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AN OVERVIEW ON THIN LAYER CHROMATOGRAPHY

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ABSTRACT

In the present article attempt has been made to explain the basic ideas and the significance of Thin layer Chromatography (TLC) in different analytical methods. As TLC is less time consuming, low cost, and can be performed with less complicated technique it has a wide application in pharmaceutical analysis. If performed precisely 32 amino acids can be separated by TLC. Also it has a wide application in identifying impurities in a compound. It can be used as a preliminary analytical method prior to HPLC. The concept of TLC is simple and samples usually require only minimal pretreatment. TLC can be used to monitor the progress of a reaction, identify compounds present in a given substance. TLC is also used to separate the identical compounds in a mixture. Many standard methods in industrial chemistry, environmental toxicology, food chemistry, water, inorganic and pesticide analysis, dye purity, cosmetics, plant materials, and herbal analysis rely upon TLC as the preferred approach.

INTRODUCTION: Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. Chromatography was discovered by M. Tswett in 1906.Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

Thin layer chromatography can be used to: Monitor the progress of a reaction, identify compounds present in a given substance, 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 (resulting in a higher Rf value). 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 Rf 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.

Principle of TLC [1] **:** 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 (the thin layer) applied to a glass or plastic plate and a liquid mobile phase (eluting solvent) that 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 [1].

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).

$$R_f = \frac{\text{Distance of centre of spot from starting point}}{\text{Distance of solvent front from starting point}}$$

The R_f value is a constant for each component only under identical experimental condition. It depends upon number of factors as;

1. **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.

2. **The mobile phase**: The purity of solvents and quantity of solvent mixed should be strictly controlled. It should be made freshly for each run if one of the solvents is very volatile or hygroscopic. Example- acetone.

3. **Temperature**: Although precise control of temperature is not necessary, the tank should be kept away from sources of heat, direct sunlight etc. As the temperature is increased, Volatile solvents evaporate more quickly, solvents run faster, and R_f values generally decrease slightly.

4. **Thickness of layer**: Standard plates approximately 250 micrometer is the preferable thickness of layer. Below 200, the R_f values vary considerably. The layers may be of higher or lower thickness in individual compounds.

5. **Developing tank**: It is important that saturated conditions are attained for running TLC plates. This is best accomplished by using small tanks with filter paper liners and sufficient solvent, and by leaving the tank to equilibrate for at least 30 minutes before running the plates. A well fitting lid is essential.

6. **Mass of sample**: Increasing the mass of sample on the plate will often increase the R_f of drug, especially if it normally tails in the system. However, if a plate is grossly overloaded, this too will give a tailing spot and will have the effect of apparently decreasing the R_f value. The two situations are normally easy to distinguish by the intensity of the spot.

7. **Chromatographic Technique**: Depending upon the development technique used i.e. ascending, descending, horizontal etc, the R_f value change for the same solvent system.

Plate preparation[1]: 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.

Capillary spotters: Place a melting point capillary and in the dark blue part of the Bunsen burner flame. Hold it there until it softens and starts to sag. Quickly remove the capillary from the flame and pull on both ends to about 2-3 times its original length. If you pull the capillary inside the flame, you will have a "piece of art", but not a good spotter. Allow the capillary to cool down, and then

break it in the middle. Make sure to break off the closed end on one of them.

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.

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- napthol, 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.

Development solvents [2]: 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.

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.

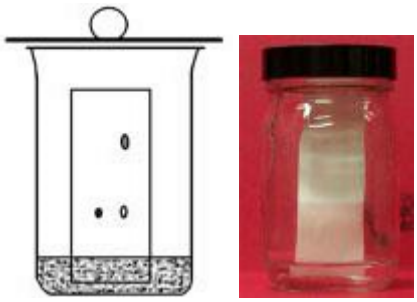
Developing a Plate [2]: A TLC plate can be developed in a beaker or closed jar. Place a small amount of solvent (mobile phase) in the container. A small spot of solution containing the sample is applied to a plate, about one centimeter from the base. The plate is then dipped in to a suitable solvent, 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*f* 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.

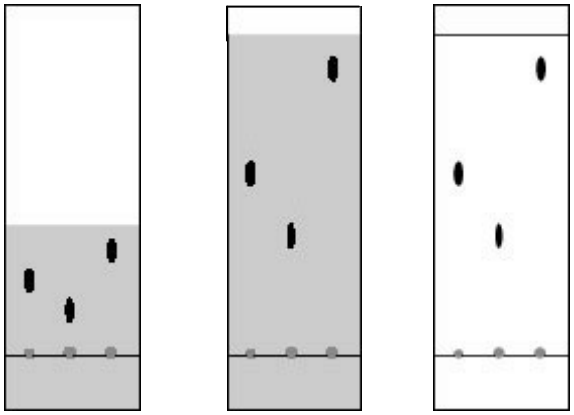
Precautions during sample application;

1. Sample should be dissolved in a nonpolar solvent as polar solvent has a tendency to spread out the starting spot.
2. Solvent used for dissolving sample should be volatile.
3. While applying sample, the surface of the adsorbent should not be disturbed as this distorts the shapes of the spots on subsequent developed chromatogram, hindering the accuracy of quantitative measurements.
4. The sample spot should be within 2-5 mm in diameter.

The TLC Experiment:



LC CHAMBER FOR DEVELOPMENT WITH A LID OR A CLOSED JAR



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.

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 (UV_{254}). 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.

Preparative TLC[8]: TLC can also be used on a small semi-preparative scale to separate mixtures of up to a few hundred milligrams. The mixture is not "spotted" on the TLC plate as dots, but rather is applied to the plate as a thin even layer horizontally to and just above the solvent level. When developed with solvent the compounds separate in horizontal bands rather than horizontally separated spots. Each band (or a desired band) is scraped off the backing material.

The backing material is then extracted with a suitable solvent (e.g. DCM) and filtered to give the isolated material upon removal of the solvent. For small-scale reactions with easily separated products, preparative TLC can be a far more efficient in terms of time and cost than doing chromatography. Obviously, the whole plate cannot be chemically developed or the product will be chemically destroyed. Thus this technique is best used with compounds that are colored, or visible under UV light. Alternatively, a small section of the plate can be chemically developed e.g. cutting a section out and chemically developing it, or masking most of the plate and exposing a small section to a chemical developer like iodine.

Applications [1-6, 9, 10]: Thin layer chromatography has been a useful tool in numerous applications of pharmaceutical importance.

1. **TLC of 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.

2. **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

   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.

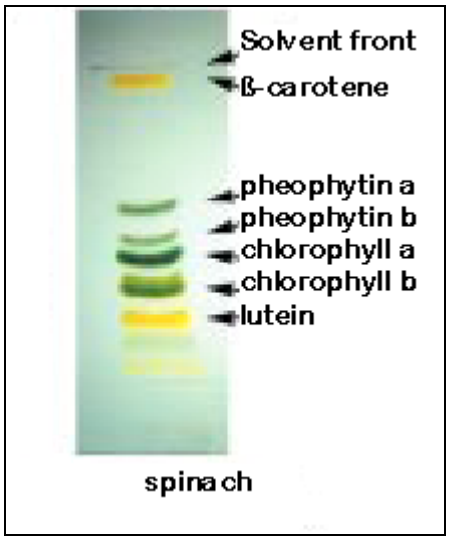
3. **Separation of multicomponent pharmaceutical formulations**: It is also used in separation of multicomponent pharmaceutical formulations.

4. **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 aluminum oxide. The spots are visualized by spraying first with an alcoholic iodine-potassium iodine solution followed by 25% HCl- 96% ethanol (1:1).

5. **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.

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

7. **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). A typical separation of dyes in spinach looks like this:



8. **Analysis of Heavy Petroleum Product**[8]: 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 preparataive variant) for a rapid determination of the group composition of heavy petroleum products (asphalts, pitches, resids), and in connection with spectroscopic studies of the chemical composition of the fractions obtained.

9. **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.

10. **Applications related to Organic Chemistry** [1-6]:

- 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.

- 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. With the help of TLC it is possible to know whether a reaction is

complete and had followed the expected course. The nature of byproducts can also be ascertained by using TLC. If the reaction does not proceed as desired or expected, then an examination of the behaviour of the spots with standard reagents may sometimes give information for the rapid identification of the products.

Problems in TLC:

Over-large Spots: Sample spots made using TLC capillaries should be no larger than 1-2 mm in diameter, because component spots in the developed plate will be no smaller than, and will usually be larger than, the size of the initial spot. If the initial spot is larger than 2 mm in diameter, then components with similar R*f* values may not be resolved because their spots will be so large that they will overlap considerably and may appear to be one large spot. Small initial spots, on the other hand, maximize the potential of complete separation of components.

Uneven Advance of Solvent Front: A common problem in TLC is uneven advance of solvent along the plate. Instead of a straight line, the solvent front may appear to bow either up or down in the center. Uneven advance of solvent leads to uneven advance of substance spots, and inaccurate R*f* values result. A frequent cause of uneven solvent advance is the use of a developing chamber that does not have a flat bottom. Glass bottles usually have bottoms that curve upward from the edges to the center. If the bottom of the TLC plate is placed on this curved surface, the shape of the solvent advance line may mirror the shape of the container bottom. It is therefore important to use flat-bottomed developing tanks in TLC. A bowed solvent front may also result if too little developing solvent is placed in the chamber; if the plate is cut improperly, so that the sides are not exactly perpendicular to the bottom edge; and if the slide is excessively tilted in the chamber. Care in choosing and using a developing chamber is the best defense against curved solvent fronts. Water is seldom used as a developing solvent because it has a tendency to produce a dramatically curved front. This may be due to its unusually high surface tension.

Streaking: Sometimes a substance will move along a TLC plate as a long streak, rather than as a single discrete spot. This is the result of spotting the plate with too much substance, more than the moving solvent can handle. The solvent moves as much substance as it can, but a substantial amount of substance is left behind. The substance is dragged along by the solvent leaving a trail of substance that may sometimes span the entire distance between the starting line and the solvent front. 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.

Specific TLC Procedures:

1. **Separartion for alanine, glycine, threonine, and proline**: TLC of amino acids is more difficult 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. Observe the spots, and decide whether or not a chosen solvent system has been effective in moving an amino acid or in separating a mixture. Therefore the process of finding an effective solvent system can be long and painstaking. As points of general information, amino acids are quite polar and tend to move on silica gel plates with polar solvents. They have R*f* values close to 1 when water or concentrated ammonia is used as the developing solvent, probably because of their high solubility in water. Diluting a polar solvent

with a less polar one results in smaller Rf values, roughly in proportion to the amount of less polar solvent used, Thus, alanine, glycine, threonine, and proline all have R*f* values of around 0.60 when developed with a 50/50 mixture of water and n-propanol, and around 0.40 when developed with a 30/70 mixture of concentrated NH_3 and n-propanol. The following procedure assumes the use of 50/50 water/n-propanol as the developing solvent, but one can try other polar/non-polar combinations.

Experimental Procedure In the hood, prepare 10 mL of a mixture consisting of 50% 1-propanol and 50% water by volume, and pour about half of this into a clean developing tank. Make sure that the level of liquid in the tank is no higher than 5 mm, and close the lid. Prepare a solution of about 0.001 g of amino acid in 0.2 mL of water. Dissolve the acid, and then draw some solution up in a spotting capillary and double-spot a properly marked and activated TLC plate. Allow the plate to dry for 5 minutes, and then lower the plate into the developing tank so that its bottom is submerged in the developing solvent. Close the lid, and allow the plate to develop until solvent has risen to the pencil line at the top of the plate.

Remove the plate from the tank and place it in an oven at 50 ^{o}C to dry. When the plate is dry, visualize it using ninhydrin spray or iodination. Circle the amino acid spots with pencil, and calculate R*f* values. Compare the measured R*f* values with the values for the amino acids. On this basis, identity of amino acid is done. In combination with other data , obtain, information which will help unambiguously to identify amino acid. Suppose that amino acid has R*f* v*a*lue similar to that of alanine, one should then prepare a small amount of alanine solution and spot it alongside amino acid on a new TLC plate. Develop, dry, and visualize the plate to confirm that amino acid indeed has exactly the same value as alanine, and that the spot is the same shape and color. Finally, it is very important to be observant of detail in doing TLC. In addition to the R*f* value for a substance, the shape of the spot produced by a particular developing solvent and the shade of color produced by iodine or ninhydrin can be characteristic of the substance. For example, when alanine, glycine, threonine, and proline are spotted side-by-side on a plate and developed with 70% n-propanol/30% conc NH_3 following observations can be made:

Amino Acid	Solvent	Spot Color after Iodination	Spot Color with Ninhydrin	R_f Value	Spot Shape
alanine	50/50 water/n-propanol	white on brown bkgrnd	purple	0.65	circle
glycine	30/70 conc NH_3/n-propanol	white on brown bkgrnd	pink	0.25	elongated oval
glycine	50/50 water/n-propanol	white on brown bkgrnd	pink	0.55	circle
threonine	50/50 water/n-propanol	white on brown bkgrnd	purple	0.57	circle
proline	50/50 water/n-propanol	white on brown bkgrnd	yellow with pink border	0.65	circle

Rf values of amino acids

2. **Separation of dyes in spinach**: On a balance weigh out 0.5 grams of fresh spinach and combine with 0.5 grams of anhydrous magnesium sulfate and 1.0 grams of sand. Transfer these materials to a mortar and using a pestle grind the mixture until a fine dry powder is obtained (grind the mixture really well). The anhydrous magnesium sulfate will remove the water from the leaves. Transfer the powder (2.0 grams total) to a small test tube and combine with 2.0 mL of acetone. Stopper

the test tube and shake vigorously for approximately one minute. You need to make sure that the solid and solvent are well mixed.

Allow this mixture to stand for 10 minutes, and then using a pipette carefully transfer the solvent above the solid into a small micro centrifuge tube. Use care not to transfer any of the solid material. The solvent extract should be green. Cap the micro centrifuge tube to minimize solvent evaporation. Obtain a TLC chamber (a 400 mL glass beaker covered with parafilm or aluminum foil) and add developing solvent (a mixture of petroleum ether, acetone, cyclohexane, ethyl acetate and methanol). The solvent should completely cover the bottom of the chamber to a depth of approximately 0.5 cm. Keep the chamber covered so that evaporation doesn't change the composition of the solvent. Allow the TLC plate to develop (separation of pigments) for approximately 10 minutes. As the solvent moves up the TLC plate you should see the different colored pigments separating.

Remove the TLC plate from the chamber when the solvent front is approximately 1.0cm from the top of the TLC plate. With a pencil, mark the level of the solvent front (highest level the solvent moves up the TLC plate) as soon as you remove the strip from the chamber (the solvent evaporates and disappears quickly). Also measure the pigment distances quickly as some pigments (especially the beta-carotene) may fade over time.

Beta-carotene is the most non-polar pigment (highest R*f*) and its band will be yellow. Chlorophyll a has a larger *Rf* than does chlorophyll b. For the following calculations mark the center of the initial pigment dot; this will be the starting point for all the following measurements. Also mark the middle point of each pigment band and the solvent front. The literature gives R*f* values of 0.61 and 0.49 for pheophytin a and pheophytin b. Use these values to help identify which spots are due to those compounds. Use the pure extract of ß-carotene to identify the location of that band.

3. **Identification of naphthodianthrones**: Hypericum perforatum extracts containing naphthodianthrones have been identified by TLC using silica gel plates with fluorescence indicator and ethyl acetate-formic acid-water(30:2:3 v/v/v) or toluene- ethyl acetate-formic acid-water(50:40:5:5 v/v/v/v) as the mobile phase. TLC can also be used for the identification of indolic alkaloids isolated from various Rauwolfia Species by using silica gel plates as stationary species; acetone-light petroleum-diethyl amine(2:7:1 v/v/v) and 1% solution of ammonium cerium (IV) sulfate for the visualization of spots.

4. **Identification of drugs: Aspirin, acetaminophen, ibuprofen, and caffeine:** Draw a light pencil line about 1 cm from the end of a chromatographic plate, and on this line spot aspirin, acetaminophen, ibuprofen, and caffeine, which are available as reference standards. Use a separate capillary for each standard. Make each spot as small as possible, preferably less than 0.5 mm in diameter. Use the blower to facilitate the evaporation of the solvent between applications. Examine the plate under the ultraviolet (UV) light to see that enough of each compound has been applied; if not, add more. On a separate plate run three of the unknowns and one of the aspirin standard. The unknown sample is prepared by crushing a part of a tablet, adding this powder to a test tube or small vial along with an appropriate amount of ethanol, and then mixing the suspension. Not all of the tablet will dissolve, but enough will go into solution to spot the

plate. The binder starch or silica will not dissolve. The 1% solutions should be prepared and ready to use. After the solvent has risen to about 2/3 of the length of the plate, remove the plate from the developing chamber. Quickly mark the solvent front with a pencil and allow the solvent to dry. Examine the plate under UV light to see the components as dark spots against a bright green-blue background. Outline the spots with a pencil. The spots can also be visualized by putting the plate in an iodine chamber made by placing a few crystals of iodine in the bottom of a capped jar. Calculate the R*f* values for the spots.

Substances:	Aspirin	Caffeine	Acetaminophen	Ibuprofen
R*f* Values:	0.45	0.08	0.24	0.60

5. **Separation of Inorganic Ions:** TLC has been used for separating cationic, anionic, purely covalent species and also organic derivatives of the metals. In order to carry out TLC of groups of cations, silica gel is first washed with acid and water to remove impurities of sodium, magnesium, calcium and iron. But this treatment removes the calcium sulphate binder. Therefore, calcium sulphate must be replaced by starch or some other suitable binder. After washing and drying of TLC plate, the spots of cations or anions to be separated are applied on this plate. The plate is then kept in a close chamber and the lower part of the plate is then dipped into a solvent. It is than removed from chamber and dried, visualized for spots by suitable visualizing reagents.

REFERENCES:

1. Singhal S., Singhal N., Agarwal S., Pharmaceutical Analysis II, Thin layer chromatography, Pragati prakashan, First edition, 2009, 98-111.
2. Kasture A.V., Mahadik K.R, Wadodkar S.G, More H.N., A textbook of pharmaceutical analysis, Instrumental methods, Nirali Prakashan, 9th edition, 2005, vol II, 18-30.
3. Quach, H. T, Steeper, R L, Griffin, G. W, Separation of plant pigments by thin layer chromatography. Journal of chemical education. 2004, 81, 385-7.
4. Skoog D. A., Holler F.J. and Nieman T.A., "Principles of instrumental analysis, Saunders college publishing, 5th edition, 2006 ,761-766.
5. Chatwal G. R., Anand S.K, Instrumental methods of chemical analysis, Himalaya publishing house,5th edition,2008, 2.599-2.616.
6. Beckett A.H, Stenlake J.B., Practical pharmaceutical chemistry, Thin layer chromatography, CBS publishers, 4th edition, 2005, 115-128.
7. Ali M. and Agrawal V., Thin-layer chromatography of aromatic amines, Separation Science and Technology, 2002, 37, 363 - 377.
8. Sharma B.K., Instrumental methods of chemical analysis, Goel publishing house, Meerut, 5th edition, 2007, 241-264.
9. Vidya Sagar, Instrumental methods of drug analysis, Pharma Med Press, First edition 2009,263.
10. Justus G. Kirchner, Thin-layer chromatographic quantitative analysis, Journal of Chromatography A ,1 August 1973, Volume 82, Issue 1, 101-115.

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