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A Comprehensive Review on HPTLC Method Development

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ABSTRACT

High-Performance Thin-Layer Chromatography (HPTLC) is a widely utilized analytical technique that offers significant advantages in the qualitative and quantitative analysis of a variety of chemical substances. This review aims to provide a comprehensive overview of HPTLC method development, focusing on its principles, instrumentation, key parameters, and applications. The paper discusses the critical steps involved in HPTLC method development, including selection of stationary phases, mobile phases, detection methods, and optimization of experimental conditions to achieve high resolution, sensitivity, and reproducibility. The review also highlights the importance of sample preparation, validation procedures, and the integration of HPTLC with other analytical techniques, such as mass spectrometry (MS) and spectrophotometry, to enhance its capabilities. Furthermore, the article explores the wide range of applications of HPTLC in pharmaceutical, environmental, food, and forensic analysis. This review aims to serve as a valuable resource for researchers and analytical chemists involved in the development and optimization of HPTLC methods for complex matrix analysis.

Keywords: HPTLC, Method development, Validation, Instrumentation, Qualitative analysis, Chromatography

INTRODUCTION

High-performance thin-layer chromatography (HPTLC) is an advanced form of instrumental TLC, which does not only include the use of high-performance adsorbent layers (e.g. silica gel with refined uniform particles, approximately 5µm in diameter, as compared to 12µm in TLC), but also adopted instrumentation e.g. the development chambers. It usually also implies a standardized methodology for development, optimization, documentation and the use of validated methods. The HPTLC technique is applied in qualitative and quantitative separations of compounds in mixtures, where the quantitative mode operates in a more optimized way (standardized with a given procedure),The compounds in samples can therefore be tested using it **[1].**Even if science and technology have never been more promising or provided so many potentials to prolong life and enhance health, both the public and commercial sectors continue to invest in these fields despite the state of the conomye. It's quite difficult to increase pharmaceutical sector success rates and offer more medications, yet there cannot be many predictive scientific and analytical methods available.medication research includes controlling the synthesis of the medication and its end product in bulk,

analyzing the drug's side effects or potential contaminants toxicologically, and tracking bodily fluids to determine how a drug and its metabolites behave in an organism. Purity, content, homogeneity, chemical and physical stability, biological availability, identification studies, and the therapeutic efficacy and quality of the pharmaceutical product and bulk medication are common standards for drug evaluation [2].

History:

Russia's history, 1872–1919 Botanist In 1906, Tswett separated plant pigments using chromatography. Because the results of the analysis were "written in color" along the length of the adsorbent column, he named the new method chromatography. The words "color" and "writing" are chroma and graphene. Each and every area of the biological and physical sciences uses chromatography. Twelve Nobel Awards were given out for research in which chromatography was essential between 1937 and 1972 alone [3].

PHASES

STATIONARY PHASE

Interaction between the target molecule and the column is known as the stationary phase. Because of the uniform dispersion of tiny particles, the HPTLC plate set exhibits smooth layers. About 10x10 or 10x20 cm is the size of the HPTLC plates. Most compounds in a typical TLC are analyzed using silica gel and a less polar mobile phase, like chloroform-methanol. Methanol-water and other more polar aqueous mobile phases are employed in reversed-phase TLC in conjunction with lipophilic chemically modified silica gel phases. These plates increase the sensitivity and resolution of detection. The pharmaceutical sector is also beginning to use them for several drug quantitative analyses, in addition to the biochemical and clinical sciences[4].

MOBILE PHASE

The target molecule is dissolved by the solvent in this phase. Thin layer chromatography's mobile phase is determined by the characteristics of the analyte. The selection of the mobile phase is primarily determined by the analyte's physical and chemical characteristics as well as the makeup of the stationary phase. In normal phase TLC, the suggested ingredients for the mobile phase are diethyl ether, methylene chloride, and chloroform. They can be used alone or in conjunction with hexane. If the device use reversed-phase TLC, the strength can be adjusted using tetrahydrofuran, acetonitrile, or even methanol diluted with water[4].

PRINCIPLE OF HPTLC

It is a potent analytical technique that works well for both quantitative and qualitative analysis. Depending on the type of adsorbents employed on plates and the solvent system utilized for development, separation may occur as a result of partitioning, adsorption, or both phenomena. The solvent in the mobile phase passes through due to capillary action. The components travel toward the adsorbent in accordance with their affinities. Slower motion can be observed by the component that has a greater affinity for the stationary phase. The component that has a lower affinity for the stationary phase moves morequickly. On a chromatographic plate, the components are so separated [5].

HPTLC operates on the same separation by adsorption concept as TLC. This method uses a mobile phase that transports different components and moves by capillary action. The components show adsorption on the stationary phase based on their affinity for the adsorbent.While components with a lower affinity for the stationary phase move quickly in the mobile phase, those with a higher efficiency for the stationary phase interact with the surface and travel more slowly. Components separate as a result of this difference in component movement [6].

Advantages [7] [8]

1. A lesser period for analysis.
2. Minimize the quantity of solvent needed.

3. Managing several samples.
4. A great deal of flexibility
5. The sample is detectable in nanograms.
6. Increased precision and sensitivity.

1. Need a lot of area.
2. Complicated equipment
3. Someone with a technical knowledge is needed.

Disadvantages[7]

CLASSIFICATIONOFHPTLC

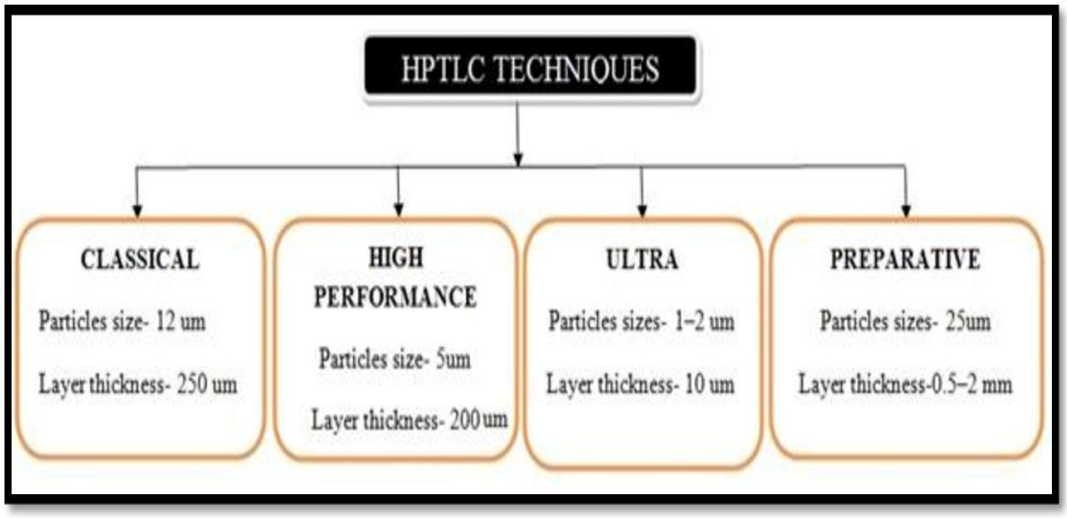

Figure no.1[9]

Difference between TLC and HPTLC [10][11]

Table No.1- Difference between TLC and HPTLC

FEATURES	TLC	HPTLC
1.Technique	Manual	Instrumental
2. Layer	Lab made / Pre – coated	Pre – coated
3.Shape of sample	Circular (2-4nm diameter)	Rectangular (6mm L x 1mm W)
4.Size of sample	Uncontrolled/Solvent dependent	Controlled/Solvent independent
5.Good Lab Practice	Not Compliant	Compliant
6.Volume range	1 to 10µL	0.1 to 500µL
7.PC connectivity	No	Yes
8.Validation	No	Yes
9.Method storage	No	Yes
10.Sample holder	Capillary / Pipette	Syringe
11.Quantitative Analysis	No	Yes
12.Wavelength range	254nm or 356nm Visible	190 or 800 nm Monochromatic
13.Analysis judgement	By analyst	By machine
14.Spectrum analysis	No	Yes
15.Vol.precision	2-10%	0.5-2%
16.Spot shape	Spot	Band
17.Plate height	30µm	12µm
18.Sample spotting	Manual spotting	Auto sampler
19.Efficiency	Less	High (due to smaller particle size)
20.Layer of sorbent	250µm	100µm
21.Analysis time	20-200min	1-3min
22.Mean particle size	10-12µm	5-6µm
23.Stationary phase	Silica gel, Alumina and Kieselguhr	Wide choice of stationary phase Like Silica gel for normal phase and C8, C18 for reversed phase modes

Difference between HPLC and HPTLC [11]

Table No.2- Difference between HPLC and HPTLC

FEATURES	HPLC	HPTLC
1.Type	Reverse Phase Chromatography	Straight Phase Chromatography
2.Stationary phase	Liquid	Solid
3.Conditioning phase	None	Gas
4.Separation by	Partition	Adsorption
5.Results	By machine	By machine + eyes
6.Analysis	Online	Offline
7.Resolution	Very high	Moderate to high
8.Time per sample	2-60 min	1-30 min
9.Chromatography system	Closed	Open
10.High temp./Pressure	High pressure	None
11.Separating medium	Tubular column	Planar layer (plate)
12.Strongly retarded fractions seen as	Broad peaks	Sharp peaks
13.Sensitivity	High to Ultra	High moderate to ultrahigh
14.Cost per analysis	Very high	Low
15.Detectors	UV, Fluor, MS	UV-Visible, MS

INSTRUMENTATION OF HPTLC

1.AUTO SAMPLER[11]: The sample application portion is the main distinction between TLC and HPTLC. The sample would be manually spotted on a silica plate using a capillary in TLC. On the other hand, an HPTLC auto-sampler might be a precise device that uses nitrogen gas to spray a predetermined amount of sample. Additionally, data on the amount of sample to be applied and, consequently, the number, width, and location of the bands of the samples are fed into a computing system that is connected to the auto-sampler. Keep in mind that, in contrast to TLC, you should use a syringe filter to filter the material before putting it on the TLC plate. If you don't, there's a good risk that the precipitate or undissolved sample would clog the HPTLC syringe, which can be expensive.

Figure No.2-sample applicator[12]

2.DEVELOPING CHAMBER[11]:

Typically**,** HPTLCplates are developed in a broad glass chamber, near similar to TLC. Here, you pour a solvent into the chamber and cover it to saturate it for at least 20 to 30 minutes. To ensure that the vapor's are distributed evenly, you will also dip a piece of paper into the solvent system. Once the plate is saturated, you want to position it vertically inside the chamber to make sure the sample areas are still above the solvent's edge. There are two primary types of vertical developing chambers: twin trough chambers, which have a ridge at the bottom that divides the chamber into two troughs, and flat bottom chambers. In twin trough

chambers, the solvent usage is lower than in the flat bottom ones**.**

Figure no.3 - Developing chamber [13]

3.Chromatogram device And Derivatizer [14]: This method includes a gas phase in addition to stationary and mobile phases. The outcome of the separation can be greatly impacted by this gas phase.

Processes: After the lower end of the plate is submerged, the developing solvent will flow up the layer by capillary action until the required distance is reached, at which point the chromatography will be stopped. Silica gel as a stationary phase and advancements, which can be characterized as adsorption chromatography, are the main focus of the following discussions. There are four kinds of processes: There are four different kinds of procedures that take place.

1. The stationary phase absorbs molecules from the gas phase when it is dry. The polar components will be withdrawn from the gas phase and loaded onto the stationary phase's surface during thisprocess, known asadsorptivesaturation.
2. The gas phase interacts with the portion of the layer that has previously been wetted with themobile phase. As a result, the liquid's less polar components are discharged into the gas phase.
3. In contrast to (1), adsorption forces rather than vapor pressure control this process.
4. Secondary fronts may arise during migration if the stationary phase separates the elements of the mobile phase under specific circumstances.

The Derivatizer introduces an automated spraying apparatus that uses a novel "micro droplet" spraying technology to set a new benchmark for consistency in reagent transfer onto TLC plates (patent pending). The Derivatizer guarantees uniform and repeatable administration of all common reagents. To accommodate the differing physicochemical properties of the various reagents, such as acidity or viscosity, four separate color-coded spray nozzles are used, and the user can choose from six spraying levels. In addition to greatly more homogeneous reagent dispersion, the Derivatizer offers the following advantages over hand spraying:

1. Environmentally friendly and safe handling through a closed system
2. Intuitive handling and easy cleaning
3. Effective operation results in low reagent use (4 mL for 20 x 20 cm plates and 2 mL for 20 x 10 cm plates).
4. Reproducible and user-independent results

Figure no.4-Derivatizer[15]

4.TLC Scanner [16]: One type of scanning densitometer is the TLC Scanner 4. It determines how well separated substances reflect in fluorescence or absorption modes. The produced densitometric data may be quantitatively evaluated thanks to the TLC Scanner 4, which is controlled by vision CATS software. It is possible to choose one or more wavelengths for scanning densitometry from the 190–900 nm range of light. As a result, detection can be adjusted to match the analyte's spectral characteristics to its ideal specificity and sensitivity**.** To determine the simplest wavelength for your molecule, you will select a multiwavelength scan (190 to 900 nm) when scanning it for the first time. The simplest wavelength will show sharper peaks than the others. You will choose that particular wavelength each time you scan your plate after you have determined which wavelength is best for your compound. After that, it just takes a few mouse clicks to calculate the amount of your chemical. The compound's number is directly proportionate to its height. You can display the information (areas) or the peaks (3D view) separately or as an overlay graphic by simply selecting the height, which generates data for every peak inside that Rf value. Additionally, you will even give name of substance to the peaks.

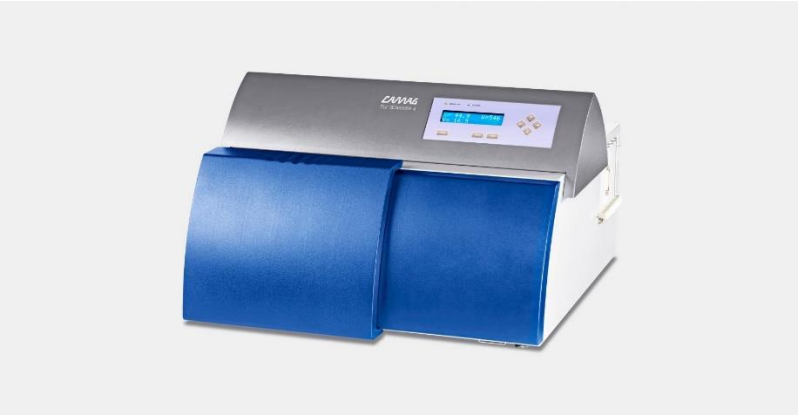

Figure no.5- TLC scanner[16]

5.DIGITAL CAMERA FOR PHOTO DOCUMENTATION [17]:

Improved designs and UV cabinets that enable the installation of a digital camera to capture plate pictures are now replacing the ancient UV cabinets. HPTLC is currently a fundamental prerequisite for any laboratory engaged in herbal analysis in order to

identify plant extracts by comparing them to extracts from Botanical Reference Materials (BRM) in order to identify adulterants or replacements and to study formulations, among other things. TLC for chemical inspection and a microscope for physical inspection are the initial points, according to forensic analysts long ago.

Figure no.6 - Digital camera [18]

SCHEMATIC PROCEDURE FOR HPTLC METHOD DEVELOPMENT

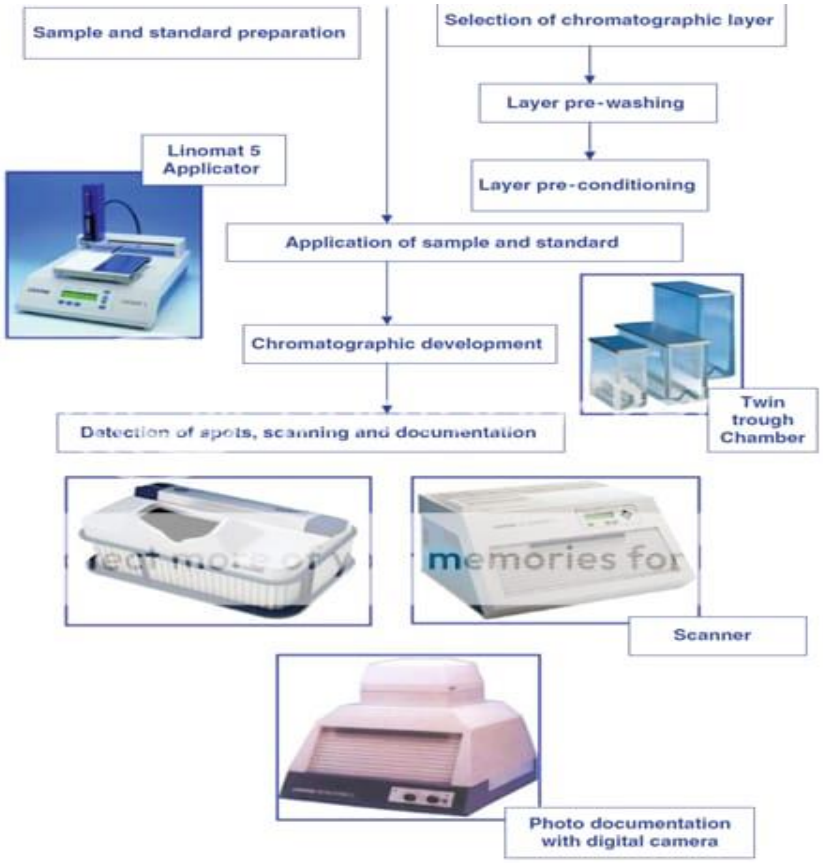

Figure no.7- schematic procedure for HPTLC method development [19]

Steps of HPTLC

1. Sample Preparation
2. Selection of stationary phase
3. Layer Prewashing
4. Mobile phase selection and optimization
5. Sample application

6. Chromatogram Development (separation)
7. Plate Labelling
8. Derivatization
9. Documentation
10. Detection
11. Quantitation

Steps involved in HPTLC[20]

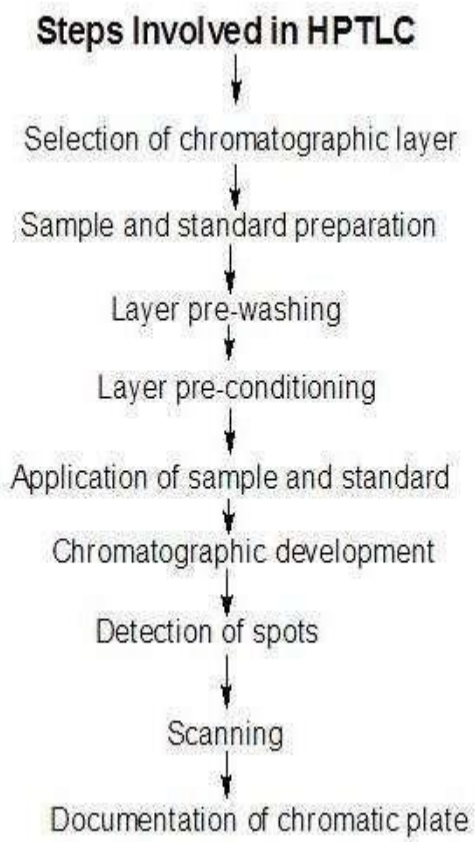

Figure No.8- Steps involved in HPTLC[20]

1.Sample preparation [21]

It requires a highly concentrated solution because very little sample needs to be administered. Solvents for normal phase chromatography utilizing silica gel pre-coated plates must be volatile or non-polar. In reversed phase chromatography, the sample is typically mixed using polar solvents.

2.Selection of stationary phase [22]

A stationary phase must be chosen before HPTLC can start in order to separate the various chemicals in a combination. Normal phase silica gel is used in approximately 90% of all pharmaceutical separations; although samples containing dissociating compounds can be separated using alumina, while samples containing ionic compounds can be separated using cellulose. For substances with high polarity, the reverse-phase HPTLC method which uses a methodology similar to reverse-phase TLC is employed. Plates are often cleaned with methanol and dried in an oven to get remove of extra solvent after the stationary phase has been chosen.

3.Layer Prewashing[21]

To get eliminate of water vapors or other volatile contaminants, plates must be cleaned. To prevent contamination, plates are handled at the top of the plate. Methanol acts as a prewashing solvent, and each trough in a 20 × 10 cm twin trough chamber (TTC) uses a combination of methanol and ethyl acetate or even mobile phase. In each TTC trough, the two 20 × 10 cm or four 10 × 10 cm plates can be developed back-to-back. After removing the plate, dry it for 20 minutes at 120°C in a clean drying oven.

Conditioning -The previously cleaned plates are dried for 15 to 20 minutes at 120°C. We call this process conditioning.

4.Mobile phase selection and optimization:

The mobile phase is determined by the analyte's physical and chemical properties as well as the adsorbent material applied as the stationary phase. Separate, precise volumetric measurements of each mobile phase component must be made in appropriate volumetric glasses and shaken to ensure proper mixing of the contents. The right solvents mobile phase needs to be selective, either via personal experience, reading, or trial-and-error techniques. In order to improve resolution, mobile phases are usually composed of polar and nonpolar solvents, either with or without an acid or basic modifier. Compared to the silica gel layer (normal-phase TLC), they are less polar. The mobile phase, which is often a solution of methanol, acetonitrile, or tetrahydrofuran with water in reversed-phase TLC, is less polar[23].

5.Sample application:

There are automated devices for applying samples; in particular, for quantitative HPTLC, use the spray-on methods with the Linomat 5 or Automatic TLC sampler (ATS) 4. A motor is usually used to empty the syringe that holds the sample. The volume and pace of delivery are managed electronically. If the syringe or the plate is moving linearly, a stream of an inert gas, like nitrogen, around the syringe tip atomizes the sample and forms a band on the TLC/HPTLC plate. Band-wise application is used to improve separation and increase densitometer availability. The best resolution and sensitivity are achieved when the sample is applied in narrow bands. There are many drawbacks to circular spots, but "line" or "band" application has benefits [5].

6.Chromatogram development:

It is most important step used in HPTLC method development. Horizontal development chambers or twin-trough chambers are used to develop HPTLC plates. The best repeatability is often provided by saturated twin-trough chambers equipped with filter paper. A twin-through chamber prevents humidity and solvent vapor preloading[24].

7.Plate Labelling:

For identification purposes, the plates must have clear labels in accordance with GMP/GLP. The number in the upper right corner is normally written using abright pen or soft pencil. The project number, the analysis date, and the subsequent plate number are required to be printed on the label. Merck additionally provides laser-coded HPTLC silica plates with unique code numbers to help with documentation for analyses carried out under GLP [25].

8.Derivatization:

Derivatization is a procedural method that mainly alters an analyte's properties to make it easier chromatographic separations. The plates can be derivatized by spraying them with an appropriate reagent or by putting them. Immersion is the recommended derivatization method for improved reproducibility [24].

9.Detection:

This is also most useful step in HPTLC method development**.** White or ultraviolet light is used for examining the chromatogram. Options include quantitative assessments installing video or scanning densitometry, as well as visual examination of digitized images[14].It is energetic and non-destructive when UV light is detected. Fluorescent compound scan spots are visible at 254 nm, or radio wavelength. Non-fluorescent patches and fluorescent stationary phases, such as colloid GF, are frequently applied. If each component responds to UV light, derivatization using a visualizing agent is necessary. White light visualization The natural color of daylight (white light) is commonly used to identify the zone that contains separated chemicals [11].

10.Documentation:

Improved designs of UV cabinets that enable the installation of a digital camera to capture plate pictures are now replacing older models. Even though this gadget does not follow GLP, small labs like it. Any laboratory engaged in herbal analysis must now use HPTLC as a primary need in order to identify plant extracts by comparing them to extracts from Botanical Reference Materials (BRM) in order to identify adulterants or replacements, conduct formulation studies, etc. TLC for chemical inspection and a microscope for physical inspection are frequently used as the beginning points by forensic analysts. It is very essential step in HPTLC method development. [26]

11.Quantitation:

Using a densitometer or scanner with a fixed sample light beam in the shape of a rectangular slit, the majority of contemporary HPTLC quantitative studies are carried out in situ by measuring the zones of samples and standards. The scanner is typically used for quantitative evaluation. The chromatogram may be scanned using fluorescence, absorbance, or

reflectance modes; the scanning speed is determined by the recording of chosen band fast spectra. It is feasible to use linear or non-linear regressions to calibrate both single and multiple levels[27].

HPTLC method validation for pharmaceutical analysis [28]:

Accuracy: How closely the value obtained complies with the recognized reference value.

Precision: The degree of consistency among a set of measurements derived from several samplings of the same homogenous sample.

Repeatability: The difference in measurements made by one person or using the same tool on the same object and in similar conditions.

Reproducibility: The variation that occurs over extended periods of time and between various instruments and operators using the same measuring procedure is known as reproducibility.

Sensitivity: The capacity to identify alterations in the quantity of a substance in a combination or its presence.

Specificity: The capacity of the intended analyte to be precisely measured free from interference from other ingredients in the combination.

Linearity: The capacity to derive test results that roughly correspond to the analyte concentration in the sample.

Range: The difference between the sample's upper and lower analyte concentrations for which it has been shown that the analytical process has an adequate degree of linearity, precision, and accuracy.

Robustness: The ability to avoid being impacted by minor, intentional changes in method parameters.

Testing for System Suitability: Verifies that the analytical system is operating as desired.

Limit of Quantification(LOQ) and Limit of Detection (LOD): the lowest concentration of analyte in a sample that can be quantitatively assessed with appropriate precision and accuracy, and the lowest concentration of analyte in a sample that can be detected but not quantified, respectively.

APPLICATION OF HPTLC:

1.Pharmacokinetics study:

HPTLC is used in bioavailability studies to assess new treatments and over-the-counter medications. As demonstrated by the co-administration of thalidomide and dexamethasone to rat plasma, HPTLC can be utilized to track drug-drug interactions. The FDA has validated the development approach employed in this study. Researchers are concerned about how long medications last. The HPTLC Bober K.'s solvent mixture of methanol, ethyl acetate, and ammonia has been successful in producing a more substantial one; use a plate of pre-coated silica gel 60F254. Details regarding the byproducts of diphenhydramine breakdown Food certification sildenafil (phosphodiesterase inhibitor) and its analogs, sibutramine, are examples of common ingredients in nutritional supplements and soft drinks that HPTLC can identify [29].

2.Forensics: Investigating cases of chemical warfare, drug and alcohol abuse, and apprehending individuals in minor and severe crimes have all been done with HPTLC. Additionally, HPTLC can detect concentration levels in the picogram range. Detecting cannabis, the most widely abused recreational drug, was made easier with HPTLC [29]

3.Takeaway message: Pharmaceutical sector greater use of HPTLC alone or in combination with other technologies, such FTIR and MS, for pharmaceutical formulation and bulk drug analysis. Additionally, HPTLC is utilized. Utilized effectively in the biomedical industry, expanding biochemistry A modern agricultural application example would be calculating the pesticide residues in fruits and vegetables [29].

4.HPTLC in natural products: The HPTLC method is quick, easy, reliable, and

incredibly flexible. It is also a great instrument for detecting adulterations and is well suited for checking stability as well as assessing and tracking the processes of cultivation, harvesting, and extraction. The aforesaid diterpenoids in the root bark of Photinia integrifolia were successfully quantitatively analyzed using a straightforward and repeatable procedure employing HPTLC. whereby Photinia integrifolia plant extracts were standardized using the diterpenoids 1β,3α,8β-trihydroxy-pimara-15-ene (A), 6α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene (B), and 2α,19-dihydroxy-pimara-7,15-diene (C) as chemical markers [30]5.

OTHER[5]:HPTLC is used in food and feed products for quality control, pesticides, additives (such vitamins), stability testing (expiration), etc.

CONCLUSION

In conclusion, High-Performance Thin-Layer Chromatography (HPTLC) is a powerful and versatile analytical technique widely used for the qualitative and quantitative analysis of various substances. Method development involves optimizing parameters like stationary phase, mobile phase, and detection methods to achieve accurate, reproducible results. HPTLC offers significant advantages, including cost-effectiveness, simplicity, and the ability to handle multiple samples simultaneously. Its applications span across pharmaceuticals, environmental monitoring, and food safety. Overall, HPTLC remains a valuable tool in analytical chemistry, with continued advancements enhancing its effectiveness and broadening its applications.

Declaration by Authors

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