Experimental Biochemistry
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Lecture - 49
Interaction study of HSA protein with Curcumin and Gallic acid using UV-VIS Spectroscopy

Hello everyone. Welcome to experimental biochemistry course. In this week we are going to study protein ligand interaction. Now from the start of this course we have seen different characteristic of protein. In the theory classes we have studied how proteins structure has been made rather how the primary structure of the protein folds to form a three-dimensional 3 d protein structure. Secondly, how proteins are expressed purified and characterized. This have been studied both in the theory as well as experimental courses. And most importantly we have seen enzymatic activity of protein.

Now, the enzymatic activity of the protein is very much important as you all know. Because it determines whether the protein will function or not in a biological medium; however, when you are talking about a biological medium, what we can see is that there are various other solutes in a biological medium apart from the ligand of our choice. Or apart from the substrate or apart from that compound which protein will catalyze. Briefly what we can say in a cellular environment as we have seen or rather schematically seen in various textbook in a cellular environment various slots of cell organal is are present. Apart from the cell organals in the cytoplasm various solutes are present. Starting from salts to sucrose molecule to lipids and all other things are present within human biological system.

So, when protein is present within a biological medium or within a cell. So, it does not interact with only one particle at a particular time. What we can say in a more easier term is that when you are considering a particular interaction with protein and a particular ligand. So, protein and ligand coming close to one another; through ligand what we are trying to express is that any sort of solute any sort of molecule which will interact with protein. So, in a biological medium when a protein and a ligand or any molecule come close to one another, it is prone to some sort of interactions. Coming to those interaction those may be some may be specific and some may be nonspecific. Regarding specific and on specific those are a bit advanced which will not discussed right now.

However, what we can say is that from your previous classes where you have studied about the enzyme kinetics, those interactions might be somewhat specific. Why? Because the enzyme interactions happen in such a way where the molecule or the substrate attaches to the catalytic site of the enzyme. And the catalytic site there are specific residues which basically react with the substrate and catalyze it to form a specific product. Now suppose a particular molecule, aparticular substrate in spite of attaching to that active site of the protein attaches to somewhere away from the active site. Well, it might happen. The region which is; which contains the active site of the protein might not interact with that particular molecule. The molecule might attach somewhere away from the active site. In that cases the interaction is not that much specific will we cannot actually decide this as nonspecific right. Now because it will be much more generalized. On a; we are note what we can say is that those interaction which does not attach in the active site where the molecule does not attach in the active site the attaching somewhere away from the active site fine.

So, where it attaches from away from the active site, we might be interested. And this gives rise to a new kind of study known as a protein ligand interaction. Where a ligand attaches to a region of a protein which may or may not be the active site or it may be far away from the active site and we are interested to find how the interactions can be determined. In order to determine such interactions various studies have been done. Some may be spectrophotometric method some may be other methods. Some studies you have seen in the theory classes. In this experimental class what you are going to see s that how we can promote UV waves techniques in order to basically find the mode of interactions or basically quantify the interaction.

In order to quantify the interactions, we need a particular protein a ligand and then we will spectrophotometric determine how the interactions can be mathematically evaluated. In order to study the protein ligand interaction the protein of our choice is the very well known serum albumin protein. Now there are two different type of serum albumin which is commonly studied in biophysical chemistry classes. One is human serum albumin or commonly known as HSA and the other is bovine serum albumin commonly known as BSA. Now this serum albumins are quite familiar with us because we have used it in our previous classes where you have studied the spectroscopic determination of proteins, amino acids and also in protein in actuation and folding studies.

Now, we are going to use human serum albumin. Why we are interested in serum albumin all of a sudden? Now serum albumin has a practical role in our biological system. In terms of serum we come might be quite familiar that it is present with a blood ok. So, it various function and one of the important function is that it maintains an osmotic pressure within our body. That is a bit detailed mechanism which we might not cover out here in details. On the gentle note what we can say is that when we are talking about HSA human serum albumin, it is basically one of the transport protein which we can consider. Transport regarding transport it is a big generalized term. So, when something goes in your blood any sort of medicine or when an injection is pushed or any medicine has been injected within our body or within our blood.

So, what happens is that those molecules or those medicines buying to some protein. So, those proteins basically carry those molecules to your target site. So, out here what human serum albumin can do is that human serum albumin can bind with those medicines those proteins, it can bind in the blood system and it can carry those molecules to the target site fine. Now suppose we take some molecules which do not bind with human serum albumin and inject those molecules.

So, it will be completely irrelevant. Because it will not bind with any protein and it only get disintegrated in the blood line. So, how to determine whether a protein or will interact with a particular molecule or not. For that we first need to see whether that particular molecule or that particular ligand which should be injected or which should biological important whether interacts with our protein of interest that is human serum albumin.

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Now, the two compounds or the two ligands which we are interested in studying is that one is gallic acid out here it is seen as gallic acid and the second one is curcumin fine. So, curcumin many of you might be familiar with curcumin. It is present in turmeric. That is we regularly use in our house fine. So, that yellow powder is actually curcumin. Curcumin has the lot and lots of application. Starting from our health benefits it has antioxidant activity and it protects neurons and all sorts of good benefits are present in curcumin. Similarly, with gallic acid; it is also beneficial for our body.

Now, when you are taking curcumin or when you are taking gallic acid not in pure form ok. So, we are generally not taking these in pure form because of certain solubility issues which will address few minutes later only. So, talking about curcumin and gallic acid when they are injected or when taken in the body or in the biological system. So, whether it is interacting with the serum albumin or not we are interested in, for that we have chosen these two ligands. And we will taking a protein that is human serum albumin. And the medium which we will use is phosphate buffer pH 7.4 and the concentration is 20 millimolar as all of you are might be familiar that phosphate buffer basically used to mimic a cellular environment.

So, we are using phosphate buffer out here. Now this two what we are going to do is that we are going to estimate separately. First we are going to estimate either gallic acid and

then we are going to compare this one with curcumin. And see which one interacts in a bitter way with human serum albumin.

Now, let us see the amount of gallic acid and curcumin we require in order to carry out our experiment. Now this is gallic acid. We are going to prepare 5 millimolar 25 ml gallic acid. I have calculated it and the weight is around as per your calculation for molarity the weight is around 0.021 gram for 5 millimolar 25 ml gallic acid. Now here it is the weighing balance. So, we have kept a butter paper out here and we have teared it so the value is showing 0 0. And now what we are going to do we are going to add 0.021 gram of gallic acid we are going to add a pinch of this gallic acid now.

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So, when you are added a small pinch of this gallic acid what we can see is that the weight has reached to almost around 0.0253. So, it is a bit higher than 0.021 gram; however, we can easily proceed with this one. Because as we have discussed previously we should take the amount a bit higher than the required one now we are going to transfer this amount of gallic acid to a falcon tube.

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Here I have taken a clean falcon tube it is the 50 ml falcon. Now this butter paper I have taken gallic acid out here. And I am going to gently put the gallic acid here a small amount of gallic acid out here as we can see this white powder; powdery substance. To it I will be adding 25 ml double distilled water.

You might be dissolving this in various solvents, but for biological experiments kindly dissolve it in double distilled water. So, I am adding double distilled water. Initially after adding 10 to 15 ml of double distilled water I will just put this cap and we will gently mix it. So, that it dissolves completely it might not dissolve completely out here. Apart of it has already dissolved; however, a small portion is still there small portion of the solid. Now I will add the remaining amount of with double distilled water.

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Now, I am adding 25 ml double distilled water. Now after adding 25 double distilled water I will again just stir it bit mix it thoroughly. And now as you can see a small amount of gallic acid is still undissolved out here.

So, what does this show? This shows that gallic acid is a bit is not completely soluble in water at this state. So, before measuring the; before taking the concentration of gallic acid kindly note down it is solubility; however, right now what I will do is that I will give it for sonication in ultrasonic bath. And after some time it will completely dissolve. Now advance experiments have been made various sorts of experiments have been made from time to time been or to solubilize this gallic acid. Due to its spores solubility in this aqueous medium sometimes taking of this gallic acid into the biological system is not at all beneficial because it is not soluble within the aqueous medium. So, it should be made soluble fine.

So, that is bit advanced research, but; however, for the time being what we can say is that it is not completely soluble in water gallic acid. So, we have to give it for sonication. So, that it mix thoroughly. So, I will giving it for sonication. And we will proceed in measurement of curcumin solution. We have measured gallic acid and made gallic acid solution with using double distilled water. Now we will measure curcumin. We will prepare around 20 millimolar 10 ml curcumin solution. Why 20 milli molar 10 ml? I will discuss bit later only for that we require basically 0.073 gram of curcumin.

So, here is curcumin I have teared it we have kept this paper out here. And the way we require is 0.073 gram. So, I will take a pinch of this yellowish powder.

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So, it is around 0.03 gram. I will require almost double this weight now it is 0.0751 gram. So, let us take this out for convenience.

Now, this one is the yellow powder or curcumin powder what we can see it is the very distinct color. So, it is very interesting to see because the turmeric which we use in our home as the same color. If you paste turmeric it has the same color fine. So, curcumin it has actually the curcumin which you are using it is this color. Now curcumin are various grades when you purchase it commercially curcumin has various mixtures mixture of other compounds. So, it is better to go for some I mean high graded curcumin compounds.

Now, this curcumin what I will do is that I will dissolve it not in double distilled water. Remember not in double distilled water, but in ethanol fine. So, I will dissolve it in ethanol.

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So, we have dissolved the curcumin in this falcon tube in ethanol. Now the various grades of ethanol which generally used. The grade which we use out here is spectroscopic grade or rather HPLC grade as you can see here. Always use this serum ethanol while making any particular chemical solution. In laboratory we can see various other sorts of ethanol which do not have written this analytical grade or spectroscopy grade

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Do not go for that ethanol because that ethanol is use other purposes like washing or sterilizing all sorts of things; however, out here ethanol which you will be dissolving it is in this type of ethanol.

So, this is the solution which we have used. Here we see intense yellow color fine. Now we will not use this solution for our analysis. What we will do is that we will take a small amount of this solution around 5 ml of the solution in a falcon tube and make it volume to around 25 ml fine. So, why we are doing this, basically curcumin is not at all soluble in double distilled water. You can try it by yourself you can add water to curcumin powder and you can see that it is not solubilizing in water. Fine whereas, on the other hand it is soluble in ethanol to a certain fraction, but of course, this one is much higher than that of double distilled water.

So, what we do, we basically take higher concentration of ethanol and higher concentration of curcumin out here fine. Higher concentration of ethanol means it is pure ethanol without any dilution. And higher concentration of curcumin means we are using 20 millimolar of curcumin. For that we were going to prepare in actual 5 milli molar of curcumin from here. For that we are going to take a small amount of this ethanol curcumin dissolved ethanol. And we are going to transfer in a falcon tube containing double distilled water.

Now, what is happening in this process? So, the final ethanol solution has basically final curcumin solution has basically ethanol water mixture fine. So, the solution which we are using for analyzing this protein ligand interaction has water ethanol mixture or ethanol water mixture; however, please keep it mind the solution which will be using for protein ligand interaction should not be pure ethanol. Why? Because you have studied previously in the theory classes that ethanol basically degrades the protein. It denatures the protein. The protein structure will disintegrate when you will increase the concentration of ethanol from time to time in a titration method as we will see in a short time fine.

So, there is again a limitation of solubility in this. For that what we are doing is that we are taking a higher amount of curcumin dissolving it in ethanol. Now we will take small amount of this ethanol dissolved curcumin. And we will transfer it into another falcon.

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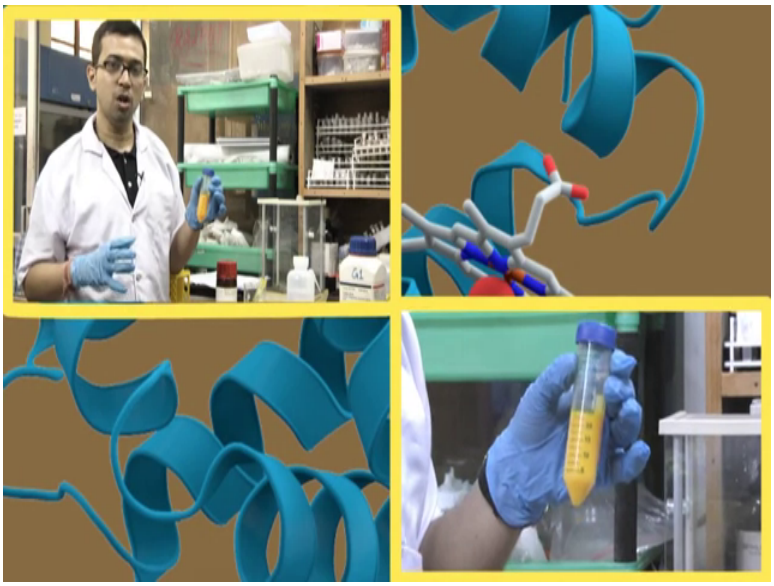

Now, we will prepare 25 ml of curcumin solution in ethanol water mixture. For that what we are going to do we are going to take out 5 ml of ethanol into this. And the volume will be 25 ml. So, 5 ml of this ethanol curcumin mixture in this falcon and making up this volume with double distilled water. Now this is the 1 ml pipette I have adjusted the volume up to 1 ml. And now I will gradually take this curcumin solution from here and we will transfer it into this falcon 1 ml, 2 ml, 3 ml, 4 ml and then 5 ml.

Now, to it I will be adding double distilled water. Now it is almost around 25 ml solution. Now this curcumin is actually 5 millimolar curcumin dissolved in double distilled water or rather in ethanol water mixture. Please note the percentage of ethanol in your solution should not exceed around 20 to 30 percent of ethanol percentage. What happens actually ethanol percentage increases in a solution. In that case the protein again will start malfunctioning and it may degrade. So, better keep the ethanol percentage low out here we have kept around 20 or less than a bit less than that.

So, we are proceeding with this one. This is curcumin dissolved in water ethanol mixture. Now we are going for measurements. For measurement what we will do we will initially proceed with UV visible spectroscopy ok. For UV visible spectroscopy will going into details why we are going to going into UV visible spectroscopy, but before that let me just give your brief recap about the UV visible spectroscopy. Now this UV visible spectroscopy have one; uses two cuvette as all know.

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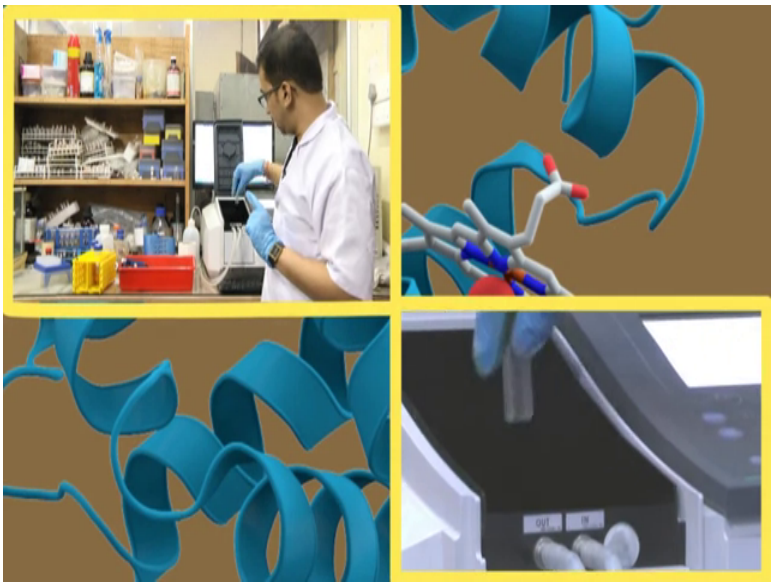

So, here this is the trans band side of this cuvette and this one is actually a 3 ml cuvette.

So, we will be taking phosphate buffer solution and the volume will be 3 ml out here. So, initially we are going adding 1 ml I am taking phosphate buffer and I am adding here 2 ml. And number 3 ml. So, as you all know in a UV machine it is shown here, these are quite familiar because we have studied in and used it for a previous experiments also. This cuvette machine has a chamber out here it contains two compartments. So now, I will take this one and keep it in one of the compartments. Now before keeping it in a compartment we should wash both of this transparent sides and keep it in such a way that the incident line phases the transparent side.

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Similarly, I will take another cuvette the first one I have kept it in the blank chamber and now I will keep it in the sample chamber. Now I am washing this one. Those two sides trans and sides I am washing. And I am holding the cuvette in the other side ok. Do not hold the cuvette in the trans band side. Now I am placing this one again in the sample part sample holder. I am closing this lid and out here this is the software we are quite familiar with this software out here.

So, in the software what we can see is that this is the method part here method we can go here.

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We can see that it ranges from 200 to 600 this is the wavelength range over which the scan has to be carried out fine. And the scanning speed is kept as medium. And the scanning interval is 1 nanometer. That is, it will take reading at each and every single nanometer and the final may ask selected a file name and rest of the parameters should be kept same. And now I am clicking.

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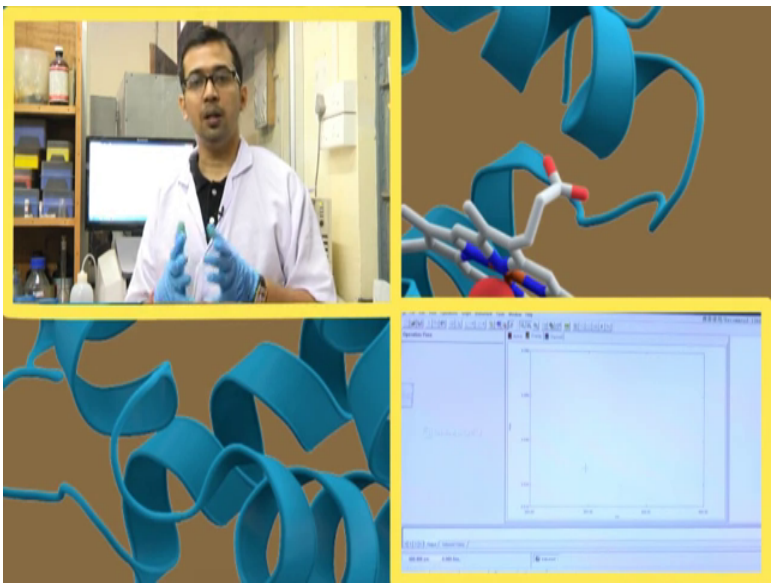

After this one when we have kept the phosphate buffer in the two cuvettes within this UV machine. First we will go for this base correction. So, we will click here this base

line. Once we click this baseline it will show the start wavelength as we have given here 600 and where it will finish it is 200 nanometer and we will click. So, baseline is actually getting initialized now for the time being why we are interested in analyzing this UV spectroscopy. So, what happens is basically we are going to take the molecule either curcumin or gallic acid in the cuvette. And we will measure it is u v spectra gallic acid or curcumin as a separate UV spectra which you can see in any sorts of paper in any textbook or online ok. So, we will do is that we will add to it HSA or the portion of our interest drop wise in a titration method.

What does that mean? That actually means is that we will be taking curcumin in a cuvette. We will take it is uv, fine it will give a catrosphic u v spectra of curcumin or gallic acid. To it we will first add small amount say 5 microliter or 2 microliter of HSA solution. The HSA solution which we have prepared actually will add it and then we will see how the spectra changes. Which spectra changes we will be interested in the spectra for gallic acid or curcumin. Now why we are interested in this case? Because what happens gallic acid or curcumin has a specific spectra UV spectra. And now we know at this stage that UV spectra or absorbance spectroscopy gives an insight into the chemical environment of the compound also.

Now, we know basically from our spectroscopic studies or UV spectroscopy which many of you might be familiar with, that when we are changing a molecule from polar to non polar environment it shifts basically the lambda max shifts in either in the red or the blue region depending up on the medium fine. So, what happens is basically when the environment the chemical environment or the surrounding of the molecule changes, the UV spectra if prone to particular change. Now in this case what you can say is that it is kept in a environment first is phosphate buffer. It gives a particular spectra and now when protein is added to it is medium changes. Now what why this medium changes because this curcumin might attach to the HSA or it might not attach properly; however, it is medium is prone to change because we are changing we are adding a foreign substance. That is protein to the phosphate buffer media.

So, it is UV spectrum might change. And again when you are in gradually increasing we are change getting a change in the UV spectra. Depending upon the intensity of this changes or the magnitude of the changes we can basically quantify the amount of interaction between the protein and the ligand. And that is why UV spectroscopy serves

as an important tool for determining protein ligand interaction I have taken out this cuvette which was kept in the sample holder fine. So, we will take curcumin solution into it. First what I will do I will take this micropipette I have adjusted it to 10 microliter if a 20 micropipette. So, I will be initially taking out 20 microliter of this solution from here into a waste beaker fine.

So, what I am doing is that I am basically preparing this curcumin solution in a 3 ml phosphate buffer solution when we will be adding directly 10 ml curcumin to it then the total volume will be 3 ml plus 10 microliter. In order to avoid that what we are doing is that we are initially taking out 10 microliter first the volume is actually 3 microliter 3 ml minus 10 microliter. Then I am again adding curcumin that is 10 microliter curcumin to make the final volume to 3 ml worth which was in the first.

So, this one is basically curcumin solution in water ethanol mixture. First stir it gently or rather mix it thoroughly before using it. Then I am going to take 10 microliter of this curcumin I am going to take 10 microliter curcumin and add it in this phosphate buffer solution.

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And I am adding this one in the phosphate buffer solution and mixing it thoroughly. As I have previously discussed in the spectroscopic related classes while taking this experiments while doing this experiment that please note air bubble or the water bubble is not formed within this solution or within this cuvette.

So, once we have taken it take this cuvette and put it in the sample compartment. After that we will go for observation.

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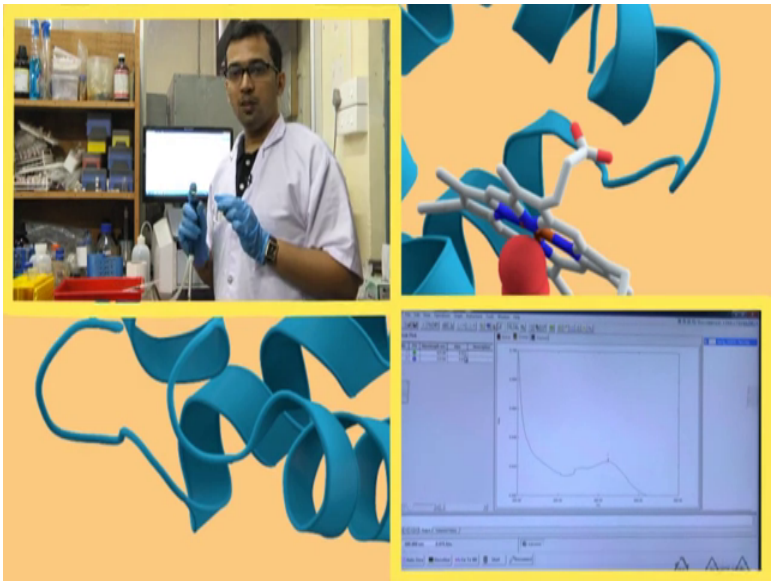

So, I have taken this curcumin within the cuvette. And now I will do auto zero, but before that what we can see a small amount of absorbance value is been shown not to remove that we will do auto zero. This auto zero basically fixes the initial wavelength to 0. So, it is around 0.01;we can proceed with this it is now 0. So, we have clicked start. And let us change this one to around 0.1. So, here we can see absorbance taking place increase in the absorbance value out here. This value again decreases a bit and out here this fluctuation is because of change in the lamp. Now it again increases what happens is basically around 340 or 350 the lamp changes from one lamp to xeon lamp to hydrorubidium lamp it changes.

So, what happens basically in during that region is a bit fluctuation in the UV spectra. So, this one is for curcumin. The intensity what we are getting out here around maximum peak is around 423 nanometers from 0.023; however, this intensity is very low as it should be expected. So, what we can do? We can actually increase the amount of curcumin out here. So, we can basically double this amount or take a bit higher amount of curcumin in this case. Now we are increasing the amount of curcumin concentration out here initially we did it with 10 microliters.

But right now we will take 10 times higher that is 100 microliters. For that we have taken this to 100 microliter pipette and made the volume 200 microliter. And I will be initially pipetting out 100 microliter from here and will be adding this curcumin. So, it is better before adding you take this solution and mix it thoroughly. You mix this thoroughly. And after that you take 100 microliter of curcumin from here and put it in this cuvette and mix it properly.

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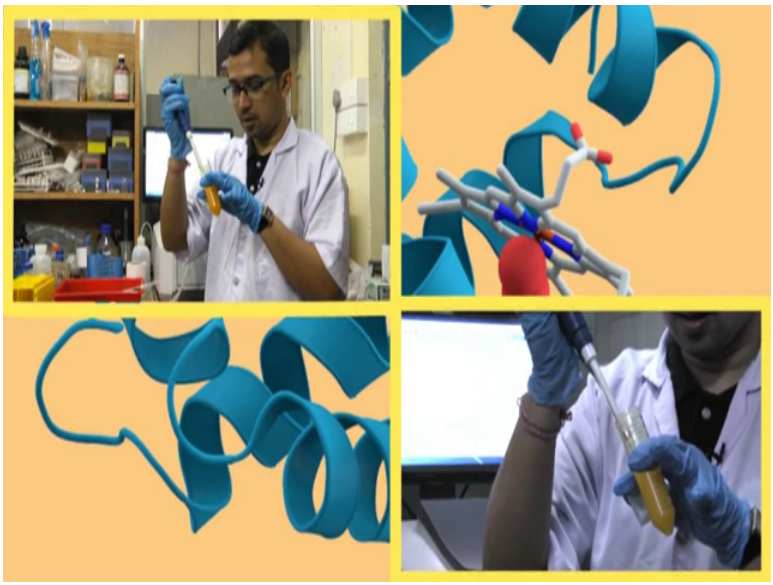

The solution will turn faint yellowish out here. So, once we have mixed it immediately you transfer this one in the cuvette holder. And after that what we will do we directly go to auto zero; after auto zero we will click start.

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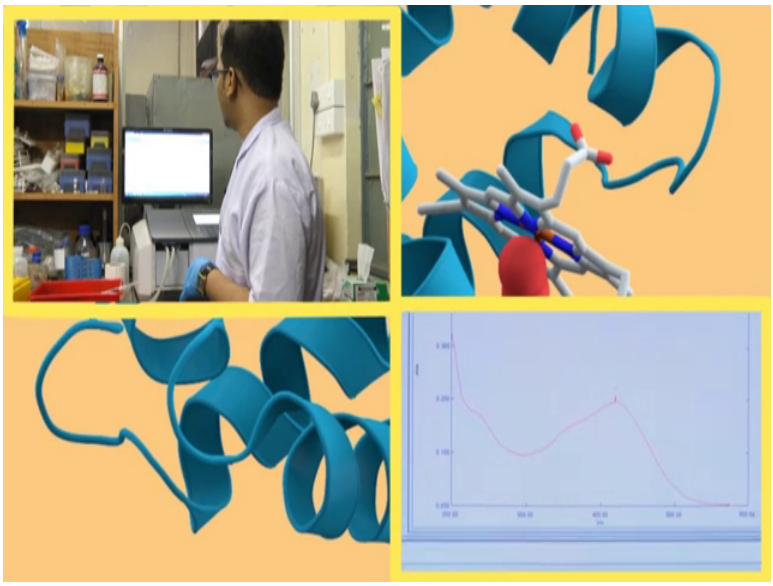

So, let us increase this one to volume value of around 0.5. So, here we can see the absorbance increases, because we have increased the concentration absorbance will increase. The maxima comes just around 0.2 which was expected basically; there were decrease in the absorbance value again around 350 or something like that. And after that there is again an increase with a small hump of around just over 230 region or in that case.

So, let us see from here we are first deactivating the initial one. And we are initially seeing that where is the hump coming. So, out here the hump is coming around given here for 119. So, this peak is around 419 nanometer and we are currently interested in this peak. Or we might be interested in some other peak that we will find out later during the experiment. For the time being what we will do we will save this one we will save as here as gallic; not gallic acid or rather curcumin and curcumin only. Saving it out here; let us saving it has a text file

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So, this is the UV visible spectra of curcumin. Now what we will do, we will slowly add HSA to it and see how this spectra changes. I have taken HSA solution; so this HSA I have prepared it is 1 mill molar HSA and to this; from this HSA I will basically take 5 microliter of HSA.

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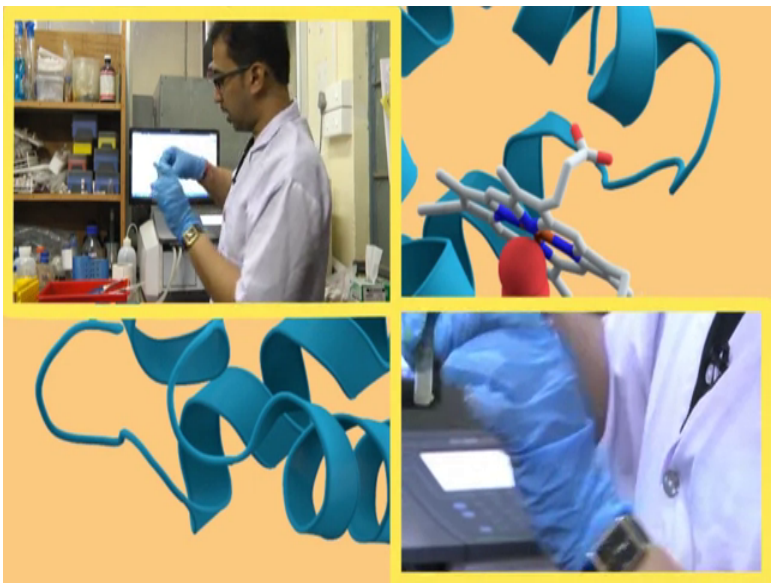

I have taken a clean micropipette once again. And I will be taking 5 microliter of HSA from it; 5 microliter of HSA ok. Now what I will do, I will take out this cuvette as you

can see this cuvette is yellowish because it has curcumin in it. And I will add directly to it HSA why; because this is a titration method actually.

In this method what we do basically go on increasing the concentration HSA like this. We keep on adding volume of HSA to it. So, initially we have added 5 microliter of HSA and mixed it thoroughly. Please note that bubbles are not formed here a few bubbles have been formed which I am actually driving out fine. So, once we have mixed this I am transferring it to this chamber. And after that what I will do? I will give this auto zero and after auto zero; I will click this start. So, let us see how it comes actually.

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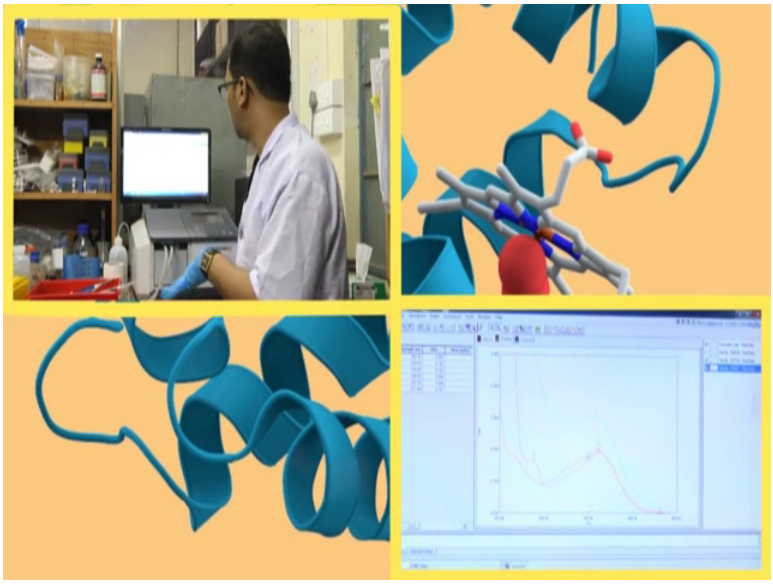

F the absorbance increases here as we can see; the absorbance value again increases in this region and shows an almost (Refer Time: 37:49) peak out here. This is actually a plateau region and it increases; let us increase this value a bit to 1.0.

So, what actually happen is that we can see that this value is the absorbance increased a bit, but this absorbance this spectrum is not the correct spectrum. Why; because we have to actually subtract the spectra for HSA from it. Now we will see this similar experiment this similar one only in a different way. In what way? Now this is the cuvette. And this is the cuvette cap I am putting it here, this is the cuvette cap as you can see.

So, it is better sometimes to actually mix it using this cuvette cap fine. So, first I will actually wash this cuvette cap a bit I have washed this previously also. Just to be safe I

am washing it currently. Wash this cuvette cap and now I am just mixing it in a different way in which I am just inverting it. There is a risk do not leave this cap otherwise entire solution will come down. And now after that transfer it in the sample holder. Close this one, give auto zero and again start this experiment.

Now, let us change this thing a bit from 0.1 to 0.5. Interestingly what we can see is that this reading changes a bit. How it changes the absorbance for the same solution; for the same solution the absorbance value was initially higher than that of curcumin. Now when I mixed it in a separate way; it coming in a lower way. So, these are what; these are actually experimental anomaly which occur from time to time. How you mix it how you prepare the solution actually determines how the experiment will carry out. So, in this case what happen is the general rule is to actually mix it properly. During protein ligand interaction take it close it with a sample compartment or the sample cap or the cuvette cap and then mix it and then go for observation out here.

So, here we can see there is a bit decrease or almost same out here this shift or the peak almost same, but; however, there is a slight shift in the peak intensity in the blue region. Or what we call sorry in the red region or we can say is the red shift. If I am not; if yes ok. So, I just confused with a red and the pink purple line. So, the red line or the orange line actually for curcumin and this purple line what we can see here this line has actually shifted in the red region that is the high wave length. And this one is for HSA plus curcumin. So, what is happened out here is that there is a shift in the lambda max value bit bathochromic shift. And this region here we can see it is a bit lower than 300 and this is somewhat around 276 nanometer. This is due to the protein present here, but again what can I say is that this is not a actual spectra of the protein ligand complex. Why? I will discuss it a bit later on

Now, I have again taken this mixture it contains now curcumin plus 5 microliter of HSA. And I will again add another 5 microliter of HSA to it and mix it thoroughly. So, a total volume of HSA out here is now 10 microliter and similarly the final volume as increased from 3000 to 3010 microliter. Now I have kept this cap again once again here this is a cuvette cap I have kept it here. And I am just inverting it mixing it carefully. Please note not to leave it this is quite a bit of what I can say a delicate process. If this slips from your hand it might be blunt. So, hold it thoroughly use your thumb in this way in the lower part keep two fingers index finger and the middle finger in this way in the cap hold

it. Put your hand in this way why because if it suddenly falls cuvette fall in your hand. So, mix it thoroughly.

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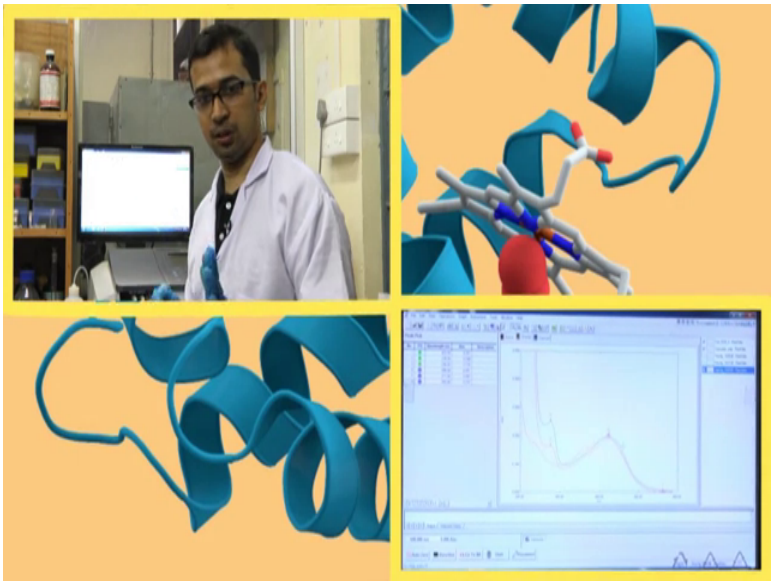

And now after that transfer this one into the sample holder. Get auto zero, auto zero and start with experiment. Here the peak comes here. Now absorbance actually, bit increase in the absorbance we can see.

What I will actually do is that your rather what is (Refer Time: 44:18) that after this one, I will be subtracting the HSA absorbance. Now why I am subtracting HSA absorbance. Basically what happens is that to which you are adding HSA. Initially you have curcumin to curcumin you have added HSA. And will increase the concentration of HSA. Now whose absorbance are we actually bothered a bit. We were actually bothered with absorbance for HSA only or rather the absorbance for curcumin only not HSA. So, we are bothered with the absorbance of curcumin only. Now this spectra as we can see here has actually the overlap of both HSA and curcumin. Again what I am saying, this has the tendency of overlapping of HSA as well as curcumin.

So, in order to remove the contribution of HSA from here. What we need to do we need to subtract the absorbance of HSA. How to subtract this one? We have to take blank absorbance after this experiment. Now what we have done out here is that I have actually taken the spectra for curcumin with HSA. It is suppose it is kept as sample one ok. Now in blank actually it is sample two which will have the absorbance for HSA only. So, your

net absorbance would be a equal to s 1 minus s 2 or on better note what we can say is that we have s 1 which is actually curcumin plus HSA minus s 2 which have only HSA. So, what you get curcumin plus HSA in 1 minus HSA is actually curcumin. So, when we will subtract the absorbance of HSA from here we will get the absorbance of curcumin at that particular concentration of HSA.

Now, when why do the control we can see how we can proceed with the blank subtraction. For the timing out here is got this green type of spectra as we can see here, the absorbance is a bit higher than that of curcumin around 420 nanometer and this peak is even higher from the previous one of the concentration of HSA (Refer Time: 46:38) much more. So, I am saving this one as curcumin HSA.

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So, I am saving this one as curcumin HAS; 10 that is 10 microliter. And also in the text file. This sort of saving the spectrum file; you are what familiar with sense saving this one and now I am proceeding for the next observation by adding again 5 microliter of HSA.

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Now, I have again taken this HSA in a fresh micropipette or a fresh micro tip. So, please note that do not use the previous this tip while you are taking as new solution. Always use a fresh tip because the previous tip might be contaminated or it should be contaminated. So, taken a new tip and taken the HSA solution 5 microliter and I am adding this one. So, the total volume comes to around your 15 microliter. So, I am again putting this cap and mixing it thoroughly. Once I have mixed it I am keeping it in this sample compartment getting auto zero done; auto zero complete.

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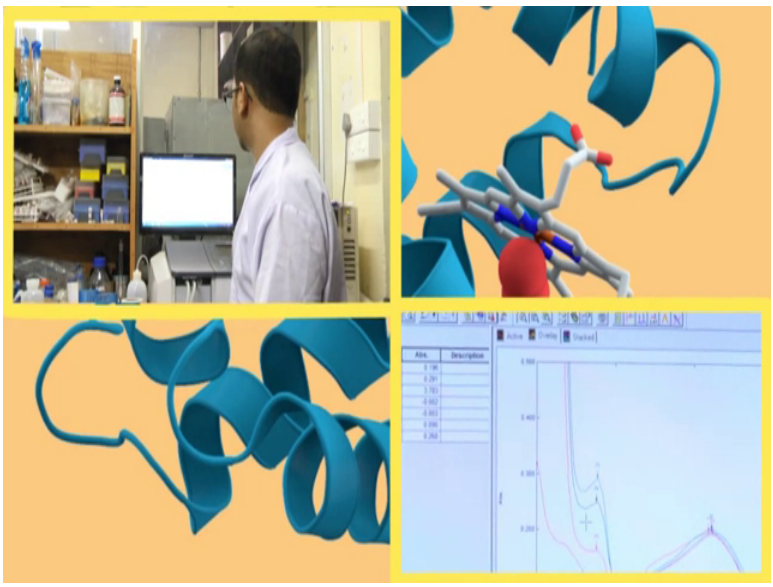

And let us start. So, if I can actually change this maxima from 0.5 to around 0.3 which is a bit closer out here. We can see the changes now this one this value is actually this blue line which is going out here.

So, more or less we can say is that, the absorbance change in the absorbance around 423 nanometer does not change that much, that much remains more or less same; sometime is increase sometime is decrease, but; however, this is not the final change once again I am saying. This is surely to change 0.5 this peak because we are increasing the concentration of HSA and this around 278 is for the (Refer Time: 49:15) concentration increases; now I am saving this one as 15.

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For 15 microliter; now I will be adding to it again 5 microliter. For that I will be taking out this cuvette taking a fresh micropipette. This is the HSA solution. I will be taking out 5 microliter of HSA from here putting it mixing carefully placing this cap once again and in up and down motion I am doing it and finally, keeping it here.

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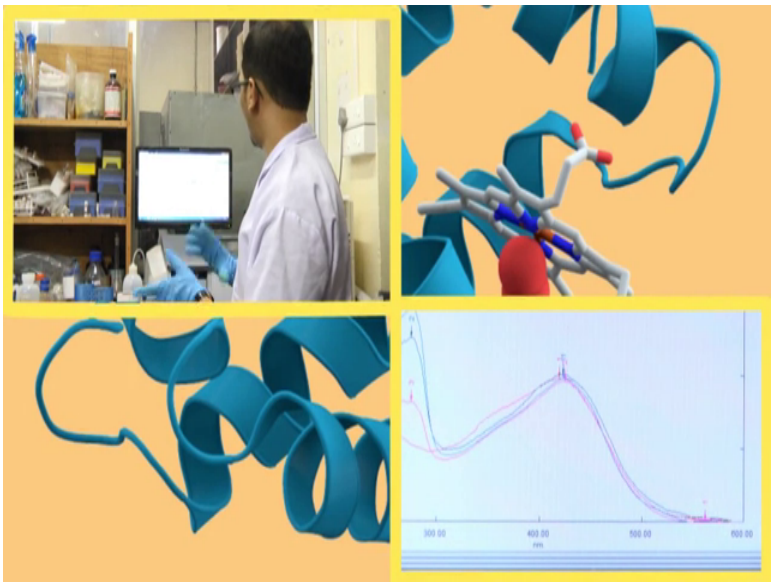

After auto zero it will give this absorbance value as 0.00 and once done ready with the scanning. So, it is a bit cumbersome out here. So, all the spectra are overlapping one above the another. So, it is quite difficult for you to see. So, I am just actually removing the other spectras which are appearing, which have appeared previously actually. So, this one is for 10 and this one is for 15. So, here we can see for this 20 microliter; this is coming and this one for curcumin only where only curcumin are present. So, again it increases the bit. So, this is for curcumin along with HSA having 20 microliter.

Now, the next HSA I will be adding is 30 microliter not 30 microliter rather I will be adding 10 microliter. So, that the total volume goes up to 30 microliter. Here I am saving this one as curcumin HSA 20 in text file also. Now I am taking again 10 microliter of HSA from here 10 microliter of HSA. In this solution I will be putting 10 microliter of HSA taking this cap and mixing it.

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So, another important some mixing is that, curcumin as I have said is not that much soluble in water. So, what happen if you look it carefully you can sometimes this residues of curcumin can be found in the lower bottom at the cuvette. So, it is better to mix it thoroughly. So, that the entire curcumin present out here comes up in the solution and interacts with protein. Otherwise we cannot actually get a proper or correct result.

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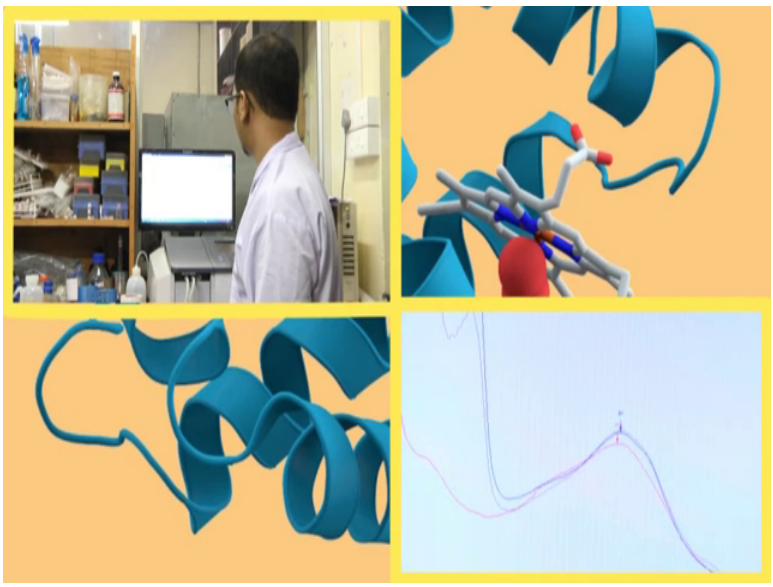

So, you have started it this one shows for the previous one that is for 20 microliter and this one goes for your 30 microliter. The next reading which you are interested in

actually 50 microliter rather. We are taking 20 microliter from here. So, that the final volume is 50 microliter. So, one interesting thing we can see out here is that; there is one cross over point where the intensity again decrease and again increase. So, there is a cross over point out here. So, this one is for yeah our only curcumin the red one; the orange one.

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So, let us do save as as 30 curcumin HSA 30 curcumin HSA a 30 and now I will be adding directly 20 microliter of HSA to it. Here I am using actually 1 millimolar of HSA for a convenience you can also go to 2 millimolar of protein.

Now, I will take this out add HSA to it, put this cap. And gently mix this one. After mixing this one I am going to transfer it into this compartment.

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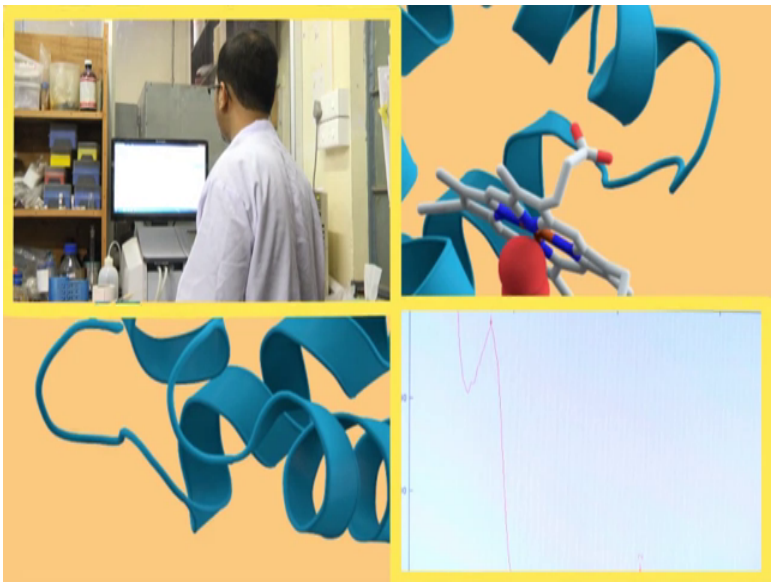

And after auto zero, we will give it for scan. Now for the time been I have completed a from starting from 0. 0 concentration is 0 volume of HSA to 50 microliter of HSA out here. Let us remove the previous one. So, it will be easier for you to visualize. So, what I will do is that now for blank or blank control, I will take a cuvette I will fill it with phosphate buffer. As we have done in the; during this 10 correction. We will take 3 ml phosphate buffer without curcumin. And then to phosphate buffer we will take 5 microliter of HSA first we will take the absorbance then again 5 microliter of HSA and again take the absorbance.

So, basically what we are doing we are getting this same titration type experiment, but without curcumin. So, at each and every concentration of each and every amount of HSA we are getting a particular absorbance graph for the protein. And what we will do; we will actually subtract this one those graph from this graph. So, for a particular concentration of a HSA, suppose for 5 will calculate the concentration later on from the total volume for the time been we can say suppose for 10 microliter of HSA, what we shall do is that we will take that spectra which has curcumin plus HSA 10 that is 10 microliter of HSAand from that we will subtract HSA only 10 that is solution without curcumin only 10 microliter HSA.

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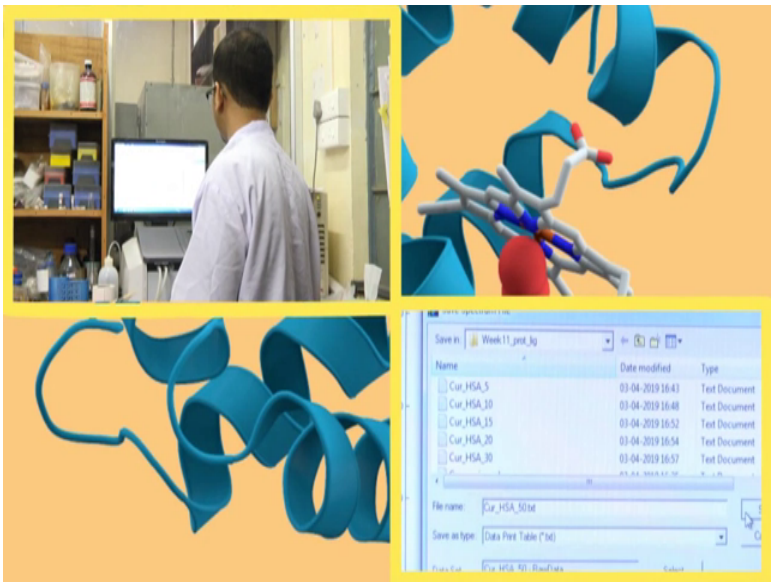

So, out here we will just extend this one to point 8 and will save it as curcumin HSA 15 and save as curcumin HSA 15 text file. So, I am now actually enabling all the files which you have currently done till now is 1.0.

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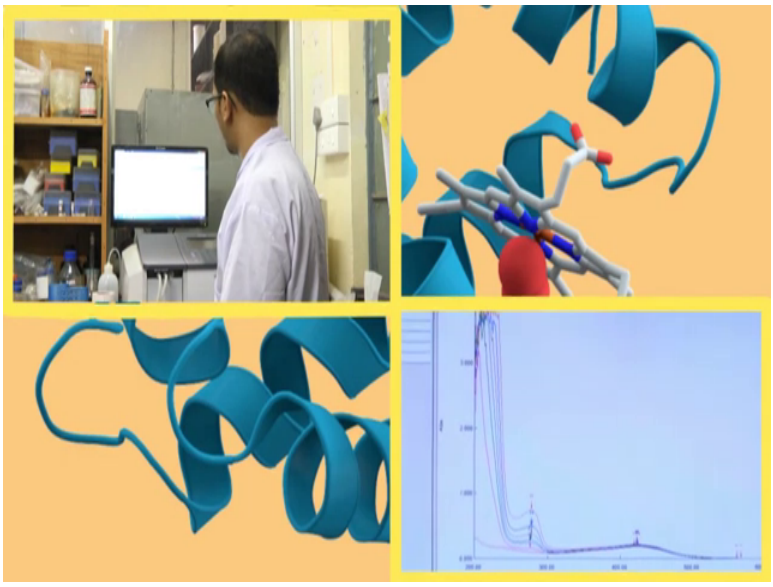

So, this one is for this one is for only curcumin. The second one is for curcumin this one HSA 5 microliter with curcumin. The third one is for 10 microliter. The fourth one out here, the fifth one for 20 microliter and 30 microliter fix and finally 50 microliter. So, what we can see is that there might be a bit decrease out here in the change in decrease in

this region, but; however, there is strong change out here. So, no need to bother regarding this because these are all protein peaks.

Now, coming to this region below 250 here at this is 200 this is 300 this comes around 276 and coming to region 200 to 220; if we just extend it to around 2.0 more than that 1 0. So, these are all actually the peptide this comes for the peptide absorbance 4.0. So, it is actually a very high absorbance value. So, here we can see for pure curcumin here. Pure curcumin this one this which one is appearing disappearing right now this one is pure curcumin. So, does not have any such peaks this peak this for peptide. And once started writing HSA all this peaks are coming. So, we have to remove all the unnecessary peak to get the absorbance of curcumin. Absorbance of curcumin in presence of protein only. So, again transferring to the 0.5.

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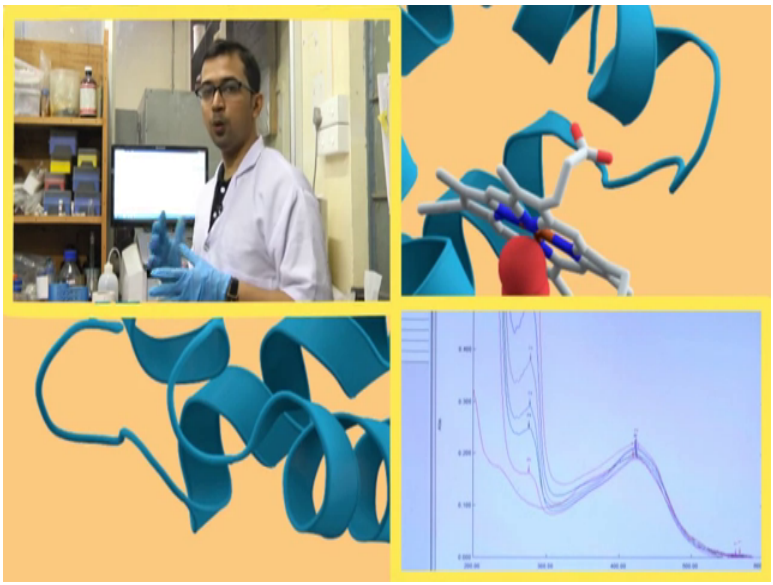

And now we will proceed for our control spectra.

Now, we are doing the experiment that absorbance for bank subtraction method. We have taken this same cuvette which have kept in the sample compartment we have washed thoroughly with double distilled water. Please make sure that curcumin is not present out in the cuvette. You can actually check it with your eyes only because if curcumin is present it will appear yellowish. If you still feel that you cannot actually remove it in double distilled water this yellowish sting add juts ethanol to it and you can add at that time your washing ethanol that is local ethanol not the high graded ethanol. And you can

wash your cuvette properly and you can proceed for this observation. And after you are washed ethanol please make it sure to dry this cuvette and wash again with double distilled water. For the time being out here the entire solution I; so, hope it is completely remove because I cannot actually see any sort of yellowish thing out here. I washed with double distilled water. And now I will pour here 3 ml of phosphate buffer.