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PG AND RESEARCH DEPARTMENT OF BIOCHEMISTRY

M.Sc. BIOCHEMISTRY

E-NOTES

SUBJECT NAME: BIOINSTRUMENTATION
SUBJECT CODE: GOBC15A

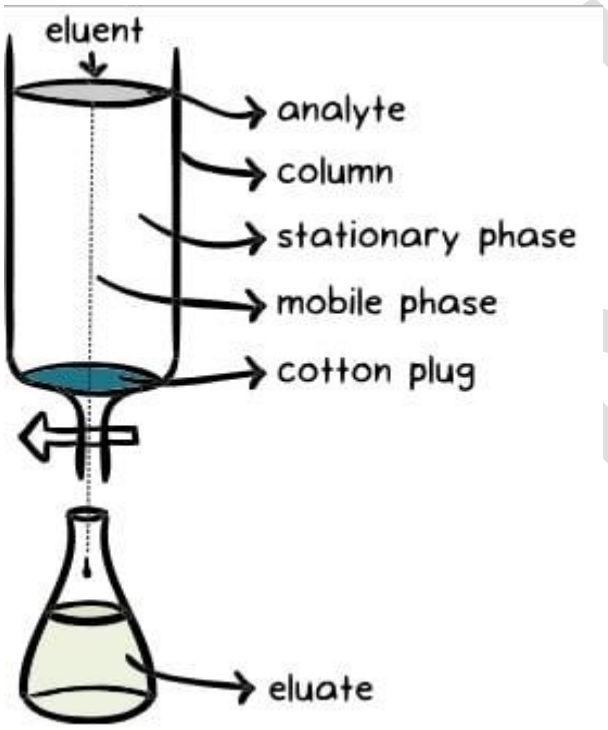
SYLLABUS

Unit-III: Chromatographic techniques Chromatographic techniques: Principles and applications of paper, TLC, ion exchange, gel filtration, affinity, GLC and HPLC .

What is chromatography?

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- The Russian botanist Mikhail Tswett coined the term chromatography in 1906.
- The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of **fatty acid** mixtures.
- A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to separate materials.
- It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures.

Principle of Chromatography



- Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase.
- The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.
- Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster.

Three components thus form the basis of the chromatography technique.

1. **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".
2. **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."
3. **Separated molecules**

The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other.

Rf value.

The Rf value is defined as the ratio of the distance moved by the solute (i.e. the dye or pigment under test) and the distance moved by the the solvent (known as the Solvent front) along the paper, where both distances are measured from the common Origin or Application Baseline, that is the point where the sample is initially spotted on the paper.

$$\text{Rf Value} = \frac{\text{Distance from Baseline travelled by Solute}}{\text{Distance from Baseline travelled by Solvent (Solvent Front)}}$$

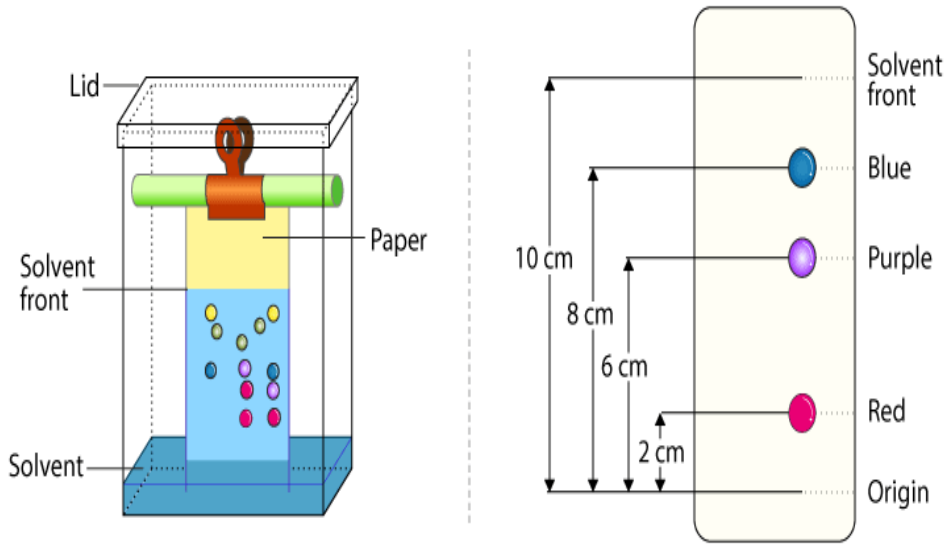
Paper Chromatography

Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

Paper Chromatography Principle

The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

Paper Chromatography Diagram



Paper Chromatography Procedure

Below we have explained the procedure to conduct Paper Chromatography Experiment for easy understanding of students.

1. **Selecting a suitable type of development:** It is decided based on the complexity of the solvent, paper, mixture, etc. Usually ascending type or radial paper chromatography is used as they are easy to perform. Also, it is easy to handle, the chromatogram obtained is faster and the process is less time-consuming.
2. **Selecting a suitable filter paper**: Selection of filter paper is done based on the size of the pores and the sample quality.
3. **Prepare the sample:** Sample preparation includes the dissolution of the sample in a suitable solvent (inert with the sample under analysis) used in making the mobile phase.
4. **Spot the sample on the paper:** Samples should be spotted at a proper position on the paper by using a capillary tube.
5. **Chromatogram development:** Chromatogram development is spotted by immersing the paper in the mobile phase. Due to the capillary action of paper, the mobile phase moves over the sample on the paper.
6. **Paper drying and compound detection**: Once the chromatogram is developed, the paper is dried using an air drier. Also, detecting solution can be sprayed on the chromatogram developed paper and dried to identify the sample chromatogram spots.

Paper Chromatography Applications

There are various applications of paper chromatography. Some of the uses of Paper Chromatography in different fields are discussed below:

- To study the process of fermentation and ripening.
- To check the purity of pharmaceuticals.
- To inspect cosmetics.
- To detect the adulterants.
- To detect the contaminants in drinks and foods.
- To examine the reaction mixtures in biochemical laboratories.
- To determine dopes and drugs in humans and animals.

Types of paper chromatography:

1. Ascending Paper Chromatography – The techniques goes with its name as the solvent moves in an upward direction.
2. Descending Paper Chromatography – The movement of the flow of solvent due to gravitational pull and capillary action is downwards, hence the name descending paper chromatography.
3. Ascending – Descending Paper Chromatography – In this version of paper chromatography, movement of solvent occurs in two directions after a particular point. Initially, the solvent travels upwards on the paper which is folded over a rod and after crossing the rod it continues with its travel in the downward direction.
4. Radial or Circular Paper Chromatography – The sample is deposited at the centre of the circular filter paper. Once the spot is dried, the filter paper is tied horizontally on a Petri dish which contains the solvent.
5. Two Dimensional Paper Chromatography – Substances which have the same r_f values can be resolved with the help of two-dimensional paper chromatography.

Thin Layer Chromatography

Thin Layer Chromatography is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel.

On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor (R_f) expressed as:

R_f = dist. travelled by sample / dist. travelled by solvent

The factors affecting retardation factor are the solvent system, amount of material spotted, absorbent and temperature. TLC is one of the fastest, least expensive, simplest and easiest chromatographytechnique.

Thin Layer Chromatography Principle

Like other chromatographic techniques, thin-layer chromatography (TLC) depends on the separation principle. The separation relies on the relative affinity of compounds towards both the phases. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast. Therefore, the separation of the mixture is attained. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.

Thin Layer Chromatography Diagram

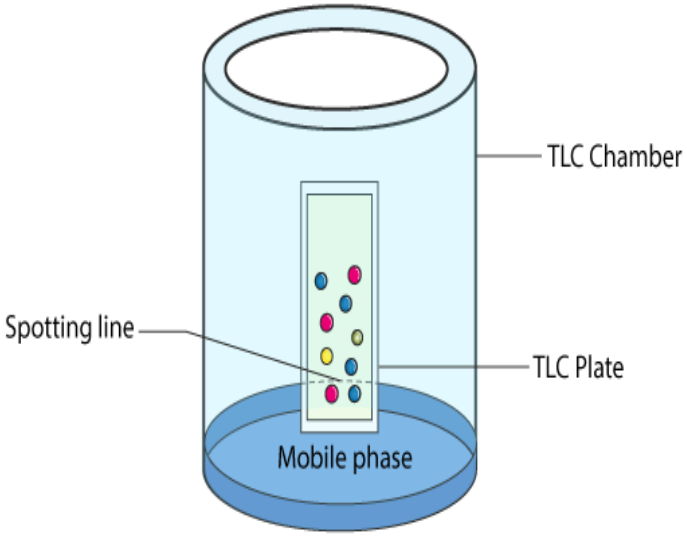


Diagram of Thin Layer Chromatography

Thin Layer Chromatography Procedure

Before starting with the Thin Layer Chromatography Experiment, let us understand the different components required to conduct the procedure along with the phases involved.

1. Thin Layer Chromatography Plates – ready-made plates are used which are chemically inert and stable. The stationary phase is applied on its surface in the form of a thin layer. The stationary phase on the plate has a fine particle size and also has a uniform thickness.
2. Thin Layer Chromatography Chamber – Chamber is used to develop plates. It is responsible to keep a steady environment inside which will help in developing spots. Also, it prevents the solvent evaporation and keeps the entire process dust-free.
3. Thin Layer Chromatography Mobile phase – Mobile phase is the one that moves and consists of a solvent mixture or a solvent. This phase should be particulate-free. The higher the quality of purity the development of spots is better.
4. Thin Layer Chromatography Filter Paper – It has to be placed inside the chamber. It is moistened in the mobile phase.

Thin Layer Chromatography Experiment

The stationary phase that is applied to the plate is made to dry and stabilize.

- To apply sample spots, thin marks are made at the bottom of the plate with the help of a pencil.
- Apply sample solutions to the marked spots.
- Pour the mobile phase into the TLC chamber and to maintain equal humidity, place a moistened filter paper in the mobile phase.
- Place the plate in the TLC chamber and close it with a lid. It is kept in such a way that the sample faces the mobile phase.
- Immerse the plate for development. Remember to keep the sample spots well above the level of the mobile phase. Do not immerse it in the solvent.
- Wait till the development of spots. Once the spots are developed, take out the plates and dry them. The sample spots can be observed under a UV light chamber.

Thin Layer Chromatography Applications

- The qualitative testing of various medicines such as sedatives, local anaesthetics, anticonvulsant tranquilisers, analgesics, antihistamines, steroids, hypnotics is done by TLC.
- TLC is extremely useful in Biochemical analysis such as separation or isolation of biochemical metabolites from its blood plasma, urine, body fluids, serum, etc.
- Thin layer chromatography can be used to identify natural products like essential oils or volatile oil, fixed oil, glycosides, waxes, alkaloids, etc.
- It is widely used in separating multicomponent pharmaceutical formulations.
- It is used to purify of any sample and direct comparison is done between the sample and the authentic sample.
- It is used in the food industry, to separate and identify colours, sweetening agent, and preservatives
- It is used in the cosmetic industry.
- It is used to study if a reaction is complete.

Disadvantages of Thin Layer Chromatography:

1. Thin Layer Chromatography plates do not have longer stationary phase.
2. When compared to other chromatographic techniques the length of separation is limited.
3. The results generated from TLC are difficult to reproduce.
4. Since TLC operates as an open system, some factors such as humidity and temperature can be consequences to the final outcome of the chromatogram.
5. The detection limit is high and therefore if you want a lower detection limit, you cannot use TLC.
6. It is only a qualitative analysis technique and not quantitative.

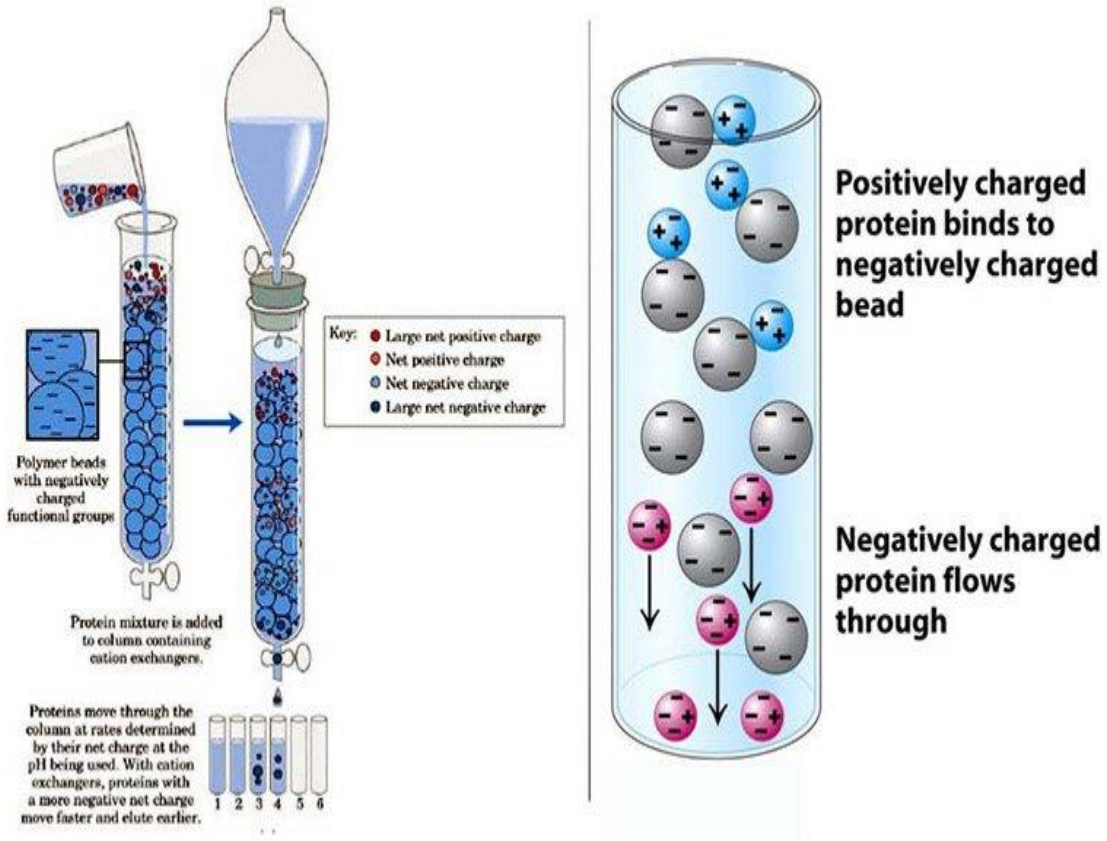
Ion Exchange Chromatography

- **Chromatography** is the separation of a mixture of compounds into its individual components based on their relative interactions with an inert matrix.

- Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.
- The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers.
- In this process two types of exchangers i.e., cationic and anionic exchangers can be used.

1. **Cationic exchangers** possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group.
2. **Anionic exchangers** have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials.

- Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thin-layer chromatographic methods that work basically based on the principle of ion exchange.



Principle of ion exchange chromatography

This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte.

- The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
- The charged groups of the matrix can be positively or negatively charged.
- When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.
- In this "ion cloud", ions can be reversibly exchanged without changing the nature and the properties of the matrix.

Instrumentation of ion exchange chromatography

Typical IC instrumentation includes: pump, injector, column, suppressor, detector and recorder or data system.

1. **Pump**

The IC pump is considered to be one of the most important components in the system which has to provide a continuous constant flow of the eluent through the IC injector, column, and detector.

2. **Injector**

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. Liquid samples may be injected directly and solid samples need only to be dissolved in an appropriate solvent. Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi).

3. **Columns**

Depending on its ultimate use and area of application, the column material may be stainless steel, titanium, glass or an inert plastic such as PEEK. The column can vary in diameter from about 2mm to 5 cm and in length from 3 cm to 50 cm depending on whether it is to be used for normal analytical purposes, microanalysis, high speed analyses or preparative work.

Guard column is placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove particles that clog the separation column

4. **Suppressor**

The suppressor reduces the background conductivity of the chemicals used to elute samples from the ion-exchange column which improves the conductivity measurement of the ions being tested. IC suppressors are membrane-based devices which are designed to convert the ionic eluent to water as a means of enhancing the sensitivity.

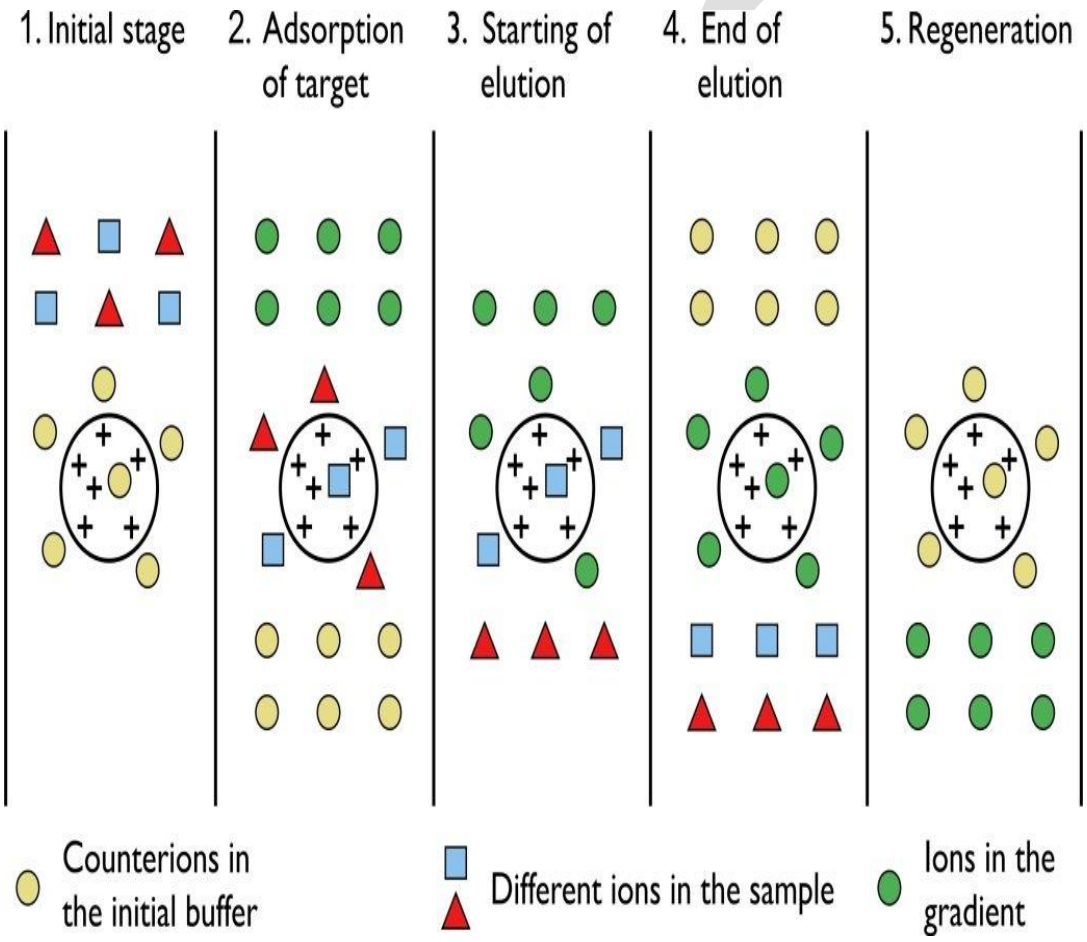
5. **Detectors**

Electrical conductivity detector is commonly use.

6. **Data system**

In routine analysis, where no automation is needed, a pre-programmed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer.

Procedure of ion exchange chromatography



- Ion exchange separations are carried out mainly in columns packed with an ion-exchanger.
- These ionic exchangers are commercially available. They are made up of styrene and divinyl benzene. Example. DEAE-cellulose is an anionic exchanger, CM-cellulose is a cationic exchanger.
- The choice of the exchanger depends upon the charge of particle to be separated. To separate anions “Anionic exchanger” is used, to separate cations “Cationic exchanger” is used.

- First the column is filled with ion exchanger then the sample is applied followed by the buffer. The tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are widely used.
- The particles which have high affinity for ion exchanger will come down the column along with buffers.
- In next step using corresponding buffer separates the tightly bound particles.
- Then these particles are analyzed spectroscopically.

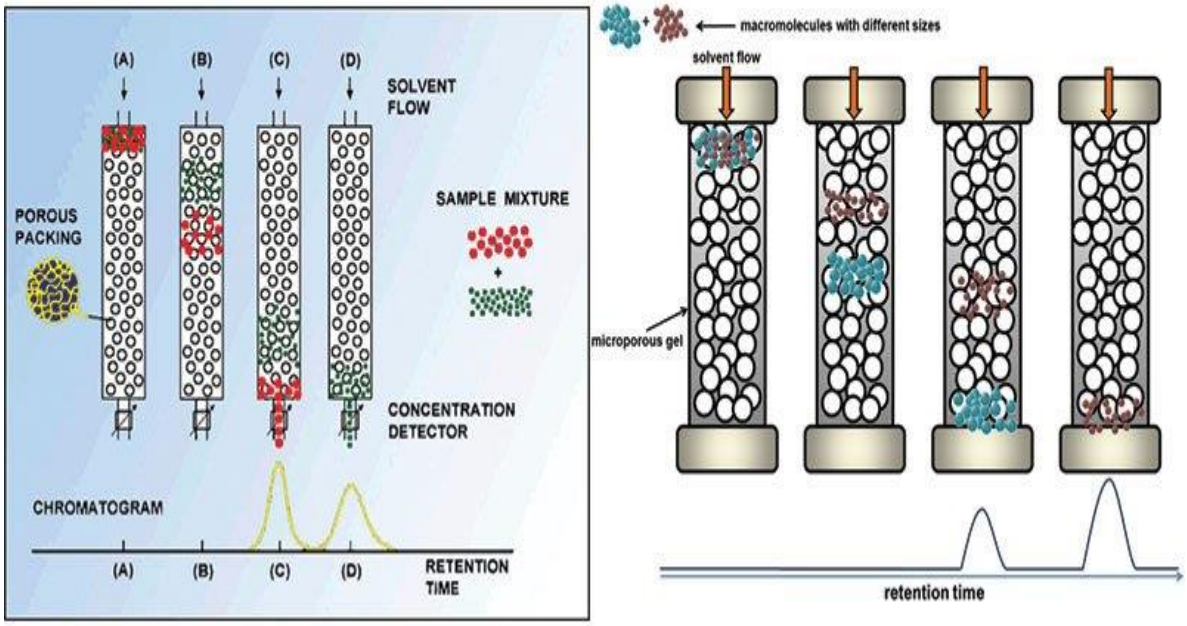
Applications of ion exchange chromatography

- An important use of ion-exchange chromatography is in the routine analysis of **amino acid** mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- This is most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by method is used for softening of drinking water.
- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.
- Chelating resins are used to collect trace metals from seawater.
- To analyze lunar rocks and rare trace elements on Earth.

Gel Permeation Chromatography

- Gel permeation chromatography is also called as gel filtration or size exclusion chromatography.
- In size exclusion chromatography, the stationary phase is a porous matrix made up of compounds like cross-linked polystyrene, cross-like dextrans, polyacrylamide gels, agarose gels, etc.
- The separation is based on the analyte molecular sizes since the gel behaves like a molecular sieve.
- This technique is used for the separation of proteins, polysaccharides, enzymes, and synthetic polymers.
- As a technique, size exclusion chromatography was first developed in 1955 by Lathe and Ruthven.

Gel Permeation Chromatography



Principle of Gel Permeation Chromatography

- It is a technique in which the separation of components is based on the difference in molecular weight or size.
- The stationary phase used is a porous polymer matrix whose pores are completely filled with the solvent to be used as the mobile phase.
- The molecules in the sample are pumped through specialized columns containing such microporous packing material (gel).
- The basis of the separation is that molecules above a certain size are totally excluded from the pores, while smaller molecules access the interior of the pores partly or wholly.
- The flow of the mobile phase hence will cause larger molecules to pass through the column unhindered, without penetrating the gel matrix, whereas smaller molecules will be retarded according to their penetration of the gel.

Components/ Instrumentation of Gel Permeation Chromatography

1. Stationary Phase
2. The Mobile Phase
3. The Columns
4. The Pump
5. Detectors

A. Stationary phase

It is composed of semi-permeable, porous polymer gel beads with a well-defined range of pore sizes.

It has the following properties:

- Chemically inert
- Mechanically stable
- With ideal and homogeneous porous structure (wide pore size give low resolution).
- A uniform particle and pore size.

Examples of gel:

1. **Dextran** (Sephadex) gel: An α 1-6-polymer of glucose natural gel
2. **Agarose** gel: A 1,3 linked β-D-galactose and 1,4 linked 3,6-anhydro-α, L-galactose natural gel
3. **Acrylamide** gel: A polymerized acrylamide, a synthetic gel

B. The Mobile Phase

It is composed of a liquid used to dissolve the bio-molecules to make the mobile phase permitting high detection response and wet the packing surface.

C. Columns

Any of the following kinds may be used:

- Analytical column- 7.5–8mm diameters.
- Preparative columns-22–25mm
- Usual column lengths-25, 30, 50, and 60 cm.
- Narrow-bore columns- 2–3mm diameter have been introduced

D. Pumps

They are either syringe pumps or reciprocating pumps with a high constant flow rate.

E. Detectors

The detectors may be concentration sensitive detectors, bulk property detectors, refractive index (RI) detector, etc.

Steps in Gel Permeation Chromatography

It involves three major steps:

A. Preparation of column for gel filtration

It involves:

1. Swelling of the gel

2. Packing the column semi-permeable, porous polymer gel beads with a well-defined range of pore sizes.
3. Washing: After packing, several column volumes of buffer solution is passed through the column to remove any air bubbles and to test the column homogeneity.

B. Loading the sample onto the column using a syringe

C. Eluting the sample and detection of components

Applications of Gel Permeation Chromatography

1. Proteins fractionation
2. Purification
3. Molecular weight determination.
4. Separation of sugar, proteins, peptides, rubbers, and others on the basis of their size.
5. Can be used to determine the quaternary structure of purified proteins.

Advantages of Gel Permeation Chromatography

- Short analysis time.
- Well defined separation.
- Narrow bands and good sensitivity.
- There is no sample loss.
- The small amount of mobile phase required.
- The flow rate can be set.

Limitations of Gel Permeation Chromatography

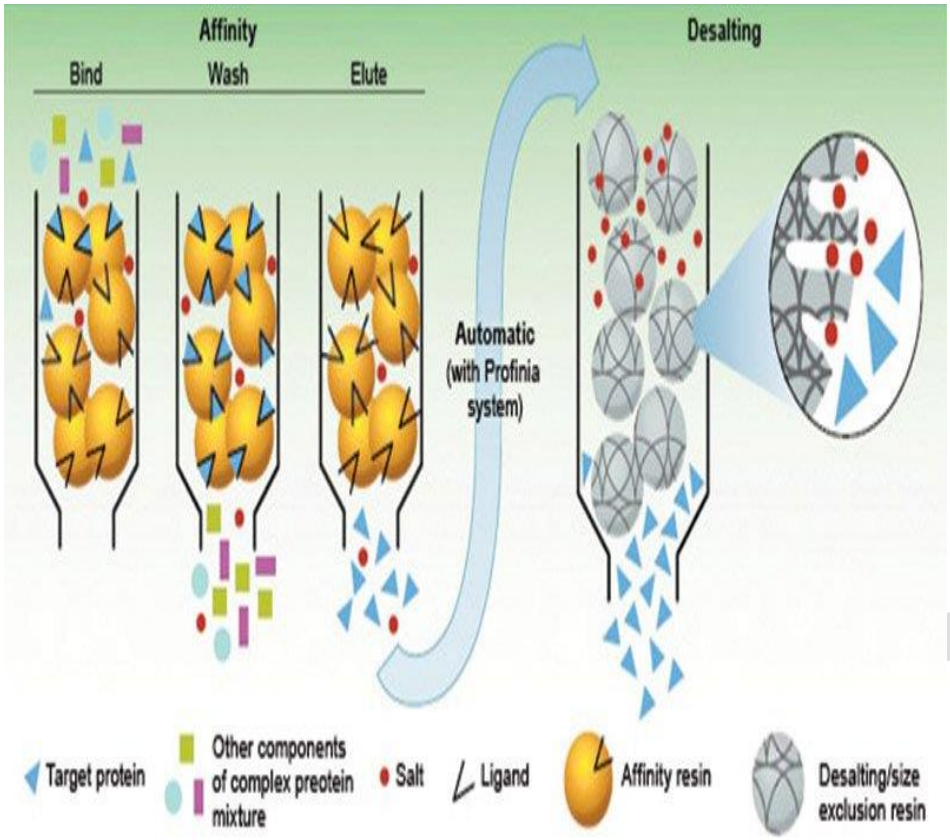
- The limited number of peaks that can be resolved within the short time scale of the GPC run.
- Filtrations must be performed before using the instrument to prevent dust and other particulates from ruining the columns and interfering with the detectors.
- The molecular masses of most of the chains will be too close for the GPC separation to show anything more than broad peaks.

Affinity Chromatography

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- It is a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase.
- Affinity chromatography is a type of liquid **chromatography** for the separation, purification or specific analysis of sample components.
- It utilizes the reversible biological interaction or molecular recognition called affinity which refers to the attracting forced exerted in different degrees between atoms which cause them to remain in combination.

Example: Enzyme with and inhibitor, antigen with an antibody etc.

- It was discovered by Pedro Cuatrecasas and Meir Wilcheck.



Principle of Affinity Chromatography

- The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way that the reactive groups that are essential for binding of the target molecule are exposed.
- As the crude mixture of the substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.
- Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the pH, ionic strength or polarity conditions.

Components of Affinity Chromatography

1. **Matrix**

- The matrix is an inert support to which a ligand can be directly or indirectly coupled.
- In order to for the matrix to be effective it must have certain characters:
- Matrix should be chemically and physically inert.
- It must be insoluble in solvents and buffers employed in the process

- It must be chemically and mechanically stable.
- It must be easily coupled to a ligand or spacer arm onto which the ligand can be attached.
- It must exhibit good flow properties and have a relatively large surface area for attachment.
- The most useful matrix materials are agarose and polyacrylamide.

2. **Spacer arm**

- It is used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance.

3. **Ligand**

- It refers to the molecule that binds reversibly to a specific target molecule.
- The ligand can be selected only after the nature of the macromolecule to be isolated is known.
- When a hormone receptor protein is to be purified by affinity chromatography, the hormone itself is an ideal candidate for the ligand.
- For antibody isolation, an antigen or hapten may be used as ligand.
- If an enzyme is to be purified,a substrate analog, inhibitor, cofactor, or effector may be used as a the immobilized ligand.

Steps in Affinity Chromatography

- Affinity medium is equilibrated in binding buffer.
- Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.
- Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.
- Affinity medium is re-equilibrated with binding buffer.

These events can be summarized into the following three major steps:

1. **Preparation of Column**

- The column is loaded with solid support such as sepharose, agarose, cellulose etc.
- Ligand is selected according to the desired isolate.
- Spacer arm is attached between the ligand and solid support.

2. **Loading of Sample**

- Solution containing a mixture of substances is poured into the elution column and allowed to run at a controlled rate.

3. **Elution of Ligand-Molecule Complex**

- Target substance is recovered by changing conditions to favor elution of the bound molecules.

Applications of Affinity Chromatography

- Affinity chromatography is one of the most useful methods for the separation and purification of specific products.
- It is essentially a sample purification technique, used primarily for biological molecules such as proteins.

Its major application includes:

- Separation of mixture of compounds.
- Removal of impurities or in purification process.
- In enzyme assays
- Detection of substrates
- Investigation of binding sites of enzymes
- In in vitro antigen-antibody reactions
- Detection of Single Nuceotide polymorphisms and mutations in nucleic acids

Advantages of Affinity Chromatography

- High specificity
- Target molecules can be obtained in a highly pure state
- Single step purification
- The matrix can be reused rapidly.
- The matrix is a solid, can be easily washed and dried.
- Give purified product with high yield.
- Affinity chromatography can also be used to remove specific contaminants, such as proteases.

Limitations of Affinity Chromatography

- Time consuming method.
- More amounts of solvents are required which may be expensive.
- Intense labour
- Non-specific adsorption cannot be totally eliminated, it can only be minimized.
- Limited availability and high cost of immobilized ligands.
- Proteins get denatured if required pH is not adjusted.

Gas-Liquid Chromatography (GLC)

Gas-Liquid Chromatography (GLC) is a separation technique in which gas (usually inert gas, such as helium or nonreactive gas, such as nitrogen), is used as a mobile phase (Figure 1) , and liquid as a stationary phase.

- Basis of separation is difference in partition coefficient of volatilized compounds between liquid and gas phases when the desired compound is carried through the column by a carrier gas.

Principle

GLC is based upon partitioning of compounds between stationary liquid and mobile gas phase. Due to its high sensitivity, reproducibility, and speed of resolution, it is widely used for several qualitative and quantitative analyses.

The gaseous compounds being analyzed interact with the walls of column, which is coated with different kinds of stationary phases.

This causes each compound to elute at different time (retention time), and then retention times are compared. This makes GLC analytically very useful.

As compound leaves the column, they pass through a detector, which is linked to chart recorder via amplifier. Chart recorder records the peaks

Applications of Gas-Liquid Chromatography

Gas liquid chromatography has a very wide field of application in the separation and analysis of multi component mixtures such as essential oils, hydrocarbons and solvents. It is one of the primary analytical techniques, which was used in forensic laboratory.

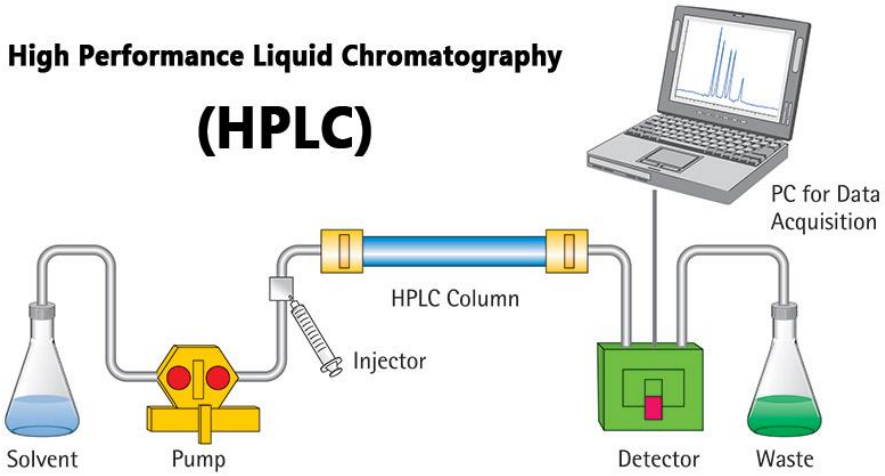
- GLC is combined with mass spectroscopy (GC/MS) for drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples.
- GC/MS is also used in airport security to detect unwanted substances. Non derivatized sugars and sugar alcohols are successfully analyzed by GC/MS using atmospheric pressure chemical ionization (APCI) in negative ion mode.
- GC is widely used by forensic scientists for an analysis of body fluids for the presence of illegal substances, testing of fiber and blood from a crime scene, and to detect residue from explosives.

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.

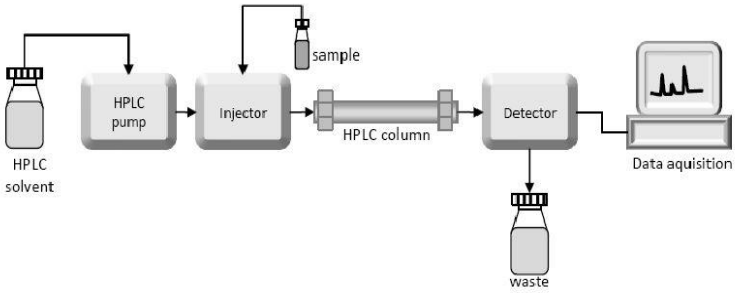
HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.



Principle of High-Performance Liquid Chromatography (HPLC)

- 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 (HPLC)



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

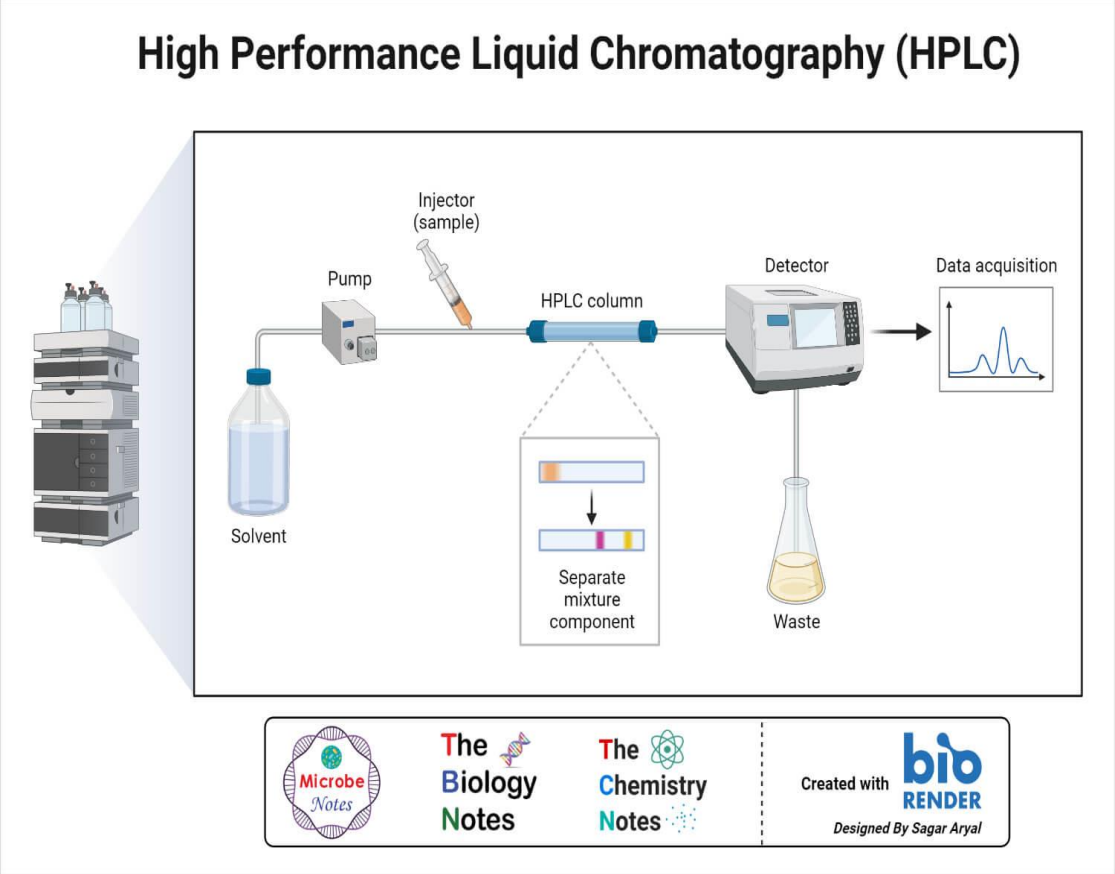
The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes.

- 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).



Types of High-Performance Liquid Chromatography (HPLC)

1. **Normal phase:**

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.

2. **Reverse phase:**

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.

3. **Ion exchange:**

Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

4. **Size exclusion:**

Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

Applications of High-Performance Liquid Chromatography (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 High-Performance Liquid Chromatography (HPLC)

1. Speed
2. Efficiency
3. Accuracy
4. Versatile and extremely precise when it comes to identifying and quantifying chemical components.

Limitations

1. **Cost:** Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics.
2. **Complexity**
3. HPLC does have **low sensitivity** for certain compounds, and some cannot be detected as they are irreversibly adsorbed.
4. Volatile substances are better separated by gas chromatography.