A study material for M.Sc. Biochemistry (Semester: III) Students
on the topic (CC-14; Unit C)

Column Chromatography

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- **Chromatography** is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification.

Chromatography

- Russian scientist Tswett in 1906 used a glass columns packed with finely divided $CaCO_3$ to separate plant pigments extracted by hexane. The pigments after separation appeared as colour bands that can come out of the column one by one.

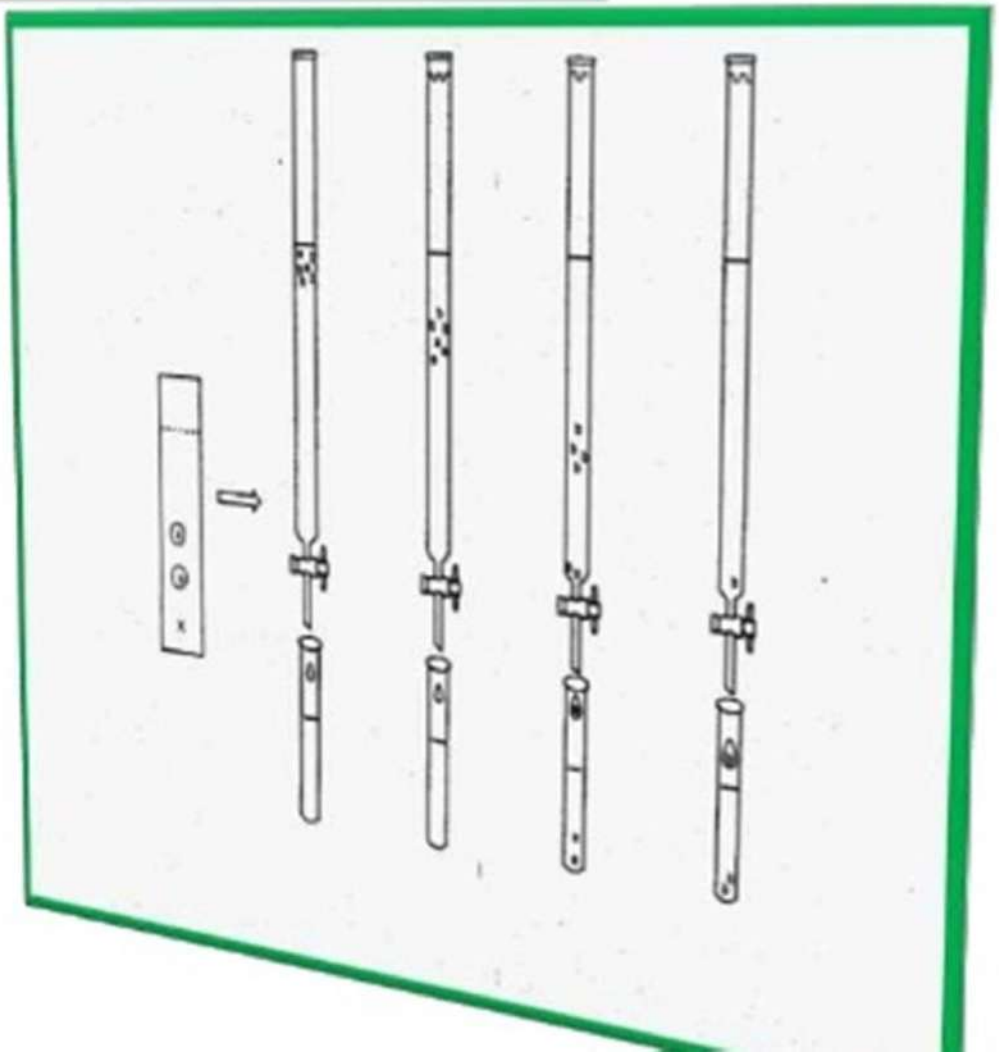
- Tswett was the first to use the term "chromatography" derived from two Greek words "Chroma" meaning color and "graphein" meaning to write.

Classification of chromatography

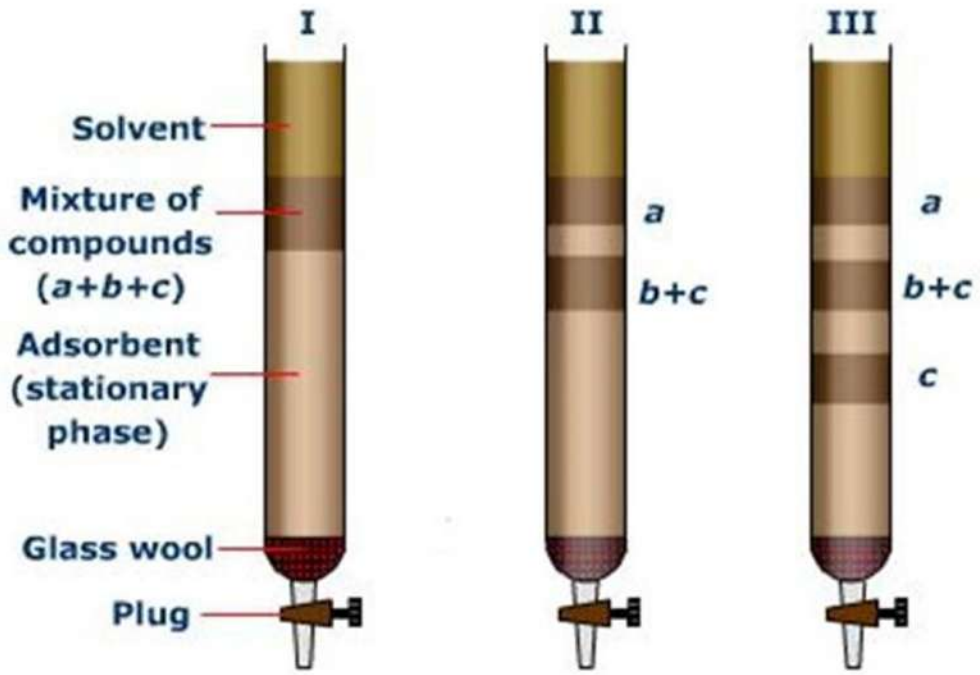
1. **Based on mechanism of separation**
   - I. adsorption chromatography
   - II. Partition chromatography
2. **Based on phases**
   - I. Solid phase chromatography
     - i. Solid-liquid chromatography
     - ii. Solid-gas chromatography
   - II. Liquid phase chromatography
     - i. Liquid-liquid chromatography
     - ii. Liquid –gas chromatography
3. **Based on shape of chromatographic bed**
   - I. Planner chromatography
     - i. Paper chromatography
     - ii. Thin layer chromatography
   - II. Column chromatography
     - i. Packed column chromatography
     - ii. Open tubular column chromatography

Column chromatography

It is defined as a separation process involving the uniform percolation of a liquid solute through a column packed with finely divided material.



Principle



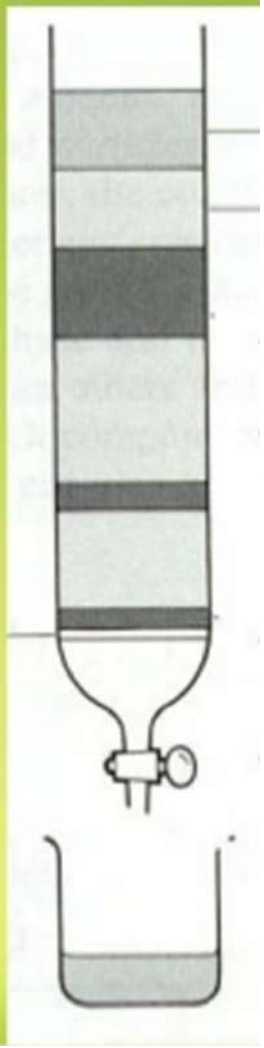
- Column chromatography makes use of a stationary phase, consisting of small particles that are packed in a glass tube to build a column and a mobile phase or eluent which is carrying the sample and moves through the stationary phase by gravity action.

- *Different* molecules are retained *differently* by the stationary phase according to their *different* physico-chemical properties. This retention is a result of mainly (1) ***ad****sorption* of a substance on a surface and (2) *partition* (or *distribution*) of a substance between physical phases.

- Contrary to thin-layer chromatography, at CC the process of chromatography is performed until the analytes have been eluted, i.e. they have been washed from the stationary phase by the eluent. The mobile phase is called eluate when it has passed the column. (At TLC, the analytes are still in the stationary phase after complete development)

COLUMN CHROMATOGRAPHY

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



TYPES OF COLUMN CHROMATOGRAPHY

S.No	Types of column chromatography	Mobile phase	Stationary phase	Sample phase
1	Adsorption chromatography	Liquid	Solid adsorbent	Solution
2	Partition chromatography	Liquid	Immiscible solvent on solid matrix	Solution
3	Ion exchange chromatography	Liquid	Ion exchange resin	Solution
4	Gel chromatography	Liquid	Solvent held in the interstices of a polymetric solvent	Solution

Stationary phase

- The *stationary phase* or *adsorbent* in column chromatography is a solid. The most common stationary phase for column chromatography is silica gel, followed by alumina. Cellulose powder has often been used in the past. Also possible are ion exchange chromatography, reversed-phase chromatography (RP), affinity chromatography or expanded bed adsorption (EBA).

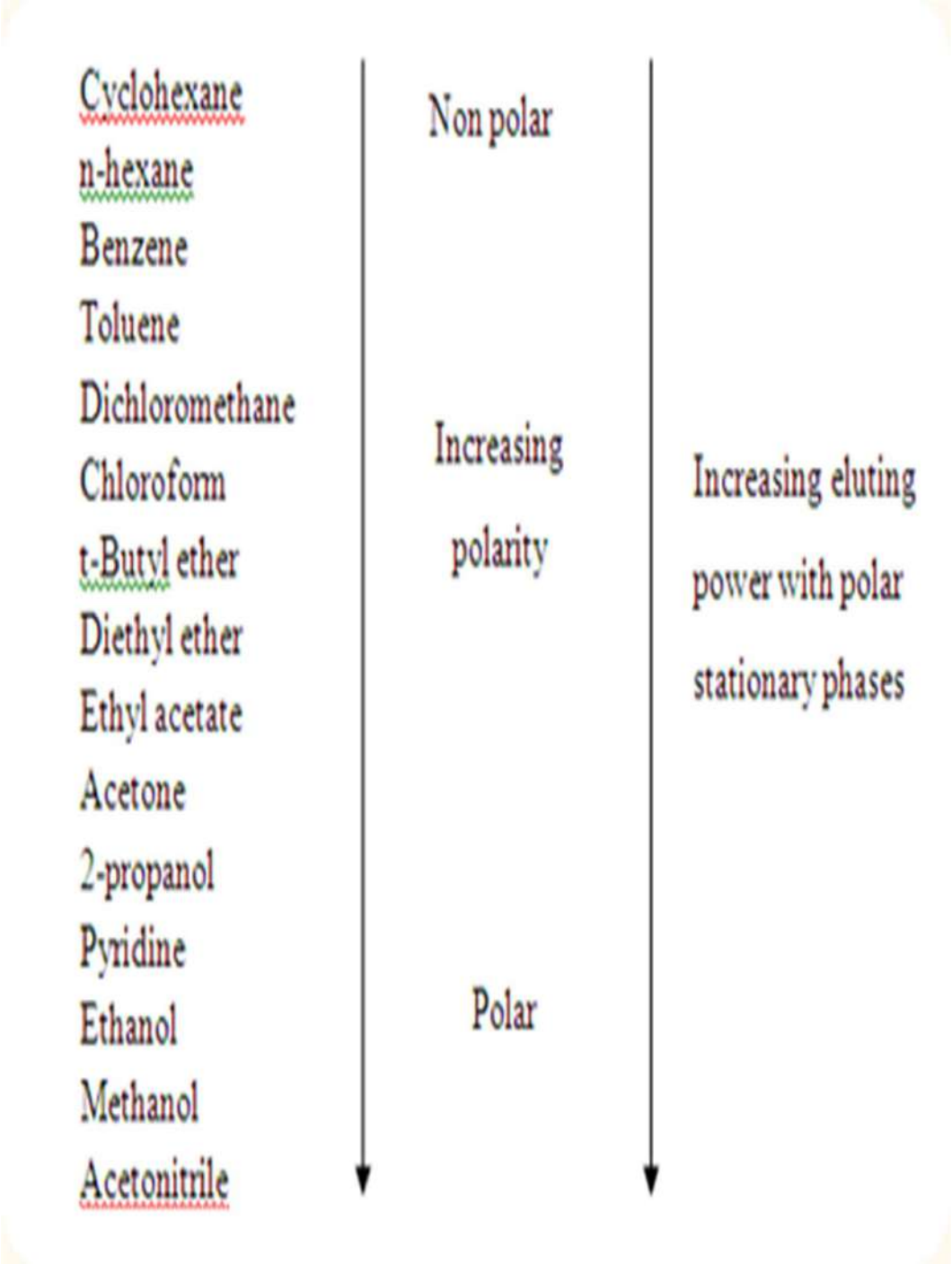
Mobile phase

The *mobile phase* or *eluent* is either a pure solvent or a mixture of different solvents. It is chosen so that the retention factor value of the compound of interest is roughly around 0.2 - 0.3 in order to minimize the time and the amount of eluent to run the chromatography.
The eluent has also been chosen so that the different compounds can be separated effectively. The eluent is optimized in small scale pretests, often using thin layer chromatography (TLC) with the same stationary phase.
There is an optimum flow rate for each particular separation. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation.
However, the maximum flow rate is limited because a finite time is required for the analyte to equilibrate between the stationary phase and mobile phase

STATIONARY PHASE USED IN CC:

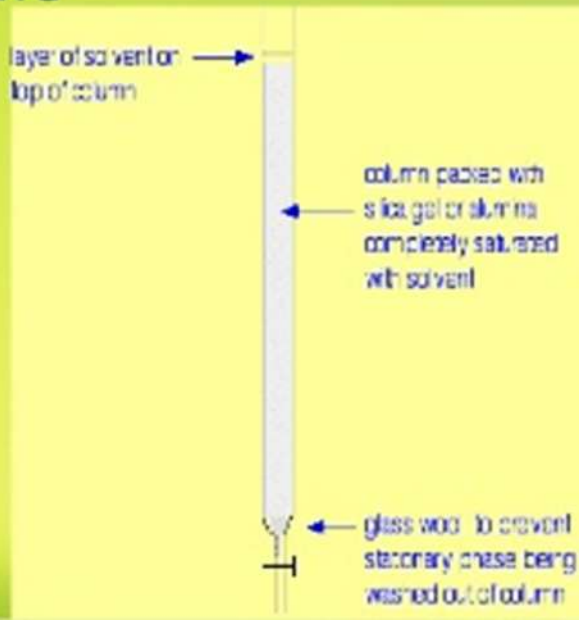
- Different adsorbents are used for this purpose such as:
- **1. Silica gel**
- **2. alumina**
- **3. Silicic acid**
- **4. Sodium carbonate**
- **5. Calcium carbonate**
- **6. Charcoal**
- **7. Fuller's earth**
- **8. Sucrose**
- **9. Starch etc.**
- The most commonly used adsorbents for column chromatography are **Silica gel (SiO_2) and Alumina (Al_2O_3).**

Commonly used mobile phase in Column Chromatography, arranged in order of increasing polarity

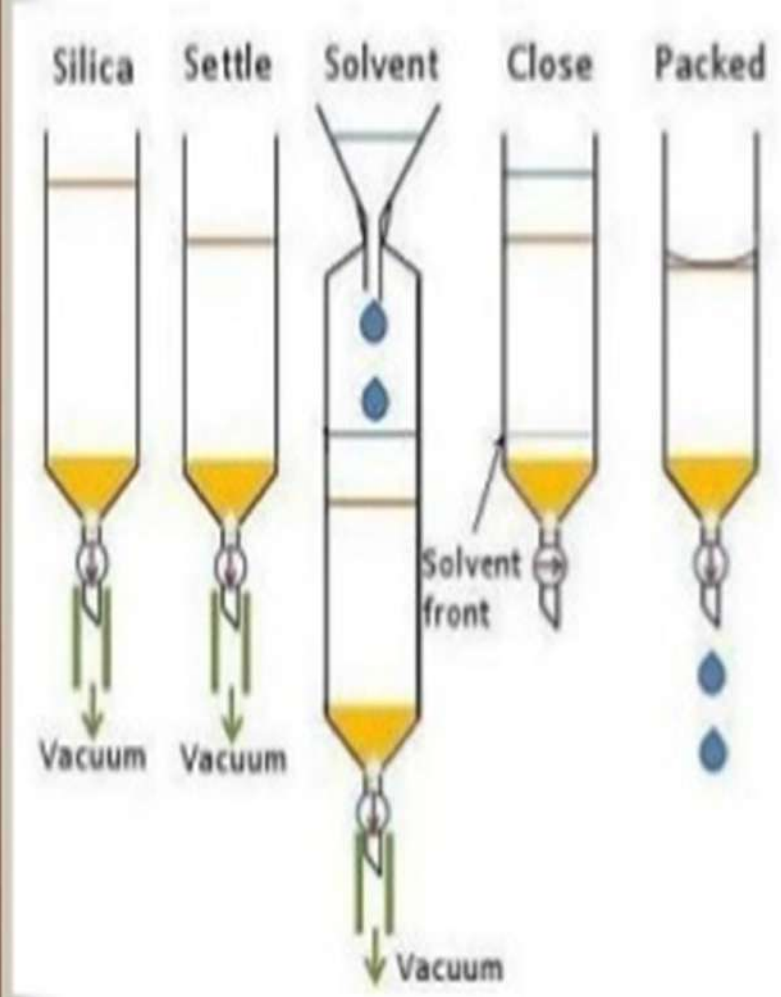


COLUMN CHROMATOGRAPHY

- PREPARATION OF THE COLUMN
- It consists of a glass tube with bottom portion of the column – packed with glass wool/cotton wool or may contain asbestos pad,
- » Above which adsorbent is packed
- » After packing a paper disc kept on the top, so that the adsorbent layer is not disturbed during the introduction of sample or mobile phase.



Methods of Column Packing



Dry Method :

- Add dry silica / Alumina to the column and apply to the bottom of the column. This will compress the silica gel and keep it compressed for the next steps. Packing can be improved by tapping the column.
- While applying vacuum; pour solvent in it.
- Allow the solvent to move though the column until reaches to the bottom. At this stage vacuum is not require.
- Allow 5-6 columns value of solvent to flow through the column to make sure it is complete packed.
- Drain the solvent till the solvent level is just even with the surface of the stationary phase

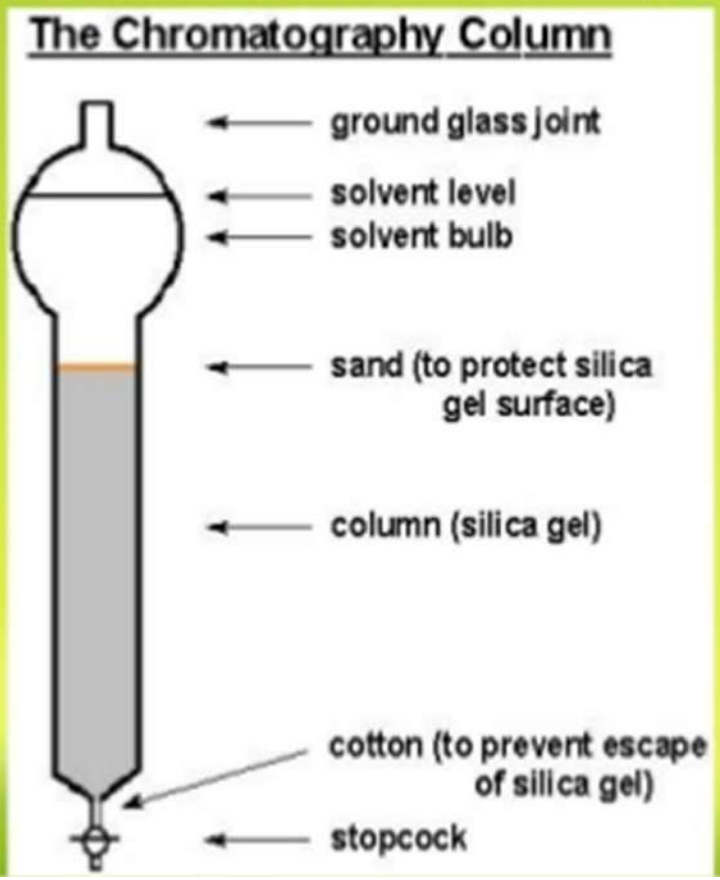
COLUMN CHROMATOGRAPHY

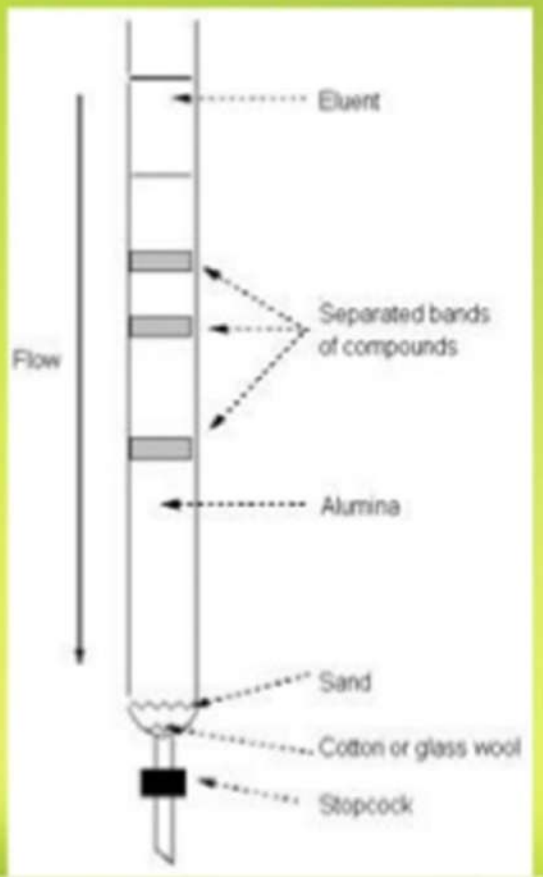
Introduction of the Sample

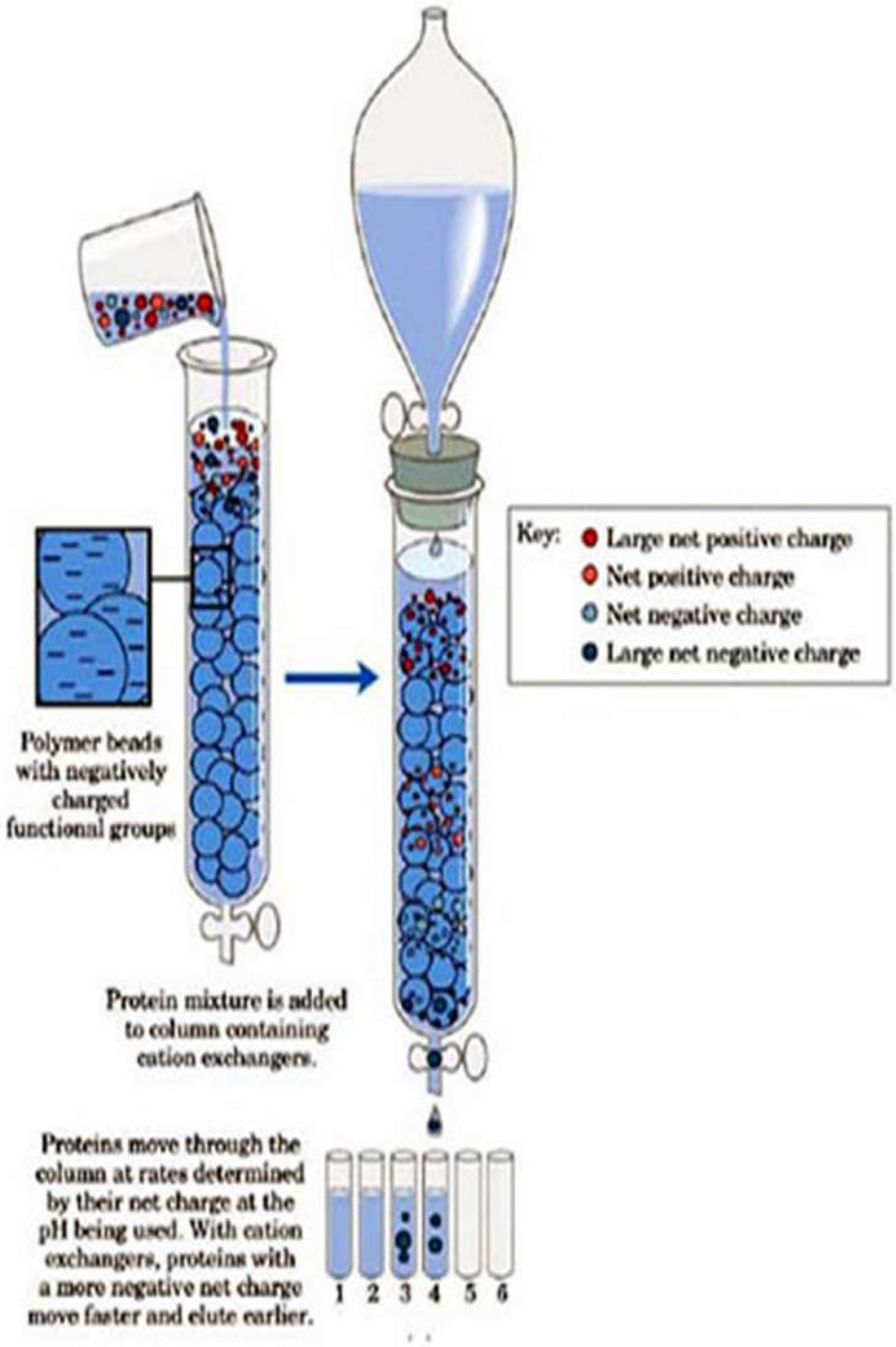
- The sample which is usually a mixture of components is dissolved in minimum quantity of the mobile phase.
- The entire sample is introduced into the column at once and get adsorbed on the top portion of the column.
- From this zone, individual sample can be separated by a process of elution.

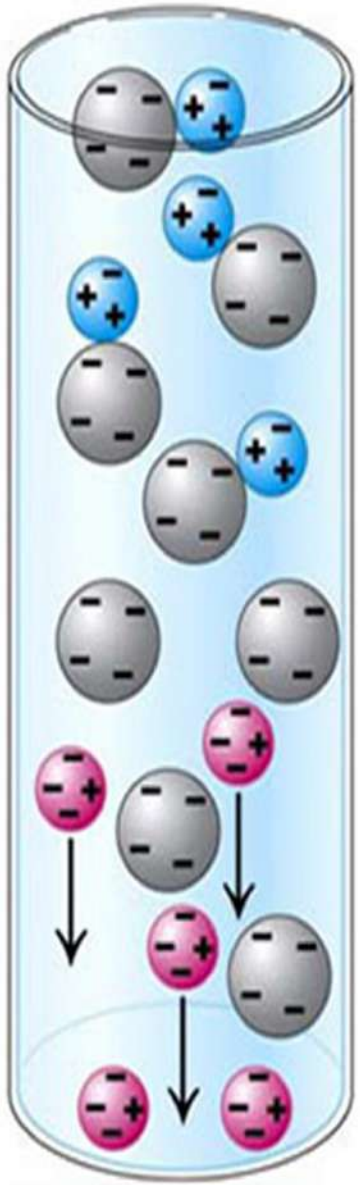
COLUMN CHROMATOGRAPHY

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





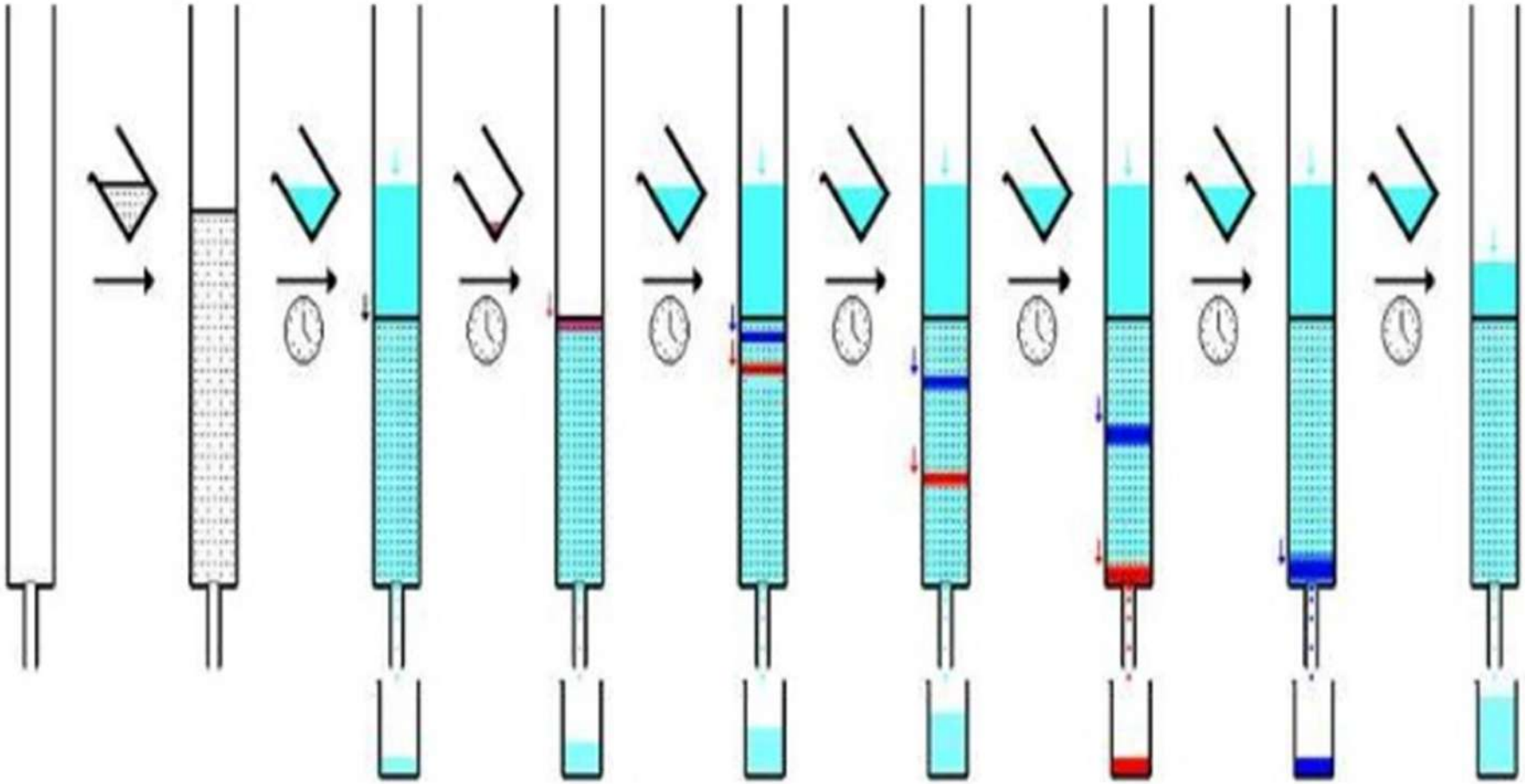




Positively charged protein binds to negatively charged bead

Negatively charged protein flows through

Column chromatography proceeds by a series of steps.



APPLICATIONS OF COLUMN CHROMATOGRAPHY

- Column chromatography is best suited to separate active principle from plant materials.
- To separate impurities along with the important constituents
- Isolation of metabolites from important components
- Used for determination of phytomenadione in tablets and injections
- Determination of flucinolone, acetonide, betamethasone in formulations.

Advantages & Disadvantages of column chromatography

Advantages

- It can be used in both analytical and preparative applications.
- It is used to identify the number of components of a mixture.
- It is also used to separate and purify important quantities of those components for subsequent evaluation
- Any type of mixture can be separated
- Any quantity of mixture can be separated
- There is wider choice of Mobile Phase (Solvents)
- It is low cost process and disposability of the stationary phase once it is used in the process
- Process can be scale up form lab scale to commercial scale
- Automation is possible

Disadvantages

- Time consuming Process
- More amounts of Mobile Phase (Solvents) required
- Scale up process will take a long time to properly prepare & use
- Automation makes the techniques more complicated & expensive

Acknowledgement and Suggested Readings:

1. Microbiology, An Introduction; Tortora, Funke and Case; Pearson Publication
2. Microbiology; Prescott, Harley and Klein; The MacGraw-Hill Companies
3. Microbiology: Principles and Explorations; Jacquelyn G Black; John Wiley and Sons Inc.
4. Brock Biology of Microorganisms; Madigan, Martinko, Stahl and Clark; Benjamin Cummings (Pearson Publication)

Thanks