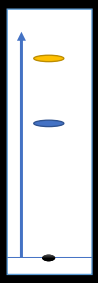
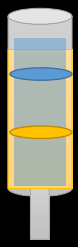
Basics of Chromatography (Part 1)

- ✓Basic Introduction
- ✓Types
- ✓Principle





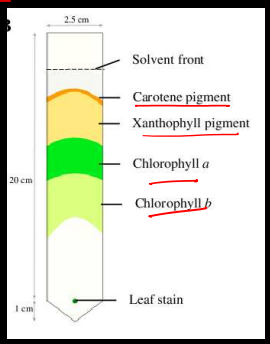
Chromatography
Instrumental Analysis

CHROMATOGRAPHY

Introduction:

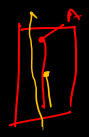
Mikhail Tswett

- **Chromatography** is a combination of two words;
  - **Chromo** – Meaning color
  - **Graphy** – representation of something on paper
- Chromatography, literally "color writing", was first employed by Russian scientist Mikhail Tswett in 1903/1906.
- He continued to work with chromatography in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls. Since these components have different colors (green, orange, and yellow, respectively) they gave the technique its name.



CHROMATOGRAPHY

Introduction:



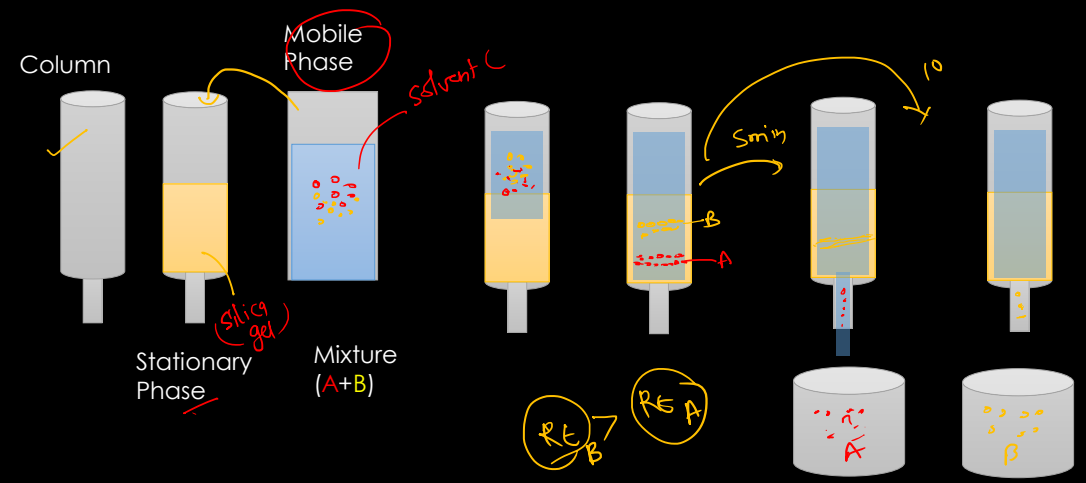
- **IUPAC definition (**International Union of pure and applied Chemistry) (1993):
- Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction.
- It is usually introduced as a technique for identification and separation of compounds from a mixture.
- Basically it consists of two phase- 1) Mobile phase 2) Stationary Phase
- And Separations occurs due to continuous distribution of component between two phases, where mobile phase moves over the stationary phase

CHROMATOGRAPHY

Introduction:

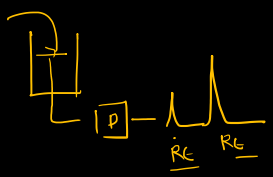
- **The stationary phase** may be a solid, or a liquid supported on a solid or gel,
- **The mobile phase** may be either a gas or a liquid.

CHROMATOGRAPHY



CHROMATOGRAPHY

Common Termns:



- **Chromatograph:** Instrument employed for a chromatography.
- **Chromatogram:** It is the visual output of the chromatograph.
- **Eluent:** Fluid mixture entering a column.
- **Eluate:** Fluid exiting the column.
- **Elution:** The process of passing the mobile phase through the column.
- **Flow rate:** How much mobile phase passed / minute (ml/min).
- **Linear velocity:** Distance passed by mobile phase per 1 min in the column (cm/min).
- **Retention time:** It is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.

CHROMATOGRAPHY

Types or Classification:

- **Based on Principle**
  - Adsorption
  - Partition
  - Ion Exchange
  - Size Exclusion
  - Affinity
- **Based on Mobile Phase**
  - **Liquid Chromatography**
    - Liquid-Liquid Chromatography- Paper, Ion Exchange, Droplet Counter Current (DDC)
    - Liquid-Solid Chromatography- Column, HPLC, TLC, HPTLC

CHROMATOGRAPHY

Types or Classification:

- **Based on Mobile Phase**
  - **Liquid Chromatography**
  - **Gas Chromatography**
    - Gas-Solid Chromatography (GSC)
    - Gas-Liquid Chromatography (GLC)
  - **Super-Critical Fluid Chromatography**
- **Based on Geometry**
  - Planner- TLC, HPTLC, Paper
  - Columnar- Column, HPLC, GC, Ion Exchange, Affinity, DDC

CHROMATOGRAPHY

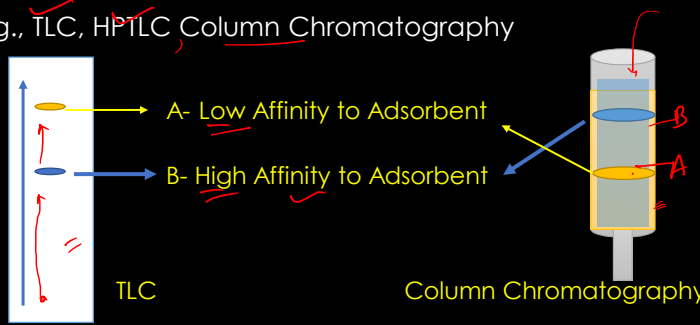
Types or Classification:

- **Based on Polarity of the Phase**
  - Normal Phase- Stationary phase is Polar & Mobile phase is Non-polar
  - Reverse- Stationary phase is Non-polar & Mobile phase is Polar, Widely used in Pharmacy

CHROMATOGRAPHY

Principles:

- **Adsorption Chromatography**
- Adsorption Chromatography involves the separation of a chemical mixture based on the interaction of the adsorbate with the adsorbent.
- Separation based on Affinity of analyte to Stationary Phase (Solid) and Mobile Phase (Liquid or Gas)
- E.g., TLC, HPTLC Column Chromatography



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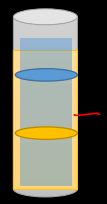
Principles:

- **Partition Chromatography**
- The separation of components between two liquid phases viz original solvent (Mobile phase-Liquid/Gas) and the film of solvent/Liquid used in the column (Stationary phase).
- This separation theory was introduced in the year 1940s which was published by Richard Laurence Millington Synge and Archer Martin
- E.g. Liqui-Liquid Chromatography and Gas-Liquid Chromatography



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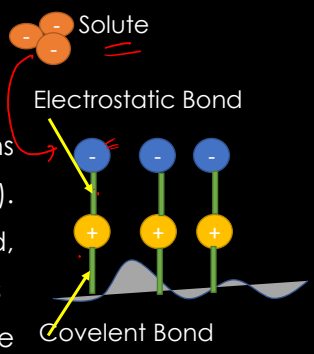
Principles:

- **Ion Exchange Chromatography**
- Ion- exchange chromatography is based on electrostatic interactions between charged protein groups, and solid support material (matrix). Matrix has an ion load opposite to that of the protein to be separated, and the affinity of the protein to the column is achieved with ionic ties
- Ion exchange chromatography is commonly used to separate charged biological molecules such as proteins, peptides, amino acids, or nucleotides



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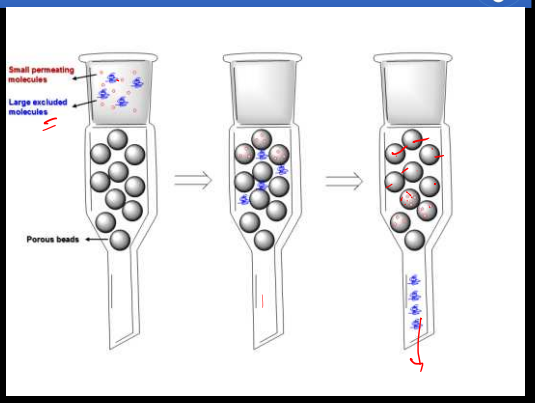
Principles:

- **Size Exclusion/Gel Permeation/Gel Filteration Chromatography**
- separates analytes on the basis of size, typically in organic solvents. The technique is often used for the analysis of polymers.
- The basic principle of this method is to use dextran containing materials to separate macromolecules based on their differences in molecular sizes. This procedure is basically used to determine molecular weights of proteins.



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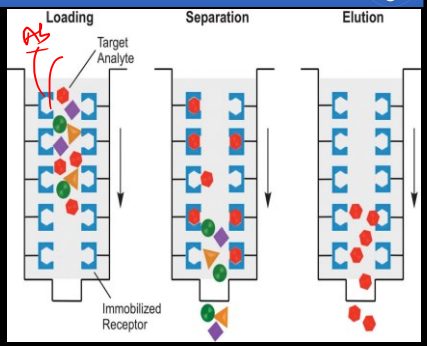
Principles:

- **Affinity Chromatography**
- Affinity chromatography can be defined as a type of liquid chromatography that uses a biologically-related agent, or "affinity ligand", as a stationary phase to selectively retain analytes or to study biological interactions like Antigen-Body interaction.
- The affinity ligand is immobilized within a column and used to selectively bind a given target or group of targets within a sample. Because of the highly selective nature of many affinity ligands, the result is a column that can be used to isolate, measure, or study specific targets even they are present in complex biological samples



Basics of Chromatography (Part 2)

- ✓Theories:
  - ✓Plate Theory
  - ✓Rate Theory

Chromatography
Instrumental Analysis

CHROMATOGRAPHY

Basic Principles:

- Chromatography is an essential technique in analytical chemistry. **M.S Tswett** first developed it in 1906 to obtain the separation of coloured substances into their components.
- Chromatography helps in separating, identifying, and purifying components from a mixture.
- The basic principle of chromatography is based on the concept of distribution of components of a mixture of organic compounds between two phases which are stationary and mobile

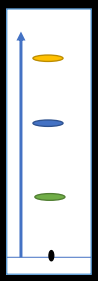
CHROMATOGRAPHY

Theories:

- There are two major theories to understand the separation:
  - Plate Theory
  - Rate Theory
- Here are a few things to know about chromatography:
  - This the Color writing so components are Colored
  - The colored solutes are soluble in the same solvent/mobile phase.
  - One of the components should be more soluble in the solvent for obtaining the separation.
  - The stationary phase is the one that is attached to the column or plate of the chromatography. The most common material used for the same is silica gel, calcium carbonate, activated charcoal, etc. It is primarily porous.

CHROMATOGRAPHY

Theories:



- There are two major theories to understand the separation:
  - Plate Theory
  - Rate Theory
- Here are a few things to know about chromatography:
  - Mobile phases are the mixture of the solute dissolved in the solvent, consisting of the solute to be separated.
  - As the mobile phase flows along with the stationary phase, it adsorbs on the stationary phase based on their affinity and attains separation.

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Plate Theory:

- The Plate Theory concept was adapted to chromatography by Martin and Synge in 1941. T
- The plate model supposes that the chromatographic column contains a large number of separate layers, they called theoretical plates.
- Separate equilibrations of the sample between the stationary and mobile phase occur in these plates. Each single equilibration termed ad Theoretical Plates
- The length of the column is required for one equilibrium is called Hight equivalent a theoretical plate (HETP)

$$HETP = L/N$$

  - L- Length of Column
  - N- No. of Theoretical plates

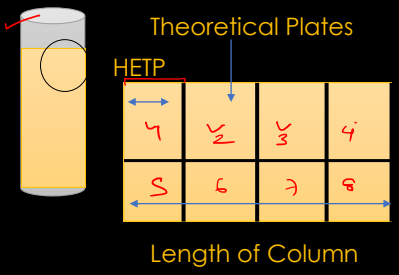

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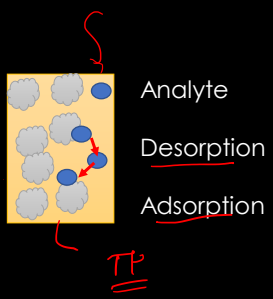
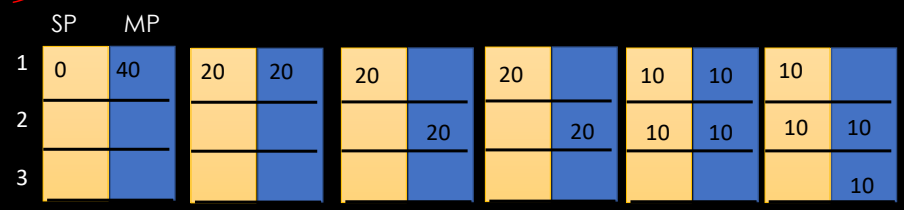
Plate Theory:

- When a compound is passing through the column, it is getting distributed between the stationary phase & the mobile phase and an equilibrium is established.
- After equilibrium, the solute is carried by the mobile phase from one plate to another and the process is continued till the solute elutes out of the column with it's characteristic retention time and peak width





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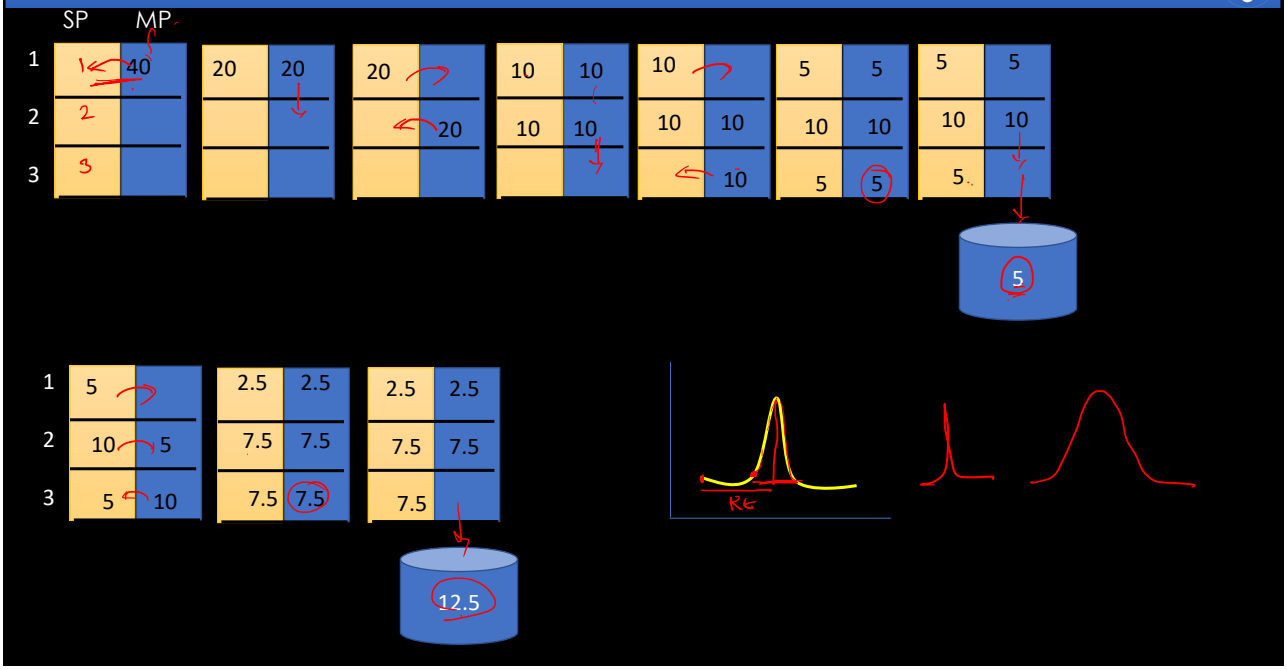

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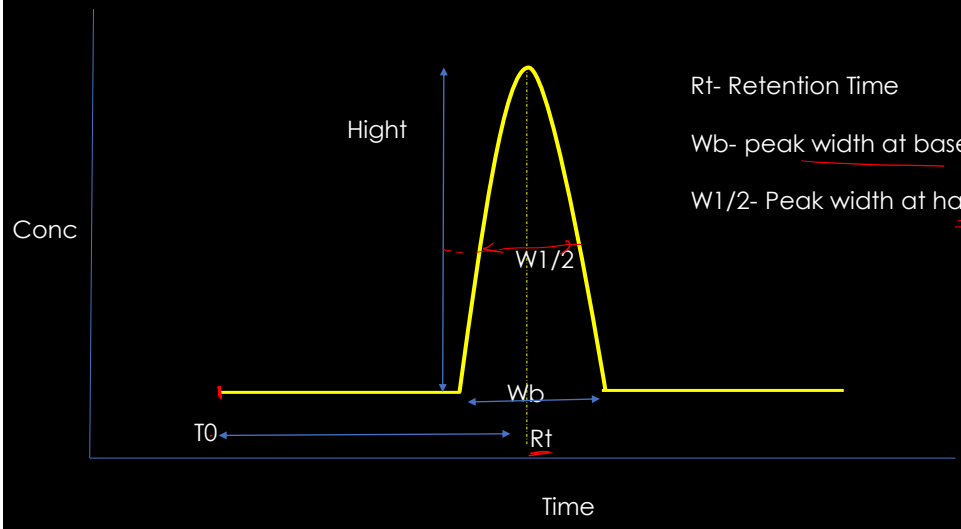

Rt- Retention Time

Wb- peak width at base

W1/2- Peak width at half Hight

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Plate Theory:

- **Retention time:** It is the time taken by a sample component peak to reach it's maximum height. All molecules spend the same amount of time in the mobile phase and this time is called the column dead volumn or hold up time, t0. This can be calculated by injecting an unretained sample into the system. The adjusted retention time of a compound is given by, $Rt' = Rt - t0$, where Rt is the retention time.

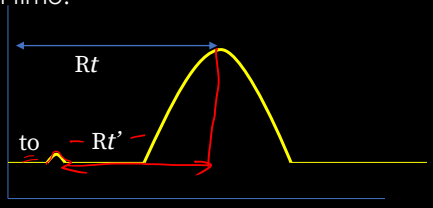

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Plate Theory:

- Relative Retention: a: This factor gives an idea as to how far two compounds can be separated by a given stationary phase. For any two compounds 1 and 2, the relative retention, a is the ratio of their adjusted retention times. Hence, $a = Rt'2 / Rt'1$. Relative retention has to be always more than 1.

$\alpha : \frac{RT^2}{RT_1}$

> 1

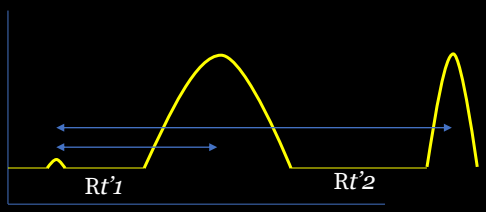

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Plate Theory:

- **Peak width:** Ideally any chromatographic peak should be symmetrical and Gaussian shaped, though this may not be the case always. Depending upon sample characteristics, many times we get tailing peaks. The common measures of peak width are: width w1/2 , measured at ½ height of the peak and width w measured at baseline between tangents drawn to the inflection points

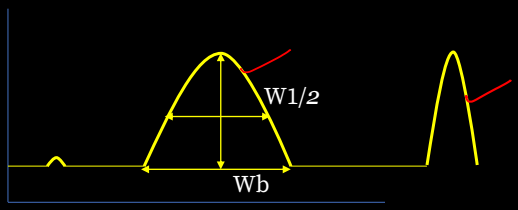

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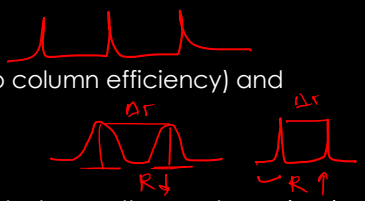
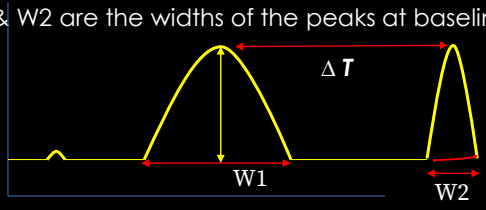
Plate Theory:

- **Resolution:** Rs: Resolution is the most important parameter in chromatography. It is resolution that provides complete information about the sample composition by way of the total number of components.
- The resolution depends on two factors:
  - 1. Width of the solute peak (it should be narrow, related to column efficiency) and
  - 2. Distance between the peaks (related to selectivity).



R: 2 Δ *T* / *(W1 + W2),*

  - where, ΔT is the distance between peak 1&2 (measured between the peak maxima) and W1 & W2 are the widths of the peaks at baseline.



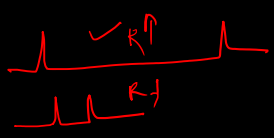


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Plate Theory:

- **Retention Factor (Capacity factor):** k: It is an important parameter which shows how long a compound can be retained by the stationary phase. Ideally, there should be sufficient difference between k values of different sample components so as to get a desirable separation.
- Retention factor can be calculated as $k = (t - t0)/t0$. A range of k values from 2 to 10 will be most desirable

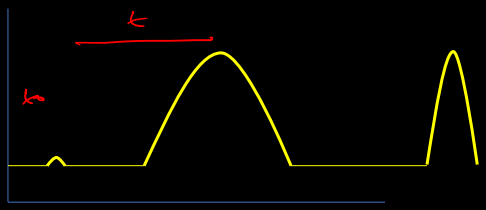

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Plate Theory:

- The chromatographic resolution is related to the number of theoretical plates of the column, the selectivity factor (α) and the retention factors (k) of two solutes by the following equation:

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

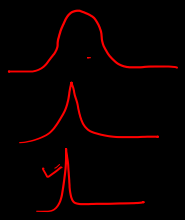
- If all the variables in the resolution equation are kept constant, except the number of theoretical plates, then the resolution is proportional to the square root of N. Therefore, increasing the number of theoretical plate by 4 will increase the resolution by a factor of 2.

$$R \text{ proportional to } (\sqrt{N})$$

CHROMATOGRAPHY

Rate Theory:

- The rate theory provides a more realistic explanation of the processes that take place inside a chromatographic column.
- It takes into account the time taken by the solute to equilibrate between the stationary and mobile phase unlike the plate model which assumes that the equilibration is infinitely fast.
- When the solute elutes out of the column, the band shape is affected by the rate of elution.
- here are various mechanisms that contribute to broadening of the band and it can be shown by ***Van Deemter equation***:



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

CHROMATOGRAPHY

Rate Theory:

- here are various mechanisms that contribute to broadening of the band and it can be shown by ***Van Deemter equation***:

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

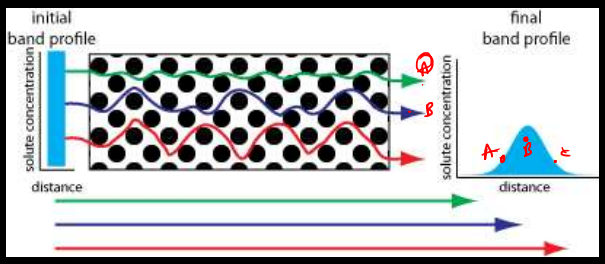
- u- average velocity of the mobile phase
- A,B&C- Factors contributing to band broadening.
- A low value for HETP will increase the efficiency of the column, To get a low value for HETP, all the three factors, A, B & C must be minimized.

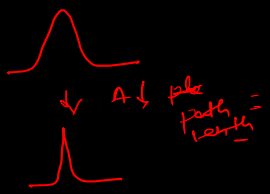
CHROMATOGRAPHY

Rate Theory:

$$HETP = A + B/u + Cu$$

- **Factor A: *Eddy Diffusion***: In a packed column, the solute molecules take different paths at random while passing through the column. This leads to broadening of the peak because different paths in a packed column are of different lengths.
- To minimize A term, reduce the particle size of the packing material and also pack the column more uniformly. Note that A term is not applicable to Open Tubular (capillary) columns.





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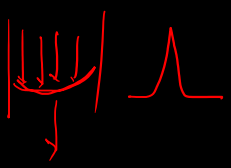
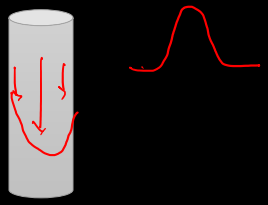
Rate Theory:

$$HETP = A + B/u + Cu$$

- **Factor B: *Longitudinal Diffusion:*** As the analyte is passing through the column, it diffuses out towards the edges of the column. Hence the concentration of the analyte is always more at the centre as compared to the edges. This leads to band broadening.
- If the velocity of the mobile phase is high, then the analyte will spend less amount of time in the column which will reduce the effect of longitudinal diffusion.





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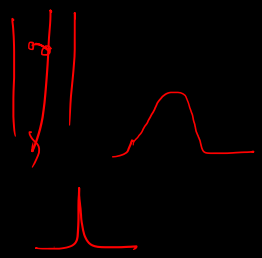
Rate Theory:

$$HETP = A + B/u + Cu$$

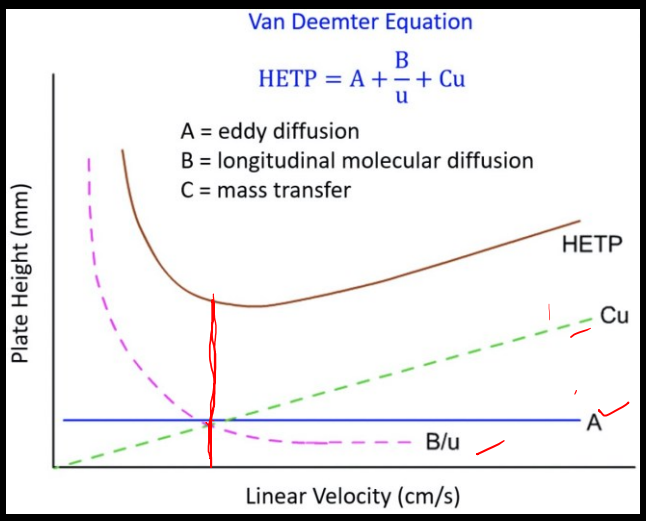
- **Factor C: *Resistance to Mass Transfer*** : The analyte takes a certain amount of time to equilibrate between the stationary and mobile phases.
- If the velocity of the mobile phase is high and the analyte has a strong affinity towards the stationary phase, then the portion of analyte in the mobile phase will move ahead of the portion of analyte in the stationary phase.
- This will lead to band broadening. The higher the velocity of mobile phase, more will be the band broadening.
- The effect of C term can be reduced by decreasing the stationary phase content (film thickness in case of capillary column), reducing the column radius and increasing the temperature.



CHROMATOGRAPHY

Rate Theory:

$$HETP = A + B/u + Cu$$



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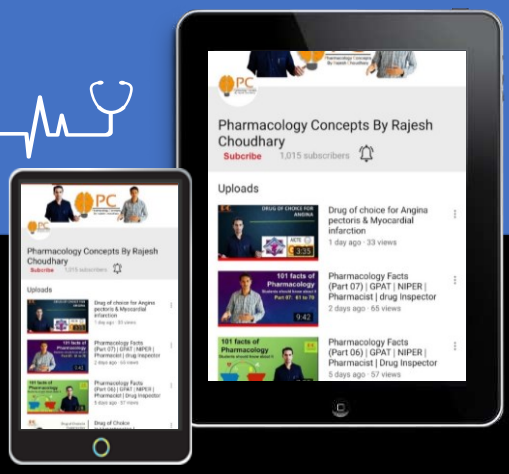

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