cold saline (0.9% NaoH) blotted, dropped into a cold beaker, and mixed well with scissors. To this 0.25M sucrose containing homogenizing medium (8ml/gm of net weight) was added. The suspension was taken in a hominines and a Teflon pestle, driven by a electric motor is passed from the suspension upto 4 to 5 times. The clearness between the pestle and the wall is the order 0.1 to 0.2mM which is sufficient to break the cell but not the internal structures. The resulting homogenate was then filter through a muslin cloth to give a cell free homogenate . the volume of filtrate was made upto give a 10% homogenate.

Purification

1. **Isolation rat liver nuclei**

The rat liver homogenate was centrifuged at 600g for 10 minutes in cold, when unbroken cell and unwanted cell debris. Pelleted out. The turbid supernatant was poured into a cold beaker. The pellet was re suspended into gently with glass rod in half the original of the medium and recentrifuged. The combined supernatant were centrifuged at 2000rpm (15000g) for 10 minutes, taking care ti increase and decrease the speed very slowly. The sediment was a mainly nucleus which was kept suspended in 2ml of 0.25M sucrose containing 0.00018M cold solution.

2. **Assessment of purity**

The presence of DNA in nuclei fraction was assessed to check its purity.

Principle:

The deoxyribose in DNA in presence of acid forms hydroxyl levvlinins aldehyde reacts with diphenyl amine to give a blue colour which is then read at 595nm.

Reagents

1g of diphenyl amine in 10ml acetic acid and by adding 2.5 ml concentrated sulphuric acid.

Procedure

0.5ml of the resuspended nuclear fraction is made up to 3ml with water. To this 5ml of the reagent was added, mixed well and heated in a boiling water bath for 10 minutes. After cooling the developed color was read at 595nm. A standard graph is plotted by pipetting different volumes of DNA (100ug/ml) into test tubes and made up to 3ml with water and proceeded as before.

The amount of DNA in the fraction is estimated from the standard graph.

Assessment of purity of nuclear fraction

Solution		**volume of distilled water (ml)**	**Volume of diphenyl amine (ml)**	**Colorimeter reading**	
Volume (ml)	**Concentration (µg)**				
Blank					
Standard					
1.0					
2.0					
3.0					
4.0					
5.0					
6.0					
Unknown					
1.0					
2.0					

Calculation

Colorimeter reading _____ corresponds to _____ of DNA

0.2ml of nuclear fraction contains _____

2ml of nuclear fraction contains : _____

Result

The amount of DNA present in 2ml of nuclear fraction: _____.

Experiment No: 14

SALTING OUT OF PROTEINS USING AMMONIUM SULPHATE PRECIPITATION

Salting out is a purification method that utilizes the reduced solubility of certain molecules in a solution of very high ionic strength. Salting out is typically, but not limited to, the precipitation of large biomolecules such as proteins.

Introduction

In contrast to salting in, salting out occurs in aqueous solutions of high ionic strength that reduce the molecule's solubility causing certain proteins to precipitate. Ideally, the type of salt being used and the concentration of the salt can be varied to selectively precipitate a the molecule. In reality, salting out is an effective means for initial molecule purification, but lacks the ability for precise isolation of a specific protein.

The Mechanism Behind Salting Out

The conformation of large biomolecules *in vivo* is typically controlled by hydrophobic and hydrophillic interactions with the cellular environment. These interactions largely govern the molecule's final confirmation by folding in such a way that most hydrophobic functional groups are shielded from the polar cellular environment. To achieve this conformation the molecule folds in such a way that all of the hydrophobic parts of a molecule are aggregated together and the hydrophillic groups are left to interact with the water. In the case of proteins it is the charged amino acids that allow selective salting out to occur. Charged and polar amino acids such as glutamate, lysine, and tyrosine require water molecules to surround them to remain dissolved. In an aqueous environment with a high ionic strength, the water molecules surround the charges of the ions and proteins. At a certain ionic strength, the water molecules are no longer able to support the charges of both the ions and the proteins. The result is the precipitation of the least soluble solute, such as proteins and large organic molecules.

The Hoffmeister Series

Salting out can be a powerful tool to separate classes of proteins that vary in size, charge, and surface area among other characteristics. One method of controlling the precipitation is the utilize the different effects of various salts and their respective concentrations. A salt's ability to induce selective precipitation is dependent on many interactions with the water and solutes. Research by Franz Hofmeister in the early 20th century organized various anions and cations by their ability to salt out.

The ordering of cations and anions is called the Hoffmeister Series (1). The cations are arranged as follows

$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$

Where ammonium has the highest ability to precipitate other proteinacious solutes. Likewise, the order for anions is

$F^- \geq SO_4^{2-} > H_2PO_4^- > H_3CCOO^- > Cl^- > NO_3^- > Br^- > ClO_3^- > I- > ClO$

Between cations and anions in solution the concentration of the anion typically has the greatest effect on protein precipitation.

One of the most commonly used salts is ammonium sulfate, which is typically used because the ions produced in an aqueous solution are very high on the *Hofmeister* series, and their interaction with the protein itself is relatively low. Other ions such as iodide are very good at precipitating proteins, but are not used due to their propensity to denature or modify the protein.

The Thermodynamics of Salting Out

Salting out relies on changes in solubility based on ionic strength. The ionic strength of a solution, I, is defined

$$I = \frac{1}{2} \sum_i m_i z_i^2$$

Where

- mi is the concentration of the ion and
- zi is the charge of the ion.

Total ionic strength of multiple ions is the sum of the ionic strengths of all of the ions. Using the Debye-Hückel limiting law given by

$$\log \gamma_{\pm} = -\frac{1.824 \times 10^6}{(\epsilon T)^{3/2}} |z_+ z_-| \sqrt{I}$$

where

- I is the ionic Strength
- z+ is the catonic charge of the electrolyte for γ±
- z− is the anionic charge of the electrolyte for γ±
- γ is the mean ionic activity coefficient
- T is the temperature of the electrolyte solution
- ϵ is the relative dielectric constant for the solution

Which can be adapted for for an aqueous solution at 298 K,

$$\log \gamma_{\pm} = -0.509 |z_+ z_-| \sqrt{I}$$

The solubility, SS, of a particular aqueous solute can be defined as

$$\log \frac{S}{S_o} = -0.509 |z_+ z_-| \sqrt{I} - K'I$$

Where

- K′ is the is a constant dependent on the size of the solute and the ions present,
- S is the solubility and
- S0 is the solubility in pure solvent.

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DEPARTMENT OF BIOCHEMISTRY

Subject	**:**	**Practical I- QUANTITATIVE ESTIMATION AND SEPARATION TECHNIQUES**	**Semester**	**:**	**I**
Subject code	**:**	**17BCP111**	**Class**	**:**	**I M.Sc Biochemistry**

TITLE OF THE EXPERIMENTS

Colorimetry

1. Isolation and estimation of starch from potato (Anthrone method)
2. Isolation and estimation of glycogen from liver (Anthrone method)
3. Estimation of Total carotenoids (Spectroscopic method)
4. Estimation of fructose in fruits (Resorcinol method)
5. Estimation of ascorbic acid (DNPH method)
6. Estimation of Vitamin E (Dipyrridyl method)

Fluorimetry

7. Estimation of thiamine from cereals or fruits
8. Estimation of riboflavin

Titrimetry

9. Estimation of lactose in milk
10. Estimation of calcium in milk

Separation techniques

11. Separation of amino acids by paper chromatography- circular, ascending & Descending.
12. Separation of plant pigments by TLC.
13. Separation of plant pigments by column chromatography.
14. Estimation of quercetin using HPLC (Demo).

Cell biology:

15. Preparation of standard buffer and determination of pH of buffers.
16. Subcellular fractionation by differential centrifugation and purity assessment with

marker enzymes (Group Experiment).

17. Salting out of proteins using ammonium sulphate precipitation

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