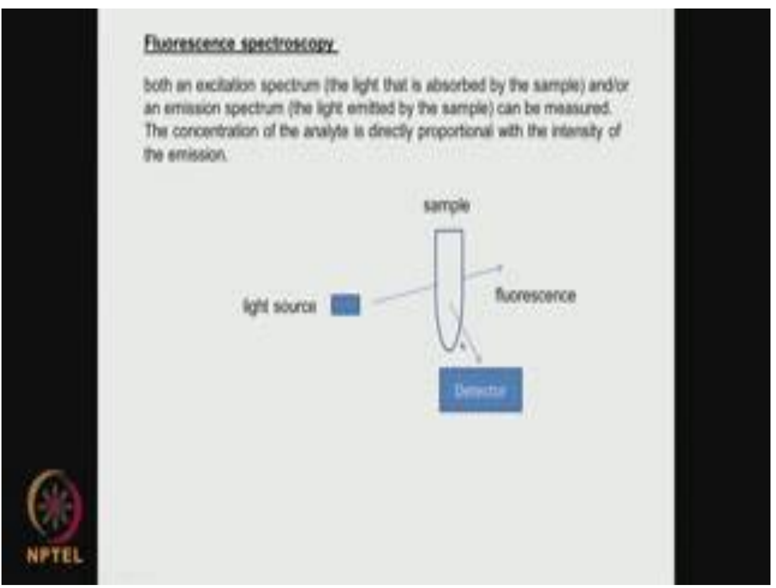
Medical Biomaterials
Prof. Mukesh Doble
Department of Biotechnology
Indian Institute of Technology, Madras

Lecture - 16
Analytical Tools

Hello everyone. Welcome to the course on Medical Biomaterials. We will continue on the topic of analytical tools. Yesterday I introduced something called spectroscopy there are different types of spectroscopies, almost 7 or 8 different techniques which is widely used in analytical chemistry, organic chemistry, but in biomaterials we do not use all those spectroscopies, but some of them are used. So, we will just introduce some of those. The one that is a quite lot used commonly is fluorescent spectroscopy.

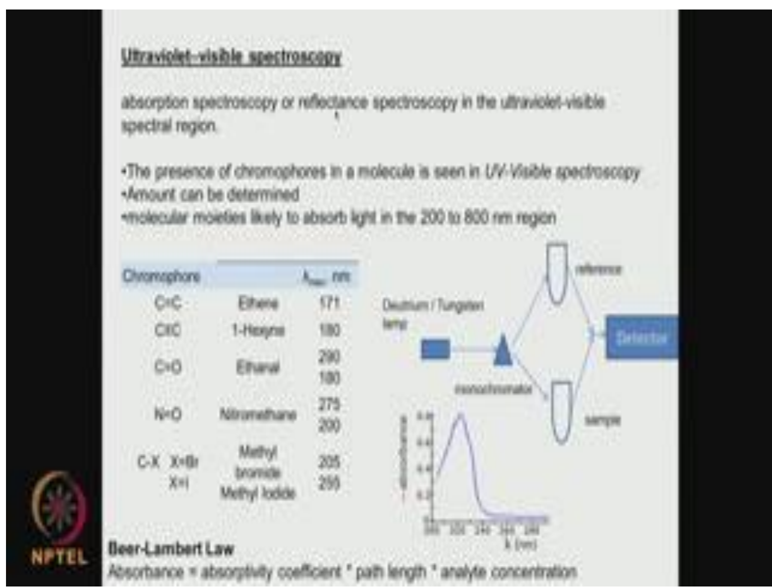
(Refer Slide Time: 00:46)



What happens is you take the sample and then there is a light source both the excitation spectrum that is the light that is absorbed by the sample and or the emission spectrum that is the light that is emitted by the sample can be measured. So, we can measure both the light excitation as well as the emission. So, we can measure the concentration of the analyte that is present in the sample, which is directly proportional to the intensity of the emission. So, at different concentrations you will get different intensities. So, we can draw a standard graph, and we can indirectly later on calculate concentration of the

analyte from the intensity of the light. So, this is useful if I want to monitor a metabolyte and so on actually.

(Refer Slide Time: 01:42)



Ultra violet visible spectroscopy, this is also very useful. What happens is absorption spectroscopy or reflectance spectroscopy in the ultra violet or the visible spectral region. So, we are looking at either at the ultra violet or the visible. So, we can monitor if there are any chromophores. What is a chromophore? A functional group like C double bond C, or C triple bond C, or C double bond O, aromatic groups, N double bond O and so on these are called chromophores. So, they have certain characteristic wavelength. So, we can monitor them. So, we can know whether chromophores are present in a sample, we can also determine what is the amount of those.

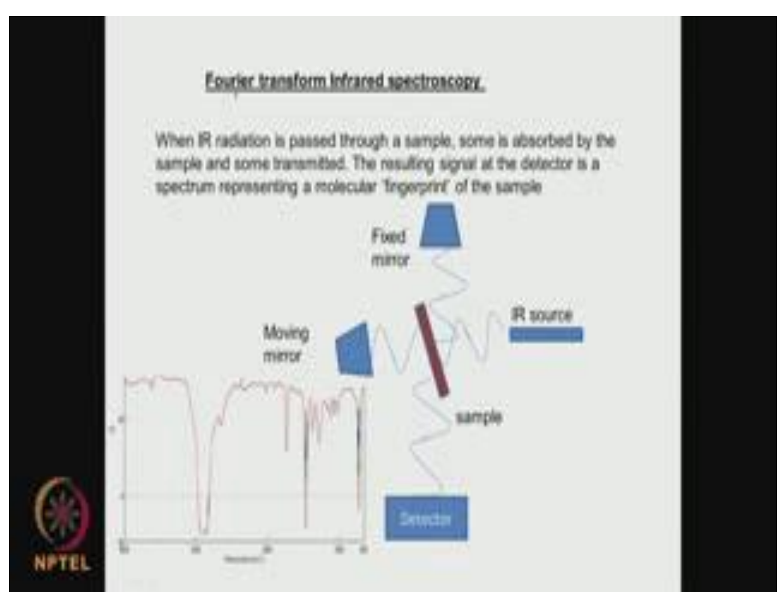
Generally, when we talk about UV and visible, we are talking in this region 200 to 800 nanometers. So, how does it work? So, we have a lamp deuterium tungsten lamp, then there is a monochromator, and light passes through the sample and its detected. We have a reference here, so with respect to the reference we can monitor whether a chromophore is present at what concentration. Generally, if you look at the spectrogram, it will look like this at a particular lambda max and as I said that lambda max is characteristic of the type of chromophore present. So, you make it a nice looking spectrogram like this.

So, we can measure from these absorbents, what is the concentration if you have a standard graph. And this lambda max is an indication of what type of chromophore that

is present. So, it uses the Beer-Lambert's law. If you remember in your schooldays 12th standard days, it is given as absorbance equal to some constant called absorptivity coefficient the path length that is this length into the concentration of the analyte. So, from the absorbance, we can calculate analyte concentration or from the analyte concentration, we can calculate absorbance. So, this is also very useful technique.

When is it used? For example, I am looking at the biofilms that are formed on implants on devices and I want to know what type of material is present and at what concentration I can scrape the biofilm into a solution I can do a UV spectroscopy. So, these are quite useful both which talked about the fluorescence as well as the UV visible spectroscopy to monitor the composition of the biofilm.

(Refer Slide Time: 04:25)



Then comes the something called the Fourier transform infrared spectroscopy - FTIR Fourier transform infrared spectroscopy. This extremely powerful we can look at material surfaces. For example, if there are any ketonic group present on a surface like on a polymer or if any hydroxyl present or amino presents either polymer or metals or even an oxide, this is very powerful. If I have a biofilm that is deposited on the polymer, I want to know whether there are amide groups that are present I can use this. So, this is very useful technique for looking at surfaces as well as the deposit the organic deposit on the surface. So, it is called FTIR - Fourier transforms infrared spectroscopy, this is very useful technique.

So, what happens is when an IR radiation is passed through a sample, some of it is absorbed by the sample and some is transmitted? The resultant signal is a molecular fingerprint because atoms which are connected through bonds start vibrating there is bending. So, these vibrational frequencies are very characteristics of the type of functional groups that are present. For example, if there is a OH type of a functional group, it will vibrate at certain frequency or wave number. If there are ketonomic group like C double bond O, it is very characteristic and so on actually.

So, this is the setup of an IR. So, we have the sample there is an IR source, there is a fixed mirror, moving mirror then there is a detector. So, this is a typical spectrogram. So, we have the wave number that is centimeter inverse, here in the x-axis, and this is called the percentage transmission. So, we have as you can see some bands around 2900, there are some bands at 1400, there are some bands at 1700, there are some bands at 700 centimeter inverse. So, all these bands are characteristic of functional group. So, I can look at from the literature, I can say what types of functional groups are present. So, if I am doing some surface modification, some of these bands could disappear, I may be forming some new bands.

So, I can tell what type of surface modification I am doing, whether the surface modification is successful, so that way IR is extremely useful. So, each functional group has certain characteristic wave number in centimeter inverse. So, I need to know that data.

(Refer Slide Time: 07:12)

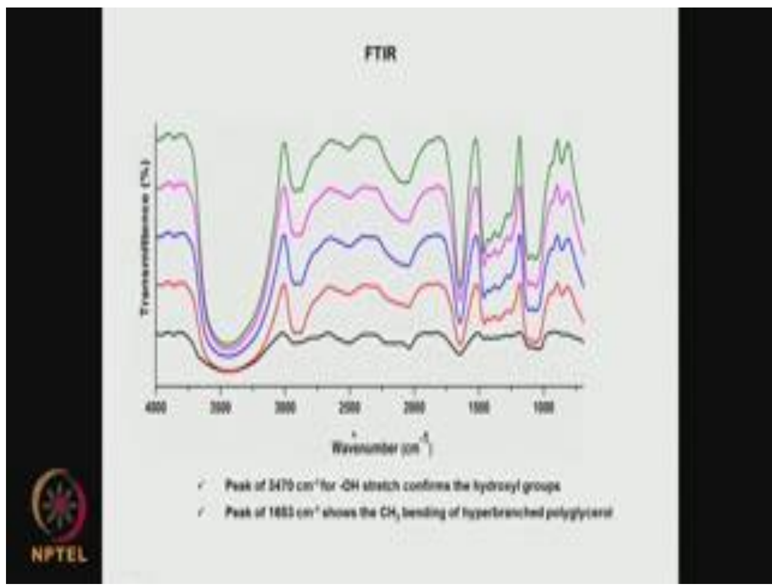
Functional Group	Characteristic Absorption(s) (cm^{-1})
Alkyl C-H Stretch	2950 - 2850 (m or s)
Alkenyl C-H Stretch	3100 - 3010 (m)
Alkenyl C=C Stretch	1680 - 1620 (v)
Alkynyl C-H Stretch	~3300 (s)
Alkynyl C≡C Stretch	2260 - 2100 (v)
Aromatic C-H Stretch	~3030 (v)
Aromatic C-H Bending	860 - 680 (s)
Aromatic C=C Bending	1700 - 1500 (m,m)
Alcohol/Phenol O-H Stretch	3550 - 3200 (broad, s)
Carboxylic Acid O-H Stretch	3000 - 2500 (broad, v)
Amine N-H Stretch	3500 - 3300 (m)
Nitrile C≡N Stretch	2260 - 2220 (m)
Aldehyde C=O Stretch	1740 - 1690 (s)
Ketone C=O Stretch	1750 - 1680 (s)
Ester C=O Stretch	1750 - 1735 (s)
Carboxylic Acid C=O Stretch	1780 - 1710 (s)
Amide C=O Stretch	1690 - 1630 (s)
Amide N-H Stretch	3700 - 3500 (m)

NPTEL

For example, as you can see alkyl, alkenyl, alkenyl C double bond O, alkenyl then you have C triple bond, aromatic, aromatic carbon, C double bond C aromatic, alcohols phenols, carboxylic acid, amines, nitriles, aldehydes, ketones, esters, carboxylic acid, C double bond O and so on actually. So, you can see on this side we have the characteristic absorption, wave number centimeter inverse. So, for example, the C double bond O of an aldehyde happens at 1740 to 1690, C double bond O of ketone is 1750 to 1680; the C double bond O of ester is 1750 to 1735. So, it may it is bit difficult to determine whether the C double bond O signal is because of carbonyl or ester or aldehyde, but if you have a peak or sorry a band at 1720, 1730 and so on, so we can be sure that there is a C double bond O.

Similarly, if you have around 3500 plus, we can be sure there is an alcohol or phenolic OH present. So, if we have around 2500 to 3000, we can say there is an OH from a carboxylic acid. If we have 3700, 3500 that range, we can be sure that is NH. So, if you have 1690, 1630 then there could be amide. If you are looking at amine you can have 3500, 3300; if you have nitrile, 2260 2220. So, by looking at the characteristic band, we can find out what type of functional groups or we can predict what type of functional groups there are present in the surface. Generally, this is the surface phenomena, which we are monitoring. And if some may do a surface modification and some bands disappear, we can say that particular functional group has taken part in the surface modification process, so that way this is very useful technique.

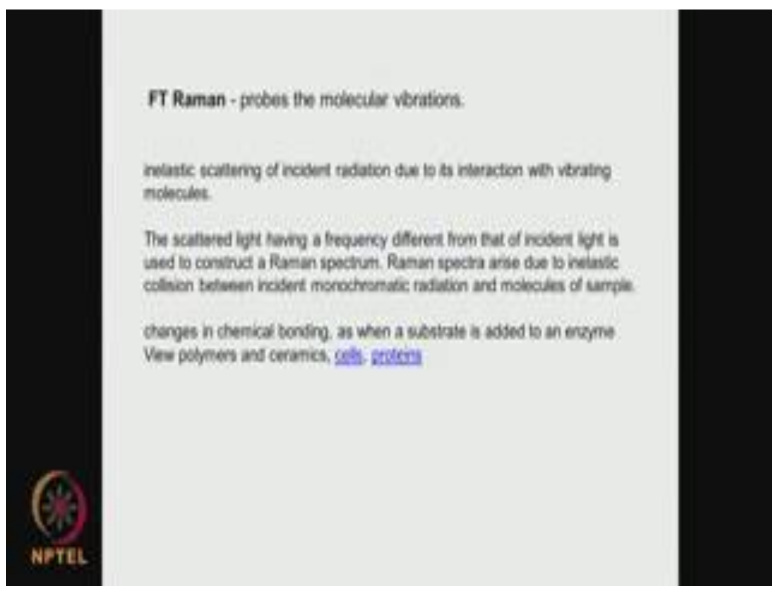
(Refer Slide Time: 09:18)



So, this is a typical say for example, FTIR, I am showing here of some hyper branched polyglycerol. So, as you can see here lot of OH because polyglycerol glycerol contains lot of o h we can see here and then 1653 here, here this is the CH 2 of the bending of the hyper branched. So, this is a very simple polymer. So, we can see this. Suppose there are ketonic group then you may have a peak around 1720 that is ketonic C double O and if you have got a nitrile and so on, you may have it much lower and so on. So, we can monitor the presence of certain functional groups from these bands.

So, here the x-axis is wave number, and the y-axis is generally transmittance. So, FTIR is very useful technique to find out what type of functional groups are present in that polymer, or a metal, or a ceramic and if I am making modifications how these functional groups get affected, sometimes when there is a bonding these bands get shifted then we can say that these bands are taking part in the reaction. So, FTIR is extremely useful in the area of bio surface measurement.

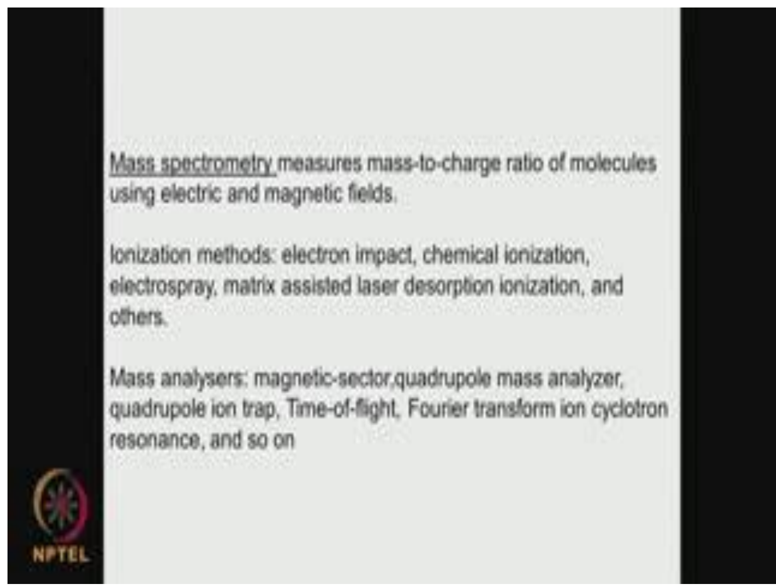
(Refer Slide Time: 10:44)



Similarly, just like FTIR, there is something called FT Raman; it also probes the molecular vibration. So, it is called inelastic scattering of incident radiation due to its interaction with the vibrating molecules. So, here this scattered light having a frequency different from that of the incident light and that is used to construct a Raman's spectrum. So, basically Raman's spectra arise due to inelastic collision between incident monochromatic radiation and the molecule. So, we have a monochromatic radiation it hits the molecule molecules are vibrating. So, it produces scattered light having a frequency different from that of the incident light, so that is the Raman's spectrum.

So, changes in chemical bonding as when the substrate is added to an enzyme we can look at polymers we can look at biomolecules peptides proteins and so on. So, FT Raman is very useful if I am looking at biofilm formation where the biofilm contains lot of proteinaceous material. So, with the help of FT Raman, FTIR we can try to probe the surfaces of several materials. So, these tools are widely used for surface engineering surface modification.

(Refer Slide Time: 11:58)

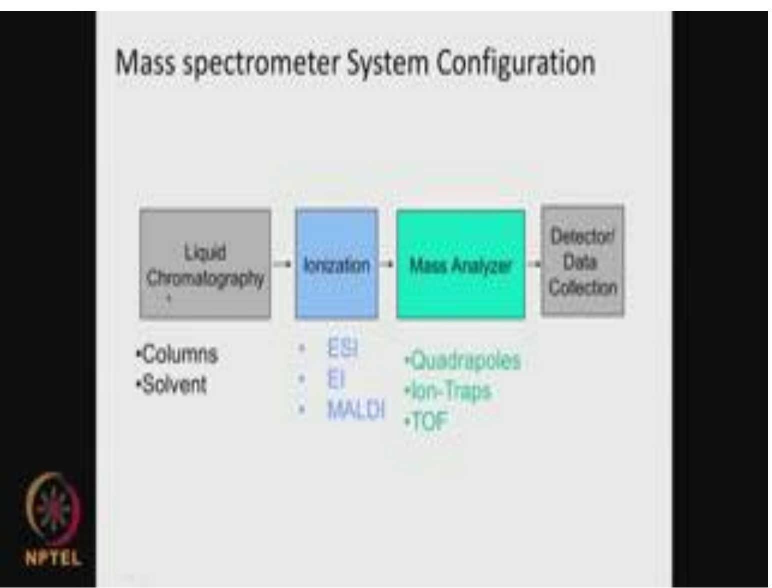


Then comes mass spectrometry. Mass spectrometry is very useful to determine the mass especially if I have a polymeric material, I can find out what is the mass of the polymer. If I have a biofilm with lot of proteins, I can get some idea about what type of protein or present on the surface. So, mass spectrometer is very useful there. So, what happens, it calculates the mass to charge ratio molecules using electrical and magnetic field. So, molecules are ionized first and then they are focused and they are sent to the detector or the mass analyzer. So, the ionization is the first step, molecules are ionized. So, there are different ways of ionizing molecules, one is you bombard the molecule with electrons or you can bombard the molecule with chemical, or we can spray the molecule at high voltage or we can use a matrix material which in turn ionizes the molecule that is called as matrix assisted laser desorption.

So, there are so many different ways by which molecules can be ionized; unless you ionize and charge the molecule, it will not be focused on to a detector using electrical or magnetic fields. So, neutral molecules cannot be focused. So, we need to ionize the molecule. So, how do we ionize different techniques electron impact that is called EI, chemical ionization CI, electron spray ESI, matrix assisted laser desorption is called MALDI. So, these are principal methods by which ionization of the molecules are done. So, once the molecules are ionized, we can be focused using electric and magnetic field onto a analyzer.

So, different types of analyzer are there magnetic, quadrapole, quadrapole ion trap, time-of-flight, Fourier transform ion cyclotron and so on actually, we will not spend too much time, time-of-flight is widely used sometimes ion trap are also used those are mass analyzers. So, molecules initially are separated using a liquid chromatography or HPLC - high pressure liquid chromatography, then they are ionized, and then they are focused onto a analyzer that way we can get molecular weight of a mixture of say enzymes or proteins or even small molecules natural products. That means, with mass spec, I can get molecular weights of in the order of 1000s or I can get molecular weight in the order of 20, 30, 40,000. So, it is very versatile instrument and it is used by organic chemists, synthetic chemists, natural product chemists, proteomics, and biologists and so on actually.

(Refer Slide Time: 15:01)

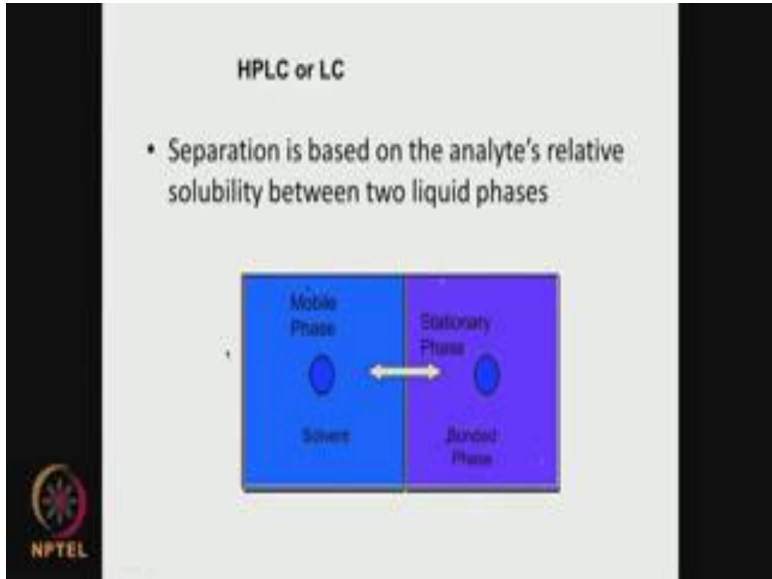


So, what does it contain there is a chromatography for separating various components. So, it is a liquid chromatography, we can also use a gas chromatography. So, it can be LC or it can be a GC. So, there could be in liquid chromatography column and solvent in a GC, it can be column and gases. Then you do the ionization, like I said you can use electron, you can by bombarding or we can use electron spray or we can use a matrix assisted laser desorption ionization technique, so all these are possible. Then you have the mass analyzer, quadrapole ion trap, time-of-flight and finally, we have the detector where it is the electronics, so the ions go and bombard current is produced. So, we get the mass of the particular molecule, it can be a protein or it can be a small molecule.

(Refer Slide Time: 15:59)

Now, let us look at each one of them little bit in more detail. So, we can have hybrid gas chromatograph mass spec, liquid chromatograph mass spec, gas chromatograph infra red, liquid chromatograph NMR. So, we can have many different types of these are called hybrid techniques hyphenated techniques. So, we can have the liquid chromatograph and the mass spec. So, both are possible.

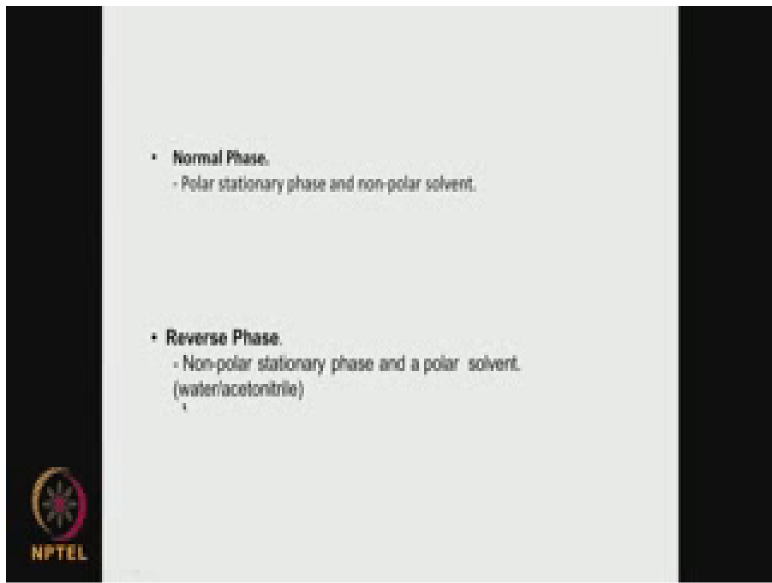
(Refer Slide Time: 16:22)



Now, how does this HPLC or liquid chromotograph works; we will not spend any time on this the separation is based on the analytes relative solubility between two phases. So,

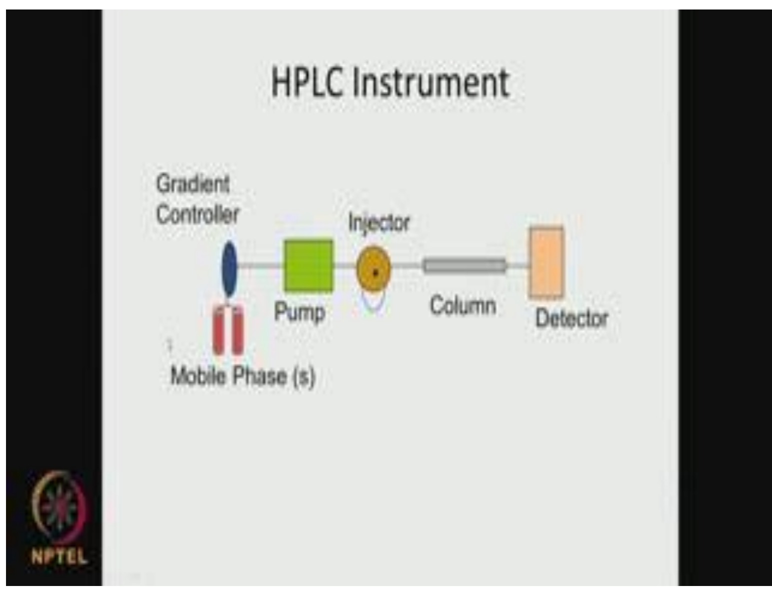
we have a bonded phase or stationary phase, we have the solvent phase or the liquid phase, so the analyte gets either adsorbed or partitioned. So, there is an interaction, so the separation of the various components takes place. So, we get each component separately which can be introduced into the mass spec and the molecular weight of that component could be determined that is how these LC or HPLC help you to separate the components.

(Refer Slide Time: 17:07)



So, in HPLC or LC we have the normal phase that means, the normal phase is a polar stationary phase; and the solvent could be a non-polar solvent, whereas when you have a reverse phase the stationary phase could be non-polar or hydrophobic material, whereas the polar solvent could be water acetonitrile combinations of these and thereof actually. So, we can have a reverse phase liquid chromatograph, or we can have a normal phase liquid chromatograph.

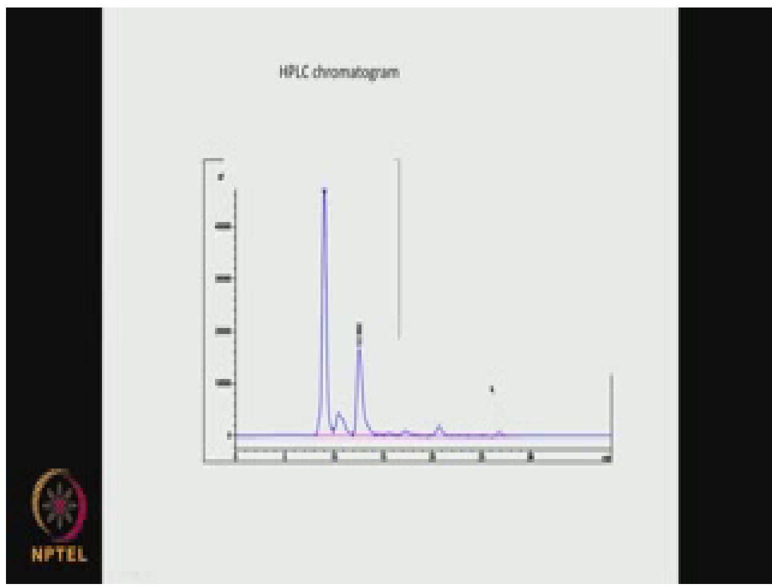
(Refer Slide Time: 17:42)



So, typically the HPLC instrument will look like this. We have a column, where we have a stationary phase we can inject the sample here the mobile phase could be one single solvent or it could be many solvents. And then it is pumped inside and it goes here to the detector after the separation. The detector could be your mass spectrometer, which I showed you before like this. This whole thing the detector could be mass spectrometer.

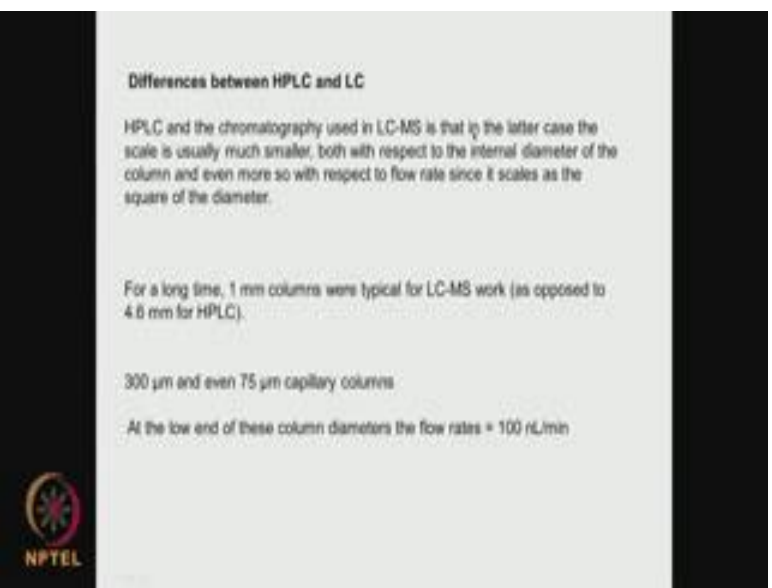
So, typically what comes out of the column HPLC column or a liquid chromatography column is peaks of various components separated out like this you know. So, this we can say a very large component A and another component B and we also have may be C, D, E and so on which are of smaller may be of smaller concentration.

(Refer Slide Time: 18:22)



So, each one is introduced into the mass spec and the molecular weight of each one of these components could be determined in the mass spec, this is how the combination of HPLC and mass spec works.

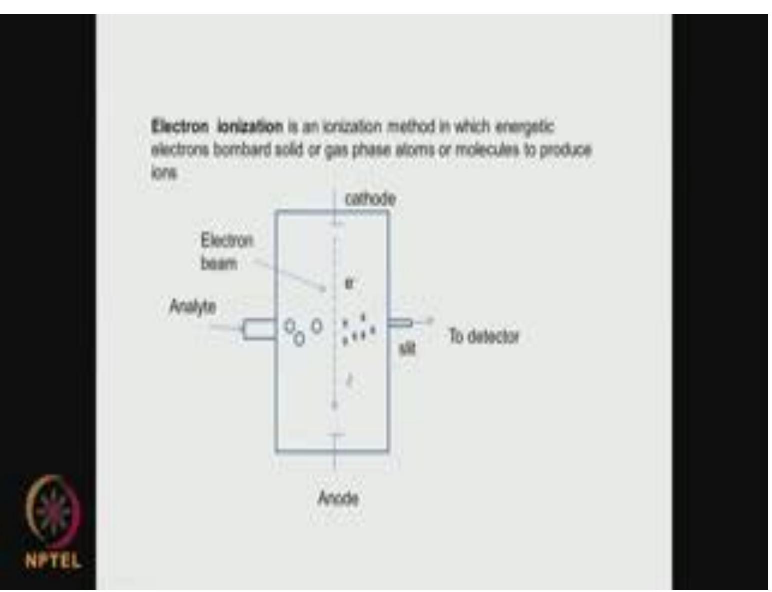
(Refer Slide Time: 18:57)



Now, what is the difference between HPLC and liquid chromatography HPLC and LC? LC is much smaller than HPLC and with respect to the internal diameter of the column and also with respect to the flow rate. Whereas HPLC may have higher flow rate and it may have larger diameter column. If you look at LC-MS, generally the column size

could be 1 mm; whereas, in HPLC the column size that is diameter could be 4.6 mm. And of course, we can also have capillary columns which is of 300 micrometer or 75 micrometer capillary columns, and the flow rate in LC could be even 100 nano liter. So, the flow rate in liquid chromatograph can be very, very low; whereas in HPLC the flow rates are much higher and the column diameter is also much higher. So, we will not spend too much time on that. So, the HPLC or the LCs initial frontend where the various components are separated out, it could be a mixture of proteins, it could be a mixture of metabolites and then these enter the gas chromatography.

(Refer Slide Time: 20:18)

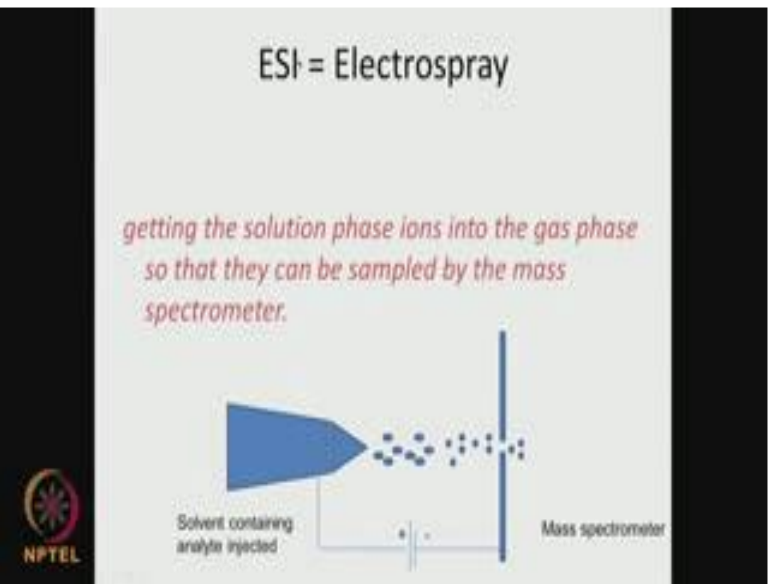


So, let us look at the various ionization techniques in mass spectrometer electron ionization. As I said initially electrons are produced it bombards the molecule and makes it into smaller ions. So, the analyte is coming here, it may be coming from HPLC or LC or GC. Electrons are produced here it bombards. So, a fragments are formed here then these fragments are focused and sent it goes to the mass spectrometer detector and so on. This is called electron ionization. So, high energy electrons bombard the sample. So, you get very small fragments.

So, in EI you get lot of small, small fragments. So, EI is not generally used for large biomolecules, because if we break the biomolecules, it may be very difficult to reconstruct and tell; what is the molecular weight of the biomolecule. So, that is the

problem with EI technique, it is good for small organic molecule, but generally this is not used for small organic molecule.

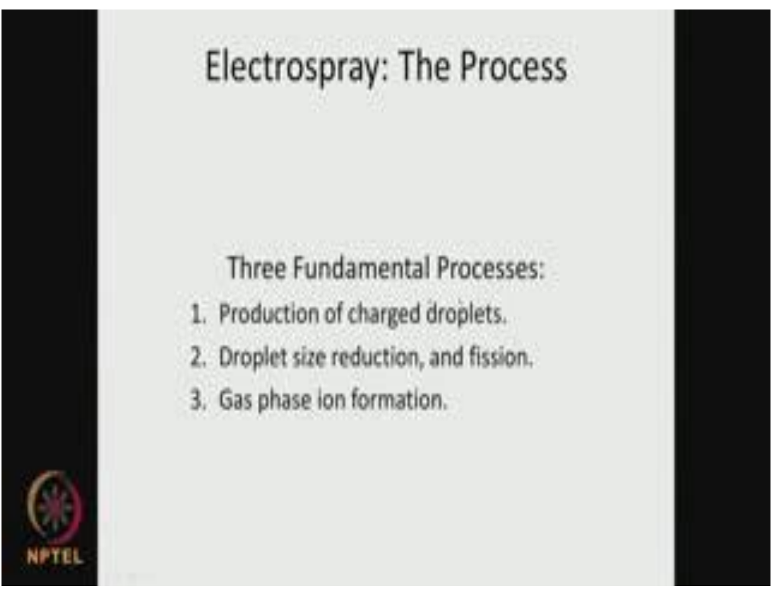
(Refer Slide Time: 21:24)



ESI is another technique; it is much milder, it is electro spray ionization. So, what we do is analyte and solvent together is injected through a small nozzle, we apply a very high voltage here. So, the particles or the stream coming out will be small diameter spray and it will be very highly charged. So, as it travels you can focus it and so these are the solvent gets evaporated, the analyte is now charged small particle, and it goes into your mass spec.

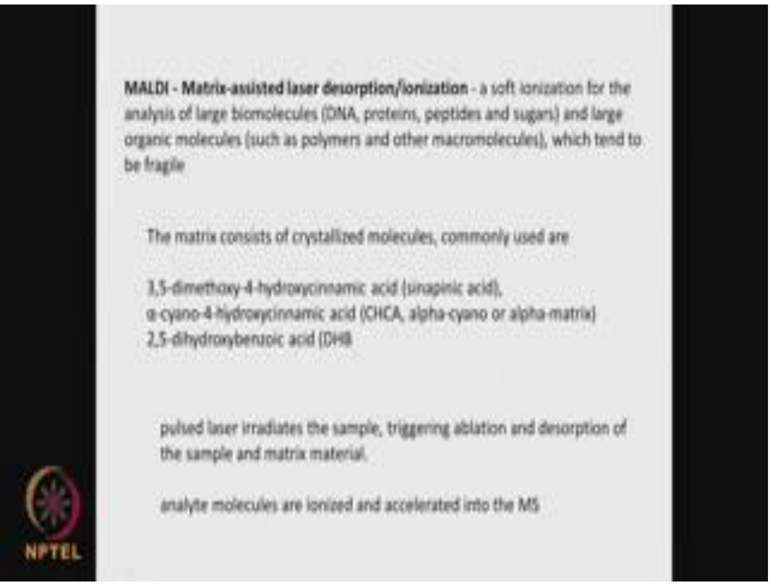
So, this technique is much milder. So, you are not actually breaking the protein into smaller bits by bombarding it. So, we will call it a milder technique here. So, ESI is quite used widely especially for protein and biomolecules, whereas EI is used if you are interested in small molecule especially in organic chemistry research.

(Refer Slide Time: 22:38)



So, this process electro spray or ESI we produce charged droplets charged droplets droplet size reduction, so the droplet gets reduced, solvent gets evaporated gas phase ion formation. So, you have ions formed here, this is called gas phase because the solvent is completely evaporated during this process.

(Refer Slide Time: 22:58)

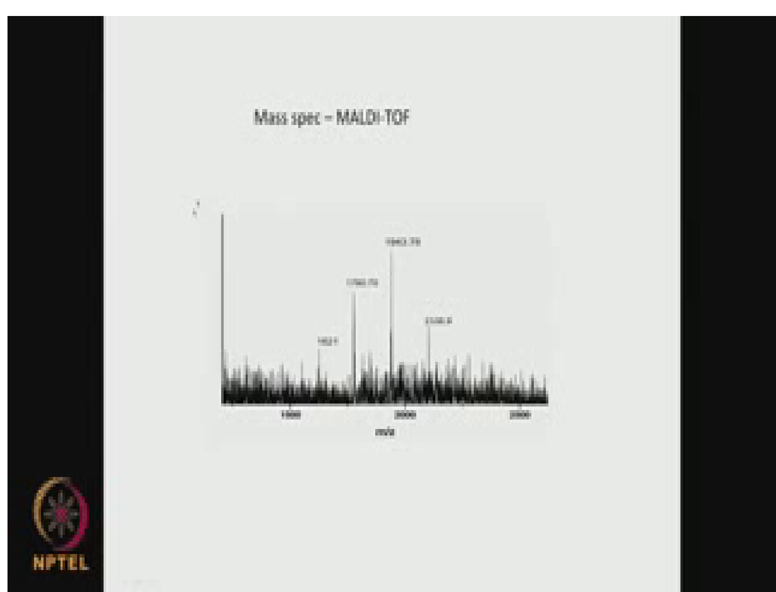


Another technique is called MALDI - matrix assisted laser desorption and ionization technique. This is also a very soft ionization technique unlike EI. So, what we do is this is very good for large biomolecules like DNA, protein, peptide, sugar or polymers or

macromolecules. So, what we do is we take the sample, mix it with a matrix material, matrix is a crystallized molecule. So, it could be 3, 5-dimethoxy-4-hydroxycinnamic acid, or alpha cyano-4-hydroxycinnamic acid, or 2, 5-dihydroxybenzoic acid and so on.. So, a laser is pulsed on the matrix not on the sample. So, the matrix gets ionized and which in turn ionizes your sample. So, it is a very mild technique.

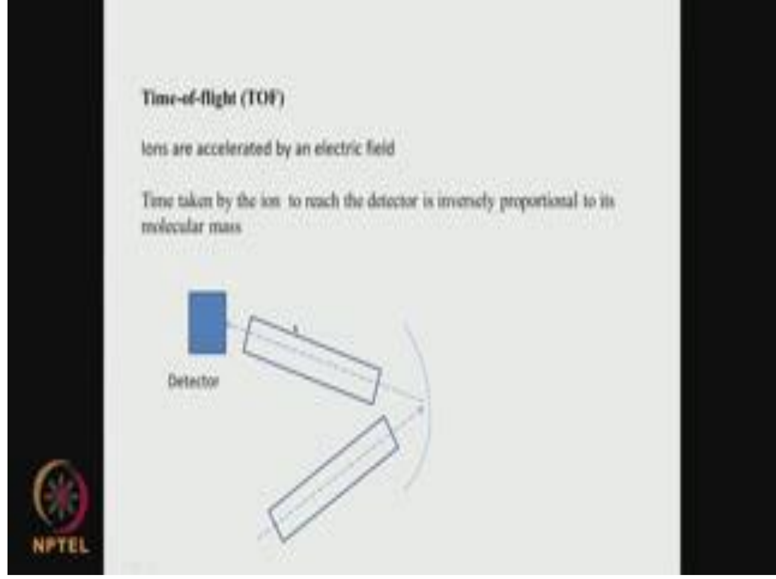
So, the matrix material and the sample which are ionized travels to your mass spec. So, the matrix gets initially ionized, which in turn ionizes your analyte. So, the analyte molecules are ionized, and accelerated into the mass spectrometer. So, this is also a very mild technique, this is very useful if we cannot want to break proteins into smaller bits and pieces.

(Refer Slide Time: 24:32)



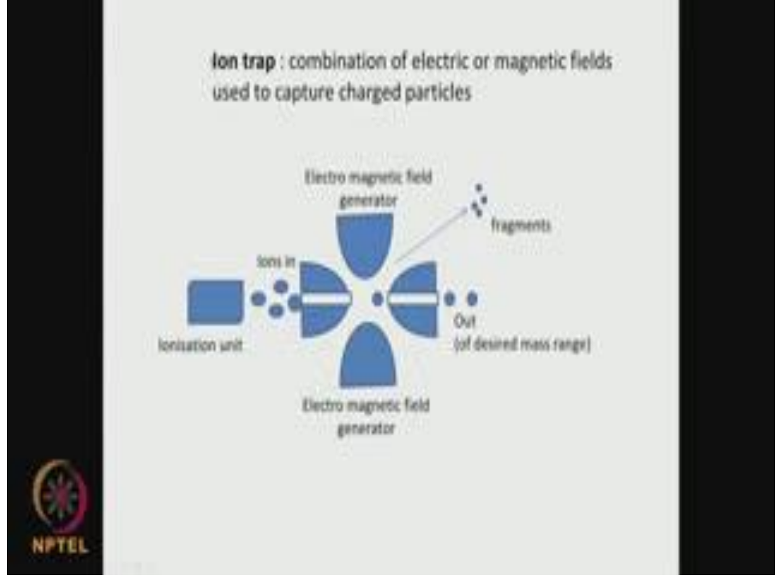
So, typical spectrum if you are using MALDI matrix assisted laser desorption ionization technique, this is called TOF – time-of-flight. So, as you can see we see the molecular weights of the material very clearly and nicely.

(Refer Slide Time: 24:54)



So, what is this time-of-flight, time-of-flight the ions are accelerated. Once you form the ions, you accelerate the ions; it goes like this, like this, like this and goes to the detector. The time these ions take to reach the detector is inversely proportional to its molecular mass. So, if we have a very large massive material ion, it will take much longer time to reach the detector. If you have a very small mass material, it will go faster. So, it is inversely proportional to molecular mass the time it takes. So, from the time you get a inverse relationship for the mass and that is how the molecular weight of material is detected. And this particular mass spectrum is from a MALDI and the detector side is called the time of flight detector.

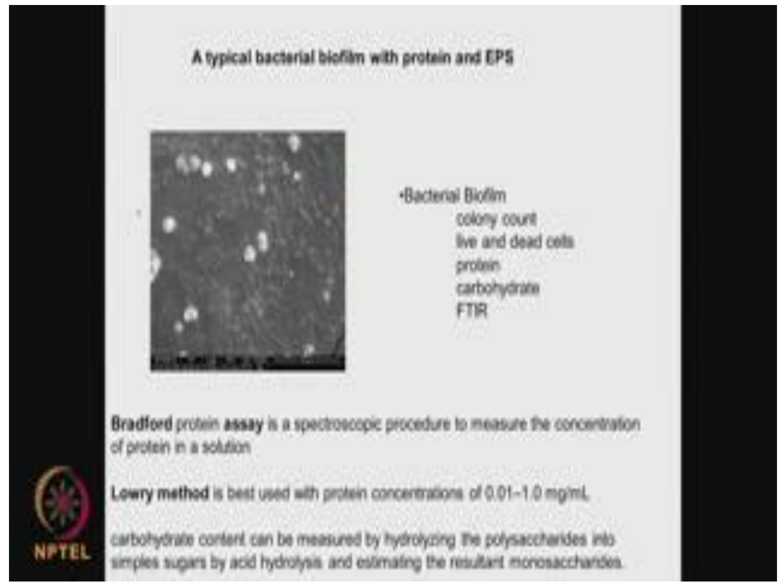
(Refer Slide Time: 25:59)



So, there are different types of the detector side. So, one is the time of flight the other one is called the ion trap. So, what it does is it is the combination of electric magnetic fields to capture charged particles of certain mass. So, you are interested in certain mass, you are not interested in any other mass, so that is why it is called ion trap. So, we have the ionization in it this could be EI, ESI or MALDI. So, ion enters a chamber ion enters. So, we apply electromagnetic field and so on, so we maintain ions of our interest all other ions smaller or bigger are thrown out, smaller is thrown out, smaller is thrown out, bigger is bombarded and made into smaller and then whatever is trapped comes out of our desired mass range that is called ion trap.

So, we trap ions of certain molecular weight which is of our interest all other smaller ones are removed or rejected the larger ones are broken into pieces, so whatever comes out is ions of our interest. So, how do you capture them using electromagnetic field and so on actually? So, these are very widely used, the ion trap and the time-of-flight these are the two things that are used to capture the ions of our interest.

(Refer Slide Time: 27:23)



For example, if you look at this picture this is a biofilm, and if I want I can take samples out I can look at the molecular weight of the proteins using MALDI type of mass spec or I can use a ESI type of a mass spec. And get an feel of what are the various molecular weight proteins that are present on my surface. So, we talked quite a lot about spectroscopy, the most important one is according to me is FTIR - Fourier transform infrared spectroscopy. And then I just gave a brief glimpse about mass spectrometer, LC-MS or GC-MS, LC means liquid chromatography. So, why do we need that we can separate out the various components present and then we can find out the mass spec mass of those components.

And how do you ionize them, we can use EI - electrons ionization or electro spray, we can spray these components and also apply very high voltage. So, they get charged, so that is another way. Another way is MALDI, so we use a matrix material which gets ionized which in turn ionizes your sample that is called MALDI. And then later on how do you get the ions of our interest, we can use ion trap type of this setup. Then there is something called time-of-flight, which tells you depending upon the time ion reaches the detector we can find out what is the molecular weight because there is inverse relationship between the time it takes to reach the detector vis a vis the mass of the fragment. So, we will continue more on these analytical tools in the next class also.

Thank you very much for your time.