Spectroscopic Techniques for Pharmaceutical and Biopharmaceutical Industries

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Lecture 21 - UV – Vis Spectroscopy & its Applications - II

Hello friends, welcome to lecture 21 of this course. In the last lecture, we discussed about theory of UV visible spectroscopy and then we started to discuss applications of UV visible spectroscopy. I will continue with that and discuss more applications of UV visible spectroscopy.

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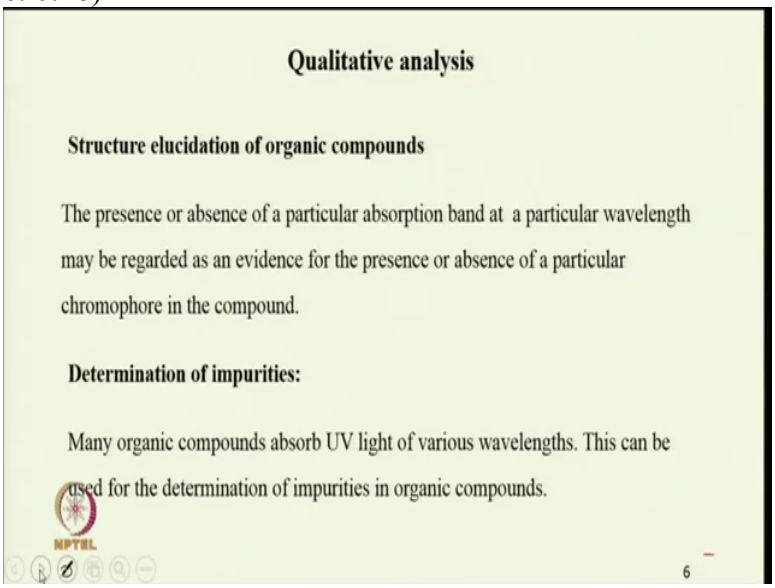

As I discussed in the last lecture, there are 2 very important applications of UV visible spectroscopy, one in qualitative analysis and another is in quantitative analysis. In qualitative analysis, we can do several different things and one of the most important thing is structural elucidation of organic compounds. When we are going for a structure elucidation of organic compounds, what we look for is the presence or absence of a particular absorption band at a particular wavelength. This band may be regarded as an evidence for the presence or absence of a particular chromophore in the compound.

So, just by looking at the band at a particular wavelength, we can tell about whether particular compound is present in this sample or not. The second important qualitative analysis is in the determination of impurities. So, whenever we are trying to synthesize a molecule or a drug, there is always a chance for different kind of impurities present in the sample. Impurities can be due to reactant or impurities can be due to side product, formation of side products.

Again, reactant and your side product which is present as impurity can have a characteristic wavelength, characteristic wavelength and based on that characteristic wavelength, we can tell whether a particular impurity is present or not. So, the different compounds absorb a different wavelength and that can be used for the determination of impurities in a sample.

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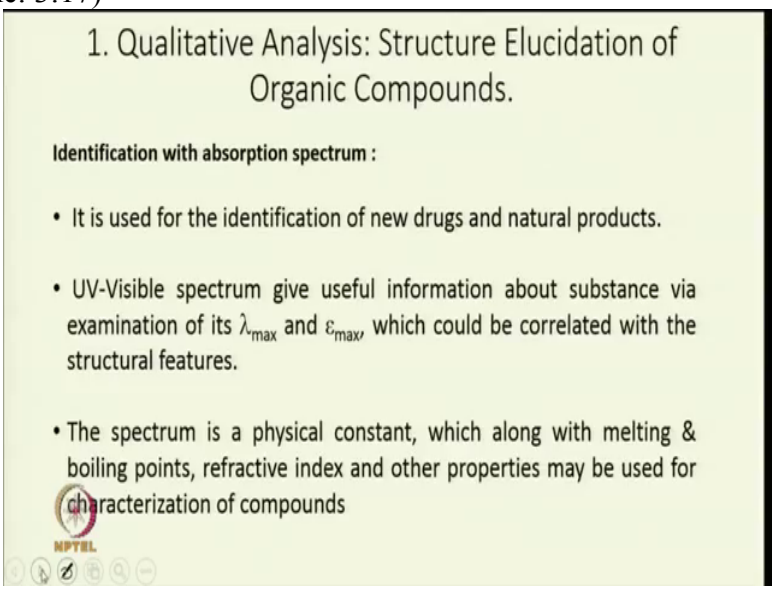

So, first application in qualitative analysis is a structure elucidation of organic compounds. So, UV visible spectroscopy is quite often used to look at the different organic compounds synthesized during a reaction, that is why it is generally used for the identification of new drugs and natural products. And as I told you, that the useful information about the substance can be obtained by looking at the lambda max and epsilon of the formed product. And based on that, we can tell which kind of structural feature is present in the particular sample. So, a spectrum along with melting and boiling point, refractive index and other properties can be used for characterization of a compound.

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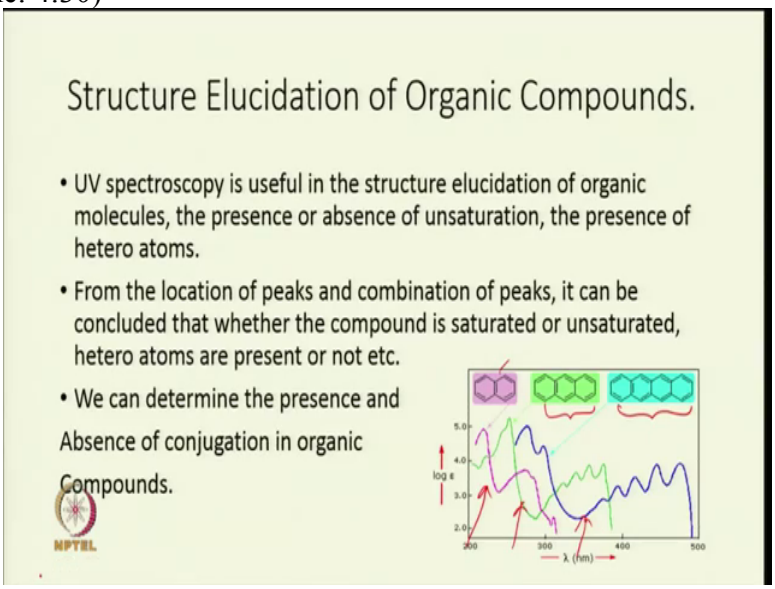

UV visible spectroscopy is particularly useful in the structural elucidation of organic molecules with conjugated double bond. So, a molecule with a conjugated double bond can be easily be identified using UV visible spectroscopy. Similarly, the presence of hetero atoms can also be identified using UV visible spectroscopy.

From the location of peaks and combination of peaks, it can be concluded whether the compound is saturated or unsaturated, whether hetero atoms are present or not. And that is why UV visible spectroscopy is quite often used during the synthesis. So, here is the 3 different aromatic compounds given and their characteristic UV visible spectra is also given. So, this is your phenolphthalein, this is the 3-benzene ring and this is with the 4-benzene ring and you can see that the characteristic spectrum of these 3 compounds are quite different, are quite different and that is why UV visible spectroscopy can be used to tell about the structural feature of the compounds.

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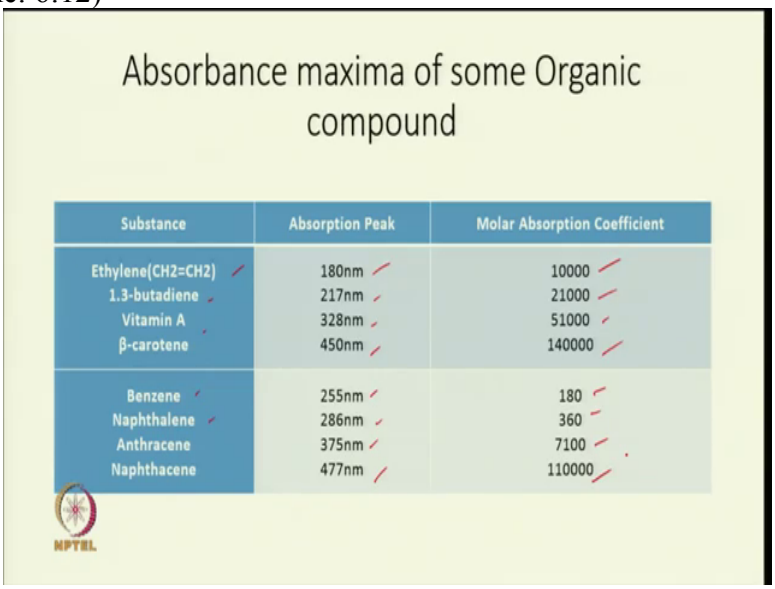

So, here is some example of absorption maxima of some organic compounds. Here first case ethylene, it has a peak around 180 nanometer and a smaller absorption coefficient is 10,000; 1, 3 butadiene 217 nanometer and epsilon value is 21,000. Vitamin A it absorbs at 328 nanometer and its epsilon value is 51,000. Beta carotene absorbs at 450 nanometer and its molar absorption coefficient is 1, 40,000. Benzene has absorption peak at 255 nanometer and epsilon value is 180.

Naphthalene has lambda max at 286 nanometer and its molar absorption coefficient is 360. Anthracene has absorption peak at 375 nanometer and its epsilon value is 7100. Napthacene has lambda max at 477 nanometer and this is its epsilon value. So, you can see that every compound has different lambda max and different epsilon value and based on that we can not only qualitatively see this is the compound we can also calculate concentration or amount of this compound using epsilon value and I will go to quantity analysis in a few minutes. So, but what you need to know is epsilon is very important when we want to get the concentration.

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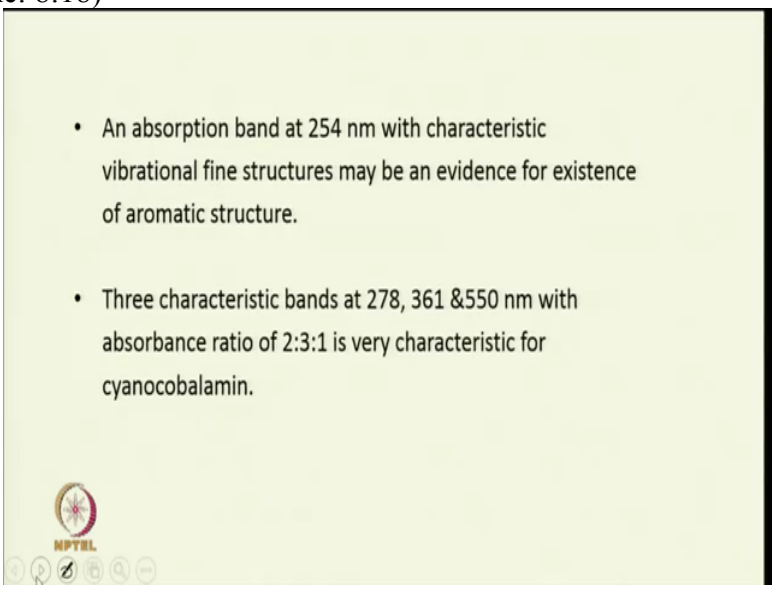

So, an absorption band at 254 nanometer with characteristic vibrational fine structure is an evidence for existence of aromatic structure. Other compound can also show their characteristic peaks. For example, cyanocobalamin has characteristic bands at 278 nanometer, 361 nanometer and 550 nanometer and their absorption ratio is 2 is to 3 is to 1, respectively. So, based on that you can identify the compound.

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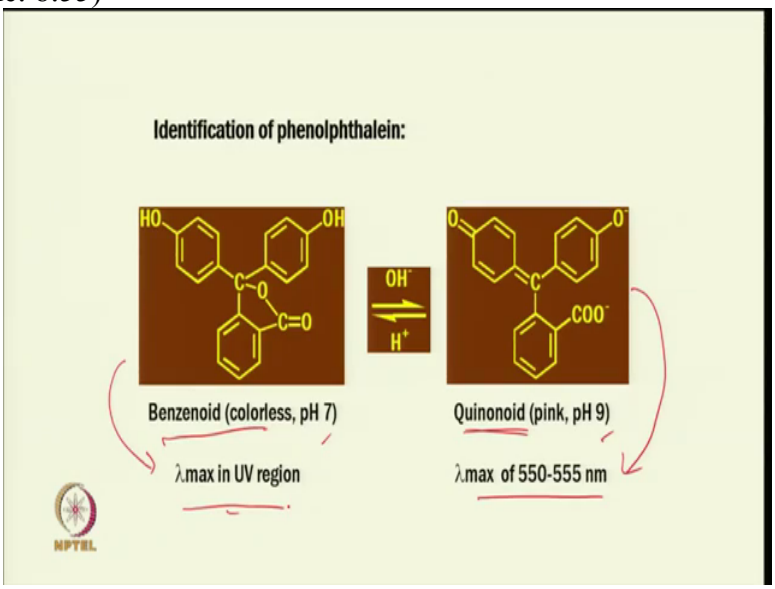

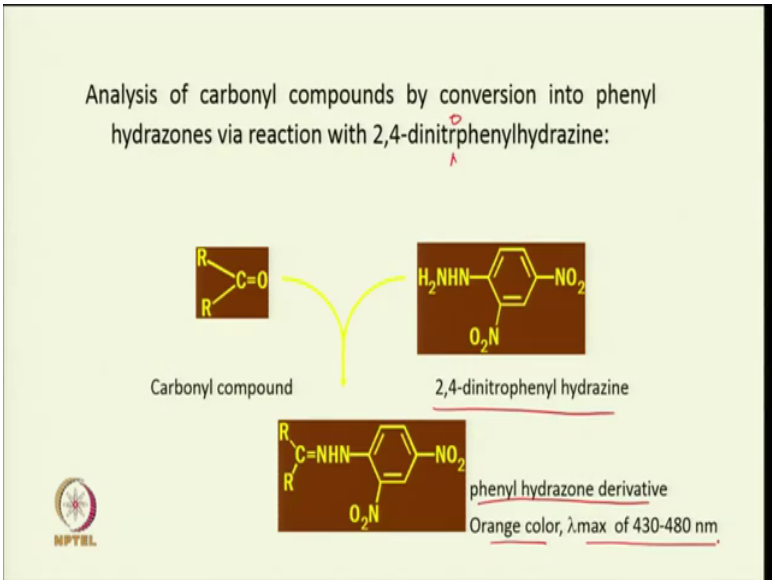

So, here is another case, for example, if you are looking at phenolphthalein, phenolphthalein is a mixture of 2 different species, benzenoid and quinonoid. One exists at pH 7 and other exists at pH 9 and this is colorless and quinonoid is pink. And based on acidic conditions or basic condition one of this form can exist or in between it can be a mixture, it can be mixture. The lambda max for this compound is in UV region whereas lambda max for quinonoid is in visible region and its lambda max is 550 to 555 nanometer.

Whereas for benzenoid, lambda max is in UV region. So, looking at the lambda max value you can identify not only the compound but you can also identify its different forms, its different forms. Sometime, we can identify a particular compound by converting it into a derivative which is colored, derivative which is colored. For example, if you take the carbonyl compound, they can be converted into phenylhydrazone via reaction with 2, 4 dinitro phenyl hydrazine.

And based on its absorbance, we can know whether carbonyl functional group is present in the molecule or not, molecule or not. So, this is your 2, 4 dinitro phenyl hydrazine and when it reacts with carbonyl compound it gives you phenyl hydrazone derivative which has orange color and lambda max of 430 to 480 nanometer. So, a compound which does not give color can also be identified, at least its functional group can be identified by reacting with a compound so that you get a product which has some color.

For example, in this case what we are doing is carbonyl compound, taking carbonyl compound and reacting it with 2, 4 dinitro phenyl hydrazine to get a colored compound which has lambda max of 430 to 480 nanometer.

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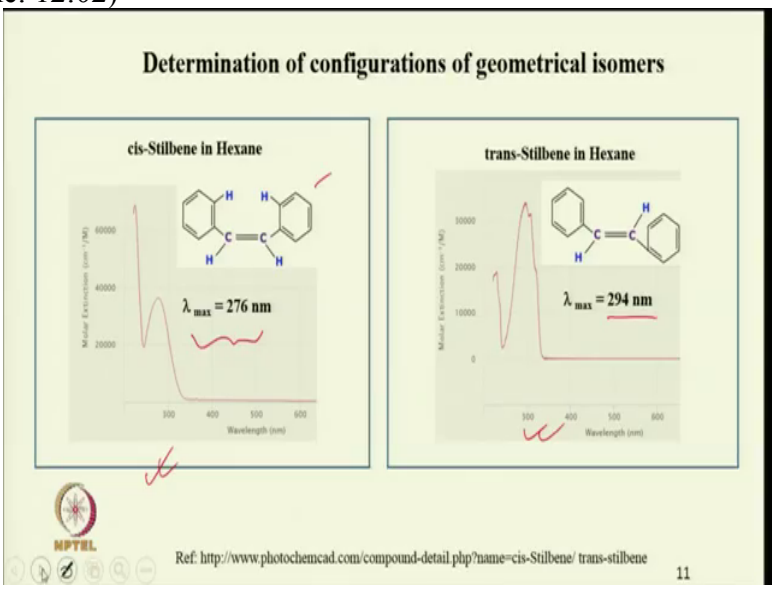

Not only that, we can also differentiate between different geometrical isomers or we can tell about configuration of geometrical isomers. For example, this is the case of cis-Stilbene and when you take a spectra of this compound in hexane what you will get is, this which has a lambda max around 276 nanometer. Whereas, if you take trans-Stilbene and try to obtain UV visible spectrum in hexane, what you will get is lambda max around 294 nanometer, lambda max around 294 nanometer.

So, just by looking at the positions of lambda max we can differentiate between different geometrical isomers, we can differentiate whether a particular compound is in cis form or in trans form. So, cis-Stilbene has lambda max of 276 nanometer whereas trans-Stilbene has lambda max of 294 nanometer.

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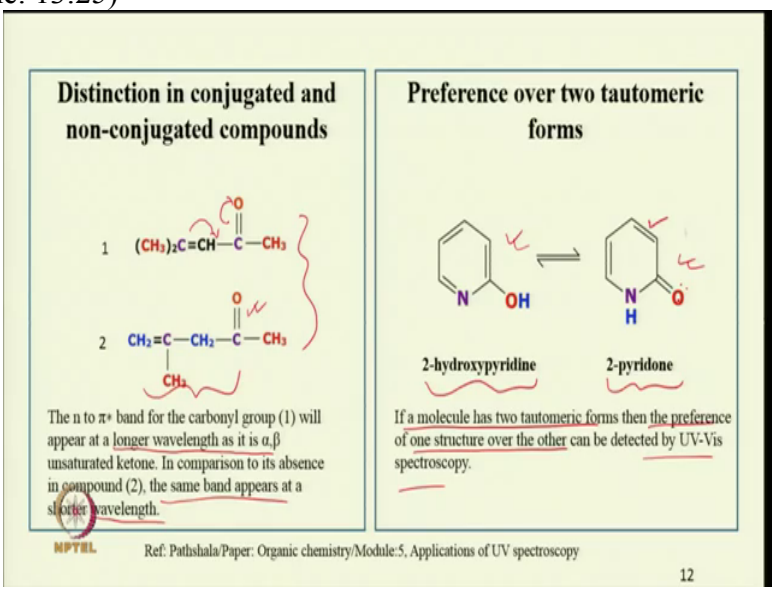

Distinction in conjugated and non-conjugated compound can be made. For example, here is the 2 different compounds. Here you see there is conjugation, there is a conjugation whereas in this case there is no conjugation, there is no conjugation. So, if we look at the spectrum of the compound 1 and 2, the n to pi star band for the Carbonyl group and the compound 1 will appear at longer wavelength as it is an alpha-beta unsaturated ketone.

So, since this keto group is in conjunction with this double bond and so, n to pi star band is going to appear at longer wavelength. Whereas in the compound 2 C double bond O this into pi star band for this compound is going to be at lower wavelength, since this C double bond O is not in conjunction with double bond and so, the band appears at shorter wavelength. Now, we can also use UV visible spectroscopy to look at which tautomeric form is present.

For example, you see the case of this versus this and so, between 2- hydroxypyridine and 2-pyridone these are the 2 tautomeric forms can also be distinguished using UV visible spectroscopy. So, in this case (2-tauto) then preference of 1 structure over the other can be detected by UV visible spectroscopy. So, here you see this lone pair n to pi star is again in conjunction with this double bond, this double bond. And so, just by looking at this we can know n to pi star band, we can know which tautomeric form is present in the sample.

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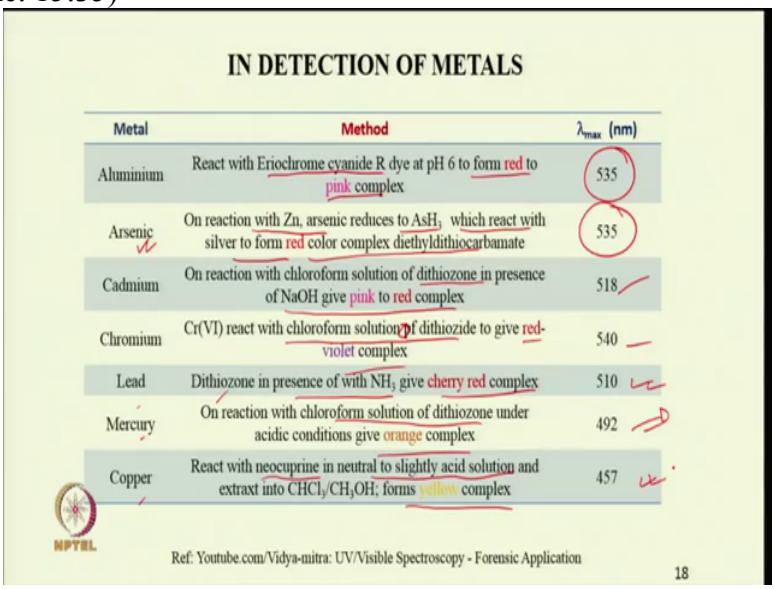

Now, UV visible spectroscopy can be used for the detection of metals. Here, we do not take a spectra of metals; what we do is we convert metal to a complex which absorbs in UV visible region. For example, aluminum can be identified by acting it with Eriochrome cyanide R dye at pH 6. What will happen is it will form a pink complex, and it will form a pink complex and lambda max is going to be 535 nanometer.

So, Eriochrome cyanide R dye is red and when it reacts with or when it forms complex with aluminum, it gives a pink complex which absorbs at lambda max of 535 nanometer. Similarly, arsenic can be identified by you know reacting it with zinc. What happens that arsenic reduces to AsH3 and this reacts with silver to form red color complex diethyldithiocarbamate, and it absorbs at lambda max of 535 nanometer.

Cadmium on reaction with chloroform solution of dithiozone in presence of NaOH gives pink to red complex. So, dithiozone is a pink complex, pink dye when it reacts with cadmium it gets converted to red complex and its lambda max is 518 nanometer. Similarly, chromium 6 it reacts with chloroform solution of dithiozide and it gives you a red violet complex which absorbs at 540 nanometer. Similarly, lead reacts with dithiozone in presence of ammonia to give you cherry red complex which absorbs at 510 nanometers.

Mercury on reaction with chloroform solution dithiozone gives you orange complex and its lambda max is 492 nanometer. Copper reacts with neocuprin in neutral or slightly acidic condition to form yellow complex which absorbs at 457 nanometer. So, just by reacting a particular metal with a dye which color changes when it reacts with metal and the metal dye complex absorbs at different lambda max, then we can predict the presence of metal in a solution, presence of metal in a solution.

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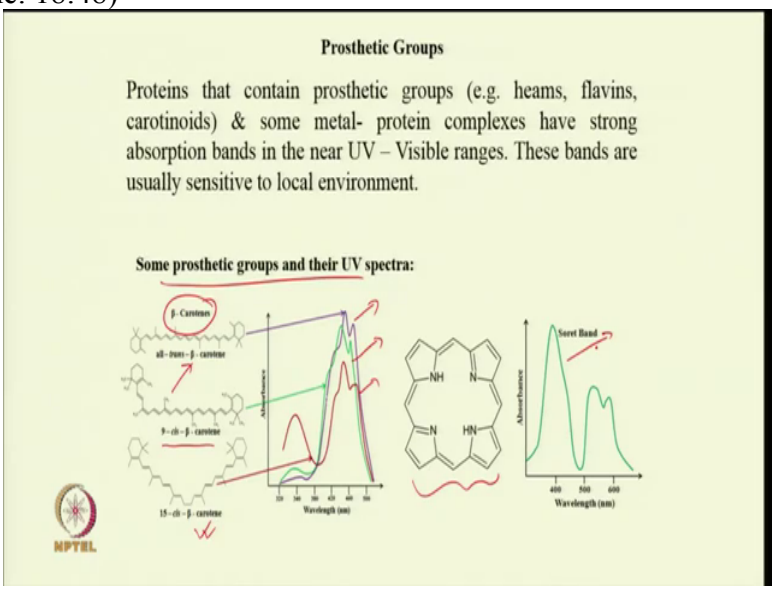

UV visible spectroscopy can also be used to identify different proteins, it is also quite useful for the proteins that contain prosthetic group. For example, heams, flavins or carotenoids. So, in that case, when there is a prosthetic group, some metal protein complexes have very strong

absorption band in near UV visible range and these bands are usually sensitive to local environment. And so, we can study the changes in protein happening.

Here is some prosthetic group and their UV spectrum. So, beta carotene can be in 3 different form when it has all trans beta carotene and you can obtain this kind of a spectrum. Whereas, if there is 9 cis beta carotene, then you will get this green spectrum and if you have 15 cis beta carotene, then you have this red spectrum. So, just by looking at spectra, you can also differentiate between different types of carotene. Heam is very important prosthetic group and it is presenting few proteins and those proteins can be characterized by looking at the absorption of this heam group, particularly solid band is quite useful. And that if you look at the solid band, and you can tell about the environment around the heam group.

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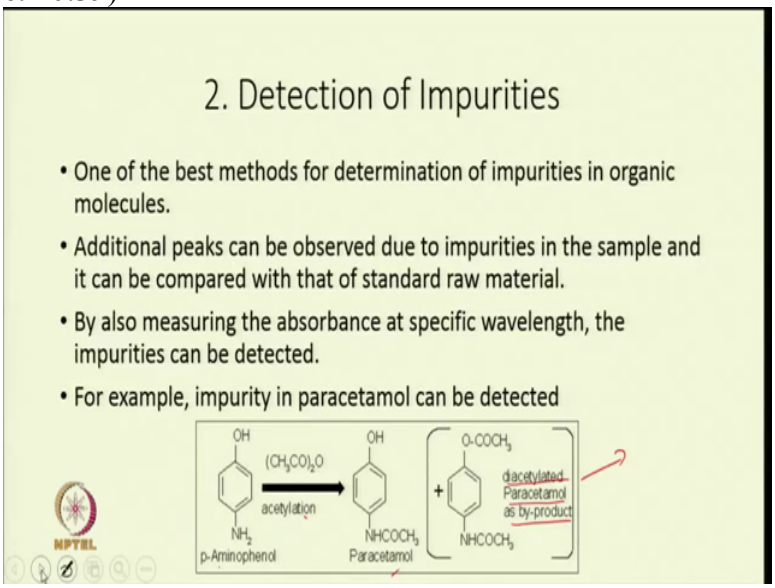

UV visible spectroscopy can also be used for detection of impurities. And basically it is one of the best methods for determination of impurities. Additional peaks can be observed which is due to impurities. And then if you compare with the standard raw material, you can tell whether that is impurity or not. You can also measure the absorbance as a specific wavelength and that can be used to identify impurities.

For example impurity in paracetamol is detected by using UV visible spectroscopy, this is the way in which paracetamol is obtained. So, this is para-aminophenol on acetylation gives you paracetamol and this is diacetylated paracetamol as a byproduct. So, impurities can be present as a byproduct or can be due to byproduct and our aim should be to remove that byproduct and for that it is important to know what is the amount of the side product and once you

purified then again you need to make sure that there is no byproduct left and for that you have to use UV visible spectroscopy.

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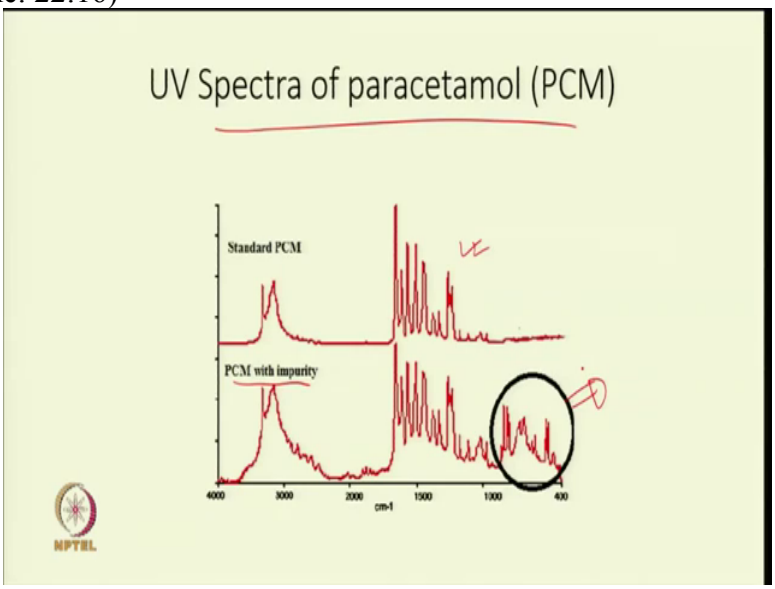

So, this is your UV spectra of paracetamol, PCM, this is the standard. And if PCM is presented with impurity then these extra peaks will be obtained and in the final paracetamol are the paracetamol which are going to be used as medicine, we need to make sure that these peaks are not present so that there is no impurities.

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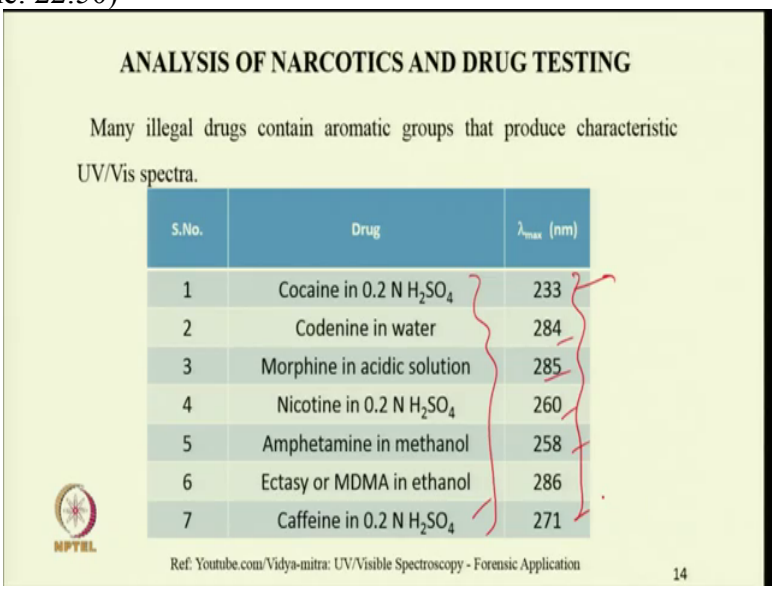

UV visible spectroscopy is quite often used for narcotics and drug testing. Many illegal drugs contain aromatic compound that produce characteristic UV visible specta. For example, these are the few drugs and corresponding lambda max value. Cocaine in 0.2 normal H2SO4, lambda max around 233 nanometer. Codenine in water has a lambda max around 284

nanometer. Morphine has lambda max around 285 nanometer. Nicotine has lambda max around 260 nanometer and amphetamine in methanol gives you peak at 258 nanometer. And similarly, caffeine in 0.2 normal H2SO4 gives you lambda max at 271 nanometer.

So, just by looking at this looking at the UV visible spectrum and identifying peak at a particular lambda max you can tell whether particular narcotics or drug is present in the sample or not.

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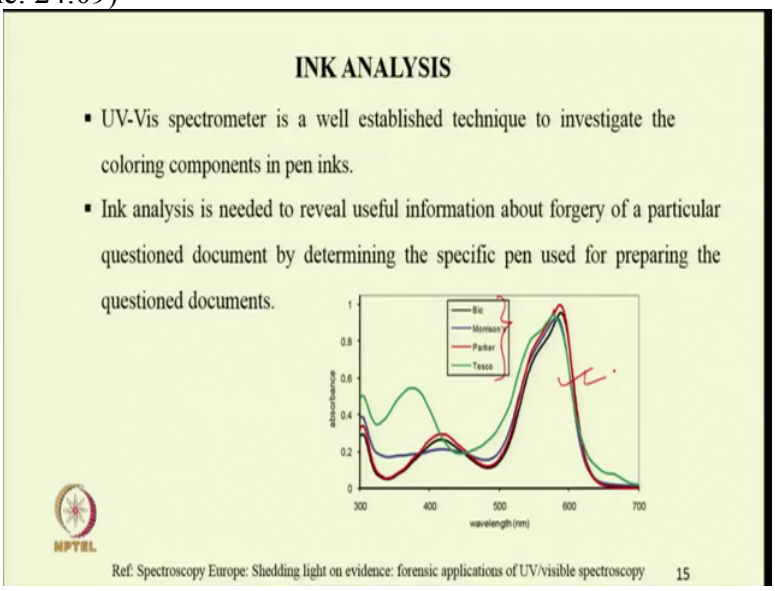

Ink Analysis can also be done using UV visible spectrometer, it is quite often used to investigate the coloring compounds in the pen ink. Ink analysis, as I told you in the first second lecture, it is needed to reveal useful information about forgery of a particular questioned document and what you try to look at is which kind of paint is used for preparing the questioned document.

For example, these are the 4 different kinds of pen and if you analyze their ink what you will get is the different spectrum and each of the spectrum is unique. What does that mean is all 4 inks used by different pens. For example, Bic or Morrison, or Parker or Tesco has different spectrum. And so, you can know which kind of ink is being used to write the document and just by looking at the ink you can know whether that document is fraud or not.

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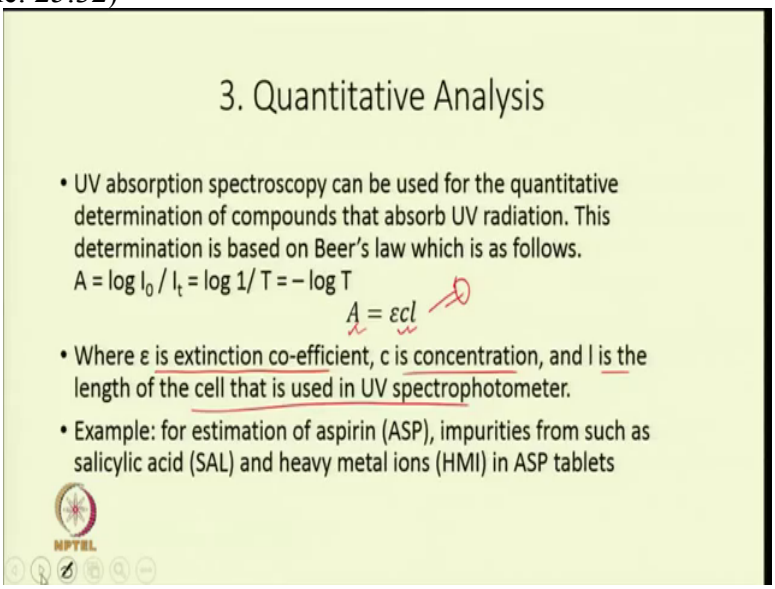

I have told you some example of you know qualitative analysis, there can be 100 different examples. And if time permits in the later lectures I will discuss more applications. But let us first see and look at other kind of applications of UV visible spectroscopy. And one of the most important application is quantitative analysis. So, UV visible spectroscopy not only used for qualitative analysis, it is also used for quantitative analysis and more important hypothesis which we used to analyze the quantity is Beer-Lambert law, which I have already discussed.

And the equation which we are generally concerned with is A is equal to epsilon c l where A is absorbance and you can look at that absorbance is proportional to concentration, absorbance is proportional to concentration. So, if you have 2 samples and a particular compound is in both samples at different concentration you can tell that what is the concentration of that particular component sample 1 and what is the concentration of that is molecule or that substance in the sample 2 using Beer-Lambert law or Lambert's Beer law.

So, here epsilon is extinction coefficient, c is concentration and l is length of the cell that is used in UV spectrophotometer. For example, in the estimation of aspirin impurity from such as salicylic acid and heavy metal ions in ASP tablets can be estimated, can be estimated, ok.

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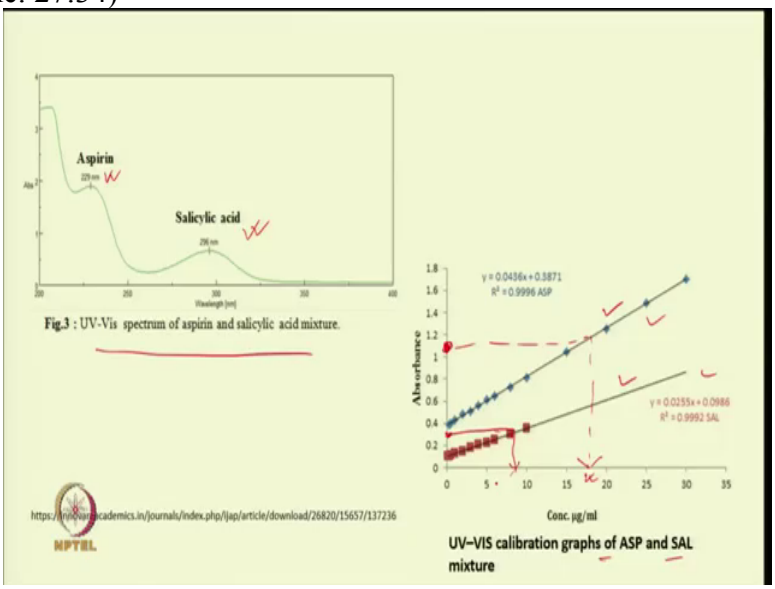

For example, look at this spectrum, this is UV visible spectrum of aspirin and salicylic acid mixture. Aspirin has lambda max at 229 nanometer and salicylic acid has lambda max at 296 nanometer. These two are calibration curve, these 2 are calibration curve, 1 for ASP and another for your salicylic acid, 1 for ASP and another for your salicylic acid and just by using calibration curve, you can know what is the concentration. For example, if absorbance is here and it was like this, then you know this is your MR. So, this is for aspirin and this is for salicylic acid.

And if I have suppose absorbance at this point, absorbance at 220 nanometer is this then concentration of your aspirin will be around 18 microgram per ml. And if suppose concentration of salicylic acid is around your this point, 0.3 then you have probably the concentration of salicylic acid is around 8 or 9, between 8 and 9. So, just by using calibration curve and measuring the concentration of the sample at the 229 nanometer or 296 nanometer we can obtain what is the amount of aspirin in that particular sample and what is the amount of salicylic acid in the particular sample.

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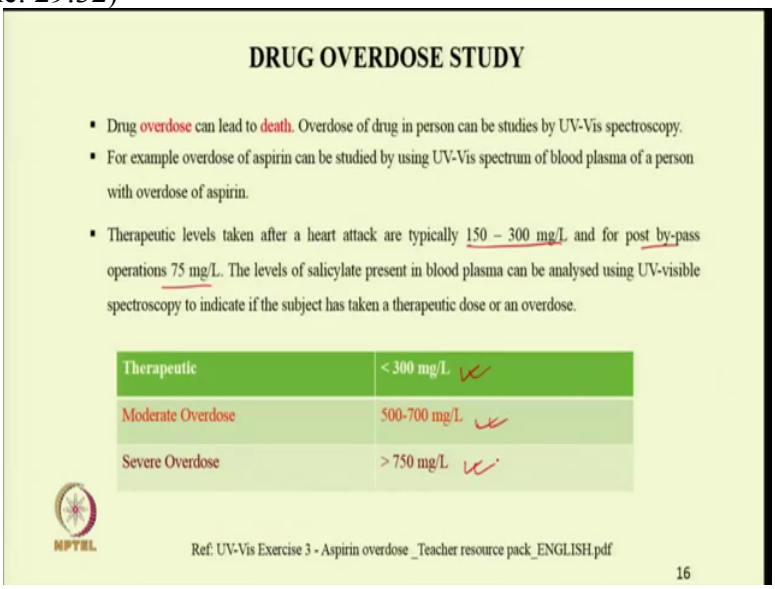

We can also look at drug overdoses study. Drug overdose can lead to death. Overdose of drug in person can be studied by UV visible spectroscopy. For example, overdose of aspirin can be studied using UV visible spectrum of blood plasma of a person with overdose of aspirin, therapeutic level of aspirin are typically 150 to 300 milligram per liter. And for post by operation, it is 75 milligram per liter. So, the level of salicylic acid present in blood plasma can be analyzed using UV visible spectroscopy to indicate it does subject has taken a therapeutic dose or a particular person has taken a therapeutic avoidance.

The moderate overdose is around 500 to 700 milligram per liter, whereas therapeutic dose is less than 300 milligram per liter and severe overdose is greater than 750 milligram per liter.

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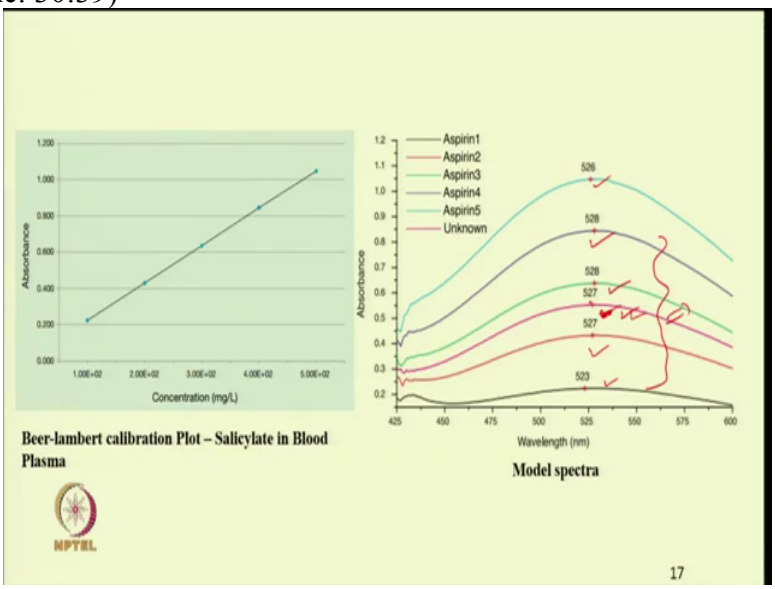

And we can calculate the amount of salicylate again using calibration plot and that will give you salicylate in blood samples. So, these are the few different samples, black one is aspirin 1, this red one is aspirin 2, this one is your aspirin 3 and this one is aspirin 4, this one is aspirin 5. So, these 5 are used to make a calibration curve because for them we know the concentration, all for this all we know the concentration. So, this one is unknown, this one is known.

So, once we make the calibration curve, then we can measure the concentration of unknown or we can take the absorbance of the unknown sample and this is the spectrum of unknown sample. So just by measuring the absorbance at 527 nanometer and using this calibration curve, we can know what is the amount of aspirin in that sample.

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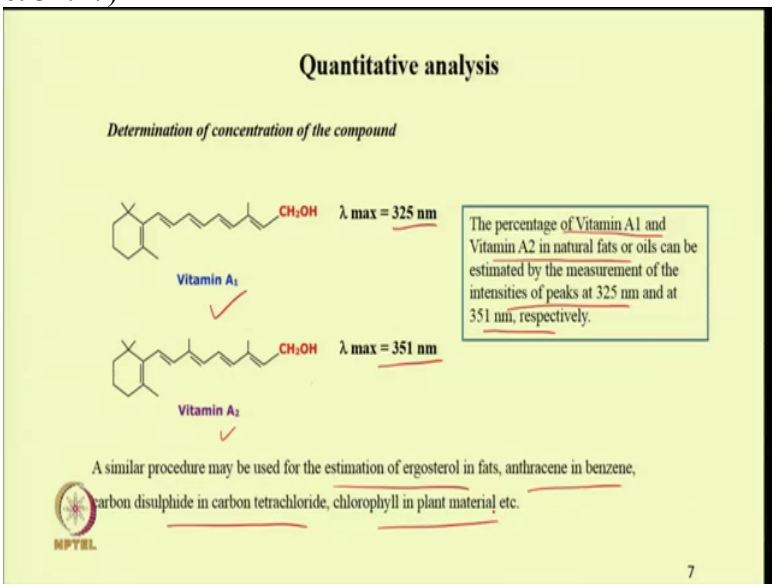

We can also calculate the percentage of vitamin A1 and vitamin A2 in natural fats or oils and that we can do it by measuring the intensities of peak at 325 nanometer and 351 nanometer. Vitamin A1 absorbs at 325 nanometer whereas vitamin A2 absorbs at 351 nanometer and that is why by measuring the intensity of peak at 325 nanometer and 351 nanometer we can know the concentration or we can know the concentration of vitamin A1 and vitamin A2. Similar process can be used for estimation of ergosterol in fats, anthracene in benzene, carbon disulfide in carbon tetrachloride and chlorophyll in plant materials.

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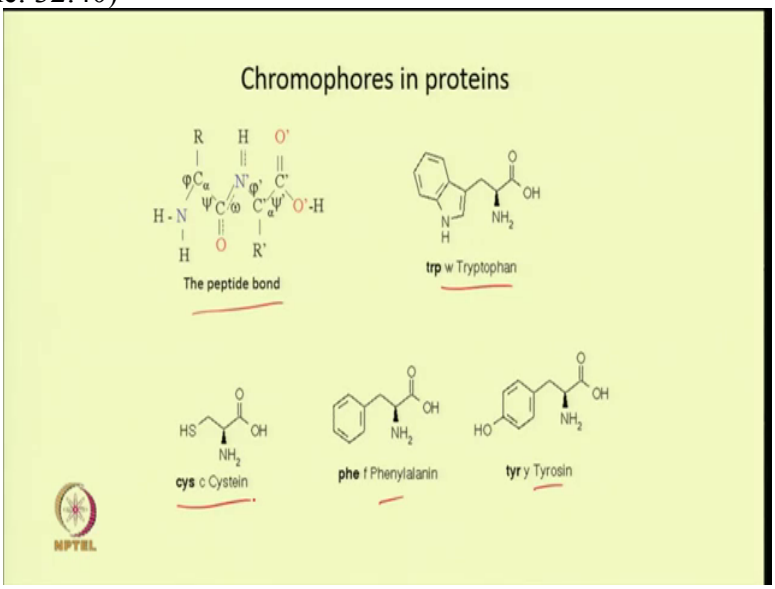

We can also use UV visible spectroscopy to study proteins. There are a lot of things we can do, some of them we will discuss and see if we have time then we can discuss more application. So, this is your peptide bond, we know that proteins have alpha amino acid attached to each other by a peptide bond. And these are the 3 chromophores, aromatic chromophores which are present in the protein apart from the system which can also act as a chromophore.

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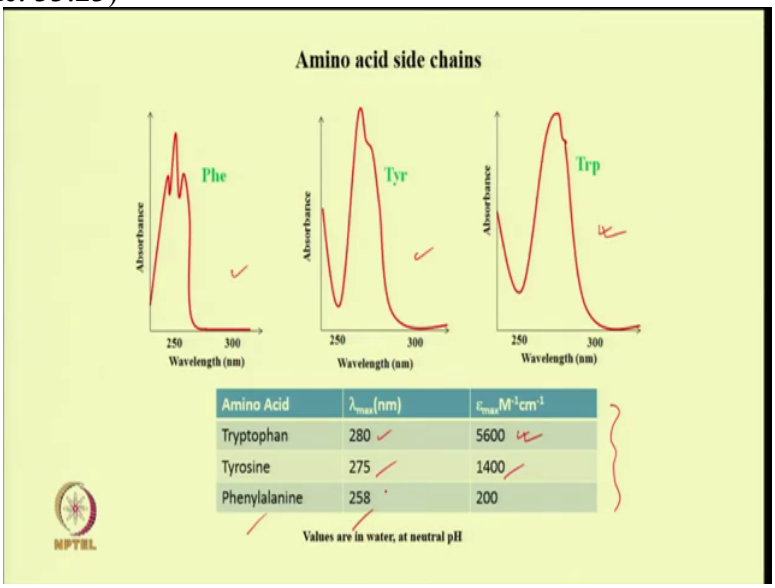

This 3 amino acid has different characteristic spectrum and it is shown here, this is for phenylalanine, this is for tyrosine and this is for tryptophan and this table gives you the lambda max and epsilon max value. So, tryptophan absorbs at 280 nanometer and has epsilon max and it is around 5600 per molar per centimeter. Tyrosine, 275 nanometer. So, this is

basically epsilon at lambda max. And phenylalanine absorbs at 258 nanometer and this is epsilon max value.

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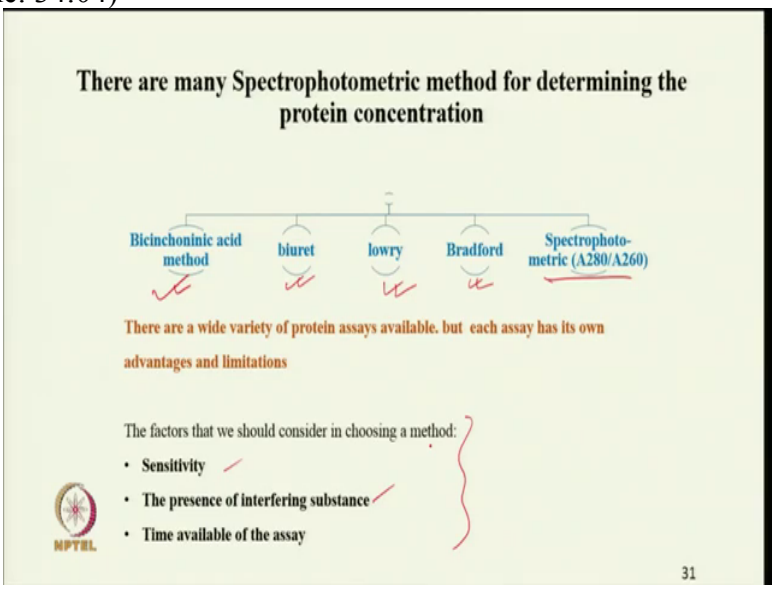

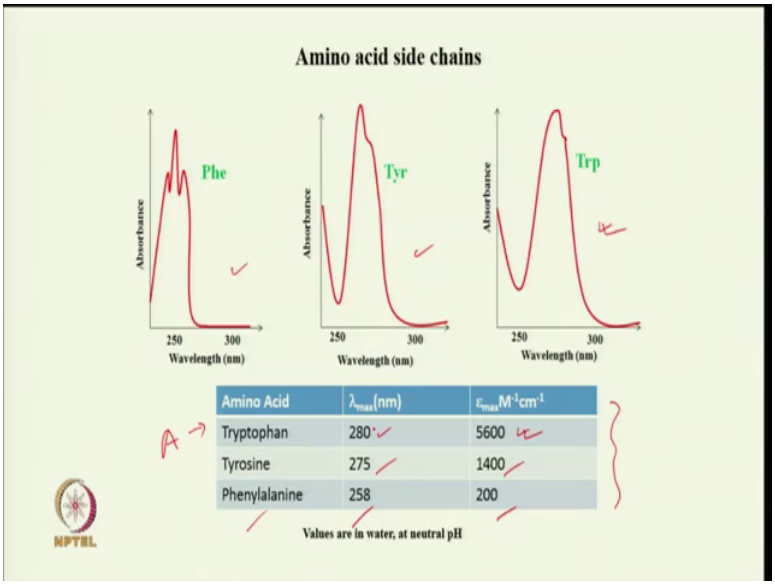

So, we can also use your, let me go back again. We can measure the protein concentration by measuring the absorbance at 280 nanometers, every protein has characteristic epsilon value, characteristic epsilon value because of presence of tryptophan, tyrosine, or phenylalanine. And since your epsilon value of tryptophan is quite high, so tryptophan absorbance is measured to know the concentration of protein. The tryptophan of absorbs at 280 nanometer and so, we measure the absorbance at 280 nanometer and then by using epsilon we can calculate what is the concentration of protein in the sample.

There are other methods, spectroscopic methods for determination of protein concentration and they are based on your combining, your protein with a dye which can give you color in visible region. So, this is the same method, there is a bluret method, lowry method, bradford method and then spectrophotometric method A280 by A260 can be used to know the concentration of protein in the sample. And the choice of method depends on the particular sample. It will depend on sensitivity required and the presence of interfering substances and time available for that.

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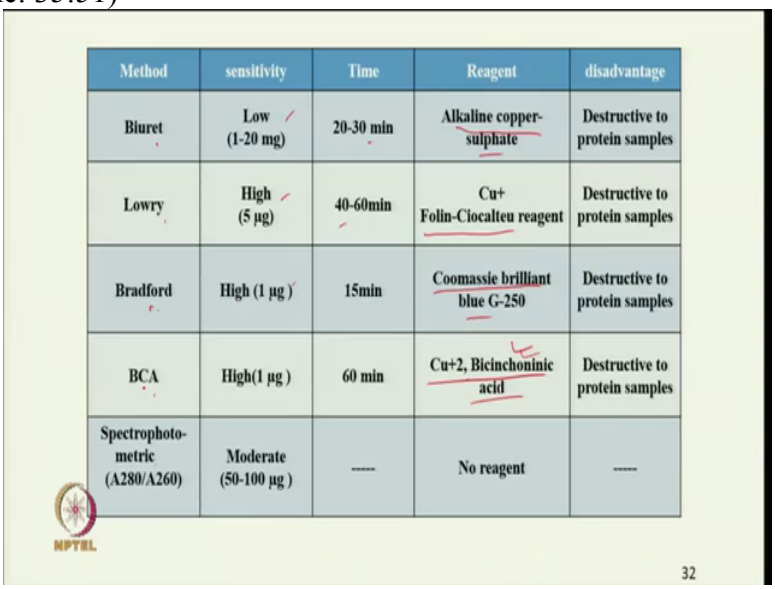

So, these are the different methods: bluret method, lowry method, Bradford, BCA method and spectrophotometric method and this is your sensitivity low, high, high, high, moderate. This takes 20 to 30 minute, this takes 40 to 60 minutes. So, these are the different time required. Here reagent is alkaline copper sulfate, here reagent is copper plus Folin–Ciocalteu reagent. Coomassie brilliant blue is used in Bradford method and this is a copper plus this dye is used for BCA method.

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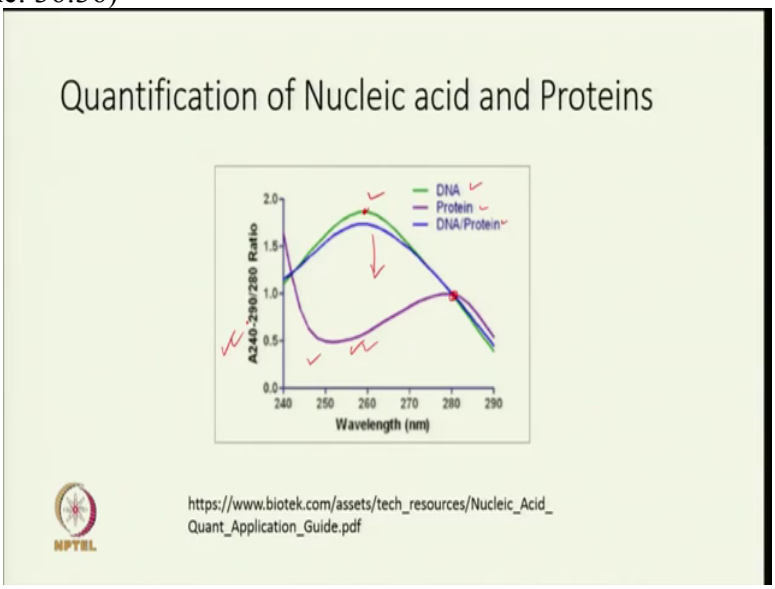

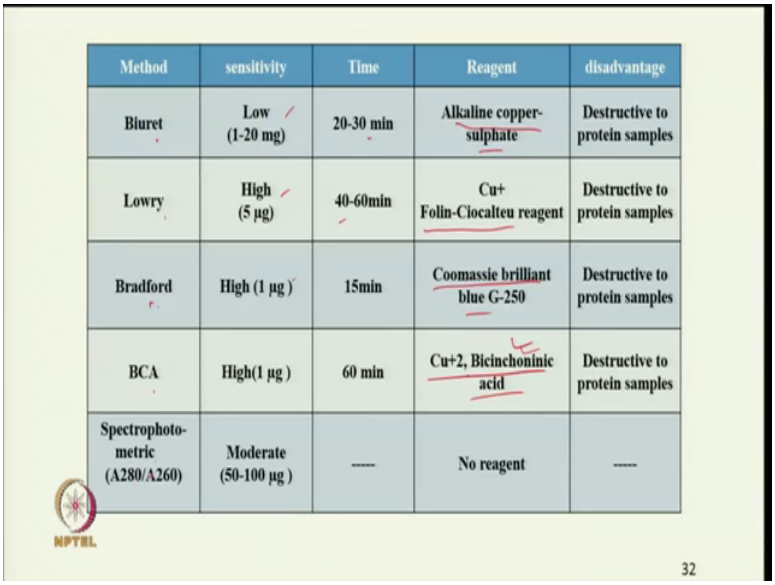

Method	sensitivity	Time	Reagent	disadvantage
Biuret	Low (1-20 mg)	20-30 min	Alkaline copper-sulphate	Destructive to protein samples
Lowry	High (5 µg)	40-60min	Cu+ Folin-Ciocalteu reagent	Destructive to protein samples
Bradford	High (1 µg)	15min	Coomassie brilliant blue G-250	Destructive to protein samples
BCA	High(1 µg)	60 min	Cu+2, Bicinchoninic acid	Destructive to protein samples
Spectrophoto-metric (A280/A260)	Moderate (50-100 µg)	-----	No reagent	-----

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So, as I told you quantification of nucleic acid and protein can be done and this is your the different spectrum of DNA protein and DNA protein. So, this is for protein, this is for DNA and this is for protein DNA mixture. And just by looking at 280 nanometer by; if you remember absorbance at 280 divided by A260 we can know what is the amount of nucleic acid and protein in the sample. Nucleic acid and protein in the sample.

So, this is spectra of protein and you can see that there is a peak around 280 nanometer which is due to tryptophan. And this is for DNA molecule. And now, if you if a mixture has both DNA and protein, what we do is we get this ratio and that can tell you about the protein DNA ratio.

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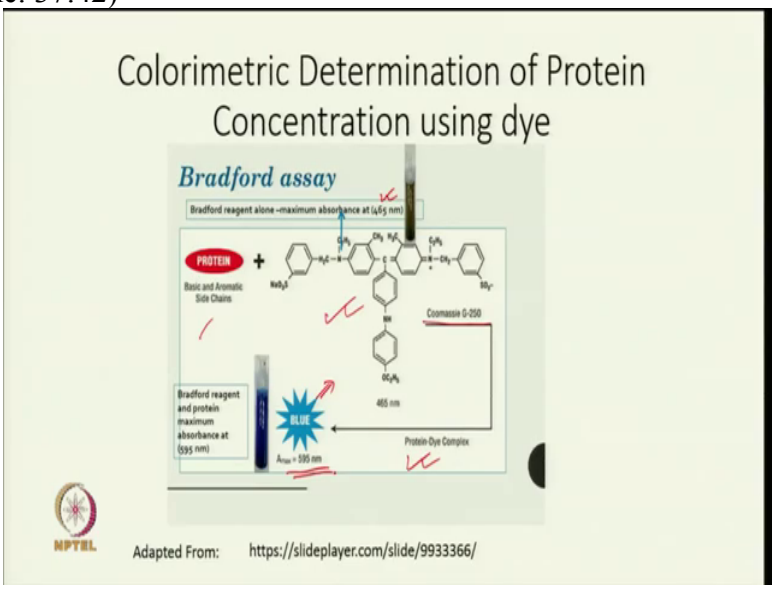

So, here is the different calorimetric methods for determination according to concentration. And I told you one of the method is Bradford assay. In Bradford assay protein reacts with Bradford reagent, this is Bradford reagent, this is Coomassie G-250 which absorbance is at 465 nanometer, is at 465 nanometer and when you add this dye to the protein it forms a protein dye complex which is blue in color and it absorbs 595 nanometer. So, just by looking at absorbance at 595 nanometer you can calculate what is the amount of protein in the sample.

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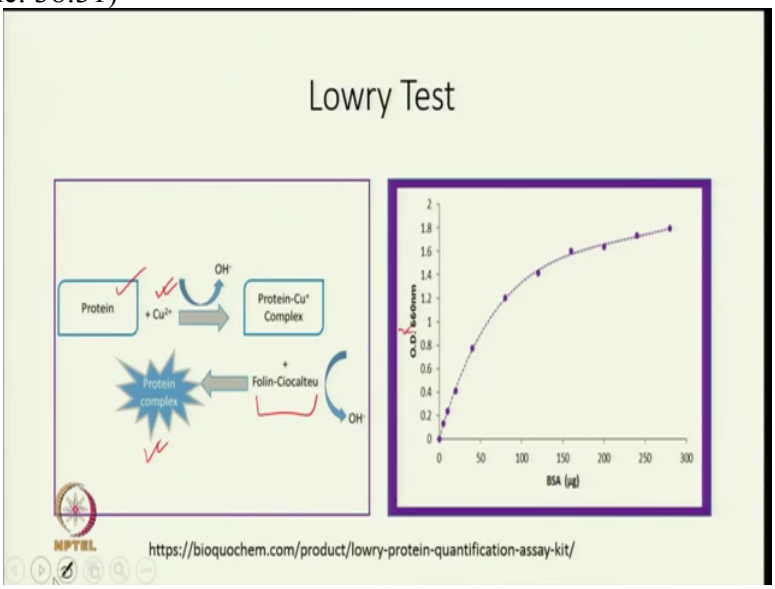

For lowry test, what we do is first protein reacts with copper 2 plus in presence of OH minus and then this complex Folin–Ciocalteuu this is the dye, which gives you a blue color, blue

protein complex and just by measuring the absorbance this protein complex at 660 nanometers, we can know what is the concentration of protein.

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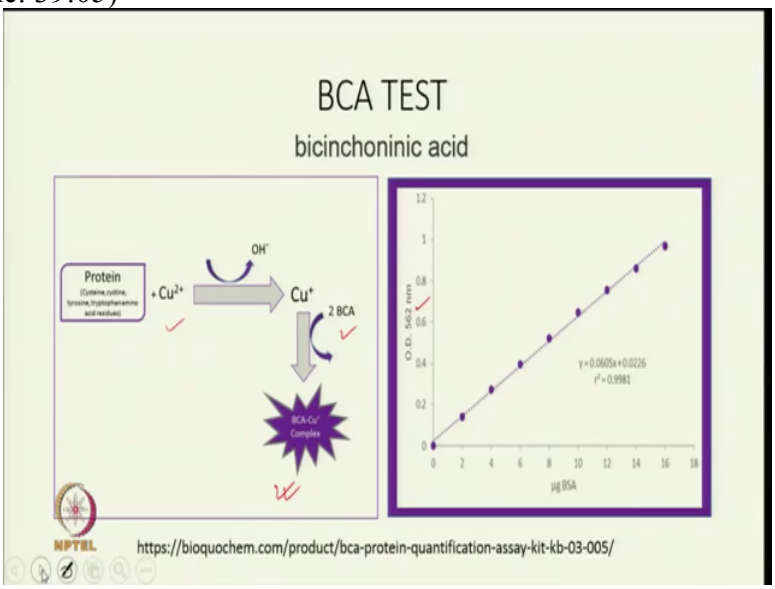

The next one is BCA test and again in this copper 2 plus and BCA is used to get this complex colored complex, which absorbs at 552 nanometer and just by measuring the absorbance at 552 nanometer we can know the concentration of the protein in the sample.

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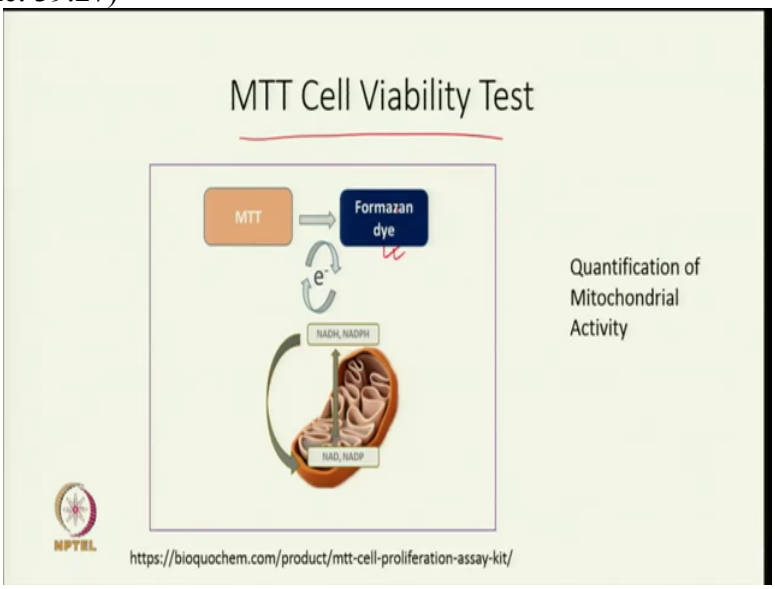

The UV visible spectroscopy is not only used for the measurement of concentration, it can be used to study the cell viability. For example, this MTT assay which is well-known viability assay done in lot of biochemistry labs. Here MTT in presence of NADH or NADPH gives you formazan dye and this absorbs at a particular wavelength. So, by looking at the formation of this dye, we can know whether cell is viable or not.

If cell is not viable, then NAD, NADP will not be converted to NADH or NADPH and then your entity will not go to formazan dye and if it is going to formazan dye, then you know the cell is viable, if not then cell is not viable. So, this is quite often used to study the cell viability assay.

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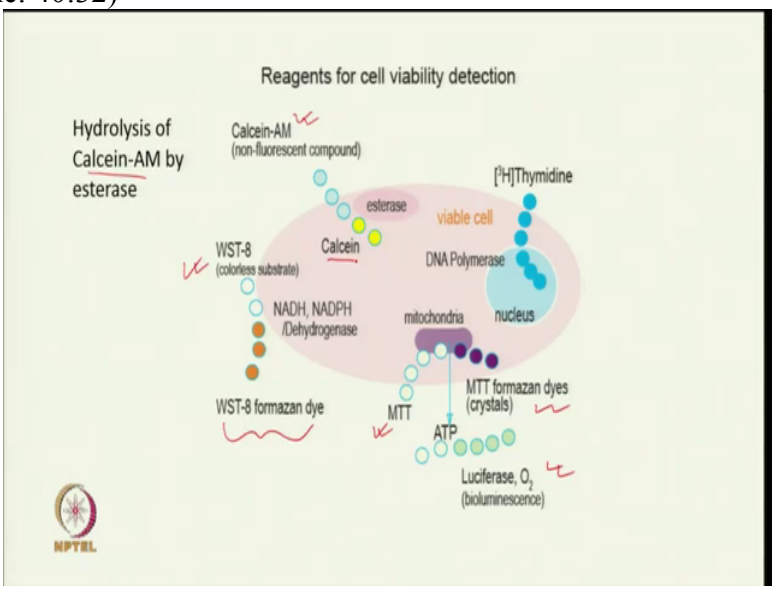

As there are other techniques of cell viability which again uses dye to get a complex which can be detected either by UV visible spectroscopy or by fluorescent. For example, you see, we have already discussed about MTT going to MTT formazan dye. Similarly luciferase activity can be checked. WST-8 which is a colorless substrate but in presence of NADH, NADPH it goes to this WST-8 formazan dye. This calcein-AM is non-fluorescent compounds when it is acted by esterase which basically hydrolyze this calcein-AM, calcein is formed which has a different color and lambda max value. So, this kind of studies can be done if you want to look at whether cell is viable or not.

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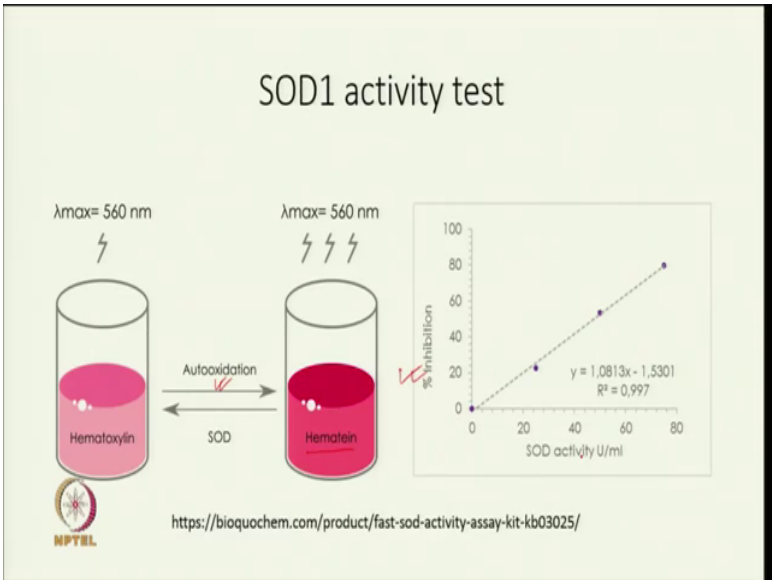

Similarly, we can also look at the SOD1 activity test. So, in this hematoxylin goes to hematein when auto oxidation happens. Auto oxidation happens, then hematoxylin goes to hematein, what SOD1 does is it inhibits this process. So, if SOD 1 is active, then it will bring hematein back to hematoxylin and percentage inhibition can be obtained by measuring the absorbance at 560 nanometer and that can tell you about SOD activity.

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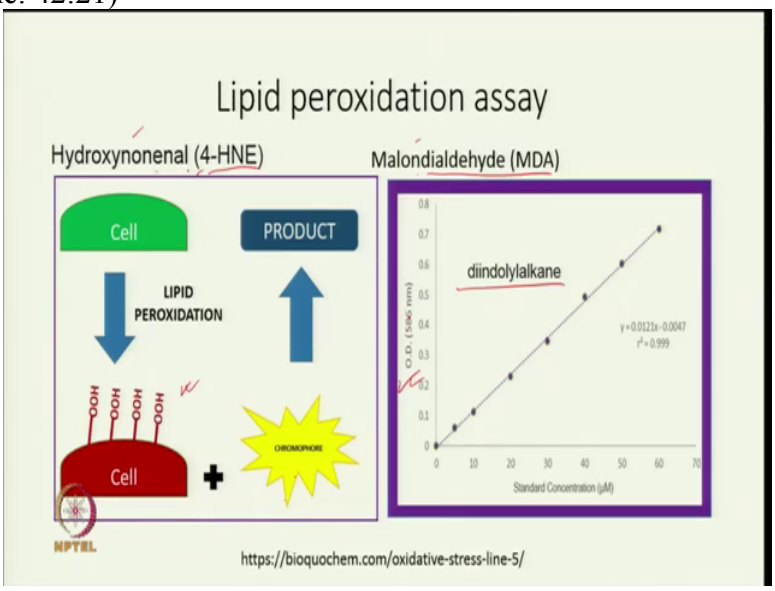

Similarly, lipid peroxidation assay is quite often done. And for that, what we do is we can look at the liquid for oxidation. When liquid for oxidation happens hydroxynonenal and malondialdehyde is formed. When they react with di-indolylalkane then a complex is formed a product is formed. And its amount can be detected by measuring OD at 586 nanometer.

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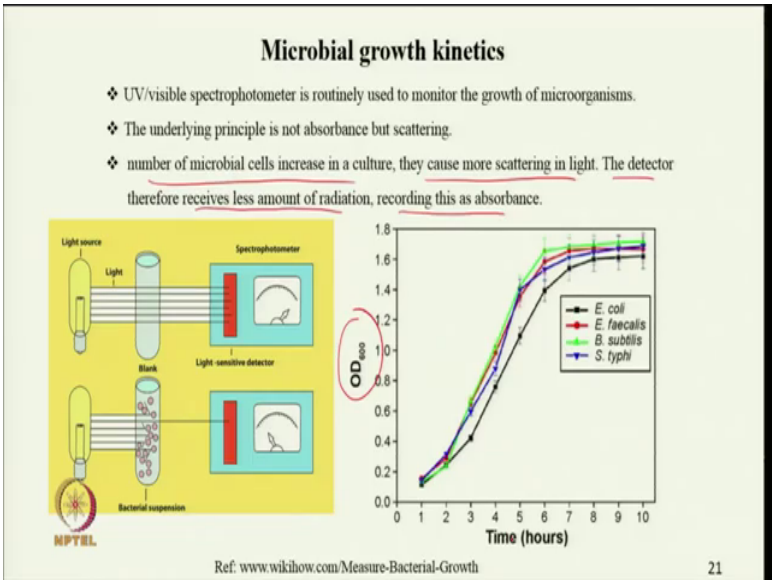

Apart from that microbial growth kinetics can also be studied. We generally use UV visible spectrophotometer for that, here the underlying principle is not absorbance, it is a scattering and since scattering also does not let light reach the detector and so, what happens that absorbance increases if there is a scattering of light and by looking at how absorbance changes, we can talk about how much it is formed. So, when number of microbial cells increase in culture they cause more scattering of light and detector therefore receives less amount of radiation and that is recorded as higher absorbance, recorded a higher absorbance.

And this is the picture, you can see in blank it is at 0 and when there is cell formation, or increase in the cell then what happens that it scatters the light and less light reaches a detector and you will see higher absorbance. This higher absorbance can be measured at 600 nanometer and that is generally called the turbidity assay, generally known as turbidity assay which is done at 600 nanometer and just by looking at you can also talk about microbial growth.

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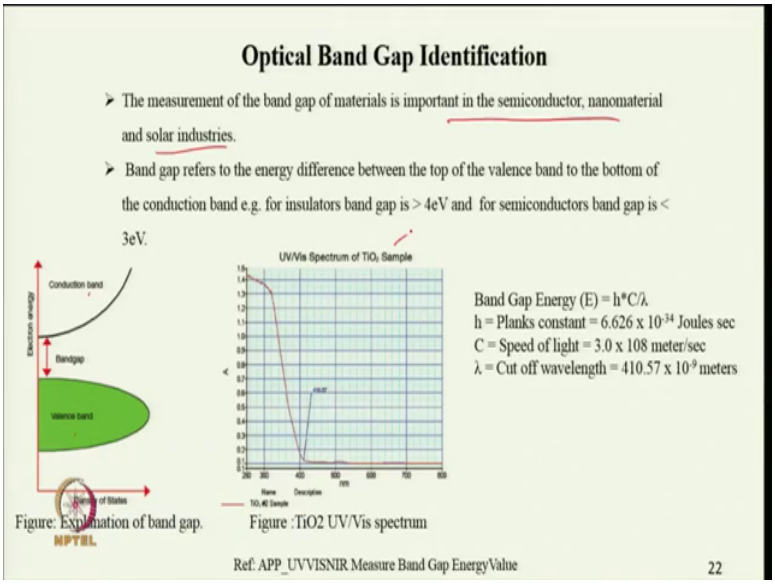

Figure: Explanation of band gap.

Figure :TiO2 UV/Vis spectrum

Similarly, optical band gap identification also can be done using the UV visible spectroscopy. The measurement of band gap of materials is important in semiconductor nanomaterial and solar industry. Band gap basically refers the gap between valence band and conduction band, this I have already told you and you can look at the UV visible spectrum of for example, TiO2 molecule, just by looking at that you can tell what is the band gap.

So, these are the few applications and there can be more application. But since now time is up, so I will stop here, I will discuss some more application in the next class and then I will move to fluorescence spectroscopy. Thank you very much. Thank you for listening. See you in the next lecture. Bye.