Genetic Engineering: Theory and Applications
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Lecture 33
Spectroscopy

Hello everybody this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT Guwahati and so far what we were discussing, we were discussing about the characterization of the product which you are going to get from the over-expressed cells.

So as we discussed in the past that these over-expressed product what you are going to get from the overexpress cells could be either the biomolecules such as DNA or protein or it could be the small molecules which are actually going to be come because your overexpressing some protein and that is actually modulating the metabolism of the organism to channelize the things for production of a particular secondary metabolites. So for so far what we have discussed let us recap what we have discussed so far.

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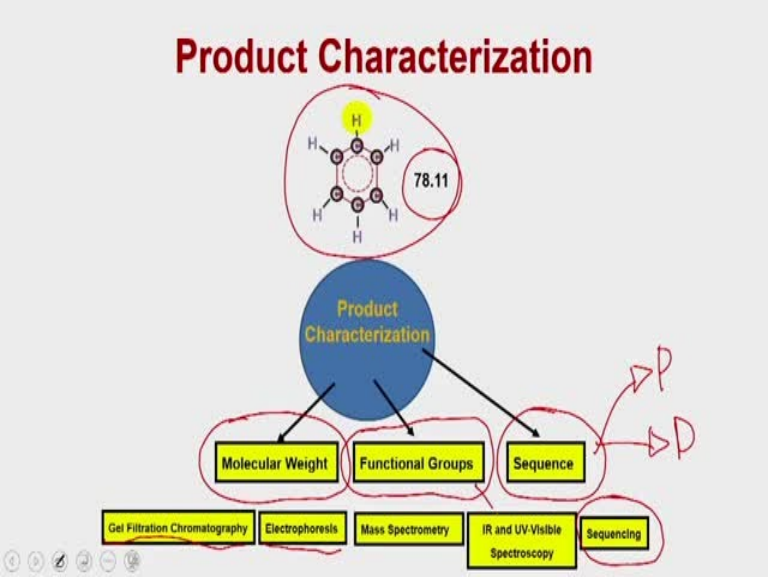

So we have this started our discussion about the characterization of a simple molecule like benzene. So what we have is information is the molecular weight, so there are 3 aspects what we have to taken-up so far in terms of the product characterization what we have said is that we are

going to characterize product in terms of the molecular weight and the molecule weight characterization can be done with the help of the gel filtration chromatography or the electrophoresis.

Then we have discussed about the sequence of the sub-units, in that case this is more applicable for the protein as well as for the DNA and in both the cases you can actually do a sequencing reaction to sequence the arrangement of the subunits in the case of the protein it will be the amino acid whereas in the case of DNA it will be nucleotide. Then the third aspect what we have also said is very important for product characterization is the characterization of the functional group and that can be done with the help of IR and the UV visible spectroscopy.

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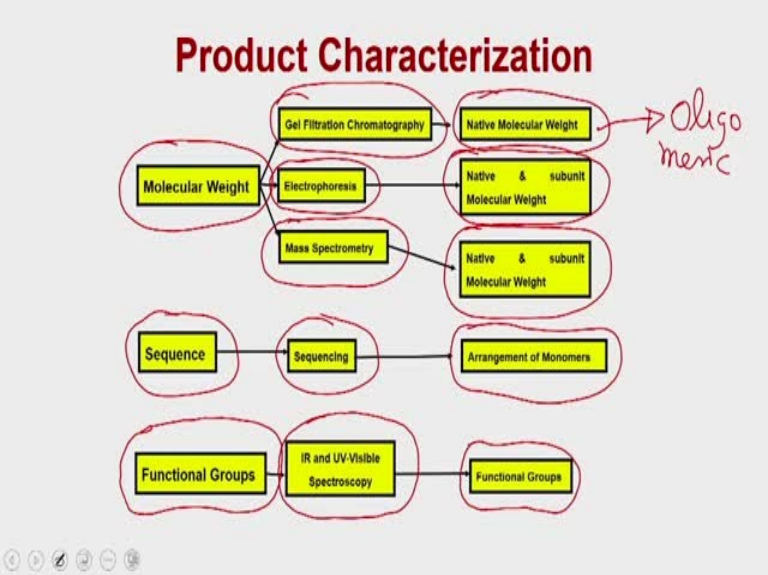

So what we are going to discuss we are going to discuss about the IR as well as the UV visible spectroscopy and that will give you the idea about the functional group as well as that can be able to help you in terms of the identifying the particular compound. But before getting into the details of the identifying the functional group you might have to do some pre-requisite analysis of the compound so that you will be able to more precisely be able to more precisely be able to determine the level the presence of the functional group. For this purpose we have taken 3 compounds.

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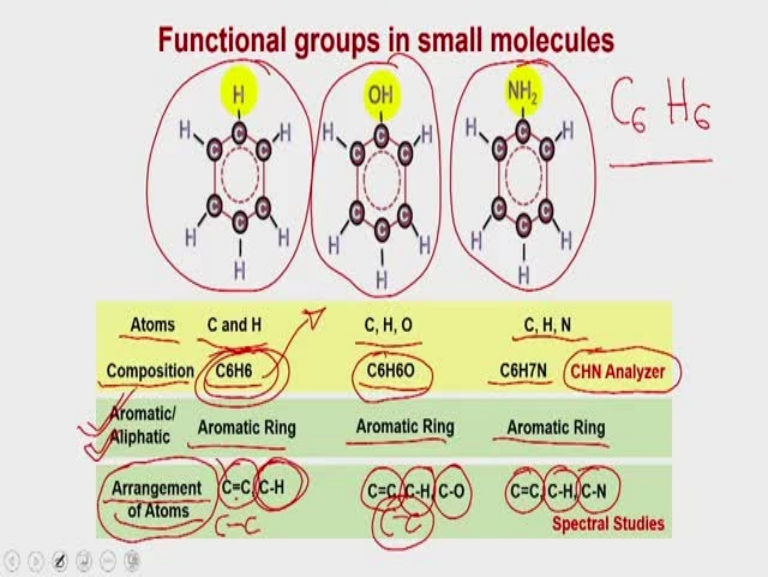

So we have taken a simple compound that is Benzene. We have taken another simple compound that is Phenol and we have taken the third simple compound that is called as the Aniline and what he can see from the structure because the structure of these 3 compounds are known that the in terms of the atom the Benzene only contains carbon and hydrogen whereas the Phenol contains carbon hydrogen and oxygen. And Aniline contains the 3 compounds that is a carbon hydrogen and nitrogen.

So the first thing what you have to do when you synthesize a new product or suppose you purify a new product from the over-expressed cells which are of non-biological in nature which means they are going to be the small molecules. Suppose some redaction metabolites or the secondary metabolites which you are getting from the over-expressed cells, the first thing what you have to do is you have to do the composition analysis of the constituents atoms which are present in that particular product or whatever the product these are the composition of that particular atoms present in the molecules.

For example in this case we said that the benzene contains carbon and hydrogen the phenol contains carbon, hydrogen and oxygen and the aniline contains carbon, hydrogen and nitrogen. Similarly you can do the composition analysis so if you do a composition analysis, the composition of the benzene is C6-S6 whereas the composition of the phenol is C6-H6O whereas

the composition of the aniline is C6-H7N which means you can do simply by giving your compound into the CHN analyzer.

So if you give your compound to the CHN analyzer it will tell you the percentage of carbon, hydrogen and nitrogen which is present inside your compound, so depending on the presence of these compound you can be able to calculate the empirical formula, you can be able to deduce what are the different atoms are present in your compound and for CHN analysis you might have to give 1 milligrams to 5 milligrams of the compound depending on the number of atoms and other groups are present.

Then the second thing what you have to ask is whether the compound is aromatic or the aliphatic? Because I can have a compound which is C6H6 but I can have a linear chain aliphatic compound instead of the cyclic aromatic compounds, so the second thing what you have to ask is, whether the compound what you are isolating or the hydrocarbon what you are isolating it is the aliphatic or the aromatic?

In these cases all the 3 compounds are having the aromatic ring but you can be able to do the set of reactions and that reactions are going to tell you whether the compound is aliphatic in nature or the aromatic in nature. Then you can also do a third analysis and that is call as the arrangement of atoms. So now we have to know that this compound actually contains carbon and hydrogen but whether the carbons are present in a single bond or whether the carbons are present in the double bond or the carbon is present in the form of single bond or whether the carbon is having a bond with the hydrogen.

What is the nature of that particular bond and so on, if you have this information that actually will allow you to identify the compound as well as that will allow you to determine the presence of the functional groups. For example, in the case of benzene you have the carbon double bond and the carbon single H. Whereas in the case of phenol you have the carbon double bond then you have the carbon to hydrogen bond as well as the carbon to oxygen bond. And in addition to that you can also may get a band for the carbon-carbon single band.

Similarly in the case of Aniline you have carbon double bond, carbon to hydrogen bond and the carbon to nitrogen bond. So how you are going to get the arrangement of the atoms onto the molecule? Let u revise once more that what you are supposed to do.

The first thing what you have to do is you have to do a CHN analysis which means you have to give your compound for the CHN analysis that will give you the composition of the molecule and that will also going to help you in terms of deducing the empirical formula. Once you have the empirical formula then you will be able to predict whether this compound is going to have a functional group or not.

For example, in the case of benzene you got the empirical formula that is C6H6 that itself says that this particular compound does not contain any functional group because the carbon and hydrogen together are not forming any functional group. Then you require a third compound either it will be nitrogen or oxygen to form the functional group.

And that is what exactly is happening here. In the case of phenol you are getting the empirical formula that is C6H6O which means now you have a oxygen and that actually can give you the functional group in terms of the aldehyde that is going to give you functional group in terms of the alcohol and that information whether it is a aldehyde that going to give you the functional group in terms of the alcohol and that information whether it is a aldehyde or whether it is a alcohol that information you will get when you will be going to study the arrangement of the atom.

But before that if you can be able to deduce or if you can be able to determine the compound and its nature whether it is aromatic and aliphatic that also can be able to help you in terms of the predicting the kind of functional group could be present if you have this particular type of empirical formula. After that you can be able to the arrangement of atoms and arrangement of atoms will going to tell you about not only what are the different bonds are presented between the different atoms but also what are the different bonds are could be possibly be exists in this particular molecule.

And once you have all these 3 information and if you can be able to know that what could be my predictable structure because everything is you are doing as a blindly because you are trying to isolate a particular type of molecule and this study what we are doing is we are just simply tries the product based on that our prior information. For example if we are trying to the benzene we know that it should be a benzene.

So it should not give you the reaction for aliphatic reaction. So it should not give you any reaction which are specific for aliphatic because as soon as it gives you reaction for aliphatic compound then that will give you a doubt whether it is a benzene or it is going to be a hexane actually.

Then if this is going to give you aromatic compound this means you are on the right track because CHN analysis already says that it is C6H6 which means it is going to be could be a benzene molecule. But once you going to get the second characterization that means whether the compound is aromatic or aliphatic it will say that okay this is benzene. Then the third is once you know the arrangement of atom then is going to be sure that you can be able to predict with the 99 percent probability that is molecule what I have isolated from the cell is actually containing the is actually the (())(11:02).

But apart from that for arrangement of the atom we can do a different series of spectral studies. And that actually will give you the information about the presence of functional group onto your molecules.

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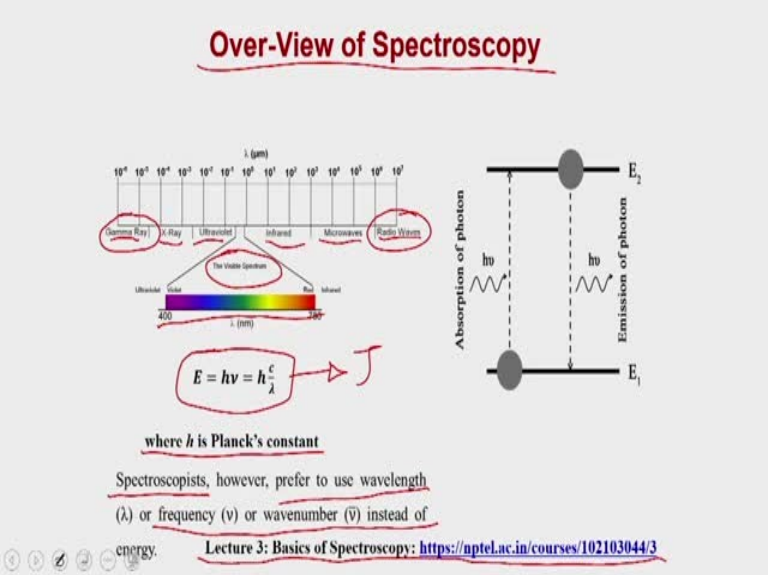

And when we talk about these spectral studies we all first have to understand the spectroscopy what is the basic understanding about the spectroscopy. So the spectroscopy means that the study of the interaction of the electromagnetic radiation with the matter. So what is electromagnetic radiation? Electromagnetic radiation is the radiation where you have a set of the lights or the set

of waves of different wavelength. It could start from the radio waves on one end and it ends on the gamma rays on the other end.

In between you have the range which is called as the visible spectrum this is the spectrum which anybody can see with the naked eye. Which means the human eye is very-very susceptible or very-very can be able to perceive only this flight which is actually falling under the visible spectra. But beyond the visible spectra you have the infrared light, you have the microwaves and then you have the radio waves.

Down to the visible range you have the ultraviolet, you have x-rays and then you have the gamma rays. And if you see the wavelength of these lights are on varying very much from the radio waves to gamma rays and if you want to calculate the energy associated with each radiation you can use the this formula which is called as E equal to h Nu or E equal to h c by lambda, where H is the Planck's constant.

So if you calculate the energy you are going to get the energy in terms of joules or kilo joules. And the joule or the kilo joule is the energy but many times that kilo joule can be converted into the electro volt and but for the spectroscopists mostly they use a term which is called as the wavelength.

So for spectroscopists those who study the interaction of the electromagnetic radiation with the matter they actually prefer to use the wavelength or the frequency or the wave number instead of the energy which means they are not going to give you the values or they are not going to give you the conclusion in terms of joules, electro volt or any other parameter. They are only going to give you the in a the analysis in terms of the wavelength that okay this wavelength of the light is interacting with your matter and so this could be the nature of this particular matter.

So this is just an overview of spectroscopy if you are interested to read more about this spectroscopy I would suggest that you can go to the one of our course that is been given on the NPTEL website and I have given the link here, so if you go to that particular website you may get the reading material, you may get the content in more in detail to study about the electromagnetic radiations and the phenomena of radiations.

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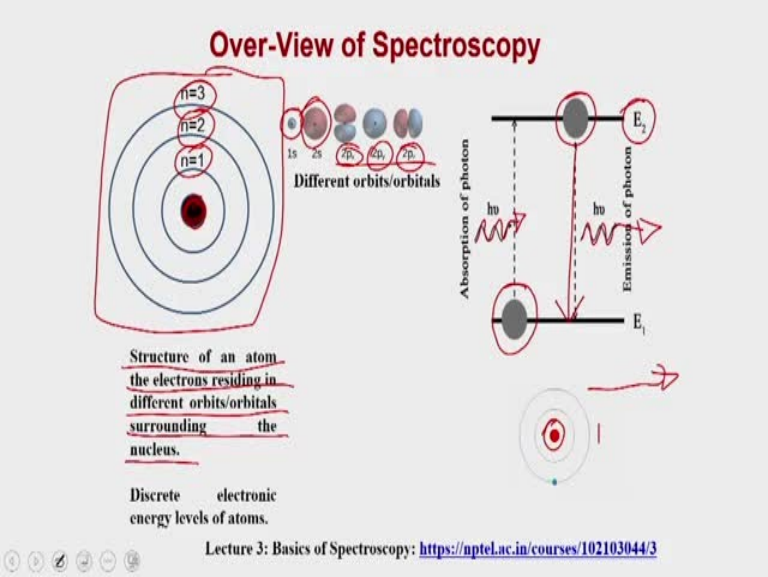

Now the question comes why the different molecules are absorbing differently. So every molecule so if you see the structure of an atom what you say is that the electrons are residing in the different orbits or the surrounding the nucleus. So what you see is this is the typical structure of an atom and where this central thing is the nucleus where the proton and the neutrons are present.

Whereas these are the different shells in which or different shells where the electrons are present so you can have the lower energy state which is n 1, n 2 or n 3. And within this you are going to have the different types of orbitals. Which means either it could be 1S or it could be 2S or it could within the 2p you can have the px, py and pz. And all these different orbitals are associated with different amount of energy. So if molecule is changing its position from the lower orbital to higher orbitals it is going to absorb the light.

If it comes back from the higher orbital to the lower orbital it is going to emit the light. Now you can see this in terms of an animation, what you see is in the center you have a nucleus and surrounding this nucleus we are showing a electron which is travelling into the two different energy levels. So you can see that when the electron is present in the outer energy level that means electron is having more energy it actually emits the light.

And as soon as it emits the energy it comes into the lower energy states now you will see that once it is in the lower energy state it is going to receive the energy and it will go to the higher

energy state. This is what is actually happening when the molecules are mowing from the lower energy state to the higher energy state they absorb the light. When they go from the higher energy state to lower energy state they are actually going to emit the light. This means you are going to have two phenomena absorption as well as the emission.

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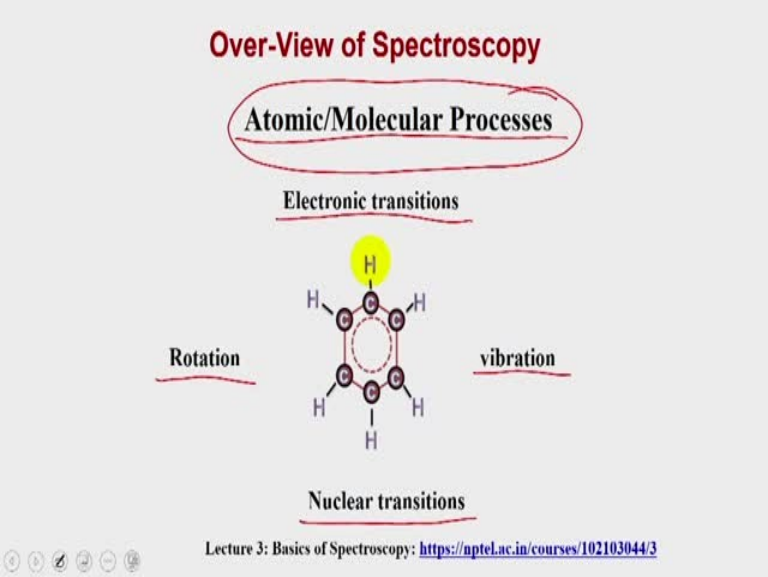

And all these absorption and emission which is actually going to be the happening and the different molecules are going to do this phenomena differentially because of the various types of atomic as well as the molecular processes which are happening within the molecule. What are these phenomena? You can have the rotations, you can have the electronic transitions, you can have the vibrations and you can have the nuclear transition.

We will discuss this in more detail in the next slide what are these phenomena or what are these processes and how you can be able to map the electronic transitions, rotations, nuclear transitions or vibrations using the different parts of the electromagnetic radiations.

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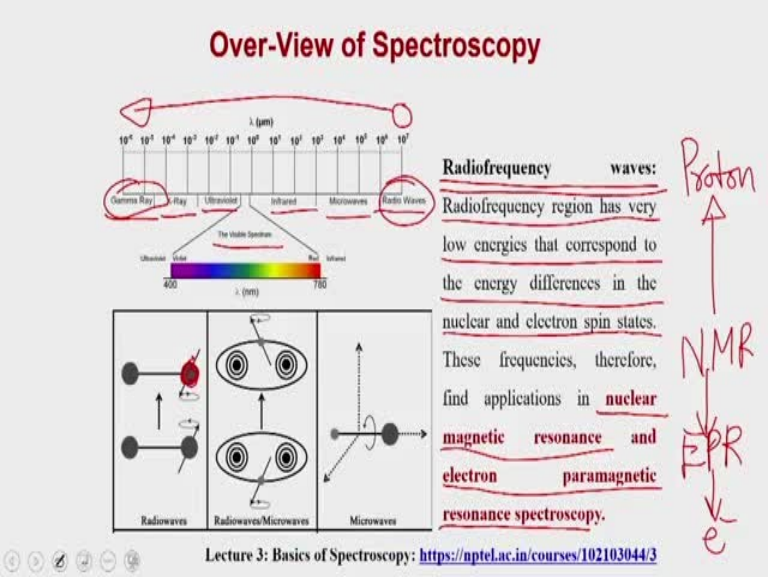

So first let us see that this is the electromagnetic radiation, this is the visible spectrum and this is the radio waves, microwaves, infrared, ultraviolet, x-ray and gamma rays. So you can imagine that actually if you put the values in terms of the energy it is this is the lowest energy and it increases in this fashion which means the gamma rays are the rays which are actually having the highest energy, whereas the radio waves are the waves which are actually containing the lower energy.

And these all these different energy or different radiations can be used to map the different molecular processes which are happening within the molecules. So the first molecular process is which can be mapped with the help of the radiofrequency or the radio waves. So radiofrequency region has a very low energy that corresponds to the energy difference in the nuclear and the electron spin states, okay. So the first phenomena is the rotation, so rotation could be on its own spin, on its own axis.

So it could be that the some molecules are spinning on its own axis and that spinning can be changed. And that is very-very small quantity of energy and that can be mapped with the help of the radio waves whether you are studying the spinning of the protons which are present inside the nucleus or whether you are studying the spinning of the electrons which are present in the outer shells.

And for these purposes you can actually use the radio frequency waves in the two phenomena you can use them for the nuclear magnetic resonance which is called as NMR. So that actually is going to study the rotation moment in terms of the proton or you can be able to use the electron paramagnetic resonance spectroscopy which actually is so the other phenomena is the EPR where you are actually can be used to study the phenomena of electrons.

So you can be able to do a study the rotation movement within the electrons using the EPR studies or EPR spectroscopy. Where as you can use the NMR spectroscopy to study the rotation moment within the protons which are present inside the nucleus..

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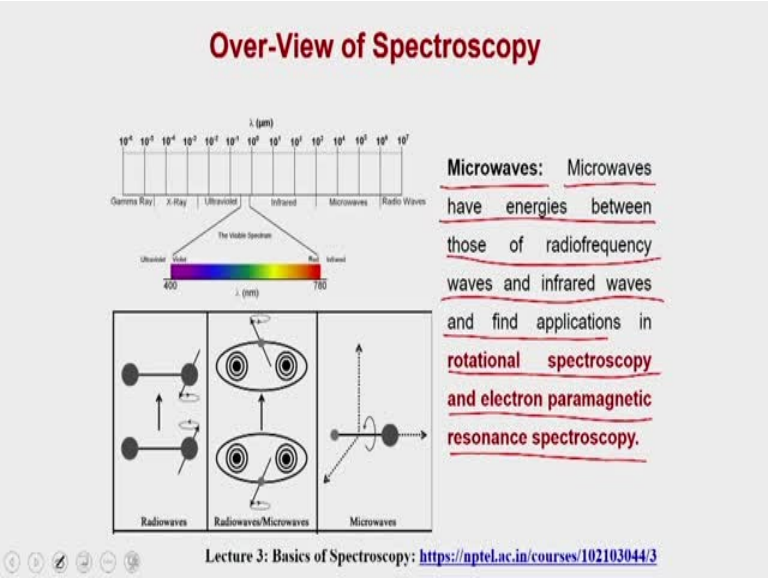

Now the second is the microwaves. So the microwaves have the energies between those of the radio frequency waves and the infrared frequency and the that can be used in terms of the rotational spectroscopy as well as the electron paramagnetic resonance spectroscopy. So both the radio waves as well as the microwaves are of very-very low energy radiations and both are can be used to study the rotations within the molecules.

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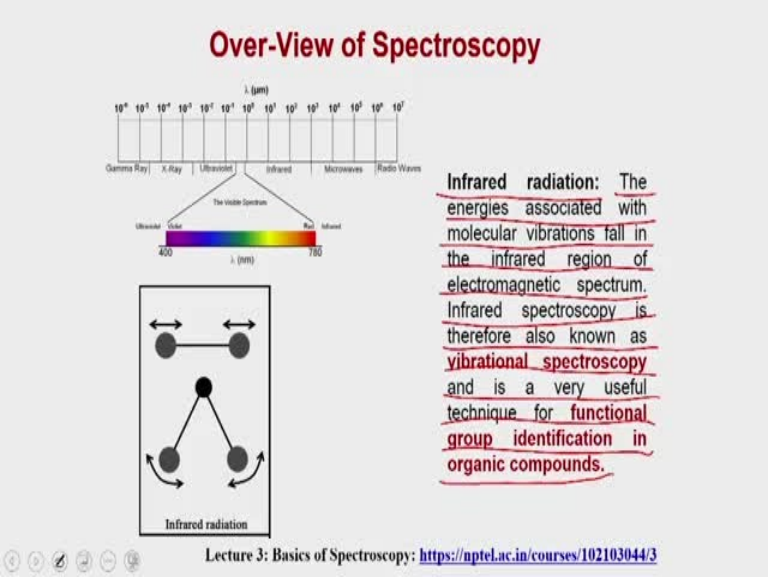

Then the third is the infrared radiations. The infrared energy associated with the in IR radiation is actually falling within the vibrational or the molecular vibrational studies and that is why the infrared region of the electromagnetic spectrum. So infrared spectroscopy is therefore also known as the vibrational spectroscopy and it is very-very useful technique for functional group identifications in the organic compounds.

So the IR radiation is going to study or IR radiation has the energy which actually can be mapped within the vibration. Which means how the molecules is how the atoms which are bound to each other are vibrating? Which means either the molecule will vibrate like this or the molecule will vibrate like this or molecule will or molecules are vibrating like this. So there are different types of vibrational moment which are happening when you are at, when you are connecting the two atom with a bond or three atoms with a bond.

So you can imagine that if I am a central atom I can have the different types of movements within the two molecules, it could be symmetric molecules, symmetric rotations or it could be asymmetric rotations or it could be symmetrical vibration or it could be asymmetrical vibration. It could be rotational vibration and on and so on. So all these different types of vibrational things we are going to study anyway when we will take up the IR spectroscopy.

But this is very-very specific in terms of the molecule as well as the kind of functional groups are present. So if you change one atom or if you change the central atom the it is going to affect the

vibrational frequencies of these other atoms and that is how it is actually going to affect in terms of in what region of the IR radiation it is going to absorb the light. And that can be mapped to tell you that okay this particular molecule contains carbonyl functional group instead of the aldehyde or alcohol or so on.

So that can be done because the alcohol is going to have its own vibrational energy, the amine may have its own vibrational energy and so on. And all these are been characterized so that can be used to map or at least can be able to use to predict whether this particular type of functional group is present in your compound or not.

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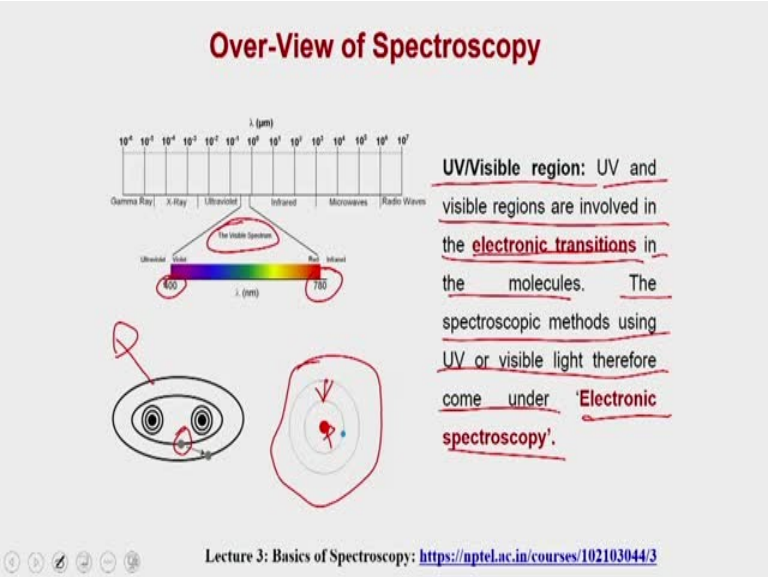

Now the fourth is the UV visible region. So UV visible region as I sat is the visible spectra, so it starts from the 400 nanometer and it goes up to 780 nanometer and UV visible region are involved in the electronic transitions, in the in the molecules. The UV spectroscopy method using UV or visible light thereafter comes under the electronic spectroscopy.

So what it means is that it is actually going to study how the atoms, how the molecules are having the electronic transitions? Which means, how the electron is making a transition within the atom? How it is going from the lower energy state to higher energy state and higher energy state to lower energy state? And if you want to study that that can be used and that can be studied with the help of UV visible spectroscopy.

And that is actually going to have an effect in terms of what is attached to this particular atom. Which means it is actually going to give you indirectly going to give you the information who is the neighbor present next to this particular type of atom which is showing this phenomena. Which means suppose you have carbon and hydrogen it may give you some different types of absorption pattern. If you have the carbon and oxygen it may give you different and that is how you can be able to use the UV visible spectroscopy also to characterize the molecule as well.

So this is exactly the same, the electronic transition means that the atom the electron is switching from high energy state to lower energy state and from the lower energy state to higher energy state.

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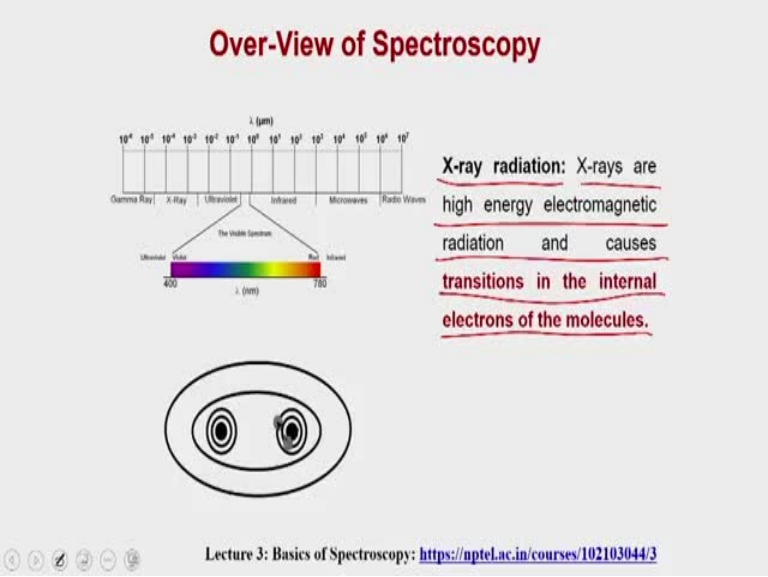

Then it comes to the x-ray radiations. So x-ray are very high energy electromagnetic radiations and they cause transitions in the internal electron of the molecule which means the electrons which are present within the atom and the electrons which are not present in the valence shell those are the atoms which are going to receive the x-ray radiations and that is how they are going to give the transitions and that is how they can be mapped using the x-ray radiations.

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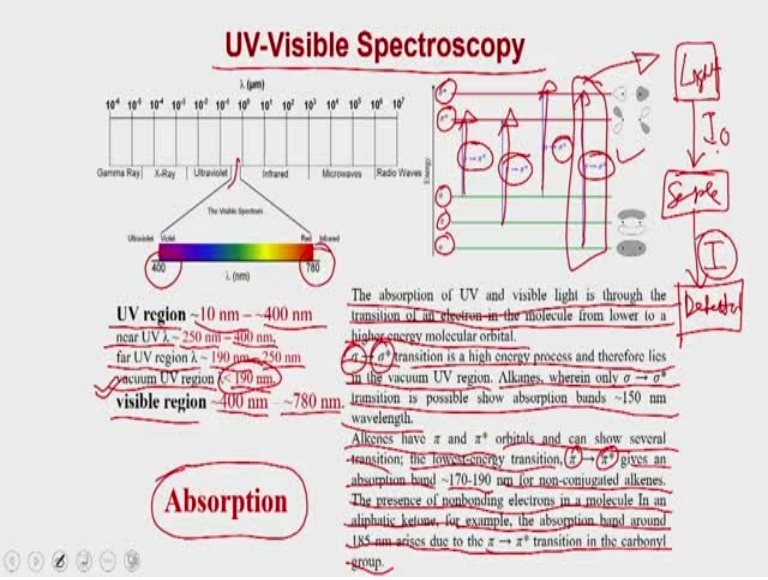

So we first star with the UV visible spectroscopy, so UV visible spectroscopy starts from the, so they has a visible range which starts from the 400 to 780 nanometer and within that before to the visible range you have the UV range which starts from the 10 nanometer to 400 nanometer, this UV range is been further divided into that three different components. One is called near UV which is from the 250 nanometer to 400 nanometer then you have the far UV which is from 190 to 250 nanometer.

And then you have the vacuum UV which is actually less than 190 nanometer whereas the visible range is from the 400 nanometer to 780 nanometer. The absorption of a UV and visible light is through a transition of an electron in the molecule from lower to a higher energy orbitals. So you can imagine that you have the sigma, Pi n, pi star and sigma star and all these are the different energy states from where the electron actually can go for higher transitions.

So if the electron goes from n to Pi star it will have certain amount of energy. If it goes from the Pi to Pi star it is going to have a different set of energy and if it goes from n to sigma star then it is going to have another energy or if it goes from sigma to sigma star then it is going to have different type of energy. So the sigma to sigma star transition which means this interaction is a high-energy process and therefore lies in the vacuum UV range.

Which means this is actually is the maximum transition possible which means from the lowest energy it is going to the highest energy and that is why this particular type of transition requires

very high energy which is actually within the vacuum UV range because you can see that the wavelength is very-very low.

And as you might have seen in the initial formula that E is equal to H C by lambda, so as the lower the wavelength the higher will be the energy. S if it, there will be a transition from sigma to sigma star then this is the higher energy process. Alkanes wherein only sigma to sigma electron transition is possible show absorption band around 150 nanometer. Whereas alkanes having Pi to Pi star orbitals and can show several transitions.

The lowest energy transition which is from Pi to Pi star gives an absorption band around 170 to 190 nanometer for a non-conjugated alkane whereas the presence of non-bonding electrons in a molecule. In an aliphatic ketone for example, the absorption band around 185 nanometer arises due to the Pi to Pi star transition in the carbonyl group.

So what you can see is that UV visible spectroscopy actually works on the principle of that the molecule is absorbing the light, going to the higher energy state and depending upon the type of the orbital are present in that particular molecule the it will absorb the energy as per the that particular specific transitions. But UV visible phenomena actually works on the basic principle of absorption. So if you have to make a device which actually can map the absorption phenomena you might have to need the couple of set of components.

So what you need actually is you need a light source, right. If I have to prepare an instrument which could be able to map this phenomena or which could be able to tell me that whether the molecule is absorbing or not? What I need is, I need a light source, I need a something so that where I can keep my samples. So I need a sample holder and then I need something so that it actually will tell me that how much is the absorption.

Which means suppose I am providing I0 as the initial light and suppose I got the I light it will should tell me that how much the i is lower to the i0 and that is actually going to be used to calculate the absorption of the light by this particular molecule.

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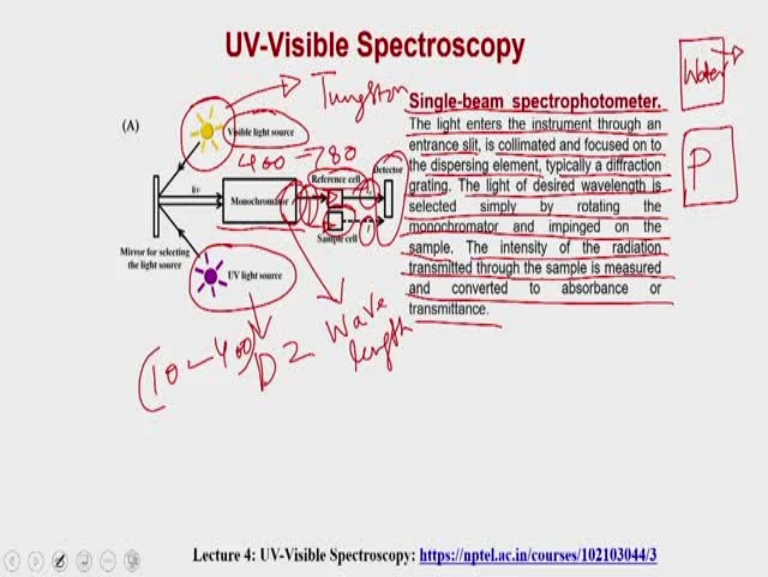

So the simple UV visible spectrophotometer could be a single beam UV visible spectrophotometer where what you have is a light source as I said you know we would need a light source. So you can have the two light source, one for the visible light source and another one is for the UV light source. So in the visible light source you can have a simple tungsten bulb and the bulb what you have in your home right, tungsten lamp or tungsten bulbs.

Whereas the UV light you can have the D2 lamps or Xenon lamps then what you need is so they both are going to give you the lights depending upon what wavelength or what region you are going to select. If you select the 400 to 780 then the only this bulb is going to be used for giving you the light. But if you use the phenomena from 10 to 400 then you are going to use the UV light. Then next two that you are going to have a monochromator.

The monochromator is going to give you the flexibility to use a particular wavelength so or a set of wavelength, so monochromator actually can scan or can give you the range of 190 to 400 or 400 to 600 or whatever the range you would like to select. Then next to that you are going to have the reference cell or the place where you can keep the samples. So you can have the reference cell as well as the sample cell and then you are going to have the detector, so detector is going to tell you that what is the I and what is I0 and what is the intensity of I and what is the intensity of I0.

So if you use those two phenomena you can be able to tell you that what is the amount of light is been absorbed by the sample. So light enters in the instrument through an entrance slit is collimated and focused on to the dispersing element typically a diffraction grating right, so monochromator could be of diffraction grating, monochromator could be a simple prism you might have seen a prism when you might be using it in your 9 standard or high school or you might be using it for you know where when you can actually split the sunlight into the seven lights and those kind of experiments.

So the monochromator could be as simple as a prism or at complicated as like monochromator gratings. The light of the desired wavelength, so the monochromator will give you a light of a desired wavelength simply by rotating the monochromator and (integrating) imprinting the samples. The intensity of the radiations transmitted through the sample is measured and converted to the absorbance or the transmittance.

But there is a problem with the UV visible single beam UV visible spectroscopy is or spectrophotometer is that it either because this is a single beam, so it actually either measures the reference sample or your test samples it does not measure the both at the simultaneously. What is mean by the reference sample is, suppose you have taken a protein solution in water. So the water is going to be a reference sample whereas the protein solution is going to be your test samples.

But if you have a single beam spectrophotometer what you are going to do is, you are going to first measure the water and then you are going to take out and use the your test sample which is protein. But in between if there will be any fluctuation in light or if there will be any fluctuation in the intensity of the light then it actually going to give you the artifacts because it will not going to adjust, because suppose the water is having some absorbance that actually it is going to convert it in terms of that okay this is 0.

But so the water absorbance it will consider as 0 and in reference to that it is going to give you the absorbance for the your protein sample. But if in between the intensity of the light is going to be reduced or increased then it is going to give you the artifacts. For example, if you do not have suppose at time 0 the water is showing an absorbance of 0.2, so the 0.2 becomes 0. But at time x if the water absorbance itself is 0.3 then there will be an error of 0.1 unit and that actually could

be very-very significant when you are actually making a suppose you are calculating the consultation of that particular protein using the UV visible spectroscopy then it is going to be a major problem.

So avoid that people have started developing or they, so to avoid that what you can do is you can have the double beam spectrophotometer. What you can do is you can just split the beam here into 2 part, one will go to your sample thing and one will go to the reference (())(36:23) so irrespective of whatever happened to your intensity, it will actually going to affect the both. So in that case the background subtraction is going to be more and more accurate.

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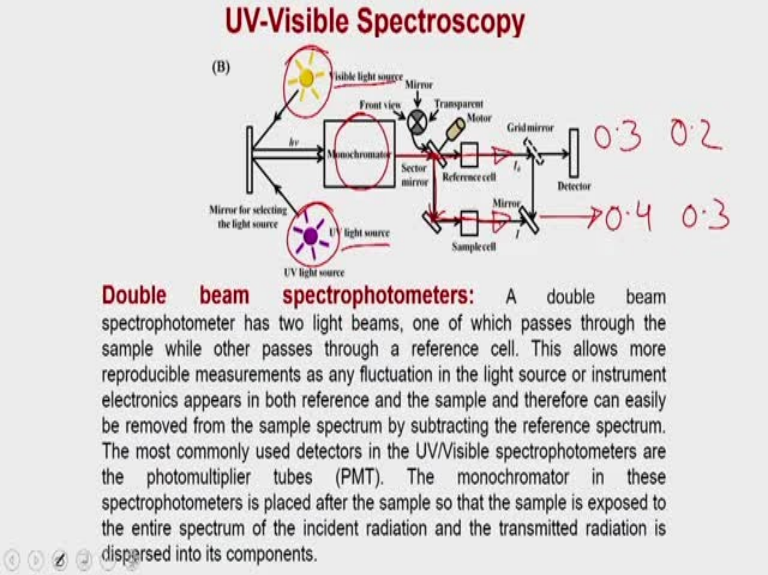

So this is what is exactly going to happen, so you have in the double beam spectrophotometer you are going to have the visible light, you are going to have the UV light source then you are going to have the monochromator and the next to the monochromator you are going to have a splitter, so that splitter is going to split the single beam into the two beams. So one is going into the test sample, the other one is directly going into the reference sample and now that problem of that reference sample is not available for the test sample to correct its absorbance it is not there.

If you, if there will be if it is in the beginning it is 0.3 and later on it becomes 0.2 then the sample also if suppose initially it was 0.4 then it becomes 0.3. So because in online times, in live time itself because it is measuring both it is actually can do the correction on its own because with the help of software you can ask that okay (keep select) keeps subtracting the reference (())(37:45)

reference sample for my test sample and keep giving me the values. And that will be going to be very-very precise.

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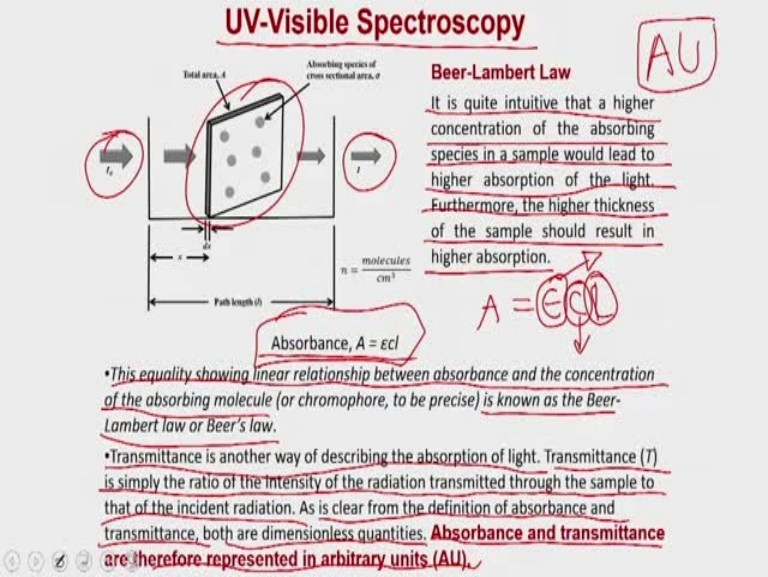

So you can imagine that you have exposed a sample with the light I0 and after the light is been passed through that sample you got the intensity which comes out as I, okay so if you try to calculate the absorbance that absorbance under the phenomena that absorbance is directly proportional to the epsilon CL, so absorbance is directly proportional to epsilon C and L and where epsilon is the molecular absorbance coefficient, the C is the concentration of the molecule but you have taken in this particular (())(38:40) and L is the path length of that particular (()) (38:44).

This equation showing linear relationship between absorbance and the concentration of the absorbing molecules is known as the Beer Lambert law or the Beer's law. And it is quite intuitive that a higher concentration of the absorbing species in a sample would lead to the higher absorption of the light. Furthermore the higher thickness of the sample should result in the higher absorption and as a result the absorbance is directly proportional to the epsilon CL and that actually can be used to calculate the absorbance of a particular sample or at a given wavelength or in a range of wavelength.

Transmittancy, so absorbance is one phenomena what is the amount of light is been absorbed the transmittance which is actually is another way of describing the absorption of light the

transmittance is simply the ratio of the intensity of the radiation transmitted through the sample to that of the incident radiation. It is clear from the definition of absorbance and transmittancy both are dimensionless quantities. Which means whether it is absorbance or transmittance both are dimensionless because both are actually the ratio, one in one case it is the ratio of the light which is been absorbed versus the total light or in other case it is the light which is the passed through the.

So but in both the cases you are actually subtracting the initial value from the final value and that is how it is actually a ratio, so that is why either absorption or absorbance or the transmittance does not have any dimensions or any unit. And that is why the absorbance and transmittance are represented in a arbitrary unit that is called AU. Which means, these values have no real sense because it can be any value it can be because these are arbitrary unit so there is no unit, so it can be just say 0.2, 0.3, 0.4 that is all arbitrary units.

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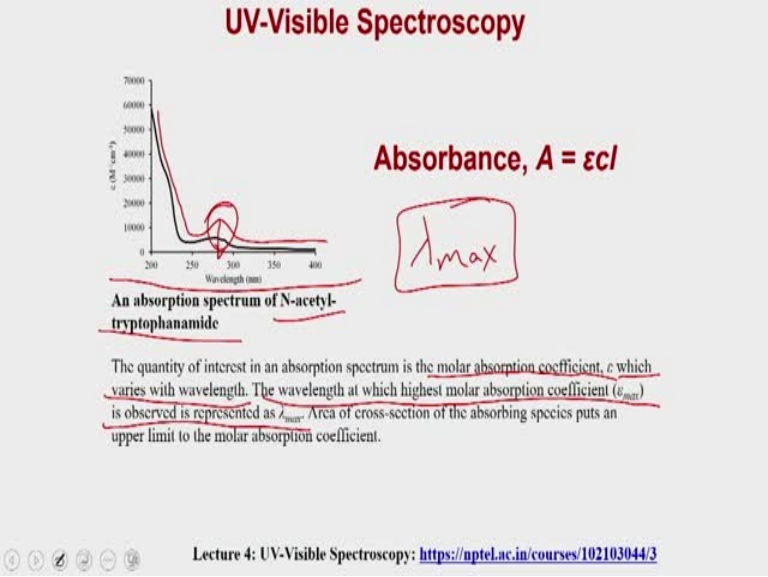

Now this is a typical absorption spectra what you see is the absorption spectra of N-acetyl l tryptophanamide and what you see is that the molecule is going like this and then it is showing you a peak at this wavelength. So at this wavelength whatever they absorption it is going to give you that is called as the molar absorption coefficient epsilon which varies with the wavelength. So at the lambda max whatever you are going to get the molar absorption coefficient that is the absorption coefficient which people are normally use.

So the wavelength at which the highest molar absorption coefficient is absorbed is represented as the lambda max and that is the molar absorption coefficient mostly people use because that is actually going to give you the more accurate values.

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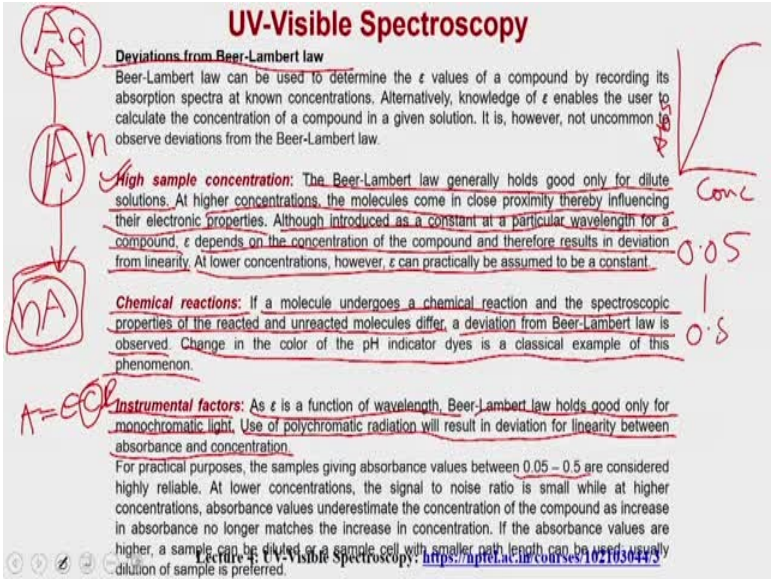

Now, because the Beer Lambert law is there, there are deviations from the Beer Lambert law. There are some conditions under which the molecule does not follow the Beer Lambert law. What are these conditions? High sample concentration, so you can imagine that if I calculate if I plot the absorbance versus concentration of a molecule it will go to the linear to some extent after that it is going to get saturated.

So the Beer Lambert law generally hold only for a dilute solution. At higher concentration the molecule comes in close proximity therefore influencing their electronic properties. So when you make the solution of very-very high concentrated solutions the molecule crowd because of the molecular crowding one molecule is going to start affecting the electronic transitions in the another molecule. And because of that it is actually going to give you very high absorbance which is not actually equivalent to as per the Beer Lambert law.

Which means it is not going to follow A equal to epsilon Cl equation and although introduced as a constant at a particular wavelength for a compound epsilon depends on the concentration of the compound and therefore results in deviation from the linearity. So because of that molecular

crowding the epsilon is also getting affected and because of that you are going to get not the linear relationship between the absorbance versus the concentration.

And as a result it is going to give you the deviation, either it is going to give you a plateau or it may give you a very high readings. That is why it is recommended that you should actually do a measurement at that diluted concentrations. Then you have the second thing is chemical reaction, if the molecule undergoes a chemical reaction and the spectroscopic properties of the reacted and the unreacted molecule differ then you will see a deviation from the Beer Lambert law is going to observe.

The change in the color of the pH indicator dye is a classical example of this phenomena. So what will happen is, if you are actually following a particular molecule which is colorless so it is going to give you some absorption phenomena but suppose in the change of pH it changes its color then it is going to give you different absorption phenomena and in that case it is not going to follow the Beer Lambert law.

Apart from that chemical reactions, if a molecule suppose you have a molecule a either it goes for polymerization reactions or it goes for the disintegration reaction which means either the A get broken down into more number of A's or A get into the polymerization that also is going to be give you the deviation from the Beer Lambert law. Because as you see that A is directly proportional to the epsilon in Cl right, if the A which is actually the N molecules will become one molecule then the concentration of the particular A is going to be reduced by the N times.

Similarly, if the A is going to be polymerized and will give you A N then also there will be a change in concentration. So that actually also going to give you the deviation from the Beer Lambert law. The third is the instrumentation factor, as epsilon is a function of wavelength the Beer Lambert law holds good only for a monochromatic light. Use of a polychromatic radiation will results in the deviation of linearity between absorbance and concentration. That is why it is recommended for the practical purposes that you do a measurement only in the range of 0.05 to 0.5.

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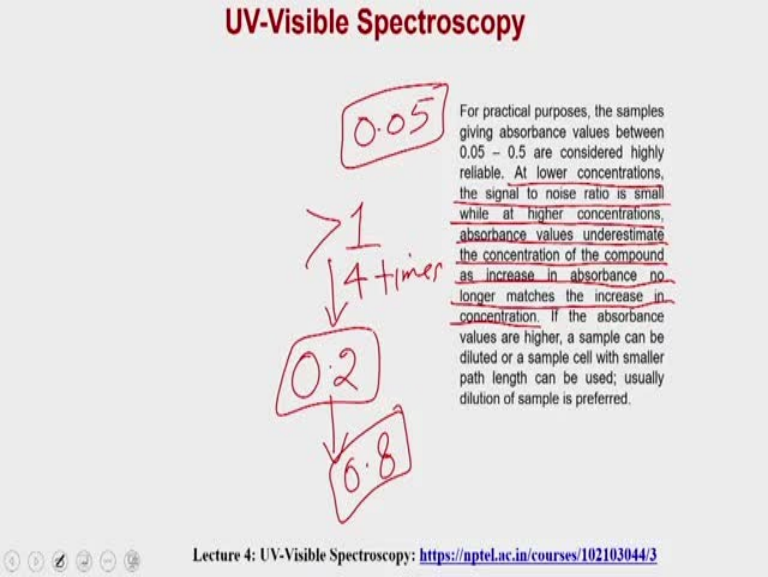

At lower concentrations, the signal to noise ratio is small while at a higher concentration the absorbance value under estimate the concentration of the compound as increased in absorbance no longer matches the increase in concentrations. So what you have to do actually? If you have an absorbance of a sample which is less than the 0.05 in that case you might have to concentrate the sample and increase in c but suppose you have the value which is above to 1 then what you are going to do?

You are going to dilutes it to 3 times, 4 times and then you are going to measure if it gives you an OD of 0.2 for example I have diluted this 4 times and I got the OD of 0.2 this means the effectively the original OD is 0.8 and that is how you are going to resolve or that is how you are going to be accurately measure the absorbance of that particular compound. So do not go with the very low readings, for example if it is showing 0.0002 that is also not reliable. If you go by 1.2 that also is not reliable.

So you try to measure the absorbance in the range of 0.05 to 0.5 because above to this value and the lower to this value you are going to see a deviation from the Beer Lambert law.

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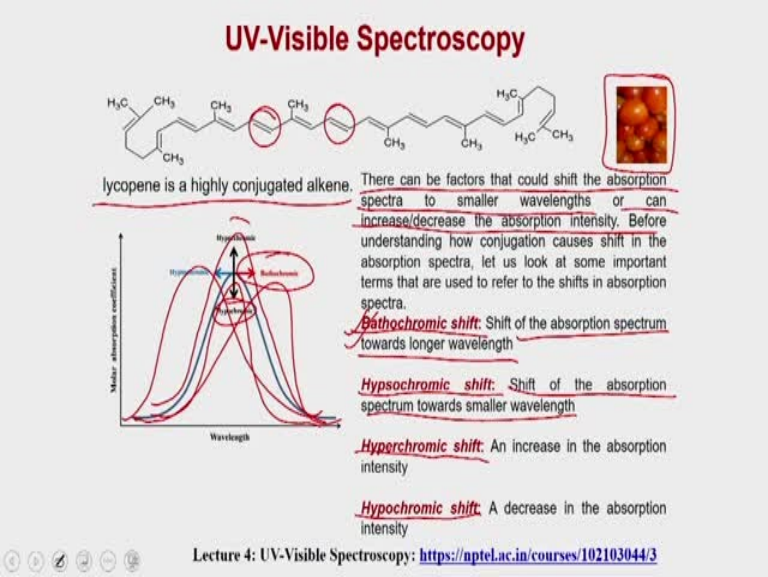

Let us take an example, so this is a simple example of a lycopene which is a highly conjugated alkene. So what you see is the double bond conjugated molecule and a lycopene is a natural product which is present in the tomato for its red color. And it has because of this highly conjugated alkene like structure it actually can give you or it can have different factors. So there are factors which can actually shift the absorption spectra to a smaller wavelength or can increase or decrease the absorbance intensity.

So what are these? So these phenomena are can be described in a different way. So when you see a shift in the absorption spectra when you see increase and decrease for all these phenomena there is a specific terminologies what people use, so what are these terminologies? You can have the Bathochromatic shift which means the shift of the absorption spectra towards the longer wavelength, so if you have a bathochromic phenomenon there will be a shift like this.

Then you have the hypsochromic (())(49:17) which means shift of the absorption spectra towards the smaller wavelength which means it is going to shift like this. Then you can have the hyperchromic shift which means the absorbance will go up or you can have the hyporchromic shift which means the absorbance will going to go down. Which means in all these phenomena in one case your lambda max is changing towards the longer wavelength which is the bathochromic shift, the other one is the hypsochromic, so in the hypsochromic shift the lambda max (will) shift towards the near wave towards the shorter wavelength.

Which means it is now the compound is start absorbing at lower wavelength. So if the compound is started at higher energy or it can actually decrease the absorbance which means it is going to have the masking effect or it is going to have the stimulatory effect. So you can have the hyperchromic as well as the hyporchromic effects.

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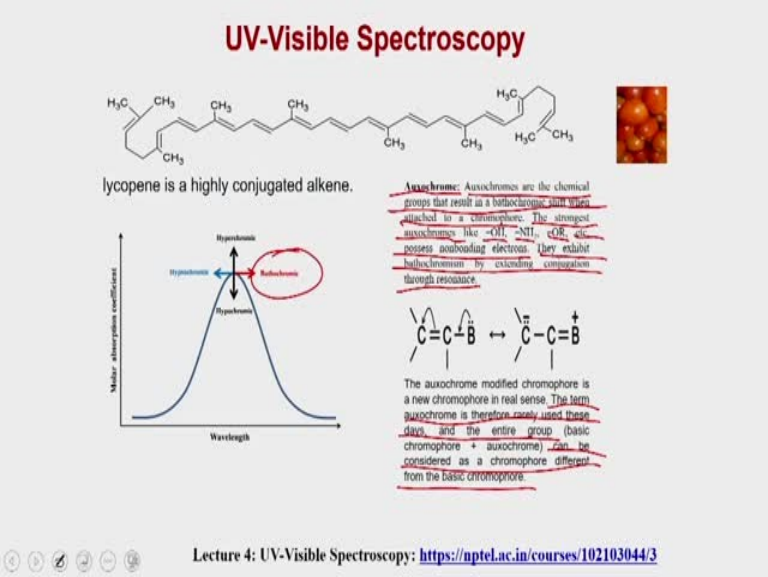

Apart from that you have a auxochrome. So auxochromes chemical group that result in a bathochromic shift which means it is going to shift towards the longer wavelength attached to a chromophore the strongest auxochrome like hydroxyl groups, amine groups or etc versus non-bonding electrons they exhibit bathochromic by extending conjugation through resonance. The auxochrome modify chromophore is a no chromophore in real sense the term auxochrome is therefore really used for these days and the entire group can be considered as a chromophore different from the basic chromophore.

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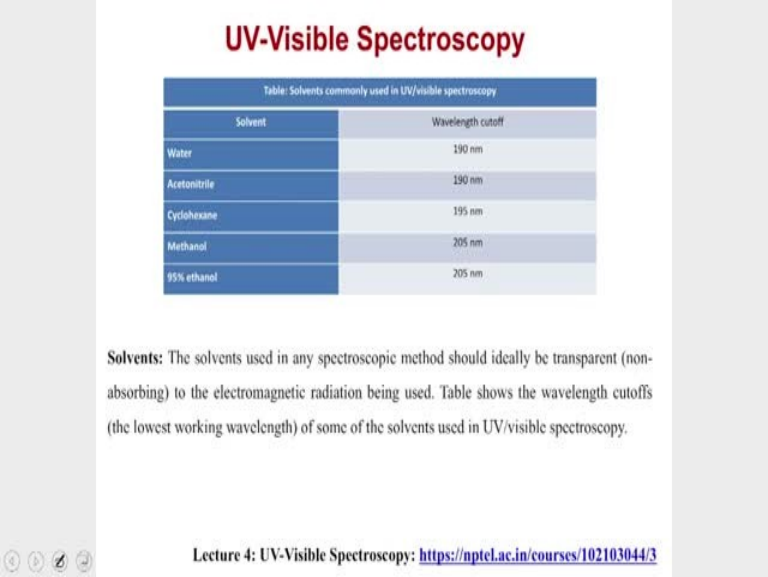

Apart from that when you do a UV visible spectroscopy you actually measure the absorbance or the transmittancy in a particular solvent system and ideally the solvent system should not absorb, it should be completely transparent, it should not absorb into the particular wavelength at where you are working a particular range of wavelength, water does not have any absorbance in the visible range that is why the water is the most suitable solvent for the visible range.

So these are the solvents what you can use in the different wavelengths. For example, the water, water can be used anytime which is above to this 190 nanometer, similarly acetonitrile, cyclohexane, methanol and 90 percent alcohol they all can be used above to this wavelength they cannot be used at lower to this wavelength because lower to this wavelength the molecules are itself are going to start absorbing very strongly.

So if you have water or the solvent which is going to start absorbing very strongly then it is going to mask the absorbance which is coming from your own sample. And that may actually give you the deviation from the Beer Lambert law that may give you some different types of artifacts. Apart from that you can have the biological chromophore.

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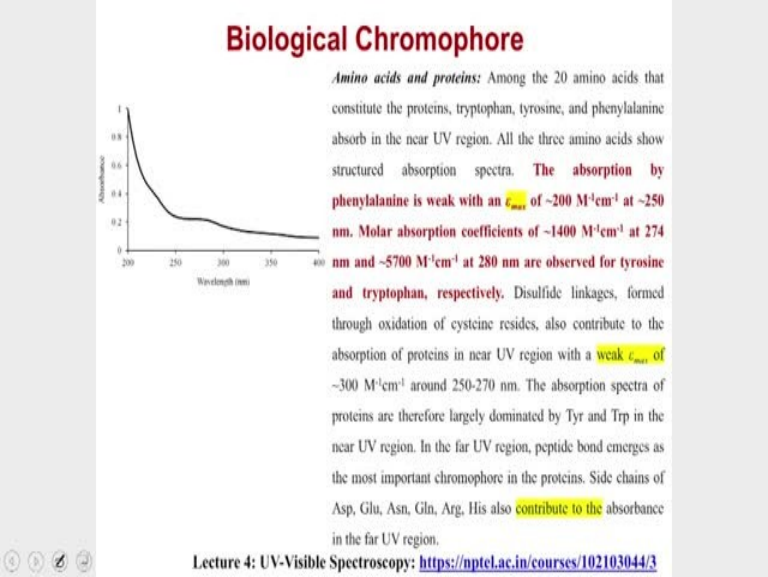

So far we have discussed about the small molecule chromophore you can have the biological chromophore, classical example is the amino acids. So the protein is made up of 20 amino acids among them tryptophan, tyrosine and phenylalanine absorbs in the near UV range all the three amino acid show the structured absorption spectra. The absorption by the phenylalanine is weak with the lambda max of 200 per mole per centimeter at 250 nanometer whereas the molecular absorption coefficient of 1400 at 274 nanometer and 5700 at 218 nanometer are observed for tyrosine and tryptophan respectively.

Similarly, you can have the disulfide linkages which are formed by the cysteine and that also is going to give you the absorption and that can be used as a chromophore within the proteins. So the disulfide linkages formed through the oxidation of cycteine residues also contribute to the absorption of protein in near UV range with a week epsilon maxima of 300 around 250 to 270 nanometer.

The absorption spectra of protein are therefore largely dominated by tyrosine and tryptophan in the near UV range, in the far UV range the peptide bond emerges as the most common important chromophore in the protein. The side chain of the aspartate, glutamate, asparagines, glutamine, arginine and histidine also contributes into the absorbance in the far UV range. So this is all about the protein apart from the protein you can have the nucleic acids.

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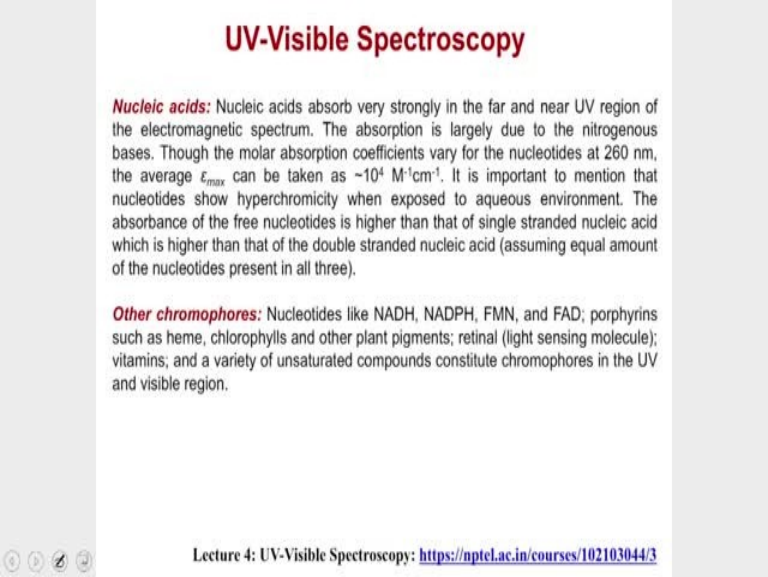

So nucleic acids also absorbs very strongly in the far and near UV range of the electromagnetic spectrum. The absorption is largely due to the bases right. It has a adenine, guanine and all those kind of nitrogenous bases, so those bases are actually absorbing very strongly in the UV range so the molecular absorption coefficient vary for the nucleotide at 216 nanometer, the average lambda max can be taken as 10 to the power 4. It is important to mention that nucleotide show hypochromicity when exposed to the aqueous environment.

The absorbance of the free nucleotide is higher than that of single standard nucleic acid which is higher than that of double standard nucleic acid. So because the nucleotide actually shows the hyperchromicity which means it is going to give you the increase in the absorbance that phenomena is always been used to follow the unwinding of a DNA molecule because once the DNA unwinds the nucleotides are getting exposed to the water and that is how they are going to give you the higher absorbance or higher absorbance and that is how it is actually can be used to monitor the percentage of the DNA which is double bond or which is present in present in the form of double helix or in the percentage of DNA which is present in the form of single standard.

Because single standard DNA is going to give you more absorbance compared to the double standard DNA. Apart from that you have the other chromophore is like nucleotides, nucleotides like NADH, NADPH, FMN, FAD porphyrins such as heme, chlorophylls and other plant pigments, retinal so which is actually a light sensing molecules vitamin A and a variety of

unsaturated compounds constitute chromophore is in the UV as well as the visible range. So this is all about the UV visible spectroscopy, the phenomena of the absorption as well as we have discussed about the Beer Lambert's law and in the subsequent lecture we are going to discuss about the application of UV visible spectroscopy and subsequent to that we will also discuss about the IR spectroscopy. So with this I would like to conclude lecture here, thank you.