Adikavi Nannaya University :: Rajahmundry
Department of Chemistry
M.Sc. (Final) Chemistry Syllabus for III-Semester
Specialization: *Analytical Chemistry*
Paper - I: Separation Methods – I
(Effective from 2016-17Admitted batch)

Unit – I Chromatography - 1

Chromatography: classification of different chromatographic methods, methods of development-Elution development, Gradient elution development, displacement development, and frontal analysis.
Principles of chromatography, different migration, adsorption phenomena, partition, adsorption coefficient, retardation factor, retention time and volume, column capacity, temperature effects, partition isotherm.
Dynamics of chromatography-efficiency of chromatographic column, zone spreading, High Equivalent Theoretical Plate (HETP), Van Deempter equation, resolution, choice of column, length and flow velocity, qualitative and quantitative analysis.

Unit - II Chromatography – 2

Column chromatography (adsorption chromatography): principles, general aspects, adsorption isotherms, chromatographic media, nature of forces between adsorbent and solutes, eluents (mobile phase), column chromatography without detectors and liquid chromatography with detectors and applications.

Gel Exclusion chromatography or Gel filtration chromatography: principles, properties of xerogels, apparatus and detectors, resolution of gel type, applications to organic compounds.

Capillary Electrophoresis : Principle, Details of the Instrument, Applications to Inorganic and Organic compounds.

Unit – III Chromatography – 3

Gas chromatography: Theory, Instrument description of equipment and different parts, columns (packed and capillary columns), detector specifications-thermal conductivity detector, flame ionization detector, electron capture detector, nitrogen-phosphorus detector, photo ionization detector, programmed temperature gas chromatography; applications in the analysis of gases, petroleum products etc., other detectors used their Principles and Applications.

Inorganic molecular sieves: structure of zeolites, crystals, types of sieves, application in the separation of gases including hydrocarbons, ion exclusion-principles and applications,

Counter current chromatography-principles and application, Affinity chromatography- principles and applications

GC-MS – Introduction

Instrumentation – GC – MS interface – Mass spectrometer (MS) Instrument operation, processing GC – MS data – ion chromatogram Library searching – Quantitative measurement – sample preparation Selected ion monitoring – Application of GC-MS for Trace constituents. Drugs analysis, Environmental analysis and others.

Unit – IV Chromatography – 4

Liquid-liquid partition chromatography: principle, supports, partitioning liquids, eluents, reverse phase chromatography, apparatus, applications

High performance liquid chromatography: Theory, Instrument description of the different parts of the equipment, columns, detectors-UV detector, refractometric detector, Fluorescence detector, Diode Array detector, applications in the separation of organic compounds, names of other detectors used their Principles and Applications.

LC-MS – Introduction – Instrumentation – liquid chromatograph – Mass spectrometer Interface – Instrumental details – Processing LC-MS data – ion chromatograms – Library searching – Quantitative measurements.
Sample preparation – selected ion monitoring. Application of LC-MS for Drug analysis, Environmental samples and others.

Text books:

1. R.P.W Scott, Techniques and practice of Chromatography, Marel Dekker Inc., New York
2. M.N. Sastri ,Separation methods, Himalaya Publishing Company, Mumbai

Reference books:

1. E. Helfman, Chromatography, Van Nostrand, Reinhold, New York
2. E. Lederer and M. Lederer, Chromatography, Elsevier, Amsterdam.
3. Chemical separation methods, John A Dean, Von Nostrand Reinhold, New York
4. R.P.W Scott, Techniques and practice of Chromatography, Marel Dekker Inc., New York
5. H.M Mc Nair and J. M. Miller, Basic Gas Chromatography, John Wiley, New York
6. W. Jeumings, Analytical Gas chromatography, Academic Press, New York
7. H. Eugelhardt (ed), Practice of HPLC, Springer Verrag, Berrin

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Separation Methods

Introduction

Separation is defined as an operation or a physical or chemical process by which a mixture is resolved into its components.

For example, in laboratory preparation of (an organic) a compound (synthetic drugs) consists a mixture containing some quantities of unreacted starting materials or by products from side reaction known as impurities. therefore the synthesized compound is not in pure state. Therefore it is vital to separate or isolate the desired compound from rest of the impurities to obtain the drug of the required quality and uniform potency.

the general goals of separation are of two kinds analytical and preparative. In the former, one aims at the isolation of the desired component in a high degree of purity for quantitative measurements. In the later, one aims at the separation of the component with the degree of purity required for a specific use of the separated compound.

Distinction between separation and purification

In separation, one would need to isolate several or all of the components (analytes) present in the mixture in the pure state. the number of analytes isolated in pure state and their relative amounts are also important in separation (Analytical method)

In case of purification method, the starting material consists mainly one compound, and the small amounts of other compounds (impurities) must be removed to get the main component as a pure entity (preparative method).

Principle of Separation Techniques

The basic principle of separation technique is that, distribution of components of the mixture between two phases. The distribution or partitioning is (brought about) due to difference in volatility, solubility, adsorption or molecular size etc. In the process of separation, one of the two phases gets considerably enriched one component, and the other phase in respect of the second component the two phases are isolated or separated and subsequently components are recovered.

The separation of two components consists in carrying a succesive series of equilibrium stales (operations). Suppose an equilibrium stali is established between two phases at the end of the first operation, the two phases (A & B) contain molar fractions of two components (1 and 2). If C_1 and C_2 are molar fractions of components 1 and 2 in phase-A, then $\left(\frac{C_1}{C_2}\right)_A$ gives the composition of phase-A. Similarly $\left(\frac{C_1}{C_2}\right)_B$ represents composition of phase-B.

$$\frac{\left(\frac{C_1}{C_2}\right)_A}{\left(\frac{C_1}{C_2}\right)_B} = \alpha \text{ (separation factor)}$$

Several purification methods and separation methods are given in the Table-I.

Method	Underlying Basis or principle
Crystallisation	- Difference in solubility at hotter and lower temp
Sublimation	- Difference in vapour pressure
Distillation	- Difference in volatility (boiling points)
Filtration	- Flow of solvent usually water through filter paper
Centrifugation	- Difference in the rates of sedimentation of particles under centrifugal fields.
Electrophoresis	- Difference in migration rates of components under applied electric field.
Chromatography	- Difference in adsorption, partition, ion-exchange molecular size etc.
Dialysis	- Differential migration across a semi-permeable membrane which allows certain types of molecules to pass through it but excludes others.

Chromatography :

Chromatography is defined as a laboratory experimental method of separating a mixture of components into individual components through a series of equilibrium distribution of components between stationary phase (solid or liquid) and mobile phase (liquid or gas). It is a simple method of separating components of a mixture i.e separation method or separating a desired compound from its impurities (purification method).

The requirement to distinguish chromatography from other separation techniques is that the separation through a distribution of analyte between two phases, one phase is stationary phase and other phase is mobile phase.

Stationary Phase The stationary phase is a solid or liquid or a mixture of solid & liquid, and is finely divided and is fixed in place like column or thin film or sheet or strip.

Mobile phase The mobile phase is liquid or gas fills the interstics of the stationary phase and is able to flow through the stationary phase which caries the components.

Principle of Chromatography The sample containing the mixture of components is introduced into the stationary phase, mobile phase is allowed to flow through the stationary phase. The components undergo a series of adsorption or partition processes between mobile phase and stationary phases as they move through the Chromatographic system i.e column, thin film or paper.

the components move through the chromatographic system at different rates, because of different physical and chemical properties of individual components and hence different affinity for stationary phase. the component having least affinity to the stationary phase moves rapid and will be eluted first. the component having strong affinity to stationary phase move slowly and will be eluted last.

Preparative / Analytical Chromatography

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture and is thus a form of purification. When chromatography is applied to the purification of a relatively large amount of a material it is known as preparative chromatography.

Chromatographic technique when it is used for the determination / estimation of composition of a sample, it is known as analytical chromatography. Analytical Chromatography is done with smaller amounts of material (Both qualitative and quantitative)

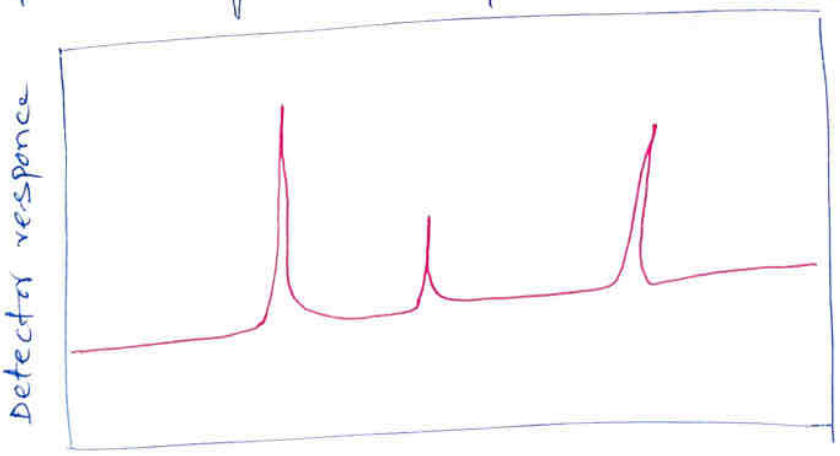
A wide range of stationary and mobile phases (i.e different combinations of stationary phase and mobile phase) are used to separate components with only small differences in physical or chemical properties of the analytes

Chromatography Terms

Chromatograph: It is an instrument that enables a sophisticated separation.

Chromatography: It is a physical method of separation that distributes components to separate between mobile phase and stationary phase moving through chromatographic system.

Chromatogram: It is the visual output of the chromatograph. In case of optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture



Eluate – Eluent – Eluite

Eluate is the mobile phase leaving the column

Eluent is the solvent that carries the analyte

Eluite is the analyte or solute eluted

Retention time : It is a characteristic time takes for a particular analyte to pass through the system under set of conditions.

Elution: The procedure of removing the components of a mixture sequentially from the column by sweeping them through with the mobile phase is known as elution

Isocratic elution: Elution using a mobile phase of constant or same composition i.e polarity is known as Isocratic elution

Gradient elution : Elution using mobile phase of progressively varied composition i.e polarity is known as gradient elution

Classification of different Chromatographic methods

1. Classification of Chromatographic methods based on physical state of the mobile and stationary phases. - There are FOUR basic types of Chromatographic methods.

Mobile phase	Stationary phase	Name of the Method
Liquid	solid	Liquid-Solid Chromatography (LSC)
Liquid	Liquid	Liquid-Liquid Chromatography (LLC)
Gas	solid	Gas-Solid Chromatography (GSC)
Gas	Liquid	Gas-Liquid Chromatography (GLC)

2. Classification of Chromatographic methods based on nature of separation / mechanism of separation of analytes between Satinary and mobile phases

Nature of the separation	Name of the method	Examples of Techniques
Adsorption process	Adsorption Chromatography	LSC GSC TLC & PC
Partition process	Partition Chromatography	LLC TLC & PC GLC

Ionic replacement reactions	Ion exchange Chromatography Ion pair " Ion Chromatography
Sieveing dependent upon molecular size	Gel Chromatography
Rapid and reversible formation of complex	Complexing Chromatography.

(iii) Classification based on the configuration of the chromatographic system.

Type of Configuration	Name of the Technique	Examples.
Paper sheet or strip	paper Chromatography	Paper-chromatography (PC)
Thin film on glass or Aluminium sheet	Thin-Layer Chromatography (TLC)	
Column	Column Chromatography. i) LLC ii) LSC iii) Ion exchange Chromatography iv) Gel Chromatography v) Complexing Chromatography vi) Ion pair Chromatography vii) Ion Chromatography viii) GSC ix) GLC	

The following table gives a system of classification based on physical state of mobile / stationary phases, nature of separation and configuration of the system.

Mobile phase	Stationary phase	Configuration	Nature of Separation	Name of the Chromatographic Technique
Liquid	Solid	column	→ Adsorption	Liquid-Solid Adsorption Chy.
Liquid	Liquid	column	→ partition	Liquid-Liquid partition Chy.
Liquid	solid	column	→ Ionic replacement	Ion-exchange chromatog.
				Ion-pair "
				Ion chromatography.
Liquid	Liquid	column	→ Sieving dependent upon molecular size	Gel Chromatography
Liquid	solid	column	→ Rapid & reversible formation of complexes	Complexing Chromatography
Liquid	solid	~~column~~ Thin film	→ Adsorption or partition	Thin-Layer Chromatog.
Liquid	solid (paper)	Sheet / Strip	→ ~~Adsorption~~ or partition	paper Chromatography.
Gas	solid	Column	→ Adsorption	Gas-solid Chromatog.
Gas	Liquid	column	→ Partition	Gas-Liquid Chromatog.
Supercritical fluid	Liquid	column	→ partition	Capillary supercritical Fluid Chromatography.

Classification of Chromatographic techniques based on relative polarity of stationary phase and mobile phase used in the separation technique

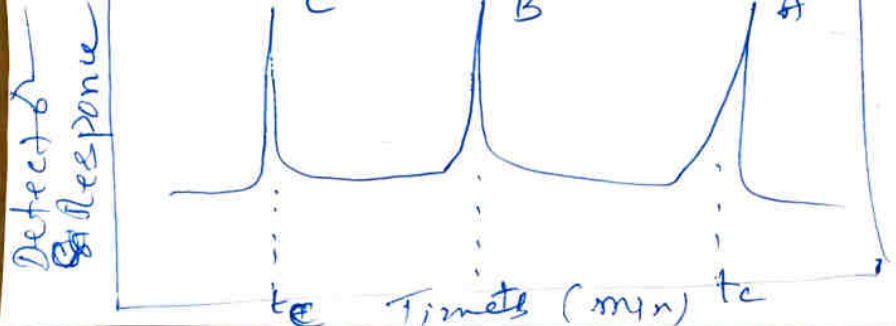
Nature of stationary phase	Nature of mobile phase	Name of the methode	Order of Elution
Polar	Non-polar	Normal phase	Separation of components in the order of Non-polar to polar (Increasing order of polarity)
Non-polar or Less polar	polar	Reverse phase	Separation of components in the order of decreasing polarity and then non polar i.e High polar - Less polar - non polar

Normal phase Chromatography

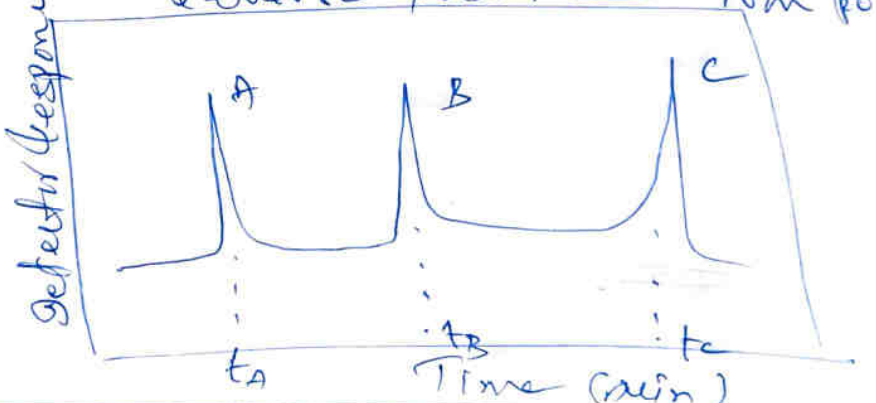
Reverse phase Chromatography

If A, B, C are polar, Less polar and nonpolar analytes. then the order of elution

Normal phase



Reverse phase



Choice of Suitable Chromatographic Technique

The choice of a particular Chromatographic method is based on the nature of the sample.

(i) If the sample is sufficiently volatile, Gas-Liquid column chromatography is normally the most convenient method. Use of heated columns and vaporisation chambers allows separation of the components / compounds that boil at temperature as high as 400°C

(ii) For non-volatile substances / samples, their separation can be carried out by Liquid-solid adsorption Chromatography or Liquid-Liquid partition Chromatography.

(iii) Thin Layer Chromatography and paper Chromatography methods are very convenient methods of separation of components irrespective of volatility

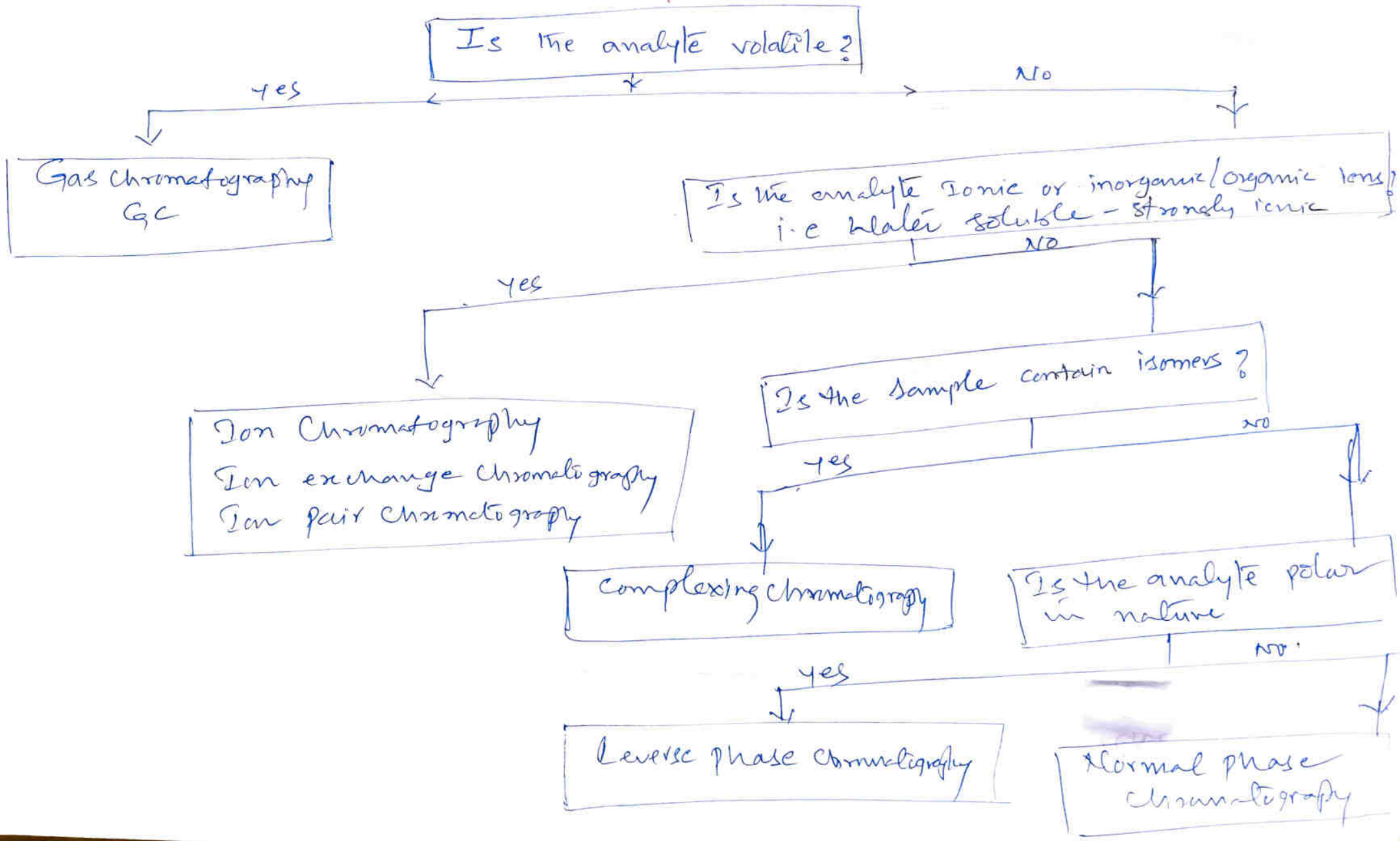
(iv) Inlaid soluble compounds which are strongly ionic in nature are separated by ion-exchange Chromatography using either an anionic or a cationic resin. Ion exchange Chromatography is suitable method for the separation of ionised and ionisable molecules present together in a mixture. Inorganic or organic ions can be separated by Ion Chromatography.

(v) Complexing Chromatography is used for the successful separation of components of mixtures of constitutional, configurational and isotopic isomers.

(vi) Gel Chromatography is used to separate compounds differing in molecular size

(10)

Flow Chart of ~~(Classification)~~ ~~Choice~~ Choose Suitable Chromatographic Techniques.



Method development is the procedure to separate and remove various components present in sample mixture by adopting a suitable chromatographic technique.

Method development:

Method Development

Sample: A mixture of two components A and B

Stationary phase: Adsorbent (Silica, Alumina, starches)

Mobile phase: solvent or mixture of solvents.

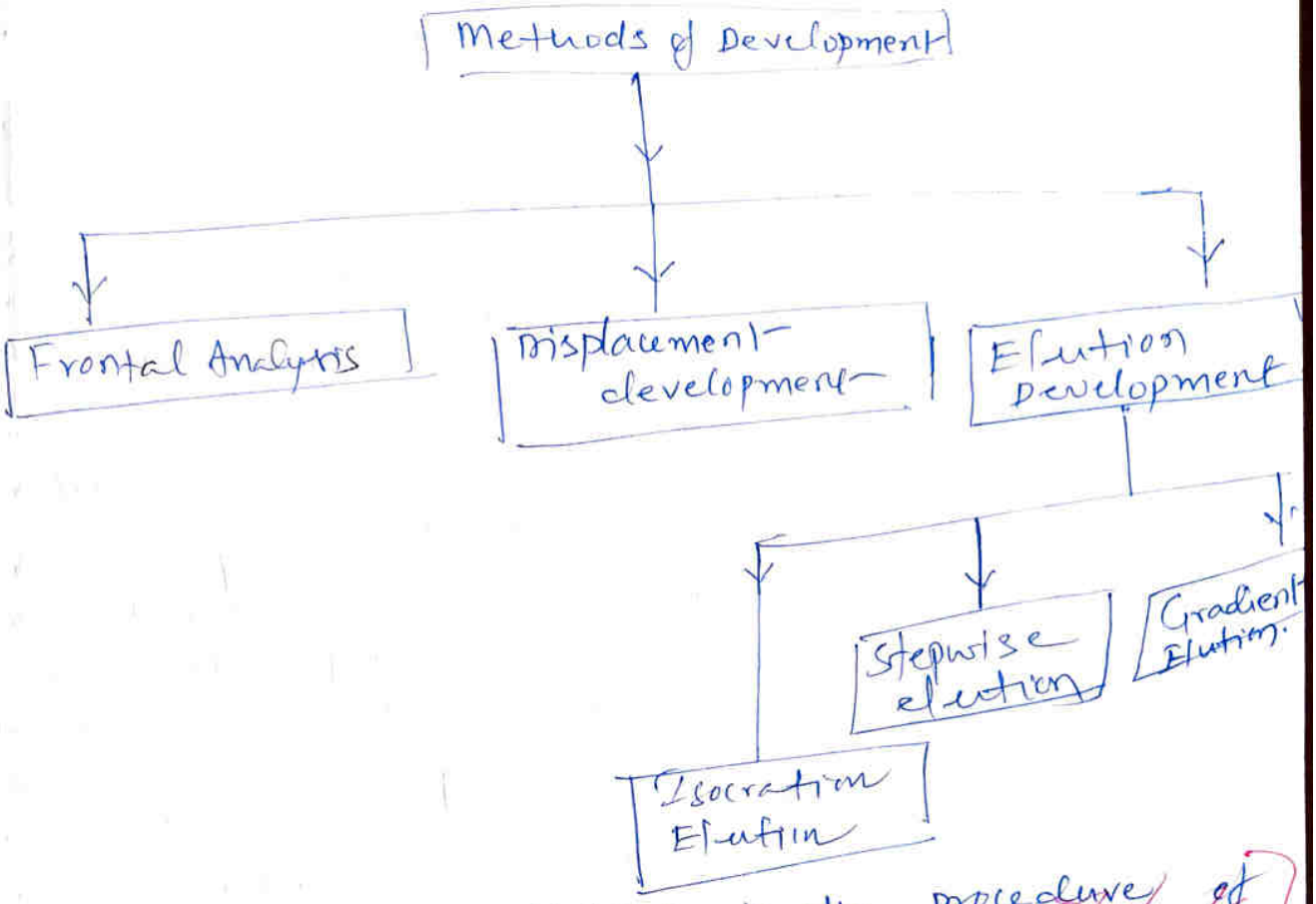
Sample preparation: the sample is dissolved in mobile phase, sonicated to dissolve and a homogeneous solution is used for the analysis.

Detector: It is used to detect separated components.

Procedure: (Adsorption column chromatography)

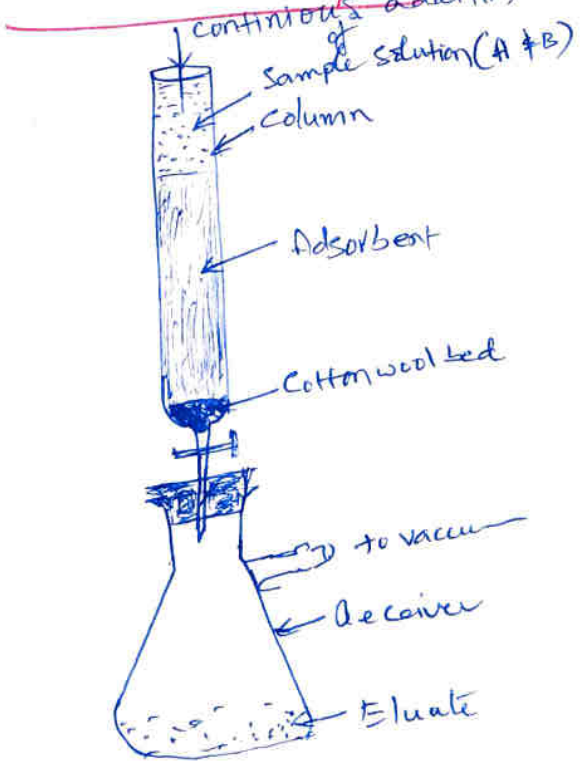
- A chromatographic column of uniform bore size is taken and holded at to a stand, cottonwool is placed at the bottom of the column.
- the adsorbent is made into slurry using solvent or mobile phase, and is introduced into the column slowly, make sure that no air gaps or bubbles inside the column, allowed to stand to settle adsorbent bed uniformly, if necessary a small amount of mobile phase is allowed to flow through it, and drain out the exess eluent by opening stopcock, and allowed it to dry.
- the sample solution is introduced at the top of the column, after some time, the mixture is adsorbed completely and the column saturated.
- the components A and B are adsorbed on stationary phase due to physical or physico-chemical interactions between components and mobile phase. Let component 'B' has strong affinity towards stationary phase than component A.
- the components come out of the column in the order of increasing affinity towards stationary phase i.e First - A and Second - B.
- the components are identified on the basis of their retention volume (V_R).

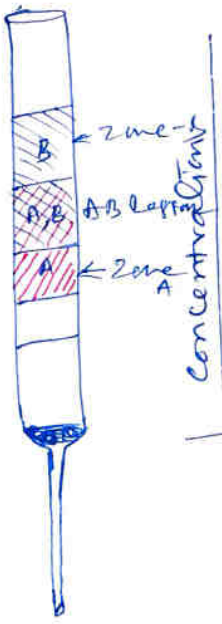
Different Methods of Development - Frontal analysis - Displacement development - Elution development

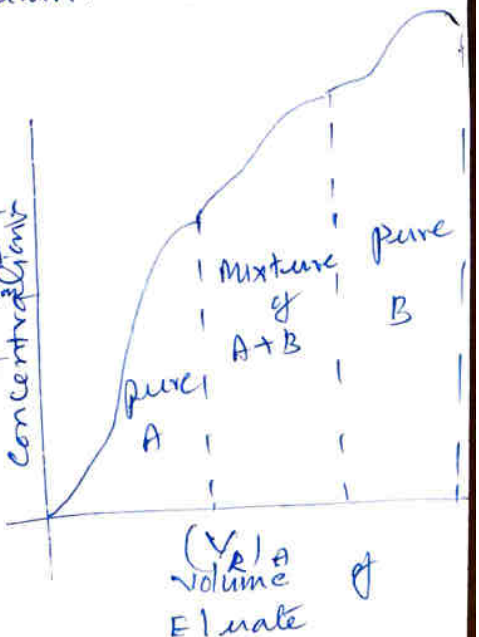


Method of development: This is the procedure of separating and removing the various components of the given mixture from a column.

Frontal Analysis



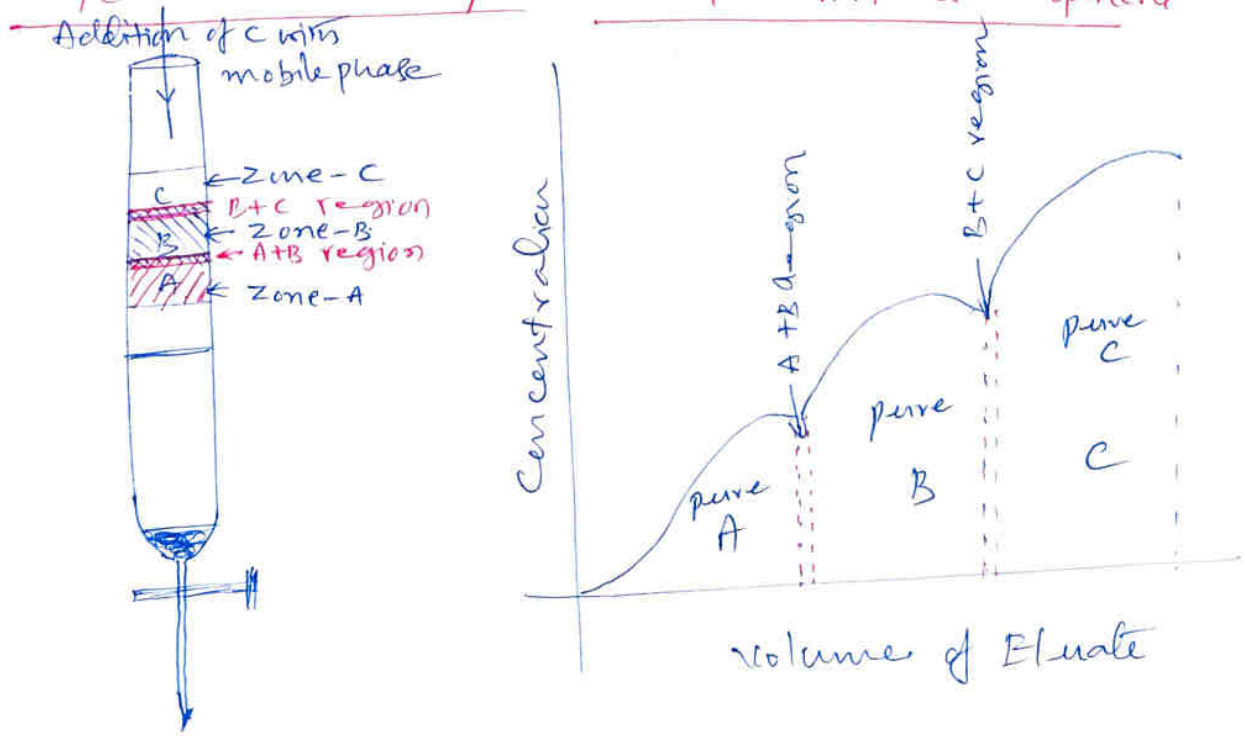




In frontal analysis, the procedure of separating and removing the various components of the given mixture is addition of the sample solution onto the column, after certain time the mixture is adsorbed completely and the column is saturated. On continuing the addition of the mixture, the less strongly adsorbed component (A) leaves the column in a pure form first. Then 'A' is eluted as a mixture with component B the amount of 'A' in the mobile phase can be calculated from the retention volume (V_R), while the other components can be determined from the volume concentration ratio.

Frontal analysis a preparative method primarily used for the separation of on component from others with greater affinity for the stationary phase.

Displacement Analysis or Displacement development

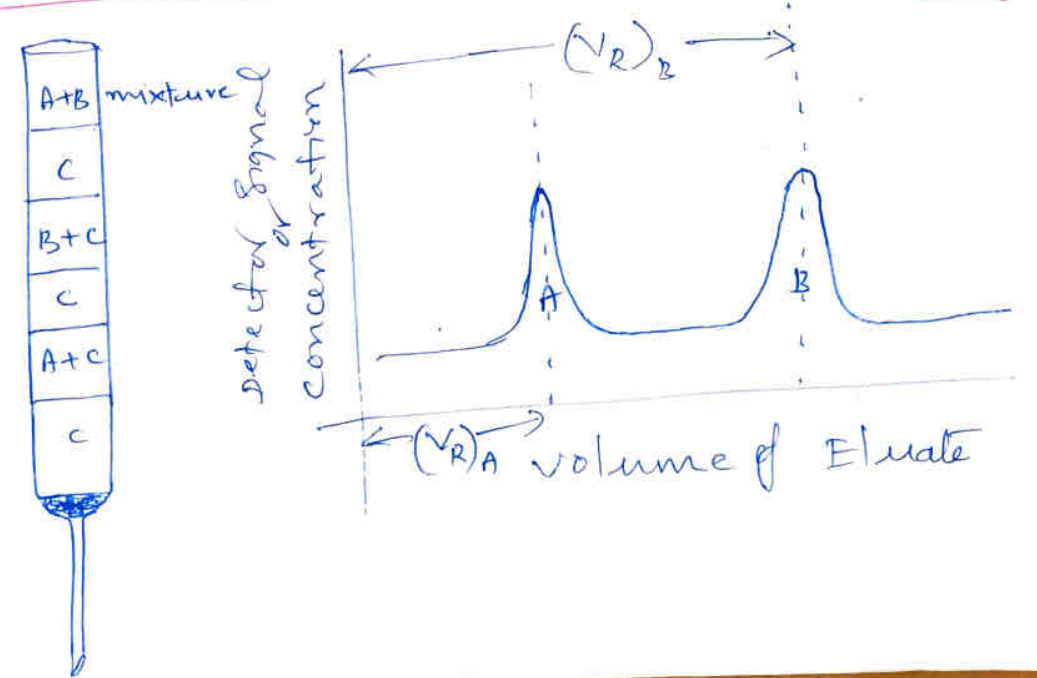


Displacement analysis involves the elution by an eluant 'C' which has a greater affinity for the stationary phase than the sample components A and B.

The sample mixture is first introduced at the top of the column where it adheres to the stationary phase. The mobile phase containing displacing agent 'C' is allowed to flow through the column. During the period of elution A and B are displaced by component C on the stationary phase. During the process, the components undergo separation due to the differencies in adsorption / partition properties.

This method does not generally produce completely separated components, between the zones of pure components there are regions containing mixtures which can be collected and treated further.

Elution Analysis or Elution Development



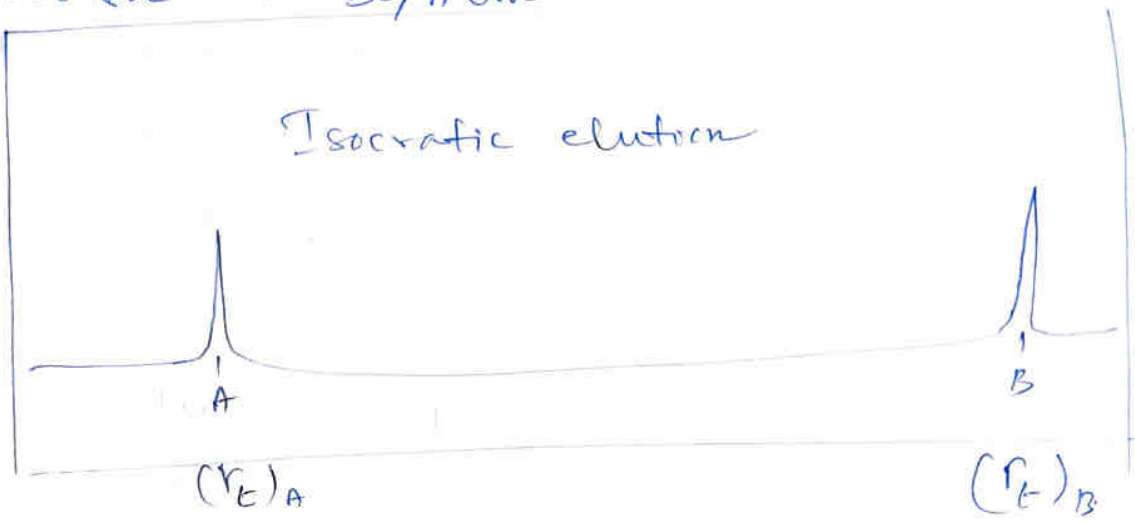
In elution analysis, a small amount of sample of the mixture containing components A and B is introduced at the top of the column and elution is carried out with an eluant 'e' which has a lesser affinity for the stationary phase than the sample components. the components migrate at a rate determined by their relative affinity for stationary phase but at a slower rate than the eluant. the components are eluted in the order of their affinities but their migration is determined by the mobile phase

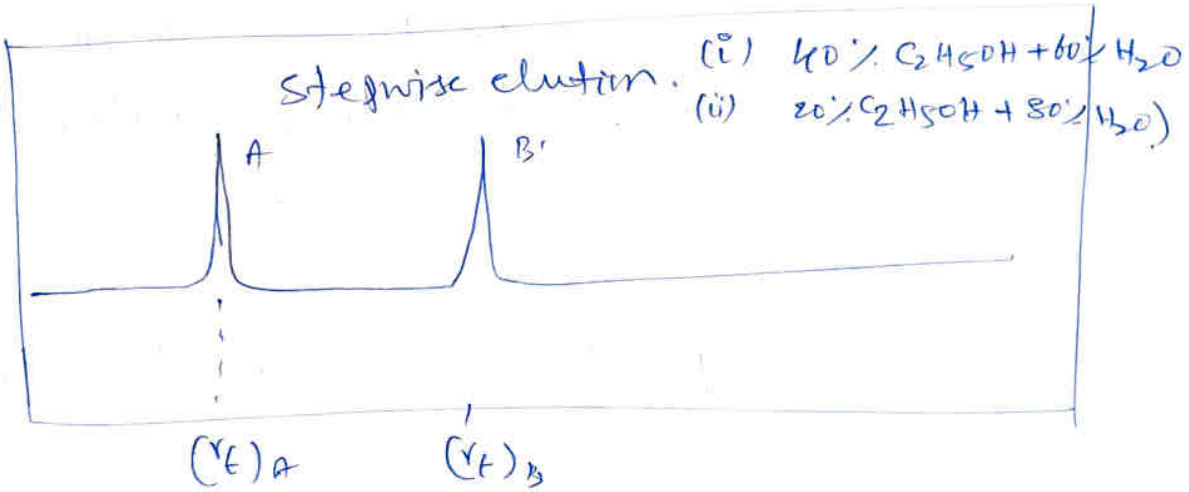
the components can be separated completely with a zone of the mobile phase between the components. the components are identified on the basis of their retention volumes. the area below the detector response is characteristic of the amount of the component.

the procedure of separating the components of a mixture sequentially by using a mobile phase of same composition i.e same polarity throughout the elution is known as isocratic elution. (40% C_2H_5OH & 60% H_2O)

Stepwise Elution the elution process can be modified by changing the eluant (i.e mobile phase) after a predetermined period of time is called stepwise elution

For example, a set of eluants with increasing eluting power can be employed with release the components having greater affinity to the stationary phase and move them through the system.





In stepwise elution $(r_t)_B$ is less when compared with $(r_t)_B$ in isocratic mode

Gradient Elution

Separation of components of widely varying affinity to the stationary phase can be achived by gradual change in the composition of the eluant i.e composition of the mobile phase is progressively varied throughout the elution process.

Forexample, if the solvent used is a mixture of C_2H_5OH and H_2O; its composition is varied continuously with time from pure water to pure ethanol.

Partition – Partition coefficient – Nature of Partition forces – Chromatographic behaviour of solutes – Retention time – Retention volume – Retardation factor (Retention ratio) – Relative Retention

Partition – Partition coefficient :

Chromatography is a simple, selective and rapid separation method comprising two phases - mobile phase and stationary phase. If both phases are in liquid state, it is known as liquid-liquid chromatography. The sample mixture is introduced into the mobile phase, ~~an~~ undergoes a series of interactions many times between the liquid stationary phase and liquid mobile phase. When sample mixture enters a chromatographic system, solutes / analytes distribute between these phases. ~~The~~ the solute / analyte is soluble in both the immiscible phases, and the extent of solubility depends upon the nature of the solute, mobile phase and stationary phase. This process is called partition. At any stage there exists an equilibrium state.

If the solute – Stationary phase interactions (polar, dipole-dipole, non-polar-non polar) are strong, then the partition or distribution of solute is maximum, and obviously solute-mobile phase interactions are weak, partition is less. therefore the concentration of solute in stationary phase $(C_x)_{sp}$ is high, and $(C_x)_{mp}$ is low.

Stationary phase (sp)
$\uparrow\downarrow$ x
Mobile phase (mp)

where x is solute particle present in the sample mixture.

Partition coefficient (K) is defined as the ratio of the concentration of solute (x) in stationary phase and mobile phase

$$\therefore \text{Partition coefficient}(K) = \frac{(C_x)_{sp}}{(C_x)_{mp}}$$

If x equally distributed between sp & mp then $(C_x)_{sp} = (C_x)_{mp}$, therefore $K = 1$

If $(C_x)_{sp} > (C_x)_{mp}$ $K > 1$, retain in the column for longer time, and elute last

If $(C_x)_{sp} < (C_x)_{mp} \Rightarrow K < 1$, x has less retention, moves along with mobile phase - less retention time and elute first.

Nature of the partition forces

- Dipole dipole forces
- Hydrogen bond forces
- Dispersion forces.

The partition of solute (x) between two phases results from the balance of forces between solute molecules and molecules of stationary phase & mobile phase. These forces may be polar in nature arising from permanent or induced electric fields associated with both solute and solvent molecules or they can be London's dispersion forces which are very weak.

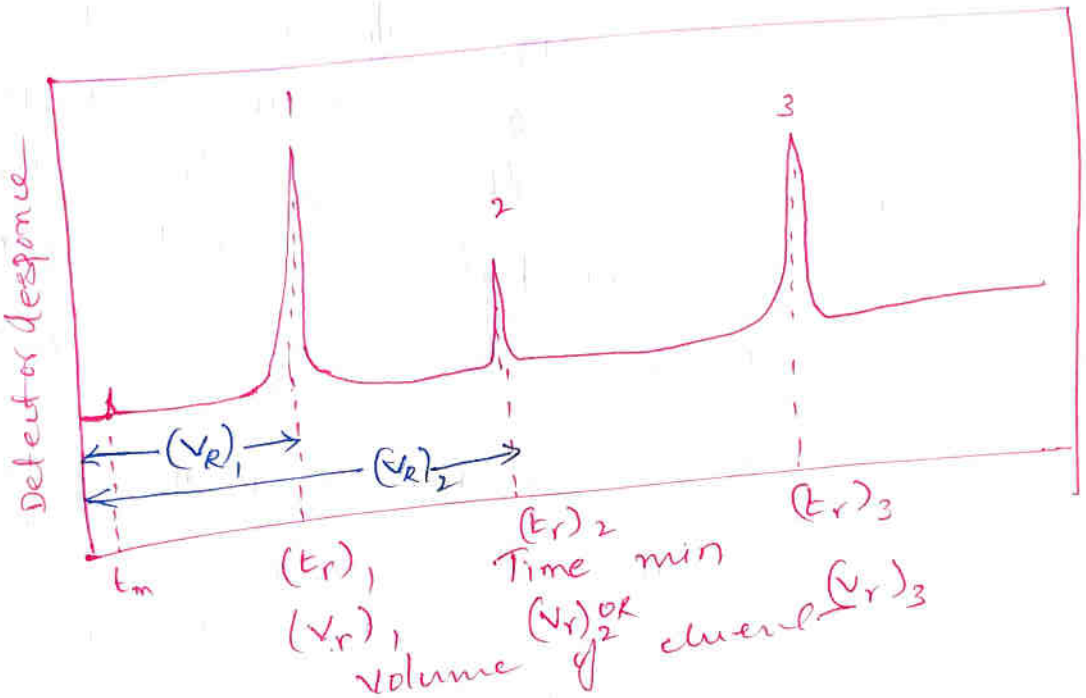
In a liquid composed of molecules with permanent dipoles, these molecules are much more strongly attracted to one another than to those of a non-polar. Thus dipole-dipole forces leads to association of liquids or liquid-solute ion pairs.

Dipoles capable of forming hydrogen bonds, bond between +ve pole of hydrogen of one molecule and -ve pole of hetero atom of another molecule. Water has tendency to form intermolecular hydrogen bonds

In non-polar liquid such as CCl_4, London's dispersion forces is the only force present between two molecules. In CCl_4 dipole forces and hydrogen bonding forces are absent.

Chromatographic behaviour of solutes

- Retention time (t_R)
- Retention volume (V_R)
- Partition Ratio (k) — Capacity factor (k)
- Retardation factor (R)



In column chromatography, the chromatographic behaviour of a solute can be described in terms of retention time of solute, retention volume of solute and retardation factor. A good chromatographic separation can be achieved by varying the mobile phase and stationary phase, pH, temperature, flow rate etc to secure the required retention of the components of the mixture

Retention volume (V_R): The volume of mobile phase (eluent) required to carry the component molecule or the band through the system to the detector is called retention volume (V_R)

Retention time : The time taken by a solute band maximum to move through the column is called retention time, denoted by (t_R). Retention time is experimentally determined. For a mixture of three components, for good efficiency and selectivity $(t_R)_1$, $(t_R)_2$ and $(t_R)_3$ are retention time of components respectively.

Relation between retention time & retention volume

Flow rate (F_m) is defined as volume of mobile phase that flows through column per one minute (ml/min).

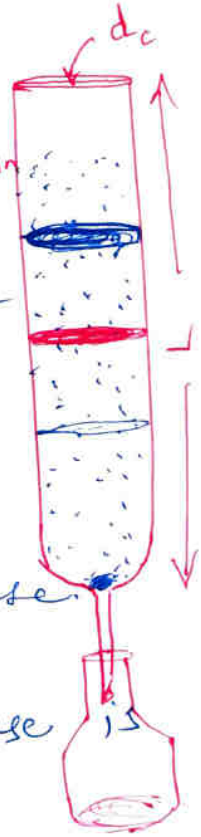
If F_m is the flow rate in ml/minute of the mobile phase, then retention volume of eluent (V_R) is obtained by multiplying retention time (t_R) with mobile phase flow rate (F_m)

$$\therefore V_R = t_R * F_m$$

Relation of Flow rate (F_m) to column diameter & length

Flow rate (F_m) in terms of column parameters is given by

$$F_m = \left(\frac{\pi d_c^2}{4}\right) \cdot \epsilon_{tot} \cdot \left(\frac{L}{t_m}\right) \text{ ml/min}$$



where d_c is diameter of the column

ϵ_{tot} is total porosity

L Length of the column

t_m Dead ~~volume~~ time. time of a non retained solute

$\frac{L}{t_m}$: Velocity of the mobile phase.

The average velocity of the mobile phase is measured by dead time t_m

the fraction $\left(\frac{\pi d_c^2}{4}\right)$ is cross section of the empty column; Cross section × Length = Volume of column

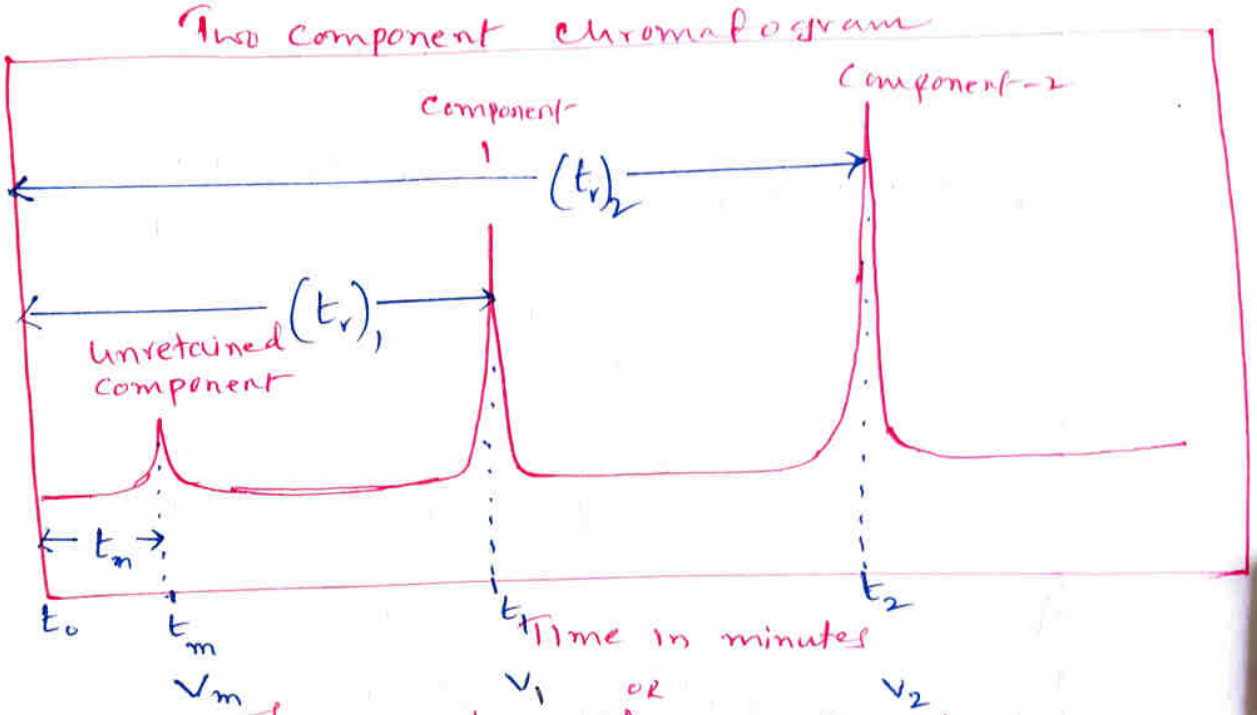
The value of total porosity for packed packings is 0.35 – 0.45
For porous packing material ϵ_{tot} is varies from 0.70 – 0.90

17(f) ∴ F_m = (Volume of column × C_{tot})/t_m time and

The correcte d retention time and retention volume are given by

$$(V_R)' = V_R - V_m$$

$$(t_R)' = t_R - t_m$$



$(t_r)_m$ or t_m : Retention time of an unretained component

$(t_r)_1$ or t_1 : Retention time of component-1

$(t_r)_2$ or t_2 = Retention time of component-2

$(V_r)_m$: Retention volume of unretained component

$(V_r)_1$: Retention volume of component-1

$(V_r)_2$: Retention volume of component-2

The corrected retention time $(t_r)' = t_r - t_m$

The corrected retention volume $(V_r)' = V_r - V_m$

$\therefore (t_r)'_1 = (t_r)_1 - t_m$ & $(t_r)'_2 = (t_r)_2 - t_m$

$(V_r)'_1 = (V_r)_1 - (V_r)_m$ & $(V'_r)_2 = (V_{r_2}) - (V_r)_m$

In gas Chromatography, the retention volume must be corrected for the compressibility of the gaseous phase arising from pressure differential along the column

∴ the net retention volume (V_N) is given by

$$V_N = j \times V_R$$

where j: compressibility factor

V_R: retention volume

Partition Ratio (k') – Relative Retention (α) – Retardation Factor (R)

The solute partition ratio (k') is the most important quantity in column chromatography. It relates the equilibrium distribution of the sample within the column to the thermodynamic properties of the column and to the temperature of the column. For a given set of operating parameters, k' is a measure of the time spent in the stationary phase relative to the time spent in the mobile phase.

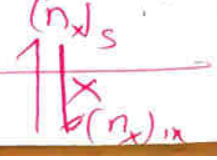
the solute partition ratio is the ratio of number moles of solute in stationary phase (n_s) and mobile phase (n_m)

$$k' = \frac{n_s}{n_m}$$

OR

k' is defined as the ratio of the total amount of a solute in the stationary phase to the total amount in the mobile phase at equilibrium

$$k' = \frac{\text{stationary phase}}{\text{mobile phase}}$$



x: component

~~Retardation Factor~~

Concentration $C = \frac{\text{No. of moles}}{\text{volume}}$

$C = \frac{n}{V}$

$\Rightarrow n = C \cdot V$

$$\therefore k' = \frac{n_s}{n_m} = \frac{C_s V_s}{C_m V_m} = \left(\frac{C_s}{C_m}\right) \cdot \left(\frac{V_s}{V_m}\right)$$

$$\therefore k' = K \frac{V_s}{V_m} \quad \text{or} \quad k' = D \cdot \frac{V_s}{V_m}$$

where K or D is distribution coefficient

k' is also known as capacity factor or column capacity ratio $\because V_R = t_R \cdot F_m$

k' interms of retention time $k' = \frac{(t_r - t_m)}{t_m}$ or $k' = \frac{V_r - V_m}{V_m}$

Relative retention (α) or Separation factor

~~Capacity factor or~~

If the sample contains minimum of two components, then we can define relative retention (α). Let us consider the sample consists two components 1 and 2, then k_1' and k_2' are capacity factors of components 1 and 2 respectively.

The solvent efficiency i.e the extent to which two components can be separated is expressed as relative retention (α) and is defined as

$$\alpha = \frac{k_2'}{k_1'}$$

k' value of a solute is given by an equation interms of retention time

$$k' = \frac{t_r - t_m}{t_m}$$

In another away partition ratio is stated as the addition of time or volume a solute band takes to elute over an unretained solute for which $k' = 0$ divided by the elution time or elution volume of unretained band.

Values of k' higher than 8 waste valuable analytical time

k' values less than unity are unfavorable due to potential interferences from non retained peaks and early peaks perhaps no analytical interest.

Desirable values of k' $\quad 1 < k' < 8$

Retention ratio (R) or Retardation factor

Partition ratio or Capacity factor k' is stated as the additional time or volume a solute band takes to elute over an unretained solute, divided by the elution time or volume of an unretained band

$$\therefore k' = \frac{t_r - t_m}{t_m} \quad \text{or} \quad k' = \frac{V_r - V_m}{V_m}$$

on rearranging $k' t_m = t_r - t_m \Rightarrow t_m(k'+1) = t_r$

$$\therefore t_r = t_m(1+k') \Rightarrow \left(\frac{1}{1+k'}\right) = \frac{t_m}{t_r}$$ ✓

the fraction of time that a sample molecule spends in a particular phase will be very close to the fraction of all molecules of that solute which are instantaneously in the same phase ✓

Thus, the average fraction of time spent by a solute molecule in the mobile phase will be

$$\left(\frac{n_m}{n_m + n_s}\right) = \frac{1}{1+k'} \quad \text{or} \quad \frac{C_m V_m}{C_m V_m + C_s V_s} = \frac{1}{1+k'} = R$$

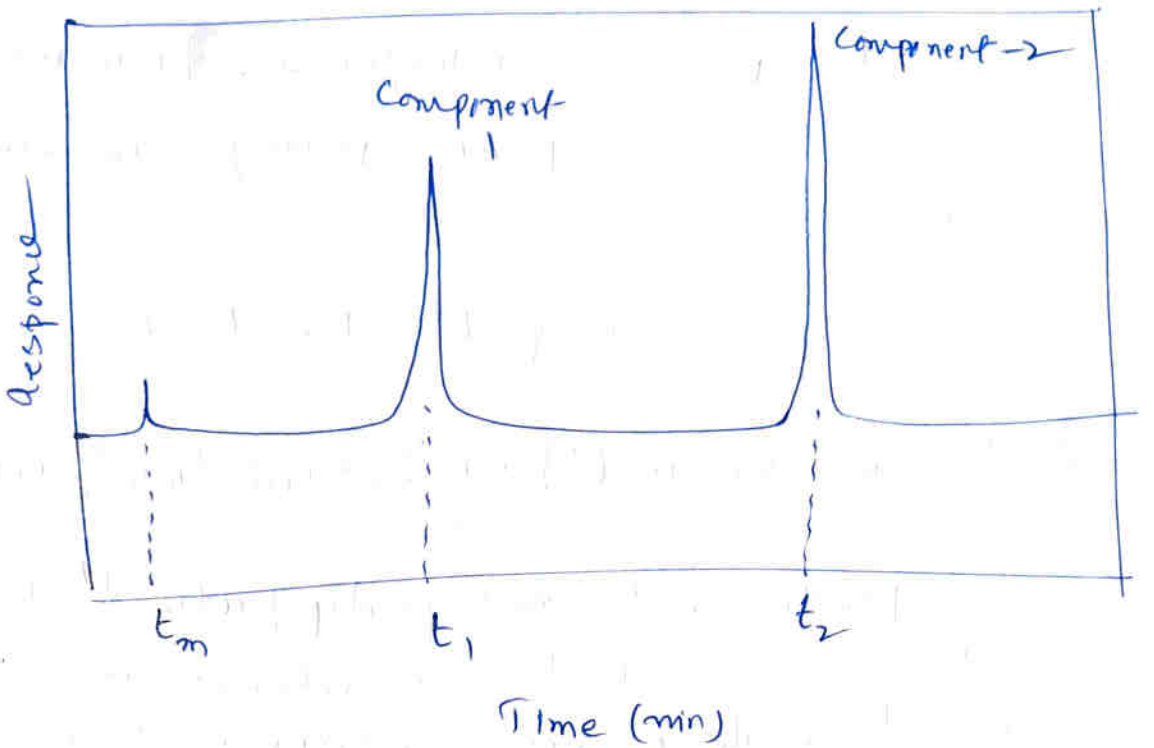
the above equation is denoted by R, called retardation factor.

$\therefore$ Retardation factor (R) is the fraction of time spent by the solute in the mobile phase

$$\therefore R = \frac{t_m}{t_m + t_s} = \frac{V_m}{V_m + K V_s} = \frac{V_m}{V_s} = \frac{t_m}{t_r}$$

where t_r is the retention time of the solute

t_m " " " unretained solute



The equation $k' = \frac{t_r - t_m}{t_m}$ gives a direct measure of k' values of solutes from the respective peaks in the chromatogram

$$\therefore k_1' = \frac{t_1 - t_m}{t_m} \quad \& \quad k_2' = \frac{t_2 - t_m}{t_m}$$

$$\therefore \alpha = \frac{k_2'}{k_1'} = \frac{t_2 - t_m}{t_m} \times \frac{t_m}{t_1 - t_m}$$

$$\therefore \alpha = \frac{t_2 - t_m}{t_1 - t_m}$$

$\because t_2 > t_1$, α is always greater than unity

i.e $\alpha > 1$

Values of k' higher than 8 waste valuable analytical time

k' values less than unity are unfavorable due to potential interferences from non retained peaks and early peaks perhaps no analytical interest.

Desirable values of k' $\quad 1 < k' < 8$

Retention ratio (R) or Retardation factor

Partition ratio or Capacity factor k' is stated as the additional time or volume a solute band takes to elute over an unretained solute, divided by the elution time or volume of an unretained band

$$\therefore k' = \frac{t_r - t_m}{t_m} \quad \text{or} \quad k' = \frac{V_r - V_m}{V_m}$$

on rearranging $k' t_m = t_r - t_m \Rightarrow t_m(k'+1) = t_r$

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Thus, the average fraction of time spent by a solute molecule in the mobile phase will be

$$\left(\frac{n_m}{n_m + n_s}\right) = \frac{1}{1+k'} \quad \text{or} \quad \frac{C_m V_m}{C_m V_m + C_s V_s} = \frac{1}{1+k'} = R$$

the above equation is denoted by R, called retardation factor.

$\therefore$ Retardation factor (R) is the fraction of time spent by the solute in the mobile phase $\quad \therefore R = \frac{t_m}{t_m + t_s} = \frac{V_m}{V_m + V_s} = \frac{V_m}{V_s} = \frac{t_m}{t_r}$

Principles of Chromatography — Different migration rates
Principle of Different Chromatographic Methods

Basic principle of Chromatography

The Sample mixture undergoes a series of interactions many times between stationary phase and mobile phase as it is moving through the system by the mobile phase

The interactions of analytes with stationary phase and mobile phase depend on nature of the analytes and ~~xxx~~ nature of the stationary and mobile phases: these differences govern the rate of migration of analytes under identical conditions.

The separated components emerge in the order of increasing interaction with the stationary phase. the least retarded analyte elutes first, the most strongly retained analyte elutes last.

Principle of Ion-exchange Chromatography

Sample: Ionic Substances such as simple inorganic, Organic ions such as enzymes, proteins, hormones, viruses nucleic acid etc.

Stationary phase: Ion-exchanger (zeolites, Ion exchange resins)

Mobile phase: Electrolyte solution.

Principle: The principle of ion-exchange chromatography is diffusion and exchange of ions from the sample solution to the site of ion-exchanger (ion exchange site) and then desorption by the eluent and diffusion of the ions into the eluent.

Principle of Adsorption Chromatography

Sample: Both polar and non polar components.

Stationary phase: The solid material or adsorbent such as alumina, ~~charcol~~ Charcoal silica gel, $MgCO_3$, $CaCO_3$, K_2CO_3, Na_2CO_3, Sucrose starch and cellulose is used as stationary phase (decreasing order of polarity)

Mobile phase: Several solvents such as fluoroalkanes, petroleum ether, carbontetrachloride cyclohexane, toluene, benzene, esters, chloroform ethyl ether, dichloroethane, methyl ethyl keton, acetonitrile, alcohols, water, Pyridine and organic acids (Increasing order of polarity)

For strongly adsorbed compounds, polar solvent such as alcohol, pyridine and weakly adsorbed compounds, normally non-polar solvents like petroleum ether cclq, or cyclohexane are recomended.

Principle: The separation of components in a mixture into individual components depends upon the adsorption of analytes on the adsorbent by physical and chemical interactions between analytes and stationary phase. Adsorption is based on purely physical in nature characterized by low adsorption energies and rapid equilibrium results good separation.

Partition Chromatography - Principle

Sample: Mixture containing polar and non polar components.

Stationary phase: The stationary phase in partition chromatography is a very thin film of liquid that is adsorbed on the surface of an inert solid material like silica gel, cellulose powder or diatomaceous earth (clay). The solid serves only a support for the liquid stationary phase, example: water and any hydrophilic organic solvents.

Mobile phase: A liquid or mixture of solvents is used as mobile phase in partition chromatography. The solvent series in the order of decreasing capacity of H-bonding is given below.

water, formaldehyde, methanol, acetic acid, ethanol, acetone, n-propanol, ethyl acetate ether, butyl acetate, chloroform, benzene toluene, cyclohexane and petroleum ether

Principle: The separation of components in the mixture is based on partition of component between stationary phase (liquid) and mobile phase (liquid). During the migration, a series of many number of partitions takes place inbetween stationary and mobile phases (immiscible liquids). Since partition is dependent on solubility of solute in two immiscible liquids, and hence a small difference in molecular weight will influence partitioning

Time on the column, the medium fixed molecules for long time and large molecules pass straight through in the eluent.

Principle in Gel Chromatography [Exclusion Chrom]

The separation of molecules present in a given mixture is based on size and shape of molecules instead of polarity. The selectivity is based on steric factors between the adsorbate and adsorbent. If the mixture contains small, medium and large size molecules being eluted through stationary phase (Gel), small molecules completely penetrate, medium sized molecules partially penetrate and large molecules do not penetrate. Thus the small molecules are retained longer

Principle in affinity Chromatography

In affinity chromatography, a ligand is covalently bonded to a solid matrix which is packed into a chromatographic column. A mixture of components is then applied to the column. The unbound contaminants which have no affinity for the ligand are washed through the column, leaving the desired components such as protein, peptide or DNA fragment bound to the matrix. ~~Elution~~ Elution is accomplished by changing the pH, applying salt solution or organic solvents or by eluting with a molecule which competes for the bound ligand.

It is based on unique interactions between biomolecules. It involves the use of a bioselective stationary phase agarose or cross linked agarose, dextrans.

Band Broadening – Van Deemter equation

Fig-1

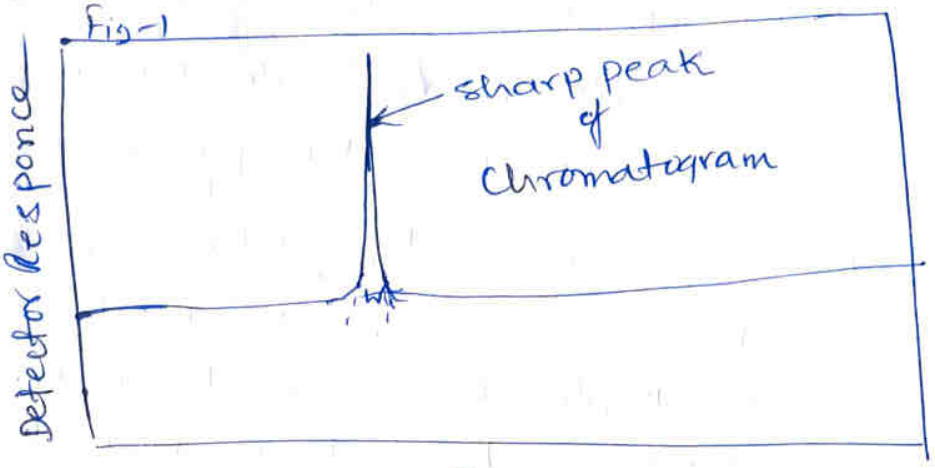
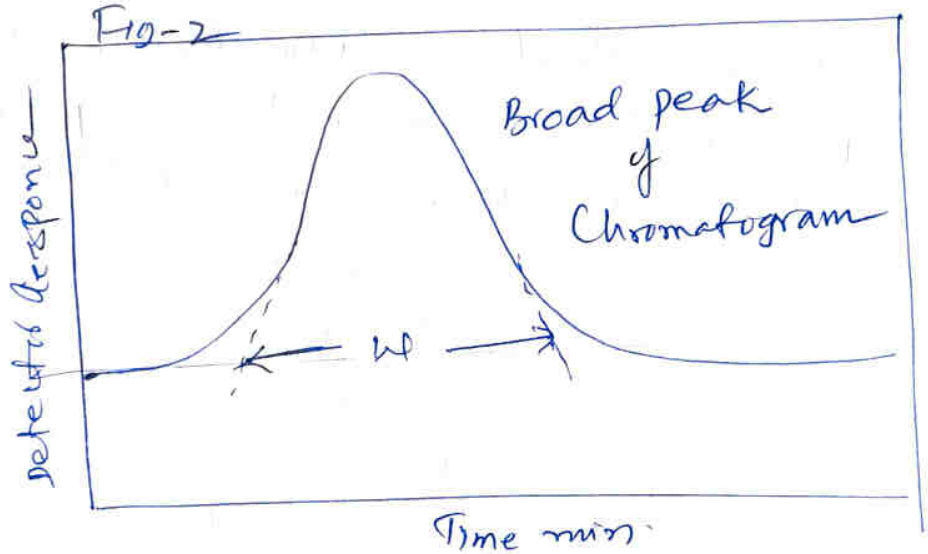


Fig-2



Best separations and obtained by achieving sharp and narrow peaks with good resolution in the Chromatogram (Fig-1)

Peak broadening for a particular solute in the Chromatogram corresponds to 'Zone broadening' for that solute in Chromatographic column. The broadening of zones migrating through the Chromatographic column is affected by the following processes

(1) Axial or Longitudinal molecular diffusion

(2) Mass transfer processes

(1) Axial or Longitudinal molecular diffusion

It is the migration of solute from regions of higher concentrations to lower concentrations in the direction of flow of mobile phase in the column It is expressed by $2\gamma D_m$, where γ is an obstructive parameter and D_m is the solute diffusion coefficient in the mobile phase. In coated capillary columns $\gamma = 1$, and in packed columns γ is about 0.6. High diffusion rates in the mobile phase cause solute bands to disperse axially along the column, particularly at low mobile phase velocities. This leads to peak broadening. The contribution of longitudinal diffusion to the plate height (H) is significant only at low mobile phase velocities.

$$\text{Longitudinal Diffusion Factor} = \frac{2\gamma D_m}{\bar{U}}$$

where $\bar{U}$ is average linear velocity i.e flow rate experimentally the average linear velocity is determined by injecting a non-retained solute on the column being tested and measuring the time taken for it to pass through the column.

$$\bar{u} = L / t_m$$

(2) Mass transfer processes:

Mass transfer process has significant contribution to zone broadening. Mass transfer processes arise due to slow equilibrium between mobile phase and stationary phase zones.

Band broadening arises because molecules in the stationary phase zone tend to get left behind when the main band passes over, while the molecules in the mobile (phase) zone move ahead.

Mass transfer process may be divided into two terms

(a) stationary phase mass transfer
(b) mobile phase mass transfer
(c) Eddy Diffusion

(a) Stationary phase mass transfer: The rate at which solute molecule transfer into and out of the stationary phase makes a significant contribution to zone broadening and hence to efficiency of separation. The rate depends mainly on diffusion for liquid stationary phases and on adsorption-desorption kinetics for solid stationary phase. Solute molecules will reside in or on the stationary phase for varying lengths of time, thus molecules diffuse more deeply into the stationary phase (liquid in case of LLC or solid in LSC). Therefore to reduce band broadening from stationary (phase) mass transfer effects, liquid layers should be as thin as possible.

(b) Mobile phase mass transfer: In LSC, when porous stationary phases are used, the intra-particle empty volume is filled with mobile phase at rest. Solute molecules must

diffuse through stagnant mobile phase in order to reach the stationary phase. Molecules that diffuse only a short distance into the pore will rapidly regain the mainstream whereas molecules that diffuse further and spend more time in the pore will be left behind the mainstream, resulting zone broadening.

(c) Eddy Diffusion: Eddy diffusion occurs in LLC and GC from the movement of the mobile phase in a packed column in tortuous channels. The flow paths available are tortuous and zig-zag, some of these are narrow ones and others wider ones. This is so on account of irregularities in the packing material and different qualities of the packing of the column.

The zig-zag path taken by a part of the mobile phase makes some molecules of a particular solute take longer paths and they lag behind the bulk of the molecules of that solute, while some molecules of the same solute which are present in another part of the mobile phase take shorter paths and move ahead of the ~~main str~~ mainstream of the molecules of that particular solute.

Also, the mobile phase moves more slowly in narrow flow paths and more rapidly in wider ones so that inequalities in the transport of the molecules of solute by the mobile phase results zone broadening.

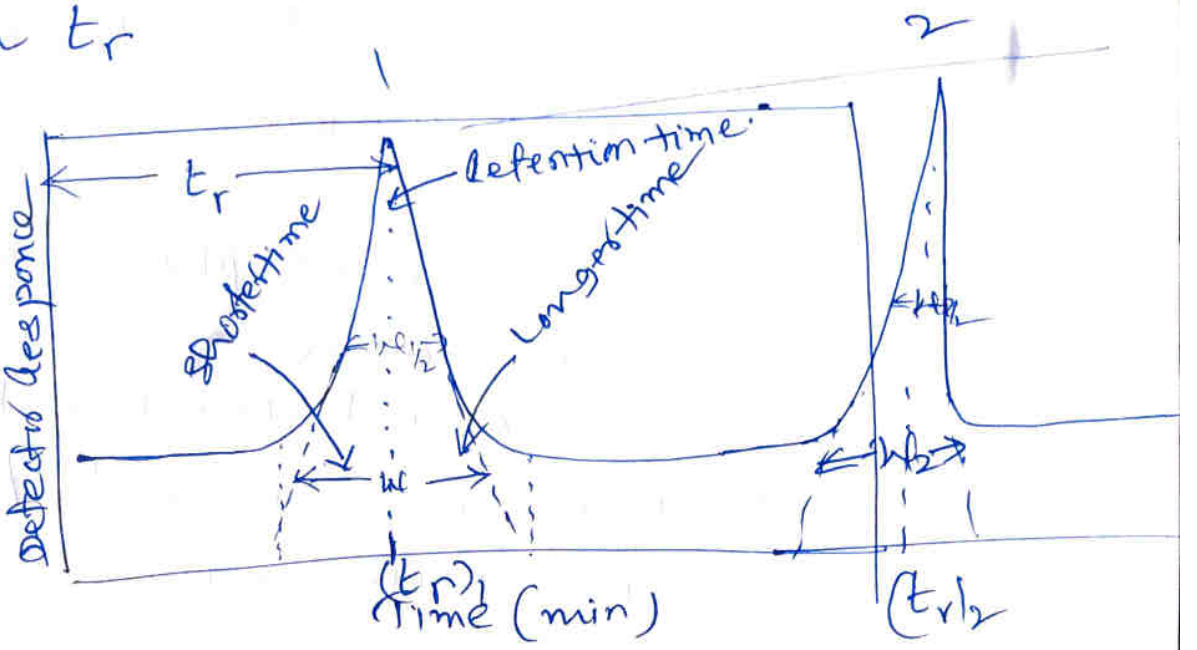
Efficiency of Chromatographic column – Hight Equivalent theoritical plates (HETP) - Van - Deemper equation

In Chromatographic separation of components present in a sample mixture, the sample is introduced into the column, mobile phase is allowed to flow over the stationary phase. the separation of the components is achieved by a series of distributions of solute molecules between mobile phase and stationary phase.

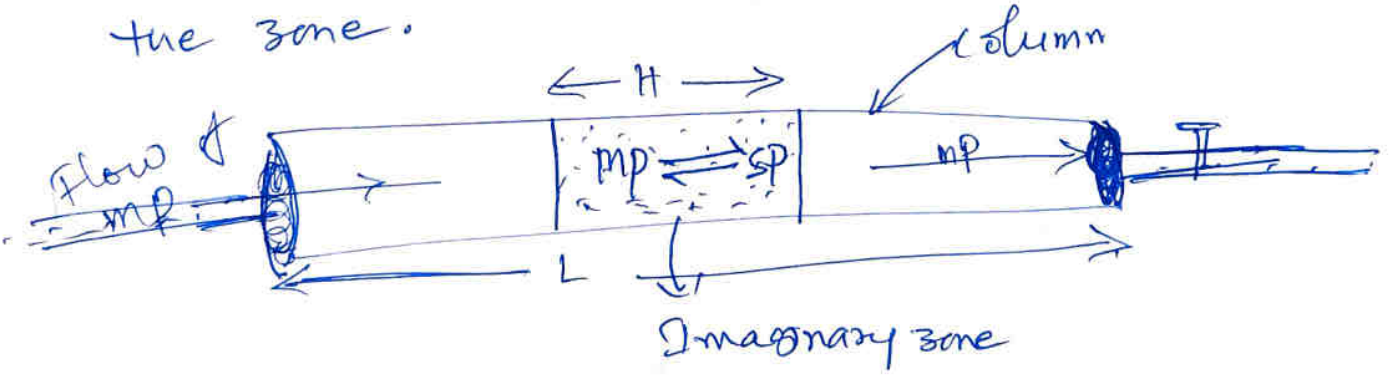
the heart of the Chromatographic separation is column. A good separation is obtained by choosing correct combination of stationary phase (column) and mobile phase the efficiency of separation is expressed in terms of number of theoritical plates (N), assymetry factor, resolution etc.

The efficiency of Chromatographic column is measured in terms of the number theoritical plates (N) in the column. column efficiency is the rate at which molecules spread out as they migrate through the column. For a single solute not all molecules elute at time t_r to give a retention volume V_r.

Some particles elute in shorter time than t_r and a few molecules elute longer time than t_r



The 'theoritical plate' is an imaginary zone or segment of the column in which the composition of mobile phase flowing out of the segment or zone is in equilibrium with the average composition of the fixed phase within the zone.



The height equivalent of theoritical plate (HEPT) or simply plate height (H) is the length within the column that is able to achieve equilibrium between phases under a particular set of conditions i.e temperature, flow rate etc.

Number of theoritical plates is calculated from the equation

$$\text{No. of theoritical plates } (N) = \frac{\text{Column Length } (L)}{\text{plate height } (H)}$$

$$\therefore N = \frac{L}{H} \qquad \text{---------- (1)}$$

But N is related to retention time of the solute eluted t_r and width of the chromatographic peak (W) by the following equation

$$N = 16 \left(\frac{t_r}{W}\right)^2 \qquad \text{---------- (2)}$$

W is obtained by extrapolation of tangents at the points of inflection to the base line.

The number of theoritical plates calculated by substituting the values of t_r and W in equation (2) obtained from experimental chromatogram. It represents the measure of efficiency of the column. The efficiency of column is characterized by high value of N (Larks).

By combining equations (1) and (2) we can calculate height equivalent theoritical plates (H)

$$\therefore H = \frac{L}{16} \left(\frac{t_r}{W}\right)^2 \qquad \text{---------- (3)}$$

This relationship shows that t_r and W must vary together to keep H as constant

It means that for several peaks in the same chromatogram, the longer the retention time (t_r), the larger will be W and broader the peaks. Since L is constant

The value of N is also determined by $N = 5.54 \left(\frac{t_r}{w_{y_2}}\right)^2$ and $H = \frac{L}{16}\left(\frac{t_r}{W}\right)^2$

Significance of H

i) plate height is a more true measure of the column efficiency (which is determined by the number of equilibrium steps and is independent of the column length)

ii) plate height reflects the sharpness of the chromatographic peak

(iii) Low value of H indicates that the sample equilibrates rapidly many more times between stationary phase and mobile phase before emerging from the column. (High efficiency of column) - sharp peak high resolution.

iv) Large value of H indicates that there are a few number equilibriums and the sample spreads inside the column and thus poor resolution

Van-Deemter equation or plate height equation

Plate height (H) is a function of thermodynamic and kinetic processes that take plane in the column.

The longitudinal and transverse diffusion in the mobile phase, finite rate of equilibrium of solute between the stationary and mobile phases (mass transfer), diffusion in the liquid stationary phase, and flow irregularities influence the plate height.

The relationship between above variables and the plate height is given by the Van Deemter equation

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{8}{\pi^2}\frac{k'}{(1+k')^2}\frac{d_f^2 u}{D_{liq}} \quad \text{---(1)}$$

OR

$$H = A + \frac{B}{U} + C \cdot U \quad \text{---(2)}$$

Where $A = 2\lambda d_p$

$B = 2\gamma D_m$

$C = \frac{8}{\pi^2}\frac{k'}{(1+k')^2}\frac{d_f^2}{D_{liq}}$

Here λ – a constant which is a measure of packing irregularities.

d_p – average particle diameter of solid

γ – a correction factor accounting for tortuosity of the channels in the column

D_m: Diffusivity of the solute in the mobile phase

U: Average velocity of mobile phase

k': Capacity factor

d_f: the stationary film thickness

D_{liq}: molecular diffusion coefficient of the solute in the stationary phase.

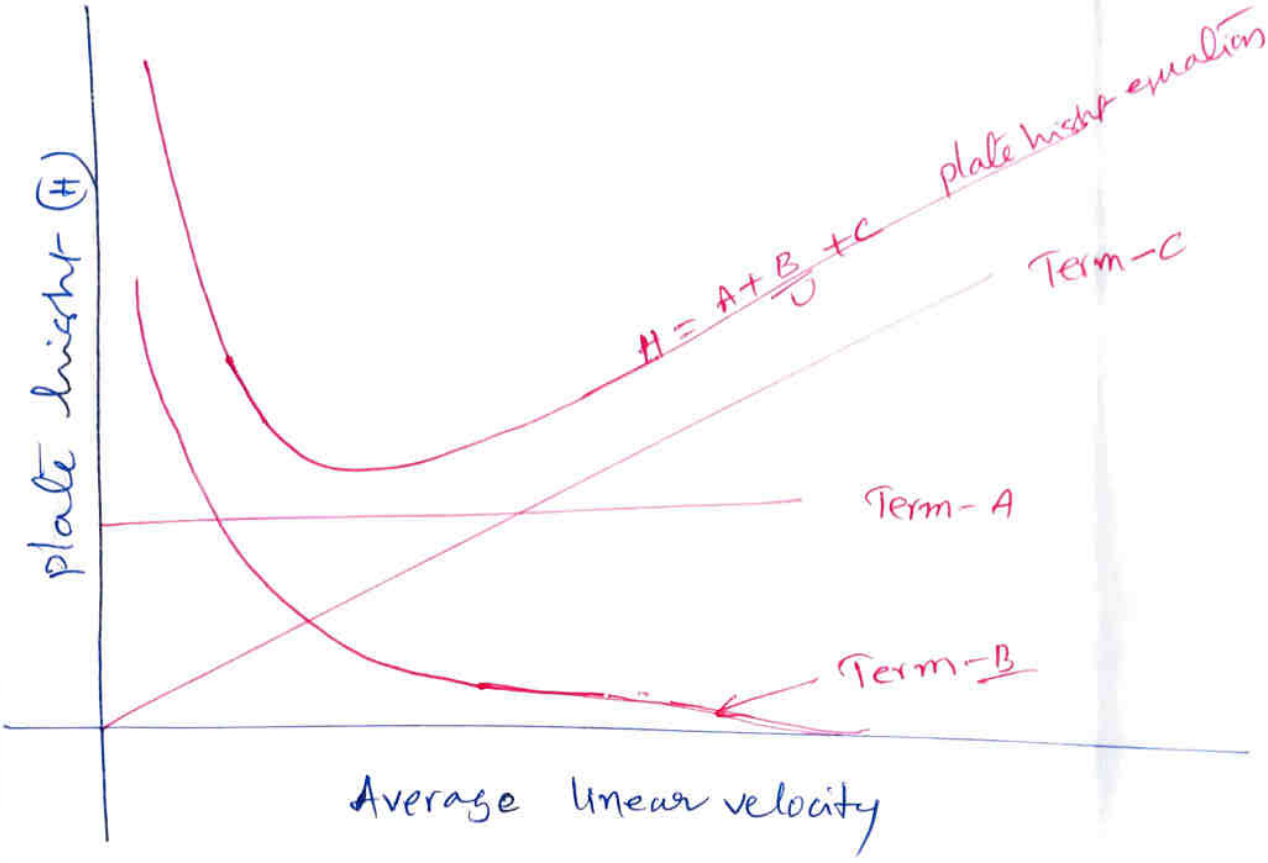
The constant A, B and C are characteristic of an individual column and vary from one colum to another column.

The term – A in van-Deemter equation is called eddy diffusion.

The term – B defines the effect of longitudinal or axial diffusion i.e random molecular motion

The term – C referes to mass transfer processes at the solute–stationary phase ~~interference~~ interface

The plot of plate height (H) against linear velocity (U) gives a hyperbola curve called van-Deemter plot



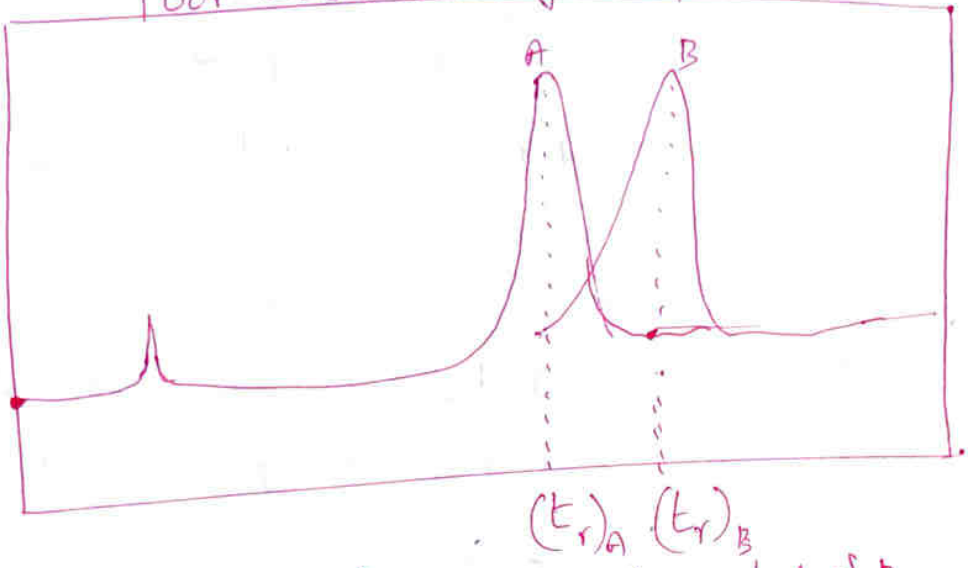
Eddy Diffusion (Term-A) :-

Longitudinal Diffusion (Term-B) :-

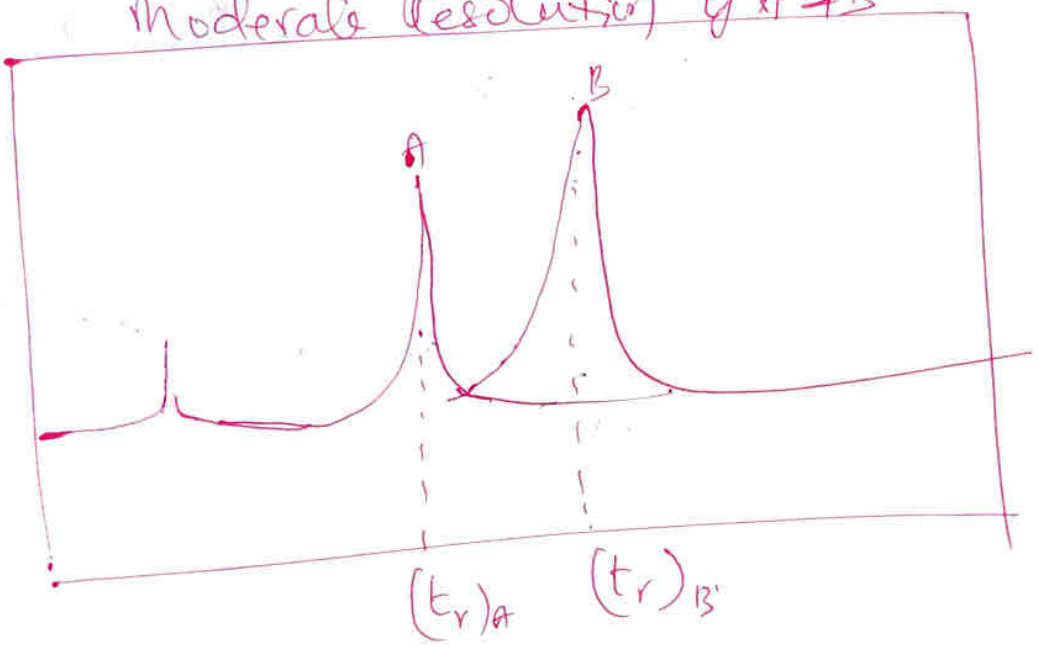
Mass Transfer (Term-C) :-

Resolution – Choice of column – Length – Flowrate

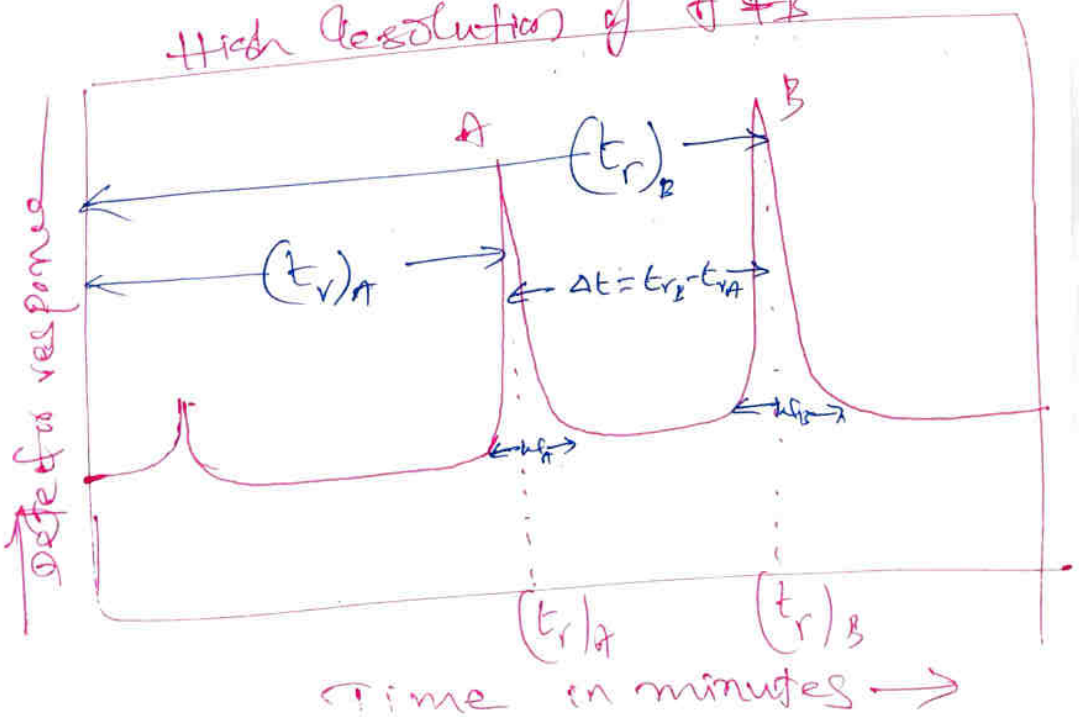
Poor Resolution of A & B



Moderate Resolution of A & B



High Resolution of A & B



In Chromatographic separation of two or more than two component sample, the analytes migrates through the Chromatographic column, and separated into discrete bands or peaks if band widths are smaller i.e peaks are sharper. In well-behaved Chromatography where the paks are Gaussian in shape, the resolution is expressed by the following equation.

$$R = \frac{(t_r)_B - (t_r)_A}{\frac{(w_A + w_B)}{2}} = \frac{2\left((t_r)_B - (t_r)_A\right)}{(w_A + w_B)}$$

OR

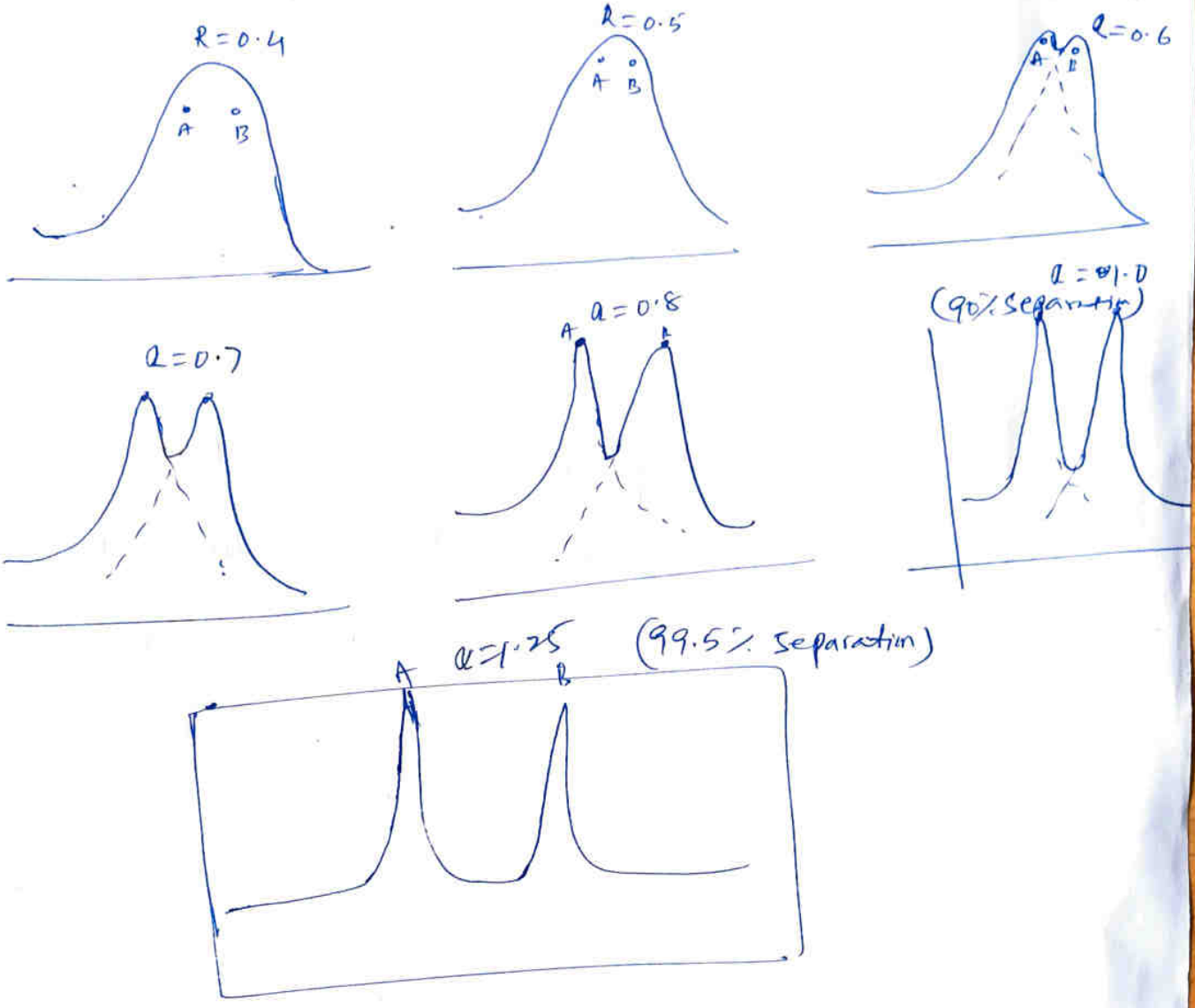
$$R = \frac{R \cdot \Delta t}{(w_A + w_B)} \quad \text{OR} \quad R = \frac{\Delta t}{0.5\,(w_A + w_B)}$$

$(t_r)_A$ & $(t_r)_B$: Retention time of component A & B respectively.

w_A & w_B : Base peak width of peak A & B respectively (time units)

$$\Delta t = (t_r)_B - (t_r)_A$$

The Standard resolution curves for peak height ratio 1:1 are shown ~~below~~ below.



For R = 0.4 and 0.5 two peaks merged. Peak shapes do not provide observable band centers

For R = 0.6, two peaks are observed, do not coincide error in measurements of t_r and W

For R = 0.7 & 0.8 two peaks clearly observed, peak centers are observed, but poor resolution.

R = 1.0, 90% of the peaks separated.

For R = 1.25 A good and qualitative separation 99.4% can be obtained.

For approximately equal quantities of two solutes in adjacent bands, the resolution equation can be approximated in more useful form as

$$R = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1+k'}\right)\cdot\left(\frac{L}{H}\right)^{1/2}$$

This equation can be divided into 3 parts

(i) Selectivity term dependent upon α

(ii) Rate of migration term depending upon k'

(iii) Efficiency term depending on L & H

Each term can be calculated directly from the recorded chromatogram

The first term selectivity depends on the molecular forces between the solute and SP & MP. Once MP, SP and temperature are chosen, the relative retention is fixed and can not be changed.

The second term k' depends on the intermolecular attractions of the system and amount of SP and surface area of SP. In practice the column loading of the SP and partition coefficient should be chosen such that $(t_r)_1 = 2t_m$: optimum range of k' 1 to 5

The third term (L/H), resolution may be improved by decreasing the plate height 'H' rather than increasing the column length. By lowering flow rates, H decreases