**Title: Chromatography Techniques for Separation and Analysis of Biological Samples: Enhancing Characterization and Quantification of Complex Mixtures**

**Name:** Shamim\*, Anshu, Rustam Ekbbal

**Designation:** Assistant Professor

**Affiliation:** IIMT College of Medical Sciences, IIMT University, Meerut, Uttar Pradesh, India-250001

Mobile No: 9506696061

E-mail id: [pharmacistshamim1@gmail.com](mailto:pharmacistshamim1@gmail.com)

**Address for correspondence**

**Name:** Shamim

**Designation:** Assistant Professor

**Affiliation:** IIMT College of Medical Sciences, IIMT University, Meerut, Uttar Pradesh, India-250001

E-mail id: [pharmacistshamim1@gmail.com](mailto:pharmacistshamim1@gmail.com)

**Abstract**

Chromatography techniques play a pivotal role in the separation and analysis of biological samples, enabling the characterization and quantification of complex mixtures. This abstract provides an overview of the various chromatographic methods employed in the separation of biological samples, including gel chromatography, affinity chromatography, ion exchange chromatographic, etc. Separation of biological samples through such techniques based on their differential partitioning between a stationary phase and a mobile phase due to their enhanced detection and identification capabilities, enabling the analysis of complex biological matrices. These methods are widely employed for the separation of compounds such as proteins, peptides, nucleic acids, carbohydrates, and small molecules and acknowledged for versatility, efficiency, and compatibility with various detectors, including UV/Vis, fluorescence, and mass spectrometry. It has gained significant attention in the analysis of biomolecules and open several ways in discovery and development of novel components. Moreover, advancements in chromatographic technology have led to the development of hybrid techniques, such as liquid chromatography-mass spectrometry (LC-MS) and their combinations offer complementary separation and detection capabilities, enhancing the identification and quantification of analytes in complex biological samples. In conclusion, chromatography techniques are indispensable tools in the separation and analysis of biological samples. The diverse range of chromatographic methods available, along with their compatibility with various detection techniques, enables the comprehensive investigation of complex mixtures, leading to significant advancements in the fields of biochemistry, pharmaceuticals, clinical diagnostics, and environmental analysis.

**Keywords:** Chromatography techniques, Biological samples, Gel chromatography, Affinity chromatography, Ion exchange chromatography, Stationary phase, Mobile phase, Detection capabilities, Complex biological matrices, Proteins, Peptides, Nucleic acids, Discovery and development

**1. Chromatographic Ion Exchange:**

Chromatography is the technique of dissolving an amalgam of chemicals into their constituent parts based on how they react with an inert matrix. Also referred to as " Ion exchange is a form of "ion chromatography." a method that separates ionic components or highly polar compounds according to their affinity for ionic affinity to get exchange (Fekete et al., 2015). Therefore, the concept of separation foundation is the exchange of ions that is reversible between the ions on the ion exchangers and the target ions in the sample solution. This procedure can make use of two distinct types of exchangers, anionic and cationic exchangers. Cationic exchangers with a negative charge are going to positive energy attractions to themselves. Additionally called "Acidic ion exchange" materials, these exchangers since the source of their negative charges are the ionization of acidic groups (Nesterenko & Paull, 2017). By using anionic exchangers, positively charged anions may be converted to negatively charged anions. Also known as "Basic ion exchange" materials. The most used technique for ion exchange chromatography is column chromatography. On the other hand, the ion exchange principle is the basis for various thin-layer chromatographic techniques. Concept (Kim & Kuga, 2001).

**1.1 The ion exchange chromatography principle.**

An analytical and an oppositely charged stationary phase, or ion exchanger, are attracted to one another in this sort of chromatography (Ec et al., 2019)

* In the ion exchangers, charged groups are effectively covalently linked to the surface of an insoluble matrix.
* The charged groups in the matrix might be positively or negatively charged.
* When the matrix is suspended in an aqueous solution, ions with the opposite charge will surround the charged groups of the matrix.
* This "ion cloud" allows for the reversible transfer of ions without changing the matrix's makeup or properties.

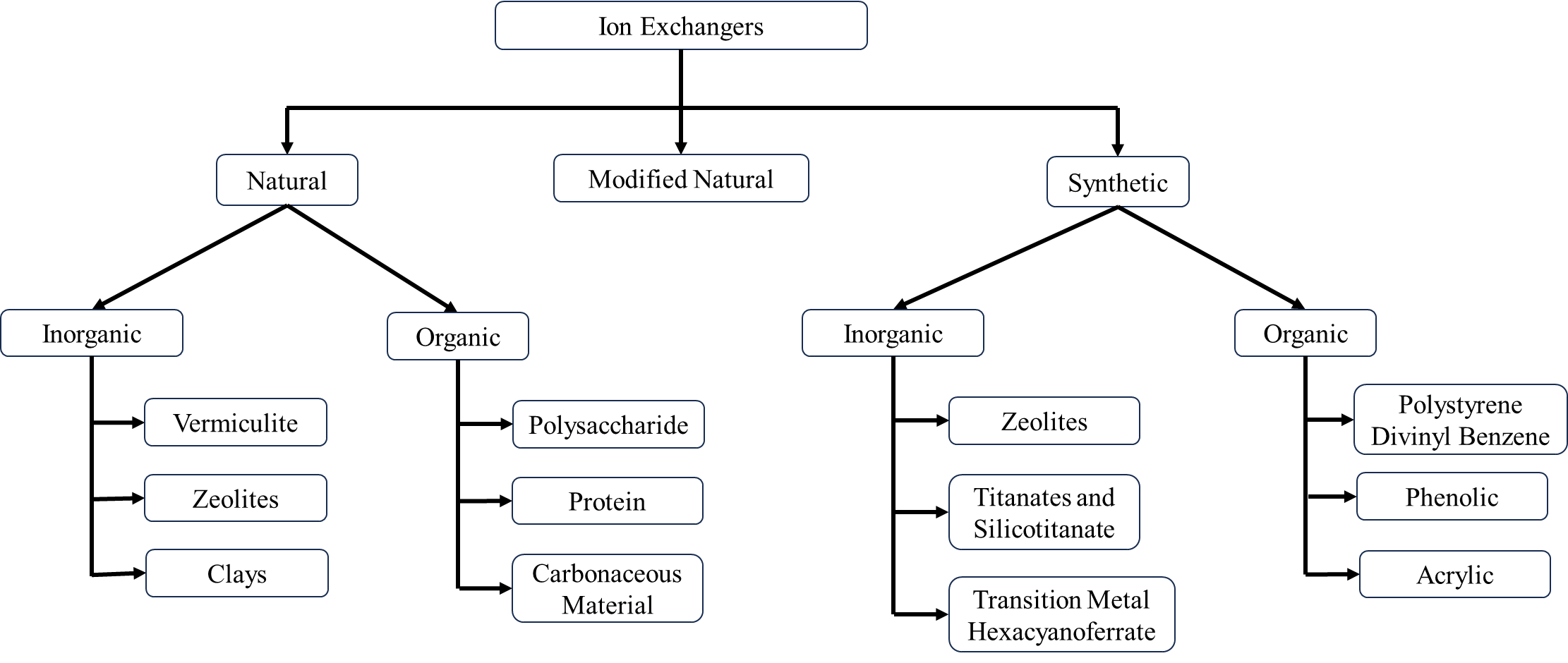
**1.2 Ion exchange chromatography properties**

Ion exchange chromatography is widely used to separate charged biological parts such as proteins, peptides, amino acids, or nucleotides. Amino acids, which are zwitterion compounds having both positively and negatively charged chemical groups, are the building blocks of proteins. (Thurston et al., 2022)

1. The inorganic solvents are not soluble including chloroform, benzene, and carbon tetrachloride as well as in water.
2. Their nature is complicated. In nature, polymers are typical.
3. They have counterions or active ions that can interchange with other ions with ease.

**1.3 Classification of Chromatography by Ion Exchange**

The process of ion exchange chromatography versatile separation technique that is widely used in various fields, including biochemistry, pharmaceuticals, environmental analysis, and industrial processes. It involves the separation of charged analyses \ according to how they interact with ion exchange resins. Ion exchange is based on many characteristics, such as the nature of the sample, chromatography may be divided into several categories. stationary phase, the mode of operation, and the charge of the analyses (Harinarayan et al., 2006).



**Fig 1: Classification of** **Chromatography by Ion Exchange**

**1.4 The equipment used in ion exchange chromatography**

A pump, injector, column, suppressor, detector, and recorder or data system are the most common types of instruments used in ICs. (Bhusnure & Mali, 2015)

**1.4.1 Pump**

One of the most vital parts of the system, the IC pump, is in charge of providing a constant flow of eluent through the IC injector, column, and detector.

**1.4.2 Injector**

Various techniques can be used to complete a sample introduction. Utilizing an injection valve is the easiest option. Samples of liquids are added right away; just need to dissolve in water solid samples with the proper solvent. A liquid sample should be able to be injected with superior repeatability and pressure (up to 4000 psi) using an injector with a volume between 0.1 and 100 millilitres.

**1.4.3 Columns**

The material based on the usage and location, the column might be made of glass, an inert material, titanium, or stainless steel material like PEEK. The size of the column can be anywhere between 2mm and 5 cm, and its length can be anywhere between 3 cm and 50 cm, based on whether it will be applied to routine analytical tasks, microanalysis, high-speed analyses, or preliminary work.

There is a watch column in front of the separating column. The separation column's lifespan and usefulness are increased as a result of this safety measure. These strong columns were created to filter or eliminate debris that blocked the separating column.

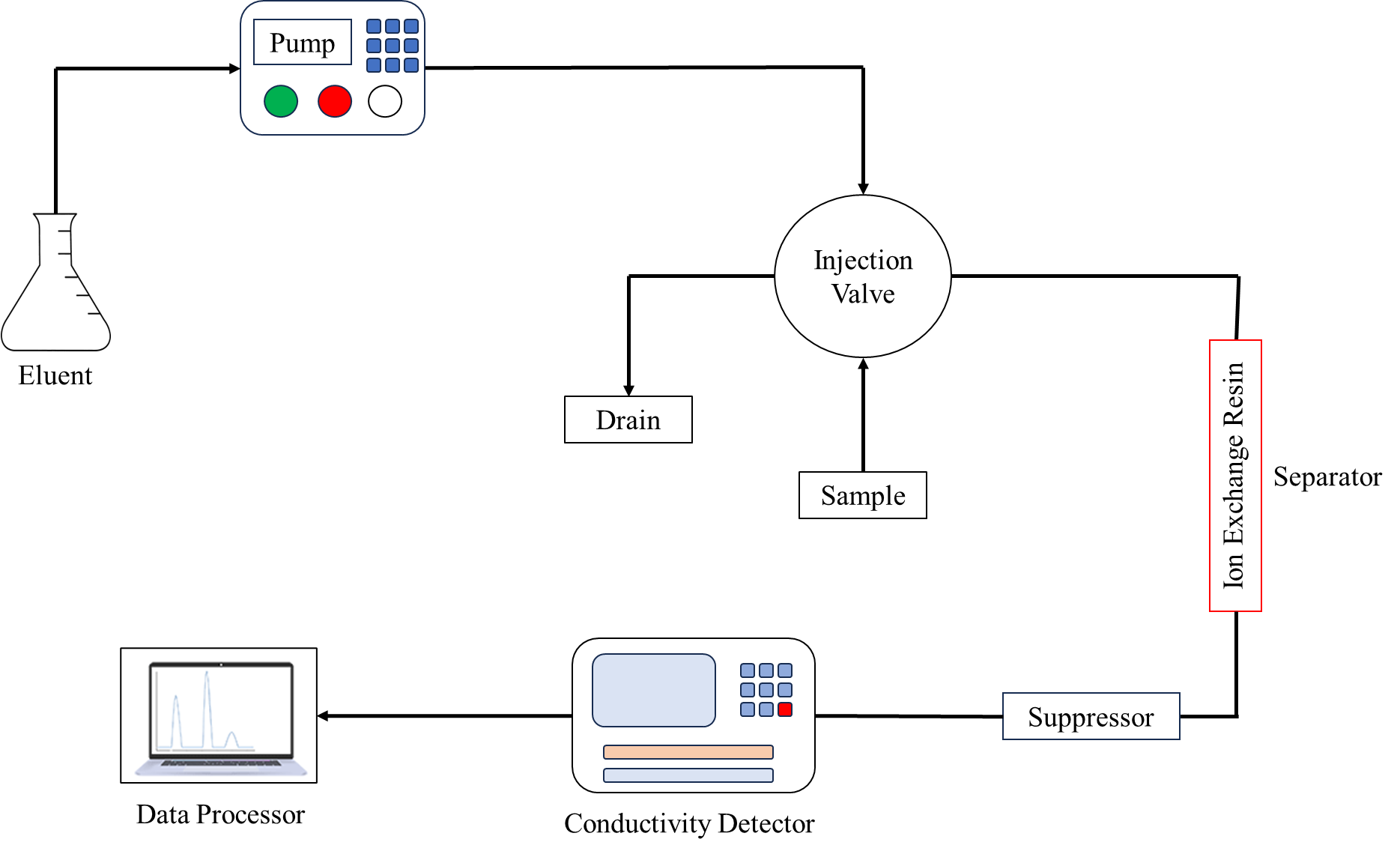
**1.4.4 Suppressor**

Enhancing the measurement of the suppressor conductivity of the ions under investigation by reducing the chemical mixtures used to elute samples from the ion exchange column has a background conductivity. By converting the ionic eluent to water, Membrane-based devices known as IC suppressors improve sensitivity.

**1.4.5 Detectors**

Commonly used as an electrical conductivity detector (Galaev, 1998).

**1.4.6 Data system**

A computational integrator with pre-programmed instructions may be adequate for routine analysis in which automation is not required. For higher control levels, a more advanced gadget is needed, like a data station or minicomputer (Ngere et al., 2023)**.**

**Fig 2: Instrumentation of ion exchange chromatography**

* 1. **Ion-exchange process mechanisms**

To track deamidation and succinimide production, the Chromatographic separation known as the "ion exchange chromatography" technique mostly dependent based on the protein's net charge, is frequently utilized. The chromatographic matrix is directly connected to molecules with positive (cationic) or negative (anionic) charge (Lenhoff, 2016)

* The ion diffuses to the surface of the exchanger. This happens in a homogeneous solution fairly rapidly.
* The ion diffuses from the matrix structure to the exchange site depending on the degree of resin cross-linkage and the Solution's concentration. This process's rate-determining phase is thought to be the ion exchange step.
* Ion exchange occurs instantaneously until equilibrium is reached at the exchange location.
* The exchanged ion diffused to the surface through the exchanger.
* By altering the pH, the ionic concentration, or affinity delusion, which makes use of the ion with the highest affinity for the exchanger, the bound molecules can be released.
  1. **Technique for sample analysis**

The sample is prepared so that certain analytical equipment can inspect it. Examples of sample preparation techniques include crushing and dissolving, chemical digestion using sample preparation, sample extraction, sample cleaning, and sample pre-concentration (Duong-Ly & Gabelli, 2014)

* Columns with an ion exchanger are where most ion exchange separations are done.
* It is possible to buy these ionic exchangers commercially. Styrene and divinyl benzene are the main ingredients. Example. In contrast to DEAE-cellulose, which is a cationic exchanger, CM-cellulose acts as an anionic exchanger.
* The chosen exchanger will depend on how charged the particles need to be separated. In contrast to the "Cationic exchanger," which is used to separate cations, the "Anionic exchanger" is used to do the same for anions.
* The sample is applied first, then the ion exchanger is added, and finally the buffer. The use of phosphate widely used buffers includes pyridine buffer, acetate buffer, citrate buffer, and tris buffer.
* Buffers and particles with a high affinity for the ion exchanger will descend the column together.
* The Next move involves employing a suitable buffer that can separate the particles that are connected firmly.
* These particles' spectroscopic analysis is then conducted.
  1. **Resins that exchange ions**

The terms "ion-exchange polymer" and "ion-exchange resin" refer to a resin or polymer that serves as a medium for ion exchange. It is a substrate constructed from an organic polymer that is a support structure or insoluble matrix. Often appearing as small microbeads (0.25-1.43 mm in radius) that are white or yellowish. The process is known as ion exchange because the beads are usually porous (having a certain appropriate size distribution alters their characteristics), offering a sizable surface area on and inside of them where the ion trapping and the concomitant additional ions are released (Kosanović et al., 2017). Ion-exchange resin comes in many different varieties. Polystyrene sulfonate is the main component of most commercial resins, followed by polyacrylate (Nesterenko et al., 2007).

Ion exchange resins are employed in a variety of distinct decontamination, purification, and separation procedures. Water filtration and water softening are the two most common examples. Ion exchange resins serve as a more adaptable substitute for the use of natural or synthetic zeolites commonly employed in these procedures. When filtering biodiesel, ion exchange resins are also quite effective. (M. & E., 2013).

**Types of resins**

1. Anion resins
2. Resin for cation exchange
3. Anion-exchange resin
   1. **Characteristics Ion exchange Resins**

The following characteristics are typically used to define ion exchange resins (Duong-Ly & Gabelli, 2014).

* + 1. **Capacity**

Indicates how many ions may be exchanged or held in one unit of resin mass. Usually expressed as milligrams ion per milligram (mg/g) of resin.

* + 1. **Swelling**

Resins may swell (increase in volume) when in contact with a solvent. A resin's chemical makeup, polymer structure, and cross-linking all have an impact on how it behaves as it swells. When compared to resins with lower levels of cross-linking, those with higher levels show less swelling tendency. When exposed to a particular solvent, swelling is commonly stated as the resin's percentage increase in volume or weight.

* + 1. **Selectivity**

This describes the resin's predilection for or capacity to exchange or adsorb specific ions over others. It is a key characteristic that governs how well the resin separates or expels particular

Ions in a solution.

* + 1. **Stability**

To determine the resin's integrity, determined by the beads' resistance to mechanical and chemical forces.

* 1. **Factors Affecting Ion–Exchange**

Ion chromatography's selectivity series appears to be best described by the interaction of two factors: electrostatic attraction (ES) and enforced-pairing (EP), which is caused by hydrophobic attraction and by water-enforced ion pairing. (Yu & Sun, 2012)

* + 1. **Nature of Exchange Resins**

The cross-linking and swelling characteristics of the exchange are critical in ion exchange. Resins stiffen and expand less when there is more cross-linking agent present, and there is less ion separation when there is less cross-linking agent present. Various sizes transform problematic since because there are so many various kinds of pores, they can't fit through the full pore, and if the swelling is bigger, the separation fails.

**Ions in the exchanger's nature**

1. When the concentration is low and the temperature is normal, the exchange increases as the valence increases.

Na<Ca<Al

1. The size of the hydrated ion decreases with a similarly charged ion exchange increasing.

Li<H<Na<NH4

1. The more polarizable ion has a greater exchange capacity.

I¯<Br¯<Cl¯<F¯

1. Polyvalent anions are frequently preferred to other ions in diluted solutions.
2. Exchange is favoured at greater concentrations, and vice versa, if the solution has a lower positive charge and the resin, has a higher positive charge.
   1. **Applications of ion exchange chromatography**

Biochemistry, pharmaceuticals, environmental studies, and industrial operations are a few examples of the many industries that employ the potent known separation technique of ion exchange chromatography. Here are a few uses for ion exchange chromatography.(Kabytaev et al., 2016).

* The regular examination of mixtures of amino acids is a significant application of chromatography using ion exchange.
* In clinical diagnostics, the 20 essential amino acids are isolated from blood serum or protein hydrolysis.
* The best method of water filtration is this one. Water (or a non-electrolyte solution) can be entirely deionized by changing the solute cations and anions into hydrogen and hydroxyl ions. A technique for softening drinking water is frequently used to achieve this.
* While analyzing the outcomes of nucleic acid hydrolysis. In this way, we learn about the biological significance of these molecules' structural makeup as genetic information carriers.
* Chelating resins are used to remove trace metals from saltwater.
* To evaluate rare traces of elements on Earth and lunar materials.
  1. **Advantages of ion exchange chromatography**

In analytical and preparative ion exchange chromatography applications is a potent separation method. It has numerous benefits, such as:(Michalski, 2014).

* It is one of the greatest methods for dividing charged particles.
* The use of almost any charged molecule with it is possible, including large proteins, tiny nucleotides, and amino acids.
* Ion exchange has analytical and preparative uses in laboratories, with analytical uses being more prevalent.
* Ion exchange chromatography can also be used to identify inorganic ions.
  1. **Disadvantages of Ion exchange chromatography**

Like each analytical method, ion exchange chromatography has some drawbacks of its own. Ion exchange chromatography has several drawbacks, some of which are listed below:(Duong-Ly & Gabelli, 2014)

* This approach can only be used for distinct charges ions or polar compounds.
* The resin used in this process is susceptible to damage from several chemicals.
* A large number of samples are required.
* The apparatus is costly and requires buffers, which are pricey chemicals.

1. **Gel Chromatography**

Gel chromatography, commonly referred to as gel filtering using variations in the speeds at which different chemical compounds flow through a bed of porous, semisolid material, is a technique used in analytical chemistry to separate chemical compounds. Proteins, peptides, amino acids, enzymes, and other low-molecular-weight substances may all be separated using this technique. from one another (Liu et al., 2013). Based on the differences in their molecular sizes, the components of a mixture can be separated using gel chromatography. Large molecules are unable to penetrate the pores and tend to flow unimpeded, whereas small molecules tend to disperse into the inside of the porous particles, restricting their flow. As a result, the molecules with the highest molecular weight exit the bed first, followed by those with decreasing molecular weight. The most used bed materials are polyacrylamide and a polymer made of dextran and epichlorohydrin. Typically, appropriate agents are used to suspend the dry polymers, creating a homogenous mixture that is semisolid (Ackers, 1970).

* 1. **Principle of Gel Chromatography**

Molecules are divided differently between a fixed phase and a dynamic phase. Which is the underlying concept of gel chromatography. In gel chromatography, a cross-linked matrix of porous polymers, such as agarose or polyacrylamide serves as the stationary phase. Many different pore sizes exist in the gel matrix, resulting in a network of interconnecting channels. (Ó’Fágáin et al., 2017)

* GPC uses the analyses’ hydrodynamic volume (radius of gyration) or size to separate the analyses. Alternative Separation techniques, on the other hand, depend on chemical or physical interactions to find analyses. We utilize a column of porous beads. For particle separation.
* Smaller analyses spend more time in pores because they can enter them more rapidly, which results in a longer retention duration. These smaller molecules elute later because they stay in the column longer. While on the other hand, larger analyses spend little to no time in the pores and are rapidly removed. In each column, different molecular weights may be split.
* Analyses that are too big won't be preserved, whilst those that are too little will be completely kept. Analyses that are not retained are eluted using the free volume outside of the particles (VO). Whereas the volume of solvent contained in the pores is used to elute analyses that are retained. The following equation may be used to calculate both the total volume, Vt, as well as the volume of the polymer gel:
  1. Vt = Vg + Vi + Vo
  2. **Equipment for gel chromatography**

An effective gel permeation chromatographic setup contains the following essential components:

**Gels**

The chemical makeup and molecular dimensions of the analyses that may be separated using the GPC method have a significant impact on the gel choice. For stationary phases in gel permeation chromatography equipment, xerogels are frequently utilized.(Wieland & Determann, 1967)**.**

The GPC process employs only organic gels in its utilization. The trade names for the many varieties of gels that are sold on the market are Polyacrylamide-containing BioGel p-2

* Sephadex G-10-200 (Dextran)
* Styrogel (modified polystyrene gels)
* Agarose

Sepharose and BioGel A are brand names for gel agarose. These gels have hydrogen bonds and can withstand temperatures of 0 to 30 °C and a pH range of 5 to 8. All of the gels used in the gel filtration procedure have the following qualities in common:

* They are inert chemically.
* They are stable mechanically.
* They have consistent pore and particle sizes.
  + 1. **Gel chromatography column**

Eluent is allowed to flow under gravity in a semi-permeable, porous polymer-filled column utilized in the GPC device. The gels in the chromatographic column have a well-defined range of pore sizes. Using a buffer solution to clean the column before testing will eliminate any air bubbles (Liu et al., 2011)

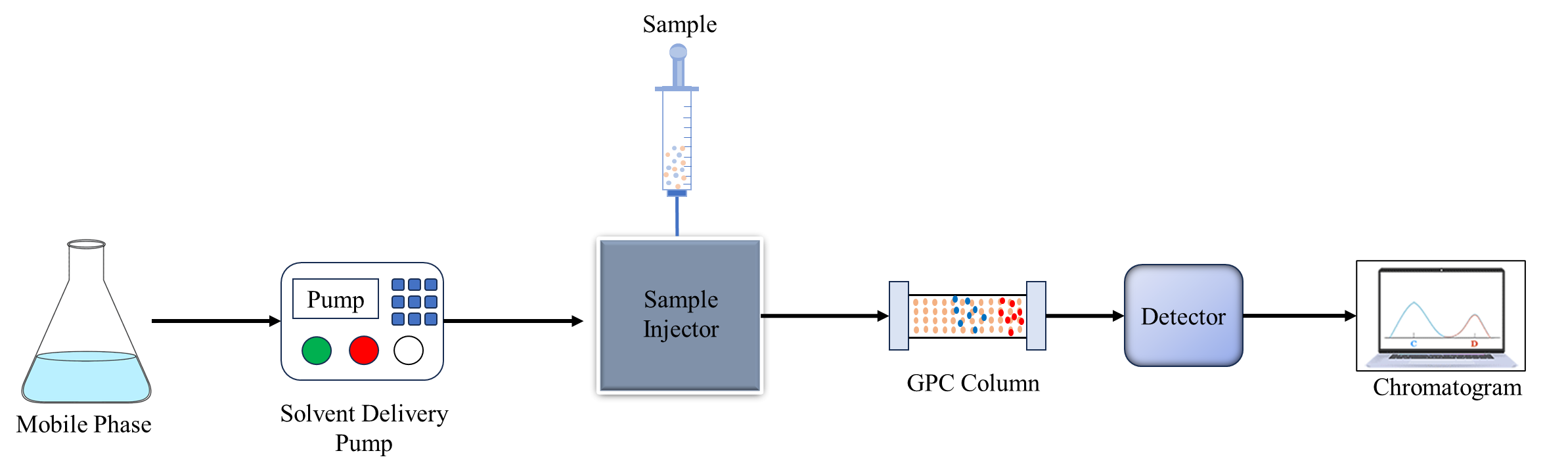
* + 1. **Eluent**

In gel permeation chromatography, the mobile phase, often referred to as the eluent should be a solvent that is appropriate for polymers. In general, organic solvents that can be used as an eluent in the GPC instrument include (Blaha & Berek, 1981)**.**

* THF, or tetrahydrofuran.
* O-Dichlorobenzene
* Trichlorobenzene
* Polyalkenes
* M-Cresol
* O-Chlorophenol
  + 1. **Pump**

In the GPC approach, piston or peristaltic pumps are employed to distribute the mobile phase uniformly or at a constant flow rate (Ó’Fágáin et al., 2017)

* + 1. **Chromatographic detector**
* The detector can measure an appropriate physical characteristic of the effluent, such as its refractive index, absorbance, fluorescence intensity, and other electrical characteristics, much as in conventional chromatographic techniques.
* The GPC analysis employs a variety of detector types. Generally speaking, they fall into two groups:
* The first group of detectors measures the absorption of light and includes concentration-sensitive devices including UV, RI, IR, and density detectors.
* Two molecular weight-sensitive detectors are included in the second category: low-angle light scattering (LALLS) and multi-angle light scattering (MALLS).(Kara & Fisher, 2012)

****

**Fig 3: Instrumentation of Gel Chromatography**

* 1. **Sample analysis procedure**
* Chromatography columns are used for almost all gel permeation chromatography. The experimental model resembles previous liquid chromatography methods quite closely. Samples are in a suitable solvent, dissolved —usually an organic one for GPC— injected into a column, and then filtered. The column-based separation of a complex mixture. Using a pump guarantees a constant flow of new eluent to the column. Because most analyses cannot be seen with the human eye, a detector is required. Several detectors are frequently employed to get more details about the polymer sample.
* The GPC stationary method is the gel. When applying the gel to a specific separation, the size of the gel's pores requires close attention. The lack of ionizing groups and poor affinity of the gel-forming agent for the components to be separated in a particular solvent are two additional desirable qualities.
* The GPC column's packing material is a microporous interior. The gel is added to the gel filtration column, which is then filled with the gel.
* To strongly activate the detector and wet the packing surface, the polymer needs a suitable solvent in the eluent (mobile phase). Most frequently, the tetrahydrofuran components of GPC polymers (THF) dissolve at room temperature.
* The two pump types for uniform distribution of relatively tiny Pumps for GPC's liquid volumes might be piston or peristaltic.
* A detector will continually track the weight-based polymer concentration in the eluting solvent in GPC. There are two main categories in which detectors may be divided. Some of the first forms of concentration-sensitive detectors include UV absorption, differential refractometer (DRI) or refractive index (RI) detectors, infrared (IR) absorption, and density detectors (Winzor, 2003)**.**
  1. **The use of gel chromatography**

The molecular weight distribution and relative molecular weight of polymer compounds are frequently assessed using GPC.GPC evaluates the Function of size and form of molecules based on their innate viscosity. This relative information may be used to calculate molecular weights with 5% precision under identical circumstances. Polystyrene standards with variations of less than 1.2 are frequently used to calibrate the GPC. (Ruzicka et al., 1968)**.**

**Advantages of Gel Chromatography**

Gel chromatography, commonly referred to as size exclusion chromatography (SEC) is a technique used in analytical chemistry to sort and purify molecules according to their size. The following are some benefits of gel chromatography: (Shen et al., 2016)

It is the ideal method for separating molecules with different molecular weights because:

* Separation may be carried out in any setting since it is unaffected by alterations in temperature, pH, ionic strength, or buffer composition.
* When compared to other approaches, zonal dispersion is reduced.
* The molecular weight and elution volume are linked.
* An important method for protein purification.
* By fractionating without the necessity for protein binding, this innovative separation method reduces the risk of protein loss.
  1. **Disadvantages of Gel Chromatography**

A common method for sorting and purifying biomolecules according to their size is gel chromatography, commonly referred to as size exclusion chromatography or gel filtration chromatography. While using gel chromatography has many benefits, it also has certain drawbacks, such as:(Caltabiano et al., 2018)

* The GFC run's brief time scale can only resolve a finite number of peaks.
* The instrument must pass through filters before being used to prevent dust and other particles from damaging the columns and interfering with the detectors

1. **Affinity Chromatography**

Chromatography is a crucial biophysical method for separating, identifying, and purifying a mixture's parts for qualitative and quantitative analyses. Analysis. This technique of separation involves coming into contact with a mobile phase that is conveying a mixture and a stationary phase that is selectively absorbent. To separate, purify, or Analyse certain components of materials, A type of liquid chromatography is used called affinity chromatography. It utilizes the affinity-based molecular recognition method, which describes the compelling force that is provided to varying amounts of atoms to keep them together. In biology, affinity uses this reversible mechanism to keep molecular links intact. An enzyme, an inhibitor, an antigen, an antibody, etc. are some examples (Rodriguez et al., 2020).

Meir Wilcheck and Pedro Cuatrecasas are the authors of the discovery.

* 1. **Principle of Affinity Chromatography**

The stationary phase in affinity chromatography is constructed of a support medium (such as cellulose beads) on which the substrate (or occasionally a coenzyme) has been covalently bonded to expose the reactive groups necessary for enzyme binding.(Ostrove, 1990)**.**

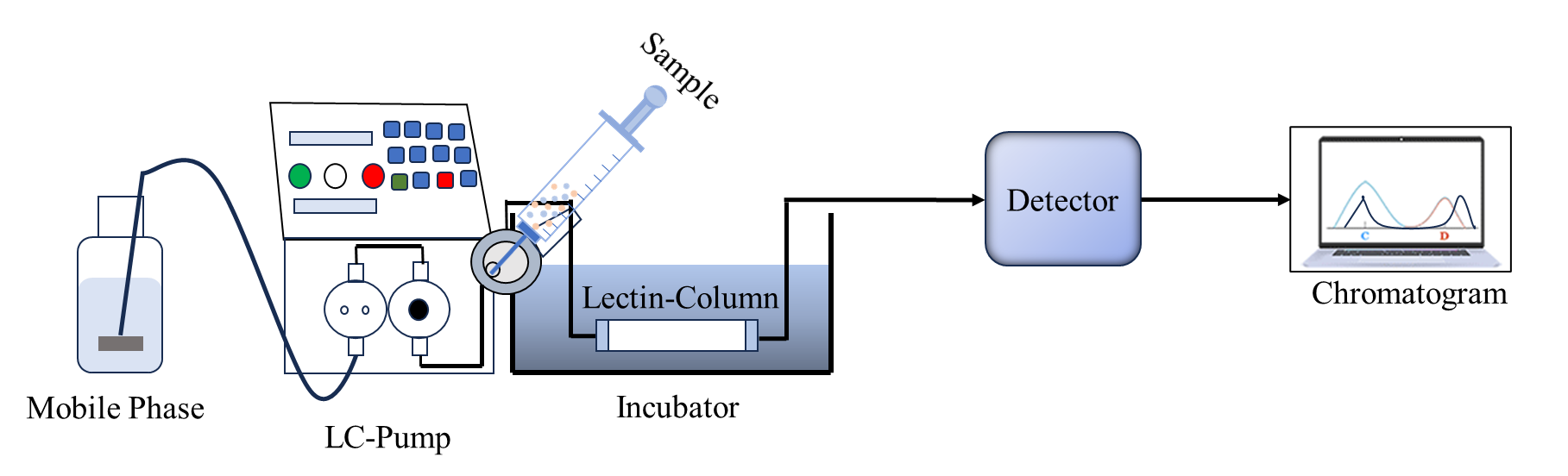
* The reactive groups necessary for the tethering of the target molecule are still accessible because, in the stationary phase, the substrate (ligand) is still affixed to the support material covalently.
* When an In a chromatography column, a crude chemical mixture is run, and only the compounds with binding sites for the immobilized substrate bond to the stationary phase; all other substances are eluted into the column's mobile phase Interior.
* The bound target molecules can be freed after the other substances have been removed using techniques such as introducing a rival ligand to the mobile phase or modifying the pH, ionic strength, or polarity of the solution.
  1. **Instrumentation of Affinity Chromatography**

Affinity chromatography is a method for separating biological mixtures that have a highly precise relationship, such as one between an antigen and an antibody, an enzyme and a substrate, or a receptor and a ligand(Hage et al., 2012)**.**

* + 1. **Matrix**
  1. A ligand can be connected to the matrix directly or indirectly as an inert support.
  2. Specific characters are necessary for the matrix to function well, including:
  3. The matrix must be inert both chemically and physically.
  4. It must not dissolve in the buffers and solvents used in the procedure.
  5. It must be stable in both chemical and mechanical terms.
  6. It must be straightforward to connect to a ligand or spacer arm that can accommodate the ligand.
  7. It must have good flow properties and a substantial surface area for attachment.
  8. Agarose and polyacrylamide are the best matrix materials.
     1. **Spacer arm**

Reducing the effects of steric hindrance helps the ligand and target molecule better bond.

* + 1. **Ligand**
  + It describes a chemical's reversible binding to a particular goal molecule.
  + Once the kind of extracted macromolecule has been chosen, the ligand can only be chosen has been identified.
  + The hormone itself is a prime choice for the ligand when a hormone receptor protein has to be purified using affinity chromatography.
  + To isolate antibodies, a ligand such as it’s possible to employ an antigen or hapten.
  + The immobilized ligand for the purification of an enzyme may be an analogue of a cofactor, an effector, a substrate, or an inhibitor (Zhang et al., 2018)**.**



**Fig 4: Instrumentation of Affinity Chromatography**

* 1. **Sample analysis procedure**

The physical removal of the sample from the target population, preservation of the sample, and preparation of the sample for analysis are the three phases that typically make up the sampling plan implementation process. It is generally easy to Analyse a sample when an analytical method is selective for the analysis (Arora et al., 2017)**.**

* + In the binding buffer, the affinity medium is balanced.
  + The application of the sample encourages the target molecule(s) to particularly bind to a complementary ligand. Exactly reversible attachment of the target compounds to the ligand takes place, and unbound material is flushed through the column.
  + By modifying the polarity, pH, or ionic strength while using a competing ligand, elution can be carried out precisely or randomly. The concentrated, purified version of the target protein is collected.
  + The affinity medium is rebalanced using a binding buffer.

The following three significant steps can be used to summarize these actions:

* + 1. **Preparation of Column**
* Sepharose, cellulose, and agarose are just a few of the sturdy support materials that are used to fill the column.
* Ligands are chosen according to what is desired isolation.
* A spacer arm is connected to the solid support and ligand.
  + 1. **Loading of Sample**

The mixture of the solution containing the chemical is added to the elution column and permitted to flow at a specified pace.

* + 1. **Elimination of the Ligand-Molecule Complex**

Modifying the environment to encourage the release of the bound molecules allows for the extraction of the target substance.

* 1. **Uses for Affinity Chromatography**
  + One of the top techniques for separating and purifying certain compounds is affinity chromatography.
  + It functions largely as a sample purification procedure for biological substances like proteins.
  + The division of a complex mixture.
  + Impurity removal or purification procedure.
  + During enzyme tests
  + Identifying substrates
  + Research into enzyme binding locations
  + In vitro antibody-antigen interactions
  + Nucleic acid analysis for mutations and single nucleotide polymorphisms (Hage, 2017)**.**
  1. **Advantages of Affinity Chromatography**
  + Using the specific interactions between a ligand and a target molecule immobilized on a solid substrate, affinity chromatography is a potent separation method. Affinity chromatography has the following benefits:(Bi et al., 2015)
  + Extreme specificity
  + Highly pure target molecules can be obtained.
  + One step of purification.
  + The matrix might be utilized right away.
  + The matrix is sturdy and is not difficult to dry or clean.
  + Provide a high yield of a purified product.
  + Certain pollutants, such as proteases, can also be removed via affinity chromatography.
  1. **Disadvantages of Affinity Chromatography**

Based on the unique interactions that biomolecules have with ligands that have been immobilized on a solid substrate, affinity chromatography is a potent technology for the cleansing and separation of biomolecules. While affinity chromatography provides many benefits, there are some drawbacks as well. Affinity chromatography has the following drawbacks:(Moaddel & Wainer, 2009)**.**

* The expense of resins can make affinity chromatography costly.
* High production costs for resin.
* Limited selection of resin.
* Resins might need to be customized for certain goals.
* Storage conditions might affect resin sensitivity**.**
  1. **Limitations of Affinity Chromatography**

To separate and purify certain target molecules from complicated mixtures, affinity chromatography is a potent technology used in molecular biology and biochemistry. It has several restrictions, nevertheless, much as any analytical or separation process. Affinity chromatography has the following typical drawbacks:(Arora et al., 2015)**.**

* + - An approach that takes time.
    - More solvents are needed, which might be pricey.
    - Prolonged labour
    - Non-specific adsorption can only be reduced, not completely removed.
    - The high price and dearth of immobilized ligands.
    - If the proper pH is not changed, proteins get denatured**.**

**References**

1. Ackers, G. K. (1970). Analytical gel chromatography of proteins. *Advances in Protein Chemistry*. https://doi.org/10.1016/S0065-3233(08)60245-4
2. Arora, S., Ayyar, B. V., & O’Kennedy, R. (2015). Affinity chromatography for antibody purification. *Methods in Molecular Biology*. https://doi.org/10.1007/978-1-62703-977-2\_35
3. AArora, S., Saxena, V., & Ayyar, B. V. (2017). Affinity chromatography: A versatile technique for antibody purification. In *Methods*. https://doi.org/10.1016/j.ymeth.2016.12.010
4. Bhusnure, O. G., & Mali, S. N. (2015). RECENT TRENDS IN ION-EXCHANGE CHROMATOGRAPHY. *International Journal of Pharmaceutics and Drug Analysis*.
5. Bi, C., Beeram, S., Li, Z., Zheng, X., & Hage, D. S. (2015). Kinetic analysis of drug-protein interactions by affinity chromatography. In *Drug Discovery Today: Technologies*. https://doi.org/10.1016/j.ddtec.2015.09.003
6. Bleha, T., & Berek, D. (1981). Gel chromatography with mixed eluents. Partition of solute due to the preferential solvation of gel. *Chromatographia*. https://doi.org/10.1007/BF02314761
7. Caltabiano, A. M., Foley, J. P., & Striegel, A. M. (2018). Aqueous size-exclusion chromatography of polyelectrolytes on reversed-phase and hydrophilic interaction chromatography columns. *Journal of Chromatography A*. https://doi.org/10.1016/j.chroma.2017.12.007
8. Duong-Ly, K. C., & Gabelli, S. B. (2014). Using ion exchange chromatography to purify a recombinantly expressed protein. In *Methods in Enzymology*. https://doi.org/10.1016/B978-0-12-420119-4.00008-2
9. Ec, E., Obbina, I., & VA, W. (2019). Applications of Column, Paper, Thin Layer and Ion Exchange Chromatography in Purifying Samples: Mini Review. *SF Journal of Pharmaceutical and Analytical Chemistry*.
10. Fekete, S., Beck, A., Veuthey, J. L., & Guillarme, D. (2015). Ion-exchange chromatography for the characterization of biopharmaceuticals. In *Journal of Pharmaceutical and Biomedical Analysis*. https://doi.org/10.1016/j.jpba.2015.02.037
11. Galaev, I. Y. (1998). New methods of protein purification. Displacement chromatography. *Biochemistry (Moscow)*.
12. Hage, D. S. (2017). Analysis of biological interactions by affinity chromatography: Clinical and pharmaceutical applications. In *Clinical Chemistry*. https://doi.org/10.1373/clinchem.2016.262253
13. Hage, D. S., Anguizola, J. A., Bi, C., Li, R., Matsuda, R., Papastavros, E., Pfaunmiller, E., Vargas, J., & Zheng, X. (2012). Pharmaceutical and biomedical applications of affinity chromatography: Recent trends and developments. In *Journal of Pharmaceutical and Biomedical Analysis*. https://doi.org/10.1016/j.jpba.2012.01.004
14. Harinarayan, C., Mueller, J., Ljunglöf, A., Fahrner, R., Van Alstine, J., & Van Reis, R. (2006). An exclusion mechanism in ion exchange chromatography. *Biotechnology and Bioengineering*. https://doi.org/10.1002/bit.21080
15. Kabytaev, K., Durairaj, A., Shin, D., Rohlfing, C. L., Connolly, S., Little, R. R., & Stoyanov, A. V. (2016). Two-step ion-exchange chromatographic purification combined with reversed-phase chromatography to isolate C-peptide for mass spectrometric analysis. *Journal of Separation Science*. https://doi.org/10.1002/jssc.201500989
16. Kara, D., & Fisher, A. (2012). Modified silica gels and their use for the preconcentration of trace elements. In *Separation and Purification Reviews*. https://doi.org/10.1080/15422119.2011.608765
17. Kim, U. J., & Kuga, S. (2001). Ion-exchange chromatography by dicarboxylic cellulose gel. *Journal of Chromatography A*. https://doi.org/10.1016/S0021-9673(01)00800-7
18. Kosanović, M., Milutinović, B., Goč, S., Mitić, N., & Janković, M. (2017). Ion-exchange chromatography purification of extracellular vesicles. *BioTechniques*. https://doi.org/10.2144/000114575
19. Lenhoff, A. M. (2016). Ion-exchange chromatography of proteins: The inside story. *Materials Today: Proceedings*. https://doi.org/10.1016/j.matpr.2016.10.038
20. Liu, H., Nishide, D., Tanaka, T., & Kataura, H. (2011). Large-scale single-chirality separation of single-wall carbon nanotubes by simple gel chromatography. *Nature Communications*. https://doi.org/10.1038/ncomms1313
21. Liu, H., Tanaka, T., Urabe, Y., & Kataura, H. (2013). High-efficiency single-chirality separation of carbon nanotubes using temperature-controlled gel chromatography. *Nano Letters*. https://doi.org/10.1021/nl400128m
22. M., Y., & E., R. (2013). Ion Exchange Chromatography - An Overview. In *Column Chromatography*. https://doi.org/10.5772/55652
23. Michalski, R. (2014). APPLICATION OF ION CHROMATOGRAPHY IN CLINICAL STUDIES AND PHARMACEUTICAL INDUSTRY. *Mini-Reviews in Medicinal Chemistry*. https://doi.org/10.2174/1389557514666141013142203
24. Moaddel, R., & Wainer, I. W. (2009). The preparation and development of cellular membrane affinity chromatography columns. *Nature Protocols*. https://doi.org/10.1038/nprot.2008.225
25. Nesterenko, P. N., Fedyanina, O. N., Volgin, Y. V., & Jones, P. (2007). Ion chromatographic investigation of the ion-exchange properties of microdisperse sintered nanodiamonds. *Journal of Chromatography A*. https://doi.org/10.1016/j.chroma.2007.02.019
26. Nesterenko, P. N., & Paull, B. (2017). Ion chromatography. In *Liquid Chromatography: Fundamentals and Instrumentation: Second Edition*. https://doi.org/10.1016/B978-0-12-805393-5.00009-9
27. Ngere, J. B., Ebrahimi, K. H., Williams, R., Pires, E., Walsby-Tickle, J., & McCullagh, J. S. O. (2023). Ion-Exchange Chromatography Coupled to Mass Spectrometry in Life Science, Environmental, and Medical Research. In *Analytical Chemistry*. https://doi.org/10.1021/acs.analchem.2c04298
28. Ó’Fágáin, C., Cummins, P. M., & O’Connor, B. (2017). Gel-filtration chromatography. In *Methods in Molecular Biology*. https://doi.org/10.1007/978-1-4939-6412-3\_2
29. Ostrove, S. (1990). Affinity chromatography: General methods. *Methods in Enzymology*. https://doi.org/10.1016/0076-6879(90)82031-V
30. Rodriguez, E. L., Poddar, S., Iftekhar, S., Suh, K., Woolfork, A. G., Ovbude, S., Pekarek, A., Walters, M., Lott, S., & Hage, D. S. (2020). Affinity chromatography: A review of trends and developments over the past 50 years. In *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. https://doi.org/10.1016/j.jchromb.2020.122332
31. Ruzicka, J. H., Thomson, J., Wheals, B. B., & Wood, N. F. (1968). The application of gel chromatography to the separation of pesticides part I. organophosphorus pesticides. *Journal of Chromatography A*. https://doi.org/10.1016/0021-9673(68)80003-2
32. Shen, Y., Roberge, A., Tan, R., Gee, M. Y., Gary, D. C., Huang, Y., Blom, D. A., Benicewicz, B. C., Cossairt, B. M., & Greytak, A. B. (2016). Gel permeation chromatography as a multifunctional processor for nanocrystal purification and on-column ligand exchange chemistry. *Chemical Science*. https://doi.org/10.1039/c6sc01301e
33. Thurston, J. R., Marshak, M. P., & Reber, D. (2022). Monitoring Ion Exchange Chromatography with Affordable Flame Emission Spectroscopy. *Journal of Chemical Education*. https://doi.org/10.1021/acs.jchemed.2c00455
34. Wieland, T., & Determann, H. (1967). Some recent developments in gel chromatography, with special reference to thin layers. *Journal of Chromatography A*. https://doi.org/10.1016/s0021-9673(01)85919-7
35. Winzor, D. J. (2003). Analytical exclusion chromatography. In *Journal of Biochemical and Biophysical Methods*. https://doi.org/10.1016/S0165-022X(03)00071-X
36. Yu, L. L., & Sun, Y. (2012). Trace adsorption of positively charged proteins onto Sepharose FF and Sepharose FF-based anion exchangers. *Journal of Chromatography A*. https://doi.org/10.1016/j.chroma.2012.07.004
37. Zhang, C., Rodriguez, E., Bi, C., Zheng, X., Suresh, D., Suh, K., Li, Z., Elsebaei, F., & Hage, D. S. (2018). High-performance affinity chromatography and related separation methods for the analysis of biological and pharmaceutical agents. In *Analyst*. https://doi.org/10.1039/c7an01469d