Downstream Processing
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Lecture - 27
Chromatography (Continued)

We will continue with the chromatography, chromatography is the most important technique for separation as well as purification. We can use it for small molecule metabolites, or we can use it for large bio-molecules even enzymes and proteins. That is why chromatography is extremely important, and we are going to spend lot of time on this chromatography, and will also review some of these fundamental principles several times.

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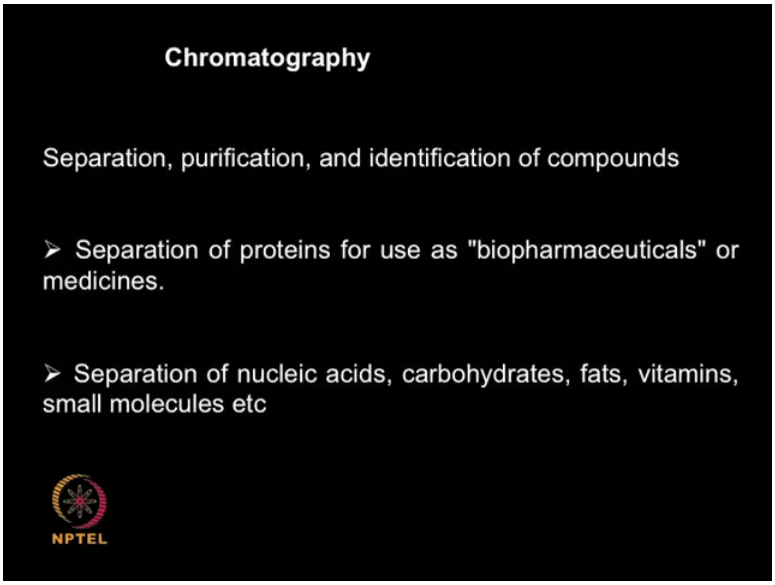

So, we use chromatography for separation, purification even for identification of compounds. We can use say the high performance chromatography for identifying some unknown metabolites. So, chromatography is are used in biopharmaceuticals or in medicine, chemistry for separating proteins, separation of nucleic acids, carbohydrates, fats, vitamins, small molecules and so on actually.

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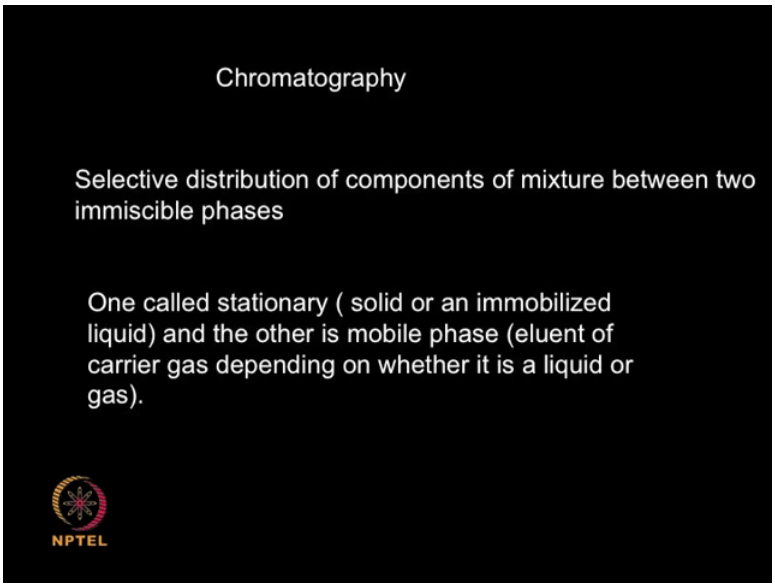

So, what does it do? Basically a solute gets distributed in the continuous phase as well as in the stationary phase that is what happened actually. So, this distribution happens because of so many factors either it is partition, either it is because of ionic forces whether it is because of hydrophobic forces, or certain types of ligands selectively buying certain proteins. So, so many different forces may be leading to a separation of a solute from the continuous phase. So, what happens, the various components or various mixture, mixture of proteins get separated because of these different forces.

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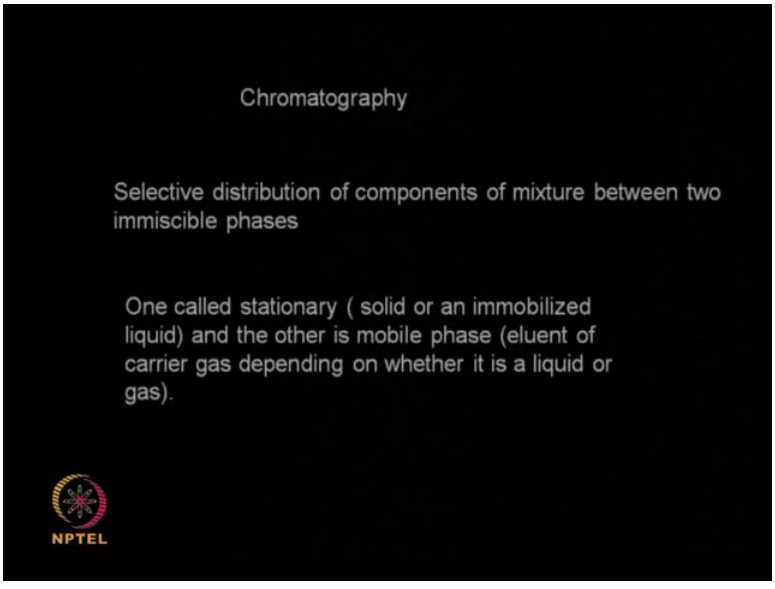

So, you have a solid phase or it is called a immobilized phase, or it is called a stationary phase then you have a liquid phase, or a mobile phase it is a eluent and the carrier gas or a fluid both can be there. So, if it is a gas then we call it a gas chromatography, it is a liquid we call it a liquid chromatography. So, what happens? This picture tells you pictorially, what is happening.

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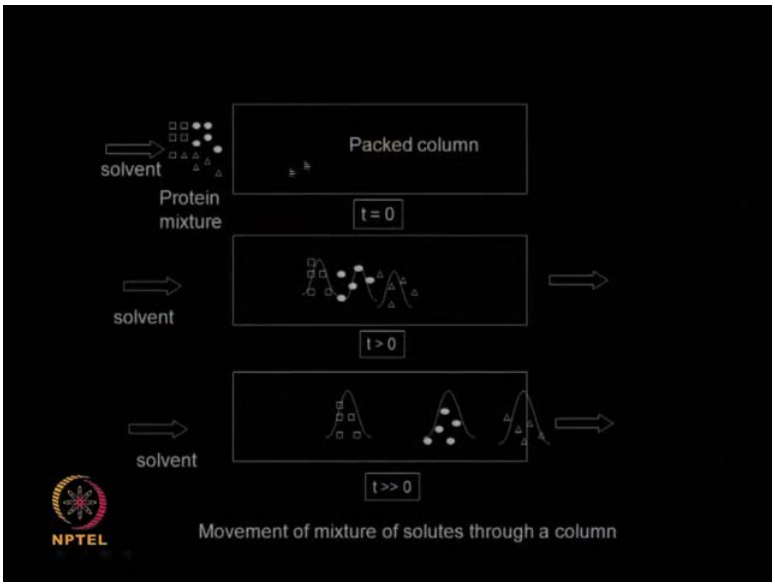

So, we have a packed column and then we have a continuous solvent flowing through. You have a mixture of material that needs to be separated, which is fed ferment is it could be a protein mixture. So, like you may being having square protein, circular protein and triangular proteins, at time equal to 0 you are injecting this particular mixture. As it travels through the column slowly separations start happening, and when the things exit at the column, you will have a very good separation. For example, the triangular proteins may be coming out first followed by the circular proteins, and followed by the square proteins at a very large time.

So, this is called the retention time elution time elution volume and so on. So, different names are there. Now, of course, you are not go into get a beautiful separation as shown here, and if you take a real life sample there could be several proteins, several tens of proteins several hundreds of protein. So, the separation of each protein might not be distinct and separate they may be sometimes over lapping. So, you may have to improve on the solvent so that you are able to get a good separation.

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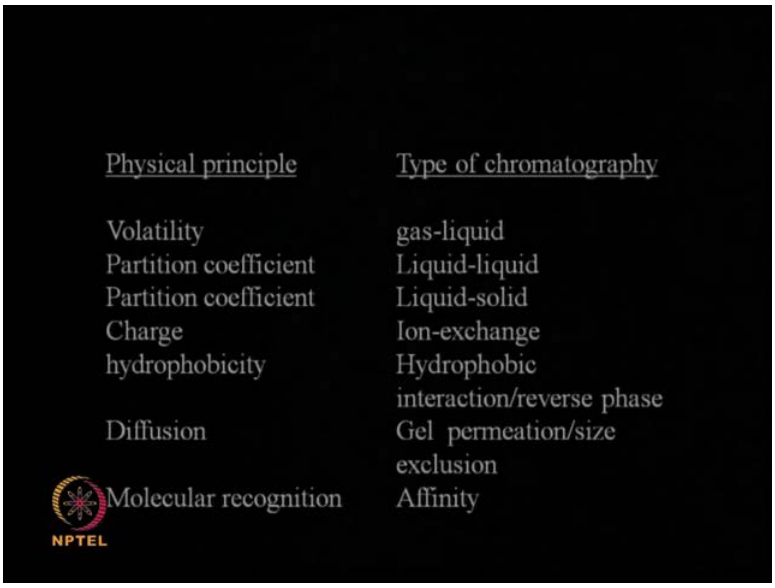

So, what are the physical principles? If it is based on volatility then you have something called a gas liquid chromatography, or GLC. So, if it is based on partition coefficient. That means, the solute partitions between the liquid in the continuous phase and the liquid that is immobilized, then we call it liquid-liquid chromatography. If it is again based on partition coefficient, you may have a liquid-solid chromatography. So, the forces of the on the solid could be because of ionic forces, or hydrophobic forces, or electrostatic forces.

Now, if the physical principals are based on charge then we call it ion exchange chromatography. For example, you want remove some salts from a protein mixture I can have a cation or anion or a combination of both cation-anion chromatography, which will separate all the salts. So, you will just have proteins. If it is because of hydrophobic forces then we have hydrophobic interaction chromatography, or reverse phase chromatography.

So, if you have very highly hydrophobic proteins it may get bound to the hydrophobic matrix, where less hydrophobic or hydrophilic proteins come out. So, that is called hydrophobic interaction chromatography. If it is based on size the size of the protein or molecular weight of the protein then that is called a gel permeation or size exclusion chromatography.

What happens there, small proteins enter certain wide space they get entrapped large proteins travel out fast. So, bigger once come out first smaller once come out very, very slowly. Then we have molecular recognition. So, you have a ligand, which will recognize only a certain protein ligand will not recognize any other protein, then it is called a affinity chromatography. So, many different principles you can have different types of chromatography.

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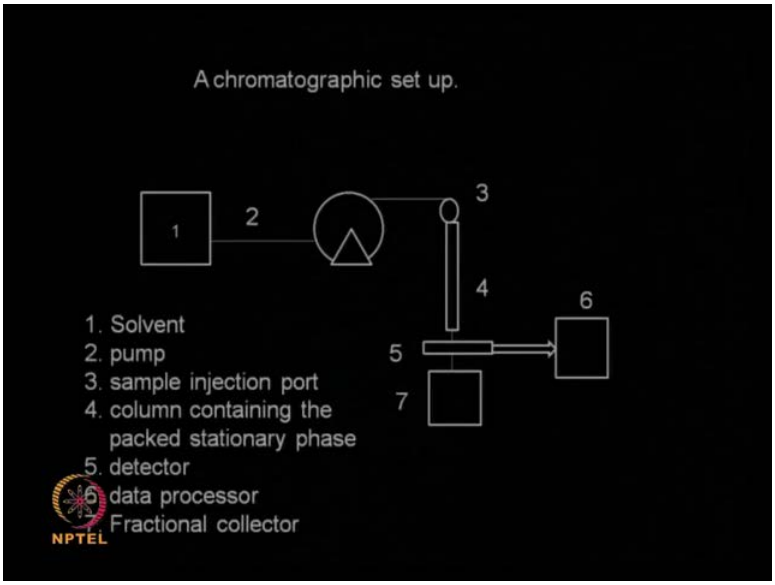

So, how does setup look like you have a solvent which is pumped, and then you have a injection port where you are introducing your mixture of proteins, or metabolites. Then there is a large long column, column could be running into hundreds of feet and then a there is a separation taking place, then we have a detector it could be a UV detector, IR detector, mass spectrometry detector. So, many types of detectors it will detect the various proteins that it coming out.

Then we can have here data logging system, which will store all the data for further processing. We can even collect each of the sample, as it is coming out and if the sample is pure we could do several other studies, which a metabolite I could do here mass spectrometer I could do enamore. So, that I can find out the structure of the molecule, if it is a protein I could do again here mass spectrometry using a maldi type of a instrument, or I could sequence the protein to understand the various amino as it present.

So, by collecting each of the fractions I could do several other analysis on this. So, this how here chromatography setup looks like.

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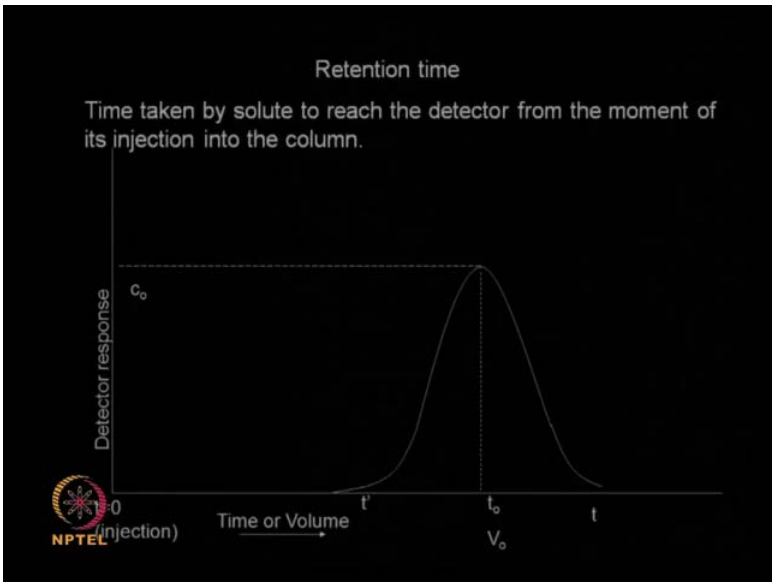

So, when the component comes out of the column, there is going to be a detector responds so it will appear like this. There is an increase in the detector responds, and then there is a decrease. Now, this time is called the retention time if it is in terms volume this is called the retention volume. And generally, we assume with this should be a very uniformly distributer normal Gaussian curve, but in reality does not happen you may have a slightly distorted curve, you may have a long start or you may have a long tail, but generally we assume it to be a normal Gaussian uniform distribution.

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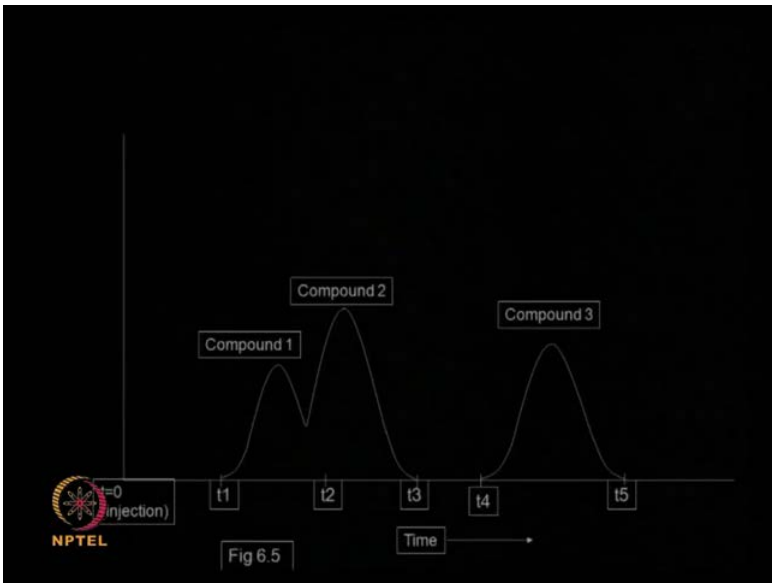

So, if you are many proteins or many components you may have the responds like that. So, this one comes out first followed by this one and then finally, followed by this one. So, this particular compound takes much longer time that compound 1 and compound 2. If you look at this chromatogram you see that compound 3 is nicely resolved whereas, compound 1 and compound 2 are not so.

So, if I start collecting compound 1, I may be even collecting compound 2 as impurity. If I am collecting compound 2 I may be collecting compound 1 also as an impurity. Whereas, if I am collecting compound 3 is going to be very, very pure there is no other impurity. So, ideally I would like to separate these two in a similar fashion, I have separated on compound 3.

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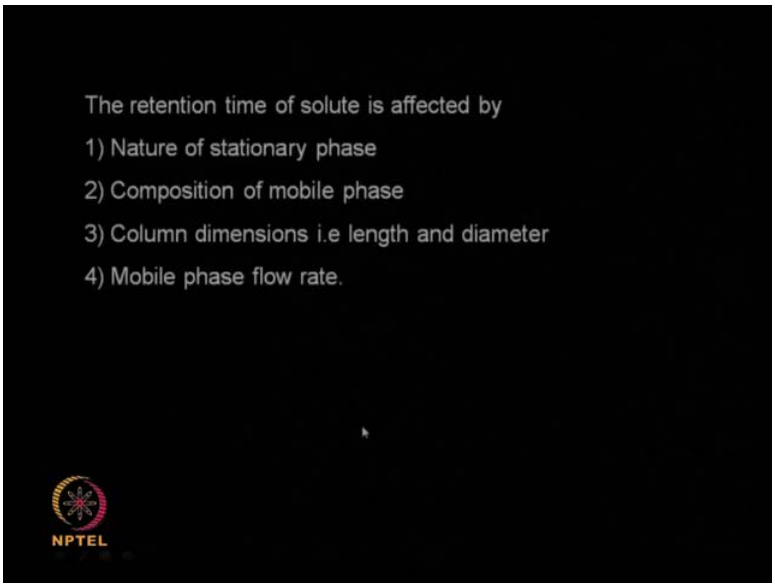

So, what are the factors that determine the retention time? Nature of the stationary phase type of stationary phase the porosity, particle size, particle size distribution, type of ions precision hydrophobicity of the surface so many factors. Composition of the mobile phase with the mobile phase these hydrophobic, is it hydrophilic solvent and so on actually. Then the column dimension, how long is the column, what is the diameter, what is the number of theoretical plates and then the flow rate of the mobile phase. So, all these factors determine the retention time of your sample.

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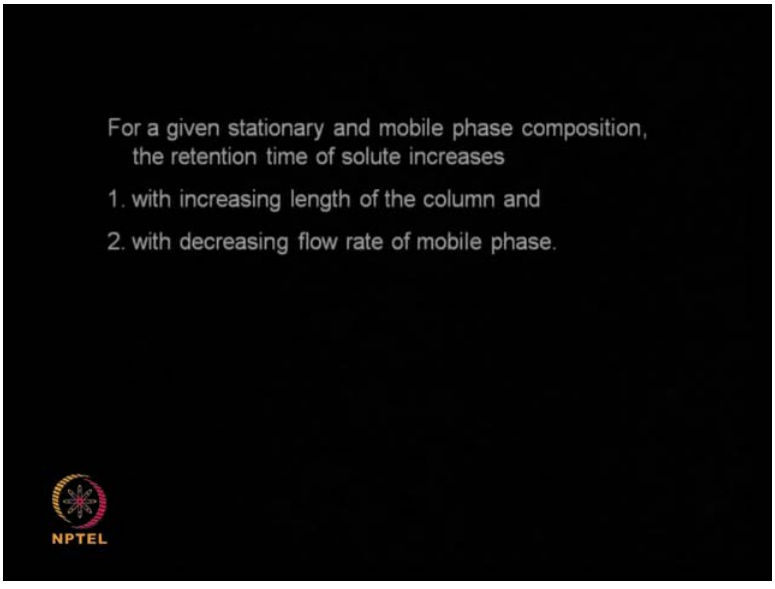

For a given stationary phase and mobile phase, the retention time of the solute increases with increasing length of the column it is obvious. So, if I have a longer column it takes much longer time for the sample to come out so this is obvious. The retention time of the solute increases with decreasing flow rate of the mobile phase. So, if the flow rate of the mobile phase is low obviously, it takes much longer time for the solute to come out. So, both these statements are very true and obvious.

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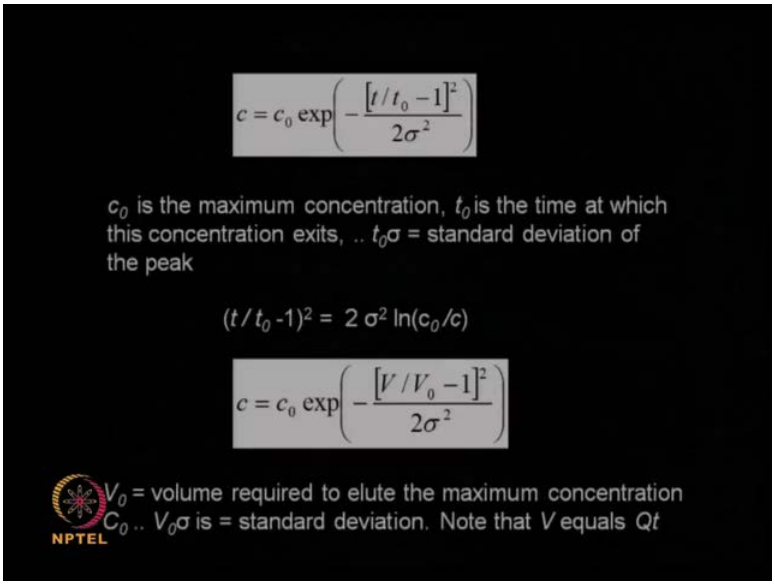

Now, let us look at or recap some of the equation which I introduce in the previous class. So, the concentration of the solute that is living the column is going to be in a form of a Gaussian distribution. So, the equation for a Gaussian distribution is c equal to c naught exponent minus t by t naught minus 1 hole square by 2 sigma square, c naught is the maximum concentration because it is a Gaussian distribution so maximum concentration.

And t naught is a time at which this concentration happens agreed, and t naught into sigma is the standard deviation of the peak, this is a typical equation for a normal distribution. So, we can take logarithm of these equation, you will end up with t by t naught minus 1 hole square is equal to 2 sigma square logarithm of c naught by c. Just like time that is retention time we can also have an equation for volume V.

So, what we say c equal to c naught exponent minus V by V naught minus 1 hole square by 2 sigma square V naught is the volume required to elute the maximum concentration. That is at that particular volume you will get V peak max and here V naught sigma will

be your standard deviation. So, of course, note V equal to Q into t Q is nothing but your flow rate, t is your time, and V is your volume. So, these two equations we saw last class and they come or the arise, because we assume the chromatogram to be a normal distribution, or Gaussian distribution.

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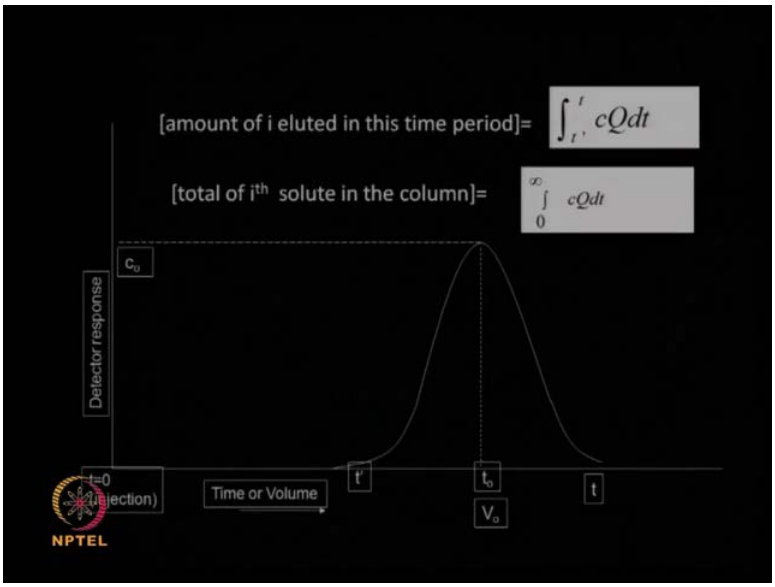

Now, the amount of i eluted in this time t and t dash will be obvious integral c into q into dt q is your flow rate, c is your concentration. So, amount eluted in this time is by this, but the total amount of this particular species, in the entire period of time will be from 0 to infinity that is also obvious. So, the that the total amount will be from 0 to infinity amount eluted in this time will be integral of t dash to t.

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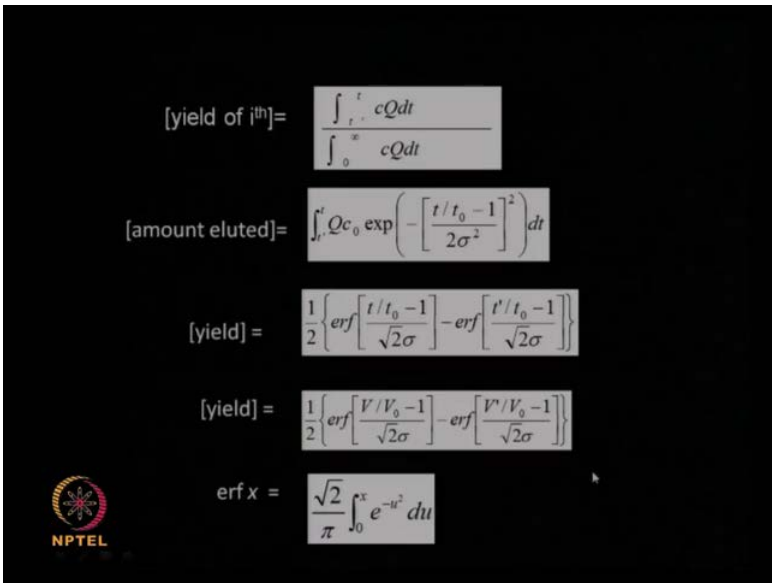

So, yield will be the amount you collect during this period t dash and t and the total between 0 and infinity. So, the amount eluted if we substitute for c I will substitute as c 0 e power minus t by t naught minus 1 square divided 2 sigma square d t. So, yield become half into error f this called an error function error f t by t naught minus 1 by square root of 2 sigma minus error f t dash by t naught minus 1 divided by 2 square root of 2 sigma.

Where error function is define like this error function x is equal to square root of 2 by pi integral 0 to x e power minus u square by d u. So, by calculating there are functions substituting here will be able to calculate what is the yield? Similarly, we can have an error yield equation for in terms of V also, we can put half error f V by V naught minus 1 divided by square root of 2 sigma minus error f V dash by V naught minus 1 divided by square root of 2 sigma.

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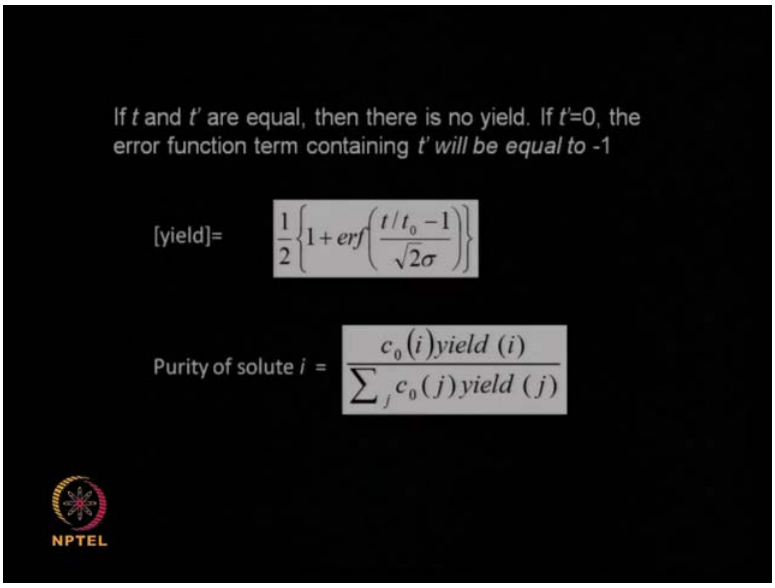

Suppose, we start collecting from the time equal to 0, then t dash will call it as 0. So, that particular term when you put t dash as 0, this particular term will be keep equal to minus 1. So, yield will become half of 1 plus error f t by t naught minus 1 by divided by square root of 2 sigma. That means, if I am collecting from a time t equal to 0 to time t and if t naught is the time at which you see the maximum concentration.

Then yield will be given by this particular relation. Now, any mixture of protein is going to have many components. So, there is always a purity factor involved in it. So, purity of solute i is given by c naught i yield i divided by various c naught yield j, j could be 1 to n. Where n could be the number of components present in your protein mixture, right? So, that is the equation for purity.

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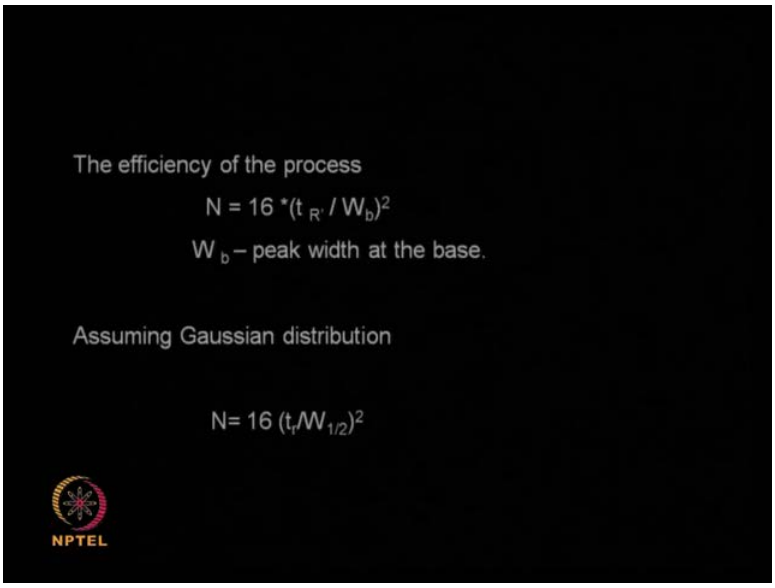

Then we also have some equations, which tell the efficiency of the process or the efficiency of your chromatogram. So, there is a term called number of theoretical plates N, N is given by this particular equation 16 t divided by W b whole square, N equal to 16 t R dash divided by W b whole square, t R dash is your corrected retention time.

W b is the peak width at the base, if I know the peak width I substitute here if I know the corrected retention time I substitute here, and I get the number of theoretical plates. So, instead of looking at the peak width, I can look at the width at half peak height that is called W half. Then I can use that equation and also, I can calculate the number of theoretical plates.

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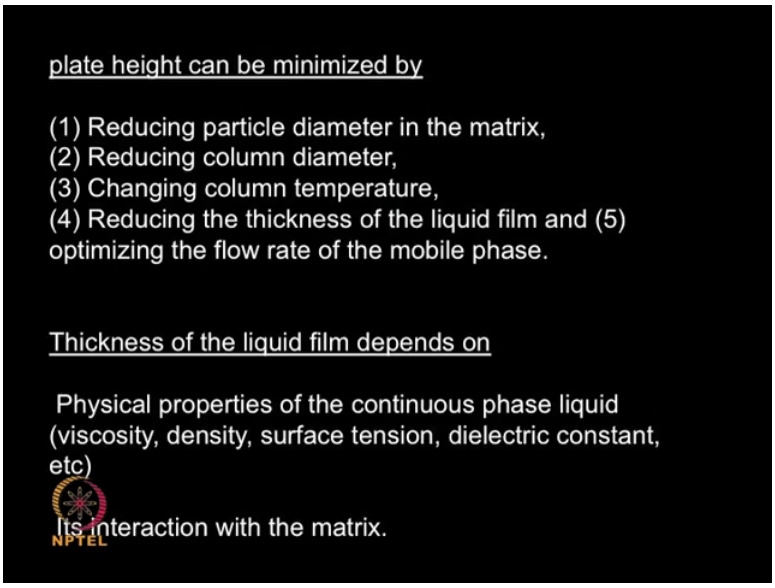

Now, the plate height can be minimized by reducing particle diameter in the matrix, reducing column diameter in the matrix, or changing column temperature, reducing thickness of the liquid film, and also optimizing the flow rate of the mobile phase. So, by doing all these I can reduce the plate height, when reducing the plate height. That means, I will have more number of plates for a given column length, more number of plates for a given column length.

Now, any particle which is their present in your stationary phase is going to be covered by a liquid film. Now, this liquid film is going to be dependent upon the physical properties of this continuous phase liquid like viscosity, density, surface tension, dielectric constant.

So, many factors actually. So, this liquid film is going to offer some resistance to the solute that is going to go and attach itself to the stationary phase. So, smaller the liquid film faster will be the attachment. That means, the resistance will be less bigger the liquid film resistance will be more.

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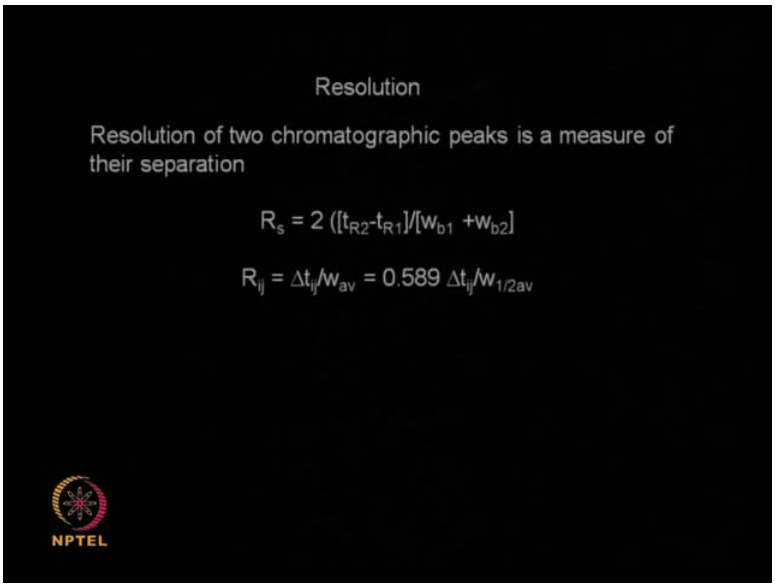

Now, there is another factor which is called the resolution. So, resolution of two chromatographic peaks is a measure of their separation, how close those two peaks are, how far those peaks are correct. So, the equation given here is resolution is given by the distance between the two retention times t R 2 minus t R 1, or delta t divided by the width of the peak at the base, or conversely the resolution is given by the difference in the retention time. That is delta t divided by the half height at half maximum, that is average of the height at maximum. So, w half average is nothing but the average at half peak height.

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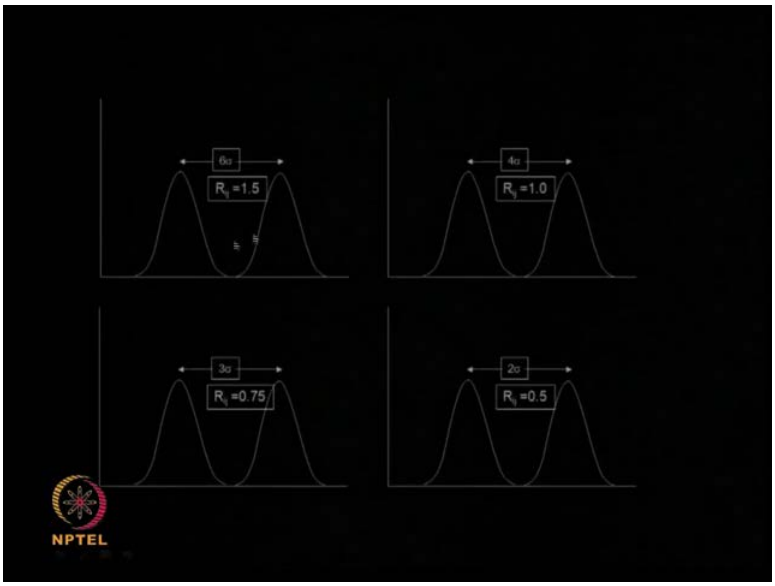

So, if you look at a 2 peaks like this you know, if they are away at a distance of six sigma then the resolution is 1.5, if they are at distance of two sigma then the resolution is 0.5 there at a distance of one resolution then there at distance of four sigma. If there distance of three sigma then I would say the resolution is at 0.75. So, depending upon the distance between the maximum of these two peeks, the resolution also varies starting from even 0.5 going up to 1.5.

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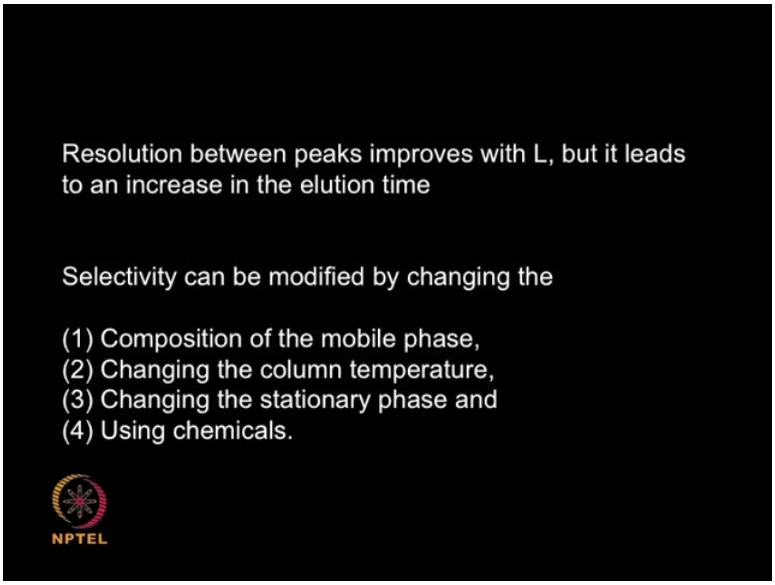

Now, resolution between peaks improves with length. So, if I have longer column, but I can have better resolution, but then if I am going to have higher resolution the elution time is also going to increase. That means, the peak will take much longer time to come out actually. So, how do you modify the selectivity, we can modify selectivity by changing the composition of the mobile phase, changing the column temperature, changing the stationary phase, or even using chemicals. We can add some chemicals which will improve the selectivity of certain solute towards the stationary phase.

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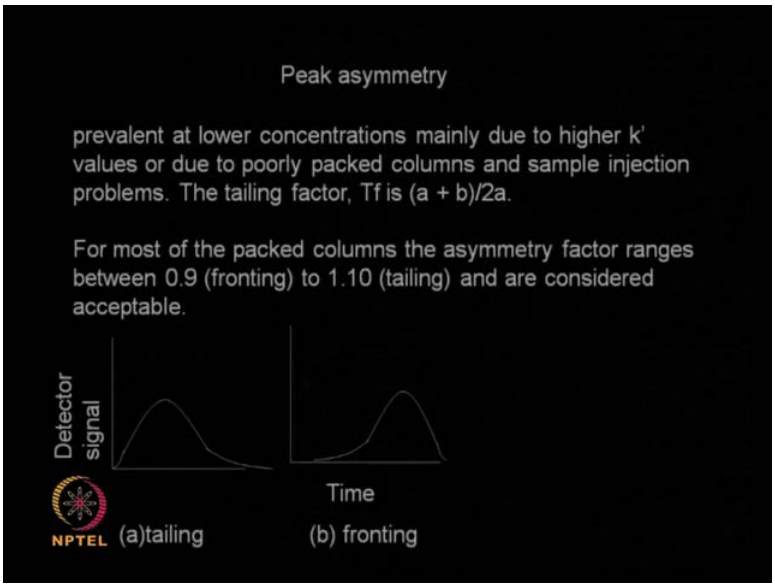

Last time you also looked at something called peak asymmetry because although, the equations which I have show shown or related to a Gaussian, or a normal distribution. In reality the peaks are not going to be a normal distributed peaks, it may either have a tailing or it may have a fronting. There something called a tailing factor which is a plus b by 2 a, where a is the distance from the start to the middle of their peak and b is the distance from the middle to the end of the peak.

Generally, you should have between 0.9 to 1.1. So, if it is 0.9 that means, you are having fronting that means, what is it you are a by b they are not uniform, or a is not same as b, but a is different from b. That is why you are having either the factor 0.9 or the factor above 1.1. So, if it is above 1.1 that we can called this as tailing if it is around 0.9 or less then we can call it as a fronting. That means in a case of fronting a is larger b is smaller in the case of tailing d is larger a is smaller.

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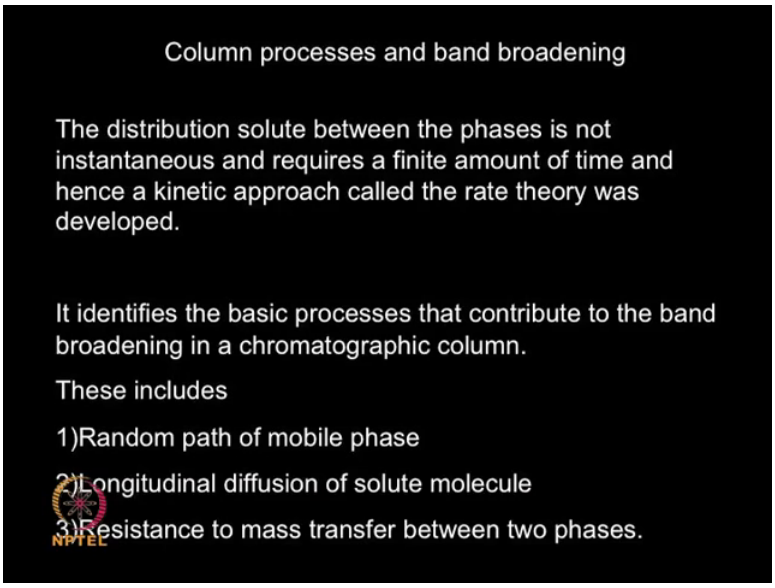

Now, what is column processes and band broadening. So, what happen is when the band keeps travelling inside your chromatogram it is starts becoming broader, broader actually because the solute is not going to get distributed instantaneously that is going to be certain time for it to distribute. So, there is a kinetic process there is a rate process and you need to consider that.

So, these processes needs to be considered when you are giving a finite time for the solute to get itself distributed between the stationary, and the mobile phase. Because the solute has to travel in a random path, through the mobile phase then there is a longitudinal diffusion of the solute molecules, then there are resistance to mass transfer between these two phases. So, all these factors come into picture hence, there is a finite time for it to get distributed, as well as there is a broadening of the peak as it travels from the entrance of the column to the exit of the column.

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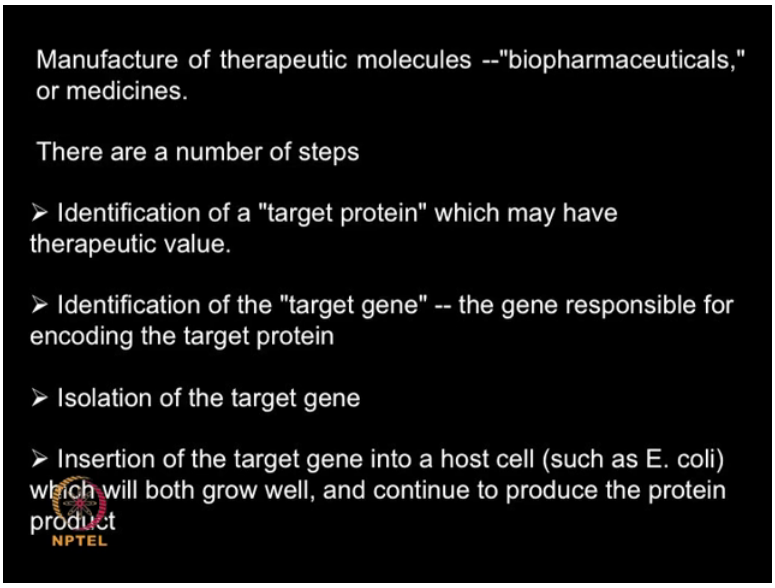

Now, let us look at a therapeutic molecule for example, you know biopharmaceutical or a medicine we need how do we go about deciding on a chromatography base separation. First of all you may identify what protein, which you want to purify from a mixture of protein. So, you may be identifying the target gene that is gene responsible for encoding the protein, you may isolate the target gene and then this gene may be inserted into a host cell more generally, E coli. So, that when it grows it continuously produces the protein product.

So, you would decide that I want to make this protein in access, you identify what gene makes that protein take the gene put it in E coli that is the host cell. So, when it grows the protein is produce in access. Generally, we like to have it in extra cellular type of situation we do not want the protein to be produced as a Golgi body or a intra cellular material. Then you have lot of problems about breaking the cells and extracting your product. So, you would like the protein to be in a extra cellular form.

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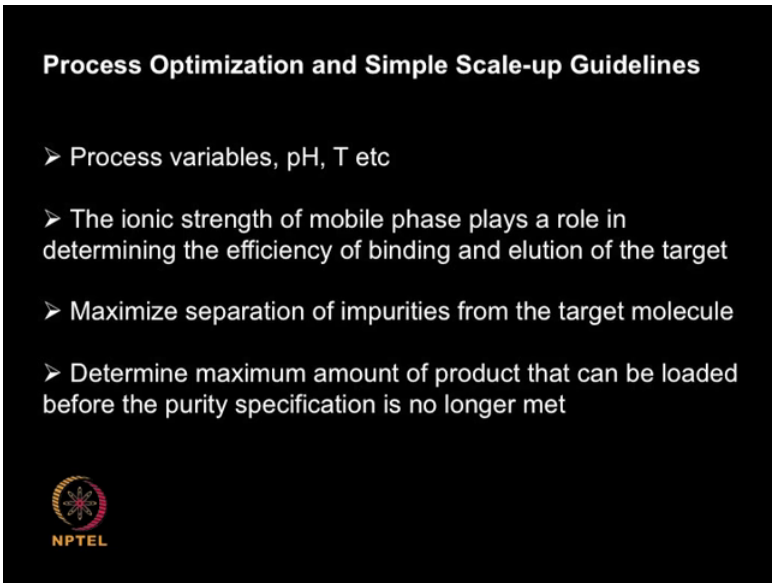

Then what do you do look at the how do you manufactures protein large quantity. So, you look at the process variables, we look at that pH temperature strength so many factors. So, all this needs to be considered, and then you go into the scale up. That means, large scale production of the protein. So, how do you go over doing the large scale production you have to consider the process to be as sufficient, as it was in the small scale. So, there are no differences when you scale it up from a large scale up to a commercial scale.

So, the ionic strength of the mobile phase plays a role in determining the efficiency of binding and elution, we also want separate the impurities from the target molecule. So, the impurities should be far away from the target molecule. And also you need to find out what should be the maximum amount of product that can be loaded before the purity specification is no longer met. Then we may have to resort to regeneration of the column.

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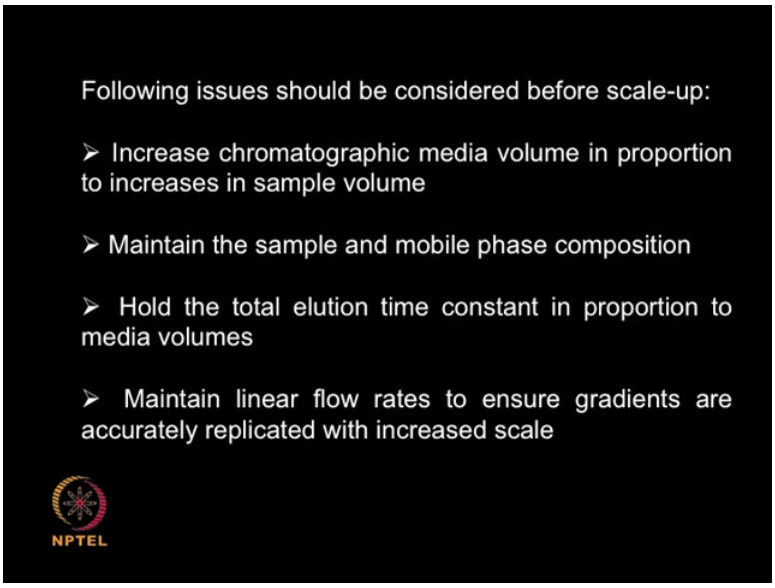

So, when you are scaling we have to consider several other factors, we have to look at increase chromatographic media volume in proportion to increase in sample volume. So, if I am going to have large sample volume. That means, I need to have more chromatographic media volume, but at the same time I need to maintain the sample and the mobile phase composition same.

Then I would like to maintain the same elution time just be if I am doing a protein purification in lab, and may elution time is 35 minutes I don't want to be running in hours, if I am going to scale up I want to see whether I can achieve the same elution time. That means, the same 35 minutes.

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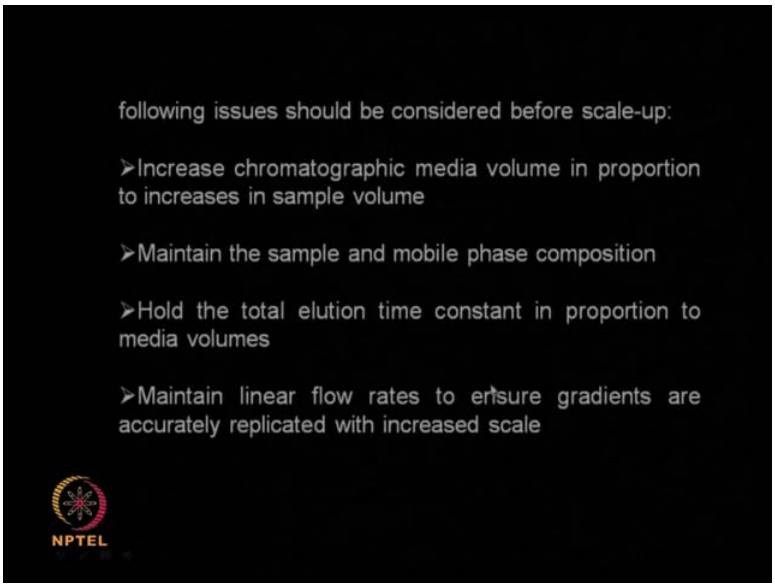

So, if I am going to increase the media volume, how much the elution time going to change. So, I need to consider that aspect also, I want to maintain linear flow rates so that when gradients are accurately replicated with increased scale. So, when I am doing a gradient or a mixture of solvent flow rates in small scale, I want to replicate the same thing in the large scale so that there is no discriminancy, when I scale it from small to large scale.

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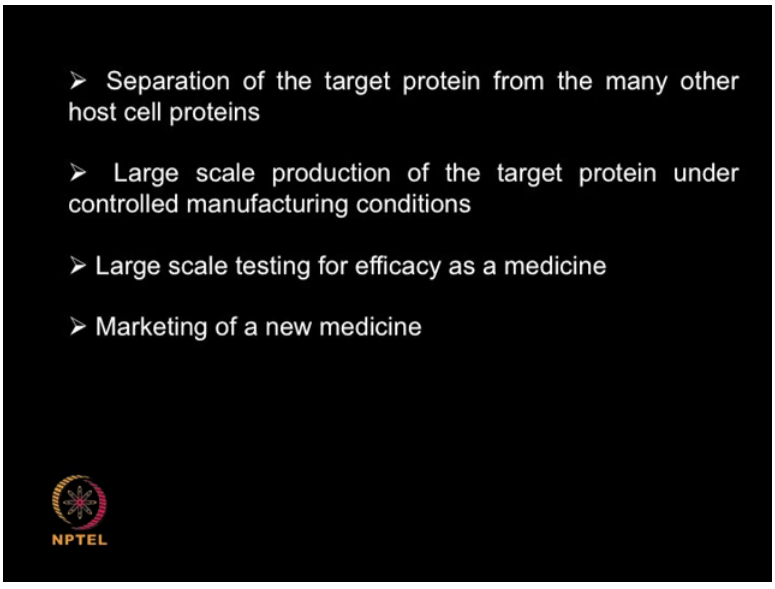

Then I need to consider this separation of the target protein from several hundreds of protein. Then I need to consider controlled manufacturing conditions, and when I say controlled you are talking about the ambient conditions ambient sterility, purity and so more so on actually. And then I should be able to do a large scale testing for efficacy as a medicine because you may make the protein in large scale, then you may have to test it out in several patients or cases to see whether, it works in that particular scale.

Then of course, once you have happy with the whole thing you are going into a marketing of a new medicine. So, all these factors need to be considered when you are thinking about a therapeutic protein, moving from the lap to the semi technical scale or moving from semi technical to full scale commercial manufacturing scale.

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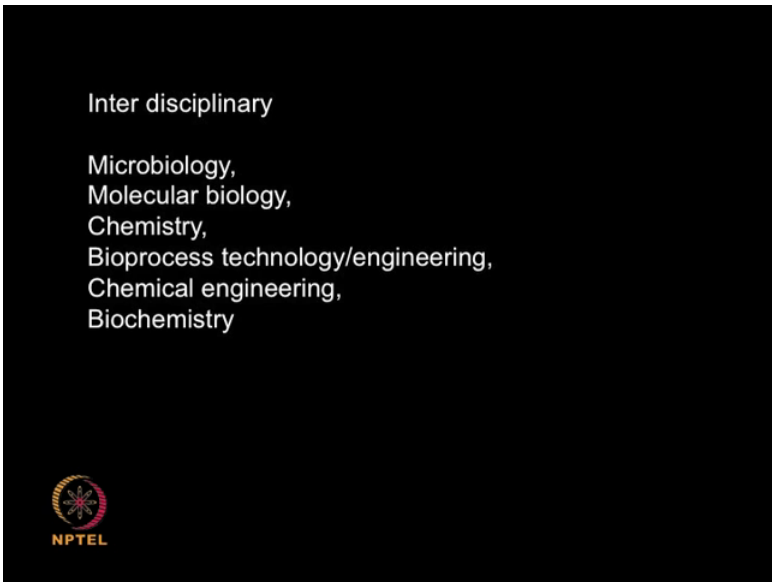

So, you see chromatography is highly interdisciplinary subject we need to have expertise of so many at different disciplines. Microbiology comes in, molecular biology, the chemistry, the bioprocess technology and engineering, chemical engineering principles, biochemistry principles and so on. So, we need to consider all these aspects, when you are designing a chromatography system for a purification or separation of a protein.

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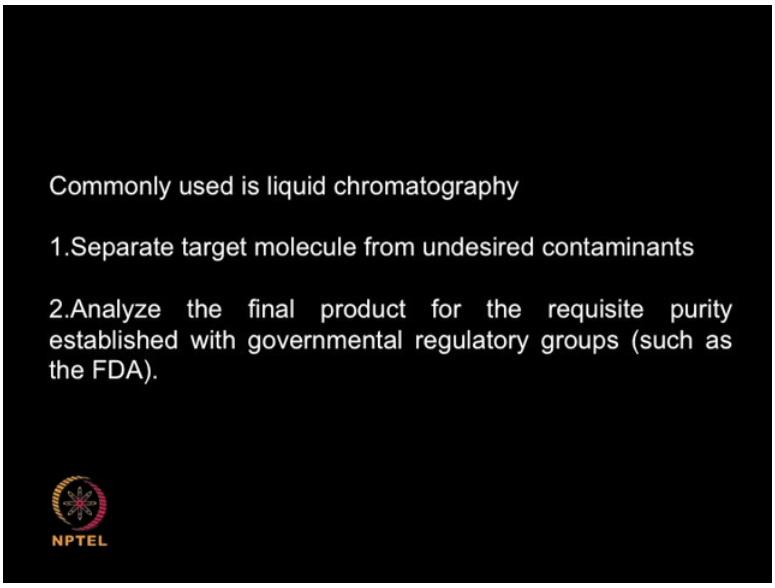

We use liquid chromatography for separating target molecules from undesired contaminants, or we can use liquid chromatography for analyzing the final product. And see whether, the requisite purity has been reached with respect to certain regulatory conditions like FDA that is the food and drug administration stipulations.

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We have the high performance liquid chromatography, or high pressure liquid chromatography it is generally, used in laboratory situations for finding out an unknown component, or finding out the concentration of the unknown component. So, you can use

it for environmental testing, we want to identify pesticide or a toxin in a sample liquid or a gas sample. It can be used pharmaceutical industry for separation of chiral molecules, it can be used in pharmaceutical industries for identifying impurities present. So, HPLC that is the high performance liquid chromatography, or high pressure liquid chromatography here has quite a lot of applications.

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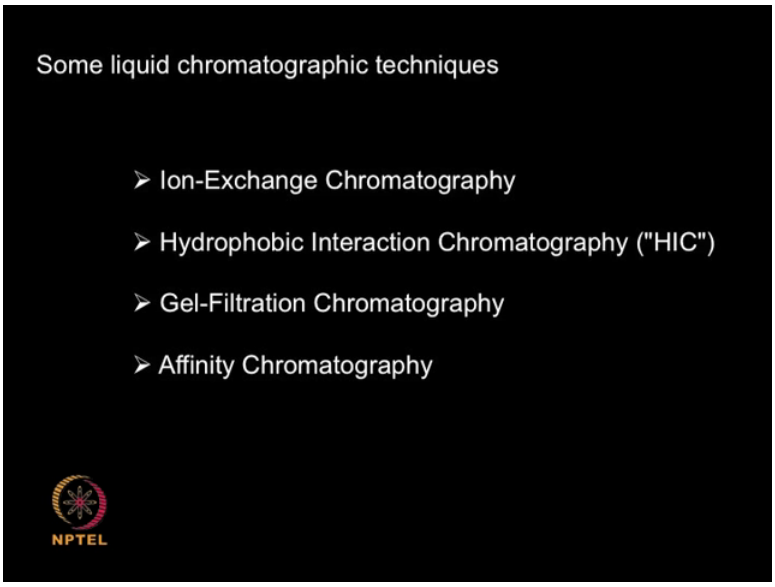

So, some liquid chromatography techniques, ion exchange hydrophobic interaction gel filtration affinity and so on. Actually, we will be spending time on each one of them in more detail.

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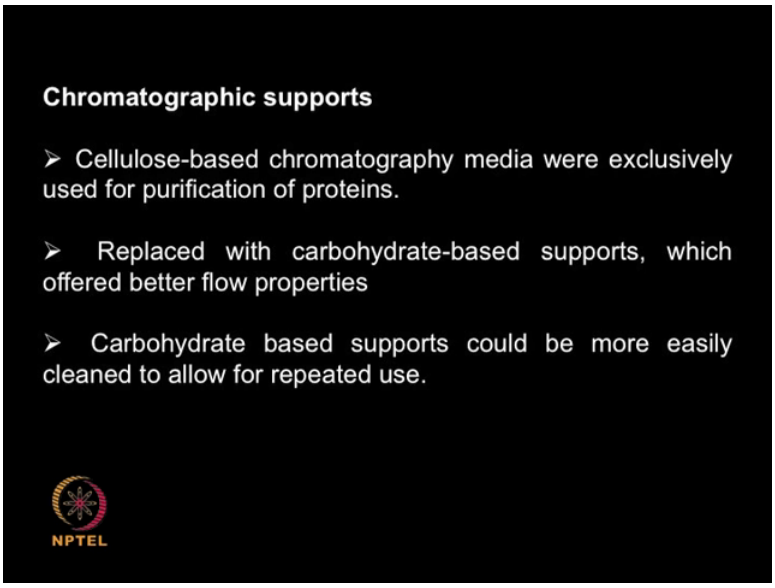

What are the supports, the stationary phases going to have or the fixed phase. Originally they use to have cellulose based media for purification of proteins. Later on they moved into carbohydrate based supports, because it offers better flow properties, because it can be clean properly after repeated use because you want to reuse, reuse many, many how thousands of hours.

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Then as time moved on they went into synthetic polymeric material, polystyrenes and different types of polymers because we can achieve very high flow rate, we can have

good designs, stability it is easy to clean and we can achieve very high purity, so currently only polymeric stationary phases are used.

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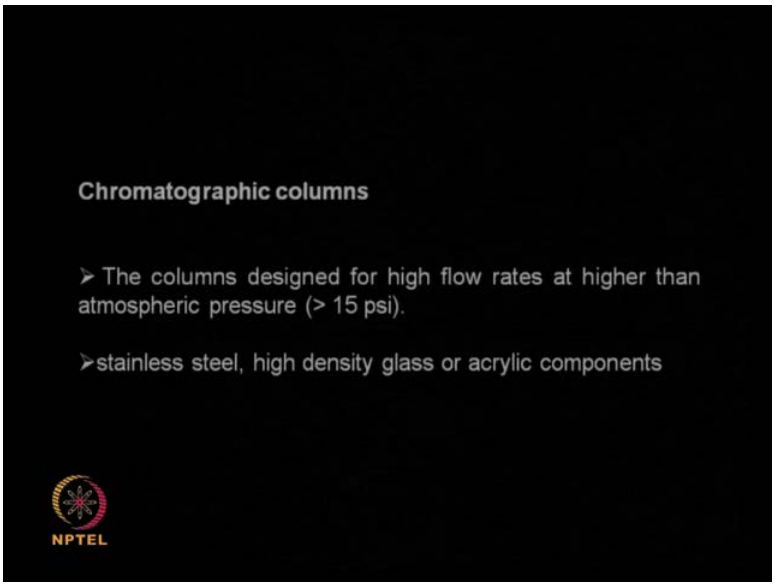

The columns if you look they are designed for high flow rates, and for atmospheric pressure or very high very high pressure. Types of columns used stainless, steel high density glass, or acrylic components and so on depending upon the system, which you are studying and the type of components you are studying.

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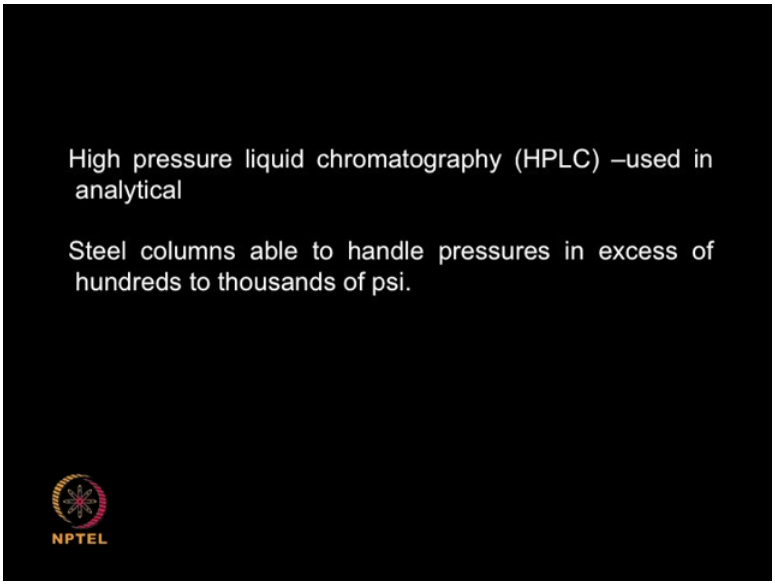

If you take high pressure liquid chromatography or a high performance liquid chromatography. We are talking in terms of hundreds and thousands of psi and the steel columns are generally used.

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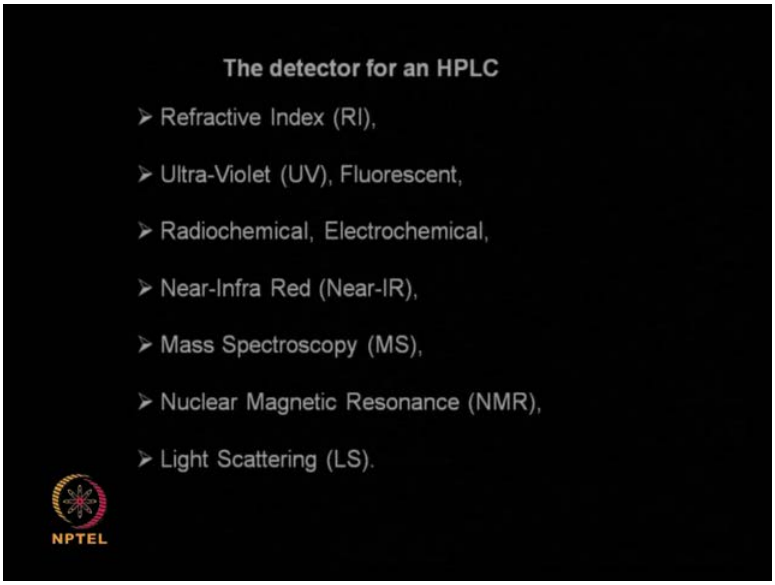

Detectors they are the most important, which detects or a particular metabolite or a protein. So, a large number of detectors are currently available in the market. Refractive index detector R I, if there is a change in the refractive index with it gives out a signal or a responds.

Then you have a ultra violet detector UV, then you have fluorescent detector if your molecule is fluorescing, then that is a very good detector. We can have a electrochemical or a radio chemical detector. So, if it is a radioactive material then detector will immediately spot.

You can have a near infrared detector, then we can have a mass spectrometry detector. Mass spectrometry detector can be used to determine the mass of the component, we can have a nuclear magnetic resonance or NMR. So, it can identify these state of the proton or state of the carbons.

We can have a light scattering detector, so what happens here in the component flows light gets scattered, the light that is going to the receiver is diminished. So, that is the measure of the concentration of the particular component. So, wide range of detectors

can be used depending upon the how, the solute behaves either it has got a fluorescent capacity, or whether it has got a mass giving out a large mass value or whether it responds at ultraviolet range and so on actually.

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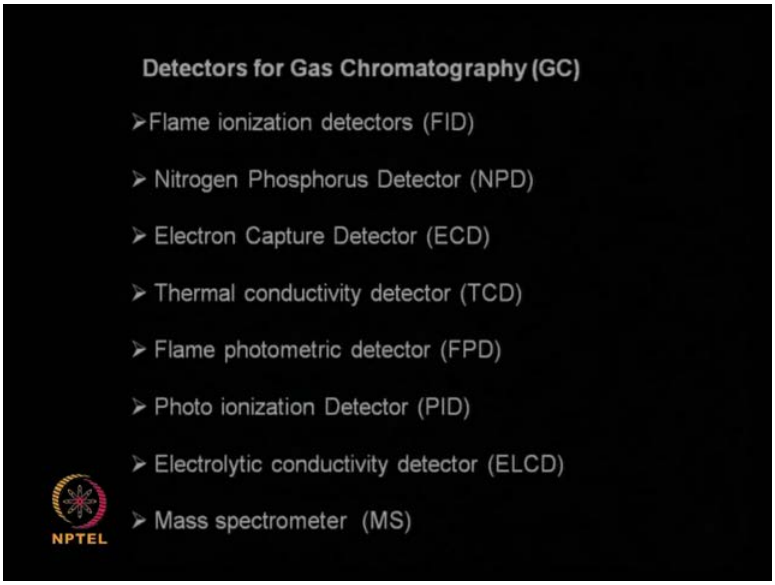

So, go to gas chromatograph then again you have several other detectors, we have the flame ionization detectors; that is called the FID. We have nitrogen phosphorus detectors, we have electron capture detectors, thermal conductivity detectors. So, if a component flows and it changes or affects the thermal conductivity of your standard, or the base material will get a responds. Flame photometric detector, photo ionization detector, electrolytic conductivity detector, mass spectrometry detector.

So, this is like a GCMS just like liquid chromatography, mass spec you also have a gas chromatograph mass spec. Flame ionization detectors are widely used very accurate. So, the component is ionized, and then that signal is red in form of a current and that is why the flame ionization detector works. So, depending upon the type of molecular studying situation, we can have different detectors possible incorporated into your system.

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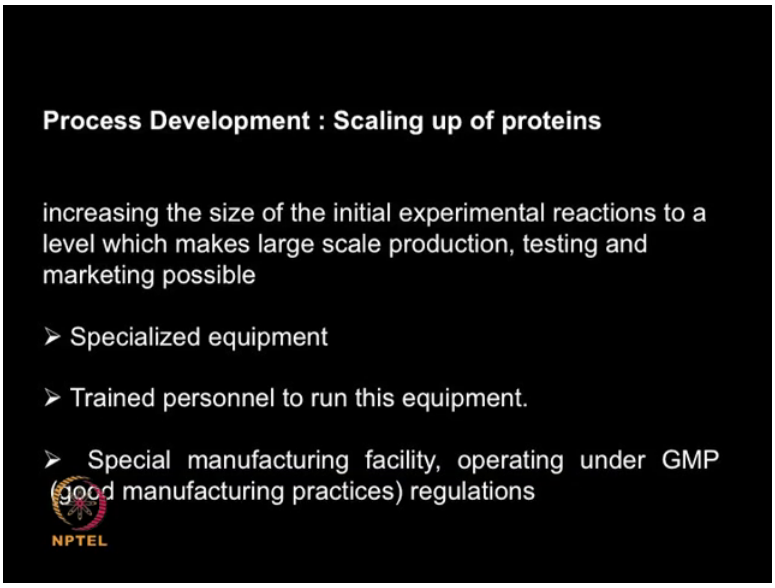

So, when you are scaling up proteins let us go back to proteins again, we want to move from a lab or experimental scale to a large scale production, testing, marketing as I talked sometime back. So, we are talking about specialized equipment trained personnel to run this equipment, manufacturing facility, GMP you must herd about GMP good manufacturing practice, GLP good lab practice and regulation. So, all these points need to be considered if you are thinking about scale up actually.

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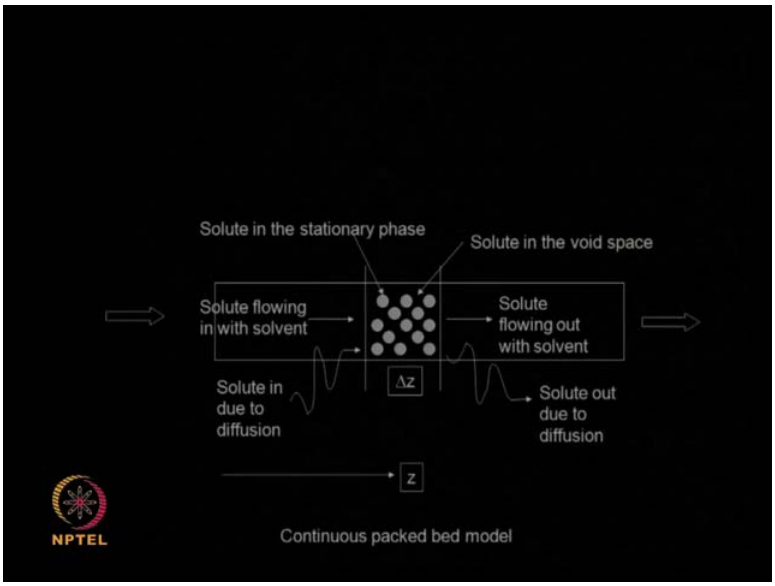

So, a chromatograph is nothing but a long tube, we have stationary phase particles here. So, a solvent flows from component solvent comes out from the other end, solute is getting carried through the solvent and there is an interaction between the solute, and the stationary phase. So, there is a separation of the various components. So, what are the things happening here solute flowing in with the solvent solute flowing out with the solvent, solute is coming in because of diffusion solute is coming out because of diffusion. There is no conversion of the solute so there are no reactions taking place. So, we can balance all the input flows with all the output flows.

So, you can have partial differential equation relating concentration as a function of distance z, and also a function of time because there is going to be buildup of concentration in this particular region delta z. So, the buildup of concentration of a solute in this region will be equal to the solute flowing in through the solvent, solute flowing in because of the fusion minus solute flowing out, with the solvent minus solute flowing out due to diffusion. So, if you are interested in using a packed bed type of approach then this is how you do the modeling. So, what terms will come flow rates will come into picture diffusion coefficient will come into picture.

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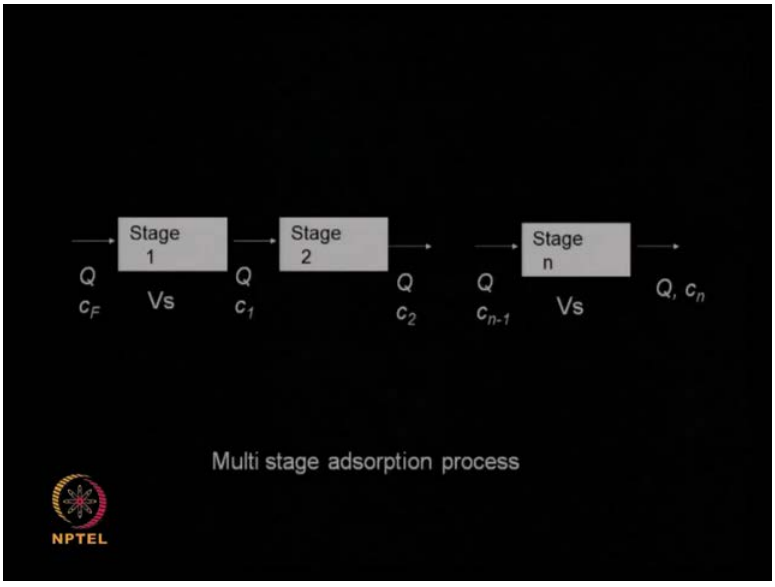

Another approach we can think of the whole column into multiple stage. So, there could be n stages each stages of equal volume V s, V s, V s. So, if you add up all these that could be the total volume of your pack bed. So, what happens, each stages well mixed

like a continuous third tank vessel. So, there is solvent flow and the solute flowing inside at a concentration c F, then again there is a solvent flow out, there is a solute flowing out at a concentration c 1, but inside things are well mixed, we can consider it as a store tank vessel.

And then this material is flowing into the second stage. So, the concentration of the solute entering c 1 concentration of the solute living a c 2 and so on. Until finally, you will have c n that is the concentration of the solute coming out at the n th stage. So, each stage could be considered as an ideal well mixed situation, and there are n stages where things are happening. So, this is a multi stage discrete stage module.

So, the previous case I said it is a pack bed model this is called a discrete stage model. So, people have attempted to model the solute partitioning, or solute adsorption from these continuous phase into stationary phase by using either here pack bed model, or a multistage ideal model. You will look at these models once again later on, in the course of this particular adsorption.

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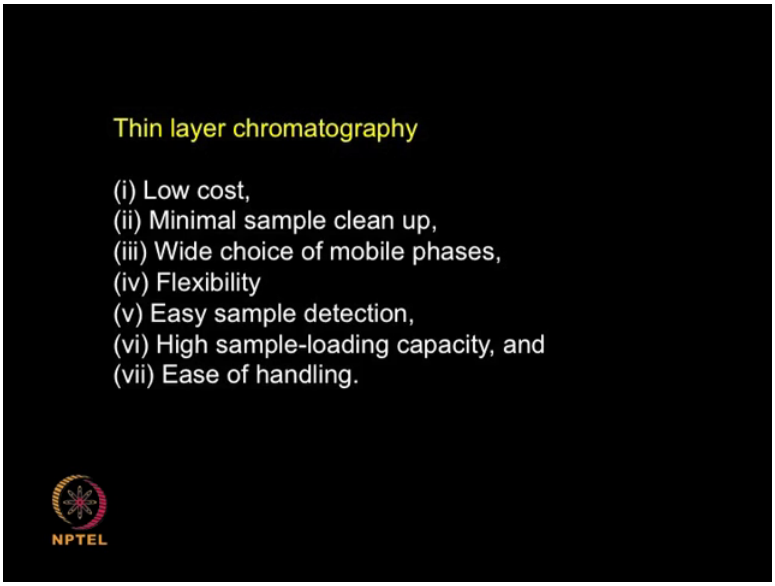

Now, a well known chromatography which is practiced by organic chemistry for the past 50, 60 years is called TLC, thin layer chromatography. It is a low cost it reads minimum sample so cleaning up is easy, we can use a wide choice of mobile phases, it is very flexible, easy sample detection, we can even load high samples, and ease of handling. So, all these are very, very advantages which leads for the use of TLC by all the organic

chemistry. So, synthetic chemistry if it is preparing something in his lab immediately, he checks whether the product has formed whether intermediates of formed by using a TLC thin layer chromatography.

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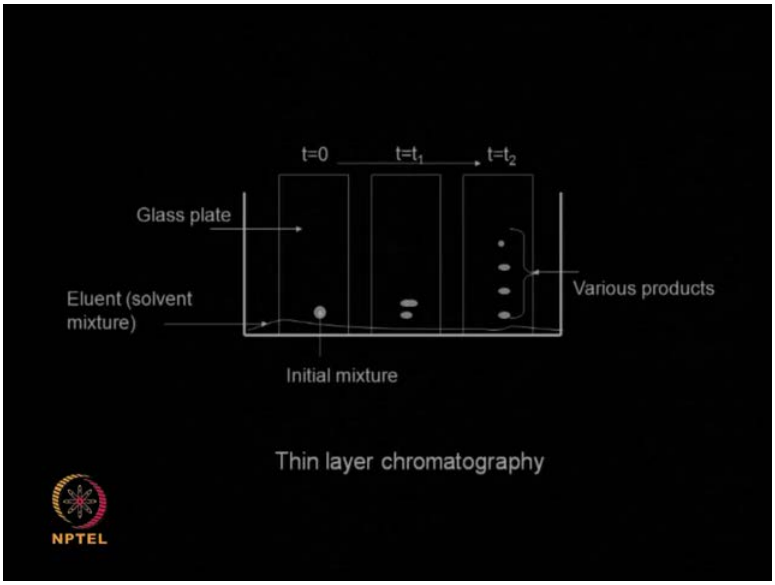

So, what does it do? He has a glass plate and then on top of the glass plate he has coated silica. Then he puts in his sample mixture, he dips it into a solvent this could be a single solvent multiple solvents. So, as the solvent travels upwards, it carries some of the components. So, over a period of time the various products are separated out. So, initially you would make a spot of your initial mixture, and it repeat in the solvent as the solvent raise because of capillary action, it separates the various components of your product, and you will end up having several spots. So, you could say each one of them is a product.

So, you have to play around with the solvent mixture so that you get good separation of the spots, this is called thin layer chromatography so simple. And if you know a particular product may be formed and if you have an authentic sample of that product from some manufacturer. What do you do? You spot it and see where it comes that can tell you whether one of the product is what the product, which you expect to form so simple, very simple requires very minimal cost and easy to test it out.

So, if you have 2 or 3 expected products and we have authentic, or pure samples of those 2 or 3 you can spot it, along a along this particular product and see where it goes and

stops. Then you can say this is possibly that product, this is possibility with the other product and so on.

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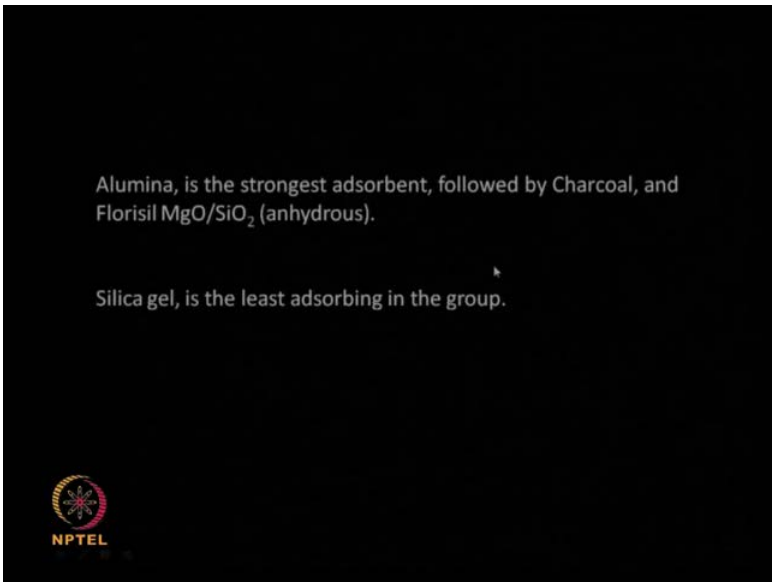

So, we use alumina strongest absorbent followed by charcoal, florisil, magnesium oxide, silica. We can use silica gel which is the least adsorbing in the group. So, different types of a coatings can be used.

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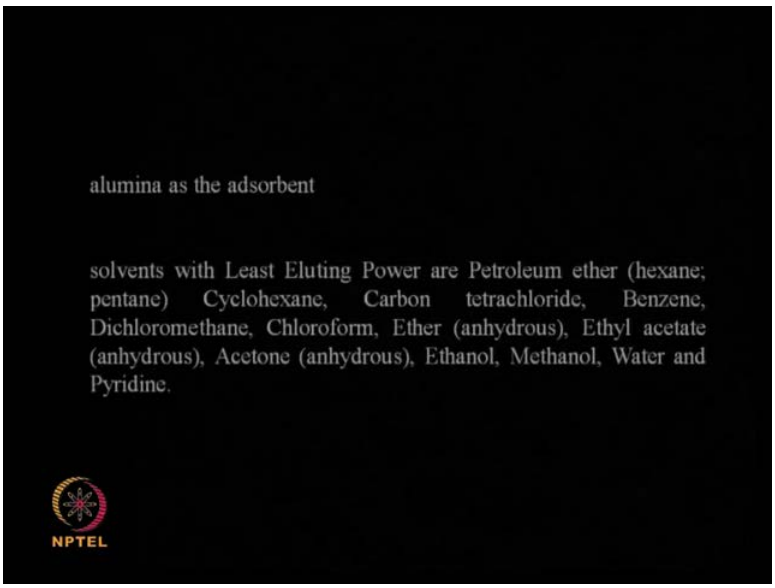

So, if alumina is the adsorbent solvents with the least eluting power are petroleum ether. That is hexane, pentane, cyclohexane, carbon tetra chloride, benzene, dichloromethane,

chloroform, ether, ethyl, acetate, acetone, ethanol, methanol, water and pyridine. So, that is how it goes solvents with the least eluting power.

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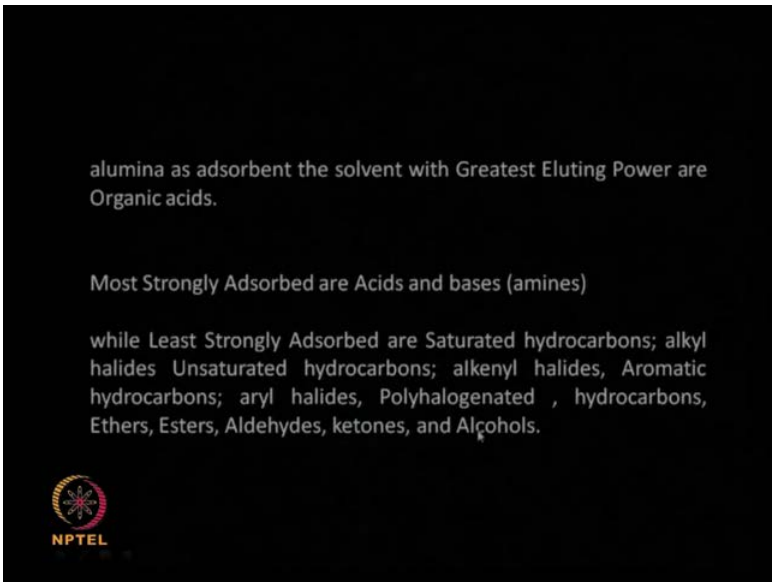

If you use alumina as adsorbent solvent with the greatest eluting power are organic acids. So, most strongly adsorbed are acids and bases while least strongly adsorbed are saturated hydrocarbons, alkyl halides, unsaturated hydrocarbons, alkenyl halides, aromatic hydrocarbons like aryl halides, polyhalogenated, hydrocarbons, ethers, esters aldehydes, ketones and alcohols.

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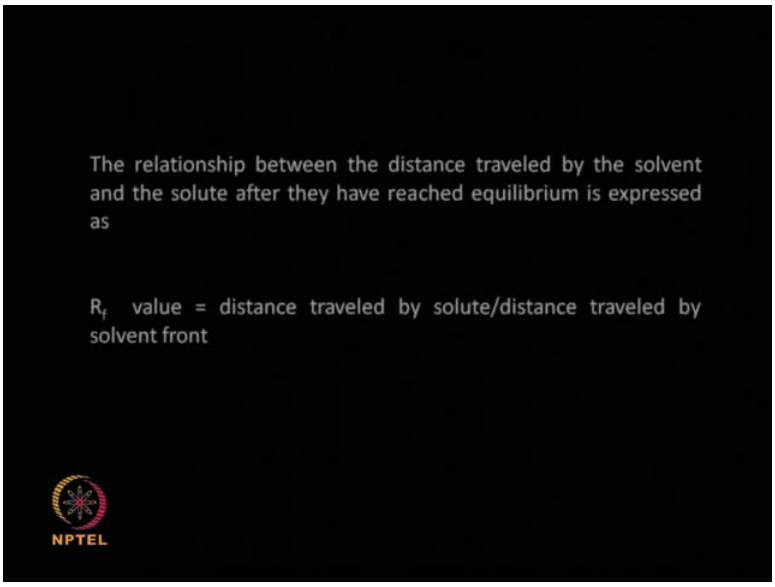

So, the relationship between the distance travelled by the solvent and the solute after they have reached equilibrium is represented as R f values. So, R f is nothing but distance travelled by the solute divided by distance travelled by the solvent front. So, by looking at this ratio, we can we can tell how fast how far the solute will travel with respect to the movement of the solvent. So, different components will have different R f values.

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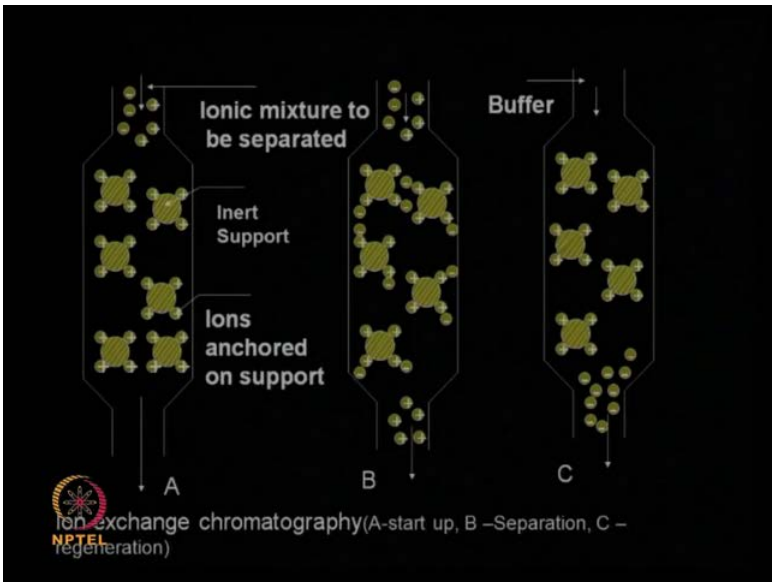

Now, let us look at ion exchange chromatography. So, the separation is based on ionic forces could be cationic forces, it could be anionic forces. So, what we have, we have a base inert support now a day's mostly, they use synthetic polymers. And then we may have a certain set of ions you know. For example, in this particular example they got lot of plus ions. Now, when you feed a mixture of plus and minus, what will happen all the minuses will bind to the pluses and the pluses will go away.

So, if there are proteins, which has got a positive charge they will not get bound to the support they will travel out. So, proteins with negative charge, will be retarder or bound later on when I use a buffer, I can remove all these negatively charge protein. So, if I have a positively charge protein, and negatively charge protein with this type of ion exchange, I can remove all the positively charge protein and retain only the negatively charge protein, which can be later separated by changing the pH or the buffer. So, now you are ion exchange chromatography is again ready for another batch of positive, and negatively charge proteins.

So, similarly we can also have negative ions anchored to the support and same thing can be performed. So, you can have positive ions anchored to the support, we can have negative ions anchored to the support. So, either you can have anion exchange chromatography, or you can have cation exchange chromatography or you can have both the ions anchored to the support. That means, such a chromatography that is a combination of anion and cation exchange chromatography can separate out all the salts from a protein.

So, if I have here salt precipitation using salting out method, you are going to have salt and proteins. So, what do you do you pass it through a anion, cation exchange chromatography which will have both anions and cations anchored to the support. And this salts will be removed and only the pure protein will flow out. So, depending upon the capacity of the chromatography, you will stop the process regenerate the column and again you do it and so on you can do that. So, a combination of anion, cation exchange chromatography can help you to separate sodium chloride, or any other salt from protein so you will get pure protein and you can remove all the salt.

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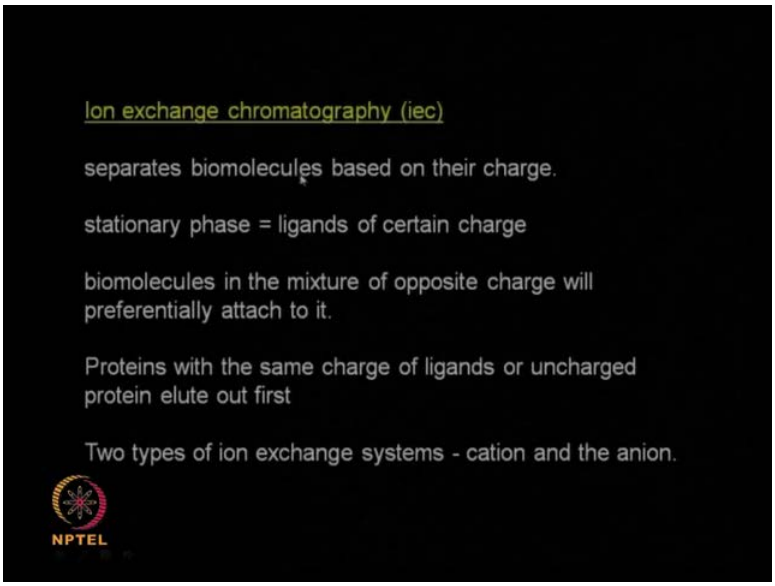

So, ion exchange chromatography is very good for bio-molecules based on charge. So, on the stationary phase, we attach ligands of certain charge. So, bio-molecule in the mixture of opposite charge will preferentially attach, proteins with the same charge of ligands or uncharged proteins elute out first. So, we can have two types of ion exchange

chromatography cation and anion, and you can have cation anion mixed chromatography is also possible.

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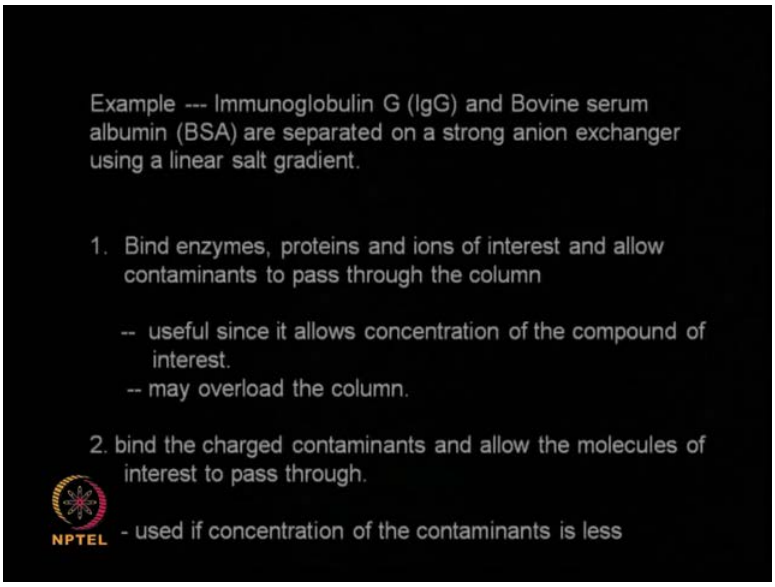

So for example, immunoglobulin G (IgG) bovine serum albumin BSA are separated on a strong anion exchange using a linear salt gradient. So, we can bind enzymes proteins and ions of interest and allow contaminants to pass through the column. So, whatever you need of interest will be held in the column rest all will pass out. This is useful it allows concentration of the compound of interest. So, that means your compound of interest will get concentrated, but it may over load the column also because compound of interest keep on binding to the stationary phase, and after some time it will not be able to bind.

Another approaches bind the charged contaminants and allow the molecules of interest to pass through, this is a very good technique if the contaminant concentration is very less. So, all the contaminant we will retained inside the column whereas, your product of interest will be living the column continuously. So, we can think of both the approaches either bind the product of interest in the column, there by getting grade of contaminant initially, or bind the contaminant and allow the product of interest to elute out first.

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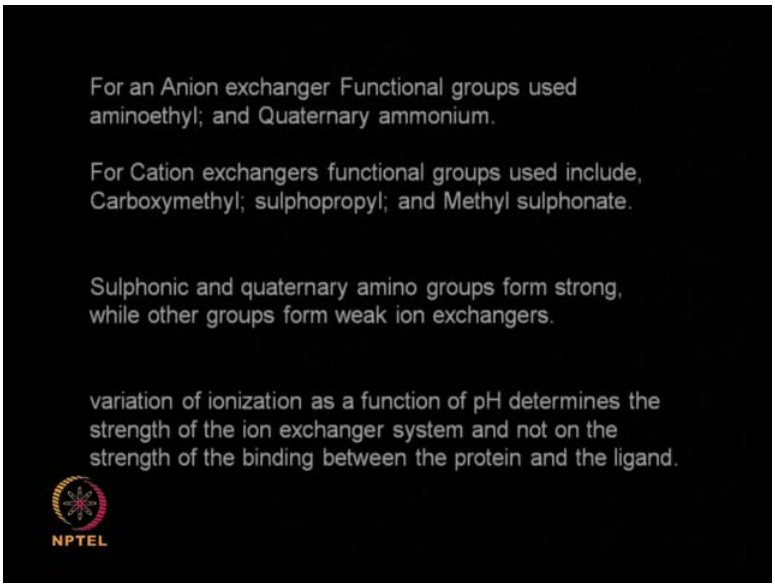

So, for an anion exchanger the functional groups used aminoethyl and quaternary ammonium. For a cation exchanger, the functional groups used include carboxymethyl, sulphopropyl and methyl sulfonate, sulphonic and quaternary amino groups form strong while other groups form week ion exchangers.

So, variation of ionization as a function of pH determines the strength of the ion exchanger system. So, the ionization as a function of pH tells you the strength of the ion exchanger, it does not tell on the strength of the binding between the protein and the ligand, it does not tell how strongly the ligand is bound to the protein, it just tells you the strength of the ion exchanger that is all.

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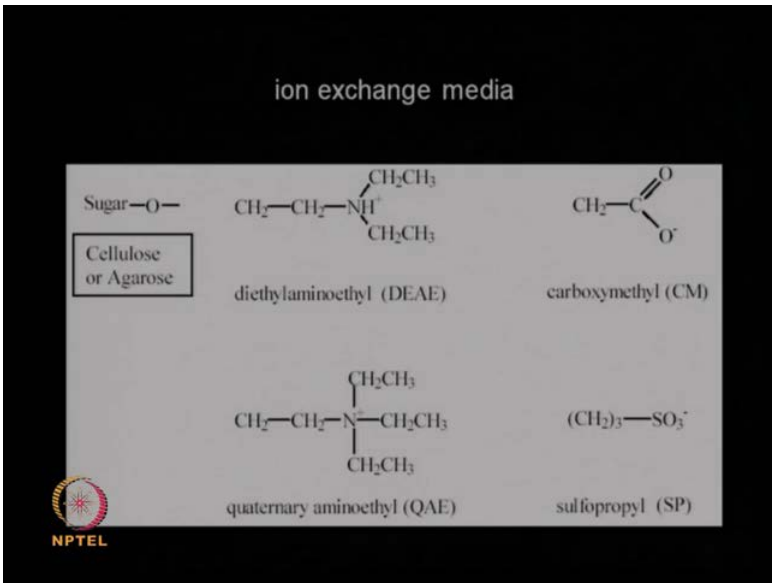

So, if we have cellulose or agarose type of medium if we have diethylaminoethyl. So, we can have positively charged groups present on the column, or you can use carboxymethyl which will have a negative charge or you can use a sulfopropyl, which will have a negative charge. If you have a quaternary aminoethyl, it will have a positive charge. So, you can generate different types of charges on the surface of the protein by using different functional groups. A nitrogen based functional quaternary groups, or o-minus carboxy groups.

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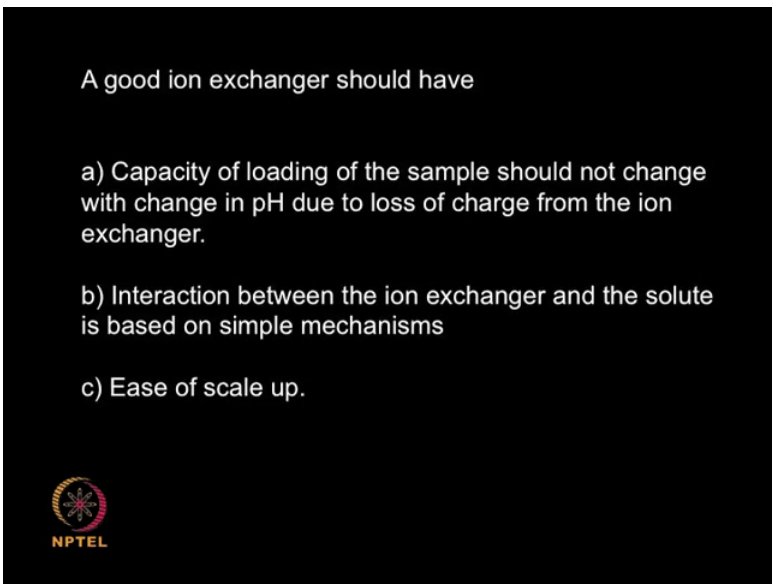

A good ion exchanger should have capacity of loading of the sample, should not change with the change in the pH. So, change in pH should not affect the capacity of the loading or due to loss of charge from the ion exchanger. Interaction between ion exchanger in the solute should be based on simple mechanism, it should be easy to scale up because I may be wanting to do ion exchange in a very large scale set up, then I want to scale it up to a very large manufacturing setup. So, it should be easy to scale up.

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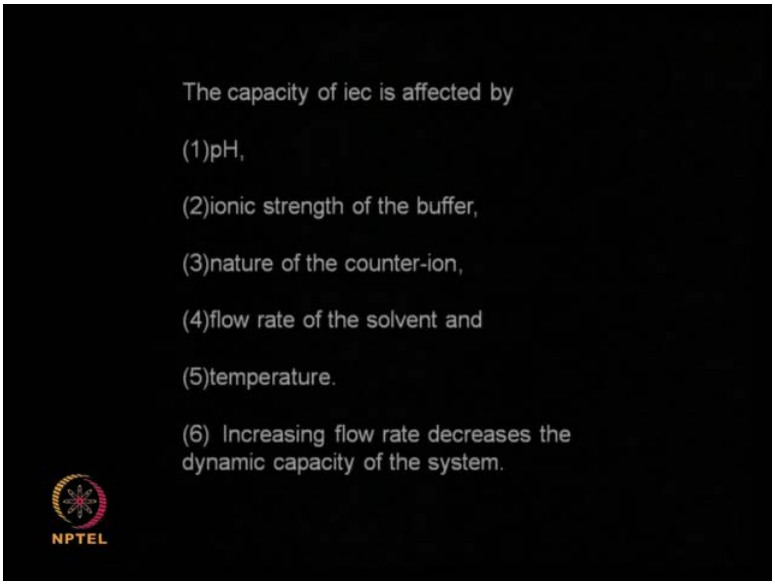

The capacity of ion exchange chromatography depends upon pH ionic strength of the buffer, nature of the counter ion, flow rate of the solvent, temperature and so on. So, if I increase flow rate it decreases the dynamic capacity of the system.

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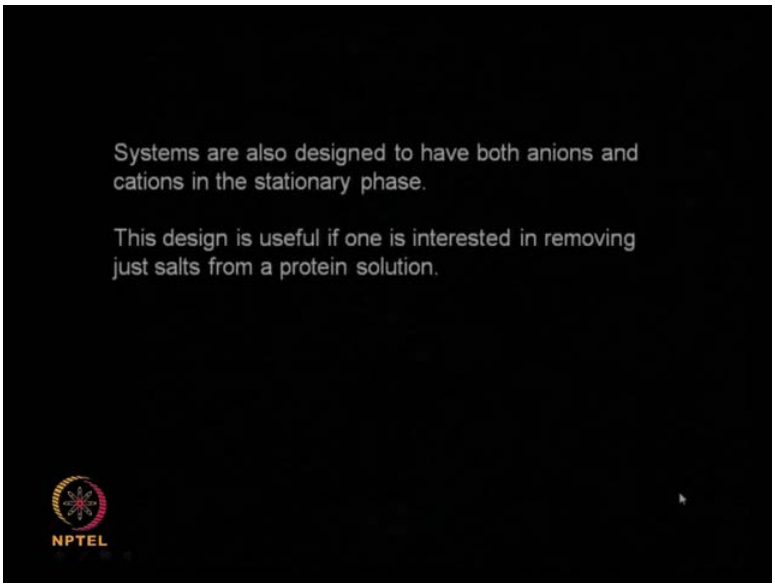

So, you can as I said originally we can have combination of both anions and cations in the stationary phase, that way we just remove all the salts that are present from a protein. So, a protein entering with protein and salt, what is living will be pure protein no salts. So, that is also a very good system. So, you may have a salting out facility followed by a anion, cation exchange combination.

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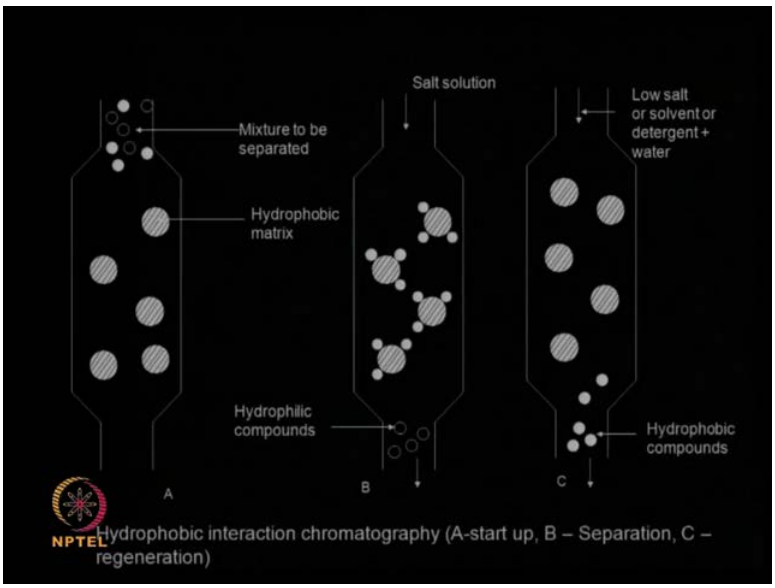

Let us look at a next chromatography system, this called hydrophobic interaction chromatography. So, we have matrix which have hydrophobic group present. So, you

may have a mixture of proteins with hydrophobic, hydrophilic. So, what happens hydrophobic proteins get attached to hydrophobic matrices, whereas hydrophilic proteins or compounds live fast.

So, from a mixture of hydrophobic and hydrophilic we may have hydrophilic components or proteins coming out first, the hydrophobic proteins or components are retained. Then we can change the salt concentration or we can change the add detergent and so on. So, whatever proteins that got attached or bound will get released, this is what is the hydrophobic interaction chromatography is all about. Now, we will look at this in more detail in the next class.