CHROMATOGRAPHY

PG Sem II
CC6 Unit IIA

Introduction to Chromatography

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- The Russian botanist Mikhail Tswett coined the term chromatography in 1906.
- The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures.
- A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to separate materials.
- It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures.

Chromatography

Thin layer chromatography is used to separate the colorful components of a plant extract

- Mikhail Tswett, Russian, 1872-1919 Botanist In 1906 Tswett used to chromatography to separate plant pigments
- He called the new technique chromatography because the result of the analysis was 'written in color' along the length of the adsorbent column
- Chroma means “color” and graphein means to “write”

- Chromatography has application in every branch of the physical and biological sciences. 12 Nobel prizes were awarded between 1937 and 1972 alone for work in which chromatography played a vital role.
- The substances in a mixture are not chemically combined, so therefore they can be separated through some physical process.
- chromatography, technique for separating the components, or solutes, of a mixture on the basis of the relative amounts of each solute distributed between a moving fluid stream, called the mobile phase, and a contiguous stationary phase.
- The mobile phase may be either a liquid or a gas, while the stationary phase is either a solid or a liquid.
- Chromatography is the ability to separate molecules using partitioning characteristics of molecule to remain in a stationary phase versus a mobile phase.
- Once a molecule is separated from the mixture, it can be isolated and quantified

Principle of Chromatography (how does chromatography work)

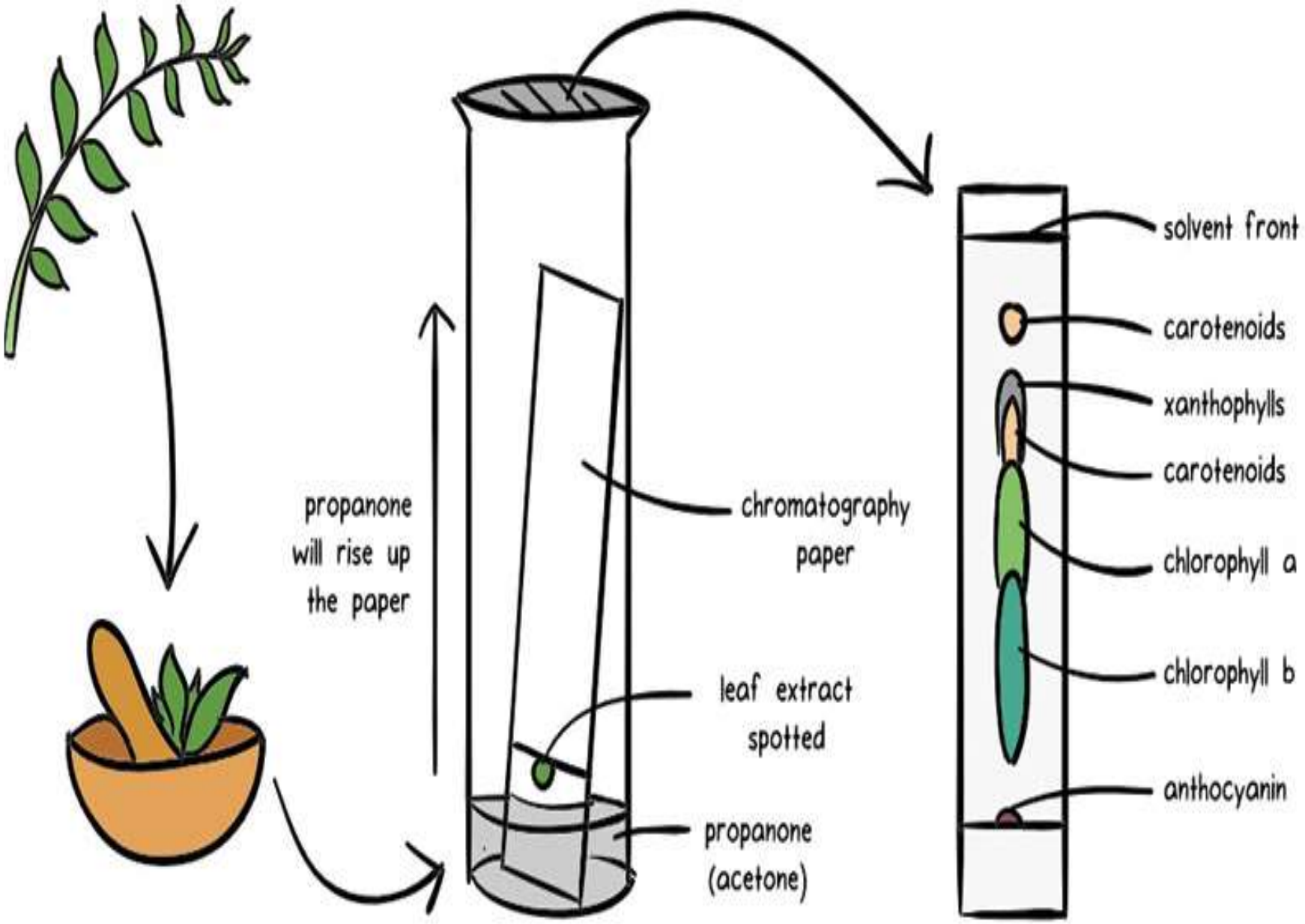
- Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase.
- The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.
- Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster.
- Three components thus form the basis of the chromatography technique.
- **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".
- **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."
- **Separated molecules**
- The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other.

Stationary Phase

- **The stationary phase in chromatography is the phase that is either a solid or liquid particle attached to a glass or a metal surface on which the components of the mixture to be separated is absorbed selectively.**
- The term stationary refers to the fact that this phase remains stationary while the other phase moves.
- Most substances used as stationary phases are porous, thus allowing the attachment of components during chromatography.
- The stationary phase to be selected for a chromatographic process depends on the nature of the components to be separated and the type of chromatography.
- Depending on the type of chromatography gel beads, thin uniform paper, silica, glass, some gases, or even liquid components are used as a stationary phase.

Mobile Phase

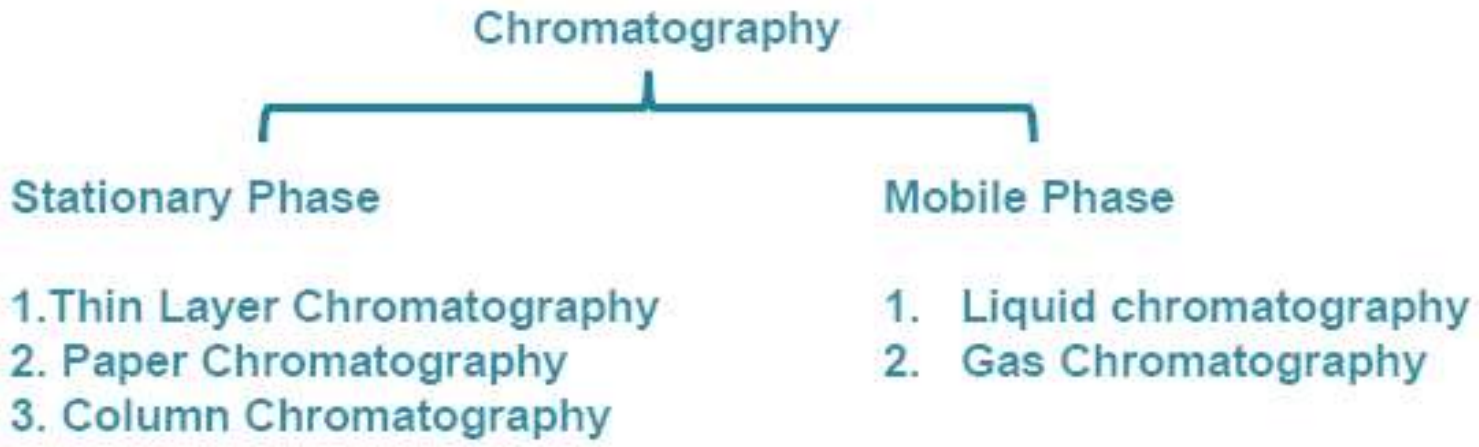
- **The mobile phase in chromatography is the phase that is either liquid or gas that is passed through a chromatographic system where the components of the mixture are separated at different rates by adsorbing them to the stationary phase.**
- The mobile phase is the solvent that carries the mixture as it moves down the stationary phase.
- The term mobile indicates that the phase is moving down the chromatographic system, whereas the other phase remains stationary.
- Substances used as mobile phases are selected for a chromatographic process depending on the nature of the components to be separated and the type of chromatography.
- Alcohol, water, acetic acid, acetone, or some gases are the commonly used mobile phase in different chromatographic techniques.


propanone
will rise up
the paper
chromatography
paper
leaf extract
spotted
propanone
(acetone)
solvent front
carotenoids
xanthophylls
carotenoids
chlorophyll a
chlorophyll b
anthocyanin

Types of Chromatography

- Substances can be separated on the basis of a variety of methods and the presence of characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase.
- This leads to different types of chromatography techniques, each with their own instrumentation and working principle.
- For instance, four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion.
- Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography.

Different Chromatographic Techniques

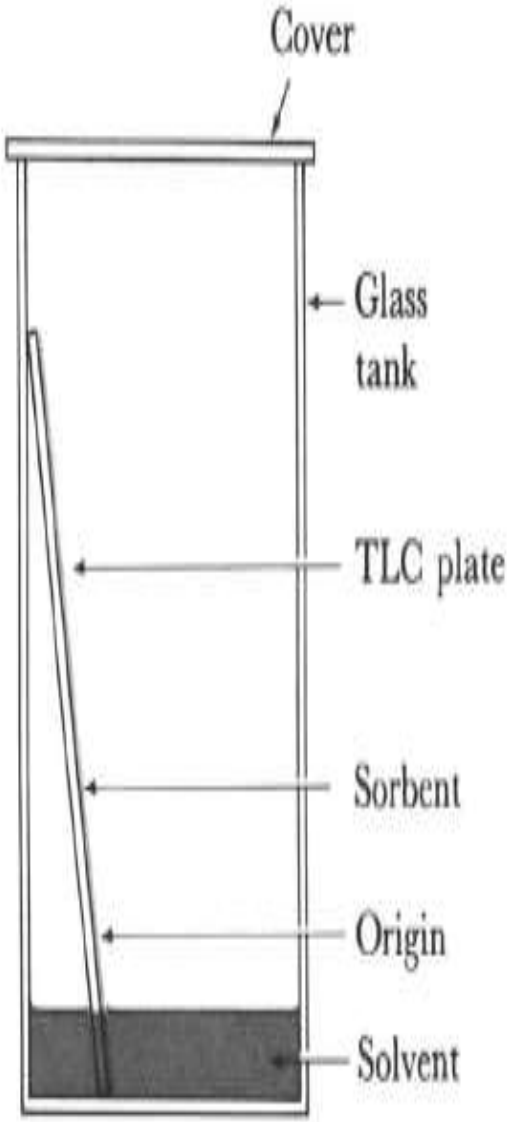


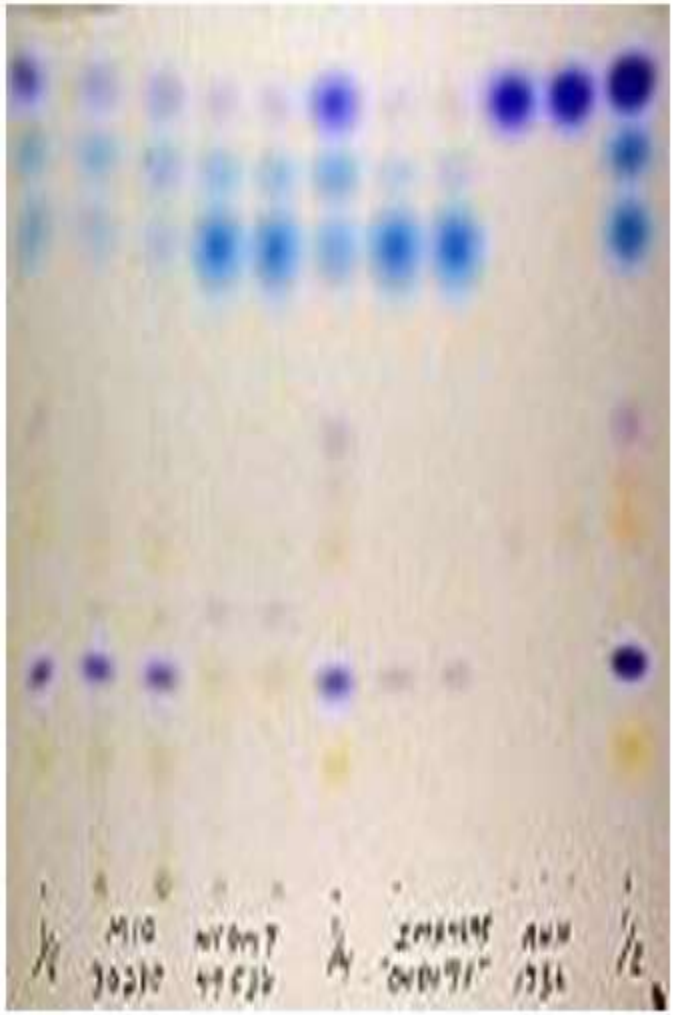
Classification according to the force of separation

1- Adsorption chromatography.
2- Partition chromatography.
3- Ion exchange chromatography.
4- Gel filtration chromatography.
5- Affinity chromatography.

Thin Layer Chromatography

- TLC is a method for identifying substances and testing the purity of compounds.
- TLC is a useful technique because it is relatively quick and requires small quantities of material.
- Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase.
- The stationary phase: is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate.
- The mobile phase: is a developing liquid which travels up the stationary phase, carrying the samples with it.
- Components of the samples will separate on the stationary phase according to how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.



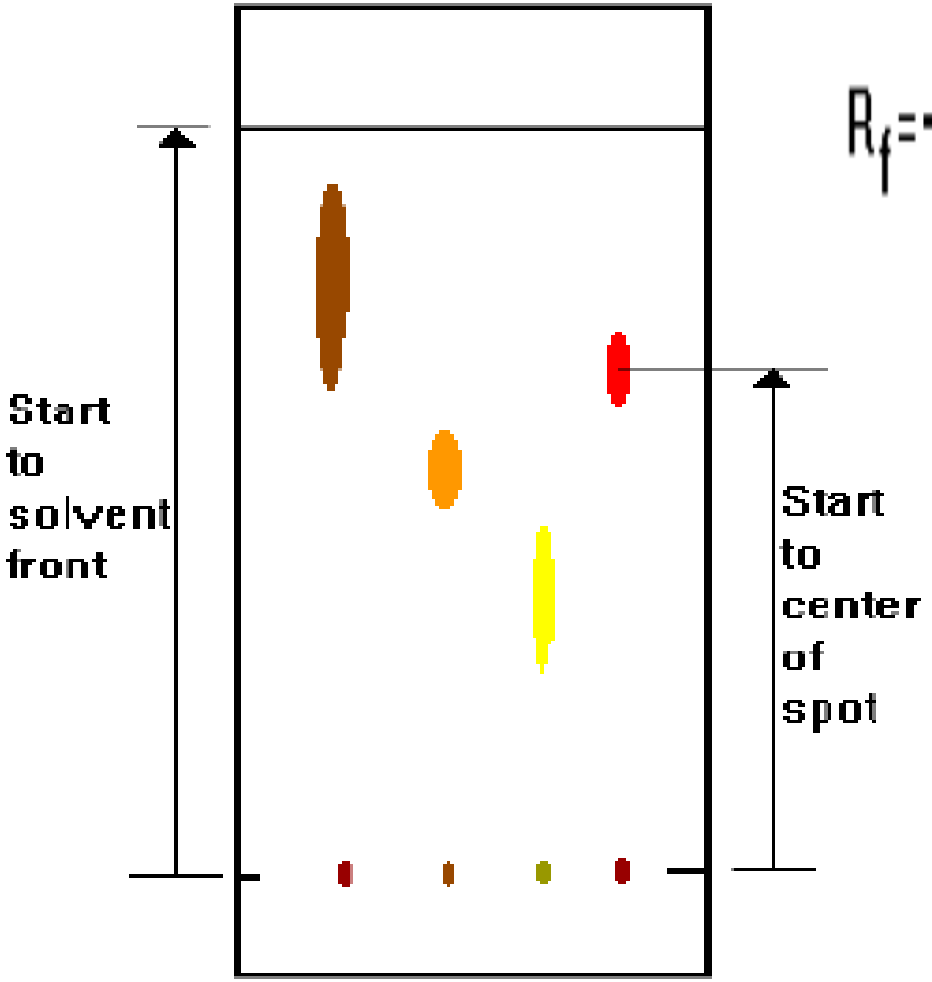


If the spots can be seen, outline them with a pencil. If no spots are obvious, the most common visualization technique is to hold the plate under a UV lamp. Many organic compounds can be seen using this technique, and many commercially made plates often contain a substance which aids in the visualization of compounds.

Visualizing Agents

Reagents	Compounds
Iodine	Aromatic compounds
UV light	Unsaturated compounds
p-Anisaldehyde	Carbohydrate
Bromocresol green	carboxylic acid
2,4-dinitrophenylhydrazine	Mainly for aldehydes and ketones
Ninhydrin	Good for amines
Sulfanilic Acid Reagent (Diazotized), Pauly's Reagent	phenolic compounds turn orange or yellow with this reagent sprayed on the TLC
Sulfuric acid	Sugar
Aniline phthalate	Cardiac glycosides
Antimony trichloride	Alkaloids
Dragendorff' s reagent	

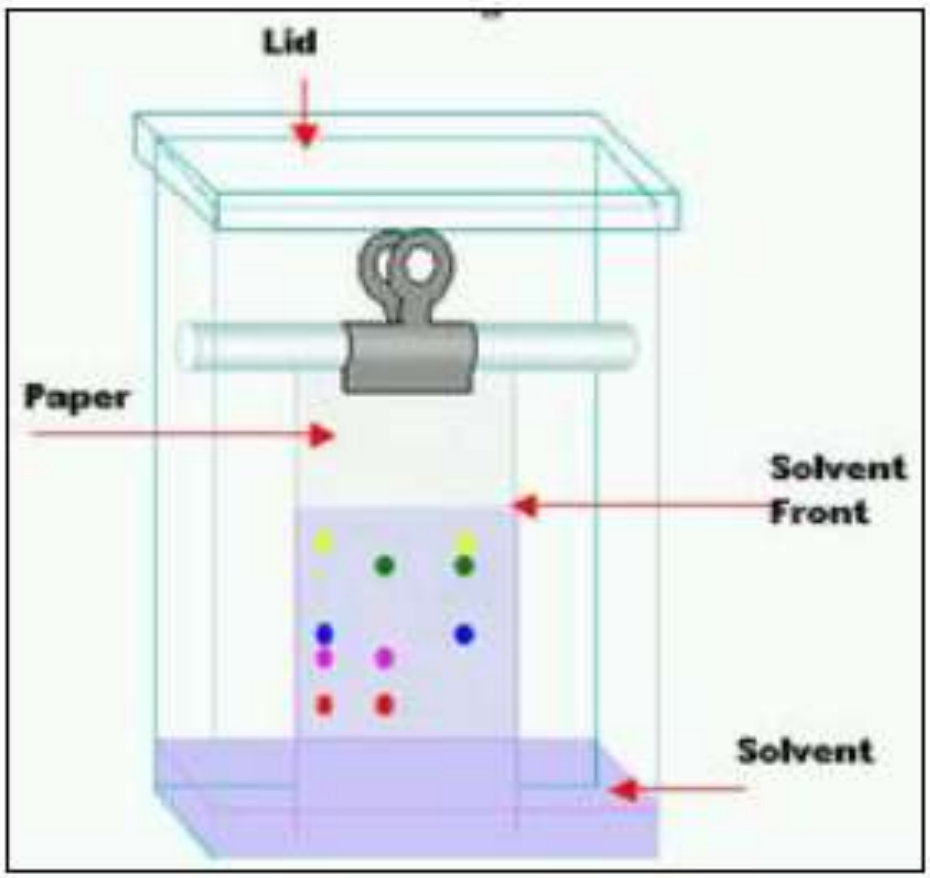
Interpreting the Data



$$R_f = \frac{\text{Distance from start to center of substance spot}}{\text{Distance from start to solvent front}}$$

The Rf (retention factor) value for each spot should be calculated. It is characteristic for any given compound on the same stationary phase using the same mobile phase for development of the plates. Hence, known Rf values can be compared to those of unknown substances to aid in their identifications.

Paper Chromatography

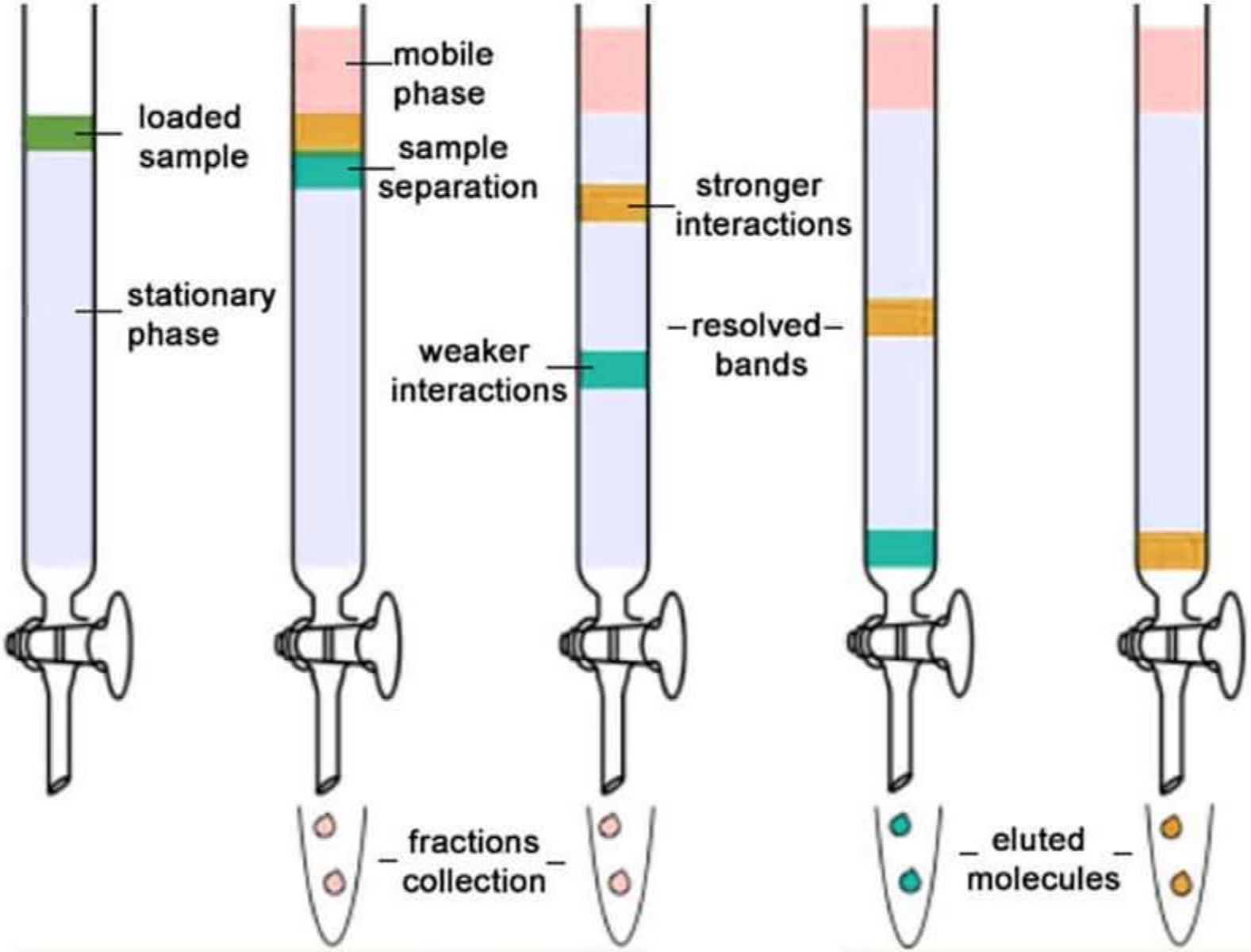


- A method of partition chromatography using filter paper strips as carrier or inert support. The factor governing separation of mixtures of solutes on filter paper is the partition between two immiscible phases. One is usually water adsorbed on cellulose fibres in the paper (stationary phase). The second is the organic solvent flows past the sample on the paper (stationary phase).

$$K = \frac{c(stationary)}{c(mobile)}$$

Column chromatography

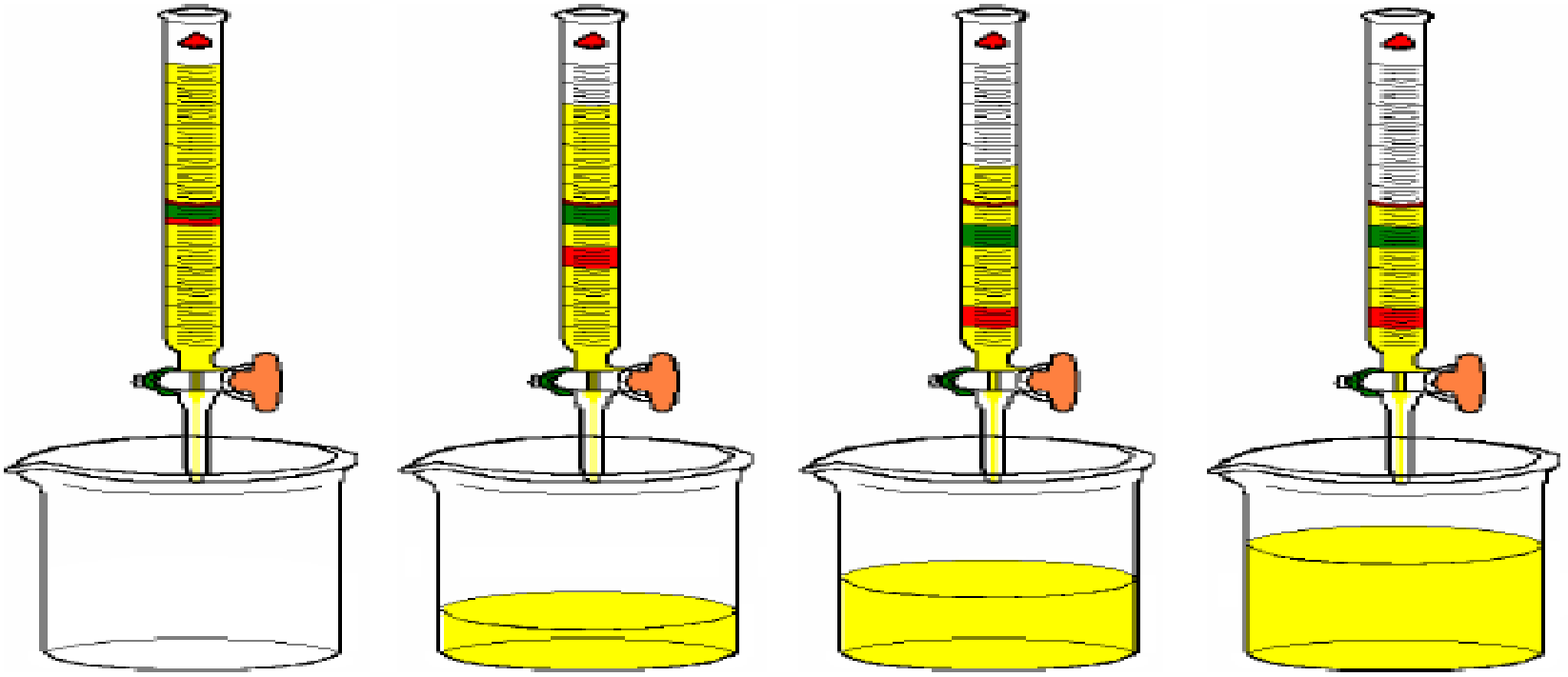
- Column chromatography is the separation technique where the components in a mixture are separated on the basis of their differential adsorption with the stationary phase, resulting in them moving at different speeds when passed through a column.
- It is a solid-liquid chromatography technique in which the stationary phase is a solid & mobile phase is a liquid or gas.
- **Principle of Column chromatography**
- This technique is based on the principle of differential adsorption where different molecules in a mixture have different affinities with the absorbent present on the stationary phase.
- The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
- However, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.
- Here, the stationary phase in the column chromatography also termed the absorbent, is a solid (mostly silica) and the mobile phase is a liquid that allows the molecules to move through the column smoothly.


loaded sample
stationary phase
mobile phase
sample separation
weaker interactions
stronger interactions
resolved bands
fractions collection
eluted molecules

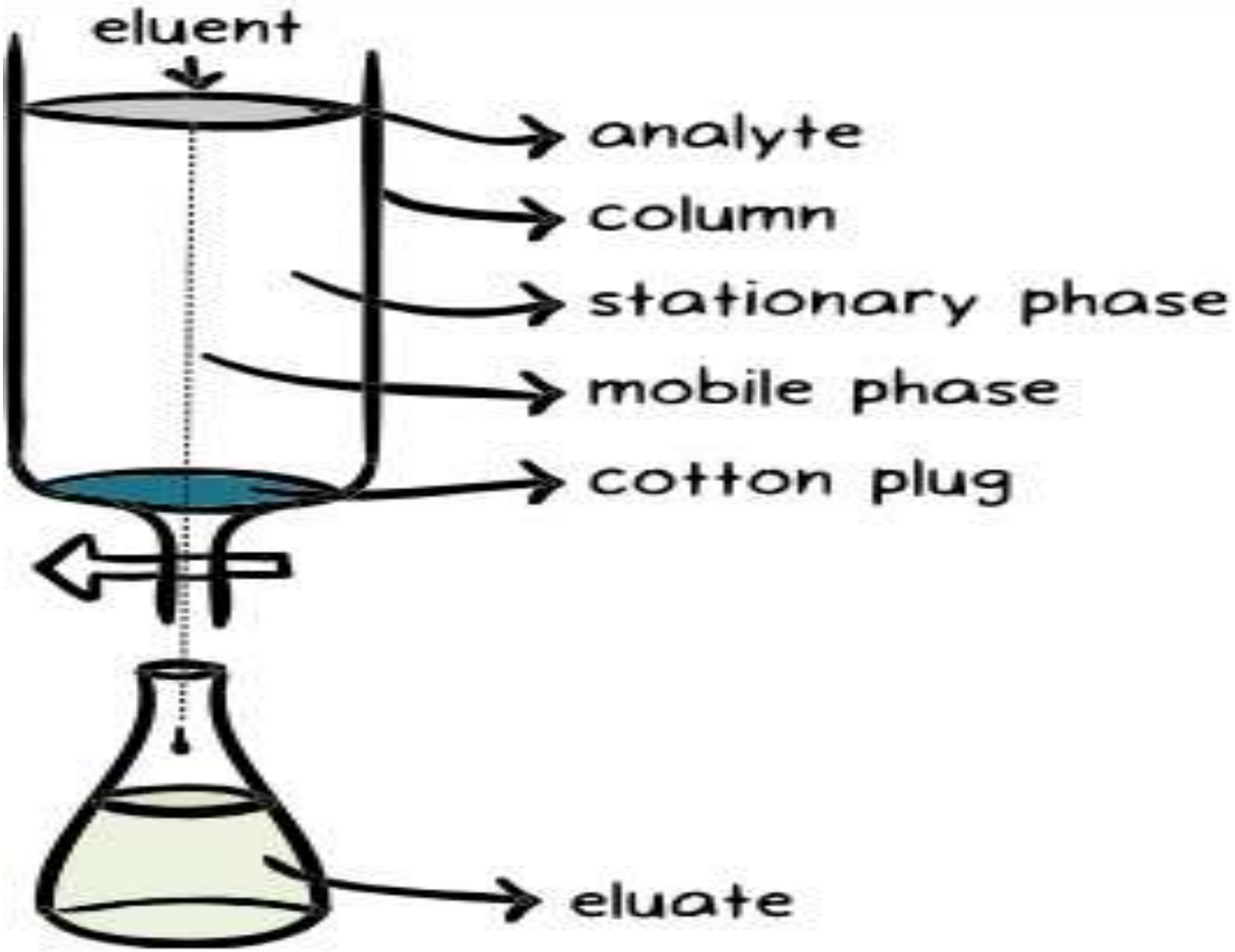
Steps of Column chromatography

- The column is prepared by taking a glass tube that is dried and coated with a thin, uniform layer of stationary phase (cellulose, silica).
- Then the sample is prepared by adding the mixture to the mobile phase. The sample is introduced into the column from the top and is allowed to pass the sample under the influence of gravity.
- The molecules bound to the column are separated by elution technique where either solution of the same polarity is used (isocratic technique), or different samples with different polarities are used (gradient technique).
- The separated molecules can further be analyzed for various purposes.

Column Chromatography



Stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or under the effect of gravity


eluent
analyte
column
stationary phase
mobile phase
cotton plug
eluate

Factors affecting solutes separation in CC

Factor	Effect
Particle size of solid stationary phase (or of support)	Decrease of size improves separation (but very small particles need high pressure).
Column dimensions	Efficiency increases as ratio length / width increases.
Uniformity of packing	Non uniform packing results in irregular movement of solutes through column & less uniform zone formation, (i.e. band broadning or tailing).
Column temperature	Increase in column temperature results in speed of elution but does not improve separation (tailing).
Eluting solvent	Solvents should be of low viscosity (to give efficient resolution) & high volatility (to get rapid recovery of the substances).
Solvent flow rate	Uniform & low flow rate gives better resolution.
Continuity of flow	Discontinuous flow disturbs resolution
Condition of adsorbent	Deactivation of adsorbent decreases separation.
Concentration of solutes	Substances of high concentration move slowly.

Types of Column Chromatography

Mode or type	Stationary phase	Mobile phase	Mechanism
Adsorption Chromatography	Solid that attracts the solutes	Liquid or gas	Solutes move at different rates according to the forces of attraction to the stationary phase.
Partition Chromatography	Thin film of liquid formed on the surface of a solid inert support	Liquid or gas	Solutes equilibrate between the 2 phases according to their partition coefficients
Ion Exchange Chromatography	Solid resin that carries fixed ions & mobile couterions of opposite charge attached by covalent bonds	Liquid containing electrolytes	Solute ions of charge opposite to the fixed ions are attracted to the resin by electrostatic forces & replace the mobile counterions.
Molecular Exclusion Chromatography	Porous gel with no attractive action on solute molecules	Liquid	Molecules separate according to their size: 1.Smaller molecules enter the pores of the gel, and need a larger volume of eluent. 2.Larger molecules pass through the column at a faster rate.
Affinity Chromatography	Solid on which specific molecules are immobilized	Liquid or gas	Special kind of solute molecules interact with those immobilized on the stationary phase

Uses of Column chromatography

- Column chromatography is routinely used for the separation of impurities and purification of various biological mixtures.
- This technique can also be used for the isolation of active molecules and metabolites from various samples.
- Column chromatography is increasingly used for the detection of drugs in crude extracts.
- **Examples of Column chromatography**
- Extraction of pesticides from solid food samples of animal origin containing lipids, waxes, and pigments.
- Synthesis of Pramlintide which is an analog of Amylin, a peptide hormone, for treating type 1 and type 2 Diabetics.
- Purification of bioactive glycolipids, showing antiviral activity towards HSV-1 (Herpes Virus).

Applications of Chromatography

Pharmaceutical sector

To identify and analyze samples for the presence of trace elements or chemicals.

Separation of compounds based on their molecular weight and element composition.

Detects the unknown compounds and purity of mixture.

In drug development.

Chemical industry

In testing water samples and also checks air quality.

HPLC and GC are very much used for detecting various contaminants such as polychlorinated biphenyl (PCBs) in pesticides and oils.

In various life sciences applications

Food Industry

In food spoilage and additive detection

Determining the nutritional quality of food

Forensic Science

In forensic pathology and crime scene testing like analyzing blood and hair samples of crime place.

Molecular Biology Studies

Various hyphenated techniques in chromatography such as EC-LC-MS are applied in the study of metabolomics and proteomics along with nucleic acid research.

HPLC is used in Protein Separation like Insulin Purification, Plasma Fractionation, and Enzyme Purification and also in various departments like Fuel Industry, biotechnology, and biochemical processes.