Principles of downstream techniques in Bioprocess – a short course
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Lecture-18
Chromatography [Continued 2]

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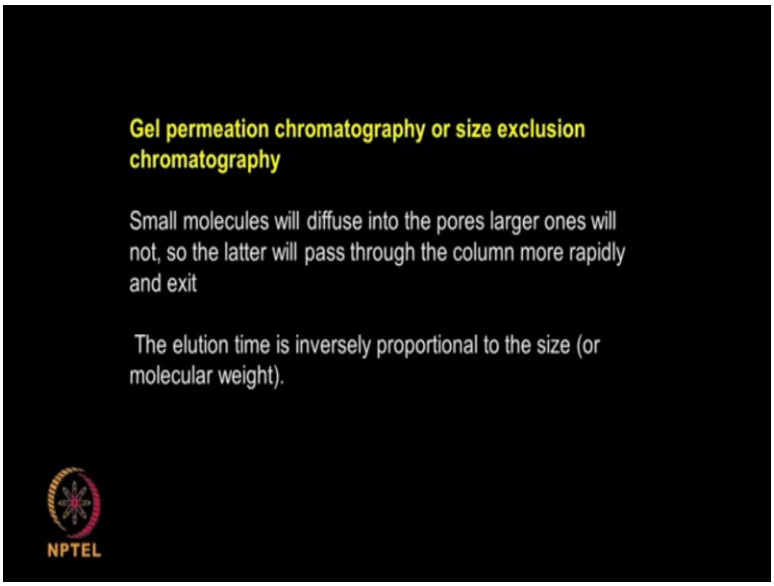

In this class also, we will continue on chromatography. The next chromatography is called gel permeation chromatography or size exclusion chromatography. So it is a very useful technique, we can use it for separating molecules based on their size or molecular weight or it can also be used as an analytical tool, to find out molecular weight of unknown proteins or polymers when you have the data for a set of a known polymers or proteins.

So the small molecules will diffuse into pores of the stationary phase, where the larger ones cannot. So the larger ones will come out of the column faster, the small ones will take little bit time. So there is inverse proportionality between the size or molecular weight and the elution time.

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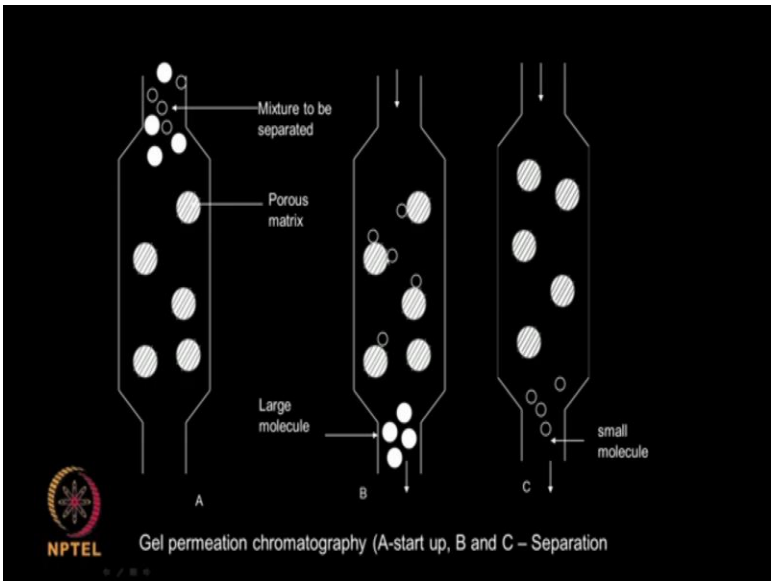

That is what it happens here, so as you see the big ones and the small proteins are travelling down and the small ones get entrapped into the small pores. So the big ones come out and later on, we can elute the small ones of these columns.

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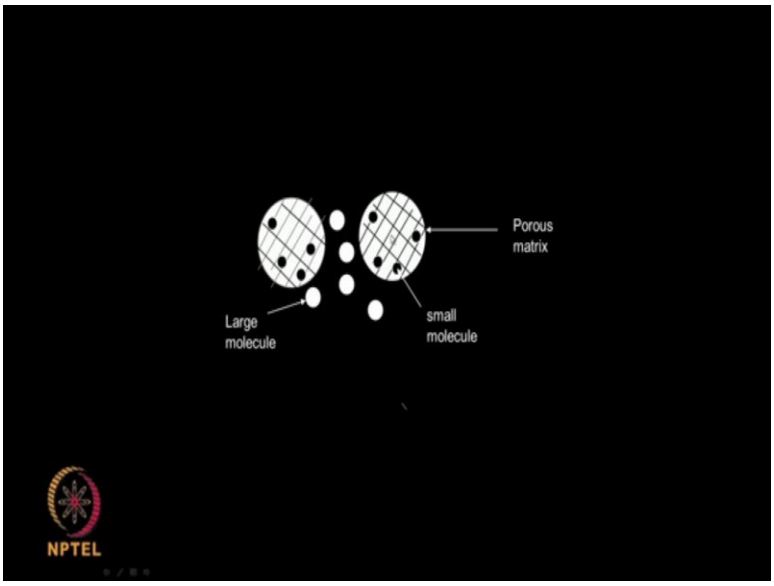

So basically if you look at those stationary phase, you have pore, pores here. It is made up of lot of pores. So the small molecules can nicely enter whereas the larger ones cannot enter and so they are very porous different porosity.

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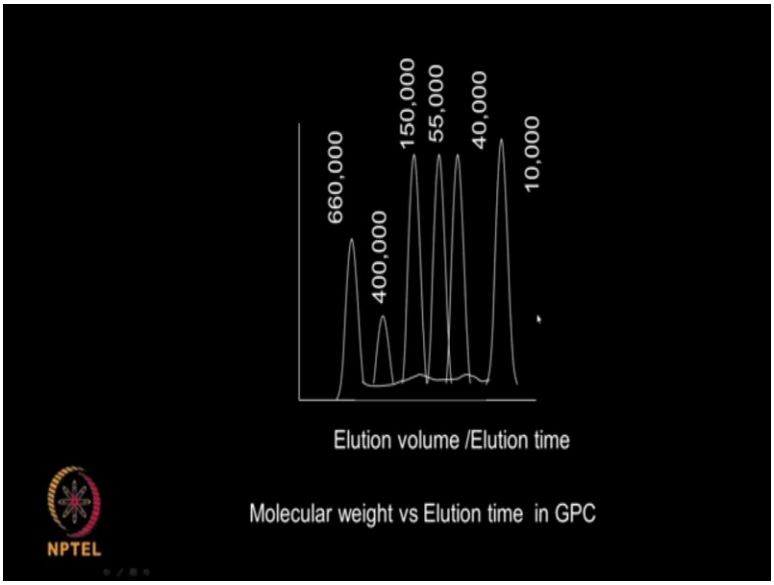

So if you look at a typical gel permeation or size exclusion chromatography, chromatography as a function of elution volume or elusion time, okay, the larger ones or the larger molecular weight comes first and the smaller ones, so this smaller molecular weight comes out last. So we can easily identify the molecules over a wide range of molecular weight. So it is a very very useful technique for doing the separation as well as for analytical purposes.

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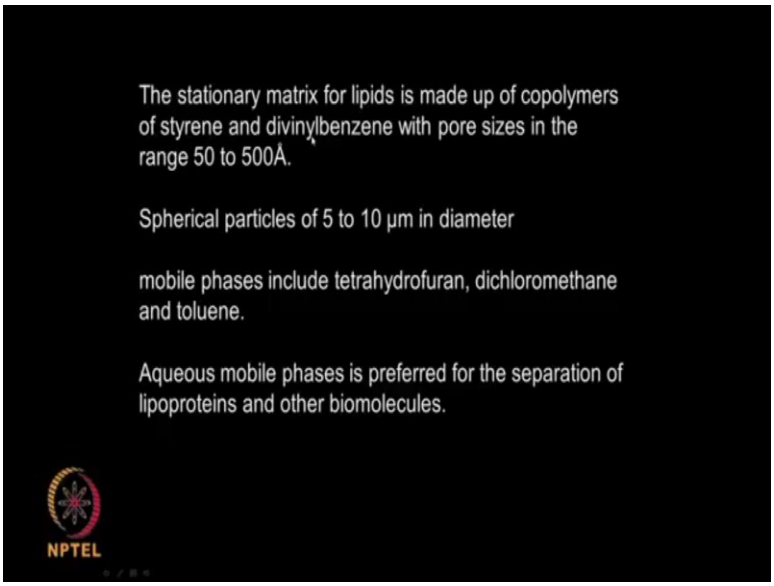

So the stationary phase lipids is made up of copolymers of styrene and divinylbenzene, whereas if it is if it is a lip a hydrophilic material, you may have hydrophilic type of polymers, so the fir

for the lipids you are having styrene or divinylbenzene and pore size varying from 50 to 500 armstrong.

There are of spherical particles of 5 to 10 micron, the mobile phase could be tetrahydrofuran, that is or dichloromethane or toluene, then if you have proteins and other biomolecules, you generally use aqueous mobile phase okay, whereas if you are using synthetic polymers, then you can use solvents.

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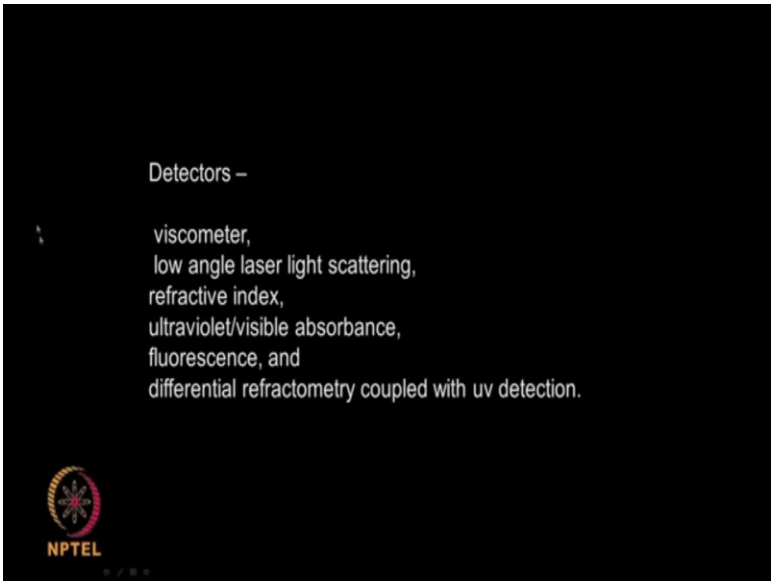

So the detectors you have viscometer detector, low angle laser light scattering detector, refractive index detector, uv visible absorbance detector, fluorescence detector, differential ref refractometer coupled with uv and so on so wide range of detectors could also be used, depending upon the type of polymers or biomolecules are separating okay.

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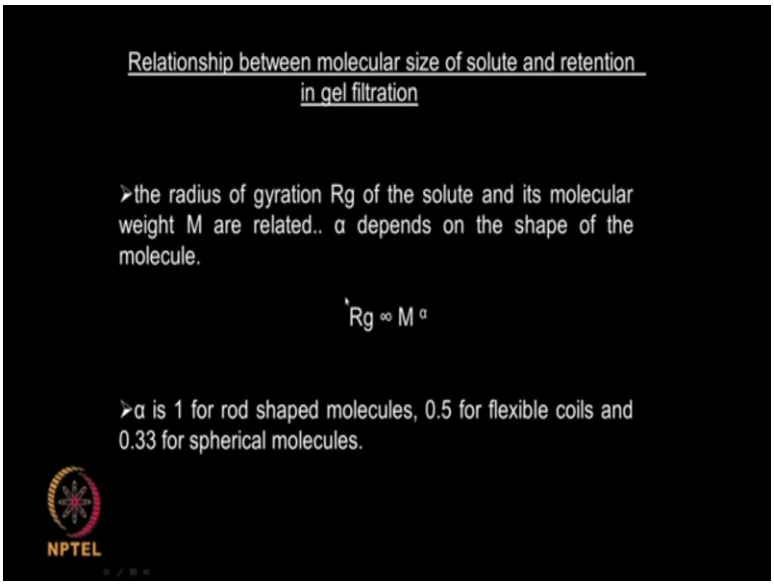

So basically if you look at how the molecular weight of the size seems to relate each other, the radius of gyration of the solute molecule, okay, that is the size or shape of a solid molecule, is proportional to the molecular weight raised to the power of alpha. Now this alpha is 1 for rod shaped molecules and 0.5 for flex flexible coils and about 0.33 for spherical molecules.

So the size is connected to the molecular weight is spherical and raised to the power 0.33. So that is the relation between molecular weight and the size or radius of gyration.

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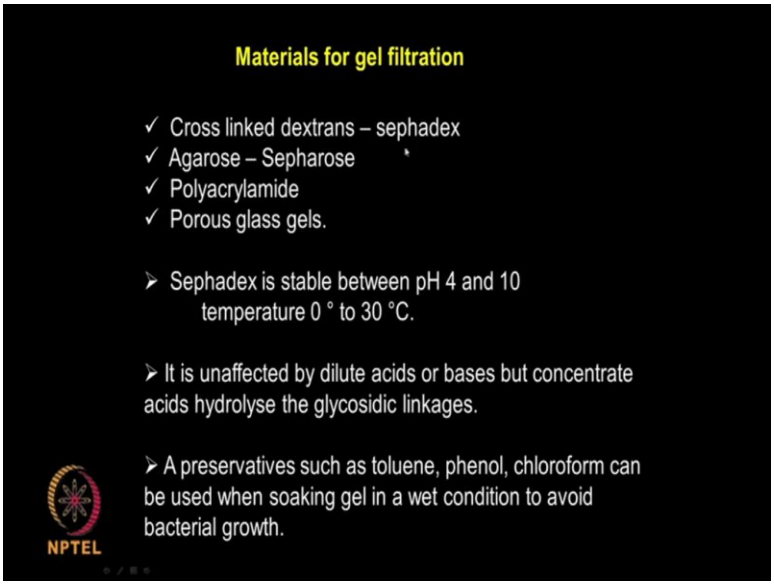

So materials used in gel filtration like cross linked dextrans, okay, if you are looking at hydrophilic polymers, agarose, sepharose, polyacrylamide, porous glass gels and so on... Sephadex is stable between ph 4 and 10 and almost 30 degrees, it is unaffected by dilute acids or bases but concentrate acids hydrolyse the glycosidic linkages.

Sometimes you add preservatives just like toluene, phenol, chloroform, so that you can soak the gel in wet condition, to avoid bacterial growth.

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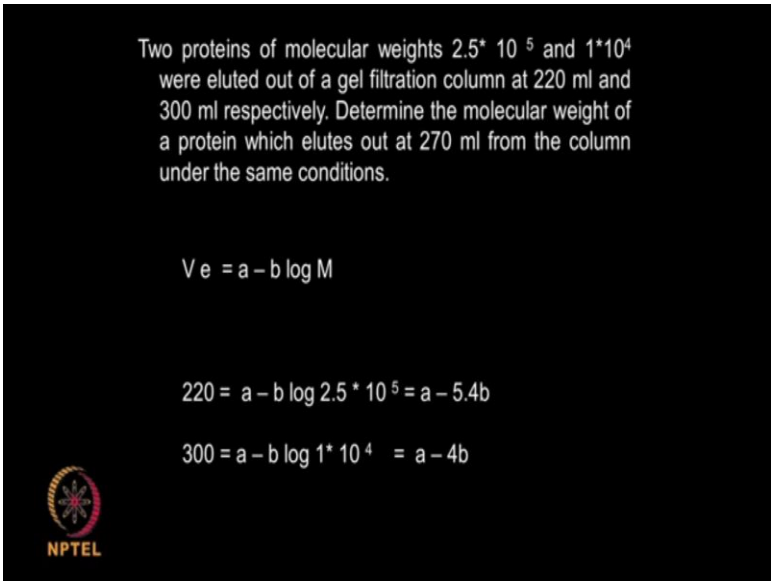

So let us look at a simple problem. 2 proteins, we have one protein with a molecular weight of this, another 10 power 5 range and another protein with a molecular rate of this, that is 10 power 4 range. They are eluted through a gel filtration column. The first one gets eluted after 220ml of this continuous phase collected the other one comes out at 300 ml.

So determine the molecular weight of another unknown protein, when it comes out at 270 ml form the column, at the same condition. So we have the elution volume and the corresponding molecular weight elution volume and the corresponding molecular weight. So now you want to calculate the molecular weight of the unknown, when we know the elution volume.

Now the relationship we need to remember. It is not, although it is proportionality, there will be a logarithm coming in okay because you remember the size is directly proportional to molecular weight raised to the power some alpha right. That is why this log comes in here. So the elution volume and log of molecular weight are linearly related. So please remember this.

It is not just elution volume and molecular weight but elution volume and log of molecular weight. So once you remember that becomes very simple. We have a - term here because as the molecular weight increases, log volume goes down right, as a molecular weight increases volume goes down.

So 220 ml you know the molecular weight 300 ml, you know the molecular weight. So you can calculate a and b from this and then for the unknown, we know the elution volume. We can calculate the molecular weight.

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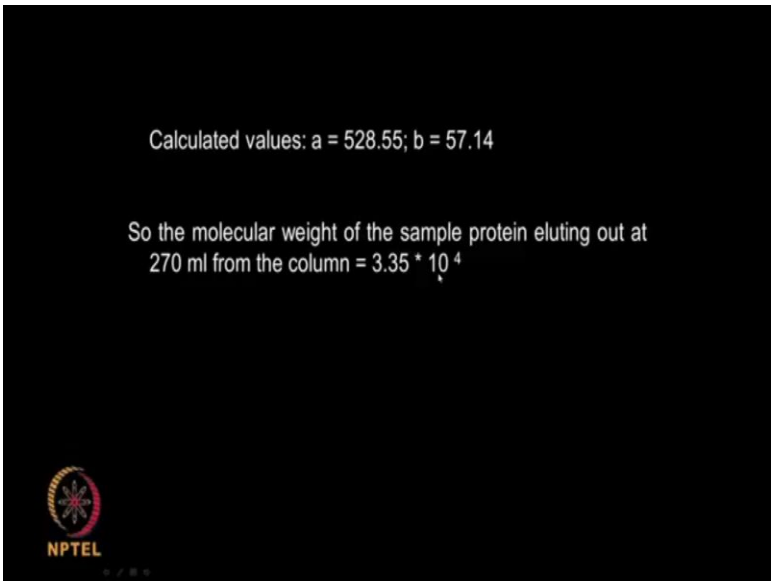

So a and b are calculated, so when it is 270 ml elution volume, the molecular weight is given by this. So it is a very powerful technique, we can find out using this type of permeation, gel permeation chromatography, to find out molecular weight of synthetic polymers, proteins and so on actually. So that way it is a very very useful technique in addition to normal which we are looking at.

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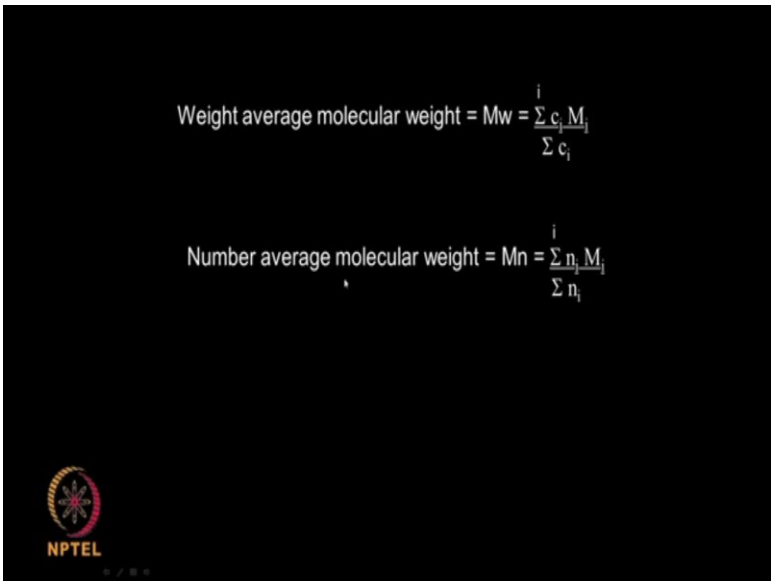

The molecular weight, there are 2 types of molecular weight, one is called the weight average, other one is called the number average. So basically it is called Mw and Mn. So Mw is given by the relation summation of ci Mi divided by ci. ci is the concentration of the polymer of molecular weight Mi and so you multiply ci Mi and divided by all the concentrations here.

So that will give you the weight average, whereas in the number average instead of ci, you use n. That is number of molecules having that molecular weight Mi. So you divide by ni okay that is total number. So this is defined as Mn and this is defined as Mw.

So Mw by Mn is also called dispersity or spread in the molecular weight dispersion and it is very very useful number to have the dispersity. The dispersion number is 1 that means it tightly packed molecules of almost same molecular weight; the dispersity is very large that means you will have molecules polymer chain length of varying sizes. Okay.

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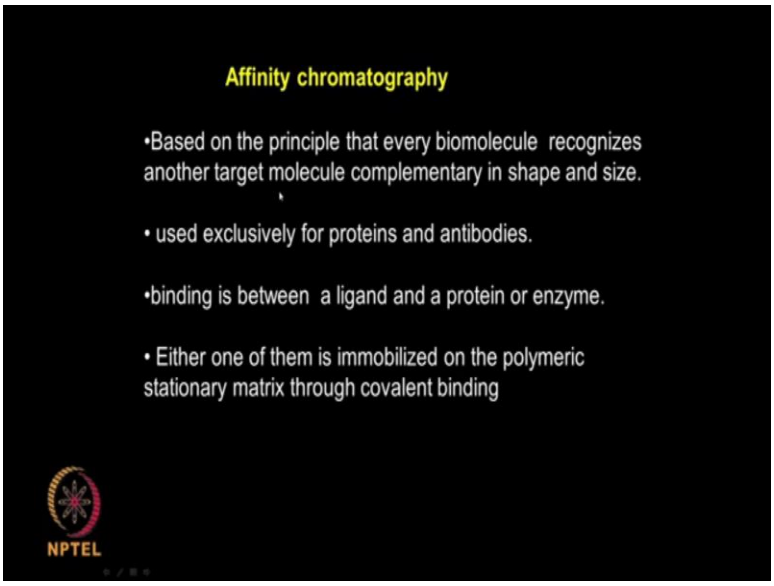

Then comes the affinity chromatography. This is based on the principle that every biomolecule recognize another, target molecule complementary in shape and size okay. It is like a protein ligand enzyme inhibitor its complementing each other. It is like a lock and a key. So it is used exclusively for proteins and antibodies. Okay. So we can have antibody antigen, protein, ligand or protein inhibitor and so binding is between a ligand and protein or enzyme.

Either one of them is mobilized. So you can have proteins immobilized to capture certain ligands, specific ligands or we can mobilize ligands to capture certain proteins of our interest on the stationary phase okay. Generally they are connected to covalent bond. Okay. So we can have the antigen and then we can collect the antibodies and vice versa.

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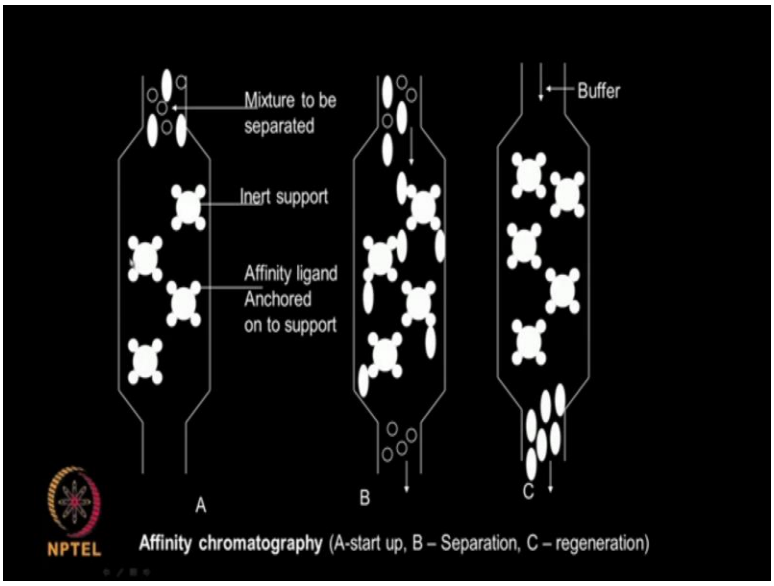

So that is what happens here. So we have the ligand bound to the stationary phase support material, so lot of proteins flow and the proteins which are very specific to that ligands only binds, so other proteins traveled down and later on we can change the condition, so that the proteins that are adsorbed get desorbed and then comes out.

So changing of condition could be either changing ph or salt concentration or ionic strength or dielectric strength and so on actually okay.

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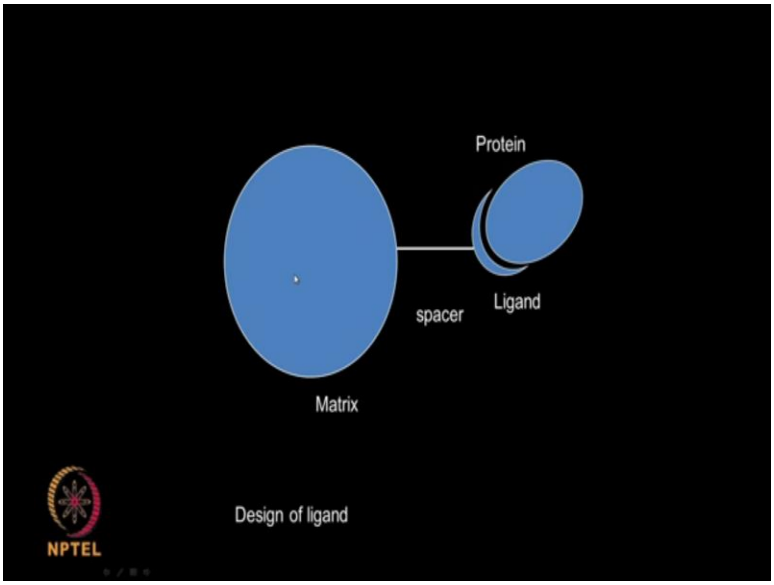

So for example, you have the ligand which is connected to the matrix with the through a spacer. Why do you need a spacer? If you do not have a spacer, the protein will start interacting with the matrix and it will not have a good binding. So there is a spacer, then the ligand. So there is a spacer, then the ligand. So the protein which is very specific to the ligand only will bind to that ligand. Okay. It is like a lock and key mechanism here.

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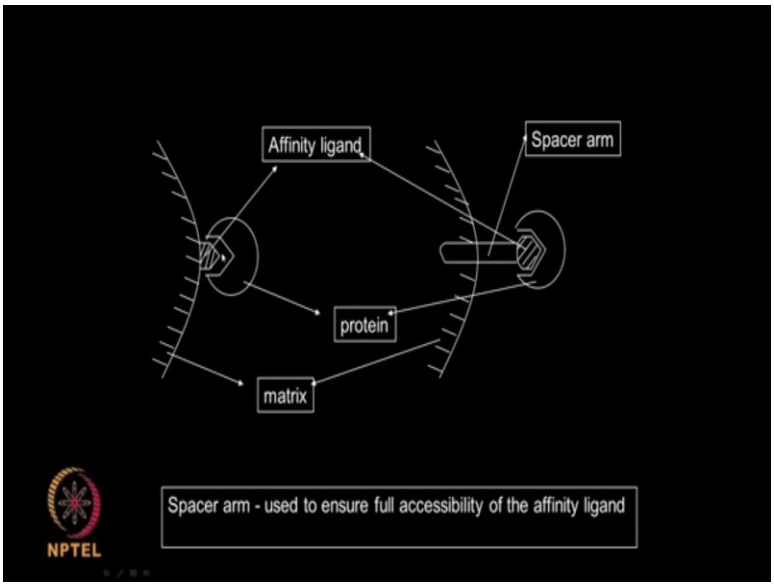

So you have affinity ligands, okay, like this and if you have as I said, if you have the space no spacer, the protein will start interacting with the support and it will not fit nicely. Whereas if you have a spacer, protein will fit nicely to the ligand. That is why you need a spacer.

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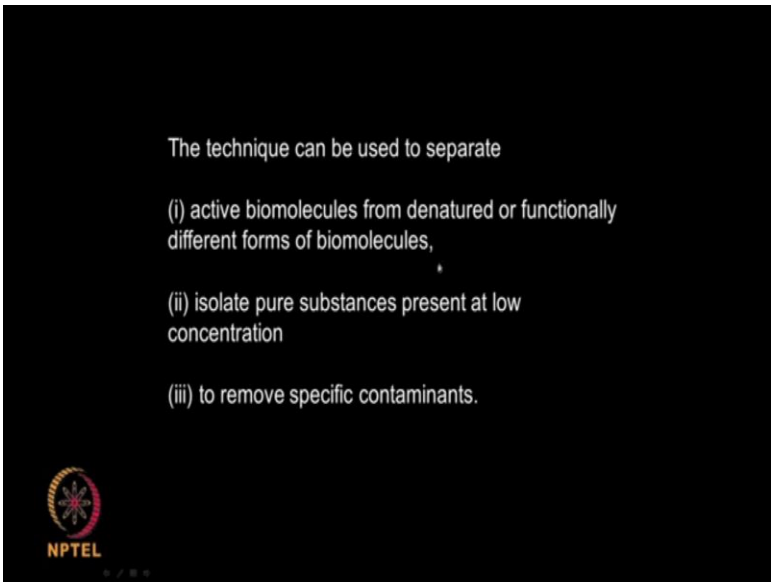

So it can be used for separating active biomolecules from denatured or functionally different forms of biomolecules, isolate pure substance present at very low concentration to remove specific contaminants.

Affinity chromatography, you use quite a lot in biopharmaceutical application, where we are interested in very very pure product and it is an expensive technique because the ligand specific ligand of interest is immobilized on the surface of the stationary phase.

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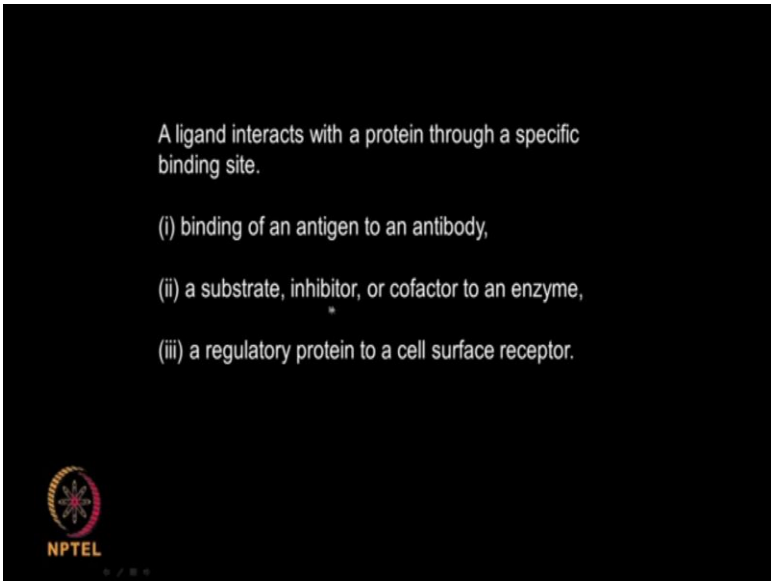

So ligand interacts with the protein through a specific binding. Binding of an antigen to an antibody, they substrate or an inhibitor or cofactor to enzyme, a regulatory protein to a cell surface receptor. So these, each one of them are complementing one another.

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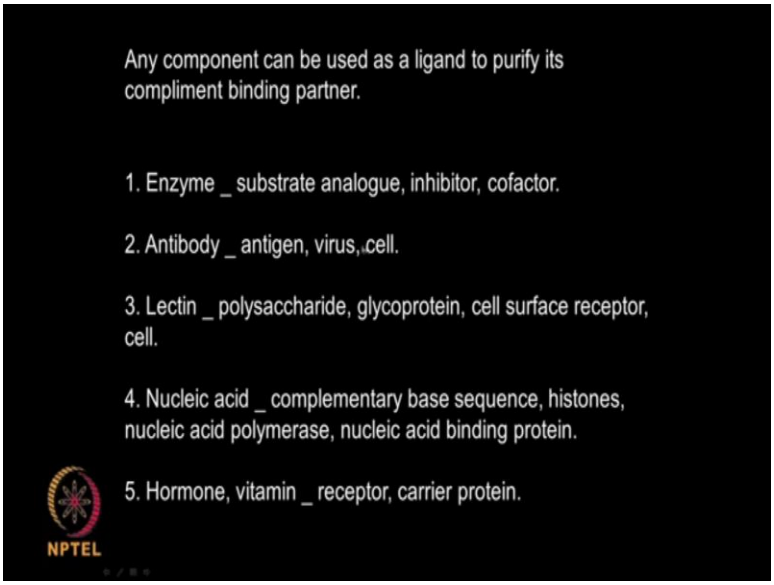

So we can have enzyme, substrate, analogue, inhibitor, cofactor, antibody-antigen, virus, cell, lectin, polysaccharide, glycoprotein, cell surface receptor, nucleic acid, complementary base sequence histones, nucleic acid, polymerase, nucleic acid, binding protein, hormone, vitamin receptor, carrier protein. So you can have different types complementing each other, one on the stationary phase and other coming out to the continuous phase.

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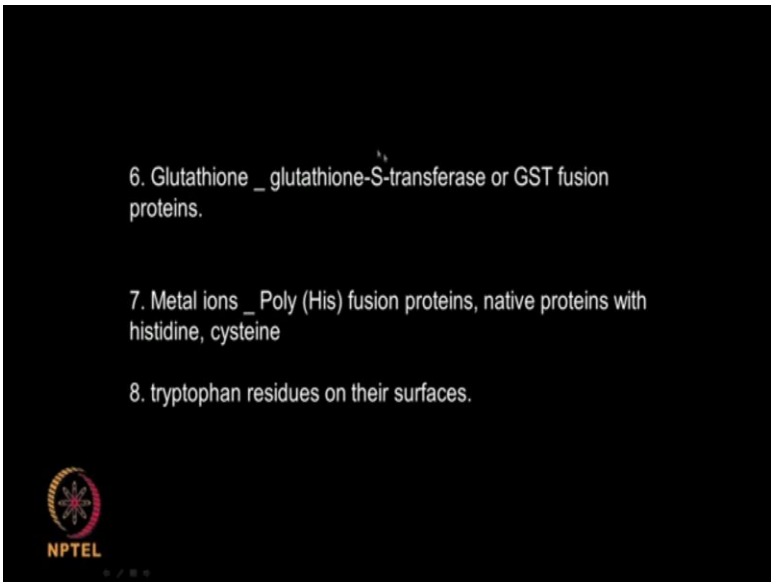

More of it glutathione, glutathione-s-transferase or GST fusion proteins, metal ions going to poly HIS fusion proteins or native proteins, with histidine tryptophan residues on their surfaces and so on actually.

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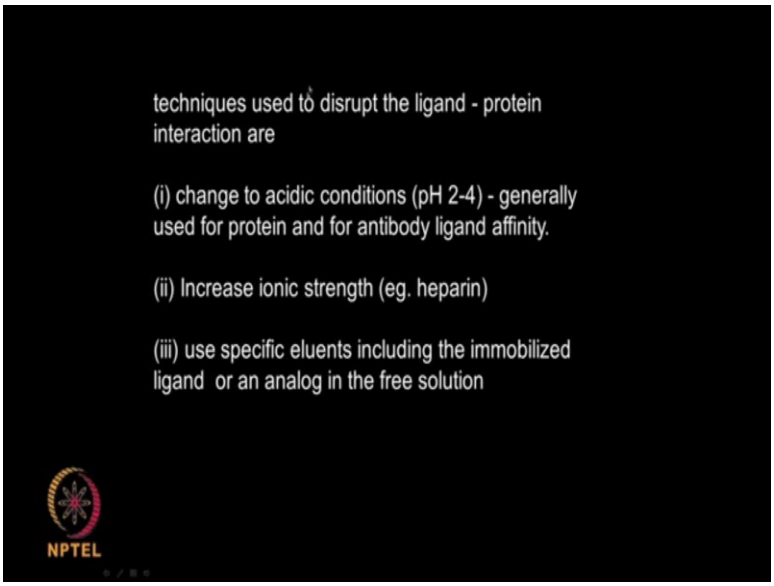

So techniques used to disrupt the ligand proteins, once you have done the job, you have isolated, you need to collect it back. So what do you do? We can change to acidic conditions pH 2 to 4 generally used for protein and antibody ligand affinity. Increase ionic strength, example as in heparin, use specific eluants, including the immobilized ligands, okay so the same ligands, we can send it okay, so that the proteins comes out or an analog in the free solution. So the analog

will go replace the original coating bound to the stationary phase. So different techniques are followed.

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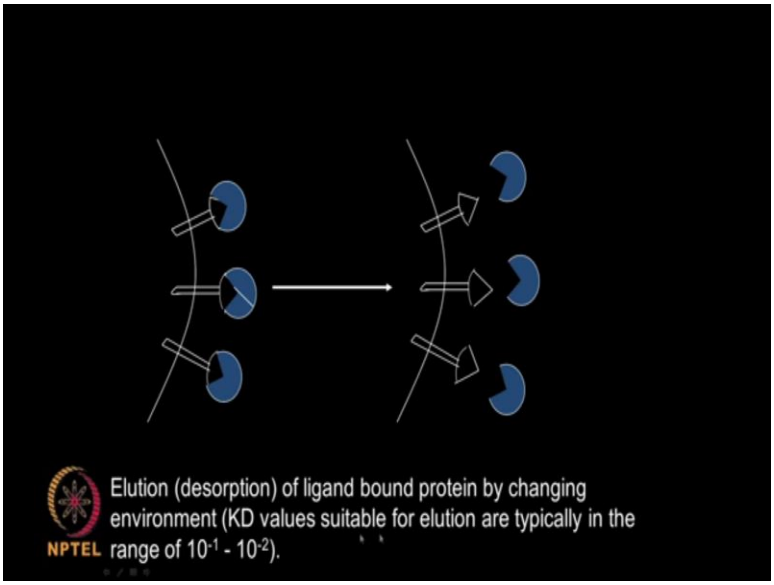

For example, so I can change condition, that is desorption of ligand bound protein by changing conditions environment. So the KD value changes okay, so ideally it should be the range of 10 power - 1 to 10 power - 2 here so that ligand desorbed.

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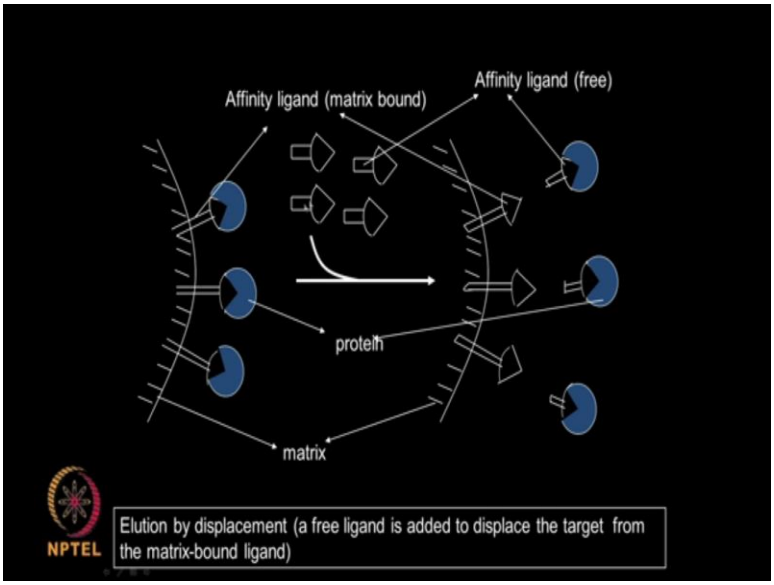

So we can have affinity ligand in the free form, so the proteins that are bound on the surface will now bind to the affinity ligand, which will get washed and it will come out of the chromatography.

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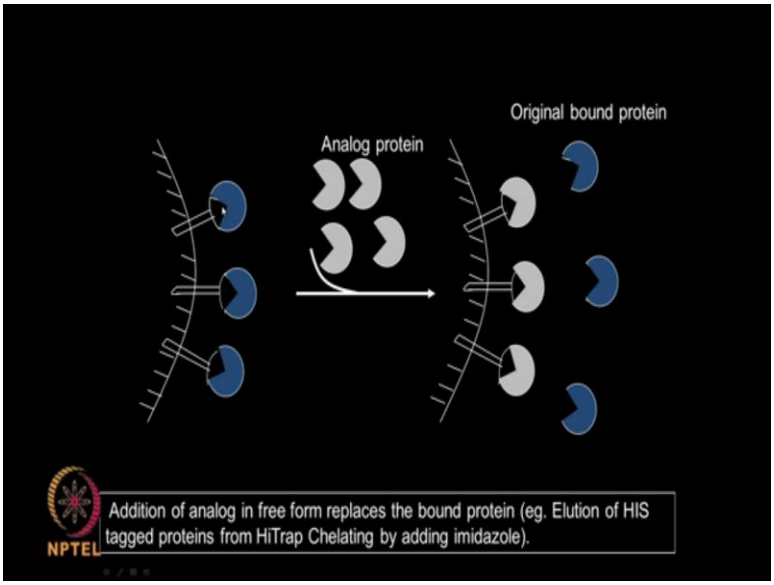

Another approach is, we can have a analog protein, okay, this may have better more affinity with the ligand, so that may leave replace the original bound protein, which can be again removed by washing. Example, for example, elution of HIS tagged proteins from hitrap chelating, by adding imidazole so imidazole will replace the HIS tagged protein.

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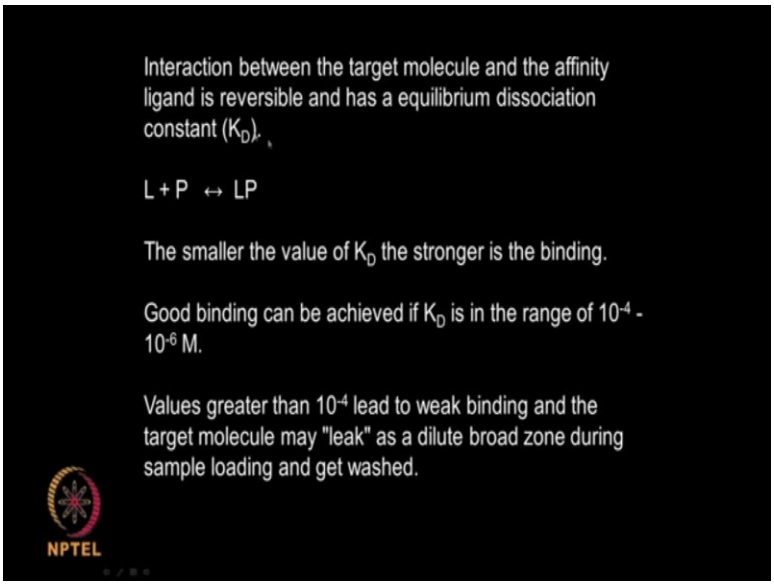

So basically when you are looking at a ligand and a protein okay there is a equilibrium constant dissociation constant, so ligand + protein gives you LP, the complex, the smaller the value of KD the stronger is the binding okay. So if good binding can be achieved, if you are in this range, 10 power - 4 to 10 power - 6 okay, so if it is greater than 10 - 4, this will lead to weak binding. That means the target molecule may slowly leak as a dilute broad zone during sample loading and it will get washed actually. So if it is greater than 10 power - 4 okay.

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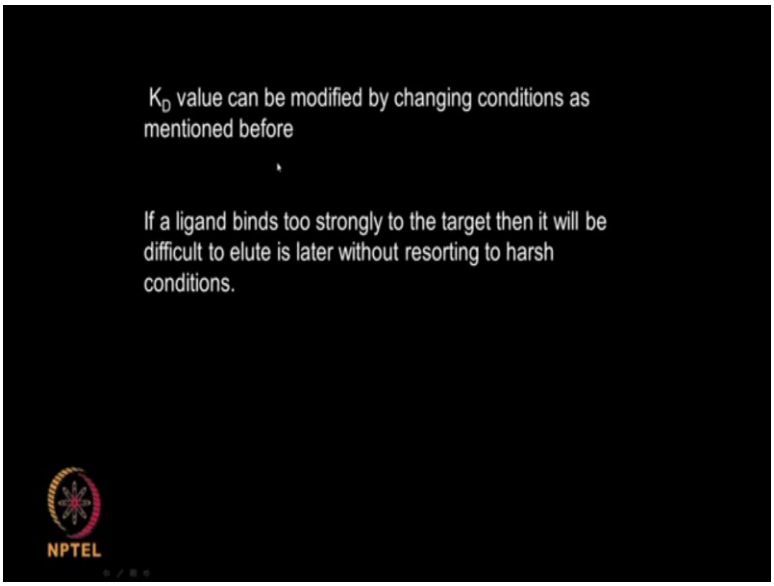

KD values can be modified by changing conditions okay, I talked about lot of conditions. If a ligand binds too strongly to the target, then it may be difficult to elute it out. So we need to balance between good binding, so that there is not leaking. At the same time we should have

enough facility or enough conditions favorable conditions for it to desorb and come out as a washing okay. So if you have larger than 10 power - 4 as your KD value, there could be leakage of target molecule. So what do we do? The residence time is too short for complete binding so it requires higher res residence time or…

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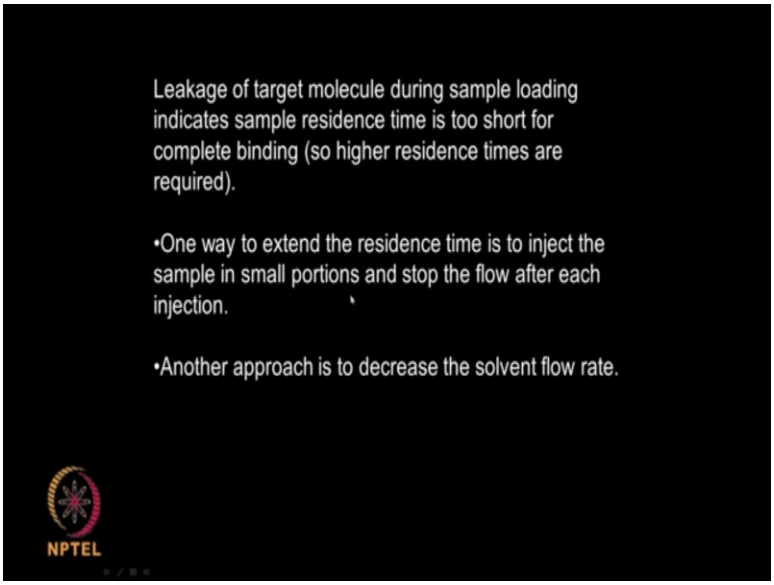

Another approach is to inject the sample in small portions, flow the samples, stop the samples, flow the sample, stop the sample, okay. Another approach is to degrease the solvent flow rate they are therefore your residence time gets increased okay.

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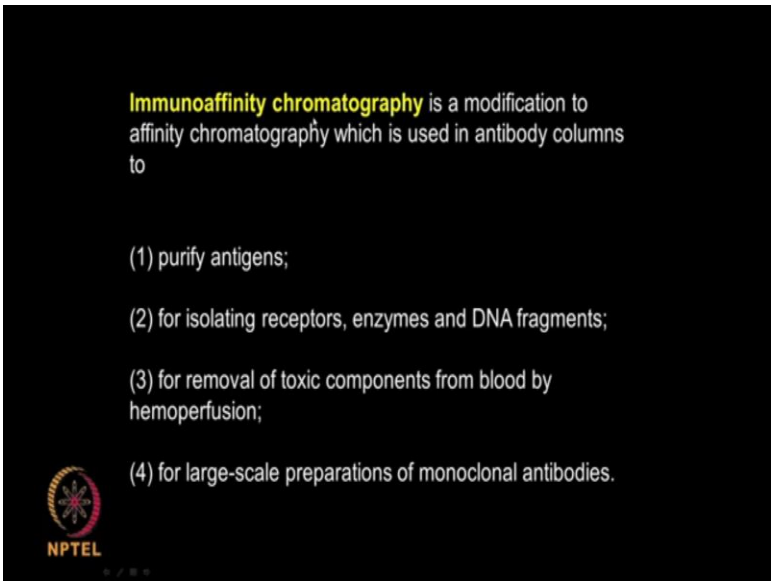

Now let us look at immunoaffinity chromatography. This is a modification of the affinity chromatography, where we are using antibody columns purify antigens that is why it is called immunoaffinity immune. Isolating receptors enzymes and DNA fragments removal of toxic components from blood by hemoperfusion, its large preparation of monoclonal antibodies.

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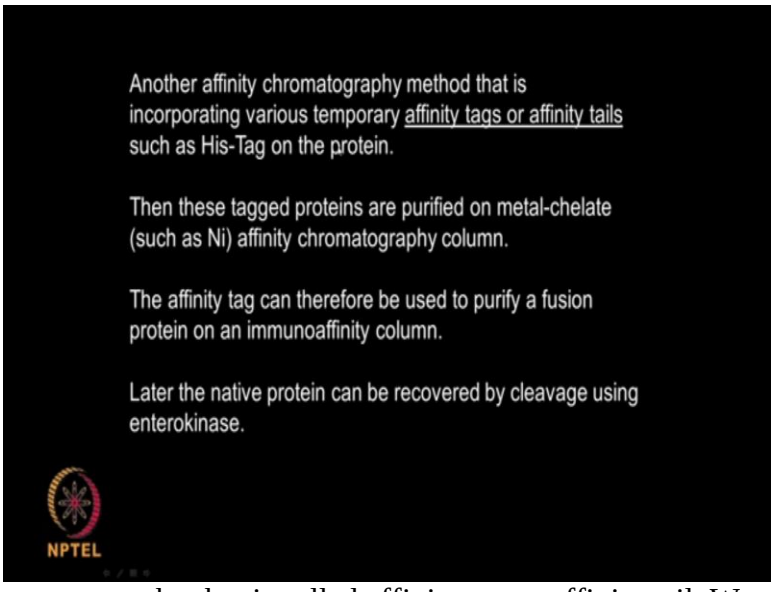

Another affinity chromatography that is called affinity tag or affinity tail. We all must have come across histidine tagged on the protein. Then these tagged proteins are purified on metal chelator such as nickel and then later on, once you do that the affinity tag can there be used to purify a fusion protein. Later the native protein can be recovered by removing this affinity tag, using some cleavage protein using enterokinase like. So you attach a tag like a histidine tag and then

use a nickel column to capture the protein and then you collect it okay and then remove the histidine tag.

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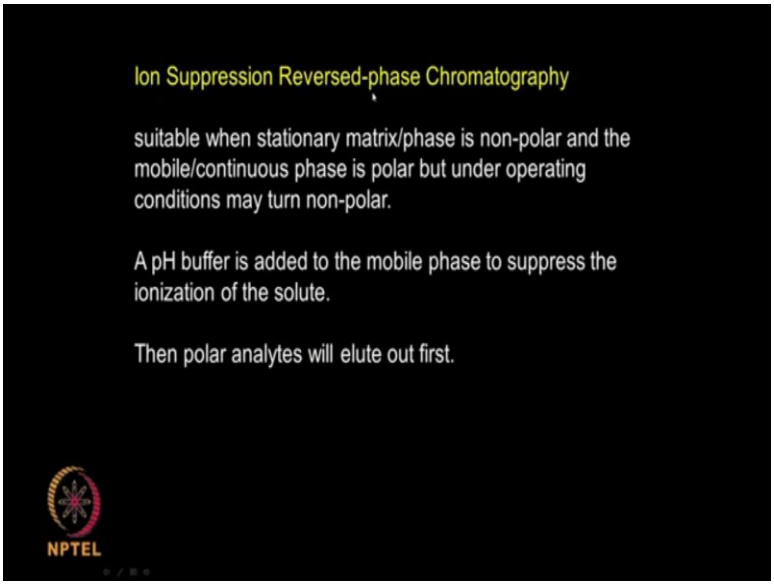

Ions separation reversed-phase chromatography, I did mention this yesterday. If a protein gets ionized, it will appear more polar. So it may not work well in a reverse phase because the reverse phase column is a hydrophobic. So what do we do? We need to add some chemical okay a pH modifier, so that it does not get ionized. That is why it is called ion suppression. So when suitable ,when stationary matrix is nonpolar like a hydrophobic material and the mobile phase is polar like water or aster nitrile, but sometimes because the proteins start ionizing. It may appear polar.

So a pH buffer is added to the mobile phase to suppress the ionization, then it it will look like a reversed-phase chromatography or a hydrophobic interaction. Okay. It is an ion suppression, these are modified versions of the basic 4 types of proteins like your gel permeate, gel filtration, affinity ion chromatography and hydrophobic interaction or reversed-phase chromatography. Okay.

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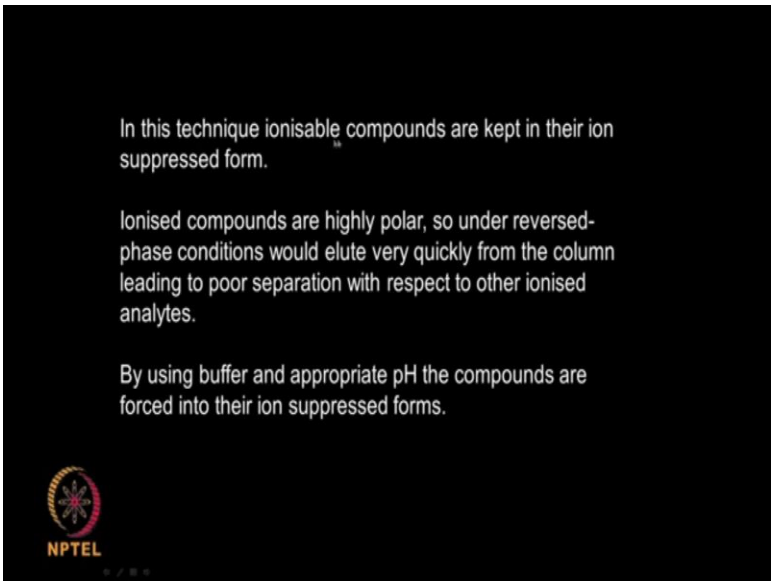

So in this technique ionizable compounds are kept in their ion suppressed form because ion ionized compounds are highly polar okay. So we can use a buffer and appropriate pH and once we know the pk value, we can keep it in the suppressed state actually.

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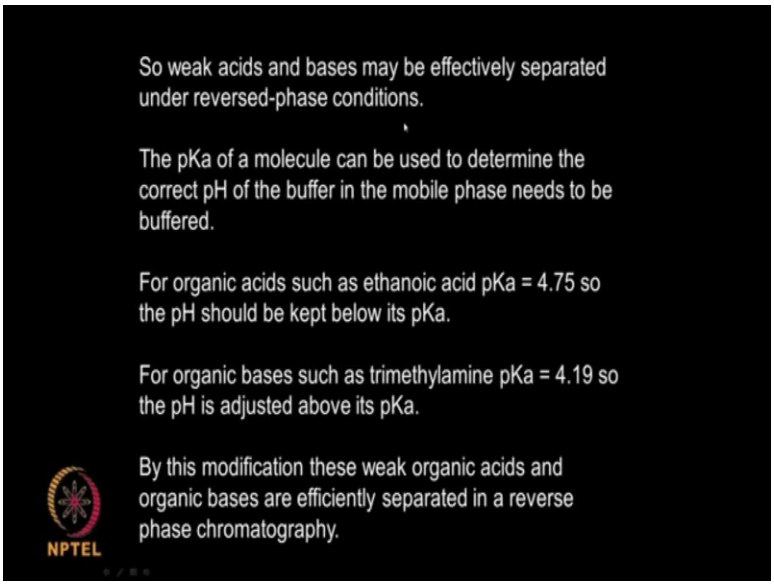

So weak acids and bases may be effectively separated under reversed phase conditions, the pKa of a molecule can be used to determine the correct pH of the buffer okay. So for organic acids such as ethanoic acid pKa is 4.75. So the pH should be kept below this pKa. For organic bases such as trimethylamine pKa is 4.19. So the pH is adjusted to above this. So by this modification, weak organic acids, weak organic bases can be suppressed. I mean it can be efficient, at the

dissociation can be suppressed, thereby we can achieve good separation using a hydrophobic interaction or a reversed-phase chromatography okay.

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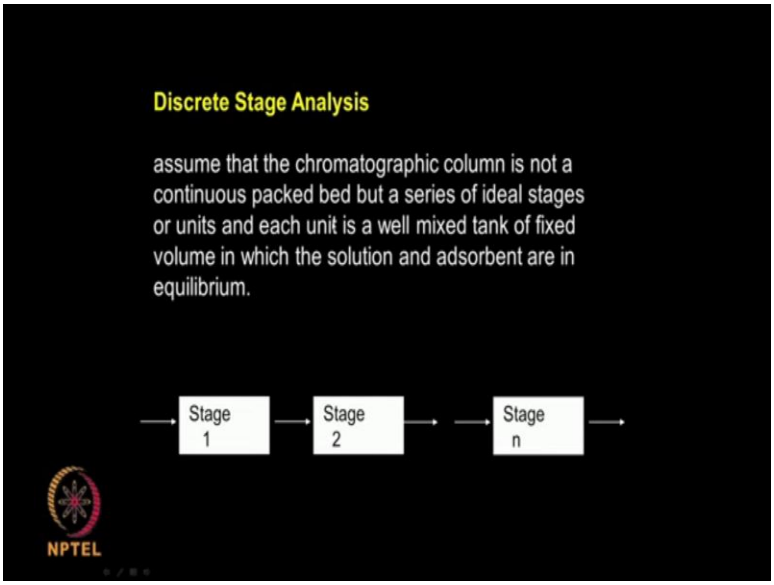

Now we looked at quite a lot of theory now, let us look at some tem analytical approaches. So the chromatography column can be considered as set of n stages, you know 1 2 3 4 and each stage is well mixed contains a stationary phase solid and the mobile phase liquid through which the solute is partitioned because of one of those physical principle which we talked about and then the continuous phase moves to the next stage.

So this almost looks like a liquid-liquid extraction, only thing is one of the stage one of the phases remain constant with inside the stage itself. So there is only movement of only one phase whereas in liquid-liquid extraction you have moment of two phases. So the concentration of the solute will keep changing as it keeps travelling here.

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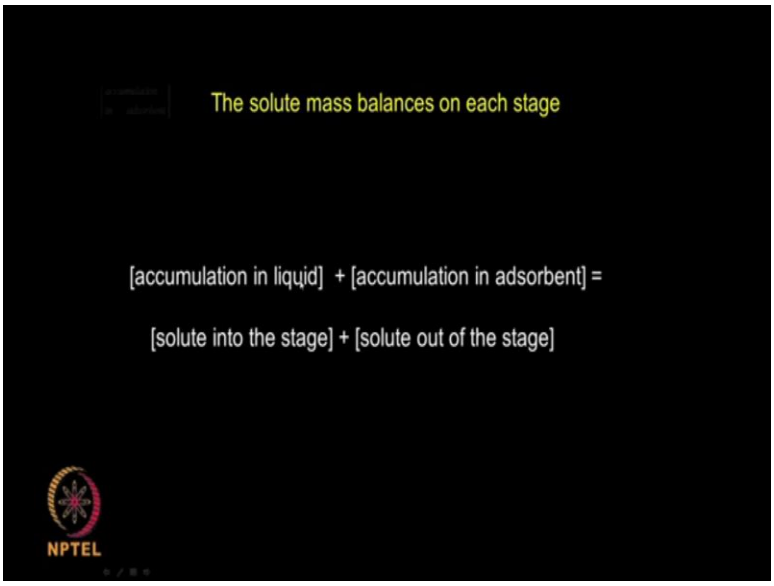

So if you want to do a mass balance of the entire stage, the accumulation in the liquid phase in one stage accumulation, in the adsorb for the stationary phase, in one stage solute into the stage solute out, of the stage right so this is the total mass balance happens. So accumulation in liquid accumulation in adsorbent solute into the stage solute out of the stage.

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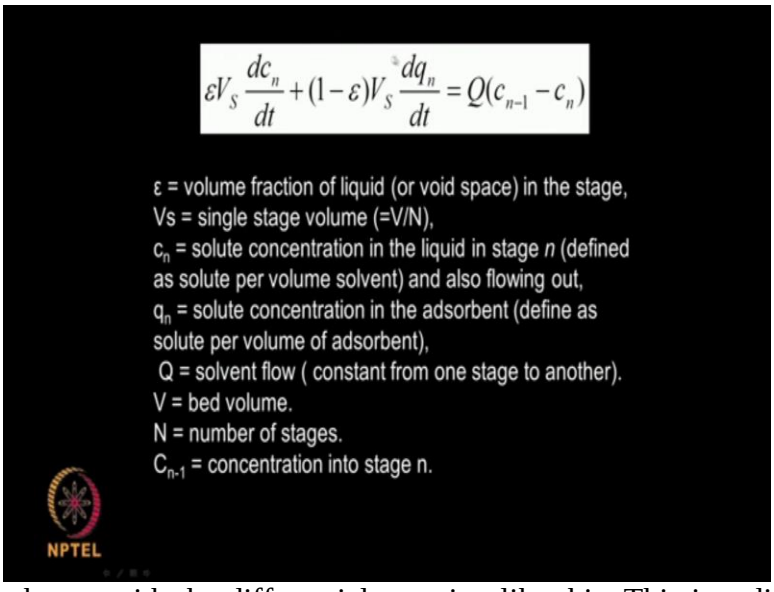

So it ends up with the set with the differential equation like this. This is a different differential equation because cn - 1 is a concentration of the solute in the mobile phase entering, cn is the concentration of the solute in the mobile phase leaving the stage n, cn is the concentration of the solute in the mobile phase or in the liquid that is inside the stage n and qn is the concentration of

the solute in the stationary phase in stage n. And epsilon is the porosity of void space okay, so epsilon is the volume fraction of the liquid.

So 1 - epsilon, Vs is the volume of the solid, so epsilon into Vs that is the volume of the stage will be the volume of the liquid. So Vs is given by total volume of the entire chromatographic column divided by n, so depending upon what is out n generally, we would like to take very large n, so that the, if the derivation or the results are much more accurate. So one point you need to note is n. So we could have n varying from 1 to capital n okay that is the total number of stages. So we could have several differential equations, we moving form c1, c2, c3, c4, c5 and so on actually okay. So this is a set of difference differential equation okay.

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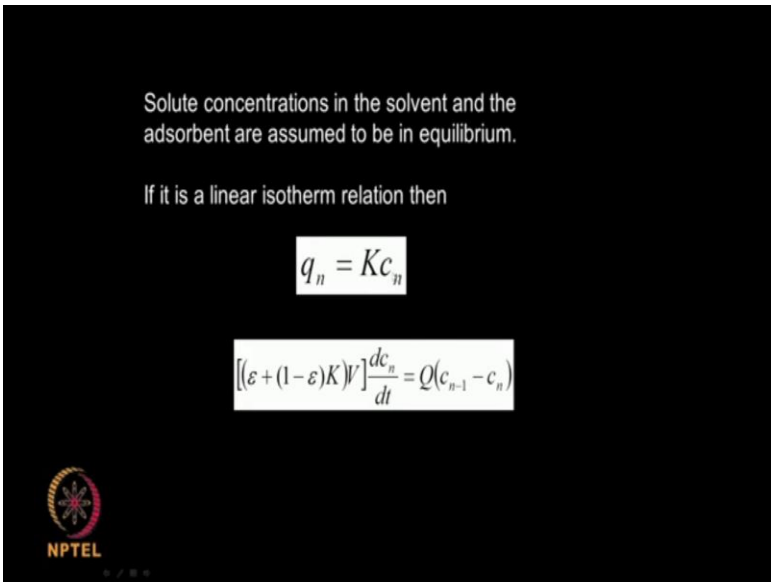

So one can solve this if the relationship between the concentration of the solute, in the mobile phase concentration of the solute in the stationary phase are related in a linear fashion. That means in a linear isotherm, then you know qn equal to KCn, that means it can be q1 equal to KC1 q2 equal to KC2 and so on actually. So when you do that, the equation becomes manageable. It looks very simple right, so this is a set of a difference differential equation but it looks very nice and clean. This is valid only when you assume linear isotherm relationship and you assume the k value remains constant and in all the stages that means q1 equal to KC1 q2 equal to KC2 and so on actually.

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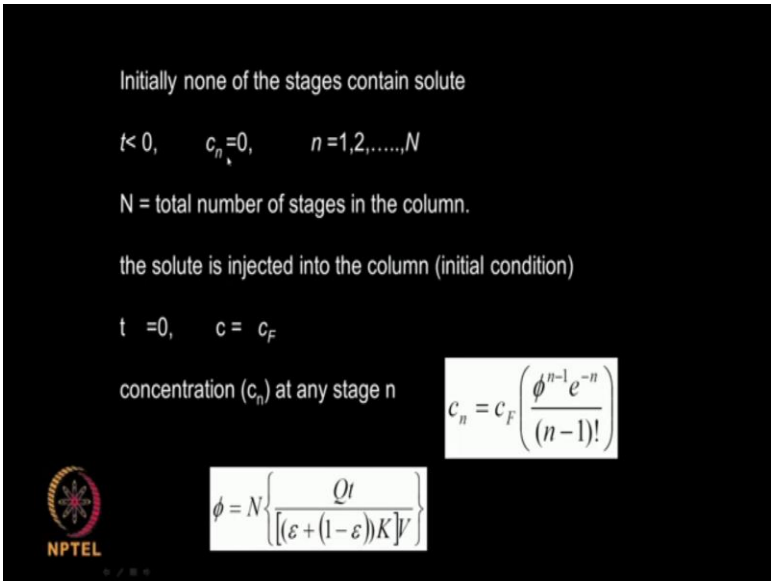

So initially at time less than zero, there is no solute inside the vessel right, inside the chromatographic column. So it will all be 0. So only at time equal to 0, you are injecting some cF okay so when you do that, it is easy to solve. Let us not go into it. You will end up with an equation with this form. So concentration of the solute leaving any stage is given by the concentration of the feed phi raised to the power of n - 1 e - raised to the power - n divided by n - 1factorial okay. You all know what is a factorial right?

Factorial 3 is 3 into 2 factorial. 5 is 5, 4… 5 into 4 into 3 into 2 like that okay. So the phi is given like this where it is function of n number of stages, epsilon is the porosity of the void volume, v is the volume of the column, k is your partition coefficient, t is your time. So that will come here. So with these equation, we can calculate the concentration of the solute leaving any stage as a function of stage as well as function of time, when I inject a sample at t equal to 0 of concentration cF okay. So you have solved this set of difference differential equation for linear isotherm okay.

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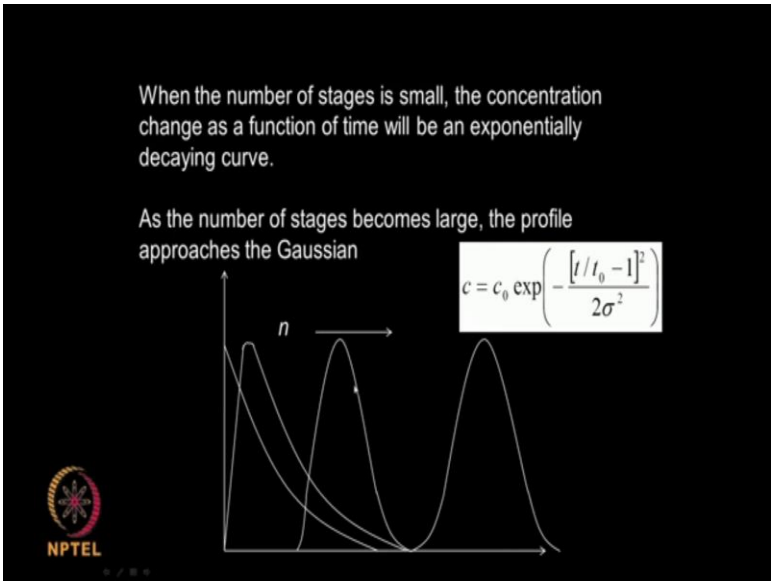

So if n = 1 then you will, when I checked the sample that means if the whole chromatographic column is assumed to be a one single stage, when you inject a sample at time equal to zero, the out lay output will appear like this output will appear like this exponentially falling that is for n = 1. So when I slowly increase the n, when I make n equal to 2 and I inject the sample in the entrance, it will go up the concentration and then it will go down as you keep increasing the number of n, total n, so the concentration will keep going like this and finely it may end up into a nice looking normal distribution or a Gaussian distribution which will obey this equation.

You remember this equation which I taught you in the previous last two classes. So for very large n hmmm, very large stage number of stages, it will, the output will appear like a nice looking normal distribution or a Gaussian distribution. Whereas if n = 1, it will appear as a exponentially decaying concentration okay. That is why you need to consider the entire chromatography column divided into several stages of type n okay.

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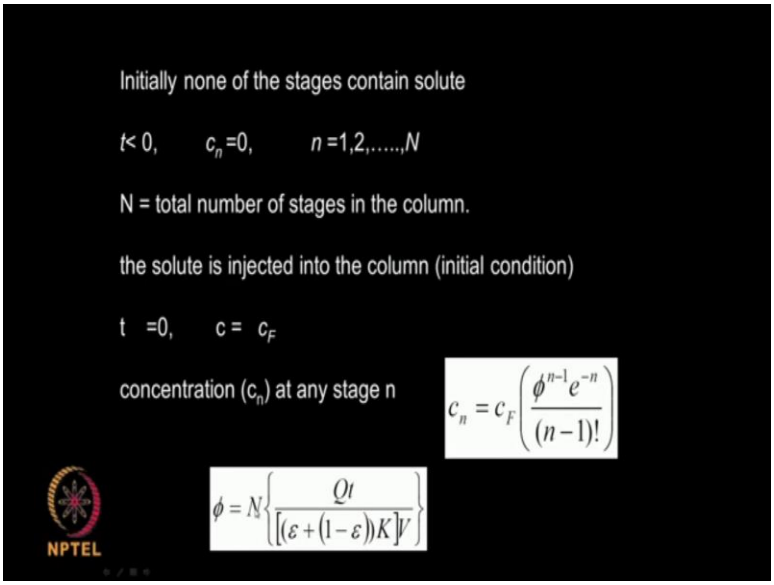

So we have this equation and we have these equation and he we can relate these two equations to get relationship between n. n is the number of stages right relationship between this and these okay.

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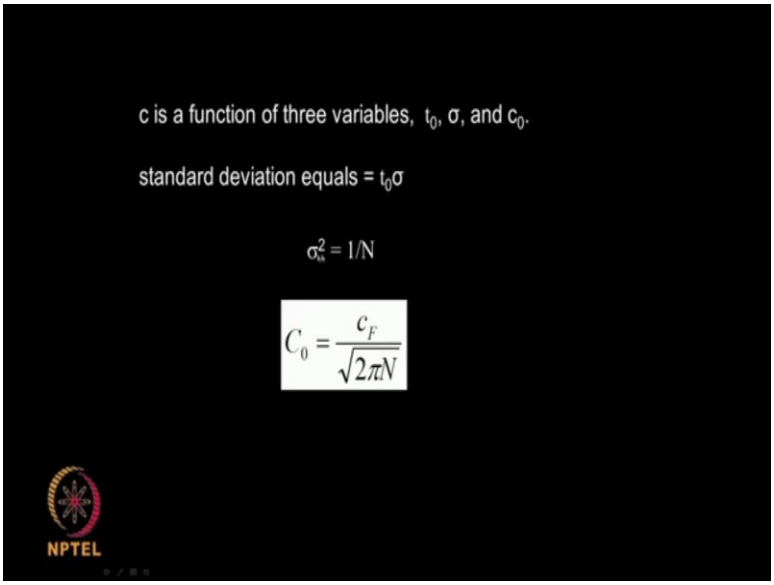

So when you do that, we find something interesting. Sigma square = 1 by n. Please remember standard deviation, I said is t not sigma, so we get the relationship between sigma square = 1 by n. So when I get a chromatographic peak okay when I get a chromatographic peak, I can calculate number of stages using the width and the base or the width at the half maximum or if I

know sigma or if I know the standard deviation and the retention time, I can calculate sigma and from there I can calculate n that is number of stages okay.

So there are different approaches by which we can calculate n, the number of stages and I also mentioned that this n is the number of theoretical plates in the previous I said why they are calling it as theoretical plates okay. Then c zero that is the maximum concentration, the peak reaches is also connected as cF divided by square root of 2 pi n okay, so the many relationships are brought in which connects all these parameters okay.

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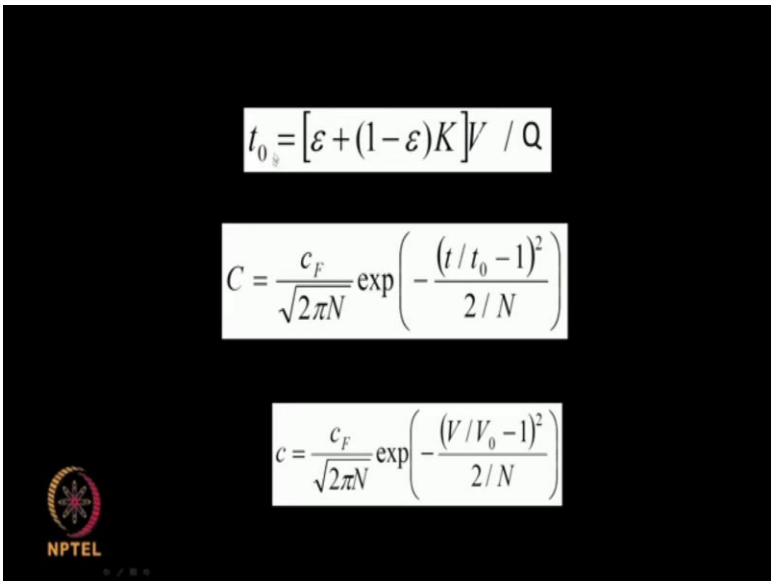

And then we can say t not that is the elution time = epsilon + 1 - epsilon k multiplied by v / q and then so c = cF square root of 2pi N exponent - t / t0- 1 whole square by 2 / n you had a sigma square before. So instead of sigma square, we are putting n and here we had c0 and instead of that, we are putting cF by square root of 2pi N. Okay. So you see all these are getting connected with each other, so we can use these equations to calculate many parameters. We can use it to calculate c0. We can use it to calculate number of stage, we can use it to calculate so many things even k, we can use it to calculate.

So if I inject a sample into a chromatographic column and I know the porosity, I know the volume I know the flow rate, I know the retention time. From the retention time, I can predict or calculate what will be the partition coefficient that is solute partitioning from the mobile to

stationary phase okay. So I can do many things with these equations. so we need to remember all these equations to do several calculations which we will do in as the course of time okay.

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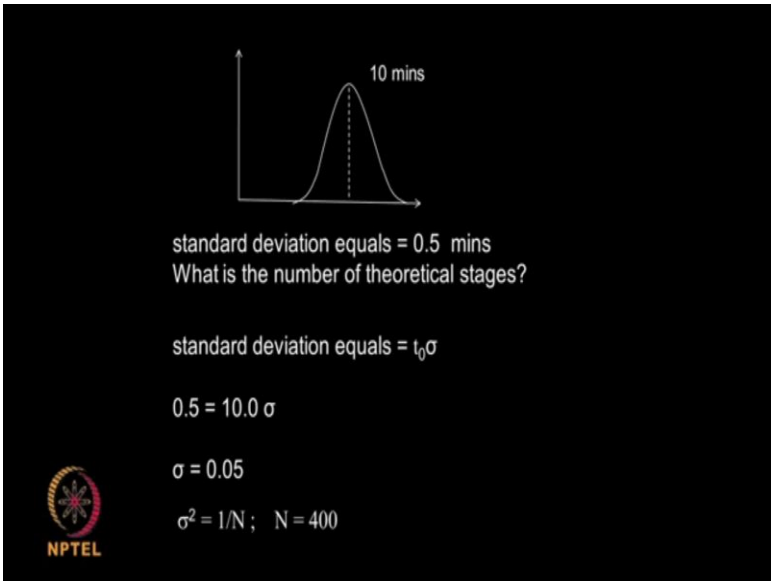

So let us do a problem, simple problem. You have a chromatographic peak coming out, retention time is 10 minutes, the standard deviation is 5 minutes, now what is the n. So it is asking you to calculate n. So remember standard deviation = t0 sigma, that means retention time into sigma okay, so t zero is given by 10 minutes, standard deviation is 0.5, so sigma is 0.05. So as you know there is a relationship sigma square = 1 / n. So n = 400, so N is the number of stages or number of theoretical plates of that particular chromatographic column and I also mentioned,

If N is large we can say the efficiency of separation is large, when N is small efficiency of separation is small. So if I am comparing 2 different chromatographic column, all I have to do is, I will inject a sample and see the peak I can look at the retention time, I can look at the base and from there I can calculate what is the number of stages of column 1 and number of stages of column 2 and then I can say this column is more efficient than another column okay. So that sort of calculations, we can nicely do using these set of equations.

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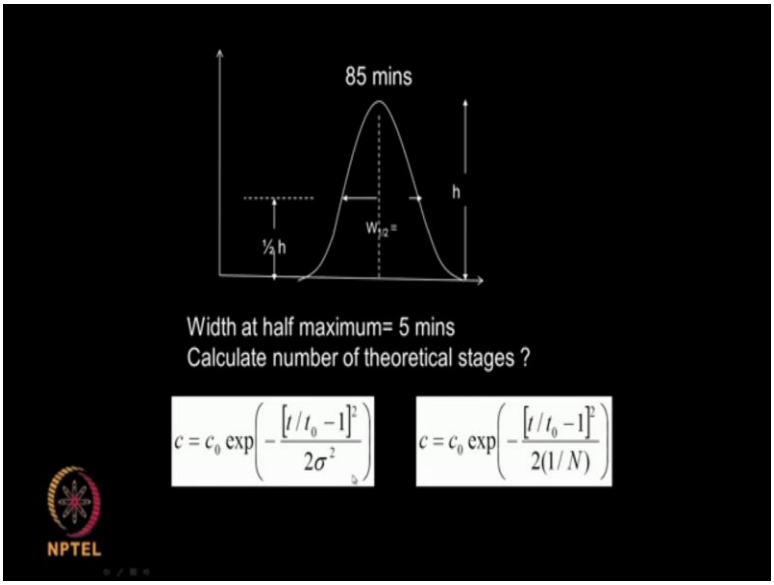

Okay let us look at another problem. So the retention time is 85 minutes, width at half the maximum is 5 minutes, okay so calculate the number of theoretical stages. Do you remember the equation which I introduced in the previous class between the width at half and the retention time and the number of theoretical plates right. So you need to recall that. So if you recall that, I can for example use or I can instead sigma square I can put 1 by n okay.

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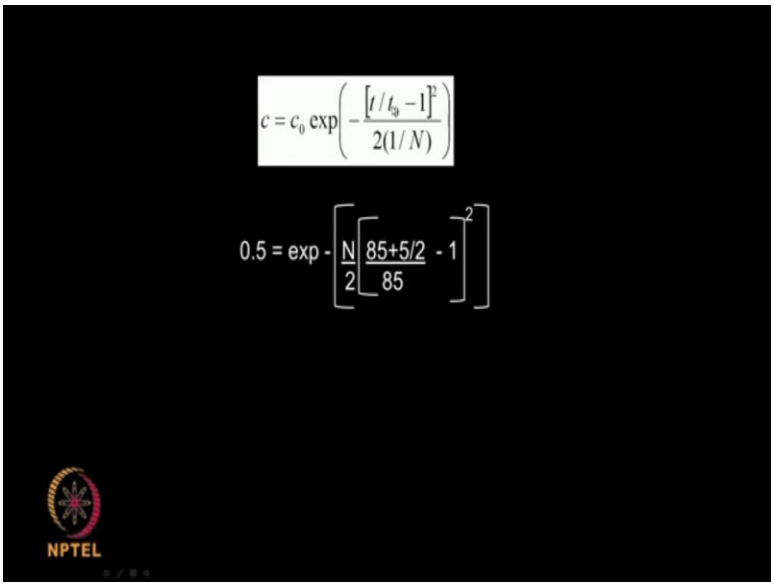

So I have this c / c0 is 0.5 and the retention time is here, okay, 85 is so from there I can calculate the number of theoretical stages okay. Do you understand okay.

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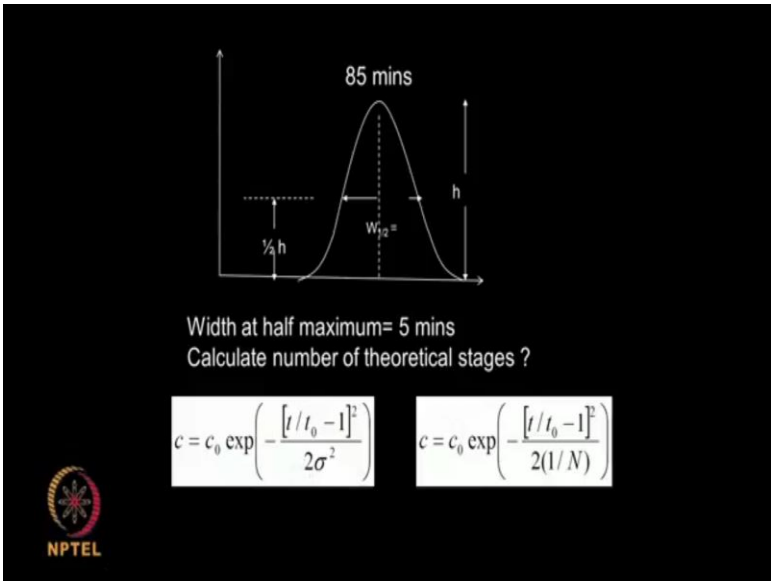

So I have this equation and then instead of sigma square, I can put it as 1 by N. So from there I substitute these terms here and then I calculate the number of stages okay.

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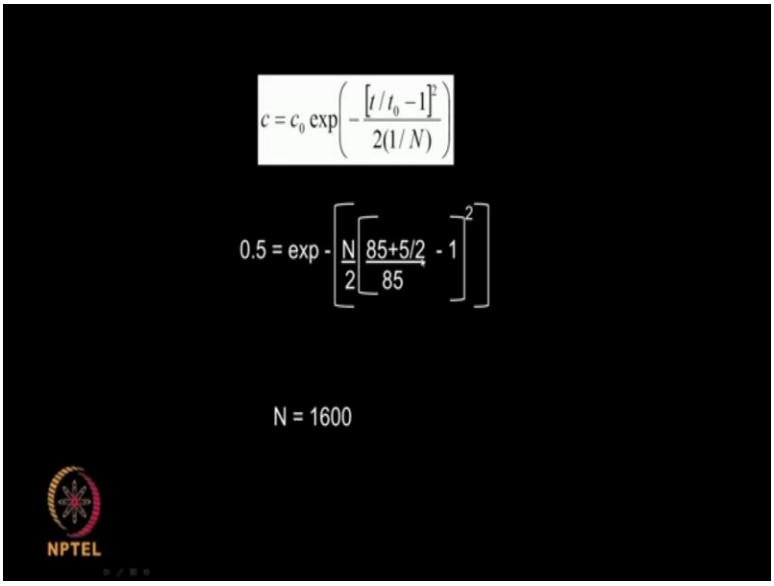

So it comes out to be 1600, okay you may wonder why I am putting 85 + 5/2 and c by c0 as 0.5.

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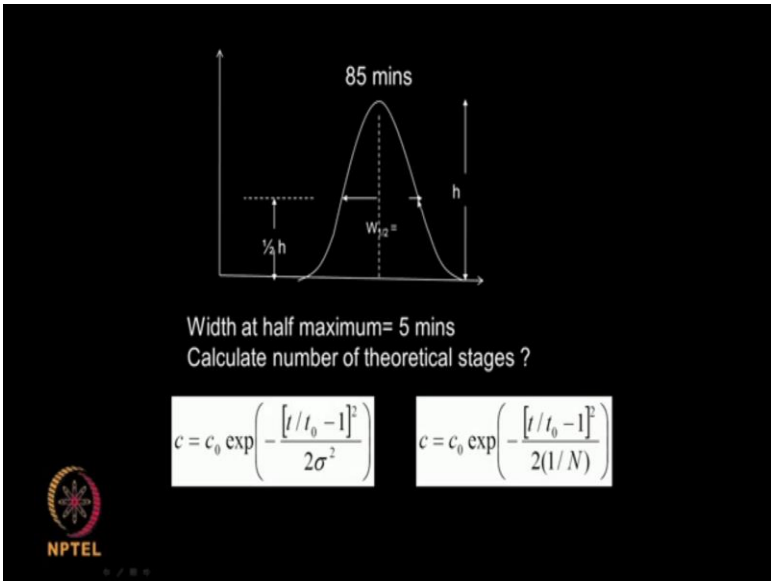

So what, why am I doing that at this place the concentration is 50 % of the c0, so the time at this place will be, how much it will be? 85 minutes + 2.5 minutes right because the width at half maximum is 5 minutes. So if you divide by 2 this will become 2.5 this will become 2.5 and this concentration is exactly half of c0.

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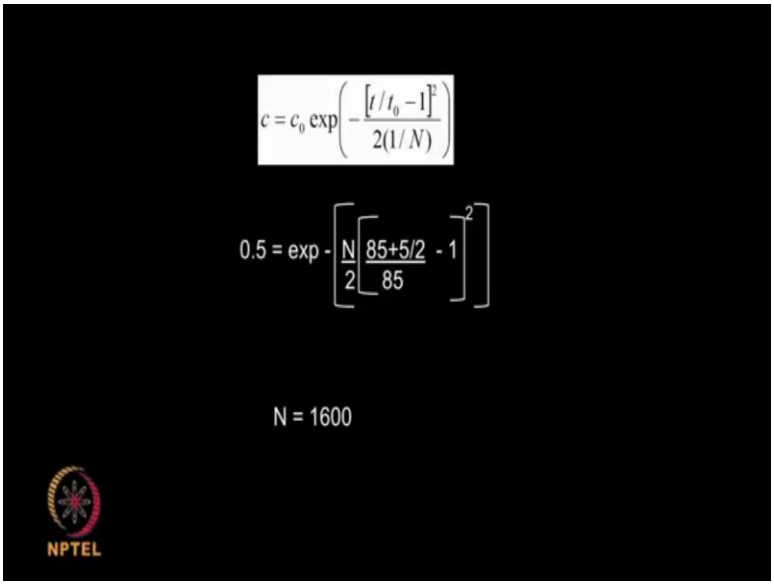

So c by c0 at this place will be 0.5 and the time will be 85 + 2.5 and that is what I am doing here 85 + 2.5 and t not is 85 okay and c instead of sigma square I will put 1 by n. That is why here I put 0.5. From here I calculate n. Understood? Little bit tricky but it is not very difficult to work

out okay. So we will look at more problems as we go along because they are very interesting problems and they are very very useful for chromatographic purposes.

Thank you very much.