Downstream Processer
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Lecture - 34
H P LC

We will continue with the topic of H P L C, because it is most important analytical tool that is being widely used both for biomolecules that means peptides or proteins or for even small molecules like acids alcohol and small esters and so an. Actually that is why you would like to spend more time of concept of H P L C. There is a relationship between this H P L C which is used for analytical purposes and this obviously normal separation techniques using chromatography, different type of chromatographies which we looked that.

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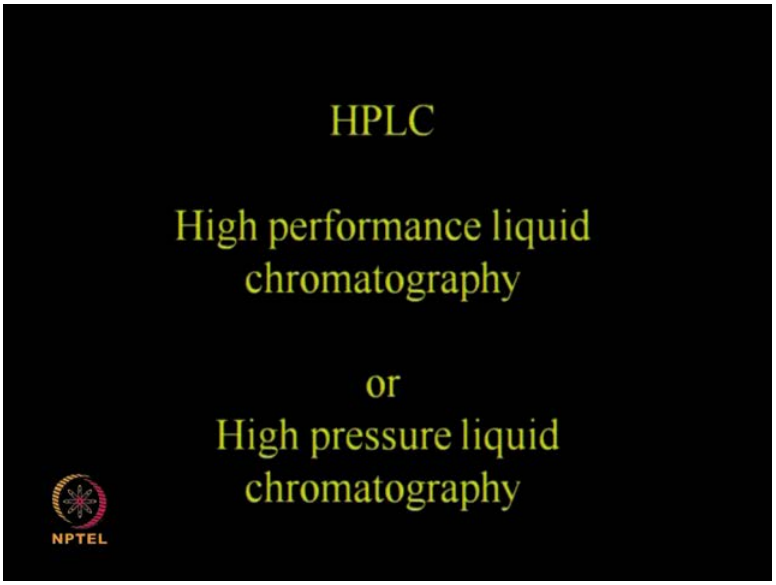

So, HPLC the P's can be for performance or it can be for pressure because it is a very high performance system as well as the pressures are very high, the back pressures are very high because the tube diameter is small, particle sizes are in micron range. So, the back pressure developed is extremely large and so you need a very high pressure pumps to operate in an H P L C.

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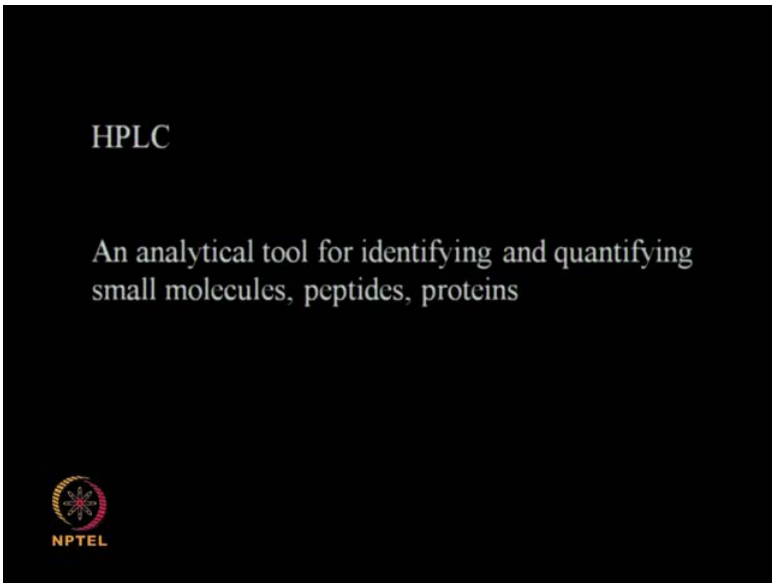

So, it is for identifying an unknown compound as well as it can used for quantifying an compound as well actually. So, that is the main advantage of H P L C we can use it for both the purposes.

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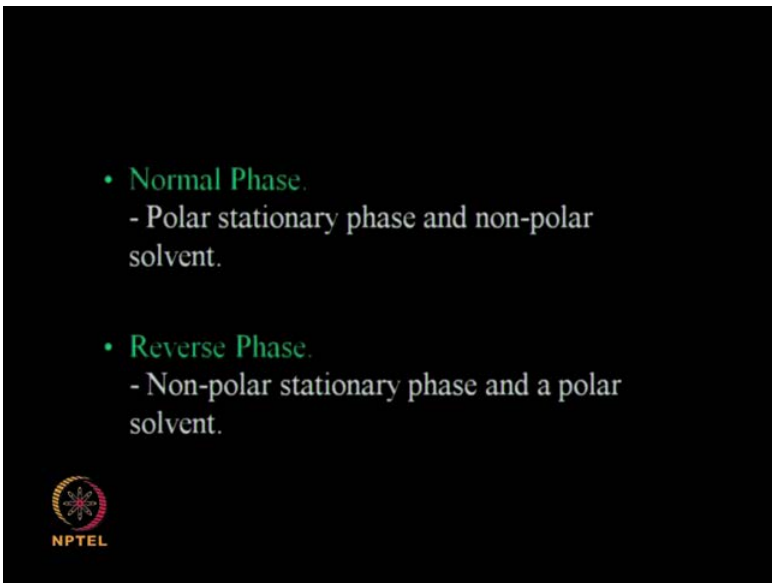

There are two types of H P L C's I talked about it in the previous class, one is called the normal phase that means you have a stationery phase which is polar like a silica and we have a non-polar solvent. Non-polar solvent means it could be hexane or any other hydrocarbon, the other types of H P L C is called the reveres phase H P L C that means you have a non-polar stationary

the phase and a polar solvent. So, non-polar stationary phase would be a long chain hydrocarbon and the polar solvent could be water, methanol acetonitrile and so on.

So, now a days the fashion is to go towards reverse phase, because the solvent which are using is extremely cheap, mostly will be using water dominantly water and it could be some other organic polar compound. So, in H P L C we consume quite a lot of solvent both for analytical purposes as well as for regeneration and cleaning, flashing and so on. So reverse phase H P L C has become extremely popular.

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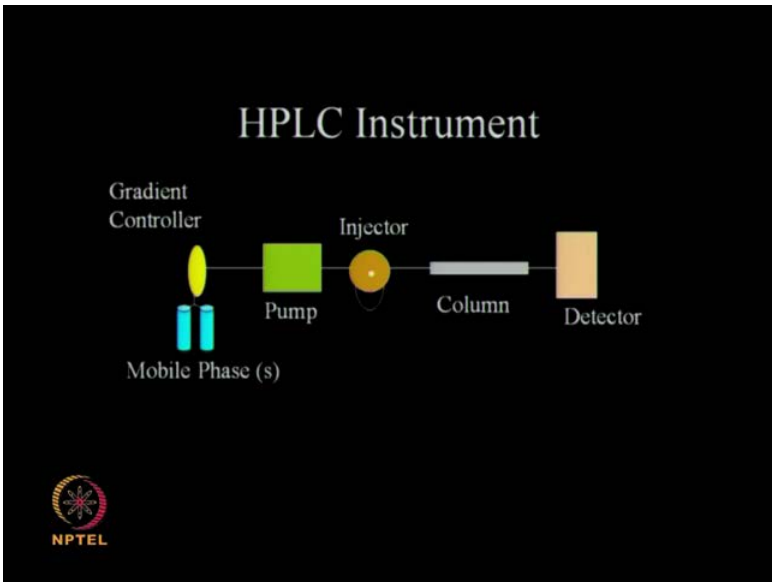

Now, a typical setup I talked about in the previous class you have a reservoir for the mobile phase. Then we have the pump. It could be two types of approaches: one is the isocratic or other is the gradient. In an isocratic we are using only one solvent so, generally we will where only one pump whereas, in a gradient system we may be using two or more solvents. You are changing the solvent that means you are changing the dipole moment so, that separations get enhanced or improved.

So, you have the pump or pumps and then we have the injector where we have injecting your solute mixture and then the column, it can be several speech and then file finally the detected. There are different types of redactors and that was going to spender today on dominantly the types of detector there advantages and dies advantages so on. So, there is a typical HPLC instruments. We sometimes always have a god column here. A god column helps as a filter unite catchers and small particles and the catchers some polymeric proxy so that the long the life of

the main column. So, that is the main advantage of using a god column and once he get we steps can god column is can replace it another god column. So, like that into separate the life of the column is prolong.

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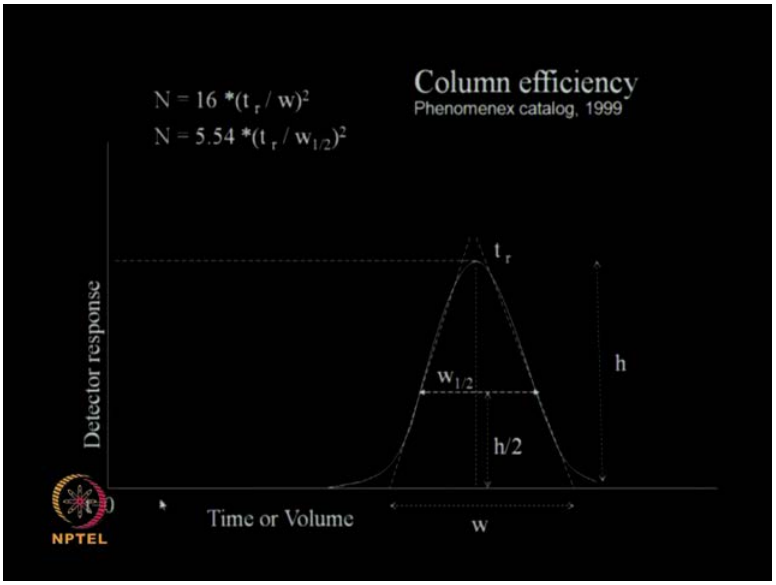

Now, a typically when I inject a sample after sometimes I am going to get the heap is going to like is reach maximum and then fall down. So, this is called the retention time that is the time he takes for the maximum of the peak to appear. So x axis is here to and he have to detect the so this maximum time is the retention time and he can also use it retention voice that means volume of the day and continues phase we collect until the peak is the maximum. Now if we drat 2 tangents and this infection point and if we measure the with as the base that is called the w, with at the base and you can also have something called half bit at the maximum

That means if this is the maximum height of the period peak how the maximum is this so, the with measured here is called with that half maximum. Why do we need have this? We can calculate a term called theoretical place. Theoretical place stele the efficient as from the column more the number of theoretical place, more is the efficient. So, if an comparing two columns and a one column has higher number of theoretical plates, then we can say that column is more efficient then the other column, all other thing being the same.

So, if we measures w that is with and the based on the standard method are if we measured with at half the maximum then the we can use whose either one of the calculate number of theoretical place. So, if you are using at the base then the formula is n is equal to 16 the r by w full square, t

r is here attention time. If we are using with that half maximum then we use n equal to 5.54 t r by w half full square. So, this is very very important formula for measuring the number of theoretical place and number of theoretical stages.

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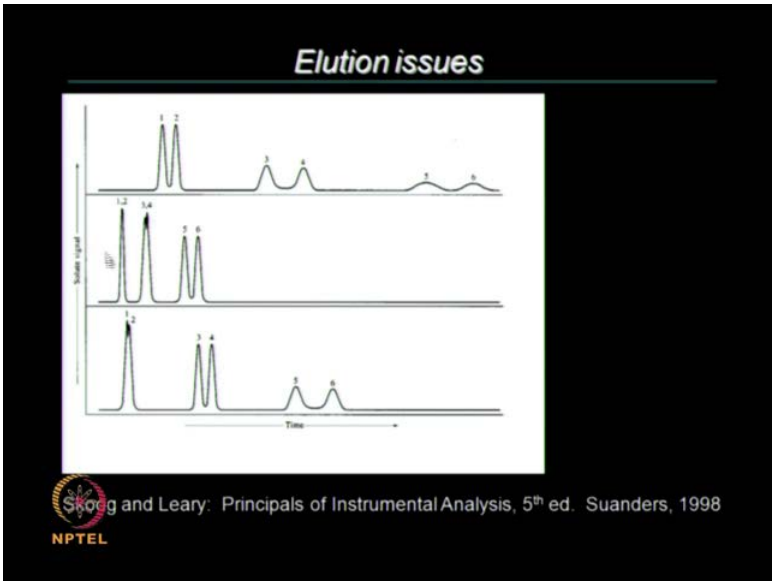

When you are doing a H P L C and if this solute mixture contains several components life is not going to be very simple that we will get individual peace well separated out and but it is going to the very problematic. Say for example, it may be like this and you get very good peace for components for 1 2 and after some time we get the component 3 and 4, but component 5 and 6 are coming very very late and it is very broad. So, we will not be able to accurately measure component 5 and 6 concentration and its happening after a very long time because as he now the base x axis is time. So, you are extending unnecessarily your analysis time.

So, I can try to bring it closer that means I can reduce the retention time of the component 5 and 6 by changing some conditions like changing the solvent system of changing the ratio of solvent. Then what happens? 5 and 6 is coming but 3 and 4 has merged we can see them hardly 1 and 2 completely merge that we cannot differentiate 1 and 2 so we again this also not deserve then we keep playing around the solvent systems and ratios and so on. You may be able to separate 3 and 4, but 1 and 2 is not separated. So, we are closer but not really the good or the best set of chromatogram. So, we have to again player on this set of condition so that 1 and 2 also get separated. So, if we on to measure accurately the concentration 1 and 1 even this is not good.

So, we may have to improve to further so that we get a good separation for 1 and 1, but at the same time 5 and 6 do not go very far away during the analysis time. So, you see the if we have a multiple components mixture it is going to be a real challenge. We may have to optimize to the condition until you get the best set of a results were all the components mixture with your interest get well separated out at the base. That is very very important that is call at the basis, because they are not separated out at the basis, neither here.

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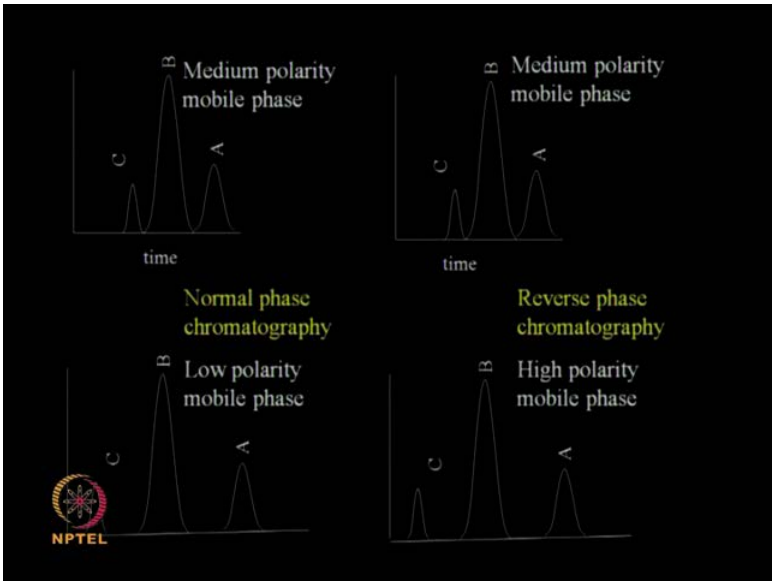

Now, when you are using here normal phase chromatograph when your easing both of them paid in different stage a principles. So, we need to see how to improve this type of separation. For example, if they are very very close, we are using in normal chromatograph a b c and the close and you want to separate. Suppose, you are operating at medium clarity what he do you? You switch over to low polarity. Low polarity means he make it more hydrocarbon that means you make it more hydrophobic then you get a good separation of these peace. Why it is so? You have the a normal phase which is the a polar so, it attract more hydrophilic group, it does not high interreget the hydrophobic group.

So, by going down in the hydrofelicity, by making it more hydro phobic we are getting a good separation. So, let us go to rewires phase from monograph were the stationary phase is a hydrophobic material like c 18 kind of material. Now, the pix are very close what we do? You change the polarity to high, from medium you go to high. So, when we go to high polarity that means polar components are retained in the solvency so, the separations jar much better. So, if

we are using normal face, if you want have an achieve better phase line separations remove to no polar you are an rewires phase autography, if you want an chive a good base line separation go to high polarity solvency. So, you see the approaches which you adopt depend upon the type of chromatography which we are using.

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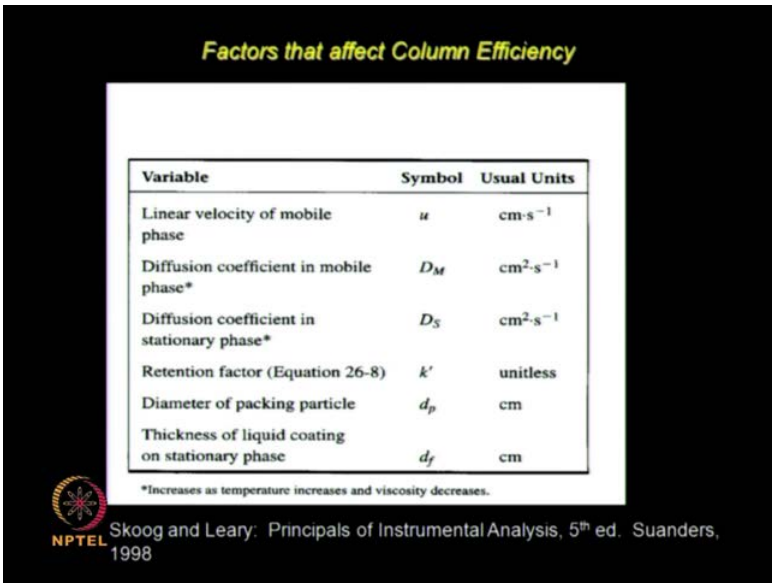

The other factor which affect your column efficiency are the velocity of the mobile face, the diffusions co efficient of solute in mobile face, diffusion co efficient in the stationary phase that means how the solute gets the defuse inside the post of your stationary face. The retention factors the diametric articles smaller the dramatics better in the separation, but then the back pressure is very very high.

Thickness of the liquid of coding on the stationary phase as we know we have the stationary phase is the soled, the mobile phase is the liquid and there was they are two faces stamp we are always going to have a liquid layer which is settled on the soled and your solute as the defuse through bat if it is have to be solid face. So, there is a mass transfer resistance for the liquid solute to move from the bulk of the liquid phase to the solid of the face.

So, smaller the thickness the resistance is less, higher is the thickness then the resistance is more and the solute will take much longer time to defuse from the bulk and comedown to the surphase of the solid. So, what are the factors that affect? Factor that may be effecting this liquid thickness may be density of your continues face, viscosity of the continues face, the size of the

stationary phase material the turbulent that happening inside, the wide age or the wide space inside at the column and your distribution practical size so many factor effete that.

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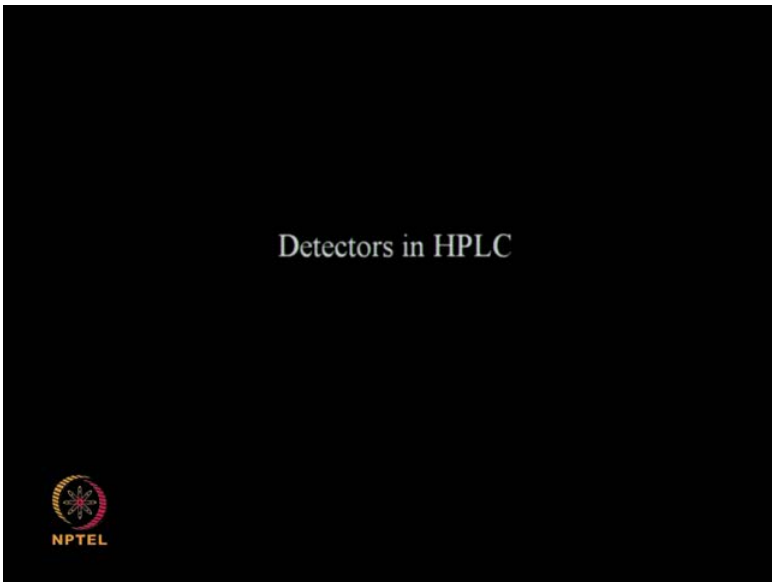

Now, that is look at detective in H P L C he said detective pay very important roll alien and there are different types of detectors depending upon the application of which you are looking at.

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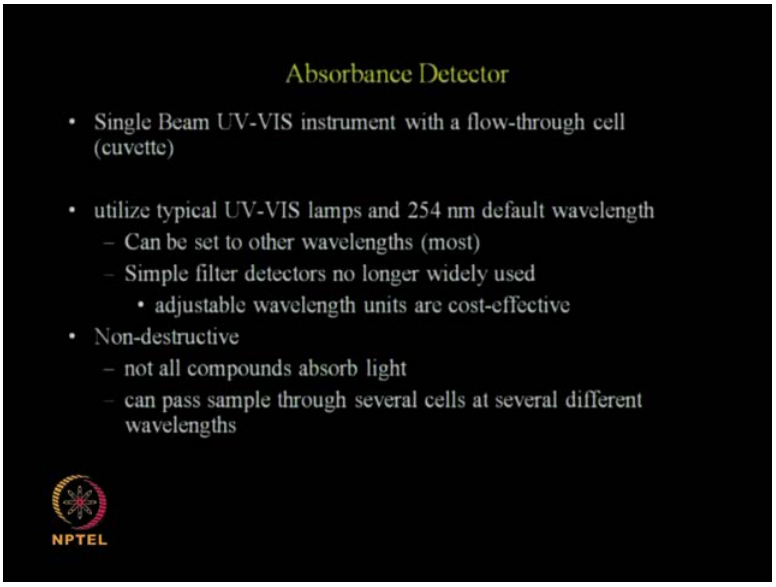

There is most simple one which I mention lost time also is the single being you be disable. So, you have a quit were you taking were solution and you we disable instruments and he measures

the lambda maximum. So, generally in a you we visible a you detect at 254 nano meters likes a d fault abler, but he will able to set it to different they lamb but, you can measures only at one sing and but, it extremely cheap such an instruments you have that is very fast effective. We can use different filters to look at different wave lenses. It is a non destructive test that means after you have done the experiment we can collect your sample when use it some other mannoses that is the name of advantage of is type of detect.

And because as he know if the quantity and of the solute and is real is scare we will not be having enough of the solute for performing large verity of analytical measurements. So, in that case we do this H P L C, collect all the sample again take it back may be perform some other hand. So, that is the main for disadvantage a not all compounds of light absolve light so, that is going to be a problems. Next detect it is also a very common tool used in HPLC and its quit versatile and we can measure it at several valance because diodarats detectors can complete range of valence every seconds so, the amount of data you collect it large and this is very ideal for r and d types of purposes.

So, initially I will be the almanac that the compound which are I am going to separate. So, in that cause use a diodaradik. I will scan the enter waveland and from that I will know what would be the lamdom axis with. But the data we will going to get for each second fix going to scan the enter wavelength is huge volume updates. That is the main disadvantage, but its ideal for research purposes so, single beaming is ideal for routine quality control, were as a dad is very good for r and d type of products. Again is the non distractive type of technique that means often they experiments we can again collect the solute back and use it for some other analytical purposes.

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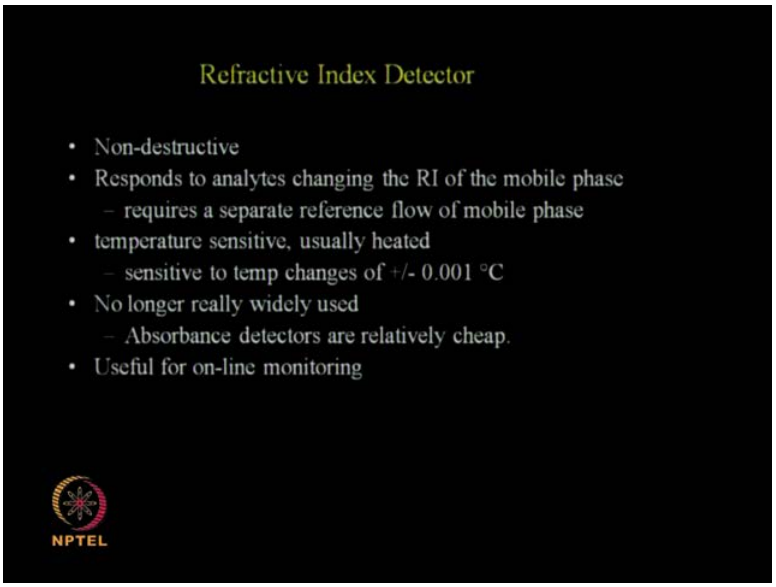

Next time is distract in that there is a change in index of the solute measure. This again in a non distractive ideal if the solute have different diffractive, but then it is extremely temperature. So, even a small change in temperature of the order of 0.001 degree can affect the result for active index so, you may start getting very spurious results. So, that is why nobody uses it now a days and its small change in temperature going to affect the R I, but it is used in online monitoring.

So, you have a manufacturing plant and there is the product which is being produce used you want to know the concentration. So, there in the pipe line you put R I detector and you cal byte the R I detector and then we star measuring the changes in R I early given indication of the changes in the concentration. So, it is ideal for online and its extremely cheap that is the main advantage of the using the R I type of detector.

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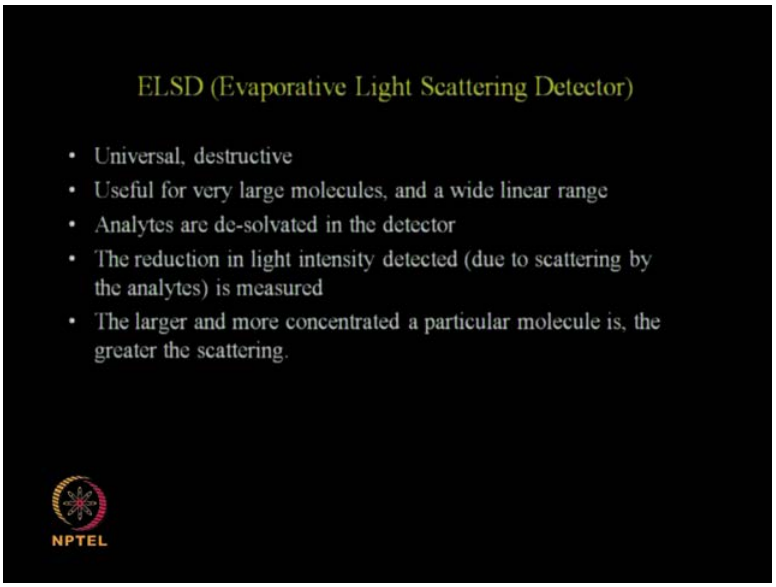

Now, another detector like which called operator lights scattering detector E L S D. This is also use now a days, but the main disadvantage is distractive ethnics that means often he have done and them measurements are analysis these products is not available for sum other analytical. It is very useful for large molecules and its linear were a for a vidal range and the analyst d solvate the detector is the solvented the operator. The reduction in the light intensity is detected due to the scattering by the analyte.

So, that is why as the name implies it is a light catering. So, the light the laser fall on the analyte are the solute and its cattle which is special. So, larger molecule are more concentrated the molecules the scatterings is also going to be more which is measure of the size of the concentration molecules. So, the scattering is measures and hence the size of the concentration of the molecule is implied. It is a very good techniques and its spite accurate.

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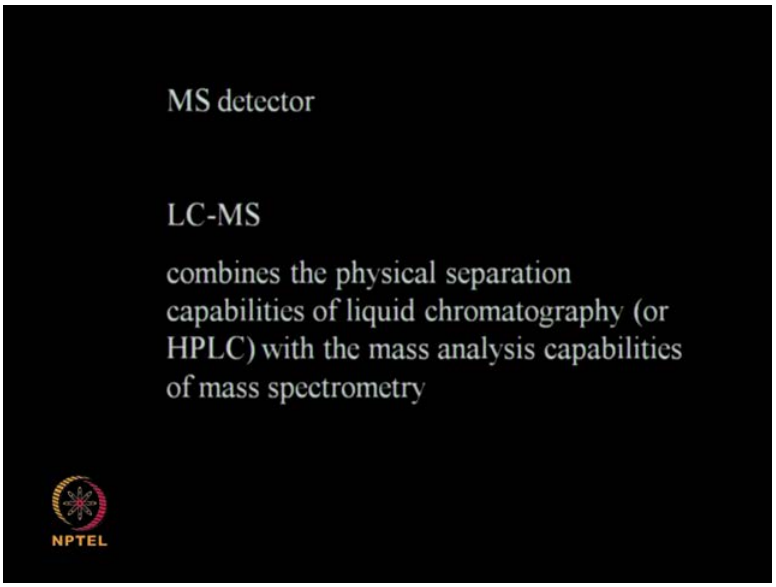

Then we have the mass sect detective M S means mass specs. Now a day the mass spectrum is detectors it will become very useful it can give a mass value for the solute or analyte. So, now a days L C M S that means liquid chromatography and mass pact are getting couple to gather and we can use the L C part the separation of the various solute of the bio molecule of protean and then M S scan taluses what is the bass number of each one of the peck.

So, it is a combination both the liquid process chromatography and the mass analysis and with extremely useful because he can even at the fragmentation of the mass and from the fragmentation patron may be able to imply what is the type of bio molecule precision. If the molecule is very small organic molecule it is very easy to detect or an implied. If large bio molecules then cakes a lot of time to understand the structure, but there are many data basis number now a day available which gives use the M S spectrum or M S fragmentation pattern and then try to implied what the molecule is.

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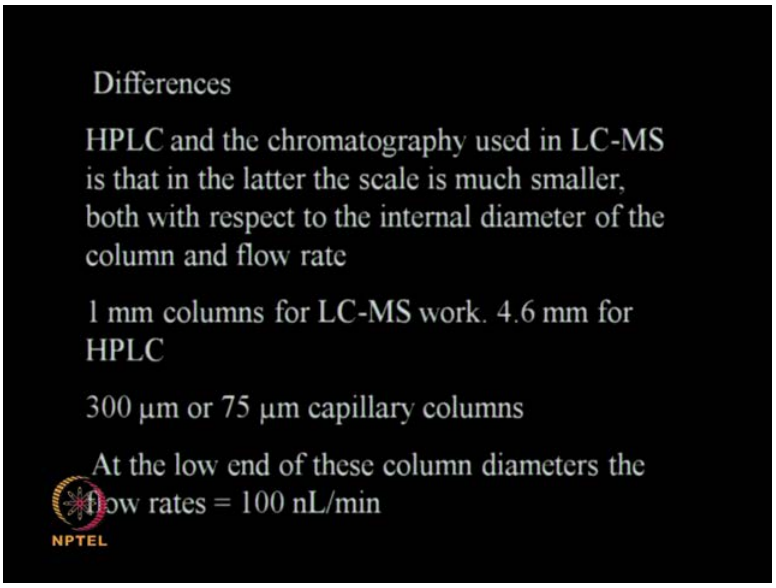

So, HPLC per se these avails the L C-M S that is liquid chromatography M S. There are slightly differences in the stub. The L C part of the L C M S is much smaller than the H P LC in the sense, the internal diameter is very small, the column is also very small, fluorite is also very less in the L C M S. Whereas, in the HPLC the length of the column very larger, flowrate is also very large. See for example, you may be use in a one mm column for l c ms work were as the HPLC may be a 4.6 mm column. You may be using very very small capillary column of 300 parameter, 75 macro meter capillary column and you may use even go down in florid even 100 nana letters per minute that is very small flow rate. So, such small miniaturized sizes can be use in L C part of the L C M S and like in the H P L C were you need to have hair florid, higher diameter, longer columns.

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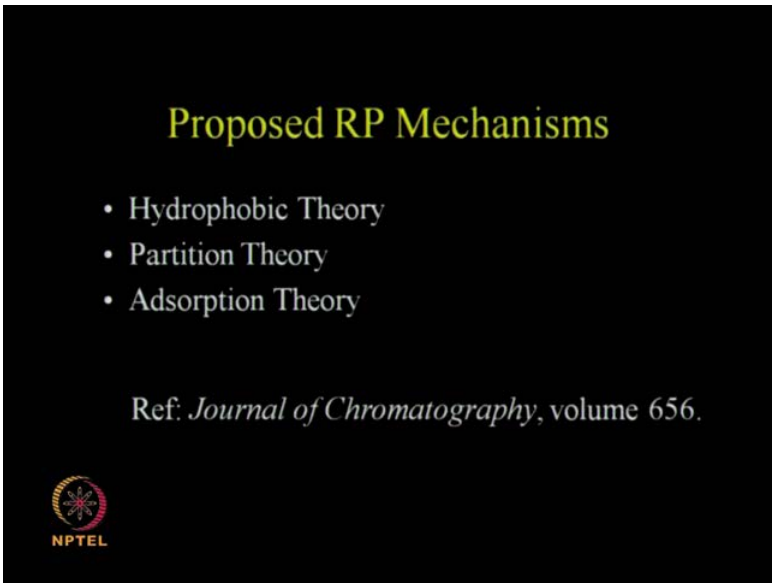

Now, how does the reveres phase chromatography of there are several mechanism and it has been proposed and in real situation all is mechanism of combined together and that is a real system works. The 3 mechanisms or theories which have been proposed for the reveres phase chromatograph towards are one is hydroponic theory, other is called partition theory at this called the absorption theory.

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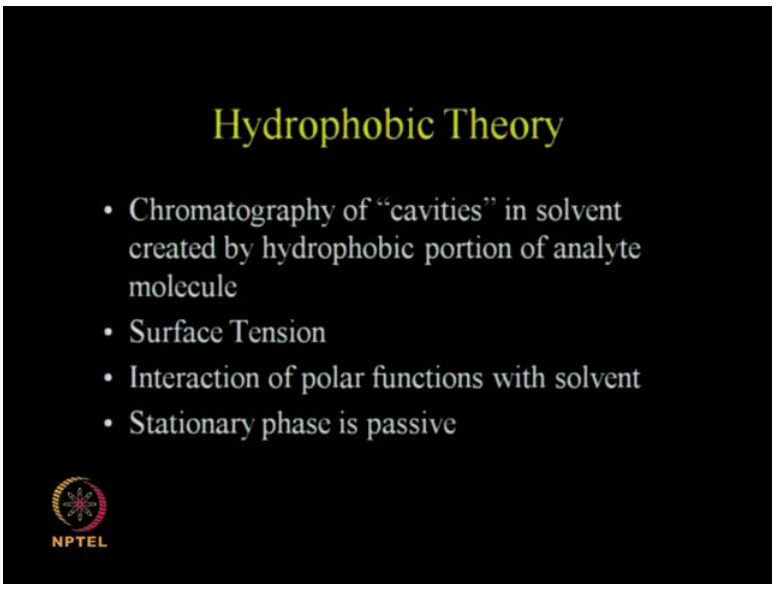

So, let us spend little bit time of on each one of these theories. What is hydrophobic theory? So, there are cavities in solvent created by hydrophobic portion of analytic molecules. So, there are

cavities which are created of the hence so, there are head folic portion sufficient are there. So there are interactions of pollard function in the molecule with the solvent. So, stationary phase is passive stationary phase very passive. So, we have the solvent and the analyte interacting and because of the cavity of hydrophobic resins that was the hydrophobic theory.

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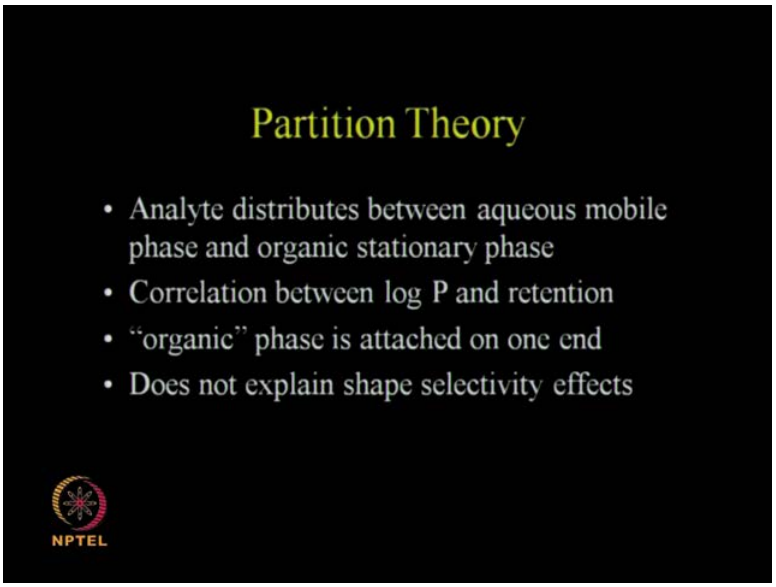

Now, partition theory this is the partition which we are been looking the we are talking about in liquid chromatography these are in extraction of and so on. So, the analyze distribute itself between the appears mobile phase on the organic stationary and so there is a partition. Now, there is a retention of these analytes in the column is corelated to the log P. Log P is nothing but logger then the partition of an analyte between Octanol and water. Octanol to be consider a hydrophobic and water is consider to be is hydro filling. So, more hydrophilic molecules it will be found more in oftener that an in water so, log p will be loge. More hydrophilic molecules at the partition, more in the water layer so, lock p will be smaller.

So, smaller the log P you can change the molecules are hydrophilic, larger the lock p you can say the molecules are hydrophobic. So, there is a correlation it will be log p and in their retention so, if the log p is large that means the hydrophobic then it is get stay put inside the column much longer. If the log P is the more that means the molecules hydrophilic so, it comes out faster. Then you have the organic phase its attached one and it does not a explain shape selectivity effect means this theory does not tell you how why the selectivity have atence because of shape. So, molecular shape does not come in to the picture. It does consider the

hydrophobicity of the molecules nothing else does not bring in the shape fat. Whereas, if we look at the previous theory of hydrophobic there are pockets created in the solvent and depending upon for analyze. So, the shape selected come in to picture there.

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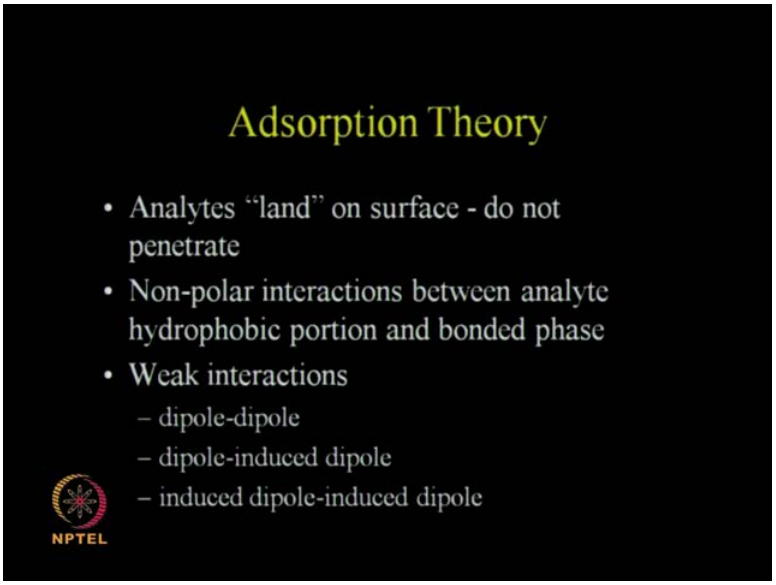

The third theory is the absorption theory. So, what happens the analytes land on the surphase of the stationary phase so, it does not any create. So, there are lot of non-polar interaction that means that means hydrophobic interaction between analyze hydroponic portion and the bonded face. Bonded phase means may be having a c 18 or c 8 or c 3 different types of hydrocarbon bonded to the and solid polymeric matrix. So, there is a non-polar interaction between the and a analyze the hydrophobic portion of the analyze and the bonded space so, the interaction would be dipole dipole, dipole indives dipole, indives dipole indives dipole so and so on.

So, all the forces week interactive forces operating on it. So, you see 3 different theories one talks about how the analytic affects the solvent that is the stationary phase does not come in to picture. The 2nd theory is partition that means the liquid in the stationary phase and the solute in the mobile phase partitions and based on lock pea. The 3rd theory is the absorption were the analyst lands because of non-polar interaction for interaction and between the analyte hydrophobic reason on the bonded phase So, these the three different theory which have been proposed to explain the reveres phase chromatography the reality there could be a combination of all is 3 theories.

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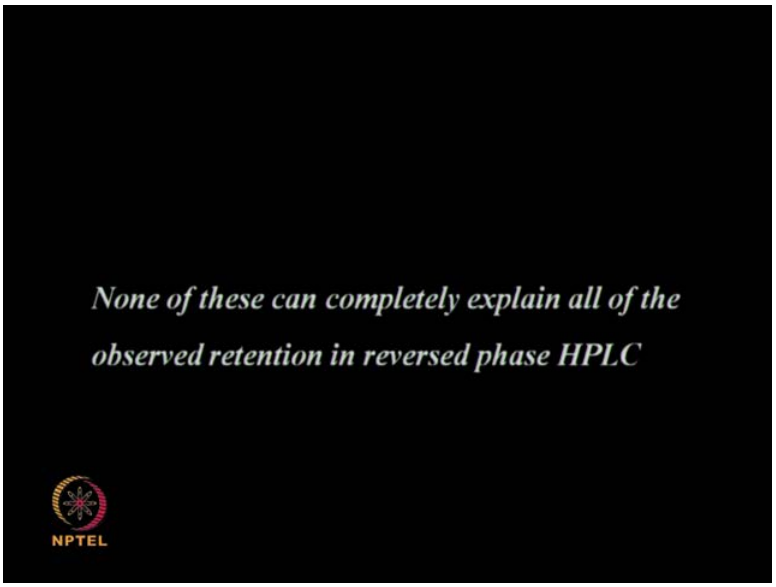

So, none of these can completely explain all of the observed retention in reserved phase H P L C. So its a combination of all these three thing.

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So, what are the parameters that are very important in reveres phase? The solvent strength that is solvent is the mobile phase. Choice of solvent: what solvent do I take? Do I take water do at a water methodol due at a aside ton trail due at a the due at a due at a combination the ph of the mobile phase and then Silanol activity. We will talk about the Silone activity as we go along because have a lot of say a h molecules which really creates lot of unwanted interactions.

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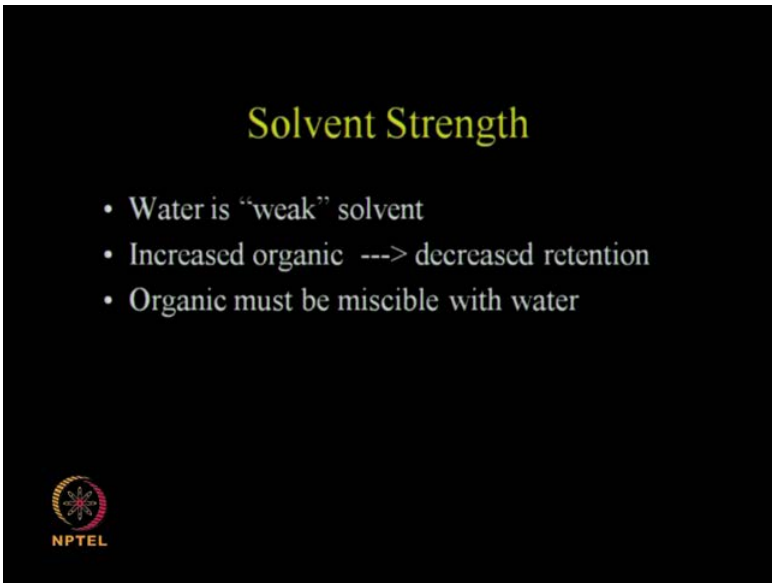

Solvent strength, water is a very week solvent. So as a keep it increase organic then there is a decrease to retention. Organic must be miscible with water that means whatever organic molecule solvent select it should be miscible with water. So, if you keep increasing the organic content then the retention keeps going down because it is become a which changing the hydrophobic nature of the continuous phase.

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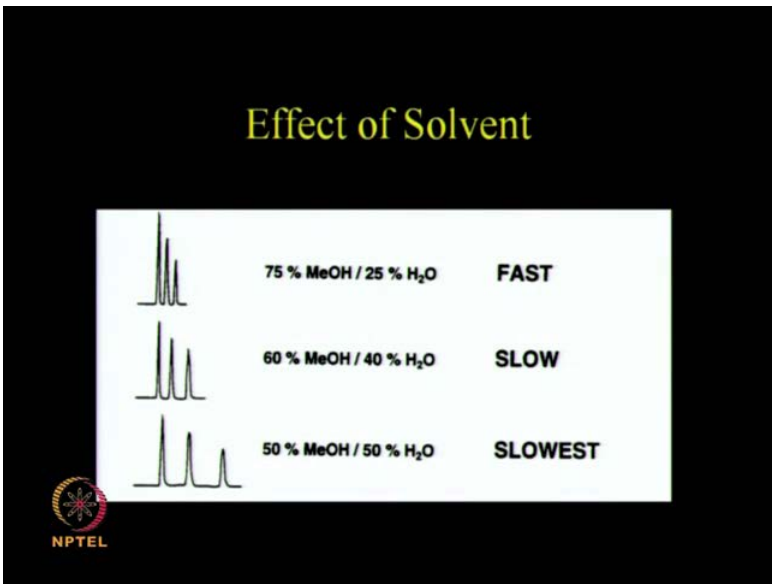

So for example, I take 75 percent methanol, 25 percent water. So, the peaks are very close so it is a fast. Now, I am reducing the methanol amount and increasing the water amount you see and

50 percent methanol and 50 percent water slowest separations are very high why? I have brought in water so, that means I have more hydrophilic. So, the separations are much higher whereas, here I am reducing the water contents that mean I am reducing the hydrophilicity.

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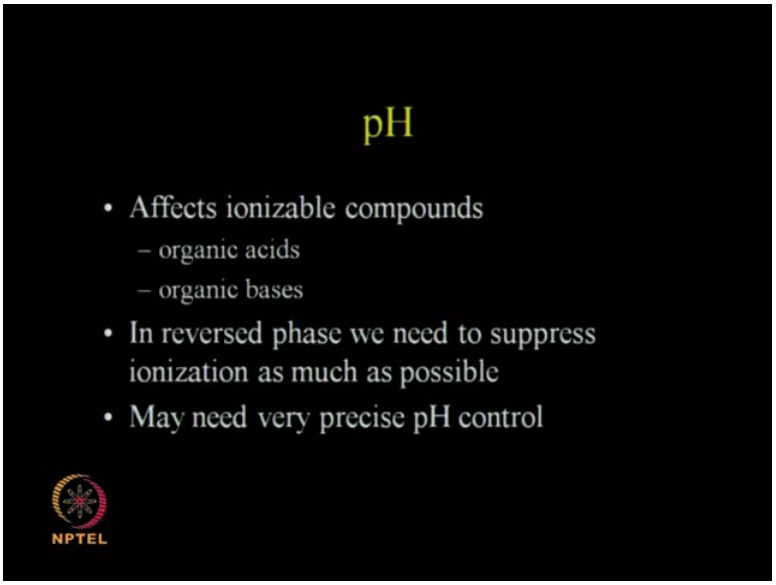

pH generally effect ionizable compounds like organic aside, organic basis and so on. But then we have to two supers their ionizable and as much as possible in a reveres space because one sedges sin ides as you know ionizable molecules the polar so the all diagnose molecules together and he will not get good separation of those polar molecules, because ionized molecules are extremely polar. So, we have to change the pH so that this pH is suppressed. Once he do that then the hydrophobic nature of the stationary phase coming to picture on the separation relatively different polar compounds are enhanced. So, we need to have very good ph control. That is very very important if you are looking at ionizable compounds like organic acids, basis and all that.

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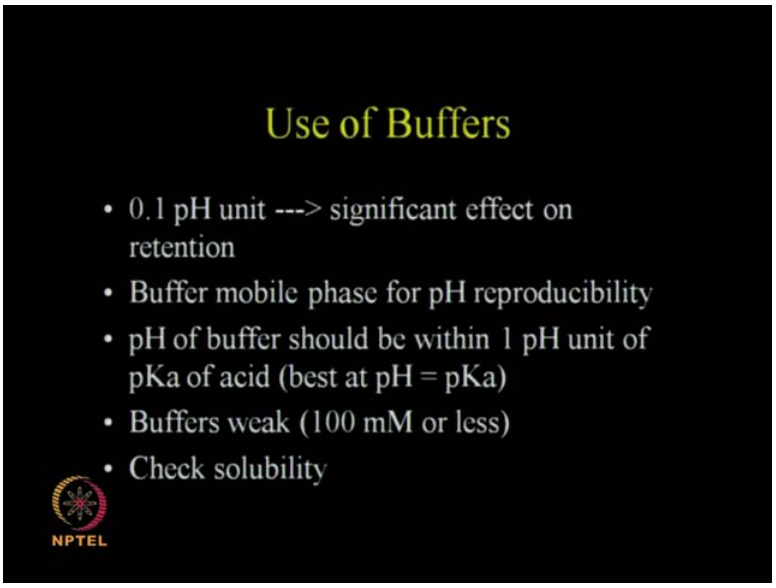

So, you can use buffers control point so 0.1 pH unit significant effect on retention. So, such a small amount will definitely effective your retention. So, buffer mobile phase for pH reproducibility we need to do. So, pH of the buffer must be within 1 pH unit of pKa of the acid so, pH should be equal to pKa for acids. So, we can say buffer are very week if you are using 100 millimolar and less than. But, most of the important thing many to see whether the buffer soluble in your continuous phase that are also very very important. So, for acid you adjust the pH that it is equal to be the pKa of the acid.

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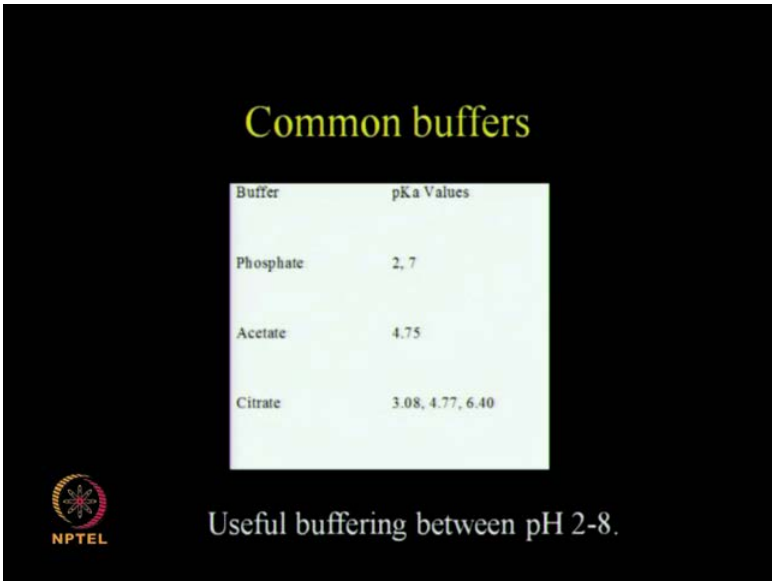

So, what are the common buffers we use? We use Phosphate, we use Acetate, we use Citrate but, then Phosphate we can work at pKa 2 or 7, Acetate at 4.75, Citrate at 3.084 or 0.77 or 6.40 pKa values. Ideally you should buffer between 2 to 8 more on the acidic side so 2 to 8 so, that you use buffer so, that we are able to took your pH in that rate.

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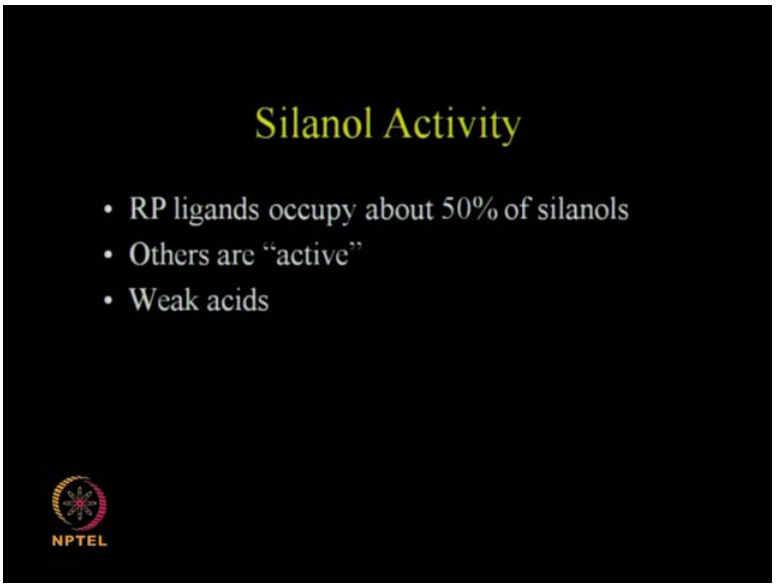

Silanol activity so, reveres phase legend occupy about 50 percent of Silanol that means there are silica on your stationary phase and then put in your long chain hydrocarbon to make the surphase obey. The long chain hydrocarbon could be c 18 or c 8 or c3 or something, but you are not going to replace all the cilice acid SiOH which is extremely acidic. So, if you are replacing only 50 percent then the remaining 50 percent is there which is going to create lot of issues. So, they are active, there week acid.

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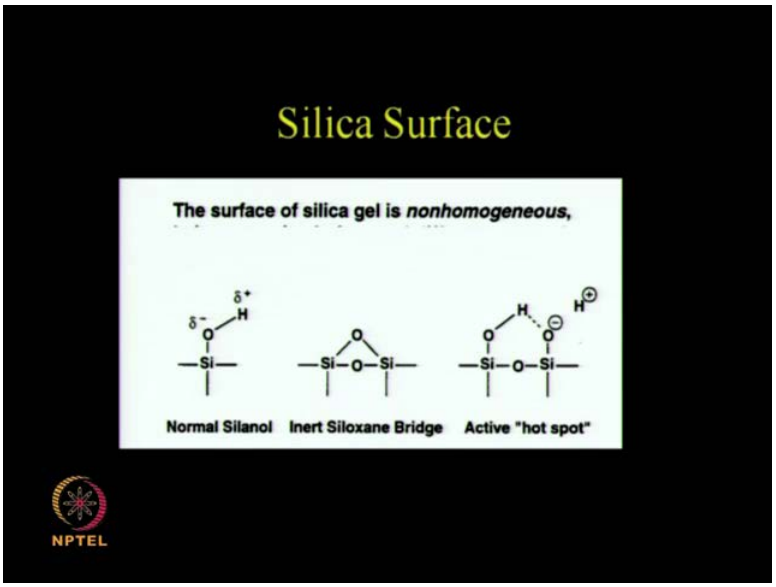

So, there are different types of Silanols. Surface will be highly non-homogeneous so, the silica if this silica next to each other form a bridge like this then this is an inert Siloxane bridge. But normally you will be like this S i O there is a negative charge here, small positive charge here. So, this is going to create problem because there is a small positive charge here. Another problem it can form this type of bonds that is the two adjacent Silanols and H plus can get release. Then you are in big trouble because you have protons in the continuous phase and you have a negative charge on the surface of your stationary phase. These are called hot spots. So, if you have solutes which are also having a certain charges then there is going to be not a hydrophobic interaction, but it is going to be more of ionic interaction. Then you are completely destroyed the hydrophobic chromatographic principle.

So, different types of Silanols and the surface will be highly non-homogeneous. Ideally, I would like to have this situation, but then many of the time you will have like this. But then if you have like this you are in big problem because proton are liberator and you are going to have O minus on the stationary phase. So, we need to understand this and see whether I can neutralize these passivating or neutralization that is what is called before you actually perform your H P L C studies.

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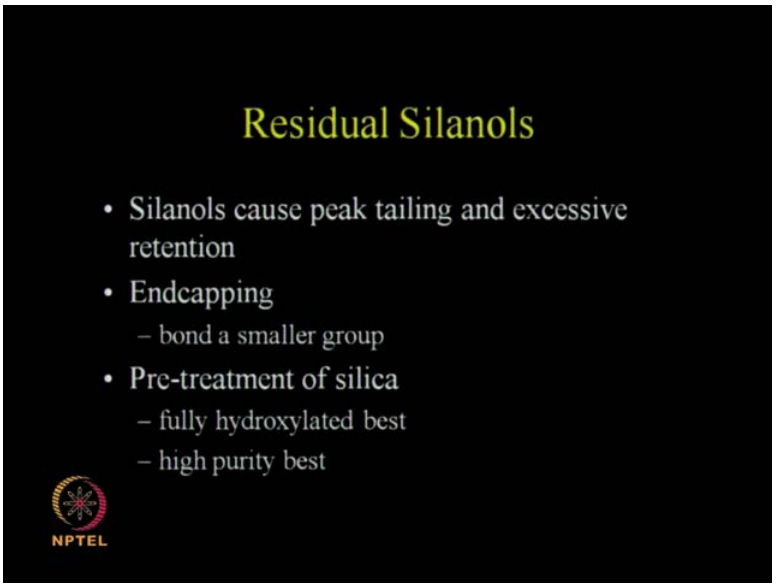

So, what does Silanols do? They cause peak tailing and excessive retention because you have sometimes O minus, we have charged so your molecules solutes get captured and slowly get released. So, we can end cap this molecule using a smaller group. Pre-treatment of silica that means they can fully hydroxylated or we can use very high purity that is the best actually fully hydroxylated or high purity material or we can end capping by having a small molecule. So, this is how you can get rid of it.

So, if you are having a peak tailing or excessive retention you know this is a Silanol problem. So, what does Silanol do? It interacts through hydrogen bonding, it create dipole dipole interaction, it create ion exchange because you have O minus on the surphase and you have H plus which is floating around in your continues phase. No pH's Silanols will get protonated or we can add basic modifier like Tetraethylammonium to compete for the sites. So, you have O minus then we can add some basic material like tetraethylammonium plus type of thing which will go and block those Silanols.

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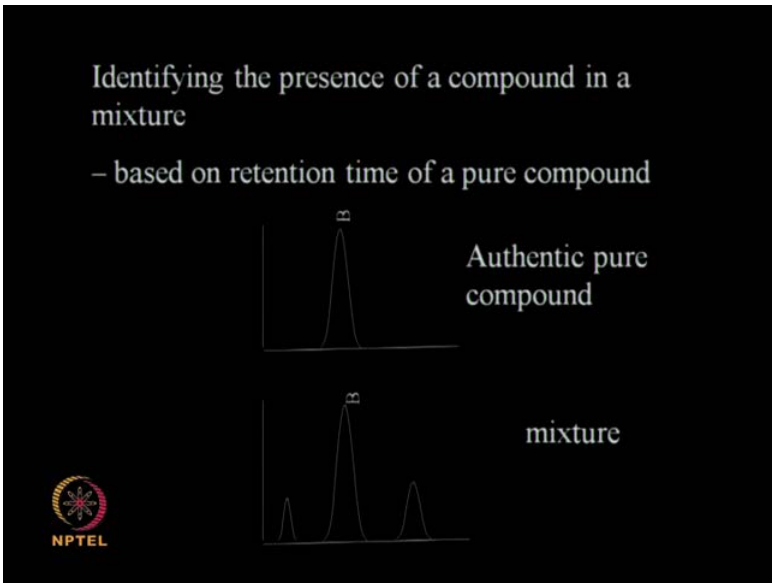

Now, how can you use the HPLC for detecting a compound in the mixture? Suppose, we know what the compound might be? So we know that possibly we are producing a fatty acid ester. So, what we do? We get an authentic pure compound other fatty acid astern and then we inject into the H P L C and note down the retention time and when inject your mixture at the same conditions and if you find a peak at that particular retention time you can be sure that the unknown peak in the mixture would be this particular fatty acid astern.

You can also call something called spiking. So, what do we do is you take this pure compound add little bit to the mixture and then again do a analysis. And if the peak height increases or the peak area increases after you spike it then you can be very sure that particular peak is the compound which you thought it was so, that is called spiking. So, in a spiking what do we do? You first get a chromatogram of your mixture and then you add the little bit of their pure compound in the mixture and see wither that peak area or peak height goes up? If it goes up then you can be sure that whatever the compound which have added pure compound is that particular peak.

So, that is how you identify the presence of a compound. So, if you have 2 or 3 compounds you do the same things for all those 2 or 3. You try to purchase these authentic components and then get there retention time and the pure component retention time and then use that number to check whether peaks are appearing near mixture. That is how you need to do here. Identification and presence of a particular compound in a mixture ok.

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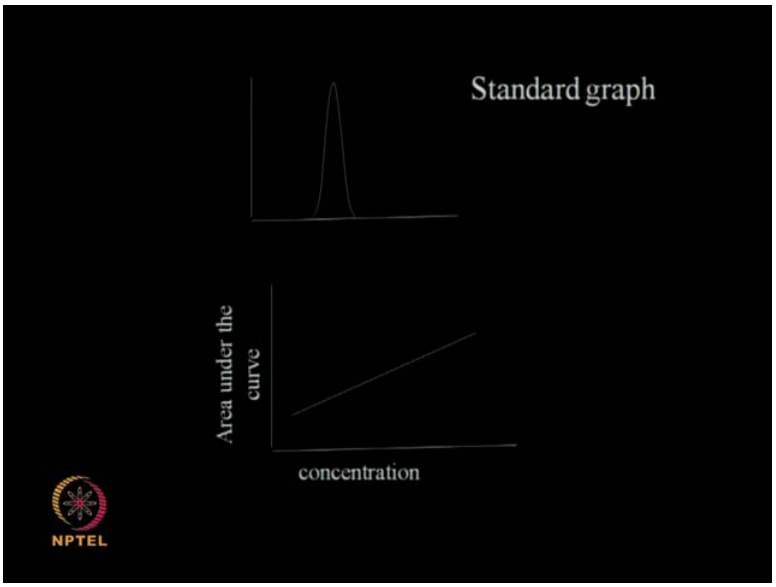

Then how do you quantify that particular component in the mixture? What do we do? you prepare something called standard graph. So, what do we do? Now you know what that particular compound in your mixture is. So you take different concentrations of that compound inject into your H P L C and get the area and the curves then you flat a graph between area and the curve and the concentration. So, once you do that you should get a good straight line. If you do not get a good straight line then that you are not operating in the levier range of the response of the H P L C. So, may be if you are using very large concentration differences you will not get linear respond so, it always good to operate in the linear range.

So, once you do different concentrations of the known compound and measure area under the curve if flat a graph between the area and of the curve and the concentration and you get a straight line connecting these two. This is called standard graph so, if you know the area under the curve for compound in the mixture using this standard graph we can read out what will be the concentration.

This is how you need to do. So, you inject your mixture calculate the area and of the curve for that particular compound in the H P L C chromatogram and then from this graphs you tell what is the concentration of that component in the mixture? So if you have 2 or 3 components then you prepare standard graphs for each one of the component and then in the mixture when you inject you get the areas of each of the component from the chromatogram of the mixture. And

then you read out the concentration for each one of the component using different standard graphs.

So this is how you measure or estimate the concentration of the component in your mixture. So, H P C L is extremely powerful to do both this operations. It can tell you wither a particular compound is present, it can also tell you: how to determine the concentration of the component? Another method by which we can do is called the internal standard method. What do we do in internal standard method? We add a known concentration of a particular new component which is not part of your mixture to this mixture and then see what is the area under the curve of that particular component.

So, by looking at the ratio of the area and the curve of that internal standard with the unknown component we can again measure or estimate what will be the concentration of your compound. So, there we are using something called a standard. A standard is another compound woes concentration is known, which we are adding and by measuring its area under the curve we know the response of that particular component and we try to extrapolated to the response of the compound which we are trying to quantify and then get the concentration of that particular component. That is called the internal standard method. Another approach as I explained is the standard graph. This are the different ways by which you can tell what is the concentration of the particular compound in your mixture?

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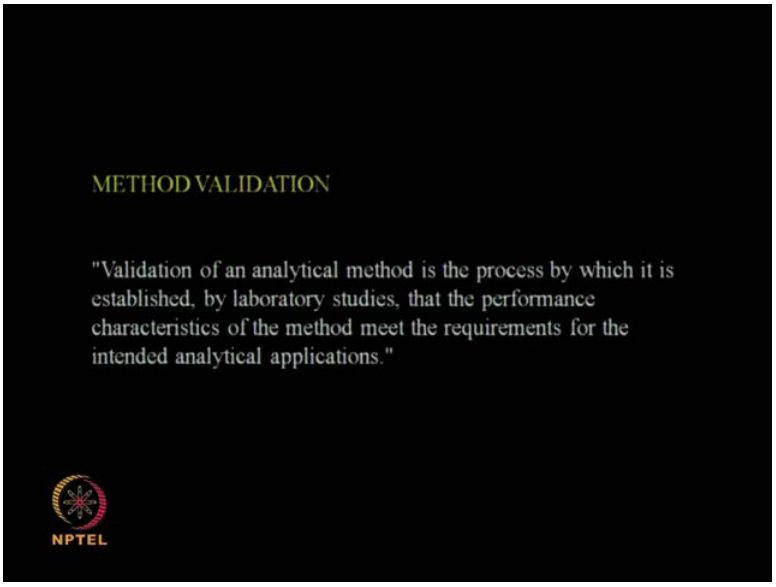

Now, in a HPLC the most important thing is the method validation, because when you developing a method any two validated totally and completely especially, if you are working on clinical trials the F D A that is the food and drug administration is very particular that whatever methods you develop is completely validated and the method validation protocols, the results, the analysis have to be put with the papers when you are applying to the food and drug administration for coming up with the new method.

So, method validation is the process by which it is established by laboratory studies that the performance characteristics of that method meet the requirements for the intended analytical application. That means if am developing a method for measuring a particular metabolite in my mixture at a concentration of 0.1millimoles per litre. Then a method which we develop should be accurate enough to measure that particular concentration that is what is called method validation and as I said in all drug testing laboratories, in all clinical trials method validations plays a very important role. It is most important because the food and drug administration that is the F DA authorities go through this method which we have developed to see whether they are accurate? Whether they are good enough? Whether they are robust to perform the particular task for which you have developed the method for.

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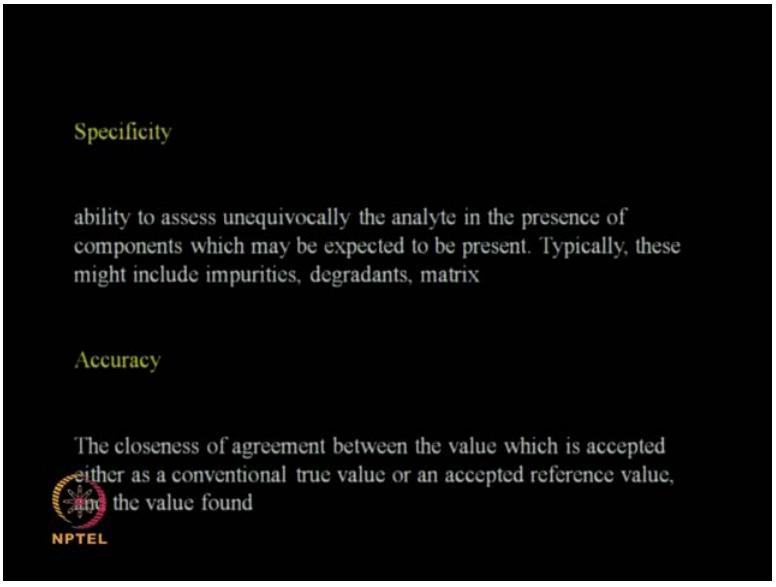

Specificity is the ability to assess without any doubt the analyte in the presence of components which may be expected to be present. That means you may have many components in your mixture, but without any doubt the installine should be able to detect your component of interest.

So, the mixture may contain impurities, mixture may contain degradants, mixture may contain matrices matrixes and so on.

So, you may have many components but you should without any doubt be able to. That means the analytical technique which you are developing should clearly show the component which you are trying to identify. Accuracy is a closeness of agreement between the value which is expected with the reference value and the value which you found. So, you are using the instrument and you are coming up with certain concentration value for a component. How close it with is respected what is accepted or what is the reference. That is what this is called accuracy.

So, when you develop a technique, the technique should be accurate enough and within the tolerance which is allowed. It could be 0.1 percent and difference is 0.01 percent difference is depending upon the type of component you are trying to measure. That is what is called the accuracy. So, above certain accuracy the method which you have developed might not be good enough for the task for which it is indicted for.

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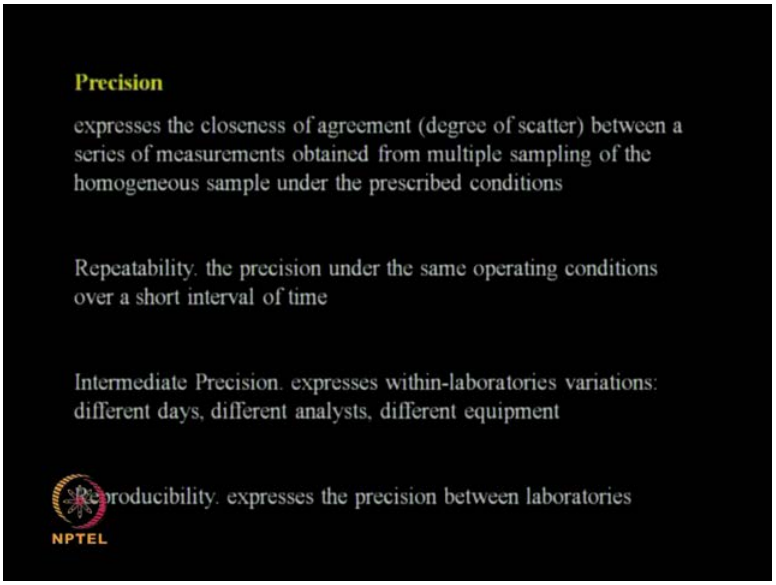

Precision: So if I am measuring a particular component in a mixture, if you do it many many times how it close or how the degree of scatter is? That is that is what is called precision. So, if I do it 3 or 4 times and am I getting a same result? Or am I getting randomly different results far apart from each other. So, if I am doing that then I am in trouble because my method is not repeatable so, which is very dangerous so each time I may get different different answers.

So, precision tells you if I do it many times how close each one of these measurement is. So, in precision you have two things: one is the repeatability, other is reproducibility. So, in repeatability if I am repeating it many times how close they are? In reproducibility if it is done in different labs then how close there is a result, that is called the reproducibility and now a days in clinical, trails as you know drugs are tested in different parts of the world, different continents, different hospitals and in each place they have measurements.

They may use the same bio acid or analytical tool they should get the same answers otherwise they are in big trouble. So, reproducibility is also very very important. Repeatability is important because if I repeat the same assay 3 or 4 or 5 times I should get almost the same answer. Then you have the intermediate precision that means within laboratories, variations on different days different analysts, different equipment and so on that is called the intermediate precision. So, precision and accuracy you can see both are very important. Precision is when I repeat many times am I getting the same answer or am I getting different answer. Accuracy is I get an answer how close is to the real answer or correct answer or expected answer? That is the accuracy.

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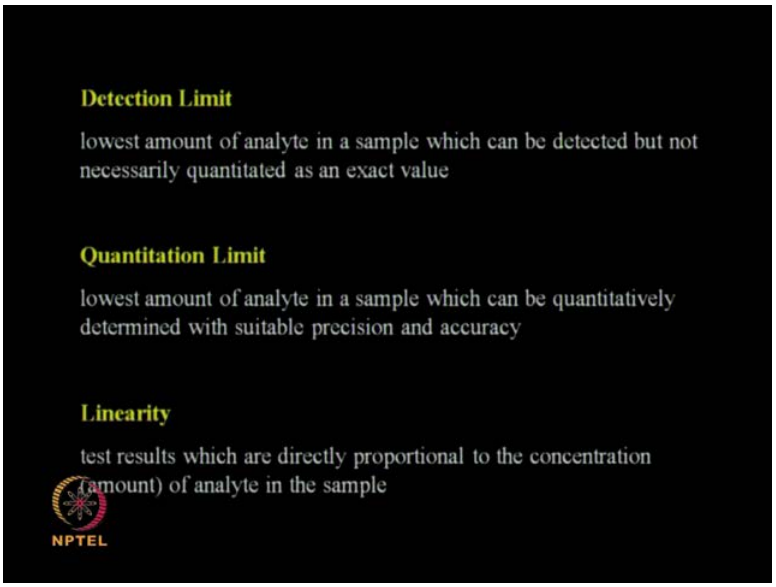

Detection limit is the lowest amount of analyte in a sample which can be detected by the instrument. So, I may have 0.1 micro molds can I detect that with my instrument? That is what is called a detection limit. But that is not necessarily the quantitation limit, because quantitation limit is exactly can I measure quantitatively the amount? Quantitation limit will be generally higher than the detector limit. I may be able to detect, there might be a small peak coming out in

a chromatograph but I will not be able to quantitatively tell how much it is. So, quantitative limits will be slightly higher than the detection limit.

So I need to know when I develop a technique can I quantitatively determine 1 micro gram per m l of a particular impurity, because the F D A may be interested to know whether the technique has a good quantitation limit or is it 10 micro grams per m l concentrations that can be quantitatively determined. Then we have the linearity as I talked about in the standard drafts the response and the concentration of the solute should be linearly related. Then only we can develop a linear regression relation between the concentration and the response. We do not like to operate in the nonlinear range so, what is the linearity range? That is very important and so if your concentration are very high you may like to dilute it so, that you operate within the linear range. So, generally the analyte concentration was the response we would like to operate in the linear range.

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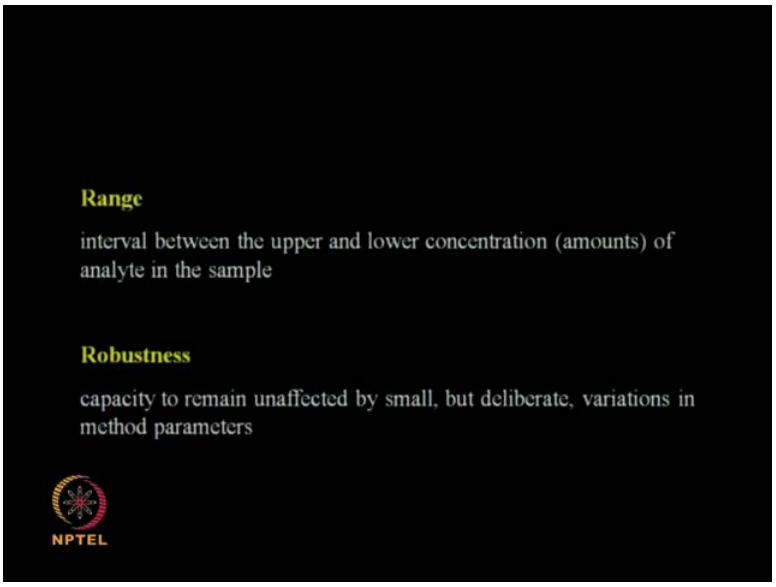

That is meant by us as the linearity. Then range is the region that is the lowest and the upper most concentration division in which I am operating because none of the instrumental techniques or accedes or the equipments can operate an entire range of concentration. So, they will always have certain range within which you can operate. So, that range also as to be specified. Robustness is how stable is a technique even when there are small small variations in your methods. I make a small mistake during preparation of the sample or there are small mistakes in the solvent flow rate. So is the method very robust or is it going to be changing

dramatically when you have small mistakes incorporated, that is what is called robustness. And ideally we would like to have a robust technique H P L C technique.

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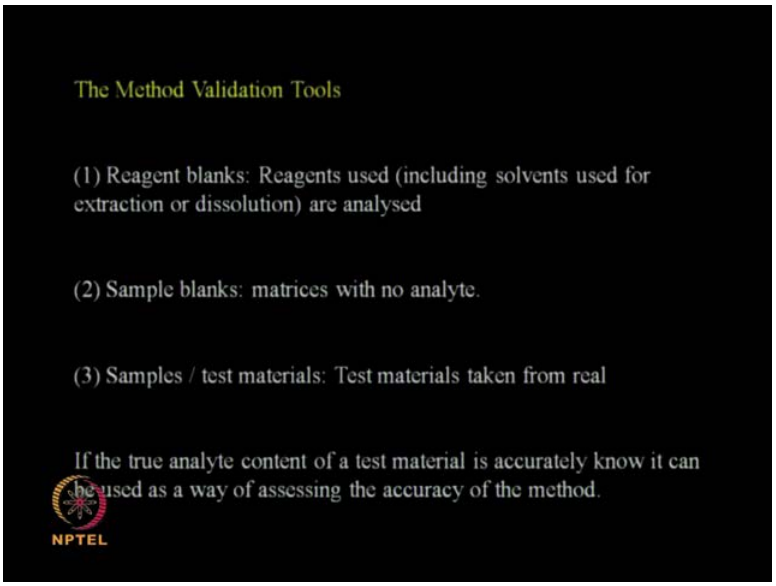

So, what are the method validation tools? We may have to use reagent blanks that means if I am using solvents for extractions or dissolution I need to test those also and see whether there are any peaks in a chromatogram coming out because of the reagents. Sample blanks that means if I am using any matrices without if I analyte I need to perform an H P L C and see whether they are giving any peaks and whether those peaks are going to interfere with my solute. Test materials that means we may take materials which are not real material, but they may be test materials. So, these are the various method validation tools which we need to adopt to before we can tell the method which we have developed is good enough actually.

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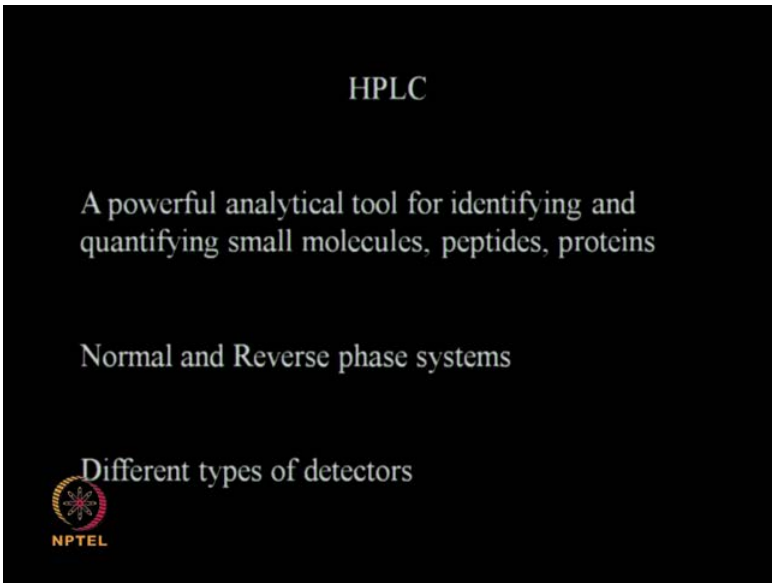

So, to conclude we have spent couple of lectures on the high performance and high pressure liquid chromatography. It is extremely powerful tool for identifying presence of a particular analyte, it could be a small molecule, it could be a metabolite, it could be a peptide, it could be a protein, it could be a amino acid it is become extremely equitize. It can be used for identifying, it can be used for quantifying. Two techniques: normal phase and reverse phase chromatography. The reverse phase makes use of hydrophobic stationary phase and a hydrophilic continuous phase and like water, acitonitride, methanol

So reverse phase chromatography is become a extremely ebiviquators because the amount of solvent, type of solvent which we use is very cheap. There are a plethora of detectors starting from u v, diode array, refractive index, electron light scattering ,mass spectrometric detector and so on depending upon the type of molecules you are trying to analyze we need to go for different detectors. Then I talked about the concept of method validation, preparation of the standard graph and how the method validation is extremely important especially if you are developing it for the food and drug administration or getting approvals for drug testing or approvals for clinical trials and so on. So, H P L C is a most powerful tool which may which one makes use of and it is becomes part and parcel of the research, the quality control, the analyses, the clinical trials and drug development and so on.