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Review On: Biotechnology Of Herbal Extract

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Abstract:

This study aims to review the fundamental principles and significance of Thin Layer Chromatography (TLC) in research, particularly in phytochemistry. TLC is a straightforward, cost-efficient, and user-friendly planar chromatographic technique that has been utilized in chemistry laboratories for many years to separate chemical and biochemical substances. Typically, chemical and optical methods are employed to detect analyte spots on the TLC plate. Additionally, TLC is widely used to identify impurities in compounds. This review emphasizes the application of TLC for the qualitative and quantitative analysis of bioactive compounds in medicinal plants.

Keywords: *Thin Layer Chromatography, Solvent Selection, Plant Extraction, Phytochemical Screening, Medicinal Herbs.*

Introduction:

Chromatography is a collection of laboratory techniques used to separate mixtures into their individual components based on their interaction with two phases: a stationary phase (solid or liquid on a solid surface) and a mobile phase (liquid or gas). The mixture is dissolved in the mobile phase, which moves through the stationary phase, causing the components to travel at different speeds and separate due to their varying affinities for each phase. Among the various types of chromatography, thin-layer chromatography (TLC) is a widely used method that employs a thin layer of adsorbent material, such as silica gel, alumina, or cellulose, coated on a flat surface like glass, metal, or plastic. Compared to paper chromatography, TLC offers faster results, better separation, and flexibility in adsorbent selection. A small sample is applied near the bottom of a TLC plate, which is placed in a developing chamber with a solvent (mobile phase). The solvent rises by capillary action, separating the mixture's components. TLC is extensively used to monitor organic chemical reactions, assess the purity of compounds, and analyze complex mixtures in fields like biotechnology and phytochemistry. To optimize separation, the solvent system is adjusted through trial and error, with higher solvent polarity increasing the movement of components and lower polarity slowing them. The best solvent system achieves optimal separation, and TLC patterns often correspond to those in column chromatography.

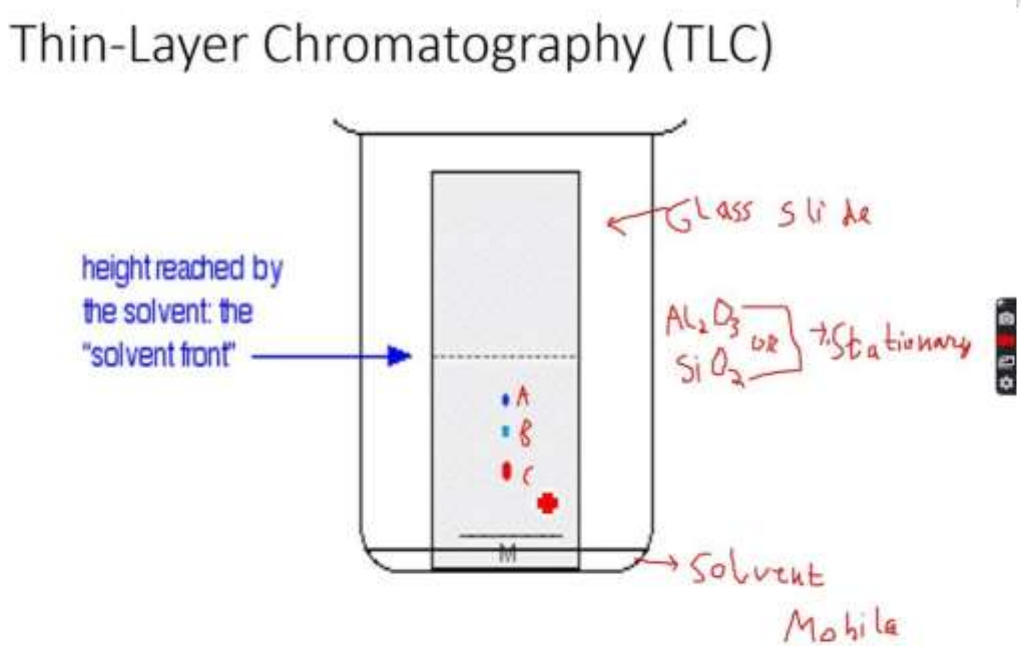
Thin-layer chromatography (TLC) is a faster technique compared to column chromatography, making it a preferred method for determining the optimal solvent system for column chromatography procedures, such as flash chromatography. The ideal solvent system for flash chromatography is one that moves the desired compound on a TLC plate to an Rf value of 0.25-0.35 while ensuring a separation of at least 0.20 in Rf values between the desired compound and its nearest neighbor. Using TLC to analyze a mixture helps identify the most suitable solvent(s) for flash chromatography.

TLC is also widely employed to monitor reaction progress, identify compounds in a sample, and evaluate the purity of substances. The separation of compounds on a TLC plate relies on the competition between the solute and the mobile phase for binding sites on the stationary phase. For example, when silica gel is used as the stationary phase, it is polar in nature. Between two compounds with different polarities, the more polar compound binds more strongly to the silica, displacing the mobile phase and therefore moving less on the TLC plate. In contrast, the less polar compound interacts less with the stationary phase and travels farther up the plate.

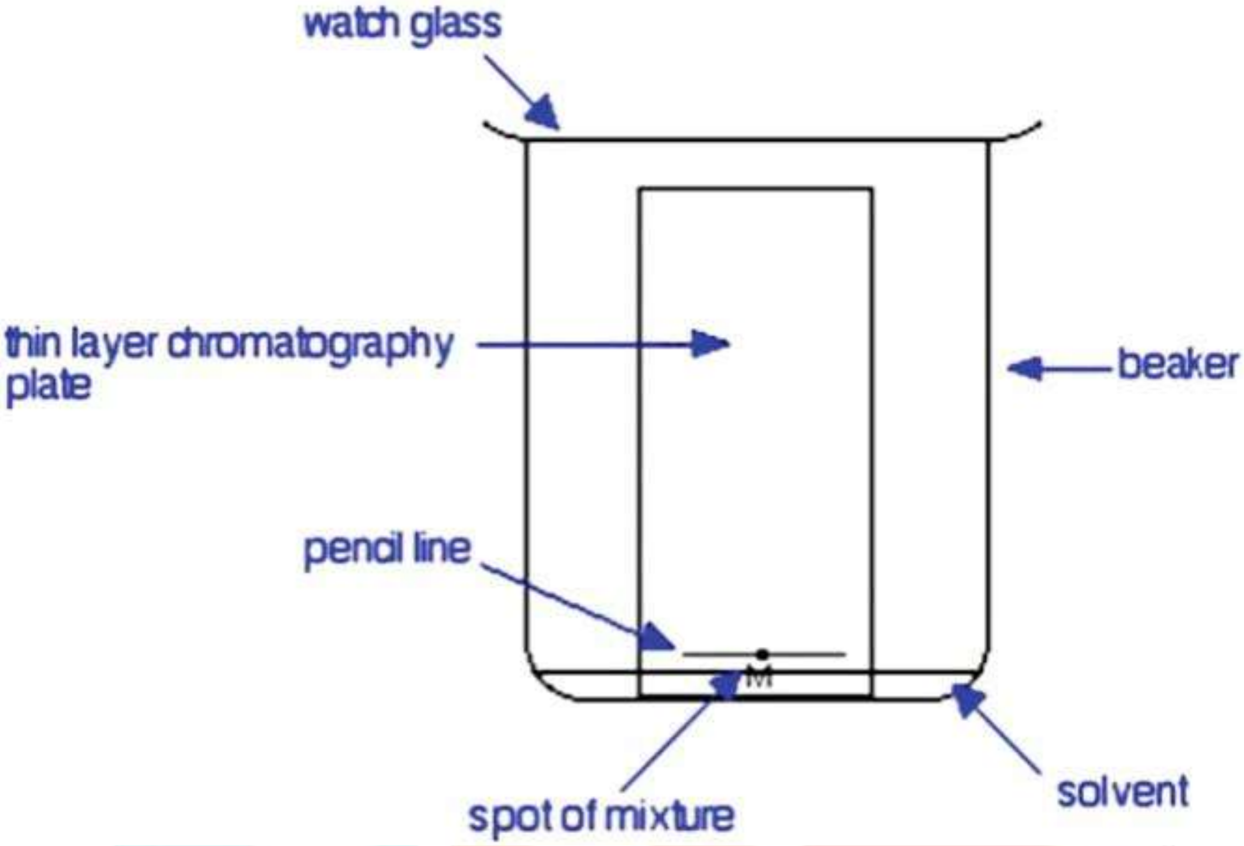
If the mobile phase's polarity is increased by using a more polar solvent or solvent mixture, it competes more effectively with the solute for binding sites on the stationary phase. This results in all compounds on the TLC plate moving higher, with higher Rf values. For instance, using a mobile phase of ethyl acetate and heptane, increasing the proportion of ethyl acetate will cause all compounds to move farther up the plate. However, changing the polarity of the mobile phase typically does not reverse the order in which compounds move on the plate.

Chromatogram:As the solvent gradually ascends the TLC plate, the various components of the dye mixture move at different speeds, causing the mixture to separate into distinct colored spots. The solvent is allowed to travel nearly to the top of the plate to achieve maximum separation of the dye components for the specific combination of solvent and stationary phase used.

Principle: Thin layer chromatography (TLC) operates on a glass plate coated with a solid phase, typically



aluminum oxide or silica gel. The mobile phase is a solvent selected based on the properties of the components in the mixture. The principle of TLC involves the distribution of a compound between the solid stationary phase on the plate and the liquid mobile phase moving over it. A small amount of the sample is applied near the bottom of the plate, and the plate is then placed in a developing chamber containing a shallow pool of solvent below the applied sample. The solvent rises up the plate through capillary action, and as it moves, the compounds in the mixture either remain with the stationary phase or dissolve in the solvent and travel upward. The movement of each compound depends on its physical properties, particularly its molecular structure and functional groups. The "Like Dissolves Like" principle applies: the more similar a compound is to the mobile phase, the further it



will travel. Compounds that are more soluble in the mobile phase will move farther up the plate, while those that are less soluble and have a higher affinity for the stationary phase will move more slowly or stay behind.

Rf value: In thin-layer chromatography (TLC), the behavior of an individual compound is described by a value known as Rf (retention factor), which is expressed as a decimal fraction. The Rf value is calculated by dividing the distance a compound has traveled from its original position by the distance the solvent has traveled from the same starting point (the solvent front). The Rf value can vary depending on the type of adsorbent used, as different adsorbents will yield different Rf values for the same solvent. To ensure reproducibility, the adsorbent must have a constant particle size and binder. TLC plates should be stored in desiccators over silica gel before use, and the sample should be applied quickly to prevent the plate from absorbing water vapor from the air. Due to the challenges involved with activating plates, it is generally better to use plates stored at room temperature without activating them.

To identify the compounds present in a sample measurements are taken from the TLC plate, including the distance traveled by the solvent and the distance traveled by each spot. Once the solvent approaches the top of the plate, the plate is removed from the beaker, and the solvent front is marked before it evaporates. These measurements are used to calculate the Rf value, which is determined by dividing the distance traveled by the compound by the distance traveled by the solvent.

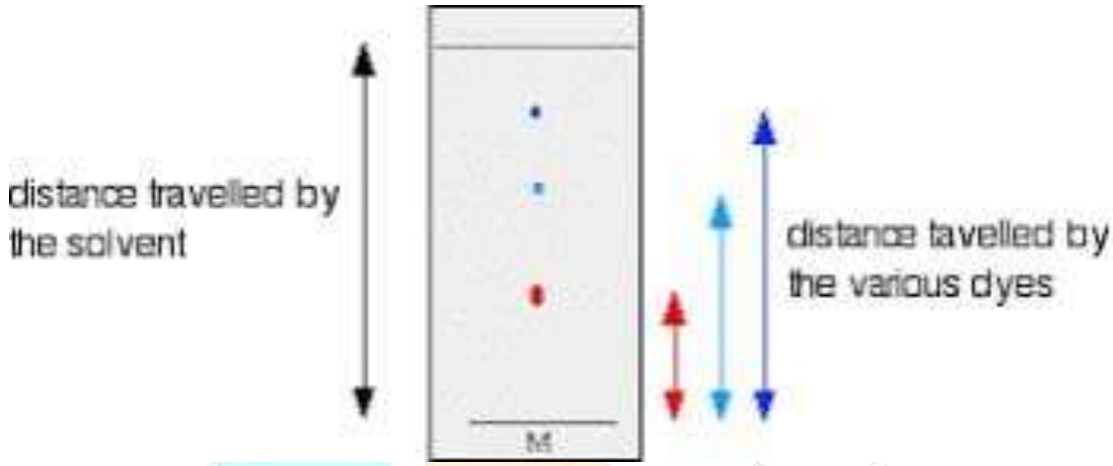
For example, if the red component moves 1.7 cm from the baseline while the solvent travels 5.0 cm, the Rf value for the red dye would be 0.34. If the experiment is repeated under identical conditions, the Rf value would remain the same. However, changes in factors such as temperature or the exact solvent composition can alter the results, so consistency is important when using TLC for identification.

The Rf value for a compound is considered constant only if the following conditions remain unchanged :

- Solvent system
- Adsorbent
- Adsorbent thickness

- Amount of material spotted

Since it is challenging to maintain these factors exactly from one experiment to the next, relative Rf values are usually reported. "Relative Rf" means comparing the Rf values of compounds run on the same plate under identical conditions. A higher Rf value indicates that the compound is less polar, as it interacts less with the polar adsorbent on the plate. Conversely, a more polar compound will have a lower Rf value.



To confirm the identity of a compound, an authentic standard or sample of the compound can be spotted and run alongside the unknown on the same plate. If the two substances share the same Rf value, they are likely the same compound. However, if their Rf values differ, they are definitely distinct compounds. It is crucial to perform this comparison on the same plate, as reproducing identical conditions across different experiments can be difficult.

Preparation of TLC Plates

TLC plates are typically commercially available, with standard particle sizes to enhance consistency. To prepare the plates, the adsorbent, such as silica gel, is mixed with a small amount of an inert binder like calcium sulfate (gypsum) and water. This mixture is applied as a thick slurry onto an unreactive carrier, usually made of glass, aluminum foil, or plastic. After application, the plate is dried and then activated by heating it at 110°C for 30 minutes in an oven. For analytical purposes, the adsorbent layer's thickness is generally between 0.1–0.25 mm, while for preparative TLC, it ranges from 0.5–2.0 mm.

Spotting the Plate

To spot the plate, dip the thin end of the spotter into the dilute solution, allowing the liquid to rise into the capillary due to capillary action. Briefly touch the plate at the start line, and allow the solvent to evaporate. Then, spot the same area again to form a concentrated, small spot. Avoid overloading the plate with material, as this can result in poor separation (leading to 'tailing'). Ensure that the spots are spaced sufficiently apart and away from the plate's edges. If possible, spot the sample along with the starting materials and any potential intermediates.

Locating Spots on TLC Plates

The positions of different solutes separated by TLC can be identified using various techniques. Colored compounds are visible directly when viewed against the stationary phase, but colorless compounds need to be made visible with specific spraying agents that produce colored spots where the substances are located. Some common methods for revealing invisible spots in TLC include:

1. Corrosive agents, which are purely inorganic, can be sprayed onto the plate to reveal spots.

2. A dilute solution of potassium dichromate in concentrated sulfuric acid. In this process, potassium dichromate (yellow) is reduced to chromic sulfate (green) by most organic compounds, often used to detect sugars.

3. Sulfur trioxide vapors, produced by heating fuming sulfuric acid, char organic compounds and turn them into dark spots.

4. A solution of potassium permanganate.

5. Iodine vapors.

Other common reagents used for detection include saturated hydrogen sulfide solution, 0.2N aqueous ammonium sulfide, 0.1% alcoholic quercetin, 0.2% methanolic 1-(2-pyridylazo)-2-naphthol, 1% methanolic oxine, and 0.5% aqueous sodium rhodizonate. If the TLC plate contains a fluorescing material, the solutes can also be viewed under ultraviolet light.

Development Solvents

The selection of an appropriate development solvent depends on the nature of the substance being analyzed and the type of adsorbent used on the TLC plate. An ideal solvent should not chemically react with the components of the mixture being examined. Carcinogenic solvents (such as benzene) and environmentally hazardous solvents (like dichloromethane) should always be avoided. Solvent systems can range from non-polar to polar solvents, with non-polar solvents being preferred since highly polar solvents can lead to the adsorption of any component of the solvent mixture. Common development solvents include petroleum ether, carbon tetrachloride, pyridine, glycol, glycerol, diethyl ether, formamide, methanol, ethanol, acetone, and n-propanol.

Mobile Phase

In silica gel chromatography, the mobile phase is typically an organic solvent or a mixture of organic solvents. As the mobile phase moves across the silica gel surface, it transports the analyte along the stationary phase particles. However, the analyte molecules can only move with the solvent if they are not bound to the silica gel surface. The retention factor of the analyte is determined by the ratio of time it spends bound to the silica gel surface versus the time it spends in the solution.

The interaction of the analyte with the silica gel surface in the presence of a specific solvent or solvent mixture can be seen as the result of two competing forces. First, polar groups in the solvent can compete with the analyte for binding sites on the silica gel. If a highly polar solvent is used, it will interact strongly with the silica gel surface, leaving fewer free sites for the analyte to bind. As a result, the analyte will move more quickly through the stationary phase. Similarly, the solvent's polar groups can interact with the polar parts of the analyte, preventing the analyte from interacting with the silica gel surface, which also leads to faster movement of the analyte.

The polarity of a solvent used in chromatography can be assessed by its dielectric constant and dipole moment—higher values for both indicate a more polar solvent. Additionally, the solvent's hydrogen bonding ability should be considered. For instance, methanol, being a strong hydrogen bond donor, can significantly inhibit the ability of most analytes to bind to the silica gel surface, except for the most polar analytes.

Developing a Plate

A TLC plate can be developed in either a beaker or a closed jar. Begin by adding a small amount of the solvent (mobile phase) to the container. A small spot of the sample solution is applied to the plate, about one centimeter from the bottom. The plate is then placed into a container with a suitable solvent, such as hexane or ethyl acetate, and sealed. The solvent rises up the plate through capillary action and dissolves the sample mixture, which is carried upward by the solvent.

The components of the sample move at different speeds due to variations in their affinity for the stationary phase and differences in solubility in the solvent. By altering the solvent or using a mixture, the separation of the components (indicated by the Rf value) can be modified. It is important that the solvent level remains below the starting line of the TLC plate, or the spots will dissolve. The lower edge of the plate is then immersed in the solvent. As the solvent (eluent) moves up the plate through capillarity, it carries the sample components at different rates depending on their interaction with the stationary phase and their solubility in the solvent. Non-polar solvents tend to move non-polar compounds to the top of the plate because these compounds dissolve easily and do not interact with the polar stationary phase. Allow the solvent to travel up the plate until it is about 1 cm from the top. Remove the plate and immediately mark the solvent front. Avoid letting the solvent run off the edge of the plate. Finally, let the solvent evaporate completely.

Visualization

Once the solvent front has moved to within about 1 cm of the top of the adsorbent (usually after 15 to 45 minutes), remove the plate from the developing chamber and mark the position of the solvent front. Allow the solvent to evaporate.

If the sample components are colored, they can be observed directly. If they are colorless, they may be visualized by shining ultraviolet light on the plate or by placing the plate in a closed container where the atmosphere is saturated with iodine vapor for a few minutes. Alternatively, the spots may be revealed by spraying the plate with a reagent that reacts with one or more components of the sample.

Analysis

The separated components, visible as distinct spots, are identified by comparing their migration distances with those of known reference materials. First, measure the distance from the start line to the solvent front. Then, measure the distance from the start line to the center of each spot. To calculate the Rf value, divide the distance traveled by the solvent by the distance traveled by the spot. This ratio is known as the Rf value.

Since the substances being separated may be colorless, several methods are available to visualize the spots. A small amount of a fluorescent compound, such as manganese-activated zinc silicate, is often added to the adsorbent, enabling the spots to be seen under UV light (UV254). The adsorbent itself will fluoresce light green, while the spots of the analyte will quench this fluorescence. Iodine vapors are a non-specific color reagent, and there are also specific color reagents that can be applied by dipping the TLC plate or spraying it. Once the spots are visible, the Rf value for each can be determined by dividing the distance traveled by the spot by the distance the solvent front traveled. These values depend on the solvent and the type of TLC plate used and are not fixed constants.

Using Thin Layer Chromatography to Identify Compounds

Imagine you have a mixture of amino acids and need to identify which specific amino acids it contains. For simplicity, let's assume the mixture could only contain five known amino acids. A small drop of the mixture is placed on the baseline of the TLC plate, and small spots of the known amino acids are placed next to it. The plate is then placed in a suitable solvent to develop as described previously. In the first diagram, the mixture is labeled "M," and the known amino acids are labeled 1 to 5. The plate is shown after the solvent front has almost reached the top, but the spots remain invisible. The second diagram shows the appearance of the plate after spraying it with ninhydrin.

There's no need to measure the Rf values, as you can simply compare the positions and colors of the spots in the mixture with those of the known amino acids. In this case, the amino acids corresponding to spots 1, 4, and 5 are present in the mixture. If the mixture contains amino acids not included in the comparison set, there will be additional spots that don't match any of the known ones. To identify these, you would need to run the experiment again with other amino acids for comparison.

Interactions Between the Compound and the Adsorbent

The strength with which an organic compound binds to an adsorbent is influenced by several types of interactions: ion-dipole, dipole-dipole, hydrogen bonding, dipole-induced dipole, and van der Waals forces. In the case of silica gel, the primary interaction between the adsorbent and the compounds being separated is of the dipole-dipole type. Highly polar molecules interact strongly with the polar SiOH groups on the surface of the silica gel and tend to adsorb more tightly to the fine particles of the adsorbent. In contrast, weakly polar molecules interact less strongly and generally move through the adsorbent more quickly than polar molecules. As a result, compounds generally follow the elution order based on their polarity, with weakly polar compounds moving faster than highly polar ones.

Applications of Thin Layer Chromatography

Thin Layer Chromatography (TLC) has proven to be a valuable tool in various pharmaceutical applications. Below are some of its key uses:

Amino Acids

TLC of amino acids is more challenging than that of colored substances like inks, as amino acids are colorless. This makes it impossible to see the spots with the naked eye after the plate has developed and dried. To visualize these spots, methods such as ninhydrin or black-light (UV) visualization are used. For example, a mixture of 34 amino acids, proteins, and peptides was successfully separated from urine using silica gel plates, with all substances testing positive with ninhydrin. The development process involved using a chloroform-methanol-ammonium hydroxide mixture followed by phenol-water.

Pharmaceuticals and Drugs

TLC is employed for the identification, purity testing, and concentration determination of active ingredients, auxiliary substances, and preservatives in pharmaceuticals. It also plays a role in process control during synthetic manufacturing. Many pharmacopoeias accept TLC for detecting impurities in drugs and chemicals. For instance, penicillins were separated using silica gel and two solvent mixtures, acetone-methanol and iso-propanol-methanol, with iodine-azide as a detecting agent sprayed on the dried plates.

Separation of Multicomponent Pharmaceutical Formulations

TLC is also widely used in separating complex pharmaceutical formulations, where it helps analyze the components and ensure quality control.

Qualitative Analysis of Alkaloids

TLC is a quick and efficient method for the qualitative analysis of alkaloids in pharmaceutical formulations and plant-based drugs. It is particularly valuable in toxicology, offering faster analysis (30-60 minutes) compared to paper chromatography (12-24 hours). TLC has been used to isolate purine alkaloids on various adsorbents such as silicic acid, silica gel, and aluminum oxide. The spots are visualized by spraying with a mixture of alcoholic iodine-potassium iodine solution followed by hydrochloric acid-ethanol solution.

Clinical Chemistry and Biochemistry

TLC is employed in clinical chemistry for detecting active substances and their metabolites in biological samples. It is used for diagnosing metabolic disorders, such as phenylketonuria, cystinuria, and maple syrup disease in infants. Additionally, TLC is useful in analyzing urinary constituents, such as steroids, amino acids, porphyrins, and bile acids. It is often used alongside other chromatographic techniques to detect minor metabolites and achieve more precise separations.

Cosmetology

TLC is used in the identification of raw materials and end products in cosmetics, including dyes, preservatives, surfactants, fatty acids, and perfume components.

Food Analysis

In food safety, TLC helps detect pesticides and fungicides in drinking water, as well as residues in vegetables, salads, and meats. It is also used for determining vitamins in soft drinks and checking for banned additives in food products, such as sandalwood extract in fish and meat. Additionally, TLC is used to ensure compliance with limit values for substances like polycyclic aromatic hydrocarbons in drinking water and aflatoxins in milk and dairy products.

Analysis of Heavy Petroleum Products

Thin-layer chromatography (TLC) is not commonly employed for the analysis of petroleum products, especially heavy petroleum products, which are among the most complex substances. However, the simplicity, cost-effectiveness, and efficiency of TLC, particularly when compared to column chromatography, make it a valuable technique. TLC has been used in a preparative variant for the rapid determination of the group composition of heavy petroleum products such as asphalts, pitches, and residues. It is also useful in spectroscopic studies to analyze the chemical composition of the fractions obtained from these products.

Separation of Aromatic Amines

In the separation of aromatic amines using TLC, cationic and non-ionic surfactant-based systems have been employed as mobile phases on silica gel plates. The mobility of amines has been examined with surfactant concentrations both below and above their critical micellar concentration. Additionally, the effect of organic and inorganic additives, such as alcohols, urea, NaCl, and NaBr, in micellar solutions on the mobility and efficiency of separation has been studied.

Applications in Organic Chemistry

a) Purification Processes: TLC is widely used to monitor and check various separation and purification processes. It is particularly useful for assessing the progress of purification, such as in molecular distillation, and for checking the purity of distillation fractions.

b) Analytical Tool: TLC is frequently used in organic chemistry for its rapid separation capability and applicability to a wide range of chemical compounds. It is particularly effective for isolating individual components from mixtures and checking the purity of samples. Beyond separation, TLC is used to identify organic compounds, study reactions, and isolate compounds like acids, alcohols, glycols, amides, alkaloids, vitamins, amino acids, and antibiotics. The technique is also valuable for examining the progress of chemical reactions by comparing the reaction mixture to the initial reagents. Furthermore, TLC plays a crucial role in evaluating the efficiency of other separation and purification methods, such as distillation and molecular distillation. The high sensitivity of TLC allows even trace impurities to be detected, making it a powerful tool for confirming the purity of a sample.

Conclusion

Thin-layer chromatography (TLC) is a straightforward, affordable, and user-friendly technique widely used in phytochemistry and biochemistry. It has numerous applications, particularly in the development of new drugs and various formulations derived from medicinal plants. However, there is a need for more comprehensive documentation to support sustainable development in education and research.

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