Downstream Processing
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Lecture - 26
Chromatography

Most important downstream process is chromatography. Chromatography can be used for separating biomolecules, proteins, carbohydrates, peptides and even small molecules metabolized small organic molecules. Because of chromatography it was possible to purify proteins to a very high degree of purity. The different types of chromatography's, each one working on a certain principle and that is what we are going to look at in the next few classes.

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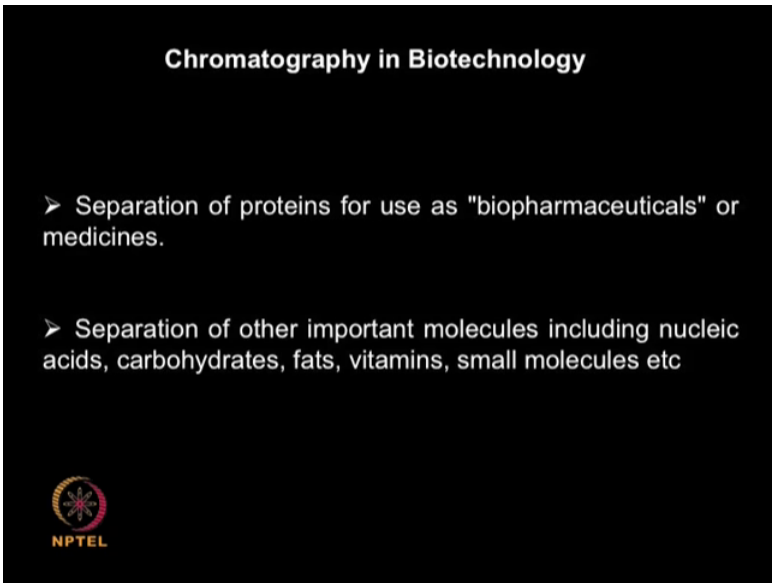

So, chromatography is widely used in biochemical engineering, bioprocess engineering, biopharmaceuticals, and medicine. It is also used for separating out carbohydrates, fats, vitamins, small molecules, and so on actually. So, it is got a very wide range of application.

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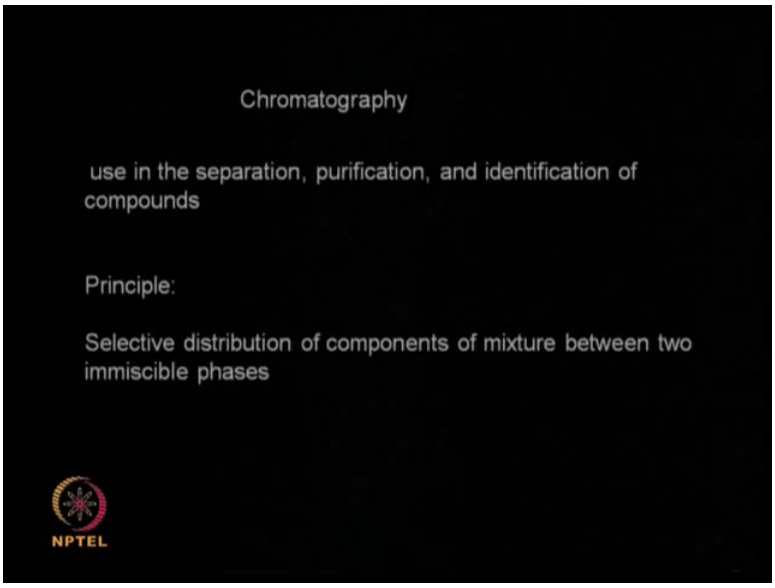

So, it can be used for separation, it can be used for purification and also it can be used for identifying compounds. There is analytical chromatography which is called high performance liquid chromatography or high pressure liquid chromatography that is h p l c, which is used for identifying compounds in a mixture.

So, basically what does chromatography do there is a selective distribution of components of mixture between two phases. So, you can have stationary phase you can have a mobile phase, so the solute gets distributed based on different principles physical principles. Hence, there is a separation of the various solutes so various solutes may have different separating or partitioning effects which may lead to a separation of components from a mixture.

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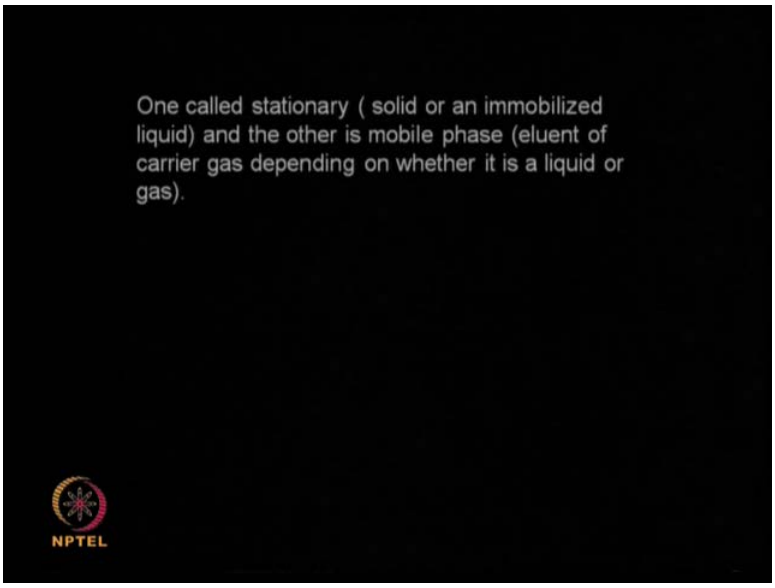

So, in a chromatography what are the various components present, we have a stationary phase that is a solid or an immobilized phase you may have a immobilized liquid or you may have ions or you may have ligands or you may have hydrophobic molecules. All immobilized on a stationary phase. Then you have a mobile phase that means it could be a solvent or a mixture of solvents.

It could be a water and miscible solvent or totally immiscible hydrocarbon. This is called a mobile phase and your solution is also sent in through the mobile phase, which gets partitioned between the mobile phase and the stationary phase. Sometimes you can also have a gas as your carrier, then it is called a gas chromatography and you can have a liquid as a carrier then it becomes a liquid chromatography.

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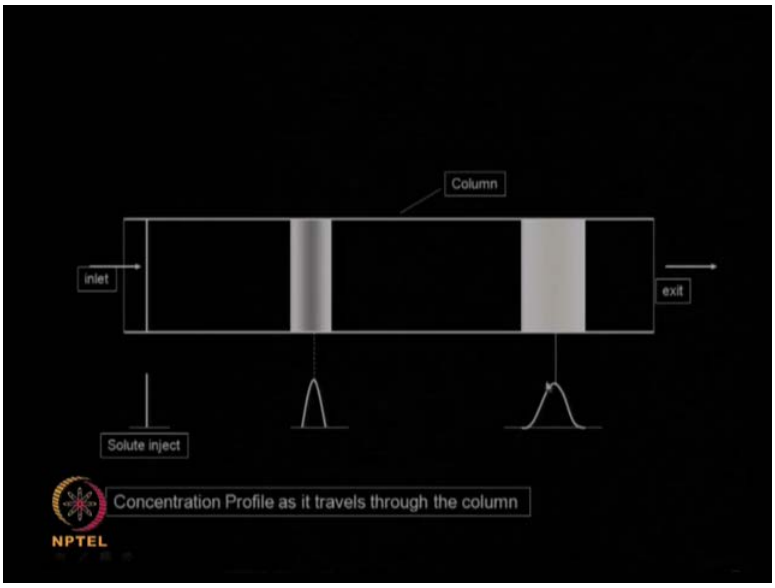

So, typically the chromatography consists of a long column could be extremely long it could be when 100 feet for example, which is packed and then you are injecting the solute so the solute is getting injected at the inlet. So, the solute keeps travelling because of the interaction of the solute with the stationary phase components.

So, there is some sort of adsorption desorption or partition takes place, so thus the concentration of the solute that is moving is going to spread. So, it may be a very sharp injection at the beginning, it is slowly spreading and as it leaves the column is going to spread out like a Gaussian peak.

So, generally we assume this to be Gaussian in shape or we can call it normal distribution or bell shape, but it is never. So, we are going to see as we go long there may be tails or there may be very long start, but generally if you look at a concentration profile as it travels, because of the interaction between the stationary phase and the liquid phase the solute forms here spread uniform distribution spread. This is very common in all chromatography.

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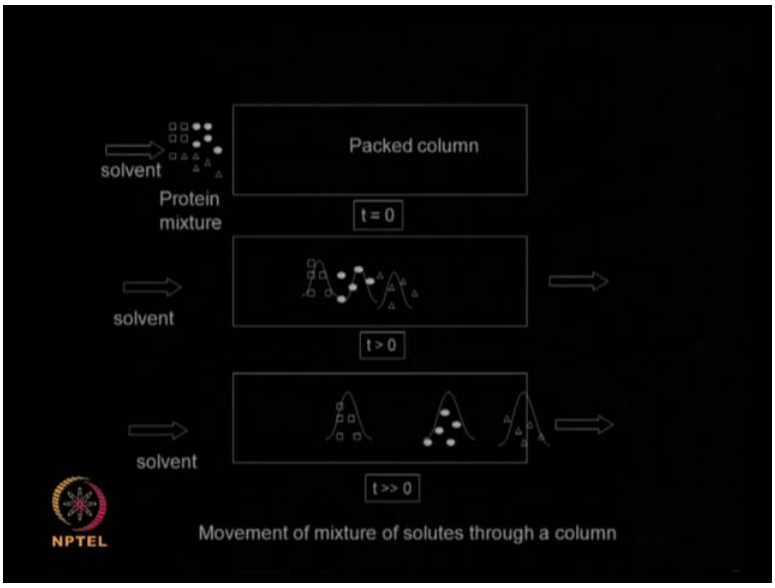

So, if we have the mixtures of protein for example, which is injected at the inlet and there is a solvent, which carries this protein and there is a fat column on which you have certain stationary phase material. The proteins are going to get separated, because of the varying interactions they have with the stationary phase. There could be ionic interaction between the solute in the mobile phase and the stationary phase. This ionic interaction could be varying depending upon the type of proteins present.

So, proteins which are having a very strong ionic interaction will travel slowly or they will get slowdown proteins, which have weak ionic interaction will travel faster there could be hydrophobic interaction. So, proteins which have high hydrophobic interaction with the stationary phase will travel slowly proteins, which will have less hydrophobic interaction with the stationary phase will travel faster or it could be here polar interaction. So, a proteins which I have very high polarity will interact much more with the stationary phase, so they will travel slowly.

Whereas, proteins which will has less polar groups on its surface will not interact much with the stationary phase so they will travel faster. So, slowly as this protein mixture travels through the column. They get separated and the protein, which has the least interaction with the stationary phase will come out first. So, the principle of interaction could be hydrophobic or polar or ionic or affinity or anything like that actually.

So, the proteins which have least interaction with the stationary phase will come out first proteins, which have most interaction with the stationary phase will get retarder. They will come last that is how you get the separation, this is how here chromatography is able to separate proteins or small molecules or metabolized or any biomolecule into various components. Of course, you are not going to get very sharp differentiation in the proteins sometimes there could be an overlap, which may be very close or it may be considerably different.

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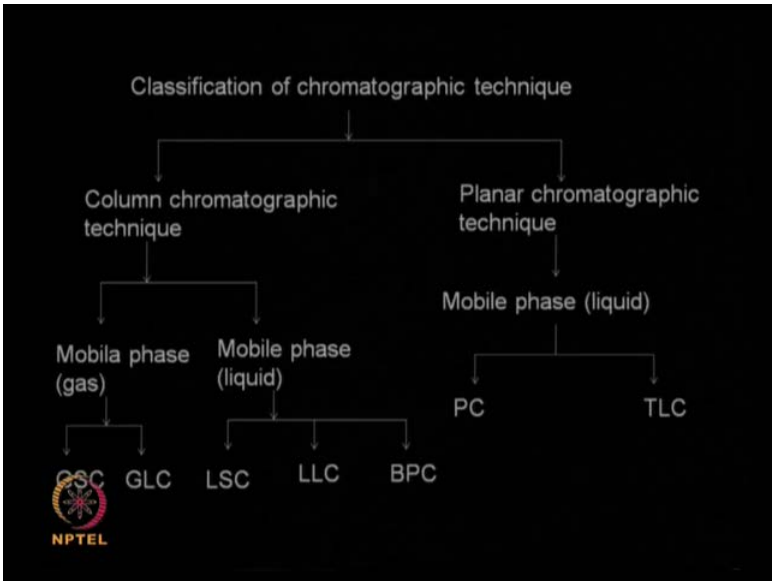

So, we are going to look at all these variations as we going along during the course of this particular topic. There are different classifications of chromatography, it can your in a very broad sense a planar chromatography or a column chromatography as the name implies in a column chromatography. We are going to use a column in a planar chromatography, we are going to have a planar surface like paper chromatography thin layer chromatography.

If you look at chromatography many years back, organic chemistry use to use something call the thin layer chromatography to separate out components in a reaction mixture. Possibly even identify whether the product has formed, if they have authentic sample of the product. They will simultaneously run the thin layer chromatography and they see whether the product has formed.

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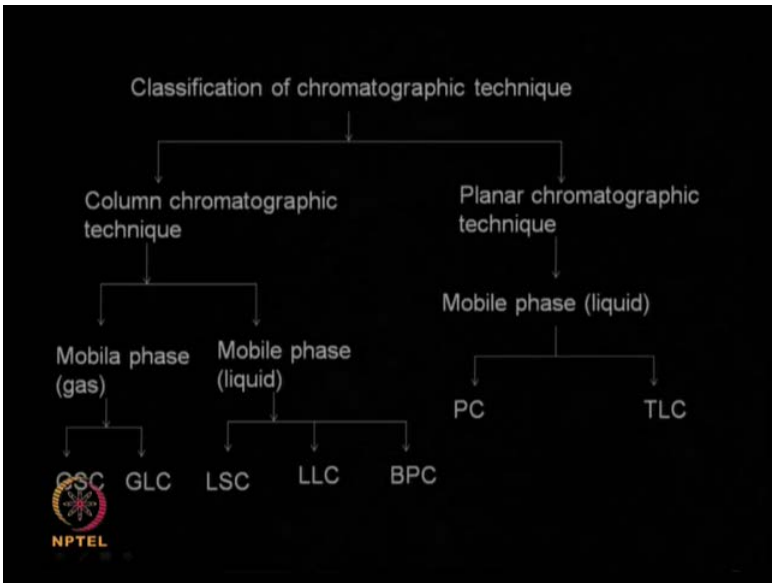

So, organic chemic have been using this chromatography for a very, very long time. If you look at the column chromatography, which uses the column, there is a mobile phase there is a stationary phase in the mobile phase. It can be a gas mobile phase, you can have a liquid so you can have a in the gas. You can have gas solid chromatography or a gas liquid chromatography. Similarly, in the mobile phase you can have liquids, so you can have a liquid-solid, liquid-liquid and so on. So, different types of chromatography's are possible.

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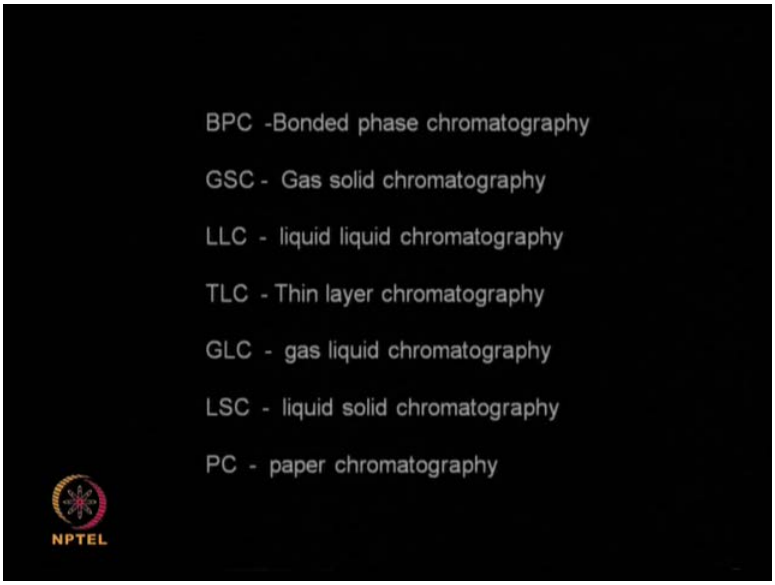

So, if you look at the stationary phase, it could be a bonded phase you can have a gas-solid. You can have a liquid-liquid you can have a gas-liquid or liquid-solid and so on. So, all these various combinations are possible, so that particular table was trying to differentiate between the structure of the chromatography, whether it is a paper or a planar or whether it is a column or a tube. It was not talking about the physical principle.

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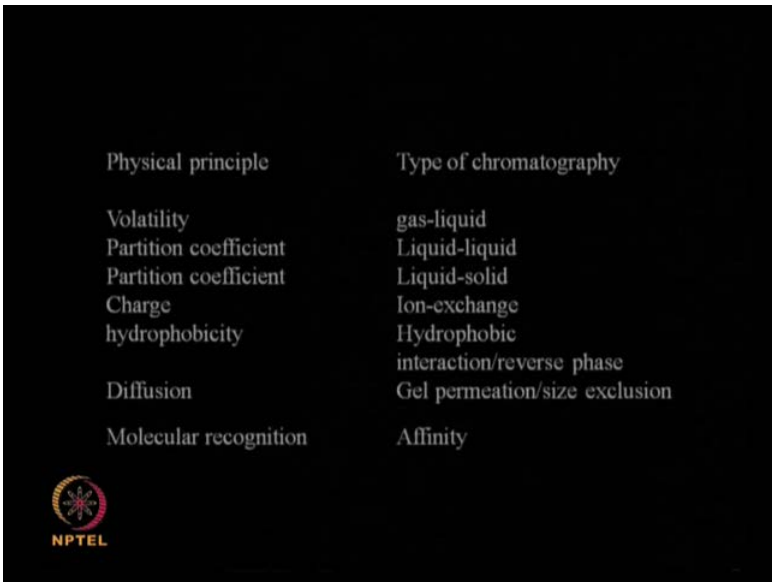

The different types of chromatography based on physical principle is what you shown in this particular slide. So, if the separation is based on volatility; that means the boiling point and so on. Then we use something called a gas-liquid chromatography, if it is based on partition coefficient. Then we have a liquid-liquid chromatography; that means the solute gets partitioned between two liquids. The liquid which is in the stationary phase and the liquid, which is in the continuous phase.

Similarly, you can have a liquid-solid chromatography; that means you have a solid stationary phase and a liquid and your solute is getting partitioned between these two. If the separation is based on charge; that means positive charge or negative charge, then it is based on ion-exchange chromatography. If the separation is based on hydrophobicity; that means because of hydrophobic nature of certain proteins, they get retardant inside the column. Then we call something called a hydrophobic interaction or reverse phase chromatography.

So, hydrophobic forces or the key in deciding on the separation, if the diffusion of the proteins through force is the key, then it is called a gel permeation chromatography or size exclusion chromatography g p c, if the molecular recognition or binding between a ligand and protein is what is important. Then it is called the affinity chromatography, so you have large number chromatography's possible and each chromatography works on a certain physical principle.

So, if you charged molecules then I will go for an ion-exchange chromatography. For example, I have salts, I have protein, I want to get of my salts, where do you get the salts from? Like you use salts for a salting out ammonium sulfate or sodium chloride, then I want to remove the salt first. What do I do? I may go into ion-exchange chromatography.

If I have proteins of different sizes or different molecular weights, then what do I do? I can use a gel permeation or size exclusion chromatography. Suppose, I want to remove a particular protein out of a mixtures of several protein, then if I know a ligand which is very, very selective for the protein, I may have the ligand immobilized on the station replace. Then what happens? The protein is bond only to that ligand rest of the proteins do not bind to the ligand, because as you know ligand protein binding is very, very selective.

So, only that particular is capture by the ligand whereas, other other proteins are not capture by the ligand. So, that is called affinity chromatography. So, depending upon the principle which you want to follow depending upon the mixture of proteins you want to separate. We may use different types chromatography's and all these chromatography's are already being practiced commercially for purifying biomolecules, bio pharmaceutical products, protein and so on actually.

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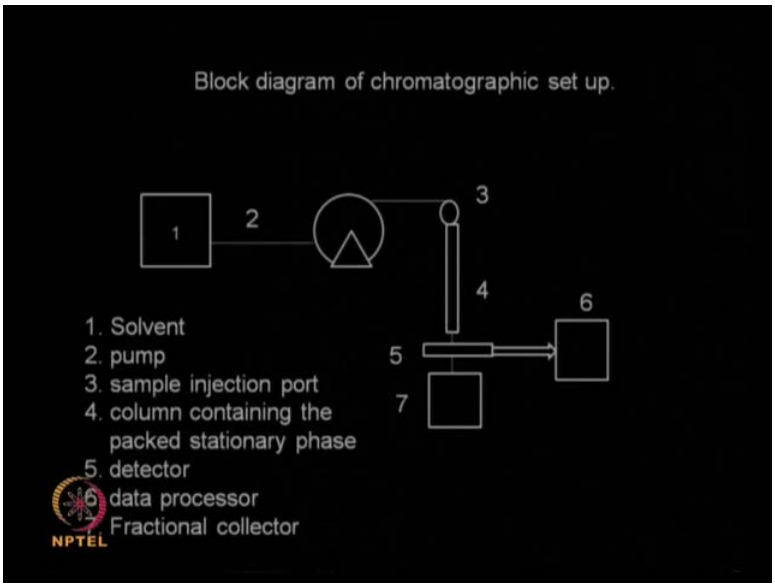

A typically block diagram of a chromatography set up. This is how it is going to look at so you have a solvent holder or a solvent tank. There is a pump, then you are injecting your sample. So, the solvent is the continuous phase then you have the column containing the packed or the stationary phase, then you have a detector there are so many types of detectors. That are available ranging from u v to refractive index to lights scattering and so on actually. Then final you will have the collector of the various fractions and you can also have a data loging and data analysis programs computer and so on actually.

So, solvent or the solvent mixture flows through the pump and it flows through the column. So, we can have two pumps and we can have two different solvents also, that way we can change the polarity of the solvent or the continuous phase. We may move from highly non-polar to polar or we can move from highly polar to non-polar type by switching from one pump to another.

So, a single pump is called an isocratic system here double pump could be a binary system. So, we can change the polarity by having two pumps and two solvent holders. We can collect each of the fraction that is coming out. Then you can do further processing of that fraction we can further purify it or we can do analytical to find out, what is the nature of the protein or we can take it for further downstream as well actually.

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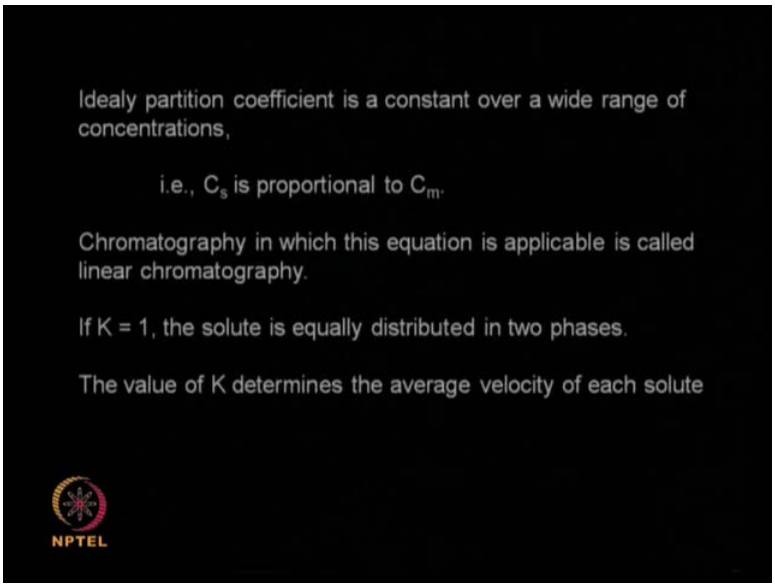

So, the important parameter that comes into picture is the partition coefficient that is the concentration of solute in each phase ratio of the concentration of the solute in each phase k. So, K is equal to C s by C m c s is the solute in the stationary phase and m is the solute in the mobile phase.

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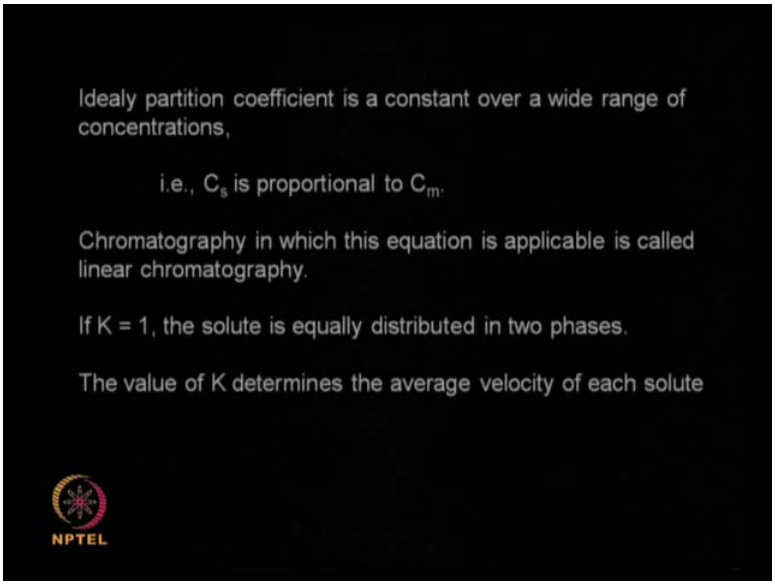

So, ideally you want very high partitioning and you also want the partition coefficient to be constant over a wide range of concentrations right. If C s is directly proportional to C m of course, we call it the linear chromatography. If k is equal to 1; that means the solute

is equally distributed between the mobile and the stationary phase, so the value of K determines the speed at which the protein travels from the inlet to the outlet. So, if the K is very large, so you the protein is slowdown or retarded by the stationary phase. So, it travels very slowly, if the protein wants to travel faster, then the partition coefficient has to be smaller.

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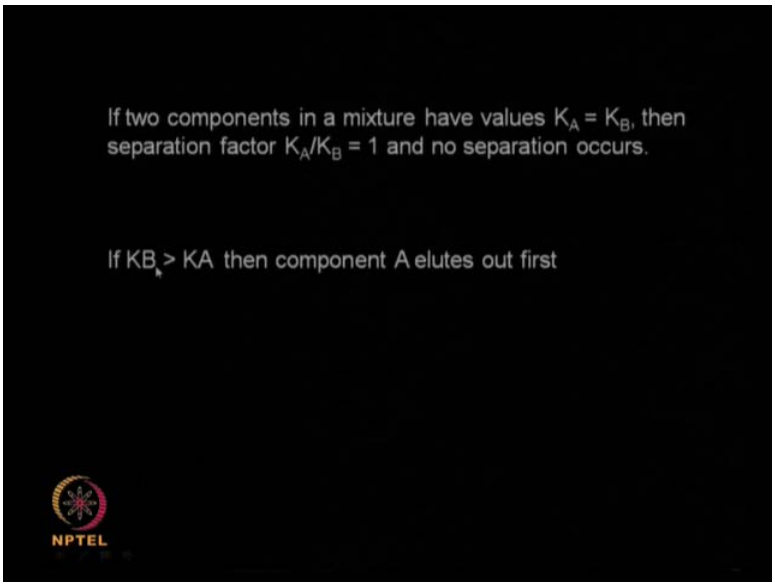

So, if two components in mixture have K equal to K B; that means I have two components in a mixture and each component will have a partition coefficient, which could be called K A and K B. If K A is equal to K B, then we are in trouble because this there is not going to be any separation happening because K A by K B is equal to 1. So, K A should not be equal to K B and the difference should be considerably large.

So, that we can get a good separation either K A can be larger than K B or K B can be larger than K A. If K B is large than K A, then what happens? That means B is slowdown or retarder by the stationary phase because more of B is going to get partition to the stationary phase.

So, B will be travelling slowly. K A relatively will be travelling faster, so if K B is greater than K A. A will be eluting out first whereas, if K B is less than K A, then B will be eluting out first. Of course, in a real protein mixture situation, we are not going to have only two proteins, you may be having even ten’s of protein or even hundreds of proteins.

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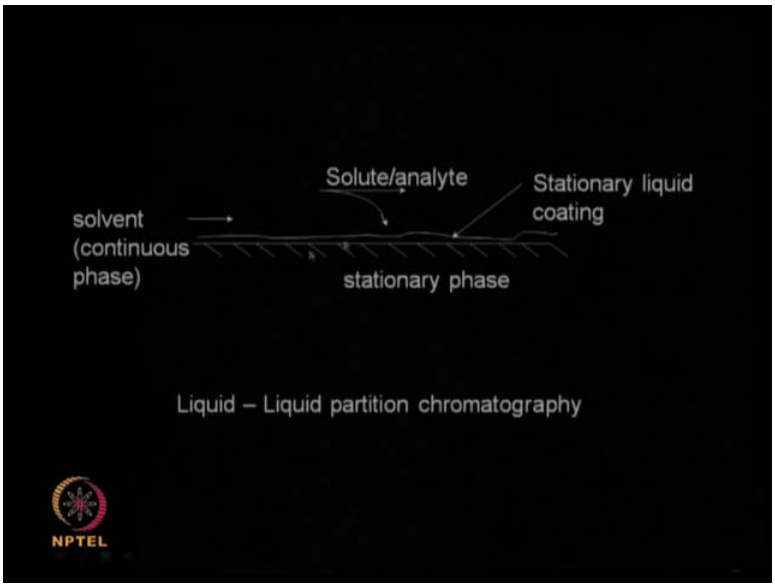

So, this just shows a pictorially. What is happening inside a chromatography? So, you have a continuous phase solvent flowing in we have a stationary phase. Suppose, we have a liquid, then this is called a liquid-liquid partition chromatography. We have a stationary liquid, so the solute gets partitioned in the stationary liquid as well as the mobile liquid or solute or analyte. This ratio as I described in the previous slide is called the K. So, this is the typical liquid-liquid partition chromatography.

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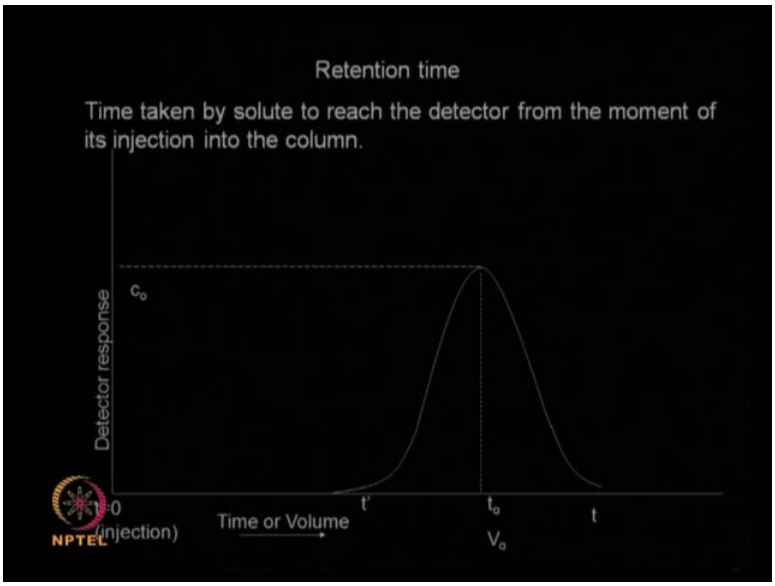

So, when the protein comes out of the column after a certain period of time. I said you may have some something like a Gaussian or a normal distribution. So, typically at a time T naught, we will call this as the retention time of the protein the protein concentration reaches the maximum.

Then it goes down, so this particular graph can be called here normal distribution or a Gaussian, so you are injecting at T equal to 0 and the protein is coming out at T equal to T naught. It will be coming out in a normally distributed shape. This could be applying for volume also just like time. We could this as volume at after you are collected a volume of V naught the protein will be reaching a maximum concentration.

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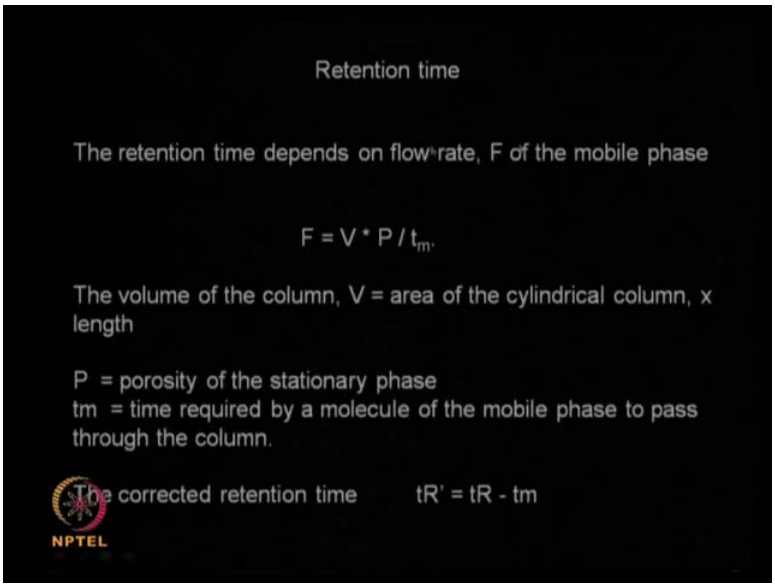

So, the retention time of the protein depends on flow rate of the mobile phase obvious right. So, the flow rate is faster retention time will be less, so flow rate is equal to V that is the volume of the column P porosity of the stationary phase divided by time. So, the retention time is a function of the volume of the column the porosity as well as the time required by a molecule of the mobile phase to pass through the column, okay?

Now, there is something called it corrected retention time which is given by t R dash equal to t R; that is a retention time. We calculate minus the t m that is the time required by a molecule of the mobile phase to pass through the column. That means if there is no interaction between the mobile phase solute and the stationary phase, then it is the time required for the molecule to just go through the column. As I said the columns are

generally very long, may be even going up to hundreds of feet. So, that time needs to be subtracted to get the corrected retention time t R dash.

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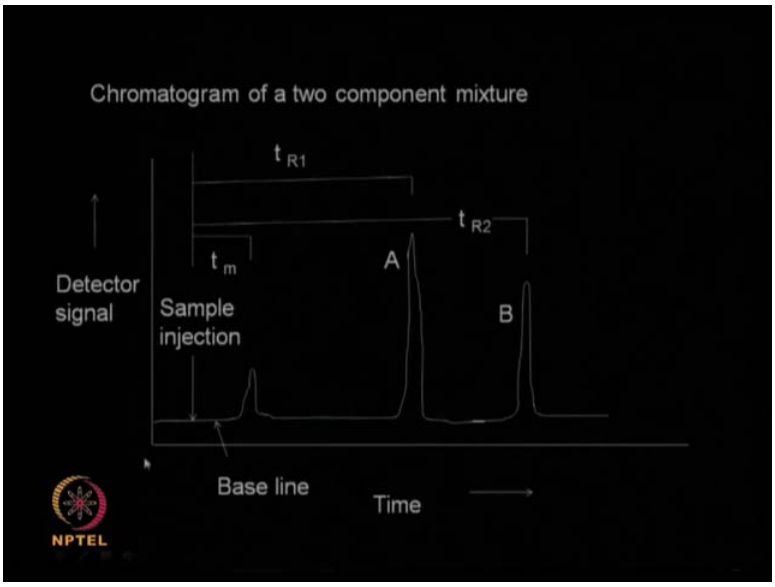

So, if you look at chromatogram of two components, so this we call it as the base line. This we call it as the detector signal, this could be your time this could be your time. So, there is peak coming out first and this is the t m; that is the time required for a molecule to travel from the inlet outlet. If there is no interaction of the molecule with the stationary phase material and then you have component A coming out component B coming out. Now, this is the retention time for component A. This is the retention time for component to the, but if you want to do the correct detected time, we need to subtract this t m from t R 1 and we need to subtract the t R from t R 2.

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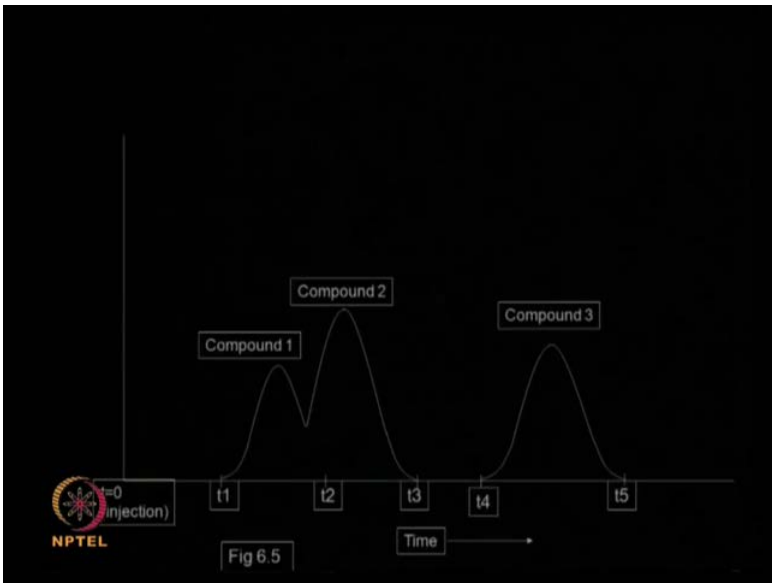

We can have different types of chromatograms possible. Imagine I have a mixture of three proteins. I am passing it through a column and I am getting an output signal like this protein; 1 protein, 2 protein, 3. So, if you look at this compound three your protein three, which well separated from one and two, so we can collect this. We can assume when we collect till the 100 percent. But let us look here, we have both compound one and compound two or protein 1 and protein 2 partially mixed together. So, when I collect samples in this region, I may get compound two also it present or when I collect here.

I may get compound one here also. Ideally, I need to get a better separation of these two proteins, if you want to collect 100 percent. Whereas, I can collect very pure compound three without any problem, there is not going to be any contamination where compound one and compound two. So, here I would say this base line separation is not good, this is the base line, so the base line separation of compound one and compound two are not good. So, when I collect in these periods of time I will be also collecting the other protein.

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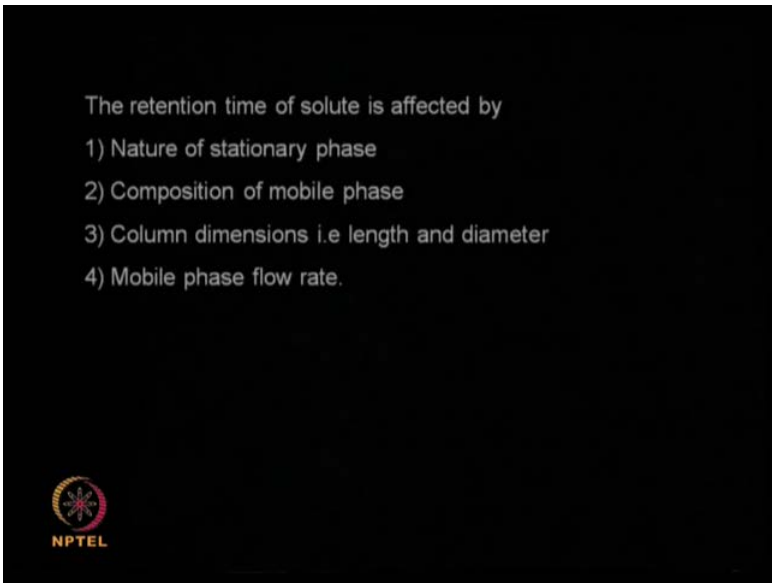

So, the retention time of solute is affected by many parameters nature of stationary phase that means what is the type of stationary phase I am using? The porosity, particle size particle size distribution so on actually, whether is a charged uncharged, whether it is called hydrophobic groups and so on. Composition of the mobile phase mobile phase is a solvent, so what is the composition of my solvent phase?

Does it have water, does it have pure solvent, does it have mixtures of solvent, what is the polarity of the solvent mixture dipole movement of the solvent mixture viscosity of the solvent mixture and so on. Column dimensions, what is the length of the column? What is the diameter of the column and finely the flow rate of the mobile phase. How fast the mobile phase is flowing or how slow the mobile phase is flowing?

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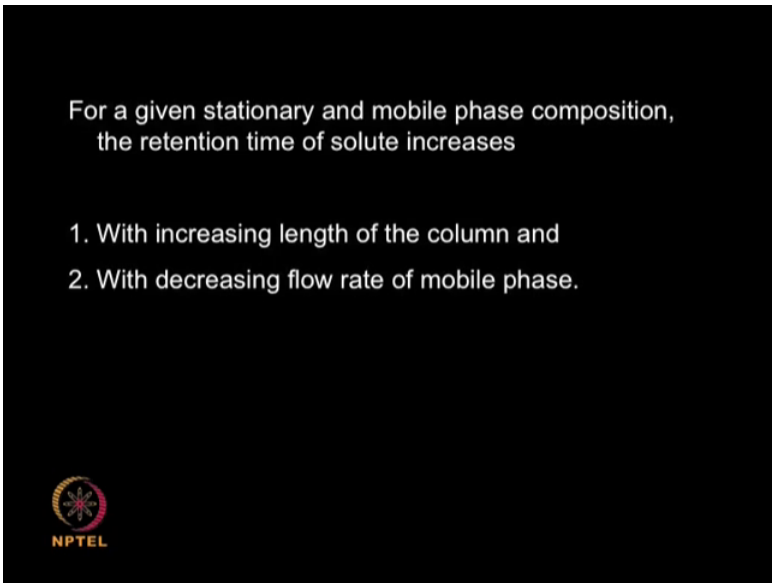

So, all these parameters affect the movement of my solute through the column, so for a given stationary and mobile phase composition the retention time of solute increases with increasing length of the column. So, if I have very long column it will take much longer time for the compound to come out, if I have very short column it will come out faster or the retention time of the solute increases. If I decrease the flow rate of the mobile phase; that means if I reduce the flow rate of mobile phase the compound will take much longer time to come out obvious is it not?

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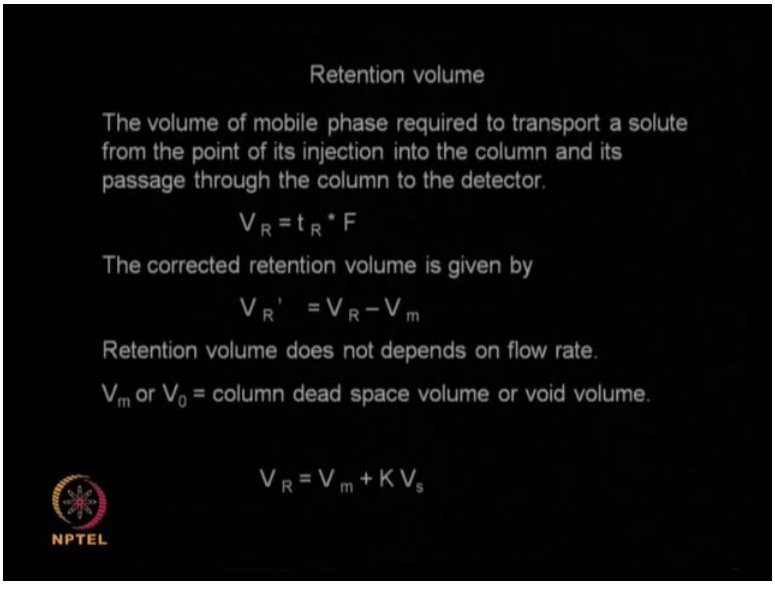

Now, again let us go back to this retention volume, which is also same as retention time so retention volume is the volume of mobile phase required to transport the solute from the point of its injection into the column. It is passage through the column to the detector right. So, V R is equal to t R into F, F is your flow rate, t is your time. Now, the corrected retention volume again just like we did corrected retention time we need to also subtract V m. This V m relates to the the dead space of or the void volume. Now, so V R is equal to V m plus K into V S.

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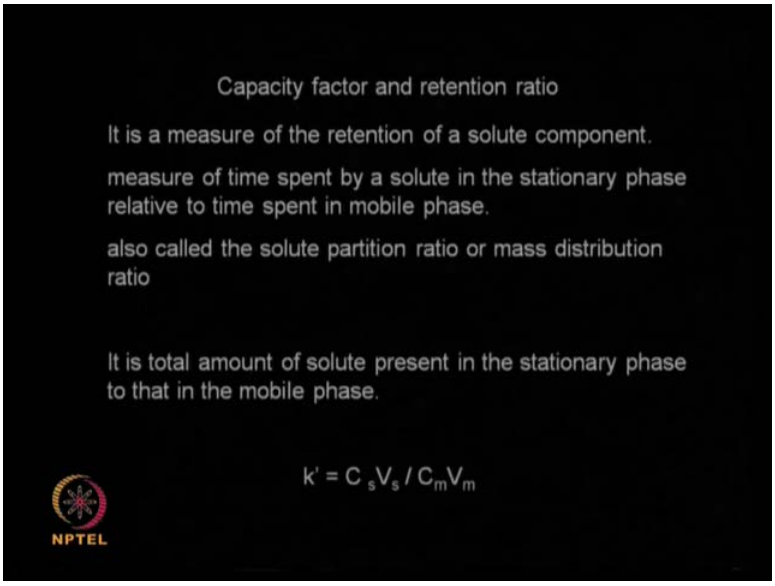

There is another factor which is called the capacity factor or the retention ratio, so it is a measure of the retention of a solute component. So, it is a measure of the retention of the solute component it is a measure of time spent by a solute in the stationary phase relative to the time spent in the mobile phase or it is also called mass distribution ratio or it is also called solute partition ratio.

So, capacity factor is given by this particular formula S relates to the stationary phase and m relates to the mobile phase. So, it is the total amount of solute present in the stationary phase divided by the total amount of solute present in the mobile phase. So, how do you calculate total?

Total is nothing but concentration into volume that is why you have C m into V m. This is the total amount present in the mobile phase this is the total amount present in the stationary phase, okay? So, this is a measure of the time spent by the solute in the

stationary phase; that is the S vis-à-vis the time spent in the mobile phase. That is the m if it spends more time on the stationary phase. Then the capacity factor goes up because it comes in the numerator, if it spends less time on the stationary space. Then the capacity factor goes down that means it spends more time in the mobile phase relatively.

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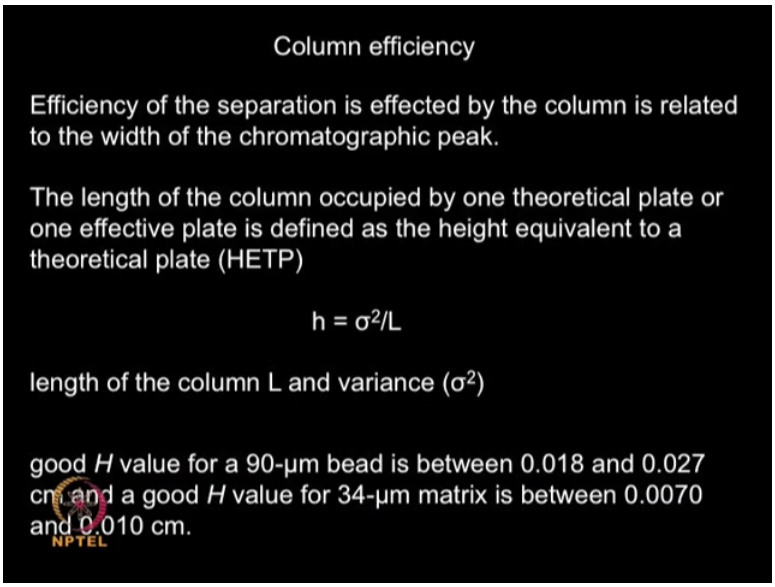

That is another term which is called the column efficiency so the efficiency of the process is related to the width of the chromatographic peak if the width is very large. Then what it means is you are having a sort of a spread of the product whereas, if they if the efficiency of the width of the peak is very narrow. That means the spread is much less the length of the column occupied by one theoretical plate is defined as the height equivalent to a theoretical plate.

So, that is here term called a theoretical plate this called the height equivalent of a theoretical plate. It is given by this formula H is equal to sigma square by l where l is the length of the column and sigma square is the variance

So, generally good H value for a 90 micron bead is between 0.018 to 0.027 centimeter, good H value for a 34 micron matrix is between 0.007 and 0.01. Ideally we would like to have the height to be very small; that means if your sigma square is very small height of a theoretical plate also will be very small. So, all these factors determine the efficiency of the column, okay?

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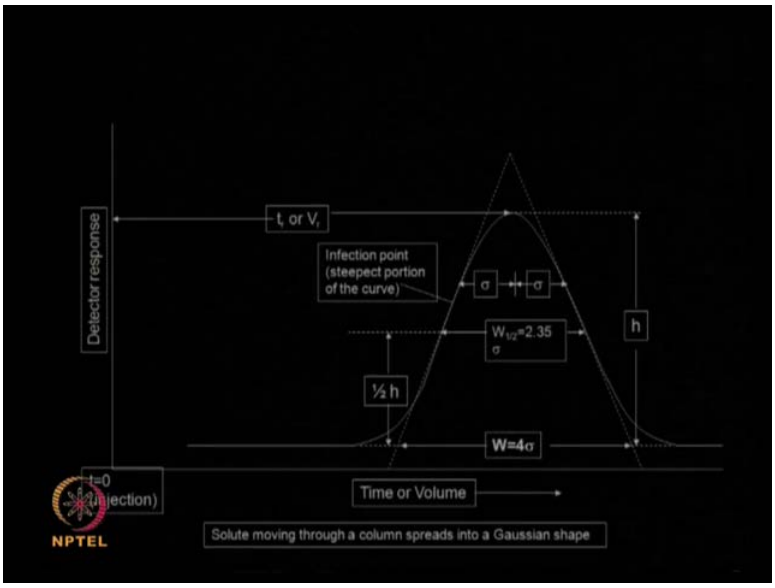

So, imagine once again Gaussian type of peak coming out, this is called the base and this is called the half width. That means the width at half the maximum. So, if this is the maximum height H, this is half H. This is called the inflection point or the steepest portion of the curve, so we can draw a tangent, we can draw another tangent. This will look like a triangle and this is the steepest point. Now, this weight W is equal to 4 sigma and this width W half is equal to 2.35 sigma. So, generally we assume the solute flowing through the column spreads into a Gaussian shape actually.

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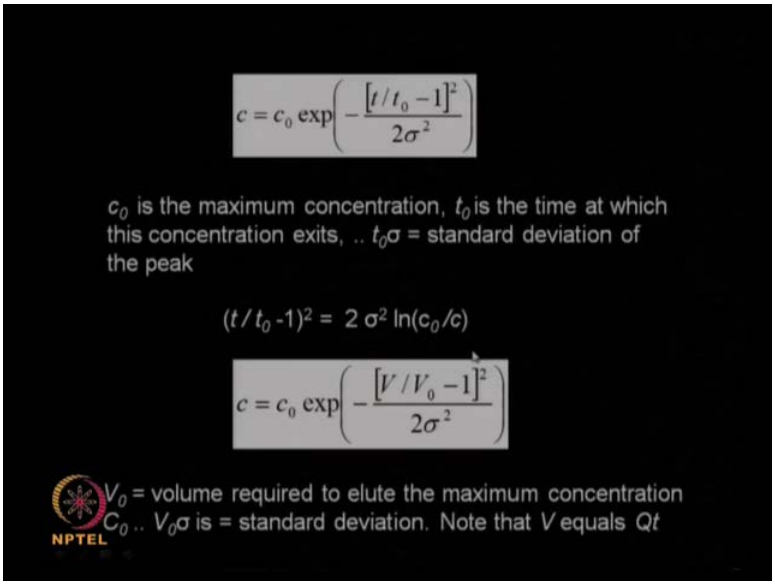

But it is not always same. So, if you assume a Gaussian shape for the solute distribution, then we can write an equation for concentration C is equal to C naught that is the maximum concentration. If power minus t by t naught minus 1 whole square by 2 sigma square. t naught is the time at which the concentration exists that is the maximum concentration reaches at time t naught and t naught sigma is the standard deviation of the peak, okay?

So, we can take a logarithm on both sides and then we end up with t by t naught minus 1 square is equal to 2 by 2 into sigma square logarithm of C by C naught, this is just a mathematical manipulation. So, we can get an equation of this form. So, this equation relates C naught that is a maximum concentration, t naught is the time at which this maximum concentration happens, C is the concentration at any time t, t naught sigma is the standard deviation of that particular Gaussian curve just like t. We can also substitute V as well, so C is equal to C naught exponent minus V by V naught minus 1 whole square by 2 sigma square.

V naught is the volume required to elude the maximum concentration, which corresponds to that t naught here. So, you see V corresponds to t here, V naught corresponds to t naught here and here V naught sigma is a standard deviation. So, please note that V equal to Q into t Q is your flow rate V is your volume. So, flow rate into time is your V that is why we have similar looking equation here, except that you replace t with V and t naught with V naught. So, we the main assumption in this is the peak that is leaving a chromatograph is a normally distributer or a Gaussian curve and once you assume it to be Gaussian you know the equation for a Gaussian distribution.

Hence, you can say C as a function of time will look like this, C is as a function of time will be C equal to C naught exponent minus t by t naught minus 1 whole square by 2 sigma square or C is equal to C naught exponent minus V by V naught minus 1 whole square by 2 sigma square. So, this equation is very useful and the main assumption here is the peak that is leaving the chromatography column is normally distributed uniform and it follows a Gaussian type of relationship.

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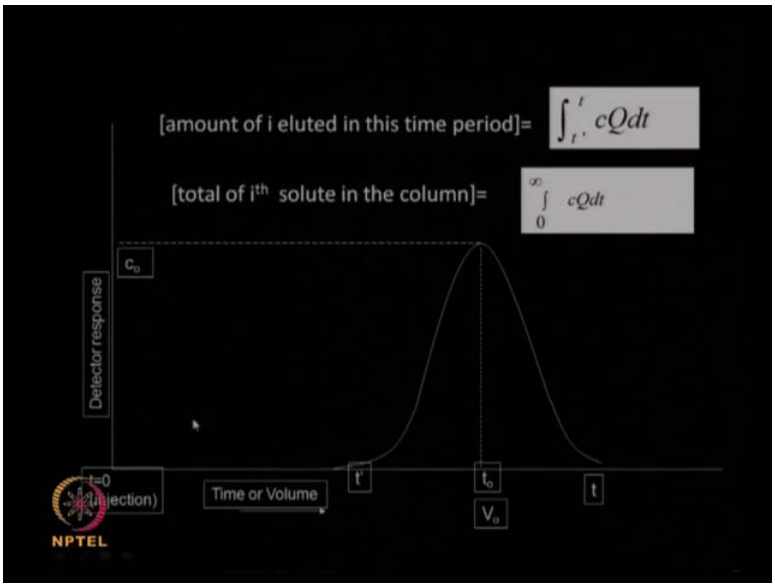

Now, let us go back again to the chromatogram. So, you are injecting your sample here and at time equal to t naught, it is showing a maximum peak and it is falling down, this is a Gaussian distribution. Now, we called is as beginning of this rise as t dash and ending as t, then amount of this particular component suppose we call it i component I, eluted in this time could be integral of C into Q into d t, Q is your flow rate, C is your concentration varying between t dash to t.

So, this is nothing but area under this curve. Total of i th solute in the column total, so we have to integrate throughout that is why we integrate between 0 to infinity c Q d t, so this is the total of i in the solute that is starting from zero right going right up to infinity. Whereas, amount of i eluted between these two times we just integrating between these two limits t dash and t, okay?

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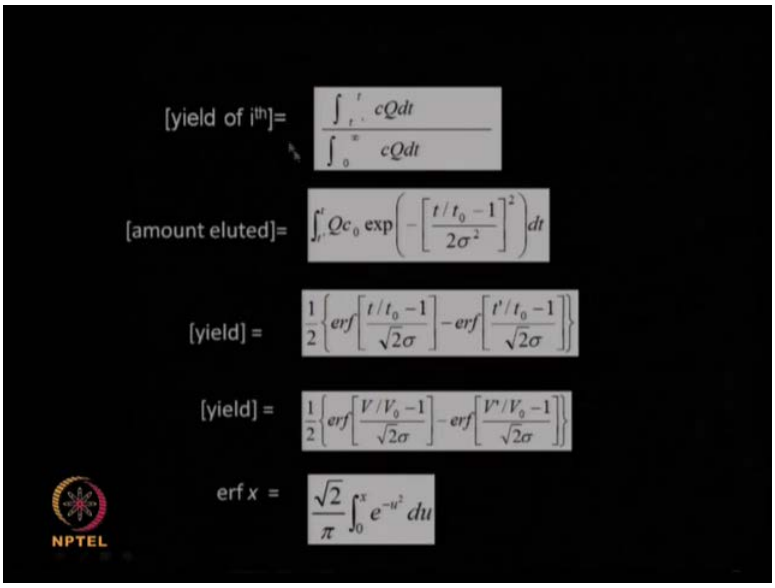

So, what do we do? Yield of i the component will be integral c Q d t between t dash to t divided by c Q d t between 0 to infinity obvious right, this is yield. The amount eluted we can integrate t dash to t Q c naught exponent minus t by t naught minus 1 whole square divide by 2 sigma square d t. So, what do we do? We substitute for c here like this and then when you integrate you end up as yield is equal to half of 1 by 2 error functioned t by t naught minus 1 divided by square root of 2 sigma minus error functioned t dash by t naught minus 1 divided by square root of 2 into sigma.

So, the yield if you are collecting the sample between time t dash and t is given by this. What is this error function? Error function is nothing but square root of 2 pi integral 0 to x e minus u square by d u, so we can get values of this in tables for different values of x. So, for different values of x, we can get this integral we can substitute here and here as well. So, the yield of the i th component if you are looking at it between time t dash and t and if the peak is coming out at t naught is given by this relation a corresponding relation for volume will be instead of t we substituted as V.

So, half error functioned V by V naught minus 1 divided by square root of 2 sigma minus error functioned V dash by V naught minus 1 divided by square root of 2 sigma. So, this is a very important equation, because we want to know how much of the protein. We are able to collect if I start collecting between t time t dash to time t protein may be present in different locations inside the column, but you are just collecting between t

dash and t. Assuming that t naught is the time at which the protein maximum concentration is observed and t naught lies between t dash and t.

Ideally when I am doing a protein purification, I want to maximize my yield. I want a collect as much of the protein as possible, but then there is a balance you needs to strike. If I try to increase my, the window of time the impurity is also may get collected, so the purity of the protein which I am collecting may be going down.

Whereas, if I decrease the window of collection the purity of my protein of interest may go up, but the yield will start going down, so I need to strike a balance between both. So, if so if t and t dash are equal, then there is no yield obvious. If t dash is equal to 0, but that means from time equal to 0 itself. I have started collecting the error function term containing t dash. That is this term, if t dash is equal to 0, the error function containing this term will be minus 1.

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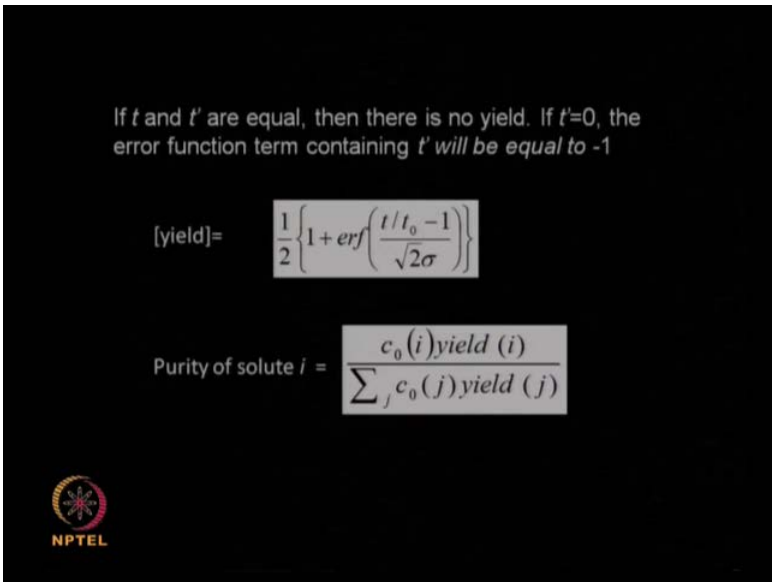

So, what will happen? yield will become half of 1 plus error function t by t naught minus 1 divided by square of 2 sigma. So, if I am collecting from time equal to 0 to sometime t, then the yield will be half of 1 plus error function t by t naught minus 1 divided by square root of 2 by sigma. Now, there is something called purity. Here protein mixture may contain several proteins n proteins, so when I am collecting the i th protein I may be also collecting some amount of the remaining protein S also right.

So, how do I calculate purity? Purity is nothing but concentration C 0 i into yield i divided by summation of C 0 j yield j of all the j's that means there are n protein. I will have n terms like that all added up together with each other, so that is called the purity. I said if my window of collection is very short I am going to get more pure protein, but my yield will keep going down. If the window of collection is very large, the yield will be large, but the purity will be going down. So, I need to strike a balance.

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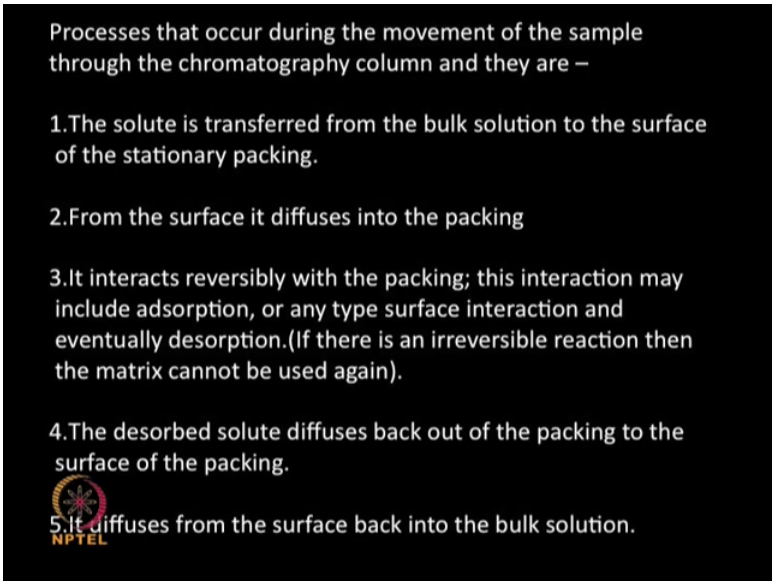

That is, so I need to work at actually. So, what is happening? You have here protein or a metabolite traveling in a solute or these sorry traveling in your continuous phase. Now, this particular solute will start interacting with the stationary phase material. So, there are many steps that is happening the solute is transferred from the bulk solution to the surface of the stationary packing solute is there in the bulk solution. So, it will come to the surface of the stationary packing. Then it diffuses into the packing it interacts reversibly with the packing, so this interaction different types of interactions are possible no it is reversible interaction.

It is not irreversible because chromatography is always reversible process. We want to collect the protein and then we want to recover it, so it is a reversible processor. So, there is an interaction between the surface and the protein. Of course, later on there is going to be a desorption of this solute it diffuses back to the packing surface.

So, it diffuses back to the top of the packing to the surface and then from there it diffuses back to the bulk solution. So, many steps are taking place here so the solute from the bulk solution comes to the surface of the stationary phase. Then there is a diffusion of the solute through the stationary phase. Then there is a irreversible interaction with the stationary phase, then there is a desorption from the bulk right up to the surface of the packing.

Then there is a diffusion from the packing surface into the bulk of the liquid. So, each one could be controlling or slowing down the process. So, depending upon which one is controlling you will have different types of processes taking place actually. So, ideally we would like to have more interaction between the stationary phase solute and the solute in the continuous phase. We do not want the other resistances to be playing a major role in this chromatography process.

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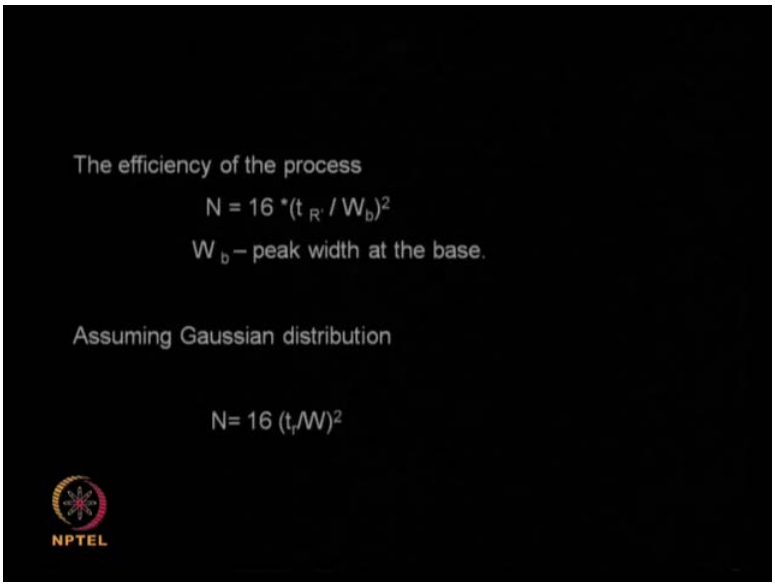

So, there is something called the efficiency of the process or the number of theoretical plates. So, the number of theoretical plates is given by n is equal to 16 t R divided by W B whole square where W B is the peak width at the base that means the width of the peak at the base. So, we are assuming a Gaussian distribution here. Do not forget that we are assuming a Gaussian distribution here.

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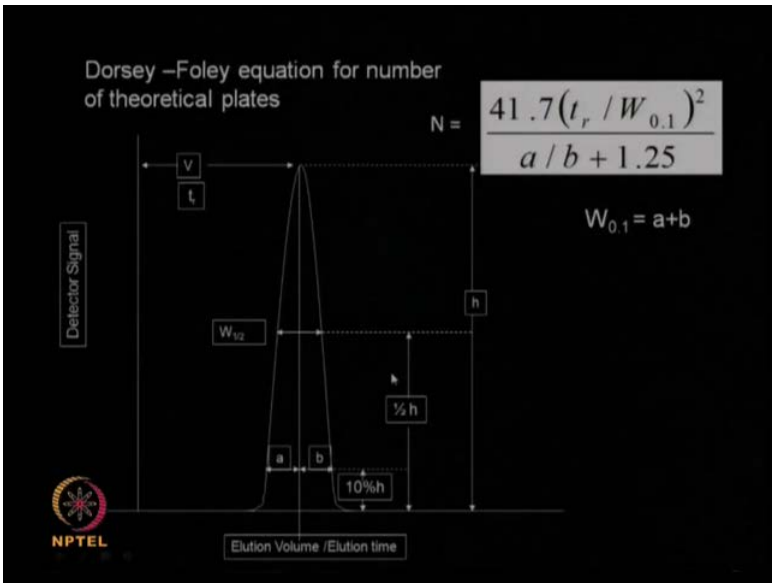

So, there are different types of equations available for this depending upon whether we are machining the base width or whether we are machining the half width maximum and so on actually.

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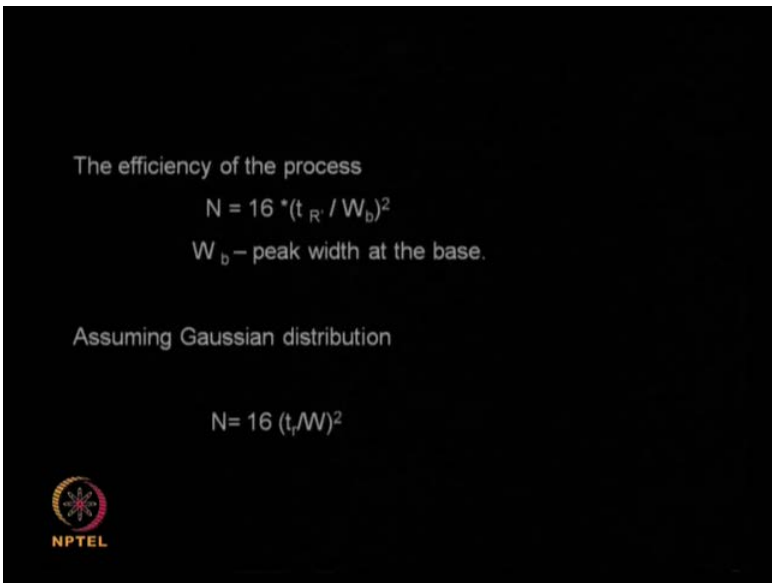

That means width at the half maximum and so on. So, all these assumptions are based on a Gaussian distribution, but what happens if it is not Gaussian.

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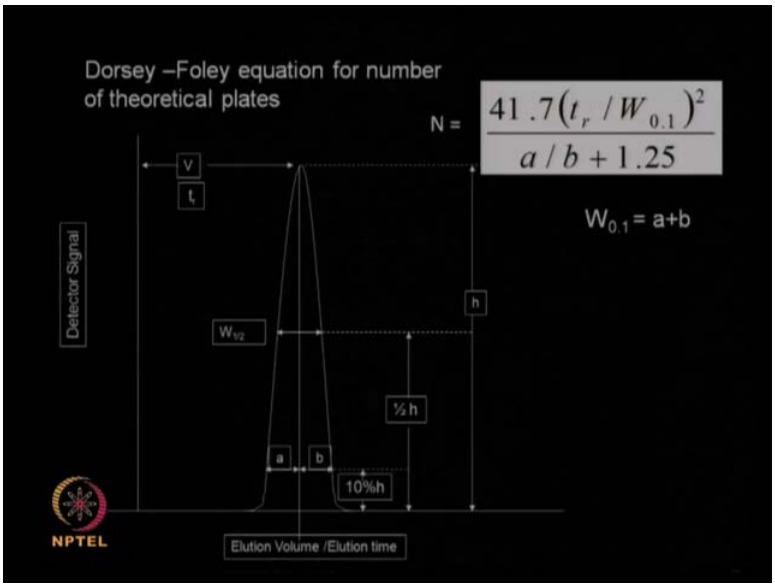

There is another relationship that is call the Dorsey-Foley equation for calculating the number of theoretical plates. That is given by this relationship and the assumption here is the peak is not uniform with respect to the centre. So, there is non-uniform region between the left side of the centre and on the right side of the centre.

So, if this width is a that is on the starting side and B is on the ending side, then the number of theoretical plates is given by 41.7 t R by W point 1 divided by A plus B A by B plus 1.25 where A plus W 0.1 is nothing but A plus B that is this width and t R is your retention time. So, this particular relationship takes care of differences in the symmetry of the chromatogram or the spectrum that is leaving the column.

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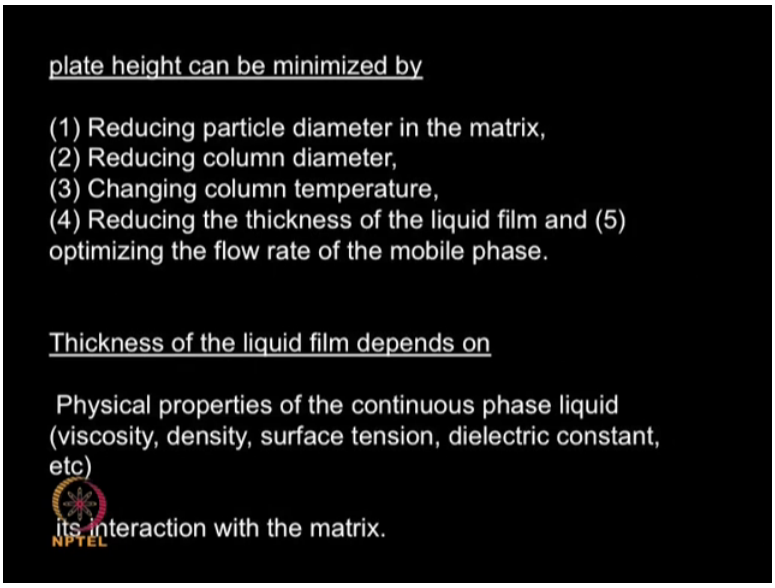

So, we talked about plate height and we talked about number of plates and we said smaller the plate height better. It is; that means it is more efficient, so how do you reduce this plate height? We can reduce it by reducing particle diameter in the matrix. So, I can make the particles very fine, but there is a disadvantage. If we make particles very fine, you are going to have the liquid flowing through encountering lot of resistance. So, the pressure drop increases; that means I need to increase the pump pressure.

Reducing column diameter when I reduce column diameter for same through put again I am going to increase the velocity of the liquid flow through the column. So, again I am going to encounter pressures and forces changing column temperature. Yes, I could change column temperature, but then again if I am handling proteins, I do not want to affect the stability of the protein by increasing column, reducing the thickness of the liquid film. So, if you have any liquid present on the stationary phase. If you reduce the thickness of the liquid film I will be able to improve the plate height optimize the flow rate of the mobile phase.

So, I can, I need to addressed with the flow rate of the mobile phase, so the plate height becomes good. So, if your thickness of the film especially comes into picture when we are talking about liquid-liquid chromatography, the thickness depends on physical properties of the continuous phase, such as viscosity, density, surface tension, dielectric

constant etcetera. Also, how it interacts with the matrix that is the stationary phase matrix?

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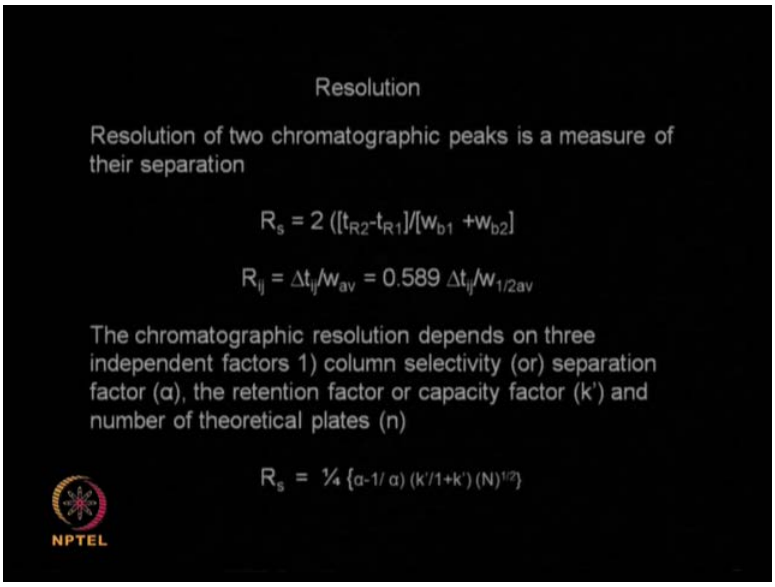

There is another term which is called the resolution. So, if I have two components present, how good the separation of these two components is? That is what resolution tells about is a separation very good; that means resolution is very good if the separation is very bad that means resolution is very poor. So, resolution is a measure of the separation of two components present in my mixture. So, it depends upon the retention time, it also depends upon the width at the base of the peak.

So, resolution is given by two times difference in the retention time divided by some of the width of the bases of the two components or we can take based on the width at half maximum W. That is W half, then resolution is given by 0.589 delta t i j. That means the difference in the retention time of component i and j divided by average of the width at half maximum. So, if the width at half maximum for component A is something width at half maximum for component B is something else.

You take an average between these two and substitute here that is what is resolution. So, the resolution depends on many factors you know it depends on column selectivity or separation factor retention factor capacity factor number of theoretical plates and so on. So, all these affect the resolution, so we can get an equation for resolution in terms of all

these. You know separation factors capacity factors theoretical plates and so on actually. So, resolution tells you how good the column is in separating two components?

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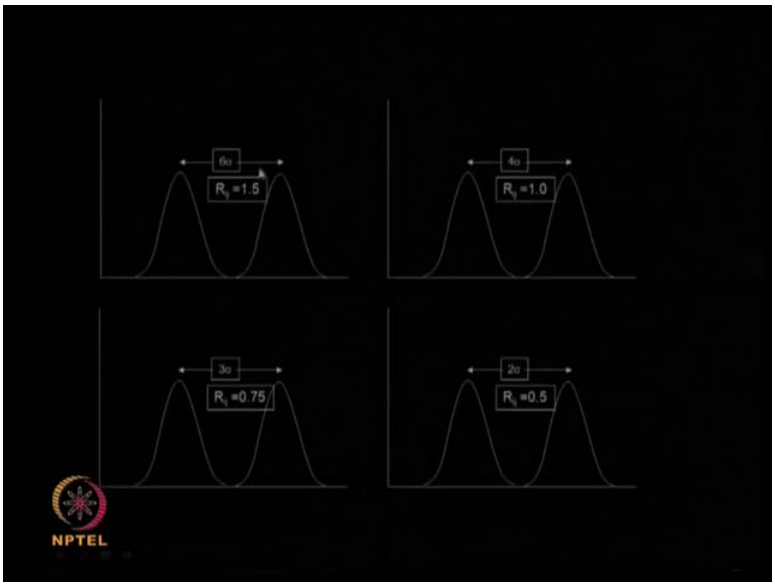

For example, I have two components and the output looks like this. You know they are well separated the distance between these two is 6 sigma. Then the resolution is 1.5 if the distance between these two maxima are 4 sigma. Then the resolution will be 1, if the distance between these two are 3 sigma, then the resolution is 0.75. If the distance between these two are 2, sigma then the resolution is 0.5. So, depending upon the distance whether it is 2 sigma or 3 sigma or 4 sigma or 5 or 6 sigma, I get different numbers ranging from 0.5, 0.75, 1, 1.5, and so on actually. So, resolution tells you how good the peak maxima are or how far apart this peak maxima R?

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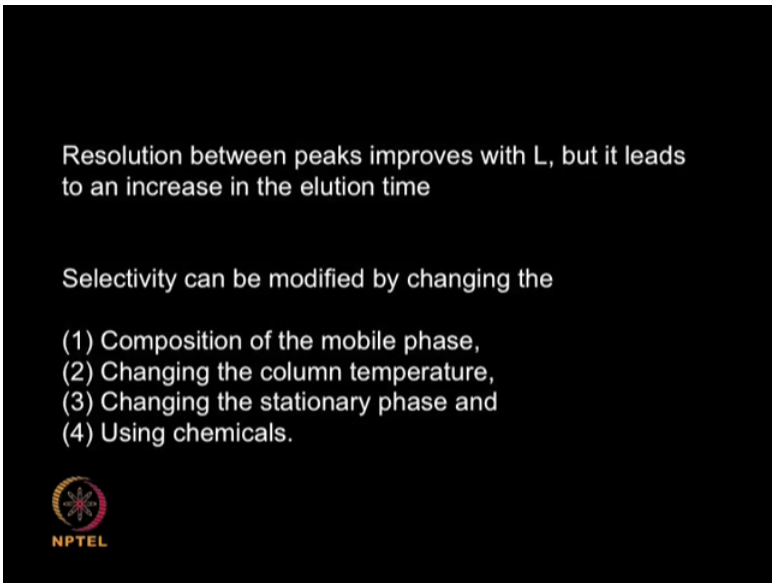

So, resolution between peaks improves with l that means if I have longer column, I can move the two peaks lightly further apart, but it also leads to an increase in the elution time. That means the component will come out much slowly, so the selectivity can be modified by changing the composition of the mobile phase. That means I can change the composition of the mobile, I can make it more polar or I can make more non-polar. I can play around with the composition changing the column temperature as I originally said Sometimes the proteins may be very sensitive to column temperature.

So, we might not be able to play too much with column temperature, but if we are handling only small molecules or metabolized or drugs, then changing column temperature is an option. Changing the stationary phase, I can use some other stationary phase. Sometimes using some chemicals, additives, all these can in also help in changing the selectivity of the chromatography process.

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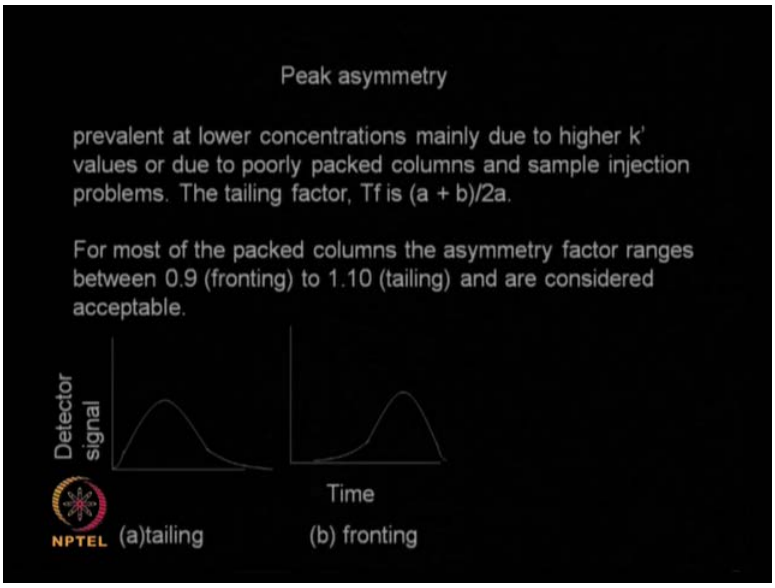

There is something called peak asymmetry. Although, we have been think saying or thinking that the peak leaving the column will look a normally distributed Gaussian extremely symmetric, but it will not be. So, it may have a front or a fronting or it may have a tailing; that means the concentration may be dropping very slowly. That is called a tailing or the concentration will be rising very slowly, that is called the fronting. So, all these are possible and all these are because of may be the polarity of the solvent or may be because of the stationary phase material, okay?

Sometimes you may have a very poorly packed columns or there could be some sample injection problems or the capacity factors may be very different. So, all these are possible actually. So, how do you calculate this tailing factor? We can we know, what is A, we know what is B. So, A plus B divided by 2 A, and if we calculate that, generally this number varies between 0.9 to 1.1. So, if it is around 1.1 that means B is more, so you get a tailing. If it is less than 1; that is around 0.9, that means A is more that means you will have the fronting.

So, if we calculate this A plus B divided by 2 A, and if it comes out to be less than 1, we can say it is a fronting type of situation. If it is greater than 1, you may call it as a tailing type of situation. So, this is possible because of so many factors as I said, because of the selection of the solvents, because of the type of packing we are using. Sometimes we make mistake during injection of the solute mixture or the protein mixture. So, all these

could cause this problem, but ideally one would like to have here Gaussian type of distribution or a normal type of distribution.