Experimental Biochemistry
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Lecture – 50
Interaction Study of HSA Protein with Curcumin and Gallic Acid Using UV-VIS Spectroscopy (Contd.)

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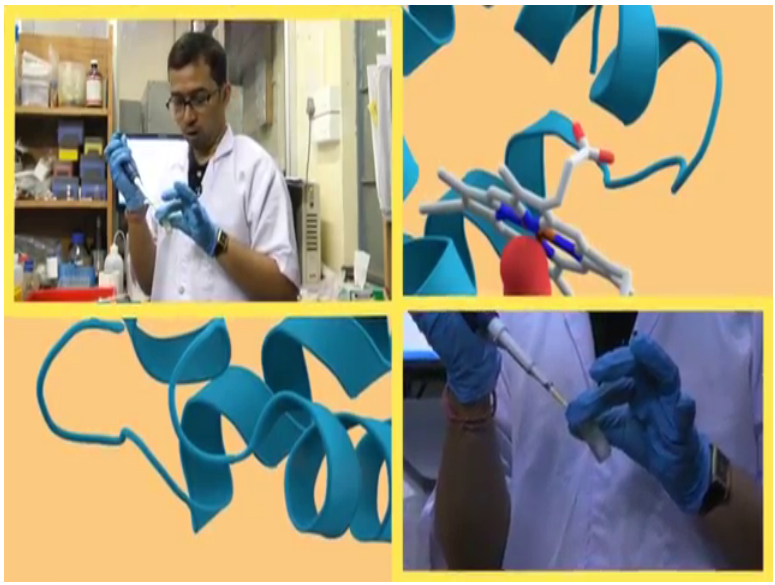

Now, am taking 3 ml of phosphate buffer this 1 ml, 2 ml and 3 ml of phosphate buffer. To this what I will do is that I will add 5 micro liter of HSA to it. Now this is the HSA, the same HSA which I have been using. I will add this 5 microliter of HSA. Now what you can do is that you can in this stage may not use the cap out here because we are not making any protein ligand interaction. This HSA actually it becomes soluble in water properly. Wants to put it out here mix it thoroughly and then am giving it for analysis.

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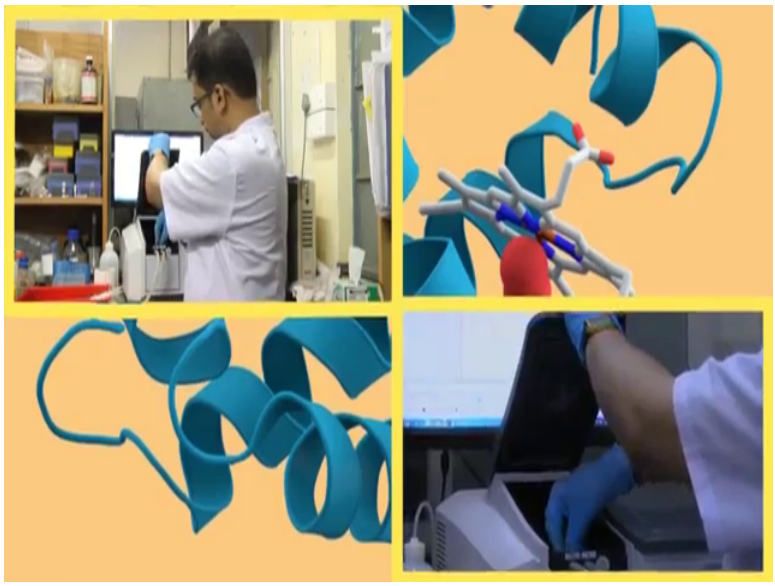

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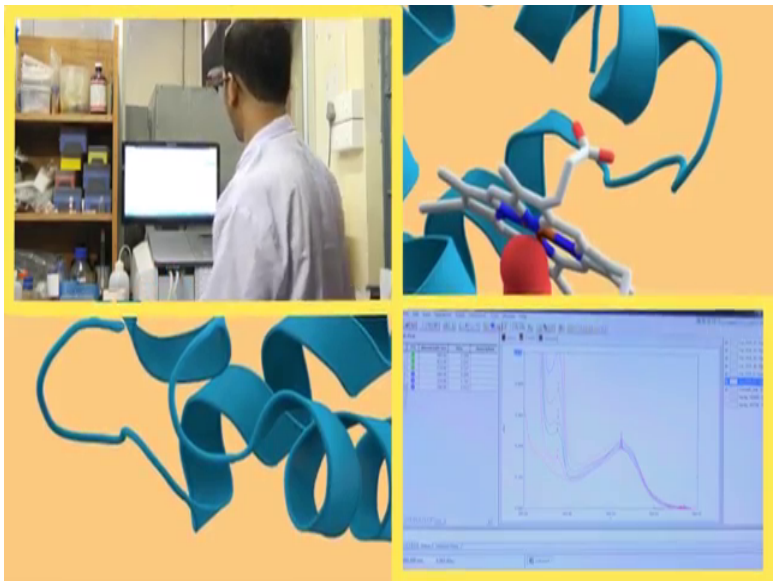

So, putting it in this chamber, doing auto 0. After auto 0, I am giving start. So, this one is for only HSA and only HSA in phosphate buffer solution.

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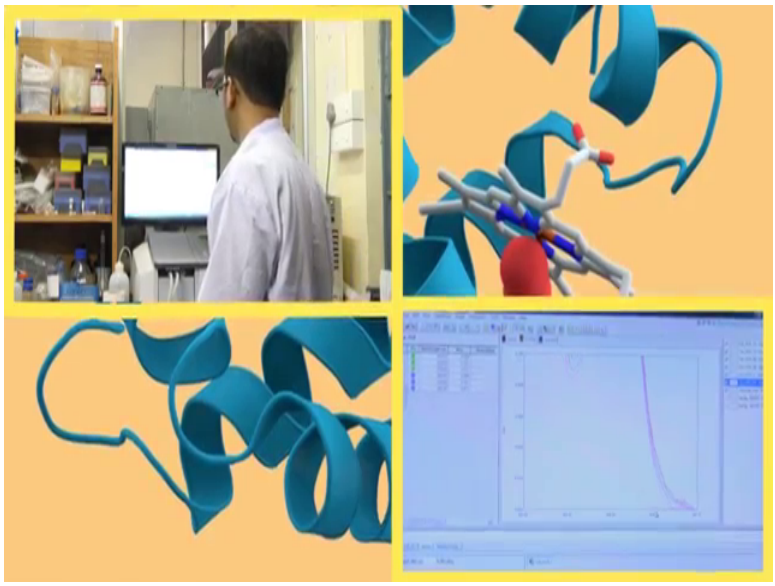

So, if we just lower the scale value to 0.1, here we can see there is a this absorbance for HSA. Now this will increase, so I am just increasing this one to 0.5. So, it is almost a straight line no such visible peaks out here in this region for curcumin is not present. Now this is the peak for HSA.

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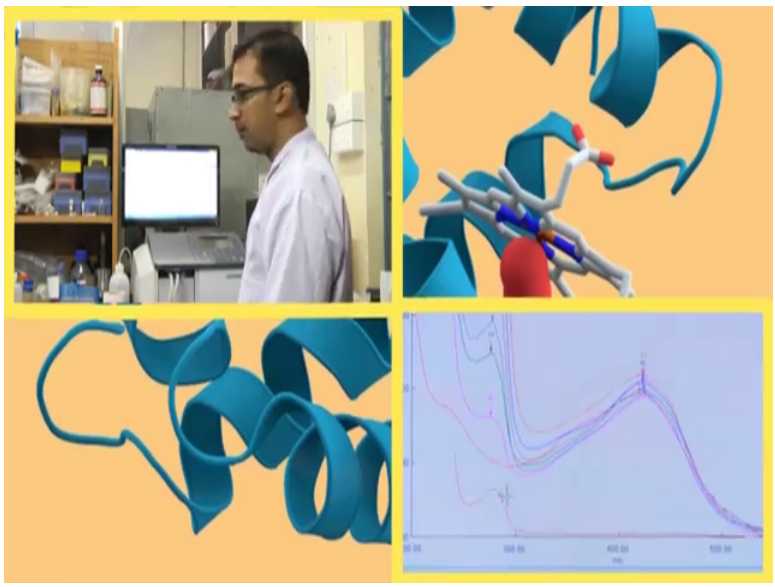

So, I am saving this one again as HSA. So, let us change this not HSA only hsa underscore 5 ok. Saving it again as a text file hsa underscore 5.

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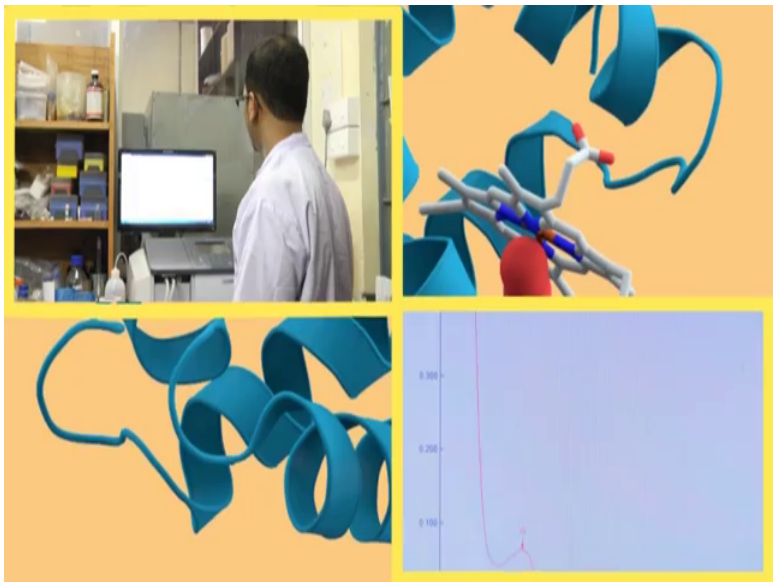

So, if we just for the time being completely remove this other spectras and enable this HSA, what we can see? It gives a peak of around; this one is around 279 nanometer. So, if it keep it here 0 and this one we change it to 0.2.

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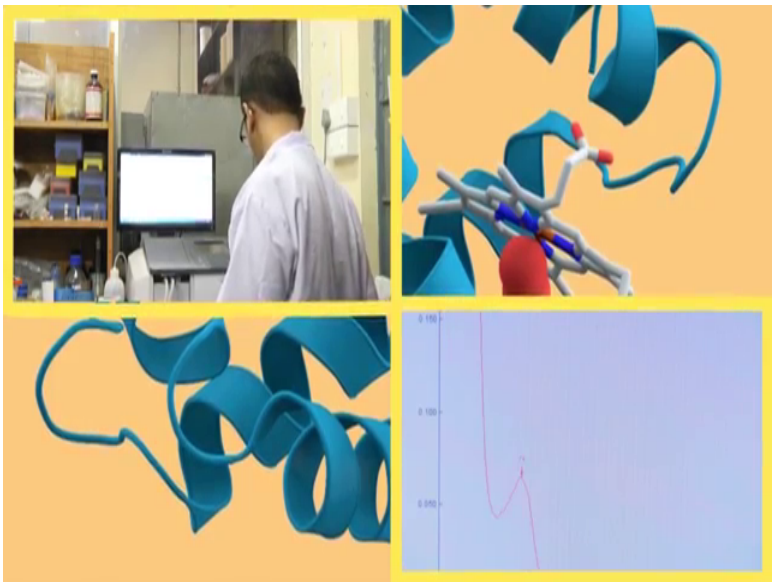

Here we can see around 280 nanometer to (Refer Time: 03:35) nanometer, it gives a lambda max value. Now what I will do is that, I will again add HSA to it. So, this one is for 5. Now I will again add again another 5 microliter of HSA. So, each and every time, please make it sure to take a fresh dip and dip it into the HSA solution ok. Now I will

take this out and I will mix it, please note that bubbles are not formed out here. Sometimes bubbles are being formed so we have to just remove the bubbles ok. Again we are transferring it; after auto 0, we will give it for measurement. Now in this region in around 600 to around 500 region, there is not any major changes in the UV spectra for HSA.

However, here we can see it the value, if we just increase this one to 0.2, here we can see again this value increases ok. So, initially what we were doing is that, we were taking this curcumin and we are putting this cap ok. The cuvette cap into it and we are mixing it and then measuring the absorbance. What was actually happening out there? Curcumin sometime precipitate in this aqueous medium. So, when we are adding HSA all of the curcumin rather the desired amount of curcumin is not coming into the aqueous solution in which HSA is actually dissolved. In order to do that, we have to just force forcefully actually bring it into the solution by like a mixing it like an up and down method. So, you can proceed with this method, if you feel like or in some advanced machines, UV machines what happened is that there is a magnetic stirrer attached to it.

So, you take a cuvette you that is much more reliable and much more accurate. What happens in the cuvette holder as we can see in here, the cuvette holder get there is a magnetic stirrer. Ok magnetic starrer is basically common in the lab, but this one is quite a micro scale level. So, in the cuvette we actually put a small magnetic bit generally known as rise bit in such a way that it does not affect the cuvette or does not break the cuvette and just turn on the switch on this magnetic stirrer. So, it will rotate it w ill stir the continuously and it will make keep the system homogeneous throughout the reading.

So, another error which I am just actually letting you know, this comes during the experiment so, no need to worry these are all manual errors which actually we face regularly. And if that while scanning from the 600 to 200, the scan speed I have kept as medium. You can keep it as slow also in for slow scan speed the absorbance values much more accurate whereas, for fast scan speed also, the absorbance value sometimes accurate, sometimes may not be accurate. But out here, I keeping it medium.

Now there might be some correlation with the change in the rate of this absorbance measurement. Because what happens while this absorbance measurement is going on from 600 to 200 nanometer? Within that time the solution was kept still ok. So, the

curcumin within the solution this unbound curcumin might settle down ok; might settle down from there where into the bottom of the cuvette. What happens in that case is that, the entire solution may not be what is we call available for absorbance measurement ok. For that might lead to some error also out here. So, these are all manual errors which comes from time to time, so it is always advisable to repeat this experiment 2 or 3 times whatever you are doing so that to get an optimum value after the end of each experiment ok. So, now, we are done with this up to HSA 5 microliter.

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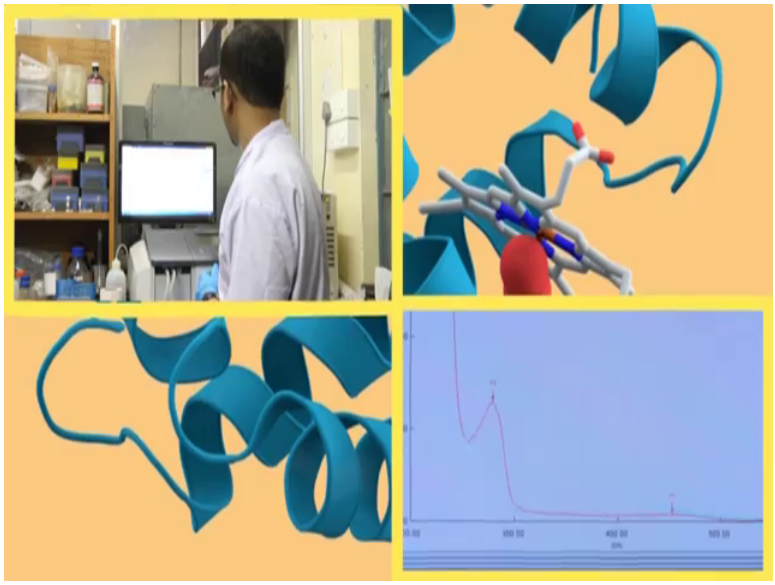

So, I will say as only HSA 5, I will change it to HSA 10. HSA 10 and I will again take around another 5 micro liter of HSA take around 5 micro liter of HSA. I am adding here, mix it thoroughly. In a similar process, we are going to do this auto 0 and after auto 0, we are keeping the start. So, here a blue line it is not visible out here, here there is a faint blue line sky blue line is coming and again if absorbance is increasing.

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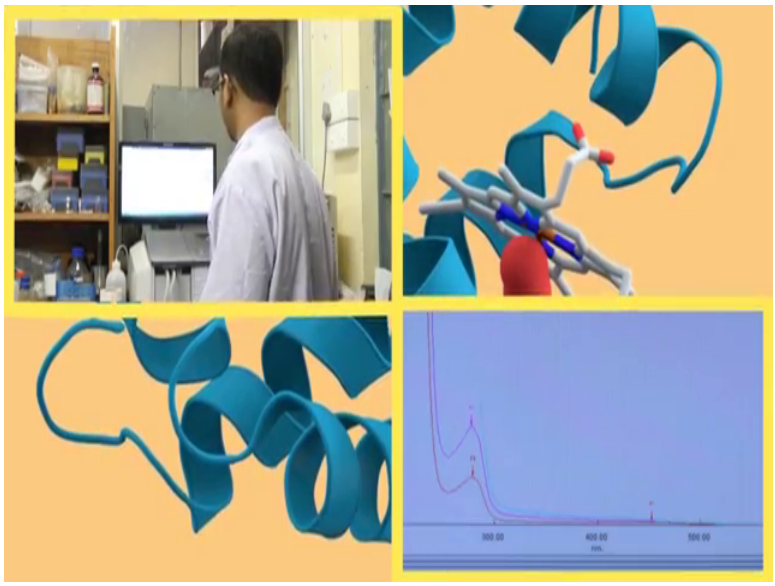

Here I guess it is now a bit not available right now this is visible here; this small into the faint sky blue line ok. So, it is increasing I am again changing those value to 0.5. So, again it increases as we have seen previously these regions, what we can see am showing you just saving this one. this one I am saving as HSA 15. So, these regions this 2.0 or rather 3; so, this peaks ok. So, we have also seen previously when HSA was added to curcumin, we can see the appearance of these sorts of peaks. So, these are actually the peptide bond, this absorbance due to peptide bond ok 5. Now this was for 15 and we will again add another 5 to make it 20 for making auto 0, then start. Well this is one of the methods of a analyzing this protein ligand binding using UV spectrum of automated method. There are various other methods which we have also seen some we have also seen in the theory classes ok.

However, apart from this one spectrophotometric method is also fluorescence. In case of fluorescence what we do actually, we measure in a just the opposite way. We take the protein ok, so just note it down we are not doing out here but just note it down, what we do is that we take a protein solution. We take the fluorescence emission of the protein ok. So, the protein meaning we are taking this HSA solution human serum albumin, it has tryptophan residues and tyrosine also. So, we are not bothering about the tyrosine ok. Because as we know if we take the fluorescence spectra of tyrosine, we will not see it is actually emission lambda max. Because due to the higher quantum value of this quantum

yield of tryptophan, it will overshadow this tyrosine. So, what will happen we will get we are interested in the fluorescence emission of this tryptophan ok.

So, what we will do is that we will take the fluorescence emission of tryptophan entire in HSA pure HSA and to it we will gradually add the ligand. So, out here what we have done? We have taken this ligand or curcumin and to it we have added HSA ok. In fluorescence, what will do? We will take HSA and monitor its fluorescence spectra that is fluorescence spectra of HSA and to it we will gradually add curcumin. People might ask that why you are not taking with curcumin or all that. So, this is actually a standardized method. You can actually extend this method in whatever way you want and we have to basically standardize it. So, this method works for all sorts of protein ligand complexes where in fluorescence spectro of spectrophotometer. So, out here what we can feel that again this absorbance value increases for protein, we have 20. Now I am taking 10 microliter.

So, initially I have taken up to 20 microliter. So, I am taking another 10 microliter the addition will give me total 30 microliter of HSA in the solution. Please note the amount of the volume or rather the amount of protein or the amount of blank pitch will take it should correspond to that similar volume which you have taken in the previous case ok. So, suppose in the previous one we have taken like 20 microliter of protein and now we are adding 22 microliter of protein like 20 microliter of protein plus curcumin and now we are taking only 22 microliter of protein. So, it will not work because you have to take the exact volume of this solution in the blank only which you have taken in the sample part.

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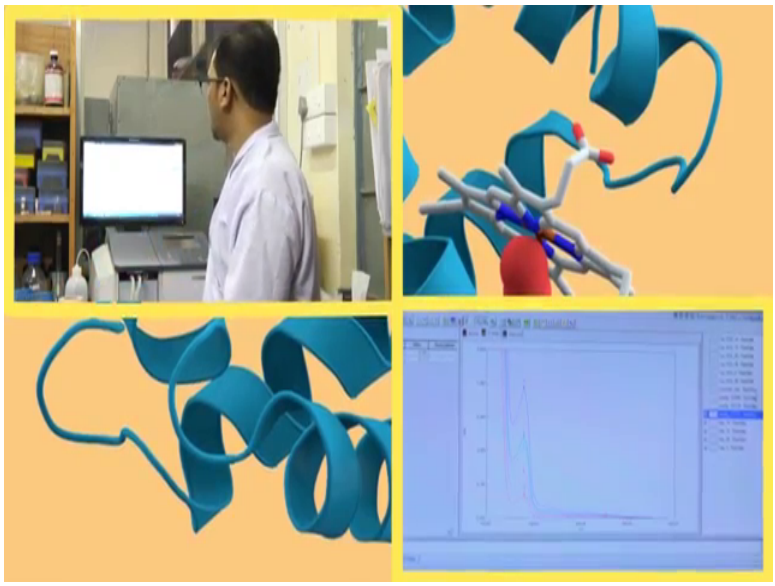

So, on a general note we are let us just revise the UV curve of this protein because you might be having your exams now and so, let us revise this one. So, the UV part of this protein it contains 1 lambda max that is around 278 nanometer or 280 nanometer ok. Now for different amino acids as we have previously seen, different amino acid for different UV absorption spectra, but we are bothered with the UV absorption spectra for aromatic amino acids only ok. Like phenyl alanine, tyrosine tryptophan, but most importantly we are interested in determining the rather analyzing the uv spectra for tyrosine or tryptophan out here.

So, this protein UV spectra do not have any remarkable absorbance or any remarkable sort of peak in the region above 300 ok. So, scanning from 600 we do not have anything above 300. It gives in a region of less than 300 in a region of 280 or 278. So, this is a characteristic of protein this is a protein spectra having a sort of amino acid residue which absorb which is the which have a chromoforb to it ok. Apart from it this region now we have seen make it to 4.0 this is for peptide absorption ok.

I am saving this one as HSA 20. So, what I have done HSA 30. So, the previous one actually I have to save the previous one also, but I am currently saving this one. Ok I am showing you a small instrumental technique suppose you forgot to save any sort of data or any sort of spectra ok. In the time well, but you will save it in the later part ok. For how to do it, but for the time being I am saving this one, this spectra 0.5 this deep violet

color spectrum, violet line this one I will save as HSA 30, but I forgot to save the HSA 20 ok. So, how to save HSA 20? Text right now, HSA 20 is actually this blue line ok. I am doing this one, this one HSA 20. I have not saved this one ok. Now once you there are 3 types of layer out here, here you can see one is active, one is overlay and one is tact. So, currently we are in overlay ok, this one of tact these are different types of screen variation which we can see and one is active.

An active part is that which we can be we are actually monitoring. The active part is the HSA 30 ok; so, the active part now if we click on this one, this one, this green line ok. This line is actually violet line ok. But if we just double click on this line, green line double clicking double clicked on it and if we come to active we can see this has turned to green line. So what, if the basic fundamental part out here? If you have forgot to save any spectra while taking measurements what we have to do it after experiment, you have to go to that spectra double click on it go to this active tab bar and see whether that color spectrum is active or not and then again you go here in the file in a similar way save as save it as for 20 ok. Let us see if I have save it or not and save it as 20 ok. I am now saving it as text file, saving it as 20. Fine now the last thing for the control is another 20 microliter. Now I am taking 20 microliter initially, it was 30 microliter in total and I am adding 20 microliter which brings me the final volume to 50 microliter of HSA.

Now while calculating the concentration of HSA, please note to take the final volume in the way which we are adding the solution like, initially we are taking 3 ml solution and to it you are gradually adding 5 microliter, 5 microliter of HSA. So, the first concentration of HSA should be calculating the volume not 3 micro 3 milli ml or 3000 microliter. It should be 3000 and 5 microliter the final volume ok. So, in v 1, s 1, v 2, s 2 what you see the final volume v 2 should be 3005 microliter. Again when you are adding 10 microliter of HSA to it and the final volume is 2010 microliter. So, this total volume should be taken into consideration while calculating the final concentration of HSA in this cuvette ok.

So, why we require the final concentration? Now how to analyze this spectras or how to analyze this interaction? Basically if you get into the theoretical part there is a equation called Benesi Hildebrand plot ok. Basically in easier terms that we can say we have to actually plot the absorbance versus concentration. Whose concentration? The concentration which you are varying, the absorbance will be on the y axis not absorbance

I am coming to it and concentration will be under x axis. Coming to absorbance, the absorbance will be one by change in absorbance. Change in absorbance means 1 divided by or rather the reciprocal of change in absorbance. The initial absorbance like initial absorbance is the or rather the change from the absorbance of the ligand which we are measuring initially. Suppose in this case we have taken curcumin ok. We now we will be measuring the change in the absorbance of curcumin and we will be taking 1 by the reciprocal to it that is one by delta a. Delta a is a change in absorbance.

And in the x axis we will be plotting from that is the ok. In the x axis we will be taking one by concentration of HSA because you are increasing the concentration of HSA so, with increasing concentration of HSA how the reciprocal of the change in absorbance of varying you have to plot it and from that plot actually if you just to do a bit calculation from that you can get the affinity value or the binding affinity value from that case we can actually use to quantitatively estimate the value of the protein ligand binding.

Now suppose we are measuring the absorbance. So, at what wavelength or which wavelength we are interested in? As I said it is one by delta a, but absorbance at what which wavelength. So, the wavelength we are considering if the wavelength where the changes will occur or rather in where the maximum change in curcumin will take place which we will we can find once we get the subtracted values. Now we will proceed for subtraction, that is will subtract the blank one that if the increasing concentration of HSA that is the e concentration of HSA from it is respective a curcumin plus HSA complex.

Here we can see this interesting spectra with increasing concentration of HSA we are getting an increasing value of the absorbance. Now these are control, what we need to do is that we need to subtract. Subtract whom? So, let us first disable all this out here from here what we have saved we will be taking curcumin HSA this is curcumin plus HSA 5 microliter HSA and this is only 5 microliter of HSA. So, what we are doing of that, now let us enhance this one a bit 0.3. So, this purple line or pink purple line is curcumin plus HSA and this red line is HSA and both the cases the HSA is 5 microliter. What we will do we will actually subtract this one from this one ok. So, how to subtract? Here is a icon known as manipulate ok. Manipulate icon give an option for subtraction ok. The type here we will select data set the data will take it from here and the operation out here we will select as subtraction. So, this is a facility for this type of software this instrument gives this facility or the instrument might have some advanced facilities also.

So, suppose what happens if your instrument does not provide this type of facility? What you can do is that what conventional people does, we will take the text file we will take the text file this text file for curcumin plus HSA 5 microliter and we will take the text file for HSA 5 microliter and we will plot it in excel and we will subtract it like the normal way. Out here we will take curcumin HSA 5 we have selected and we will subtract it from HSA 5. Value we just get it giving our random names sub 5 and this is the value for sub 5 ok. So, am just saving this one and saving this as SUB C 5 which means subtracted curcumin for 5 micro molar of HSA. SUB C 5 I am saving again this one as text file. Now I am turning this off this is su subtracted file ok. The second thing, we need we are going to subtract is curcumin HSA 10 minus HSA 10.

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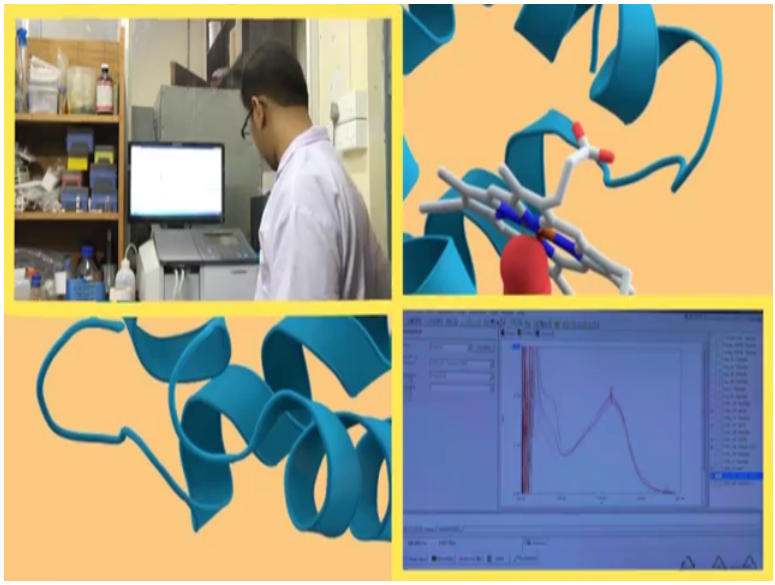

So, SUB 10 again a random name. So, it gives another value ok. So, this value is actually a bit higher than this one ok. You are saving this one as SUB here C 10 ok. And now what we will go we will shift for 15. Curcumin HSA 15 minus HSA 15 is calculated as SUB 15. So, this one is for 15. We will save as again su sub in this way only similarly we have to go on subtracting ok. Subtracting 15 and each and every time you subtract you make sure to save these files. Next what I am doing is that I am doing 20. So, curcumin is HSA 20 and HSA only 20 I am subtracting this one. SUB 20 SUB C 20 and now we are taking this 30 curcumin HSA 30 subtracting from HSA 30 and calculating it. We are saving as again SUB C 30 and finally, we have curcumin HSA 50 and we are subtracting from HSA 50. So, let us have this one again as not text file this is curcumin C 50, SUB C

50 save as this one ok. And now let us enable this one. So, what we can see out here these peaks there is a bit distribution some are increasing some are decreasing ok.

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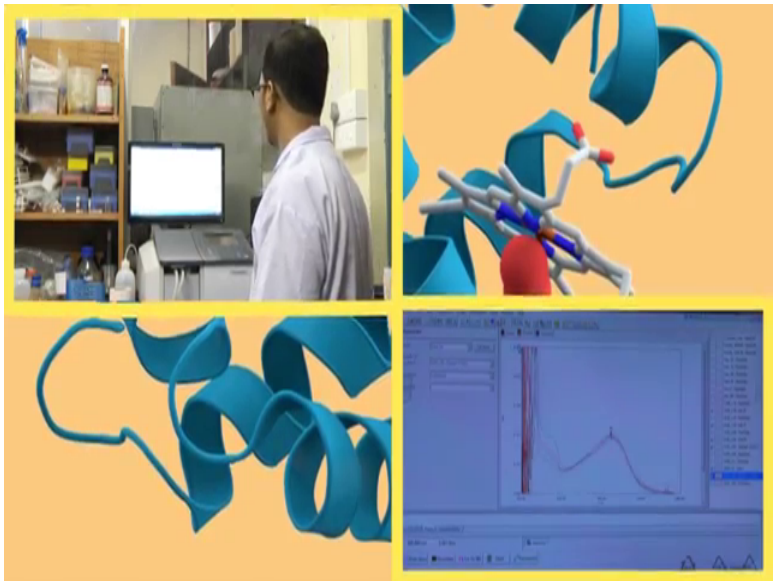

If we take it point 50; so, in this region 1, 0.

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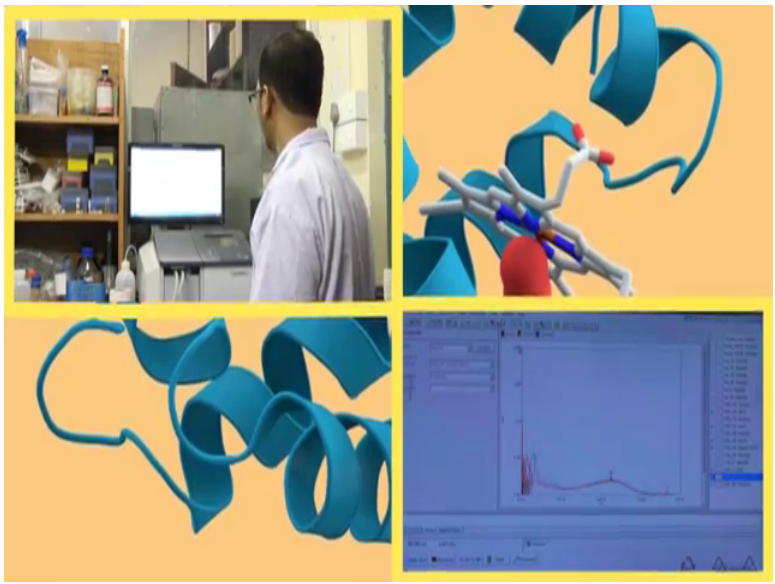

So, this region is quite not a very happy region it is quite distributed in uneven process. So, we can change this one from 250 then visualize this one from 250 and then we can change it well again to 0.25 ok. So, let us analyze this one. So, we are now disabling all those spectras and this one is the spectra for curcumin 0.3. Ok there is spectra for pure

curcumin. Now once we have added HSA the actual value is actually not this one ok. Subtracted ch 5 ok. So, the next value is the subtracted value ok. So, this one is a (Refer Time: 31:23) decreases.

Now again when you are adding 10 microliter of HSA what is happening? You will be adding another decrease in value, in this way what we can see is that this value again decreases here. We can see bit decrease in this value. we cannot this one, again this one now this one is actually the raw data this one and finally, this one.

So, what we can see is that initially there is a bit decreasein no this one is actually have protein. This one have actually protein. So, ya this one where actually all those names are the similar names subtraction so, it will be a bit difficult to find out. for convenience I am showing that this line this red line this red line watch what you can see out here has a particular absorbance value. Now some absorbance are getting decreased some are increased ok.

In this region some are getting decreased some are getting increased, where as in this region what you can see out here in around 300 region everything is getting decreased. So, for taking this delta absorbance value delta a value which region we are actually interested in fine. Which again we are interested in? We are interested in that region where the decrease or the change in the absorbance values are quite smooth ok. In all those cases in all the cases that we can say is that this particular region ok. This on 400 nanometer region should be ideal. But as we all know there might be some manual error while doing experiment whereas, in the next experiment if you proceed what you can see is that, it shows a gradual decrease.

Here we can we have seen that from correspond to curcumin spectra at 420 nanometer when you have added HSA it gradually decreases ok. Up to 10 microliter 15 microliter, but again it increases. So, there might be some anomalies might arise ok Where the anomalies lies when I am saying you telling this to you and anomaly there might be anomaly there might be logic ok. I am saying this to a bit later. But; however, for calculation you can go it in a region around three hundred nanometer or this region which you feel is good enough for calculation. However, from here we cant actually quantify the extent of interaction until and unless you plot the equations and get the ka values ok. Now what happens why you are getting this uneven distribution? Number 1:

there might be a manual error ok. That is a very easy solution or very easy problem to address and number 2: another thing might happen is that the curcumin which I have first told that beginning I am telling that curcumin might be actually not completely soluble in water ok. There is a problem with curcumin it is not completely soluble in water. So, what happens when you are taking curcumin in there all you are mixing it up some of the fractions during the scan is getting precipitated ok.

So, while it is getting precipitated as you know from Lambert Pearson the concentration or the light or the concentration of the solution you measured for those particular solvent only or solute only which is homogeneously mixed in water ok, or in the solvent. Out here it is not homogeneously mixed. So, since this one is not homogeneously mixed and something is coming down in the solution. So, what is happening? So, the absorbent value what we are getting is much lower. What we can say, initial curcumin absorbent value what we have got without in absorb HSA should have been a bit higher. If we had to completely mixed it and dissolve it in water or if you are taking a bit lower concentration of curcumin, there are some there are some actually errors which come time to time and you need to actually do an experiment to find out where the errors are. You cannot actually address the error in the beginning only.

So, we need to do the entire experiment to find the errors are and then you can repeat the next experiment only with the same condition. So, out here what is happening they curcumin is actually settling down, but in the bottom ok. Now when you are adding HSA when you are adding HSA what is happening your HSA is combined with curcumin. So, it is very reported that HSA combines with curcumin. You can find in a paper HSA curcumin interaction paper online it is available. HSA is combined with curcumin it is forming an HSA curcumin complex.

So, one interesting happens what you know? When an insoluble component comes in contact with a soluble component and the soluble component is of a much higher volume a much higher dimension then the insoluble component. So, what happens out there is that the insoluble components becomes soluble. So, I am explaining this in terms of protein ligand, curcumin is very small it is just a molecule and whereas, this HSA is a vast molecule it is a protein composed of hundreds and hundreds of small molecules small amino acids. So, when this small curcumin attaches with the HSA that curcumin

might have been initially insoluble in the solution at that moment. But when it is coming in contact with this HSA it is becoming soluble.

So, let us absorbance increases the absorbance of curcumin increases. Now again when you are adding more and more concentration of HSA to it more and more curcumin are coming in contact with HSA and more and more curcumin are getting solubilize in the solution initially there is no one there is no one to solubiiize as the curcumin. So, it was getting precipitated.

So, as we are increasing the concentration of HSA. So, what is happening? More and more curcumin are coming in contact with HSA and its absorbance is increasing. On the other hand something other might happen ok. So, when curcumin is getting attached with HSA what is happening? Those curcumin which were initially soluble. So, I am explaining this for those curcumin which was initially soluble. So, when it was initially soluble we have added HSA to it. So, what is happening? Those solubilized curcumin are giving contact with HSA and up in when it is coming in contact with HSA what is happening its exposure to solution is decreasing. So, this curcumin is no more exposed in the solution because it is coming in contact with our HSA ok.

So, when it is in contact with HSA it is no more exposed in the solution rather it is exposed in the solution to a much lower extent and as a result what is happening its absorbance value is decreasing a bit. And now when you are adding more and more concentration of HSA to it those curcumin which were not solubilize in water is becoming more and more solubilize as a result it absorbance again increases. So, that anomalies absorbance which you might be getting in there for those components which are not properly soluble in water if maybe due to this reason where the protein ligand interaction might result in solubility issues ok.

So, in this way what we can say that it is safe to take the absorbance in this region where there is a constant decrease ok. So, in this region is the constant decrease in the absorbance value and from that we can actually measure the change in the absorbance value. Now we will see how the changes in the gallic acid occur in presence of HSA. We had seen how the changes in the curcumin spectra occurs in presence of HSA and the next ligand which we are interested in is gallic acid. Here we will see a few changes in HSA spectra ok. For first we have to take the uv absorbance of gallic acid now in the

similar way we are taking 100 microliter of gallic acid there is a cuvette I have washed it properly the same cuvette with double distilled water and then ethanol and then I have taken three ml of phosphate buffer in a similar process. Now I am pipetting out 100 microliter of my phosphate buffer and again putting it 100 microliter of gallic acid that is 5 milli molar of gallic acid which I have prepared. Mixing it thoroughly and then doing auto 0. After that start.

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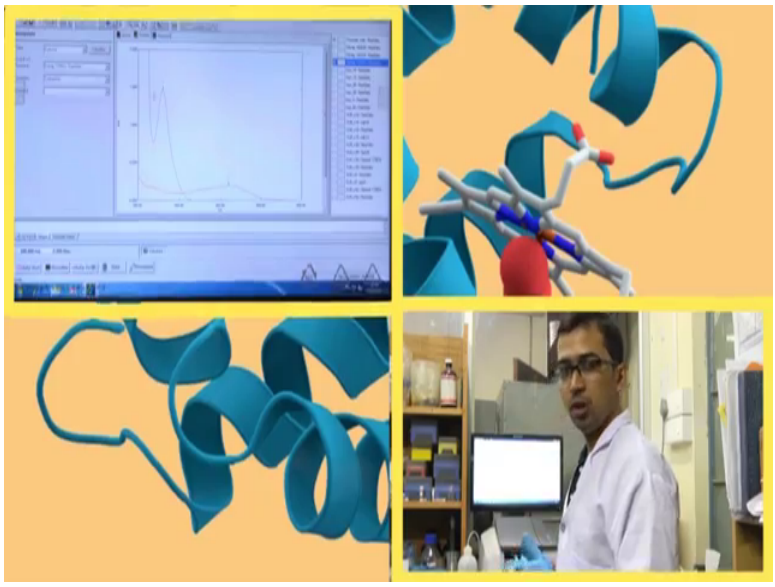

So, gallic acid has a separate spectra should have a separate spectra and this spectra is for curcumin. I have kept it for a reference. So, it is already a 476 so, no notable changes in this region gallic acid absorbs in a region lower wavelength region. Here we can see that if its peak is emerging out here. For this one if the peak for gallic acid ok, now this concentration is a bit higher because it is above 1 for that what I will be doing is that I will be taking a slightly lower concentration of gallic acid in this case.

So, you can actually proceed with this one, but it is better not to take the absorbance value above 1, but during experimental cases if this type comes and you will get a proper spectra we can proceed with it ok. Let me take under dilute solution, then I will just show you how the absorbance come and where are the peaks. For out here we have taken a bit dilute solution of gallic acid fine. Now for the timing what we can feel that it does not show any notable peaks in the higher wavelength region, unlike curcumin. So, we may not take the higher wavelength scan at this moment ok. Why because, it very much

necessary to not to waste this lamp intensity because the lamp will be scanning from 600 to 200 and none of the peaks will be coming at this region that is why we are actually shifting this peak from here go to method from 600 we will going to have 500 ok. Just to actually retain the efficiency of this lamp and not to waste this lamp ok.

So, we are again doing the start ok. We have to do auto 0 fine for that let us do auto 0 and one thing is that if you change this wavelength range sometimes some softwares might ask for again base time collection within that range also ok. So, you might proceed with base line correction first suppose we change from 600, 200 region from 600 to 200, 500 to 200 then again you can actually go for 500 200 base line correction and again go for analysis now whatever is done we will go for start. Here we can see it as change to 500 the scanning is taking place out here, peaks were not observed if there is you know absorption in this region. Here absorption has started ok. Here we can see in a new peak emerges it is just below one, and this peak is around. let us say 3 point no it is around it is around 3.o ok. 3, 3.5.

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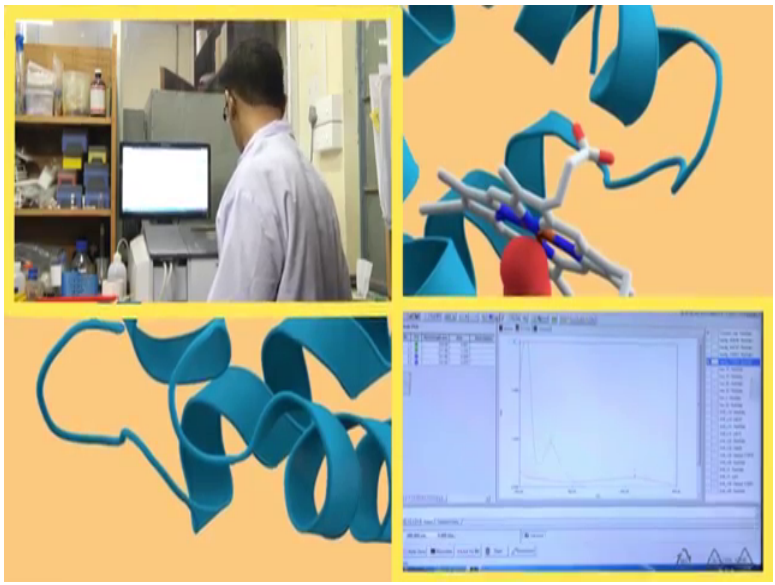

So, here we can see generally two peaks of arises. So, this peak is below 250 region around 210, 211 here 211 is shown, but a peak of interest we are we are interested in is actually this peak which comes around 259 or 260 actually gallic acid absorbs around 260 nanometer. So, an another peak not peak a small hump which you cannot actually see from here, but it is shown around this 250 or something like that is not properly

visible. But out here this 260.;260 region gives an intense peak and if we compare between curcumin and gallic acid. We can see that they have distinct absorbance peak.

So, as we have said that UV absorbance can be used to detect the type of compounds it can this v spectroscopy can actually discriminate types of compound it is very much chemically dependent structure dependent. So, rather functional group dependent also so, this is the UV spectra for gallic acid. So, I am saving this as a gallic acid only. In this file I am saving it also as text file and for the time being I am disabling this curcumin 1 and enabling this gallic acid.

Now what I will do I will add to it HSA to it and see how it changes, but for the time being since we have first seen how the HSA changes in case of curcumin. Out here we are taking selectively a few volumes of HSA few concentration of HSA like 10, 20 and 15 or 30 and we will see how the spectra changes ok. Now will shift to HSA and add it in curcumin or rather gallic acid solution. So, we have taken this gallic acid to it and measured its spectra and now we will add HSA to it. The HSA we will take here is 10 microliter of HSA.

Here we are taking 10 microliter of HSA is in a clean tip microtip. Here we have gallic acid and we are adding here HSA. Mixing it. And again I am taking this cap ok. Now this cap has been cleaned the cap which you will use for curcumin 1 it is better to use a different cap or if we use the same cap make sure to make it properly clean ok. For now I am mixing it, transferring out here and doing auto 0, after the start. So, as we have seen this one is also expected to come in a region near to it because protein absorbs around 270 to 280. So, overlapping region might take place. Now here I will be doing 10, 20, 30 or 50 as let us see how can we proceed. However, out here we have actually the control 1 or rather the absorbance or HSA only or like HSA only 5 microliter or 10 microliter those will be used for subtraction ok. Now why I am using those because am using this at a at a stretch.

Now if we use any experiment like here we are keeping the same temperature same condition same experimental part at a stretch we are doing it. So, those control we can use for subtraction out here ok. Now suppose what happens, if we use this experiment we do the curcumin experiment on 1 day and we do this gallic acid experiment in a second day. So, at for each and every day make sure to take the contour separately ok, but if we

are doing at a stretch then you can carry out, but it is always better to if you do it separately.

So, this one we can see 0.2 this one again it increases the value we can label this one as gallic acid HSA 10 and as well as this one as also in text file. Now, what I will do? I will again add 10 microliter in order to make the final volume of HSA in it as 20 microliter. Now I will be adding again 10 microliter to it taking this cap mixing it thoroughly and after mixing. Doing auto 0 please make sure that the solution does not spills out when you are mixing it.

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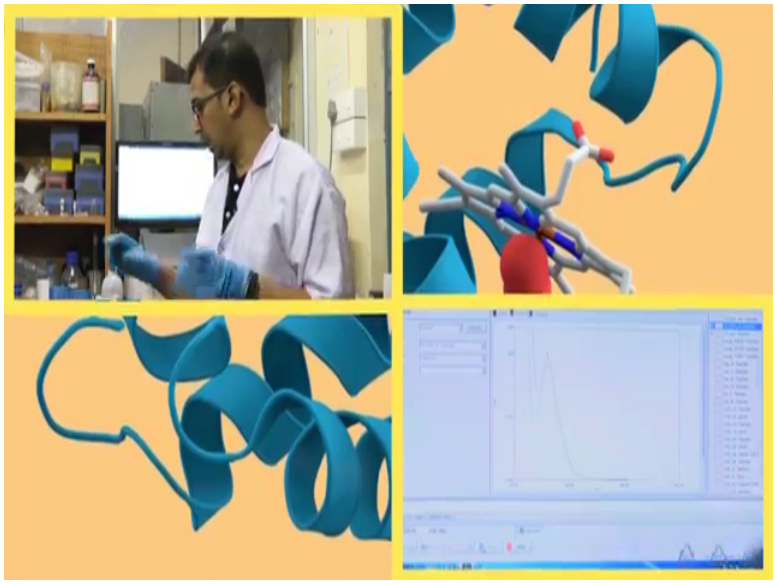

Now for convenient what you can do is that you can use a bigger apparel like micro pipette, like you can use this type of micro pipette ok. Whichever greater tip micro tip this one has a bigger micro tip you can actually put this one within the cuvette chamber or rather the cuvette and you can actually mix it you can actually do this one up and down. If you do this up and down it will just take solution from here and it will just mix it if you are not comfortable using that up and down method using hand, you can do it using a micro pipette. So, let us see how this observation comes. So, again here we can see it is increasing.

So, what happens basically there is a overlap region. So, as an increase in the concentration of HSA so, it absorbance also increases. Now we are saving this one as gallic acid HSA 20. Gallic acid HSA say 20 ok. After that you have to wait I will be

adding another 10 microliter so, it will be 30. So, generally some confusion arises how many readings should one take? Ok. It is always better to take minimum 8 to 10 readings out here due to tutorial class since we were bothered with the nature of the curve. So, we are not going into details into the number of readings out here I am taking 5 to 6 readings for curcumin a bit lesser in this case ok. But while you are doing an experiment make sure to take minimum 8 to 10 readings.

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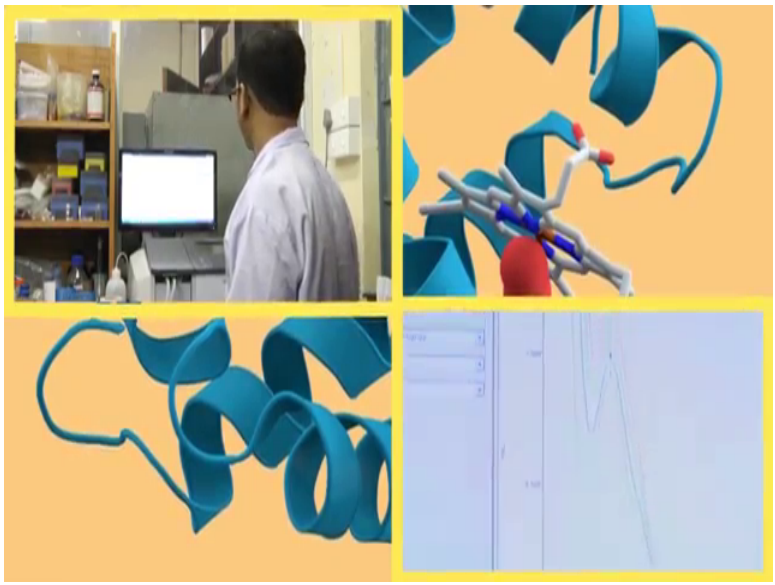

Why? Because when you will be plotting out there. So, what happens you might see that some reading might have errors ok. So, out there minimum from 8 to 10 readings you can see 3 to 4 readings might have error ok. So, the change is not in a regular fashion in which it should have been expected ok. So, in that case you have to just omit those readings ok. But if you have only 5 readings and you omit 3 of them from that only. So, we are left with only 2 points and you can actually draw any straight line or acceptable straight line or you can draw a straight line using 2 points, but not for any experiment. For an experiment you need minimum 5 to 6 points.

So, if you take at least 8 to 10 points from that easily you can deduce you can subtract or you can leave 3, 4 points which are having error and then you can plot it using 5 to 6 points. So, here again the absorbance is increasing. So, 1.5 absorbance has increased and this is gallic acid and HSA 30 micro molar. Now we are going finally, into 50 micro

molar that is the final concentration. So, we will set it at around 20 microliter and we will add. 20 microliter of HSA to it you are setting this micro pipette to 20 microliter ok.

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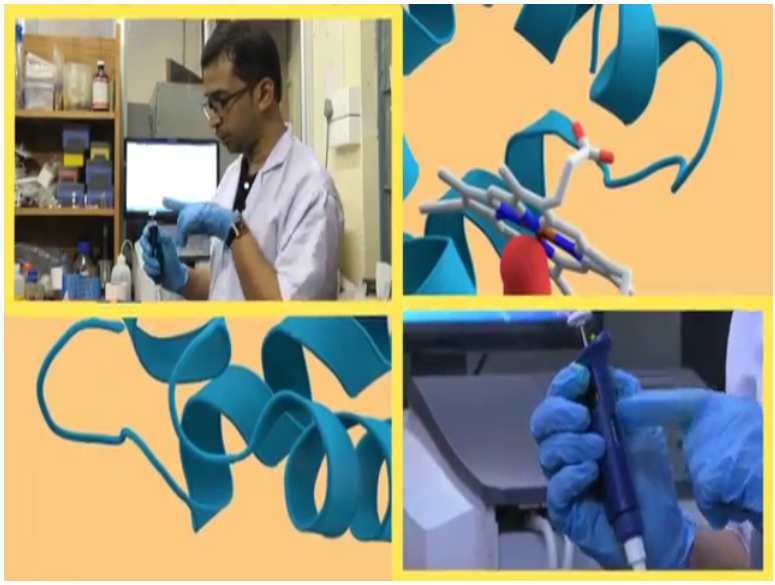

This is also saying this is a revision class. So, in this way what you can see is that this is the micro pipette this is the 20 microliter micro pipette 20 microliter and here the readings are there do you have you have already studied in the first class. So, just a reminder this is a 20 microliter pipette and here you can see the graduation this 2 0 0 indicates here it is actually 20 microliter. Now I will be taking 20 microliter of HSA from it. Now I will be taking 20 microliter of HSA and please remember to use the same HSA solution for sample and control ok. Do not change the HSA solution or the concentration.

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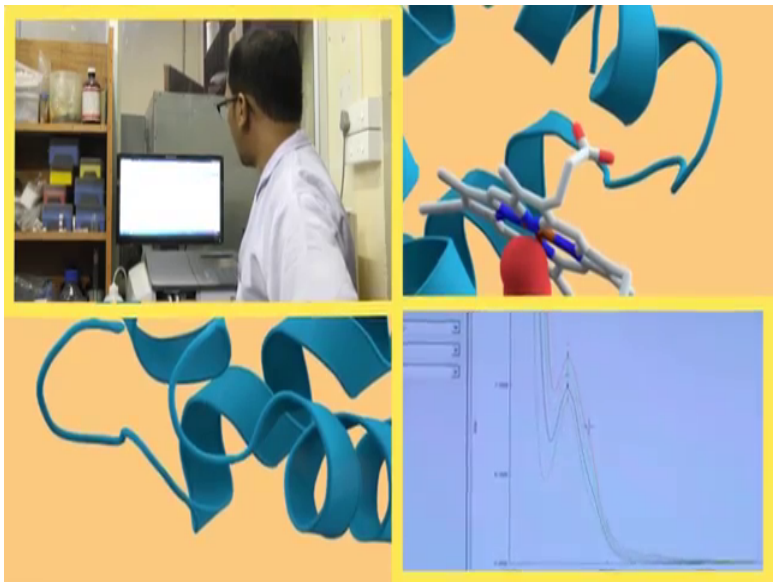

This is for 50 auto 0 and now starting this one. Here we can see the value gradually increases. So, this part is actually well appear we can see a small hump this one this red line and blue line green line here you can see a small hump is arriving here. A small hump followed by another peak. So, this hump is basically due to your absorbance of HSA out here. So, HSA gives is hump comes around 280. So, HSA gives around 270 to 280 for HSA. So, I am saving this file again GA HSA 50 and HSA 50 text file. Now we will do the subtraction and see in which range the values come and how the changes out here ok.

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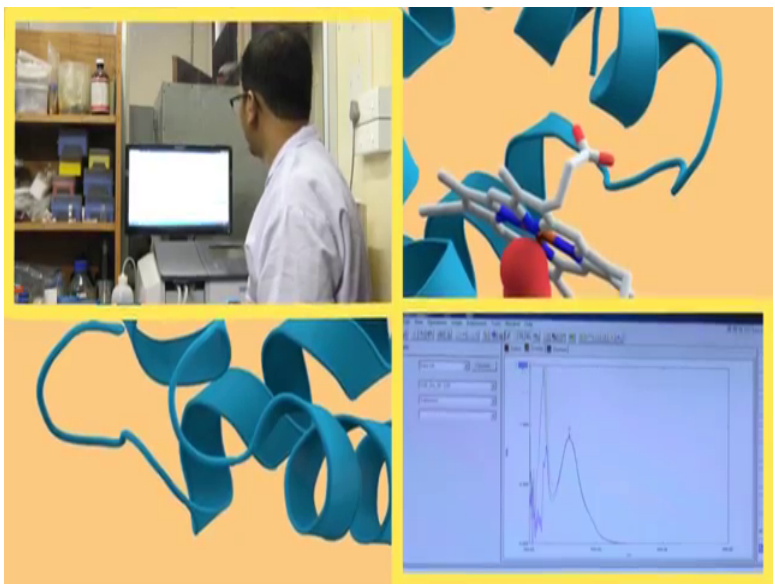

So, again for the subtraction we will go to this manipulate option we will select dataset already subtraction operation is activated from there we will select GA HSA 10 and we will subtract from our HSA 10. It is somewhere out there; HSA 20. So, we have to search it properly so, HSA 10 calculating it S 10 ok. So, it is coming I am going into details about the changes spectra after a few minutes. I am saving it as SUB GA 10 ok. So, for 10 micro molar of HSA if this is a change and again I am filling in the text file on a next thing, I will be doing is I am taking GA HSA 20 and I am subtracting this one from HSA 20 ok. So, just subtract. So, what I am getting S 20 ok saved as SUB GA 20 text file ok.

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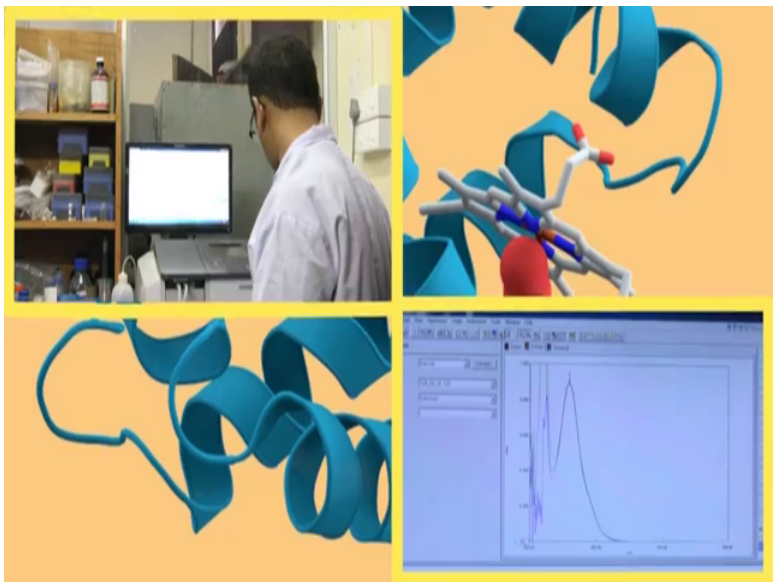

So, now, what I am observing out here is that if we just highlight this one and zoom this point. So, let us put 1.0 and the lower part let us enhanced around 0.6

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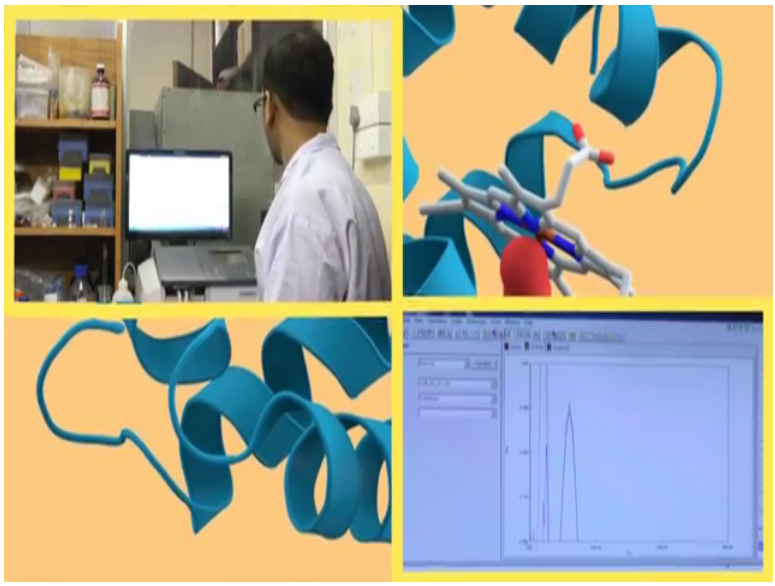

If it is not clear that extent let us change this one as 230 and this one as 300 ok.

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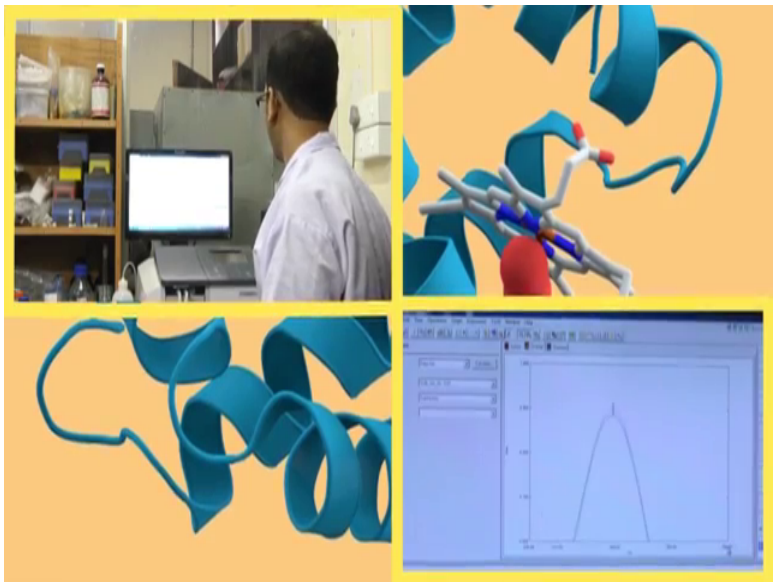

So interestingly what you can see is that or let us say this one as 280 and this one as 240. So, this is the region where the change is occurring around 260. So, here we can see there is not a major change in the intensity, but this one where it is gone?So gallic acid only. So, this is the green line where you can see the gallic acid only the green line ok. The green line appearing and disappearing this is for only gallic acid not HSA. Now when I am giving this 10 microliter of HSA and 20 microliter of HSAso, you can see

here in microliter 20 microliter HSA. So, it is decreasing in the first 2 cases. Now let us see what happens when you subtract for higher concentrations. Say 30 HSA, 30 calculate S 30 now again here we can see this S 30 decreases saved SUB GA 30. 30 and the final one out here is 50.

So, we have to search from here. So, how this files come? Files basically comes out here in the way that you are arrange the names and the according to the names this come ok. So, you will be searching for GA it just went up here. GA HSA 50 and from there HSA only 50 where it is HSA 50 S, 50 for it again increased a bit ok. Save as S, if we change a bit like 0.7, 0.92 ok. So, this one here we can see we have completed the subtraction and this one is for only gallic acid. Where it has gone? Here only gallic acid ok. Out here the absorbance in addition of HSA we now subtracted the control that is only HSA decreases. however, here we can see 1 or 2 points the decrease is not in a regular trend like 3 are decreasing and again 2 are increasing again, but the absorbance value is lower than gallic acid ok.

So, what is happening out here? So, gallic acid is actually soluble in water, we all know solubility has some issues, but is in a soluble in a better extent than curcumin. So, when it is soluble in water more or less most of the gallic acid is actually distributed homogeneously in water and it is showing absorbance. Now it is binding with this protein HSA what is happening? Gallic acid is not at all exposed. Now why this absorbance is decreasing is a very primary question. What happens is that absorbance? Why absorbance is shown? Because when a molecule is exposed to the light incident light it absorbs this incident light. Suppose the molecule is actually bound by this light ok.

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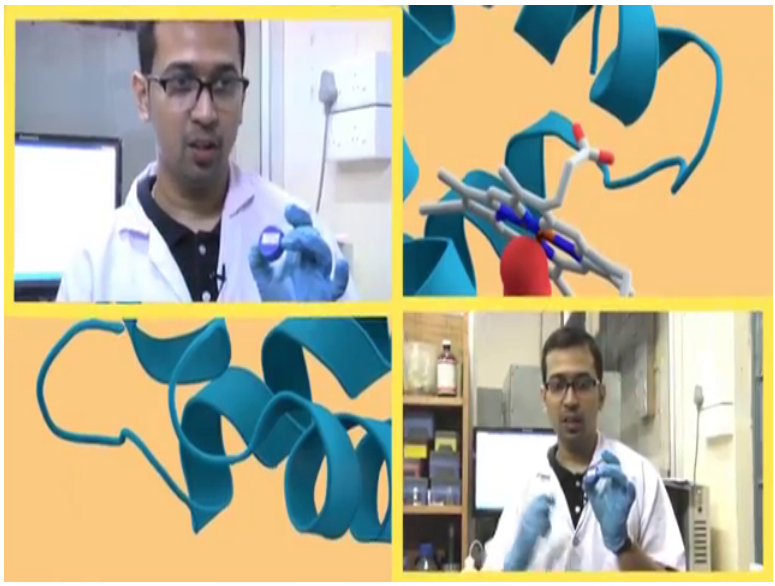

Suppose this is if I give you an example if I give you a some sort of small example out here. What you can see is that suppose this is a molecule suppose this is a molecule this is actually a lead of the falcon or cap of the falcon the molecule which shows a uv absorption ok. So, the absorption is shown in the region around 260 as in gallic acid. So, this is for just for gallic acid in this region. Now it is absorbed it is light is emitting, entire part of this gallic acid is exposed in this room as we can see. Suppose this room is actually the uv chamber or the cuvette ok.

Here this is the gallic acid molecule it is exposed to the light this molecule. Now suppose my hand my hand is actually the protein ok. This HSA protein suppose my hand is actually the HSA protein which is coming and this is the molecule ok. Now once it is coming here and it is attaching this in this way here you can only see my hand ok. So, the incident light is actually only seeing my hand or incident light is only seeing the protein molecule it is no longer seeing the gallic acid it is seeing the gallic acid, but at a much lower extent.

In the cuvette, what is happening? The gallic acid is actually facing the light ok. So, suppose this one this is the cap of the falcon I am taking this as a example suppose this room this entire room is a cuvette ok. And this is actually a cuvette in which this is a molecule of gallic acid. That is how covering here throughout during brownian motion all those things right. So, now what is happening out here, this gallic acid is absorbing

light. So, the light actually supposed any light which is incident on it this is the uv light ok. So, the uv light is absorbing absorbed by this gallic acid. It was free initially it was happy. Now when we are adding HSA molecule protein molecules are coming, suppose my hand is actually this hand is actually the protein molecule now when protein molecule is coming this suppose this gallic acid this is absorbing from this side, it is absorbing from this side here there throughout the molecule throughout the surface it is absorbing ok.

So, now, what is happening is that when this protein molecule is coming in it is attaching to this spot this gallic acid is getting attached to this protein molecule. So, when this gallic acid is getting attached to this protein molecule one part of this gallic acid is facing the uv light or something like that or rather the uv light is not at all being exposed to this gallic acid. So, here what we can see that more or less you can see my hand only and you cannot actually see the gallic acid anymore or rather you can see small fragment of small portion of this gallic acid.

So, what is happening when the protein is getting bound with this ligand only a small part of this ligand is exposed. So, the entire ligand cannot actually absorb from there ok. So, what is happening it actually the extent of absorbance or a with the amount of absorbing power of this ligand actually decreases. Because it is not attached it is not at all exposed to uv light and as a result its absorbance value decreases.

Now what is happening in the actual case is that, suppose this due to this one there is a lots of beadings are attached got functional models are attached which are responsible for absorbance. Suppose one ring is getting attached to a protein molecule which was responsible for absorbance and that absorbance correspond to that ring is decreasing ok. Several things might happen in the medium, but out here due to this protein ligand direction and this is the reason in which this molecule the absorbance is decreasing. Where does in case of curcumin, what is happening?

In curcumin like this is hovering here and there curcumin is settled down like this way. So, curcumin is down it is not there it was not hovering in this region. But when protein came it rescued curcumin curcumin is a long molecule one part of the curcumin is actually becoming into solution whereas, the other part is not. So, due to change in the functional moiety actually curcumin and gallic acid are different from functional moiety

function compounds different functionality change in absorbance might differ. From this way can actually estimate what is the extent of interaction? What is the extent of interaction between the ligand and the complex and the protein.

Now, this is the change in the structure change in absorbance of our gallic acid in presence of HSA. Now from here we can actually create take the delta a in the region around 260 because it do not have any other absorbance region and here we can see this region is quite what we can say it decreasing in a very systematic way on a regular way and here we can change take this one with increase in concentration of HSA and we can plot this one ok.

And from this plot we can take actually the ratio of the slope or ratio of the actually we have to take the intersect and slope ok. So, intersect by slope if we just take we can get the k a value. And if we compare the ka value between the curcumin and the gallic acid we can know which one interacts with protein and we can actually quantify the extent of interaction. So, we have seen how to carry out the experiments concerning protein ligand interaction.

Initially we have taken HSA solution 1 millimolar HSA and we are prepared gallic acid as well as curcumin ok. We have taken gallic acid we have dissolved it in double distilled water and you have taken curcumin initial dissolved it in ethanol ok. If this ethanol is actually hplc bred hybrid ethanol and then from that ethanol we have taken a small amount of ethanol in double distilled water and made up the volume and the water in the water ethanol mixture the percentage of ethanol is better to keep it around 10 to 20 or around 10 is better ten percent ethanol is good do not increase the concentration of ethanol then it will harm your protein.

Now after we have prepared it we have shifted to this uv instrument where do we have initially taken a cuvette done the different correction with phosphate buffer ph 7.4 and your concentration is around 20 milli molar. And now to it we have gradually added HSA, but before that we have taken curcumin to it we have actually studied the UV absorbance of curcumin. You have seen how the peaks of absorbance retro curcumin comes and we have added HSA to it and it gives particular spectra and we have taken another blank which contains only phosphate buffer HSA. You have subtracted that absorbance HSA from curcumin plus HSA solution and you have obtained only HSA

solution ok. Only curcumin solution fine. And after that we have plotted it and again we have taken this gallic acid taken its absorbance added HSA to it, taken it absorbance or gallic acid HSA solution after you have taken only HSA and subtracted it.

So, in a nutshell what you have seen we have taken the ligand and taken its uv added the protein and finally, got the spectra and subtracted it from the control which is only the protein. In this way we actually can estimate the protein ligand interaction and observe it. Now there are some other ways in which we can do it monitor it using this uv tool only. Suppose we want to monitor with change in temperature how the interaction varies. So, what we can do we can actually increase the temperature and actually we can for that part what we need to do we need to take our ligand and we need a protein and different temperature we can study the protein ligand interaction. We can start a protein ligand interaction in different th we had taken phosphate buffer ph 7.4 we can do it at phosphate buffer ph 6 also.

So, in different way we can actually regulate the protein ligand interaction why at different region because in a body the ph varies at some region temperature varies, some region environment varies. So, in order to mimic a biological system or a biological process we have to change the medium, this is a most generalized medium in which we are using actually a phosphor buffer if we change your different temperature and other ph conditions in this way what we can do we can actually get a outlook of how a protein ligand direction might take place in a biological system. So, I hope this experiment was helpful in giving you an idea how ligand or how solutes might interact with protein and how that absorbance of the solutes might change in presence of protein.

So, thank you.