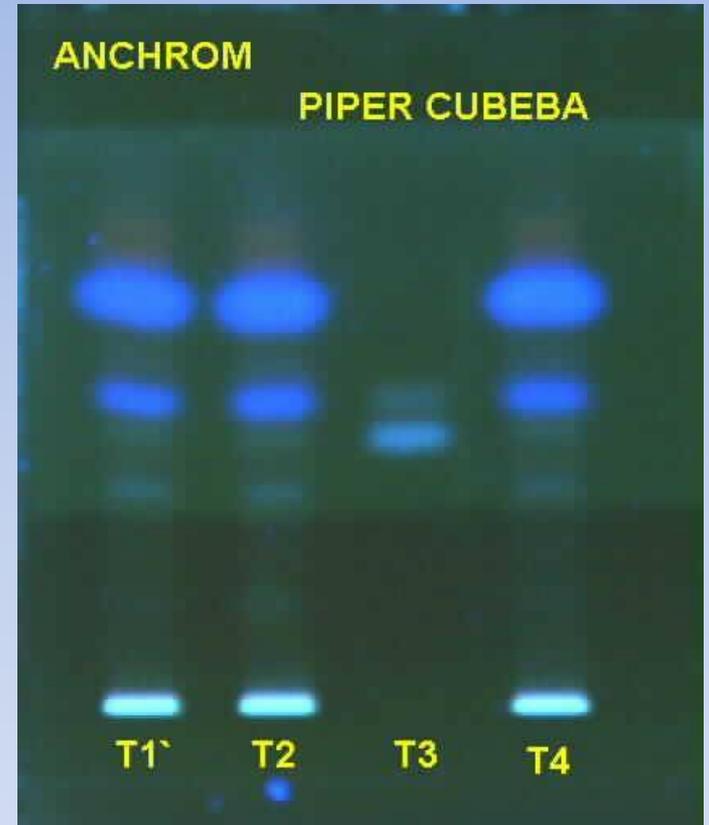


# HPTLC -Theory and Instrumentation

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# Introduction

The term chromatography means “ to write in colour ” in greek and was first introduced by the Russian botanist “ Michel Tswett ” described his results by saying that the solvents according to the adsorption sequences are resolved into variously colored zones ”. Such a preparation is termed as chromatogram and the corresponding method is the chromatographic method

## PRINCIPLE

*"The separation of the components of a mixture is due to their different affinities for a stationary phase such as a solid or a liquid and their differential solubility in a moving phase such as a liquid or gas"*

# What is Chromatography?

- The enthusiast could define it as *“the only method for obtaining chemically pure compounds”*.
- The cynic could say *“the best method for a young would-be –scientist to publish a large number of papers rapidly”*.

# *But what is chromatography?*

We cannot state a single definition for chromatography because of its various techniques and applications. There are many possible definitions for chromatography:

*“It is a method of analysis in which the flow of solvent or gas (mobile phase) promotes the separation of substances by differential migration in a porous absorptive medium”.*



- **Chromatography comprises a group of methods for separating molecular mixtures that depends on the differential affinities of the solutes between two immiscible phases.**
- **One of the phases is a fixed bed of large surface area while the other is fluid, which moves through, or over the surface of, the fixed phase.**

- The components of the mixture must be of molecular dimensions, which requires that they be in solution or in the vapor state.
- The relative affinity of the solute of each of the phases must be reversible to ensure that mass transfer occurs during the chromatographic separation.

- The fixed phase is called The stationary phase, and other is termed The mobile phase.
- The stationary phase may be a porous or finely divided solid, or a liquid that has been coated in a thin layer on an inert supporting material.

- It is necessary that the stationary phase particles be as small as possible in order to provide a large surface area so that sorption and desorption of the solutes will occur frequently.
- The mobile phase may be a pure liquid or a mixture of solutions (e.g. buffers) or it may be a gas (pure or a homogenous mixture).

- Chromatographic methods can be classified according to the nature of the stationary and mobile phases.
- If the stationary phase is solid, the process is called **Adsorption chromatography**, where as if the stationary phase is a liquid, it is termed **Partition chromatography**.

- In adsorption chromatography, the mobile phase containing the dissolved solutes passes over the surface of the stationary phase.
- Retention of the components and their consequent separation depends on the ability of the atoms on the surface to remove the solutes from the mobile phase and absorb them temporarily by means of electrostatic forces.

- If the mobile phase is a liquid . the process is called liquid solid chromatography(LSC) but when the mobile phase is a gas the method is called gas solid chromatography(GSC).

- In partition chromatography, an inert solid material such as silica gel, serves to support a thin layer of liquid, which is the effective stationary phase.
- As the mobile phase containing the solute passes in close proximity to this liquid phase, retention and separation occur due to the relative solubility of the analytes in the two fluids as determined by their partition coefficients.

- If the mobile phase is a liquid this type of partition chromatography is called Liquid Liquid chromatography (LLC) and if the mobile phase is a gas the process is termed gas-liquid chromatography (GLC).
- Two other modes of chromatography in which the stationary phase is a solid are classified differently from LSC and GSC because of the unique nature of their separation processes.

- In ion exchange chromatography the stationary phase consists of polymeric matrix onto the surface of which ionic functional groups, e.g. carboxylic acid or quaternary amines, have been chemically bonded.
- As the mobile phase passes over this surface, ionic solutes are retained by forming electrostatic chemical bonds with the functional groups.
- The mobile phase used in ion-exchange chromatography is always liquid.

- In size exclusion chromatography, the stationary phase is a polymeric substance containing numerous pores of molecular dimensions.
- Solutes whose molecular size is sufficiently small leave the mobile phase to diffuse into the pores.
- Larger molecules which will not fit in to the pores remain in the mobile phase and are not retained.

- This method is most suited to the separation of mixtures in which the solutes vary suited to the separation of mixtures in which solutes vary considerably in molecular size.
- The mobile phase in size exclusion chromatography may be either liquid or gaseous.

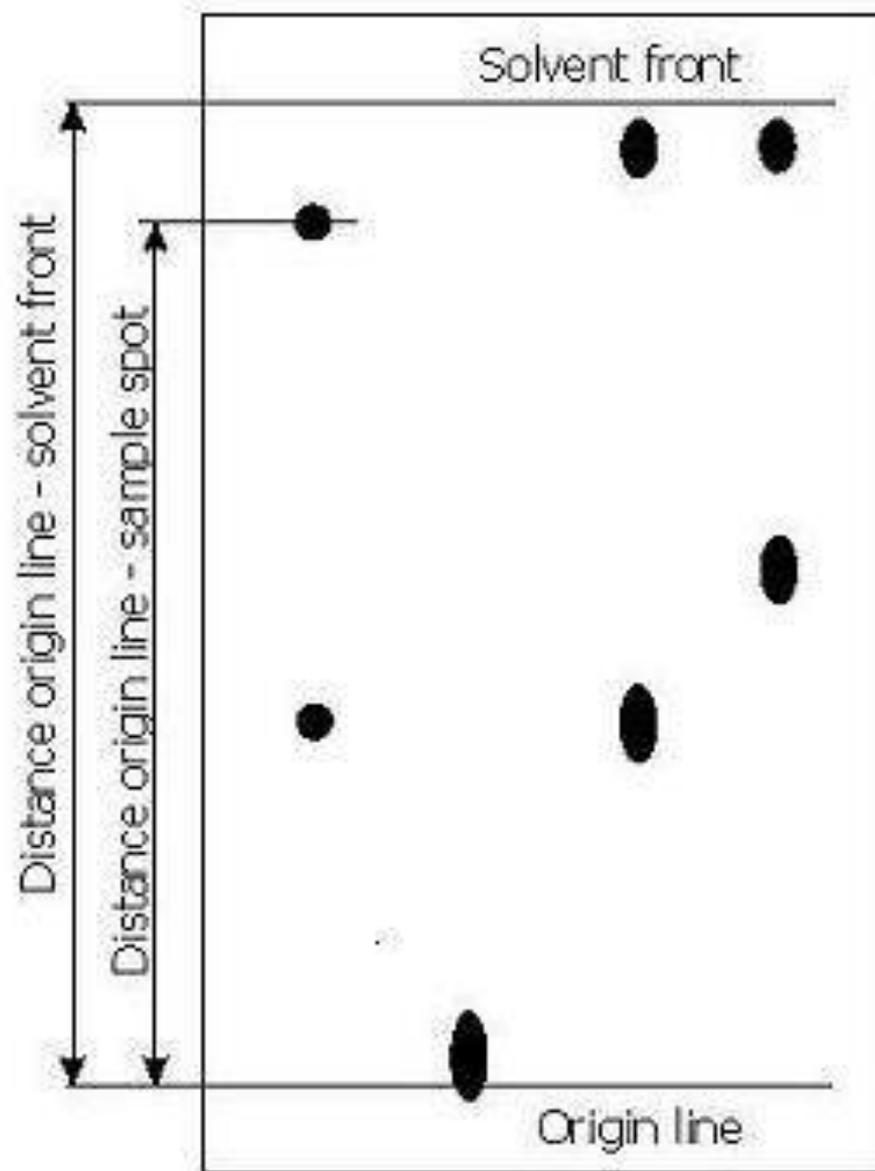
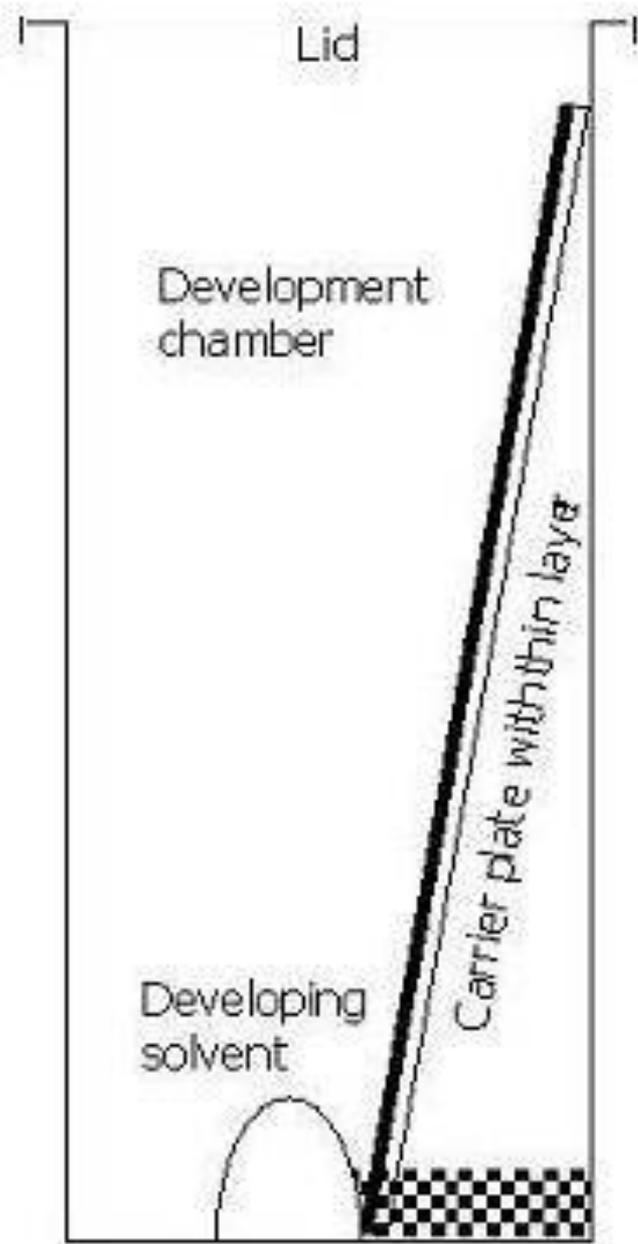
- The classification given above for the various type of chromatography can be deceptive in their simplicity.
- Except in isolated cases pure adsorption or partition chromatography rarely occurs.
- The ultimate success of a chromatographic separation depends on the ability of the analyst to recognize the limitations of the methods and adjust his experiments accordingly.

# Thin Layer Chromatography

In this, the mixture is spotted on a thin layer of adsorbent coated on a plate. The mobile phase is allowed to flow through the adsorbent due to capillary action. The components move according to their affinities towards adsorbent. The component with more affinity towards stationary phase travels slower and the component with less affinity towards stationary phase travels faster.

- The flow rate is calculated by

$$R_f = \frac{\text{Distance traveled by component}}{\text{Distance traveled by Solvent front}}$$



**Mobile phase :** Liquid solvents like Petroleum ether, Carbon tetrachloride, Acetone, Benzene, Ethyl acetate, Chloroform, Methanol, Ethanol, Water.

**Stationary phase :** Silica gel G, Alumina, Cellulose, Keiselghur etc

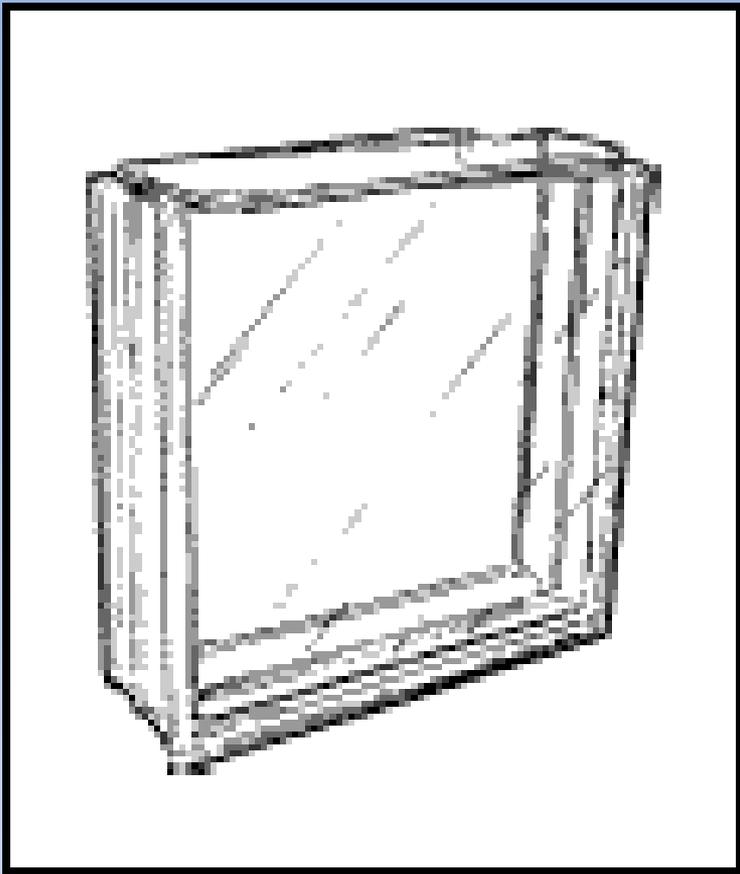
**Detecting system :**

Iodine chamber for polysaccharides

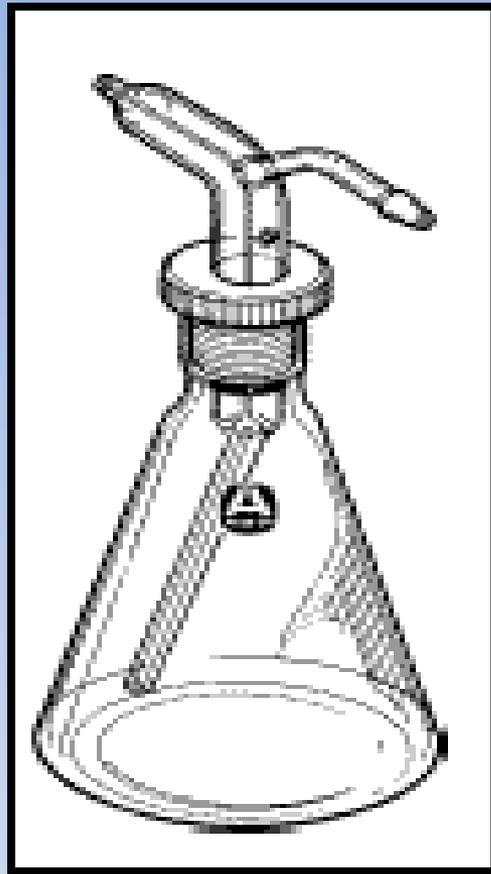
Ninhydrin reagent for amino acids

Dragendroff's reagent for alkaloids

3,5 Dinitro Benzoic acid for cardiac glycosides



**TLC Chamber**



**TLC Spray Flask**



**TLC Spray bottle**

Fill position

Spread position

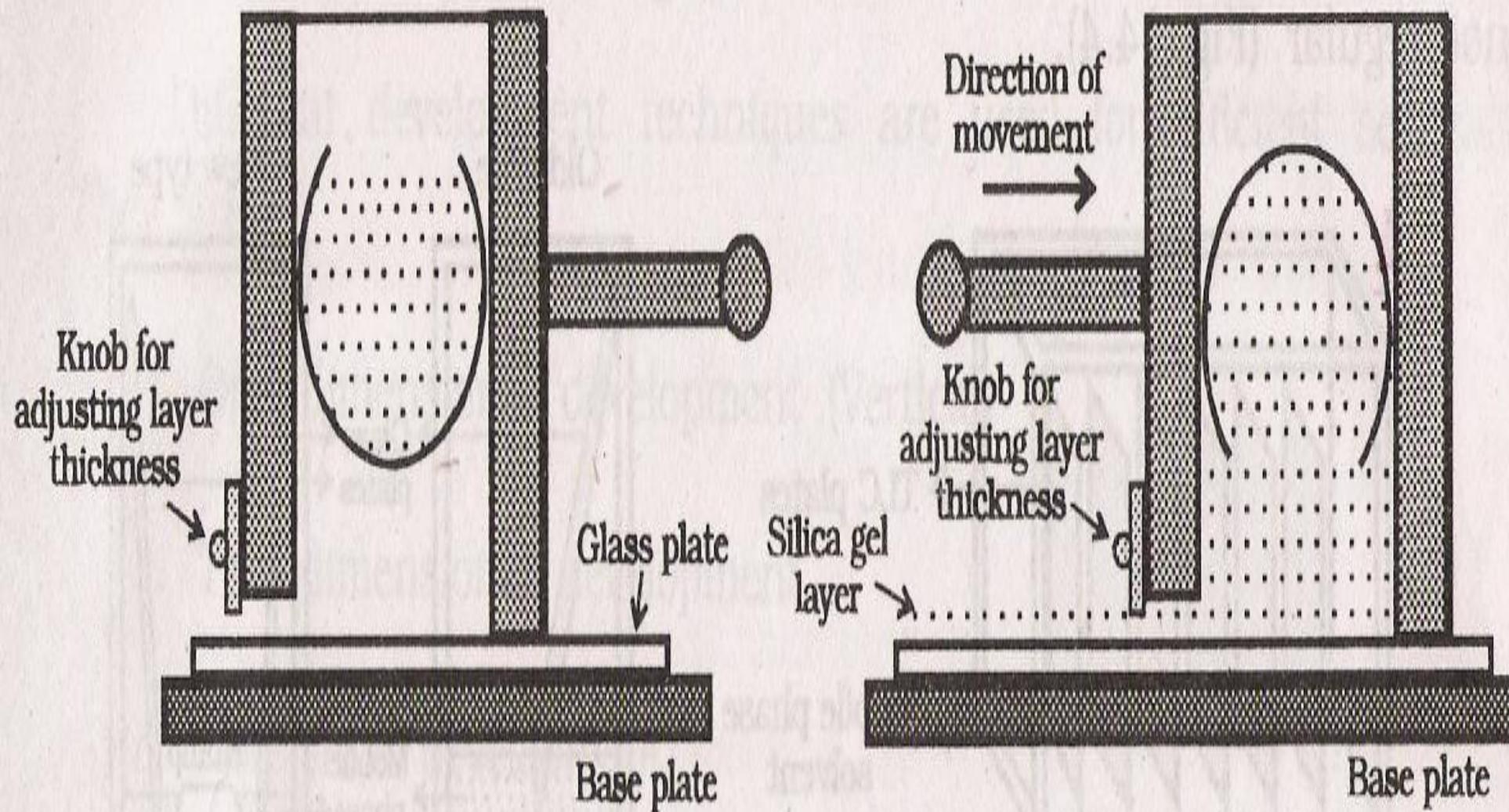
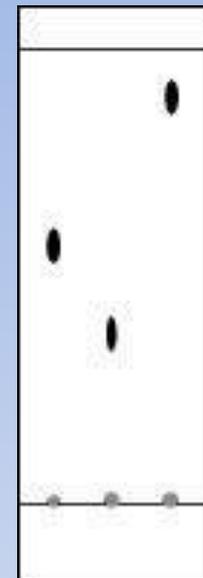


Fig 14.1. TLC Spreader



TLC chamber for development e.g.  
beaker  
with a lid or a closed jar

after ~5 min

after ~10  
min

after drying

# *Applications of TLC*

- It is employed for both qualitative and quantitative analysis of plant extracts.
- Identification of Digitoxin present in Digitalis purpurea leaf:  
Stationary phase : Keiselghur  
Mobile phase : Xylene : Ethyl methyl ketone :  
Formaldehyde (50:4:4)  
Detecting system : Trichloroacetic acid +  
Chloraxime.
- Separation of amino acids
- Analysis and identification of carotenoids
- Separation of alkaloids (nicotine, nornicotine, nicotone, nicotyrine) present in tobacco leaf.

- Separation of Tropane alkaloids ( Tropane, Scopoline, Belladonnine, Hyoscyamine etc) present in belladonna plant.
- Separation of Opium alkaloids (codeine, morphine, papaverine, thebaine) present in opium plant.
- Separation of phenols in plants.
- Separation of Ergot and Rauwolfia alkaloids

- Determination of Ursolic acid from *Oscimum sanctum* leaves.
- Isolation of a gum resin, Saussurine from *Saussurea lappa* roots.
- Separation of antibiotic principles such as alliins & alkylcysteine derivatives from *Allium sativum* rhizomes.
- Differentiation of different species of Aloe plant.
- Isolation and estimation of capsaicine from *capsicum annum*

- Separation of carotenoids like  $\beta$ -carotene and lycopene.
- Separation of Cholerectic principles – diferuloylmethane from *curcuma longa* rhizomes
- Isolation of hallucinogens like cannabiniol, cannabidiol from resin of *cannabis sativa*

# HPTLC

H.P.T.L.C. is very useful qualitative analysis method; it combines the art of chromatography with quickness at a moderate cost. It is a major advancement of TLC principle with short time duration and better resolution.

# The important steps involved are,

- **Sample Preparation**
- **Selection of Chromatographic layers**
- **Plates**
- **Pre washing**
- **Conditioning**
- **Sample Application**
- **Pre conditioning**
- **Mobile Phase**
- **Chromatographic Development**
- **Detection of spot**
- **Scanning and Documentation**

# 1. SAMPLE PREPARATION:

- It needs a high concentrated solution, as very less amount of sample need to be applied. For normal phase chromatography using silica gel pre-coated
- Plates solvents should be non polar of volatile type. For reversed phase chromatography usually polar solvents are used for dissolving the sample.

## **2. SELECTION OF CHROMATOGRAPHIC LAYERS**

- Layer of H.P.T.L.C. are available in the form of pre coats silica gel of very fine particle size is widely used as adsorbent.

### **PLATES:-**

- The plates are similar to conventional T.L.C. plates. Here silica gel of very fine particle size is widely used as adsorbent. The use of particle size helps in greater resolution and sensitivity.
- Plates are produced from 4 to 5 mm silica gel with an inert binder to form a 200mm layer. Plates of 20x20cms are 5x7.5cms is used. Silica gel F254 having a pore size of 6 mm with fluorescent indicator is a coat material. The difference between T.L.C. and H.P.T.L.C. plates is particle size of coated material, which is 5 to 20 mm of T.L.C. and 4 to 8 mm for H.P.T.L.C.

## **1.PRE WASHING:-**

- Plates need to be washed to remove water vapors or volatile impurities. The plates are cleaned by methanol.

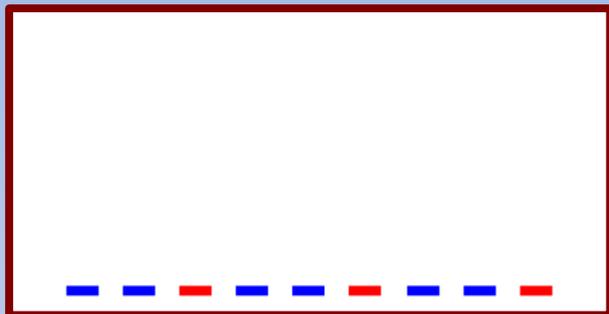
## **2.CONDITIONING:-**

- The pre washed plates are placed in oven at 120°C for 15 to 20 mins. This process is known as conditioning.

# 3.SAMPLE APPLICATION

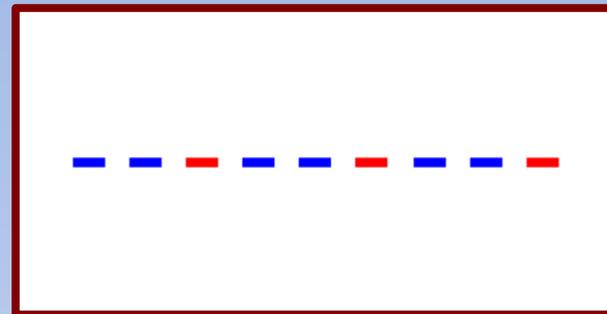
- The size of the sample spot applied must not exceed 1mm in diameter.
- There are different techniques for the spotting of sample; one of them is self-loading Capillary in which small volume of samples may be applied to the plate. Surface using platinum- iridium tubing fused into the end of a length of glass tubing.

# Linomat 5 Sample Application Techniques



## Quantitative Analysis

Most often used. Usual application volume 6 – 20 ul.  
Usual band size 6 mm. For aq. samples 8 mm. Distance from side, 12 – 15 mm, distance from bottom 8 mm.



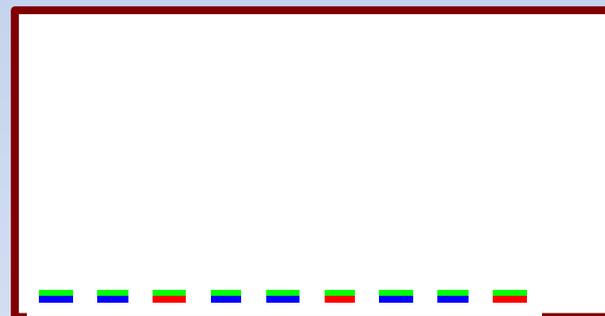
## In – situ clean – up

Used to separate ( extracted ) fatty matrix from the samples. Samples applied 110 mm from bottom, developed in ether, plate dried, cut 120 mm from bottom, turned 180° and developed with appropriate mobile phase. The ether moves the fatty matter to the top of the plate, which is then cut off.



## Micro – prep isolation

For isolation of fractions on a mg scale (for identification by spectroscopy). Usual band length – 190 mm. Usual volume applied 500 ul ( = 5 – 20 mg ). Several similar plates need to be chromatographed.



## Superimpose

Required for overlapping of internal std. or spiking or derivatizing reagent or improving quantification; Single method for application and superimpose as per GLP.

## **4.PRE CONDITIONING (Chambers Saturation):**

- For low polarity mobile phase there is no need of saturation. However saturation is needed for highly polar mobile phases.

## **5.MOBILE PHASE:**

- The solution of appropriate mobile phase is by trial and error in which chemical properties of solute and solvent solubility of analytic absorbent layer are considered

## **6.CHROMATOGRAPHIC DEVELOPMENT**

- **The linear development method is most familiar technique in H.P.T.L.C. here the plate is placed vertically in solvent system in a suitable container. The solvent is usually fed by capillary action and chromatogram can be developed from the both sides.**
- **Circular development, anti circular device and multiple development are some of others methods which are used for chromatographic development.**

# AUTOMATIC DEVELOPING CHAMBER



In ADC2 chromatogram development is fully automatic and reproducible, 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 the ADC2. Two modes of operation are possible: stand-alone with input of parameters via keypad or remote operation from winCATS with process monitoring, documentation of operating parameters and reporting.

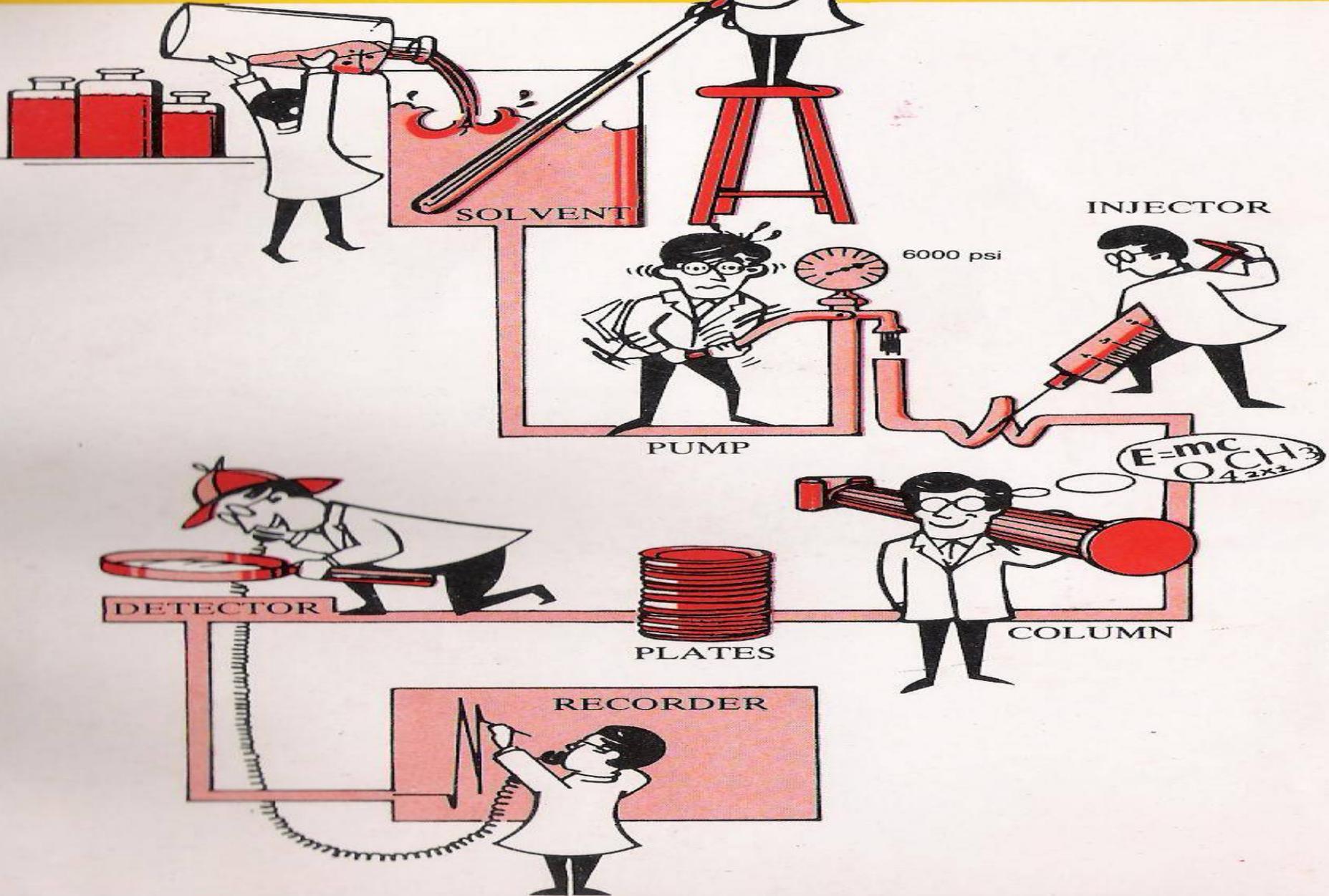
# 7.DETECTION OF SPOTS

- **Immediately after the development is completed, the plates are removed from the chamber and dried to remove the traces of mobile phase. Generally detection can be known by iodine vapor in iodine chamber.**

# 8. SCANNING AND DOCUMENTATION

- **The H.P.T.L.C. equipments are supplied with computer and data recording and storing devices. The development of H.P.T.L.C. plates scanned at selected UV regions wavelength by the instruments and the detected spots are seen on computers in the form of peaks. The scanner converts bond into peak and peak heights or area is related to the concentration of the substance on the spot. The peak heights and the area under the spot are measured by the instrument and are recorded as percent on the printer.**

# Summary..



# REFERENCES

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  - **HPTLC**, By- Dr. P D Sethi, 1<sup>th</sup> edition, 1996.
  - Pharmaceutical Analysis, By- Gurdeep Chatwal, 5<sup>th</sup> edition.
  - Pharmaceutical Analysis By- Kasturi, Wadodkar, Kadam, Nirali publications.
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*Thank you*