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UNIT-3

INSTRUMENTAL METHODS OF ANALYSIS

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INSTRUMENTAL METHODS OF ANALYSIS

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Unit – 3

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1 Classify different types of chromatography.

Technique	Stationary phase	Mobile phase
Column/Adsorption Chromatography	Solid	Liquid
Partition Chromatography	Liquid	Liquid
Paper Chromatography	Liquid	Liquid
Thin Layer Chromatography (TLC)	Liquid/Solid	Liquid
Gas – Liquid chromatography (GLC)	Liquid	Gas
Gas – Solid Chromatography (GSC)	Solid	Gas
Ion Exchange Chromatography	Solid	Liquid

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2 Define retention time, Rf value, and resolution.

1. Retention Time

Retention time is the time taken by a compound to travel from the **injection point** to the **detector** in a chromatographic system.

Explanation:

- It is measured in **minutes or seconds**.
- Each compound has a **unique retention time** under fixed conditions, which helps in **identifying the compound**.
- It depends on the **interaction of the compound** with the stationary and mobile phases.

Example:

In **HPLC (High-Performance Liquid Chromatography)**, if a drug elutes (comes out) at 5.2 minutes, then its retention time is **5.2 minutes**.

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2 Define retention time, Rf value, and resolution.

Rf Value (Retention factor or Ratio to front):

Rf value is the ratio of the distance travelled by the substance to the distance travelled by the solvent front in thin-layer chromatography (TLC) or paper chromatography.

Formula:

Rf=Distance travelled by the solute / Distance travelled by the solvent front

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2 Define retention time, Rf value, and resolution.

3. Resolution:

Resolution is a measure of how well two compounds are separated in a chromatographic system.

$$Rs = 2(tR2 - tR1) / w1 + w2$$

Where:

- t_{R1} and t_{R2} =Retention times of two peaks
- w_1 and w_2 =Widths of the two peaks at the base

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3 Explain the principle and procedure of column chromatography Describe stationary and mobile phases used.

Principle:

- Column chromatography works on the principle of **differential adsorption** of compounds.
- Different compounds in a mixture have different affinities (attractions) towards the **stationary phase** and **mobile phase**.
- Components that are **more strongly adsorbed** on the stationary phase move **slowly**.
- Components with **less adsorption** move **faster**.

Thus, the compounds are separated as they pass through the column.

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3 Explain the principle and procedure of column chromatography Describe stationary and mobile phases used.

Mobile Phase:

The mobile phase is a liquid solvent (or mixture of solvents) that flows through the column.

Common mobile phases:

Non-polar to moderately polar solvents like:

- Hexane
- Chloroform
- Ethyl acetate
- Methanol

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Procedure of Column Chromatography:

Preparation of the Column:

- A glass column is filled with the stationary phase (silica or alumina) either by wet packing (with solvent) or dry packing.
- A small piece of cotton or glass wool is placed at the bottom to prevent loss of adsorbent.

Sample Loading:

- The sample mixture is dissolved in a small amount of solvent and added carefully on top of the packed column.

Elution:

- The mobile phase (solvent) is added from the top.
- As the solvent flows down by gravity or pressure, the components of the mixture start to separate based on their adsorption.

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Procedure of Column Chromatography:

Collection of Fractions:

- Different components come out (elute) at different times and are collected in separate containers.

Detection:

- The collected fractions are analyzed using TLC, UV spectroscopy, or HPLC to identify the compounds.

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4 Explain the principle, procedure, and applications of TLC.

Principle:

- TLC works on the principle of **adsorption chromatography**.
- Different compounds in a mixture get adsorbed to the **stationary phase** (usually silica gel) at different rates based on their polarity and affinity.

  The compounds are carried up by a **mobile phase** (solvent) through capillary action, leading to **separation**.
- **More polar compounds** stick to the stationary phase and move slowly.
- **Less polar compounds** travel faster with the solvent.

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Procedure:

- Preparation of the TLC Plate:

A TLC plate is made of glass, aluminum, or plastic coated with a thin layer of stationary phase (usually silica gel or alumina).

- Sample Application:

A small drop of the sample solution is applied near the bottom of the plate (about 1–2 cm from the edge) using a capillary tube.

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➢ Development (Running the Plate):

- The TLC plate is placed vertically in a development chamber (a closed jar or beaker) containing a small amount of mobile phase (solvent).
- The solvent rises up the plate by capillary action, carrying the components of the sample.

➢ Drying the Plate:

- After the solvent front reaches near the top, the plate is removed and dried.

➢ Detection:

The spots are visualized using:

- UV light
- Iodine chamber
- Chemical spray reagents (e.g., ninhydrin for amino acids)

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- **Advantages of TLC:**

- Simple and inexpensive
- Requires a small sample size
- Quick results
- Can analyze multiple samples simultaneously

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5 Explain column chromatography.

Column chromatography is a **separation technique** used to isolate individual components from a mixture based on their **differential adsorption** onto a **stationary phase** as they are carried by a **mobile phase** through a column.

Principle:

- Column chromatography works on the principle of **differential adsorption**.
- Different compounds in a mixture have different affinities towards the **stationary phase** (adsorbent) and **mobile phase** (solvent).
- Compounds that are **strongly adsorbed** move slowly.
- Compounds that are **weakly adsorbed** move faster.

This difference in movement causes the **separation** of compounds as they pass through the column.

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5 Explain column chromatography.

Component	Description
Stationary Phase	Solid adsorbent like **silica gel** or **alumina**
Mobile Phase	Liquid solvent like **hexane**, **chloroform**, **ethyl acetate**, etc.
Column	Glass or plastic tube with a stopcock at the bottom

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Procedure:

Column Preparation:

- The column is packed with the stationary phase (silica or alumina) using either dry or wet packing method.
- A cotton plug or glass wool is placed at the bottom to hold the adsorbent.

Sample Loading:

- The sample mixture is dissolved in a small amount of solvent and applied carefully to the top of the packed column.

Elution:

- The mobile phase (solvent) is added from the top.
- The solvent flows downward by gravity or under pressure, carrying the components of the mixture.

Separation:

- Components separate into different bands/zones based on their affinity.

Detection:

- The collected fractions are analyzed using TLC, UV spectroscopy, or other methods to identify the compounds.

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6 Differences between paper chromatography and TLC.

Feature	Paper Chromatography	Thin Layer Chromatography (TLC)
1. Stationary Phase	Cellulose paper (water in paper acts as phase)	Thin layer of silica gel or alumina on a plate
2. Mobile Phase	Solvent (organic or aqueous)	Solvent (organic or aqueous)
3. Support Material	Filter paper	Glass, aluminum, or plastic plate
4. Separation Principle	Partition chromatography	Adsorption chromatography
5. Speed of Separation	Slow	Fast
6. Sensitivity	Less sensitive	More sensitive
7. Rf Value Accuracy	Less accurate	More accurate
8. Sample Size	Requires larger sample	Requires very small sample
9. Detection	Usually with chemical reagents or UV light	UV light, iodine vapors, or chemical sprays
10. Resolution	Lower (poor separation of close compounds)	Higher (better separation and resolution)
11. Reusability	Not reusable	Usually not reusable, but more durable
12. Applications	Used for simple or educational experiments	Used in labs for qualitative analysis, purity check

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7 What are the advantages and limitations of paper chromatography?

Advantages of Paper Chromatography:

- **Simple and Inexpensive**
  - Requires basic materials like filter paper and solvents.
  - No expensive instruments are needed.
- **Easy to Perform**
  - Ideal for beginners and educational labs.
  - Procedure is straightforward.
- **Minimal Sample Requirement**
  - Only a small quantity of the sample is needed for analysis.
- **Good for Polar Compounds**
  - Effective in separating amino acids, sugars, and other polar compounds.
- **Useful for Qualitative Analysis**
  - Helps in identifying compounds and checking purity.
- **Non-destructive Technique**
  - Sample can be recovered (in some cases) after separation.

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Limitations of Paper Chromatography:

- Limited Quantitative Use.
- Limited Detection Sensitivity.
- Cannot detect very small amounts of compounds.
- Only for Water-Soluble Substances.
- Not suitable for non-polar or volatile compounds.
- Not Suitable for Large-Scale Separation.
- Best for small-scale or analytical purposes only.
- Variation in paper type can affect reproducibility.

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8 What are the differences between electrophoresis and chromatography?

Feature	Electrophoresis	Chromatography
1. Principle	Separation based on **electrical charge and size** of molecules	Separation based on **differential affinity** toward stationary and mobile phases
2. Driving Force	**Electric field**	**Capillary action** or **solvent flow**
3. Type of Substances	Mostly used for **charged biomolecules** like proteins, DNA	Used for **organic, inorganic, polar, and non-polar** compounds
4. Separation Basis	Charge, size, and mass	Polarity, adsorption, partition, or molecular size
5. Medium Used	Gel or paper (e.g., agarose, polyacrylamide)	Solid stationary phase (e.g., silica) and liquid/gas mobile phase
6. Detection Method	Staining, UV light, or fluorescence	UV, iodine chamber, chemical spray, etc.
7. Equipment Required	Requires **power supply** and **electrophoresis chamber**	Simple setup (TLC, column) to advanced instruments (HPLC)
8. Application Area	Biotechnology, molecular biology, clinical diagnostics	Pharmaceutical analysis, organic chemistry, drug testing

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9 What is electrophoresis? State its principle?

- ✓ **Electrophoresis** is a laboratory technique used to **separate charged molecules**, such as **proteins, DNA, or RNA**, based on their **size** and **electrical charge**, by applying an **electric field**.
- ✓ It is widely used in **biotechnology, molecular biology, and clinical laboratories** for analytical and diagnostic purposes.

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9 What is electrophoresis? State its principle?

Principle of Electrophoresis:

Electrophoresis is based on the **movement of charged particles** in a fluid or gel under the influence of an **electric field**.

When an electric current is applied, **negatively charged molecules** (like DNA) move toward the **positive electrode (anode)**, and **positively charged molecules** move toward the **negative electrode (cathode)**.

The **rate of migration** depends on:

- **Size** of the molecule
- **Shape** of the molecule
- **Charge** of the molecule
- **Strength of the electric field**
- **Type of supporting medium** (e.g., agarose or polyacrylamide gel)

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10 Write note on theory and working of gel electrophoresis.

Gel electrophoresis is a technique used to **separate charged biomolecules** (such as **DNA, RNA, and proteins**) based on their **size and charge**, using a **gel matrix** and an **electric field**.

Basic Principle:

When an electric field is applied, **charged molecules migrate** through a gel.

- **Negatively charged molecules** (like DNA and RNA) move toward the **positive electrode (anode)**.
- The **gel acts as a molecular sieve**, allowing **smaller molecules** to move faster than larger ones.

The **separation** occurs due to differences in:

- **Size** of molecules
- **Charge**
- **Shape**
- **Pore size** of the gel

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Working of Gel Electrophoresis:

1. Preparation of Gel:

A gel is prepared using substances like:

- Agarose gel → for DNA/RNA
- Polyacrylamide gel → for proteins

The gel is poured into a tray with wells at one end for sample loading.

2. Sample Loading:

The sample (e.g., DNA) is mixed with a loading dye and added into the wells of the gel.

3. Buffer System:

A suitable electrophoresis buffer (e.g., TAE or TBE for DNA) is added to cover the gel.
The buffer conducts the electric current and maintains pH stability.

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Working of Gel Electrophoresis:

4. Running the Gel:

- The gel tray is connected to a power supply.
- Electric current is applied: Negative electrode (cathode) near the wells
  Positive electrode (anode) at the opposite end
- The negatively charged molecules move toward the positive electrode.

5. Separation:

Molecules migrate at different speeds based on size:

- ✓ Smaller fragments move faster and farther
- ✓ Larger fragments move slower and shorter distances

6. Visualization:

After electrophoresis, the gel is stained using:

- ✓ Ethidium bromide (for DNA/RNA, viewed under UV light)
- ✓ Coomassie blue or silver stain (for proteins)

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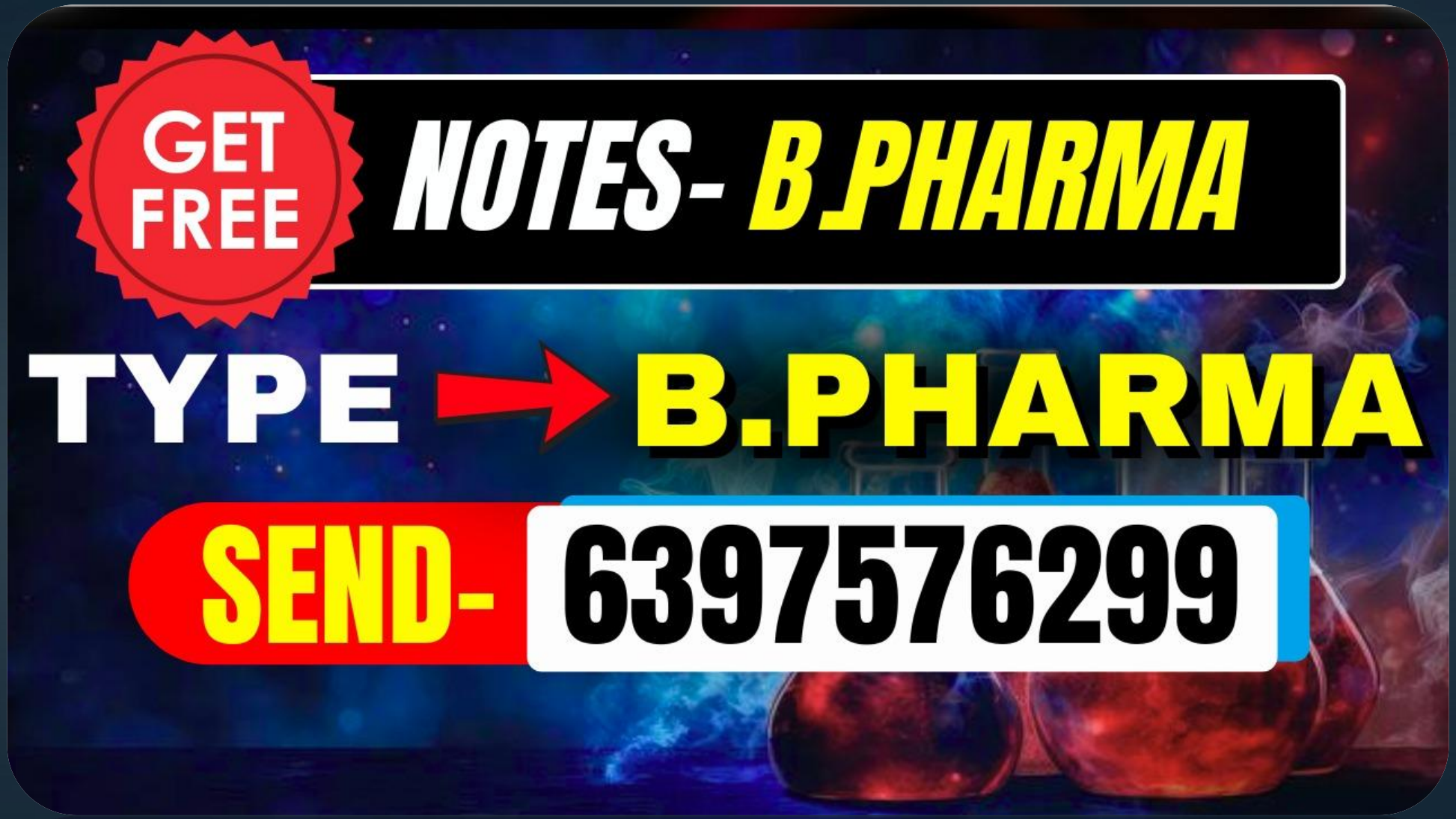

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