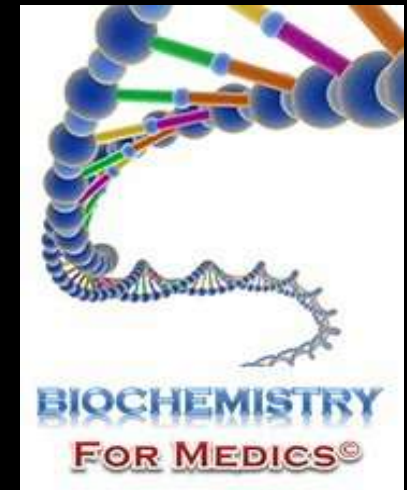


# CHROMATOGRAPHY

Biochemistry for Medics  
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# Chromatography

The word Chromatography is derived from two Greek words – *Chroma* means – colour and *graphein* to write.

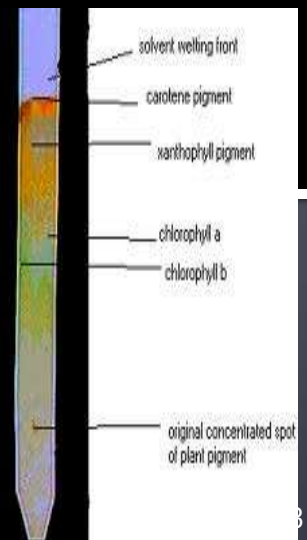
Chromatography is the collective term for a family of laboratory techniques for the separation of mixtures.

# Chromatography

**Chromatography**, literally "**color writing**", was used—and named—in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll.

New forms of chromatography is useful for a wide range of separation processes and chemical analysis tasks, especially in biochemistry.

Chromatography is a misnomer since it is no longer limited to the separation of the colored substances.



# Chromatography- Principle

Chromatography is based on the principle of the partition of the solute between two phases/solvents. Chromatography usually consists of a **mobile phase** and a **stationary phase**.

**1) The mobile phase** usually refers to the mixture of the substances to be separated dissolved in a liquid or a gas.

**2) The stationary phase is a porous solid matrix** through which the sample contained in the mobile phase percolates. The interaction between the mobile and the stationary phases result in the separation of the compounds from the mixture.

**3) These interactions include the physico chemical principles such as the adsorption, ion- exchange, molecular sieving and affinity.**

# Chromatography- Classification

Two ways to classify methodology of chromatography:

1) Based on interactions between sample component and stationary phase:

i) Partition

ii) Adsorption

iii) Ion-exchange

iv) Gel-filtration

v) Affinity

vi) High performance liquid chromatography

# Chromatography- Classification(Contd.)

b) Base on nature of stationary phase or mobile phase

It is of two types

i) Planar- It may be Paper or Thin layer

ii) Column- it may be Gas or Liquid

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane.

Column chromatography is a separation technique in which the stationary bed is within a tube.

# Partition Chromatography

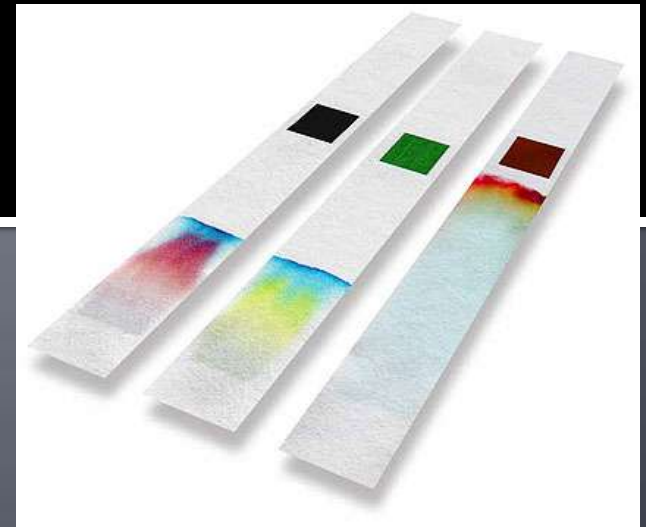
It is used for the separation of mixture of amino acids and peptides. The molecules of a mixture get partitioned between the stationary and the mobile phase depending on the relative affinity of each one of the phases.

**It is undertaken in two ways:**

- 1) Paper chromatography**
- 2) Thin layer chromatography**

# Paper chromatography

- An analytical technique for the separation and identifying mixtures that are either coloured or can be made coloured
- It is a liquid partition chromatography
- Used for separation of amino acids, sugars, sugar derivatives & peptides





# Paper chromatography

- 1) **The stationary phase** is water held on a solid support of filter paper (cellulose).
- 2) **The mobile phase** is a mixture of immiscible solvents which are mixtures of water, a non polar solvent and an acid or base.e.g.Butanol, acetic acid water or phenol-water-ammonia.

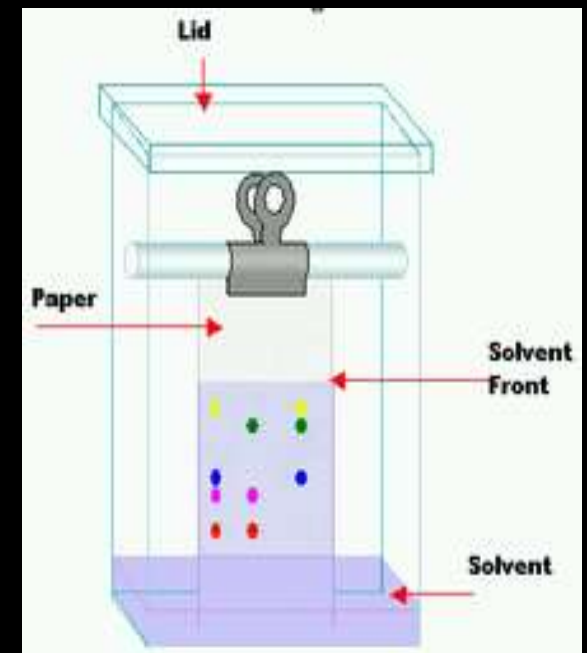
# Paper chromatography- Procedure

- 1) A small **spot of sample** is applied to a strip of chromatography paper about two centimeters away from the base of the plate. This sample is absorbed onto the paper and may form interactions with it.
- 2) The paper is then dipped in to a suitable solvent, such as ethanol or water, taking care that the spot is above the surface of the solvent, and placed in a sealed container.
- 3) **The solvent moves up the paper by capillary action** and dissolves the sample mixture, which will then travel up the paper with the solvent solute sample.

# Paper chromatography- Procedure

-Different compounds in the sample mixture travel at **different rates due to differences in solubility in the solvent, and due to differences in their attraction to the fibers in the paper.**

-Paper chromatography takes anywhere from several minutes to several hours



# Ascending and Descending Paper Chromatography

## Ascending Chromatography

In this method, the solvent is in pool at the bottom of the vessel in which the paper is supported. It rises up the paper by capillary action against the force of gravity.

## Descending Chromatography

In this method, the solvent is kept in a trough at the top of the chamber and is allowed to flow down the paper. The liquid moves down by capillary action as well as by the gravitational force.

**Substances that cannot be separated by ascending method, can be separated by the above descending method.**

# Paper Chromatography -Analysis

After development, the spots corresponding to different compounds may be located by their color, **ultraviolet light, ninhydrin** or by treatment with **iodine** vapors.

The paper remaining after the experiment is known as the **Chromatogram**.

The components which have been separated differ in their **retention factor** i.e Ratio of distance traveled from the **spot or origin** by the solute component to that of the distance traveled from the **spot or origin** by the solvent.

# **$R_f$ value**

$R_f$ , Distance traveled by sample/ distance traveled by solvent.

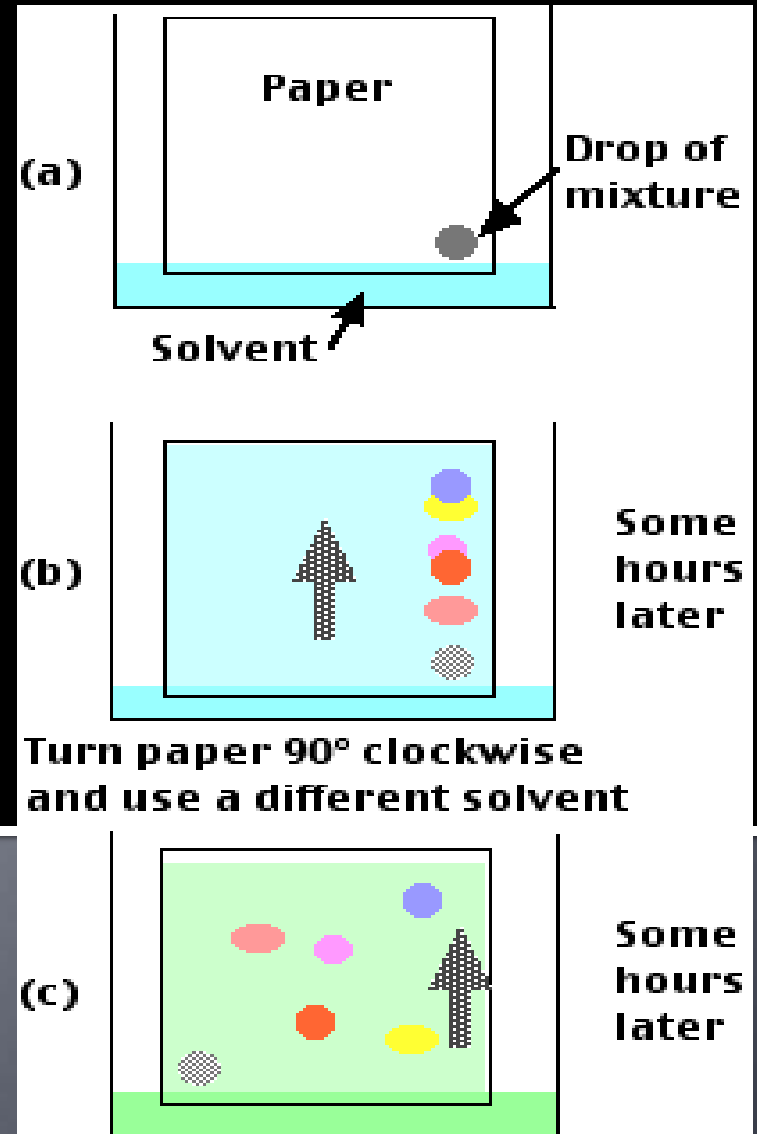
If  $R_f$  value of a solution is zero, the solute remains in the stationary phase and thus it is immobile.

If  $R_f$  value = 1 then the solute has no affinity for the stationary phase and travels with the solvent front.

Retention Factor can never be greater than one.

# Two dimensional chromatography

Sometimes, it is difficult to separate a complex mixture of substances by a single run with one solvent system. In such a case, a second run is carried out by a different solvent system, in a direction perpendicular to the first run. This is referred to as- **two dimensional chromatography** which enhances the separation of a mixture into individual components.



# Significance of Paper chromatography

- 1) Paper chromatography is a very easy, simple, rapid and highly efficient method of separation.
- 2) Can be applied even in microgram quantities of the sample.
- 3) Can also be **used for the separation of a wide variety of materials like amino acids, oligopeptides, sugars, oligosaccharides, glycosides, purines and pyrimidines, steroids, vitamins and some alkaloids like penicillin, tetra cyclin and streptomycin.**
- 4) Not preferred for separating proteins because they are not soluble in many of the solvent systems and are also denatured by them.
- 5) Paper chromatography is inferior to thin layer chromatography in resolving power.



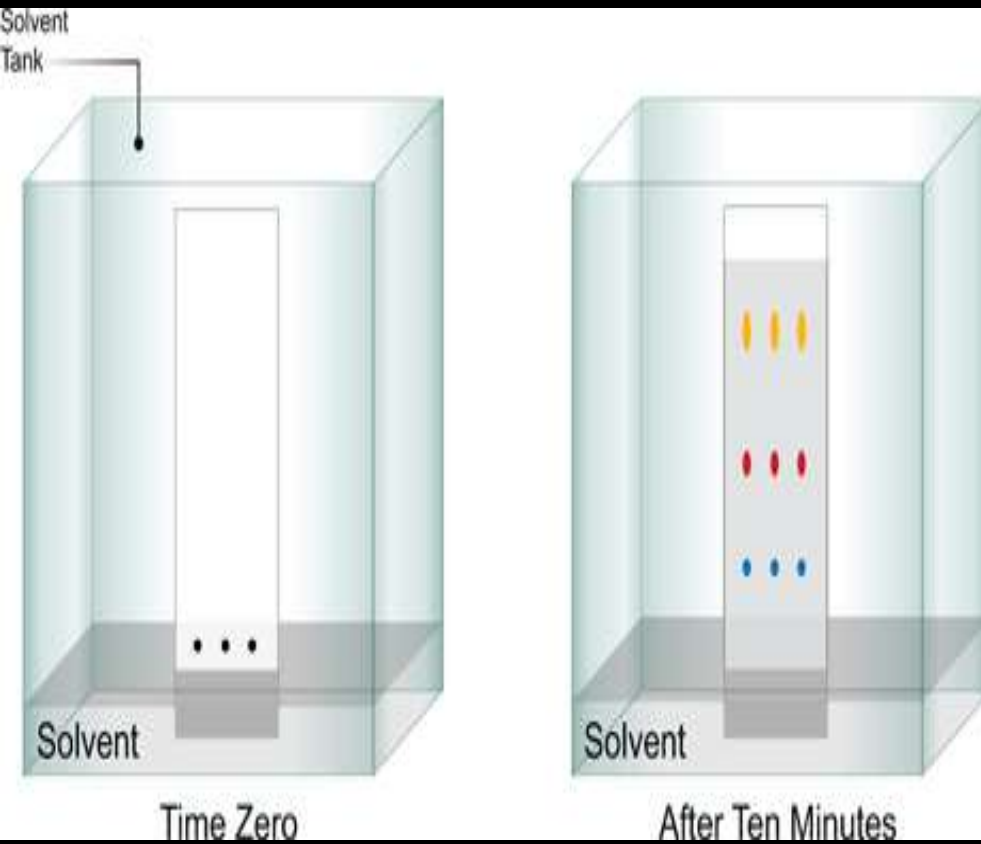
# Thin layer chromatography (TLC)

- It is a chromatographic technique used to separate mixtures.
- It involves a *stationary phase* consisting of a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose immobilized onto a flat, inert carrier sheet.
- A *liquid phase* consisting of the solution to be separated which is dissolved in an appropriate solvent and is drawn up the plate via capillary action, separating the solution based on the polarity of the components of the compound in question.

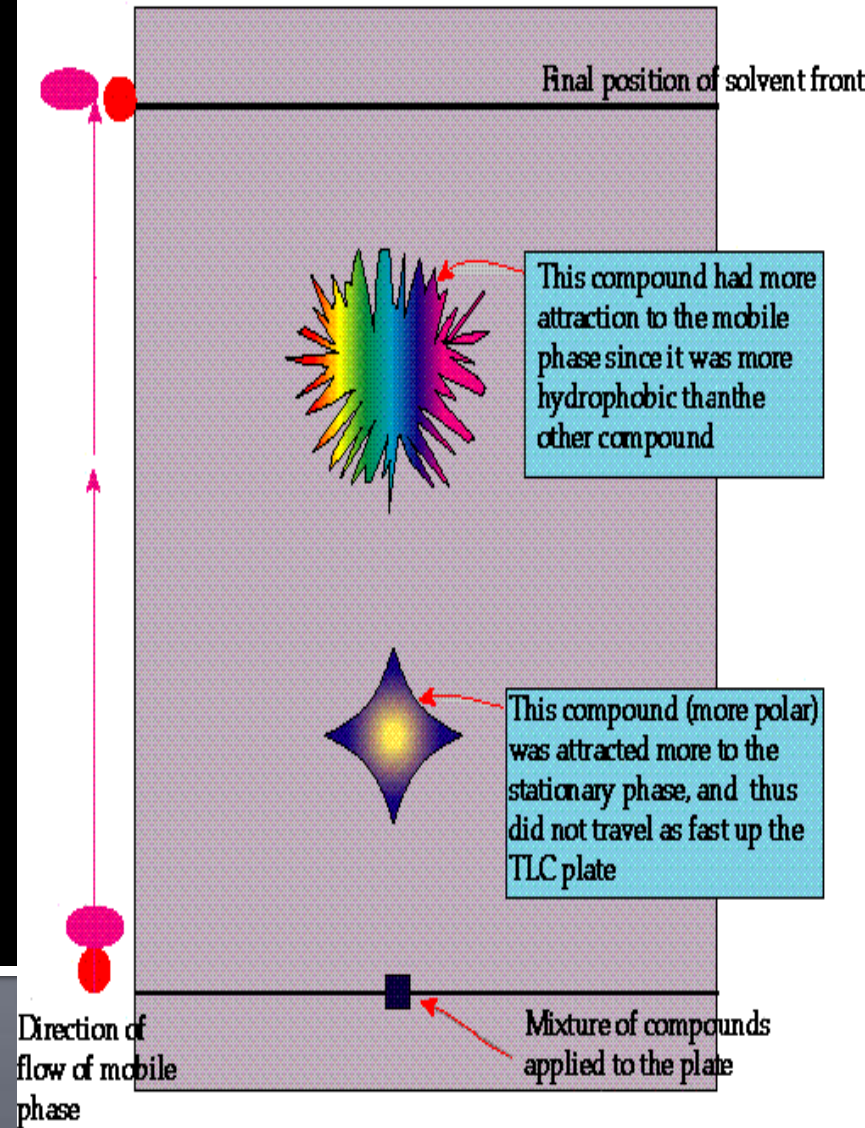
# TLC- Procedure

- The process is similar to **paper chromatography** with the advantage of faster runs, better separations, and the choice between different stationary phases.
- A small spot of solution containing the sample is applied to a plate, about one centimeter from the base. The plate is then dipped in to a suitable **solvent**, such as **hexane** or **ethyl acetate**, and placed in a sealed container. The **solvent** moves up the plate by **capillary action** and meets the sample mixture, which is dissolved and is carried up the plate by the solvent.
- Different **compounds** in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility in the solvent.

# TLC -Overview



# Thin Layer Chromatogram



The most non-polar compounds will travel further up the plate than the more polar compounds.

# Significance of TLC

Its wide range of uses include

- determination of the pigments a plant contains
- detection of pesticides or insecticides in food
- identifying compounds present in a given substance
- monitoring organic reaction

# Advantages of TLC over paper chromatography

- 1) In case of paper chromatography , it takes 14-16 hrs for the separation of the components, but in TLC, It takes only 3-4 hrs.
- 2) TLC has the advantage that the corrosive reagents like sulphuric acid can also be used which pose a limitation for the paper chromatography.
- 3) It is easier to separate and visualize the components by this method.
- 4) It has the capacity to analyze multiple samples in a single run.
- 5) It is relatively a low cost.

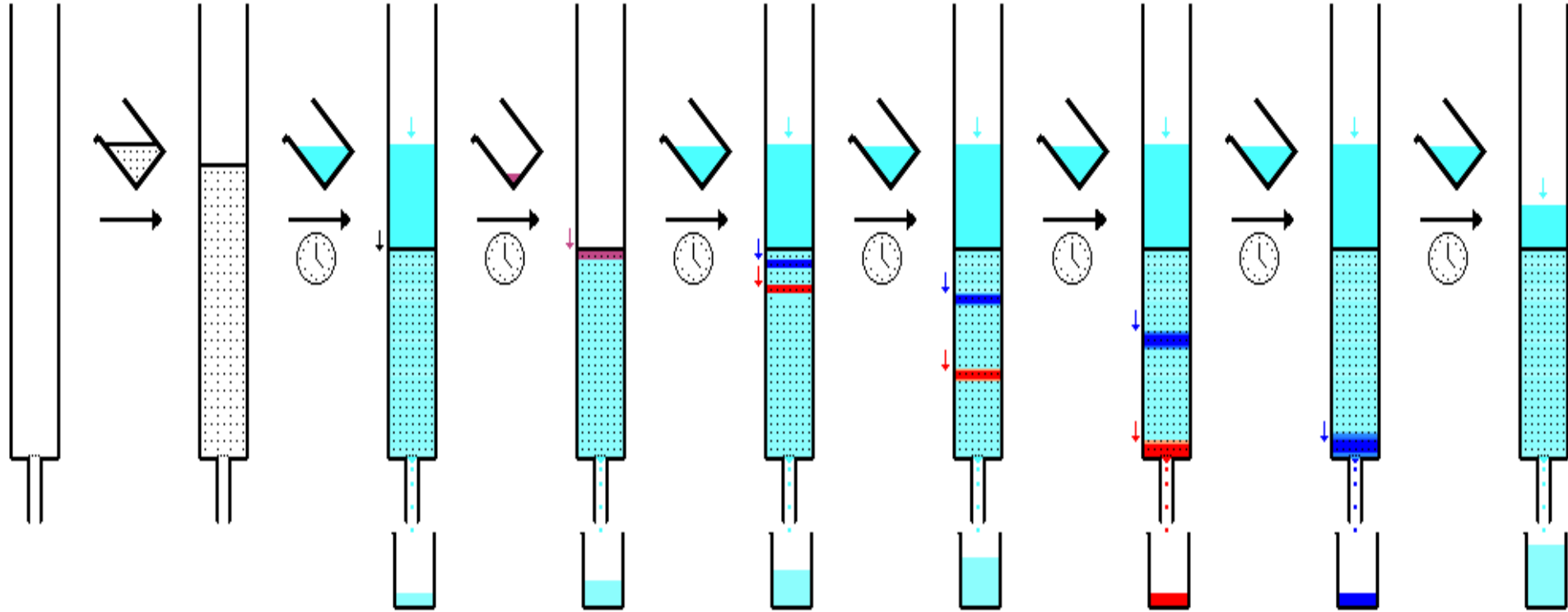
## 2) Adsorption chromatography

- In this technique the separation is based on differences in adsorption at the surface of the solid stationary medium.
- The adsorbents such as **silica gel, charcoal powder and calcium hydroxyapatite** are packed in to a column in a glass tube. This serves as the stationary phase.
- The sample mixture in a solvent is loaded on this column.
- The **individual components get differentially adsorbed** on to the adsorbent.

## 2) Adsorption chromatography- Elution

- The elution is carried out **by a buffer system (mobile phase)**. The most weakly held fraction moves fastest, followed by others, according to the order of tightness in adsorption.
- The **individual compounds come out of the column at different rates** which may be separately collected and identified.
- Amino acids may be identified by **Ninhydrin colorimetric method**.
- An Automated column chromatography apparatus- **fraction collector** is frequently used now a days.

# Adsorption chromatography



The substance loosely adsorbed to the stationary medium comes in the earlier fraction.



# Ion – Exchange chromatography

- 1) Ion-exchange chromatography (or *ion chromatography*) is a process that allows the separation of **ions** and **polar molecules** based on the charge properties of the molecules.
- 2) It can be used for almost any kind of charged molecule including large **proteins**, small **nucleotides** and **amino acids**.
- 3) The solution to be injected is usually called a **sample**, and the **individually separated components** are called **analytes**.
- 4) It is often used in protein purification, water analysis, and quality control.

# Ion – Exchange chromatography

- Ion exchange chromatography retains **analyte** molecules based on **coulombic** (ionic) interactions. The stationary phase surface 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**.

i) Cation exchange chromatography retains positively charged **cations** because the stationary phase displays a negatively charged functional group:

ii) Anion exchange chromatography retains **anions** using positively charged functional group:

# **Ion – Exchange chromatography- Procedure**

A mixture of amino acids or proteins can be conveniently separated by ion exchange chromatography. The amino acid mixture (at p H around 3.0 )is passed through a cation exchange and the individual amino acids can be eluted by using buffers of different p H. Suppose, a mixture of Arginine and Aspartic acid is passed through a cation exchange column. Arginine has extra positive charge and so adheres to the column. But negatively charged Aspartic acid molecules will not adhere and come out first from the column

# **Ion – Exchange chromatography- Procedure (Contd.)**

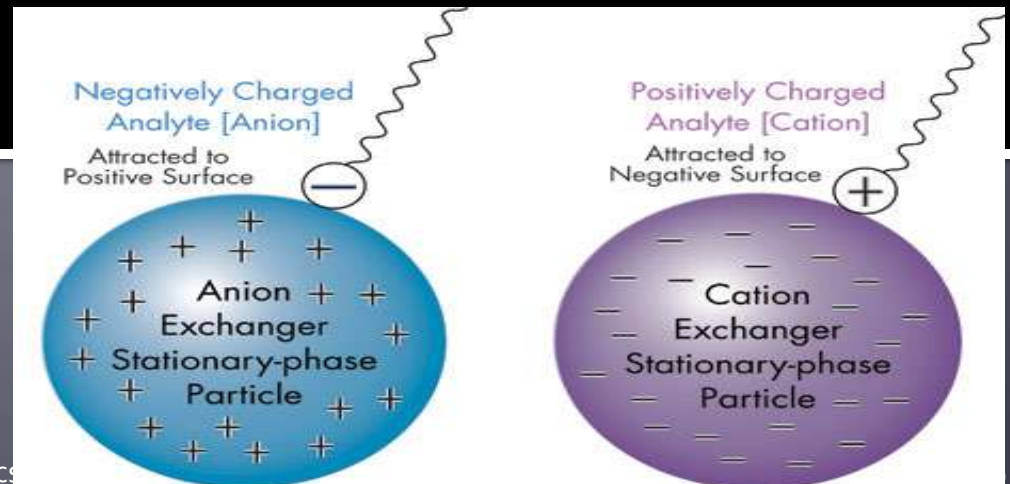
when weak NaOH is passed, the positive charge of Arginine is neutralized. Na<sup>+</sup> will replace Arginine in the column, thus Arginine is eluted finally. The various fractions eluted, containing individual amino acids, are allowed to react with Ninhydrin reagent to form colored complexes. This is continuously monitored for qualitative and quantitative identification of amino acids. The amino acid analyzer is based on this method.

# Types of ion exchanges resins

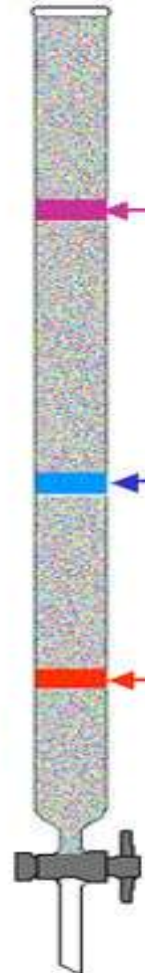
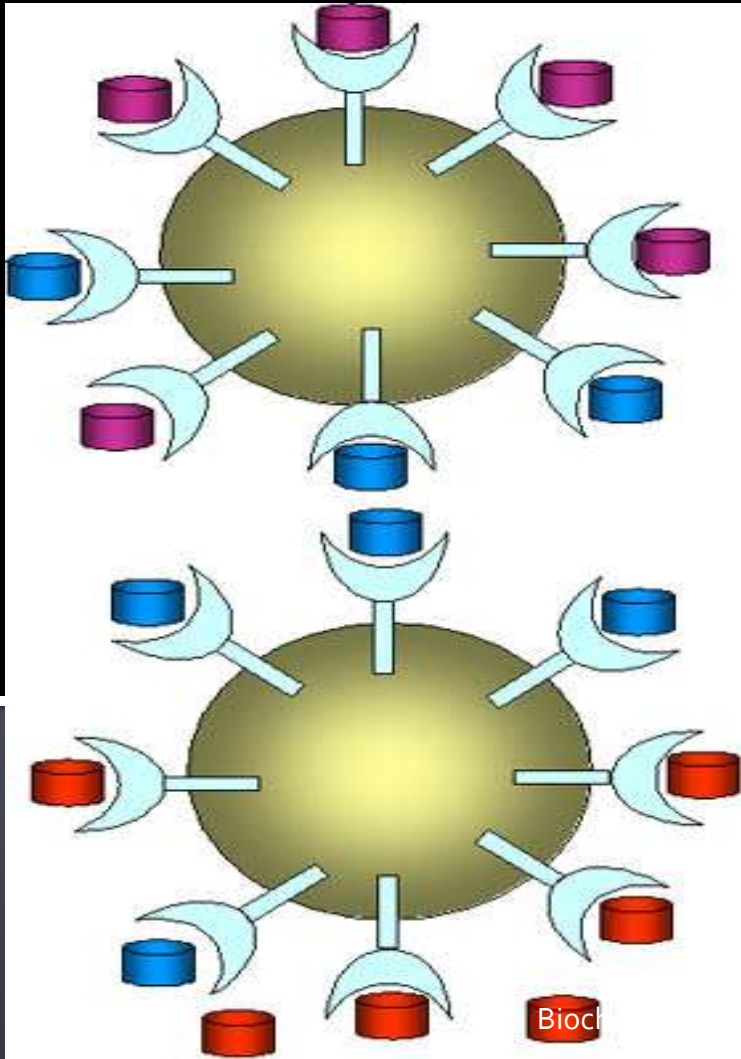
Various types of ion exchanges resins are commercially available-

**Cation Exchange resin-** Polystyrene sulfonate resins, CM-Sephadex gel, CM – cellulose, These bear acidic groups and immobilize cations from adjacent solutions.

**Anion exchangers-** DEAE cellulose, Trimethyl amino Polystyrene, DEAE- Sephadex. All these bear basic groups ionizing into fixed positions and immobilize anions from neighboring solutions.



# Ion – Exchange chromatography- An Overview



more highly charged molecules are more tightly bound to the resin, and so travel slowly and are eluted later

moderately charged molecules equilibrating between the resin and the moving buffer more readily

Less charged molecules bind less strongly to the resin, equilibrate with the moving buffer more readily, and so travel rapidly and are eluted sooner

## 4) Gel filtration chromatography or Molecular Sieve chromatography

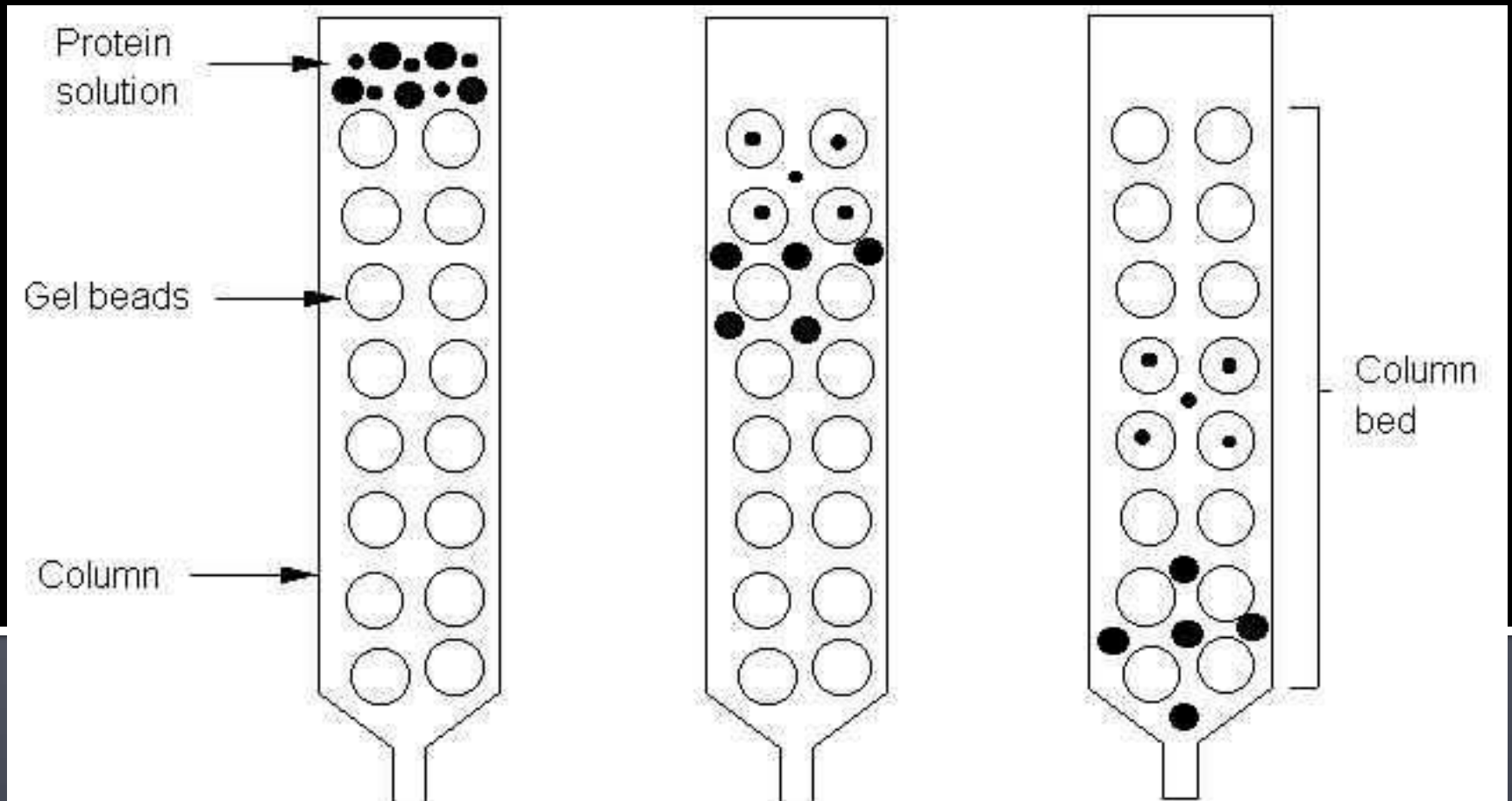
- This is extremely useful in separating ribosomes, viruses, nucleic acids and proteins depending on their particle sizes and shapes.
- In Gel filtration chromatography, the separation of the particles is based on their size, shape and molecular weight.
- This technique is also referred to as molecular exclusion chromatography.
- The apparatus consists of a column packed with sponge like gel beads( usually cross- linked polysaccharides) containing pores.
- The gels serve as molecular sieves for the separation of smaller and bigger molecules.

# Gel filtration chromatography or Molecular Sieve chromatography

- The solution mixture containing molecules of different sizes( say protein) is applied to the column and eluted with a buffer.
- The larger molecules can not pass through the pores of a gel and therefore move faster.
- The smaller molecules enter the gel beads and are left behind which come out slowly. By selecting the gel beads of different porosity, the molecules can be separated.
- The gel filtration chromatography can be used for an approximate determination of molecular weights.
- This is done by using a calibrated column with substances of known molecular weights.



# Gel filtration chromatography-An Overview



Larger particles come out first, while smaller particles come in later fractions

# 5) Affinity chromatography

-The principle of affinity chromatography is based on the property of specific and non-covalent binding of proteins to other molecules, referred to as substrates or cofactors.

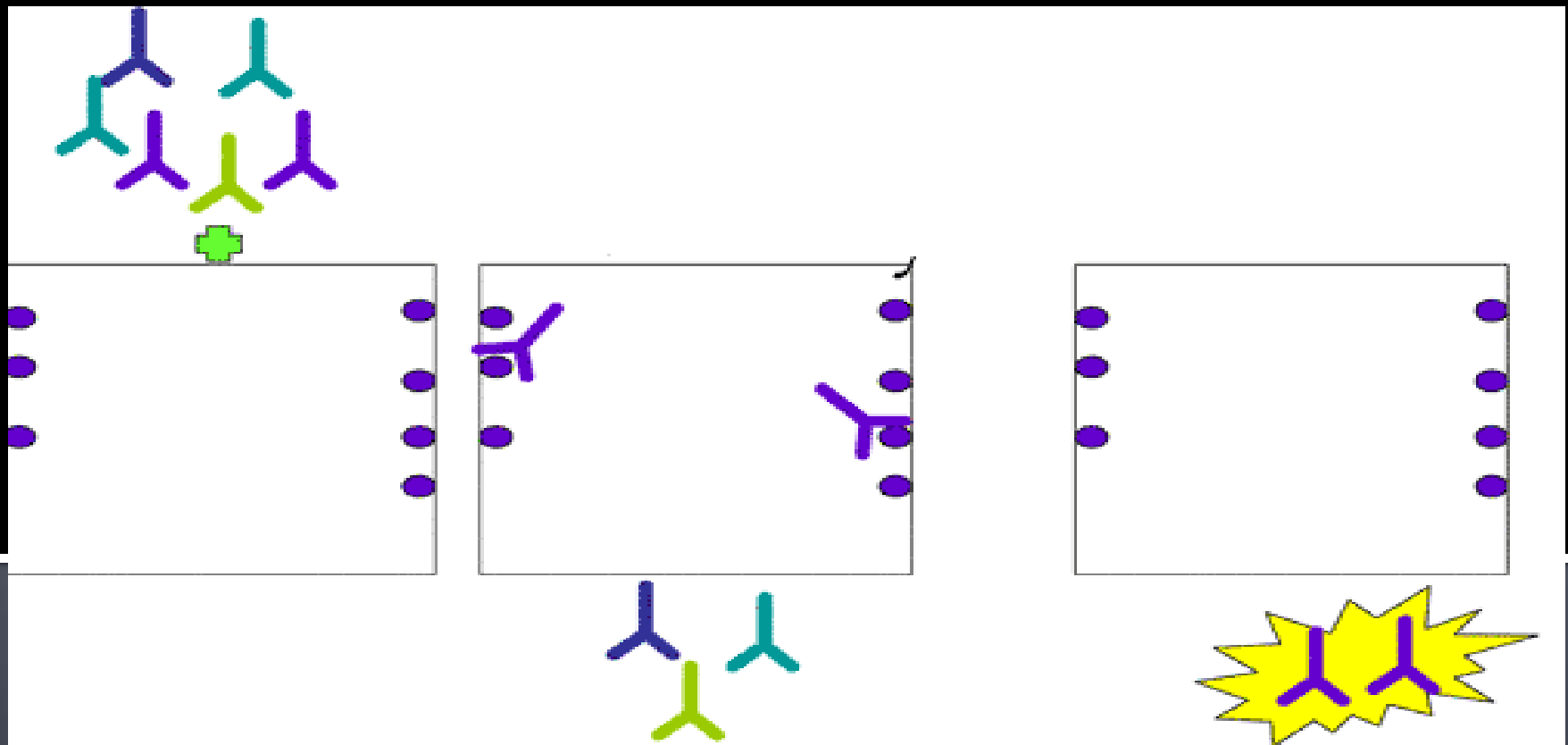
The technique involves the use of ligands covalently attached to an inert and porous matrix in a column. The immobilized ligands act as molecular hooks to selectively pick up the desired protein while the remaining proteins pass through the column. The desired protein captured by the ligands, can be eluted by using free ligand molecules. Alternatively, some reagents that can break protein ligand interactions can also be employed for the separation.

# Affinity chromatography- Significance

- 1) Affinity chromatography is useful for the purification of enzymes, vitamins, nucleic acids, drugs, hormone receptors, antibodies etc.
- 2) It is also widely used for the estimation of Glycated Hb. Normal Hb does not bind and comes out first, while glycated Hb binds with the Boronic acid used as a ligand. Sorbitol is then added to elute the Glycated Hb which can be quantitated then.

# Affinity chromatography- Significance

By using antibodies, antigens can be easily separated. Conversely, antibodies can be purified by passing through a column containing the antigen.



Specific Antibody binds to a specific antigen attached to the stationary medium, the remaining antibodies come out in earlier fractions.

## 6) High performance liquid chromatography-

Chromatographic techniques are slow and time consuming. The separation can be greatly improved by applying high pressure in the range of 5000-10,000 pounds per square inch, hence this technique is also referred to as **high pressure liquid chromatography**.

**HPLC requires the use of non compressible resin materials and strong metal columns. The eluents of the columns are detected by methods such as UVabsorption and fluorescence. It can be applied in the form of partition, adsorption, ion exchange or molecular sieve chromatography.**

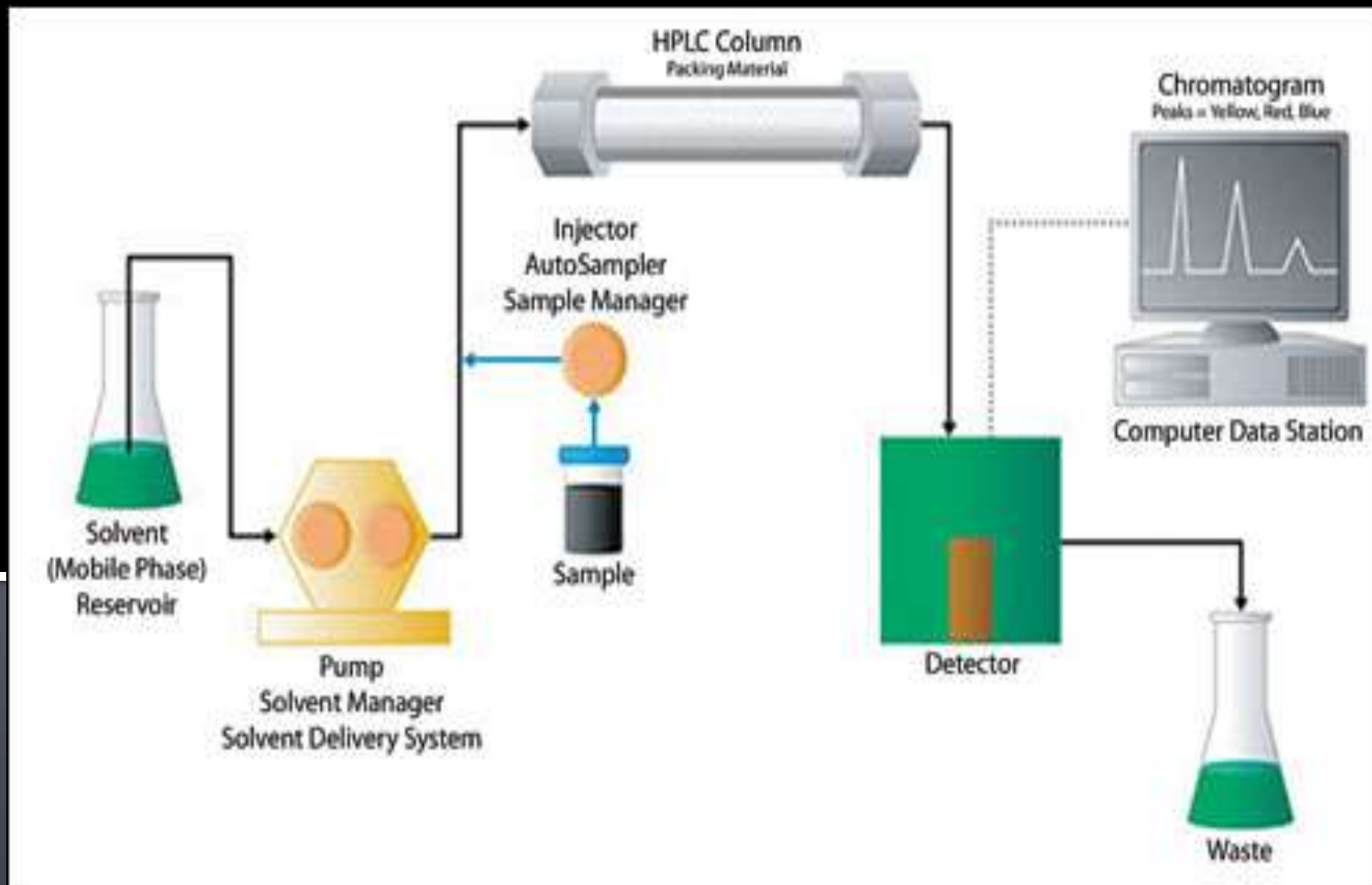
# High performance liquid chromatography-

- 1) **The stationary phase**- consists of an immobilized thin layer of a liquid on the micro glass or plastic beads, tightly packed in to a narrow column.
- 2) **The mobile phase** -consists of a buffered solvent system which is passed under high pressure through the column for eluting the solutes of the sample.

# High performance liquid chromatography- Significance

Due to rapidity in action it is used for assaying amino acids, peptides, proteins, carbohydrates, lipids, nucleic acids and related compounds, vitamins, hormones, metabolites and drugs such as antiarrhythmics, antibiotics, antiepileptics, analgesics, bronchial smooth muscle relaxants and anti-depressants.

# High performance liquid chromatography- Apparatus





## 6) Gas liquid chromatography-

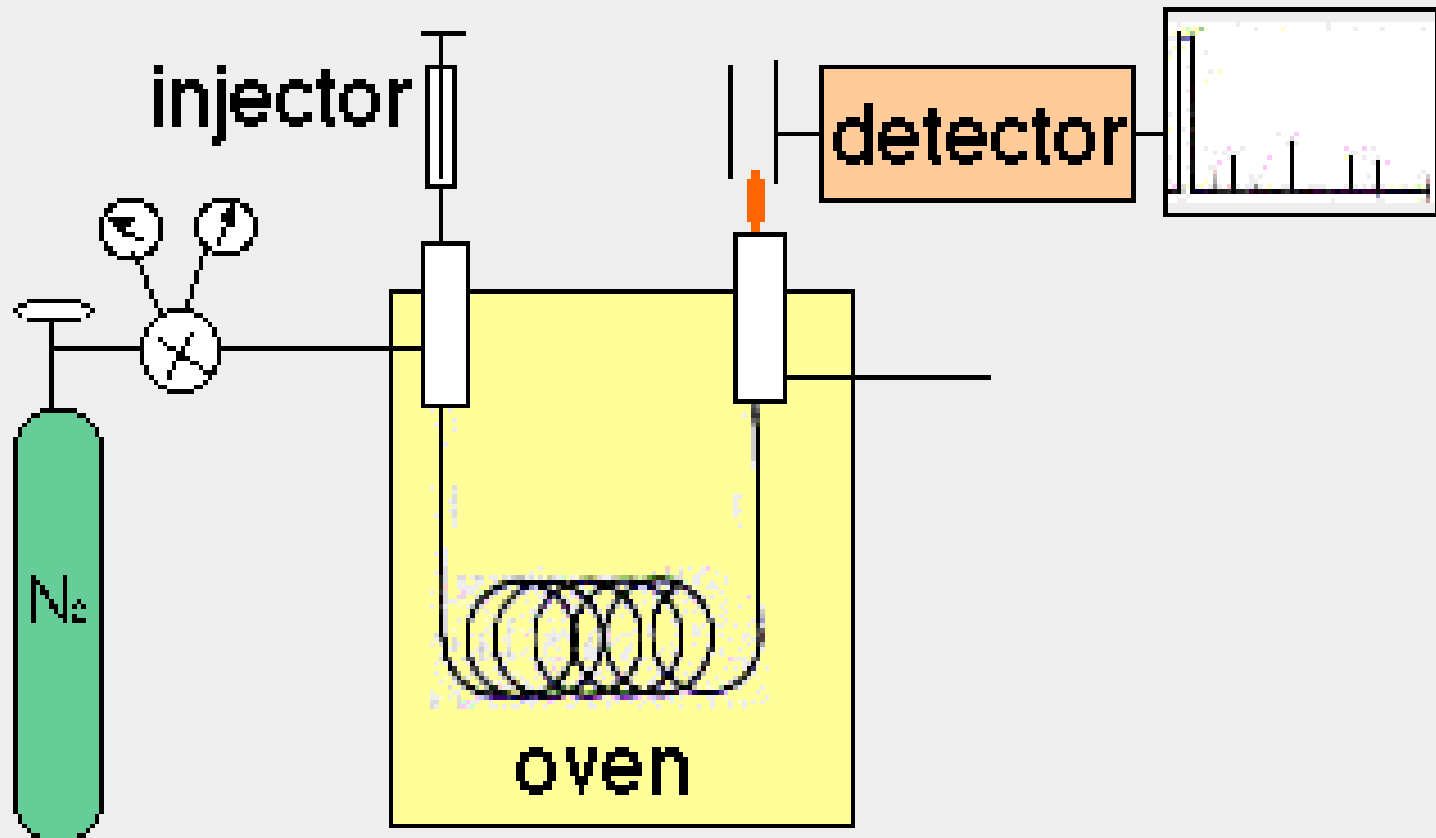
- The method of choice for the separation of volatile substances or volatile derivatives of certain in volatile substances.
- In GLC. the stationary phase is an inert solid material(diatomaceous earth or powdered firebrick), impregnated with a non volatile liquid(silicon or polyethylene glycol).
- This is packed in a narrow column and maintained at high temperature (around 200 degree C).
- A mixture of volatile material is injected in to the column along with the mobile phase, which is an inert gas (argon, helium or nitrogen).

# Gas liquid chromatography-

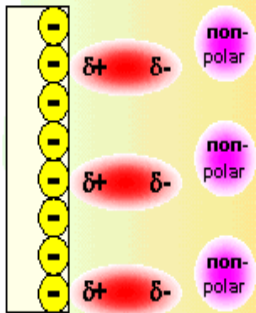
-The separation of the volatile material is based on the partition of the components between the mobile phase(gas) and stationary phase( liquid), hence the name gas liquid chromatography.

-The separated compounds can be identified and quantitated by a detector. Gas liquid chromatography is sensitive, rapid and reliable. It is **frequently used for the quantitative estimations of biological materials such as lipids, drugs and vitamins.**

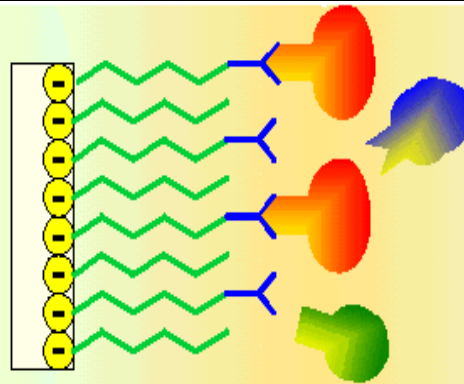
# Gas liquid chromatography-Apparatus



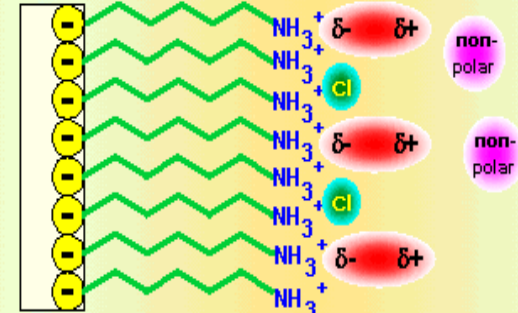
# Summary



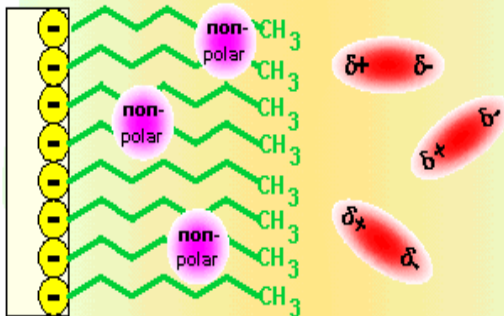
Adsorption Chromatography



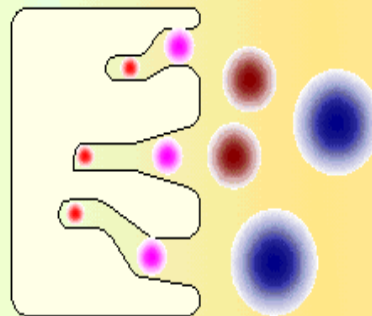
Affinity Chromatography  
( covalent binding )



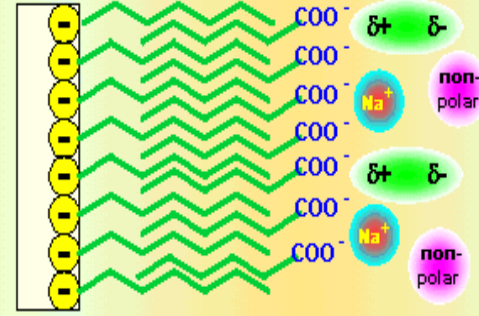
Ion-Exchange Chromatography  
( cation or anion resins )



Partition Chromatography  
( reversed phase )



Molecular Exclusion Chromatography  
( gel filtration, gel permeation,  
molecular sieve )



Ion-Pairing Chromatography  
( liquid cation or anion resins )