Analytical Technologies in Biotechnology
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Module - 6
Spectroscopic Techniques
Lecture - 3
Infrared and Fluorescence Spectroscopy

In this lecture, we are going to discuss two techniques; one is Infrared Spectroscopy and next one is Fluorescence Spectroscopy. Now, infrared spectroscopy is an example of absorption spectroscopy, whereas fluorescence spectroscopy is an example of emission spectroscopy. Now, infrared spectroscopy, which falls under absorption spectroscopy, here transitions which are taken into account are the ones, which happens between the vibrational labels in the ground state. That is when a particular molecule or atom absorbs energy equivalent to the differences in the two vibrational labels, then they will absorb that particular form of energy.

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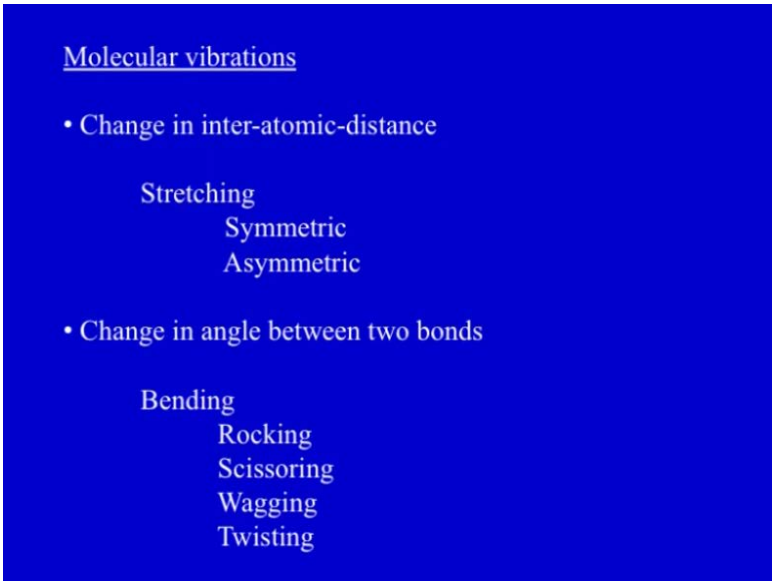

So, infrared spectroscopy exploits the fact that molecules have specific frequencies at which they rotate or vibrate, now when we consider molecules and they are like bonds between these molecules. These are like they acts like a spring actually, which can stretch bend or rotate a bit around, they can rotate and you know produce differences or you can say there is a constant movement of these atoms around these bonds. And they

are either vibrating, due to certain absorptions and these transitions are measured in IR spectroscopy.

So, if we have to determine, if some compound is IR active or not then, if you consider for example, carbon dioxide then, carbon dioxide has 2 Oxygen, which are double bonded to the central carbon. So, here dipole moment from the symmetric stretch of the left oxygen will be offset by the symmetric stretch of the right oxygen and therefore, it is considered as IR inactive.

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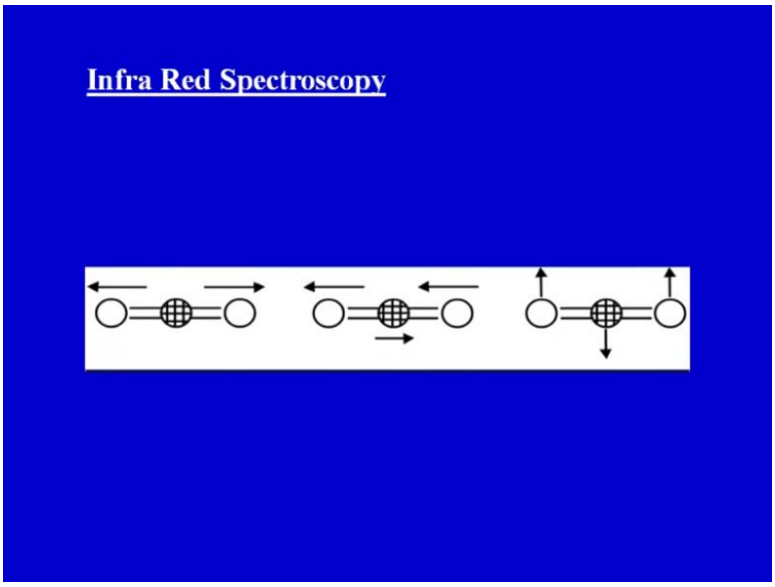

So, if you can see here, they are like stretches which are in offset by each other, which like and they could be different kinds of stretches in here. Now, in very simple terms, infrared spectroscopy can be defined as absorption measurement of different IR frequencies by a sample, which is positioned in the path of an IR beam. So, if a sample is put in IR beam, there will be absorptional and as I said, there will be transition will be measured in vibrational labels of ground state.

Now, the photon energy, which is associated with this part of the infrared spectrum is around 1 to 15 kilocalorie per mole and it is not it is like only, you can say large enough to excite electrons and induce vibrational excitation of covalently bonded atoms and groups. Now, the covalent bonds in the molecules, these are not rigidistics, these are not like static here, but rather they could be in molecular model terms, like they are mostly

like springs actually or you can say stiff springs and these could be stressed bend and other motions could happen in there.

The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample, so different chemical groups like, when IR spectra is taken and in the sample, if they are different types of chemical groups. Each group depending on it is chemical environment conformation and chemical structure and chemical environment means environment around it like solvents and other parameters.

That will affect it is motions like, stretching or bending and that particular characteristic feature of these every type of bonds will give a characteristic spectra, which is a very helpful in determining these, chemical functional groups in a particular sample or particular molecule. Now, using various sampling accessories IR spectrometers can accept a wide range of sample types, which could be range from gases liquids and solids and they are certain limitations also for these.

So, IR spectroscopy is a very important and very popular tool, for structural elucidation and compound identification, now here, when we say structural elucidation it does not mean 3 dimensional structure. But, it will tell about, the functional groups, it will tell about also IR spectroscopy like FT IR spectroscopy can tell about, secondary structural information and also changes in it is structure, like say in different environmental conditions.

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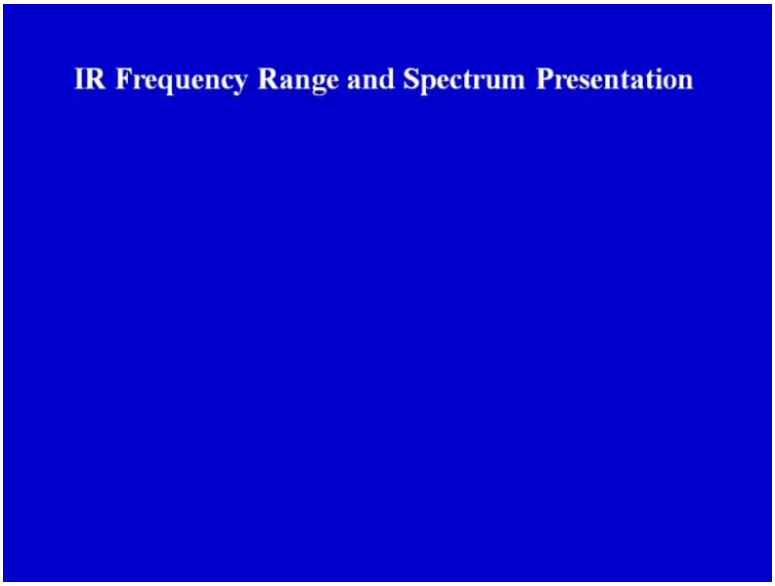

If we consider IR frequency range or the spectrum here, it lies between the microwave and visible parts of the electromagnetic spectrum, this we have discussed in the last chapter actually. Now, far IR has low energy and may be used for rotational spectroscopy and mid IR may be used to study the fundamental vibrations and associated rotational vibrational structure. Then, there is near IR can excite overtone or harmonic vibrations.

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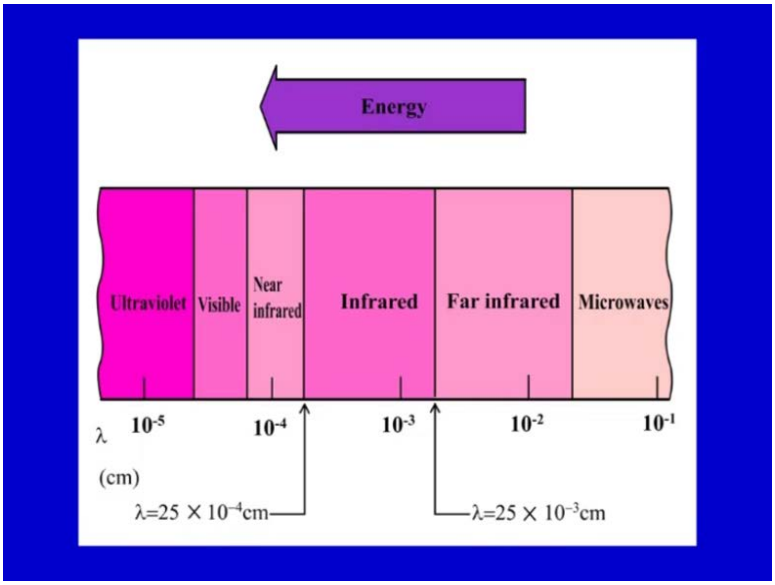

So, if you can see here, this is the spectrum in electromagnetic spectrum infrared lies between visible, that is near infrared and then, this is far infrared and this is the between the microwave and visible regions actually. Also like here, if you can see this lies between 10 is to minus 4 to around here, 10 minus 2 centimeters or you can measure it in different forms actually.

Now, infrared radiations spans a section of the electromagnetic spectrum and have a wave numbers from roughly 13000 to 10 per centimeter, now wave number is inverse of wavelength actually and that is the most commonly used in here or frequency is utilized here, to plot absorption spectrum. So, or we can say it in terms of wavelengths is around 0.78 to 1000 micrometer, now it is bound by the red end of the visible region at high frequencies and the microwave region at low frequencies.

So, IR absorption positions are generally presented as either, like I said it is mostly presented as wave numbers, rather than wavelengths, now wave number defines the

number of waves per unit length actually. So, thus wave number are directly proportional to frequency, as well as the energy of the IR absorption. The wave number unit that is per centimeter or reciprocal centimeter is more commonly used in modern IR instruments, that are linear in the per centimeter scale, in contrast wave lengths could be used, which are inversely proportional to the frequency.

And their associated energies wave numbers and wave lengths, can be inter converted like, wave number is in reciprocal centimeter and wave length will be in centimeter actually, so that way, you can convert that. Now, IR absorption information is generally presented in the form of spectrum with wave number as on the x axis and absorption intensity or transmittance as the y axis like, in U V visible also we have seen, it is absorption is on the y axis as I have shown you and the wave length or here wave number is on x axis.

And also transmittance could be taken, which is the ratio of the radiant power transmitted by the sample to the radiant power incident on the sample, as you have seen in the beer's law in U V visible spectroscopy. So, here also, so same principles applies, now the transmittance spectra provide better contrast between intensities of strong and weak bends, because transmittance ranges from 0 to 100 percent whereas, absorbance ranges from infinity to 0.

So, one should be aware about or the one, who is doing analysis should be aware that same sample will good give quite different profiles for the IR spectrum, which might be linear in wave number and IR plot where, which is linear in wave length. So, that has to be taken into accounts, so it will appear, as if some IR bands have been contracted or expanded, if you compare the wave number spectrum to wave length spectrum. And the frequency used in mid IR region is around between like 4000 to 400 per centimeter or 2.5 to 25 micrometer.

The far IR requires the use of specialized optical material and sources, it is used for analysis of say organic, inorganic and organometallic compounds involving heavy atoms, it provides useful information to structural studies, such as the conformation and lattice dynamics of a samples. Near IR spectroscopy needs minimal or no sample preparation, it is very typical, it offers high speed quantitative analysis, without consumption or destruction of the sample. And the instrument can often be combined

with U V Vis and coupled with fiber optics device for remote analysis, near IR spectroscopy has gained quite increased interest as a particularly in process control applications.

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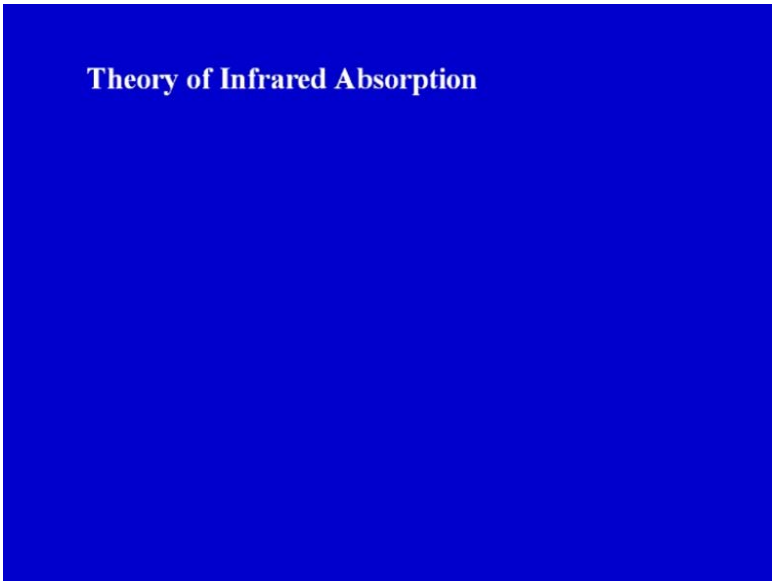

Now, if we consider theory of infrared absorption here, I think all of you are aware that, at very low temperatures that is temperatures above, absolute 0, all the atoms and the molecules will be like at any temperature for that matter, if you are above a particular like lowest temperature that is absolute 0. Then atoms or the molecules, they are not static, they are in continuous vibration with respect to each other and their vibration can be in different directions actually and in different ways.

So, when the frequency of a particular specific vibration is equal to the frequency of the IR radiation, directed on the molecule, the molecule absorbs the radiation and each atom has here 3 degrees of freedom actually, corresponding to motions along any of the 3 Cartesian coordinates that is x y and g. Now, major types of molecular vibrations, that will be there are they stretching and bending and they could be others also more complex 1 and infrared radiation is absorbed and associated energy is converted into these types of motions, so that is a continuous process, which keeps on happening.

Absorption will involve discrete quantized energy levels like, as you have seen in vibrational labels within the electronic label, however the individual vibrational motion is usefully accompanied by other rotational motions. And these combinations will lead to

the absorption bands not the discrete lines, commonly absorbed in mid IR region, like we have discussed earlier also that, there are lot of discrete lines, but these are closely spaced lines, which appears as bends. In simple terms, if we say how it works actually, the IR spectra obtained by detecting changes in transmittance or absoption intensity, as a function of frequency. And most commercial instrument separate and measure IR radiation using, either dispersive spectrometers or Fourier transformed spectrometers.

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Now, dispersive spectrometers, they were introduced in mid forties and they were widely used spectrometers and they provide the robust like, these are instrumentation, required for the extensive application of this technique.

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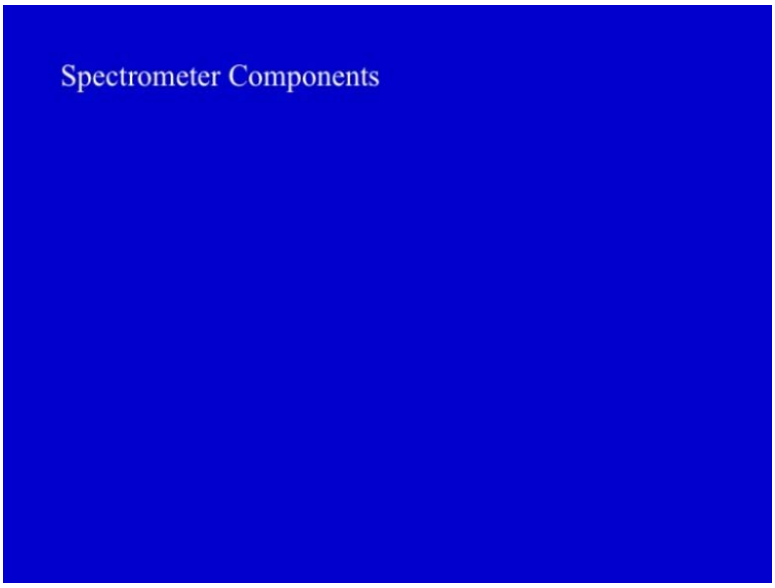

Spectrometer components, if we take the IR spectrometer consists mainly of 3 basic components, as in other spectrophoto meter, as we have discussed earlier, one should be like in any other spectrometer, one is radiation source monochromator and detector and then a place for sample to be put in. Now, the common radiation source for the IR spectrometer is an inert solid heated material electrically to say from 1000 to 18000 degree celcius.

Now, 3 popular types of sources are, these are nursed galore constructed of rare earth oxides, then globa constructed of silicon carbide and there is a nychrome coil actually. So, these are different radiation sources and these all produce continuous radiations, but differentradiation energy profiles. Then, monochromator is a device used to disperse a broad spectrum of radiation and provide a continuous calibrated series of electromagnetic energy bands of determinable wavelengths of frequency range.

Then, prisms or gratings are the dispersive components used in conjuction with the variable slit mechanisms mirrors and filters, for example a grating rotates to focus a narrow bend of frequencies in a mechanical slit. Narrower slits, enable the instrument to better distinguish more closely spaced frequencies of radiations and will result in better resolution, whereas wider slits allows more light to reach the detector and provide better system sensivity.

So, there has to be a compromise in the settings, for desired slit with and to get good results, most detectors used in dispersive IR spectrometers, can be categorised into 2 classes, one is thermal detectors and photon detectors. Now, thermal detectors will include thermocouples thermistors or neumatic devics and the major heating effects produced by infrared radiation, other and photon detectors will measure photons actually.

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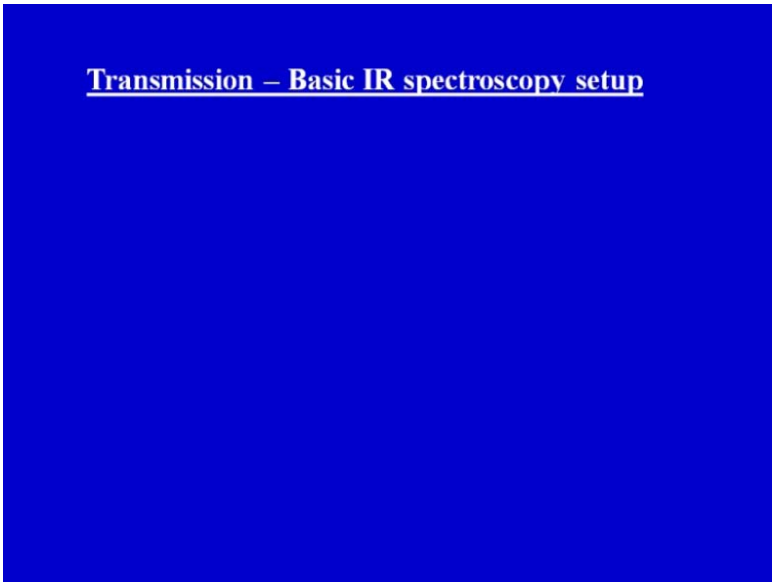

Now, basic IR spectroscopic setup here, in this particular one that, IR source emits an IR beam and IR beam here, in very basic way IR beam here is split into 2 identical beams. Now, one beam of the split beams goes through the sample and other will go through the reference cells, so that is how it will occur, now reference cell typically consists of the solvent that sample is dissolved in and IR used to measure the amount of energy absorbed in the frequency of the infrared light is varied.

In the pulsed fourier transform IR a single pulse is send through the sample and this will contain many frequencies, which will allow a for much faster test. In a typical dispersive IR spectrometer, radiation from a broad band source passes through the sample and is dispersed by monochromator in 2 component frequencies, then the beams fall on the detector which generates an electric signal and results in a recorder response. Most dispersive spectrometers have a double beam design and 2 equivalent beams, from the same source pass through the sample and reference chambers respectively.

So, using an optical choper, such as sector mirror or other, the reference and sample beams are alternatively focused on the detector. And commonly the change of IR radiation intensity, due to absorption by sample is detected as an optional signal that is translated into the recorder response, through the actions of synchronous motor.

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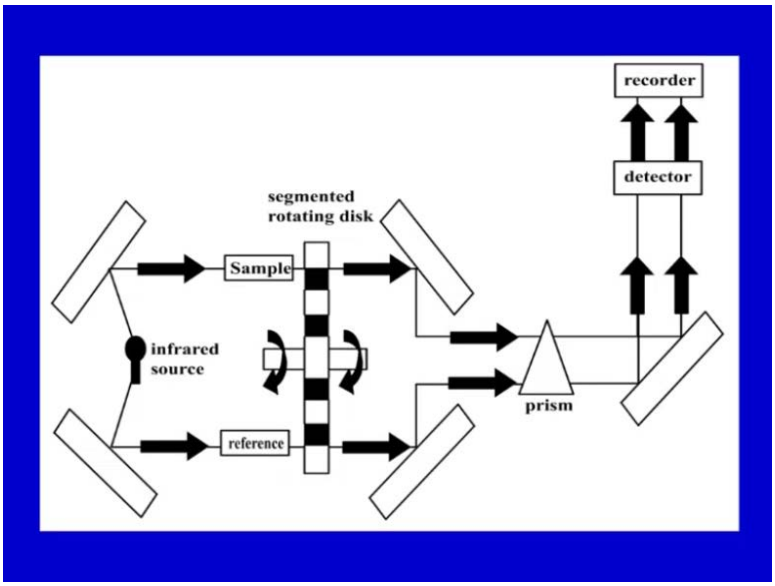

So, these are very simple schematic here that, you have an infrared source, this beams are then divided into 2 here, one goes to the sample another to reference. Then finally, it goes through the prism and to detector and recorder as we have there are lot of like filters and like prism and other monochromators, which are put in here, as we have discussed just now.

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So, this is very simple schematic of how the transmission occurs, as far as fourier transform spectrometers are concerned, fourier transform spectrometers have replaced kind of replaced, not completely dispersive instruments for many application due to the superior speed and sensitivity. And they have greatly extended the capabilities of infrared spectroscopy and have been applied to many areas, that are very difficult or near impossible to analyze by dispersive instruments.

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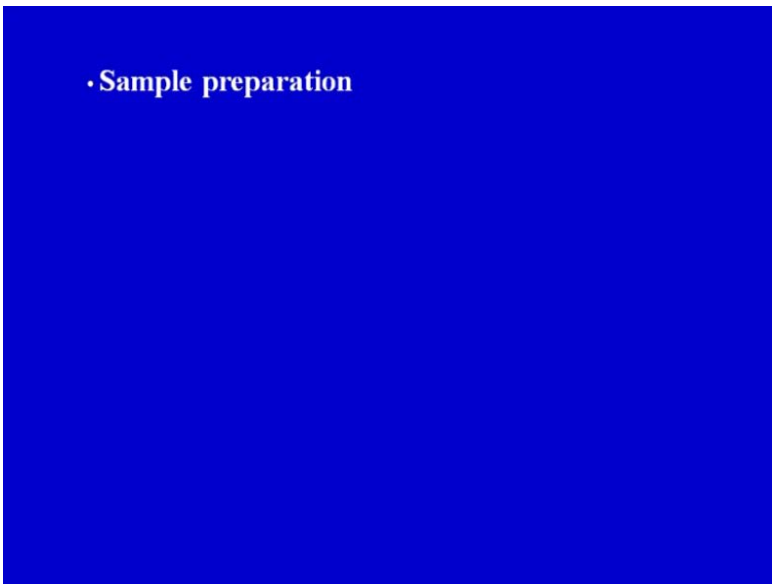

So, instead of viewing each component frequency sequentially as in a dispersive IR spectrometer, all frequencies are examined simultaneously in F T IR spectrascopy, 2 main forms of preparation for here sample preparation. For solids, begin with grinding the material to a fine powder and then dispersing it in a matrix potassium mostly potassium bromide is the widely used matrix.

The ground material can be dispersed in a liquid, usually like mineral oil or nujol to form a mal typically, no more than 20 milligram of solid is grounded and then, 1 or 2 drops of nujol are used to create a paste, which then spread between the 2 IR transparent windows. Then like the mixture will be like transferred to a dye, that will have a barrel of a particular diameter and they will be pressed and finally, like at a particular pressure and then a material, which is recrystallization of the K B R results in the clear glass heaters, which can be analyzed by transmission.

Now, liquid preparation is a easier than solids and is traditionally prepared as thin film cells, whereas cell consist of IR transparent windows and it is important to have constant path length for the laser, to travel through and that the sample is homogenous here. Now, it is possible to obtain an IR spectrum from samples and many different forms, like liquids solids and gases, but there are problems, many materials like are opaque to IR radiation and must be dissolved or diluted in a transparent matrix to obtain a spectra.

Alternatively it is possible to obtain reflectance or emission spectra, from opaque sample then, typically solutions in like particular concentrations like 105 to 10 percent concentrations are handled in IR cells of 0.1 to 1 millimeter in thickness. Concentration of 10 percent and cell path length of 0.1 millimeter represents very one practical combination. In a double beam spectrometer compensatic cell is filled with pure solvent and placed in the reference beam, as we have discussed.

In the single beam instruments the solvent bends are mostly removed by obtaining the different spectra, through substraction of the solvent, that is zeroing of that particular spectra. Both fix thickness and variable thickness liquid cells are available, commercially and normally consist of metal frame plates, IR transmitting windows and gaskets, they determine the path length of the cells these are all available.

Now, salt plates or IR transmitting materials can be used for semi volatile and non volatile liquid samples, like sodium chloride discs are the most popular and economical choice for this purpose. Silver chloride or barium floride plates may be also used for substances that will dissolve or react with anatial plates. So, the sample preparation here is also in a similar way drop of the sample is squeezed between the 2 salt plates to form a film of very fine thickness like 101 millimeter so.

Then plates can be held together in a capiliary attraction or may be clamped in a screw tightened holder and then, it is possible to place the film of samples on salt plates by melting or relatively low melting solid. And squeezing it in between it 2 plate the solid sodium chloride crystal salt plate can usually be cleaned with dry methylene chloride or acetone and this is smear technique is one of the simplest way to obtain IR spectra. So, they are various methods and like I said like, we discussed like, there are 2 important instruments here dispersive and fourier transform IR spectroscopy.

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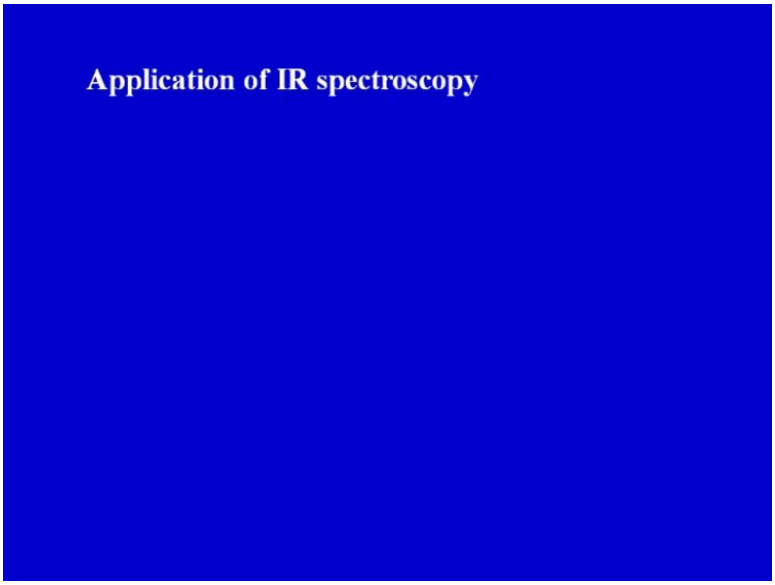

There are lot of applications of IR spectroscopy it mostly gives much of analytical information, which could be qualitative, that is the combination of the fundamental vibrations or the rotations of various functional groups. And subtle interactions of these functional groups with other atoms of molecule will result in the unique, generally complex IR spectrum, for each individual compounds.

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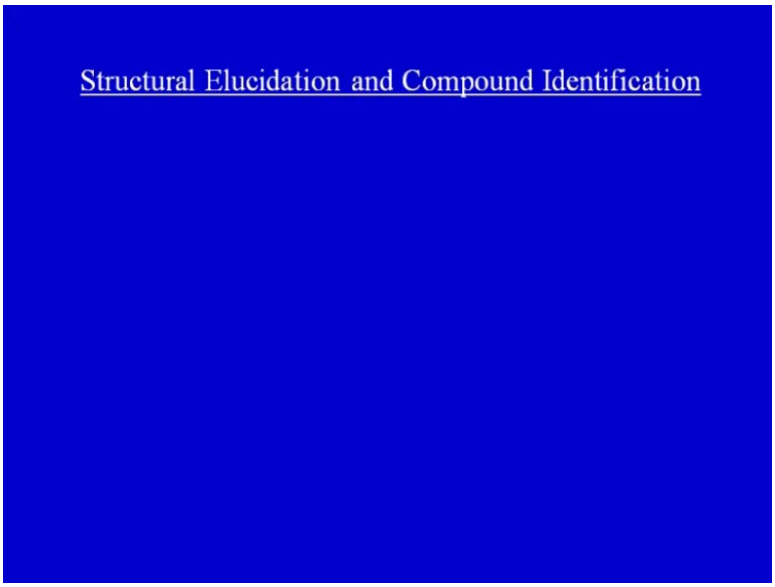

So, these are characteristic spectras are obtained and it could be utilized for structural elucidation and compound identification, in a structural elucidation, because of the complex interactions of the atoms within the molecule, IR absorption of the functional groups may vary over a wide range. However, it has been found that many functional groups give characteristic IR absorption at specific narrow frequency ranges, regardless of their relationship with the rest of the molecules. This both ways it could happen that spectra could be affected by the chemical environment, but in certain cases, it might be as it is not affected. So, much there are generalized tables or the positions and relative intensities of the absorption bands and have been established by certain groups.

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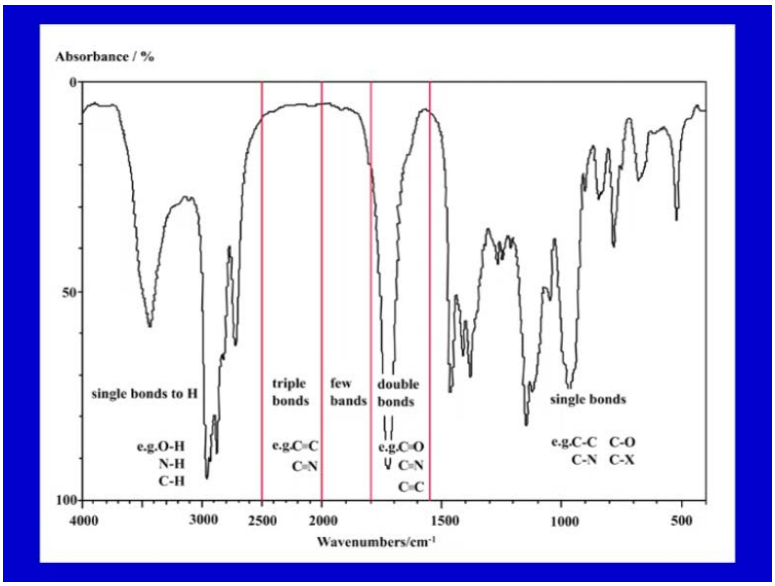

So, these strong absorption bands or the characteristic spectra, in particular region of the like say wave numbers, usually comes like say from 4000 to 2500 percent emitter, usually comes from stretching vibration between hydrogen and some other atoms with a mass with a particular mass, which is a less. The O H and N H stretching frequencies fall in 3700 to 2500 per centimeter then, various intensity with various intensities the hydrogen bonding has a significant influence on the peak shape and intensity.

So, that is also very important then the C H stretching bands, occur in region of 3300 to 2800 per centimeter and there are various like, we can go on where different kinds of like aliphatic saturated or double bond compound. They have a characteristic spectra, different regions could be taken, there are certain regions called finger print region, from 1300 to 910 per centimeter and absorption in this region includes the contribution from complex interacting vibrations giving rise to generally, unique finger print for each compound.

A good match between the IR spectra of 2 compounds and all frequency ranges particularly in the finger print region, strongly will strongly indicate that, they have the same molecular structure. So, that way the 2 molecules could be compared, if they have same structure or an unknown molecule, if IR spectra matches to certain expert wave numbers, they could be also identified. So, there are whole lot of like, tables or standard values, to which they IR absorption in different regions can be corelated and it could be found out.

So, structural elucidation in like, I said structures of say proteins, secondary structures could be determined, it could be complimentary to circular dichroism technique and this is because of differential hydrogen bonding patterns and they reflect on IR spectrum, which could be observed.

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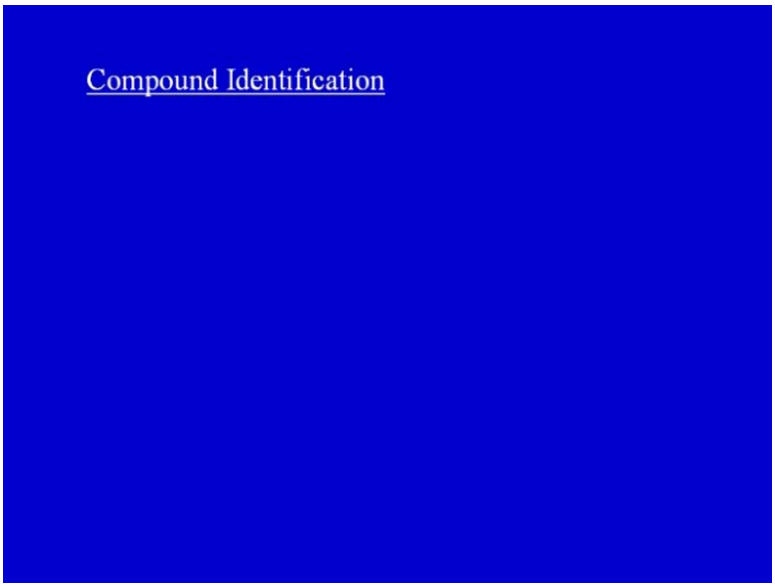

The compound identification is another important application and here since IR spectrum of every molecule is unique. And so, identification methods often say organic compound or other compounds or say amide bond or particular functional groups can be performed, through comparing it with large number of reference spectra in various conditions actually. And that way one could like, this database could be utilized, for different areas in biotechnology like forensics, biochemicals, biochemical analysis or polymer analysis or even forensic science, it could be utilized.

There are computer search programs, which can facilitate the matching process and many cases, for exact match to the spectrum of an unknown material cannot be found, they will list the reference compound that match the unknown most closely. So, this kind of information is very, very important. Certainly this is very useful information and when it is combined with say N M R or mass spectrometry, positive identification with high confidence could be achieved.

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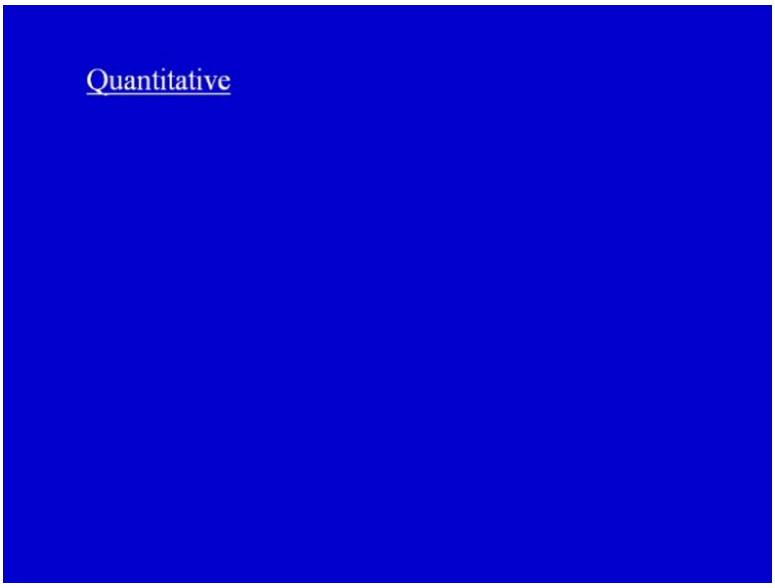

They could be quantitative information also, where IR spectroscopy mostly was considered qualitative, but quantitative and semi quantitative analysis, it is considered as qualitative and semi quantitative only. But, like this could also be like with F T IR instrumentation and strong computerised, data processing capabilities, have greatly improved the performance of this quantitative IR work. And this model infrared spectroscopy has gained acceptance, as a reliable tool for quantitative analysis also, the basis for quantitative analysis of absorption spectrometry is again the beer's law, which is bouguer beer lambert's law.

And for a single compound or homogenous medium, it will be expressed as we have done where it will be a molecular absortivity, path length and the concentration multiplied to each other gives the absorbance. So, law basically, states that the intensities of absorption bands are linearly proportional to the concentration of each component in a homogenous mixture or solution. And deviations of beer's law occur, more often in infrared spectroscopy than u v vis, but still it is a it is a way to measure or to quantitate particular material.

So, like we have discussed, these applications of IR spectroscopy can be in various areas biology and other areas, where like we have seen that, different different kinds of analysis right from identification of functional groups or structural information about molecules. In different forms could be derived from IR spectroscopy, even titrations like

P H titrations could be performed, hydrogen bonding in different environments, could effect IR spectroscopy and that could go structural information.

Totomeric groups could be identified then, there is lot of applications in, like other industries like, petroleum oil and or greese like, to analyze these contents or other applications. It could be also applied like, I said in forensics or boichemistry or other areas, it could be also have lot of applications. So, IR spectroscopy is one very important analytical tool, which could be applied, which could have applications in the field of in biotechnology for various kinds of to gain information for various kinds of problems actually. And it could be combined with other techniques like N M R or mass spectrometric, so this completes the section on IR spectroscopy.

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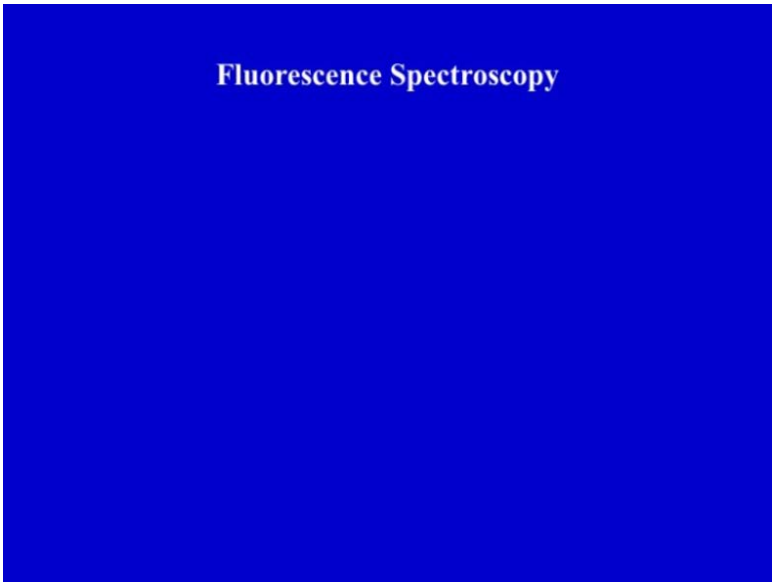

Let us move on to the next one, that is fluorescence spectroscopy, now as we have discussed about, earlier for U V Vis that is the absorption spectroscopy, but fluorescence spectroscopy is a emission spectroscopy, it is complementary to the absorption spectroscopy. Now, here molecules have like, as we see very states, which are energy labels actually, in fluorescence spectroscopy, what is done is like, there is like excitation. It is concerned primarily with electronic and vibrational states, as we have shown in the energy diagram, let me show you here, very simple way the principle of fluroscence spectroscopy.

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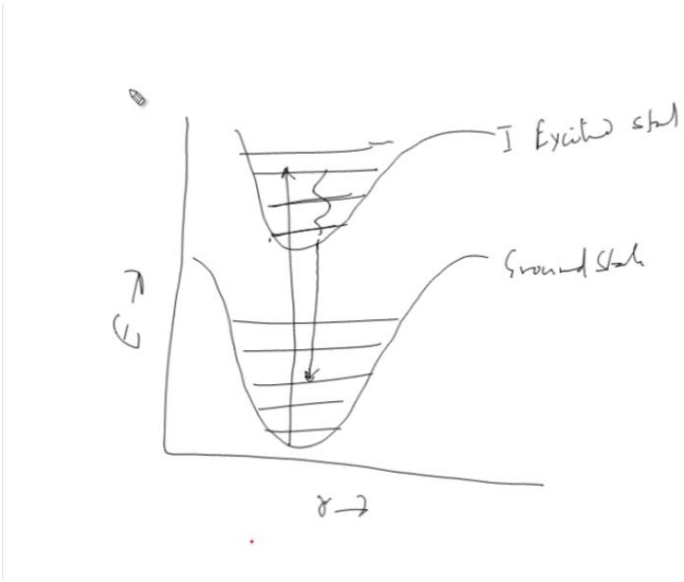

So, if you could recall the energy diagrams, which is, which we have made in these forms, now if you could recall, they were vibrational labels in here, different vibrational labels and this is the ground state and this is the first excited excited state. Now, these are the electronic state and these one, which are thin lines are vibrational states, so what happens is there is an excitation, which occurs. Now, in absorbance this will be like different thing, but in fluorescence, what happens is the the excited atom or electron will first fall through these vibrational labels here.

And then it will come down to a particular vibrational label in a ground state, but it comes to higher to a different label where, there is certain emission of certain photons, which the and the phenomenon is known as a fluorescence all right. So, the very in very simple terms, we have tried to understand the phenomenon of fluorescence. So, here what is happened that species being examined has a ground electronic state, that is a low energy state of interest.

And an excited electronic state of higher energy in fluorescence spectroscopy, the species is first excited, as I have shown you by absorbing a photon from, it is ground electronic state to one of the various vibrational states in the excited electronic state. And then, with it comes back like through different interactions may be, it will reach the lowest vibrational state of the excited electronic state and then, molecules may drop down into any of the several vibrational labels in the ground state.

Now, the emitted photons will have differenced energy and thus frequencies and therefore, by analysing the differenced frequencies of light emitted in fluorescence spectroscopy along with their relative intensities, the structure of different vibrational labels can be determined. Now, fluorescence is an emission phenomenon, rather than absorption, where in energy transition from a higher to lower state is accompanied by a radiation. Only molecules in their excited forms are able to emit fluorescence and thus they have to be brought into a state of higher energy rather to the emission phenomenon. So, if there is a chromophore, there is a fluorescing material, first radiation for absorption has to be provided or it should be excited.

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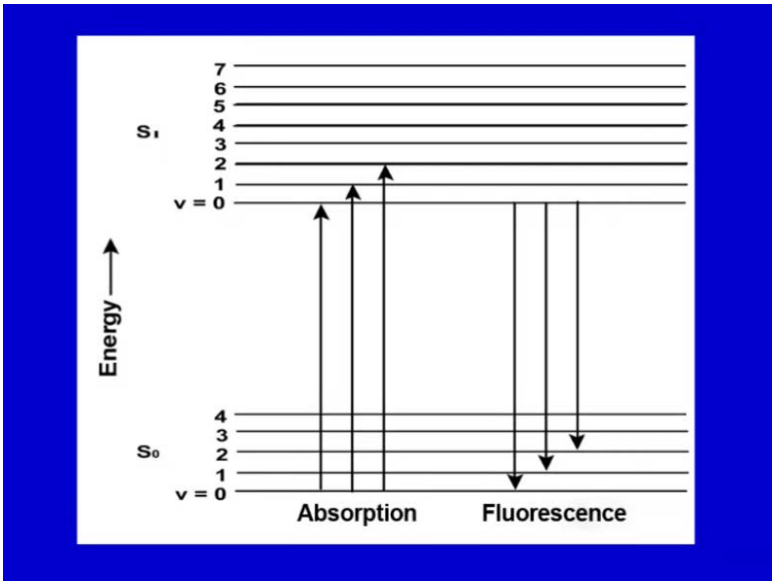

So, here if we can see here, the fluorescence in this case, if these are going from ground state to one of the vibrational state and when, they come back the one, which is not like when, they come back they will emit certain photons like in here and they will show the fluorescence. So, only those, which have a difference in energy will show the fluorescence, not all of them like, when they are excited and when, they come to ground state will show fluorescence, but only certain compounds will show fluorescence.

The fluorescence properties of a molecule are determined by properties of the molecule of itself that is internal factors and as well as the environment of the say particular say, if we consider protein, then external factors. The fluorescence intensities emitted by a molecule is dependent on the lifetime of the excited state. So, if we consider protein, the

fluorescence properties would be determined by the properties of the molecule, as well as the environment of the protein as well.

So, the transition from the excited to the ground state can be treated like D K process of first order, that is number of molecules in the excited state decrease, exponentially with time. And it could be like, it like anology to kinetics, where exponential coefficient, which is called rate constant is the reciprocal of the lifetime. The lifetime is the time, it takes to reduce the number of fluorescence emitting molecules to a particular label and they are proportional to lambda Q.

Now, the ratio of photons emitted and photons absorbed by a fluorophore is called quantum yield actually and the quantum yield is the dimensionless quantity and most importantly, the only absolute measure of fluorescence of a molecule. Measuring the quantum yield is a difficult process and requires comparison with the fluorophore of known quantum yield. In biochemical applications the measurements is really done and most commonly the fluorescence emissions of 2 or more related samples are compared and their relative differences are analyzed. So, this was a little bit about, the principle or you can say theory of fluorescence.

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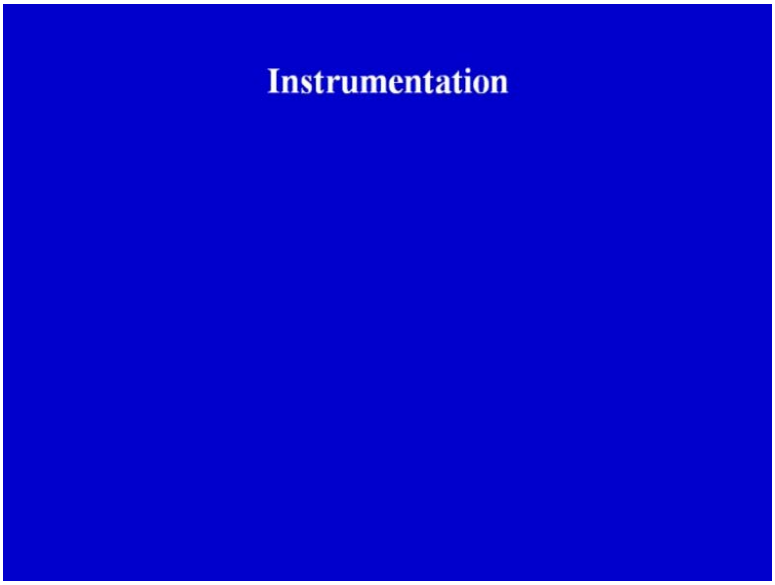

Instrumentation, if we consider, there are in general 2 types of instruments adjust, which are filter fluorometers, which use filters to isolate the incident light and fluorescent light and there are spectro fluorometers, which use diffraction gratings monochromators to

isolate the incident light and the fluorescence light. Now, both types use the scheme, where light from an excitation source passes through a filter or monochromator and strikes the sample.

Now, upper portion of the incident light is absorbed by the sample and some of the molecules in the sample will, so remember, you have to have fluorescent compound in the sample, which could be intrinsic or extrinsic. Intrinsic means, inherent property of the molecule to fluores and extrinsic means, you are putting a dye or other fluorescing material attached to the sample.

Now, the fluorescent light is emitted in all directions and light also passes, through a second filter or a monochromator and each is detector and this is usually, placed at 90 degrees to the incident light to minimize the risk of a transmitted or reflected incident light reaching the detector. Now various kinds of light sources may be used excitation source, including lasers photo diodes and lamps like xenon arcs and mercury vapour lamps are in particularly used.

Laser only emits light of high radiance at a very narrow wave length interval typically, under 0.01 nanometer, which makes an excitation monochromator or filter unnecessary here. Disadvantage of this method is that, the wavelength of a laser cannot be changed much, a mercury vapour lamp is alligned, lamp meaning, it emits light near peak wavelength by contrast xenon arc has a continuous emission spectrum with nearly constant intensities in the range say from 300 to 800 nanometer.

And sufficient radiance for measurements down to just above 200 nanometer, so different kinds of sources could be utilized in here and which, we also discussed in fluorescence microscopy some of these things. Filters or monochromators may be used in fluorometers like, I said monochromator transmits light often adjustable wavelength with an adjustable tolerance. And the most common type of monochromator utilized is a diffraction grating, that is collimated light illuminates a grating and exits with a different angle depending on the wave length.

The monochromator can then be adjusted to select, which wave length to transmit and for alloying anisotropy measurements, they could be also addition of polarizers or polarization filters, one alters the excitation monochromator and one before the emission monochromator or filter. So, that is fluorescence polarisation could be achieved and the

fluorescence is most often measured at 90 degree in spectro photometers, if you could recall in microscopy, it was not, so relative.

So, here it is at 90 degree to relative to the excitation light and the geometry is like here, this geometry is used instead of placing the sensor at the line of excitation at 180 degree angle. So, there could be different kinds of arrangements in here, now no monochromator is perfect and it will transmits some stary light, that is light with other wave lengths then the targeted. Further the fluorescence can also be measured from the front, which is often done for as a opaque or turbid sample, then the detector can either be, he has single chanelled or multi channelled.

The single channel detector can only detect the intensity of one wave length at a time, while the multi channel detectors can detect the intensity at all wave lengths simultaneously, making the emission monochroamtor or filter unnecessary. So, the different types of detectors both have advantages and disadvantages and also in terms of cost factor. So, that could be chosen as per requirement here, the most versatile fluorometers with dual monochromators and a continuous excitation light, source can required both an excitation spectrum and a fluorescence spectrum.

So, when measuring fluorescence spectra, the wave length of the excitation light is kept constant, preferably at a wave length of high absorption and the emission monochromator scans the spectrum, for measuring excitation spectra. The wave length passing, through the emission filter or monochromator is kept constant and the excitation monochromator is scanning. So, the excitation spectrum genearlly is identical to the absorption spectrum as the fluorescence intensity is proportional to the absorption. So, there could be different arrangements in here.

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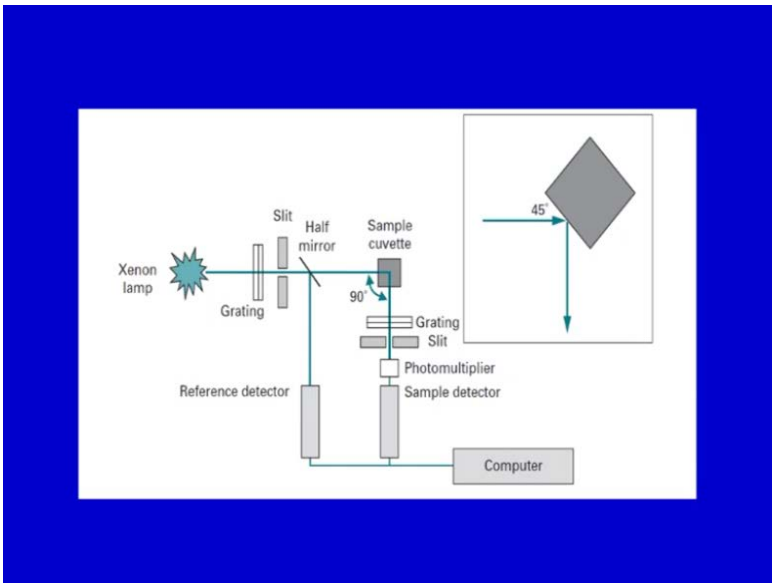

This is a very simple scematic of spectroflouorometer, there is a lamp here and the grating slit, there is a mirror, which reflects light to reference detector and there is a sample cuvette also. So, both will like through again grating slit and there is a excitation filter and emission filters here and finally, data is collected on a sample detector and then, send to the computer. So, this is like as spectrophotometers are this is a here detection is at 90 dgree as opposed to in a straight line.

So, fluorescence spectroscopy is a very useful technique and works most accurately at a very low concentration of emitting fluorophore, the better fluorescence spectrometers in laboratories will have a photo counting detector yielding a very high sensitivity. Fluorescence is a very sensitive technique and there should be temperature control is required for accurate work, as the emission intensity of fluorophore is dependent on the temperature. So, that is also very important for spectrofluorometer, as well as for other works also the temperature control is a very important.

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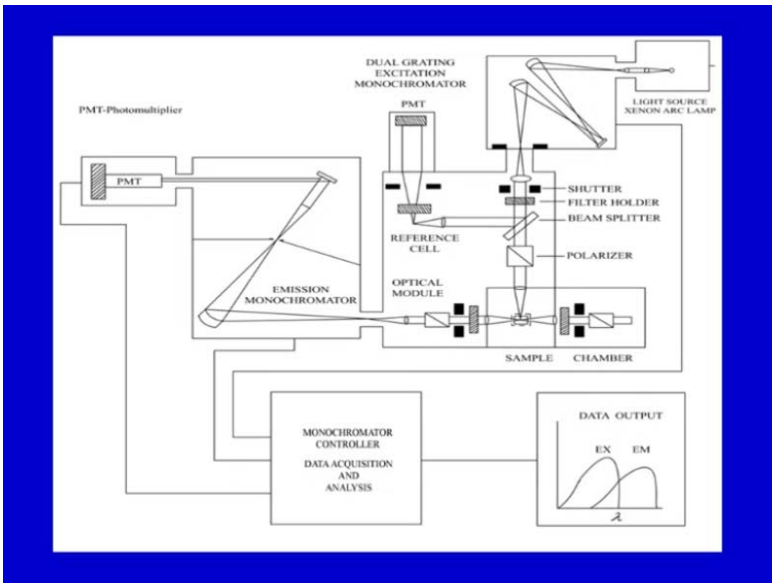

This is a very, this is the schematic of fluorescence spectrometer, as we have shown earlier, so you can see like it starts from here, there is a light source, there is a dual grating excitation monochromator the shutters filter holders beam splitters. And then, there is a sample chamber here and there is a emission monochromator and finally, photo multiplier for detection. So, it contains in essence, it contains both excitation as well as emission monochromators, sample holder it for say polarisation effect, it would have 2 polarizers as well attached to it. So, there could be fluorescence polarization could be observed.

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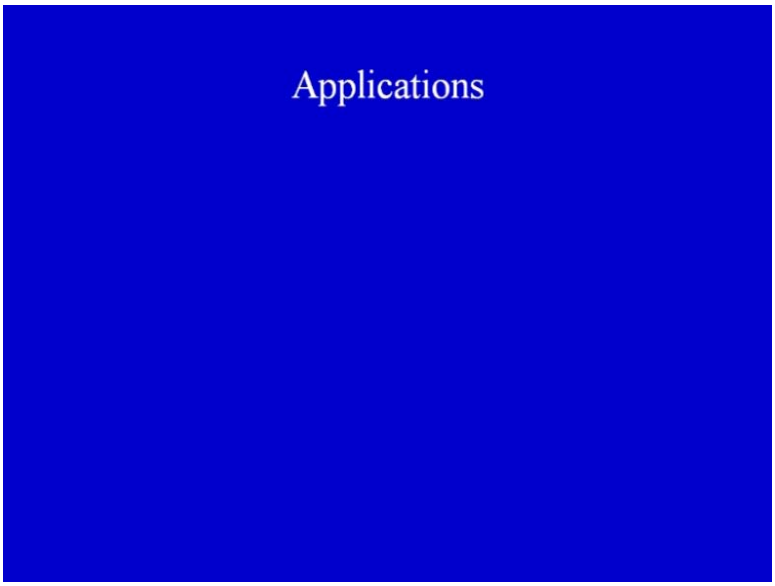

Now, there are lot of applications of fluorescence spectroscopy, in biotechnology and otherwise, fluorescence spectroscopy is used, for biochemical medical, chemical research and various other fileds for analysing say organic compounds and lot of other compounds. Then there is like also has been there are pods, which is used in differentiating malignant skin tumours from atomic fluorescence spectroscopy techniques are useful in other kinds of analysis measurements of compounds present in air, water or other media like heavy metals detection could be performed like mercury.

Fluorescence can be used for to redirect photons, there are many and highly varied applications, fluorescence, despite there is a problem here that in biology few compounds exhibit the phenomenon of fluorescence. And so, if there is a problem like say in proteins tryptophan exhibits that, so that could be used like riboflavin and others could exhibit, but again problem is there are not many of these. In that case in extrinsic fluorescence or certain fluorescing componds can be attached to these molecules and then, they could be monitored.

Effects of P H solvent composition and the polarization of fluorescence may all contribute to structural elucidation. Non fluorescent compounds like, I said can be often labeled with fluorescent probes, to enable monitoring of molecular events like following it in a cell or for say structural information or certain chemical reactions all these things could be utilized in here, like a exchanging of groups and others.

So, extrinsic fluorescence is a as distinct from intrinsic fluorescence, where you have a native compound exhibiting that, property fluorescing dyes are sensitive to the presence of metal ions and these could be used to track changes of these ions in vitro samples. And if you could recall, we have discussed about, many applications in microscopy of fluorescing dyes like say for calcium detection and other lot of different ions could be detected in here.

Proteins passes 3 intrinsic fluro force mainly, the tryptophan tyrosine and phenyl aniline have very low quantum yield, particularly phenyl aniline has very low quantum yield and contribute and does not contribute much to that. So, but usually trpptophan fluorescence can be done in 295 to 305 nanometer range, the main application of intrinsic protein fluorescence, aims at conformational monitoring, that is conformational changes in different conditions or in various environments, where say tryptophan is exposed or it is

present or it is not exposed in the structure, frequently molecules of interest for biochemical studies are non fluorescent and many of this cases like, we said external fluorophores could be attached here. So, that could be coupled in different forms like for example, green fluorescent protein, which could be cloned with the protein of interest and it could be, used for fluorescence experiments.

There are lot of different extrinsic chromophores or fluorescing compounds one, like green fluorescent protein, there are dyes, another one is A N S or one anilino 8 naphthelene sulphonate, which is emits a weak fluorescence in polar environment, but when it combines with proteins in hydrophobic environment it fluoresces. So, those properties could be differential properties, could be exploited, there are lot of other like, we have discussed earlier for calcium, there are fura 2 or indo one dyes.

There are intrinsic fluorescence for nucleic acid, if we consider there are very weak and require excitation wave lengths are too far in the U V region, for practical to be used for practical applications. So, numerous extrinsic fluorescent probes could be combined with D N A, for enhanced emissions. So, as we have seen and we have discussed earlier also, fluorescence spectroscopy could be utilized, for lot of applications like, we have discussed in the microscopy sections.

Whether it could be applied for a localization or for a in like in spectroscopy, it could be utilized for structural information, for localization, for exchange of groups, for quantitative in qualitative terms, knowing metal ions and their exchanges. So, there are whole lot of applications, it is a very sensitive technique and a phenomenon of fluorescence is very quick, very fast, we have decay rates, as we have discussed earlier is very very fast.

So, this technique is one another very important technique, which is used in biotechnology for different applications. So, this completes 2 techniques here in this lecture, one is IR spectroscopy, another is fluorescence spectroscopy, one can always refer to literature, for more information. These lectures, we have tried that you can understand, basic concepts and what are, what could be the applications and used in different areas of biotechnology.

Thank you.