Experimental Biochemistry
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Lecture - 12
UV And Visible Spectroscopy

In this lecture we will be starting a discussion on Spectroscopy. The first lecture will be on UV and Visible Spectroscopy and the second lecture will be related to Fluorescence Spectroscopy.

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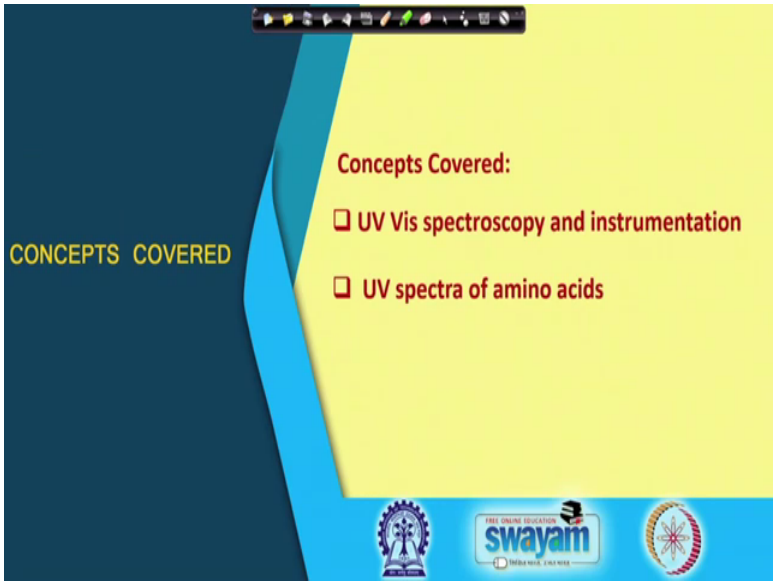

The concepts that we are going to cover in this lecture include UV Visible spectroscopy and the instrumentation associated with it and the UV spectra of amino acids, we will see which amino acids contribute to getting a UV spectrum for proteins and how we can use the UV spectra for specific studies. Later on when we consider studies related to proteins we will see in the instrumentation itself how we can understand structural changes of proteins related to spectroscopy both UV visible and fluorescenc.

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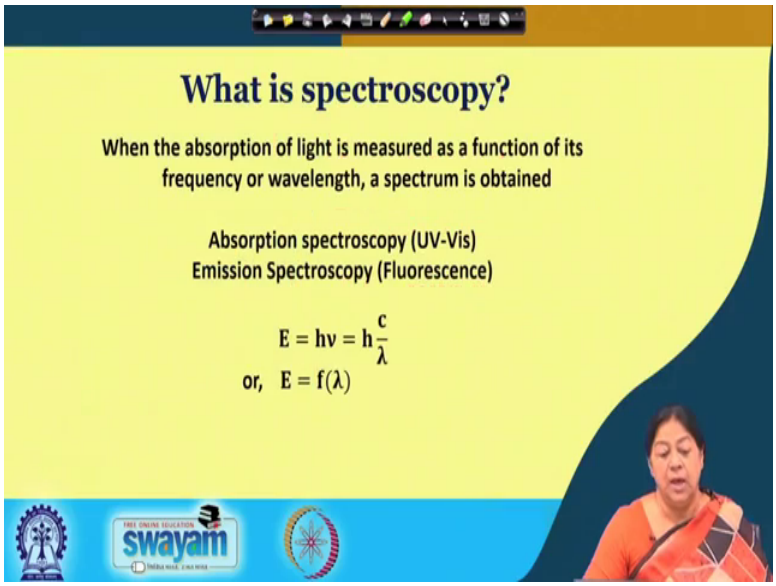

When we consider spectroscopy we have to understand that there is absorption of light, this light has energy that corresponds to a specific frequency and a specific wavelength. The equations that we have are E is equal to h mu where mu is the frequency or E is equal to h c by lambda c being the velocity of light and lambda the wavelength of light which means that this energy that we have is a function of lambda.

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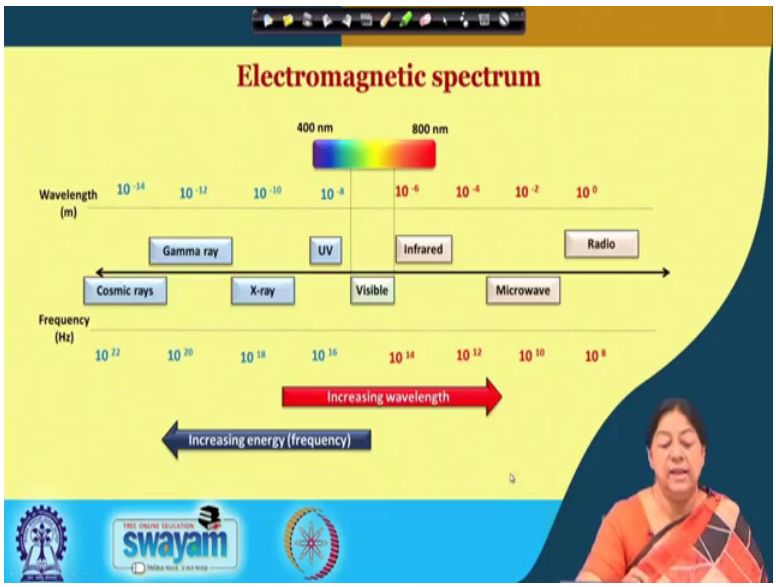

In the electromagnetic spectrum we see that we have in this red arrow increasing wavelength going from UV visible infrared to microwave to radio waves. This means if

this is increasing wavelength it means lesser energy this is increasing energy means increasing frequency as well. Now we are interested in this region we all know that the visible light that we see spans 400 nanometer around 800 nanometer and this is the spectrum vibgyor the rainbow colors that we know. The ultra violet form is just before the visible and the infrared region away from the visible range. Now we will be concerned with UV visible spectra we will see why we need to consider only UV and visible cases.

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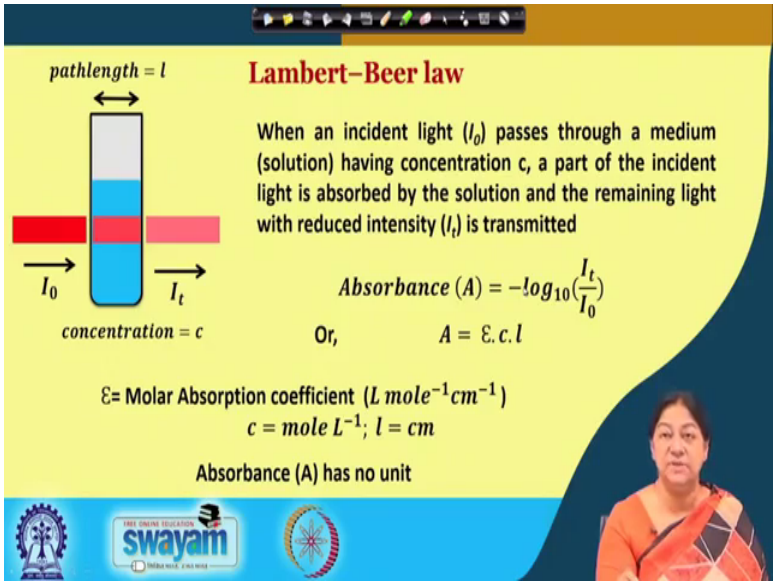

There is a specific law that tells us that when we have a specific incident light that shines upon our solution or our sample, the incident light has intensity equal to I 0 when it passes through our sample that has a concentration c part of the light is absorbed and part of it is transmitted with reduced intensity now which is I t. Now when we consider this reduced intensity so we have an absorbance that is given by this expression that relates the transmitted light to the incident light and we get a specific expression that tells us the absorbance is equal to epsilon C l, where epsilon is the molar absorption coefficient C is the concentration and l is what is called the path length. So, we have a concentration a path length and absorbance that is associated with some property of this solution that is the Molar Absorption Coefficient that tells us how much light this solution is actually absorbing because the more it absorbs the less it will transmit.

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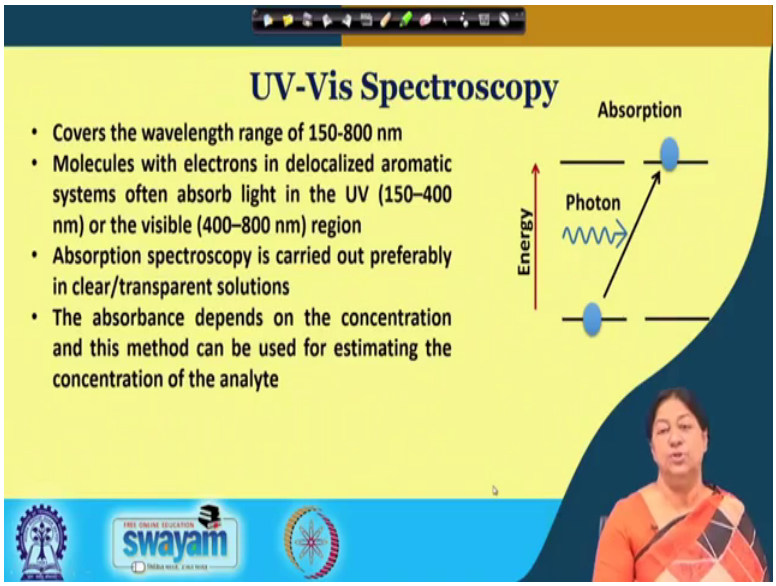

This Lambert Beers law is used for understanding concentrations for determining concentrations of proteins that we realize from the equation that we need know the Molar Absorption coefficient to determine the concentration of a solution given once we know what the absorbance is. UV visible spectroscopy covers the wavelength 150 to 800 nanometers as we saw in our electromagnetic spectrum, what happens is the molecules with electrons in the delocalized aromatic systems which you will see what this means they absorb light in the UV region and this absorption spectroscopy is preferably carried out in clear or transparent solution.

As mentioned before the absorbance depends upon the concentration and this method can be used to determine the concentration of the analyte. So, if we just look at a very simplistic way of understanding this we have a specific electron here a substance that is going to be put up into the higher excited level. So, we have a ground level an excited level and we have photon absorption.

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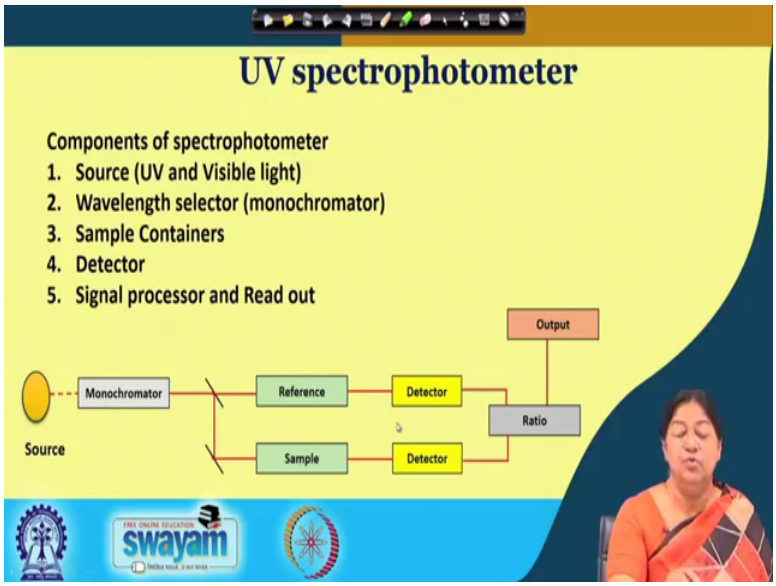

When we look at now the UV visible spectrophotometer what does it look like what does it have, it has a source it has what is called a monochromator what is a monochromator do it selects a wavelength. Now we have to understand which wavelength is required to be selected will depend upon on our sample and for proteins we will have a specific wavelength that will tell us whether we have a protein solution or not. In this kind of a spectrophotometer we have a sample and we have a reference we have then a detector and we have an output. Now from this information we will be able to determine the absorbance of our sample.

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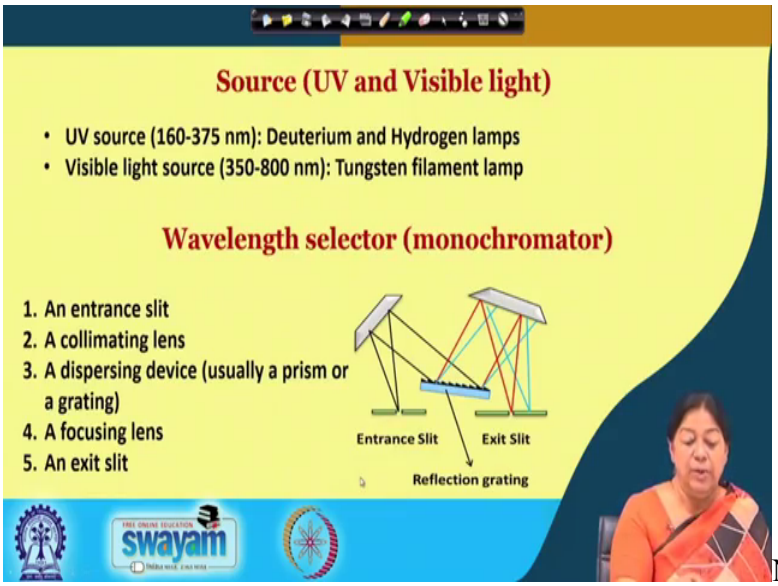

Now, when we look at the UV and visible lights we have a UV source that is present in the machine and the visible light source that covers 2 different wavelength regions. So, we have the deuterium lamp usually that is from 160 to 375 nanometer and then the visible light source just overlapping a bit which is the tungsten filament lamp. We look at the wavelength selector that tells us basically that it is basically a grating or reflection grating that will select the specific wavelength of our interest that we are going to use for our measurements.

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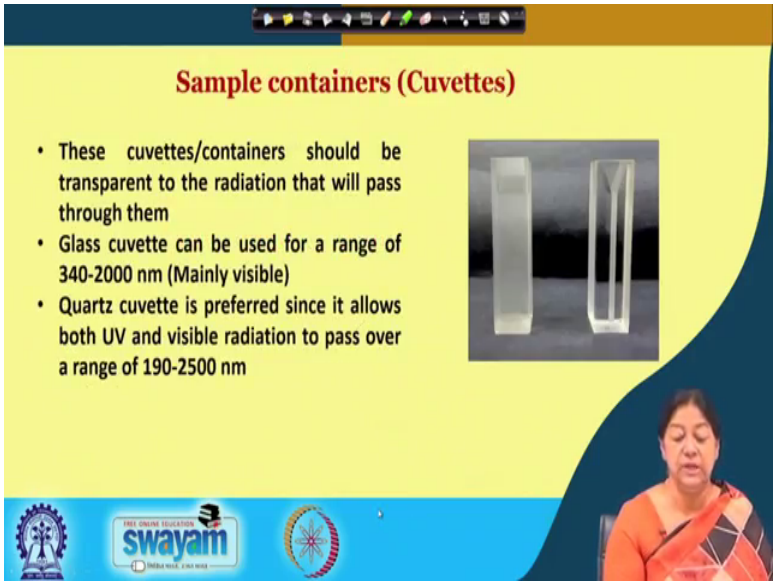

The sample containers are called cuvettes and in this cuvettes we will need transparence cuvettes glass cuvettes or quartz cuvettes which will have minimum absorption of light,

because we do not want the specific sample cell holder to absorb any light. Because, our sample is to absorb the light which will tell us how much light is being transmitted to give as a value of the absorbance of the sample.

When we use these specific there are specific kinds of cuvettes where we can have one with extremely narrow bore. So, that because when we have a lower amount of solution then we do not need to use the cuvette that is of a larger volume. There are different volume cuvettes that can be used can be purchased for the equipment and they are usually 1 centimeter by 1 centimeter so the path length is 1 centimeter.

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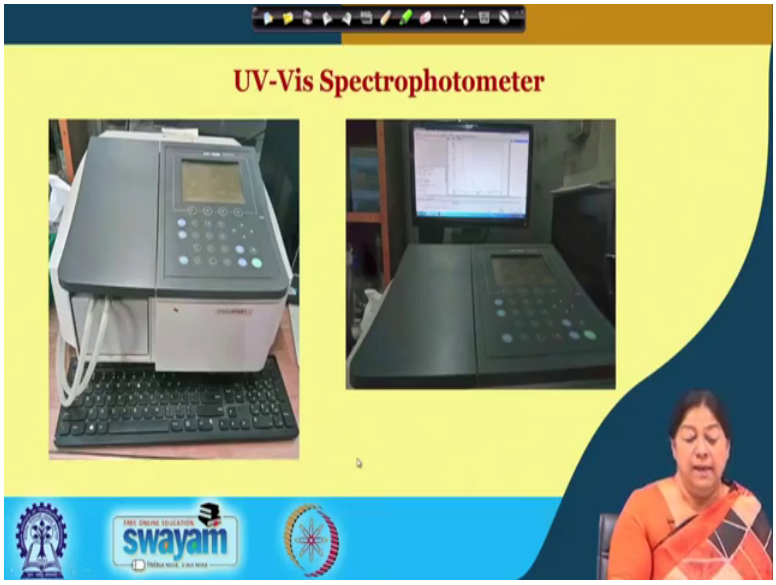

This is a typical UV visible spectrophotometer and with a spectrometer here.

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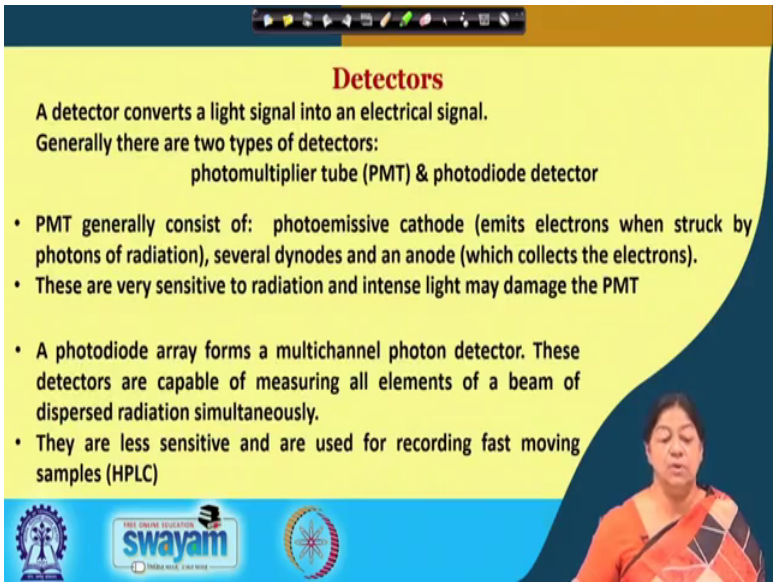

When we consider now what we mean by a Detector. So, when we consider what a detector is it converts a light signal into an electrical signal and we have 2 types of detectors. We have to realize that when we have a specific photo emissive cathode that emits the electrons when it is struck by the photons of radiation and this anode collects the electrons and these are extremely sensitive to the radiation and intense light may damage the photo multiplier tube.

Another way of detecting is called a photodiode array that actually forms a multichannel photon detector because, we have to see how many photons are being excited. How many what is the intensity of the light that is being absorbed, because from a knowledge of the wave length that we use we know the incident light intensity.

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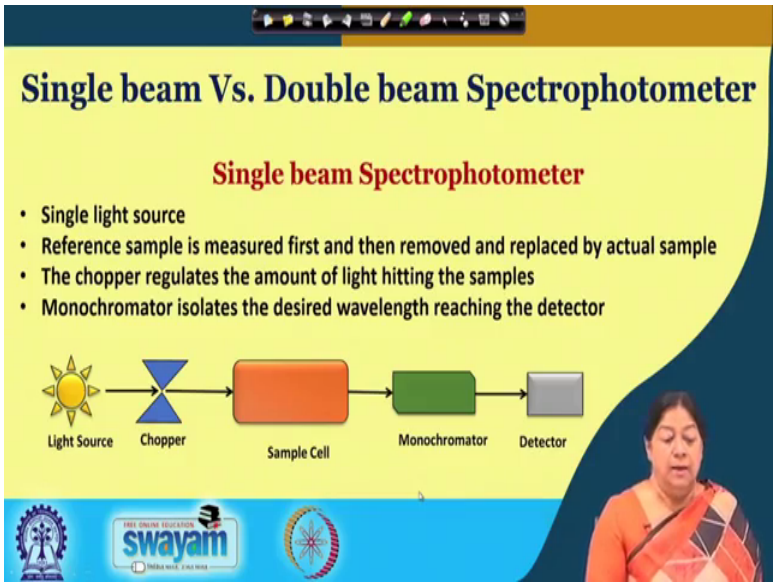

There are 2 types of spectrophotometer that are used one is a single beam one is a double beam. In a single beam case we have a sample cell and we have a reference sample what do we mean by that, if the protein is dissolved in a buffer we discussed about buffer solutions before when the protein is dissolved in a buffer, then this means that the buffer may have some components that absorb light.

To eliminate this component or this contribution of the buffer to understand only the absorbance that is given by the protein, we first have to put the reference cell in the sample holder and then determine or rather auto zero the instrument, knowing that this buffer is not going to contribute to the overall absorbance. We then put in the sample holder with our protein solution in the same buffer and then whatever absorbance is observed since it has already been auto zeroed is due to the protein or the sample alone.

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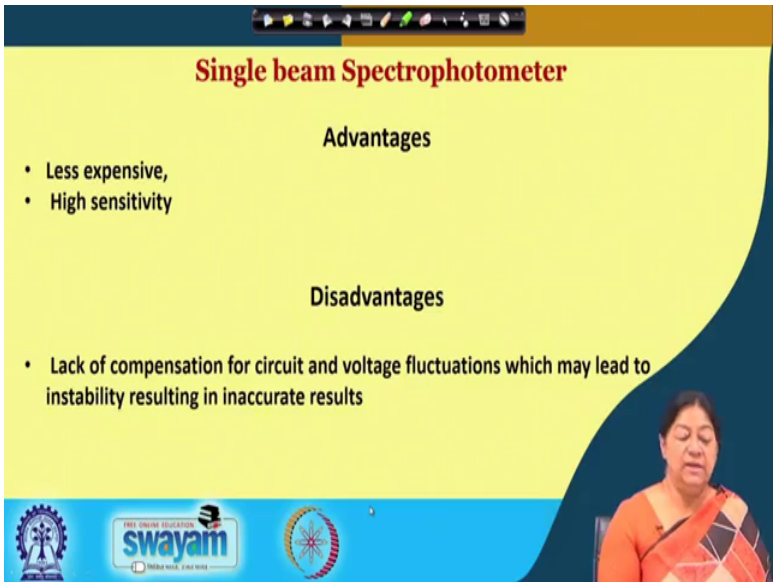

The advantage of this is it has it is less expensive even though it does have high sensitivity, but it has sometimes it gives inaccurate results.

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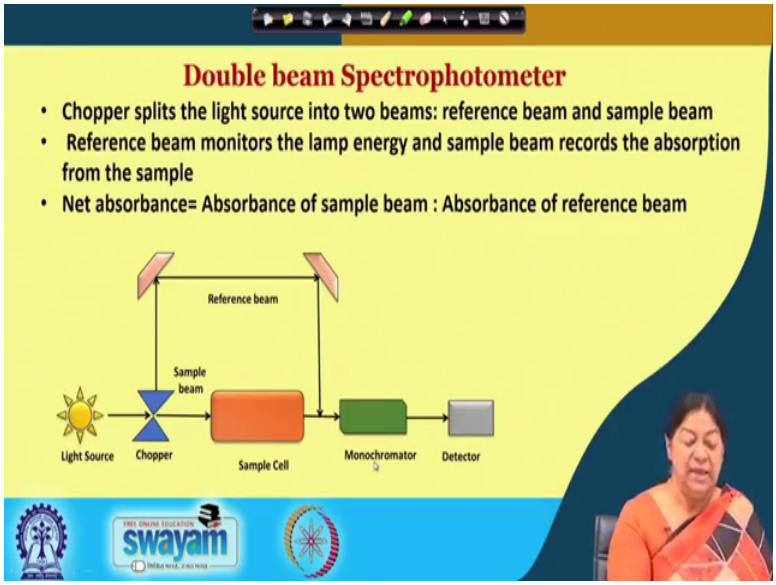

In a double beam spectrophotometer we have what is called a chopper that splits the light source and we have 2 cells we have a reference cell holder and we have a sample cell holder and it automatically looks at the difference between these or the ratios of these and then it finds out the contribution of the sample alone.

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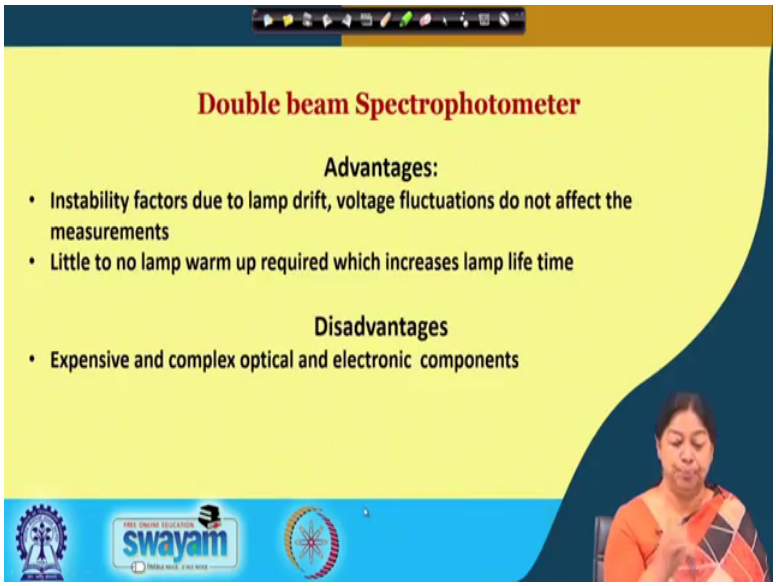

The advantage of this is that their voltage fluctuations do not affect it and we have specific absorbance values that can be read off the instrument and use for calculations directly.

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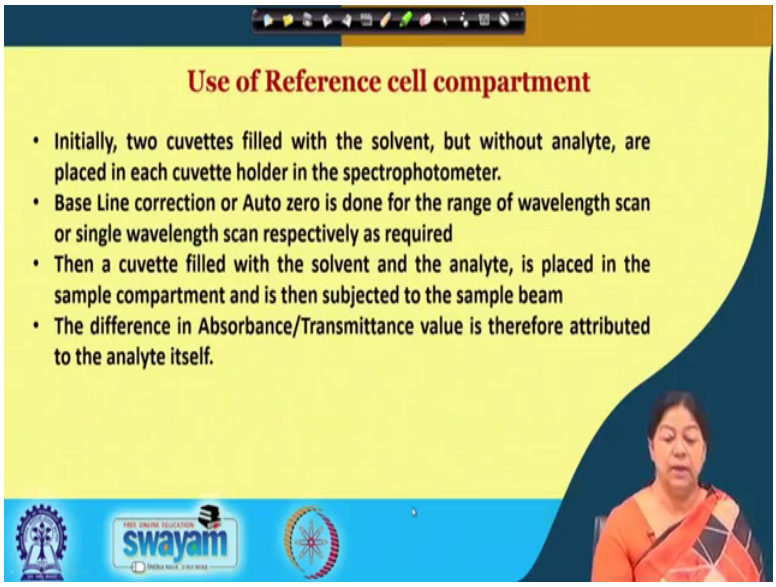

Now when we look at the reference cell compartment these 2 cuvettes as I mentioned are filled with the solvent or the buffer and placed in the cuvette we auto zero the instrument and then we place or replace the sample cuvette with our sample and then we can find out the absorbance values of the analyte or our specific sample of interest.

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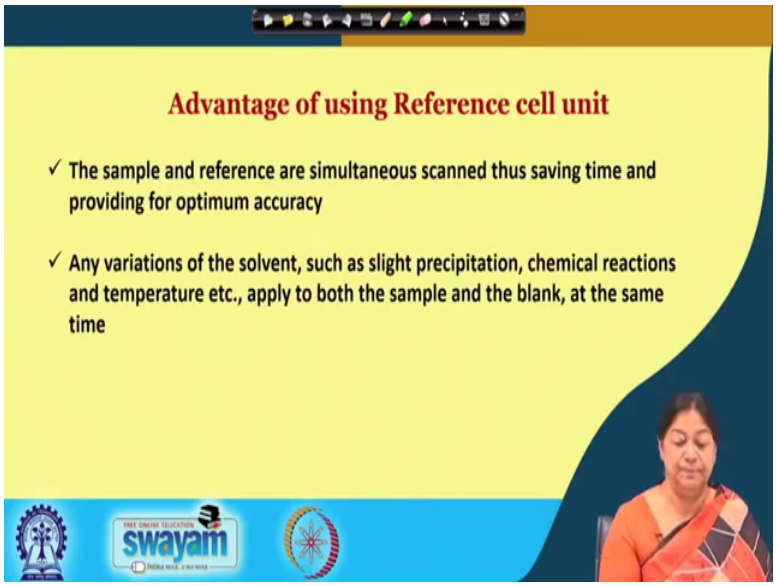

The sample and this reference in this case as I mentioned are scanned simultaneously and they provide optimum accuracy, so that any other changes that occur in the buffer simultaneously occur in the sample cell as well as.

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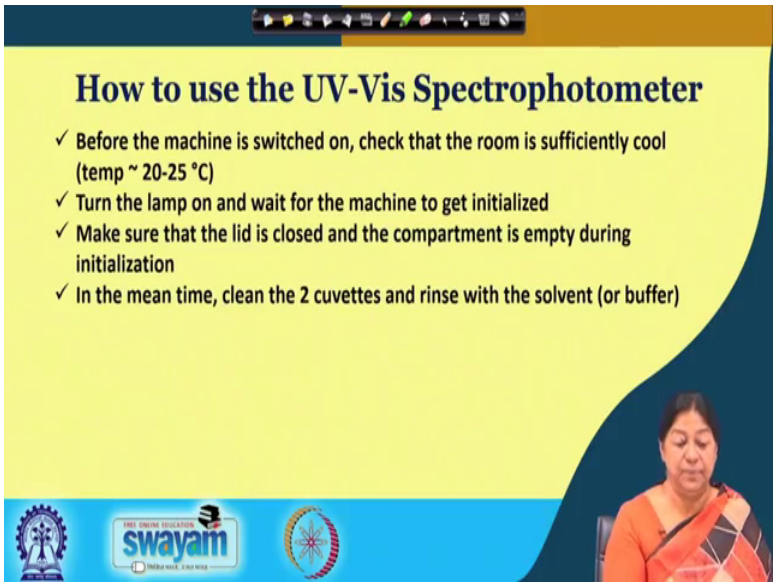

Now before the machine is switched on or is initialized the room has to be sufficiently cooled and we have to wait for the machine to be initialized. This will all be demonstrated to you in the experimental component of the lecture and we have to rinse the cuvettes very carefully and handle them also very carefully.

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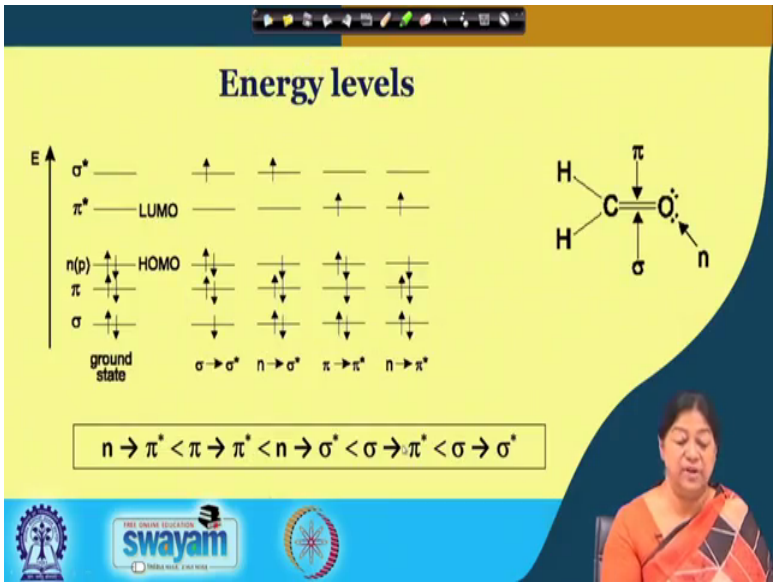

Now, what we have here is we have a specific CO bond I am going to talk about is now what we mean by ground states and excited states and show you why we are going to be considering the UV visible region for our region of interest. We have a ground state here as you can see we have the sigma the pi and the non bonding we have the pi star and the sigma star.

In the highest occupied molecular orbital and the lowest unoccupied molecule orbital, we can now have energy electrons go to the different levels. So, this corresponds to we can see one has is missing from the sigma it has gone up to the sigma star. So, this is a sigma to sigma star the energy difference is the most the wavelength is the least.

Here we are considering an n to sigma star a pi to pi star and n to pi star. Now we know the energy values for the UV range, the question that we ask is if I consider a peptide bond if I consider the specific wavelength of the particular aromatic amino acid residues which we are going to talk about in a moment which range do they fall into the lambda or the wavelength that they do fall into corresponds to that of the UV range.

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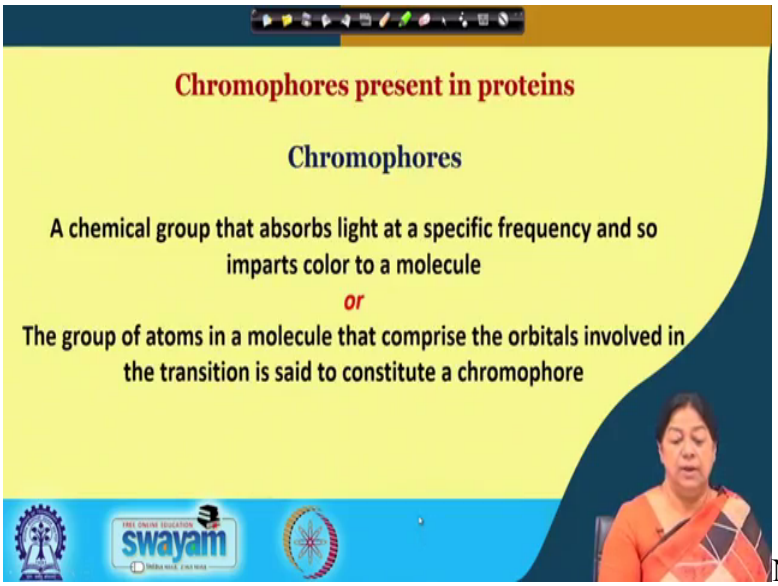

Now, the component present in the sample that is going to absorb the light is called a Chromosphores. So, the chemical group that absorbs the light at a specific frequency which means that a specific wavelength and imparts color to the molecule, in this case we will see that it absorbs at a specific wavelength and this will. So, the group of atoms in a molecule that comprise the orbital's involved in the transition that we just spoke about is going to be the chromophore corresponding to that specific energy and that specific wavelength.

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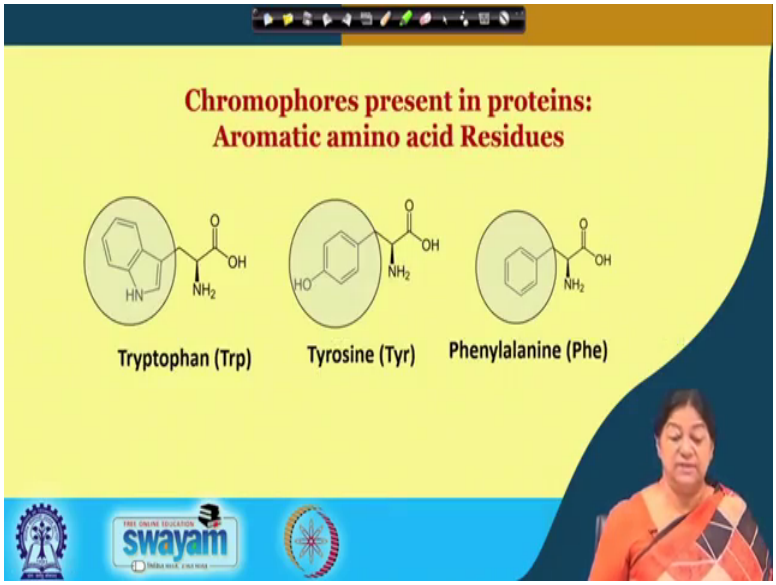

So, when we look at our specific chromophores presents in proteins these are the Aromatic amino acid residues tryptophan Tyrosine Phenylalanine.

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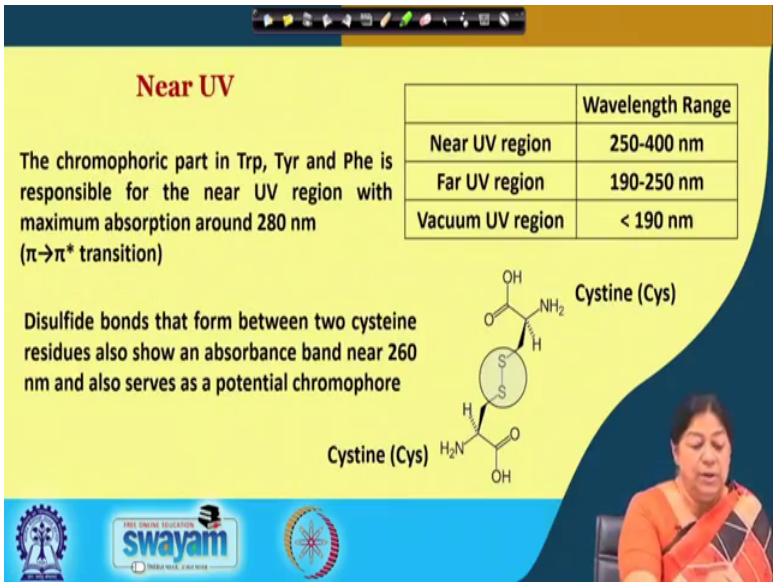

If we look so we have a specific chromophoric part in Tryptophan Tyrosine and Phenylalanine which is responsible for the near UV region with a maximum absorption around 280 nanometer that corresponds to the pi to pi star transition. If we look at the regions here we have the near UV region we have the far UV region and we have something called the vacuum UV region. Another component or another chromophoric component of a potential chromophore is the disulfide linkage between 2 cysteine residues. So, the disulfide forms between 2 cysteine residues are shown this is called a cysteine.

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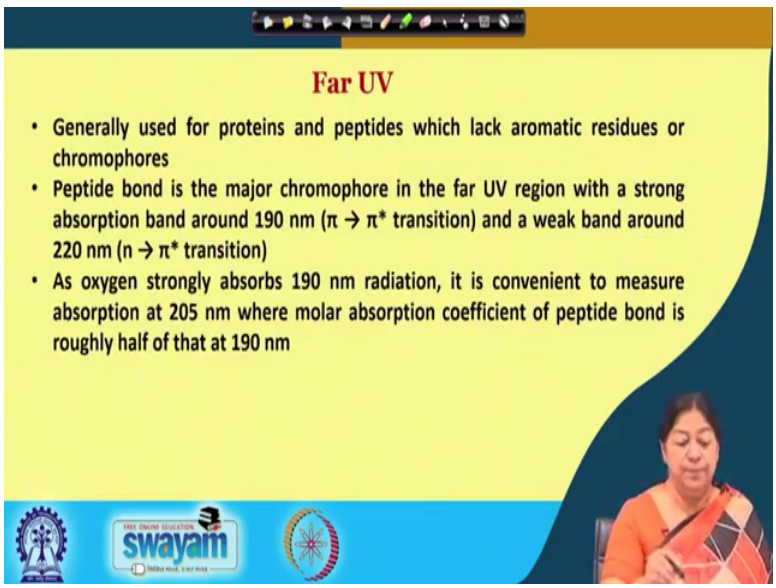

When we consider now the far UV this is generally used for proteins and peptides that lack aromatic residues, this chromosphores present in this position that is going to absorb the light is the peptide bond which is present in proteins. The peptide bond is a major chromophore in the far UV region with a strong absorption band around 190 nanometer corresponding to the pi to pi star transition and a weak band around 220 nanometer corresponding to the n 2 pi star transition.

Now since oxygen usually also absorbs around the 190 nanometer region we use 220 nanometer when in the case that there are no aromatic amino acid residues present. But ideally at the aromatic amino acid residues will have a specific absorbance because they have specific extinction coefficient that will make them absorb the light.

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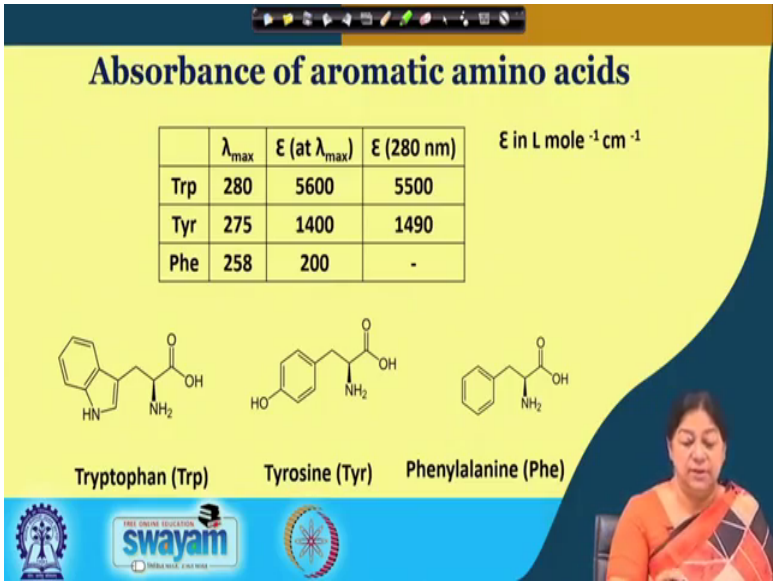

So, what is this absorbance so we have what is called the lambda max, what do you mean by this we mean by this is the maximum wavelength at which the specific amino acid absorbs light the spectrum we will see what the spectrum looks like. We have the molar extinction coefficient at the lambda max and the molar extinction coefficient at 280 nanometer.

So, when we look at the molar extinction coefficient we see that the tryptophan has the highest extinction coefficient, what does this mean that is if any protein molecule has a large number of tryptophan residues it will absorbs a large amount of light. Similarly if

you have Tyrosine or Phenylalanine which are weaker chromophores compared to Tryptophan.

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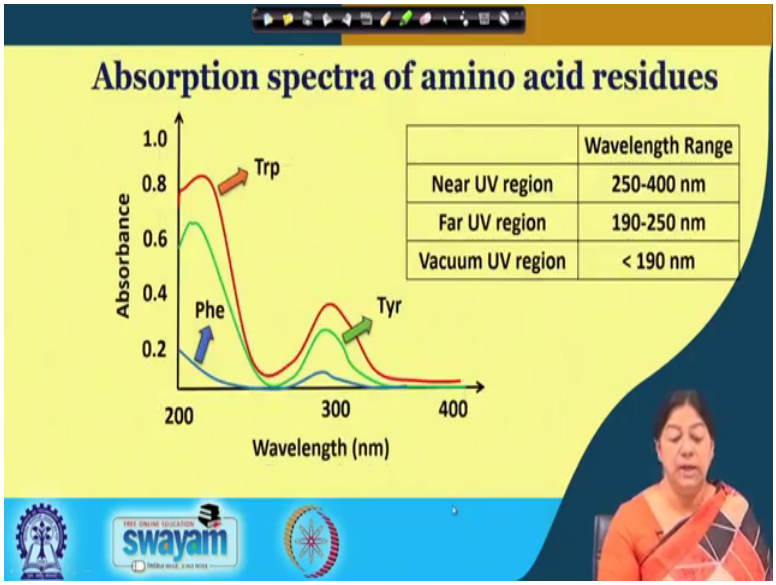

This is the absorption spectra of amino acids residues, you can see that we have the Tryptophan given in red the Tyrosine in green and the Phenylalanine in blue and what do we see we see the absorbance value here that does not have any units the wavelength in nanometers. We see that the absorption of the phenylalanine because the extinction is low is less compared to the tyrosine which is lesser than that of the tryptophan.

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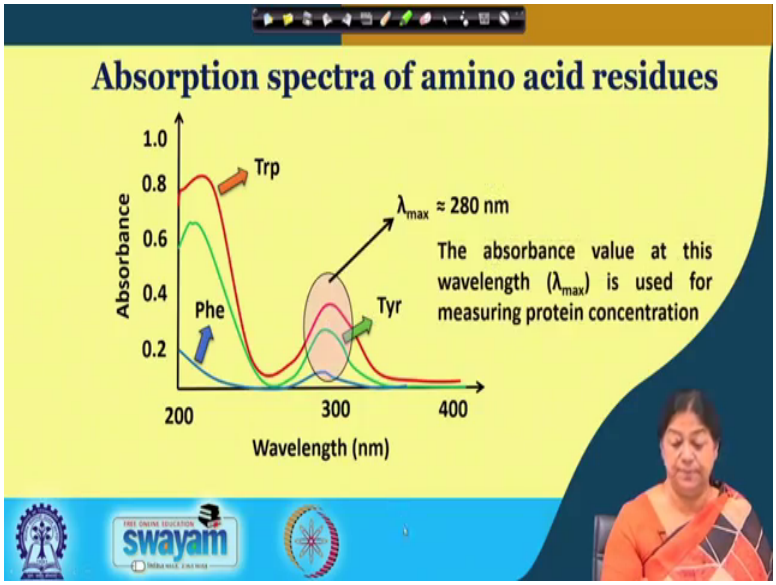

This is the absorbance at 280 nanometers where each and every amino acid aromatic amino acid shows a peak. So, this is the absorbance value at this wavelength that is the lambda max is used for measuring protein concentration. So, if a protein has aromatic amino acid residues present and we use a wavelength of 280 nanometers what does this mean, it means that in our selection of wavelength we select 280 nanometer and we set our machine to check whether our protein sample is present or not. So, if we have to say 2 test tubes 1 is a protein solution and one is just buffer then how do we determine which one is the protein solution.

We use a UV spectrophotometer for this purpose we set the wavelength we initialize the machine set the wavelength at 280 nanometers once this wavelength is set 280 nanometers and we auto zero the machine we set the one with the buffer that will not show any absorbance at 280 nanometer. If it is a protein solution it will show a definite absorbance at 280 nanometer and we can from our knowledge of the molar extinction coefficient or rather the extinction coefficient of the protein.

The epsilon value of the protein we can determine the concentration from Lamberts Beers law what is that the absorbance is equal to the extinction multiplied by the concentration multiplied by the length and as I mentioned the length is normally 1 centimeter.

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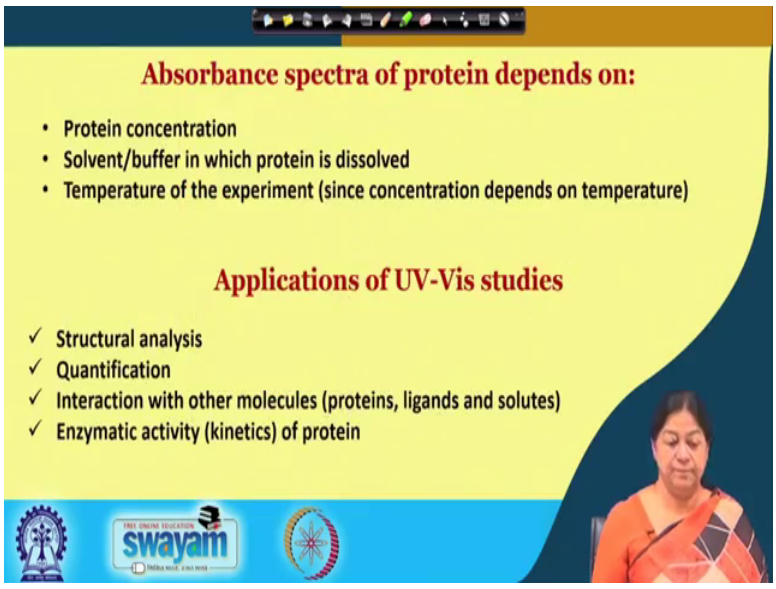

So, if you look at the absorbance spectra of proteins, what it does it depend upon it depends upon the protein concentration the absorbance is equal to epsilon C l, the solvent or the buffer in which it is dissolved and the temperature of the experiment because we know that the concentration depends upon the temperature.

What we do for applications of UV visible studies, we use it for structural analysis for quantification for interaction with other molecules if it. So, happens that we have a specific interaction of what is called a ligand with the protein and it changes the structural aspects of the protein.

Then this if this affects the aromatic amino acid residues there is going to be a change in the spectra of the protein, for structural analysis which we will see later on when we consider protein and proteins and protein degeneration, we will see how the tryptophan or the tyrosine mostly their absorbance changes as a protein unfolds and the enzymatic activity or kinetics of proteins can also be studied with a use of UV spectroscopy. If we have a colored protein then we can use the visible region for a look at the absorbance of the protein determination of concentration and any such associated methods.

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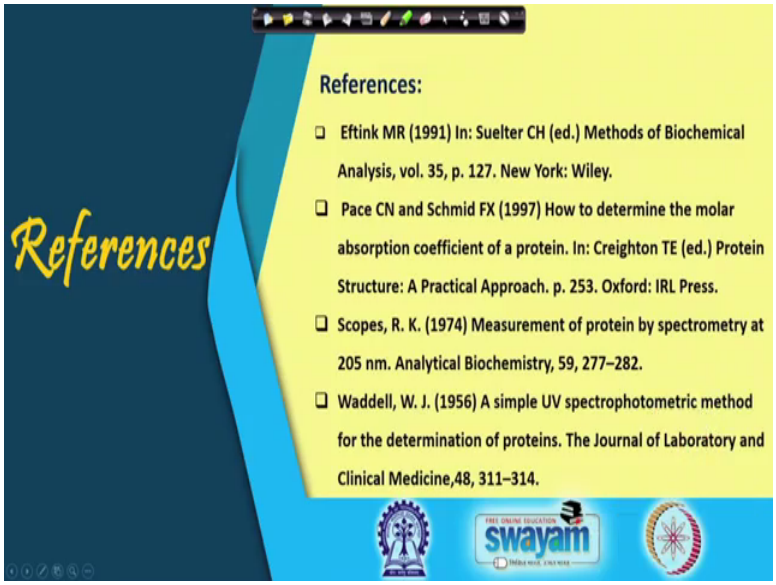

The references that I have mentioned here are Eftink there are pace and Schmid that tells you how to determine the molar absorption coefficient of a protein. This can also be done theoretically which we will show you in the laboratory portion and a measurement of the protein component or the protein concentration by spectroscopic methods. If you look at

these references these are references are a bit old, but there are new books or specific books that actually tell you how you can determine the absorption and the how it relates to the concentration of the protein the specific methodologies that will be used and also demonstrate to you during the course.

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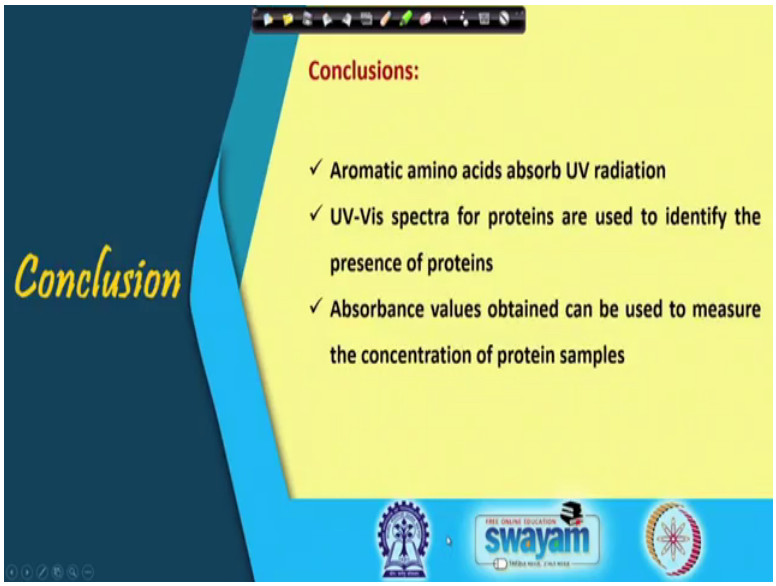

So, what did we learn is that when we consider UV radiation, the aromatic amino acids the tryptophan tyrosine phenylalanine are those that absorb UV radiation and when they absorb UV radiation what does it mean we are talking about the n to pi star or a pi to pi star transitition. This transitition corresponds to a specific energy level and this energy level corresponds to a specific wavelength which is the wavelength of interest.

The UV visible spectra of proteins are used to identify the presence of proteins, it is also used to determine structural changes interactions with ligands as well as most commonly the concentration of protein samples using Lambert beers law which is absorbance is equal to epsilon C l with the knowledge of the epsilon value the molar absorption coefficient, a knowledge of the path length which is usually 1 centimeter and once we determine the absorbance from the UV machine we can find out the concentration of the protein involved.

In many cases to find out the concentration of unknown proteins, a standard sample may be used to determine a calibration curve from which the concentration of an unknown

protein can also be determined. We will study Fluorescence spectroscopy in our next lecture.