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High performance thin layer chromatography: Principle, working and applications

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Abstract
This review article gives knowledge of about High-Performance Thin Layer Chromatography, HPTLC-based analytical method development and its applications in food industry, in accordance to practical evaluation. Among the modern Analytical tools, HPTLC is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks. HPTLC is on TLC, but with enhancements that tend to increase the resolution of the compounds to be separated and allows both quantitative and qualitative analysis of the compounds.

Keywords: HPTLC, working, quantification, characterization, applications

1. Introduction
Chromatography has grown in significance and popularity to become a leading type of analysis in instrumental analytical chemistry. HPTLC (High performance thin layer chromatography) is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. Some of the enhancements such as the use of higher quality TLC plates with finer particle sizes in the stationary phase which allow better resolution (Reich and Schibli, 2005) [32].
HPTLC is robust, simplest, rapid, and efficient tool in quantitative analysis of compounds (Attimarad *et al*., 2014) [3]. It is a type of planar chromatography which utilizes a flat (planar) stationary phase for separation unlike column chromatography. Among the modern Analytical tools HPTLC is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks (Andola and purohit, 2010), because of its suitability for high-throughput screening, sensitivity and reliability in quantification of analytes at nanogram levels (Vundac *et al*., 2005) [41]. It is one of the most widely applied methods for the analysis in pharmaceutical industries, clinical chemistry, forensic chemistry, biochemistry, cosmetology, food and drug analysis, environmental analysis, and other areas (Attimarad *et al*., 2014) [3]. This review focuses on the principle, working and applications of HPTLC in food industry.

2. Principle and working
It is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks. Separation may result due to adsorption or partition or by both phenomenon, depending upon the nature of adsorbents used on plates and solvents system used for development (Bairy, 2015) [4]. The mobile phase solvent flows through because of capillary action. The components move according to their affinities towards the adsorbent. The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster. Thus the components are separated on a chromatographic plate. Difference between TLC and HPTLC are listed in Table 1.

Table 1: Difference between TLC and HPTLC

Feature	TLC	HPTLC
Technique	Manual	Instrumental
Plates	Lab Made/Pre-coated	Pre-coated
Plate Height	30 um	12 um
Layer of sorbent	250 um	100 um
Stationary phase	Silica gel, Alumina & Kiesulguhr	Wide choice of stationary phases like silica gel for normal phase and C8, C18 for reversed phase modes
Analysis time	20-200min	1-3min
Separations	10 - 15 cm	3 - 5 cm
Mean particle size	10-12 um	5-6 um
Efficiency	Less	High due to smaller particle size
Sample holder	Capillary/pipette	Syringe
Sample spotting	Manual spotting	Auto sampler
Size of sample	Uncontrolled/Solvent dependent	Controlled Solvent independent
Shape of sample	Circular (2-4 nm dia)	Rectangular (6mm L X 1mm W)
Sample tracks per plate	≤ 10	≤ 36 (72)
Vol. range	1 to 10 µL	0.1 to 500 µL
Development chamber	More amount	New type that require less amount of mobile phase
Wavelength range	254 or 366 nm, visible	190 or 800 nm, Monochromatic

Detection Limits (Absorption)	1-5 pg	100-500 pg
Detection limits (Fluorescence)	50-100 pg	5-10 pg
PC connectivity, Method storage, Validation	No	Yes
Quantitative analysis	No	Yes
Scanning	No	Use of UV/ Visible/ Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer
Analysis judgment	By analyst	By machine

Source: Patel and Patel, 2008; Shivatare *et al*., 2013 [38]

Schematic procedure for HPTLC method development is given in Fig. 1

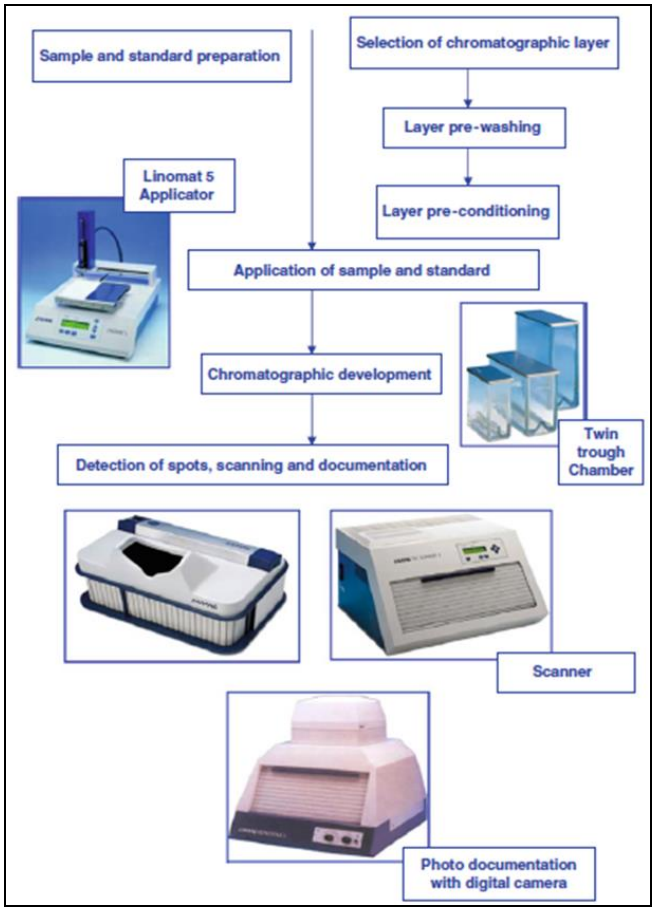

Fig 1: Schematic procedure for hptlc method development

2.1 Selection of stationary phase (chromatographic layer)

Smaller plates (10*10 or 10*20 cm) with significantly decreased development distance (typically 6 cm) and analysis time (7–20 min) are used (Shivatare *et al*., 2013). [38] Following are stationary phases that are mostly used:

- Silica gel GF - with a less polar mobile phase, such as chloroform– methanol, has been used for more than 90% of reported analysis
- Pre-coated plates - different support materials - different Sorbents available like;
  - Aluminum oxide - Basic substances, alkaloids and steroids
  - Cellulose - Amino acids, dipeptides, sugars and alkaloids
  - RP2, RP8 and RP18: Chemically bonded octa decyl silane (ODS), an n-alkane with 18 carbon atoms, is the most frequently used stationary phase. C8 and shorter alkyl chains provide other alternatives. These are used for separation of non-polar substances like fatty acids, carotenoids, cholesterol, etc (Bairy, 2015) [4].

2.2 Sample and Standard Preparation (Layer washing)

To detect damage and impurities in the adsorbent, plates need to be inspected under white and UV light before use. In order to improve the reproducibility and robustness of the results, It is advisable to prewash the plates. Generally, methanol is used as a prewashing solvent; however, a mixture of Chloroform: Methanol (1:1), Ethyl acetate: Methanol (1:1), Chloroform: Methanol: Ammonia (90:0:1), Methylene chloride: Methanol (1:1), 1% ammonia or 1% acetic acid or even mobile phase of the method may also be used. Dry the plates and store in dust free atmosphere. Layer washing is done to avoid interference from impurities and water vapours (Jain *et al*., 2014; Patel *et al*., 2010) [17, 26]

2.3 Activation of pre-coated plates

- Freshly open box of plates do not require activation
- Plates exposed to high humidity or kept on hand for long time to be activated placing in an oven at 110-120ºC for 30min prior to spotting.
- Aluminum sheets should be kept in between two glass plates and placing in oven at 110- 120ºC for 15 minutes.

2.4 Application of sample and standard

The most critical step to obtain good resolution for quantification in HPTLC. Sample application technique depends on factors such as the type of sample matrix, workload and time constraints. Usual concentration range of sample is 0.1-1µg / µl because the concentration above this range results in poor resolution of samples. There are 2 ways in which sample and standard can be applied;

- Automated instruments are available for sample application; especially for quantitative HPTLC, apply samples using the spray-on technique with Automatic TLC sampler (ATS) 4 or Linomat 5. The sample is typically contained in a syringe, which is emptied by a motor. Delivery speed and volume are electronically controlled. A stream of an inert gas such as nitrogen around the tip of the syringe atomizes the sample and creates a band on the TLC/HPTLC plate if either the syringe or the plate is moving linearly. Band wise application is done in order to achieve better separation and high response to densitometer. Sample application in the form of narrow bands provides the highest resolution and sensitivity (Patel *et al*., 2010) [26].
- Circular spots have numerous disadvantages while "line" or "band" application is advantageous.

2.5 Selection of mobile phase

- The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte (Patel *et al*., 2010) [26].
- The elution power of the mobile phase depends on a property called eluent strength which is related to the polarity of the mobile phase components.
- Multi component mobile phase once used not recommended for further use and solvent Composition is expressed by volumes (v/v) and sum of volumes is usually 100 (Jain *et al*., 2014) [17].
- Only 10 -15 ml of mobile phase is required while using twin trough chambers.

2.6 Chamber saturation

HPTLC plates are developed in chambers (flat-bottom chambers, twin-trough chambers, or horizontal-development chambers) and generally, saturated twin-trough chambers are used for the best reproducibility. Chamber saturation is carried out as follows: Development chamber is lined on three sides with filter paper. Appropriate volume of mobile phase is prepared and carefully poured into chamber in order to wet the filter paper is thoroughly so that it adheres to rear wall of chamber. Tilt chamber to the side (about 45°) in order to equalize solvent volume in both troughs. Replace the lid and let chamber equilibrate for 20 min (Patel *et al*., 2010) [26].

2.7 Chromatogram development

Desired developing distance that is 70 mm from lower edge of plate is marked on the right edge of the plate with a pencil. Lid should not be removed but slide off to the side and plate should be placed in the front trough in such a way that the layer and filter paper face each other and the back of the plate is resting against front wall of chamber. Lid is replaced and plates are developed to the mark. After development, plates are removed from chamber and dried vertically in the direction of chromatograph for 5 min in a stream of cold air. Remaining mobile phase and filter paper should be discarded after each development. Vacuum desiccators can also be used for drying (Patel *et al*., 2010) [26].

2.8 Detection

- Detection under UV light is first choice because it is non-destructive. Zones with fluorescence or quench fluorescence are viewed in cabinets that incorporate short-wave (254 nm) and long-wave (366 nm) UV lamps.
- Spots of non-fluorescent compounds are visualized using fluorescent stationary phase. For example, silica gel GF has been used for visualizing non UV absorbing compounds like ethambutol, dicylomine (Jain *et al*., 2014; Patel *et al*., 2010) [17, 26].

2.9 Derivatization

- Derivatization is necessary for some compounds that do not respond to UV light and involves reaction with a specific reagent.
- Derivatization is performed either by immersing the plates or by spraying the plates with a suitable reagent.
- Immersing: Tank of immersion devices is charged with 200 ml of reagent and plate is placed in the holder of the immersion device, parameters should be set according to method to start the process. The plate is removed from plate holder and dried in stream of cold air.
- Spraying: Charge the bottle of the sprayer with up to 50 ml of reagent. Place the plate in spray cabinet against a filter paper. Spray plate with horizontal and vertical motion until it is homogeneously covered with reagent. The plate is dried with cold air.
- To induce or optimize the derivatization reaction, it may be necessary to heat the plates (Patel *et al*., 2010) [26].

2.10 Quantification

Quantitative analysis are performed in situ by using a chromatogram spectrophotometer (densitometer or scanner) with a fixed sample light beam in the form of a rectangular slit that measures the zones of samples and standards. Generally, TLC Scanner 3 using win CATS software is used that scans the chromatogram by reflectance, transmittance, absorbance or by fluorescent mode. Scanning speed is selectable up to 100 mm/s. Spectra recording is fast. Concentration of analyte in the sample is calculated by considering the sample initially taken and dilution factors (Patel *et al*., 2010) [26].

2.11 Documentation

Each developed plate is documented using digital documentation system under UV light at 254 nm, UV light at 366 nm, and white light. If a type of light does not produce usable information, that fact must be documented. If a plate is derivatized, images are taken prior and after derivatization.

3. Applications

HPTLC finds application such as Quality control, Additives (e.g. vitamins), Pesticides, Stability tests (expiration), etc in food and feed stuff (Sonia *et al*., 2017) [39].

3.1 Compositional evaluation

3.1.1 Carbohydrates

High performance thin-layer chromatography (HPTLC) has

remained valuable and preferred method for the analysis of carbohydrates due to the ease of handling, suitability for rapid and simultaneous analysis of multiple samples and lower equipment cost than standard HPLC (Li *et al*., 2013; Robyt, 2000) [20, 34]. For separation of carbohydrates, silica gel has been principal stationary phase (Robyt, 2000) [34]. In order to improve resolution of sugar analytes, silica gel plates have often been reported to be pre-treated with suitable buffers, like phosphate or borate while as acetonitrile: water (85:15 v/v) is frequently the first choice as developing solvent and in order to enhance resolution, it is often modified by addition of organic solvents or acids

Wua *et al*. (2015) [44] carried out the characterization and comparison of polysaccharides from Lycium barbarum in China using saccharide mapping based on PACE and HPTLC, suggested that saccharide mapping based on PACE and HPTLC analysis could be a routine approach for quality control of polysaccharides.

3.1.2 Proteins

Fingerprinting of amino acids (free as well as protein bound aminoacids) is required for evaluating quality, stability and provenience of the product. Traditionally, detecting and quantifying proteins on HPTLC plates can be performed with fuorescamine or ninhydrin as staining reagents (Hakanson, *et al*., 1974) [13]. Meisen *et al*. (2004) [22], showed an antibody based detection on HPTLC plates for analyzing Shiga toxin binding glycosphingolipids. Furthermore, we already developed an immunostaining protocol for the investigation of phosphopeptides using commercially available antibodies in a HPTLC assay.

3.2 Detection of toxins

Scussel (2003) [35], has compared TLC and HPTLC for determination of aflatoxin M_1 in milk and B_1 in eggs and reported that HPTLC technique provides a better spot shape and much smaller size than TLC leading to a more accurate fluorescence detection than the visual (the extract is manually applied on the plate). Studies have indicated that the toxicity of AFM_1 is of the same order of magnitude as that of AFB_1 (Wogan and Paglialunga, 1974) [43] and that AFM_1 is also a potent hepatocarcinogen in laboratory animals (Shih and Marth, 1971) [37]. Analysis of feed samples, for aflatoxin B_1 has been carried out by usig HPTLC method and out of 59 samples of feed analysed by HPTLC, 47 samples have been reported to be positive for Aflatoxin B_1 representing 79.66% with a concentration of 25.53±5.31ppb (mean±S.E.) and 0.5 ppb Limit of detection (Ramesh *et al*., 2013) [28]. Dhand *et al.*, (1998) [11] have reported 75% of feed samples (21 of 28) contaminated with aflatoxin B_1.

3.3 Quality control

HPTLC has been widely used in the authentication and quality control of herbal substances (Reich and Schibli, 2007) [31].

3.3.1 Fruit juice

A fast quantitative method for quality control of cranberry juice has been developed using HPTLC-densitometry (Boudesocque *et al*., 2013), which allows the rapid quantitation of quality markers that is catechin and proanthocyanidins (PAC-A_2 and PAC-B_1) in one step without tedious sample conditioning like thiolytic degradation (Kimura *et al*., 2011) or acidic degradation and derivatization with phloroglucinol (Hümmer & Schreier, 2008) [15].

3.3.2 Milk

Rani *et al*. (2014) [30], developed a HPTLC-MS (mass spectroscopy) based method for the quantification of melamine using on Silica gel 60 F254s HPTLC plates and optimized mobile phase (iso-propanol/dichloromethane/ water, 5:2.5:3, v/v/v) in a twin trough chamber saturated (5 min) at pH 6.8 (Fig. 3). Densitometric scanning at 200 nm resulted into a single peak of standard melamine at Rf value 0.57± 0.02. Visualization was carried out at 254 nm and image was recorded. After optimization, adulterated test samples 15 µL were loaded on the same plate and quantified for accuracy. The matching of UV spectra of standard melamine among the observed peaks in the adulterated test samples was considered a basis for the presence of melamine adulteration (Fig. 3).Melamine (2, 4, 6-triamino-1, 3, 5-triazine, $C_3H_6N_6$) is used in the manufacturing of plastics, fertilizers, melamine-formaldehyde resins for surface coatings, laminates, fire retardant, and adhesives (Wen *et al*. 2010). Melamine being rich in nitrogen (66.7%), is sometimes added to food in order to increase the protein content, which is not measurable by Kjeldahl or Dumas method (Chan *et al*., 2008) [7] and is associated with several health issues like urolithiasis, tissue injury, and even bladder cancer (Newton and Utley, 1978) [23].

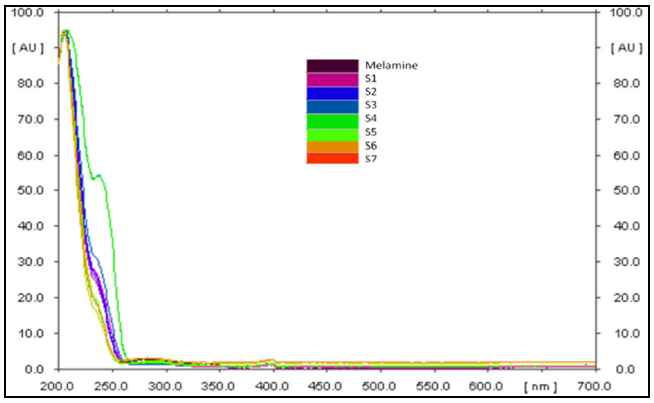

Fig 2: UV Spectra for standard melamine and S1–S7 were spiked milk samples (Rani *et al*., 2014) [30].

3.4 Separation, identification and quantification of bioactive components

Many researchers have used HPTLC for separation, identification and quantification of bioactive components. Kruger *et al*. (2013) developed HPTLC for determination of anthocyanins from pomace, juice and wine. HPTLC method has been used to estimate piperine in Ayurvedic formulations containing plant ingredients of Piperaceae family and has been reported to be successfully employed for standardization and quantitative analysis of piperine in Ayurvedic formulations (Hazra *et al*., 2014). Reim and Rohn, (2015) [33] carried out HPTLC (coupled to mass spectrometry) characterization of peas and reported presence of saponins in the pea samples. HPTLC has also been used to study the chemical profile of secondary metabolites of medicinally important plant Zanthoxylum rhetsa (Roxb.) by Alphonso and Saraf (2012) [1]. High-performance thin-layer chromatography (HPLTC) has also been used to identify the compounds responsible for the

antioxidant, anti-inflammatory, and xanthine oxidase activities of garlic extracts, using a mobile phase of ethyl acetate: formic acid: water, 85:5:10 (v/v/v) and spray drying plates with anisaldehyde reagent. They reported maximum absorbance at 254 nm and revealed qualitative presence of gingerol (6G), 6-shogaol (6S), and 6-paradol (6P) on HPTLC plates under CAMAGTLC Scanner (Nile and Park, 2015). HPTLC has also been used to determine oligomeric Pas (proanthocyanidind), alkaloids and anthocyanins through the entire value chain (from fresh cocoa beans, through roasted cocoa, cocoa mass and up to molded chocolate bars) and established method has been proven as a suitable tool for comprehensive compound analysis in laboratories with high sample throughput (Pedan *et al*., 2018).

3.5 Detection of Drug residues in food

HPTLC allows the qualitative and quantitative detection of multi-residues in meat (Shankar *et al*., 2010) [36]. Reported uses of HPTLC applied to meat include the detection of residues like clenbuterol and other agonists in meat tissues (Degroodt *et al*., 1991) [10], nitroimidazol in pork and poultry (Gaugain and Abjean, 1996) [12], sulphonamides (Bukanski, 1988) [6] and thyreostatic drugs in animal tissues. Argekar *et al*. (1996) [2] developed method for HPTLC determination of six fluoroquinolones (ciprofloxacin, enrofloxacin, lomefloxycin, norfloxacin, ofloxacin and perfloxacin) on silica with butanol-ethanol-ammonia 20:5:11 and reported the linearity range to be 10-150 ng, Rf values in the range of 0.35 to 0.40 and found the method comparable with other official methods.

HPTLC has also been applied for the detection of flumequine in milk (Choma *et al*., 2002) [8], analysis of corticosteroids (Hoebus *et al*., 1993; Vanoosthuyze *et al*., 1993) [40] and antibiotics in milk (Choma *et al*., 1999) [9]. Ramirez *et al*. (2003) developed an analytical method to identify and quantify multiple antibiotic residues in cow's milk by combined application of HPTLC with bioautography. Oka *et al*. (2000) [25] have designed an improved HPTLC method for analysis of tetracyclines.

4. Advantages of HPTLC

The use of HPTLC is particularly useful, when a laboratory has to analyse very high number of similar samples, searching for classes of molecules. In fact, HPTLC can be easily applied as a screening approach to define the presence/absence of certain analytes in a series of samples. After screening, only positive samples are included in further analyses, reducing significantly both costs and time spent for reaching the final results.

1. HPTLC allows a parallel separation and quantitative determination of many samples at the same time
2. HPTLC is simpler, more flexible, more accessible, and cheaper than other commonly used methods.
3. Sample clean-up can be omitted or greatly reduced, because the plate is disposable, and the chromatography itself is effective solid phase purification
4. Planar chromatography is an optimum tool for national and international standards to keep analysis inexpensive - especially nowadays (Locher *et al*. 2017).

5. Conclusion

Nowadays, Planar chromatography has become an optimum tool for national and international standards to keep analysis inexpensive. The methods could be utilized for compositional evaluation, quality control, source determination, etc of various foods and food products. HPTLC method has been recommended for fast control of food labelling in food inspection or for inexpensive quality control in food production.

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