Modern Instrumental Methods of Analysis

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Lecture No. # 13

Fluorescence and phosphorescence Spectrophotometry 3 Applications

So, in the last class we had discussed about the optics involved in the fluorescence instrumentation.

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OPTICS

A filter photometer usually employs a mercury lamp as an excitation source. Therefore a primary filter is required to transmit the desired excitation wavelength through the optical cell. Another filter known as secondary filter is required to transmit the fluorescence radiation on to the detector for measurement. The secondary filter also serves to absorb the scattered excitation radiation.

1

As I told you there are primary filters used to transmit the desired excitation wavelength through the optical cell. Another filter is known as secondary filter is required to transmit the fluorescence radiation onto the detector for measurement. The secondary filter also serves to absorb the scattered excitation radiation.

This is the major difference between the optics in spectrophotometer and spectrofluorometer. In spectrophotometer, you do not need the second filter whereas, in the spectrofluorometer, you would need two optics, one before and one after. So, excitation filters are generally band pass types, which transmit a comparatively broadband of radiation as against single mercury lines, from interference filters. In the

later case, stray radiation levels are usually quite high, owing to the pinholes and defects in the film coatings. Emission filters are usually of the sharp cutoff type, which pass long wavelengths and attenuate shorter wavelengths. So, band pass filters have more than one transmission windows.

If glass filters are used they frequently fluoresce themselves and hence stray radiation is more in such cases. That is why we always prefer quartz cells in spectrofluorometry. However, filter photometer optics also cannot resolve any Rayleigh and Raman scatter peaks, so we would get end up with higher fluorescence, if there is a component of Rayleigh and Raman scattering. So, in spectrophotometers, fluorometers, grating monochromators are used in place of excitation filters.

Instead of the filters, they use monochromators, which permits us the freedom to use 200 to 800 nanometer of scanable wavelength. That means, wherever excitation occurs, we can go to that wavelength and fix it, so that excitation occurs.

This arrangement is advantages for observing scattered radiation also, by comparing the reference and sample spectra. Distortion of fluorescence peaks or the presence of additional peaks is observed when gratings are employed. So, this is an advantage. Usually, gratings of 600 grooves per mm are blazed for 300 nanometers are used for excitation. For emission gratings are blazed for first order for 500 nanometers, their focal length is approximately 0.25 meters and f by 4 are f by 5 apertures are used.

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Gratings of 600 grooves/mm blazed for 300 nm are used for excitation. For emission, gratings are blazed for first order for 500 nm. Their focal length is 0.25 m and f/4 or f/5 apertures are used. Filters are used to block higher order diffraction.

The excitation monochromator is located between the source and sample while the emission monochromator is located between the sample and the detectors. To obtain good spectral sensitivity and fine structure of the spectra, the emission monochromator should be able to resolve of 0.1 nm wavelength.

4

Usually, filters are used to block higher order diffraction that is their main function. So, it need not be exact like interference filters, where the orders are well separated. The excitation monochromator is basically located between the source and the sample. That is very logical, because after the source, we need to choose the excitation wavelength. While the emission monochromator should be located between the sample and the detectors, because there again we are choosing on the emission wavelength and intensity of the emission wavelength, has to be measured. So, it has to between the sample and detectors.

To obtain good spectral sensitivity and fine structure of the spectra, the emission monochromator should be able to resolve up to 0.1 nanometer wavelength. Then you can get small nuances for the emission spectra and better resolution. Now, we move on to cells and cell compartment. So, just like in spectrophotometry, we use one centimeter quartz cells or glass cells, you can use rectangular cell or cylindrical cell, fabricated from glass or silicon, for fluorescence measurements also. The surfaces through which the incident radiation and the emitted radiation pass much be nicely polished and more care must be taken to avoid fingerprints.

Leaving the fingerprints is always a problem on such fluorescence measurements because some of the skin oils often flourish. So, if you touch the polysurface with your fingers, it is going to be used slightly more fluorescence, which may be unaccounted. Low volume microcells capable of handling very small samples are available commercially. That means the sample volumes to be handled in spectrofluorometry can be very small volumes. So, flow cells are useful in continuous flow analysis and room temperature phosphorescence also and for the measurement of chemiluminescence. Usually, cell compartments are just like in spectrophotometry, designed to reduce the amount of stray radiation reaching the detector. Now, there sometimes baffles are obtained for this purpose.

Sample cell geometry also is very important in spectrofluorescence measurements. There are usually three types of popular arrangements for viewing the fluorescence; one is you can view it at right angle. That is the emission radiation is coming like this, you put a cell and measure the absorbance at the measure the fluorescence at the right and left. This we have already discussed earlier. This is a very popular method; the right angle method, it is known as and this is used mostly in commercial instruments. In this mode, excitation

beam passes through a long solution path, so that there is an upper concentration limit is absorbed before attenuation of the exciting radiation disrupts the linear relationship, between the luminescent power and the solvent concentration.

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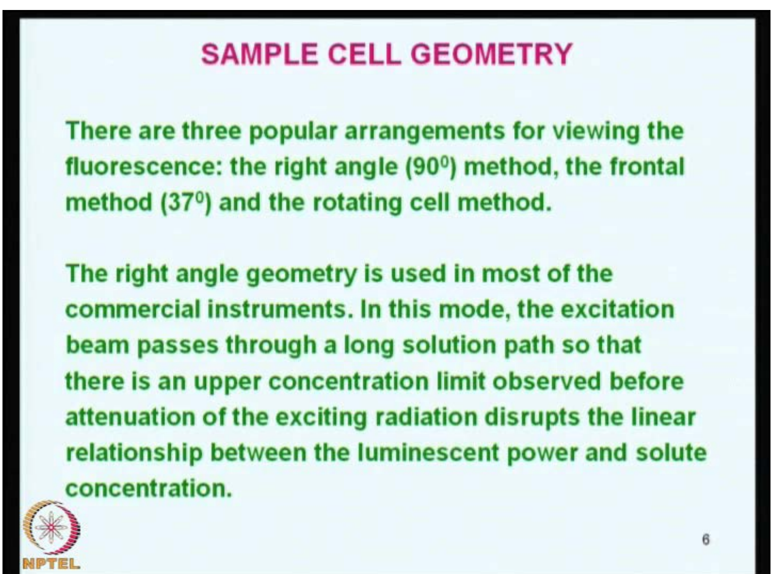

Usually, this is the most preferred arrangement. in this arrangement, none of the (( )) surfaces are directly eliminated by the excitation beam, because they are viewed by the emissions monochromator. Scattered radiation, of course, it originates from the bulk of the solution itself, so that cannot be helped. Now, you can use another arrangement. This is known as frontal geometry; the frontal method of cell elimination is used.

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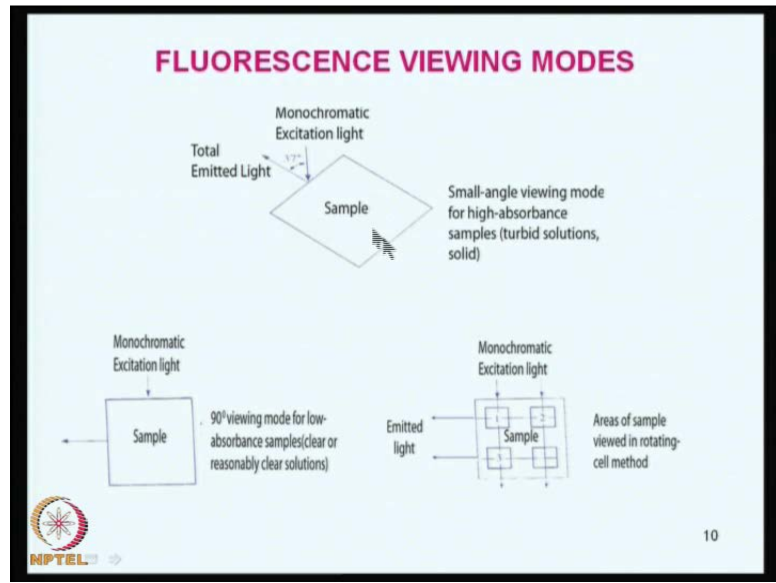

You can see here, in this figure that the first one is monochromatic excitation sample at 90 degree viewing mode. Here the radiation is coming from the top and measurement is made at the right angles. That is towards the exit beam, which is at right angles to the incident beam. This is 90 degrees and is very common and this second one is frontal method that is used for 37 degree, is used for measuring the total emitted light. Here, the monochromator is coming like this, falling on the sample, and the detector is located at 37 degrees like this, and that is a viewing angle. This is an essentially very small angle viewing mode for high absorbance sample, turbid solutions and solids etcetera.

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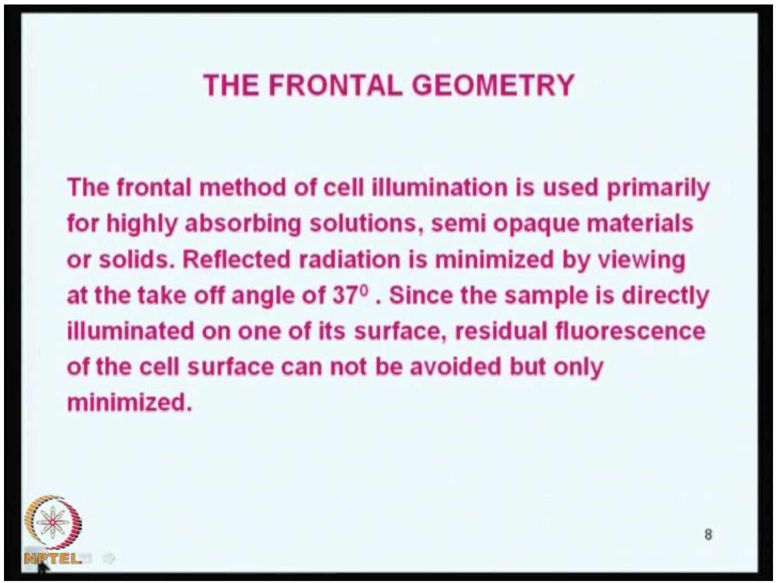

Now, you can see in the frontal geometry, reflected radiation is minimized, by viewing at the take off angle of 37 degrees. You can use other angles also but, this is the optimum. Since, the sample is directly illuminated on one of its surfaces, residual fluorescence of the cell cannot be avoided but, it can only be minimized.

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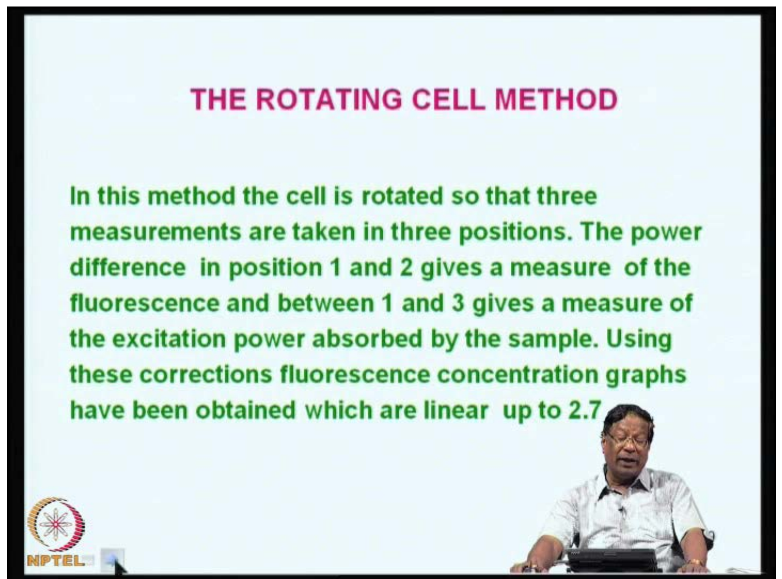

Now, another arrangement is rotating cell method. In this cell (Refer Slide Time: 10:05) what we do is we measure the sample 1 and sample 2, sample 1 and sample 3, and then after you measure sample 1 and sample 2 reference, you change the angle, the viewing angle, so 1 and 2 is like this 1 and 3 is like this. So, the emitted radiation comes like this and then another radiation is coming like this.

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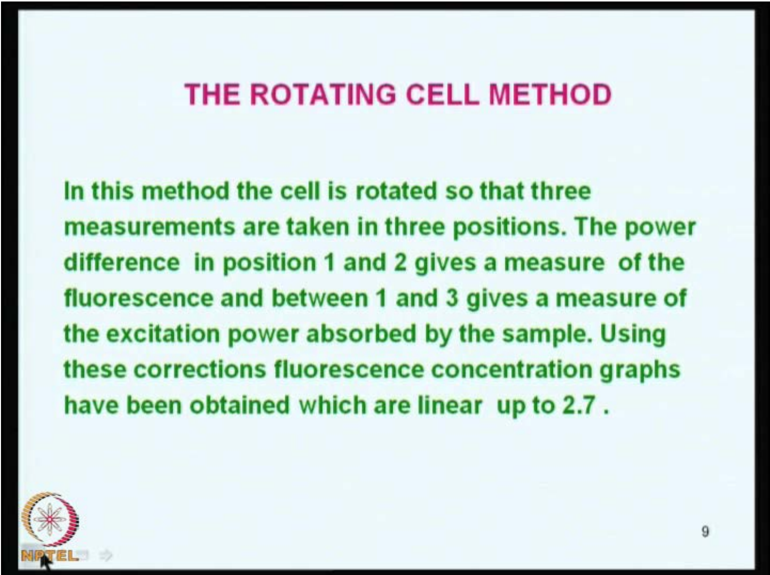

So, power difference between position 1 and 2 gives a measure of the fluorescence whereas, the power difference between position 1 and 3, gives a measure of the excitation power absorbed by the sample. That means just by rotation you will be measuring both the parameter.

These things can be corrected for fluorescence concentration and graphs have been obtained, which are linear up to 2.7. So, these are the different arrangements, (Refer Slide Time: 11:10) I want you to remember that you have to measure the fluorescence usually at right angles, 90 percent of the commercial angles are here and commercial instruments are of this type. Viewing angle, if it is 37 degree or small angle, and it gives high absorbance samples for turbid solution, solids etcetera. Other one is to go for rotation; this gives you a chance to correct the fluorescence for abrasions.

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DETECTORS

Luminescence emission signals are typically low in intensity. Therefore photomultiplier tubes are most suitable detectors. Often these are operated in photon counting mode to obtain improved signal to noise ratios. Sometimes PMTs are cooled to obtain better signal to noise ratios.

CCDs are also used in spectrofluorometry as it permits the rapid recording of both excitation and emission spectra. Such systems are useful in chromatography and electrophoresis.

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11

Now, let us discuss about the detectors. Obviously, we have already discussed most of the detectors in our previous discussion on spectrophotometry. Same detectors are used here also. The luminescence emission signals are typically very low in intensity, because basically you are taking from the incident radiation; all the incident radiation. You are putting a filter, taking out a small excitation filter, excitation wavelength and you are allowing it to fall on the sample. After the sample, again you are going to choose the wavelength that is coming out and then putting it on to the detector. So, a large percentage of the incident radiation is no more there that reaches the detector. Only the emitted radiation corresponding to particular frequency will be there in the detectors.

Therefore, photomultiplier tubes are most suitable detectors for fluorescence. Barrier layer cells and phototubes are not convenient. Obviously, because the there is no possibility for amplifying the signal. Often these are operated in photon counting modes to obtain improved signal to noise ratio. Sometimes PMTs are cooled to obtain better signal to noise ratios also. Then CCDs are also used in spectrofluorometry, as it permits rapid recording of both excitation and emission spectra; simultaneous recording almost.

Such systems are useful in chromatography and electrophoresis, not in spectrofluorescence, as such, because in chromatography CCDs will act only as a small component of the total chromatography arrangement. So, there if fluorescence of a particular sample separated has to be determined, we use CCD instead of PMT. So, data

handling; now after you collect the sample, you need to collect the data and usually nowadays microprocessor controlled instruments are available and these incorporate several features such as signal correction for the blank, corrected excitation spectra, corrected emission spectra.

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Difference and derivative spectra, also can be obtained and then peak detection is possible and processing deconvolution is possible. Three dimensional luminescence spectra can be obtained, calibration plots can be exhibited on the screen, and then curve fitting can be done by linear least square methods, and calculation of the statistical parameters and smoothing of spectra, all such features are available in modern spectrofluorometers. But, it all depends upon how much the cost you are ready to invest in a good spectrofluorometer and again that is dictated by the requirement.

So, if it for research purposes etc, you should go for a high end instrument, which offers all these features, otherwise it is not necessary. You can go for filter fluoremeter, if it is a routine analysis lab, which is doing analysis for only a few parameters. Otherwise, we can go for spectrofluorimeter. Other features include kinetic measurements, high performance liquid chromatography and time resolved measurements; these things are also being offered by commercial instruments. Most of the data handling has to be done by microprocessors.

So, among the commercial instruments, you can have a filter fluorometer like for excitation and emission and they provide a relatively simple low cost quantitative fluorescence analysis. They use mercury lamp for excitation source and a pair of PMTs as transducers. The source beam is usually split into a reference beam and a sample beam and the reference beam is attenuated by an aperture disk, so that it is intensity is roughly the same as that of the fluorescence intensity. This is important, because they should not go out of the range for measurement. So, if the incoming radiation is very strong, we reduce the aperture slightly smaller and then try to match the signal within the performance parameters of the detector.

The reference beam usually passes through the primary filter and reaches the detector and the sample beam passes through the sample, and emitted fluorescence is passed through a secondary filter and reaches the detector. So, the electrical outputs from the PMT are used to compute the ratio as usual, just like in spectrophotometry; ratio of the sample to reference. Filter photometers are essentially single beam instruments and cost around fifty thousand rupees and easily available in the market.

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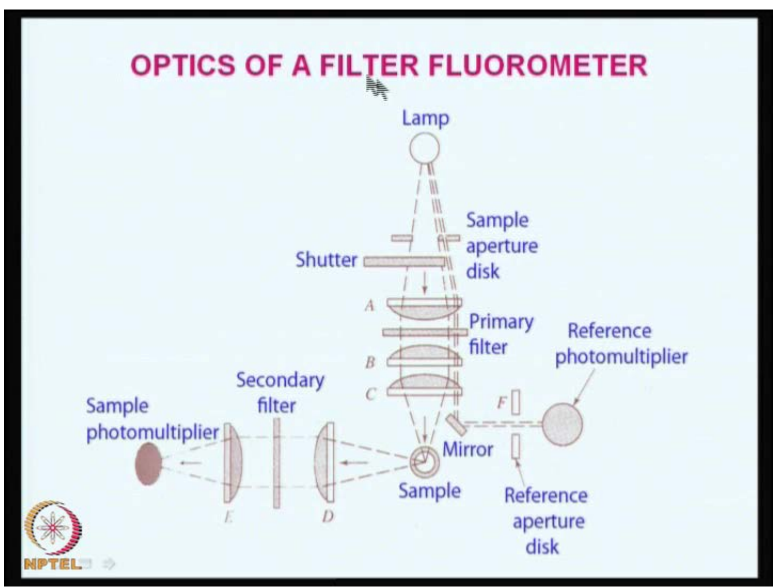

This is the arrangement of the optics of a filter fluorometer and this is the lamp and then the sample aperture is here, and then we have a shutter, which can reduce the aperture. If you close the shutter then it is total complete blocking. Otherwise, if you keep on

opening, it becomes more and more and this is the arrangement what I was talking about when we were discussing the adjustment of the emission fluorescence incident radiation.

Then we have a primary filter and then we have a mirror, from the mirror we have, it falls onto the sample. from the sample, viewing at right angle, I have shown here, the signal will pass through like this through a collimator and then we need a secondary filter, and then another lens, and then to focus, and then we have a photomultiplier tube dedicated to the sample.

The other one is here; I put a mirror and take out part of the radiation coming from the source. Simply, put a mirror and take it through an aperture and take it to the reference detector. Once you have the data for the reference photomultiplier and sample photomultiplier, you can determine the ratio and go for the measurement directly. that is for filter photometers.

Now, let us discuss about spectrofluorometers. Obviously, they have to be made of prisms and gratings. That is a main difference between a spectrophotometers and filter photometer anyway. So, spectrophotometers are capable of providing both excitation and emission spectra. This is very important, because sometimes you may like to know for a new compound where the excitation wavelength, and where is the emission wavelength. So, you can scan both. They employ two grating monochromators. Obviously, because one for excitation, another for emission.

They provide satisfactory spectra for quantitative analysis. So, however the emission spectra obtain in such instrument do not necessarily compare well with instruments of other manufacturers, because the output depends not only on the fluorescing molecule, but also it depends upon the lamp intensity, and lamp life, and then wear and tear, and then it depends upon the transducer characteristics and monochromator. So, 99 percent of the time, fluorescence measurements obtained in one instrument do not necessarily match the readings taken in other spectrofluorometers.

This we have to understand very clearly, because one should not compare the spectra from one instrument to another instrument, and say that we got higher absorbance, fluorescence readings, in our instrument, so what has been reported earlier in some other lab, in some other instrument are different from ours. So, it is a known fact and you have to account for that. Usually, spectra obtained in such instrument are referred as

uncorrected spectra. So, in uncorrected spectra variations are always expected. Such spectra can still be used for rough laboratory comparison and quantization within one laboratory.

You can do the quantization in your own laboratory based on the availability of the fluorescing molecule, for the same molecule, same calibration, same instrument and that is the idea. So, this can be used, but not for inter laboratory, because some other instruments, some other characteristics, some other fluorescence, you will be getting. So, it should not be possible for you to measure, compare, measure and compare, both the fluorescence in inter laboratory. But in intra laboratory, yes it can be done provided the instruments are of the same age having almost same characteristics, but still you could end up having a rough comparison rather than the exact comparison.

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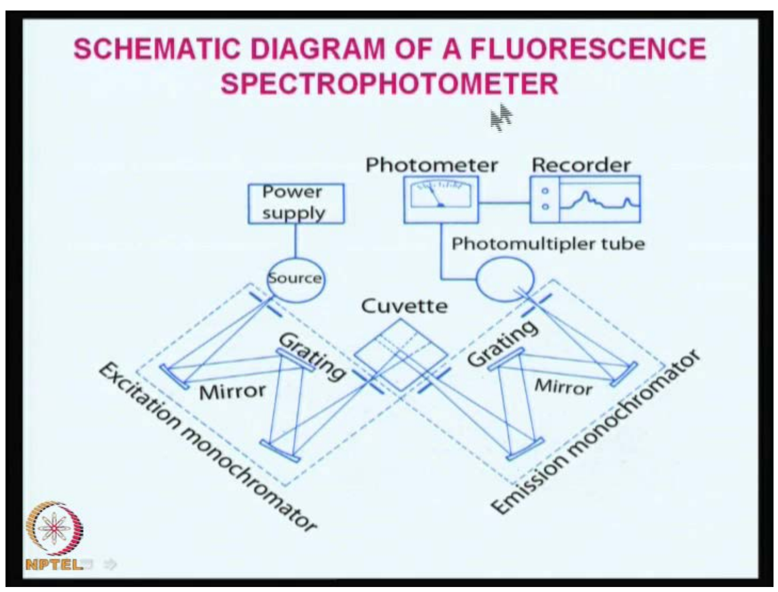

This is the schematic diagram of a fluorescence spectrophotometer. What we have here is we have a source and a power supply for the source. From the source, we are collecting the excitation… There is an aperture here, immediately after the source and we have an excitation monochromator that is a grating. From the grating, it falls onto another mirror and then through an aperture it reaches the cuvette. From the cuvette, weaving at right angles, again with an aperture, and we enter into the second optics that is for emission monochromator. So, it goes to the mirror and then grating and then onto another mirror and then it reaches the photomultiplier tube.

So, from the photomultiplier tube whatever current is given, it is taken to the photometer and recorder and other paraphernalia that are usually part and parcel of fluorescence spectrophotometers. This is in general, the optics of a spectrofluorometer; you will see here that instead of simple filters, the gratings are used. Obviously, the optics gets more complicated, but the readings would be much more reliable. Sometimes, spectrofluorometers with double monochromators are equipped it 4 gratings. Four gratings are used to reduce this scattered radiation. they also permit the use of larger slits to achieve the same resolution, as in conventional monochromators that have smaller slits widths. So, larger slits allow more radiation to fall onto the sample with a consequence that samples can be analyzed at lower concentration; higher the beam intensity, lower is the detection limit. This point you will have to remember.

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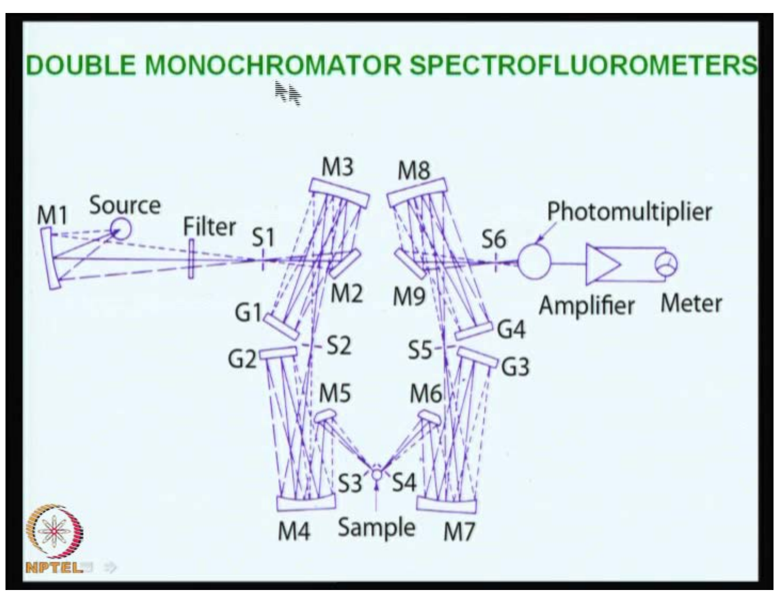

See here, in this case, we have a source, a mirror, filter, and then this part is double monochromators, and this part M 8 and M 7 containing gratings G 1, G 2, G 3 and G 4. This is G 1, this is G 2, this is G 3 and this is G 4. So, 4 gratings are used and corresponding mirrors M 3, M 2, M 4, M 5, M 6 and M 4, like that, there are other mirrors involved. Obviously, the optics gets more complicated in this case but, again after the sample, they are directed on to different gratings.

Then it is the signal is collected on the photomultiplier tube, which is further amplified and sent onto a read out meter. So, double monochromators, are also available in the

market and double monochromators with PMT are available. Another possibility is spectrofluorometer based on array detectors. So, we have already discussed about array detectors. Only thing is in this case if PMTs are replaced by array detectors. These spectrofluorometers permit fluorescence spectra to be obtained within a fraction of a second. If you use an array detector, within a fraction of a second, immediately both are recorded and the records can be obtained either on the computer screen or onto the recorder.

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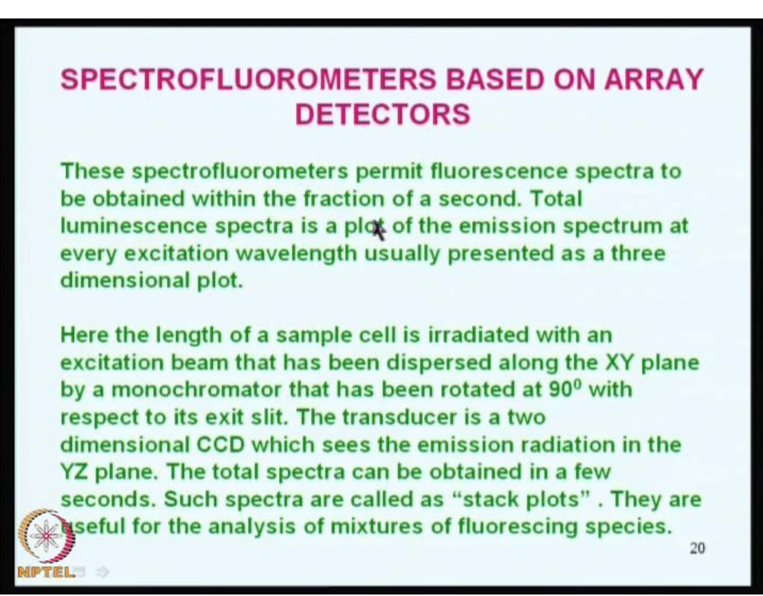

So, total luminescence spectra, what you get in this case is a plot of the emission spectrum at every excitation wavelength, usually presented as a 3-dimensional plot. One is the intensity; another is the excitation intensity, luminescence as well as intensity as well as the luminescence wavelength. So, you will have 3 parameters varying and information is collected at every second or every fraction of a second, presented as a 3 dimensional plot. Here, the length of the sample is irradiated with an excitation beam that has been dispersed along the XY plane by a monochromator and that has been rotated at 90 degrees with respect to the exit slit.

The transducer in this case is a 2 dimensional CCD, which sees the emission radiation in the YZ plane. the total spectra can be obtained in a few seconds, as I told you earlier, and such spectra are called as stack plots, because they are linear 3 D plots and they are useful for the analysis of mixtures of fluorescing species. Suppose, there are 2 or 3

fluorescing substances in the same mixture, then you should be able to analyze using such stack plots.

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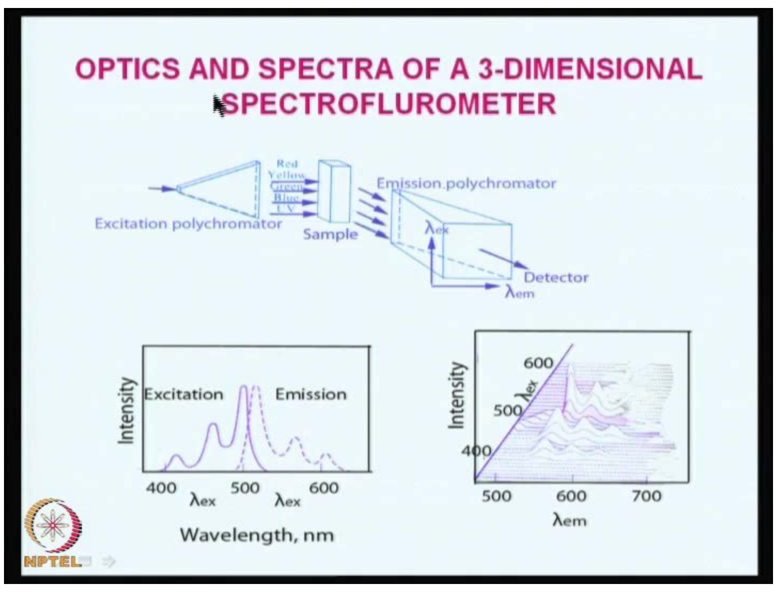

This is the optics and spectra of a 3-dimensional spectrofluorometer. Here, what we have is an excitation polychromator is here. It will give you red, yellow, green, blue, etcetera colors and then it is directed onto the sample holder containing the sample, followed by emission polychromator. Detector is placed across the monochromator. So, here you can see that the excitation and emission. In the bottom figure, we have excitation spectrum showing in the continuous line that is one peak around 400, another around 450 and then 500 and then it reaches the zero.

This is the excitation spectrum, the one with dotted line shows emission spectrum, and it extends from 548 to almost 630. This is a hypothetical compound. It is not necessarily a actual sample, but the point to be noted here is that the excitation spectrum is almost a mirror image of the emission spectrum and vice versa. See, for example, this peak, a solid dotted peak, is almost same as the solid dashed peak, and then this peak corresponds to this peak, and the one around 400 is almost identical with that of 600 nanometer.

The same thing, if you see, if you plot in 3 D, then you have excitation in one axis, emission in another axis and intensity as the third axis; z axis. So, you can see the plots like waves in a lake or waves in sea. You could see the plots like this and they are useful

for the analysis of mixtures as I have told you. Usually, spectrofluorometer and spectrophosphorimeters, now I am coming talking about spectrophosphorimeters, not fluorescence phenomena, but spectrophosphorimeters, where the emission occurs in a higher time frame, lasting from several seconds to minutes as we had discussed in our introduction and such instruments are also available.

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SPECTROPHOSPHORIMETERS

Phosphorimeters need two more additional components than fluorometers. The first is a device to irradiate the sample alternately and measures the phosphorescence after a time delay . The time delay is required to differentiate between fluorescence emission and phosphorescence. Both mechanical and electronic devices are used.

A gated scheme for the delay works satisfactorily. A pulsed xenon arc lamp is used for excitation. After a specified delay time, the data acquisition system is activated to obtain phosphorescence.

22

Phosphorimeters need two more additional components than fluorometers. This is very important, because the first is a device to irradiate the sample alternately and measures the phosphorescence after a time delay. First you have to irradiate, give some time for the phosphorescence to occur, emit the radiation and that radiation has to be measured. In fluorescence, immediately it is measured. So, there is no time delay, so in this case the time delay is required to differentiate between fluorescence, emission and phosphorescence.

In general, the phosphorimeter metric output has got an absorption spectrum, a fluorescence spectrum and phosphorescence spectrum. So, all the three are available in this case, so you have to differentiate between all the three. In fluorescence measurements, absorbance is already taken care of. Whereas, in phosphorescence, fluorescence does not record absorbance and in phosphorescence fluorescence has to be eliminated, because the transitions to higher excited states in singlet to singlet, may be occurring even in a phosphoring molecule. So, that has to be eliminated.

For this, you can use both mechanical and electronic devices. So, in general, a gated scheme for the delay works satisfactorily. It is something to do with electronics. We are not going into the details, but basically it is a question of arranging a time delay for the measurement and a pulsed xenon arc lamp also can be used for excitation. So, after a delay, the time data acquisition system is activated to obtain phosphorescence. So, the instrumentation basically defers only in these two aspects and measurement has to be done with a time delay given by gated circuitry or some other mechanism.

So, when the lamp is off, fluorescence has decayed to a very small value, and the signal can be integrated. So, pulse xenon lamps, you put it on, off, on, off, on, off, and when the lamp is off, fluorescence has decayed, because it occurs within a fraction of a time; 10 raised to minus 4, so you give a time delay. By the time, the pulse, the lamp is off; fluorescence would have already decayed to minimal level, so that the measurement has to be made when the lamp is off. When the lamp is on, fluorescence is also measured. So, you can even correct for the difference between fluorescence and phosphorescence by subtracting the two signals.

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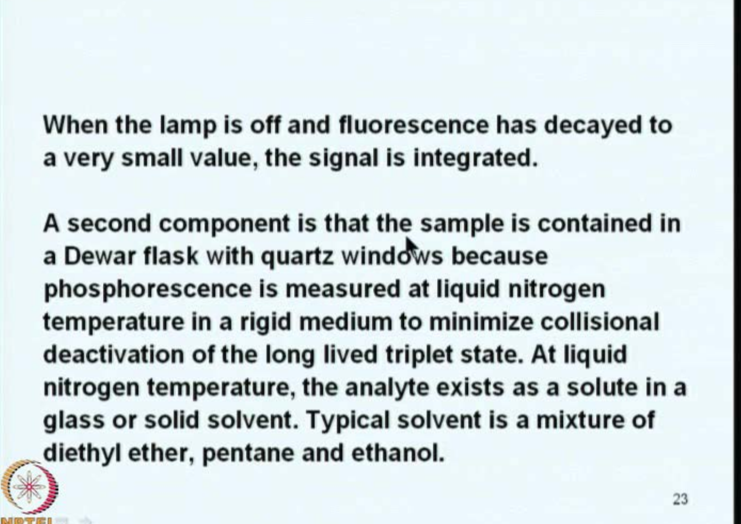

So, a second component that is important in phosphorimetry is that the sample is contained in a Dewar flask. That is with quartz windows, because phosphorescence has to be measured at liquid nitrogen temperature. If you remember, our previous discussion on the measurement of phosphorescence, and we know that at liquid nitrogen

temperature or very low temperature, phosphorescence remains stabilized. So, you need to cool the whole system in a Dewar flask; Dewar flask essentially does the cooling.

The Dewar flask, when it contains the sample should also have windows made of quartz through which the radiation can pass through. That is very important, so with that triplet state, in general, will last longer in phosphorescence measurement and the measurement can be completed at liquid nitrogen temperature. The analyte exists as a solute or a solid solvent. Obviously, at liquid nitrogen temperature, many of the phosphorescing compounds solidify. So, it either exists as a solute in a glass container or quartz container or it will become a solid solvent. So, typical solvent is a mixture of diethyl ether pentane and ethanol.

You can use these solvents either singly or in multiples or in mixtures; you can use multiple solvents systems in different ratios containing diethyl ether pentane and ethanol. Then measure the fluorescence reduce the… Put all of them in a Dewar flask, pass the nitrogen, they will cool down, and when the cooling down takes place, they will remain a solids or solids solvents. Then a measurement can be done easily. But the phosphorescence has not taken off as strongly as fluorescence, as an analytical instrumental technique.

Obviously, because of the need for cooling of the substances to such low temperature and it requires slightly more involved and dedicated requirement to measure the fluorescence. So, obviously fluorescence is the more practiced technique and it can be used very easily, because of the availability of the instrument. For phosphorescence, they are slightly more specialized instrument useful for research purposes.

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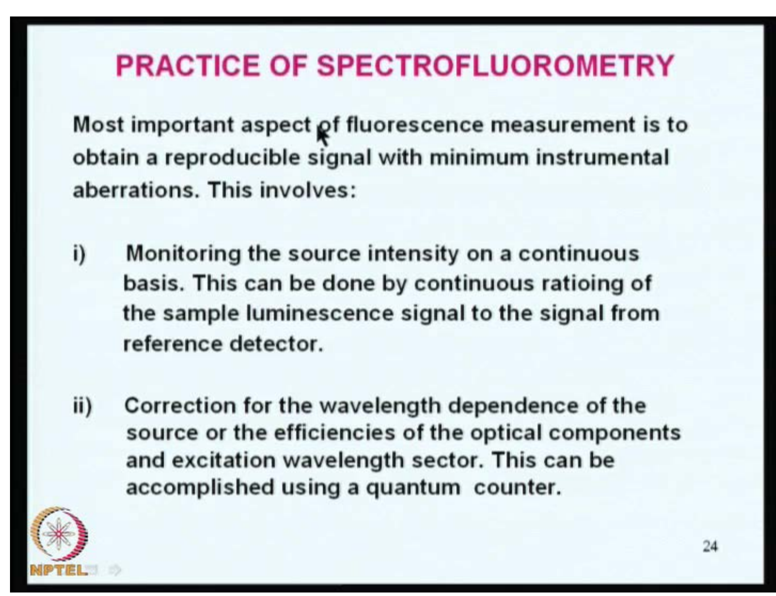

Most of the important aspect of fluorescence measurement is to obtain a reproducible signal with minimum instrumental aberrations. So, how do you measure the fluorescence? How do you practice fluorescence?

The important aspect is to measure the fluorescence to obtain a reproducible signal, if there is no reproducibility, there is no analytical technique. You cannot measure anything because the reproducibility is not there means you cannot rely on you results. The most important aspect is to get a reproducible signal for a given concentration of the analyte. If that is not done there is absolutely no point in going ahead with the fluorescence. So, what does it involve? It involves monitoring the source intensity on a continuous basis, because when we are making the measurements, and if intensity of the lamp itself is wavering, then there will be differently changes in the fluorescence output.

This fluorescence output has to be corrected to the changes in the lamp intensity. This can be done by continuous rationing of the sample luminescent signal to that of reference detector. So, long as you keep on continuously making the ratio measurement, there will not be any change in the fluorescence signal. Basically, very simple analytical technique and electronic technique also, all you got to do is keep on measuring the ratio because ratio will remain same. If you make absolute measurement, there could be changes, but if you make the ratio measurements, then there will be there will be reproducible.

Sometimes you have to correct them for the wavelength dependence of the source or the efficiencies of optical components also. Excitation wavelength sector, this can be accomplished using a quantum counter, but this is not very important, because in general most of the instruments offer dependable, wavelength dependable intensity of the sources. Only the source may vary in intensity for that rationing has to be done. Now, in some instruments, the reference spectrum is stored in the computer memory; microprocessor memory. So, you can continuously do the rationing by from the stored memory itself.

After the sample spectrum is scanned, the correction is made by just calculating the ratio of the spectrum to that of the reference stored in the memory. This is another way of doing it instead of continuously like doing the rationing. To obtain corrected and uncorrected spectra, you need to use a standard. Usually, a quinine sulphate is a very good standard and it is used in 0.2 molar H2SO4; sulfuric acid, 0.2 molar sulfuric acid, if you make a solution, you will be able to determine the quinine sulphate. It gives you very reproducible result and if there are any changes in the source intensity, you can increase the aperture to get the same fluorescence value for quinine sulphate. That is the idea of a standard.

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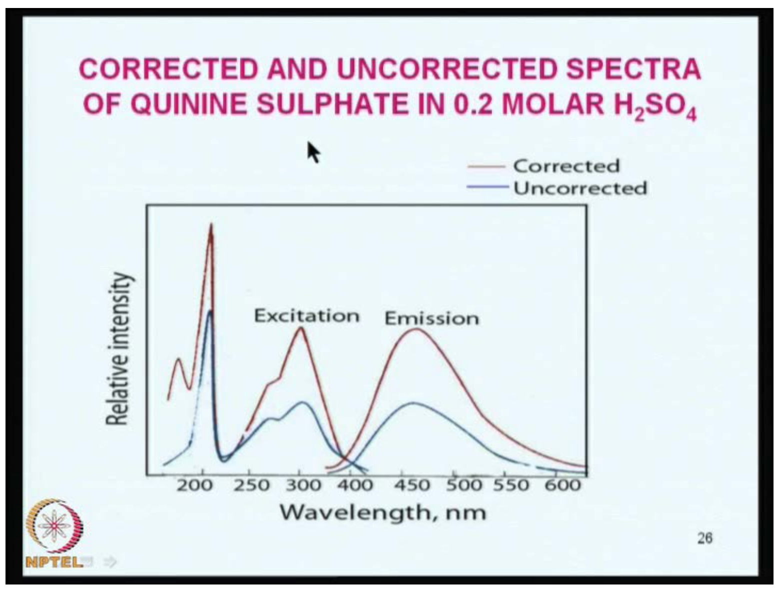

So, the standard is quinine sulphate. This quinine sulphate is used as a solution in 0.2 molar solutions, and you can see this spectrum here. Now, you can see relative intensity

has been plotted versus wavelength. In spectrophotometry, we plot not the relative intensity, but we plot the absorbance; negative log of transmittance or the absorbance. Here, we have plotting relative intensity and here there is a blue spectrum figure at the bottom, you can see here. The spectrum in blue color shows you the excitation and emission spectra and corrected samples. So, you can see that uncorrected sample has got lower intensity. In all the cases, whereas, spectra in the red shows you higher intensity because it has been corrected for other losses.

So, this peak blue, the peak around 200 is obviously having about of 25 times more intensity in the corrected spectra. Same thing is true of the excitation as well as emission. You can see they increase in the sensitivity when we correct the spectrum. This is the reason why we go for correction of these spectra. So, if you use corrected spectra, then what happens is that you will be working at lower concentration, especially in some of the applications involving the measurements of pikogram level or nanogram levels of environmental pollute and etcetera. The signal would be so small that you have to use corrected spectra to obtain good sensitivity.

So, corrected spectra are useful for such purposes and the concentration of the substance are very near the detection limit. Above the detection limit if they would give you a good signal anyway, but very near the detection limit, there could be the electrical noise and other signal noises, have to be filtered. In that case, if you go for corrected spectra, then it is simpler to work at still lower concentrations. To correct the emission spectrum, what you should do? We have to use a calibrated light source to determine the calibration factors for the emission monochromator and transducer also this is very important.

The information is stored in the computer memory and again you can take a normal spectrum, fluorescence spectrum, and obtain the correction using the computer stored memory data that is stored in the computer memory. So, uncorrected emission results are multiplied. All you have to do is take the uncorrected emission results and then multiply with an appropriate correction factor to determine the corrected emission spectrum. This is offered as a standard feature in most of the spectrofluorimeters. So, regarding the instrument standardization, we have already discussed that variations in the source intensity, and other instrumental factors, it is impossible to obtain the same readings for a solution or the same set of solution from day to day. So, it is a common practice to

standardize an instrument to present to a preset value using a standard solution of stable fluorophore.

This arrangement even helps in publications, suppose you want to publish your results in a journal, in a research journal. Then what you should do? You should take readings using 0.2 molar quinine sulphate and say that using this aperture; let us say about 0.2 nanometer aperture, we have adjusted the quinine sulphate to 80 percent intensity. Compare to that, our results are like this. So, that somebody else who wants to work on the same system, they can also use 0.2 molar quinine sulphate, adjust it to that 80 percent intensity and get the same results. This is in a way of known standardizing as well as presentation of the same data, which can be used for inter laboratory comparison.

So, the inter laboratory comparison must be and must always accompany the standardization results, in the publication. So, any spectrofluorimetric method adopted for the measurement of a substance, in an environmental sample or sea water sample or a lake water sample or any other pharma compound, pharmaceutical molecule, etcetera if you want to publish, you should always say yes we have done this standardization using quinine sulphate. Basically, to about 80 percent transmittance, so that in other instruments, in some other laboratory, they can also adjust the aperture to the same level and compare the concentrate. Then inter laboratory comparisons can be made.

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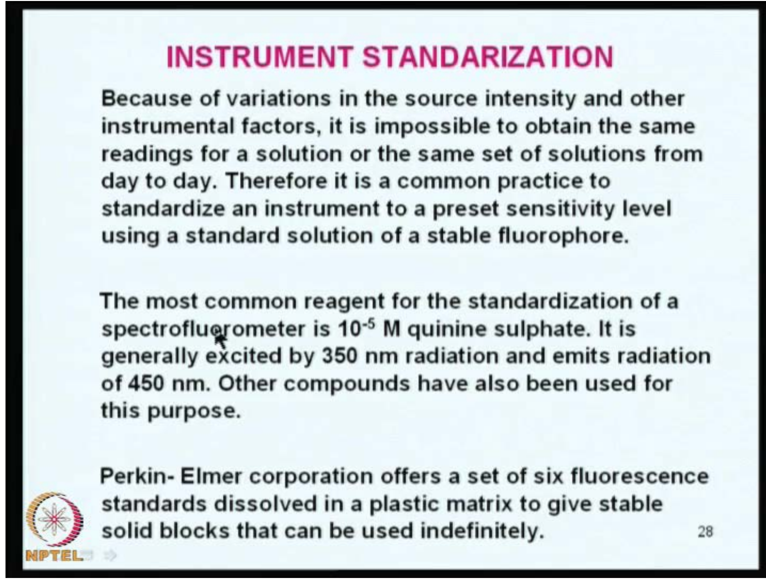

So, the most common reagent for this standardization of a spectrofluorometer is 10 raise to minus 5 molar quinine sulphate. Earlier, we had in the figure what I had shown is quinine sulphate in 0.2 molar H_2SO_4. That is the solvent, and now what we are saying is 10 rise to minus 5 molar quinine sulphate also should be there. So, you prepare 10 raise to minus 5 molar quinine sulphate in 0.2 molar sulfuric acid and adjust the sample fluorescence to a particular value and then you can report your results. It is generally excited using 350 nanometer radiation and it emits radiation also around 450 nanometers.

These two numbers are fixed for quinine sulphate, excitation occurs at 350 and emission occurs at 450. So, whenever you are doing the publication of your results, you should say that we have used 10 raise to minus 5 molar quinine sulphate in 0.2 molar concentrations of H_2SO_4 and using the excitation wavelength of 350 and measured the emission wavelength at 450 nanometers. Then other compounds can be measured. You can use other compounds also for standardization; you need not restrict yourself to quinine sulphate alone.

There are other compounds and they have been used. Perkin-Elmer Corporation offers a set of 6 fluorescence standards dissolved in a plastic matrix. What they do is they dissolve and take fluorescence quinine sulphate and standardize it, make different concentrations in a solid solvent and then put it in a block. Then they give you this is the standard and that block you need not wash, you need not prepare, you need not do anything, except use that block, adjust your aperture, adjust the excitation wavelength to 350 nanometers, and then continue, measure the excitation at 450 nanometers and that is one standard.

So, they will give or offer you about 5 or 6 standards for fluorescence measurements, for day to day measurements and for inter laboratory comparison. So, all of it is put in a single glass block solid matrix and it is easy to give. You do not have to prepare so many substances; you just take the sample and put it and then adjust it and then make start your measurements. This is only a convenience arrangement, so you can save the cost, if you can prepare your own solutions. So, what are the analytical aspects of fluorescence? In spectrophotometry, basically what we are doing is an incre we measure the increase in the intensity of the incident radiation that results in a proportional increase in the transmitted radiation.

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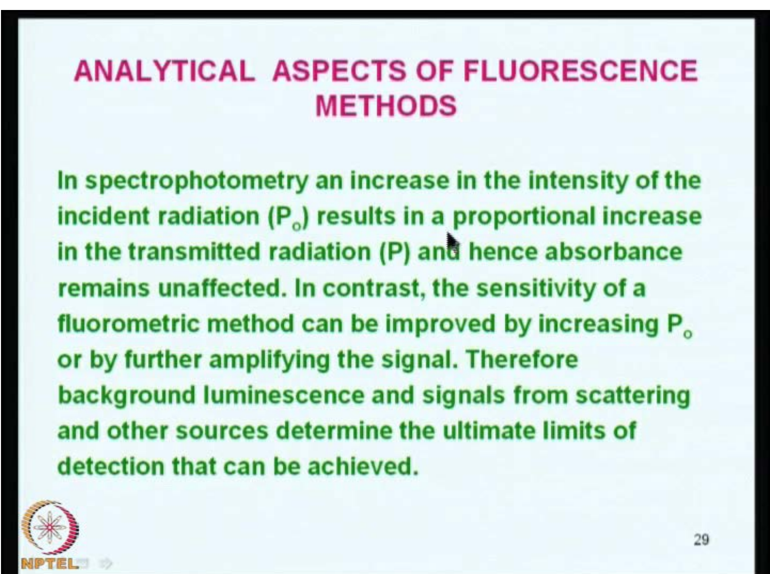

So, the absorbance is always a ratio unaffected by the intensity of the incoming light, because it is only the ratio. In contrast the sensitivity of a fluorometric method can be improved by increasing the incident radiation. We have discussed the theoretical aspects of this. You showing a figure in the last class in the window journal and by further amplifying, we can amplify the signal further and we can improve the intensity.

Therefore, for the same analytical process, there is no minimum detection limit for a fluorescing compound, because you can always increase the intensity, fluorescence intensity, gets a higher reading. So, this is a major difference in spectrophotometry compared with the spectrofluorometry. Therefore, background luminescence and signals from scattering and other sources only determine the ultimate limits of the deduction in fluorescence.

I want you to understand that this difference is, so because we are saying that the fluorescence intensity of a given substance, of a given concentration, can always be increased, by increasing the source input. So, I just now, I made a statement that there is no limit to the measurement of the sample fluorescence, but it is not you can go down to higher levels or lower levels for measurement. But, it is slightly wrong statement, because the measurement, here in last sentence here in this (Refer Slide Time: 53:33) what it says is the background luminescence and signals from the scattering and other sources determine the ultimate limits of deduction that can be achieved.

So, there is a limit of deduction, but that limit is controlled by the scatter, as well as background luminescence. Suppose, the substance is fluorescing, glass cell itself is fluorescing. What you can do? We have to just subtract it, so background luminescence at that wavelength is automatically limited to that. To have a good reading, you have to increase the fluorescence at least about 3 times that of the background and that is a standard practice.

The precision of fluorescence methods is often limited only by the background noise, source flicker noise and drift. In general, the precision and accuracy of fluorescence methods are usually 2 to 5 times higher than the spectrophotometric determinations. That means the precision and accuracy are also important now. So, they are 2 to 3 times higher in spectrofluorometric measurements.

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The accuracy is again often limited by the concomitants. What do you mean by concomitants? Concomitants are the other substances present in the sample, in which you may or may not be interested; 99 percent you may not be interested, but suppose they give fluorescence at the same wavelength, same this thing, then it can be construed as interference.

The interference, in spectrophotometry, spectrofluorometry always occurs by the presence of particles present in the sample, as well as that the particles that cause increased fluorescence. The substance could be having higher fluorescence or it could be

quenching the existing fluorescent that is also interference only. So, the interference has to be always determined whether it has increased or decreased from a standard and that standard again is in turn standardized by quinine sulphate. So, depending upon the nature of the chemicals species, the fluorescence has to be measured.

Luminescence methods have linear dynamic ranges also, comparable to spectrophotometric methods. That is you can determine the substances in 10 raise to minus 4 molar, minus 5 molar concentrations, in ppm levels, as well as parts per billion level. Sometimes, ppb, parts per billion levels, 10 raise to minus 12 nano micrograms of the substances can be determined by spectrophotometry. They also show better sensitivity and selectivity, because not many molecules would show similar fluorescence. So, in fluorescence in general you will end up always with lower interferences, compare to spectrophotometry, because many molecules will not fluoresce at the same wavelength. Even if the flourish they may not be emitting the fluorescence at the same wavelength. So, the selectivity of a fluorescence method is always better than the better than the spectrophotometry.

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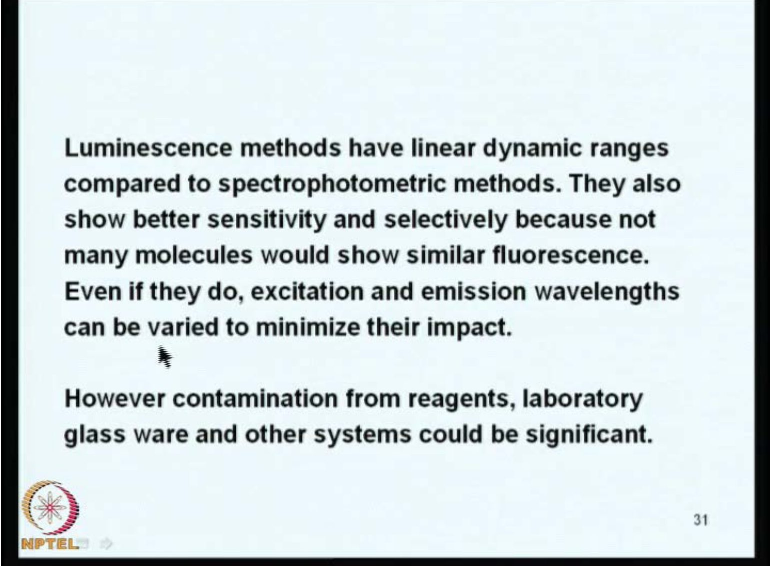

So, even if they do fluoresce, excitation emission lengths, measurements, wavelength itself, we can vary to reduce the interferences of other fluorescing compounds. However, contamination from reagents, laboratory glassware, and other systems, could also be significant and one has to take care that we will have to reduce the contamination

whenever you are working with fluorescence. We will continue our discussion on fluorescence measurement in the next class.