Column Chromatography

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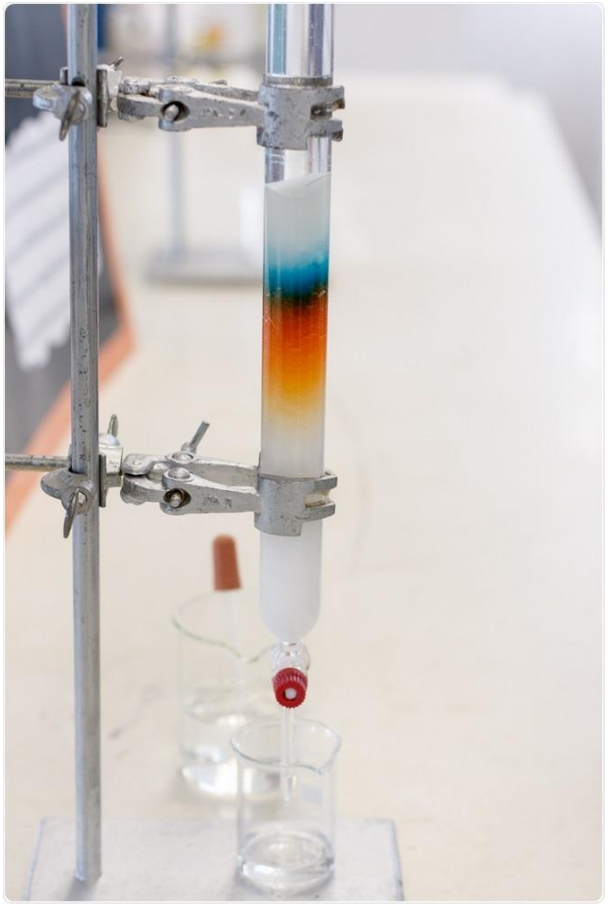


The principle of separatation

Concentration of separated compound in these phases (stationary and mobile) is determined by distribution coeficient $\mathbf{K_d}$

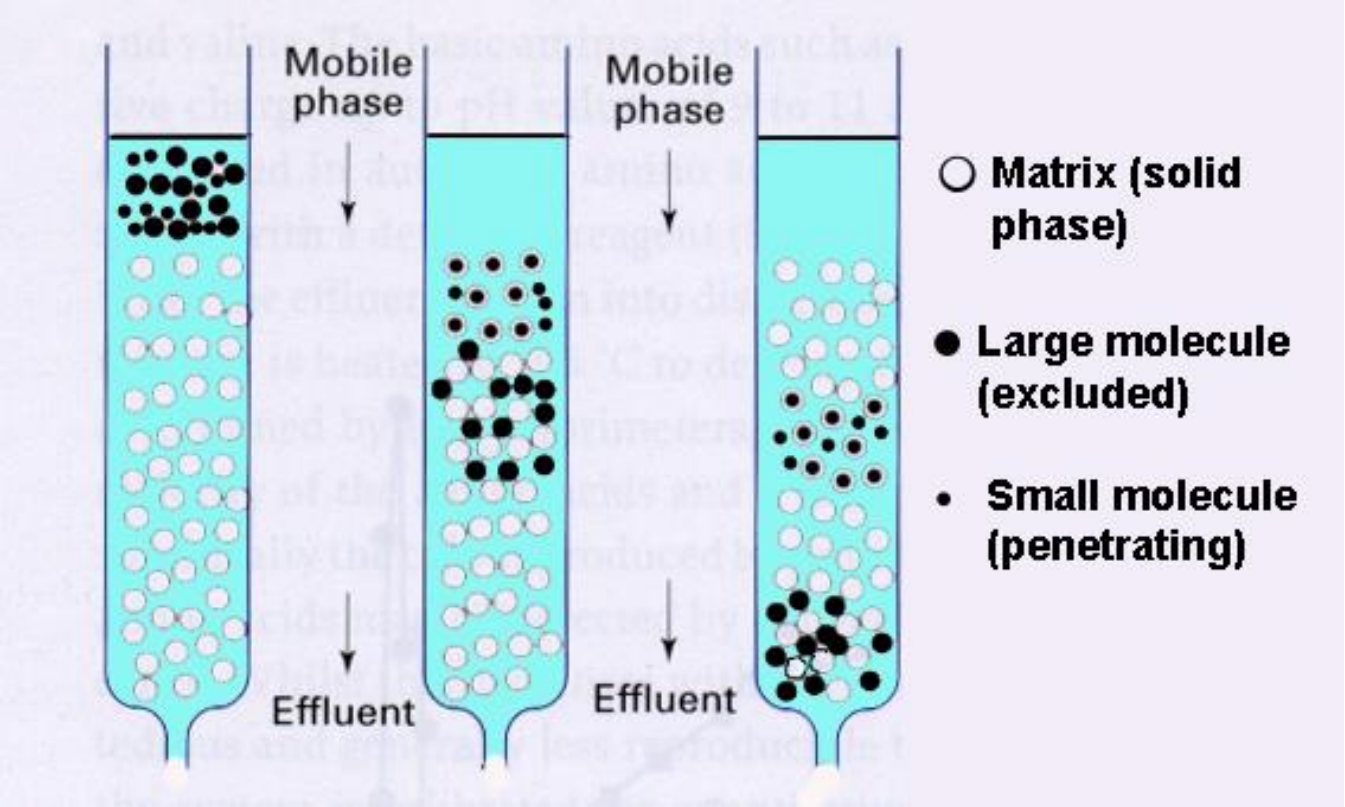
$$\mathbf{K_d = c_s / c_m}$$

Compounds are sepatared when their distribution koeficients in choosen mobile and stationary phase are different

Liquid chromatography

1. **an adsorption equilibrium** (between statinary solid phase and mobile liquid phase) - adsorption chromatography, hydrophobic chromatography
2. **a partition equilibrium** (between stationary liquid phase and mobile liquid phase) - partition chromatography, reversed-phase liquid chromatography

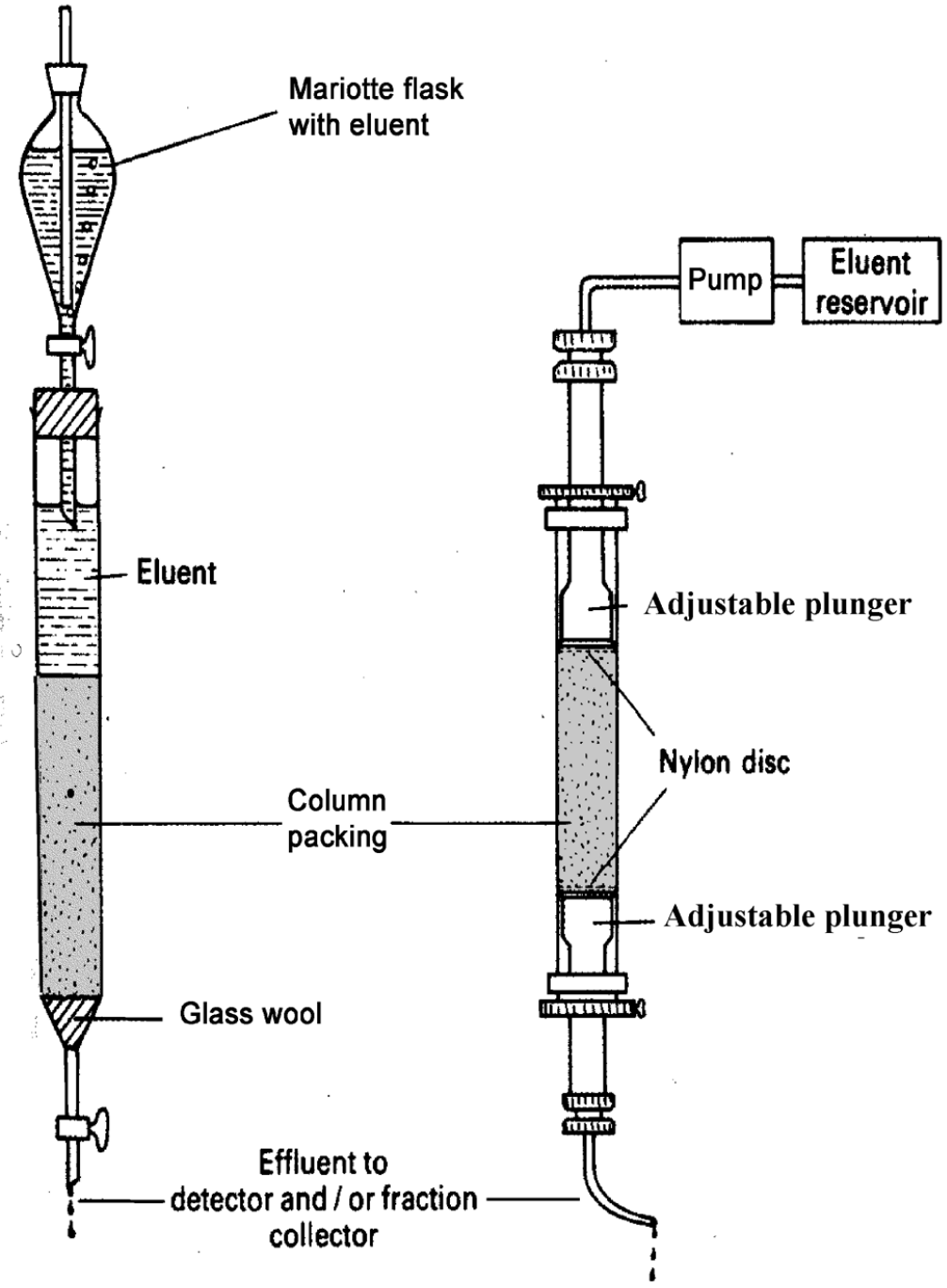
3. **<u>an equilibrium between liquid phase trapped inside pores of stationary porous material and mobile liquide phase</u>** - permeation chromatography or molecular exclusion chromatography



4. **an ion-exchange equilibrium** (between stationary ion exchanger and mobile electrolyte phase) - ion-exchange chromatography
5. **an affinity equilibrium** (between stationary immobilised ligand and mobile liquid phase) - affinity chromatography (e.g. immunoaffinity chromatography, lectin affinity chromatography, dye-ligand chromatography)

Models of liquid chromatography

- column chromatography - stationary phase attached to suitable matrix (insoluble support) is packed in glass or metal column and mobile phase is passed through column by gravity or pump
- planar chromatography - suitable matrix is coated in thin layer onto a glass, plastic or metal plate (special case is a filter paper) and mobile phase is passes across the thin layer by capillary action - thin-layer chromatography, paper chromatography



Column chromatography

Development modes of chromatography

Sample is dissolved in a suitable solvent and applied to stationary phase as a narrow dicrete band. Sample component is called analytes.

- **elution (zonal) development** - mobile phase called elluent flows continously over the stationary phase and the analytes with higher solubility in the mobile phase move along the stationary phase more rapidly. Analytes are eluted when they have been removed from column.
- **displacement (affinity) development** - mobile phase contains specific solutes with higher affinity for stationary phase than separated analytes

Retention time and Retention volume

- "Retention time (t_r) is the elapsed time from the introduction of sample to the peak maximum". or in other words "t_r is the time a solute takes from the point of injection to the detector.

Retention volume (V_r) → The volume of mobile phase eluting between solute's introduction and appearance of solute to the peak maximum. In other words.

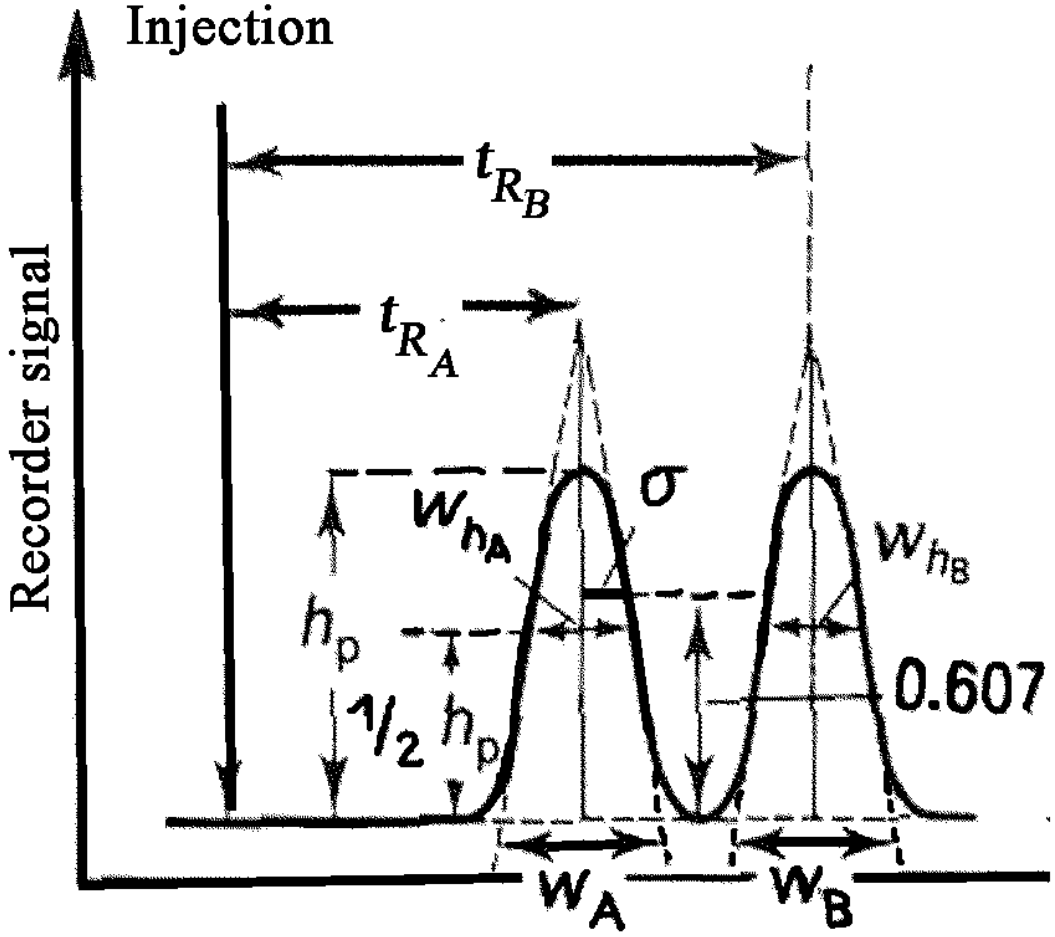
Retention volume is the volume of mobile phase needed to move a solute from ~~introduction to the~~ point of injection to the detector.

Baseline width (W) → The width of a solute's Chromatographic band measured at the baseline. Baseline width is measured in units of time or volume

A small peak results from the solutes that move through the column at the same rate as the mobile phase. These solutes do not interact with the stationary phase and are considered as nonretained.

The time or volume of mobile phase required to elute nonretained components are called void time (t_M) or void volume respectively.

Theory of chromatographic separation



t_R retention time

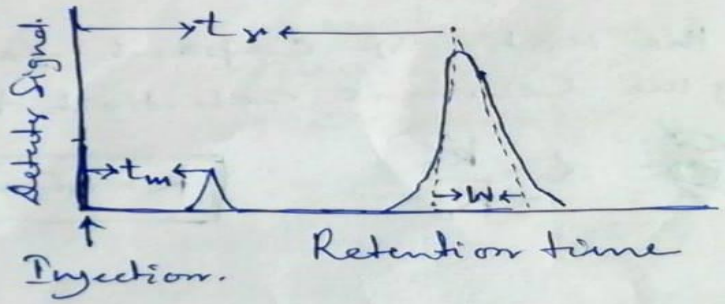
h_P height of peak

σ - standard deviation of Gaussian peak

Void volume V_0 - volume of mobile phase in column (analyte that does not interact with stationary phase is eluted in this volume)

Dead time t_M - the time taken to pass throuhg void volume of column

Resolution

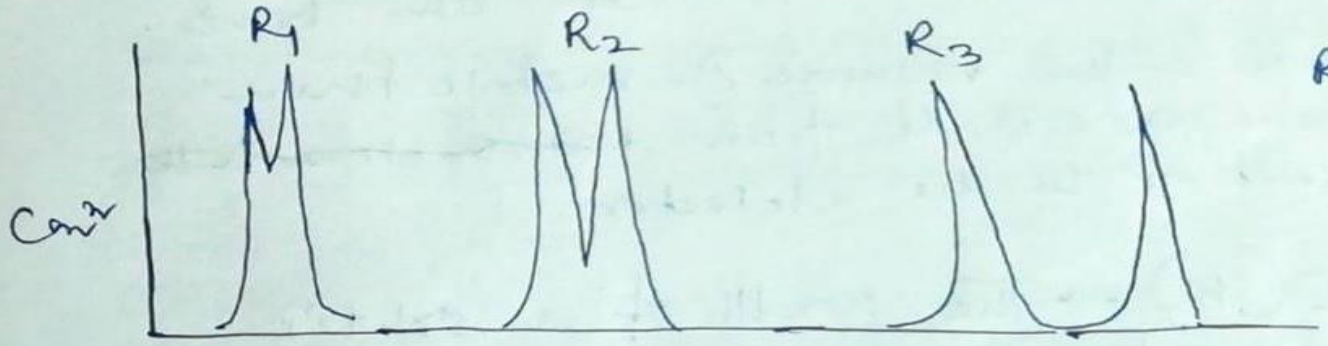


Resolution ⇒ Resolution is the quantitative measure of the degree of separation between two peaks (A & B) and represents as.

$$R = \frac{t_{r(B)} - t_{r(A)}}{0.5(W_B + W_A)} = \frac{2\Delta t_r}{W_B + W_A}$$

High R value indicates better separation.

From the eqn it is clear that R can be increased by increasing Δt_r or by decreasing W_B or W_A.

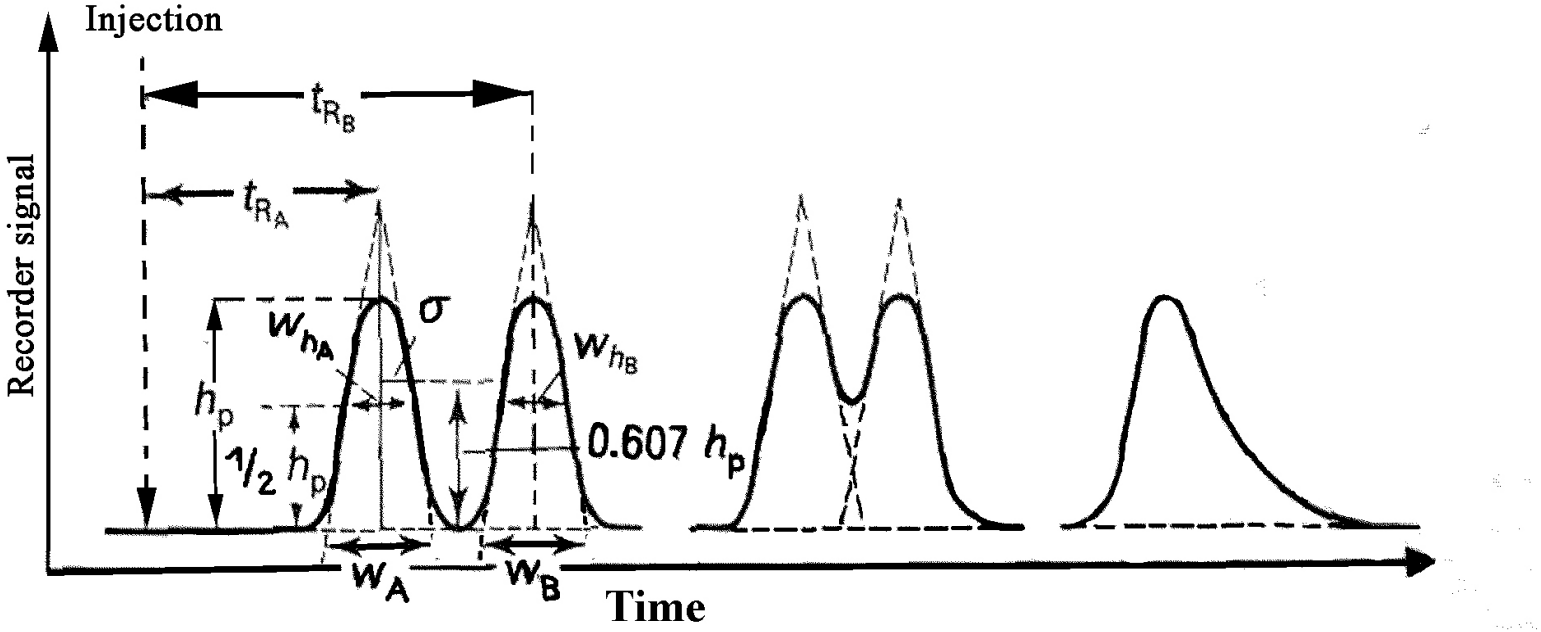


$R_3 > R_2 > R_1$

Resolution R_S

is ratio of the difference in retention times between two peaks to mean of their base width

$$R_S = \frac{2(t_{R_A} - t_{R_B})}{w_A + w_B}$$



Capacity factor & Selectivity factor

- **Capacity factor (k')** → A measure of how strongly a solute is retained by the stationary phase, this can be measured by measuring the column's void time t_m and solute's retention time t_r.

$$k' = \frac{t_r - t_m}{t_m}, \text{ or } k' = \frac{t_r'}{t_m}$$

- $(t_r - t_m) = t_r'$ is known as adjusted retention time.

- **Selectivity factor (α)** → The ratio of capacity factors for two solutes showing the column's selectivity for one of the solutes

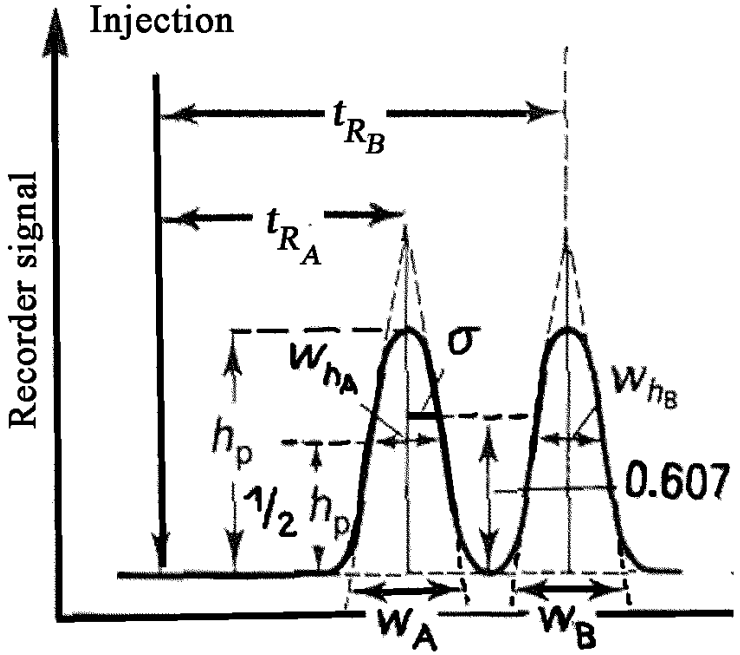
$$\alpha = \frac{k'_A}{k'_B}$$

k = Small k

$$\text{or } \alpha = \frac{t_{r_B} - t_m}{t_{r_A} - t_m}$$

α is equal to 1 when solutes elute with identical retention time.

Theory of chromatographic separation
capacity and separation factor



Capacity factor k´

factor expressing proportion of mass of the analyte in the stationary and mobile phase

$k' = M_S/M_M$

$$k' = \frac{t_R - t_M}{t_M}$$

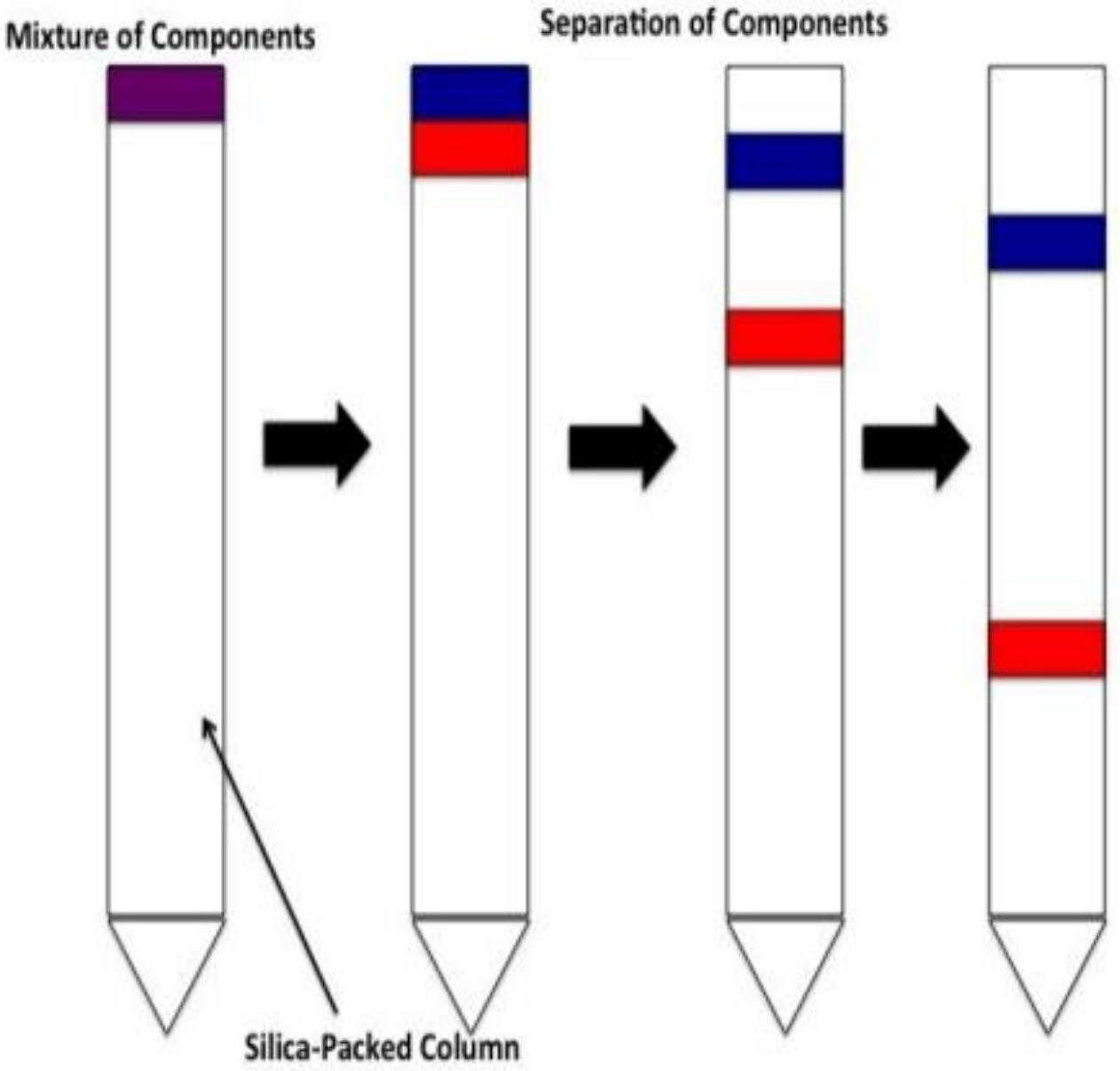
Selectivity (separation factor) α

ratio of capacity factors of two analytes

$$\alpha = \frac{k'_A}{k'_B} = \frac{t_{R_A} - t_M}{t_{R_B} - t_M}$$

Plate Theory

- The theory assumes that the column is divided into a number of zones called *theoretical plates*.
- At each plate equilibrium of the solute between the mobile phase & the stationary is assumed to take place.
- The partitioning of a solute between the phases takes plate at each theoretical plate.
- Thus, the number theoretical plates in the column is used as a measure of efficiency of the column to separate the components from each other


Mixture of Components
Separation of Components
Silica-Packed Column

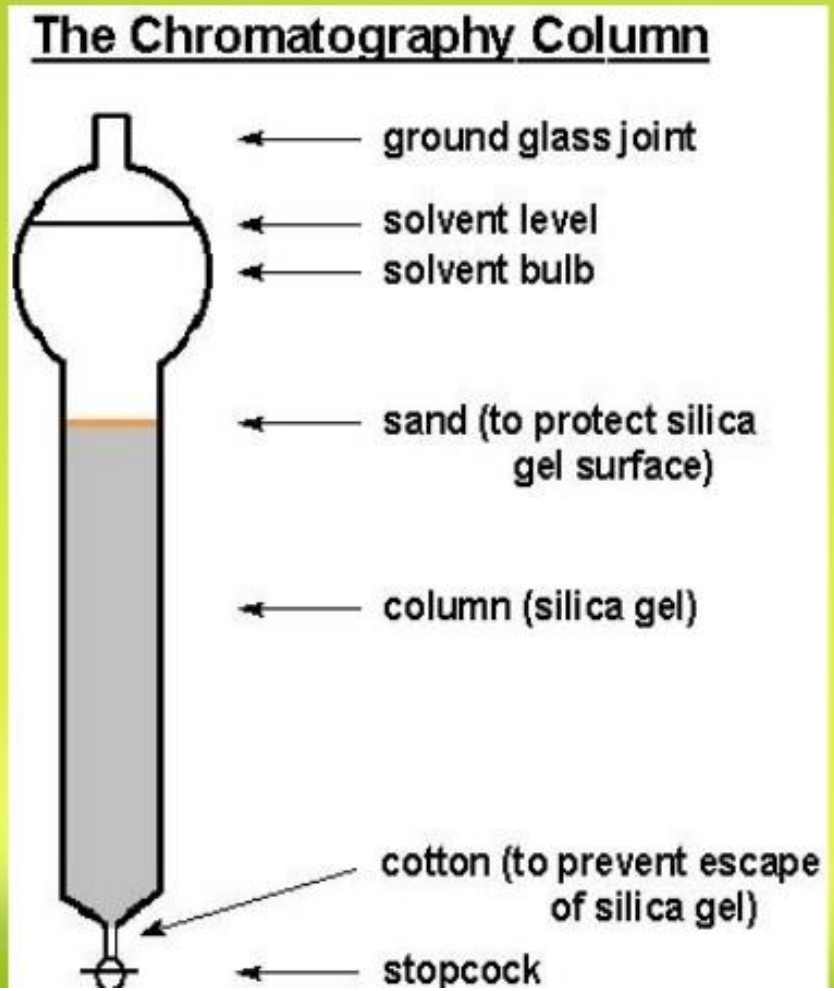
COLUMN CHROMATOGRAPHY

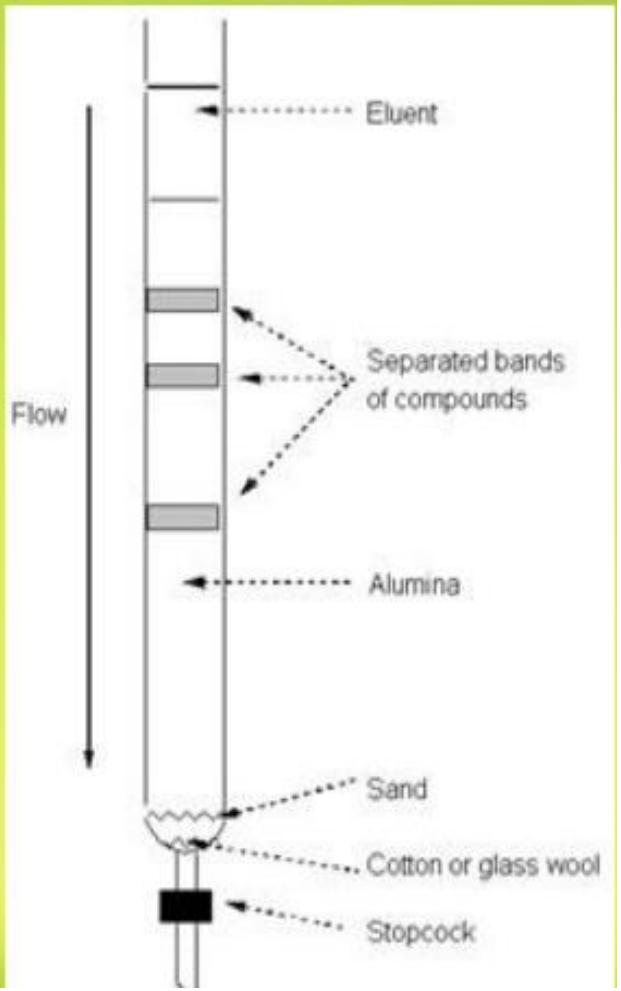
- Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids.
- This is a solid - liquid technique in which the stationary phase is a solid & mobile phase is a liquid.

PRINCIPLE

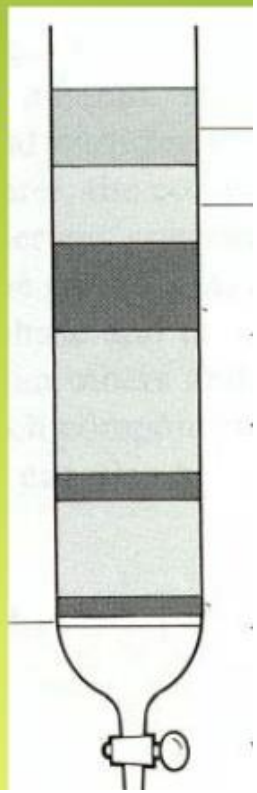
- **Adsorption**
- Mixture of components dissolved in the M.P is introduced in to the column. Components moves depending upon their relative affinities.

Adsorption column chromatography, the adsorbent, packed in a glass column, and a solvent, the mobile phase, that moves slowly through the packed column. A solvent used as a mobile phase is called an eluent.





- A compound attracted more strongly by the mobile phase will move rapidly through the column, and elute from, or come off, the column dissolved in the eluent.
- In contrast, a compound more strongly attracted to the stationary phase will move slowly through the column.



- **Adsorbents:** The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc.,
- Alumina is generally suitable for chromatography of less polar compounds. Silica gel gives good results with compounds containing polar functional groups.

- **Adsorbent in C.C should meet following criteria**

  - Particles should be spherical in shape & uniform in size
  - Mechanical stability must be high
  - They shouldn't react chemically
  - It should be useful for separating for wide variety of compounds
  - It should be freely available & inexpensive

  (The particle size of the commercially available grade is in the range 50 – 200 μm.)

Selection of Stationary Phase

- Success of chromatography depends upon proper selection of S.P, it depends on the following:

1. Removal of impurities
2. No. of components to be separated
3. Length of the column used
4. Affinity differences b/w components
5. Quantity of adsorbent used

Mobile Phase

- They act as solvent, developer & eluent. The function of a mobile phase are:
- As developing agent
- To introduce the mixture into the column – as solvent
- To developing agent
- To remove pure components out of the column – as eluent

The choice of the solvent is depend on the solubility characteristics of the mixture. The solvents should also have sufficiently low boiling points to permit ready recovery of eluted material.

However, polarity as seen the most important factor in adsorption chromatography.

Different mobile phases used: (in increasing order of polarity)

Petroleum ether, carbon tetrachloride, cyclohexane, ether, acetone, benzene, toluene, esters, water, etc

It can b e used in either pure form or as mixture of solvents

COLUMN CHARACTERISTICS

- The main function of all the columns is to support the stationary phase.
- The material of the column is mostly good quality neutral glass since it shouldn't be affected by solvents. An ordinary burette can also be used as column for separation.
- Column dimensions - length & diameter ratio (10:1,30:1 or 100:1)

- The length of the column depends upon:
- Number of compounds to be separated
- Type of adsorbent used
- Quantity of the sample
- Affinity of compounds towards the adsorbent used

- Better separation will be obtained with a long narrow column than short thick column because number of plates will be more.

Let’s watch the video

Video on column chromatography

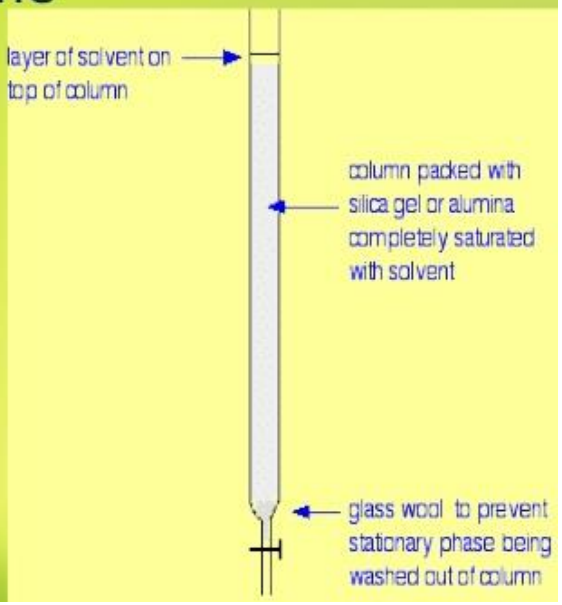
https://youtu.be/frmPbmXmvaQ

Preparing the column

It consists of a glass tube with bottom portion of the column – packed with glass wool/cotton wool or may contain asbestos pad,

Above which adsorbent is packed

After packing a paper disc kept on the top, so that the adsorbent layer is not disturbed during the introduction of sample or mobile phase.



Packing technique

- There are two types of preparing the column, they are:
- **i. Dry packing / dry filling**
- **Ii. Wet packing / wet filling**

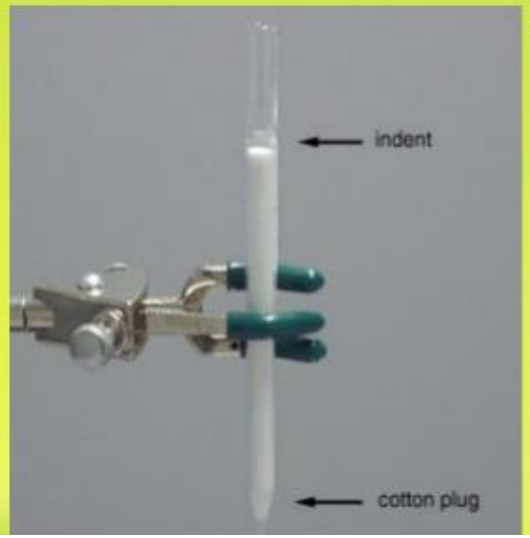
- The column should be free from impurity, before using column, it should be washed properly and dry it.
- Before filling column with stationary phase, cotton/glass wool is kept
- It should be uniformly filled

Dry packing

- Adsorbent is packed in the column in dry form
- Fill the solvent, till equilibrium is reached

DEMERIT: Air bubbles are entrapped b/w M.P & S.P→ cracks appear in the adsorbent layer.

- After filling tapping can be done to remove void spaces.


indent
cotton plug

Wet packing

» ideal & common technique

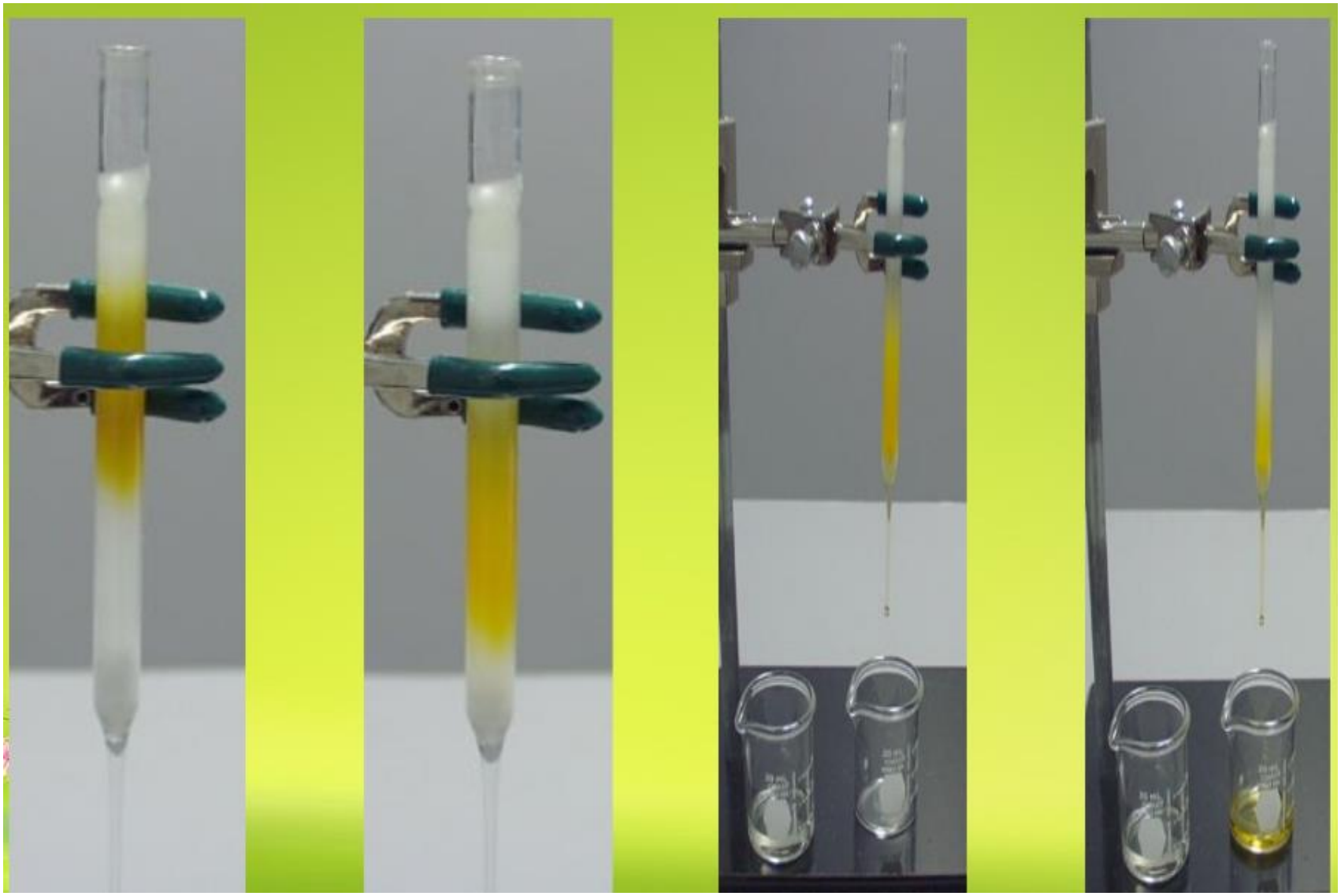
The material is slurried with solvent and generally added to the column in portions.

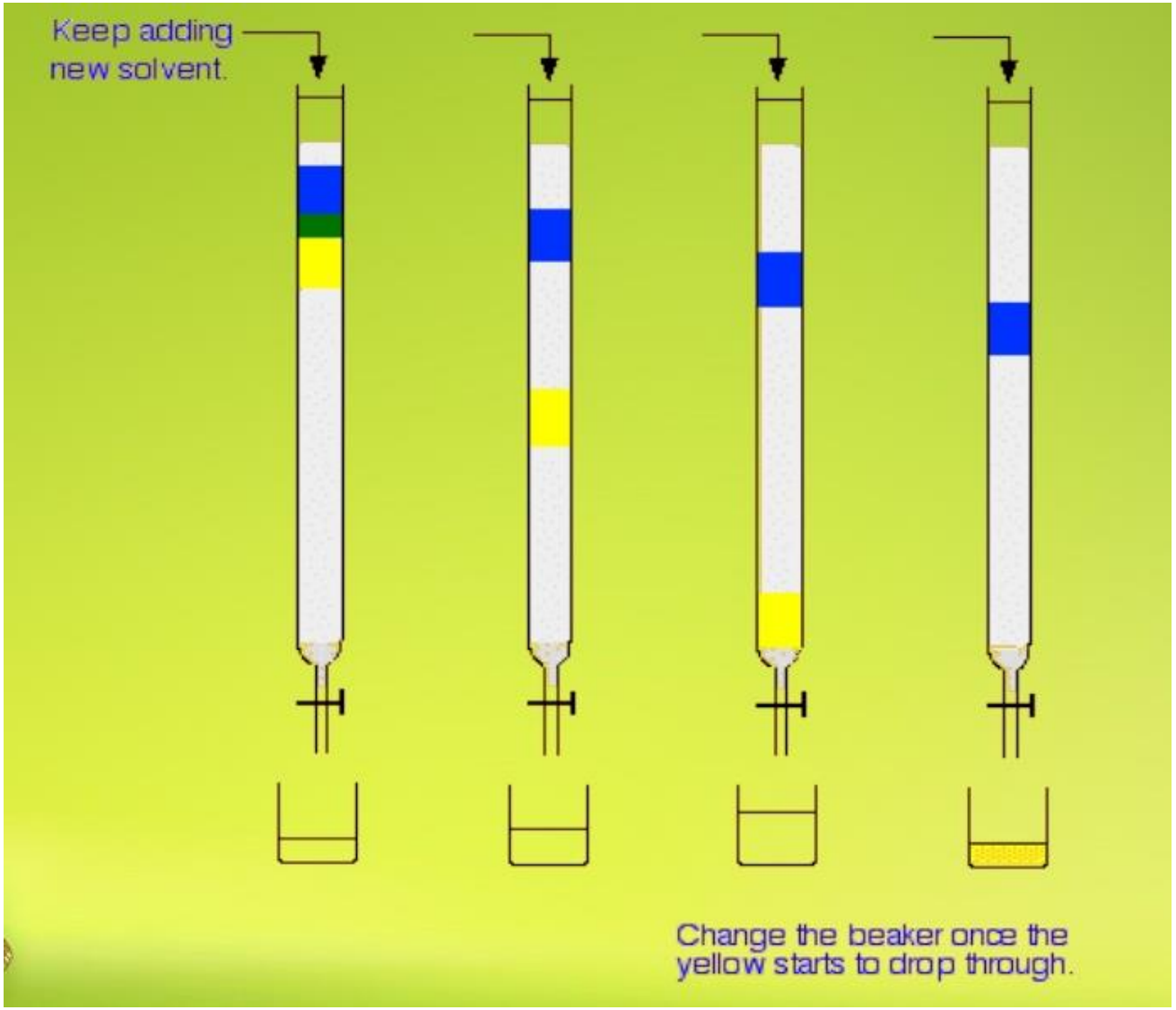
◊ S.P settles uniformly & no crack in the column of adsorbent.

» solid settle down while the solvent remain upward.

» this solvent is removed then again cotton plug is placed.

- The sample which is usually a mixture of components is dissolved in minimum quantity of the mobile phase.
- The entire sample is introduced into the column at once and get adsorbed on the top portion of the column.
- From this zone, individual sample can be separated by a process of elution.




Keep adding new solvent.
Change the beaker once the yellow starts to drop through.

Isocratic Vs Gradient elution

separated out from the column. The two techniques are:

- (i) Isocratic elution technique : in this elution technique , same solvent composition or solvent of same polarity is used throughout the process of separation.
- Example: chloroform only

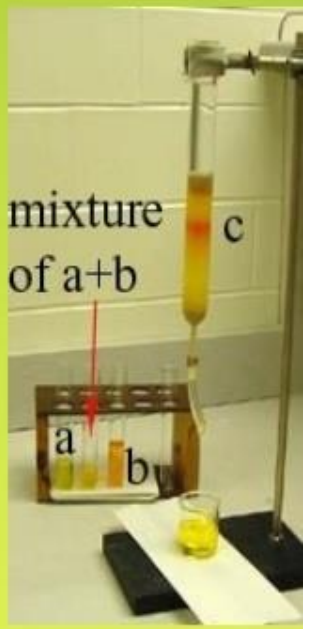
(ii) Gradient elution techniques:
(gradient – gradually)

- Solvents of gradually ↑ polarity or ↑ elution strength are used during the process of separation.
- E.g. initially benzene, then chloroform, then ethyl acetate then chloroform

- DETECTION OF COMPONENTS
- If the compounds separated in a column chromatography procedure are colored, the progress of the separation can simply be monitored visually.

- If the compounds to be isolated from column chromatography are colorless. In this case, small fractions of the eluent are collected sequentially in labelled tubes and the composition of each fraction is analyzed by TLC.

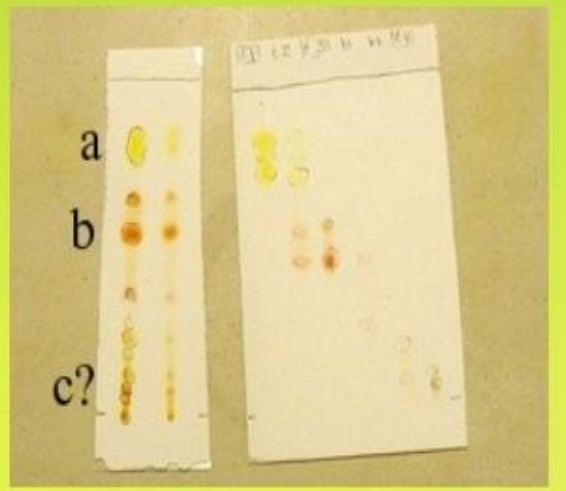
- **Eluting the sample:** Components a, b, and c as column progresses.



- Fractions can be collected in test tubes, beakers, or Erlenmeyer flasks.

Analyzing the fractions:

Analyze the fractions by thin-layer chromatography



Factors affecting column efficiency

Dimension of the column: column efficiency has been improved by increasing length/width ratio of the column.

Particle size of column packing: separation to be improved by decreasing the particle size of the adsorbent.

Activity of the adsorbent

Temperature of the column: The speed of the elution increases at higher temperatures.

Packing of the column

Quality of solvents: solvents having low viscosities is giving better results.

Applications

Separation of mixture of compounds

Purification process

Isolation of active constituents

Estimation of drugs in formulation

Isolation of active constituents

Determination of primary and secondary glycosides in digitalis leaf.

separation of diastereomers

- Advantages of C.C

» Any type of mixture can be separated

» Any quantity of mixture can be separated

» Wider choice of Mobile Phase

» Automation is possible

- Disadvantages of C.C

» Time consuming

» more amount of Mobile Phase are required

» Automation makes the techniques more complicated & expensive