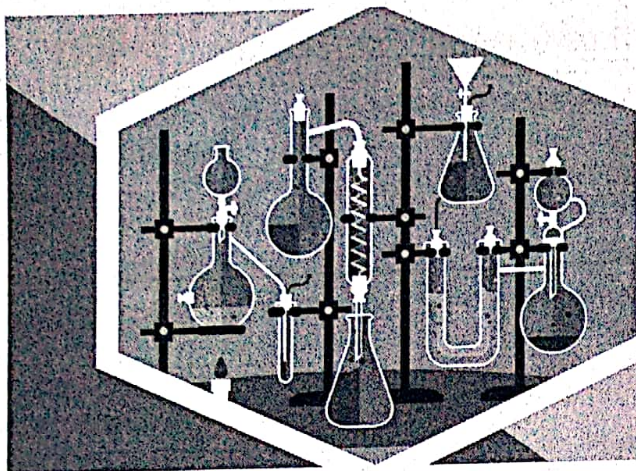

Chromatographic Separation of a Mixture of Compounds

Chapter 7

7.1 Basic Concepts

Chromatography is a versatile laboratory technique for the separation, identification and purification of the constituents of a mixture. The mixture is dissolved in a fluid. This constitutes the mobile phase. This mobile phase carries the mixture through a network of another substance, known as the stationary phase. Various constituents of the mixture bind with different strengths with the stationary phase. So these constituents travel through the stationary phase with different velocity which causes them to separate. The separation originates from differential partitioning between the mobile and the stationary phases. Depending on the physical states of the mobile and the stationary phases a chromatography may be liquid-liquid, solid-liquid or gas-liquid. The following section deals with three types of chromatography *viz*. Thin layer chromatography (TLC), Paper chromatography (PC) and Column chromatography (CC).

7.2 Thin Layer Chromatography (TLC)

Thin layer chromatography is a widely used laboratory technique for the separation and identification of the components of a mixture, even present in minute quantity, on the basis of their relative affinities to the stationary and mobile phases. The stationary phase is composed of a thin layer of an adsorbent like silica gel, alumina etc adhered to a flat surface of an inert substance. For the preparation of the chromatoplate a glass plate or a piece of aluminum foil is coated with a thin film of silica gel or alumina on one side. The chromatoplate is then spotted with a very dilute solution of the mixture taken for separation using a fine capillary tube near one end of the plate. The chromatoplate is then dipped in the developing solvent (the mobile phase) in such a way that the spots are not immersed in the solvent. The developing solvent is allowed to rise a suitable distance along the plate which develops the chromatogram. The plate is then dried and sprayed with appropriate reagents to figure out the location of the separated components. If the separated components impart colour on the chromatogram spraying with the suitable reagent is not necessary. The separated components may be identified by comparing the respective R_f values with those of standard samples.

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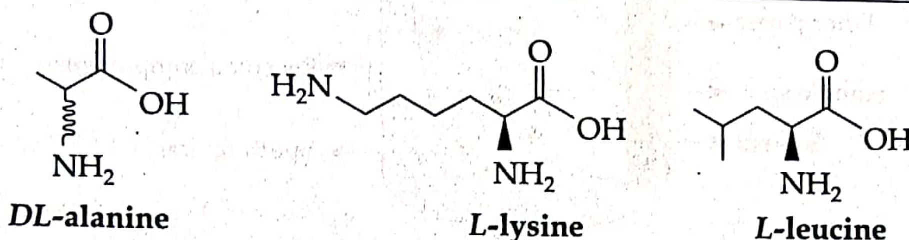
Experiments based on Thin Layer Chromatography (TLC)

7.2.1 TLC Separation of a Mixture Containing 2/3 Amino Acids [*DL*-Alanine, *L*-Lysine and *L*-Leucine]



Chemicals required	Apparatus required
1.DL-alanine	1.Glass plates (12 cm × 4 cm)
2.L-lysine	2.Capillary tubes for TLC
3.L-leucine	3.Solvent chamber
4.Silica Gel for TLC or precoated TLC plates	4.Air oven (100-110°C)
5.Distilled water	
6.*n*-butanol	
7.Glacial acetic acid	
8.Ninhydrin	
9.Ethanol	
10. Chloroform	

Procedure :

1. **Preparation of TLC chromatoplate :** Prepare a moderately thick slurry of TLC silica gel in chloroform in a gas jar. Stir the slurry well with a glass rod to ensure homogeneity as far as possible. Dip the TLC plate into the slurry keeping the gas jar in a slightly inclined position. Take the plate out of the slurry and hold it vertically on the jar so that extra slurry adhered to the plate falls into the jar. Then place the plate horizontally on a rack and allow to dry in air for 10-15 minutes. Scrap off the silica gel from the bottom and backside* of the TLC plate into the jar.

2. **Preparation of the solution of amino acids :** Dissolve 10-15 mg of each of the pure amino acids and the unknown mixture in ~1 ml of distilled water in four different fusion tubes. Label the fusion tubes properly.

3. **Preparation of the developing solvent :** Mix *n*-butanol, glacial acetic acid and distilled water in (4 : 1 : 1, v/v/v) ratio i.e. 80 ml of *n*-butanol and 20 ml each of glacial acetic acid and distilled water.

* TLC glass plates are usually smooth on one side and rough on the other side. The rough side holds the layer of silica gel much better than the smooth side. So here 'backside' refers to the smooth side.

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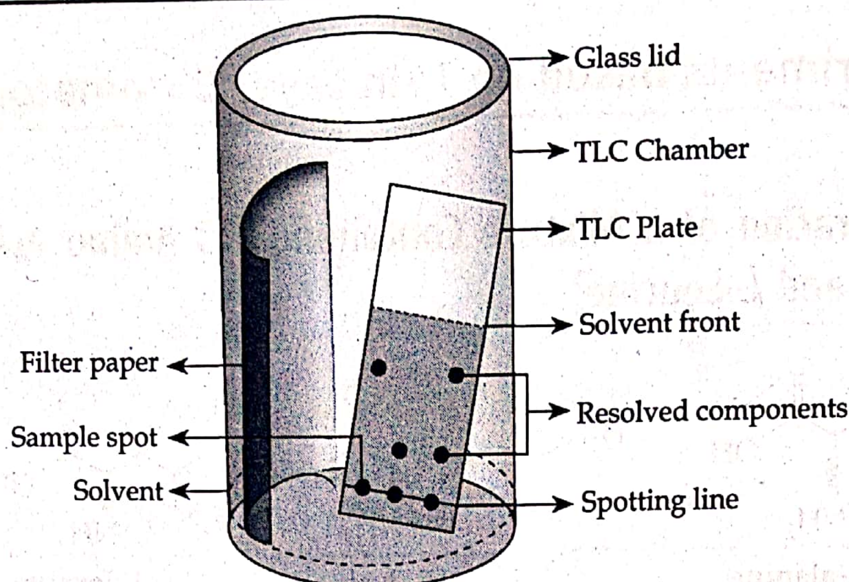

Figure-7.1 Thin layer chromatography

4. **Application of the spots :** Spot the chromatoplate with the three pure amino acids and the unknown mixture using four different capillary tubes. The spots should be ~1 cm above the lower edge of the TLC plate. Try to keep the diameter of the spot within ~2 mm. Dry the spots in air (shown by invisibility of the spots leaving only the marks of capillary pricking on the TLC plate).

5. **Development of the chromatogram :** Dip the dried TLC plate in glass jar containing the developing solvent (*n*-butanol + glacial acetic acid +distilled water in 4:1:1,v/v/v ratio) in such a way that the spots are not immersed into the developing solvent. Cover the jar with lid and allow the developing solvent to rise 7-8 cm. Remove the plate and allow it to dry in air till the wet appearance of the plate disappears.

6. **Identification of the spots :** Spray the dried plate with the ninhydrin reagent (0.5% ethanolic solution of ninhydrin) (spray from a slightly remote distance otherwise some parts of the layer of silica gel may be detached from the plate) and heat the plate in air oven (100-110°C) for 5-10 minutes. Take out the plate from the oven and encircle the purple spots with a pencil. Mark the centre of each of the purple spots with a pencil. Measure the distance travelled by the compounds and the solvent with a scale. Then find out the respective R_f values. Identify the amino acids present in the unknown mixture by comparing the R_f values of the components of the mixture with those of the standards. Draw a sketch of the chromatogram showing the parameters you have measured.

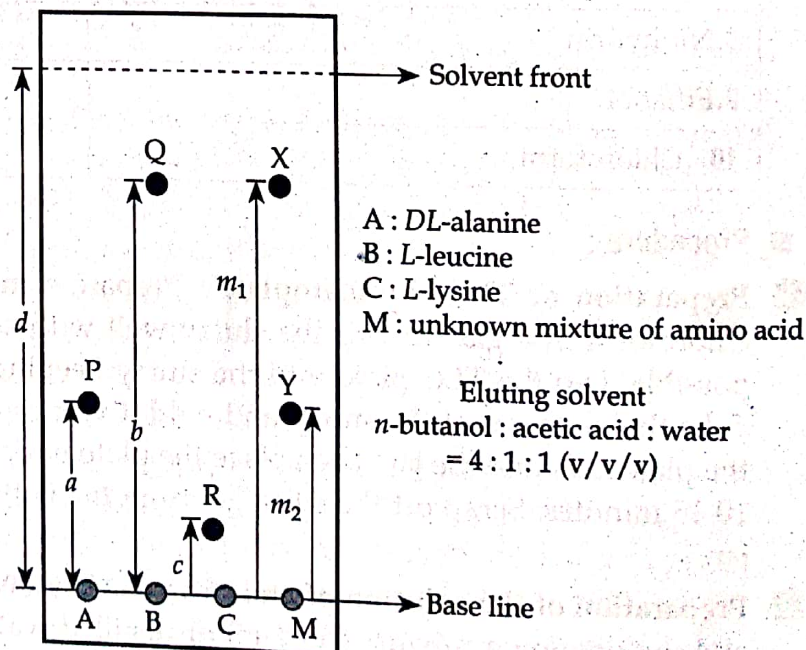

Figure-7.2 Sketch of the thin layer chromatogram : Separation of a mixture of aminoacids

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Experimental Results :

Calculation of R_f values :

[R_f = Distance travelled by the solute/Distance travelled by the solvent front]

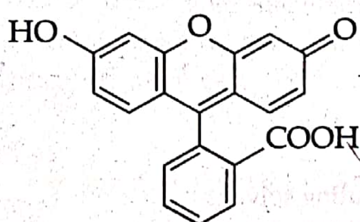
Mobile phase composition : *n* - Butanol : Acetic acid : Water = 4 : 1 : 1 (v/v/v)

Distance travelled by the solvent front from the base line = *d* cm

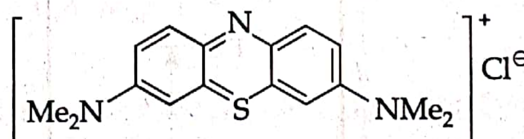
Name of the sample	Distance travelled by the solute from the base line	R_f value
1. *DL* – alanine	AP = *a* cm	a/d
2. *L* - Leucine	BQ = *b* cm	b/d
3. *L* - Lysine	CR = *c* cm	c/d
4. Unknown mixture	XM = m_1 cm YM = m_2 cm	m_1/d m_2/d

By comparing the R_f values of the components X and Y of the unknown mixture of amino acids with the R_f values of the pure amino acids, it is found that the mixture contains ***DL*-alanine** and ***L*-leucine.**

7.2.2 TLC Separation of a Mixture of Dyes (Fluorescein and Methylene blue)



Fluorescein



Methylene blue

Chemicals required	Apparatus required
1. Methylene blue	1. Glass plates (12 cm × 4 cm)
2. Fluorescein	2. Capillary tubes for TLC
3. Silica Gel for TLC or precoated TLC plates	3. Solvent chamber
4. Methanol	4. Air oven (100-110°C)
5. Ethanol	
6. Benzene	
7. Chloroform	

Procedure :

1. **Preparation of TLC chromatoplate :** Prepare the chromatoplate following the procedure as described under the heading of "TLC Separation of a Mixture Containing 2/3 Amino Acids [DL-Alanine, L-Lysine and L-leucine]"(Section 7.2.1).

2. **Preparation of the solution of mixture of dyes :** Prepare a solution of a mixture of fluorescein and Methylene blue by dissolving ~10 mg of each of the compounds in 5ml of ethanol in a test tube.

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3. **Preparation of the developing solvent :** Prepare a mixture of chloroform and methanol in 9 : 1 ratio (v/v) as the developing solvent.
4. **Application of the spots :** Spot the chromatoplate with the mixture of dyes using a capillary tube. The spots should be ~1 cm above the lower edge of the TLC plate. Try to keep the diameter of the spot within ~2 mm.
5. **Development of the chromatogram :** Dip the dried TLC plate in glass jar containing the developing solvent (chloroform + methanol in 9:1 v/v ratio) in such a way that the spots are not immersed into the developing solvent. Cover the jar with lid and allow the developing solvent to rise 7-8 cm. Remove the plate and allow it to dry in air till the wet appearance of the plate disappears.
6. **Identification of the spots :** The upper yellow spot is of that of fluorescein (R_f ~ 0.56) and the lower blue spot corresponds to Methylene blue ((R_f~0.16).

   Determine the R_f values from a measurement of the distance travelled by the dyes and the developing solvent.

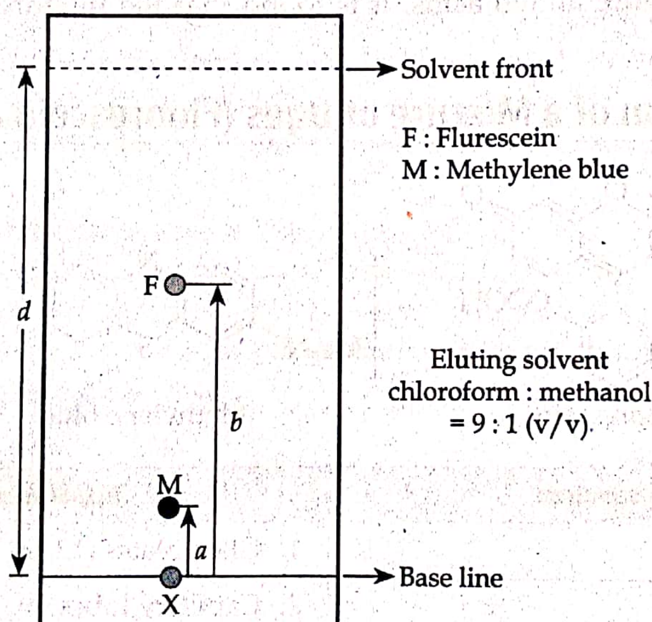

Figure-7.3 Sketch of the Thin layer chromatogram : Separation of Fluorescein and Methylene blue in a mixture

Experimental Results :

Calculation of R_f values :

[R_f = Distance travelled by the solute/Distance travelled by the solvent front]

Mobile phase composition : Chloroform : Methanol = 9 : 1 (v/v)

Distance travelled by the solvent front from the base line = d cm

Name of the sample	Distance travelled by the solute from the base line	R_f value
1. Methylene blue	XM = a cm	a/d
2. Fluorescein	XF = b cm	b/d

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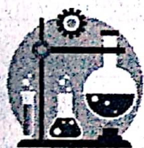
7.3 Column Chromatography (CC)

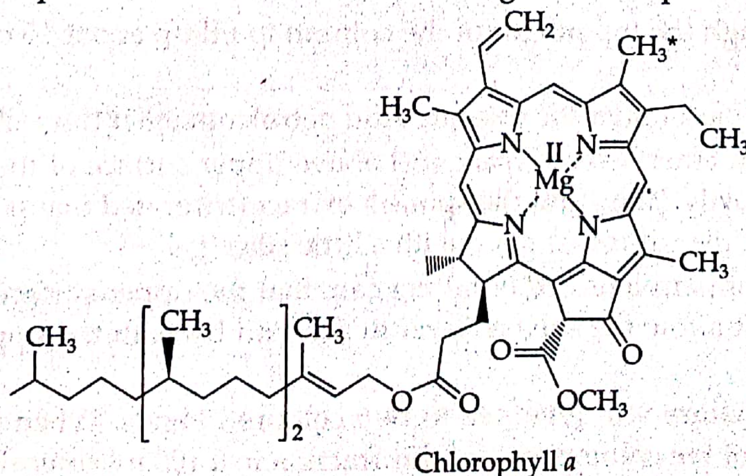
In column chromatography the stationary phase is kept within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle of the tube (open tubular form). The sample is dissolved in minimum volume of a solvent with as low polarity as possible. If the mixture dissolves in a highly polar solvent it is preferentially triturated with silica gel and the triturated, almost dry mass is poured into the column. The column can be packed in two ways. In one way a solvent is poured into the column and then the stationary phase is added on to the solvent to fill up the column (Wet pack). If the reaction mixture is difficult to be stripped off the solvent due to the very high boiling point of the solvent, it is directly poured on the dry bed of adsorbent stationary phase. (Dry pack).The column is then eluted with suitable solvent and fractions are collected from time to time. Elution of different components of the mixture can be monitored by TLC. After elution of one component the eluant may have to be changed for the elution of other components. However if the components under investigation can form different coloured zones(bands) on the chromatogram, the bands may be separated by elution with appropriate solvents and monitoring by TLC may not be needed. However performing a TLC of the mixture can give a clue to the choice of solvents for eluting out the different components. Usually upper spots in the TLC are eluted out with relatively low polar solvents and the lower spots in the TLC are eluted out with relatively high polar solvents. After completion of elution the fractions having identical spots on TLC are combined and the solvent is evaporated to obtain the component in the pure form.



Experiments based on Column Chromatography (CC)

7.3.1 Column Chromatographic Separation of Leaf Pigments from Spinach Leaves

Two categories of molecules are primarily responsible for photosynthesis in plants: the chlorophylls and the carotenoids. Chlorophylls, the green pigments, absorb certain wavelengths of light that are then converted into chemical energy. Examples of chlorophylls are chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and pheophytin *b*. The structure of chlorophyll a is shown below. In chlorophyll *b*, the starred (*) methyl group is replaced by a –CHO group. Pheophytin *a* and *b* are identical to chlorophyll *a* and *b*, respectively, except that in these molecules, the Mg^{2+} ion is replaced by $2H^+$ ions.



Chlorophyll *a*

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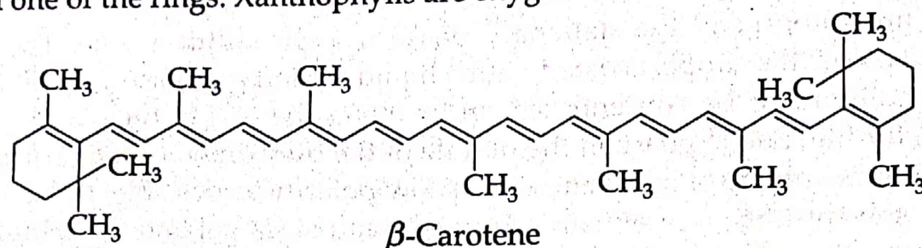
Carotenoids, the yellow pigments found in spinach, are also involved in photosynthesis. The structure of β-carotene is given below. α-Carotene differs from β-cretonne in the placement of the double bond on one of the rings. Xanthophylls are oxygen-containing derivatives of the carotenes.



β-Carotene

Chemicals required	Apparatus required
1. Fresh spinach leaves (20 g)	1. Mortar and pestle
2. Silica gel (60-120 mesh) for column chromatography	2. Glass chromatographic column
3. Petroleum ether	3. Stand and clamp
4. Acetone	4. Measuring cylinder (100 ml)
5. Distilled water	5. Conical flasks (100 ml)
6. Sodium sulfate	6. Beaker (50 ml)
	7. Funnel
	8. Dropper
	9. Spatula
	10. Cotton

Procedure :

① Cut ~20 g of fresh spinach leaves into fine pieces and put in a clean, dry porcelain morter. Add a mixture of 20-25 ml of petroleum ether and acetone (3:2, v/v) to the morter and grind thoroughly with a pestle till the formation of a dark green liquid. Filter the liquid in a 100 ml conical flask and add 2-3 g of anhydrous sodium sulfate to remove traces of water. Decant off the supernatant liquid in a beaker. Wash the sodium sulfate with 2-3 ml of petroleum ether and mix the washings with supernatant liquid. Carefully concentrate the contents of the beaker to about half its volume on a water bath. Add silica gel and stir with a spatula to form a free flowing powder (triturated mass).

② Fix the glass chromatography column to a stand with a clamp. Insert a small piece of cotton at the bottom of the column using a long glass rod.

③ Pour silica gel through the top mouth of the column to fill up about 15 cm of the column. Keep the stopper open.

④ Put a 100 ml conical flask under the column. Add petroleum ether through the top of the column.

⑤ Allow the petroleum ether level to reach just above upper surface of the bed of silica gel when the column drips slowly. Then add the spinach extract (triturated mass) into the column. Cover the upper surface of the triturated mass with a little silica gel.

⑥ Continue addition of petroleum ether taking care that the upper surface of the silica gel bed is not disturbed. Don't allow the solvent level to descend beneath the upper surface of the silica gel bed.

⑦ Original spinach mixture will give rise to two coloured bands. When the lower yellow band reaches the bottom of the column, collect this fraction in a 100 ml conical flask.

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⑧ After complete elution of the yellow band allow the petroleum ether level to drop below the upper surface of the silica gel bed. Start adding acetone and keep the solvent level always above the upper surface of the silica gel bed. The green band will move down the column. When this green band reaches the bottom collect it in another 100 ml conical flask.

⑨ Careful evaporation of the extracts gives the pigments in the solid state.

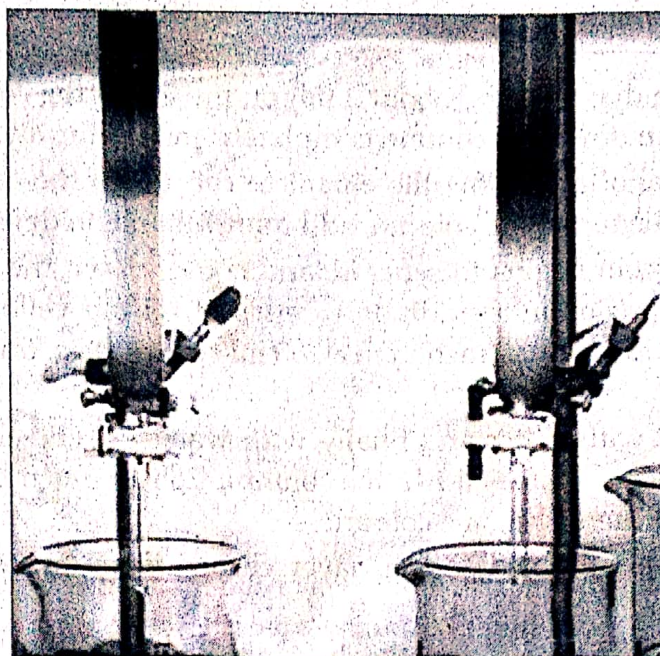
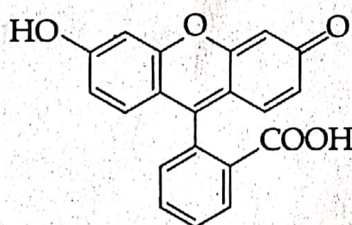
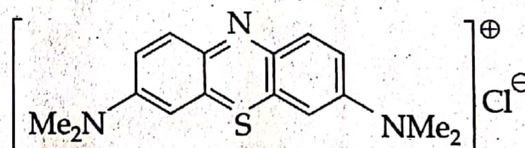


Figure-7.4 Separation of bands of pigments of spinach leaves in column chromatography

7.3.2 Column Chromatographic Separation of Mixture of Dyes (Fluorescein and Methylene blue)



Fluorescein



Methylene blue

Chemicals required	Apparatus required
1. Fluorescein	1.Test tube
2. Methylene blue	2.Glass chromatographic column
3. Silica gel (60-120 mesh) for column chromatography	3. Stand and clamp
4. Ethanol	4. Measuring cylinder (100 ml).
5. Water	5. Conical flasks (100 ml), 3 pieces
6. Acetic acid	6. Cotton
	7. Dropper
	8. Spatula

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Procedure :

1. Prepare a solution of the dye mixture by dissolving ~25-30 mg of each of fluorescein and Methylene blue in 10 ml of ethanol in a test tube.
2. Fix the glass chromatography column to a stand with a clamp. Insert a small piece of cotton at the bottom of the column using a long glass rod.
3. Pour silica gel through the top mouth of the column to fill up about 15 cm of the column. Keep the stopper open.
4. Add the solution of the dye mixture (about 2 ml) on the top of the column with a dropper and allow the solution to run down the column completely and uniformly.
5. Elute with 5 ml of ethanol and allow the eluant to run down the column. When the ethanol level is 2-3 mm above the top of the column, add more ethanol to develop the coloured bands.
6. Yellow band of fluorescein begins to separate and comes down the column while methylene blue remains at the top. Continue addition of ethanol till the yellow band reaches near the bottom of the column. Collect this fraction in a 100 ml conical flask until the lower end becomes colourless.
7. Then elute the column with water and finally with water - acetic acid (7 : 3, v/v) when the blue band of methylene blue comes down the column. Collect this fraction in a separate 100 ml conical flask until the effluents become colourless.
8. Separated dyes may be subjected to thin layer chromatography to check the efficiency of separation.
9. Careful evaporation of the extracts gives the dyes in the solid state.

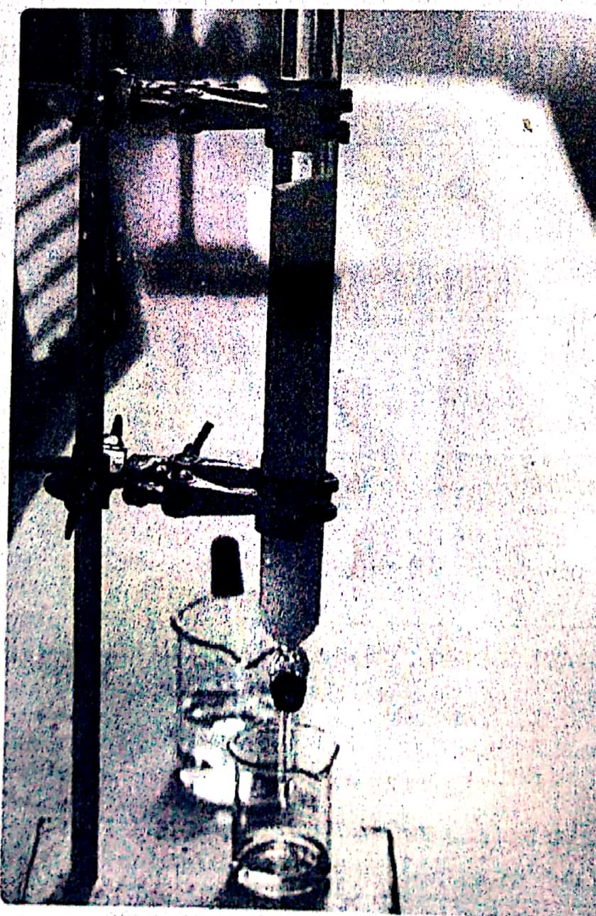

Figure-7.5 Separation of bands of fluorescein and methylene blue in column chromatography

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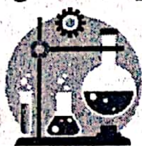
NOTE:
Fluorescein contains acidic functionality. So its binding with silica gel, which is acidic in nature, is not strong. Basic nature of methylene blue causes strong binding with silica gel. This is why fluorescein comes out of the silica gel column earlier than Methylene blue. Use of basic alumina instead of silica gel just reverses the order of elution. Acidic fluorescein strongly binds with basic alumina and is retained in the column for a longer period than methylene blue which weakly binds with basic alumina.

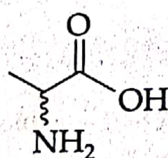
7.4 Paper Chromatography (PC)

In paper chromatography adsorbed water molecules present in the filter paper supported by the cellulose molecules of the paper constitute the stationary phase. A paper strip made from Whatman No. 1 filter paper is spotted with a very dilute solution of the mixture taken for separation using a fine capillary tube near one end of the paper. The paper is then hanged in a glass jar containing the developing solvent in such a way that the spots are not immersed in the solvent and the lower end of the paper does not touch the bottom of the jar but the bottom of the paper remains in contact with the solvent. The developing solvent is allowed to rise a suitable distance along the paper which develops the chromatogram. The paper is then dried and sprayed with appropriate reagents to figure out the location of the separated components.(If necessary the paper has to be kept in a hot air oven for the development of spots) If the separated components impart colour on the chromatogram spraying with the suitable reagent is not necessary. The separated components may be identified by comparing the respective R_f values with those of standard samples.

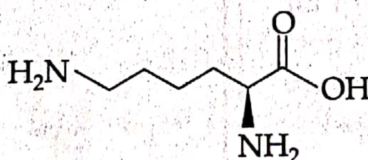


Experiments based on paper Chromatography (PC)

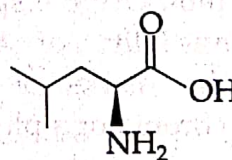
7.4.1 Paper Chromatographic Separation of a Mixture Containing 2/3 Amino Acids [*DL*-Alanine, *L*-Lysine and *L*-Leucine]



DL-alanine



L-lysine



L-leucine

Chemicals required	Apparatus required
1. *DL*-alanine	1.Whatman No.1 chromatography paper strip (20 cm × 4 cm)
2. *L*-lysine	2.Capillary tubes
3. *L*-leucine	3.Solvent chamber
4. Distilled water	4.Air oven (100-110°C)
5. *n*-butanol	
6. Glacial acetic acid	
7. Ninhydrin	
8. Ethanol	

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■ **Procedure :**

1. **Preparation of the developing solvent :** Mix *n*-butanol, glacial acetic acid and distilled water in (4:1:1, v/v/v) ratio i.e. 80 ml of *n*-butanol, and 20 ml each of glacial acetic acid and distilled water.

2. **Preparation of the solution of amino acids :** Dissolve 10-15 mg of each of the pure amino acids and the unknown mixture in ~1 ml of distilled water in four different fusion tubes. Label the fusion tubes properly.

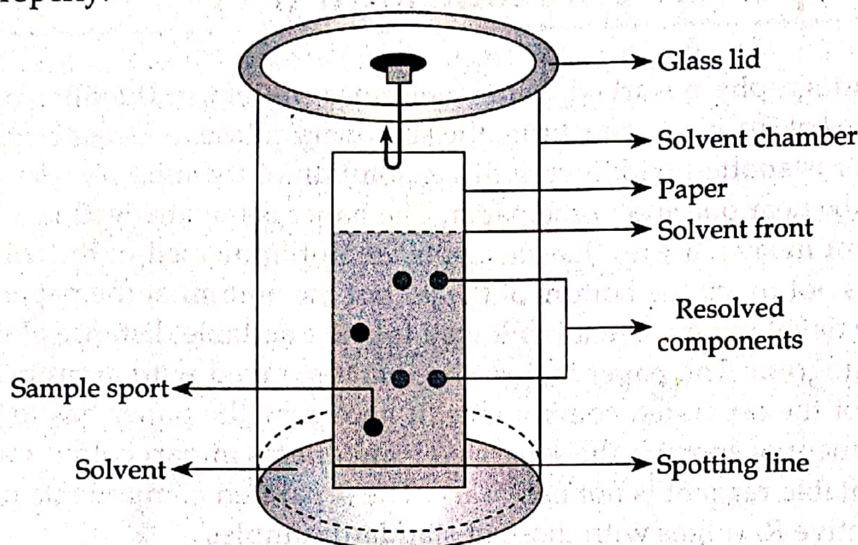

Figure-7.6 Paper chromatography

3. **Application of the spots :** Draw a faint straight line with a pencil ~3 cm above the lower end of the chromatography paper. Spot the chromatography paper with the three pure amino acids and the unknown mixture using four different capillary tubes on the straight line. Keep a distance of ~0.7-0.8 cm in between the spots. Try to keep the diameter of the spot within ~2 mm. Dry the spots in air (shown by invisibility of the spots leaving only the marks of capillary pricking on the chromatography paper).

4. **Development of the chromatogram :** Hang the dried chromatography paper in glass jar containing the developing solvent (*n*-butanol + glacial acetic acid + distilled water in 4:1:1, v/v/v ratio) in such a way that the spots are not immersed into the developing solvent but the lower end of the paper touches the developing solvent. Cover the jar with lid and allow the developing solvent to rise 8-10 cm. Remove the paper and allow it to dry in air till the wet appearance of the paper disappears.

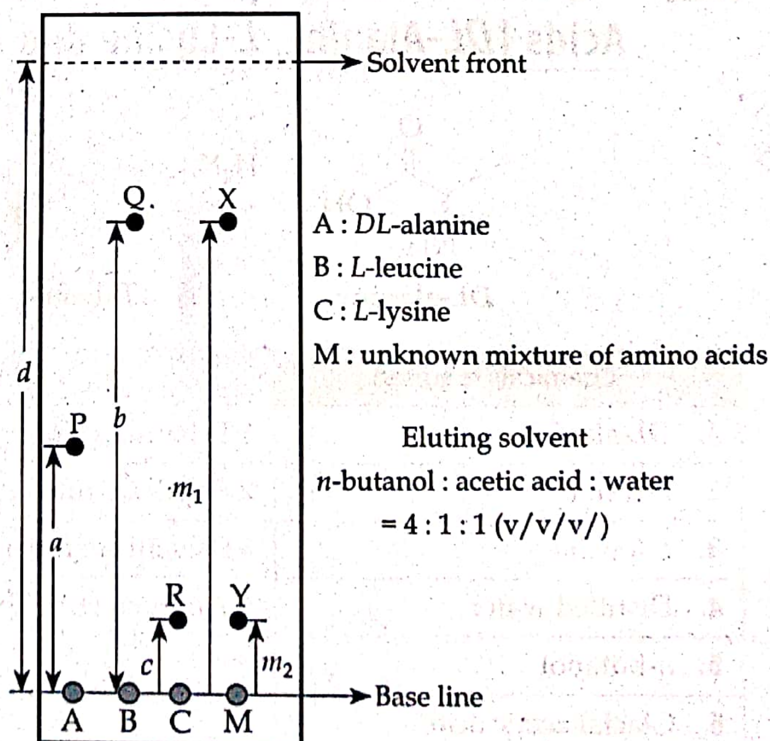

Figure-7.7 Sketch of Paper Chromatogram : Separation of a mixture of amino acids

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5. **Identification of the spots :** Spray the dried chromatography paper with the ninhydrin reagent (0.5% ethanolic solution of ninhydrin) and heat the paper in air oven (100-110°C) for 5-10 minutes. Take out the paper from the oven and encircle the purple spots with a pencil. Mark the centre of each of the purple spots with a pencil. Measure the distance travelled by the compounds and the solvent with a scale. Then find out the respective R_f values. Identify the amino acids present in the unknown mixture by comparing the R_f values of the components of the mixture with those of the standards. Draw a sketch of the chromatogram showing the parameters you have measured.

Experimental results :

Calculation of R_f values :

[R_f = Distance travelled by the solute/Distance travelled by the solvent front]

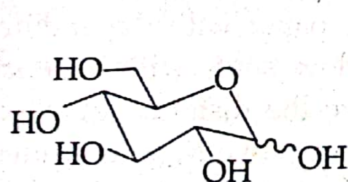
Mobile phase composition : *n*-butanol : Acetic acid : Water = 4 : 1 : 1 (v/v/v)

Distance travelled by the solvent front from the base line = *d* cm

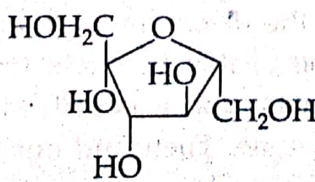
Name of the sample	Distance travelled by the solute from the base line	R_f value
1. *DL* – alanine	AP = *a* cm	a/d
2. *L* - leucine	BQ = *b* cm	b/d
3. *L* - lysine	CR = *c* cm	c/d
4. Unknown mixture	XM = m_1 cm YM = m_2 cm	m_1/d m_2/d

By comparing the R_f values of the components X and Y of the unknown mixture of amino acids with the R_f values of the pure amino acids, it is found that the mixture contains **L-leucine and L- lysine.**

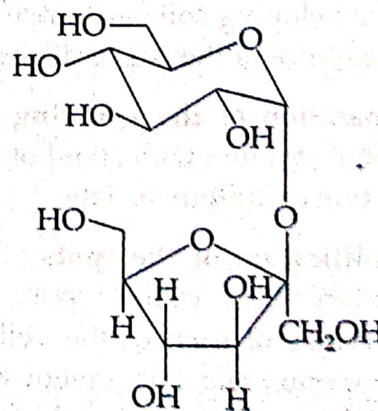
7.4.2 Paper Chromatographic Separation of a Mixture Containing 2/3 Sugars [Glucose, Fructose and Sucrose]



D-Glucose



D-Fructose



D-Sucrose

Chemicals required	Apparatus required
1. *D*-Glucose	1. Whatman No.1 chromatography paper strip (20 cm × 4 cm)
2. *D*-Fructose	2. Capillary tubes
3. *D*-Sucrose	1. Solvent chamber
4. Distilled water	2. Air oven (100-110°C)
5. *n*-Butanol	
6. Glacial acetic acid	
7. Ethanol	
8. Aniline	
9. Oxalic acid	

Procedure :

1. **Preparation of the developing solvent :** Mix *n*-Butanol, glacial acetic acid and distilled water in (4:1:5, v/v/v) ratio i.e. 80ml of n-butanol, 20 mlof glacial acetic acid 100 ml of distilled water.

2. **Preparation of the solution of sugars:** Dissolve 30-35 mg of each of the pure sugars and the unknown mixture in ~0.5ml of distilled water in four different fusion tubes. Label the fusion tubes properly.

3. **Application of the spots :** Draw a faint straight line with a pencil above the lower end of the chromatography paper. Spot the chromatography paper with the three pure sugars and the unknown mixture using four different capillary tubes on the straight line. Keep a distance of ~0.7-0.8 cm in between the spots. Try to keep the diameter of the spot within ~2 mm. Dry the spots in air (shown by invisibility of the spots leaving only the marks of capillary pricking on the chromatography paper).

4. **Development of the chromatogram :** Hang the dried chromatography paper in glass jar containing the developing solvent (*n*-butanol + glacial acetic acid +distilled water in 4:1:5, v/v/v ratio) in such a way that the spots are not immersed into the developing solvent but the lower end of the paper touches the developing solvent. Cover the jar with lid and allow the developing solvent to rise 8-10 cm. Remove the paper and allow it to dry in air till the wet appearance of the paper disappears.

5. **Preparation of the spraying reagent :** Dissolve 10-12 drops of aniline in 50 ml of absolute alcohol and mix with 50 ml of 0.2 (M) aqueous solution of oxalic acid. The resulting solution is a solution of aniline oxalate.

6. **Identification of the spots :** Spray the dried chromatography paper with the aniline oxalate solution when yellow spots will be visible. Encircle the yellow spots with a pencil. Mark the centre of each of the yellow spots with a pencil. Measure the distance travelled by the compounds and the solvent with a scale. Then find out the respective R_f values. Identify the sugars present in the unknown mixture by comparing the R_f values of the components of the mixture with those of the standards. Draw a sketch of the chromatogram showing the parameters you have measured.

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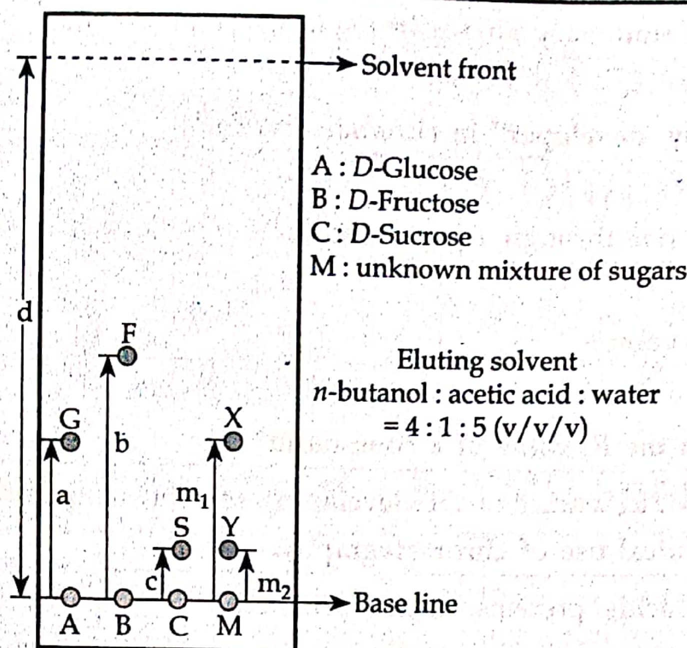

Figure-7.8 Sketch of Paper Chromatogram : Separation of a mixture of sugars

Experimental results :

Calculation of R_f values :

[R_f = Distance travelled by the solute/Distance travelled by the solvent front]

Mobile phase composition : *n*-butanol : Acetic acid : Water = 4 : 1 : 5 (v/v/v)

Distance travelled by the solvent front from the base line = *d* cm

Name of the sample	Distance travelled by the solute from the base line	R_f value
1. *D*-Glucose	AG = *a* cm	a/d
2. *D*-Fructose	BF = *b* cm	b/d
3. *D*-Sucrose	CS = *c* cm	c/d
4. Unknown mixture	XM = m_1 cm YM = m_2 cm	m_1/d m_2/d

By comparing the R_f values of the components X and Y of the unknown mixture of sugars with the R_f values of the pure sugars, it is found that the mixture contains D-Glucose and D-Sucrose.

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