**Advance Analytical Course**

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**Lecture No. #17**

Instrumental techniques for chemical analysis - time and again, I have been talking about why and how, these instruments are important in chemical analysis. And the course actually deals with advanced analytical techniques for analysis.

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So, it is very appropriate to sum up, all that we have learnt so far, and to see, how each one plays a very vital role in the analytical field.

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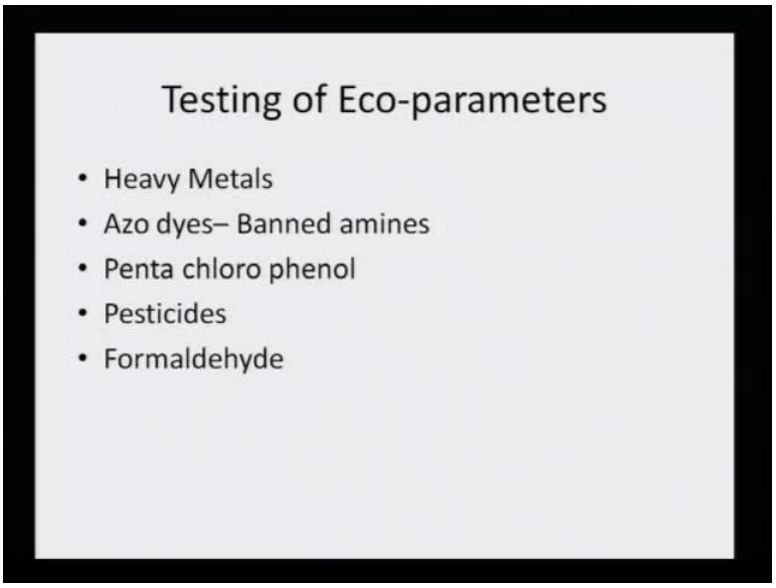


Testing of Eco-parameters - when we say that any commodity has to be exported, it has to be free and it should be eco parameter should be tested. And the eco-parameters that are tested are: heavy metals, azo dyes that is the banned amines, penta chloro phenol, pesticide remedies and formaldehyde residues.

If a commodity has any one of these at a level which is not permitted, the consignment will not go for export; it will be returned back, and therefore, the industry is becoming very conscious; particularly the textile industry, the food industry and the leather industry - they need to test their commodities for these eco-parameters.

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List of toxic and harmful substances used by textile and leather industries - they use banned pesticides, such as DDT, Dieldrin, Aldrin and Pentachlorophenol as preservative. They also use chlorinated products like Hypochlorite for chlorine bleaching. Azo-dyes containing aromatic amines, although they have been banned, but still in our country, a lot of dying houses are still continuing to use azo-dyes.

Dyes containing trace amounts of heavy metals, such as Arsenic, Lead, Cadmium, Mercury, Nickel, Copper, Chromium, Cobalt and Zinc. And Formaldehyde which is used as a mordant, formaldehyde is also used as a cross linking for finishing in urea, and formaldehyde is also used as a pesticide finishing plasticizer finishing.

Stain removers containing chlorinated products, wooden boxes treated with insecticides are the other areas which are coming in the list of toxic and harmful substances.

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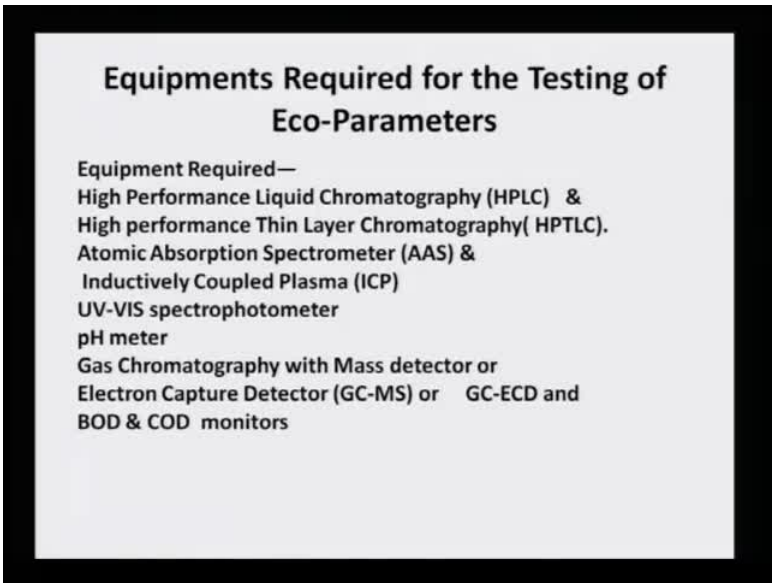


Equipments that are required for the testing of eco-parameters – obviously, if there are parameters that need to be tested, there should be an equivalent machine that requires to be used for that particular analysis.

Equipments that are required are: High Performance Liquid Chromatography that is HPLC, we have already learnt about it; High Performance Thin Layer Chromatography that is HPTLC, Atomic Absorption Spectrometer AAS, we a little while ago we were talking about that, and Inductively Coupled Plasma that is ICP spectrometer, UV-VIS spectrometer, pH meter, gas chromatography with mass detector or electron capture detector that is GC-MS, or GC with ECD and BOD and COD monitors. These are mandatory equipments that should be present in an analytical laboratory, which uses to analyze eco-parameters.

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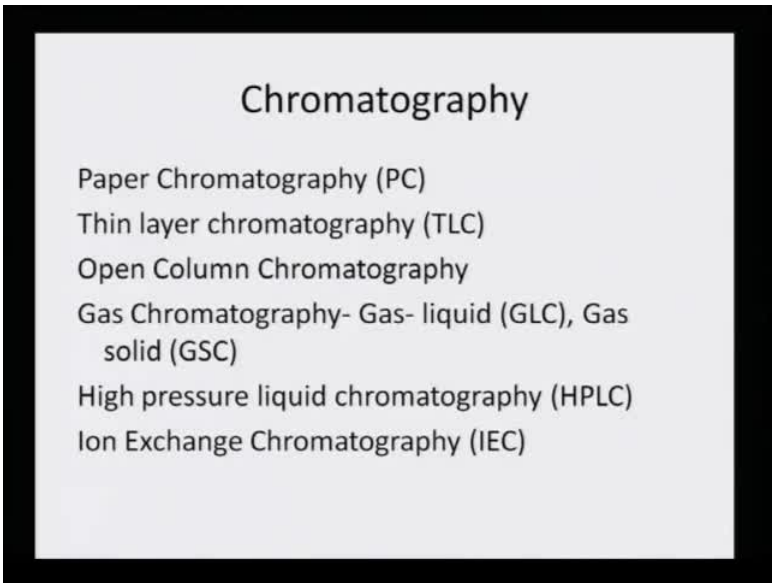


Among the chromatographic techniques, let us do a little of recap Paper Chromatography which has become a little absolete; Thin Layer Chromatography which is the most starting point for any chromatographic analysis; open column chromatography; Gas Chromatography with Gas-liquid or Gas-solid, which could be GLC or GSC; High Pressure Liquid Chromatography which is HPLC and Ion Exchange Chromatography or IEC.

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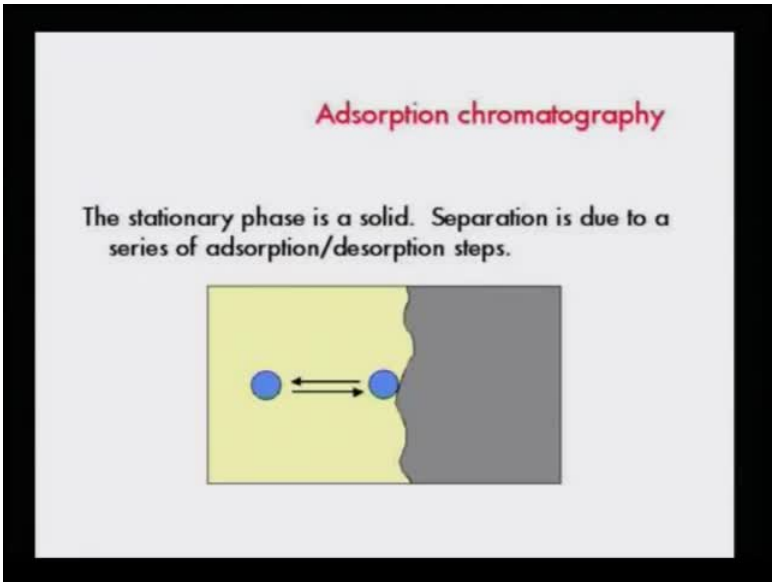

Adsorption chromatography - how it works? The stationary phase is a solid. Separation is due to a series of adsorption and desorption, adsorption and desorption, and that is how it passes on to the column.

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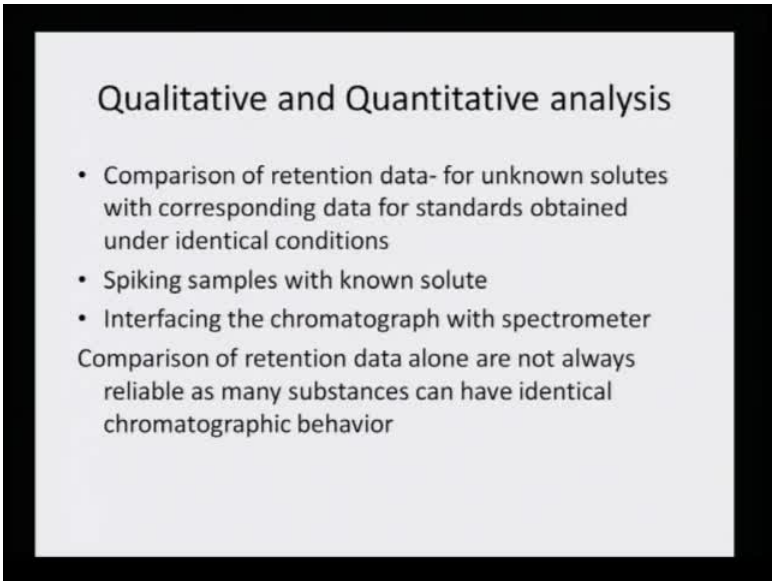


Qualitative and quantitative analysis - comparison of retention data for unknown solutes with corresponding data for standards obtained under identical conditions; little while ago, I was talking about calibration method. Unless and until we have a standard, it will not be able to possibly, compare any two substances. When we say that this person is taller than this person, we are making a comparison because we are taking one as the standard, and against that standard, the other person is made to see whether he is taller or shorter. Similarly, when we take in a GC, a retention data graph, we have to have standard or scale against which, the other or the unknown is measured. Spiking sample with known solute interfacing the chromatograph with spectrometer; comparison of retention data alone are not always reliable as many substances can have identical chromatographic behavior.

I would like to draw your attention to the fact ,that suppose, if we run a GC of a standard and unknown, and we find that the retention time is the same, can anyone with surety say that it is the same compound? No. Why? Because different compounds also can have the same retention time, according to the gas chromatographic conditions or the parameters that have been set for the operation conditions of the GC machine. But when we try to

look at the inter case, and we take a GC MS, then the splitting mass, splitting pattern, or the mass fragmentation pattern gives us an idea, whether these two compounds which were having the same retention point or retention data are same or different.

So, that is what is meant by saying, that although GC gives a roundabout idea, that retention time, if they are matching, may be that they are same compound. It is necessary to have another process to validate that. It is the same and GC for GC MS is a good validating a machine.

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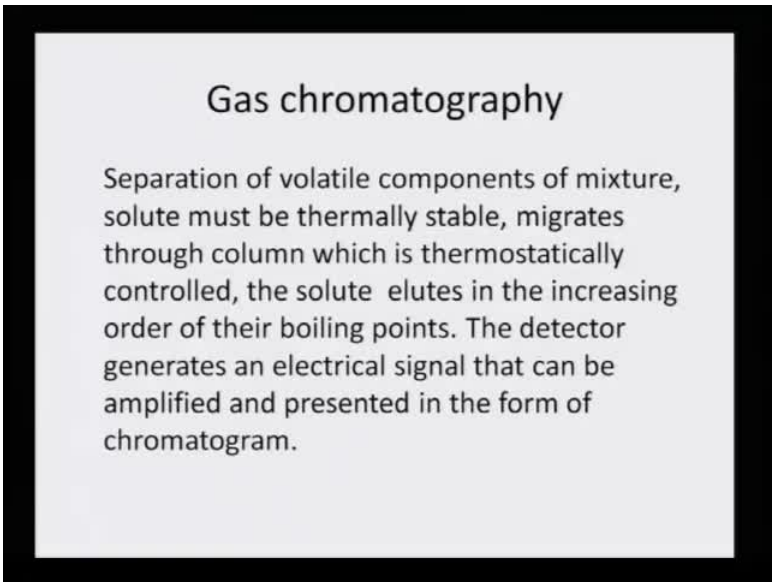


Gas chromatography - this is more like recapping all that we have studied because all these machines are used in the eco-parameter analysis.

Separation of volatile compounds of mixture, solute must be thermally stable, migrates through column which is thermostatically controlled, the solute elutes in the increasing order of their boiling points, and the detector generates an electrical signal that can be amplified and presented in the form of a chromatogram. So, it clearly shows that this machine has certain mandates, and it has certain qualities, and only if a compound befits those qualities, it can be analyzed.

First thing is that the separation can only take place for compounds which can become vapors or which have low boiling point or volatile compound plus these compounds should be thermally stable at that temperature, and should migrate through the column

which is being controlled. The temperature of the column is controlled thermostatically. And when they are eluting, first the low boiling compounds will come to the detector, and then slowly, the medium boiling points will come and then the higher boiling points will reach. So, it is all a role of their boiling points, through which they reach one by one to the detector and then the detector then amplifies the signal and a chromatogram is received.

This is how a GC machine looks like and we have already learnt about it; so, I will not spend much time talking about this particular slide, but I will spend some time in this slide. Why? Because I have chosen this pictorial description mainly to show you that how this is a complex mixture, and as and when the mobile flow, the mobile phase is passing through the column, and the time is passing through different types of compounds shown in a different geometrical structure, though they are not like that in real life, but still I am trying to show you this depiction because it will give you an idea, how this separation takes place.

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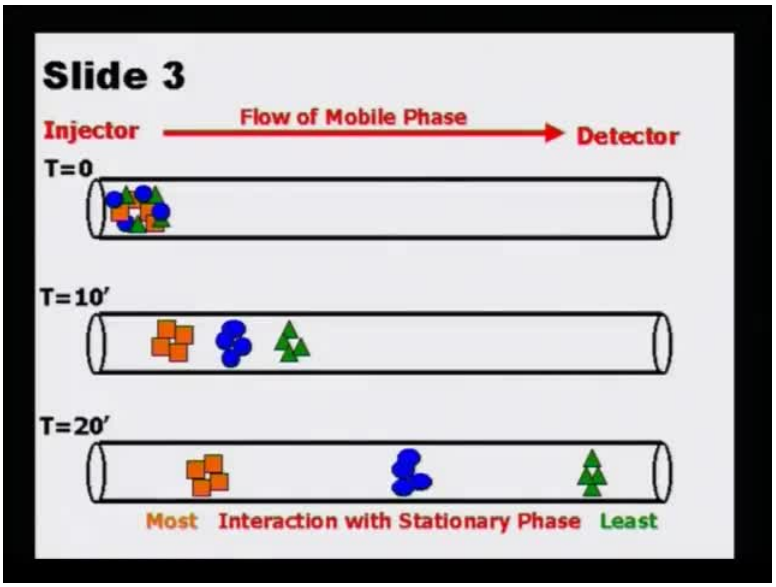


When I say with my hands that the separation is taking place like this, probably it is not so clear, but when I show it with this kind of a pictorial slide, it makes it much more clear. And here you see, in the third one, after 20 minutes of time, the components have completely separated and the one which is lowest boiling that is the green one has almost reached the other end near the detector.

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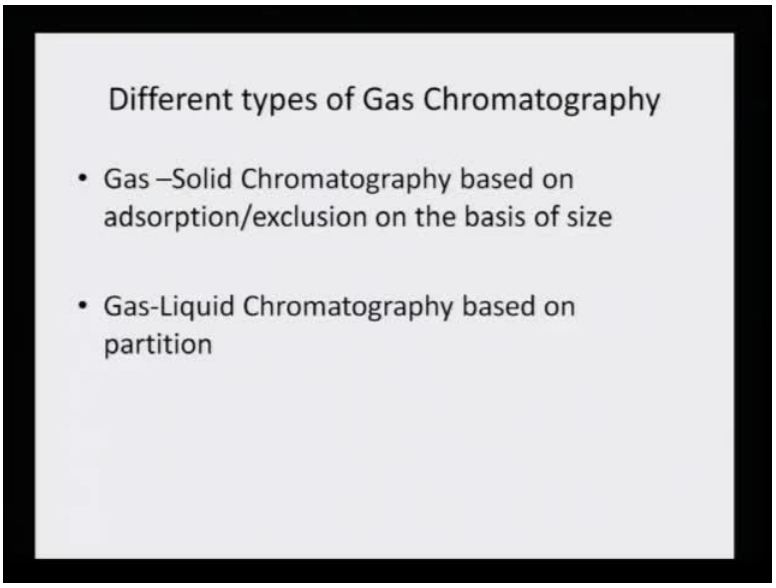


Different types of gas chromatography - there could be gas-solid chromatography based on adsorption and exclusion on the basis of the size or it could be gas-liquid chromatography based on the partitioning of these different components that I showed you in the previous line.

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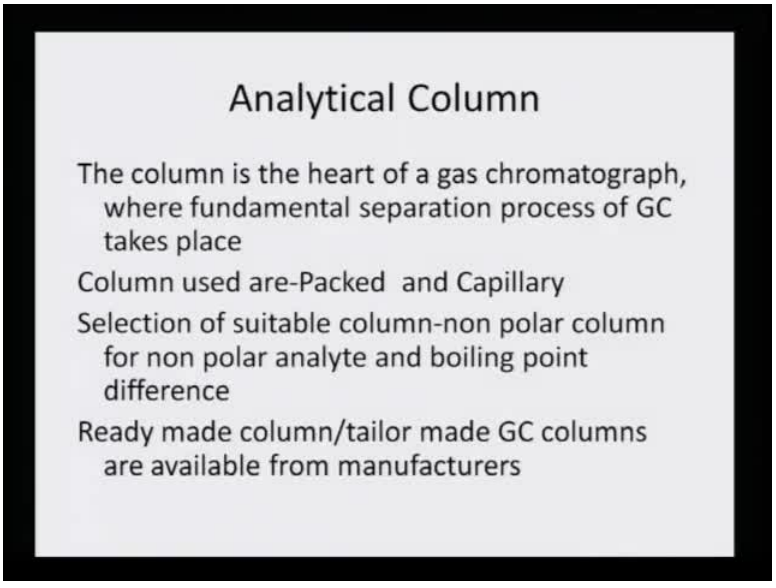


Analytical column obviously plays a very important role. The column is the heart of the gas chromatograph where, fundamental separation processes of GC takes place. Column used are packed or capillary and selection of suitable column; non-polar column polar

column are meant for non-polar analyte, and polar columns are meant for polar analytes and their boiling point differences are the ones which make it a very useful. Readymade columns or tailor made GC columns are available from the manufacturer. Now, science has advanced so much that the manufacturers are making columns which are truly useful for the particular analysis.

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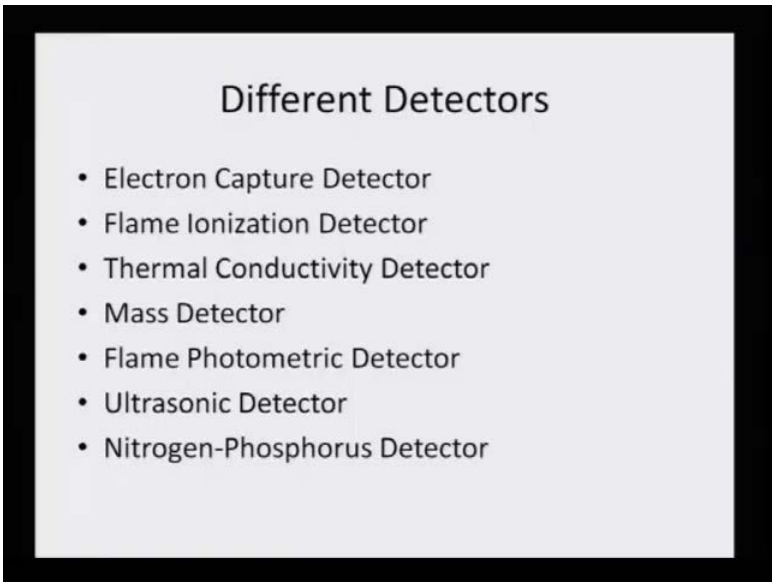


Different detectors - we have talked about GC detectors, but I will still like to recapitulate; that is, we have electron capture detector, flame ionization detector, thermal detector that is thermal conductivity detector, mass detector, flame photometric detector, ultrasonic detector nitrogen- phosphorus detector and many more. This is just a list of few.

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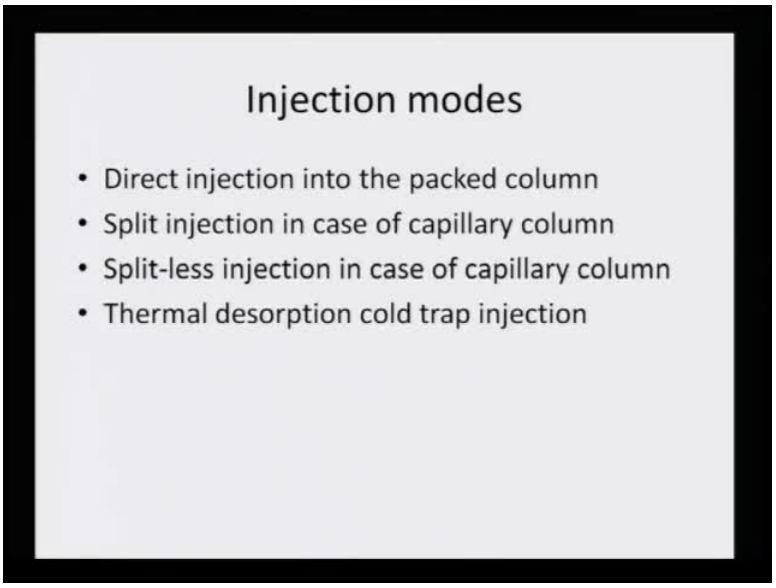


There are different injection modes also. There could be a direct injection mode on the packed column or capillary column; there could be a split injection in the case of capillary column. Why do we need a split injection? Because the entire amount need not necessarily passed through the capillary column.

Capillary column is the size of a hair, and you see that it will be only overloading the column, if too much of compound is passed through it, and that is why, since very small quantities cannot be injected, so, if 1 micro litre is injected, one-tenth, one-hundredth of that 1 micro litre can pass through this capillary column. Split-less injection, where it is a packed column or a capillary column but, it will not the compound will not cause clogging can also be carried out. Thermal desorption and cold trap injections are mainly for gaseous samples.

We had discussed this in the previous lecture, that how these gasses are adsorbed, and then they are on the canisters, and the canisters are then thermally disrobed on to the GC capillaries or GC columns.

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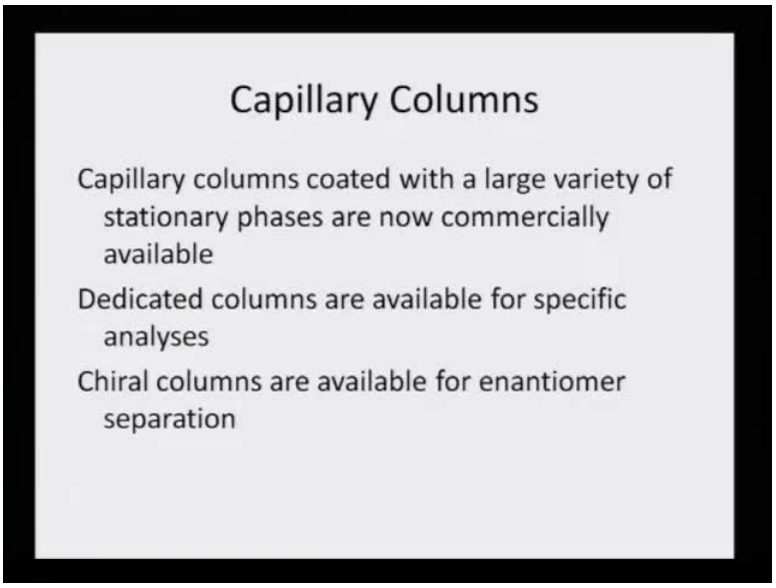


Capillary columns - capillary columns coated with large variety of stationary phases are now commercially available. Dedicated columns are available for specific analysis. Chiral columns are available for enantiomeric separation.

So, you see capillary columns there is a huge variety; not only that they are of different polarities, there are very dedicated columns for very very particular type of analysis, and so much, so that that enantiomers that is chiral compounds can also be selectively separated using the enantiomer or the chiral columns.

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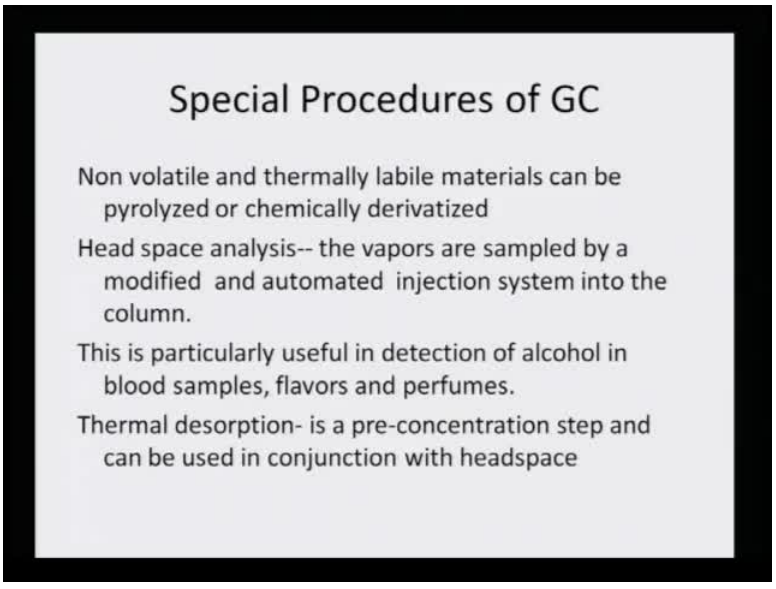

Special procedures for GC for non-volatile and thermally labile materials can be pyrolyzed or chemically derivatized. Head space analysis - the vapors are sampled by a modified and automated injection system into the column. This is particularly useful in detection of alcohol in blood samples, flavors and perfumes. Thermal desorption is a pre-concentration step and can be used in conjunction with headspace. All these we have already learnt, but I am trying to recap, and to make you aware that these processes are used time and again, and they are very useful in the analysis of all the eco parameters that I mentioned a while ago.

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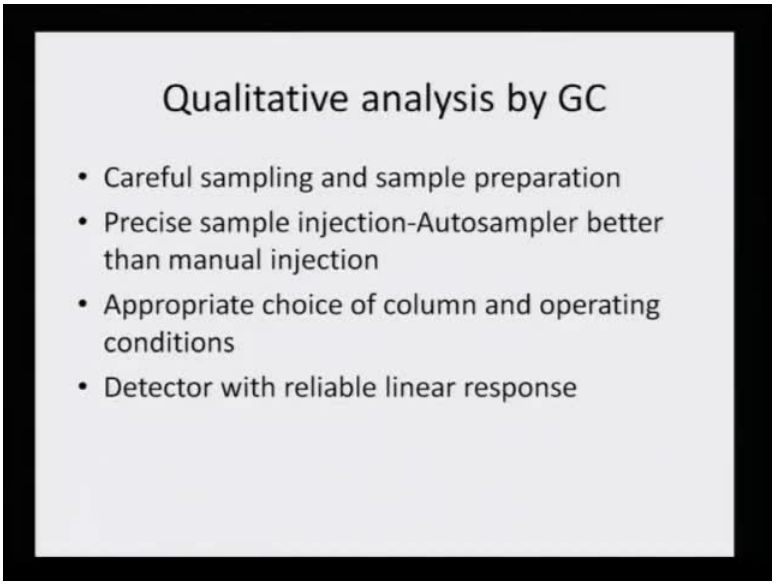


Qualitative analysis can be carried out by GC - Careful sampling and sample preparation; precise sample injection - auto sampler is better than manual injection; appropriate choice of column and operating conditions; detector with reliable linear response. Now, this particular slide requires a little more attention.

We need to do very careful sampling and the sample preparation. Once the sample is prepared, we can either use a manual injector if we only a couple of samples to analyze in a day, but suppose if it is a commercial testing laboratory, it would be receiving hundreds of sample in a day. In order to make the analytic procedure faster, autosampler can be used because if these samples are all alike and they have to be analyzed on the same GC program, a GC sampler with an auto-sampler will be one of the best options; rather, a GC with in auto-sampler will be the best answer.

Similarly, appropriate choice of column; what kind of polarity should be used at what stage, is what is very important and crucial in a GC analysis. And, of course, the detector should have a very linear response; otherwise, if it does not show any good response that means that there is a problem at the detector end. So, even though the separation has taken place, the results cannot be obtained in the right manner.

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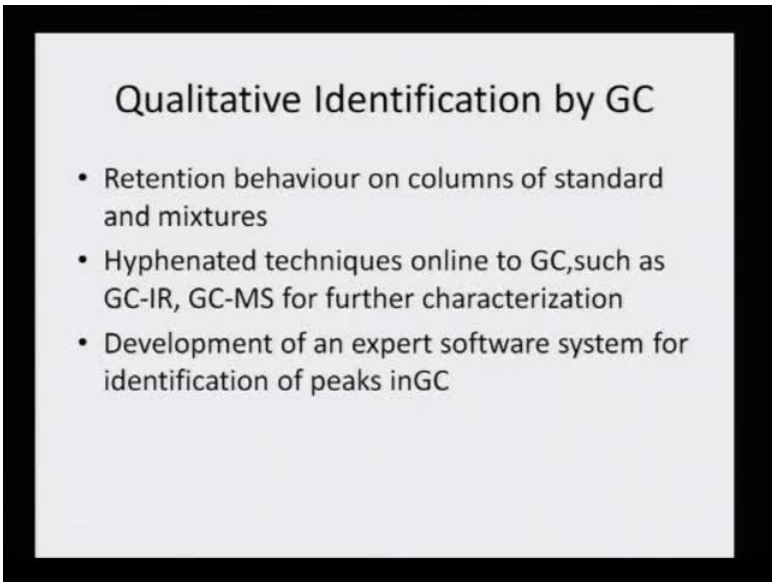


Qualitative identification by GC - all the eco parameters that I mentioned, particularly the pesticides, the formaldehydes, the PCBs and the other eco compounds can be identified through GC's retention behavior on column of standard and mixtures; hyphenated techniques online to GC, such as GC-IR, GC-MS for further characterization; development of expert software for identification of GCs.

So, these are various qualitative methods of identification of GCs. We have already seen that GC, when it is coupled with an MS, which gives us further more information. GC when it has done its separation and is coupled with IR, gives us very very detailed account of the types of compound that are present.

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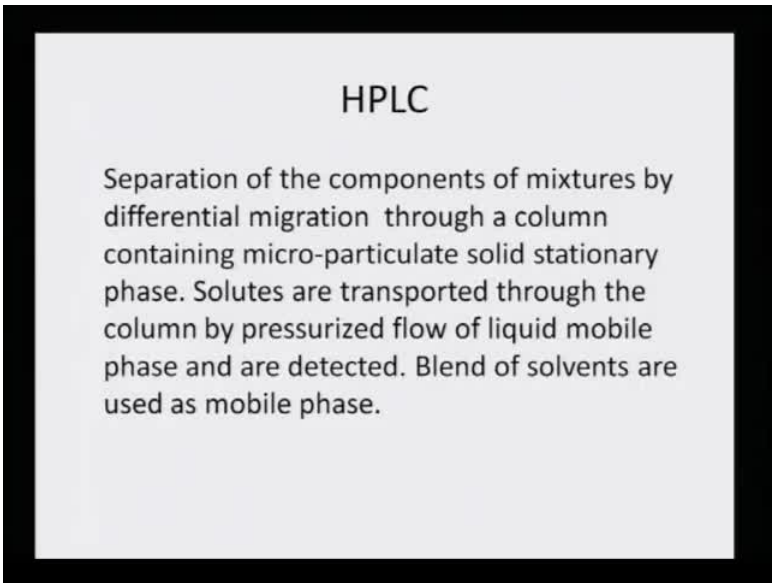


HPLC - coming to HPLC the other major chromatographic technique, separation of components of mixture by differential migration through a column containing micro-particulate solid stationary phase. Solutes are transported through the column by pressurized flow of liquid mobile phase, and are detected. Blend of solvents are used as a mobile phase.

So, here, the non-volatile compounds are identified and it is because they have similar kind of different rates of movement with the mobile phase on the stationary phase that causes the separation, and by pressurized flow of the liquid they are made to flow towards the detector end.

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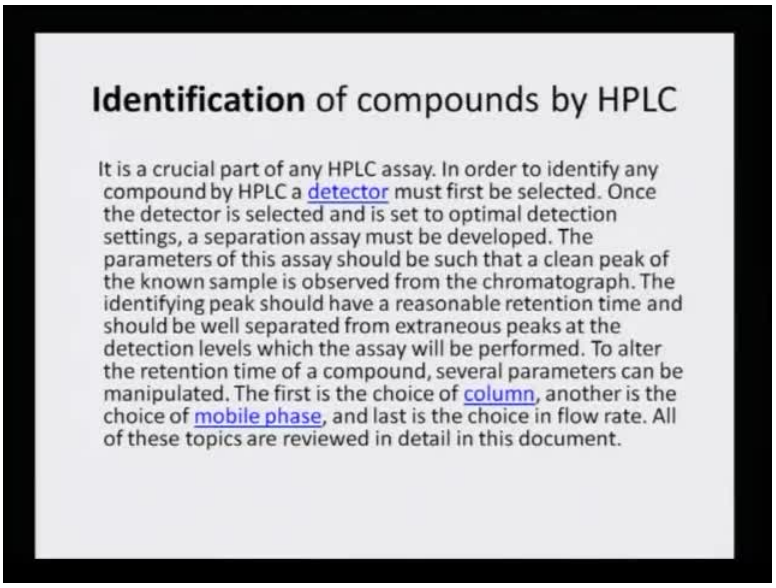


Identification of HPLC or by the help of HPLC - it is a crucial part of any HPLC assay. In order to identify compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay may be developed. The parameters of this assay, assay means test, should be such that a clean peak of the known sample is observed from the chromatograph.

The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels, which the assay will be performed. To alter the retention time of a compound, several parameters can be manipulated. The first is the choice of the column, another is the choice of mobile phase, and the last is the choice of flow rate. All of these are reviewed.

Now, you see that there are three things which have to be remembered while working with HPLC. One is that the detector should be of choice and the other thing is that we can we need to choose the right polarity of the column. If we want to do RPLC and if we use normal phase column, that will not give correct result. Similarly, the mobile phase that is the polarity of the mobile phase also plays a very important role, and if we try to speed up that is the flow rate also has to be manipulated very correctly.

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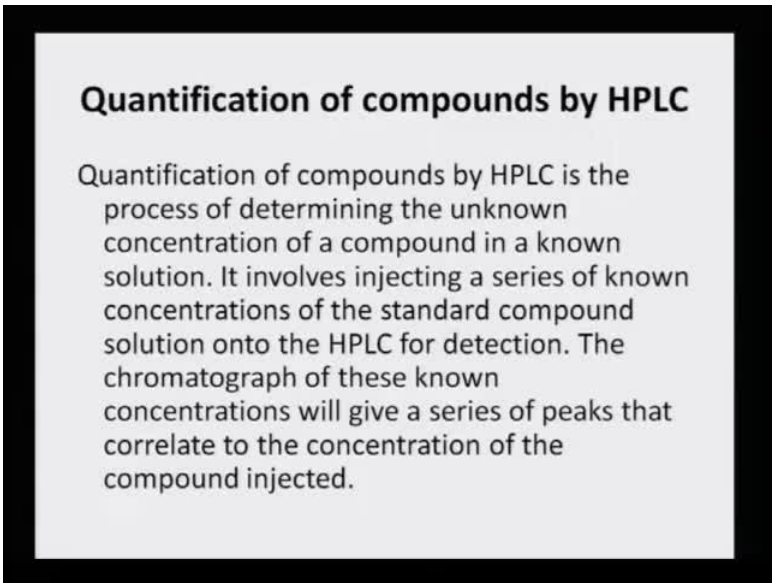


Quantification of compounds by HPLC - Quantification of compounds by HPLC is a process of determining the unknown concentration of the compound in a known solution. It involves injecting a series of known concentrations of standard compound solution onto the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected.

And once we have formed this calibration curve, then the unknown is injected and seen, where the concentration lies. If the concentration of the unknown is very low as compared to the calibration graph that is prepared, then also, it will not give correct result; if the concentration is very high, then also, it will have to be manipulated and brought to the range of the calibration curve; otherwise, quantification will not be accurate.

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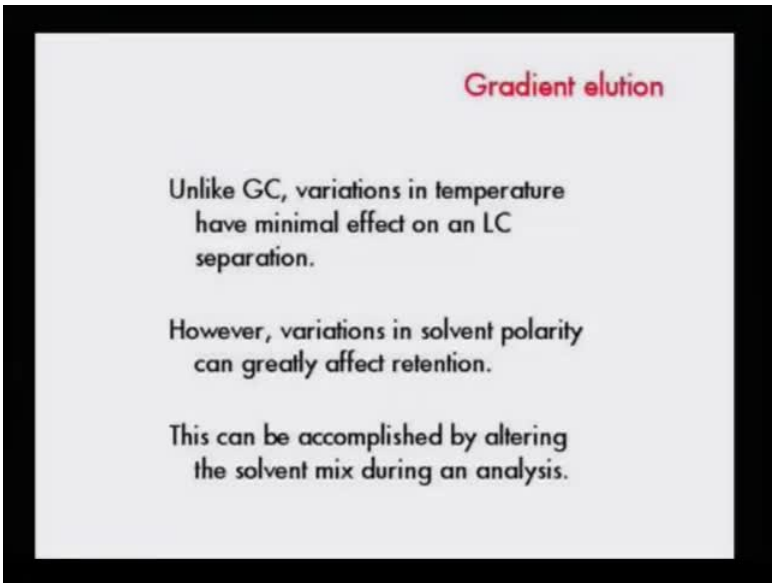


Gradient elution - unlike GC, variation in temperature has minimal effect on the liquid chromatographic separation. However, variation in solvent polarity can greatly affect retention. This can be accomplished by altering the solvent mixture during an analysis. I will give you an example. Suppose, if I have got a chromatogram in which, I find a peak at 2 minutes; it is too fast and it comes in the beginning, and it comes as a broad peak. If I would not know whether this is the real peak or it is a combination of one or two peaks, but if the peak is coming somewhere between 5 to 10 minutes and if the peak is very sharp, I certainly know that this peak represents one compound only. Therefore, one can manipulate the flow rate, or one can manipulate the combination and the polarity of the solvent in such a manner that the height of the peak and the shape of the peak comes as though it is just one compound, if it is so.

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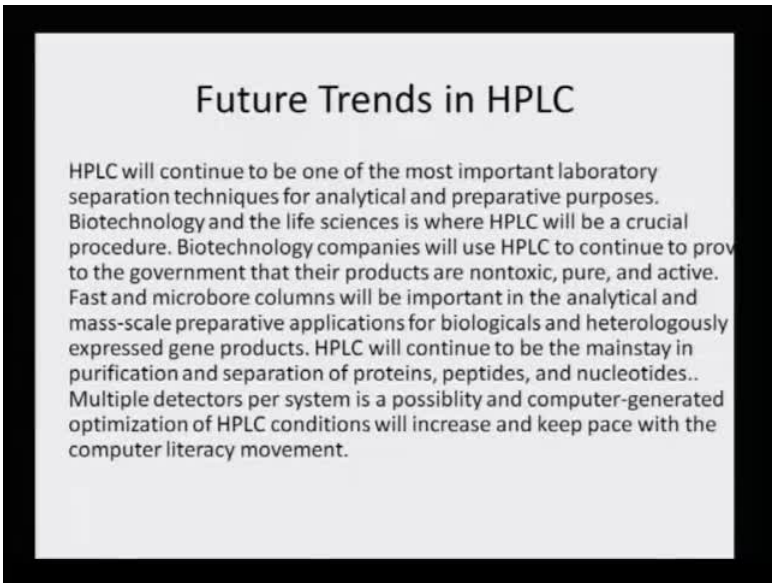


Future trends in HPLC - HPLC will continue to be the most important laboratory separation techniques for analytical and preparative purposes. Biotechnology and life sciences is where HPLC will be a crucial procedure. So, for those who are pursuing biotechnology and life sciences, not only as a chemical analysis, but even they would be requiring a lot of use of HPLC.

So, biotechnology companies will use HPLC to continue to prove to the government that their products are nontoxic, pure, and active. Fast and microbore columns will be important in the analytical and mass-scale preparative applications for biological and heterologously expressed gene products.

HPLC will continue to be the mainstay in purification and separation of proteins, peptides, and nucleotides. Multiple detection per system is a possibility and computer-generated optimization of HPLC conditions will increase and keep pace with the computer literacy movement.

So, you see, these are all computer driven machines; they are not manual machines anymore. And so, those who understand how to operate computers can be good analyst because they will be able to understand, but the softwares are very user friendly. So, it is not difficult to be able to understand or to be able to maneuver, or a learn these softwares of the softwares that are used with GC and HPLC

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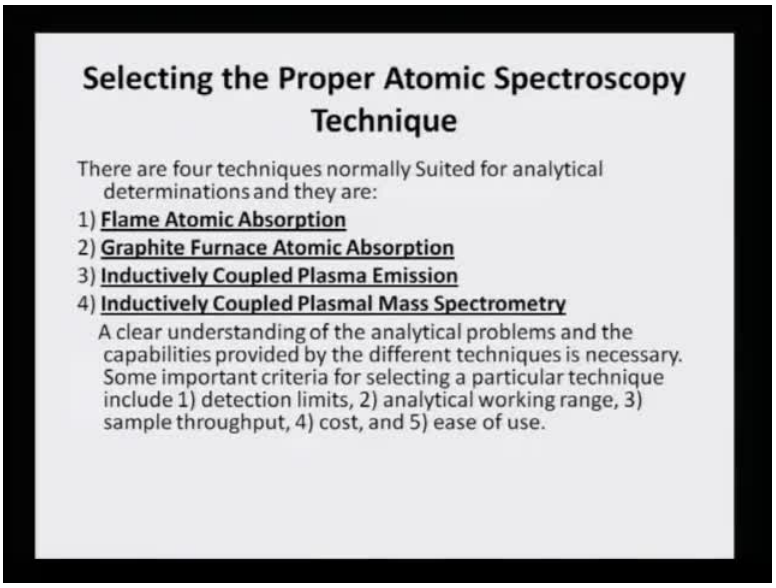


Coming to the metal analysis of the eco parameters, the different types of spectroscopic methods that are used for metal analysis are by selecting the proper atomic spectroscopic technique.

There are four major techniques that are suited for analysis of metals. Flame atomic absorption, graphite furnace atomic absorption, inductively coupled plasma emission, and inductively coupled plasma mass spectrometry. A clear understanding of these analytical problems and the capacities provided by the different techniques is necessary. Some important criteria for selecting a particular technique would include: detection limits, analytical working range, and sample throughput, cost, and the ease of use.

Now, the question is for an analyst to decide, whether I should use flame atomic absorption? or I should use graphite furnace atomic absorption? or should I use ICPE or should I use ICP MS? is a big debatable issue. Why? because many criteria have to be kept in mind. For a routine analysis, time and again, I have mentioned that flame atomic absorption is the most ideal machine; however, when PPB or PPT level of trace elements, but very crucial elements have to be analyzed, Graphite furnace atomic absorption machine is the answer, and ICP, as I said, is a costlier machine, but again, if the sensitivity and the trace analysis is required, then one needs to use ICP. And ICP MS is still costlier machine and the operational cause, are still higher because it has a mass detector attached to it. Finds much wider use, but it is very expensive for a day to day

analytical work. So, the analyzer must take these points into consideration while considering which instrument to use and when to use.

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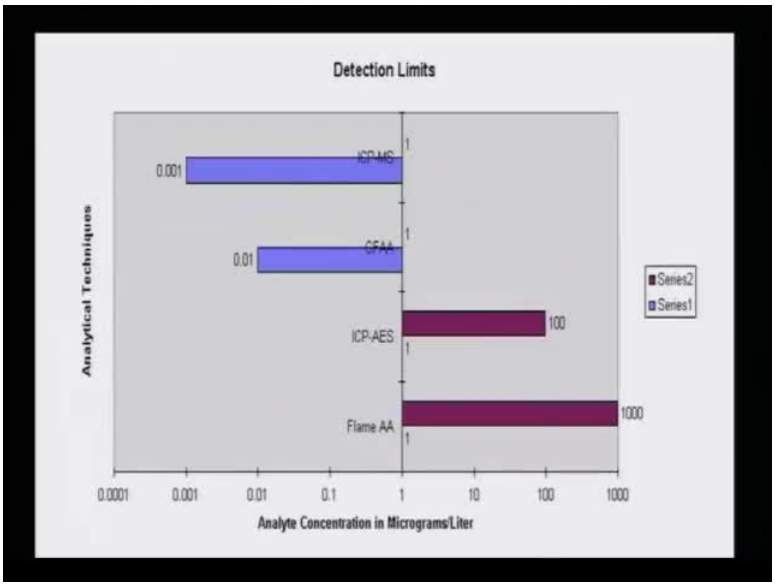


If one looks only at the detection limits, here is a slide which clearly shows that flame atomic absorption is very poor in its detection limit; that is, it only takes care of PPM level, not more sensitive. However, ICP emission takes care of PPB level, whereas graphite takes care of PPB and PPT, and ICP MS takes care of PPB and PPT levels of trace quantities, parts per billion and parts per trillion. You imagine that how these sensitivities and detection limits have been confined to these particular instruments and that gives an idea for an analyst to be able to make their right choice.

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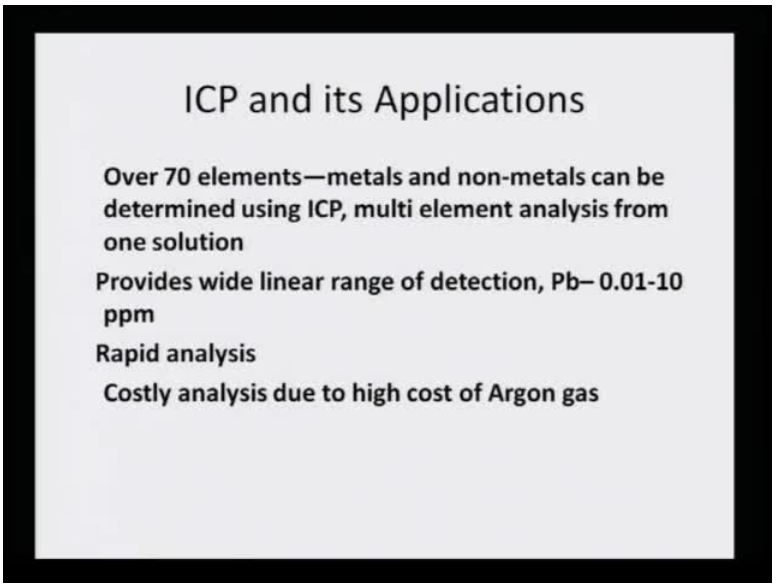


ICP and its applications - over 70 elements, metal and non-metals can be determined using ICP, multi element analysis from one solution. So, you see that their hair is a machine which can do miracle. One can just take one solution and an analyze seventy elements in one go whereas, in atomic absorption one had to analyze one by one; it was more time consuming, but there are always advantages and there are limitations.

It provides wider linear range of detection that means that for Lead, for example, from 0.1 to 10 PPM that is the kind of range it has. The analysis is very rapid, but it is costly analysis due to high cost of Argon gas because the Argon gas is the one which creates the plasma or the flame in this particular machine.

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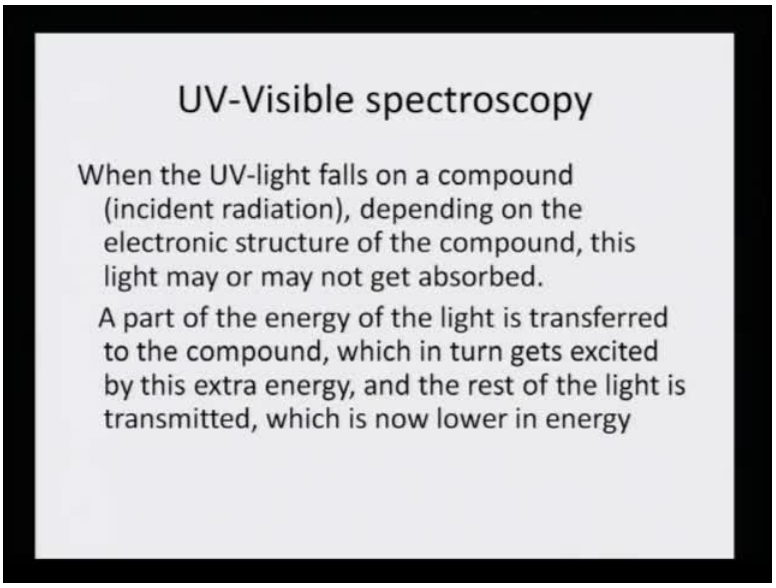


UV spectroscopy - I will fleetingly inform you that it is a tool which needs to be dealt much in detail at a latest stage, but here, I am introducing you to this because it is one of the parameters which is used for the analysis of eco compounds or eco friendliness of a compound. When the UV light falls on a compound, incident radiation depending on the electronic structure of the compound, this light may or may not get absorbed. A part of the energy of the light is transferred to the compound, which in turn gets excited by this extra energy and the rest of the light is transmitted, which is now low in energy.

So, there is an incident light. If there is a UV active compound present, then that light will be a part of that light will be absorbed and the incident light which will come out will be lower in energy. Simple funda that there is a light falling, if there is something eaten up, then whatever remains comes out. So, that is the idea, but for that criterion, the analyte must have a UV absorbing capacity containing compound. That is what is the main criteria.

Similarly, in infrared spectroscopy, I am again introducing this very fleetingly because you should know that if these tools are used for the analysis of eco parameters, then how are they are used.

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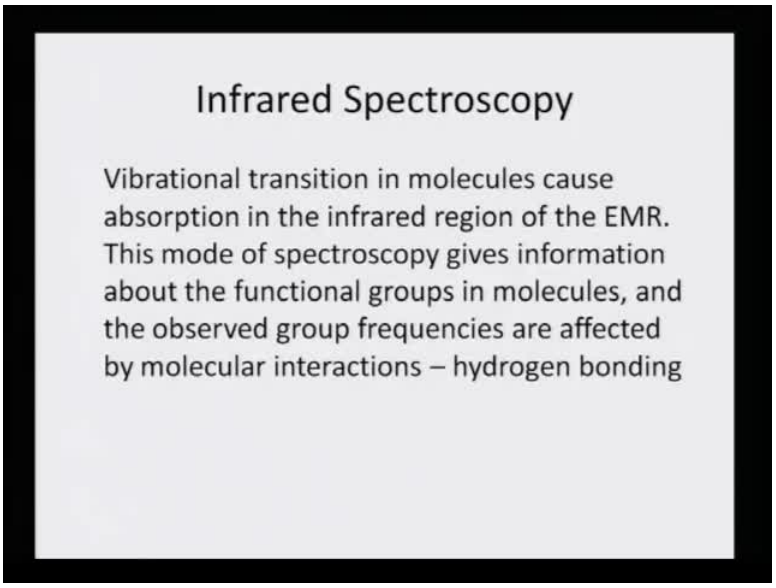


Vibrational transitions in molecules cause absorption in the infrared region of the electromagnetic radiation. the This mode of spectroscopy gives information about the functional groups in molecule and the observed group frequencies are affected by molecular interaction, that is the hydrogen bonding.

Now, what happens? Every bond in chemical compounds are in a state of vibration and when there is this infrared light, the vibrational energy of the bonds is equivalent to the infrared energy or light energy. And when they are equivalent, a part of it is absorbed and its starts vibrating even faster because they are matching. If they were not matching, they would have not made any difference and that is what has been used to identify the functional groups, and even it shows that there are molecular interactions like hydrogen bonding.

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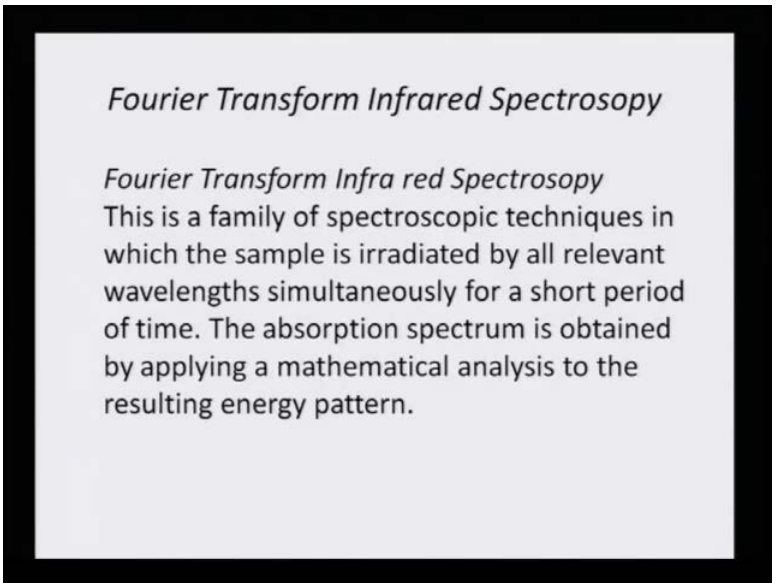


The more advanced level of infrared is the Fourier transform infrared spectroscopy. The Fourier transform infrared spectroscopy - this is a family of spectroscopic technique in which the sample is irradiated by all relevant wavelengths simultaneously, for a short period of time.

The absorption spectrum is obtained by applying a mathematical analysis of the resulting energy pattern. So, what is done? A huge lot of different wavelengths of infrared is incident, made to be incident on the sample, and the sample quickly then does a lot of absorption at the same time simultaneously, and then these spectra are over laid one above the other to show the sharpness of the peak, and it is all a very mathematical application of the energy patterns.

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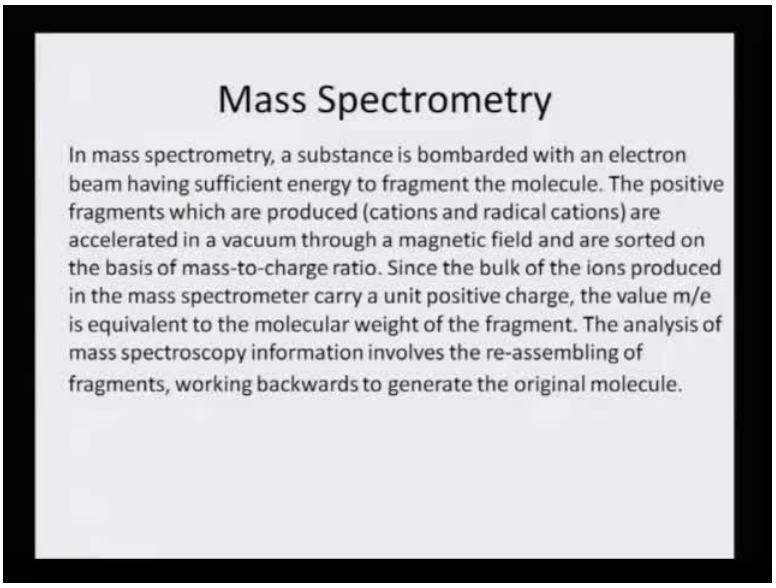


Mass spectrometry - we have been talking about GC-MS, ICP-MS everywhere; the word MS, which is the word which can be elaborated as Mass spectrometry. In mass spectrometry, a substance is bombarded with an electron beam having sufficient energy to fragment the molecule. The positive fragments which are produced, that is the cations and the radical cations are accelerated in vacuum through a magnetic field and are sorted on the basis of mass-to-charge ratio.

Since the bulk of the ions produced in the mass spectrometer carry a unit positive charge, the value e by m is or rather m by e is equivalent to the molecular weight of the fragment. The analysis of mass spectroscopy information involves the re-assembling of fragments, working backwards to generate the original molecule.

I have, time and again, told you that molecules break in a particular fragment pattern, and once they break in that pattern, it is always a set rule to break in that manner, and though the first peak that appears is the molecular ion peak, and backwardly all the fragrance come. And when they are collected and the information is collated, then only the total molecular structure can be ascertained.

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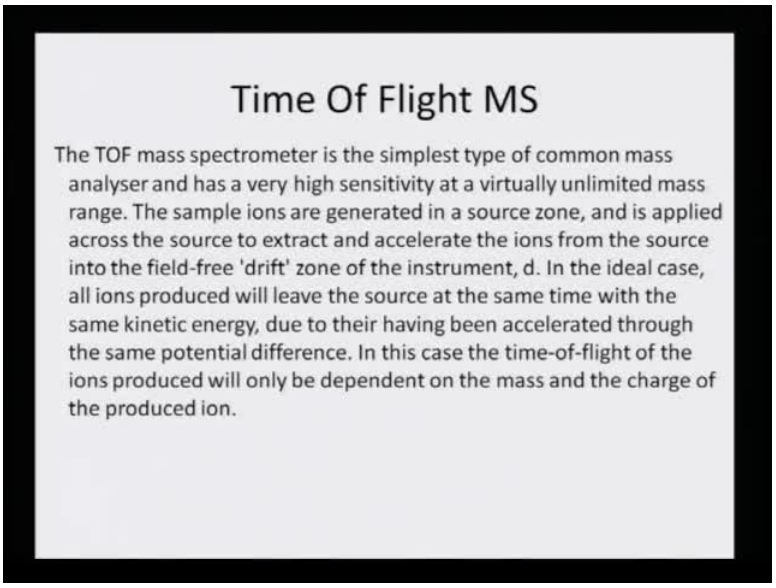


Time of flight is one of the common mass analyzer. We have talked about it in the previous class. The time of flight mass spectrometer is very special and highly sensitive, and therefore, it is one of the most latest techniques of today's mass analyzers.

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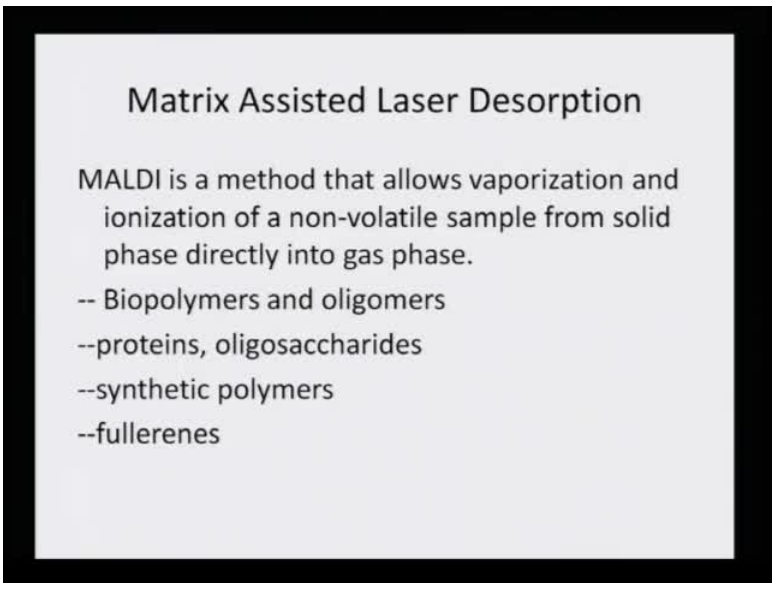


Matrix assisted laser desorption - this is a technique which is called MALDI, is a method that allows vaporization and ionization of non-volatile samples from solid phase directly into the gaseous phase. Biopolymers and oligopolymers can be analyzed by MALDI, this option method; proteins and oligosaccharides, synthetic polymers and fullerenes can be

analyzed. I will take these into details. I am just introducing the names at this point of time because you should understand the name and should be able to associate as to what is MALDI. MALDI is associated with mass spectrometry.

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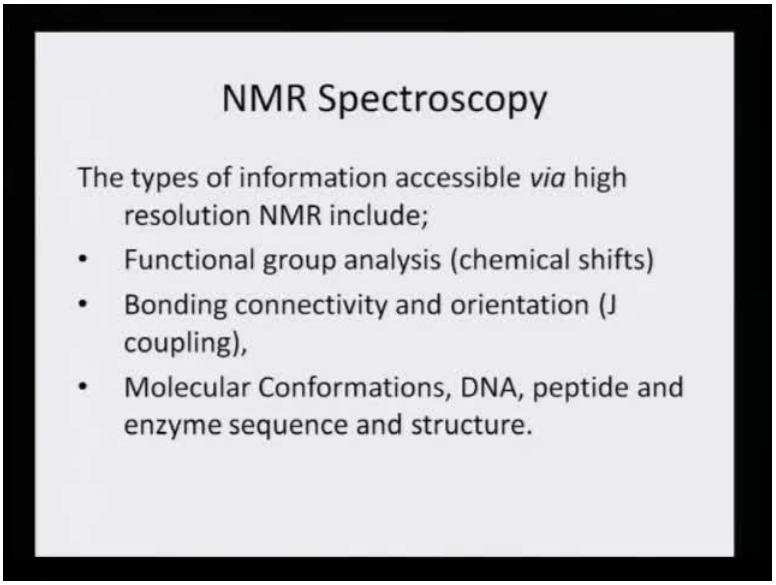


Similarly, coming to NMR, the types of information that are accessible via high resolution NMR are that it gives the idea of functional group analysis, that is the chemical shifts, bonding connectivity and orientation in a molecule that is the J coupling, and molecular conformations that is DNA, peptide enzyme sequencing and structures.

You see, these are the types of information that these spectroscopic methods give us. So, with this, we have come to an end of overview of all the different types of chromatographic and spectroscopic techniques, which need to go hand in hand, to be able to find out the structure and to be able to analyze the culprit eco compounds or the banned chemicals for analyzing the eco parameters.