Shree H.N.Shukla College of Science

(Affiliated to Saurashtra University& G.T.U)

Behind Old Marketing Yard Nr. lalpari lake Amargadh, Bhichri
Rajkot-360001. Mobile No: 97277 53360

S.Y.B.Sc. SEM-4

Subject: Biochemistry

Paper-401: BIOPHYSICAL & BIOCHEMICAL TECHNIQUES

Unit -4

CHROMATOGRAPHY

UNIT – 4 CHROMATOGRAPHY

- Chromatography is a technique used for separation, purification and identification of compounds from a mixture. Such method can be used for separation of large amount (several grams) or small amounts (pictograms quantities) of materials.
- The first detailed description of chromatography is generally credited to Michael Tswett, a Russian botanist, who separated chlorophyll from the plant pigment in 1906. The various colored plant pigments were separated calcium carbonate column using petroleum ether for elution. Thus the name of the process was coined from Greek word for colour (chroma) and to write (graphy).

PRINCIPLE

The word chromatography means a family of closely related extremely powerful separation methods. The common features in all chromatography techniques are:

- Two mutually immiscible phases are brought into contact with each other.
- One of the phases is stationary and other is mobile.
- The mobile phase either moves or percolates through the interstices of the stationary phase
- The sample mixture introduce into the mobile phase undergoes repeated interactions (partition) between the stationary and mobile phase, while being carried through the system of the mobile phase.
- Different components of the mixture interact differentially on the basis of small differences in their physico-chemical properties. The compounds which interacts more with the mobile phase and least with the stationary phase, migrates faster and vice a versa.

  The basis of all forms of chromatography is the partition or distribution co-efficient (Kd) which describes the way in which a compound distributes itself between two immiscible phases. The value for the coefficient is constant at a given temperature and given by expression:

  Kd = Cs/Cm

  Where, Cs= concentration of substance in stationary phase

  Cm = concentration of substance in mobile phase

- The distribution of compound can be between lipid phases, solid-lipid phase or liquid-gas phase.

- The distribution coefficient of compound between cellulose (stationary phase and carbon tetrachloride (mobile phase) is 0.2; it means that the concentration of the compound in carbon tetrachloride is five times than in cellulose.
- All chromatography systems consist of two phases. The stationary phase may be solid, gas or liquid which is immobilized. The mobile phase may be liquid or gaseous. The choice of the stationary or mobile phase is made so that the compounds to be separated have different distribution coefficients. This may be achieved by setting up:

1. Adsorption equilibrium between a stationary solid and a mobile liquid phase (Adsorption Chromatography).
2. A partition equilibrium between a stationary liquid (or semi liquid) a mobile liquid phase (Counter Current Chromatography and Partition Chromatography).
3. Partition equilibrium between a stationary phase and mobile gaseous phase (Gas-Liquid Chromatography).
4. An ion exchange equilibrium between an ion exchange resin stationary phase and a mobile electrolyte phase (Ion-Exchange Chromatography).
5. An equilibrium between a liquid phase inside and outside a porous structure or molecular sieves (Exclusion Chromatography)
6. An equilibrium between am macromolecule and a small molecule for which it has a high biological specificity and hence affinity (Affinity Chromatography).

ADSORPTION CHROMATOGRAPHY

- In adsorption chromatography; some commonly used adsorbents in order of increasing adsorbing power are: cellulose, starch, calcium carbonate, magnesia, silica gel and alumina. adsorption can be fairly selective so that one solute may be adsorbed selectively from a mixture.
- Two differing factors are exploited in separation of components by adsorption chromatography. One is the different degree of adsorption of various components on the adsorbent surface and the other is varying solubility of different components in the solvent used (mobile phase). sometimes adsorption of water by the adsorbents can affect the separation, so the water is removed by heating the adsorbent for the several hours at 110°C in oven.
- In some case however, presence of water result in better separation since adsorption is coupled with partition due to presence of water. Varying the amount of water it contains can actually control the adsorption activity of an adsorbent.
- Generally, **water is used as a stationary phase** and any **organic solvent can be used either along in a form of mixture as the mobile phase**. Some common solvent in order

of increasing eluting power from alumina are: petroleum ether, carbon tetrachloride, toluene, benzene, chloroform, ether, acetone, ethanol, methanol, organic acid and bases.

- ♣ The adsorption chromatography may be carried out on a plane mode (paper and thin layer chromatography) or on a column mode. In case of columns chromatography, the glass column of various sizes and diameter are used.
- ♣ The lower end of the column is blocked with the glass wool so that only the solvent can pass and the adsorbent gel cannot pass through it.
- ♣ The adsorbent is mixed with appropriate buffer to make gels and the glass column is filled with gel and care is taken to have homogenous bed.
- ♣ There should not be any bubbles cracks or space between the adsorbent and the walls of the column. The sample is applied on the top of the adsorption bed of column and the component are resolved by the application of eluting solvent on top of the column by means of a continuous flow at a fixed flow rate.

One Word Question

Sr. No.	Question	Answer
1	In thin layer chromatography, the stationary phase is made of _____ and the mobile phase is made of _____	Solid, liquid
2	In which type of chromatography, the stationary phase is held in a narrow tube and the mobile phase is forced through it under pressure	Column chromatography
3	What is Eluent?	Solvent
4	some commonly used adsorbents	cellulose, starch, calcium
5	The process of passing a mobile phase through a chromatography column is called which one of the following?	Elution

PAPER CHROMATOGRAPHY:

PRINCIPLE

- ♣ The cellulose fibres of chromatography paper act as the supporting matrix for the stationary phase.
- ♣ The stationary phase may be water, a non-polar material such as liquid paraffin or impregnated particles of solid adsorbents like alumina, silica gel or ion exchange resin .while these modification lead to gel or ion exchange resin.
- ♣ While these modification lead to different mechanisms for separation the technique remains the same. The paper may be washed with acid (0.1N HCL) to removed impurities like inorganic substance, adsorbed salts, mineral matters etc. and then dried before the chromatography is carried out.

Apparatus and Paper Development

- ♣ The sample is applied to the paper as a small spot or a narrow streak. There are two main techniques, which may be employed for development of paper chromatograms- ascending, or descending techniques.
- ♣ In both cases the solvent is placed at the base of a sealed tank or a glass jar to allow the chamber to become saturated with solvent vapour. After equilibrium of chamber is achieved, the development of paper chromatography is started.
- ♣ In ascending mode the base of the paper is in contact with solvent at the base of the chamber. In descending techniques the upper end of the paper is in the contact with the trough with solvent and the lower end is allowed to hang vertically but not in contact with the base of the tank.
- ♣ The development is continued until the solvent front approaches the end of the paper. The paper is then removed and solvent is allowed to evaporate either at room temperature or a by keeping in an oven. After drying, the spots of resolved sample components can be subjected to detection.

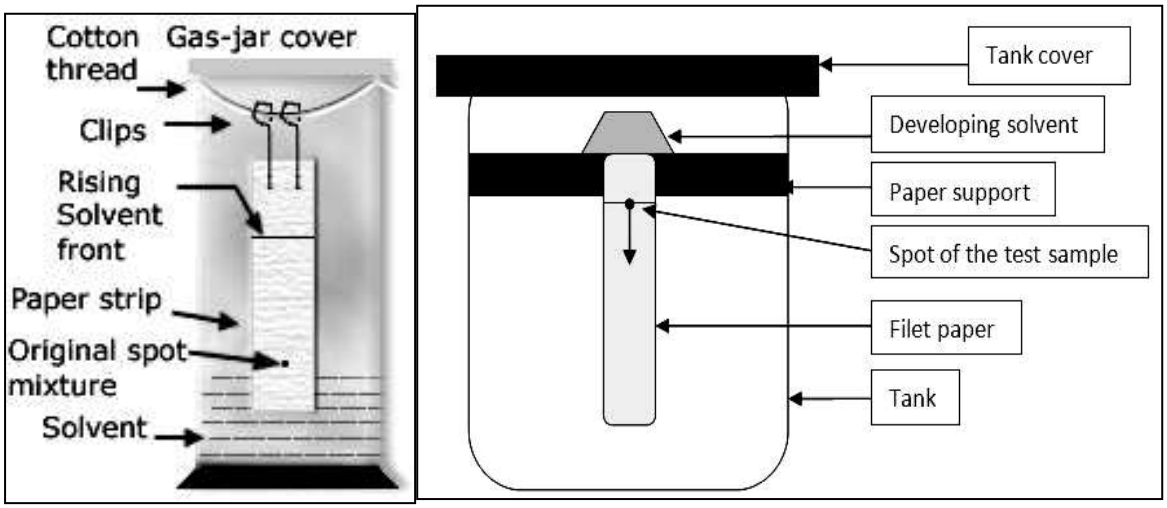

Figure1: Apparatus for ascending type and Descending type Paper Chromatography

Ascending technique has two advantages over descending technique:

- ♣ The setup required is sample.
- ♣ The resolution of sample components by ascending technique is somewhat better because two forced are acting on the solute. The capillary force makes it moves up and the gravitational force opposes this movement.

  The disadvantage of ascending technique is that it is very slow.

Choice of Solvent System:

- Usually in paper chromatography the stationary phase is water since it is very well adsorbed by cellulose.
- The mobile phase which is less polar, flows over the polar stationary phase. Mobile phase is usually a mixture of various solvent such as alcohols, acids, esters, ketones, phenol, amines, hydrocarbons etc.
- The solvent are selected in such a way that the resolution of sample components is satisfactory, e.g. For separation of amino acids the solvent system is satisfactory, e.g. for separation of amino acid the solvent system used are **methanol : pyridine: water** in a proportion **25 : 12 : 63** or **acetic acid : butanol : water** :in a proportion of **3 : 12 :5.**

Detection of Spot:

- ♣ If the sample components are colored, the detection is easiest. The spot can be observed visually. Usually the components are colorless and hence they can be detected by using specific coloring reagents.
- ♣ Amino acid can be detected as blue/purple colored spots after spraying the paper with Ninhydrin reagent.
- ♣ Compound with double bonds (unsaturation) can be detected as yellow/brown colored spots after subjecting the paper chromatogram to iodine vapors.
- ♣ If compounds show adsorption of light they can be detected by UV or infrared adsorption.
- ♣ Direct or indirect fluorescence measurement; compounds may have inherent fluorescence e.g.: riboflavin or they can be made fluorescent by using specific fluorescent dyes.
- ♣ If the compounds are radioactive the spots can be detected by autoradiography.

The components may be extracted/eluted from the paper chromatography and quantitatively estimated or other chemical or physical test may be performed.

The identification of a given compound may be made on the basis of the distance travelled by the solute relative to the distance travelled by the solution front. The ratio, which reflects the distribution coefficient of the given solute is known as the retardation factor (also known as relative flow) Rf, and is constant for a given compound under standard condition.

$$\mathbf{Rf} = \frac{\boldsymbol{distancetravelledbythesolutefromorigin}}{\boldsymbol{distancetravelledbythesolventfrontfromorigin}}$$

Application of Paper Chromatography

- o Paper chromatography is widely used for separation of various organic compounds from the mixture. Some of the specific applications are listed below:

- ♣ To control of purity of pharmaceutical products.
- ♣ Detection of adulterants and contaminants in foods and drinks.
- ♣ The studies of food ripening and fermentation.
- ♣ Detection of drugs and dopes in animals and humans.
- ♣ Analysis of cosmetic.
- ♣ Analysis of reaction mixture in bio chemical labs.

THIN LAYER CHROMATOGRAPHY:

Thin layer chromatography (TLC) was developed to separate classes of compounds, initially in the lipid field where paper chromatography had not been successful. As TLC developed it was found to have wide applicability for many classes of compounds and to have many advantages over paper chromatography.

PRINCIPLE:

Partition, adsorption, exclusion and high performance liquid chromatography may all be carried out in the thin layer mode.

Thin Layer Preparation:

- ♣ The methods involve the use of finally powdered adsorbents such as silica gel, celite, alumina, cellulose and cellulose derivates. The powdered solid adsorbent (stationary phase) usually mixed with binder (10% $CaSO_4$).
- ♣ Slurry is prepared in water and is applied to a glass, plastic or foil plate as a uniform layer by means of plate spreader starting at one end of the plate and moving progressively towards the other .generally the thickness of the layer is adjusted between 0.2 to 0.5 mm. For rapid screening tests, small TLC plates of microscope slide size are also commercial available. In case of adsorbents dryings of TLC plate is carried out in an oven at 100 to 120 °C. This also serves to activate the adsorbent.

Sample Application:

- ♣ The sample is applied as a spot by means of auto pipette, syringes, or automatic applicator.
- ♣ The spot of sample is generally placed 2.0 to 2.5 cm above the lower edge of the plate. For preparative TLC, the sample may be applied as a band across the plate rather than a spot.

Plate Development:

- ♣ Separation takes place in a glass tank (TLC chamber), which contents the developing solvent to the depth of about 1.5 cm. This is allowed to stand for 1hour with a lid over the top of the tank to ensure that atmosphere within the tank becomes saturated with solvent vapor. After this equilibration, the TLC plate is placed in the tank vertically so that it stands in the solvent.
- ♣ The lid is closed and separation of the compounds occurs as the solvent travels up the plant.
- ♣ In order to improve resolution, the technique two dimensional TLC may be used. The material is to be chromatographed is placed towards one corner of the plate as a single spot and the plate is developed in one direction and then removed from the tank and dried. Then it is developed with other solvent system in a direction at right angle to the first direction.

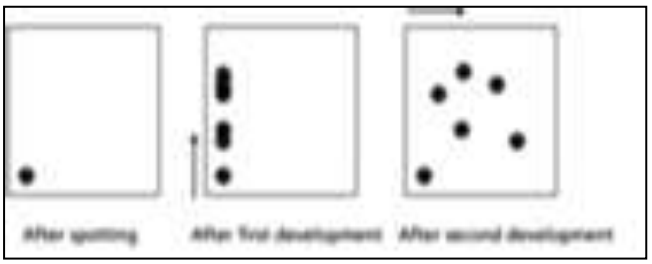

Figure 2:Two dimensional Thin Layer Chromatography (2D TLC)

Detection of the Components:

Most of the detection methods are same as described earlier for paper chromatography (for example: UV absorption, fluorescence, auto radiography or production of colors by specific chemical treatment). The Detection methods for specific for TLC are:

- ♣ Spraying the plate with 25 to 50% sulfuric acid in ethanol and heating. This results in charring of most of the compounds, which show up as brown colored spots.
- ♣ Iodine vapour is used as a universal reagent for the detection of organic compound having ring structure of double bonds.

Application of TLC:

TLC is more versatile, faster and more reproducible. It is used for analytical as well as preparative purpose. TLC is widely used to separate various compounds like polysaccharides, amino acids, plant pigments, hormones, drugs, lipids, phospholipids, vitamins etc.

Advantages of TLC over paper chromatography:

- ♣ It offers wide choice of adsorption.
- ♣ Resolving power is greater than the paper chromatography.
- ♣ Separation is faster in TLC (10-30 min) than paper chromatography (2-3 hours).
- ♣ In paper chromatography the spots are diffused because of the fibrous nature of cellulose, which produces capillary diffusion. Inorganic absorbents are used in TLC does not have fibrous structure, so capillary diffusion is eliminated.
- ♣ Because of inert nature of inorganic adsorbents in TLC, sensitive and simplified detection method like charring with sulfuric acid can be used.
- ♣ In TLC the spot are compact and therefore the material is more concentrated in the smaller spot, thus giving a high level of detection sensitivity. The lower limit of the amount of material that can be detected on the thin layer chromatography is 2-4Microgram while in paper chromatography it is 20-40 microgram.

- ♣ TLC can be used for analytical and reparative purpose to isolate large quantities of any compound.

One Word Question

Sr. No.	Question	Answer
1	The size of a thin layer of adsorbent is about _____	0.2 mm
2	The chromaplate or thin layer chromatography plate is made up of _____	Glass
3	The relative adsorption of each component of the mixture is expressed in terms of its _____	RF Factor
4	Amino acids detected by spraying the plate with ninhydrin solution is an example of _____	Paper Chromatography
5	When there are to complex mixture are separated which development technique of paper Chromatography is used?	Ascending Descending

❖ ION-EXCHANGE CHROMATOGRAPHY:

Ion-exchange chromatography is a variation of adsorption chromatography in which the solid adsorbent has charged groups chemically linked to an inert solid.

PRINCIPLE:

- ♣ The principle of the ion-exchange chromatography is the attraction between oppositely charged particles. Ions are electro statically bound to charged groups; these ions may be exchanged for other ions in an aqueous solution.
- ♣ Ion exchange are most frequently used in columns to separate molecules according to charge. Many biological materials, for example amino acid and proteins, have ionizable groups and they may carry net negative charge or positive charge depending on their **Pka** Value and on the pH of the solution.
- ♣ The Charged molecules bind to the ion-exchangers reversibly; the molecules can be eluted by changing ionic strength or pH of the eluting solvent.
- ♣ Two types of ion exchangers are available:
- ♣ Cation Exchange Resin: Cation exchangers have chemically bound negative charges and they exchange reversibly the positive charged ions (cations).
- ♣ Anion Exchange Resin: Anion Exchangers have chemically bound positive charges and they exchange reversibly the negatively charged ions (anions).
- ♣ The inert materials used in ion-exchange chromatography are: crossed-linked polystyrene, cross-linked dextran and cellulose etc. to this solid support, charged groups are covalently attached to prepare ion-exchange resin. Di ethyl amino ethyl (DEAE) or carboxy methyl (CM) groups are chemically linked to cellulose to give anion and cation exchange resin respectively.

- ♣ All exchangers are generally supplied with appropriate counter ion, normally sodium or chloride. In some cases, the ion-exchanger may need pre-treatment with acid or alkali to generate the desired salt form salt form and than it can be filled into the column.

Process of Ion-Exchange:

The actual ion-exchange mechanism is thought to be composed of five distinct steps.

1. Diffusion of the ion-exchange surface. This occurs very quickly in homogeneous solutions.
2. Diffusion of the ion through the matrix structure of the exchanger to the exchange site. This is dependent upon the degree of cross linkage of the exchanger and the concentration of the solution. This process is thought to be feature, which controls the rate of whole ion-exchange process.

3. Exchange of ions at the exchange site. This is thought to occur instantaneously and is an equilibrium process.

Cation Exchanger:

$R\text{-}COO^{-}.....Na^{+}$ +	**$H_3N^{+}\text{-}R`$**	**↔**	**$R\text{-}COO^{-}.....N^{+}H_3\text{-}R`$ +**	**Na^{+}**
Exchanger	**counter ion**		**bound molecular ion**	**exchanged ion**

Anion Exchanger:

$(R)_4\text{-}N^{+}....Cl^{-}$ +	**$^{-}OOCR\text{-}R`$**	**↔**	**$(R)_4\text{-}N^{+}...^{-}OOC\text{-}R`$ +**	**Cl^{-}**
Exchanger	**counter ion**		**bound molecular ion**	**exchanged ion**

The more highly charged the molecule to be exchanged, the tighter it binds to the exchanger and less readily it is displaced by other ions.

1. Diffusion of the exchange ion through the exchanger surface.
2. Selective desorption by the eluant and diffusion of the molecules into the external solution .The selective desorption (elution)can be achieved by several ways: by change in pH and/or ionic strength or by affinity elution, in which case an ion which has greater affinity for the exchanger than has the bound molecules is introduced into the system.

Application of Ion-Exchange Chromatography:

- ♣ Ion exchange chromatography has several applications, some of which are listed below:
- ♣ Amino acid auto analyzer used to determine amino acid sequence of proteins is based onion exchange principle.
- ♣ Ion exchange has been extensively used to determine the base composition of nucleic acids i.e. content of adenine, thymine, guanine and cytosine.
- ♣ It is commonly used to produce deionised water in laboratory as well as in industries. The solute cations are exchanged for hydrogen ions and solute anions are exchanged for hydroxyl ions.
- ♣ Biological samples having trace quantities of metal ions can be passed through the ion exchange resin, which holds the metal ions, hence these metal ions gets concentrated, eluted and detected easily.
- ♣ Both cations and anions exchange resins are used into purify the proteins and enzymes based on the overall charge on them. If the overall charge preset on the proteins is negative then anion exchanger resins are used; and if the overall charge present on the proteins is positive then cation exchanger resins are used.
- ♣ Ion-exchange chromatography has also been used for separation of many vitamins, hormones, other biological amines, drugs, organic acids and bases.
- ♣ Besides the used of ion exchange chromatography in column mode, it can also be used on TLC or HPLC (high performance liquid chromatography).
- ♣ Ion exchange chromatography has also been used for separation of nucleic acids.

❖ GEL PERMEATION (EXCLUSION) CHROMATOGRAPHY:

PRINCIPLE:

The separation of molecules based on their molecular size and shape using molecular sieve properties of a variety of porous gel permeation chromatography. A column of gel particles is in equilibrium with a suitable solvent for the molecules to be separated. Large molecules, which are completely excluded from the pores, will pass through the interstitial spaces, while smaller molecules will be distributed between the solvent inside and outside the molecular sieve and will pass through the column at a slower rate.

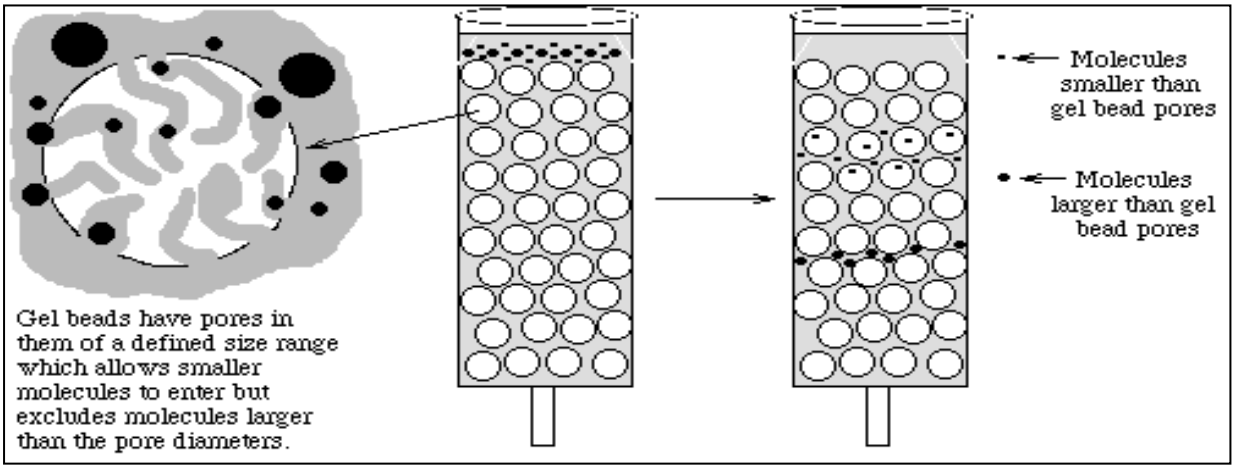

Figure 3: Gel Permeation Chromatography

- ♣ The distribution of a solute in a column of a swollen gel is determined by the total volume of solvent, both inside and outside the gel particles.
- ♣ For a given type of gel, the distribution coefficient Kd, of a particular solute between the inner and outer solvent fraction is a function of its molecular size. If the solute is large and completely excluded from the **solvent within the gel**, **Kd = 0**; where as if the solute is sufficiently small to gain complete accessibility to the **inner solvent, Kd = 1**.
- ♣ The **elution volume (Ve**) of a given solute depends on the **void volume (Vo**), the **distribution coefficient (Kd)** and the volume inside the **gel matrix itself (Vi).**

$$Ve = Vo + Kd \times Vi$$

- ♣ The inner volume (Vi) can be calculated from the known dry weight of the gel (a) and water regain value.

$$Vi = a \times Wr.$$

- ♣ It is also possible to undertake gel permeation chromatography using thin layer mode (TLG) in which a thin layer of swollen gel spread on to a glass plate. The gel beads forms stationary phase and the interstitial fluid forms mobile phase.

- In TLG the layer is not dried as in case of TLG and in case of TLG there is no solvent front. TLG is used for separation of hydrophilic substance like proteins, peptides, nucleic acids etc.
- The great advantage of TLG over column gel permeation chromatography is that large number of samples can be chromatographed at same time under identical condition with very small amounts of samples.

Materials used in gel permeation chromatograph:

- Gels that are commonly used include cross linked dextran (Sephadex), agarose (Sepharose), polyacrylamide (Biogel P) and polystyrenes (Bio-Beads S).
- **The dextran gels** are obtained by cross linking the polysaccharide dextran with epichlorhydrin. In this way the water soluble dextran is made water insoluble, but it retains its hydrophilic character and swells rapidly in aqueous media, forming gel particles for gel permeation. By varying the degree of cross linking several types of sephadex gels have been obtained.
- They differ in porosity and consequently are useful over a different molecular size ranges. Each types of **sephadex** are characterized by its water regain capacity (the amount taken up in the completely swollen gel granules by one gram of sephadex).
- **Agarose gels** are produced from agar, are linear polysaccharides consisting of alternating residues of **D-galactose** and **3, 6-anhydro L-galactose** units. Their gel forming properties are attributed to hydrogen bonding in both inter and intra molecular type. Due to their hydrophilic nature and the nearly complete absence of charged of groups , agarose gels like dextrans gels, cause very little denaturation and sensitive biochemical substances.
- **Sephadex gels** are used for used for fractionation of molecules having molecular weights up to 8,00,000 Daltons. The agarose gels may be used to separate molecules and particles having molecular weights of several million Daltons. The agarose gels are therefore used widely for the study of viruses, nucleic acids and polysaccharides.
- **Polyacrylamide gels** are produced by the polymerization of acryl amide monomer and methylene bis acryl amide (cross linking agent). By varying the proportions of two monomers, gels with differing pore sizes may be obtained. They have an exclusion limits ranging from 1,800 to 4,00,000 Daltons and properties similar to dextran and agarose gels.

Application of Gel Permeation Chromatography:

- **Purification of biological macromolecules** such as viruses, proteins, enzymes, antibodies, nucleic acid and polysaccharides. Low molecular weight compounds also can be separated from the high molecular weight substances e.g. amino acids, peptides from proteins; oligonucleotide from the nucleic acids etc.
- **Molecular weight determination:** The elution volumes of globular proteins are largely determined by their molecular weight. It has been shown that over a considerable range, the elution volume is approximately a linear function of the logarithm of

molecular weight. Hence using calibration curve of proteins with similar shape and known molecular weight, it is easy to determine molecular weight of other proteins.

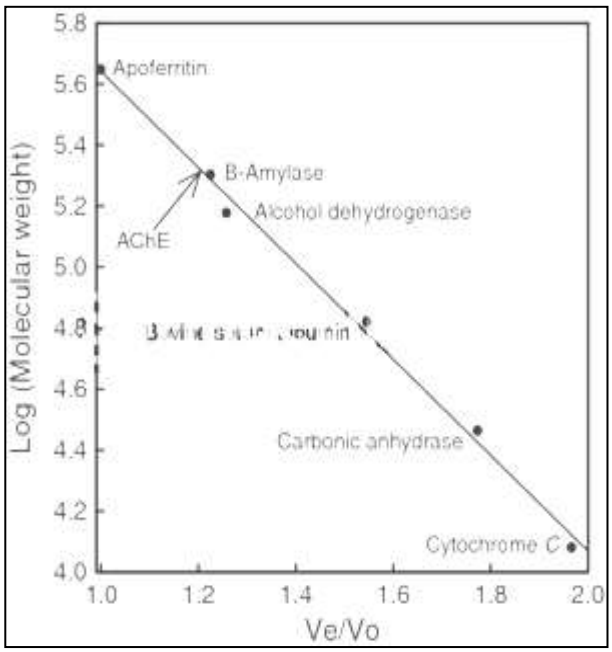

Figure 5: Determination of molecular wt. of proteins by gel permeation chromatography.

- **Solution Concentrations:** solution of high molecular weight substances can be concentrated by addition of dry Sephadex G-25. The swelling gel adsorbs water and low molecular weight substances while the high molecular weight substances remain in the solution, upon centrifugation the gel will sediment and the supernatant will have high molecular weight substances concentrated in the solution.
- **Desalting:** By using a column of Sephadex G-25 solution of high molecular weight compounds may be desalted. The desalting method is more efficient and faster than dialysis. Application includes removal of phenol from nucleic acid preparations, ammonium sulfate from protein preparation, monosaccharide from polysaccharides and amino acids from protein.
- **Protein - ligand binding studies:** for doing receptor assays by separating bound and free ligand i.e. hormone assays.

One Word Question

Sr. No.	Question	Answer
1	_____ is a liquid column chromatographic method of separating solute molecules according to differences in molecular size	Gel filtration
2	Which of the following technique separates the sample by percolating it through a bed of porous, uncharged particles?	Gel filtration
3	Which of the following is not a gel filtration media used in gel filtration?	Silica Gel
4	Ion exchange chromatography is based on the	electrostatic attraction
5	The cost of the resins used in the ion exchange process is _____	High

❖ AFFINITY CHROMATOGRAPHY:

PRINCIPLE:

- ♣ This technique exploits the most unique and specific property of biomolecules, their biological function. The specificity or affinity of macromolecule with a small molecule (a ligand) is utilized for separation of the macromolecule of interest from the heterogeneous mixture.
- ♣ The small molecule is attached (immobilized) to a gel matrix and the mixture containing desired macromolecule is allowed to pass (percolate) through the matrix. The vast majority of the molecules have no affinity for the bound molecules or ligand and flow through the matrix unretarded.

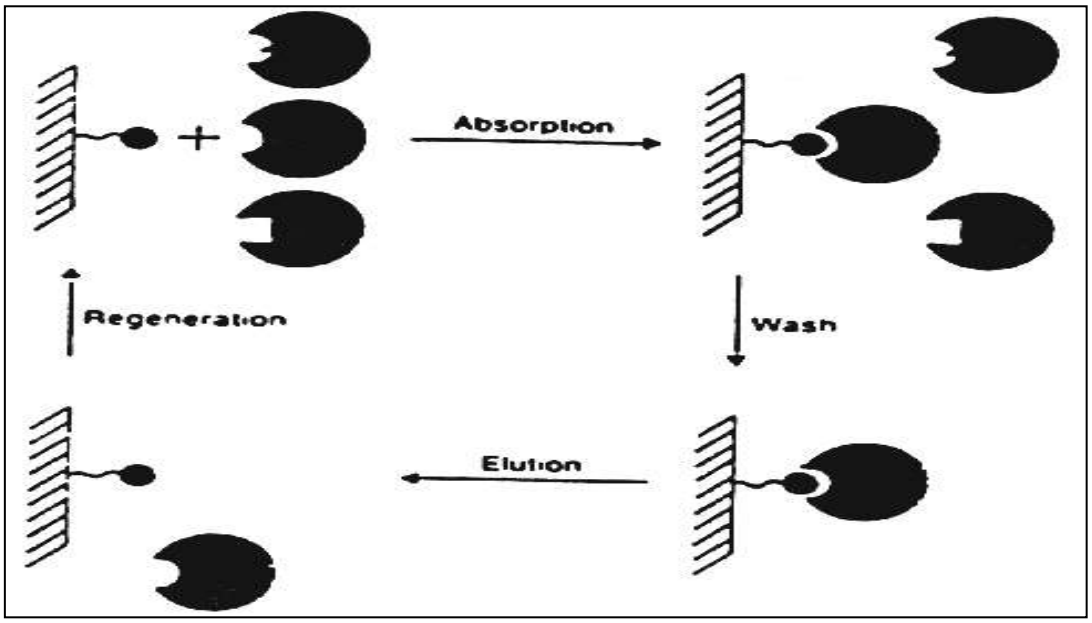

Figure 6: Purification of enzyme by Affinity Chromatography

- ♣ The desired macromolecules, however, recognize the bound molecules, bind to it and thus retarded. After all the undesired components have been flushed from the column the conditions of the wash solution are altered to bring about dissociation of consequential gets eluted out and appears in the affluent largely purified from the original mixture.

This technique gives highest degree of purity and yield in comparison to the other chromatographic techniques that separates the biomolecules from the mixture based on small difference in physic-chemical properties.

Table 1: Use of different ligand in purification of various macromolecules using affinity chromatography

Enzyme /protein/Biomolecules	Immobilized ligand
Adenosine deaminase	Adenosine
Amino peptidase	Hexametylenediamine
Avidin	Biocytin
Carbonic anhydrase	Sulfanilamide

Chymotrypsin	Tryptophan
Glycerol-3P dehydrogenase	Glycerol-3Phosphate
Thrombin	Benzamidine
Xanthine oxidase	Allopurinol
Coagulation factor	Heparin
Follicle stimulating hormone	Concavlin A
Interferon	Antibody
Thyroxin binding globulin	Thyroxin
Serine proteases	L-Arginine
Immunoglobulin G	Protein A
m-RNA	Polydeoxythymidylic acid
Endotoxins	Polymixin

Applications of Affinity Chromatography:

In order to achieve highest degree of purity using conventional chromatographic procedures it takes lot of time and several methods have to be used .while with the help of affinity chromatography it is possible to obtain highest degree of purity in a single step.

Because of this reason this technique is becoming increasingly popular for purification of large number of macromolecules including enzyme, antibodies, nucleic acid, vitamin-binding protein, transporter protein, and drug and hormone receptors.

One Word Question

Sr. No.	Question	Answer
1	The chromatographic method of separating biochemical mixture of compounds, based on highly specific biological interactions is referred to as _____	affinity chromatography
2	Which of the following is not a highly specific biological interaction to be used in affinity chromatography?	Cations-anions
3	Acetylcholine esterase can be purified by using affinity chromatography with the use of _____ as a ligand.	Acrdinium
4	The purity of an enzyme at various stages of purification is best measured by:	Specific activity of the enzyme
5	Which would be best to separate a protein that binds strongly to its substrate?	Affinity chromatography
	Affinity chromatography separation is based on…	specific interaction between the analyte and the ligand

❖ HPLC (HIGH PERFORMANCE LIQUID CHROMATOGRAPY):

- ♣ The resolving power of the chromatographic technique increases with the length of the column or the surface area of the stationary phase.

  Therefore the smaller the particle size of the stationary phase betters the resolution.

  Unfortunately, the smaller the particles size the greater is the resistance to flow of mobile phase.

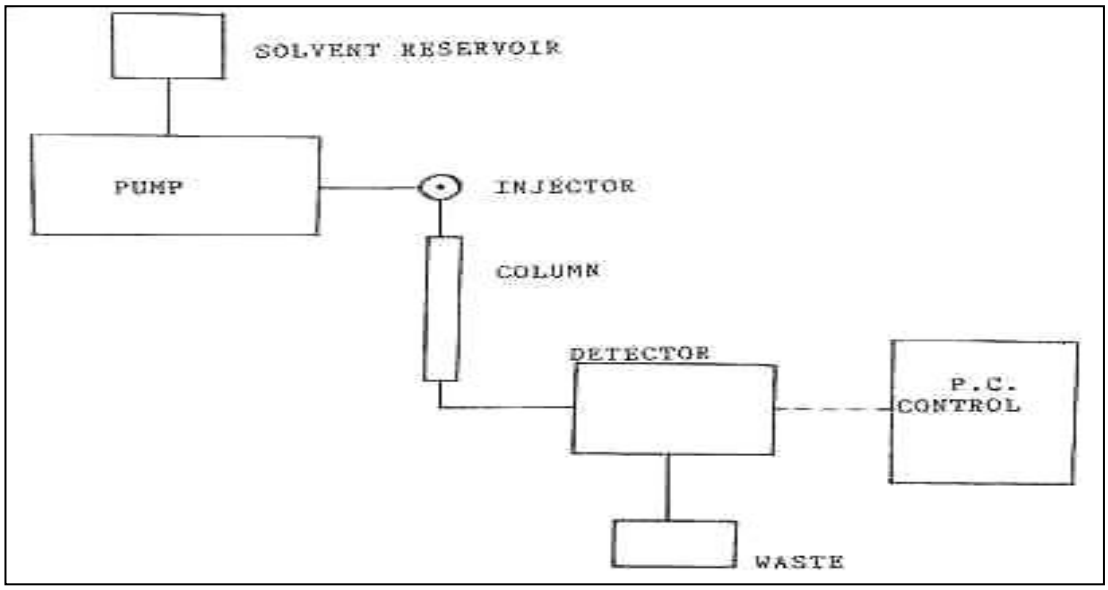

Figure 7: A diagram showing different components of HPLC

- ♣ This creates back pressure in the column that is sufficient to damage the matrix structure of the stationary phase thereby actually reducing flow of mobile phase and impairing resolution.
- ♣ In the recent past there has been a dramatic development in column chromatography technology that has resulted in the availability of new smaller particle size, stationary phases that can withstand this pressure.
- ♣ The high pressure mobile phases delivery systems (pumps) have been developed that are capable of driving the mobile phase through small pore matrices at pressures exceeding 7000psi. Hence high performance liquid chromatography is also known as high pressure liquid chromatography.
- ♣ The specialized column is available for adsorption, partition, ion-exchange, exclusion and affinity modes of HPLC. This offers faster separation and better resolution of compound from the mixture. Therefore the HPLC has emerged as the most popular powerful and versatile technique of chromatography. Many commercially available HPLC systems are microprocessor controlled.
- ♣ The separated components on HPLC can be detected by using the detectors. The UV adsorption, fluorescence and electrochemical detector are very sensitive and therefore used in HPLC. Depending on sample we have to use the detector.

Application of HPLC:

- ♣ The wide applicability, speed and sensitivity of HPLC has resulted in it becoming the most popular form of chromatography and virtually all types of biology molecules have been identified, assayed or purified using the techniques. Any molecules hydrophobic, charged or uncharged, large or small can be purified by using HPLC.

- Some examples of compounds that can be purified by using HPLC are: amino acid, peptide, vitamins, drugs, carbohydrates, various plants pigment, dyes, organic acids and bases, nucleoside and nucleic acids, charged ions etc.

❖ GAS-LIQUIED CHROMATOGRAPHY (GLC OR GC)

- This technique is based upon the partitioning of compounds between a stationary liquid and mobile gaseous phase. It is widely used for the qualitative and quantitative analysis of large number of compounds because it has high sensitivity, reproducibility, speed and resolution.
- A stationary phase of a high boiling point liquid material such as silicon grease or polyethylene glycol is supported on an inert granular solid.
- This material is packed into a narrow coiled glass or steel column 1 to 100 meter long and 0.03 to 4 mm internal diameter. Through this column an inert carrier gases (the mobile phases) such as nitrogen, helium or argon is passed.
- The column is maintained in an oven at an elevated temperature, which assure that the compounds to be separated are kept in the vapour state and that analysis times are reasonable.
- As the compound leaves the column, they pass through a detector that is linked via an amplifier to a chart recorder which in turn record the detector. The flame ionization detector is most widely used in GLC but use of some other types of detectors such as infrared spectrophotometer, nuclear magnetic resonance spectrophotometer and atomic emission spectrophotometer can spectra that can help to identify the unknown compounds.

Applications of Gas Liquid Chromatography:

The technique is widely used for separation for volatile and non-polar compound like different hydrocarbons, pesticides etc.

The polar compounds like carbohydrates, fatty acids. Possessing polar groups such as –OH, -NH_2, -COOH can be converted into non polar compounds by derivatization like methylation, silanisation or perfluroacylation and then they can be separated by GLC.

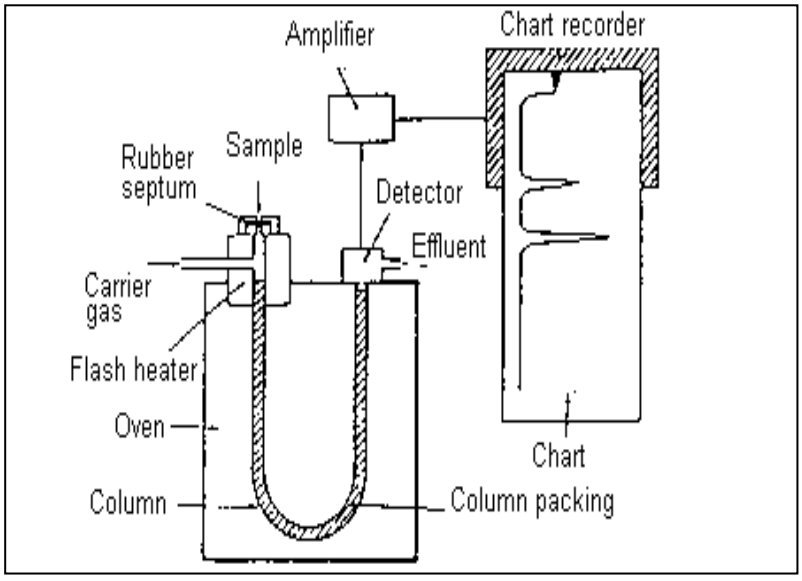

Fig: 8:A diagram showing different components of GLC.

Typical uses of GLC: Mainly use for testing the purity of particular substance and also used identify the compounds

One Word Question

Sr. No.	Question	Answer
1	HPLC stands for	High Pressure Liquid Chromatography
2	HPLC methods include	Liquid/liquid (partition) chromatography
3	In normal phase HPLC, there is a	Normal polar phase
4	Which of the following is not a feature of carrier gas used in gas chromatography?	Argone
5	In which of the following methods are liquid samples injected into the column in gas chromatography?	Micro-syringe
6	GLC use for the sample contain	Volatile compound