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UNIT-5 CHAPTER-1 ION-EXCHANGE CHROMATOGRAPHY

Introduction

- Ion-Exchange chromatography is the process by which a mixture of similar charged ions can be separated by using an ion-exchange resin which exchanges ions according to their relative affinities.
- This method is used for any kind of charged molecule including large proteins, small nucleotides and amino acids.
- Ion-exchange is based on adsorption.
- It is basically a reversible chemical process.

CLASSIFICATION

Principle

1. Cation exchange chromatography
2. Anion exchange chromatography.

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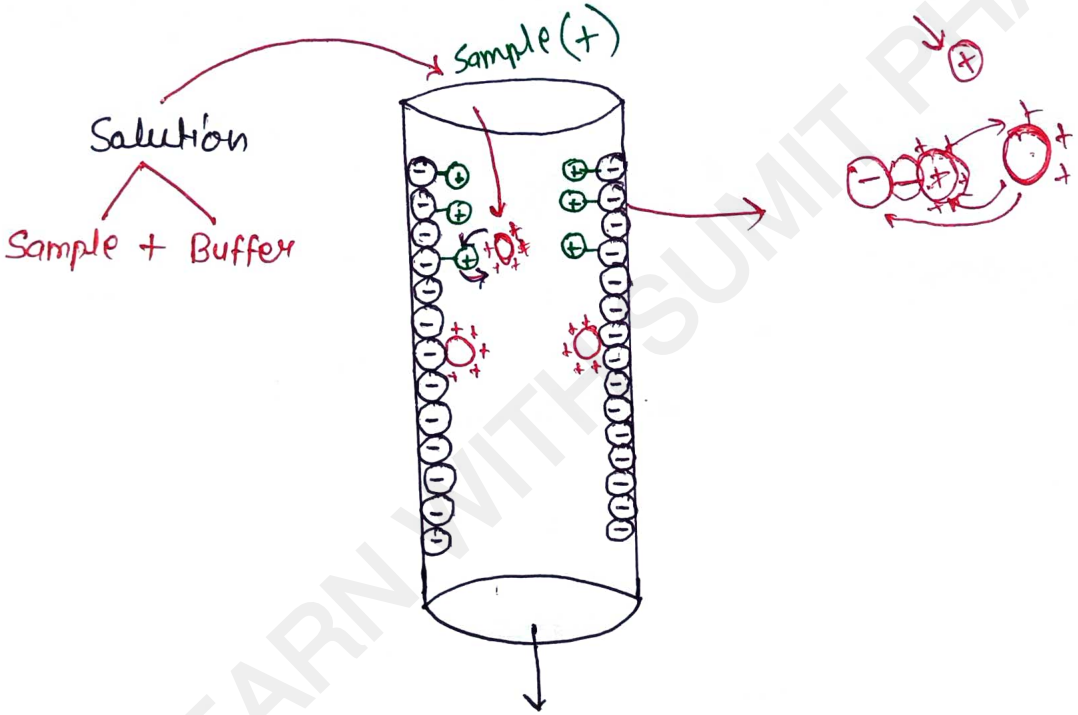
① Cation Exchange Chromatography

In Cation exchange chromatography, the Solid support with desired functional group is prepared with various beads differing in flow rate, stability, binding capacity. [buffer pH b/w 4-7]



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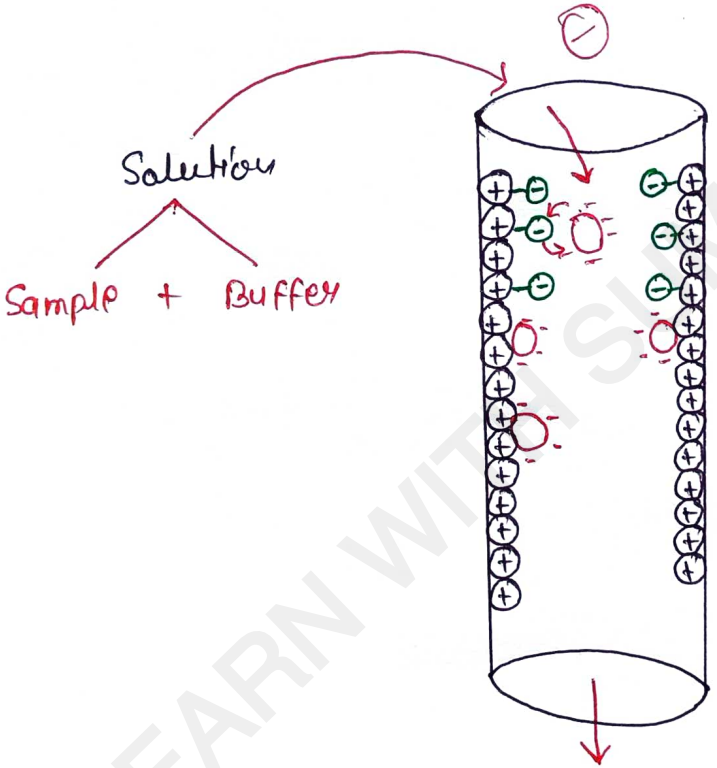
② Anion Exchange chromatography

In this method, Solid Support with desired functional group is prepared with various beads, differing in flow rate.

[buffer pH b/w 7-10]

Mechanism



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Classification of Ion-Exchange Resins

(A) According to chemical nature
- Strong cation exchange resin.
- Weak cation exchange resin.
- Strong anion exchange resin.
- weak anion exchange resin.

(B) According to the Source :-

① Natural :- Cation → Zeolytes, clay etc.
Anion → Dolomite.

② Synthetic :-

Class of Resin	Nature	PH range	Applications.
Cation (strong)	Sulfonated polystyrene	1-14	- fractionation of cations. - Inorganic Separations. - Peptides, amino acids, B. vits
Cation (weak)	Carboxylic methacrylate	5-14	- fractionation of cations - Biochemical separations - organic bases, antibiotics.

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Anion (strong)	Quaternary ammonium Polystyrene	0-12	- Fractionation of anions - Alkaloids, vitamins - Fatty acids.
Anion (weak)	Polyamine phenol formaldehyde	0-9	- Fractionation of anionic complexes - Anions of diff. valency. - Vitamins, amino acids.

Inorganic and organic resins

⇒ Functional groups present in different resins -

① Strong cation exchange resin - SO_3H ✓

② weak cation exchange resin - $COOH$, OH, SH, PO_3H_2 ✓

③ Strong anion exchange resin - N^+R_3, NR_2 ✓

④ Weak anion exchange resin - NHR, NH_2 ✓

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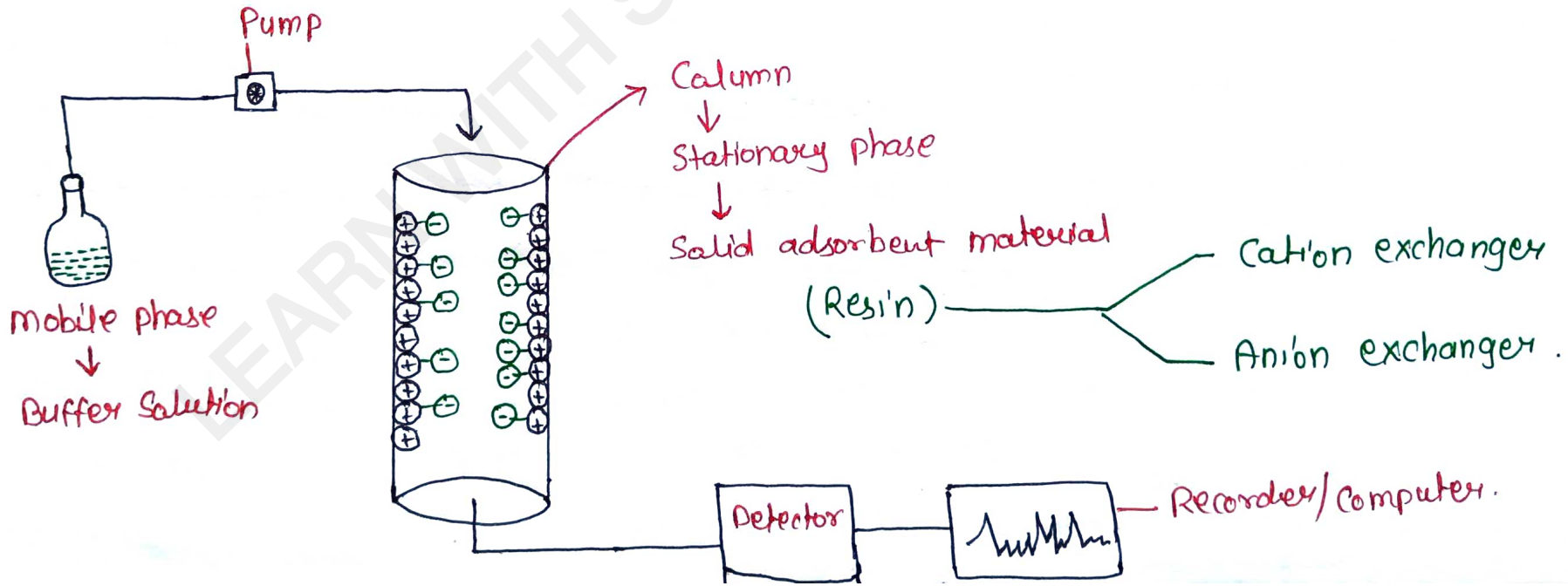
Properties of all ion exchangers

① They are almost insoluble in water, benzene, CCl_4, ether etc.

② Complex in nature i.e. they are polymeric.

③ Contain active or counter ions that will exchange reversibly with other ions in a surrounding solution without any substantial change in the material.

Instrumentation



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Practical Requirements

① Column :- Glass, Stainless steel or polymers.

② Packing the Column :- wet packing method → A slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. Care must be taken to avoid air bubbles.

③ Application of the Sample :- • After packing, Sample is added to the top of the stationary phase, use syringe or pipette.
- The layer is usually topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the velocity of newly added eluent.

④ Mobile phase :- Acid, alkali's, buffers.

⑤ Stationary phase :- Ionic compound consisting of cationic (+) & anionic (−) species

⑥ Elution :- • Components of mixtures separate and move down the column at diff. rates depending upon the affinity of ion for ion exchanger.
- The elutes are collected at diff. stages.

⑦ Analysis of elute :- Spectrophotometric, flame photometry, polarographic etc.

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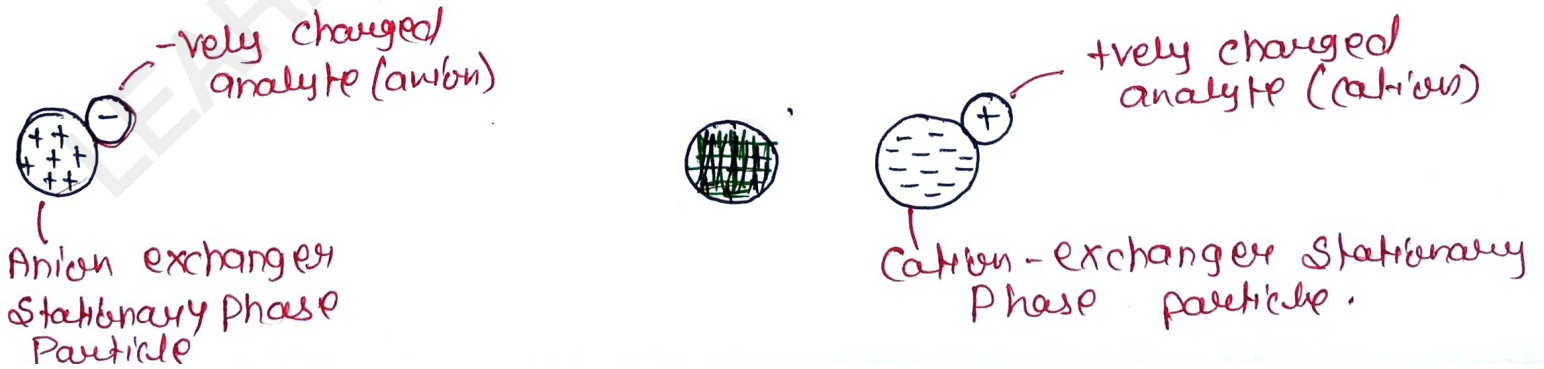
Mechanism of Ion-Exchange process

⇒ Ion-exchange chromatography is involved in the separation of ionisable compounds (having diff. charges) and comprises of mobile and stationary phase like other column-based liquid chromatography technique.

- The mobile phase in this method is an aqueous buffer system, and the stationary phase is an inert organic matrix, and carrying both are oppositely charged ions.

So the negatively charge analyte (anion) are are attached with Anion-exchanger stationary phase particle and the positively charged analyte (cation) are attached with cation exchanger stationary phase particles.



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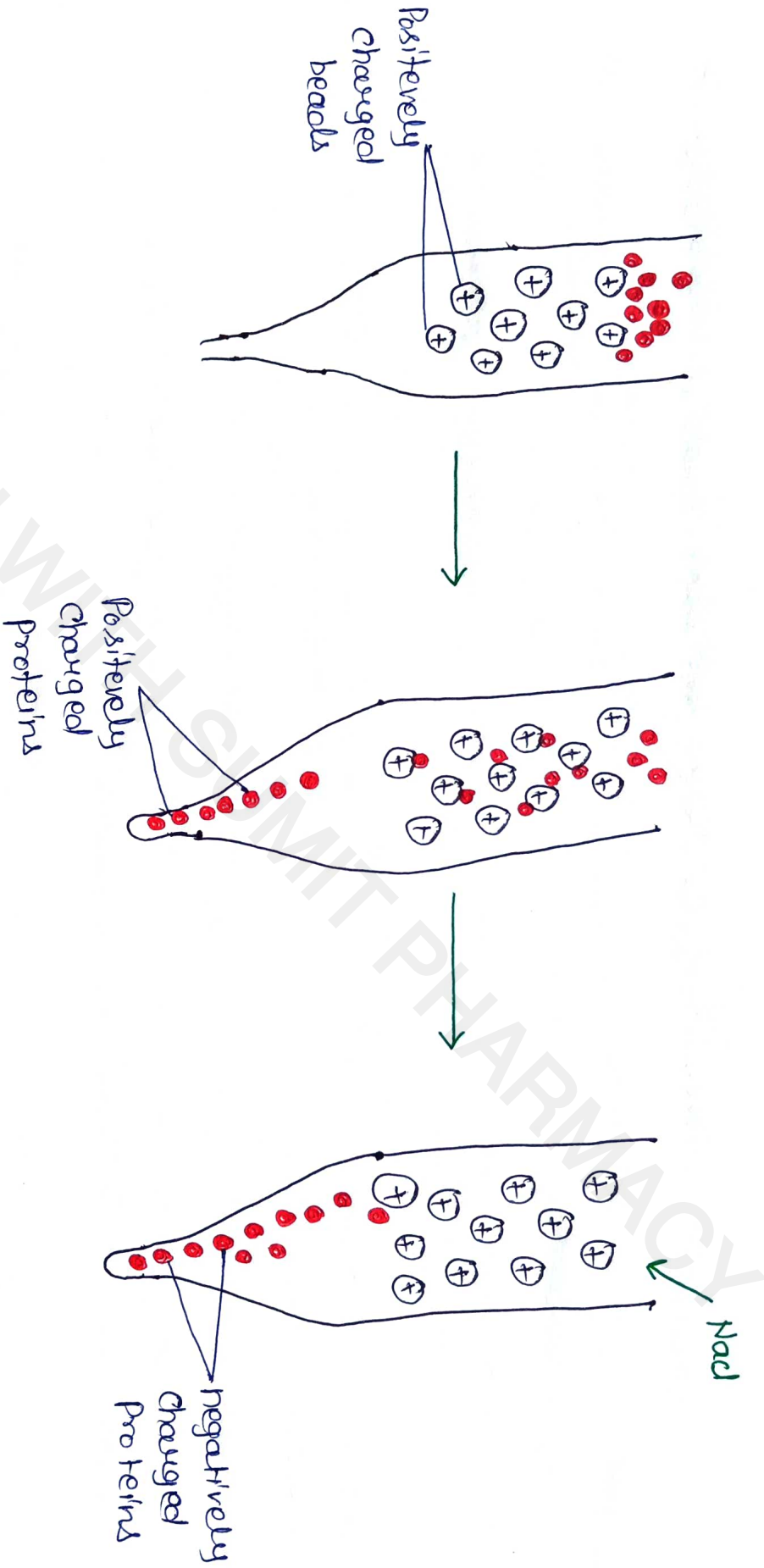

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Factors Affecting Ion Exchange Separations

① Nature and properties of Ion exchange resins :-

- Cross linking and swelling is important.
- If more cross linking, they are more rigid but swelling is less.
- Swells less → Separation of ions of different sizes is difficult.

② Nature of exchanging ions :-

① Valency of ions,
② Size of ions,
③ Polarizability,
④ Conc.ⁿ of solution,
⑤ Concⁿ and charge of ions,

③ pH of mobile phase.
④ Ionic strength.
⑤ mobile phase modifiers.
⑥ Temperature.

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Methodology of Ion-Exchange chromatography

The following two techniques are used to bring the solution and ion exchange resins in contact –

1. Batch method
2. column method.

① Batch method

It involves single step equilibrium process.

- Resin + Solution are mixed in vessel.
- Filter the solution.
- The extent to which the ions from the solution are exchanged for those on the resin depends on the selectivity coefficient.
- The batch method is used for softening of water and production of de-ionized water.

ex → Exchange of calcium and magnesium ions which causes hardness –

$$2RSO_3^- Na^+ + Ca^+ \rightleftharpoons (RSO_3^-)_2 Ca^{+2} + 2Na^+$$ ✓

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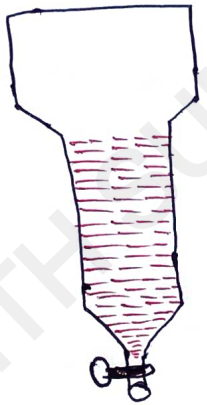
② Column method

- It involves in separation of components of a mixture by selecting different coefficient of resin.
- The difference in selectivity coefficient leads to different migration rate on ion-exchange column.



- This involves frontal analysis, elution analysis, displacement development.

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Applications

1. Softening and determination of water.
2. For extraction of enzymes from tissues.
3. Purification of solutions free from ionic impurities.
4. Separation of inorganic ions.
5. Separation of sugars, amino acids and proteins.
6. Ion-exchange column in HPLC.

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UNIT-5 CHAPTER-2 GEL CHROMATOGRAPHY

Introduction

- Gel chromatography is a chromatographic method in which molecules are separated based on their size.

Gel chromatography →
- Size Exclusion chromatography
- Gel permeation chromatography
- Gel filteration chromatography
- Molecular Sieve chromatography.

Principle

- In gel chromatography, the molecules are separated based on their size, therefore it is also known as molecular - sieve chromatography.
- Smaller molecules penetrate the particles to varying extents depending upon their shape and size.

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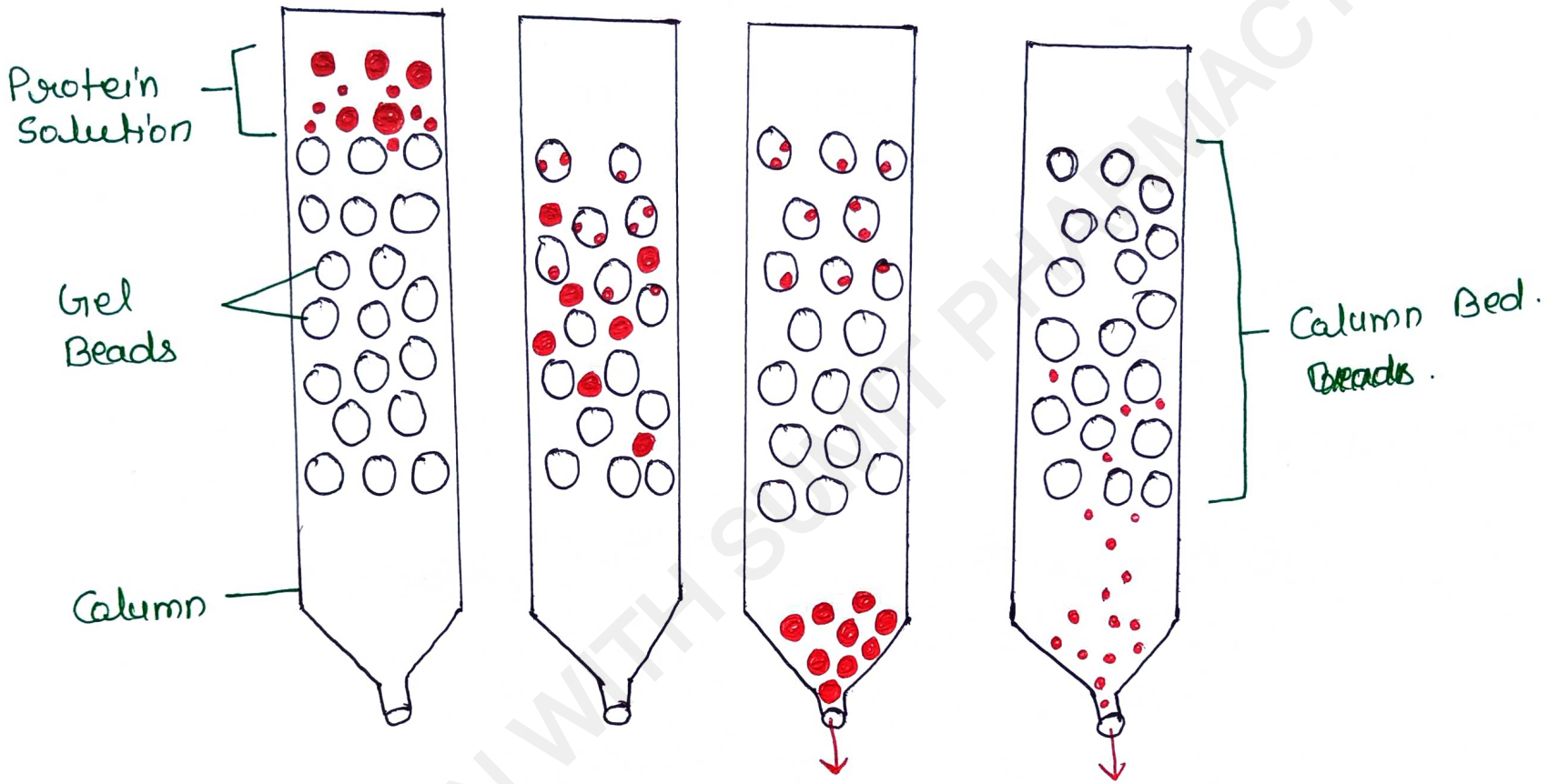

- There is thus a partition of the molecules b/w the liquid inside the gel particles and that outside.
- The smaller the molecules, the larger the percentage of liquid within the particles that is available to them.

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- Molecules therefore leave the column in the order of decreasing molecular size.
- The larger size will leave the column first followed by smaller sizes depending on their partition (shape and size) ranges.

Theory

Total volume of column packed with a solid matrix that has been swelled by water and/or other solvent is given by -

$$V_t = V_g + V_m + V_s$$

V_t = Total bed volume
V_g = Volume occupied by solid matrix
V_m = Void volume of mobile phase i.e. unbound solvent in interstices b/w the solvent loaded porous particles.
V_s = Volume of solvent held in pores.

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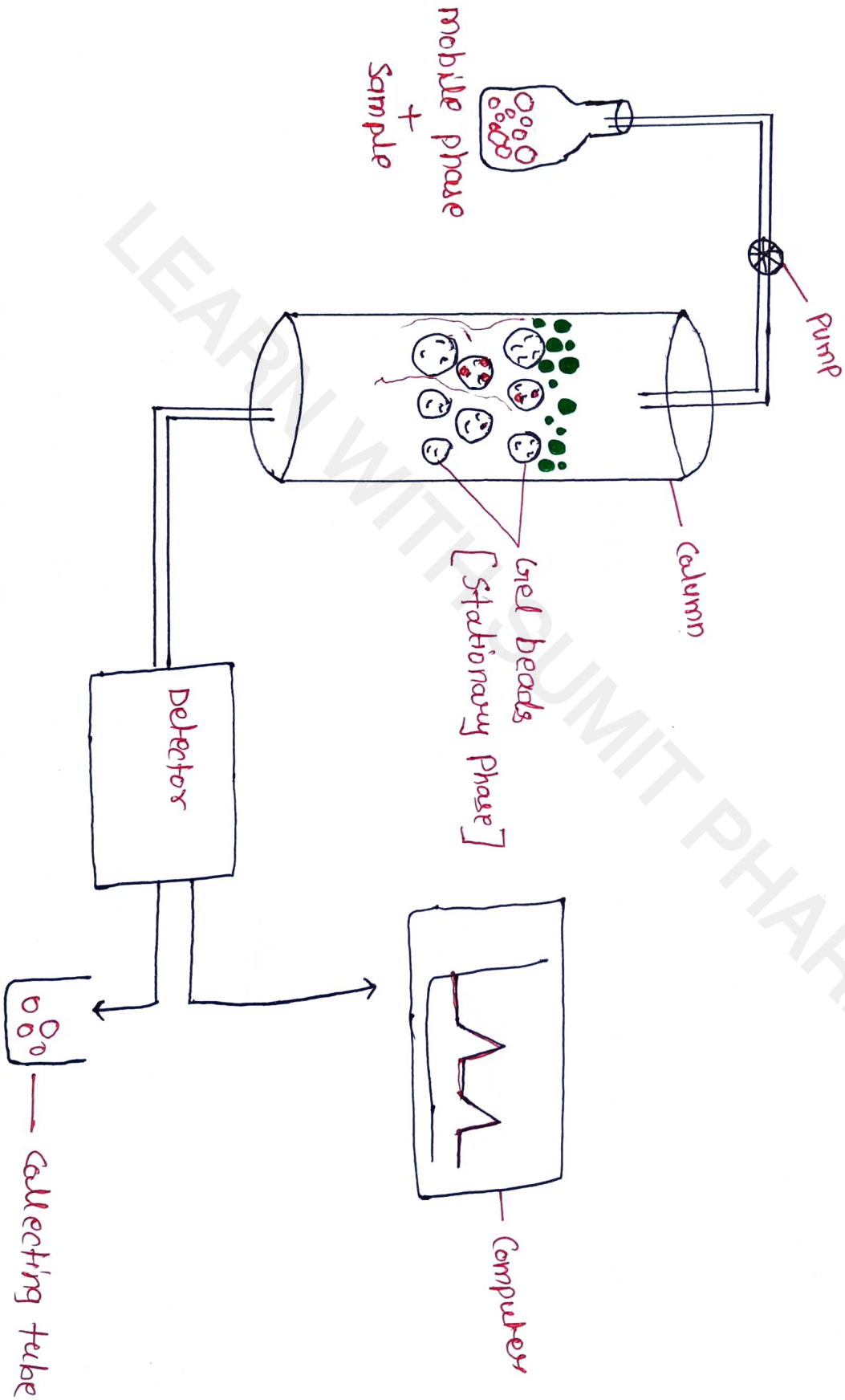
Instrumentation



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Working Principle of Instrument

① The Separation is based Strictly on the size of the sample in solution.

② There should be no interaction with the column Packing.

③ The mode of Separations is not based on molecular weight, But on the size of the material being analyzed (usually a polyme) in solution.

④ In other words, To do GPC, correctly, the sample must be dissolved in a suitable Solvent.

materials and method for sample analysis.

① Gel

- Gels are used as stationary phase for GPC.
- The pore size of a gel must be carefully controlled in

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order to be able to apply the gel to a given separation.

- other desirable properties of the gel forming agents are the absence of ionizing groups and in a given solvent, low affinity for the substances to be separated.
- Commercial gels like PL gel, Sephodex, Bio-gel (Polyacrylamide), agarose gel are often used based on different separation requirements.

Classification of Gel

- Rigid gel
- Semi-rigid gel
- Soft gel.

Choice of gel :- In GC two types of separation are done -

- **Desalting** → Separation of high molecule substances from low molecular weight substance.
- **Fractionation** → Similar substance are eluted closer to one another.

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② Eluent

- The eluent (mobile phase) should be a good solvent for the polymer.
- It should permit high detector response from the polymer and should wet the packing surface.
- The most common eluents in for polymers that dissolve at room temperature GPC are Tetrahydrofuran (THF).

③ Particle Size

- The gel in the powder form with particle size of 70 μ in diameter is used, But the use of finer grade material gives further improvement in the resolution.
- The material with particle diameter lower than 40 μ can be used in many cases.

④ Gel preperation

- Dry powder is allowed to swell in liquid and then use.

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OR

- warm the gel slurry in boiling water at 100°C.

⑤ Drying of Gel

- Gel can be stored very well in wet state and there is no need of drying.

⑥ Column

- The column used for HPC is straight glass column with bed support at the bottom.
- The bed support is such type that it retained stationary phase and allows liquid to pass through it.
- Glass wool or filter paper used as bed support which is then covered with quartz, sand or glass beads.
- The diameter of the column generally larger than adsorption or partition chromatography.
- The larger column diameter and greater column lengths are Preffered for high resolution.

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⑦ Packing of the Column

- The procedure for packing depends on the nature of the gel.
- Special efforts are needed to maintain uniform slurry.
- with soft gels more careful packing is needed.
- while hard gel, do not require so much precaution.
- The gel is allowed to swell, and then deaerated under vaccum.
- The gel is then allowed to settle and the supernatural liquid is taken off.
- The gel is remixed and then poured in the column, Packing in many steps should be avoided as it gives uneven packing.
- The liquid should be carefully added, otherwise, the gel surface will be disturbed.
- with hard gels the principle of the packing remains same with slightly modified procedure.

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- Agarose gels which are too thick to be packed directly into the column are mixed with buffer solution, deaerated under vaccum and then packed.

⑧ Pump :-

There are two types of pumps available for uniform delievery of relatively small liquid volumes for GPC.

⑨ Preperation of Sample

- The sample is dissolved in proper solvent so that there will not be any solid particle or other, substances which may be strongly adsorbed on the gel.
- In analytical applications, sample of 1.3% of bed volumes used.
- However in group separations sample of 25.30% of total

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Column volumes used.

- The smaller the sample volume, the greater will be reduction of the component conc? in the elute.
- The dilution effect must also be taken into account in deciding the column and sample sizes.

(10) Application of the Sample

- The use of pipette with bent tip is preferable for the application.
- Viscous samples are introduced with the help of a valve loop.
- Commercial plunger type column have special inlet arrangements for the sample.

(11) Detector

(i) Differential refractometer.
(ii) UV photometer.

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Applications

① Molecular weight determination :- Gel filteration chromatography is widely applied for determination of the molecular weight of proteins.

② Separation of Macromolecules :- Gel filteration chromatography is commonly applied in research laboratories for separation of proteins and peptides.

③ Group Separation :- Gel filteration chromatography is also applied for fractionation of crude samples into low and high molecular weight protein groups.

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UNIT-5 CHAPTER-3 AFFINITY CHROMATOGRAPHY

Introduction

- Affinity chromatography is a technique used to separate and purification of the biochemical mixture.
- Affinity column is a highly specific technique.
- Affinity chromatography technique depends on the specific affinity b/w stationary phase and the analyte (Ligand).
- Affinity chromatography is a reveresible bio-chemical reaction.

Principle

- The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way that the reactive groups that are essential for binding of the target molecule are exposed.

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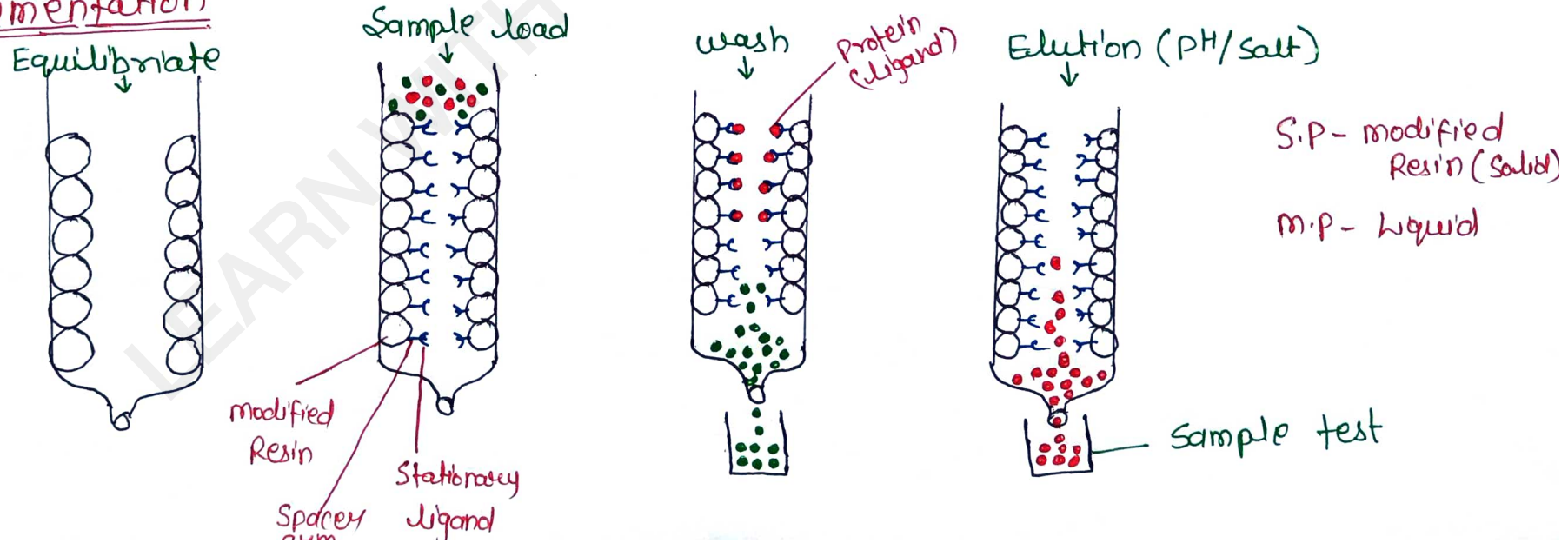
- As the crude mixture of substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.

- Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the pH, ionic strength or polarity conditions.

Instrumentation



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Examples :

Stationary phase ligand	Purified protein.
Protein A	IgG
Antigen peptide	Antibody (IgG, IgY., etc)
Antibody	Antigen
gelatin	Fibronectin
GST	GST - Tagged Protein
Lectin	Sugars / glyco proteins
heparin	Growth factors
Amino acid	Amino acid binding proteins.

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Steps → Choose S.P ⟶ Resin+Ag — Ab
Resin+Ni — (for His-tag protein)
etc.
Genetic engineered protein.

Components of Affinity chromatography

① Matrix

- The matrix is an inert support to which a ligand can be directly or indirectly coupled.
- In order to for the matrix to be effective it must have certain characters ↓
  - matrix should be chemically and physically inert.
  - It must be insoluble in solvents and buffers employed in the process.
  - It must be chemically and mechanically stable.

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- it must be easily coupled to a ligand or spacer arm on to which the ligand can be attached.
- it must exhibit good flow properties and have a relatively large surface area for attachment.
- The most useful matrix materials are agarose and polyacrylamide.

② Spacer Arm

- it is used to improve binding b/w ligand and target molecule by overcoming any effects of steric hindrance.

steric hindrance: non bonding interactions that influence the shape and reactivity of ions and molecules.

③ Ligand

- It refers to the molecule that binds reversibly to a specific target molecule.

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- The ligand can be selected only after the nature of the macromolecule to be isolated is known.
- when a hormone receptor protein is to be purified by affinity chromatography, the hormone itself is an ideal candidate for the ligand.
- For antibody isolation, an antigen or hapten may be used as ligand.
- If an enzyme is to be purified, a substrate analog, inhibitor, cofactor, or effector may be used as a immobilized ligand.

Steps in Affinity Chromatography

- Affinity medium is equilibrated in building buffer.
- Sample is applied under conditions that favor specific binding of the target molecules to a complementry binding substance (ligand).

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- Target material substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.
- Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity.
- Target protein is collected in a purified, concentrated form.
- Affinity medium is re-equilibrated with binding buffer.

These events can be summarized into the following three major steps :-

① Preperation of Column

- The column is loaded with solid support such as Sepharose, agarose, cellulose etc.
- Ligand is selected according to the desired isolate.

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- Spacer arm is attached b/w the ligand and Solid Support.

② Loading of Sample

- Solution containing a mixture of substances is poured into the elution column and allowed to run at a controlled rate.

③ Elution of ligand-molecule complex

- Target substance is recovered by changing conditions to favor elution of bound molecules.

Applications

- Separation of mixture of compounds.
- Removal of impurities or in purification process.
- Detection of Substrates.
- Investigation of binding sites of enzymes.
- In in-vitro antigen-antibody reactions etc.

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