Proteins and Gel-Based Proteomics
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Mod 02 Lecture Number 5

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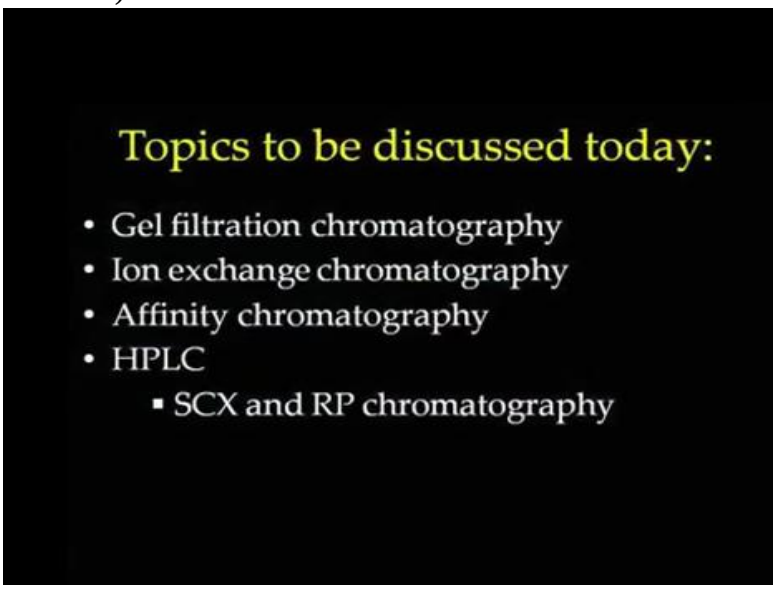

Welcome to the Proteomics course. Today we will talk about protein purification and peptide isolation using chromatography methods. The chromatography techniques have always been considered prominent for the separation of enzymes and proteins.

Protein purification by conventional chromatography is usually achieved by combining chromatography methods such as gel filtration, ion exchange and affinity chromatography. From complex proteome it is challenging to purify a protein in a single chromatographic step. Therefore sequential prefractionation steps involving different modes and types of chromatography methods are becoming necessary for proteome level analysis.

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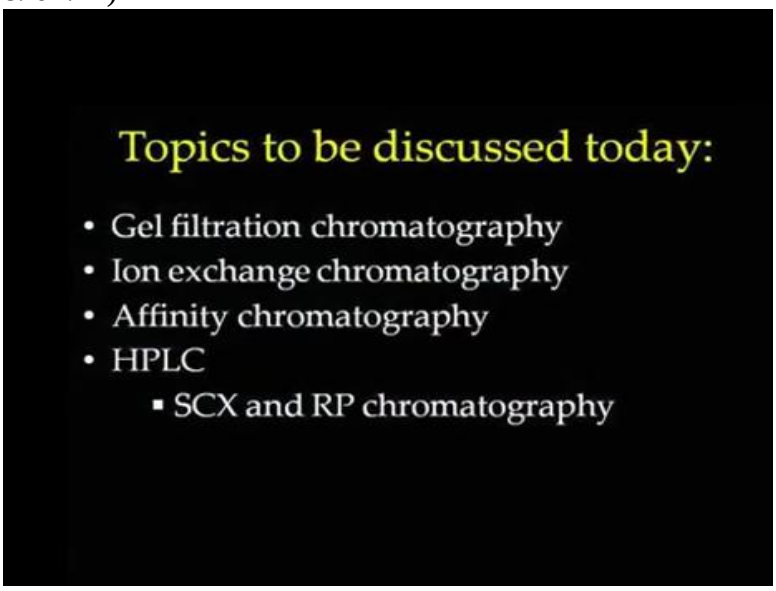

The lecture outline, we will talk about gel filtration chromatography, ion exchange chromatography, affinity chromatography. These methods will be discussed in light of protein purification and then we will talk about few methods such as strong cation exchange and reversed phase chromatography for peptide isolation using high performance liquid chromatography methods.

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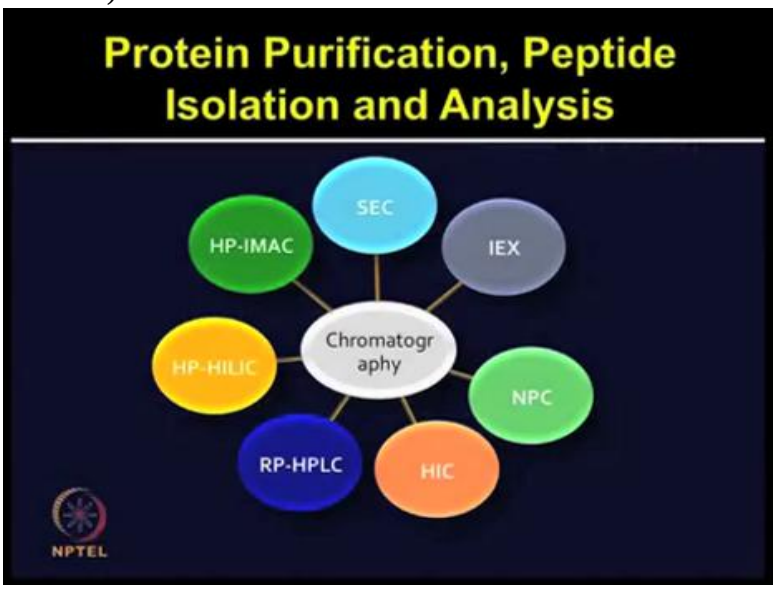

Protein purification, peptide isolation and analysis: It can be performed by many methods but there are certain chromatography methods which are commonly used such as size exclusion chromatography SEC, ion exchange chromatography IEX, immobilized metal ion chromatography IMAC, normal phase chromatography NPC, reversed phase HPLC or RP-

HPLC, hydrophobic interaction chromatography HIC and hydrophilic interaction chromatography HILIC.

These are only few chromatography methods but there are many more which are also used for different types of applications.

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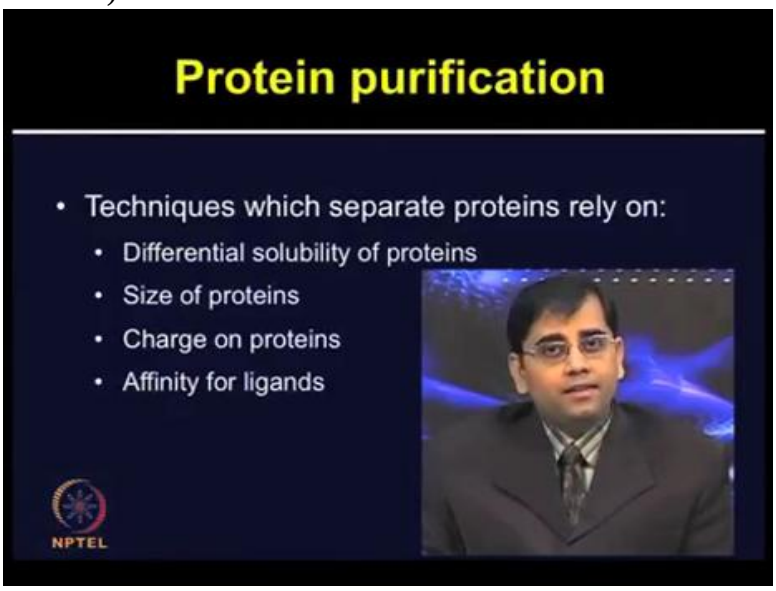

So if your aim is to purify the proteins, there are various techniques which can separate proteins and they rely on different type of principles such as differential solubility of the proteins, size of protein, charge on the given protein and affinity for various ligands.

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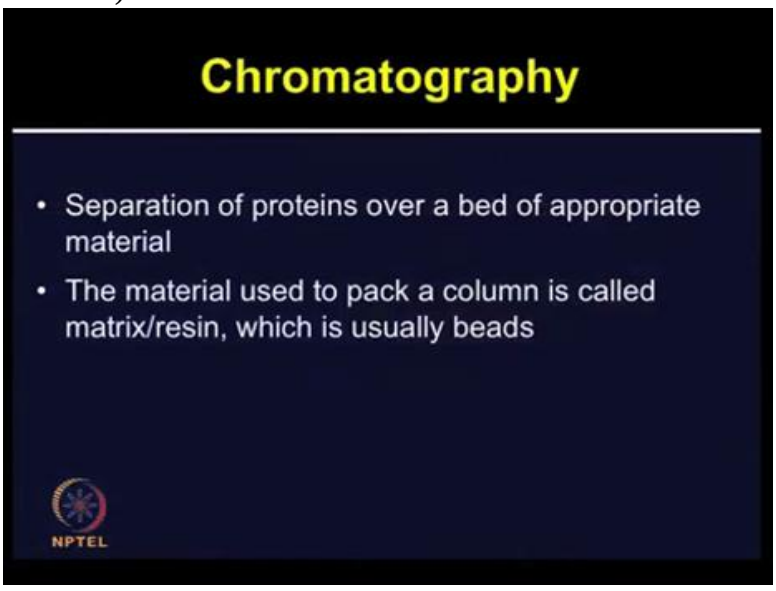

So let's talk about what is chromatography? So, chromatographic methods involve separation of proteins over a bed of appropriate materials. These materials are usually packed in a given column and known as matrix or resin. So these matrix and resins, they are usually beads with or without attached chemical groups. Now the binding and interactions of proteins with the column matrix is an important feature of chromatography.

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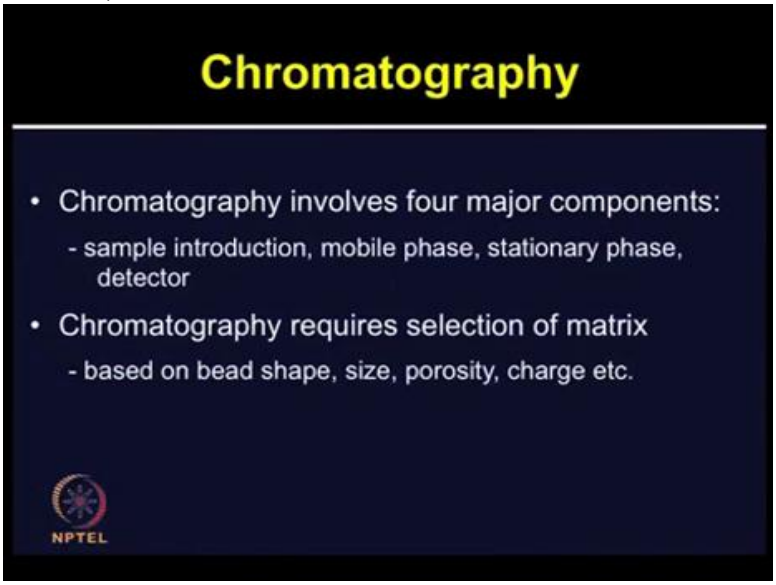

The chromatographic technique involves 4 major components; an inlet for sample introduction, the mobile phase, stationary phase and a detector. Each type of chromatography requires very educated and informed choice of matrix considering the bead shape, size and porosity. In addition to the functional group type, the charge, distribution and density as well as elution conditions such as the pH, the ionic strength and gradient shape.

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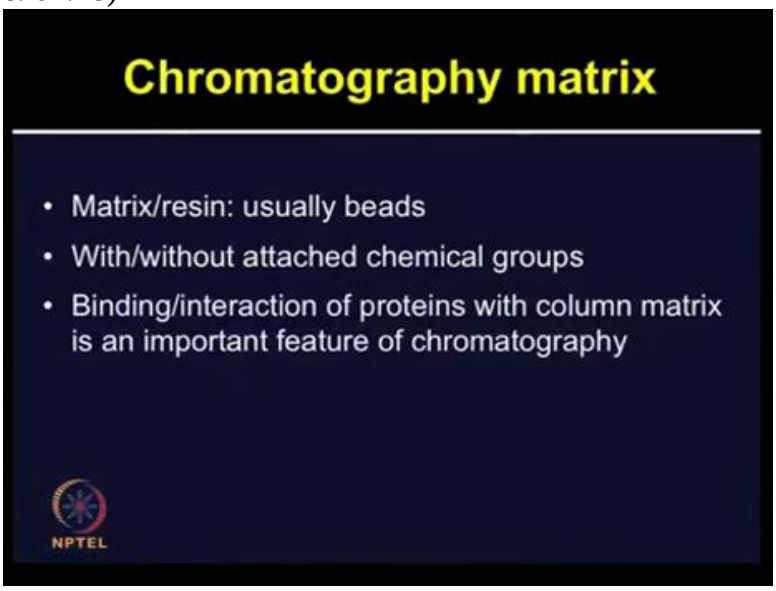

So, as we just talked the chromatography involves the selection of right matrix. These matrix are very important for binding and interaction of proteins and they can determine the kind of achievement which we want to achieve from these types of chromatographic methods.

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So let's move on to more specific chromatography techniques such as gel filtration chromatography. The gel filtration chromatography, it separates proteins on the basis of difference in size. It is used to separate a protein of interest from protein mixture that are larger or smaller in size.

If proteins are having similar sizes, then the gel filtration or the size exclusion chromatography is not an appropriate choice for doing the protein purification. So this

method is also used for various proteomic applications when there is need to remove even contaminants such as salts and low molecular size detergents.

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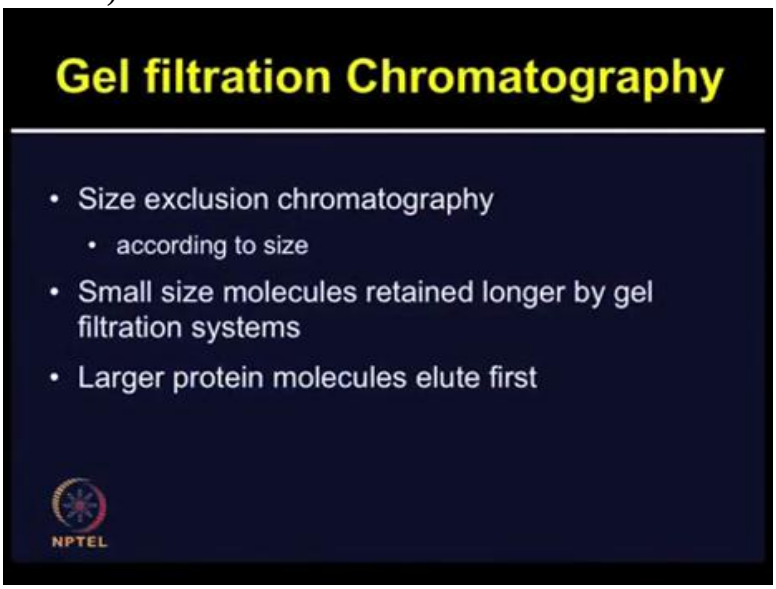

So gel filtration chromatography is also known as size exclusion chromatography. It means the molecules are separated according to the given size. The small molecules, or even if you are separating small molecules such as salt, those will be retained longer by the gel filtration system and larger molecules such as proteins will elute first because they cannot enter inside the porous...these beads and they can be used to separate proteins based on the size.

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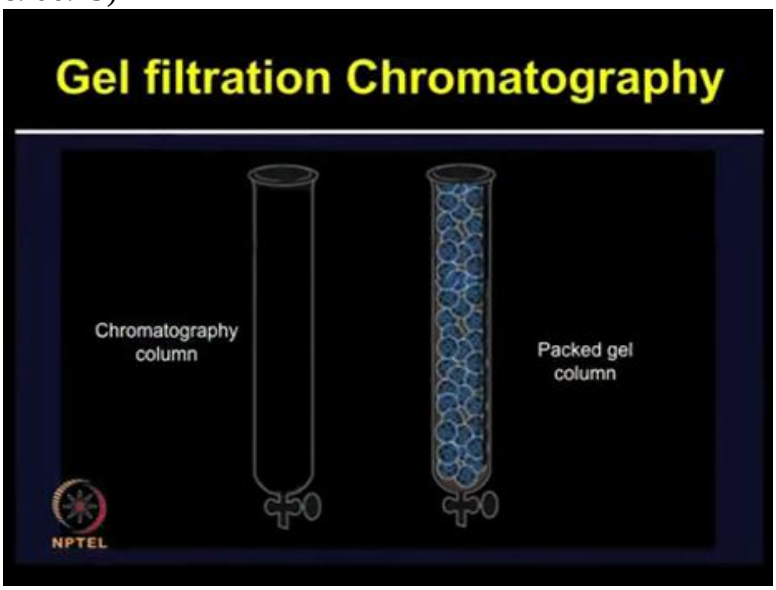

The gel filtration column is composed of porous beads which are made from polyacrylamide, dextran or agarose. Now these columns are packed with hydrated porous gel matrix.

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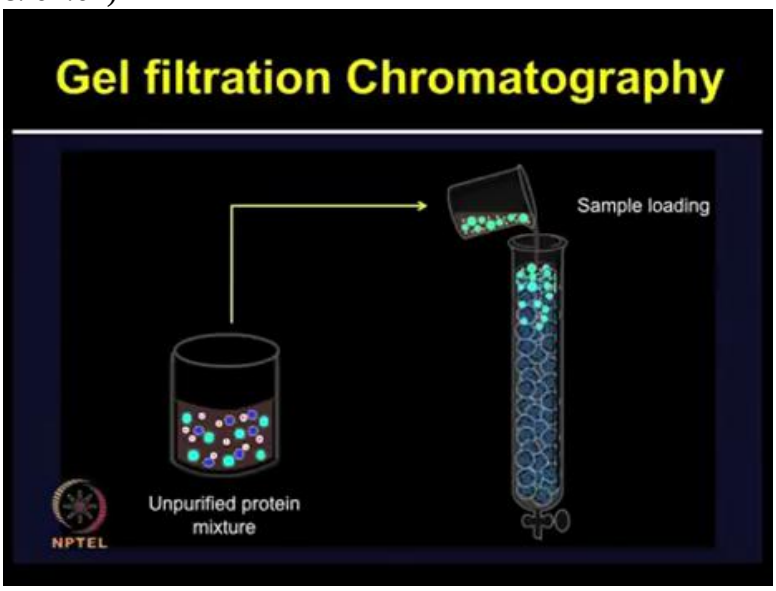

The protein sample which contains a mixture of unpurified proteins of different size is then loaded on these columns.

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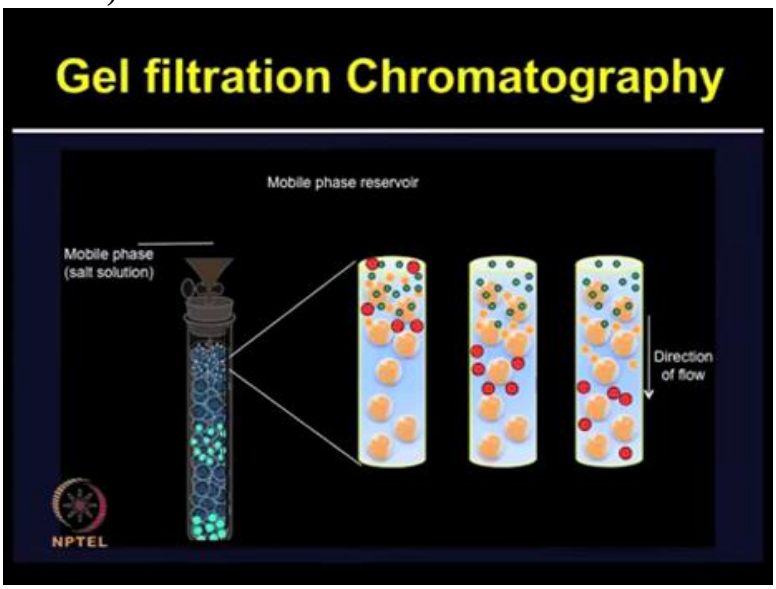

Now when protein sample is applied to the column the small proteins pass from the pores of the beads while the large proteins are excluded. Therefore this technique is also known as molecular exclusion chromatography.

The beads of different pore sizes or increasing retention time by adjusting the increase in column length or decreasing the flow rate can be adjusted to achieve higher resolution of proteins.

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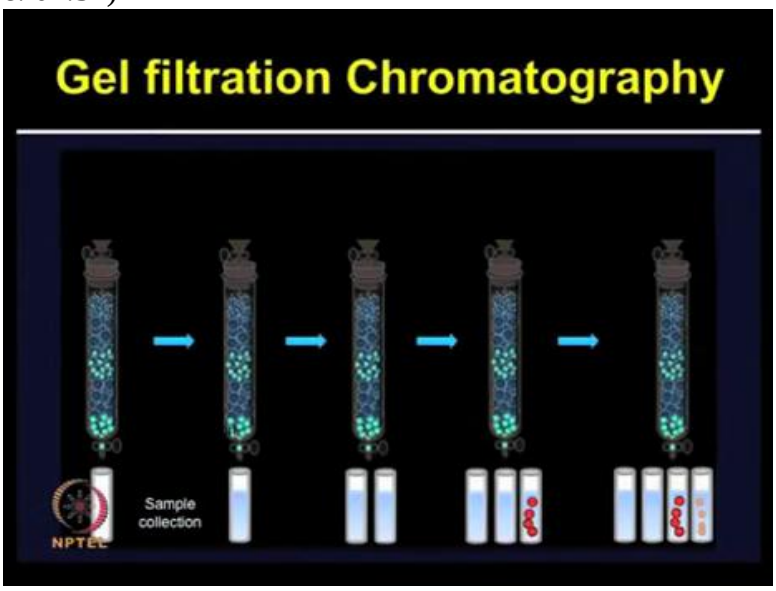

After these steps, the fractions are collected and analyzed for the protein content. So you can expect that larger proteins shall elute first, and then the small molecules will be eluted later. So gel filtration chromatography is useful for removal of even contaminants, doing desalting steps as well as for buffer exchange. However its drawbacks are its low capacity, broad distribution of pore size and small sample volumes to be analyzed.

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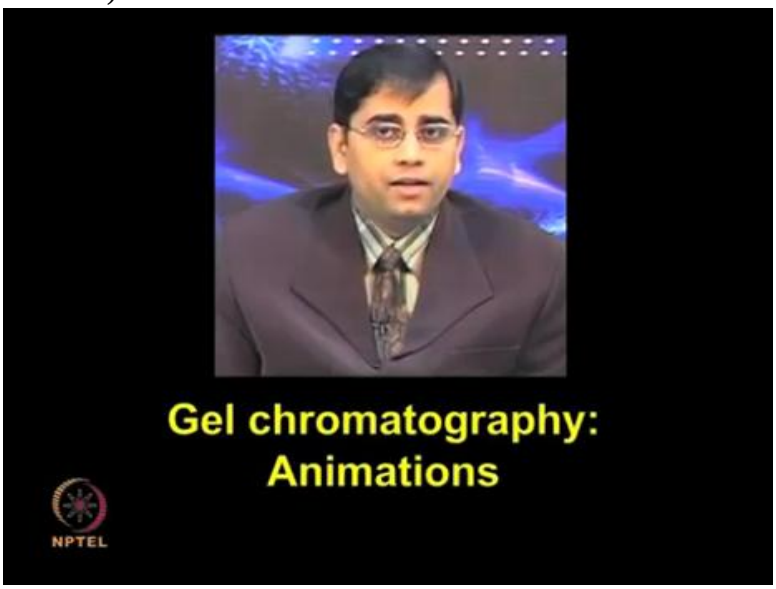

So let me describe the gel chromatography technique in following animation.

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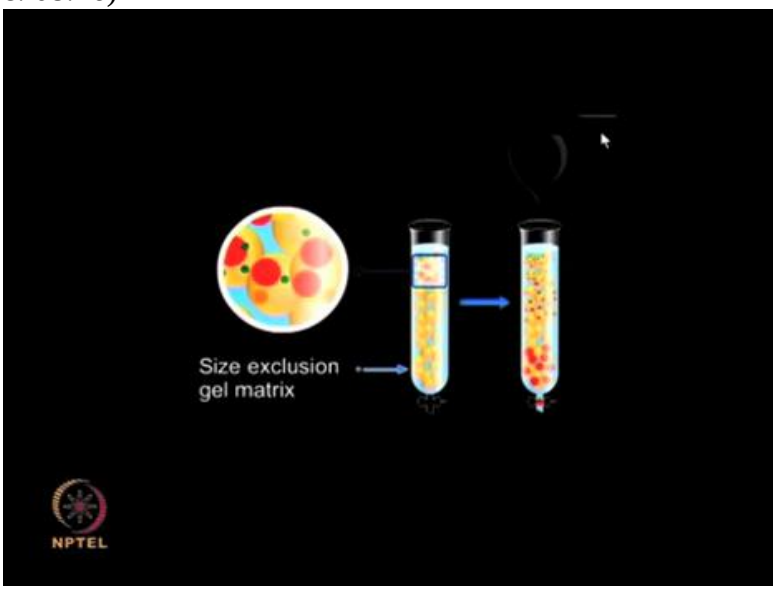

Let’s first give the definition of few components. Size exclusion gel matrix, the matrix filling the gel filtration column consists of a highly hydrated polymeric material commonly dextran, agarose or polyacrylamide.

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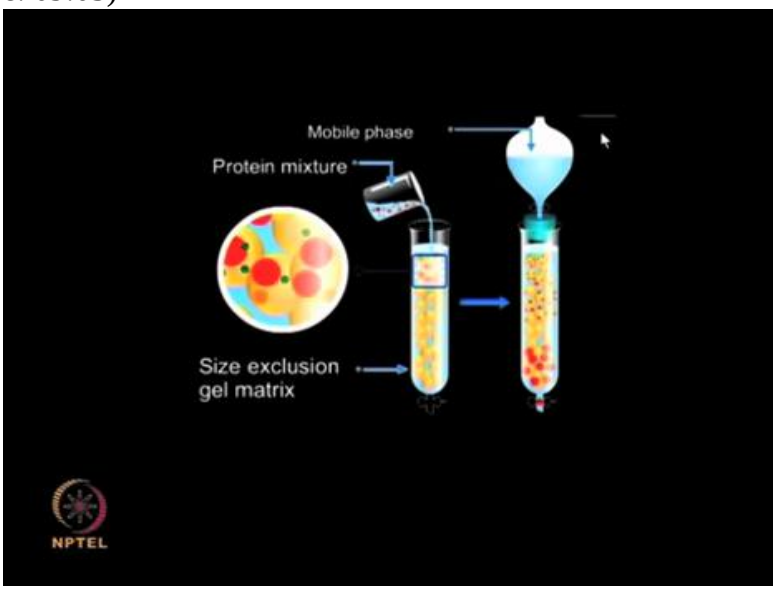

The protein mixture is a mixture of unpurified proteins of different sizes which is applied on the top of the column.

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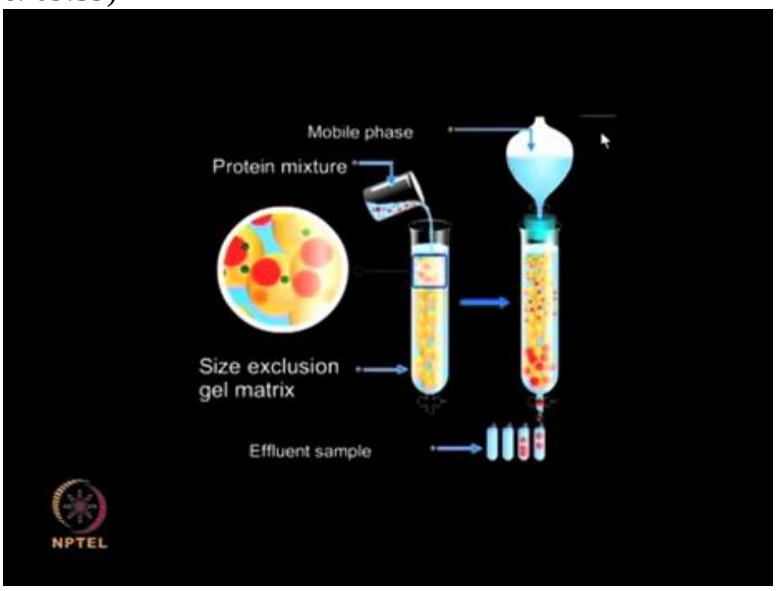

Mobile phase, the proteins are eluted out of the column by using a suitable mobile phase that carries the protein out for elution. For gel filtration, a salt solution of appropriate strength is commonly used so that it will not have any effect on properties on the proteins being purified.

Solvent or buffer system are often used in other types of chromatography. Effluent sample fractions, the solutions leaving the column are collected in suitably sized fractions. Initial fractions will contain only the mobile phase while later fractions will have purified proteins.

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Let me give an analogy of gel filtration with sieve. This process is very similar to separation of small particulate matter from food grains using a sieve. The larger grains remain behind

the sieve while the smaller sand or stone particles pass through them and are removed. In keeping with this, the gel filtration technique is also commonly referred as molecular sieve.

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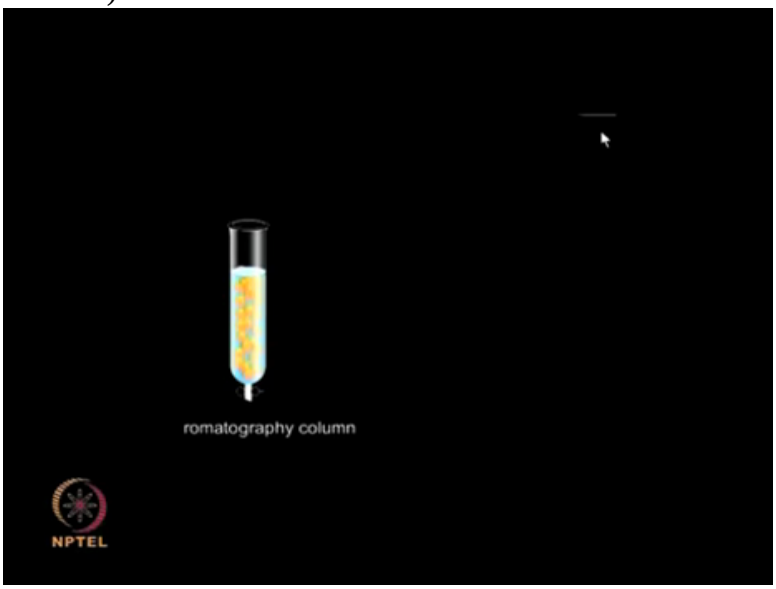

Now let me show you how this process works. So first the cortex which is suitable for required protein separation

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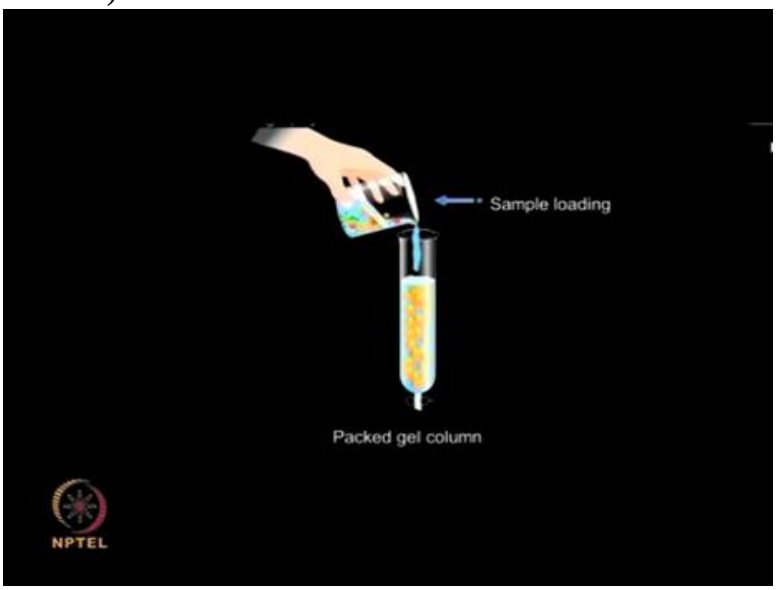

This packed gel matrix is then loaded with the protein sample containing mixture of unpurified proteins of different sizes.

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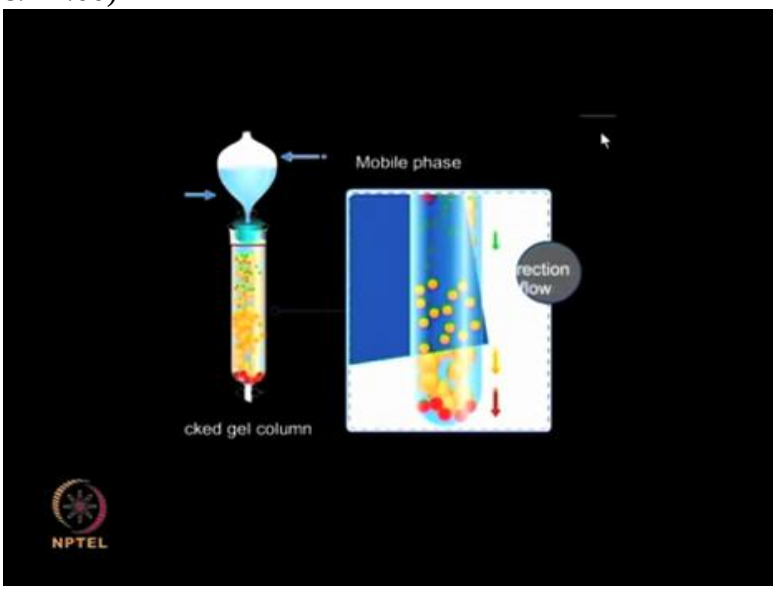

The column is then eluted with a salt solution of appropriate concentration.

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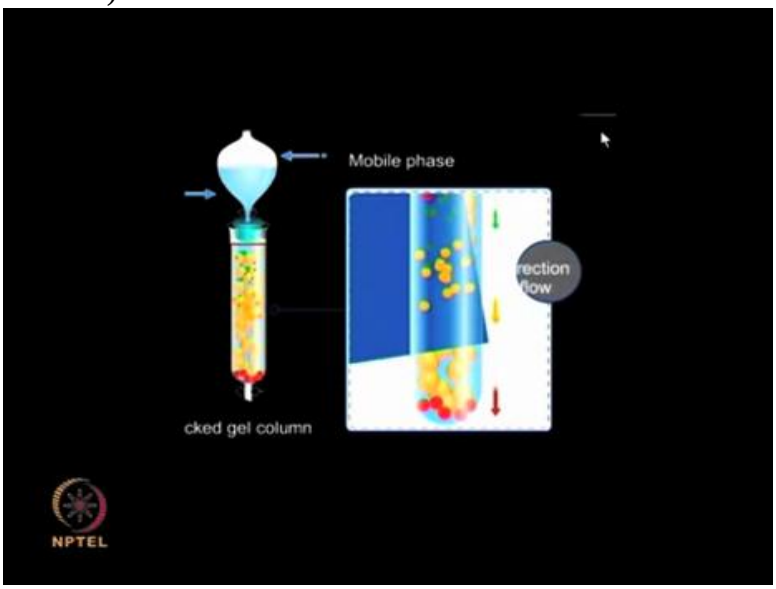

Large proteins that cannot enter the pores of the gel move down through the interstitial spaces at a faster rate and are eluted first. The smaller proteins move in and out of the pores thereby taking longer time to be removed from the column.

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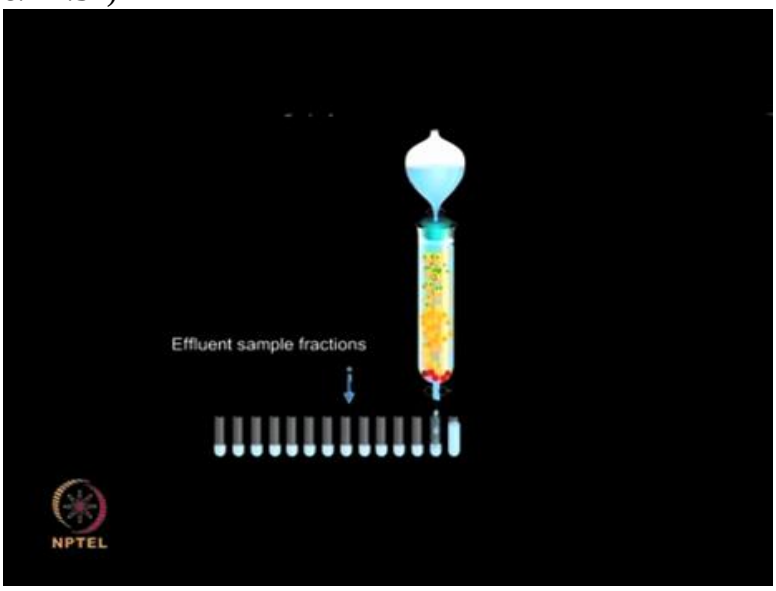

The fractions of appropriate size should be collected and analyzed for their protein content.

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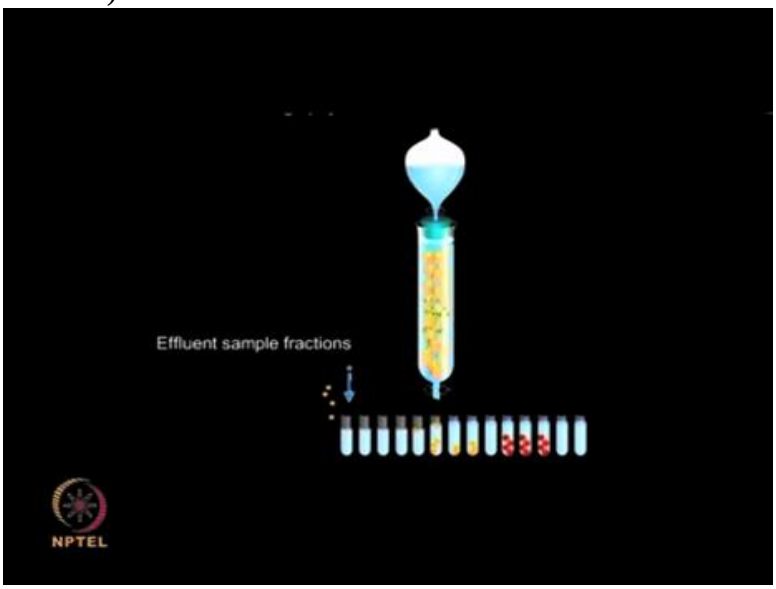

The largest proteins eluting out first will be present in the initial fractions while the smaller fractions which elute out later will be present in the later fractions. Once all these fractions are collected, then it can be analyzed for their protein content by using a UV visible spectrophotometer.

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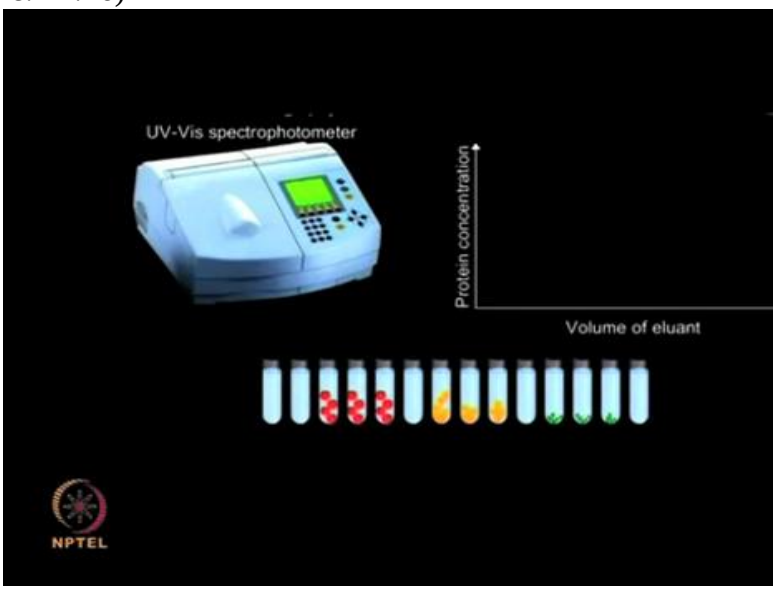

Now analyze the protein content by using UV visible spectrophotometer at 280 nanometers.

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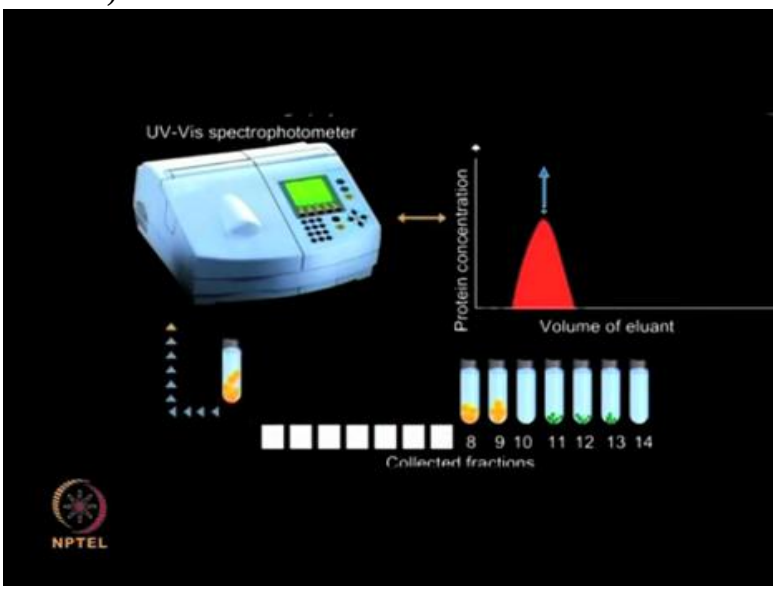

Once all the absorbance values are recorded, a graph of eluent volume versus protein concentration can be plotted.

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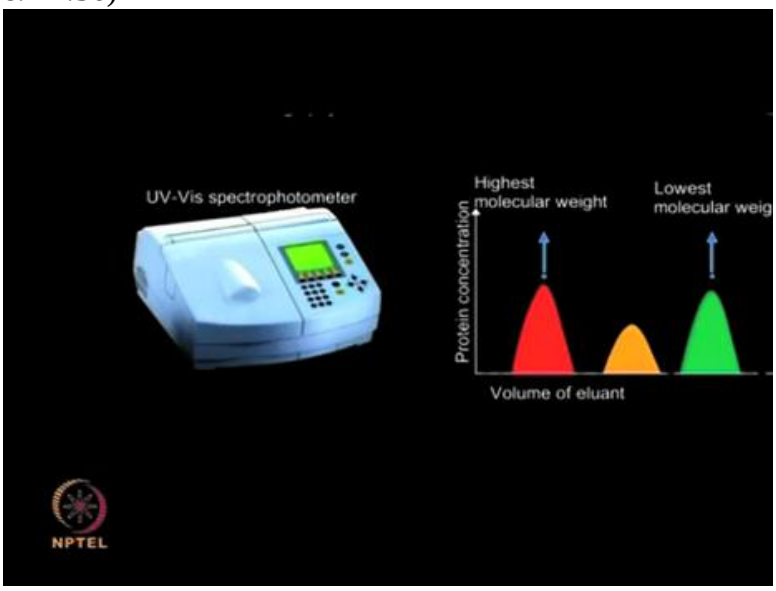

The highest molecular weight is in the beginning and lowest molecular weight towards the end.

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Let's now move on to next chromatography method which is ion exchange chromatography. This is one of the most versatile chromatographic separation method which relies on differences between number of charges and distribution of charged groups in defined pH and solvent conditions.

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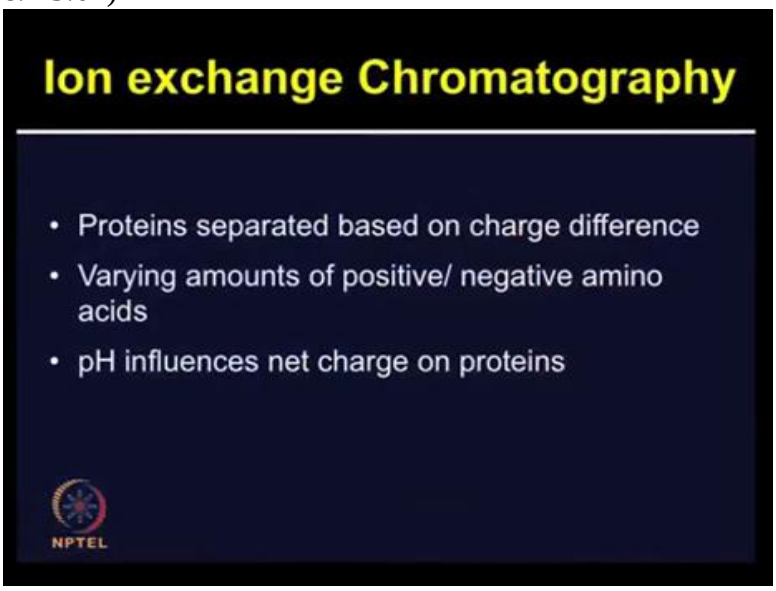

In ion exchange chromatography, the proteins are separated based on charge difference. The proteins with overall negative charge will interact with positive charges or the vice versa. So by varying the amount of positive and negative amino acids and even pH can influence the net charge on proteins.

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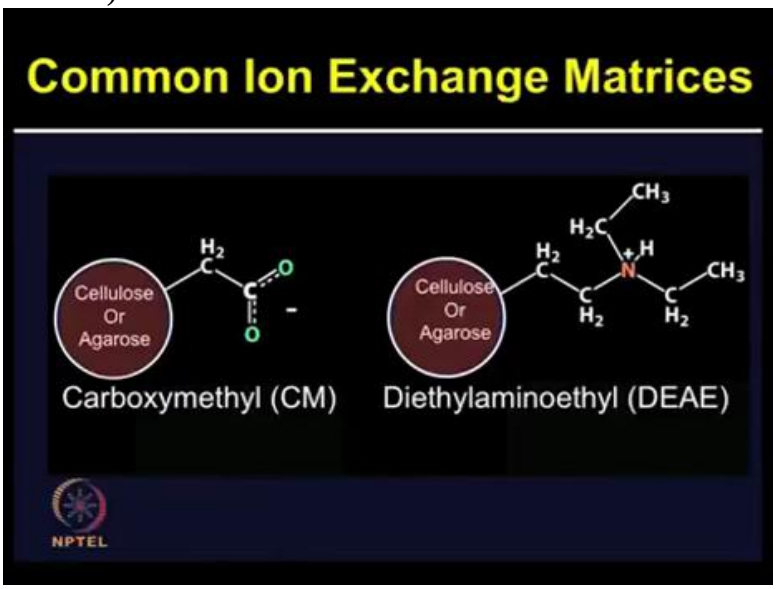

So in this slide, some of the common ion exchange matrices are shown like CarboxyMethyl CM, and DiEthyl AminoEthyl DEAE. So when a desired protein is positively charged, the cat-ion chromatography should be used, when a desired protein is negatively charged, the an-ion exchange chromatography method should be used.

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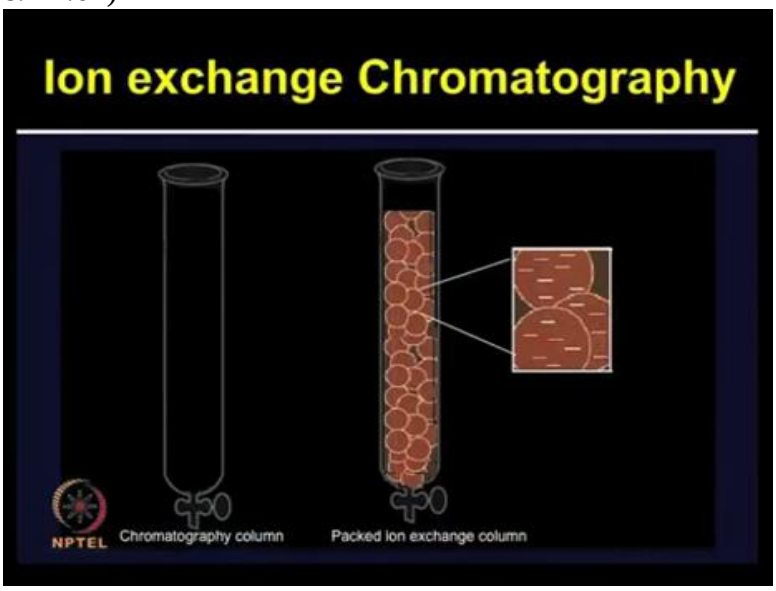

So ion exchange chromatography, the column is packed with the resin, whether it is cat-ion or an-ion exchanger depending upon the charge of the protein that needs to be bound to the column and purified.

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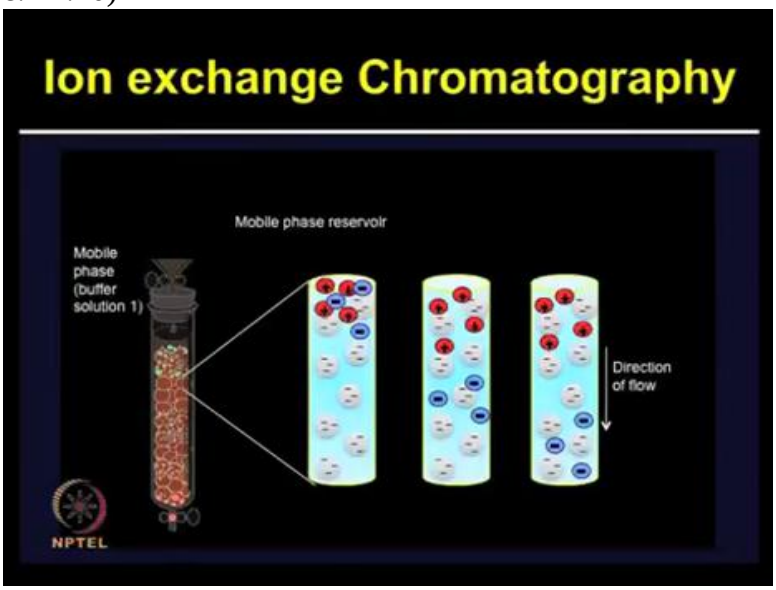

So proteins are adsorbed to the ion exchange column and then it can be de-adsorbed by increasing the salt or altering the pH of the buffer which can change the charge on protein. So various anionic buffers such as acetate and phosphate are used for cat-ion exchange and cationic buffers such as Tris chloride or ethanolamine are used for the anion exchange.

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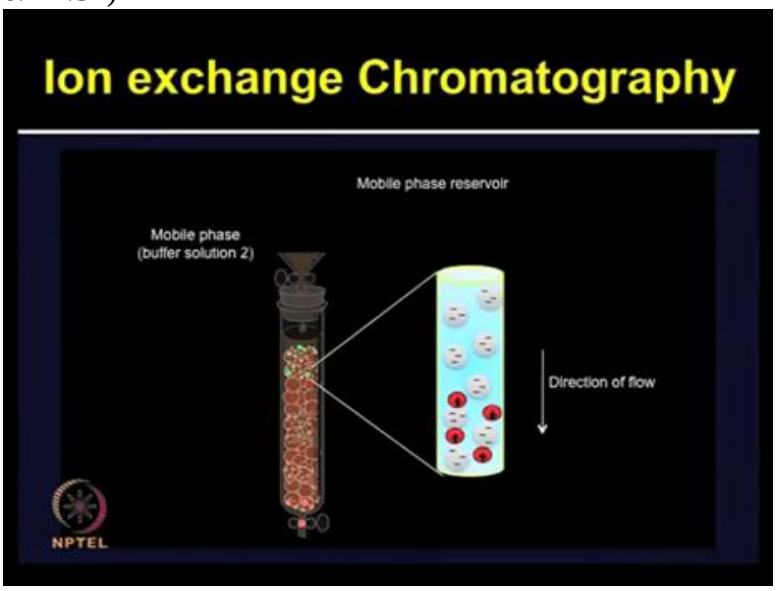

Now the buffer solution is changed so that the net pH of the protein of interest can be modified and it no longer binds to the ion exchange resin.

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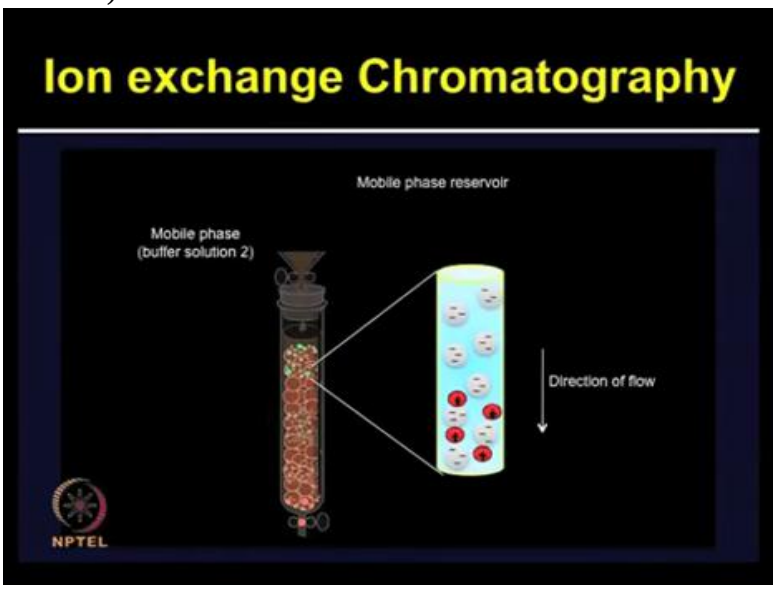

Therefore the bound protein can be eluted out as shown in this slide.

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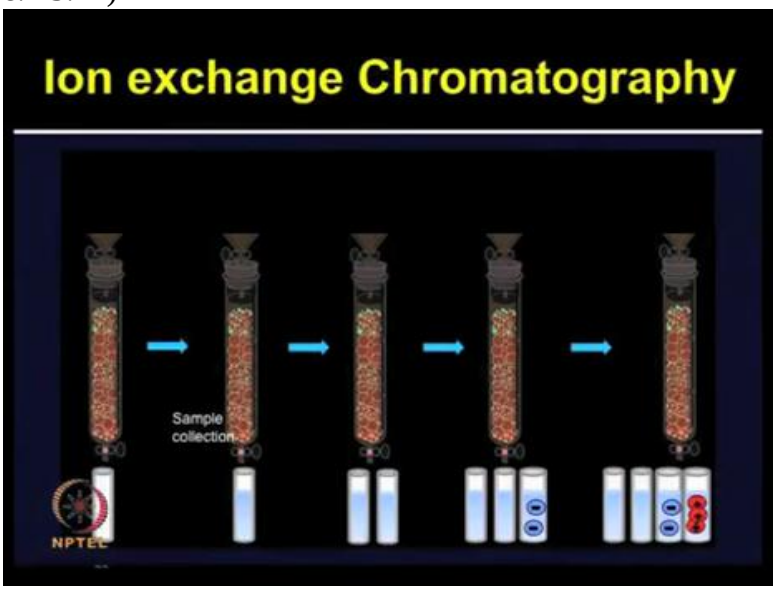

So if you have negatively charged protein which gets eluted first will be present in the initial fractions while the positively charged protein that bound to the column will be eluted in the later fraction or it will be vice versa.

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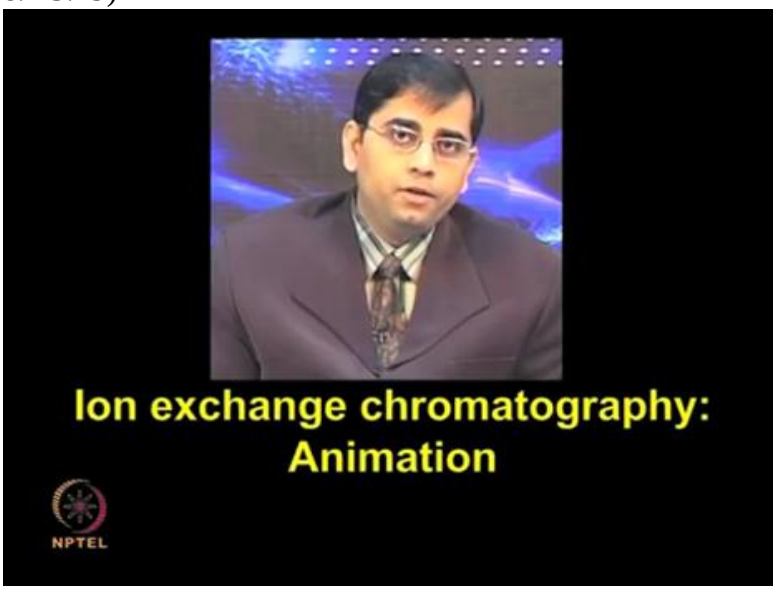

So let me describe how ion exchange chromatography works step by step in following animation.

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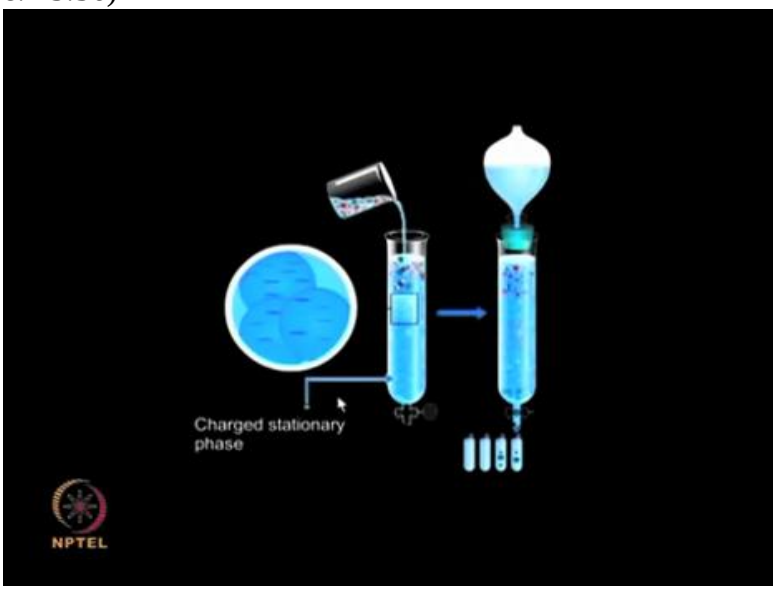

The charge in stationary phase, the column in stationary phase consists of a positively charged or negatively charged polymeric matrix which will bind molecules of the opposite charge. Commonly used ion exchangers include negatively charged Carboxy Methyl cellulose or CM cellulose which is a cation exchanger and positively charged DEAE cellulose which is anion exchanger.

The protein mixture, the unpurified protein mixture which consists of proteins of different net charges are loaded on to the column. The proteins having charges opposite to that of stationary matrix will bind to it while remaining proteins will be eluted.

Mobile phase, the proteins are eluted out of the columns by using suitable mobile phase, and then samples are collected by using different sample fractions. The solution leaving the column can be collected in suitably sized fractions for further analysis.

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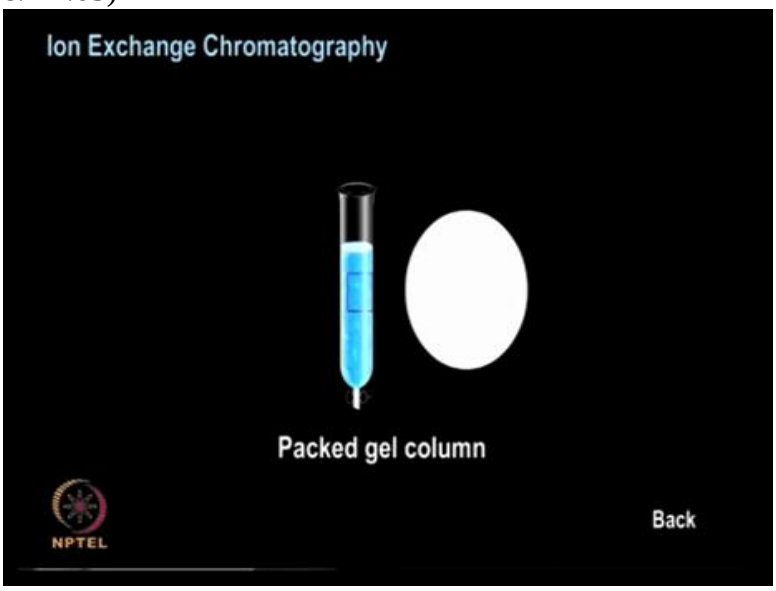

After giving you brief description of the components let me show you the process in animation. The column is packed with the suitable cation or anion exchange resin depending upon the charge of the protein that needs to be bound to the column and purified.

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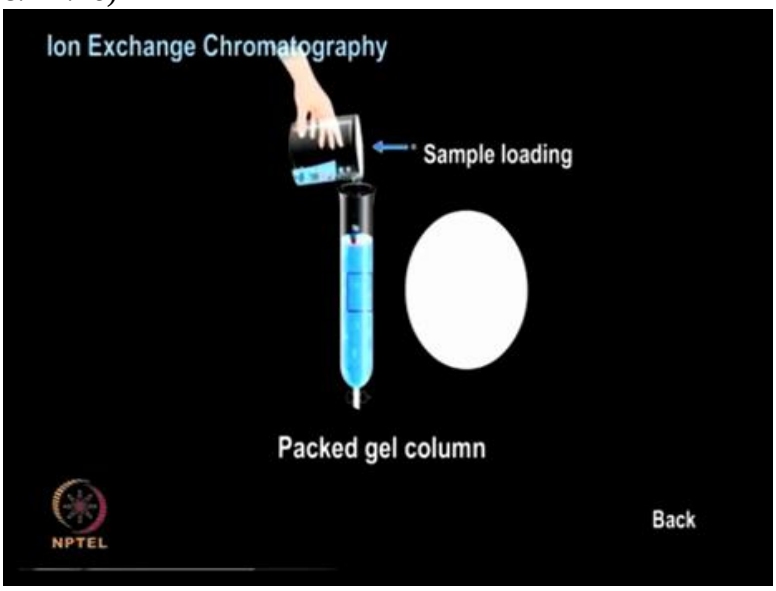

The an-ion exchange column is then loaded with the impure protein mixture consisting of various positively and negatively charged proteins.

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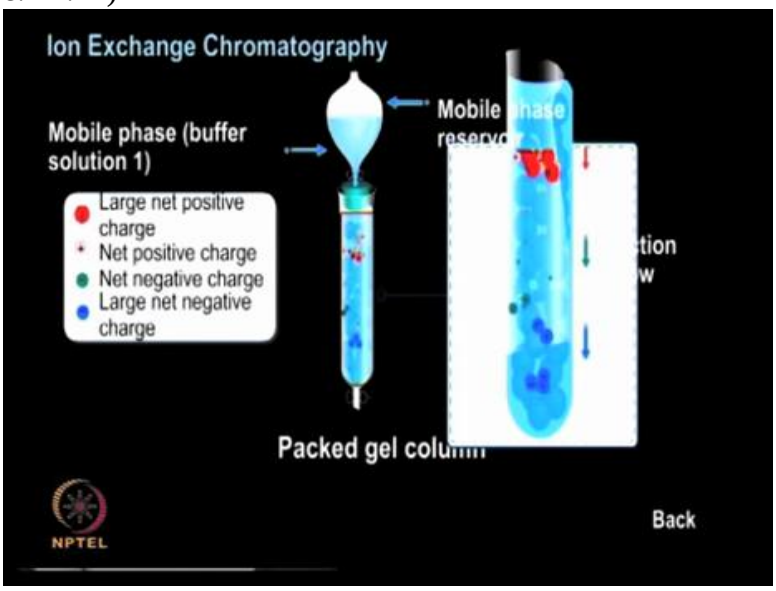

The column is eluted with a buffer solution of suitable pH such that the negatively charged molecules are removed from the column while the positively charged molecules remain bound to the anion exchange resin.

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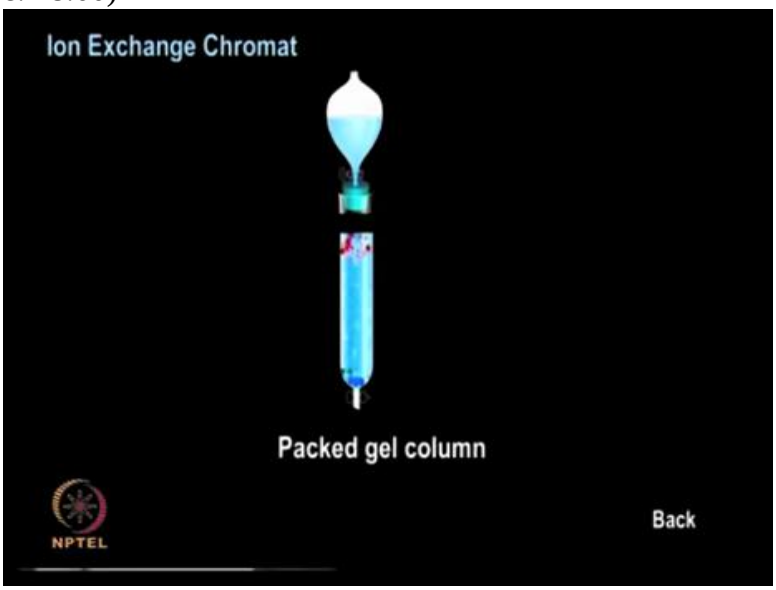

The buffer solution is changed such that net pH of the protein of interest is modified and no longer binds the ion exchange resin.

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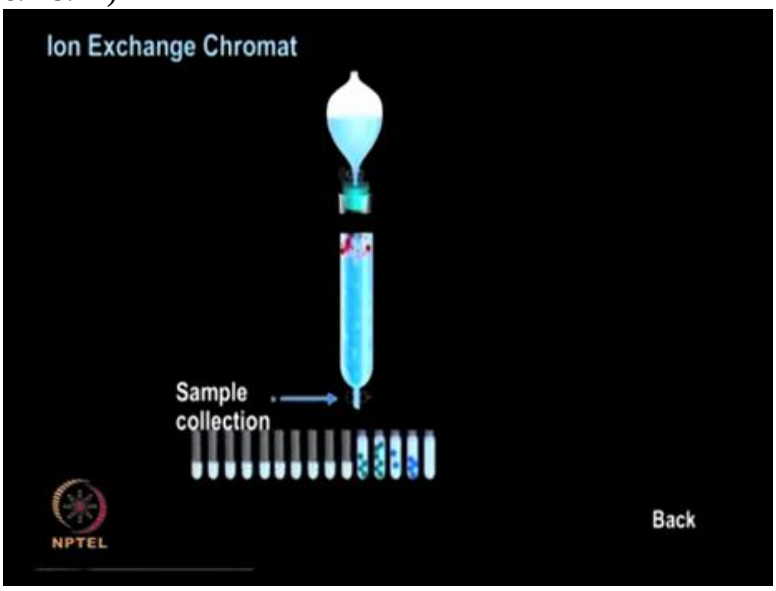

Therefore the bound protein also gets eluted out of the column in this manner. The fraction of appropriate size must be collected and analyzed for their protein contents. The negatively charged proteins which get eluted first will be present in the initial fractions…

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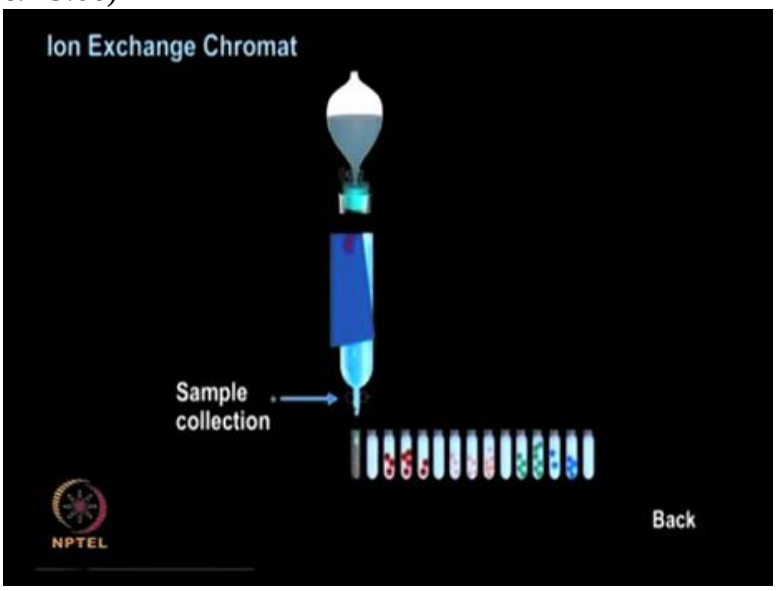

… while positively charged proteins that bound to the column are eluted in later fractions. Once all the fractions are collected then the protein content can be analyzed by using a spectrophotometer.

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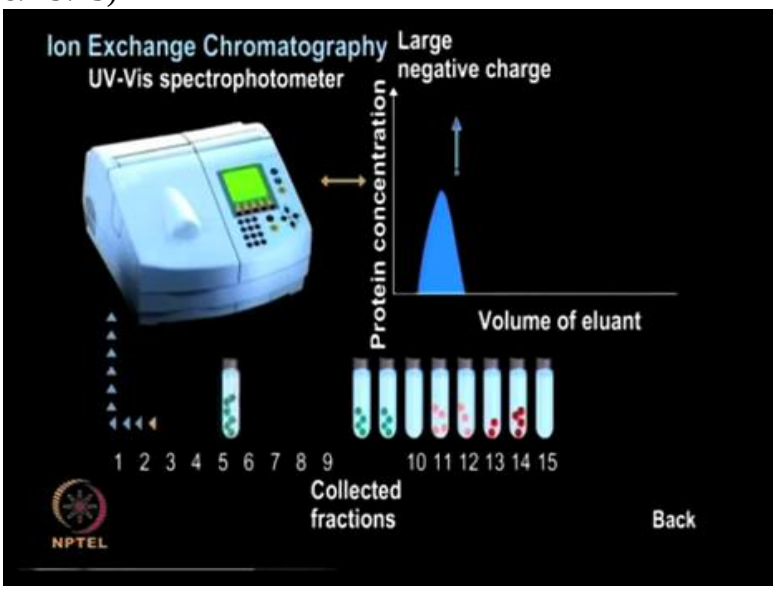

Analyze the contents, these fractions for their protein content by using a UV visible spectrophotometer .at 280 nanometers. A graph of eluent volume versus protein concentration can then be plotted.

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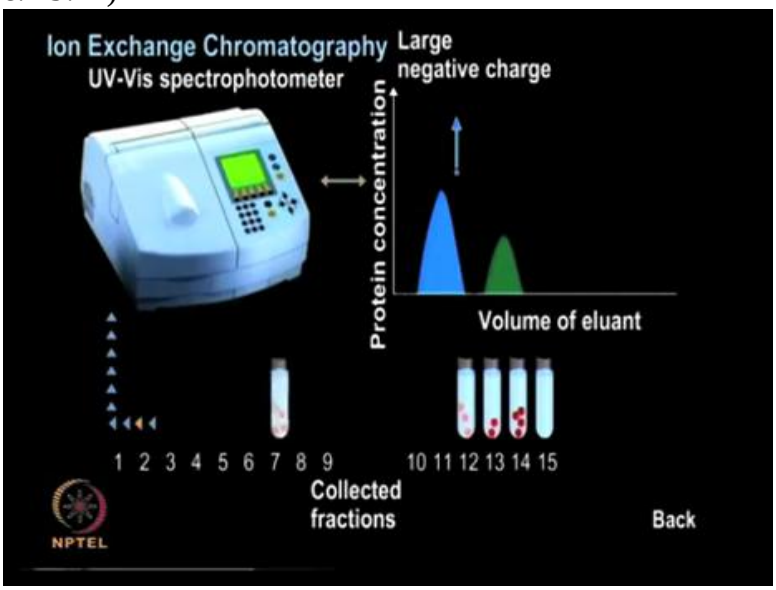

In this particular example, the negatively charged large molecules coming first and then positively charged fractions are coming later.

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Let's now talk about another very important method which is affinity chromatography which is a desirable method for protein purification to the homogeneity. Due to the selectivity of immune recognition, it is possible to purify a protein in a single step under the favorable conditions and affinity chromatography is one of the methods of choice for protein purification.

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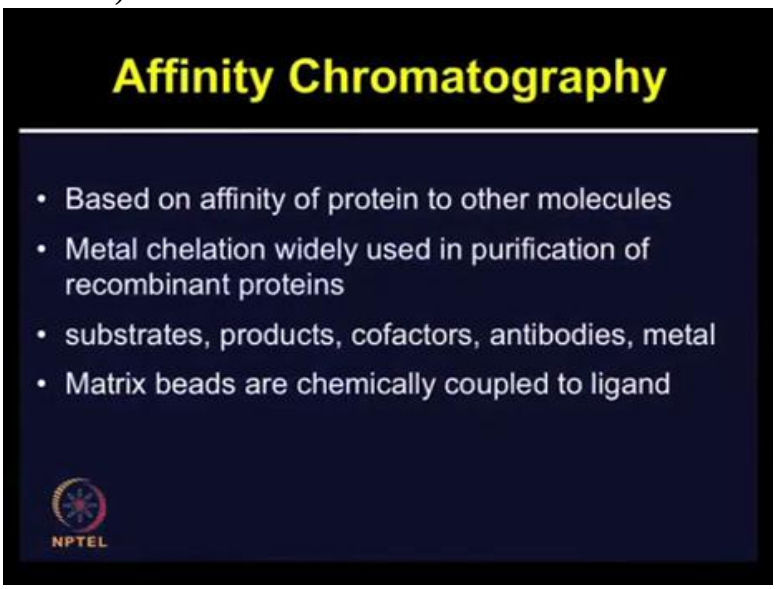

So affinity chromatography is based on affinity of proteins to its ligands or other molecules. The metal chelation is widely used in purification of recombinant proteins where substrates, products, cofactors, antibodies or metals, these can show affinity for given protein and this is used for strategy to purify the proteins based on its affinity. The matrix beads are chemically coupled to these ligands.

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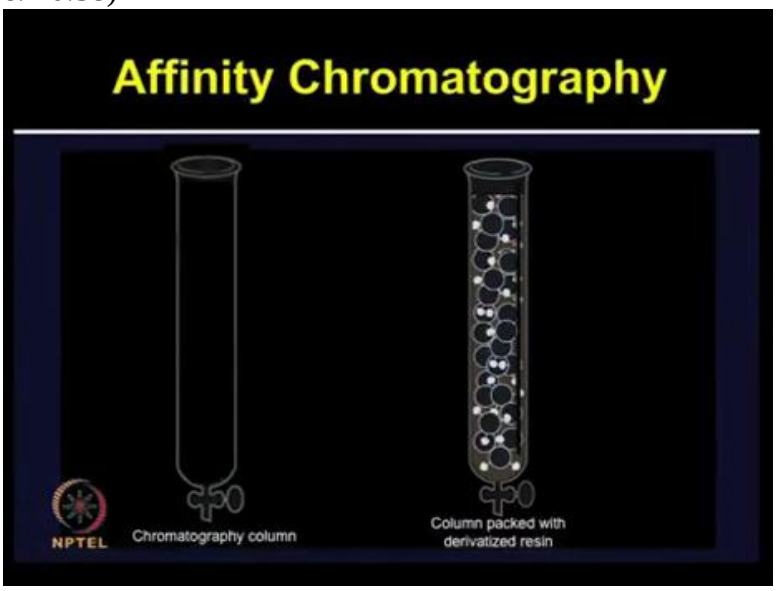

So in affinity chromatography, the column is packed with resin which is covalently coupled to the ligand specific to the protein of interest. The protein mixture is passed over the derivatized affinity column.

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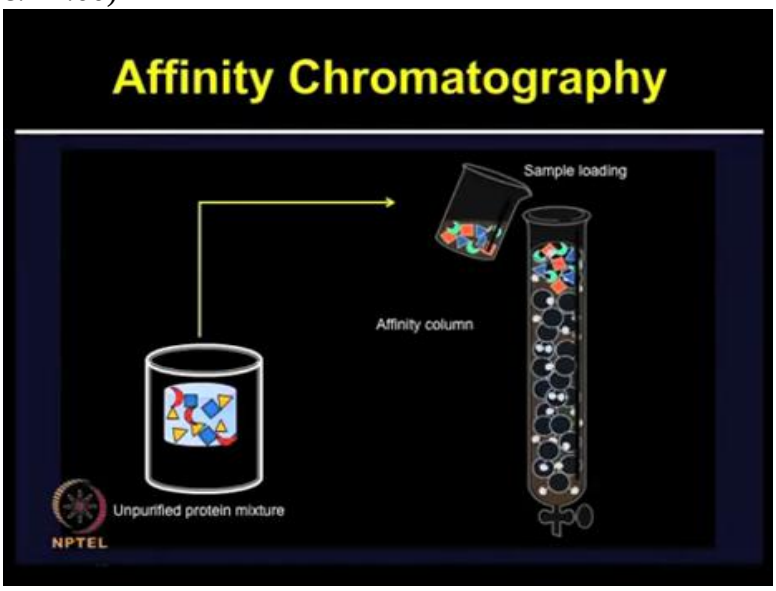

The protein of interest binds through a specific interaction while all other proteins which do not interact will not bind.

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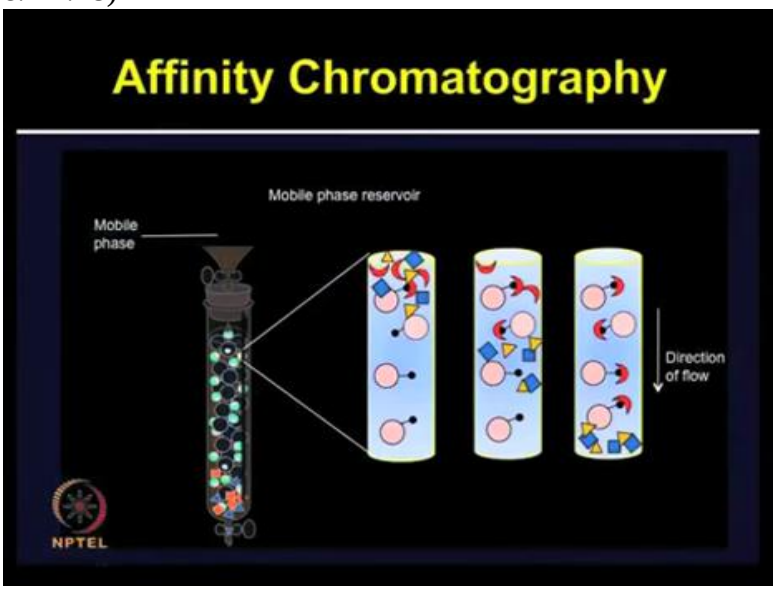

Now the column is washed with a suitable mobile phase to remove the unbound protein and protein of interest which has higher affinity for the ligand remains bound to the derivatized column matrix and is not removed during the washing step.

Now these proteins of interest can be desorbed by applying excess ligand in the solution. So due to the specific interaction, the affinity chromatography achieves very high degree of protein purification.

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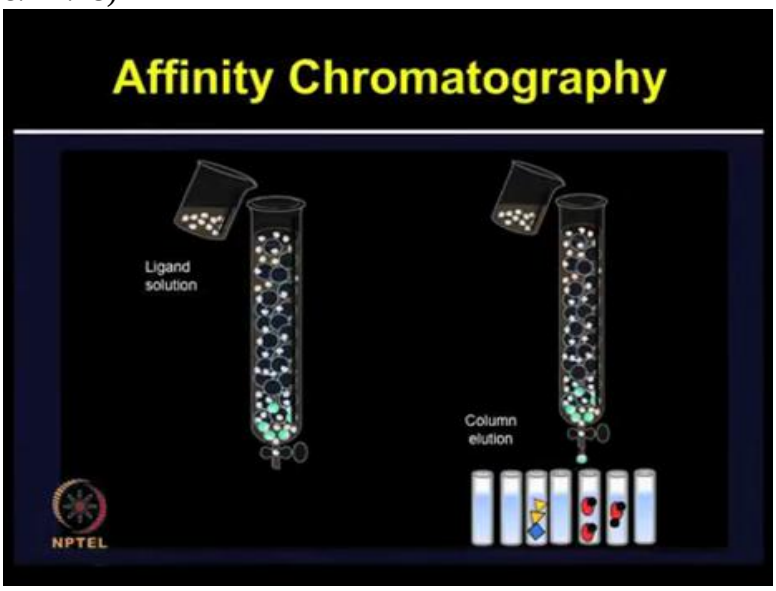

It is not limited by the sample volume which is the case of gel filtration chromatography and because of its superiority in achieving the pure protein, it is usually considered as the final step for the protein purification.

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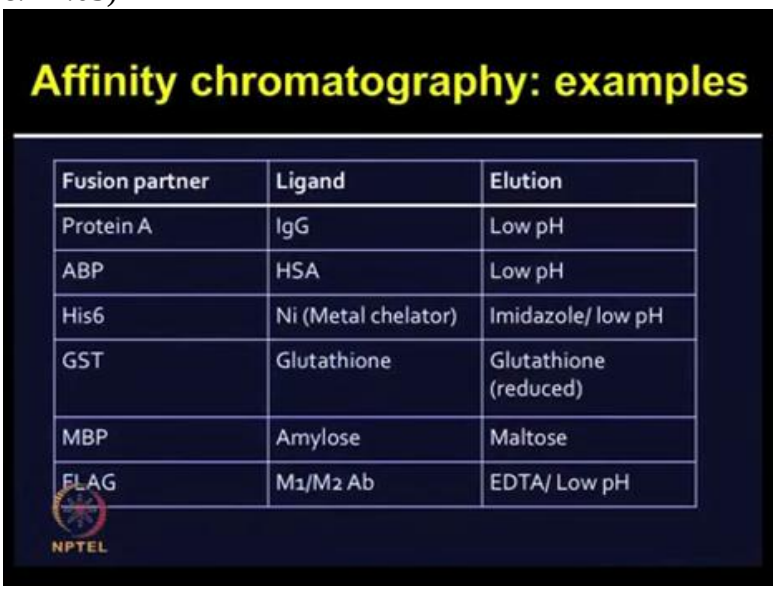

There are various examples where affinity chromatography can be used especially the antigen-antibody pairs, they are commonly used for bio-affinity pairs.

The matrix containing protein A which is used for the IgG purification. So in this case the protein A recognizes the Fc region of IgG and this interaction is being used for protein purification using affinity chromatography.

Another strategy is Concanavalin A protein binds to the glucose molecule. So by adding concentrated solution of glucose, the glucose can display the column where these molecules are attached on the binding site of Concanavalin A.

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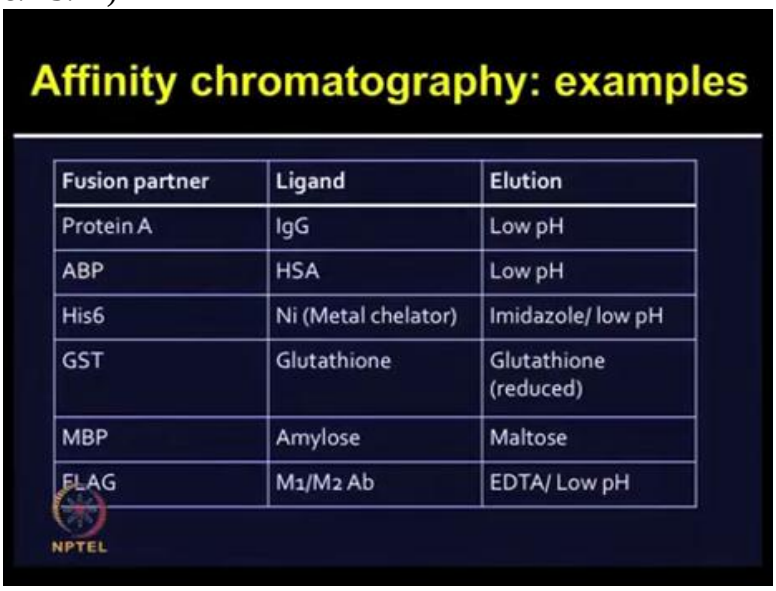

But these are only few examples. There are many other examples where different type of fusion partners and ligands are used for affinity chromatography method as shown in this slide.

The protein A binds with IgG and the proteins can be eluted by lowering the pH, ABP binds with HSA and elution is again with the low pH.6-Histidine tag binds with Nickel NTA columns, the metal chelators and imidazole or low pH condition can be used for elution.

GST, glutathione s-transferase binds with glutathione and reduced glutathione is used for elution. The Maltose-Binding Protein MBP binds with amylose and it can be eluted with maltose. Then FLAG proteins can be bound to M1 or M2 antibodies and EDTA or low pH elution can be used for eluting the proteins.

There are various other examples but these are common strategies being employed for protein purification.

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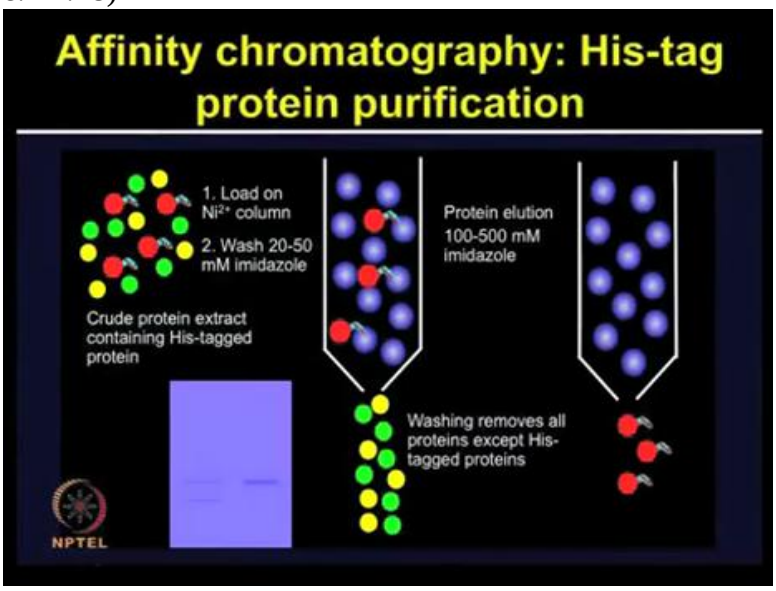

So we just discussed...there are various strategies, by adding a tag or by applying some affinity interaction, the proteins can be purified. The genetic engineering methods have made it possible to create the fusion proteins which show a strong affinity between the fused protein and the ligand.

So, for example as shown in this slide the affinity of histidine tag with Nickel nta column. Now because of interaction of histidine tag with Nickel, the proteins which contain histidine tag will bound to these resins of Nickel nta.

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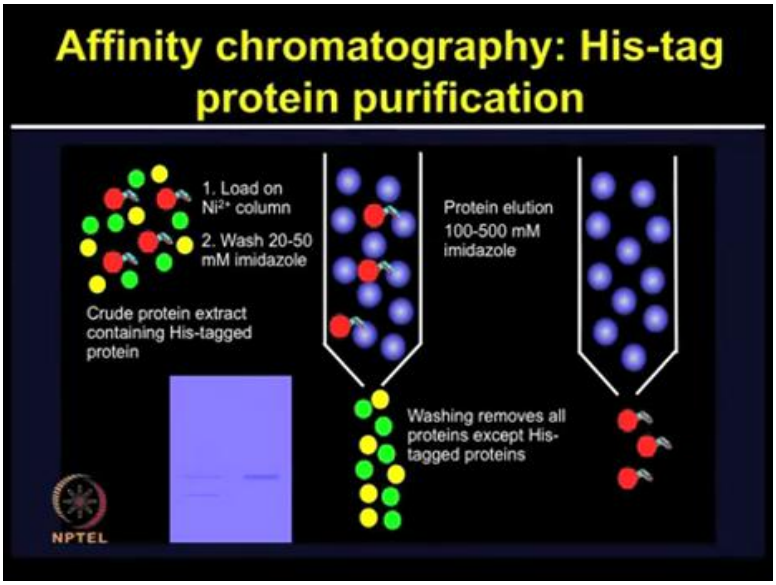

Now after washing with the mild imidazole such as 20 to 50 milli-molar, unbound residues will come out but by increasing the concentration such as 100 to 500 milli-molar of imidazole, the proteins can be eluted out.

The effectiveness of protein purification should be further assessed on SDS PAGE gel where one needs to see whether one pure band is shown or even some contaminating bands is also seen. So one case is shown on the left side where contaminating bands are seen where as in the other case, only a pure band can be seen.

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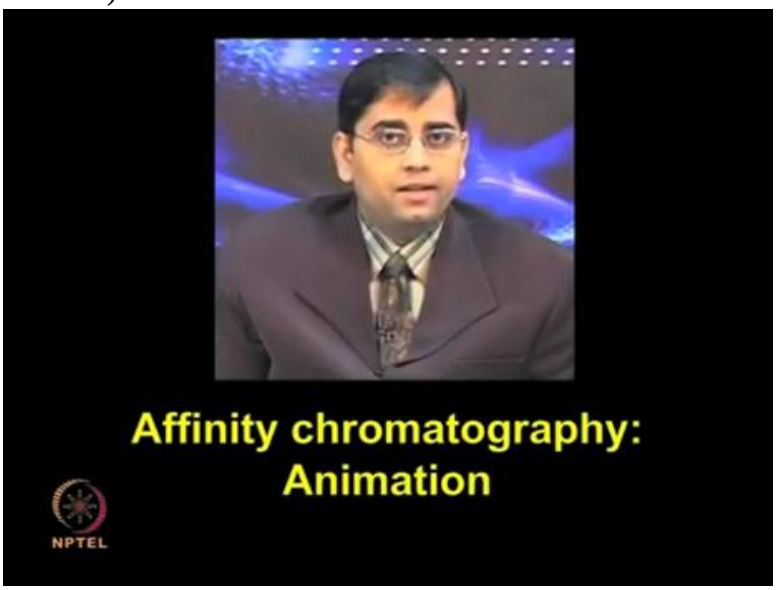

So let's discuss the affinity chromatography methods step-by-step in following animation.

Let me give you definition of few components.

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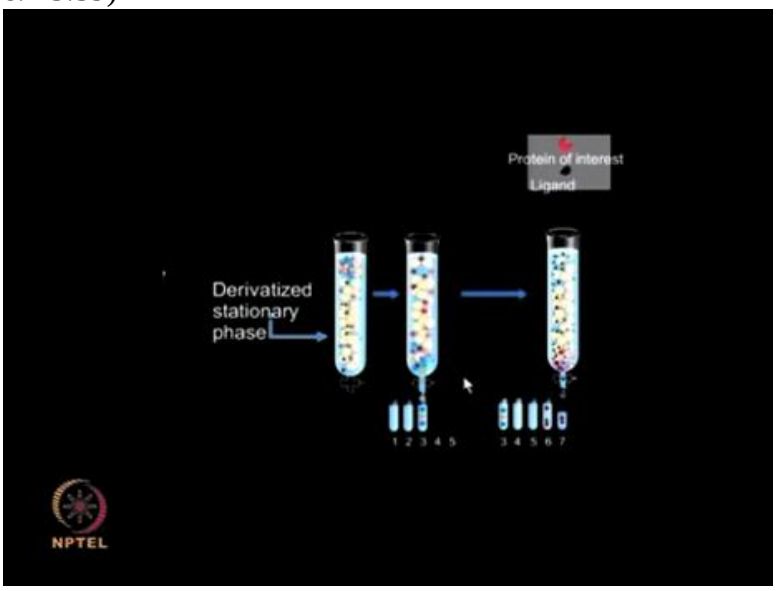

The derivatized stationary phase: The stationary phase resin in affinity chromatography consists of a covalently bound ligand that will specifically bind the protein of interest by interacting it.

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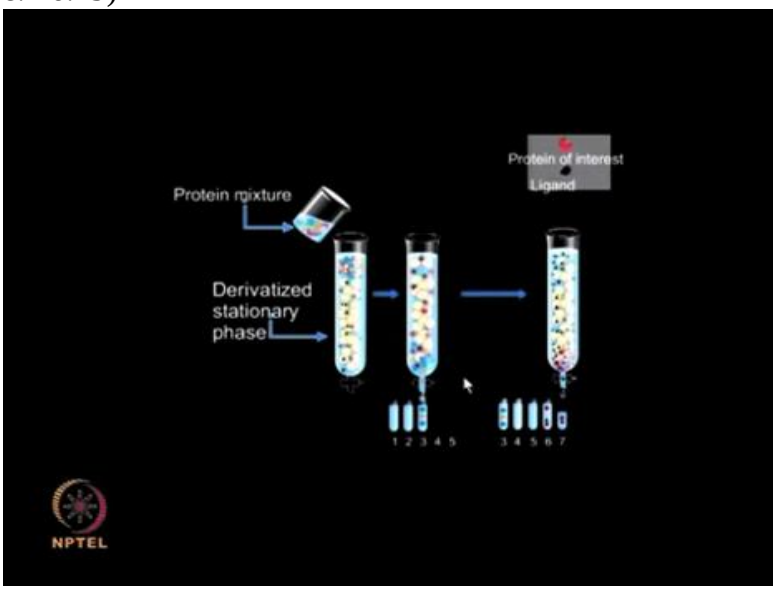

The protein mixture: It is unpurified protein mixture which consists of proteins having different properties and interaction specificity for the ligand bound to the column matrix.

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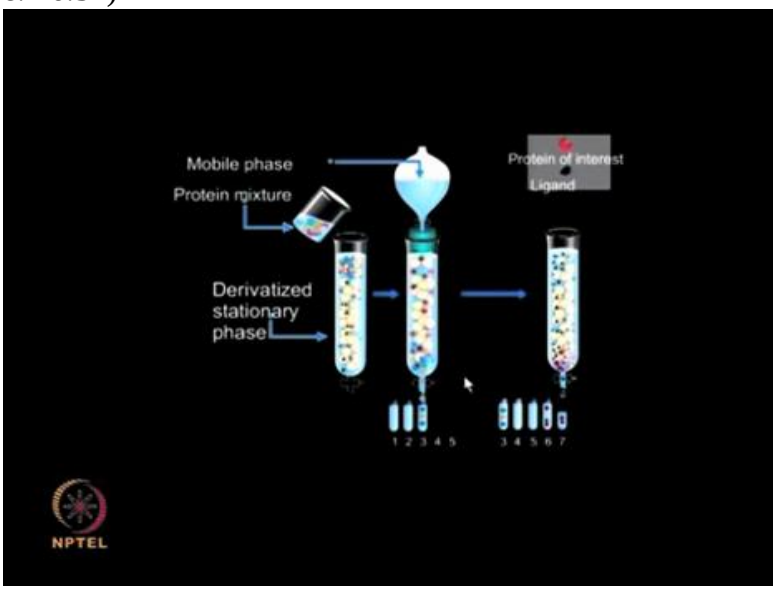

Mobile phase: Following the sample loading, the unbound proteins are washed out of the column using suitable mobile phase. Depending on protein of interest, this could be either water or sometimes salt solution.

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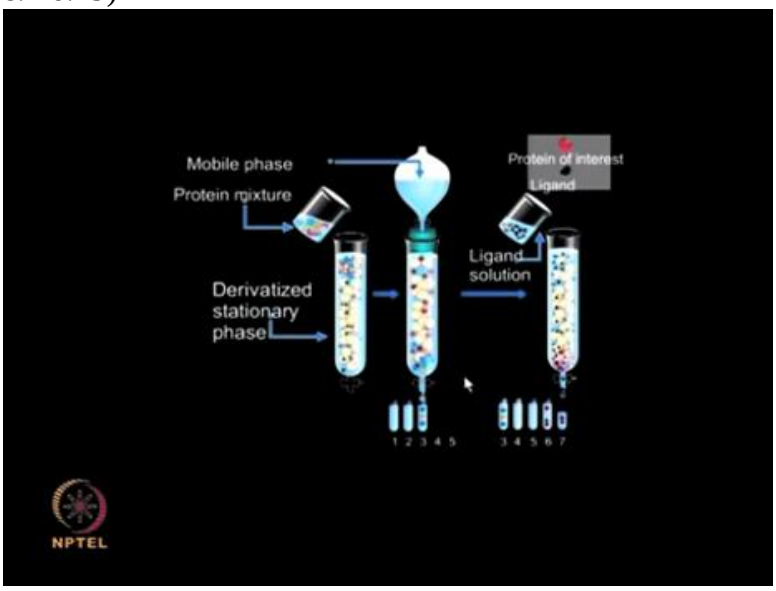

The ligand solution: The solution is passed through the column to elute the bound protein of interest since it contains the same ligand that is bound to the column matrix. It is capable of eluting the proteins by interacting with it.

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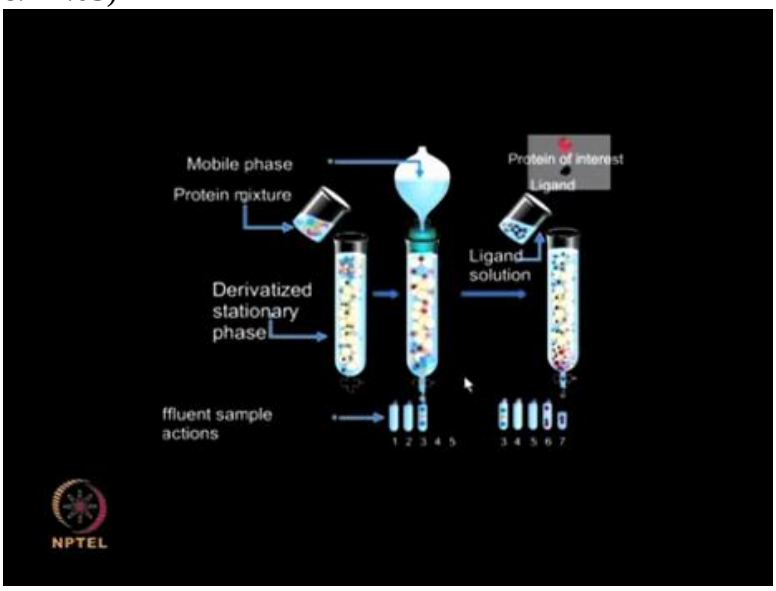

The effluent sample fractions: The solution leaving the column is collected in suitably sized fraction for further analysis. The unbound proteins are eluted from the column first followed by the bound fractions which are removed after washing with the ligand solution.

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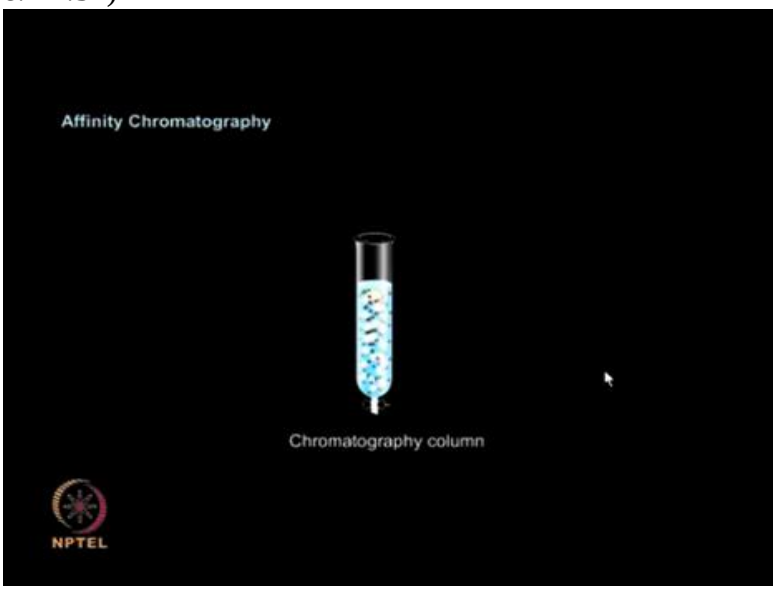

Now let's see the process in the following animation. The column is first packed with a suitable resin that has been covalently coupled to the ligand specific to the protein of interest.

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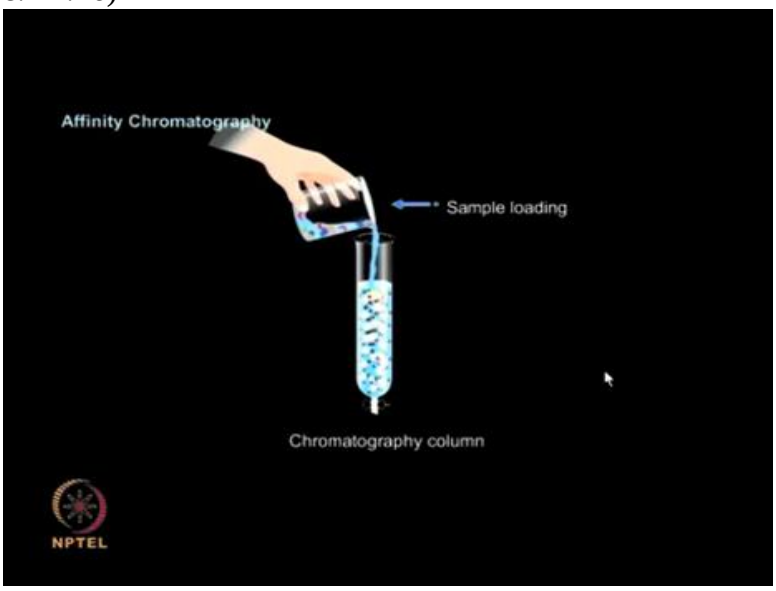

This derivatized affinity column is then loaded with the protein mixture containing various proteins having different properties and interaction specificity.

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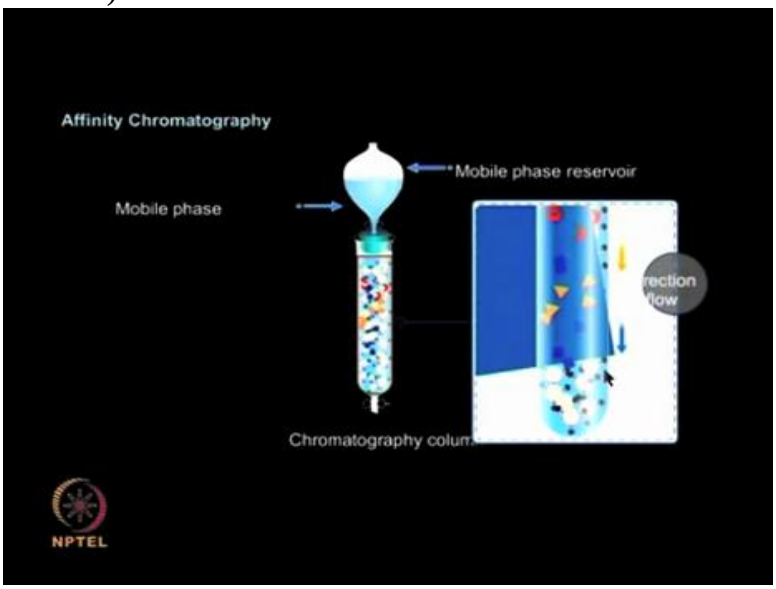

The column is washed with a suitable mobile phase to remove all the unbound proteins.

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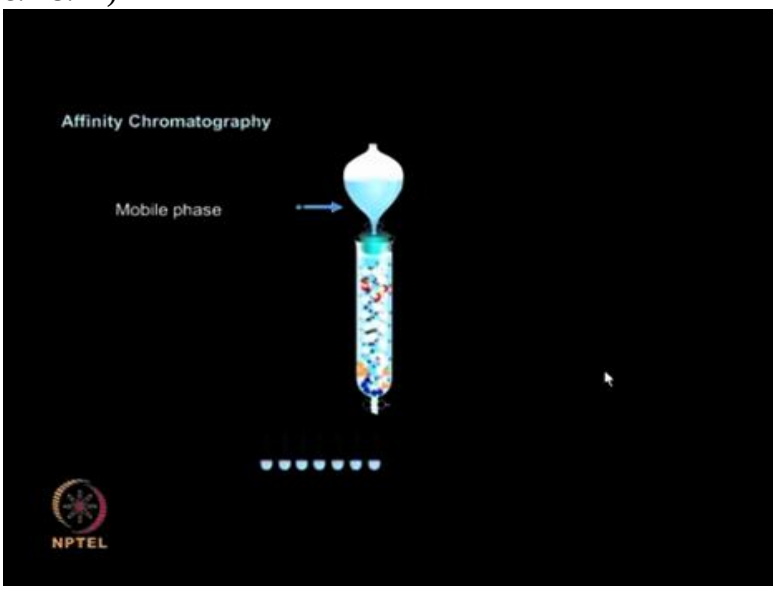

The protein of interest which has higher affinity for the ligand remains bound to the derivatized column matrix and is not removed during the washing.

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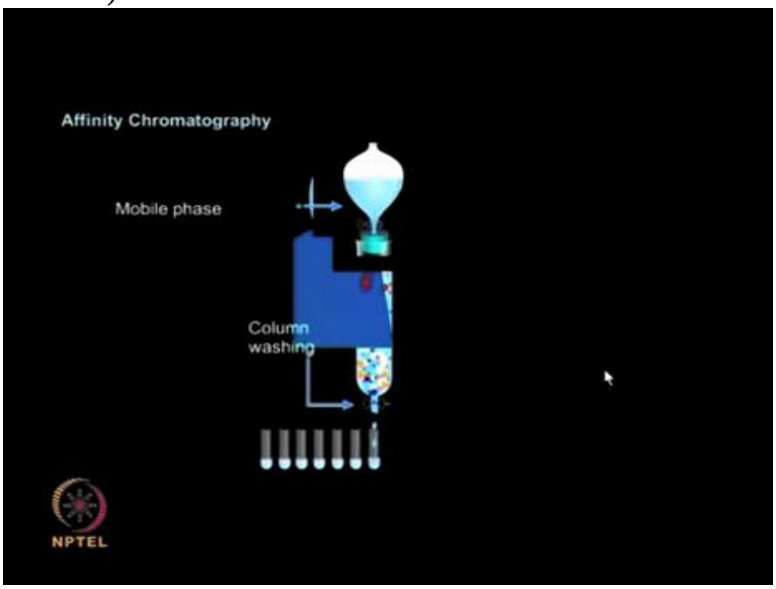

The fractions collected during washing can be analyzed…

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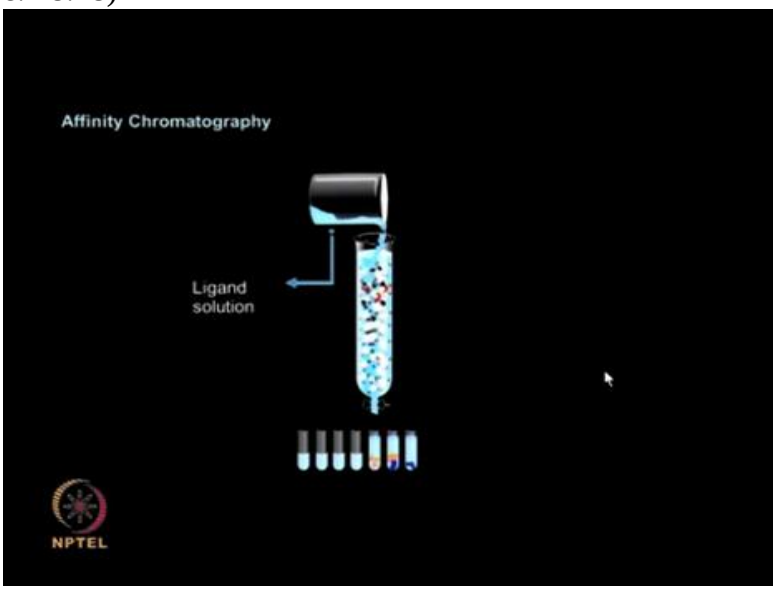

… assessed and discarded if they are not required.

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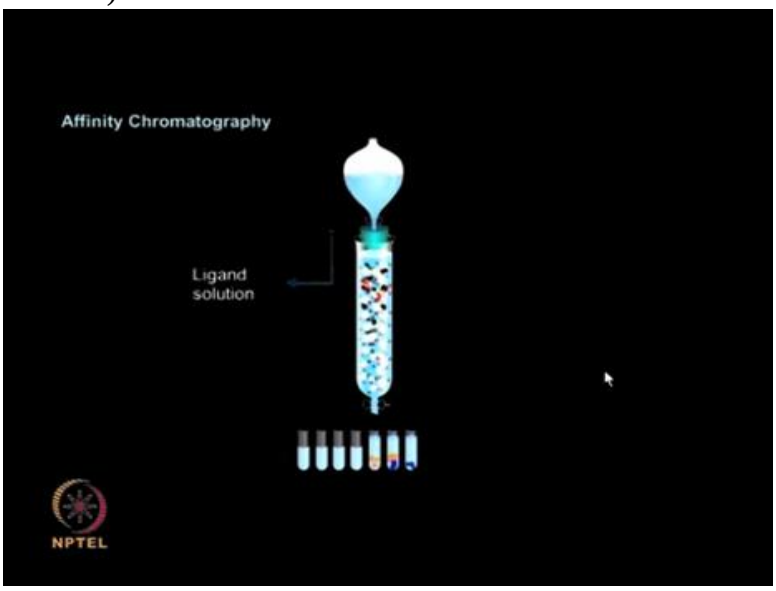

After the column has been washed thoroughly, the protein of interest is eluted by passing a ligand solution which binds to the matrix-bound protein and removes it from the column.

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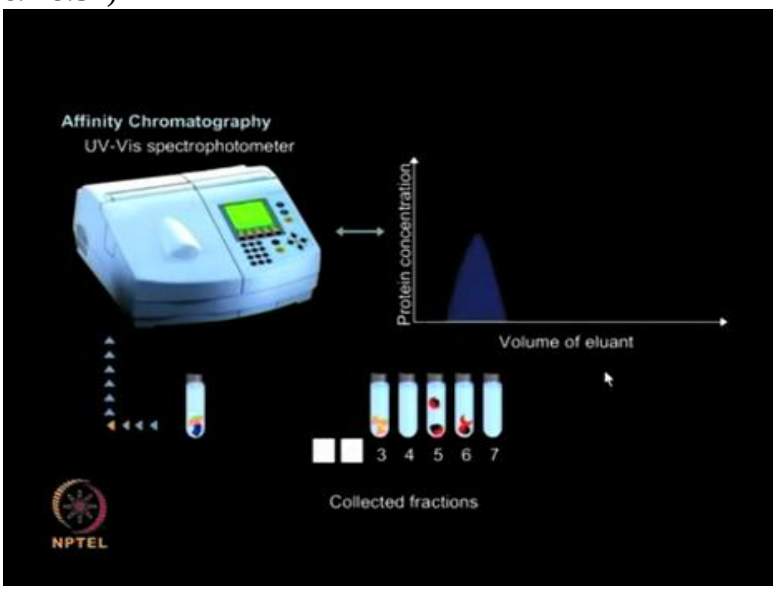

The fractions are then analyzed for their protein content using a UV visible spectrophotometer at 280 nanometers.

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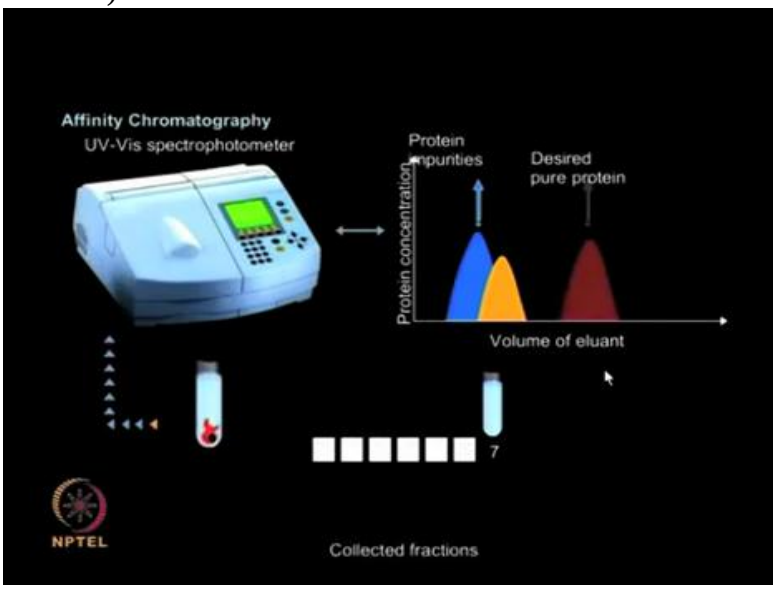

A graph of eluant volume versus protein concentration

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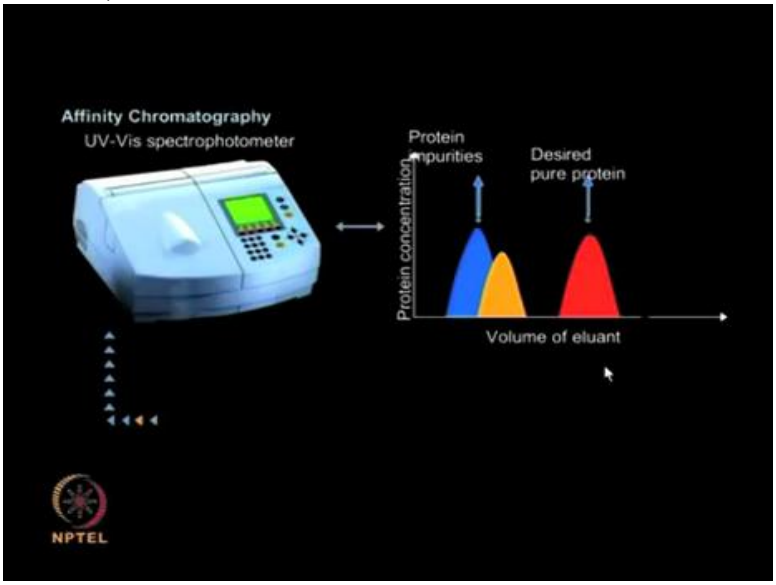

...can then be plotted

(Refer Slide Time: 29:13)

Let's now talk about High Performance Liquid Chromatography HPLC. HPLC is a separation technique that separates molecules based on their differential adsorption and desorption between stationary phase matrix in column and mobile phase. Better separation and resolution of the components can be achieved by HPLC.

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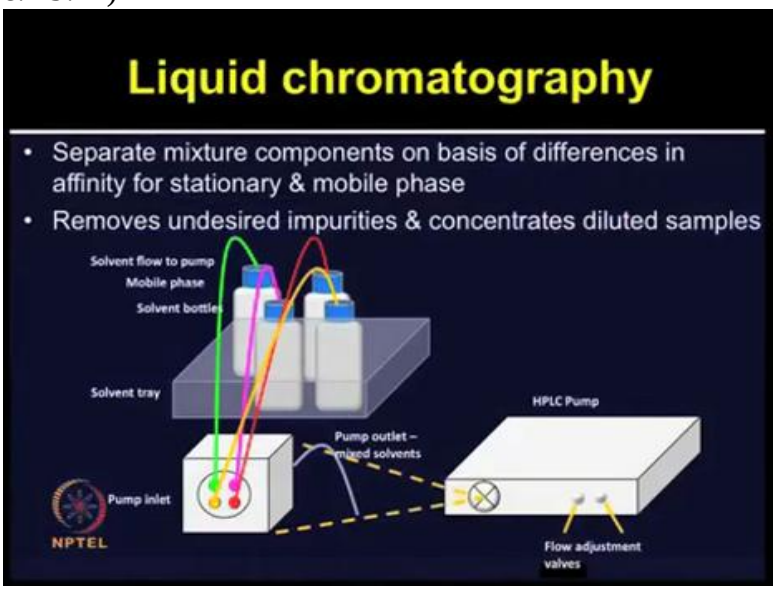

So what is Liquid Chromatography? The Liquid Chromatography separates mixture components on the basis of differences in the affinity of stationary and mobile phase. It can remove the undesired impurities.

Therefore, for various types of mass spectrometry based applications, it increases the reproducibility and robustness for analysis of peptides and proteins. It also concentrates the diluted samples and therefore it increases the sensitivity detection of low level proteins by using...when you are applying the different types of proteomic technologies.

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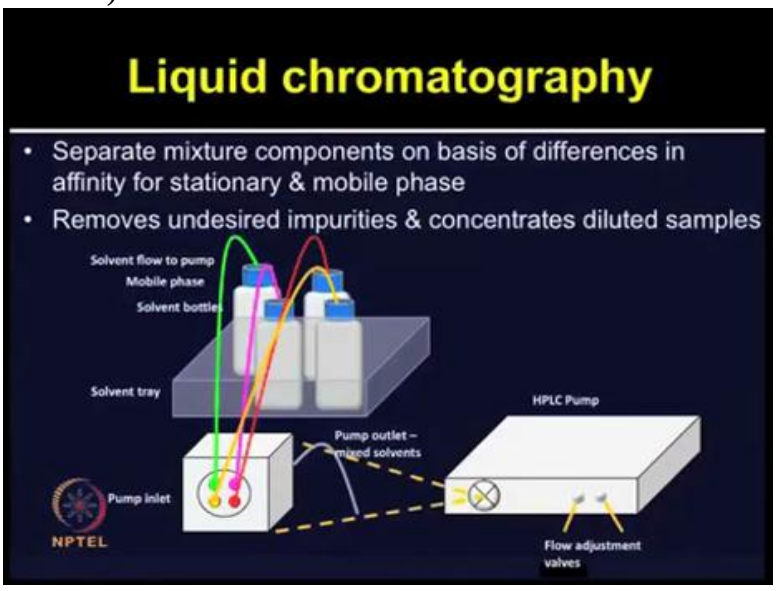

It is mostly used for separating the peptide mixture. One of the diagrams is shown here; which is a typical Liquid Chromatography setup which consists of solvent bottles,

degassifiers, dual or quaternary pumps, the sample injector column and detectors. Different solvents can be placed in the solvent bottles depending upon the purification requirements.

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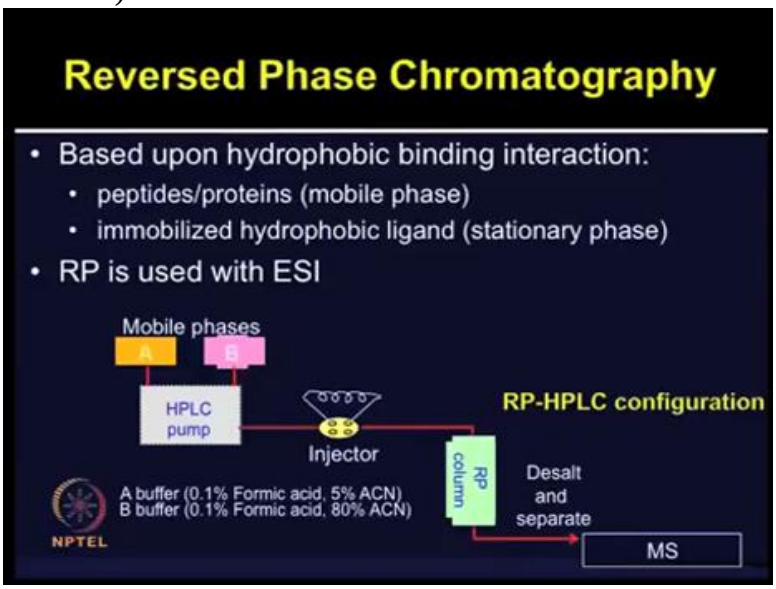

Let's first talk about Reversed phase RP Chromatography. The Reversed-phase chromatography is based upon the hydrophobic binding interaction between the peptides or proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase.

In reversed phase, the stationary phase consists of the long aliphatic carbon chains which is highly hydrophobic in nature. Molecules are bound on the column by means of hydrophobic interactions and are eluted out when the solvent polarity is modified.

The Reversed-phase chromatography is mostly used with electrospray ionization in mass spec due to its compatibility of acidic, aqueous and polar mobile with ESI. It is also used for desalting the peptides before injecting for electrospray ionization.

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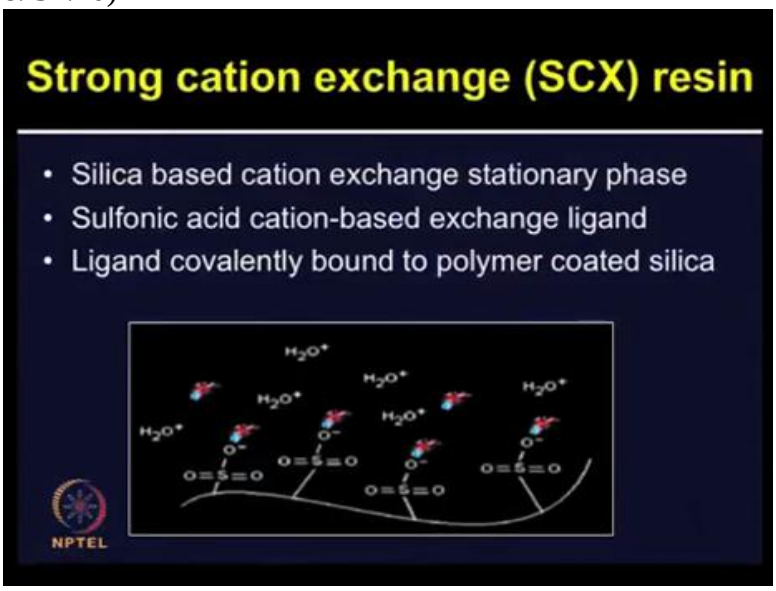

Let's now talk about strong cat-ion exchange or SCX chromatography. SCX consists of a stationary phase matrix which is made up of negatively charged sulfonic acid groups which bind the oppositely charged peptide molecules. These molecules can be eluted out using a positively charged mobile phase which binds the analyte molecules more firmly.

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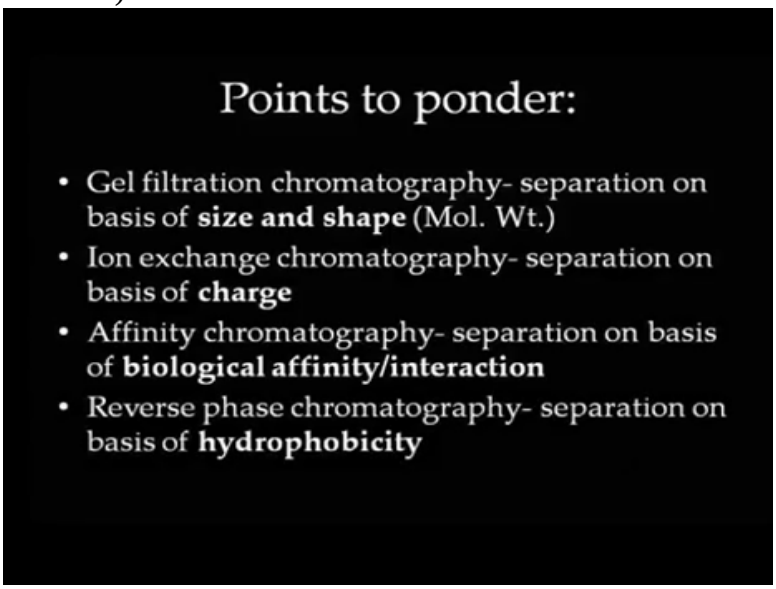

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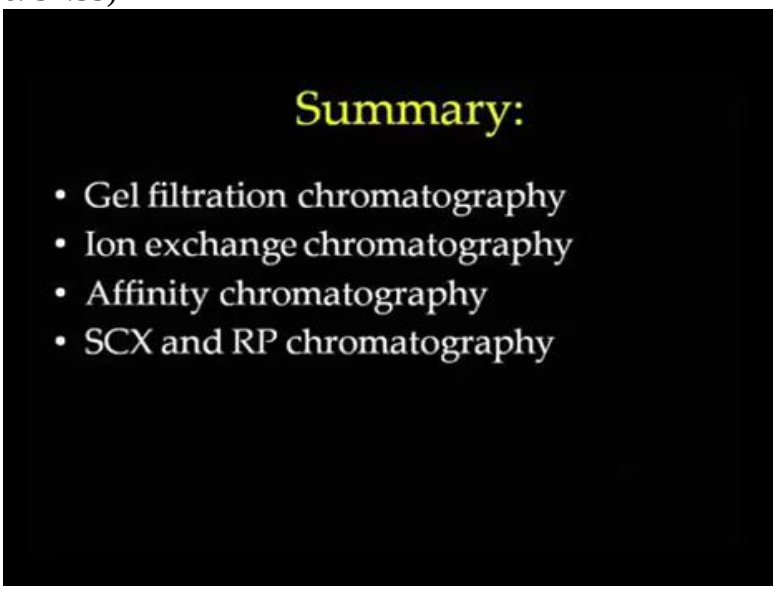

In summary, we have talked about principle of different types of chromatography methods commonly being employed for the proteins and peptides and we looked at some of these chromatographic methods in more detail. Thank you.