Shree H. N. Shukla Institute of science

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STUDY MATERIAL

Subject Name: ANALYTICAL TECHNIQUES AND BIOINFORMATICS

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UNIT II PRINCIPLES AND THEORIES OF CHROMATOGRAPHY

2.1 Chromatography: Theory and Principle
2.2 Paper and Thin Layer Chromatography
2.3 Affinity and Ion Exchange Chromatography
2.4 Partition and Size Exclusion Chromatography
2.5 Gas Chromatography, GC-MS, LC-MS
2.6 High Performance Liquid Chromatography (HPLC) and FPLC

Chromatography is a difficult enough technology to fathom at the best of times and the number of technical terms used can be daunting. This handy guide clarifies some of the more frequently used terms to aid understanding.

Absorption

In chromatography, absorption signifies the process by which a solute partitions into a liquid-like stationary phase.

Adsorption

The process by which a chemical entity is accumulated on a surface.

Mobile Phase

The eluate moving through the column. In gas chromatography (GC) this will be a gas, and in liquid chromatography (LC) a liquid.

Stationary Phase

The substance that remains in one place in the column. In GC this will be a liquid of high-viscosity, which clings to the inner walls of the column; in LC it will be some sort of packing, either solid or gel-based.

Capillary Column

A column whose inner diameter is under 0.5 mm.

Eluate

The mobile phase exiting a column.

Elution

- The passage of the mobile phase through the column to transport solutes.
- High Performance Liquid Chromatography (HPLC)
- The name given to the one of the most modern forms of column liquid chromatography. It is characterized by high flow rates and high back column pressure.
- Heart-Cutting
- The term used in preparative chromatography and column switching for the collection of the centre of the peak of a solute.
- Robustness
- The extent to which a method of chromatography and its results will remain unaffected by minor changes in the parameters of the method (equipment, environment, etc.).
- Partition Chromatography
- A type of chromatography based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.
- Flow Rate
- The amount of mobile phase that has passed through the column per unit time. The units are millilitres per second (mL/sec) or, more commonly, millilitres per minute (mL/min).
- Linear Velocity
- The time it takes for the mobile phase to pass through the column. Generally expressed as either centimetres per minute (cm/min) or metres per second (m/s). It is estimated as the column length divided by the time it takes for a non-retained compound to pass through the column.
- Interstitial Volume
- The volume between the particles in a packed column.
- Solute
- The term for the sample components being analysed.

Validation

A programme which guarantees the accuracy, specificity, precision and robustness of a method or process. To learn more about the benefits of validation, read the article: **Why are Methods Validated?**

Void Volume

The volume of the empty column before the mobile phase is passed through it. Ideally this volume should be exactly the same as that of the mobile phase itself.

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.

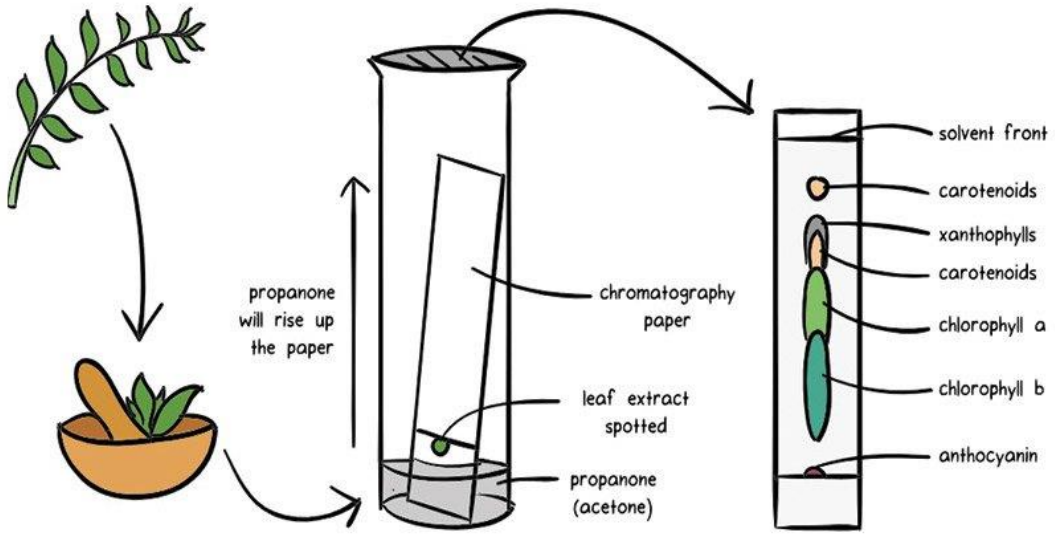
Principle of Chromatography (how does chromatography work)

- 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.



Types of Chromatography

- Substances can be separated on the basis of a variety of methods and the presence of characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase.
- This leads to different types of chromatography techniques, each with their own instrumentation and working principle.
- For instance, four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion.
- Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography.

Commonly employed chromatography techniques include:

1. Column chromatography
2. Ion-exchange chromatography
3. Gel-permeation (molecular sieve) chromatography

4. Affinity chromatography
5. Paper chromatography
6. Thin-layer chromatography
7. Gas chromatography (GS)
8. Dye-ligand chromatography
9. Hydrophobic interaction chromatography
10. Pseudoaffinity chromatography
11. High-pressure liquid chromatography (HPLC)

Applications of Chromatography

Pharmaceutical sector

- To identify and analyze samples for the presence of trace elements or chemicals.
- Separation of compounds based on their molecular weight and element composition.
- Detects the unknown compounds and purity of mixture.
- In drug development.

Chemical industry

- In testing water samples and also checks air quality.
- HPLC and GC are very much used for detecting various contaminants such as polychlorinated biphenyl (PCBs) in pesticides and oils.
- In various life sciences applications

Food Industry

- In food spoilage and additive detection
- Determining the nutritional quality of food

Forensic Science

- In forensic pathology and crime scene testing like analyzing blood and hair samples of crime place.

Molecular Biology Studies

- Various hyphenated techniques in chromatography such as EC-LC-MS are applied in the study of metabolomics and proteomics along with nucleic acid research.

- HPLC is used in Protein Separation like Insulin Purification, Plasma Fractionation, and Enzyme Purification and also in various departments like Fuel Industry, biotechnology, and biochemical processes.

1. Paper Chromatography

Paper chromatography definition explains that is an inexpensive and powerful analytical technique, which requires a piece of paper or strips serving as an adsorbent in the stationary phase across which a particular solution is allowed to pass.

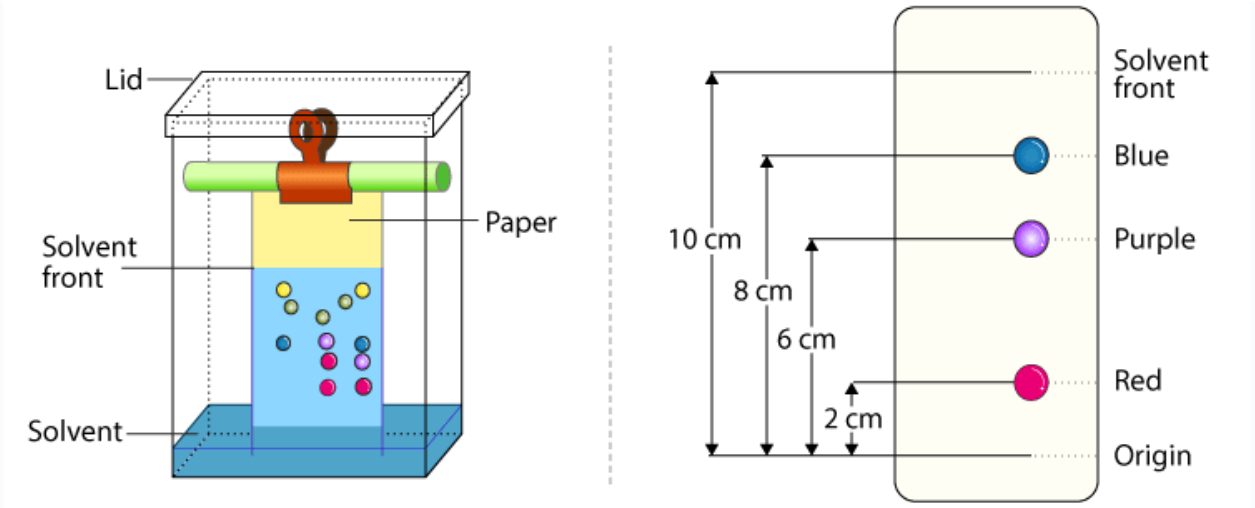
For the separation of dissolved chemical substances and lipid samples (in particular), paper chromatography is found to be very trustable. This analytical tool employs very few quantities of material.

Principle of Paper Chromatography

Paper chromatography is a form of liquid chromatography where the basic principle involved can be either partition chromatography or adsorption chromatography.

In paper chromatography separation of component is distributed between phases of liquid. Here, one phase of liquid is water that is held amidst the pores of filter paper and the other liquid is the mobile phase that travels along with the filter paper. Separation of the mixture is the result that is obtained from the differences in the affinities towards the water and mobile phase when travelling under capillary action between the pores of the filter paper.

Though in a majority of paper chromatography applications, the principle is based on partition chromatography but sometimes, adsorption chromatography can take place where the stationary phase is the solid surface of the paper and the mobile phase is the liquid phase.



Paper Chromatography procedure

1. **Selection of the ideal type of development:** Based on factors such as the complexity of the solvent, mixture, paper, etc. the development type is chosen. Mostly either Radial or Ascending type of paper chromatography is employed because of the easiness they offer while handling and performing which ultimately leads to obtaining the chromatogram faster within a shorter duration of time.

2. **Selection of Filter paper:** As per the pores' size and the sample quality.
3. **Sample preparation:** This involves the dissolution of the sample in an ideal solvent that is being utilized in developing the mobile phase.
4. **Sample loading or spotting on the paper:** With the help of a capillary tube, micropipette, the sample is spotted on the paper at an accurate position. This promotes the interpretation of the chromatogram more quickly and easily.
5. **Chromatogram development:** This is carried by the paper immersion in the mobile phase. The mobile phase crosses over the sample on the paper because of the capillary action of the paper.
6. **Drying of paper and detection of the compound:** With the aid of air drier, the paper is dried as soon as the chromatogram is developed. On the chromatogram developed paper, the

detecting solution is sprayed and dried thoroughly for the identification of the sample chromatogram spots.

Types of Paper Chromatography

Ascending Paper Chromatography

As per the name, the developing solvent is found to be moving in an upward direction. Here, a sufficient quantity of mobile phase is poured into the development chamber. Sample and reference are spotted on the line drawn a few centimetres from the bottom edge of the paper suspended from a hook or clip at the top.

Descending Paper Chromatography

Here, the solvent front travels down the length of paper suspended from the top inside the developing chamber. The mobile phase is kept in a trough in the upper chamber. The paper with spotting on the line drawn a few centimetres from the top is clamped to the top. Before elution, the jar is covered and equilibrated with the mobile phase vapour.

Ascending – Descending Chromatography

It is a mixed type of chromatography where the solvent first travels upwards on the paper that is folded over a rod and after crossing the rod it moves downwards.

Horizontal or Circular Paper Chromatography

This allows the separation of sample components in the form of concentric circular zones through the radial movement of the liquid phase.

Two-Dimensional Chromatography

This helps in resolving substances that have similar Rf values. Where, Retardation factor (Rf) = The distance travelled by the solute/ distance travelled by the solvent front

Applications of paper chromatography

In the analysis of different classes of compounds namely:

- Amino acids and organic acids
- Alkaloids
- Polysaccharides
- Proteins and peptides
- Natural and artificial pigments
- Inorganic cations
- Plant extracts

2.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 chromatography technique.

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

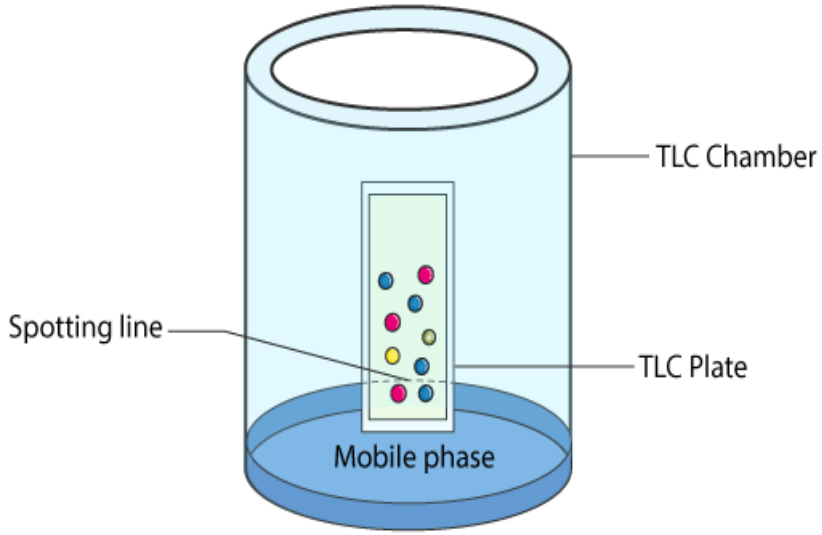


Diagram of Thin Layer Chromatography

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 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.

3.Column Chromatography

In chemistry, Column chromatography is a technique which is used to separate a single chemical compound from a mixture dissolved in a fluid. It separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates which allow them to get separated in fractions. This technique can be used on a small scale as well as large scale to purify materials that can be used in future experiments.

Column Chromatography Principle

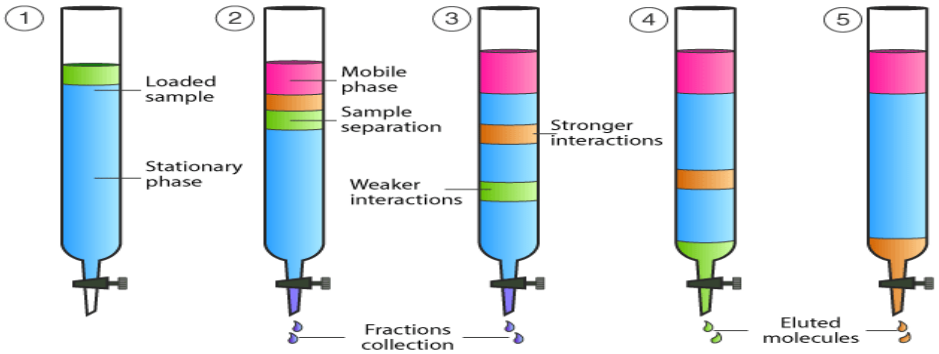
When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slowly are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as:

R_f = the distance travelled by solute/ the distance travelled by the solvent

R_f is the retardation factor.

Column Chromatography Diagram



Column Chromatography Diagram

Column Chromatography Procedure

Before starting with the Column Chromatography Experiment let us understand the different phases involved.

Mobile phase – This phase is made up of solvents and it performs the following functions:

1. It acts as a solvent – sample mixture can be introduced in the column.
2. It acts as a developing agent – helps in the separation of components in the sample to form bands.
3. It acts as an eluting agent – the components that are separated during the experiment are removed from the column
4. Some examples of solvents used as mobile phase based on their polarity are – ethanol, acetone, water, acetic acid, pyridine, etc.

Stationary phase – It is a solid material which should have good adsorption property and meet the conditions given below:

1. Shape and size of particle: Particles should have uniform shape and size in the range of 60 – 200μ in diameter.
2. Stability and inertness of particles: high mechanical stability and chemically inert. Also, no reaction with acids or bases or any other solvents used during the experiment.
3. It should be colourless, inexpensive and readily available.
4. Should allow free flow of mobile phase
5. It should be suitable for the separation of mixtures of various compounds.

Column Chromatography Experiment

- The stationary phase is made wet with the help of solvent as the upper level of the mobile phase and the stationary phase should match. The mobile phase or eluent is either solvent or mixture of solvents. In the first step the compound mixture that needs to be separated, is added from the top of the column without disturbing the top level. The tap is turned on and the adsorption process on the surface of silica begins.
- Without disturbing the stationary phase solvent mixture is added slowly by touching the sides of the glass column. The solvent is added throughout the experiment as per the requirement.
- The tap is turned on to initiate the movement of compounds in the mixture. The movement is based on the polarity of molecules in the sample. The non-polar components move at a greater speed when compared to the polar components.
- For example, a compound mixture consists of three different compounds viz red, blue, green then their order based on polarity will be as follows blue>red>green
- As the polarity of the green compound is less, it will move first. When it arrives at the end of the column it is collected in a clean test tube. After this, the red compound is collected and at last blue compound is collected. All these are collected in separate test tubes.

Column Chromatography Applications

- Column Chromatography is used to isolate active ingredients.
- It is very helpful in Separating compound mixtures.
- It is used to determine drug estimation from drug formulations
- It is used to remove impurities.
- Used to isolation metabolites from biological fluids.

Types of Column Chromatography:

1. Adsorption column chromatography – Adsorption chromatography is a technique of separation, in which the components of the mixture are adsorbed on the surface of the adsorbent.

2. Partition column chromatography – The stationary phase, as well as mobile phase, are liquid in partition chromatography.

3. Gel column chromatography – In this method of chromatography, the separation takes place through a column packed with gel. The stationary phase is a solvent held in the gap of a solvent.

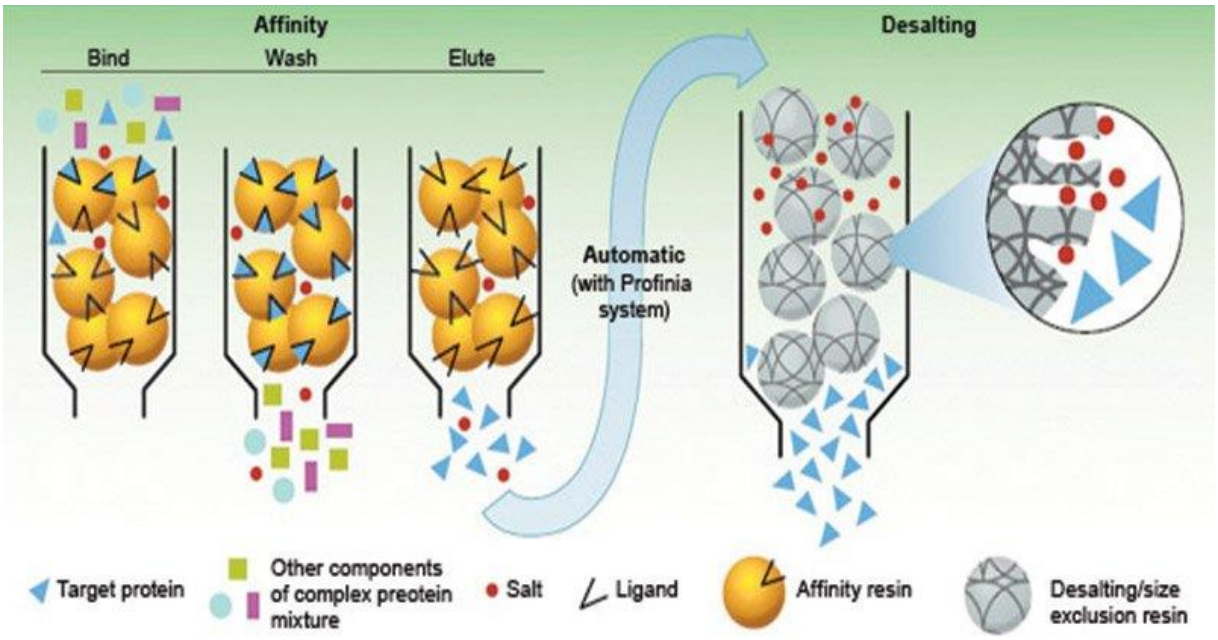
4. Ion exchange column chromatography – A chromatography technique in which the stationary phase is always ion exchange resin.

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.

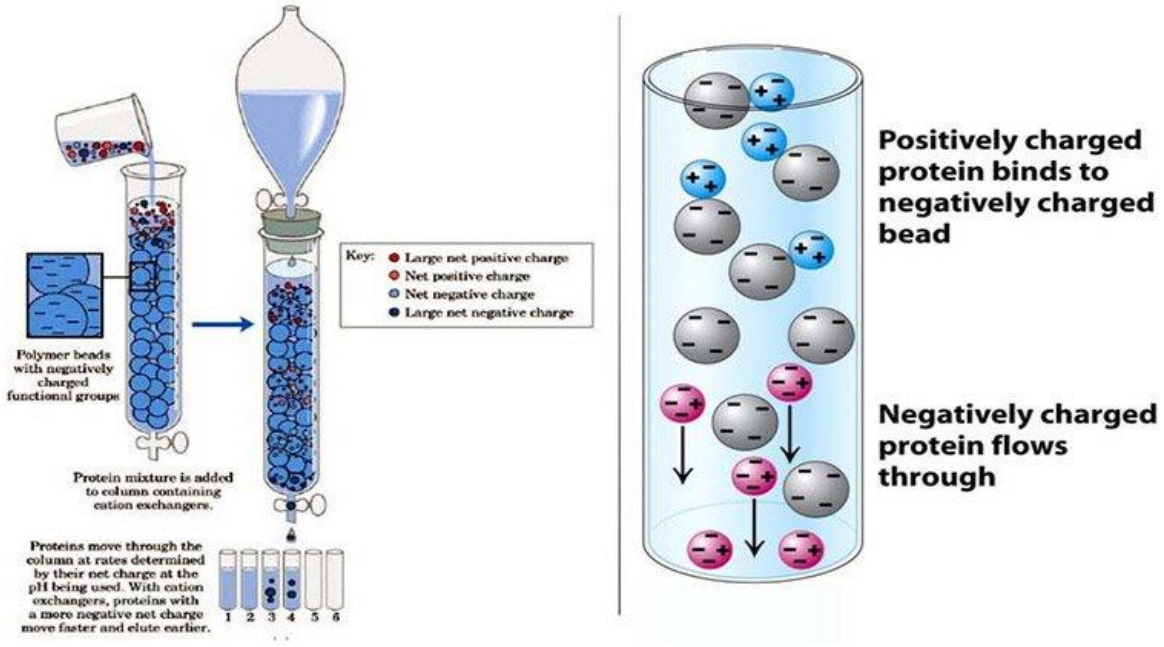
- Limited availability and high cost of immobilized ligands.
- Proteins get denatured if required pH is not adjusted.

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.



working 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.

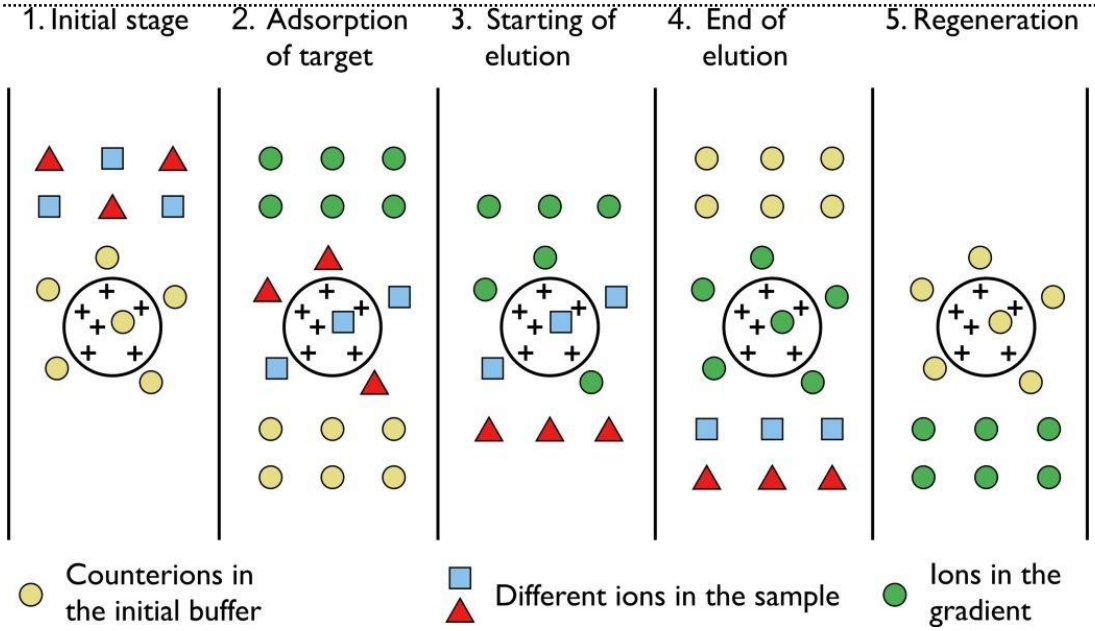
5. **Detectors**

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.
- 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.

Advantages of ion exchange chromatography

1. It is one of the most efficient methods for the separation of charged particles.
2. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids.
3. Ion exchange is used for both analytical and preparative purposes in the laboratory, the analytical uses being the more common.
4. Inorganic ions also can be separated by ion-exchange chromatograph.y

Limitations of ion exchange chromatography

- Only charged molecules can be separated.
- Buffer Requirement

What is Partition Chromatography?

Partition Chromatography technique is defined as

the separation of components between two liquid phases viz original solvent and the film of solvent used in the column.

This separation theory was introduced in the year 1940s which was published by **Richard Laurence Millington Synge and Archer Martin**. It is also known as *Liquid-liquid chromatography (LLC)*. Or if gas is the mobile phase it is called *Gas-liquid chromatography (GLC)*.

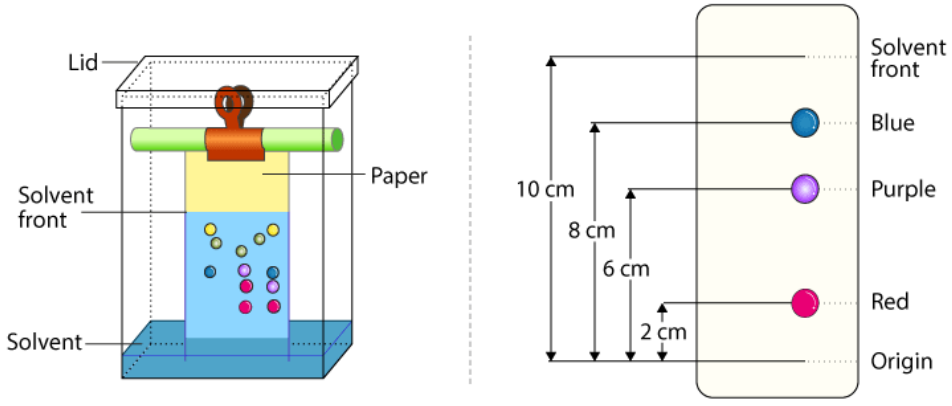
Principle of Chromatography

- Chromatography is a separation method where the analyte is contained within a liquid or gaseous mobile phase, which is pumped through a stationary phase.
- Usually, one phase is hydrophilic and the other lipophilic. The components of the analyte interact differently with these two phases.
- Depending on the polarity they spend more or less time interacting with the stationary phase and are thus retarded to a greater or lesser extent.
- This leads to the separation of the different components present in the sample.

Partition Chromatography Principle

The separation of the components from the sample mixture is carried out by the process of partition of the components between 2 phases. Both phases are in liquid form. In this process, the immiscible solid surface coated with the liquid surface on the stationary phase is in the mobile phase. The liquid surface is immobilized by a stationary phase which results in making its a stationary phase. The mobile phase moves from the stationary phase and components get separated. The separation depends on different partition coefficient.

Partition Chromatography Diagram



Partition Chromatography Procedure

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

Apparatus required – chromatography jar, liquid impregnated paper (stationary phase), capillary tube (to apply sample mixture), mobile phase (example chloroform, methanol, acetone, ethanol).

1. Take a clean and dry chromatography jar.
2. To make sure that the environment of the jar is saturated with solvent vapours, a paper impregnated in mobile phase is set to the walls.
3. Add mobile phase to the jar. Around 0.5 cm to 1 cm from the bottom of the jar.
4. Close the jar.
5. Allow attaining equilibrium.
6. Mark the baseline on adsorbent.
7. Apply sample to the paper with the help of capillary tube.
8. Air-dry the sample spot.
9. Place the paper in the jar and close it.
10. Allow the system to stand till the solvent moves to some distance from the baseline.
11. Take out the paper and dry it.
12. If the sample components are separated showing colours then dry it in ordinary light. If it is a colourless component then dry it in UV lamp.
13. Store the chromatogram
14. Calculate R_f value.

Partition Chromatography Applications

There are various applications of Paper Chromatography. Some of the applications are mentioned below:

- To separate and identify amino acids.
- To separate and identify tannins.
- To separate and identify alkaloids.

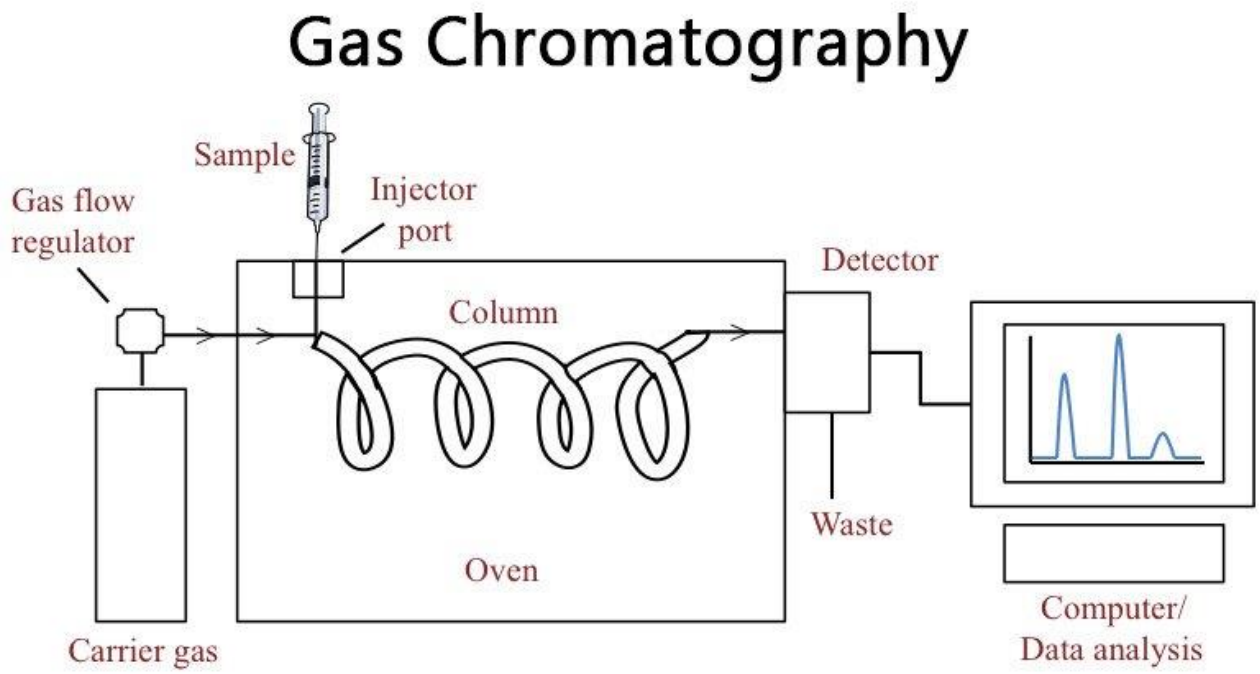
- To separate and identify carbohydrates.
- To separate and identify glycosides.

Types of Partition Chromatography

1. Liquid-liquid Chromatography – It is a chromatography technique where a sheet of blotting paper, is used instead of adsorption column. The components are separated based on their differential migratory velocities. On separating they are stained to make the chromatogram visible.
2. Gas-liquid Chromatography – A chromatography technique in which the separation of the mixture is done by an inert gas along a tube. The tube is filled with finely divided inert solid. The solid is coated with a nonvolatile oil. The migration of each component occurs at a rate determined by its solubility in oil as well as its vapor pressure.

gas chromatography

- Gas chromatography differs from other forms of **chromatography** in that the mobile phase is a gas and the components are separated as vapors.
- It is thus used to separate and detect small molecular weight compounds in the gas phase.
- The sample is either a gas or a liquid that is vaporized in the injection port. The mobile phase for gas chromatography is a carrier gas, typically helium because of its low molecular weight and being chemically inert.
- The pressure is applied and the mobile phase moves the analyte through the column. The separation is accomplished using a column coated with a stationary phase.



Principle of Gas chromatography (how does gas chromatography work)

The equilibrium for gas chromatography is partitioning, and the components of the sample will partition (i.e. distribute) between the two phases: the stationary phase and the mobile phase.

Compounds that have a greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer **retention time (Rt)** than samples that have a higher affinity for the mobile phase.

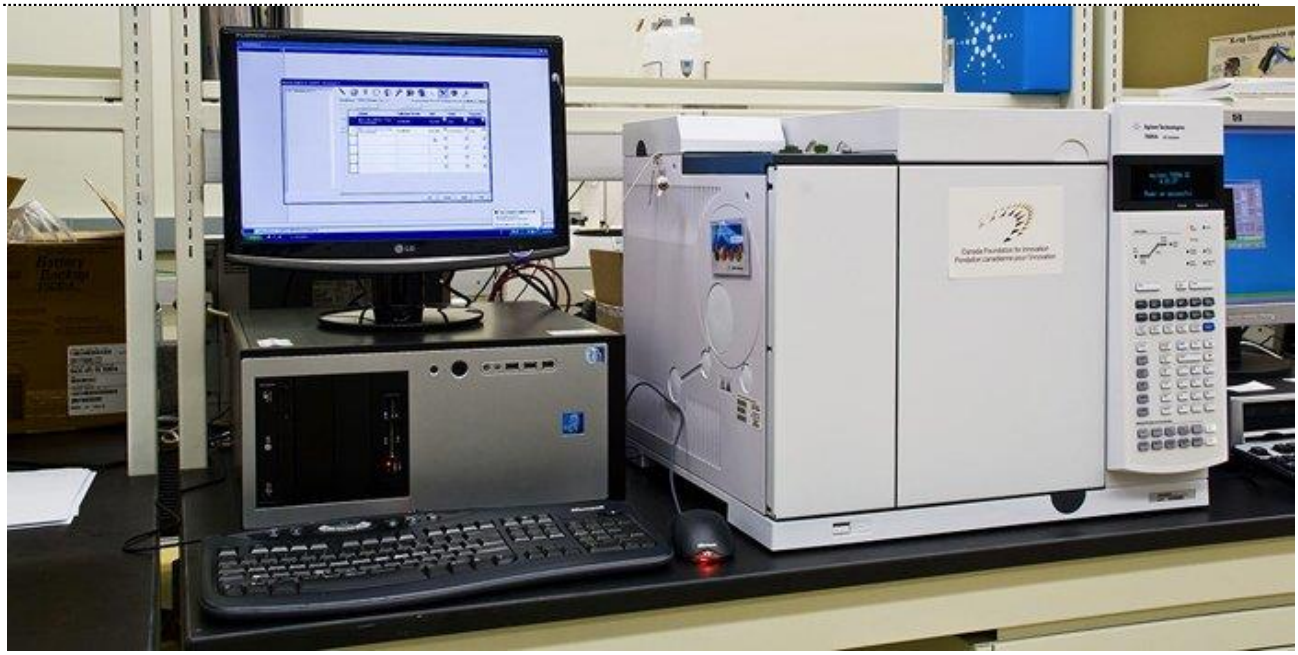
Affinity for the stationary phase is driven mainly by intermolecular interactions and the polarity of the stationary phase can be chosen to maximize interactions and thus the separation.

Ideal peaks are Gaussian distributions and symmetrical, because of the random nature of the analyte interactions with the column.

- The separation is hence accomplished by partitioning the sample between the gas and a thin layer of a nonvolatile liquid held on a solid support.
- A sample containing the solutes is injected into a heated block where it is immediately vaporized and swept as a plug of vapor by the carrier gas stream into the column inlet.
- The solutes are adsorbed by the stationary phase and then desorbed by a fresh carrier gas.
- The process is repeated in each plate as the sample is moved toward the outlet.
- Each solute will travel at its own rate through the column.

- Their bands will separate into distinct zones depending on the partition coefficients, and band spreading.
- The solutes are eluted one after another in the increasing order of their kd, and enter into a detector attached to the exit end of the column.
- Here they register a series of signals resulting from concentration changes and rates of elution on the recorder as a plot of time versus the composition of carrier gas stream.
- The appearance time, height, width, and area of these peaks can be measured to yield quantitative data.

Parts of Gas chromatography



Gas chromatography is mainly composed of the following parts:

1. **Carrier gas in a high-pressure cylinder with attendant pressure regulators and flow meters**

- Helium, N_2, H, Argon are used as carrier gases.
- Helium is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapors.
- N_2 is preferable when a large consumption of carrier gas is employed.
- Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 ml/min), capillary restrictors, and a pressure gauge (1-4 atm).

- Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.
- The operating efficiency of the gas chromatograph is directly dependant on the maintenance of constant gas flow.

2. **Sample injection system**

- Liquid samples are injected by a microsyringe with a needle inserted through a self-scaling, silicon-rubber septum into a heated metal block by a resistance heater.
- Gaseous samples are injected by a gas-tight syringe or through a by-pass loop and valves.
- Typical sample volumes range from 0.1 to 0.2 ml.

3. **The separation column**

- The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape.
- Copper is useful up to 250^0
- Swege lock fittings make column insertion easy.
- Several sizes of columns are used depending upon the requirements.

4. **Liquid phases**

- An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support.
- No single phase will serve for all separation problems at all temperatures.

Non-Polar – Parafin, squalane, silicone greases, apiezon L, silicone gum rubber. These materials separate the components in order of their boiling points.

Intermediate Polarity – These materials contain a polar or polarizable group on a long non-polar skeleton which can dissolve both polar and non-polar solutes. For example. diethyl hexyl phthalate is used for the separation of high boiling alcohols.

Polar – Carbowaxes – Liquid phases with a large proportion of polar groups. Separation of polar and non-polar substances.

Hydrogen bonding – Polar liquid phases with high hydrogen bonding e.g. Glycol.

Specific purpose phases – Relying on a chemical reaction with solute to achieve separations. e.g AgNO3 in glycol separates unsaturated hydrocarbons.

5. **Supports**

- The structure and surface characteristics of the support materials are important parameters, which determine the efficiency of the support and the degree of separation respectively.
- The support should be inert but capable of immobilizing a large volume of liquid phase as a thin film over its surface.
- The surface area should be large to ensure the rapid attainment of equilibrium between stationary and mobile phases.
- Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.
- Diatomaceous earth, kieselguhr treated with Na 2CO 3 for 900^0 C causes the particle fusion into coarser aggregates.
- Glass beads with a low surface area and low porosity can be used to coat up to 3% stationary phases.
- Porous polymer beads differing in the degree of cross-linking of styrene with alkyl-vinyl benzene are also used which are stable up to 250^0

6. **Detector**

- Detectors sense the arrival of the separated components and provide a signal.
- These are either concentration-dependent or mass dependant.
- The detector should be close to the column exit and the correct temperature to prevent decomposition.

7. **Recorder**

- The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.
- An integrator may be a good addition.

The procedure of Gas Chromatography

Step 1: Sample Injection and Vapourization

1. A small amount of liquid sample to be analyzed is drawn up into a syringe.
2. The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.

3. The injection of the sample is considered to be a "point" in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
4. The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporize.
5. The vaporized components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.

Step 2: Separation in the Column

- Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will, therefore, have the longest retention time (Rt). It will emerge from the gas chromatograph last.
- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.
- If we consider a 2 component mixture in which component A is more polar than component B then:

1. component A will have a **longer retention time** in a polar column than component B
2. component A will have a **shorter retention time** in a non-polar column than component B

Step 3: Detecting and Recording Results

1. The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.
2. The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
3. The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last.

Applications

- GC analysis is used to calculate the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water.
- Gas chromatography is used in the analysis of:

(a) air-borne pollutants

(b) performance-enhancing drugs in athlete's urine samples

(c) oil spills

(d) essential oils in perfume preparation

- GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- Gas Chromatography is used extensively in forensic science. Disciplines as diverse as solid drug dose (pre-consumption form) identification and quantification, arson investigation, paint chip analysis, and toxicology cases, employ GC to identify and quantify various biological specimens and crime-scene evidence.

Advantages

- The use of longer columns and higher velocity of carrier gas permits the fast separation in a matter of a few minutes.
- Higher working temperatures up to 5000C and the possibility of converting any material into a volatile component make gas chromatography one of the most versatile techniques.
- GC is popular for environmental monitoring and industrial applications because it is very reliable and can be run nearly continuously.
- GC is typically used in applications where small, volatile molecules are detected and with non-aqueous solutions.
- GC is favored for non-polar molecules.

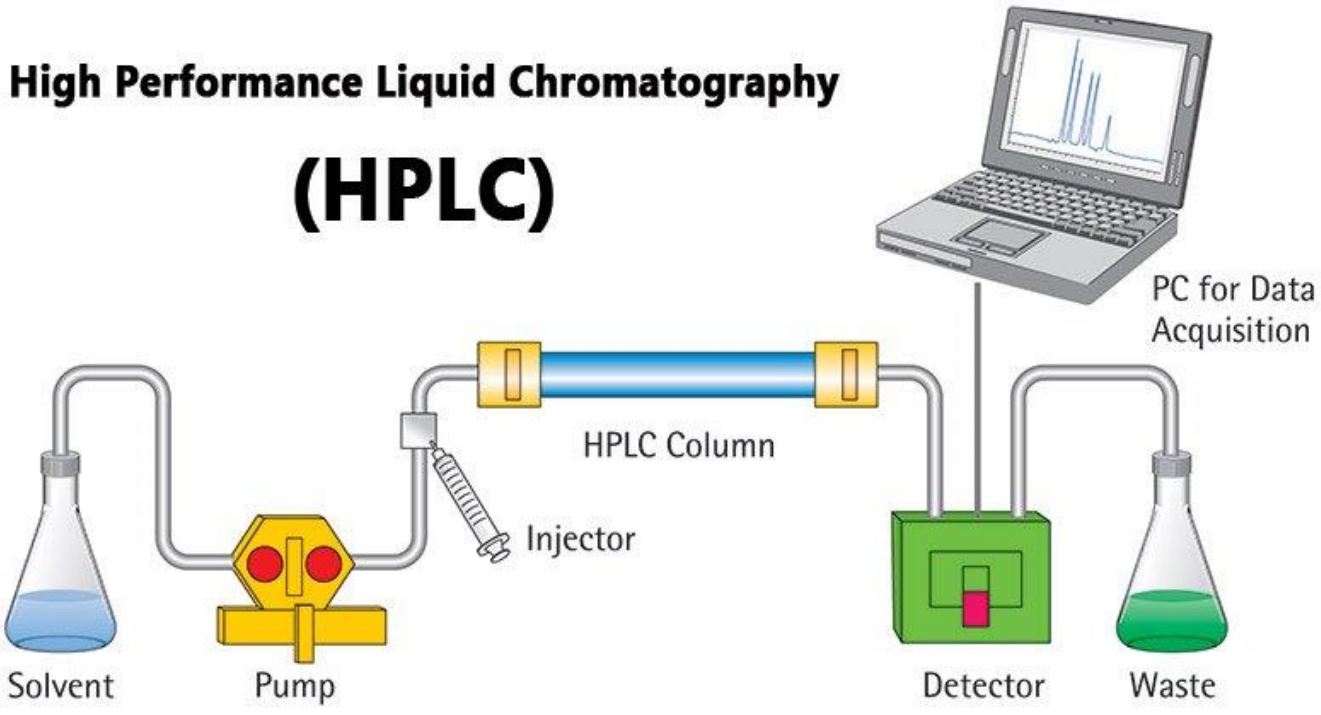
Limitations

- Compound to be analyzed should be stable under GC operation conditions.
- They should have a vapor pressure significantly greater than zero.
- Typically, the compounds analyzed are less than 1,000 Da, because it is difficult to vaporize larger compounds.
- The samples are also required to be salt-free; they should not contain ions.

- Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

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.

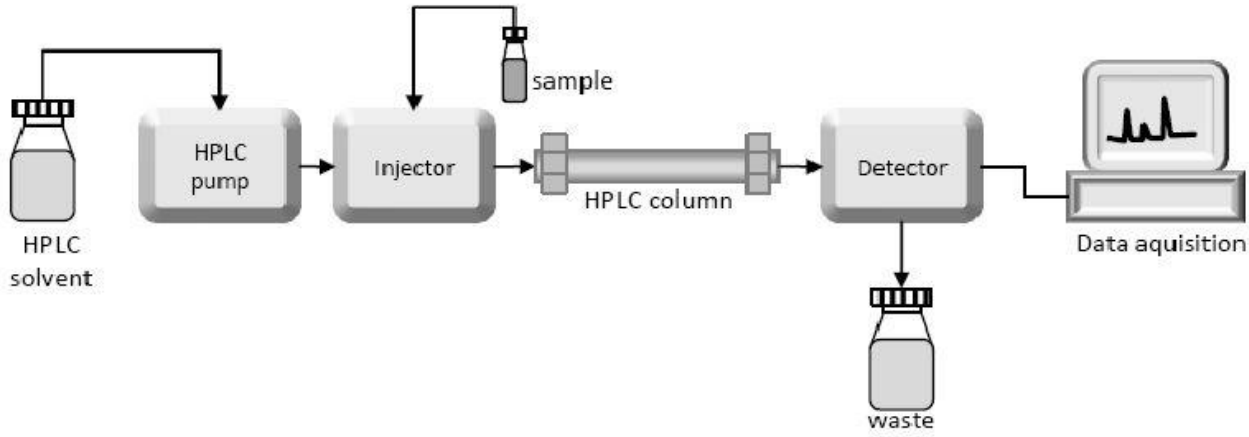


Principle of High-Performance Liquid Chromatography (HPLC)

- 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.

Fast protein liquid chromatography

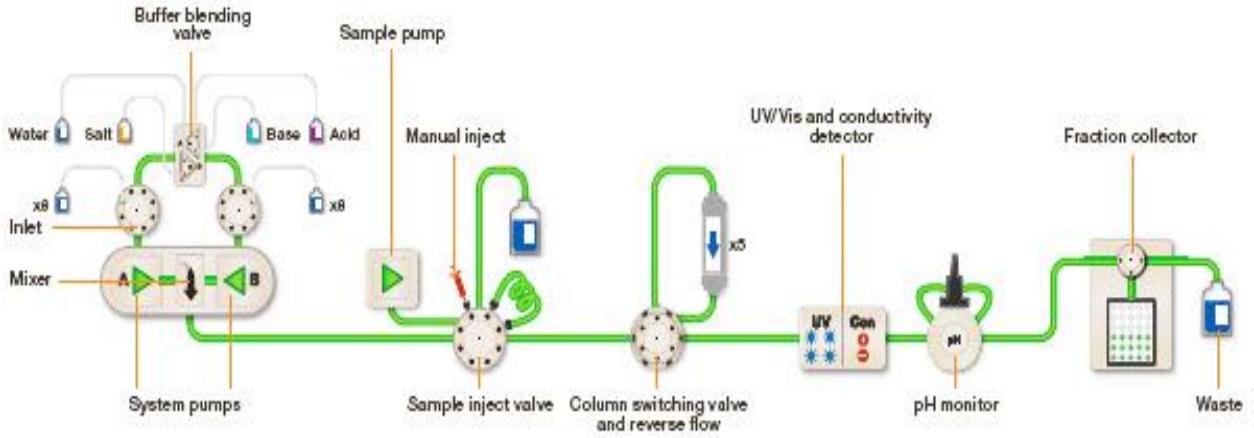
Fast protein liquid chromatography (FPLC) is a type of liquid chromatography that provides high resolution by small-diameter stationary phases for protein characterization and separation. The technique features high loading capacity, biocompatible buffer systems, fast flow rates, and stationary phases for common chromatography modes (e.g., gel filtration, ion exchange, reversed phase, and affinity chromatography). The system enables reproducible separation by incorporating a high level of automation including samplers, gradient program control, and peak collection. FPLC allows the users to monitor several parameters at a time such as UV level, pH, and conductance and it allows for multiple columns to be run in tandem; therefore, minimizing the time needed to isolate pure protein. The method is applicable to proteins as well as other kinds of biological samples including oligonucleotides and plasmids.

Principle

In fast protein liquid chromatography, the solvent velocity is controlled by a microprocessor through a software interface to maintain the constant flow rate of the solvents. The mobile phase is an aqueous buffer, and the flow rate is kept constant by a positive-displacement pump while the buffer composition can be varied by drawing fluids from external reservoirs. The stationary phase is composed of beads, usually of cross-linked agarose, packed into a cylindrical column. The eluant is passed through the detectors to measure the salt concentration (by conductivity) and protein concentration (by absorbing the ultraviolet light at a wavelength of 280nm).

Apparatus

The FPLC system consists of a control unit, high-precision pumps, a column, detection system, and a fraction collector. The pump allows the fraction of each buffer entering the column to be continuously varied. The injection loop, a segment of tubing of known volume, is filled with the sample solution to be injected in the column. The sample loading is done by the injection valve which links the mixer and sample loop to the column. The FPLC column is a glass or plastic cylinder packed with beads of resin. It is mounted vertically with the buffer flowing downward from top to bottom. The eluant from the column passes through the flow cells for protein concentration measurements. The detector records the salt and protein concentration.



Protocol

Sample preparation

1. Aspire Sephadex G-25 resin as a slurry in water into the column before packing.
2. Equilibrate the column in 3-4 column volumes of buffer A (10 mM Tris-HCl, pH 7.0).
3. Load the sample (50 mL) under gravity flow.
4. Pass buffer A through the column and monitor the protein by measuring A_{280}, and collect it as a single large peak.
5. Measure the conductivity using the conductivity meter.

FPLC modes

Ion-exchange FPLC

1. Prime the pumps A and B with filtered and degassed buffers A (10 mM Tris–HCl, pH 7.0.) and B (10 mM Tris–HCl, pH 7.0, 1 M NaCl), respectively.
2. Set the pressure limits on both the pumps below the maximum for the column in use.
3. Equilibrate the Mono Q column (1 mL volume) with 5 volumes of buffer A and 10 volumes of buffer B and then with 5 volumes of buffer A.
4. Wash the sample loading loop with buffer A.
5. Load the sample (0.5–10 mL) (approximately 1 mg/mL) and wash the column with buffer A. Collect the flow-through and evaluate for the protein of interest.

Note: If protein not bound then replace the Mono Q column with Mono S column.

6. Regenerate the column by washing it with 10 volumes of buffer B, then with 5 volumes of buffer A.

Scouting FPLC methods

1. Create gradients of shallowness by varying the concentration of buffer B at different time-points.
2. Identify the column to which the protein binds and perform chromatography at different pH values using various buffer systems.

Gel-filtration FPLC

1. Prime the pumps A and B with buffer C (50 mA/Tris-HCl, pH 7.0, 100 mM KCl) with no column in the system.
2. Set the pressure limits on both P-500 pumps.
3. Connect Superose 12, and operate the system at 0.5 mL/min flow rate for 90 minutes for column equilibration.
4. Wash the sample loading loop with buffer C.
5. Load 200 µL of the sample at the rate of 2 mg/mL.
6. Monitor A_{280}; collect peaks with the fraction collector and record the elution volume of peak.

Applications

Diagnosis of β-thalassemia

Separation of plasma proteins in urine and cerebrospinal fluid

Assessment of protein unfolding

Precautions

- Use neutral pH to allow the assessment of both anion- and cation-exchange columns at the same pH.
- Avoid pH extremes to prevent protein denaturation.
- The FPLC procedure should be performed at 4°C for the purification of labile proteins.
- To avoid NaCl precipitation, prime pumps with water to remove 20% ethanol.
- Decreased flow rates should be used for improved resolution in gel-filtration chromatography.
- Wash the system with water after the procedure.

Strengths and limitations

- The fast-protein liquid chromatography is a simple and reproducible separation technique with efficient resolution.

- The chromatography columns have longer lifetime because of the inert construction against the very high salt concentrations and corrosive liquids.
- The FPLC supports a wide range of columns as the procedures are carried out under low pressure.
- The wide flow range makes it a suitable technique for analytical and preparative chromatography.
- The technique needs glass columns and cannot withstand high pressures.
- The method is not sensitive to thermolabile proteins.