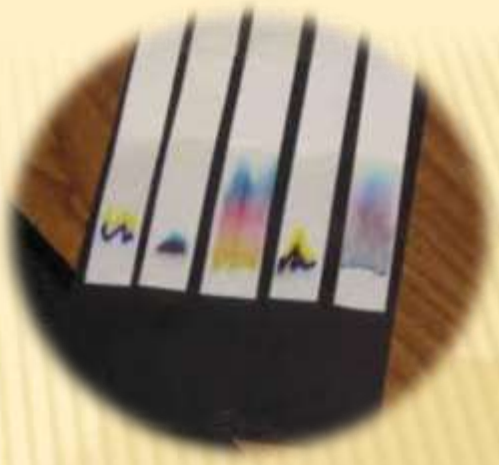


CHROMATOGRAPHY

T.Y.B.PHARMACY

SUBJECT: PHARMACEUTICAL ANALYSIS-IV

2015 PATTERN

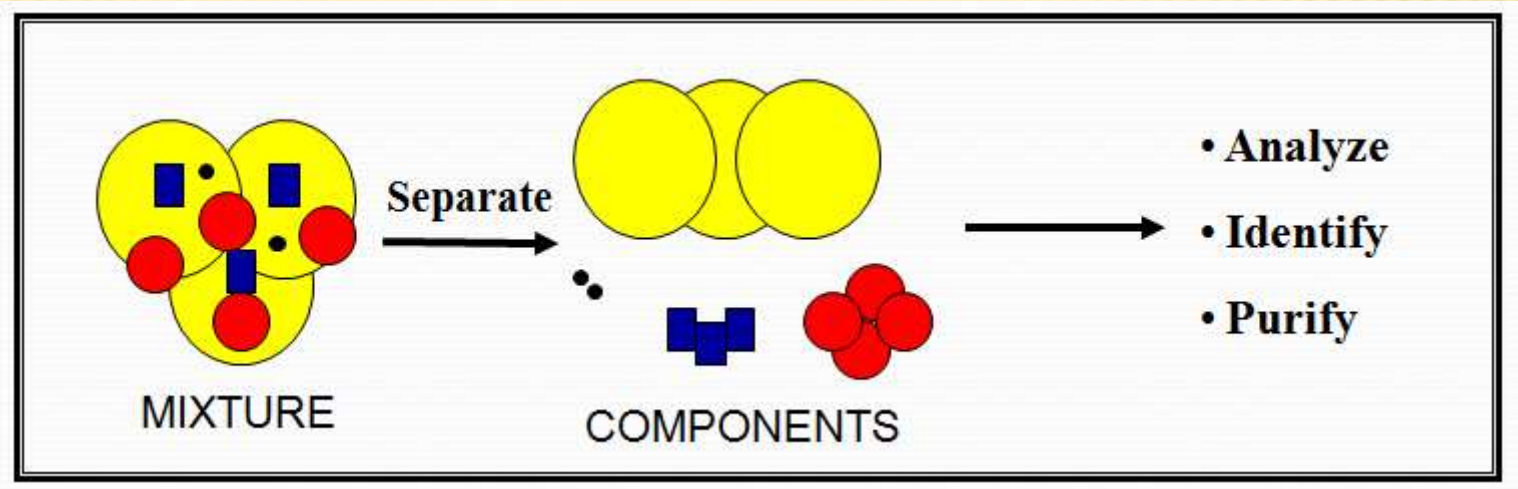
Prepared by-Prof.Gade S.T.

M.Pharm (Pharmaceutical Chemistry)

gadesonali0808@gmail.com

MES's College of Pharmacy, Sonai

WHAT IS CHROMATOGRAPHY ?



Based on

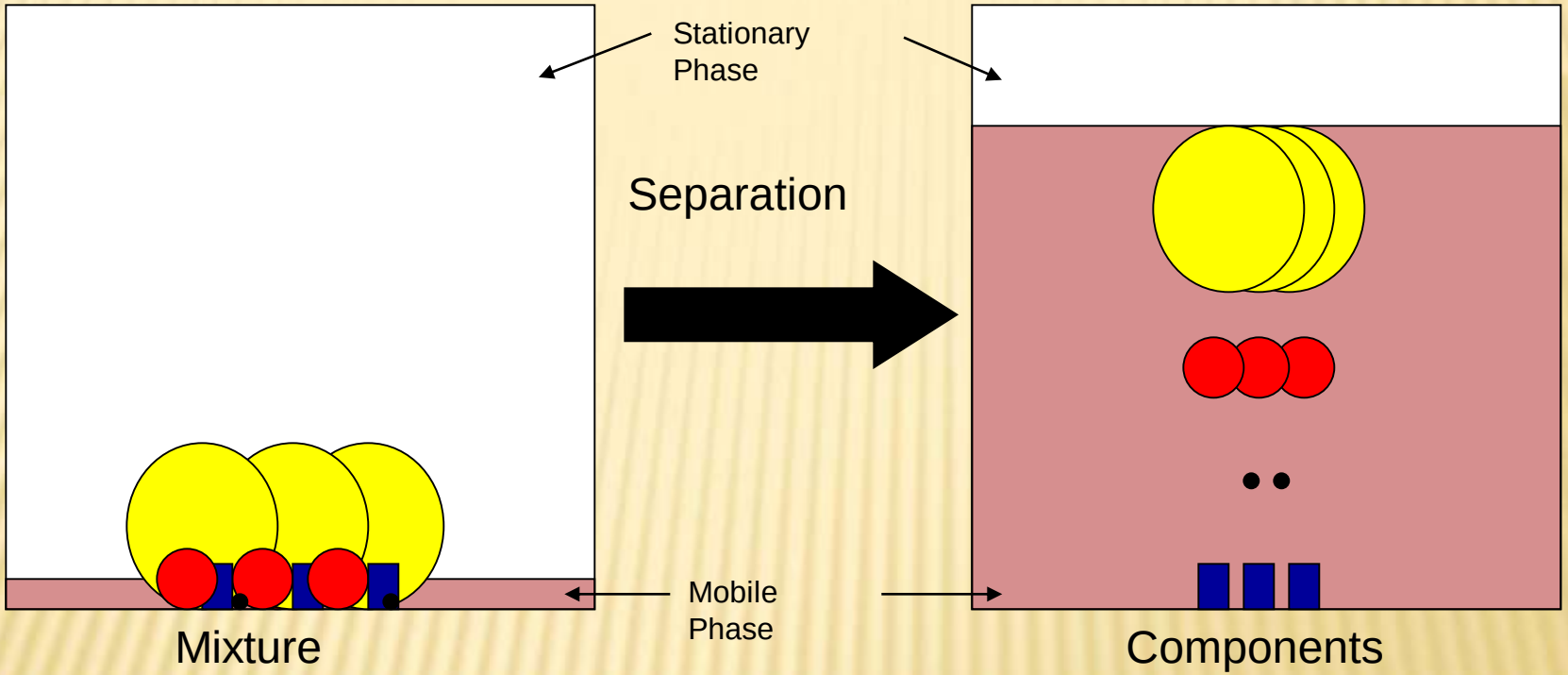
- polarity
- boiling point
- ionic strength
- size

USES FOR CHROMATOGRAPHY

Chromatography is used by scientists to:

- **Analyze** – examine a mixture, its components, and their relations to one another
- **Identify** – determine the identity of a mixture or components based on known components
- **Purify** – separate components in order to isolate one of interest for further study
- **Quantify** – determine the amount of the a mixture and/or the components present in the sample

ILLUSTRATION OF CHROMATOGRAPHY



HISTORY

- In The Year 1906 , Mikhail Tswett (A Russian Botanist , 1872 – 1919) used a New Technique to Separate Plant Pigments.
- He Called This Technique “Chromatography”. It was called so Because the Results of The Analysis were ‘Written In Color’ along the length of the Absorbent Column.

Here,

‘*Chroma’* Means ‘Color’

‘*Graphein’* Means ‘Write’ .

CHROMATOGRAPHY

- It is the 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 Moves (Mobile phase) through it in a definite direction.

- The Chromatographic Process Occurs due to Differences in the Distribution Constant of the individual sample components.

Distribution Coefficient

the way in which a compound (the analyte) distributes between two immiscible phases.

Definition:

$$\frac{\text{Concentration of component A in stationary phase}}{\text{Concentration of component A in mobile phase}}$$

STATIONARY PHASE

DEFINITION :

- Fixed in place either in a column or on a planar surface

FUNCTION :

Act as adsorbent

ADSORBENT :
atoms that accumulate on the surface of the material

TYPES OF STATIONARY PHASE

PARTICLE	WALLS OF TUBE	FIBROUS MATERIAL
• Porous or solid	• Capillary Tube	• Paper

EXAMPLE:

Silica gel, alumina and cellulose powder

MOBILE PHASE

- DEFINITION :
- Carries the analyte through the stationary phase.

FUNCTION :
Act as eluent

ELUENT :
solvent that carry the analyte in elution.

MOBILE PHASE

- In the form of :
- Gas, liquid

COMPARISON OF THE PHASE

MOBILE PHASE	STATIONARY PHASE
GAS **GAS CHROMATOGRAPHY** (GC)	**LIQIUD** **GAS-LIQUID CHROMATOGRAPHY** (GLC) **SOLID** **GAS-SOLID CHROMATOGRAPHY** (GSC)
LIQUID **LIQUID CHROMATOGRAPHY** (LC)	**LIQUID** **LIQUID-LIQUID CHROMATOGRAPHY** (LLC) **SOLID** **LIQUID-SOLID CHROMATOGRAPHY** (LSC)

CLASSIFICATION OF CHROMATOGRAPHY

ACCORDING TO THE PACKING OF STATIONARY PHASE

- **Thin layer chromatography (TLC):** The stationary phase is a thin layer supported on glass, plastic or aluminum plates. layer of silica gel roughly 250 mm thick.
- **Paper chromatography (PC):** The stationary phase is a thin film of liquid supported on an inert support.
- **Column chromatography (CC):** Stationary phase is packed in a glass column.

➢ ACCORDING TO THE FORCE OF SEPARATION

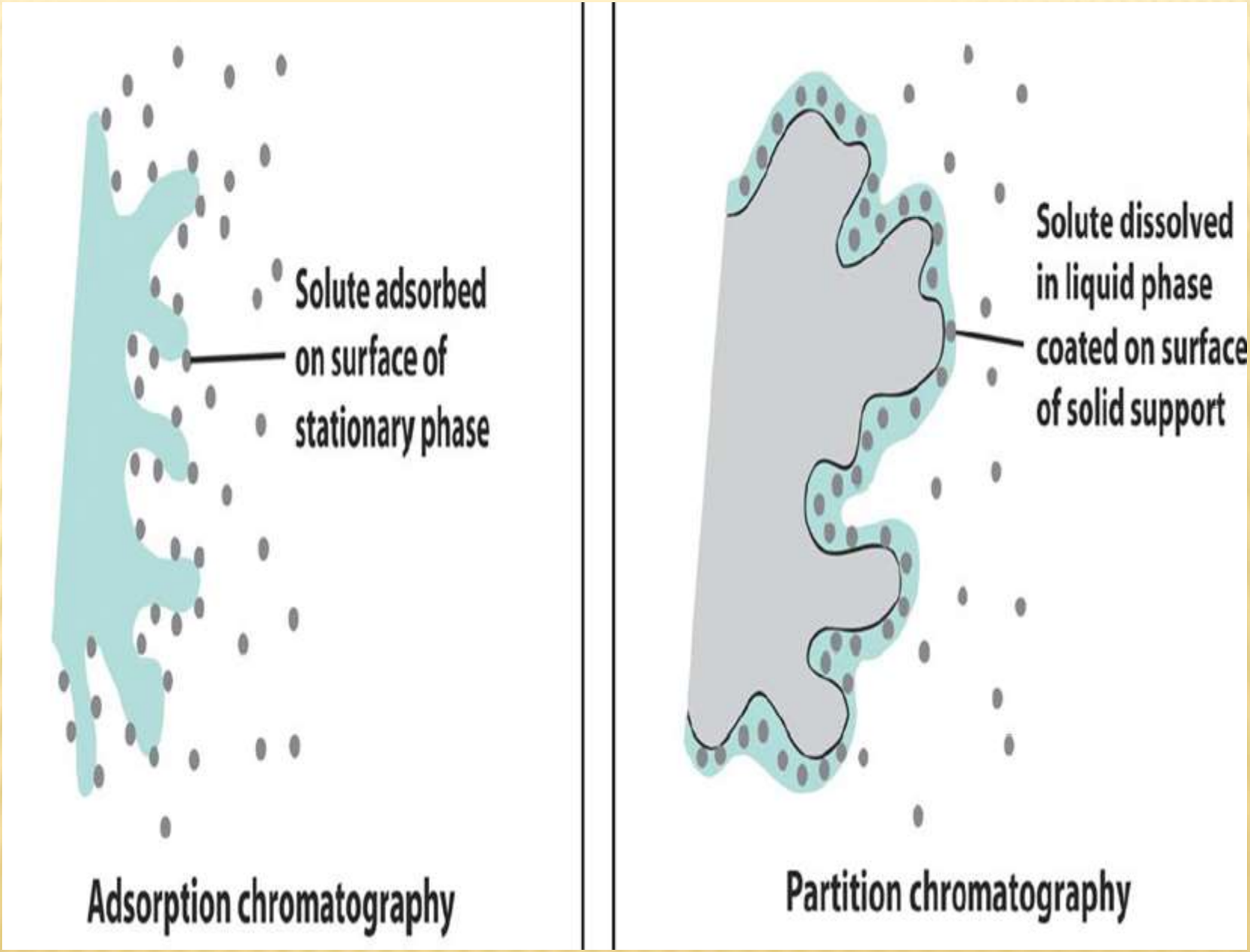
- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Gel filtration chromatography
- Affinity chromatography
- HPLC

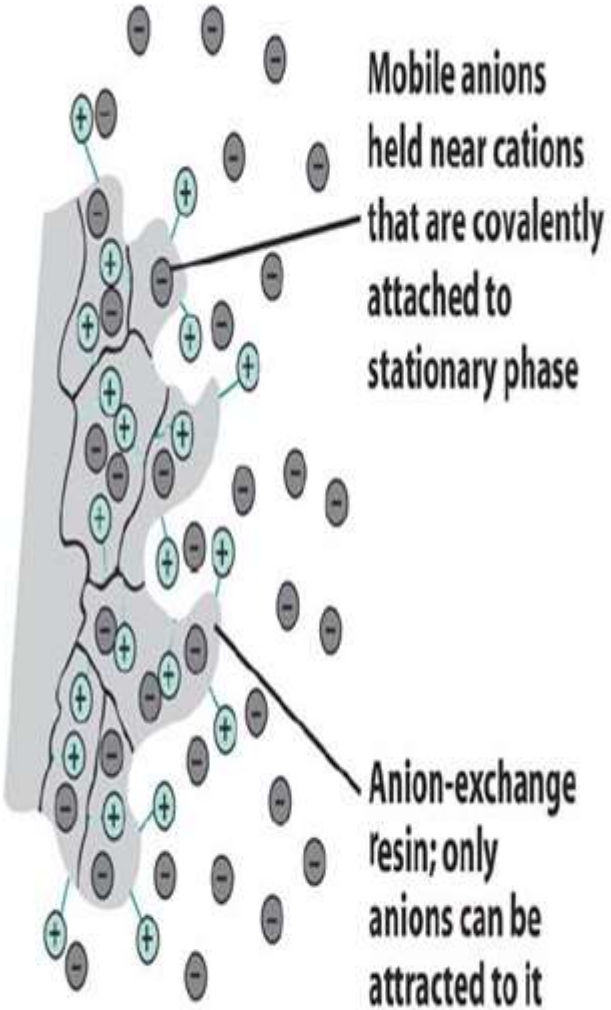
Adsorption chromatography

- Stationary phase = solid
- Mobile phase = liquid or gas
- Mixture of components are adsorbed on the surface of the stationary phase
- Example: column chromatography, thin layer chromatography (TLC)

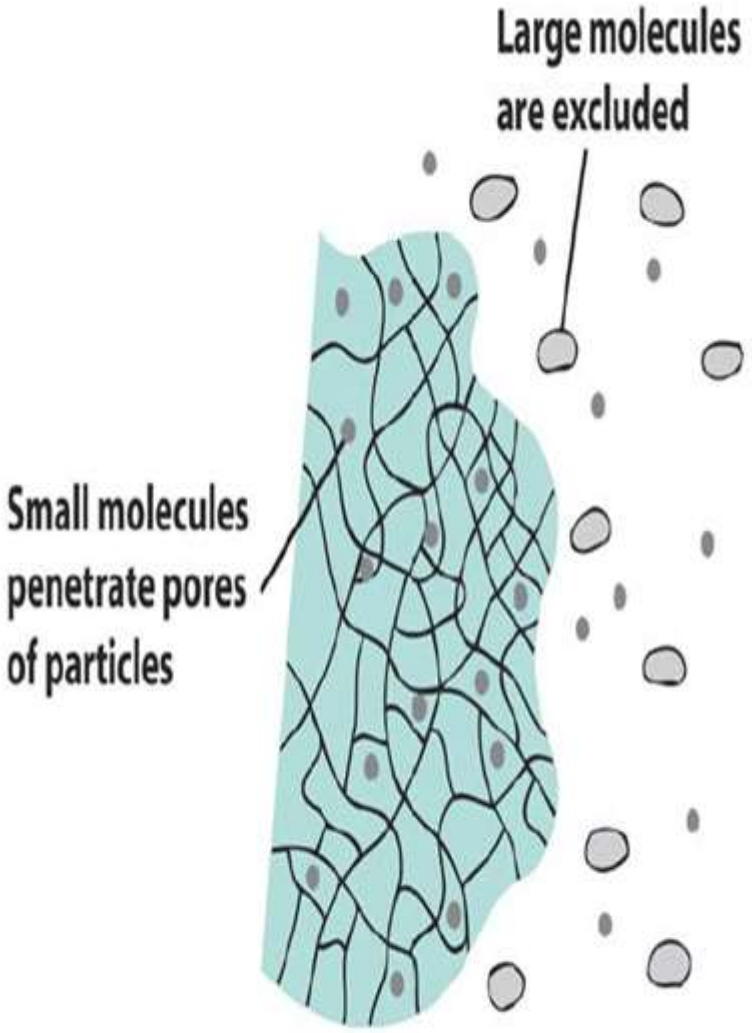
Partition chromatography

- Stationary phase = non-volatile liquid film supported on an inert solid
- Mobile phase = liquid or gas
- Mixture of components are partitioned between the liquid film and the mobile phase (Used partition coefficient / distribution coefficient, K)
- Example: paper chromatography, gas-liquid chromatography (GLC)

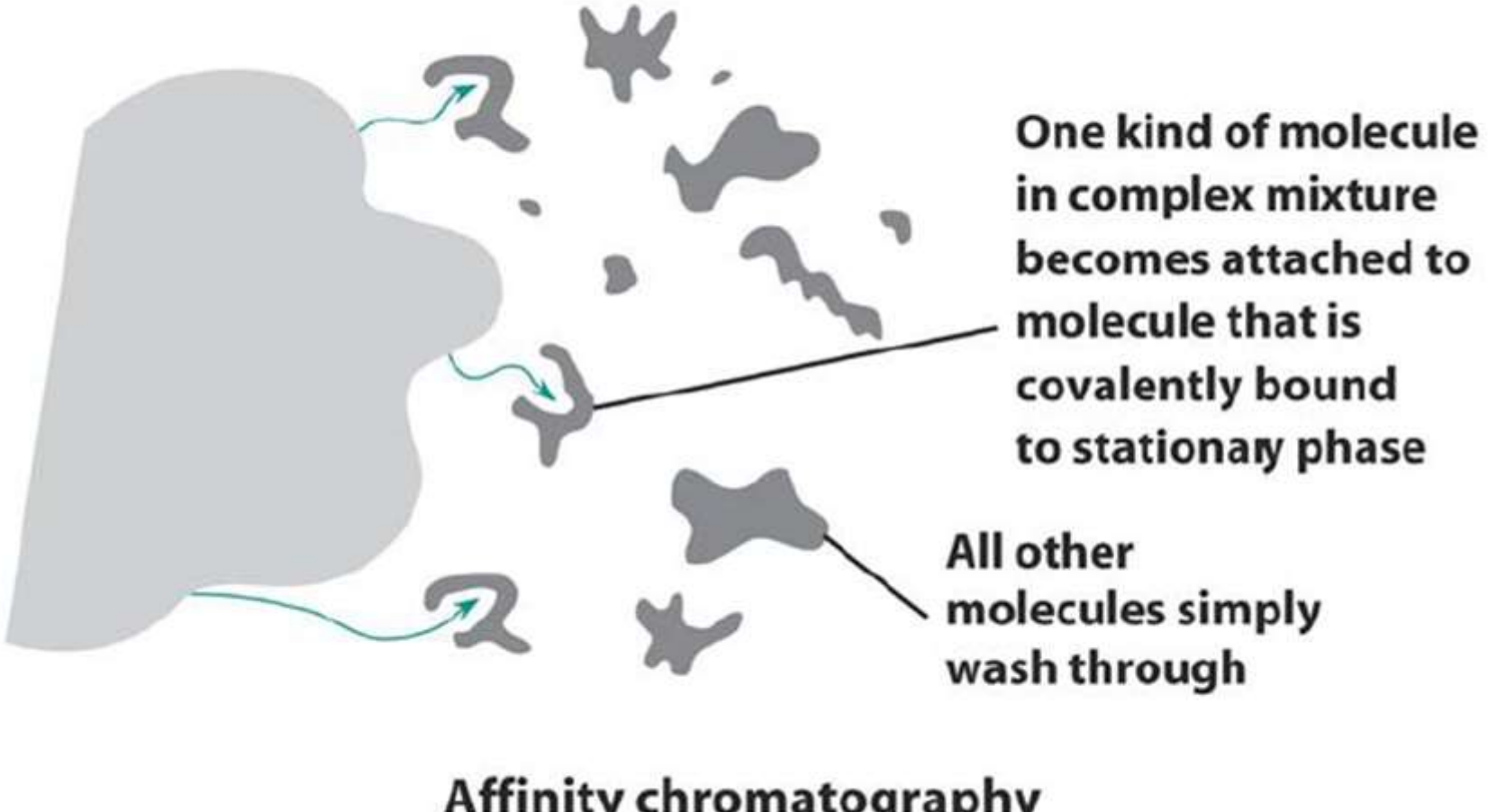

Solute adsorbed
on surface of
stationary phase
Adsorption chromatography
Solute dissolved
in liquid phase
coated on surface
of solid support
Partition chromatography



Ion-exchange chromatography



Molecular exclusion chromatography



Affinity chromatography

CHROMATOGRAPHY TERMS

Chromatogram:

It is the visual output of the chromatograph.

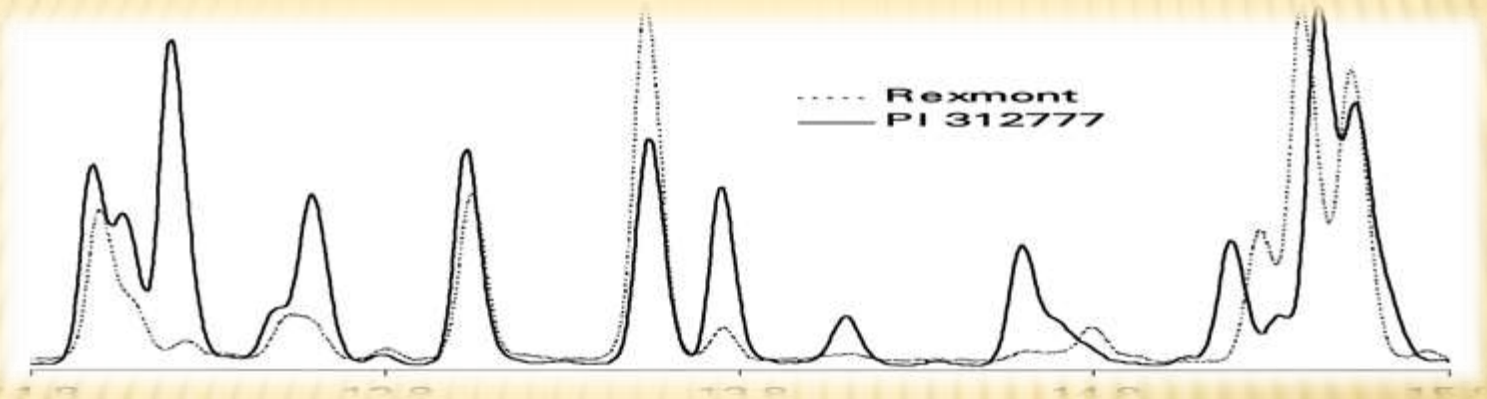
Chromatograph:

It is equipment that enables a sophisticated Separation.

Stationary phase (bounded phase):

It is a phase that is covalently bonded to the support particles or to the inside wall of the column tubing.

CHROMATOGRAM



- The **visual output** of the chromatograph. **Different patterns** on the chromatogram correspond to **different components** of the separated mixture

CHROMATOGRAPHY TERMS

Mobile phase:

It is the phase which moves in a definite direction.

Analyte (Sample):

It is the substance to be separated during chromatography.

Eluate:

It is the mobile phase leaving the column.

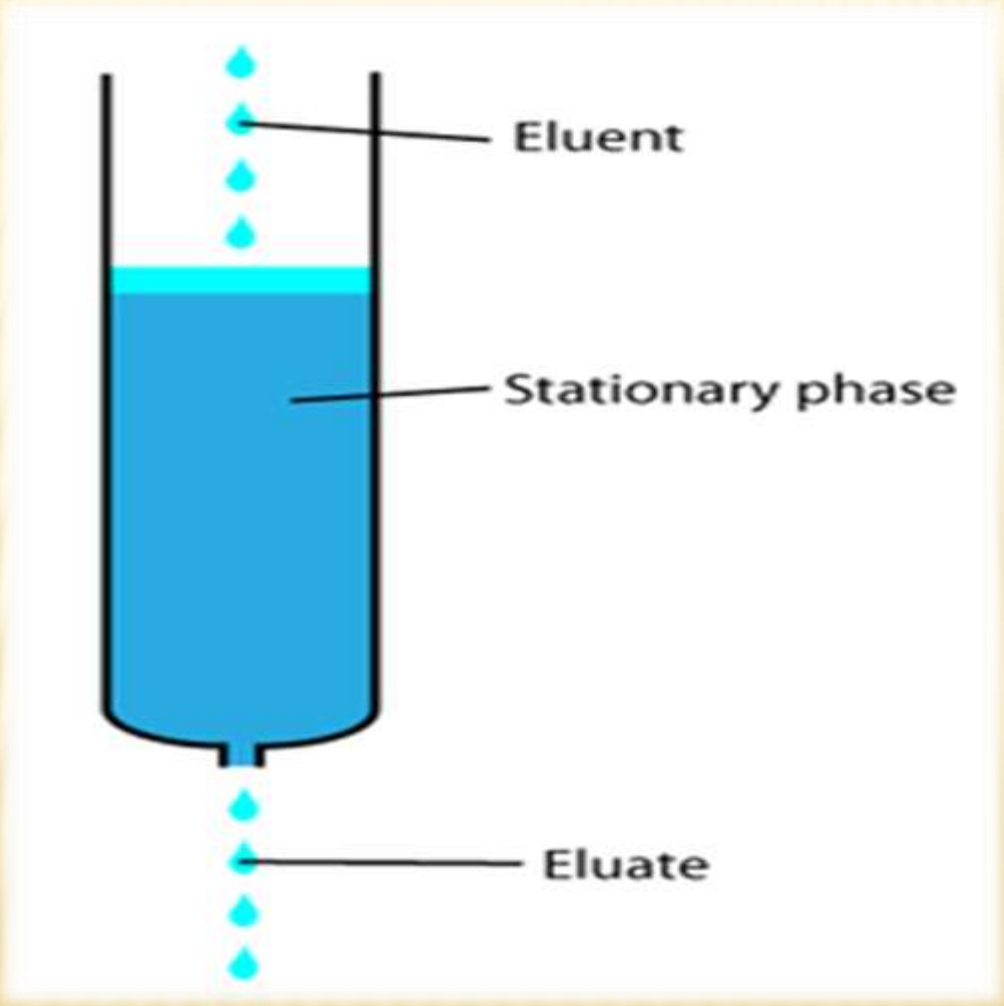
Retention time:

It is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.

Eluent:

It is the solvent that will carry the analyte.

ELUTION



TYPES OF CHROMATOGRAPHY…

Paper

Thin layer

HPLC

Gas

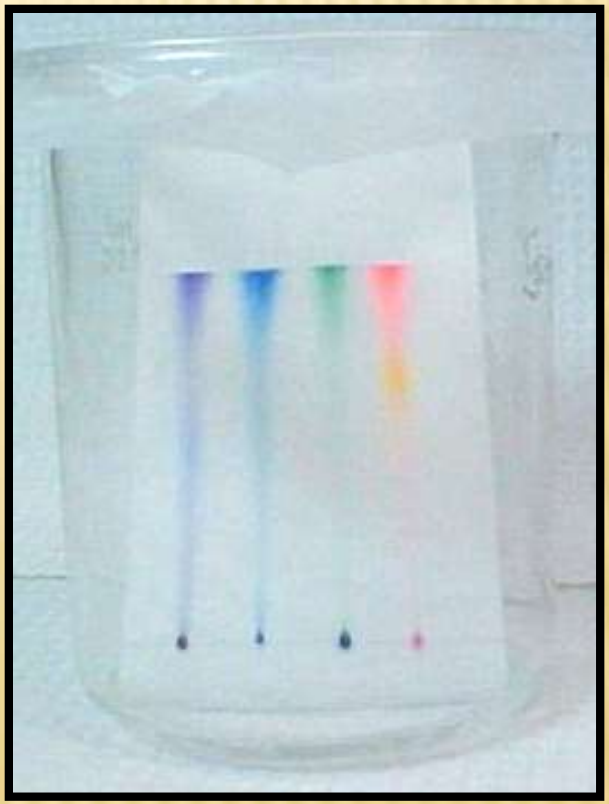
Column

PAPER CHROMATOGRAPHY

Principle:

The principle involved in separation by paper chromatography is largely by partition coefficient phenomenon.

Separation of components depends on both their solubility in the mobile phase and their differential affinity to the mobile phase and stationary phase.



PRACTICAL REQUIREMENTS

- Choice of paper
- Modification of paper
- Preparation of paper
- Preparation of sample
- Application of sample
- Solvents
- Chromatographic chamber
- Development of chromatogram
- Drying of chromatogram
- Detecting or visualizing agents

• Choice of paper

Whatman filter papers of different grades like No.1, No.2, No.3, No.4, No.20, No.40, No.42 etc are used. In general this paper contains 98-99% of α-cellulose, 0.3 – 1% β –cellulose

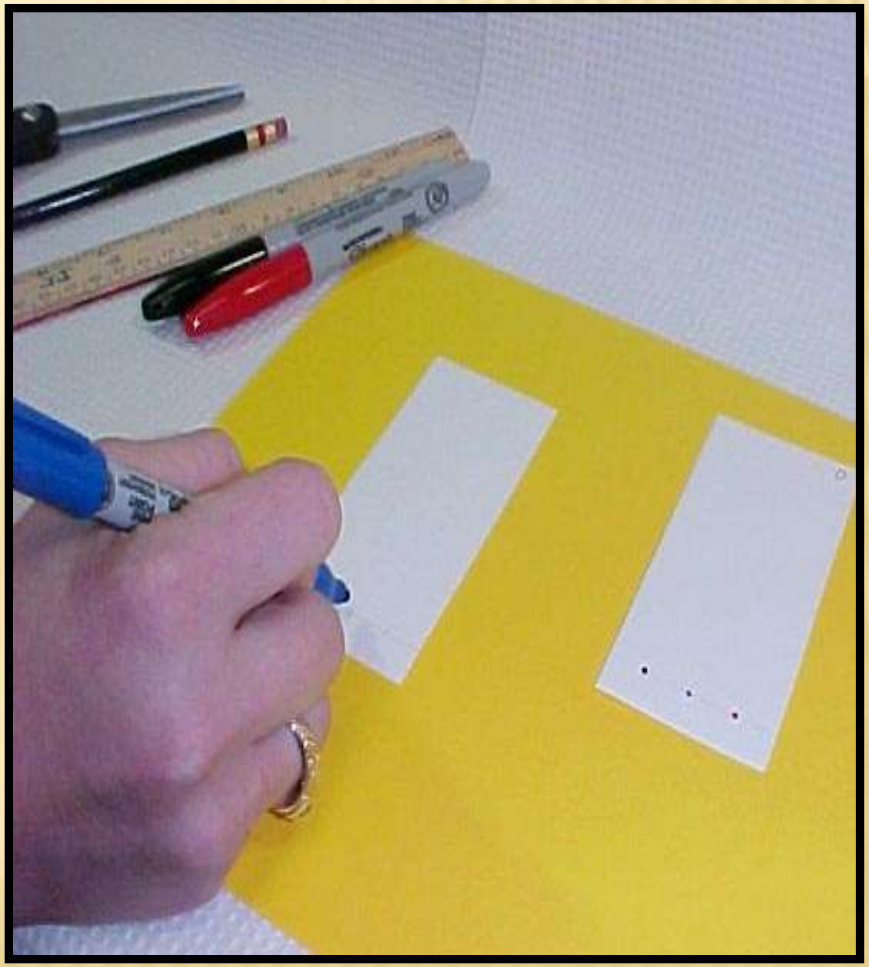
- Factors that governs the choice of paper:
  - » Nature of Sample and solvents used.
  - » Based on Quantitative or Qualitative analysis.
  - » Based on thickness of the paper.

•MODIFICATION OF PAPER

- **Modified Papers** – acid or base washed filter paper, glass fiber type paper.
- **Hydrophilic Papers** – Papers modified with methanol, formamide, glycol, glycerol etc.
- **Hydrophobic papers** – acetylation of OH groups leads to hydrophobic nature, hence can be used for reverse phase chromatography.
- Impregnation of silica, alumna, or ion exchange resins can also be made.

•PREPARATION OF PAPER

- Cut the paper into desired shape and size depending upon work to be carried out.
- The starting line is marked on the paper with an ordinary pencil 5cm from the bottom edge.
- On the staring line marks are made 2cm apart from each other.



•PREPARATION OF THE SAMPLE

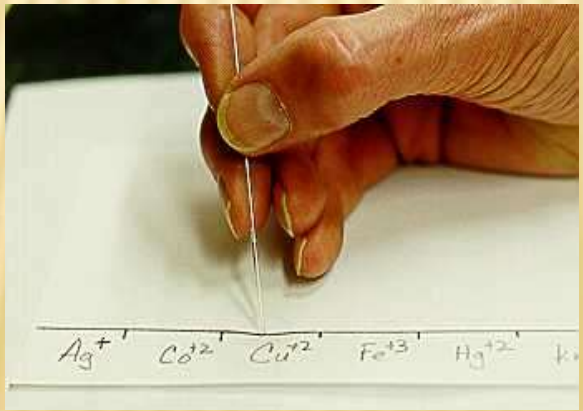
- Choice of suitable solvent for making solution is very important. Pure solutions can be applied direct on the paper but solids are always dissolved in small quantity of a suitable solvent.
- Biological tissues are treated with suitable solvents and their extracts obtained. Proteins can be precipitated with alcohol and salts can be removed by treatment with ion exchange resin.

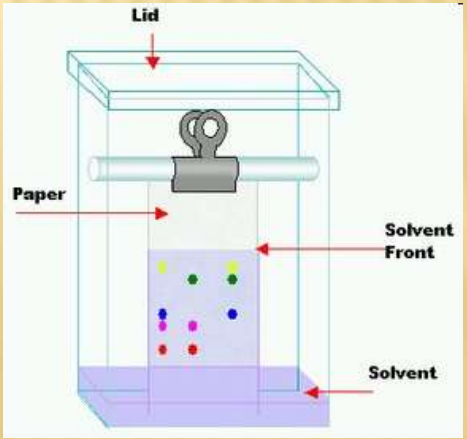
•APPLICATION OF SAMPLE

The sample to be applied is dissolved in the mobile phase and applied as a small spot on the origin line, using capillary tube or micropipette.

very low concentration is used to avoid larger zone

- The spot is dried on the filter paper and is placed in developing chamber.





•CHOICE OF THE SOLVENT

- The commonly employed solvents are the polar solvents, but the choice depends on the nature of the substance to be separated.
- If pure solvents do not give satisfactory separation, a mixture of solvents of suitable polarity may be applied.

- **Mobile Phase**
- Pure solvents, buffer solutions or mixture of solvents
- Examples- **Hydrophilic mobile phase**

  Isopropanol: ammonia:water 9:1:2

  Methanol : water 4:1

  N-butanol : glacial acetic acid : water 4:1:5

Hydrophobic mobile phases

dimethyl ether: cyclohexane

kerosene : 70% isopropanol

•CHROMATOGRAPHIC CHAMBER

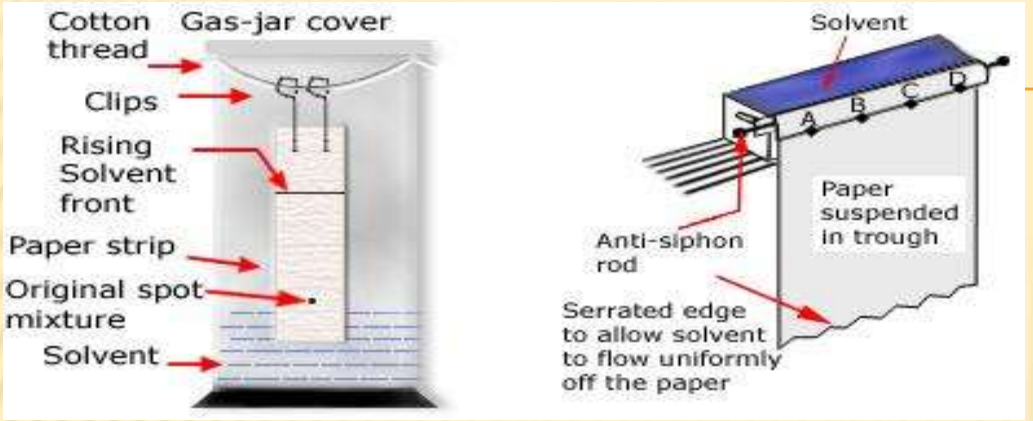
The chromatographic chamber are made up of many materials like glass, plastic or stainless steel.

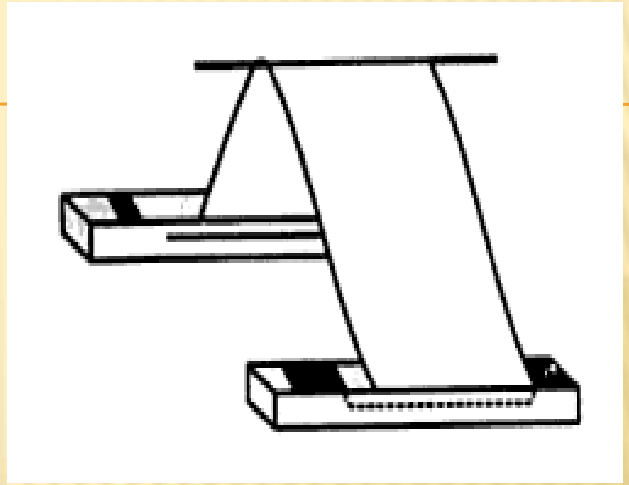
Glass tanks are preferred most. They are available in various dimensional size depending upon paper length and development type.

The chamber atmosphere should be saturated with solvent vapor.

•DEVELOPMENT TECHNIQUE

- Paper is flexible when compared to glass plate used in TLC, several types of development are possible which increases the ease of operation.
- The paper is dipped in solvent in such a manner that the spots will not dip completely into the solvent.
- The solvent will rise up and it is allowed to run $2/3^{rd}$ of paper height for better and efficient result.

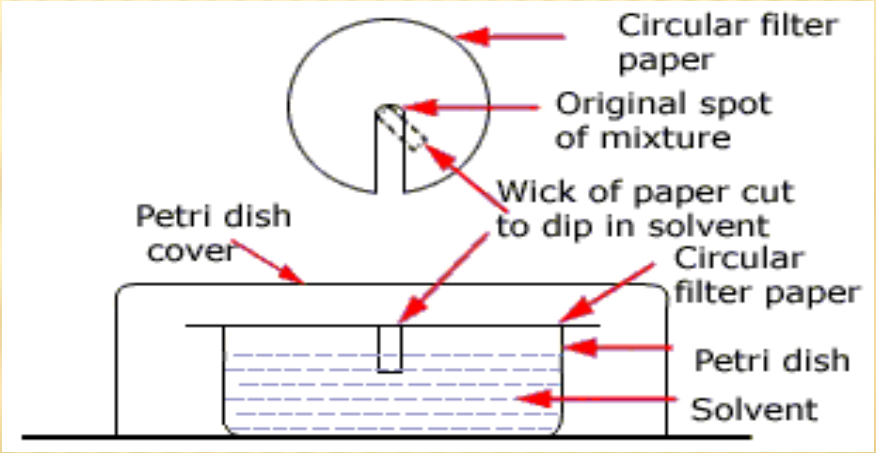




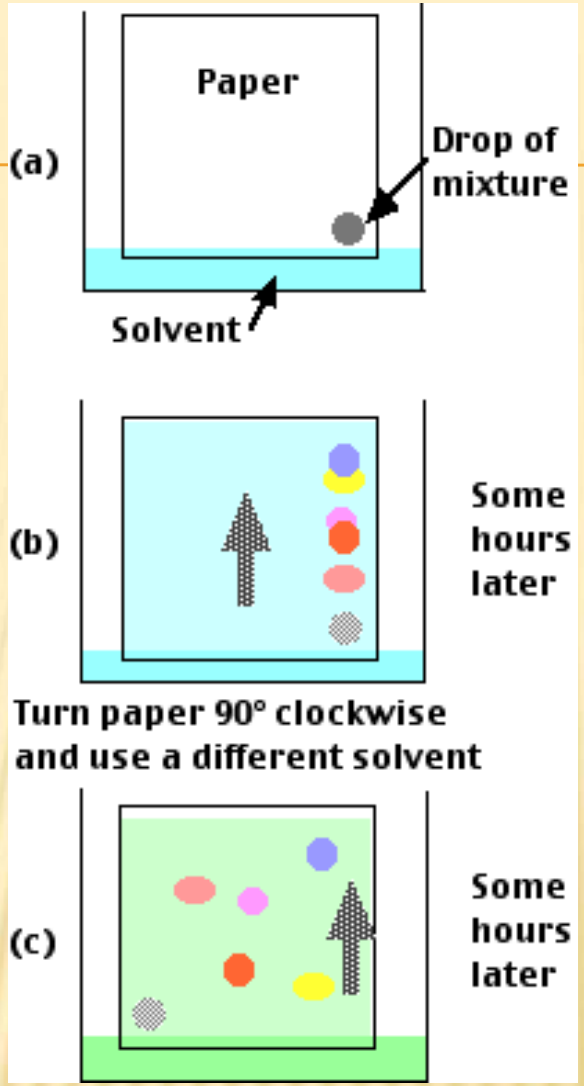
Ascending

Descending

Ascending-Descending



Radial



TWO DIMENSIONAL

Drying of chromatogram

- After the solvent has moved a certain distance for certain time the chromatogram is taken out from the tank & position of the solvent front is marked with a pencil.

- They are dried by cold or hot air depending on volatility of solvents. A simple hair dryer is a convenient device to dry chromatograms.

- **Detecting /visualizing agents**

If the substance are colored they are visually detected easily.

But for colorless substance, Physical and chemical methods are used to detect the spot.

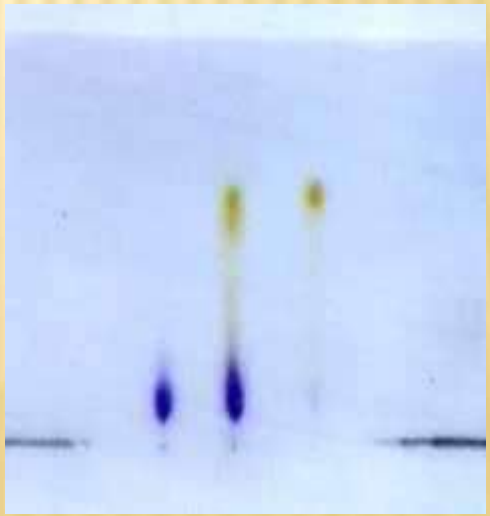
(a) Non specific methods (Physical methods)

E.g. iodine chamber method,

UV chamber for fluorescent compounds – at 254 or at 365nm.

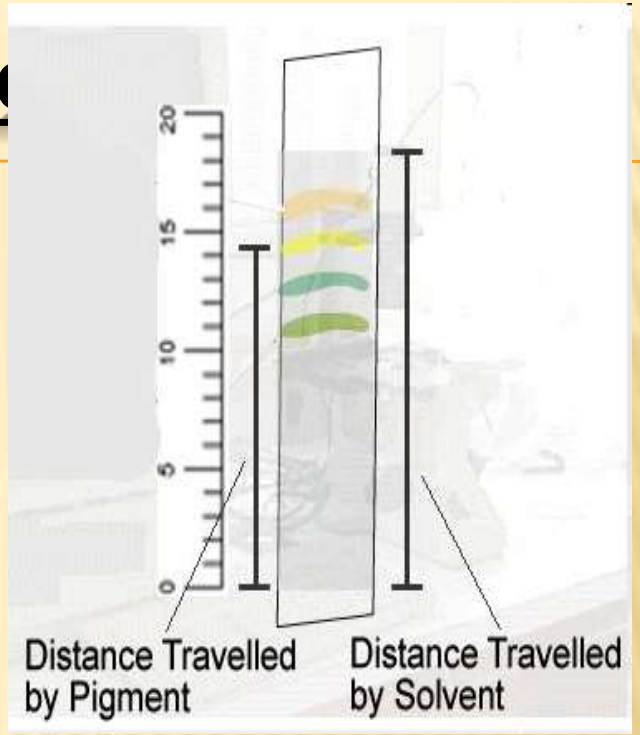
(B) SPECIFIC METHODS (CHEMICAL METHODS) OR SPRAYING METHOD - EXAMPLES,

- Ferric chloride
- Ninhydrin in acetone
- Dragendroff's reagents
- 3,5 dinitro benzoic acid
- Phenolic comp. & tannins
- Amino acids
- Alkaloids
- Cardiac glycosides



RF VALUE (RETARDATION FA

In paper chromatography the results are represented by Rf value which represent the movement or migration of solute relative to the solvent front.



The Rf value is calculated as :

$$\frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front}}$$

APPLICATIONS

- Separation of mixtures of drugs
- Separation of carbohydrates,vitamins,antibiotics,proteins,….
- Identification of drugs
- Identification of impurities
- Analysis of metabolites of drugs in blood , urine ….
- Used in the separation of various organic mixture.
- Used in almost all area to solve complicated problems in chemistry, biology, biochemistry.
- Used for both qualitative and quantitative analysis.

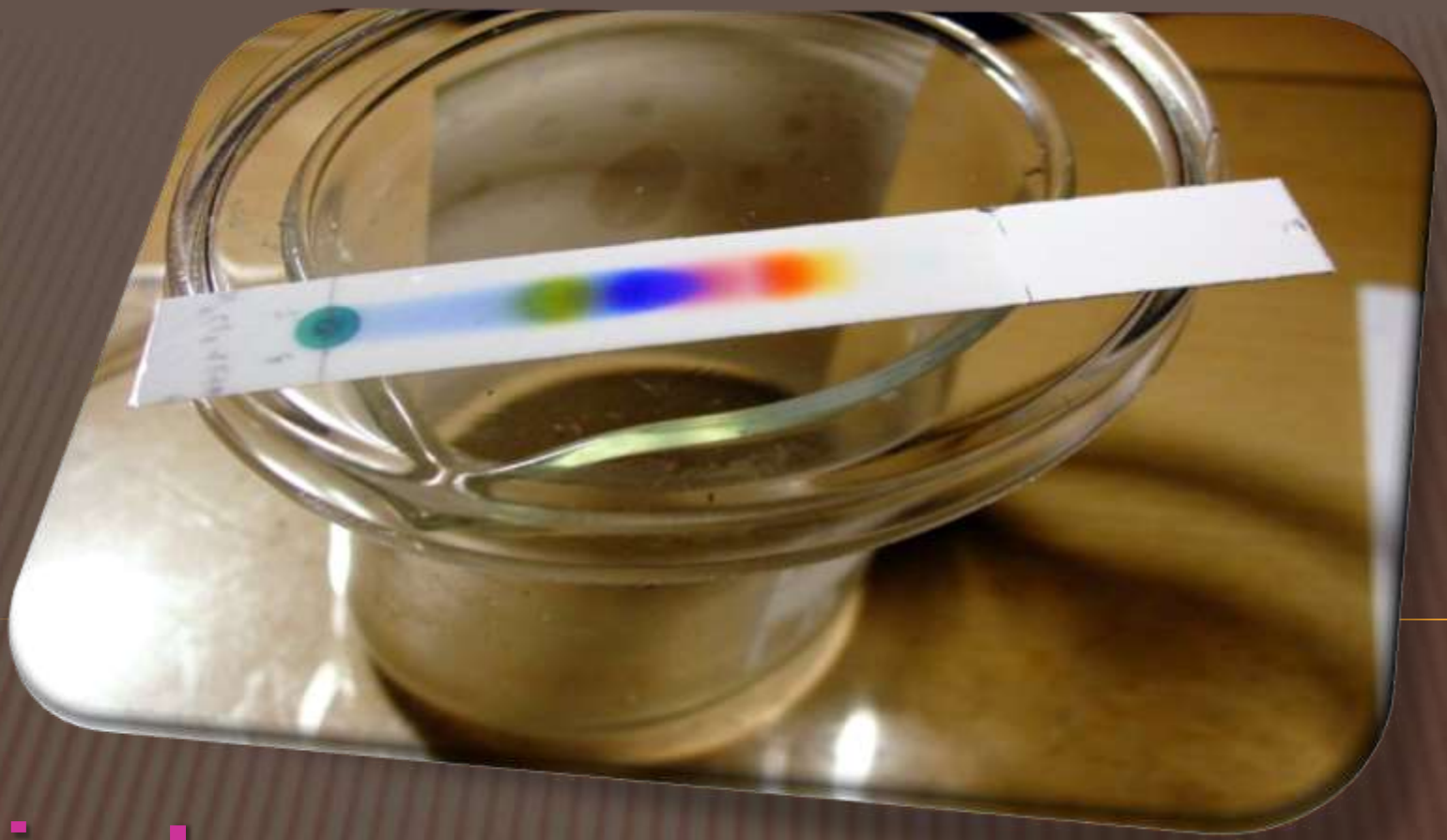
ADVANTAGES OF P.C

Simple ,rapid ,inexpensive ,excellent resolving power

PRECAUTIONS IN P.C

Establishing the vapor solvent equilibrium

Stability of solvent mixture is first ensured



Thin Layer chromatography

INTRODUCTION

In 1944, Consden,Gorden & Martin used filter papers for separating the Amino acids.

In 1950, Kirchner identified terpenes on filter paper.

In 1958,**STAHL** develop standard equipment for analysing by **THIN LAYER CHROMATOGRAPHY** .

PRINCIPLE

The Principle is based on ADSORPTION Chromatography

The component with more affinity towards the stationary phase travels slower.

The component with lesser affinity towards the stationary phase travels faster.

REVERSE PHASE CHROMATOGRAPHY

In this the stationary phase is Non-polar & mobile phase is polar & it is widely used in pharmaceutical analysis.

1) Polar compound get eluted first
2) Non-polar compounds are retained for long time

Comparison of Normal phase & Reverse phase :

	Normal phase	Reverse phase
Stationary phase	Polar	Non-polar
Mobile phase	Non-polar	Polar
Compound eluted first	Non-polar	Polar
Compound eluted last	Polar	Non-polar
Example of stationary phase	Silica gel	C_4 ,c_8 –bonded phase

STATIONARY PHASE

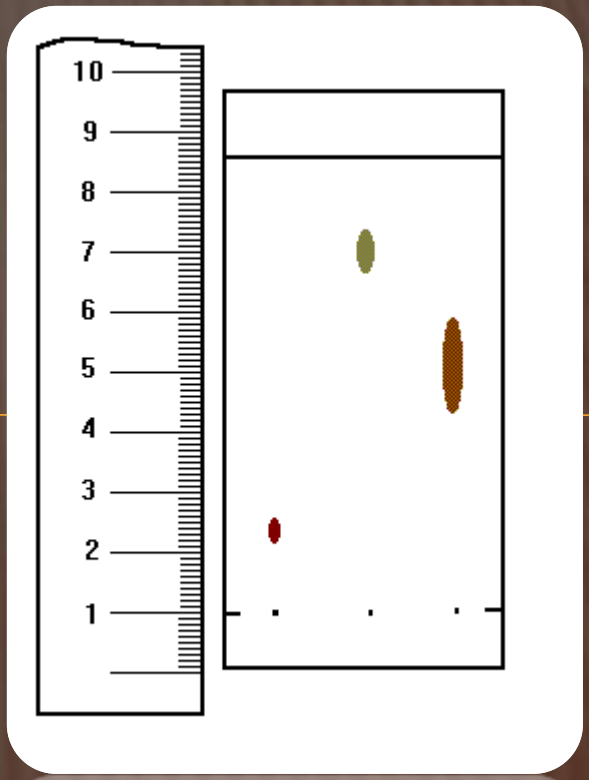
NAME	COMPOSITION
Silica gel H	Silica gel without binder
Silica gel G	Silica gel + $CaSO_4$
Silica gel GF	Silica gel + Binder + fluorescent indicator
Alumina	Al_20_3 Without Binder
Al_20_3 G	Al_20_3 + Binder
Cellulose powder	Cellulose Without Binder
Cellulose powder	Cellulose With Binder
Kieselguhr G	Diatomaceous earth + binder
Polyamide powder	Polyamide
Fuller's earth	Hydrous magnesium alumina
Magnesium Silicate	magnesol

MOBILE PHASE

1) Nature of the substance to be separated i e weather it is polar or non-polar.
2) Mode of Chromatography.
3) Nature of Stationary phase.
4) Mode Separation i e Analytical or Preparative.

Examples: 1) Petroliam ether 2) Cyclohexane
3) Acetone 4) Toluene
5) Ethyl acetate 6) Benzene
7) Alcohols 8) Water
9) Chloroform 10) Pyridine

GLASS PLATES



Three types : they are ,

1) Full plate : 20cm × 20 cm.
2) Half plate : 20cm × 10 cm.
3) Quarter plate : 20cm × 5 cm.

Microscopic slides can also be used for Monitoring the progress of a chemical reaction.

PREPARATION & ACTIVATION OF PLATES

The T L C plates can be prepared by following techniques :

1) Pouring.
2) Dipping.
3) Spraying.
4) Spreading.

Activation :

It is nothing but removing of water/ moisture & other adsorbed substance from the surface of any adsorbent by heating.

APPLICATION OF SAMPLE

The concentration of the sample should be 2-5µl of a 1% solution.

Sample is spotted using a capillary tube or micropipette.

Spots can be placed at random process .

Spots should be kept atleast 2cm above the base of the plate.

Spotting area should not be immersed in the Mobile phase.

Go for development.

DEVELOPMENT TANK

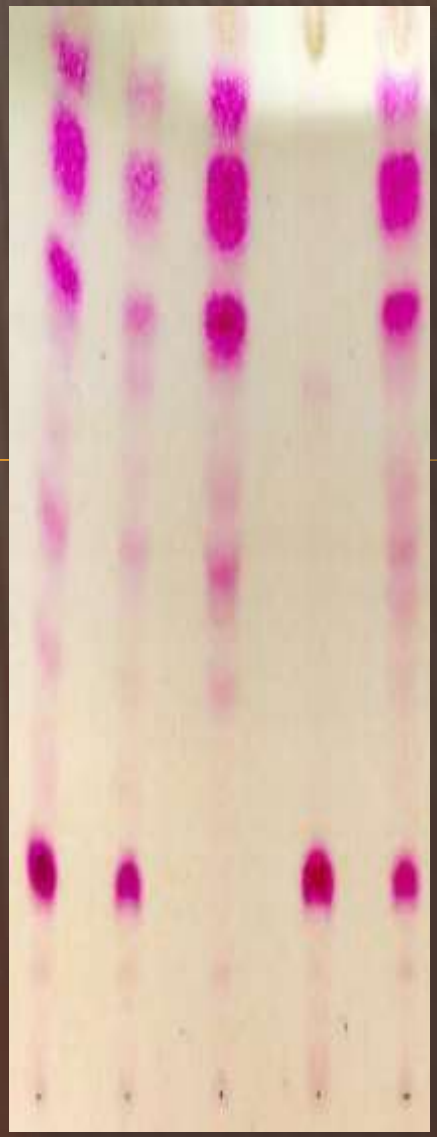
The development tank should be lined Inside with filter paper moistened with mobile phase to saturate the atmosphere& also prevent the

“ EDGE EFFECT ” .

DEVELOPMENT TECHNIQUE

Different development techniques are :

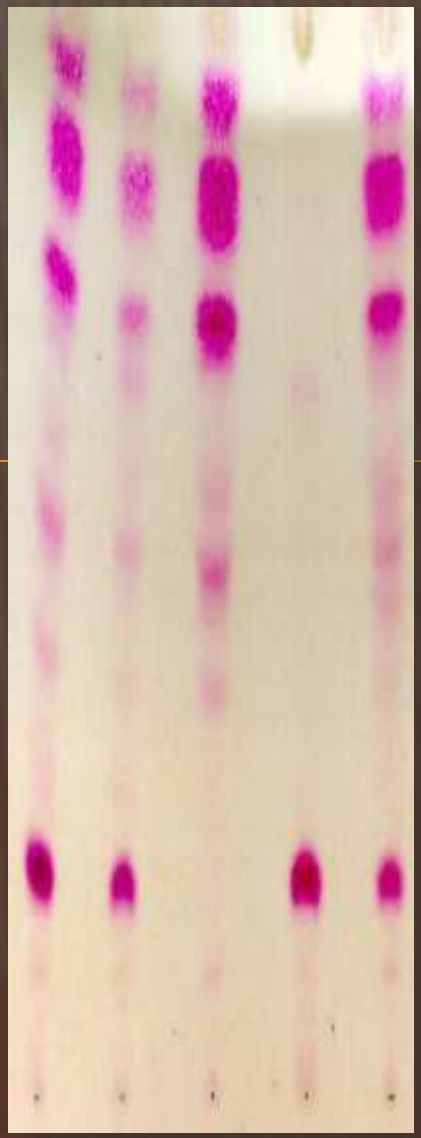
1) One dimensional development.
2) Two dimensional development.
3) Horizontal development.
4) Multiple development.



DEVELOPMENT TECHNIQUE

Different development techniques are :

1) Ascending development.
2) Descending development.
3) Horizontal development.
4) Multiple development.



DETECTING AGENTS

Detecting agents are two types. they are,

(A) Non-Specific method

1) Iodine chamber method.
2) Sulphuric acid spray method.
3) UV chamber for fluorescent compounds.
4) Using fluorescent stationary phase.

(B) Specific method

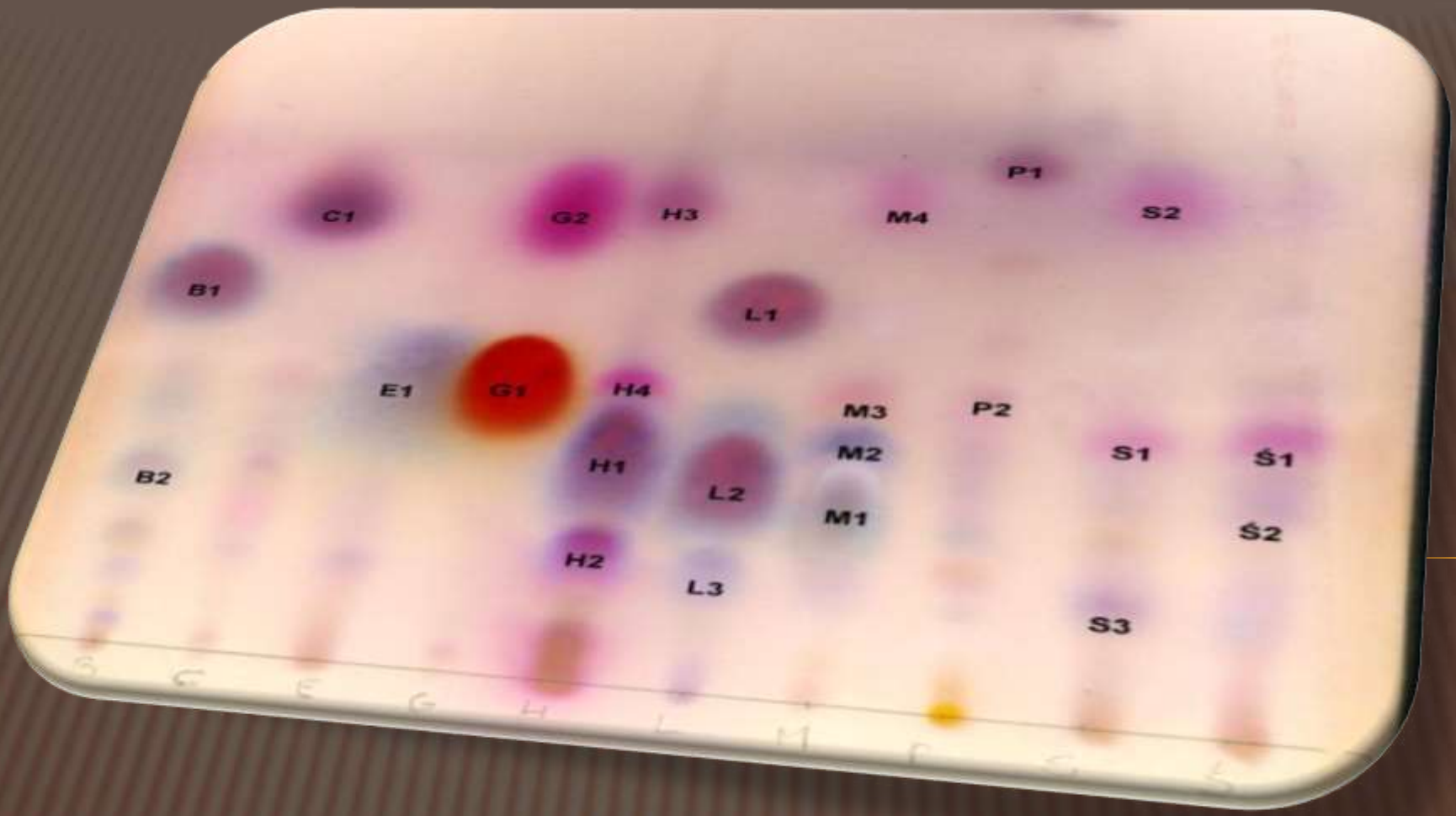
1) Ferric chloride.
2) Ninhydrine in acetone.
3) Dregendroff reagent.
4) 3,5 – Dinitro benzoic acid.
5) 2,4 - Dinitro phenyl hydrazine.

DETECTION

The R_f value is calculated for identification "Rf value is the ratio of distance travelled by

The solute to the distance travelled by the solvent front"

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$



DEVELOPMENT OF T L C

APPLICATION

1) **Separation of mixture of drug of chemical,biological,plant origin.**
2) **Separation of Carbohydrates, vitamin, antibiotics, proteins, etc.**
3) **Identification of drug. Ex :Amoxicillin, Levodopa**
4) **Detection of foreign substances.**
5) **To detect the decomposition products of drug.**

COMPARISON OF TLC & PAPER

TLC CHROMATOGRAPHY	PAPER CHROMATOGRAPHY
It requires less amount of the substance.	It requires more amount of the substance.
Less time consuming	More time consuming
Sharpness of separation is good	Sharpness of separation is less than TLC
Capacity of thin layers of adsorbent is higher.	Less in paper chromatography.
Strong acids can be safely identified.	It is not possible in paper
Corrosive reagents may be coated on glass plate.	Also not possible in paper
Physical strength is more	Physical strength is less
It can be heated in oven	Not possible
Sensitivity is more	Sensitivity is less

THANK YOU