Experimental Biochemistry
Prof. Pritam Roy
Department of Chemistry
Indian Institute of Technology, Kharagpur

Lecture - 21
Denaturation of HSA By GdnHCl Studied by Trp Fluorescence

We have previously seen in the first part of the video how to do protein, denaturation using temperature, elevated temperature and in preference of urea. Those studies were done using the visible spectrophotometer. In those studies what we have seen is that we have used HSA that is Humans Serum Albumin protein, and we have subjected it to two different stressful conditions; number one is urea that is.

We have subjected the protein HSA that is human serum albumin to two different conditions. Number one was urea, where we have gradually increase the concentration of urea in case of HSA and we have seen that UV visible spectra of the protein. Actually, we monitor that UV spectra of tryptophan residue and it showed the gradual change in the UV spectrum which indicates that the tryptophan residue somewhere affected or on the other hand we can say that the protein is somewhat affected presence of urea.

On the other hand, when we have used heat treatment that had some elevated temperatures starting from room temperature to 45, 60 degree and even 70, 80 degrees centigrade when you use those higher temperature, there we have seen the UV spectra also changes in case of HSA protein. From our previous studies it has shown that UV spectra suggest denaturation in case of HSA protein.

Now, in our previous week we have seen apart from UV spectra there is another spectrophotometer which is commonly known as the fluorescent spectrophotometer. In this case also we can monitor the change in the tryptophan residue or tryptophan environment of HSA protein in presence of denaturating agent. However, the denaturating agent we will be using out here is guanidine hydrochloride.

Now, we can see how guanidine hydrochloride affects the protein, that is HSA when added in concentration dependent manner. For this what we will do? We will take a particular concentration of HSA protein in a qubit and we will gradually increase the concentration of guanidine hydrochloride. Now, guanidine hydrochloride is an well

known denaturating agent which actually disrupt the structure of protein. When it will act to HSA it should disrupt the structure of HSA also and they are by a fluorescent spectra should give some other types of signal as compared to native protein.

Now, we will proceed with our experimental section. So, this is the fluorescent spectra instrument the fluorimeter, where we will carry out the denaturation studies. In this instrument as we are commonly familiar with there is a chamber like the UV instrument and this is the qubit chamber.

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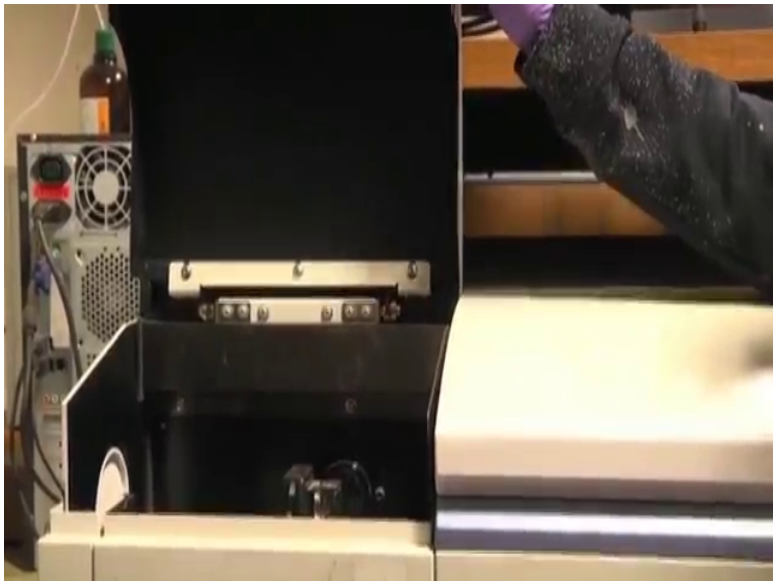

We can open this qubit chamber; this is the lead actually of the qubit chamber. Once you open it, we can see there is a place where we can keep the qubit and this is the entire chamber, the light enters from here and the sample is kept in this chamber.

Now, one thing to note about this instrument is that about its lamp and the fan.

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So, this is the instrument where we can see two options are there, one is run and one is Xe lamp. So, as we know from a theoretical classes that Xe menuphenon lamp is there in the fluorescence spectra instrument. So, in the fluorometer this run indicates actually in case of this instrument the fan. So, a major component of the instrument is the fan. So, why fan is important out here? Basically, when we turn on this lamp of the instrumental, lamp gets heated from time to time when it is lit in the instrument because once we lit in a lamp we always know that it is get heated up and it heats the instrumental other parts. Many crucial parts are there, many small parts are there in this instrument which might be affected when its heated to a very high temperature, in order to compensate that there is actually this run part which is symbolizing the fan.

So, the fan is it makes some sort of sound you can indeed we can understand that the fan is running it makes the sound of a common fan. So, this fan keeps the instrument cool and once you turn on this instrument kindly note that the run part and the xenon part both are blinking. And if the xenon lamp this part is blinking wait for a few minutes until this run part is blinking because this indicates that the fan is working and now you can proceed with the experiment. And if the sound is not working for an instrument for an fluorometer if the fan of not working kindly do not proceed until unless the fan is on, and if it does not turn on then kindly refer to some engineer or some sort of that part. Otherwise, until unless this lamp and fan part is not turning on kindly do not proceed

with this experiment. So, here we can see that both are blinking out here. So, the xenon lamp is on and the fan is also running. So, we can start with this experiment.

Now, we are going to open the software for this instrument. The software is fl solutions for this particular instrument, and once you click it we can see here this initialization is taking place.

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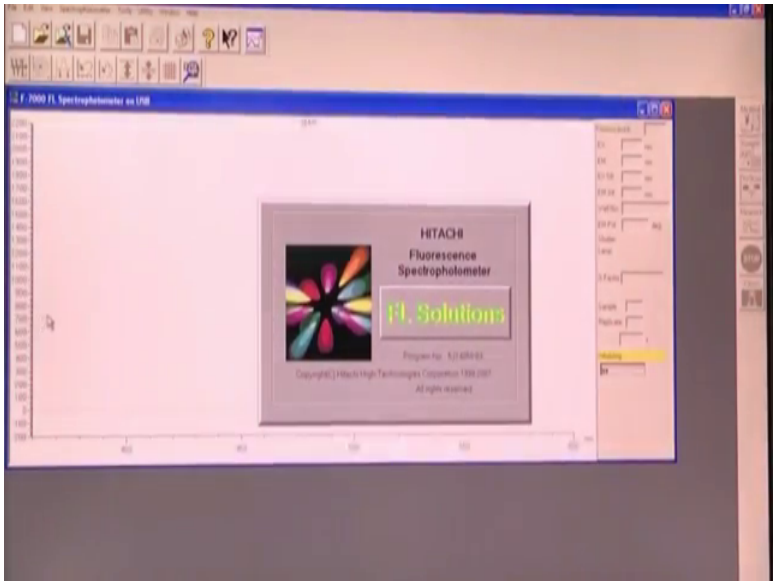

Kindly remember do not proceed with any experimental reading or something like that when initialization is going and do not open the lid also. After that parameter setting is taking place and we can see these are all inactive out here because initialization is still going on, and once this menus are active we can be sure that the initialization is been completed. Now, here we can see the excitation emission exits lead, emissions lead all are given out here, but in this case, we have to change it to our required parameters.

Now, you are going to see the fluorescence spectra of HSA in presence of guanidine hydrochloride, but before that we have to take the fluorescence spectra for native HSA. Now, before proceeding we have to take the sample in the fluorescence qubit.

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So, this fluorescence qubit as we have seen in our previous week the 4 sides of this qubit are clear and is transparent and kindly remember to wash this qubit properly before proceeding with the experiment. I have washed this with double distilled water several times and now we have to clean the 4 sides of this qubit with a clean tissue paper and after cleaning this 4 sides of this qubit, now we could add double distilled water into it.

Now, in this puppet I have taken HSA solution. Now, I will be adding this HSA in this double distilled water, once I have added the HSA in this distilled water make sure to make it homogeneous. Please see that no air bubbles are formed in this qubit. If the air bubbles are formed kindly take necessary steps to drive out the air bubble from the solution, and the solution is clear either we can say it is completely transparent solution of HSA and this is native HSA for whom we will see the fluorescent spectra. So, this is the HSA solution whose fluorescence I am going to examine.

Now, I am going to transfer this qubit into the qubit chamber. So, basically, I am opening this lid and here we can see the qubit chamber present here.

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We will carefully place the qubit in this chamber, only one qubit holder or qubit chambers present here this unlike UV 1 where two qubit holders are present out here only one is present. So, light will enter in this way and the fluorescence will be detected at, right angles 90 degree. As we can see from the theory classes of fluorescence spectra the fluorescence emission is detected at 90 degree to the incident light. Now, this is the entire qubit chamber. Now, I will close this lid before proceeding with the experiment. So, have close this lid now we will set the parameters for our experiment.

Now, we will proceed with the experiment before that we have to set up the methods or rather the parameters which is pretty much important for our study. Now, here we can see methods, sample, pre-scan measure all are given. We will proceed to hear later on, first we will go to method.

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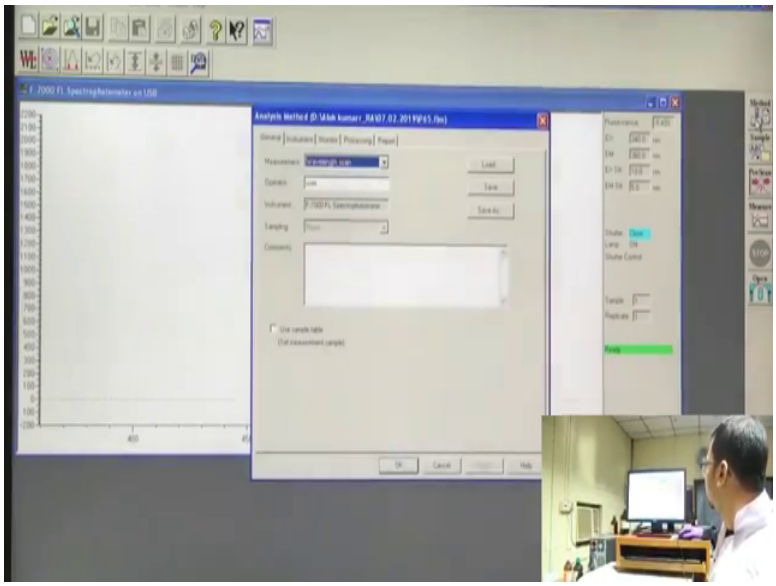

In the method, we can see various terms are icons are there like general instrument monitor. In the measurement part we can see various options are there, out here we will do the wavelength scan, so we will select this wavelength scan.

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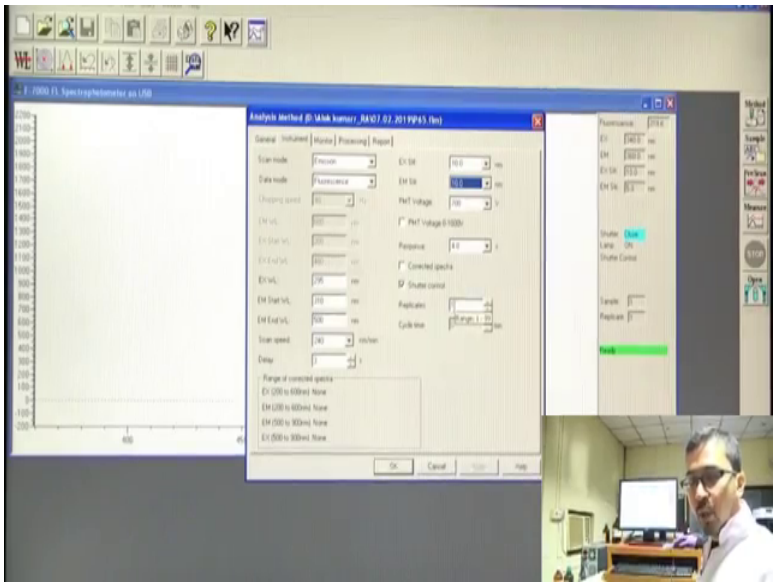

Now, this is the important portion, out here the scan mode we will set as a emission. Kindly note that it is excitation synchronous all are present there, but however, we were bothering with fluorescence emission.

Data mode fluorescence, now come into here the excitation wavelength. Talking about excitation wavelength we will excite it for tryptophan residue. Now, HSF tryptophan residue, so we will excite for tryptophan; as we have seen it the previous week if we excite any protein having both tryptophan and tyrosine for tyrosine residues that excitation wavelength for tyrosine residues, the emissions spectra comes for mainly tryptophan because due to its higher quantum yield the tryptophan gives greater intense permission spectrum which overshadows the emission spectrum for tyrosine.

So, that is why we will give the excitation wavelength for tryptophan that is 295 and this is the emission wavelength. Emission wave length will start from a few wavelength away from the excitation here we can see as 310, and it will go up to 450 or we can set it as 500 whatever. So, let us give it 500 in this case. So, this is the scan speed. We can monitor it at very low scan speed that is 30 or very high scan speed, the scan speed is nanometer per minute. Now, talking about scan speed if you set it as a very low scanspeed the accuracy will be much more then at highest scanspeed, but however for lower scan speed it will take much more time than the highest scan speed in order to compensate both higher and lower scan speed will settle at a scan speed which is in a mid value here let us say for 240.

This is a delay, now they are talking about the excitation emission slit. The excitation slit is set as 10 and nanometer kindly note the unit it is nanometer, and emission slit is also 10 in our case. And these are pmt voltage which you are not currently bothered with, replicated one that is the reading will be repeated only ones.

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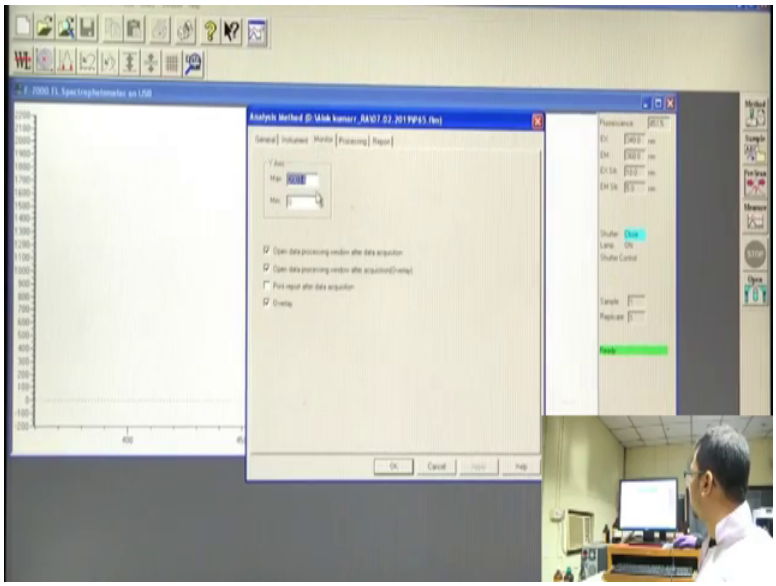

After that monitor, this is the maximum x and y value. So, this the y value basically maximum and minimum part, and the rest of the things we are currently not interested in. So, this one is the main thing that is the excitation wavelength, emissions scan range and the excitation and emission slit with. Now, we are clicking, ok.

After clicking ok, we can see the parameters option is coming which shows that it is getting initialized with the new parameters. After that we can actually set the sample name out here.

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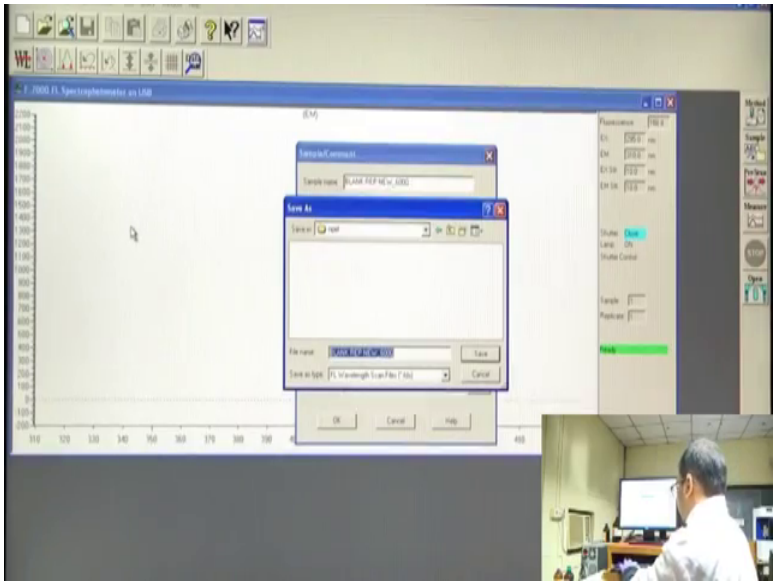

We can go to a file of our choice, we can go to desktop and select anyone like NPTEL, and go there and HSA fl indicating has florescence of file name.

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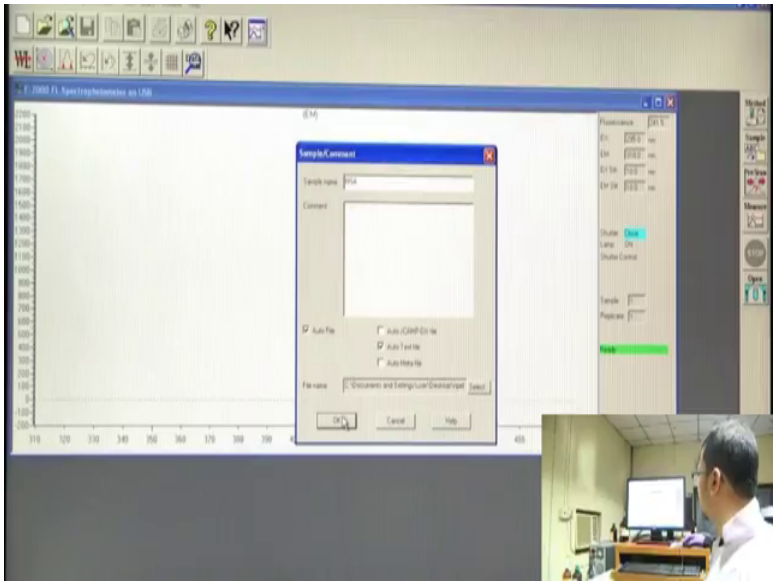

Now, the sample name you can give as HFA, ok. And here we can see auto text file which means that a text file will be generated automatically, and auto file is actually mission file, and now I am clicking ok, so we will start with the experiment.

Now, we will proceed with the scanning part of the sample before that will go to pre-scan, we are clicking pre-scan, this mission require this pre-scan mode, in some mission this pre-scan may not be present and once pre-scan is there again the options come up here it is becomes active. Now, we can click measure.

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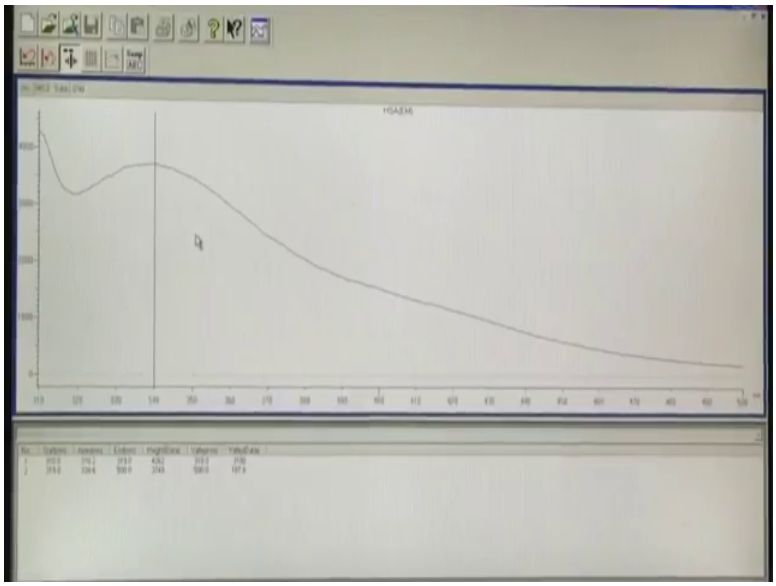

Now, we can see scanning is going on. Here we can see a curve is coming like this one around 340 or 350. Basically, this one is actually the emission for tryptophan residue out here we can see this emission spectra of tryptophan.

Now, from this part we can actually measure the emission intensity from here emission intensity corresponding to particular wavelength. Here the emission intensity maximum emission intensity is the in this range here we can see the maximum intensity at around 340 nanometer and from this data we can get the intensity of value this is arbitrary unit intensity value, we can get intensity value from here. So, this is the emission spectrum for native HSA. Here we can also see the value properly if we turn on the full screen, we can see this value out here. So, this is the emission spectra for HSA. Now, we will add the denaturant that is got in a hydrochloride and see how the spectra changes.

Now, we have seen this is the spectra for native HSA in order to plot this in our computer or desktop we need the values at your text value. So, where we can get the text values? The text file is there in the NPTEL folder.

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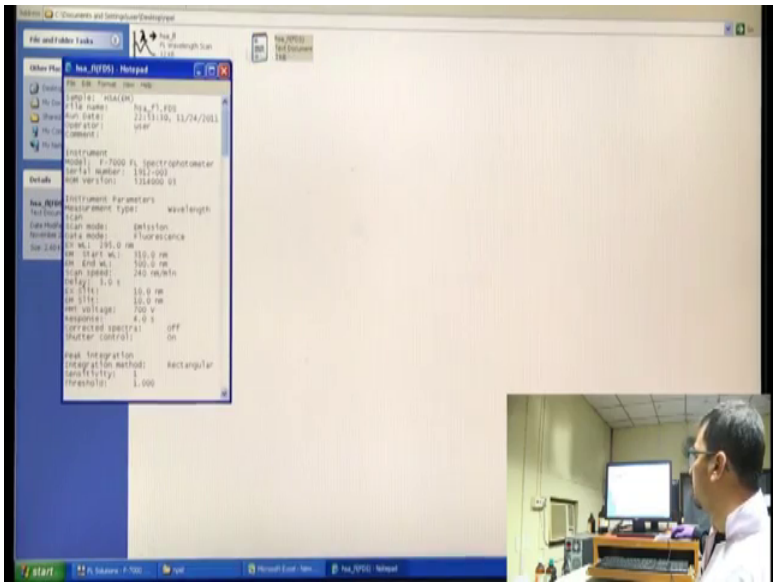

Actually, the folder which you have created for this one. So, here we can see the text document is written for our file.

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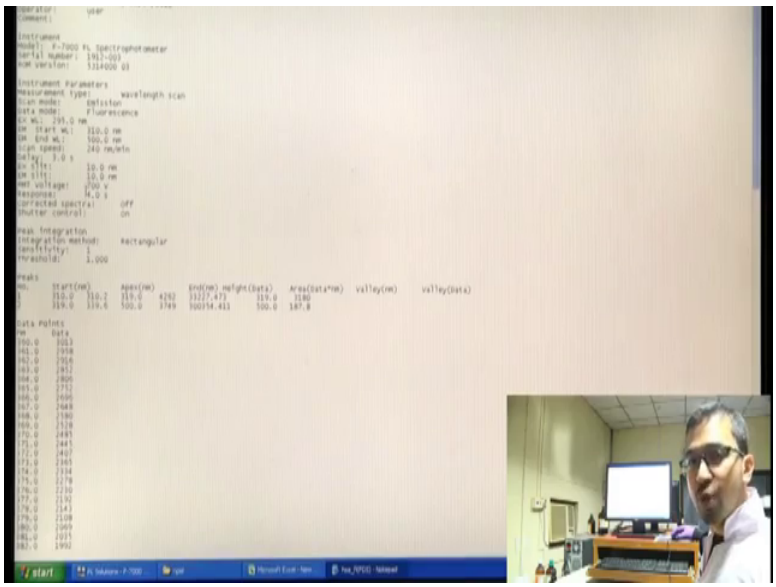

If you click this text document, the text document appears like this. It opens in a notepad, and out here we can see all the parameters which you have given in the method part for this instrument and from here it comes in that values which you are interested in. So, from here we can actually take the text values and plot it and we can get the emissions spectra.

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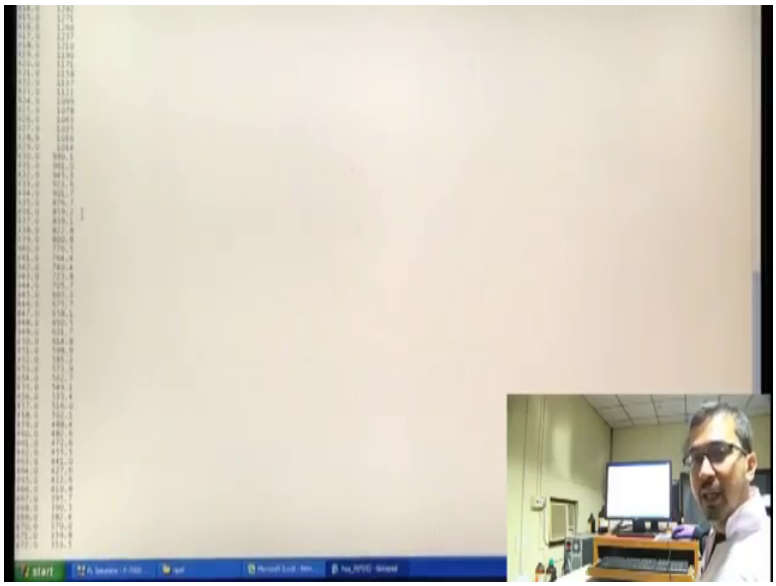

And if we want to copy this one what we can do? We can actually select all those data parameters, we can select the values, we can control C and we can paste in excel, ok.

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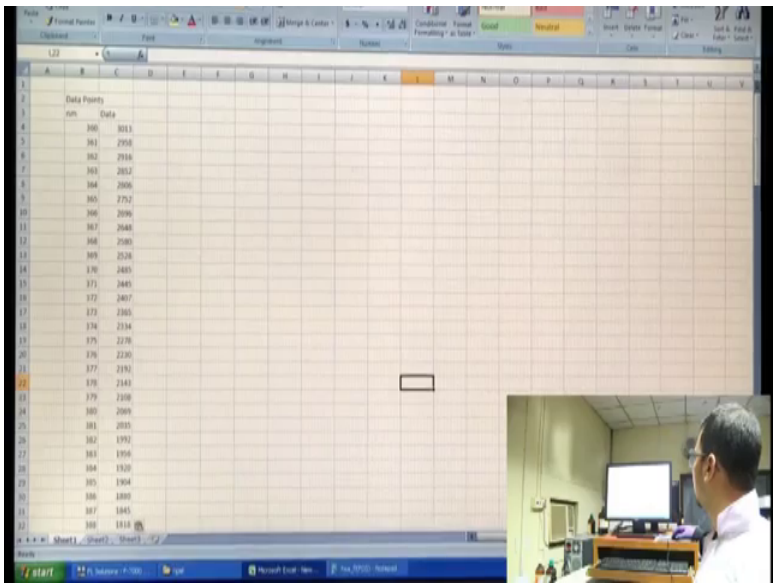

So, these are certain simple things which you might be knowing out here, but the basic thing is you have to first see whether you get a proper hump like this one which indicates we are getting a proper emission spectra. Sometimes you can see that the instead of a hump it comes like a flat type of line. So, in that case you can increase the concentration and proceed with the experiment.

Now, we are going to add guanidine hydrochloride to HSA solution. We are prepared 6 smaller stock solution guanidine hydrochloride. Now, we are adding the small amount of guanidine hydrochloride to HSA solution. After adding kindly make sure to make this sample homogeneous like in this up and down way, after that with a clean tissue paper rub all 4 sides of the qubit and one thing here we can see a small bubble is being formed which we will try to remove here, done. And before using this qubit kindly make sure not to touch this part of the qubit to where sample is there. And kindly hold that part of the qubit was sample is not there, ok.

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So, I am transferring this thing again it is qubit compartment. Now, I am closing this lid and we will proceed with the experiment, and see how the fluorescence spectra of HSA changes upon adding a small amount of guanidine hydrochloride. Now, we will set a sample name out here.

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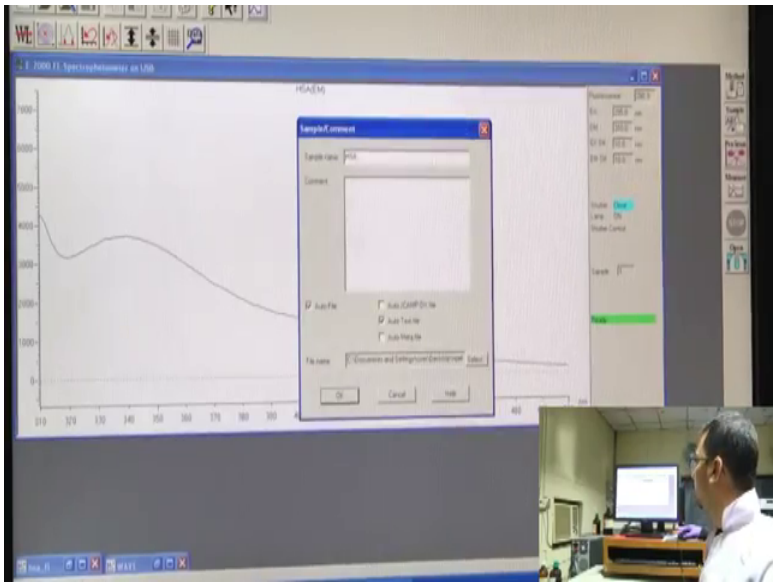

Initially it was only HSA, now we will set as HSA and the score g, g indicates guanidine hydrochloride. For the first sample we will set it as g 1 and, and we will go to measure directly.

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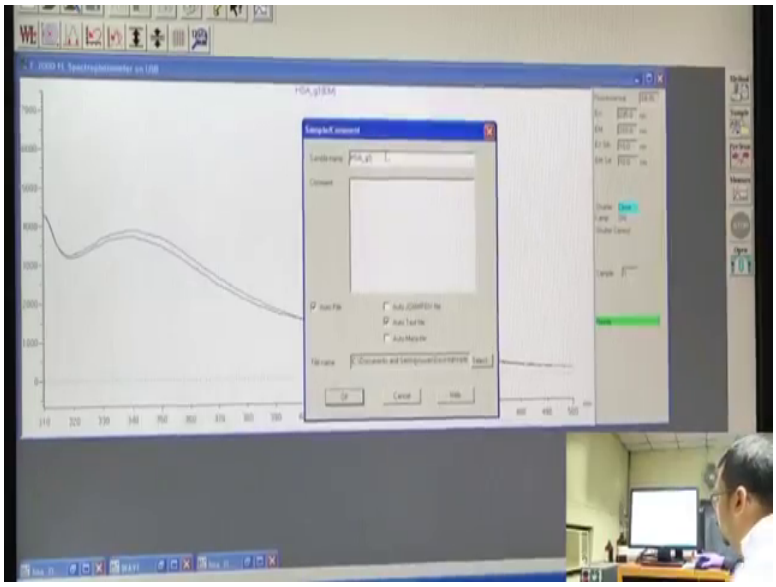

Here we can see the spectra appears that is the emission spectra of tryptophan. Interestingly, we see a slight increase in the emission spectra, this change in the emission spectra is only due to the change in the tryptophan environment which indicates that the protein is somehow affected in presence of guanidine hydrochloride. Now, here if we

open this one, we can go to the value, the maximum one. It comes again around 340. Now, we have to note down the emission value that is the intensity of emission corresponding to this maximum region.

Now, one thing to note out here is that while we will be plotting here actually you may not be able to see this in experiment while doing this experiment, but when we were plotting in excel, so what you can see? You may see that the wavelength out here might shift depending upon the concentration of guanidine hydrochloride use. Out here we can say that it is maxima for a emission comes around 340 and this gives the maxima out here is looks around 340, but it might happen in cases that with addition of denaturant the maximum might shift in a red region which is around 340 to 3 45 or in a blue shift region that is in a lower wavelength region.

Now, it can be clearly understood once we add higher concentration of guanidine hydrochloride in this solution. Now, we will add higher concentration of guanidine hydrochloride to it. We will mix the solution properly and kindly see that bubbles are not formed, and I will place this in the chamber and will wash, we will clean this qubit properly and place this in the chamber. Now, we will set the sample name out here as g 2, and carry out with the measurement.

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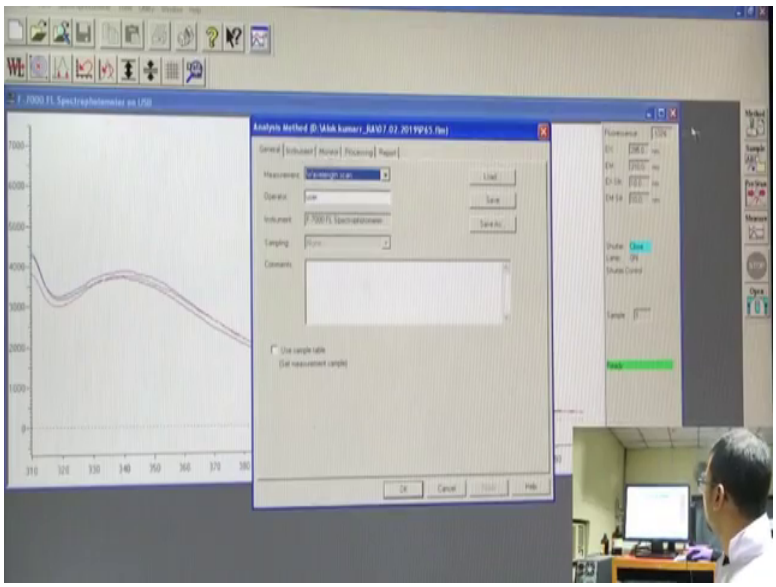

One interesting out here we can see is that the spectra the emission spectra again decreases, ok. So, why this decreases happening? Basically, what is happening in the

solution is that we are adding solution of guanidine hydrochloride. So, the HSA solution which have a specific concentration out here say 2 micro molar or 4 micro molar, in the qubit when added to guanidine hydrochloride or rather when guanidine hydrochloride added to it a specific volume of guanidine hydrochloride is added which results in the change in the concentration of HSA.

So, the concentration of HSA basically decreases when a large volume of sample is added to it. So, what we should do in aware to remove this confusion? Because we cannot be sure whether the tryptophan intensity actually increases or decreases. So, once we are done with this experiment we have to take only double distilled water without HSA and to it add guanidine hydrochloride the exact amount or rather the exact volume from the exact concentration which we are using out here, ok. We will explain this again once we start with the control part. For the time being let us take this spectra for HSA along with guanidine hydrochloride, ok.

We can see there with change again in the emission spectra out here the change might not be might not that be I mean significant; however, the changes happening out here. From here we can actually gather the emission maxima once again, but it is better to calculate the emission maxima after we have carried out the control part. Now, you will add again higher concentration of guanidine hydrochloride. One small thing to note from here is that the emission maxima out here is coming at a value of our own 4000 to 5000, here we can see the y axis. So, in order to get this spectra in a better view in a better angle we can change those maximum y value out here.

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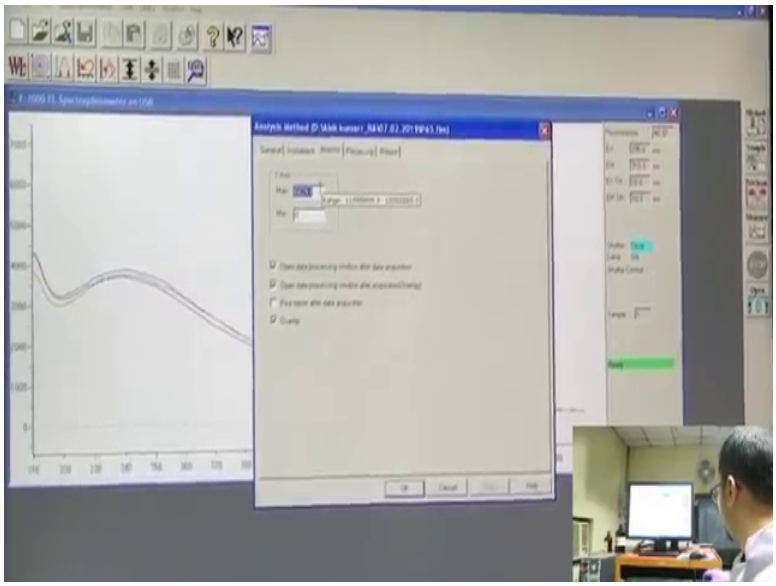

Once you go to this method we go to monitor here the y max we can see it set up to something around 6000 and we can actually change it to 5000. So, we can say that y max has been changed to 5000. In doing so what has happened if that your basically the previous, what we can see, the previous spectra vanishes do not need to bother about this one because all the text files are being saved out here. As you can see one and two and all those text files are being saved out here. So, the thing you need to bother is the text file which I will show you later on.

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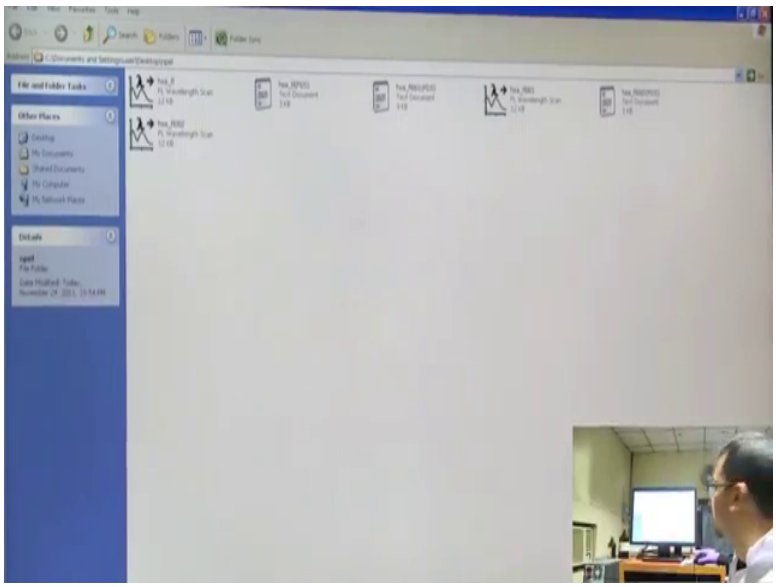

So, out here we have added again the concentration of guanidine hydrochloride and we will go to sample change it to again, 3 and we will click measure.

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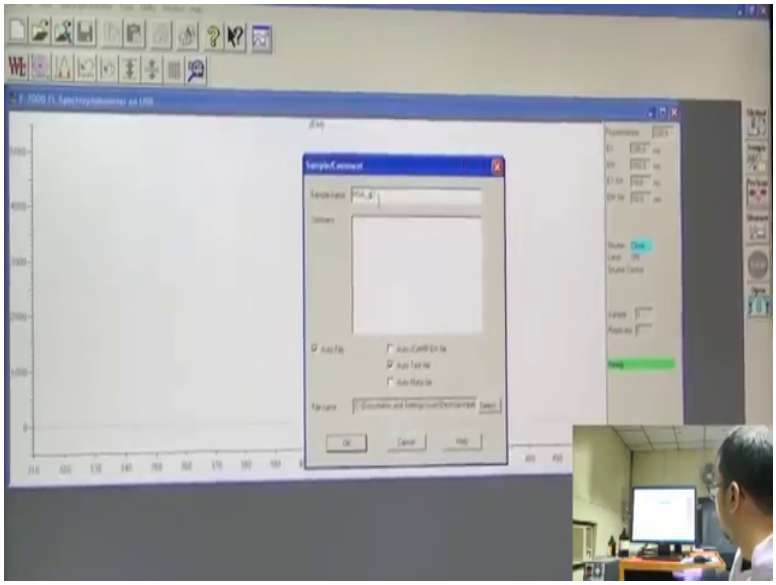

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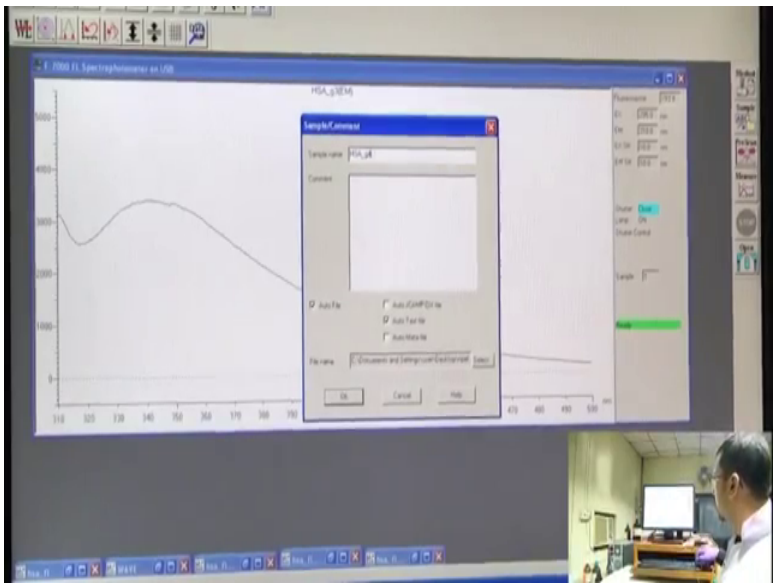

Here, we can see a better dissolution of the emission maxima you can point out here. You can reduce it to around 4000, time to time you have to monitor whether the maxima is coming at 340 or it shifting from here. So, let us see where the maxima comes in this case.

Once it reaches the 500 nanometer value. So, here we can see where is the emission maxima the emission maxima is around 3401 are yeah, 341.4. So, the emission maxima for native was around 340 its 341.8 or 4. So, it does not deviate that much until unless you see it at guanidine hydrochloride concentration. So, let us proceed with guanidine hydrochloride. You have added 3 different concentration, let us let us move to the 4th one.

Now, we will again go to sample we will change it to g 4 and give measure.

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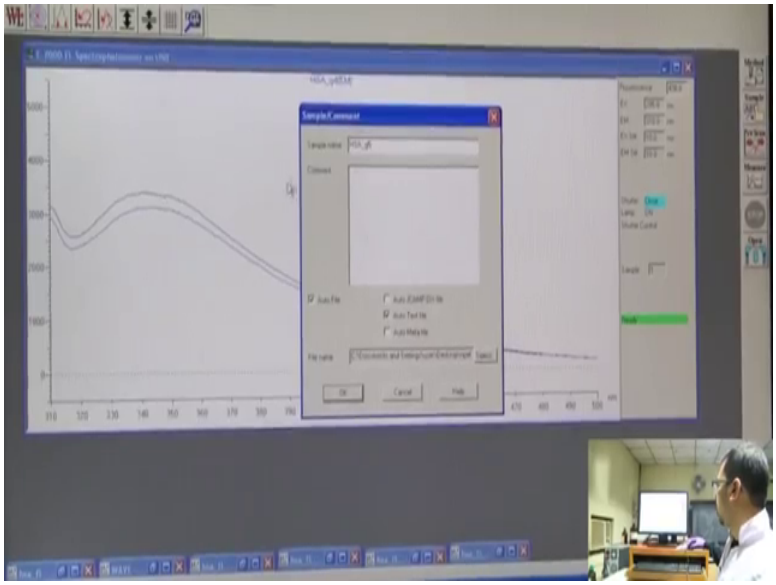

Again, we can see the intensity decreases, then decrease intensity might be again due to dilution or due to changing tryptophan environment we can pretty sure right now, whether the dilution is affecting it or tryptophan environment is changing in some way which is lowering a fluorescence intensity until unless we subtract the control.

Again, we can see them emission maxima is almost the same out here. Let us complete. If we move here, we can see that although emission spectrum which you have carried out here is accumulated in this window. So, we can actually select any of the data and see how we will carry out with a range of concentration of guanidine hydrochloride. Again measure.

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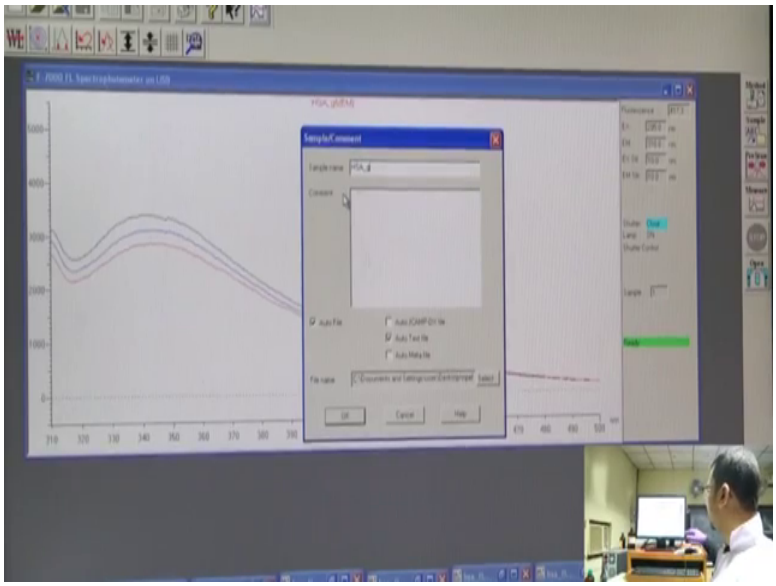

And out here in the screen you can actually a few things to note down in the screen is that this y axis is indicated the fluorescence intensity, the x axis is the wavelength the wavelength we have chosen out here is 310 the starting emission wave length around 500 that is the emission wavelength where it will complete. All the slit with the mentioned out here. So, this is basically the window of a fluorescence emissions spectra. And again, we can feed over decrease, the fluorescence emission again decreases, but this one again maybe due to dilution or maybe due to soul effect of tryptophan.

Now, this is the last concentration of guanidine hydrochloride we are taking minimum you should take around 5 to 6 reading or its better if you take around 8 readings. So, we are taking up till here 6 reading, and measure.

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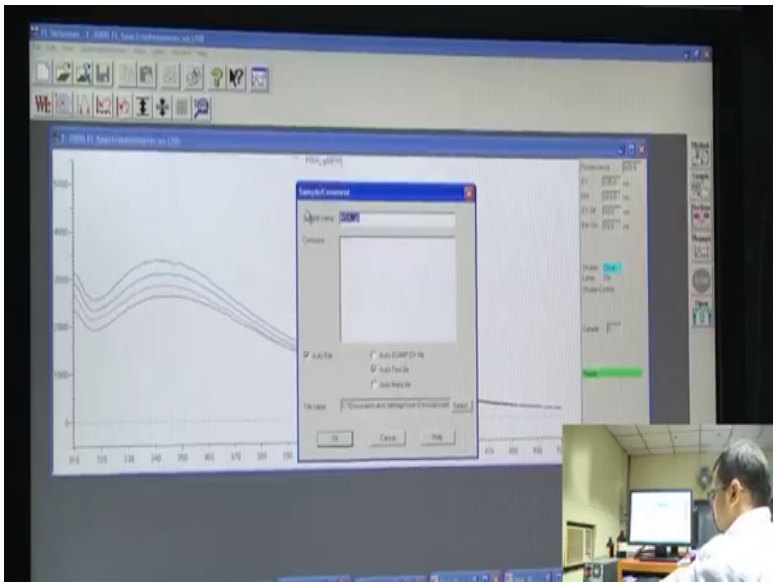

One thing you can be pretty sure from this spectra is that the effect is mainly due to dilution out here. So, how can you conclude from here? Actually, what happens is that tryptophan you really in an environment when protein is denaturates, tryptophan basically opens up. So, as we have seen in the absorption spectra the absorption it is increases.

So, once a tryptophan residue opens up in this medium upon denaturation of protein, it fluorescence intensity will again increase because tryptophan residue will face the solvent. However, there might be acquainting fluorescence intensity once again due to solvent exposure, but which we can be pretty sure after subtracting the control. So, here we are done with for only HSA along with guanidine hydrochloride.

Now, we will shift to only guanidine hydrochloride spectra. You can see a guanidine hydrochloride do not exhibiting in sort of fluorescence spectra. Well it might be that guanidine hydrochloride may not exhibit any sort of spectra, but however, some background fluorescence might be present their which we should subtract or some fluorescence intensity decrease through dilution should be subtracted from sample which have only double distilled water and will add guanidine hydrochloride to it.

Here we have taken again double distilled water in this qubit and we will say check and we will check fluorescence emission of double distilled water. It is quite upset to hear that double distilled water will give fluorescence, but let us see what spectrum does it

give in this region. And will transfer this thing into the qubit chamber again and we will close the lid carefully.

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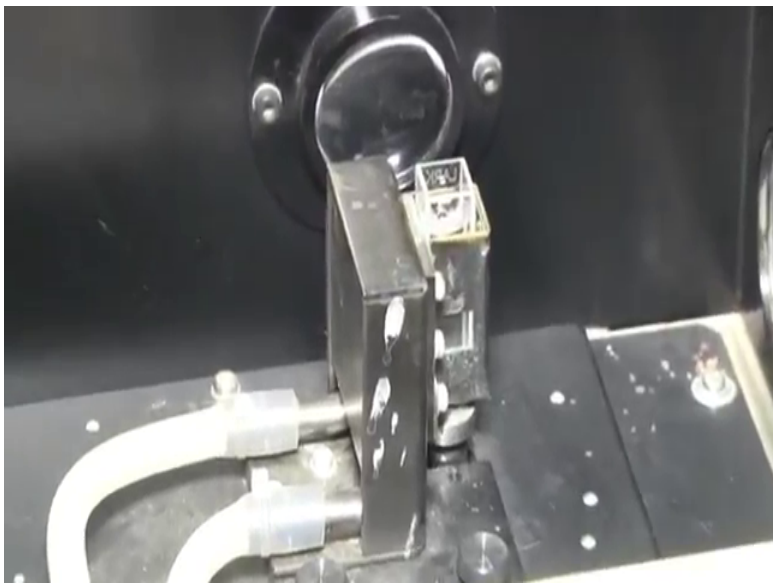

Now, when we are taking the measurements for control, the control is also for double distilled water because initial H 2 O HSA was dissolved in double distilled water. So, we are taking for double distilled water also and the parameters should be the same as that of HSA that is in case of sample.

So, what are the spectra? What are the backgrounds spectra this solvent imparts on the native protein or the protein which is created with guanidine hydrochloride? We have to check from this control. So, control is pretty much important while taking any sort of UV or any sort of fluorescence reading. So, let us see how the spectra for H 2 O double distilled water appears in case of our current parameters settings. We will go to sample will change this to the w a t indicating for water, ok.

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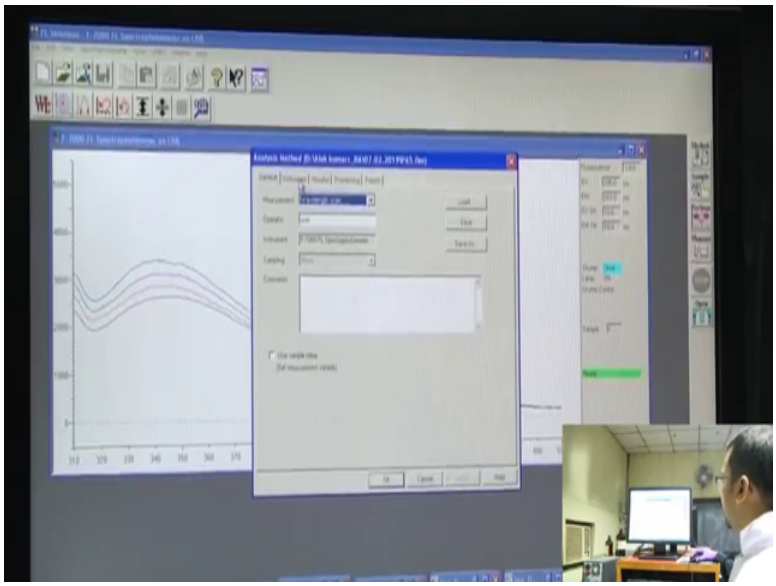

Now, we can actually set the excitation out here to 95, its same as this and emission out here is 310, we can go to method basically, we can again go to method here is an instrument, we can check again once we had been controlled whether the excitation emission is same.

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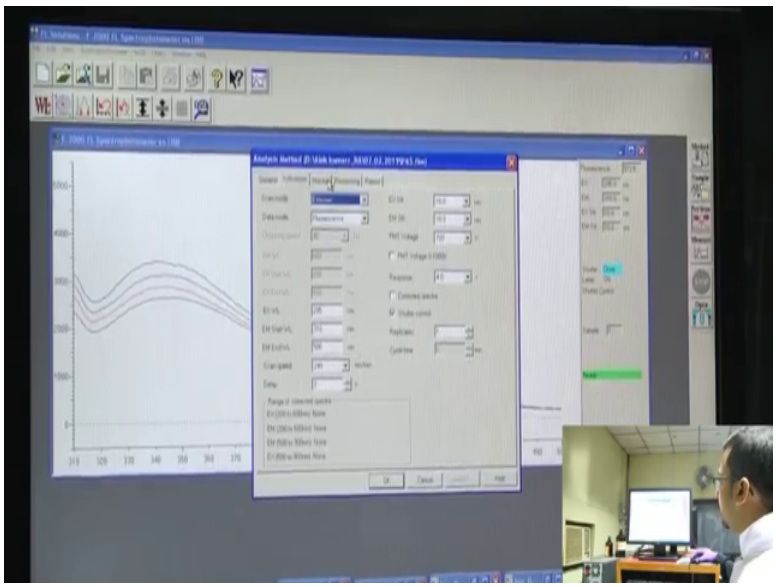

In a monitor part we can actually adjust the value to a lower value suppose around 4000 since we are doing for control and rest of the thing are same as it is and let us press ok.

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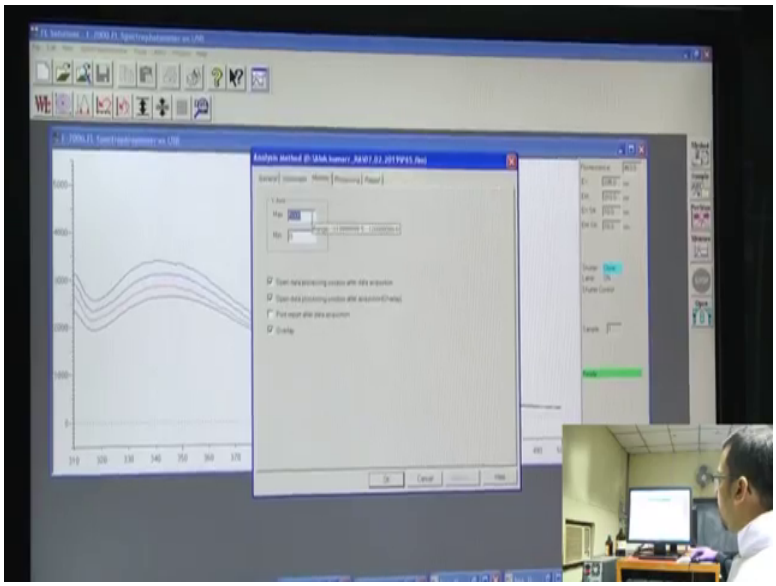

So, it is better to start the control from a fresh page rather fresh spectrum page. Now, the instrument is ready, let us hit a pre-scan. Once getting a fresh sample we can go with the pre-scan, after pre-scan we will hit measure and let us see how the spectra comes.

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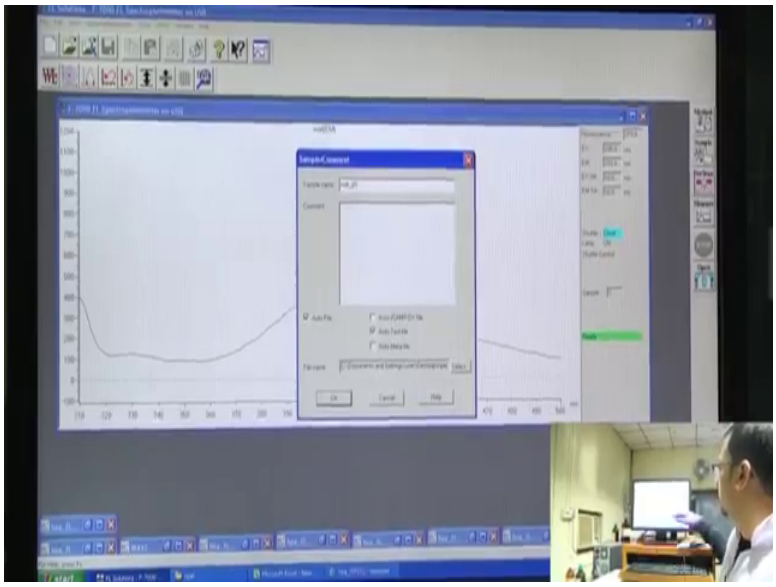

So, we can basically see there is no such spectra out here; however, one small trailing peak is present here, ok. It again rises out here in the region of around 400, but this value is very much insignificant as compared to the native protein. So, this is the spectra for pure double distilled water.

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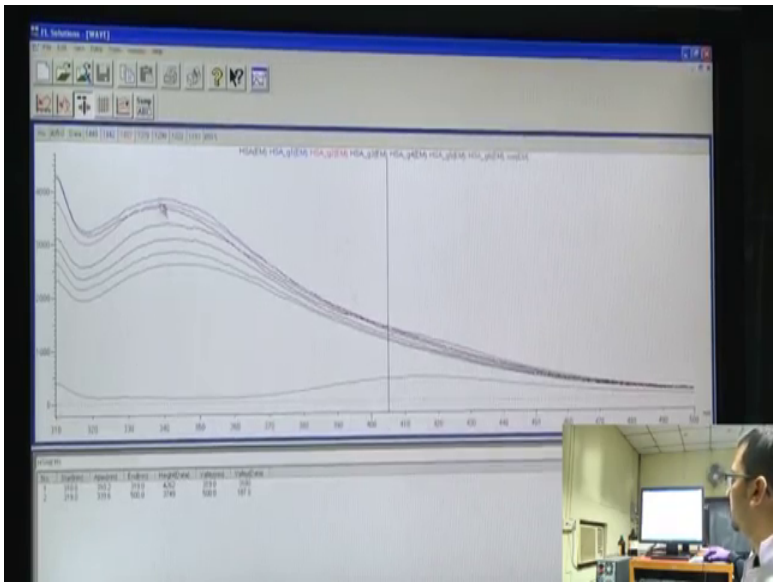

Here, we can see these are for HSA and guanidine hydrochloride and this is for pure double distilled water, and we can see the intensity is pretty low I mean it is very lower than that of the native protein because no such fluorescence thing is present out here. However, we can say that this thing the trailing part in the region around 310 or 300 or lower than that is also is coming due to this water path in this water out here. So, once you subtract this one, we have to subtract this and this that you have to subscribe this one, it is a double distilled water for this one and then we can actually get the true emissions spectra of HSA. Now, in double distilled water we will add the same concentration of guanidine hydrochloride step by step.

Now, we have taken out this double distilled water and to it will be adding guanidine hydrochloride the same volume which you have added previously. Remember which we have given as g 1, the g 1 the volume corresponding to that we will be adding that exact value of guanidine hydrochloride to this one. And we will mix it properly note down that no water bubbles are formed out here, for the solution is again clean, no visible color changes, so it will transfer this thing again in the sample compartment and close display gently. Now, we will change the sample name from water to we will give water g 1, initially it was HSA g 1 and here we will give water g 1, ok, now directly measure.

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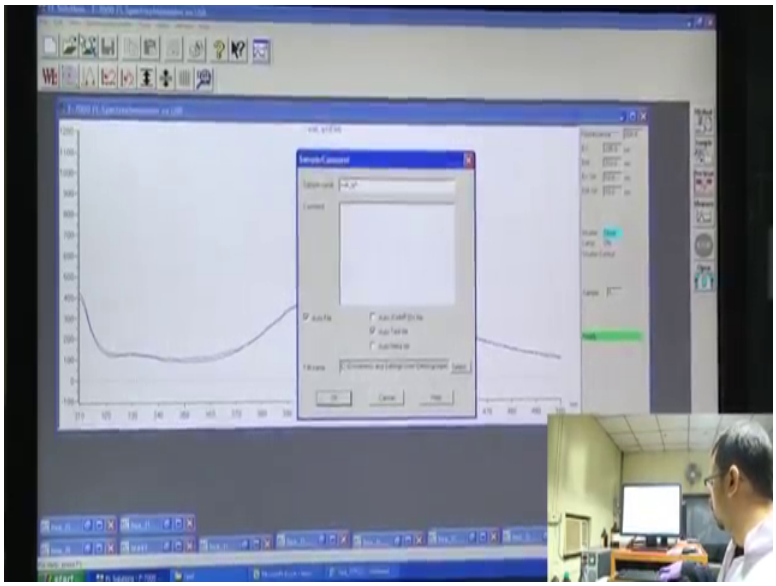

Let us see the spectra for small amount of guanidine hydrochloride added. Here, we can see that the spectrum is more or less similar. So, guanidine hydrochloride do not have that much effect in the fluorescence spectra both are almost overlapping over one another. Now, the first scanning is being done what will use with the value. So, the text file from here that is for water g 1, we will subtract this from HSA g 1. So, HSA g 1 minus water g 1 gives your only HSA value which has been affected due to g 1, that is particular concentration of guanidine hydrochloride when added at that stage.

Now, we will increase the concentration of guanidine hydrochloride and see how the spectra of the control changes. Now, again in the sample compartment part we will change this to g 2, and we will proceed like with g 3, g 4, and measure.

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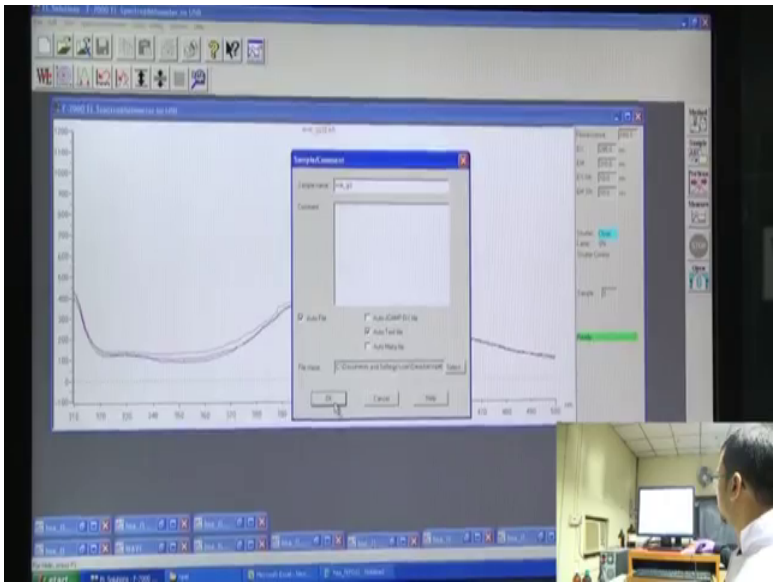

So, again we can see this slight change or not that much significant. Once you see the spectrum of HAS, do not be too much sure about the changing spectra upon adding any sort of legando any sort of molecule or any sort of denaturation agent until and unless you have done the control.

Now, we can see in this region in the region 320 to 370 or 390, there is a change in the spectra that even increases a bit. However, in this region that is around 400 to 430 the change is not that much more coming into rather it overlaps. Again, we will be adding higher concentration. Now, we will see for g 3 whether any changes comes, in this region we can see or only a change in this region is observed. Let us see we have clicked measure.

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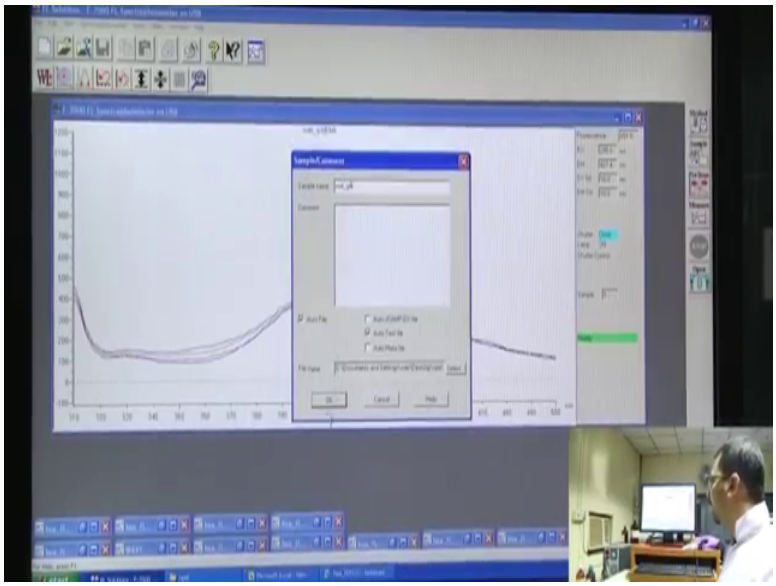

A slight change in this part that is in the region of 330 to 340. So, the slight change sometimes might be very much significant once you subtract this some HSA path. I hope it will begin overlap with the part, yes more or less it as the same intensity range.

Now, let us check till here what is the status of our experiment. So, from here we can see with change in the concentration guanidine hydrochloride ever change in the spectra, initially it has increased the bit but again decreases. However, once we have added this guanidine hydrochloride on to water only the change is not that much prominent, ok. So, now we have to add again the g 4 and g 5 and g 6. Now, for sample g 4 please make sure to add the exact amount of guanidine hydrochloride in control also that the same we have added in HAS, otherwise wants to subtract it you will not get the proper value.

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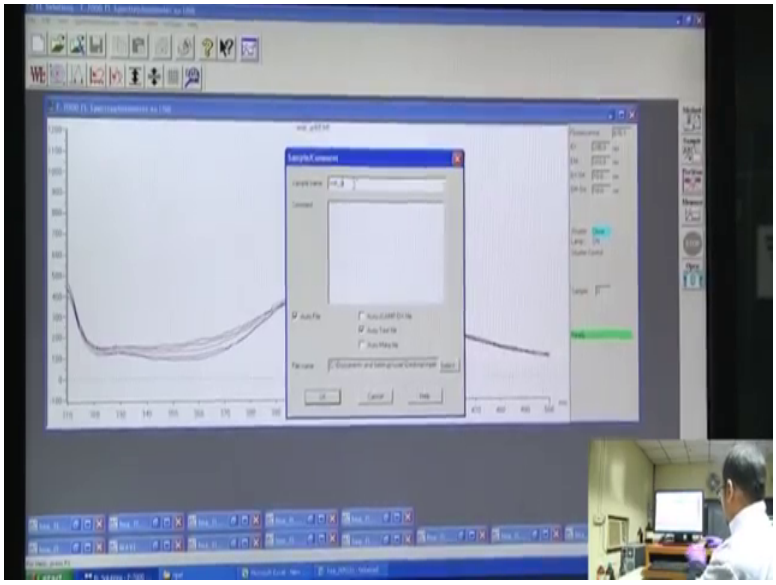

So, here we can see almost same not that change. Well, this data indicates that there might have been some changes in a tryptophan environment, since the solvent that is guanidine hydrochloride. Do not have that much of effect out here, it do not have any sort of fluorescence peak or fluorescence intensity. So, the change in the fluorescence spectra of HSA is only due to the denaturation of protein. So, let us check the other two values up till here we have done up to g 4.

Now, the remaining two g 5 and g 6, and we changed this name to g 5, measurement. One important thing while doing this fluorescence experiment is that while scanning is going on make sure not to touch other part of this instrument like in this option this option do not try to click on this things, ok. Why? Because once the instrument is going on it might clicking on other icons might affect this measurement.

However, if for some reason you think that you have clicked the wrong icon or the spectra is not correct or you need serious, you have serious reason to abort this reading, so you can click stop directly out here. Do not click this cross icon while the spectra scan is going on. Now, we will see for g 6. So, this is the last part of the control. We have started with around 2.5 ml solution. It was still here, in this part this 2.5. Once you started adding our guanidine hydrochloride to it we can see the level or the solution the volume of the solution in this qubit increases. So, the last thing we are adding out here is the concentration of or the volume of guanidine hydrochloride which you have added in case of has g 6, the similar volume and similar concentration of guanidine hydrochloride we are adding in this control that if this is double distilled water.

We will be cleaning all the 4 sides make sure to hold this qubit in such a way that your finger doesnt touch on this transparent side where sample is there, but hold it very carefully. And I am transferring this to the holder, closing this lid, and the last part was g 6, and measure. And here again we can see that it goes in a value which is almost similar to the last one. So, the control for g 5 and g 6 almost similar out here. Actually, what happens is that the intensity out here is pretty low here in this range. So, the changes are not that much significant which was shown in case of HSA. So, the sample scanning is going on here we can see 493, 496 and 500 and its complete.

So, here we can go and check this one we have done some has g 1, g 2, g 3, g 4, g 5, g 6 HSA and for corresponding to this HSA we have taken water, corresponding to this HSA g 1 we have taken water g 1, HSA g 2 water g 2. So, what we were going to do? From HSA we are going to subtract water. So, basically it was HSA solution in double distilled water, upon subtracting only the background sector for water we will get the emission spectra for pure HAS. And again, how pure HSA was affected in presence of guanidine hydrochloride? Once we add the guanidine hydrochloride in presence of only water we can get this HAS.

Now, again HSA g 2 minus water g 2, HAS g 3 minus water g 3, HSA g 4 minus water g 4, g 5 HSA minus water g 5 and lastly HSA g 6 and water g 6 minus water g 6. So, this subtracted value you have to plot it, we have to plot it you have to subtract the value of it from each and individual wavelength and then the final spectra you have to plotting in an excel and find the change, and from there one can understand how the spectra for HSA change is in presence of guanidine hydrochloride.

In this study we will see the effect of temperature on protein denaturation. So, we have used HSA and we have heated it to several temperatures are like 37, 45, 60 and 80 and compared with the fluorescence spectra of native HAS, that is HSA kept at around 25 degree centigrated. Rather, (Refer Time: 47:43) HSA that is the HSA which is kept in refrigerator maybe also used. Basically, the native HSA and is used to compare the fluorescence spectra of HSA which has been treated with temperature. So, now will see the fluorescence spectra of temperature treatment of HSA.

In our previous studies we have seen how denaturating agents like guanidine hydrochloride and urea affects the tertiary structure of protein. Due to the tertiary

structure of protein rapture due to presence of guanidine hydrochloride or in case of HSA we can see that when addition of urea, it damage a 3-dimensional structure. It affects actually the increasenic fluorescence or rather it affects the amino acid residues which is reflected from the change in fluorescence spectra of tryptophan residues or also the change in absorption spectra of tryptophan residues.

Now, apart from denaturating agents like urea, guanidine hydrochloride, other grateful stressful agents like temperature might also affect the protein in 3-dimensional structures. So, how does it affect? Actually, when you heat a protein sample the hydrogen bonding presence in the protein is ratchet, due to which the 3-dimensional structure that protein opens up and it actually exposes all the most of amino acid residues. As a result, the environment of tryptophan residue again changes for due to heating which you can be reflected from the fluorescence spectra.

Now, we will see the fluorescence spectra of HSA upon treatment of heat. For this what you have used is that we have used native HSA and we have heated it at 37 degrees centigrade, 40 degrees centigrade, 65 and 80 degrees centigrade, and at different temperatures we will see its fluorescence spectrum. Here we have taken again double distilled water and to it will add native HSA. Please ensure that bubbles are not formed in the solution, and we have to mix it thoroughly, and while mixing you can see sometimes bubbles are formed and so avoid this rapid mixing mix it very gently in like with the micro puppet. And once bubbles are formed make sure to remove this bubbles and after that we have to clean this one this are a regular step. All the 4 sides are cleaned, and we will transfer the solution in the qubit chamber, this is the holder, we will place it here and close this lid.

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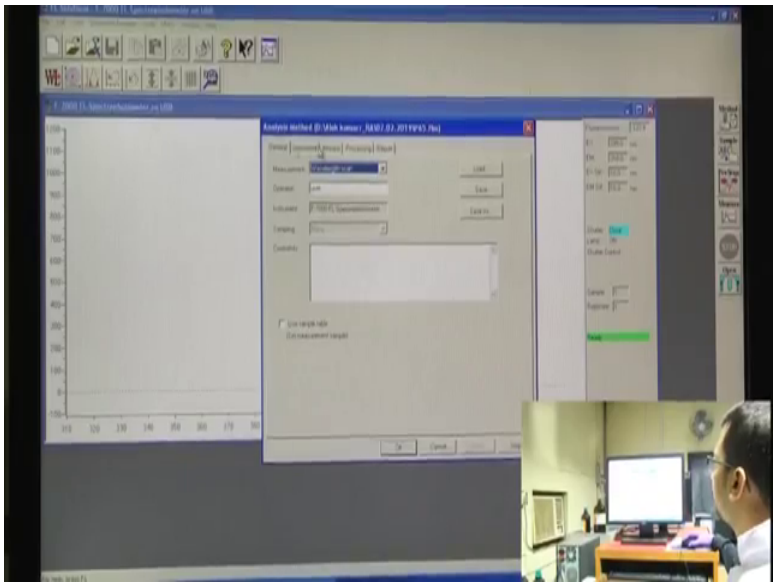

Now, here we have to go to method again.

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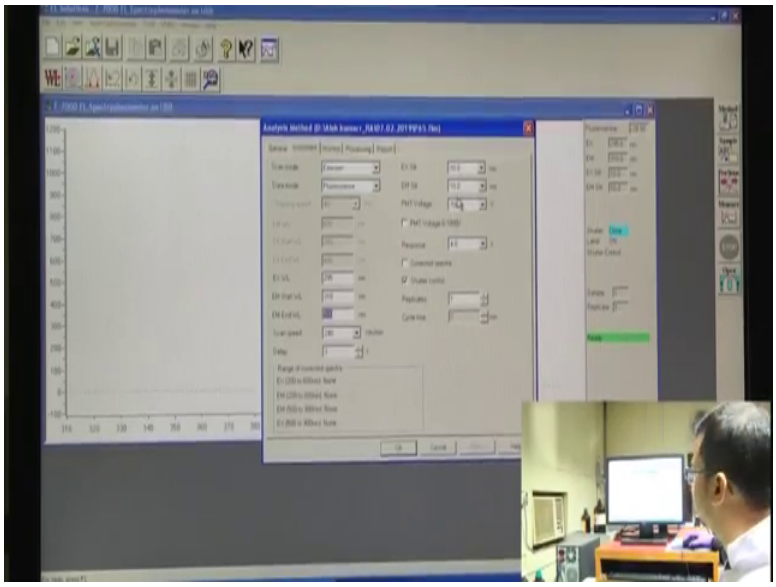

And here we can see the excitation wavelength is same 295 that is for tryptophan. The emission wavelength start from 310 and ends to 500, the scan speed is 240 and excitation emissions slit border 10 nanometer, and we will change this maxima to something of around suppose 5000 for y axis and we will click ok.

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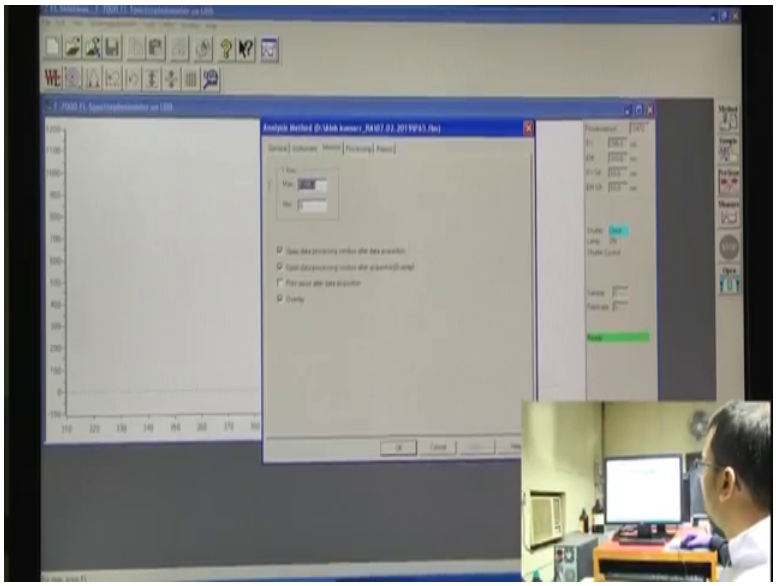

Parameter initialization has occurred, now you will do pre-scan, once pre-scan is there it will show ready and then we can click measure. But before doing measure, what we can do? We can actually change the sample name. We can change the sample name is nat HSA.

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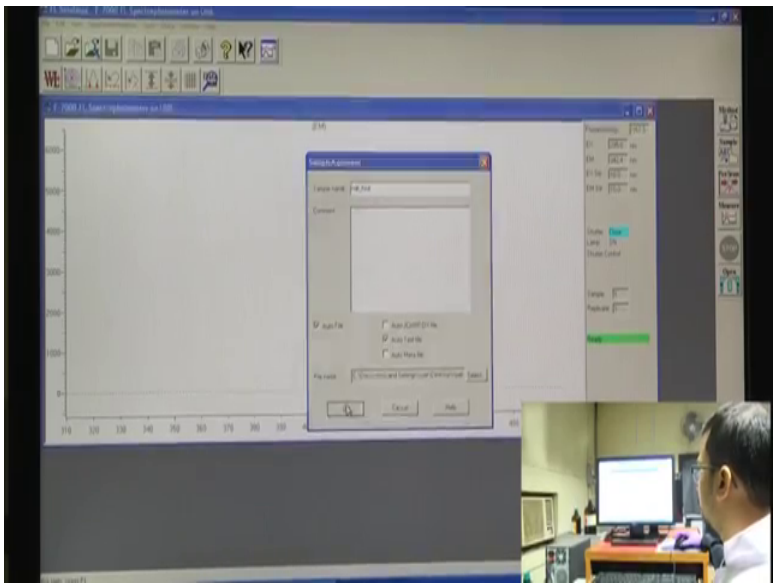

So, what does this indicate? This is actually native HSA, ok. And now, we can go to measure.

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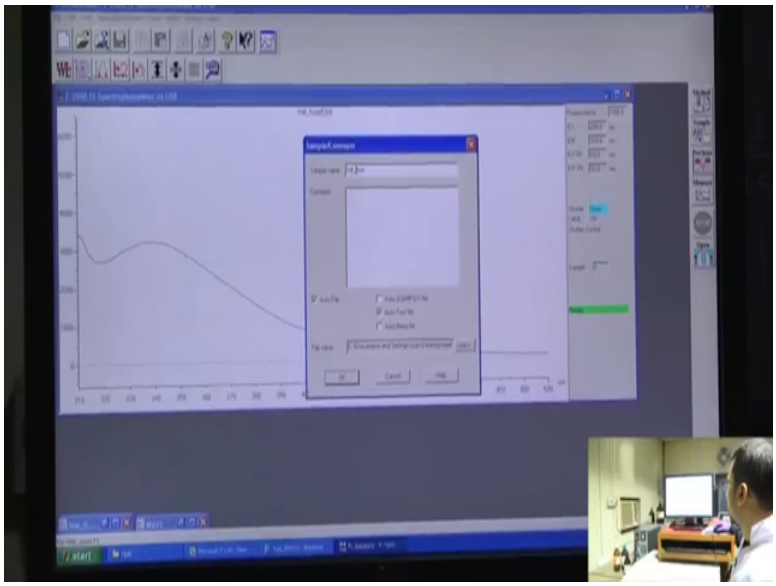

In this case also for each and every sample we have to subtract double distilled water or rather the solvent out here. And how to make the control? For example, once you have used heated sample like HSA heated at 40 degrees centigrade, you should control should be actually double distilled water heated at 40 degrees centigrade. Here we can see a beautiful emission spectra obtained out here. This is a native HSA.

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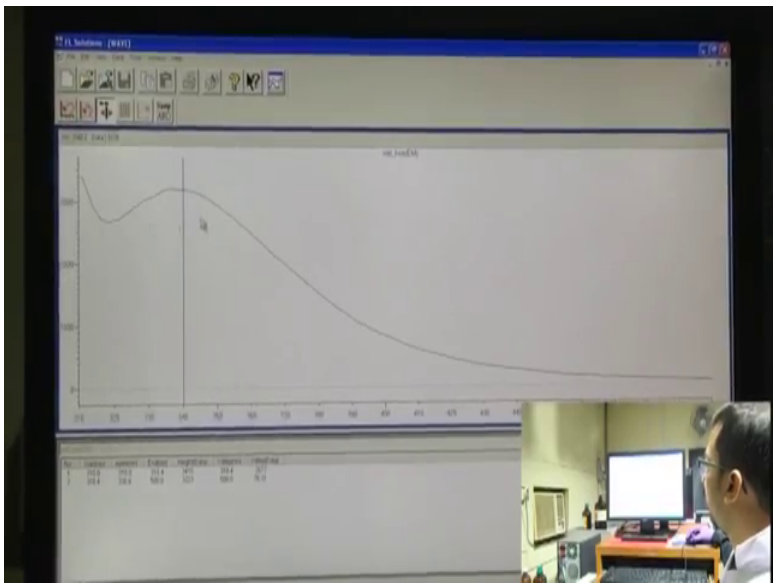

From here we can actually calculate the emission maxima, the intensity of the emission maxima it ranges around 340 or something like that. So, this is the value of the emission maxima we have to note it down.

Now, we will see for sample which is heated at 40 degrees centigrade, this HSA in 40 degrees centigrade. For that we have to first throughout, this solution that if the solution for which is the native HSA take the qubit, again clean it and pour the sample solution for 40 degrees centigrade HAS. Out here we have taken this qubit which was initially filled with HSA solution that is native HSA solution and we will add again heated HSA solution. Now, we will add around 37 degrees centigrade if HSA has been heated to 30 degrees centigrade. And 37 degree centigrade is actually the HSA present in our body, so we can see what actually happens when we heat HSA 37 degrees centigrade because in generally what happens is that HSA use is native HSA. So, let us see what about bit amount of temperature or body temperature does to this protein.

So, for that we will be adding HSA to again to double distilled water. We have cleaned all the 4 sides, and now we are transferring it to this sample holder and closing this lid. Now, will change it to around HSA t 1 that is the temperature which we have imported out in the solution its t 1 and the measure.

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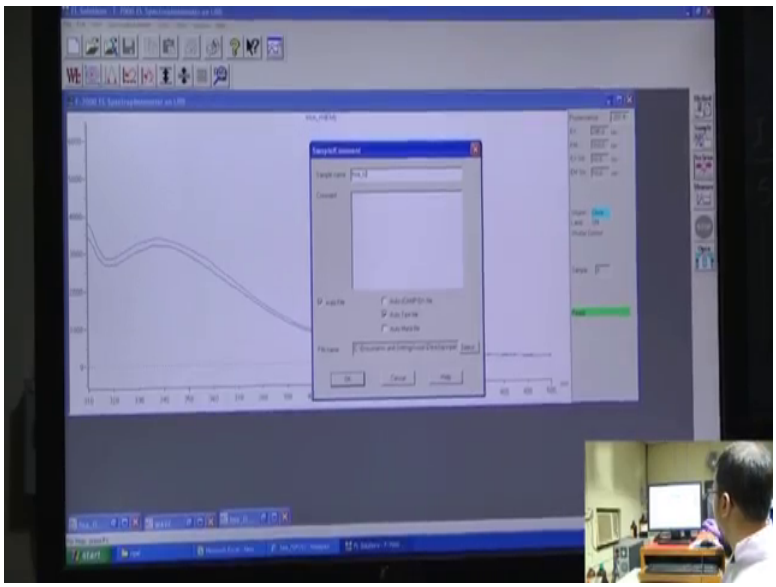

Here, we can see a small increase in the intensity a fluorescence intensity, a small increase in fluorescence intensity actually occurs out here which actually indicates that

the environment of HSA from differs some native. So, does it signify that HSA in our body actually denaturates or is not properly structured or the 3-dimensional structure in our bodies not retained out there in our body temperature? Actually this is not the case, because what happens in our body is that HSA in our body within a pH of around 7.4, ok.

So, the solution which we are doing out here even solvent water. Solvent plays a very crucial role in fluorescence spectrum. So, often changing the solvent if we would have done in a pH solution of different pH value of around pH 7.4 or something like that then we could have seen that is spectra might not have been same to what we are seeing it now. So, actually what happens in our body? Due to pH and other different condition all the enzymes in our body are pretty well retained. So, it is not that much affected what we are seeing in this instrument.

Basically, the native structural protein or rather the native protein under defense native and the heated protein differs on the fact that in case of the heated protein or rather than 37 degrees centigrade due to certain environmental stress, certain environmental stress while heating this protein may cause depression structure and such sort of stress is not present in your body at normal condition. So, in our body at 37 degrees centigrade and a buffer condition or rather a body pH condition of around 7.4 the protein is completely retained. So, let us see at high temperature that is around 45 degrees centigrade.

Now, we are doing this HSA solution at 45 degrees centigrade, the second temperature t 2, and then measurement.

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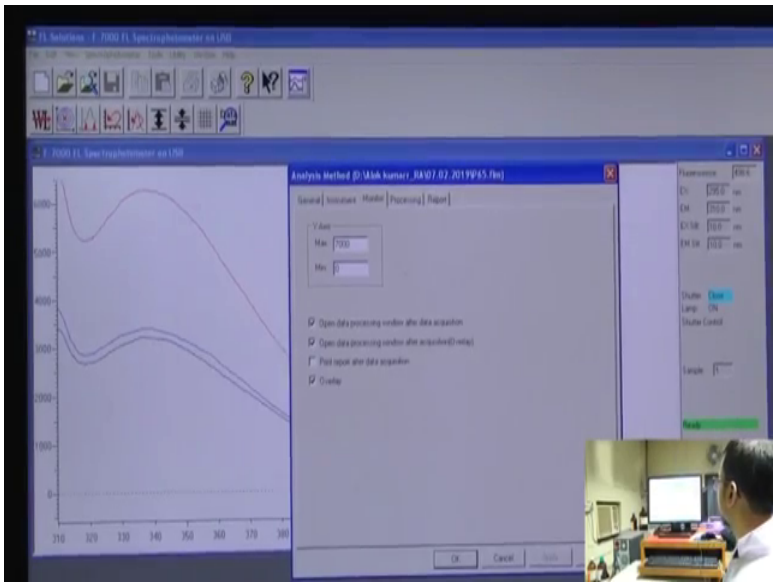

Well, one interesting to note down out here is that the change in the spectra is pretty higher in this case compared to 37 degree centigrade. So, what basically happen is there from compared to native protein and protein at 37 degrees centigrade the change in fluorescence spectra is not that much, ok. But when it is heated could have a much higher temperature, as here we can see it around 45 degrees centigrade here the fluorescent emission the increase in fluorescence emission is much higher as compared to native protein when it is compared with HSA at 37 degrees centigrade.

Now, we can actually change this one this 6000 part, we have given up to 5900 we can change it up to 7000. Despite actually your all the other pics are gone temporarily, but things will be accumulated out here.

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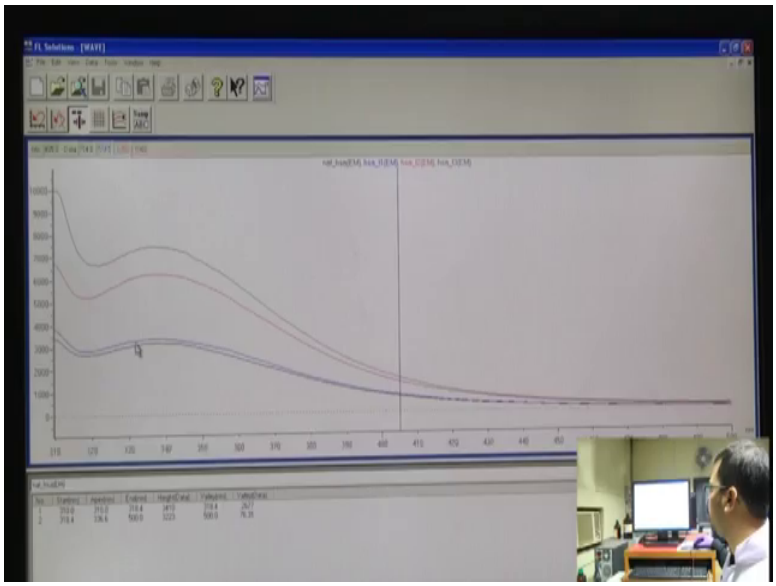

So, now, we will add HSA heated at 60 degrees centigrade. Now, we will check at temperature 6 degree centigrade. We will click this one, (Refer Time: 58:47) t 3, already it is 3.

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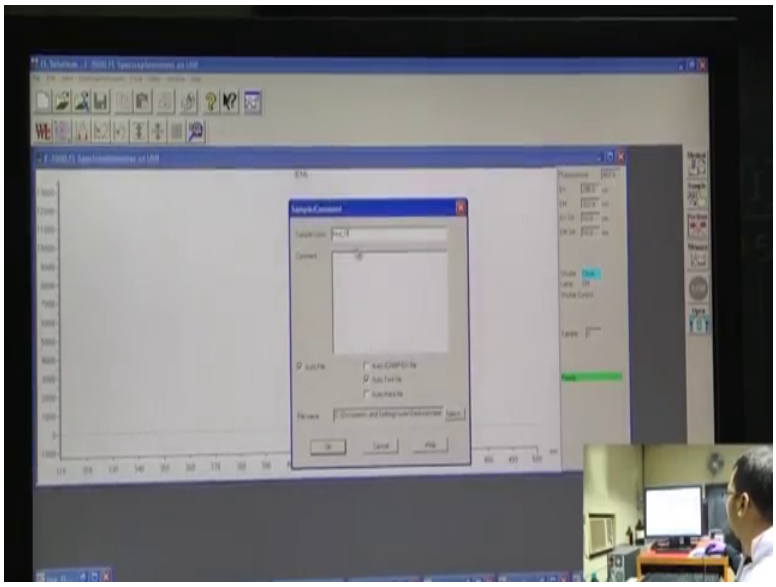

And we have measure, there again we can see the emission spectra.

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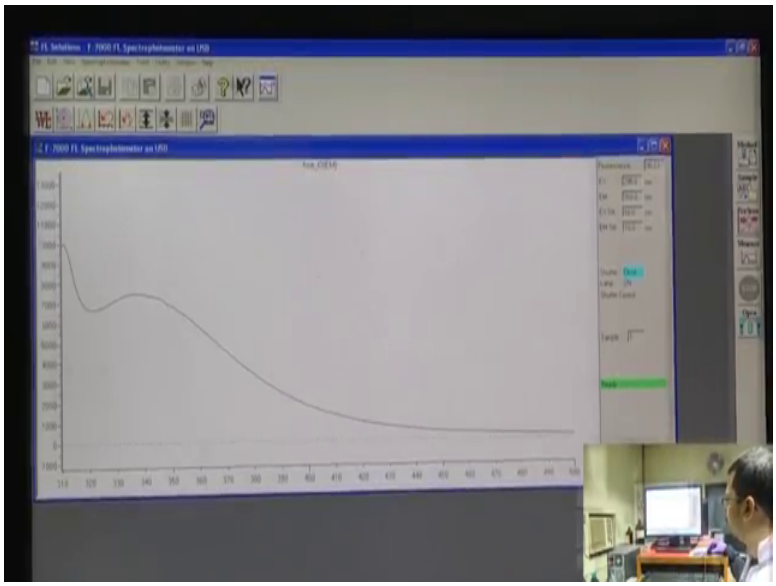

Now, this part of the spectra we can actually eliminate by subtracting it from the solvent whichever solvent it is. The main part of the spectra we should be bothered with this intensity around this 340 to 50 which is for tryptophan.

So, the reading has been taken. So, this reading has been taken. Now, we will go to this part and we will see how it changes. Now, again this one is 25 or rather native HSA on this 37 and this one is 35 and this is 60. So, we can expect that more or less a major amount of protein denaturation has taken place out here in range of 45 to 37 to 45. And now up to 60 we can see again it increases at tryptophan fluorescence which shows that again protein undergo certain confrontational destabilization.

Now, we will check at 80 degrees centigrade how the spectral looks like. Now, we will check this overlay spectrum of various fluorescence emission of HSA and various temperature, starting from here we can say it is around native protein the black one. The blue one just over it is actually the protein at 37 degrees centigrade. From here not much difference in the fluorescence spectra as we was bothering initially there might be a difference, as compared to the protein heated at 45 degrees centigrade.

So, what we can expect as of the body temperature increases, the protein denaturation might take place. As we can see the protein started denaturating or rather the denaturation extent is much more out here from the difference in the fluorescence spectra. Again we increase to 60 degree centigrade the fluorescence emission increases

and it increases to high greater extent and 80 degrees centigrade. Now, from here to here we can see a major increase in the fluorescence emission which will circle indicates that HSA undergoes denaturation in on heat treatment and the heat treatment applied out here is temperature from native protein to 30, 70 to 80 degrees centigrade which causes the denaturation of HSA.

Now, one small thing to point out here is that once we have started heating HSA at 60 or 70 or 80 degrees centigrade you can see the solution turns the HSA protein turns turbid or rather it turns actually white, ok. So, what basically happens is that, actually when HSA heated at higher temperature it precipitates and as we know protein precipitate is actually white and so it appears as whitish color.

So, on the other hand one small thing which might not be required at your stage or it is an advanced stage, HSA is a such a protein when heated it in high temperature might be in present a buffer or any sort of solvent at high temperature like 65, 70 or 80 degrees centigrade it causes protein fibrillation. So, protein fibrillation is basically a separate topic which is one of the one of the causes are one of the outcomes of protein denaturation. So, you can search protein fibrillation in google and you can come across videos new sort of ideas which is associated with the denaturation study.

Till yet what you have seen is that how protein behavior changes or how the behavior protein changes when treated with denaturating agents, like guanidine hydrochloride, or urea. And both are studied in UV visible spectrometer, both are studied in UV visible spectrometer and fluorescence spectrometer. Now, we can see the changes in the tryptophan fluorescence in both cases, for heat treatment and in case of guanidine hydrochloride. And also, in case of UV we have also seen how the absorption spectra of tryptophan changes.

From this case we can conclude that the fluorescence spectra of intrinsic fluorophore present in protein is affected mainly due to the denaturation of protein and the change in the medium of the fluorescence tryptophan residue.