Proteomics: Principles and Techniques
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Lecture No. # 20
Fundamentals of Mass Spectrometry

Welcome to the proteomics course.In today's lecture, we will talk about fundamentals of mass spectrometry. So, let us switch grades on the previous module on gel based proteomics, and now switch on to mass spectrometry and its various types of applications.

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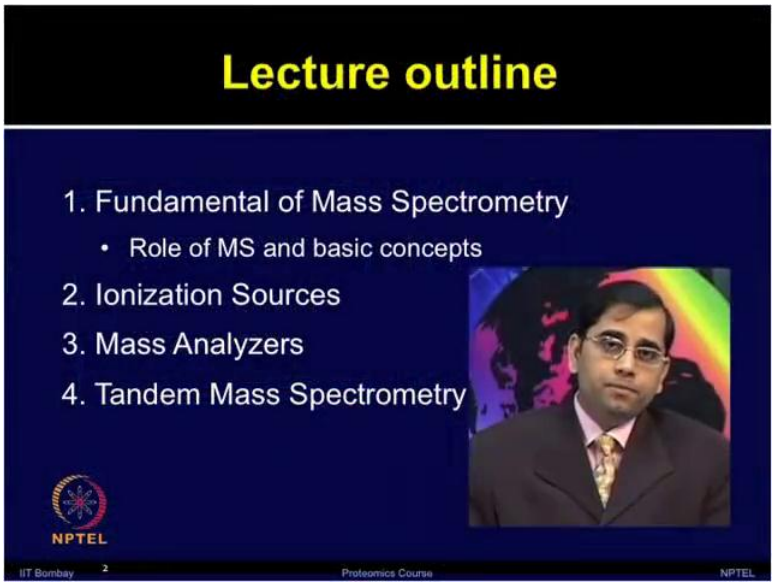

So, today first we will talk aboutfundamentals of mass spectrometry, I will describe you role of MS, and various basic concepts involved in understanding the mass spectrometry. We will look at the individual componentssuch as ionization source, mass analyzers, as well as other components, then,we will talk about tandem mass spectrometry. So, in the previous module we talked about gel based proteomics, the gel based proteomic techniques resolved several hundred proteins. However, the scale at which we want to study the proteome requires much more analytical instrument capability, and mass spectrometry has ability to provide that platform for comprehensive coverage of proteome. MS has become an important analytical toolin biology in general, and proteomics during the last decade, and now various applications have emerged out by

using MS based platform. It offers high throughput, sensitive and specific analysis for many applications.

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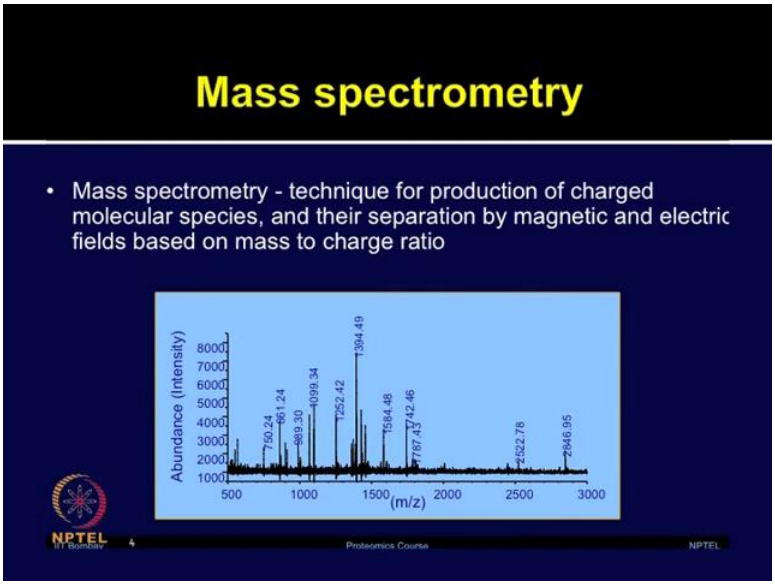

Let us first look at some of the basic concepts of mass spectrometry. So, first of all what is mass spectrometry? It is an analytical technique to measure the molecular mass of individual compounds, and atoms accurately by converting them into the charged ions. So, by definition this is a technique for production of charged molecular species in vacuum, and there separation by magnetic and electrical fields based on mass to charge ratio. You can see the MS spectrum shown in the slide M by Z, and intensity abundance plotted on the y axis. So, what are the unique features of mass spectrometry molecular specificity?

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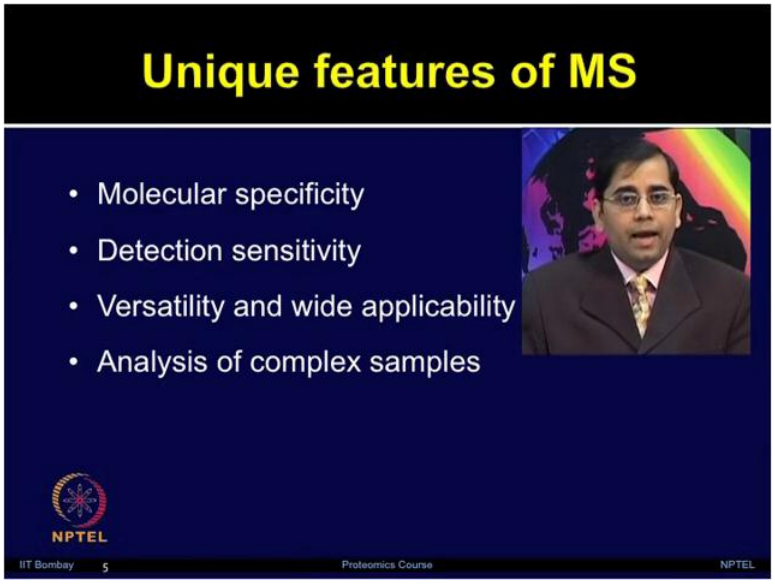

Due to its unique ability to accurately measure molecular mass, and provide fragment ions of analyte mass spectrometry offers molecular specificity, it provides ultra high detection sensitivity. So, in full MS can detecteven a single molecule, and even sensitivity at the atomole and zeta mole has been also demonstrated. It provides a versatile platform to determine thestructure of compounds, and it is applicable to all the elements, all type of samples: whether it is volatile, non volatile, polar, non polar, as well as solid, liquid or gaseous. So, analysis of complex samplessuch asproteome is very much possible by using MS.

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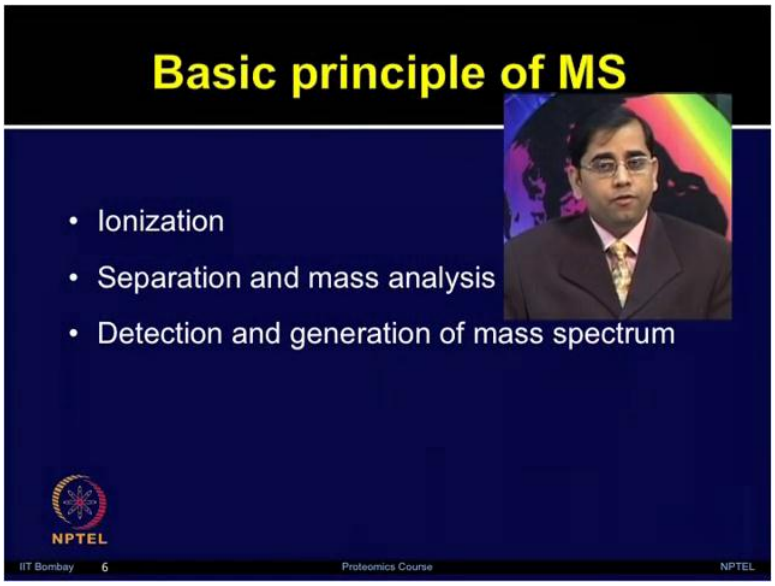

And what is the basic principle of mass spectrometry? So, first step is ionization, to convert analyte molecules or atoms into the gas phase ionic species, it removes or adds electrons or protons. The second step is separation, and mass analysis of molecular ions and charge fragments on the basis of mass to charge ratio. The final step is detection and generation of mass spectrum, these are the main steps involved in the mass spectrometry operation.

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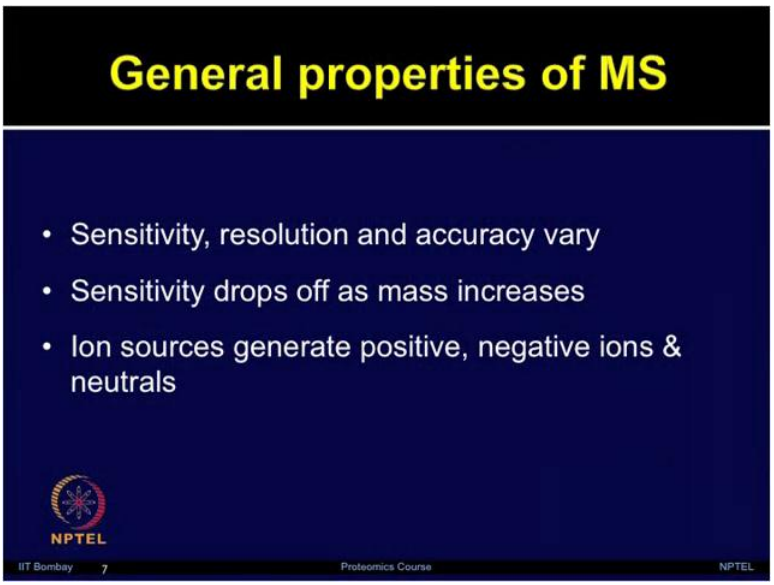

Now, let us discuss about general properties of MS, with sensitivity, resolution and accuracy. They vary among various mass spectrometers, the sensitivity drops off as a mass increases, and as I mentioned the sensitivity for protein detection can be as low as in the attomolar or femtomolar range. Ion sources they generate positive, negative and neutral. The neutral or basic cannot be focused, or accelerated by the ion optics. So, one can analyzeeitherpositive or negative ions, the positive ions have an adductwhich is typically a proton, and sensitivity for negative ions generally lower.

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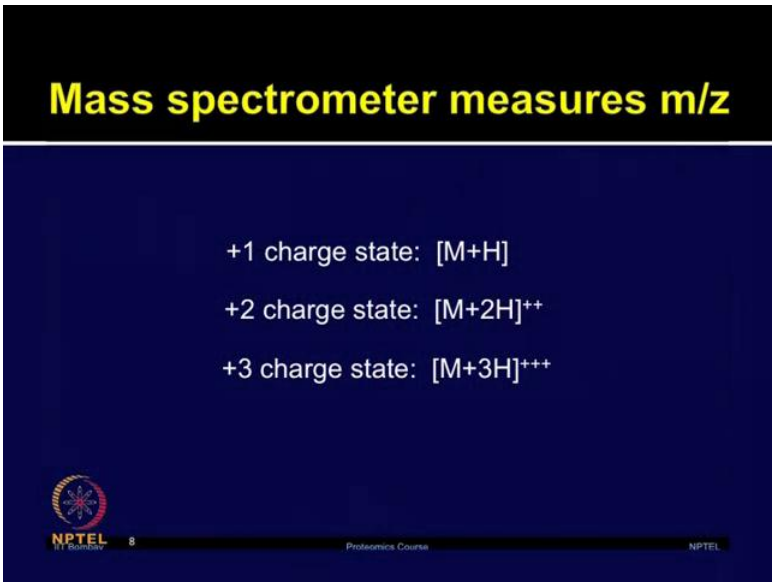

So, mass spectrometer measures M by Z, the MS data is presented as mass to charge ratio which is mass of an ion m divided by the number of charges Z it carries, so total charge on ion is represented by Q equals to Z E, where e is the charge on an electron.

Now, how you can calculate M by Z of any peptide? So, as I mentioned here you can have multiple charge states, plus one charge state, plus two, plus three, or multiple charge states are possible. So, if you need to calculate the M by Z, you need to add M plus H, or M plus 2 H, or M plus 3H as shown here, and then divide by 2 or 3 depending on the how many charge states carries

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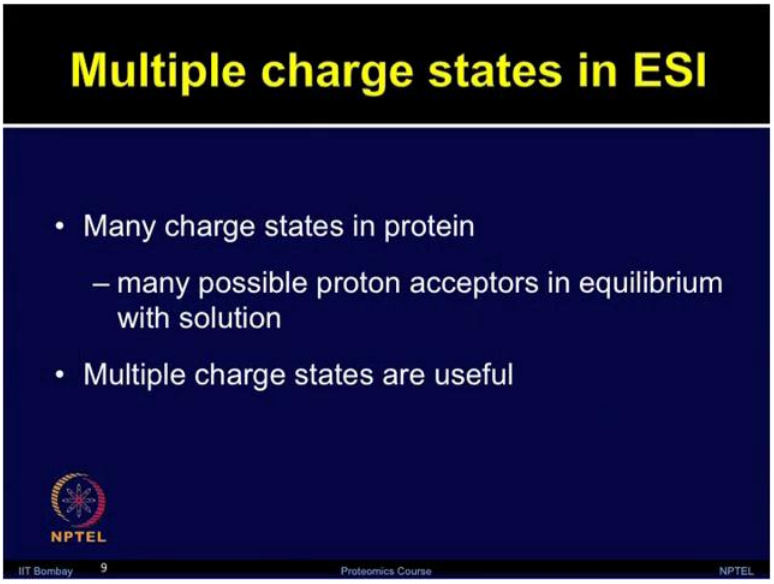

I will talk in more detailabout how various types of ionization source works, that will be much later part of the lecture, but just in this context I would like to mention that there are multiple charge states present in the electrodes preionization.

So, many charge states in proteinsthere many possible proton acceptors in the equilibrium with the solution, so the multiple charge states are quite useful, because they forms ions which are in the mass range of mass analyzers, such as to quadrupoles ion traps, etcetera. Now, during the initial part we are trying to cover some of the basic terminology, and basic concepts involved in the mass spectrometry.

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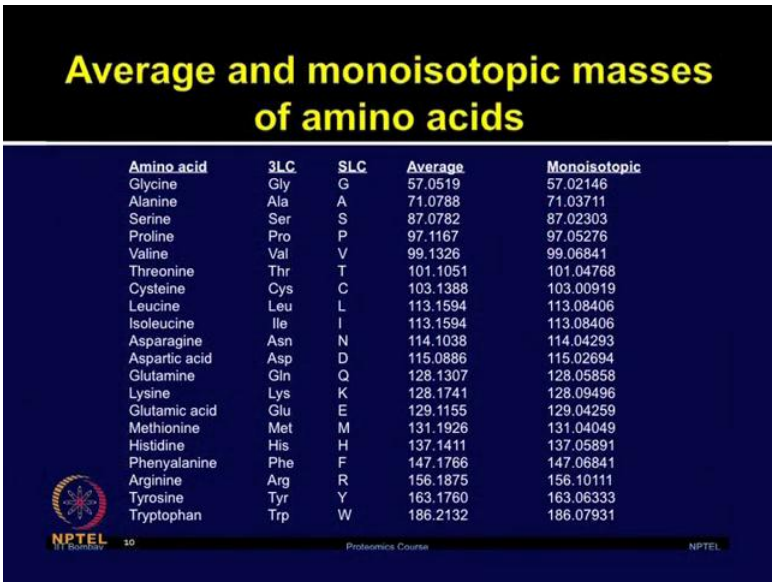

So,let us talk about what is average and monoisotopic mass of amino acids? I have shown you a table here, which showsamino acids 3 letter codes, single letter code, average and the monoisotopic masses, you can use this for your reference later on, which can be used for the data analysis in the calculations. So, what is monoisotopic mass of a protein? It is sum of masses for most abundant isotope of each element, the average mass of an element is a average of the isotopic masses of each element waited for theisotopic abundance. I hope you are able to distinguish the average and monoisotopic masses of amino acid.

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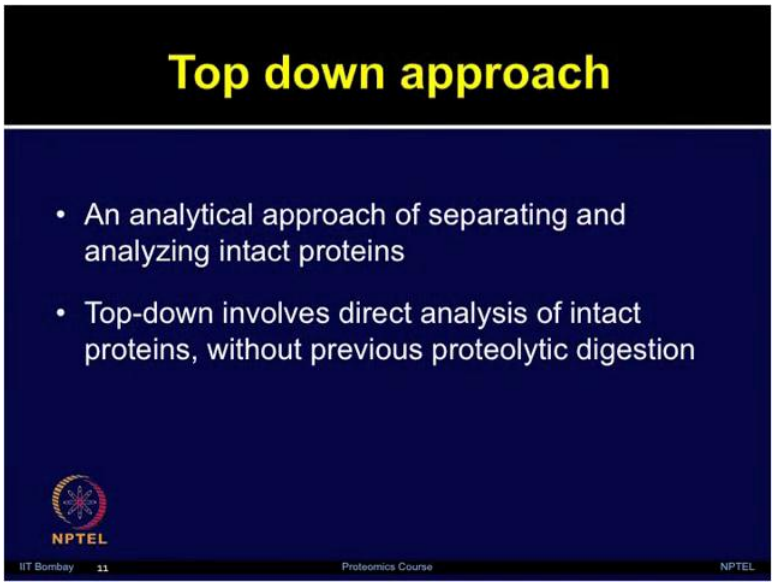

Now, by employing mass spec different type ofapproaches people use in the proteomics, top-downand bottom-up. So, what is top-down approach? Top-down is analytical approach of separating and analyzing intact proteins without any previous proteolytic digestion. So, let me give you brief idea about the procedure, what are the steps involved in top-down approach.So, proteins are first introduced in to MS directly, because we are not doing the in-gel digestion or tryptic cleavage, proteins are broken into fragments inside the mass spectrometer, the fragments are analyzed by either MS or MS MS, and then proteins can be identified using databases.

So, advantage of the top-down approach is that it retains information on protein isoforms, sequence and modifications. Where as, the disadvantages that sensitivity and proteome coverage is very limited.

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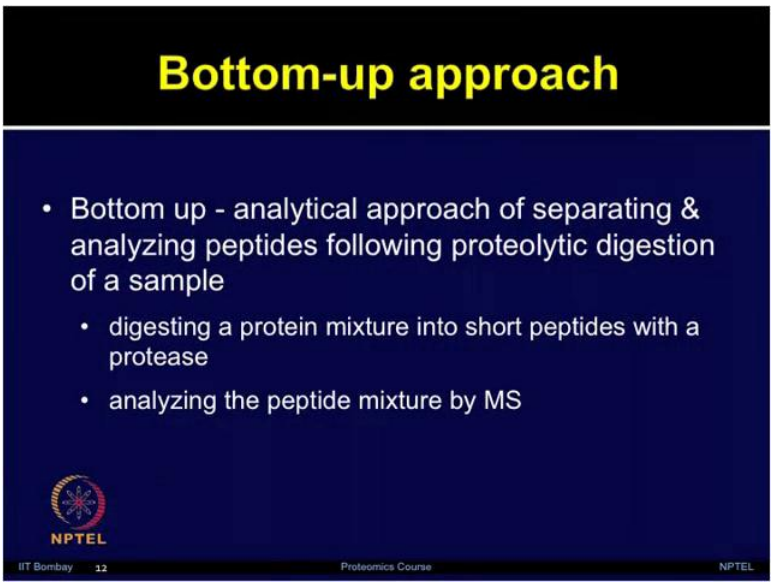

So, let us now discuss what is bottom-up approach? This is an analytical approach of separating, and analyzing peptides following the proteolytic digestion of sample. So, let me give you what are the various steps involved in doing the bottom-up approach?

So, first of all proteins are digested by using trypsin or some other proteolytic enzymes, peptides are separated by using liquid chromatography and introduced inside the mass bead, peptide fragment inside the MS, and proteins can be identified by using databases. The advantage of this approach is that one can obtain more sensitivity, and higher

proteome coverage. The disadvantage includes, the information about the protein isoforms is lost, and also it is unable to distinguish very close sequence homologous.

So, both top-down and bottom-up approaches have their own advantages and disadvantages. Now, let us talk about different parts of the mass spectrometer, so major components include the sample inlet, ion source, mass analyzer, detector, signal processing components and data output.

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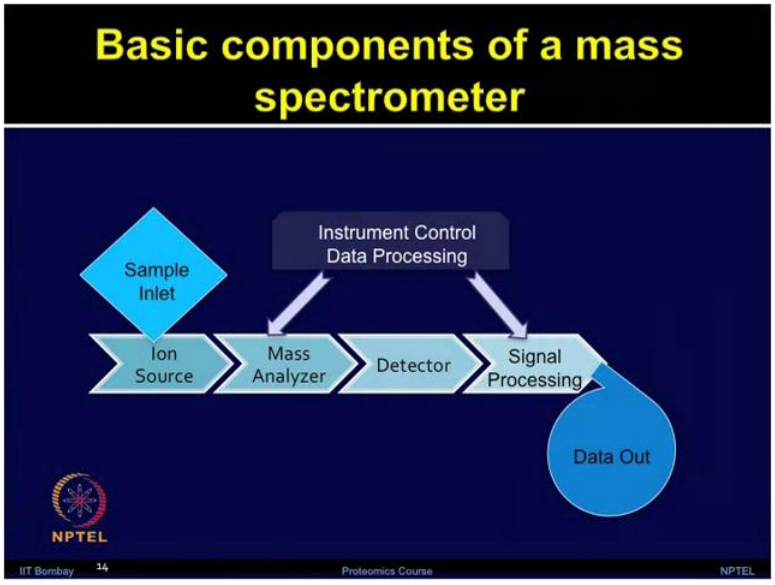

Let us look at each of these components in little more detail. Sample inlet: it transfer a sample into the ionization source, the ion source or ionization source: it converts neutral sample molecule into the gas phase ions, mass analyzers: it separates an analyses mass of the ionic species. The various type of mass analyzers available, which we will discuss in more detail during the subsequent part of the lecture. Now, one need to maintain the vacuum condition, a very low pressure is maintained inside the mass spectrometer, detected measures and amplifies the ion current of mass reserve ions, and then we need electronics to control the operation of various units. The data system: it records, processes, it stores and help to display the data output.

So, although there are three major components involved: ionization source, mass analyzer and detector, but then there are some accessory component, which are also equally important for doing the mass spectrometry based experiments. So, various

components includes sample introduction, one can couple that with HPLC or chip based technologies for doing the liquid chromatography based separations, sample ionization.

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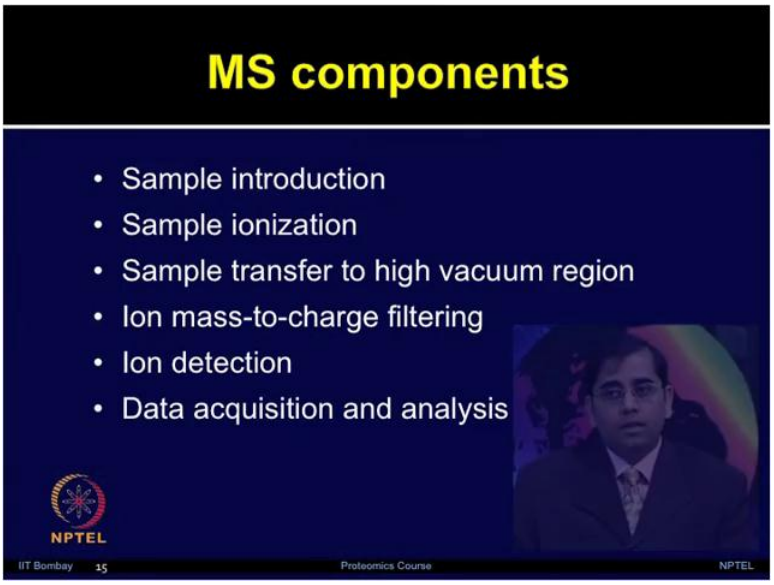

The various types of ionization sources currently available, sample transfer to the high vacuum region. So, the ion mass to charge filtering can be performed by the mass analyzers, ion detection by using detectors, and then data acquisition and analysis by using data system, all these are integral part of the MS components. The slide shows you an overview of workflow involved in performing in MS based experiment.

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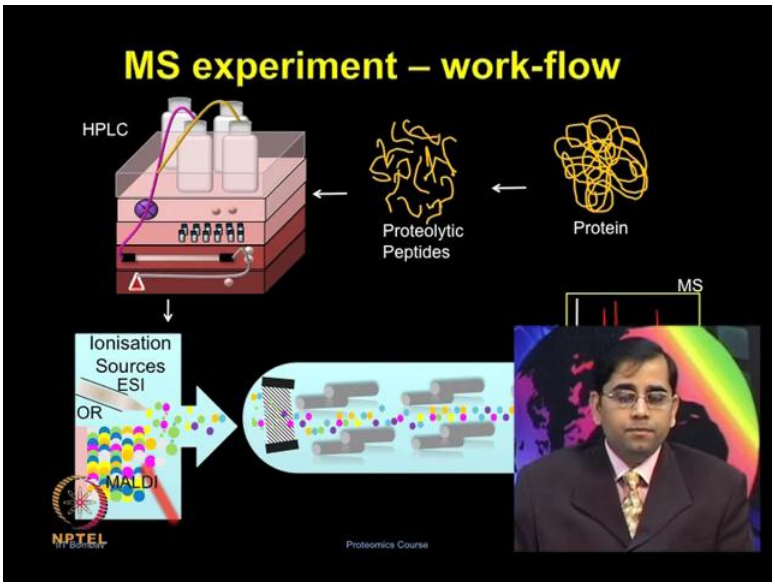

First of all, you can prefractionate your sample by using liquid chromatography, or one can also trydifferent methods of doing the prefractionation, and then applying that for doing the for their MS analysis. After prefractionation, then in-gel digestion or proteolytic cleavage can be performed by using various enzymes such as trypsin, and then the sample can be injected into the ionization source, whether it can be ESI or Maldi, most commonly used for proetomics, as well as other type of ionization sources. And then these ions can be further resolved into themass analyzers, again there are various type of mass analyzers,and then data can be analyzed in MS or MS MSmode. This slide gives you an overview of in-gel digestion process.

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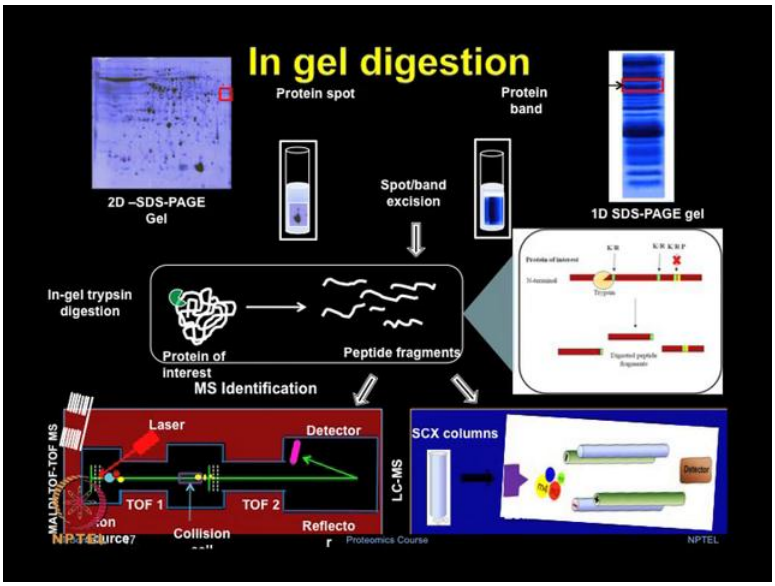

Well we talk in more detail about each of these steps, as we go along in the next lectures talking more specifically about a specific type of mass spectrometry techniques. But, today I am giving you a sort of an overview various type of concepts, and various steps involved in performing the mass spectrometry experiments. So, in-gel digestion regardless of, you have done the gel based proteomics or you want to perform the gel free proteomics. You can resolve the proteins on the gels, simplify the proteome, it lies either this spots or the bands of your interest, and then subject based to the in-gel digestion or in solution digestion, so that proteins can be cleaved into the small peptide, and these can be then further analyzed by using ionization source and mass analyzers.

So, what are different type of ionization sources involved in the MS analysis? So, success of mass spectrometry experiment lies in efficiency of converting a neutral compound to a gas phase ionic species. So, you have various type of options currently available, you can select what type of ionization source you want for your specific application. So, the choice of particular ionization source is dictated largely by the nature of sample, which one wants to investigate.

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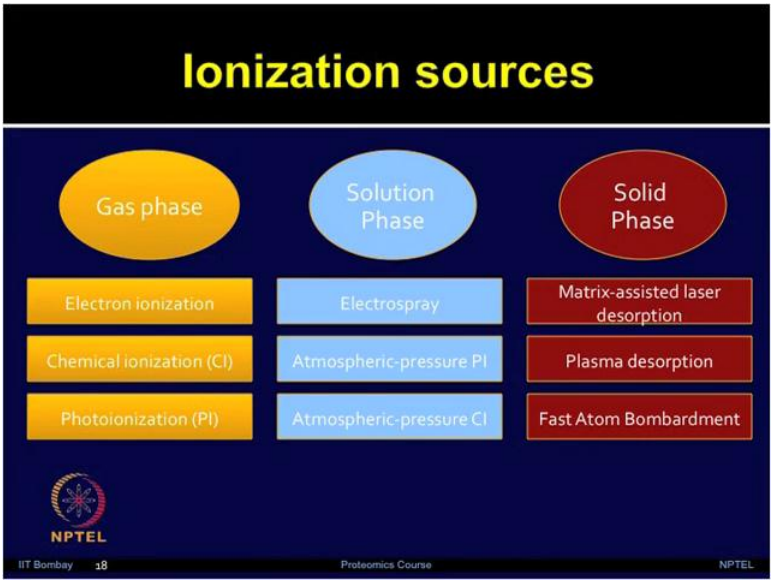

So, the gas phase: electron ionization, chemical ionization and photoionization, these are the more commonly used ionization sources. With solution phase: electrospray, atmospheric-pressure and atmospheric-pressure CI, these are the more commonly used ionization sources with solid phase: Maldi or matrix assisted laser desorption ionization, plasma desorption are the more commonly used solid phase ionization sources. The traditional ionization sources used for the small molecule chemical application, relied on the chemical or electrical ionization.

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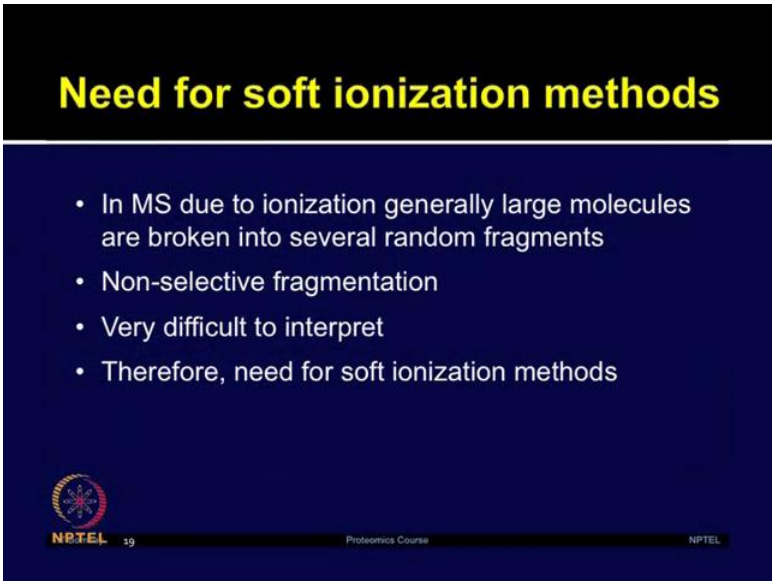

But, these processes are too energy rich to ionize intact large biomolecules, and the lead to the unpredictable analyte decomposition. So, for proteomic application there was need for the soft ionization methods in mass spectrometry, these are non-selective fragmentation, the hard ionization is very difficult to predict.So therefore, it led to the need of soft ionization methods in proteomics.

What are different properties of ionization source? The main function of an ion source is to convert sample molecules, or atoms into the gas phase ion species. Now, in the animation I will show you,two most commonly used soft ionization methods: Maldi and ESI.

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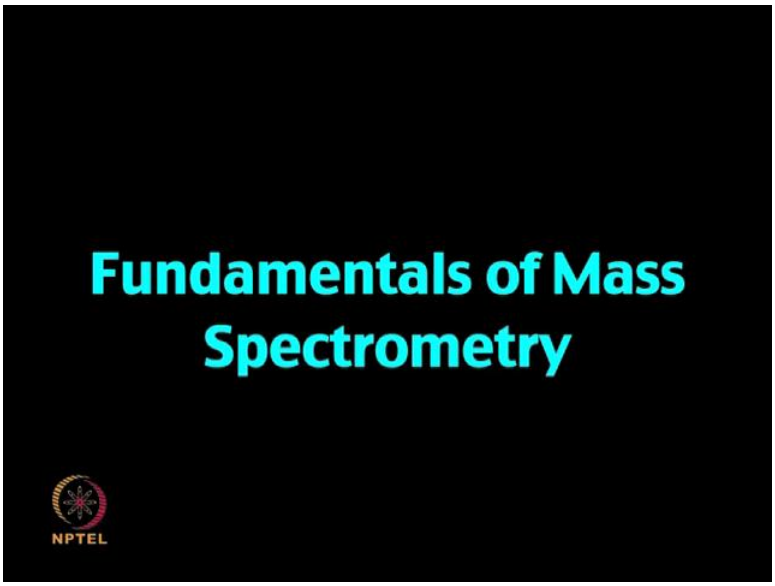

Fundamentals of mass spectrometry, mass spectrometry is the technique for protein identification, and analysis by production of charge molecular species in vaccum, and their separation by magnetic and electric fields based on mass to charge ratio. MS has increasingly become the method of choice for analysis of complex protein samples in proteomic studies, due to its ability to identify thousands of proteins.

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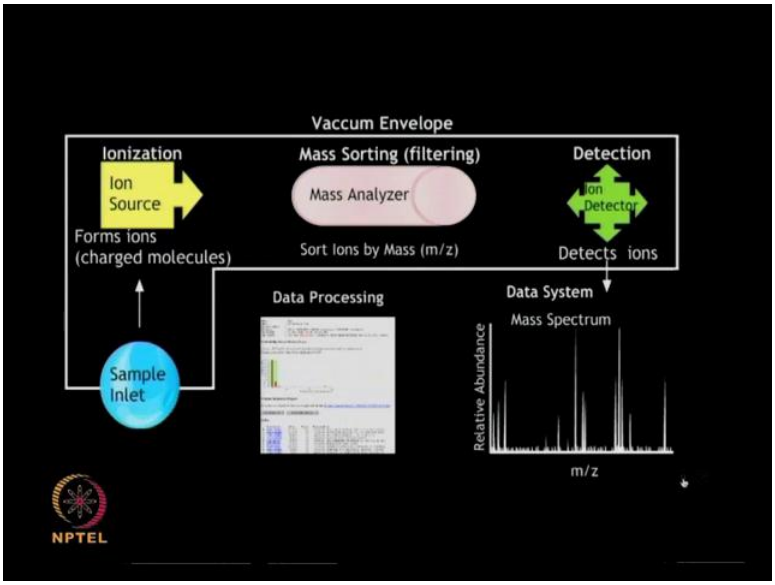

Mass spectrometer is an instrument that produces charged molecular species in vaccum, separates them by means of electric and magnetic fields, and measures the mass to

charge ratio, and relative abundance of the ions that is produced. It is being increasing used for detection and analysis of proteins from the complex samples. The various components which are involved in the mass spectrometry experiment are shown here, starting from the sample inlet, the ionization source, mass analyzer, detector, and then data analysis and data processing. Let us first define these terms, so that our understanding for each component becomes more clear, even if when we come to the advanced concepts. Sample inlet, this is first point of contact where the sample is introduced within the mass spectrometer, either as liquid nanodroplets or as a mixture with the matrix ionization source.

The ionization source is responsible for converting the analyte molecules into gas phase ions and vaccum. Ionization source enables the ionization, which can be further integrated with the mass analyzers, the technology that enables is known as the soft ionization for its ability to ionize non volatile biomolecules, while ensuring minimum fragmentation and thus easy easier interpretation.The commonly used ionization source include Maldi, matrix assisted laser desorption ionization, and ESI or electrospray ionization. Mass analyzers, the mass analyzers resolves the ions produced by the ionization source on the basis of their massed charge ratios,there are various type of mass analyzers available including time of flight, quadrupole, ion trap, etcetera. Detector, the ion detector determines the mass of ions that are resolved by the mass analyzer, and generates data which can be further analyzed, the electron multiplieris the most commonly used detection technique. Now, let us look at the function of each of these components in more detail.

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Let us first start with the ionization source, the ionization source is responsible for converting analyte molecules into gas phase ions in vaccum, this has been made possible by the development of soft ionization techniques, which ensures that the non volatile protein sample is ionized without completely fragmenting it, most commonly used ionization sources are Maldi and ESI.

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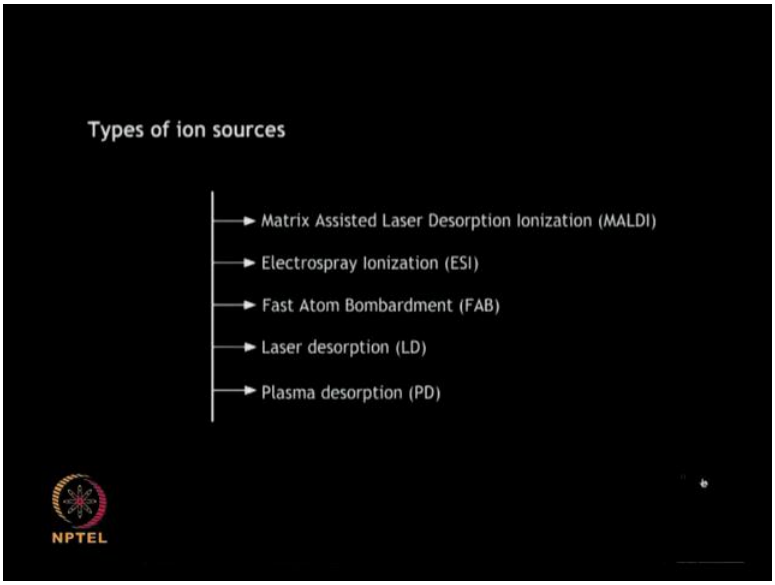

Additionally, there are other ionization sources such as, fast atom bombardment:FAB, laser desorption:LD, plasma desorption: PD.

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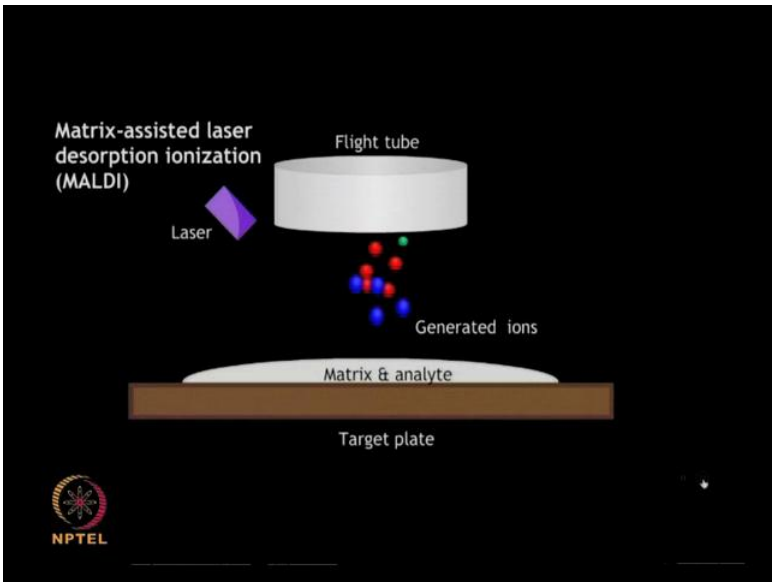

Let us discuss the two most commonly used soft animation techniques, Maldi and ESI in more detail. In Maldi the analyte of interest is mixed with an aromatic matrix compound such as, alpha cyano four hydroxycinnamic acid or sinapinic acid. This is dissolved in an organic solvent, and placed on a metallic sample plate. The evaporation of solvent leave the analyte embedded in the matrix, target plate is placed in a vaccum chamber with high voltage, and short laser pulses are applied.The laser energy gets absorbed by the matrix, and it is transferred to the analyte molecules, which undergo rapid sublimation resulting in gas phase ions.These ions then accelerate towards the mass analyzer based on their mass to charge ratios.

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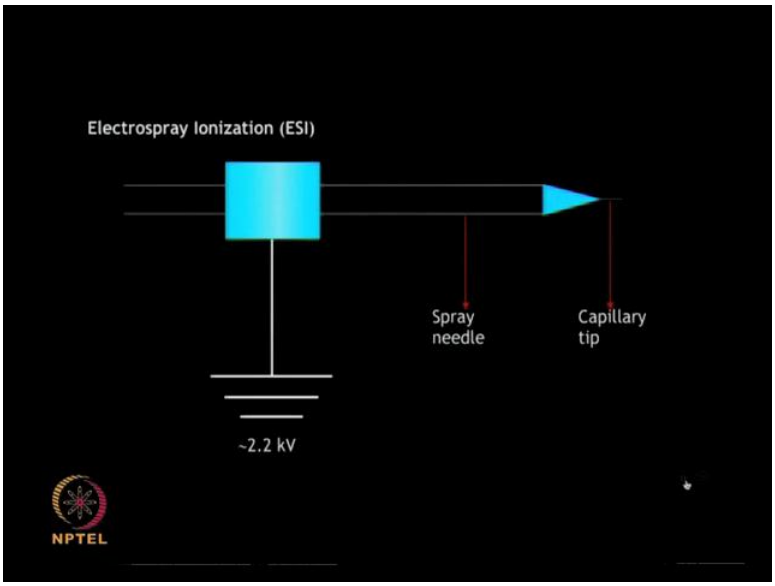

In electrospray ionization the sample is present in the liquid form, and ions are created by spraying a dilute solution of the analyte at atmospheric pressure from, the tip of a fine metal capillary creating a mest of droplets.

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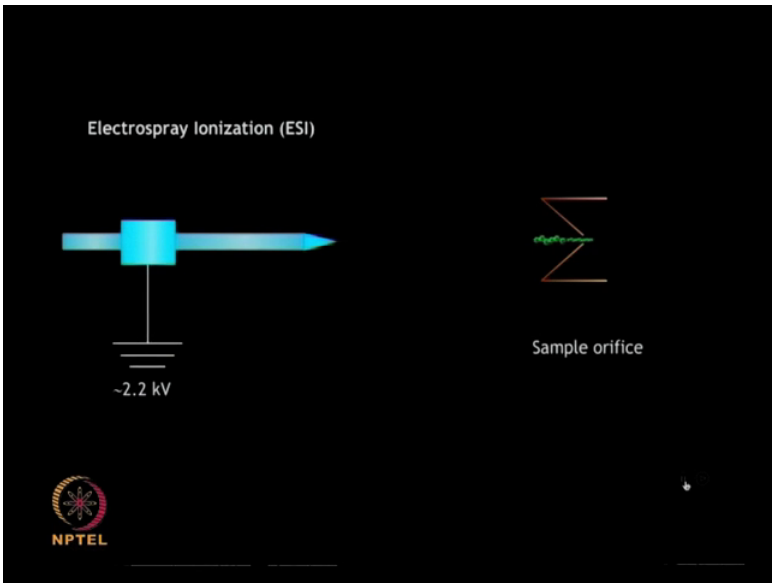

The droplets are formed in a very high electric field, and becomes highly charged. As the solvent evaporates, the peptideand protein molecules in the droplet pick up one or more protons from the solvent to form charged ions.

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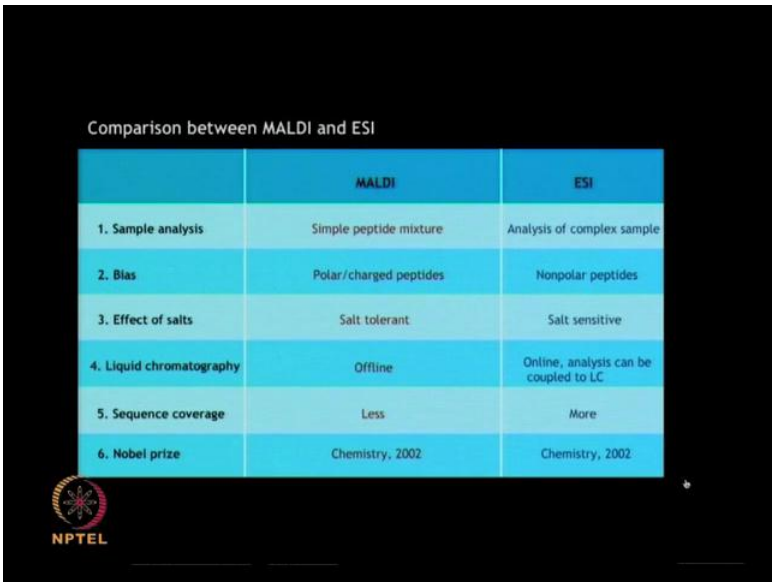

These ions are then accelerated towards the mass analyzer, depending upon their mass and charge. Let us have a comparison between Maldi and electrospray ionization, and discuss their pros and cons which can be used for the analysis of different types of protein sample. In Maldi the sample analysis is for the simple peptide mixture, where as in ESI it can be used for the analysis of complex samples. There is a bias towards the polar or charged peptides in Maldi whereas, it is for the non polar peptides in ESI. Maldi is more salt tolerant whereas, ESI is more salt sensitive. Liquid chromatography can be performed offline whereas, in ESI it is online and analysis can be coupled to the liquid chromatography. For the proteomic applications the sequence coverage is less in Maldi, as compare to the electrospray ionization. Both, Maldi and ESI developments were avoided with the nobel prize.

So, we are talking about an MS experiment, and I am trying to give you an overview of various steps involved, first of all we looked at liquid chromatography based prefractionation, in-gel digestion, different type of ionization sources, and now let us move on to the mass analyzers. A mass analyzer plays two most important function.

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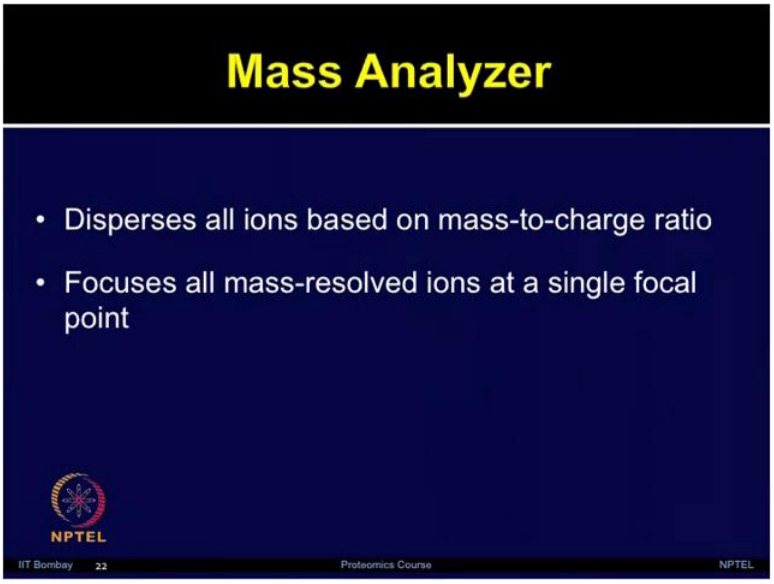

First of all, it disperses all the ions based on their massed charge or m by c ratio. Second, it focuses all the mass-resolved ions at a major single focal point. So therefore, all the ions inter in the mass spectrometer, it can maximize their transmission.

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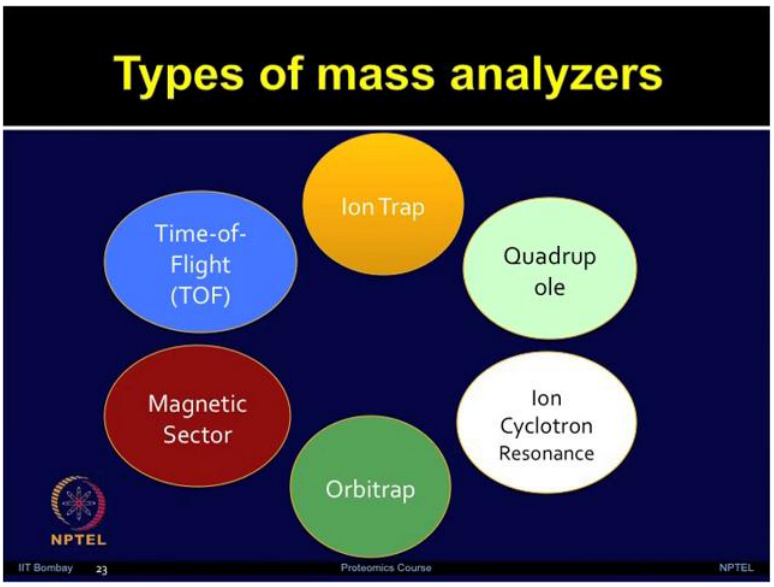

There are several different types of mass analyzers are currently available, that choose the same basic properties which we discussed. Some of the popularmass analyzer configurations are shown in the slide, which include time of flight or TOF, ion traps, quadrupole, magnetic sector, orbitrap and ion cyclotron resonance.

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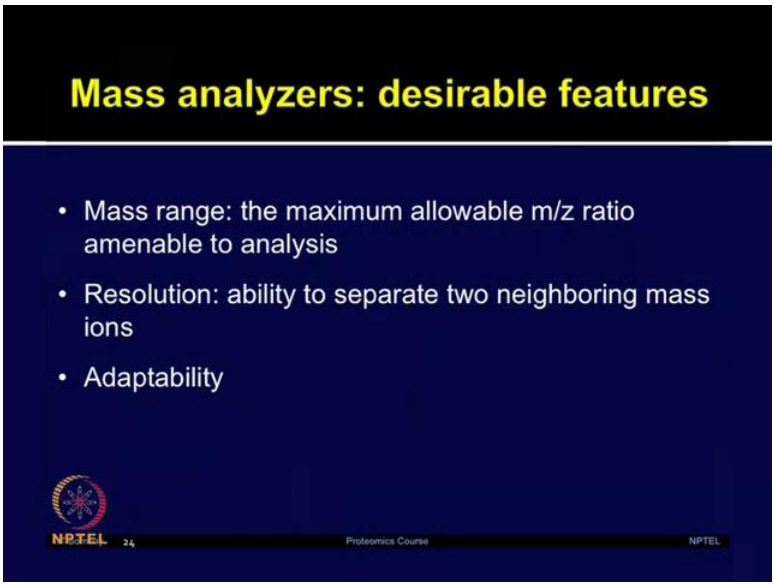

What are the desirable features of a mass analyzer? The performance of mass analyzers can be evaluated on the basis of various desirable characteristics. Mass range: which is maximum allowable m by z ratio, which is amenable to the analysis, so if you have higher value that will be useful for analysis of the high mass compounds. Resolution or ability to separate two neighboring mass ions, so how well is the mass of interest can be separated from the other species that describes mass resolution. Adaptability: the possibility of outfitting the mass analyzer with certain ionization techniques, as well as other devices such as, chromatography systems, and multichannel array detectors also desirable feature of the mass analyzer.

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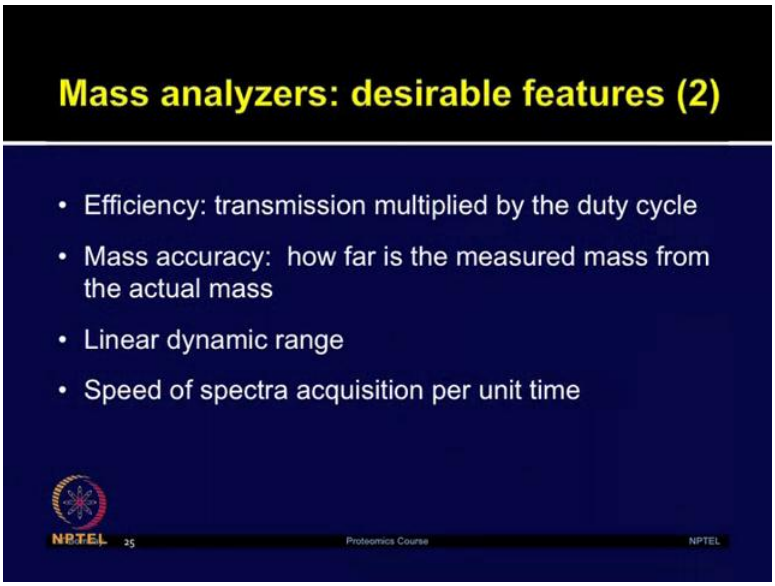

Efficiency: the transmission multiplied by the duty cycle, which is defined as the fraction of ions of interest formed in a single ionization event. The mass accuracy: how far is the measured mass from the actual mass, it is expressed in the p p m units, linear dynamic range. The range over which an ion signal is linear with the analyte signal. Speed: how many spectra can be acquired per unit time? All these are desirable features of mass analyzers, again in the desirable features

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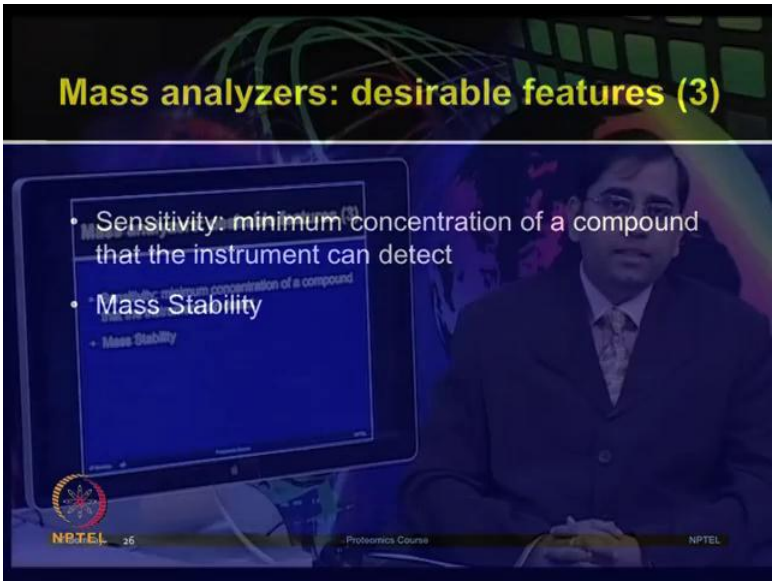

One need to look at the sensitivity, the minimum concentration of a compound that the instrument can detect with a particular signal to noise ratio, so detection sensitivity is the smallest amount of an analyte that can be detected at a certain defined confidence level. Mass stability: how reproducible is the measured mass? So, while you are comparing various type of mass analyzers, one need to look at all of this parameters, and then only one can decide for what application they can select, which type of mass analyzers.

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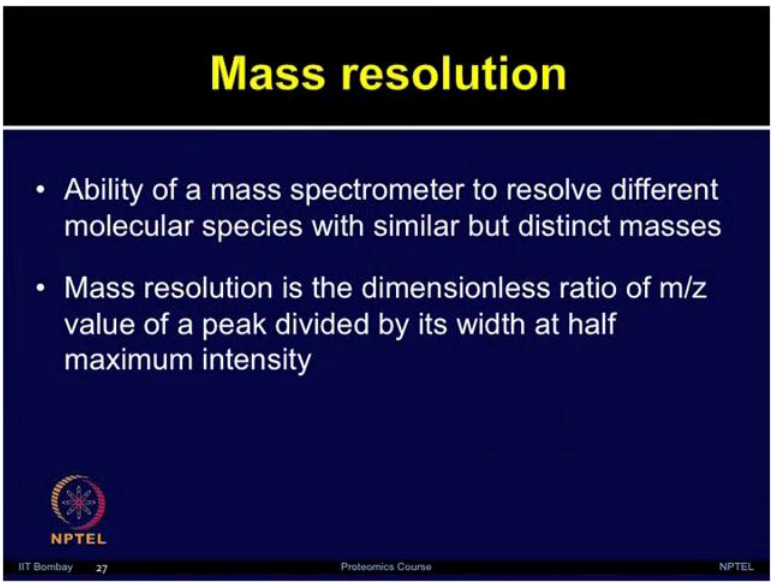

While we discussed various properties which are desirable for the mass analyzers, let us talk about mass resolution and mass accuracy in little more detail. So, mass resolution, it is ability of a mass spectrometer to resolve different molecular species with similar, but distinct mass. Mass resolution is dimensionless ratio of the m by z value of a peak divided by its width at half maximum intensity, it can ridge from 1000 to 100000 in different mass spectrometers. Now, if you have resolution of 1000 that would mean, that an instrument can resolve 2 peptides that differ by 1 unit at a mass of 1000.

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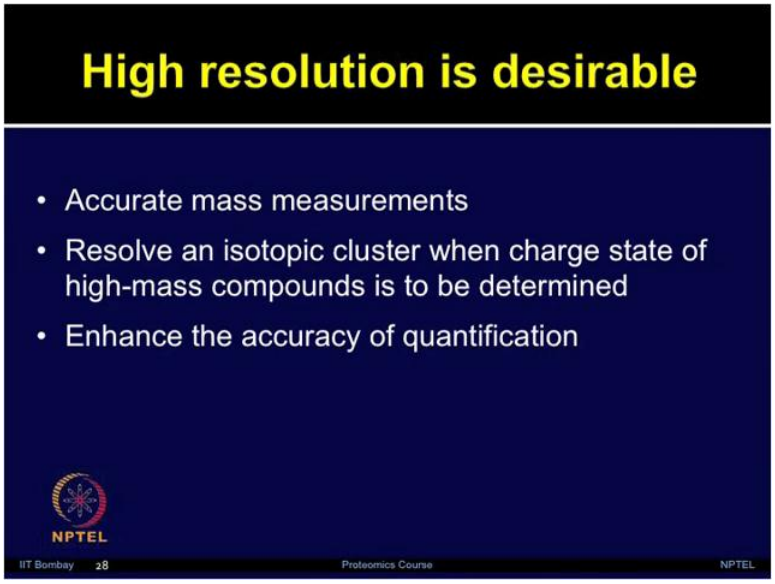

Obviously, high resolution is desirable for your application, because it can help to perform accurate mass measurement, it resolves isotropically labeled species when the percentage incorporation of the label is to be determined. It resolves an isotope cluster when the charge state of high mass compound is to be determined. It enhances the accuracy of quantitation, and mass selected precursor ions in the MS experiments can be unambiguously established.

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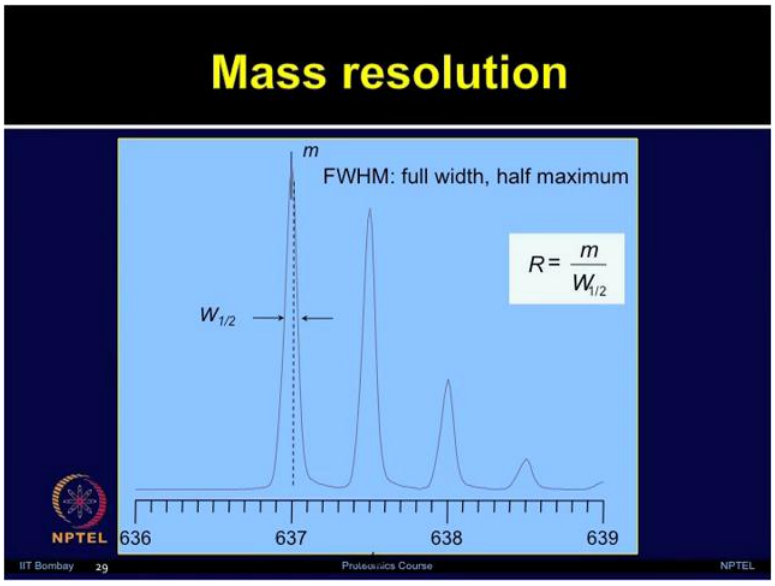

This slide shows you the full width, half maximum or FWHM. So, mathematically mass resolution is inverse of resolving power RF shown as r equal to M by W half, where m is the average of mass, and W half is the difference in the accurate mass of the 2 neighboring ions.

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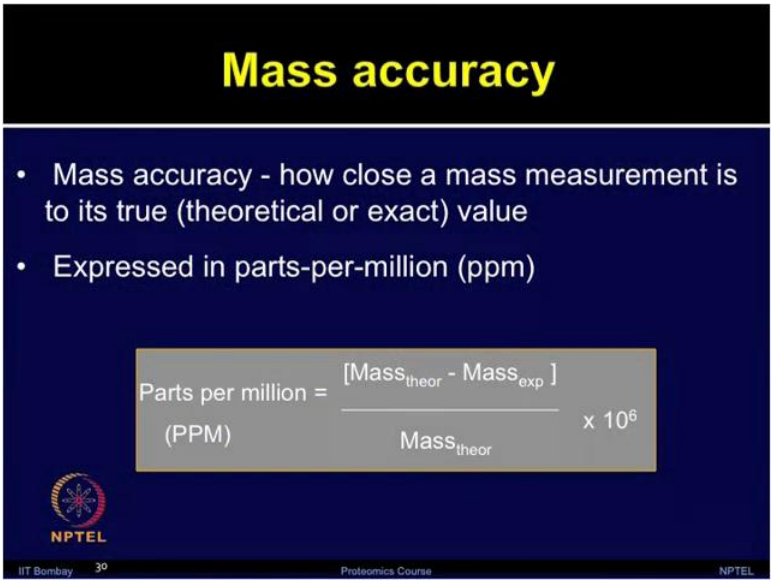

Now,let us talk about mass accuracy. How close a mass measurement is to its true theoretical or exact value? It is expressed in parts per million or ppm, absolute value is mass dependent. The equation for the parts per million the mass accuracy shown here, which is theoretical mass minus experimental mass divided by the theoretical mass times 10 to the power 6. So, mass accuracy affects the number of peptides in a data base with similar masses, and so lowered the measured ppm values, then the few are the possible matches in the data base. Now, currently there are many mass analyzers, which can measure both in MS and MS MS mode less than one ppm mass accuracy.

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So, let me show you few available mass analyzers in following animation. It resolves the ions produced by the ionization source on the bases of their mass to charge ratio, various characteristics such as: resolving power, accuracy, mass range and speed determine the efficiency of these mass analyzers.

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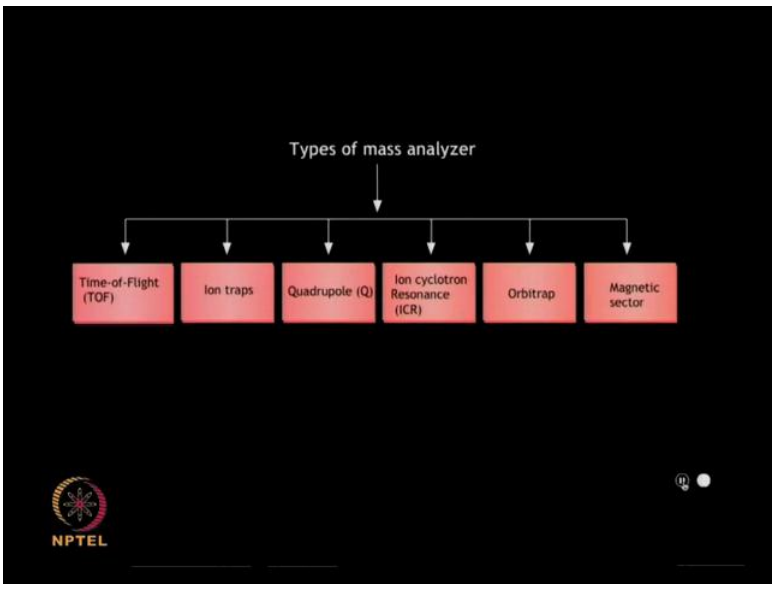

Let us discuss few most commonly used mass analyzers for the proteomics applications. Currently various type of mass analyzers are available including: time of flight, ion traps, quadrupole, ion cyclotron resonance, orbitrap and magnetic sector.

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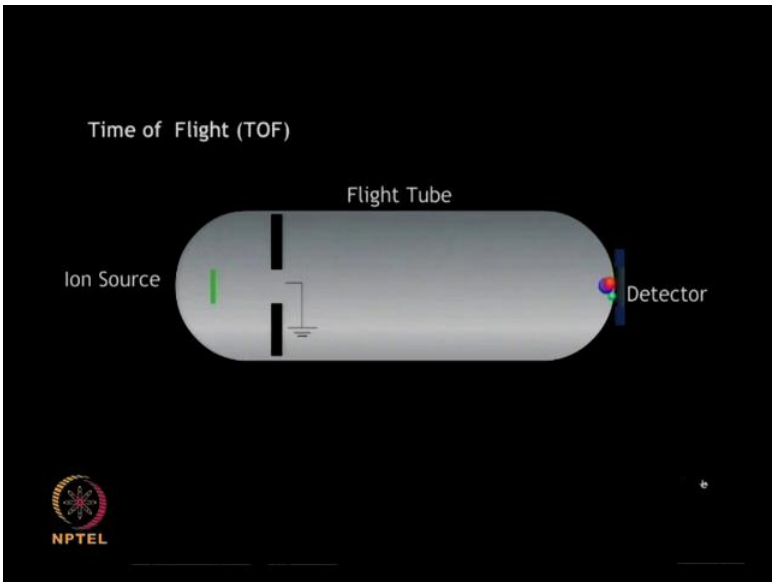

The time of flight analyser accelerates charged ions generated by the ionization source, Maldi along along tube, known as the flight tube or TOF.Ions are accelerated at different velocities depending on their mass discharge ratios, ions of lower masses are accelerated to higher velocities, and reached to the detector first,the time of flight under such circumstances is inversely proportional to the square root of molecular mass of the ion.

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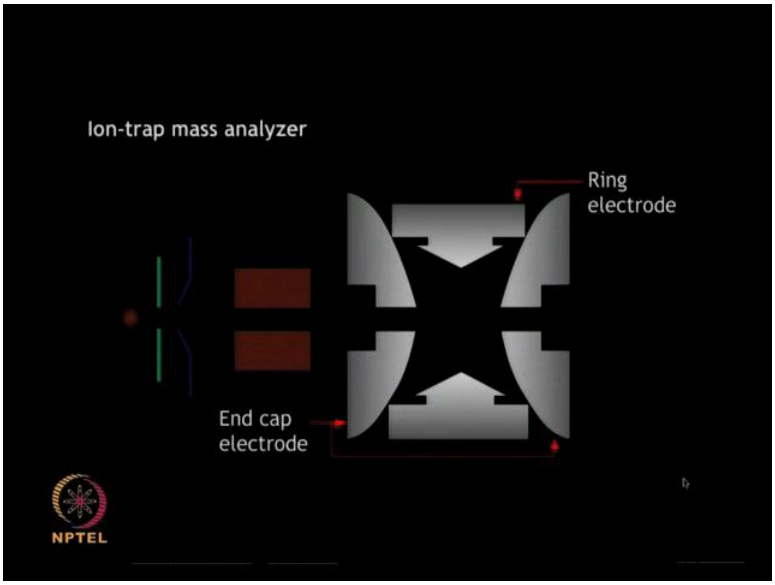

The TOF analyser has several applications in the proteomics. Now, let us discuss the next mass analyser, ion trap. An ion trap makes use of a combination of electric and magnetic fields, and captures ion in a region of a vacuum system or tube.

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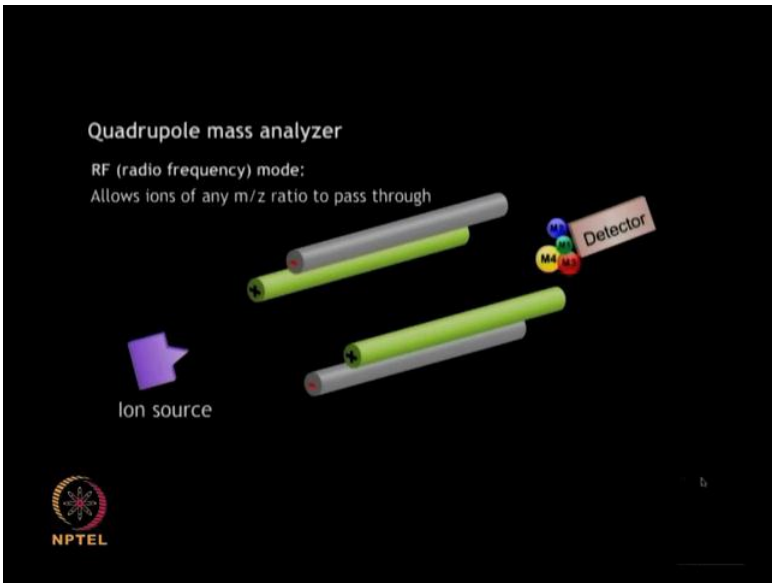

Ion trap, trap the ion using electric field, and measure the mass by selectively ejecting them to a detector. Quadrupole: the quadrupole mass analyzers use oscillating electric fields to selectively stabilize, or destabilise the paths of ion passing through a radio frequency quadrupole field. The quadrupole mass analyser can be operated in either radio frequency or scanning mode.In RF mode or radio frequency, ions of all M by Z are allowed to pass through which are the detected by a detector.

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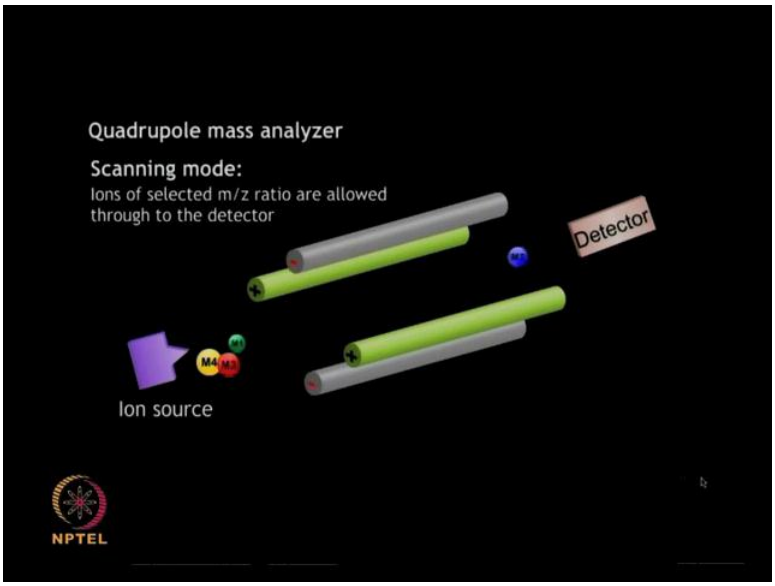

In the scanning mode the quadrupole analyser selects ions of a specific M by Z value as set by the user, a range can also be entered in which case only those specific ions, which satisfy the criteria will move towards the detector and rest can be filtered out.

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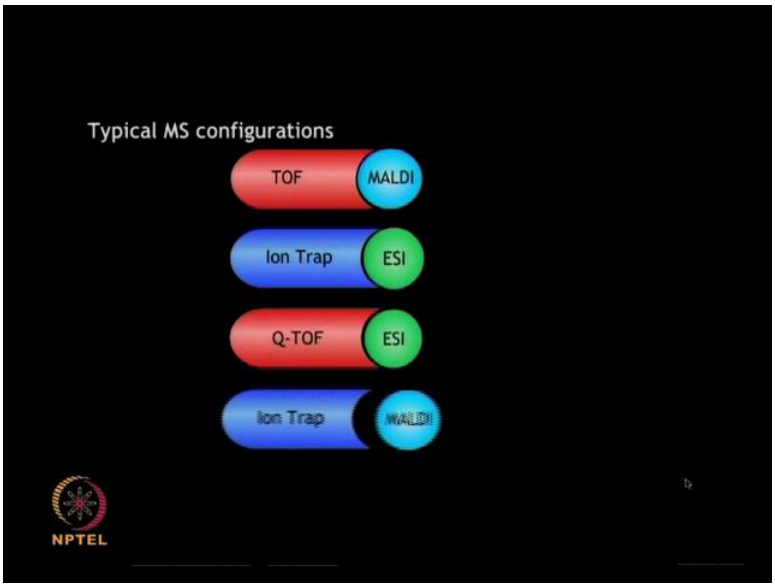

The arration source, and mass analyzers can be combined in different ways to give various configurations for the mass spectrometer. Some of the most commonly used MS configurations are Maldi, with time of flight TOF. Electrospray ionization, ESI with ion trap, ESI with quadrupole time flight Q-TOF, and Maldi with ion traps.

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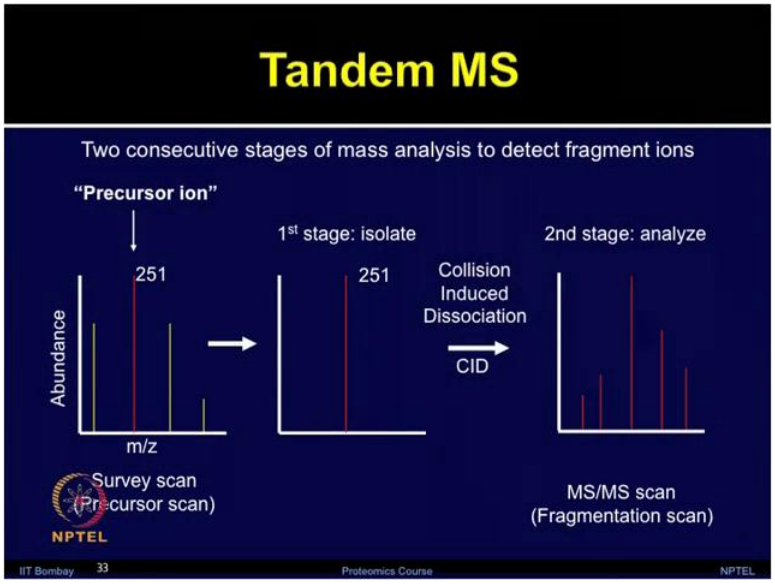

Now, let us talk about tandem mass spectrometry or tandem MS.Tandem MS involves two mass spectrometry system, the first m s performs mass selection of a desired target ion form a stream of ions produced in the ionization source, as you can see in the slide the precursor ion 251 is selected, this mass selected ion undergoes fragmentation or a chemical reaction, and then the second MS system performs mass analysis of the product ions that are formed in the intermediate step. The various type of fragmentation methods currently available.

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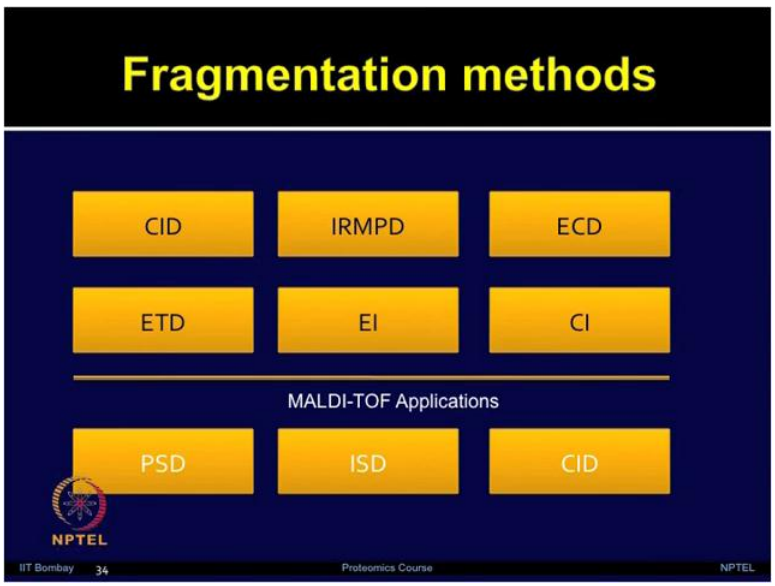

I have given a list ofthese methods on the slide: CID; collision induced dissociation, IMRPD; infrared multiphoton dissociation, ECD; electron capture dissociation, ETD; electron transfer dissociation, EI; electron impact, which is used for the small molecule, CI; chemical ionization, again used for the small molecule. For the Maldi, more specifically, PSD or post source decay, ISD in source decay, as well as CID or collision induced dissociation are commonly used fragmentation methods. So, most commonly means of ion activation, and dissociation in organic and bioorganic materials is collision induced dissociation.This slide gives you a comparison of MS versus MS MS analysis.

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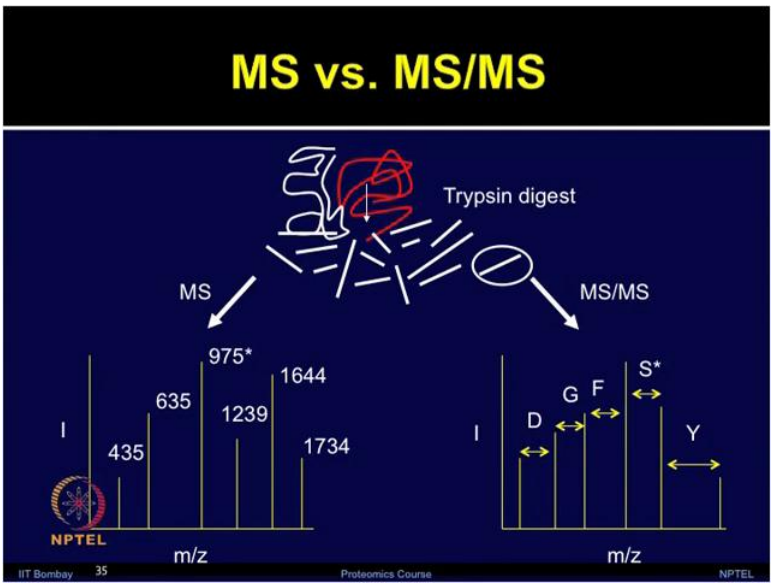

In MS/MS a peptide is fragmented, and masses of the fragment ions are recorded in a spectrum, with tandem MS or MS/MS uses two stages of mass analysis as I talked earlier, the first stage which can do selection of an ion for its subsequent fragmentation, and then second stage this ion is fragmented by using different type of fragmentation methods, such as CID or ECD, and then sequence information may be obtained via de novo analysis of the spectrum. So, for some of them are commonly used tandem MS configurations, let me show you this animation.

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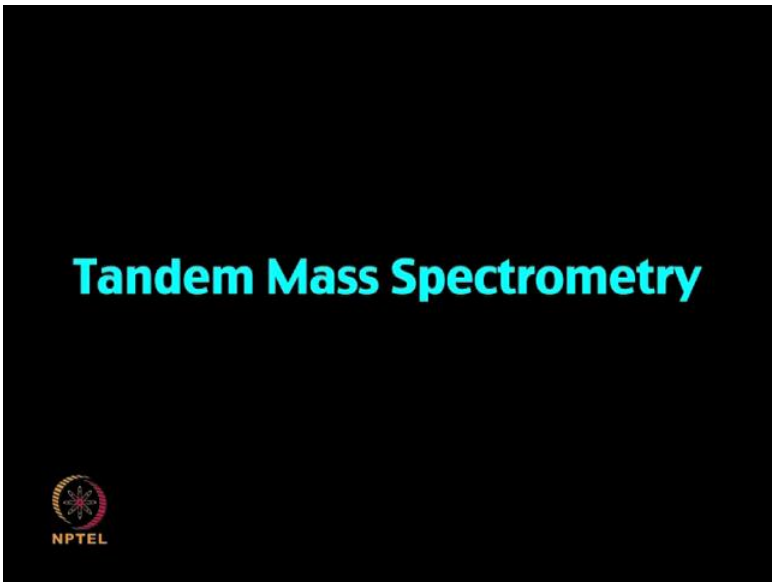

Tandem mass spectrometry: Combination of various mass analyzers in tandem gives rise to the tandem mass spectrometry.

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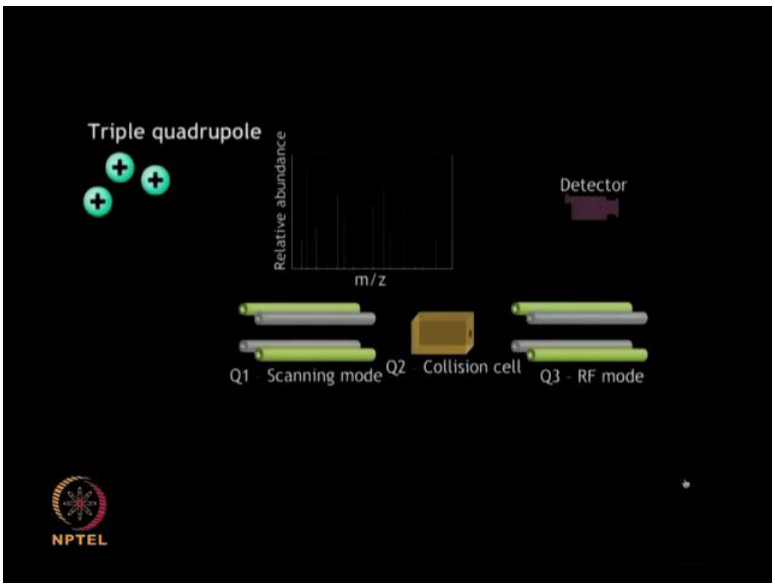

The triple quadrupole consist of two sets of parallel metallic rods, which are interspersed by a collision cell. The first quadrupole scans the ions coming from the ionization source, and allows only ions of a particular M by Z ratio to pass through, these ions enter the collision cells where they are fragmented by collision against an inert gas like argon. The smaller fragments can then enter the third quadrupole Q three, which scans all the

ions in a radio frequency or RF mode, which generates a spectrum based on the varying behavior of ions in an oscillating electrical field.

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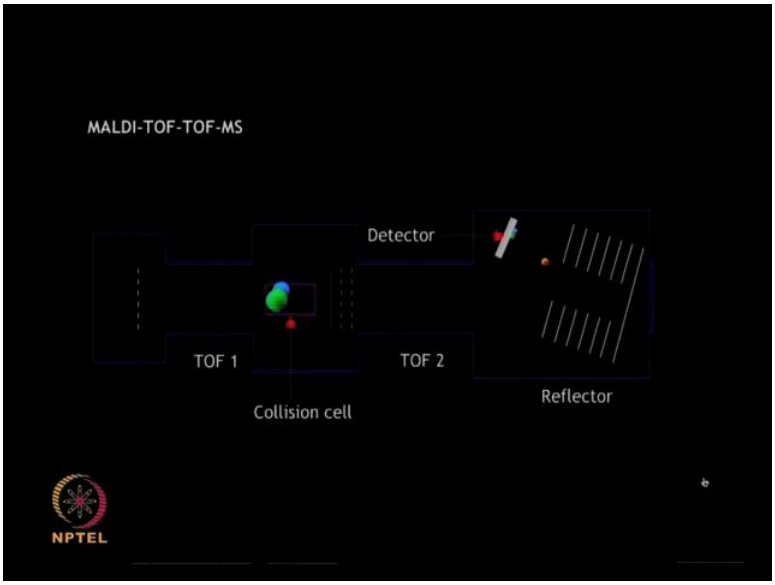

Now, let us talk about some of the very commonly used tandem mass spectrometry configurations. In addition to triple quad, Maldi-TOF-TOF it is another common tandem m s configuration, in which the ions are first resolved on the basis of their time of flight In first TOF analyzer, TOF one the selected ions enter the collision cell where they are further fragmented, and these fragmented ionsare accelerated and further resolved on the basis of the m by z values in the second TOF flight of tube, TOF tube after which they can be detected.

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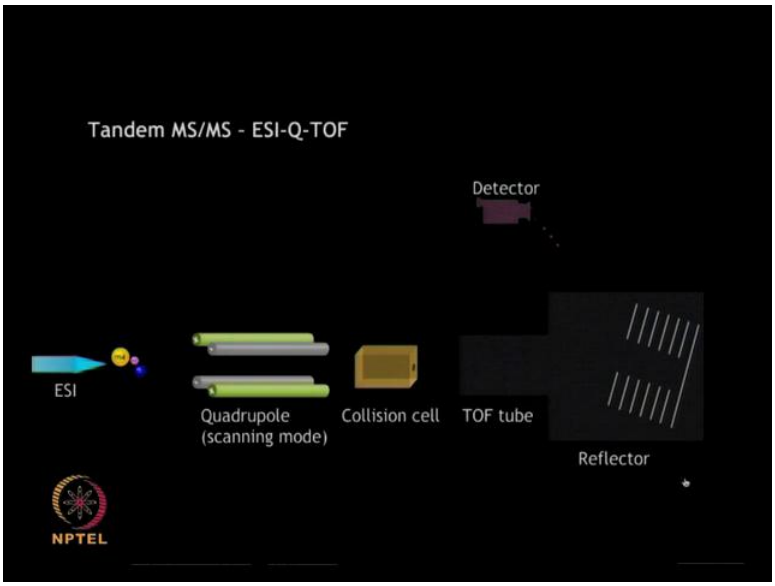

ESI-Q-TOF is another commonly used tandem MS configuration that first selects ions in quadrupole, the selected peptide is then fragmented in the collision cell, and resulting ions are accelerated and resolved on the bases of the time of light.

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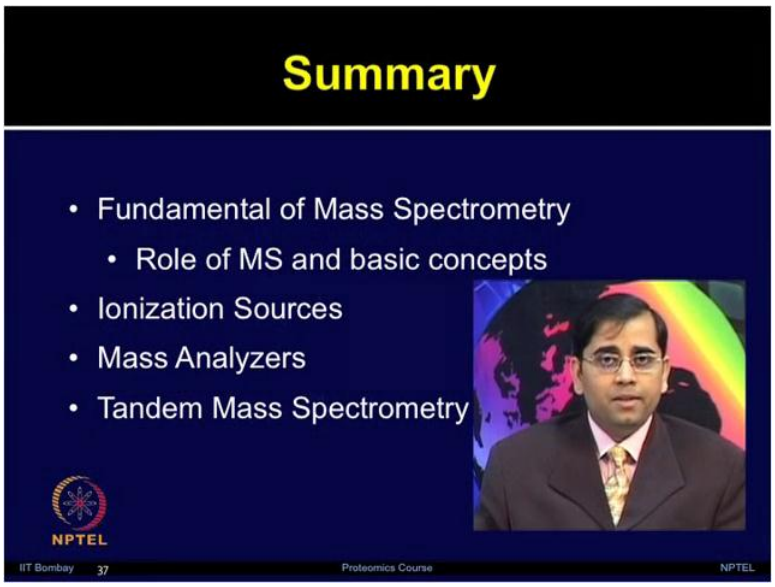

So, in summary today we talked about some of the fundamental concepts involved in the mass spectrometry, we talked about different type ionization sources, mass analyzers, detectors, different type of terminology involved in evaluating the performance of these instruments. So, what an ideal MS should be? It should posses wide mass range, high

sensitivity, high resolution, high mass accuracy, true MS/MS, and MRM capabilities, wide linear dynamic range, multiple charge separation capability, polarity switching capability with rapid or low, complimentary ionization, one can also use the modular where different type of ionization sources can be combined, such as ESI and ENCPI, targeted analysis can be performed for PTM, label free quantification or MRN type of acids. So, from your ideal mass spectrometer, you would like to have wide range of applications, and that is only possible if it has very high specifications.

So, from today's lecture I hope you are able to understand some of the fundamental concepts involved in the mass spectrometry, very briefly we touched upon ionization sources and mass analyzers, and then we talked about tandem MS configurations. In the subsequent lectures, we will talk in more detail about some of these ionization source, mass analyzers and different type of mass spectrometry, configurations and its applications.Thank you.

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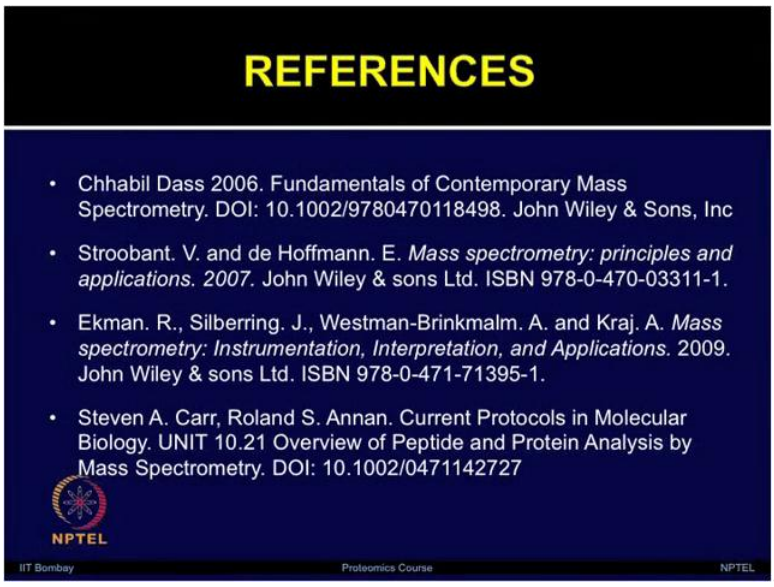

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