Principles of downstream techniques in Bioprocess – a short course
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Lecture-16
Chromatography

We are starting a new topic called chromatography. Chromatography is most important in bioprocess downstream operations because it is very well suited for biomolecules like proteins, enzymes and those which are very temperature sensitive. Whereas in chemical engineering downstream process, they use distillation as a purification technique. Whereas here we cannot use distillation as a technique because of the temperature sensitivity nature of the biomolecules.

So chromatographies are extremely efficient and there are different types of chromatography operating on different principles and that is what we are going to spend some time on and we will be spending almost 4 classes on chromatography because it is a very involved topic.
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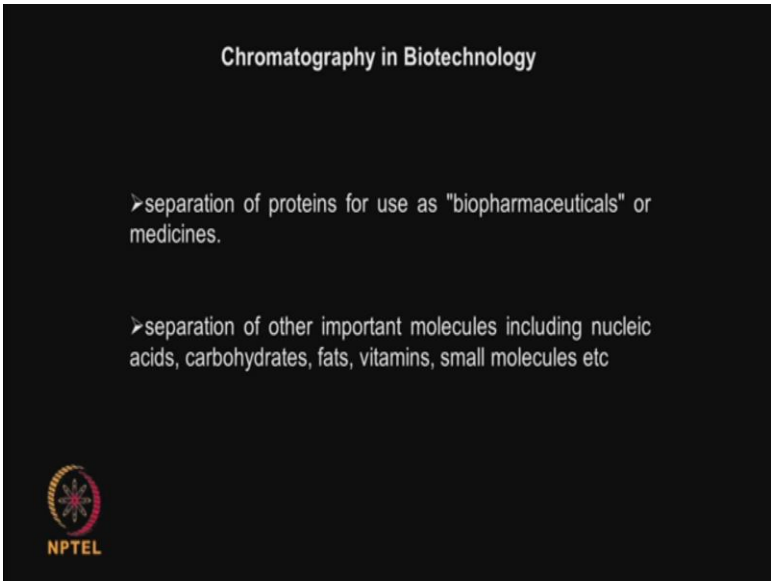

So it is used for separation of proteins especially in biopharmaceutical industries for medicines. It is used for isolating pure enzymes. So it is widely used for example nucleic acids, carbohydrates, fats, vitamins, even small molecules can be separated using chromatography. And there are different types of chromatography like I said ion exchange chromatography, size exclusion chromatography and then reverse phase chromatography and so on actually. We will

talk about each one of them and we will also look at some problems involved in chromatography.

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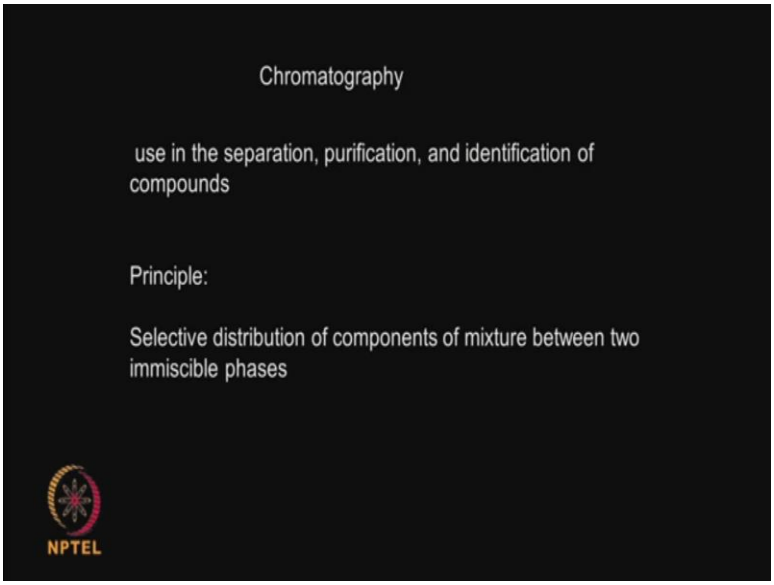

So where do you use it? It can be used for separation purification, it can be used for identification of compounds also, as you know must have all heard about hplc right? High pressure liquid chromatography, I high-performance liquid chromatography. So generally hplc is used for analytical purposes for identifying unknown molecules, for finding out the concentration of unknown molecules in the mixture.

So it is become very very useful the hplc. So will look at that also. So in chromatography, the selective distribution of components from a mixture between 2 phases, you may have a stationary phase, you may have a mobile phase. As the name imp implies stationary phase is a solid and mobile phase is a liquid or sometimes it is a gas okay.

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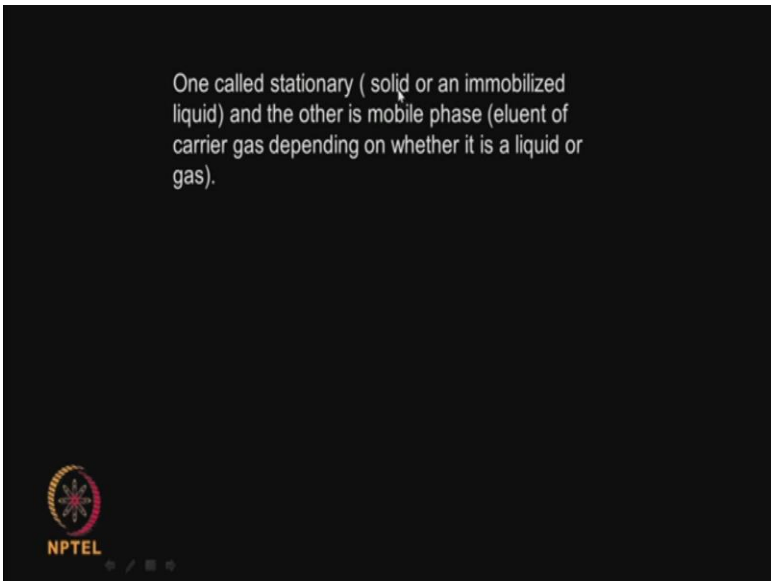

So one is called the stationary or solid or immobilized liquid. So you can have pure solid alone or you can have a liquid immobilized on that. the other one is the mobile phase, it could be gas or it could e a liquid okay and mixtures gases and so on actually.

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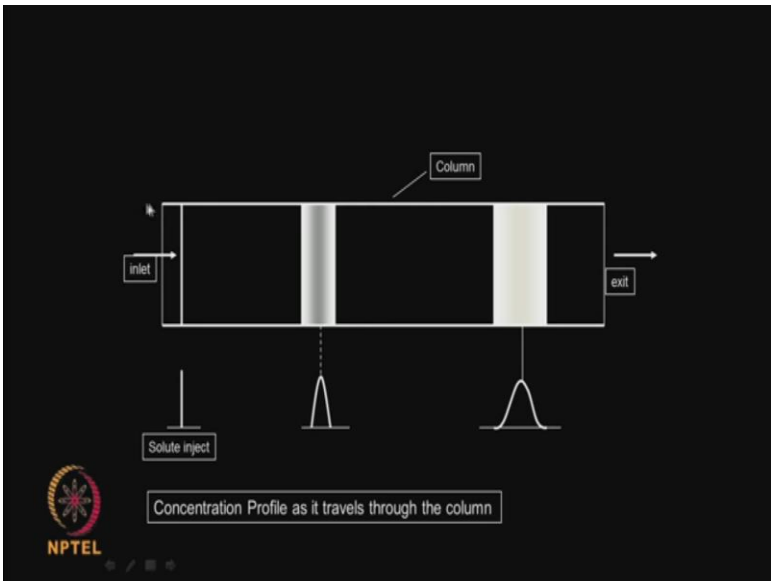

So what do we do? You have a long column and the stationary phase is packed inside this long column and a mixture is injected from one end. Okay. It will be generally a very sharp peak and then as it travels through it and the solute gets partitioned solute gets adsorbed on the surface, solids start interacting with the stationary phase material. So. So many things can happen. Different types of non-bonded interactions happening between the solute molecules in the liquid phase and on the solid phase.

So slowly they start spreading out okay and finally when it comes out, it comes out like a Gaussian peak or it comes out like a normal distribution at the end of it. So if there are many solutes traveling through it, you will get many peaks corresponding to it. Each one may be overlapping with one another but basically when you inject a very sharp peak, it comes out like a normal distribution. Why does it happen? It happens because of the interaction between the solute with these stationary phase and partitioning of the solute between the stationary and the mobile phase and so on…

Because of that the age distribution of this solute varies. Okay. That is called this is called dispersion. So the shape of the curve is generally assumed to be normal and the height and the width makes lot of difference and those parameters, we can tell how efficient is your separation process and so on. So we will look at some problems related to the height of these normal distribution curve as well as the width.

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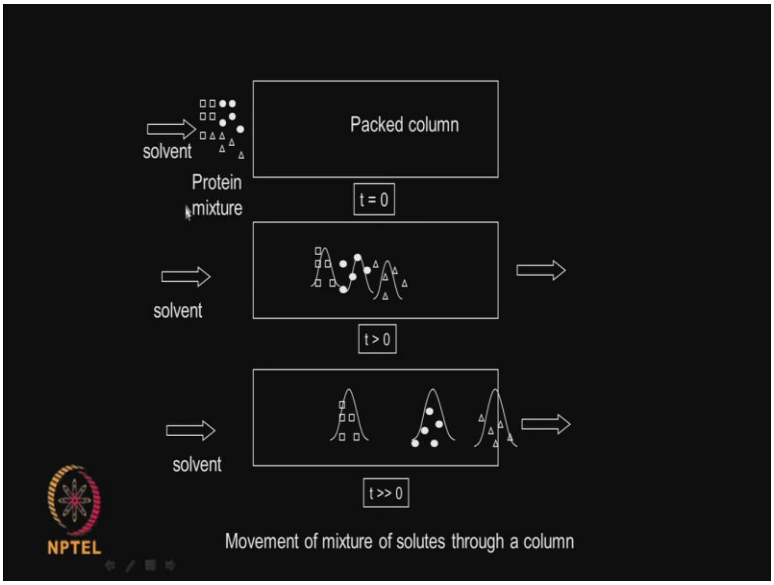

So we have a mixture of solutes here and there is a solvent or sometimes if it is gas, it will be a gas carrier gas here. So the proteins gets separated like this and as it travels and travels they get separated one after the another, after another and that is what happens in a chromatographic system okay. So as the time increases slowly they get they get separated more and more and as

they travel, they get separated out. So the concentration of anyone solute varies as a function of length as well as a function of time as well okay.

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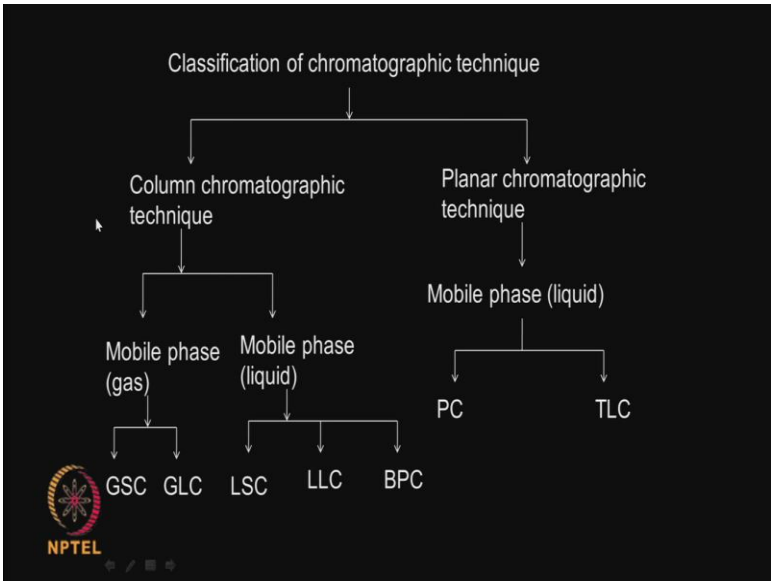

Now if you look at, you must have heard about gas chromatograph. Gas chromatograph have been there for a very very long time. So if this mixture of solutes are vaporizable, the gas chromatograph is also very cheap and a very powerful technique. So the mobile phase there is a gas, okay. So this solid phase or the stationary phase could be a solid, completely solid or a liquid which is immobilized on a solid phase. Okay.

So this is a gas chromatograph h and then you have different types of mobile phase gas or liquid and so on actually and the solid the stationary phase could be just solid or it is a liquid immobilized on solid or plain solid okay. So you have the Gas Solid Chromatograph, the Gas Liquid Chromatograph and so on actually. Vapor Chromatograph, Thin Layer Chromatography. So lot of different type of chromatographs are there, each one making use of different physical principles okay.

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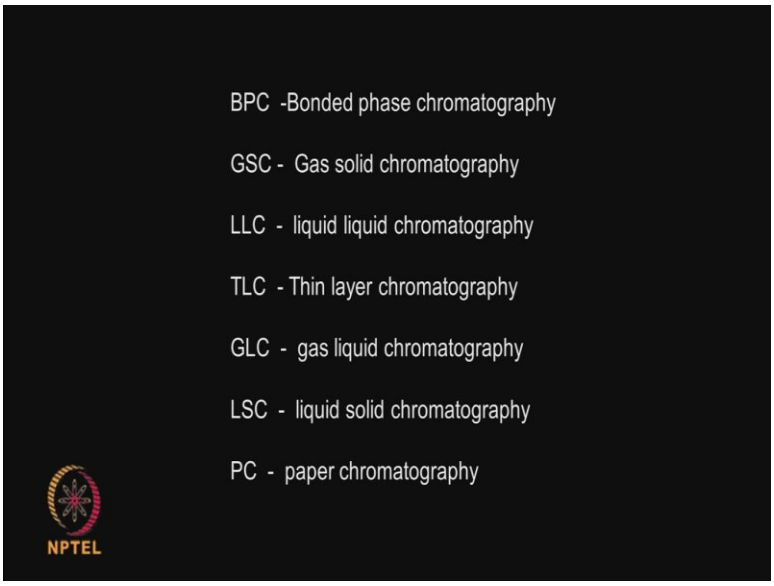

So you must have heard about Thin Layer Chromatography. We all use Thin Layer Chromatograph or regular chemistry experiment, where you want to identify some components. This is a very simple technique and very extremely powerful and very cheap also. Then we have the Gas Liquid Chromatography, Liquid Solid Chromatograph, Paper Chromatograph, Liquid-Liquid Chromatograph, Gas Solid Chromatograph, Bonded Phase Chromatograph. So different types of chromatographs are there actually.

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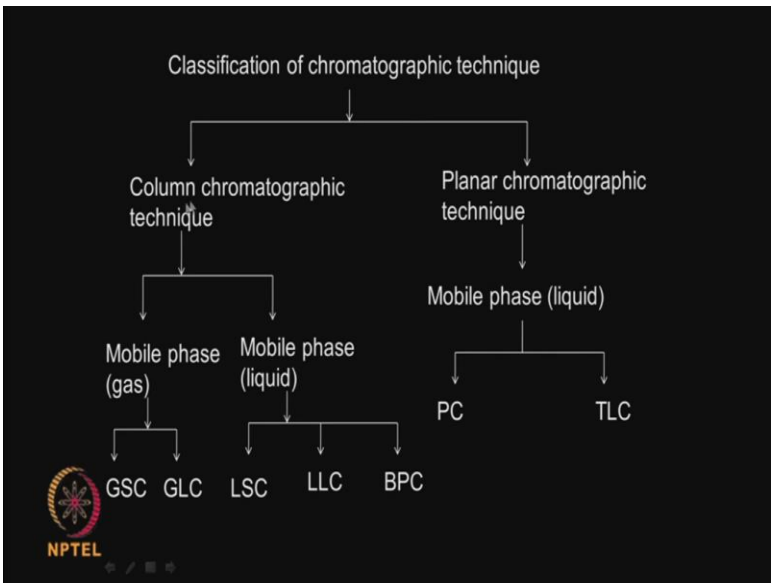

These are based on column. So you have a column in which your stationary phase is packed, where these are based on planar or paper. For example chromatograph and thin layer chromatograph, they are planar. So the solute and the solvent travels over thin planar paper okay.

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So what is the physical principle? If you look at gas-liquid chromatograph, the volatility of the various components present in the mixture, that is the principle. If we look at liquid-liquid chromatograph, we are looking at partition coefficient, that means the partition coefficient of the solute in the stationary liquid (())(07:42) the mobile liquid. Okay. That is what it is. Same thing if you look at liquid-solid chromatograph again, partition coefficient come into picture.

If we look at ion-exchange chromatograph, charge of the molecules could be a biomolecules or it could be a proteins. So the charge determines the separation process, if we look at hydrophobic interaction chromatograph, the hydrophobicity as you know proteins different proteins will have extent of hydrophobicity and this particular chromatograph makes use of that principle for separating out proteins. This is also called reverse phase chromatograph or inver interaction chromatograph. Okay.

Then gel permeation chromatograph or size exclusion chromatograph, they are based on diffusion size of the molecules, okay smaller the size or bigger the size, they get separated. So this is basically based on sizes. Then we have the affinity chromatograph. It its sort of recognizes the molecular ligand protein interaction. So you may have ligand immobilized on a stationary phase. So proteins which exactly bind to that ligand only or bound whereas other proteins are washed out. So we can specifically extract proteins which are very selective for that ligand.

So this is a very powerful technique for separating out a single protein, from a large mixture okay. So like a antibody antigen, separation ligand protein exactly we look at each one of them little bit in more detail later.

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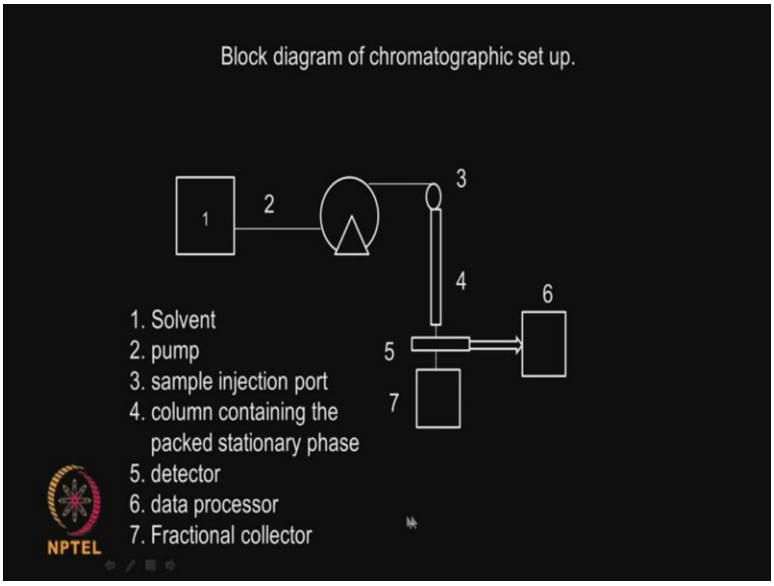

So basically this is how it will look like. You may have the carrier gas or a carrier solvent or the mobile phase, okay and then you have the pump. The pump injects the mobile phase through this column and then you may inject the sample here and then the material travels through that and then each one of them come out. There is a detector here and then here you have the collector here okay.

The detector will tell you which peak is coming out. So we can collect the products on different collectors. That is why it is called fractional collector. So typically in the chromatographic setup will have this type of assembly.

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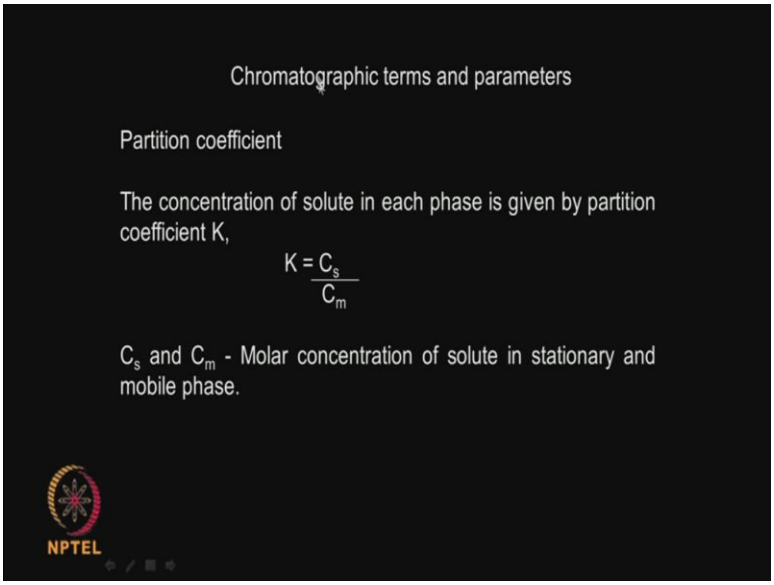

So the most important parameter that is called partition coefficient, that is the concentration of the solute in each phase here. We have seen partition coefficient in where have we have seen it in liquid-liquid extraction, that is the partition coefficient right. So same thing comes here k the partition coefficient, okay that is the concentration of the solute in the stationary phase and in the mobile phase concentration okay.

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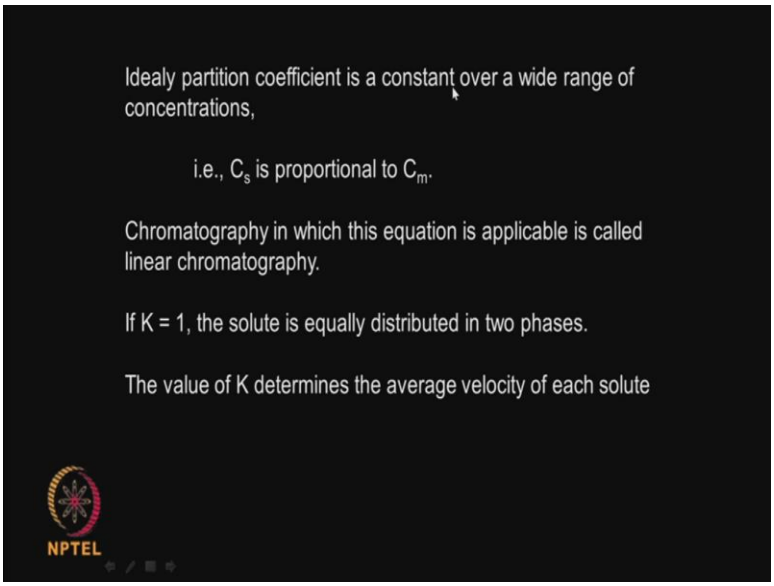

See ideally partition coefficient is a constant over wide range of concentrations. So irrespective of concentration, partition coefficient should be constant actually. Okay. If the value of K determines the average velocity of each solute, okay so if K is very very large, what happens? The solute gets more adsorbed, I mean more partitioned on the stationary phase. So it travels

very slowly okay. K = 1 that means solute is equally distributed in 2 phases and that is not good continuous phase material because you will not achieve any separation.

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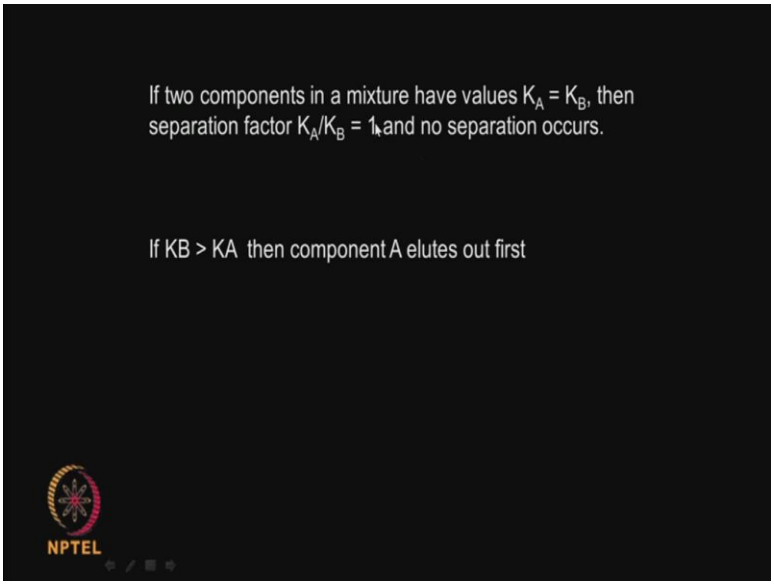

If 2 components in a mixture have KA = KB, then the separation factor is 1, so no separation okay. If KB > KA then what happens, KB travels slowly on the stationary phase, KA travels faster on the continuous phase. So A elutes out first okay. If KB and KA are equal, both will get partitioned at the same ratio on to the stationary phase. So you will not get any separation at all okay.

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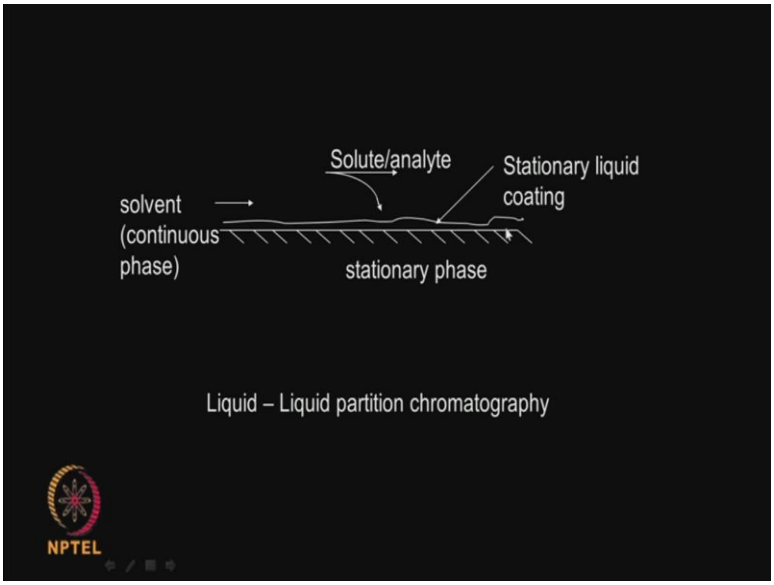

Okay so if you look at liquid-liquid partition chromatography, so we have the stationary phase and then there is a stationary liquid on top of it, the solvent comes, that is the continuous phase of the solute partition. So this is exactly like extraction, only thing is one liquid is immobilized on the surface is exactly like the extraction.

So you will have the term partition coefficient coming into this picture, especially for liquid-liquid partition chromatography. So stationary phase is will have the solid matrix and a liquid that is coated on to the stationary phase. So this is exactly like liquid extraction only thing is one of the liquid is immobilized on the surface.

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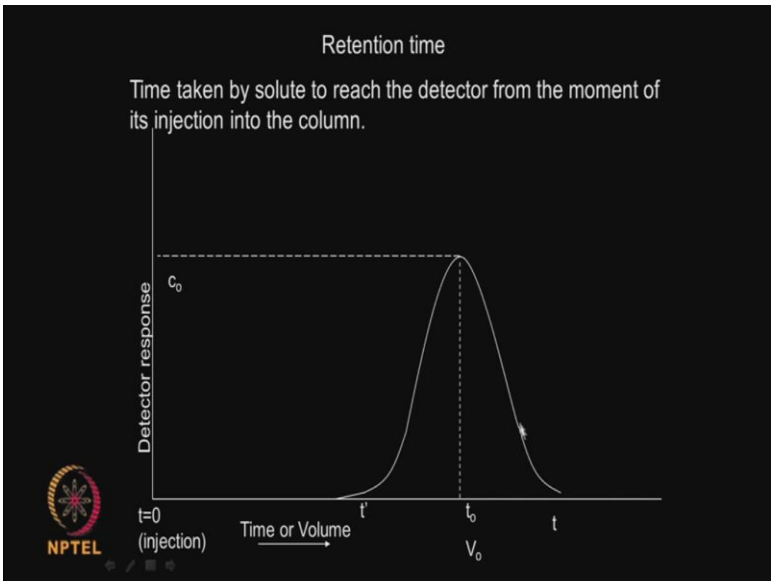

So typically as I said the solute will come out in this fashion, we will call this a normal distribution or uniform distribution or Gaussian distribution and so on… okay. So this the x-axis could be time, so I am injecting it at time = 0 okay, so at time = some time, we call it the elution time t0, then it will come out the maximum that could be c0 okay. So okay but generally you will not get this type of graph, there could be some skewed either tail or fronting and so on can be happening some, many times it will not be like a normal distribution like a bell curve the shape may be different.

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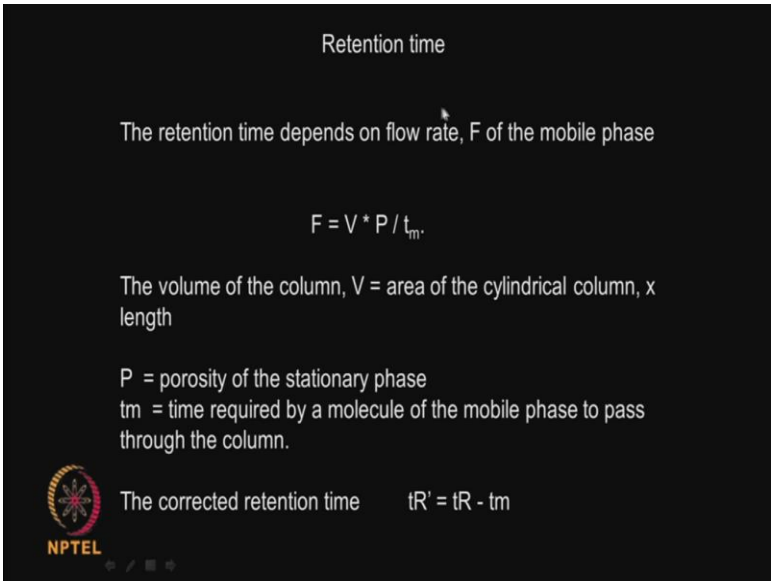

There is something called the retention time. This is called the retention time, okay the time it takes for the liquid peak to archive that maximum here. It is called the retention time. Okay. Retention time depends on the flow rate f of the mobile phase, so F = Volume*P, that is porosity of the stationary phase/tm. Okay. So that means tm will be VP /m. P is the porosity, tm is the time required for the molecule of the mobile phase to pass through the column. Okay there is something called corrected retention time because it is a as soon as you inject.

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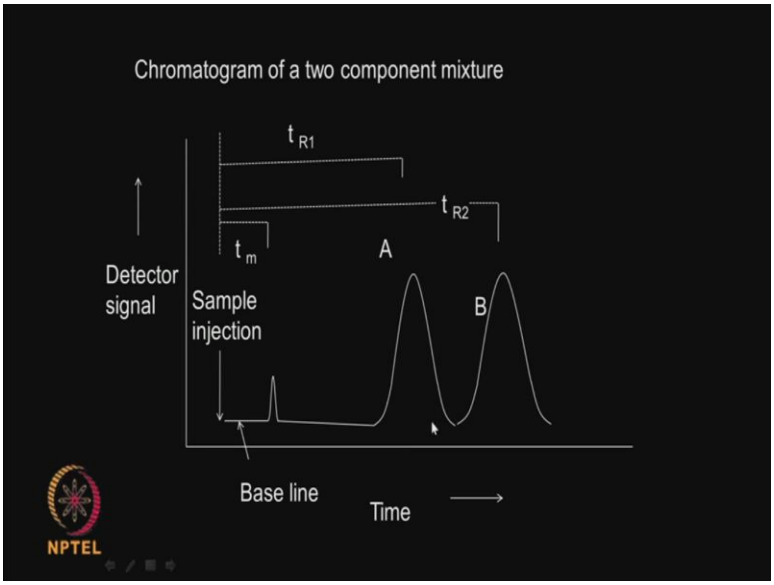

There will be some peak coming out okay. Okay. That is called the tm. So if you get a retention time okay, we need to generally subtract this time, with this time okay to get the actual retention time. Okay. So we need to subtract.

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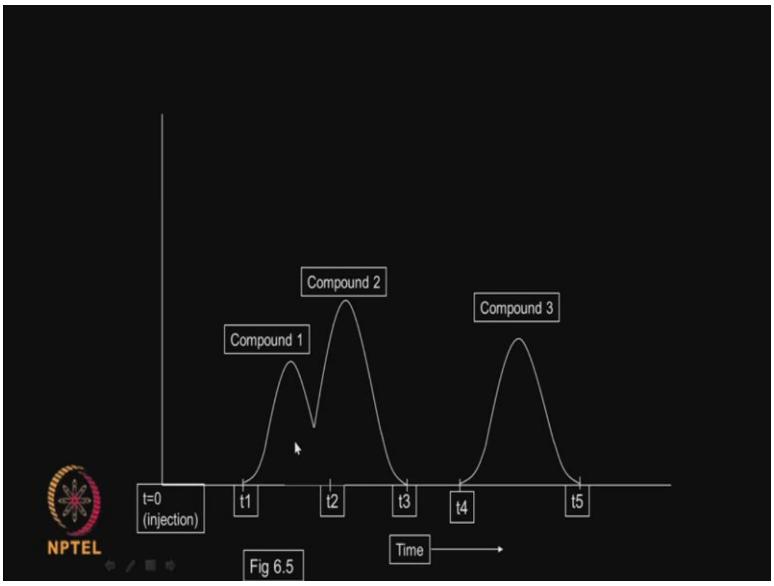

So if you have many components, then the output may look like this. Okay. We can say the component 3 is separating out very well because it is clear. When the baseline is very clear whereas in this particular phase, component 1 and component 2 are not very clear. You can see a overlap here. So if I am collecting, if I am collecting component 2 at from at this time, you could get component 1 also, may be after this you may get only component 2 but here you may get component 1 and 2. So that problem is always there.

Whereas here with respect to component 3, there is no contamination from either 1 or 2. So ideally you would like to have a peak of this h shape and size whereas you would like to have like this, where 2 of these peaks are overlapping with each other .

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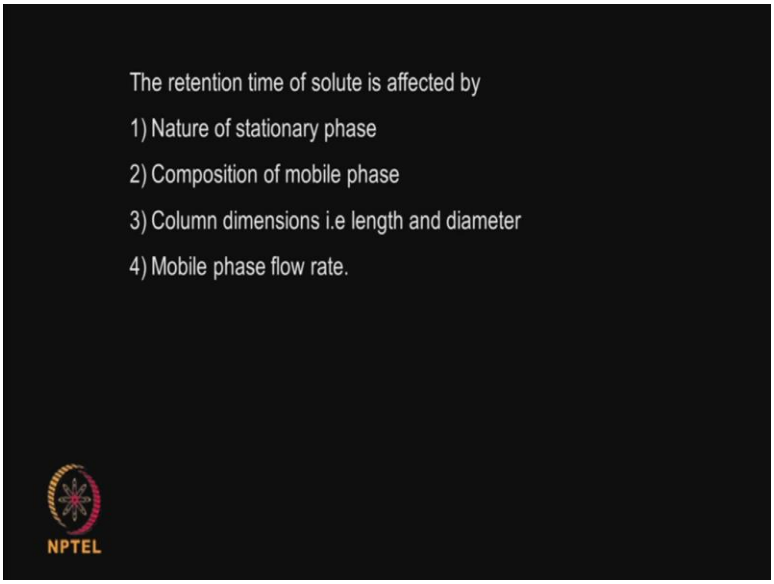

So the retention time of solute is affected by the nature of stationary phase that means what type of stationary phase I am using, what is the particle size of the stationary solid, what is the porosity and so on. Composition of the mobile phase, am I using a hydrophilic mobile phase? Am I using a hydrophobic? I am using a mixture of them, what is the dielectric constant and so on.

Then column dimension, what is the length of the column, what is the diameter of the column, then mobile phase flow rate, how fast or how slow the mobile phase is flowing through. So all these affects the retention time, I am not talking anything about the separation efficiency but I am just talking about the retention time that means what time it will take for a particular solute to come out.

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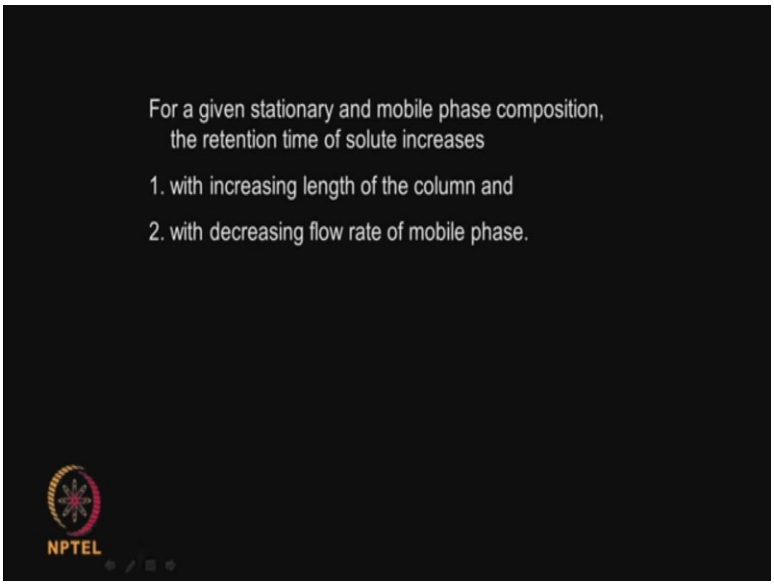

For a given stationary and mobile phase composition that retention time of solute increases with the increase in length of the column. It is obvious if I have a very long column; it will take much longer for the stationary phase to come out obvious. With decreasing flow rate of mobile phase, so if I decrease the flow rate then it will take much longer time. So if I increase length also it will take much longer time. If I decrease flow rate also it will take much longer time.

I am not talking about, please I am not talking about the separation efficiency but I am talking only about the retention time. If I have 2 components then both the components will also feel the same. If I reduce the flow rate both the components will come slower, if we increase the length both the components will come out slower okay.

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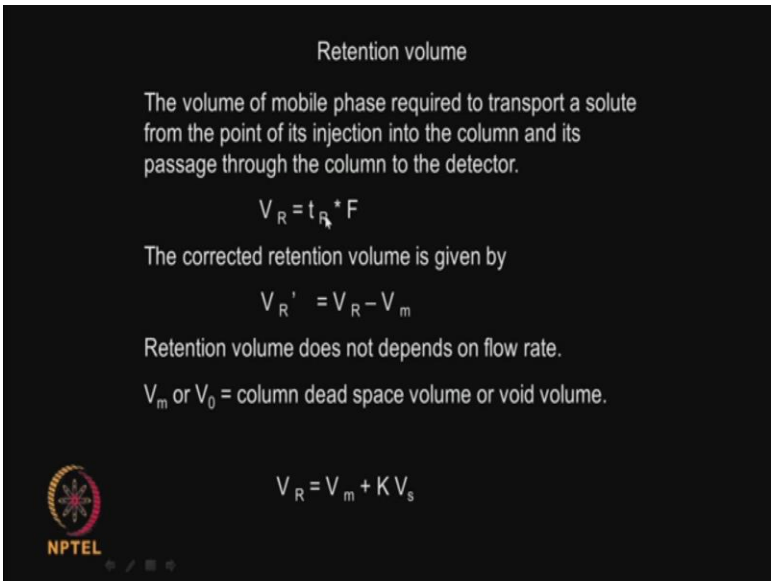

Now let similar to retention time you also have retention volume. So retention volume is given by retention time * flow rate that is obvious right. So that is the volume of the mobile phase required to transport the solute from start injection point right up to the detector point n right. So that.

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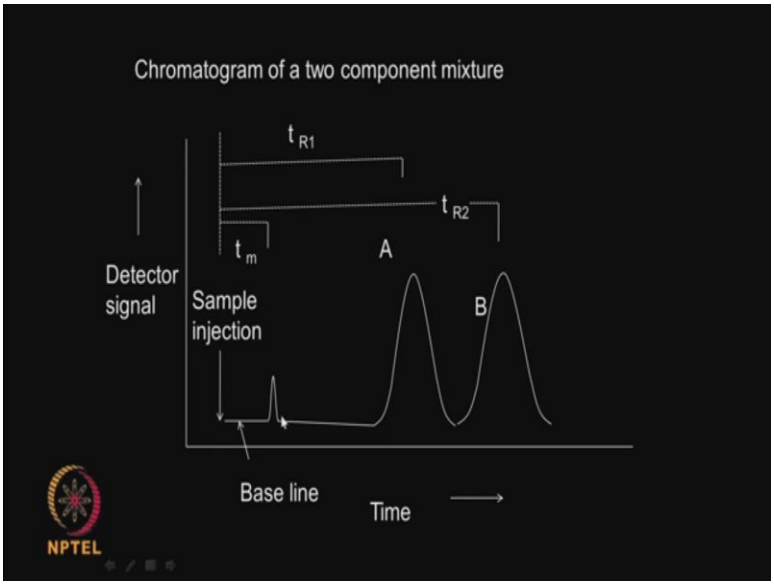

Similarly if you want to do corrected retention volume, we need to subtract with respect to Vm like if you remember, I mentioned something called tm here right, because if you just m inject you will get a small peak corresponding h to the dead volume of your pipe packed material. So actual retention if you want to corrected then you need to subtract this retention time with the tr.

Similarly if you want to know the actual retention volume you may have to subtract with respect to Vm okay. So Vm is the column dead space or void volume you can say okay. Remember that. So Vm is the column dead space or the void volume.

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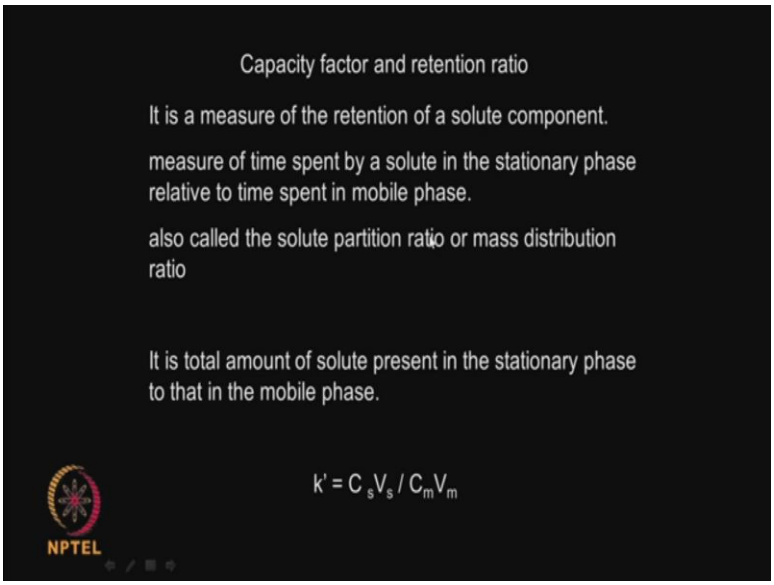

So there is something called capacity factor and retention ratio. Let us look at this. What is the capacity factor? It is a measure of the retention of a solute component, how much of the solute can be taken in that particular column. So it measures time spent by a solute in the stationary phase related to time spent in the mobile phase okay. This is also its called something to do with partition ratio right because mobile phase and stationary phase.

If you are looking at ratios then partition coefficient also comes into picture or it is coming as mass distribution ration also. So it is the total amount of solute present in the stationary phase to that in the mobile phase. So obviously so if vs is the volume of the stationary phase, Vm is volume of the mobile phase inside that column, C is the concentration of the solute in the stationary phase, cm is the concentration of the stationary phase in the mobile phase, then k' = Cs Vs.

This gives you total amount of solute in the stationary phase inside the column, total amount of solute in the mobile phase inside the packed column. That ratio is called capacity factor okay.

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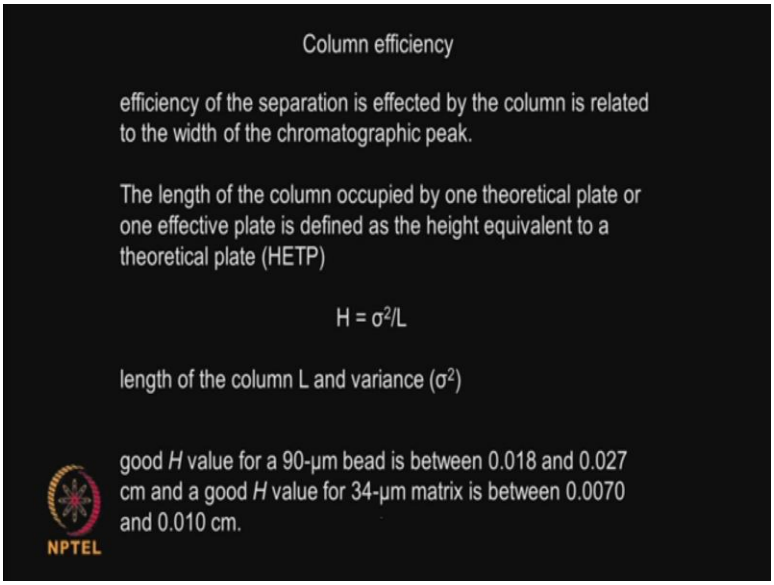

Then we have something called column efficiency. Now we come to the efficiency of separation okay. So far we looked at retention, retention time, all those things but then when you have many peaks ideally I would like to have a good separation between 2 peaks. That is what I want ultimately in a chromatography I want to achieve that. So column efficiency, efficiency of the separation, it is related to the width of the chromatographic peak.

The width of the chromatographic peak is large, if I had 2 peaks and both of them are very large, then they will be overlapping one or another so obviously the column efficiency is very poor. The width of the peak is very very small or very sharp, then you may have a good column separation, that means good column efficiency. There is a term called theoretical plate okay. This comes from chemical engineering, there is a term called theoretical plate okay.

Number of theoretical plates so a column is divided as consisting of lot of theoretical plates and separation is taking place in each theoretical plate okay. So more the number of theoretical plates, better is the efficiency expected. So each called column it can be converted to certain number that is called the theoretical plates okay. So if then corresponding to theoretical plates, we also have another term that is called height equivalent to a theoretical plate.

So ideally if I have a very small height equivalent then I can say it is a very good efficient column. So if I have very large number of theoretical plates then I can say it is a very good

column with good efficiency. If the height equivalent of a theoretical plate is very small then I would say the column I very good and it can do a good separation.

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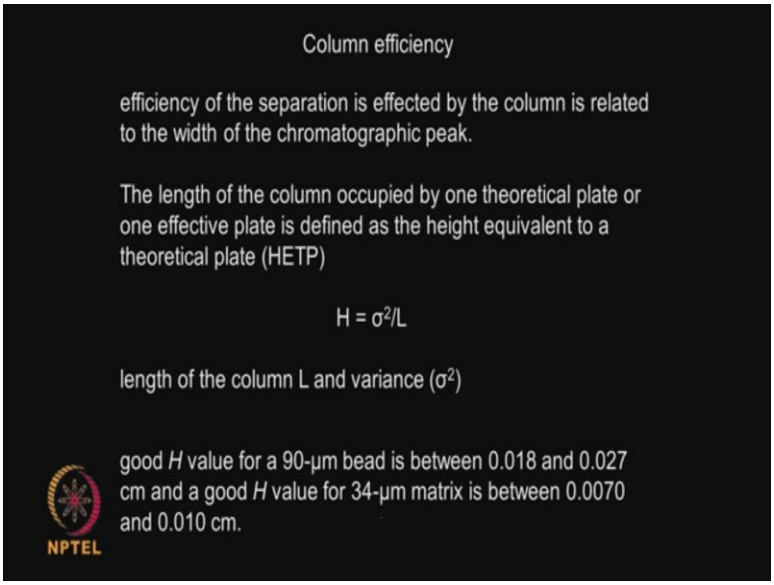

So 2 terms, these 2 terms comes from chemical engineering mostly from distillation column. So if you have large number of theoretical plates then you can say the column is very efficient. If you have a very small height equivalent of the theoretical plate then you can say you have a efficient column okay. How do we calculate the number of theoretical plates? We will come to that as we go along, there is a formula for height equivalent of a theoretical plate h.

Height equivalent of theoretical plate is given by sigma square/l, l is the length of the column and sigma square is the variance, as you know the spread variance is the spread of the normal distribution curve which we are talking about. So good h values for a 90 micron bead is between 0.018 and 0.027 centimeter and good h value for a 34 micron matrix between 0.007 and 0.010 centimeter. So these are some figures experience-based figures again they are come from engineering because in chemical engineering,

You have distillation column, packed distillation column, where you have packing of beads of different sizes. So these terms come from there. So h is given like this h = sigma square / l. h is called the height equivalent of a theoretical plate. So smaller the h suppose if I am comparing 2 columns and h of one column is lower than h of another column, then I can say that particular

column is more efficient than the other column. If I have 2 columns and if the number of theoretical plates of one column is higher than that of other, I can say that column has better separating efficiency. How to calculate number of theoretical plates, we will come to that soon.

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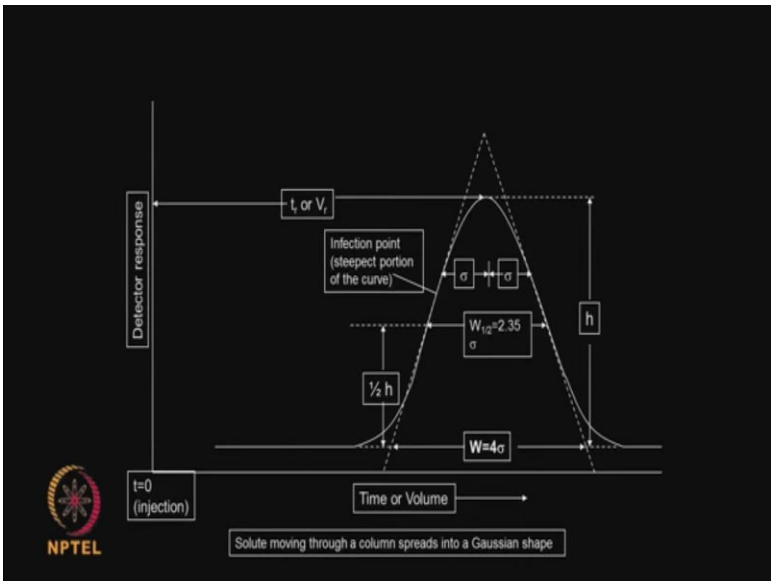

Okay so you have as I said a peak like this coming out as you inject at time = zero okay, this x-axis could be time retention time or it could be retention volume. As I said retention volume is nothing but retention time into flow rate okay. This is called the width at the base. How do you estimate? You draw a line a, a tangent at the maximum place okay, steepest portion into another tangent and it takes this. This is called the width at the base, this is called the height. Okay.

This is called the half width, half width is half of the height. Now if you have h here we take half h this is the half height okay. Now there are some correspondence but half width, base width, this is called base width and this is called half width. So there are some relationships which connect these numbers with theoretical plates efficiencies and so on. So will look at it actually. So you understand? So as the separation happens when you inject at time = 0 and at the retention time or retention volume, we get a peak of this form.

You draw a tangent okay at the maximum inflection, you draw another tangent at the maximum inflection point and you look at the base, that is the width at the base and then this is the height and half height. If you measure the width, that is called half width okay. There are some

relationships as I said connecting with these efficiency of your column because as I originally said if the separation efficiency is good, the width will be smaller. So there will be an inverse relationship between efficiency of separation and the width because width indicates spread, spread indicates inefficiency. Okay.

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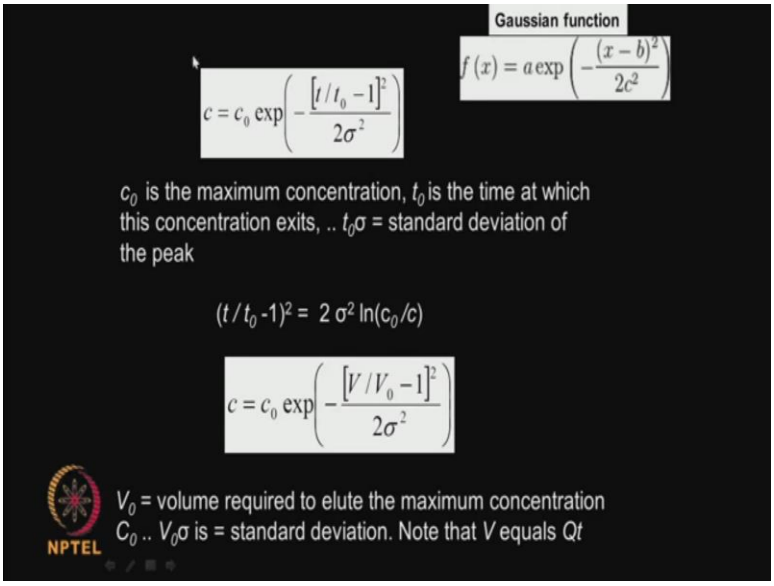

So as I said this is a normal distribution, these pictures. So just to recap if you remember you must have studied long time back, this is the equation for a normal distribution, some number a exponent x - b / 2c square right. x – b. x could be. b could be the average value right. if you remember long time back and c is called the standard deviation okay if you remember. So similar equation we could derive for the change in concentration as a function of time for the Gaussian peak that is coming out of the chromatographic column.

Same. So it will be of this form c is c = c0 exponent t - t0 - 1 / 2 sigma square. t0 is your retention time, okay so t0 sigma is equivalent to your standard deviation. So t0 sigma is the standard deviation of the peak and t0 is equivalent to the mean or that means the constant in the time at which the peak is maximum or it is the retention time correct. Similarly we can get another equation just like retention time, we can get for retention volume.

That is the volume required to elute the maximum concentration. So similar so here the standard deviation will be V0 *t sorry V0* sigma just like t0*sigma okay. So because am comparing with

the Gaussian equation, this we must have really studied long time back in your statistics and so on Normal distribution and Gaussian distribution. So if you compare that we will get this as t0 sigma as the standard deviation or V0 sigma as the standard deviation okay.

So that is how the a equations, that is how the concentration varies with time here and this is how the volume varies with time. Okay. t0 is your retention time V0 is the retention volume okay. So we have an equation this equation is very useful, we can do many things with this equation and we will do some problems later on actually.

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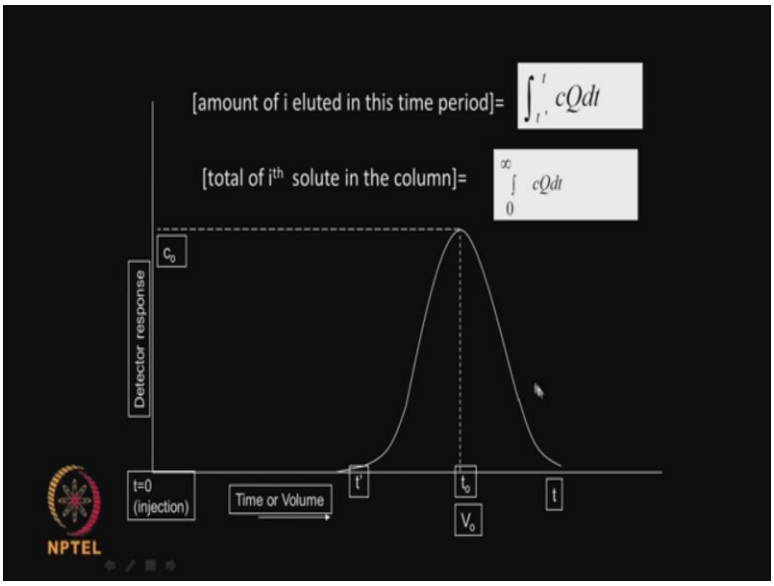

Okay so I have a curve like this going up. So if I want to know amount of I a solute I solute eluted in this time period that means between t and t0, so what do I do? I do an integration of that right c*Q. Q is flow rate concentration *Q*dt. For the c I have an equation in the previous page which I showed you right as a function of t0 and sigma and so on.

If I want to know the total I then I need to integrate between 0 and infinity okay. So that is why I have 0 but if you want to know how much between these 2 times, I will integrate between t and t0, t'. So if I collect the sample between this time to this time, I will get only this much amount. If I collect the same sample from 0 to infinity, I will collect the entire solute but then I will I could be having impurities also as I go along.

So generally as we collect when the peak is rising and in-between the rising and the falling right okay. So we will not be collecting the entire solute I but you may be collecting only when it is showing maximum concentration. So you will be losing the solute in this in this period of time or in this period of time right. So the efficiency of collection depends upon the time minimum time and the maximum time within which I am collecting these 2 for the solute and that is given by this.

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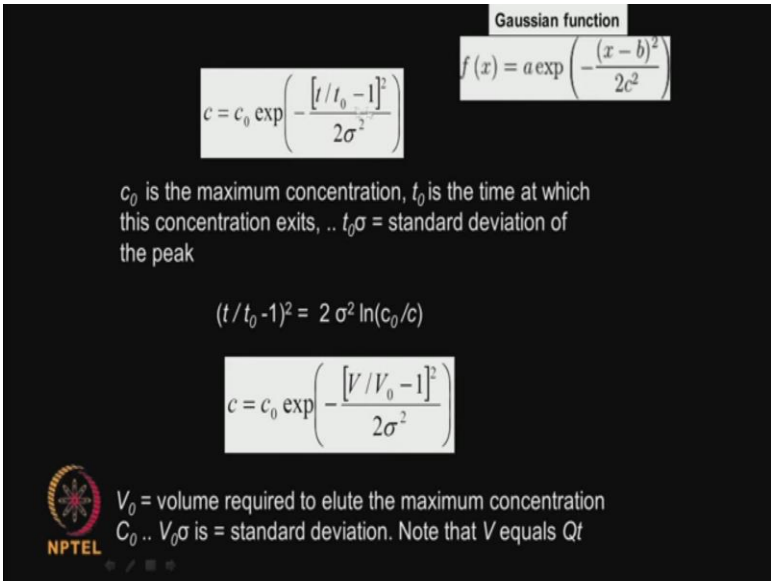

So we could integrate this equation, if you remember this is the c0 c equation, I could put there. I could integrate and I will end up with slightly complicated looking equation. We will look at it later on and if I can integrate between t' to t or I can integrate between 0 to infinity and so on actually okay. So we will look at it after sometime.

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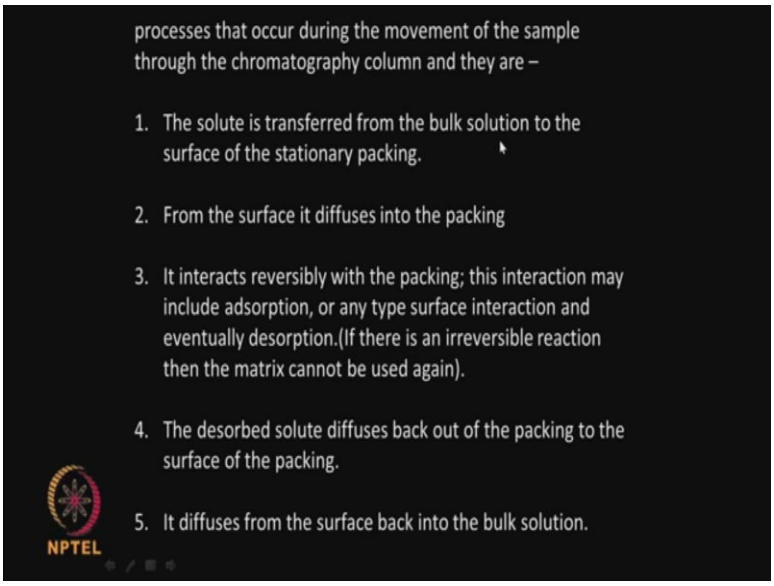

So the process that occur during the movement of the sample through the chromatography column, so many things happen the solute is transferred from the bulk solution to the surface, from the surface it diffuses into the packing. It interacts reversibly with the packing. So they, it could be adsorption, it could be interaction, non-bonded interaction, so many things okay. Then it gets desorbed, it diffuses out from the packing and then it goes out back into the bulk solution.

So it is moving from the bulk to the surface and then it moves and goes to this surface of the packing material or the stationary phase, then it could be getting desorbed form the packing on to the from away from the surface. Then it could be moving into the bulk. So all these are retarding your solute passage, so it comes become slower and slower. So the more it comes slower it comes, the retention time is longer.

So each of these solutes have different retention time that is why the partitioning of these material takes place okay. So with that we will stop on chromatography and we will continue in the next session further and as I said there are many types of chromatography and we will look at each one of them as we go along. Thank you very much.