# HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

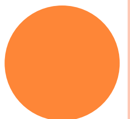

**B.V. Rao**

Associate Professor

**Chebrolu Hanumaiah Institute of Pharmaceutical Sciences**

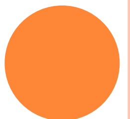
## DEFINITION:

- **Chromatography** is a physical process of separation in which the components to be separated are distributed between 2 immiscible phases -a **stationary phase** which has a large surface area and **mobile phase** which is in constant motion through the stationary phase.



## INTRODUCTION:

- HPTLC is the improved method of TLC which utilizes the conventional technique of TLC in more optimized way.
- It is also known as **planar chromatography** or **Flat-bed chromatography.**
- The basic difference between conventional TLC & HPTLC is only in the particle size & pore size of the sorbents.
- It is very useful in quantitative & qualitative analysis of pharmaceuticals.

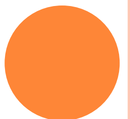


## PRINCIPLE :

- The principle of separation is adsorption.

  Stationary phase - solid

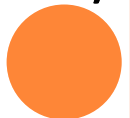
  Mobile phase - liquid



## Advantages Of HPTLC Over Other Chromatographic Methods

HPTLC is the most simple separation technique today available to the analyst due to-

- Simultaneously analysis of sample and standard
- Lower analysis time and less cost per analysis.
- It is very simple to learn and the instrumentation is very easy to operate.
- The sample preparation is very simple.
- It involves very low maintenance cost.
- Solvents used in HPTLC needs no prior treatment like filtration and degassing.
- HPTLC allows the use of corrosive and UV absorbing mobile phases.
- In HPTLC ,the mobile phase consumption for sample is extremely low.



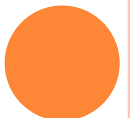
# DIFFERENCES BETWEEN TLC AND HPTLC

| Parameters | TLC | HPTLC |
|---|---|---|
| TYPE OF CHROMATOGRAPHIC PLATE | HAND MADE / PRECOATED | PRECOATED |
| PARTICAL SIZE DISTRIBUTION | WIDE | NARROW |
| PARTICAL SIZE RANGE | 5 – 20 µm | 4 – 8 µm |
| SHAPE | SPOT | SPOT / **BAND** |
| SPOT SIZE | 2 – 4 mm | 0.5 – 1 mm |
| LAYER THIKNESS | 250 µm | 100 – 200 µm |
| SOLVENT CONSUMPTION | 50 ml | 5 – 10 ml |
| NO. OF SAMPLES | MAXIMUM 12 | 36 – 72 |
| OPTIMUM DEVELOPMENT DISTANCE | 10 - 15 cm | 5 – 7cm |
| SAMPLE VOLUME | 1-10 µl | 0.1-2µl |
| NO. OF SAMPLE PER PLATE | 15 – 20 | 40 - 50 |

# INSTRUMENTATION :

- ***Steps involved***

1. Selection of the HPTLC plates & sorbents
2. Sample preparation including prewashing & pre chromatographic derivatization
3. Application of sample
4. Pre-conditioning
5. Development techniques(separation)
6. Detection including post chromatographic derivatization
7. Quantitation
8. Documentation



## PROCEDURE:

SAMPLE AND STANDARD PREPARATION

SELECTION OF CHROMATOGRAPHIC PLATES

PLATES PRE-WASHING

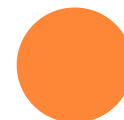
PLATES PRE-CONDITIONING

APPLICATION OF SAMPLE

CHROMATOGRAPIC DEVELOPMENT

DETECTION OF SPOTS

SCANNING AND DOCUMENTATION OF CHROMOPLATE USING PC CATS SOFTWARE

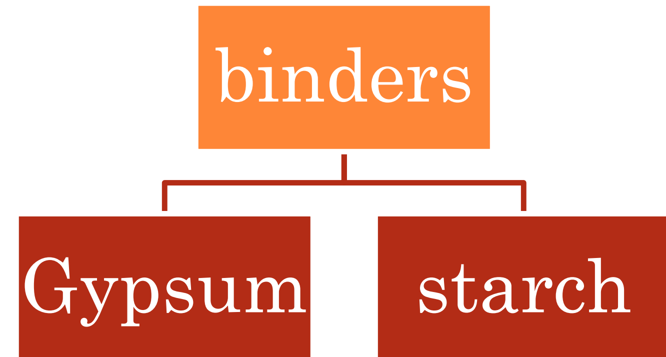


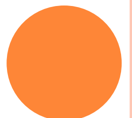
# 1.SELECTION OF HPTLC PLATES:

TYPES OF PLATES

- HAND MADE
  - made up of cellulose
  - support materials like glass, alluminium, plastic
- PRE COATED
  - sorbent layers with different format and thickness are used

sorbent thickness of 100 – 250 μm are used for qualitative & quantitative analysis



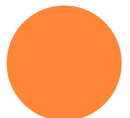



# *Pre washing of pre coated plates*

The main purpose of the pre-washing is to remove impurities which include water vapours and other volatile substances from the atmosphere when they get exposed in the lab environment.

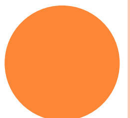
Common methods involved pre washing are as follows

1. Ascending method
2. Dipping method
3. Continuous method



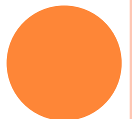
## SOLVENTS USED FOR PRE-WASHING

- Methanol
- Chloroform: methanol ( 1:1 )
- Chloroform: Methanol: Ammonia (90:10:1 )
- Methylene chloride: Methanol ( 1:1 )
- Ammonia solution (1%)



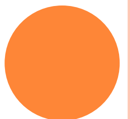
## ACTIVATION OF PLATES:

- Plates exposed to high humidity.
- Plates are placed in oven at $110^{o}$-$120^{o}$c for 30 min prior to the sample application.
- If the plates are not dried properly, the moisture is present on the plates & the principle changes to **partition chromatography**. (stationary phase- liquid, mobile phase- liquid)



## 2.Sample Preparation:

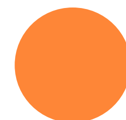
- For normal chromatography: Solvent should be non-polar and volatile.
- For reversed chromatography: Polar solvent is used for dissolving the sample
- Sample and reference substances should be dissolved in the same solvent to ensure comparable distribution at starting zones.



## ***3. Application of sample***

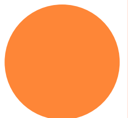
1. Sample application is the most critical step for obtaining good resolution for quantification by HPTLC.
2. Some applicators used for spotting are:
   a) Capillary tubes
   b) Micro bulb pipettes
   c) Micro syringes,
   **d) Automatic sample applicator.**

**The major criteria is that they shouldn’t damage the surface while applying sample.**



## Automatic applicators used:

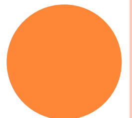
1) **CAMAG Nanomat:** Samples applied in the form of spots. The volume is controlled by disposable platinum iridium of glass capillary which has volume of 0.1-0.2µl.



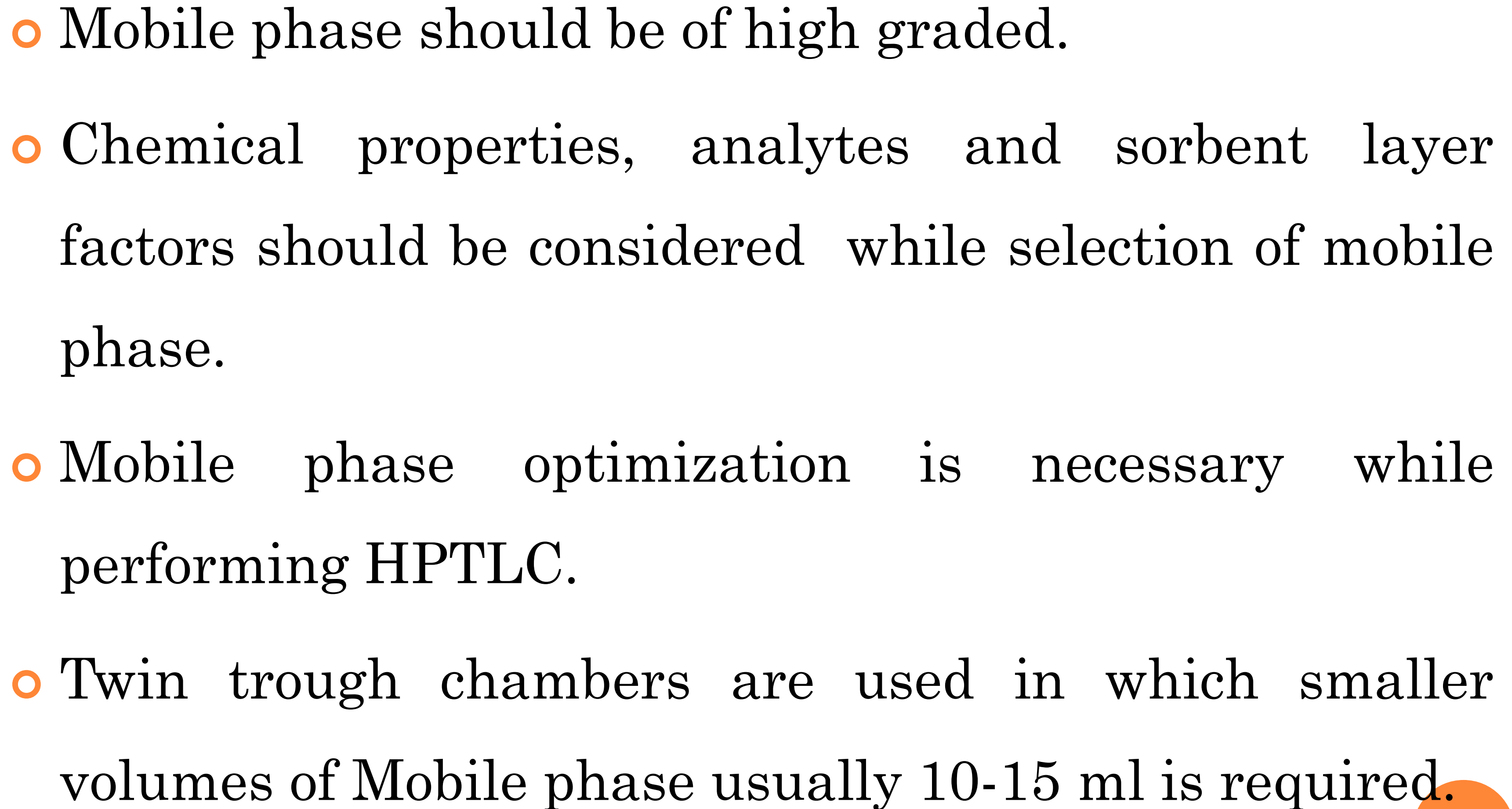
## *2) CAMAG Linomat:*

Automated sample application device. Sample is loaded in micro syringe (Hamilton Syringe) 1µl capacity. Sample can apply either as spot or band by programming the instrument with parameters like spotting volume ,band length etc.

Glass: Borosilicate



## MOBILE PHASE:

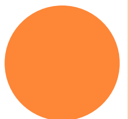


- Mobile phase should be of high graded.
- Chemical properties, analytes and sorbent layer factors should be considered while selection of mobile phase.
- Mobile phase optimization is necessary while performing HPTLC.
- Twin trough chambers are used in which smaller volumes of Mobile phase usually 10-15 ml is required.

- Various components of Mobile phase should be measured separately and then placed in mixing vessel and then transferred to developing chambers.. This prevents contamination of solvents and also error arising from volumes expansion or contraction on mixing.

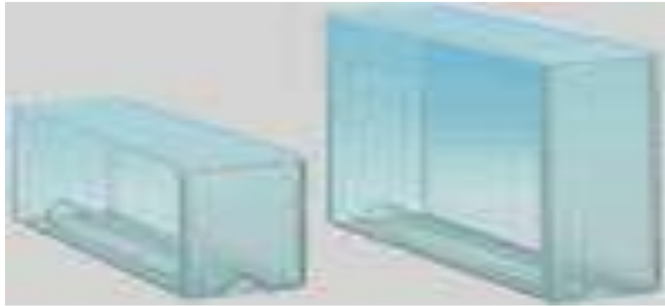
**Development Chambers:**

1. **Twin trough chamber**
2. **Rectangular chambers**
3. V-shaped chambers
4. Sandwitch chamber
5. Horizontal development chamber
6. Automatic development chamber (ADC)



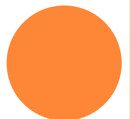
# DEVELOPMENT CHAMBERS:

## 1. Twin trough chamber:

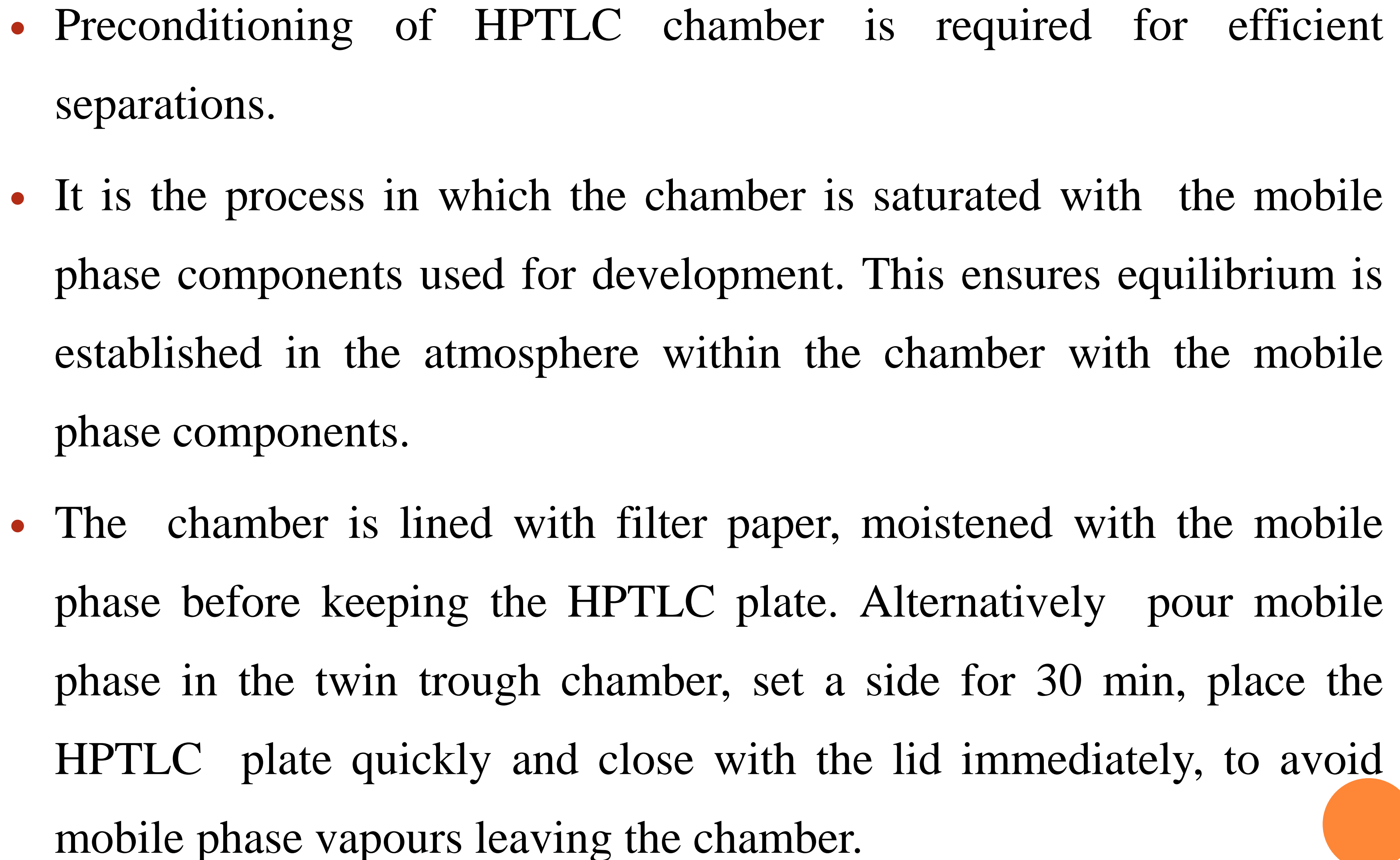


Rectangular chambers

Automatic development chamber (ADC)



# 4.PRE-CONDITIONING (CHAMBER SATURATION):



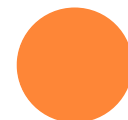
- Preconditioning of HPTLC chamber is required for efficient separations.
- It is the process in which the chamber is saturated with the mobile phase components used for development. This ensures equilibrium is established in the atmosphere within the chamber with the mobile phase components.
- The chamber is lined with filter paper, moistened with the mobile phase before keeping the HPTLC plate. Alternatively pour mobile phase in the twin trough chamber, set a side for 30 min, place the HPTLC plate quickly and close with the lid immediately, to avoid mobile phase vapours leaving the chamber.

## 5.DEVELOPMENT TECHNIQUES:

- The different methods used for development of chambers are like- Ascending, descending, 2-dimentional, horizontal, multiple overrun, gradient & forced flow planar chromatography.
- Plates are spotted with sample and air dried then placed in the developing chambers.
- After the development, plate is removed from chamber and mobile phase is removed under fume cup-board to avoid contamination of laboratory atmosphere.
- The plates should be always laid horizontally because when mobile phase evaporates the separated components will migrate evenly to the surface where it can be easily detected.

## DRYING:

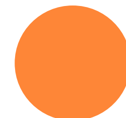
- Drying of chromatogram should be done in vacuum desiccators with protection from heat and light.
- If hand dryer is used there may be chances of getting contamination of plates, evaporation of essential volatile oils if any present in the spot or compounds sensitive to oxygen may get destroyed due to the rise in temperature.



# Factors influencing separation and resolution of spots:

- Type of stationary phase
- Type of pre-coated plates
- Layer thickness
- Binder in the layer
- Mobile phase composition
- Solvent purity
- Size of developing chamber
- Sample volume to be spotted
- Size of spot diameter
- Solvent level in chamber
- Gradient elution
- Relative humidity
- Temperature
- Separation distance
- Mode of development

Greater the difference between two spots and smaller the spot diameter of sample and better will be the **resolution**.



## 6. Detection and visualization

Detection are of two types:

- Qualitative
- Quantitative

- ***Qualitative detection:***

  HPTLC is routinely used for qualitative analysis of raw materials, finished products, plant extracts etc. It involves the identification of unknown sample from mixture by comparing the Rf values of the sample components with the standards.

- ***Quantitative detection:***

  Quantitative of the chromatogram by HPTLC basically involves direct and indirect methods.

## ***Indirect method:***

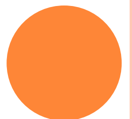
It involves removal of analyte from the plate followed by quantitation.

Eg :

Scrapping and elution which is followed by analysis of eluant by convenient methods like

1) Spectrophotometry
2) Flourimetry
3) Colorimetry

Collection of samples from scrapping will results in the loss of sample, so vaccum devices and elution chamber are used.



# *Densitometry*

•Visualization of spots/bands done directly on plate.

•Amount of compound unknown is measured by comparing standard curve from reference chromatogram with same condition.

❖**Densitometer basically consists of :**

Light source

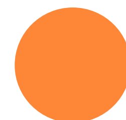
Visible Radiation

UV Radiation

Wave length selection device.

Condensing and focusing system.

Photo-sensing detectors.

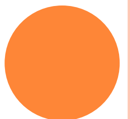


DETECTION METHODS

SPRAYING

DIPPING

GAS PHASE



## Derivatization by Spraying:

- It comes with a rubber pump but may also be operated from a compressed air or nitrogen supply.

- It also consists of two kinds of spray heads.
- Spray head type A is for spray solutions of normal viscosity , e.g. lower alcohol solution.

- Spray head type B is for liquids of higher viscosity.

  Eg: sulphuric acid reagent

## Derivatization by Dipping

- For proper execution of the dipping technique, the chromatogram must be immersed in reagent and withdrawn at a controlled uniform speed.

## *Derivatization through gas phase:*

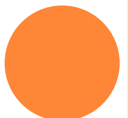
- It offers rapid and uniform transfer of the reagent.
- Only few reagents are suitable they include I,Br,Cl, as well as volatile acids, bases and some other gases like $H_2S$ , NO.
- In gas phase derivatization can be easily accomplished in twin trough chambers where the reagent is placed or generated in the rear trough , while the plate facing the inside of the chamber is positioned in the front trough.

# DOCUMENTATION

HPTLC plates have been evaluated quantitatively and qualitatively should be documented as per guidelines of GLP.

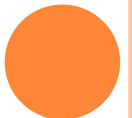
Common methods of documentation are:-

- PHOTO DOCUMENTATION.
- VIDEO DOCUMENTATION.



## ***APPLICATIONS***

* Food analysis
* Pharmaceutical industry
* Clinical applications
* Industrial applications
* Forensic applications
* Cosmetics analysis



## Food analysis:

Estimation of vitamins, pesticides ,food substances.

Curcumin-curcuminoids in turmeric powder.

Sudan-1,2,3 etc in chilli powder.

Cholesterol in edible oil.

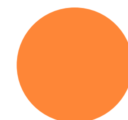
Saffron in food.

## Forensic applications:

Detection and estimation of traces of drugs or poisonous compounds.

## Cosmetic analysis:

Presence of colouring agents, preservatives, trace materials and prohibited substances



| Features | HPLC | HPTLC |
|---|---|---|
| Stationary phase | Liquid/solid | Solid |
| Mobile phase | Liquid | Liquid |
| Conditioning phase | None | Gas |
| Sample should be | | Non volatile |
| Results | By detector | Detector + eyes |
| Analysis | On-line | Off-line |
| Resolution | Very high | Moderate to high |
| Chromatographic sys | Closed | Open |
| Seperating medium | Tubular column | Planar layer(plate) |
| Analysis in parallel | No | Yes |
| High pressure | Yes | Up to 100 samples |
| Time per sample | 2-60min | 1-3min |
| Data obtained | Little to high | Very high |
| Post-chromatography | Limited possibilities | Simple,possible for every sample |
| Fraction collection | Requires prep.scale | Simple no special |

# PARAMETERS:

- **Separation Efficiency**:
- Position of the mobile phase at time t as it moves through a sorbent layer

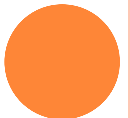
$$(Z_f)^2 = \kappa t$$

- Zf - distance moved by the mobile phase from the sample origin
- k - velocity constant.

k is dependent on:

$$\kappa = 2K_o d_p(\gamma/\eta)\cos\theta$$

- $K_o$ - permeability constant, $d_p$ -average particle diameter,
- g -surface tension of the mobile phase,
- η- viscosity of the mobile phase, and
- θ- contact angle.



## Plate Height:

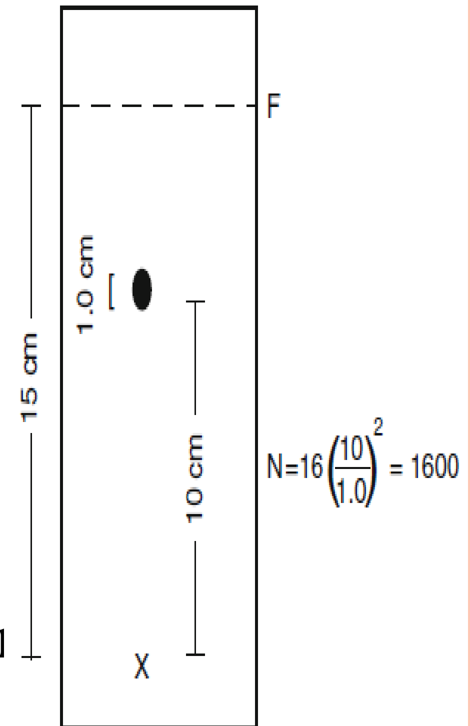
- Broadening of a chromatographic spot can be expressed in terms of the theoretical plate number 'N' of the given chromatographic system:

$$N = 16 Z_f Z_s / W_s$$

- $Z_f$ and $Z_s$ are the migration lengths of the mobile phase and solute, and $W_s$ is the chromatographic spot width in the direction of the mobile phase migration.

$$N = 16 (Z_s / W_s)^2$$

- N is proportional to the migration length of the mobile phase $Z_f$, when Zs /Ws ratio remains constant.
- Increase in $Z_f$ -increase of N and better separation

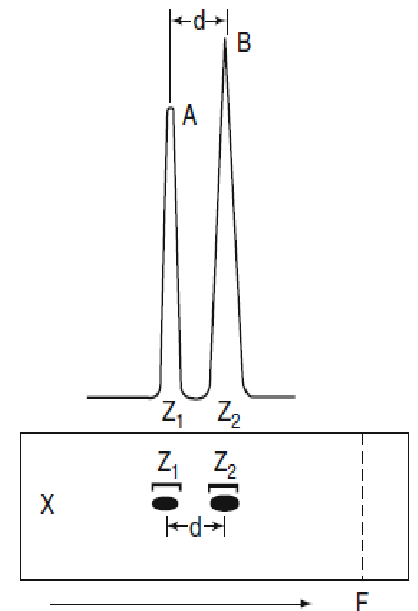


- **Resolution:**
- The separation between two spots is measured by the quantity $R_s$, and is called resolution.
- The $R_s$ of two adjacent chromatographic spots 1 and 2 is defined as the distance between the two spot centers divided by the mean spot widths.

$$R_s = z_2 - z_1/0.5(w_1 + w_2)$$

- When $R_s$=1, the two spots are reasonably well separated.
- $R_s$ values larger than 1 indicates better separation and smaller than 1 suggest a poor separation.
- $R_s$ is also given by the equation

$$R_s = R_{f(2)} - R_{f(1)}/0.5(w_1 + w_2)$$




$R_f(1)$ and $R_f(2)$ are the $R_f$ values of chromatographic spots 1 and 2

## CAPACITY FACTOR/ RETENTION FACTOR

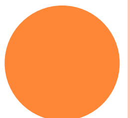
- The ratio of its retention time in the stationary phase to that in the mobile phase.

$$k = t_s / t_m$$

- Measures the degree of retention

$$R_f = 1/k + 1 \text{ or } k = (1 - R_f)/R_f$$

- Ratio of total number of moles of analytes in each phase.
- If a substance doesn't migrate, its $R_f$ =0 and k=1
- If a substance migrates with solvent front, its $R_f$=1 and k=0
- If k< 1, more faster elution ( lesser $r_t$)
- If k > 5, Lesser elution ( Longer $r_t$)
- Ideally k for an analyte **between 1 & 5**.

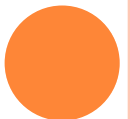


## Spot Capacity

- Separation number or spot capacity is defined as the maximum number of substances, which are completely separated between $R_f$ 0 and 1, provided that the separation conditions are isocratic
- measures the efficiency of separation.

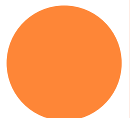
$$SN = (Z_f / b_0 + b_1) - 1$$

- Separation number is given by the expression
- $Z_f$ - migration distance of the front
- $b_o$ - width of the spot at Rf =0
- $b_1$ - width of the spot with Rf = 1



# REFERENCES:


1. www.spinger.com/978-3-642-1404-2
2. www.camagproducts.com
3. P.D Sethi, HPTLC- quantitative analysis of pharmaceutical formulations, published by CBS publishers and distributors, pg no 3-23.
4. Snyder and Kirkland, introduction to modern liquid chromatography, 2nd edition, published by Wiley and intersceince, pg no 247.
5. G. vidyasagar, instrumental method of drug analysis, published by pharmamed press, pg no 137-149.
6. www.camag.com
7. Ashutoshkar, pharmaceutical analysis volume 2, published by CBS publishers and distributors, pg no 299-314.
8. B.k.sharma, chromatography, GOEL publishing house, Meerut pg no 5-16.

Thank You!