Course: ACCE 3111

Instrumental Analysis

Chromatographic Techniques

Section-A

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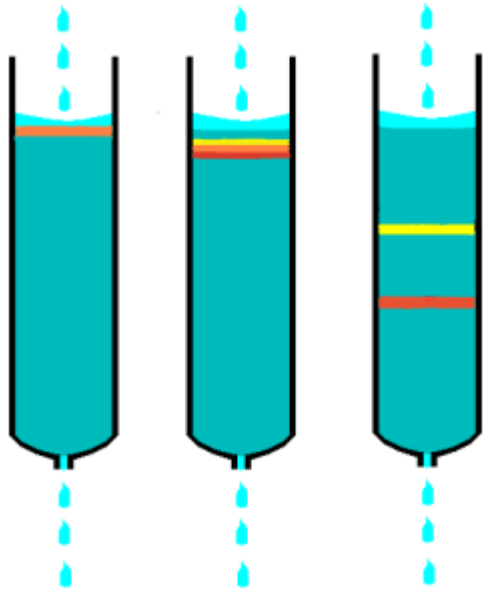
Course outline

1. Principles of chromatographic analysis
2. Thin layer chromatography
3. Ion exchange chromatography
4. High performance liquid chromatography
5. Normal phase, reversed phase, ion-exchange and ion paring techniques
6. Applications of HPLC in analysis of drugs and industrial products.

Chromatography

Chromatography is a laboratory technique used to separate the components of a mixture based on their different affinities for a stationary phase and a mobile phase.

- **Mixture:** A combination of two or more substances that are not chemically bonded.
- **Stationary Phase:** A solid material (or a liquid coated on a solid) that remains fixed in place.
- **Mobile Phase:** A liquid or gas that carries the mixture through the stationary phase.
- **Affinity:** The tendency of a molecule to be attracted to either the stationary or mobile phase.



Types of Chromatography

There are many different types of chromatography, each with its own specific applications. Some common types include:

- **Paper Chromatography:** Uses a strip of paper as the stationary phase.
- **Thin-Layer Chromatography (TLC):** Uses a thin layer of adsorbent material on a plate.
- **Gas Chromatography (GC):** Uses a gaseous mobile phase and a column packed with a stationary phase.
- **High-Performance Liquid Chromatography (HPLC):** Uses a liquid mobile phase and a column packed with a stationary phase, often with high pressure.
- **Size Exclusion Chromatography:** Separates molecules based on their size and shape.
- **Affinity Chromatography:** Uses specific interactions (like antibodies) to isolate target molecules.

Application of Chromatography

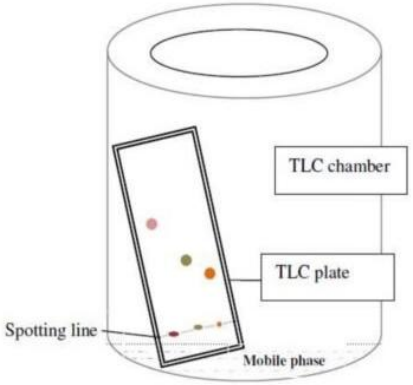
Chromatography is used in a wide variety of fields:

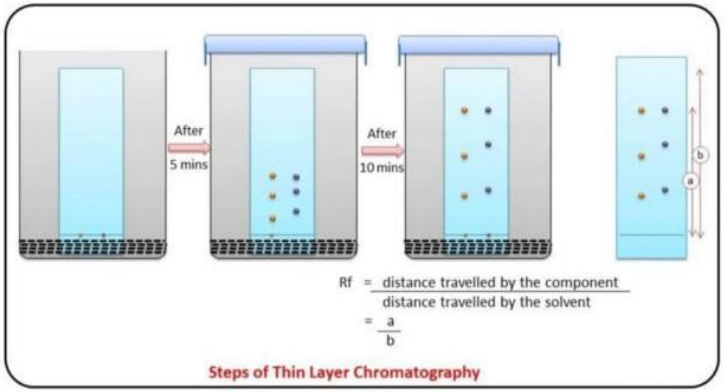
- **Food Industry:** To analyze food colorings, flavors, and additives.
- **Pharmaceuticals:** To analyze drug purity, identify impurities, and study drug metabolism.
- **Environmental Science:** To detect pollutants in air, water, and soil.
- **Biotechnology:** To separate and purify proteins, enzymes, and other biomolecules.
- **Forensic Science:** To identify and analyze evidence.
- **Clinical Laboratories:** To diagnose metabolic disorders.

Thin Layer Chromatography

Currently the most planar chromatography is based on the thin layer technique, which is faster, has better resolution and is more sensitive than its paper chromatography equivalent.

TLC is a chromatography technique used to separate non-volatile mixtures. It works by distributing compounds between a stationary phase (a thin layer of adsorbent material on a plate) and a mobile phase (a liquid solvent that travels up the plate)





Principle of TLC

- The sample is dissolved in a volatile solvent.
- Sample is applied with the help of capillary on to the base line drawn on the solid adsorbent.
- The plate is dipped into the solvent working as mobile phase
- As the mobile phase rises up the TLC plate by capillary action, the components dissolve in the solvent and move up the TLC plate.
- Separation will occur on the basis of differences in adsorption. Weekly adsorbed component will separate first and strongly later on.

Principle of TLC

TLC principle relies on the differential migration of compounds based on their varying affinities for a stationary phase and a mobile phase.

- **Stationary Phase:**
  Typically, a thin layer of silica gel, alumina, or cellulose is coated on a solid support like glass, plastic, or aluminum.

- **Mobile Phase:**
  A suitable solvent or solvent mixture travels up the stationary phase via capillary action.

- **Separation:**
  As the mobile phase moves, different compounds in the mixture interact with the stationary phase to varying degrees. Compounds with a higher affinity for the stationary phase will travel slower, while those with a higher affinity for the mobile phase will travel faster.

- **Visualization:**
  Once the mobile phase has traveled a certain distance, the plate is removed, and the separated components are visualized using various techniques, such as UV light or chemical reagents, revealing distinct spots at different levels.

TLC

TLC is a versatile technique used for:

- **Separating and identifying compounds in a mixture:** Determining the number of components and their purity.
- **Monitoring the progress of a reaction:** Observing the disappearance of reactants and the formation of products.
- **Purifying small amounts of compounds:** Separating compounds for further analysis or use.

Advantages:

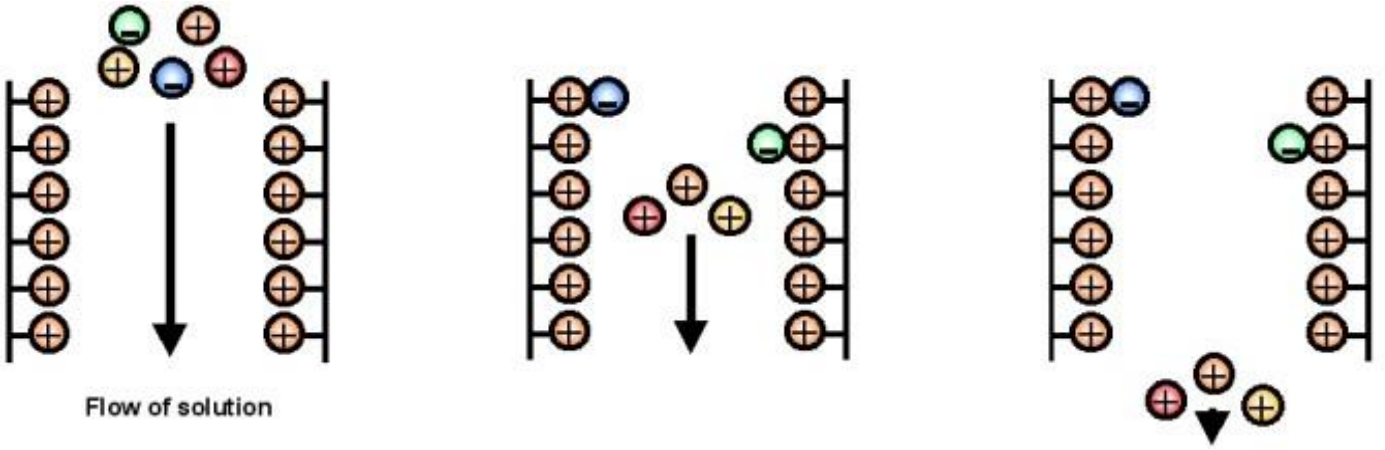
- **Simplicity and speed:** TLC is a relatively simple and fast technique.
- **Low cost:** TLC plates and solvents are relatively inexpensive.
- **High sensitivity:** TLC can detect very small amounts of compounds.

Limitations:

- **Not suitable for volatile compounds:** The mobile phase needs to be non-volatile for TLC to work.
- **Limited capacity:** TLC is generally used for separating small amounts of material.

Ion-Exchange Chromatography

Ion exchange chromatography (IEC) is a separation technique that utilizes the differences in charge between molecules to separate them. It works by selectively binding charged molecules to a stationary phase (an ion exchanger) based on their attraction to oppositely charged functional groups. The separated molecules are then eluted using changes in buffer conditions.



Ion-Exchange Chromatography

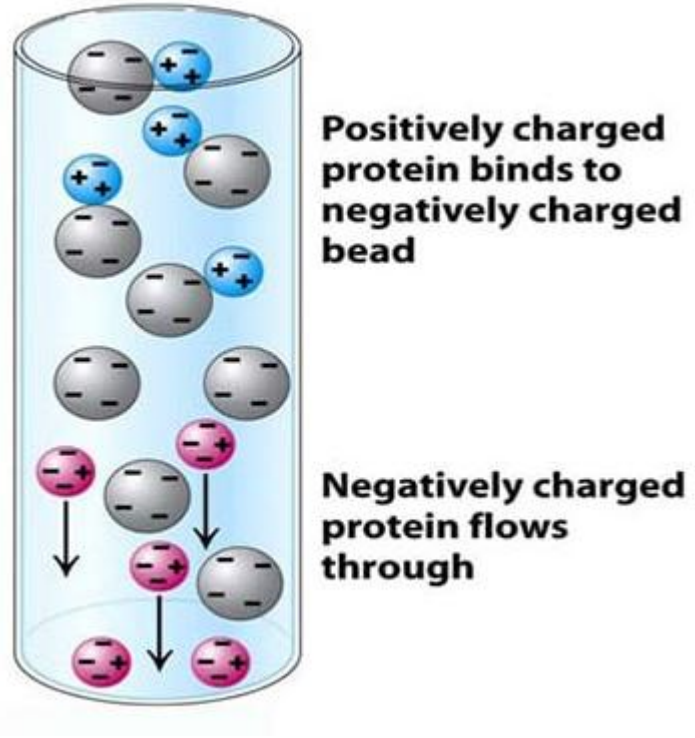
- **Working Activity**

- **Stationary Phase:**
A solid support (resin) with charged functional groups (anionic or cationic).

- **Mobile Phase:**
A liquid (buffer) that carries the sample through the column.

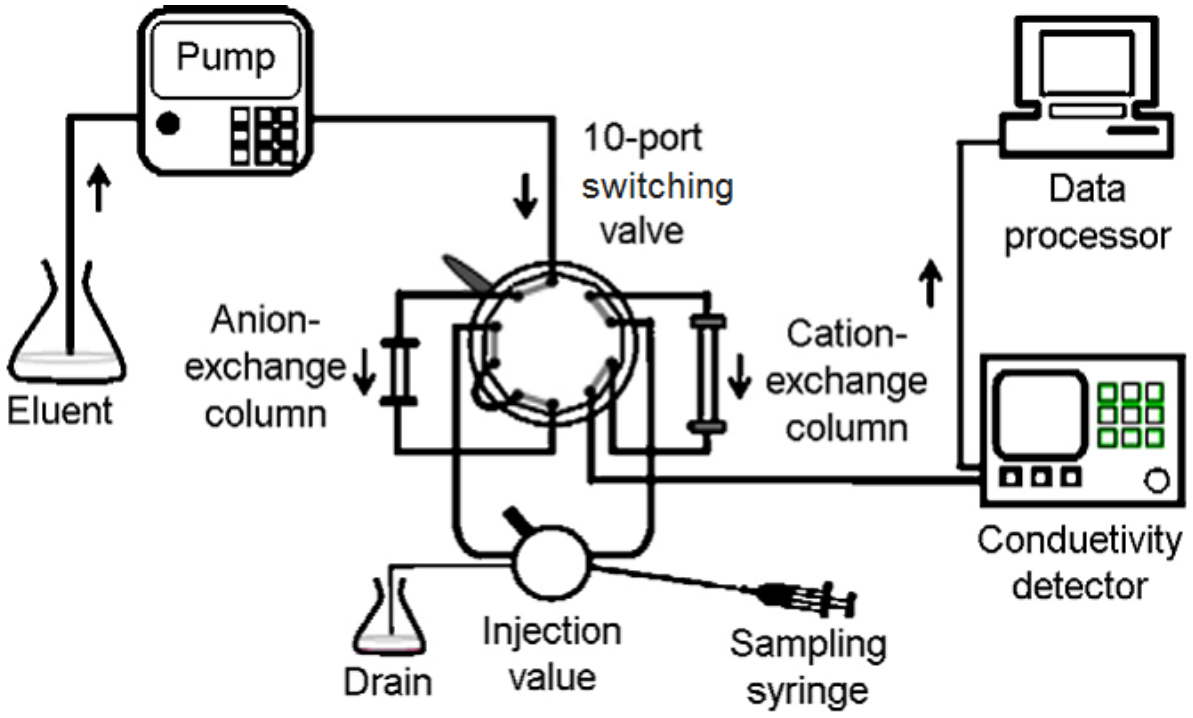
- **Analytes:**
The molecules being separated, which have either a positive or negative charge.

- **Electrostatic Interactions:**
The attraction between oppositely charged molecules, driving the separation process.



Ion-Exchange Chromatography

Ion exchange chromatography (IEC) setup involves a column packed with a charged resin, a mobile phase (buffer solutions), and a system to deliver and monitor the sample and eluent. The process separates molecules based on their charge interactions with the resin.



Ion-Exchange Chromatography

Cation Exchange Resins:

•Strong Acid Cation Resins

These resins have sulfonic acid groups ($-SO_3H$) and can exchange cations across a wide pH range.

•Weak Acid Cation Resins

These resins have carboxylic acid groups (-COOH) and are effective for removing cations associated with alkalinity.

Anion Exchange Resins:

•Strong Base Anion Resins

These resins have quaternary ammonium groups (NR^{4+}) and can exchange anions across a wide pH range.

•Weak Base Anion Resins

These resins have amine groups and are typically used for removing weakly acidic ions.

Ion-Exchange Chromatography

Common Resin Matrices:

•Polystyrene-divinylbenzene (PS-DVB):

This is a widely used matrix for both cation and anion exchange resins.

•Cellulose, agarose, polymethacrylate, and polyacrylamide:

These are also used as matrices for ion exchange resins.

Ion-Exchange Chromatography

Buffers for Cation Exchange

Phosphate buffers: Effective at neutral to slightly alkaline pH and are suitable for separating a wide range of cations.

- **Acetate buffers:** Useful for separations at slightly acidic pH.
- **Citrate buffers:** Provide good buffering capacity at slightly acidic to neutral pH.
- **Tris buffers:** Commonly used for separations at slightly alkaline pH.

Ion-Exchange Chromatography

Buffers for Anion Exchange

Tris buffers: A versatile buffer that can be used across a wide pH range, making it suitable for many anion exchange applications.

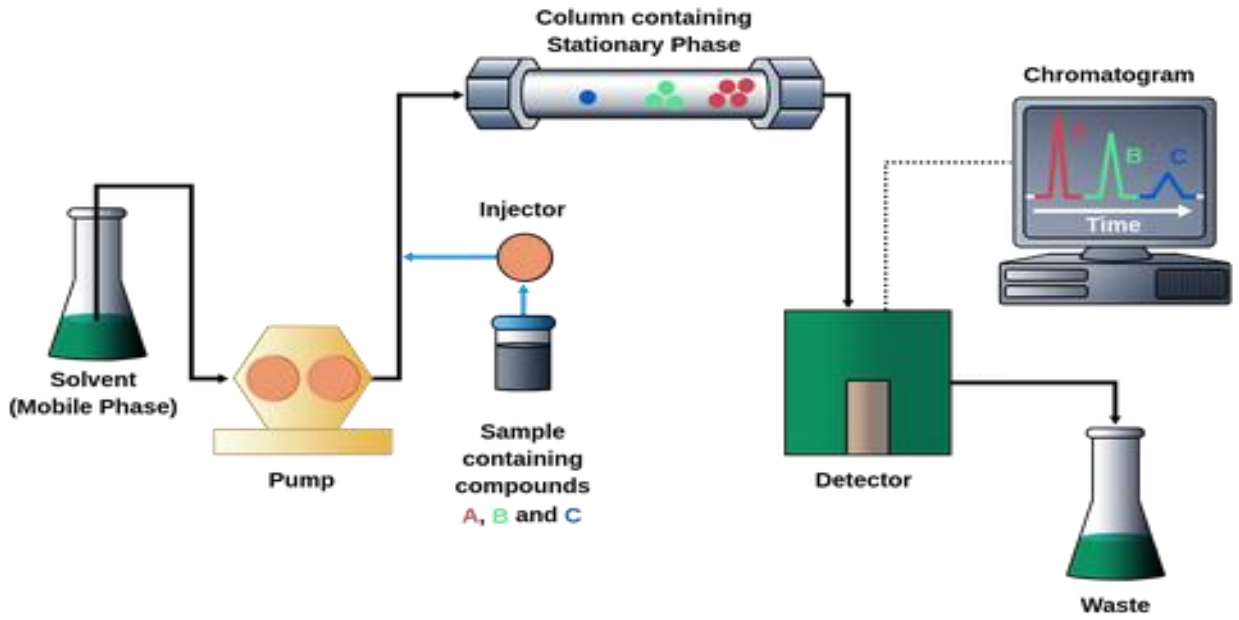
- **Piperazine buffers:** Effective at slightly alkaline pH.

- **Diethylamine buffers:** Can be used at higher pH values for separating strong anions.

- **N-Methyl-piperazine, Bis-Tris, and Bis-Tris propane buffers:** Also used in anion exchange, covering a range of pH values from slightly acidic to slightly alkaline.

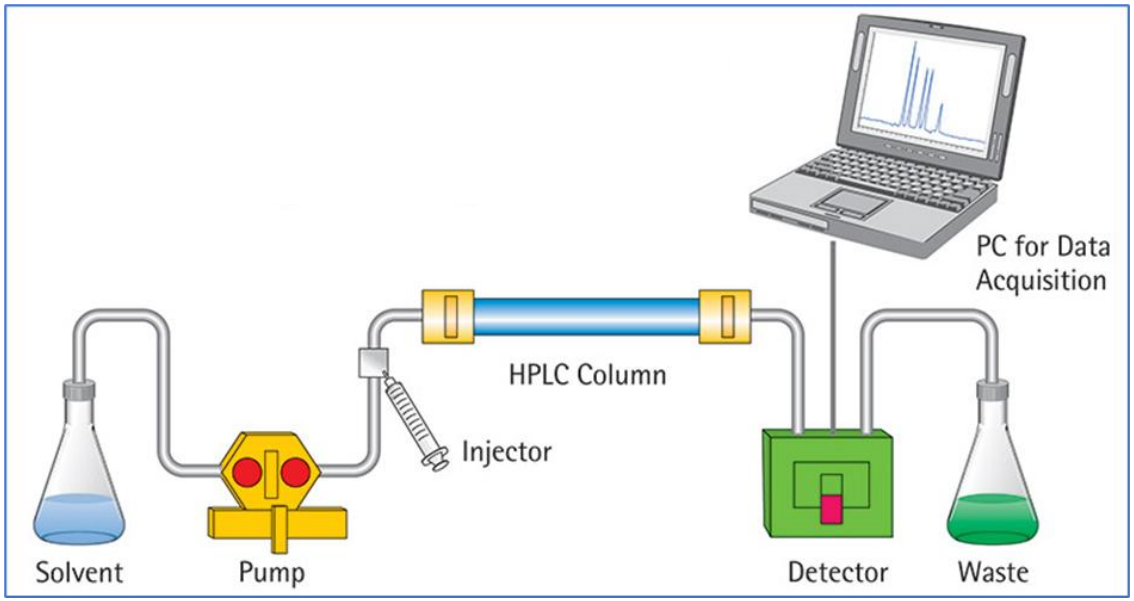
HPLC

High-Performance Liquid Chromatography (HPLC) is an analytical chemistry technique used to separate, identify, and quantify the components of a mixture. It works by forcing a liquid (mobile phase) through a column packed with a solid material (stationary phase) under high pressure, separating the mixture's components based on their interactions with the two phases.



HPLC: Principle

High-Performance Liquid Chromatography (HPLC) works on the principle of differential migration of compounds based on their varying affinities for a stationary phase and a mobile phase. The sample mixture is introduced into a flowing stream of liquid (mobile phase) that is forced through a column packed with a solid material (stationary phase). Components with stronger interactions with the stationary phase move slower, while those with weaker interactions elute faster, leading to separation.



HPLC

•Separation:

•HPLC separates compounds based on differences in their physical and chemical properties, such as polarity, size, and charge, as they interact with the stationary and mobile phases.

•Mobile Phase:

•A liquid solvent or a mixture of solvents that carries the sample through the column.

•Stationary Phase:

•A solid material packed inside the column, typically a porous solid with specific properties that interact with the sample components.

•Pressure:

•HPLC uses high pressure to force the mobile phase through the column, enabling faster and more efficient separations than traditional liquid chromatography.

HPLC: How it Works:

Sample Introduction:
The sample is dissolved in a suitable solvent and injected into the HPLC system.

Column Separation:
The mobile phase carries the sample through the column, where the different components of the sample interact with the stationary phase to varying degrees.

Detection:
As the separated compounds elute (emerge) from the column, they pass through a detector that measures their concentration.

Data Analysis:
The detector's output is recorded as a chromatogram, which shows peaks corresponding to the separated compounds. The peak positions and areas can be used to identify and quantify the components of the sample.

HPLC: Mobile Phase

Several factors are considered when choosing a mobile phase:

- **Solvent Strength/Polarity:** The mobile phase's polarity is a key factor in determining the retention of analytes. Reversed-phase HPLC, the most common mode, uses a polar mobile phase (like water, methanol, or acetonitrile) and a nonpolar stationary phase.
- **Inertness:** The mobile phase should be inert towards the sample and the stationary phase to avoid unwanted reactions or interactions.
- **Detector Compatibility:** The mobile phase should not interfere with the detector's response, ensuring accurate detection of the analytes.
- **Miscibility:** The solvents in the mobile phase mixture should be miscible (mixable).
- **Viscosity, Refractive Index, Toxicity, and Cost:** These factors are also considered during mobile phase selection.

HPLC: Stationary Phase

Types of Stationary Phases:

•Normal-Phase:
Uses a polar stationary phase (like silica) with a non-polar mobile phase, good for separating polar compounds.

•Reverse-Phase:
Uses a non-polar stationary phase (like C18, where beads have long carbon chains attached) with a polar mobile phase, commonly used for separating non-polar compounds.

•Ion-Exchange:
Uses a stationary phase with charged groups, allowing separation based on the charge of the sample components.

•Size Exclusion:
Separates molecules based on their size, using a porous stationary phase where larger molecules can't enter the pores and elute faster.

HPLC: Stationary Phase

Importance of Stationary Phase:

•Separation Efficiency:

The choice of stationary phase is crucial for achieving effective separation of sample components.

•Retention Times:

It determines how long each compound will be retained in the column, which affects the overall analysis time.

•Sample Suitability:

Different stationary phases are designed for different types of samples, and selecting the appropriate one is essential for accurate results.

HPLC: Detector

Characteristics of Ideal Detector:

- Adequate sensitivity, the sensitivities lie in the range of 10^{-8} to 10^{-15} g solute/s.
- Good stability and reproducibility
- A linear response to solutes that extends over several orders of magnitude
- A short response time independent of flow rate
- High reliability and ease to use
- Similarity in response toward all solutes
- The detector should be nondestructive
- Detector should have minimal internal volume to reduce zone broadening and should be compatible with liquid flow.

Applications of HPLC

HPLC) is widely used in both the pharmaceutical and industrial product sectors for **analysis, quality control, and research**. **HPLC is also widely used in various fields, including:**

•Pharmaceuticals:
Analyzing drug purity, identifying impurities, and quantifying drug concentrations.

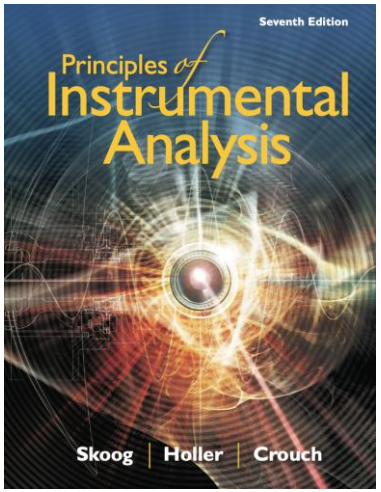
•Food Science:
Analyzing food components, detecting contaminants, and ensuring quality control.

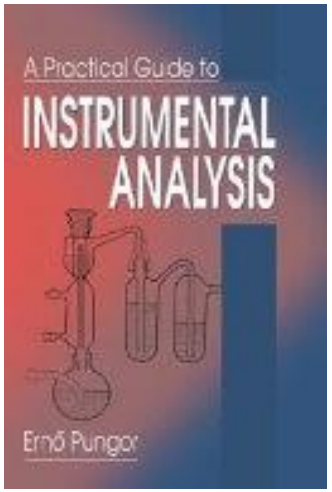
•Environmental Science:
Monitoring pollutants in water and soil.

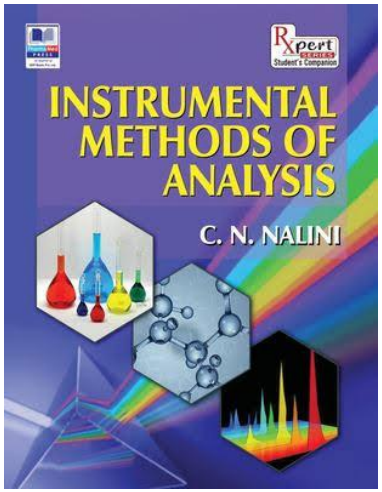
•Biochemistry:
Separating and analyzing proteins, nucleic acids, and other biomolecules.

•Forensic Science:
Analyzing samples in crime investigations.

Recommended books and sources







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