Thin Layer Chromatography

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Chromatography

- **Chromatography** is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.
- **Types of Chromatographic Techniques:**

Technique	Stationary	Mobile Phase
Column/Adsorption Chromatography	solid	Liquid
Partition Chromatography	Liquid	Liquid
Paper Chromatography	Liquid	Liquid
Thin Layer Chromatography (TLC)	Liquid/Solid	Liquid
Gas – Liquid chromatography (GLC)	Liquid	gas
Gas – Solid Chromatography (GSC)	Solid	gas
Ion Exchange Chromatography	Solid	Liquid

Introduction

TLC is one of the simplest, fastest, easiest and least expensive of several chromatographic techniques used in qualitative and quantitative analysis to separate organic compounds and to test the purity of compounds.

TLC is a form of liquid chromatography consisting of:

- A mobile phase (developing solvent) and
- A stationary phase (a plate or strip coated with a form of silica gel)
- Analysis is performed on a flat surface under atmospheric pressure and room temperature

Definitions

- **Thin Layer Chromatography** can be defined as a method of separation or identification of a mixture of components into individual components by using finely divided adsorbent solid / (liquid) spread over a glass plate and liquid as a mobile phase.
- Synonyms: Drop, strip, spread layer, surface chromatography and open column chromatography

- **Adsorption** or retention or **partition** or both or any other principle of a substance (s) on the stationary phase
- Separation of the adsorbed substances by the mobile phase
- Recovery of the separated substances by a continuous flow of the mobile phase (elution)
- Qualitative and quantitative analysis of the eluted substances

History

- Michael Tswett is credited as being the father of liquid chromatography. Tswett developed his ideas in the early 1900's.

- 1938:- Izmailov & shraiber described basic principle and used it for separation of plant extracts.

- 1944:- Consden, Gorden & Martin started using filter papers for separation of amino acid.

- 1950:- Kirchner who used impregnated glass plate coated with alumina, identified terpenes.

- 1958:- Ergon stahl introduced a standard equipment for preparing uniform thin layers of known thickness

When TLC used ?

TLC is used if

- the substances are nonvolatile or of low volatility
- the substances are strongly polar, of medium polarity, nonpolar or ionic
- a large number of samples must be analyzed simultaneously, cost-effectively, and within a limited period of time
- the samples to be analyzed would damage or destroy the columns of LC (liquid chromatography) or GC (gas chromatography)
- the solvents used would attack the sorbents in LC column packings
- the substances in the material being analyzed cannot be detected by the methods of LC or GC or only with great difficulty
- after the chromatography, all the components of the sample have to be detectable (remain at the start or migrate with the front)
- the components of a mixture of substances after separation have to be detected individually or have to be subjected to various detection methods one after the other (e.g. in drug screening)
- no source of electricity is available

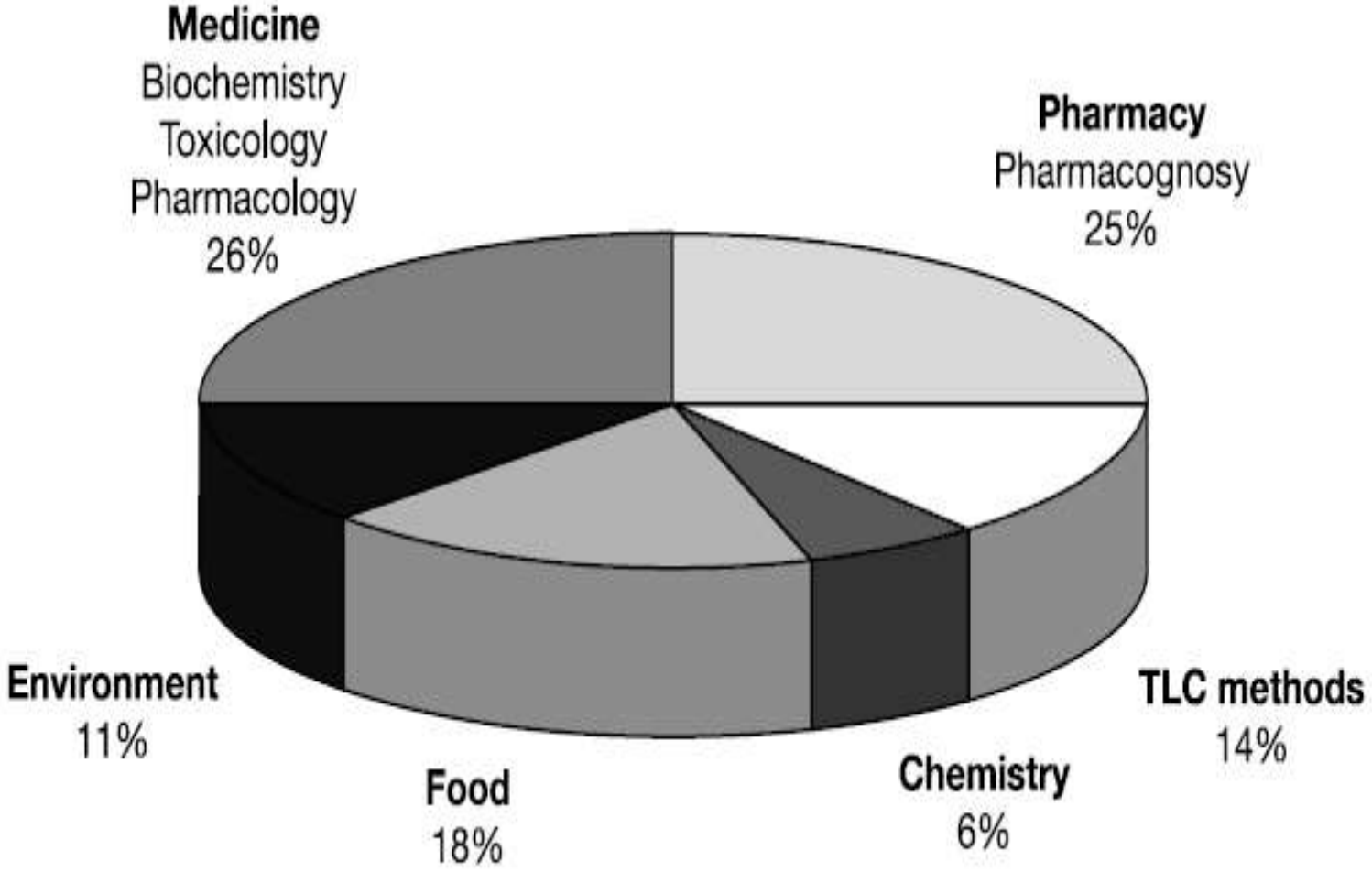

Figure 1. Fields of application of thin-layer chromatography (TLC/HPTLC) over the years 1993–1994

Where TLC used

Pharmaceuticals and Drugs
Identification, purity testing and determination of the concentration of active ingredients, auxiliary substances and preservatives in drugs and drug preparations, process control in synthetic manufacturing processes.

Clinical Chemistry, Forensic Chemistry and Biochemistry
Determination of active substances and their metabolites in biological matrices, diagnosis of metabolic disorders such as PKU (phenylketonuria), cystinuria and maple syrup disease in babies.

Cosmetology
Dye raw materials and end products, preservatives, surfactants, fatty acids, constituents of perfumes.

Food Analysis
Determination of pesticides and fungicides in drinking water, residues in vegetables, salads and meat, vitamins in soft drinks and margarine, banned additives in Germany (e.g. sandalwood extract in fish and meat products), compliance with limit values (e.g. polycyclic compounds in drinking water, aflatoxins in milk and milk products).

Environmental Analysis
Groundwater analysis, determination of pollutants from abandoned armaments in soils and surface waters, decomposition products from azo dyes used in textiles.

Analysis of Inorganic Substances
Determination of inorganic ions (metals).

Principle of TLC

It is based on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvents employed

The components with more affinity towards stationary phase travels slower.

Components with less affinity towards stationary phase travels faster

Continue…….

- In TLC, a solid phase, the **adsorbent,** is coated onto a solid support (thin sheet of glass, plastic, and aluminum) as a thin layer (about 0.25 mm thick). In many cases, a small amount of a **binder** such as plaster of Paris is mixed with the absorbent to facilitate the coating.
- The mixture (A + B) to be separated is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom. A solvent, or mixture of solvents, called the **eluatant, is allowed to flow up the plate by capillary action.** At all times, the solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. Any one molecule will spend part of the time sitting still on the adsorbent with the remainder moving up the plate with the solvent. A substance that is strongly adsorbed (say, A) will have a greater fraction of its molecules adsorbed at any one time, and thus any one molecule of A will spend more time sitting still and less time moving and vice versa**.**

Continue……..

- Separation of mixtures in microgram quantities by movement of a solvent across a flat surface; components migrate at different rates due to differences in solubility, adsorption, size or charge; elution is halted when or before the solvent front reaches the opposite side of the surface and the components examined *in situ or removed for further analysis*.

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Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase

1.The stationary phase:

is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate.

2.The mobile phase:

is a developing liquid which travels up the stationary phase, carrying the samples with it.

Components of the samples will separate on the stationary phase according to:

how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase

Basic Theory

The basic parameter used to describe migration in TLC is the R_f value, where

$$R_f = \frac{\text{distance moved by the solute}}{\text{distance moved by mobile phase front}}$$

R_f values vary from 1 to 0, or from 100 to 0 if multiplied by 100 (hR_f).

The capacity factor, k', is the ratio of the quantities of solute distributed between the mobile and stationary phases, or the ratio of the respective times the substance spends in the two phases,

$$k' = \frac{t_s}{t_m} = \frac{\text{retention time in stationary phrase}}{\text{retention time in mobile phase}}$$

The capacity factor and R_f are related by the equation

$$k' = \frac{1 - R_f}{R_f}$$

Continue……

The flow constant or velocity constant (κ) is a measure of the migration rate of the solvent front. It is an important parameter for TLC users and can be used to calculate, for example, development times with different separation distances, provided that the sorbent, solvent system, chamber type and temperature remain constant. The flow constant is given by the following equation:

$$\kappa = \frac{Z_F^2}{t}$$

where

κ = flow constant [mm^2/s]
Z_F = distance between the solvent front and the solvent level [mm]
t = development time [s]

The following example illustrates the usefulness of the flow constant in laboratory work. In a TLC, if the development time for a migration distance of 10 cm was 30 min and the Z_0 distance is 5 mm, the κ value is 6.125 mm^2/s.

Retardation Factor

The position of a substance zone (spot) in a thin-layer chromatogram can be described with the aid of the retardation factor R_f. This is defined as the quotient obtained by dividing the distance between the substance zone and the starting line by the distance between the solvent front and the starting line (see Fig. 3):

$$R_f = \frac{Z_S}{Z_F - Z_0}$$

where

R_f = retardation factor

Z_S = distance of the substance zone from the starting line [mm]

Z_F = distance of the solvent front from the solvent liquid level [mm]

Z_0 = distance between the solvent liquid level and the starting line [mm]

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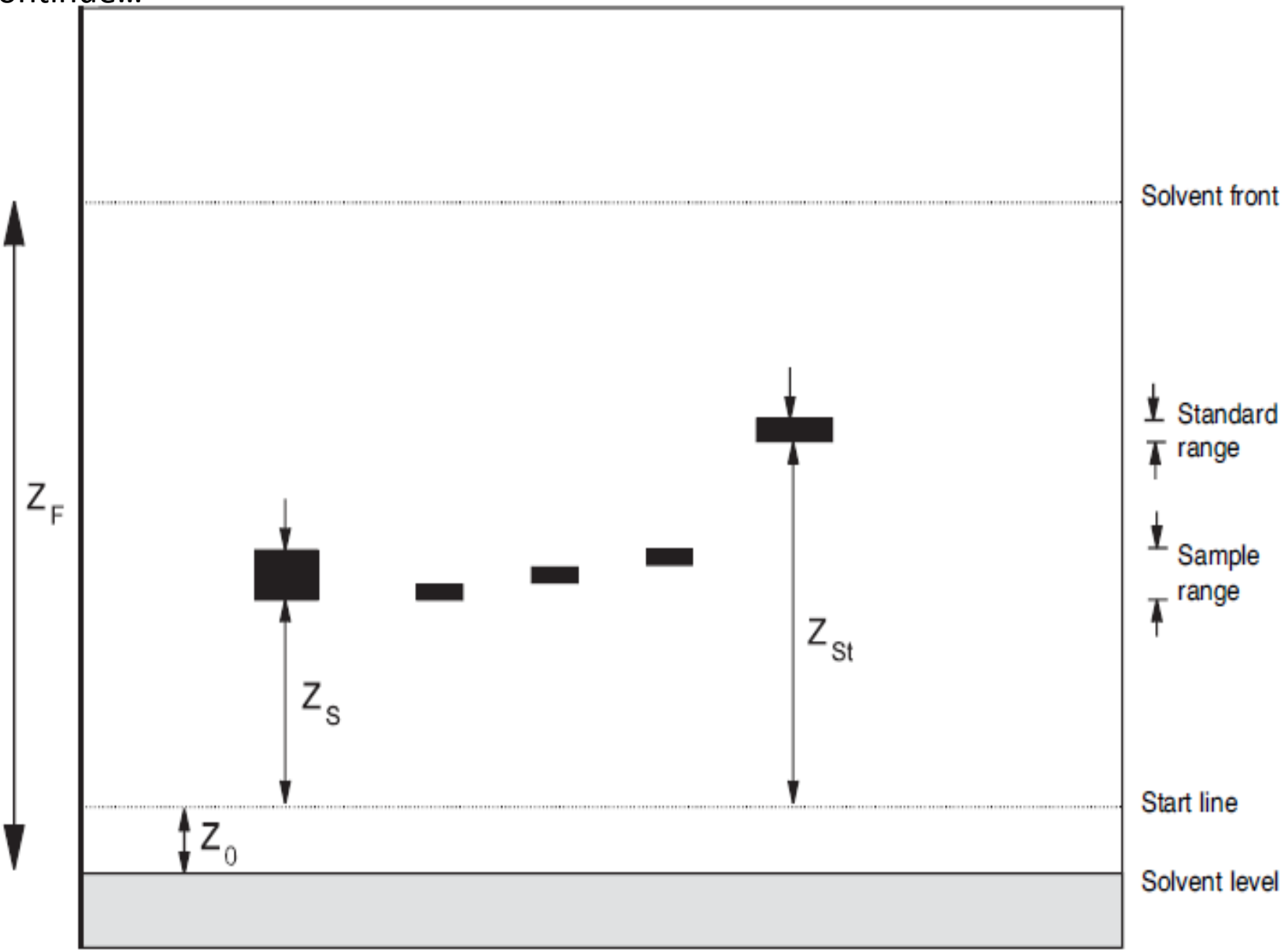

Solvent front
Standard range
Sample range
Z_F
Z_S
Z_{St}
Start line
Z_0
Solvent level

Factors affecting R_f value

It depends on following factors:

- Nature adsorbent
- Mobile phase
- Activity
- Thickness of layer
- Temperature
- Equilibrium
- Loading
- Dipping zone
- Chromatographic techniques

Selection of Stationary Phase

The choice of the stationary phase for a given separation problem is the most difficult decision in TLC

The chose of stationary Phase in following characters considered.

The chemical composition of the stationary Phase and in particular that of its surface, must be suitable for the task. To obtain satisfactory separation efficiency, the mean particle size, the particle size distribution and the morphology of the particle must be considered

Stationary phases for thin-layer chromatography

Stationary phase	Predominant sorption process	Use
silica gel	adsorption or partition	general
modified silica gels	adsorption or partition	similar to bonded phase HPLC
alumina	adsorption or partition	general
cellulose powder	partition	inorganic, amino acids, nucleotides, food-dyes
kieselguhr	partition	sugars
modified celluloses e.g. DEAE and CM	ion-exchange	nucleotides, phospholipids
Sephadex gels	exclusion	macromolecules

Mobile Phase

The choice of mobile phase is largely empirical but general rules can be formulated. A mixture of an organic solvent and water with the addition of acid, base or complexing agent to optimize the solubility of the components of a mixture can be used. For example, good separations of polar or ionic solutes can be achieved with a mixture of water and *n-butanol. Addition of acetic acid to the mixture allows more* water to be incorporated and increases the solubility of basic materials, whilst the addition of ammonia increases the solubility of acidic materials. If the stationary phase is hydrophobic, various mixtures of benzene, cyclohexane and chloroform provide satisfactory mobile phases. It should be emphasized that a large degree of trial and error is involved in their selection. For TLC on silica gel, a mobile phase with as low a polarity as possible should be used consistent with achieving a satisfactory separation. Polar solvents can themselves become strongly adsorbed thereby producing a partition system, a situation which may not be as desirable

Least Eluting Power (alumina as adsorbent)

-Petroleum ether (hexane; pentane)
-Cyclohexane
-Carbon tetrachloride
-Benzene
-Dichloromethane
-Chloroform ; -Ether
-Ethyl acetate (anhydrous)
-Acetone (anhydrous)
-Ethanol ;
-Methanol
-Water ; -Pyridine

Greatest Eluting Power (alumina as adsorbent)

- Organic acids

Mobile Phase

- The eluting solvent should also show a maximum of selectivity in its ability to dissolve or desorbs the substances being separated.
- A more important property of the solvent is its ability to be itself adsorbed on the adsorbent.
- A number of common solvents in approximate order of increasing adsorb ability, and hence in order of increasing eluting power.
- Mixtures of solvents can be used and, since increasing eluting power results (0.5 to 2% by volume)
- solvents to be used in chromatography should be quite dry

Selection of adsorbents

- Solubility of compound e.g, hydrophilic or lipophilic
- Nature of substance to be seperated i.e whether it is acidic, basic or amphoteric
- Adsorbent particle size
- Adsorbent should not adhere to glass plate
- Reactivity of compound with the solvent or adsorbent
- Chemical reactivity of compounds with binders

Chromatographic media-coating material

Sorbents

That in experiments performed to solve various problems by the adsorption method the use of various sorbents would be necessary. They tested various substances, including aluminum oxides, aluminum silicates, calcium carbonate,kaolin, kieselguhr, magnesium oxide, powdered sugar, silica gels, starch and talc

The separation efficiency obtained in TLC is essentially determined by the **mean particle size and the size distribution** of the sorption agent used in the preparation of the layer. As can be seen from Fig. Below, the mean particle size of silica gel of a quality suitable for HPTLC is 5 m, that of TLC quality ca. 11 m and that of PSC quality over 20 m.

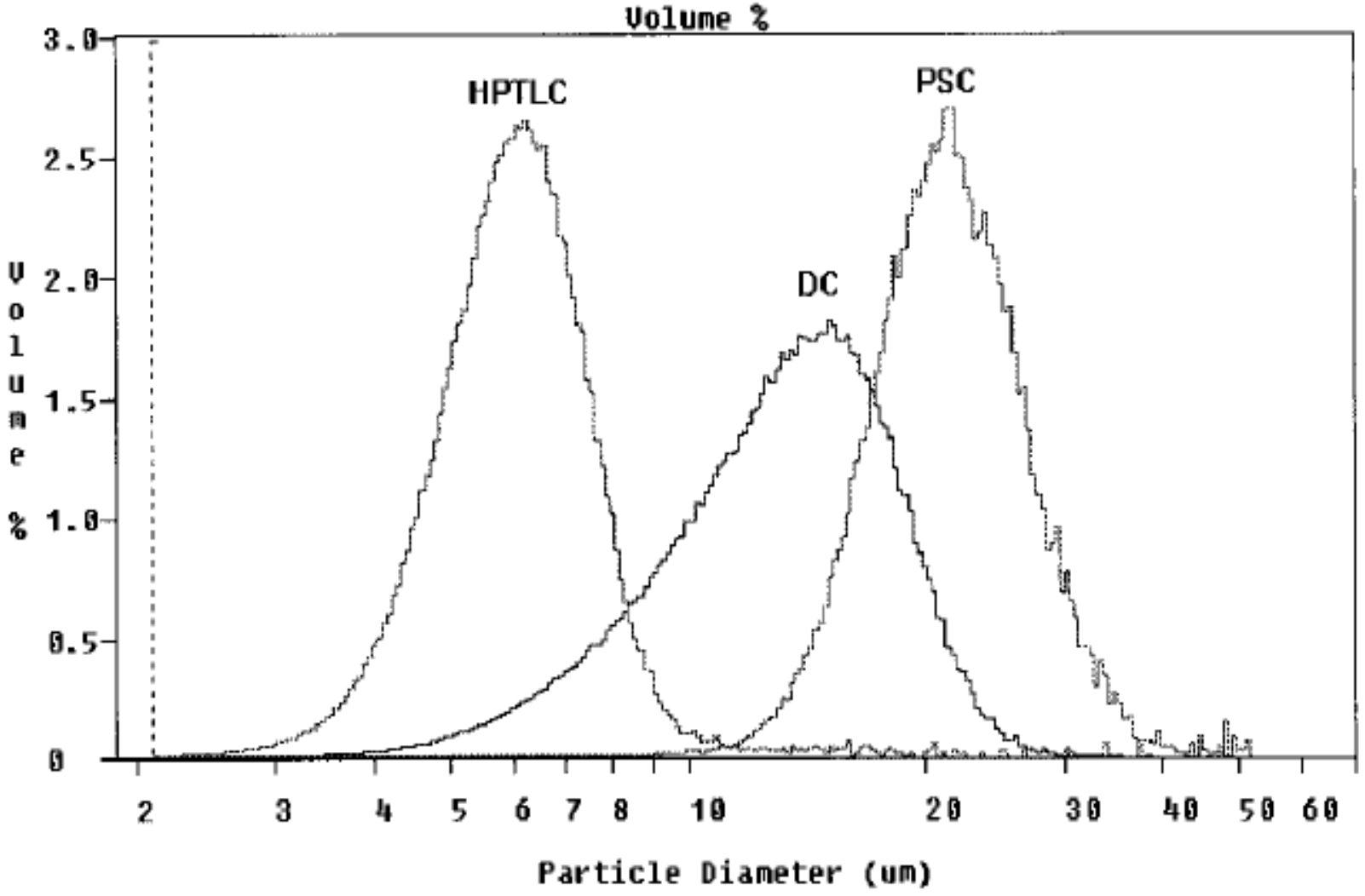

Volume %
3.0
2.5
2.0
1.5
1.0
0.5
0
Volume %
HPTLC
DC
PSC
2
3
4
5
6
7
8
10
20
30
40
50
60
Particle Diameter (um)

Table 1: Types of sorbents and supports for precoated layers

Sorbent material	Support
Aluminum oxide 60, 150	Aluminum foil, glass plate, plastic film
Cellulose (unmodified)	Aluminum foil, glass plate, plastic film
Cellulose (acetylated)	Glass plate, plastic sheet
PEI-Cellulose	Glass plate, plastic sheet
Silica gel 40	Glass plate
Silica gel 60	Aluminum foil, glass plate, plastic film
Kieselguhr	Aluminum foil, glass plate
LiChrospher® Si 60	Glass plate
Si 50000	Glass plate
Si 60 RAMAN	Aluminum foil
Silica gel, modified	
CHIR (chiral)	Glass plate
CN (cyano)	Glass plate
DIOL	Glass plate
NH_2 (amino)	Aluminum foil, glass plate
Silica gel 60 caffeine-impregnated	Glass plate
Silical G ammonium sulfate-impregnated	Glass plate
Silica gel 60 silanized (RP-2), RP-8	Glass plate
RP-18	Aluminum foil, glass plate

Mixed layers	
Aluminum oxide/acetylated cellulose	Glass plate
Cellulose/silica	Glass plate
Cellulose 300 DEAE/cellulose 300 HR	Glass plate
Silica gel 60/ kieselguhr	Aluminum foil, glass plate
Two-zone layers[a)]	
Si 50000 (conc.), silica gel 60 (sep.)	Aluminum foil, glass plate
Si 50000 (conc.), RP-18 (separ.)	Glass plate
Kieselguhr (conc.), silica gel 60 (sep.)	Glass plate
Silica gel 60 (1st sep.), RP-18 (2nd sep.)	Glass plate
Special layers	
IONEX (ion exchange resin)	Plastic sheet
Polyamide 6	Plastic sheet
Polyamide 11	Aluminum foil, glass plate

This table does not claim to be fully comprehensive

[a)] conc. = concentrating zone
sep. = separation zone

Table 3a: Important commercially available precoated layers and examples of typical applications

Sorbent material	Chromatographic principle	Typical applications
Aluminum oxide	Adsorption chromatography due to polar interactions	Alkaloids, steroids, terpenes, aliphatic, aromatic and basic compounds
Cellulose		
Unmodified cellulose	Partition chromatography due to polar interactions	Amino acids and other carboxylic acids as well as carbohydrates
Acetylated cellulose	Depending on acetyl content transition from normal phase to reversed phase chromatography	Anthraquinones, antioxidants, polycyclic aromatics, carboxylic acids, nitrophenols, sweeteners
Cellulose ion exchangers	Anion exchange	Amino acids, peptides, enzymes, nucleic acids constituents (nucleotides, nucleosides) etc.
Mixed layers Cellulose DEAE/cellulose HR	Ion exchange	Mono- and oligonucleotides in nucleic acid hydrolyzates
Ionex ion exchangers	Cation and anion exchange	Amino acids, nucleic acid hydrolyzates, amino sugars, antibiotics, inorganic phosphates, cations; racemate separation in peptide synthesis

Sorbent material	Chromatographic principle	Typical applications
Kieselguhr	Commonly impregnated for reversed phase separations	Aflatoxins, herbicides, tetracyclines
Polyamide	Partition chromatography due to polar interactions (e.g. hydrogen bonds)	Phenolic and polyphenolic natural substances
Silica		
Unmodified silica gel		
Standard and nano silica gel, also with concentrating zone	Normal phase chromatography	Most frequent application of all TLC layers
High purity silica gel 60		Aflatoxins
Silica gel G, impregnated with ammonium sulfate		Surfactants, lipids (neonatal respiratory syndrome)
Silica gel 60, impregnated with caffeine for PAH determination	Charge transfer complexes	Polycyclic aromatic hydrocarbons (PAH) acc. to German drinking water specification (TVO)
Chemically modified layers: CHIRalplate	Enantiomer separation based on ligand exchange chromatography	Chiral amino acids, α-hydroxy-carboxylic acids and other compounds which can form chelate complexes with Cu(II) ions

Table 3a: Continued

Sorbent material	Chromatographic principle	Typical applications
Cyano-modified layer CN	Normal phase and reversed phase chromatography	Pesticides, phenols, preservatives, steroids
DIOL-modified layer		Steroids, hormones
Amino-modified layer NH_2	Anion exchange, normal phase and reversed phase chromatography	Nucleotides, pesticides, phenols, purine derivates, steroids, vitamins, sulfonic acids, carboxylic acids, xanthines
RP layers:		
RP-2, RP-8, RP-18		Nonpolar substances (lipids, aromatics)
Silica gel 60 silanized		Polar substances (basic and acidic pharmaceutical active ingredients)
RP-18 W/UV_{254}, wettable	Normal phase and reversed phase chromatography	Aminophenols, barbiturates, preservatives, nucleobases, PAH, steroids, tetracyclines, phthalates
Spherical silica gel		
LiChrospher® Si 60	Normal phase chromatography	Pesticides, phytopharmaceuticals

Sorbent material	Chromatographic principle	Typical applications
Mixed layers		
Aluminum oxide/acetylated cellulose	Normal phase and reversed phase chromatography	Polycyclic aromatic hydrocarbons (PAH)
Cellulose/silica gel	Normal phase chromatography	Preservatives
Kieselguhr/silica gel	Normal phase chromatography, reduced Adsorption capacity compared to silica gel	Carbohydrates, antioxidants, steroids, photographic developer substances

Table 3b: New precoated layers

Name of the plate	Particularities	Typical applications
Adamant® (Macherey-Nagel) **Lux®** (Merck)	Both: Increased amount of fluorescence indicator	Universal
UTLC (Merck)	Ultra thin monolithic silica gel	Steroids, azepams, amino acids, phthalates and phenols
ProteoChrom® (Merck)	a) HPTLC silica gel 60 F_{254s}, 20 × 10 cm glass plate b) HPTLC cellulose, 10 × 10 cm aluminum sheet	Amino acids, peptides (from protein digest) Amino acids, peptides, 2D-TLC
HPTLC Premium Purity Plate (Merck)	Wrapped in a special plastic-coated foil	All pharmacopoeia applications

What Are the Uses of Precoated Layers

TLC investigations are mainly concerned wit the determination of

- Identity
- Purity
- Assay
- or a combination of these parameters

PREPARATION OF CHROMATOPLATES

- Glass plates or flexible plates are commonly used for adsorbent. Size used depends on type of separation to be carried out, the type of chromatographic tank and spreading apparatus available.
- The standard sizes are 20 x 5 cm, 20 x 10 cm or 20 x 20 cm .
- The surface should be flat without irregularities.
- The standard film thickness is 250um

Methods for application of adsorbent.

- Pouring
- Dipping
- Spraying
- Spreading.

- **Pouring**: The adsorbent of finely divided and homogeneous particle size is made into slurry and is poured on a plate and allowed to flow over it so that it is evenly covered.

- **Dipping :** This technique is used for small plates by dipping the two plates at a time, back to back in a slurry of adsorbent in chloroform or other volatile solvents. Exact thickness of layer is not known and evenness of layer may not be good.

- **Spraying :** Slurry is diluted further for the operation of sprayer. But this technique is not used now a days as it is difficult to get uniform layer.

- **Spreading :** All the above methods fail to give thin and uniform layers. Modern methods utilize the spreading devices for preparation of uniform thin layers on glass plates. Commercial spreaders are of two types (a) Moving spreader, (b) Moving plate type.

  It gives layer thickness from 0.2 to 2.0 mm.

ACTIVATION OF PLATES

- After spreading plates are allowed to dry in air and further dried and activated by heating at about 100^{0}c for 30 mins.

- By removing the liquids associated with layer completely, the adsorbent layer is activated.

Solvent Systems

The solvent system performs the following main tasks:

- To dissolve the mixture of substances,
- To transport the substances to be separated across the sorbent layer,
- To give hRf values in the medium range, or as near to this as possible,
- To provide adequate selectivity for the substance mixture to be separated.

They should also fulfill the following requirements:

- Adequate purity,
- Adequate stability,
- Low viscosity,
- Linear partition isotherm,
- A Vapor pressure that is neither very low nor very high,
- Toxicity that is as low as possible

SOLVENT SYSTEM continue

- The choice of the mobile phase is depends upon the following factors:-

1. Nature of the substance to be separated
2. Nature of the stationary phase used
3. Mode of chromatography (Normal phase or reverse phase)
4. Separation to be achieved- Analytical or preparative.

Solvent system continue

- The organic solvent mixture of low polarity is used Highly polar solvents are avoided to minimize adsorption of any components of the solvent mixture. Use of water as a solvent is avoided as it may loosen the adhesion of a layer on a glass plate.

- Solvents with an increasing degree of polarity are used in liquid-solid or adsorption chromatography. The solvents listed in elutropic series are selected.

Storage of solvents

Storage of solvents is **unnecessary** if they are used in a TLC chamber immediately after they have been prepared. However, it is sometimes that certain solvent systems can be stored for several months. In this case, the best advice is to store them in a **dark bottle in a cool place**. The “daily quota” of a solvent system should also be kept cool in the summer, e.g. if laboratory temperatures exceed 25 °C. Care must be taken to adjust the temperature to room temperature before the development

Some type of solvents

1 *n-Heptane* 2 *n-Hexane* 3 *n-Pentane* 4 Cyclohexane 5 Toluene 6 Chloroform 7 Dichloromethane 8 Diisopropyl ether 9 *tert-Butanol* 10 Diethyl ether 11 Isobutanol 12 Acetonitrile 13 Isobutyl methyl ketone 14 2-Propanol 15 Ethyl acetate 16 1-Propanol 17 Ethylmethyl ketone 18 Acetone 19 Ethanol 20 1,4-Dioxan 21 Tetrahydrofuran 22 Methanol 23 Pyridine

- n-Hexane
- Cyclohexene
- Toluene
- Benzene
- Diethyl ether
- Chloroform
- Dichloromethane
- 1,2 dichloroethane
- Acetone
- Ethyl acetate
- Acetonitrile
- Propanol
- Methanol
- Acetic acid
- Water.

Increasing polarity

APPLICATION OF SAMPLE

- Sample solution in a non polar solvent is applied.
- The concentration of a sample or standard solution has to be minimum of a 1% solution of either standard or test sample is spotted using a capillary tube or micropipette.
- The area of application should be kept as small as possible for sharper and greater resolution.

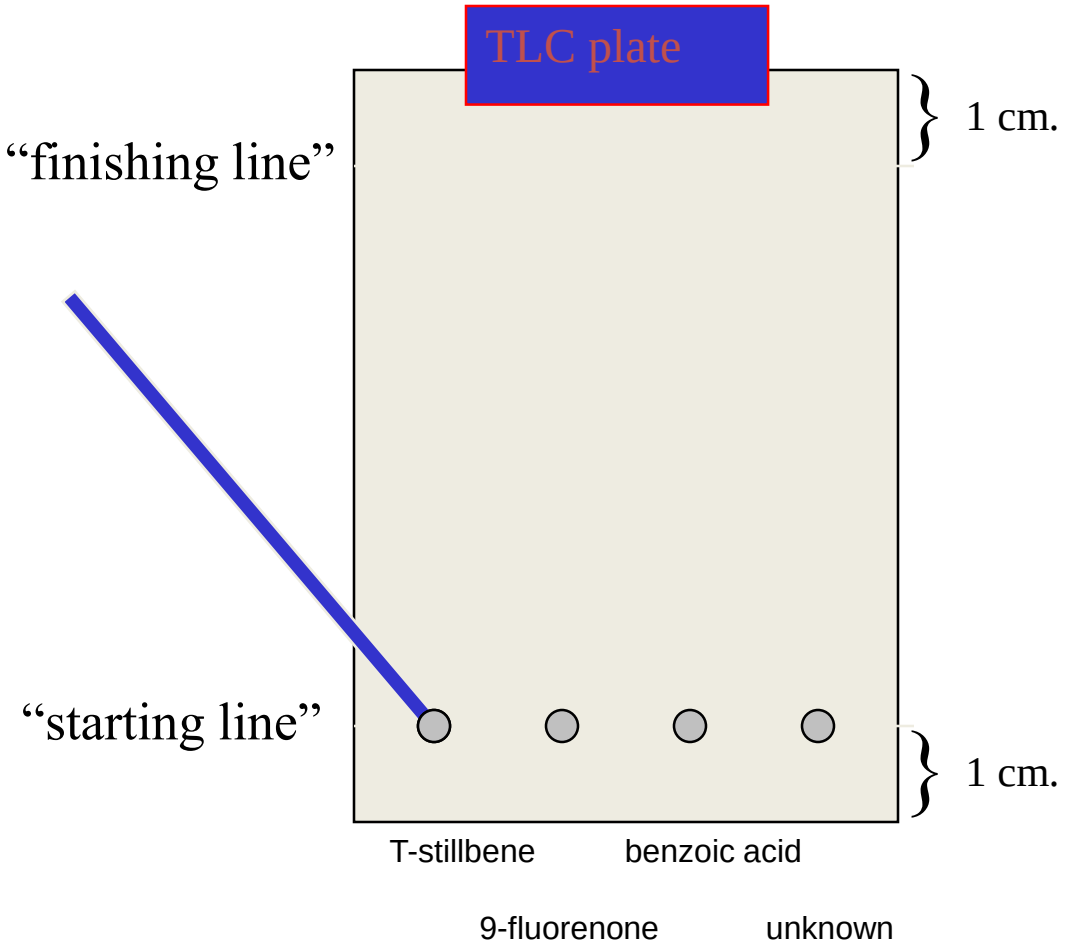
Sample Application (spotting)

Process

A. Draw “guide lines” lightly with pencil

B. Dissolve solid sample in CH_2Cl_2

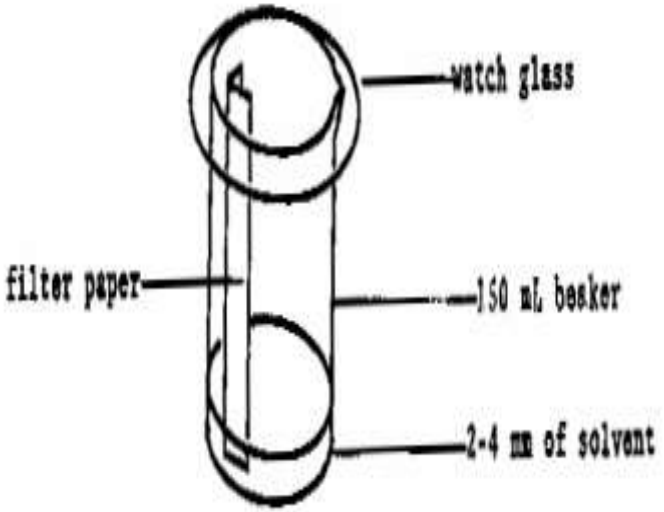
C. Use TLC capillary to transfer and spot dissolved sample



How to Run Thin Layer Chromatography

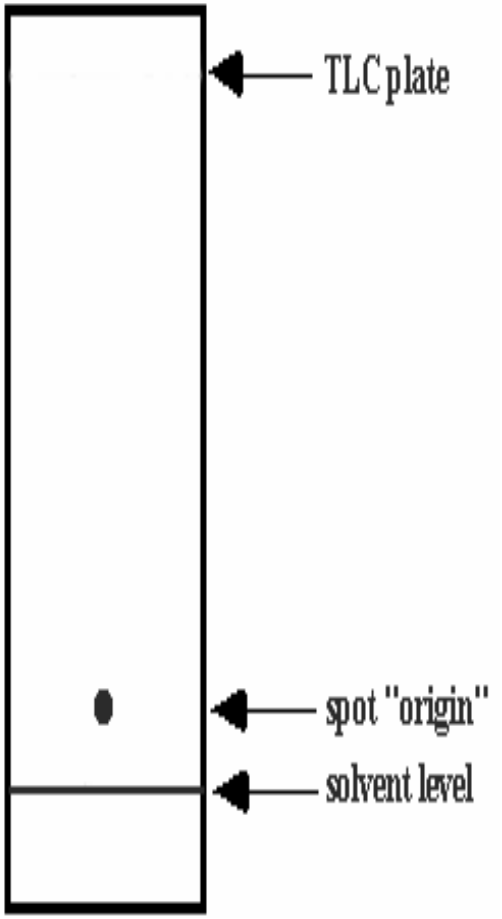
- Step 1: Prepare the developing container
- Step 2: Prepare the TLC plate
- Step 3: Spot the TLC plate
- Step 4: Develop the plate
- Step 5: Visualize the spots

Preparation of the developing container



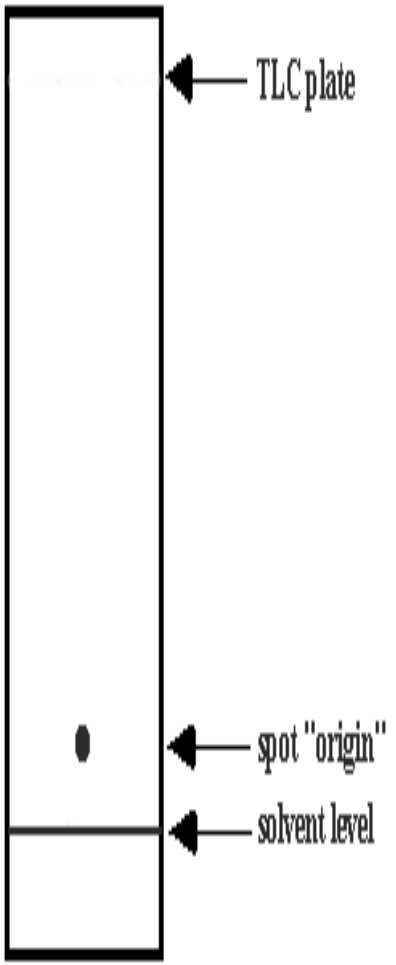
- It can be a specially designed chamber, a jar with a lid, or a beaker with a watch glass on the top
- Pour solvent into the chamber to a depth of just less than 0.5 cm.
- To aid in the saturation of the TLC chamber with solvent vapors, you can line part of the inside of the beaker with filter paper.
- Cover the beaker with a watch glass, swirl it gently.
- Allow it to stand while you prepare your TLC plate.

Preparation of the TLC plate



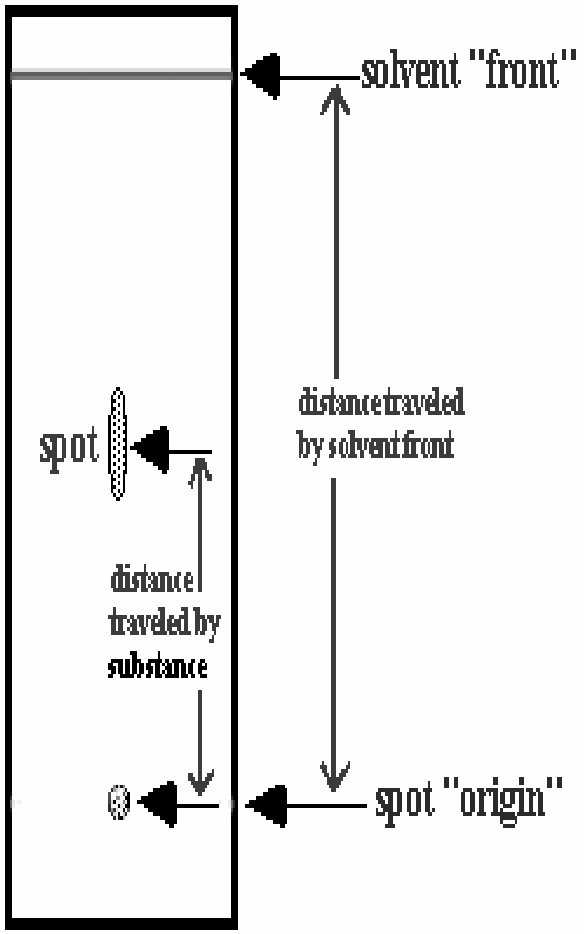
1. Pouring, Dipping, Spraying, Spreading
2. TLC plates used are purchased as 5 cm x 20 cm sheets. Each large sheet is cut horizontally into plates which are 5 cm tall by various widths;
3. Handle the plates carefully so that you do not disturb the coating of adsorbent or get them dirty.Measure 0.5 cm from the bottom of the plate.
4. Using a pencil, draw a line across the plate at the 0.5 cm mark. This is **the*origin***: the line on which you will spot the plate. Take care not to press so hard with the pencil that you disturb the adsorbent.
5. Under the line, mark lightly the samples you will spot on the plate, or mark numbers for time points. Leave enough space between the samples so that they do not run together; about 4 samples on a 5 cm wide plate is advised.

Spot the TLC plate



- Prepare 1% solution of drug dissolving in volatile solvents like hexanes, ethyl acetate, or methylene chloride.
- Dip the microcap or microcapillary into the solution and then **gently** touch the end of it onto the proper location on the TLC plate.
- Don't allow the spot to become too large - if necessary, you can touch it to the plate, lift it off and blow on the spot. If you repeat these steps, the wet area on the plate will stay small.
- This example plate has been spotted with three different quantities of the same solution and is ready to develop

Thin Layer Chromatography Column Development



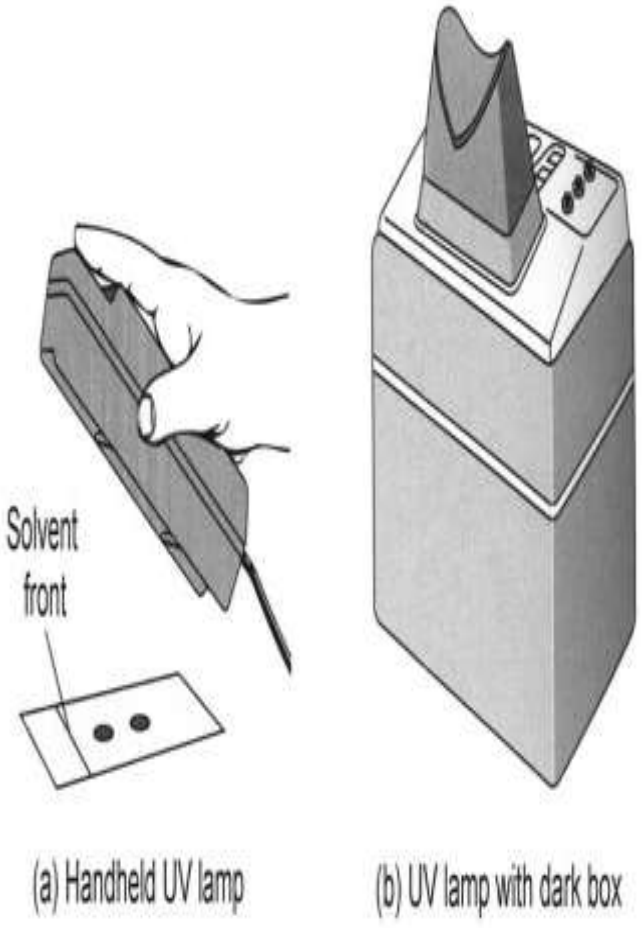
- Place the prepared TLC plate in the developing beaker, cover the beaker with the watch glass, and leave it undisturbed on your bench top.

The solvent will rise up the TLC plate by capillary action. Make sure the solvent does not cover the spot.

Allow the plate to develop until the solvent is about half a centimeter below the top of the plate.

Remove the plate from the beaker and immediately mark the solvent front with a pencil

Visualize the spots



- If there are any colored spots, circle them lightly with a pencil.
- Most samples are not colored and need to be visualized with a UV lamp.
- Hold a UV lamp over the plate and circle any spots you see.
- Make sure you are wearing your goggles and do not look directly into the lamp. Protect your skin by wearing gloves

Chromogenic reagents for visualizing thin-layer chromatograms

Reagent	Applications
iodine vapour	general organic, unsaturated compounds
phosphomolybdic acid	general organic
fluorescein/bromine	general organic
sulphuric acid	general organic (TLC only)
ninhydrin or isotin	amino acids
2,4-dinitrophenylhydrazine	ketones and aldehydes
H_2S water, diphenylcarbazide or	metals
rubeanic acid	metals
aniline phthalate	sugars
antimony trichloride	steroids, essential oils
chloroplatinic acid	alkaloids
bromothymol blue	lipids

General Review of preparation of materials

- The thin layer chromatography plates are commercial pre-prepared ones with a silica gel layer on a glass, plastic, or aluminum backing. Use the wide plates for spotting several compounds on the same plate. This allows for more precise comparison of the behavior of the compounds.

- The samples are spotted on the thin layer plates using fine capillaries drawn from melting point capillaries. You will need to draw several spotters.

- Samples for spotting are prepared by dissolving approximately 0.1 g (the amount on the tip of a spatula) of the compound in less than 0.5 mL of a solvent (ethyl acetate, dichloromethane, or ether work well).

General Review of preparation of materials

- When spotting samples on the TLC plates, it is a good idea to check if enough sample has been spotted on the plate. Allow the solvent to evaporate and then place the plate under a short wavelength ultraviolet lamp. A purple spot on a background of green should be clearly visible. If the spot is faint or no spot is apparent, more sample will have to be applied to the plate.

- The chromatograms are developed in a 150-mL beaker or jar containing the developing solvent. The beaker is covered with a small watch glass. A wick made from a folded strip of filter paper is used to keep the atmosphere in the beaker saturated with solvent vapor.

General Review of preparation of materials

- When the plates are removed from the developing solvent, the position of the solvent front is marked, and

- the solvent is allowed to evaporate. The positions of the spots are determined by placing the plates under a short wavelength ultraviolet lamp.

- The silica gel is mixed with an inorganic phosphor which fluoresces

  green in the UV light. Where there are compounds on the plates, the fluorescence is quenched and a dark purple spot appears

TLC Developing Chambers

- Ascending development,
- Descending development,
- Horizontal development.

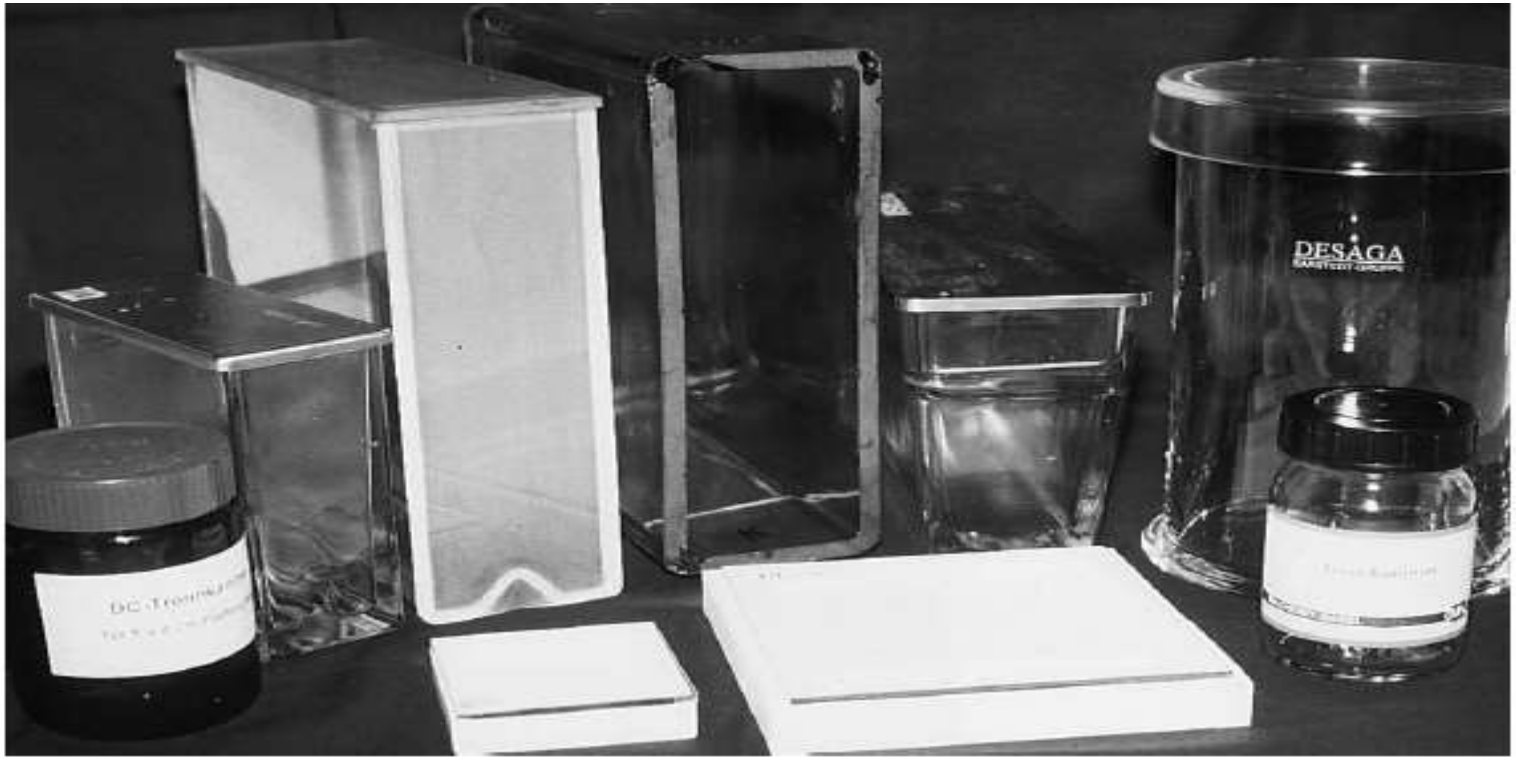


Figure 56. Selection of developing chambers for TLC

Back row from left to right:
Screw top jar (brown glass) for maximum plate size 5 × 8 cm
Double-trough 10 × 10 cm chamber with stainless steel lid (CAMAG)
Double-trough light-weight 20 × 20 cm chamber with glass lid (CAMAG)
Flat-bottomed 20 × 20 cm chamber placed on its side for drying
Chamber, 20 × 10 cm, with V-shaped bottom and stainless steel lid (DESAGA)
Cylindrical 10 × 20 cm developing chamber with glass lid (DESAGA)

Front row from left to right:
Horizontal 5 × 5 cm chamber (DESAGA), common name: "baby chamber"
Horizontal 10 × 10 cm chamber (DESAGA)

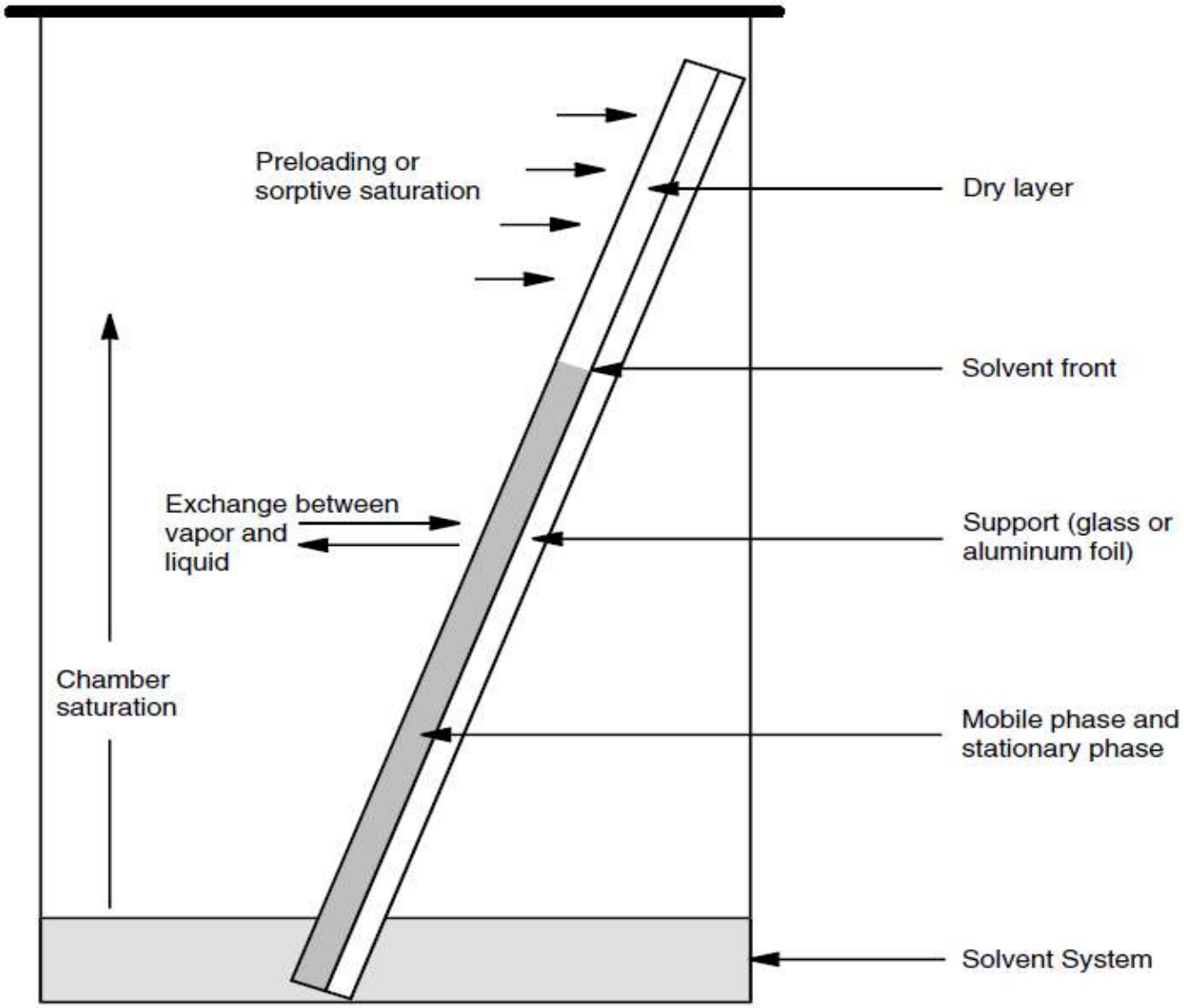
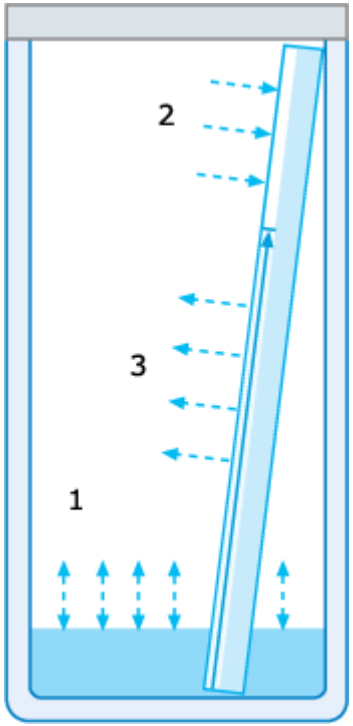


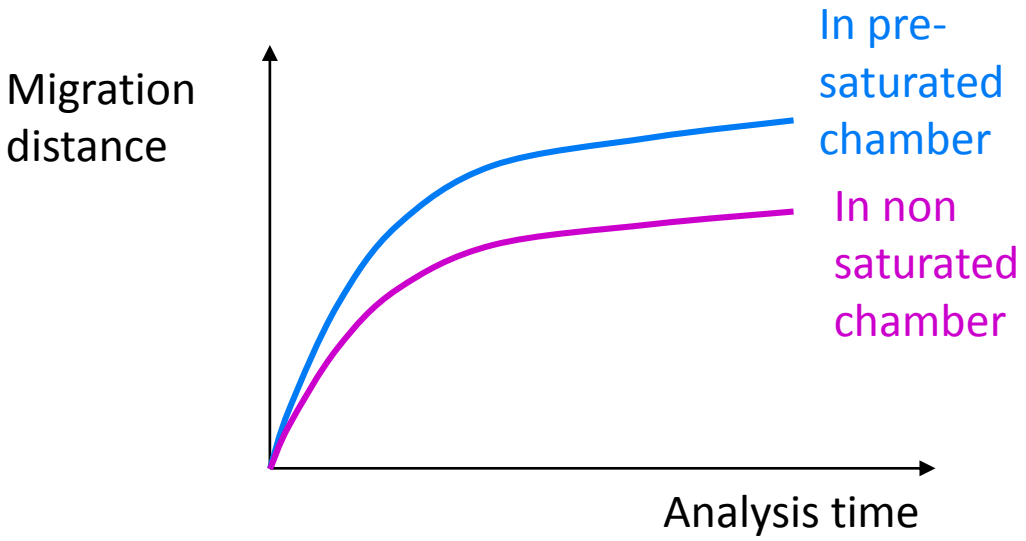
Figure 57. Terms used in connection with chamber saturation
Schematic representation of the exchange processes between the gas space, the solvent system reservoir and the TLC plate before and during development.

Vertical Development

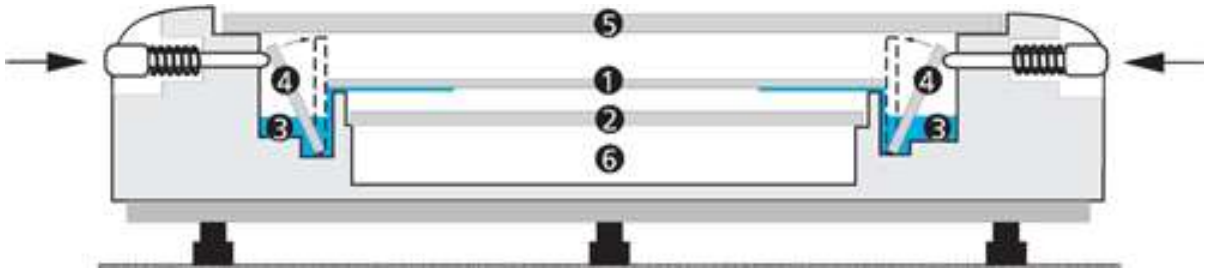
1. Solvent in Liquid-Vapour equilibrium
2. Solvent in Vapour adsorbs on the layer
3. Solvent migrating in the layer vaporizes



Effect of gravity



Horizontal Development



1. HPTLC plate (layer facing down)
2. glass plate for sandwich configuration
3. reservoir for developing solvent
4. glass strip
5. cover plate
6. conditioning tray

No effect of gravity
Migration speed is constant
Better resolutions can be achieved

Better control of the operating conditions (saturation, evaporation)
Possibility to develop both sides of the plate = Twice more samples

Development of Thin-Layer Chromatograms

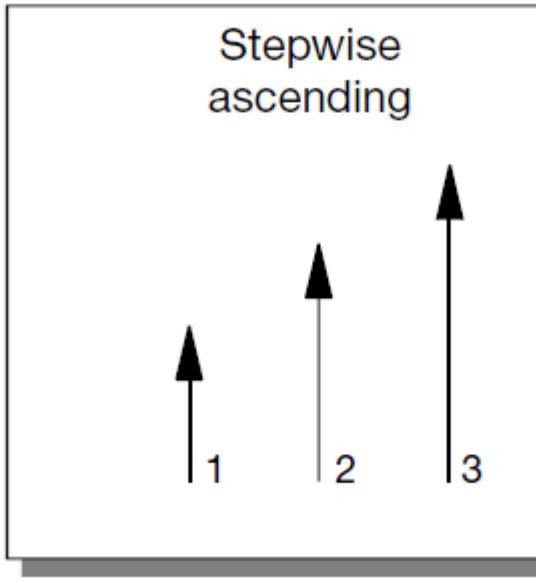
- **1. One-dimensional development**
- Single development
- – vertical
- – horizontal, in one direction
- – horizontal, in opposite directions
- – circular
- – anticircular
- **Multiple development**
- – separate runs over the same migration distance
- – stepwise, increasing
- – stepwise, decreasing
- – automated multiple development, stepwise with solvent gradient

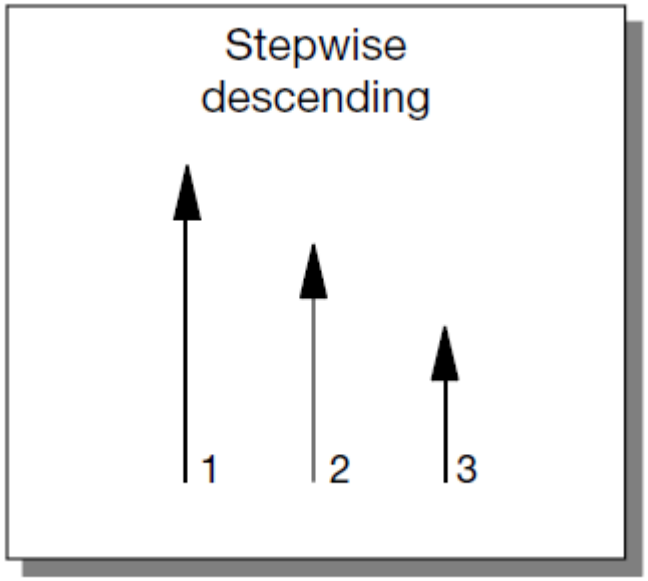
2. Two-dimensional development

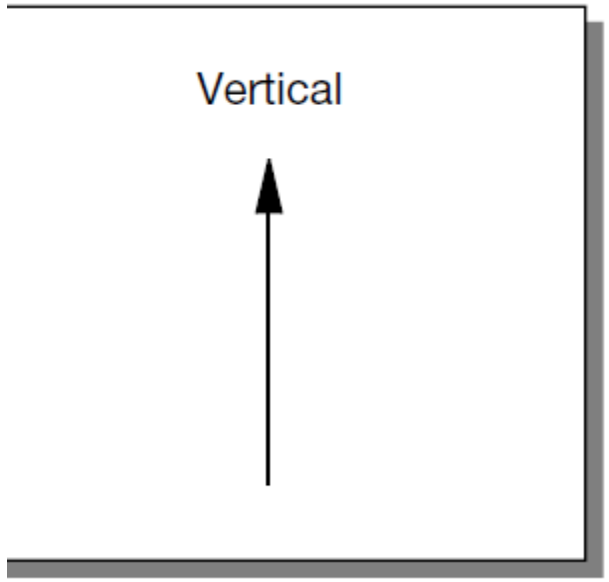
- Two dimensions, one solvent system
- Two dimensions, two solvent systems
- SRS (separation in 1st dimension chemical reaction separation in 2nd dimension)

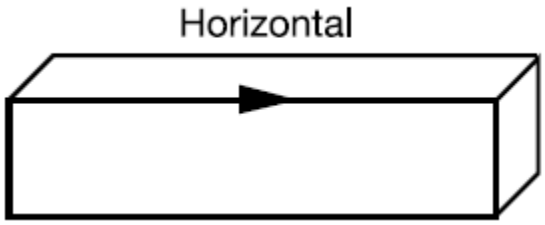
One-Dimensional Development

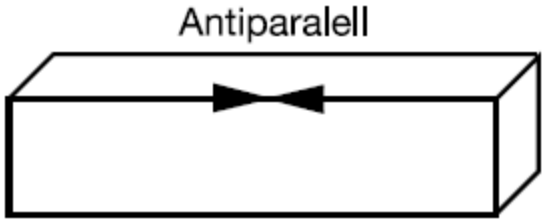
Most thin-layer chromatograms are produced in one dimension, and in fact even today it is very difficult to obtain quantitative results from plates developed in more than one dimension. All present-day commercially available TLC scanners therefore operate on the principle of a one-dimensional chromatographic lane.


Stepwise
ascending
1
2
3


Stepwise
descending
1
2
3

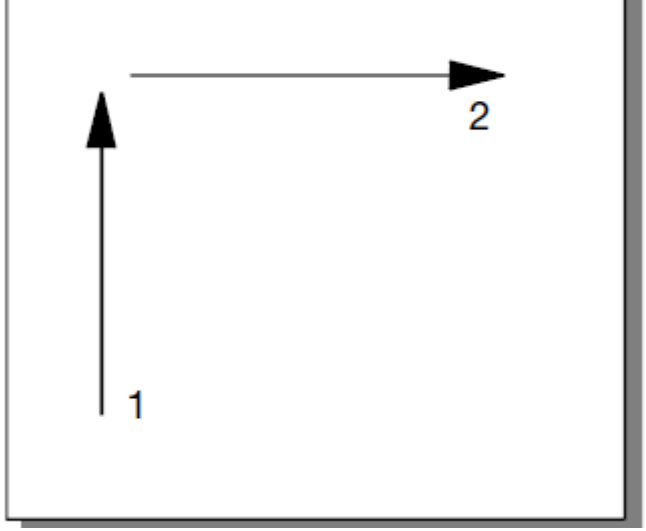

Vertical

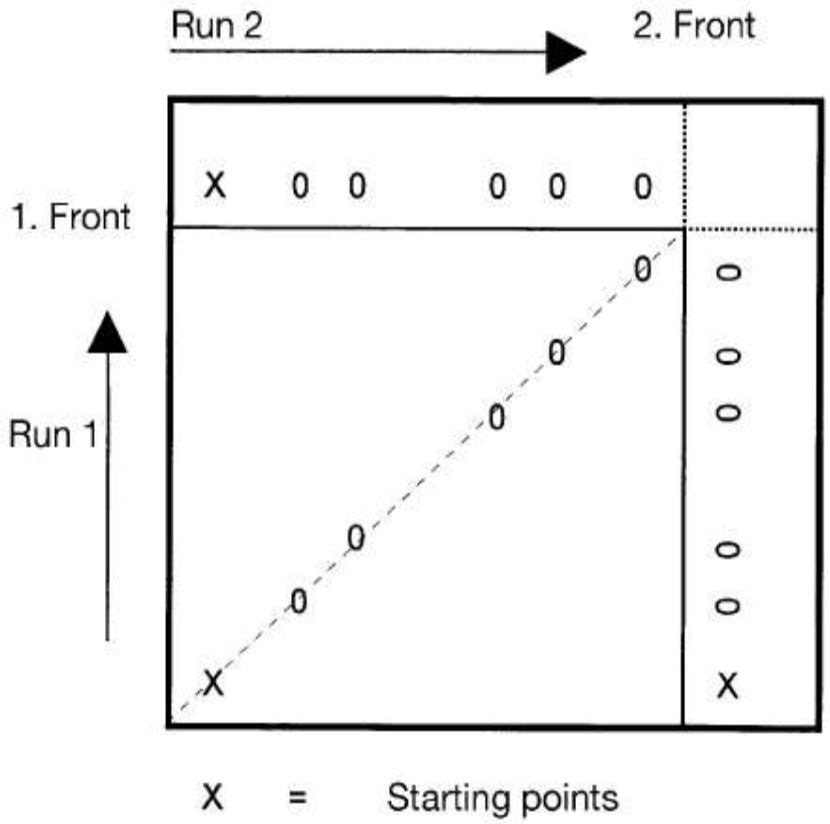

Horizontal


Antiparalell

Two-Dimensional Development

- More complete separation of sample components can be achieved by two-dimensional development. In this process, the plate is developed normally and following complete drying, it is turned 90^{o} and the development of the plate is continued. This second development is performed using a different mobile phase with very different selectivity (otherwise little further separation would result).





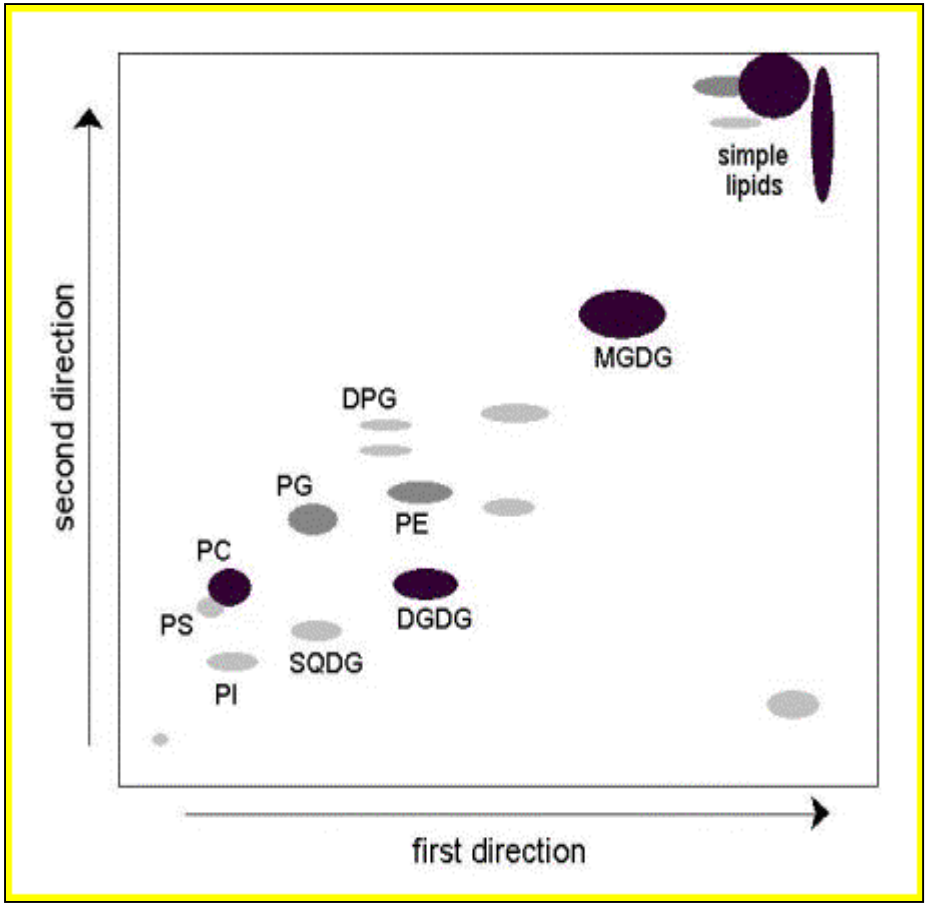
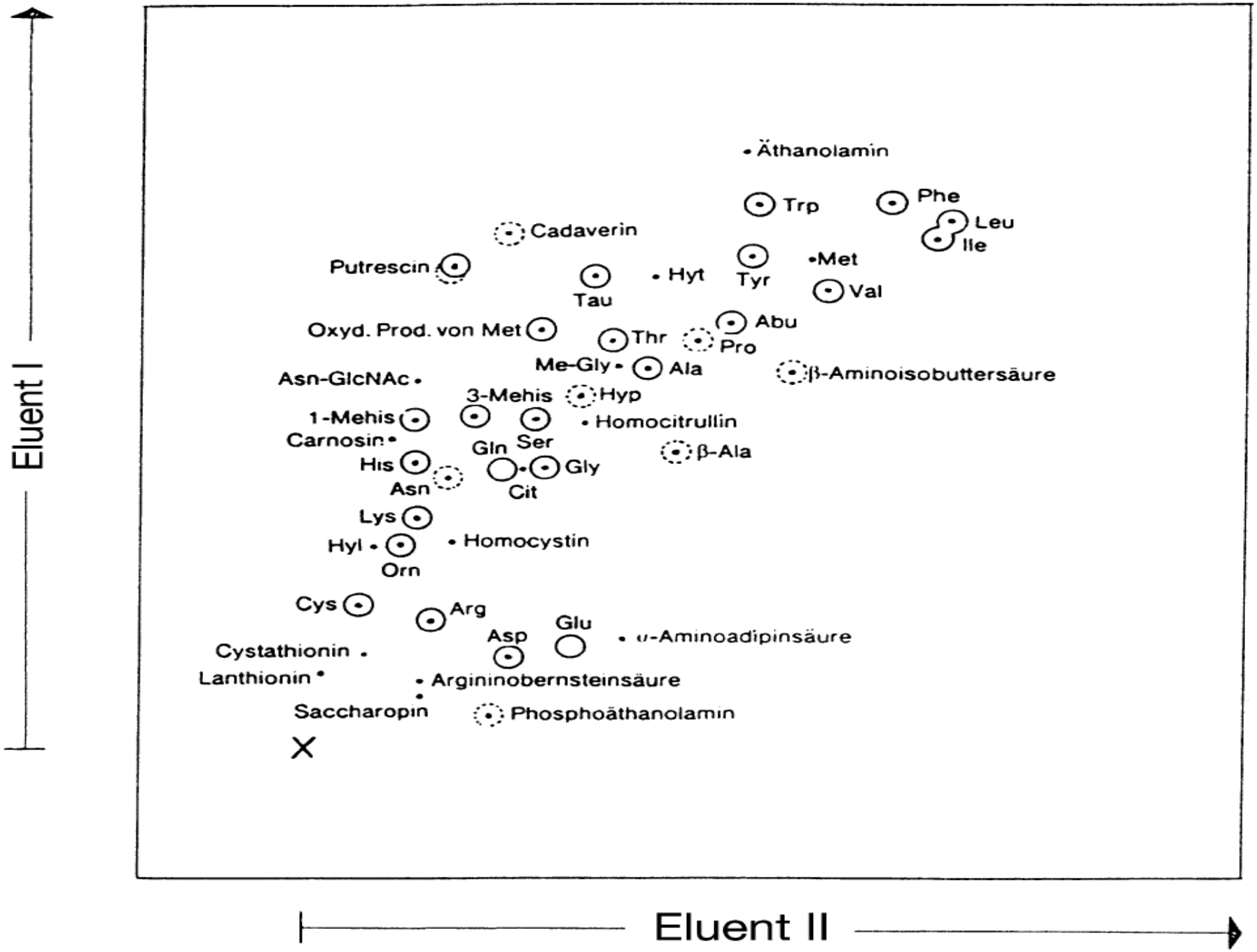


Figure 69. Schematic diagram of a 2D-TLC in which with the same solvent system was used for developing in both dimensions
The direction of flow in the two runs is indicated by arrows.


Eluent I
Äthanolamin
Trp
Phe
Leu
Ile
Cadaverin
Putrescin
Tau
Hyt
Tyr
Met
Val
Oxyd. Prod. von Met
Thr
Abu
Pro
Me-Gly
Ala
β-Aminoisobuttersäure
Asn-GlcNAc
3-Mehis
Hyp
1-Mehis
Homocitrullin
Carnosin
Gln
Ser
β-Ala
His
Gly
Asn
Cit
Lys
Hyl
Homocystin
Orn
Cys
Arg
Glu
Asp
ω-Aminoadipinsäure
Cystathionin
Lanthionin
Argininobernsteinsäure
Saccharopin
Phosphoäthanolamin
Eluent II

Above Figure. Position of the urine amino acids on a 20 × 20 cm TLC plate after a two-dimensional development and subsequent derivatization with the ninhydrin reagent

- x Start point
- ⊙ Position of substances present in the standard solution and not visible after the first heating process
- ◌ Position of substances present in the standard solution and in general only visible after the second heating process
- ○ Position of substances that become visible in most urine chromatograms but are not present in the standard solution for technical reasons
- • Position of substances that do not usually occur in urine and are also not present in the standard

Applications of TLC

- It is used for separation of all classes of natural products and is established as an analytical tool in modern pharmacopoeias.

  - E.g. Acids, alcohols, glycols, alkaloids, amines, macromolecules like amino acids, proteins and peptides, and antibiotics

  - for checking the purity of samples

  - as a purification process

  - examination of reaction

  - for identifying organic compounds

- Extensively used as an identification test and test for purity.
- As a Check on process – checking of distillation fractions and for checking the progress of molecular distillation.

Applications of TLC

- Applications of TLC for separation of Inorganic Ions – Used for separating cationic, anionic, purely covalent species and also some organic derivatives of the metals.

- Separation of Amino Acids- two dimensional thin – layer chromatography

- Separation of vitamins – vitamin E, Vitamin D3, vitamin A

- Application of TLC in quantitative analysis

HPTLC

INTRODUCTION

HPTLC is a sophisticated & automated form of TLC
Efficient separation in short time

Introduction

- HPTLC is a form of thin-layer chromatography (TLC) that provides superior separation power using optimized coating material, novel procedures for mobile-phase feeding, layer conditioning, and improved sample application.

- The basic difference between conventional TLC and HPTLC is only in particle and pore size of the sorbents.

- The principle of separation is similar that of TLC adsorption.

- It is very useful in quantitative and qualitative analysis of pharmaceuticals.

PRINCIPLE

Adsorption

Advantages of HPTLC Over Other Chromatographic Methods

1. In HPTLC, simultaneous processing of sample and standard – better analytical accuracy & precision
2. Lower analysis time & less cost per analysis
3. HPTLC is very simple
4. In HPTLC, the sample preparation is simple

5. Solvent used in HPTLC needs no prior treatment like filtration & degassing
6. In HPTLC, the M.P consumption for sample is extremely low
7. HPTLC allows the use of corrosive & UV absorbing M.P

Advantages of HPTLC

8. It promotes high separation efficiencies/ resolution of zones due to higher number of theoretical plates.
9. Shorter developing times or analysis time
10. Lower amounts of mobile phase / solvent consumption
11. Enormous flexibility
12. Parallel separation of many samples with minimal time requirement
13. Simplified sample preparation due to single use of the stationary phase.
14. Efficient data acquisition and processing

STEPS INVOLVED IN HPTLC

1.Sample preparation
2.Selection of chromatographic layer
3.Plates
4.Pre-washing
5.Conditioning
6.Sample application
7.Pre-conditioning
8.M.P

9. Chromatographic development
10. Detection of spots
11. Scanning & documentation

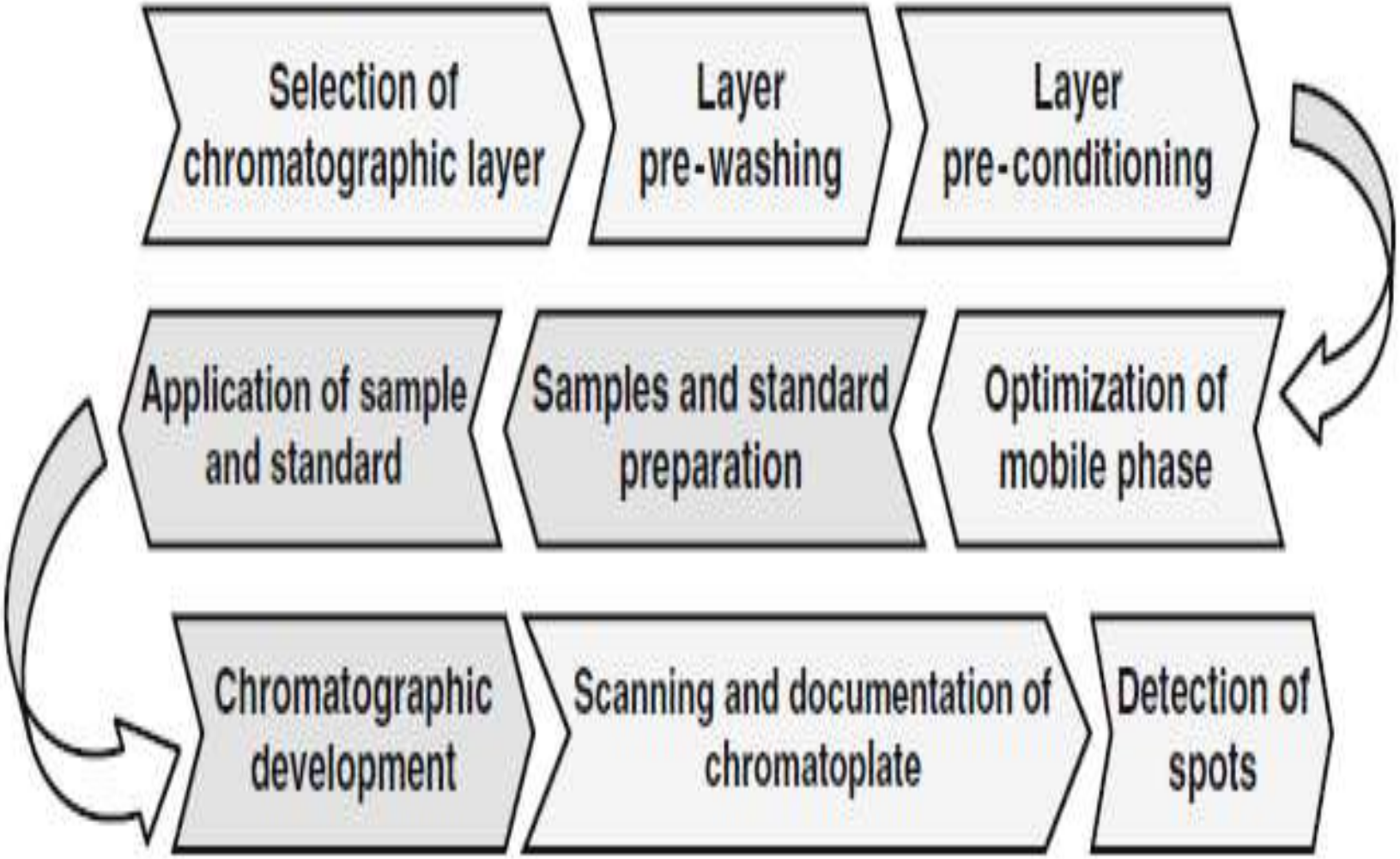


Fig. 1.3 Schematic procedure for HPTLC method development

HPTLC: Separation and Resolution

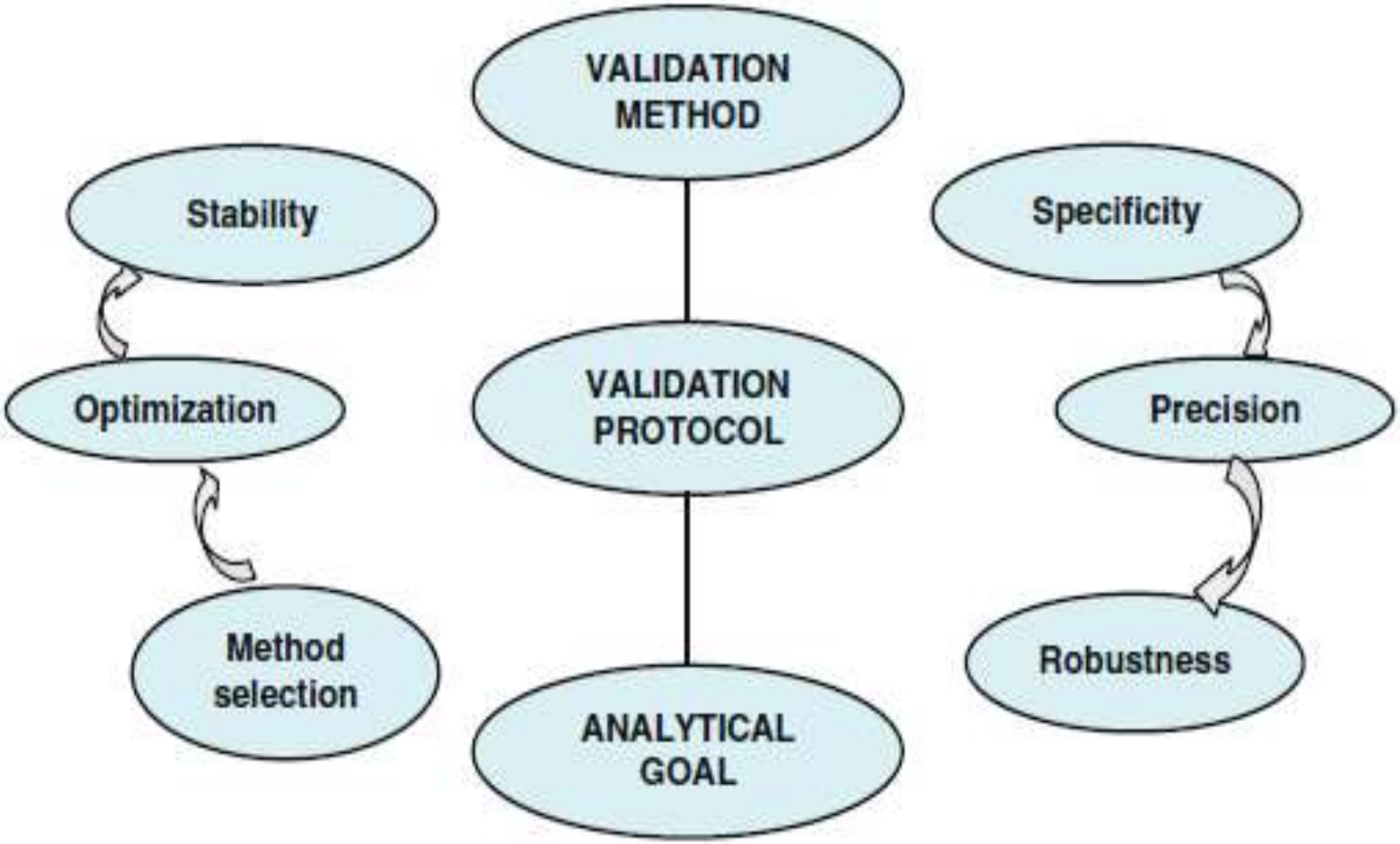
To which extent various components of a formulation are separated by a given HPTLC system is the important factor in quantitative analysis. It depends on the following factors:

- Type of stationary phase
- Type of precoated plates
- Layer thickness
- Binder in the layer
- Mobile phase
- Solvent purity

Above slide Continue

- 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

Validation process involved in HPTLC



Type of analytical procedures and required validation characteristics

Type of analytical procedure	Identification	Assay/in vitro release study/ dissolution study/content/potency	Testing for impurities	
Characteristics			Quantitative	Limit test
Linearity	−	+	+	−
Range	−	+	+	−
Specificity[a]	+	+	+	+
Accuracy	−	+	+	−
Precision				
Repeatability	−	+	+	−
Intermediate precision	−	+[b]	+[b]	−
Limit of detection	−	−	−[c]	+
Limit of quantification	−	−	+	−

+ Characteristic normally evaluated, − characteristic normally not evaluated

[a]Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure

[b]If reproducibility has been performed, intermediate precision is not needed

[c]Evaluated in certain conditions

Basic acceptance criteria for evaluation validation experiments-

(Ferenczi-Fodor et al. 2001; Patel et al. 2010)

Characteristics – parameter		Impurity testing Acceptance criteria	Assay
Linearity			
Residual plot		No trend	No trend
Correlation coefficient		$r \geq 0.99$	$r \geq 0.998$
Y-axis intercept		≤25%	≤2%
RSD residuals			≤1.5%
	Impurity level ≤0.5%	≤10%	
	Impurity level ≥0.5%	≤5%	
Range		From limit of quantification to 120% of the specified limit of impurity	80–120%
Precision			
Repeatability			
	Impurity level 0.1–0.2%	RSD ≤ 20%	RSD ≤ 2% ($n \geq 6$)
	Impurity level 0.2–0.5%	RSD ≤ 10%	
	Impurity level ≥0.5%	RSD ≤ 5%	
Intermediate precision		1.5 × RSD of repeatability	RSD ≤ 3% ($n \geq 6$)
Accuracy			RSD ≤ 3%
	Impurity level ≤0.5%	RSD ≤ 10% ($n = 3$)	
	Impurity level ≥ 0.5%	RSD ≤ 5%	
Limit of detection		RSD ≤ 10–20%	–
Limit of quantification		RSD ≤ 20–50%	–

SCHEMATIC PROCEDURE FOR HPTLC METHOD

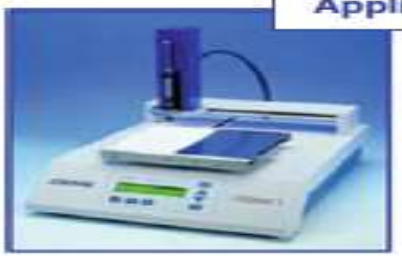
Sample and standard preparation

Selection of chromatographic layer

Layer pre-washing

Layer pre-conditioning

Linomat 5 Applicator



Application of sample and standard

Chromatographic development

Twin trough Chamber

Detection of spots, scanning and documentation

Scanner

Photo documentation with digital camera

Sample preparation

1.For normal phase chromatography using silica gel / alumina pre-coated plates, solvents – non polar

2.RP chromatography , usually polar solvents

Selection of Chromatographic layer

» Depends on the nature of material to be separated

Commonly used materials are Silica gel 60F, Alumina, Cellulose etc

Pre-washing

- to remove water vapors
- volatile impurities

Which might get trapped in the plates

To avoid this, plates are cleaned by using methanol as solvent by ascending or descending etc.

Conditioning

Plates activated by placing them in an oven at 120°C for 15 to 20 minutes.

Sample Application

Application of 1.0 - 5µl for HPTLC

Application carried out by Linomat applicator on the plates which give uniform, safe & std. results

Sample Application

❖ Usual concentration of applied samples 0.1 to 1 µg / µl for qualitative Analysis and quantity may vary in quantization based on UV absorption 1 to 5 µl for spot and 10 µL for band application.

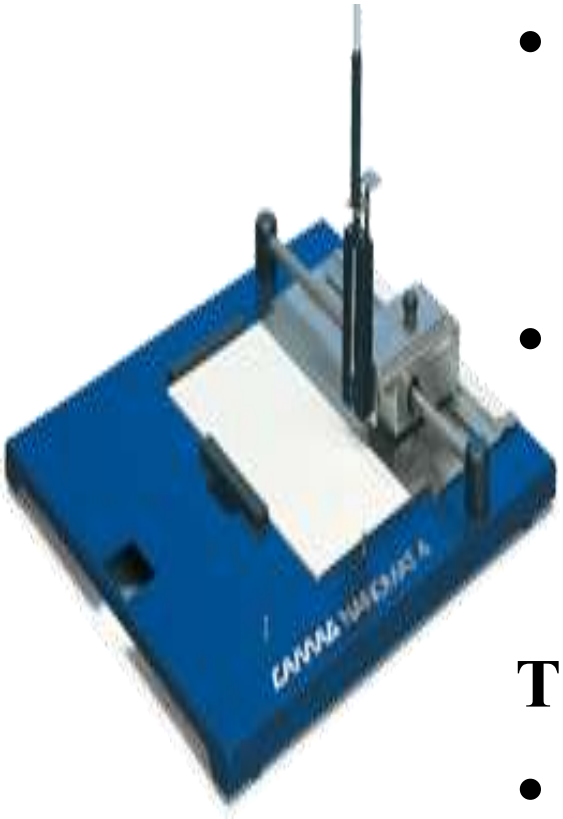
- MANUAL , SEMI-AUTOMATIC , AUTOMATIC APPLICATION

  Manual with calibrated capillaries

  Semi and auto-application through applicators

- Applicators use spray on or touch and deliver technique for application.

Manual Sample Applicator



- The Nanomat serves for easy application of samples in the form of spots onto TLC and HPTLC layers .
- The actual sample dosage performed with disposable capillary pipettes , which are precisely guided by the capillary holder.

The nanomat is suitable for

- Conventional TLC plates including self-coated Plates up to 20 × 20cm
- HPTLC plates 10 × 10 cm and 20 × 10 cm
- TLC and HPTLC sheets up to 20 × 20 cm

Semi automatic sample applicator

- The instrument is suitable for routine use for medium sample throughout . In contrast to the Automatic TLC sampler , changing the sample the Linomat requires presence of an operator.
- With the linomat , samples are sprayed onto the chromatographic layer in the form of narrow bands.
- During the spraying the solvent of the sample evaporates almost entirely concentrating the sample into a narrow band of selectable length.

Automatic Sample Applicator

- Samples are either applied as spots through contact transfer (0.1-5 micro lit) or as bands or rectangles (0.5->50 micro lit) using the spray on techniques.

- Application in the form of rectangles allow precise applications of large volume with out damaging the layer.

- ATS allows over spotting.

Sample Application parameter on HPTLC plate

Parameter	HPTLC
Distance from lower edge of plate for use in TTC	8 mm
Distance from lower edge of plate for use in horizontal development chamber (HDC)	5 mm
x-position of first track	15 mm
Minimum space between bands/spots	2 mm
Maximum diameter of application spot	5 mm
Band length	8 mm
Maximum number of tracks on a 10 × 10 cm plate	7
Maximum number of tracks on a 20 × 10 cm plate	16

Chromatographic development

Ascending, descending, horizontal, continuous, gradient, multidimensional…

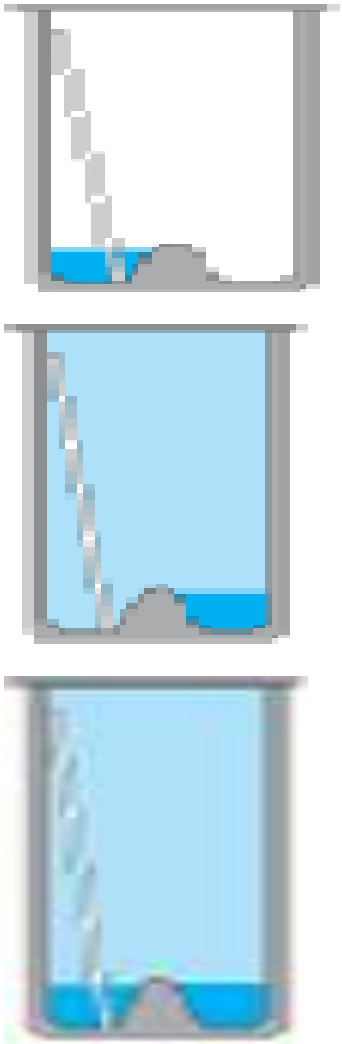
HPTLC – migration distance of 5-6mm is sufficient, after development, plates removed & dried.

Common problems encountered during chro. Development are as follows…

1. Tailing: due to the presence of traces of impurities, this can be reduced by buffering the M.P

2.DIFFUSION: This is seen as zones on chromatographic plates. This may arise due to non-uniformity of M.P

DEVELOPING CHAMBER - Twin trough chamber



- **Low solvent consumption:** 20 mL of solvent is sufficient for the development of a 20x20cm plate.

  This not only saves solvent , but also reduces the waste disposal problem

- **Reproducible pre –equilibrium with Solvent vapor:** For pre-equilibration, the TLC plate is placed in the empty trough opposite the trough which contains the pre-conditioning solvent. Equilibration can be performed with any liquid and for any period of time.

- **Start of development** : It is started only when developing solvent is introduced into the trough with the plate.

Automatic developing chamber (ADC)

- In the ADC this step is fully automatic and independent of environmental effects.

- The activity and pre-conditioning of the layer , chamber saturation developing distance and final drying can be pre-set and automatically monitored by ADC.

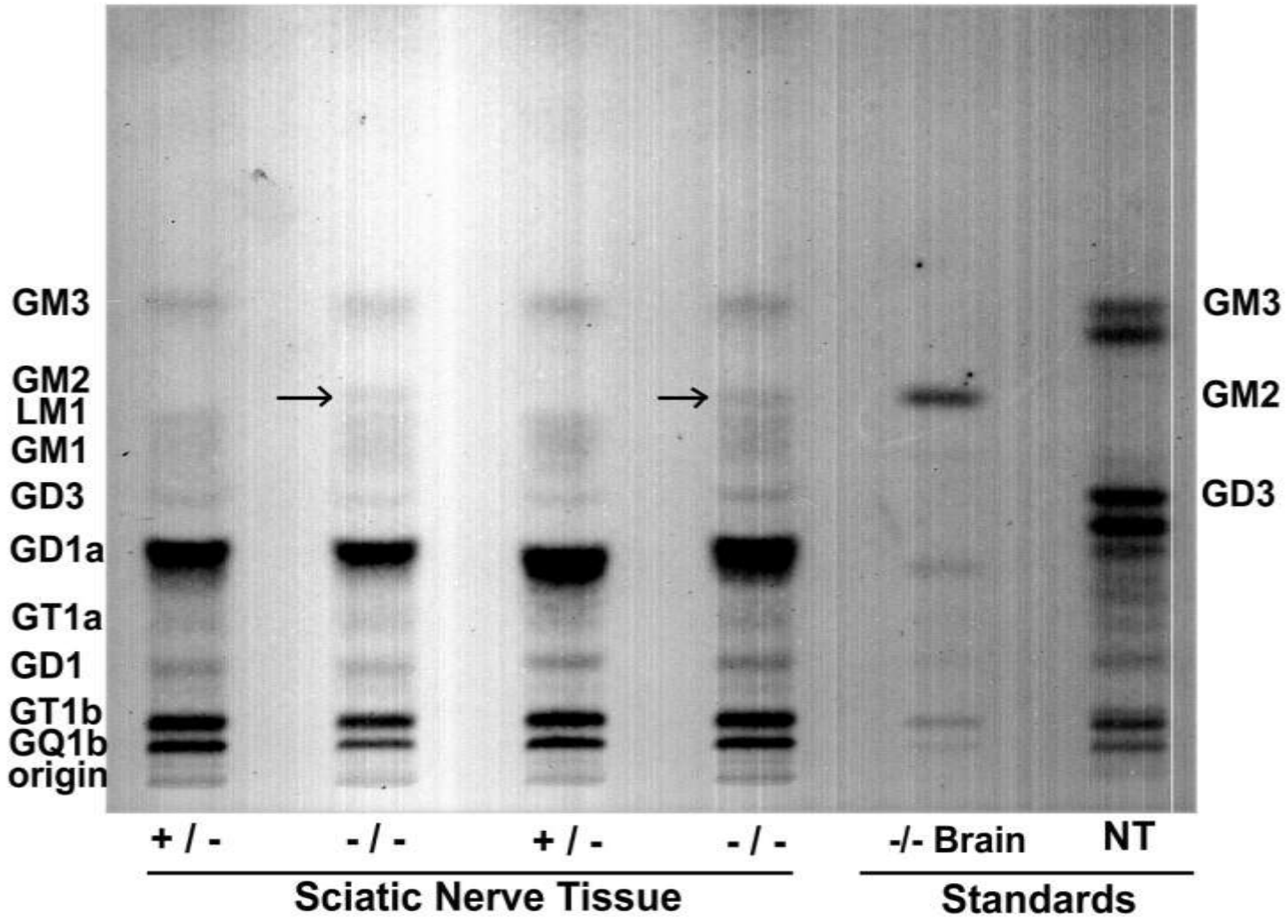
DETECTION OF SPOTS

Detection can be done by iodine vapor in iodine chamber. Visual inspection at 254nm of UV region in UV cabinet

Scanning & Documentation

1.HPTLC plates are scanned at selected UV regions WL by the instrument & the detected spots are seen on computer in the form of peaks.

2.The scanner converts band into peaks & peak height or area is related to the concentration of the substance on the spot.


GM3
GM2
LM1
GM1
GD3
GD1a
GT1a
GD1
GT1b
GQ1b
origin
GM3
GM2
GD3
+ / -
- / -
+ / -
- / -
-/- Brain
NT
Sciatic Nerve Tissue
Standards

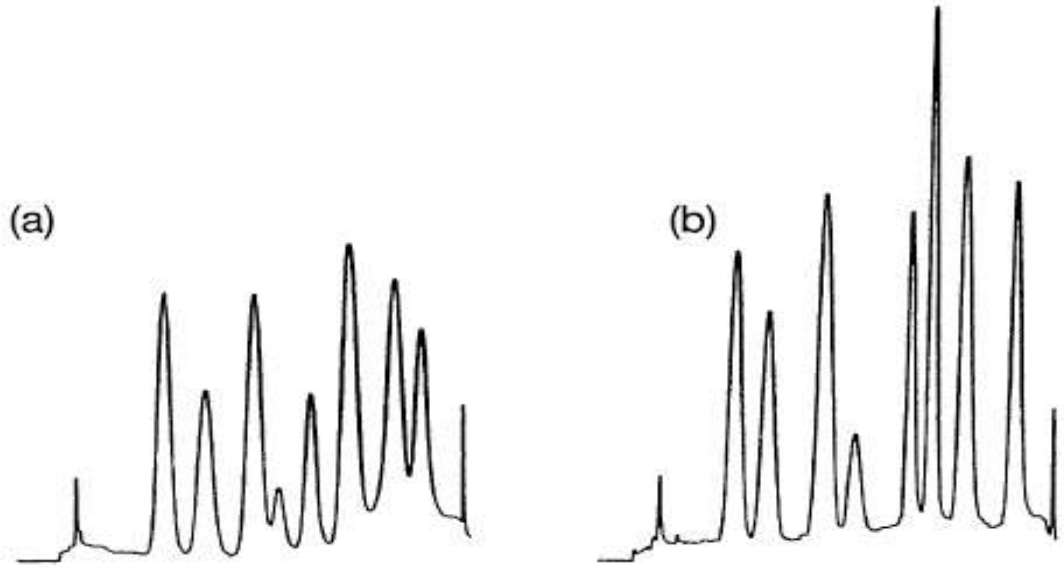
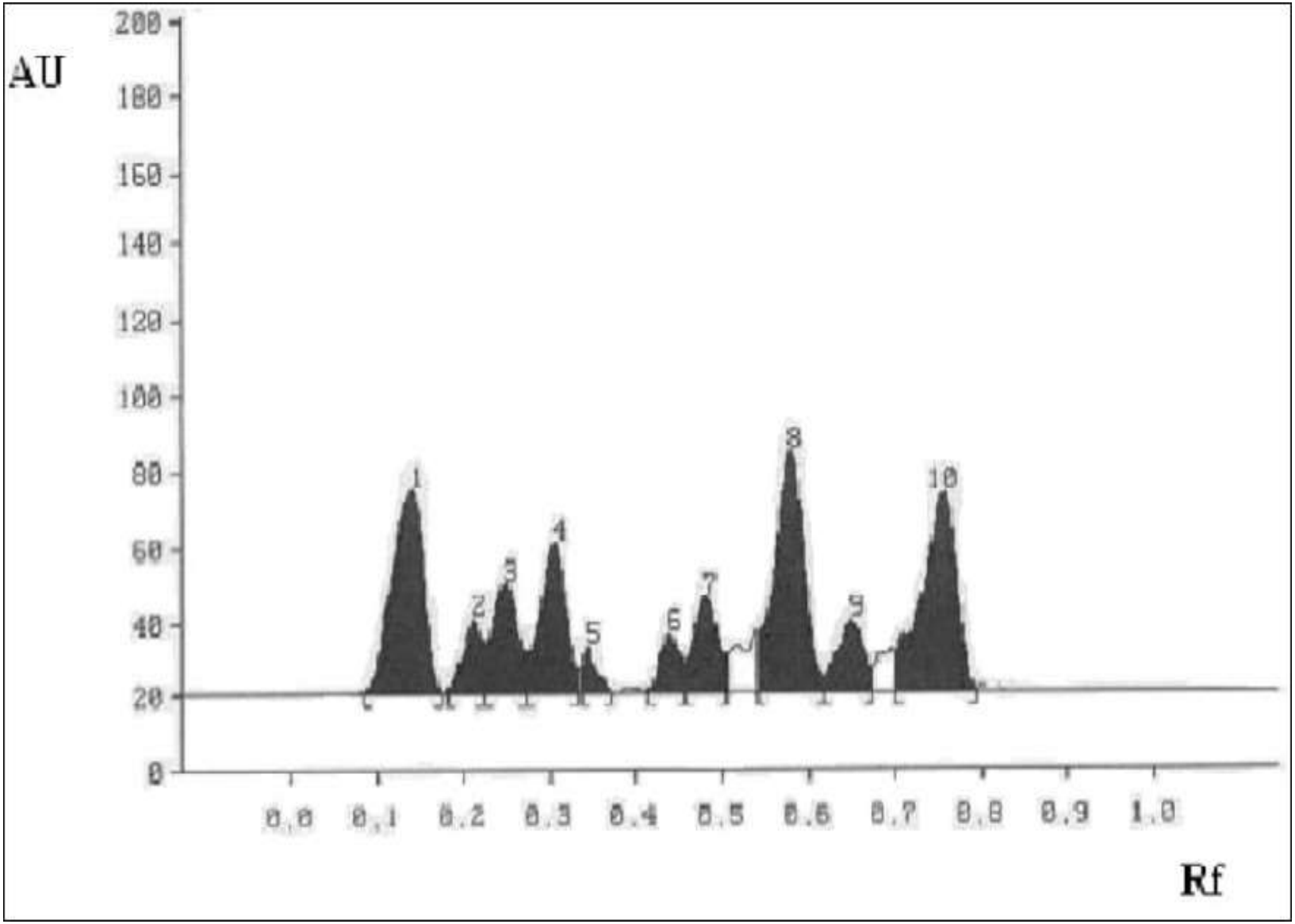
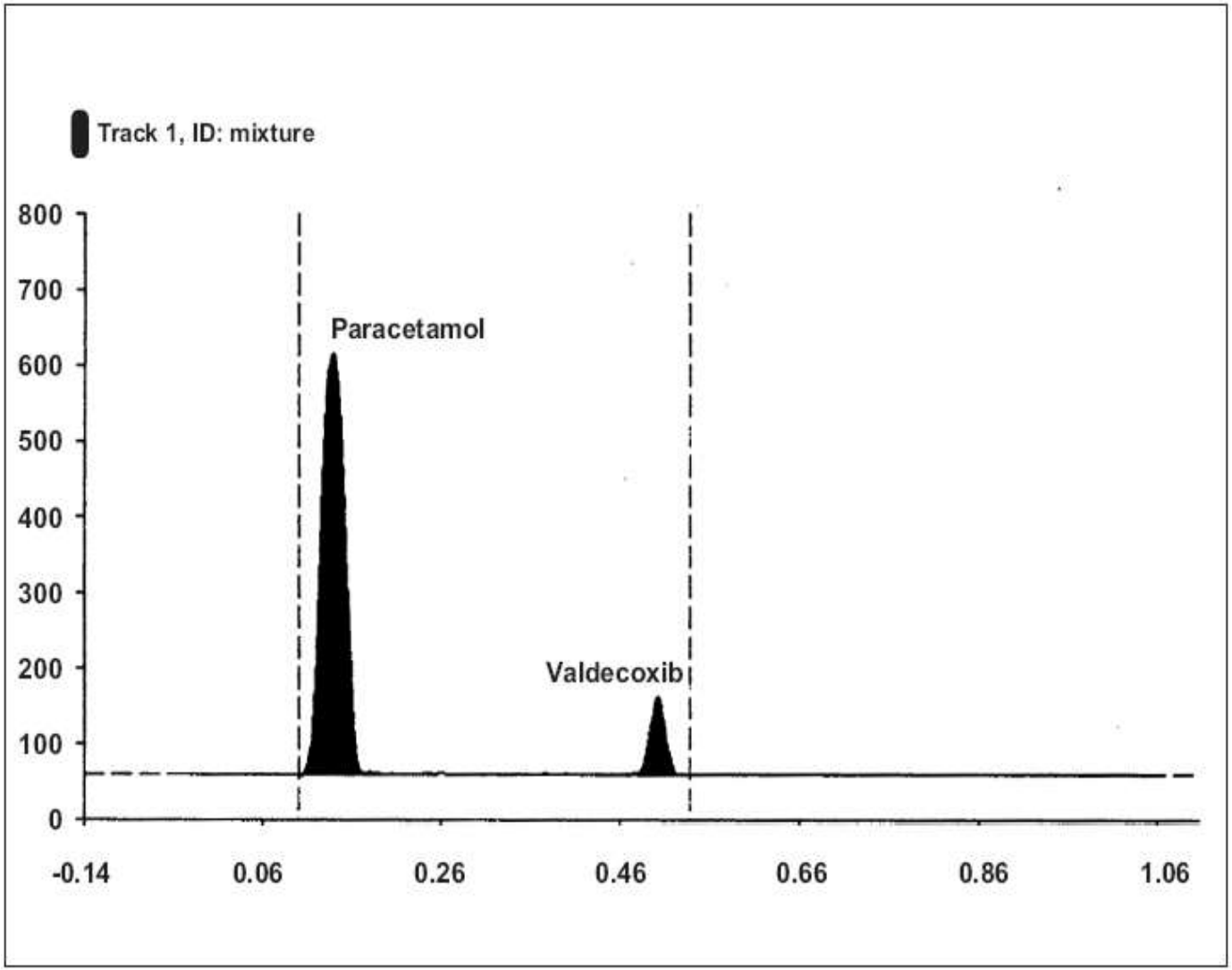


Figure 12. Comparison of separation efficiencies of HPTLC silica gel 60 F_{254} and HPTLC LiChrospher® Si 60 F_{254s} precoated plates of one manufacturer
(a) HPTLC silica gel 60 F_{254} GLP precoated plate (Merck Article No. 1.05613)
(b) HPTLC LiChrospher® Si 60 F_{254s} precoated plate (Merck Article No. 1.15445)

Detection: Direct optical evaluation under 254-nm UV light using the TLC-Scanner II (CAMAG)
The scans show the following pesticides from *left to right:*
Hexazin, Metoxuron, Monuron, Aldicarb, Azinphos methyl, Prometryn, Pyridat, Trifluralin
Sample volume: 50 nl, normal chamber without chamber saturation, solvent system: petroleum ether (40–60 °C) + acetonitrile (70 + 30 v/v), migration distance: 7 cm.


AU
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf


Track 1, ID: mixture
800
700
600
500
400
300
200
100
0
Paracetamol
Valdecoxib
-0.14
0.06
0.26
0.46
0.66
0.86
1.06

Application of HPTLC Seperation

- Multidimensional and multimodal seperation by HPTLC in photochemistry
- Stability-indicating HPTLC determination of imatinib mesylate in bulk drug and pharmaceutical dosage
- A Quality control for authentication of herbal photochemicals
- Herbal drug quantification
- Determination of artemisinin and its derivatives in bulk pharmaceutical dosages
- Biomedical application.

Comparison between HPTLC and TLC on the basis of parameters

Parameters	HPTLC	TLC
Technique	Automated/instrumental	Manual
Mean particle size	5–6 µm	10–12 µm
Layer thickness	100 µm	250 µm
Plate height	12 µm	30 µm
Efficiency	High due to smaller particle size generated	Less
Separations	3–5 cm	10–15 cm
Analysis time	Shorter migration distance and the analysis time is greatly reduced	Slower
Solid support	Wide choice of stationary phases like silica gel for normal phase and C8, C18 for reversed-phase modes	Silica gel, alumina, and Kiesulguhr
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting
Sample volume	0.1–0.5 µl	1–5 µl
Starting spot's diameter	1–1.5 mm	3–6 mm
Separated spot's diameter	2–5 mm	6–15 mm
Sample tracks per plate	≤36 (72)	≤10
Scanning	Use of UV/visible/fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible
Separation time	3–20 min	20–200 min
Detection limits (absorption)	100–500 pg	1–5 ng
Detection limits (fluorescence)	5–10 pg	50–100 pg

Features of HPLC & HPTLC

Feature	HPLC	HPTLC
Stationary phase	Liquid/solid	Solid
Mobile phase	Liquid	Liquid
Conditioning phase	None	Gas
Samples should be		Nonvolatile
Results	By detector	Detector + eyes
Analysis	On-line	Off-line
Resolution	Very high	Moderate to high
Chromatography system	Closed	Open
Separating medium	Tubular column	Planar layer (plate)
Analysis in parallel	No.	Yes.
	Only one at a time	Up to 100 samples
High pressure required?	Yes	No
Time per sample	2–60 min	1–3 min
Data obtained from chromatography	Little to very high (detector dependent)	High to very high (detector dependent)
Post-chromatography derivatization	Limited possibilities Cumbersome	Simple, possible for every sample, gives additional information
Fraction collection/micro-preparative chromatography	Requires prep. scale chromatograph and fraction collector	Simple, no special requirements
Sensitivity	High to ultra-high	Moderate to ultrahigh
Fluorescence data	Possible, optional	Possible, built-in
Abs. spectra for identification	Yes (PDA)	Yes
Detectors	UV, Fluor, Electrochem light scatter, MS, etc.	UV–Vis, bioluminescence, MS
Chromatogram image documentation	No	Yes, at 254 and 366 nm and visible
Sample cleanup	Thorough column reusable	Not so imp Layer disposable
Chromatographic fingerprint	Yes, but limited	Yes, comprehensive
Cost per analysis	Very high	Low
Eqpt. maintenance	Very high	Low
Analyst's skills required	High to very high	Low (TLC) to high (HPTLC)