Unit-3

Instrumental Methods of Analysis

B.Pharma 7 Sem Notes

Unit: 3

Introduction to chromatography:

- **Adsorption and partition column chromatography-** Methodology, advantages, disadvantages and applications.
- **Thin layer chromatography-** Introduction, Principle, Methodology, Rf values, advantages, disadvantages and applications.
- **Paper chromatography-** Introduction, methodology, development techniques, advantages, disadvantages and applications.
- **Electrophoresis**– Introduction, factors affecting electrophoretic mobility, Techniques of paper, gel, capillary electrophoresis, applications.

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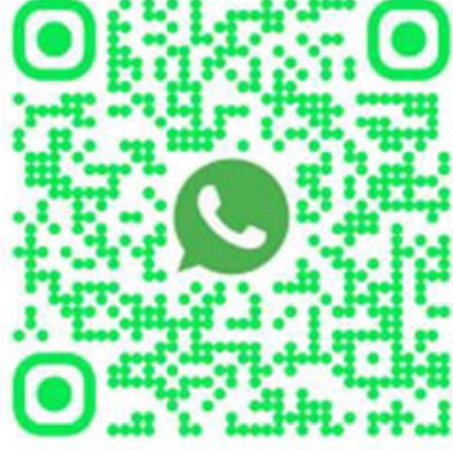

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INTRODUCTION TO CHROMATOGRAPHY

What is Chromatography?

Chromatography is a powerful physical separation technique used to separate, identify, and quantify the components of a mixture. The word comes from Greek: 'chroma' (colour) + 'graphein' (to write) — because it was first used to separate plant pigments.

Simple Analogy — How Chromatography Works
Imagine you place a drop of ink on a paper and let water flow through it. Different colour dyes in the
ink travel at different speeds — some move fast, some move slow. After some time, the dyes are separated
into distinct coloured bands. This is exactly what chromatography does — but with great precision!
The 'runner' is the mobile phase; the 'track' is the stationary phase.

Basic Principle of Chromatography

Chromatography works because the components of a mixture have DIFFERENT affinities (attractions) for two phases:

Phase	What It Is	Role in Separation
Stationary Phase	A solid or liquid that stays fixed in place (e.g., silica, paper, gel)	Retains components with high affinity — they travel SLOWLY
Mobile Phase	A liquid or gas that moves through/over the stationary phase (e.g., solvent, gas)	Carries components with high affinity — they travel FAST

Components that prefer the stationary phase move slowly. Components that prefer the mobile phase move faster. This difference in speed causes separation.

Terms in Chromatography

Term	Definition
Rf Value (Retardation Factor)	Distance travelled by compound / Distance travelled by solvent front. Value is always between 0 and 1.
Resolution (Rs)	Measure of how well two adjacent peaks/spots are separated from each other.
Retention Time (tR)	Time taken by a compound to travel from injection to detection (in column chromatography).

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Term	Definition
Eluent / Eluate	Mobile phase used = Eluent. The solution coming out of the column = Eluate.
Elution	Process of washing a compound off the stationary phase using mobile phase.
Adsorption	Adhesion of molecules to the surface of the stationary phase.
Partition	Distribution of solute between two immiscible liquid phases.

Classification of Chromatography Methods

Basis of Classification	Types
Mechanism of separation	Adsorption, Partition, Ion-exchange, Size-exclusion (gel), Affinity
Physical state of mobile phase	Liquid Chromatography (LC), Gas Chromatography (GC)
Nature of stationary phase	Column, Planar (Paper, TLC)
Technique of development	Frontal, Displacement, Elution chromatography

COLUMN CHROMATOGRAPHY

Column Chromatography

Column chromatography is the classical form of chromatography in which a vertical glass column is packed with a solid stationary phase (adsorbent or support). The sample mixture is loaded at the top, and a liquid mobile phase (eluent) is passed through the column. Components separate as they travel down the column at different rates.

A. Adsorption Column Chromatography

In adsorption chromatography, the stationary phase is a solid adsorbent (e.g., silica gel, alumina). Separation occurs because different components adsorb onto the surface of the stationary phase with different strengths.

Principle
Sample molecules compete with mobile phase molecules to adsorb onto the surface of the solid stationary phase.
Molecules with STRONGER adsorption to the stationary phase move more SLOWLY (retained more).
Molecules with WEAKER adsorption move more QUICKLY (less retained) through the column.

The order of elution depends on polarity: Non-polar compounds elute first when non-polar solvent is used.
This is because silica/alumina are POLAR adsorbents — they hold polar compounds more tightly.

Common Stationary Phases (Adsorbents)

Adsorbent	Polarity	Activity	Used For
Silica Gel (SiO_2)	Polar	High	Most widely used; separates polar and non-polar compounds
Alumina (Al_2O_3)	Polar	High	Alkaloids, vitamins, lipids; acidic, basic or neutral grades
Charcoal (Activated)	Non-polar	Variable	Decolourisation; removing pigments
Cellulose	Polar (mild)	Low	Similar to paper chromatography; for hydrophilic compounds
Calcium Carbonate	Polar	Mild	Plant pigments, carotenoids

Eluotropic Series — Solvents in Order of Increasing Polarity

The eluting power of a solvent depends on its polarity. More polar solvents compete better with the sample for adsorption sites and elute compounds more quickly.

Increasing Polarity →	Solvents (Weak to Strong Eluents)
Non-polar (weak eluents)	Petroleum ether → Hexane → Cyclohexane → Carbon tetrachloride
Intermediate	Benzene → Toluene → Chloroform → Diethyl ether
Polar (strong eluents)	Ethyl acetate → Acetone → Ethanol → Methanol → Water

Methodology of Adsorption Column Chromatography

Step 1: Selection of Adsorbent and Solvent

- Choose adsorbent based on sample type (silica for most organic compounds).
- Choose solvent system based on polarity — start with a non-polar solvent and gradually increase polarity (gradient elution).
- Rule of Thumb: Use TLC first to find the best solvent system (Rf ≈0.3–0.4 on TLC is ideal for column work).

Step 2: Preparation of the Column

- Take a clean, dry glass column with a stopcock at the bottom. Place a small cotton plug or glass wool at the bottom to prevent adsorbent loss.
- Wet Method (most common): Make a slurry of the adsorbent with the starting solvent. Pour slowly into the column while opening the stopcock to allow solvent to drain. Tap gently for uniform packing. No air bubbles should form.
- Dry Method: Pour dry adsorbent in small portions while tapping. Then wet with solvent.
- Amount of adsorbent: Usually 30–100 times the weight of the sample.

Step 3: Sample Application

- Dissolve the sample in a small volume of the starting (weakest) solvent.
- Carefully layer the sample solution on top of the adsorbent using a pipette. Do NOT disturb the adsorbent surface.
- Allow sample to be adsorbed onto the top of the column. Wash with a small amount of starting solvent to move the sample down slightly.

Step 4: Elution (Development)

- Pass the mobile phase (eluent) through the column in order of increasing polarity (step-gradient or linear gradient).
- Components separate into distinct bands as they travel down the column at different rates.
- Collect separate fractions (portions) of the eluate in numbered test tubes or vials using a fraction collector.

Step 5: Detection and Collection

- Coloured compounds: Visible as coloured bands in the column.
- Colourless compounds: Detect fractions by UV lamp (UV-active), spray reagents (e.g., ninhydrin for amino acids), or TLC of each fraction.
- Pool fractions containing the same pure compound. Evaporate solvent to get the pure isolated compound.

Advantages of Adsorption Column Chromatography

- Large amounts of sample can be separated (preparative scale — grams to kilograms).
- Relatively inexpensive equipment (simple glass column).
- Wide variety of adsorbents and solvents available.
- Can separate structurally similar compounds effectively.
- Both polar and non-polar compounds can be separated.

Disadvantages of Adsorption Column Chromatography

- Time-consuming process — can take hours to days.
- Requires large volumes of solvents — costly and wasteful.
- Irreversible adsorption: Some compounds bind too tightly and cannot be eluted.
- Band tailing: Peaks spread out, causing overlapping and poor resolution.
- Activity of adsorbent can vary — affected by moisture content (especially alumina).
- Not suitable for thermally labile or unstable compounds.

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B. Partition Column Chromatography

In partition chromatography, separation is based on the DIFFERENTIAL PARTITIONING (distribution) of sample components between two liquid phases — a stationary liquid phase and a mobile liquid phase. The stationary liquid is coated on an inert solid support.

Principle — Partition Coefficient
Each solute distributes itself between the stationary liquid and the mobile liquid according to its
Partition Coefficient (K) = Concentration in stationary phase / Concentration in mobile phase.
Components with HIGH K (prefer stationary phase) → move SLOWLY.
Components with LOW K (prefer mobile phase) → move QUICKLY.
The stationary liquid and mobile liquid MUST be IMMISCIBLE with each other.

Types of Partition Chromatography

Type	Stationary Phase	Mobile Phase	Features
Normal Phase (NP-LC)	Polar liquid (e.g., water on silica)	Non-polar solvent (e.g., hexane)	Polar compounds retained longer; non-polar elute first
Reversed Phase (RP-LC)	Non-polar liquid (e.g., C18 on silica)	Polar solvent (e.g., water + methanol)	Non-polar retained longer; polar elute first; most widely used today
Liquid-Liquid Partition	Pure liquid stationary phase	Immiscible liquid mobile phase	Classic Craig countercurrent extraction system

Common Support Materials

- Kieselguhr (Diatomaceous earth) — most common inert support; holds stationary liquid without retaining sample.
- Celite, Chromosorb — other common supports.
- Modified silica (C18, C8) — in modern HPLC reversed-phase partition.

Methodology

- The support material is coated with the stationary liquid phase (e.g., water or a high-boiling oil).
- Packed into a glass column. The mobile phase (immiscible with stationary phase) is passed through.
- Sample components distribute between the two liquid phases as they move down the column.
- Fractions are collected and analyzed as in adsorption column chromatography.

Advantages of Partition Column Chromatography

- Better for separating water-soluble, hydrophilic compounds (amino acids, sugars, vitamins).

- Milder conditions — suitable for thermally labile and biologically active compounds.
- Good resolution for compounds that differ in polarity.
- Less risk of irreversible adsorption compared to adsorption chromatography.

Disadvantages of Partition Column Chromatography

- The stationary liquid phase can dissolve in or bleed into the mobile phase, reducing reproducibility.
- Not suitable for non-polar compounds (poor retention).
- Limited choice of solvent systems (must be mutually immiscible).
- Slower than modern HPLC; requires careful control of solvent composition.

Applications of Column Chromatography (Both Types)

Application Area	Example
Isolation of natural products	Separating alkaloids (quinine, morphine) from plant extracts
Pharmaceutical analysis	Purification of drug substances; isolation of active constituents
Protein purification	Ion-exchange column chromatography for enzymes and proteins
Petroleum industry	Separation of hydrocarbons by adsorption on silica/alumina
Food industry	Separation of food colours, flavours, vitamins
Forensic science	Analysis of drugs of abuse, poisons in biological samples

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THIN LAYER CHROMATOGRAPHY (TLC)

Thin Layer Chromatography (TLC)

Introduction

Thin Layer Chromatography (TLC) is a planar (flat-surface) chromatographic technique in which the stationary phase is a thin layer of adsorbent coated on a flat, rigid support (glass plate, aluminium foil, or plastic sheet). The mobile phase (solvent system) moves up the plate by capillary action. It is one of the most widely used techniques in pharmaceutical and organic chemistry laboratories.

Historical Note
TLC was first introduced by Egon Stahl in 1956, who standardised the technique and developed
pre-coated glass plates. Today, TLC is used daily in almost every chemistry laboratory as a
quick, inexpensive, and versatile separation tool.

Principle of TLC

TLC is primarily an ADSORPTION technique (when silica or alumina is used). Separation occurs because different components of the sample have different affinities for the stationary phase (adsorbent) and the mobile phase (solvent).

- **Components with HIGHER affinity for the stationary phase → adsorb strongly → travel SHORT distances (low Rf).**
- **Components with LOWER affinity for the stationary phase → move quickly with the solvent → travel LONG distances (high Rf).**

The mobile phase moves UP the plate by CAPILLARY ACTION. As it rises, it carries the sample components with it, separating them into distinct spots.

Materials Used in TLC

Stationary Phase (Adsorbent Coating)

Adsorbent	Support / Binder	Used For
Silica Gel G (Gypsum binder)	$CaSO_4$ ½ H_2O as binder	Most common; for most organic compounds
Silica Gel GF_{254}	Silica + fluorescent indicator	Compounds visible under 254 nm UV light (appear dark)
Alumina (Neutral/Acidic/Basic)	Starch or gypsum	Alkaloids, vitamins; depends on pH of compound
Cellulose	Natural	Hydrophilic compounds, amino acids, nucleotides

Adsorbent	Support / Binder	Used For
Kieselguhr	Inert support	Partition TLC for hydrophilic substances

Plate Supports

- Glass plates (most common): 20×20 cm, 10×20 cm — rigid, heat-stable, can be sprayed with corrosive reagents.
- Aluminium foil-backed plates: Flexible, can be cut to any size; less costly.
- Plastic-backed plates: Light, inexpensive; but cannot withstand high-temperature charring.

Mobile Phase (Solvent System)

The solvent or mixture of solvents must be chosen carefully. The ideal solvent gives Rf values between 0.2 and 0.8 for the compounds of interest.

- Single solvents: Hexane, chloroform, methanol, ethyl acetate.
- Solvent mixtures: Chloroform:methanol (9:1), Ethyl acetate:hexane (1:1), Butanol:acetic acid:water (4:1:5) — commonly used for amino acids.
- More polar solvent → higher Rf values (moves compounds up faster).

Methodology of TLC

Step 1: Preparation of the TLC Plate

- Use pre-coated plates OR prepare by spreading a slurry of silica gel G in water uniformly on the glass plate using a spreader to a thickness of 0.25 mm (analytical) or 0.5–1 mm (preparative).
- Dry plates at room temperature for 30 min, then activate by heating in an oven at 105–110°C for 30–60 min. This removes adsorbed moisture and increases adsorbent activity.

Step 2: Sample Application (Spotting)

- Draw a pencil line (baseline) 1.5–2 cm from the BOTTOM edge of the plate.
- Apply a small, CONCENTRATED spot of sample (1–10 μL) using a micro-capillary tube or micropipette onto the baseline. Spot diameter should be ≤1–2 mm.
- Apply reference standards alongside the sample spots for comparison.
- Allow the spot to dry completely before development (use a hair dryer or warm air).

Step 3: Development (Running the Chromatogram)

- Pour the mobile phase (solvent) into the TLC chamber (a closed jar or glass tank) to a depth of LESS than the spotting line (about 0.5–1 cm). This is critical — if the solvent level is above the spot, the sample will dissolve directly into the solvent.
- Line the chamber with filter paper soaked in solvent to saturate the atmosphere. This ensures consistent, reproducible results.
- Wait 10–15 minutes for the chamber to become saturated with solvent vapour (pre-saturation).
- Place the plate VERTICALLY in the chamber. The bottom edge dips into the solvent (below the baseline). Cover the chamber immediately.
- Allow the solvent to rise by CAPILLARY ACTION until it is 1–1.5 cm from the TOP of the plate (this is the solvent front).

Step 4: Detection of Spots

- Remove the plate from the chamber and immediately mark the solvent front with a pencil.
- Dry the plate. Then detect spots using one or more methods:
  - UV Light (254 nm): Spots appear dark on a fluorescent background (if plate contains GF indicator). At 366 nm, fluorescent compounds glow.
  - Iodine Chamber: Place plate in a closed jar with iodine crystals. Most organic compounds form brown spots.
  - Spray Reagents: Ninhydrin (amino acids → purple spots), Dragendorff's reagent (alkaloids → orange spots), $FeCl_3$ (phenols → blue/green), H_2SO_4 + heat (charring — universal).
  - Biological Detection: For antibiotics — bioautography.

Step 5: Calculation of Rf Value

Rf = Distance travelled by the spot (from baseline) / Distance travelled by the solvent front (from baseline)

Rf Value — Key Points
Rf is always between 0 and 1.
Rf = 0 means the compound did not move at all (stayed at baseline).
Rf = 1 means the compound moved with the solvent front.
Ideal Rf for identification: 0.2 to 0.8.
Rf values are REPRODUCIBLE under identical conditions (same adsorbent, solvent, temperature, humidity).
Two different compounds can have the same Rf in one solvent system but different Rf in another.
Rf values in BP/USP/IP are given for specific solvent systems on silica gel G plates.

Factors Affecting Rf Value

Factor	Effect on Rf
Polarity of mobile phase	More polar solvent → higher Rf (faster movement)
Activity of adsorbent	More active (dry) adsorbent → lower Rf (stronger retention)
Thickness of adsorbent layer	Thicker layer → slightly lower Rf
Temperature	Higher temperature → higher Rf (less adsorption)
Chamber saturation	Unsaturated chamber → uneven, irregular Rf values
Amount of sample applied	Overloading → streaking, tailing, incorrect Rf
Nature of the compound	More polar compound on silica → lower Rf

Advantages of TLC

- Simple, quick and inexpensive — results in 30–60 minutes.
- Small amounts of sample required (a few micrograms).

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- All components (including those remaining at the baseline) are visible simultaneously.
- Many samples can be run simultaneously on the same plate.
- Versatile — a wide range of detection reagents can be applied directly on the plate.
- Can be used for both qualitative (identification) and quantitative (densitometry) analysis.
- Preparative TLC can isolate small amounts of compounds (scraping the zone off the plate).

Disadvantages of TLC

- Not suitable for volatile compounds (they evaporate before detection).
- Rf values are not absolute — vary with conditions (humidity, temperature, batch of plates).
- Limited to relatively small quantities — not suitable for large-scale preparation.
- Irreversible adsorption of some compounds can occur.
- Less sensitive than HPLC or GC for quantitative analysis.

Applications of TLC

Application	Details
Identity testing of drugs	Compare Rf of sample with authentic standard — used in IP/BP/USP
Purity testing	A single spot on TLC indicates absence of impurities
Monitoring reactions	Check progress of a chemical synthesis reaction (spot reactant vs product)
Column chromatography guidance	Choose correct solvent system for column work using TLC
Detection of adulterants	Identify foreign substances in food and drugs
Analysis of plant extracts	Separation of alkaloids, flavonoids, terpenes from plant material
Limit test for related substances	Detect and semi-quantify impurities in pharmaceuticals (IP/BP)

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Paper Chromatography

Introduction

Paper chromatography was developed by Martin and Synge in 1944 (for which they won the Nobel Prize in 1952). It is a planar chromatographic technique in which special chromatography paper serves as both the support AND the stationary phase. It was the first modern chromatographic technique and revolutionised the analysis of biological compounds.

Principle

Paper chromatography is a PARTITION technique (not adsorption). The cellulose fibres of the chromatography paper hold water firmly by hydrogen bonding. This adsorbed water acts as the STATIONARY LIQUID PHASE.

How Separation Occurs
Stationary Phase: Water held by cellulose fibres of the paper.
Mobile Phase: An organic solvent or solvent mixture that is partially miscible with water.
When the mobile phase moves through the paper, each solute distributes itself between the
stationary water phase and the moving organic phase according to its Partition Coefficient.
Hydrophilic (water-loving) compounds prefer the water (stationary phase) → LOW Rf (move slowly).
Lipophilic (organic solvent-loving) compounds prefer the mobile phase → HIGH Rf (move fast).

Materials Used

Chromatography Paper

Paper Type	Description	Use
Whatman No. 1	Standard grade; medium flow rate	General purpose separations
Whatman No. 2	Thicker; slower; more capacity	Preparative separations
Whatman No. 3MM	Very thick; high sample capacity	Preparative work; electroblotting
Whatman No. 4	Very fast flow rate	Quick separations when speed matters
Whatman No. 40, 41, 42	Hardened; acid-washed	Quantitative analysis; gravimetric work

Mobile Phase (Solvent Systems)

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Compound Type	Recommended Solvent Systems
Amino acids	n-Butanol : Acetic acid : Water (4:1:5) — BAW system
Sugars	n-Butanol : Acetic acid : Water (4:1:5) or Ethyl acetate : Pyridine : Water
Alkaloids	Chloroform : Methanol (9:1)
Inorganic ions	2M HNO_3 or other aqueous systems

Methodology of Paper Chromatography

Step 1: Preparation of Paper

- Cut Whatman chromatography paper to the required size (e.g., 20×20 cm or 20×40 cm).
- Handle paper with GLOVES only — fingerprints contain amino acids that will appear as spots!
- Draw a baseline (pencil line) 2–3 cm from one edge.

Step 2: Sample Application

- Apply sample as a small, round spot (2–3 mm diameter) on the baseline using a capillary tube or micropipette.
- Apply 2–10 µL of a dilute solution. Allow to dry between applications.
- Apply reference standards alongside. Leave at least 2–3 cm between spots.

Step 3: Development

- Place the spotted paper in a chromatographic tank containing a small amount of mobile phase.
- Saturate the tank atmosphere with solvent vapour for 30–60 min (place filter paper soaked in solvent around the walls of the tank).
- Develop using the appropriate technique (see Development Techniques below).
- Remove the paper when the solvent front reaches the desired distance (leaving 2 cm from the top). Mark the solvent front immediately.

Step 4: Detection

- Dry the paper thoroughly at room temperature or in a warm oven.
- Use appropriate detection reagents:
  - Ninhydrin reagent (0.2% in acetone) sprayed and heated at 105°C → Purple/pink spots for amino acids.
  - Silver nitrate → dark spots for reducing sugars.
  - Dragendorff reagent → orange-red spots for alkaloids.
  - UV light (254 or 366 nm) for UV-absorbing or fluorescent compounds.
  - Iodine vapour → brown spots (reversible detection).

Step 5: Measurement and Calculation

- Measure the distance from the baseline to the CENTRE of each spot (= distance moved by compound).
- Measure the distance from the baseline to the solvent front.
- Calculate Rf = Distance moved by compound / Distance moved by solvent front.

Development Techniques

Different development techniques are used depending on the complexity of the mixture:

A. Ascending Development (Most Common)

- The solvent travels UPWARD by capillary action against gravity. The paper is placed vertically with the bottom edge dipping into the solvent (below the baseline). Simple, most widely used.

B. Descending Development

- The solvent travels DOWNWARD under the combined effect of capillary action and gravity. The trough containing solvent is at the TOP; the paper hangs down from it. Faster than ascending; allows longer development (solvent can drip off the end of the paper). Better for compounds with low Rf in ascending development.

C. Radial (Circular) Development

- A circular paper disc is used. The sample is spotted at the CENTRE. Solvent is applied at the centre (through a wick) and spreads outward. Compounds separate as concentric rings. Quick and simple; gives good resolution of complex mixtures.

D. Two-Dimensional (2D) Development

MOST POWERFUL technique. The sample is spotted at ONE CORNER of a square paper. Development is done with SOLVENT 1 in one direction. The paper is removed, dried, then rotated 90°, and developed again with SOLVENT 2 in the perpendicular direction.

- Each compound now has an x-coordinate (from solvent 1) and y-coordinate (from solvent 2).
- Greatly increases resolution — compounds that had the same Rf in one solvent system may separate in the other.
- Used for complex mixtures like amino acids (up to 20 amino acids can be resolved).

Advantages of Paper Chromatography

- Extremely simple technique — no sophisticated equipment needed.
- Very inexpensive — cheap paper and solvents.
- Small sample quantities sufficient (micrograms).
- Excellent for separating and identifying hydrophilic compounds (amino acids, sugars, organic acids).
- 2D development gives very high resolution for complex biological mixtures.
- Both qualitative and quantitative analysis possible.

Disadvantages of Paper Chromatography

- SLOW technique — ascending paper chromatography can take 4–12 hours.
- Less reproducible than TLC — paper quality and humidity affect results significantly.
- Not suitable for hydrophobic, non-polar compounds (lipids, hydrocarbons) — prefer TLC or GC.
- Rf values are approximate; not suitable as sole identification method.
- Spray reagents may react with and damage the paper, making quantitative analysis difficult.
- Largely replaced by TLC and HPLC in modern laboratories.

Applications of Paper Chromatography

Application Area	Example
Amino acid analysis	Separation and identification of amino acids from protein hydrolysates (classic use)
Sugar analysis	Identification of monosaccharides and disaccharides in biological samples
Drug identification	Identification and purity testing of vitamins, antibiotics, plant drugs
Inorganic ion analysis	Separation of metal ions using specific solvent systems
Forensic analysis	Detection of drugs, inks, dyes, poisons
Food adulteration	Detection of synthetic colours in food items
Clinical biochemistry	Analysis of urinary metabolites, bile pigments

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ELECTROPHORESIS

Electrophoresis

Introduction

Electrophoresis is a separation technique based on the MOVEMENT of charged particles (ions, molecules) through a medium under the influence of an APPLIED ELECTRIC FIELD. Molecules with different sizes, shapes, and charges migrate at different speeds and are thus separated.

Basic Principle
When an electric field is applied across a medium containing charged molecules:
CATIONS (positive charge) → migrate toward the CATHODE (negative electrode).
ANIONS (negative charge) → migrate toward the ANODE (positive electrode).
The SPEED of migration depends on the charge, size, and shape of the molecule.
Higher charge → Faster migration. Larger size → Slower migration.
Smaller charge → Slower migration. Streamlined shape → Faster migration.

Factors Affecting Electrophoretic Mobility

Electrophoretic mobility (μ) is defined as the velocity of migration per unit electric field strength:

$$\mu = v / E = q / (6\pi\eta r) \quad \text{[Stokes equation]}$$

Where: v = velocity of migration; E = electric field strength; q = net charge on particle; η = viscosity of medium; r = radius of particle

Factor	Effect on Electrophoretic Mobility	Explanation
Net Charge of Molecule	MORE charge → FASTER migration	Depends on ionisation of groups at the given pH
pH of Buffer	Changes net charge on the molecule	At isoelectric point (pI), net charge = 0 → NO movement
Size and Shape of Molecule	LARGER or globular → SLOWER migration	Larger molecules experience more frictional resistance
Electric Field Strength	HIGHER voltage → FASTER migration	More force acting on the charged molecule
Viscosity of Medium	HIGHER viscosity → SLOWER migration	Greater resistance to movement through thick medium
Temperature	HIGHER temp → FASTER (lower viscosity)	But excessive heat causes band distortion (Joule heating)
Ionic Strength of Buffer	HIGH ionic strength → SLOWER migration	Counter-ions shield the charge on the molecule

Factor	Effect on Electrophoretic Mobility	Explanation
Electroosmotic Flow (EOF)	Moves both charged and neutral species	Water flow from anode to cathode in capillary electrophoresis
Molecular Sieving Effect	Gel pores sieve molecules by size	Smaller molecules move faster through gel pores
Adsorption	Adsorption to support medium slows migration	Paper may adsorb some compounds

Isoelectric Point (pI) — Important Concept
The isoelectric point (pI) is the pH at which a molecule (e.g., a protein or amino acid) has a NET CHARGE of ZERO.
At pH = pI → No migration in electrophoresis (molecule stays stationary).
At pH < pI → Molecule is POSITIVELY charged → migrates to cathode.
At pH > pI → Molecule is NEGATIVELY charged → migrates to anode.
By choosing the buffer pH, we can control the direction and speed of migration.

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Techniques of Electrophoresis

A. Paper Electrophoresis

Paper electrophoresis uses a strip of chromatography paper (Whatman No. 1, 3, or 3MM) moistened with buffer as the support medium. It was one of the earliest forms of electrophoresis.

Instrumentation and Setup

- An electrophoresis tank/chamber with two buffer reservoirs (one for each electrode). The electrodes (Pt wire or graphite) are immersed in each reservoir.
- The paper strip is moistened with buffer and placed across the two reservoirs, bridging them.
- Sample is applied as a spot or a streak at the CENTRE or designated origin of the paper.
- DC power supply is connected (typically 200–300 V; 5–10 mA).
- Cooling is needed to prevent overheating (high-voltage paper electrophoresis uses cooling plates).

Types of Paper Electrophoresis

- Low-Voltage Paper Electrophoresis (LVPE): 200–500 V/m. Slower; takes several hours. Used for proteins, amino acids.
- High-Voltage Paper Electrophoresis (HVPE): 1000–5000 V/m. Very fast (30–60 min). Better resolution. Needs cooling.

Procedure

- Soak paper strip in appropriate buffer (e.g., pH 8.6 barbiturate buffer for serum proteins).
- Blot excess buffer with filter paper to remove surface moisture (paper should be moist, not dripping).
- Apply sample as a thin band at the marked origin.

- Connect to power supply and run for the required time.
- Remove paper, dry it, and stain with appropriate dye (e.g., Coomassie blue for proteins, ninhydrin for amino acids).
- Destain background, scan or measure intensities for quantitation.

Applications

- Serum protein electrophoresis: Separation of albumin, α_1-, α_2-, β-, γ-globulins.
- Amino acid analysis and identification.
- Separation of inorganic ions, nucleotides, nucleosides.
- Detection of abnormal proteins (paraproteins in multiple myeloma).

B. Gel Electrophoresis

Gel electrophoresis uses a GEL (a porous, sieve-like material) as the support medium. The gel not only carries the electric current but also acts as a MOLECULAR SIEVE — separating molecules by both CHARGE and SIZE.

Types of Gels

Gel Type	Material	Pore Size	Best Used For
Agarose Gel	Polysaccharide from seaweed	Large pores (1–300 nm)	DNA, RNA, large proteins (>50 kDa)
Polyacrylamide Gel (PAGE)	Acrylamide + bisacrylamide polymer	Small, controllable pores (1–10 nm)	Small proteins, peptides, DNA fragments
Starch Gel	Hydrolysed starch	Medium pores	Isoenzyme analysis; older technique

SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)

What is SDS-PAGE?
SDS is a detergent that denatures proteins and coats them uniformly with negative charge.
The negative charge is proportional to the mass of the protein (approximately 1.4 g SDS per gram protein).
This ELIMINATES the effect of the protein's own charge and shape.
All proteins now migrate based on SIZE ONLY — smaller proteins migrate faster through the gel pores.
By comparing with molecular weight markers (protein ladder), we can determine the MW of unknown proteins.
Most widely used technique for protein molecular weight determination.

Procedure for SDS-PAGE

- Prepare the gel: Mix acrylamide/bisacrylamide solution with buffer, SDS, ammonium persulphate (initiator), and TEMED (catalyst). Pour between glass plates and allow to polymerise.
- Sample preparation: Mix sample with SDS sample buffer (contains SDS + β-mercaptoethanol + loading dye). Heat at 95°C for 5 min to fully denature proteins.
- Load samples into wells (formed by a plastic comb during gel preparation). Load molecular weight markers in adjacent lanes.
- Run electrophoresis at constant voltage (100–200 V) in Tris-glycine running buffer until the dye front reaches the bottom.
- Staining: Soak gel in Coomassie Brilliant Blue R-250 for 1 hour, then destain to remove background.
- Visualise bands. Use a ruler or imaging software to calculate molecular weights by comparing with standard curve.

Other Important Gel Electrophoresis Techniques

- Native PAGE: No SDS used. Proteins separated by charge AND size. Native (non-denatured) conformation is preserved.
- Isoelectric Focusing (IEF): Separation based on pI. A pH gradient is established in the gel. Each protein migrates to the position in the gradient where pH = pI (net charge = 0) and stops. Excellent resolution.
- 2D Gel Electrophoresis: IEF (by pI) in first dimension + SDS-PAGE (by MW) in second dimension. Can resolve >1000 proteins from a single sample. Gold standard for proteomics.
- Agarose Gel Electrophoresis: Standard for DNA analysis. DNA is always negatively charged (phosphate backbone) — no SDS needed. Smaller DNA fragments migrate faster. Ethidium bromide (or SYBR Green) is used to visualise DNA bands under UV.

Applications of Gel Electrophoresis

- Protein molecular weight determination (SDS-PAGE).
- DNA fingerprinting / DNA profiling (forensic, paternity testing).
- Quality control of recombinant proteins and vaccines.
- Western blot: After SDS-PAGE, proteins are transferred to membrane and detected with specific antibodies.
- PCR product analysis: Confirm correct size of amplified DNA fragments.
- Isoenzyme analysis (e.g., LDH isoforms in cardiac diagnosis).

C. Capillary Electrophoresis (CE)

Capillary Electrophoresis is a modern, high-performance separation technique that uses a narrow-bore capillary tube (50–100 µm internal diameter) filled with buffer as the separation medium. It combines the high resolution of electrophoresis with the automation, speed, and quantitative capabilities of HPLC.

Feature — Electroosmotic Flow (EOF)
The fused silica capillary wall has silanol groups (-SiOH) that ionise at neutral/high pH to give $-SiO^-$ (negative).

This attracts a layer of positive counter-ions from the buffer, forming an electrical double layer.
Under the applied electric field, this layer of positive ions (and the water surrounding them) flows
toward the CATHODE — this is called Electroosmotic Flow (EOF).
EOF is a plug-like (flat) flow — unlike the parabolic flow in HPLC pumps. This gives VERY SHARP peaks.
EOF carries BOTH cations AND neutral species toward the cathode. Anions migrate AGAINST EOF but are
still swept toward cathode if EOF is faster than their backward migration.

Components of a CE Instrument

Component	Description
Capillary	Fused silica capillary, 25–100 µm ID, 20–60 cm length; coated or uncoated
Buffer Reservoirs	Two vials containing background electrolyte (BGE); capillary ends dip into them
High Voltage Power Supply	Typically 10–30 kV DC; generates the electric field
Sample Vial	Sample is introduced (injected) into the capillary by pressure or electrokinetic means
Detector	UV-Vis detector (most common, on-capillary); also fluorescence, MS
Data System	Records electropherogram; calculates migration time and peak area

Modes of Capillary Electrophoresis

CE Mode	Abbreviation	Separation Basis	Applications
Capillary Zone Electrophoresis	CZE	Charge-to-size ratio in free solution	Amino acids, drugs, inorganic ions, proteins
Micellar Electrokinetic Chromatography	MEKC	Partitioning into SDS micelles + electrophoresis	Neutral and charged molecules including drugs
Capillary Gel Electrophoresis	CGE	Size sieving in gel-filled capillary	DNA sequencing, oligonucleotides, small proteins
Capillary Isoelectric Focusing	CIEF	pI-based focusing in pH gradient	Protein isoforms, haemoglobin variants
Capillary Electrochromatography	CEC	Combination of HPLC + CE	Complex mixtures requiring both retention and charge separation

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Procedure for Capillary Electrophoresis

- Condition the capillary: Flush with NaOH (1 M), water, then running buffer (BGE) each day before use. This ensures reproducible EOF.
- Sample injection: Hydrodynamic injection (pressure difference) OR electrokinetic injection (apply voltage). Inject a very small volume (≈1–5 nL).
- Run: Apply high voltage (10–30 kV). Migration time for each component is recorded.
- Detection: On-capillary UV detection at 200–280 nm. Peak area is proportional to concentration.
- Wash between runs: Flush capillary with NaOH, water, and buffer to regenerate the capillary surface.

Advantages of Capillary Electrophoresis

- Extremely high resolution — can separate enantiomers, isoforms differing by a single charge.
- Very small sample volume (nanolitres) — essential when sample is limited.
- Very fast — separations in 5–20 minutes.
- Highly automated — suitable for routine pharmaceutical quality control.
- Efficient — generates very high theoretical plates (100,000–500,000/m) far exceeding HPLC.
- Aqueous buffers used — minimal organic solvent waste; environmentally friendly.
- Can analyse both small molecules and large biomolecules (proteins, DNA).

Disadvantages of Capillary Electrophoresis

- Lower sensitivity than HPLC (short path length for UV detection).
- Limited loadability — very small injection volumes reduce sensitivity for trace analysis.
- Migration time reproducibility can be affected by EOF variability.
- Not easily scaled up for preparative separations.

Applications of Electrophoresis

Application Area	Technique Used	Example
Clinical diagnosis	Serum protein electrophoresis (paper/gel)	Diagnosis of paraproteinaemia, cirrhosis
Molecular biology / genetics	Agarose gel electrophoresis	DNA fingerprinting, PCR analysis, gene mapping
Proteomics	SDS-PAGE, 2D-PAGE	Protein identification, biomarker discovery
Pharmaceutical QC	Capillary electrophoresis	Purity of peptides, proteins, oligonucleotides (biopharmaceuticals)
Haemoglobin analysis	Alkaline / acid gel electrophoresis	Detection of sickle cell anaemia, thalassaemia
DNA sequencing	CGE (automated CE)	Genome sequencing projects (Human Genome Project used CE)

Application Area	Technique Used	Example
Forensic science	DNA profiling by gel/CE	Identification from crime scene samples
Food analysis	CE (CZE, MEKC)	Detection of food additives, vitamins, toxins

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