Thin Layer Chromatography

Aim:

To separate a mixture of amino acids by Thin Layer Chromatography (TLC) and identify the test amino acids by measuring their Rf values.

Introduction:

Chromatography is the process through which biomolecules are separated and analysed from a complex mixture. This separation process consists of two phases: a stationary phase and a mobile phase. The mobile phase consists of the mixture to be separated which percolates through the stationary phase. These two phases can be solid-liquid, liquid-liquid or gas-liquid. Thin Layer Chromatography (TLC) is a solid-liquid form of chromatography where the stationary phase is a polar absorbent and the mobile phase can be a single solvent or combination of solvents.

Principle:

Thin Layer Chromatography (TLC) is a type of chromatography which is based upon the distribution of biomolecules between two immiscible phases. TLC was originally developed to separate lipid molecules and can be used to identify components in a sample, and for preparative purposes. In TLC the stationary phase is a polar absorbent, like finely ground alumina (Al2O3) or silica (SiO2) particles which are coated on a glass slide or plastic sheet to create a thin layer of the particular stationary phase. Silica contains some free – OH groups which form hydrogen bonds or other Van-der-Waals interactions with the analyte components and as a result adsorption takes place. Sometimes a small amount of a binder such as plaster of Paris is mixed with the absorbent to facilitate the coating.

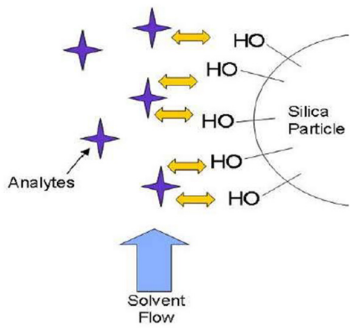


Fig 1: The free OH groups of silica interacts with the analytes (materials to be separated)

The mixture to be separated is dissolved in a solvent and the solution is spotted at one end of the coated TLC plate next to the reference material. In order to determine whether an unknown substance is the same as a substance of known structure, it is necessary to run the two substances side by side in the same chromatogram, preferably at the same concentration. The plate is placed with spotted end down in a covered jar containing a shallow layer of suitable solvent. The solvent (mobile phase) is allowed to move up the plate by capillary action through the adsorbent at its own rate and as a result differential partitioning occurs between the components of the mixture dissolved in the solvent and the stationary adsorbent phase. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate. When the solvent front has moved to within about 1 cm of the top end of the adsorbent, the plate should be removed from the developing chamber.

If the components of the sample are coloured, they can be observed directly. If not, they can sometimes be visualized by shining ultraviolet light on the plate or by spraying the plate with a reagent (e.g. ninhydrin) that will react with one or more of the components of the sample. Sometimes the spots can be visualized by allowing the plate to stand for a few minutes in a closed container in which the atmosphere is saturated with iodine vapor. The ninhydrin reaction is used to detect the presence of amino acids. Amino acids contain a free amino and carboxyl group which reacts together with ninhydrin to produce a characteristic blue colour (or occasionally pale yellow). In this reaction first an amino group is attached to the first or alpha carbon of the amino acid's carbon chain and then the nitrogen atom of the amino group reacts with ninhydrin to give a blue-purple product known as Ruhemann's purple as shown in figure 2. Some amino acids (e.g. proline, secondary amine) give yellow-orange colour.

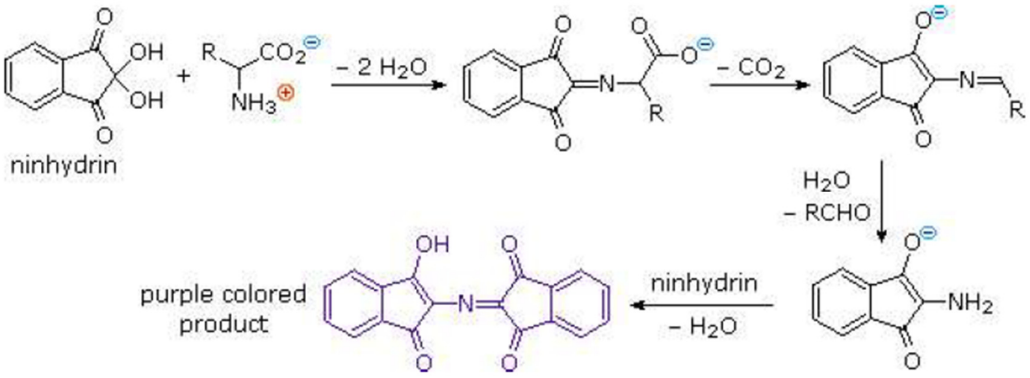


Fig 2: During ninhydrin reaction amino groups of proteins react with ninhydrin to form blue-violet compound called Rhumann's purple.

In addition to qualitative results, TLC can also provide chromatographic separation of biomolecules because the distance travelled by a substance relative to the distance travelled by the solvent front depends upon the molecular structure of the substance. The relationship between the distance travelled by the solvent front and the substance is usually expressed as the Rf value which is also called 'retardation factor' and expressed as following:

R_f = distance travelled by substance/ distance travelled by solvent front

The Rf values strongly depend upon the nature of the adsorbent and solvent. Therefore, for the separation and subsequent identification of amino acids in a given mixture, Rf values of individual amino acids have to be calculated by performing Thin Layer Chromatography.

Requirements:

1. Isoleucine
2. Histidine
3. Tryptophan
4. Glycine
5. Test Sample
6. TLC plate
7. Developing reagent
8. Solvent for TLC
9. **Glasswares:** TLC Chamber (with lid) or a beaker that can fit the TLC plate
10. **Other requirements:** Micropipette, Tips, Hot air oven/Incubator

Important Instructions:

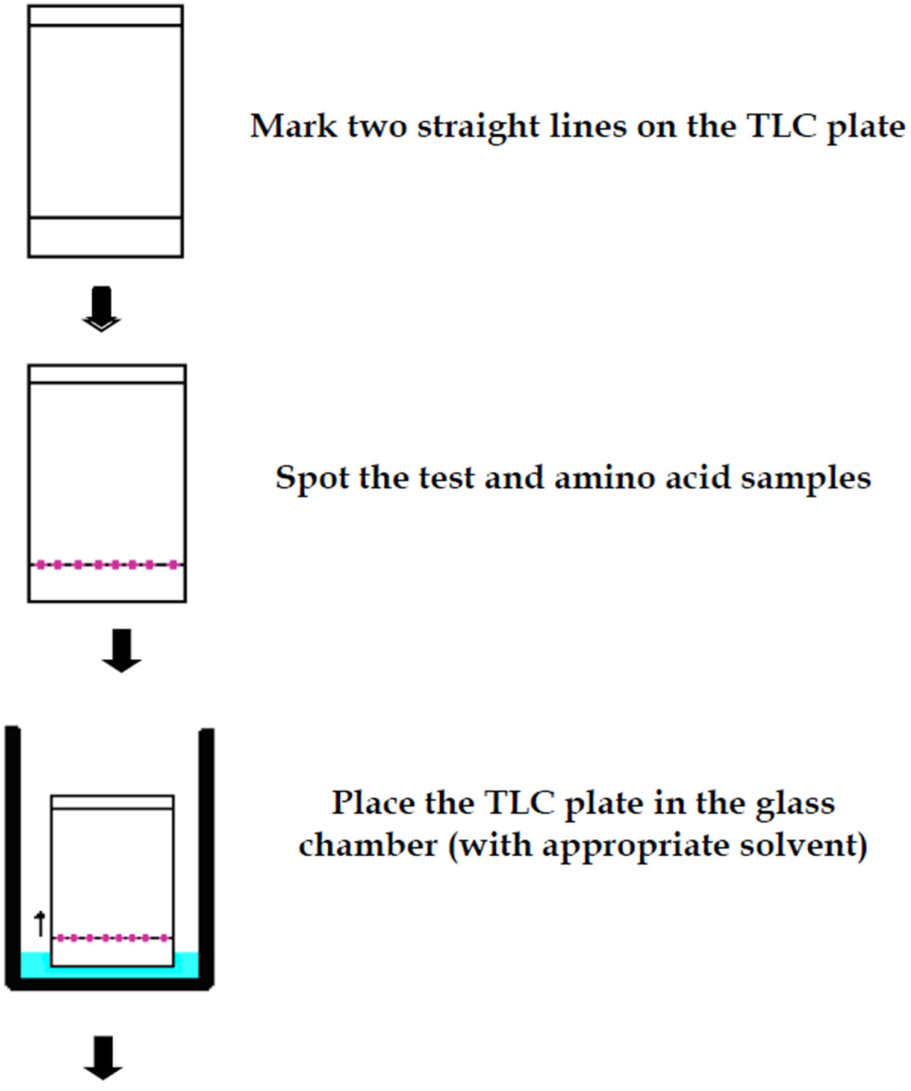
1. Before starting the experiment the entire procedure has to be read carefully.
2. Allow all the components to come to room temperature before starting the experiment.
3. Change the tip while spotting each amino acid.
4. Always wear gloves while handling reagents and TLC plates.

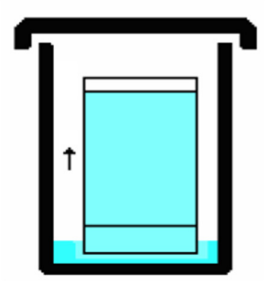
Procedure:

1. Take a TLC plate and with the help of pencil draw two straight lines on the white surface of the plate: one 2 cm from the bottom of the plate and another 1 cm from top of the plate. Never use a pen as dyes (used in ink) may interfere with the results by developing spots on the plate.
2. Mark 10 equidistant points on the bottom line for loading of amino acids samples and test sample. While marking the lines and points do not make a trough with the pencil.
3. Allow all the amino acid samples and test sample to come to room temperature. Then spot 1 µl of each amino acid and test sample along the bottom line on the TLC plate. While spotting use separate tips for each sample.
4. Allow the plates to air dry (~ 10 minutes). Further drying should be done by keeping the TLC plate at 70□C in a hot air oven or incubator for 2 – 3 minutes.
5. Take 10 ml of solvent system in the TLC chamber (with lid) and keep for 10 minutes at room temperature.

6. Place the TLC plate inside the chamber with clean forceps. While keeping the plate make sure that the spotted samples are near the solvent. Furthermore, the TLC plate should be in a straight position so that the solvent phase can move uniformly along the plate.
7. Allow the solvent front to reach the top line of the plate. After that take it out with the help of clean forceps and air dry the plate for 15 – 20 minutes. Keep the plates at 70□C for 2 minutes for further drying.
8. Add 1 ml of the Developing Reagent on the plate and swirl the plate very carefully. Look for the development of the coloured spots of different amino acids and the test sample.

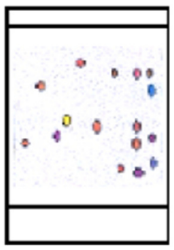
Flowchart:





Allow the solvent front to reach the top line

Dry the TLC plate and apply the developing reagent



Look for the appearance of different coloured spots

Troubleshooting Guide:

Sr. No.	Problem	Possible Cause	Solution
1	Appearance of over-large spots after development	Initial spot is larger than 2 mm in diameter	While spotting the samples on TLC plate ensure that the spots are not larger than 1-2 mm in diameter
2	Solvent front advances unevenly	Use of a developing chamber that does not have a flat bottom	It is therefore important to use flat-bottomed developing tanks during TLC
3	Substance moves along the TLC plate as a long streak, rather than as a single discrete spot	Spotting the plate with too much of sample, more than the moving solvent can handle	Streaking can be eliminated by systematically diluting the spotting solution until development and visualization show the substances moving as single spots, rather than elongated streaks