# PAPER-I

## SEPARATION METHODS (UNIT-I)

**SEPARATION :-** It is an operation by which a mixture is resolved into individual components.

⇒ Separation based on size, charge, structure.

⇒ In the separation Technique, we can observe Two phases.

1) Stationary phase
2) Mobile phase

**CHROMATOGRAPHY :-** It is a method of separating mixture of components into individual components through separation process.

In the word chromatography "chroma" means "colour" and "graphy" means "To write". It was 1st invented by "Mikhail Tswett" (1906).

In Chromatography, the components to be separated are distributed between two phases, is a Stationary bed (or) large surface area (or) Bulk area, It is known a Stationary Phase. It may be either solid (or) liquid Coated onto an inert solid support.

The other fluid percolates through the stationary phase, it is known as Mobile phase.

It may be either liquid (or) gas. The transfer of mass between the mobile phase and a stationary phase occurs by adsorption on stationary phase surface are absorbed into partical pores.

Separation of components in a sample is based on the fact that the rate of travel of an individual solute molecule through a column is directly related to partision of that molecule between mobile phase and stationary.

The partition coefficient of each component determines how much of it is in mobile phase at any time. Therefore, the overall time spent in the stationary phase determines the retardation (or) tension of the solute each partical will travel at a rate dependent upon retardation

Then separation takes place, each component emerge from the volume get difficult time interval, the overall process is considered as differential migration phenomenon.

## Principle :-

Chromatography may be defined as a method of separationg a mixture of components into individual components through equilibrium distribution between Two phases. one of the

phase is known as stationary phase and the other phase is known as mobile phase.

Stationary phase is either solid or liquid coated on to an solid support mobile phase may be either liquid (or) a gas.

Separation of compounds in a sample is based on the fact that the rate of travel of the individual solute molecules through a column, the rate of travel depends on the distribution of solute molecule between the mobile and the stationary phase.

distribution is expressed interms of Partition coefficient. Each component will travel through a column at a rate depending upon Partition coefficient, when separation takes place each component emerges from the column at a different time interval.

The Partition coefficient is denoted by "Kd" and is given by

$$K_d = \frac{\text{Con. of solute in stationary phase}}{\text{Con. of solute in Mobile phase}}$$

(or) $K_d = \frac{C_S}{C_M}$

where $C_S \rightarrow$ Con. of solute in stationary phase

$C_M \rightarrow$ Con. of solute in Mobile phase

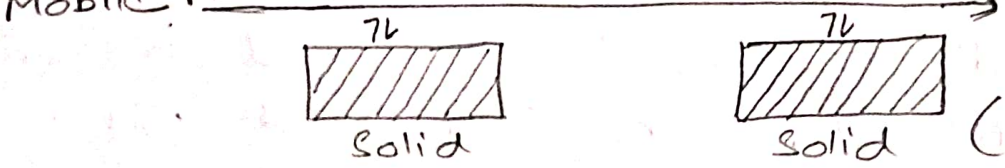
## Classification of chromatographic methods :-

⇒ In chromatography, there are two phases, one of the phase is stationary phase and the other is mobile phase.

⇒ stationary phase may be either solid (or) liquid coated on inert solid materials.

⇒ Mobile phase may be either liquid (or) gas.

⇒ If the stationary phase is solid, the physical principal of separation is the Adsorption, Hence the Technique is called Adsorption chromatography




⇒ If the stationary phase is liquid, the physical principal of separation is Partition. Hence the technique is called Partition chromatography.


⇒ If separation Takes place on the basis of exchange of ions, the technique is called Ion-exchange chromatography.

⇒ If the separation is based on size, the technique is called size-exclusion chromatography.

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→ classification based on stationary phase materials

Chromatographic methods

- Solid stationary phase (Adsorption chromatography)
  - liquid mobile phase
    - eg: 1) CC (column chromatography)
    - 2) TLC
  - gas mobile phase
    - eg: GSC
- liquid coated on a inert support material. (Partition chromatography)
  - liquid M.P
    - eg: 1) PC
    - 2) HPLC
  - gas M.P
    - eg: GLC

→ classification based on mobile phase materials.

Chromatographic methods

- liquid M.P
  - Solid stationary phase (Adsorption chromatography) (LSC)
    - eg: TLC
  - liquid coated on a inert material (Partition chromatography) (LLC (or) LL Partition)
    - eg: PC, HPLC
- Gas M.P
  - Solid stationary phase
    - eg: GSC
  - liquid coated on a material.
    - eg: GLC

1) If only 1 phase is indicated as gas chromo, it is mobile phase.

2) Stationary phase consist of a solid support with a covalently attached anionic ($SO_3^-$) (or) cationic($N(H_3)^+$) functional groups are used in ion-exchange chromatography

3) Porous gels are used as S.P in size-exclusion Chromatography in which seperation is due to differences in the size of the solutes.

## Methods of development :-

The process by which the solute is carried through the stationary phase by mobile phase is known as development.

1) Frontal Analysis :- [No development, No mobile phase]

This consists of a continous addition of mixture sample A&B. In this, first the column is filled with a known amount of stationary phase (or) Adsorbent. After a certain time the solid phase is completely saturated by Adsorbing the mixture. If in the mixture A&B, A has greater Affinity towards stationary phase than B. then "A" is more strongly Adsorbed on a solid stationary phase than B. So, B is eluted first.

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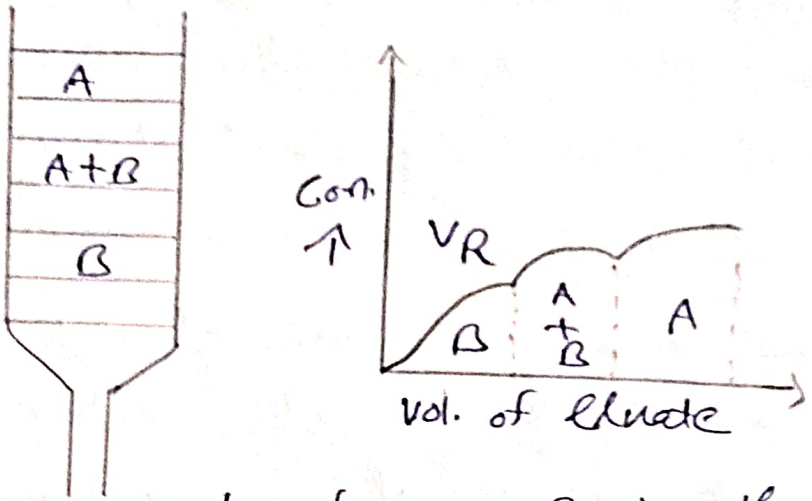
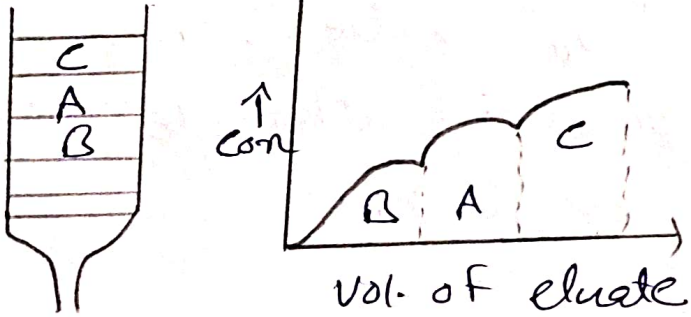



The amount of A or B in the M.P can be Calculated from the Retention volume while from other components can be determined from the volume concentration ratio.

<u>displacement development</u>:-

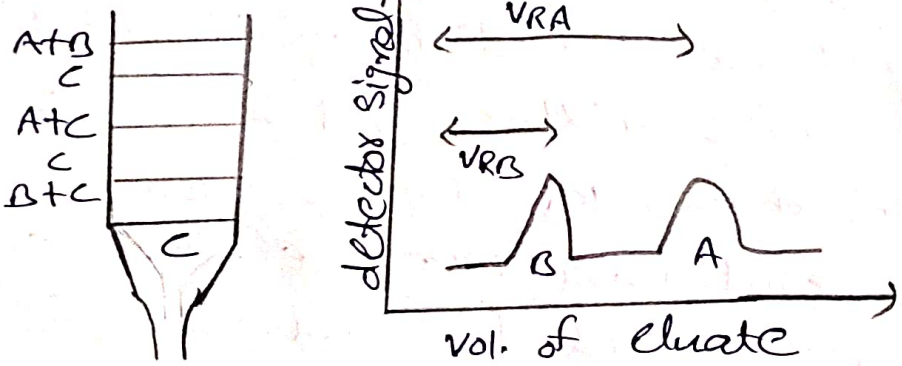
In this, an eluant "c" which has greater Affinity towards stationary phase than A & B is used. The sample mixture A & B is first introduced on the (mobile phase) Top of the column where it adheres to the stationary phase elution occurs. when the displacing eluate containing 'e' is passed through the column displacing on stationary phase during this process the components are separated due to their difference in Partition (or) adsorption Properties.



This method doesnot generally produce, completely separated components between the zones of pure components these are zeligious containing mixture.

Elution development :-

In this also an eluant "e" is used but it has low affixity towards Stationary phase than A&B. Small sample of the mixture is introduced at the Top of the column, and this eluated with an eluant "c", which has lesser affinity from A&B. The components are migrate at a rate determined by their relative affixity from the sop but at a slower rate than the eluant.




The components are eluted by the order of their affixities but the migration is determined by M.P. The components are identified on the basis of their "$V_R$" values. The area below the detector signal is characteristic of amount of compounds present.

(5) Gradient elution :-

The elution process can be modified by changeing the eluant after a predetermined period of time. This can be achieved by a set a eluants which increasing eluting powes can be employed which release the components having greater affixity to the stationary phase. This is called stepwise dilution.

separation of components widely varying affixity to the stationary phase through gradual change in the composition of eluant solvent is called gradient elution.

This is for increasing eluting power of the M.P the solvent composition gradient may be linear, steadily increasing (or) decreasing with conc$^{n}$, p$^{H}$, polarity (or) ionic strength.

Partition coefficient :-

The concentration of solute in each phase is given by the partition coefficient ($k_d$).

The ratio of concentration of solute in a stationary phase and mobile phase is called partition coefficient. It is denoted by $k_d$ and given by

$$K_d = \frac{\text{Conc}^n \text{ of Solute in Stationary phase (g/m)}}{\text{Conc}^n \text{ of Solute in mobile Phase (g/m)}}$$

$$= \frac{C_S}{C_m}$$

Adsorption Coefficient :- In adsorption Chromatography the Partition Coefficient is replaced by adsorption Coefficient.

Adsorption Coefficient may be defined as the ratio of Concentration of Solute is adsorbed phase and Concentration of Solute in Solution phase.

∴ Adsorption Coefficient = $K_d$

$$K_d = \frac{\text{Conc}^n \text{ of Solute in adsorbed phase (g/g)}}{\text{Conc}^n \text{ of Solute in solution phase (g/ml)}}$$

Retardation factor (R) :-

The retardation factor, R is the ratio of displacement velocity of a Solute to that of an ideal standard substance (often the mobile phase) which doesnot dissolve in (or) which is not absorbed by Stationary phase [$K_d = 0$]

It may also be defined as the fraction of time spent by a Solute molecule in the mobile phase.

⑥ $$R = \frac{t_M}{t_m + t_s}$$

where, '$t_m$' and '$t_s$' are the times the molecule remains in the mobile and stationary phases respectively.

"R" is also an equilibrium property. It is a function of Partition coefficient.

If "R" is the equilibrium fraction of solute in mobile phase, then $(1-R)$ is the equilibrium fraction of solute in the stationary phase [as $R_{mob} + R_{sta} = 1$] then the fraction $R/1-R$ is same as the fraction of molecule in the mobile phase divided by the fraction in the stationary phase.

$$\frac{R}{1-R} = \frac{C_m V_m}{C_s V_s}$$

$$\Rightarrow \frac{1-R}{R} = \frac{C_s V_s}{C_m V_m} \qquad \left[ K_d = \frac{C_s}{C_m} \right]$$

$$\Rightarrow \left(\frac{1}{R} - 1\right) = K_d \frac{V_s}{V_m}$$

$$\Rightarrow \frac{1}{R} = 1 + K_d \frac{V_s}{V_m}$$

$$\Rightarrow \frac{1}{R} = \frac{V_m + K_d V_s}{V_m}$$

$$\Rightarrow \boxed{R = \frac{V_m}{V_m + K_d V_s}} \text{ (or) } \boxed{R = \frac{1}{1 + K_d \left(\frac{V_s}{V_m}\right)}}$$

## Retention Volume :-

Retention volume is the amount of mobile phase which has left the column at the instant the maximum of solute zone emerges from the column.

(or)

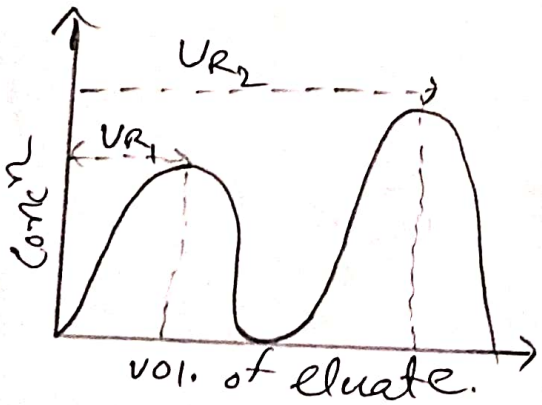
It is the volume of mobile phase passes through the column, through the insection point and Peak maximum.

At the Appearance of the peak maximum one of half of the solute is eluated in the retention volume. "$V_R$" and one half remains in the column in the mobile phase. plus the volume of stationary phase.

Therefore $V_R C_m = V_m C_m + V_s C_s$

$$\therefore V_R = V_m + \frac{V_s C_s}{C_m}$$

$$\boxed{V_R = V_m + K_d V_s}$$




## Retention Time :- ($t_R$)

It is the time elasped b/w the injection point and Peak maximum.

④ The retention time is related to the retention volume through the equation.

$$V_R = t_R \cdot F_E$$

$V_R, T_R$ are characteristic prop of chromatography.

where, $F_E \longrightarrow$ rate of flow of mobile phase.

Column Capacity :-

Column capacity is expressed in the terms of capacity factor (or) partition ratio (k). It is the ratio of amount of solute in the two phases.

It is given by

$$K = \frac{C_s V_s}{C_m V_m} \qquad \left\{ K_d \frac{V_s}{V_m} = K/V_m/V_s = K_d/\beta \right\}$$

$\frac{V_m}{V_s}$ is called volumetric phase ratio

It is denoted by "$\beta$" character.

$$K = K_d/\beta$$

The partition ratio (K) is related 'R' by

$$R = \frac{1}{1+K}$$

K is also related to retention value by

$$K = (V_R - V_m)/V_m$$

## Temperature effect :-

The most important parameter in the operation of Chromatographic columns is Temperature. The Partition coefficient Kd depends on Temperature, gas and liquid volume, gas & liquid diffusities also change with temperature.

The Partition Coefficient Kd changes exponentially with absolute temperature in the following way.

$$Kd = e^{\Delta S^\circ/RT} \cdot e^{\Delta H^\circ/RT}$$

where, "$\Delta S^\circ$, $\Delta H^\circ$" are standard entropy & enthalphy functions and "R" is a gas constant.

Temperature also influences resolution, the temperature is usually established as a compromise between better resolution [when it is lowered] and high speed [when it is raised]

## Partition Isotherms :-

The graph which gives the stationary phase concentration of the solute as a function of mobile phase concentration is called Partition Isotherms.

In normal situations, Isotherms are

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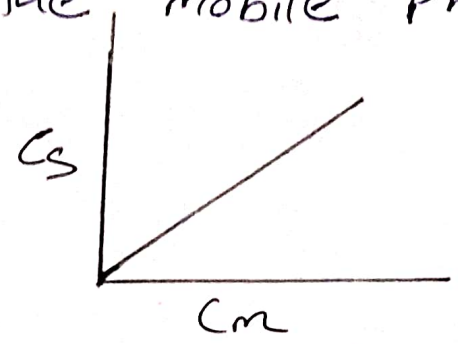
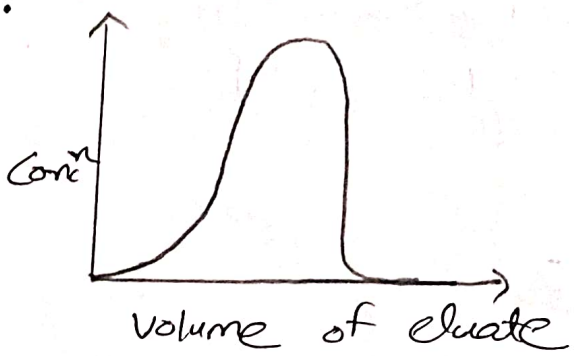
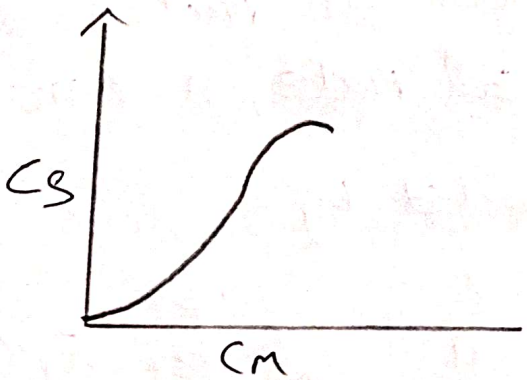
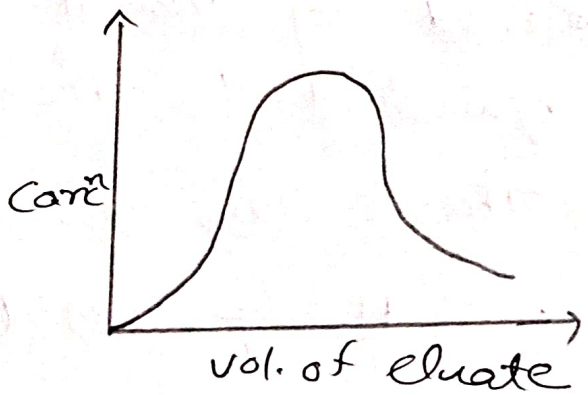
Symmetrical bell shaped peaks of normal (or) gathssian error function. under this conc$^{n}$, the concentration of solute in the stationary phase is directly proportional to the conc$^{n}$ of solute in the mobile phase.



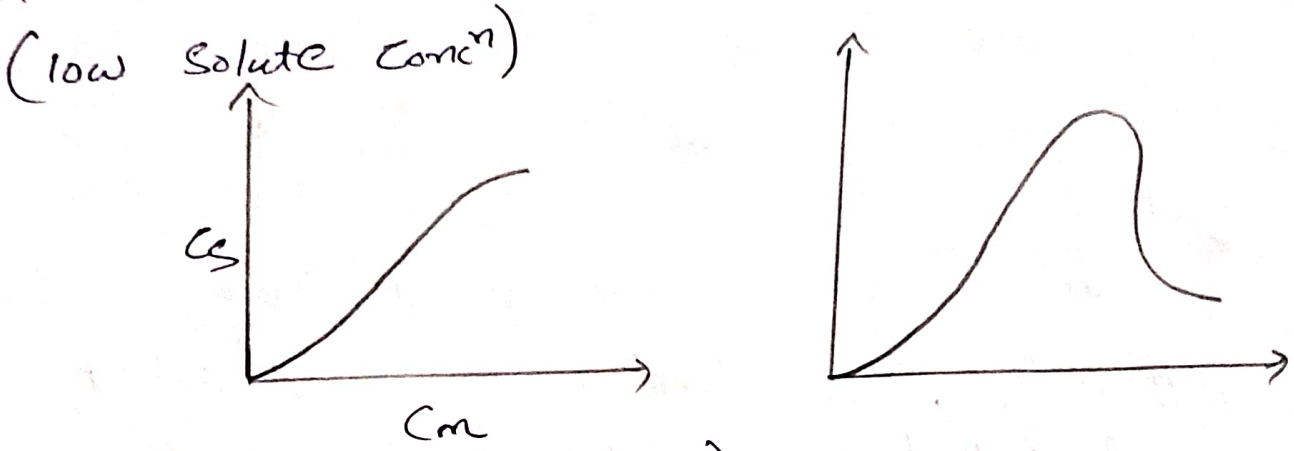

generally, the isotherms are deviated from normal situation, peaks with sharp front and daily rare boundary are caused by Langmuir type isotherms. The peak is caused by the main portion of the solute is eluated rapidly than the end zone. This is due to limited no. of sides avaliable for adsorption normal narrow peaks are obtained with micro samples.



Peaks with sloping front boundaries and sharp rare boundaries are caused by the non-linear isotherms. This is due to the low solubility of solute.

In the stationary liquid phase Normal narrow peaks are obtained with micro samples.




## Efficiency of chromatographic column :-

The efficiency of a chromatographic column is a measure of its ability to separate the components in a given mixture. The efficiency is expressed by no. of theoritical plates (or) Height equivalent to theoritical plate, H (HETP)

These two are related by

$$H = \frac{L}{N} \quad (or) \quad N = \frac{L}{H}$$

[where, L → Length of the column]

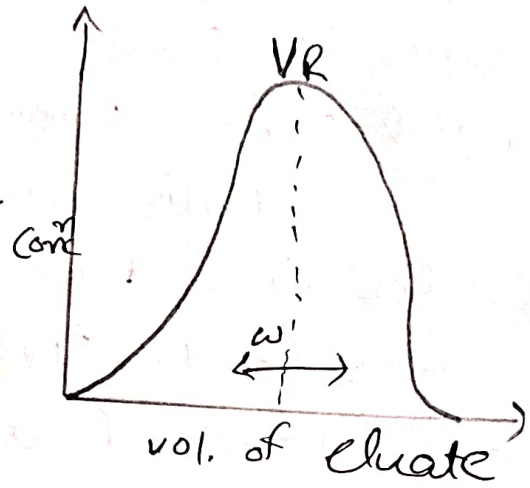
④ In general, theoritical plate can be defined as the length of the column in which the solute undergoes one complete equilibrium between the two phases.

The Number of plates is given by

$$N = 16 (V_R/w)^2$$

where, $V_R$ → Retention volume

$w$ → Peak width in volume units.




The value of "H" is a measure of the efficiency of chromatographic system. The smaller the value of "H" the more efficient and better resultation. "N" is inversely related to "H" an increase in "N" improves the efficiency and resolutio

The length of the column can lt be increased without optimum ~~results~~ limits. The limits are the result of practical rather than theoritical considerations increase in the length excessively leads to broad bands.

The solute is diluted, so it is more difficult to detect. The separation can be very time

consuming and wasteful to solvents.

## Zone Spreading (or) Zone Broadening :-

When the sample is introduced at the top of the column, the components are separated as compact zones. The dimensions of the zone increase in all directions, as compared to initial zone when development continues.

Both longitudinal spreading of each solute in the flow direction of the mobile phase and lateral (back) spreading occurs. The increase is greater in the flow direction. The column efficiency is decreased due to zone spreading.

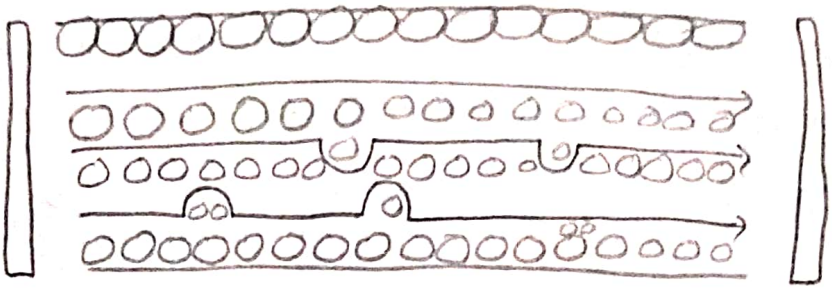
Zone spreading arises from 3 main sources. They are

1) multiple pathways
2) molecular diffussion in mobile phase.
3) Resistance to mass transfer between two phases.

## Multiple Pathways :-

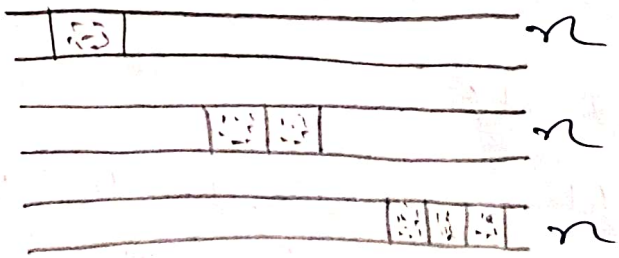
multiple pathways means the flow of molecules of the sample will travel through the column at different rates.

(10) Eddy diffusion:- Eddy size, shape and packing of the particles will determine the kinds of pathways available for travel. This effect can be minimised using small particles of uniform size.



Molecular diffusion in mobile phase:-

Molecular diffusion describes the movement in a longitudinal fassion away from a compact zone. when it travels through the column. This is due to the random motion of the molecules. This effect can be minimised by adjusting the flow rate of mobile phase.

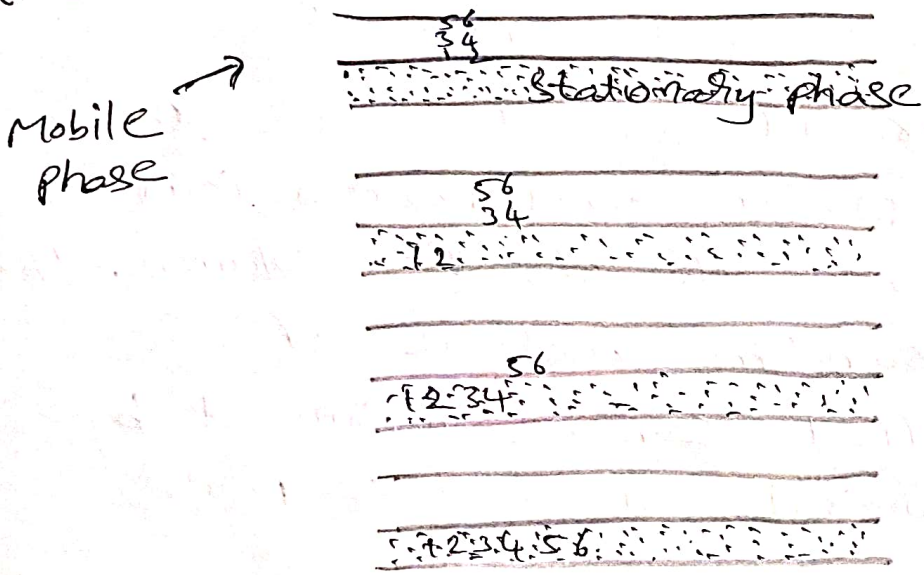


Resistance to mass transfer b/w two phases:-

Mass transfer effects are the important contributor to zone bordering the solute molecules are continously and reversibily transfer b/w

stationary & mobile phase. This mass transfer process is not instanteous. A finite time is required for the molecules to transfer through the mobile phase to read the interface and enter the stationary phase.

In this process, some molecules will have faster rates than others because of this the zone will broaden when it moves down the column.




## Vandeemter equation:-

The height equivalent to theoriticaplate is effected by the ratio of time spent by the solute in mobile phase, stationary phase.

⇒ The ratio of time spent is affected by.

i) affixity blw the solute of substrate

ii) the Molecular weight of the solute
iii) the flow rate of the M.P and
iv) the length of the column.

⇒ upto a certain increase in the length only results an increase in the value of N but with large ↑, the diffusion of solute becomes difficult.

⇒ other factors that affect the spreading of the solute are

i) diffusion in M.P
ii) eddy currents in mobile phase
iii) the flow rate of geometry of packing

The relation between these variables and the plate height and hence the column efficiency is given by vandeemter equation.

$$H = 2\lambda d_p + \frac{2\gamma D_m}{\mu} + \frac{8}{\pi^2}\frac{k'}{(1+k')^2}\frac{d_f^2 \mu}{d_{eq}}$$

$$= A + B/u + C$$

⇒ The term "A" describes the eddy diffusion and relates to the variable unequal diffusion

and wide fluctuation of the solute through particle bed.

These can be reduced by using small particles of uniform size.

⇒ The term B describes the band broade due to longitudinal diffusion during the random motion of molecules.

⇒ The term "C" relates to the resistance to the rate of mass transfer at which the solute species are adsorbed (or) desorbed and diffuse into each phase.

The term u → linear velocity of M.P. A plot of HEV gives a hyperbola called vandeemter plot.

Vandeemter equation :-

$$A + \frac{B}{U} + C.U$$

$$H = 2\lambda dP + \frac{2\gamma Dm}{U} + \frac{8}{\pi^2} \cdot \frac{k'}{(1+k')^2} \cdot \frac{df^2 u}{d\mu}$$

HETP

$\lambda$ → a constant which is a measure of packing irregularity.

$\gamma$ → a correction factor accounting for the Tortuosity of the channels in the column.

$d_p$ → average particle diameter of the column solid support

$u$ → (Avg. M.P velocity) linear velocity of the mobile phase.

$d_M$ → diffusivity of the solute

$D_M$ → in the mobile phase

$k'$ → capacity factor

$d_f$ → the stationary film thickness

$d_{MZ}$ → molecular diffusion coefficient of the solute in the S.P.

Simplest form

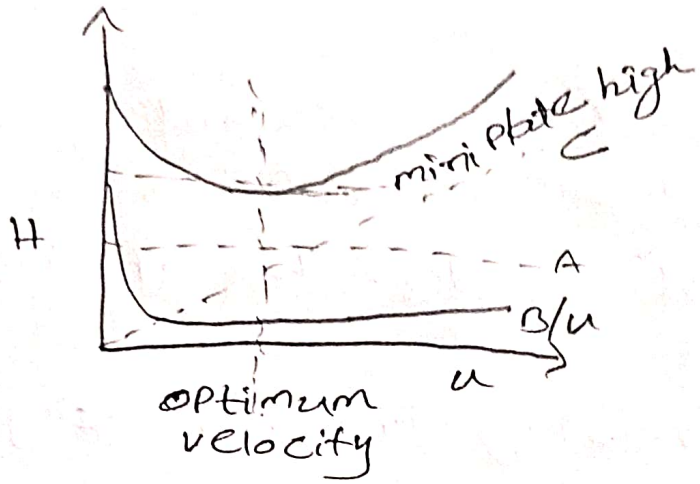
$$H = A + \frac{B}{u} + C$$

where, $A = 2\lambda d_P$

$$B = 2\gamma d_M$$

$$C = \frac{k'}{(1+k')^2} \cdot \frac{d_P^2}{d_{MZ}}$$

(Total $A$ & $\frac{B}{u}$ & $C$)



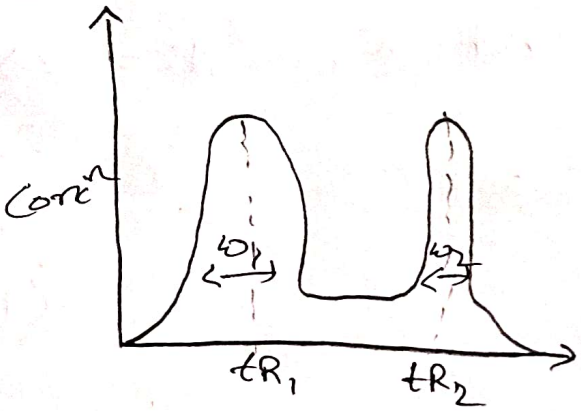

(By useing this graph we can detect the velocity of M.P)

## Resolution (Rs):-

Resolution is a measure of separation efficiency of a pair of solutes on a column over a reasonable period of time. Adequate resolution between two bands is important in chromatographic separation. The Rs Resolution Rs b/w two peaks is given by the equation.

$$R_s = \frac{t_{R_2} - t_{R_1}}{\left(\frac{w_1 + w_2}{2}\right)}$$

$$= \frac{2\Delta t}{w_1 + w_2}$$




where, $\Delta t = t_{R_2} - t_{R_1}$

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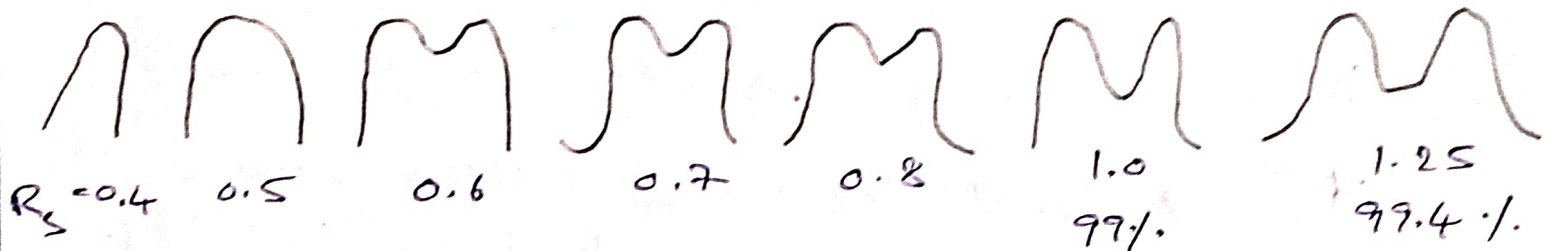
$t_{R_2}, t_{R_1}$ are the retention times of solute.

$\Delta t$ is diff. blw retention times.

$w_1, w_2$ peak width in time units.

## The Standard resolution curves:-

The standard resolution curves for peak height ratio (1:1) equal size peaks are shown below.




For $R = 0.4$ (or) 0.5 the peaks are merged.

For $R = 0.6$ the peaks are observable.

where "R" is increased to 0.7 & 0.8 the peaks are observed but (complete) separation is not completed.

when $R = 1.0$, 99% of the compound is separated.

when $R = 1.25$ % a good separation can be obtained. (99.4 %)

For complete separation, the value of R should be greater than 1.25.

The relation b/w R and No. of theoritical plates is given by the equation.

$$R = \frac{1}{4} \frac{(\alpha-1)}{\alpha} \frac{k'}{(1+k')} \sqrt{N}$$

(or) $$N = \left[\frac{4R\alpha}{(\alpha-1)}\right]^2 \left\{\frac{1+k'}{k'}\right\}^2$$

where $\alpha \longrightarrow$ relative retention (separation factor)

$k' \longrightarrow$ capacity factor (column capacity)

$N \longrightarrow$ No. of Theoritical plates choice of column length and flow velocity.

After the No. of theoritical plates required for a desired degree of resolution has been calculated, the next step is fixing of operating conditions. The time required for separation is the time $t_p$ required to travel the solute through one plate multiplied by the No. of plates required.

$\therefore$ Separation time $t = t_p \cdot N$

"$t_p$ is given by the length (or) height of plate divided by zone velocity.

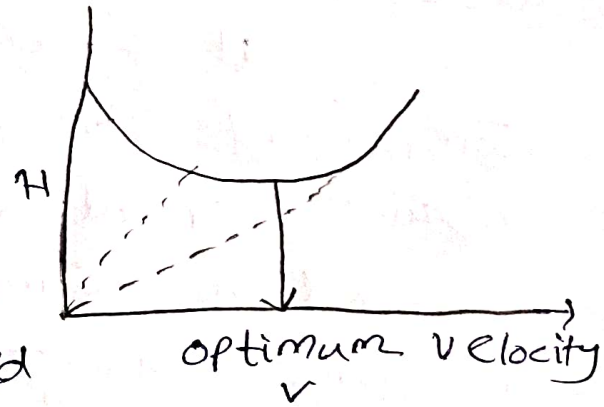
$$t_p = \frac{\text{length of height of Plate}}{\text{Zone velocity}}$$

$$t_p = \frac{H}{Rv}$$

$$\therefore \text{Separation time} = \frac{HN}{Rv}$$

H/v ratio can be obtained directly from vandeemter plot. It is simply the slope 'm' of the line drawn from the origin to a point on the graph.

once the velocity has been selected, the column length necessary for the separation is obtained as NH.




For practical work one should start with a column. which is as long as possible. Then the flow velocity is increased until the resolution is Adequate.

## Qualitative and quantitative analysis :-

Chromatography is a versatile method for separating closely related chemical species.

In addition, it can be used for qualitative identification and quantitative determination of separated components.

Qualitative Analysis :-

A chromatogram provides a single point information about each compound in a sample. {Retention Time (or) Retention volume}. The No. of data points obtained by chromatography is small compared with the number provided by a single IR, N.M.R (or) mass spectrum. Spectral data can be determined with better accuracy than chromatography, lacks imp qualitative applications.

It is widely used for recognising the components in a mixture. However confirmation will require spectral (or) chemical analysis of the separation components. The spectroscopic identifications will not be possible without a priliminary chromatographic separation.

Hence, chromatography is precursor to qualitative spectroscopic analysis.

## Quantitative analysis:-

Two general techniques are used to determine the components which are separated by Chromatographic Technique in the first each component is collected in a separate container and determined by a suitable chemical (or) Instrumental method.

The 2nd is based on the fact that the area under the peak is directly proportional to the conc$^n$ of the components. once the areas are determined, Calibration Curve can be prepared by plotting area Vs - conc$^n$ of the standard.

The unknown mixture is subjected to separation under similar conc$^n$s with peak area, it's conc$^n$ is determined from the Calibration Curve.

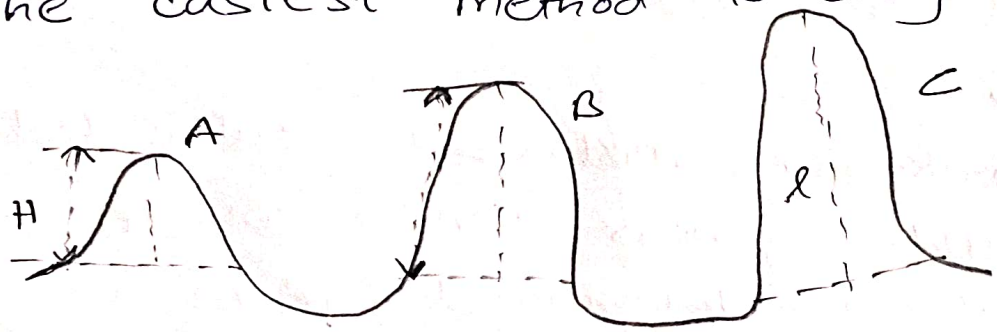
These are several methods for determining Peak are and relating this to conc$^n$. These are

1. Peak height
2. Triangulation

③ Planimetry
④ cut and weight
⑤ Disc and electronic digital integration.

**Peak height :-**

Peak height is measured from the base line to the peak maximum for best results, the peak should be a well defined, gausian shape [ ∩ ] and completely resolved from other peaks. The peak height is proportional to the area which is proportional to the conc$^{n}$. this is the fastest and greatly the easiest method to carry on.




**Triangulation :-**

this method is based on the fact that the peak is considered as a triangle. The area is calculated by multiplying the peak height by the width of the peak at half the height of the peak.

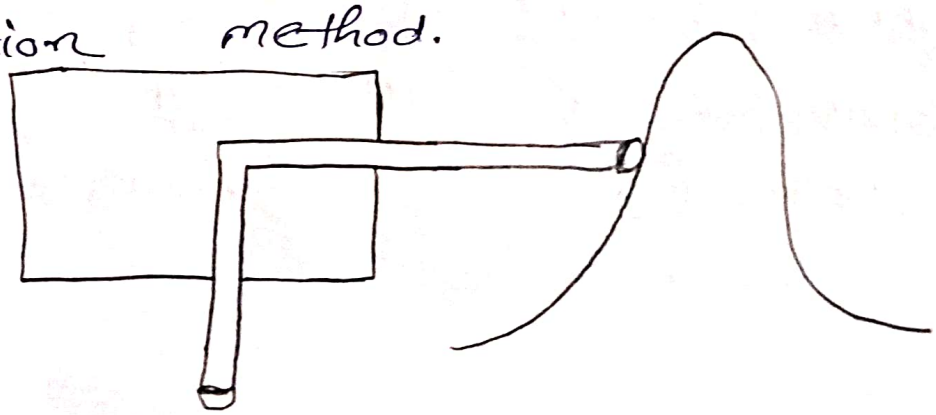
(3)

- Area = Peak height
  x Peak width at half weight


In the 2nd method the width at the base of the peak is used. The area is calculated by


$$\text{Area} = \frac{1}{2} \times \text{Peak heights} \times \text{Peak width of the base.}$$

Planimetry :- A Planimeter is a mechanical device that is used to trace the perimeter of the Peak. The result is an integration of the peak with its area and recorded, directly on a dian. (using OC). Planimetry is a technique and requires not only skill but also patience by the operator unlike the triangulation methods the true peak area is measured Non-gaussian type peaks can be measured accurately than triangulation method.

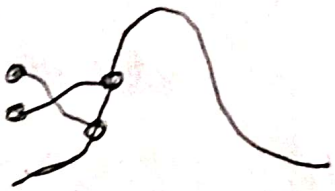


Cut and weigh method :-

The cut and weight technique is a Perimeter technique the peak is cut out and weighted on the analytical balance. A Calibration Curve is Prepared by Plotting the Peak weight, Against Conc^n of the Standard.

This method is Preferred over triangulation if Peaks are Non-gaussian however it is more time Consuming than the triangulation method.

The accuracy & Precision of the method the accuracy & Precision of the method depend on the quality & uniformity of the recorded Paper.



V Disc and electronic digital integration :-

The disc integrator is a mechanical device which essentially does automatically what planimeter does manually the recorder equipped with the disc integrator and a pen