Thin Layer Chromatography (TLC)

Thin layer chromatography is a type of partition chromatography.

- A technique used routinely by the researchers in the field of phytochemicals, biochemistry, and so forth, to identify the components in a compound mixture, like alkaloids, phospholipids, and amino acids.
- It is a semi-quantitative method consisting of analysis.
- High-performance thin-layer chromatography (HPTLC) is the more sophisticated or more precise quantitative version.

Principle

Similar to other chromatographic methods, thin layer chromatography is also based on the principle of separation.

1. The separation depends on the relative affinity of compounds towards stationary and the mobile phase.
2. The compounds under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, the separation of components in the mixture is achieved.
3. Once separation occurs, the individual components are visualized as spots at a different level of travel on the plate. Their nature or character are identified using suitable detection techniques.
4. Suppose the physical property of compound from mixture is similar to the mobile phase the compound will remain longer in mobile phase and will travel a longer distance on TLC plate. The compounds that are not as much soluble in mobile phase will have an affinity for stationary phase and will travel to a smaller extent than the soluble compounds. An R_f value is “retardation factor” or “ratio to front” which can be calculated by using the formula.

$$Rf = \frac{Distance\ traveled\ by\ the\ compound}{Distance\ traveled\ by\ solvent\ front}$$

These R_f values can be calculated by observing spots on TLC plates under UV transilluminator at 365nm.

System Components

TLC consists of TLC glass plates, stationary phase, solvent system, aluminum foil, chromatographic chamber;

1. **TLC plates,** preferably ready-made with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. TLC plate is prepared by using approximate silica powder (polar) in appropriate ml of distilled water and is continuously stirred using glass rod to form a slurry. Thin slurry is good for analytical TLC and silica layer on the glass plate must be thick for preparative TLC analysis. When stationary phase is silica, which is polar then it is known as standard, and if it is non polar, then it is known as reverse phase.
2. **TLC plate size,** truly, the size of TLC plate is not an important issue, and it doesn't have any relation with resolution of compounds. Conversely, it will affect the developing time of TLC. Large-size plate will take more time and solvent to complete run on TLC and small time will have reverse effect. 6.5 cm length by 2.5 - 5 cm width is enough to produce good results in a minimum amount of time. A plate larger than 6.5 cm length will require more time to run. Preparative separation of bile acids by adsorption chromatography, has been achieved successfully using the plates of dimensions 5 x 20 size in measuring jar of 550 cm^3.
3. **TLC chamber.** This is used for the development of the TLC plate. The chamber maintains a stable environment inside for proper development of spots. It also prevents the evaporation of solvents and keeps the process dust-free. Once the crude sample is spotted on TLC plate. The plate is ready to transfer in developing chamber. This developing chamber previously equilibrated with suitable solvent system and its vapors. TLC previously equilibrated chamber in vertical position such that stationary phase is in contact with mobile phase. Small, medium, large size large-size 0ml, 500ml, and 1000ml may be used as developing chamber. To the top of beaker aluminum foil is wrapped and cover it with glass plate.
4. **Mobile phase.** This comprises of a solvent or solvent mixture. The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. Water (polar) is universal solvent dissolving most of the compounds in it. Chloroform is non polar. Ethyl's acetate is mid polar. Separating chamber is usually allowed to get saturated with vapors of solvent / mobile phase. The phenomena of like dissolve like applied here.

   Compound that is polar will be dissolved in polar solvent, compounds that are non-polar will dissolved in non-polar solvent system and most of the compound, which are polar and non-polar has an ability to get dissolved in mid polar solvent such as ethyl acetate. Sometime combinations of mobile phase are used to separate polar, nonpolar, mid polar compounds. Solvent system used for separation of stress induced metabolites is chloroform: ethyl acetate: benzene: glacial acetic acid (25: 15: 2:10) has been optimized by [28] for medicinal important *passiflora foetida*.

   Optimizing good solvent system is very important and the most difficult level of TLC. Basically start optimizing with non-polar solvent and observe the separation if

compounds does not move to fast add polar solvent. Now compare this plate with the previous plate. If the spot stays at its original site add more of the polar solvent conversely if the spot runs with solvent front try adding non polar solvent. Once solvent front reaches the maximum end of TLC plate this means sample are fully run on TLC. TLC plates are removed from developing chamber and are allowed to dry of solvent/ mobile phase completely.

5. **A filter paper.** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.
6. **Visualization:** Crude extract is initially dissolved in suitable solvent in which extract dissolved completely. Test crude sample (10 µl) spotted onto the silica plates. Plates were developed in the saturated vertical chromatographic chamber saturated by 1-100ml solvent system (as per requirement) [12]. After 30-90, min plates were removed from developing chamber and immediately visualized under 365 nm in (UV) Ultra-violet transilluminator. Numbers of distinctive colors spotted on a plate, each indicates distinct compound. If extract contains to dye or inks, then the visualization would be very easy, however, organic compounds are color less. In this case, the plate is kept in jar containing iodine crystal's most organic compounds absorb iodine vapors within 2 minutes and become visible.
7. **Analysis:** Individual band traveled distance is measured under UV transilluminator and R*f* factor retardation factors (R*f*) were measured against solvent front and compared with the standard retardation factor values. Alternatively, the standard compound with known R*f* value is applied along with the separating samples. Further screening & selective separation for antimicrobial activity on TLC may be performed.
8. **Analytical TLC:** It is used for analysis and identification of various compounds that are run on TLC plate observed under UV transilluminator. Quantity of sample loaded is very less (5µl-10µl) due to preliminary analysis of identification of secondary metabolites and other compounds

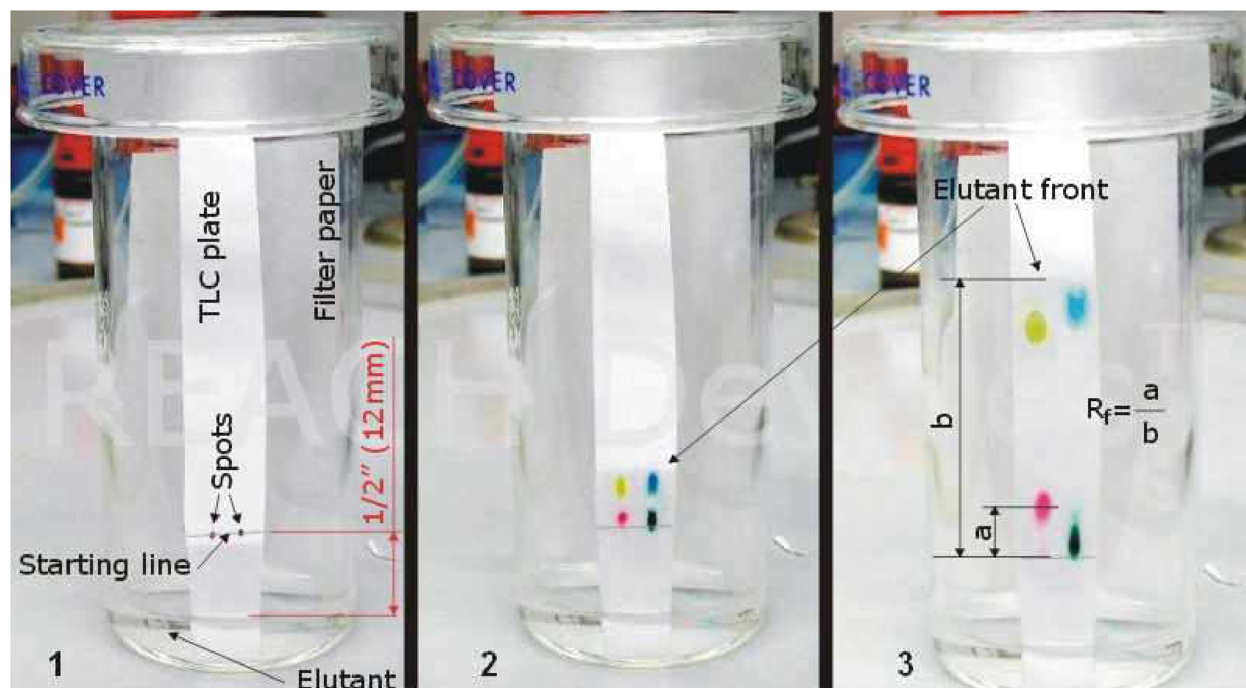

Figure : Setup of Thin layer chromatography analysis

Procedure

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are preferred.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom. A moistened filter paper in the mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect this way).
4. Now, the plate prepared with sample spotting is placed in the TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
5. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent — as shown in the picture) for development.

6. Allow sufficient time for the development of spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber or any other methods as recommended for the said sample.

Advantages of TLC

- Microscale techniques: only few milligrams of extracts is enough to run on TLC plate for analysis and identification.
- Rapid identifier: Sample identification in short time.
- Easy to monitor: a chromatography separation reaction.
- Easy determination: Number of compounds in a mixture could be easily determined.
- Identification of antimicrobial compounds can be done readily of TLC profiled plates. Here pouring potential test microbes (*E.coli, S. aureous*) cultures along with agar on resolved TLC plates can yield the inhibition band on TLC this will confirm an antibacterial activity.
- Spray reagents: The developed, dried TLC plates are used for spraying. 5 – 10 ml solution of spray is sprayed from 10- 15 cm distance in the even manner over the surface of TLC. Excessive spraying of reagent is not recommended.
- Resolved TLC plates can be stored for long duration time.
- Separated compounds may be subjected to FTIR, IR, MS GCMS, LCMS, NMR.

Disadvantages/Problems in TLC

- Large spots huge spotting of crude sample causes inappropriate separation of
- compounds that will result in smear formation and smudging.
- Bands pungent curves: many times compounds on TLC plate are separated with a very pungent curving instead of proper straight bands. This may be due to concentrated sample or may be due to previously used solvent system.
- Uneven leveling of spots: results in unevenly distribution of sample on stationary phase.
- Uneven advancement of solvent front: TLC plates that are not made evenly, and silica gel is not spread evenly then the solvent front run in disturb manner affecting
- movement and separation of compounds.
- Smear formation: a developed TLC plate sometimes shows smear formation this may be due to the unequal distribution of molecules between stationary and movable phase or may be due to mix solvent/ mobile phase.
- Plate position: it is very important to keep the plate in vertical position in the
- chromatography chamber. Slight dash may led to fall of TLC plate in solvent system spoiling whole plate and sample.

- Uneven level of developing chamber: the baseline of developing chamber must be a plane, if it is unlevel the solvent front will not travel in equal level over TLC affecting separation of molecules.

Applications

TLC has been used for various analyses in pharmaceutical and drug industries. Some of the few examples are a detail below;

1. **Identification of secondary metabolites:**

Plant secondary metabolites are naturally producing compounds, which are not of earliest importance to plants, on other sides, they are subordinate means they are not involved in original metabolism. Secondary metabolites are produced on external stimuli, abiotic stress biotic stress, injury, heat, cold, etc. some of the important secondary metabolites are alkaloids, esters, flavonoids, isoflavonides, Phytoalexins, Tannins, Salicylic acid, lignin. All these metabolites are identified from plant explants by using TLC.

Stress conditions on plant's results in production of secondary metabolites from plant such as amino acid example proline, quaternary amines such as polyamines, different sugar and alcohol. All these secondary metabolites could be analyzed using TLC. Different kinds of the metabolites are used for formulation of medicines and cosmetology. Secondary metabolites isolated and purified may be used for antimicrobials test, compound structure identification, MS, FTIR, and GCMS. It can also be used for improving organic farming for better crop results.

2. **Separation of amino acid:**

Various chromatographic systems were developed for analysis amino acids. TLC of secondary metabolites is easy as compare with amino acids because secondary metabolites are colored, however, amino acids are colorless making them tougher to be visualized by naked eyes. The ninhydrin or the black-light visualization techniques is basically used for observing amino acids. Several amino acids, proteins and peptides have been successfully separated and isolated from urine using silica gel plates. All these substances were found to be ninhydrin positive. The developments were carried out first with chloroform-methanol-20%ammonium hydroxide (2:2:1) and then with phenol-water describes a simple fast procedure for extraction, separation, and quantitative estimation of amino acid from plant tissue.

3. **Analysis of phospholipids:**

Phospholipids and glycolipids from plants have been analyzed by using TLC with solvent system chloroform-methanol-water (75:25:2.5) from plant. Same solvent system with varying ratio has been used to analyses Phospholipids pooled rat livers. Other common mobile phases are Triethylamine, ethanol, hexane and isopropanol. To improve

the separation of phospholipids a couple of chemical modifications can be made to the silica gel. Many reagents can be used to detect phospholipids such as 3% Copper acetate, Iodine, **8%** phosphoric acid and Molybdenum blue [37] Method is simple, rapid and reliable for determination of phospholipids with average recovery of 100-8%.

4. **Separation of Prostaglandins:**

TLC has been used for separating prostaglandins derivatives, for example, prostaglandin E1, (PGE1) and prostaglandin FI, (PGF1,) from sheep vesicular glands and Prostaglandins EI. E2, and EI were obtained from sheep vesicular glands and are separated using TLC.

5. **Pharmaceutical and Drugs:**

Antibiotics Penicillin's have been separated on silica gel 'G' [12,3] by using the two solvents, acetone- methanol (1:1) and isopropanol-methanol (3:7). As the detecting agent, the iodine-azide reaction could be employed by spraying the dried plates with a 0.1 % iodine solution containing 3.5% of sodium azide. Several other drugs have been separated and isolated using TLC.

6. **Analysis in Cosmetology:**

In the identification of dye raw materials and end products, preservatives, surfactants, fatty acid, constituents of perfumes TLC has been used in cosmetology.

7. **Clinical Chemistry and Biochemistry:**

A useful tool in analysis of the urinary constituent derived from lipids in analysis of many urinary constituents such as steroids, amino acids, porphyrins and bile acids. Urinary analysis by TLC is most effective when done in conjunction with other chromatographic processes, so that minor metabolites can be detected and resolved completely free of other components.

8. **Food Analysis:**

TLC is used for the determination of pesticides and fungicides in drinking water, residues in vegetables, salads and meat, vitamins in soft drink sandalwood extract in fish and meat products) aflatoxins in milk and milk products explain TLC analysis of products, foods, beverages, and plant constituents explaining in details of a solvent system used and mode of detection.

9. **Analysis of Heavy Petroleum Products:**

Thin-layer chromatography (TLC) is commonly used for analysis petroleum products and coal products. In particular, For such a data, TLC is the simplicity and economy.

10. **Separation of aromatic amines:**

Aromatic amines have the main role in formation of variety of dyes used in textile, leather, plastic and paper products. Cationic and non-ionic surfactant mediated systems shall be use as mobile phases in thin-layer chromatographic for separation of aromatic amines on silica gel layers.

High-performance thin layer chromatography (HPTLC) is one of the sophisticated instrumental techniques based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, and so on enable it to be a powerful analytical tool for chromatographic information of complex mixtures of pharmaceuticals, natural products, clinical samples, food stuffs, and so on.