Advance Analytical Course

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Lecture No. # 37

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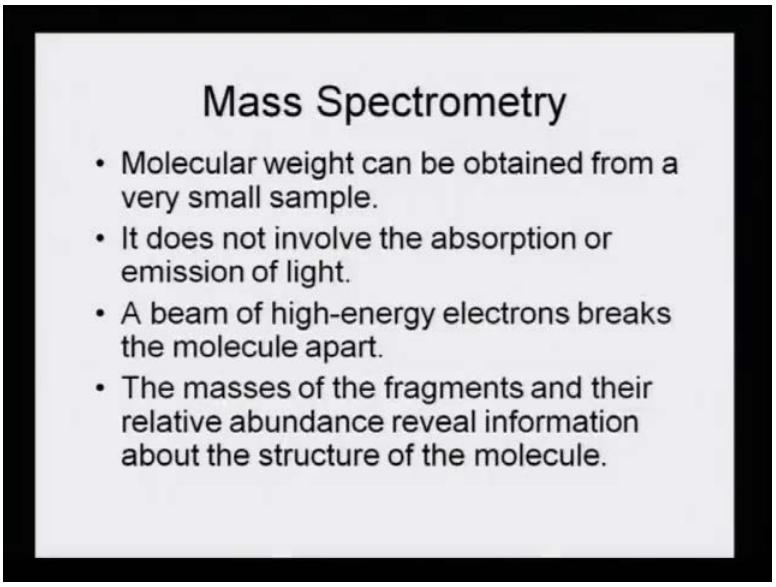

When we try to look at mass spectrometry, there are certain points, that needs to be kept in mind and those points will also make you understand, that mass spectrometry is much, much different than the other spectroscopic methods, that we discuss, particularly the UV, visible spectroscopy, IR spectroscopy, NMR spectroscopy and so what are the differences, that make mass spectrometry different from these spectroscopic methods?

First thing is that mass spectrometry helps us to find out the molecular weight and can be obtained from a very small sample. So, the sample size can be as small as possible and yet, we can get the molecular weight of the compound. It does not involve the absorption or emission of light, so it is not a true spectroscopy method; why? Because there is no absorption of light or emission of light.

So, what, how does it function? What is the role that excites a molecule? And how does the spectrum, mass spectrum come into picture?

A beam of high-energy electron breaks the molecule apart. The masses of the fragments and their relative abundance reveal information about the structure of the molecule. So, time and again, ever since I have been discussing GC-MS and later on, even in the beginning of this lecture, I had said that any organic molecule consists of bonds. Now, these bonds can be, you know, some of them are strong bond and some of them are weak bond. It is only the weak bonds, that will break in the fragmentation pattern initially and as and when the molecule breaks into smaller fragments, still smaller fragments will be formed under high-energy system.

So, the initial breaking of the molecule to molecular ion is only possible from the beam of electron bombarding, the atom, the molecule, so that is very important to be understood and this initial bombardment actually, creates molecular ion peak. And then, the molecular ion peak, according to its bonding, the weak bonds or the labile bonds first break up and give fragments.

Then comes, why the lines are short and long? The relative abundance of these fragments would depend upon all the rules, that you have learnt about the stability of that particular cation and therefore, one must remember, that there are, the more stable the cation or the fragment, the more long will be the line, or the relative abundance of that particular fragment will be higher than the others.

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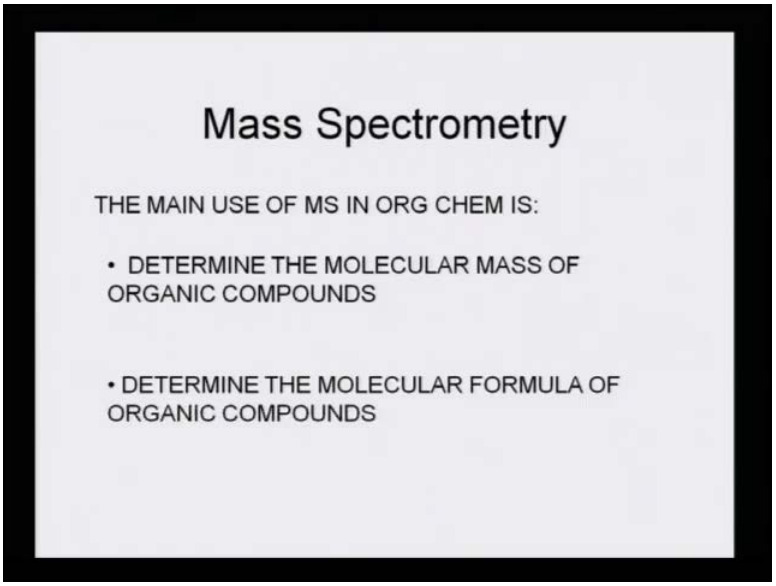

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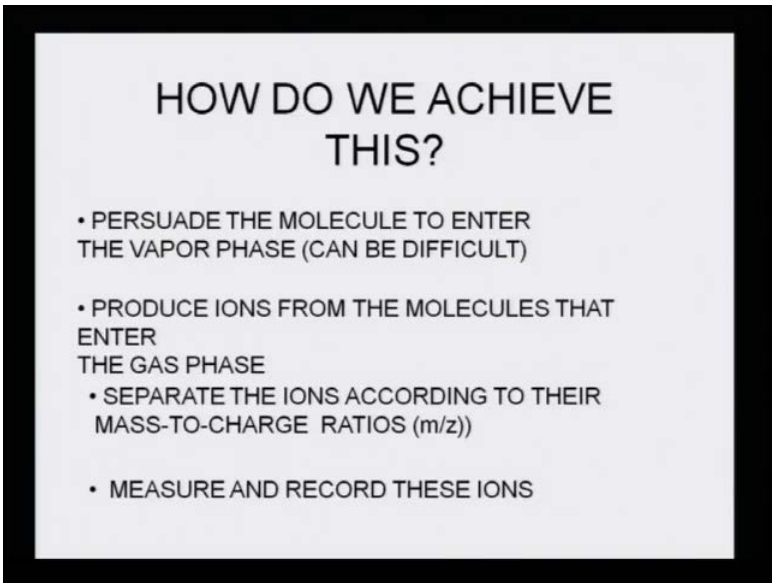

Mass spectrometry - the main use of mass spectrometry is in organic chemistry to determine the molecular mass of an organic compound; to determine the molecular formula of the organic compound and therefore, how is it achieved? So, the 2 functions are very important - one is to find out, what is the molecular formula and the other thing is that, what is the molecular mass of a substance?

How do we achieve this? Persuade the molecules to enter the vapor phase at this, which can be a little difficult. Produce ions from the molecules that enter the gas phase and then, separate the ions according to their mass to charge ratio, that is, m by z.

So, these are the main functions that happened in a mass spectrometer. We have learnt a while ago, that there is an ion source, which bombards the molecule. Then, there is an analyzer, which analyses these different fragments according to their mass to charge ratio and then, there is a detector.

So, separate the ions according to their mass to charge ratio, that is, m by z and measure and record these ions. So, these are the 4 functions that happen in a mass spectrometer ionizing methods. We have already discussed, but I would like to repeat here, that you know, in order for you to recap and to be able to comprehend, what is a hard method, what is a soft method, and so on.

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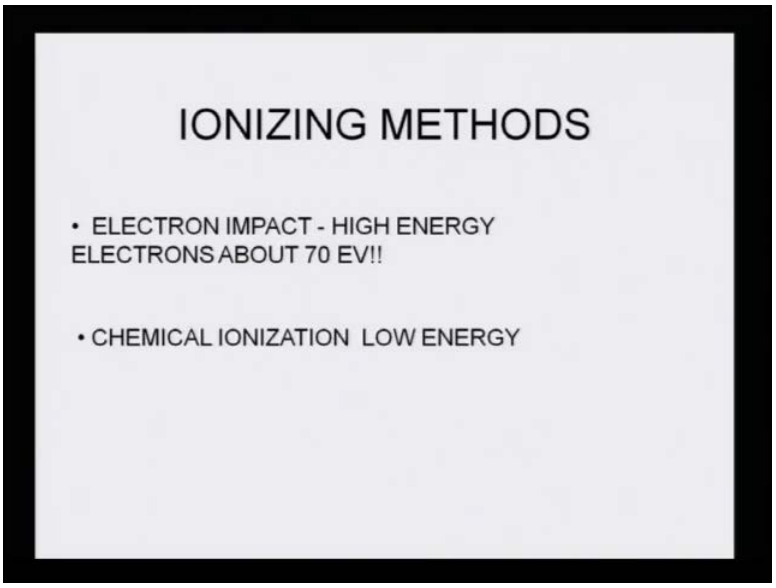

The first and the most popular method, which most of the mass spectrometers have as an ionizing method, is the electron impact. It is high energy, electrons at about 20 electro volt, the beam of electron is made to bombard the molecule and that is why, it is very harsh method. Chemical ionization or low energy methods are also possible.

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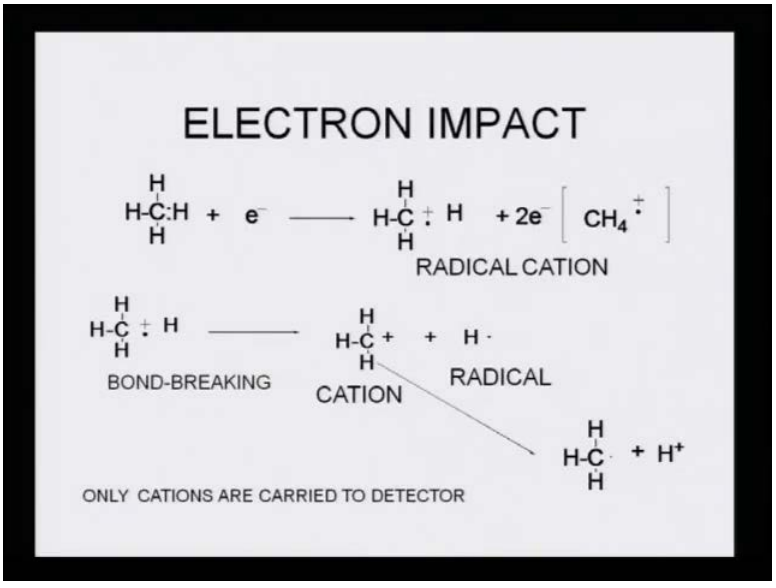

Electron impact method - when there is a molecule, which is methane, methane, when it is bombarded with electron, it produces CH 3 plus dot, which is a methyl cation, radical

cation and 2 electrons are ejected out. These 2 electrons, then participate in bombarding the other particles as well.

Therefore, this, now, methyl radical cation, then gives away hydrogen radical and becomes methyl cation alone. And that is how, the methyl cation, then further is gives away its charge to hydrogen radical, and that is how, the impact actually breaks the molecule into different fragments.

Of course, because it was a simple molecule, methane it appears very simplified. I chose this example mainly to make you understand, that how a radical cation is first formed with the initial bombardment of the electron and then the radical cation, then gets converted into cation and radical and finally, there is a propagation process that takes place.

The bond breaking occurs and the only cations are carried to the detector, why? Because charge species tend to move and when they move, they generate an electrical and magnetic field, and because of their mass to charge ratio, they are deflected towards the detector.

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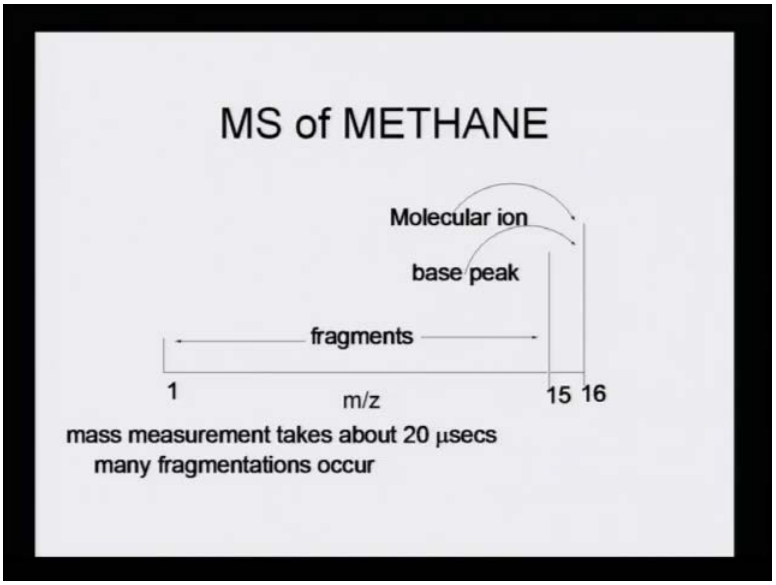

Mass of methane - one would find a molecular ion or a base peak at 16 because CH 4, C is 12 and H is 4, so it makes 14, sorry, 16 and then from that, when 1 hydrogen is

eliminated, it forms a peak at 15. So, they, one would find, according to methane, only 2 major peaks and those would be at 16 and 15 amu.

Now, from this, you will also understand, that just the way where we had an NMR at TMS, which was the starting point to, from TMS, all others protons were towards the left hand side. Similarly, molecular ion peak is the actual molecular weight of the substance because only 1 electron has been lost. It is a radical cation and therefore, from this molecular ion peak, all other fragments will fall on the left hand side and will be smaller in number in terms of their mass to charge ratio.

Mass measurements take about 20 micro seconds; many fragmentations occur during this process. So, you see, that the time required for this bombardment and the fragments to be formed is very, very small and therefore, it is one method, which is very fast and very efficient for the fragmentation of molecules.

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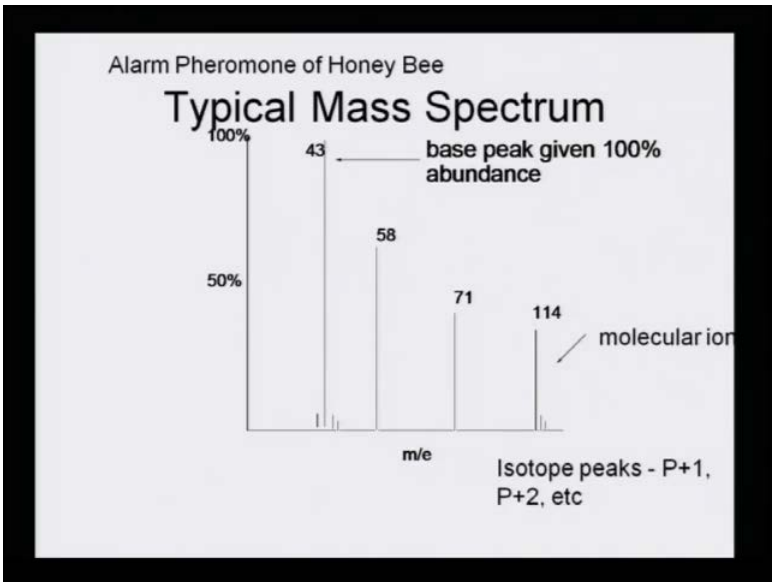

So, what does a typical mass look like? Let us try to take an example of alarm pheromone of honey bee. And a typical mass spectrum shows that it has a relative abundance base, peak gives 100 percent abundance and there are other examples or other situations. Now, you will see that molecular ion peak is at 114 and it shows 2 small peaks attached to 114, which will be at 1 mass unit extra and 2 mass units extra.

Now, this is actually arriving from the fact, that this pheromone has such an element, which appears in 3 isotopic concentrations in nature and that is why, the peak does not come as a single molecular ion peak, but is attached to 2 small ones, which are due to the isotopic effect and therefore, they are called isotope peaks.

So, note, that when 114 is the molecular ion peak and when 71 is deducted from it, the m by z fragment, that is obtained is 43 and that is the most stable fragment of this alarm pheromone of honey bee. Why? Because it, all the stability rules of this particular moiety holds good and makes it a very stable fragment. Note, that 43 is the mass of the radical, which is most stabilized.

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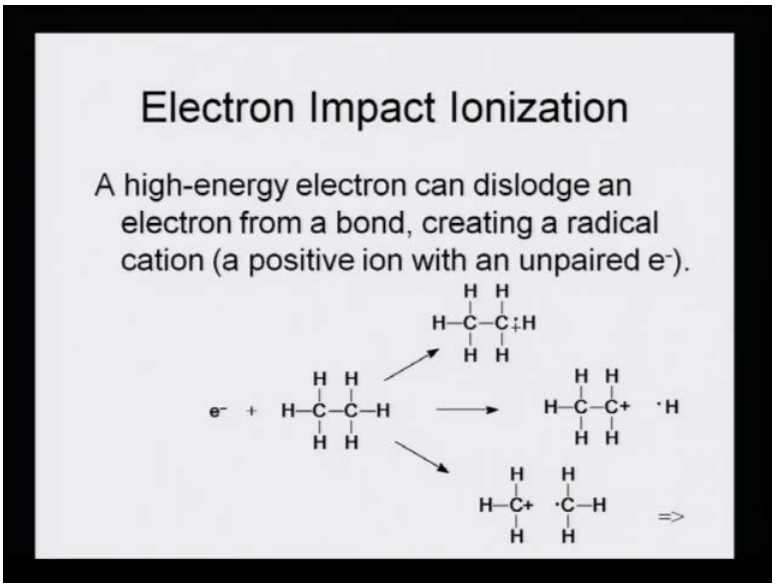

Now, electron impact ionization - a high-energy electron can dislodge an electron from a bond, creating a radical cation, a positive ion with an unpaired electron. So, what is a radical cation? A radical cation is a cation having 1 electron, unpaired electron, and that is what is generated first and the foremost, when an electron beam impacts on the molecule. Therefore, only 1 electron is ejected out and they, it gives the molecular ion peak.

So, if we take an example of ethane now and there is an electron impact, it will give rise to ethyl radical cation and subsequently, it will give rise to ethyl cation and therefore, the ethyl cation will then stabilize and form methyl cation and radical, methyl radical, and that will go on.

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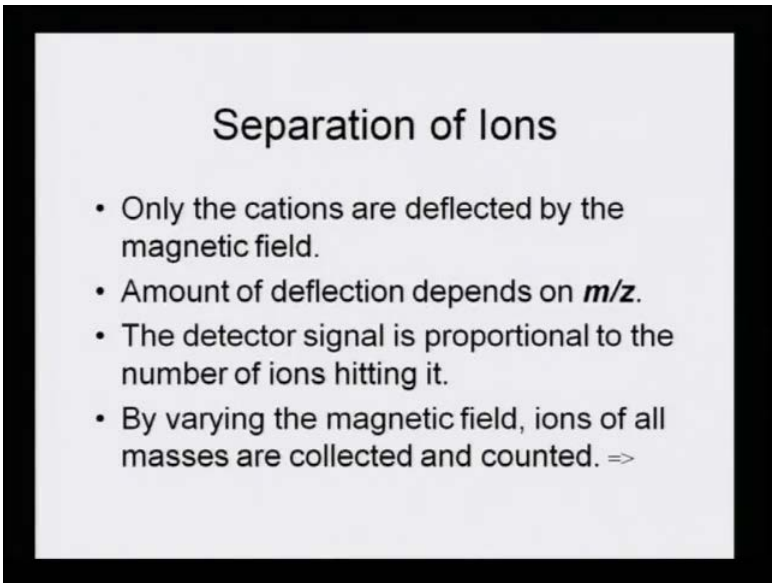

Separation of ions - now what happens, because we have been talking, that separation of ions and reaching to the detector is governed by the fact, that the mass to charge ratio, the smaller ones will reach first, then the medium ones and then the highest ones. Only the cations are deflected by the magnetic field, why? Because charged particles have a tendency to take a trajectory movement because of the electrical and the magnetic field that they create around themselves, and that helps them to move towards the detector.

The amount of deflection depends on the m by z. As I said, the smaller will first reach the detector and therefore, the medium will go after that and the highest will reach at the end. So, that discriminates the smallest particle and the amount of all those smallest particles that are generated through the fragmentation pattern, reaching the detector.

The detector signal is proportional to the number of ions hitting it. So, the detector is a very sensitive detector and any signal, which is coming is registered and it, the number of registries that are made, are actually dependent on the number of impacts that occur. So, it is proportional to the number of ions that are reaching the detector.

By varying the magnetic field, ions of all masses are collected and counted. So, what happens is that when the magnetic field is altered, the ions of all the masses gradually get detected; move towards the detector and they get collected; and they are accounted or they are numbered.

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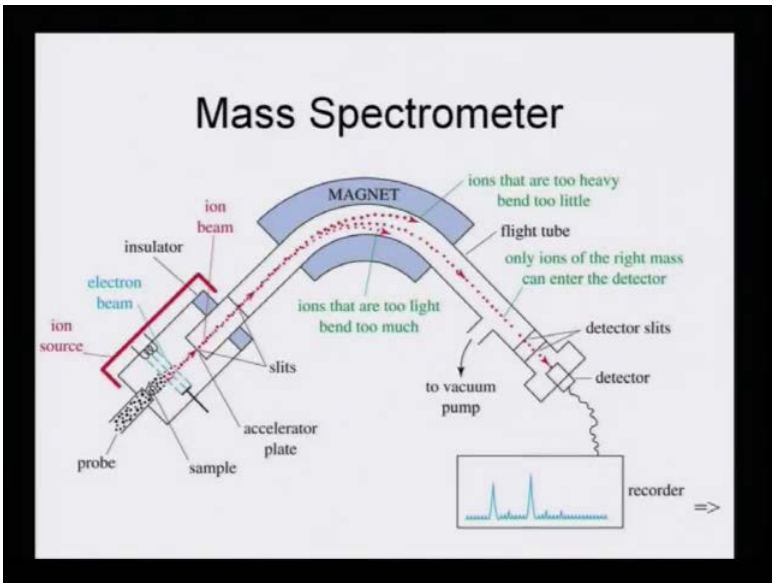

Now, this is how the mass spectrometer looks like. There is a probe, there is an ion source and here is a beam of electron, and the beam of electron is actually bombarding the ion source. Now, I must also tell you, that samples must be gaseous phase or vapor phase in order to have a very efficient impact with the electron beam. And then the electron, the ion beam, that is generated, is passed through a magnet, where it is deflected because of the electrical charge that these ions are carrying.

Ions that are too light bend too much and ions that are too heavy bend too little. So, there is a flight tube through which it passes. Only ions of the right mass enters the detector; so, if they are too light, too heavy, they will get deflected, they will not reach the detector properly. Only the ions with the right mass at a time reach the detector.

Then there are detector slits through which they enter the detector and then, they are amplified and the recorder then, actually, records the spectrum. Now, the whole system has to be under very high vacuum otherwise this deflection will not take place in such a precile manner.

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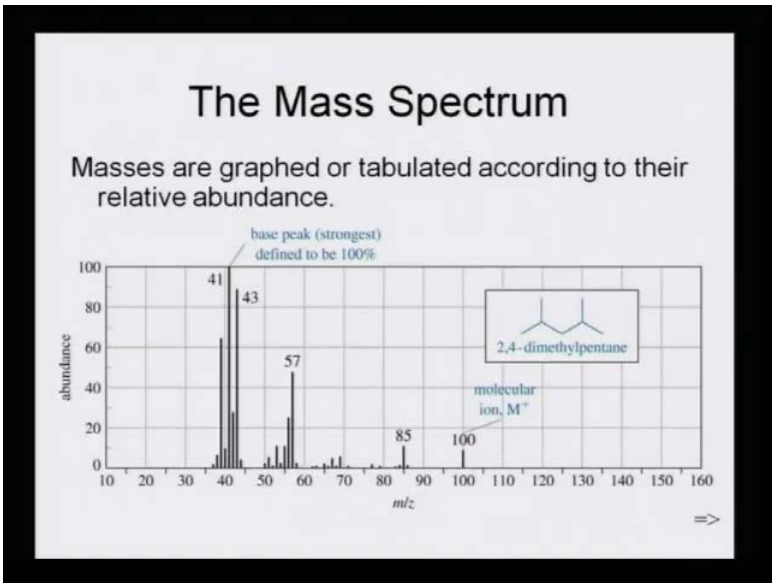

A typical mass spectrum, you will see, that is first and the foremost that you see in a spectrum of mass spectrometry is that it is a line spectrum. Masses are graphed or tabulated according to their relative abundance. 2nd thing, that you must remember is that they are appearing according to their relative abundance; relative abundance is related to the stability of that fragment.

So, the more stable the fragment, the higher will be the peak in the spectrum. So, when we look at the base peak or the strongest peak, almost like 100 percent, that particular fragment is expected to be more stable, then comes 57. And the molecular ion peak appears at 100, it is a, the compound is 2, 4-dimethyl pentane. 2, 4-dimethyl pentane loses 15 amu to become 85 and this is by the loss of CH 3 group.

So, one of the methyl gets chopped off and finally it gets further chopped off and further chopped off, till it comes to relative abundance highest peak of 41 and 43, and that is the more stable configuration of the fragment and therefore, it shows relative abundance as 100 percent almost.

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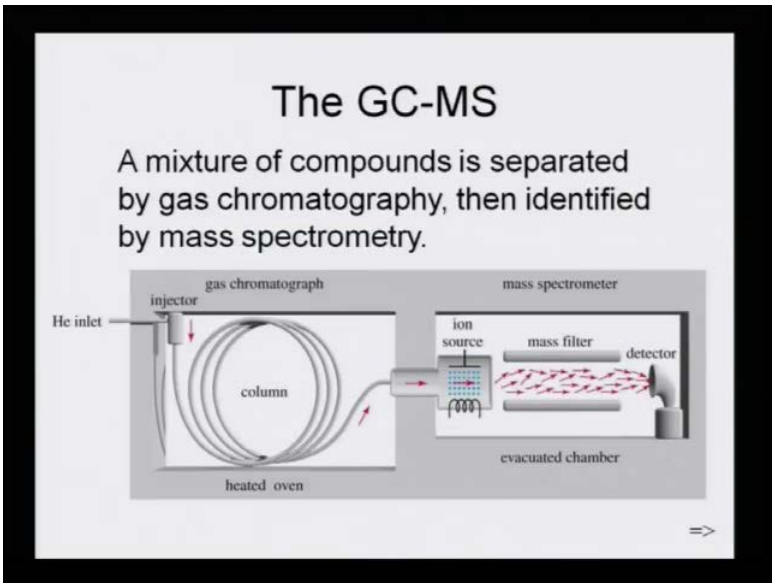

Now, we had talked about GC-MS, what exactly is the arrangement? How is the GC attached to the mass spectrometer is shown pictorially here. A mixture of compound is separated by gas chromatography, particularly the volatile compounds, then identified by mass spectrometry. And you will find that the helium is the carrier gas and it, the compound is injected through the injector in GC, and then, it goes through the column for its separation process. And at the point of interface, it is connected to the mass spectrometer and enters the ion phase. At this point, there is an electrical, there is an electron beam, which is bombarding the molecule and then through the mass filter, different sized fragments, that is, the differently m by z fragments start moving towards the detector. And as what I mentioned, that smaller ones will reach faster than the medium ones and the medium ones will reach faster than the heavier ones.

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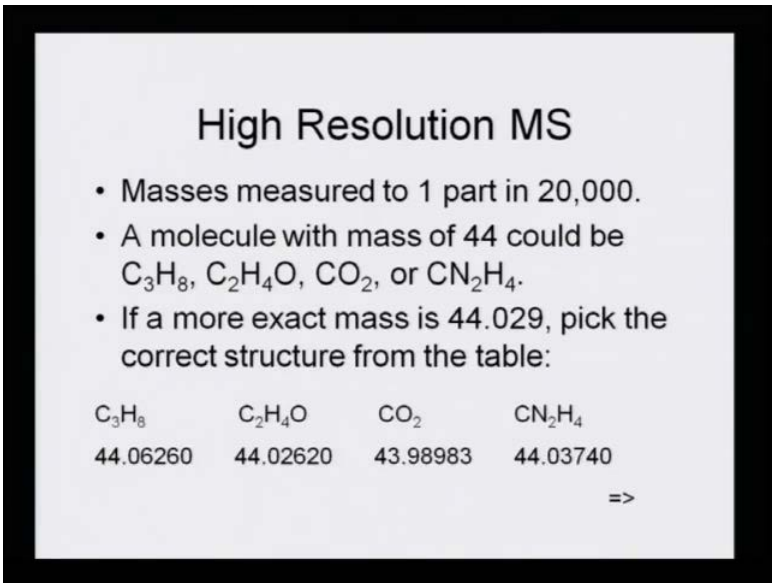

High resolution mass is also possible. Masses are measured to 1 part in 20000. A molecule with masses of 44 could be C 3 H 8, it could be C 2 H 4 O or CO 2 or CN 2 H 4. So, if there is a mass, which is appearing at 44 amu, these are the 4 or there are even more possibilities, why? Because when you make a summation of C 3, it is 36 plus 8 is 44. Similarly, if you make a summation of C 2 H 4 O it will be 24 plus 4 plus 16 and similarly, for CO 2 it will be 12 plus 32 and so on and so forth. One can add up and find that it totals up to 44 amu.

If a more exact mass is 44.029, pick the correct structure from the table. Now, you will see, that C 3 H 8, the total, the accurate up to 4 or 5 places of decimal works out to be 44.06; for C 2 H 4 O, the most accurate value comes to 44.026; similarly, for carbon dioxide, it works out to be 43.9898 and for CN 2 H 4, it works out to be 44.037. So, obviously, it is the molecule closest to the C 2 H 4 O.

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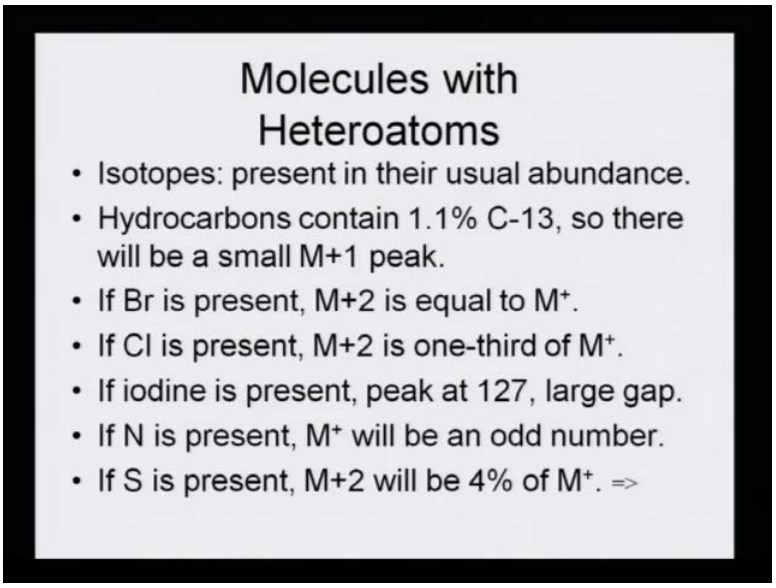

Molecules with heteroatoms - now the isotopes, I just mentioned you a while ago, will also contribute and will be shown on the mass spectrum. One cannot escape seeing the isotopes if they are present in substantial quantity. Isotopes present in their usual abundance. Hydrocarbons contain 1.1 percent of C-13, so there will be a very small M plus 1 peak, which will be almost negligible, like 1 percent of carbon-13.

We have already seen the relative intensities of these 2 isotopes, where we are doing NMR spectroscopy and therefore, for hydrocarbons this M plus 1 peak will be almost non-traceable. Nevertheless, it will appear as a small noise, but if Br is present, the M plus 2 is almost equal to M because the isotopes are almost equivalent. Similarly, for chlorine, the M plus 2 is almost 1-3rd of the molecular ion peak.

So, one has to remember, that in the case of Br and chlorine, that is, bromide and chloride, one cannot neglect the isotopic peaks and they are characteristic. Also, it is an additional or an added advantage, that if the compound has a bromine atom in the molecule or if the compound has a chlorine atom in the molecule, the mass spectrum will definitely reflect these in the molecular ion peak and subsequently, in the daughter fragments also, till these groups are completely eliminated from the fragment.

If iodine is present, peak at 127, large gaps will be obtained and if nitrogen is present M plus will be an odd number. So, these are certain very, very important and very characteristics of mass spectrum.

So, therefore, it is also diagnostic to be able to identify, that the compound has a bromine group or a chlorine group or an iodine group and so much so, that if contains nitrogen, the M plus will always be an odd number; if sulfur is present, M plus 2 will be almost 4 percent of the molecular ion peak.

So, you see that whether we have heteroatoms, which are of the halogen series or whether we have heteroatoms like nitrogen and sulfur, the molecular ion peak shows some kind of special characteristic and therefore, one can find another line or it will be an odd number as mentioned to you.

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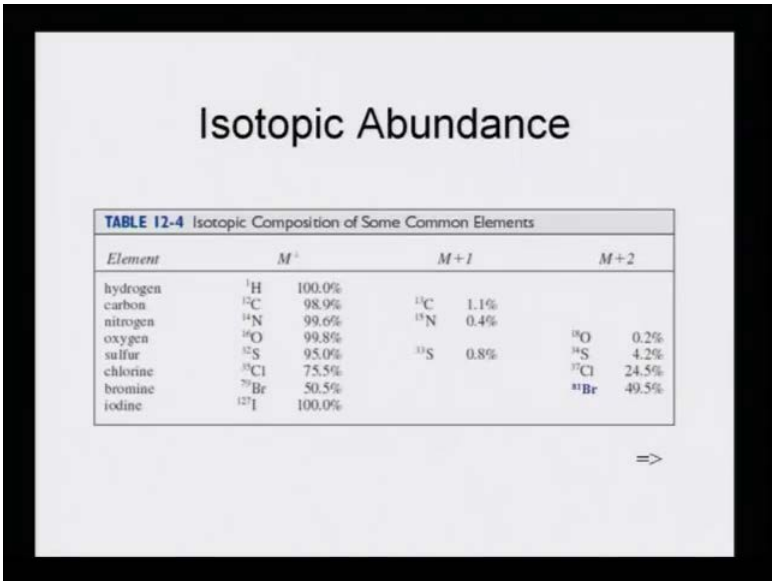

Now, when we try to look at the isotopic abundance of some elements, it is seen, that hydrogen does not show any relative abundances. The M ion is only 1 because the M plus 1 and the M 2 are in very trace quantities, that is, deuterium and tritium.

Hydrogen exists mainly as the proton; carbon has a relative abundance of C-12, 98.9 percent and C-13 as 1.1 percent. Similarly, nitrogen-14 is 99.6 percent and N-15 is 0.4 percent; oxygen-16 is 99.8 percent, it does not have another isotope; sulfur-32 is 95 percent and sulfur-33 is 0.8 percent and there is a third isotope, which is 4.2 percent, sulfur-34; chlorine-35 is 75.5 percent and chlorine-37, M plus 2 is 24.5 percent; similarly, bromine-79 is 50.5 percent and bromine M plus 2-81 is 49.5 percent, that is why, 2 equal size peaks will be seen for bromine. Iodine is only 1 isotope that one will find, that it has 127 as 100 percent.

Therefore, these give an idea, that if in a mass spectrum, if we find these kinds of small peaks appearing with the molecular ion peak, one can expect the presence of these heteroatoms.

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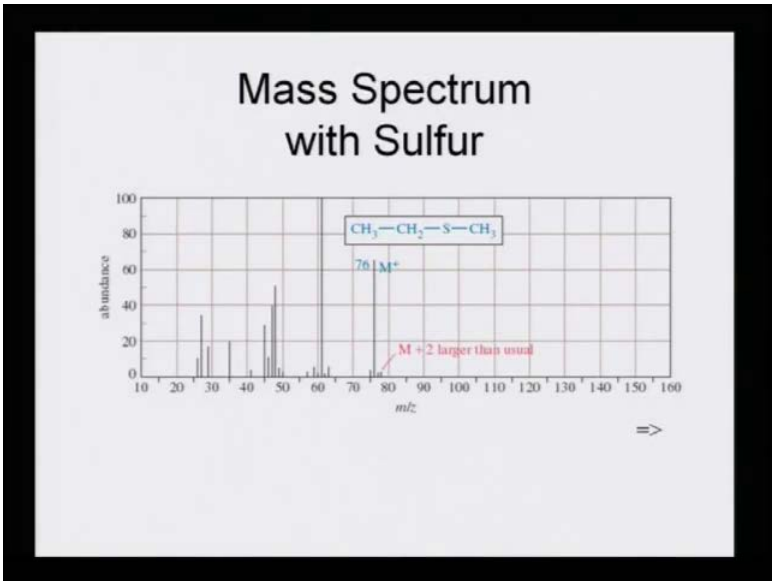

Now, we have taken a typical example of a compound containing sulfur. You will see, that it is a sulphide, it is an ethyl, methyl sulphide and you will find, that the M plus 2 appears fairly and it is very apparent because the molecular ion peak, which is appearing at 76, which is remark by the M plus sign, shows 2 significant small lines at M plus 2, large enough to be noticed.

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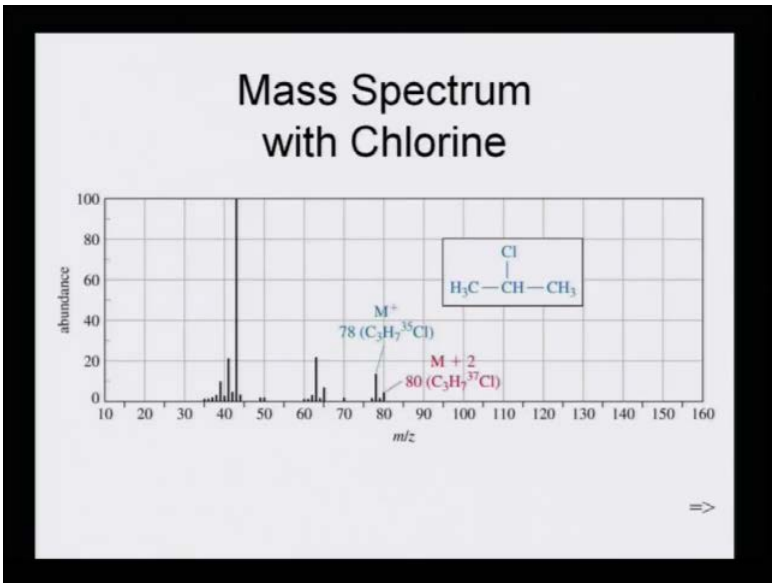

Similarly, if we try to look at the mass spectrum with chlorine, we will find, that a chloride which is, dimethyl pentane, sorry, dimethyl propene chloride, the molecular ion peak is at 78, but at M plus 2 there is a peak, which is appearing almost 1-3rd the size of the M plus. So, therefore, it is rightly so, that the difference between the M and the M plus 2 is in the ratio of 3 is to 1 and if they also appear in relative abundance of their isotopic concentration.

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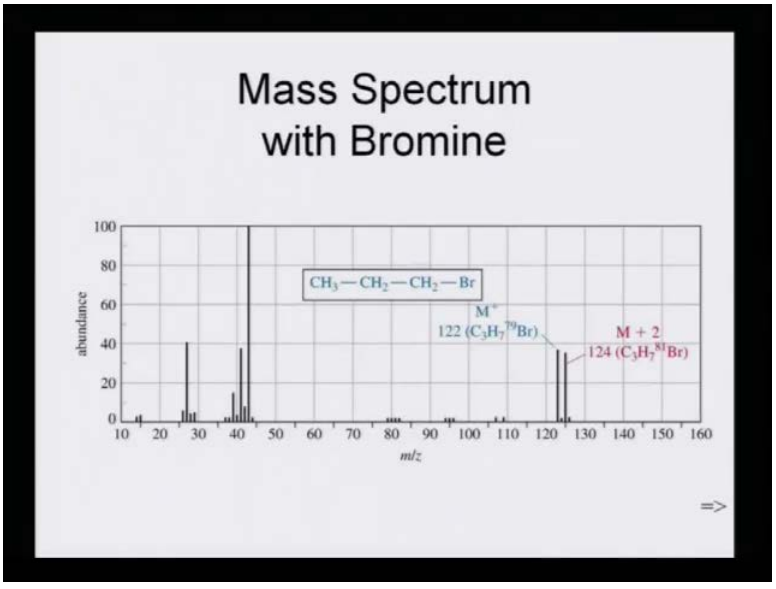

So, if we try to look at the mass spectrum of a bromide molecule with molecule having bromine, we should expect the molecular ion peak and the M plus 2 almost equivalent, and rightly so, this compound CH 3 CH 2 CH 2 Br shows a molecular ion peak at 122, and there is a M plus 2 almost of the equivalent height, why? Because the relative abundance of the 2 isotope is 50.5 and 49.5.

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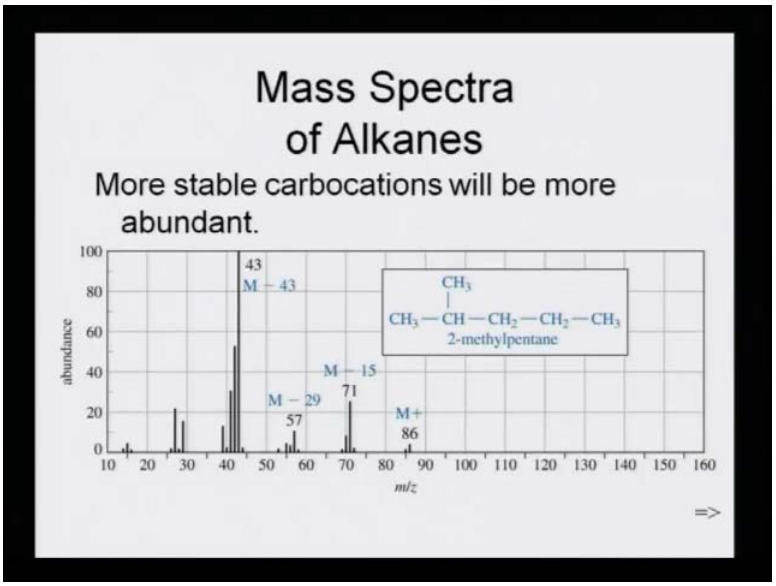

Then, when we try to look at the mass spectra of alkane, more stable carbocations will be more abundant. I just explained to you, that all the rules of stability will be applicable for these cationic fragments and so, whatever you have learnt as the stabilizing factor for any carbocation, will be applicable even when we are studying mass spectrum. And therefore, the relative abundance of that particular carbocation will be highest because it has the stabilizing factor and therefore, does not lose the charge very easily.

When we try to look at the molecule 2-methylpentane, you will see, that the first fragmentation that takes place, the molecular ion peak is at 86 and the first fragmentation that takes place is M minus 15, why? Because the methyl group gets chopped off and that is the most labile bond, that will take place to be broken off and then, subsequently, there is a loss of 29, which is CH 3 CH 2 and so the, the, the, the derivative from the 86 molecular ion peak, the most stable fragment will be 43. So, this is how the rule of stabilization of carbocation are truly applicable in the case of these fragments.

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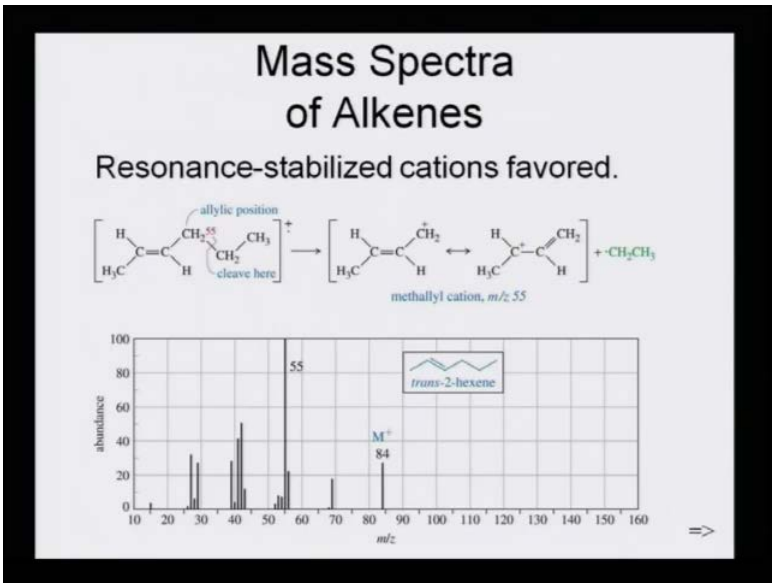

Mass spectra of alkane - they are stabilized by resonance, stabilized cations are more favored and therefore, when there is an allylic position, there is CH 2, that will break and forms the allyl cation rather, because the positive charge on the CH 2 can be easily, it is in conjugation with the electrons of the double bond and therefore, it is most stable cation.

And when we try to look at the example of trans 2 hexene, the molecular ion peak appears at 84, which is M plus and then, the subsequent loss is by the loss of ethyl group, which is then giving a very relatively high, relative abundance of 55 is 100 percent, which means, that there is an additional stabilizing factor and the stabilizing factor for this kind of allylic cations is due to resonance.

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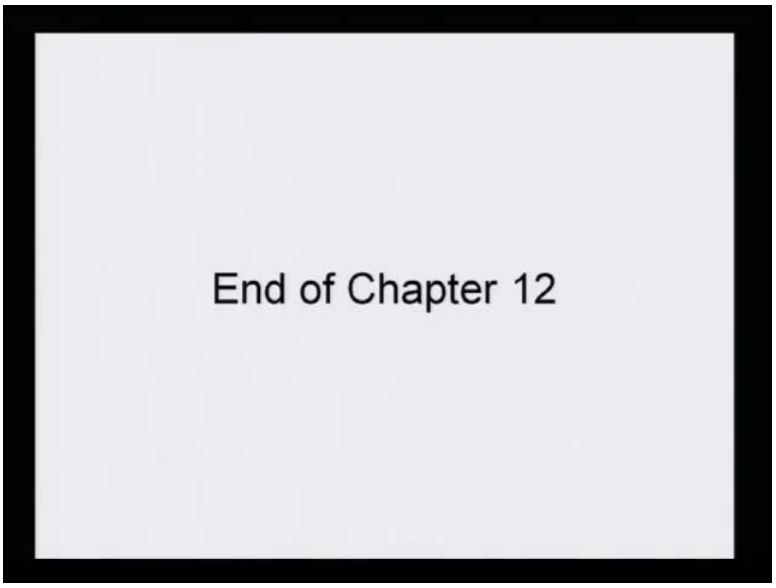

So, with this, we have come to an end of the mass spectroscopic method. Nevertheless, the story does not end here. Several, several compounds can be analyzed by this mass spectrometric method.

I will try to take you to another course of quickly going through an array of compounds, which will give you an insight about how this mass spectrometric analysis can be extended to proteomics.

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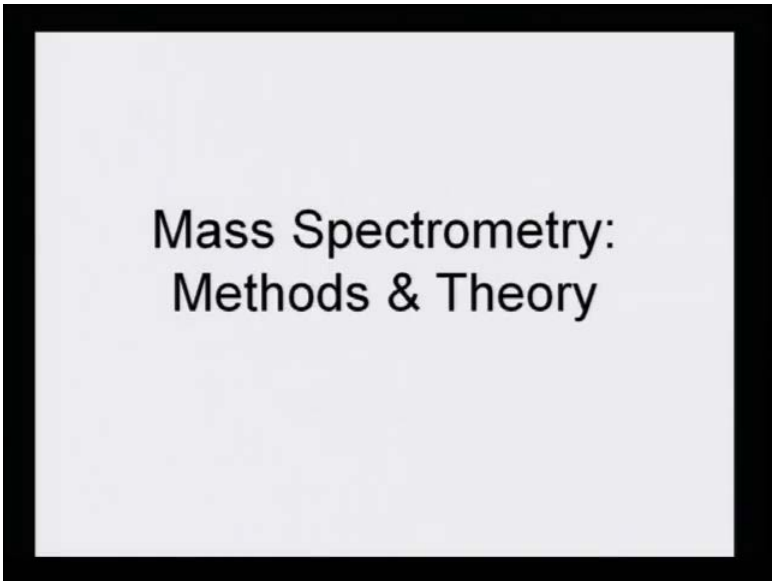

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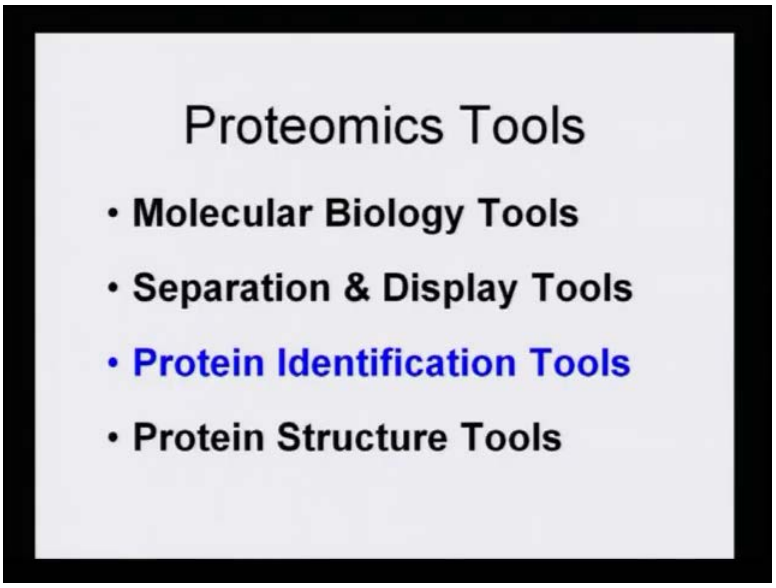

If we try to quickly look at the mass spectrometric methods and theory, you will find, that these can be even extended very extensively to proteomics tools, molecular biology tools, separation and display tools, protein identification tools and protein structure tools.

So, it is not that only organic compounds can be analyzed by this method. Very, very intricate, very large macro molecules, like proteins, peptides also can be analyzed with lot of great specification. Mass spectrometry of course, here needs little bit of manipulation because now, the task is more difficult.

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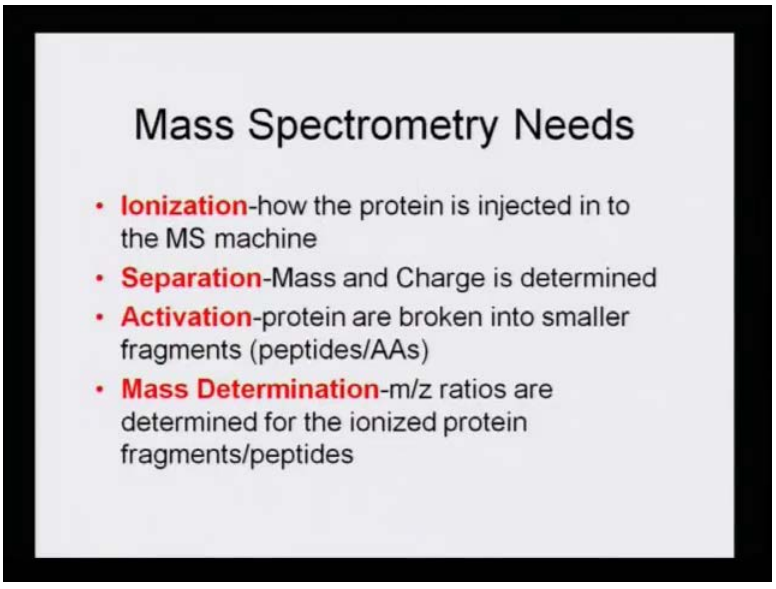

Ionization - how the protein is injected in the MS machine. Because as I was telling, organic compounds are transferred to the machine mass spectrometer by converting the compound in to the gaseous state, that is, the vapor phase, but how can a protein then be transferred, that is a big challenge. Then, how does the separation take place? The mass and charge, how are they determined on the protein molecule?

Activation - protein are broken into smaller fragments, that is, they are broken down into peptides and subsequently, these peptides are then broken down into amino acids. Therefore, how does the mass determination really take place? m by z ratios are determined for the ionized protein fragments and peptides. So, still they are very large fragments, but they are able to identify because they are ionized in their molecular state.

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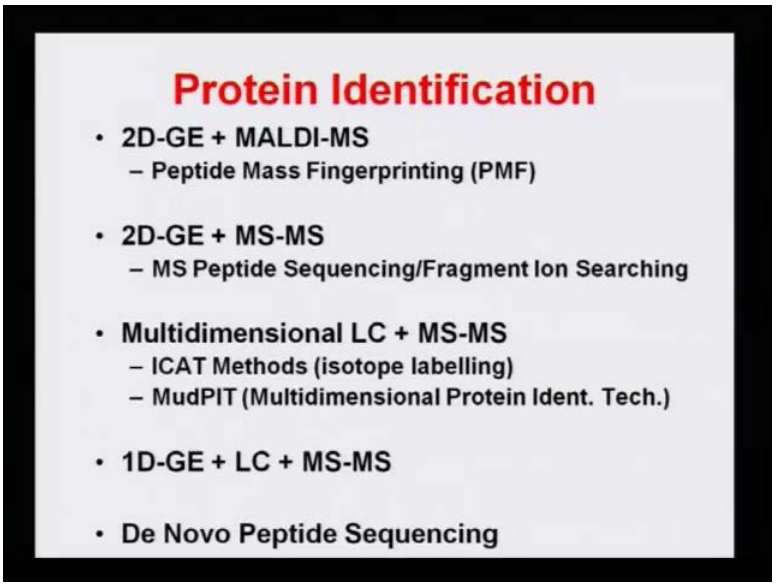

Several methods have been identified and developed for protein identification. The MALDI-S, that means, the 2D-GE plus MALDI-MS is actually used for peptide mass fingerprinting. Then, another method that is used, where MS and MS, I had mentioned a while ago, are coupled for peptide sequencing fragment ion searching. Multidimensional LC, when it is coupled with MS and MS - it is able to do a more intricate study, and De Novo peptide sequencing is possible.

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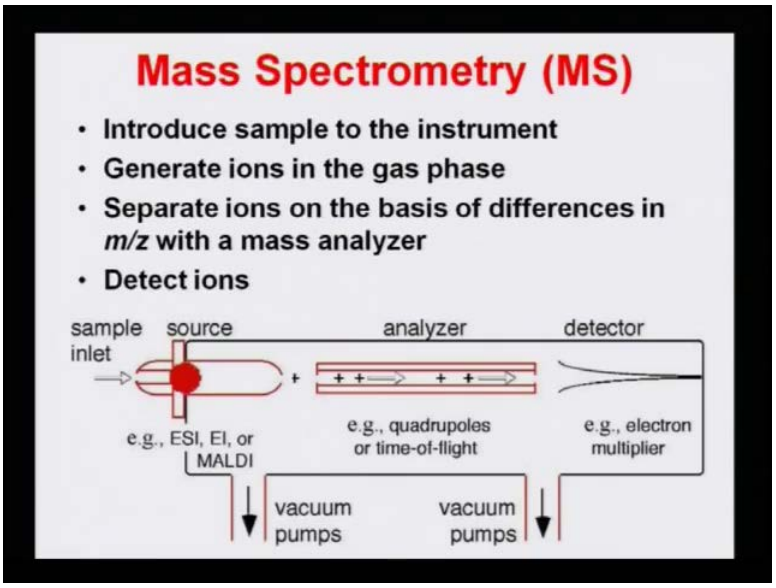

Mass spectrometer remains the same. Introduce sample to the instrument, generate ion in gas phase, separate ions on the basis of the differences in m by z with a mass analyzer and this is how, thus, the, you know, the breaking up or the ionization should be done either by ESI method or EI method, or MALDI method or FAB method, these are the only possible methods. And then, the ions are made to pass through an analyzer, which could be a quadrupole, which could be time of flight, which could be ion trap and subsequently, it reaches the detector where the information is then passed on to the recorder. The entire system, as what you know, must be under very high vacuum.

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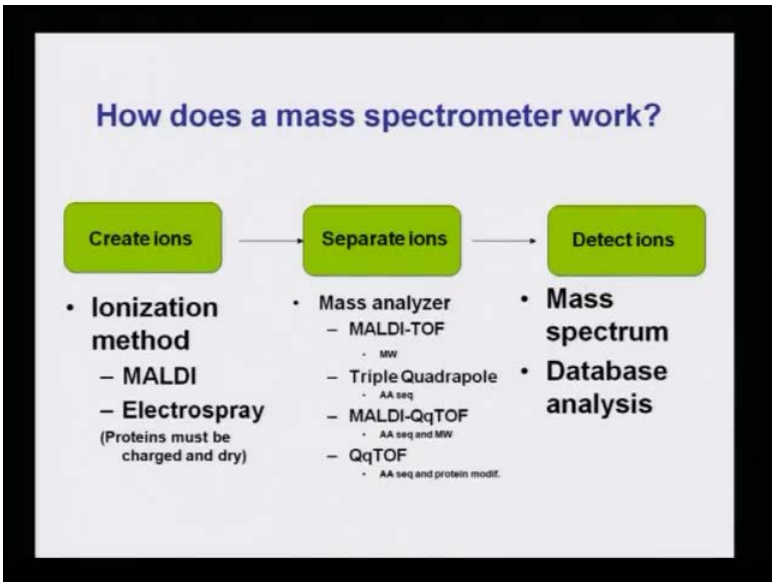

How does mass spectrometer work? We have already looked at it, but will take a quick look to have a recap. Ions have to be created and for creation of ions from a neutral molecule, the substance must be first changed into vapor phase or gaseous phase and then, the ionization methods could be for, particularly for protein only. 2 soft methods are recommended - one is the MALDI, that is, matrix assisted laser desorption ionization or electrospray ionization.

Then separation of these ions can be done by using mass analyzers, that is, MALDI time of flight or triple quadrupole or MALDI quadrupole and TOF put together, and several types of analyzers may be used for a particular purpose. If only the molecular weight has to be determined, MALDI and time of flight analyzer is good enough, but if the amino acid sequencing has to be done, then triple quadrupole is what is the basic requirement. Similarly, if the atom, if the amino acid sequencing, as well as, molecular weight has to be calculated, then MALDI with a quadrupole and time of flight analyzers must be used.

And then, finally, they are detected by the detector; mass spectrum is created and then, data base is also generated and kept for further analysis.

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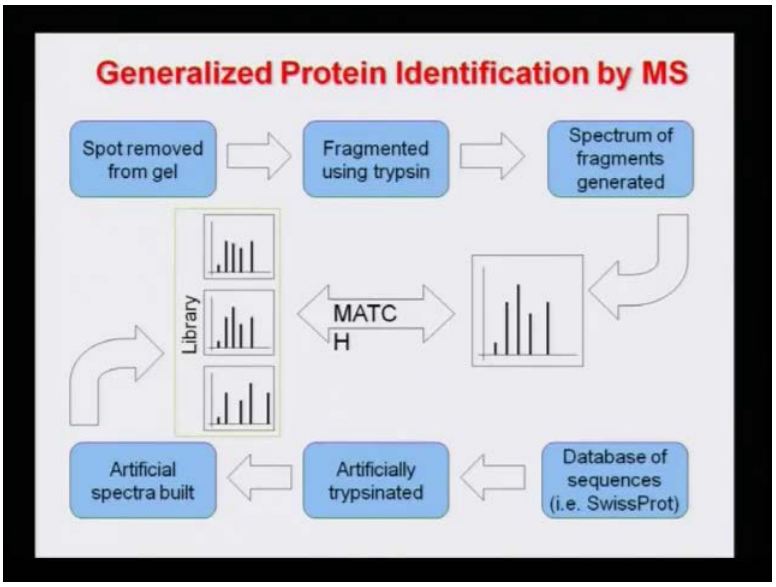

Generalized protein identification by MS - so one can make a lot of adjustments to do this complicated protein molecule analysis and therefore, one has to make certain adaptation. So, it is spotted on the, from the gel fragment, fragmented using trypsin and then, the spectrum of the fragments are generated and furthermore, they are then matched

with the library and therefore, it is seen, whether the sequence is matching with any of the known peptide sequences or whether it is a completely new kind of molecule.

So, you see, that I tried to give you an overview of the entire use of mass spectrometry including the organic molecules, the simplified molecules and even the toughest of the tough biological molecules, like peptides, which, and proteins, which are very intricate system of huge number of amino acids and monomers. So, one can appreciate all these spectroscopic methods in a very fine manner and as, what I will repeat once again, that no single method is a full proof method. It is with the help of all these spectroscopic method, that one can determine the structure of any compound, whether be it organic compound or biological sample.