Downstream Processing
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Lecture - 30
Chromatography (Continued)

We have been looking at that Chromatographic separation, the past few lectures. Chromatography is the most important downstream, purification step. The main advantage of chromatography is it can be used for small molecules, metabolites, bimolecular like's proteins and peptides that is why lot of time is spent in taking this chromatography. There are different types of chromatography; we talked about an exchange chromatography, we talked about gel permeation chromatography, we talked about reverse face or hydrophobic interaction chromatography. And recently I started talking about the affinity chromatography.

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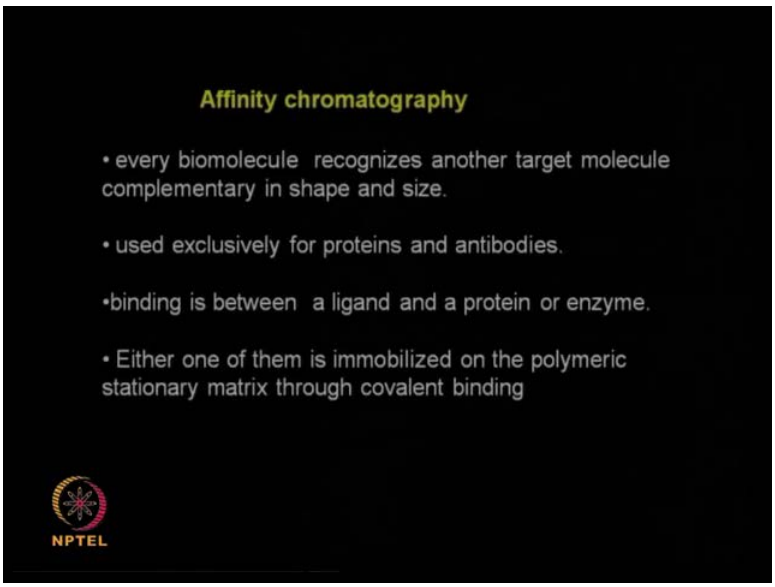

So, affinity chromatography is looking at a affinity between a ligand and protein; that means, a biomolecule recognizes, another target molecule complementary in shape and size. So, you have a protein antibody a ligand and an enzyme and so on actually. So, either the protein can be immobilized on the stationary phase or the ligand can be immobilized on a stationary phase. So, it tries to capture whatever is complementary to it, both with respect to the shape and size.

So, affinity chromatography is a the most perfect chromatography in all these techniques which is very, very selective, unlike the other type of chromatography that is why affinity chromatography is very, very expensive tool. And it is used in highly expensive bimolecular separation, may be form a ceutical products and so on. It cannot be used for a normal bulk chemicals, because the cost of affinity chromatography is very high and it is very exact type of technique.

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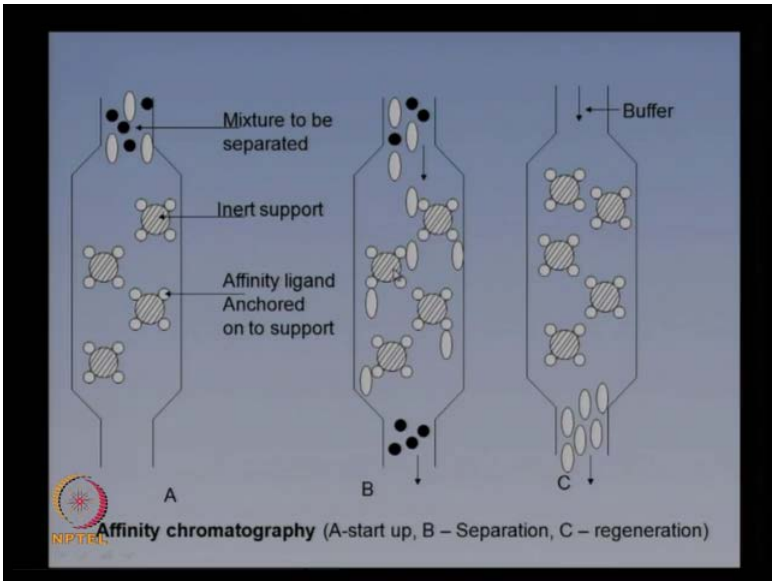

So, we saw this picture in the previous class as well, so what you have is you have a inert support here, we have the affinity ligand anchored to this inert support. Now, imagine we have a mixture that needs to be separated, we have a those molecule, which are complementary to this affinity ligand and those molecule, which are not complementary to the affinity ligand.

So, what happens as the mixture flows here, those molecule which are complementary to the affinity ligand get bound and remaining molecules flow out. So, we are separating those, which are complementary and those which are not complementary. And how do you later on separate those which are bound, we can change the p H we can change the buffer conditions, we can change the hydrophobicity, there by dissolving those molecule which are bound to the affinity ligand.

So, this can be used very selectively for capturing only those proteins, which are interested in. So, if we have ligands which are inhibitors to those proteins, those ligands

could be anchored here and those proteins will bind remaining proteins will just travel through because they are not complementary. So, we have concentrated our desired protein this is how the affinity chromatography works actually. So, let us look more about the affinity chromatography.

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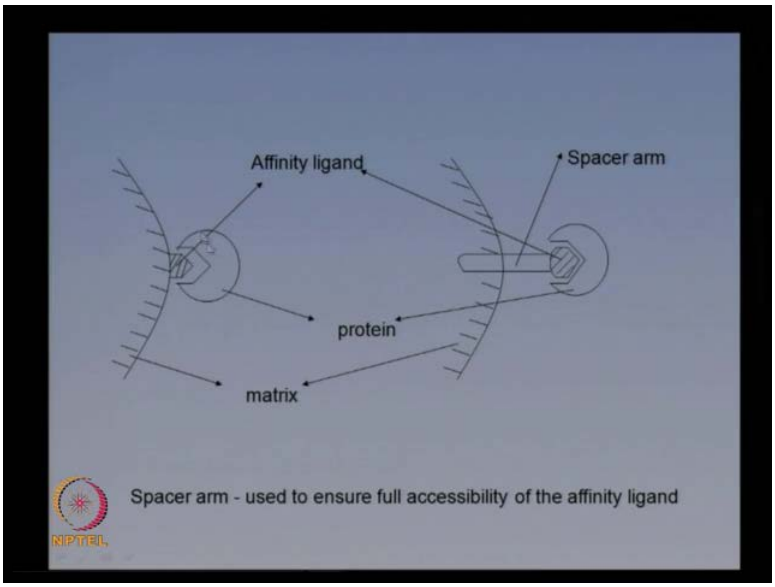

So, we have the affinity ligand, but then there is also something called spacer arm we need a long spacer arm, so that the protein is bound only to the ligand, it does not have any interaction at the matrix. If it has an interaction at the matrix, sometimes if it is the protein may get deactivated or denatured, so we do not want that happens. So, we have a very long spacer arm, may be a long chain hydro carbon. And at the end of this chain you may have the ligand fixed here.

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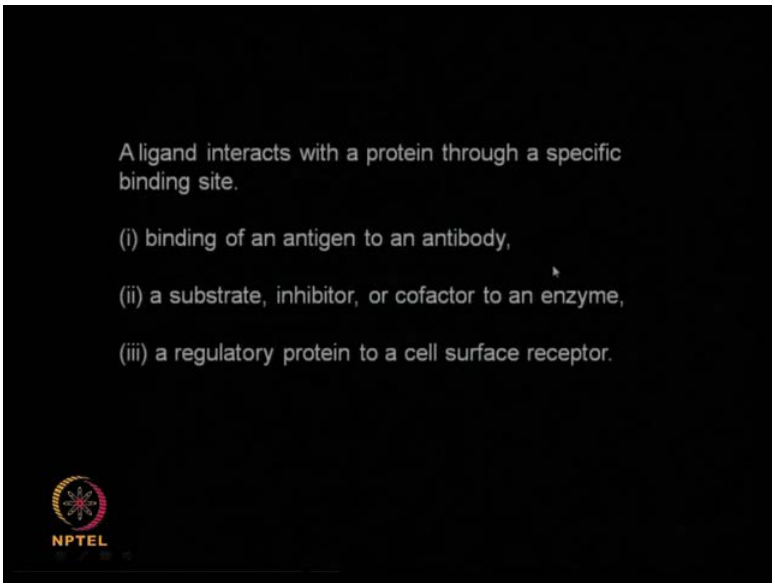

Now, the ligand interacts with a protein through a specific binding site. So, as you know all proteins have a binding site, whose shape and size matches exactly with the ligand. So, the binding could be between antigens an antibody. So, you see it becomes very, very specific and no other antibody will bind to this antigen. You can have a substrate or a inhibitor or a cofactor binding to an enzyme.

So, the substrate could be very specific to the enzyme, other enzymes will not bind to this substrate. It could be a regulatory protein to a cell surface receptor. So, the protein and cell surface receptor are complementary to each other. So, this is how you achieve this specificity in the affinity chromatography.

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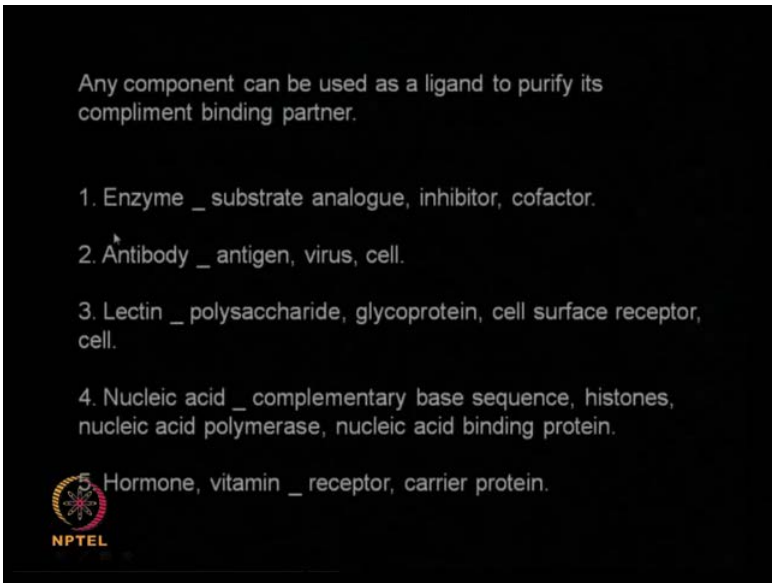

So, any component can be used as a ligand to purify it is compliment binding partner. So, enzyme means, it could have a substrate analogue, it could have an inhibitor, it could be a cofactor which in antibody means it could be an antigen, it could be a virus, it could be a cell. So, if I am going to separate a virus present in a mixture I run through an affinity chromatography where the antibody is my affinity ligand. So, only that particular virus will get captured by this antibody.

So, I want to purify, here in a material inter cellular material, but I want to remove the virus. So, we can use an affinity chromatography. You can have a lectin you can have a polysaccharide or glycoprotein or cell surface receptor or cell, we have a nucleic acid, we can have a complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein and so on. So, if we have a hormone or a vitamin, we can have a receptor or we can have a carrier protein. So, complementary type of a systems.

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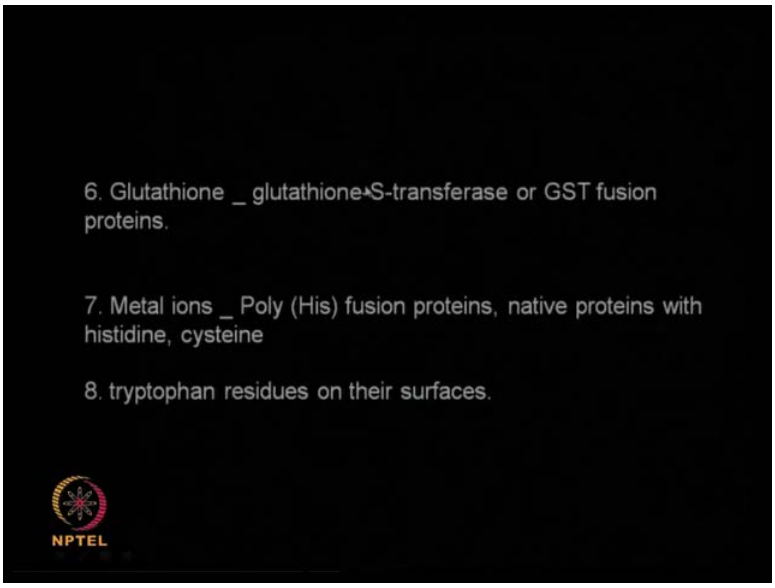

If we have a glutathione, we can have a glutathione-S-transferase which is exactly complementary to glutathione or we can have a aglutathione S transferase fusion proteins which are again complementary to glutathione. So, the glutathione could be bound to the stationary phase and you can selectively capture this type of enzymes. We have metal ions we can have poly his fusion protein, we can have native proteins with histidine, cysteine and so on. So, if we have tryptophan residues on their surfaces. So, all these types of complementing systems could be considered for purification.

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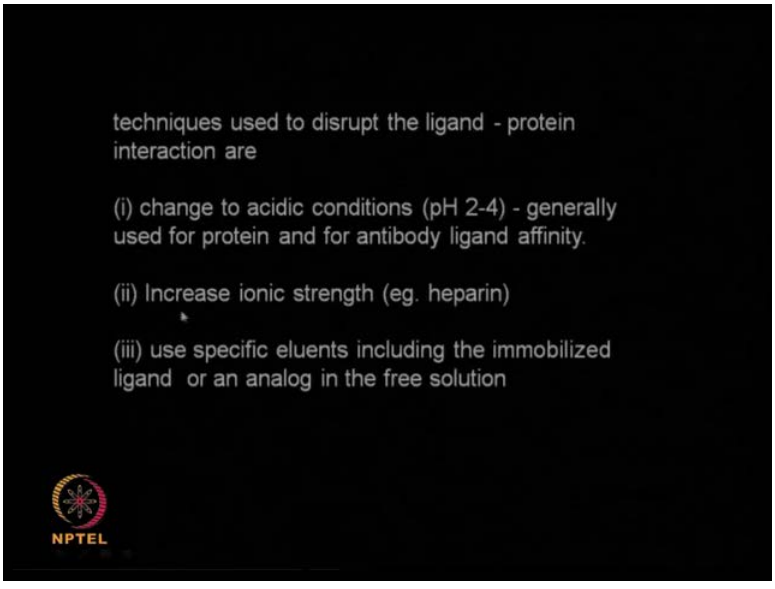

So, once you have captured these proteins or viruses. Now, how do you disrupt this ligand protein interacton because we need to reactivate the column how do you do that, we can change to acidic conditions like we can bring down the pH 2 to 4. So, generally this type of approach is used if you want a separate protein and antibody ligand affinity. We can increase the ionic strength for example, in the case of heparin, by increasing the ionic strength heparin which is bound can be liberated.

You can use specific elements for example, we can have immobilized ligand; that means, the ligand, which is immobilized also in the eluents or we can have analog in the free solution; that means, we can have a similar looking protein, which will displace the original protein which is bound to the affinity ligand. So, all these techniques can be used for dissolving or distructing the ligand and protein interaction.

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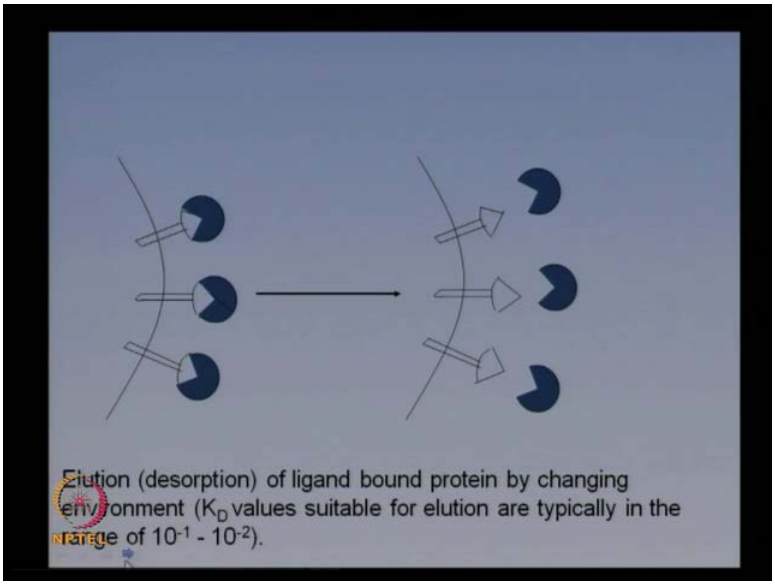

So, for example, we have the affinity ligand here, with the spacer arm. Now, there is a protein which is now, adopt. Now, if you want it to dissolve, we have to change the environment. So, the K D values suitable for elution generally between 10 power minus 1 to 10 of minus 2 in that situation you can dissolve this a protein which is bound to the affinity ligand.

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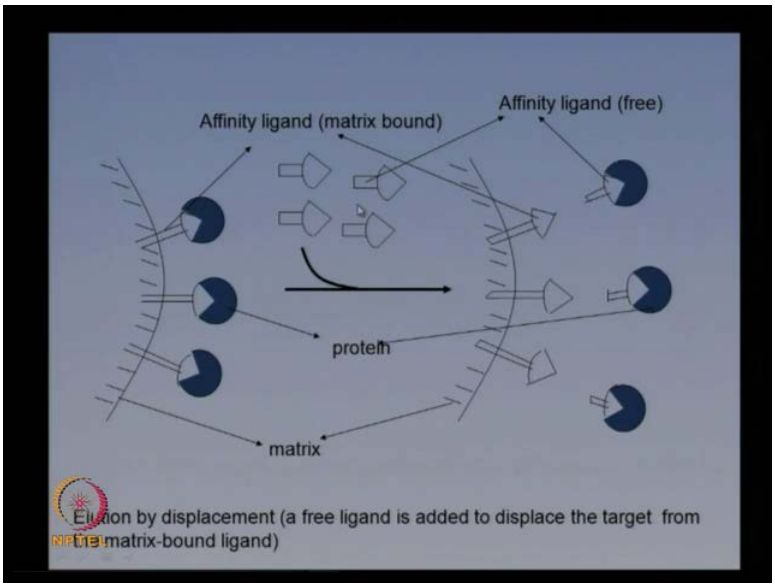

We can also have, this same affinity ligand which is in the matrix bound form in the free form or in the element. So, what happens, the protein which is bound now, will get unbound and then it will bind to this free form of affinity ligand. So, it is like a displacement. So, the protein which is bound, will get unbound and then it will bind to the free form of the affinity ligand, which is flowing down through the column . So, it is like a displacement type of approach. So, this is another technique by which, we can dissolve whatever protein that has been observed on the affinity ligand.

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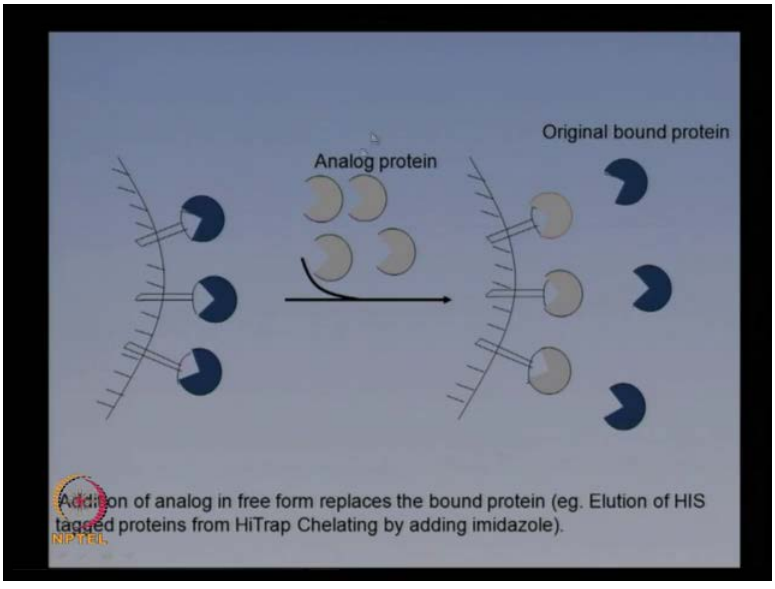

Another approach, we can have a analog protein, which is almost similar to the protein that is bounds. So, what happen? This analog protein displaces and the original bound protein goes in into the free form. So, for example, elution of his tagged protein from Hi Trap Chelating by adding imidazole. So, we add imidazole. So, whatever tagged protein and that is bound gets displaced. So, that is another approach.

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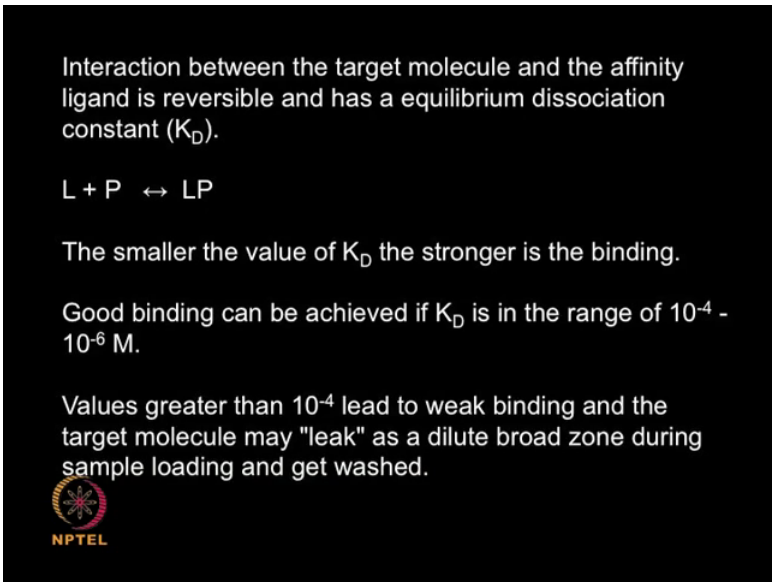

So, we see. So, many different approaches, we can change the pH, we can have a protein which is analog to the original protein, we can have this ligand which is immobilized in the free form and we can change salt concentration. So, by doing all these we are distructing whatever, has been adopt from the observed state in to the free form. So, the interaction between the target molecule and an affinity ligand is always a reversible process and it is in a equilibrium process.

So, there is something called a dissociation constant. So, if l is the free ligand, p is the protein it forms a complex called L P and this is a reversible reaction. And this equilibrium dissociation constant is called K D if K D is very small, then the binding is very strong. So, the K D is very large, binding is very weak. So, smaller the K D stronger is the binding. So, good binding if you want to achieve generally K D should be in the range of 10 power minus 4 to 10 power minus 6.

So, the values are greater than 10 power minus 4 then you are going to have weak binding and the target molecule may leak as a dilute broad zone; that means, the binding

is not going to be very strong and the target molecule will slowly start leaking out, that is called leaking during sample loading and they start getting washed actually. So, the best values of K D should be in the range of 10 power minus 4 10 power minus 6.

So, we can do a small experiment in the lab, we can take the affinity ligand, immobilize it and then we can run some simple batch protein experiments. And then see what is the disassociation constant and then from the disassociation constant, we can tell whether the binding is going to be strong or whether the binding is going to be weak.

And generally, as I mention here, would like to have a K D value between 10 power minus 4 to 10 power minus 6. So, if we have 10 power minus 4; obviously, the binding is going to be weak and if it is around 10 power minus 6 the binding is going to be strong.

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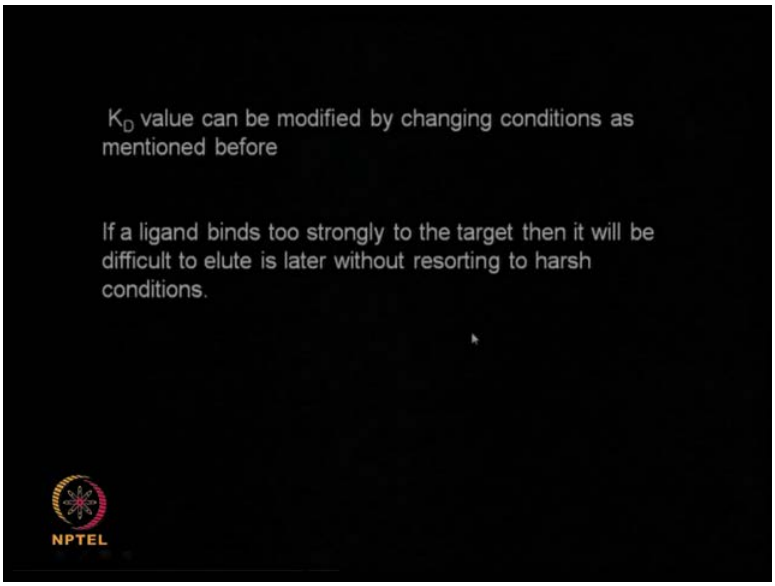

So, how do you modify this K D values, it can be modify by changing conditions as mention before, we talked about changing the conditions. So, initially you want a good binding because you are trying to capture the protein, later on you change the environments. So, that whatever has been captured is dissolved so; that means, you are modifying the K D value.

But, one may big problem in it consider is, if the ligand binds too strongly to the target. Then it becomes very difficult for it to elute out when you are doing the washing or when you are doing the regeneration. So, you need to consider, this particular point, we do not

want too strong a binding then you need a very strong conditions to, un bound whatever has been bound to the ligand.

And if we, use a very harsh condition in a deactivate protein or the protein may get unfolded it may lose it is activity. So, in it keep that point in mind. So, basically you are talking about how strong the binding is. So, that there is no leakage of protein these are this how easy it is to distrupt the protein which has been bound. So, in it player on the these two values actually.

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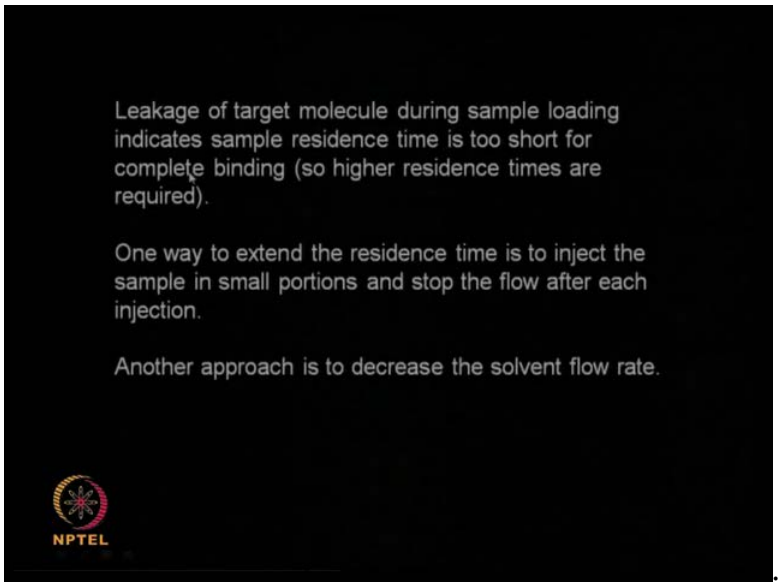

So, leakage of target molecule during sample loading, indicates sample residence time is too short for complete binding. So, slowly the target molecule is coming out; that means, you are not giving enough time for the ligand and the protein to interact. So, one way of doing that is the inject little bit. So, we are injecting then stopping the flow, then again we are injecting and then stopping the flow.

So, multiple small injections, that is one approach, another approach is to decrease the solvent flow rate of course, is the third approach is to modify the system. So, that the K D values becomes smaller. So, that the binding is stronger. But process wise what can we, do we can inject little bit stop inject little bit stop and so on. So, that is one approach, other approach is to reduce the solid flow rate, because you are residence time is too short, that is why you are target molecule is getting leaked out.

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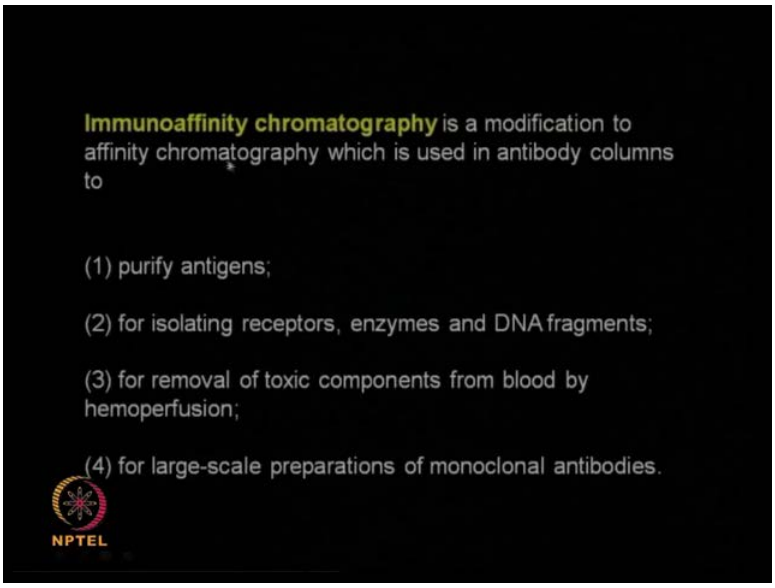

There is another type of chromatography that is called immunoaffinity chromatography. Is a modification affinity chromatography, it is used generally in antibody columns, if you want a purify antigens for isolating receptors, enzymes and DNA fragments, for removal of toxic components from blood for large-scale preparations of monoclonal antibodies.

So, for such antibody type of purification, we use this immunoaffinity chromatography. So, if you want a purify antigens, you want a isolate receptor enzymes DNA s on remove toxic components from blood, that is called hemoperfusion or if you want to do preparation of monoclonal antibodies.

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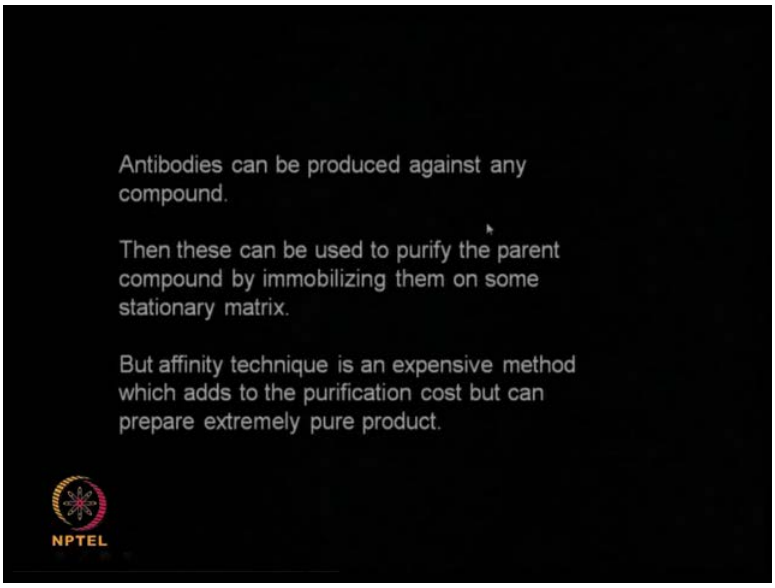

Then what you do you use something called immunoaffinity chromatography. So, basically what you do we can produce antibodies for any compound, then what do you do these antibodies or immobilized on a stationary matrix. So, we purify the parent compound. So, if there is a mixture which contains your parent compound with other compounds, these antibodies which have been prepared from the parent compound will we very selective to words these parent compound and it will not bind rest of the compound.

So, that way we can achieve very high purity, but this is a very expensive technique, it adds to the purification cost. So, if you are interested in preparing very pure components or products.

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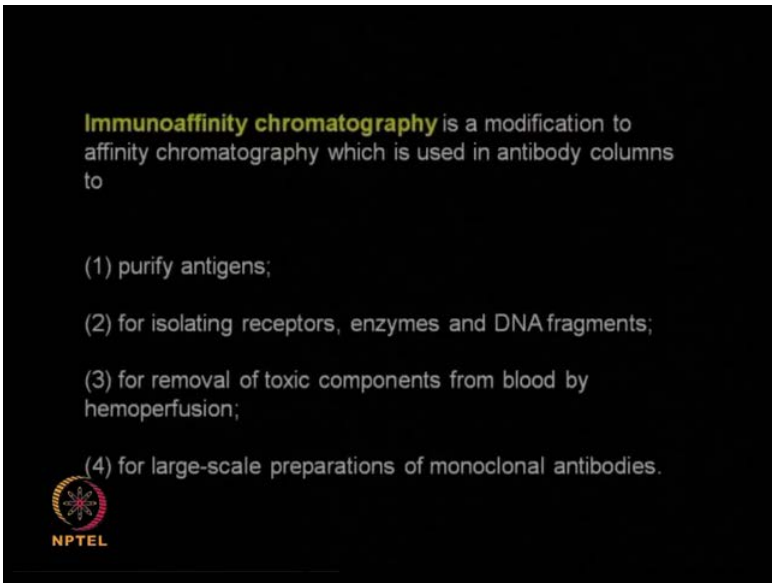

Like monoclonal antibodies or antigens or we want purification of blood, where you do not want any toxic compounds present Then this type of technique are adopted.

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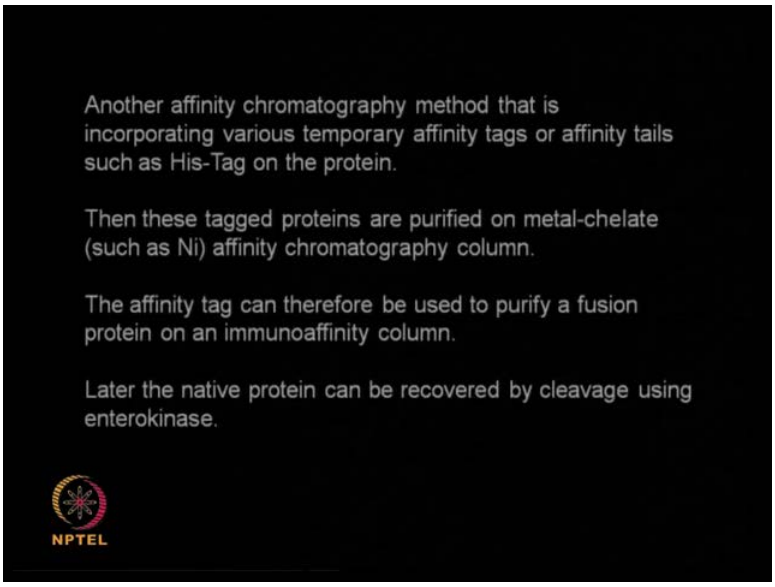

So, another affinity chromatography method is incorporating various temporary affinity tags or affinity tails. So, we attach a affinity tag and then we perform this affinity chromatography and then we remove the tag as, simple as that for example, everybody knows his tag. So, we attach a his tag on to a protein, then these tag proteins are purified on metal chelate, such as nickel in an affinity chromatography column.

Then the affinity tag can therefore, be used to purify a fusion protein or a immunoaffinity column, then the native protein can be recovered by cleavage using enterokinase. So, we are using enzyme. So, that the his tag on the protein gets removed. So, initially you attach histadine tag to the protein. And then we used some sort of a nickel affinity column, purify the protein and then we remove the histadine tag using a enzyme to cleave the attached histadine.

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Then there is another type of chromatography, which is called ion suppression reversed phase chromatography. So, the name implies that we are trying to suppress the ionization of a particular protein, once you suppress then you use the reverse phase system to purify. So, this is very suitable when you have the stationary matrix or the phase is non polar and the mobile or continuous phase is polar, but under operating conditions may turn non polar.

So, what do you do we add a p H buffer. So, that the mobile phase is suppress the ionization. So, by adding a p H buffer we are preventing the ionization of the solute in the mobile phase and then we are using a non polar stationary phase. So, what happens the polar analytes will elute out first, non polar analyte will elute out later, because your stationary phase is non polar, that is why you have the reverse phase.

So, the ion suppression is achieved by adding a p H buffer. So, it prevents the ionization of the solute and then you have a reverse phase stationary phase. So, the polar analytes

will be coming out first. So, if the analyte or the solute is in ionized form, then you are going to have problem suppressions own be very good. So, by suppressing the ionization we have now, polar non ionizable product and non polar material. And the stationary phase is a non polar or reverse phase material. So, the ion suppressed polar material will come out first. So, this is called the ion suppression reverse phase chromatography.

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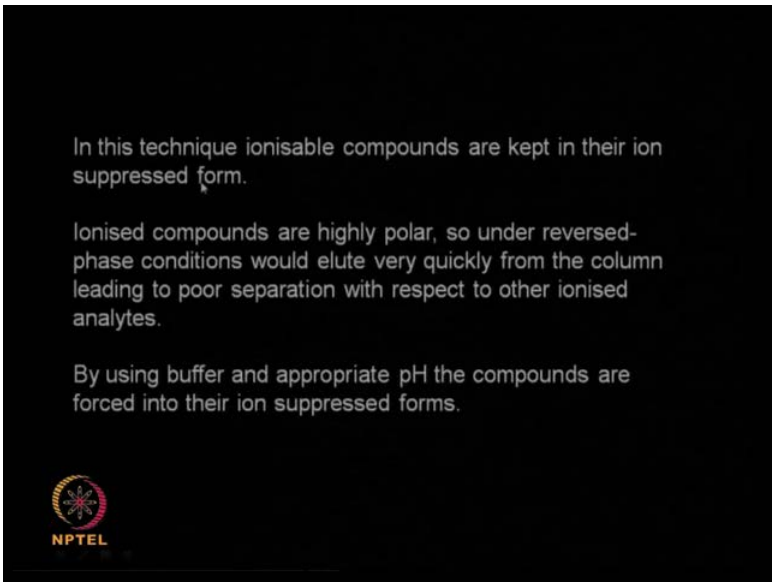

So, in this technique ionisable compounds are kept in there, ion suppressed form. Now, ionized compounds are very, very polar as you know. So, under reverse phase condition they will elute out very quickly from the column, leading to poor separation with respect to the ionized analytes.

So, if you have many ionized analytes they will never that separated out at all they will just come out of the column very fast because they are highly polar. So, what do you do, you suppress the ionization by adding a buffer, then there will be some interaction between the non ionized polar compounds on the surface which is reverse phase. So, some separation will happen in the various polar analytes. So, we are using a buffer and appropriate p H. So, that the ionization is suppressed.

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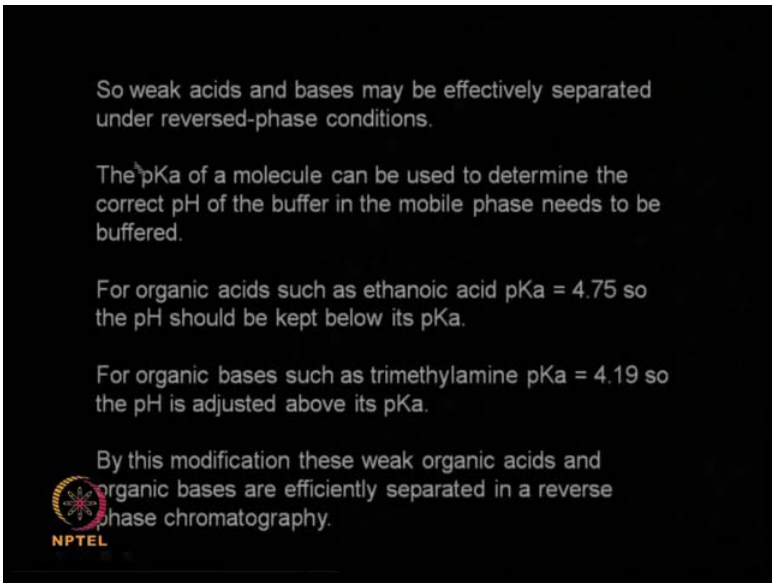

So, week acids and bases may be effectively separated under this reverse phase condition. So, the p K a of a molecule can be used to determine the correct p H of the buffer in the mobile phase. So, based on the p K a we can adjust the p H in the buffer. So, for example, for organic acid such as ethanoic acid, you know p K a is 4.75. So, the p H should be kept below it is p K a. So, we have to use lower p H. So, that you are preventing the ionization.

So, organic bases such as trimethylamine p K a is 4.19. So, the p H is adjusted above it is p K a. So, we go to higher p H, so that it does not get ionized. So, by this modification, these week organic acids in organic bases are efficiently separated in a reverse phase column otherwise, if we do not suppress the ionization these acid or these bases will get ionized and their separation efficiency of these compounds, are extremely poor in a reverse phase chromatographic system. So, this is called a ion separation reveres phase chromatography.

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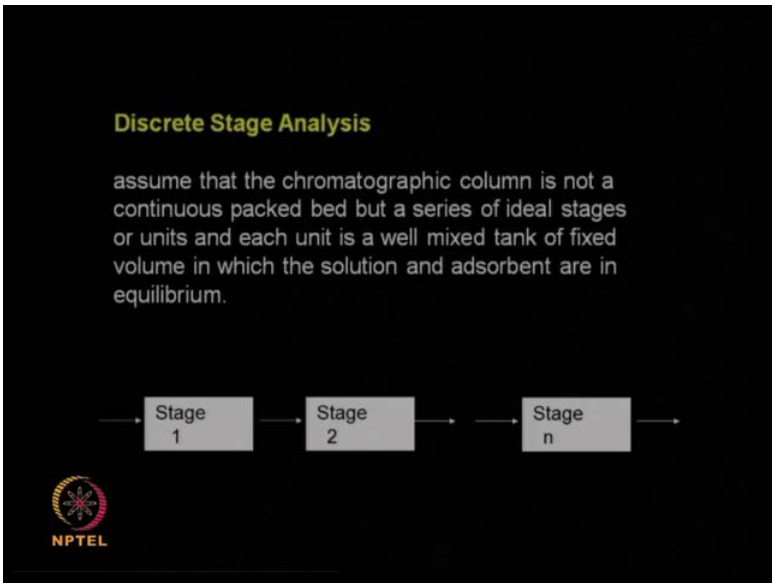

Now, we talked quite a lot about different types of chromatography. We talked about hydrophobic or reverse phase chromatography, we talked about ion exchange chromatography, we talked about the gel permeation or gel filtration chromatography, then we talked about these legand based affinity chromatography. All these are the basic chromatography and there are many modifications to these where, we are talking about ion suppression chromatography and so on.

Now, let as again go back to the mathematics of this column operation and see, how we can build up some more relationships within various parameters in the flow. Now, we have a long stationary phase and we have the mobile phase travelling over it. Now, this stationary phase can be divided into multiple stages I originally is mention that in engineering, some of the unit operations are viewed as staged process, equilibrium staged process.

Like adsorption, extraction, distillation, chromatography all these are considered as equilibrium staged process; that means, you have many fluids they come in contact with each other then they at it equilibrium they get separator So, the solute concentrations that are living the stage or an equilibrium with each other, then they go to the next stage and again there is a mixing of a various fluids and again the fluids gets separated and the fluids that are living, each of the stage or an equilibrium and so on it happens.

So, by using this type of concept by, using the concept of mass balance, we build up lot of a equations in staged processes. And you can see that is a similarity between many of these stage processes justiculation or adsorption or extraction or chromatography. And we have a mass balances one of the equation and we have the isotherm or relationship between this streams leaving out for the stage as another equation and we can perform many calculations.

So, that is what we are going to do. So, consider a chromatographic column as a stage process. So, we have the stationary phase in small, small stages. We are called like a n stage process. Now, we had the mobile phase travelling from one direction to the another direction. So, the solute gets absorbed, disturbed list on whatever be the principle it could be based on hydrophobic interactions it would be based on ionic interaction, it could be based on affinity interactions and so on. And, so we will try to model each one of the stage and extend it to the entire chromatography; that is what we are going to look at.

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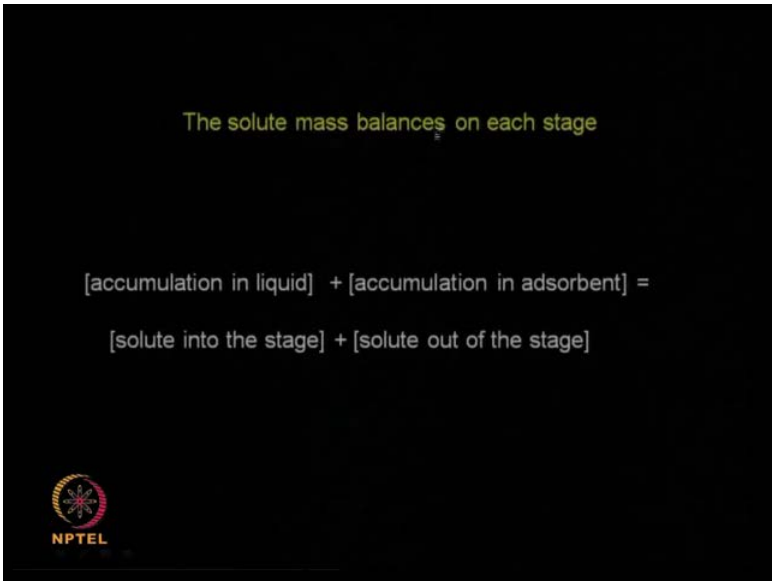

So, how do you do the mass balance on each stage.

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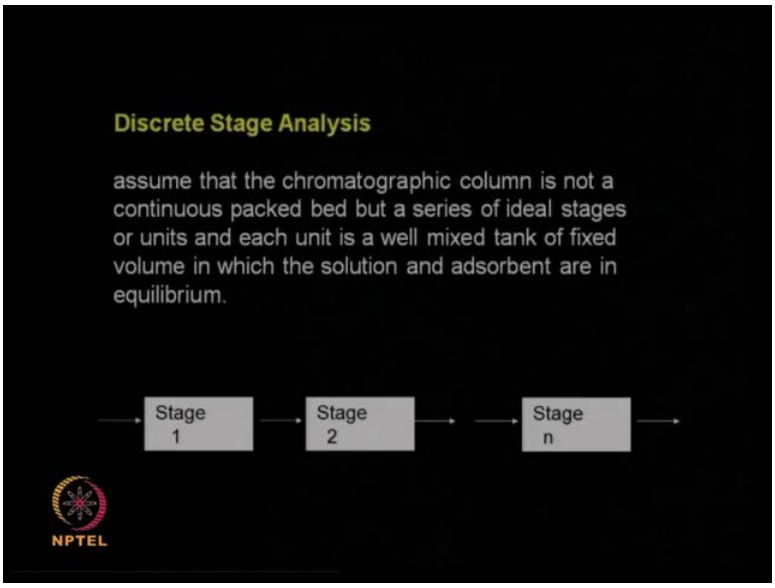

So, we as you can see, we have stream coming in, stream going on, that is all in chromatography it is very simple, we have the stationary phase, stationary inside the stage and what is flow there is a only one single stream coming in there is a another stream going out.

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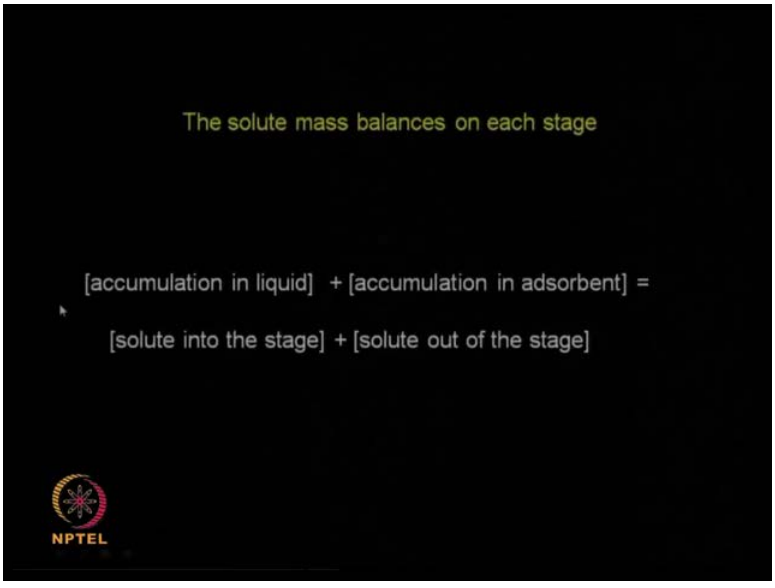

Now, the stream that is coming in has some solute, under stream that is going out also has some solute. So, accumulation in the liquid because of the material coming in the liquid and the plus accumulation in the adsorbent. Now, this will be equal to the solute

inside the stage plus the solute out of the stage. So, this is the accumulation and then whatever is in and whatever is out.

So; obviously, you should have a negative term here because we are talking about out. So, material in, material out is equal to accumulation. Now, accumulation; there are two types of accumulation, one accumulation of the solute in the continuous phase, another is the accumulation of the solute in the stationary phase or the adsorbed phase. So, we have the solute coming in because of the stream that is travelling inside minus solute going out because of the stream that is going out that is equal to the accumulation, accumulation is of two types one is the accumulation in the liquid accumulation in the absorber.

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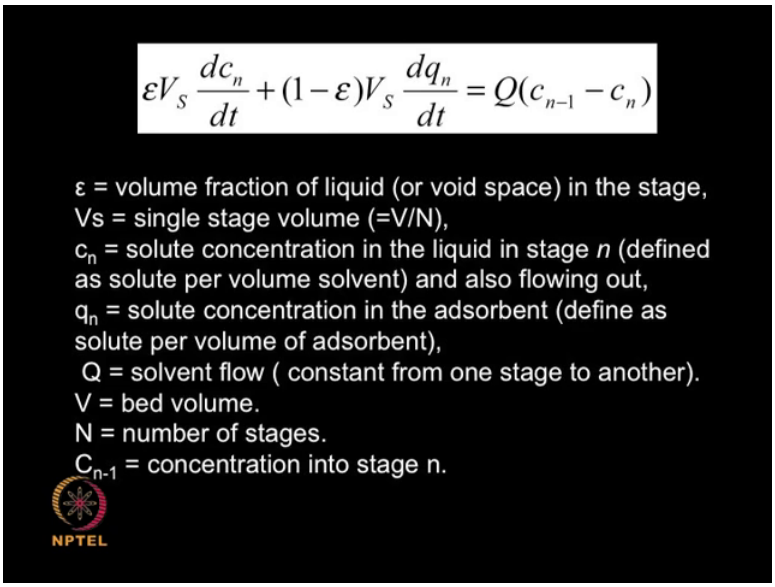

So, when you do that, let us look at a stage n. So, whatever is coming in this Q into C n minus 1 that is the C n minus 1 is the concentration of the solute entering in from the n minus 1'th stage. C n is the concentration of the solute that is living the n'th stage, Q is your solvent flow rate. We assume that the solvent flow rate is constant in each of the stages.

So, Q into C n minus 1 is the solute coming into the stage Q into C n is the solute going out of the stage there is a minus term so; obviously, this is the mass balance, on the left hand side we have the accumulation, there are two types of accumulation I mentioned, one accumulation is related to the accumulation of the solute in the mobile phase, other

one is the accumulation of the solute in the stationary phase. Now, again let us go to the accumulation in the mobile phase.

What is the equation, epsilon that is the volume fraction or void space in the stage into V s is the volume in single stage. So, this could be volume of the entire column divided by n stages, suppose there are n stages capital N single stage volume will be V by n we have to multiply by the void age because you have solids also present inside. So, epsilon is your wide age. So, e into the s is the volume of the liquid space inside the stage multiplied by d c n by d t.

So, this is the accumulation of the solute, in the liquid part inside a stage plus there is accumulation in the solid part also. So, if epsilon is the void space 1 minus epsilon will be fraction of solids present in a stage multiplied by V s, V s is the volume of each stage into d q n by d t Q small q is the solute concentration in the adsorbent or solute concentration in the stationary phase in that particular stage n.

So, please note we have C n q n C n minus 1. So, C n is the concentration of the solute in the mobile phase inside the stage as well as concentration of the solute in the mobile phase living this stage small n C n minus 1 is the concentration of the solute, in the mobile phase entering the stage small n, q n is the concentration of the solute in the stationary phase inside stage n.

Very straight forward equation Q C n minus 1 is the concentration of solute entering stage n minus Q C n is the concentration of solute living stage n, this is equal to the accumulation there are two types of accumulation, one accumulation is the concentration of the solute in the mobile phase, other accumulation is the accumulation because of the stationary phase. For stationary phase concentration, we use Q for the mobile phase concentration we use small c.

Epsilon is your void age V s is the volume of single stage; that means, volume of the entire column divided by n is your number of stages capital N. So, you see we will have many differential equations like this, for each one of the stage. So, we can have c 2 minus c 3 here, will have c 3 we can have c 3 minus c 4 here we can have a c 4 and so on. So, for each stage, we can have this type of differential equation.

Now, how would you relate c n and q n, we talked long time back there could be a linear adsorption type of relationship between c n and q n.

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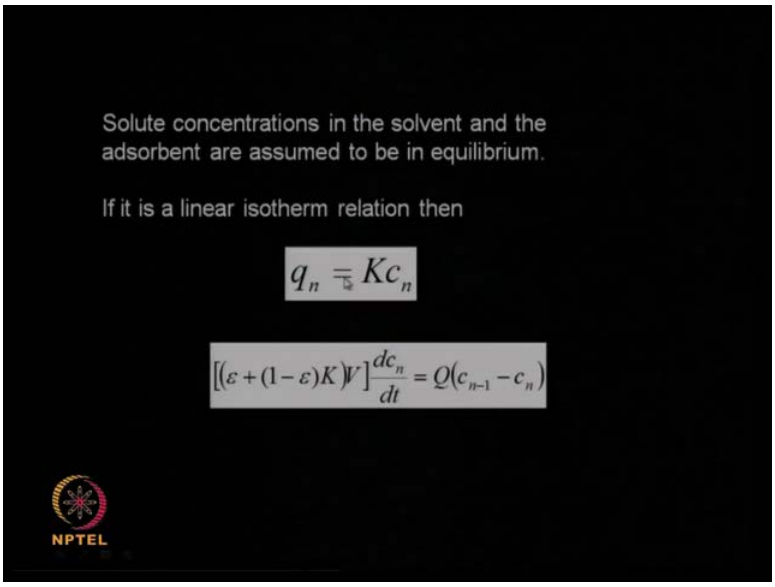

So, q n that is the concentration of the solute that is adsorbed and C n is the concentration of the solute in the mobile phase. If they are in equilibrium, they are related as a linear isotherm relation. We can have non-linear relationship also, but here we are assuming a linear relation, so q n equal to K into c n. This is a simple relationship, we can have a non-linear type of relationship we can have a lamer type of relationship and so on.

In the simplest is if the solute that is adsorbed on the stationary phase and the solute that is there in the mobile phase or linearly related. So, by putting in the value for K n like this, we can rearrange the equation to get a differential equation like this. So, Q into c n minus 1 is the amount of solute entering stage n Q into c n is the amount of solute live in stage n this is equal to a big number here d c n by d t Now, this is called a differential, difference equation because I can have many, many equation like this for each of the stage.

So, stage 1, stage 2, stage 3, stage 4. So, inside stage one this will be c 1 and this will be feed concentration, inside stage 2 this will be c 2 this will become c 1 inside stage 3 this will become c 3, this will become c two this will become d c 3 by d t and so on. So, first stage c n minus 1 will be c f, c f is the feed concentration.

So, this is called a differential, difference equation because you are going to have many, many equations like this, the this is differential part, this is difference part. So, there are many techniques to a solvent. So, please note that we hit considered linear isotherm, that is why we are able to combine all this into single term. If we consider non-linear equations, we cannot solve them analytically, but when you once consider a linear isotherm relation, we get a series of differential, difference equation then it is very, very simple to analytically solvate.

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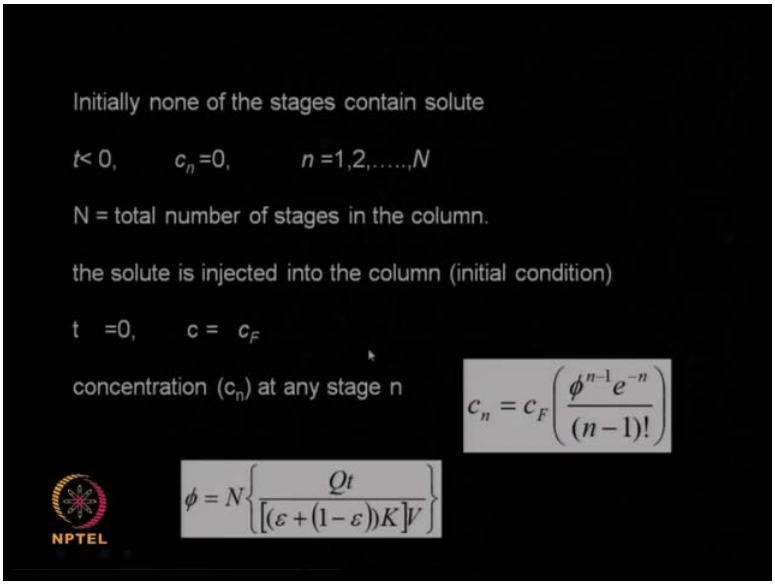

So, let us not go too much into the analytical solution procedure, but I need some initial conditions as well, if you want to solve any differential equation you need initial conditions. So, what happens initially there is no solute; that means, at time less than 0, we have no solutes. So, c n 0 to 0 at all n's 1 to n where time less than 0. Where capital N is the number of stages total number of stages. Now, at time equal to 0, I am injecting the solute. So, at time equal to 0 I am injecting the solute, in the beginning in the start of my react in my tubular reactor or tubular chromatography column.

So, these are initial condition. So, by using these initial conditions, one can get a relationship for c n, that is the concentration inside the stage n or the concentration living the stage n this is given by, this particular relation c n equal to c f phi raise to the power n minus 1 e minus n divided by n minus 1 factorial where phi is given by capital N capital

N is the number of stages Q into t Q is the flow rate of the continuous phase divided by epsilon plus 1 minus epsilon K V.

So, you see V is the total volume of the chromatographic column n is the number of the stages in the chromatographic column, Q is your flow rate t is the time, epsilon is your porosity K is the linear adsorption, isotherm which relates q n c. So, q n is equal to k into c n. So, this equation is very, very useful, let us look at our original chromatographic equation, which assumes a Gaussian type of distribution correct.

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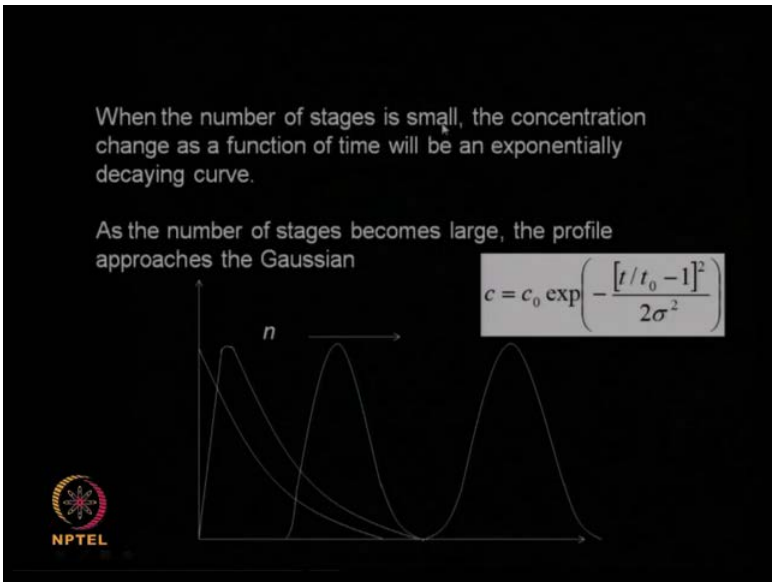

Before that let us go back when the number of stages is small, the concentration change as a function of will be an exponentially decaying curve. So, if stages number of stages are very small, you are going to get as time goes we are going to get equations like this, but as the number of stages become larger and larger, you are going to have a Gaussian type of distribution like this.

Now, if the stages are very small, if the stages are one then you are going to have this type of reduction in the stages are more than one slowly, slowly you are going to get, but this is not a uniform Gaussian distribution, but a stages become extremely large, you are going to get uniform Gaussian distribution. And the equation for a Gaussian distribution is what c equal to c naught exponent minus t by t naught minus 1 whole square divided by 2 sigma square.

This is the equation for the Gaussian distribution, where t is your time t naught is this particular thing. That is the time at which you get a maximum in the peak and then t naught sigma is your standard deviation of this particular relationship. I hope you remember this equation, we talked about this equation many, many classes back this is your standard equation for a Gaussian distribution. So, t naught is the time at which the maximum of the peak appears. So, it is called the elution time c naught is this maximum concentration.

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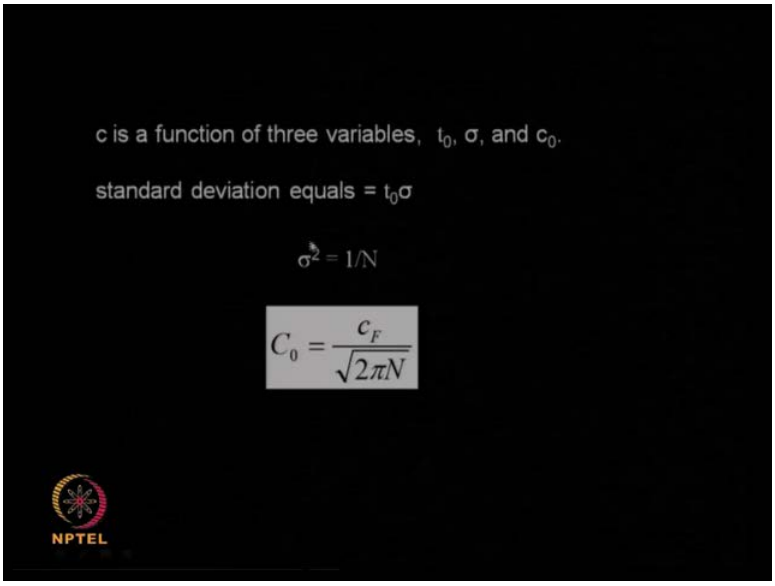

Now. So, C is a function of 3 variables t naught, sigma and c naught. And standard deviation is given by t naught sigma. So, by comparing this relationship, with the previous relationship which, we prepared or which we derived for a discrete stage model, we can say that sigma square is equal to 1 by n. So, you see that there is a relationship between sigma and number of theoretical stages or theoretical plates. And what is sigma, t naught sigma is this standard deviation.

So, if I know the standard deviation, I can calculate what is the number of theoretical stages of a column? So, what do you do I inject a sample and I see the t naught is the time at which the elution is maximum. Then I get the standard deviation of the Gaussian curve, then what do I do from this equation I can calculate sigma and then from this equation I can calculate n that is the number of theoretical stages or number of

theoretical plates of the column. So, it tells you how efficient column is going to be; that means, it tells you the total number of stages in that particular column.

So, this is going to be a very useful equation, we will look at some problems relating to this equation. Now, c naught that is the concentration maximum, will be a function of the feed concentration and the number of theoretical stages in this fashion. So, again if I know c f that is a feed concentration and if I know the number of theoretical stages, I can tell what will be the maximum concentration of that particular peak that is going to elute out. So, you see these equations are very, very useful for doing plenty of calculations, we are going to look at it as time progresses.

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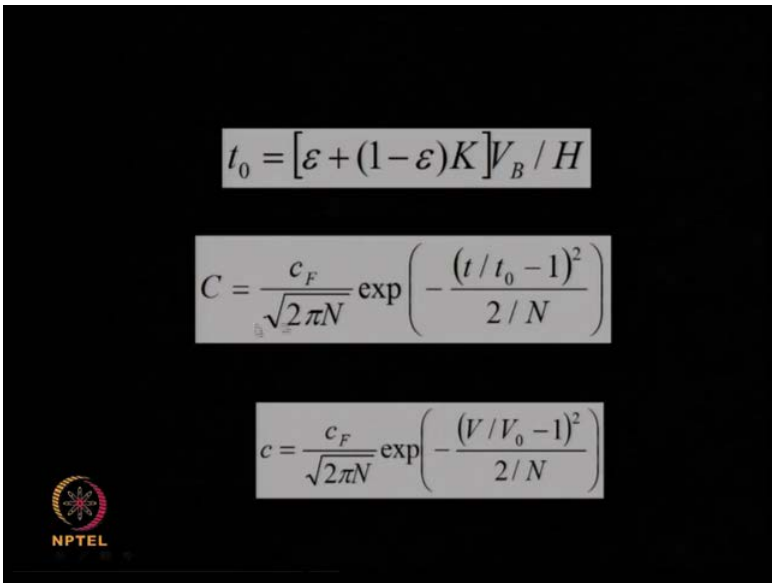

And then t naught, that is the elution time that is the time at which the peak is maximum, is related to your wide age, it is related to your linear adsorption isotherm K volume of the bed and height of the bed, this fashion. So, by substituting all these into our Gaussian relation, we get c is equal to here in a Gaussian relation if you remember we had c naught, c naught is c f by square root of 2 pi n exponent of minus t by t naught minus 1 here, we had sigma square.

So, we have 2 by N, N is the number of theoretical stages or number of theoretical plates. Now, instead of t naught elution time we can also use elution volume, that is instead of t we can substitute as v. So, we get v by v naught, v naught is the maximum elution volume, the volume at which you get the maximum in that p. So, these two equations are

very useful, here we have the number of theoretical stages, we have the feed concentrations, we have the elution time coming into the picture and here we have the concentration coming on the left hand side.

So, these equations again can be used for design calculations, as we are going to see some more problems as we go along.

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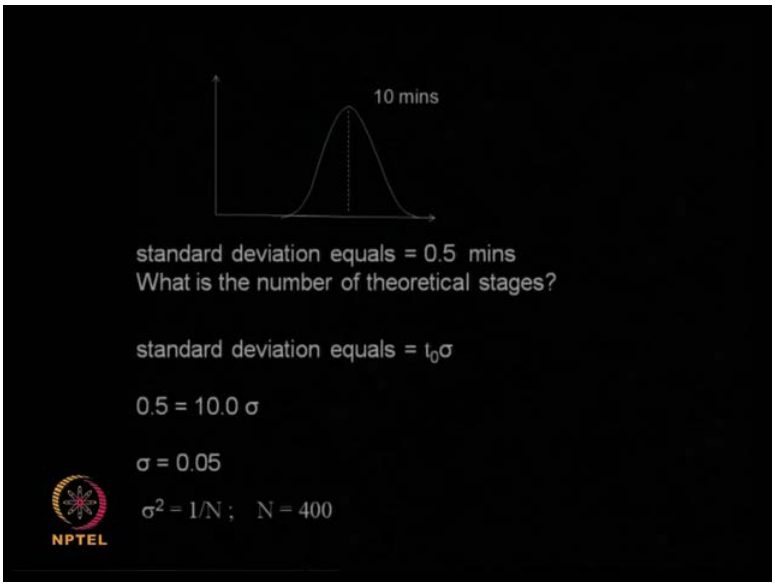

Now, let us look at a problem, imagine I am injecting a solute at time equal to 0 and at 10'th minute I get this peak maximum will assume this to be a Gaussian distribution. So, t naught will be 10 minutes. Now, the standard deviation is 0.5 minutes. So, what is the number of theoretical stages or number of theoretical plates for that particular chromatography, so which equation to be use. So, we can use that the equation which connects sigma with n.

So, what is the equation, standard deviation equals t naught sigma. So, here t naught is given here 10 minutes, standard deviation is given here 0.5. So, I can calculate sigma once I calculate sigma, then I use another equation which is relating sigma and number of stages, what is the equation sigma square equal to 1 by N. So, N is equal to from sigma I take n is equal to 1 by 0.05 square. So, N is equal to 400. So, it means this particular chromatographic column has 400 stages.

So, you see if your sigma is smaller, your number of theoretical plates is larger. So, if sigma has to be smaller, than standard deviation also has to be smaller; that means, if the curve is very broad, your theoretical plates are very small. If the curve is very sharp, your standard deviation is low, then sigma is low, then number of theoretical plates will be large. So, when I inject the sample and I get a beautiful sharp curve, then I can be sure the theoretical stages are large if I am getting a broad curve, then I can be sure my standard my theoretical stages are less.

So, you see by looking at the shape of the curve that is eluting out, we can tell what type of column it is how effective the column is does it have large number of stages or does it have small number of stages. So, if you are buying packed column from vendor, we generally ask how many stages does it have 400 stages, 500 stages, 1000 stages, 2000 stages.

So, by looking at what the vendor is saying, and when we know the number of theoretical stage, we will be able to tell what will be the width of this particular peak why is it is important suppose, I have two curves and if the width is very, very small the separation will be good if the width is very broad you are going to have over lap of one curve with another; that means, there is going to be over lap of one solute with another. So, they you are going to produce large amount of mixed solute.

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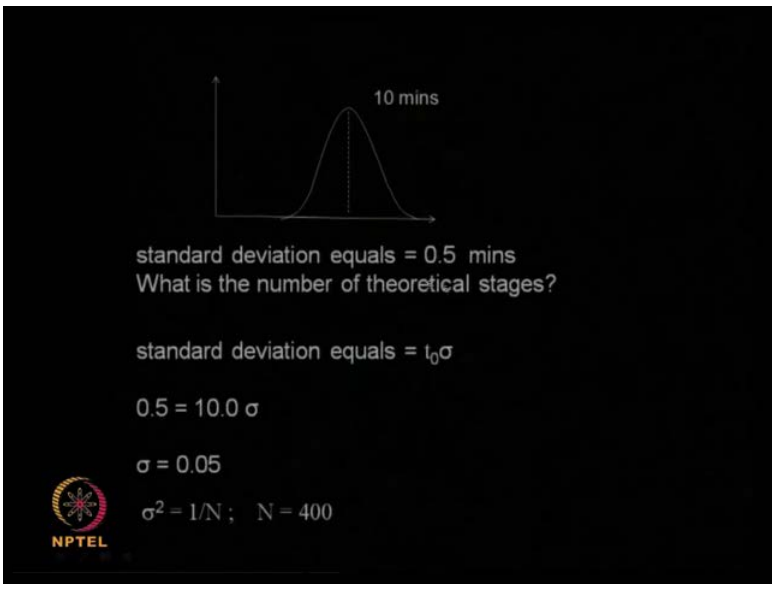

So, this problem tells you how efficient this particular column is based on the elution time and based on the standard deviations.

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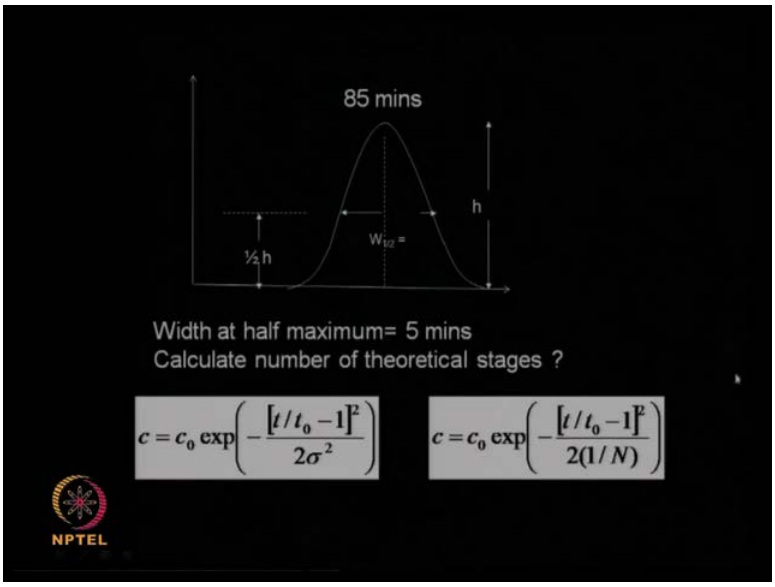

Let as look at another problem. This is in a different way. So, we know the elution time it is 85 minutes, the width at half maximum, that is this place where the maximum is half. So, if you assume the overall height of the particular peak is h at half h if this width is given that is 5 minutes can you calculate the number of theoretical stages. So, this width is given, in the previous problem t naught is given and standard deviation is given, you are asked to calculate the number of theoretical stages.

In this particular problem, the t naught is given width at half the maximum is given you are asked to calculate theoretical stages. So, how do you do this, think how do you do this problem we need to use the c equation. So, we can use this particular equation c is equal to c naught exponent minus t by t naught minus 1 whole square divided by 2 sigma square, t naught is known sigma we do not know, but then we can substitute sigma square is equal to 1 by n right.

So, by substituting this if, we know all these we can calculate n. Now, c by c naught is half at these places c by c naught is half, correct at this place c by c naught is 1 and this is 5 minutes. So; obviously, this place is 85 plus 5 by 2 that is 87.5. So, at this place the concentration c divided by c naught will be 0.5 at this place c by c naught is 0.5 and the

time will be 87.5 minutes because this is 85 minutes and this width is 5 minutes so; obviously, this is 5 by 2 plus 87.5.

So, the time is here 87.5 t naught is 85 c by c naught is 0.5. So, only unknown is n. So, you can calculate the number of theoretical stages.

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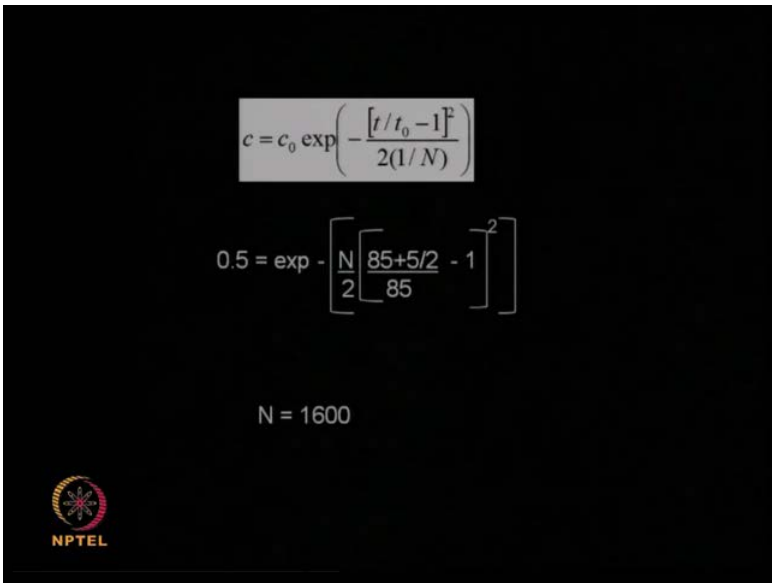

So, t is equal to 85 plus 2 by 2 as I said your c by c naught is 0.5. So, everything is known except your number of stages. So, we can calculate from this relationship the number of stages comes out to be 1600. So, in this problem what we did, we know the width at half the maximum, the time then we try to calculate the number of stages. So, we used this particular relationship. So, you remember, we use to have sigma square here sigma square is equal to 1 by N.

So, we are using c is equal to c naught exponent minus t by t naught minus 1 whole square divided by 2 into 1 by n, we note t naught which is 85 t is 85 plus 5 by 2 c by c naught is 0.5. So, we substitute everything the only unknown here is N. So, we can calculate the N. So, it is very, very straight forward. So, you see we did two problems both calculating the number of theoretical stages, both is based on a simple experimental procedure.

So, what you do you inject your solute at time equal to 0 and see, when the solute is coming out or eluting out you know the time if you know the bit at half maximum also

we can calculate the number of theoretical stages, using this particular relationship or if we know the standard deviation, we know that sigma t 0 is equal to standard deviation. So, from there we can calculate sigma and as you know sigma square is equal to 1 by N. So, we can get the value of N.

So, both the ways, we can calculate the number of theoretical stages or number of theoretical plate. Why is it, so important this number of theoretical stages or number of theoretical plates is very important because it tells you that quality of the chromatographic column, higher the stages; that means, better will be the resolution and better will be the separation, sharper will be the peak. So, the Gaussian peak will have a very good sharp appearance. So, if you have multiple solutes present the overlap of the solutes will be minimal.

So, that is the main advantage. So, we need to know the number of theoretical stages, even if you are purchasing a column, we always tell give me a column with, so many theoretical stages. So, we can substitute the theoretical stages into our equation and tell how good these separations of various solutes are going to be or how sharp the peak is going to be.

So, main assumption in this equation is the peak that is a getting eluted out of my column is a Gaussian distribution; that means, it is an uniformly looking curve. So, we make use of the mathematical representation of a Gaussian relationship. And what does a Gaussian relationship look like it is like this c is equal to c naught exponent minus t by t naught minus 1 square into 2 sigma square that is how a Gaussian distribution is going to look like.

So, making use of that relation, we can do several calculation and also by making use of the discrete stage analysis we are able to develop just based on mass balance, develop relationship between the solute concentration going in, the solute concentration going out and equate it to the accumulation. And if you assume a linear adsorption relationship, we can get quite simple relationship between various parameters, which can be used for all these type of a analysis. Now, we will continue further in this mathematical analysis and how do you make use of these mathematical, equations for designing columns, looking at performance of columns and so on. So, we will continue them in our next class.