CHROMATOGRAPHY

(BASICS)

DR AMIT KUMAR SINGH
Assistant Professor
Department of Botany,
University of Lucknow,
Lucknow-226007.

Historical Perspective:

Modern chromatography originated in the late Nineteith and early twentieth centuries from independent work by David T. Day, a distinguished American geologist and mining engineer, and Mikhail Tsvet (Tswett) a Russian botanist(1906).

Day developed procedures for fractionating crude petroleum by passing it through Fuller's earth, and Tsvet used a column packed with chalk to separate leaf pigments into colored bands.

Because Tsvet recognized and correctly interpreted the chromatographic processes and named the phenomenon chromatography, he is generally credited with its discovery.

Chromatography began to evolve in the 1940s due to the development of column partition chromatography by Martin and Synge and the invention of paper chromatography. The first publication on Gas chromatography (GC) appeared in1952. By the late 1960s, GC, because of its importance to the petroleum industry, had developed into a sophisticated instrumental technique, which was the first instrumental chromatography to be available commercially.

Since early applications in the mid-1960s, HPLC, profiting from the theoretical and Instrumental advances of GC, has extended the area of liquid chromatography into an equally sophisticated and useful method. Supercritical fluid (SFC), first demonstrated in 1962,is finally gaining popularity.

Modern chromatographic techniques, including automated systems, are widely utilized in the characterization and quality control of food raw materials and food products.

CHROMATOGRAPHY

Technique used to separate and identify the components of a mixture

PRINCIPLE: Works by allowing the molecules present in the mixture to distribute themselves between a stationary and a mobile medium. Molecules that spend most of their time in the mobile phase are carried along faster.

Components:

mobile phase: a solvent that flows through the supporting medium

stationary phase: a layer or coating on the supporting medium that interacts with the analytes

supporting medium: a solid surface on which the stationary phase is bound or coated

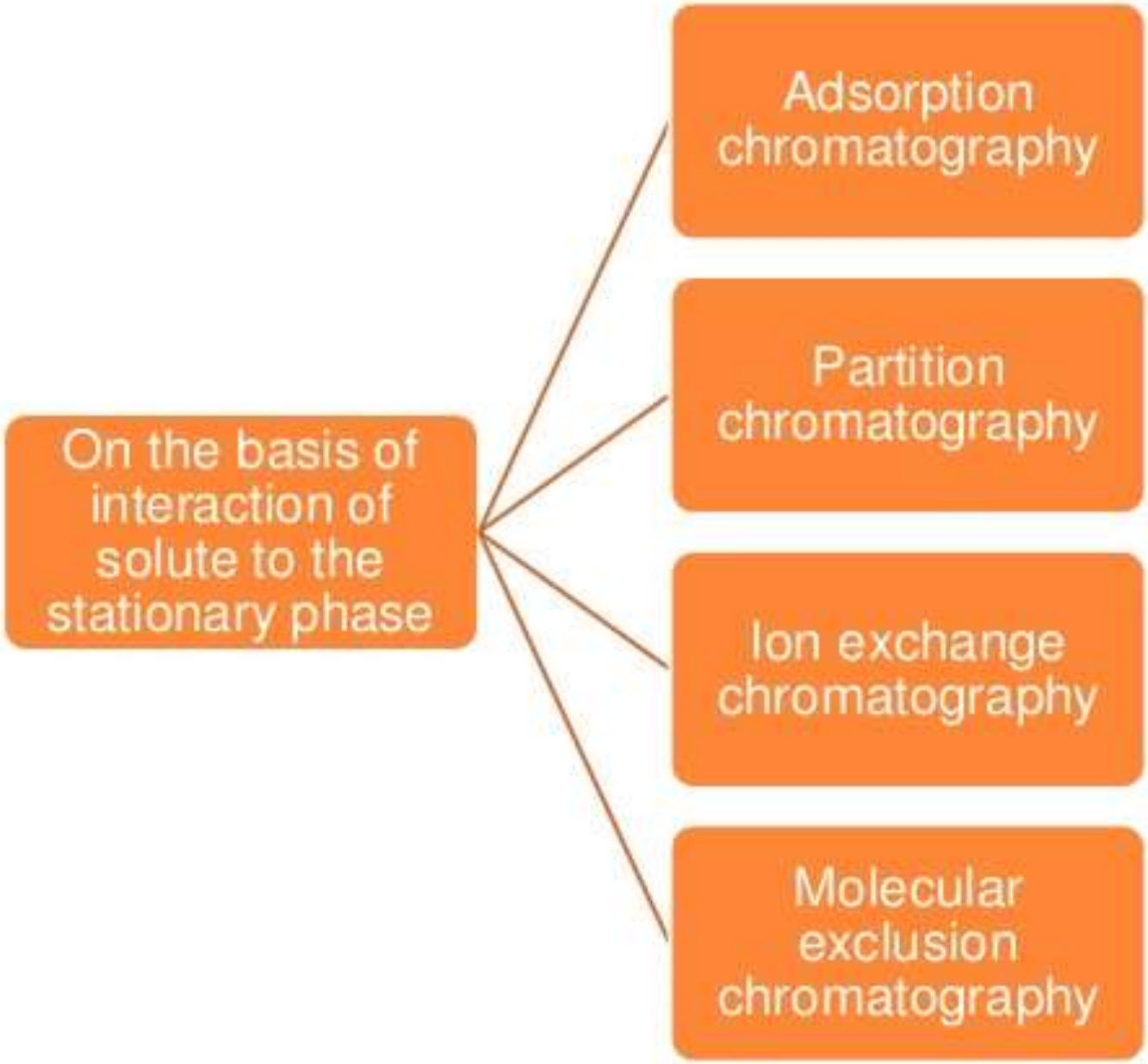
BASIC TERMS….

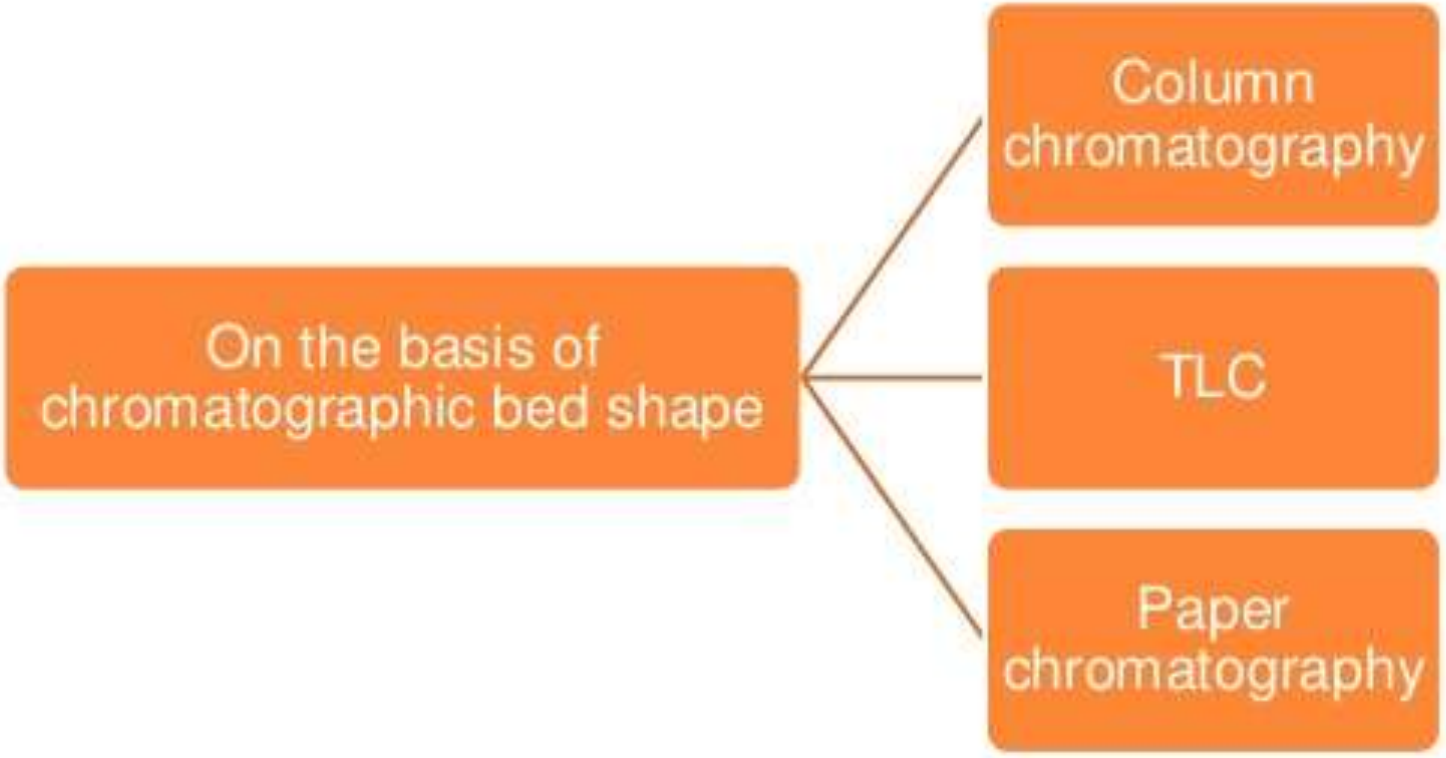
- **Adsorbtion:** Interaction of solute molecules with the surface of the stationary phase
- **Eluent:** The mobile phase
- **Elution:** Motion of the mobile phase through the stationary phase
- **Elution time:** The time taken for a solute to pass through the system. A solute with a short elution time travels through the stationary phase rapidly, *i.e. it elutes fast*
- **Stationary phase**: The part of the chromatography system that is fixed in place

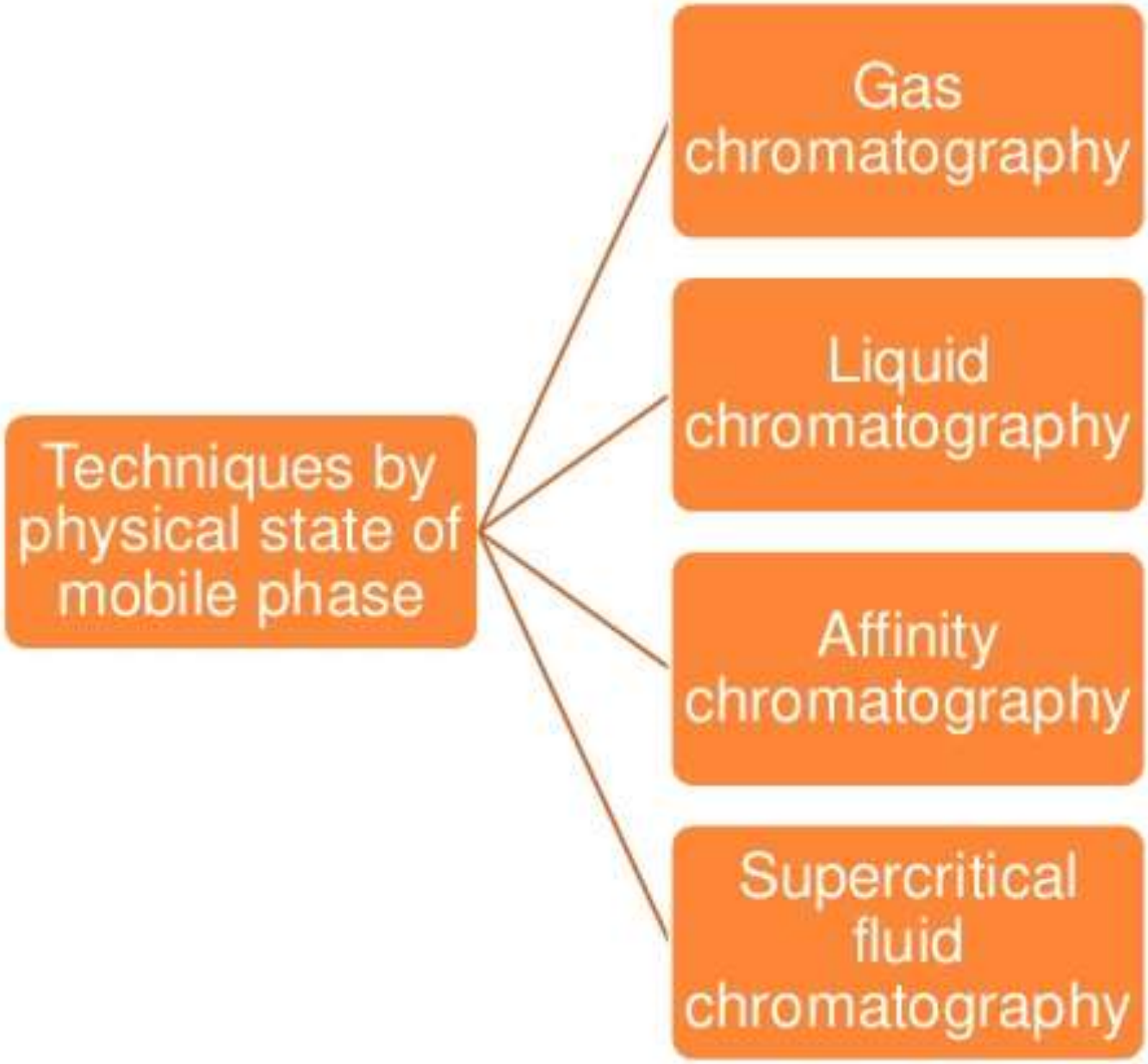
- **Normal phase:** "Unmodified" stationary phase where POLAR solutes interact strongly and run slowly
- **Reverse phase:** "Modified" stationary phase where POLAR solutes run fast *i.e. reverse order*
- **Resolution:** Degree of separation of different solutes. In principle, resolution can be improved by using a longer stationary phase, finer stationary phase or slower elution.
- **Rf value**: $\frac{\text{distance travelled by solute}}{\text{distance travelled by solvent}}$
- **Rf =** retardation factor. The Rf value has to be between 0 and 1, and it is typically reported to two decimal places.

CLASSIFICATION

- On the basis of interaction of solute to the stationary phase
- On the basis of chromatographic bed shape
- Techniques by physical state of mobile phase


On the basis of interaction of solute to the stationary phase
Adsorption chromatography
Partition chromatography
Ion exchange chromatography
Molecular exclusion chromatography


On the basis of chromatographic bed shape
Column chromatography
TLC
Paper chromatography

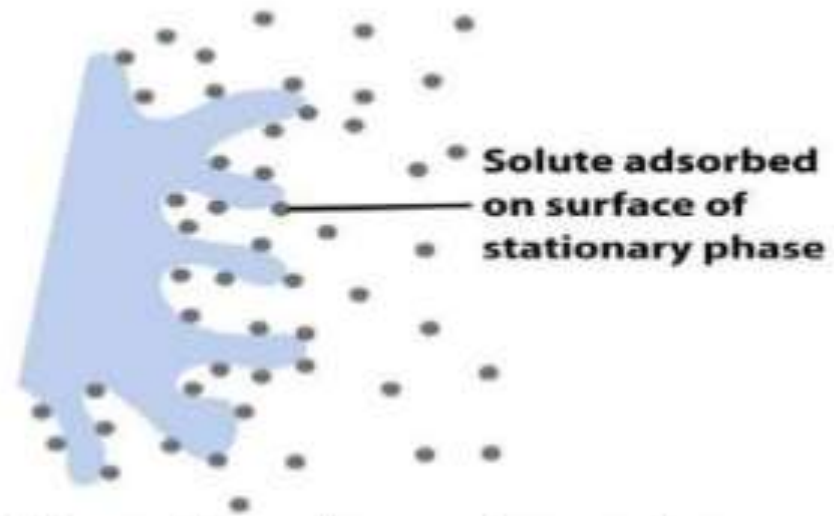

Techniques by physical state of mobile phase
Gas chromatography
Liquid chromatography
Affinity chromatography
Supercritical fluid chromatography

ADSORPTION CHROMATOGRAPHY

- Principle of separation:

utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase

- Stationary phase: adsorbent filled in a tube (column)
- Mobile phase: various solvents (eluents)



Adsorption chromatography

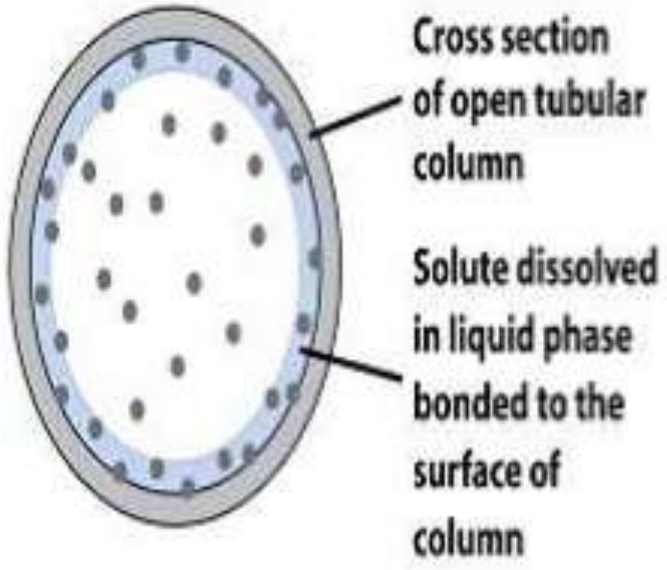
- One of the oldest type
- solute molecules bond directly to the surface of the stationary phase
- Stationary phases may contain a variety of adsorption sites differing in the tenacity with which they bind the molecules and in their relative abundance

PARTITION CHROMATOGRAPHY

PRINCIPLE

partiton of component of sample between sample and liquid/gas stationary phase retard some components of sample more as compared to others. This gives the basis of separation

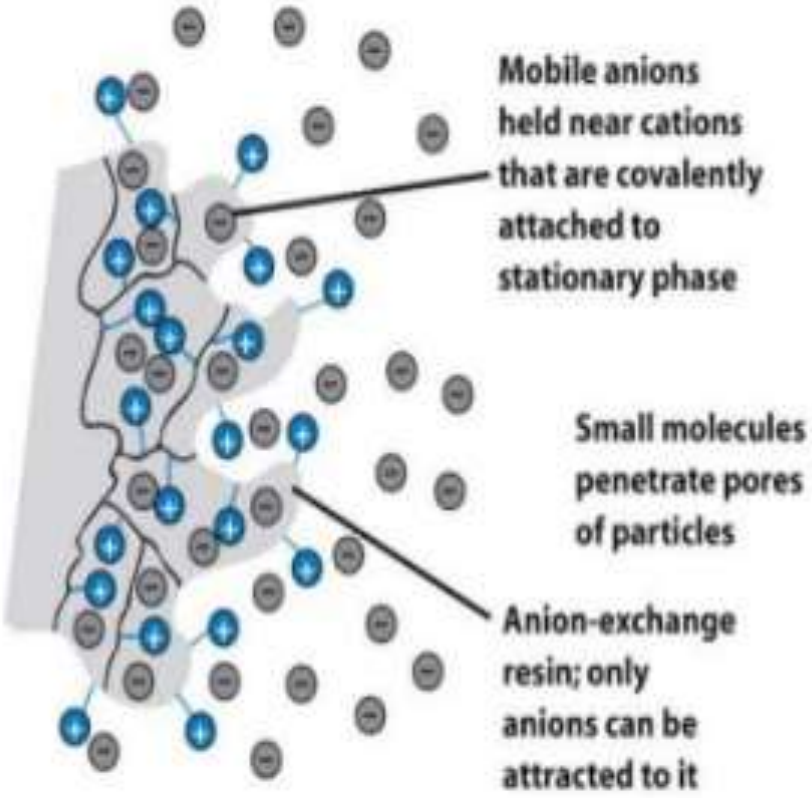
- Based on thin film formed on the surface of a solid support by a liquid stationary phase
- Solute equilibrates between mobile phase and stationary liquid phase



Partition chromatography

ION EXCHANGE CHROMATOGRAPHY

- Resin(stationary phase) used to covalently attach anions or cations onto it
- Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces
- Ion exchange mechanism separates analytes based on their respective charges



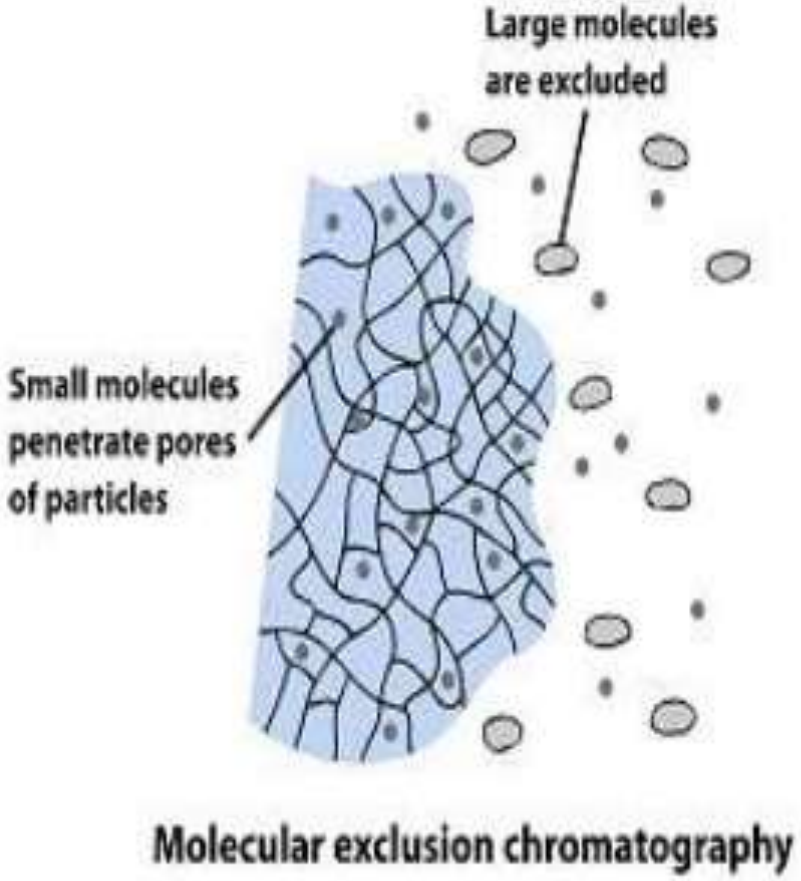
Ion-exchange chromatography

MECHANISM

- To optimize binding of all charged molecules, the mobile phase is generally a low to medium conductivity (i.e., low to medium salt concentration) solution
- adsorption of the molecules to the solid support is driven by the ionic interaction between the oppositely charged ionic groups in the sample molecule and in the functional ligand on the support
- The strength of the interaction is determined by the number and location of the charges on the molecule and on the functional group
- By increasing the salt concentration the molecules with the weakest ionic interactions start to elute from the column first
- Molecules that have a stronger ionic interaction require a higher salt concentration and elute later in the gradient
- The binding capacities of ion exchange resins are generally quite high

MOLECULAR EXCLUSION CHROMATOGRAPHY

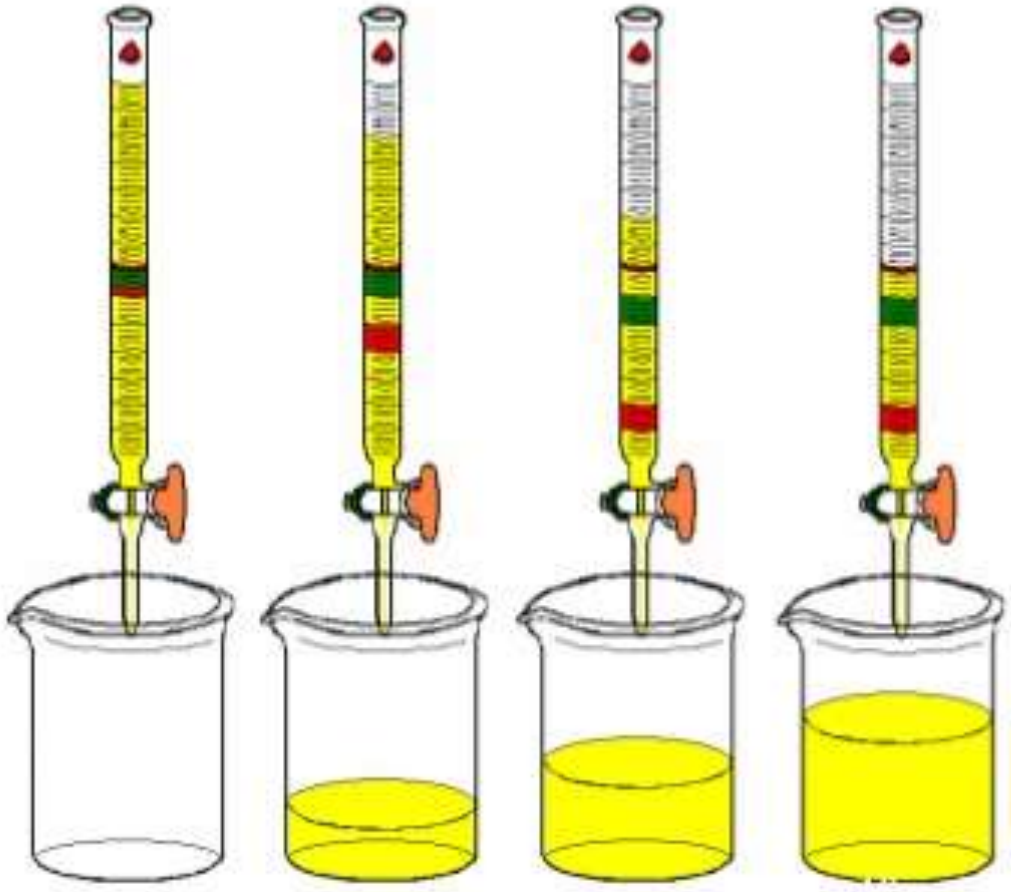
- Also known as gel permeation or gel filtration chromatography
- Lacks attractive interaction between solute and stationary phase



- Liquid or gaseous phase passes a porous gel which separates the molecule according to its size
- The pores are normally small and exclude the larger solute molecules, but allows the smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through column at faster rate than smaller ones
- It is generally low resolution technique and thus it is often reserved for the final "polishing" step of a purification

Column Chromatography

Stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or under the effect of gravity



Factors affecting solutes separation in CC
(Factors affecting column efficiency)

Factor	Effect
Particle size of solid stationary phase (or of support)	Decrease of size improves separation (but very small particles need high pressure).
Column dimensions	Efficiency increases as ratio length / width increases.
Uniformity of packing	Non uniform packing results in irregular movement of solutes through column & less uniform zone formation, (i.e. band broadning or tailing).
Column temperature	Increase in column temperature results in speed of elution but does not improve separation (tailing).
Eluting solvent	Solvents should be of low viscosity (to give efficient resolution) & h igh volatility (to get rapid recovery of the substances).
Solvent flow rate	Uniform & low flow rate gives better resolution.
Continuity of flow	Discontinuous flow disturbs resolution
Condition of adsorbent	Deactivation of adsorbent decreases separation.
Concentration of solutes	Substances of high concentration move slowly.

Elution techniques

Technique	Procedure
Isocratic elution	**Addition of solvent mixture of fixed composition during the whole process.**
Gradient elution	**Continuous or linear elution: in which there is continuous change in the composition of the mobile phase over a period of time (e.g. polarity, pH or ionic strength).**
	Step wise or fractional elution: in which the change is not continuous i.e. a sudden change in the composition of the mobile phase is followed by a period where the mobile phase is held constant.

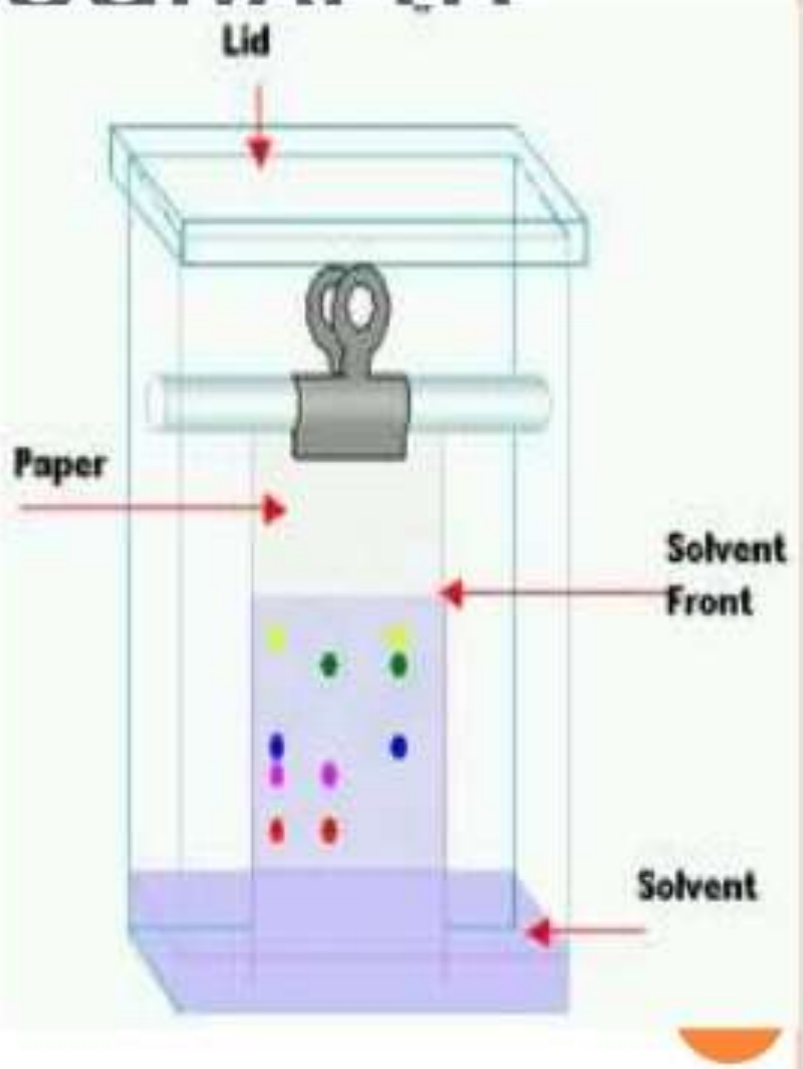
PAPER CHROMATOGRAPHY

uses filter paper strips as carrier or inert support.

The factor governing separation of mixtures of solutes on filter paper is the partition between two immiscible phases.

One is usually water adsorbed on cellulose fibres in the paper (stationary phase).

The second is the organic solvent flows past the sample on the paper (stationary phase).



PAPER CHROMATOGRAPHY

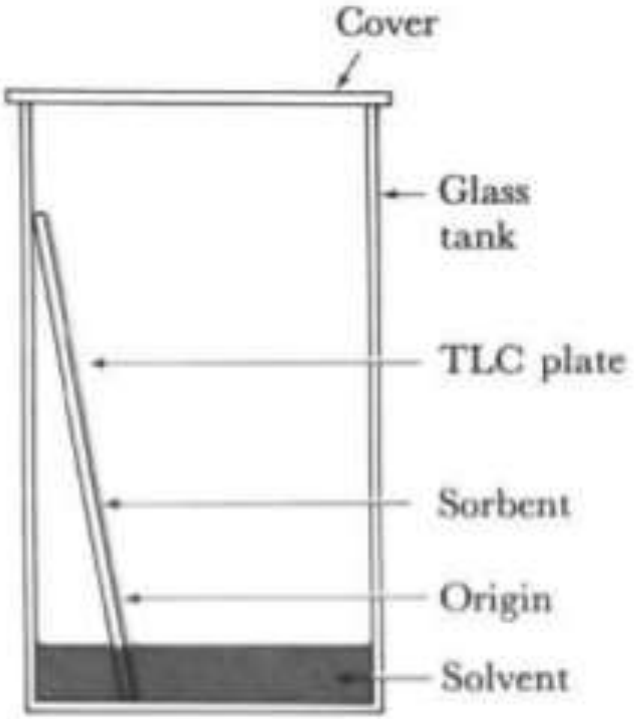
- a small dot or line of sample solution is placed onto a strip of chromatography paper
- The paper is placed in a jar containing a shallow layer of solvent and sealed
- As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent.
- This paper is made of cellulose, a polar substance,
- and the compounds within the mixture travel farther if they

are non-polar

More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far

THIN LAYER CHROMATOGRAPHY (TLC)

involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate



HPTLC

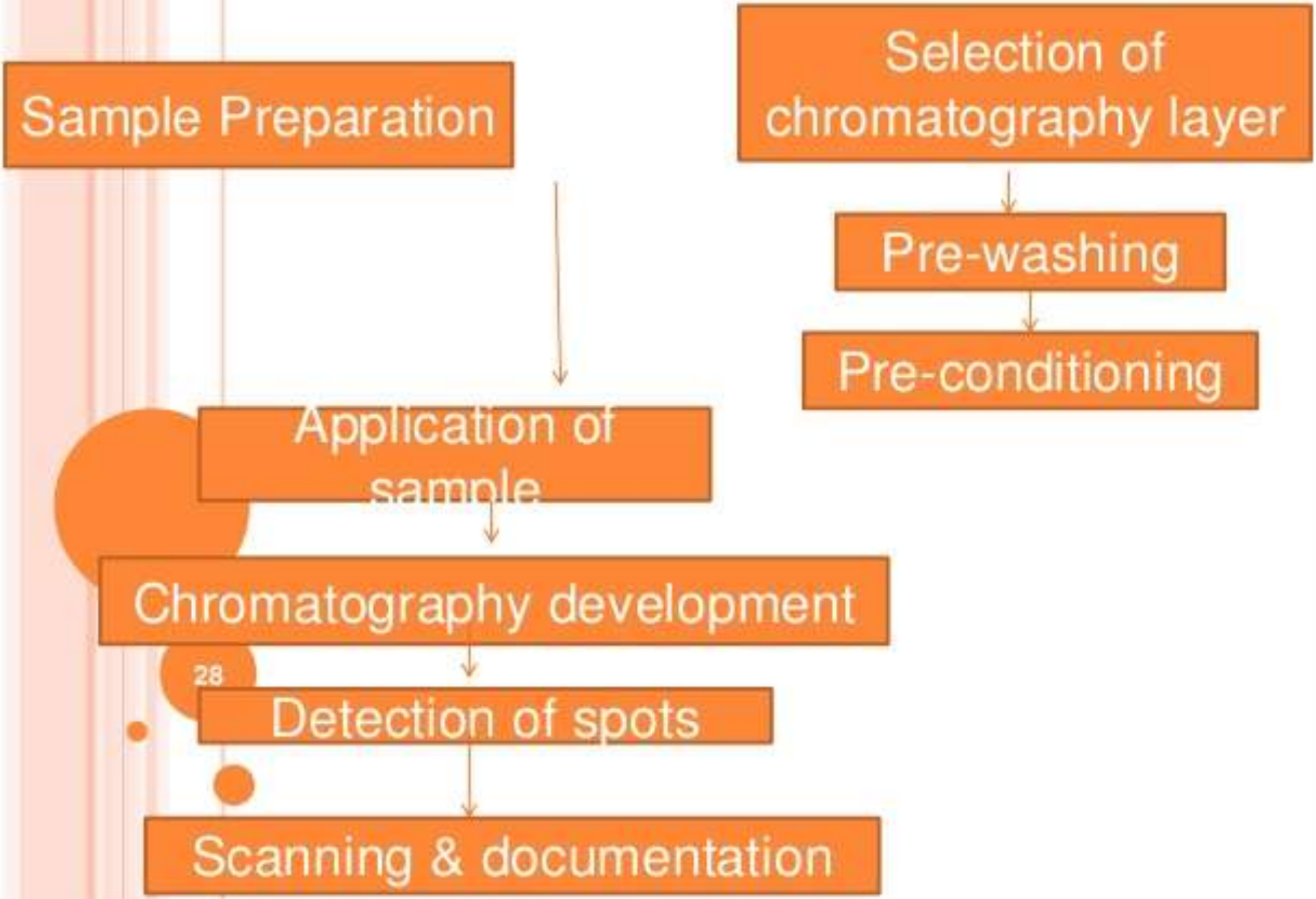
- Sophisticated form of thin layer chromatography. It involves the same theoretical principle of thin layer chromatography.
- It is also known as planar chromatography or Flat-bed chromatography.
- Traditional Thin Layer Chromatography & its modern instrumental quantitative analysis version HPTLC are very popular for many reasons such as
  - visual chromatogram
  - simplicity
  - multiple sample handling
  - low running and maintenance costs, disposable layer etc.

PRINCIPLE

- HPTLC have similar approach and employ the same physical principles of TLC (adsorption chromatography) i.e. the principle of separation is adsorption.
- Solvent flows through because of capillary action
- Components move according to their affinities towards the adsorbent Component with more affinity towards the stationary phase travels slower
- Component with lesser affinity towards the stationary phase travels faster

Thus the components are separated on a chromatographic plate

STEPS INVOLVING IN HPTLC



Pre washing of pre coated plates

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.

Silica gel 60F is most widely used sorbent

major disadvantage of this sorbent is that it contain iron as impurity

This iron is removed by using Methanol : water in the ratio of 9:1.This is the major advantage of the step of pre-washing.

ACTIVATION OF PLATES

- Freshly opened box of HPTLC plates doesn't need activation.
- Plates exposed to high humidity or kept in hand for long time require activation.
- Plates are placed in oven at 110°-120°c for 30 min prior to the sample application.

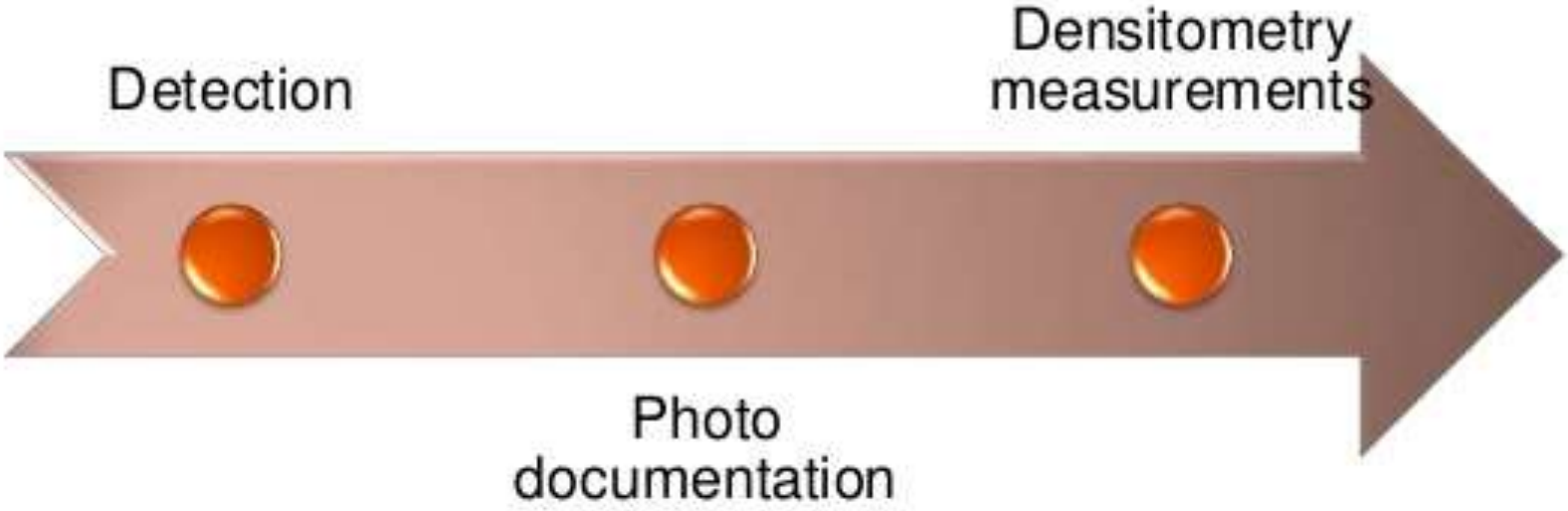
PRE-CONDITIONING

- Also called Chamber Saturation
- Un- saturated chamber causes high Rf values

Sample application

- Usual concentration range is 0.1-1µg / µl
  Above this causes poor separation
- sample and standard application from syringe on TLC plates as bands
- Band wise application - better separation

POST CHROMATOGRAPHY STEPS



DETECTION

Detection under UV light is first choice - non destructive

- Spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length)
- Spots of non fluorescent compounds can be seen - fluorescent stationary phase is used - silica gel GF

UV CABINET

DENSITOMETRY MEASUREMENTS

- Measures visible, UV absorbance or Fluorescence.
- Convert the spot/band into chromatogram consisting of peaks

Instrumentation of HPTLC consists of following:

- Lamp selector
- Entrance lens slit
- Monochromator entry slit
- Grating
- Mirror
- Slit aperture disc
- Mirror
- Beam splitter
- Reference photo multiplier
- Measuring photo multiplier
- Photo diode for transmission measurements.

DIFFERENCES BETWEEN TLC AND HPTLC

Parameter	TLC *Qualitative*	HPTLC *Quantitative*
Chromatographic plate used	Hand made /pre-coated	Pre-coated
Sorbent layer thickness	250 µm	100-200µm
Particle size range	5-20µm	4-8µm
Pre-washing of the plate	Not followed	Must
Application of sample	Manual/Semi automatic	Semi automatic/Automatic
Shape	Spot	Spot/Band
Spot size	2-4mm	0.5-1mm
Sample volume	1-10µl	0.2-5µl
Application of larger volume	Spotting which leads to over loading	Can be applied as bands
No. of samples/plate (20X20)	15-20	40-50
Optimum development distance	10-15 cm	5-7 cm
Development time	Depends on mobile phase	40% Less than TLC
Reproducibility of results	Difficult	Reproducible

REFERENCES:

1. Porath J. From gel filtration to adsorptive size exclusion. J Protein Chem. 1997;16:463–8. [PubMed] [Google Scholar]

2. Harris DC. Exploring chemical analysis. 3rd ed. WH. Freeman&Co; 2004. [Google Scholar]

3. Gerberding SJ, Byers CH. Preparative ion-exchange chromatography of proteins from dairy whey. J Chromatogr A. 1998;808:141–51. [PubMed] [Google Scholar]

4. Donald PL, Lampman GM, Kritz GS, Engel RG. Introduction to organic laboratory techniques. 4th ed. Thomson Brooks/Cole; 2006. pp. 797–817. [Google Scholar]

5. Harwood LM, Moody CJ. Experimental organic chemistry:Principles and Practice. Oxford:Blacwell Science; 1989:180–5. [Google Scholar]

6. Das M, Dasgupta D. Pseudo-affinity column chromatography based rapid purification procedure for T7 RNA polymerase. Prep Biochem Biotechnol. 1998;28:339–48. [PubMed] [Google Scholar]

7. Karlsson E, Ryden L, Brewer J Protein purification. Principles, High Resolution Methods, and Applications. Ion exchange chromatography. 2nd ed. New York: Wiley; 1998. [Google Scholar]

8. Amercham Biosciences. Ion Exchange chromatohgraphy, Principles and methods, Amercham Pharmacia. Biotech SE. 2002;751 [Google Scholar]