Advance Analytical Course

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Lecture No. # 36

Recent Advances in Mass Spectrometry

The next method of spectroscopy is mass spectrometry. And, this is one of the most conclusive method for the structure determination, because it gives an idea about the molecular weight, and subsequently, how the molecule has been formed, what are the various parts of a molecule, because as when I was discussing GC-MS, I had clearly mentioned that the MS part gives us a very important information about the fragmentation of a molecule. The particular pattern of fragmentation of every molecule will remain the same. Whether we use it as a separate mass spectrometry machine or spectrometer or we couple it with GC as a hyphenated machine of GC-MS or if we hyphenated with the LC-MS, the mass spectrometry machine has its own very precise role to play.

And this role needs to be understood more elaborately although I had fleetingly mentioned about the mass spectrometry, about the three parts of the mass spectrometer, where first, the ionization, the first bombardment of the molecule takes place with an electron beam, and then there is an analyzer, and then there is a detector. So, according to the mass-by-charge ratio, the parts or the fragments keep reaching the detector, and the entire system is under vacuum. So, all this we had studied when we were looking at the GC-MS machine, but now, we will talk specifically about mass spectrometry and the recent advances in mass spectrometry. We will try to take a look and understand what mass spectrometry is.

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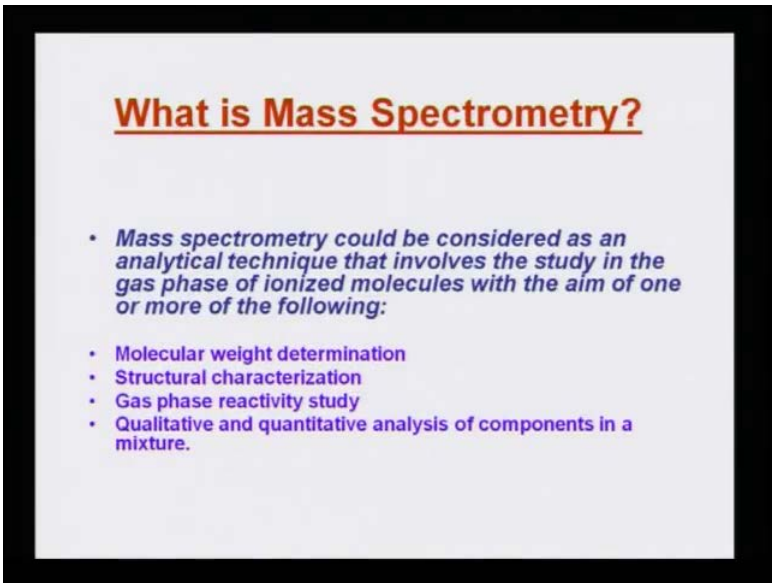

Mass spectrometry could be considered as an analytical technique that involves the study in the gas phase of ionized molecules with the aim of one or more of the following: molecular weight determination; structural characterization; gas phase reactivity study; qualitative and quantitative analysis of components in a mixture. So, these are the four major things that mass spectrometry can yield. And, by doing a mass spectrometric analysis, one can find out the molecular weight of the substance; that means the purified compound when it is fragmented in the mass spectrometer, it will give an idea about its molecular weight, because the molecular ion peak is the molecular weight. It also gives an idea about the structure. So, it is also helpful in the determination of structural characterization, plus any kind of gas phase reactivity studies can also be studied by the help of mass spectrometry. It can also help us in the qualitative and quantitative analysis of various components in a mixture. So, these are the four major functioning or roles of mass spectrometry and that is the reason why mass spectrometry is used.

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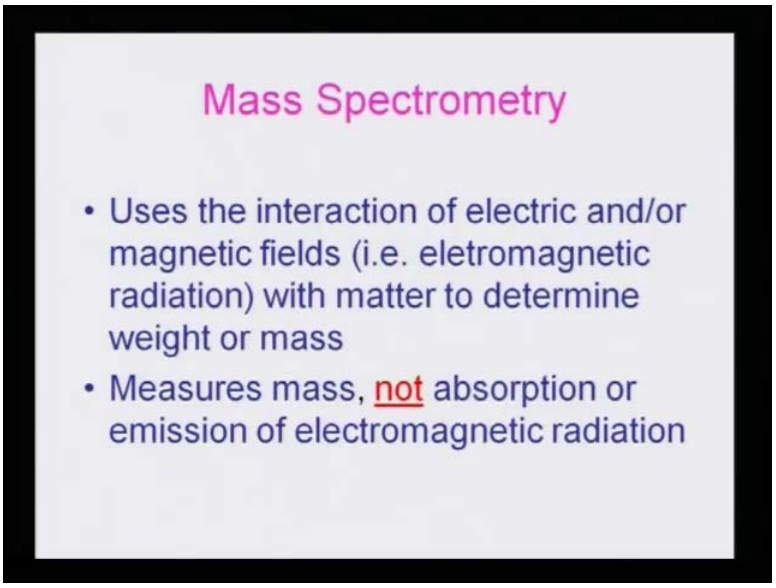

Now, when we try to look at the functioning, a mass spectrometry uses the interaction of electrical and/or magnetic fields, that is, the electromagnetic radiation with matter to determine the weight and mass although no waves are actually used here. Unlike the other cases like UV, IR, NMR, there were different waves of different regions of the electromagnetic radiation. Here, what happens is that the charged fragments that are formed are deflected towards the detector, because of this electrical and magnetic field that is generated. So, in a way, it is connected to electromagnetic movement, but it is not truly any method of absorption. Measure mass, not absorption or emission of electromagnetic radiation. But, the little difference that exists here is that it only measures the mass; it is not an absorption or an emission spectroscopic method. That is why it is called spectrometry, because it analyzes only the mass of the analyte.

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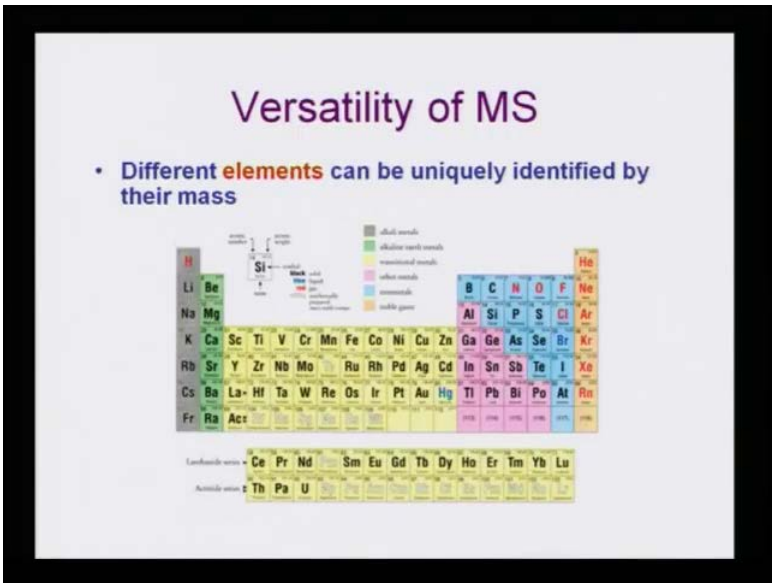

Now, the versatility of mass spectrometry is that – this particular slide I had shown earlier also – different elements can be uniquely identified by their masses. So, it has the capability of identifying all the elements of the periodic table, be it alkali metal or alkaline earth metal or transition metals or lanthanides series or actinide series or the inorganic boron family, chlorine family, and so on and so forth. So, it has that uniqueness of identification of all the different elements that are present in the periodic table, because of their masses.

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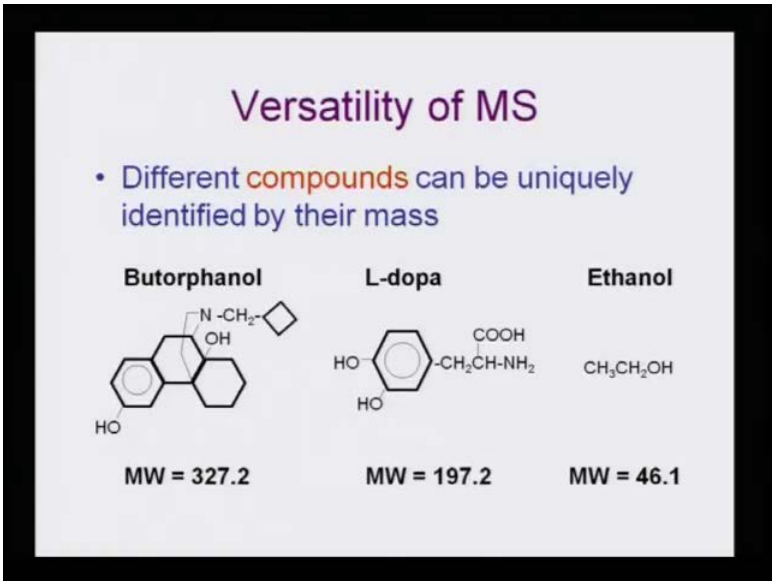

The versatility also extends to the fact that it is not only the elements of the periodic table that can be identified by mass spectrometry; even different compounds can be uniquely identified by their masses. If you take different types of molecules, like for example, I have taken the butorphanol or the l-dopa or ethanol, whatever be the complexity of the molecule or it could be as simple a molecule as ethanol, all can be identified; their molecular weight can be identified very effectively, because their masses can be identified.

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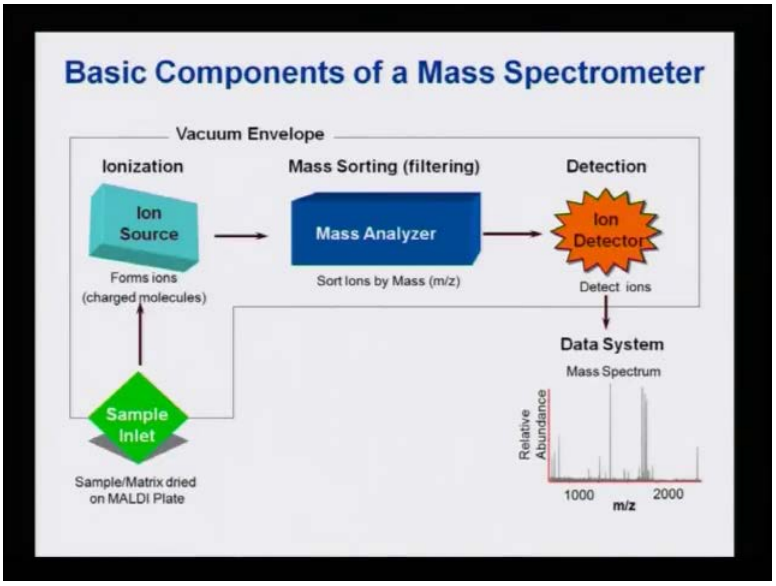

Now, if we try to recap the basic component of a mass spectrometer, let us try to look at only the mass spectrometer now. The entire envelope of the ion source mass analyzer and ion detector is under vacuum. So, it is called that it is under the vacuum envelope. There is a sample inlet obviously into the ion source, where ionization occurs. And then, after the ionization, mass sorting of filtering or analyzation takes place, because the sorting of different masses m by z has to be done by the analyzer. And then, according to the electromagnetic force that exists there, because these are all charged particles, which are moving. So, there will be an electrical field; there will be a magnetic field, and due to that, they are deflected towards the detector. And, these all must happen under vacuum, and therefore, the detector then collects the data and there is a mass spectrum that is generated.

You will also see that the mass spectrum is again a line spectrum quite similar to the carbon 13 NMR that we had taken a look while ago. So, these are very typical situations. And, you will see that the graphical representation shows that the x-axis shows the m by z, that is, the mass values in amu (atomic mass unit) and the y-axis actually shows the relative abundance. Now, what is this relative abundance? Relative abundance means how stable is the fragment; if the fragment is unstable, the line will be small or its relative abundance will be short. And therefore, the longer the line in the mass spectrum, the more stable is that fragment. And, when we try to correlate, we try to take the farthest end, that is, the left-hand side as the molecular ion peak, and from the molecular ion peak, the daughter nuclei or the daughter fragments breakdown. Now, I had also told you that the most labile bond will break first, and therefore, the fragmentation pattern remains the same. We may use any and every mass spectrometer, but the fragmentation pattern of a molecule would always remain the same.

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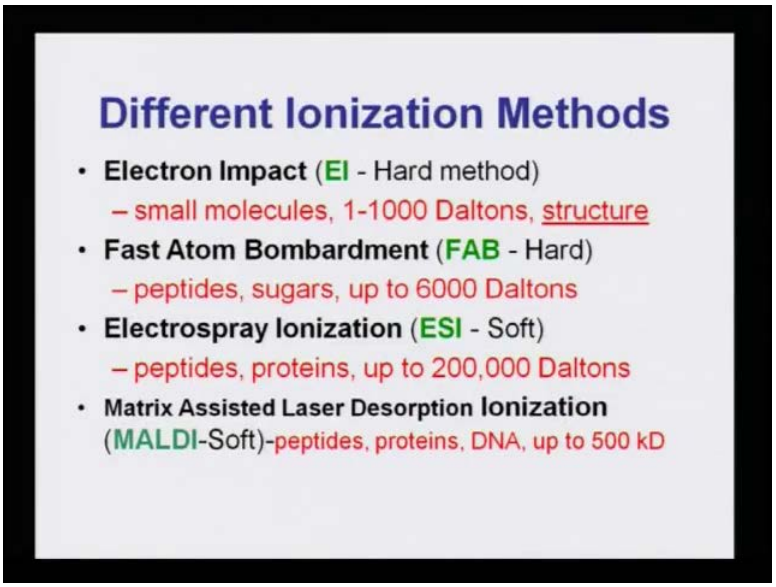

Different ionization methods have come up, because over a period of time, first, it was only electron impact, the one which I had described when I was talking about GC-MS, because there is an electron beam of almost 70 electro volt, and that first bombards the molecule, and then molecular ion peak is generated. Now, this is a very hard method. Sometimes, such methods are not very suitable for molecules, which are very sensitive. Therefore, there was a need to come up with more advancement and newer methods

came into existence, and the recent methods that have been developed for very specialized molecules.

The second method that was developed was fast atom bombardment or FAB. This is also a very harsh method or a hard method; and, it is suitable for peptides, sugars, and up to almost 6000 Daltons or atomic mass unit it can be used. The third method that was developed for simply ionization method; right now, we are discussing different ionization methods; the third method is called electrospray ionization or ESI method, which is a soft method. This is also used for peptides, proteins, and almost up to 200,000 Daltons (amus) or atomic mass units or molecular mass units. The last method, which is the most significant method and most latest method, is the matrix assisted laser desorption ionization or the MALDI method. This is also a soft method. So, we have two methods, which are hard methods, that is, electron impact and FAB method; and, there are two methods, which are soft method, that is, electrospray ionization and matrix assisted laser desorption ionization. So, these are the four methods of ionization of a molecule.

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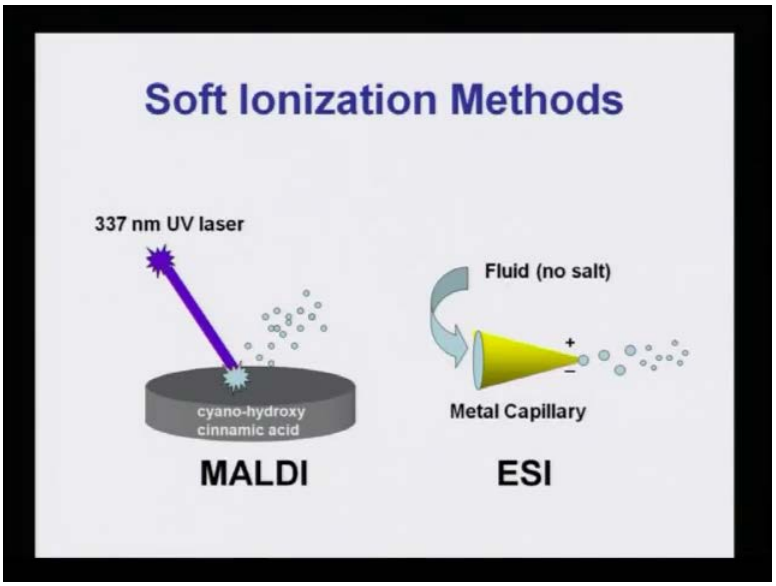

Now, what exactly happens when we talk that this is a soft method? How does the soft ionization method actually function? Now, in MALDI there is a cyano-hydroxy cinnamic acid surface, and on that, a UV laser is impacted and that creates the fragments or the ions. So, that is why it is called a soft method, because a laser beam of 337 nanometer,

which is of a UV region is impacted on the surface; whereas, in the ESI method, that is, electron spray ionization method, there is a cone and there is a fluid. And, because of the charge that is generated at the tip of the cone, that is, the positive charge and the negative charge, it gives an aerosol; or, a spray kind of ionization takes place.

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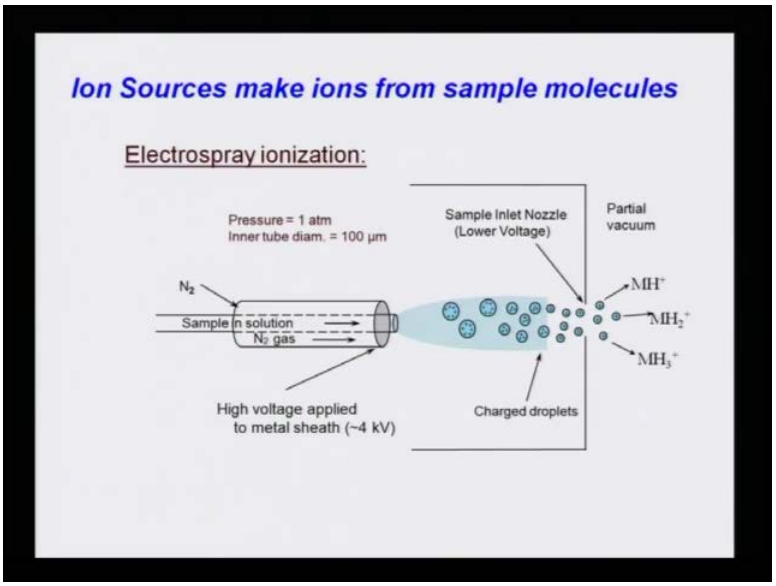

Electrospray ionization can be more elaborately understood and seen in this particular slide – ion source makes ions from the sample molecules. So, electrospray ionization – the sample is introduced with the nitrogen gas, and through the nozzle, there is a very high voltage that is applied on this sheet. And through the nozzle, the charge droplets are formed. So, that is how the electrospray ionization takes place. And, in the case of MALDI, it is the laser beam of 337 nanometer, which is made to react on the cyano-cinnamic acid surface.

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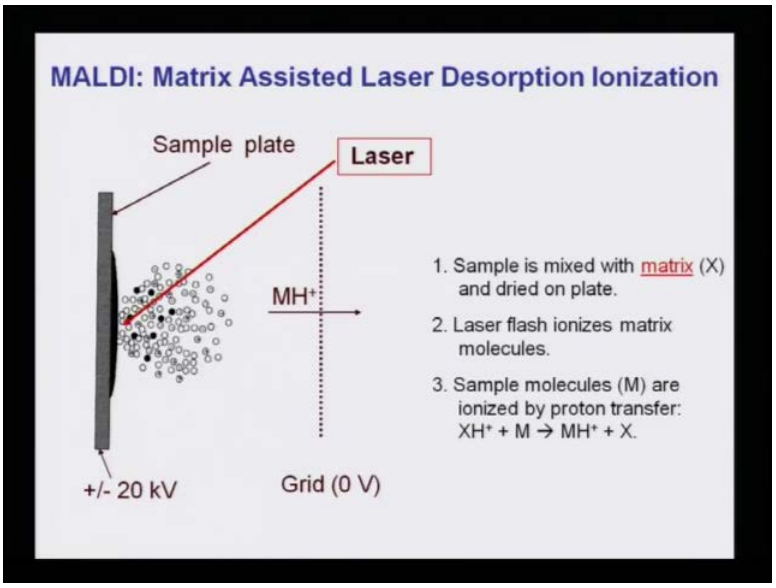

MALDI: Matrix assisted laser desorption ionization – you can take a look – the sample is placed on that particular coating and the laser is then impacted on it. Sample is mixed with the matrix. The matrix is actually the cyano-cinnamic acid and dried on plate. Laser flash ionizes matrix molecules. Sample molecules are ionized by proton transfer. So, that is how the ionization process actually takes place. And, both the MALDI method and the ESI method are soft methods of ionization, because they are used for peptides and proteins, which get denatured very easily with very harsh methods.

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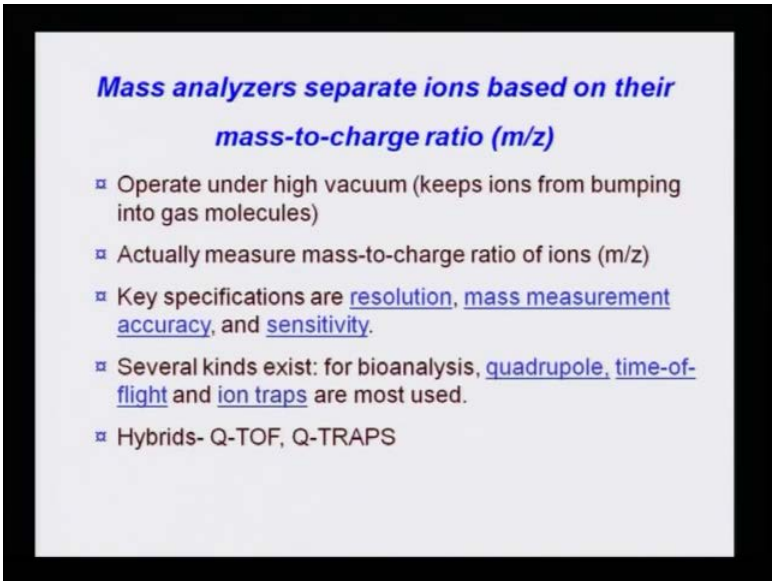

Then comes the role of the analyzer; because once the ionization has taken place, now, the second part or the middle part of the spectrometer is the analyzer part. The mass analyzers separates ion based on their mass-to-charge ratio, or which is called m by z. It operates under high vacuum; keeps ion from bumping into gas molecules; actually measures mass-to-charge ratio of ions. Key specifications are resolution, mass measurement accuracy and sensitivity. Several kinds exist: for bio analysis, quadrupole, time-of-flight and ion traps are most used. There are other types of hybrid quadrupole and time-of-flight or quadrupole ion traps; they are coupled together. So, this complicated system also exists, but the simplest of the simple, a mass analyzer is the quadrupole. And, more advanced is the time-of-flight, and then comes the ion traps. So, these are the three main mass analyzers; out of which, only one will be present. Only very rarely under very specific conditions, hybrid ones are utilized.

Now, what do they do and how do they work? First thing is that the entire situation should be at a very high vacuum, because it keeps the ions from bumping with the gas molecules, that is, the carrier gas. Actually measures the mass-to-charge ratio of the ions. So, what it does, as soon as the fragmentation takes place, according to the mass-by-charge, they are separated out, and that is what the role of the analyzer is to be more specific. Key specifications are that it resolves these different types of fragments; thus the mass measurements with accuracy and sensitivity.

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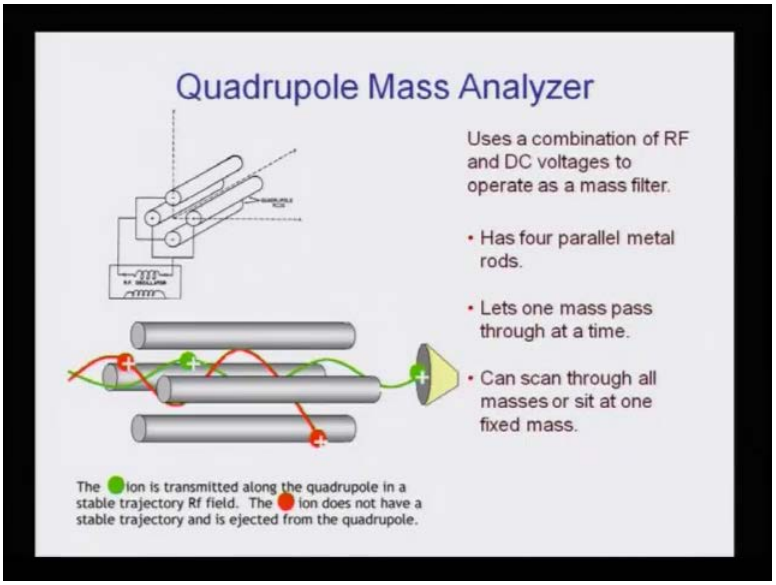

How does a quadrupole mass analyzer look like? As the name suggests, quadrupole means there will be four poles. And, in between the four poles, there is an electrical and magnetic field. And, when the ion is transmitted along the quadrupole in a stable trajectory radiofrequency field, the ion does not have a stable trajectory and is ejected from the quadrupole. It uses a combination of radiofrequency and DC voltages to operate as a mass filter; has four parallel metal rods; lets one mass pass at a time; can scan through all masses or sit at one fixed mass. So, it can actually then, start analyzing them; there is an RF and DC voltages that are applied to it, so that there is a electrical current. And, because of the electrical field, there is a magnetic field that is generated. These are all charged particles. So, they are propelled and have four parallel metal rods, which kind of direct them to go between these four parallel rods, and only allow one mass to pass through at a time; it is not that all of them will rush to the detector. So, that is the advantage of a quadrupole mass analyzer.

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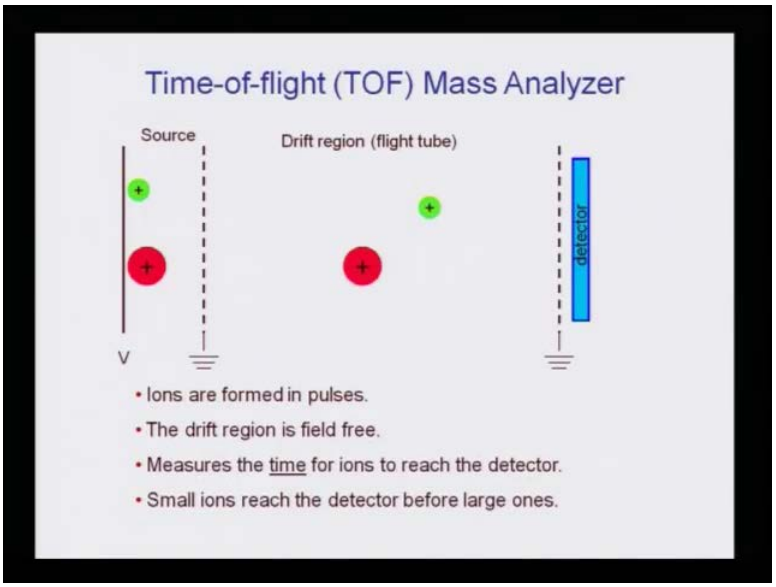

In the case of time-of-flight or TOF mass analyzer, the source generates both small and large. And, as the common sense would prevail and show you that the smaller ones will go faster towards the detector and the larger ones because of the larger mass will drift behind. So, ions are formed in pulses; the drift region is field free; measures the time for ions to reach the detector; small ions reach the detector before the large ones. So, it is very simplified model to explain you how the source generates these different types of ions, and then these charged ions how they drift in the flight tube; the name time-of–

flight, because the smaller will reach faster. So, it is a matter of time of reaching the detector.

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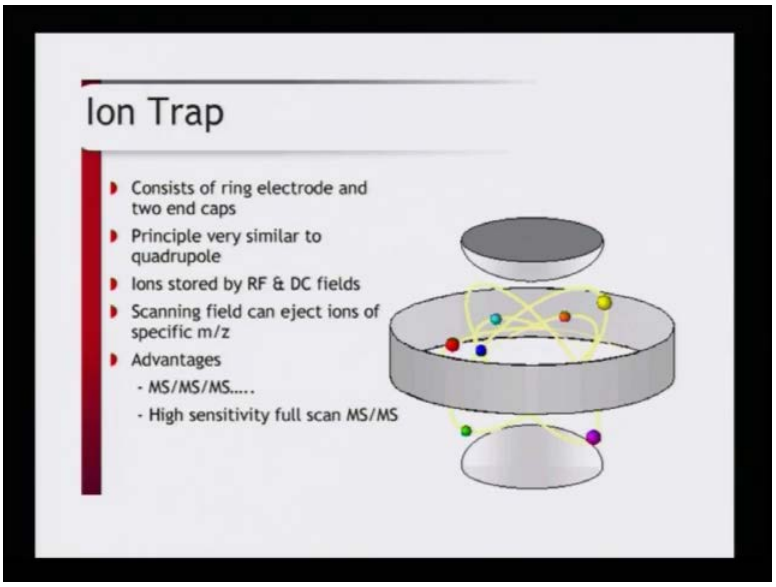

Ion trap consists of ring electrode and the two end caps; principle very similar to quadrupole; ion stored by the RF and DC field; scanning field can eject ions of specific mass-by-charge. And, advantages are that it can be applied to a system, where MS/MS/MS are hyphenated; high sensitivity full scan MS/MS hyphenated ones use ion trap, because this is consisting of two electrodes, and that is where the ions are actually generated; and then, they are regulated with the help of the RF and the DC voltages, so that a specific mass only reaches. It does a scanning and then ejects the ions only one-by-one. So, that is the added advantage.

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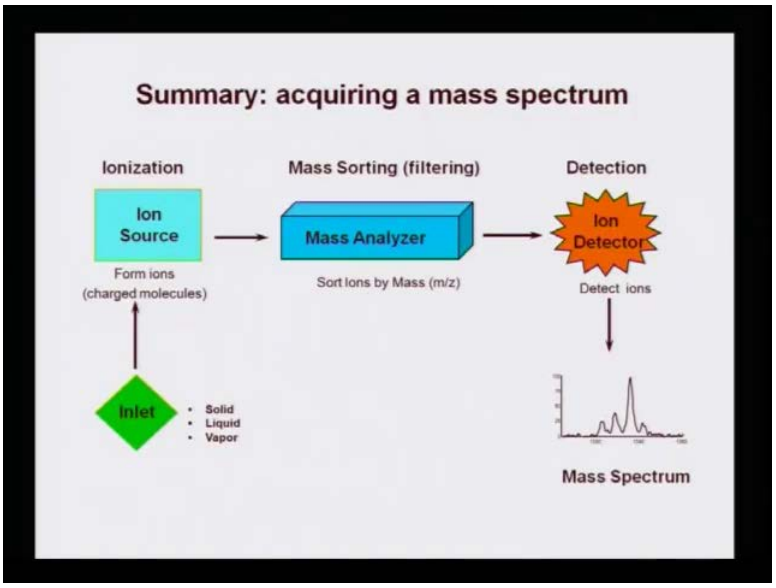

Now, if we have to take a summary of what the mass spectrum looks like or what exactly happens in a mass spectrometer, you know that there has to be an inlet. Now, inlet can allow all types of sample: vapor-phase sample, liquid sample, solid sample. Once it is injected, it goes into the ion source chamber, where it is ionized. It can use any of the ionization methods, the hard ones or the soft ones, once the ions are formed. These are charge molecules. So, they get sorted out by the mode of filtering in the mass analyzer and they are sorted by the m by z ratio. And then, they are sent towards the detector, where the ions are detected, and a mass spectrum is then amplified and given as a data. So, the data is collected as a spectrum.

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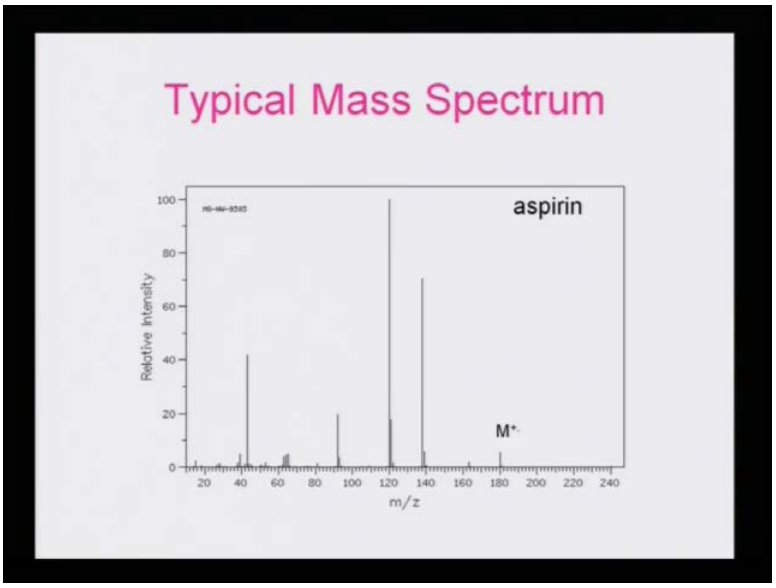

A typical mass spectrum of aspirin – you will see that it has a molecular ion peak, that is, M plus dot at 180, and then there are peaks at 139 and 120, and so on and so forth. So, these are various fragments. Now, the peak at 120 is the tallest; whereas, the peak at 180 seems to be the shortest. So, the mass molecular ion peak does not have a very high relative abundance, which means that it quickly fragments into a fragment, which is 139 and subsequently, 139 breaks up into another fragment, which is 120. Now, this 120 fragment can have some stabilizing effect, and therefore, its relative abundance is high. It is so stable that it takes a while to break it further down, and therefore, its relative abundancy or relative intensity is much higher than the molecular ion peak.

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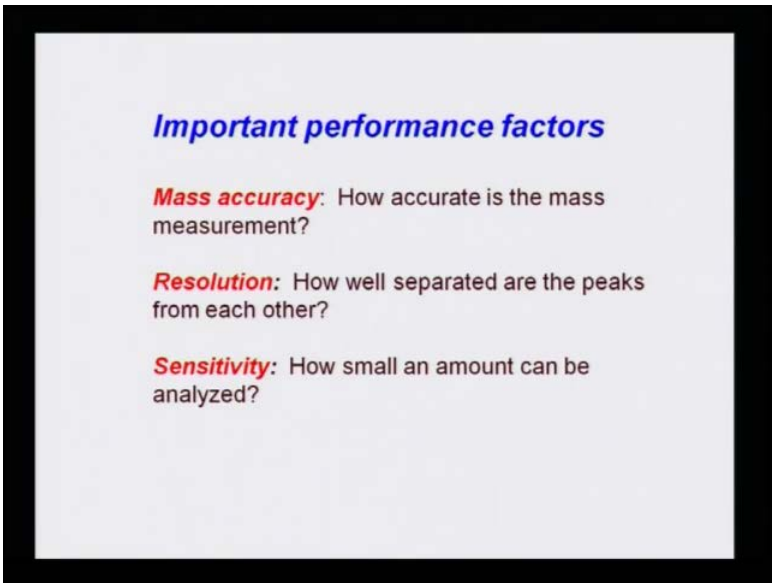

Important performance factors – now, there are three things that a machine should definitely have. Mass accuracy: how accurate is the mass measurement? Resolution: how well separated are the peaks from each other? And, sensitivity: how small an amount can be analyzed? Now, it is important to understand that these machines are very sensitive. Only a small spec, a microgram compound is enough to be analyzed; kgs and bulks and grams of compounds are not required; why, because they are highly sensitive and they can resolve these peaks very carefully, so that each peak can be depicted to a fragment and very accurate mass measurements can be taken.

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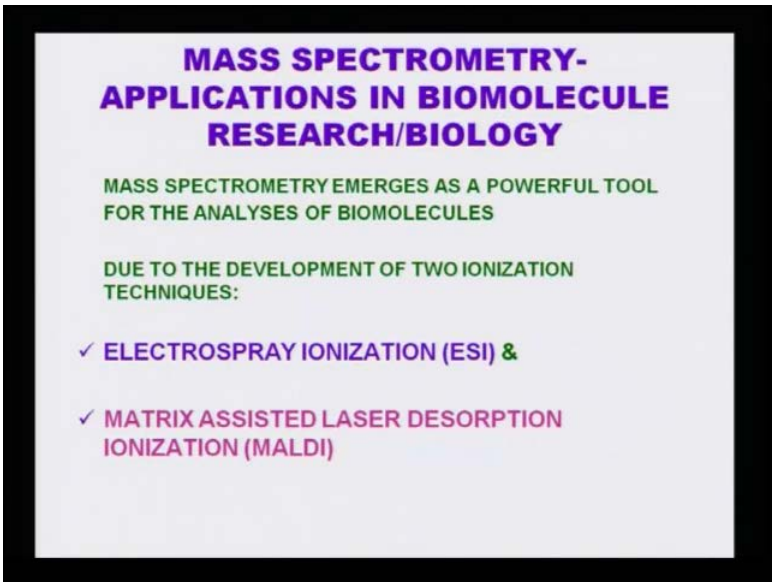

Mass spectrometry – its application in biomolecule research and biology – in today's science, of course, we can take mass spectrometry of the entire range of organic compounds and inorganic compounds, but it becomes very complicated to handle very large macromolecules, which are biological molecules, such as proteins, peptides and amino acids, and so on. Mass spectrometry emerges as a powerful tool for the analyses of biomolecules. Due to the development of two ionization techniques, that is, the electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI); these are two soft methods. We just discussed these methods a while ago. It was possible to analyze biomolecules, especially the ones, which we get denatured very easily by the hard method, that is, the electron impact method and the FAB method. There, these two methods: the ESI method and the MALDI method come very handy.

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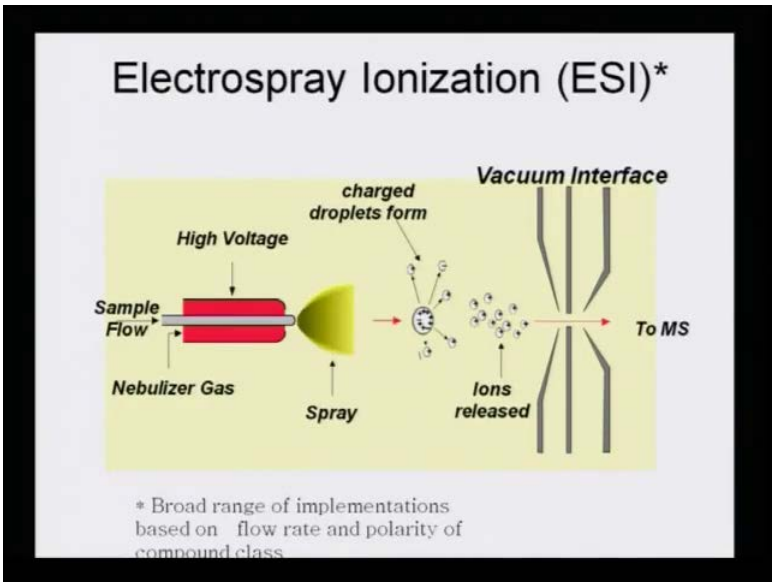

Electrospray method – we have just understood, there is another pictorial description to show you that the broad range of implementation based on flow rate and polarity of the compound class can be taken into consideration. The sample is made to flow through the nebulizer gas and there is a nozzle, there is a spray formation. And, these sprays then generate charge droplets. And then, they are at the interface, because of their ion, the vacuum that is there. These ions are then moving towards the interface to the mass detector.

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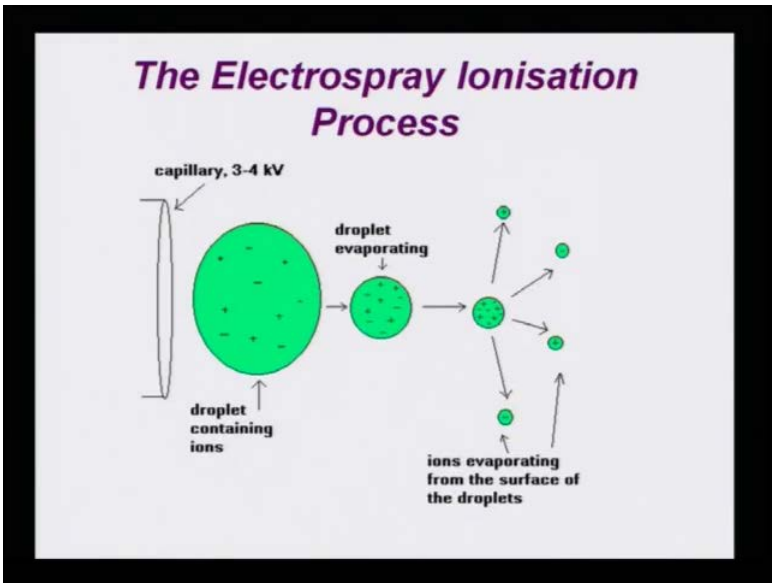

Now, the electrospray ionization process – that the droplet containing the ions is a segregate. From that, the droplet gets evaporated and the ions are spread out. So, that is where the spraying effect or the ions evaporating from the surface of the droplets actually release the ions, and that is what moves towards the detector.

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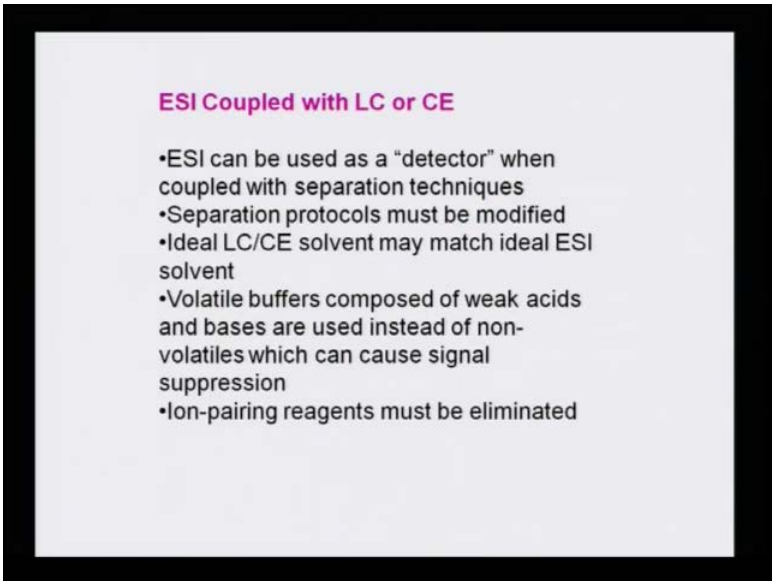

ESI coupled with LC or CE – that means this electrospray ionization method can be easily coupled with liquid chromatography or capillary electrophoresis machine. ESI can be used as a detector when coupled with separation techniques. Separation protocols

must be modified. Ideal liquid chromatography and capillary electrophoresis solvent may match ideal ESI solvent. Volatile buffers composed of weak acids and bases are use instead of non-volatile, which can cause signal suppression. Ion-pairing reagents must be eliminated. So, it is very well-suited for liquid chromatography separation method or capillary electrophoresis method; why, because the kind of solvents that are involved in LC and CE match with the ones, which are used in ESI for the droplet formation. Again, the separation protocol must be very slow; that means the procedure cannot be very fast, because this takes some time for the ESI to act as a detector. And, volatile buffers can be easily used; buffers which are made up of weak acids and bases instead of non-volatile; why, because this evaporation must take place. Unless the droplets evaporate, the release of ions will not take place. And, there should be no reagent, which can cause ion pairing in the process. So, these things have to be born in mind; only then, ESI or electrospray ionization can be coupled with liquid chromatography and capillary electrophoresis.

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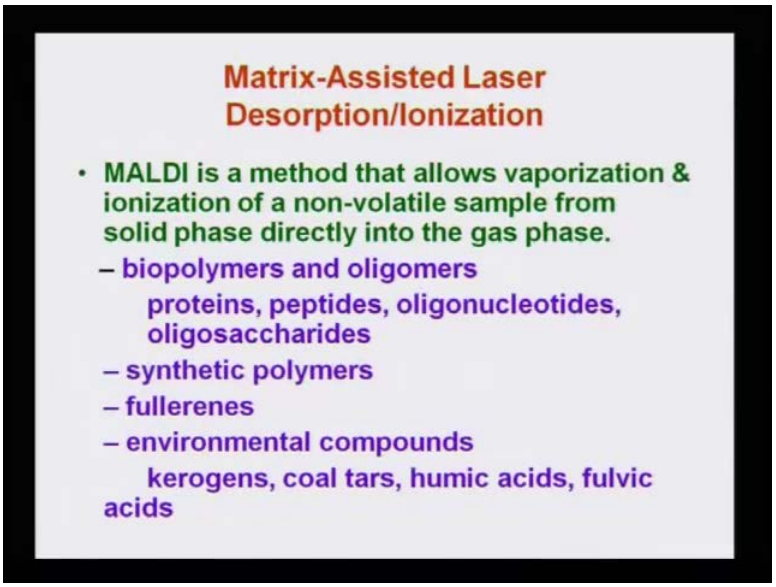

Matrix-assisted laser desorption/ionization – MALDI is a method that allows vaporization and ionization of a non-volatile sample from solid phase directly into the gas phase. So, that is the beauty that from a solid phase, it is immediately changed into the gaseous phase. And, biopolymers, oligomers, proteins, peptides, oligonucleotides, oligosaccharides – these are different substances, which were hard to volatilize and they can be changing to gaseous phase very easily by MALDI method. Even synthetic polymers can be ionized; fullerenes can be ionized; environmental compounds –

kerogens, coal tars, humic acid, fulvic acid, and many such compounds, which were not very easy to volatilize can be ionized by the MALDI method or matrix-assisted laser desorption ionization method.

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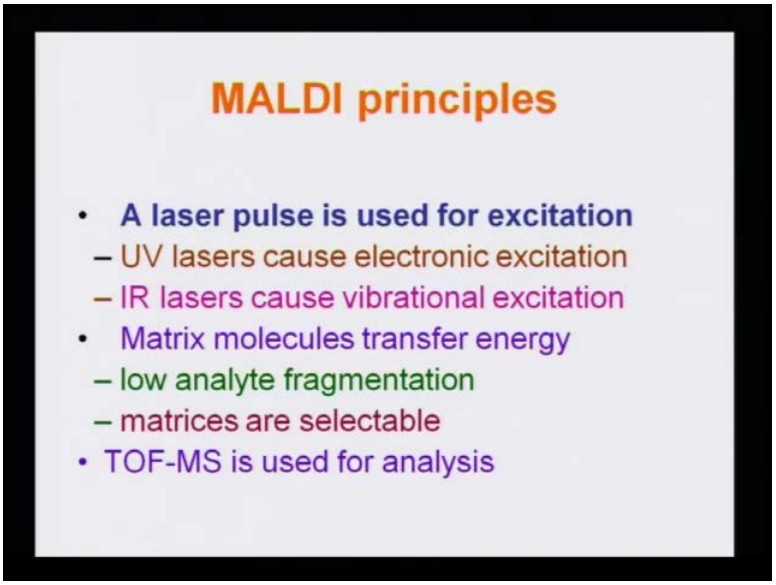

What is the basic principle of MALDI? A laser pulse is used for excitation. I told you, laser pulse of 337 nanometer; UV lasers cause electronic excitation; IR lasers also can be used which cause vibrational excitation. Matrix molecules transfer energy – low analyte fragmentation; matrices are selectable. And then, it can be connected to a TOF-MS, that is, time-of-flight analyzer-MS can be used for the analysis. So, MALDI is well-suited with TOF. So, where ionization is done by MALDI, time-of-light is the ideal analyzer of these fragments.

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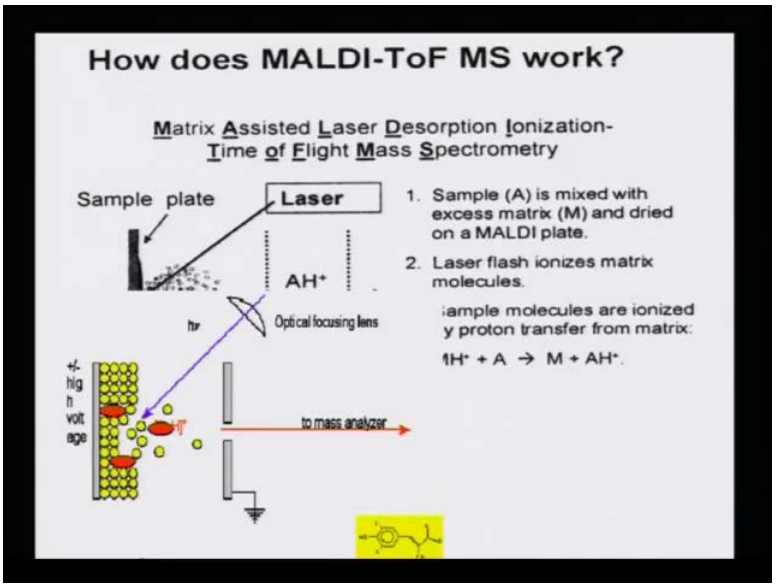

How does MALDI-ToF-MS work? Matrix-assisted laser desorption ionization coupled with time-of-flight mass spectrometry – sample A is mixed with excess matrix M and dried on the MALDI plate. Laser flash ionizes matrix molecules. Sample molecules are ionized; y proton transfer from the matrix. So, first, they are ionized and then there is a proton transfer from the matrix. And, that is how the molecular ion peak is generated and the molecule is… The fragments subsequently are formed because of the time-of-flight ionization method.

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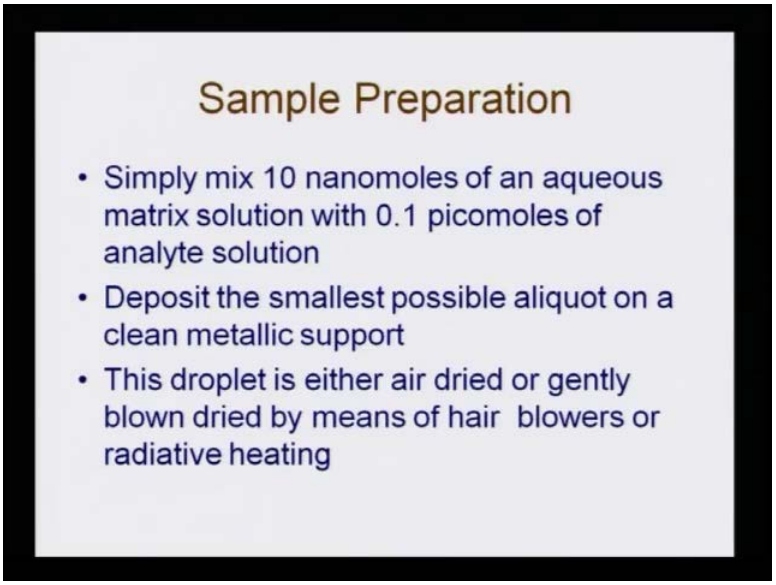

Now, sample preparation – as like any other method that we have been discussing in the spectroscopic method, sample preparation is a very crucial part; simply mix 10 nanomoles of an aqueous matrix solution with 0.1 picomoles of analyte solution. So, sample preparation for MALDI is very simple; very small quantity, 10 nanomole of the aqueous matrix solution is mixed with 0.1 picomole of the analyte solution. Deposit the smallest possible aliquot on the clean metallic support. This droplet is either air dried or gently blown dried by means of air blower or radiated heating. And, once it is dried, then it is exposed for the identification process.

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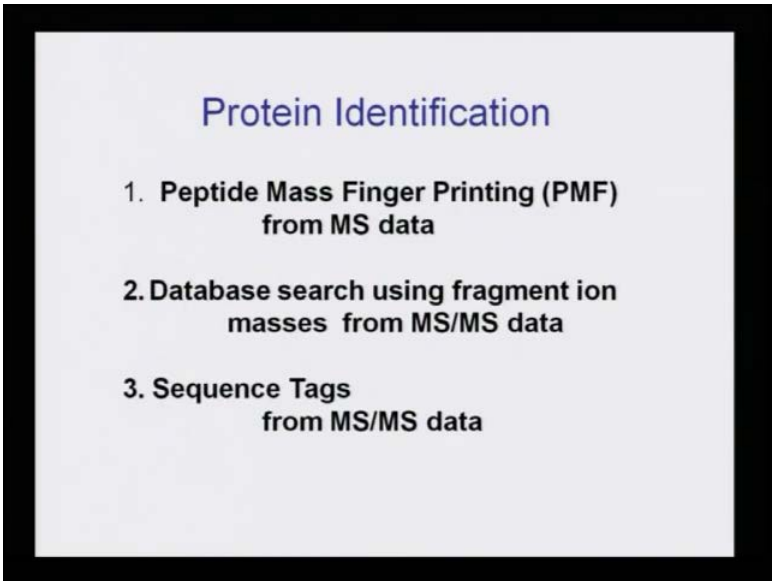

Protein can be identified: peptide mass finger printing, that is, PMF from MS data; database search using fragment ion masses from MS/MS data; and, sequence tags from MS/MS data. So, not one MS can now function completely to identify protein. In order to be able to identify protein completely, another MS must be sequenced to it. And then only, the peptide mass finger printing or PMF can be brought about. Then, because there is a lot of data that has already been collected, a library has been set up. So, there is a database search using these fragments; whether this fragment is matching with any of the fragments that are given in the database; and then, a sequence tagging is done.

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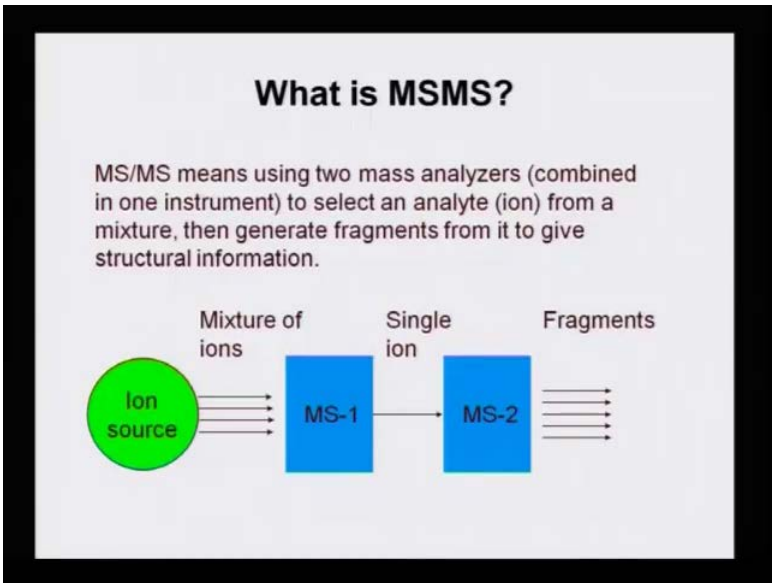

Now, if one wants to understand how the MS/MS works, how this is hyphenated, one MS is doing a part of the fragment and then the subsequent further fragmentation is done by the second MS. The MS/MS means using two mass analyzer combined in one instrument and both the MS are actually placed or housed in one machine casing to select an analyte ion from a mixture, then generate fragment from it to give structural information. So, what does the ion source do? It takes and generates the mixture of ions and that is first detected by the first MS. And then, one of the ions is then selected and further fragmented into the second MS, and the further fragmentations are obtained from this.

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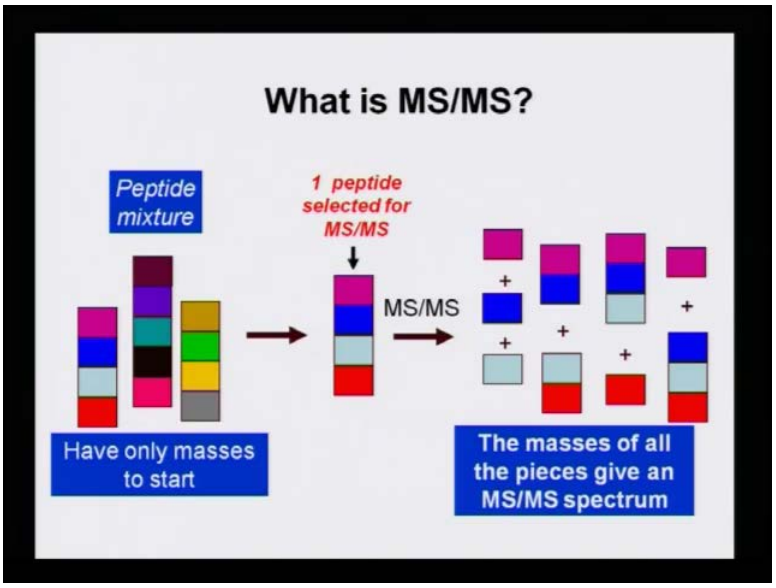

It is something like this. If I have to show you by block diagram and there is a peptide mixture, I have only masses to start with, one peptide is selected for MS/MS detection, and then slowly it breaks down into various possible fragments. The masses of all the pieces give an MS/MS spectrum. And, when all these information are correlated, then we are able to get to the actual structural determination of the peptide.

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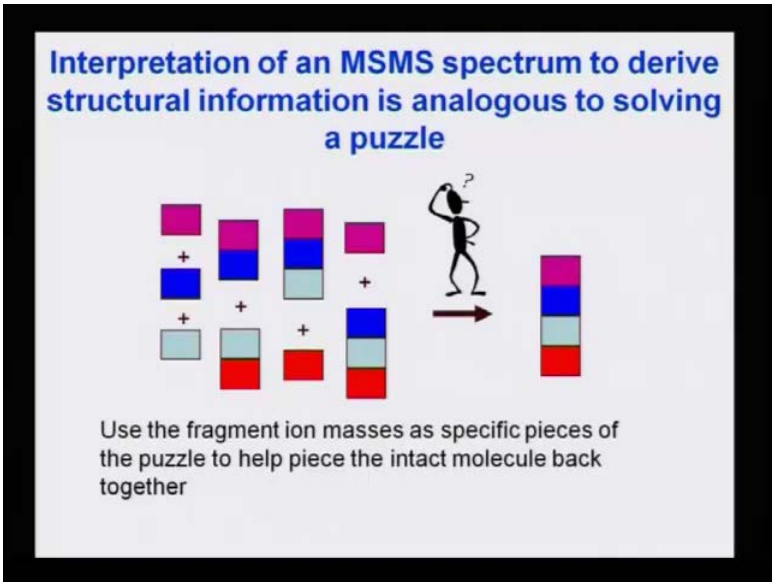

Interpretation of an MS/MS to derive structural information is analogous to solving a puzzle – it is true that only very specialists can do this. Use the fragment ion masses as

specific pieces of the puzzle to help piece the intact molecule back together. So, in order to be able to understand this, you have to put back all these blocks together to understand; which is a very intriguing problem. And, it is not a very easy for any common analyst to do it; only, very specialist, who have gained sufficient amount of experience can interpret this. But, nevertheless, it can be done and that is what I wanted to introduce you people.

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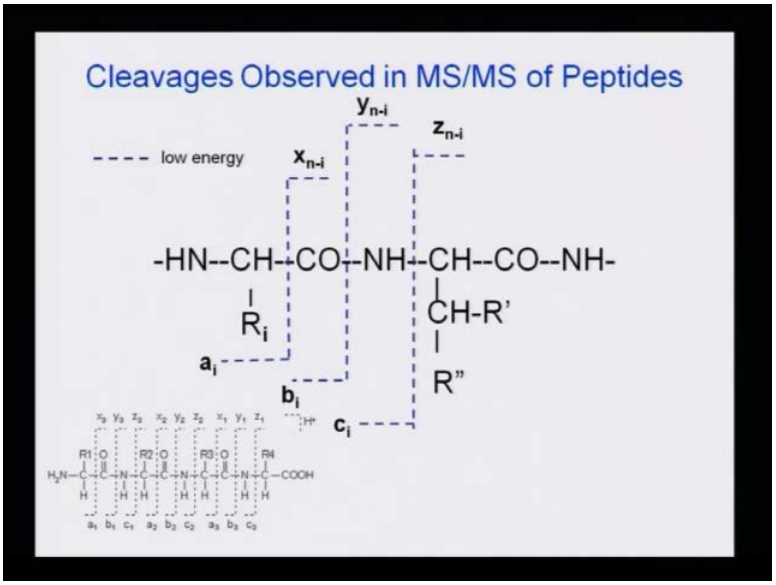

Now, you look at this particular peptide. Cleavage observed can be from various parts and these are the only points, which are most labile for the molecule to break at this point. And, I told you that the more labile the bond is, the more easy it is to break from those points only, and only those points are the ones which are labile. So, they will break definitely from those points only. So, certain fragments only will be obtained whichever be the mode of ionization. Whether we are using hard method or soft method or ESI method or MALDI method or ion impact method or FAB method, molecule will break from those particular bonds only; why, because the bond is polarized, the bond is weak, and those weak bonds are the points of actual breaking or cleavage.

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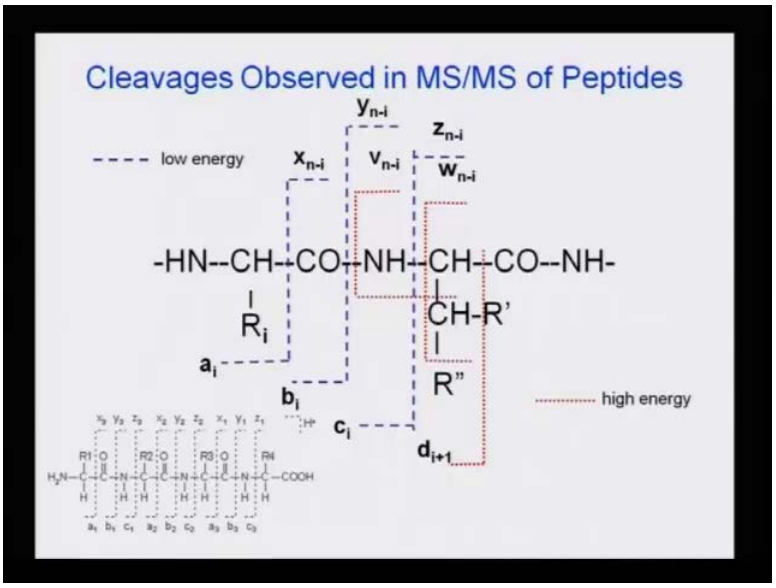

So, this is how finally, the high energy breaks them.

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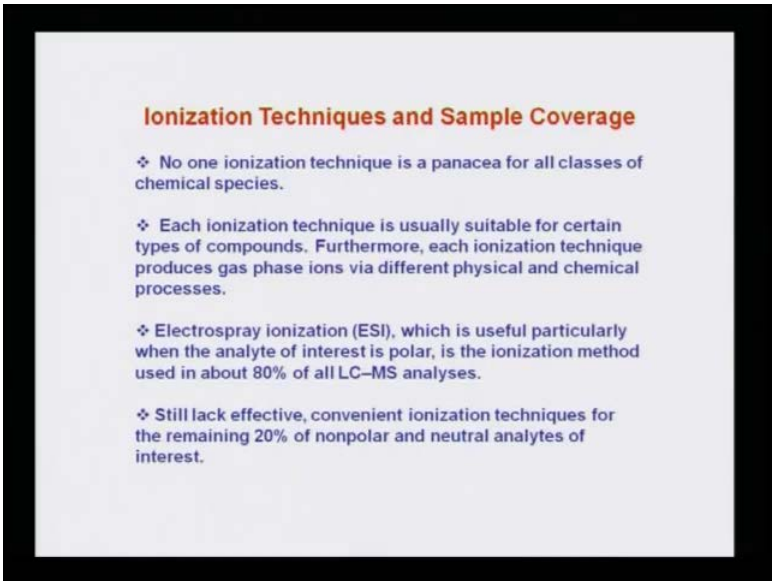

Ionization techniques and sample coverage – no one ionization technique is a panacea of all classes of chemical species. Each ionization technique is usually suitable for certain types of compounds. Furthermore, each ionization technique produces gas phase ions via different physical and chemical processes. Electrospray ionization (ESI), which is useful particularly when the analyte of interest is polar, is the ionization method used in about 80 percent of all LC-MS analyses; still lack effective, convenient ionization techniques

for the remaining 20 percent of nonpolar and neutral analytes of interest. There is no one ionization technique, which will cover all the types of compounds; first thing is that. Therefore, the more sensitive the species, the easier will be the method of ionization. Each ionization technique is usually suitable for certain types of compounds, and that is why the new methods, soft methods came into existence, because the hard methods were already existing; electron impact method was already there; then, FAB method was developed. So, further on, the ESI method and the MALDI methods came into picture.