
CHROMATOGRAPHY
TECHNIQUES
(Principle & Applications)

History of Chromatography

The first detailed description of chromatography is generally credited to Michael Tswett, a Russian biochemist, who separated chlorophyll from a mixture of plant pigments in 1906 and because of the nature of the pigments in the sample, each band had a distinctive colour. Thus, the name of the process was coined from the Greek words for "chromo" means colour and "graphy" means to write.

Chromatography became developed substantially as a result of the work of Archer John Porter Martin and Richard Laurence Millington Synge during the 1940s and 1950s. They established the principles and basic techniques of partition chromatography and their word encouraged the rapid development of several types of chromatography methods such as Paper chromatography, Gas chromatography and what would become known as High performance liquid chromatography. Since then, the technology has advanced rapidly.

What is Chromatography?

- *The term chromatography bunches together a family of closely related extremely powerful separation methods.*
- *The feature common to them all is that two mutually miscible phases are brought into contact with each other. One of these phases is stationary, while the other is mobile; the mobile phase either moves over the surface or percolates through the interstices of the stationary phase*
- *The sample mixture, introduced into the mobile phase undergoes repeated interactions between the stationary and mobile phases while being carried through the system by the mobile phase.*

- *Different components of the sample mixture interacts with the two phases differentially on the basis of small differences in their physico-chemical properties.*
- *Since these different rates of interaction govern the migration of the sample components through the system, each one of the components migrate at a different rate.*
- *The compound which interacts more with the mobile phase and least with the stationary phase migrates fast.*
- *The component showing least interaction with the mobile phase while interacting strongly with the stationary phase migrates slowly.*
- *This differential movement of the components is responsible for their ultimate separation from each other.*

Chromatography Terms

- **ANALYTE** : Substance to be separated during chromatography.
- **BONDED PHASE** : Stationary phase that is covalently bonded to support particles of the column.
- **ELUATE** : Mobile phase leaving the column.
- **ELUENT** : Solvent that will analyte.
- **CHROMATOGRAPH** : Equipment that enables the sophisticated separation.
- **CHROMATOGRAM** : Visual output of chromatograph.
- **EUTROPIC SERIES** : List of solvents ranked according to their eluting power.
- **IMMOBILIZED PHASE** : Stationary phase which is immobilized on the inner wall of

- **MOBILE PHASE** : The phase which moves in a definite direction.
- **RETENTION TIME** : The characteristic time it takes for a particular analyte to pass through the system under set conditions .
- **SAMPLE** : Matter analyzed in chromatography.
- **SOLUTE** : The sample components in partition chromatography.
- **SOLVENT** : Any substance capable of solubalizing other substance and expecially the liquid mobile phase in

TECHNIQUES OF CHROMATOGRAPHY

There are two basic techniques of chromatography:

- **PLANE CHROMATOGRAPHY :**

  - **Paper Chromatography :**

    The stationary phase is supported by cellulose fibers of paper sheet .

  - **Thin Layer Chromatography (TLC) :**

    Stationary phase is located into a glass or plastic surface.

- **COLUMN CHROMATOGRAPHY :**

  Stationary phase is packed into a glass or plastic column .

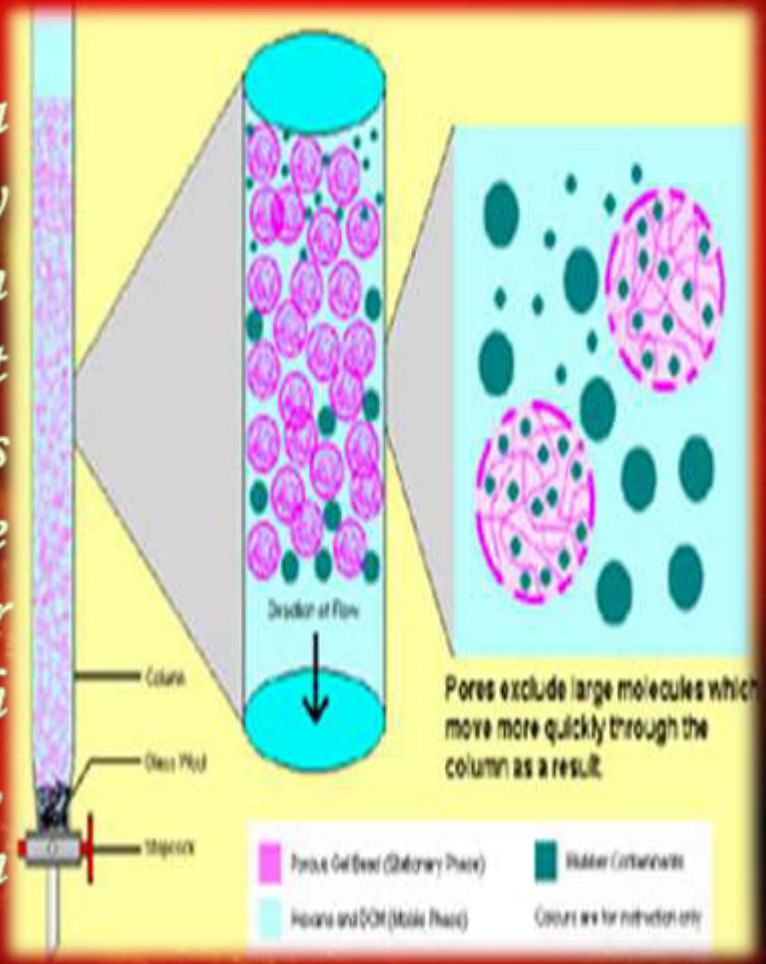
It is important to distinguish between types and techniques of chromatography. Adsorption chromatography, partition chromatography, ion-exchange chromatography etc., are types of chromatography based on different principles which can be carried out using any of the techniques.

GEL PERMEATION CHROMATOGRAPHY

Gel permeation chromatography (GPC) is a separation method dependent upon molecular size. This method is also known as gel filtration.

Principle :

Gel permeable chromatography provides a physical means for separating molecules by their size in solution. There is a distribution of pore sizes within the packing such that small molecules can enter most of the pores and are therefore, retained for the longest time period. While lager molecules enter fewer pores and are retained for the shortest length of time. By proper calibration of the columns, size, molecular weight can be deduced from the elution volume.



APPLICATIONS OF GPC

- GPC is chiefly used for the purpose of separation of biological molecules leading to their ultimate purification such as proteins, enzymes, hormones, antibodies, nucleic acids, polysaccharides and even viruses have been separated in various experiments which have used different types of gels .
- This method is especially useful for the separation of 4s and 5s t-RNA and it is also most satisfactory method for separating DNA from usually gram positive bacteria from the invariable contaminants, the teichoic acids.
- Removal of salts and small molecules from macromolecules can be easily performed using GPC since the distribution coefficients of salt molecules will be largely different from those of macromolecules. For example, in a Sephadex G-25 column, molecules above 5000 Daltons will elute very quickly but the molecules less than 1000 Daltons will be retarded.

- Dilute solutions of macromolecules with molecular weights higher than the exclusion limit may be readily concentrated by utilizing the hygroscopic nature of the dry gel. Sephadex G-200 absorbs 20 times its weight of water, although G-25 is referred for its rapid action. This treatment leaves the macromolecular solution concentrated but at the same time unaltered pH or ionic strength.

- One of the most important applications of GPC is the determination of molecular weight of macromolecules.

ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography is a process that allows the separation of ions and polar molecules based on their charge.

PRINCIPLE:

Ion exchange chromatography retains analyte molecules on the column based on ionic interactions. The stationary phase displays ionic functional groups that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography. The ionic compounds consisting of the cationic species and the anionic species can be retained by the stationary phase.

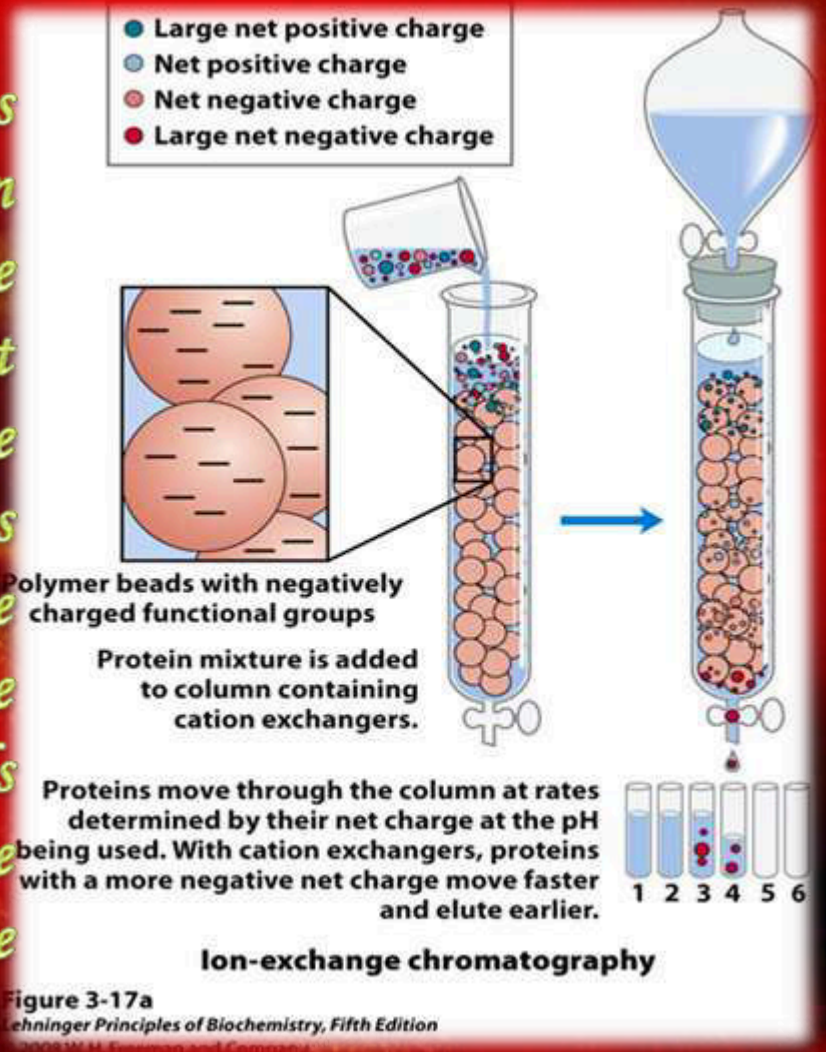


Figure 3-17a
Lehninger Principles of Biochemistry, Fifth Edition

APPLICATIONS OF ION EXCHANGE CHROMATOGRAPHY

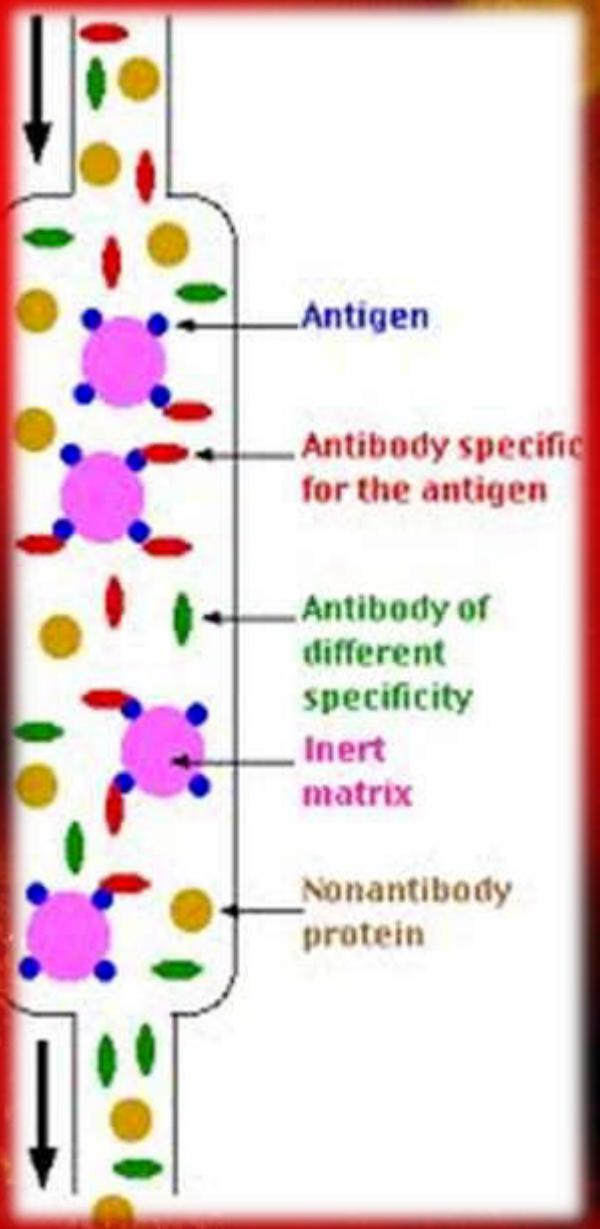
- *The most stimulating use of ion-exchange chromatography is in amino acid analysis. The amino acid "autoanalyser" is based on ion-exchange principle.*
- *The mixture of nucleotides as a result of treatment with DNAses and RNAses can be readily separated by ion-exchange chromatography. Chargaff throughout his experiments which established the equivalence of adenine and thymine, guanine and cytosine used this technique for the purpose.*
- *For many biological applications, ultrapure, metal ion free reagents are needed. This is commercially performed by ion-exchange chromatography.*

- The complete deionization of water or non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by treating water in a mixed bed of anion and cation exchanger. The same method is used for softening of drinking water.
- There are many situations where the concentration of trace metals in biological samples is below the limit detected by atomic absorption spectrometry. These samples are passed over an ion exchange resin that holds the metal ions, after enough sample have been passed over the resin, enough trace metal ions become concentrated over the exchanger. These ions can now be eluted and their concentration in the eluent is considerably higher than that in the sample. These solutions can now be read by absorption spectrometry.
- Apart from the above applications, ion-exchange chromatography has been used for the separation of many vitamin, other biological amines, organic acids and bases.

AFFINITY CHROMATOGRAPHY

PRINCIPLE:

The basic principle is that a biospecific ligand is immobilized to a solid support or resin to which a solution containing the protein of interest is passed over. Ligands are always based on biological functional pairs such as enzymes and substrate or antigens and antibodies. The specific ligand binds the protein of interest and all the non-specific molecules are washed away. The protein is eluted in a specific pH buffer by competitively displacement elution.



APPLICATIONS OF AFFINITY CHROMATOGRAPHY

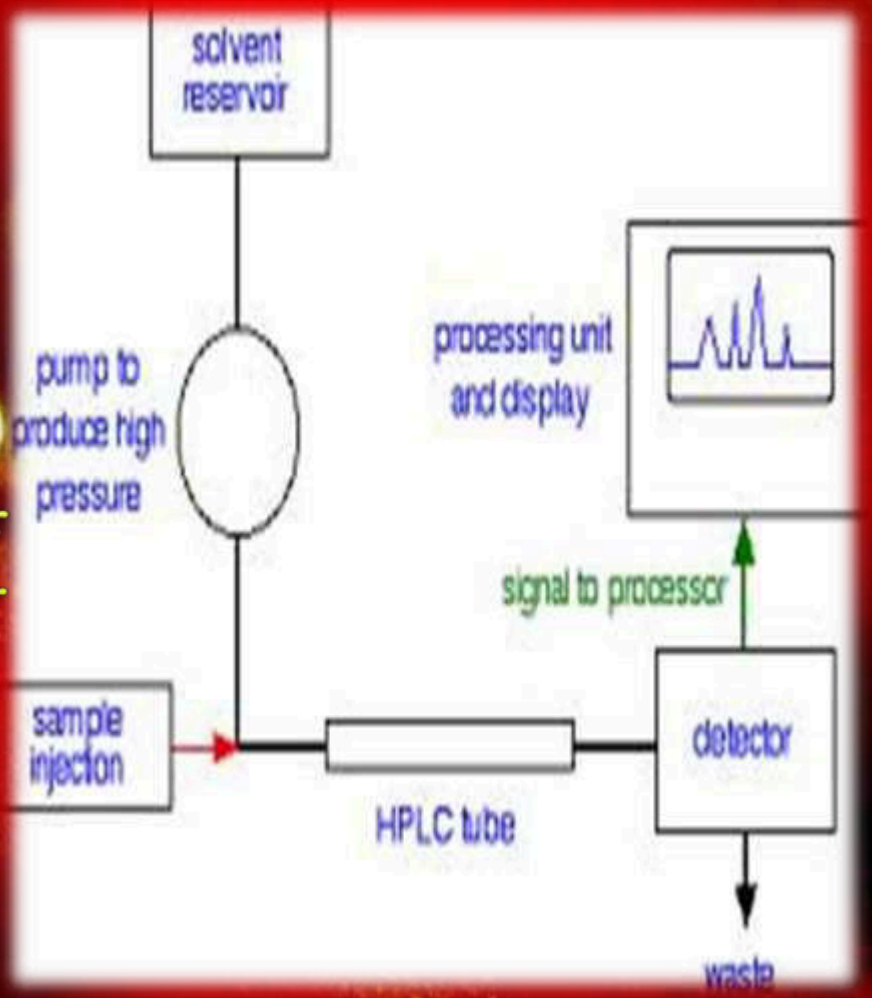
- *Affinity chromatography has been used to purify a large variety of macromolecules such as enzymes, immunoglobulin's, membrane receptors, nucleic acids and even polysaccharides'.*
- *Cells separated by using affinity fractionization include fat cells, T and B lymphocytes, spleen cells, lymph node cells, oocytes and chick embryo neural cells.*
- *Metal chelate affinity chromatography is a logical extension of the basic technique. Many proteins which have similar molecular weights and even isoelectric points cannot be separated even by such high resolution techniques as gel filtration chromatography.*
- *Use of magnetic gel beads is another extension of affinity chromatography. Immunoglobulin negative thymocytes and neurolbastoma cells have been purified by this method. The method gives the routine purity of about 95%.*

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography(HPLC) is basically a highly improved form of column chromatography.

PRINCIPLE:

In HPLC instead of solvent being allowed to drip through a column under gravity, it is forced through under high pressure of up to 400 atmospheres which makes it much faster. It allows a use of very much smaller particle size for the column packing material which gives a much greater surface area of interaction between the stationary phase and the molecules flowing past through it. This allows much better separation of the components of the mixture. These methods are highly automated and extremely sensitive



- In recent times HPLC has emerged as a method of choice for analytical purposes. The biggest advantage that it has over other techniques is the speed of analysis which is many times more than the other techniques except, perhaps, for Gas Liquid Chromatography(GLC).
- The sample requirement is also very low for this technique and as less as a few fetograms of the sample will be analyzed satisfactorily.
- On top of all this the detectors that are employed in HPLC are non-destructive in nature and thus the separated components can be recovered for further study.

APPLICATIONS OF HPLC

HPLC has been successfully applied to the separation of proteins, nucleic acids, animal and plant hormones and also complex lipids.

SOME SPECIALIZED TECHNIQUES

- Hydroxyapatite Chromatography.
- DNA-Cellulose Chromatography.
- Chiral Chromatography.
- Covalent Chromatography.