Experimental Biotechnology
Prof. Dr. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati

Module No # 03
Lecture No # 15
Basics of Chromatography (Part – 1)

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(Video Ends: 01:00)

Hello everyone, this is Doctor Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. And in the course experimental biotechnology we are dealing with the different techniques so that you can be able to utilize these techniques for tackling the different scientific problem through designing the better experiments and so on. So in this series today we are going to start the discussion about the chromatography.

So chromatography is technique which actually allows you to separate the molecules and when you have a crude mixture you can able to purify the molecule of your interest. So let us discuss about the chromatography.

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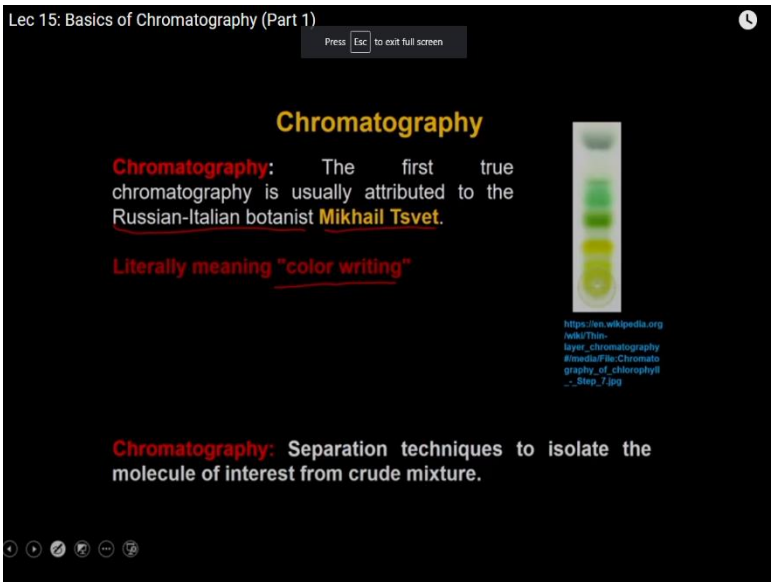

So chromatography was first discovered by a Russian Italian botanist Mikhail Tsvet and the chromatography literally means that you are writing in the color. So as you can see what the Mikhail has performed that he has applied a small plant extract on to a column, and when he has

dissolve this sample what he could found that the sample is being dissolve into the different bands of different color.

And that is how he has given the name as the chromatography or the color writing. The chromatography is a technique which is being used to separate the molecules. But before getting into the details of discussing the different aspects of chromatography let us discuss what is the basic; principles of separation?

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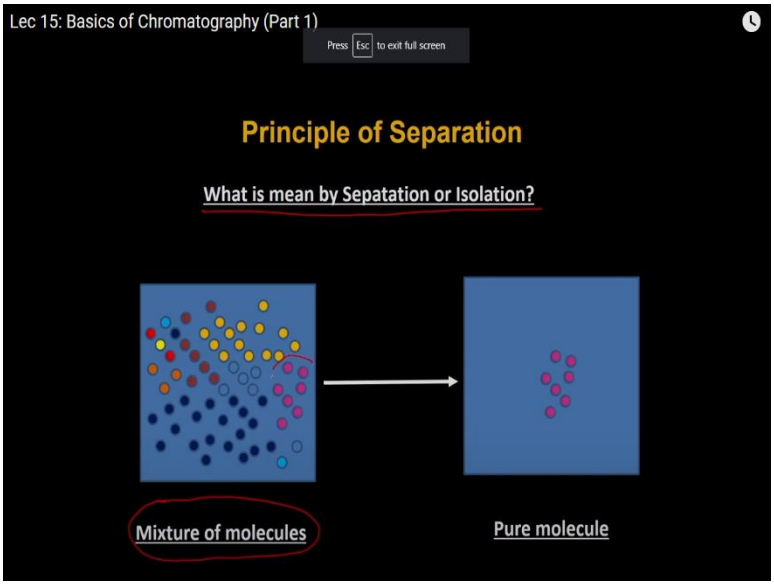

So what is mean by the separation or the isolation, so you can imagine that you have a mixture of the molecules, you can have the molecules or the different colors some are yellow in color, some are red, some are blue, some are pink. And what you have to achieve is the isolation of these pink color compounds. So that is what is called as the separation or the isolation of the compound.

So how you can achieve that suppose we give you the balls of the different colors and we ask you to separate the balls of the pink color then what you have to do is? You have to use some ways so that you can be able to recognize or you can be able to distinguish the pink color from the rest of the color and that is how you can able to separate these pink color spots. That is what exactly is this separation techniques.

The separation techniques always utilize a exclusive parameter which can be utilized or which can be exploited to isolated the compounds.

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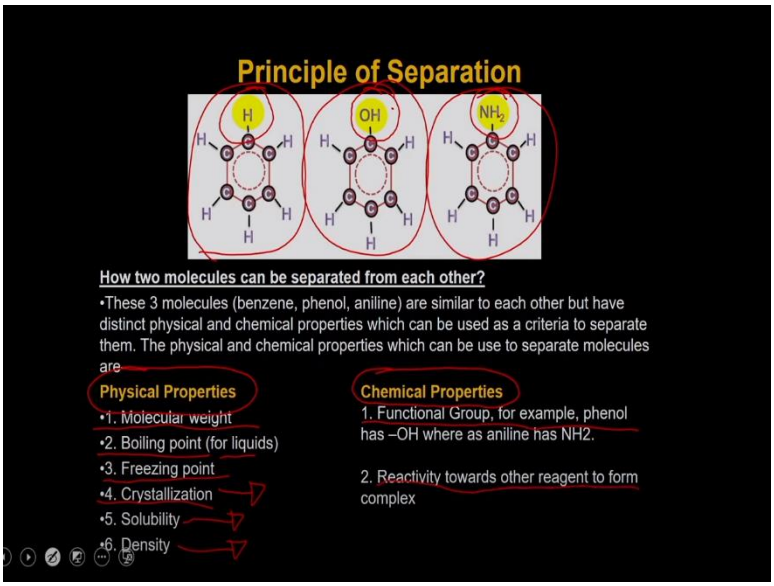

Now you can take an example of these 3 molecules. So these 3 chemical molecules are being present and we have taken these compounds only to discuss the basic principle of separation. So suppose you have the mixture of any of these 3 compounds then you can be able to separate them exploiting the different physical as well as the chemical properties. What are the chemical properties you can exploit?

You can exploit the molecular weight. You can exploit the boiling points in case these molecules are of liquids. Then you can use the freezing points, you can use the crystallization, you can use you can do something that the one compound is going to be crystallized. Whereas the other compound is going to be present in the solution and that is how the crystallized compound can be filtered out and can be separated from the rest of the molecules.

Similarly you can actually play very nicely with the solubility as well as the density parameters. So all these falls under the physical properties exactly the same as you can actually also play with the chemical properties, for example you can have the different types of functional groups. For example for this case the phenol as a functional group whereas the aniline has NH2 as a functional group.

So these 2 functional groups are very different from the benzene so you can actually exploit the presence of functional group. And once the functional groups are different their reactivity of one or other reagent is also going to be different. So when you react them some of the molecules will react the other molecule will not react. So the molecule which will react to form the complex this complex can either change the physical properties in such a way so that the complex is going to be more soluble or the less soluble.

And because of this you can be able to purify your desire compounds. Let see how you can exploit these physical or chemical properties for these three set of molecules.

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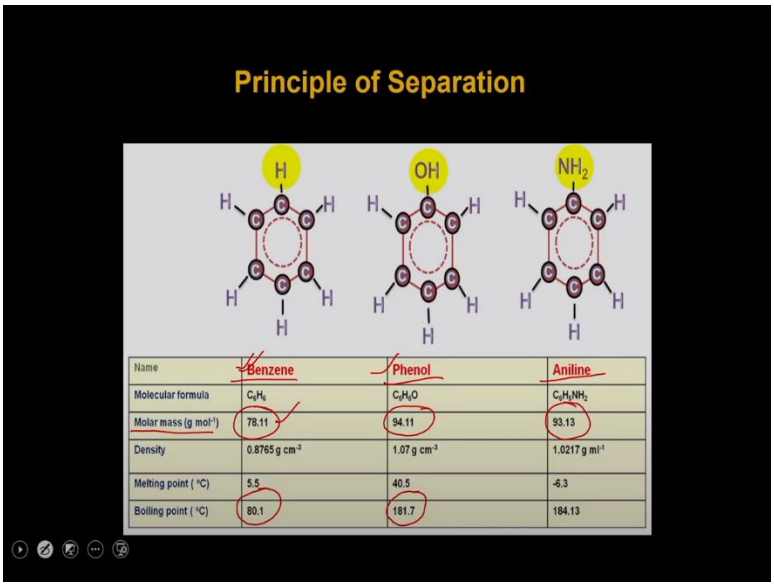

Name	Benzene	Phenol	Aniline
Molecular formula	C_6H_6	C_6H_6O	$C_6H_5NH_2$
Molar mass (g mol^{-1})	78.11	94.11	93.13
Density	0.8765 g cm^{-3}	1.07 g cm^{-3}	1.0217 g ml^{-1}
Melting point (°C)	5.5	40.5	-6.3
Boiling point (°C)	80.1	181.7	184.13

So these 3 set of molecules benzene, phenol and aniline now you can see that the molecule weight, the molecule weight of the benzene is 78:1. Whereas phenol is 94.1 and the aniline is 93. So if I have to purify the benzene and the phenol I can actually exploit the differences between the molecular weight then you can also see the boiling point, the boiling point of the benzene and phenol is different. So that also can be utilized.

Let see show the boiling point can be exploit to separate the 2 molecules using the technique called fraction distillations.

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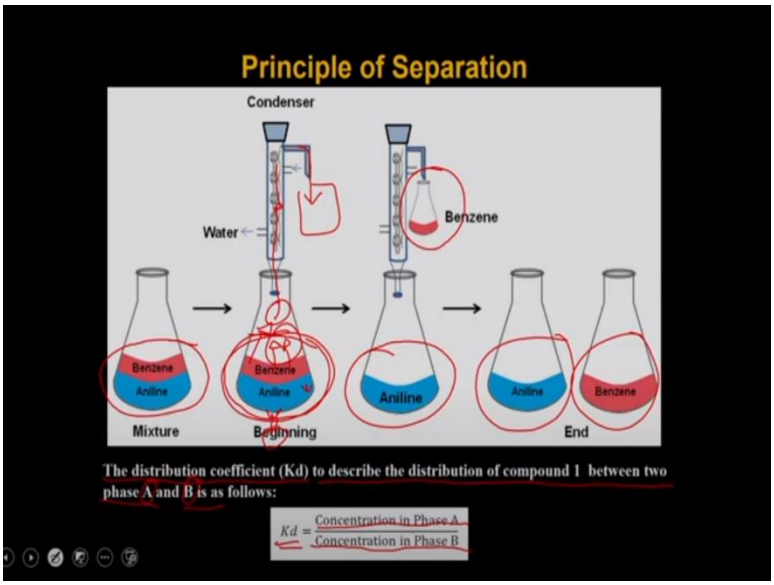

So initially what we have? We have a mixture of benzene and the aniline. So when you have a mixture of benzene and aniline what you have to do is you have to keep this sample in a flask and then you connect this flask to a condenser. If you all do not know about the condenser, the condenser is a instrument which actually allows you to distill the compound. So what happened is that the water vapors or the vapor of the compound goes into this central tubing and then this central tubing is surrounded by the water jacket.

So when the water comes goes out and comes out it this this tubing becomes cooled. So when the vapor goes in to this central tube it at actually get cooled and because it get cooled it turns in to the liquid face and that is how this water vapor can be collected in the second in the separate flask. So this distillation pretest can be used for distillation of different liquids or the mixture of a different liquid.

So what you have to do is you have a mixture of the benzene and aniline. You start heating these with the help of split lamp or the Bunsen burner. And as the temperature will go up the benzene which is actually having the lower molecule boiling point will have the tendency to go and remain in the vapor phase. Whereas the aniline which; is having the higher boiling point will remain with the liquid phase.

Which means; that the benzene will go and go with the vapor phase whereas the aniline will remain with its own phase. So as a result what will happen is when you go with the first round of

distillation you are going to have more and more amount of benzene in the upper flask, whereas you are going to have the aniline in the lower flask. So if you repeat this whole exercise multiple times or if you use the fractional distillation operators you could be able to separate these 2 molecules.

Because they are having a very huge separation in terms of their boiling point the benzene has a lower boiling point whereas aniline has a higher boiling point. And because of the difference in the boiling point the benzene will evaporate faster and evaporate sooner. So that benzene will remain the vapor phase whereas the aniline will tend to remain in the liquid phase. Another result you will collect the aniline in the lower chamber and lower flask.

And whereas the benzene you will collect in the upper flask. So at the end of these distillations you will be able to separate the aniline from the benzene. If you can be able to distribute the molecules into the 2 different phases whereas for example in this case you have the 2 phases one is the vapor phase and the other one is the liquid phase. This whole this whole phenomenon can be monitored or can be used to separate the molecules and it is.

So the distribution coefficient which actually explains this distribution is to describe the distribution of compound one between the 2 phases A and B, which means in this case the you have the distribution coefficient of benzene for vapor phase and a liquid phase. Whereas you are; also going to have the distribution for aniline which is different. So in this case the phase A is going to be the vapor phase, phase B is going to be the liquid phase.

So the Kd is going to be the concentration of compound in phase A verses the compound concentration of the compound in phase B. So as the names as the distribution coefficient is a ratio of the concentration of compound in phase A verses the phase B it actually does not have any unit and it is actually also a virtual number because as soon as you change the pairs of the compounds or as soon as you change the condition the distribution of the molecules are also going to be different between the different phases.

And as a result, the distribution coefficient always depends on the number of counter ions or number of compounds which are present in the mixture. And it is also a not a fixed number it is a

dynamic number which actually depends on what a way the compound is going to be distributed into the different phase.

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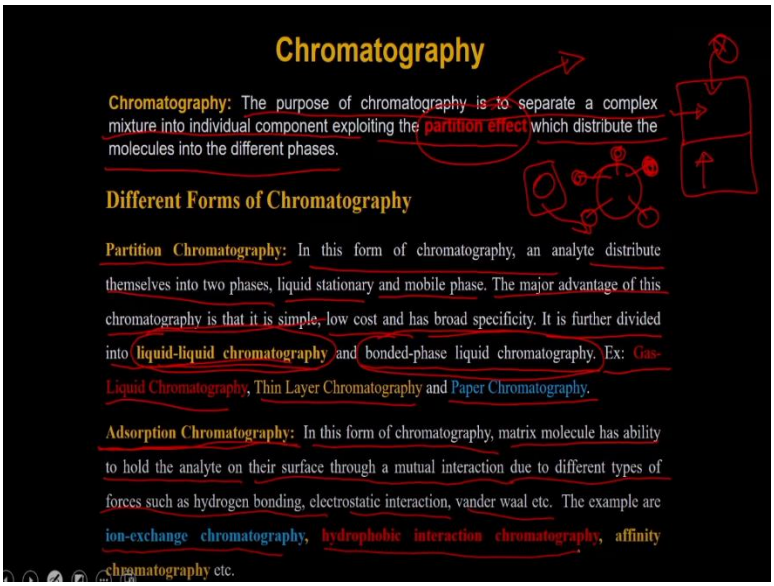

Now let us come to the chromatography. So chromatography the purpose of the chromatography is to separate a complex mixture into the individual component exploiting the partitioning effect. So partitioning effect means you are distributing the molecule between the 2 phases which distribute the molecules into the different phases and because you can do the partitioning with the help of during the chromatography.

The partitioning can be done in 2 different ways and that is how the chromatography can be divided into the 2 different types. One is called as the partition chromatography, the other one is called as the absorption chromatography. So in the partition chromatography the analyte distribute themselves into 2 phases. One is liquid stationary phase and the other one is the mobile phase.

So in the partition chromatography always occurs in a places where you have the 2 liquids which are not immiscible or the 2 liquids which are not mixing with each other, and you can put a compound and then the compound is going to distribute between the these 2 liquids. Which means if I have a, 2 liquids and if I put a compound X what will happen is the X will have the lower solubility in this one or it can have the higher solubility in to the solvent 2.

In that case what will happen is the X is going to partition between the 2 compounds. So the partition chromatography, always exploit the partitioning of the molecule between the 2 phases. In these 2 phases the 2 phases are always been liquid. The major advantage of this chromatography is that it is simple low cost and has a broader specificity. It is further divided into the liquid liquid chromatography or the bonded phase liquid chromatography.

So the in the liquid-liquid chromatography you are actually utilizing the 2 different liquid of the different solubility and the compounds are going to be partitioned between the 2 liquids. For example, if I can take the mixture of hexane and water. So suppose I my compound is hydrophobic and it is not soluble in water then what I can do is I can just simply add the hexane mix it into the very vigorously.

So in that process what will happen is the compound will going to partition between the aqueous phase as well as the hexane phase. And the sum of my compound which is actually more soluble in hexane phase will transfer from the aqueous phase into the hexane phase. On the other hand the compound which, are more of polar in nature probably will remain the aqueous phase and that is how you can actually be able to separate the molecules utilizing the different partitioning.

And that you can do in the liquid-liquid chromatography. Whereas in the bonded phase liquid chromatography you have the liquid layer which is actually been absorbed on to the solid support and then the molecules are been portioned between the liquid which is bonded on to a support verses the liquid as a mobile phase. The classical example of the liquid-liquid or the partition chromatography is the gas liquid chromatography, thin layer chromatography and the paper chromatography.

In all these 3 cases some places you are using the liquid-liquid partition chromatography and some places you are using the bonded phase liquid chromatography. Now the second way in which you can actually the partition the molecule is called as the adsorption chromatography. In this form of chromatography, the matrix molecule has the ability to hold the analyte on their surface through a mutual interaction due to the different types of the forces such as the hydrogen binding, electrostatic interactions, vander waal etc.,

So in this adsorption chromatography what you are doing is? You have the small beads on which you have the functional groups these functions group may facilitate the different types of the forces. For example you they can have the hydrogen bonding they can do the vander wall interaction or they can have the electrostatic interaction or they can have the hydrophobic interaction with the groups present on to the protein. And as a result the molecules are going to be absorbed on to these groups.

So molecules are present in free flowing into the mobile phase whereas the molecules are also being absorbed onto these beads. And then ultimately these molecules are going to be removed when you do the washing and the subsequent steps. Whereas beads absorb molecule can be removed from the column using the different types of elution techniques. And that is how the absorption chromatography is very much different from the partition chromatography

So the partition chromatography is simple low cost and it is broad specificity the partition chromatography is not very much towards the specific molecules. Whereas the absorption chromatography can be modified or can be adjusted in such a way that it should be it is going to be even for the specific molecule. So it actually provides the specificity if becomes the user friendly and it is more convenient to perform compared to the partition chromatography.

The classical example of the absorption chromatography is ion exchange chromatography, hydrophobic interaction chromatography and the affinity chromatography. Irrespective of whether you do the partition chromatography or the absorption chromatography the basic principle of the chromatography remains the same that you are actually distributing the molecule between the 2 different phases.

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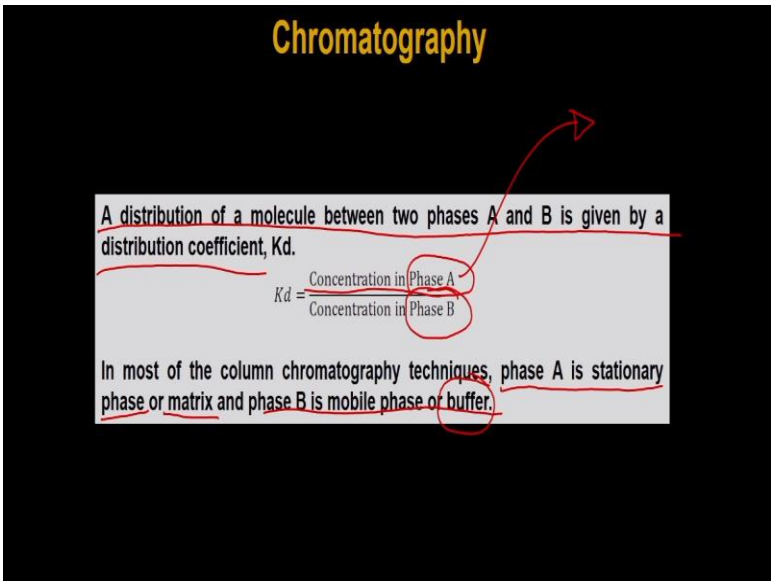

So the distribution in the case of chromatography is also been defined by the same formula that is the concentration of the molecule in phase A verses the concentration of the molecule in the phase B. In the case of column chromatography it is well understood that the phase A is always been the stationary phase or the matrix whereas the phase B is the mobile phase or the buffer. So in the case of other cases the distribution coefficient can have the multiple phases can have any phase in the phase A or phase B.

But in the case of column chromatography for the for purpose of convenience, we always keep the stationary phase as the phase A and we keep the mobile phase as the phase B to calculate the Kd values.

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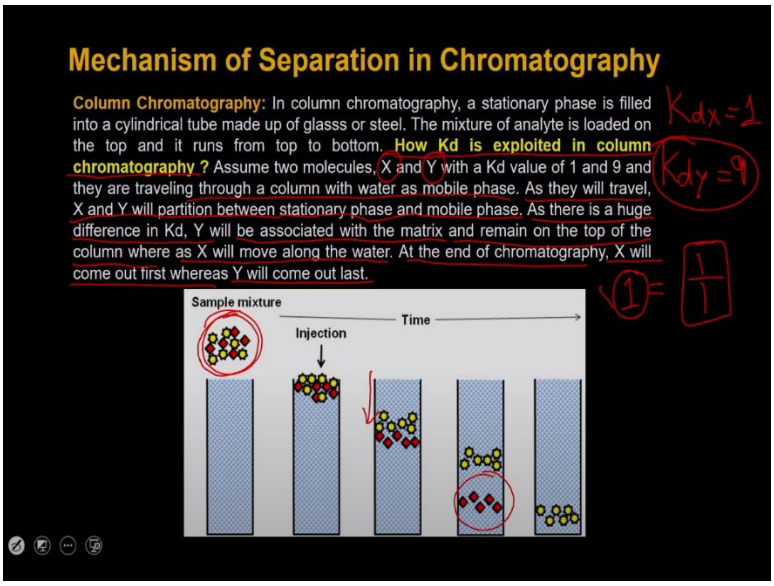

Now let us see how the molecules are being separated when they are being subjected to a chromatography. So we have taken a mixture of the compound and we would like to understand how the Kd is being used in a column chromatography to separate the 2 molecules. So we had taken a example of a molecule which is called X and Y. And the Kd value of the X is 1. Whereas the Kd value of Y is 9 ok.

If the Kd value is 1 which means if every ml of the mobile phase the molecule is going to be distributed evenly between the stationary phase as well as the mobile phase. Which means if I have the X and Y if I loaded the X and Y mixture on to the column, as the molecule will run to the column it will actually going to distribute between the matrix as the on the mobile phase.

And because the X has the Kd value of 1 it is actually going to distribute always in terms of 1 is to 1, which mean if I pass through the 1 ml of the buffer the X is going to distribute 50-50 between the matrix as well as the stationary phase. Whereas the Y is going to be distributed in terms of 1 is to 9 which means the Y is going to be remain with the stationary phase whereas the X will also is going to remain with the mobile phase.

So as the sample will run through the column with the water as a mobile phase as they will travel the X and Y will partition between the stationary phase and the mobile phase. As there is a huge difference in Kd values the Y is always been associated with the matrix or the stationary phase

and remain on top of the column. Whereas the X will move along the water phase because as you can see that the X has a lower Kd value so it will always going to be partitioned.

And that partition will going to be keep distributing it towards the mobile phase. At the end of the chromatography X will come out first whereas Y will come out at the end. So this is the X molecule and this is the Y molecule and because they have the very wide separation or wide difference in terms of the Kd values the Y is going to be separated from the X. And that is how you an actually exploit the distribution of the 2 molecules based on the distribution coefficient.

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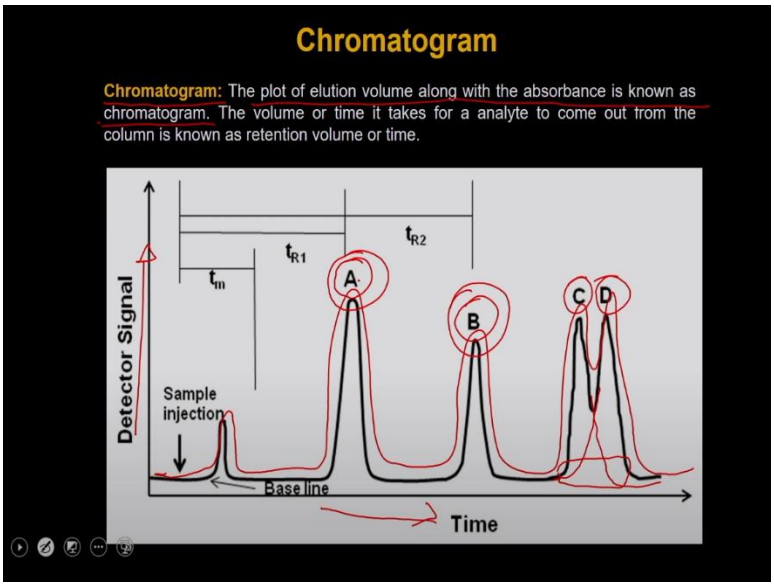

Now once you monitor the elution of these molecules from the column you can use the any parameter to monitor. For example, you can monitor it simply by taking the absorbance. So what you will see is that you will see a pattern in terms of the multiple peaks ok. And what you can see in this and this pattern where you are going to see the pattern of elution with multiple peak is called as the chromatogram.

So the plot of elution column along with the absorbance or any other parameter is known as the chromatogram. So on the Y axis you will put the detector signal which means it could be a absorbance, it could be fluorescence, it could be a refractive index and so on. And on the other hand on this side you are actually going to put the elution volume or the elution time and this pattern is known as the chromatogram.

So in the chromatogram what you can see is that you have the 4 peaks A peak, B peak, C peak and the D peak. So A and B are the 2 separate peaks which means the column is good enough to separate the A from the B. Whereas the C and D are actually are the fused peaks and how the C and D are fused peak because the C is eluting like this. Whereas the D is eluting like this which means they are actually sharing a broad base and because their base is broad they are interacting with each other and as a result they are actually being eluted as the fused peaks.

And why it is happening so? It is happening because these column is good enough to separate the A and B but it is not good enough to separate the C and D which means the column has the lesser ability to separate the peaks C and D and the ability of the column to separate the 2 peaks and give you the 2 molecules into 2 separate peak is known as the resolution.

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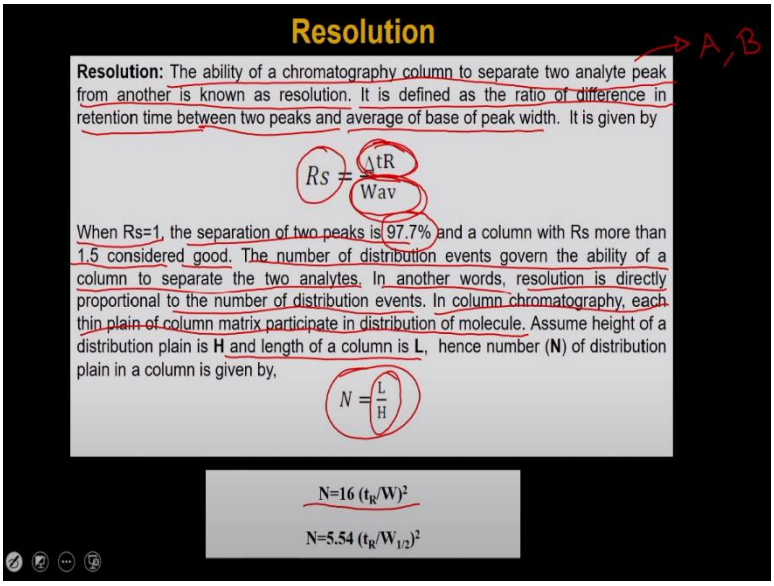

So what is mean by the resolution? The ability of a chromatography column to separate the 2 analyte peak from one another is known as the resolution, which means in the earlier figure the A and B the column is separating the peak A and Peak B, and that is called as a resolution. And it is defined as the ratio of difference in the retention time between the 2 peaks and the average of the base of the peak width.

Which means the resolution is directly proportional to the difference of the retention time between the 2 peaks and it is the inversely proportional to the average of the base of the peak width. So when you have the resolution of 1 which means the retention time and the average

width of the peak is going to be equal the separation of the 2 peak is going to be performed with an efficiency of 97.7%.

But if you have the resolution which is more that, 1.5 it will actually going to give you the separation of the 2 molecules by more that, 99%. The number; of distribution event govern the ability of a column to separate the 2 analytes. In another words resolution is directly proportional to the number of distribution events. In column chromatography each thin layer of the column matrix participates in distribution of the molecules.

So you can assume that the height of the distribution plain is H which means the beads what you are using has a diameter of the H. And the length of the column what you are taking is L. If that is the case the number of distribution plain is going to be equivalent to the length divided by the diameter of the individual beads. Which means those many number of beads are going to be present in the column.

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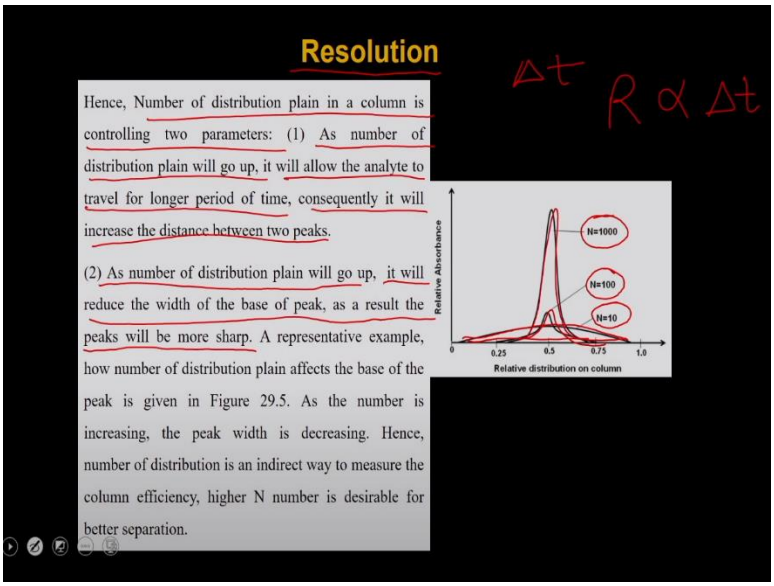

And the N is the directly proportional to the 16 tR / W whole square or N is directly proportional to the 5.54 tR / W average the whole square. Let us see how the distribution plain, are going to improve the resolution of a column. So number of distribution plain in a column is controlling 2 parameters. Number of distribution as the number of distribution plain will go up it will allow the analyte to travel for longer period of time consequently it will increase the distance between the 2 peak.

Which means as the distribution plain will go up or as the number of distribution plain will go up that the length or the distance between the 2 peak is going to be more and more which means the delta t is going to be on a larger scale. And if you remember the r is directly proportional to delta t. On the other hand, as the number of distribution plain will go up it will reduce the width of the base of the peak as it results into the peak of the more sharp peaks.

You can take the example imagine that I have packed a column with the varying number of distributions plain. So if have the distribution plain of n = 10 I am going to see a peak width with the base of this much. Whereas if I increase the number by n by = 100 I am going to reduce the base and it is going to give me the shrink base. And if I increase the distribution plains further up which means if I increase the distribution plain by another 10 times, I am going to see a sharp peak with a very, reduced base.

So that actually proves that as you increase the distribution plain you are actually going to reduce the average peak width of the base. And that actually is going to increase the resolution because the R is inversely proportional to the W average. So as the number of numbers is of the tripling plain is increasing the peak is increasing. Hence the number of distribution plain is indirect way to measure the column efficiency. Higher the number is desirable for better separation.

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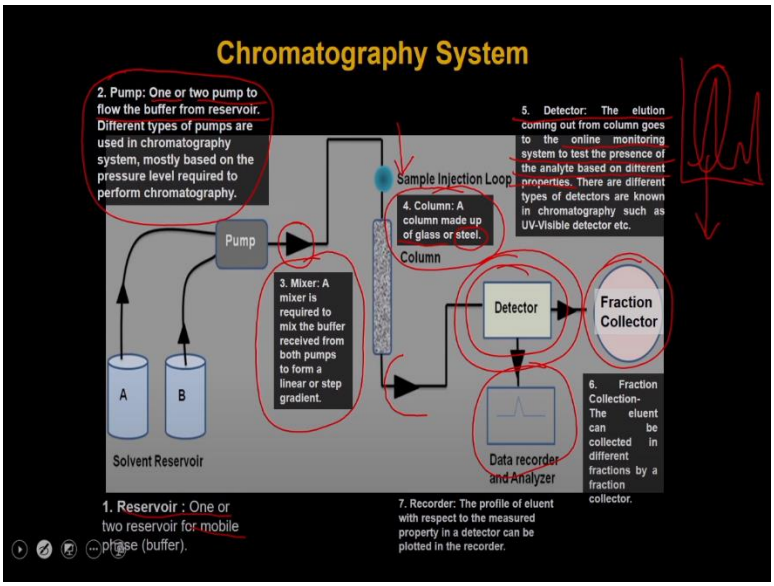

Now if you would like to perform the chromatography you can utilize a chromatography system. And in a typical chromatography system you are going to have the multiple components. Let see what are these components? So you can have the reservoirs. The reservoirs you can have 1 or 2 reservoirs where you are going to keep the mobile phase, the buffers. In some cases, even you can have the 4 reservoirs. So that you can be able to perform or you can be able to prepare the gradients between the 2 buffers.

Then next to the reservoir you are going to have the pumps depending on the type of the chromatography system you can have the 1 pump or the 2 pumps. And they can individually be connected to the individual reservoirs. So that you can be able to utilize, the different pumps to produce the very precise gradients. Depending on the pressure level you can be able to utilize the pumps which are made up of the glass or which are made up of steel.

Then next to the pump you have the mixture. The mixture is the place where you are going to mix the liquid which are coming from the individual reservoir. So that you can have the homogeneous mixed buffer and that actually will go into the column. And column is made up of a glass or the steel depending on the pressure limit. In most of the low pressure or the middle pressure chromatography system you can be able to attach the glass columns.

But when you go to the high-pressure columns you have to use the column which is made up of steel. Next to the steel next to the column you are going to have the detectors. Detector is something which actually detects the analyte which are coming out from the column the detector could be or utilize the multiple parameters. Detector could utilize the UV visible spectroscopy UV visible absorbance.

The detector could utilize the fluorescence, detector could utilize the refractive index or detector can utilize many other parameters like you can even attach the mass spectrometry to the, as the detector. And then in that case the chromatography system will it will turn into the liquid LCMS or liquid chromatography mass spectrometry system. So the detector could be off depending on what kind of molecule you are analyzing you can actually choose the detector.

And that actually is going to do the online monitoring system to test the presence of the particular analyze based on the different type of properties. There are different types of detectors

such as UV visible detector fluorescence detector or RI detector and all that. Once you detect the molecule using the detector because detector is going to give you the pattern or detector is actually going to give you the chromatogram you can actually ask the machine to collect the peaks and that you will do in the fraction collectors.

And the fraction collectors will collect these eluting molecules into the different fractions. And once and on the other hand detector will also give the signal to the recorder and the recorder is going to show you the profile of these eluting molecules in the form of a chromatogram. So this is all about the brief overview of the chromatography system. Let see how a real chromatography system look like.

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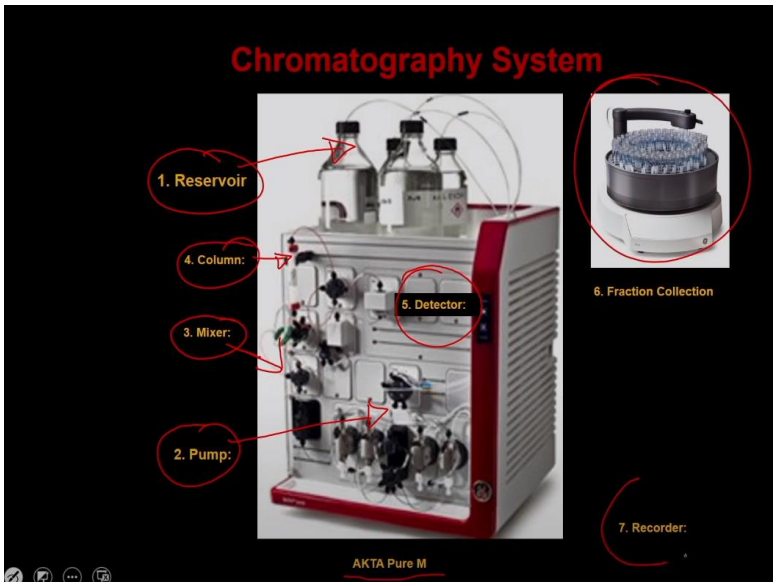

A real chromatography system for example in this case we are showing the AKTA pure M. So the AKTA pure M has the different components like you have the reservoir. So you can have the reservoirs then you have the column. So next to the reservoir you have the pump so in the AKTA pure M you have the 2 different pumps to and you can see the you have the 4 reservoirs. Then you have the mixer, so you have the mixer. Then you have the column so in this case you will see it has a in the picture we are showing a high trap high resolution column.

Then you have in this place you have a detector and next to the detector whatever is coming out can be collected in the form a different fractions, or you can actually be able to utilize a computer with the interface to the system to visualize. What is visualize the profile of the eluting

molecule in the form of chromatogram. So this is all about the chromatography system. So when you would like to utilize the chromatography to purify the molecule you have to decide a strategy and a strategy will let you to purify or isolate the protein of your interest.

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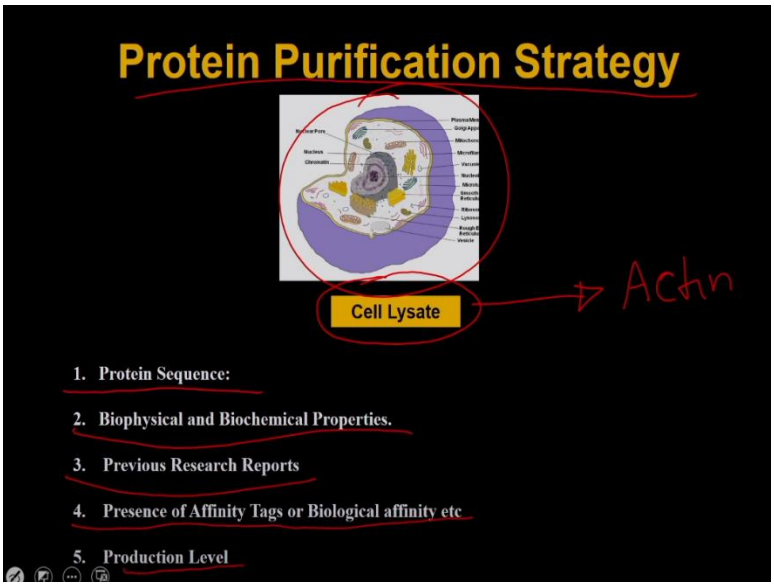

And when you would like to design a strategy you have to consider the many parameters. So when you want to do a protein purification strategy. So suppose you are working with the mammalian cells as soon as you break open the mammalian cells you are going to get the cell lysate. And once you are getting the cell lysate and you would like to isolate the protein like actin. Now if I have to purify the actin I should know the protein sequence.

I should know the biophysical and biochemical properties. I should know how the other people have purify the actin in the previous research article. And then I should also know what are; the affinity tag or the biological affinity of the actin towards a particular matrix so that I can utilize that particular matrix in the absorption chromatography. And lastly, I should also know what kind of projection level is our requirement.

So if I; have a requirement of milligram level or if I have a requirement of simply to isolate the actin for immunization purpose and so on. Depending on what kind of what kind of the production level you want you have to divide the strategy. Because if you are looking for the protein in milligrams or gram level then you have to devise a strategy where you should preserve each and every loses because when you perform the chromatography technique.

You are going to lose the molecules or you are going o lost the sample because some of the fraction you have to discard when they do not have the essential or enough proteins of enough quality ok. So in this module we are going to discuss about the different chromatography techniques. How these chromatography techniques can be utilize to purify the proteins. And on the other hand we also be going to discuss how you can utilize these chromatography techniques to answer your specific experimental or the scientific questions.

So with this I would like to conclude our lecture here. In the subsequent lecture we are going to discuss more about the different chromatography techniques. Thank you.