Enzyme Science and Technology
Prof. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati

Module - V
Enzyme Production (Part 3: Purification)
Lecture - 24
Basics of Chromatography

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bio engineering IIT, Guwahati. And what we were discussing, we were discussing about the different properties of the enzyme in the course Enzyme Science and Technology. And so far what we have discussed, we have discussed about the history of the development of the enzymology, we have also discussed about the naming and as well as the classification of the different enzymes.

And then previous two modules, we are discussing about how you can be able to produce the protein or the enzyme in the bulk quantities. In this context, we have discussed about the cloning of the enzyme from the genome, either you use the genomic library or the cDNA library to select the gene of your interest or you can use the polymerase chain reaction to amplify the gene of your interest using the site specific primers.

Once you have the gene of your interest, then you can you know digest that with the restriction enzyme and you can clone that into a suitable vector. Once you got the clone into a suitable vector, then you can transform that into the, you know, the into the host and that is how you can devise the different strategies to over express the protein into the host. So, all these processes, what we have discussed so far are called as upstream events or upstream processes.

Where you are going to clone the protein or your, then you are going to transform the protein and then you are going to over express the protein in to the host cells. Now, what we are going to discuss today is once you have the protein in the host cells you, how you can be able to recover that protein and how you can be able to verify that protein using the different chromatography techniques.

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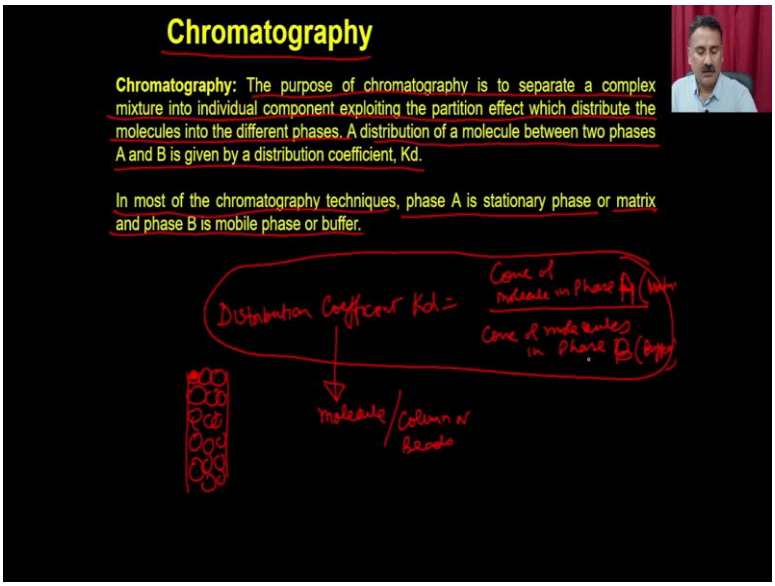

Now, what is chromatography? Chromatography, the purpose of the chromatography is to separate a complex mixture into the individual component exploiting the partition effect which distribute the molecule into the different phases. A distribution of a molecule between the two phases A and B is given by the distribution coefficient. So, distribution coefficient is distribution coefficient Kd is equal to concentration of molecule in phase 1 divided by the concentration of molecule in phase 2, ok.

So, this is actually going to be not a fixed value, it is actually going to vary and it is going to be related to the molecule what you are talking about and it also depends on the different types of partitioning agents. So, it can also depends on the column what you are going to use or the beads. So, you can imagine that if I am taking the beads and if I am taking a column right, I can actually be able to fill the different types of beads right and that is how I can actually be having the different types of distribution.

So, molecules actually can distribute from the first layer to last layer and that is how the molecules are actually going to be separated. So, in most of the chromatography techniques, the phase A is a stationary phase. So, in the concentration of the phase 1 or I will say phase A actually and the phase B right or a matrix and the phase B is the mobile phase or the buffer actually.

So, you can imagine that if I am talking about the distribution coefficient of a protein in a column; that means, the concentration of the molecule in phase A that is the matrix and this is actually going to be the buffer.

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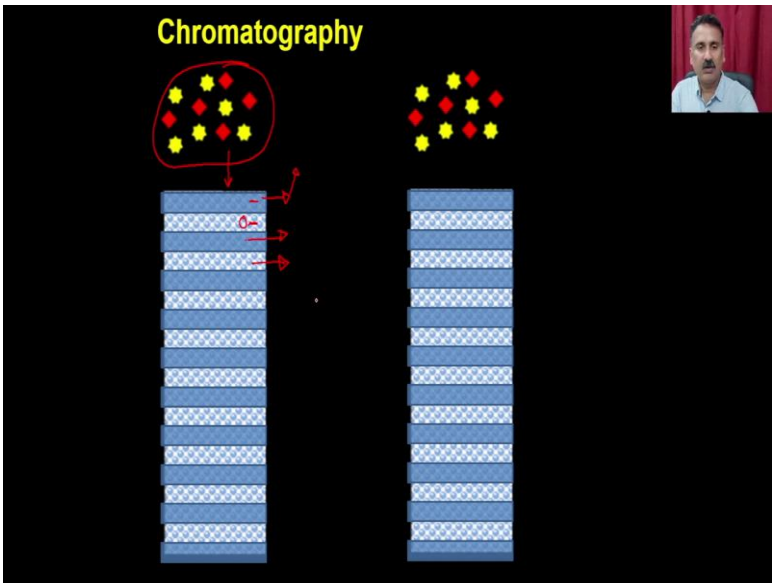

Now, this is all about if you have the mixture of the proteins and you if you are loading them, it is actually going to have the different layers right you can imagine that I have different layers. And all these layers are actually going to interact with these molecule and that is how they will actually going to distribute between the this phase or this phase.

Similarly, when they will enter into this phase it is actually going to partition between this phase or this phase and that is how these molecules are actually going to be separated.

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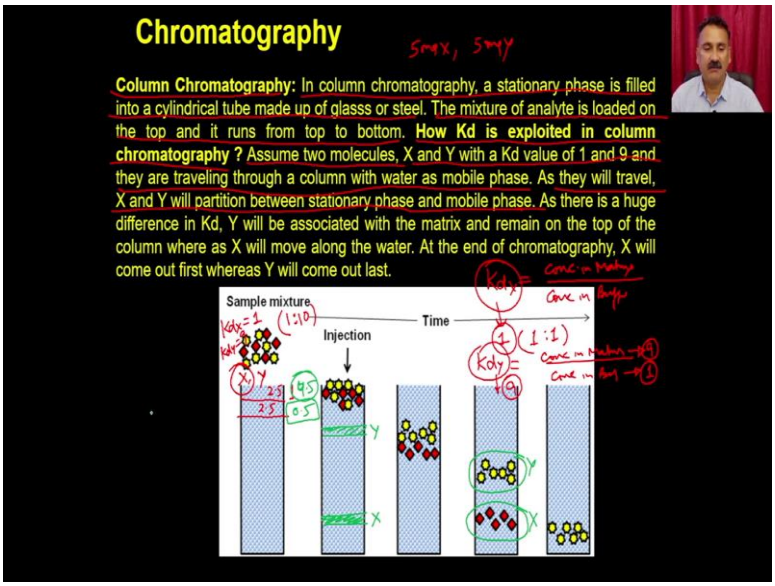

So, how the chromatography is separating the molecules? So, column chromatography in a column chromatography at stationary phase is filled into the cylindrical tube made up of glass or steel. So, this is the cylindrical tube where you have filled the beads right they can be made up of glass or steel. The mixture of analyte is loaded onto the top and it runs from the top to bottom.

Now, how the distribution coefficient is exploited in the column chromatography assume that you have two molecule X and Y. So, if you imagine that you have two molecule X and Y and the Kd the Kd X is 1 and Kd Y is actually the 9 ok this means they are actually going to be get separated in a 1 is to 10 ratio.

So, x and y with the Kd value of 1 and 9 and they are traveling through a column with a water as a mobile phase. As they will travel X and Y will partition between the stationery phase and the mobile phase. So, you can imagine that the X is actually going to be partitions ok, what is if I write the Kd X what is mean by is concentration in matrix divided by concentration in buffer.

And since the Kd value is 1; that means, the concentration in the matrix and the concentration in buffer is going to be 1 is to 1 which means if I imagine that if I loaded the 5 mg of X and 5 mg of Y then what will happen is that if it runs for 1 ml the 5 mg out of 5 mg the 2.5 mg of X is going to be on top and 2.5 mg would be in the second layer ok.

Whereas for the case of Y where the Kd X Kd Y is actually concentration in matrix versus concentration in buffer right and Kd Y is what 9 actually. So, Kd 9 is 9 this means the concentration in the matrix is going to be 9 times than the concentration in the buffer. So, it is going to be 9 is to 1 ok this means if I loaded the 5 mg.

So, this is the condition for the X right if I change the color. So, this is for Y actually. So, ok so, if it is for the Y for Y it is going to be 0.5, 4.5 right in the matrix phase and 0.5 in the water phase ok. Now, you can imagine that if this continues what will happen is that the Y is actually going to be immobilized on top of the column and the X is actually going to immobilize at the bottom of the column.

Because X is actually trying to stay with the buffer phase and Y is trying to remain with the matrix phase and so, this is actually going to Y and this is actually going to be X and that is how it is actually going to be get separated. So, what you see here is this is actually going to be Y and this is actually going to be X and that is how when you elute the X is going to come separate first and the y is going to be get later on.

So, as there is a change in that is there is a huge change in the difference in Kd Y will be associated with the matrix and remain on top of the column whereas, the X will move along the water. At the end of the chromatography X will come out first whereas, the Y will come out later on.

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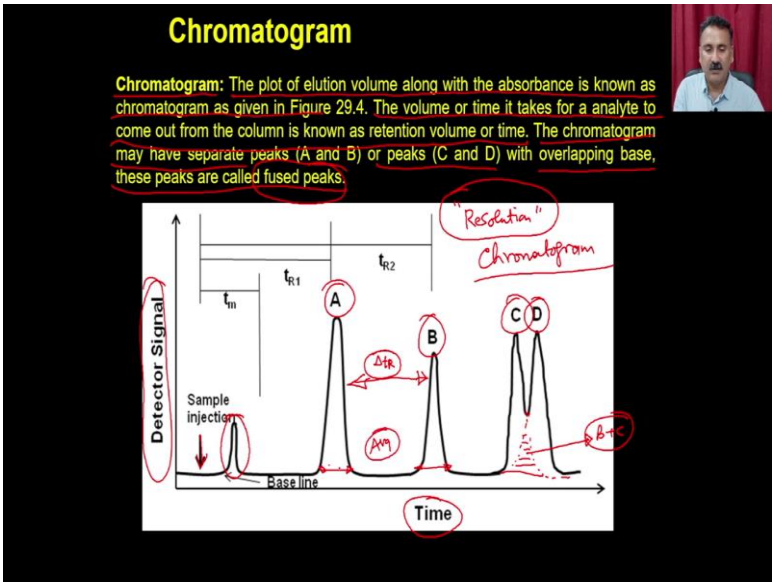

Now, we will discuss about the chromatogram the plot of the elution volume along with the absorbance is known as the chromatogram. So, if you plot the detection signal along with the time then what the pattern what you are going to see is that is going to be called as the chromatogram.

And this chromatogram is going to be give you the information about when you have injected the sample when you are actually going to see the first elution of the first protein or elution of the second protein, third protein fourth protein and fifth protein. And based on this you can be able to decide how well the column is actually separating the two different peaks.

For example, in this case the A peak and the B peaks are getting separated from each other whereas, the C and the D are actually getting merged. And why the C and D are getting merged because the C is eluting like this and D is eluting like this ok. And because of that it is actually say it is actually they are both are sharing a small area and in this small area both the B plus C are mixing to each other.

So, indirectly what we can say is that they both are actually having the overlapping base and these peaks are called as the fused peaks. So, the volume or the time is taken for the analyte to comes out from the column is known as the retention volume or retention time the chromatogram may have separate peaks, such as peak A and B or the peak C and D with the overlapping base and these peaks are called as the fused peaks.

So, in other word we can also say that the resolution or the ability to separate out the two peaks for C and D is also low for this particular column. So, how we can be able to calculate the resolution of this particular column ok or how you can be able to define the resolution of a column.

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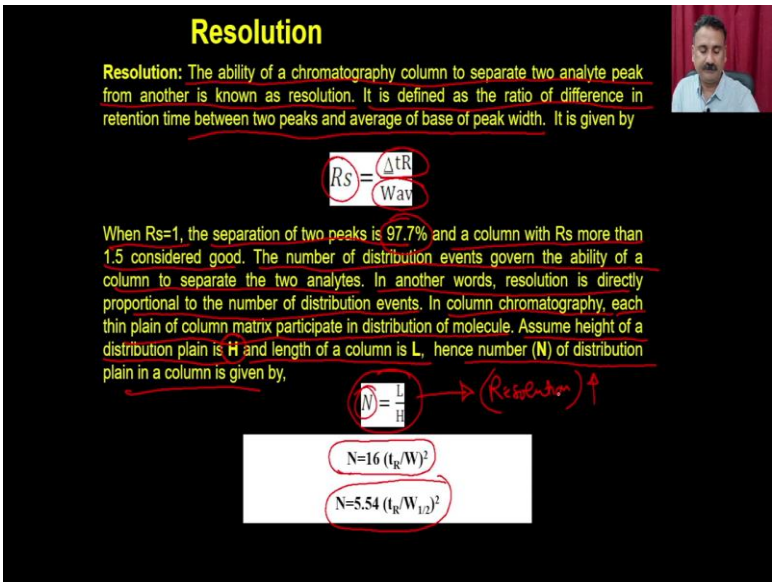

So, resolution is the ability of a chromatography column to separate two analyte peak from another and it is known as resolution. It is defined as the ratio of difference in the retention time between the two peak and average of the base of the peak, which means the resolution is directly proportional to the difference between the two peaks and it is going to be inversely proportional to the average of the base of the peak.

So, what you see in this particular experiment, this is actually the delta TR, right this is the difference in the retention time between the A and B and what you see here is that if I measure the base of the this A wavelength and the B, I can make an average of these and that is actually going to give me the average of the base of the those two peaks and that is actually going to give me the resolution of this particular column.

When the resolution is 1, the separation of the two peak is 97.9 percent and a column with r resolution more than 1.5 is considered the good. Number of distribution event governs the ability of a column to separate the two analyte. In another word, the resolution is directly proportional to the number of distribution events.

In column chromatography, each thin plane of column matrix participate in the distribution of the molecule. Assume that the height of a distribution plane is H which means the peak of the diameter of the individual beads what you are using is H and the length of the column is L, then the number of distribution plane in a column is given by L by H, ok.

Because and if you do this. So, and if you do a mathematical calculation, what you are going to see is N is equal to 16 t R by W whole square, which means the N is 5.54 t R by W under root whole square. So, that is actually going to define the resolution of a column ok and you can be able to indirectly say if this number is very high, it is actually going to say that the resolution is very high.

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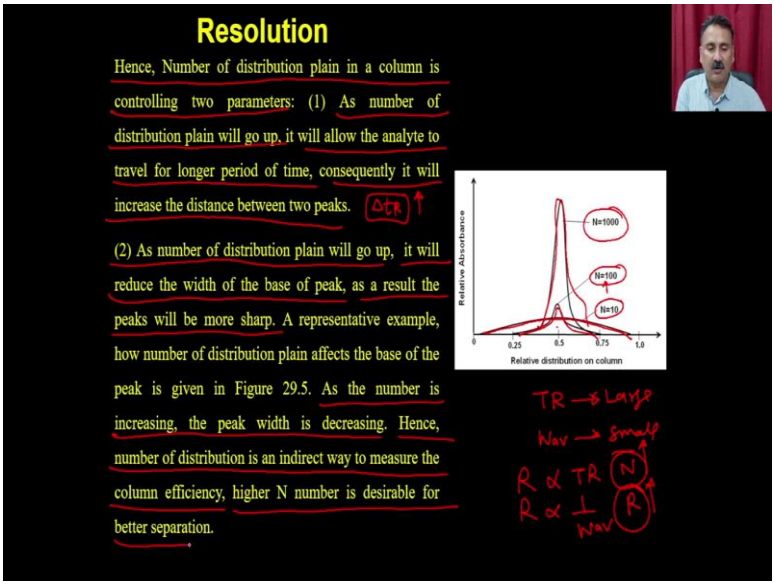

Now, hence the number of distribution plane in a column is controlling two parameters and how the resolution is getting affected by the number of distribution plane. Because as the number of distribution plane will go up, it will allow the analyte to travel for a longer period of time.

Consequently, it will increase the distance between the two peak, which means the delta t R is actually going to be on a higher side. Whereas as if the number of distribution plane will go up, it will reduce the width of the base of the peak as a result the peak will be more sharper ok. You can imagine that this is the word representative image.

So, if you have the N is equal to 10, you are going to see a flat peak actually. If you increase the number only by 10 times, you are going to see a peak actually, ok. But if you increase the number further up by 10 times, ok you will see that the width of the peak base of the peak is reducing which means that if you increase the number of distribution plane, you are actually going to have the larger TR.

Right So, TR is going to be large and the Wav is going to be small. And you know that the resolution is directly proportional to TR and the resolution is inversely proportional to the Wav, which means if N will go up, the resolution is also going to increase. And that is how the we are always saying that when you are packing a column, you should be able to ensure that the packing should be very compact.

So, that the number of distribution plane should go up. As the number is increasing, the peak width is decreasing. Hence the number of distribution in a way, the indirectly indirect way in the number of distribution plane is an indirect way to measure the column efficiency higher N number is desirable for a better separation.

So, apart from the column chromatograph columns, what you are going to use in a column chromatography, you are also going to use a chromatography system which you are going to use to run the column and as well as to monitor the illusions.

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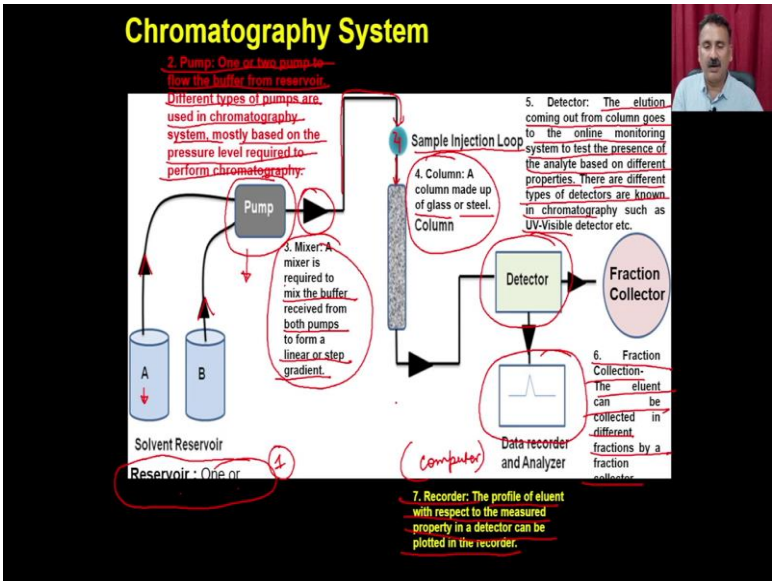

So, in a typical chromatography column, you have the multiple materials, you are going to have the reservoirs. So, number 1, you are going to have the reservoir number 2, you are going to have the pump number 3, you are going to have the mixer number 4, you are going to have the sample injection groups. And number 4, you are going to have a column, right.

So, this is the column number 5, you are going to have the detectors and number 6, you are going to have the fraction collector and number 7, you are going to have the recorder. So, reservoir is nothing but the. So, you can have the one or more reservoirs in the in the chromatography system. You can have the two reservoirs A for so, both for the individual buffers in some of the chromatography system you will find the four reservoirs. And then you can also have the pump.

So, you can have one or two pump to flow the buffer from the reservoir different types of pumps are used in the chromatography system mostly based on the pressure level required to perform the chromatography. So, chromatography systems will have the one or two pumps and they are actually going to be connected to the reservoirs. So, one pump is going to take the liquid from the A buffer, A reservoir and the other pump is going to take the liquid from the other pump.

And depending on the pressure levels the pumps can be of low pressure columns or the high pressure columns. Then you can have the mixer. So, just after the pump you are going to have a mixer. This mixer is required to mix the buffers, but is received from both the pump to form a linear or the step radiance. Then we can also have the column.

So, once you have the you know the buffer what is going to be prepared it is going to flown into the column. This column can be made up of glass or the steel. So, depending on the pressure what you are going to use you can have the for the low pressure chromatography or middle pressure chromatography you can use the glass columns, but for the high pressure chromatography you will have to use the steel chromatography steel columns.

And after this column you are going to have the detectors right. So, detectors are actually going to detect when the liquids are passing through that particular detector. So, the elution comes out from the column goes into the online monitoring system to test the presence of the analyte based on the different properties.

These are different types of detectors are known in chromatography. You can have the UV visible detectors, you can have the fluorescence detector, you can have RI detectors, you can have the mass spectrometry and so on. Once your detector is going to give you and detector is going to give you the pattern in terms of the you know the chromatograms.

At chromatogram you are going to visualize on the data recorder and analyzers and or the recorders actually. So, the profile of the eluent with respect to the measured property in a detector can be plotted in a recorder. So, recorder is nothing but a computer nowadays.

So, on the computer screen you are going to you know monitor the recorders and based on this you can actually be able to collect the fractions to collect the particular protein into a different fractions using the fraction collectors. So, in a fraction collector the eluent can be collected in different fractions by a fraction collectors.

So, this is all about the chromatography system and I would like to take to my lab for a small demo. So, that student will actually explain you how to connect the column, how to perform the chromatography using a chromatography system from the GE Health Sciences. In this video we will show you how to operate a FPLC instrument and the basic principle laying mechanism of separation of the protiens using FPLC.

FPLC is nothing but fast protein liquid chromatography we can say it is a derived version of HPLC. The main difference between HPLC and FPLC is FPLC can only be used for the separation of the protiens and sometimes small molecules also if we have a columns available, but in HPLC we can use columns separation of small molecules ah.

Suppose if you have enantiomers that also can be separated using HPLC. The columns what we will use for HPLC and FPLC are also differs. In case of FPLC we will use plastic columns, but in case of HPLC columns we will use steel columns. Because the main reason why we are using like this is if you use stainless steel columns in FPLC because of the salts and high concentration of the salts and different materials we are using. Let me pour out the steel.

So, that is why there may be improper separation of the component which we want to separate. So, that is why we will use only plastic columns in this one. The separation of the proteins in through FPLC is based on the size and shape of the protein. So, if you have like in gel filtration chromatography or size exclusion chromatography in all those mechanisms like size exclusion gel filtration can be applicable in FPLC also.

So, it depends on what column you are using. So, if you want to purify histone proteins, you can use nickel NTA column pre-peptide column. Suppose if you want to only

separate high to low molecular weight proteins, you can use any gel filtration column suitable for your protein. But the principle behind the separation is same.

So, these details of like size exclusion or gel filtration we have shown in previous video. In this video we will show how this instrument can be operated.

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We are in our lab we are using octa blue this is from GE Health Sciences. So, all the component whatever we show it is similar in other instrument or other companies instruments also, but only the architecture of the instrument changes. So, let us see what are the parts it contains. So, this is the instrument it is connected to a system for observation purpose.

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So, in this system it contains this stationary phase this is the column, this is the mobile phase; that means, buffers this is the area where we will keep all the buffers. So, it starts with the pumps actually. So, these two are pumps this is one pump and two pump these two sets whatever buffers are coming from this buffer spray it will enter here ok.

These pumps whatever the pressure they are getting they can be regulated here this is the pressure monitor this. So, once here the pressure is monitored it will go to mixer this is the mixer where two different buffers. Suppose if you are using if you are purifying through nickel NTA column.

So, in that case you need imidazole in separate buffer prepared and one is the equilibration buffer. In that case if you want to elute that protein particular (Refer Time: 22:47) protein you have to mix both the buffers A and B for instance. So, those buffers can be mixed here once the mixing is done it will directly goes to the inlet loop.

So, once it entered into inlet loop it will go to this chamber where it can be connected to the column. So, the top portion of the column it will connect here we will show you how to connect the column in coming video. So, after that whatever it comes it will enter here it will enter here and it will come directly into the UV chamber where the eluted component will be detected.

So, starting the instrument there is a power button right side of the instrument you have to just turn on that instrument then you can see a white light is blinking here; that means, the system just started. So, after that we will go to this software part. For analysis of any elution, we can use this it comes with the instrument it is the unicorn software. We use for analysis purpose.

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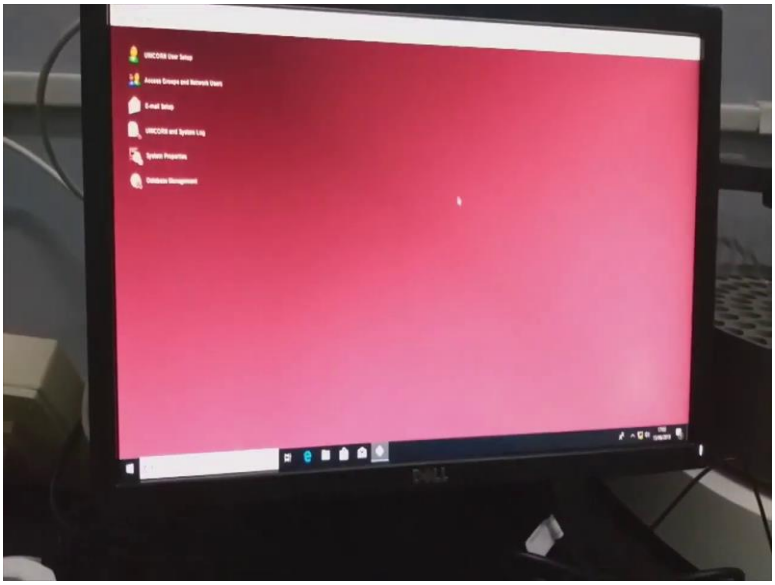

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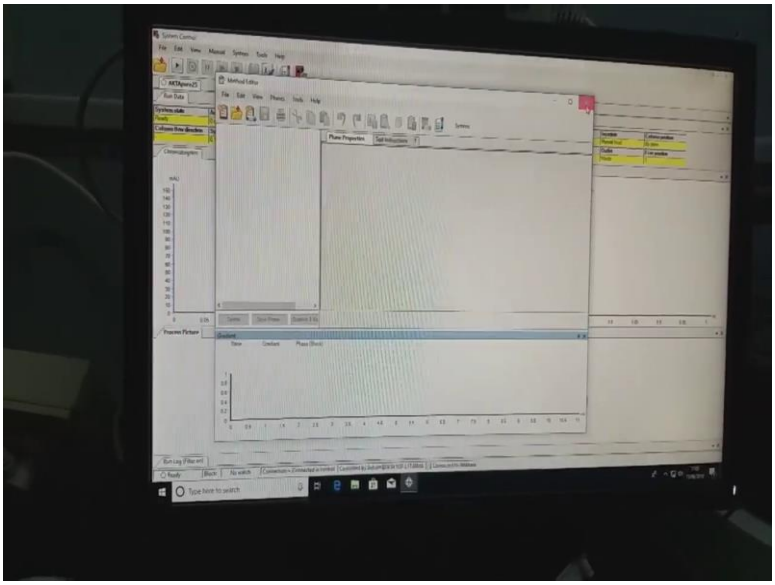

So, just to double click on that one and it will take you to the software.

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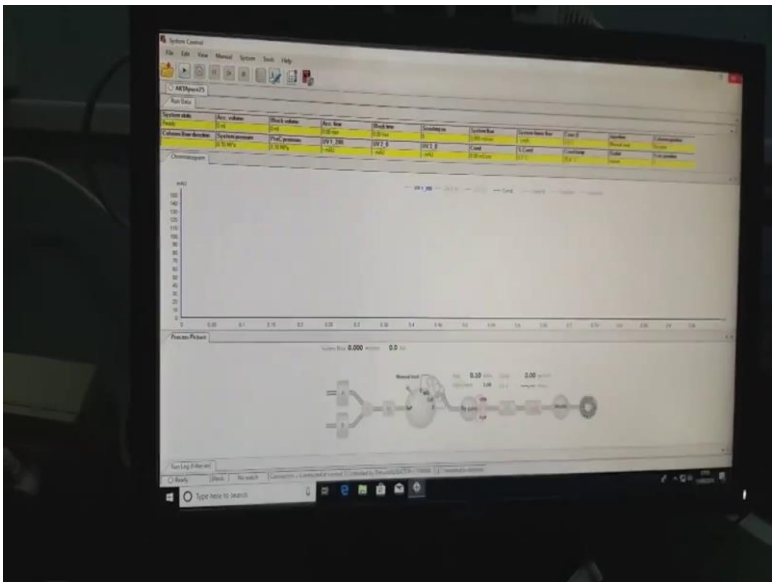

So, it will give you three pop-up windows one is method editor, another one is system control and evaluation classic. So, this is system editor where you can see chromatograms.

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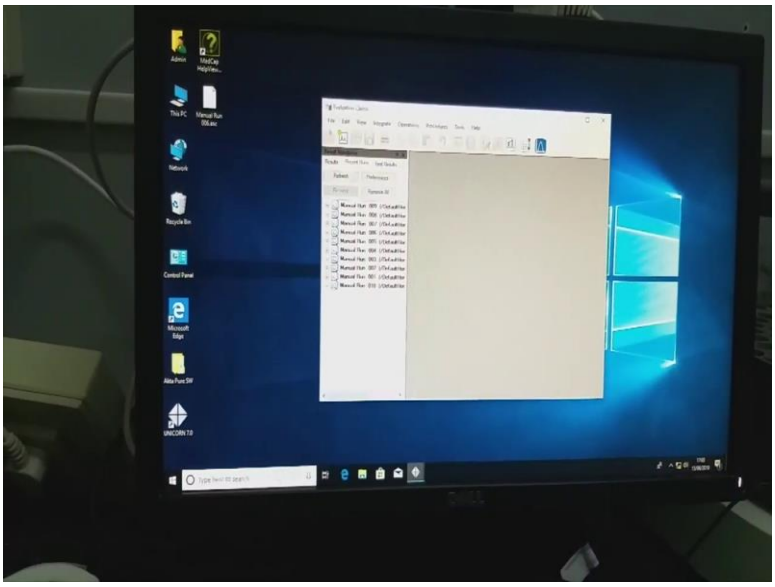

And the other one is evaluation classic where you can analyze your chromatogram.

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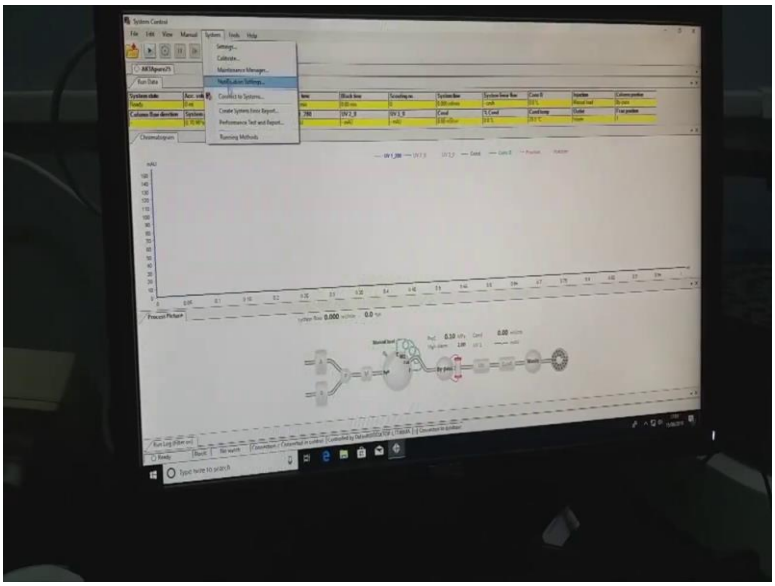

Just go for system control the first thing we have to do is connect the instrument.

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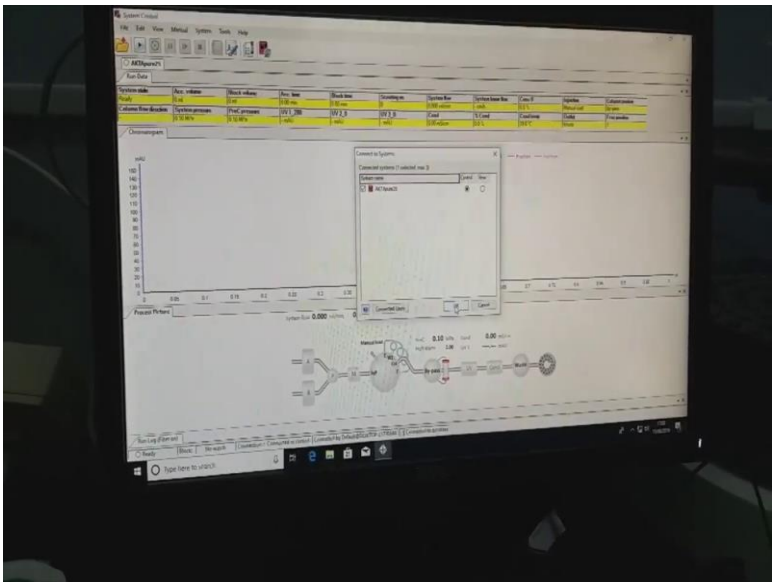

So, here you can see the connected instrument octa pure 25. So, just say ok. Now it will connects the system.

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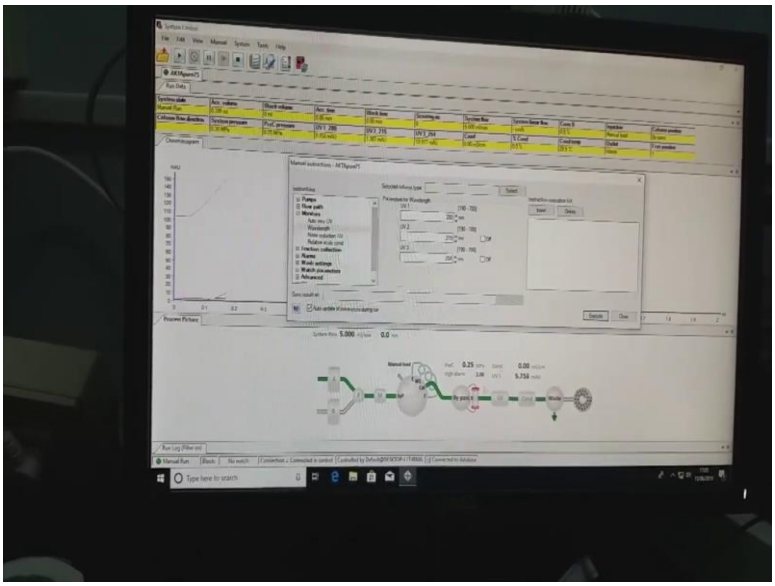

So, this will give different we can change a different commands using the software just go to manual execute manual. So, this is the manual instruction software or dialog box where you can change things. So, here different parameters you can change through this pop-up window like pumps flow path and various parameters such as monitors next to go to pumps.

So, here you can change the system flow. So, we can keep up to 20 ml if there is no column connected. So, normal condition you we can keep 5 ml also. So, you just say insert this thing in order to executed by system. Now, here it is monitors very important thing we have 3 wavelengths here we can monitor at 3 different wavelengths. So, your choice you can give. So, we are giving 280 259 254 just insert and say execute.

So, it started you can see the green path is highlighted and also chromatograms appearing in the chromatogram area. So, it will give 3 different chromatograms. So, one correspondings to blue that is 280 nanometer for tryptophan, tyrosine fluorescence. Second one is 254 nanometer for RNA or DNA related and third one is 215 for peptide.

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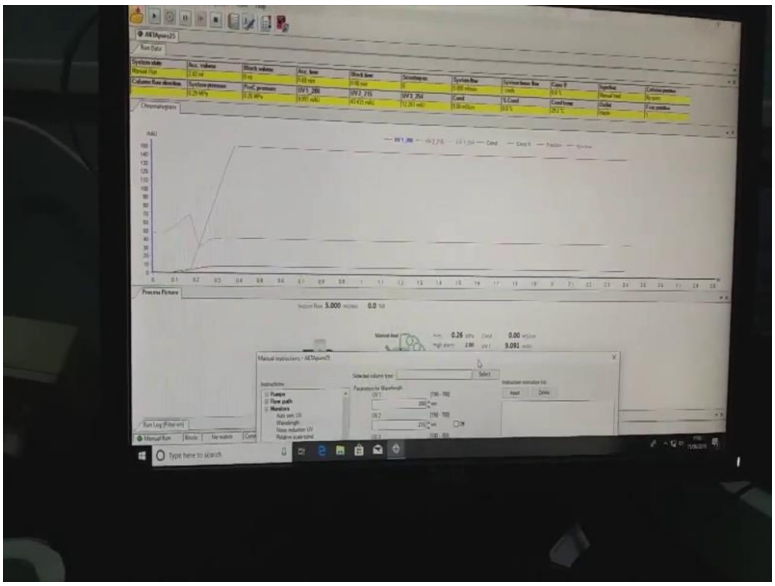

So, here we can see the path of the flow how it is connected starting from buffer A. So, here buffer A and it will go through the pump and mixing through mixer it will go to all the way to waste. So, here different parameters we can change during running we can change B also. If you want to change B you just say a start pump B. So, see we can see highlighted area.

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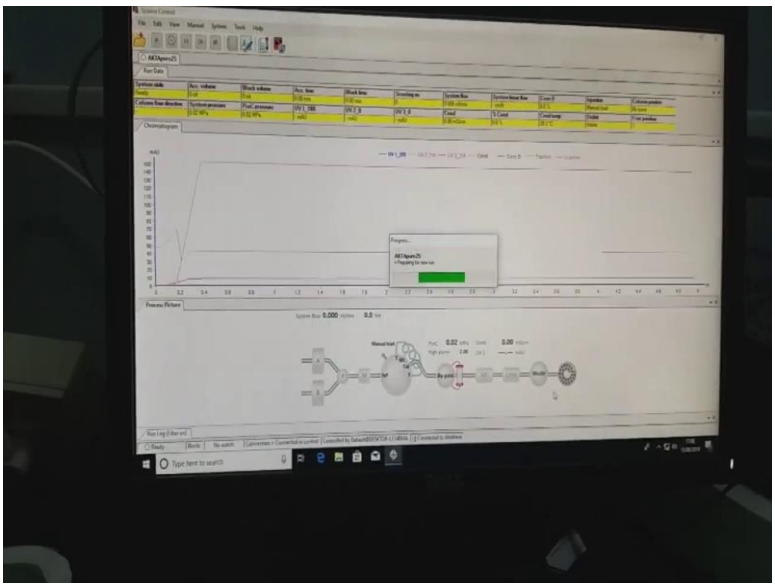

If you stop the program it will say, automatically see you can see a some dialog box appears preparing for neuron.

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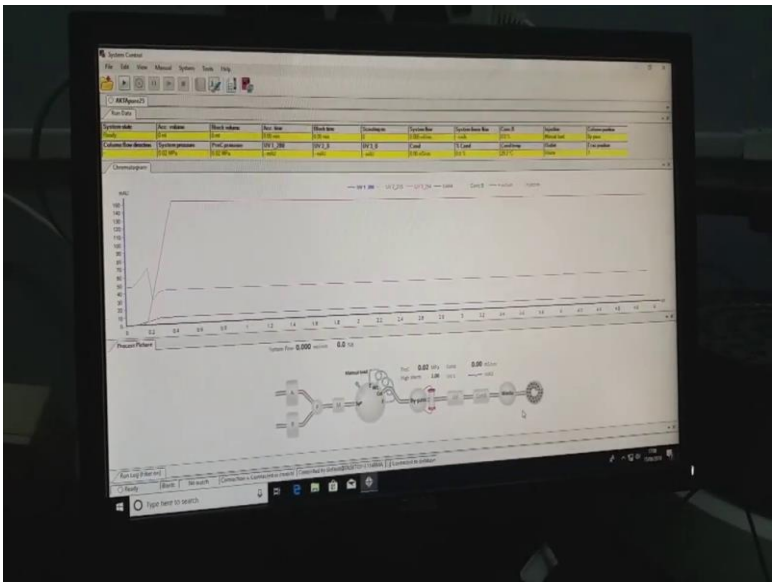

This is software introduction. This is these are the buffers we are going to use for this demo. The buffers need to be filtered through 0.2 micron filter and also degassed for the degassing purpose we will use bath sonicator. So, it will remove any air any air for a bubbles present in the buffers it will remove those things.

So, we are using most common buffer that is phosphate buffer with having pH 7.4 and this is the (Refer Time: 30:03) and this is 20 percentage ethanol all the buffers were filtered through 0.2 micron filter paper and also degassed. So, we have washed already the system already being washed. So, now what we will do we will connect the card. So, here precautions need to be taken while connecting this thing.

So, if you have any air bubbles through these loops or the or the piping system it will directly enter into column which will destroy it. So, to prevent that we have to make sure all the loops and pumps got washed thoroughly and then we will connect in running condition. Before connecting the column we have to remember few point.

This column whatever the beads are there this is in 20 percentage ethanol. So, if you directly connect it already ethanol is there whatever the flow rate, we are giving it will give more back pressure. So, the width between the distance between the column filter and the beads a settled beads may increase. So, that will reduce efficiency of the column.

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So, what we will do we have to change this pumps into water. So, we changed into water. Now, we can connect to the column. Here also some of the precautions needs to be taken. If you are using chilled buffers ok suppose you need cold buffer. So, that means, you have to bring those all the components of the system to the temperature which you want to use for your purification.

Otherwise, if you having a chilled buffer which directly enter into column that may clog or precipitate some of the salts present in the buffer inside the column. So, that will also reduce the efficiency. So, this is also need to be taken care while running the FPLC. Before connecting the column, we need to adjust few parameters.

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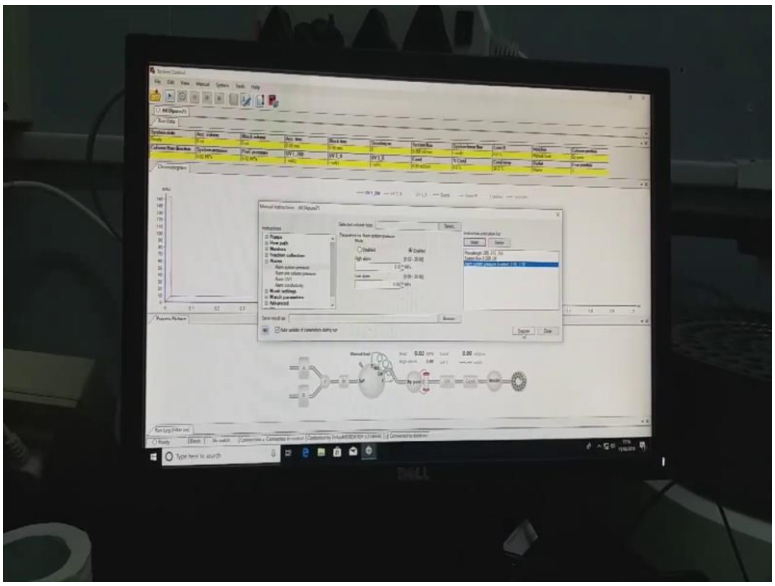

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So, here this is the software system flow I am keeping 0.5 ml per minute since we are going to connect the column. So, if the flow rate is more then the pressure alarm may come. So, after that we have to set the monitors. So, this is also I am going to set system flow 0.5 insert and now we have to set alarms.

At what pressure you need to get alarm. So, I am keeping this point 8 is fine. So, once it is done you can insert then execute. So, next here this is the column connecting portion.

So, where here this is the upward portion where we have to connect with the column. Now, we are not using column.

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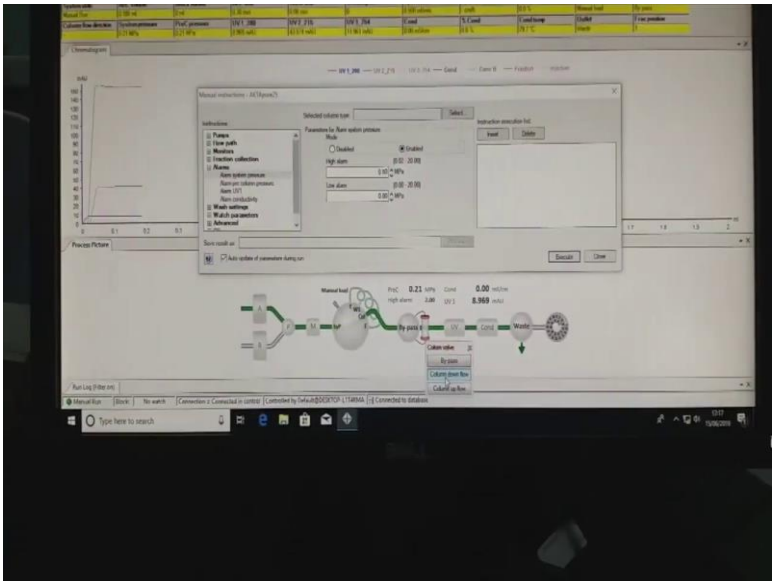

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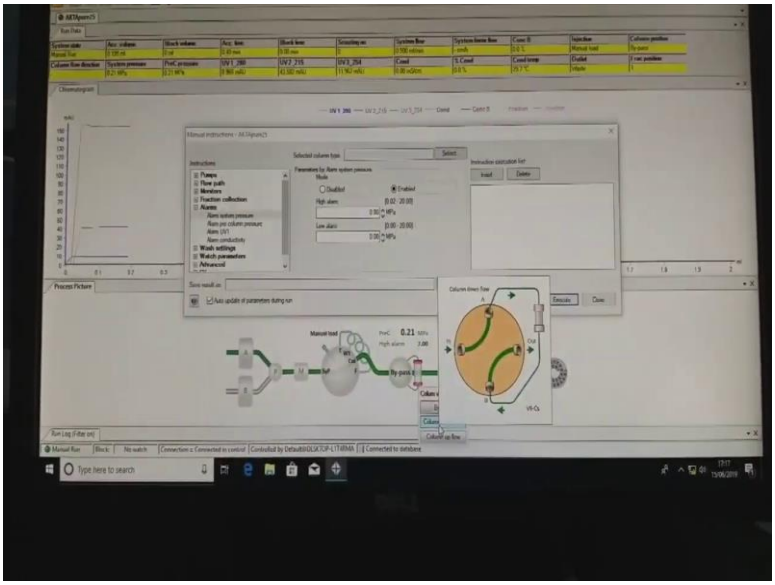

So, just we have to go to the column we just click this one column down flow so, from top to bottom. Now, you can see highlighted one. So, you cannot directly connect the column. First you have to fill the buffer or water in this loop. So, that there is no air bubbles. Just open the top of upside up the column ok.

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With this buffer itself you just directly connect. After connecting you have to take out the lower portion of the column otherwise it may burst also, but it is not the case.

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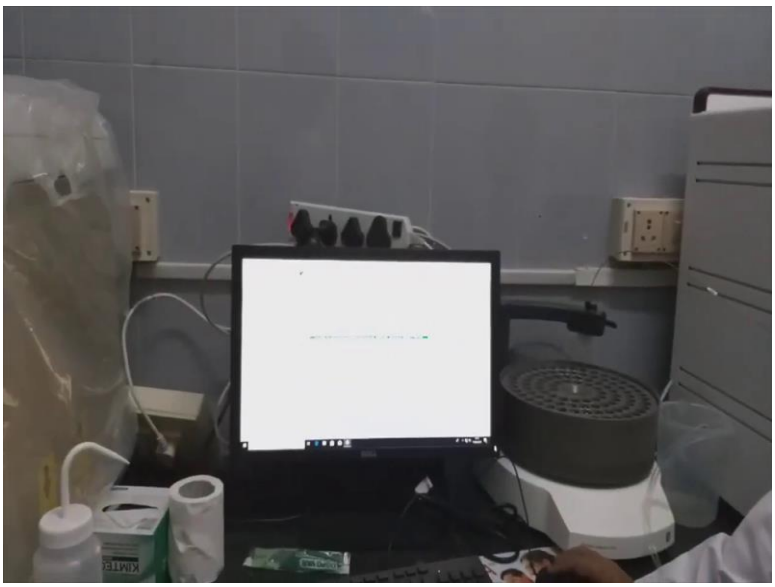

Because if is if there is any high pressure you will get alarm.

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So, here this is the bottom portion of the alarm. You can see the buffer here if you if the buffer is passing through the column. So, as we can see there is a fill up of water in this thing. So, once you see complete fill up of the this loop or this knob you can directly insert the lower portion.

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Once you see the buffer filling in you just have to connect with the downward portion. So, now the column is connected to the system and you need not to touch anything everything will be operated on the software.

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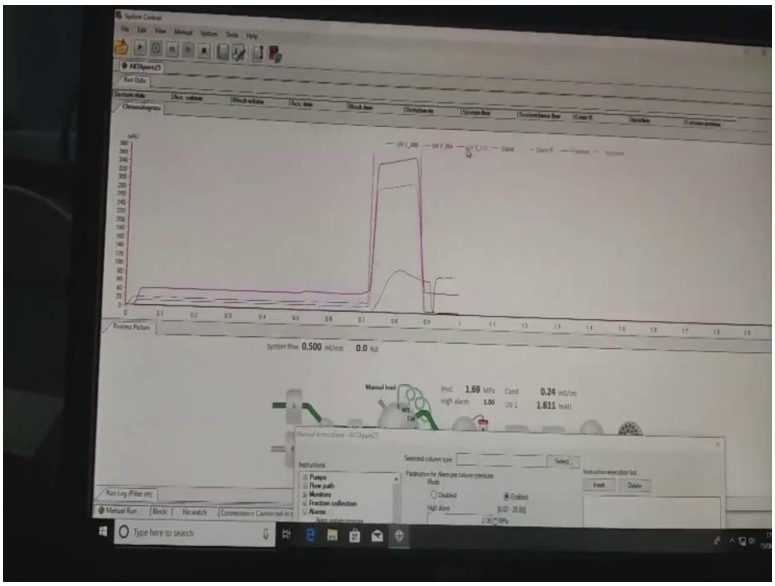

So, here see once the column is connected you can see there is a change in the different UVs and conduction of the buffer. This is the red one is the sorry this. Gray one is the per conduction and that green one is the concentration of the and these are three different units. Now, we are washing with the water. So, after once completely we removed 20 percent ethanol then we will equilibrating the buffer.

So, that the main purpose of the equilibration is suppose if you are prepared your protein solution in a suppose say phosphate buffer. So, you have water you are not equilibrated with the phosphate buffer. Then you cannot expect good resolution of good separation of the proteins and also the proteins may not be stable in the other condition like in water.

So, they may degrade or they may not be useful if you are interested in the enzymatic reactions. So, that is why we always do equilibrate with the equilibrate the column in the same buffer which our protein of interest is desirable. So, this will also helps good resolution and keep the intact of the structure of the protein.

So, we are indirectly we are providing similar conditions for the protein. So, it will behave in native condition. So, we completely washed out the ethanol whatever present inside the column. Now, we will equilibrate in the phosphate buffer. So, as we can see here the conductance is completely comes to 0.

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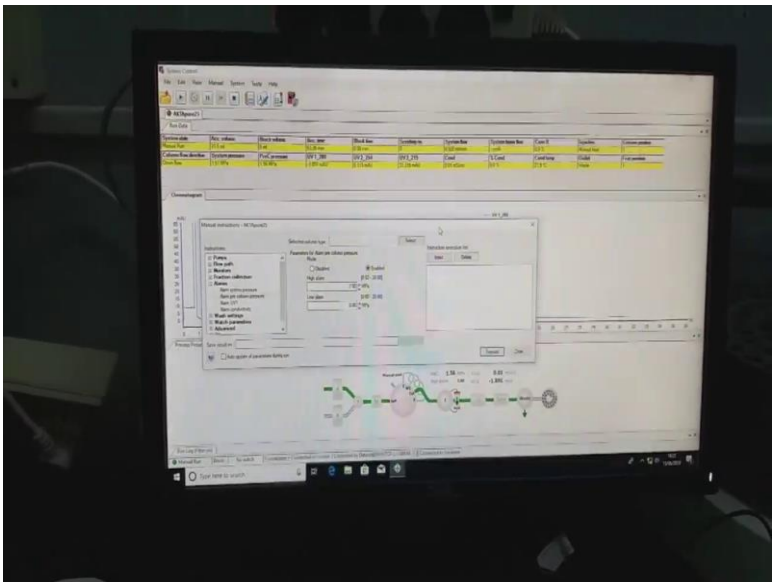

And we can see there completely flat line flat signal corresponding to UV 280 So, that means, there is no ethanol inside the column. In addition to that we already washed 30 ml of water. So, the total column volume inside the column is it is around 25 ml. So, we also washed with 5 ml extra. So, we can be sure that it is completely removed.

Now, what we have to do is we have to just pass the things whatever we said already without disturbing anything. So, here we can see a pause symbol.

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We just pause then come here and change these two buffer. So, once that is done, we will reset these things to continue mode.

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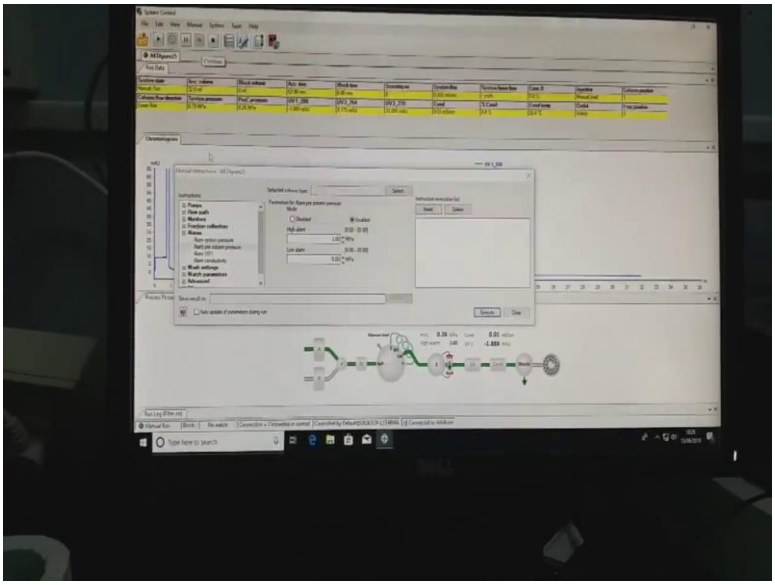

So, we can see it is again activated. So, we have equilibrated the column using the equilibration buffer. So, as we can see here there is a stable line corresponding to UV 280 and there is no other elution coming out. So, with this we can confirm that we equilibrated the column properly. So, it is time to inject the protein solution and (Refer Time: 40:16)

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So, for injection purpose this is the port where we are going to inject the solution protein solution and this is the loop Whatever we inject through this injection valve it will be stored inside the slope. The size of the loop depends on how much protein you want to inject and the column capacity.

So, we have 13000 HR column also it is it can it can be used up to you can inject up to 1ml. So, in this column we cannot inject that much. If you want to inject, we have the compatible metals tube these kind of loops. So, this we will connect as shown in here and we will use for the injecting the protein solution. So, what we will do is we have to set few parameters here.

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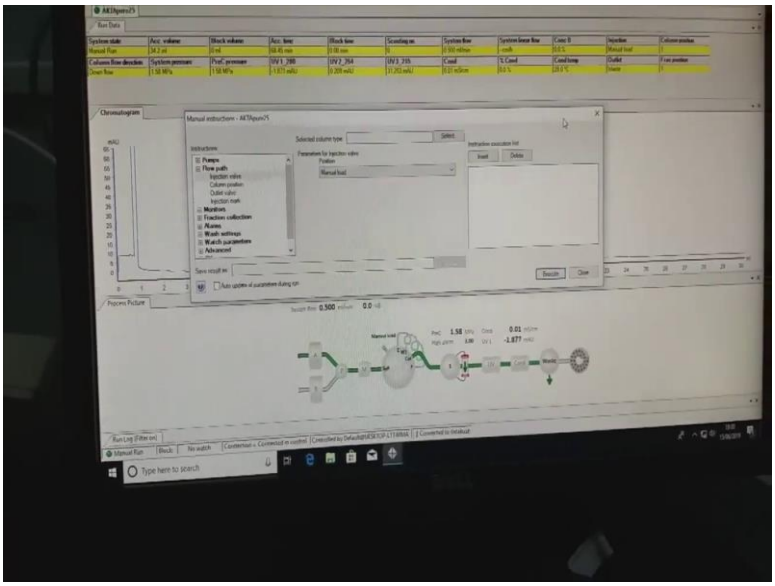

So, here flow path. So, injection valve you have to show it inject here. So, insert this one. Once that is over, we just have to insert the protein solution and execute the command.

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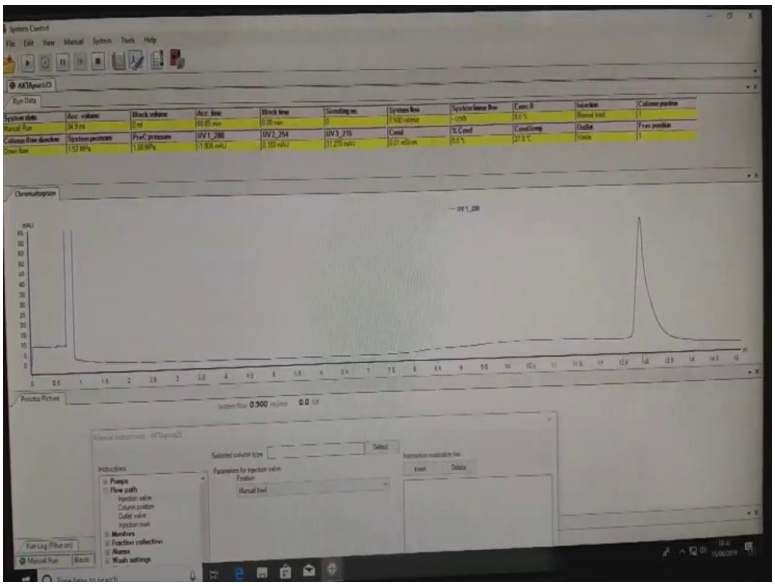

So, as we can see here the chromatogram here the protein is eluted so, at 13 ml of retention volume. So, if you want to say if you want to identify the protein molecular weight determine the unknown proteins molecular weight you have to run this kind of analysis like you need to be known that what is the protein molecular weight known protiens molecular weight.

So, which is available actually I mean commercially available. So, you have to take that protein and just inject based on that you have to construct a calibration curve between large molecular weight and the KV that is partition coefficient which is calculated based on the elution volume, subtracted with wide volume divided by total volume, subtracted with the wide volume that will give partition coefficient.

So, whatever you will get? You will get a graph straight line and based on that straight line you can get unknown proteins molecular weight. So, that can be discussed in size exclusion chromatography video.

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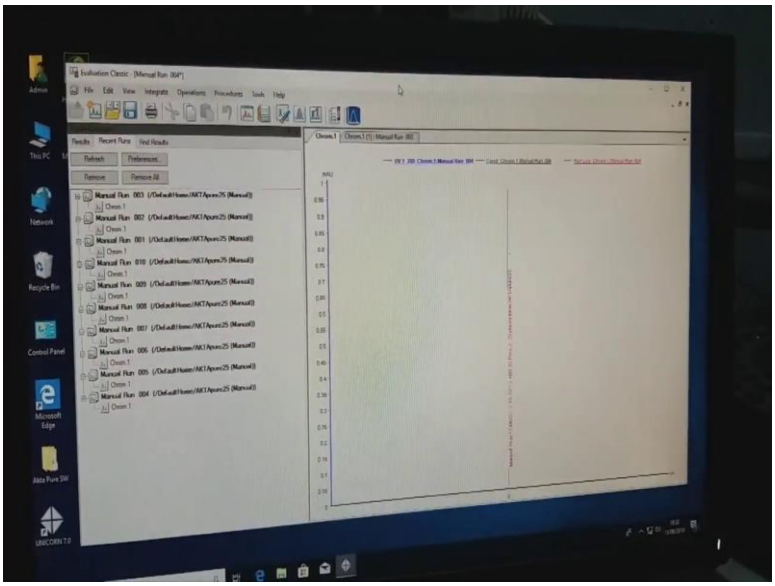

Now, we will show you how to analyze the results. So, this is the software used for evaluation purpose, evaluation classic.

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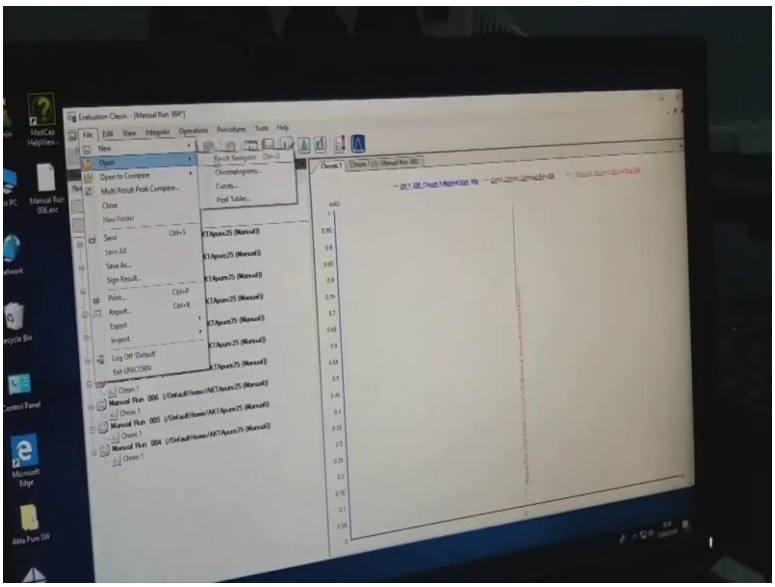

So, you have to go to file open chromatograms.

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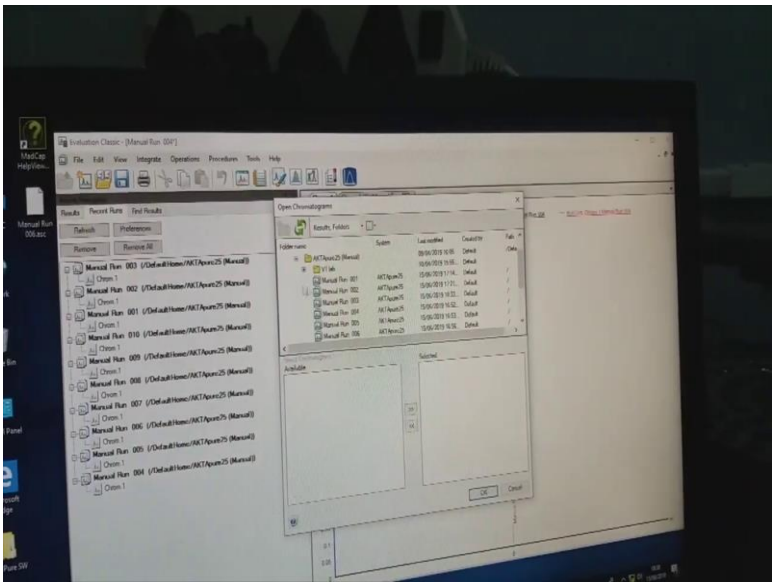

So, you have to look at where your file is kept just open that one and say ok.

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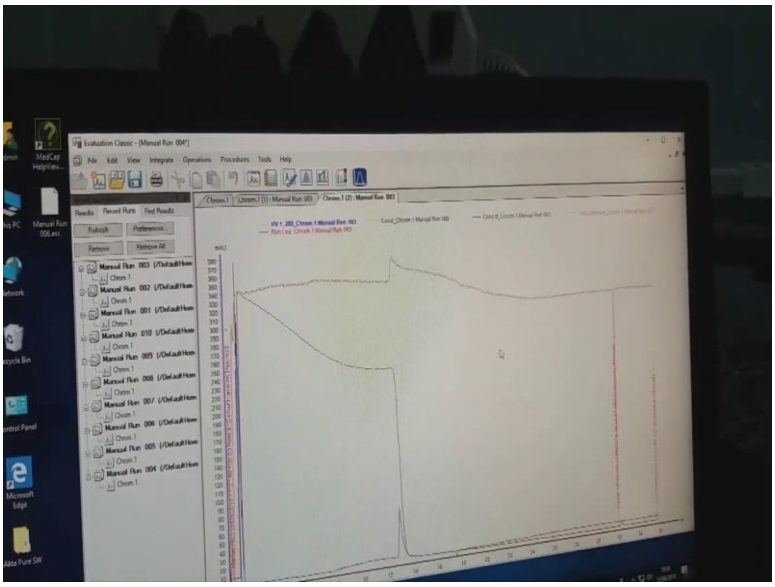

So, here you can you are seeing so many things this one correspond to pressure and conductance.

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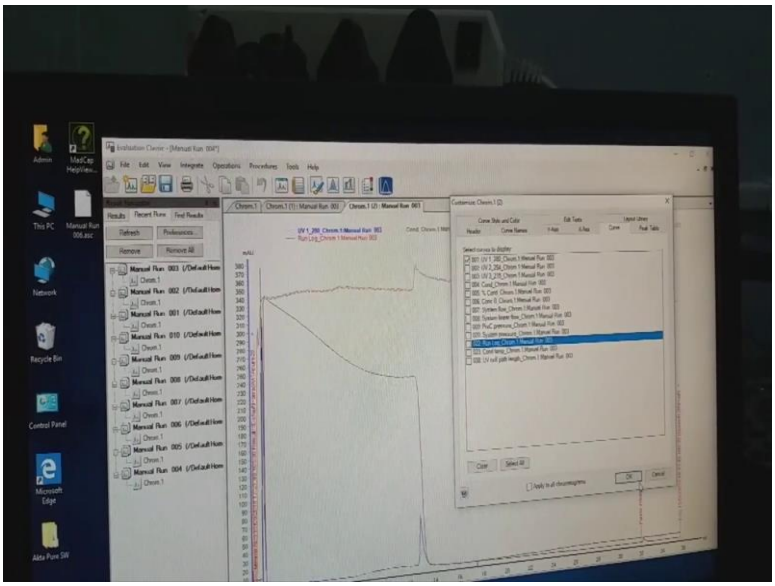

So, you can customize the things like what you want to see in the chromatogram is only UV 280 So, you just keep those things and remove all those things ok.

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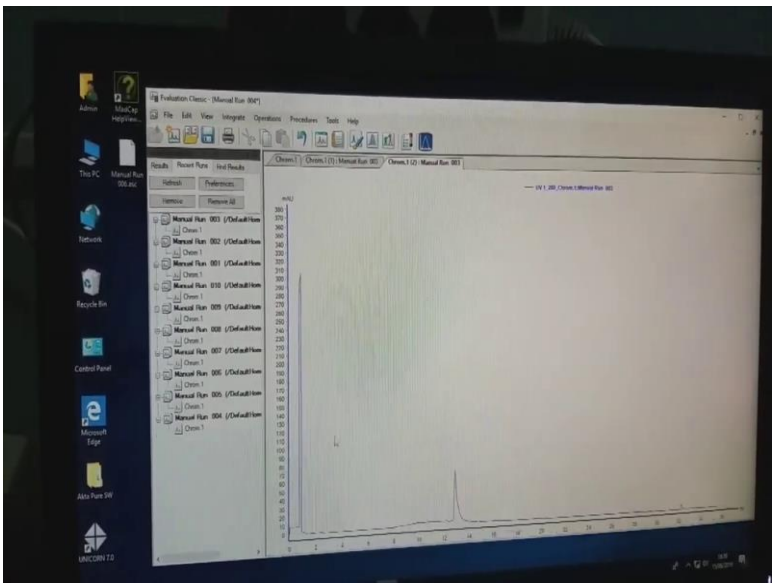

And here also it is showing 380 you do not need 380 you want to take up to 65.

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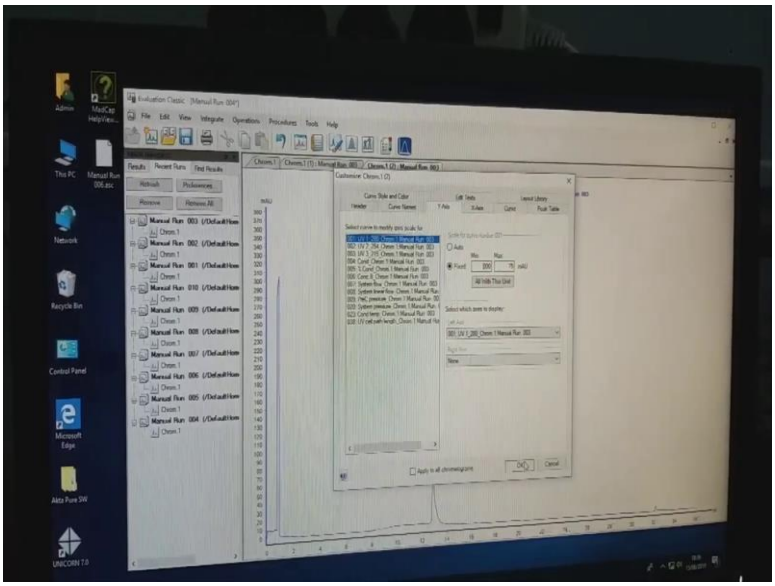

So, you just go and change the y axis.

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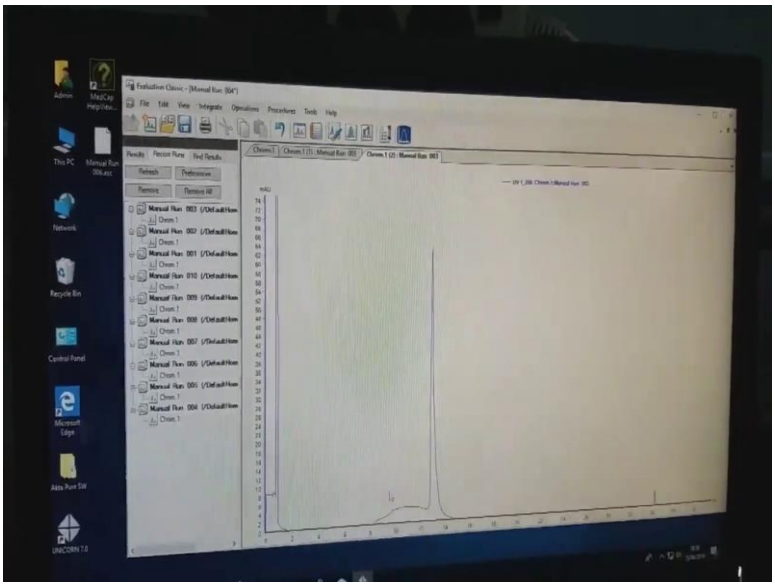

So, like so, you can see this is the chromatogram peak is very sharp. So, you can also integrate the peaks.

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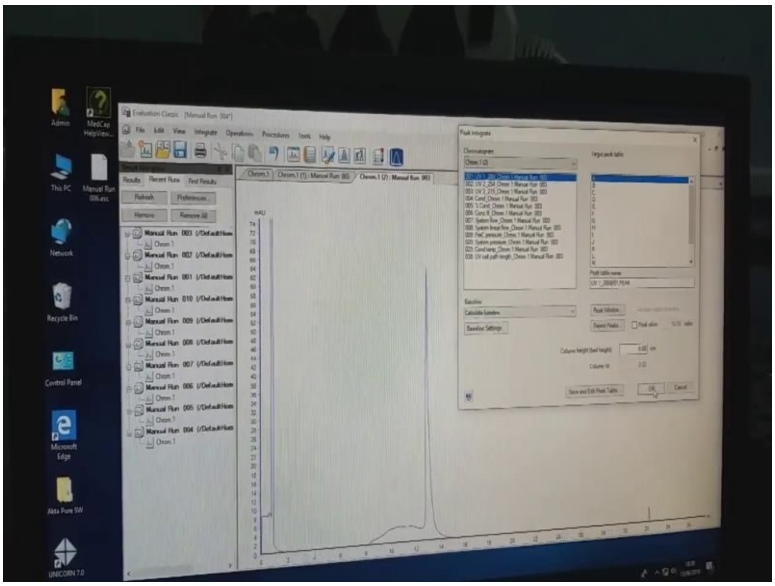

So, this is UV 280 integration.

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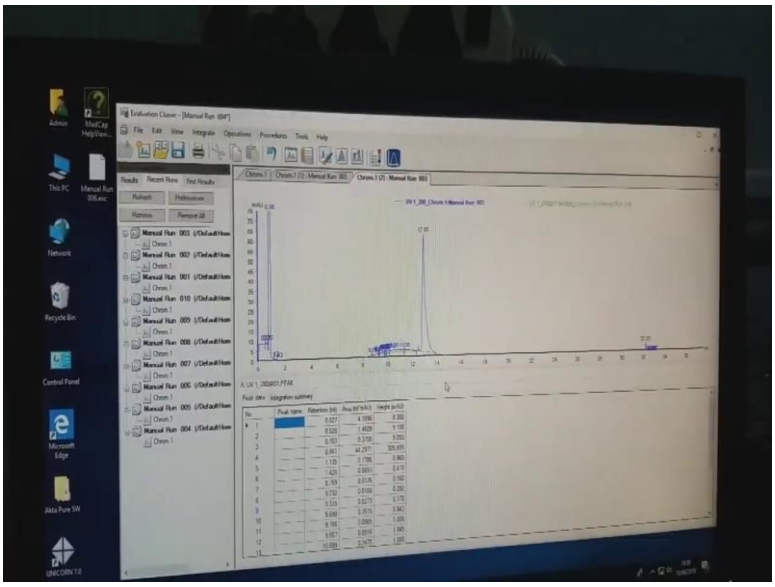

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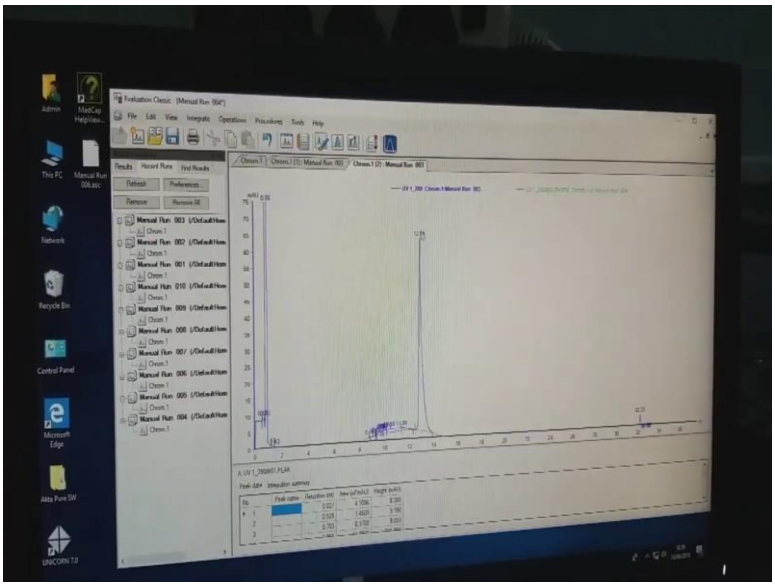

So, just say so, it will give the exact retention volume of the each and every peak how many peaks are present.

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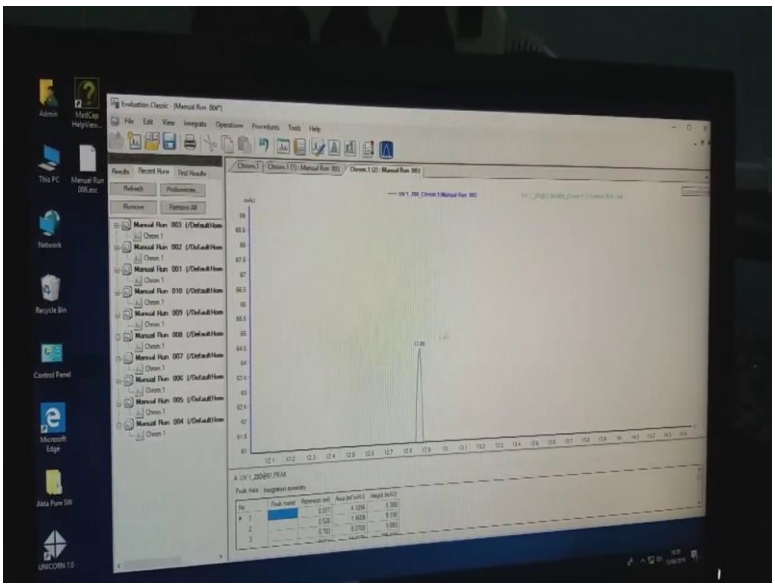

So, here we can see 12.86 this is the major peak what we have. So, with this you can analyze the results. So, if you want to calibrate you have to calculate retention volume for all the proteins whichever you are using for calibration and construct the calibration curve between partial coefficient and lab molecular weight.

So, with this you can identify unknown proteins molecular weight. So, in this particular FPLC demo we showed you how the instrument works what are the different parts and what are the precautions we need to take while running the instrument and how the software works and how to analyze these things.

So, another point we for that actually this is the fraction connected. So, while your protein is eluting suppose your protein is eluting at 12. So, started at 12 you want to collect fractions from that time onwards till the end of the elution So, what you can do you can use fraction character also from main window this is the fraction character it will automatically moves. So, here it contacts the sensor, but you need not to touch anything ok.

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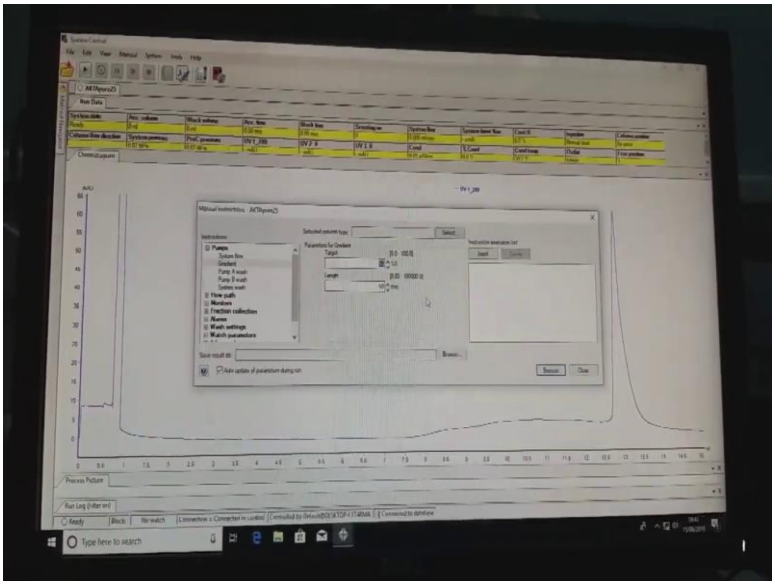

So, in main system control here fraction collector there is a option for fraction collector. How many fractionation you need to be done when you need to stop fractionation and how much feed to fractionation outlet wall all these things you can set there. In addition to that you can also set system gradient flow.

Suppose you want to elute a protein with the gradient you do not know at what concentrate at what particular concentration of imidazole if you are using nickel NTA column or at what particular concentration the protein higher or lower molecular weight elutes.

So, with this you can just adjust the concentration and length you have to give in suppose 60 minutes. So, what system will do it will over 60 minute of time it will increase 0 to 50 percentage So, you can do this one reverse gradient also. So, first you will give 15 and time you can keep just suppose 1 minute.

So, from time when you start the system it will starts with the 50 percent of the B and reduces to 0. So, all these things will make you familiarize how the system works with the first protein liquid chromatography. So, hope these things will help you to achieve your goals in your research.

Thanks for watching. Now, we understand how a fast protein liquid chromatography works. So, after using the instrument we have to from buffer to water we have to change the valves. Because if you are directly keeping in the 20 percent ethanol it may the ethanol whichever present in the buffer and the proteins or salts present in the column then it get precipitated and clog the column.

So, it is a better practice first you change this buffer system to water then wash thoroughly whatever the salts present inside the column it got eluted then again you change it to the 20 percent ethanol for preserving purpose that need to be remembered for better performance of the system.

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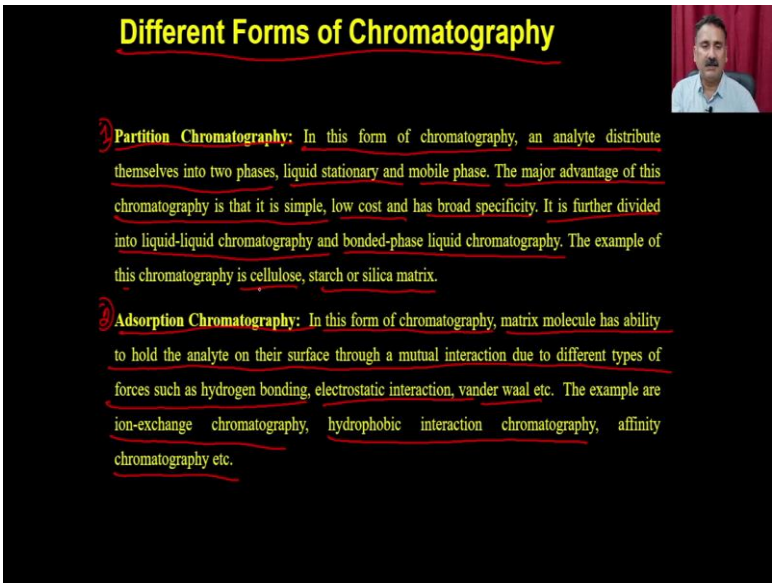

Now, we have the different forms of the chromatography you can have the partition number 1 you can have the partition chromatography or number 2 you can have the adsorption chromatography. In a partition chromatography in this form of chromatography an analyte distribute themself into the two phases, liquid stationary phase and the mobile phase.

The major advantage of this chromatography is that it is simple low cost and had a broad specificity. It is further divided into the liquid-liquid chromatography and bonded phase liquid chromatography. The examples of in this chromatography is cellulose starch and silica matrix.

Whereas, in the adsorption chromatography in this form of chromatography matrix molecules has ability to hold the analyte on their surface through a mutual interaction due to the different types of forces such as hydrogen bonding, electrostatic interactions, Vander wall forces and so on.

The examples are ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography and so on. So, these are the different forms of chromatography what you can actually be able to use you can have the partition chromatography where the molecule are actually going to be partitioned between the liquid stationary phase and the mobile phase.

And you can also have the absorption chromatography where the molecule is going to be absorbed onto the matrix molecules and they will actually going to hold that molecule for a very, very long time. Because the groups what is present onto the matrix are going to perform going to show a different types of interaction with the analyte molecules like such as hydrogen bonding, electrostatic interaction, and Vander waal forces.

So, this is all about the how you can be able to process a sample and how you can be able to recover the protein from the cell by the different types of cell disruption methods. And once you got the crude mixture where you are going to have the enzyme of your interest and the other proteins you are going to perform the chromatography techniques.

And so, very briefly we have also discussed about the basic principle of chromatography and how different form of chromatography can be used to purify the proteins. In our subsequent lecture we are going to take up some of the chromatography techniques and

we are also going to discuss how you can be able to utilize them to purify the enzyme of your interest. So, with this I would like to conclude my lecture here.

Thank you.