Natural Dyes

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Lecture No. # 08

Continuing to talk about thin layer chromatography; there are a few points that need to be clarified, that silica gel is the main coating material which is used, and it is the reaction of the dye molecules with the silica, whether it is adhering to this silica or not; So, adhering will decide the polarity of the dye, and accordingly the polarity of the solvent will then start be chosen, and once the desired chosen solvent system is taken that dyes starts moving at a different RF.

RF means the Retardation Factor and that Retardation Factor for dye one or dye two will always be different, in different polar solvents. So, therefore, the solvent polarity is of prime importance and secondly, the RF of the dye is of importance; it can be actually, because these are all colored molecules, they can be seen by the naked eye, by the use of visible light or sometimes these TLC plates are then developed in iodine chambers. So, they become brown spots, and one can actually see the dye molecule on the TLC.

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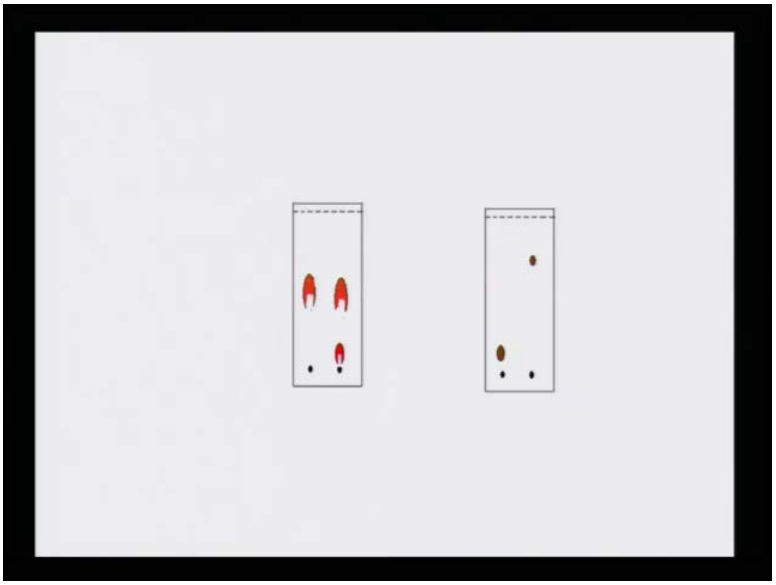

If it is a mixture of two dyes, it will show that no there is an example that I have taken that there a was a mixture of RED dye; and on one side I have spotted and which is showing only one spot, whereas on the right hand side, the spot shows two RED colors; which means that is the right hand side dye mixture, which was also a RED dye actually contains two components; and the left hand side dye is just a single component dye. Now, when I was telling you that the dye can either be seen visually, that is because you can see the RED color; otherwise it is put in the iodine chamber and all the dyes appear as brown dyes or brown spots. So, that is what I mean.

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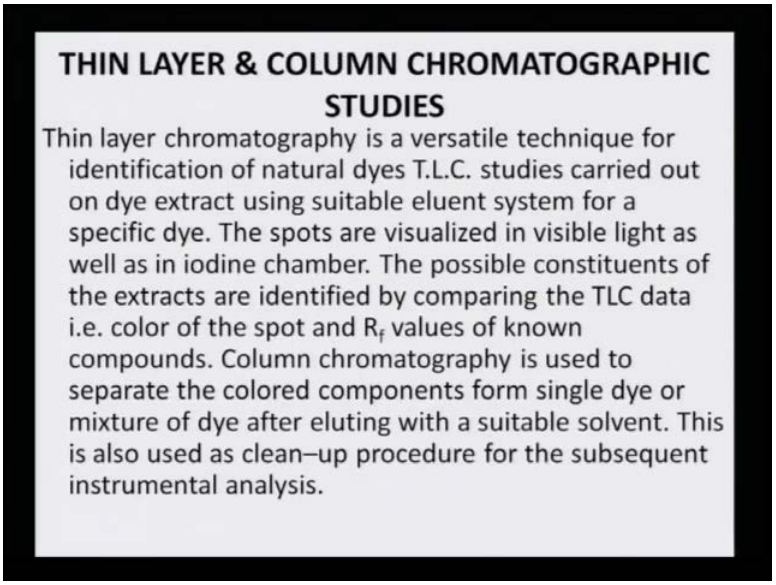

Now going to back column chromatography; column chromatography are like to be a structures, which have a stop cock at the end; and the column is filled up with the silica gel; of course, the silica gel that is used in this case is different from the silica gel that is used for TLC. So, for because on the TLC, a very even smearing of the silica gel coating needs to be done, therefore it is finer in particle. But this silica gel, which is used in column is slightly coarse, and once the silica gel is filled with the help of by making it into a slurry, then mixtures of dyes can be separated on them. So, that is the purpose of… But the basic fundamental of a separation or identification is the same as what it is for thin layer chromatography. So, if I have to explain a little more in detail.

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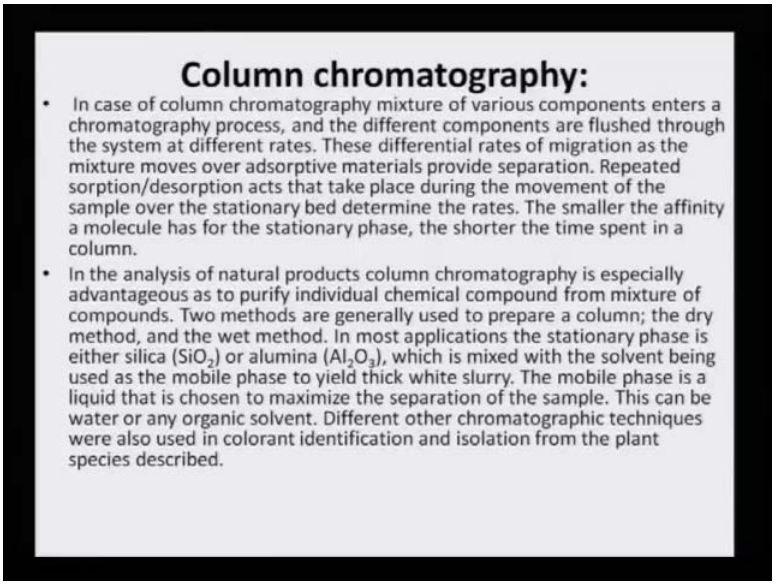

In case of Column Chromatography, mixtures of components enter a column chromatographic process, and the different components are flushed through the system at different rates in different polarity of solvents. These differential rates of migration as the mixture moves over adsorptive materials provide separation. Repeated sorption desorption acts that take place during the movement of the sample over the stationary bed determines the rate. The smaller the affinity a molecule has for the stationary phase the shorter time spent in the column. So, it is something, which is a valid for any and every organic compound.

So, it is how these a organic compounds are adsorbing and desorbing and absorbing and desorbing on the column that the rate of illusion will be decided; and because there is a long traversing area, the rate of traversing becomes larger and larger, so they get separated; you although it all starts with the mixture, but then gradually because each dye has a different rate of traversing on the column, so, therefore, it moves separately; and when it moves separately, it would be dependent on the fact; the movement will be dependent on the fact that how nicely or how not so nicely it is having an affinity of the silica gel. And if it is having a good affinity for silica gel it would be adhering to the silica gel and therefore, it will not elute very fast, but other components will elute.

So, therefore, it one can come to a conclusion that it is all a process of absorption and desorption that decide the rate of its flow onto the column and finally, out of the column. In the analysis of natural products, column chromatography especially advantageous as to purify individual chemical compounds from mixture of compounds. Two methods are generally used to prepare a column that dry method and the wet method. In most application the stationary phase either silica gel or alumina which is mixed with the solvent being used as a mobile phase to yield thick white slurry.

The the mobile phase is a liquid that is choosen to maximize the separation of the sample. This can be water or any other organic solvent. So, it is important that what kind of a solvent we should use the same solvent, in which the silica gel slurry has been made. And if the polarity of the slurry and the polarity of the you know solvent that will be subsequently used are very different; it will cause some kind of heretic movement in on the column; therefore, one should remember that one should not abruptly change the polarity. Gradually a the polarity should be changed, so that the rate of traversing does not altered. Different other chromatography techniques were also used in colorant identification and isolation from plant species and will be described. So there, this is one of the basic methods of separation if it is a mixture of dyes.

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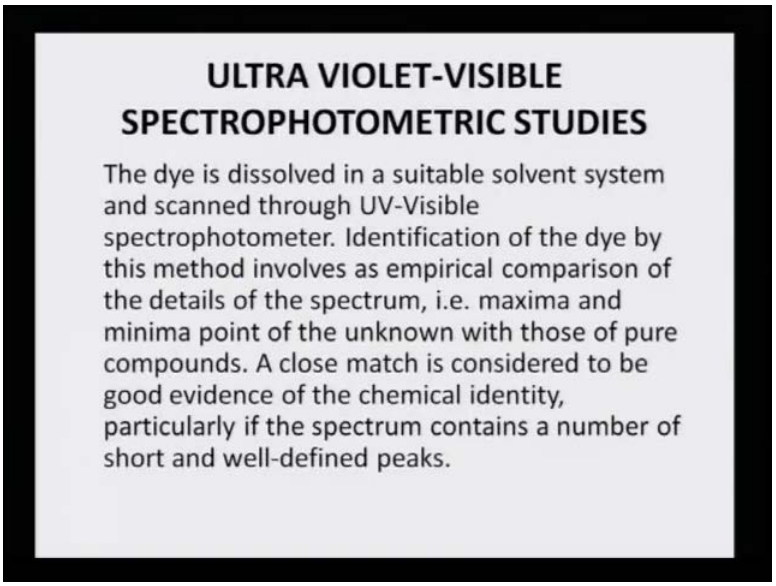

Ultra Violet-Visible Spectrophotometric Method: This we will do in a little bit more detail, because this is one of the quickest and most a you know easily doable kind of

technique to identify the dye molecule. A dye is dissolved in suitable solvent system and scanned through UV visible spectrophotometer. Identification of dye by this method involves an empirical comparison of the details of the Spectrum that is the maxima and the minima point of unknown with those of the pure compounds. A close match is considered to be good evidence of the chemical identity particularly, if the Spectrum contains a number of short and well-defined peaks.

So, this is one of the best methods as what I mentioned, why because the lambda max is the main criteria, because every colorant molecule or every chemical molecule may have a visible or a UV region lambda max, and that would depend on its chromophoric group. And So, it is a significant of its structure as well.

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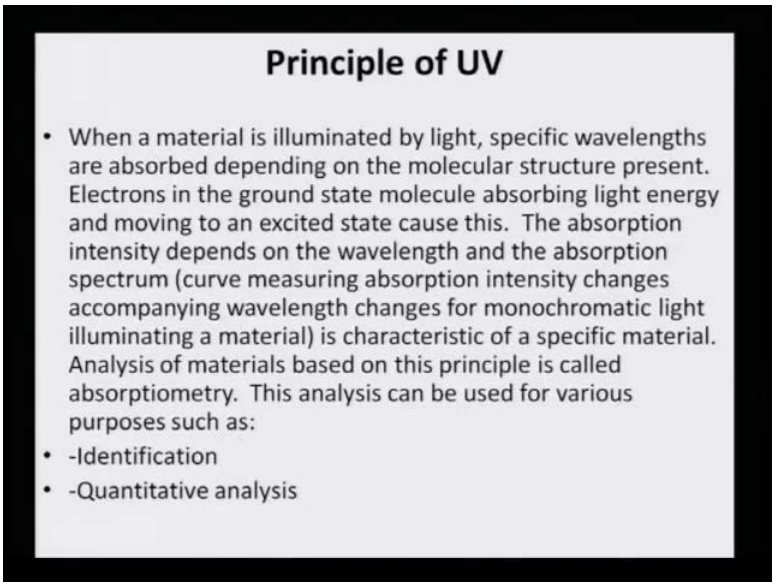

Principles of UV: When a material is illuminated by light, specific wavelengths are absorbed depending on the molecular structure present, Electrons in the ground state molecule absorbing light energy and moving to an excited state cause this. The absorption intensity depends on the wavelength and the absorption spectrum, that is the curve measuring absorption intensity changes accompanying wavelength changes for monochromatic light illuminating a material; in… And it is characteristic of a specific material; analysis of material based on this principle is called absorptiometry. This analysis can be used for various purposes that is identification as well as quantitative analysis.

Right now, let us only concentrate on the identification, because we are trying to learn various techniques, which can be used for the identification of dyestuff; be it natural dye or synthetic dye. So, the best way of doing that is that light - a monochromatic light is passed through the dye or the dyestuff and the lambda max is absorbed, which is a very characteristic of a dye; for example, I will tell you that AR18 and DR80 showed a lambda max of 509 and 543 nanometers respectively.

By whichever, UV spectrophotometer, you take this or you evaluate the UV Spectrum of these two dyes, the lambda max will always show this; which goes to prove that the structural details are such that the chromophore groups are in such a manner, that it would absorb light related to these two wavelengths only, when these dyes are present. So, that is how one tends to identify dyes, and this is valid for both synthetic dyes as well as natural dyes.

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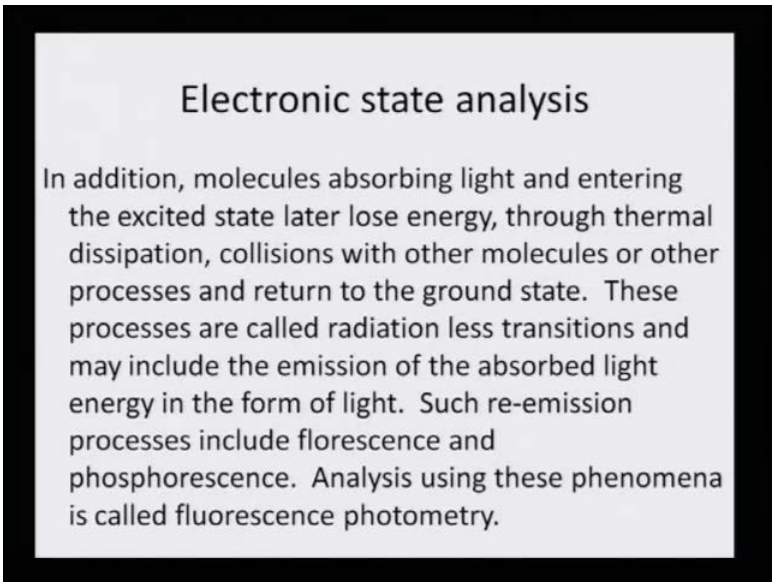

The Electronic state analysis: In addition molecules absorbing light and entering the excited state later loose energy, through thermal dissipation, collisions, another molecules or other processors and return to the ground state. These processes are called radiation less transitions and may include the emission of the absorbed light energy in the form of light. Such re-emission processes include florescence and phosphorescence. Analysis using these phenomena is called florescence photometry. So, this is another kind of study where you know, the glowing of the light is observed then that is because it

is not usually transferring to the ground state in a very normal manner there are some other things that are happening along the process, and what is exactly happening that there is some amount of radiation, while this a ground… When the electrons are coming back to the ground state, some radiations are given out, which causes florescence.

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Now, this is how the UV spectrophotometer looks like. And I just took a picture for you, because you should be able to at least identify that this, a model of UV spectrophotometer.

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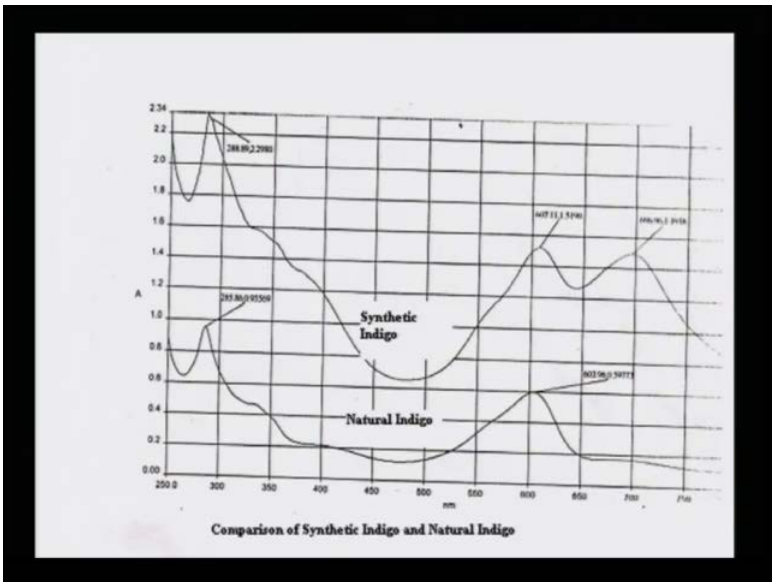

Now, when a spectrum is evaluated, this is a typical UV Spectrum of synthetic indigo and natural indigo. Now, you will see that if our layman looks at these two peaks, a these two lines; one indicating the synthetic indigo and the other indicating the natural indigo. You will say that more or less they look similar, but they are not really similar, what makes them dissimilar is the fact that the peaks at 285 as shown by natural indigo, is slightly different in the case of synthetic indigo; and it shows a 288 nanometer, which is in the UV region; and in the visible region, if you see the natural indigo shows a peak at 603, and the synthetic indigo shows a peak at 607 nanometer.

So, there are very small variations, but nevertheless with the help of UV itself, I told you with the help of TLC we could identify, that whether there is a natural indigo or synthetic indigo; similarly with the with the help of UV machine, one can identify looking carefully at the lambda maxes. So, the lambda max at 288 and 285 are definitely different. Similarly the lambda max at in the visible region, that is 603 and 607 are different. So, they make these two molecules, quite difference from each other in terms of UV spectrum.

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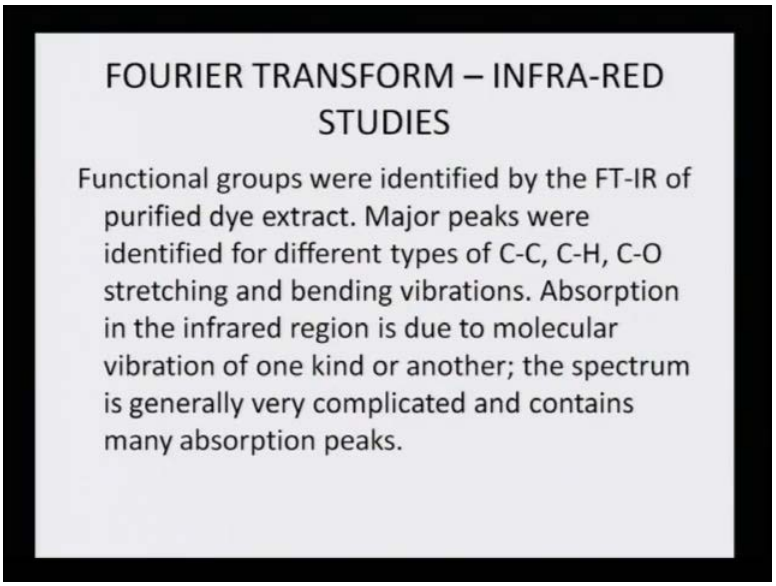

Similarly, the next machine that we come to know or look at it which is used for the identification of dyes, is Fourier transform - infra-red spectrometry. And the study show that functional groups were identified by FT-IR of purified dye extract; major peaks were identified for different types of C-C, C-H, C-O stretching and bending vibrations.

Absorption in the infrared region is due to molecular vibration of one kind or another; the Spectrum is generally very complicated and contains many absorption peaks; nevertheless there is a region, which is for the interest of the analyst; and there is one region which is very, very specific, which need not be you know, understood fully. But other half of the Spectrum give sufficient information, so that one can at least derive at some kind of conclusion.

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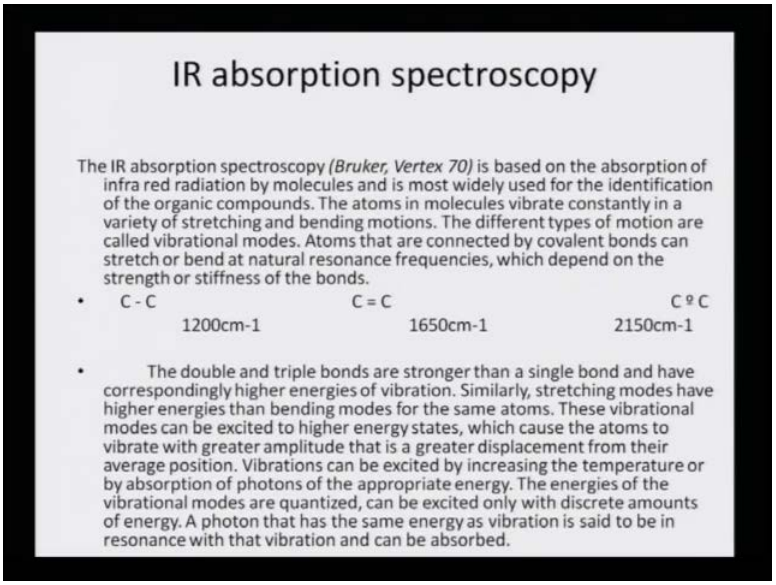

IR absorption spectrometry or spectroscopy: The IR absorption spectroscopy is based on the absorption of IR radiation by molecules; I am all along mentioning molecules, because dyes are also molecules - organic molecules. And is most widely used for the identification of organic compounds, the atoms in molecules vibrate constantly in a variety of stretching and bending motions. The different types of motions called vibrational modes; atoms that are connected by covalent bonds can stretch or bend at natural resonance frequencies, which depend on the strength or stiffness of the bonds.

So, a C - single bond C will show a vibration mode at 1200 centimeter inverse, carbon-carbon double bond will show at 1650 centimeter inverse, and carbon-carbon triple bond will show at 21 or 2200 centimeter inverse. Now you see that if if we only look at the C-C bonds, C-C bond single bond, C double bond C, C triple bond C, the variation is so prominent. If we simply take these three even then the molecules can show whether they have this particular moiety or or functional group in their structure or not.

So, part information can be obtained, and the in the beauty of this is that infrared radiation cannot cause Electronic transition as the UV light or the visible light can do. So, what it does? It only plays around with the covalent bonds stretching bending and all kinds of vibrational modes; and by that itself, that change will only occur, if the infrared radiation of the matching frequency hits the molecule not otherwise. So, this compatibility of the infrared radiation matching with the requirement of the bond C-C bond or any covalent bond has to be similar, only then vibration modes will occur; the double and the triple bonds are stronger than a single bond, and have correspondingly higher energies of vibration. Similarly, stretching modes have higher energies than bending modes of the same atoms. These vibrational modes can be excited to higher energy states, which cause the atoms to vibrate with great amplitude, that is greater displacement from their average position.

So, you you just imagine that there is a pendulum; and it is kind of go as high as the energy, as high as the energization would take place. Vibrations can be excited by increasing the temperature or by absorption of photons of the appropriate energy; the energies of the vibrational modes are quantized, and can be excited only with discrete amount of energy; a photon that has the same energy as vibration is said to be a resonance with that of vibration and can be adsorbed. So, when it is in resonance with the requirement energy or vibration for a particular bond or when it is matching or when it is compatible, you can say either of them. That is the time, when it will affect the body and that affection or that implication will cause the the the absorption peaks to come in the spectrum.

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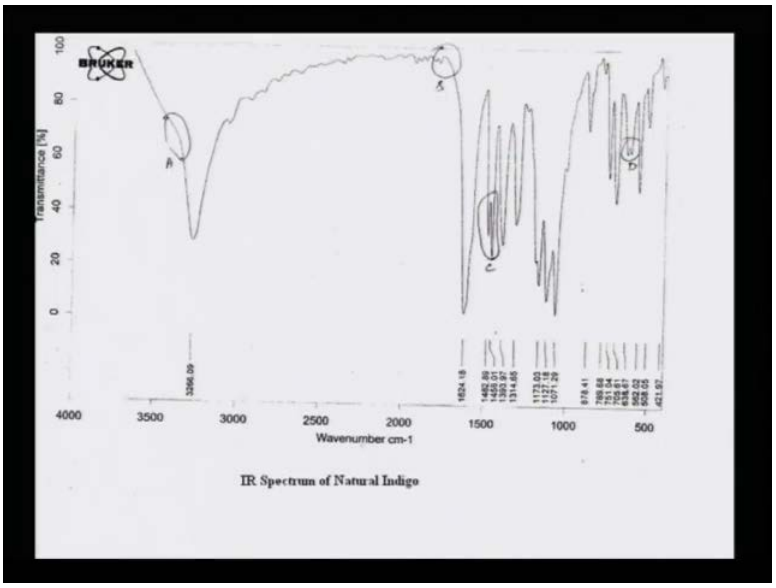

So, if I show you now, an IR Spectrum of natural indigo; you will clearly see, there are certain portions marked with A, B, C and D; and these were found to be quite different from the synthetic indigo IR spectrum. Although in one go, they both look alive, but if one looks at it very carefully the regions of A, B, C, D marked in the synthetic indigo, is quite different from the regions that are marked in the natural indigo A, B, C, D. So, that is how IR helps us to identify these molecules in a little more detailed method; and you see all these peaks are nothing but absorption peak.

It is the region between 4000 to 1500, which is a prime importance for an organic chemist to identify; the other region, which is from 1000 to 4000 is the region, which is the finger print region, which is of course, for a very specialist to a take care and understand these peaks. But the functional group area that is from 4000 centimeter inverse to 1500 centimeter inverse, is where we can find the functional groups containing C-C bond, C double bond, C-C triple bond, C-C triple bond nitrogen, C double bond O and so on and so forth. Now, let us come to another chromatographic technique, which is very popularly used for dye molecules.

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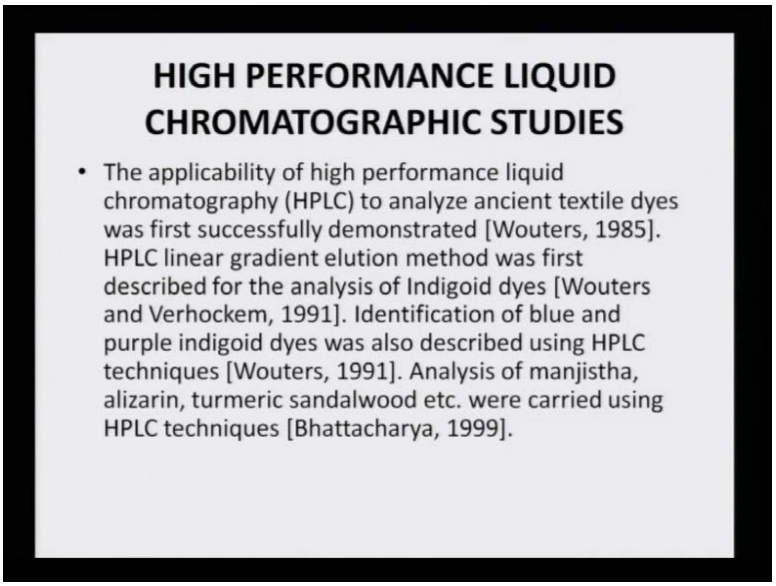

High Performance Liquid Chromatographic Studies: The applicability of high performance liquid chromatography that is HPLC, to analyze ancient textile dyes was first successfully demonstrated by Wouters in 1985. That is, it is not a very old technique; it is a very recent technique, which has been applied to dye molecules. HPLC linear gradient illusion method was first described by the analysis of Indigoid dyes by Wouters and Verhockem 1991. Identification of blue and purple indigoid dyes was also described using HPLC techniques by Wouter. Analysis of manjistha, alizarin, turmeric sandalwood etcetera, were carried out much later in the year 1999 by Bhattacharya; and the he also used the HPLC techniques. So, you see that how useful it is for the identification of natural dyes, and various different types of indigoid dyes, anthraquinoid dyes and even you know, turmeric and such dyes have been identified with the help of HPLC.

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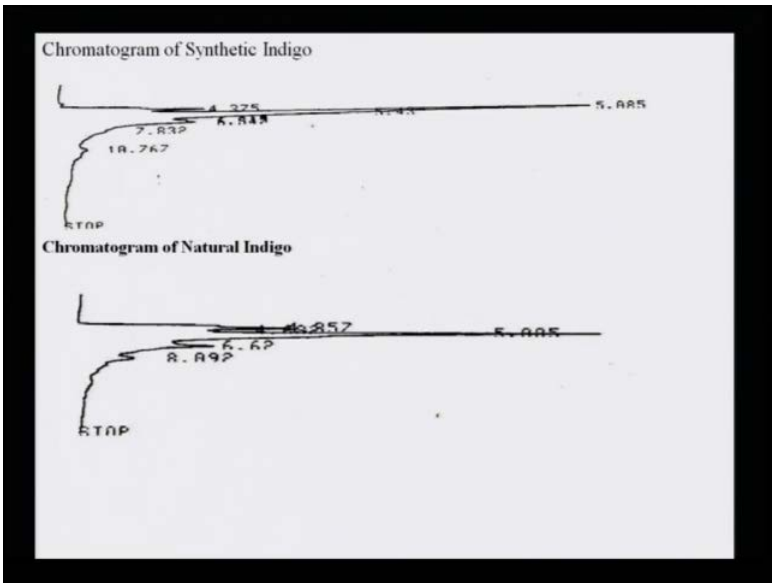

Now, you see I have taken an example of a chromatogram; the graph that is generated from a chromatographic technique is called chromatogram. And the graph that is generated out of a spectroscopic method is called spectrum. So, that should be clear now a chromatogram of synthetic indigo and a chromatogram of natural indigo has been shown here; you will see that there are small, small differences, but nevertheless one can make out that they are at least two different compounds; and the peak at 4.375 is very small in the case of synthetic indigo, whereas the peak at at 4.85 is fairly large in the case of natural indigo; and that is, because the iso indigo is also associated indigo molecule; therefore, these are you know very minute differences that one can figure out from the chromatographic technique.

Now, when we are trying to look at chromatographic technique, there is one thing which is very important to tell you, and that is that one needs a standard, because in it is a comparative analysis; whereas UV is an absolute analysis, IR is an absolute; you do not need another standard to compare; it give functional group information, it gives chromophoric group information and that is it. But in the case of chromatography techniques, one needs to have a standard molecule of the same; suppose if we want to evaluate the HPLC of alizarin in a manjistha sample.

Now, in order to be able to identify alizarin in manjistha, we should have a pure sample of alizarin and that is called as a standard. So, this kind of method is called a calibration

method. And therefore, one needs to have very pure samples of these standards in order to identify dyes in mixtures; and in natural dye, there is also another problematic situation, because no dye comes as a single molecule. They are always 4 or 5 or may be more dyes of similar structures, structurally very little difference will be there, but nevertheless they are different entities. So, therefore, a standard is a must in the case of HPLC usage.

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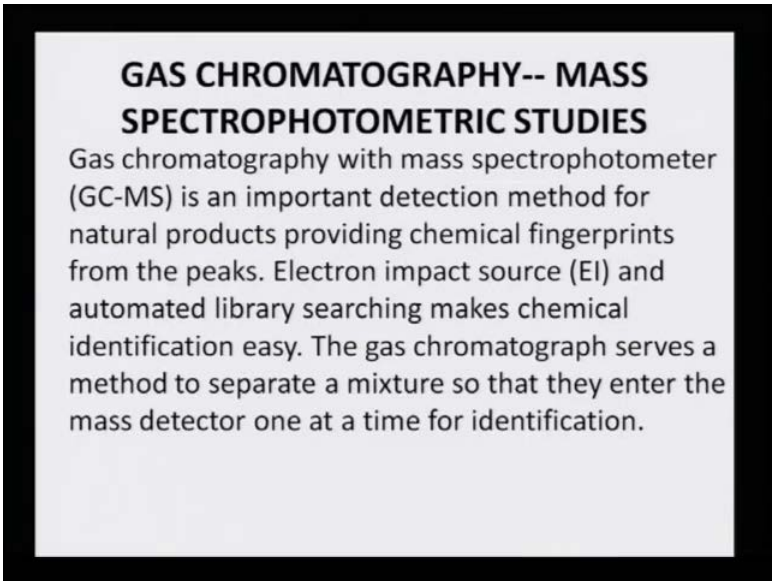

Gas chromatography: One can use gas chromatography alone or one can use it with the help of a mass spectrometric studies; gas chromatography with mass spectrometer, that is GC-MS is an important detection method for natural product, providing chemical finger prints for the peaks, from the peaks. Electron impact source (EI) and automated library searching makes chemical identification very easy. The gas chromatography serves a method to separate a mixture, so that they enter the mass detector one at a time for identification. So, the chromatography actually only separates them and the mass detector then starts detecting them and finding out whether the fragmentation pattern is of the known compound or it is an unknown compound or a different compound; and that can be done with the help of library search.

Now, here I would also like to emphasize that all organic molecules, whether they are dye molecules or they are colorless compounds, they all have a set fragmentation pattern. And therefore, the Mass fragmentation pattern is a very characteristic of the particular

compound itself. Two compounds on a gas chromatography can show the same retention time, but they may or may not be the same, just based on the retention time, but when the mass fragmentation is checked and tallied, that is the ultimate determination, whether the molecule is same or not; and therefore, it is one of the most serious kind of diagnostic test for identification of any dye molecule, again and again I am telling it can be applied for both synthetic and a natural dyes.

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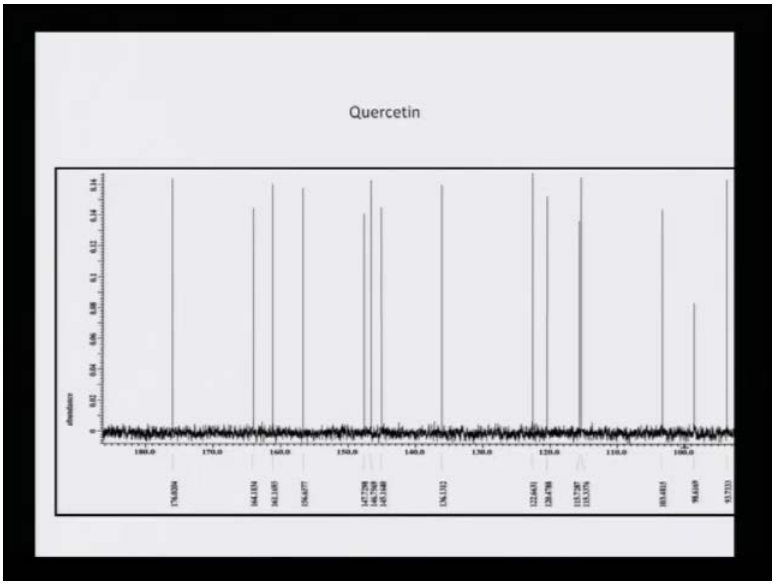

Now if I have to show you a mass spectrum of Quercetin; this is how it shows, you know the line of shows here, as the graph for different fragments that are a broken from the parent molecule. So, the the last line on the right hand side is the molecular ion peak, and then molecule breaks at the bonds, which are more facile and gives daughter moieties, which then can be correlated to find the structure; and this is the mass fragmentation pattern of a of the dye called Quercetin - It is a falconoid dye.

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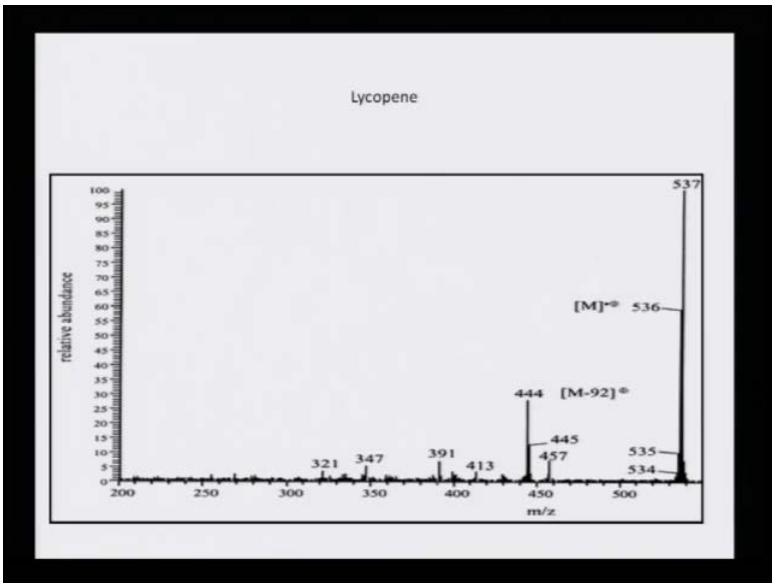

Similarly, I have taken another example of a dye, which is called Lycopene; and the molecular ion peak at 537, and there is a peak at 536 as well. So, the main molecular ion peak is 536, and M plus 1 is sometimes such peaks arrives due to the fragmentation pattern, which appears at 537; and when 537 breaks, it gives a peak at 444, that is with the loss of 92 amu - Mass atomic mass unit; and then subsequently, a peak at 391 and then at 347 and at 321, these are the different fragment or daughter molecule, that are deriving from the main Lycopene. One thing you will see that the length of these peaks are different, that is arising because the stability factor of these fragments make the line longer. So, the relative abundance is higher for the one's, which are more stable and which break after some time and not immediately.

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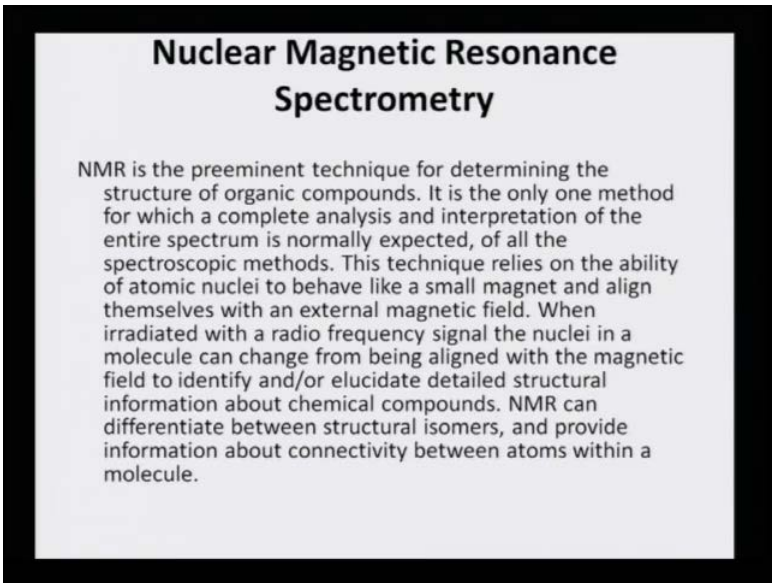

Nuclear Magnetic Resonance Spectrometry: NMR is the preeminent technique for determining the structure of organic compound. It is the only one method, for which a complete analysis and interpretation of entire spectrum is normally expected of all the spectroscopic methods. This technique relies on the ability of atomic nuclei to behave like a small magnet and align themselves with an external magnetic field; when irradiated with the radio frequency signal, the nuclei in the molecule can change from being aligned with the magnetic field to identify and or elucidate detailed structural information about chemical compounds. NMR can differentiate between structural isomers and provide information about connectivity between atoms within the molecule.

So, it gives proton NMR, gives the number of hydrogen that are present, and the carbon NMR, gives the number of carbon that are present, and the entire spectrum can be interpreted; therefore, it gives a quite a discrete information about the structural details of a molecule; be it a organic molecule, be it a dye molecule. Coming to Mass Spectrometry, we did understand a bit of mass spectrometry, when we were doing the gcms part, but to be able to explain to you in a more detail manner, because this is also one of the tools of identification of dye molecules.

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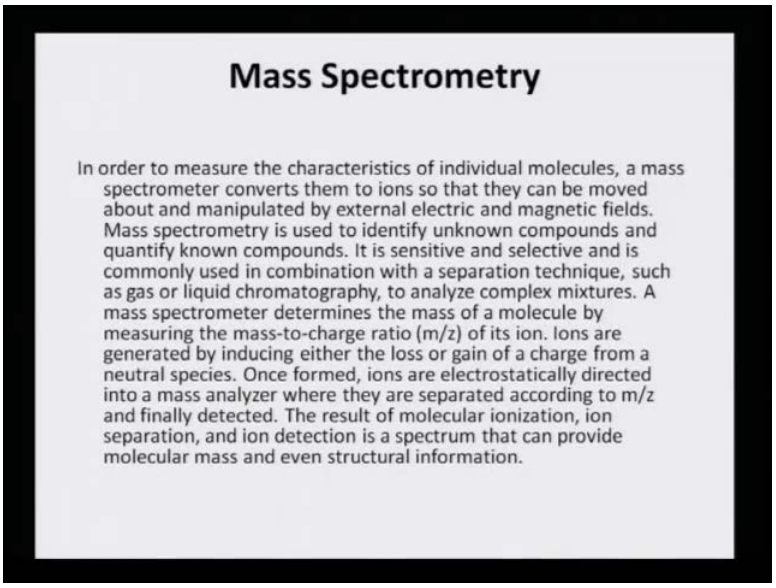

In order to measure the characteristics of individual molecules, a mass spectrometer converts them to ions, so that they can be moved about and manipulated by external electric and magnetic fields. Mass spectrometry is used to identify unknown compounds and quantify known compounds. It is sensitive and selective and is commonly used in combination with the specific with the separation technique, such as gas or liquid chromatography, to analyze complex mixtures. A mass spectrometer determines the mass of a molecule by measuring the mass-to-charge ratio that is the m by z of its ion. Ions are generated by including either the loss or gain of a charge from a neutral species. Once formed, ions are electrostatically directed into the mass analyzer where they are separated according to the m by z and finally, detected the result of molecular ionization, ion separation, and ion detection is the spectrum that can provide molecular mass and even structural information.

So, you see that this is an ultimate method for the determination of structure of an organic compound, and therefore, it is although called a spectrometric method, but it does not use any energy from the electromagnetic radiation region, unlike the UV, FT-IR and the NMR; because in UV, the UV light or visible light of the electromagnetic radiation were used, in FT-IR, IR radiations were used, in NMR, the radio frequencies were being used; but this is one spectroscopic method, where the molecule is a organic molecule, which is a which can be also a dye is bombarded with an electronic beam, and

then the molecule starts fragmenting. This fragmentation pattern is all always, always the same; and therefore, it is like the only method of fragmentation of the molecule.

And therefore, when the subsequent parts are formed, one can go back and calculate what has broken from where? And if certain amount of amu is broken, as what I had shown here in the case of Lycopene that 444 was generated from 536 or 537, only because there was loss of a fragment of 92 amu. So, this kind of correlation can only take place, because these burns break at a very particular time. So, we would not conclude that all these techniques are put together, the tools for evaluation of or identification for dyestuff, which are nothing but organic molecules.