EXPERIMENT 6
THIN LAYER CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF AMINO ACIDS

Structure

6.1 INTRODUCTION

In the last two experiment you have studied about the paper chromatography. In this part you will learn how to perform thin layer chromatography for the separation of amino acids.

Thin layer chromatography (TLC) is an efficient method of separating complex mixtures. It is a sensitive, fast, simple and inexpensive analytical technique in carrying out small scale experiments.

One of the important applications of TLC is the separation of amino acids. Amino acids contain both the amino groups as well the carboxylic groups. The most important are the $\alpha-$ amino acids as these are the units from which proteins are made.

In this experiments you will learn the movement of some simple amino acids on silica gel coated plates. TLC of amino acids is based on their distribution between a finely divided powder of an adsorbent and an organic mobile phase.

Objective

After studying and performing this experiment, you should be able to:

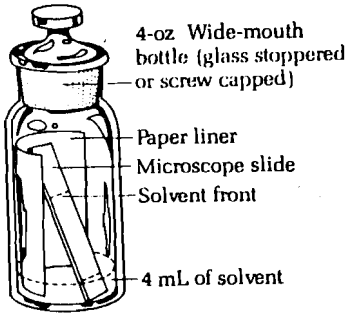
- explain the basic principle of TLC, and
- separate amino acids by TLC

6.2 PRINCIPLE

TLC is similar to PC in that the sample is spotted near one end of a plate of glass or plastic coated with a thin layer of an adsorbent. The TLC plate is place in a covered jar containing a shallow layer of developer. The developer rises up by capillary action and the solute is distributed between the stationary (absorbent) phase and the mobile phase. A solute which is more strongly adsorbed onto the stationary phase, will spend less time in the mobile phase, and hence it will migrate more slowly up the TLC plate. The sample is subsequently separated by development (elution). Treatment with a detector forms the coloured zones of the solutes. The components of a mixture are identified by the calculation and comparison of R_f values.

6.3 Requirements

Apparatus		Chemicals
TLC jar		Propanol-1
(An alternative is a beaker covered		Conc. Ammonia solution
by a watch glass or aluminum foil)		Any three amino acid from the following:
Spotting capillaries		L-alanine
Measuring cylinder100 cm^3		L-Leucine
TLC plates (Either arranged from a	5	L-Lysine
supplier or prepared by the teacher)		L-Aspartic acid
Spraying bottle	1	Methionine

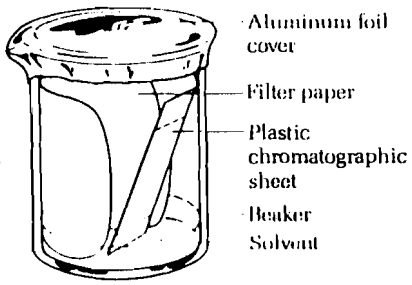


Apparatus for TLC chromatography

Preparation of TLC Plates

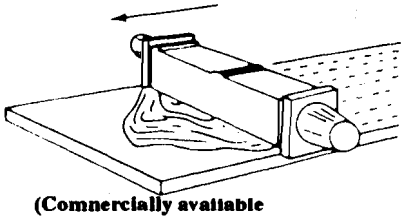
TLC plates can be prepared by one of the following methods:

A. Dipping : Combine 33cm^3 of methanol and 67 cm^3 of chloroform in a 125 cm^3 screw-cap jar, stir in 35g of Silica Gel G, and shake the capped jar vigorously for about a minute, Stack two clean microscope slides back-to-back, holding them together at the top. Without delay dip them into the slurry for about 2 seconds, Touch the bottom of the stacked slides to the jar to drain off the excess slurry, let them air dry a minute or so to evaporate the solvent, separate them and wipe the excess adsorbent off the edges with a tissue paper. Activate the slides by heating them in oven at 110°C for 15 minutes, or by placing them in a covered beaker heated to that temperature.



An alternate method for developing TLC plate

B. Spreading : Take a clean glass plate Mix about 10 g of Silica Gel G with 20 cm^3 of water (stirring and shaking well to get out any lumps) and pour on the glass plate. Spread it out with the help of TLC applicator. Let the plate air dry for ten minutes and put the plate at 110°C in a oven for at least 30 minutes to activate the adsorbent.



(Comnercially available applicator)

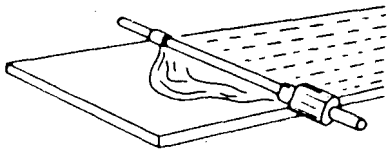
Solutions provided

1. **Sample Solution:** Provide solution of any three amino acid as above. Make one of amino acid solution as unknown sample.

   Their solution can be prepared by dissolving 15 mg of each amino acid separately in 1 cm^3 of distilled water. Warm if a particular amino acid is not soluble in cold.

2. **Detector:**

   Ninhydrin Reagent (0.2%): Take 100 cm^3 of 1-butanol and 100 cm^3 of water in a separatory funnel. Shake gently and allow it to from the layers. Remove the lower aqueous layer. Transfer the upper organic layer to a spraying bottle and to this add 0.2 g of ninhydrin, shake well and use as the detector for amino acids.



(A lab made applicator)

6.4 PROCEDURE

Proceed according to the following steps:

1. Preparation of Developer: Prepare the developer by mixing 1- propanol and concentrated ammonia in the proportion of 7:3 respectively by measuring the required volumes with the help of a measuring cylinder.
2. Take 4 silica gel coated TLC plates from your teacher.
3. Dry the plates in an oven for 30 minutes at 100°C, so that they have been activated for adsorption chromatography.
4. Take a plate and make a light pencil line across it, 1 cm above the bottom of the plate and put a short mark at the line centre where a known or unknown amino acids will be spotted.

Remove a thin strip of the layer from the edge of the TLC plate by means of a thumb nail or a spatula.

5. Label the plate at the top end to indicate known or unknown amino acids.
6. Hold the plate in the left hand cautiously, so that the fingers do not touch the adsorbent layer. Take a capillary and place in the solution of amino acid to be spotted, let the solution rise into the capillary, take out the capillary from the solution and gently touch the capillary to the layer side of the TLC plate at the marked centre. Allow to flow the solution on the plate for a short duration so that a spot of the solution is formed but not larger than 2 mm in diameter.

While marking the plates with pencil, do not press so hard on the pencil that you remove the silica gel.

Due to the limited solubility of various amino acids, great care must be taken in the preparation of the sample before applying.

(**Note:** The teacher is supposed to demonstrate this technique).

7. Allow the spot to dry. You can blow in order to aid evaporation. Apply more solution at the same place (if required). The aim is to apply a small but visible and built up spot.

The spot applied should be kept as small as possible. Application of too much of the solute should be avoided, as this will result in an elongated zone and will affect the correct calculation of R_f values.

8. Apply the unknown solution on a separate TLC plate in a similar manner.
9. After spotting all the known and unknown solutions, insert the plates into the developing jars (one plate in each jar).
10. Pour the mobile phase into the chamber, with the help of a pipette till the developer level reaches nearly at 1 cm height of the lower edge of the adsorbent layer on the plate (Remember that the spot should be above this level).
11. Cover the jar and allow the devloper to ascend along the plate. The position of the solvent front can be seen visually as the damp portion of the plate appears darker than the dry portion.
12. When the developer ascends to a required height on the plate, remove the plate from the developing chamber, mark the solvent front and dry the plate at 100°C for about 10 minutes.
13. After the plates have been dried, spray the detector on the plates with the help of a spraying bottle. The detector is 0.2% ninhydrin solution in butanol saturated with water.
14. Heat the plates at 110°C, either in an oven or on a hot plate, for 5-10 minutes, till the zones of amino acids appear as coloured spots on the plates.
15. Mark the periphery of the coloured spots and their centres.
16. Measure the distance of each spot-centre from the starting line and also the distance by which the solvent front, has moved. Calculate the R_f values.
17. From the comparison of R_f values of known and unknown samples you can determine which amino acids are present in your unknown.

6.5 OBSERVATIONS AND CALCULATIONS

Observe the colour of the spots of various amino acids on TLC plates. Measure the distance to which the centre of the amino acid has moved from the original (ds), and the distance which the solvent front has moved from the point of application (dm) on the chromatoplate.

Calculate the R_f values by the relation

R_f=ds/dm, for each known amino acid and for the unknown. Present the data in a table below.

Observation Table
Separation of amino acids by TLC

Amino Acid	ds	dm	R_f=ds/dm Remark
Leucine			
Lysine			
Alanine			
Methonine			
(known)			
Unknown			R_f resemble with .....
1.			R_f resemble with .....
2.			

6.6 RESULT AND DISCUSSION

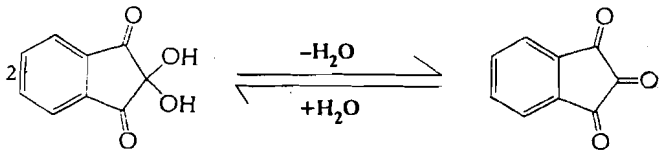
The unknown sample contains:

1.

2.

The mobile phase rises up along the plate by capillary action, rapidly at first, and then more slowly as the solvent front rises. The movement of an amino acid along the TLC plate depends on its adsorptivity, on the adsorbent layer, the solubility in the mobile phase and a number of other factors. Therefore, the different amino acids move along the plate at different rates and may have different R_f values. The significant differences in R_f values of certain amino acids results into a clean separation.

The most widely used reagent for detecting amino acids is ninhydrin. Ninhydrine is the 2-hydrate of indane-1, 2, 3 trione (or triketohydrindene hydrate) with the following formula.



Hydrate form of Ninhydrin

Unhydrated form of Ninhydrin

It reacts with amino acids to yield highly coloured products.

Amino acid + Ninhydrin $\xrightarrow{\text{heat}}$ coloured product.

The formation of visible colour with ninhydrin has limits of detection that may vary from 0.01-0.5 μg depending on the particular amino acids.