Downstream Processing
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Lecture - 33
HPLC

Today, we are going to talk about the HPLC. HPLC is an analytical tool, which is used for identifying unknown metabolite or a protein or a peptide as well as can be used for quantification. So, HPLC has been the short form of high performance liquid chromatography or high pleasure liquid chromatography.

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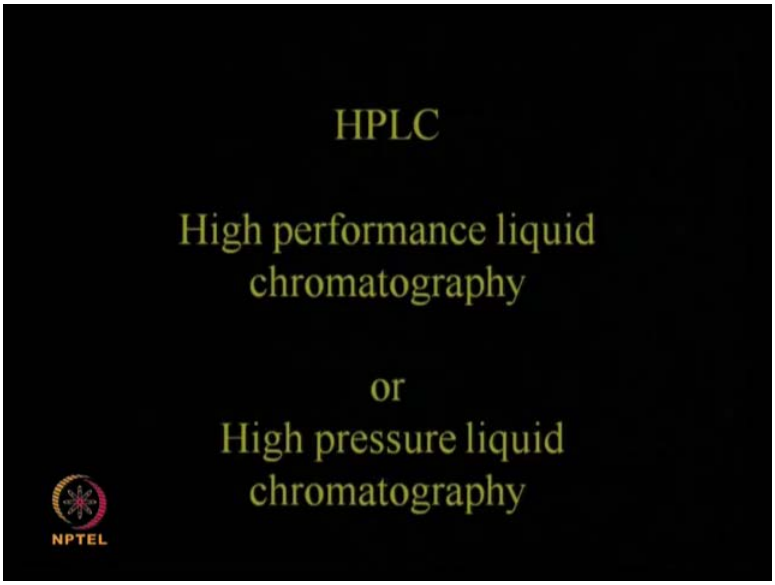

So, HPLC also uses the techniques of chromatography. So, you have different types of HPLC's which operate using different principles as we discussed the various principles of chromatography. So, the separation is achieved either through hydrophobic interactions or polar interactions or ionic forces and so on actually. So, we are going to look at the HPLC today which is a very important tool which can be used for both small molecules as well as large protein as well.

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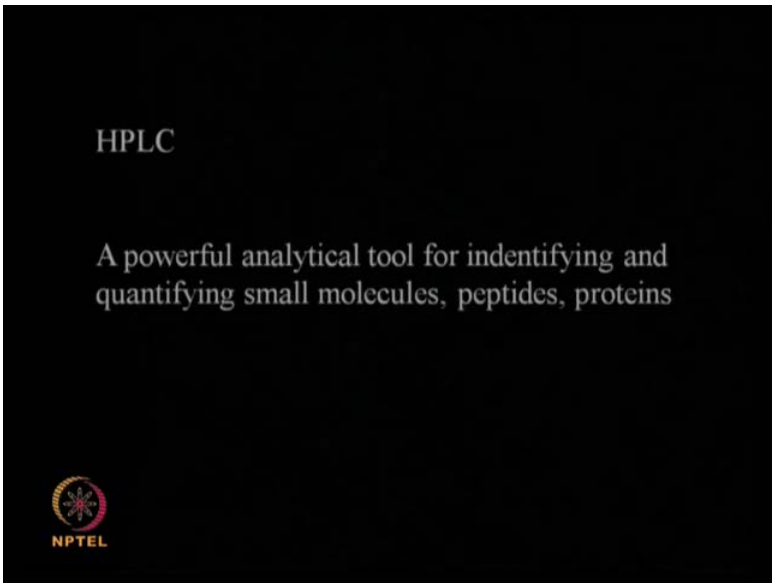

So, it is a very powerful analytical tool for identifying and quantifying small molecules, peptides, proteins, metabolides, drugs, natural chemicals, natural products, phyto chemicals and so on actually. It is extremely powerful and you achieve very good separation by changing the composition of the mobile phase for changing the stationary phase and so on actually.

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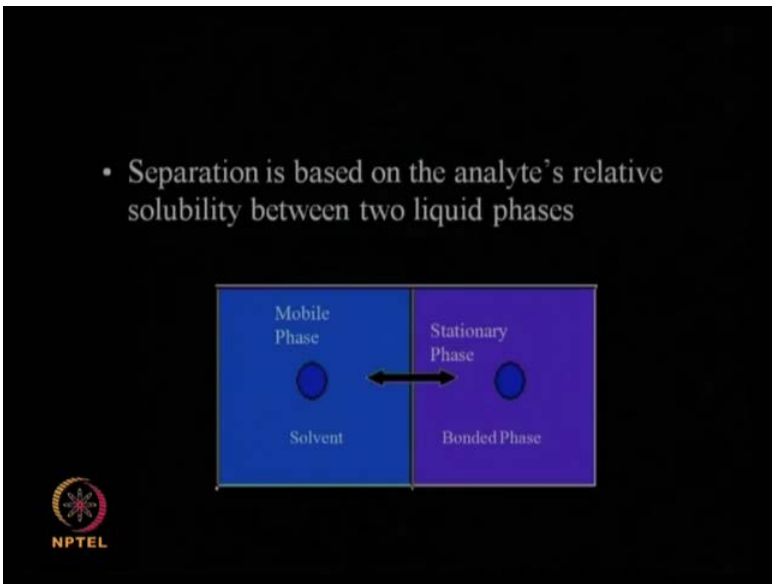

So, what happens in HPLC? You have a set of a solutes and solute partitions between the the stationary phase and the mobile phase. So, it is basically relate to solubility between

two phases actually. So, the solvent which is you are a continuous phase or the mobile phase and you have the bonded phase of the stationary phase.

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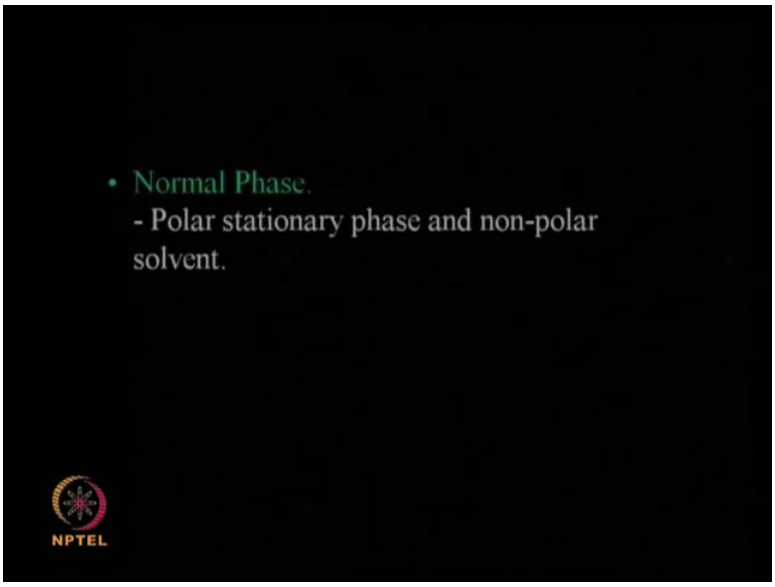

So, you're compound gets equilibrated between these two phases. And the separation happens because of forces that act on the solute and hence you have a equilibrium process taking place. So, we have two types of HPLC's, the normal phase and the reverse phase. So, what is a normal phase? Normal phase has a polar stationary phase whereas here reverse phase has a non polar stationary phase. So, in a normal phase the stationary phase is a polar or hydrophilic compound and the solvent is a non polar.

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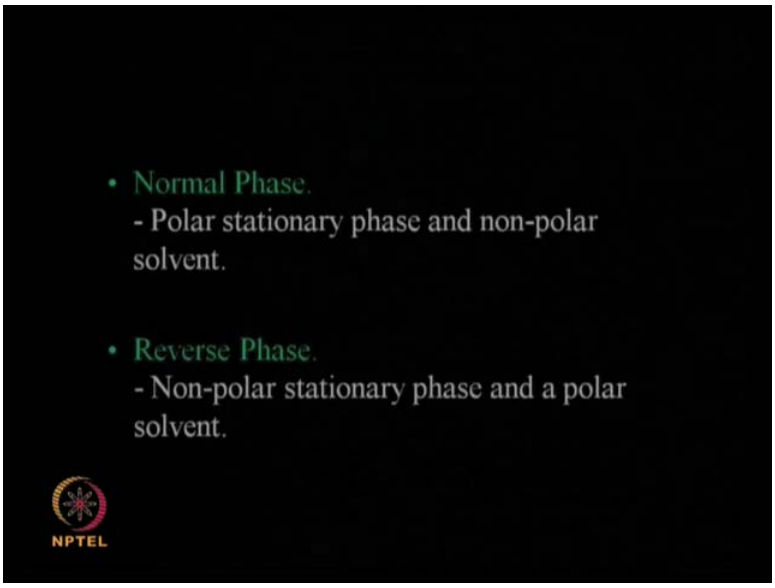

Whereas in reverse phase you have a non polar or hydrophobic compound on the stationary phase and we use the polar solvent. So, nowadays the reverse phase chromatography has become very popular whereas, originally we had the normal phase chromatography being that popular. That means in normal phase you have hydrophilic stationary phase. So, hydrophilic compounds get adsorbed onto the hydrophilic stationary phase. Whereas in a reverse phase hydrophobic compounds get adsorbed or get more partitioned and hydrophilic compounds travel faster.

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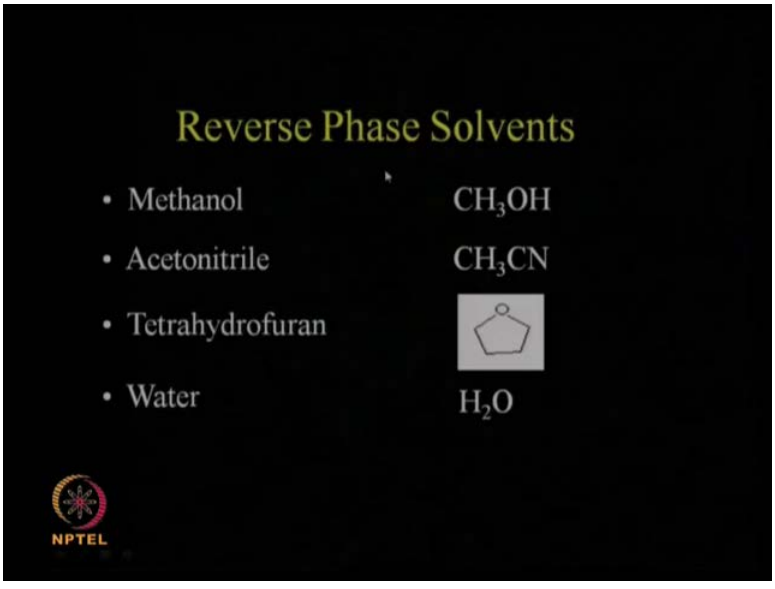

So, in reverse phase what are the solvents used? As I said in reverse phase the stationary phase is a hydrophobic compound. So, this the continuous phase is a hydrophilic for a polar. So, you use methanol, you use acetonitrile, you can use that tetrahydrofuran. Tetrahydrofuran is also called THF or you use water or even combinations of these two. Sometimes we use acetonitrile water combination if you want play around with the certain dielectric constant or other parameters.

So, if we look at this solvent in a reverse phase, predominantly you will be using water and water is very cheap. That is why reverse phase chromatography has become very popular whereas, if we use a normal phase chromatography, this stationary phase is polar like silica for example then you have to use a hydrophobic solvent like hydro carbon. So, that is very expensive and normally in this type of HPLC systems the amount of solvent which we use is quite a lot. So, the solvent cost is extremely high and then generally we try to recover the solvent.

In a reverse phase chromatography we use acetonitrile water and so you can use predominantly water and small amount of acetonitrile. So, you will loose only small amount of acetonitrile. So, operating cost may reverse phase chromatography is much less when compared to the operating cost in a normal phase chromatography. And sometimes we also use three solvents.

As I said if you want to play around with the dielectric constant in separations we sometimes use say methanol, acetonitrile, water and so on actually. We will look at some of those solvents. As we proceed in scores. So, what are the columns used? Suppose, you have solid support usually about 10, 5 or 3 micron silica or polymeric particles.

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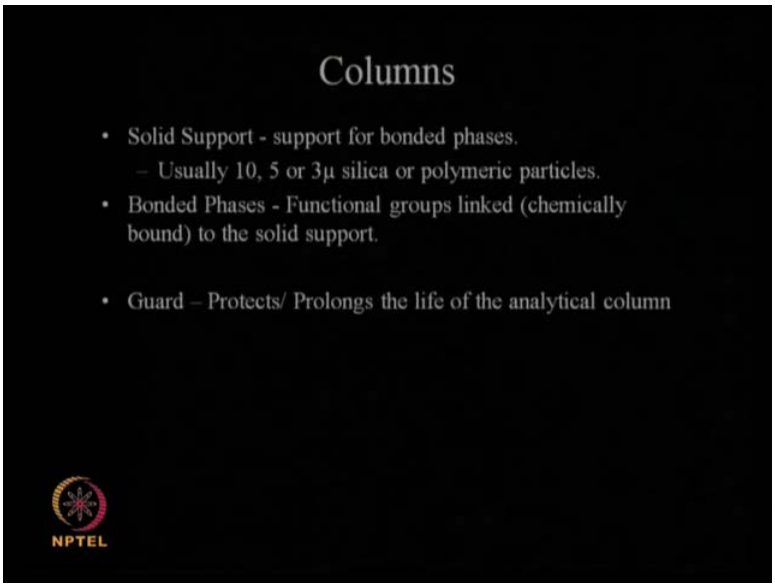

So, on top of that we have bonded phases, functional groups linked. The functional groups are covalently linked to the solid support. You also have a guard column; this is a small column, which is placed before the original large column. So, this guard column acts as a filter so it captures particles, it captures polymeric materials and other unwanted materials. Thereby, it is prolonging the life of the analytical column. After sometime after about 10,000 hours of use something you can throw away the guard column, replace it with another guard column.

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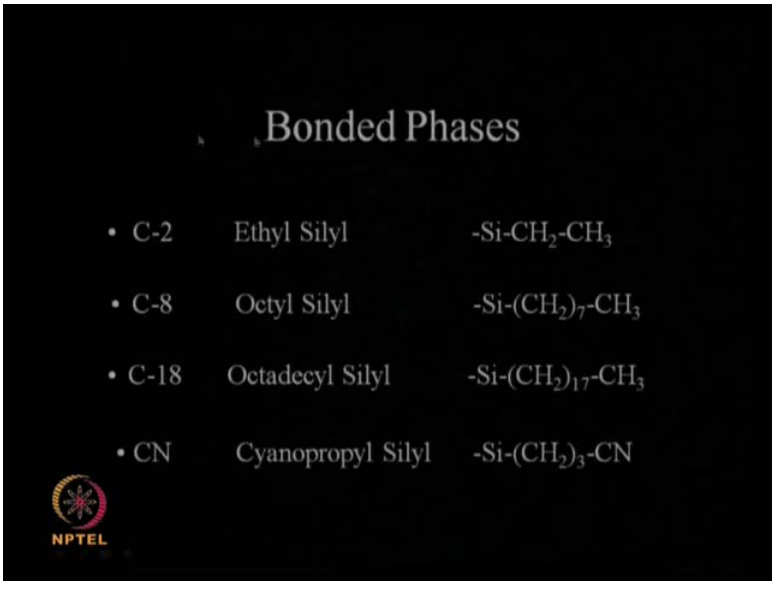

So, what are the bonded phases we use in a reverse phase chromatography? We use the ethyl silyl. So, the ethyl is a hydrophobic or you can use octyl silyl or you can use octadecyl silyl that is C 18 or we can even use cyanopropyl silyl. So, what we have? We have ethyl or octyl or octadecyl, so as we go down the series we are making it more hydrophobic, unhydrophobic. So, that is the advantage of it and C 18 type of column having become extremely popular and it is become (( )) that means it is used in quite a lot of applications practically, separation of many fighter chemicals or even organic metabolites.

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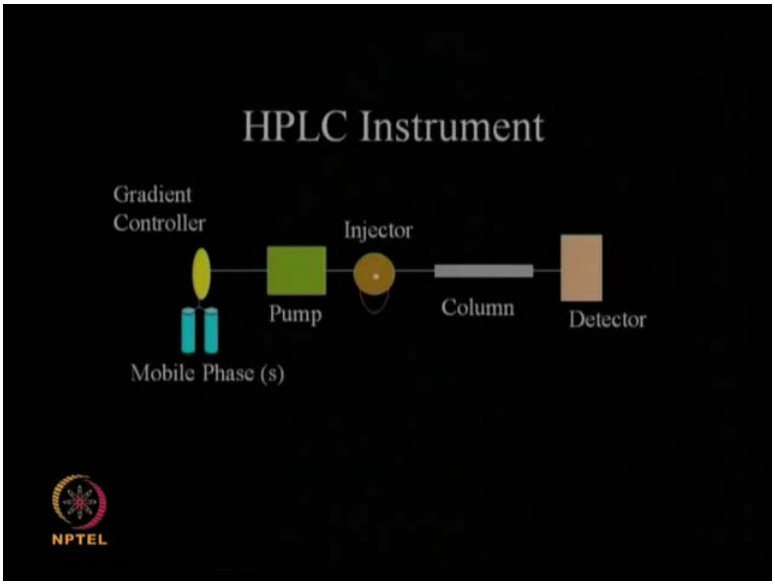

So, typical set up of an HPLC. So, we have the column here. Column will be in the order of several meters then we have a pump here and we have several tanks having various mobile phases. Either, we can run one single mobile phase that is called an isocratic system or we can have two mobile phases. We can shift from one mobile phase to another over a period of time

So, that we get some sort of a gradient which can improve the separation efficiency. So, there may be one pump if it is a isocratic or we may have two pumps pumping two different solvents. So, we have the pump and then there is a injector, that is where you are in injecting you're solute mixture. Then finally, you have the detector. There are different types of detectors that are used in HPLC. Depending upon the type of component you are separating we can have refracting index detector, we have UV

detectors and we have diode array detectors, light scattering detector, even mass spectrometric detectors.

So, you can have a HPLC mass spectrometric connected to each other, that is called LCMS. So, mass spectrometric will detect the mass of the anilide that is coming out. And the LC or the HPLC does the real separation of the various components. So, there is a typical setup.

The pump is the most important in the sense it has to deliver very high pressure may in HPLC the pressures are extremely high 30 40 50 bars because you are using very fine particles in micron size. So, the back pressure developed is also very large. So, we are going to look at different types of detectors, there advantages and disadvantages during the course of this lecture.

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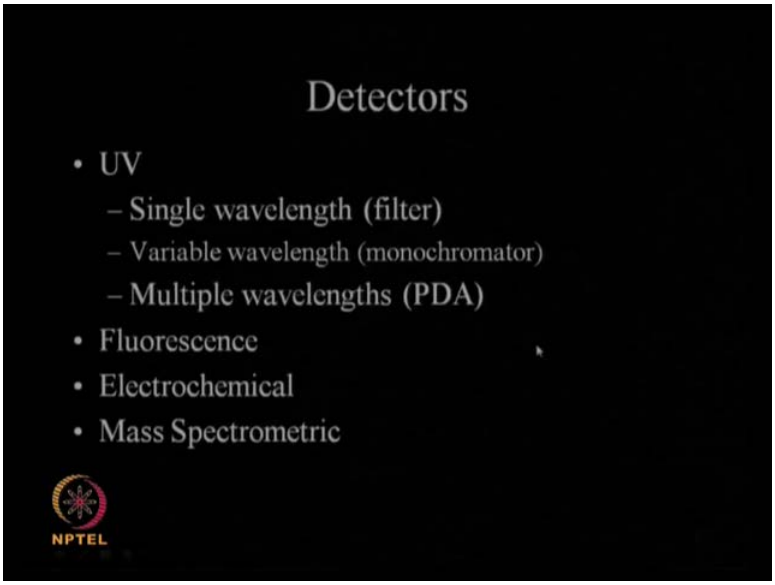

So, you may have a UV detector that means a single wavelength UV detector. Exactly matching one particular wavelength, if we know the components and come at this particular wavelength we just have a single UV detector. It is very cheap or we can have a variable wavelength detector using a monochromator or you have a multiple wavelength detector.

So, these are more expensive than a single wavelength. You can have a fluorescence detector if you know the compounds are fluorescence. We can have a electrochemical

detector, we can have a mass spectrometric detector. So, lot of detectors depending upon the type of compounds and how and they behave.

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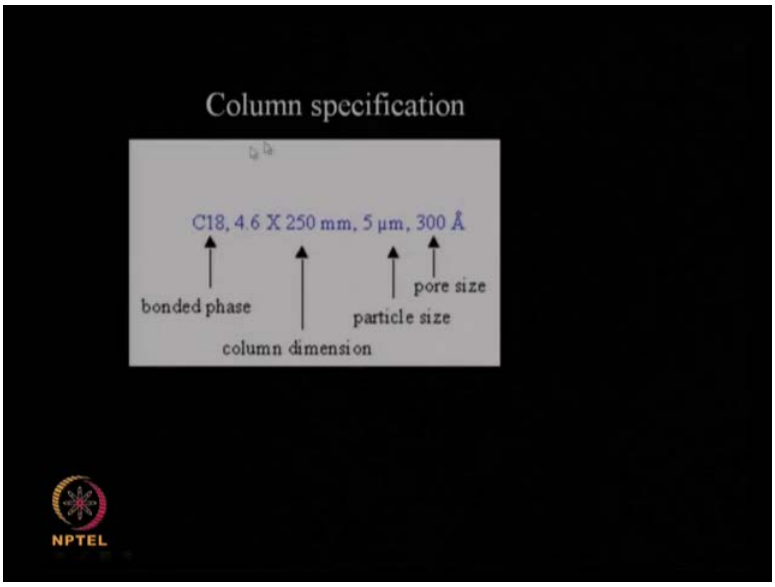

Typically, a column specification look like this, you know the C 18 column. This is the column dimension which are 4.6 meter long column, 255 which are particle size 5 micron and we have 30 angstrom pore size. That means the pore size of the particles which are supported are 300 angstrom's. This is the typical specification for a column.

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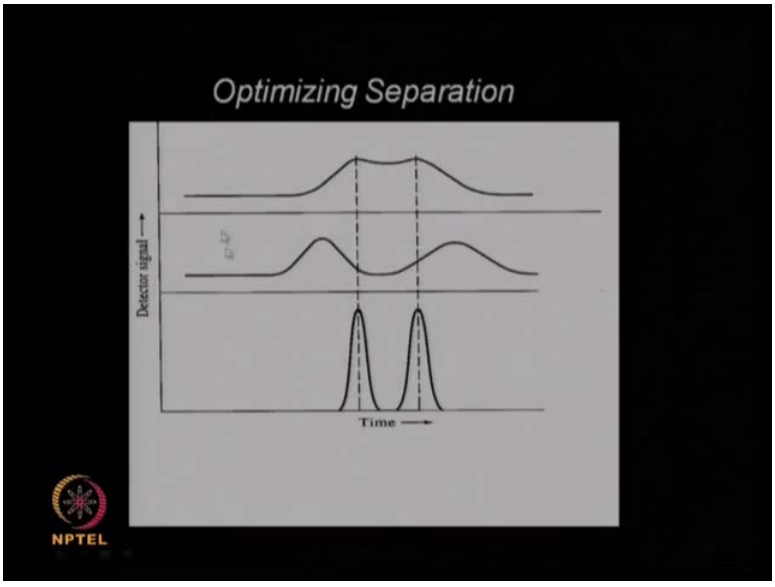

Now, the output from HPLC will be a chromatograph. It will be like a Gaussian distribution or normal distribution and so on. But if you have multiple components and if you're separation is not very good you may have peaks coming out like this. This is a peak, this is another peak, but both are over lapping so much we cannot differentiate the area of this peak or area of this peak or you may have a reasonable separation. But still this is not good separation.

Again, you have some overlap in this region and this is the best separation one hope for. You get a very good base line separation these two components are well separated.

So, we can measure the area under this curve, we can measure the area under curve independently without any error. So, we can determine what is the concentration of this species or what is the concentration of this species. So, ideally one would like to have the this type of separation, but one may end up like this and this is extremely poor. And imagine if you have any components you are going to have many peaks overlapping, some of the overlapping, some of them well spread out.

So, you the challenge is to separate each one of the component and clearly at the base line, so that you can measure the area under the curve and hence, the composition of the particular component of interest. So, the x axis you have the time and the y axis will be your detection signal. So, this is called the retention time that is the time at which we get the maximum of this particular peak.

So, the retention time is very characteristic of a particular component for a given HPLC hardware as well as given solvent and solvent fluoride. If we change solvent fluoride you are going to change your retention time or if I use different column I am going to get different retention time.

So, the retention time is very, very characteristic in a HPLC system. So, if I want to detect a compound and if I know what the compound probably is, what do I do? I get a pure authentic sample from a manufacturer injected in HPLC and see the retention time and then I inject the mixture and I see compound coming out at the same retention time and I can tell probably this particular compound is present in that mixture.

So, that is how we do identification of components. So, suppose we are looking at 3 or 4 and what we know and what we expect, it will be there in my mixture. I will buy those 3

or 4 authentic samples from a manufacturer, inject each one of the independently and check its retention time. And when I inject the mixture and if I get those 3 or 4 peaks at those retention times then I can tell that my mixture contains these components. So, that is the identification part in HPLC. How about quantification? In a quantification what we do? We inject authentic sample at certain concentration and we identify the area under the curve.

So, using that relationship between area under the curve and concentration I can tell how much concentration of another component is present in the mixture by measuring the area under the curve.

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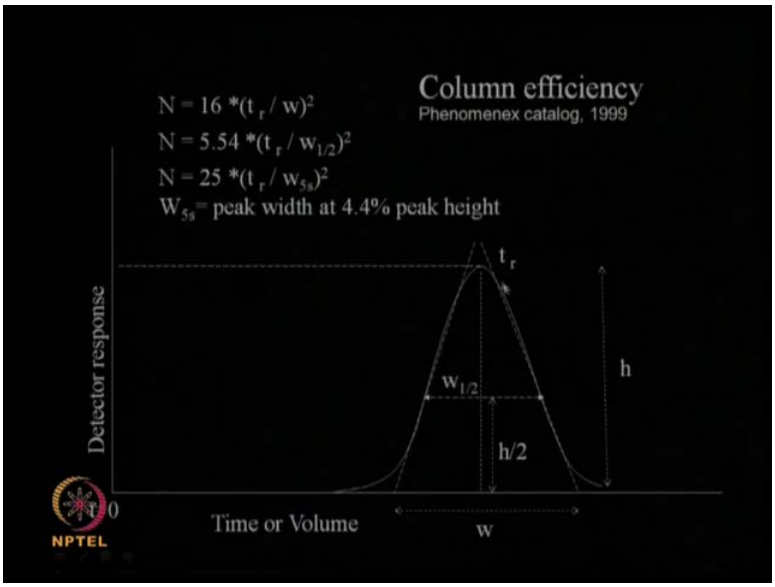

So, it typically the output from HPLC will look like this. You will have nice looking Gaussian distribution. We talked quite a lot about Gaussian distribution and the type of equation a normal distribution will have and so on. So, we can apply those knowledge and those equations in HPLC as well.

So, normally you will get a nice looking Gaussian distribution and this is called the retention time and this is called the height of the peak and this is called the half the height and this is called the width at half height. So, here this is called the width of the peak which is done by drawing two parallel lines.

You get the width at the base and the width at half the height. That means you will check what is the half the height of this and then measure the width. That is called width at half by. Now, there is a parameter called theoretical plates just like we studied theoretical plates of the chromatography. Here also, you have the concept of theoretical plates and the theoretical plates could be estimated by measuring the retention time as well as either the width at the base or width at the half maximum.

Theoretical plates is a measure of the efficiency of the HPLC column. So, if a column has large number of theoretical plates as against another column which has small number of theoretical plates we can say the one which has large number of theoretical plates to be more efficient in separating components.

So, these are some formulae which can be used to measure the theoretical plates. N is the theoretical plates, t r is the retention time, w is the width at the base. So, you have N equal 16 into t r by w whole square or we can use these equation if you are measuring w per half.

That means if you are measuring the width at half maximum then we use instead of 16 here you will get 5.54 or there is another equation, this called n equal to 25 into t r by w 5 s whole square where w 5 s is the peak width at 4.4 percent of the peak height. If this is the peak height 4.4 will come somewhere there. So, this width will be like this, you know big width.

So, if you have that then you have to multiply by 25. So, w 5 s will be width somewhere here, whereas w is the width when you draw two tangents to this Gaussian distribution. So, we can use different equations to calculate the number of theoretical plates and there will be small differences depending upon the equation, which we use. Because although we say this is a very Gaussian looking distribution which never show, there will be small differences in its normal distribution shape.

So, the N which will be calculate from this equation or this equation or this equation will be slightly different. But we can use the same equation, if you want to compare different column. So, that we do not get confused with the value of N which we get. So, all you have to do is inject the sample, determine its retention time, determine its width at half maximum, use this equation and calculate your number of theoretical plates. Then if you take another column do the same job, get the number of theoretical plates. So, we can use

this equation to compare quantitatively between two different columns and say which column is more efficient in separating a mixture and which column is less efficient.

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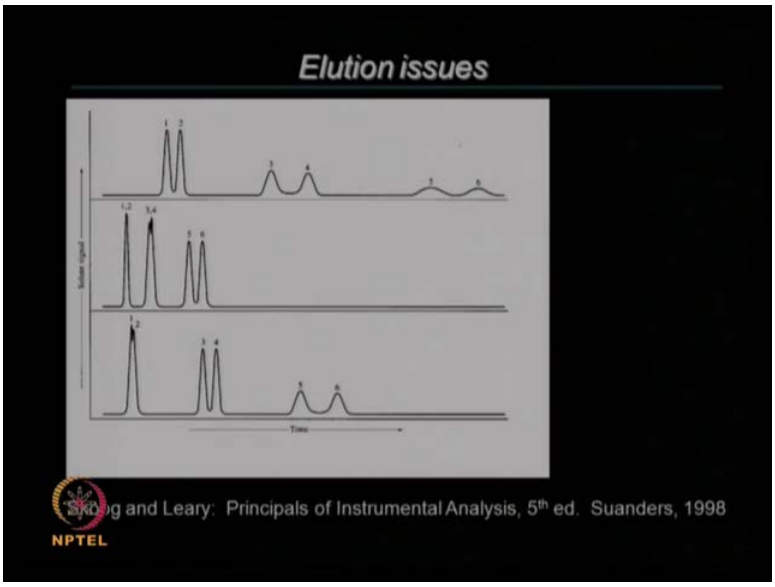

Now, there are many issues if you are performing in HPLC, it is not so easy. This is, this example is taken from a book, Skoog and Leary Principles of Instrumental Analysis. You see we are having six components. So, we are getting two nice peaks and after sometime we get slightly broader peak and after a very very long time we get extremely shallow peaks, peak number 5 and 6.

So, these peaks maybe coming at very long time, number one. Number two, there are so broad, area under the curve when we measure there will be lot of errors. So, to overcome the problem I may change the solvent conditions that means I may change type of solvents.

When we do that I may get 5 and 6 closer, these are nice and sharp peaks. But then you see 1 and 2 has overlapped and 3 and 4 has overlapped. We cannot differentiate 1 and 2, but we can slightly differentiate 3 and 4. So, again we play around with the solvent mixture and so on. 5 and 6 look reasonably good, 3 and 4 very good separations, but still 1 and 2 is not very very good separation because there is a large overlap. So, if you are measuring the area under curve for 1 and 2 we are going to have large error. So, again this is also not very good.

So, we need to again work on the solvent optimization until we get reasonably good base line separation for 1 and 2. So, these are some issues we call it elution issues. So, we need to play around with the various solvents in the continuous phase. That is very very important. There is another chromatography which we use quite a lot in synthetic organic chemistry and I also talk about it for the past 10 lectures. That is called thin layer chromatography.

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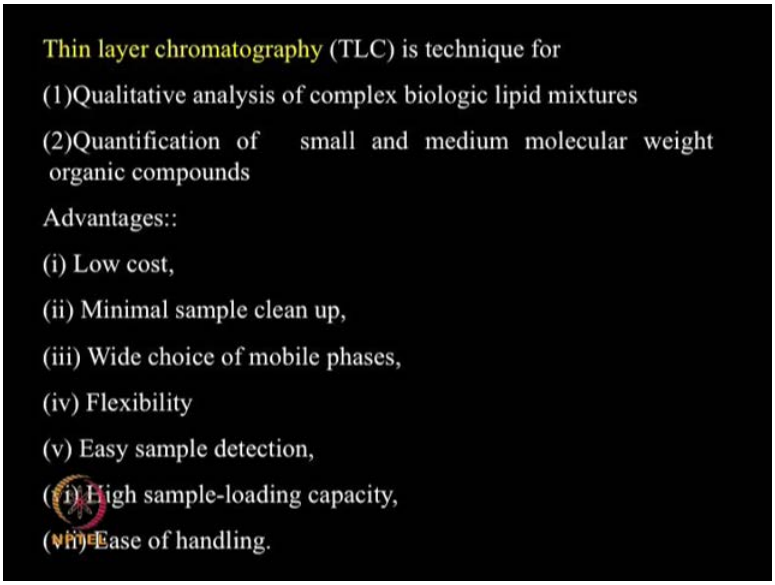

So, we can use it for qualitatively looking at mixture of biological or small molecule, chemical components. The advantages are it is a low cost, you need very little sample for that, we can use different mobile phases to perform, it is very flexible, very easy simple detection.

We can even go for high sample loading. Ease of handling is all good. So, if I am doing synthetic organic chemistry where I am performing a reaction a plus b and I want to know whether c is formed or d is formed, all I have to do is random thin layer chromatography and see weather I get new spots. So, thin layer chromatography is based on spots which I find on the plate, chromatographic plate. So, that is the main advantage.

We can monitor a reaction. Suppose, I have a raw material and I am slowly getting a product. Initially raw material will give one spot, as the product is formed I will get two spots. One for the raw material as well as one for the product, after sometime when the raw material completely gets converted and the raw material spot will disappear and I

will have only the product spot. So, beautifully we can monitor reactions. So, organic chemists use this TLC technique very extensively, practically every day for all their synthetic organic chemistry and synthesis work.

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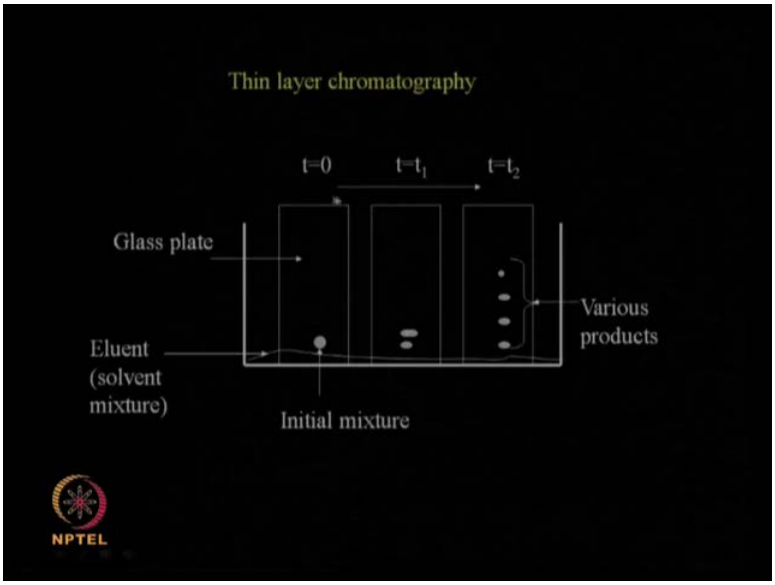

So, how does it work? So, you may have a glass plate and a coated with silica on the glass plate. Then I put a initial mixtures spot and then take a solvent which is the eluent. So, now it is a silica. So, it is a normal phase chromatography, silica is a hydrophilic compound or a polar compound. So, I have another solvent which moves as it travels upward due to capillary effect. It carries components with it. Component which are tightly bound silica remain at the bottom, component which are loosely bound will start traveling.

So, over a long period of time I am going to get various products in the mixture as various parts. Ideally I would like to get clean sharp separated spots, but sometimes what happens is you may get over lap of spots. So, generally simple system all we need is a glass plate and I coat just silica. And I put it in a solvent mixture. So, I can play around with different types of solvent mixture. So, that I change the polarity and hence, the movement of the solute from bottom to top. This layer is called thin layer chromatography and it is as I said widely used in synthetic organic chemistry.

It is also used in downstream processing if I am looking at separation of small peptides or bio molecules. So, how does it happen? You have a capillary action. Just so the

solvent and the solution mixture, solute mixture flows upwards. So, based on the partition coefficient solid will absorb, fraction of each component of the mixture. Reminder, will be in the solution will be travelling up. So, if it is slightly bound and silica it try, becomes very hard for it to travel will upward whereas if it is loosely bound and silica it becomes much easy for it to go upwards.

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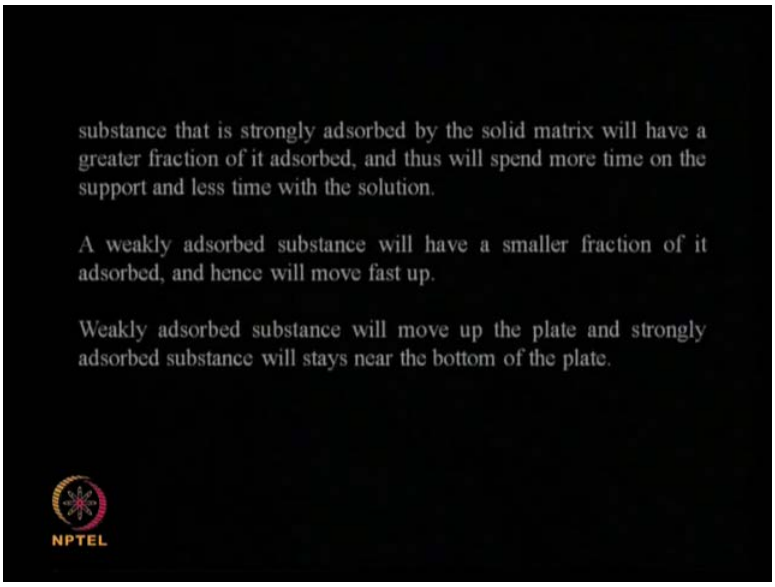

So, substance that is strongly adsorbed by the solid matrix will have a greater fraction of it adsorbed. So, it will spend more time on the support and less time with the solution, whereas weakly adsorbed substance will have a smaller fraction of it adsorbed. Hence, it will move fast. So, weakly adsorbed substance will move up the plate and strongly adsorbed substance will stay near the bottom of the plate. So, and from the location of the various spots we can tell components which are strongly adsorbed on silica, components which are weakly adsorbed on silica.

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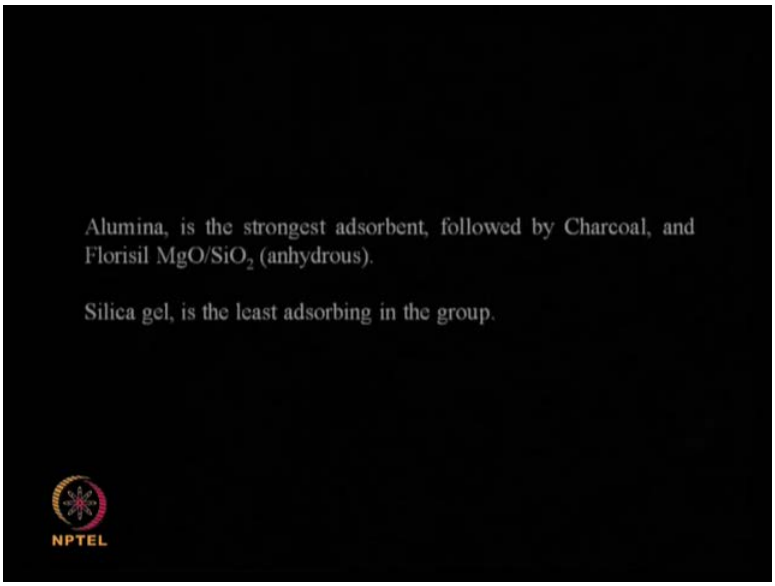

So, alumina is the strongest adsorbent followed by charcoal and florisil, magnesia, silica anhydrous and so on. Silica gel is the least adsorbing in this particular group of components actually.

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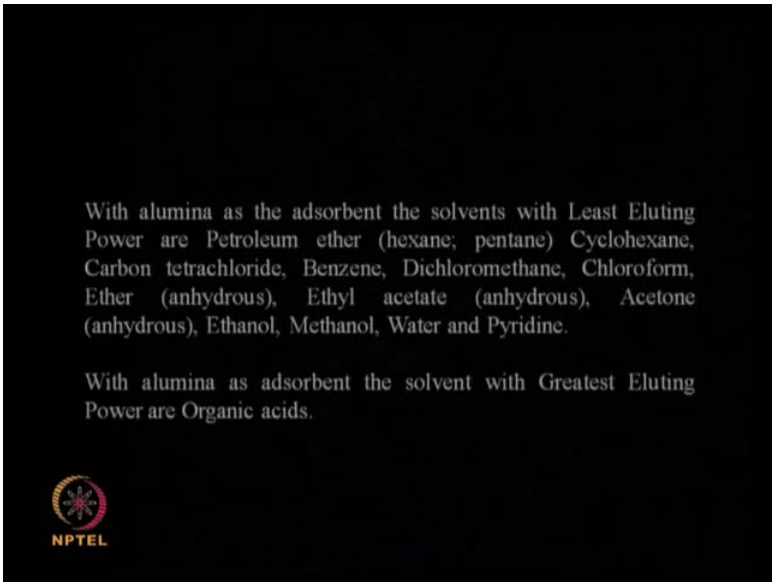

With the alumina as the adsorbent we can use different type of solvents depending upon whether I want least eluting power or whether I want strong eluting power. So, with the alumina is the adsorbent, solvent with least eluting power are petroleum ether like hexane, pentane and then go cyclohexane, then goes carbon tetrachloride, then goes benzene and dichloromethane, chloroform, ether, ethyl acetate, acetone, ethanol, methanol, water and pyridine.

So, with alumina as adsorbent the solvent with greatest eluting power will be organic acids. So, if I am using alumina I need to play around with the solvents depending upon whether I want least eluting or I want a greatest eluting power.

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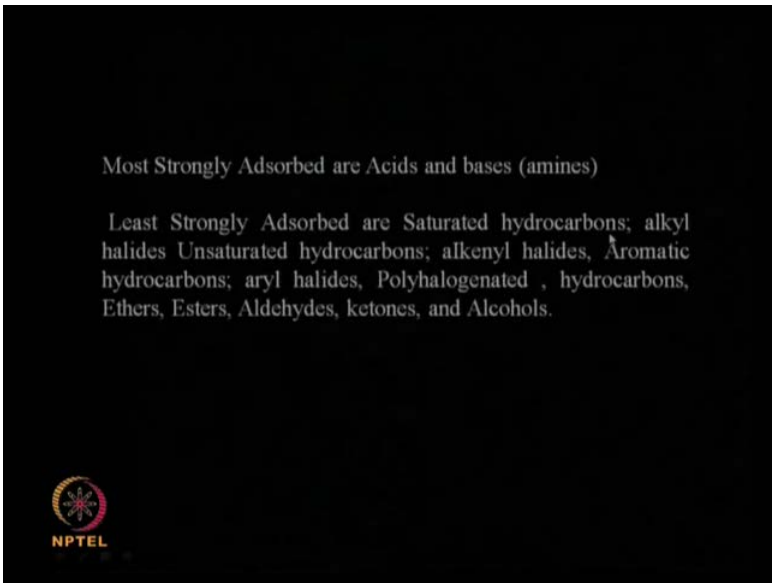

So, most strongly adsorbed are acids and bases and least strongly adsorbed are saturated hydrocarbons because you are using a stationary phase like silica or alumina which are polar or hydrophilic. So, least strongly adsorbed are saturated hydro carbons, alkyl halides, unsaturated hydro carbon, alkaline halides, organic hydro carbons, aryl halides, polyhalogenated, then comes hydro carbons, ethers, esters, aldehydes, ketones and alcohols. So, alcohols because they are hydrophilic they will get strongly adsorbed, whereas, hydro carbons which are extremely hydrophobic are leastly adsorbed.

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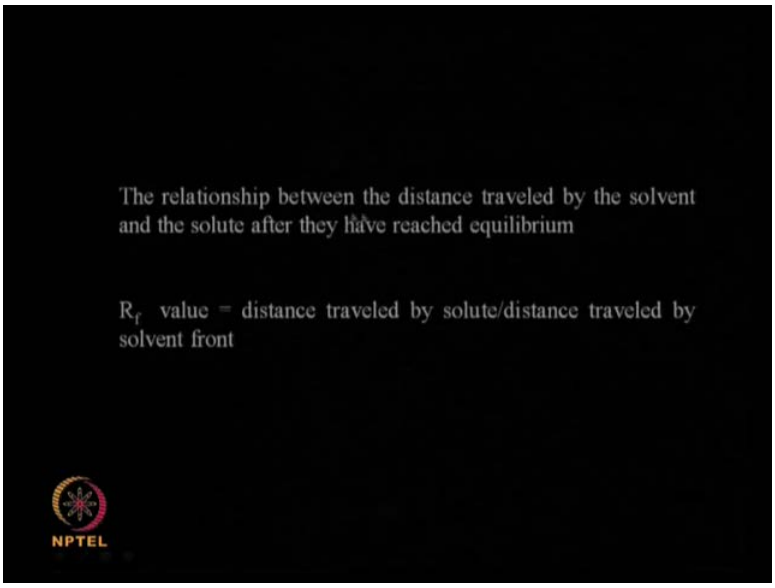

So, the relationship between the distance the solutes travel because of the solvent after the each equilibrium is given something called R f value. So, R f value is characteristic of the compound for a given stationary phase. So, R f value is the distance traveled by the solute divided by distance traveled by the solvent front. So, it is distance travelled by the solute divided by distance travelled by the solvent. So, if the R f value is very very large obviously it, the compound travels fast or very far after it has achieved equilibrium. If the R f value is very small then you can say that compound has not travelled very far in the system.

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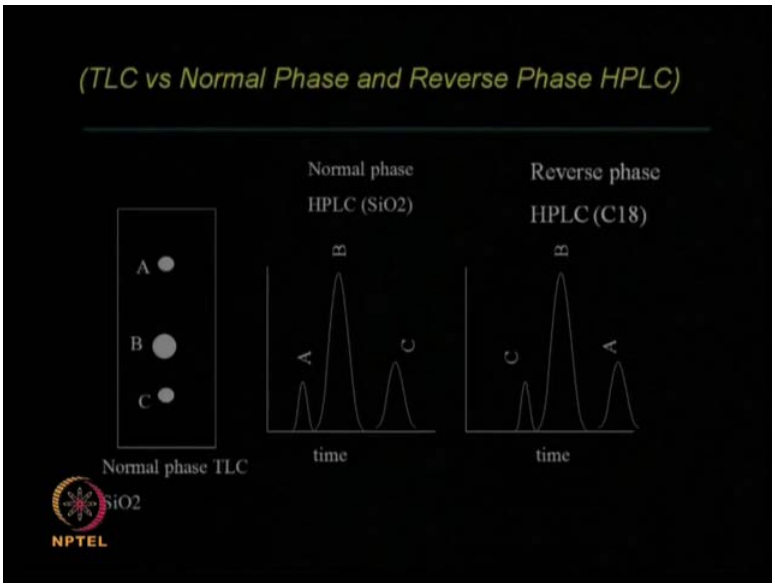

So, for example, I have TLC then I go to reverse phase HPLC. How do I connect the spots I get on TLC with the peaks that I get in the HPLC. So, that depends upon the type of HPLC whether I am using normal phase in HPLC or whether I am using a reverse phase HPLC. Now, in a TLC we use generally silica. So, it is almost like a polar stationary phase. Now, if I take a normal phase chromatography I will again use the polar stationary phase. So, the order of the components in a TLC which uses silica as against your normal phase chromatography will be the same.

So, if I take a TLC and I see a spot C which is right at the bottom and then I see B and then I see A; that means here C is very very polar and A is the least polar. So, in a normal phase what will happen? A will come out first, B will come out second, C will come out third.

That means A will have the least elution time or retention time. B will have the next retention time and C will have the highest retention time. So, in a normal phase HPLC we are using silica. So, that is also a polar. So, they will behave in the same fashion as a normal phase TLC which are also uses silica whereas, if I use a reverse phase HPLC where I am using a hydro carbon like a C 18 as the stationary phase.

Now, this stationary phase is hydrophobic or lipophilic. Then what happens? So, in a hydrophobic reverse phase chromatography I use a polar as the mobile phase. So, what will happen? C which is more polar will come out first, B which is next will come and

finally, A which is hydrophobic or non polar or lipophilic will come out last. So, you will see the order of way these three component A B C come in a normal phase, these, all these reverse phase are very very different. So, you should not get confused. You should know if I get in a TLC three components A traveling the furthest, B in the middle, C travelling the least, if you are using a normal phase you will have A coming out first.

That means A having the lowest retention time followed by B and C will have the highest retention time, whereas if I use reverse phase chromatography C will have the least retention time and A will have the highest retention time. So, we see that there is a completely reverse happening in the normal phase, in the reverse phase chromatographies.

So, you need to understand this concept very very well, so that if I do TLC curing initial downstream processing and the later on I am going to HPLC, I should be able to tell depending upon the type of HPLC I am using when each of the components will come with respect to the spots which I observe in my TLC.

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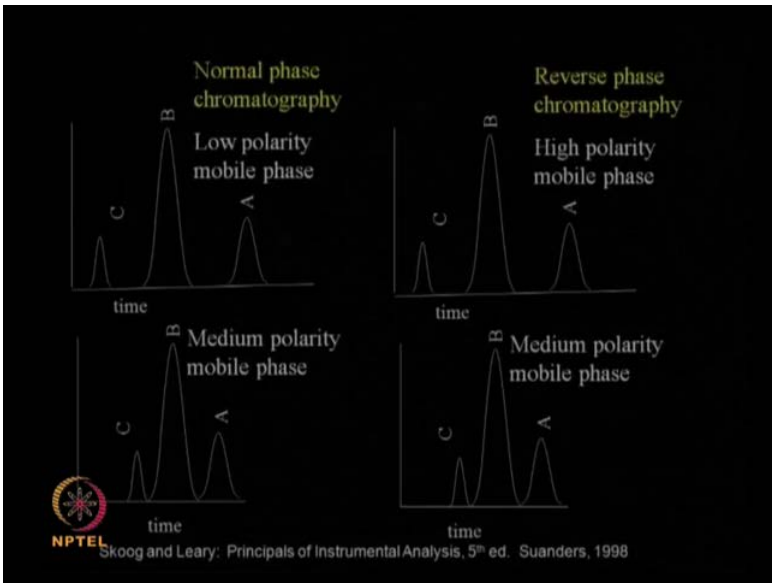

Let us go forward. So, you have a normal phase chromatography. I have a low polarity mobile phase. It is a normal phase chromatography. So, I am in the stationary phase is polar, do not forget that. So, I have components like this C B A. So, if I increase the polarity what happens? I am having a polar stationary phase and I am increasing the polarity. So, obviously these three will come together. So, that means if I am using

normal phase chromatography and if the peaks are very close I want to move peaks further apart, I reduce the polarity.

That means relatively make it more hydrophilic sorry hydrophobic. So, a normal phase chromatography if the peaks are very closed and I want to move the peaks further apart I use a slightly more hydrophobic system that means I reduce the polarity of these mixtures. Now, let us go to reverse phase. Suppose, I have a reverse phase chromatography and my peaks are like this, C B and A. Now, my solvent mixture or mobile phase is a high polarity, if I reduce to polarity to medium then they will come closer.

So, in a reverse phase chromatography if the peaks are very very close, all I have to do is increase the polarity of the mobile phase then the peaks will get separated out. So, in a normal phase chromatography if the peaks are closer I reduce the polarity for the peaks to separate out, whereas in reverse phase chromatography if the peaks are closer I increase the polarity, so that the components get separated out. So, you see the strategy which you adapt in a normal or a reverse phase changes dramatically. So, if you want move from a close to a further separated system.

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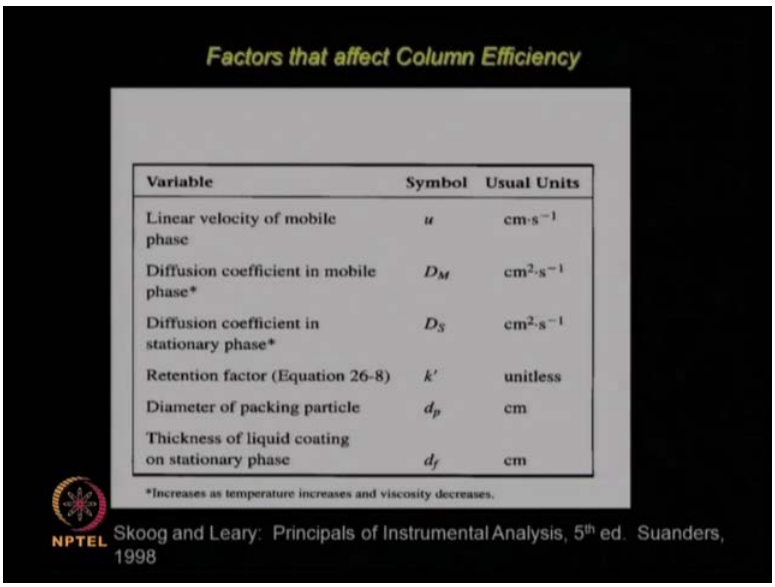

So, what are the various factor that affect the column efficiency? Factors which affect the column efficiency or the linear velocity of the mobile phase. So, if the velocity is high then obviously my retention time goes down relatively where diffusion coefficient in the

mobile phase, diffusion coefficient in the stationary phase. That means how the components diffuse inside the pores of the stationary phase. Then we have the retention factor that means how much of the solute is taken up by the stationary phase.

Diameter of the packing material, thickness of liquid coating on the stationary phase, so if you have thick material you are going to have mass transfer resistances, if you have very thin coating we are going to have less mass transfer resistance. So, all these factors affect the column efficiency. So, you can play around with many other factors, but once I select my column when some of the factors get fixed, whereas I can still play around with the solvent fluoride or I can play around with mixtures of solvent. So, that I affect my retention factor as well as I will affect my linear velocity of the mobile phase.

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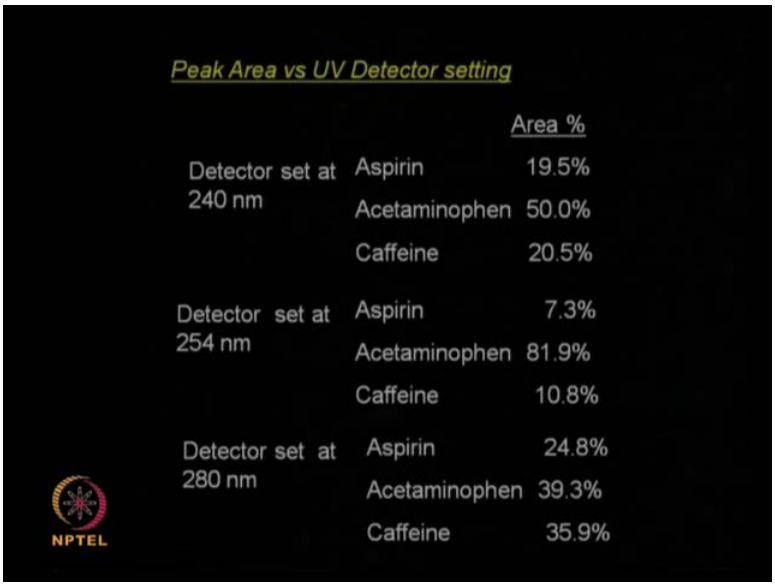

So, imagine I have three components and I am using UV detector that means the components are detector using a UV. So, if I set the detector to 240 nanometers and suppose I have three components like aspirin, acetaminophen and caffeine I may get area under the curve for aspirin 19.5, the acetaminophen as 50, caffeine as 20. Now, if I change the detector setting to 254 nanometers then this become 7.3, area under the curve goes down dramatically and this becomes very very large 81.9, caffeine becomes 10.8.

Now, if I set the detector to 280 nanometers aspirin becomes 24.8, acetaminophen become 39.3, caffeine becomes 35.9. Now, how much is the real amount? We do not know because depending upon detector setting I am getting different area under the

curve, percentage area under the curve. Why is it so? That is because of the lambda max. If the lambda max for each of the component very different depending upon where I said the lambda max that particular component will show a very high area. So, that is another big challenge when you are doing HPLC.

So, I may get mistaken whether the component acetaminophen is very very large or it is equal to caffeine. So, you will see that I am able to get different area under the curves for aspirin depending upon the lambda max of my UV detector. So, I need to have some idea about the lambda max for the components with which I am measuring in my solution or in my solute mixture.

If I do not know then I may end up with this type of misunderstanding on the whole system.So, you see UV max for aspirin is 225 and 296 nanometer, acetaminophen is 248. So, at 254 I get a very very large number. So, when I go away from 248 you see the acetaminophen keeps going down. Now, for caffeine it is 272. So, as I keep increasing, see the caffeine percentage goes up.

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So, that main problem here is the lambda max aspirin has low lambda max, caffeine has the next one, sorry acetaminophen as the next one and caffeine has the third largest.

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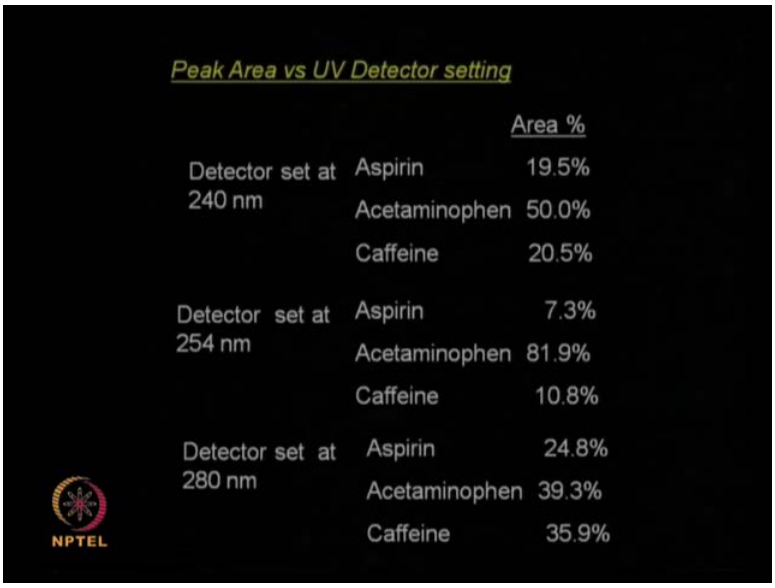

So, the numbers keep grammatically changing. So, see the aspirin it goes down and again increases because aspirin has two different lambda maxes. So, there is one lambda max here that is why you get some number. As we move out from that particular number the values go down and again it goes up, it peaks up because aspirin has two lambda max. So, the first thing is to you will use variable system just to find out the lambda max for, of various component present in my mixture.

Then you decide which lambda max to use if I want to use constant UV detector. And then do on your standardization, measurements and so on actually. So, understanding the lambda max for each one of the component is very very important. That is what this particular example tells you. Otherwise, you can totally get mistaken.

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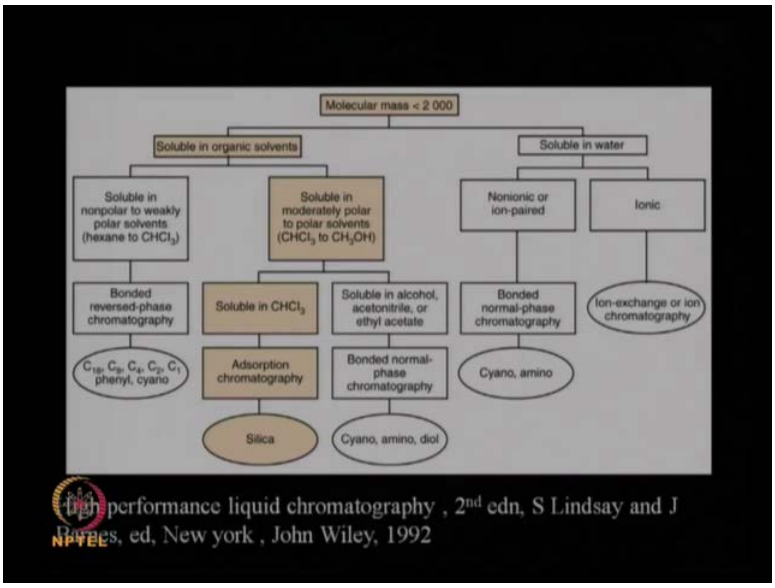

Now, there are different types of liquid chromatography's we can use depending upon the type of molecule which you are trying to detect. This is taken from a book by Lindsay and Barnes from John Wiley publication. It is called High performance liquid chromatography.

Now, if it is small molecule like molecular mass less than 2000, it could soluble in water that means it is hydrophilic or it could be soluble in organic solvent. That means it is hydrophobic. Now, in soluble in water it could be ionizable or it could be nonionic or ion paired type of this system. So, with an ionic then I can use ionic exchange or ion chromatography.

It is a non ionic or ion paired then I can use bonded normal phase chromatography. So, it is a normal phase that means the stationary phase will be hydrophilic. Now, soluble in water, now let us go to the system. If the system in solvent organic solvents, that means it hydrophobic.

Now, here we can have two different things. One is soluble in non polar to weakly polar solvents, soluble in moderately polar to polar solvent. So, if it is non polar to weakly then we will go to reverse phase chromatography. Here, we can use C 18, C 8, C 4, C 2 or phenyl or cyano. This is completely hydrophobic material.

So, we go for reverse phase chromatography whereas it is moderately polar to polar solvents. So, it may be soluble in chloroform or methanol. So, if it is soluble in chloroform you go to adsorbtion type of chromatography that means the stationary phase forces are just based on adsorptive forces or it soluble in alcohol or acetonitrile or ethyl acetate.

So, here we use bonded on normal phase chromatography. Again, we use in normal phase chromatography base cyano or amino or diol as the stationary phase. So, depending upon, this is for small molecules, depending upon whether molecule is soluble in water or whether it is soluble in organic solvent. If it is soluble in water is it easily ionized or its nonionic or ion paired, we may have two different types of chromatography either ion exchange or normal phase.

Now, on the other side if it soluble in organic solvents then one could be soluble only in weak polar or completely non polar or the other one could be moderate polar solvents. Completely non polar we may be using a reverse phase chromatography, whereas in a moderately polar we can have two systems, one is the chloroform soluble or other one is alcohol soluble or acetonitrile soluble.

So, in a chloroform soluble you may be using a silica based chromatography whereas in the other we may be using a normal phase chromatography like cyano, amino, diol and so on actually. Now, let us look at molecules which are larger than 2000 that means they are much larger molecules, whereas so far we looked at molecules which are smaller than 2000.

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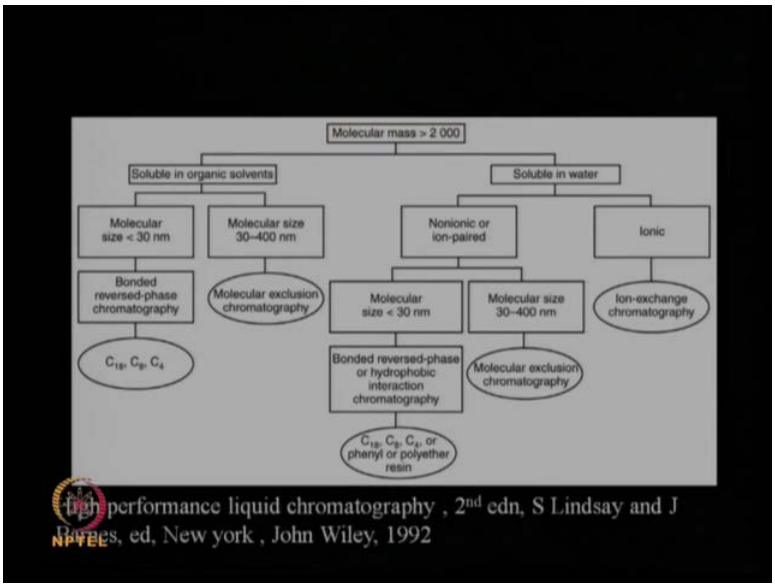

So, again this is taken from the same book High performance liquid chromatography, second edition, Lindsay and Barnes. Again, we have soluble in water, soluble in organic solvents. So, soluble in water again it can be ionic, it can be nonionic or ion paired. If it is ionic we can use a ionic exchange chromatography. If it is nonionic it could be small molecular size or large molecular size.

So, if it is a small molecular size we can use a reverse phase or hydrophobic interaction chromatography like different types of stationary phases C 18, C 8, C 4. If it is a large molecular size 30 to 400 nanometers then we can use a molecular exclusion chromatography. So, size exclusion chromatography we talked about it long time back. It is called size exclusion or gel permeation. So, it is based on size or molecular weight alone.

So, larger size polymer to smaller size polymer, larger size metabolize to smaller size metabolize or larger molecular weight metabolized to smaller molecular weight. Now, that is do with the solubility in water. What happens if they are not soluble in water, but they are soluble in organic solvents? Again, you have small molecules and large molecules. Small means less than 30 nanometers, large means 30 to 400 nanometers. So, large molecules we can again go for the exclusion chromatography like size exclusion or gel filtration chromatography, whereas small molecule we can go for reverse phase chromatography C 18, C 8, C 4.

So, again large systems we again have water soluble large systems or solvent, organic solvent soluble large systems. If the size of the molecules or molecular weight of the molecules are very large we can go for gel permeation chromatography. Of course, if it is ionic we cannot use gel permeation chromatography because as you can see here we can use only ion exchange chromatography.

Gel permeation is good for nonionic or ion paired systems actually which is based on just play size separations. So, smaller molecules will get entrapped in the pores of your stationary phase whereas larger molecules do not get entrapped, so they travel faster.

So, larger molecules will come out of the column faster, whereas smaller molecules will take much longer time because they interact. So, this table gives you what type of chromatography it use depending upon whether it is a small molecule or whether it is a large molecule. So, if the molecule are large we can go for ion exchange chromatography or we can go for gel permeation chromatography or we can go for hydrophobic interaction or reverse phase chromatography.

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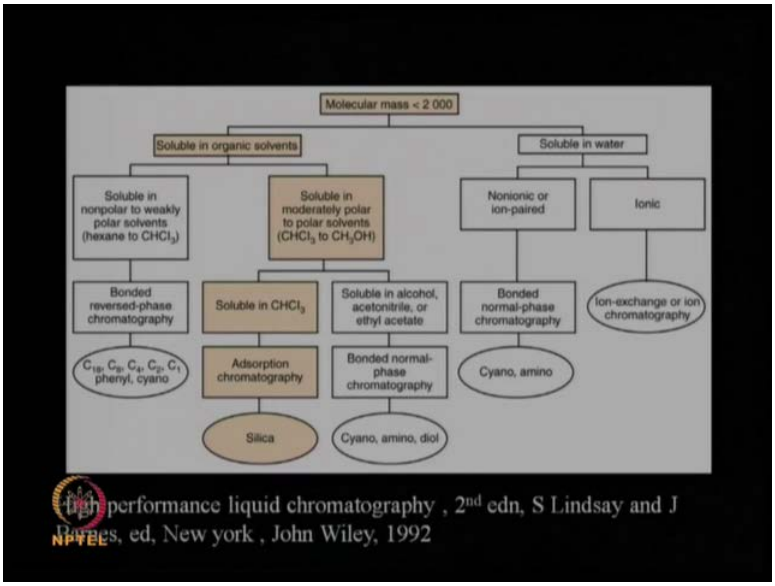

If the size of molecular sizes are very small, again we go for an exchange chromatography, normal phase chromatography, absorption chromatography and reverse phase chromatography. So, selections based on the ionizability, selection based on the size of the molecule, selections based on the solubility of the molecule.

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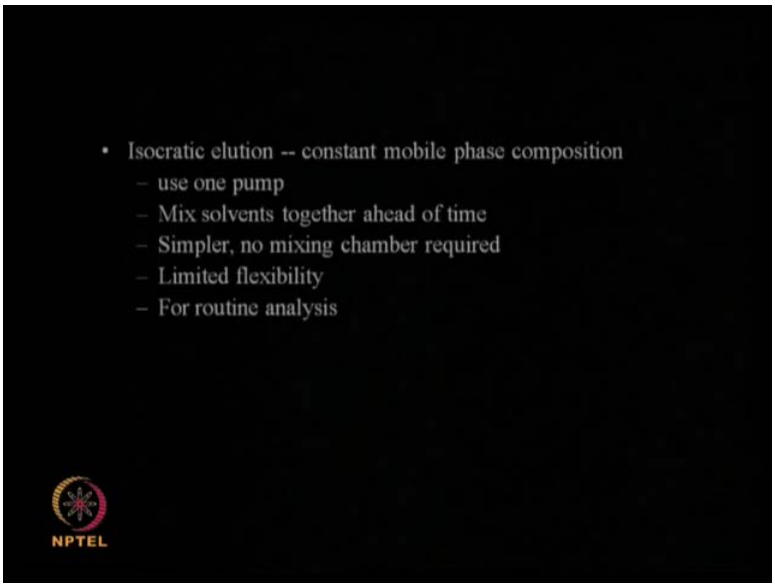

There is something called isocratic elution that means I use only one mobile phase. It is very simple, very cheap I use only one solvent, I use only one pump. The pump is very very expensive in a HPLC. Now, using only one pump and able to bring down the cost or if I want to use two solvents, I mix the solvents and make it in a container and use that mixed solvent for elusion.

But still I use only one pump. So, it is simpler. You do not need a mixing chamber. If I have two solvents then obviously I need a mixing chamber. But it has got limited flexibility because I cannot change the dielectric constant of the operation. It is good for routine operation. So, if I am going to do the same system like a (( )) where you are going to do the same thing day in and day out for very long time to come, this is the best system.

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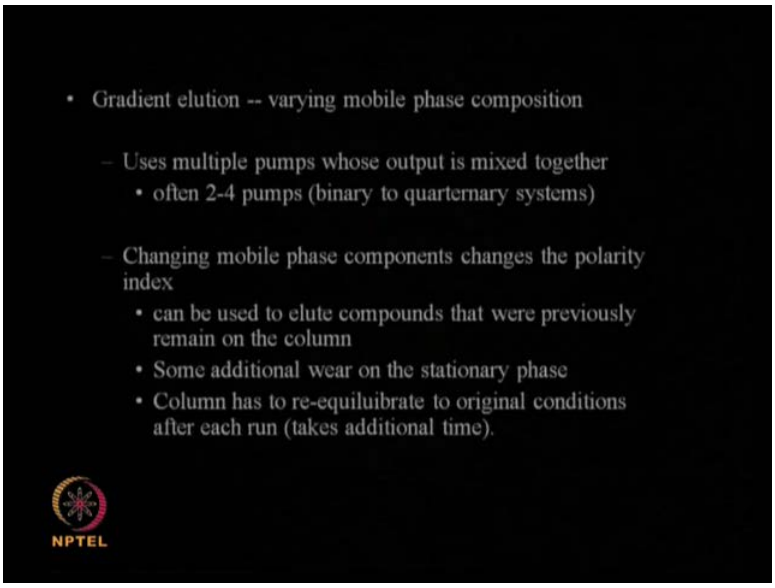

Gradient elution where the mobile phase compositions are changed as a function of time. So, initially you may start with one solvent. As time proceeds I change the solvent to something else. This is just like gas chromatography. In gas chromatograph either you can do the chromatography in one single temperature value that means the column is placed inside the oven and the temperature maintained constant or as a function of time I can change the temperature of the column. So, I may start with say 150 and in 10 minutes I may go to 250 and then maintain at the temperature.

So, exactly similar to changing the temperature in a gas chromatograph I am changing the solvent dielectric constant in a HPLC system. So, I may start with one solvent, I may go into another solvent or I may start with one solvent mixture then I move to some other solvent mixture. So, that is called the gradient elusion which is opposite to your isocratic.

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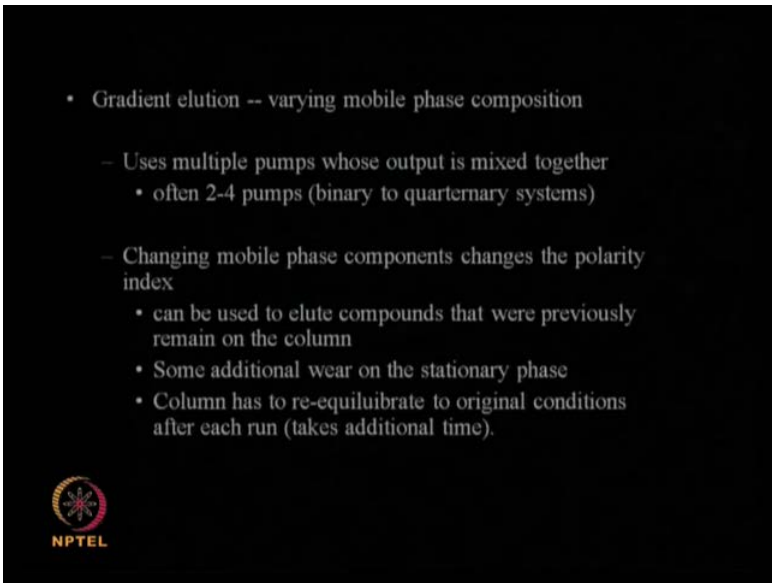

So, you will be using multiple pumps. So, you can use two pumps or you can use four pumps that means it can be a binary system or it can be quarternary system. Of course, the cost is very high because pump costs are very high. So, more the pumps, more is the cost. So, when I change the mobile phase components I am changing the polarity index. So, I can use it to elute compounds that are not coming out of my column, they stick inside, they stay inside. So, they, but the problem is that there could be some additional wear on the stationary phase. After each run I have to again bring it back to the original condition.

So, each run will take longer. So, I do a run and then I will bring it back to the original condition which requires again several minutes. So, in a gradient elution normally the time taken is much longer than a isocratic elution because in isocratic we are not changing the composition of the solvent over a period of time. So, you do not have to again re equiluiberate the system. So, that is one of the disadvantages of gradient elution. It takes much longer time, but we can get excellent separations using two solvents or three solvents or mixtures of solvents. That is the main advantage of this type of gradient elution.

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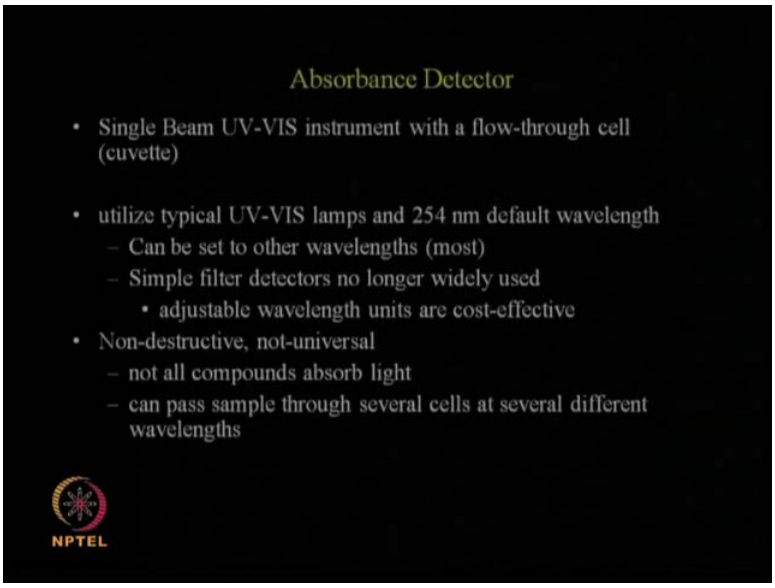

There are various types of detectors that are possible in HPLC depending upon the properties of the solutes, properties of the amino acids or proteins or peptides which are separating. And we are going to now look at some of the detectors, look at some of the advantages, disadvantages and so on actually. The most cheapest one is the single beam UV visible with the flow through cell, cuvette; that means you take your sample inside a cuvette and there is a UV which measures the absorbents of the solute mixture. Generally, it operates at 254 nanometer default wavelength, but we can also set it for other wavelengths also, know.

Some UV detectors you cannot set it then it is very cheap. There may be UV detectors where you can pre set it to some fixed value 3 4 different wavelengths or we can use even filters. We can also have adjustable wavelength units which are cost effective. The main advantage is the cost, it is very cheap. And then it is non destructive. So, the sample we can take it back and use it for some other purposes. So, especially if the sample amount is very small you cannot have, afford to have a sample which is totally destroyed after the analysis.

So, that is the main advantages. The disadvantage is not all compounds will absorb the light. So, you will not be able to detect a compound which is not UV detectable at all. So, that is a big problem or we can have several cells with different wavelengths, fixed wavelengths. So, we can look at various wavelengths in each one of the cell. So, that is

other approach by which we can slightly improve the flexibility of this type of UV detectors. Now, we will look at more detectors in the next classes and as I said depending upon the detector we can look at the compounds that are present in the mixture to very simple compounds to very complex compounds. So, we shall continue this particular topic in the next class.