Proteins and Gel-Based Proteomics
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Mod 05 Lecture Number 20

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Let's switch gears from the previous module on gel-based proteomics and switch on to mass spectrometry and various types of applications.

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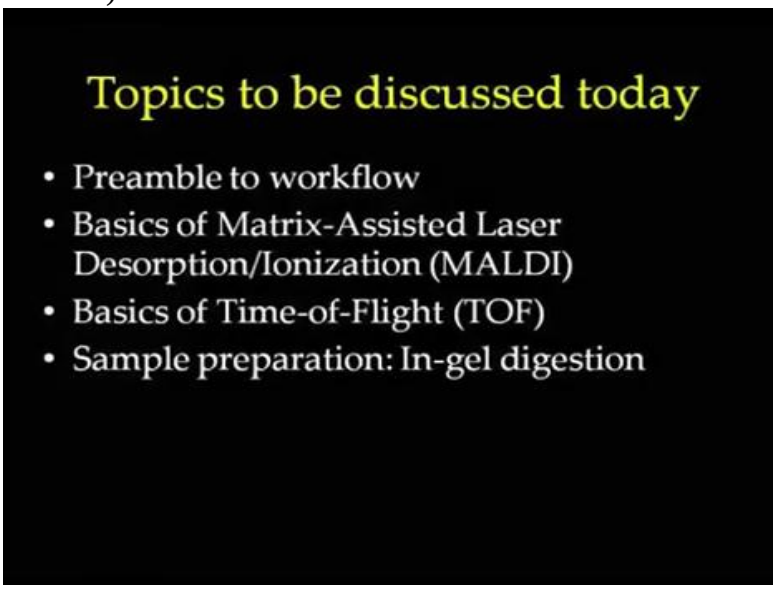

I will describe the role of MS and various basic concepts involved in understanding this mass spectrometry.

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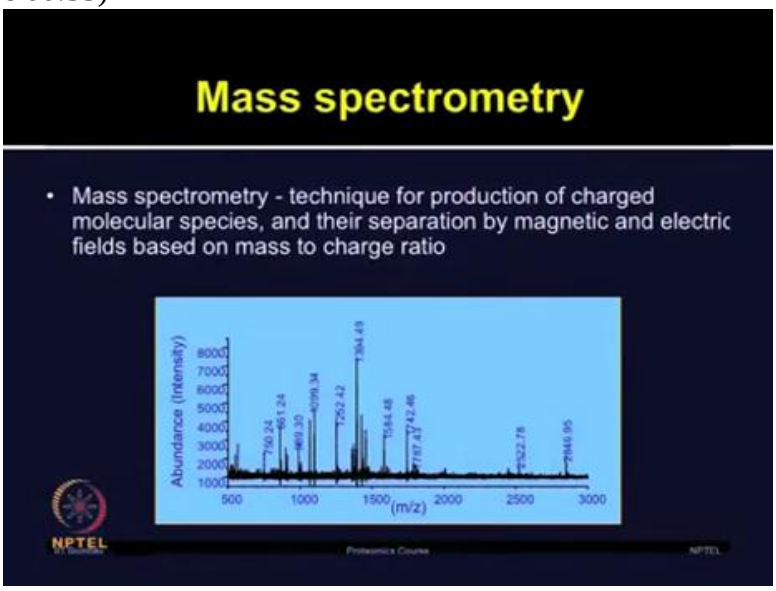

So first of all, what is mass spectrometry? It is an analytical technique to measure the molecular mass of individual compounds, and atoms accurately by converting them into the charged ions.

So, by definition this is a technique for production of charged molecular species in vacuum, and their 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.

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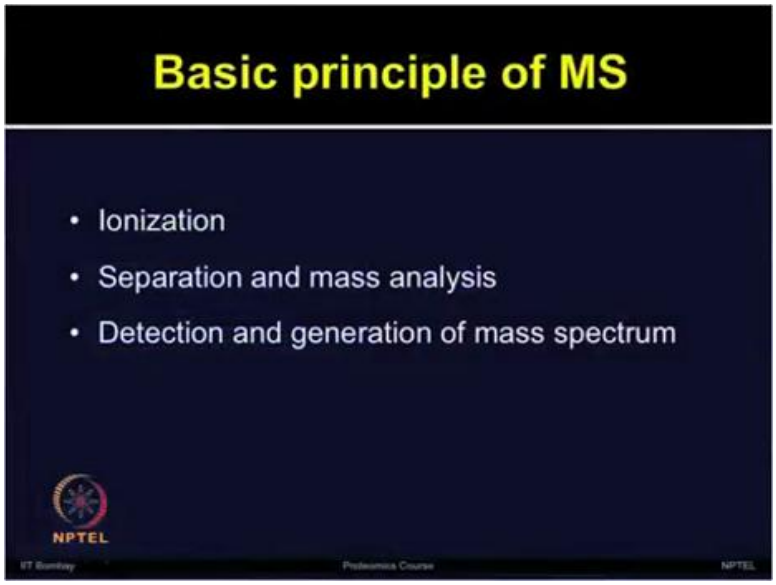

Now is the basic principle of mass spectrometry? So, first step is ionization. To convert analyte molecules or atoms into the gas phase ionic species. It removes or adds electrons or protons.

The second step is separation and mass analysis of molecular ions and charge fragments on the basis of mass to charge ratio.

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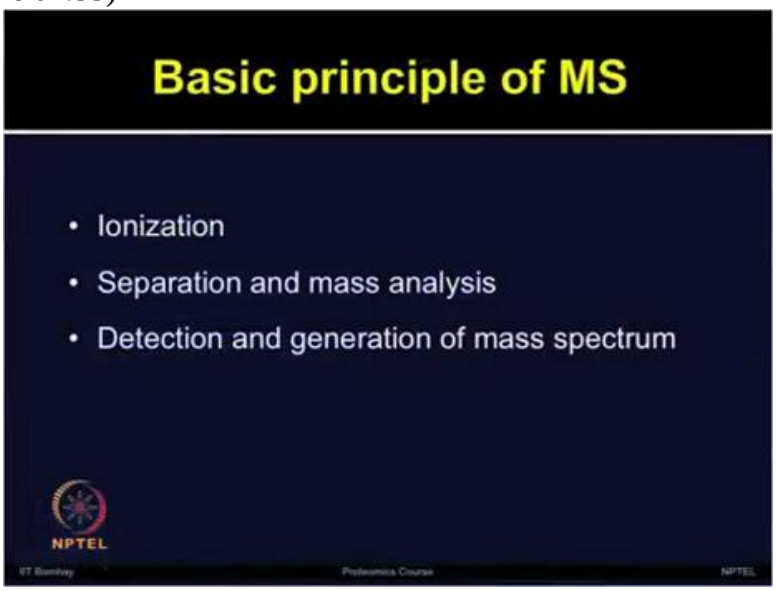

The final step is detection and generation of mass spectrum. These are the main steps involved in the mass spectrometry operations.

So today let's focus on the MALDI TOF which is one of the very widely used techniques in proteomics. This provides a high throughput platform for several applications, including molecular weight determination, protein identification as well as post translational modification studies.

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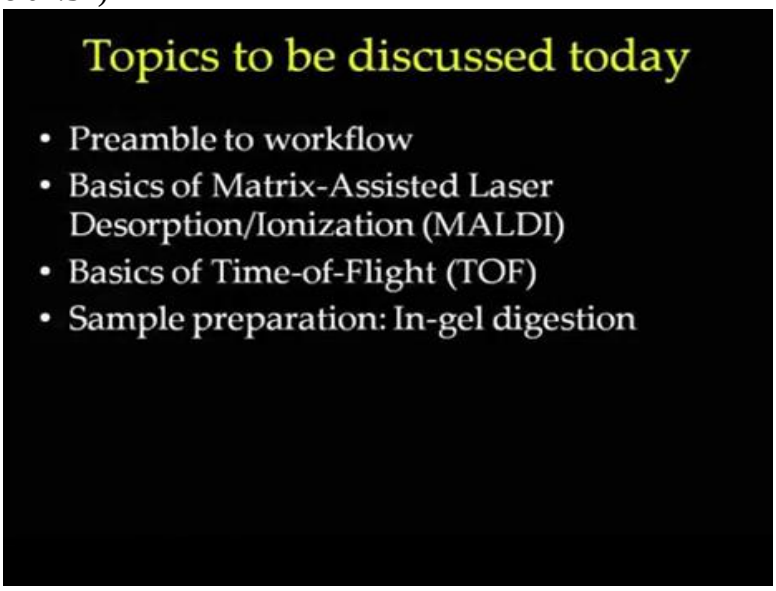

So, in today's lecture, we will talk about basics of MALDI-TOF, how to prepare the sample for MALDI TOF or TOF/TOF analysis which will include in-gel digestion, you want to do the photolytic digestion of the samples

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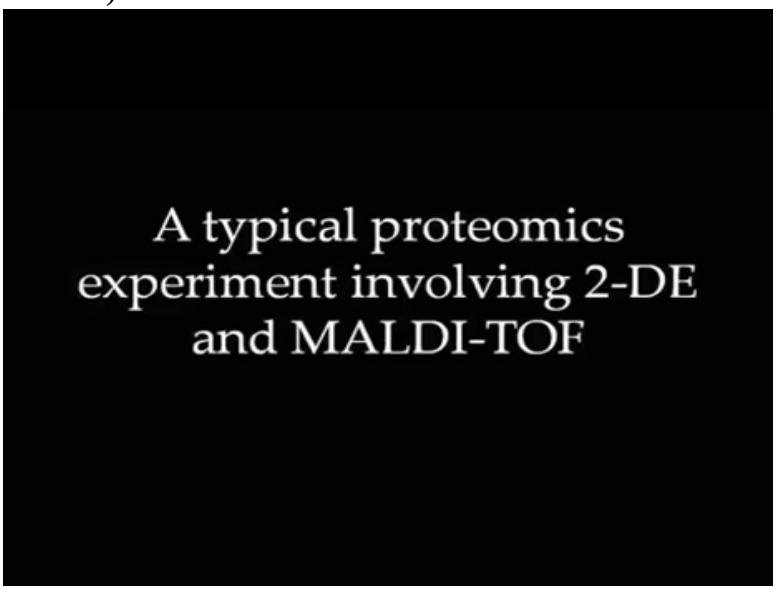

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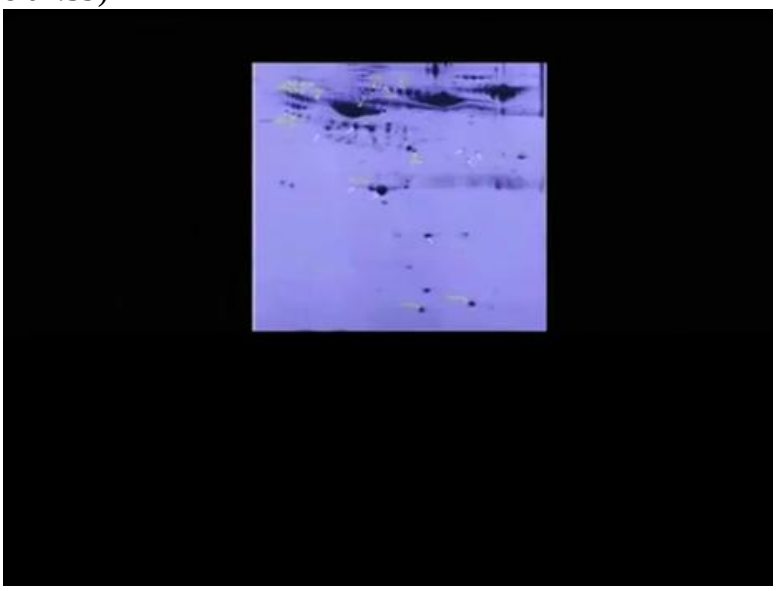

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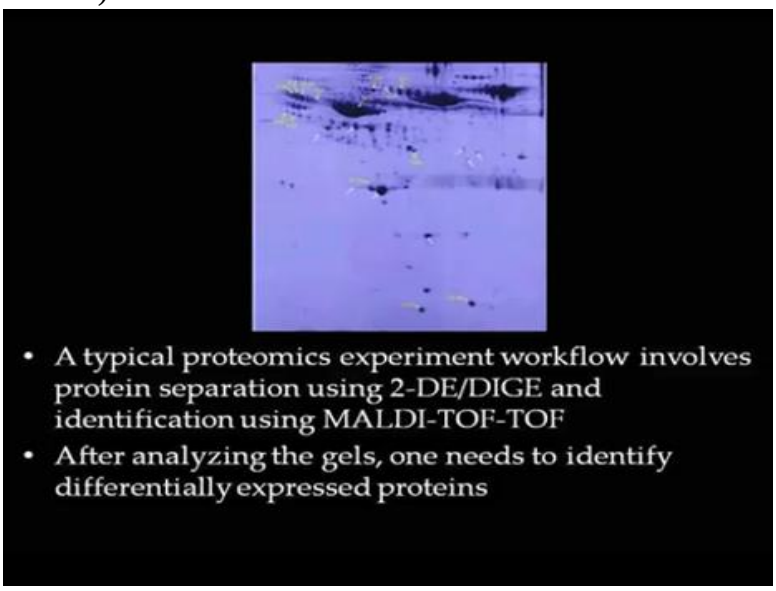

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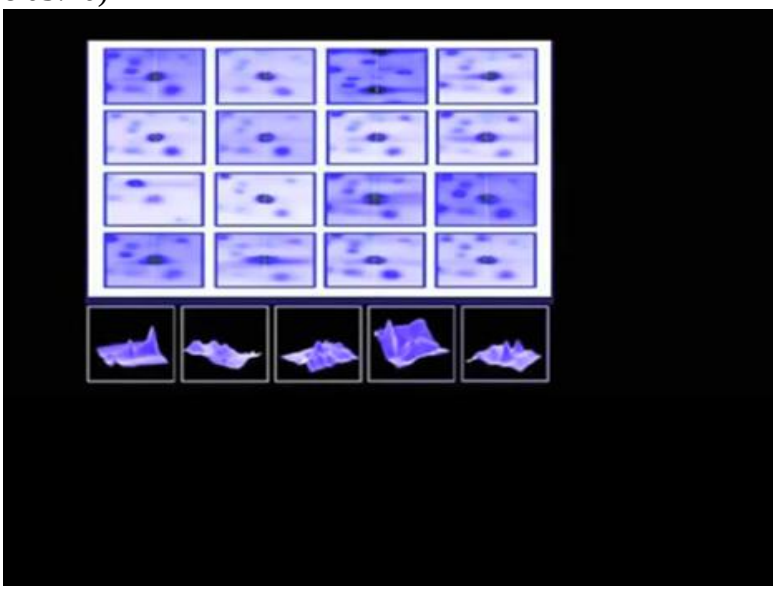

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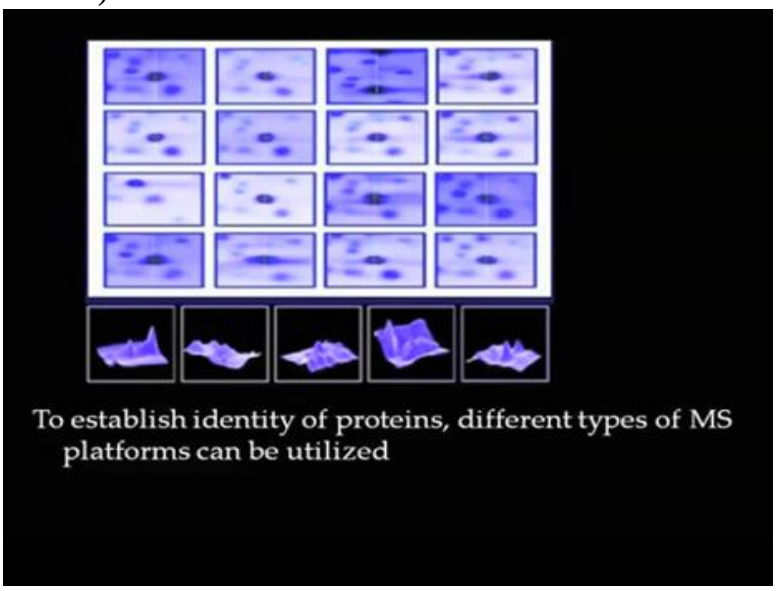

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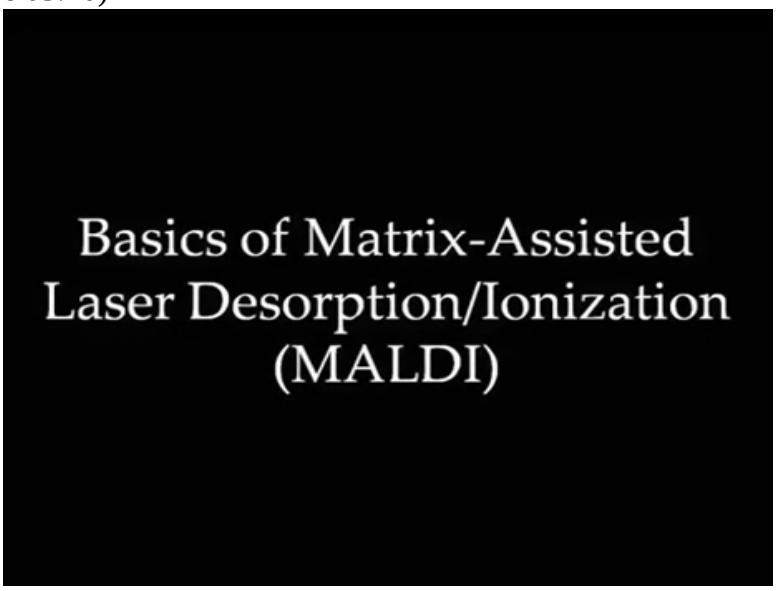

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Let us first start about basics of MALDI. So, MALDI is an efficient process of generating gas phase ions of peptides and proteins for mass spectrometric detection. MALDI is one of the most widely used ionization technique, currently applicable in the proteomics area.

This ionization method was independently developed by two scientists Koichi Tanaka and Hillenkamp. Tanaka also received the Nobel Prize for his novel contribution into soft ionization technique such as MALDI.

So, let us go through the some of the basic concepts involved in the MALDI TOF. We can explain that in two parts one is MALDI which is ionization source another is TOF which is a mass analyzer

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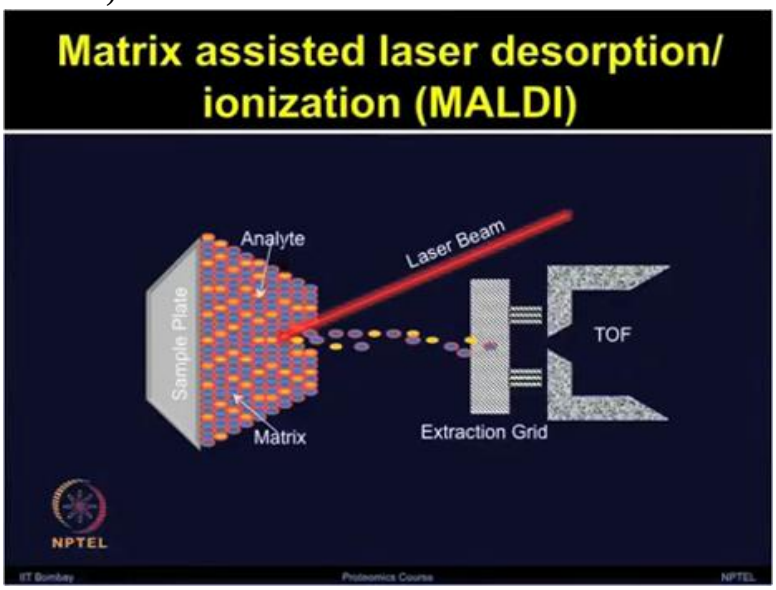

Let us first talk about Matrix Assisted Laser Desorption Ionization or MALDI. So, analytes or the proteins of interest are mixed with the matrix, which is usually an aromatic compound.

There are various type of matrixes available which we will talk in more detail when we come to the sample preparation and matrix selection, but just for your reference we can use 2,5-Dihydroxybenzoic acid , we can use Sinapinic acid and there are several other choice.

Once you selected a matrix for the experiment, then analyte and matrix can be dissolved in a organic solvent. After which then it can be placed on the metallic target. As you can see in the slide the first left section shows you how to place the analyte and matrix together on the sample plate.

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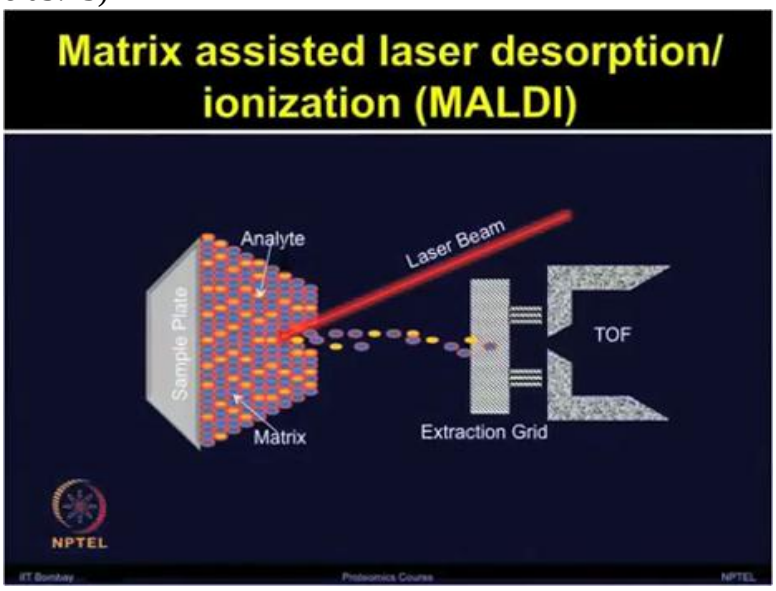

Now, once you have placed the matrix and the analyte on the target plate, you can put that in the vacuum chamber and apply high voltage. Now these crystals are targeted with the short laser beams, as you can see in the slide, then rapid sublimation can convert analyte into the gas phase ions.

Now these ions once generated they can accelerate away from the target plate through the mass analyzer, which is time of flight TOF tuned and they can reach towards the detector. Now this process is shown in the right hand side of the slide

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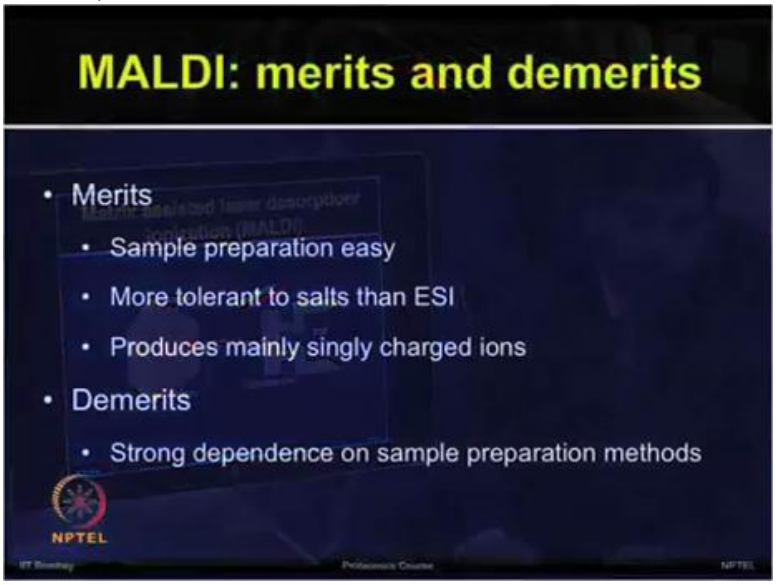

There are various advantages and disadvantages of using MALDI as an ionization source. The sample preparation is very easy; the MALDI provides high tolerance to salt as compared

to the electrospray ionization methods. The MALDI produces single charged species. Most analytes can accept the single photon. The single charge characters can result in some molecules having large mass to charge values.

So, therefore, the MALDI is typically integrated with the TOF mass analyzers, which can provide the m by z range for the large ions as well. Now these are various methods of using MALDI; obviously, it has to be connected with the TOF.

Now, there are various demerits of using the system. There is a strong dependence on how to prepare good sample for this analysis. So, sample preparation methods here will influence the spectrum generated from these experiments.

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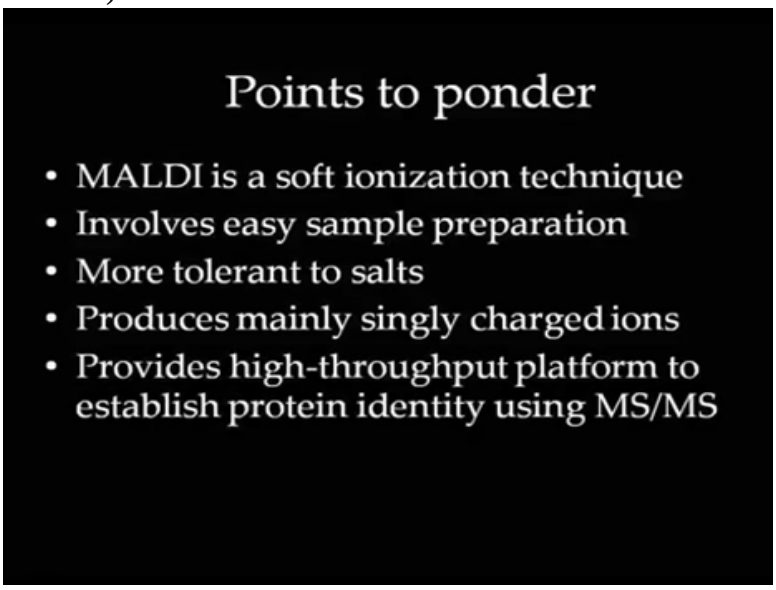

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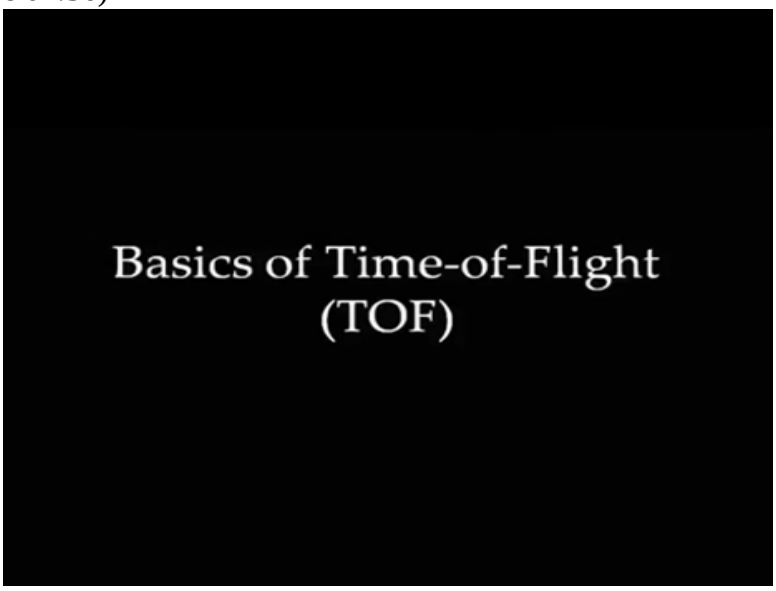

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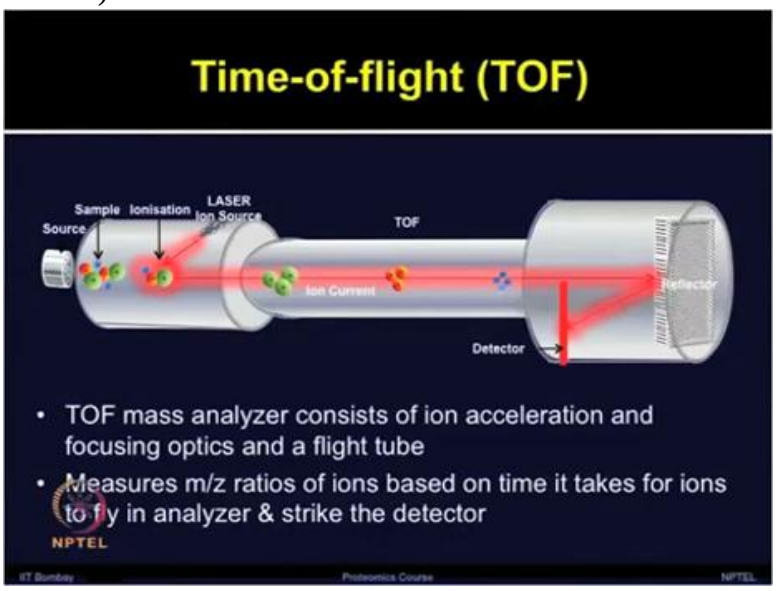

TOF mass analyzers they consist of ion acceleration and focusing optics and a flight tube. As shown in the slide you have a source where sample ionization is occurring due to the laser beam bombardment. When ions are moving in the Time of Flight tube, and reaching towards the detector.

Now often we can also add the reflector and ion mirror which can increase the path length. So, this time of flight tube it measures the mass to charge ratio of ions, based on time it takes for ions to fly in the analyzer and strike to the detector.

Now, the mass is exponentially proportional to the flight time, how much time it takes to travel in the time of flight tube. So, ions of the lower masses are accelerated to the higher velocities. Now time of flight tubes often outperforms these scanning mass analyzers in its sensitivity and scan speed.

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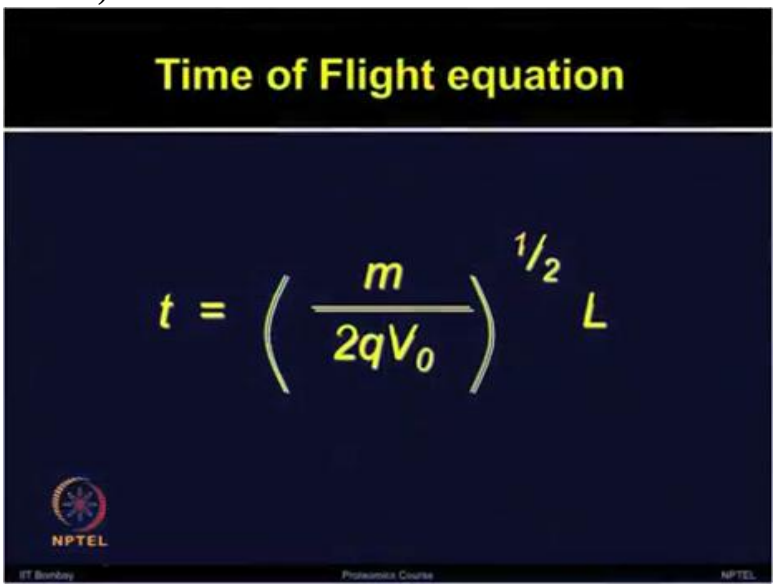

The time of flight of a charged ion can be calculated by using the equation shown in the slide. The flight time is directly proportional to the square root of mass of the ion. Now in this equation t represents time of flight, m is mass of the ion, q charge on ion, V 0 is accelerating potential and L is the length of flight tube.

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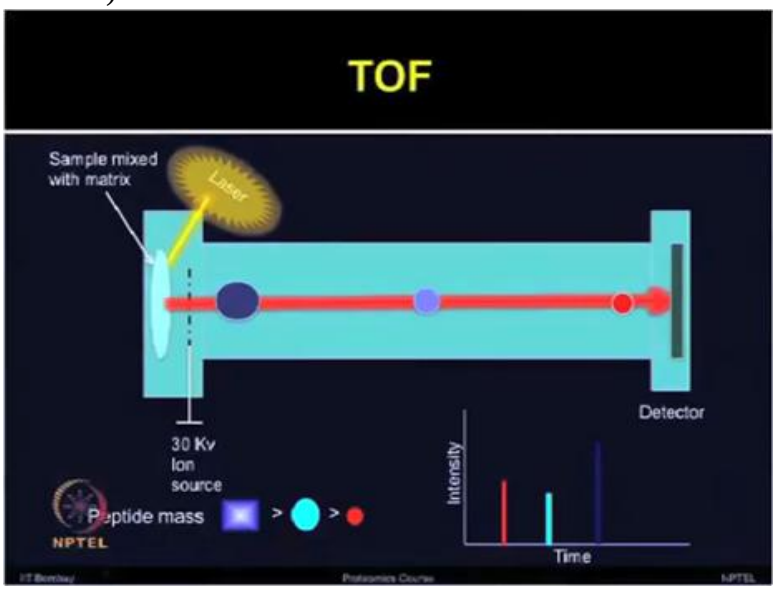

In time of flight tube the ions are accelerated to high kinetic energy, and due to the different velocities they are separated in a flight tube.

As I mentioned earlier, by adding the reflectron or a reflector, the ions can turn around in the reflector that can compensate for minor differences in the kinetic energy. Now, if you take an example where you have three ions as shown in the dark blue, light blue, and the red color in the slide.

Now you will expect that the small ion which is the red one will show the first peak, followed by the blue ion, and then the dark blue ion.

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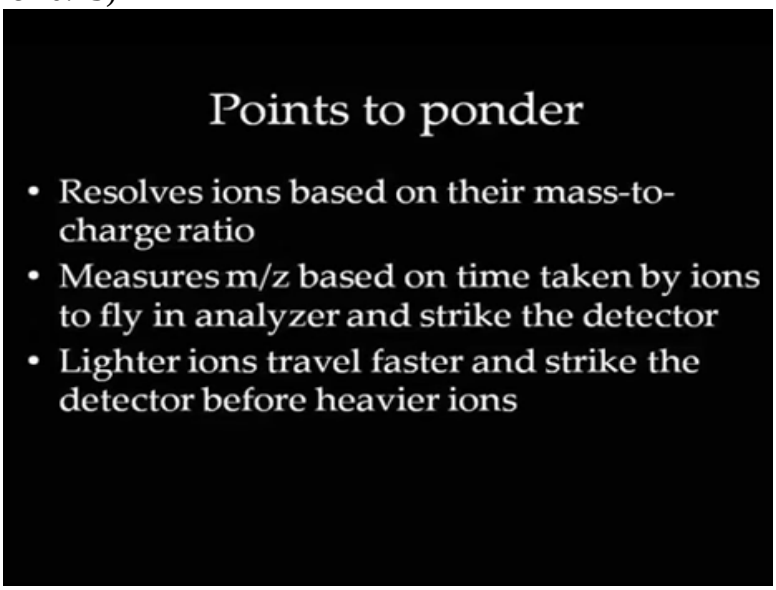

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After discussing some of the basic concepts of using MALDI and TOF, now let me give you an overview of entire MALDI TOF experiment by showing you the following animation.

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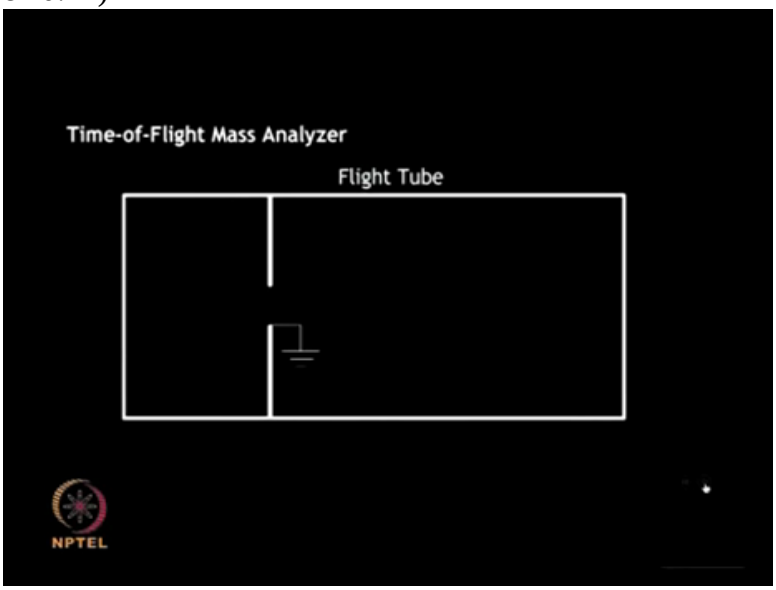

The time of flight analyzer

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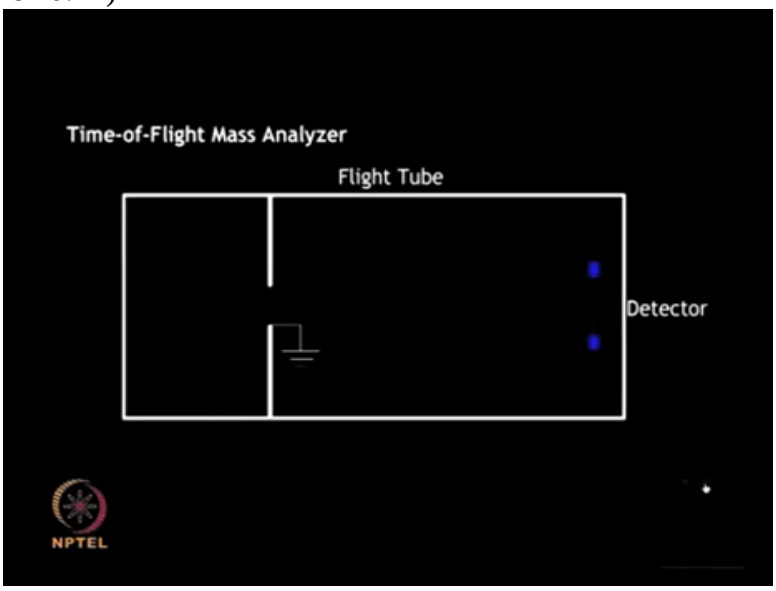

…. resolves ions

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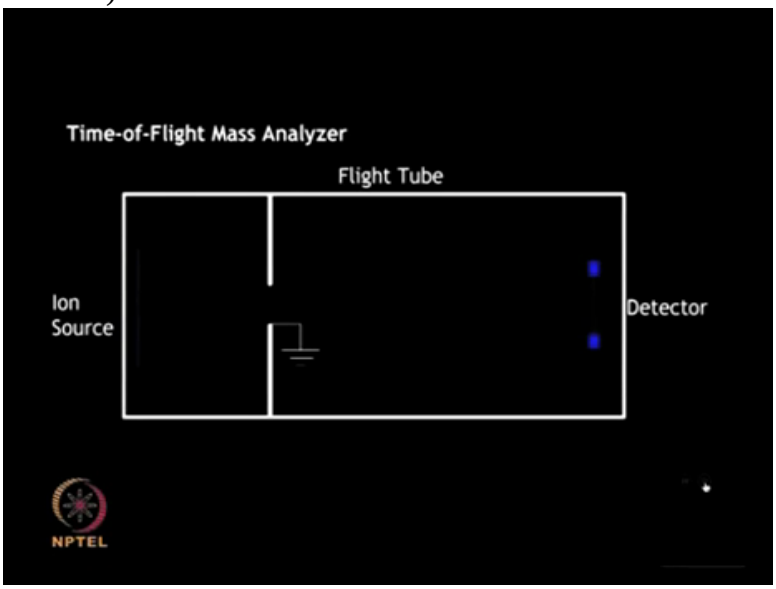

, produced by the ionization source, on the basis of their mass to charge ratio. The time of flight tube can be operated

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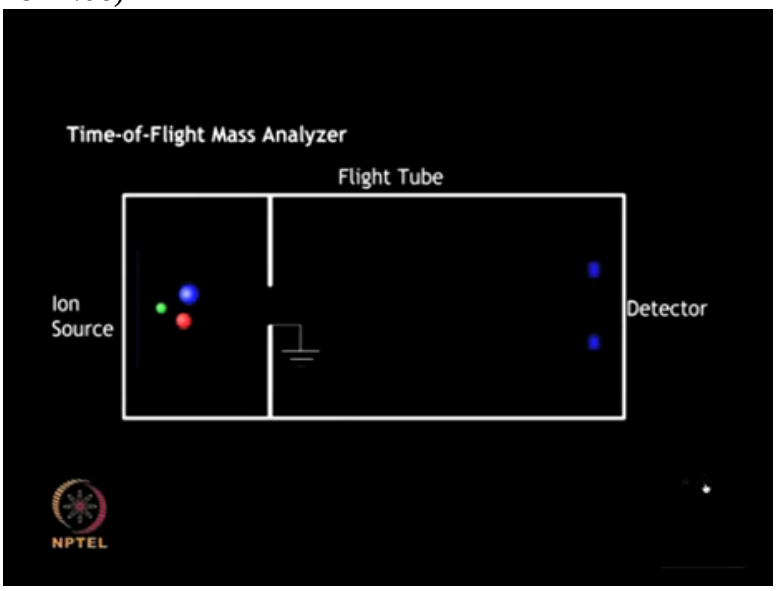

… in the linear mode or the reflectron mode which depends on the sample to be analyzed. In case of small molecules this mode usually provides sufficient resolution.

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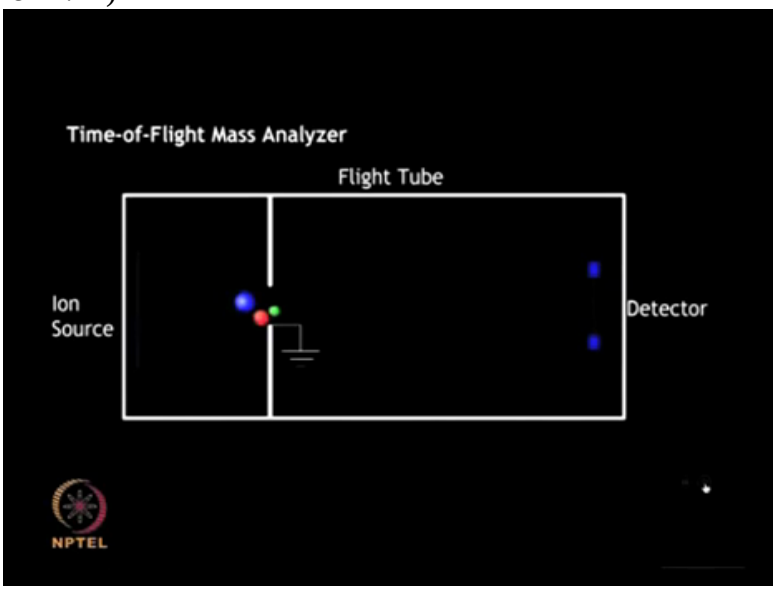

The generated ions are accelerated towards the detector, with the lighter ion travelling

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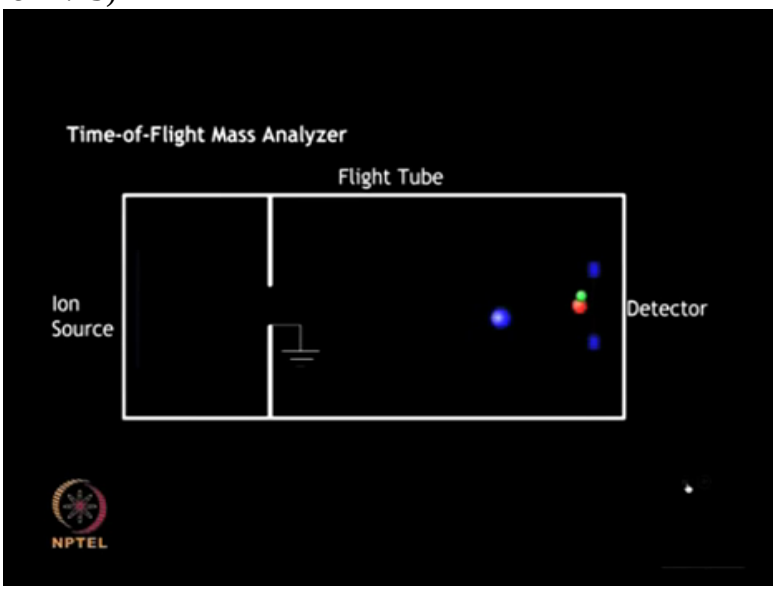

….through the TOF tube faster than the heavier ions So, the lighter ion travels faster and it strikes the detector before the heavier ion reaches to the detector.

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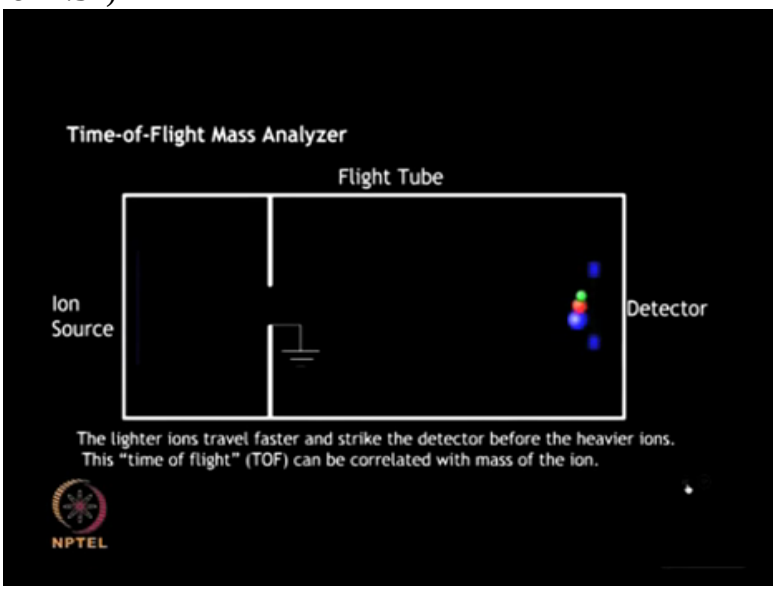

The time of flight or the TOF tube can be correlated with the mass of the ion. So, the flight of time of the ions can be correlated with the mass to charge ratio. As we talked earlier the TOF analyzer can also be operated in the reflectron mode.

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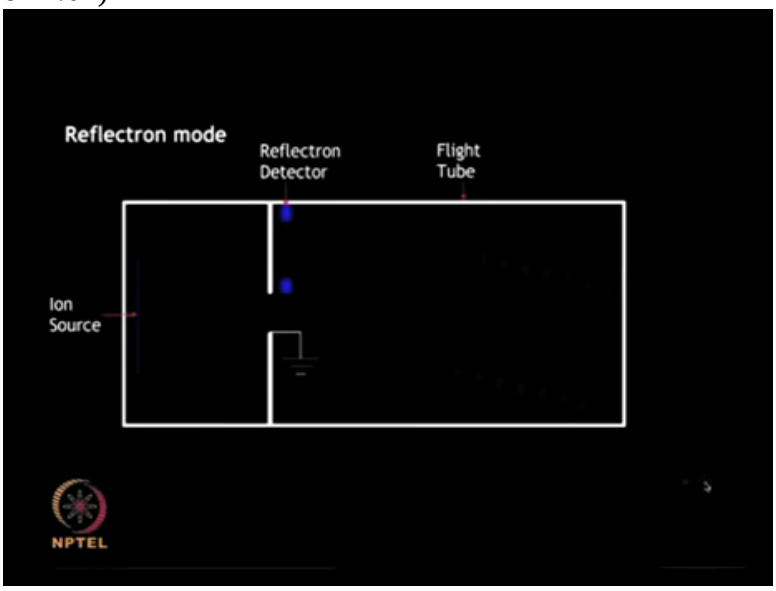

So, this is more commonly used for the proteomic studies.

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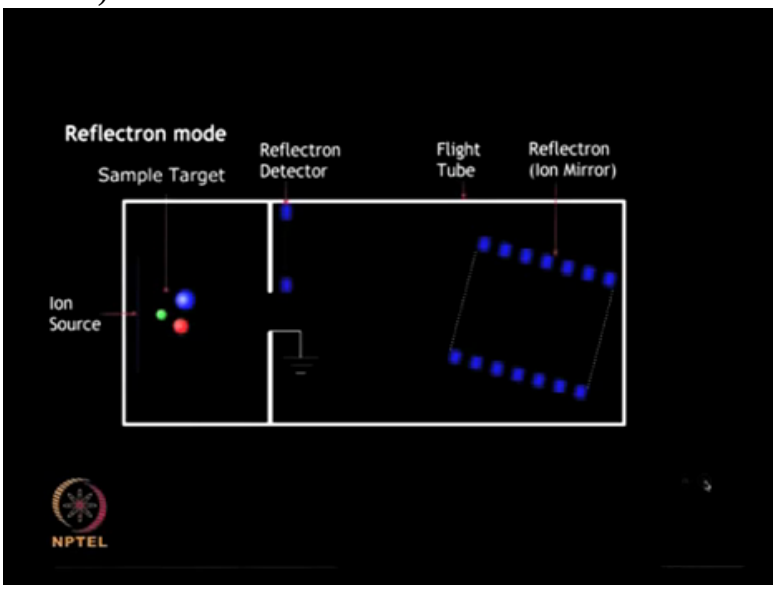

A reflectron which acts as a ion mirror is incorporated at one end of the time of flight tube.

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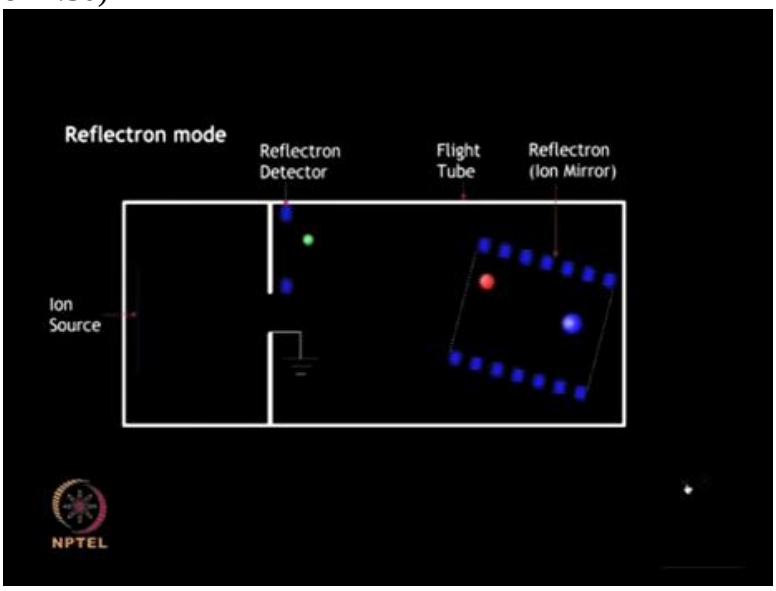

This helps in extending the path length and in turn the flight time of the ion without having to increase the actual size of the instrument. So, rather than using very long Time of Flight tubes by including the reflectron ion mirrors, we can increase the path length.

This helps to even out any kinetic energy differences between ions having the same mass and thereby improving the resolution.

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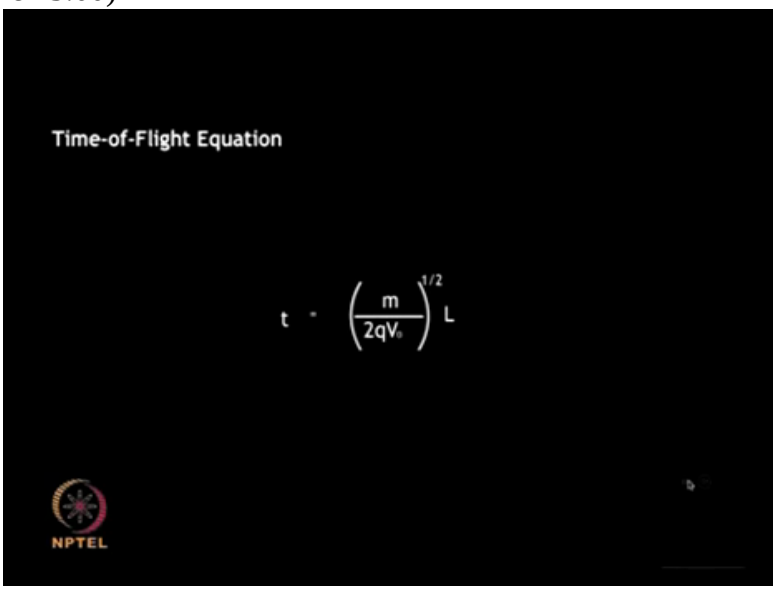

The time of flight of a charged ion can be calculated by a means of the equation shown here. The flight time is directly proportional to the square root of mass of the ion.

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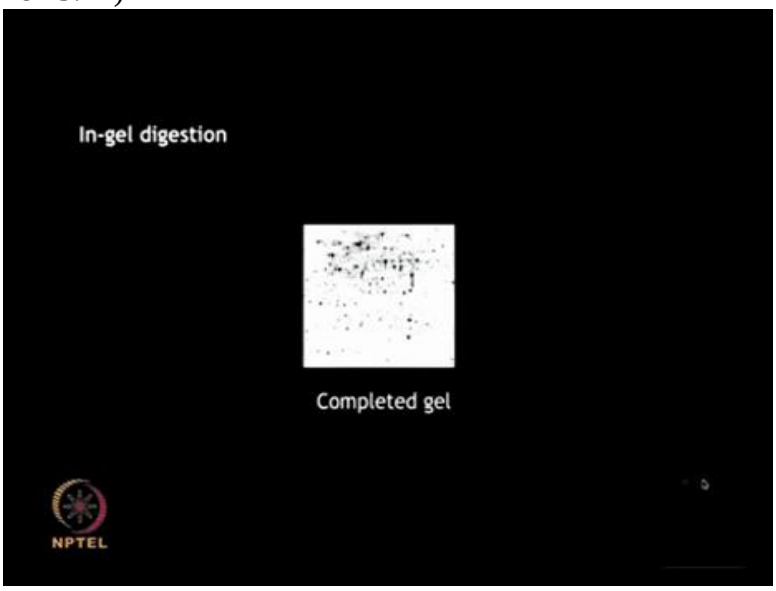

Sample preparation and spotting

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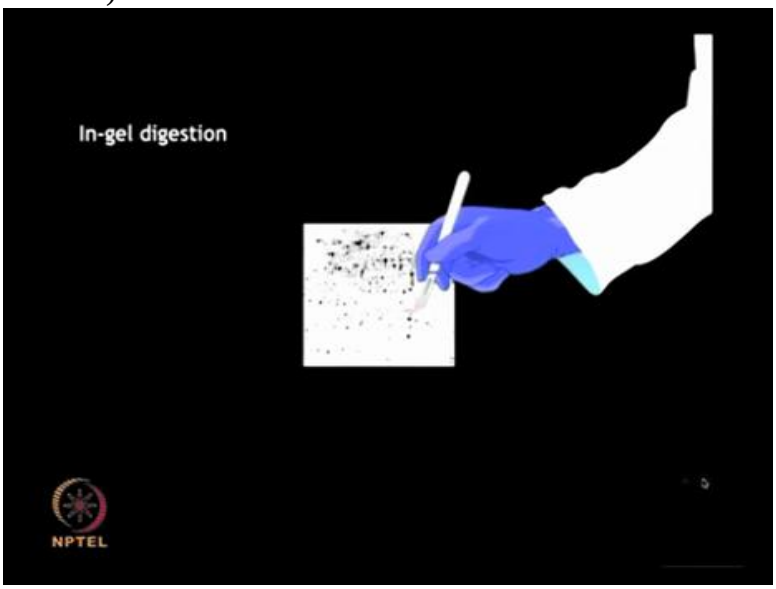

: The protein sample must be prepared suitably before it can be analyzed by the mass spectrometer.

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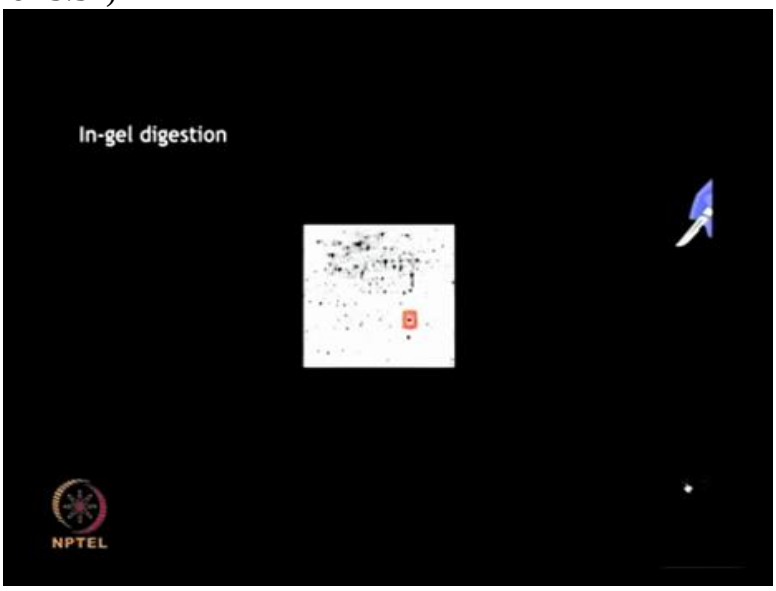

If you have run a 2D gel, so, first of all the purified protein of interest need to be excised from the gel on which it has been electrophorsed and dissolved in the suitable buffer.

So depending upon the application if you have purified a protein you can separate that on the gel, and the cut that band. Or if you have a mixture of proteins in 2D gel, you can just excise that particular spot.

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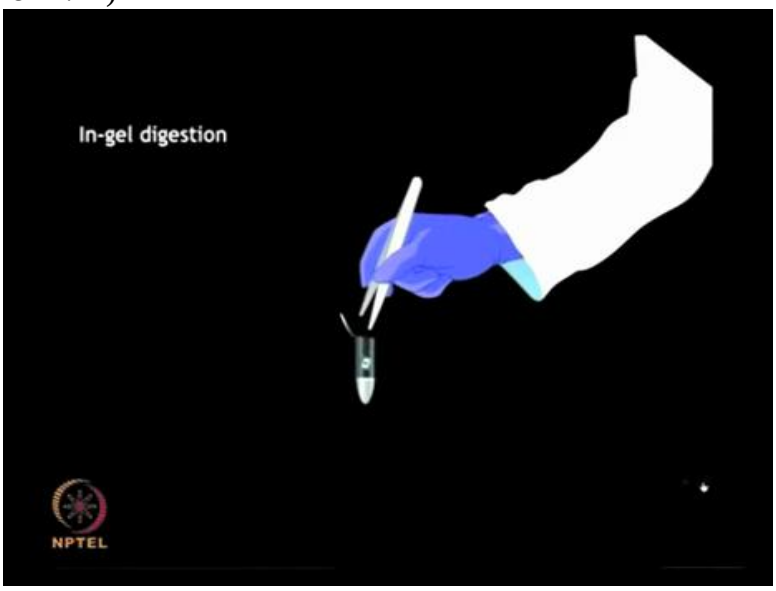

Spot can be dissolved in a suitable buffer,

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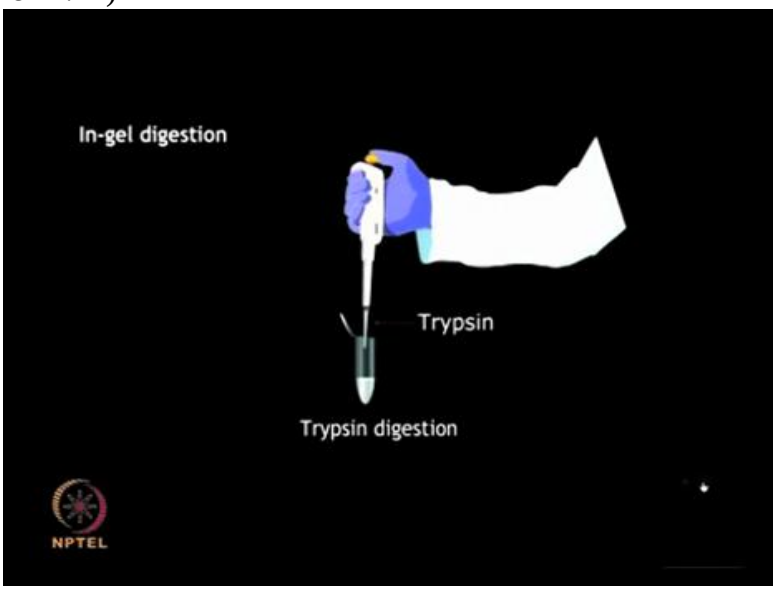

Trypsin is then added to this mixture in order to carry out digestion of the protein

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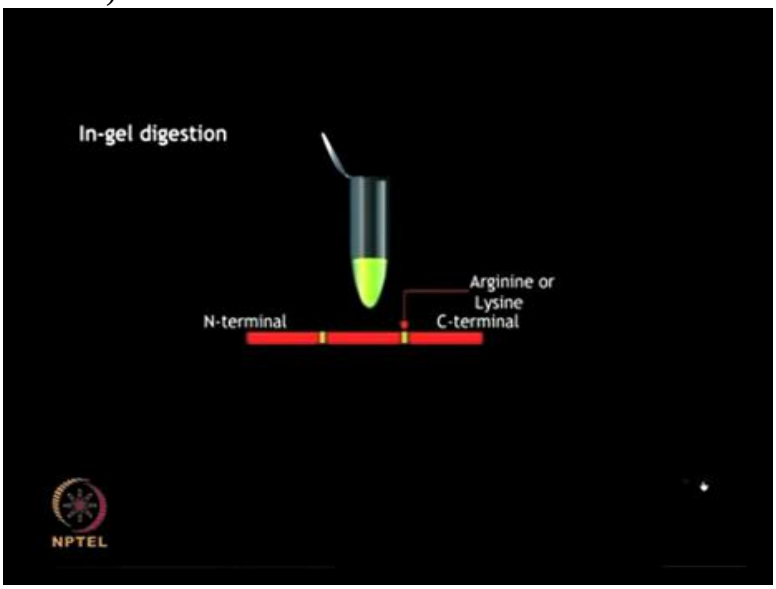

Trypsin cleaves the protein at the C terminal of the Arginine and Lysine residues, but that is not always universal.

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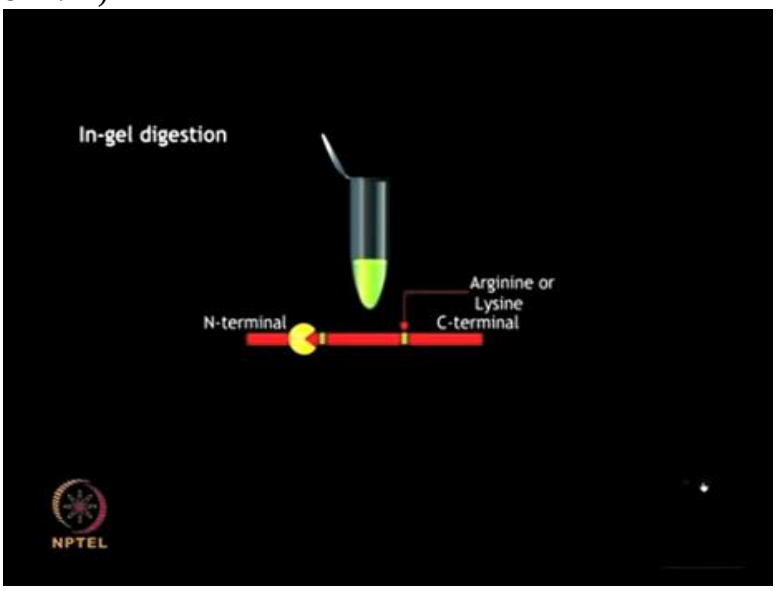

If you have a Proline present immediately after,

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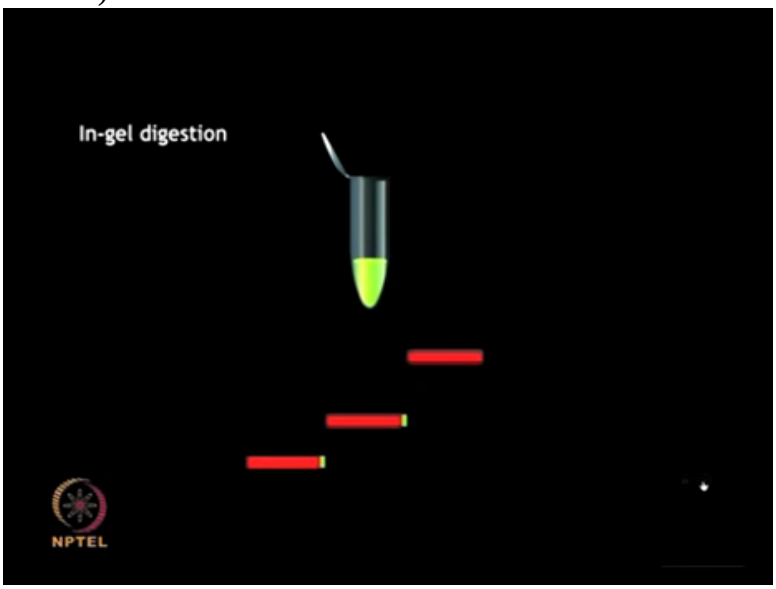

then it will hinder that, but overall the protein is digested into smaller fragments of manageable size

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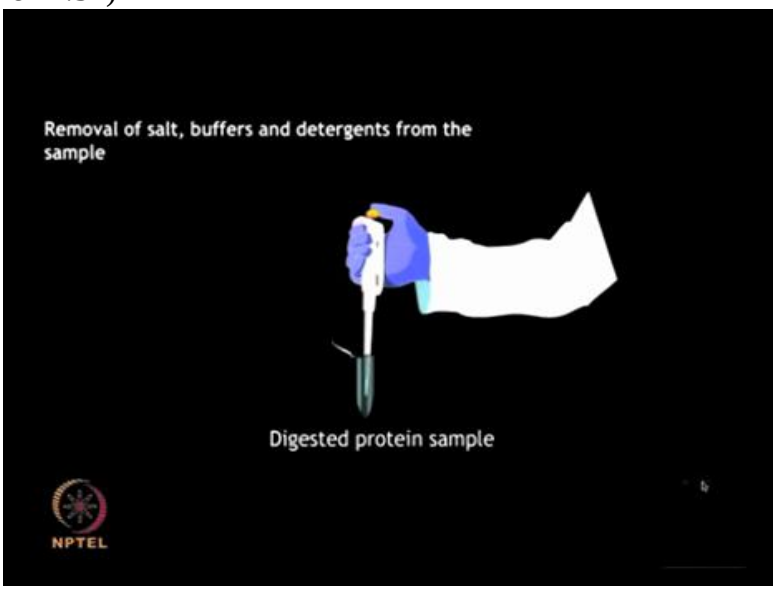

Once the protein sample has been digested, all the salt, buffer and any detergent must be removed from the sample. So, after doing in gel digestion and before proceeding for the mass spectrometry analysis,

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in between an efficient step is to use some filters

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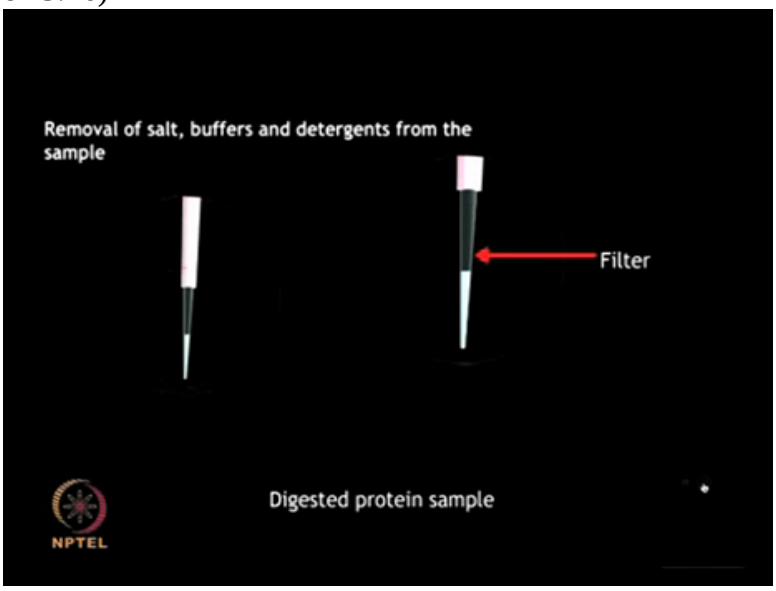

or ZipTip which can eliminate some of these contaminants and salts It offers several advantages such as quick verification, sample enrichment and ensures that there is no contamination.

So, there are multiple advantages of using ZipTips. However, it can purify only limited volume of the sample, and also it adsorbs some amount of the protein sample thereby leading to losses.

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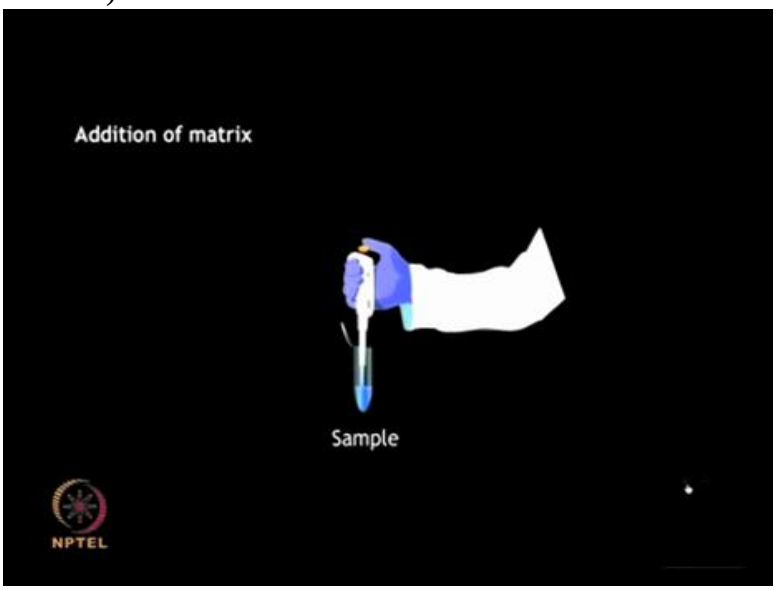

The purified protein sample can be mixed with

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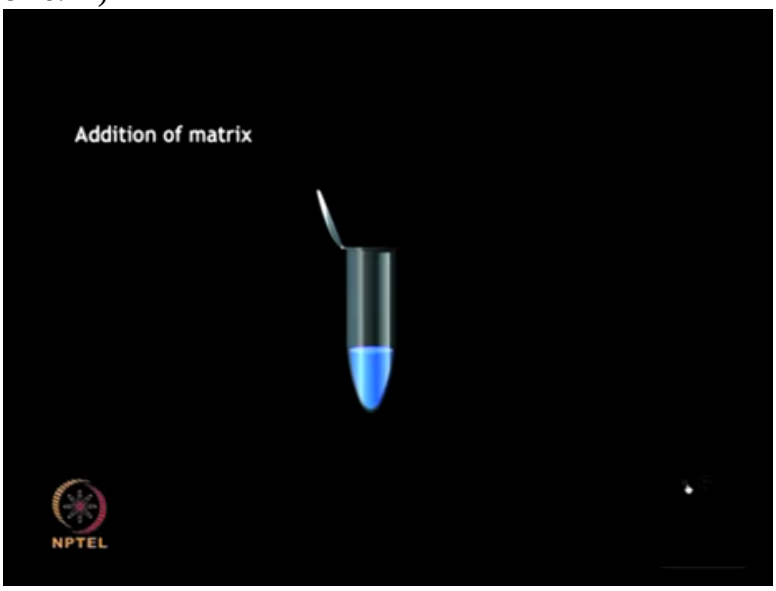

an aromatic matrix compound such as alpha alpha-cyano-4-hydroxy-cinnamic acid or Sinapinic acid in the presence of an organic solvent

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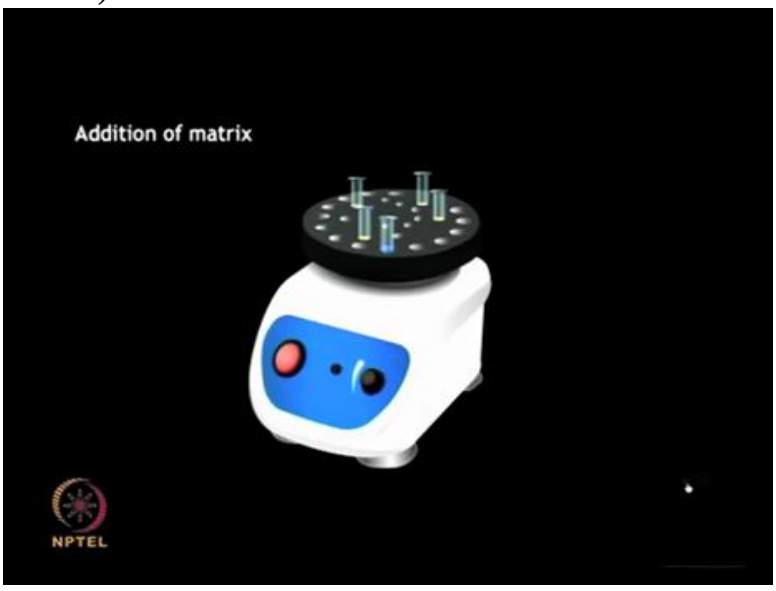

The components are mixed thoroughly

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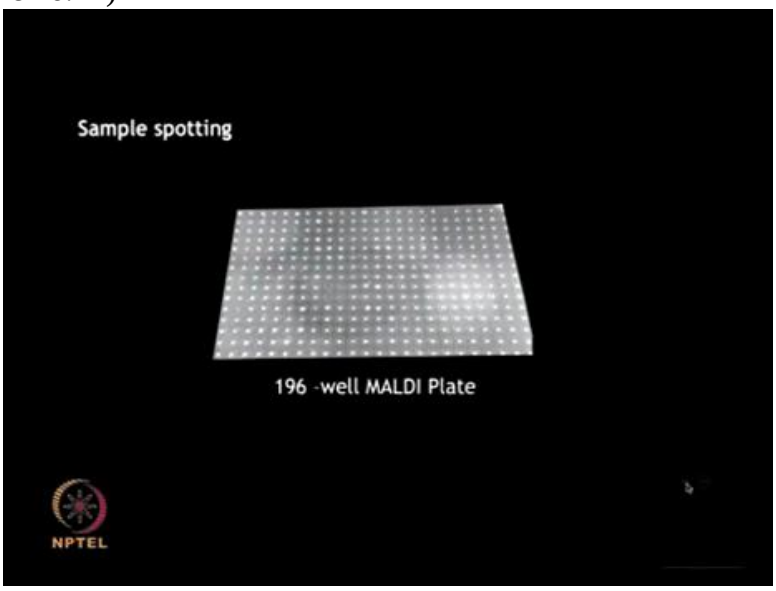

and then the solution containing the organic matrix with embedded analyte of interest can be spotted onto a metallic MALDI sample plate.

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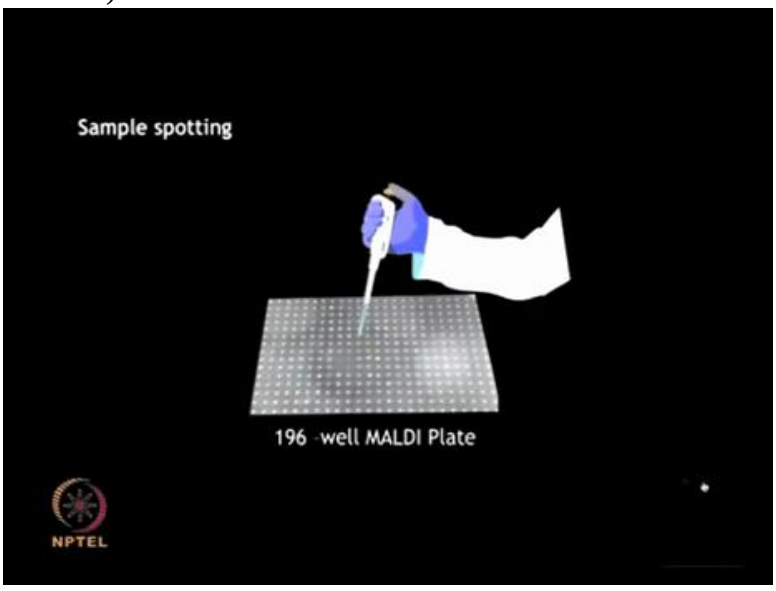

MALDI gives you an opportunity to analyze

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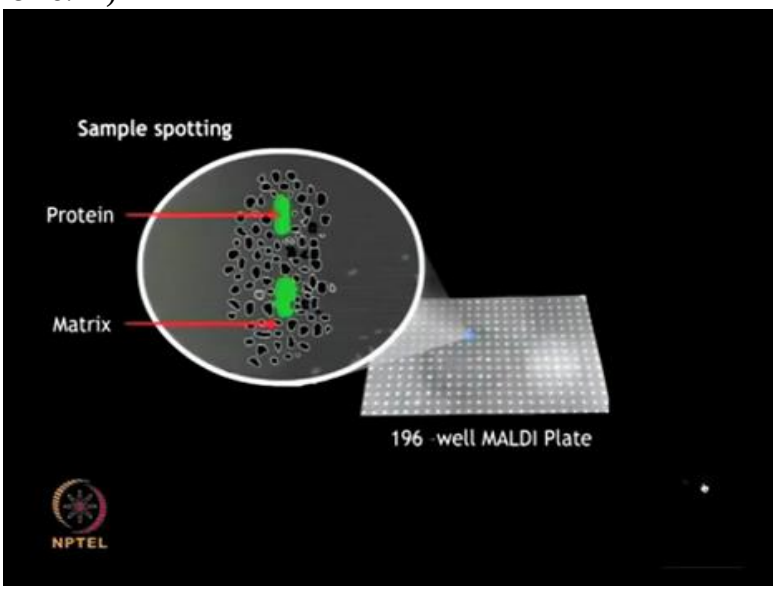

large number of samples in high throughput fashion.

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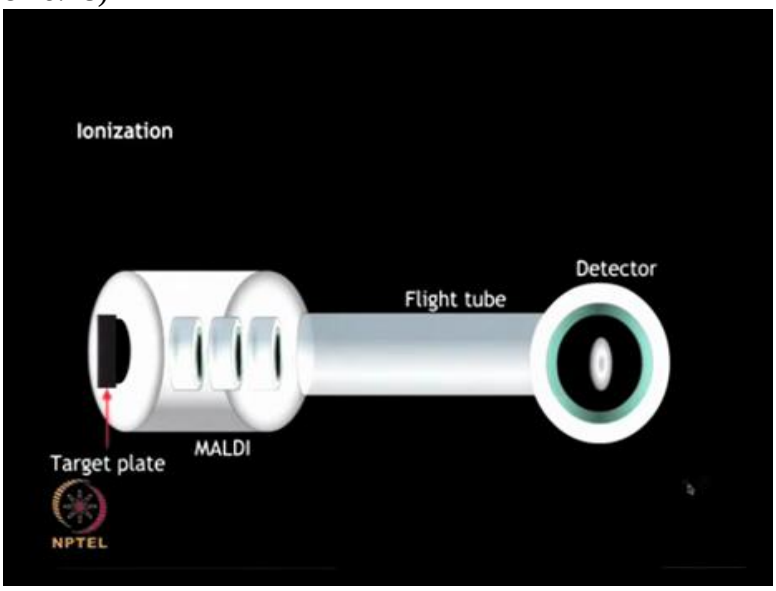

The target plate

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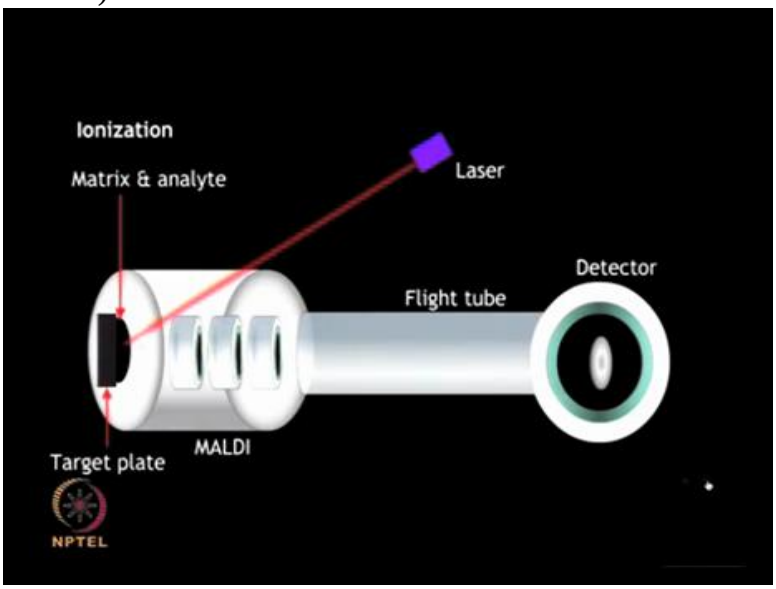

containing the spotted matrix and analyte

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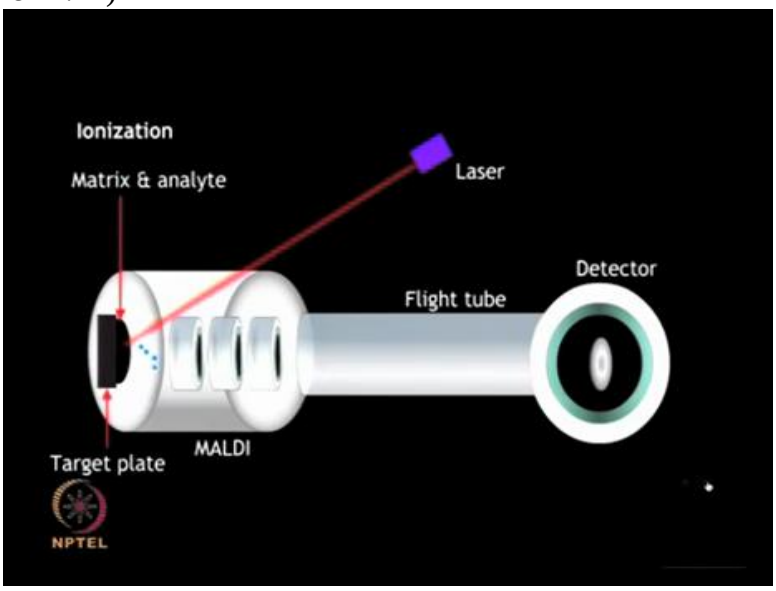

The laser energy gets absorbed by the matrix and is transferred to the analyte molecules

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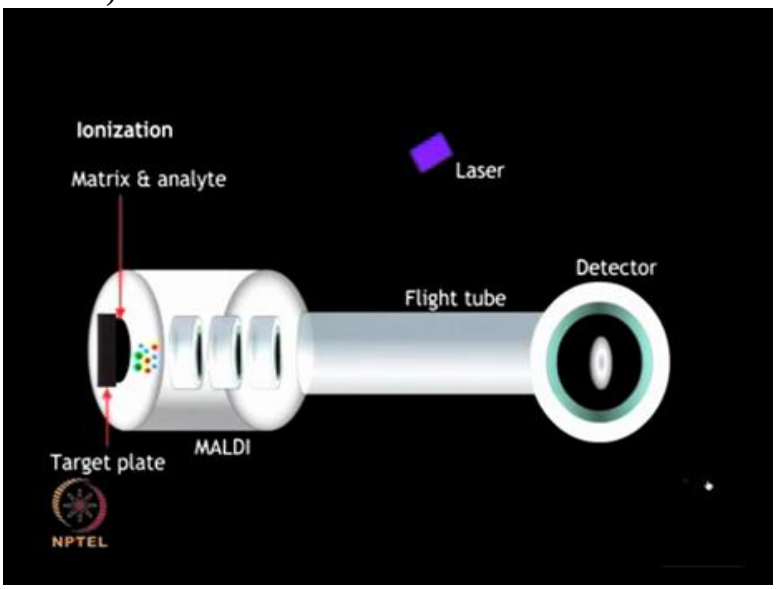

…which undergo rapid sublimation resulting in gas phase ions

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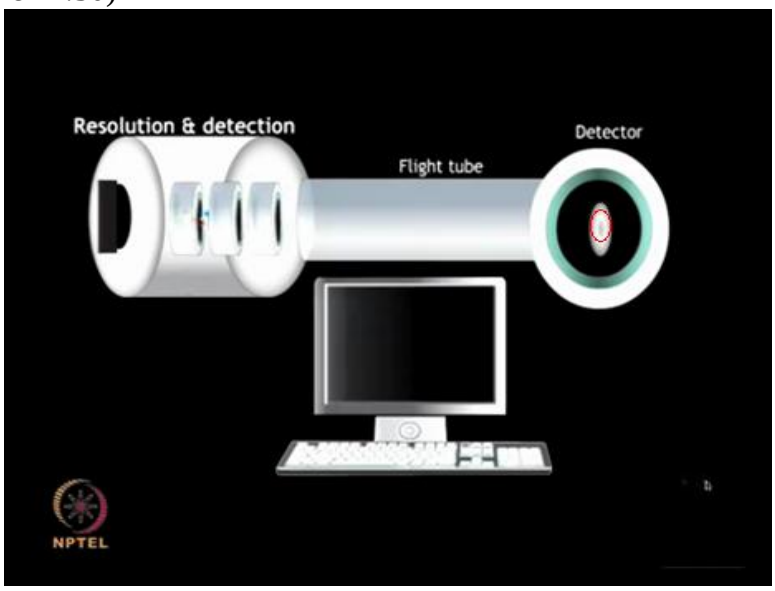

The gas phase ions generated are accelerated and travel through the side tube at different rates.

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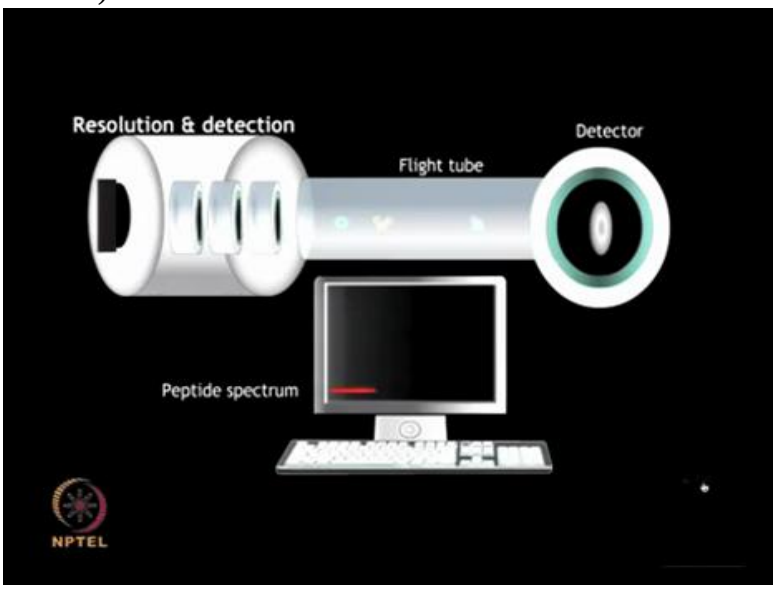

The lighter ion moves rapidly and reaches the detector first,

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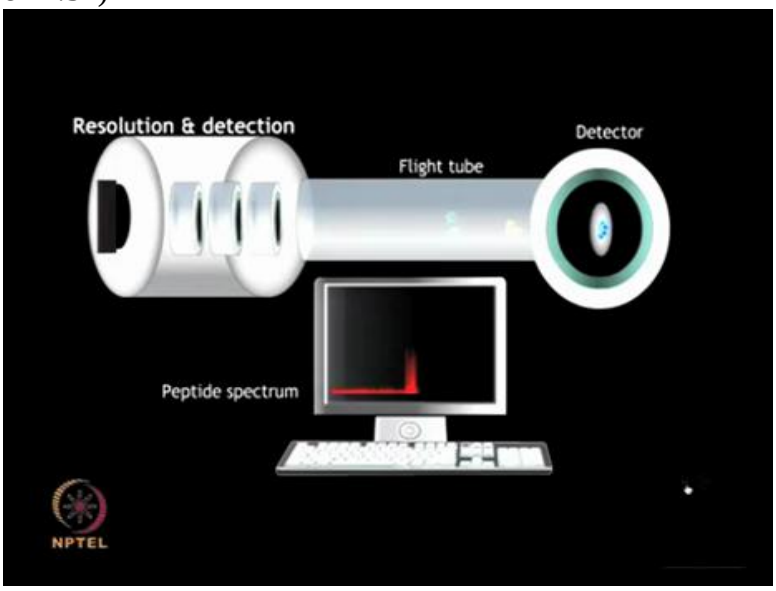

while the heavier ions migrate slowly These ions are resolved and detected on the basis of their mass to charge ratio and a mass spectrum is generated. Parameters such as

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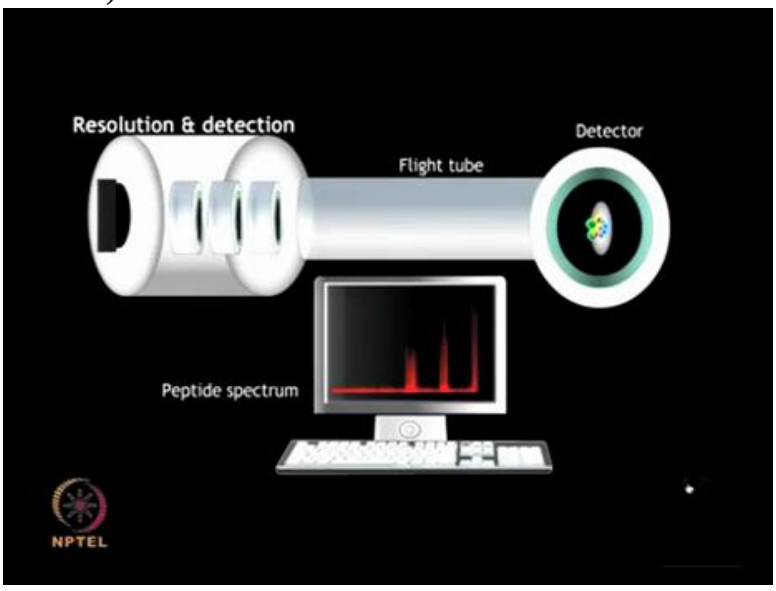

geometry design, power supply quality, calibration method, sample morphology, ion beam velocity etc all of these factors affect the accuracy of mass detection.

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After looking at the animation now let us talk about how to prepare the sample let discuss these steps in more detail.

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The first part will be in gel digestion of the protein samples. So, the mass spectrometric identification of target protein greatly depends on the efficacy of in gel digestion process that generates a mixture of peptides from the target protein through proteolysis digestion.

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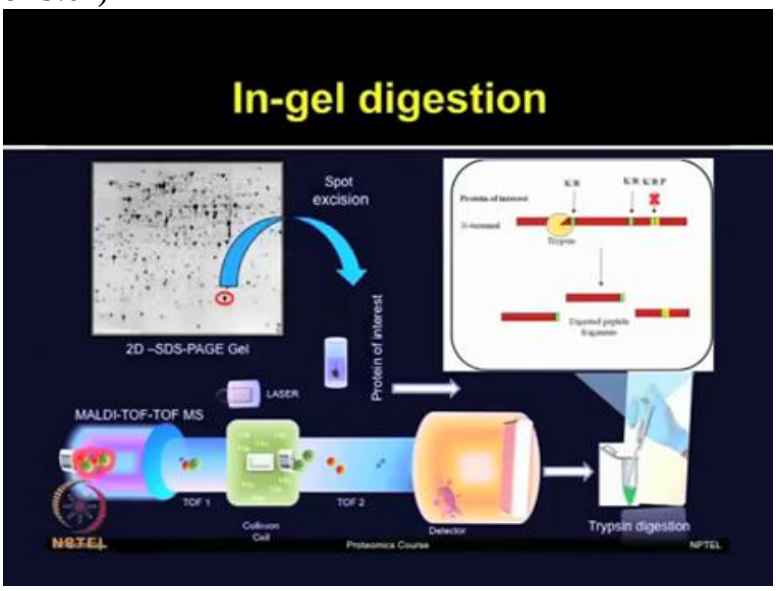

This slide gives you an overview since in the last module we discussed about, two dimensional gel electrophoresis. It shows that if you have a spot of interest, you can excise that spot from the 2D gel and then subject that to in gel digestion followed by the mass spectrometric analysis.

In gel digestion is a multistep procedure which includes spot selection, spot excision, removal of stain, reduction, alkylation, proteolysis cleavage as well as peptides extraction. So, multiple steps are involved and how good your in gel digestion is, is going to ensure the success of the spectrum generated from the mass spec.

Now although this overview shows you the process to start with the 2D gel, but same can be also applied for even the gel-free proteomic techniques.

If you want to analyze a sample even from the gel-free proteomic based approaches, it is often good idea to separate those protein complex mixture on the SDS page gel, excise the bands and then extract the proteins, on that perform the in gel digestion.

So, that you can simplify the proteome and then you can increase the overall proteome coverage. So, similar protocol can be modified and used for various types of applications in the proteomics. So, this slide gives you various recipe for performing in gel digestion.

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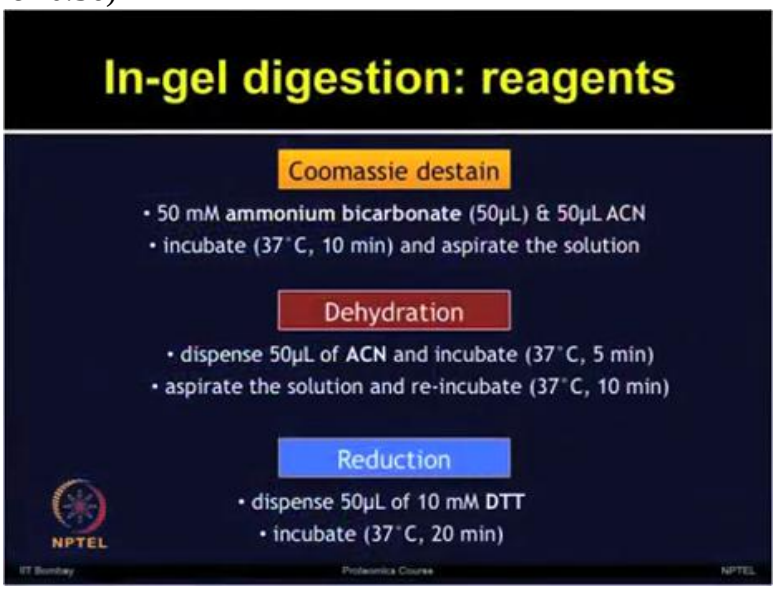

The first step is the de-staining of the spots or the band, because you have stained the gels with the Coomassie brilliant blue or some other or some other stains. And first of all you would like to remove these stains.

So, stain removal is essential prior to the mass spectrometric analysis, the excise gel pieces should be washed with the bicarbonate buffer and acetonitrile for removal of the staining agent.

You can see the recipe in the slide; we will talk more about how to do these experiments when we come to the animation of these steps.

Acetonitrile reduces the hydrophobic interaction between protein and the stain while the ionic solution decreases the ionic interaction between negatively charged Coomassie brilliant blue dye and the positively charged protein.

Once the Coomassie is destaining or the destaining step is performed then we need to dehydrate the gel pieces, which can be done by using addition of acetonitrile.

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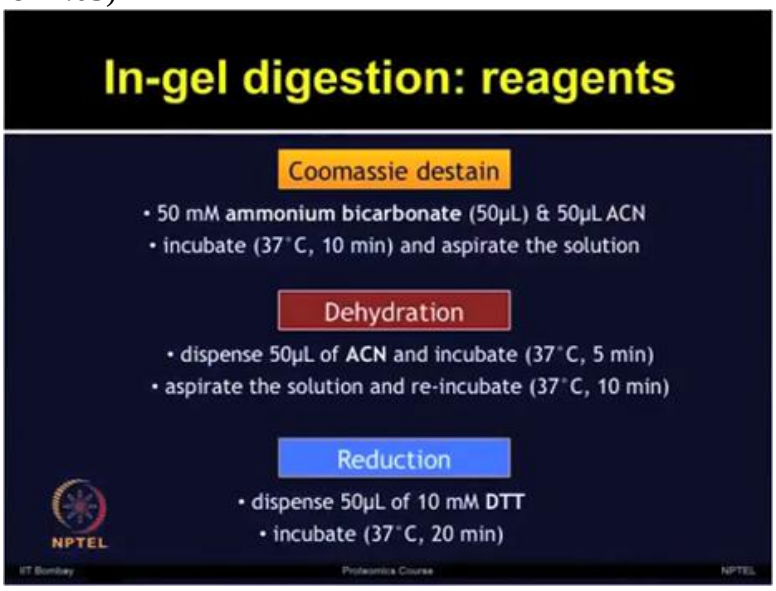

After this incubation is done then you are ready for performing reduction step. Now, why reduction step is required? So, after this stain removal the next steps are including reduction as well as alkylation of protein residues.

So, that you can denature the protein into it is primary structure, continuing on to the same theme of in gel digestion and various steps required performing such experiment. let's now look at the next step which is alkylation.

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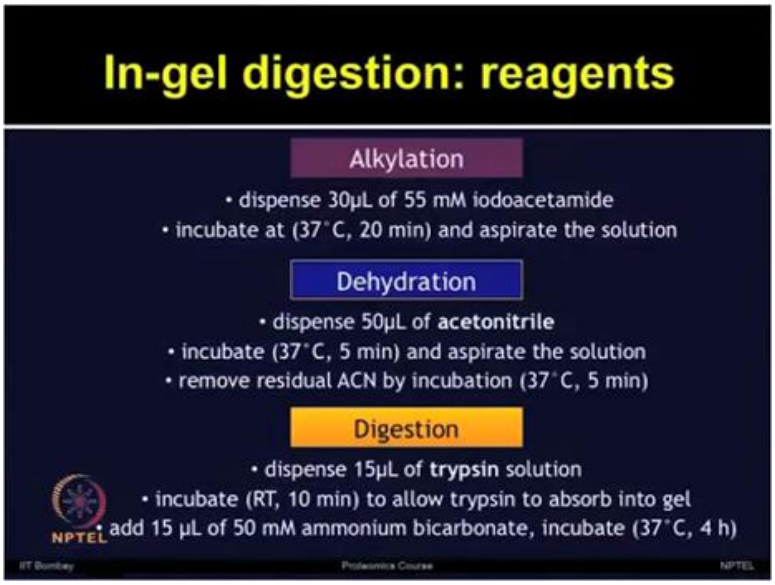

So, in the alkylation you need to add the iodoacetamide. The reformation of disulphide bonds may occur. So, to prevent that iodoacetamide which is an alkylating agent is used here.

Again you need to do the dehydration step, as recipe is mentioned in the slide, you can add the acetonitrile and then we are ready perform the digestion which usually done by using Trypsin.

So, prior to the MS identification proteins are digested to generate peptides, there are various enzymes which one can use for performing this step, but Trypsin is most widely used proteolytic enzyme, used for the protein digestion. It breaks a peptide bonds at the carboxyl terminals of basic amino acids, such arginine and lysine.

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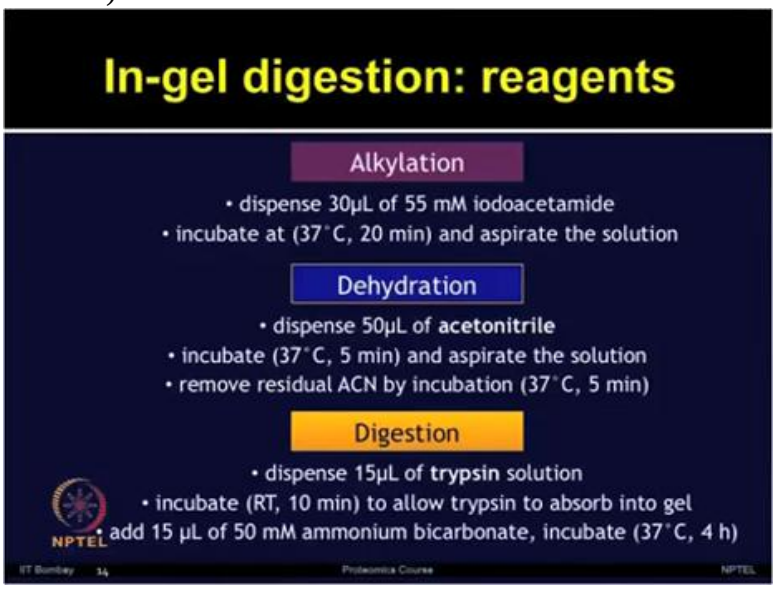

Once digestion is done which is usually the overnight step then one need to do the peptides extraction of the digested proteins. So, peptides generated through the proteolytic cleavage can be extracted by using recipe including formic acid or trifluoroacetic acid TFA in the 50 percent acetonitrile solution.

Now coming back to the importance of reduction and alkylation of the proteins; we mentioned that we need to add DTT and IAA in various steps during the in gel digestion process

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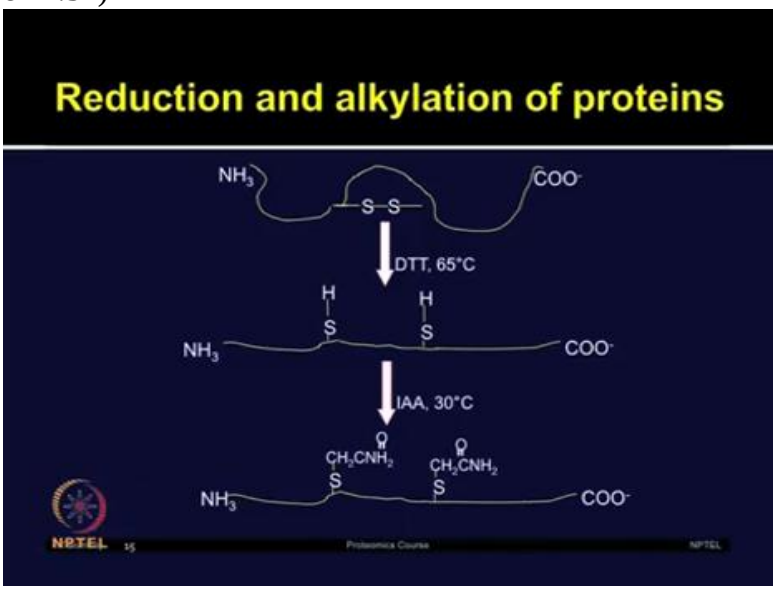

So, DTT is important for treatment of proteins to break the disulphide bonds, which we have also talked in the sample preparation when we discussed earlier. Now iodoacetamide it adds the iodoacetamide group to the sulfhydryl group and prevents disulphide bond formation. So, these steps are quiet important in gel digestion process.

So coming to the specificity of Trypsin; first of all let discuss why we need to do the proteolysis digestion? See, you want to generate the peptides with the molecular weight within the mass range of mass spectrometer; we always want to simplify the process for even very superior analytical instruments so that you can increase the efficiency of the process.

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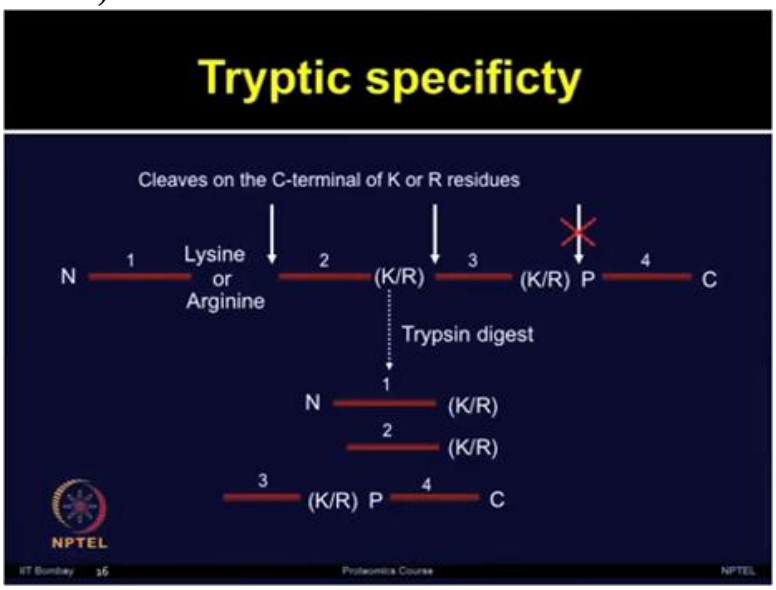

The enzymatic digestion are performed with the various enzymes, but typically with the Trypsin which cleaves at the c terminal of lysine and Arginine residues, but exceptions can occur with the proline, if proline is present then that breakage will not happen.

So, one can use the modified Trypsin which is a serine endopeptidase; however, it cleaves at the proline- lysine and the proline-arginine bonds at the much slower rate.

You can see the cleavage process and the specificity in the slide, where it shows if you have the lysine or arginine residue it can break the bonds, but when there is a proline residue present there then it cannot cleave.

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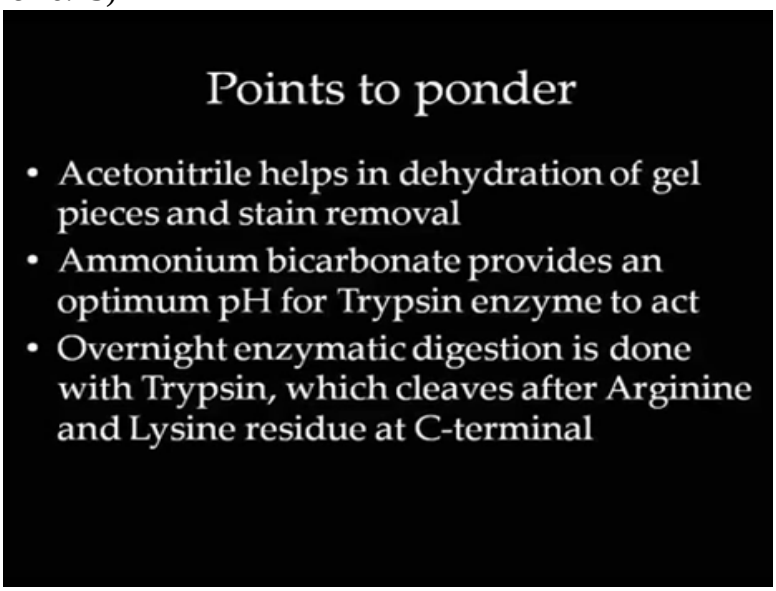

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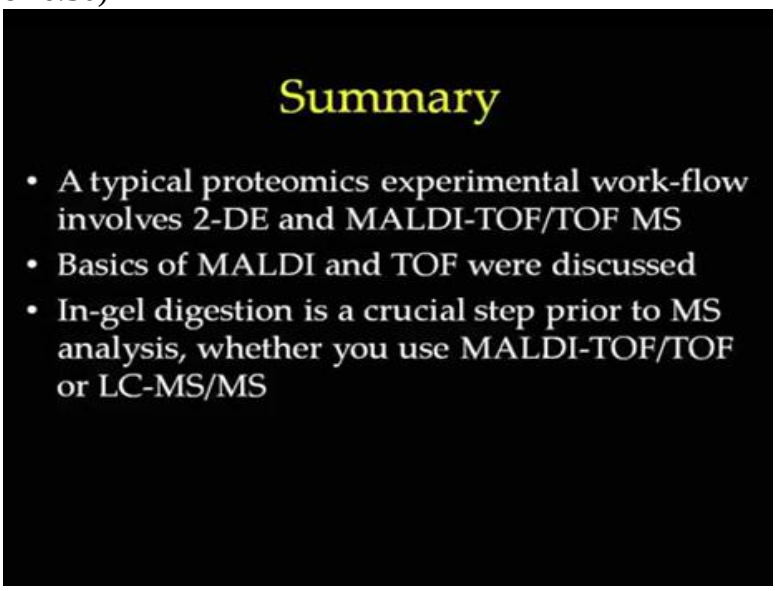

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