Bio-Physical Chemistry
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Lecture – 42
Infrared Spectroscopy of Proteins
(Contd.)

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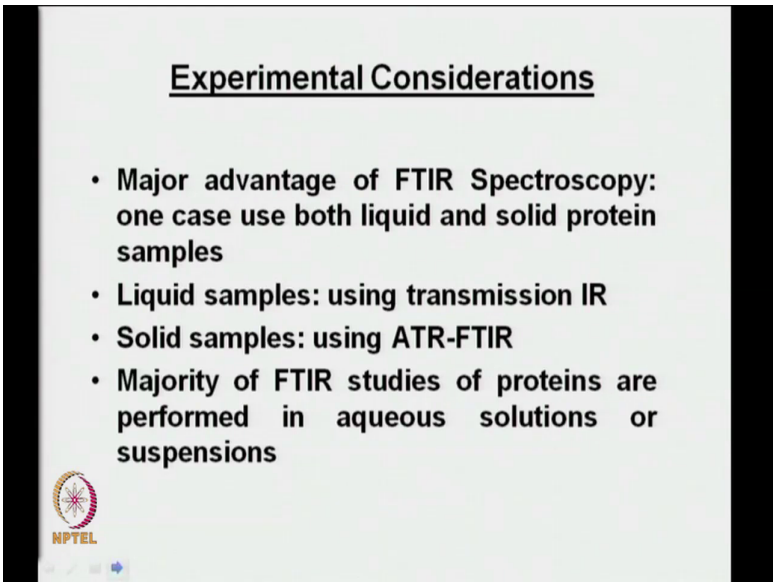

So, we will start our class today, to quickly recap now experimental considerations we talked about the advantage of FITR spectroscopy, you can use both liquid and solid protein samples. The liquid samples you can use transmission IR solid samples ATR-FITR. And as I said majority of the FITR studies of proteins are performed in aqueous solutions.

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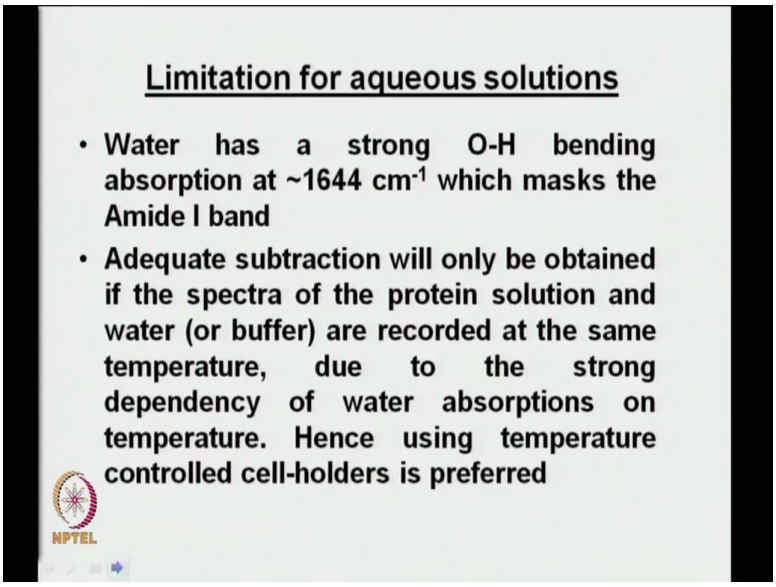

But then what can be the problems? You know we talked about this water bending mode at about 1645 centimeter inverse and this can be really strong and hence background subtraction might not be a good idea or well performed.

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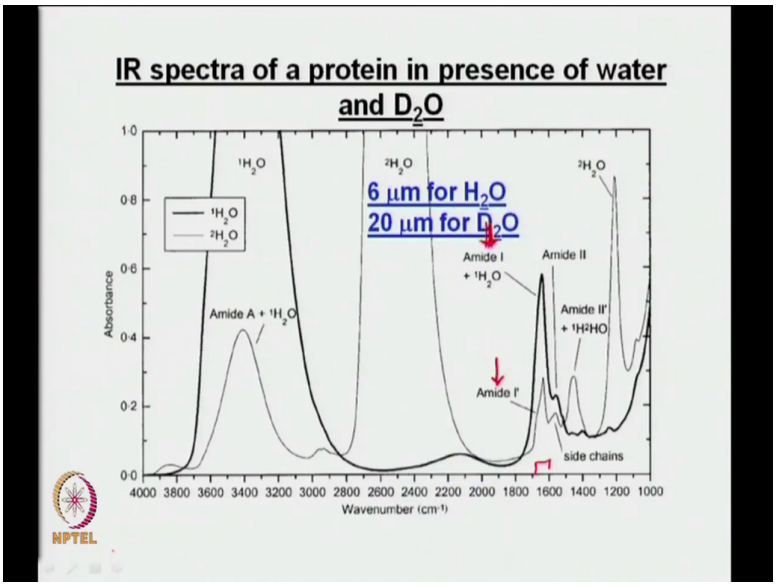

And then we looked at this IR spectrum, we saw you know this amide I prime is for the protein dissolved in D 2 O, where all the hydrogens have been exchanged to D and the amide I is obviously, the one protein the protein dissolved in aqueous solution which is water only.

So, you can see the difference in absorbance right you were having from the corresponding samples; though the path length for water is smaller than the path length for D 2 O ok.

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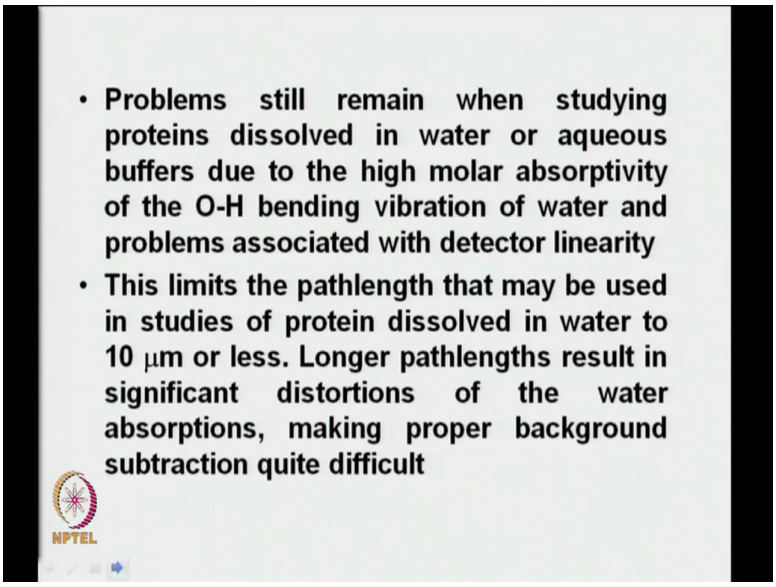

Then, we said that problems still remain due to the high molar absorptivity of the bending mode of water. So, what this does is, it limits the path length which in this as you can see its written on the slide, its it has to be less than 10 micron, it cannot be more than that otherwise the water absorbance would be taking over.

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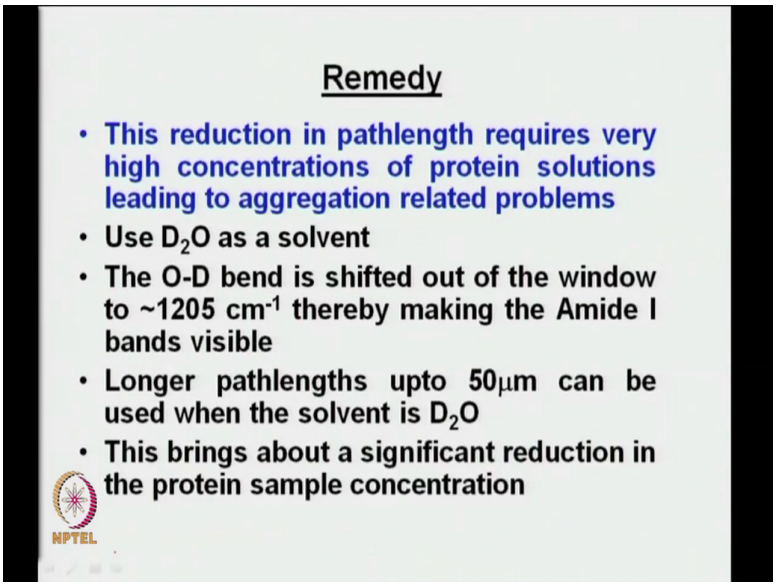

Then we did small calculation, we also talked about the remedy of a reduction in path length, then the remedy was you know using D 2 O as a solvent and then we described why because the bending mode for the O-D vibration is shifted for out from the amide 1 window.

And hence it not only does this reduce the background problem, but what it does also is it gives you a flexibility in terms of increasing your path length so, that you can use lower protein concentrations right. Because you know this is transmission absorbance which is based on Lambert Beers law and there obviously, the absorbance is proportional to your concentration and path length.

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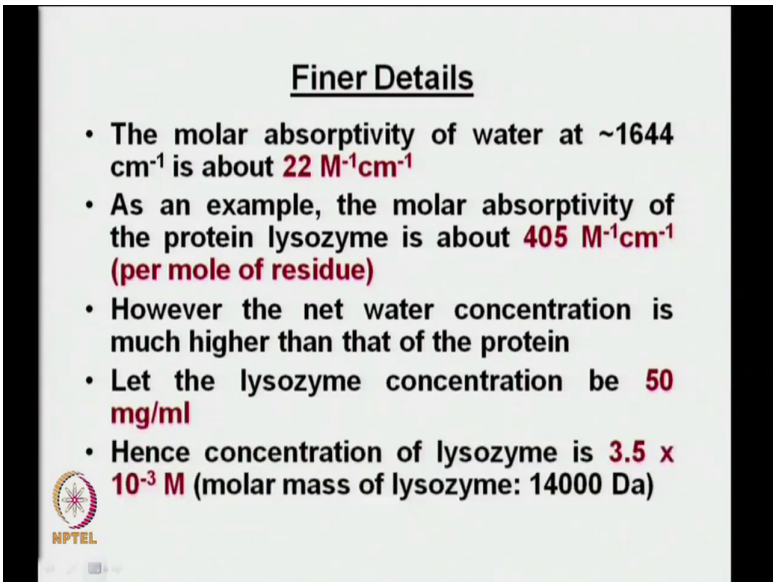

Now, when we talk to our finer details, we made a comparison between water and another protein lysozyme and what we saw was this that the molar absorptivity of water at that 1644 centimeter inverse band is about 22 mole inverse centimeter inverse. For lysozyme it was about 405 mole inverse centimeter inverse per mole of residue; that means, that per mole of amino acid involved out there.

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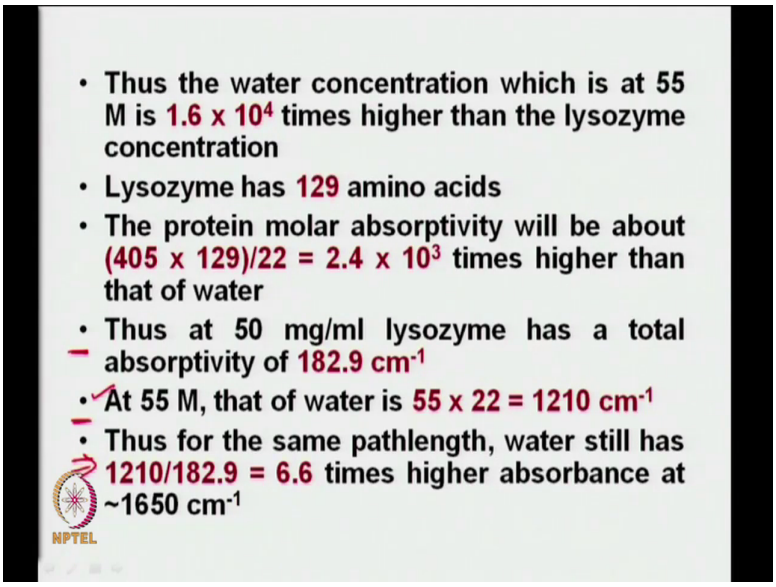

And then we did a calculation based on 50 milligrams per mole, 50 milligrams per liter of lysozyme if you are going to take that, you know try to acquire an FITR spectrum what are you going to get?

So, if we look at this at 55 milli molar. So, this is what I would bring your attention to again. So, this is kind of the last thing we were discussing right in the previous class. At 55 molar there are water that there is molar absorptivity of water is 55 times 22 which is 1210 centimeter inverse. So, you remember last time I said that I had made a mistake it was 20 at that time and I moved it 22 or correct it 22.

Now, the molar absorptivity for 50 grams per milliliters of lysozyme is 182.9 per centimeter of path length. Now this centimeter inverse is not the frequency please keep that in mind. Thus for the same path length; that means, if you are taking the same path length water still has you

can see the ratio 6.6 times higher absorbance at 1650 centimeter inverse right see this is still high ok.

So; that means, no matter what you do, in this case your problem is still not solved; that means, lysozyme might be having a much higher molar absorptivity you can see almost like you know close to you know 20 times higher also 18 19 times higher, but still it does not help right because the net concentration of water is so, high which is 55 mole ok.

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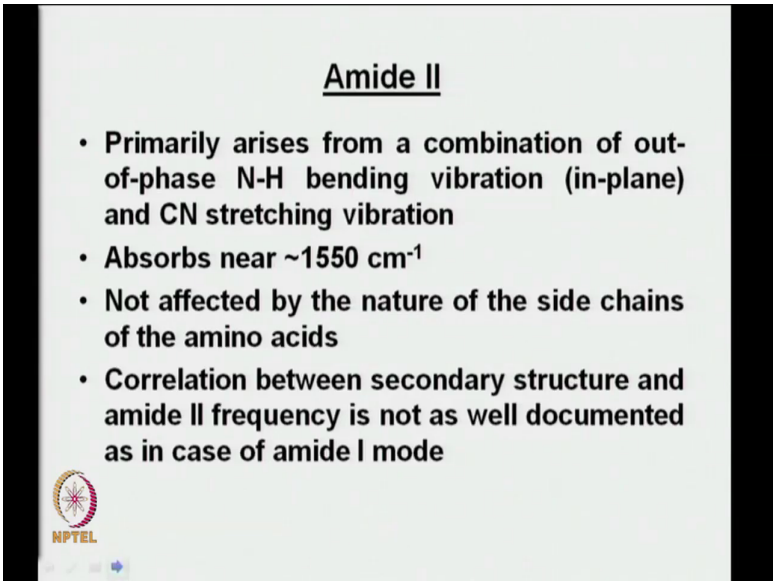

So, that was the amide I band, but let me tell you this again I mean this I have to emphasize and emphasize that the amide I band is one of the most popular modes or the most popular vibration modes at people use to probe the changes in conformations are special the secondary structure of protein ok.

Now, before we went to do the amide I band we actually you know talked about or rather just I put up a slide, where you could see the different modes of vibrations. So, one was amide A and amide B which was N-H mainly this was amide I and I said, amide I band is the one which you are going to spend some time on then what are amide other bands? Then we have the amide II band it primarily arises from a combination of out of phase N-H bending vibration in plane and CN stretching vibration.

So, we have we already know about in plane out of plane bending vibrations are and stretching vibrations are. So, you can kind; you can kind of you know try to formulate in your head that you will be having a coupling between these two different modes or two different stretching and bending and then you will be getting a certain vibrational mode.

Say, it absorbs near 1550 centimeter inverse, it is not affected by the nature of the side chains of the amino acids which is good, but then also the problem is so the correlation between the secondary structure and amide II frequency is not as well documented as in case of the amide I mode. Now this is the case and you are looking at a conformational change then possibly amide II mode is not the best one for you to look into ok.

But there are obviously, you know applications of this amide II mode, but I will we will not go into this, you can look it up I will give you references later where you can you know very good references about FITR spectroscopy proteins, where many of these things are settled or laid out in a lot more details.

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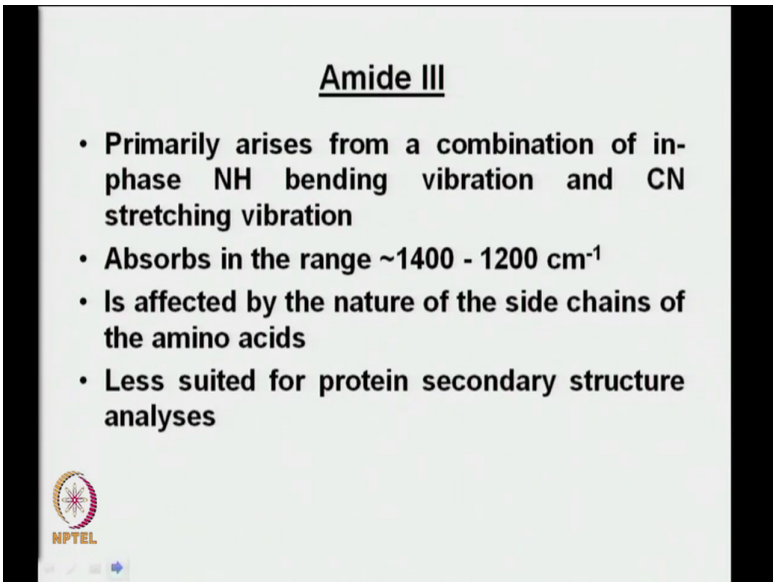

Now, whatever the amide III then? So, the amide III is primarily arises from a combination of in phase NH bending vibration and CN stretching vibration right. So, the other one was out of phase so this in phase, it absorbs in the range of 1400 - 1200 centimeter inverse, it is affected by the nature of the side chains of the amino acids.

Now that is that can make it a little more complicated, but it is less suited for protein secondary structure analyses again for the same reason ok. Now, this were the different modes of vibration that can be present you know different modes means main modes. There are also some other modes known as the skeletal vibration all those things which can be present.

Now, you know before we end this discussion on IR, I would like to spend the rest of this class and I think it would consume the rest of the class on the instrumentation. Because, if you remember we spent some time on the UV visible spectrophotometer instrumentation, where

we discussed how we have the double beam versus single beam, why you need the double beam, why it gives you the absolute absorbance or absorption spectrum, then we went into fluorescence we talked about tried to at least discuss about the different components of the fluorometer.

So, you know once you talk about and I repeat this, once you talk about or once you understand an instrument at least you know the basic components which part is doing what, at least to a certain extent then you have a much better feeling about the spectrum you are recurring right.

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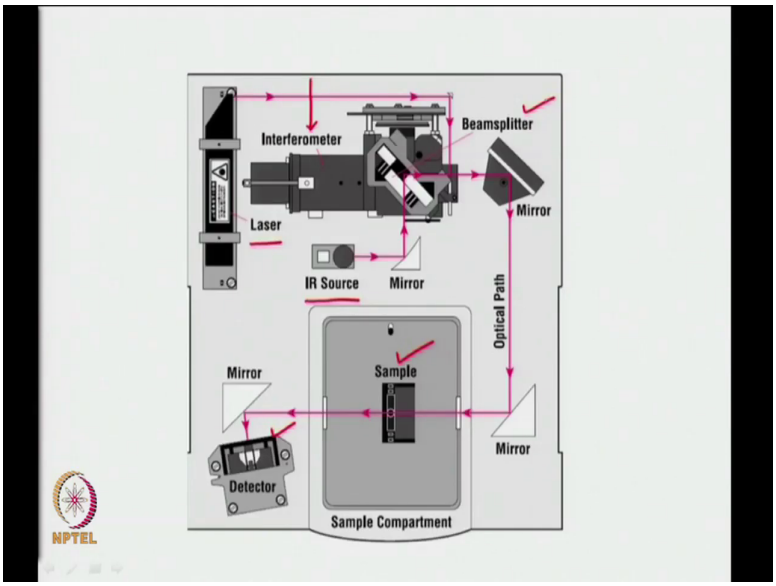

So, let us see that. So, what about the instrumentation? Let us look at this is kind of a schematic layout. So, see what are the different components. Let us look at this. One component is your IR source; remember this is an IR spectrum. So, you should be having an

IR source which is coming out. So, then this is the IR source. So, that is one which is very important the IR source.

Now then there is a one very important thing which is known as the interferometer and I will discuss it in some detail later, with this interferometer is probably the most important part of an FITR spectrometer of Fourier transform infrared spectrometry that is what we are talking about. I will tell you the significance of this word where this word comes from as we go on to the class, but just take it from you right now.

Now, then you have many mirrors beam splitters and all these things ok. Now, the other thing is if we have to place a sample somewhere. So, this is your sample compartment right and then you have a detector because detector is the one where you would be detecting your signal and then you process it with the computer whatever you have on hand.

Now, this is you can see out here it is a laser we have on the extreme left of your slide or the schematic layout. Now why do we need a laser? Just keep in mind if it is an FITR spectrum. So, this laser what it does it helps in an internal calibration because this laser there is a fixed wavelength it is a monochromatic laser, it is a fixed wavelength. So, you kind of know. So, the instrument itself calibrates it according to that helium neon laser which is the laser which is used according to that monochromatic radiation.

So, these are the main components what? We have the IR source which we need, we have an interferometer you will soon understand the significance of this, then you have a sample compartment, you have a detector and you have a laser which helps in the internal calibration and you know these different beams are passed to different optics like a beam splitter you have its a beam splitter.

Now, beam splitter I will tell you what it means, beam splitter simply means it splits a beam into two or multiple components whatever, here it is a mirror, mirror is essentially just to guide your IR light into the sample compartment or whatever direction you wanted to be guided into.

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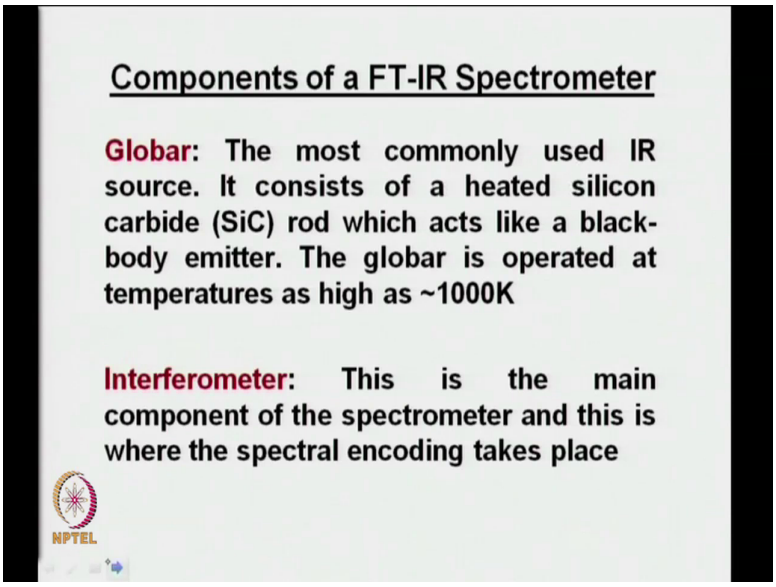

So, now let us look at the components of the FITR spectrometer. So, what is the IR source? The IR source is the globar ok. So, the IR source is a globar now remember we talked about the IR source right and this is you need an IR source. So, what is this globar? This globar consists of a heated silicon carbide rod which acts like a black body emitter its emits radiation and the globar it is operated about you know 1000 Kelvin pretty high temperature right.

Then we have the interferometer, now this is the main component as I said and this is where your spectral encoding takes place; that means, whatever spectral information you need, you need to extract from the sample is stored in this interferometer or stored in the sense is what do you are getting through this interferometer, I am should not say stored you know it is not a memory. So, whatever spectral information your getting is because of this interferometer, this is the one which helps you get this spectral information.

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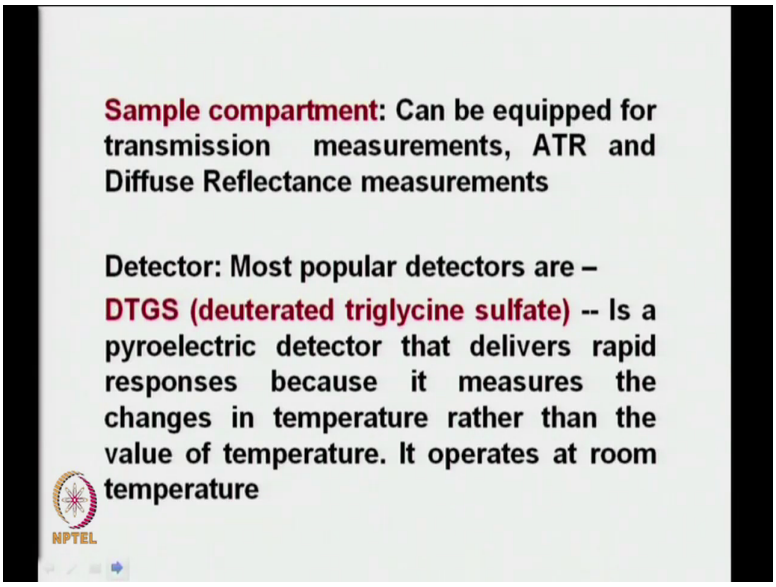

Now, how does it do it will soon see. Then you obviously, you have the sample compartment. Now remember the sample compartment one of the advantage of FITR was if you would remember that it would you know you can do liquid samples, you can do solid samples, you can do suspensions.

So, then obviously, these would be having different sample holders. So, then in one case you have transmission measurements; that means, you are just a doing a transmission measurement, then one case you have ATR its called Attenuated Total Reflection, we will look at that later and then you also have diffuse reflectance measurements you know diffuse reflectance measurements some mainly carried on powder samples. Now this is this diffuse reflectance measurement is something will not spend much will spend time on, but this is the only time we are going to talk about it ok.

Then obviously, need to detect your signal IR signal. So, what are the most popular detectors? One is referred to as DTGS its a Deuterated Triglycine Sulfate Detector, its a pyroelectric detector now its I leave it to be your responsibility figure out what a pyroelectric detector means, what how does it function. It delivers rapid responses because it measures the changes in temperature rather than the value of temperature and it operates at room temperature.

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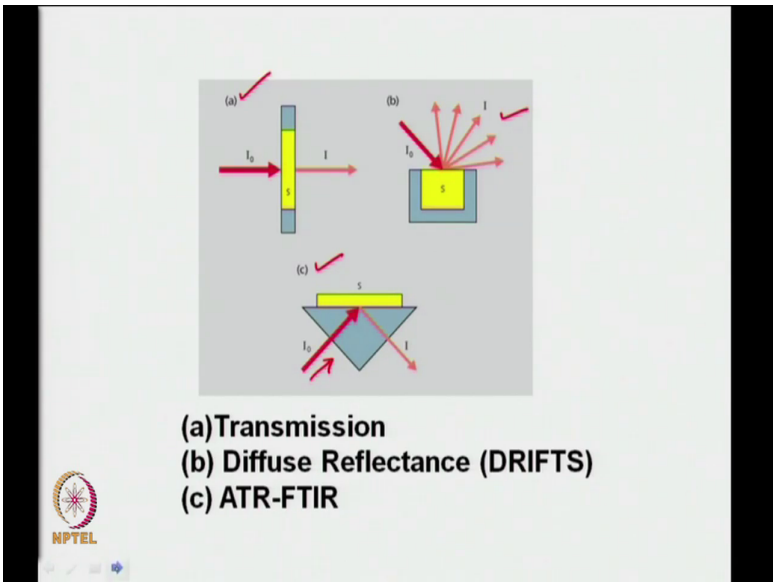

Now, before I go to another type of detector, let us look at as I said the different ways of doing these or you know the sample holders or sample measurements. So, here a is a transmission you can see a is a transmission, you can see I 0 which is the incident light and I which is the transmitted light and obviously, there is a difference between these two because some of the light has been absorbed by the sample.

Then what is b? b is referred to as the diffuse reflectance accessory its called DRIFTS. So, you can see its a powdered sample its a rough and it can you know scatter or scatter light in many directions and then you have the ATR FITR. So, ATR. So, this is c is ATR FITR we will talk about this later, but one thing you can see this is your I 0 which is going like this and there is your I.

So, it is almost you are looking at almost total internal reflection; that means, not much of this light is going through into the sample right, but then how do we get the spectrum well we will come to that later. But this is what I meant by these different sample measurements so sample types how we can measure them?

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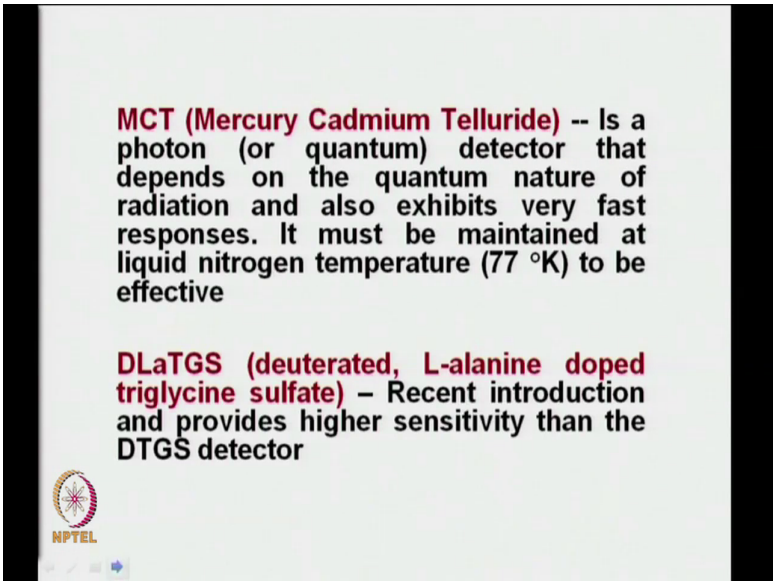

So, now again coming back to the detector, its a photon or a quantum detector what it is its a mercury cadmium telluride detector, this is also very popularly used. As I said its a photon

detector, it depends on the quantum nature of the radiation and also exhibits very fast responses. But one of the problems is it must be maintained at liquid nitrogen temperature which you know can be a problem if you do not have ready access to liquid nitrogen facilities ok.

Now, one of the very recent introductions is a variant of this DTGS referred to as dLaTGS where you dope it with L-alanine. So, as I said; as I said it is a recent introduction, but it is slowly gaining importance or prominence because it has a higher sensitivity than the DTGS detector ok. So, here now you have some idea of the type of detectors are used.

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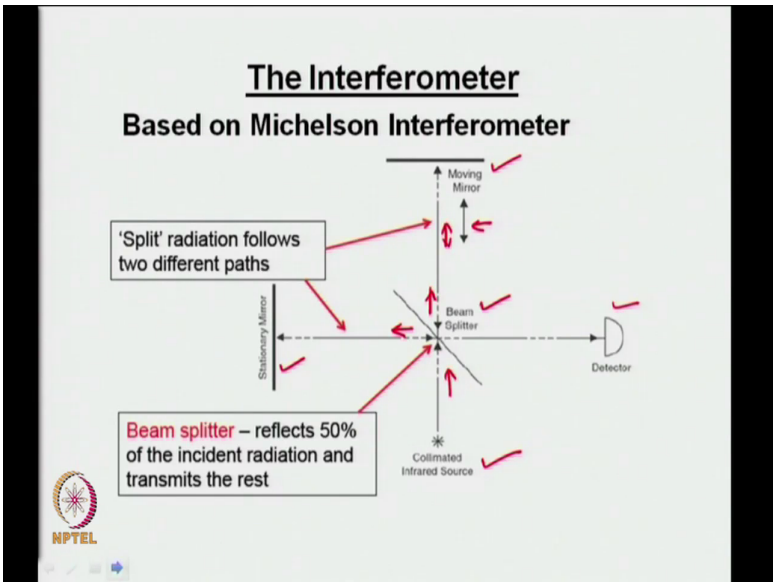

So, now let us talk about the interferometer. As I said this is the main component of your FITR spectrophotometer or spectrometer rather not photometer spectrometer. So, what is this

interferometer based on? It is based on the Michelson interferometer I am sure you have you know this from a physics.

So, now, what happens is if you look at this, if you look at this they are here this IR source let us talk about the IR source which is the globar, this is emitting the radiation. Now after this IR source is emitted what happens is, we can see here the beams beam splitter out here. As I said the beam splitter it is called a splitter because it splits the beam into two in this case. So, what does it do? You can see what here has happened the collimated IR sources gone in this direction and after that it has split into these two directions.

Now, after splitting what you know where have these beams gone. So, one beam one portion of the beam goes through this moving mirror. Moving mirror means as this one arrow shows you moving mirror means that this mirror can move in forward and backward directions along the arrow, along this it can move in forward and backward directions.

Now, what about the stationary mirror? Now as the stationary mirror suggests stationary mirror remains stationary; that means, its a fixed mirror, it does not move ok. Now this is the basic component of an interferometer.

Now obviously, after this what you have is on the right side you have a detector which is going to detect water is coming out from the interferometer. Now to also just to bring it into perspective this beam splitter is a 50 percent beam splitter what it means is that deflects 50 percent of the light in one direction and rest of the light in the other direction as I said. So, 50 percent goes to the fixed mirror and 50 percent goes to the moving mirror.

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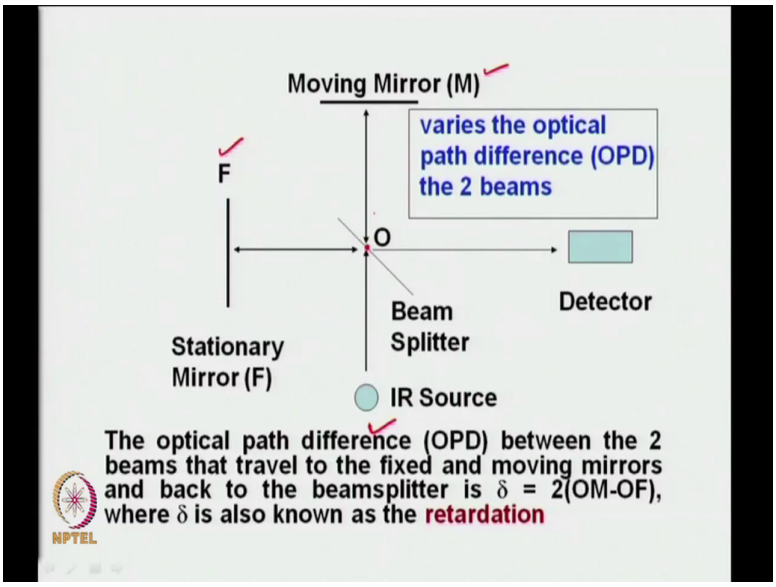

So, this is again a schematic of the same thing. So, what you can see out here the main components? Now what I have done is here for the moving mirror, I have labeled it as M, for the stationary mirror I have labeled it as F right. This is my IR source and this is the beam splitter and the point at which all these meet all these are going through in the beam splitter I have referred to as o or origins ok.

Now, see what the moving mirror does. What the moving mirror does is now suppose you have this fixed mirror, suppose you have this fixed mirror and you have the moving mirror right. Now if they are fixed at definite path say the fixed mirror the fixed mirror is fixed right.

Now the moving mirror what you can do it is you can move as I said backward and forward. Now why would you do that? You would do that because you would by moving the mirror

you would be changing the optical path difference now what do I mean by that? Let us go ahead and see what I mean?

The optical path difference between the two beams, the travel to the fixed and moving mirrors and back to the beam splitter is given by delta equal to 2 times O M minus O F what is O M? O M is the distance from the origin which is O in this case the middle of the beam splitter to the moving mirror, O F is the distance from the beam splitter that is O to F the fixed mirror ok.

So, this delta this is optical path difference is twice the difference between these two because you can understand it why it would be twice, I will leave that up to you, but delta is known as the retardation. So, this optical path difference is also referred to as retardation or this delta is also referred to as retardation ok.

Now, why is this important? Now this optical retardation is the key characteristic of FITR spectrometer how is this so? Let us look at this ok.

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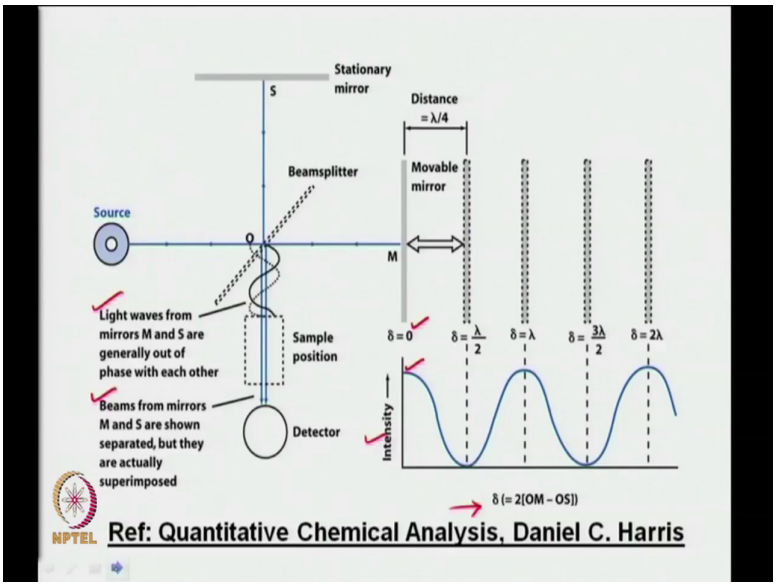

So, here again you see what happens now. Now suppose there is no optical path difference, no optical path difference means delta is equal to 0 that means, O M is equal to O F; that means, whatever distance F is from O, M is also at the same distance from O right. So, this is what we are talking about.

Now see how we have moved the mirror the mirror can be moved by units of lambda by 4 where lambda is the certain wavelength ok. So, this is how I move the mirror. Now see what happens. What you are doing is you have the fixed mirror and the moved mirror right.

So, both of these are having the IR beam bouncing back and forth; that means, one IR beam goes to the fixed mirror bounces back comes to the beam splitter. In other case you have the

moving mirror where the IR beam bounces back and comes to the point O that is the beam splitter right.

Now, then these two combine, when these two combine what they give you? These two give rise to an interferogram. So, this is essential what you are looking at which is the intensity, which is the intensity versus the optical path difference or the retardation. Now as you can understand if there is no path difference which is at delta equal to 0 that means, no optical retardation; obviously, your intensity would be at the highest right there is no phase difference nothing intensity is the highest that is the maximum.

Now, what happens is at an non integral number of wavelength; that means, when you go to delta is equal to lambda by 2, now remember when I say delta is equal to lambda by 2; that means, the distance I have moved is you know lambda by 4, it is twice that if that is so, now, that is so, you can understand what you can see what is happened.

In this case you can see when they meet when these two beams meet that is one beam from bouncing from the fixed mirror and the other beam bouncing from the moving mirror, when they meet you can see it is the minimum; that means, they interfere destructively.

So, at delta is equal to 0 you have constructive interference at delta is equal to lambda by 2 you have destructive interference. Now then you again move by lambda by 4 which is 2 times; that means, again lambda by 2 that means, you come to delta is equal to lambda again you have constructive interference right. So, this is how it goes on so; that means, you kind of give rise to an interferogram. So, it is an interference pattern right.

Now, so, this is what its being shown out here again you can see, in a normal FITR what you are going to have is if you would remember this schematic layout, you have these mirrors then you the sample in between and then you have the detector.

So, once you have the sample it will be absorbing, then because it is transmission or absorbance measurement, then you have to have a background; that means, you know you

have to do the blank. So, you will be putting a blank and there will be doing some mathematical processing which will talk about later.

But as its seen here the light waves from mirrors M and S are generally out of phase with each other right and what this one is showing is, the beams from mirrors M and S are shown separate, but they are actually superimposed we are just trying to make the point that both the beams come to the detector, but you what you are actually doing is you are actually looking at the interference pattern ok.

So, this is the main component of the FITR spectrometer which is your interferogram; that means, while you are moving the mirror, you are changing the optical retardation of the optical path length then by doing that what you are doing is your you are bringing about constructive and destructive interferences and that is a being recorded by the you know that is give being split out or being given by the interferogram it is being recorded by the detector.

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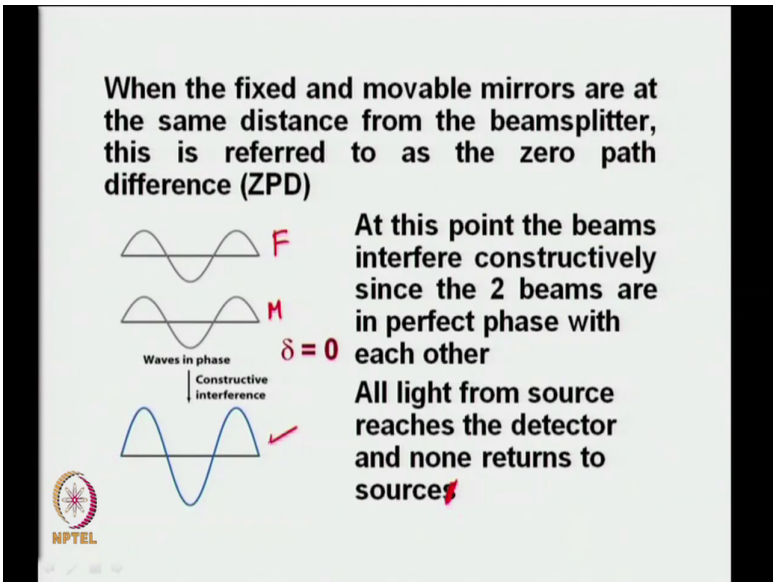

Now, moving forward as I said when the fixed and movable mirrors are at the same distance from the beam splitter this is referred to as the zero path difference are ZPD. So, here as I say you can see this is a case for delta is equal to 0 you will soon see this is a case for delta is equal to 0.

So, this is say from the fixed mirror, the top one is from the fixed mirror, the bottom one is from the moved mirror ok. So, say this is from the fixed mirror, this is from the moving mirror, now both of these are in phase constructive interference and their intensity is respectively add up good.

So, at this point as I said the beams interfere constructively, since the two beams are in perfect phase with each other now that is so, what is going to happen? So, all light from source

reaches a director and none returns to the sources or in this case I should say source I should get rid of this sources right.

So, anyway bottom line is that whenever you are having a zero path or there is zero path differences zero retardation zero optical path difference; that means, these two beams coming from the two mirrors are going to interfere constructively ok.

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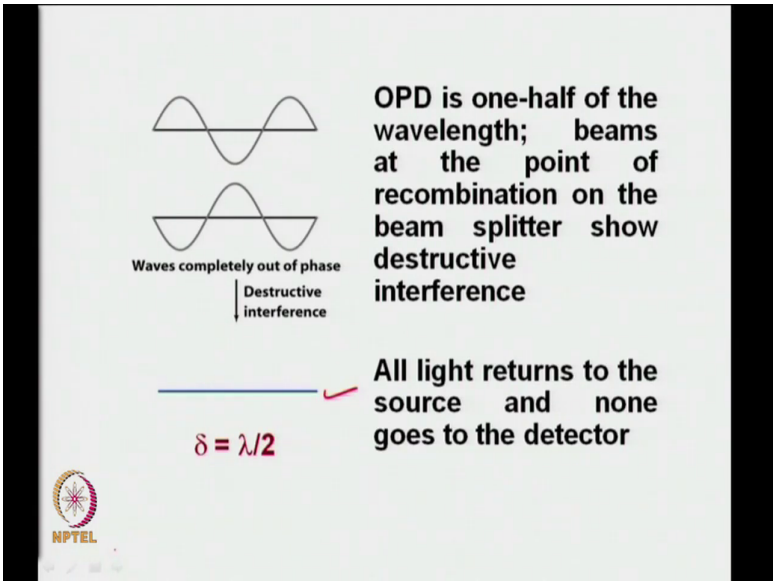

Now, then what happens if they do not do that? So, let us look at another extreme. So, here is an extreme where the waves are completely out of phase right the waves are completely out of phase. So, this happens for delta is equal to lambda by 2 if you remember that figure that slide.

So, in this case the optical path difference is one of the wavelength, beams at the point of recombination on the beam splitter show destructive interference; that means, they destroy each other because they destroy each other you can see at those points I mean there is almost no, I mean the resultant this is your resultant right this resultant there is almost no intensity coming out not almost actually none.

If you compare this with the previous slide you can see this is your constructive interference where you had enhancement; that means, these two were adding up, but in this case as you can see there is almost nothing alright.

So, now in this case all light returns to the source and none goes to the detector; obviously, what will go? Because there is nothing everything as you know there is a destructive interference, you do not have anything to go through the detector ok.

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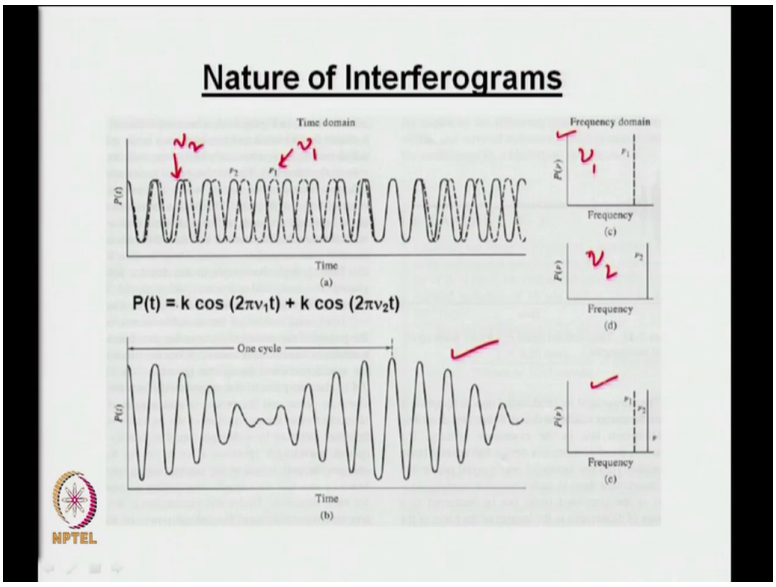

Now, let us ramp it up a notch. So, when you are talking about a globar you know IR source, you are not going to have one frequency you are going to have many frequency is coming together now how would it look?

So, let us look at this. So, this is on the left you have a typical interferogram ok; that means, the interference pattern that is coming from the interferometer and you can see on the right out here this is a frequency domain. Frequency domain means you know what the what frequency of light your putting in.

So, if you look at the frequency domain in this case you are frequency of a nu 1. Say, nu centimeter inverse I do not know what their frequencies let it be nu 1. So, because it is nu 1

what you have is, you have a certain pattern for nu 1 you can see this dotted lines, so this is these dotted lines are nu 1.

Now, what I do is, I take another monochromatic source which has nu 2. So, this is nu 2 this is nu 2, now this i superimpose on

Student: Nu 1.

Nu 1 so; that means, the solid line this is the solid line which is nu 2 right. Now what will happen is you can see how the addition is done is the cosines of these two curves and the resultant interference pattern is this. So, this is now your interferogram ok.

And obviously, think about the reverse, now if you do not look at the bottom spectrum at the top spectrum what you have done is you have taken nu 1 and nu 2, this nu 1 and nu 2 are you have put in you have added up, but suppose you do not know what they were. You had the interferogram then you would do a certain mathematical operation called the Fourier transform and what you will get? You will get from this superposition you will get these two frequencies nu 1 and nu 2 that is essentially what the idea is.

So, what the spectrometer is doing for you is, the spectrometer is getting the interferogram and from the interferogram you are doing a mathematical transformation called the Fourier transform ok. Will come to this later and then you are getting the corresponding spectrum. Your spectrum essentially is what your intensity versus your frequency ok.

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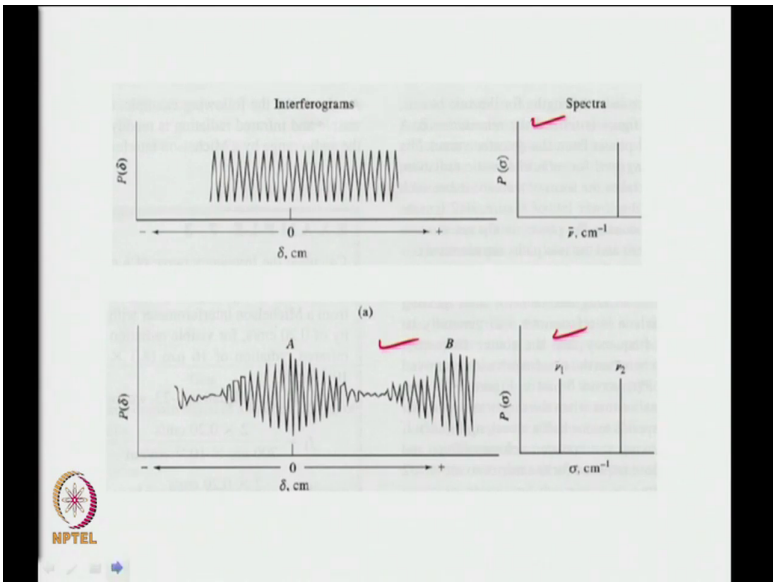

So, here again the same thing here interferogram for a monochromatic radiation this is an interferogram for two radiations superimposing on each other nu 1 and nu 2.

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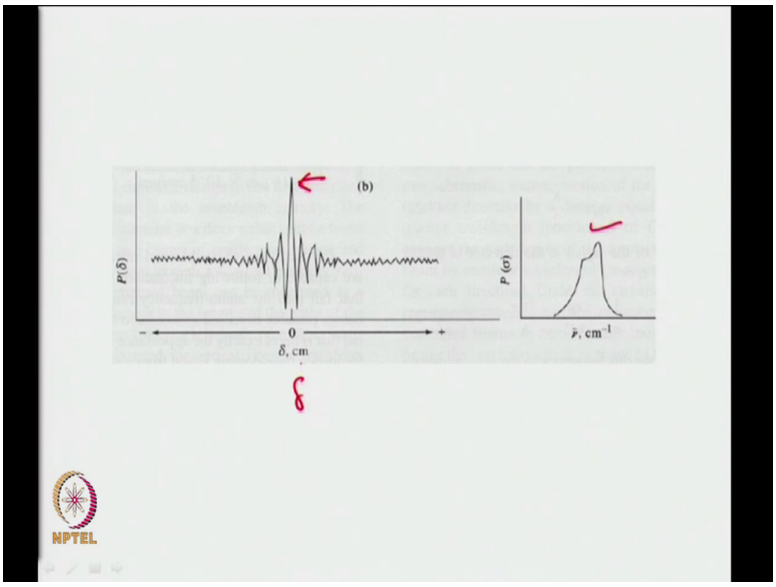

Now, see what happens. This is a typical radiation from a globar as I said the globar is a continuous radiation, its not having discrete radiations its many overlapping radiations that is why its called the continuous radiation.

Now, because it is a continuous radiation see what have, because it is a continuous radiation see what the corresponding interference pattern is like right or the interferogram looks like.

You can see there is a one center burst this is really high, remember at zero path difference where delta is equal to 0 each and every wavelength will be in phase because each and every wavelength will be in phase at 0 optical difference each and every wavelength. That means, light of wavelength of light would be constructively interfering with each other as a result you are going to have the maximum at the zero path difference which is delta equal to 0.

Now, depending upon how many wavelengths you have in the continuous radiation, because they will be having you know different natures different superimpositions that is what that is why what you can see is, you have this center and as soon as you move away from this delta is equal to 0 because your x axis is delta right as it says is delta.

So, delta is in centimeter that is the amount by which your moving the mirror, you can see it dies down very fast. You just means the mode number of you know the mode number of wavelengths you have out there in terms of a continuous radiation the faster will it decay from that center delta is equal to 0, it means the more will be the destructive interference now that is understandable ok.

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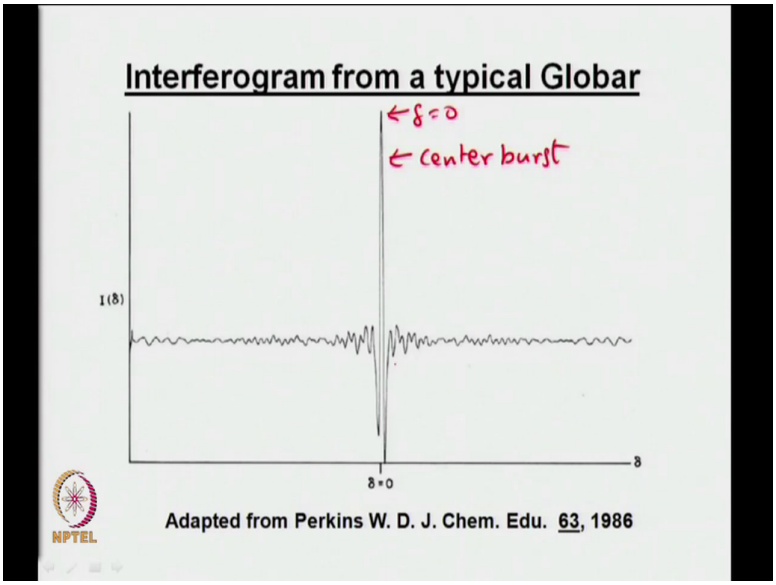

Now, let us look at this, this is a typical interferogram you get from a globar. You can see what is you can see what it means, you see again here this is the peak at zero path difference

and after that everything else almost you see nothing right there is almost everything just died down. So, quickly right and because globar has a huge range of frequencies, this is typically what is happening there is almost nothing at the flanks of this most intense thing. So, this is again delta is equal to 0 ok.

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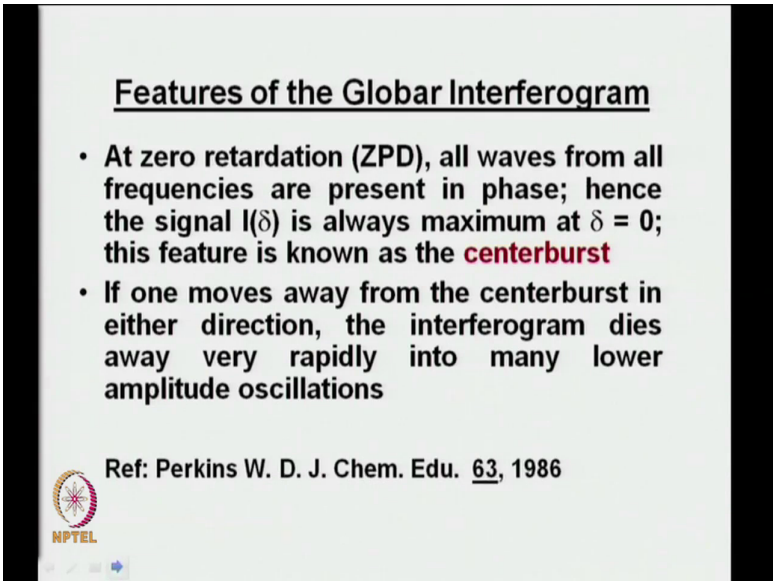

So, then what are the features of globar interferogram let us now look at it. So, at zero retardation all waves from all frequencies are present in phase that is what I was telling you. Hence the signal I delta is always maximum at delta is equal to 0 I delta means the intensity of the interferogram at delta which is the path difference. So, this feature is known as the center burst; that means, at the center you have a certain burst in signal good.

Now, if you are with that then what happens is, if one moves away from the center burst in either direction the interferogram dies away very rapidly into many lower amplitude

oscillations that is why if you would remember that is way if you would remember the previous slide, you can see this is your center burst right. So, this is your I can write this is your centre burst and then on the flanks you almost have nothing so; that means, there oscillations have totally died down because of destructive interference ok.

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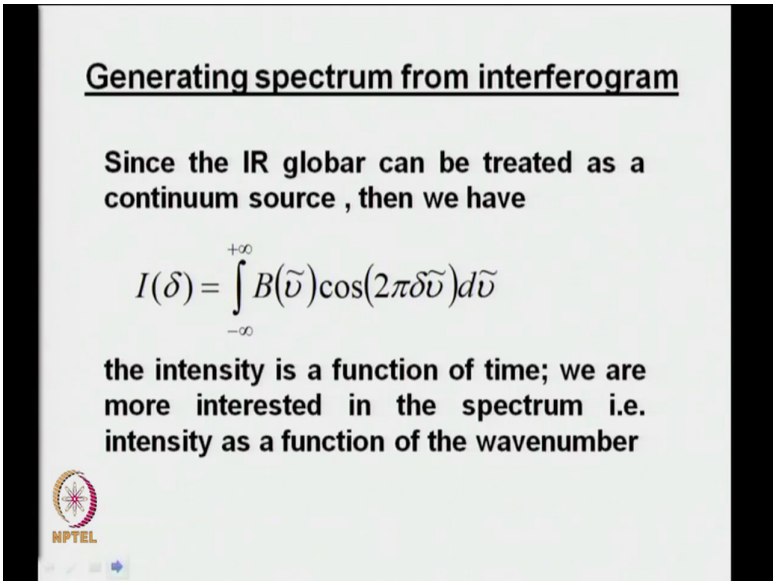

Now, how do you generate this spectrum from the interferogram right? So, this is what I was telling you, I have the interferogram now how do I get the spectrum because that is what I am interested in. I do not want to know they interferogram that is fine the instrument uses it, but I want the IR spectrum I want the spectrum of the vibrating bonds and everything.

Now, since the IR globar can be treated as a continuum source as I said its a black body emitter its feeding I means its emitting continuous radiation, then what we can say is then we can say that the intensity as a function of the path difference delta is equal to the integral over

the all space whatever distance it can move times B nu bar, where nu bar is your frequency in wave numbers times cosine of 2 pi delta nu bar d nu bar ok.

Now, the intensity I delta is a function of time why do I say it is a function of time because remember your path difference is centimeter and the beam is travelling that path difference and because it is travelling with path difference taking up some time to travel that path difference right and hence it is this interferogram is essentially a function of time that is why it is called the time domain.

So, the interferogram is in the time domain, but what do we need? We need the spectrum not as a function of time, but we needed as in the frequency domain. So, we have to convert to frequency.

So, what do we do? As it said out here we are more interested in the spectrum. Again the intensity is a function of time or the interferogram is an intensity I delta which is a function of time, but we are more interested in this spectrum that is intensity as a function of the wave number remember we talked about this wave number centimeter inverse ok.

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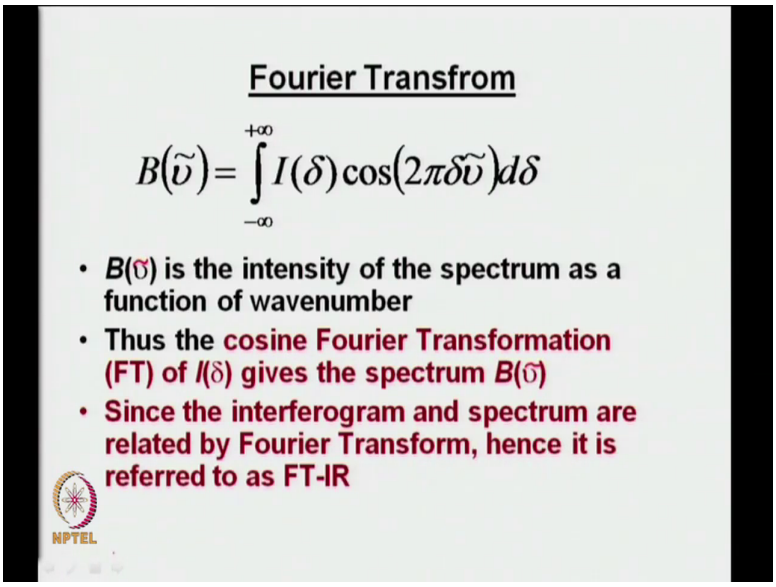

So, what do we do in that regard? What do we do is we do a mathematical transformation a mathematical (Refer Time: 30:31) known as Fourier transform. We says that B nu bar is equal to minus infinity to plus infinity I delta cosine of 2 pi delta nu bar d delta. So, before it was d nu bar now it is d delta before it was I delta now it is B nu bar.

Now, you can see what is B nu. So, this should read as nu bar. So, B nu bar is the intensity of the spectrum as a function of the wave number because this is what I want. I want the intensity as a function of wave number what was I delta? I delta was the intensity of the function of the path difference right delta I do not need that, I need the spectrum ok. So, that is what B nu bar is giving to you.

So, B nu bar what is it? Then I can say that the cosine Fourier transformation which is FT of I delta gives the spectrum B nu bar thus the cosine Fourier transformation FT of I delta gives

the spectrum B nu bar; that means, initially this interferometer is giving you I delta and then you are doing a Fourier transformation on the I delta to get what?

B nu bar which is the one you want to deal with which is the what you need that is why since the interferogram and the spectrum are related by Fourier transform hence it is referred to as FITR. I hope it is now clear to you why it is refer to an FTIR.

Now, the other you know the other way of doing IR is called as dispersive IR. Dispersive IR means the same thing you do in case of fluorometers absorption spectrophotometers where you have monochromators, they will disperse and you know you collect the corresponding or accrue the corresponding IR spectrum, but that is dispersive there is your dispersing with the help of monochromators, but in this case you are not doing that.

What you are doing is, you are doing a mathematical trick at your Fourier transforming your interferogram which has the spectral information encoded that is what it means by encoded which is encoded in the interferogram and your extracting it by doing a mathematical trick which is the Fourier transform to get B nu bar.

Now, will not go into this, this has many advantages ok. So, FITR has many advantages over the normal dispersive IR and that is why nowadays you will see when people are talking about you know protein folding and rather IR spectroscopy, then mostly people use FITR spectroscopy not the normal dispersive IR ok.

So, I hope I have made the point clear why FITR spectroscopy or why it is known as FITR not only IR.

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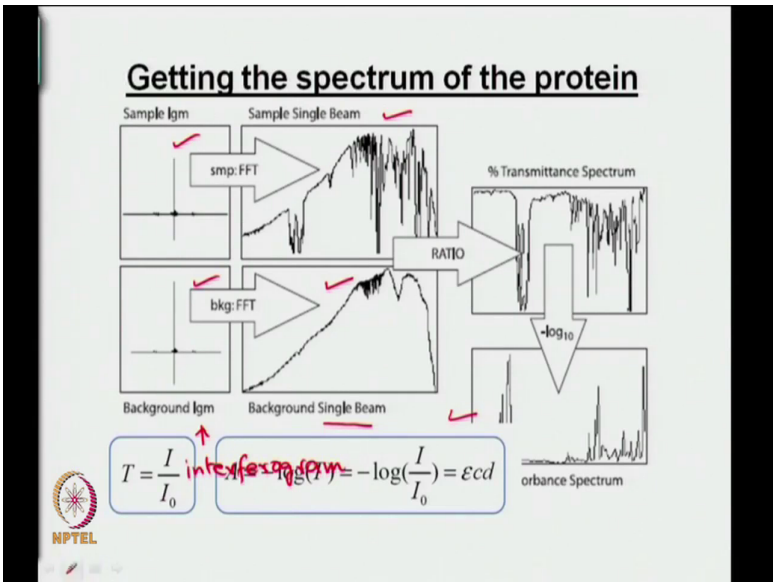

So, now, how to get the spectrum of the protein? So; that means, let us look at these two. So, you have this sample this Igm is referred to as interferogram is interferogram it is interferogram. So, you can see you have the sample out here, this is the corresponding interferogram of the sample you do a you do a Fourier transform FFT refers to as Fast Fourier Transform, but do not worry about that and then this is the corresponding the sample spectrum ok.

Then you have the background remember its a transmittance or an absorbance because we are we need the actual you know spectrum of your sample which is the protein, then you have to subtract sorry you have to get rid of the background right. So, then after the sample is done what you do is, you replace the sample with a background or either way you can do the

background first and then take the sample. So, then this is your background this is the corresponding spectrum from a background.

So, now what you do is, a ratio of this gives you the person transmittance and from the person that transmittance you to take a minus log of that you get the corresponding absorbance and this is what you see out here ok. So, this is what you see out here this is your corresponding absorbance spectrum.

Now, what is the deal? So, the deal is this, the deal is why is it called a sample single beam you can see this is referred to the single beam. The single beam is it is a single beam because simultaneously not being able to measure both. Once you are looking at the sample once you are looking at the what the background right or the blank and then you are doing you are mathematical analysis, then you are doing this ratios and converting in absorbance you needs to get the actual spectrum.

So, this is what I mean by writing this. So, T is equal I by I 0. So, let me see its I will erase this part just to make sure that you understand since I had it before I hope you will remember it.

So, here transmission is I by I 0 that means, the intensity of the sample over the intensity of the background which is incident light I 0. So, then A is equal to minus log of T and after that you get your absorbance spectrum ok.

So, that is typically how you will get is protein spectrum and in case of protein you background will either be buffer in H 2 O if you doing an transmission measurement or buffer in d two whatever.

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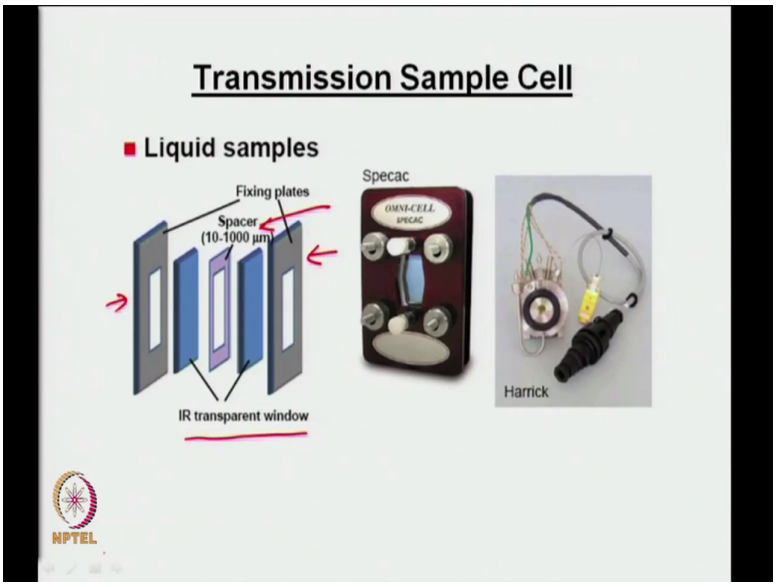

Now, what is a transmission sample cell look like? This is how it looks like, this is a liquid samples you can see it is for liquid samples you have these IR transparent windows, this is IR transparent windows they have to be IR transparent right because otherwise they will absorb all your IR radiation.

Then you have a spacer. Spacer this spacer what does this spacer mean? The spacer means that you can by changing the thickness of the spacer you can change the path and see you can from 10 to 1000 micron you can go and these are two fixing plates. So, these are two fixing plates which actually hold your sample cell together right ok.

And this is typically how sample holder would look like, this is as you can see from specac and then from Harrick scientific. So, you know sample cells can have also different connections

say for example, if you doing a protein folding study or unfolding study, you are going to ramp the temperature up.

So, you would need some accessory like a temperature jacket and a temperature controller and if it would be connected to something which should probe the temperature. So, all those things can be incorporated on a regular basis ok.

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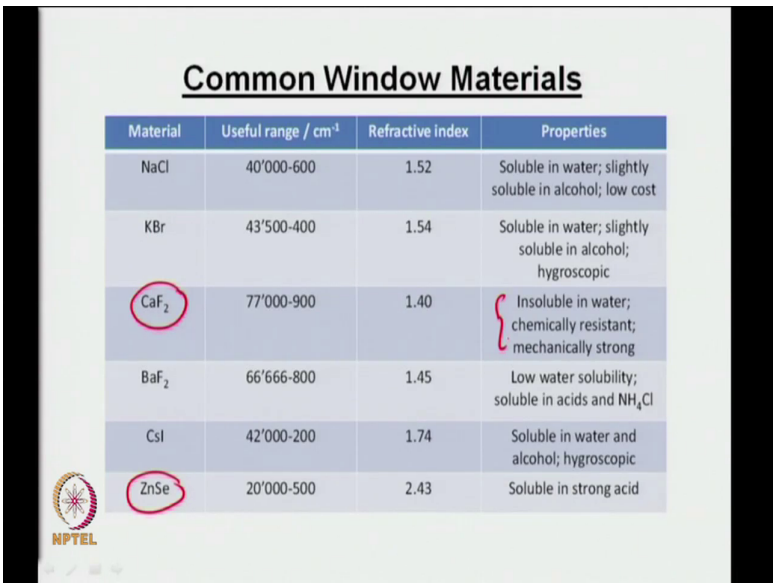

What are some of the common window materials which are being used for your sample cells? If you are doing a transmission measurement right remember the transmission measurement we talked about one of the materials is NaCl, one is KBr, one is calcium fluoride, barium fluoride, cesium iodide, zinc selenide.

Now two the most which are mostly use are one is calcium fluoride and the other one is zinc selenide and if you have look if you can correspond look at the useful range. So, these are in centimetre inverse you read it as. So, for NaCl it is 40600 you know something like that is how you read.

Now, the problem with NaCl and KBr that they are soluble in water, calcium fluoride is one of the things which is one of the substance which is mostly used because it is in soluble in water chemically of resistant and mechanically strong.

So, this is very important, this is an advantage. Now look at zinc selenide it is soluble in strong acid. So, if you have a very corrosive acid or something like that then zinc selenide would not be the proper one to do good. So, these are the common window materials and one of the most common ones which is being used nowadays is calcium fluoride right.

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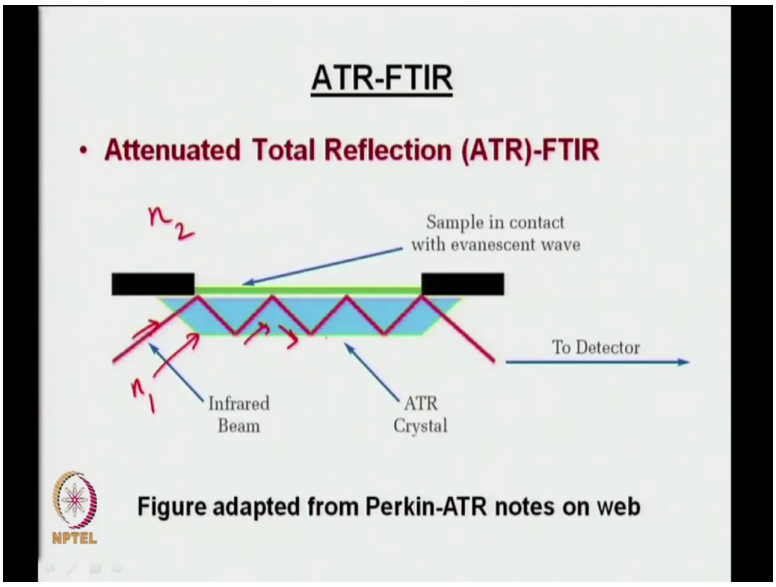

Now, remember this is the last leg of our IR discussion and we are going to talk about ATR FITR right. Now why do we need ATR FITR? Where did we first encounter this? Now let me tell where you first encounter this let me remind you.

We were talking about this problem of water background subtraction and we said that if I can remove or if I can decrease the path length, then the background subtraction is obviously, minimized because the path length is small because of the small path length, the water will not be absorbing that much right. So, the path length is small.

Now, however, for that the problem was that I would need a higher protein concentration ok. Now ATR is a way of doing or you know measuring protein absorbances or FITR spectra in

eco solutions or even suspensions right; that means, where the sample is not fully soluble in water.

What is the principle of this or what is the first the meaning of ATR? So, again ATR as I mentioned briefly before is refer to as attenuated total reflection and because its done with the FITR, it is done with applying the Fourier transform in the IR range its called ATR FITR.

So, this is what it is. Now its attenuated total reflection if you go back to your physics principles remember something known as a critical angle. If your angle is beyond the critical angle between two metrics or different refractive indices then nothing will go out so; that means, your matter will not be reflected refracted rather all ways would be totally internal reflected into the same medium.

So, this is typically what you see out here. So, this is say n 2 this is say n 1 right. So, n 1 in this case is this one which is your IR internal reflection element I will come to that later. So, you can see what happens to this IR beam it goes, but it never it never goes up right its always is following this it is always following this is always staying in side the element, it is never going towards n 2 so; that means, you have a total internal reflection right and finally, this goes to the detector.

So, this blue one is referred to your ATR crystal so; that means, you will need an ATR crystal by which you will make sure that this IR light is totally internal reflected right so; that means, critical angle it is going to play a big role out here.

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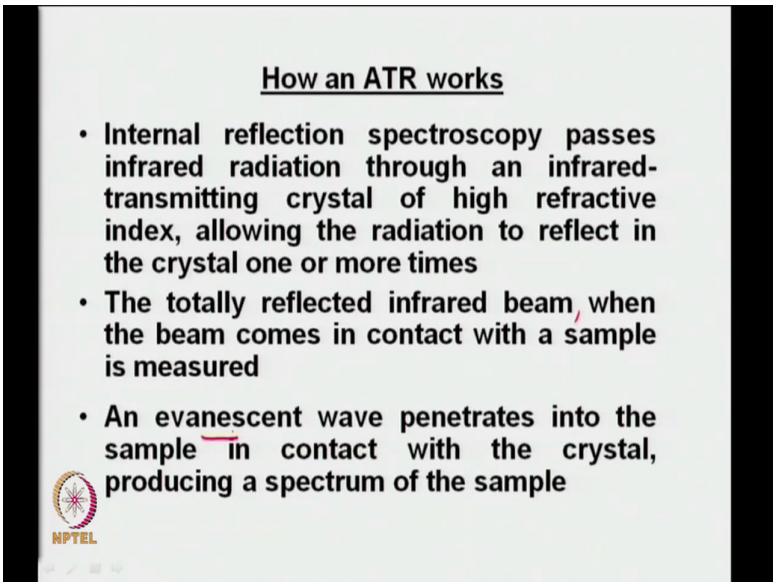

So, how does an ATR work? Internal reflection spectroscopy passes the infrared radiation through an infrared transmitting crystal of high refractive index right allowing the radiation to reflect in the crystal one or more times. So, one if its one time it is called a single bounce ATR, if it is more time this called a multiple bounce ATR.

Now, the totally reflected infrared beam when the beam comes in contact with a sample is measured right. So; that means, once you have the totally internal reflected beam and you put the sample there then you measure that.

An evanescent wave now this is important an evanescent wave what i mean by an evanescent wave? An evanescent wave penetrates into the sample in contact with the crystal producing a spectrum of the sample ok. So, couple of things one you have an ATR crystal which is the

very high refractive index higher than your sample that you need to have otherwise you will not be having this critical angle ok.

Number 2, once you have a sample then you will you pass your IR light you collect whatever IR light is coming out and then you use that to get spectral information for your sample, but then how do you get the spectral information for your sample if everything is internal reflected? You know that is not the case right otherwise you will not be getting in information.

So, what happens is, during this internal reflection a part of this actually goes in; that means, goes into the sample very small depth will see how much the depth is typically or how you know what parameters are depends upon and then whatever small path length whatever small path length, it goes through the sample absorbs it right and then you collect the corresponding spectrum the interferogram and then finally, you get the IR spectrum.

Now, what is the good part? The good part is no matter how much water you have you will see soon that the penetration depth is in the order of a few microns right; that means, the penetration one micron, two micron depending on that. So, it is very small and because if it is very very small the background of the water would not be that high, but still you will be getting a decent signal if your protein concentration is relatively high.

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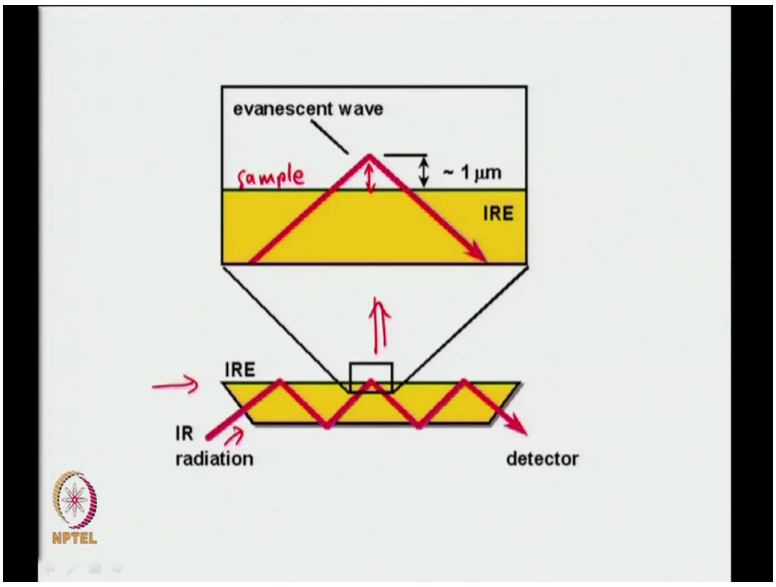

So, this is what I was meaning. So, you can see this is the this yellowish one is your IRE called the Internal Reflection Element which helps in internal reflection. So, this is of high refractive index. So, the IR radiation goes like this, this is the IR radiation now what you have done is, what we have done is here we have zoomed into this portion. Once we have zoomed into this portion you look at this portion once you have zoomed into this portion what do we see?

What do you see out here is a little bit of this evanescent wave which is gone into the sample because the sample is here this is your sample and its your its your out of a micron. So, this is the evanescent wave which actually gives you or which finally, gives you your spectrum because this is the one which the sample is seeing and absorbing right.

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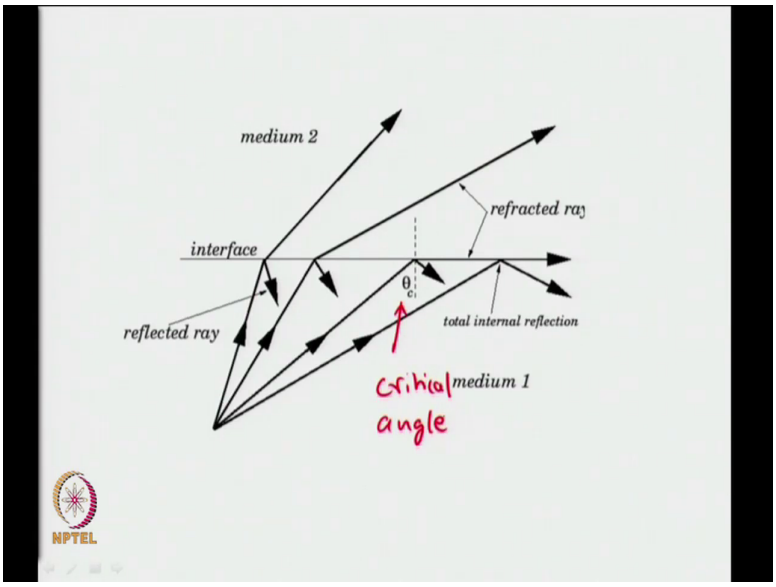

So, this is what I was meaning by this critical angle. So, you can see if this is medium 2 this is medium 1 right. You can have these rays coming from medium 1 to medium 2 at different angles, now once you reach a certain angle known as critical angle. So, this your critical angle, this is your critical angle at the critical angle and after that.

So, at the critical angle this is the angle where you start to have this total internal reflection and beyond the critical angle; obviously, the further you go; that means, the lesser or the more and more is the amount of total internal reflection inside the medium 1 itself ok. So, this is essentially what it is based on ok.

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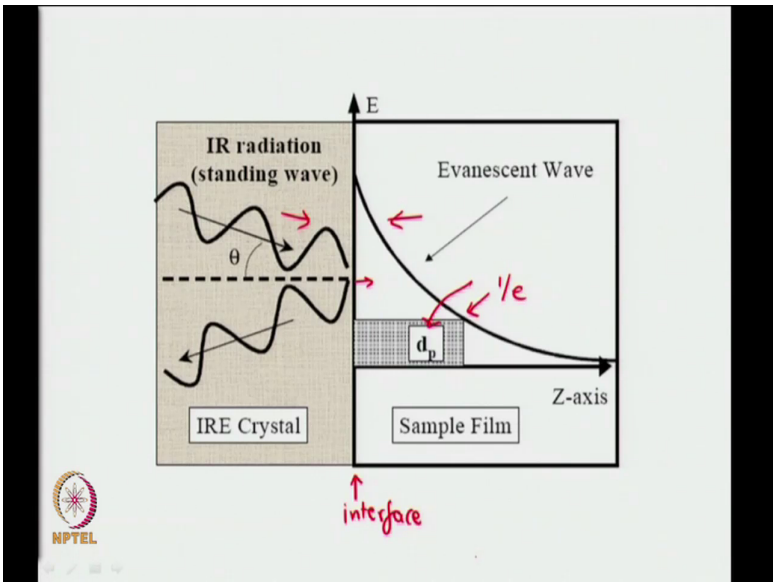

Now, when we were talking about this evanescent wave, you can see what I was meaning. This is the IR standing wave which is coming like this right now look at this evanescent wave. So, this is evanescent wave, look at the look at how the evanescent wave is decaying. So, what you are seeing.

So, this is your sample film right this is your crystal and sample interface right this is the interface, if I write the interface out here. Now once you at the interface now what is happened is this evanescent wave gone into a little bit right as you saw in the previous slide. Now, after that it will not still like that forever or I mean how would it depend how; that means, the further you move away from the from the interface how would this how would the profile of this electric field look like?

So, you can see it kind of falls down exponentially, as we move along the z axis; that means, as we move along the z axis means as your moving away from the interface; that means, you are moving more and more into the sample and what is d p? d p is something referred to as the penetration depth you will soon see. So, this is a very useful parameter d p, you will soon see this in the next slide.

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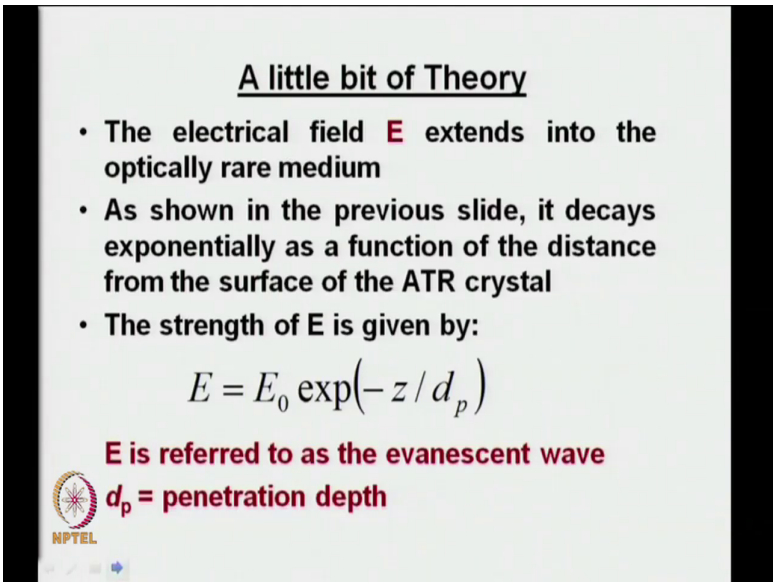

So, d p is referred to as the penetration depth ok. So, as I said as I promised you because you have been talking about this penetration depths and a little bit of it going into the sample, you know let us look at some theory just the basic theory which will keep you going.

So, the electrical field E which is coming from the IR extends into the optically rare medium right. Remember the ATR crystal n 2 or whatever n 1 of the ATR crystal of the internal reflection element n 1 is having a higher refractive index than n 2 which is optically rare

medium. So, even you are at the critical angle of beyond a little bit of it actually goes into right.

As shown in the previous slide it decays exponentially as a function of the distance from the surface of the ATR crystal that is what we just saw, it was decaying exponentially as you moved away from the interface now the what is the strength of the E given by this E?

So, the strength of the E is given by E 0 E 0 times E to the power which is exponent. So, what is exponents? So, E to the power minus z by d p. So, what is z? z is a variable your moving along the z axis right and what is d p? d p is the one which was telling you to be the penetration depth in the previous slide.

So, as I said E is referred to as the evanescent wave and d p is the penetration depth.

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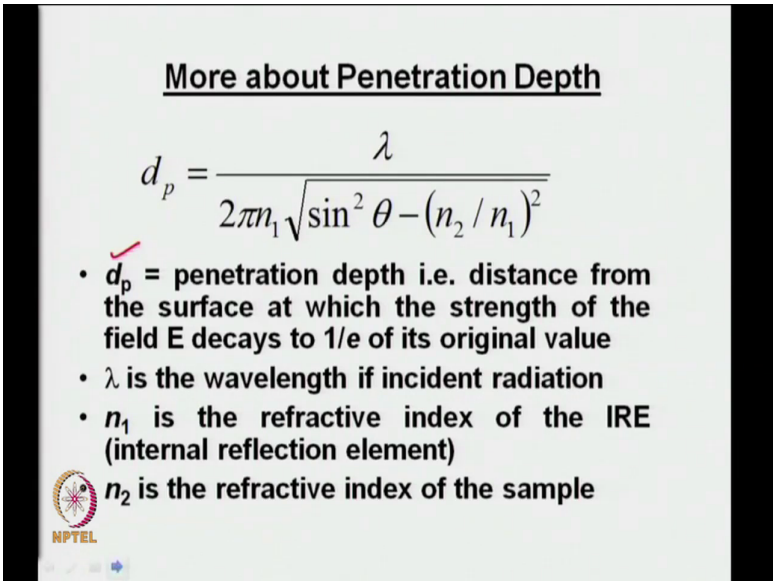

So, little more about penetration depth; obviously, you are going to ask what is d p depend upon? So, this is what d p depends upon. It depends upon it is equal to lambda; that means, its linearly proportional to lambda so; that means, higher the lambda; that means, longer the lambda more is the penetration depth. Now what you have on the denominator you can see of n 1 and you have n 2.

So, again d p is the penetration depth we discussing this one d p, it is the penetration depth what is it? It is the distance from the surface at which the strength of the field E, the field E which has which is the evanescent wave field it decays to 1 by E of its original value it decays to 1 by E of its original value. So, what I mean by that this I can show you here. So, this is what I mean. So, here this is what d p is that means, your evanescent wave has decayed to about 1 by E of its original value ok.

What is lambda? Lambda is the wavelength of the incident radiation what is n 1? n 1 is the refractive index of the IRE or Internal Reflection Element and what is n 2? n 2 is the refractive index of the sample. So, the bottom line is n 2 has to be greater than n 1 rather your n 1 which is the n r n IRE or n 1 of the internal reflection element has to be greater than n 2 otherwise you will not be having this internal reflection.

Please brush up your notes on Snell's law and this critical angle of reflection it is very useful for this for understanding this attenuated total reflection, I will not be having time to go through it.

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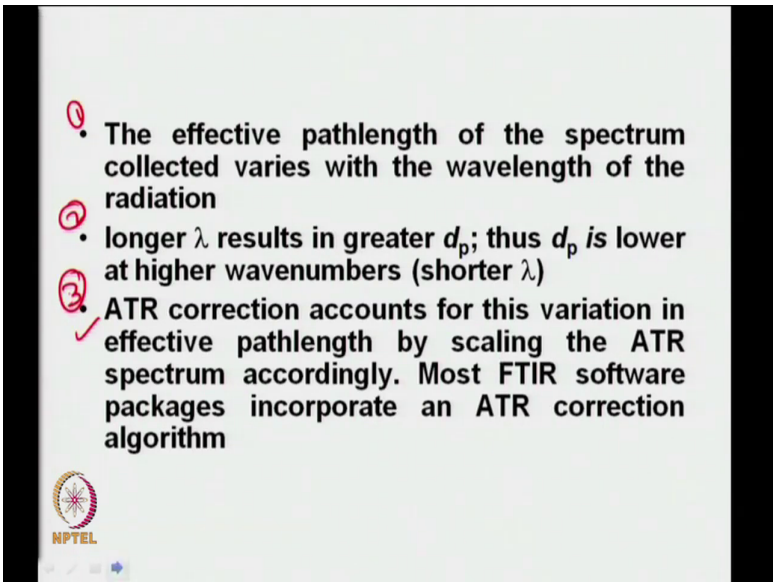

Now, as you can see the effective path length of the spectrum collected varies with the wavelength of the radiation ok. So, longer lambda results in greater d p thus d p is lower at higher wave numbers.

So, what it means is, first of all the effective path length of spectrum collected varies with the wavelength of the radiation, it does because d p is proportional to lambda longer lambda, higher lambda more will be the d p, smaller lambda means higher wave number because smaller lambda means high energy and wave number means proportional to energy; that means, smaller or lower lambda will result in lower penetration depth.

So, you what your having is you are having a variation in penetration depth, as you look at the different IR frequencies right now that might be a problem it can be a problem. So, what know the presently the instruments include is, the include something known as ATR correction.

So, this ATR correction if you read this last point, this ATR correction accounts for this variation in effective path length by scaling the ATR spectrum accordingly. The most FITR software packages incorporate an ATR correction algorithm ok.

So, the you know this slide is very informative, the first it says that. So, this is point number 1 what does it say? It says that the path length of the spectrum collected varies as the wave length of the incident radiation that is going to happen now how does it vary? So, this is how it varies longer lambda results in greater d p. So, obvious inverse the shorter lambda will result in smaller d p and then to correct for that to correct for that, nowadays the instruments they have this ATR correction factor which is in built ok.

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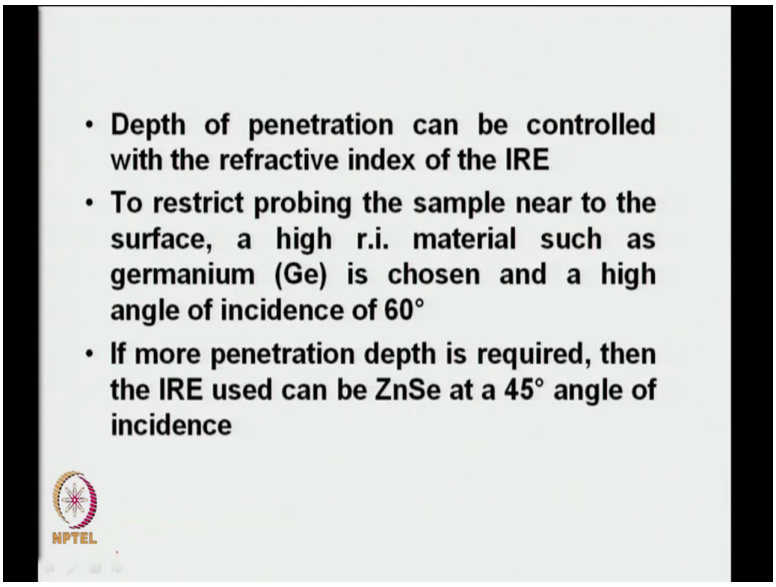

Now, we moving to the next slide how can you control the depth of penetration? That means, see if it depends upon the IRE the refractive index that was in the denominator it depends upon the lambda; that means, you can control the penetration depth. So, depth of penetration can be controlled with the refractive index of the internal refraction element.

Now, to restrict probing the sample near to the surface; that means, if you want only surface information not too far into the sample a high refractive index material such as germanium is chosen and a high angle of incidence of 60 degrees ok. The high angle of incidence makes sure that you are having that your beyond the particular angle and that you are not having any of this going into except the evanescent wave ok.

If more penetration depth is required then the IRE used can be zinc selenide at a 45 degree angle of incidence. See suppose you want to go a little more into the bulk get bulk information. So, you have to use a different crystals says zinc selenide ok.

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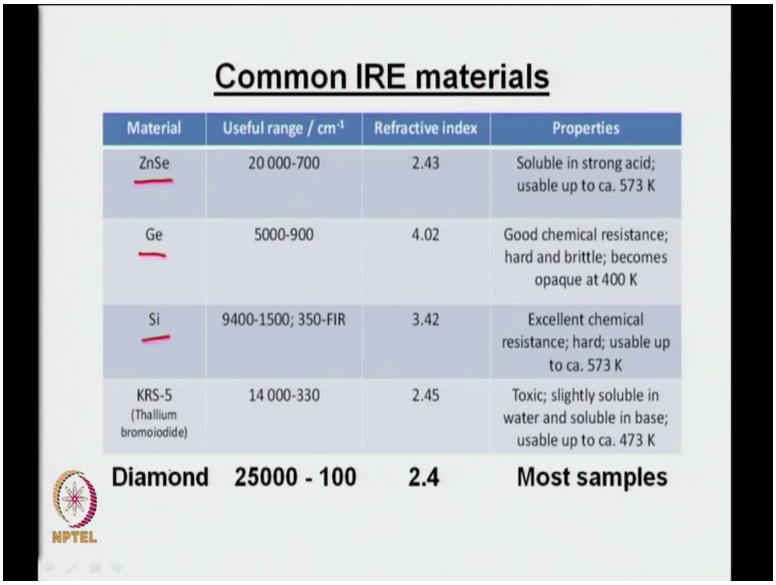

So, these are the common IRE materials as I said one is zinc the zinc selenide, this is useful range, we can see the properties it soluble in strong acid that can be a problem, look at germanium, the useful range in this case the less 5000 to 900, the refractive index pretty high you know that is what you need, it is very good chemical resistance is hard and becomes opaque at 400 Kelvin that might be a problem if you are doing with high temperature studies ok.

Now, silicon it has excellent chemical resistance ok, but it is not that much used, the other one is thallium bromoiodide I will not have talked about that, but let us look at the last one which

is diamond. Diamond is nowadays more coming into existence or being used by this different companies a manufacturing FITR spectrometers.

So, the range is from 25000 to 100 centimeter inverse and most of the samples. You know diamond is extremely raise into raise into correlations very hard and hence we can use that right. It would be very good substitute for either zinc selenide or germanium and people are using that.

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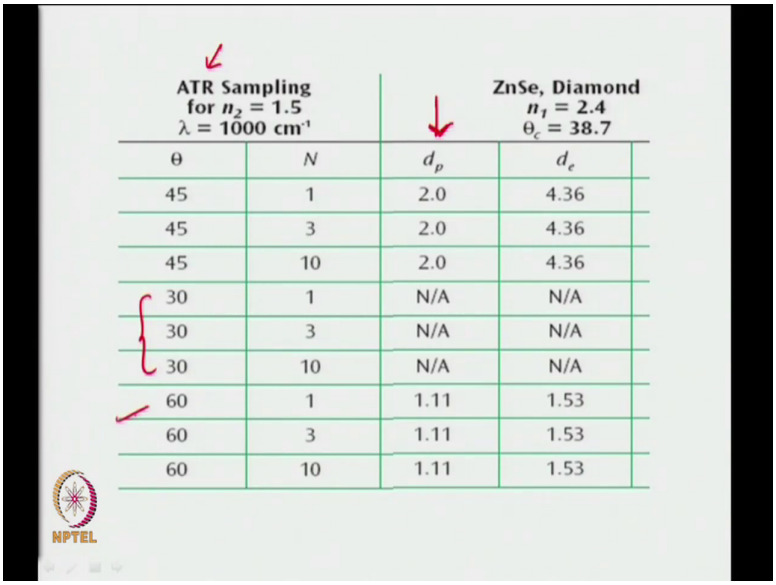

I would I am coming close to the end of the class, I just look at you know some comparative studies. So, here what we are looking is we are looking at an ATR sampling. So; that means, we are looking at some ATR measurements for n 2.

n 2 means the sample refractive index n 2 is 1.5 and what is lambda? Lambda it see lambda is equal to 1000 centimeter inverse it does not mean this lambda it says that lambda, which corresponds to 1000 centimeter inverse you can convert and see what micron units corresponds to ok.

So, here on the right side we are talking about the zinc selenide and diamond right both of them have the safe refractive indices and hence that the same critical angles you can see at theta is equal to 45 degree. So, see theta c is equal to 38.7 critical angle is 38.7.

So, theta 45, 45, 45 you can see what the d p is. So, do not worry about n write now, the penetration depth is 2, 2, 2 for zinc selenide this did not take care of do not worry about this, just look at the penetration depth do not worry about this d e I forgot to get rid of it because I am not discussing it, but this d p is the penetration depth this is the table your going to be looking at ok.

Now, see what happens to 30 when you come to 30. When you come to 30 what happens is this? When you come to 30 you can see this d p is not applicable why is it so? It is not a applicable because 30 is less than the critical angle. See if it is less than the critical angles then what you are not having anyway evanescent wave huge part of a wave is actually refracting into that right. So, refracting into the sample; so, it is no longer your internal refraction.

Then again you come to 60 right and you correspondingly see how the depth of penetration are varies, at theta is equal to 45 the depth of penetration was typically 2, at theta is equal to 60, the depth of penetration is typically 1.1 ok.

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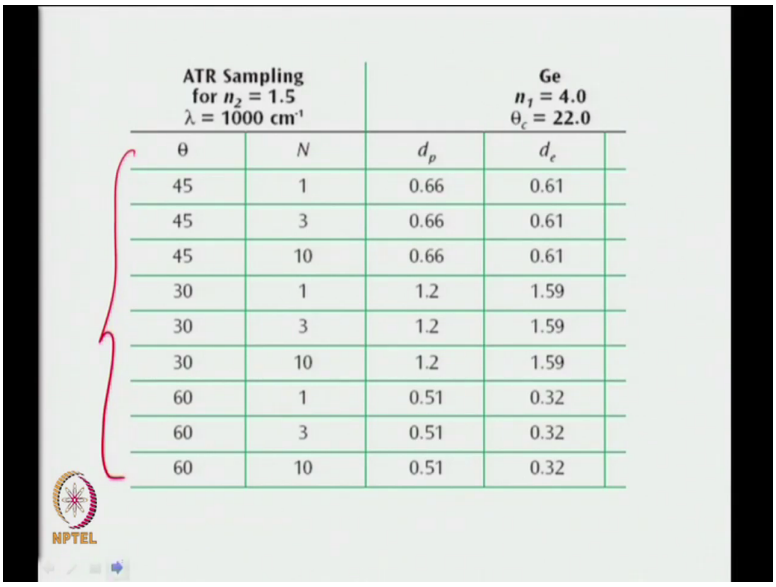

Now, for the germanium if you are using germanium as a ATR crystal now see what happen in this case. So, germanium theta c is 22. So, because theta c is 22 all these angles all these angles are greater than the critical angle and because 45, 30 and 60 are greater than the critical angle I get all these angles you are going to have internal reflection ok.

But however, see what is happened. For germanium at 45 this depth is 0.66 its in terms of micron its 0.66, in case of zinc telluride diamond it was about 2 micron right when we come to 60 its about 0.51, at 30 which you cannot do for the diamond and zinc away it is about 1.2.

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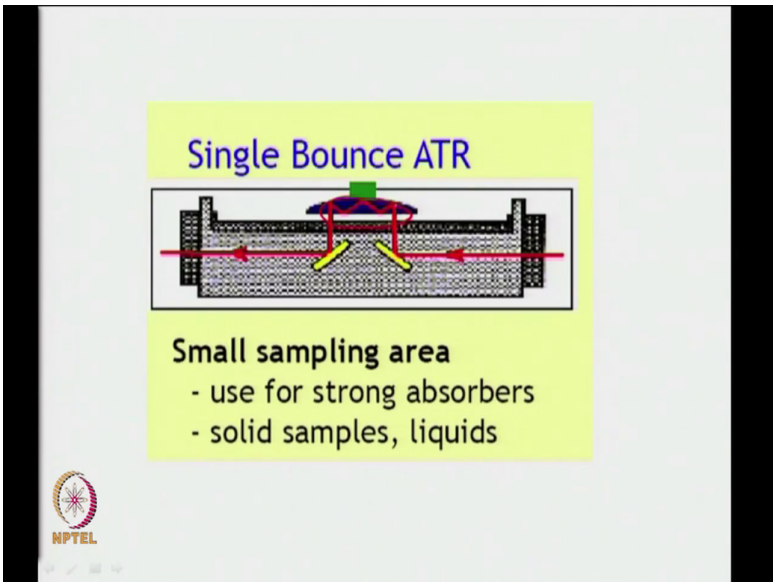

So, this is just to have an idea and finally, I just want to tell you these things what is a single bounce ATR, the single bounce ATR is one case where you can see it is bouncing only one time right it does not spend much time within the sample, it just bounce is one time often then goes into your detector.

So, it is generally use for strong absorbers and solid samples liquids strong absorbers if it absorbers very strong, then it might be a problem see you would not be getting a lot of stuff coming through.

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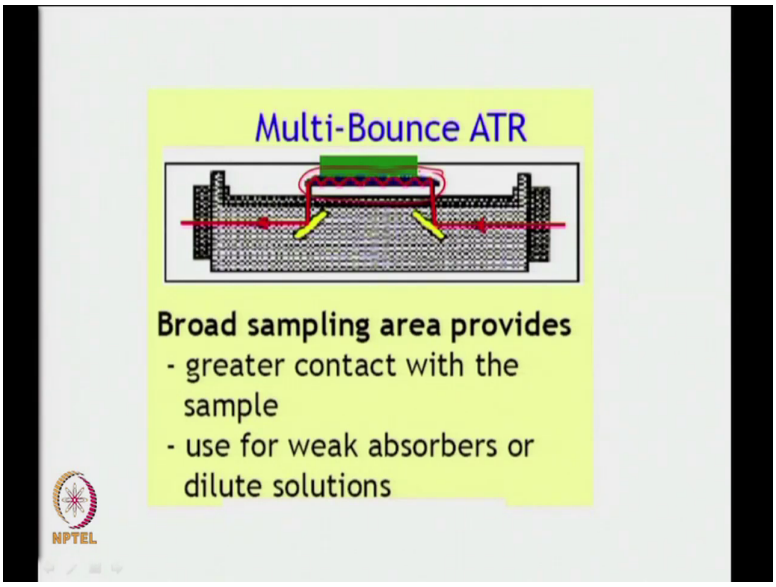

And then this is the case from multi bounce ATR you can see how many bounce is it is making. So, how many times its traversing. So, it is a broad sampling area, it provides greater contact with the sample and it used for weak absorbers and dilute solutions ok.

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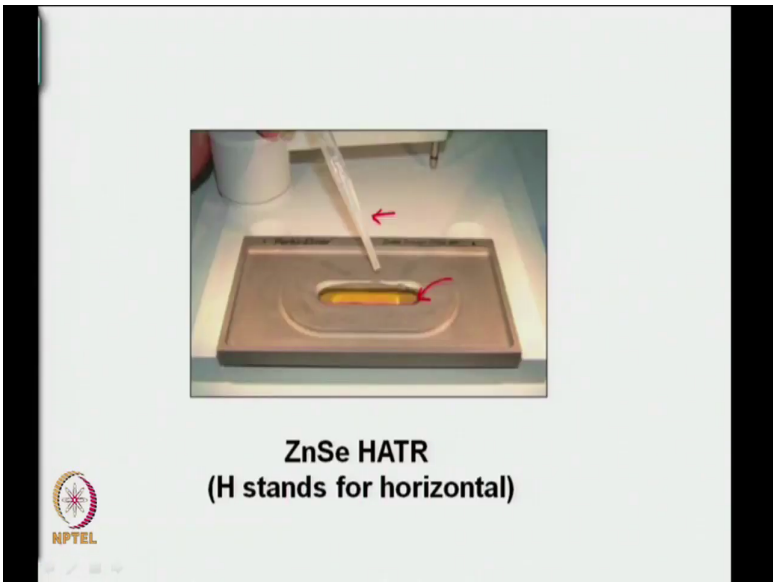

And finally, before I end the class I just what do you take a look at this ATR element. So, this is the zinc selenide H ATR it is referred to as horizontal ATR its horizontal ATR because it is kept like this on your FITR spectrometer and the sample jacket and you can see your adding someone is adding the sample on this zinc selenide crystal and after that you are going to get the spectrum.

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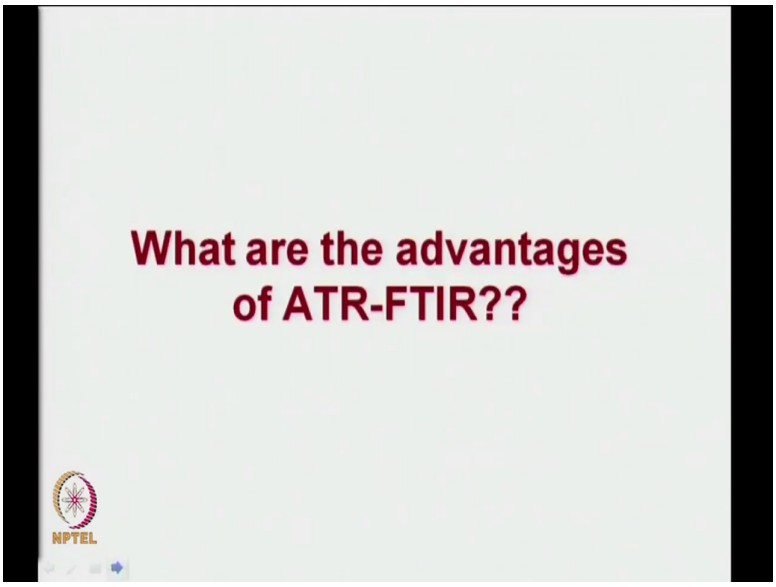

So, I would leave or stop this class with this question, you solve what this ATR is you know this is what the ATR looks like, this is a standard ATR measurements someone is doing and then what are the different aspects of an ATR, you have to keep in mind critical angle, you have to take care of the IRE that is the internal reflection element, you know what your penetration depth is it will depend upon what? Your lambda your n 1, your n 2 and all these things.

Now, the question I want to leave you with this or leave you with is what are the advantages of ATR FITR right. I have mentioned some, but what I want you do is think about it right and let me know in the next class. So, what it does is, you know it brings us to the end of the IR or whatever IR related stuff I want to talk about, I did spend some time on instrumentation

because I want to have a you to have a feel of it because these instruments are being readily you know not one readily there being frequently used in studying protein spectra ok.

So, next class what I will do is I will start with c d which is one of the very important spectroscopic tool for measuring protein conformation or doing protein conformation analysis.