Chapter 1
An Overview of HPTLC: A Modern Analytical Technique with Excellent Potential for Automation, Optimization, Hyphenation, and Multidimensional Applications

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Abstract High performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, etc. enable it to be a powerful analytical tool for chromatographic information of complex mixtures of inorganic, organic, and biomolecules. The chapter highlights related issues such as journey of thin-layer chromatography, basic principle, protocol, separation, resolution, validation process, recent developments, and modifications on TLC leading to the HPTLC, optimization, process control, automation, and hyphenation. It explains that HPTLC has strong potentials as a surrogate chromatographic model for estimating partitioning properties in support of combinatorial chemistry, environmental fate, and health effect studies.

Analytical chemists work to improve the reliability of existing techniques to meet the demands for better chemical measurements which arise constantly in our society. They adapt proven methodologies to new kinds of materials or to answer new questions about their composition. They carry out research to discover completely new principles of measurement and are at the forefront of the utilization of recent discoveries for practical purposes. Modern analytical chemistry is dominated by instrumental analysis. Analytical chemists focus on new applications, discoveries and new methods of analysis to increase the specificity and sensitivity of a method. Many methods, once developed, are kept purposely static so that data can be compared over long periods of time. This is particularly true in industrial quality assurance, forensic, and environmental applications. Analytical chemists are also equally concerned with the modifications and development of new

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instrument. The types of instrumentation presently being developed and implemented involve analytical tools including vibrational, rotational, optical, absorption, colorimetric and scattering spectroscopy, mass spectrometry, chromatography, electro chemicals, acoustics, laser, chemical imaging, light-induced fluorescence, light scattering, etc.

At this point, we will talk about chromatographic techniques. Chromatography, defined as the group techniques used for the separation of a complex mixture of compounds by their distribution between two phases, was invented in 1901 by Russian botanist Mikhail Semyonovich Tswet, during his research on plant pigments. No other separation method is as powerful and applicable as in chromatography. It is the most versatile and widespread technique employed in modern analytical chemistry. The fact has genuine reasons. First, very sensitive methods of detection are available to all types of chromatography and small quantities of material can be separated, identified and assayed. Second, chromatographic separations are relatively fast and an analysis can be completed in a short interval of time. Another advantage of chromatography is its relative simplicity and ease of operation compared with other instrumental techniques. Finally, if the established procedure is well controlled and the apparatus is well maintained, good accuracy and precision can be achieved.

Thin-layer chromatography, among various chromatographic techniques, score high over other chromatographic techniques where altogether a new problem, one might not have encountered or solved. It is a valuable tool for reliable identification providing chromatographic fingerprints.

The feature that distinguishes TLC from other physical and chemical methods of separation is that two mutually immiscible phases are brought in to contact while one phase is stationary and the other mobile. A sample is loaded on the stationary phase and is carried by the mobile phase. Species in the sample undergo repeated interaction between the mobile and stationary phase. When both phases are properly selected, the sample components are gradually separated into bands or zones. Figure 1.1 explains the facts involving the separation of the sample.

The common method of development in thin-layer chromatography employs capillary forces to transport the mobile phase through the layer. These weak forces arise from the decrease in free energy of the solvent as it enters the porous structure of the layer. For fine particle layers, capillary forces are unable to generate sufficient flow to minimize the main sources of zone broadening. Firstly, the mobile-phase velocity varies as a function of time and migration distance. Secondly, the mobile-phase velocity is established by the system variables and is otherwise beyond experimental control. This results in a slow and variable mobile-phase velocity through the layer with separation times that is longer than required. Separated zones are broader than they would be for a constant and optimum mobile-phase velocity and the zone capacity limited by the useful range of mobile-phase velocities. Multiple developments with an incremental increase in the development length and a decreasing solvent strength gradient is the basis of separations by automated multiple developments (AMDs). Results from phenomenological models indicate that further improvements over those already realized are

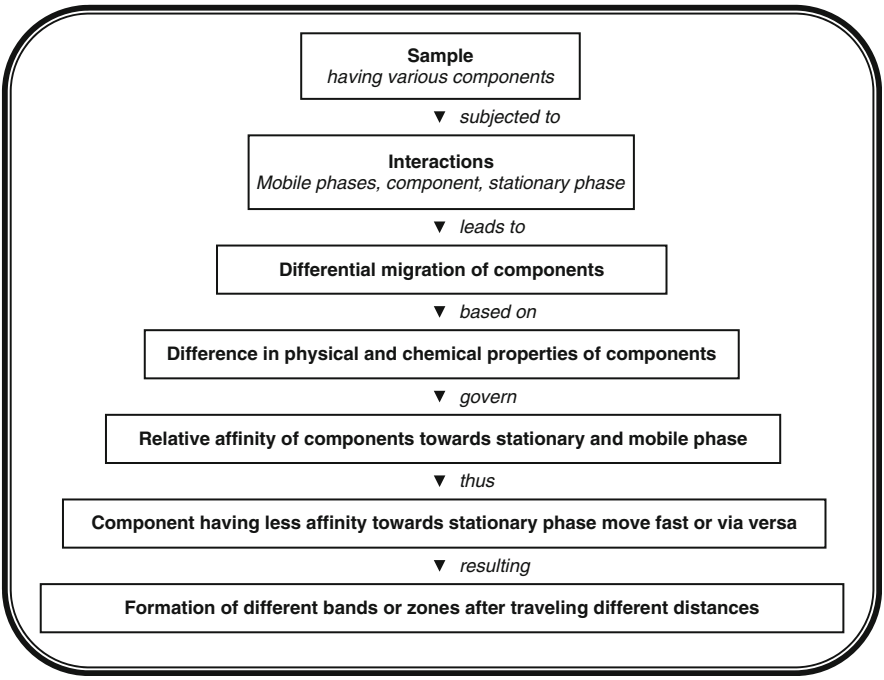

Fig. 1.1 Separation of bands on thin-layer chromatographic plate

unlikely for capillary flow systems and there is no solution to the significant increase in separation time. The magnitude and range of capillary flow velocities fundamentally limit separations in thin-layer chromatography. Faster separations with an increase in zone capacity require a higher mobile-phase velocity than in capillary flow as well as a velocity that is independent of the solvent front migration distance.

The attractive features of TLC are low-cost analysis of samples requiring minimal sample clean up and allows a reduction in the number of sample preparation steps. TLC is also preferred for the analysis of substances with poor detection characteristics requiring post-chromatographic treatment for detection. Thin-layer chromatography retains a historic link with the characterization of dyes and inks and the control of impurities in industrial chemicals. It is used for the identification of drugs and toxic substances in biological fluids, unacceptable residue levels, maintaining a safe water supply by monitoring natural and drinking water sources for crop projecting agents used in modern agriculture, and confirmation of label claims for content of pharmaceutical products. It remains one of the main methods for class fractionation, speciation and flavor potential of plant materials. It continues to be widely used for the standardization of plant materials used as traditional

medicines. It is frequently selected as the method of choice to study the metabolism and fate of radiolabeled compounds in the body and environment.

Journey of Thin-Layer Chromatography

In order to separate inorganic ions, Meinhard and Hall (1949) used a starch binder to give some firmness to the layer and described as surface chromatography. Advances were made by Kirchner et al. (195l) who used the now conventional ascending method using a sorbent composed of silicic acid. Reitsema (1954) used much broader plates and was able to separate several mixtures in one run. However, from 1956 a series of papers from Stahl appeared in the literature introducing thin-layer chromatography as an analytical procedure. Since then, silica gel nach Stahl became well known as a stationary phase. Plaster of Paris (calcium sulfate) was used as a binder and TLC began to be widely used. First book on thin layer chromatography was published by Kurt Randerath (1962), followed by those of Stahl and co-workers and second edition of Stahl's book (1969). These authors showed the wide versatility of TLC and its applicability to a large spectrum of separation problems and also illustrated how quickly the technique had gained acceptance throughout the world. Stahl (1965) could quote over 4,500 publications on TLC works. Stahl's publication highlighted the importance of factors such as the controlling of the layer thickness, the layer uniformity, the binder level, and the standardization of the sorbents as regards pore size, volume, specific surface area and particle size. Commercialization of the technique began in 1965 with the first precoated TLC plates and sheets. TLC quickly became very popular with about 400–500 publications per year appearing in the late 1960s. It was recognized as a quick, relatively inexpensive procedure for the separation of a wide range of sample mixtures. It soon became evident that the most useful sorbents was silica gel, particularly with an average pore size of 60 Å. Modifications to the silica gel began with silanization to produce reversed-phase layers. This opened up a far larger range of separation possibilities based on a partition mechanism, compared with adsorption. Until to this time, quantitative TLC was fraught with experimental error. However, the introduction of commercial spectro densitometric scanners enabled the quantification of analytes directly on the TLC layer. Initially, peak areas were measured manually, but later, integrators achieved this automatically.

Halpaap (1973) was the first to recognize the advantage of using a smaller average particle size of silica gel (5–6 mm) in the preparation of TLC plates. He compared the effect of particle size on development time, R_f values and plate height. Commercially the plates were first called nano-TLC plates but soon changed to the designation HPTLC plates with the recognition that HPTLC has added a new dimension to TLC. It was demonstrated that less amount of mobile phase, precision (tenfold) and reduction in analysis time (similar factor) could be achieved. The first major HPTLC publication was made by Zlatkis and Kaiser (1977). Halpaap and Ripphahn described their comparative results with the new 5.5-cm HPTLC plates

versus conventional TLC for a series of lipophilic dyes. Reversed-phase HPTLC was reported by Halpaap et al. (1980). Jost and Hauck (1982) reported an amino (NH2−) modified HPTLC plate which was soon followed by cyano-bonded (1985) and diol-bonded (1987) phases. The era of 1980s also saw improvements in spectro-densitometric scanners with full computer control including options for peak purity and the measurement of full UV/visible spectra for all separated components. AMD made its appearance because of the pioneering work of Burger (1984). This improvement enabled a marked increase in the number and resolution of the separated components.

Recent Developments

The multiple developments and its combination with other analytical techniques have dramatically increased the use of thin-layer chromatography for the characterization of complex mixture. TLC has strong potential as a surrogate chromatographic model for qualitative and quantitative analysis. To convert these opportunities in to the practice, several modifications have been carried out on the conventional TLC system.

Over-Pressured Layer Chromatography

Forced flow separations in the overpressured development chamber involves the sealing of the layer on its open side by a flexible membrane under hydraulic pressure and a pump is used to deliver the mobile phase to the layer. A constant mobile-phase velocity independent of the solvent front migration distance is obtained as long as the hydraulic pressure applied at the membrane maintains an adequate seal with the layer. When a solvent is forced through a dry layer of porous particles sealed from the external atmosphere, the air displaced from the layer by the solvent usually results in the formation of a second front (β front). The space between the α and β fronts is referred to as the disturbing zone and consists of a mixture of solvent and gas bubbles. In practice, the disturbing zone can be eliminated or minimized by predevelopment of the layer with a weak solvent in which the sample does not migrate. The solvent dislodges trapped air from the layer before starting the separation and consists of a mixture of solvent and gas bubbles.

Planar Electrochromatography

Electro-osmosis provides a suitable alternative transport mechanism to pressure driven flow in open tubular and packed capillary chromatography. Electro-osmotic

flow in packed capillary columns is the basis of capillary electrochromatography. The plug-like flow profile reduces *trans*-axial contribution to band broadening as well as providing a constant and optimum mobile-phase velocity. In addition, the mobile-phase velocity is independent of column length and average particle size up to the limits established by double-layer overlap. The general interest created by the rapid development of capillary electro chromatography as a useful separation method has trickled over to thin layer chromatography. Electroosmotically driven flow could provide an effective solution to the limitations of capillary flow. The current status of electroosmotically driven flow in thin-layer chromatography is probably more confusing. Recent studies have brought some enlightenment to this technique. Enhanced flow is caused by forced evaporation of the mobile phase from a solvent-deficient region at the top of the layer. Because of drainage in vertically mounted layers, electrical resistance is highest at the top of the layer and the increase in heat production drives the evaporation of solvent, pulling additional solvent through the layer. In an open system like thin-layer chromatography, evaporation of mobile phase from the layer surface competes with electro osmotic flow along the layer. The voltage, pH, and buffer concentration must be optimized to minimize either excessive flooding or drying of the layer to avoid degradation of the separation quality. These processes are probably better controlled by enclosing the layer and improving the thermostating of the system. Since high pressures are not involve, mechanisms for enclosing the layer could be relatively simple compared to pressure-driven forced flow and new approaches suggest that effective temperature control is possible. Thinner layer may also help to contain temperature gradients in combination with adequate thermostating.

Image Analysis

Slit-scanning densitometry is the dominant method of recording thin-layer separations for interpretation and quantification. This technology is now relatively mature although limited to absorption and fluorescence detection in the UV–visible range. It has adequately served the needs of thin-layer chromatography for the last two decades. Evolution of slit-scanning densitometry is now largely progressive and major changes in operation and performance seem unlikely. A possible exception is the development of scanners employing a fiber optic bundle for illumination of sample zones and collection of reflected light in conjunction with a photodiode array detector for simultaneous multi-wavelength detection and spectral recording. This approach simplifies data acquisition for some applications and affords the possibility of facile application of modern chemometric approaches for data analysis. This approach may improve the quality of available data from thin-layer separations, but does not overcome the principal limitations of slit-scanning densitometry.

For video densitometry, optical scanning takes place electronically, using a computer with video digitizer, light source, monochromators, and appropriate

optics to illuminate the plate and focus the image onto a charge-coupled device (CCD) video camera. The main attractions of video densitometry for detection in thin-layer chromatography are fast and simultaneous data acquisition, a simple instrument design without moving parts, increase in sensitivity, longer acquisition times and compatibility with data analysis. Video densitometry cannot compete with slit scanning densitometry in terms of sensitivity, resolution and available wavelength-measuring range.

Two-Dimensional Separations

Multidimensional separations employing two or more coupled orthogonal separation systems represent the preferred approach in chromatography to obtain a high peak capacity for the separation of complex mixtures. Two-dimensional separations are easily performed using planar separation systems. Even capillary flow separations can be expected to afford a zone capacity of a few hundreds rising to a few thousands for forced flow developments. In most cases, the two-solvent systems differ only in their intensity for a given set of intermolecular interactions and are not truly complementary. Such systems are responsible for the low success of two-dimensional separation systems to provide a significant increase in the separation potential apparent in many applications. Recent reports are encouraging and recognize the importance of the orthogonal nature of the retention mechanisms if a high separation capacity is to be achieved. Bilayer plates with a smaller reversed-phase strip along one edge of the plate adjacent to a larger silica gel layer have provided the most popular approach for the implementation of two-dimensional separations with a high separation capacity. Chemically bonded layers can also be used in the reversed-phase and normal phase mode and allow the use of buffers as a further means of adjusting selectivity. The awaited breakthrough in general detection for two-dimensional planar separations is likely to come from video densitometry. Data acquisition is straight forward since the whole plate is imaged simultaneously, but a problem remains with quantification that has still to be addressed.

High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC allows fast, inexpensive method of analysis in the laboratory as well as in field. Modern quantitative HPTLC, when properly performed by well-trained analysts, can be advantageous compared to high-performance liquid-column chromatography in many analytical situations. The modern HPTLC technique, combined with automated sample application and densitometric scanning, is sensitive and completely reliable, suitable for use in qualitative and quantitative analysis. HPTLC is a valuable tool for reliable identification because it can provide chromatographic fingerprints that can be visualized and stored as electronic images. To

fully take advantage of this unique feature inherent to HPTLC, reproducible results and images must be ensured. Special advantages of HPTLC include high sample throughput and low cost per analysis; multiple samples and standards can be separated simultaneously, and sample preparation requirements are often minimal because the stationary phase is disposable. Other advantages include static, off-line detection of zones using a great variety of complementary post-chromatographic universal and selective detection methods that are often applied sequentially, and storage of the separation, containing all sample components, on the layer for identification and quantification at a later time by in situ or elution methods (Fig. 1.2). At the present time, all steps of the TLC process can be computer controlled.

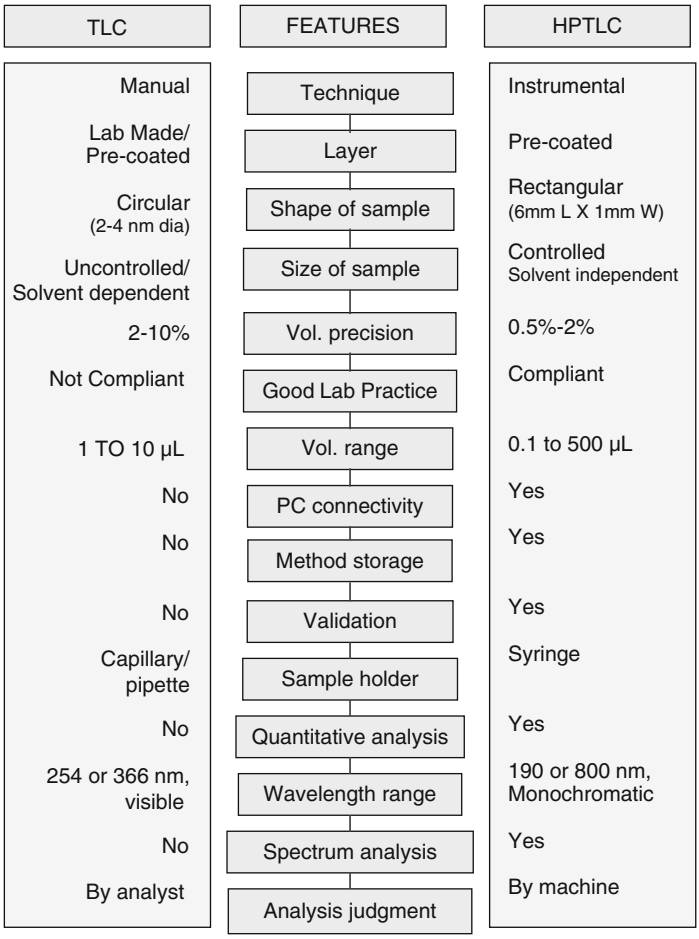

Fig. 1.2 Advancements made on TLC leading to the development of HPTLC

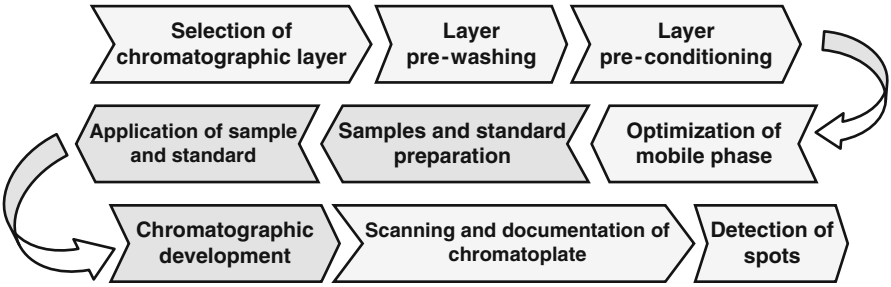

Fig. 1.3 Schematic procedure for HPTLC method development

The use of highly sensitive (CCD) cameras has enabled the chromatographer to electronically store images of chromatograms for future use and for direct entry into reports at a later date.

HPTLC-based separations involves several steps shown in Fig. 1.3. The details of each step have been discussed in the preceding chapters.

HPTLC: Separation and Resolution

To which extent various components of a formulation are separated by a given HPTLC system is the important factor in quantitative analysis. It depends on the following factors:

- Type of stationary phase
- Type of precoated plates
- Layer thickness
- Binder in the layer
- Mobile phase
- Solvent purity
- Size of the developing chamber
- Saturation of chamber
- Sample's volume to be spotted
- Size of the initial spot
- Solvent level in the chamber
- Gradient
- Relative humidity
- Temperature
- Flow rate of solvent
- Separation distance
- Mode of development

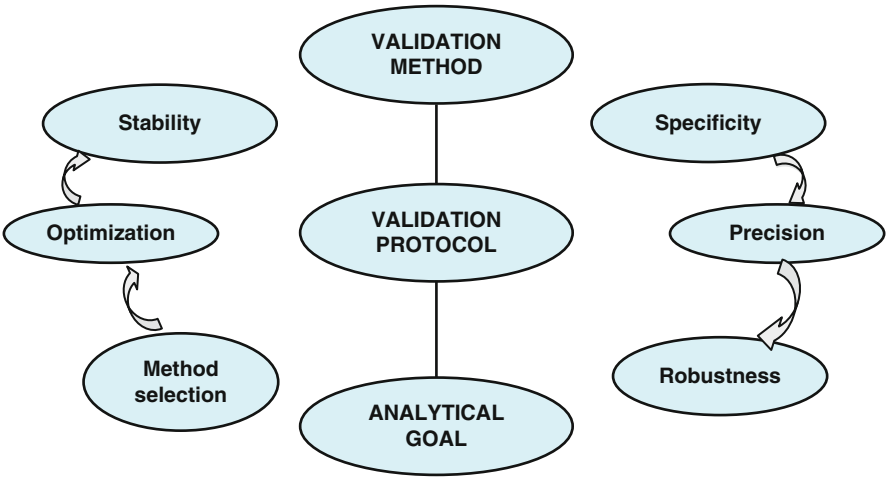

Fig. 1.4 Validation process involved in HPTLC

HPTLC: Validation Process

Validation should not be seen separately from the development of a method. The entire process can be visualized with the scheme in Fig. 1.4. It starts from a clearly defined analytical goal, method selection, optimization, and development, which is called prevalidation considerations before arriving at the elaboration of a validation protocol and is the starting point of the actual validation. After performing all the experiments described in the validation protocol, obtained data are evaluated and compared with the acceptance criteria. If all criteria are met, the method can be regarded as valid. In a less-formal approach, some validation data may be incorporated from experiments, which were conducted previously as part of the method development.

The above approach is widely accepted for validation of qualitative HPTLC methods for identification during routine use. It is possible that the validation method in different situations may require some changes in the standard validation protocol. Such changes may include restrictions with respect to relative humidity, waiting times, precision, etc. The validation protocol is a key instrument for structuring, regulating and documenting the validation processes, depending on the quality management system. The following elements must be included:

Selectivity

Ability of the developed analytical method is to detect analyte quantitatively in the presence of other components which are expected to be present in the sample matrix. Results are expressed as Resolution. If the expected impurities or related substances are available, they should be chromatographed along with the analyte to check the system suitability, retention factor, tailing factor, and resolution.

Sensitivity

Ability of the method within a given range to obtain test results in direct proportion to the concentration of analyte in the sample – calibration curve for the analyte.

Precision

Precision provides an indication of random error. Its results should be expressed as relative standard deviation (RSD) or coefficient of variation (COV). Precession is observed in terms of *replication*: precision under same conditions, same analyst, same apparatus, short interval of time and identical reagents using the same sample; *measurement of peak area*: RSD should not be greater than 1%, based on seven times measurement of same spot; *peak position*: RSD should not be greater than 2% based on seven times repositioning the instrument after each measurement; *sample application*: equal volume applied as seven spots and RSD should not be greater than 3% and under different conditions, different analyte, different laboratory, and different days and reagents from different sources using the same sample. RSD should not be greater than 10% within laboratory reproducibility.

Accuracy

Accuracy of an analysis is determined by systematic error involved. It is defined as closeness of agreement between the actual value and mean analytical value obtained by applying the test method a number of times. The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. This parameter is very important for formulated pharmaceutical dosage forms as it provides information about the recovery of the analyte from sample preparation and effect of matrix. It the recovery rate is found to be 100%, it implies that the proposed analytical method is free from constant and proportional systematic error. A blank matrix and known impurities must be available to test the accuracy of the method.

Ruggedness

This is one of the most important parameters for validation of HPTLC method. Experiments are usually recommended to evaluate ruggedness of a HPTLC method like *sample preparation*: composition, quantity of solvent, pH, shaking time, temperature and number of extractions; *sample application*: volume applied, spot shape and size, band and spot stability; *separation*: at least on three different plates; *chromatographic conditions*: chamber saturation, eluent composition, eluent volume, temperature, humidity and development distance; *spot visualization*: post-chromatographic derivatization, spraying, dipping, reaction temperature and time; *quantitative evaluation*: drying of plates, detection and wavelength.

Once the analytical method is developed, it should be performed independently by three analysts well conversant with practical aspects of the technique, analyzing the same sample under same experimental conditions to check reproducibility of the method.

Limit of Detection

Lowest amount of analyte that can be detected is not greater than 10% of the individual impurity limit. If this is not possible, then amount of analyte to be applied has to be increased. Limit of detection (LOD) is determined on the basis of signal to noise ratio. Mean of 15 noise peak areas and their absolute SD values are determined. LOD is the amount of applied sample producing a peak area which is equal to the sum of mean blank area and three times standard deviation.

Stability

Analyte should not decompose during development of the chromatogram and should be stable in solution and on the sorbent for at least 30 and 15 min, respectively. The intensity of the spot on the chromatogram should be constant for at least 60 min while optimization of the extraction/purification procedure and one must keep in mind the chemical properties and purity of the extraction solvent. Chemical reaction of the solvents and their impurities may produce extra spot/peak, thus leading to false assay values. Other important factor is pH of the aqueous phase used for extraction/purification which may lead to hydrolysis, oxidation and isomerization. The complete removal of organic solvent should be avoided.

HPTLC: Optimization and Process Control

A standard methodology is applied for optimization. Sample preparation, in most cases, a 5-min sonication with methanol, followed by centrifugation and using the supernatant as test solution, yields satisfactory results. Derivatization is optimized with the goal of convenience, safety, and reproducibility. Botanical Reference Materials (BRM) of known adulterants are used to ensure sufficient specificity of the method. Small modifications of the mobile phase composition are applied to fine-tune separation. Each step of the optimization process is documented for complete traceability. The optimization of the chromatographic mobile phase proved to be possible when the number of experimental determinations of separation parameters for each compound is obtained for more than one distinct compositions of mobile phase, at least equal with the number of variable use in the mathematical model. A mobile phase optimization program based on an original mathematical approach is to be developed for its performances by applying on three sets of compounds. The original optimization procedure starts from the idea that

into a mixture of three solvents the quantitative measure of the choused chromatographic parameter is dependent on composition of mobile phase through an equation of dependency with six or seven parameters, taking into consideration the molar fraction of the solvents. The optimization procedure is included in a program and applied on three sets of previously studied compounds through high-performance thin-layer chromatography with three solvents. The mobile-phase optimization process proved to be able to provide accurate, precise, and reproducible method of characterization and analysis of chromatographic parameters.

HPTLC: Automation

For the past 50 years, both automatic and automated instruments have been used to monitor and control process stream, such as density, viscosity, and conductivity. It is necessary to distinguish between the characteristics of automatic and automated devices. According to the current definitions of the International Union of Pure and Applied Chemistry (IUPAC), both devices are designed to replace, refine, extend or supplement human effort and facilities in the performance of a given process. The unique feature of automated devices is the feedback mechanism, which allows at least one operation associated with the device to be controlled without human intervention. An automatic photometer might continuously monitor the absorbance of a given component in a process stream, generating some type of alarm if the absorbance exceeds a preset value. By contrast, an automated system could transmit absorbance values to a control unit that adjusts process parameters (temperature and amount of additional reagent) to maintain the concentration of the measured component within preset limits. In spite, of this fundamental difference, the terms automatic and automated are often interchanged.

The use of automated sample processing, analytics and screening technology for profiling absorption, distribution, metabolism, excretion, and physicochemical properties is becoming more widespread. The use and application of these technologies is both diverse and innovative. High throughput screening technologies have been utilized enabling the profiling of an increased number of compounds. Although the drivers for using these technologies are common, different approaches can be taken. Control Systems, Safe, efficient, and economical operations of chemical processes are ever more dependent in the use of online analyzers. The use of analytical measurements of component properties in near real time for process control during manufacturing is becoming more common. The combination of online analyzers and advanced control technologies holds an enormous economic potential. As a result, the number of existing applications of HPTLC is growing steadily.

Advances in science and technology have raised an increasing demand for control analyses and posed various challenges to analytical chemists such as the need to develop new methods exhibiting as much selectivity, sensitivity, sample and reagent economy, throughput, cost-effectiveness, simplicity, and environmental

friendliness as possible. The large of number of samples, with which analysts can be confronted, imposes the use of expeditious automatic methods. Despite the major conceptual and operational differences between partly and fully automated methods, the two are frequently confused. Thus, a fully automated method allows the whole analytical process to be completed with no intervention from the analyst; also, it can by itself make the decision as to whether the operating conditions should be altered in response to the analytical results. All methods are deemed automated simply because one or several steps of the analytical process are performed in an automated manner. However, an automated method should be capable of completing all steps including sampling, sample preparation and dissolution, interference removal, aliquot withdrawal, analyte measurement, data processing, result evaluation, and decision making, and also of restarting the whole process in order to adapt it to the particular needs of a new sample if needed.

A fully automatic method is very difficult to develop especially for solid samples, the first steps in the analysis of which can rarely be performed in an inexpensive manner. Usually, the operations posing the greatest difficulties among those involved in such steps are those requiring some mechanical handling, automation of which is only possible in most cases by using robot arm adapted to the particular chemical operations to be performed. Because this equipment is too expensive for most analytical applications, fully automated methods for the analysis of solid samples are very scant and largely restricted to the control of manufacturing processes. The automation of analyses involving fluid samples is facilitated by their usually adequate homogeneity and easy mechanical handling by the use of peristaltic or piston pumps, or some other liquid-management devices. This is not the case with solid samples, analysis of which frequently involves their prior conversion into liquids by dissolution. The dissolution step is the bottleneck of analytical processes involving solid samples as it is frequently slow and must be performed manually. The earliest automatic methods used dedicated devices suited to the particular application. This restricted their scope to very specific uses such as the control of manufacturing processes or in those cases where the number of samples to be analyzed was large enough to justify the initial effort and investment required. The computer-controlled techniques have introduced a great number of advantages to HPTLC systems mainly a dramatic decrease of the needed sample and reagents volumes, and have allowed the introduction of the concept of unit laboratory operations.

HPTLC: Hyphenation

Over the past several years, the concept of hyphenation has gained rapid growth in the pharmaceutical industry because of its ability to produce a large number of compounds with a wide range of structural diversity in a short time. The combinational approach (hyphenation) has received a significant recognition compared to a traditional one-compound-at-a-time approach.

The various steps having potential for the advancements on the thin-layer chromatography are methods to provide a constant and optimum mobile-phase velocity, video densitometry for recording multidimensional chromatograms, in situ scanning, and monitoring for selective detection. These improvements dramatically increased the use of thin-layer chromatography in the form of HPTLC. Today, thin-layer chromatography has been successfully hyphenated with high-performance liquid chromatography (HPLC), mass spectroscopy (MS), Fourier transform infra-red (FTIR), and Raman spectroscopy to give far more detailed analytical data on separated compounds. Even the UV/visible diode array technique has been utilized in TLC to determine peak purity or the presence of unresolved analytes.

Liquid Chromatography–Thin-Layer Chromatography (LC–TLC)

The most general interface for coupling column liquid chromatography to thin-layer chromatography (LC–TLC) is based on different modification to the spray-jet applicator. Flow rates typical for mobile phase can be applied to the layer. A splitter in the transfer line to the spray-jet applicator is required to accommodate higher flow rates from wider-bore columns. The column eluent is nebulized by mixing with nitrogen gas and sprayed as an aerosol onto the layer. The spray head is moved horizontally on one line within a defined bandwidth. Contemporary interest in LC–TLC remains weak. The main problems are more on the detection and data handling side than separations. It is simpler to obtain mass spectral information from the solution phase using liquid chromatography–mass spectrometry (LC–MS) than to either quantify or identify separated bands by thin layer chromatography–mass spectrometry (TLC–MS).

High-Performance Thin-Layer Chromatography–Mass Spectrometry (HPTLC–MS)

The combination of chromatographic separations with mass spectrometric detection is considered an indispensable tool for problem solving in analytical chemistry and increasingly for routine analytical methods. Mass spectrometric detection brings an added level of information, complementary to the chromatographic process that improves the certainty of identification and the specificity of detection. Mass spectral information can generally be obtained from sample sizes typical of common analytical methods. HPTLC–MS is mainly a research tool available to a small number of research groups. The evolution of HPTLC–MS has been slow compared with LC–MS. The challenge was to develop an automated system for in

situ acquisition of mass spectral data directly from layers with retention of the spatial integrity of the chromatographic separation. This is certainly not a simple problem but is a problem of some importance, since it restricts the range of applications that HPTLC is considered suitable.

For more than 20 years, efforts have been made to hyphenate HPTLC with mass spectrometry, similar to that of HPLC and MS. Dr. Luftmann Head of the Mass Spectrometry Department at the Institute of Organic Chemistry of the University of Munster, Germany, developed an interface (ChromeXtractor) which allows such HPTLC–MS hyphenation. Dr. Morlock, assistant professor at the Institute of Food Chemistry of the University of Hohelnheim in Stuttgart, Germany, modified ChromeXtractor and demonstrated the performance of this versatile interface in comparison to other technical solutions for hyphenation. The substance of interest is eluted directly form the HPTLC plate and is transferred online into the mass spectrometer.

Component mixtures, even with heavy matrix load, can be separated cost efficiency on HPTLC plates. If the target zone is not visible, it can be marked either under UV 254 nm or UV 366 nm, by extrapolation of the adjacent zone made visible by derivatization. The HPTLC–MS interface is operated in semiautomatic mode which means that after manual positioning of the zone the piston is lowered at the push of a button. Moving a lever starts the solvent flow through the layer and extracts the zone. Previously, data acquisition has to be started by flow injection analysis/direct flow infusion/placebo injection or the direct data acquisition window, followed by the cleaning procedure and the HPTLC–MS interface can be used for next analysis. Hyphenating HPTLC with MS appears to hold considerable promise for those analysts who previously have had reservations towards the use of planar chromatography. The hyphenation opens a new dimension for the technique and makes it more prestigious from the scientific view. Recently, HPTLC–MS has been successfully used for the identification and quantification of amino acid in peptides, fast identification of unknown impurities, problem-solving technique in pharmaceutical analysis, identification of botanicals, screening for bioactive natural products in sponges, determination of ginkgolides A, B, and C and bilobalide in *Ginkgo bilodes* and identification of *Hoodia gordonii* a popular ingredient of botanical slimming products.

High-Performance Thin-Layer Chromatography–Infrared Spectroscopy (HPTLC–IR)

In recent years, much effort has been devoted to the coupling of high-performance thin-layer chromatography (HPTLC) with spectrometric methods. It is because of the robustness and simplicity of use of HPTLC and the need for detection techniques that provide identification and determination of sample. Infrared (IR) is one of the spectroscopic methods that have been coupled with HPTLC. IR spectroscopy

has a high potential for the elucidation of molecular structures and characteristic absorption bands. Almost all chemical compounds yield good IR spectra that are more useful for the identification of unknown substances and discrimination between closely related substances. The HPTLC and FTIR coupling can be divided into two groups – indirect and direct methods. Indirect coupling involves either the transfer of the substance from a TLC spot to a nonabsorbing IR material (KBr or KCl) or in-situ measurement of excised HPTLC spots when the spectra are recorded directly from the plate. The direct methods are based on the direct hyphenated HPTLC–FTIR technique introduced in 1989 by Glauninger and co-workers. Until then, the combination of HPTLC and ultraviolet–visible (UV–VIS) spectroscopy was the only on-line coupling method available in planar chromatography. The information content of UV–VIS spectra is rather poor and rarely enables unambiguous identification of a substance; furthermore, a chromophore is needed for UV detection. The HPTLC–FTIR spectra make possible the detection and quantification of even non-UV absorbing substances on HPTLC plates. These reasons make this hyphenated technique more universally applicable. The direct on-line coupled HPTLC–FTIR offers advantages relative to other hyphenated techniques (HPTLC–Raman spectroscopy, HPTLC–PA, and HPTLC–MS), such as: the ease of operation and the optimized operational aspects of on-line coupling. The HPTLC–FTIR coupled method has been widely used in modern laboratories for qualitative and quantitative analysis. The potential of this method is demonstrated by its application in various fields of analysis such as drug, forensic, food, environmental, and biological analysis, etc. The hyphenated HPTLC–FTIR technique will continue to be developed in the future with the aim of taking full advantage of this method's capabilities.

HPTLC: Laser

For the purpose of investigation, atmospheric pressure-matrix-assisted laser desorption/ionization is chosen for a certain applications depending on the analytes. Combination of laser desorption and APCI was recently developed in which desorption (laser) and ionization (APCI) were well decoupled. This combination was easily incorporated into HPTLC/MS system. Such a system benefits from the high spatial resolution of the laser, simple transfer of analyte molecules, compatibility with modern mass spectrometric systems and less fragmentation under atmospheric pressure. One drawback of such a system is that the cost for a traditional pulsed laser system is relatively high which somehow counteracts the advantage of TLC in the low costs. The size of the laser system is also not ideal for a miniaturization of the whole analytical system.

The initial efforts were carried out on a graphite plate (photon-absorbing material). A continuous wave diode laser replacing traditional pulsed lasers was employed for this purpose. The thermally desorbed analyte was ionized in the gas phase by a corona discharge device at atmospheric pressure and detected mass

spectrometerically. Both essential processes, desorption and ionization of analyte molecules are separated. The technique was subsequently applied to thin-layer chromatography to realize the combination of TLC and mass spectrometry. Thus, a graphite suspension was employed to couple the laser energy and improve the desorption efficiency. The power density for desorption was decreased by two orders of magnitude. In addition, a TLC plate-scanning device was developed, by which the chromatography on a TLC plate can be recovered and rapid screening for numerous analytes on a TLC plate was obtained. The device can also be applied for the identification of unknown compounds and overlapping sample spots. Finally, a quantification method for this system was developed. An internal standard was added into the mobile phase to yield a "background" signal, which was used as a reference signal for the quantification. If TLC plates with embedded graphite particles would become commercially available in the future, laser diode desorption-APCI-MS analysis would be facilitated and the pretreating time shortened.

HPTLC: Multidimensional Applications

HPTLC is now frequently used in the identification of hydrocarbon, alcohols, phenols, carbohydrates, ethers, epoxides, organic acids and lipids, organic peroxides, steroids, steroid glycosides, saponins, terpenoide glycoside, alkloides, nitro and nitroso compounds, amino acids and peptides, proteins, enzymes, nucleic acids, organic sulfur and phosphorus compounds, organometallic compounds, vitamins, growth regulators, antibiotics, pesticides and agrochemicals, synthetic and natural dyes, plastic and their intermediates and also in pharmaceutical, environmental, toxicological, forensic, and food chemical applications. TLC is a routine tool in the monitoring of synthesis processes, HPTLC offers several advantages over the present methods; such as fast, simple, and inexpensive analysis of many samples simultaneously; a disposable stationary phase, possibility to use a number of nondestructive detection methods; and cost-effective reagents. In recent years, HPTLC research has entered the chiral-separation field using a number of chiral selectors and chiral-stationary phases.

Notes

HPTLC is a most versatile technique and is known for uniformity, purity profile, assay values and precision and accuracy of results. It can handle several samples of even divergent nature and composition. It is accepted as a time-saving and most economical machine practically with minimum trouble shootings. It speeds up analysis work which is usually not possible with other parallel chromatographic techniques available. The scope of hyphenation of HPTLC with other analytical techniques appears to hold considerable promise for the analysts who previously

have had reservation towards the use of planar chromatography. Its hyphenation with mass/infra red/laser spectroscopy, etc. opens a new dimension which makes it the most prestigious among the analytical chemists in the present perspective. Undoubtedly, HPTLC is a modern analytical separation method with extensive versatility, although already much utilized, is still with great potential for future development into areas where research apparently is only just beginning.

Suggested Readings

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