UNIT 3 – BIOPHYSICAL TECHNIQUES – 2 – NOTES

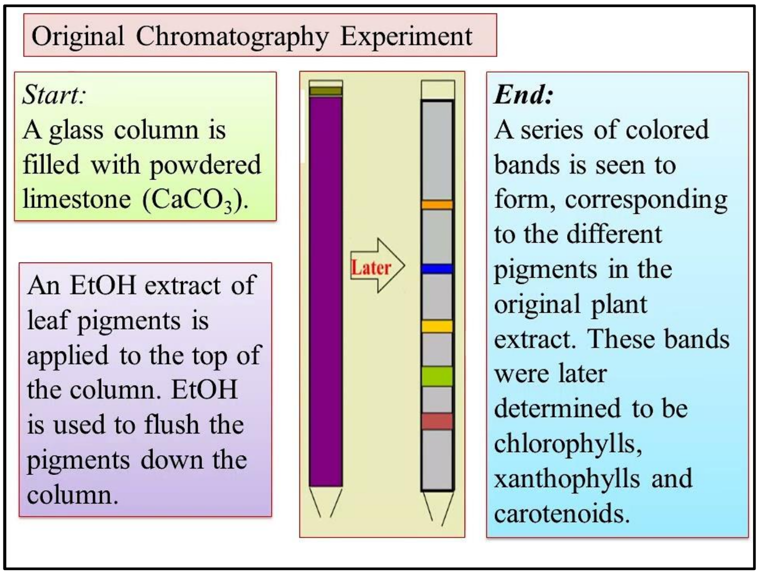
Chromatography

Michael Tswett Experiment

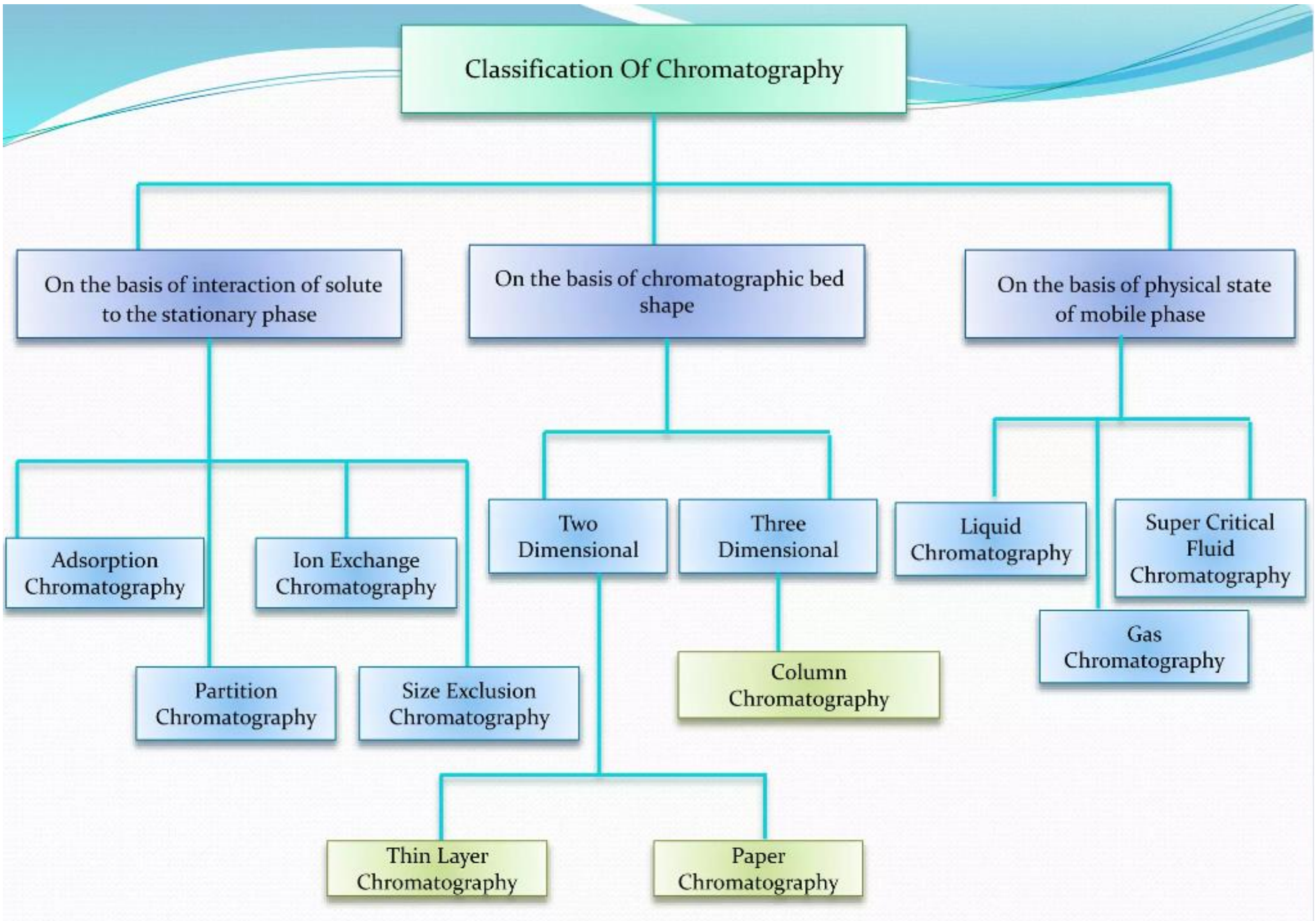
Mikhail Tswett, a Russian botanist, is credited with the invention of chromatography in 1901 during his research on plant pigments. His innovative approach to separating chlorophyll and carotenoid pigments laid the groundwork for modern chromatographic techniques. He used liquid-adsorption column chromatography with calcium carbonate as adsorbent and petrol ether/ethanol mixtures as eluent to separate chlorophylls and carotenoids.

Key Points of Tswett's Experiment

- **Objective**:
  - To separate different pigments found in plant leaves, specifically chlorophyll and carotenoids.
- **Materials Used**:
  - **Column**: A vertical glass column filled with calcium carbonate as the stationary phase.
  - **Solvents**: A mixture of ether and alcohol was used to extract the pigments from the leaves.
- **Experimental Procedure**:
  1. **Extraction**: Leaves were crushed, and pigments were extracted using a solvent mixture of ether and alcohol.
  2. **Column Packing**: The column was packed with calcium carbonate, which served as the stationary phase for adsorption.
  3. **Sample Application**: The pigment extract was introduced at the top of the column.
  4. **Elution Process**:
     - As the solvent moved through the column, different pigments were eluted at different times based on their affinity for the stationary phase.
     - The separation occurred due to varying interactions between the pigments and calcium carbonate.
- **Observations**:
  - Different pigments appeared as distinct colored bands along the column.
  - The experiment demonstrated that multiple pigments could be separated simultaneously, which was groundbreaking at the time.
- **Significance**:
  - Tswett's work established that chromatography could effectively separate complex mixtures based on differential adsorption properties.
  - His findings contributed to a broader understanding of plant biochemistry and opened avenues for further research in analytical chemistry.



Mobile Phase	Stationary Phase	Principle of Separation (Differences in …)	Container	Name of the Chromatography technique
Liquid	Solid	Adsorption	Column	Liquid – solid adsorption column chromatography
Liquid	Solid	Adsorption	TL	Liquid – solid adsorption thin layer chromatography
Liquid	Liquid	Partitioning	Column	Liquid – liquid partition column chromatography
Liquid	Liquid	Partitioning	TL	Liquid – liquid partition thin layer chromatography
Gas	Liquid	Partitioning	Column	Gas – liquid partition column chromatography
Gas	Solid	Adsorption	Column	Gas – solid adsorption column chromatography



Liquid Chromatography

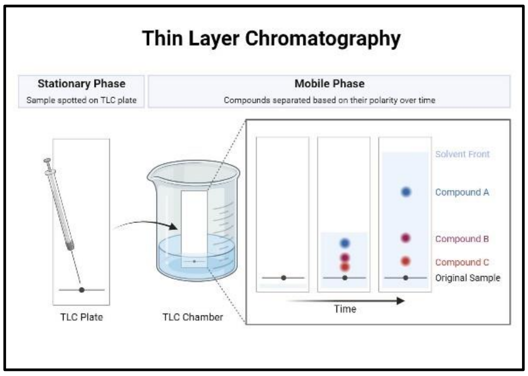
Thin Layer Chromatography and HPTLC

TLC

Thin Layer Chromatography (TLC) is a widely used analytical technique for separating and identifying components of a mixture. It employs a thin layer of adsorbent material on a flat plate, allowing for the separation of compounds based on their different affinities for the stationary and mobile phases.

Principle of TLC

- **Separation Mechanism**: TLC operates on the principles of **adsorption chromatography** and **partition chromatography**. The separation is based on the differential affinities of compounds for the stationary phase (the adsorbent) and the mobile phase (the solvent).
- **Stationary Phase**:
  - A thin layer of an adsorbent material, typically silica gel or alumina, is coated on a flat, inert substrate such as glass, plastic, or aluminum foil.
  - The stationary phase interacts with the components in the mixture based on their polarity and chemical properties.
- **Mobile Phase**:
  - A solvent or solvent mixture that moves up the plate via capillary action.
  - The choice of solvent affects how far and how quickly each component moves up the plate.



Working Mechanism

1. **Preparation**:
   - A TLC plate is prepared by applying a thin layer of adsorbent material.
   - The plate is marked with a pencil to indicate where samples will be spotted.
2. **Sample Application**:
   - Small spots of the sample solution are applied near the base of the plate using a micropipette or capillary tube.
   - The spots should be small to prevent overlapping during separation.
3. **Development**:
   - The TLC plate is placed in a developing chamber containing a small amount of mobile phase solvent.
   - The solvent rises up the plate by capillary action, carrying the sample components with it.
4. **Separation**:
   - As the solvent ascends, different components of the mixture travel at different rates due to their varying affinities for the stationary phase and mobile phase.
   - Components that interact more strongly with the stationary phase move slower, while those that have a higher affinity for the mobile phase move faster.
5. **Visualization**:
   - After development, the plate is removed from the chamber and dried.
   - Spots can be visualized under UV light or by using chemical stains to identify separated components.
6. **Rf Value Calculation**:
   - The retention factor (Rf) is calculated for each component using the formula:

$$R_f = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by solvent front}}$$

   - Rf values help in identifying compounds based on their movement relative to the solvent front.

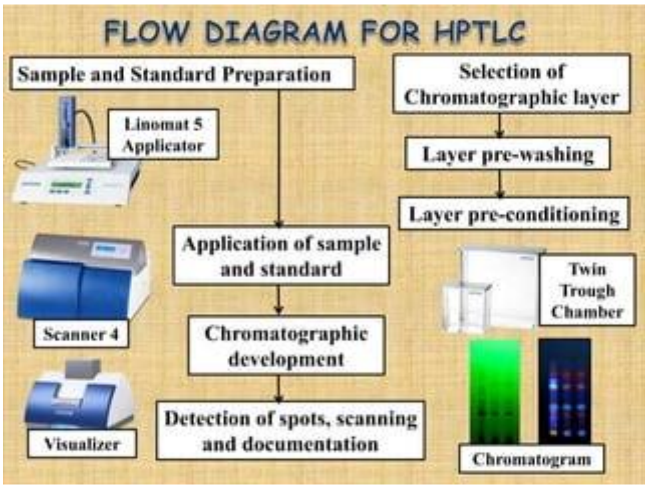
HPTLC

High-Performance Thin Layer Chromatography (HPTLC) is an advanced form of traditional Thin Layer Chromatography (TLC) that enhances the separation efficiency, speed, and sensitivity of the analysis. HPTLC is widely used for both qualitative and quantitative analysis in various fields such as pharmaceuticals, environmental testing, and food safety.

Principle of HPTLC

- **Separation Mechanism**: HPTLC operates on the same principle as TLC, which involves the separation of compounds based on their differential adsorption to a stationary phase and their solubility in a mobile phase.
- **Stationary Phase**:
  - HPTLC uses plates coated with a thin layer of optimized silica gel with a smaller particle size (typically 5-6 µm) compared to traditional TLC plates (10-12 µm).
  - The smaller particle size allows for higher packing density and smoother surfaces, reducing sample diffusion and resulting in sharper bands.
- **Mobile Phase**:
  - The choice of solvent or solvent mixture as the mobile phase is critical. It is selected based on the nature of the analytes and their interactions with the stationary phase.
  - The mobile phase travels up the plate by capillary action, carrying the sample components with it.

Working Mechanism



1. **Plate Preparation**:
   - HPTLC plates are prepared with a thin layer of silica gel optimized for high performance.
   - The plates are generally pre-conditioned in a saturated atmosphere to ensure uniform solvent vapors during development.
2. **Sample Application**:
   - Samples are applied to the plate using an automated sampler that precisely spots small volumes (0.1 - 0.5 µL) in a controlled manner.
   - This automation helps maintain reproducibility and accuracy in sample application.
3. **Development**:
   - The HPTLC plate is placed in a developing chamber containing the mobile phase.
   - Different development methods can be used, including vertical, circular, or anticircular modes, depending on the desired outcome.
   - The mobile phase ascends through capillary action, carrying the sample components along.
4. **Separation**:
   - As the solvent moves up the plate, components separate based on their affinities for the stationary phase versus the mobile phase.
   - Components with higher affinity for the stationary phase will move more slowly than those with higher affinity for the mobile phase.
5. **Visualization and Analysis**:
   - After development, the plate can be visualized using UV light or by applying specific reagents to reveal separated components.
   - Densitometric scanning is often employed for quantitative analysis, allowing for precise measurement of component concentrations.
6. **Data Storage**:
   - HPTLC systems can store chromatographic data electronically, enabling easy retrieval and comparison for future analyses.

Handling of Instrumentation

- **Equipment Required**:
    - **Chromatography Plates**: High-quality HPTLC plates with optimized silica gel.
    - **Automated Sampler**: For precise sample application.
    - **Developing Chamber**: Can be manual or automatic; twin-trough chambers are commonly used for optimal results.
    - **Scanner/Detector**: For densitometric analysis and visualization of separated components.
- **Safety and Maintenance**:
    - Regular maintenance is essential to prevent contamination and ensure accurate results.
    - Proper handling of chemicals and biological samples should be observed to ensure safety.

Applications of HPTLC

HPTLC has numerous applications across various fields:

- **Pharmaceutical Analysis**: Purifying and analyzing active pharmaceutical ingredients (APIs) and excipients.
- **Environmental Testing**: Detecting pollutants and contaminants in soil and water samples.
- **Food Safety**: Analyzing food products for additives, contaminants, or nutritional content.
- **Biochemical Research**: Studying complex mixtures such as proteins, peptides, and nucleic acids.
- **Quality Control**: Ensuring consistency and compliance in manufacturing processes.

High Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is a sophisticated analytical technique used to separate, identify, and quantify components in a mixture. It is widely utilized in various fields such as pharmaceuticals, environmental analysis, food safety, and biochemical research.

Principle of HPLC

- **Separation Mechanism**: HPLC operates on the principle of partitioning or adsorption chromatography, where components of a mixture are separated based on their interactions with a stationary phase and a mobile phase.
- **Stationary Phase**:
  - The stationary phase is typically a packed column containing small particles (usually silica) that provide a large surface area for interactions with the analytes.
  - The choice of stationary phase can vary depending on the type of HPLC being performed (e.g., normal phase, reverse phase).
- **Mobile Phase**:
  - The mobile phase is a liquid solvent or mixture of solvents that carries the sample through the column.
  - The composition of the mobile phase can be adjusted to optimize separation and is delivered at high pressure by pumps.

Working Mechanism

1. **System Components**:
   - **Solvent Reservoirs**: Hold the mobile phase solvents.
   - **Pumps**: Deliver the mobile phase at high pressure and constant flow rate.
   - **Degasser**: Removes dissolved gases from the mobile phase to prevent bubble formation in the system.
   - **Injector**: Introduces the sample into the mobile phase stream.
   - **Column**: Contains the stationary phase where separation occurs.
   - **Detector**: Monitors eluted components and generates signals for analysis.
2. **Sample Injection**:
   - A precise volume of the sample is injected into the mobile phase stream using an injector valve.
   - The sample mixes with the mobile phase and flows into the column.
3. **Separation Process**:
   - As the sample passes through the column, different components interact with the stationary phase to varying degrees based on their chemical properties (polarity, size, etc.).
   - Components that have stronger interactions with the stationary phase will move more slowly than those with weaker interactions, leading to separation.
4. **Detection**:
   - After exiting the column, components enter a detector (commonly UV-Vis or fluorescence).
   - The detector generates a signal proportional to the concentration of each component as they elute from the column over time.
5. **Data Analysis**:
   - The signals from the detector are recorded as a chromatogram, which displays peaks corresponding to different components in the sample.
   - The area under each peak is proportional to the amount of each component present in the sample.
6. **Retention Time**:
   - Each component has a specific retention time, which is used for identification and quantification.

Adsorption Chromatography

Adsorption chromatography is a separation technique that relies on the differential adsorption of compounds onto a solid stationary phase. This method is widely used for the separation and purification of various chemical mixtures.

Principle of Adsorption Chromatography

- **Adsorption Mechanism**: The fundamental principle of adsorption chromatography is based on the interaction between the adsorbent (stationary phase) and the adsorbate (components of the mixture). Molecules in the mixture adhere to the surface of the adsorbent through weak intermolecular forces such as van der Waals forces and hydrogen bonding.
- **Separation Process**: As a mobile phase (liquid or gas) flows over the stationary phase, different components of the mixture are retained to varying degrees based on their affinities for the adsorbent. Components with stronger interactions will move more slowly, while those with weaker interactions will elute faster.

Working Mechanism

1. **Column Preparation**:
    - The column is packed with an adsorbent material, typically silica gel, alumina, or activated carbon. The choice of adsorbent depends on the nature of the compounds to be separated.
2. **Sample Application**:
    - A mixture is introduced at the top of the column. The sample can be in liquid or dissolved form, depending on whether liquid-solid or gas-solid chromatography is being performed.
3. **Elution**:
    - A mobile phase is continuously passed through the column. As it moves, it carries the components of the mixture along with it.
    - Different components interact differently with the stationary phase; those with a higher affinity for the adsorbent will be retained longer than those with lower affinity.
4. **Separation**:
    - As components travel down the column, they separate into distinct bands based on their adsorption characteristics.
    - The retention time for each component depends on its interaction with both the mobile and stationary phases.
5. **Detection and Analysis**:
    - After elution, components can be detected using various methods (e.g., UV-Vis spectroscopy) to generate a chromatogram, which displays peaks corresponding to different components.
6. **Rf Value Calculation**:
    - The retention factor (Rf) can be calculated for each component using:

$$R_f = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by solvent front}}$$

Partition Chromatography

Partition chromatography is a separation technique that relies on the differential partitioning of compounds between two liquid phases: a stationary phase and a mobile phase. This method is particularly useful for separating components in a mixture based on their solubility and affinity for each phase.

Principle of Partition Chromatography

- **Separation Mechanism**: The principle behind partition chromatography is based on the distribution of analytes between two immiscible liquid phases. The stationary phase is typically a liquid that is coated onto a solid support, while the mobile phase is another liquid that moves through or over the stationary phase.
- **Partition Coefficient**: The separation of components depends on their partition coefficients, which measure the ratio of concentrations of a compound in the stationary phase to that in the mobile phase. Compounds with higher partition coefficients tend to remain longer in the stationary phase, while those with lower coefficients move more quickly with the mobile phase.

Working Mechanism

1. **Column Preparation**:
    - In liquid-liquid partition chromatography, a solid support (like silica gel or alumina) is coated with a liquid stationary phase. In paper chromatography, cellulose fibers in the paper act as the stationary phase, holding water or another solvent.
2. **Sample Application**:
    - A small amount of the sample mixture is applied at the baseline of the stationary phase (e.g., on chromatography paper).
3. **Development**:
    - The mobile phase (a solvent or solvent mixture) is introduced into the system. In paper chromatography, this involves placing the paper in a developing chamber containing the solvent.
    - The solvent rises through capillary action, carrying the sample components with it.
4. **Separation**:
    - As the solvent moves up, different components of the sample separate based on their affinities for the stationary and mobile phases.
    - Components that are more soluble in the mobile phase will travel further than those that prefer to stay in the stationary phase.
5. **Visualization**:
    - After development, separated components can be visualized using UV light or chemical stains.
    - Each component appears as a distinct spot or band on the chromatogram.
6. **Data Analysis**:
    - The distance traveled by each component relative to the solvent front can be measured to calculate retention factors (Rf values), aiding in identification and quantification.

Affinity Chromatography

Affinity chromatography is a powerful and highly specific technique used for the separation and purification of biomolecules based on their specific interactions with a ligand. This method exploits the natural binding affinity between biomolecules, making it an essential tool in biochemistry and molecular biology.

Principle of Affinity Chromatography

- **Specific Binding**: The fundamental principle of affinity chromatography is based on the highly specific interactions between a target biomolecule and an immobilized ligand on a solid support (stationary phase). These interactions can include:
    - Antigen-antibody interactions
    - Enzyme-substrate relationships
    - Receptor-ligand binding
    - Protein-nucleic acid interactions
- **Stationary Phase**: The stationary phase consists of a matrix (commonly agarose or polyacrylamide) that is chemically modified to covalently attach a ligand. This ligand is specifically chosen for its ability to bind the target molecule.
- **Mobile Phase**: The mobile phase typically contains a mixture of biomolecules, including the target and non-target molecules. As this mixture passes through the column, only the target molecules will bind to the stationary phase due to their affinity for the ligand.

Working Mechanism

1. **Matrix Preparation**:
    - The matrix (e.g., agarose beads) is prepared by covalently attaching a specific ligand that will interact with the target biomolecule.
    - The choice of ligand depends on the biomolecule of interest (e.g., antibodies for antigens, metal ions for histidine-tagged proteins).
2. **Column Setup**:
    - The prepared matrix is packed into a chromatography column.
    - The column is equilibrated with a buffer that mimics physiological conditions to maintain optimal binding.
3. **Sample Application**:
    - A crude sample containing the target biomolecule is loaded onto the column.
    - Molecules that do not bind to the ligand are washed away, while those that have an affinity for the ligand remain bound.
4. **Washing**:
    - A wash buffer is applied to remove weakly bound contaminants without eluting the target biomolecule.
5. **Elution**:
    - The bound target molecule is eluted using an elution buffer that disrupts the interaction between the target and the ligand. This can be achieved by altering conditions such as pH, ionic strength, or by adding a competing ligand.
    - The eluted fractions are collected for further analysis or use.
6. **Recycling Matrix**:
    - After elution, the matrix can be regenerated and reused for subsequent purification cycles.

Handling of Instrumentation

- **Equipment Required**:
    - **Chromatography Column**: A glass or plastic column where the matrix is packed.
    - **Pumps**: To deliver buffers at controlled flow rates.
    - **Fraction Collector**: To collect eluted fractions automatically.
    - **UV Detector**: To monitor the elution of biomolecules based on absorbance.
- **Safety and Maintenance**:
    - Regular maintenance of equipment is essential to prevent contamination.
    - Proper handling of chemicals and biological samples should be observed to ensure safety.

Applications of Affinity Chromatography

Affinity chromatography has numerous applications across various fields:

- **Protein Purification**: Isolating specific proteins from complex mixtures, such as cell lysates or serum.
- **Enzyme Assays**: Purifying enzymes for kinetic studies or functional assays.
- **Antibody Isolation**: Separating antibodies from serum or hybridoma cultures.
- **Nucleic Acid Purification**: Isolating DNA or RNA based on specific binding interactions.
- **Biopharmaceutical Production**: Purifying therapeutic proteins, vaccines, and monoclonal antibodies.
- **Research Applications**: Studying protein-protein interactions, enzyme-substrate relationships, and receptor-ligand dynamics.

Ion – Exchange Chromatography

Ion exchange chromatography (IEC) is a powerful analytical technique used to separate and purify charged molecules, such as ions and polar molecules, based on their affinity for ion exchangers. It is widely employed in biochemistry, environmental analysis, and pharmaceuticals.

Principle of Ion Exchange Chromatography

- **Separation Mechanism**: The principle of ion exchange chromatography is based on the reversible exchange of ions between the target ions in the sample solution and the ions present on an insoluble stationary phase (ion exchanger).
- **Ion Exchangers**:
  - **Cation Exchange**: The stationary phase contains negatively charged groups that attract positively charged ions (cations). For example, a cation exchange resin may contain sulfonic acid groups that bind cations.
  - **Anion Exchange**: Conversely, the stationary phase consists of positively charged groups that attract negatively charged ions (anions). An anion exchange resin might contain quaternary ammonium groups.
- **Equilibrium**: As the sample passes through the column, ions in the mobile phase compete with counter-ions on the stationary phase for binding sites. The equilibrium can be shifted by adjusting the ionic strength of the mobile phase.

Working Mechanism

1. **Column Preparation**:
   - The column is packed with an ion exchange resin that has been equilibrated with a buffer solution containing counter-ions. This ensures that the stationary phase is ready to interact with incoming sample ions.
2. **Sample Application**:
   - A sample containing a mixture of cations or anions is introduced into the column. Under low ionic strength conditions, target ions will bind to the oppositely charged sites on the resin while non-target ions pass through.
3. **Washing**:
   - A wash buffer is applied to remove any unbound or weakly bound species from the column without eluting the target ions.
4. **Elution**:
   - The bound target ions are eluted by increasing the ionic strength of the mobile phase or by changing its composition (e.g., adding a competing ion). This disrupts the interaction between the target ions and the stationary phase, allowing them to be released in a controlled manner.
   - Two common elution methods are:
     - **Gradient Elution**: Gradually increasing salt concentration to elute bound molecules based on their binding strength.
     - **Step Elution**: Using discrete concentrations of salt to elute different fractions.
5. **Detection and Analysis**:
   - After elution, components can be detected using various methods such as conductivity detection or UV-Vis spectroscopy. The resulting chromatogram displays peaks corresponding to different ions based on their retention times.

Handling of Instrumentation

- **Instrumentation Components**:
  - **Pumps**: Deliver buffers at controlled flow rates.
  - **Injector**: Introduces samples into the flow path.
  - **Column**: Contains packed ion exchange resin.
  - **Detector**: Monitors eluted components.
  - **Fraction Collector**: Collects eluted fractions for further analysis.
- **Safety and Maintenance**:
  - Regular cleaning and maintenance of equipment are crucial to prevent contamination and ensure accurate results.

- Proper handling of chemicals should be observed to ensure safety in laboratory environments.

Applications of Ion Exchange Chromatography

Ion exchange chromatography has numerous applications across various fields:

- **Protein Purification**: Isolating proteins based on their charge properties during biochemical research.
- **Water Quality Testing**: Analyzing water samples for heavy metals and other contaminants.
- **Pharmaceutical Analysis**: Purifying active pharmaceutical ingredients (APIs) and excipients.
- **Food Safety Testing**: Detecting food additives, preservatives, and contaminants.
- **Biotechnology Research**: Studying enzyme kinetics and protein interactions.

Gas Chromatography

Gas chromatography (GC) is an analytical technique used to separate and analyze volatile compounds in a mixture. It is widely utilized in various fields, including environmental monitoring, pharmaceuticals, food safety, and forensic science.

Principle of Gas Chromatography

- **Separation Mechanism**: GC relies on the partitioning of compounds between a mobile phase (carrier gas) and a stationary phase (coating inside the column). The components of the sample are vaporized and carried through the column by the inert gas. The separation occurs based on differences in volatility and interaction with the stationary phase.
- **Mobile Phase**: The mobile phase is typically an inert gas such as helium, nitrogen, or hydrogen. Helium is commonly used due to its inertness and suitable thermal conductivity.
- **Stationary Phase**: The stationary phase can be a solid or liquid coating on the inner walls of the column. The choice of stationary phase depends on the chemical properties of the analytes being separated.

Working Mechanism

1. **Sample Injection**:
    - A small volume of the sample is injected into the heated injection port using a microsyringe. The sample must be vaporized immediately upon injection.
    - The injection can be performed in split or splitless mode:
        - **Split Injection**: Only a portion of the sample is introduced into the column; excess is vented to waste.
        - **Splitless Injection**: The entire sample is introduced into the column for low-concentration samples.
2. **Vaporization**:
    - The injected sample is rapidly vaporized in the hot injection port, mixing with the carrier gas.
3. **Separation in the Column**:
    - The vaporized sample travels through a long, narrow column (typically 10-150 m) coated with a stationary phase.
    - As compounds interact with the stationary phase, they are separated based on their boiling points and affinities for the stationary phase.
4. **Detection**:
    - After elution from the column, components are detected using various detectors (e.g., Flame Ionization Detector (FID), Thermal Conductivity Detector (TCD)).
    - The detector generates a signal for each component, producing a chromatogram that shows peaks corresponding to different analytes.
5. **Data Analysis**:
    - Retention times and peak areas are analyzed to identify and quantify components in the mixture.

Key Parameters in Gas Chromatography

1. **Retention Time (Rt)**:
    - The time taken for a compound to travel through the column and reach the detector. It is specific to each compound under given conditions.
2. **Retention Time of an Unretained Compound**:
    - This refers to compounds that do not interact with the stationary phase and pass through quickly; it helps establish baseline conditions.
3. **Retention Factor (k)**:
    - Indicates how long a compound is retained relative to an unretained compound.
4. **Distribution Constant (K)**:
    - Represents the ratio of concentrations of a compound in the stationary and mobile phases at equilibrium.
5. **Phase Ratio (β)**:
    - The ratio of the volume of stationary phase to that of mobile phase within the column.

6. **Separation Factor (α)**:
   - Indicates how well two compounds are separated based on their retention times.
7. **Number of Theoretical Plates (N)**:
   - A measure of column efficiency; higher values indicate better separation efficiency.
8. **Height Equivalent to a Theoretical Plate (HETP)**:
   - Indicates how tall each theoretical plate would be if stacked; lower values indicate better efficiency.
9. **Carrier Gas Linear Velocity**:
   - Refers to the speed at which carrier gas flows through the column; affects retention time and resolution.
10. **Cold Injection Techniques**:
    - Methods like on-column injection allow samples to enter without vaporization at high temperatures, preserving sensitive compounds.
11. **Headspace Extraction**:
    - A technique used for volatile compounds where only vapors above a liquid or solid sample are analyzed, minimizing matrix interference.
12. **Selection of GC Column**:
    - Columns vary by length, diameter, and stationary phase; selection depends on analyte properties and desired resolution.
13. **Column Temperature**:
    - Controlled heating during analysis affects volatility and separation efficiency; typically ranges from 50°C to 300°C depending on analytes.

Detectors Used in Gas Chromatography

1. **Flame Ionization Detector (FID)**:
   - Detects organic compounds by measuring ions produced in a flame; highly sensitive for hydrocarbons.
2. **Thermal Conductivity Detector (TCD)**:
   - Measures changes in thermal conductivity as analytes elute; suitable for both organic and inorganic compounds.
3. **Flame Photometric Detector (FPD)**:
   - Detects specific elements like sulfur or phosphorus by measuring light emitted during combustion.
4. **Flame Thermionic Detector (FTD)**:
   - Similar to FID but more sensitive for certain compounds due to thermionic emission.
5. **Electron Capture Detector (ECD)**:
   - Highly sensitive for electronegative compounds; detects changes in current caused by analytes capturing electrons.

Applications of Gas Chromatography

- **Qualitative Analysis**: Identifying unknown compounds based on retention time compared to standards.
- **Quantitative Analysis**: Measuring concentrations using peak areas or heights from chromatograms.
- **Standard Addition Method**: A technique used for quantifying analytes by adding known quantities to samples.
- **Environmental Monitoring**: Analyzing pollutants in air, water, and soil samples.
- **Pharmaceuticals**: Quality control and purity testing of drugs.
- **Food Safety Testing**: Detecting contaminants and additives in food products.
- **Forensic Science**: Analyzing substances from crime scenes or toxicology reports.

<u>Troubleshooting GC Problems</u>

1. **Ghost Peaks**:
    - Caused by contamination from previous runs or carryover from injection port; cleaning or replacing parts may be necessary.
2. **Peak Tailing**:
    - Often due to interactions with active sites on the stationary phase; can be minimized by optimizing temperature or using different columns.
3. **Possible Causes for No Peaks**:
    - Issues may include improper injection technique, blocked columns, or incorrect temperature settings.
4. **Possible Causes for Changes in Retention Time**:
    - Changes can arise from variations in carrier gas flow rate, temperature fluctuations, or degradation of stationary phase over time.

Centrifugation Techniques: Principal, type of centrifuge and application

Centrifugation is a laboratory technique that utilizes centrifugal force to separate components in a mixture based on their size, density, shape, and viscosity. This method is essential in various fields, including biochemistry, molecular biology, and clinical diagnostics.

Principle of Centrifugation

- **Centrifugal Force**: The principle of centrifugation relies on the generation of centrifugal force when a sample is spun at high speeds. This force causes denser particles to move outward and settle at the bottom of the container, forming a pellet, while lighter components remain in the supernatant.
- **Sedimentation**: The rate of sedimentation depends on several factors, including the size and density of the particles, the viscosity of the medium, and the speed of rotation. The higher the centrifugal force (measured in g-forces), the faster the separation occurs.

Working Mechanism

1. **Sample Preparation**:
    - Samples are placed in centrifuge tubes or containers that are balanced to prevent vibration during operation.
2. **Centrifuge Selection**:
    - Depending on the application, different types of centrifuges are chosen based on their speed capabilities and temperature control features.
3. **Operation**:
    - The centrifuge is set to a specific speed (RPM) and time duration. As it spins, centrifugal force acts on the particles within the sample.
4. **Separation**:
    - Denser particles sediment at the bottom (forming a pellet), while less dense components remain suspended in the liquid above (supernatant).
5. **Post-Centrifugation**:
    - After spinning, the centrifuge is stopped, and the supernatant can be carefully decanted or pipetted off without disturbing the pellet.

Applications of Centrifugation

- **Clinical Diagnostics**: Used for blood separation to obtain serum or plasma for analysis.
- **Biotechnology Research**: Isolating DNA/RNA from samples or purifying proteins.
- **Environmental Testing**: Analyzing soil or water samples for contaminants.
- **Food Industry**: Separating emulsions or clarifying juices and beverages.

Technique	Principle	Steps	Uses	Examples
Analytical Centrifugation	Measures sedimentation behavior of particles	1. Sample placed in centrifuge 2. High-speed spinning 3. Monitor sedimentation	Determine molecular weight and size distribution of macromolecules. Analyze protein-protein interactions.	Measuring the sedimentation coefficient of proteins.
Density Gradient Centrifugation	Separates particles based on density	1. Prepare density gradient 2. Layer sample on top 3. Centrifuge until particles reach equilibrium	Separating cellular components and organelles.	Isolating ribosomes or DNA from cellular extracts.
Differential Centrifugation	Separates components based on size and density	1. Centrifuge at low speeds to pellet larger particles 2. Collect supernatant and centrifuge at higher speeds	Fractionating cell components for biochemical analysis.	Isolating nuclei, mitochondria, and other organelles from homogenized tissues.
Isopycnic Centrifugation / Equilibrium Centrifugation	Separates particles based on buoyant densities	1. Prepare density gradient 2. Add sample and centrifuge until particles settle	Purifying nucleic acids and proteins based on buoyant density.	Separating plasmid DNA from genomic DNA using cesium chloride gradients.
Rate-Zonal Density Gradient Centrifugation / Moving Zone Centrifugation	Separates particles based on size and shape	1. Prepare density gradient 2. Layer sample on top and centrifuge	Separating proteins or viruses by size.	Fractionating viral particles from cell lysates.
Moving Boundary (Differential Velocity) Centrifugation	Monitors movement of a boundary between two phases	1. Place sample in centrifuge and spin 2. Monitor sedimentation to determine particle characteristics	Analyzing particle size distributions in real-time.	Studying sedimentation behavior of colloidal suspensions.
Equilibrium Density Gradient Centrifugation	Separates particles until they reach an equilibrium position in a density gradient	1. Establish density gradient 2. Add particles and centrifuge	Purifying macromolecules based on buoyant density without altering their structure.	Isolating different RNA species from complex mixtures.
Sucrose Gradient Centrifugation	Uses sucrose solutions to create a density gradient	1. Prepare sucrose gradient 2. Apply sample to top and centrifuge	Separating cellular components like ribosomes or organelles based on size and density.	Isolating ribosomal subunits or separating proteins from cell lysates using sucrose gradients.

Electrophoresis: AGE and PAGE

Electrophoresis is a laboratory technique used to separate charged molecules, such as DNA, RNA, and proteins, based on their size and charge. The technique involves applying an electric field to a gel matrix, causing the charged molecules to migrate at different rates.

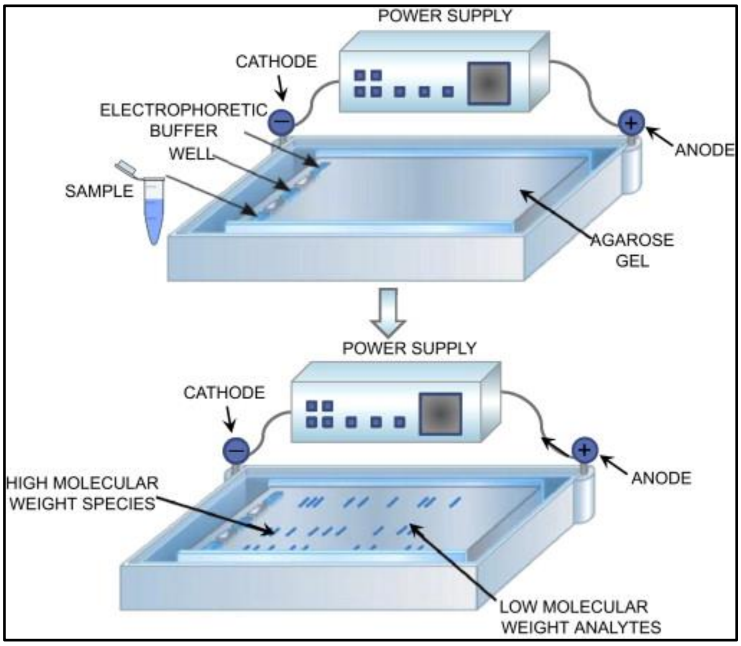
Principle of Electrophoresis

The principle of electrophoresis is based on the movement of charged particles in an electric field. When an electric current is applied, negatively charged molecules (anions) migrate toward the positively charged electrode (anode), while positively charged molecules (cations) move toward the negatively charged electrode (cathode). The rate of migration depends on several factors:

- **Charge**: More highly charged molecules will migrate faster.
- **Size**: Smaller molecules can move through the gel matrix more easily than larger ones.
- **Gel Composition**: The concentration and type of gel affect the pore size and, consequently, the separation efficiency.

Agarose Gel Electrophoresis (AGE)

Agarose Gel Electrophoresis (AGE) is a widely used method for separating nucleic acids (DNA and RNA) and some proteins. It utilizes agarose, a polysaccharide derived from seaweed, to create a gel matrix.



Working Mechanism

1. **Gel Preparation**:
    - Agarose powder is mixed with a buffer solution and heated until dissolved.
    - The solution is poured into a mold to solidify, forming a gel with pores that act as a sieve for separating molecules.
2. **Sample Loading**:
    - DNA or RNA samples are mixed with a loading dye and loaded into wells created in the gel.
3. **Electrophoresis**:
    - An electric current is applied across the gel. The negatively charged nucleic acids migrate toward the positive electrode.
    - Smaller fragments move faster and travel further through the gel than larger fragments.
4. **Visualization**:
    - After electrophoresis, the gel is stained with a DNA-binding dye (e.g., ethidium bromide) and visualized under UV light to observe distinct bands corresponding to different sizes of nucleic acids.

Applications

- Analyzing PCR products.
- Checking the integrity of RNA samples.
- Separating restriction enzyme digests of DNA.

Polyacrylamide Gel Electrophoresis (PAGE)

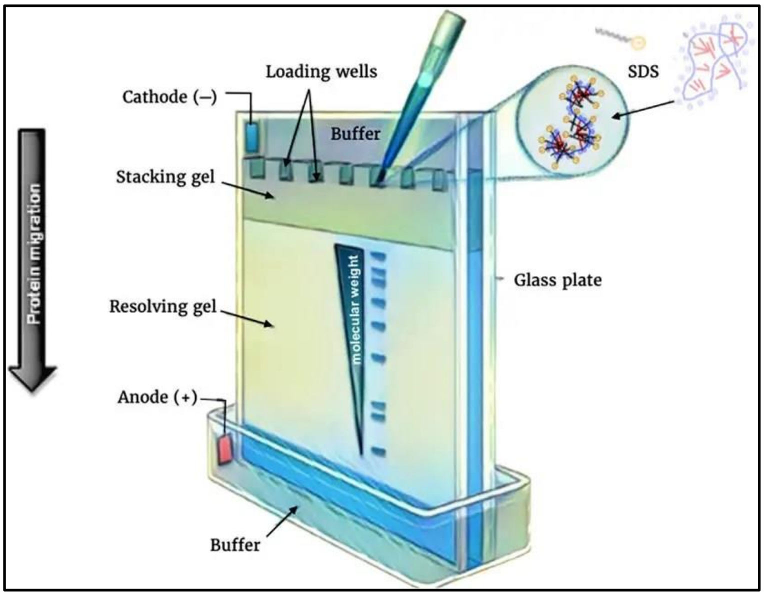
Polyacrylamide Gel Electrophoresis (PAGE) is another electrophoretic technique primarily used for separating proteins based on their size and charge. It employs polyacrylamide as the gel matrix.

Working Mechanism

1. **Gel Preparation**:
   - A solution of acrylamide and bis-acrylamide is polymerized to form a gel with controlled pore sizes.
   - The concentration of acrylamide can be adjusted based on the size of proteins being analyzed.
2. **Sample Loading**:
   - Protein samples are mixed with a loading buffer containing SDS (sodium dodecyl sulfate) to denature proteins and impart a negative charge proportional to their size.
   - Samples are loaded into wells in the gel.
3. **Electrophoresis**:
   - An electric current is applied, causing proteins to migrate through the polyacrylamide matrix.
   - Smaller proteins move faster than larger ones due to less resistance in the gel.
4. **Visualization**:
   - After electrophoresis, gels are stained using Coomassie Brilliant Blue or silver stain to visualize protein bands.

Applications

- Analyzing protein purity and molecular weight.
- Performing western blotting after separation.
- Studying protein-protein interactions.



2-D Electrophoresis

Two-dimensional electrophoresis (2-DE) is a powerful analytical technique used primarily for the separation and characterization of complex protein mixtures. It allows researchers to resolve thousands of proteins in a single gel, making it a fundamental tool in proteomics.

Principle of 2-D Electrophoresis

The principle of 2-DE is based on the separation of proteins in two dimensions:

1. **First Dimension**: Proteins are separated according to their isoelectric points (pI) using **Isoelectric Focusing (IEF)**. In this step, proteins migrate through a pH gradient until they reach the point where their net charge is zero (their isoelectric point).
2. **Second Dimension**: Proteins are then separated based on their molecular weight using **SDS-PAGE** (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). SDS denatures the proteins and imparts a uniform negative charge, allowing separation by size.

This two-step process significantly enhances the resolution of protein separation compared to one-dimensional methods.

Working Mechanism

1. **Sample Preparation**:
    - The sample containing proteins is prepared by solubilizing and denaturing the proteins, often using a buffer that contains urea and β-mercaptoethanol.
    - This ensures that proteins are unfolded and maintain their intrinsic charges.
2. **First Dimension - Isoelectric Focusing**:
    - A gel strip with a pH gradient is prepared.
    - The protein sample is loaded onto the gel strip, and an electric field is applied.
    - Proteins migrate through the gel until they reach their pI, where they stop moving due to having no net charge.
3. **Second Dimension - SDS-PAGE**:
    - After IEF, the gel strip containing focused proteins is placed on top of an SDS-PAGE gel.
    - An electric current is applied again, causing proteins to migrate through the polyacrylamide gel based on their molecular weight.
    - Smaller proteins move faster than larger ones, resulting in distinct bands.
4. **Visualization**:
    - After electrophoresis, the gel is stained with dyes such as Coomassie Brilliant Blue or silver stain to visualize protein spots.
    - Each spot corresponds to a different protein, and the position of these spots provides information about their pI and molecular weight.

Applications of 2-D Electrophoresis

- **Proteomics Research**: Analyzing complex protein mixtures from tissues or cells.
- **Biomarker Discovery**: Identifying potential biomarkers for diseases.
- **Post-Translational Modifications**: Studying modifications that occur after protein synthesis.
- **Quality Control**: Assessing the purity of protein samples in pharmaceuticals.

Isoelectric Focusing

Isoelectric focusing (IEF) is a powerful analytical technique used primarily for the separation of proteins and other biomolecules based on their isoelectric points (pI). The isoelectric point of a protein is the pH at which the molecule carries no net electrical charge. This unique characteristic is crucial for the precise separation and analysis of proteins in complex biological samples.

Principle of Isoelectric Focusing

The fundamental principle behind IEF lies in the concept of the pI of proteins. At their isoelectric point, proteins have an overall net charge of zero. As proteins migrate through a pH gradient under the influence of an electric field, they will gain or lose protons, causing their charge to change. When proteins reach the pH that corresponds to their pI, they stop migrating because there is no net electrostatic force acting on them. This results in the focusing of proteins into sharp bands at their respective pIs.

Working Mechanism

1. **Preparation of the Gel**:
    - A gel strip with a pH gradient is prepared, often using ampholytes that create a stable gradient when subjected to an electric field.
    - The pH gradient typically ranges from acidic (low pH) to basic (high pH).
2. **Sample Application**:
    - The protein sample is loaded onto the gel strip.
    - An electric field is applied across the gel.
3. **Migration**:
    - Proteins begin to migrate toward the electrode of opposite charge. Positively charged proteins move toward the cathode (negative electrode), while negatively charged proteins move toward the anode (positive electrode).
    - As they traverse the gradient, proteins encounter varying pH levels.
4. **Focusing**:
    - As proteins move through regions with different pH values, they either gain or lose protons.
    - When a protein reaches its isoelectric point, its net charge becomes zero, and it stops migrating. This process results in sharp bands forming at specific positions corresponding to each protein's pI.
5. **Visualization and Analysis**:
    - After focusing is complete, the proteins can be visualized using various staining techniques such as Coomassie Brilliant Blue or silver staining.
    - The resulting pattern of bands corresponds to different proteins, each focused at its unique pI.

Applications of Isoelectric Focusing

- **Proteomics Research**: Analyzing complex protein mixtures from tissues or cells.
- **Biomarker Discovery**: Identifying potential biomarkers for diseases.
- **Quality Control**: Assessing the purity and consistency of therapeutic biological products.
- **Clinical Diagnostics**: Diagnosing genetically transmitted disorders by identifying abnormal proteins associated with diseases.

ADSORBENTS

Adsorbents are solid materials that play a crucial role in chromatography, particularly in the separation and purification of chemical compounds. They form the stationary phase in various chromatographic techniques and are responsible for the adsorption of analytes from the mobile phase.

Criteria for Selection of Adsorbents

When selecting an adsorbent for chromatography, several criteria must be considered:

1. **Surface Area**: A higher surface area allows for more interaction sites, enhancing the adsorbent's ability to separate compounds effectively.
2. **Pore Size**: The pore size of the adsorbent should be appropriate for the size of the molecules being separated. Smaller pores are suitable for smaller molecules, while larger pores accommodate larger molecules.
3. **Chemical Compatibility**: The adsorbent must be chemically compatible with the analytes and solvents used to prevent unwanted reactions or degradation.
4. **Polarity**: The polarity of the adsorbent should match or complement that of the analytes to achieve effective separation based on adsorption characteristics.
5. **Stability**: The adsorbent should be stable under the conditions of use, including temperature and solvent composition.
6. **Cost and Availability**: Practical considerations such as cost-effectiveness and availability also influence the choice of adsorbents.

Resin	Description	Applications	Importance
Silica Gel	A porous form of silicon dioxide with a high surface area.	Widely used in normal-phase chromatography and reverse-phase chromatography.	Effective for separating a wide range of organic compounds due to its polar nature.
Alumina (Aluminum Oxide)	A porous form of aluminum oxide available in acidic, basic, and neutral forms.	Used in column chromatography and flash chromatography.	Versatile for separating various organic compounds, especially aromatic compounds.
Ion-Exchange Resins	Polymers with charged functional groups that can exchange ions with sample components.	Commonly used in ion-exchange chromatography for separating charged molecules like proteins and nucleic acids.	Allows selective separation based on charge interactions.
Size-Exclusion Resins	Porous materials that separate molecules based on size.	Used in gel filtration chromatography to separate proteins or polysaccharides.	Ideal for purifying large biomolecules without altering their structure.
Cellulose Microcrystalline	A biodegradable polymer used as an adsorbent.	Employed in thin-layer chromatography (TLC) and paper chromatography.	Useful for separating a variety of organic compounds based on their interactions with cellulose.

Importance of Adsorbents in Chromatography

- **Separation Efficiency**: The choice of adsorbent directly affects the resolution and efficiency of separations, allowing for precise analysis of complex mixtures.
- **Versatility**: Different types of adsorbents can be used to tailor chromatographic methods to specific applications, enhancing the versatility of analytical techniques.
- **Reproducibility**: High-quality adsorbents contribute to reproducible results across different experiments, which is crucial for both research and industrial applications.
- **Scalability**: Adsorbents allow for scaling up from analytical to preparative chromatography, enabling larger quantities of materials to be processed without loss of resolution.