Experimental Biochemistry

Prof. Pritam Roy
Prof. Aditya J.Basak
Prof. Snigdha Maiti
Department of Chemistry
School of Bioscience
Indian Institute of Technology, Kharagpur

Lecture – 14
UV/Visible Spectra of Amino Acids and Proteins

Hello every one welcome to Experimental Biochemistry course, now today we are going to study the spectroscopic techniques. The spectroscopic techniques required in detection and estimation of amino acids and proteins. Generally proteins have anomatic amino acid residuals like tyrosine, tryptophan, phenylalanine which can be detected using spectroscopy tools.

About spectroscopic tools you have studied in your theoretical classes, there are broadly 2 types of basic spectroscopic techniques which are UV visible spectroscopy, second one is fluorescence spectroscopy. Apart from there other types of spectroscopy which we will get to know after learning all this, but currently for this lab we will get acquainted with UV visible spectroscopy and fluorescence spectroscopy. So, let us start with our experimental methods, before starting this experiment we have to get acquainted with the instrument.

Now let us see how the UV instrument looks; for UV visible spectroscopy we need an UV instrument basically UV instrument various types and one of this type is out here, this is the UV chamber basically. And, this UV chamber record the absorbance of samples of protein or amino acid samples and the chamber actually give result which can be detected using this software, this computer gives the UV spectrum where we can see the observance values. Now, let us see how the UV chamber actually looks like; with this is the UV chamber.

(Refer Slide Time: 01:51)

Now, here it is the lid of the UV chamber.

(Refer Slide Time: 01:59)

Here we can open this lid and inside we can see there are 2 compartments where, we can placed the UV cuvette, what is cuvette will come after this. So, this one is compartment number 1, this one is compartment number 2 and out here the UV light will pass through this compartment in this direction and out here in this direction. So, here will put our cuvette so, again after putting the cuvette we will close this lid.

(Refer Slide Time: 02:25)

For measuring the absorbance of a solution either protein or amino acid we have to take the solution in clean and washed cuvette. So, what is the cuvette? You have seen in the theory classes that cuvette is like a transparent glass material, where one side is frosted out here and the other side is smooth. So, basically this is the UV cuvette.

So, cuvette all of various type 1 of the glass cuvette the other is require cuvette. So, it depends on the quality. So, for glass cuvette and plastic cuvette, but problems that basically they are not transparent to all the light within the UV region. So, it that percentage of transparent is not that much compared to quartz, let us in case of quartz it is all though expensive, but it is around 80 percent transparent to light in the region around 200 to 400 wavelength. So, for that we are advised to use this quartz cuvette. So, before using this quartz cuvette we have to note down a few points number 1, do not touch this clean side or the smooth side of the UV quartz cuvette.

So, for this UV quartz cuvette has 2 sides and clean and one frost side. So, when you are touching this frost side. So, what is the reason for bringing these two different sides? So, basically what happens during and UV like eradiation the light enters from here and passes out here, sometimes actually the solution is there which is turbid so, due to turbid solution scattering sometimes take place. So, if scattering occurs some light may be loss. So, as a result in order to prevent such loss of intensity the other two surfaces are made frost. So, we have to use the clean cuvette, do not touch cuvette with bare hand, always

use a gloves for a cuvette. When before using that clean this cuvette at least 3 to 5 times with double distilled water I have just done it right now and try to make it dry.

So, you can just invert it like this for a few minutes like keep it out here in plate or something like that in a balance like this one. So, what will happen if that water will come out of this and it will become dry, but generally this technique is not recommended; sometime people if it is not recommended because it met contaminate the sides of this cuvette. So, better keep this cuvette dry for sometime let it get dried in the air and start over experiment.

Now let us start with the UV experimental part for that we have to do 2 steps; for that you have to follow 2 steps, number 1 if the baseline correction and number 2 we have to do the sample absorption. For baseline correction we need to fill both the cuvettes with water and with the baseline will see it how it will done, but initially we have to fill this cuvette with the solution in which protein is dissolved.

Basically protein or amino acids are dissolved in buffer or polar solvents basically those solvents should be such that it should mimic the biological physiological pH. However, in this case you are using double distilled water as we know the water is neutral for the time being we are using double distilled water.

(Refer Slide Time: 05:46)

So, let us fill this cuvette with water.

(Refer Slide Time: 05:59)

Now, we have now we can see that we have filled around 50 percent of this cuvette with water, now we have to fill at least 70 to 78 now we have to fill at last 70 to 75 or 80 percent of the cuvette with double distilled water. So, we can clearly see here I am holding the frost side not the clear side out here, now we have taken the cuvette now you will feel the other cuvette with 3 ml of double distilled water. Now before taking this cuvette into the UV chamber we have to be sure that we clean this both side of the cuvette with a clean tissue paper.

(Refer Slide Time: 06:32)

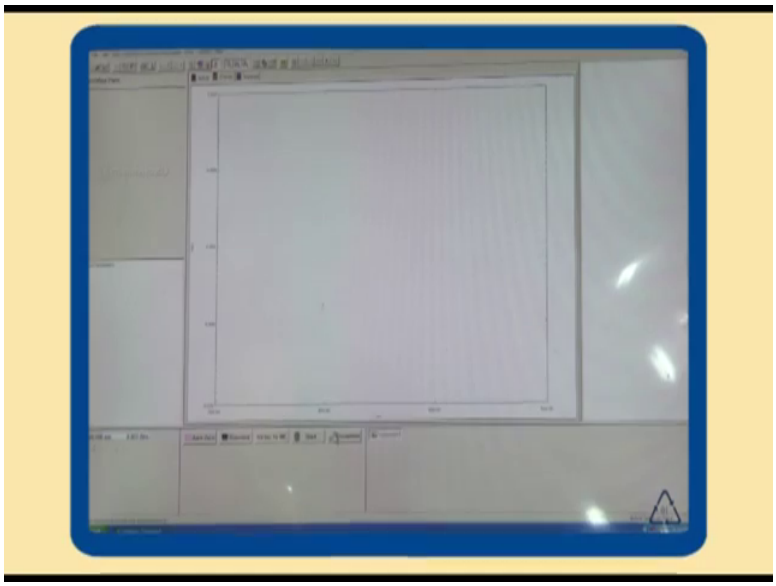
Now, we have to clean the clear side out here and here carefully, be careful with the cuvette try not to like put this cuvette here and there. Because of a slight crack or slight damage in this cuvette might change the intensity or might give incorrect results.

(Refer Slide Time: 06:55)

Now, we have taken this cuvette and filled with double distilled water. This is the reference cuvette we are filling the reference cuvette in this reference chamber and this is the sample chamber. Now we have place in such a way that the clear sight faces the light; the light is passing from here to here so, that it should go through this clear path.

For placing in this cuvette, placing this cuvette in this chamber gently; similarly we are taking the reference cuvette this is a reference 1, it has been cleaned both side and similarly we are facing it in such a way that the clear side is facing this light; the light if entering from here and passing through here and filling in the reference part. Now let us move towards analysis before that, before beginning with our experiment we have to just close this lid. Please note that do not keep the lid open while doing the experiment.

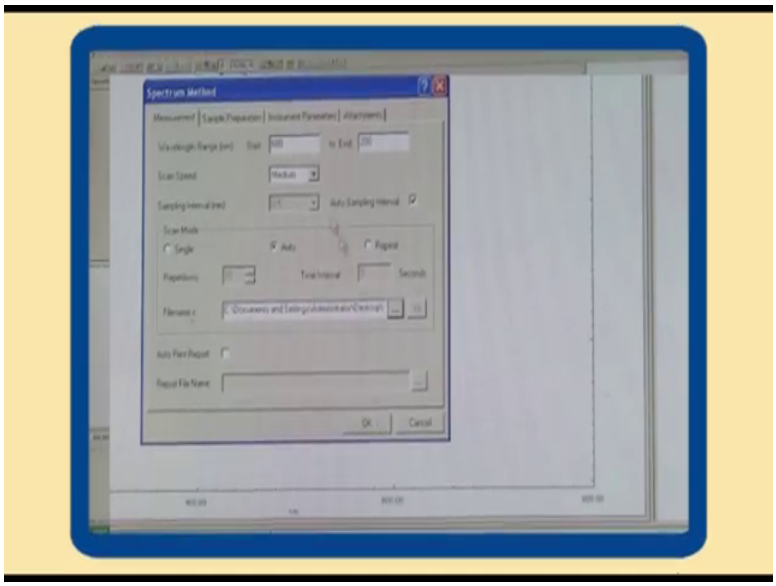
(Refer Slide Time: 07:54)



Now, let us get familiar with this software where we will measure the UV experiment, this is UV props software. Here once open this software we can see various icons are there, no need to worry about all this. The main thing we need to bother out here are this part and this method part given by m.

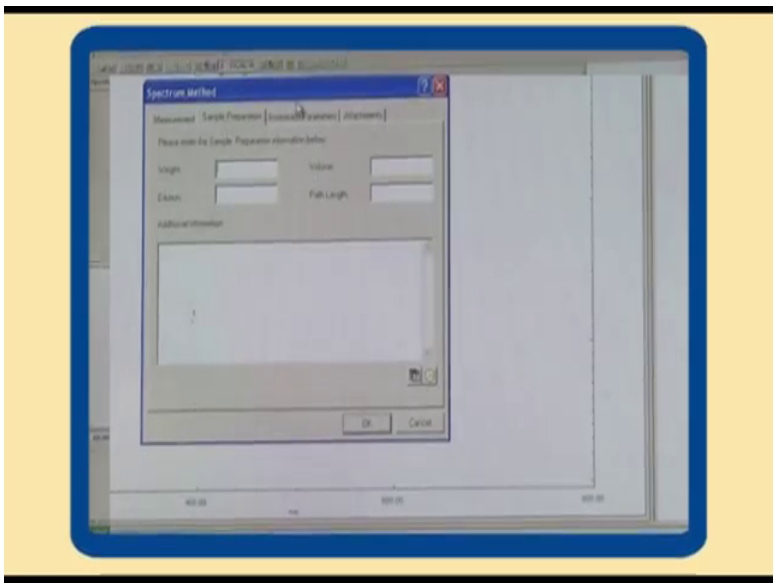
Now, here we can see graph is there here; in the graph this y axis is basically the absorbance part and the x axis is the wavelength part, here we can see nanometer is given 200 to 800 it is there. Now if you want to change our requirement like suppose we do not need from 200 to 800, we need around 200 to 600 in we can how to change this things.

(Refer Slide Time: 08:44)



Now, we have to go to method out here, here we can select the wavelength range for time being we do not need to bother about 800, we need to do from 200 to 600. The scan speed there are three types of scans speed out here fast, medium, slow, very slow. It is often recommended to go for slow to medium not fast, but better to keep it medium. Now the file name you should go to the appropriate folder where you want to save, currently the filename we are bothered with is out here. This file NPTEL UV and the sample we are marking as S 1 and open.

(Refer Slide Time: 09:31)



Now, these are certain other instrumental parameters which are default which are better to be kept default and one thing out here this lead with it is kept 2 for this instrument. Now let us press ok, now here we can see the wavelength range is up to 800 only it has not been changed; although we had changed to 600 but it will change once you start with the experiment.

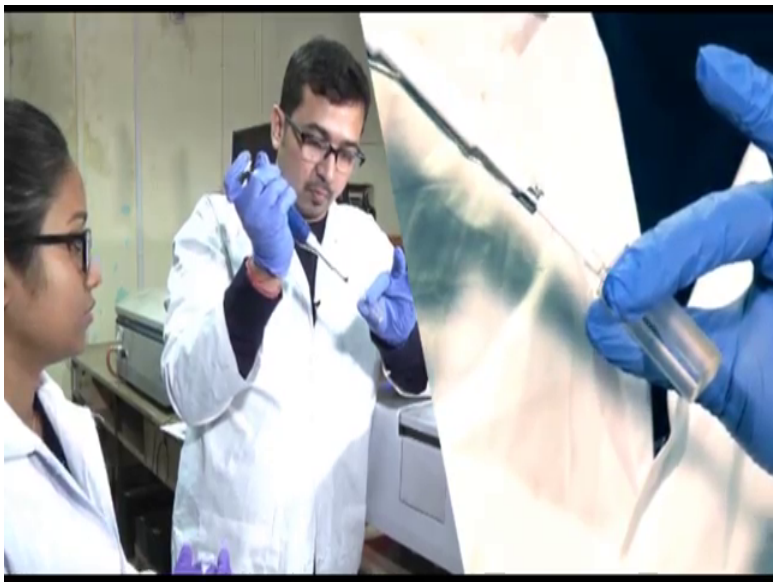
Now, let us get familiar with these icons one is the auto 0 and is the baseline and start disconnect. So, disconnect appears because the machines is currently connected, once we need to disconnect this machine from this CPU then we need to place this one. For the time being kept 2 cuvettes in this chamber and now we will go to the baseline correction. So, why baseline basically in order to correct in order to remove any error due to solvent or any error due to the background solvent we have to do baseline. Now let us click this baseline. Here we are given the option that start 600 and end 200, now it has by in default it has change to 600 from 800, now let us press ok.

So, the baseline correction will go by itself nothing will come in the graph, it will take a bit time. So, for baseline correction we have to click this baseline icon out here, it will show this 2 values which you have fed in the method part, it will start at 600 and end at 200. So, the machine is being currently initialized for baseline correction, here we can see gradually it has certain calibrating; no it is taking individual wavelength like 300, 350, 40 like this one.

While, this is scanning we can see the other icons are currently disabled, except the stop one. It is commented not to open this lead during this initialization, the chamber should be closed while baseline correction is going on. Now it has done the baseline correction up to from 600 nanometer to 200 nano meter. And, once the baseline correction is over we can see that these icons appear once again, now we are ready for sample analysis.

Now, we are done with the baseline correction let us shift to the absorption spectra. We are beginning with the amino acid which is tyrosine as we all know tyrosine is anomatic and it exhibit anomatic. And, due to anomatic group it exhibit an absorption spectra; for beginning with absorption spectra we need to take out the reference cuvette.

(Refer Slide Time: 12:31)



Once you take out the preference cuvette we will be adding 20 micro litre of tyrosine solution, we have prepaid tyrosine solution will take 20 micro litres of the tyrosine solution. Now, for this what we need to do is that, we need to take a pipette of 20 micro litre with a clean tip, pipette out 20 micro litre of the solvent or basically water out here.

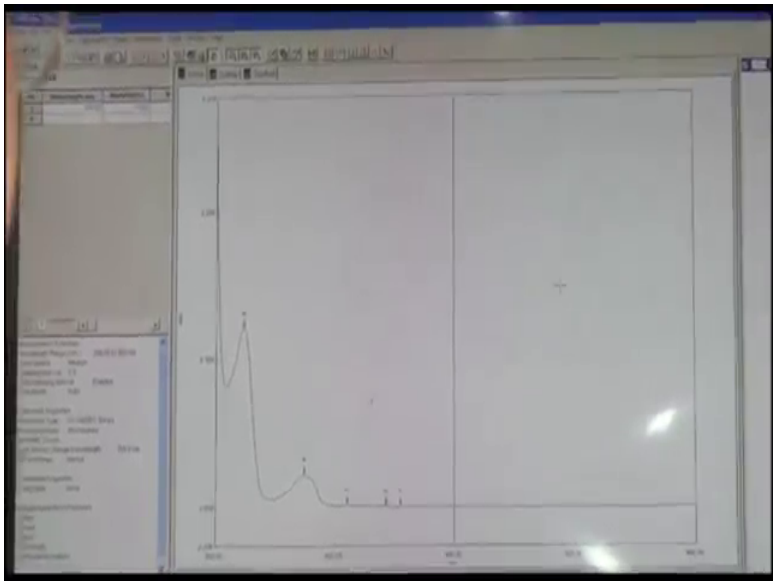
Now, once we have pipette out 20 micro litre we will be taking this tyrosine solution 20 micro litre tyrosine solution and we will be adding here. Once, you have added here we have to mix this solution, mix it carefully, note down that bubble does not appear here in the cuvette. Now why we have taken 20 micro litre of solution and again added this 20 micro liter, because in the reference solvent it we have taken 3 ml of water. Now out here we have also taken 3 ml of water and done the baseline correction. Now, once we have added this 20 micro litre of tyrosine solution the volume would change to 3 ml plus 20 micro litre.

So, all though this is not a major change, but for spectroscopic techniques the small changes are reflected in the intensity of absorption. For that what you have done, you have taken 20 micro litre out and again added this one in other to maintain the volume up to 3 ml. Now, once you have done with this one we will take again this solution and place it in the reference holder like the previous step where, the clear side will be facing where the light will go so, that the absorption can take place.

So, we have seen the absorption spectra of tyrosine solution. Now from there what we can do, we can calculate the concentration of the tyrosine solution, for evaluating concentration we have to follow the Lambert Beer's law which you have studied in the theoretical class where absorbance a equal to epsilon into c into l, where epsilon is a molar absorption coefficient. Now what you need to know if that, we need to know the molar absorption coefficient at a specific wavelength. For this we can know the molar absorption coefficient either at 275 or 278 or 280 whatever.

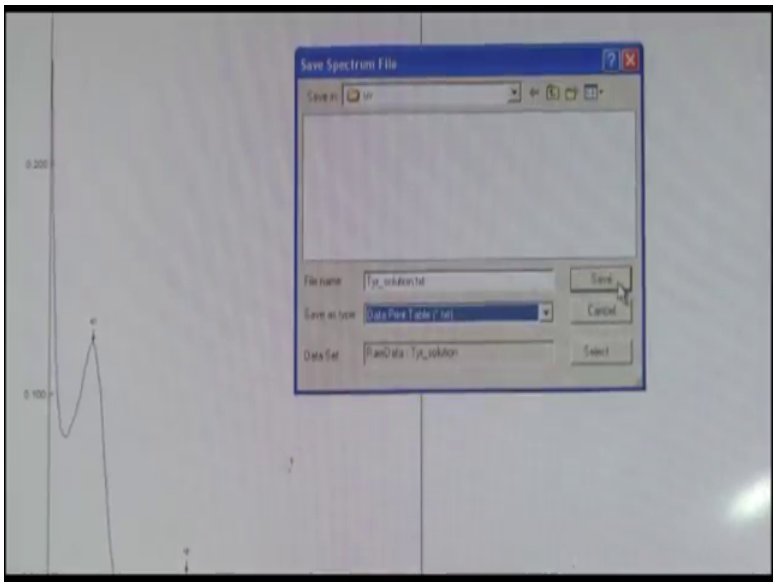
If you know the molar absorption coefficient at a particular wavelength we have to know the absorbance value at that particular wave length. Now, if we take the molar absorption coefficient at around 275 nanometer we cannot actually consider the absorption value at 278 nanometer; for that we have to consider the molar absorption coefficient of 278 nanometer. So, the absorption value the absorption value should correspond to the molar absorption coefficient wavelength. For this we can take the molar absorption coefficient around 275, 276 and calculate the concentration from the Lambert Beer's equation and from this we can know what is the concentration of a stock tyrosine solution.

(Refer Slide Time: 15:44)



Now, the important part out here we have to save as a text file.

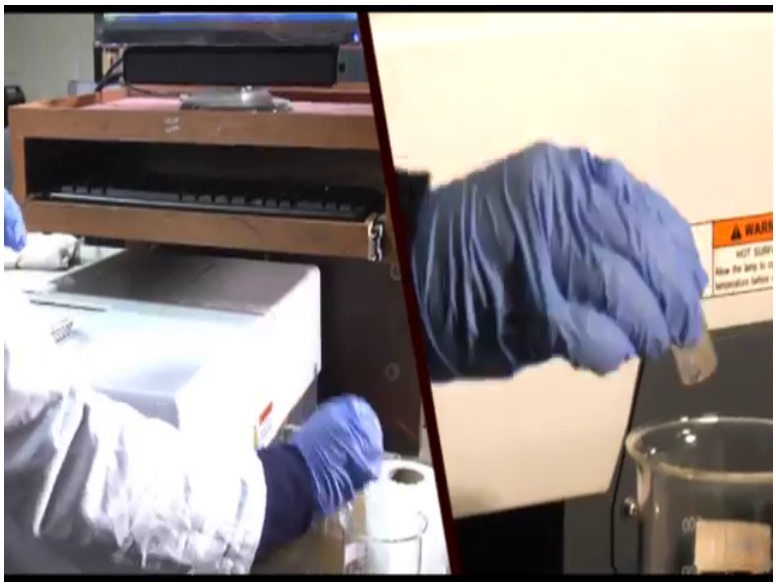
(Refer Slide Time: 15:50)



For this one we have taken go to save as out here and name will be same like this tyrosine solution. We have to go to save type out here, in the save type we have to click this one, out here we can see various extension are given. We have to go to this extension data print table dot txt and now we have to select the data printable extension, out here we can see the type changes from dot if see the data dot txt and now we have to go to save. What is the reason for this one because, once we need to plot this graph in origin or excel whatever we want we need this text file. For this we have to save have an extension of dot txt, now click let us click save.

So, once have taken a spectra remember to save it as a system file and also as text file. So, we are done with the absorption spectra for tyrosine. Now, we will shift to another amino acid (Refer Time: 16:48) the tryptophan, but before that we have to take a clean cuvette once again, for this what we need to you have to take the sample cuvette out here.

(Refer Slide Time: 17:01)



We have discard the solution in a weft beaker.

(Refer Slide Time: 17:06)

Now, you have to take again double distilled water and clean this one, clean at least 5 to 6 times; generally use this wash beaker having the water jet outlet and one recommendation do not clean it with tap water. Because, tap water would not clean this in rather it may make it impure with other components which are not required. So, I washed it quite a few times now, I will gently rub this outer side with the clean tissue paper. Whatever you do, do not touch the clean side of the cuvette always hold the frost

side. Now, we will take again 3 ml of water in the solution and carry out with our next absorption spectra.

Now, we have poured again 3 ml, we have poured 3 ml of double distilled water here in the cuvette. Now, we will again keep it out 20 micro litre of this one with fresh tip. Now, please do not use the tip you had used for tyrosine solution, because the solution here we are using tryptophan solution. So, each time you take a new solution kindly change the tip.

(Refer Slide Time: 18:44)

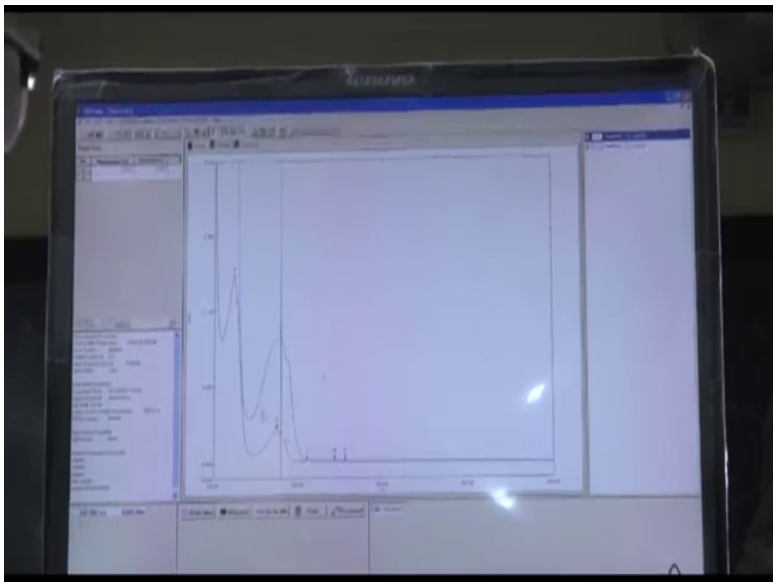
So, I will take 20 micro litre of this solution.

(Refer Slide Time: 18:49)

And again put it here tip and mix it well. Please note a few things out here, do not put the entire this tip out here in the solution just keep 50 percent of the tip in the solution. Now let us see there are few bubbles here we have to remove it, now just touch with the tip the solution and the bubble will be removed so, almost the bubbles are gone out here.

Now, let us proceed for this experiment, now this step also we have taken the solvent as double distilled water. So, in case of tyrosine solution the background is double distilled water, for tryptophan also double distilled water. So, we do not need to make another baseline; in the further experiment will do for this lab will do it as water as a solvent.

(Refer Slide Time: 20:00)



Now, have taken the tryptophan solution in the sample holder, now in the previous one we have just click the start button; however, in this case we can also start in the other way. Here in this part we can see the absorbance at around 600 nanometers showing a value which is negative, although this is not a negative one. But however, it has machine has calibrated it to be negative. However, we are all sometimes recommended to start from 0 so, machine should be calibrated to 0 for this wave length 600 nanometer.

For calibrating the machine to 0 we have to auto 0 first we can do this is optional, but it is recommended initially auto 0, when we click out 0 we can see the change out here; on the machine is calibrating it will take a bit time. Here, we can see it has adjusted to 0 value; now no value of no other numbers are coming out. Here we can now start with experiment now let us clicks start. So, we cannot basically recognize out here, once we uncheck this line here we can see there a tyrosine solution. Once you uncheck this line here we can see the gradual appearance of tyrosine spectra, current spectra is not here, it is scanning. Here we can see the peak rises out here, followed by the other 2 peaks.

Now, the machine has done with the absorption part; what we see out here this one is one peak and this is the other peak. So, basically what happened this peak is generally for the peptide bond and take place peptide bond and this one is for the anomatic part. So, here we can just click, we can take this bar and place it out. Here we can around 279 or rather around 280 or 279 we can see absorption maximum appears out here. So, what is

absorption maximum? Absorption maxima is basically the maximum, the absorption maximum basically the wavelength where we can calculate the maximum (Refer Time: 23:58). What is absorption maxima? Absorption maxima is actually the wavelength where the absorption intensity is maximum.

However, one thing we can notified here is the absorption maxima intensity out here you can claim it to be the maximum, but currently we are considering the anomatic part we generally gives absorption in this region 278 to 280 region. So, this part will be calculated here as absorption maxima. So, here we can see the absorption value that is 0.028. Now, we have to save this data once again. For this will go to file, save as now here we have to save it as trp that is tryptophan solution, initial type will be system file save. Now, again we have to go here the save as, the second save will be done in text format script even solution save.

Now, let us compared between tryptophan and tyrosine solution; out here if we click this tyrosine part here we can see this is tyrosine and this is tryptophan, two things to note down here. Before, that what we need to do we need to this enhance this part. For this we have to change the scale, for this we can change it to the scale the value which is around 0.2. So, we can change it to 0.2 thus, we can actually clearly see the values out here.

A few things to note from tyrosine and tryptophan observances is that the tyrosine absorbs around here the maxima for the tyrosine as we can see, it is around 270 value is around 274 to 275 whereas, the maximum value for the blue one tryptophan is around 280. So, there is a shift in the absorbance value and apart from that we have taken almost similar concentration of tyrosine and tryptophan; actually a prepared solution was quite similar concentration, of a intensity of tryptophan is much higher than that of tyrosine residual.

Now, we have seen videos absorption spectra for tyrosine solution and tryptophan solution. Now, this solution fully contents tyrosine residuals or tryptophan residuals; when we will shift protein. So, what is protein? Protein is basically a mixture of amino acid residuals, it composed of different types of amino acid residuals. So, apart from tyrosine or tryptophan various other amino acid like lysine, arginine, leucine, isoleucine etcetera might be there in the protein.

So, what the absorption spectra will look for a protein which have amino acids other than tyrosine and tryptophan present in it. So, we have to find out the spectra of different protein solution. Now, the protein having a tyrosine residuals might not match with the conventional tyrosine residuals. The tyrosine residuals spectra which you have seen just here might not match with the protein which are having only tyrosine residuals or, because other residuals might act over it; the spectra might be same or might differ a beat.

However, the absorption maxima is expected to remain the same; however, on the other hand the tryptophan residual, the spectra for the tryptophan residuals might not match with the protein which are having tryptophan residuals because, the protein having a tryptophan residual might also have tyrosine residuals. So, what is the conclusion out here? So, the conclusion out here is that the spectra we are seeing here in this conventional tyrosine and tryptophan solution might or might not match with the protein which are having multiple tyrosine or single or multiple amino acid residuals in it.

So, let us see with define protein which are having tryptophan and someone having tyrosine someone having mixture of this anomatic residuals and see how the absorption look like. So, let us start with a protein solution, the first protein solution we will use is ribonuclease RNase, the feature of RNase is that it has only tyrosine residuals, it has basically 6 tyrosine residuals and no tryptophan residuals. So, again we have taken this cuvette and washed it properly with double distilled water, filled it with 3 ml of double distilled water here.

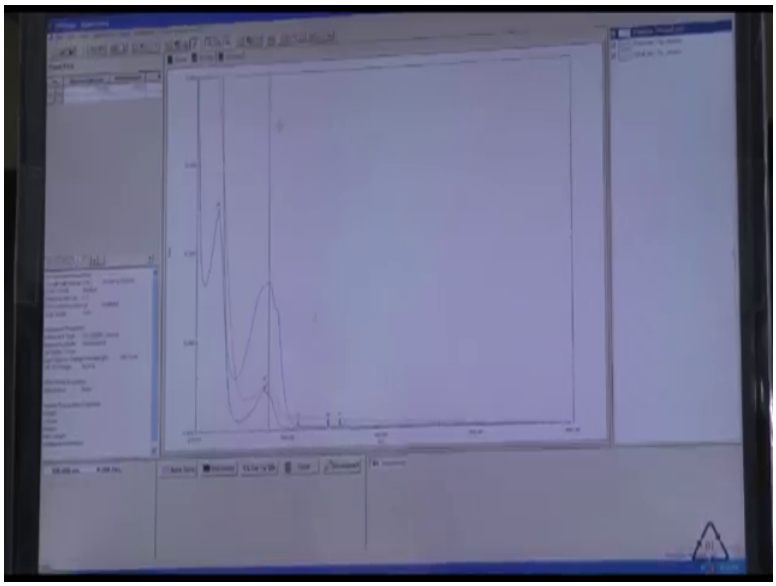
(Refer Slide Time: 28:18)

Now, we will again pipit out 20 micro litre of the solution and take a RNase.

(Refer Slide Time: 28:24)

I have taken 20 micro litre RNase and now I will at this in this cuvette and again mix it properly like the previous solutions. Kindly, note that bubbles are not present out here. Once you are done with it clean this clear side of the cuvette with tissue paper and again let us keep it in the reference compartment.

(Refer Slide Time: 28:57)



So, have taken RNase solution in the cuvette and let us start the experiment. Before, that let us click on auto 0, click on auto 0 will change this absorbance value at around 600 nanometer to 0. So, it is again 0; now let us start. Here we can see the experiment has completely start, it has started scanning. Here we can see appearance of a peak, a small hump is there, in the absorption maxima out here is around 277, one is 77 278 nanometer. So, this is for RNase. Now, we have to save this one once again save as RNase solution 1 and again let us save it as a data file.

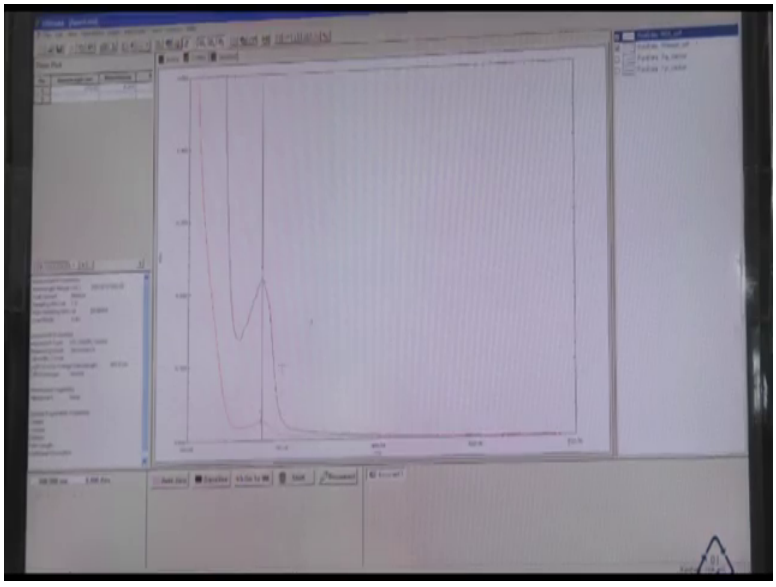
Now, as we have say earlier that RNase has only tyrosine residuals so, let us compare with tyrosine solution. Here we can see this is a tyrosine solution and this one is the red one is for RNase, the black one is for tyrosine solution. Now, let us put a proper scale and compare with here. Here we can see basically the intensity might differ because the concentration might be different in for pure tyrosine solution and RNase. However, one important (Refer Time: 31:55) note down out here is that the tyrosine residual give an absorption maxima at around 274 or 275 nano-meter whereas, it changes slightly by 2 to 3 nanometer in case of RNase which give an absorption value of around 277 or 278.

So, what conclude from this experiment is that, due to the presence of other amino acid residuals in RNase, the absorption spectrum might differ a bit, but not that much. The absorption maxima is still almost the same. Now if you compare with the tryptophan it is a well large intensity let us change it to 0.2. Now, comparing with tryptophan we can see

it is highly different from tryptophan residuals absorption spectrum. So, this is generally for tyrosine residuals. Now let us come to another protein so, you are done with the RNase absorption spectra now shitting to another protein this is serum albumin one of the very common albumin known as human serum albumin broadly known as HSA.

So, in HSA we have one tryptophan residuals. So, after tyrosine residue protein, now let us see how a protein with tryptophan residue gives an absorption spectra. Now will keeping this in the sample compartment.

(Refer Slide Time: 33:21)



Now, we have taken the solution in this UV chamber here we can see it is almost 0, but we can gain do out of 0 correction; now the intensity is quite high. So, we will change the scale and see how it looks, do not touch or do not stop this one until and unless the machine the experiment is over.

So, we are changing the scale to 0.5 now, first before analyzing let us save this one; save as HSA solution 1 in the spectrum file extension and again will take it and save as data printable format save. Now, let us scroll this bar and see here we can see the absorption maxima is around 278 and it is around 0.218 the absorption maximum. However, coming to this only tryptophan solution here we can see the tryptophan solution give the broad peak out here, but; however, in case of protein the nature of the peaks slightly change of not that much. However, the absorption maxima remains almost same with plus minus 1 change in its lambda max value plus minus 1 change is quite even the error range. So, no

need to bother much about that a few things to note down from here is that this is a spectrum for our HSA solution.

Now, if we compare between HSA and RNase solution, here we can see the following the 2 spectra varies. How it varies, number 1 if that peak the absorption peak is not that prominent out here as compared to this one. This might be caused due to 2 reasons; number 1 no here we can see the 2 absorption spectra one is for the blue line let 2 absorption spectra the upper part is for HAS, the lower one the red one is for RNase. Now can see basically 2 absorption spectra have different characteristic, number 1 the intensity values, number 2 the lambda max for both of them shift a bit up and down the wavelength.

So, why basically why there is a different number 1 of that a simple answer of that two different protein for of course, I have two different properties and deferent amino acid residues present into different proteins that is the absorption spectra value yeah it is right one. But however, one thing we did not discuss in the RNase case is that the concentration, the concentration for HSA might be higher than that of RNase and that is why it is intensity is much higher.

So, how to calculate the concentration, like the previously explained in the tyrosine part that if we know the epsilon value that is the molar absorption coefficient value at the specific wavelength that is supposed 278 for HSA or 280 for HSA or 278 for RNase; whatever it may be if the literature absorb molar absorption coefficient value are available we can actually calculate the concentration of the particular protein.

So, UV experiment is basically required for evaluating the concentration of protein. So, apart from studying the structure, studying the nature of the amino acid present in the protein one of the basic primary need of UV spectroscopy is determining the concentration of a particular protein. And, the second part is that we will go to our fluorescence experiment for fluorescence experiment as you have seen in the slide or rather in fluorescence experiment as we have seen in the theory classes that you need a excitation wavelength and animation range.

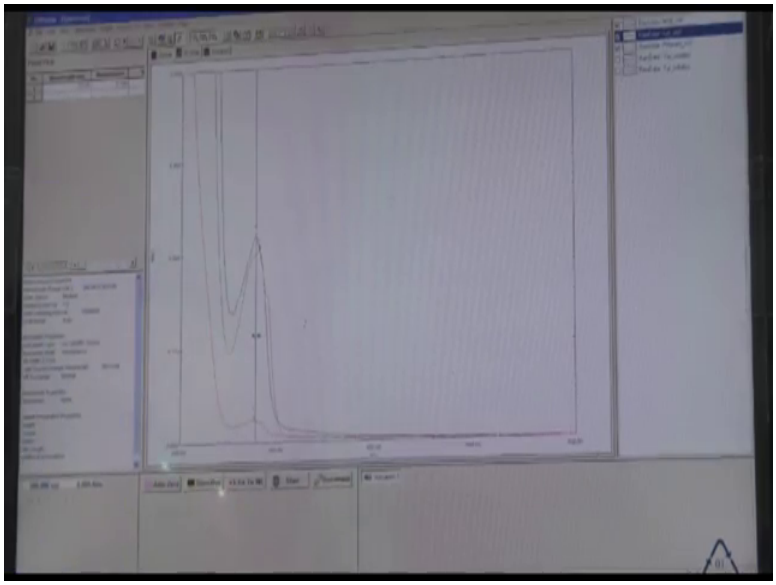
And what is excitation wavelength, basically the excitation wavelength if the maximum absorption of the protein which can be seen in the UV visible spectrometer. Now, for obtaining the fluorescence spectroscopy of fluorescence spectra of a particular protein

and fluorescence spectra, basically for a particular amino acid as we have seen in the theatrical classes; basically here we need to know that excite. We cannot actually arbitrary excite any orbited peaks and get the emission spectra.

We have to excited particular range and where to excite we can actually calculated it by using the UV spectrometer. Now, the absorption value where we can get the maximum intensity which is often called the lambda max we should excite here at that wavelength only. Now, for RNase where getting an excitation around to 278 we can excite around to 278. However, tryptophan we can get an excitation wavelength of around 278 and 280; however, the excitation wavelength or tryptophan vary bit it is around 295 nano meter.

Now, we have taken the third protein we have done with RNase and HSA and other third protein is lysozyme, this lysozyme contains tyrosine as tryptophan and also. Now, let see how the absorption spectra for this lysine protein looks like.

(Refer Slide Time: 38:55)



Now, let us do auto 0, now we can see the machine of calibrated itself. Now, let us start the scanning for lysozyme, the protein we have using in egg white lysozyme protein. Here we can see the scanning is taking place out here and one important thing to mention if do not click stop or do not open the chamber of the lid which has shown earlier while the scanning of going on this surely effect the lamp. And, if you are sure that you need to stop this one then only otherwise casually do not click the stop button it almost scanning is over; however, it will again get enabled here.

Now, we can see this part is around absorption value 1.2 and where us it is cross the upper limit let us see how much it is gone 1.5 is our 1.5, one important thing to note here kindly do not cross the absorbance value of around 1. As you have seen from your theoretical classes Lambert Beer law is applicable, do not cross the value of above 1; as we have seen from theoretical classes Lambert Beer law if only applicable for absorption value less than 1. So, better try to keep your solution below this one value and if it sometime crosses 1 dilute it, so that the absorption value for this part is below 1. And, we are not bothering about this one because currently we are interest and find the concentration from the spectra this peak.

So, this one here we have got it for lysozyme it is coming to around 282 we can see abroad peak out here. So, for first let us save this one lysozyme solution 1 in the spectrum file again save as in the data printable file. Now we are enabling lysozyme along with RNase and HSA solution interesting to note down here is that in both cases HSA and lysozyme. So, first let us consider with lysozyme and HSA we are disabling this RNase. So, lysozyme HSA we can see this upper part this black one is for our lysozyme part. So, if we change it 1.4 here we can see this part is lysozyme this upper part and the lower one is for HSA.

And, now coming to our RNase you are we can see this is RNase so, 3 defined proteins, 3 define absorption spectra and important thing we are from this absorption spectra we can calculate the concentration or we can and note the excitation wavelength from the (Refer Time:42:19). Now, we are done with the UV visible absorption spectroscopy part and after you complete this experiment, two things we notice that whether you have save the data in the computer as we have seen. So, it is better to save the data after once you are taken the absorption spectra and number 2 the most important part which many of tends forget is that we need to clean the cuvette which we have used for our measurements. So, we have to open this lid take out the 2 cuvettes, the front one and the back one and now discard the solution out here.

(Refer Slide Time: 43:12)

So, place the cuvette in such a way that the frost site touches the tissue paper, now one thing to recommend is that whenever you are using this cuvette, whenever you are placing this cuvette always try to place it on a tissue paper. This is generally known as the tissue bed for cuvette, do not place it in the table or in any sort of glass plate or whatever with the case always place it on a clean tissue paper.

(Refer Slide Time: 43:32)

Now, after that we have to clean all this quite few times with ethanol solution and followed by acetone and dry this one, otherwise after washing this one we have to gently keep it, keep this cuvette it in a tissue paper for drying.