IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Thin Layer Chromatography

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1) INTRODUCTION

- Thin layer chromatography (TLC) is one of the simplest chromatographic techniques used for the separation and identification of compounds. This is also used to monitor the progress of chemical reaction at every step. We can also check the purity of synthesized compounds at very short time span. Thin layer of some inert material such as; alumina (Al,O), silica gel (SiO2), magnesium oxide (MgO), etc. is uniformly spread on glass plate either manually or mechanically and the solution of mixture is applied on it. After the development of plate with suitable mobile phase, components of mixture get separated as spot at different places on the plate.

- Thin layer chromatography is similar to paper chromatography only instead of paper thin layer of stationary material is used. The advantage of using layer of stationary mateha' over paper is that, we can use very corrosive solvents such as hydrochloric acid and sulphuric acid as mobile phase without destroying the stationary phase. These solvents are very useful to separate and identify high molecular weight biological compounds. This technique is also useful to study variety of compounds such as natural extracts, sugars, amino acids, dyes, biological fluids, food colourings etc. Inorganic cations and anions also get separated by thin layer chromatography.

- Izmailov and Shraiber in 1938 were first to introduce this technique. They used alumina as coating material and applied over a flat glass sheet and allowed to run in suitable solvent for the separation of plant extract. Later on scientist Williams used sandwiched method for the preparation of chromatographic plate and used for the same. As study went on; scientist Kirchner in 1950 introduced

new hybrid version of stationary material ie. glass-fibre paper coated with alumina or silicic acid. Modern analytical thin layer chromatography was later introduced by Egon Stahl in 1958. He introduced modern equipment for the application of thin layer of stationary material on the plate.

- TLC also called by other names such as; open column chromatography, strip chromatography, spread or drop chromatography.

2) DEFINITION

- Thin layer chromatography (TLC) is an important technique for identification and separation of mixtures of organic compounds. It is useful in:

1) Identification of components of a mixture (using appropriate standards)
2) Analyzing fractions collected during purification.
3) Analyzing the purity of a compound

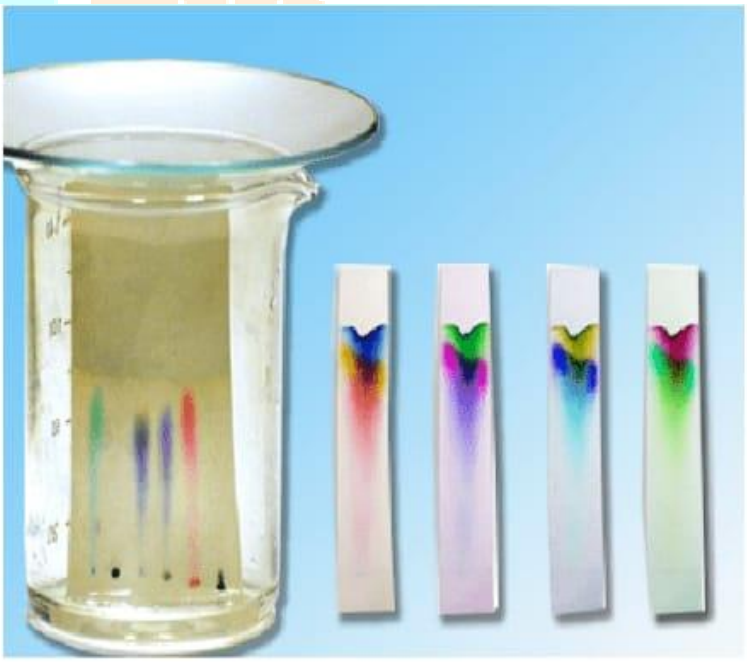


Fig.1: Thin Layer Chromatography

3) PRINCIPLE OF THIN LAYER CHROMATOGRAPHY

1. The principle involved in TLC is adsorption. One or more compounds are spotted on a thin layer of adsorbent coated on a chromatographic plate. The mobile phase solvent flows through because of capillary action.

2. The compound with greater affinity for stationary phase moves slower rate and compounds with lesser affinity moves fast.

3. Identification of components is done by calculating the R_f value for each compound,

4. R_f = distance travelled by component/ distance travelled by solvent.

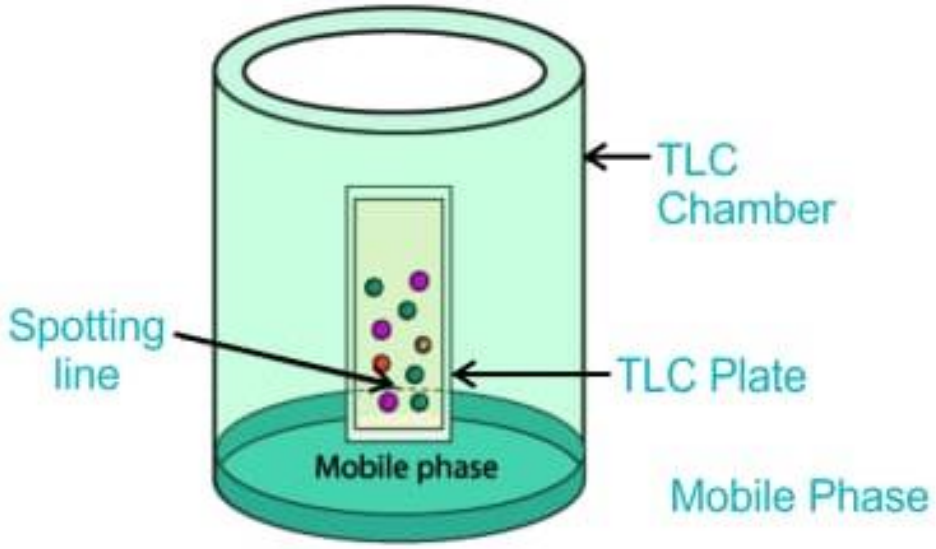


Fig.2: TLC Chamber

4) ADVANTAGES OF THIN LAYER CHROMATOGRAPHY

1. Simple method and cost of the equipment is low.
2. Rapid technique and not time consuming like column chromatography.
3. Separation of mg of the substances can be achieved.
4. Any type of compound can be analyzed.
5. Efficiency of separation Very small particle size can be used which increases the efficiency of separation. Flow rate is not.
6. Altered because of the particle size since it is not a closed column. It is plamir type having thing layer of adsorbent.
7. Detection is easy and not tedious.
8. Capacity of the thin layer can be altered. Hence analytical and preparative separations can be made.
9. Corrosive spray reagents can be used without damaging the plates.
10. Needs less solvent, stationary phase and time for every separation when compared to column chromatography.

5) DISADVANTAGES OF THIN LAYER CHROMATOGRAPHY

1. In TLC, the plate length is limited and hence separation takes place only upto certain length.
2. TLC is not applicable for separation of volatile substances, thus limiting its use.
3. It is applicable for soluble mixture components only.
4. Results getting from TLC are difficult to reproduce.
5. In TLC, only qualitative analysis possible, not quantitative.
6. TLC is not automatic process.
7. It is difficult to reproduce the findings obtained from the experiment.
8. Applicable for components of soluble mixtures only.
9. Qualitative analysis, not the analysis in quantitative terms.
10. It is not an automatic mechanism.
11. A thin layer of chromatography operates in an open system, and humidity and temperature can influence the outcomes.
12. As the plate length is limited, the separation process takes place up to a certain length.

6) PRACTICAL REQUIREMENT'S

1. Stationary Phase

2. Glass Plates

3. Preparation And Activation Of TLC Plates

4. Mobile Phase

5. Spotting

6. Development Technique

7. Detecting Or Visualizing Agent

1. Stationary phase :

- There are several adsorbents which can be used as stationary phase. Some of the stationary phases, their composition and the ratio in which they have to be mixed with water or other solvents to form a slurry for preparing thin layer chromatographic plates

2. Glass plate :

- Glass plates which are specific dimensions like **20 cm X 20 cm** (Full plate), **20 X 10** cm(Half plate)**20 cm X 5 cm** (Quarter plate) can be used.These dimensions are used since the width of the commercially available TLC spreader is **20 cm**.

- **Microscopic slides** can also be used for some applications like **monitoring the progress of a chemical reaction.** The development time is much shorter like 5 minutes.

- Glass plates of different dimensions can also be used when the TLC plates are prepared without the use of TLC spreader. In general, the glass plates should be of good quality and should withstand temperatures used for drying the plates.

3. Preparation and activation of TLC plates :

- The slurry, which is a mixture of stationary phase and water is prepared by using the ratio mentioned earlier. After preparing the slurry, the TLC plates can be prepared by using any one of the following techniques**; pouring, dipping, spraying and spreading**

a) Pouring technique:

- The slurry is prepared and poured on to a glass plate which is maintained on a leveled surface. The slurry is spread uniformly on the surface of the glass plate. After setting, the plates are dried in an oven is used for spotting. The disadvantage is that uniformity in thickness can not be ensured.

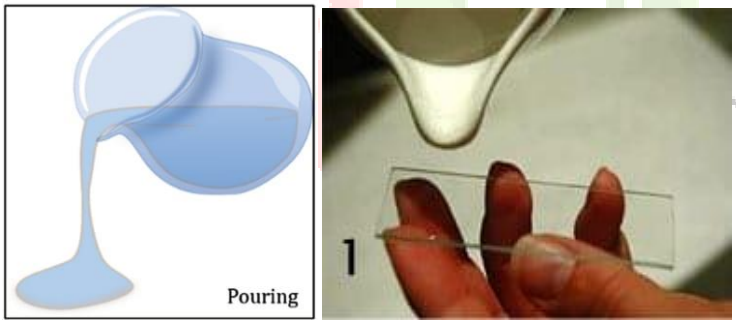


Fig.3: Pouring Technique

b) Dipping technique:

- Two plates (either of standard dimensions or microscopic slides) are dipped in to slurry and are separated after removing from slurry and later dried. The disadvantage is that a larger quantity of slurry is required even for preparing fewer plates.

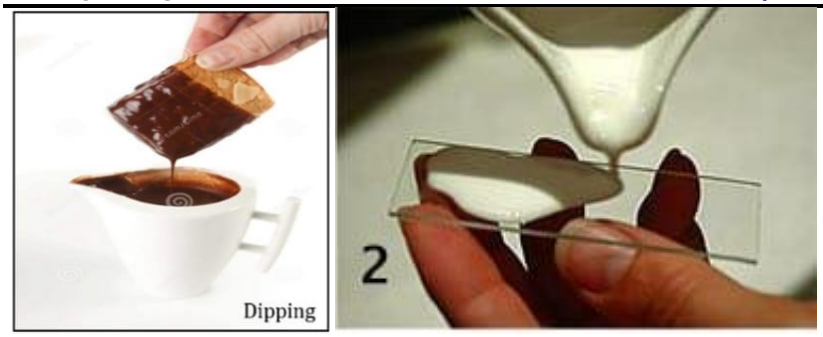


Fig.4: Dipping Technique

c) Spraying technique:

- Resembles that of using a perfume spray on a cloth. The suspension of adsorbent or slurry is sprayed on a glass plate using a sprayer. The disadvantage is that the layer thickness cannot be maintained uniformly all over the plate.

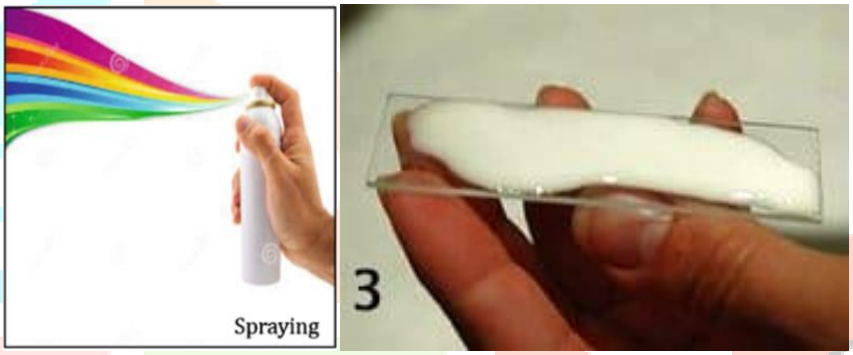


Fig.5: Spraying Technique

d) Spreading:

- Is the best technique where a TLC spreader is used. The glass plates of specific dimensions (20cm X 20cm/10 cm/5cm) are taken. The prepared slurry is poured inside the reservoir of TLC spreader. The thickness is adjusted by using a knob in the spreader.

- Normal thickness of 0.25cm is used for analytical purpose and 2 mm thickness for preparative purpose. Then the spreader is rolled only once on the plate. The plates are allowed for setting(air drying). This is done to avoid cracks,

- The plates are activated by keeping in an **oven at 100°C to 120°C for 1 hour**

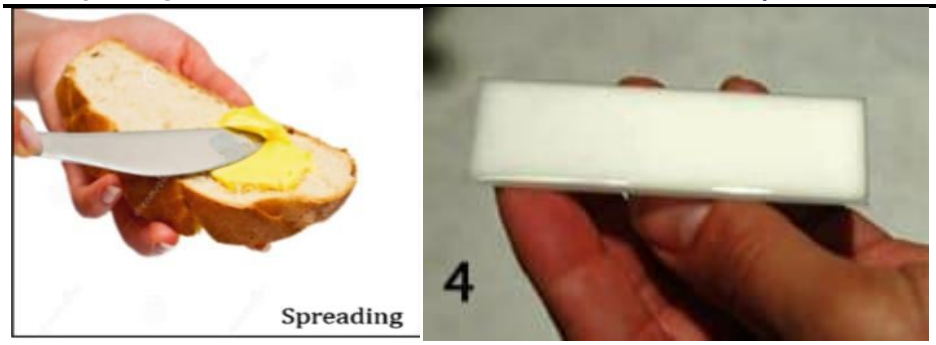


Fig.6: Spreading Technique

e) Activation of TLC plates:

- Activation of TLC plates is nothing but removing water/moisture and other adsorbed substances from the surface of any adsorbent, by heating at high temperature so that adsorbent activity is retained. The activated plates can be stored in thermostatically controlled oven or in desiccator and can be used whenever required.

4. Mobile phase :

- It is a developing liquid which travels up the stationary phase, carrying the samples with it. It depends on:
- Nature of the substance to be separated i.e polar or non polar.
- Nature of stationary phase used.
- Mode of chromatography.
- Solvent used should be of high purity.

Solvents used:-petroleum ether, Benzene carbon tetrachloride chloroform

Fig.7: Mobile Phase (Solvent)

5. Spotting :

- 2-5 (---) of a 1% solution of sample or standard is spotted using a capillary tube or micropipette. The spots should be kept at least 2cm above the base of plate and the spotting area should not be immersed in mobile phase in a developing chamber.
- The sample is applied on the narrow-band.
- The width of the band must be as narrow as possible.

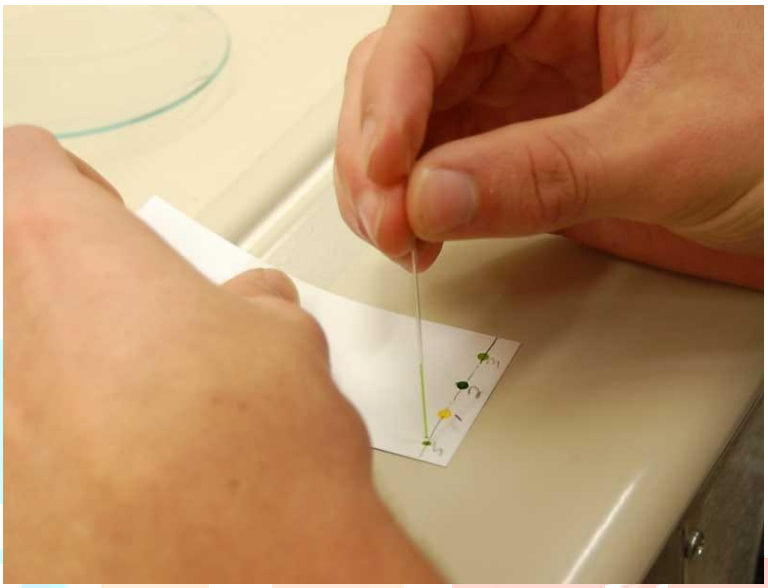


Fig.8: Spot The TLC Plate

6. Developing chamber :

- It is used for the purpose of "TLC plate run in mobile phase."
- After the mobile phase is poured into the chamber it is kept closed with lid.
- This is done to equilibrate the atmosphere of empty space in chamber with the mobile solvent.
- This is also known as saturation of TLC chamber.
- Edge effect occurs when the solvent front in the middle of TLC plate moves faster than that of edge edge of plate.

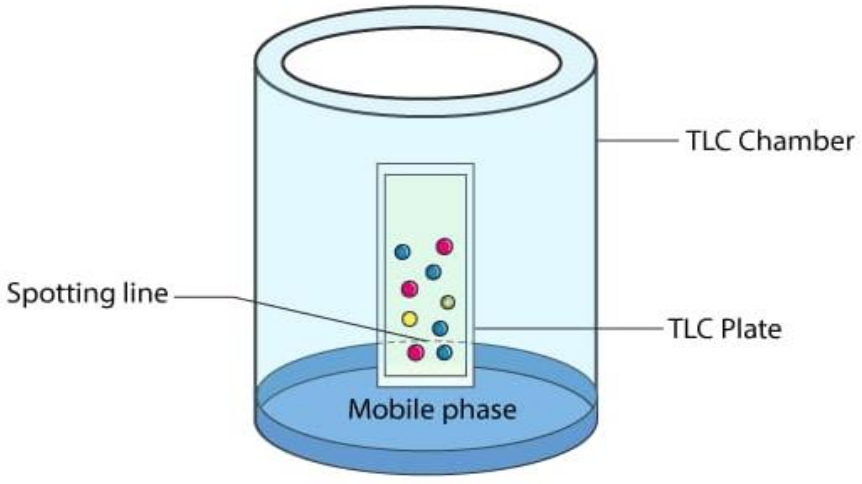


Fig.9: TLC Chamber

7. Development of TLC plate :

- Different development techniques are used for efficient separations. They are

a) One dimensional development (ascending or descending technique).

b) Two dimensional development.

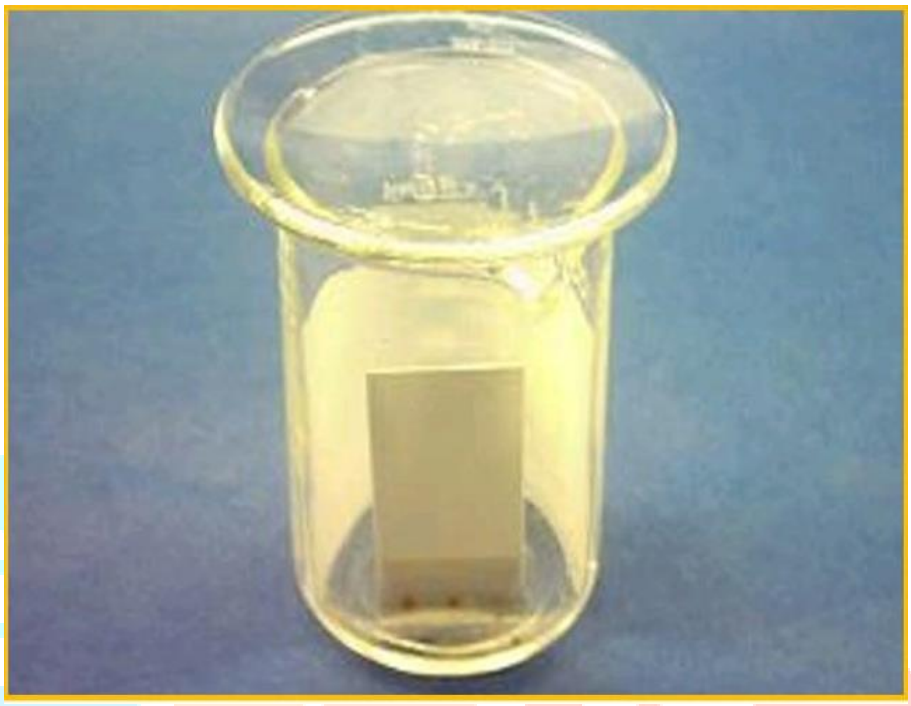


Fig.10: Development of TLC plate

a) One dimensional development (vertical) :

- Like conventional type, the solvent flows against gravity. The spots are kept at the bottom portion of paper and kept in a chamber with mobile phase solvent at the bottom.

b) Two dimensional technique

- This technique is similar to 2-Dimensional TLC. The paper is developed in one direction and after development, the paper is developed in the second direction allowing more compounds or complex mixtures to be separated into individual spots. In the second direction, either the same solvent or different solvent system can be used for development.

8. Detecting agent:

- After the development of chromatogram, the spots should be visualised. Detecting coloured spots can be done visually. But for detecting colourless spots, any one of the following techniques can be used.

a) Non specific method:

- Where the number of spots can be detected but not exact nature of compound.

Example

1. Iodine Chamber Method:

- Where brown or amber spots are observed when the paper is kept tank with few iodine crystals at the botto.

2. UV Chamber for fluorescent compounds:

- When compounds are viewed under UV chamber at 245 nm or at 365 nm fluorescent compounds can be detected.

7) HOW TO RUN THIN LAYER CHROMATOGRAPHY

1. **Step : Prepare the developing container.**
2. **Step : Prepare the TLC plate.**
3. **Step : Spot the TLC plate.**
4. **Step : Develop the plate.**
5. **Step : Visualize the spots.**

1. Step : Prepare the developing container :

- Take a beaker with a watch glass on the top.
- Required quantity of solvents are taken into the beaker.
- Cover the beaker with watch glass and mix the solvents.
- Keep them aside until the plate is prepared.

2. Step : Prepare the TLC plate :

- Take a TLC plate and cut it to required length and width.
- Now Mark a line about 1 cm from the bottom.
- On the line place two dots at equal space.

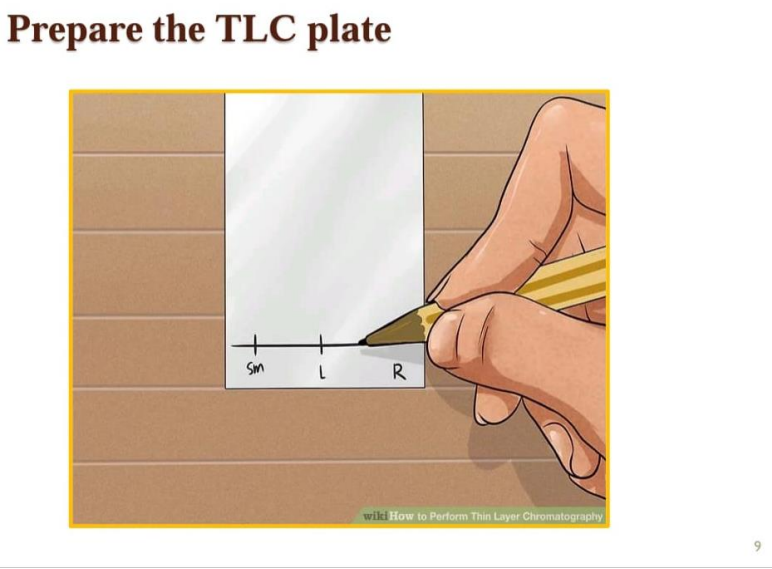


Fig.11: Prepare the TLC plate

3. Step : Spot the TLC plate :

- Take the capillary tube and by the help of heat make it into two, so that the end of the capillary tube will be thin.
- It helps to place a small amount of sample.
- Take the required solutions and spot them at the marked points.

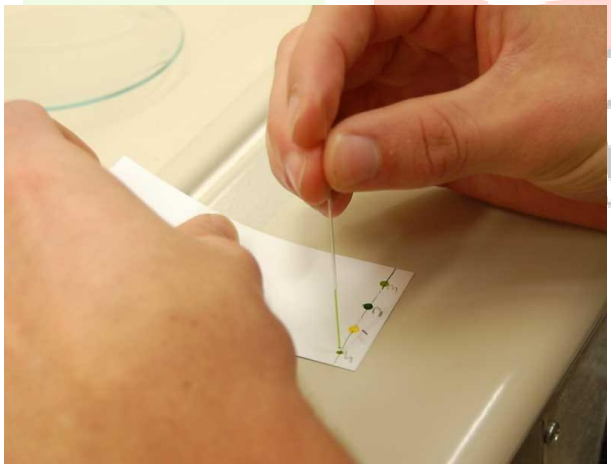


Fig.12: Spot the TLC plate

4. Step : Develop the plate :

- Put the TLC plate carefully into the beaker.
- The solution should not touch the marked line.
- Close the beaker with watch glass.
- Do not allow the solvent to run off the top of the plate.

Fig.13: Develop the plate

5. Step : Visualize the spots :

- Take off the TLC plate from the beaker carefully.
- Mark the solvent front level.
- Let it dry.
- Spray.....solution.
- Observe the spot and round it with pencil carefully.

Fig.14: Visualize the spots

8) QUANTITATIVE ANALYSIS

1) Direct technique :

2) Indirect technique :

1) Direct technique :

- Densitometer is an instrument which measures quantitatively the density of the spots. When the optical density of the spots for the standard and test solution are determined, the quantity of the substance

can be calculated.

2) Indirect technique :

- In this technique, the spots are cut into portions and eluted with solvents. This solution can be analysed by any conventional techniques of analysis like spectrophotometry, electrochemical methods etc.,

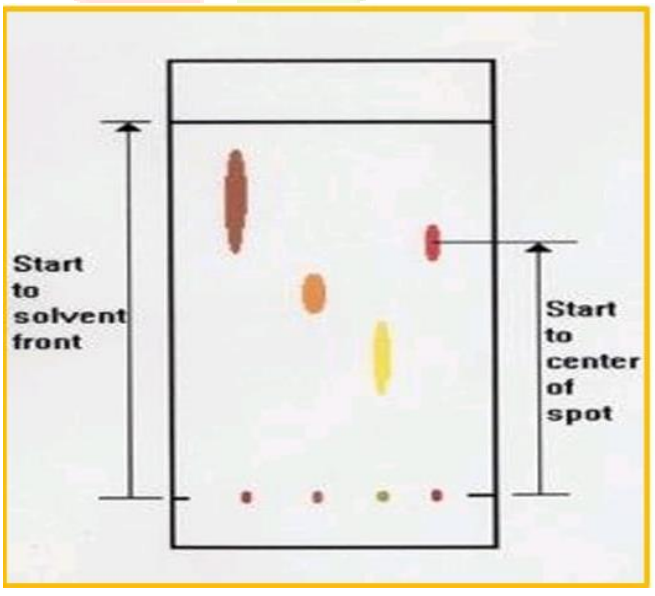
9) QUALITATIVE ANALYSIS

Rf Value

- The Rf value (Retardation factor) is calculated for identifying the spots ie, in Qualitative analysis. Rf value is the ratio of distance travelled by the solute to the distance travelled by the solvent front.

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

- The Rf value ranges from 0 to 1. But ideal values are fro 0.3 to 0.8. Rf value is constant for every compound in a particular combination of stationary and mobile phase. When the Rf value of a sample and reference compound is same, the compound is identified.



10) APPLICATION OF THIN LAYER CHROMATOGRAPHY

- Thin layer chromatography has wide range of applications in pharmaceutical analysis such as: purity testing, identification, stability testing, assay, and content uniformity testing of intermediates, raw materials, and drug products, with the analysis of sample analytes. Often degradation products, synthetic intermediates, and process related impurities do not have chromophores hence cannot be detected by the UV detector. Thus, these types of impurities are frequently specified by the TLC analysis. Sometimes impurities are eluted at the solvent front in the HPLC, and that may be complicated to quantify and monitor. Modification in the mobile phase or HPLC column could not be sufficient to solve them adequately.

- On the other hand, occasionally the impurities take more time to elute from the column and they can't be detected, but TLC method is open in which whole samples are evaluated Thin layer chromatography is used in the early stage of drug development. Some of the applications of thin layer chromatography are described below:

1) To Check Purity of Sample :

- Purity of sample can be determined with TLC Direct comparison is done between the sample and the standard or authentic sample; if any impurity is detected, and then it shows extra spots and this can be detected easily.

2) TLC in Compound Identification :

- Thin layer chromatography can be employed in purification, isolation and identification of natural products like; volatile oll or essential oil, fixed oil, waxes, alkaloids, glycosides, steroids, etc.

3) Monitoring of Chemical Reactions :

- Reaction mixture can be examined by Thin layer chromatography to access whether the reaction is complete or not. This method is also used in checking other separation processes and purification processes like distillation, molecular distillation, etc.

4) TLC in Biochemical Analysis :

- Thin layer chromatography is extremely useful in Isolation or separation of biochemical metabolites or constituent from body fluids. blood plasma, serum, urine, etc.

5) In Chemistry:

- TLC methodology is increasingly used in chemistry for the separation and identification of

compounds which are closely related to each other. It is also used for identification of cations and anions in inorganic chemistry.

6) In Pharmaceutical Industry :

- Various pharmacopoeias have adopted TLC technique for detection of impurity in official monographs.

7) Various medicines like :

- Anti-histaminic, sedatives, anticonvulsant, tranquillizers antibiotics, analgesics, local anaesthetics, steroids have been tested qualitatively by TLC method.

8) In Food and Cosmetic Industry :

- TLC method is used for separation and identification of colours, preservatives, sweetening agent, and various cosmetic products.

11) CONCLUSION

- In the thin layer chromatography experiment, as the mobile phase rose up on the TLC plate it dragged the ink from the marked dot up along the TIC plate. The pigment that was closer to the marked dot was more attracted to the stationary phase, the silica gel. The pigment that traveled the farthest up on the TLC plate more attracted to the mobile phase, the hexane and ethyl acetate solvent. From the results, out of the top four pigments that traveled the farthest, the light pink pigment whose Rf was 0.98 had the greatest affinity to the mobile phase. The dark pink spot that had an Rf of 0.72 was the closest to the marked dot therefore had a greater affinity to the stationary phase. In the column chromatography experiment the dye that separated first was the royal blue dye. This meant that the royal blue dye was more attracted to the ethanol solvent thus separating faster. The green dye in test tube three represented the dye component in the original dye mixture that took more time to separate as it was more attracted to the alumina powder. The first color dot on the TLC plate had the longest retention factor of 0.98. This meant that the light pink dye in the black ink mixture had the highest affinity to the mobile phase, which was the hexane and ethyl acetate solvent,

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