UNIT-IV: Analytical Techniques

Chromatography: Classification of chromatography methods, Principles and Applications of: Paper Chromatography, Thin Layer Chromatography (TLC), Column Chromatography, Ion-exchange Chromatography, Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Supercritical Fluid Chromatography.

Introduction:

- Mikhail Tswett in 1900, Russian botanist (referred to as Father of chromatography) is credited for the development of chromatography.
- During his research on plant pigments, primarily he separated the plant pigments such as chlorophyll (green), carotenes (orange), and xanthophylls (yellow).
- He used liquid-adsorption column chromatography with $CaCO_3$ as adsorbent and ether/ethanol mixture as eluent to separate plant pigments. Analyte is ether extract of plant.
- The word chromatography is derived from two Greek words: *Chroma* means color and *Graphein* means writing.
- Chromatography may be defined as 'A method of separating a mixture of components into individual components through equilibrium distribution between two phases'.
- *Principle of chromatography:* The sample to be examine or analyzing is termed as analyte is allowed to interact or react with two immiscible phases-mobile phase and stationary phase.
- *Stationary Phase:* The solid or liquid supported on a solid, on which the material to be separated is selectively adsorbed. The phase is immobile it means it does not move.
  Ex: Silica gel, Alumina, Charcoal etc.
- *Mobile Phase:* The mobile phase migrates the sample at different rates over the stationary phase.The mobile phase may be either liquid or gas. In general, the mixture to be separated is dissolved in a suitable solvent and allowed to pass over the stationary phase. This phase is also referred to as eluent or developer.
  Ex: Ethanol, Benzene, Hexane, Carbon tetrachloride etc.

Classifications of chromatography:

- Chromatography techniques can be explained into three fundamental ways:
  (1) Based on the shape of chromatographic bed: 2 types
  In planar chromatography, the stationary phase is spread on a flat, planar surfaces. The plane can be paper acting as a stationary phase (paper chromatography), or stationary phase spread

on glass, metal or plastic plate (thin layer chromatography). Planar chromatography also known as open-bed chromatography.

In column chromatography, the stationary phase is within a tube.

(2) Based on the physical nature of the stationary and mobile phases: 2 types

On the basis of physical nature of mobile phase:

- Gas chromatography
- Liquid chromatography

On the basis of physical nature of stationary phase:

- Gas-solid chromatography
- Gas-liquid chromatography
- Liquid-solid chromatography
- Liquid-liquid chromatography

(3) Based on the mechanism of the separation: The method of chromatography uses various types of mechanisms to separate analyte.

- Partition chromatography
- Adsorption chromatography
- Ion exchange chromatography

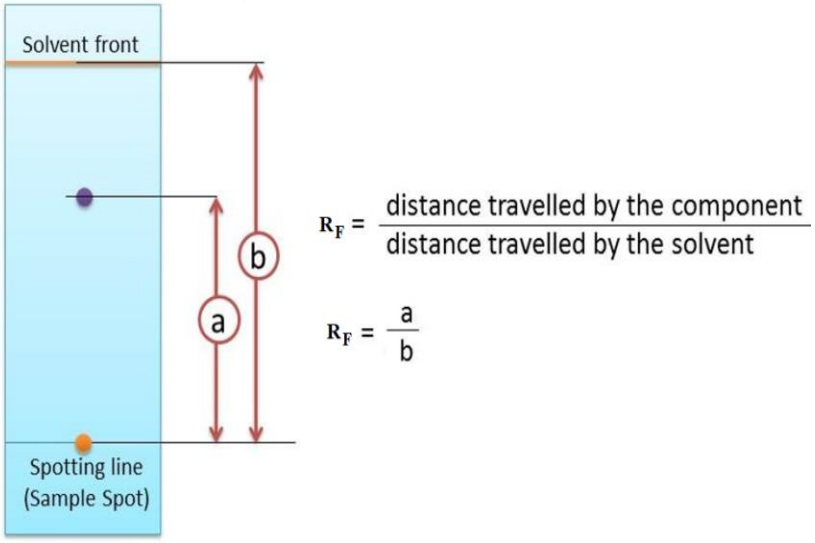
Nature of adsorbents (Stationary phase):

- An adsorbent is a substance, usually porous in nature and with a high surface area then can adsorb substances onto its surface by intermolecular forces. The ideal adsorbent must fulfill the following requirements:
- Insoluble in mobile phase, inert to solutes and homogeneity
- Colorless especially when worked with colored mixtures
- Suitable particle size enough to give good separation and reasonable flow rate.
- Adsorbents do not adhere to glass plates and hence binders like gypsum, starch are added.
- Inorganic adsorbents: Silica gel, Alumina, Magnesia, $MgSiO_3$, $CaSiO_3$ etc.,
- Organic adsorbents: Cellulose and its acetylates, charcoal and activated carbon.
- On the basis of polarity, the adsorbents may be
- Less polar: Starch, Sucrose
- Intermediate polar: $CaCO_3$, $Ca(OH)_2$
- More polar: Alumina, Silica gel

Nature of solvent system (Mobile phase):

- The choice of the mobile phase depends upon the: nature of the substance to be separated, nature of the stationary phase used and mode of chromatography (normal or reverse phase).
- The sample must dissolve in the solvent.
- The sample should satisfy suitable viscosity, purity and stability etc.
- Mixtures of solvents can be used.
- Solvents used in chromatography should be quite dry.
- Selection of solvents requires a balancing act between solvent and compound polarities.
- For most separations, the solvent should be less polar than the compounds.
- If the solvent is much more polar than the compounds, the compounds will remain in the mobile phase, and separation will not occur.
- If the compounds are much more polar than the solvent, no compounds will elute since the solvent is unable to move compounds from the adsorbent sites.
- Order of polarity for silica gel and alumina is: Hexane < petroleum ether (C_6H_{14}) < carbon tetrachloride < toluene < dichloromethane < chloroform < diethyl ether < ethyl acetate < acetone < propanol < ethanol < methanol < acetic acid < water.
- **Normal phase and reverse phase chromatography:**
- Normal phase chromatography: very polar stationary phase and a non-polar mobile phase. Stationary phase: Pure polar silica; mobile phase: non-polar solvent such as chloroform
- Reverse phase chromatography: non-polar stationary phase and a polar mobile phase. Stationary phase: Modified silica with long hydrophobic chains; mobile phase: water, methanol or acetonitrile.
- **Retention factor (R_F):**
- The sample is spotted on the filter paper at the base line or the spotting line. This filter paper is then placed in the suitable solvent.
- When the solvent rises up via the capillary action, the individual components in the sample get separated. Different components travel at different rates.
- The solvent travels the farthest on the filter paper and it leaves the line called as ‘solvent front’.
- To identify the components of the mixture, the retention factor or the R_F value can be calculated.

- The distance traveled by a particular component is constant relative to the solvent as long as all other factors like, the type of paper and the exact composition of the solvent are kept constant.
- R_F is the distance travelled by the component divided by the distance traveled by the solvent.



- The R_F value vary from '0' to '1'.
- Low R_F value means that the compound is very polar and strongly attracted to the polar stationary phase.
- High R_F value indicates relatively weak attraction to the stationary phase and good solubility in the mobile phase.
- Retention factors are useful in comparing the results of one chromatogram to the results of another.
- If the conditions in which the chromatogram run are unchanged (same mobile and stationary phases), the retention factor for a given material should remain constant. This allows unknowns to be compared to known materials.
- *Factors which affect R_F values:*
- Temperature
- The quality of the paper (or) medium used for separation
- The quality and nature of the solvents used
- The polarity of components (mixture)

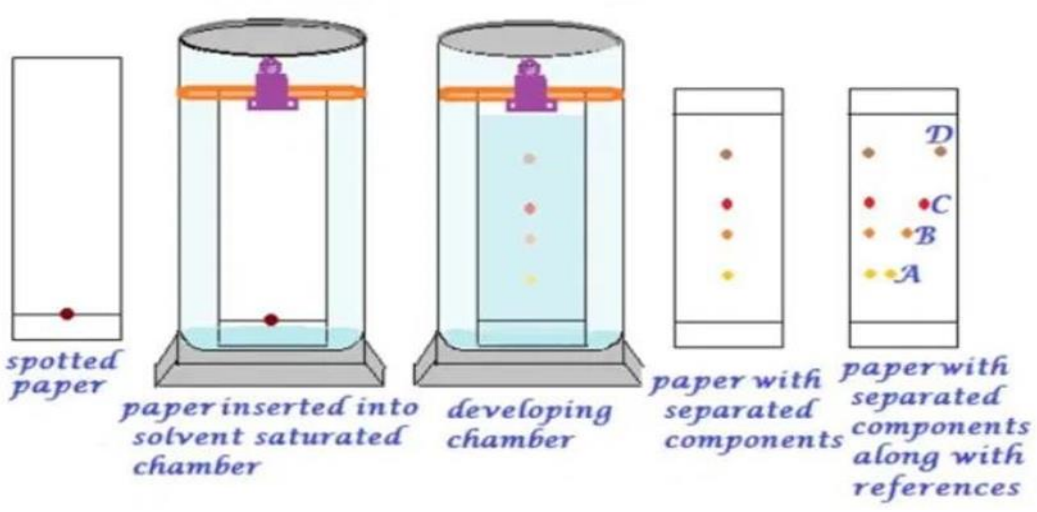
- **Principles of differential migration:**
- Chromatography is a separation method where the analyte is combined within a liquid or gaseous mobile phase, which is pumped through a stationary phase.
- Usually one phase is polar and the other non-polar. The components of the analyte interact differently with these two phases.
- Depending on of their polarity they spend more or less time interacting with the stationary phase and are thus retarded to a greater or lesser extent. This leads to the separation of the different components present in the sample.
- Each sample component elutes from the stationary phase at a specific time, its retention time.
- As the components pass through the detector their signal is recorded and plotted in the form of a chromatogram.

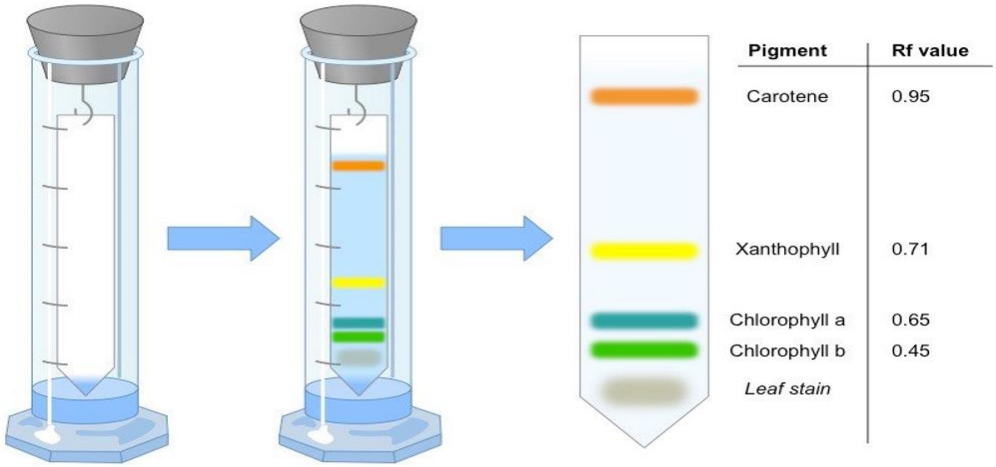
Principles and applications of:

Paper Chromatography:

Principle:

- The principle involved can be partition chromatography or adsorption chromatography.
- *Paper Adsorption Chromatography:* Paper impregnated with silica or alumina acts as adsorbent (stationary phase) and solvent as mobile phase.
- *Paper Partition Chromatography:* Moisture/water present in the pores of cellulose fibers present in filter paper acts as stationary phase and solvent as mobile phase.
- When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase under the capillary action of pores in the paper.





Applications:

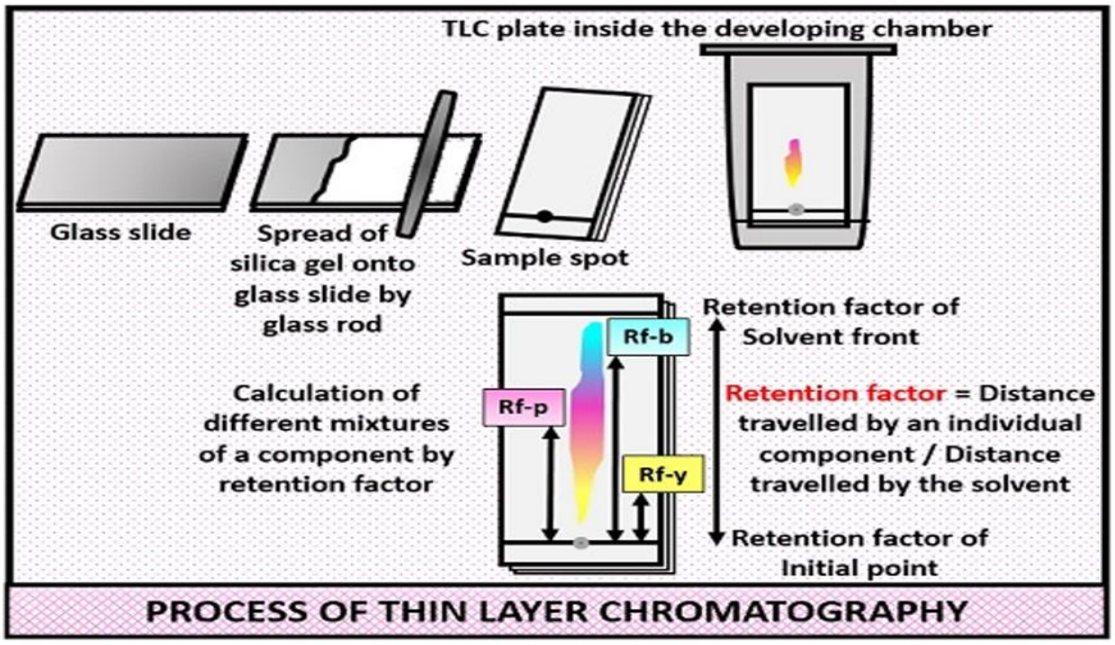
- To check the purity of pharmaceuticals
- To detect the contaminants in foods and drinks
- For the detection of drugs and dopes in animals & humans
- In analysis of cosmetics
- Analysis of the reaction mixtures in biochemical labs

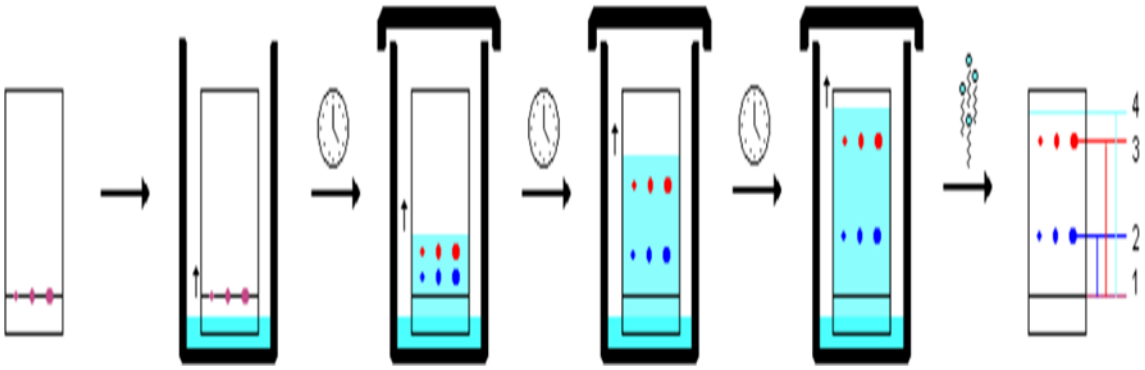
Thin Layer Chromatography:

- A separation technique used to isolate non-volatile mixtures. TLC is performed with a sheet of plastic, aluminum foil, or glass coated with a thin layer of adsorbent material, such as silica gel, alumina, and cellulose is called thin-layer chromatography.

Principle:

- The stationary phase is prepared by coating a thin layer of alumina or silica on metal, plastic or glass. The mixture of compounds moves along with the mobile phase via the stationary phase.
- The separation process depends on the relative affinity of the compounds towards the mobile phase and stationary phase.
- During this movement, the compounds with lower affinity to stationary phase travel fast while the others move slowly. Therefore, the separation is achieved.
- On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.





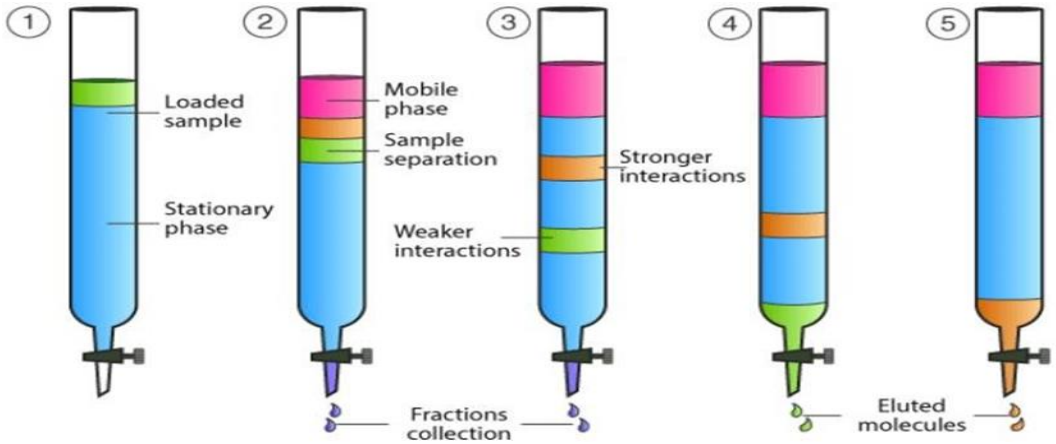
Applications:

- Compounds such as alcohols, alkaloids, antibiotics, acids, proteins, amines etc. are identified.
- To know the level of purity of the sample.
- The qualitative testing of various medicines such as sedatives, local anesthetics, tranquillizers, analgesics (used to relieve pain), antihistamines (used to treat symptoms of allergies), steroids, hypnotics.
- Isolation of biochemical metabolites (amino acids, organic acid and bases, fatty acids, lipids, and carbohydrates etc) from its blood plasma, urine, body fluids, serum, etc.
- To identify natural products like essential oils, glycosides, alkaloids, etc.
- It is used in the food industry to identify colors, sweetening agent, and preservatives.
- It is used in the cosmetic industry.

Column chromatography:

Principle:

- Mobile phase along with the mixture to be separated is introduced from the top of the column. The rate at which individual components move in the column during the separation process depends on their polarity.
- The components with lower adsorption affinity to stationary phase travel faster when compared to the greater adsorption affinity with the stationary phase. The components that move fast are removed first whereas the components that move slow are eluted out last.
- As the mobile phase comes out of the column it is collected in small fractions in the test tube. Then it is allowed to isolate and purify.



Applications:

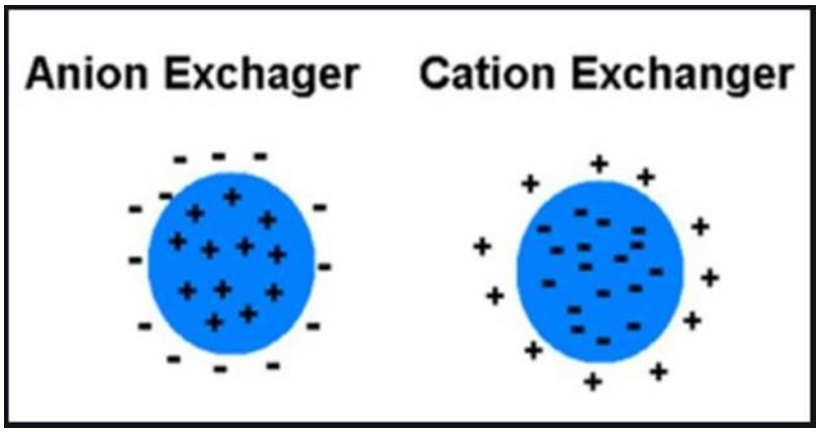
- This technique is used for the separation of diastereomers, geometrical isomers, Tautomeric mixtures etc.
- Isolation of metabolites from biological fluids
- Purification of β-ketoester, alcohols, polychlorinated biphenyls (PCB), phenols etc. Biomolecules such as Proteins, glycolipids, nucleic acids etc.

Ion-exchange chromatography:

Principle:

- Ion-exchange chromatography (IEC) is an important analytical technique for the separation of ionic compounds.
- The stationary phase consists of an immobile matrix that contains charged ionizable functional groups.

- The two types of ion chromatography are anion-exchange and cation-exchange.

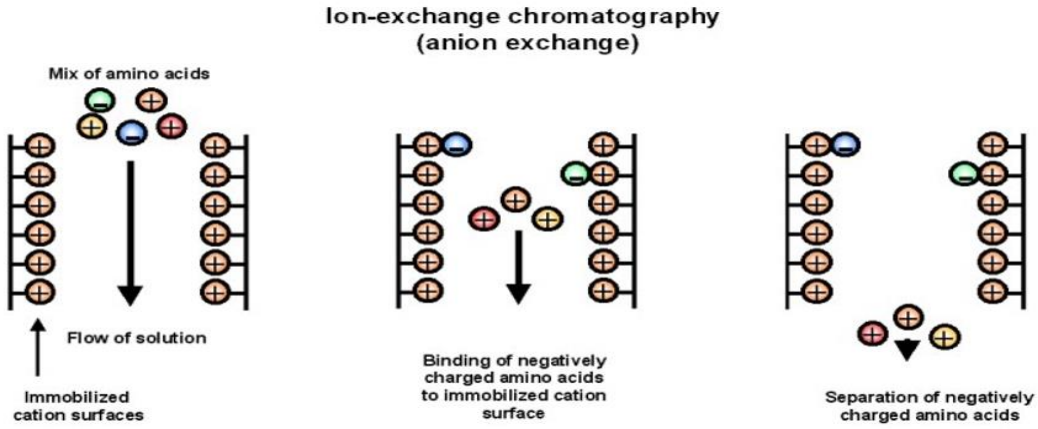


- Cation-exchange chromatography is used when the molecule of interest is positively charged. In cation-exchange chromatography, the stationary phase is negatively charged and positively charged molecules are loaded to be attracted to it.
- Cation-exchanger retains cations because the stationary phase has a negatively charged functional group.

$$\text{R-X}^{-}\text{C}^{+} + \text{M}^{+}\text{B}^{-} \rightleftarrows \text{R-X}^{-}\text{M}^{+} + \text{C}^{+} + \text{B}^{-}$$

- Anion-exchange chromatography is used when the molecule of interest is negatively charged. In anion-exchange chromatography, the stationary phase is positively charged and negatively charged molecules are loaded to be attracted to it.
- Anion-exchanger retains anions because the stationary phase has a positively charged functional group.

$$\text{R-X}^{+}\text{A}^{-} + \text{M}^{+}\text{B}^{-} \rightleftarrows \text{R-X}^{+}\text{B}^{-} + \text{M}^{+} + \text{A}^{-}$$



Applications:

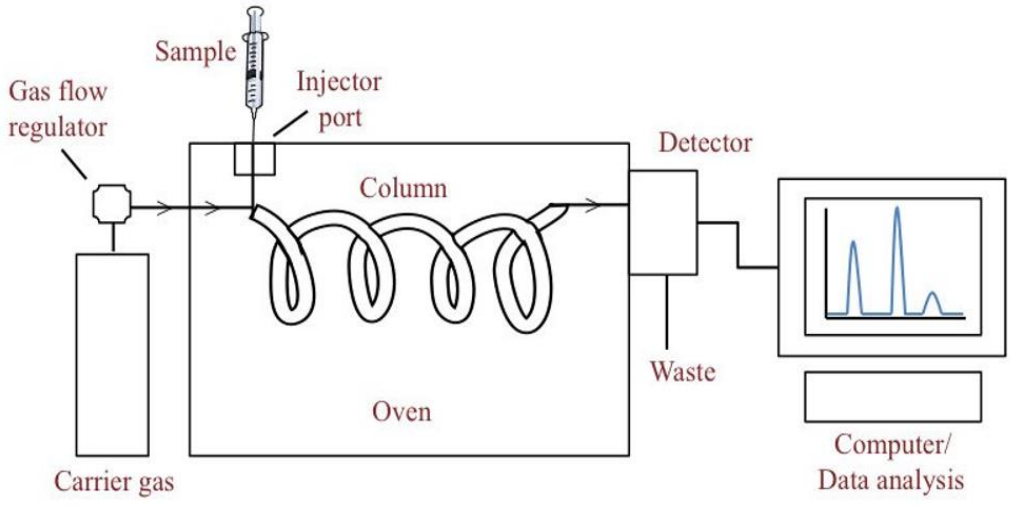
- It is often used in protein purification.
- In the analysis of amino acid mixtures.
- This is most effective method for water purification.
- In the analysis of nucleic acids.
- To collect trace metals from seawater.
- To analyze rare elements on Earth.

Gas Chromatography (GC):

- In gas chromatography, the mobile phase is a gas and the components are separated as vapors. Helium, N_2, Argon are used as carrier gases.
- Gas chromatography can be classified as gas-solid chromatography (GSC), where the stationary phase is a solid, and gas-liquid chromatography (GLC) that uses a liquid as stationary phase. GLC is more widely used than GSC.
- In GSC, the stationary phase is a solid like silica, alumina or activated carbon.
- In GLC, the stationary phase is non-volatile liquid such as Parafin oil, squalane, silicone oil, apiezon L grease, diethyl hexyl phthalate, Carbowaxes (poly glycols) etc.

Principle:

- The sample is either a gas or a liquid that is vaporized in the injection port. The mobile phase for gas chromatography is a carrier gas.



- The components of the mixture are distributed between the two phases in the column and separated based on their abilities to bind on to the stationary phase.

- A component with lower adsorption affinity to the stationary phase will spend the least time in the column and will emerge from the column first.
- A component with greater adsorption affinity to the stationary phase will spend the most time in the column and will emerge from the column last.
- A detection unit recognizes the sample components after leaving the column.
- The detector sends a signal to the recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last.
- The appearance time, height, width, and area of these peaks can be measured to yield quantitative data.

Applications:

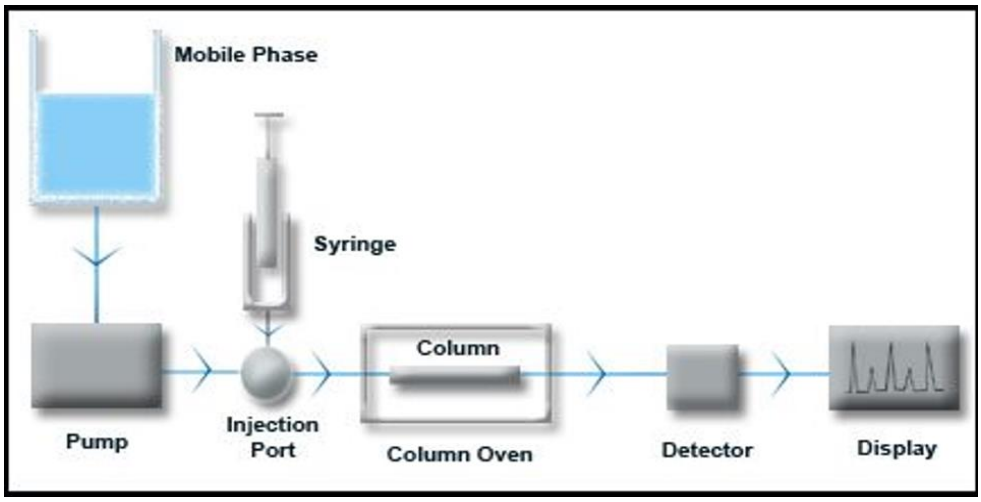
- GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- GC analysis is used to measure toxic substances in soil, air and water.
- Gas chromatography is used in the analysis of:
  - air-borne pollutants
  - performance-enhancing drugs in athlete's urine samples
  - essential oils in perfume preparation
- Gas chromatography is used extensively in forensic science.

High Performance Liquid Chromatography (HPLC):

- It gives high performance due to smaller particle size of the stationary phase (3.5-10 μm).
- High pressure is applied for rapid separation of the compounds i.e., 1000 – 5000 psi (68 to 340 atm).
- In HPLC, the stationary phase is an adsorbent (solid) and the separation is based on adsorption.
- Normal phase chromatography: The stationary phase is strongly polar (silica gel) and the mobile phase is non-polar (hexane or tetrahydrofuran).
- Reversed Phase chromatography: The stationary phase is strongly non-polar while the mobile phase is polar (as a mixture of water and methanol).

Principle:

- The mobile phase is delivered by the pump at high pressure and constant speed. The sample is introduced at the injection port.
- The sample components are distributed between the mobile phase and stationary phase in a column.
- The interactions between the sample components and the stationary phase, define their time "on-column". Different components of a sample are eluted at different times. Thereby, the separation of the sample components is achieved.
- A detection unit recognizes the sample components after leaving the column.
- The detector sends a signal to the recorder which results in a peak on the chart paper (chromatogram). The component that is detected first is recorded first. The component that is detected last is recorded last.
- The appearance time, height, width, and area of these peaks can be measured to yield quantitative data.

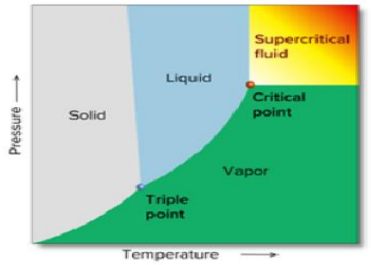


Applications:

- Water purification
- Detection of impurities in pharmaceutical industries
- Quantity of drug determination from pharmaceutical dosage forms
  Ex: Paracetamol determination in panadol tablet
- Quantity of drug determination from biological fluids
- Forensic test: Determination of steroid in blood, urine & sweat
- To separate biomolecules such as carbohydrates, lipids, nucleic acids, proteins, steroids etc.

Supercritical Fluid Chromatography:

- Supercritical fluid chromatography (SFC) is a separation technique similar to gas and liquid chromatography, but using a supercritical fluid as mobile phase.



Moves like a gas and dissolves things like a liquid

Increasing pressure, phase boundary disappears

Solvent	T_c (K)	P_c (Mpa)	Critical Density kg/m^3
Methane	192	4.60	162
Ethylene	283	5.03	218
Carbon Dioxide	304	7.38	468
Ethane	305	4.88	203
Propylene	365	4.62	233
Propane	370	4.24	217
Ammonia	406	11.3	235
Water	647	22.0	322

- Stationary phase: A column with silica gel as a packing material (similar to normal phase chromatography). It is also possible to use packed columns for reversed-phase chromatography.
- Mobile phase: Carbon dioxide has the same polarity as hexane and is suitable for the separation of non-polar substances.
- A modifier (alcohol) is added to CO_2 to separate highly polar substances.

Principle:

- Principle is similar to High Performance Liquid Chromatography (HPLC).
- Supercritical fluids are less viscous than liquids and have more diffusivity than a liquid. Therefore, a solute can show better diffusivity in a supercritical fluid than in a liquid.

- In addition, density of a supercritical fluid is closer to that of a liquid.
- The dissolving effect of a supercritical fluid is dependent on its density value. As the density of a supercritical fluid increases, its solvating power increases.
- Therefore, as the density of the supercritical fluid (mobile phase) is increased, solute solubility increases, components retained in the column can be made to elute.
- SFC can achieve high separation efficiency and allows substances to be separated at a higher speed than is possible using HPLC.

Applications:

- Common industrial applications include the pharmaceutical and biochemical industry, the polymer industry, natural product chemistry, and the food industry.
- SFC can be applied to the materials such as polymers, oils, carbohydrates, pesticides, organic pollutants, volatile toxins, poly-aromatic hydrocarbons, flavors, pharmaceutical metabolites, explosives, organometallics, plastics, PVC, paper, wood etc.
- Examples of materials analyzed in environmental applications: oils and fats, pesticides, alkanes, organic pollutants, volatile toxins, herbicides, nicotin, fatty acids, samples from clay to petroleum waste, from soil to river sediments.
- Drug metabolites, enzymes, steroids are extracted from plasma, urine, serum or animal tissues in biochemical applications.