Applications of HPLC :-

① Separation of Aspirin, Phenacetin and Caffeine in a mixture :-

HPLC is used for the quantitative determination of aspirin and caffeine in the common analgesic tablets, using phenacetin as internal standard.

Sample mixture :-

The sample mixture is prepared by weighing out accurately 0.601 g of aspirin, 0.076 g of Phenacetin and 0.092 g of caffeine and dissolve the mixture in 10 mL of ethanol, add 10 mL of 0.5 M ammonium formate solution and dilute to 100 mL with distilled water.

Mobile Phase :-

0.05 M Ammonium formate in 10% volume of ethanol – water

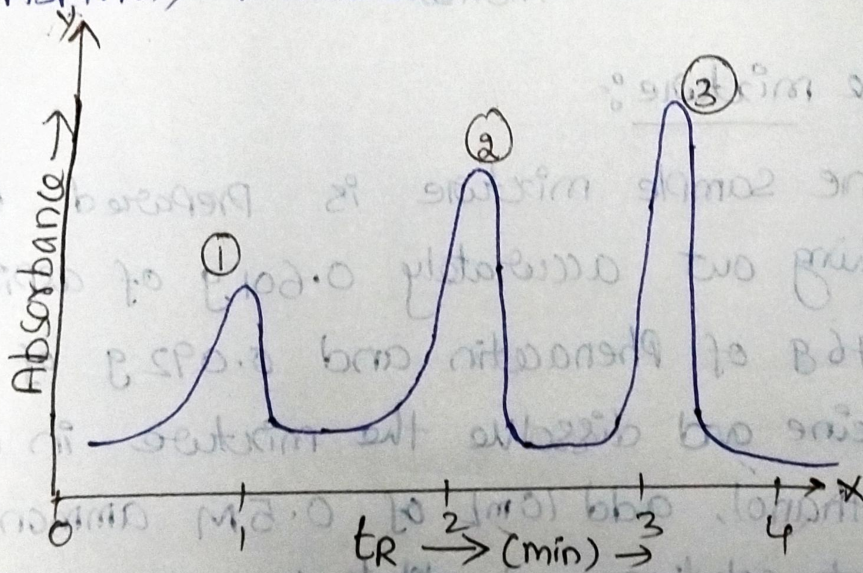
Flow rate :- 2 mL/min.

Column: $L = 15$ cm,
$id = 4.6$ mm

Packing & SP: 5 µm Silica SCX bonded phase.

Detector: UV - Detector, at absorbance → 244 nm

Procedure: inject 1 µL of the sample solution and obtain a chromatogram. Under the given conditions the compounds are separated in about 3 min, the elution sequence is Aspirin, Phenacetin and Caffeine respectively



① Aspirin
② Phenacetin
③ Caffeine.

② Separation of organic acids :-

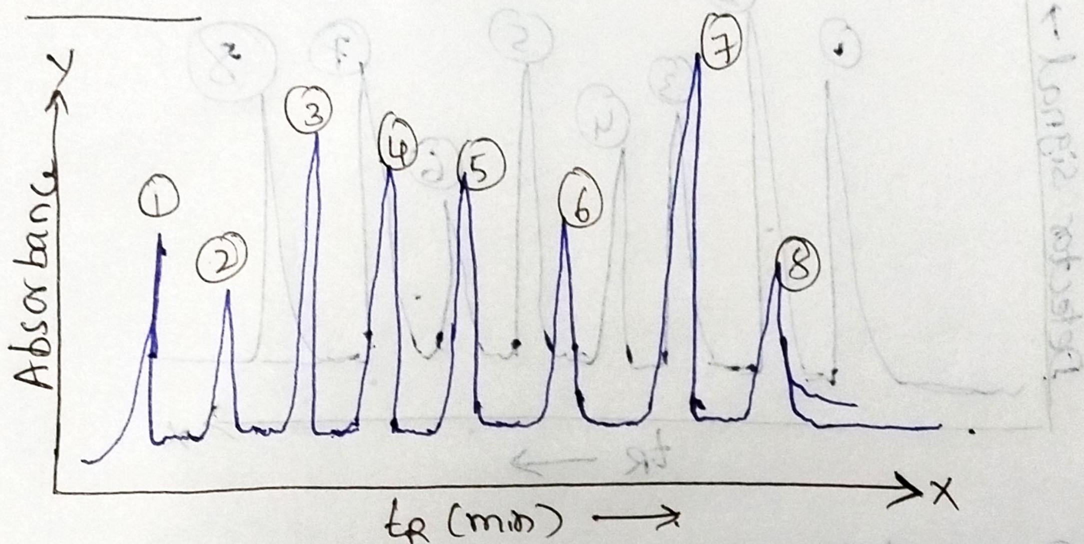
Column :- L = 30 cm,
id = 7.8 mm.

SP :- Sulphonated polystyrene (or) Divinyl benzene (DVB)

MP :- 0.1 % phosphate buffer

Flow rate :- 0.5 mL/min , T = 30°C

Detector :- UV - Detector, λ_{max} = 210 nm.



① oxalic acid
② citric acid
③ Tartaric acid
④ Malic acid
⑤ succinic acid
⑥ Fumaric acid
⑦ Acetic acid
⑧ propanoic acid.

③ Determination of carbohydrates :

Column : L = 30 cm, id = 7.8 mm

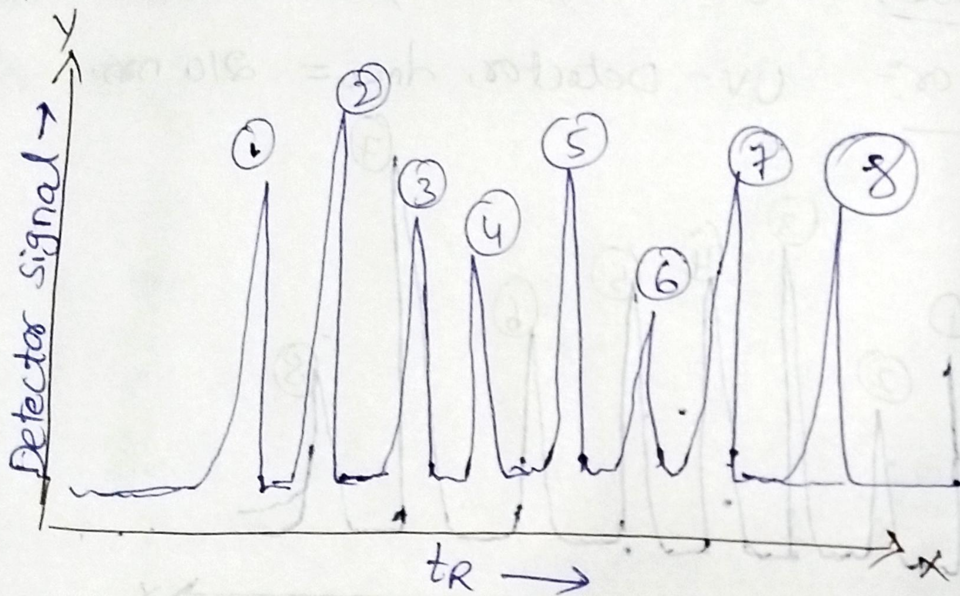
SP : Divinyl benzene (DVB).

Packing : MicroPak – NH_2

MP : Acetonitrile – water.

Flowrate : 2 mL/min , T = 35°C

Detector : Refractive index – RI detector



① Fructose
② Maltose
③ Glucose
④ Xylose
⑤ Arabinose
⑥ Ribitol
⑦ Arabitol
⑧ Xylitol.

Column chromatography

Column chromatography is a **separation technique** used to isolate and purify individual components from a mixture based on **differential adsorption** of compounds on a solid stationary phase and their movement with a liquid mobile phase.

Principle of Column Chromatography

The principle of column chromatography is based on **differential migration** or **partitioning** of the components between two phases:

- **Stationary phase**: A solid (usually silica gel or alumina) packed in a vertical glass column.
- **Mobile phase**: A solvent or a mixture of solvents (liquid phase) that percolates through the stationary phase.

Key Principle: Adsorption

Mixture of components dissolved in the MP is introduced in to the column, components move depending upon their relative affinities. Different compounds have different affinities for the stationary and mobile phases. A compound with higher affinity for the stationary phase will move slower, while a compound with higher solubility in the mobile phase will move faster down the column.

Theory of Column Chromatography

The theory is explained by the concept of **adsorption** and **partition equilibrium**:

- The solutes are adsorbed onto the surface of the solid stationary phase.
- The degree of adsorption depends on the **polarity** of the compound and the **stationary phase**.
- More polar compounds are retained longer on polar stationary phases like silica or alumina.

Elution Process

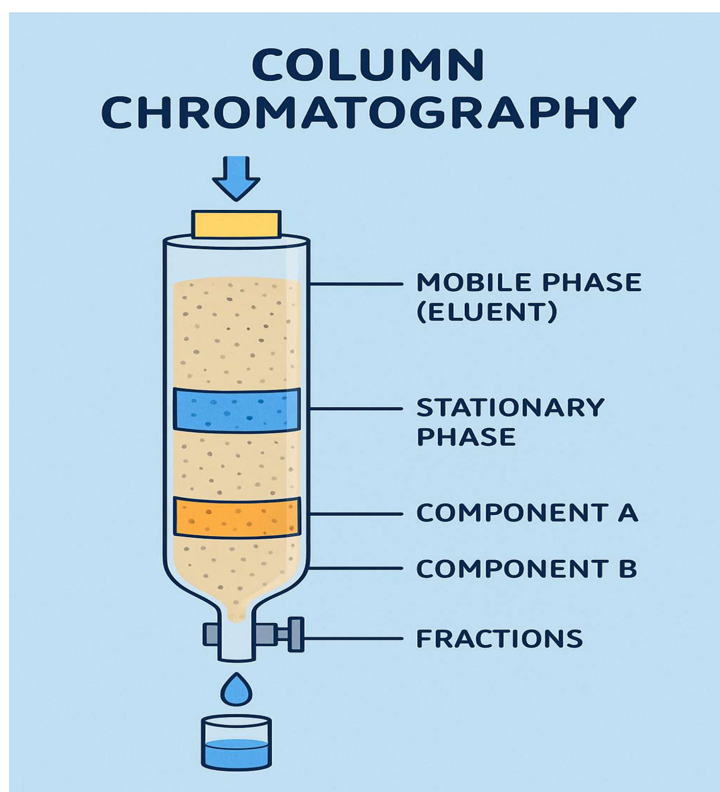
- The mobile phase (eluent) is added from the top and flows down by gravity or pressure.
- As the solvent flows, compounds interact with the stationary phase and travel at different speeds.
- This results in **separation into bands** along the column.

Types of Elution

- **Isocratic elution**: The same solvent composition is used throughout.
- **Gradient elution**: Solvent composition is gradually changed (e.g., increasing polarity) to improve separation of compounds with different affinities.

Factors Affecting Separation

- Nature of stationary phase (polarity, particle size)
- Polarity of mobile phase
- Flow rate
- Column dimensions (length and diameter)
- Amount of sample loaded



Experimental Procedure of Column Chromatography:

1. Column Preparation

- **Choose the column** based on sample size (glass column with a stopcock at the bottom).
- **Plug the bottom** with cotton or glass wool to prevent loss of adsorbent.
- **Add the stationary phase** (usually silica gel or alumina):

- Use either **dry packing** or **wet packing** (preferred):
  - Wet packing: Slurry of stationary phase in eluent poured into the column to avoid air bubbles.

2. Sample Application

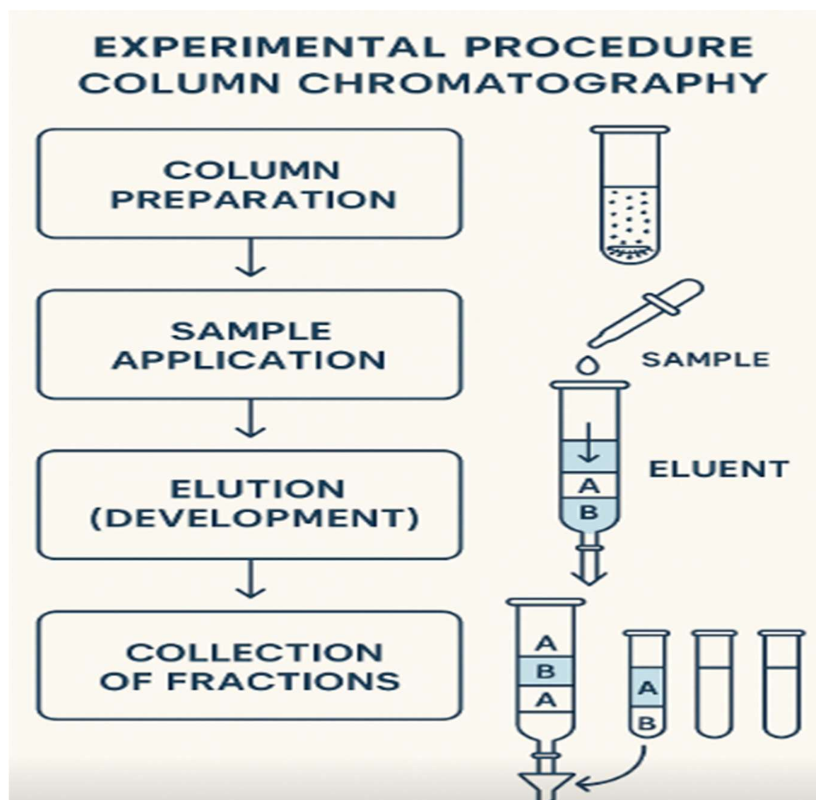
- **Dissolve the sample** in a small amount of eluent (mobile phase).
- Carefully **load the sample** on top of the stationary phase using a pipette or dropper.
- Add a thin layer of sand on top to prevent disturbance during eluent addition.

3. Elution (Development)

- Add the **eluent (mobile phase)** slowly on top of the column.
- Open the stopcock to allow eluent flow by gravity.
- The compounds will **separate into bands** based on their interaction with the stationary and mobile phases.

4. Collection of Fractions

- Collect the eluate in small, labeled test tubes or beakers.
- Use **UV light**, **TLC (thin-layer chromatography)**, or **color observation** to monitor separation.



Instrumentation and Apparatus Used

Component	Description
Glass Column	Long cylindrical tube with a stopcock for flow control.
Stationary Phase	Adsorbent like silica gel or alumina; determines the separation efficiency.
Mobile Phase (Eluent)	A suitable solvent or solvent mixture to carry the sample through the column.
Sample Loader	Pasteur pipette or dropper for careful sample addition.
Stopcock	To regulate flow of solvent and control elution rate.
Fraction Collector	Set of test tubes or beakers for collecting eluates.
TLC Plate and Chamber	To monitor the progress and completion of separation.
UV Lamp	For detection of colorless compounds in fractions.
Evaporation Setup	Rotary evaporator or water bath to concentrate purified product.

Precautions

- Avoid air bubbles during packing.
- Ensure uniform and slow elution rate.
- Use appropriate solvent polarity for desired separation.
- Prevent the column from drying during the experiment.

Detection of Components in Column Chromatography:

Once the components of a mixture are eluted and collected in fractions, identifying them is crucial. Detection can be done by physical observation, analytical techniques, or chromatographic tools.

1. Visual Detection

- **Colored Compounds**: Some components are naturally colored and can be seen as distinct bands on the column and in the fractions.
- **UV-Active Compounds**: If the compounds absorb UV light, shine a **UV lamp (254 or 365 nm)** on the collected fractions or TLC plates to detect them.

2. Chemical Detection (Spray Reagents)

After spotting on TLC, the plate can be sprayed with reagents specific to functional groups:

- **Ninhydrin** – Detects amino acids (gives purple color).
- **Iodine vapors** – Detects most organic compounds.

- **Anisaldehyde** – Detects alcohols, phenols, sugars.
- **$KMnO_4$** – Detects alkenes, alcohols, and aldehydes (gives brown spots).

3. Spectroscopic Methods

- **UV-Visible Spectroscopy**: Useful if the compounds have chromophores.
- **Infrared (IR) Spectroscopy**: Confirms functional groups.
- **NMR Spectroscopy**: Structural elucidation of isolated fractions.
- **Mass Spectrometry (MS)**: For molecular weight and fragmentation analysis.

Types of Column Chromatography:

1. Adsorption Column Chromatography

- **Stationary phase**: Solid (e.g., silica gel, alumina)
- **Mobile phase**: Liquid (organic solvents)
- **Mechanism**: **Adsorption** – solutes adhere to the solid surface.
- **Common for**: Non-polar to moderately polar compounds.

2. Partition Column Chromatography

- **Stationary phase**: Liquid (coated on an inert solid support)
- **Mobile phase**: Liquid
- **Mechanism**: **Partitioning** – solute distributes between two immiscible liquid phases.
- **Common for**: Polar compounds, amino acids, pharmaceuticals.

3. Ion-Exchange Column Chromatography

- **Stationary phase**: Ion-exchange resin (cationic or anionic)
- **Mobile phase**: Aqueous buffer solution
- **Mechanism**: **Electrostatic interaction** between charged analytes and resin.
- **Common for**: Amino acids, proteins, inorganic ions.

4. Gel Filtration / Size-Exclusion Chromatography

- **Stationary phase**: Porous gel beads (e.g., Sephadex)
- **Mobile phase**: Aqueous or organic solvent
- **Mechanism**: **Size-based separation** – larger molecules elute first, smaller enter pores and elute later.

- **Common for**: Proteins, polysaccharides, polymers.

5. Affinity Column Chromatography

- **Stationary phase**: Matrix-bound ligand specific to target molecule
- **Mobile phase**: Buffer
- **Mechanism**: **Biological specificity** – enzyme-substrate, antigen-antibody.
- **Common for**: Purifying enzymes, antibodies, nucleic acids.

6. Chiral Column Chromatography

- **Stationary phase**: Chiral material
- **Mobile phase**: Organic solvent or buffer
- **Mechanism**: **Stereochemical interaction** – enantiomers are separated.
- **Common for**: Enantiomeric purity analysis.

Applications of Column Chromatography:

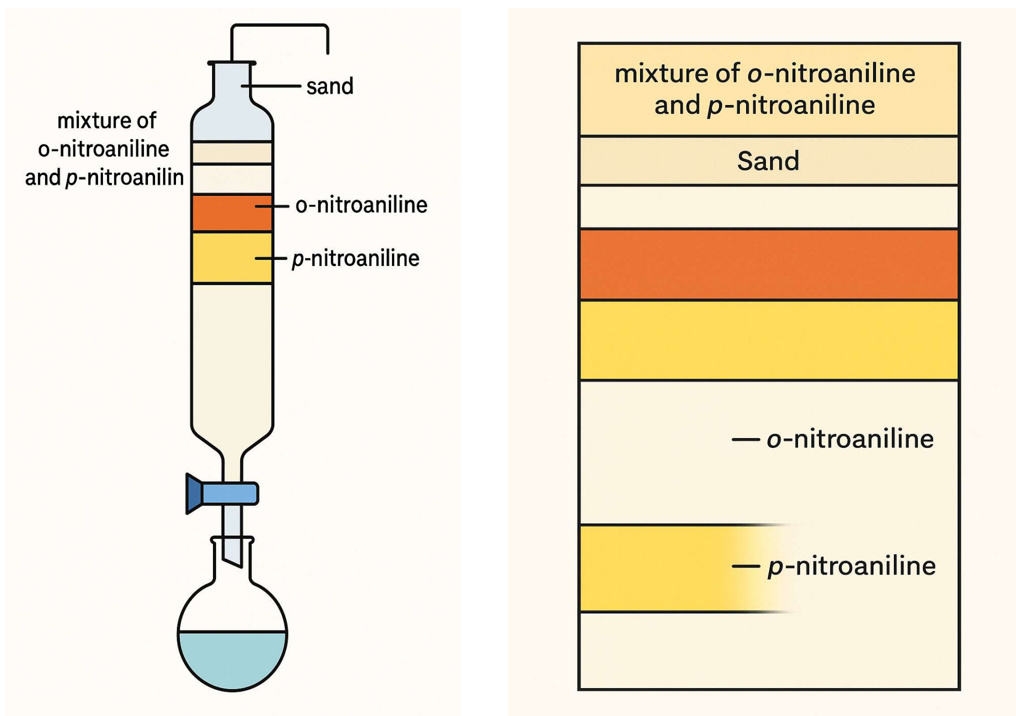
1. Separation of o-Nitroaniline and p-Nitroaniline using Column Chromatography:

- Stationary Phase: Polar solid – Silica gel or Alumina
- Mobile Phase: Solvent or solvent mixture – typically non-polar to moderately polar solvents like hexane: ethyl acetate
- Principle: Differential adsorption based on position of substituents:
- o-Nitroaniline (more polar due to intramolecular H-bonding and steric interaction) will interact more strongly with the stationary phase and move slower.
- p-Nitroaniline (less polar) will be eluted faster.

Materials Required

- Mixture of o- and p-nitroaniline
- Glass column with stopcock
- Silica gel (60–120 mesh)
- Cotton plug/glass wool and sand
- Solvents: Hexane and ethyl acetate (or petroleum ether and acetone)
- TLC plates and UV lamp (for monitoring)

- Capillary tubes, test tubes/beakers



2. In Organic and Analytical Chemistry

- **Purification of organic compounds** after synthesis.
- **Separation of isomers** (e.g., positional, geometric, enantiomers).
- **Isolation of reaction intermediates** and by-products.
- **Detection and quantification** of mixture components using preparative methods.

3. In Natural Product Chemistry

- **Separation of plant-based compounds** such as alkaloids, flavonoids, tannins, terpenoids.
- **Isolation of essential oils** and natural pigments.
- **Purification of bioactive constituents** for drug discovery or herbal formulations.

4. In Pharmaceutical Industry

- **Purification of drug molecules** during development.
- **Separation of enantiomers** for chiral drugs.
- **Quality control** of pharmaceutical ingredients.

- **Removal of impurities or degradation products** from active pharmaceutical ingredients (APIs).

. 5In Biochemistry and Molecular Biology

- **Separation of biomolecules** such as amino acids, peptides, proteins, and nucleotides.
- **Protein purification** using affinity or ion-exchange chromatography.
- **Enzyme isolation** and purification from crude extracts.
- **Desalting and buffer exchange** using gel filtration columns.

6. In Environmental and Industrial Chemistry

- **Separation of pollutants** and monitoring trace contaminants in soil or water.
- **Analysis of food additives**, preservatives, or colorants.
- **Purification of dyes, pigments, and polymers** in chemical industries.
- **Isolation of catalysts** or by-products in industrial reactions.

High-performance liquid chromatography (HPLC)

- High-performance liquid chromatography or commonly known as HPLC, is an analytical technique used to separate, identify or quantify each component in a mixture.
- The mixture is separated using the basic principle of column **chromatography** and then identified and quantified by spectroscopy.
- In the 1960s, the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.
- HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

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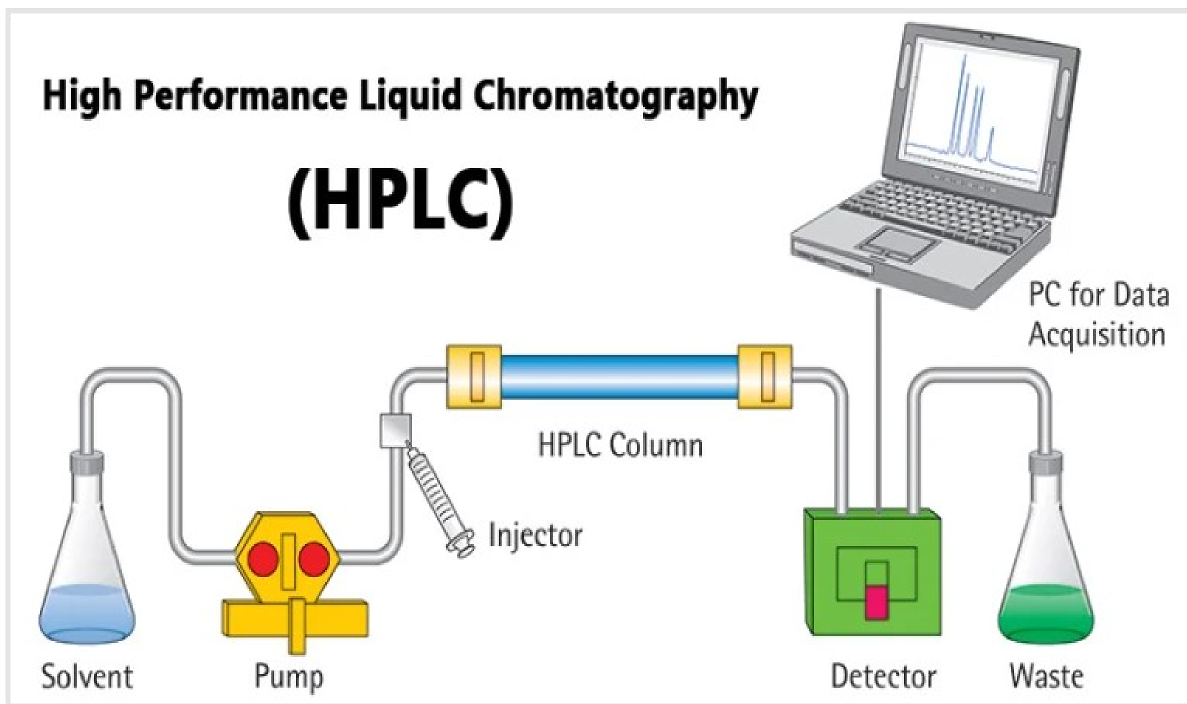
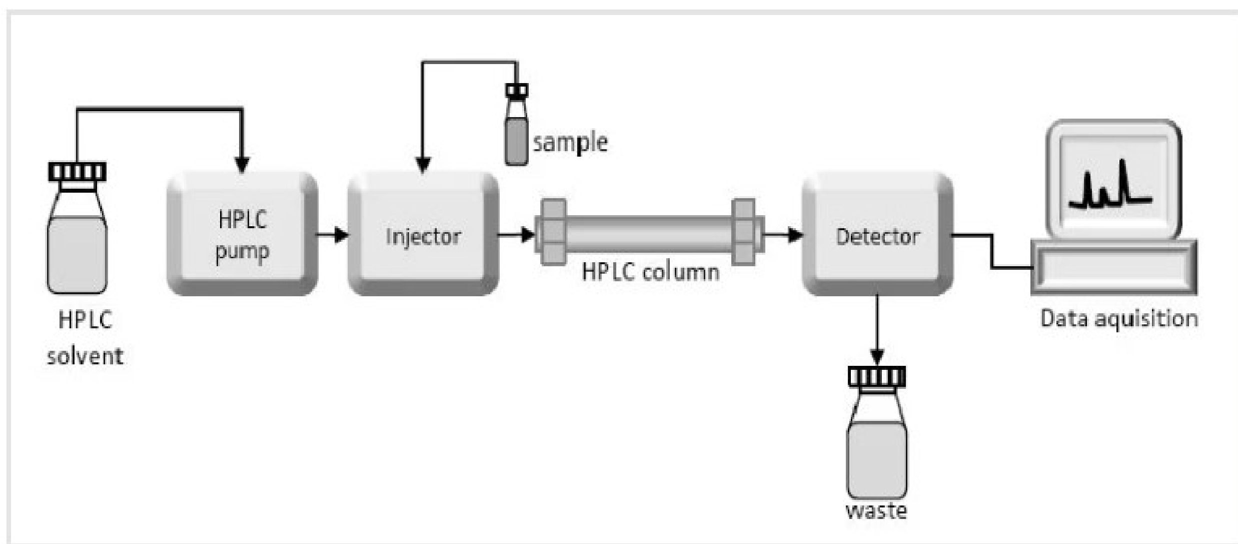


Principle of High-performance liquid chromatography:

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.

- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.

Instrumentation of High-performance liquid chromatography:



The Pump

- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses"

Injector

- An injector is placed next to the pump.
- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.
- The most widely used injection method is based on sampling loops.
- The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Column

- The separation is performed inside the column.
- The recent columns are often prepared in a stainless steel housing, instead of glass columns.
- The packing material generally used is silica or polymer gels compared to calcium carbonate.
  The eluent used for LC varies from acidic to basic solvents.
- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents.

Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation.
- The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.
- This difference is monitored as a form of an electronic signal. There are different types of detectors available.

Recorder

- The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes.
- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common.
- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are

specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes.

- When gas is present in the eluent, this is detected as noise and causes an unstable baseline.
- Degasser uses special polymer membrane tubing to remove gases.
- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore.

Column Heater

The LC separation is often largely influenced by the column temperature.

- In order to obtain repeatable results, it is important to keep consistent temperature conditions.
- Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C).
- Thus columns are generally kept inside the column oven (column heater).

Normal phase Chromatography:

Column packing is polar (e.g silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers, and chiral compounds.

The column is filled with tiny silica particles, and a non-polar solvent, for example, hexane. A typical column has an internal diameter of 4.6 mm or smaller and a length of 150 to 250 mm. Non-polar compounds in the mixture will pass more quickly through the column, as polar compounds will stick longer to the polar silica than non-polar compounds will.

Reverse phase Chromatography:

The column packing is non-polar (e.g C18), the mobile phase is water+ miscible solvent (e.g methanol). It can be used for polar, non-polar, ionizable, and ionic samples

The column is filled with silica particles which are modified to make them non-polar. This is done by attaching long hydrocarbon chains (8–18 C atoms) to its surface. A polar solvent is used, for example, a mixture of water and an alcohol such as methanol. Polar compounds in the mixture will pass more quickly through the column because a strong attraction occurs between the polar solvent and the polar molecules in the mixture. Reversed phase HPLC is the most commonly used form of HPLC.

	Stationary Phase	Mobile Phase
Normal Phase	Polar	Non polar
Reverse Phase	Non polar	Polar

Applications of HPLC:

The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components

- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

Advantages of HPLC:

- Speed
- It has a high sensitivity while a low sample consumption
- Efficiency: The most important aspect of HPLC is the high separation capacity
- Accuracy
- Versatile and extremely precise when it comes to identifying and quantifying chemical components.
- HPLC has one advantage over GC column that analysis is possible for any sample can be stably dissolved in the eluent and need not to be vaporized

Gradient Elution vs. Isocratic Elution:

- In isocratic elution, the mobile composition of the mobile phase is kept constant throughout the elution process. In gradient elution, the composition of the mobile phase is increased gradually during the elution process.
- In isocratic elution, a mixture of mobile phase or a solvent system used to separate the sample components and it is consistent over the complete testing time. There are no changes in the mobile phase composition that can be made between the entire run of the isocratic system.
- If the composition of the mobile phase remains constant throughout the HPLC separation, the separation is deemed an **isocratic elution**.

- The composition of the mobile phase in the gradient system varies throughout the chromatographic run and therefore affects the retention of the analysis. Separation in gradient mode can be either accelerated or decelerated.
- The key difference between isocratic (I) and gradient (G) systems is that the isocratic elution uses a single mobile phase composition having the same polarity, whereas the gradient elution uses more than one mobile phase and it can gradually increase or decreases the polarity of the mobile throughout the process of separation.

Detectors used in HPLC:

A chromatography detector is a device used to detect components of the mixture being eluted off the chromatography column. The detector senses the presence of the individual components as they elute the column. The detector converts a change in effluents into an electric signal that is recorded by data system.

HPLC detectors can be broadly divided into the following two classes:

(a) Bulk property detectors or Universal detectors: which measure the difference in some physical property of the solute in the mobile phase compared to the mobile phase alone. They are generally universal in application.

Ex: Refractive index and conductivity detectors, Light scattering detectors

(b) Solute property detectors or Selective detectors*:* These respond to a particular physical or chemical property of the solute, being ideally independent of the mobile phase. They generally provide high sensitivity and a wide linear response range.

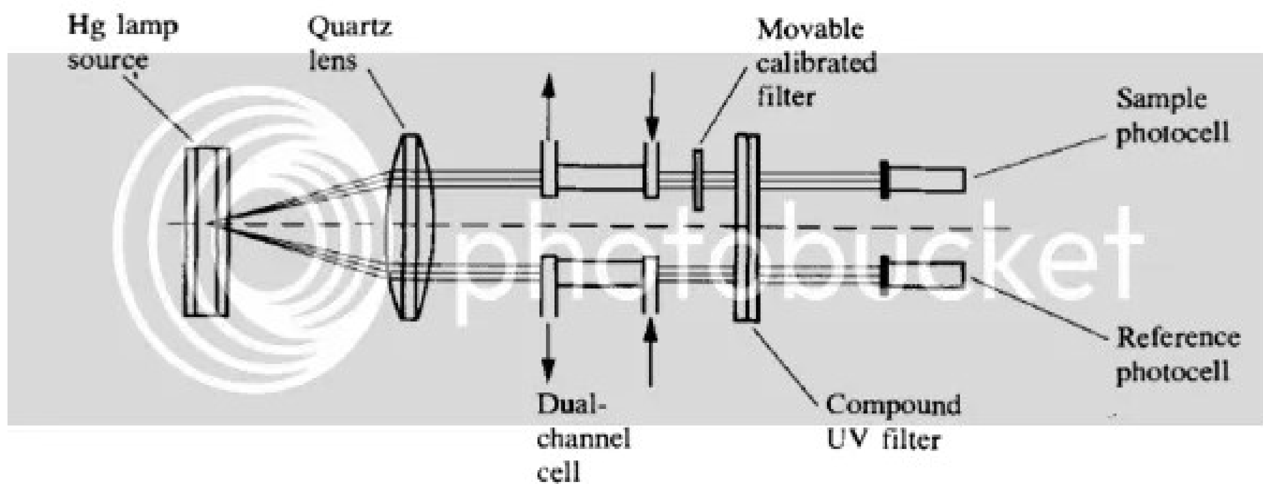
Ex: Absorbance detectors: UV – visible detectors, Fluorescence detectors, electrochemical detectors, Mass spectrometric detectors

1. **Ultraviolet/visible spectroscopic detectors:**

The UV-VIS detectors are categorized as absorbance detectors. UV detector is a very commonly used detector for HPLC analysis. During the analysis, sample goes through a sample cell. When UV light is irradiated on the sample cell, sample absorbs a part of UV light. Thus, the intensity of UV light observed for the mobile phase (without sample) and the eluent containing sample will differ. By measuring this difference, the amount of sample can be determined. Since the UV absorbance also differs depend on what wavelength is used, it is important to choose an appropriate wavelength based on the type of analyte. A standard UV detector allows user to choose wavelength between 195 to 370 nm. Most commonly used is 254 nm.

There are mainly 3 types of UV-Vis detectors are used,

- Fixed wavelength detectors: HPLC detectors which does not allow to change the wavelength of the radiation called fixed-wavelength detectors
- Variable wavelength detectors
- Diode array detectors



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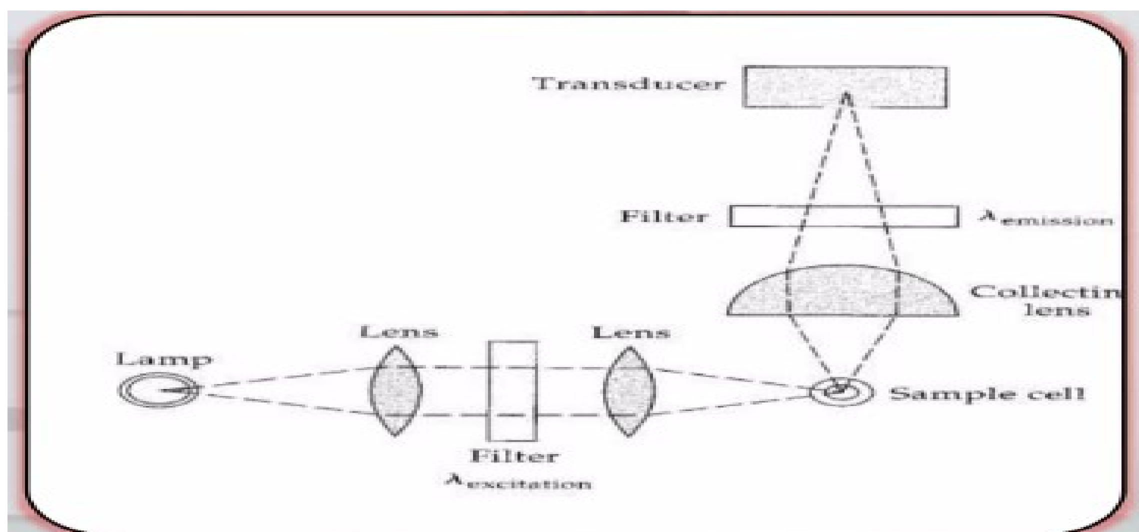
2. **Fluorescence detectors:**

By using a specific wavelength, analyte atoms are excited and then emit light signal in the form of fluorescence light. The intensity of this emitted light is monitored to quantify of the analyte concentration. Most pharmaceuticals, natural products, clinical samples, and petroleum products have fluorescent absorbance.



3. **Refractive Index Detectors:**

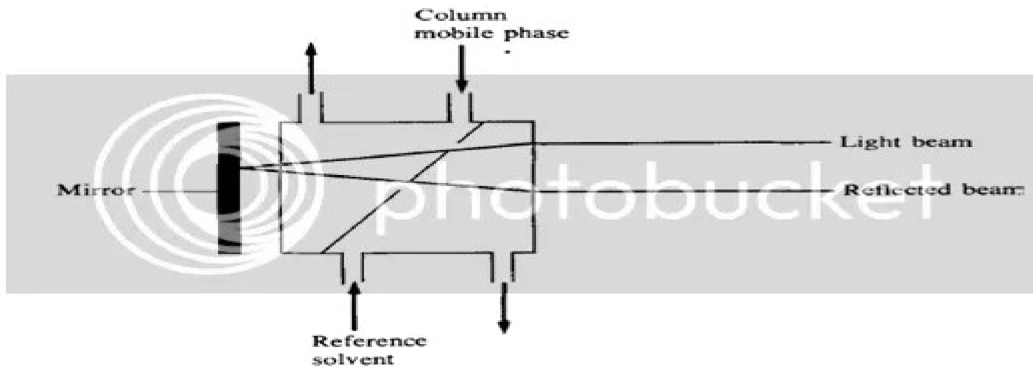
These bulk property detectors are based on the change of refractive index of the eluant from the column with respect to pure mobile phase.

The two chief types of RI detector are,

- The Deflection refractometer
- The Fresnel refractometer

The deflection refractometer, which measures the deflection of a beam of monochromatic light by a double prism in which the reference and sample cells are separated by a diagonal glass divide. When both cells contain solvent of the same composition, no deflection of the light beam occurs; if, however, the composition of the column mobile phase is changed because of the presence of a solute, then the altered

refractive index causes the beam to be deflected. The magnitude of this deflection is dependent on the concentration of the solute in the mobile phase.



4. Electrochemical detectors:

It is based on the measurement of the current resulting from an oxidation or reduction of the analyte at a suitable electrode. The level of current is directlty proportional to the analyte concentration. Three electrodes are employed which are,

- Working electrode,
- Auxiliary electrode
- Reference electrode

Electrical Conductivity Detector: Used in for the detection of ionic compounds. Detector measures the ability of the mobile phase to conduct a current when placed in a sample cell between two electrodes. Conductivity detectors measures electronic resistance and measured value is directly proportional to the concentration of the ions present in the solution