



## Thin Layer Chromatography: A Tool of Biotechnology for Isolation of Bioactive Compounds from Medicinal Plants

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### ABSTRACT

In this study, an attempt was made to review the basic principles and the importance of Thin Layer Chromatography (TLC) in research in general and in phytochemistry in particular. Thin layer chromatography is a simple, cost-effective, and easy-to-operate planar chromatographic technique which has been used in general chemistry laboratories for several decades to routinely separate chemical and biochemical compounds. Traditionally, chemical and optical methods are employed to visualize the analyte spots on the TLC plate. Also it has a wide application in identifying impurities in a compound. Study highlights the review on TLC and its application of qualitative and quantitative estimation of bio-active compounds from medicinal plants.

**Keywords:** Thin Layer Chromatography, Principle of TLC, Advantages of TLC, Applications of TLC.

### INTRODUCTION

Chromatography is the collective term for a set of laboratory techniques for the separation of mixtures into their components. All forms of chromatography work on the same principle. They all have a



Figure 1: Inventor of Chromatography

stationary phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The mobile phase flows through the stationary phase and carries the components of the mixture with it. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. There are different types of chromatography such as Column chromatography, Paper chromatography etc. among them Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between

different adsorbents. Thin layer chromatography (TLC) is among the most useful tools for following the progress of organic chemical reactions and for assaying the purity of organic compounds in phytochemistry and Biotechnology. Like all chromatographic methods, TLC takes advantage of the different affinity of the analyte with the mobile and stationary phases to achieve the separation of complex mixtures of organic molecules. A TLC plate is a sheet of glass, metal or plastic which is coated with a thin layer of a solid adsorbent. A small amount of the mixture to be analyzed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid<sup>1</sup>. A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action. To determine the best solvent or mixture of solvents (a "solvent system") to develop a TLC plate or chromatography column loaded with an unknown mixture, vary the polarity of the solvent in several trial runs: a process of trial and error. Carefully observe and record the results of the chromatography in each solvent system. You will find that as you increase the polarity of the solvent system, all the components of the mixture move faster (and vice versa with lowering the polarity). The ideal solvent system is simply the system that gives the best separation. TLC elution patterns usually carry over to column chromatography elution patterns. Since TLC is a much faster procedure than column chromatography, TLC is often used to determine the best solvent system for column chromatography. For instance, in determining the solvent system for a flash



chromatography procedure, the ideal system is the one that moves the desired component of the mixture to a TLC  $R_f$  of 0.25-0.35 and will separate this component from its nearest neighbour by difference in TLC  $R_f$  values of at least 0.20. Therefore a mixture is analyzed by TLC to determine the ideal solvent(s) for a flash chromatography procedure. Thin layer chromatography can be used to Monitor the progress of a reaction, identify compounds present in a given substance, and determine the purity of a substance. Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. For instance, if normal phase silica gel is used as the stationary phase it can be considered polar. Given two compounds which differ in polarity, the more polar compound has a stronger interaction with the silica and is therefore more capable to dispel the mobile phase from the binding places. Consequently, the less polar compound moves higher up the plate. If the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of dispelling solutes from the silica binding places and all compounds on the TLC plate will move higher up the plate. Practically this means that if you use a mixture of ethyl acetate and heptane as the mobile phase, adding more ethyl acetate results in higher  $R_f$  values for all compounds on the TLC plate. Changing the polarity of the mobile phase will normally not result in reversed order of running of the compounds on the TLC plate<sup>1,2,4,6,7</sup>.

### Chromatogram

As the solvent slowly travels up the plate, the different components of the dye mixture travel at different rates and the mixture is separated into different coloured spots.

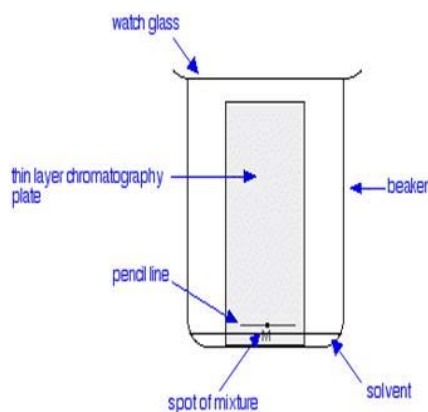


Figure 2: Chromatogram

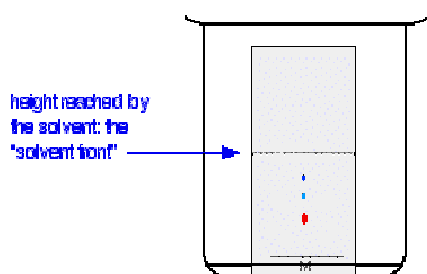


Figure 3: Developing Tank

The solvent is allowed to rise until it almost reaches the top of the plate. That will give the maximum separation of the dye components for this particular combination of solvent and stationary phase.

### PRINCIPLE OF TLC

Thin layer chromatography uses a thin glass plate coated with either aluminum oxide or silica gel as the solid phase. The mobile phase is a solvent chosen according to the properties of the components in the mixture. The principle of TLC is the distribution of a compound between a solid fixed phase applied to a glass or plastic plate and a liquid mobile phase, which is moving over the solid phase. A small amount of a compound or mixture is applied to a starting point just above the bottom of TLC plate. The plate is then developed in the developing chamber that has a shallow pool of solvent just below the level at which the sample was applied. The solvent is drawn up through the particles on the plate through the capillary action, and as the solvent moves over the mixture each compound will either remain with the solid phase or dissolve in the solvent and move up the plate. Whether the compound moves up the plate or stays behind depend on the physical properties of that individual compound and thus depend on its molecular structure, especially functional groups. The solubility rule "Like Dissolves Like" is followed. The more similar the physical properties of the compound to the mobile phase, the longer it will stay in the mobile phase. The mobile phase will carry the most soluble compounds the furthest up the TLC plate. The compounds that are less soluble in the mobile phase and have a higher affinity to the particles on the TLC plate will stay behind<sup>16</sup>.

### $R_f$ values

The behavior of an individual compound in TLC is characterized by a quantity known as  $R_f$  and is expressed as a decimal fraction. The  $R_f$  is calculated by dividing the distance the compound traveled from the original position by the distance the solvent travelled from the original position (the solvent front). Nature of adsorbent: Different adsorbents will give different  $R_f$  value for same solvent. Reproducibility is only possible for given adsorbent of constant particle size and binder. Plates should be stored over silica gel in desiccators before use and the sample should be applied quickly so that the water vapor in the atmosphere is not adsorbed by the plate. Because of the difficulties associated with activation procedures, it is far better to use plates stored at room temperature and not to activate them.

### Measuring $R_f$ values

Measurements are often taken from the plate in order to help identify the compounds present. These measurements are the distance travelled by the solvent, and the distance travelled by individual spots. When the solvent front gets close to the top of the plate, the plate is removed from the beaker and the position of the solvent

is marked with another line before it has a chance to evaporate.

These measurements are taken as:

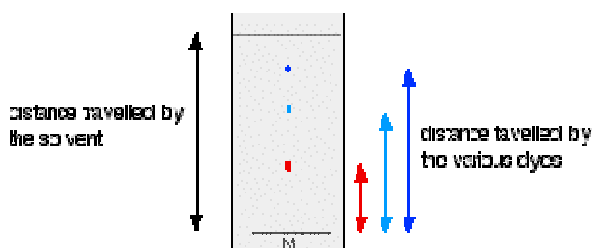


Figure 4: Spots as compounds

The  $R_f$  value for each dye is then worked out using the formula:

$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}$$

For example, if the red component travelled 1.7 cm from the base line while the solvent had travelled 5.0 cm, then the  $R_f$  value for the red dye is:

$$R_f = \frac{1.7}{5.0} = 0.34$$

If we could repeat this experiment under *exactly* the same conditions, then the  $R_f$  values for each dye would always be the same. For example, the  $R_f$  value for the red dye would always be 0.34. However, if anything changes (the temperature, the exact composition of the solvent, and so on), that is no longer true. We have to bear this in mind if you want to use this technique to identify a particular dye. We'll look at how you can use thin layer chromatography for analysis further down the page.

The  $R_f$  for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant:

- Solvent system
- Adsorbent
- Thickness of the adsorbent
- Amount of material spotted

Since these factors are difficult to keep constant from experiment to experiment, relative  $R_f$  values are generally considered. "Relative  $R_f$ " means that the values are reported relative to a standard, or it means that you compare the  $R_f$  values of compounds run on the same plate at the same time. The larger an  $R_f$  of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger  $R_f$  is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. Conversely, if you know the structures of the compounds in a mixture, you can predict that a compound of low polarity will have a larger  $R_f$  value than a polar compound run on the same plate. The  $R_f$  can provide corroborative evidence as to the

identity of a compound. If the identity of a compound is suspected but not yet proven, an authentic sample of the compound, or standard, is spotted and run on a TLC plate side by side (or on top of each other) with the compound in question. If two substances have the same  $R_f$  value, they are likely (but not necessarily) the same compound. If they have different  $R_f$  values, they are definitely different compounds. Note that this identity check must be performed on a single plate, because it is difficult to duplicate all the factors which influence  $R_f$  exactly from experiment to experiment<sup>5, 7,8,14,15</sup>.

### Plate preparation

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and activated by heating in an oven for thirty minutes at 110 °C. The thickness of the adsorbent layer is typically around 0.1- 0.25 mm for analytical purposes and around 0.5- 2.0 mm for preparative TLC<sup>6,10,11</sup>.

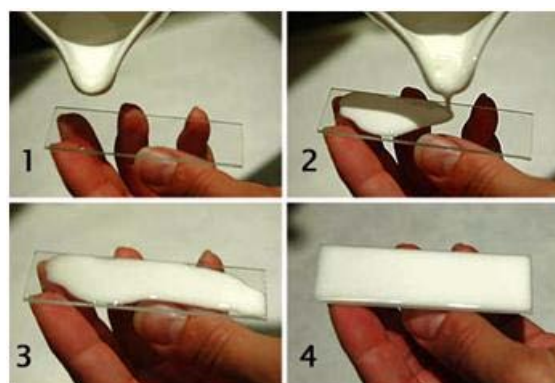
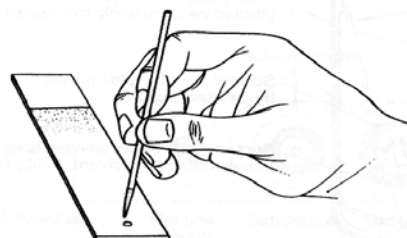


Figure 5: Steps of Preparative TLC

### Spotting the plate

The thin end of the spotter is placed in the dilute solution; the solution will rise up in the capillary (capillary forces).



Touch the plate briefly at the start line. Allow the solvent to evaporate and spot at the same place again.

This way you will get a concentrated and small spot. Try to avoid spotting too much material, because this will deteriorate the quality of the separation considerably ('tailing'). The spots should be far enough away from the edges and from each other as well. If possible, you should spot the compound or mixture together with the starting materials and possible intermediates on the plate<sup>1,12,13</sup>.

### Location of spots

The position of various solutes separated by TLC can be located by various methods. Colored substances can be seen directly when viewed against stationary phase, while colorless substances can be detected only by making them visible by making use of some spraying agent, which produces colored areas in the region which they occupy. Specifically in TLC following can be used for spraying the invisible spots:

1. Being purely inorganic in nature, corrosive agents may also be used for spraying on the invisible spots.
2. Dilute solution of Potassium dichromate in concentrated sulfuric acid. In the process, potassium dichromate (yellow) is reduced to chromic sulfate (green) by most of the organic compounds, particularly used for sugars.
3. Vapors of sulfur trioxide, produced on warming fuming sulfuric acid, chars organic compound and makes them visible as dark spots.
4. Solution of potassium permanganate.
5. Iodine vapors.

Other common reagents include saturated solution of hydrogen sulphide, 0.2N aqueous ammonium sulphide, 0.1% alcoholic quercetin, 0.2% methanolic 1-(2-pyridylazo)-2-naphthol, 1% methanolic oxine, and 0.5% aqueous sodium rhodizonate. If the adsorbent used for the TLC plate contains a fluorescing material, the solutes can be viewed under ultraviolet light<sup>1,14,18</sup>.

### Development solvents

The choice of a suitable solvent depends upon: Nature of substance, and adsorbent used on the plate. A development solvent should be such that, does not react chemically with the substances in the mixture under examination. Carcinogenic solvents (benzene etc) or environmentally dangerous solvents (dichloromethane etc) should always be avoided. Solvent systems range from non-polar to polar solvents. Non-polar solvents are generally used, as highly polar solvents cause the adsorption of any component of the solvent mixture. Commonly used development solvents are petroleum ether, carbon tetrachloride, pyridine, glycol, glycerol, diethyl ether, formamide, methanol, ethanol, acetone, and n-propanol<sup>1,4,16</sup>.

### Mobile Phase

For silica gel chromatography, the mobile phase is an organic solvent or mixture of organic solvents. As the mobile phase moves pass the surface of the silica gel it transports the analyte pass the particles of the stationary phase. However, the analyte molecules are only free to move with the solvent if they are not bound to the surface of the silica gel. Thus, the fraction of the time that the analyte is bound to the surface of the silica gel relative to the time it spends in solution determines the

retention factor of the analyte. The ability of an analyte to bind to the surface of the silica gel in the presence of a particular solvent or mixture of solvents can be viewed as a the sum of two competitive interactions. First, polar groups in the solvent can compete with the analyte for binding sites on the surface of the silica gel. Therefore, if a highly polar solvent is used, it will interact strongly with the surface of the silica gel and will leave few sites on the stationary phase free to bind with the analyte. The analyte will, therefore, move quickly pass the stationary phase. Similarly, polar groups in the solvent can interact strongly with polar functionality in the analyte and prevent interaction of the analyte with the surface of the silica gel. This effect also leads to rapid movement of the analyte pass the stationary phase. The polarity of a solvent to be used for chromatography can be evaluated by examining the dielectric constant and dipole moment of the solvent. The larger these two numbers, the more polar is the solvent. In addition, the hydrogen bonding ability of the solvent must also be considered. For example methanol is a strong hydrogen bond donor and will severely inhibit the ability of all but the most polar analytes to bind the surface of the silica gel<sup>1,8,12,14</sup>.

### Developing a Plate

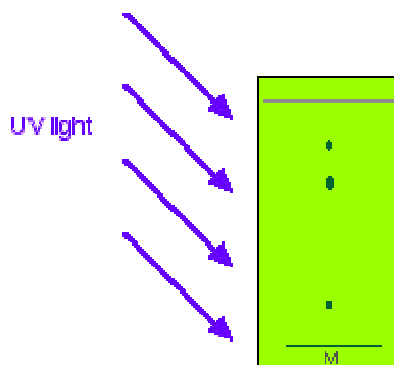
A TLC plate can be developed in a beaker or closed jar. Place a small amount of solvent (mobile phase) in the container. A small spot of solution containing the sample is applied to a plate, about one centimeter from the base. The plate is then dipped in to a suitable solvent, such as hexane or ethyl acetate, and placed in a sealed container. The solvent moves up the plate by capillary action and meets the sample mixture, which is dissolved and is carried up the plate by the solvent.

Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility in the solvent. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the R<sub>f</sub> value) can be adjusted. The solvent level has to be below the starting line of the TLC, otherwise the spots will dissolve away. The lower edge of the plate is then dipped in a solvent. The solvent (eluent) travels up the matrix by capillarity, moving the components of the samples at various rates because of their different degrees of interaction with the matrix (stationary phase) and solubility in the developing solvent. Non-polar solvents will force non-polar compounds to the top of the plate, because the compounds dissolve well and do not interact with the polar stationary phase. Allow the solvent to travel up the plate until ~1 cm from the top. Take the plate out and mark the solvent front immediately. Do not allow the solvent to run over the edge of the plate. Next, let the solvent evaporate completely<sup>1</sup>.



## Visualization

When the solvent front has moved to within about 1 cm of the top end of the adsorbent (after 15 to 45 minutes), the plate should be removed from the developing chamber, the position of the solvent front marked, and the solvent allowed to evaporate.



If the components of the sample are colored, they can be observed directly. If not, they can sometimes be visualized by shining ultraviolet light on the plate or by allowing the plate to stand for a few minutes in a

closed container in which the atmosphere is saturated with iodine vapor. Sometimes the spots can be visualized by spraying the plate with a reagent that will react with one or more of the components of the sample<sup>1,16</sup>.

## Analysis

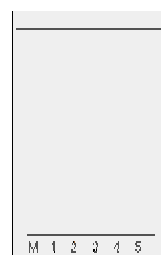
The components, visible as separated spots, are identified by comparing the distances they have traveled with those of the known reference materials. Measure the distance of the start line to the solvent front. Then measure the distance of center of the spot to the start line. Divide the distance the solvent moved by the distance the individual spot moved. The resulting ratio is called  $R_f$ -value. As the chemicals being separated may be colorless, several methods exist to visualize the spots. Often a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualization of spots under a blacklight (UV254). The adsorbent layer will thus fluoresce light green by itself, but spots of analyte quench this fluorescence, iodine vapors are a general unspecific color reagent, Specific color reagents exist into which the TLC plate is dipped or which are sprayed onto the plate. Once visible, the  $R_f$  value, or retention factor, of each spot can be determined by dividing the distance traveled by the product by the total distance traveled by the solvent (the solvent front). These values depend on the solvent used, and the type of TLC plate, and are not physical constants<sup>1,5,8</sup>.

## Using thin layer chromatography to identify compounds

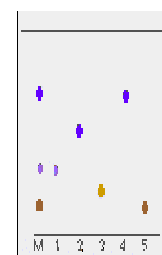
Suppose you had a mixture of amino acids and wanted to find out which particular amino acids the mixture contained. For simplicity we'll assume that you know the mixture can only possibly contain five of the common amino acids. A small drop of the mixture is placed on the base line of the thin layer plate, and similar small spots of the known amino acids are placed alongside it. The plate is then stood in a suitable solvent and left to develop as before. In the diagram, the mixture is M, and the known

amino acids are labelled 1 to 5. The left-hand diagram shows the plate after the solvent front has almost reached the top. The spots are still invisible. The second diagram shows what it might look like after spraying with ninhydrin.

There is no need to measure the  $R_f$  values because you



before spraying with ninhydrin



after spraying with ninhydrin

can easily compare the spots in the mixture with those of the known amino acids - both from their positions and their colours.

In this example, the mixture contains the amino acids labelled as 1, 4 and 5. And what if the mixture contained amino acids other than the ones we have used for comparison? There would be spots in the mixture which didn't match those from the known amino acids. You would have to re-run the experiment using other amino acids for comparison<sup>1,12,16</sup>.

## Interactions between the Compound and the Adsorbent

The strength with which an organic compound binds to an adsorbent depends on the strength of the following types of interactions: ion-dipole, dipole-dipole, hydrogen bonding, dipole induced dipole, and van der Waals forces. With silica gel, the dominant interactive forces between the adsorbent and the materials to be separated are of the dipole-dipole type. Highly polar molecules interact fairly strongly with the polar SiOH groups at the surface of these adsorbents, and will tend to stick or adsorb onto the fine particles of the adsorbent while weakly polar molecules are held less tightly. Weakly polar molecules generally tend to move through the adsorbent more rapidly than the polar species. Roughly, the compounds follow the elution order given above.

## APPLICATIONS

Thin layer chromatography has been a useful tool in numerous applications of pharmaceutical importance.

### Amino acids

TLC of amino acids is more difficult than TLC of inks, because amino acids are colorless. Therefore, one cannot see the spots with the naked eye once the plate is fully developed and dried. To see the spots, it is necessary to use either the ninhydrin or the black-light visualization techniques. E.g., Amino acids, proteins and peptides 8: A mixture of 34 amino acids, proteins and peptides has been successfully separated and isolated from urine using silica gel plates. All these substances were found to be ninhydrin positive. The development were carried out first with chloroform-methanol-20% ammonium hydroxide (2:2:1) and then with phenol-water<sup>1</sup>.

## Pharmaceuticals and drugs

TLC is used in the identification, purity testing and determination of the concentration of active ingredients, auxiliary substances and preservatives in drugs and drug preparations, process control in synthetic manufacturing processes. Various pharmacopoeias have accepted TLC technique for the detection of impurity in a drug or chemical n.e.g., Antibiotics: Penicillin's have been separated on silica gel 'G' by using the two solvents, acetone-methanol (1:1) and iso-propanol-methanol (3:7). As the detecting agent, the iodine-azide reaction was employed by spraying the dried plates with a 0.1 % iodine solution containing 3.5% of sodium azide<sup>1</sup>.

## Separation of multicomponent pharmaceutical formulations

It is also used in separation of multicomponent pharmaceutical formulations.

## Qualitative analysis of alkaloids

It is used in qualitative analysis of alkaloids in control phase of both pharmaceutical formulations and vegetable drugs. TLC has been used for the isolation and determination of alkaloids in toxicology where the 30-60 minute runs give a great advantage in comparison to the 12-24 hours required for paper chromatography. Purine alkaloids have been separated by TLC on silicic acid, silica gel and aluminium oxide. The spots are visualized by spraying first with an alcoholic iodine-potassium iodine solution followed by 25% HCl- 96% ethanol (1:1)<sup>1</sup>.

## Clinical chemistry and Biochemistry

For the determination of active substances and their metabolites in biological matrices, diagnosis of metabolic disorders such as phenylketonuria, cystinuria and maple syrup disease in babies. It serves as an useful tool in analysis of 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.

## Cosmetology

In the identification of dye raw materials and end products, preservatives, surfactants, fatty acids and constituents of perfumes.

## Food Analysis

For the determination of pesticides and fungicides in drinking water, residues in vegetables, salads and meat, vitamins in soft drinks, banned additives in Germany (e.g. sandalwood extract in fish and meat products), compliance with limit values (e.g. polycyclic compounds in drinking water, aflatoxins in milk and milk products)<sup>1</sup>.

## Analysis of Heavy Petroleum Product

Thin-layer chromatography (TLC), which is commonly used in the analysis of complex mixtures, is seldom used in the investigation of petroleum products, maybe the most complex objects. In particular, with respect to heavy petroleum products, no such information has been found in the literature. At the same time, the simplicity, economy, and efficiency of this technique in comparison with column chromatography are advantages that are widely known. TLC technique used (in the preparative variant) for a rapid determination of the group composition of heavy petroleum products (asphalts, pitches, residues), and in connection with spectroscopic studies of the chemical composition of the fractions obtained<sup>1</sup>.

## Separation of aromatic amines

Cationic and non-ionic surfactant-mediated systems have been used as mobile phases in thin-layer chromatographic separation of aromatic amines on silica gel layers. The effect of surfactant concentration below and above its critical micellar concentration on mobility of amines was examined. The influence of organic and inorganic additives such as alcohols, urea, NaCl and NaBr in micellar solutions on the mobility and separation efficiency of amines is also assessed<sup>1</sup>.

## Applications related to Organic Chemistry

- a) It has been widely used for checking number of other separation processes. TLC has also been applied successfully in various purification processes, checking of distillation fractions and for checking the progress of purification by molecular distillation.
- b) TLC has been used as an analytical tool in organic chemistry due to its high speed of separation and its applicability in a large number of chemical compounds. It's important use is in the separation and isolation of individual components of a mixture, but in organic chemistry it has also been used for: Checking the purity of samples, as purification process, for identification of organic compounds, for studying various organic reactions, in characterizing and isolating a number of compounds such as acids, alcohols, glycols, amides, alkaloids, vitamins, amino acids, antibiotics, food stuffs and examination of reaction. The reaction mixture is examined by TLC to assess whether the reaction is complete or otherwise. The method is also used in checking other separational processes and purification processes like distillation, molecular distillation etc.

High sensitivity of TLC is used to check purity of sample, because high sensitivity enables impurities to be observed in so called pure samples<sup>3,17</sup>.



**CONCLUSION**

Thin layer chromatography is simple, cost-effective, and easy-to-operate technique in phytochemistry and biochemistry with numerous applications which use in the development of new drugs and various types of formulations from medicinal plants. Further needed detailed documentation for the sustainable development in education and research.

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