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Learning Outcome of Module-V

LO	Learning Outcome (LO)	Course Outcome Code
LO1	To understand the chromatographic separation and analysis of drugs.	BP701.1, BP701.4, BP701.6
LO2	To understand the chromatographic separation using Ion exchange chromatography	BP701.1, BP701.4, BP701.6
LO3	To understand the chromatographic separation using Gel chromatography	BP701.1, BP701.4, BP701.6
LO4	To understand the chromatographic separation using Affinity chromatography	BP701.1, BP701.4, BP701.6

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ION EXCHANGE CHROMATOGRAPHY (IEC):

Chromatography is a technique for carrying out separation of a mixture of compounds into its individual components. The decades of 1930s and 1940s witnessed rapid developments in the field of chromatography that resulted in the designing of various different types of chromatographic techniques and instruments. These are column chromatography, planar chromatography which can be further classified into paper and thin layer chromatography, gas chromatography (where the mobile phase is a gas), liquid chromatography such as high performance liquid chromatography i.e. HPLC, supercritical liquid chromatograhy, size exclusion chromatography and ion exchange chromatography. Chromatography is used both for the purification and quantification of separated components or analytes. An analyte is a substance that is to be separated from the mixture. The basic principle in chromatography is the binding or adsorption affinity of different analytes in the chromatograph. In general there are two phases involved in chromatogarphy: a stationary phase and a mobile phase. Stationary phase is fixed in place for carrying out chromatographic procedure. A very simple example of stationary phase is silica which is used in thin layer chromatography. In this phase the substance that is to be separated is selectively adsorbed. During chromatographic procedures usually a combination of solvents are introduced into the system as mobile phase. The mixtures of compounds that are to be separated are introduced into a chromatograph via mobile phase. The mobile phase can be either a liquid or a gas depending on the type of chromatograph being used. According to the binding affinity of analyte in mobile phase it elutes from the column. The different compounds have different affinity towards stationary and mobile phase. This basic principle helps in carrying out separation of compounds. The compound which has higher affinity in mobile phase compared to stationary phase will elute first compared to those compounds which have higher affinity in stationary phase.

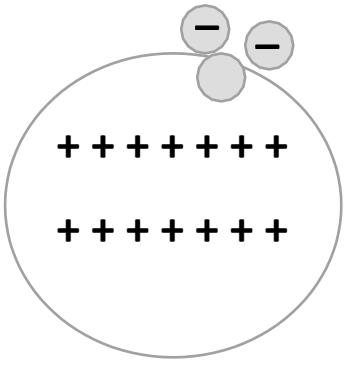
Ion exchange chromatography (IEC) technique is a type of chromatographic technique where separation of analytes is on the basis of net surface charge of the molecules. The main advantage of using this technique is that the separation is dependent only one one interaction i.e electrostatic interaction. This technique was introduced in 1960s and is still sought after for the purification of proteins, peptides, nucleic acids and other charged biomolecules. In this technique the stationary phase is usually a resin which is coated with charged moieties.

1. Principle of ion exchange chromatography

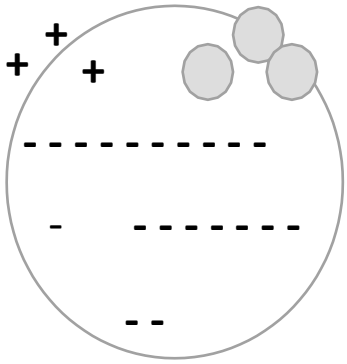
Molecules possessing the opposite charge as the resin will bind tightly to the resin, whereas molecules having the same charge as the resin will flow through the column and elute out first. In IEC the stationary phase is a solid resin (either cationic or anionic) whereas the mobile phase is a liquid. On the basis of type of resins, ion exchange chromatography can be classified as cationic or anionic exchange chromatograph. A cation exchange resin carries a net negative charge and therefore ions carrying net positive charge bind to the resin. The impurities or the components which are neutral or carry negative charge do not bind to the cation exchange resin and elute with the mobile phase. When an anion exchange resin is used, negatively charge analytes bind to the resin. The other species i.e. either carrying no charge or positively charge elute from the column without binding to the resin. The following

figure represents a pictorial representation of the charged stationary phases where the oppositely charged ions are adsorbed. In figure 'A' we see a positively charged matrix of ion change chromatograph where the negatively charged ions of compounds are adsorbed. Similarly in figure B, the matrix is negatively charged and cations are adsorbed. The adsorbed ions are of the compounds that are present in the mixture which is to be separated. These compunds, as discussed earlier are introduced into the chromatograph system via mobile phase.

A Anion exchange resin (A)



B Cation exchange resin (B)



2. Factors affecting the performance of IEC

There are many factors affect the performance of ion exchange chromatograph. Among them, factors such as ionic charge and ionic radius play a significant role during the separation in ion exchange chromatography. As discussed above, the separation of compounds in ion exchange chromatography is based on the electrostatic interaction between the analytes an dthe resin of the ion exchange chromatograph. These interactions depend on the bond energies of ions in

solutions which further are related to the ionic charge, *z and* ionic radius, *r*. The *z* and *r* combine to form ionic potential, z/r. Ions that carry high charge and are smaller in size have high ionic potential, hence attach strongly to oppositely charge solid surface i.e. the resin. However, the ions with low charge and large radius form weak ionic bonds with the solid matrix or resin. Using this difference in bond energies of different ions one can vary the chemistry of mobile phase to obtain preferential elution.

The difference in binding affinity of analytes i.e. ions to be separated from the mixture can be used to calculate the theoretical effectiveness of the separations in ion exchange chromatography. This can be done by comparing the distribution coefficients (D) of analyte ions adsorbed on the resin. The distribution coefficient is the ratio of the concentration, at steady state, of ion on the stationary phase (i.e. resin) to the concentration of ions in the mobile phase. The equation of distribution coefficient is as follow:

$$D = Cs/ Cl$$

where, D= Distribution coefficient, Cs = concentration of ions on solid support, Cl= concentration of ions in the liquid

From the above equation, we understand that at high value of D (i.e greater than 100) the analyte ions will remain bound to the stationary phase (i.e. the resin), whereas at low D (< 0.1) the ions will seldom bind to the resin and remain in the mobile phase. This measurement of D helps in carrying out the purification or separation process in ion exchange chromatography. Generally, in the ion exchange chromatographic procedures, the value of D for analyte ions that are to be separated should be very high (usually greater than 1000) so that unwanted ions can be removed from the system. After the removal of undesired components, the chemistry of mobile phase is modified so that the value of D becomes less than one. At this stage the ions which had earlier bound to the resin now get detached and come into the mobile phase and are eluted. The ions which have near about same ionic potential that is the ratio between charge and radius have similar D values. Separation of these types of ions by ion exchange chromatography becomes a challenge.

An important criteria for ion exchange chromatography is that the resin and mobile phases should be chemically compatible that is to say that the only reactions that take place during chromatography are reversible.

3. Stationary Phase

In ion exchange chromatography the stationary phase is a resin which has an organic coating to provide a charged surface. These resins are usually made up of inert material. The commonly used inert material is polystyrene spherical beads. Depending upon the charge present on the resin the ion exchange chromatograph is classified as cation exchange or anion exchange chromatograph. When the resins are coated with negatively charged surfaces the analytes with net positive charge i.e. cations bind to them, hence they are used for the separation of cations. This type of ion exchange chromatographic technique is known as cation exchange chromatography. In case the resin has positively charged surface it is known as anion exchange chromatography as the negatively charged or anions species bind to the resin.

The stationary phase in ion exchange chromatograph can also be categorized as either strong or weak matrices. Strong ion exchange matrices remain ionized across a wide range of pH levels once the column is equlibrated. In these exchangers a wide range of buffers can be used as mobile phase. Weak ion exchangers remain charged or ionized only in a certain range of pH. In these exchangers if the pH of the buffer used under any circumstances changes then the charge distribution on the column will change leading to poor or no separation of the molecule of interest. The smaller pH ranges of weak ion exchangers make them more specific compared to strong ion exchangers and hence are used to obtain very pure and specific analytes.

Properties of stationary phase affecting the ion exchange chromatography

(a) Surface area

One of the important properties of stationary phase that affects the ion exchange chromatography is the surface area of the stationary phase (here this refers to the size of the resin bead). As the bead size decreases, the surface area per unit volume increases, thereby providing better separations.

(b) Density or crosslinkages present on the matrix

The density of exchange sites on the stationary phase surface also affects the separation procedure in ion exchange chromatography. Here the density refers to the cross-linking groups present in the matrix of stationary phase. Higher the crosslinkage higher will be the binding affinity of the analytes that is more quantity of analytes will bind to the resin surface. The cross-linkage of the resin can be modified by altering the organic coating so as to provide varying degree of exchange sites per unit area.

Groups present on the stationary phase

There are different types of charged groups used for coating on the resin in ion exchange chromatograph. Some of the functional groups used are as follows:

- Aminoethyl (AE)
- Diethylaminoethyl (DEAE)
- Trimethylaminoethyl (TAM)
- Triethylaminomethyl (TEAE)
- Diethyl-2-hydroxypropylamine ethyl (QAE)
- Carboxy (C)
- Carboxymethyl (CM)
- Sulphonic acid (S)
- Sulphomethyl (SM)
- Sulphopropyl (SP)

Amongst these the commonly used anion exchangers are Diethylaminoethyl i.e DEAE and Diethyl-2-hydroxypropylamine ethyl i.e QAE. Carboxymethyl known as CM, Sulphopropyl i.e SP and Sulphonic i.e. S are commonly used as resins in cation exchange chromatograph.

4. Mobile Phase

The mobile phase in ion exchange chromatography is a liquid which can be a buffer or a mixture of solvents or solutions where additives such as salts and zwitterions can be present. Mobile phase combines with the mixture of ions that are to be separated from compounds and are pushed into the charged column for purification or separation. Therefore, the different mobile phases employed can be a mix of aqueous acid & base solutions, organic and non- aqueous solutions. The choice of mobile phase is made depending upon the mixture of compounds that are to be separated. The mobile phase used for ion exchange chromatography can be categorized into loading, wash and elution buffers or mobile phase. The composition of each of these buffers is crucial for efficient purification and quantification of analytes by ion exchange chromatography. Also, these buffers affect the quality and life of column i.e. the resin or matrix used for chromatography. If the buffer contains the wrong counterion then it may prevent the adsorption or binding of the analyte to the column matrix. As a rule, the ions present in the buffer should be

positively charged when the resin in use is positively charged. Similarly the ions in buffer should be anionic when an anionic charged resin or matrix is used in ion exchange chromatography. For example, if phosphate buffers are used for purification of analytes then they are not appropriate for positively charged resin as the phosphate ions will bind strongly to the resin leading to poor or improper purification of analytes.

Properties of mobile phase affecting performance of ion exchange chromatography

There are certain properties of mobile phase that affects ion exchange chromatography. These are:

(a) The flow rate of the mobile phase

Flow rate of the mobile phase refers to how fast the mobile phase is being passed over the resin matrix. The flow rate therefore determines the residence time of a particular column. This time actually refers to the time which proteins take for interaction with the column resin. Flow rate affects resolution of separation and also the capacity of a column to carry out the purification process. A longer residence time implies a high level of resolution and increased capacity of the column needed for carrying out chromatographic procedure. The performance of column resin depends on the flow rate. With increase in flow rate the pressure on the resin also increase. In case the backpressure is very high then it may lead to damage in the resin constitution. Therefore, as safety measure manufacturers provide a pressure limit for all of their commercially available resins which has to be kept in check during the chromatographic procedure. In routine and normal circumstances that flow rate is selected which will give the desired capacity and resolution. If as lower flow rate is selected it may lead to in some circumstances decrease in protein activity as many proteins are known to lose activity with time in chromatographic procedures.

(b) Chemistry of the mobile phase (ionic strength of the sample solution, concentration of mobile phase).

The buffer strength, its pH, presence of additives such as salts and zwitterions greatly affect the performance of ion exchange chromatography.

5. Optimization of ion exchange chromatograph

Prior to starting the chromatographic procedure it is imperative to optimize the chromatograph instrument to obtain pure and quantified analytes. One should consider certain parameters that

affect the purification procedure in ion exchange chromatography which are pH of the buffer or mobile phase, buffers ions, salts, detergents, zwitterions, organic solvents and temperature. Before the sample is loaded, the column is first equilibrated with an equilibration buffer. When selecting the equilibration buffer the pH and ionic strength should be considered so as to ensure that, when sample is loaded, analytes of interest bind to the matrix resin and as many impurities as possible do not bind and elute out from the system. The molecules that bind to the column are effectively concentrated onto the column depending on the flow rate employed for the mobile phase and the cross linking groups present on the resin while the contaminants or other unwanted substances that do not have the correct surface charge only pass through the column along with the buffer and elute out either just after sample loading or during the chromatographic procedure.

pH

When carrying out ion exchange chromatography it is necessary to control pH prevailing in the system which actually depends on the components of buffer that is used and additives such as salts. The separation efficiency also depends on the sample components, organic solvents used and temperature employed. For optimum separation each of these variables should be considered individually and in relation to each other. The molecules with the lowest net charge at the selected pH will be eluted first from the column as ionic strength increases. In a similar way the molecules with the highest charge at a certain pH will be most strongly retained. These will be eluted last. The higher the net charge of the molecule, higher is the ionic strength, as discussed earlier. Therefore, by controlling changes in ionic strength which can be done by using different gradient levels, molecules of interest are eluted differentially in a purified and concentrated form. In ion exchange chromatography, maintenance of pH is is very crucial as ionic interactions occurring during purification process vary greatly with change in pH. The buffer components themselves are ionic and may be taking part in the ion exchange process. An ideal buffer used in ion exchange chromatography must be within ± 0.5 pH units of the desired value of pH. In situations where there are chances of change in pH during the experiment, then the pH of the buffer should be adjusted to that side of pKa which will minimize the change.

Salts

During elution in ion exchange chromatography usually a non-buffering salt, usually NaCl, is added to the buffer for elution of biomolecules such as proteins from the matrix. The counterions of salt displace the sample ions from the matrix. In general, the divalent ions are stronger

displacers than the monovalent species. If we compare ions from the same periodic group it has been observed that the smaller ions have higher elution strength than the larger ions.

Detergents

Detergents are usually used as additives to increase protein solubility during their purification in ion exchange chromatography. An ideal detergent for use in ion exchange chromatography must be neutral or carry the same charge as the ion exchange support. The detergent must not contain salts. Before using detergents for ion exchange chromatography the column must be equilibrated with the buffer containing detergent so as to obtain reproducible results.

Zwitterions

Zwitterions are used to decrease protein aggregation during the purification process. They are used to elute samples closely to their isoelectric points. The commonly used zwitterions are betaine and taurine. Researchers have reported that the solubilities of protein samples at low salt concentrations are improved by the addition of zwitterions such as betaine or taurine to the buffer solutions.

Temperature

The temperature plays an important role in ion exchange chromatography. The pKa of a buffer varies with the change in temperature. Therefore, it is very important to use freshly prepared buffers at the temperature of their preparation. For example the buffering capacity of Tris buffer i.e. (tris[hydroxymethyl]aminomethane) fluctuates with change in temperature

TYPES OF ANALYSIS

Reactive biological substances can be fractionated by the method of ion exchange and has been considered as one of the most suitable method for them. There is a major role of ion exchange chromatography, from the introduction of the technique to the development of modern high performance media, in the separation and purification of biomolecules and it has contributed a lot to understand many biological processes.

IEC can be used for many types of analysis. Qualitative analysis of cations, anions,halides etc. Quantitative analysis of cations, anions,halides etc.

Trace analysis and ultra trace analysis of heavy metals etc.

Ion exchange can be combined with other advanced techniques which work on different aspects

and parameters such as size (known as gel filtration), hydrophobic property (known

as hydrophobic interaction chromatography or RPC) or biological activity (known as affinity chromatography), etc.

APPLICATIONS OF ION EXCHANGE CHROMATOGRAPHY

ENVIORNMENTAL ANALYSIS

Ion exchange chromatography is strongly entrenchedtechnique for identification of inorganic anions in environmental samples. Earlier, stages of growth in IEC was considered as apparatus for the determination of elementaryinorganic species category, evaluation of inorganic cations and anions in environmental samples viz. soil extracts, air- filter extracts, natural water samples and drinking water. As the scope of solutes that could be resolved by the IEC so did the utilization areas in which the approach was applied.

Ion exchange chromatography is widely used in the area for environmental sample analysis and a number of samples can be analyzed. It has a primary role in analysis of inorganic anions. The alternative methods are lacking due to non-availability of appropriate techniques. As a result concurrent analysis of inorganic anions in consumed water and waste water remains the necessary applications of IEC.

- Softening Hardness of water
- Demineralization of water
- Removal of interfering radicals

It plays a major role in softening the hard water owing to the presence of Calcium (Ca), Magnesium (Mg) and other divalent ions. The cation exchanger charged with sodium (Na) ions and water is passed through it. Calcium& Magnesium ions are retained in the column while sodium is exchanged. Complete demineralization of water is carried out by removing both cations and anions. The steps consists of passing water through basic anion exchanger – Chloride (Cl-),Nitrite (No2-), Sulphate (SO4-) is exchanged by OH- ions and in another step water is passed through acidic cation exchanger – Calcium (Ca), Magnesium (Mg) &sodium(Na)ions are exchanged by Hydrogen (H+) ions.

One of the significant examples of this technique is theabsolute cyanide evaluation by IEC. The Cyanide is complicit to metals such as iron and for liberatingcyanide,substantial digestion of

acid in the existence of magnesium chloride catalyst is the requisite to the process. The hydrocyanic acid thus resulted is evacuated by reflux distillation from the digestion matrix and absorbed by NaOH solution which can be investigated for cyanide utilizing Ion exchange chromatography with ampere metric detection. Similar technique can be utilized for the analysis of fluoride & sulfide in solid samples. The samples which are to be evaluated for cations utilizing IEC technique are prepared by acid digestion. Absolute Nitrogen as the ammonium ion for instance, has been investigated in environmental samples utilizing IEC by direct evaluation of the Kjedahl digest matrix. Subsequently, rare earth metals and earth metals are evaluated utilizing post column reaction.

Safe drinking water and waste water analysis are ensured by this integral technique of Ion exchange chromatography. The ingestion of inorganic ions viz. fluorides, nitrates and nitrites are fatal for the health of infants. The reduction of ill health factors from the intake of these anions is requisite demand of human body. As a result,in drinking water these anions can be determinedfollowing the approach of IEC.

Acid rain and rain water analysis are important applications of Ion exchange chromatography. The conviction of ionic constituents in rain water is periodically used to evaluate the acidificationeffects on urban and natural environments caused due to acid rain. The method to determine both cations and anions in atmospheric aerosols and rain water has been developed by the researchers.

Ion exchange chromatography has been used to estimate the terrestrial waters and ice cores from intact environments of Antarctica measuring the extent of impurities.The estimation of inorganic solutes, metal species from surface water bodies and ability of quantification of distinct oxidation states viz. Fe (II) or Fe (III), stable complexes of metals clarifies the potentiality of the IEC technique.

The determination of inorganic ions in soil is the vital ability to analyze the toxicity of metal ions in soil, solid waste and sludge. There is a great potentiality of IEC to quantify various stable metal complexes and metal oxidation states in extracts of soil for determination of the toxicity, fate and transport of metals in soil.

Determination of phosphorus, sulfur, nitrogen and their analogous oxide anions viz. phosphate sulfate and nitrate emphasizes in soil fertility and its conditioning.

PHARMACEUTICAL INDUSTRY

Ion exchange chromatography has as essential role in pharmaceutical industries for the evaluation of integrity of medical mixing, determination of selected constituents and in control production of drugs.

IEC is well established process that ensures the drug has been manufactured as per sanctioned procedures and also certifies cleaning of the residues from the equipment utilized during the process of manufacturing.

Some of the examples of the use of Ion exchange chromatography in pharmaceuticals:

- Separates the cations in water solution for injectibles.
- Analyzing final drug formulation.
- IEC provides simple approach manufacturing process of drugs.
- IEC provides the easiest approach for the segregation of anions in decongestant and analgesic drugs.

IEC has been occupied its place in drugs in the form of capsules and tablets in order to evaluate the content of drug dissolve with time period. It also detects and quantifies the inactive ingredients utilized in medicine formulations. It has made the detection of impurities or constituents that are not chemical entity easy. It provides the insights about the contents of drugs that should be given to the patient per day.

IEC is entrenched approach for the investigation of charged species viz. vigorous acid anions, sulfate chloride, fluoride, phosphate etc. This technique is suitable for the separation of charged particles of weak nature viz. carboxylic acids. The uncharged species are separated on the basis of three mechanisms viz. Donnan exclusion, Adsorption and steric exclusion/segregation process. The charge species are passed through the column unrefined.

Manufacturing process of drugs utilizes various excipients viz. citrate buffers or phosphates in incogitables, fillers viz. calcium phosphates & calcium sulfates. Ion exchange chromatography process provides a simple method for these manufacturing processes of drugs.

BIOACTIVE NATURAL PRODUCTS

These products are the chief source of functional foods, food additives and new types of drugs.

They consist of secondary metabolites viz. alkaloids, sterols, flavonoids, sterols etcof animals and plants generated through biological processes. IEC is one of the most often utilized techniques for the separation natural products in pure form. It is the core fundamental and efficacious technique as it is inexpensive and offers high recoveries of key analytes.

1. The applications of IEC are as stated under:
2. The cation exchange column is utilized to segregate thiamine from riboflavin.
3. IEC is broadly used for the separation of biological large molecules viz. proteins, polysaccharides and proteins.
4. It has the potentiality to extract and isolate mycotoxins produced by the fungus viz.
5. Aspergillus niger.
6. Recovery of basic and acidic compounds from plant extracts utilizing solid phase reagents.
7. The classic application is the extraction of noxious indolizidine alkaloid, swainsonine from plant constituents.
8. Ion exchange chromatography is utilized for the extensive extraction of natural products from limonin, glucosides and agriculture source.
9. It results in valuable recovery yield from citrus molasses.

CLINICAL ANALYSIS

Ion exchange chromatography provides well established applications in clinical perspectives. It provides a huge range for the prospects of selection in mobile and stationary phases. It aids in solving distinct separation problems when combined with various detection techniques. It can be utilized to evaluate various ions and substances in the pharmaceuticals and clinical aspects. It contributes the availability of great extent stationary phases and precise detectors. IEC has marked its presence in clinical aspects due to various reasons:

- Elementary sample preparation.
- Reduced sample volumes.
- Prevention of hazardous chemicals.
- Flexibility in utilizing reaction options so on variation of sample matrix to be investigated.

IEC provides aid in determining various inorganic and organic constituents used in pharmaceutical and clinical samples. Ion exchange chromatography methodology provides the maximal rates of diagnosis and permits the diagnosis of aminoacidopathies as compared to other

techniques viz. thin layer chromatography declined to detect as the concentration of few amino acids was very little. IEC has considerably improved diagnostic rate in clinical perspectives to a large extent.

COSMETICS

The accuracy, subtlety and suitability for the investigation of organic acids have remarked the existence of Ion exchange chromatography in cosmetic industry. The detection of organic acids viz. formic acid, glycolic acid, lactic acid, citric acid, tartaric acid etc in cosmetic samples by ion exchange chromatography methodology has abolished conductivity detection.

One of the significant example is the determination of thioglycolic acid in cosmetic samples by IEC is remarkable in suppressing conductivity detection.

Ion exchange chromatography benefits whenever the analytical problem consist of elimination or isolation of an ionic compound Ion exchange can be used for the selective desorption of individual anions or cations in the mixtures of ion. One of the leading feature of this technology is the constituents of shampoo are deserted in an unaltered chemical form. IEC has well established its utilization in herbal and ayurvedic cosmetics.

IEC has been used for the segregation of distinct ionic or the mixture of non ionic and ionic compounds such as surface active agents. It is highly sensitive technique for the detection and evaluation of nitrosoamines in our environment. NDELA (N Nitrosodiethanol amine) in cosmetics is the impurity in samples of cosmetics. It is adsorbed by strong basic anion exchange resin. IEC facilitates the clean up process of cosmetics by recovery of various other compounds utilized in formulations of cosmetics.

SCIENTIFIC RESEARCH

Ion exchange chromatography has established the leading role in scientific research area. For instance, the purified form of immunoglobulin G is efficaciously utilized in passive immunization process. IEC proves to be effective in using DEAE Sephadex A50 to yield pure Immunoglobulin G.

With the new advancements in technology, a quantitative research of evolution of amino acids in humans is made easy by Ion exchange chromatography technique.

- Investigation of amino acids in the urine and plasma is one of the remarkable features of

Ion exchange chromatography.

- Determination of amino acids in physiologic fluids by IEC.

IEC has facilitated a lot in the scientific area not only in medical but also in various other fields.

Advantages of Ion exchange chromatography

This approach has been utilized for the evaluation of anions and cations, implicating metal ions, poly hydroxyl compounds, peptides, organic acids, nucleotides, alcohols, aminoglycosides etc. It has been auspiciously applied to the investigation of deteriorated

products, raw materials, impurities; bulk active ingredients etc at various stages of production steps in addition with the evaluation of the cleaning solutions of production apparatus, desolated streams, container compatibility including other utilizations. Ion exchange chromatography has become one of the chief chromatography as compared to other liquid chromatographic approaches:

- Average cost.
- Huge sample handling capability.
- Easy automation as well as scale-up.
- Dynamic resolving ability.
- Extensive applicability in addition with high through put application scheme and broad applicability.
- It can be used at all the phases and proportions of purification.
- It is a non denaturing process.
- It can be maintained by altering the salt concentration, pH and the ion exchange media.
- It provides high selectivity and can resolute molecules with little variations in charge.
- It can serve as intensifying step. A huge volume of diluted sample can be enforced to a media and the absorbed proteins afterwards eluted in petite volume.
- Eco friendly technique as it deals with substances existing in water.
- Longer life of resin.
- Economical maintenance.

Disadvantages of Ion exchange Chromatography

Despite of broad use of IEC the mechanism of segregation has not completely been enlightened. An extensive effort has been made to depict the ion exchange process more theoretically. One of

the major disadvantages of IEC technique is that it presents no direct information on the situation occurring at the stationary phase surface as the ion exchange symmetry is always resolute by the balance between the eluent and solute interaction with the active site resins.

The ion exchange chromatography has many other disadvantages. Binding to ion exchange resins requires electrostatic interactions between the target proteins and stationary phase;

henceion exchange columns are to be packed in low-salt buffers.To carry out some of the applications, exchange step for a buffer is needed prior to ion exchange chromatography.Weak ion exchangers highly depend on pH and these resins rapidly lose capacity, and resolution when working outside of their optimal pH range which is a limitation of ion exchange chromatography

Major disadvantages are depicted as:

- Properties and nature of ion exchange resins.
- Chemicals & equipments are expensive.
- Turbidity should be maintained below 10ppm.
- Nature of ions exchanged.
- Substances viz. organic matter or resins can filthy effect to resin.

SIZE EXCLUSION CHROMATOGRAPHY (SEC)

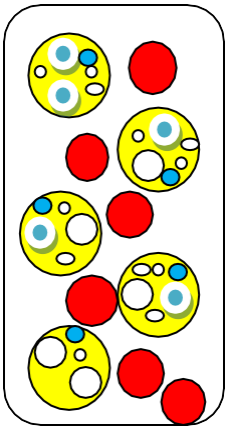
1. Description

Size exclusion chromatography has various applications like semi-preparative purifications and analysis. It separates the molecules on the basis of the difference in their size. In this technique, molecules do not bind the stationary phase instead the molecules percolate as per their size and shape. Moreover, use of buffer, adjustment of pH, and amounts of metal ions or cofactors is not necessary for the resolution in SEC. This technique avoids all the harsh conditions, therefore is usually used for biomolecules. The separation of biomolecules in aqueous systems by SEC is referred to as gel filtration chromatography (GFC), while the separation of organic polymers in non-aqueous systems is called gel permeation chromatography (GPC).

Principle

Size exclusion chromatography involves separation of components of a mixture based on their molecular size. The components separate due to their differential exclusion when they pass through stationary phase having different pore sizes in the cross linked polymeric gels or beads. The components elute from the stationary phase with different permeation rates. It involves moderate interaction with the components thus enabling high retention of

biomolecular activity.



In SEC, the column filled with gel particles or porous stationary phase is equilibrated with mobile phase. The introduction of sample percolates the components with the mobile phase. The large molecules do not enter into the pores and are excluded through the vacant space not occupied by the particles. The smaller molecules are distributed in the mobile phase and the porous molecular sieves. This results in the fast movement of the larger particles as compared to the smaller particles.

The exclusion and inclusion of particles depends upon the size range chosen during the experiment. As per the parameters selected, all the components having size equal or larger than the critical mass behave identically and are excluded from the column. These components are eluted as 'excluded volume' of the column. The particles having size lower than the critical mass are captured by the poresand are eluted as 'included volume'.

2. Mechanism of size exclusion chromatography

There are two possible mechanisms on the basis of which separation takes place. In SEC, the components are distributed within two liquid phases; 1) liquid present in the porous gel, 2) liquid present outside the gel. The distribution follows Steric exclusion mechanism, small components can enter in smaller and larger pores both, however, the larger components enter only in larger pores. Therefore, the different pore fractions are available to the molecules of different sizes. This means the distribution coefficients of components with different sizes differ in two liquid phases. The total volume (V_t) of the column packed with a gel that swell by the solvent is given by;

$$V_t = V_g + V_l + V_o$$

Where V_g is the volume occupied by the solid matrix of the gel V_i is the volume of the solvent held in the pores or interstices V_o is the free volume inside the gel particles

The diffusion equilibrium and the retention time of the component is given by

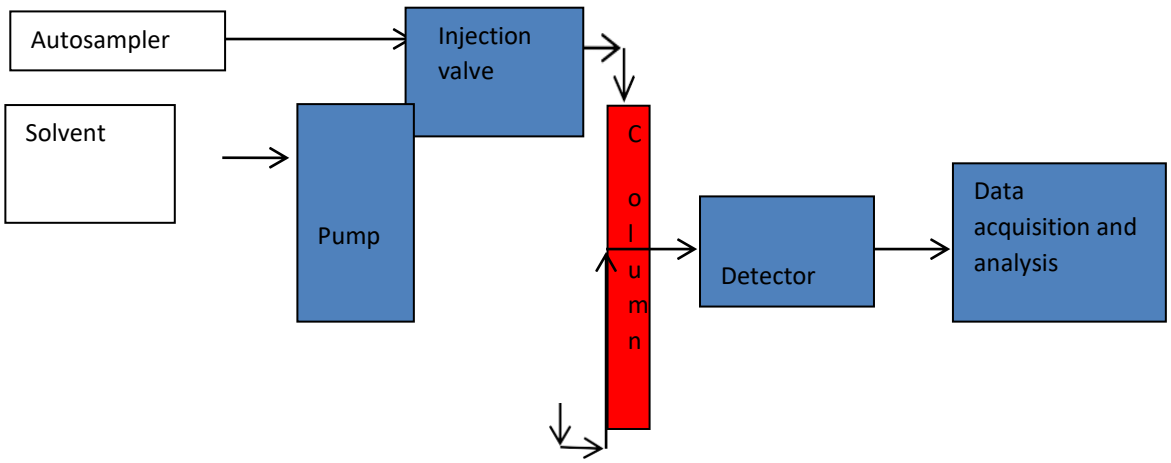
$$VR = V(int.) + KdV(int.) \quad Kd = Vi(acc)/V(total)$$

where Vi(acc) is the accessible pore volume V(total) is the total pore volume V(int.) is the interstitial volume

In secondary exclusion mechanism, the small molecules diffuse rapidly into the pores of the gel while larger molecules compete for vacant sites. The larger components move down and find relatively more unoccupied sites downwards. This result in the separation of smaller components collected at theupper end and larger components collected at the lower end

Instrumentation of SEC

The instrument of SEC consists of same components as other chromatographic techniques. The block diagram of the instrumentation is given below.



Stationary phase

The stationary phase used in SEC is semi-permeable cross linked polymer with porous nature. It has well defined pores and the size of pores is controlled during cross linking. The stationary phases with smaller pore size are used for the purification or desalting of proteins. The medium size is used to separate relatively small proteins and large sized pores are used for biological samples. Usually, dextran (Sephadex^{TM}) polyacrylamide and dextranpolyacrylamide (Sephacryl^{TM}) are used for SEC. These are available in different pore sizes and are chosen on the basis of the size of the macromolecules to be separated. The main features of a stationary phase include inertness, inexpensiveness, mechanical stability, uniform size and shape and non-reactive towards various components of the sample. Some of the good stationary phases commonly used in various applicationsare discussed below.

Dextran: This gel is homopolysaccharide of glucose residues and can be prepared by varying the cross-linking to control the pore size. It is available as dry beads under the trade name Sephadex, which swell in the presence of water. Mainly, it is used for the separation of small peptides and globular proteins with small to average molecular mass.

Polyacrylamide:

It is also a gel and can be prepared by cross-linking N,N-methylenebisacrylamide. Its separation tendency is same as dextran and is available in various sizes. It is available as bio-gel P.

Agarose:

It is a gel composed of linear polymer of D-galactose and 3,6-anhydro-1-glalactose. It shows dual nature i.e. dissolved in boiling water and is converted to gel in cold water. Its pore size is larger as compared to the gels discussed before. Therefore, it is used for the separation of globular proteins and long molecules of DNA.

Mobile phase

The mobile phase is selected on the basis of the nature of components to be separated. Most commonly THF, chloroform, toluene, dimethyl formamide and mixed solvents are used to separate various components. In case of proteins and polysaccharides, aqueous buffers are used as mobile phase.The solvent should be able to dissolve the components of a mixture and must be inert towards various components. The detectable properties of the solvent must be different from the sample components. It should not be corrosive. Before using a mobile phase for the analysis, high purity solvent is passed through a filter paper of size 0.5 micron to remove the dust, insoluble salts etc.

Preparation of sample

A sample is dissolved in an appropriate solvent to get its dilute solution usually less than 1mg/mL. Sometimes higher concentrations are required for the samples with diverse molecular weight of components. The samples are filtered before loading into the instrument to prevent the clogging.

Columns

The columns of various lengths and diameters are available commercially. However, small columns are recommended to save the time and solvent. The particle size is selected depending upon the analyte. Generally particles with 5mm size are selected but small sized particles are

more sensitive towards contamination. For high molecular weight, large particle size is recommended because small size of particles may damage the large sized analytes. The porosity of the column is also responsible

for the separation of the components. Before operation, a column is saturated with the mobile phase and is handled with care.

Pump

The pumps are used to maintain the constant flow rate during the analysis. A change in the flow rate may cause error in the results. The in-line filters prevent the entry of particles and save the valves and pump deals from damage. These are of two types; syringe pump and reciprocating pump.

Detector

The detectors used with SEC are either concentration sensitive detectors like refractive index detectors, UV-Vis detectors and evaporative light scattering detectors, or molar mass sensitive detectors like low angle light scattering detectors, multiangle light scattering detectors and viscosity detectors.

3. Applications of SEC

1. Purification
2. Desalting
3. Protein-ligand binding studies
4. Protein folding studies
5. Concentration of sample
6. Copolymerisation studies
7. Relative molecular mass determination
8. Separation of sugars, peptides, rubbers and others

AFFINITY CHROMATOGRAPHY

1. Introduction

Since the inception of affinity chromatography 50 years ago traditional purification techniques based on pH, ionic strength, or temperature have been replaced by this sophisticated approach. It has been stated that over 60% of all purification techniques involve affinity chromatographyThe wide applicability of this method is based on the fact that any given biomolecule that one wishes to purify usually has an inherent recognition site through which it can be bound by a natural or artificial molecule. Thus, we can say that

affinity chromatography is principally based on the molecular recognition of a target molecule by a molecule bound to a column.

Affinity purification involves 3 main steps:

a. Incubation of a crude sample with the affinity support to allow the target molecule inthe sample to bind to the immobilized ligand.
b. Washing away non-bound sample components from the support.
c. Elution (dissociation and recovery) of the target molecule from the immobilized ligand by altering the buffer conditions so that the binding interaction no longer occurs.

Since the beginning of this technique, the term affinity chromatography has raised many controversies among researchers. Some say it would be more accurate if termed bioaffinity chromatography (O'Carra et al, 1974) or hydrophobic affinity (Shaltiel, 1974). Nonetheless, the term affinity chromatography has been expanded to describe a potential method of separating biomolecule mixtures on the basis of specific biological interactions. Recently, a modern form of liquid chromatography referred to as "flash chromatography" was introduced.

2. Fundamental principles of affinity chromatography

Separation of a desired protein using affinity chromatography relies on the reversible interactions between the protein to be purified and the affinity ligand coupled to chromatographic matrix. As stated earlier, most of the proteins have an inherent recognition site that can be used to select the appropriate affinity ligand. The binding between the protein of interest and the chosen ligand must be both specific and reversible.

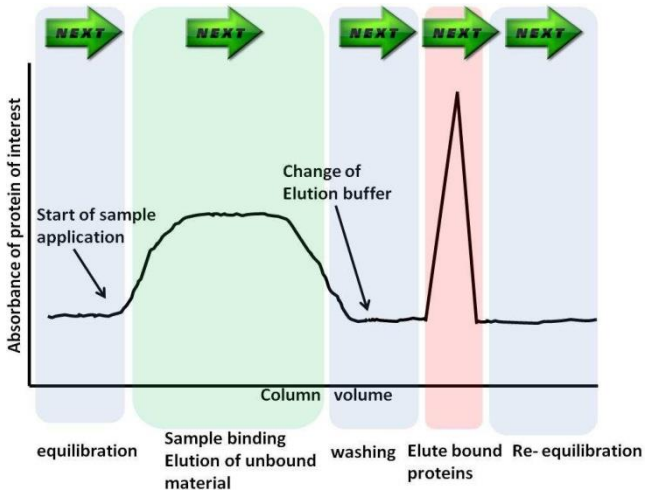


Fig. 1. Typical affinity chromatography purification

A typical affinity purification is shown in Figure 1 and involves several steps. First, samples are applied under conditions that favor maximum binding with the affinity ligand. After sample application, a washing step is applied to remove unbound sustances, leaving the desired (bound) molecule still attached to the affinity support. To release and elute the bound molecules, a desorption step is usually performed either 1) specifically using a competitive ligand or 2) non-specifically by changing the media atmosphere (e.g. changing the ionic strength, pH or polarity) (Zachariou, 2008). As the elution is perfomed, the purified protein can be collected in a concentrated form.

Biomolecules purified by affinity chromatography

Antibodies were first purified using affinity chromatography in 1951 when Campbell et al. used affinity chromatography to isolate rabbit anti-bovine serum albumin antibodies (Campbell et al, 1951). For their purification, bovine serum albumin was used as the affinity ligand on a cellulose support. Two years later, this technique was expanded to purify mushroom tyrosinase using an immobilized inhibitor of the enzyme (azophenol) (Lerman, 1953). Since then, affinity chromatography is commonly used to purify biomolecules such as enzymes, recombinant proteins, antibodies, and other biomolecules.

affinity chromatography based on their polarity and volatility.

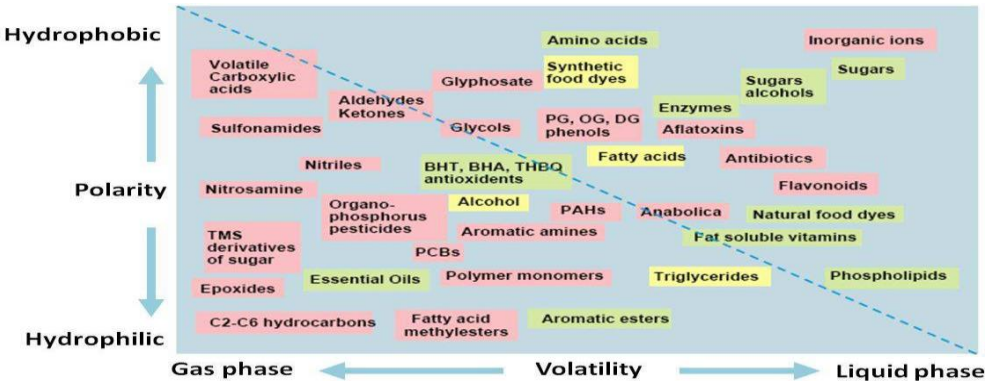


Fig. 2. Illustration showing different molecules that can be purified using affinity chromatography.

Affinity chromatography is often chosen to purify biomolecules due to its excellent specificity, ease of operation, yield and throughput. In addition, affinity chromatography has the ability to remove pathogens, which is necessary if the purified biomolecules are to be used in clinical applications. The purity and recovery of target biomolecules is controlled by the specificity and binding constant of the affinity ligand. In general, the association constants of affinity ligands used for biomolecule purification range from 10^3 – 10^8 M^{-1}

(Janson, J-C, 1984). A common affinity ligand used in these purifications is an antibody, but other affinity ligands such as biomimetic dye-ligands, DNA, proteins and small peptides have been used as well. Figure 2 shows a wide variety of molecules that can be purified by

Components of affinity medium

When affinity chromatography is used for the purification and separation of large biomolecules from complex mixtures, the support (matrix), spacer arms, and ligand must be considered.

Affinity supports (matrix)

Traditionally, affinity chromatography support materials have consisted of porous support materials such as agarose, polymethacrylate, polyacrylamide, cellulose, and silica. All of these support materials are commercially available and come in a range of particle and pore sizes. Some supports may be available with common affinity ligands already immobilized (e.g. protein A, Cibacron Blue, heparin). Other types of support materials are being developed including nonporous supports, membranes, flow-through beads (perfusion media), monolithic supports, and expanded-bed adsorbents.

Nonporous support materials consist of nonporous beads with diameters of 1- 3 μm. These supports allow for fast purifications, but suffer from low surface areas when compared to traditional porous supports. Membranes used in affinity chromatography also lack diffusion pores which limits surface area, but like the nonporous beads allow for fast separations. Flow-through beads or perfusion media (originally developed for ion-exchange chromatography) have both small and large pores present. The addition of the large flow-through pores allows substances to be directly transported to the interior of the particle which means only short distances are required for diffusion. Monolithic supports are based on the same principle as perfusion media – they contain both large flow-through pores and small diffusion pores. Expanded-bed adsorbents were designed to prevent column clogging and utilize a reverse in flow to allow for the expansion of the column bed which allows for particulates to flow freely through the column and prevent column fouling. See Figure 3. More information about expanded-bed chromatography can be found in (Mattiasson, 1999). In this type of chromatography, elution is performed in a normal packed-bed, but during the adsorption-wash step, the flow is reversed and the column bed expanded. This allows for particulate contaminates to pass freely through the column and prevent column clogging.

Regardless of the type of support used in the affinity purification, several factors must be considered when choosing a support material. These include chemical inertness, chemical stability, mechanical stability, pore size, and particle size

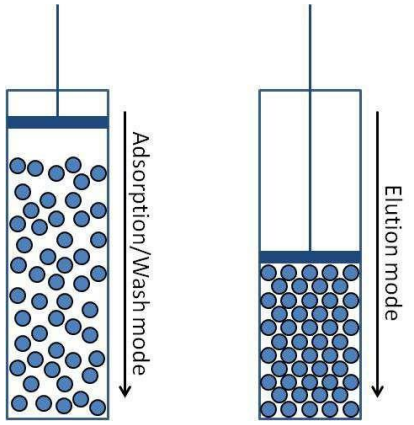


Fig. 3. Expanded-bed chromatography.

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Chemical inertness of the support material requires that the affinity support bind only the molecule of interest and have little or no nonspecific binding. While the specificity is related to the affinity ligand immobilized onto the support, the properties of the support must be chosen to limit the nonspecific binding of other molecules. Supports which have little or no nonspecific binding mimic the properties of the aqueous mobile phase. Therefore, chemically inert support materials are hydrophilic. In addition, most separations are performed in low ionic strength media. As a result, the number of charges on the support should be minimized to prevent nonspecific ionic interactions.

In addition, a support material must be chemically stable under normal operating conditions. This includes resistance to degradation by all enzymes and microbes, elution buffers, regenerating solvents, and cleaning agents that will be used within the column. These stability considerations must also be expanded to the stability of the affinity ligand- matrix linkage. Agarose-based support materials meet all of these requirements as they can be used between pH 3 and 12, are not attacked by enzymes, and are not affected by most aqueous eluants. However, ligand attachment in agarose support materials is often not as stable, depending on the type of linkage used.

Mechanical stability is another consideration when choosing a chromatographic support material for affinity chromatography. Support materials must be able to withstand the backpressures encountered during normal separations without compressing. While most commercial packing materials meet this requirement, the build-up of particulate contaminants may restrict column flow and lead to high backpressures. Under these pressures, soft porous gel supports such as agarose beads will compress and increase the pressure even further causing collapse of the support structure. More mechanically stable

supports (e.g. silica and heavily cross-linked polymers) are able to withstand these high pressures, but the build-up of particulate contaminants should be avoided if at all possible.

Particle size is an additional consideration when choosing a support material. Ideally, small particle sizes are desired to limit mass transfer effects and limit band broadening. In addition, smaller particle sizes tend to offer greater surface area of the support material and allow for a larger number of affinity ligands to be immobilized on the surface of the support. Unfortunately, as particle size is decreased, backpressures are increased. In addition, when using smaller particles, the potential for the build-up of particulate contaminants and column fouling is increased. For this reason, in preparative applications large particles (30 –

100 μm) are often used. An alternative method to avoid the potential build-up of particulates is to use an expanded-bed support material as discussed earlier and seen in Figure 3. Pore size is another item that must be considered when using affinity chromatography since the biomolecules of interest must be able to not only pass through the column but also be able to fully interact with the affinity ligand. Based on the Renkin equation which allows the estimation of the effective diffusion coefficient (Renkin, 1954), the pore diameter should be at least 5 times the diameter of the biomolecule being purified (Gustavsson & Larsson, 2006). Therefore, a typical protein with a 60 Å diameter would need a support with at least a 300 Å

pore size. Often, the optimal pore size takes into account the ability of the affinity ligand to interact with the biomolecule as well as the surface area of the column since increasing pore size leads to a decrease in the surface area which limits the number of affinity ligands which can be immobilized to the support material.

Spacer arms

Due to the fact that binding sites of the target molecule are sometimes deeply located and difficult to access due to steric hindrance, a spacer arm is often incorporated between the matrix and ligand to facilitate efficient binding and create a more effective and better binding environment. See Figure 4.

The length of these spacer arms is critical. Too short or too long arms may lead to failure of binding or even non-specific binding. In general, the spacer arms are used when coupling molecules less than 1000 Da.

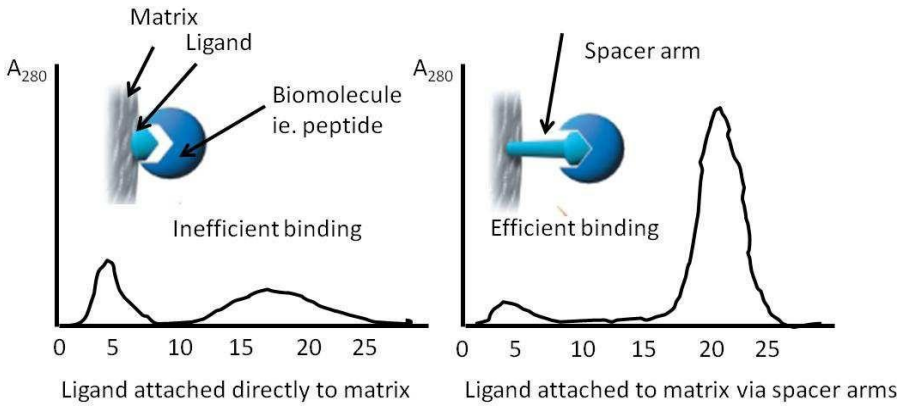

Fig. 4. Chromatogram showing better ligation and elution when spacer arms are introduced between the ligand and matrix

Ligands used in affinity chromatography

Antibodies have several advantages including their high specificity and relatively large binding constants. Antibodies or immunoglobulins are a type of glycoprotein produced when a body's immune system responds to a foreign agent or antigen. Due to the variability of the amino acid sequence in the antibody binding sites (F_{ab} regions shown in Figure 5), it has been estimated that antibodies can be produced for millions or even billions of different foreign agents.

Antibodies which are produced by separate cell lines are referred to as polyclonal antibodies. Monoclonal antibodies are produced when a single antibody producing cell is combined with a carcinoma cell to create a hybridoma which can be grown in a cell culture. Monoclonal antibodies are often more desirable than polyclonal antibodies in affinity chromatography due to their lack of variability which allows for the creation of a more uniform affinity support.

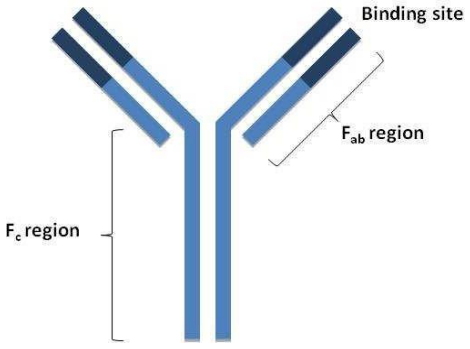

Fig. 5. Typical structure of an antibody.

Applications and uses of affinity chromatography

a. Immunoglobulin purification (antibody immobilization)
b. Recombinant tagged proteins
    i. GST tagged purification
    ii. His-tagged protein purification
c. Protein A, G, and L purification
d. Biotin and biotinylated molecules purification
e. Affinity purification of albumin and macroglobulin contamination

Long answer type Questions (10 Marks)

1. Principle, classification and mechanism of ion exchange process in ion exchange chromatography.
2. Define and classify ion exchange resins and explain the manufacture of cation exchange resin and anion exchange resin.
3. What are ion exchange resins? Classify and explain the ideal properties of ion exchange resins.
4. Principle, ligands used and applications of affinity chromatography.
5. Discuss the principle, instrumentation and applications of affinity chromatography

Short answer type questions (5Marks)

1. Give a detail account on ion exchange chromatography.
2. What is the principle of ion exchange chromatography? Write an account on various ion exchange based stationery phase and its applications.
3. Write a short note on ion exchange chromatography.
4. Write the principle and mechanism of Ion Exchange Chromatography.
5. Define and classify Ion Exchange resins. Add a note on factors affecting Ion exchange.
6. Write the theory & applications of Affinity Chromatography.
7. Write a note on affinity chromatography with special emphasis on ligands used.

Very Short answer type questions (2 Marks)

1. Write the properties of ideal Ion exchange resins.
2. What is regeneration of ion exchange resins
3. Why the buffers are used in ion exchange chromatography.
4. Mention the various factors affecting ion exchange chromatography
5. Define Guard column and its significance.
6. What is affinity chromatography?
7. Enumerate the applications of affinity chromatography
8. What is the principle involved affinity chromatography?