

CHROMATOGRAPY & ITS TYPES



1

Dr. Shabana Naz Shah
PhD. in Pharmaceutical Chemistry
Sr. Manager Technical Services
Martin Dow Limited

CHROMATOGRAPHY



- Is a technique used to separate and identify the components of a mixture.
- Works by allowing the molecules present in the mixture to distribute themselves between a stationary and a mobile medium.
- Molecules that spend most of their time in the mobile phase are carried along faster.
- Chroma - "color" and graphein - "to write".
- Colour bands - separation of individual compounds

HISTORY



Chromatography

(from Greek :*chromatos* -- color , "graphein" -- to write)

- 1903 Tswett - plant pigments separated on chalk columns
- 1931 Lederer & Kuhn - LC of carotenoids
- 1938 TLC and ion exchange
- 1950 Reverse phase LC
- 1954 Martin & Synge (Nobel Prize)
- 1959 Gel permeation
- 1965 instrumental LC (Waters)

PURPOSE OF CHROMATOGRAPHY

- • **Analytical**

- Determine Chemical composition of a sample

- **Preparative**

- Used to purify sufficient quantities of a substance



CHROMATOGRAPHY TERMS

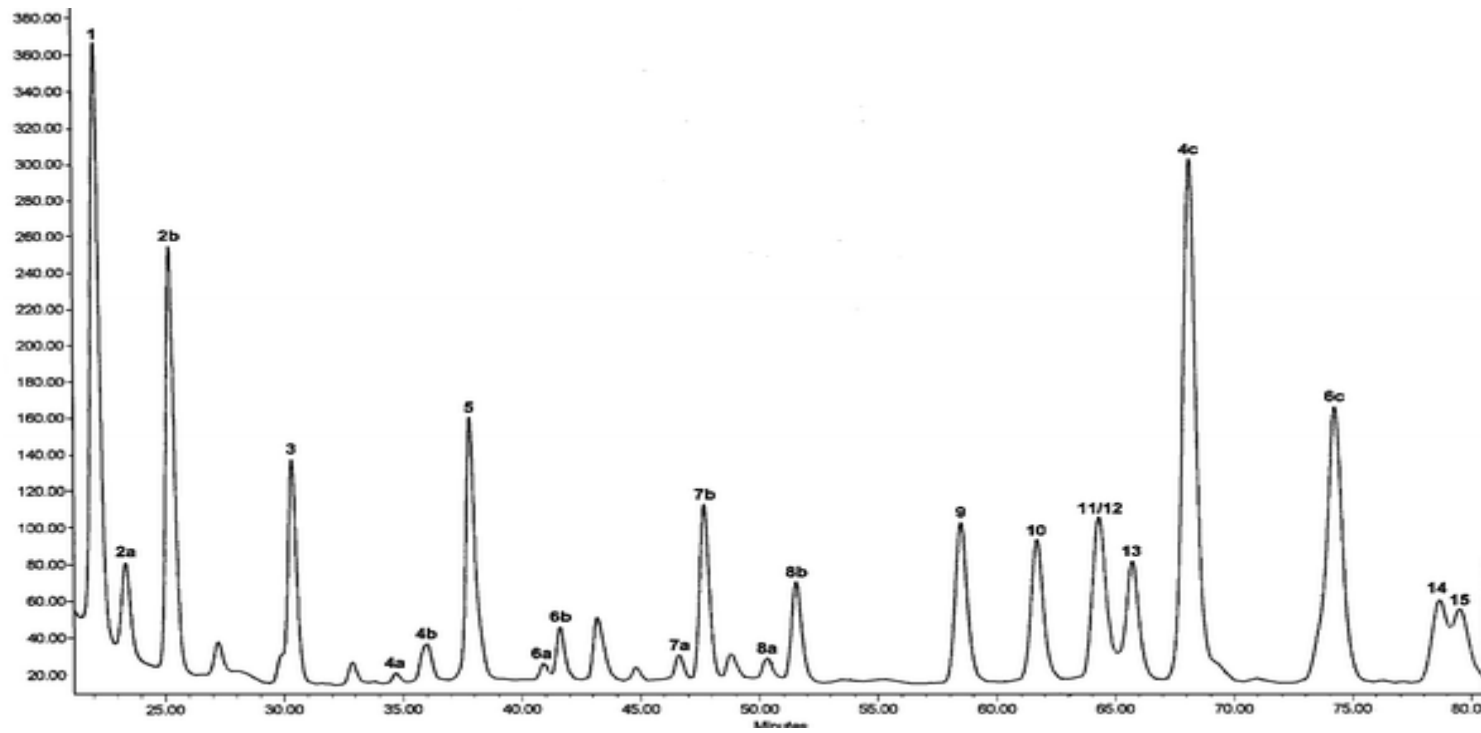
- **Chromatograph** - equipment that enables a sophisticated separation EX. Gas chromatography or Liquid chromatography
- **Eluent** - Fluid entering column/ solvent that carries the analyte.
- **Eluate** - Mobile phase leaving the column.
- **Stationary phase - Immobilized phase**
 - Immobilized on the support particles or on the inner wall of the column tubing.
 - Examples : Silica layer - Thin Layer Chromatography

CHROMATOGRAPHY TERMS

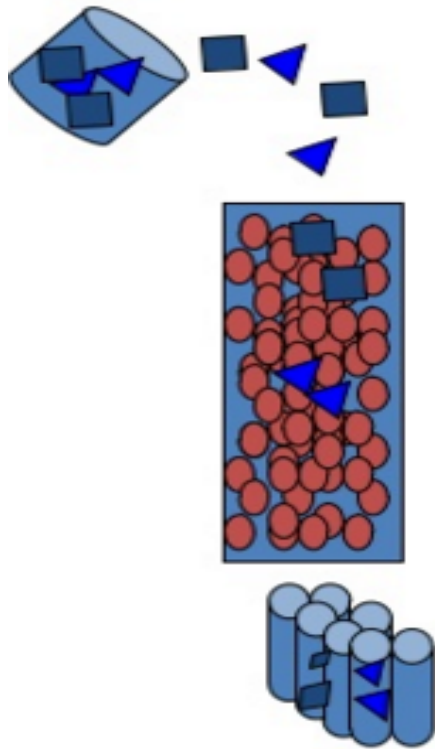
- **Mobile phase** - Moves in a definite direction. Liquid (LC), Gas (GC). The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
- **Retention time:** Time takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.
- **Sample (Analyte) :** Substance analyzed in chromatography.
- **Solvent:** Any substance capable of solubilizing another substance.

CHROMATOGRAM

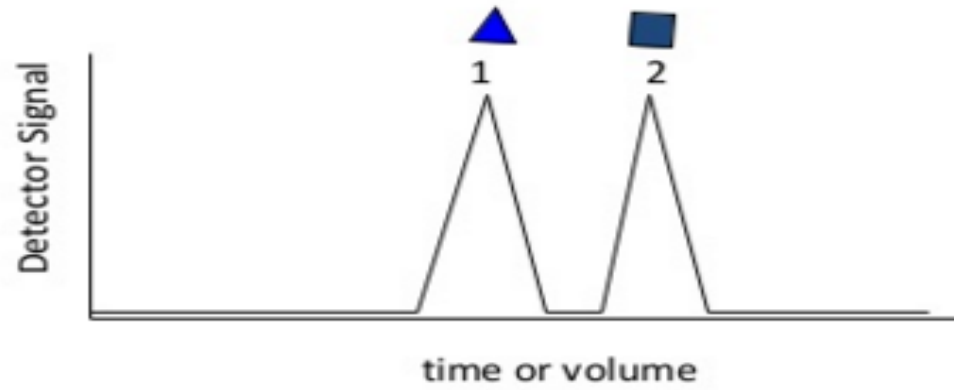
- Visual output of the chromatograph.
- Separation - Different peaks or patterns on the chromatogram correspond to different components of the separated mixture.



CHROMATOGRAM



Chromatogram - Detector signal vs. retention time or volume



HOW TO DESCRIBE A CHROMATOGRAM

- **Chromatogram** – Response of a detector **vs** time.
Shows when various components come off a column.
- **Retention time (t_1)** – The time at which a component elutes from a column.

- **Theoretical Plates:**

Assume a chromatographic peak has a Gaussian shape

H is height of peak $W_{1/2}$ is width at $1/2$ Height

(If true Gaussian $W_{1/2} = 2.35\sigma$

Where σ = standard deviation)

Width at baseline should be 4σ

HOW TO DESCRIBE A CHROMATOGRAM

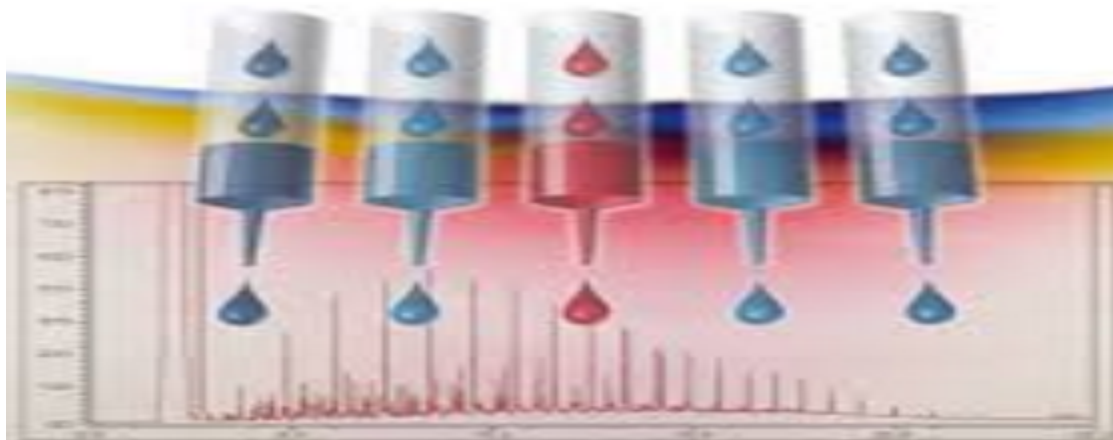
- For chromatography we retain the name Theoretical Plate, but calculate it using the retention time and the width at 1/2 height.

$$N = \frac{555t_r^2}{W_{1/2}^2}$$

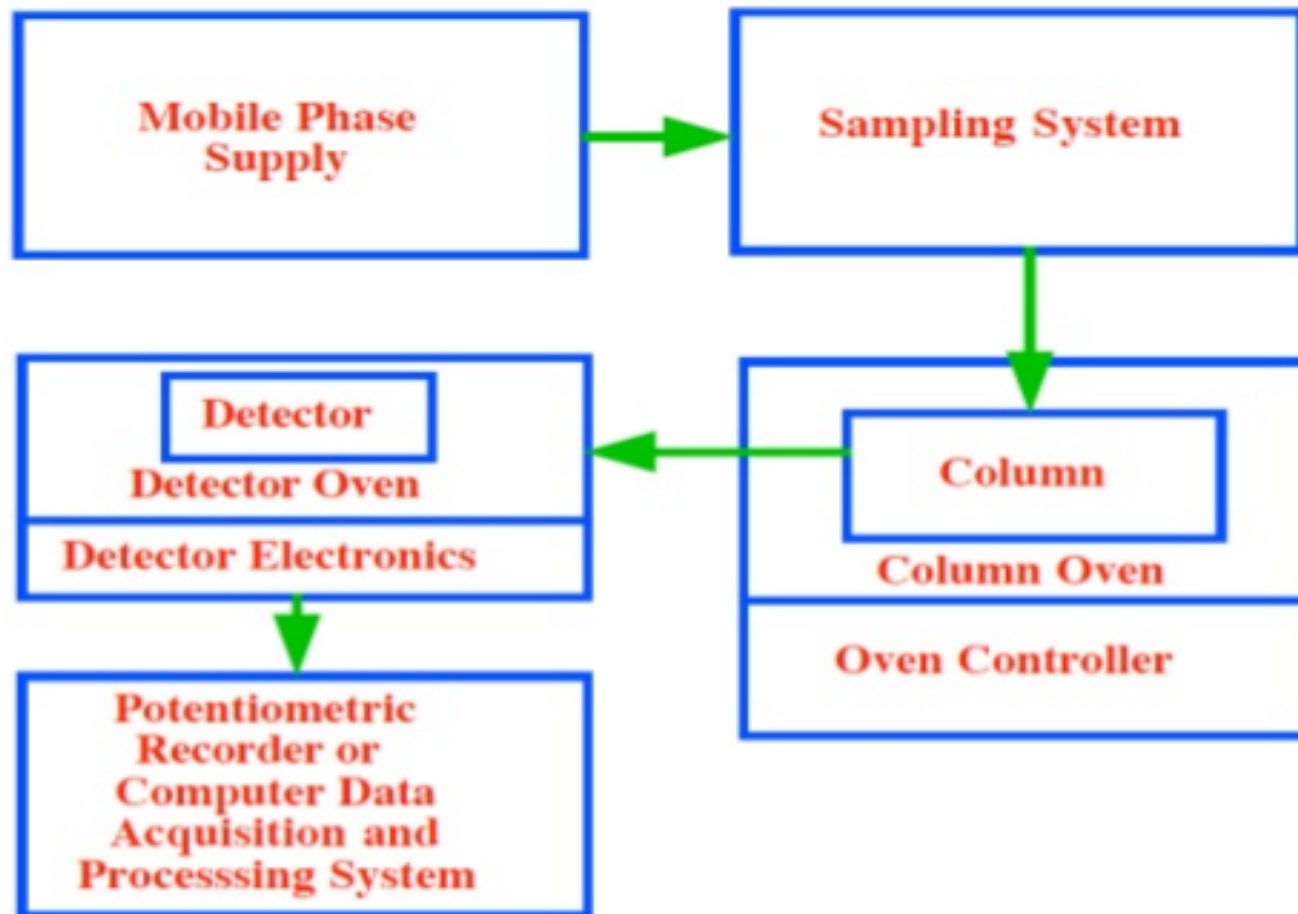
- N is number of Theoretical Plate. Again the bigger the N the better, because that means the width of the peak is small compared to its retention time.

PRICNIPLS OF CHROMATOGRAPGHY

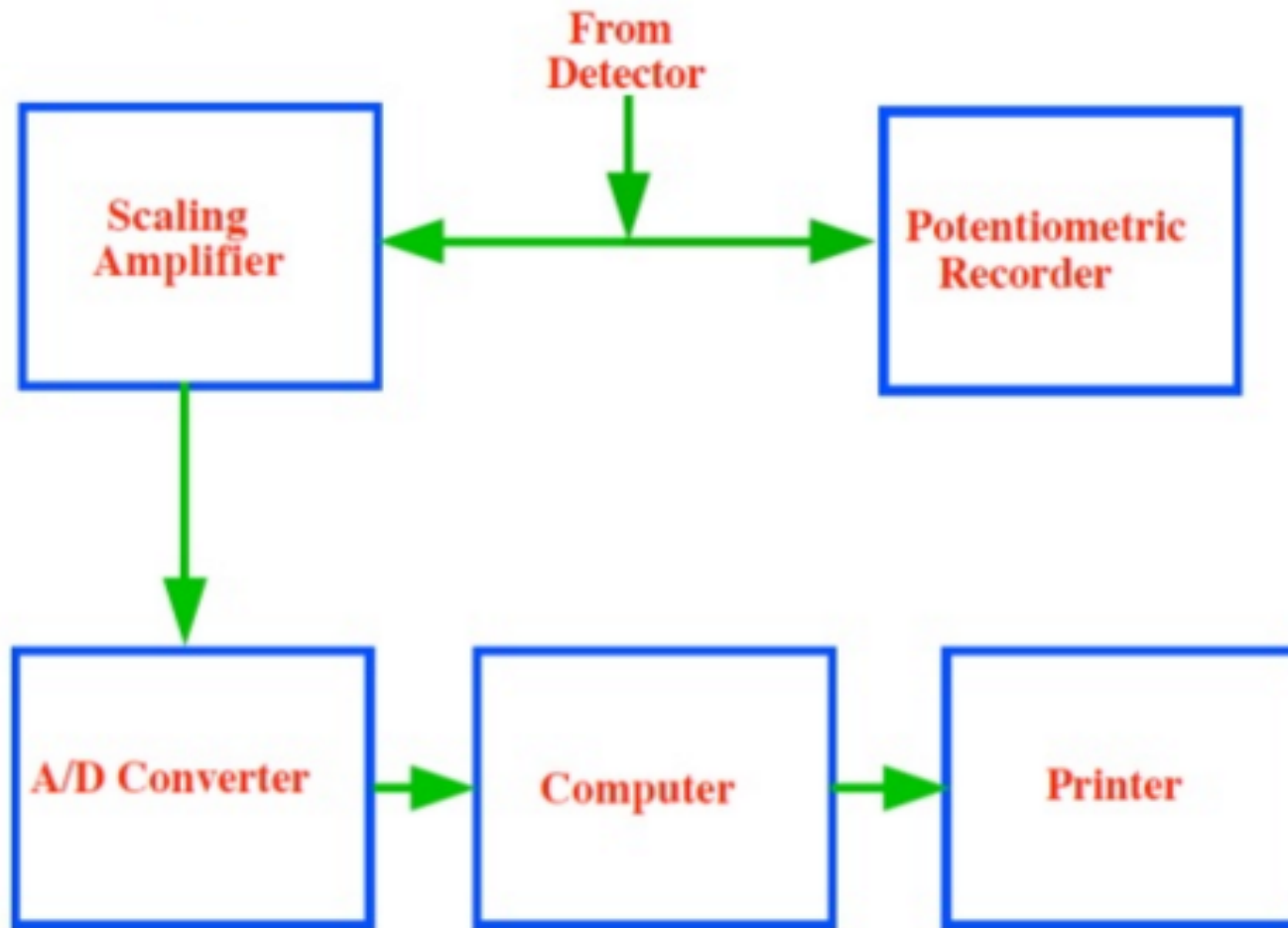
- Physical method of separation that distributes components to separate between two phases moves in a definite direction.
- Substances are separated based on their differential distribution between two phases.
- Substances will move with the mobile phase at different rate depending upon their **Partition or Distribution co- efficients.**



PRICNIPLS OF CHROMATOGRAPGHY

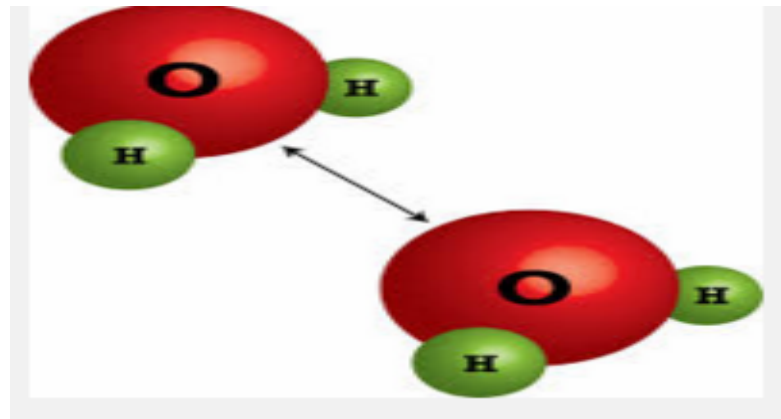


PRICNIPLS OF CHROMATOGRAPGHY



FACTORS AFFECTING THE SEPARATION

- Intermolecular interaction between the two phases.
- Extent of dispersion of solute molecules over the stationary phase.



USES FOR CHROMATOGRAPHY

- **Real-life examples of uses for chromatography:**
- **Pharmaceutical Company** – determine amount of each chemical found in new product
- **Hospital** – detect blood or alcohol levels in a patient's blood stream
- **Law Enforcement** – to compare a sample found at a crime scene to samples from suspects
- **Environmental Agency** – determine the level of pollutants in the water supply
- **Manufacturing Plant** – to purify a chemical needed to make a product

CLASSIFICATION OF CHROMATOGRAPHY

There are two classification schemes:

- ➡ Mobile phase
- ➡ Attractive forces



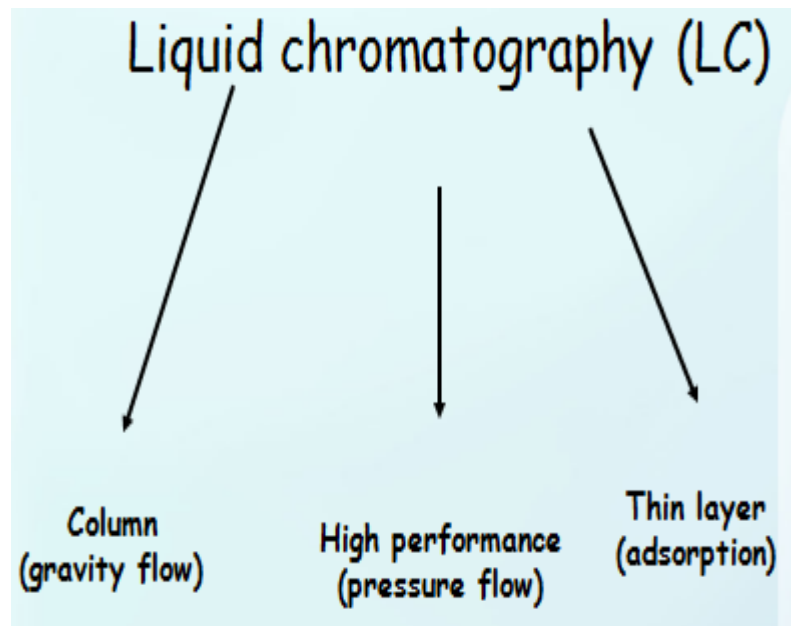
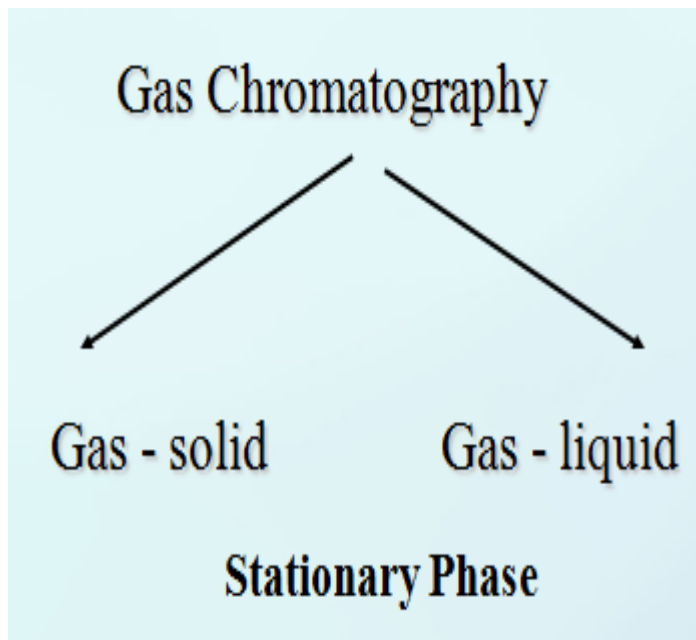
CLASSIFICATION OF CHROMATOGRAPHY

Mobile Phase:

- ✦ Gas (GC)
- ✦ Water (LC)
- ✦ Organic solvent (LC)
- ✦ Supercritical fluid (SCFC)



CLASSIFICATION BASED ON MOBILE PHASE



CLASSIFICATION BASED ON ATTRACTIVE FORCES

1. Adsorption
2. Ion Exchange
3. Partition
4. Size Exclusion

CLASSIFICATION OF CHROMATOGRAPHY

■ **Techniques by Chromatographic bed shape.**

- Column chromatography
- Planar chromatography
 - Paper chromatography
 - Thin layer chromatography

■ **Techniques by Physical state of mobile phase.**

- Gas chromatography
- Liquid chromatography

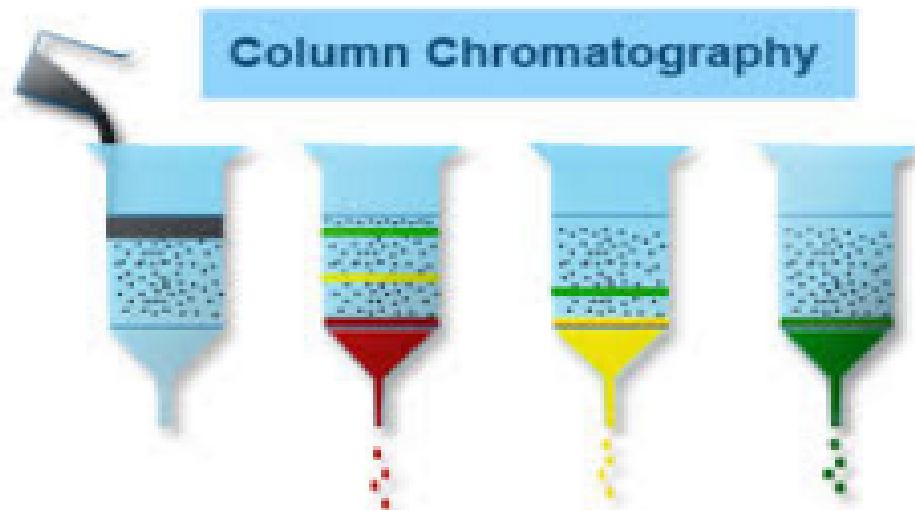
■ **Affinity chromatography**

- Supercritical fluid chromatography

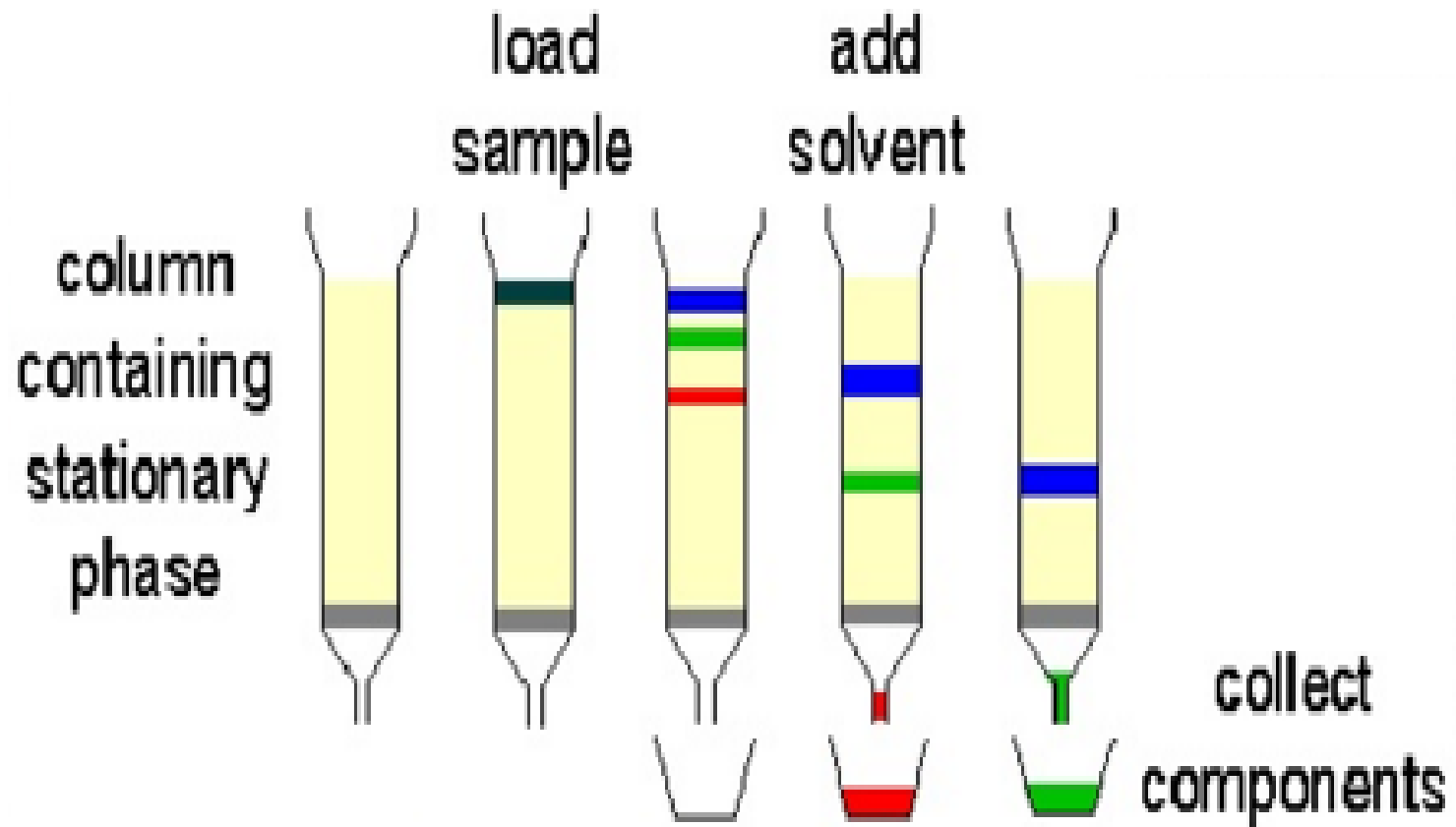
COLUMN CHROMATOGRAPHY

■ Column chromatography involves the following:

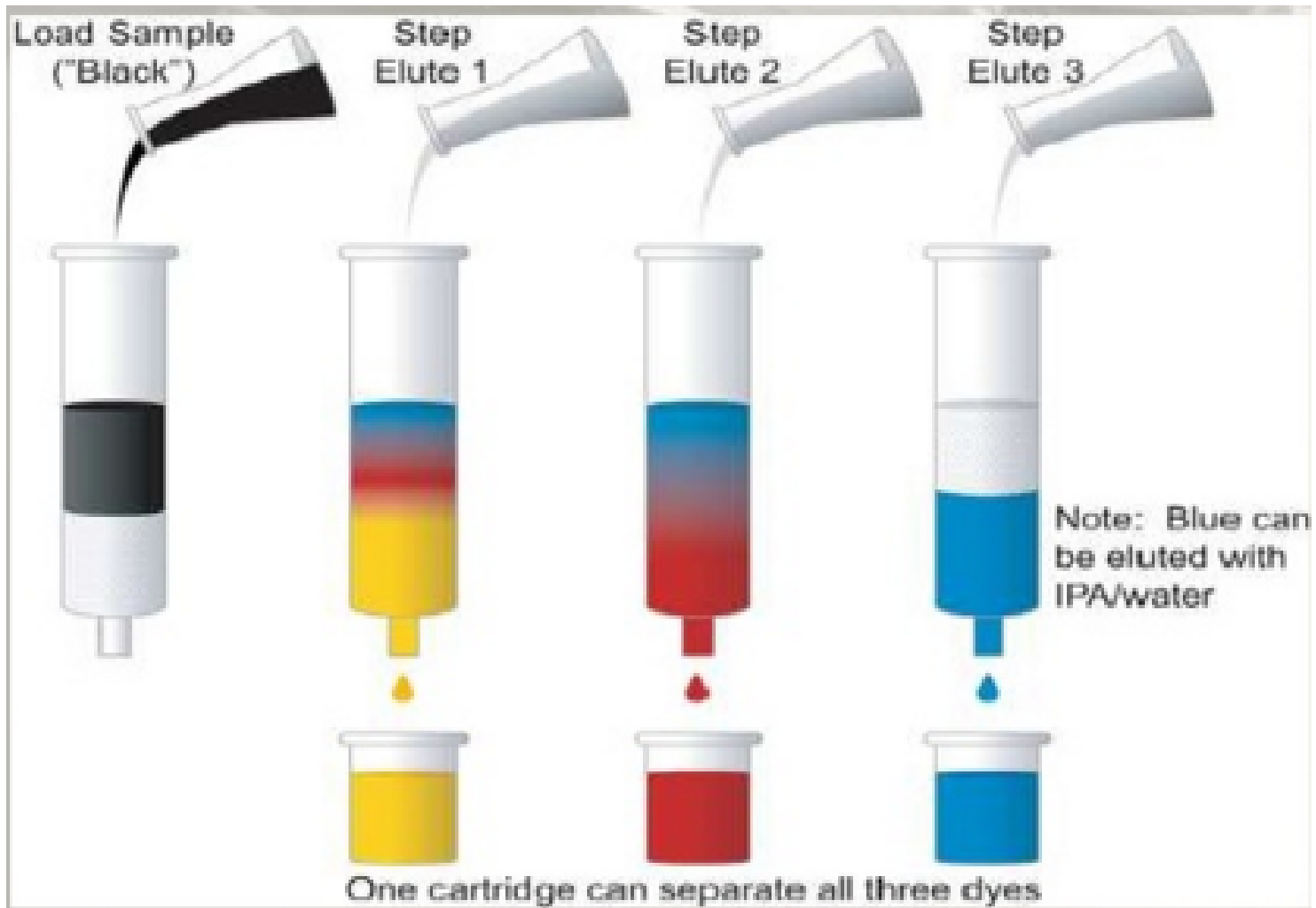
1. Adsorption/retention of substance on stationary phase
2. Separation of adsorbed substance using mobile phase.
3. Recovery of individual components by continuous flow of mobile phase.
4. Quantitative and qualitative analysis of solute and the components which are recovered.



COLUMN CHROMATOGRAPHY



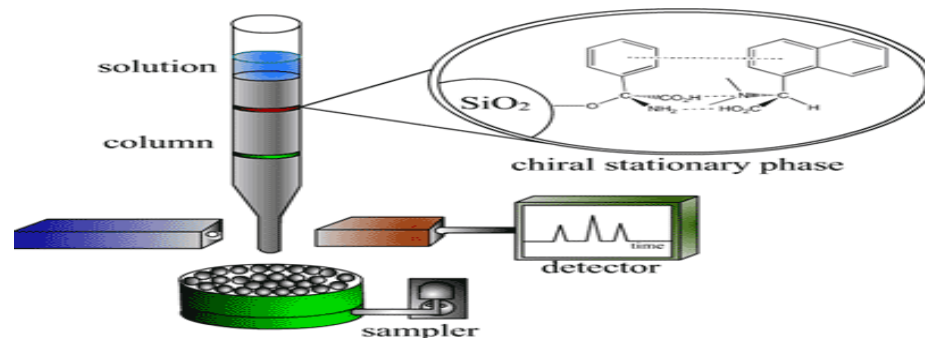
COLUMN CHROMATOGRAPHY



PLANAR CHROMATOGRAPHY

- ✚ Separation technique - Stationary phase is present as or on a plane.
- ✚ Paper – Paper Chromatography.
- ✚ Layer of solid particles spread on a support such as a glass plate - Thin layer Chromatography.
- ✚ Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase.

✚ Retention factor (R_f).



PAPER CHROMATOGRAPHY



PRINCIPLE:

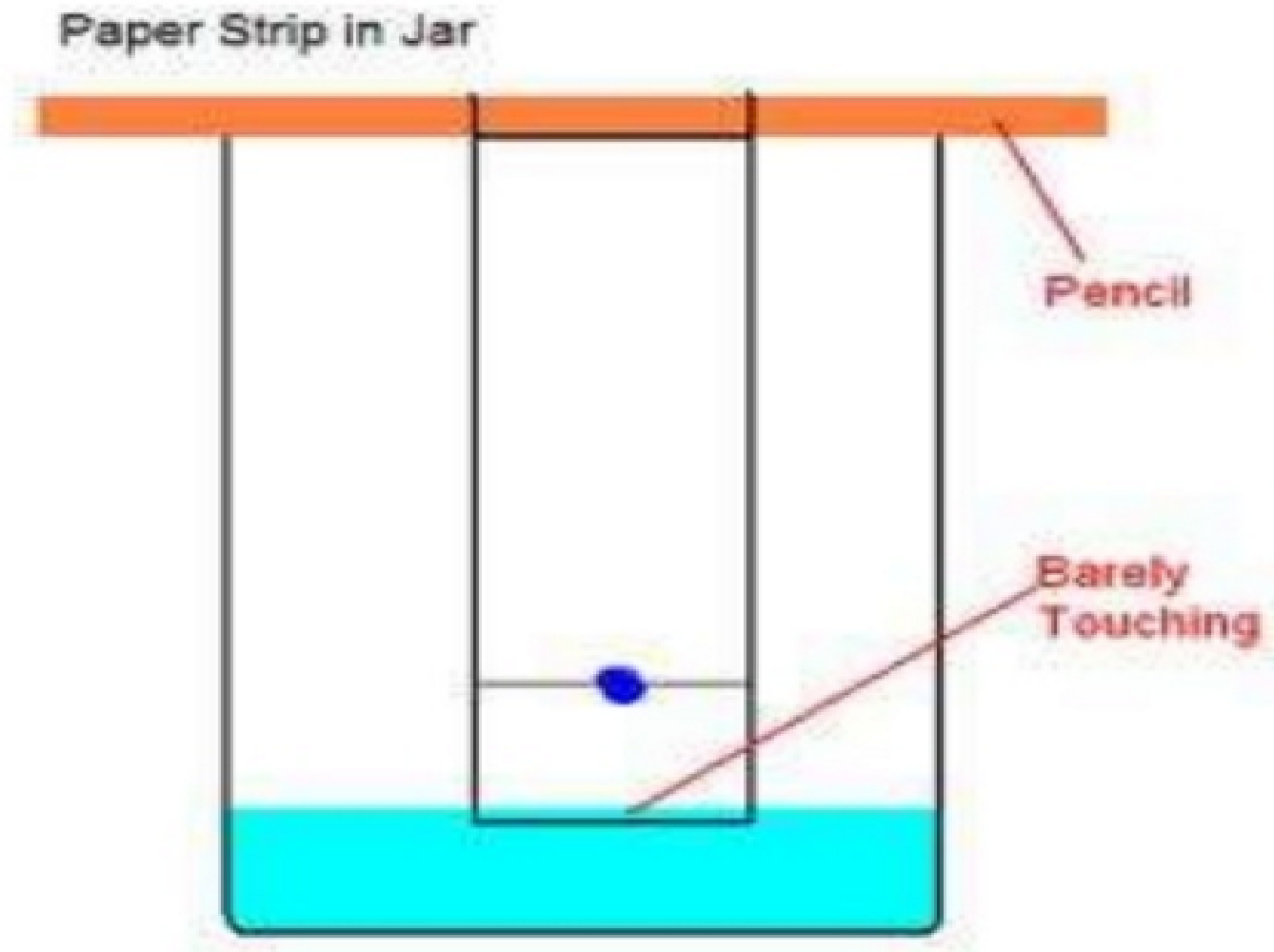
- ✚ This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non- polar.
- ✚ More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.
- ✚ • Retention factor :

$$R_f = \frac{\text{Distance travelled by a Solute}}{\text{Distance travelled by a Solvent}}$$

$R_f = \text{zero}$, - Solute remains in the stationary phase and thus it is immobile.

$R_f = 1$ - Solute has no affinity for the stationary phase and travels with the solvent front.

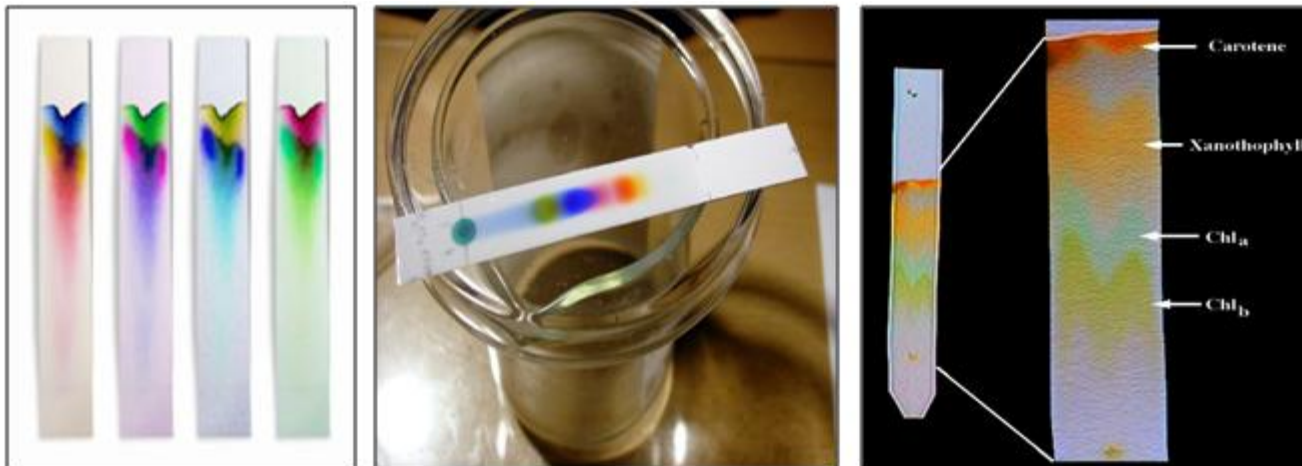
PAPER CHROMATOGRAPHY



THIN LAYER CHROMATOGRAPHY

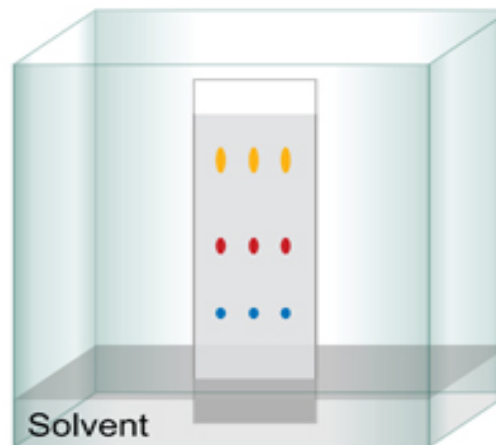
- Widely employed laboratory technique.
- Stationary phase - Adsorbent - Silica gel, Alumina, Cellulose.
- Widely used in pharmaceutical & food stuff industry

- Advantages :
 - ❖ Simple, Rapid and Cheap
 - ❖ Faster runs.
 - ❖ Better separations.
 - ❖ Choice between different adsorbents.
 - ❖ Better resolution
 - ❖ Allow for quantification

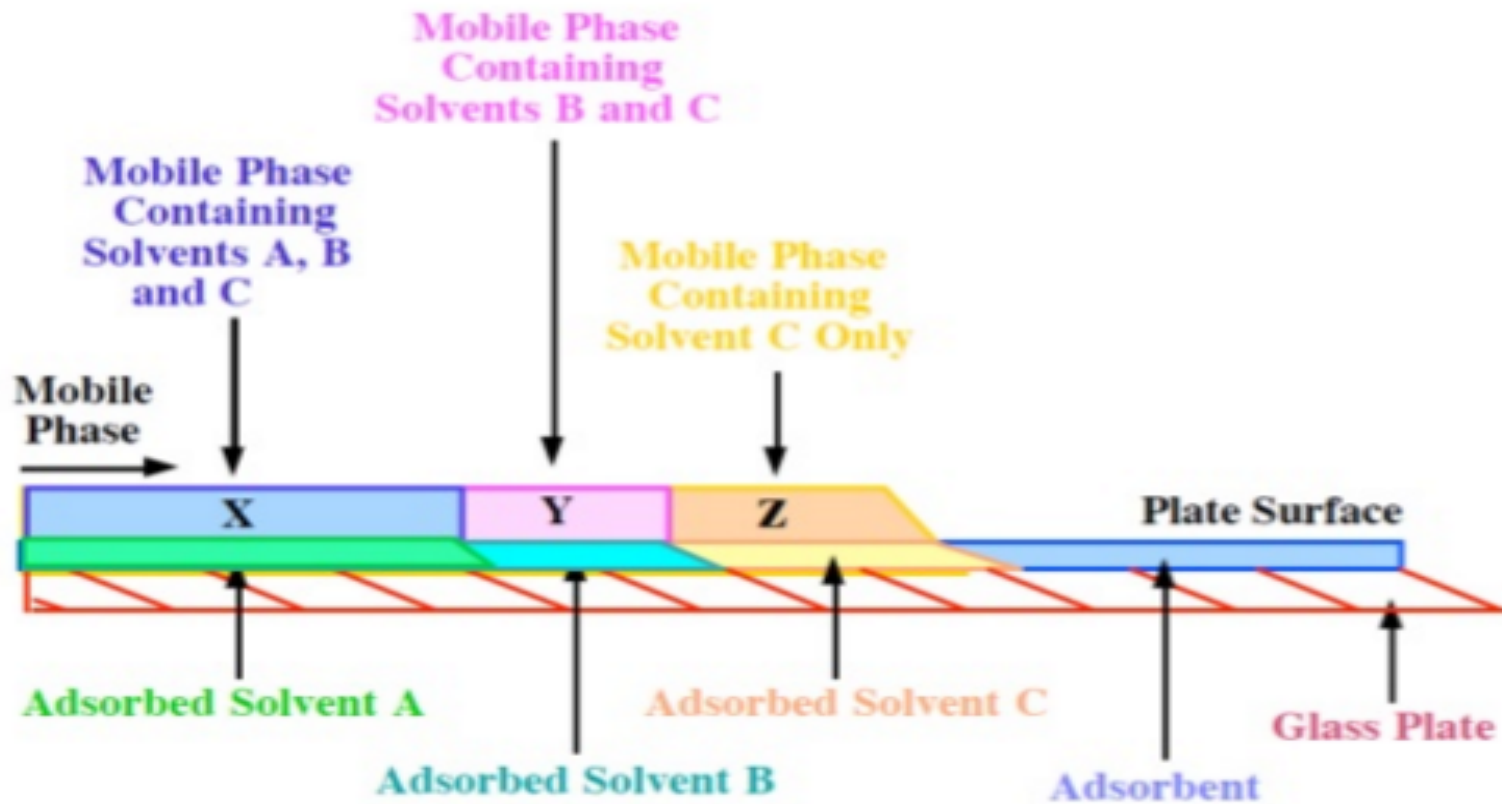


THIN LAYER CHROMATOGRAPHY

- Used to identify the unknown compounds and to determine the purity of mixture.
- TLC Plate - Aluminium or glass - coated by stationary phase.
- Coated material : 0.1-0.3mm in thickness.
- Fluorescent indicator that will make it fluorescence during the UV light exposure.
- STATIONARY PHASE: Silica gel, Alumina, or Cellulose on a flat, inert substrate.
- MOBILE PHASE: Volatile Organic solvents



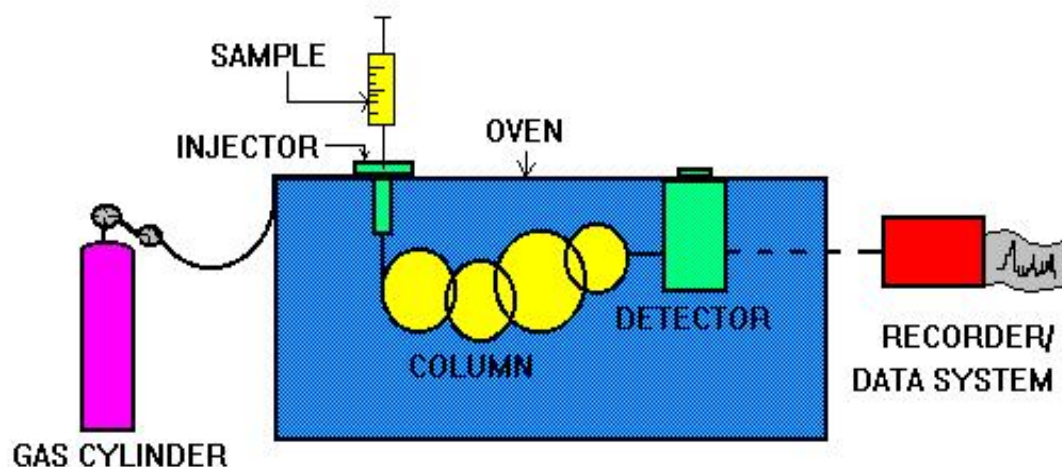
THIN LAYER CHROMATOGRAPHY



The Development of a Thin Layer Plate

GAS CHROMATOGRAPHY

- ❖ Gas-Liquid chromatography, (GLC).
- ❖ Mobile phase – Gas (Helium) Carrier Gas Pressure = 4 kg/cm²
- ❖ Stationary phase - Column, which is typically "packed" or "capillary".
- ❖ The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column).
- ❖ Partition Coefficient of Volatile analyte between a solid stationary phase (Silicone) and a mobile gas (Helium).



GAS CHROMATOGRAPHY

Advantages:

- High sensitivity.
- High Resolution.
- High speed.
- High Accuracy.
- Highly Quantitative.

APPARATUS:

Gas Chromatograph, GC analyzer, Normal syringes and one micro syringe, Beakers, Sample bottles and Electronic weight.

CHEMICALS:

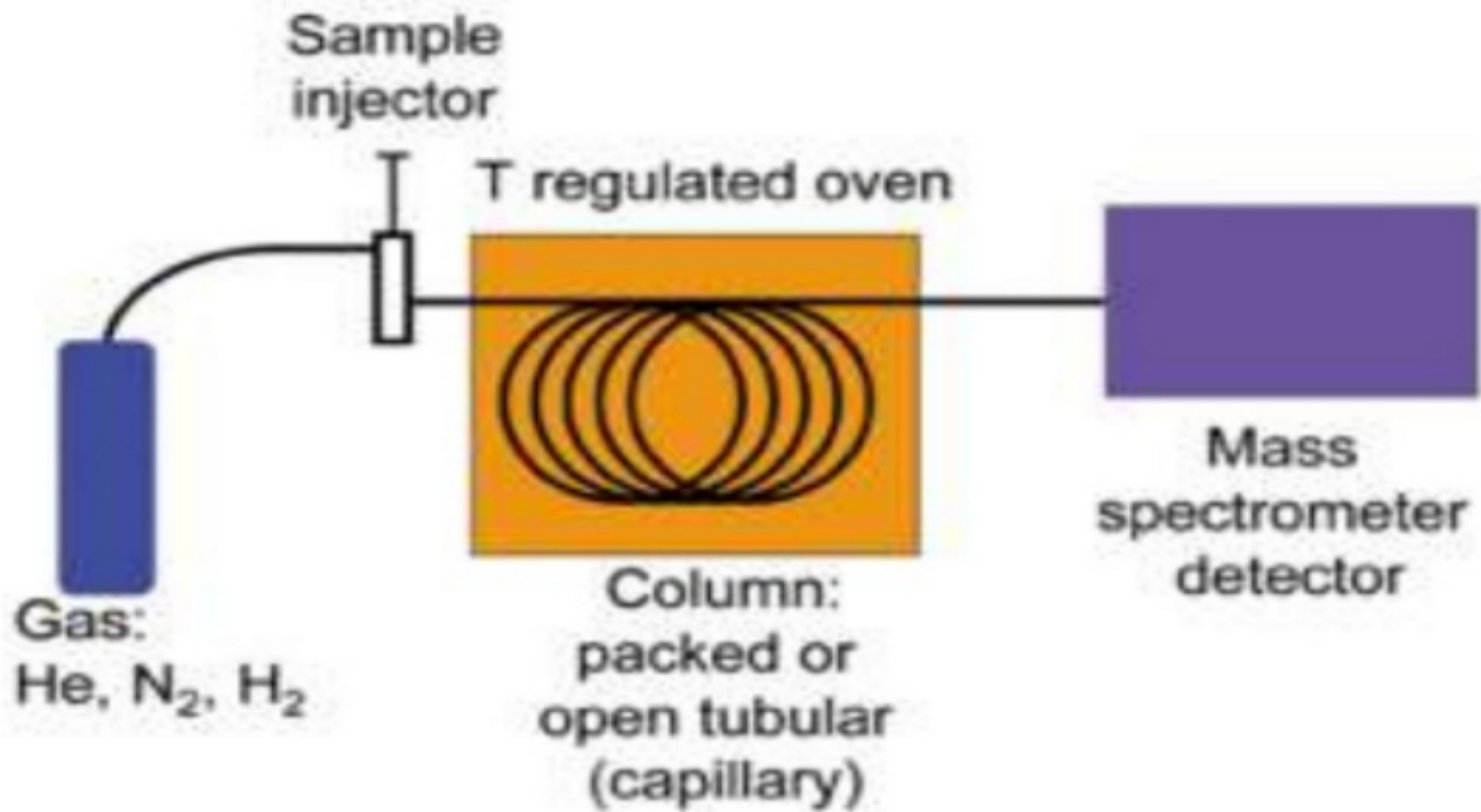
Methanol, Isopropyl Alcohol and water.

SAMPLE:

- Gases, Liquid, Solids.
- M. Wt: 2-800.
- Volatile.



GAS CHROMATOGRAPHY



APPLICATION OF GAS CHROMATOGRAPHY

- Quantitative & Qualitative analysis of low polarity compounds.
- Analytical chemistry, Biochemistry, Petrochemical, Environmental monitoring.
- Measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- Measuring toxic substances in soil, air or water.



APPLICATION OF GC- MS

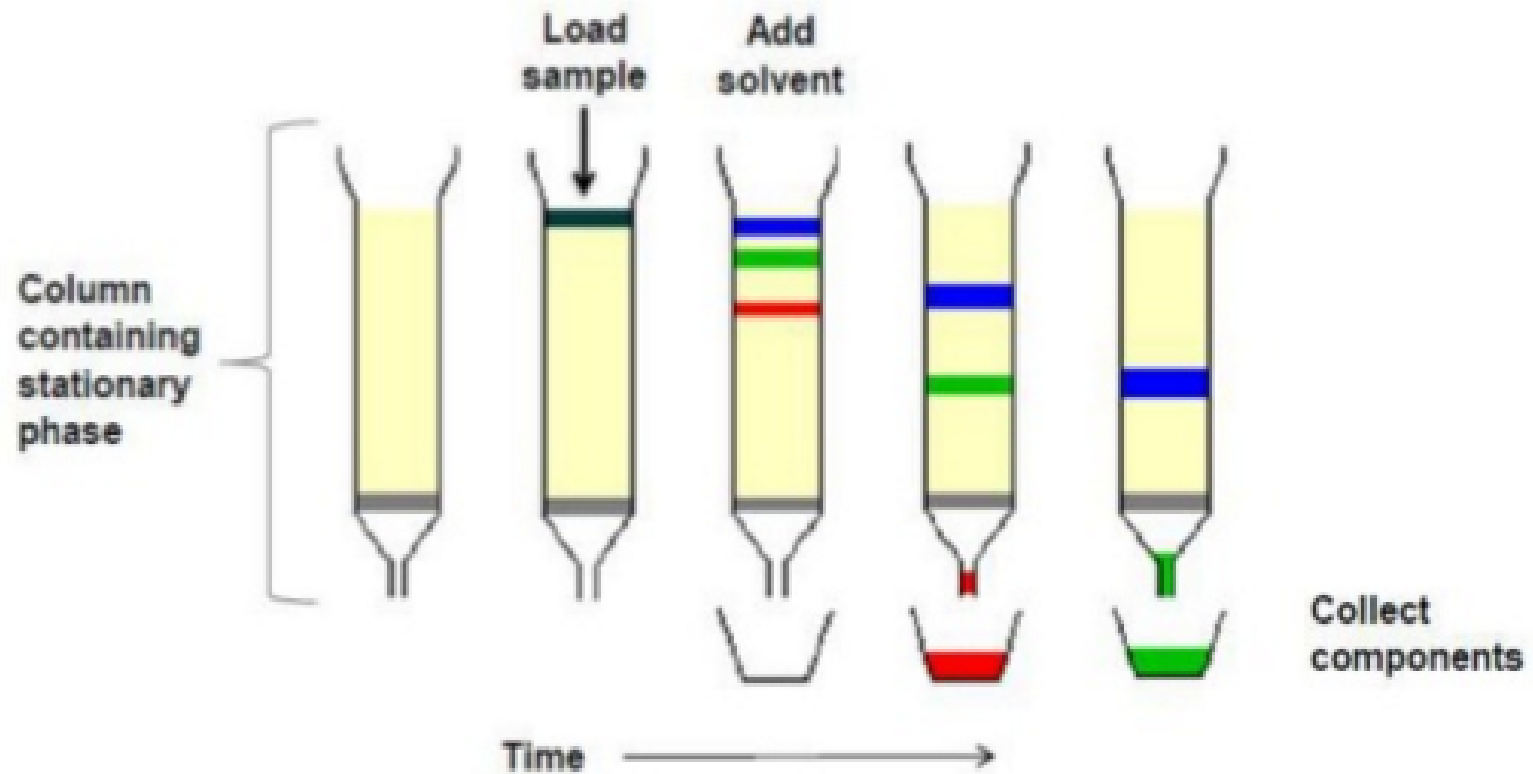
- ④ **Environmental Monitoring** : Organic Pollutants.
- ④ **Criminal Forensics** : Analyze the particles (Fiber) from a human body in order to help link a criminal to a crime.
- ④ **Law Enforcement** : Detection of illegal narcotics.
- ④ **Forensic Toxicology** : Find drugs and/or poisons in biological specimens of suspects, victims, or the deceased.
- ④ **Sports Anti-doping Analysis** : Test athletes urine samples.
- ④ **Security** : Explosive detection (September 11 development) systems have become a part of all US airports.
- ④ **Food, Beverage and Perfume** : from spoilage or Adulteration - aromatic compounds, esters, fatty acids, alcohols, Aldehydes, terpenes
- ④ **Medicine** : Congenital metabolic diseases In Born error of metabolism

LIQUID CHROMATOGRAPHY

- ❑ Mobile phase - Liquid.
- ❑ Column or a plane.
- ❑ Very small packing particles and a relatively high pressure - High Performance Liquid Chromatography (HPLC).



PRINCIPLE LIQUID CHROMATOGRAPHY



SOLVENT TYPES USED IN LIQUID CHROMATOGRAPHY

➤ ORGANIC

- ✓ Methanol
- ✓ Acetonitrile
- ✓ THF
- ✓ Methylene Chloride
- ✓ Hexane

➤ AQUEOUS

- ✓ Water

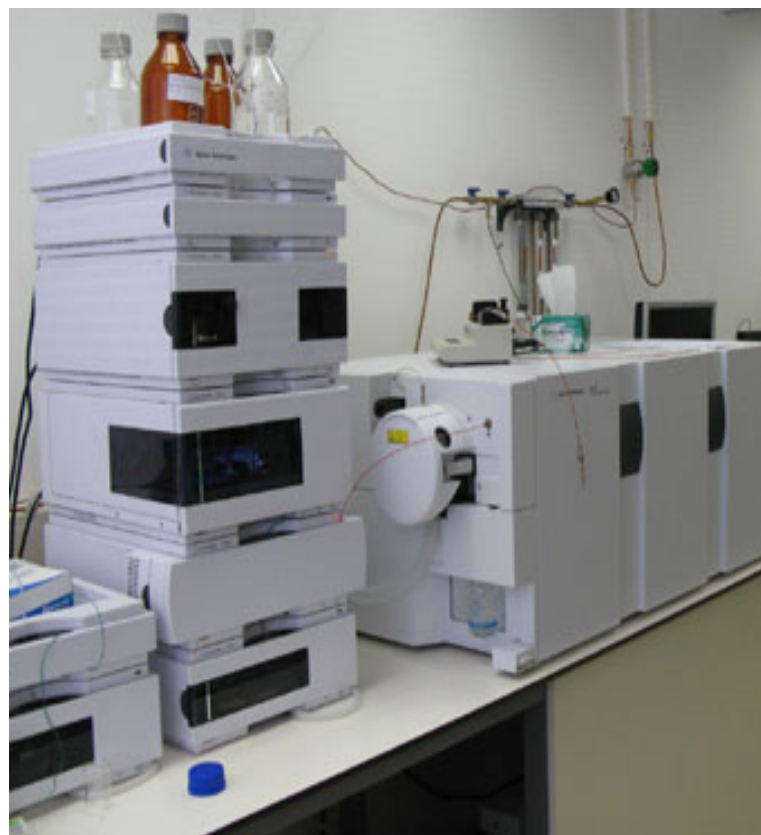
➤ SOLVENT MIXTURES

- ✓ CHCl_3 / ETOH
- ✓ MeOH / H_2O
- ✓ MeOH / CAN / H_2O
- ✓ Hexane / IPA



LC- MS

- Mass spectra is obtained rapidly.
- Small amount of material is required to form the spectra.
- Data collected is highly informative with respect to molecular structure.



APPLICATION OF LC- MS

- **Pharmacokinetics** : How quickly a drug will be cleared from the hepatic blood flow and organs of the body.
- **Proteomics** : Peptide mass fingerprinting.
- **Drug development**: Peptide Mapping, Glycoprotein Mapping, Natural Products Dereplication, Bioaffinity Screening, In Vivo Drug Screening, Metabolic Stability Screening, Metabolite Identification, Impurity Identification, Degradant Identification, Quantitative Bioanalysis, and Quality Control.
- **Fungal toxins.**
- **Pesticides, Herbicides.**

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

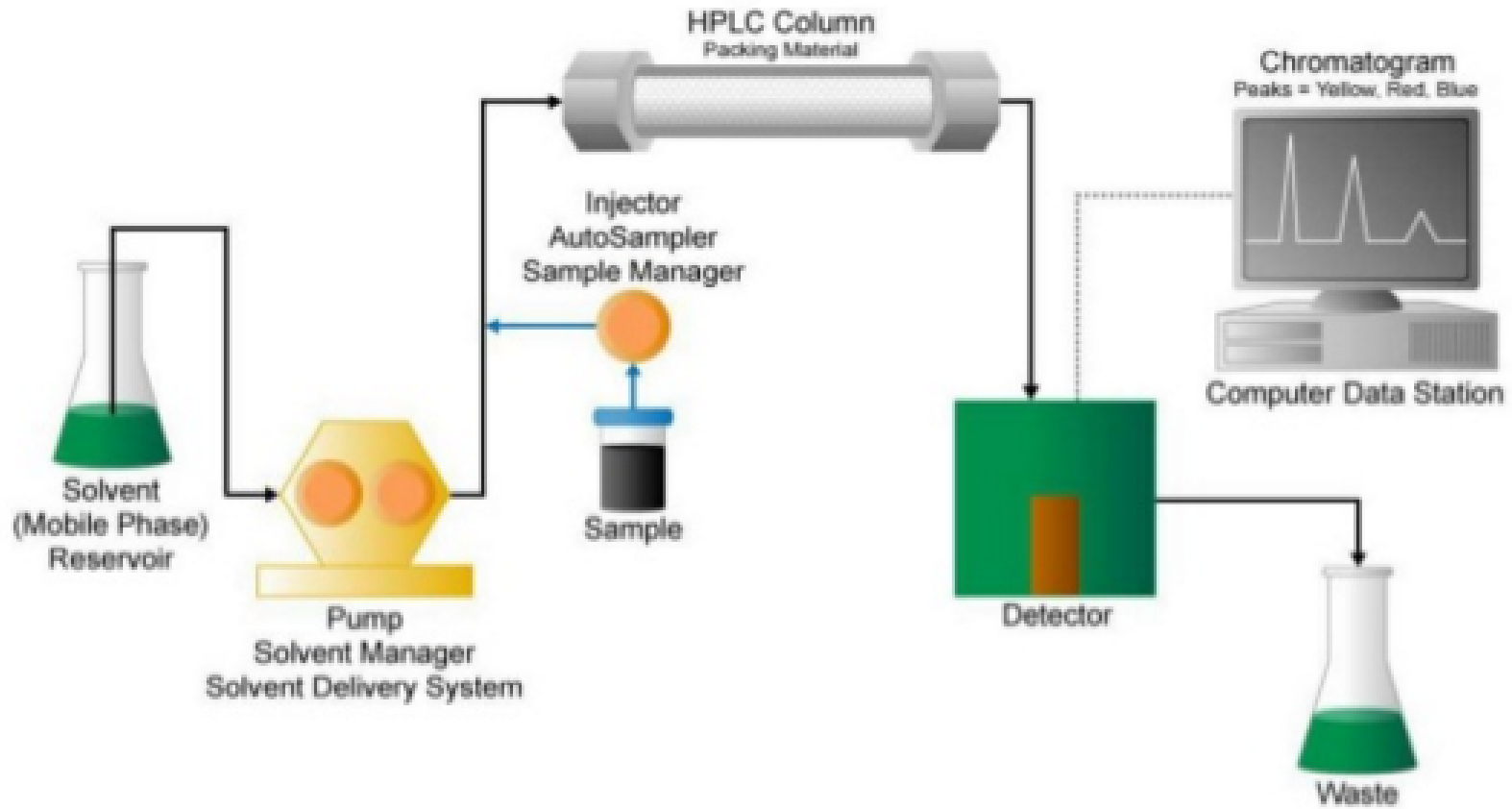
- Columns : Small diameter (4.6 mm), stainless steel, glass or titanium.
- Column packing with very small (3, 5 and 10 μm) particles.
- Relatively high inlet pressures and controlled flow of the mobile phase.
- Detecting very small amounts
- High resolution.
- Rapid analysis.
- Speed, efficiency, sensitivity and ease of operation.
- High degree of versatility.
- Easily separate a wide variety of chemical mixtures.
- 400 atmospheres.



PUMP PRESSURE:

- "Ultra High Performance Liquid Chromatography" systems 1000 atmospheres.)

HPLC SYSTEM



ELUTION OF HPLC SYSTEM

Isocratic and Gradient.

ISOCRATIC :

- ◆ ISO = SAME
- ◆ Solvent Composition Stays the Same for the Entire Run Example : 60:40 (Alcohol:Water)

GRADIENT:

- ◆ Solvent Composition Changes Throughout the Run



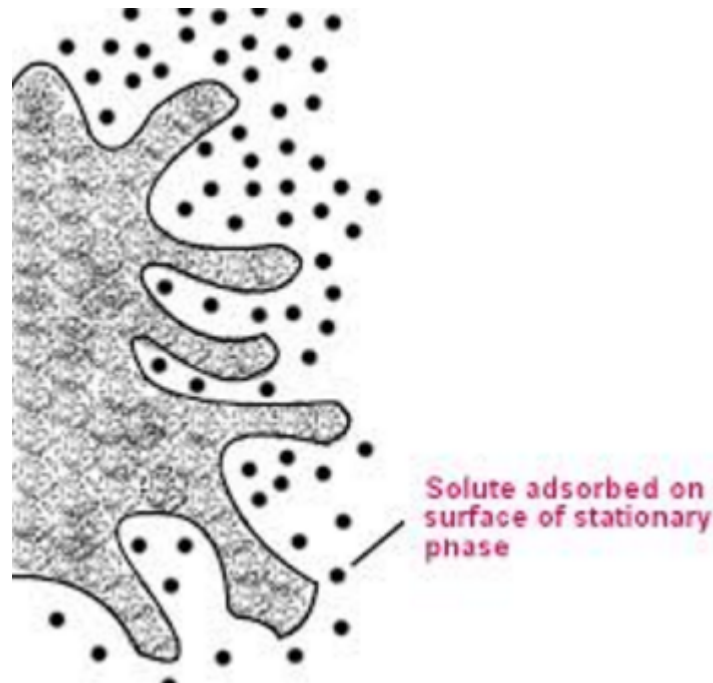
TYPES OF HPLC

- Nature of the stationary phase
- Separation process
- Adsorption chromatography
- Ion-exchange chromatography
- Size exclusion chromatography



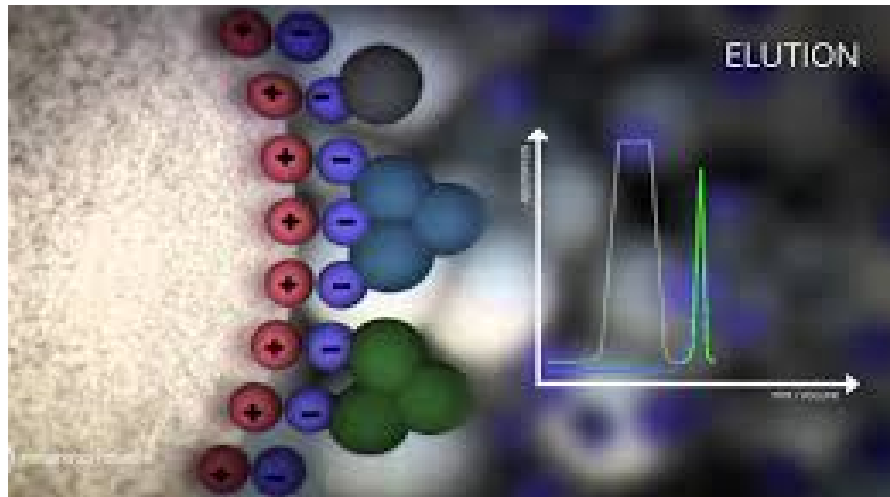
ADSORPTION CHROMATOGRAPHY

- Adsorption chromatography is probably one of the oldest types of chromatography around.
- It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.



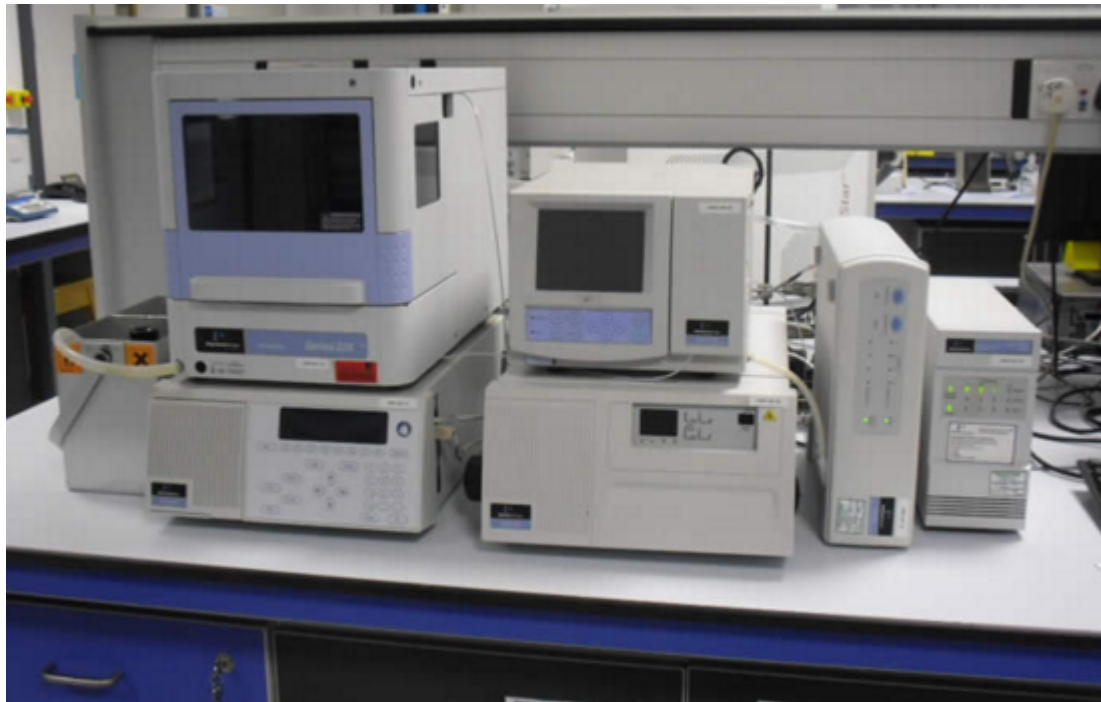
ION EXCHANGE CHROMATOGRAPHY

- In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it.
- Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.



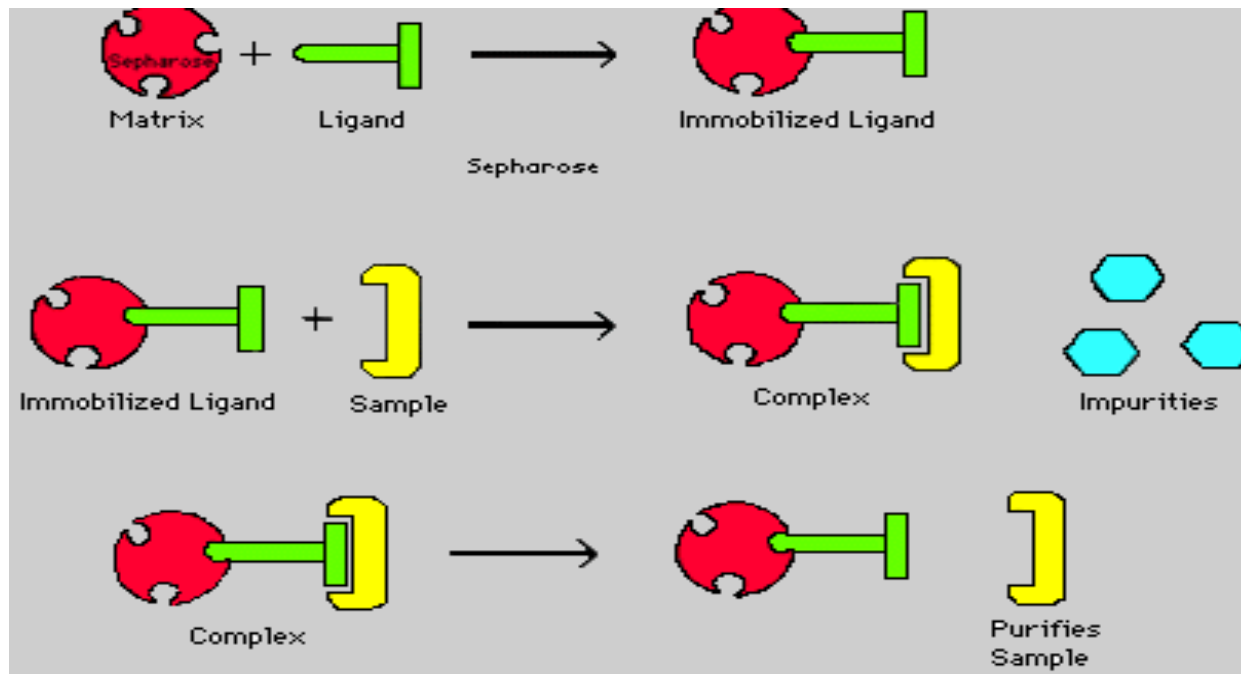
SIZE EXCLUSION CHROMATOGRAPHY

- Gel filtration or gel permeation chromatography.
- Separation-Molecular size of its components.
- Larger molecules are rapidly washed through the column, smaller molecules penetrate inside the porous of the packing particles and elute later.



AFFINITY CHROMATOGRAPHY

- Based on specific & non-covalent binding of the proteins to other molecules-Ligand.
- Physical properties of the analyte.
- Biochemistry in the purification of proteins bound to tags.
- After purification, some of these tags are usually removed and the pure protein is obtained.



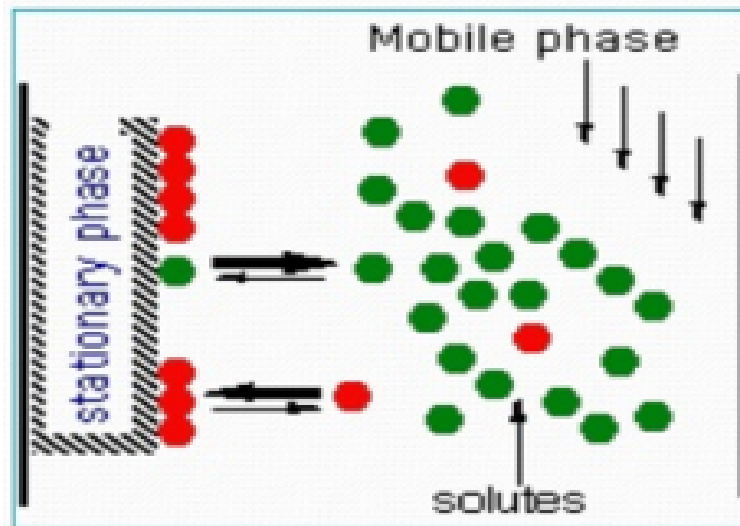
SUPERCritical FLUID CHROMATOGRAPHY

- Used for the analysis and purification of low to moderate molecular weight, thermally labile molecules.
- Principles are similar to those of HPLC.
- Mobile phase – High pressure liquid or super critical Carbon dioxide.
- Modifiers – Methanol, ethanol, isopropyl alcohol, acetonitrile and chloroform.
- Application- Use in industry primarily for separation of Chiral (Asymmetric Carbon atoms) molecules likes serine, soman, glyceraldehydes, phosphorus, sulfur metal, cobalt, etc.



PARTITION CHROMATOGRAPHY

- This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.



MOLECULAR EXCLUSION CHROMATOGRAPHY

- Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute.
- The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size.
- The pores are normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume.
- This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

