Introduction to Proteomics
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Lecture - 23
Liquid chromatography

Today we will talk about Liquid Chromatography, Mass Spectrometry or LC-MS/MS.

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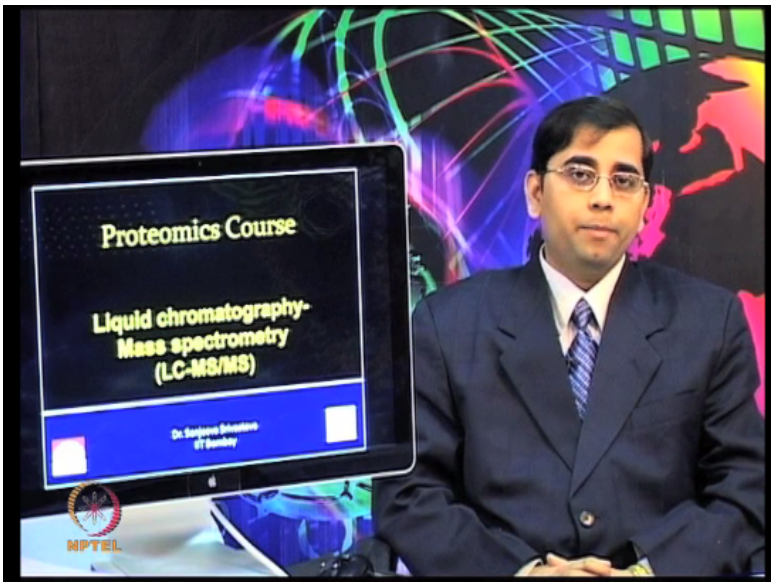

MS are based on production of ions which are subsequently separated according to the M/Z ratio. The resulting mass spectrum provide a plot of relative abundance of generated ions as a function of M/Z. MS provides most versatile platform and comprehensive analytical technique for the proteomic scientist for wide variety of applications.

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So today's lecture I will first give you an overview of the mass spectrometry workflow. We will then talk about individual component in some more detail such a liquid chromatography, ionization sources.

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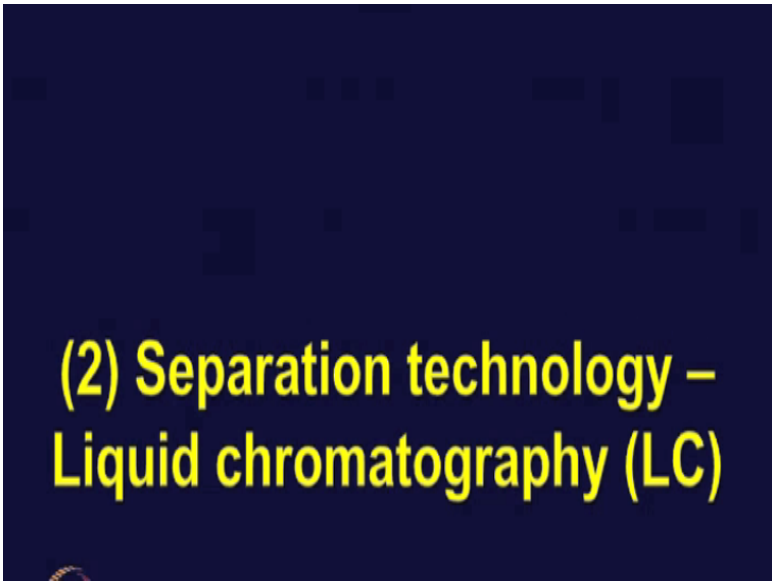

Software doing the (()) (01:22) digestion, now let us move on to second part the separation technology, liquid chromatography or LC. So chromatography in general is a physical separation method in which component for Separation selectively distributed into immiscible phases, (()) (01:42) phase flowing through the stationary phase. Now depending upon the mobile phase the technique is termed as either liquid chromatography or gas chromatography etcetera.

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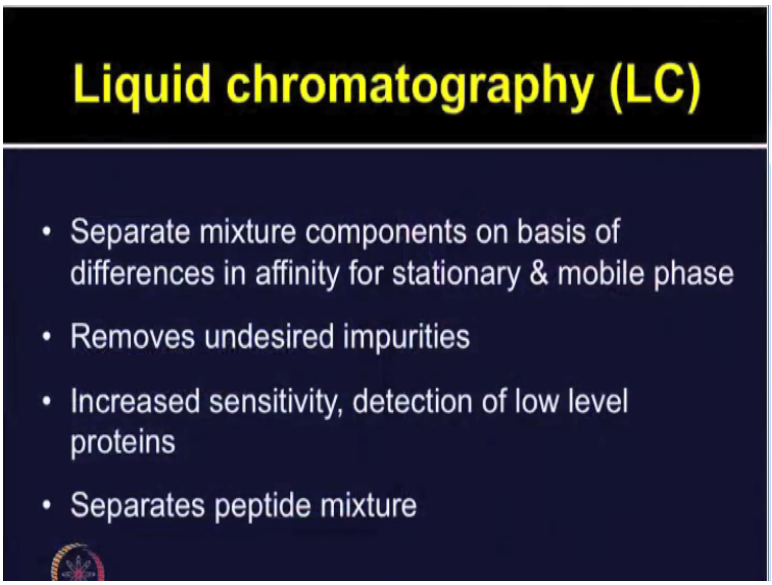

So what is liquid chromatography? The peptide mixtures can be fractionated in-line with the instrument prior to the introduction into mass spectrometer, that I think one of the advantage for doing the proteomic applications. So in LC it can separate mixtures or components on the basis of differences in the affinity for stationary and mobile phase. Liquid chromatography is also useful for removing the undesirable impurities.

It also increases reproducibility in the sample as well robustness of the MS measurement. LC along with further enrichment can help into the concentration of the available examples. It also helps in increase sensitivity, detection of low level proteins and further it can separate peptide mixture.

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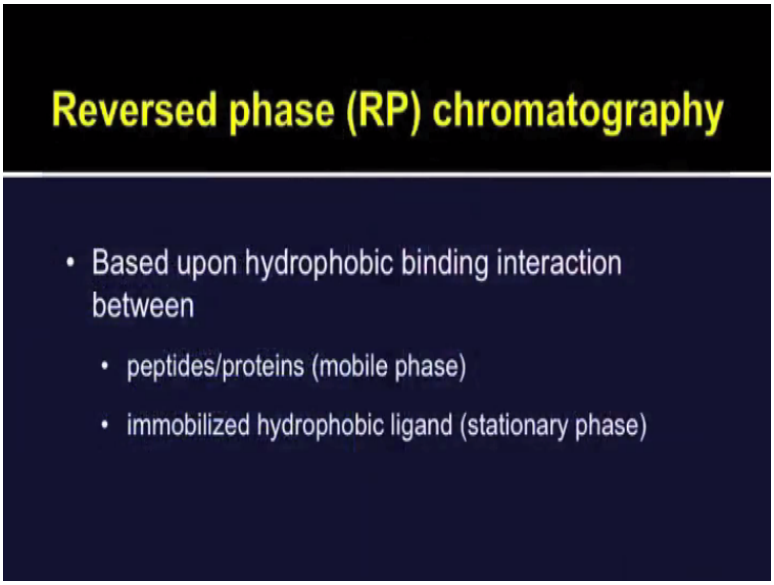

There are various type of chromatography one can use for different application. In Proteomic, the most commonly use method for the peptide fractionation is Reversed-phase liquid chromatography or RPLC which separates peptides based upon the hydrophobic binding of interaction between the peptides or proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase.

By utilizing this hydrophobic one can separate the peptides. However, if your Proteo mixture or the peptides are very complex then can one can further use another type of chromatography method such as strong cation exchange as well as different type of multi-dimensional separation can be employed.

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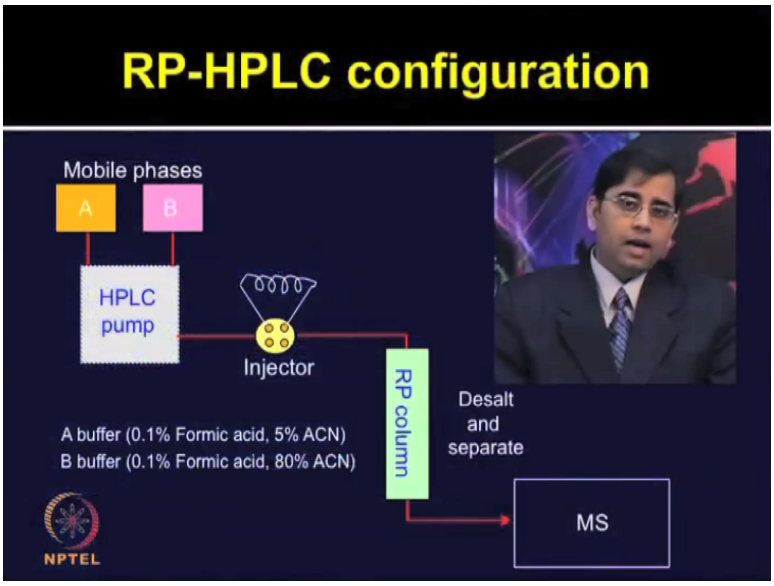

In this slide, I have shown you the configuration for Reversed-phase HPLC. As you can see the two components in the mobile phase A and B link with the HPLC pump. A buffer can provide between the range of 0.1% of Formic acid to 5% of Astro Nitrate and in B 0.1% to 80% of Astro Nitrate. So first of all equate a system in buffer A then load the peptides and wash those then run the gradient of increasing mobile phase B, now wash the reversed-phase with buffer B and then re- equate in A, in that way the peptides can be separated.

And prior to MS analysis it can be desalted so that there is no inference of salt. Now reversed-phase is most commonly employed with the electrospray ionization because of its compatibility of reversed-phase acidic acquire and polar mobile with electrospray ionization.

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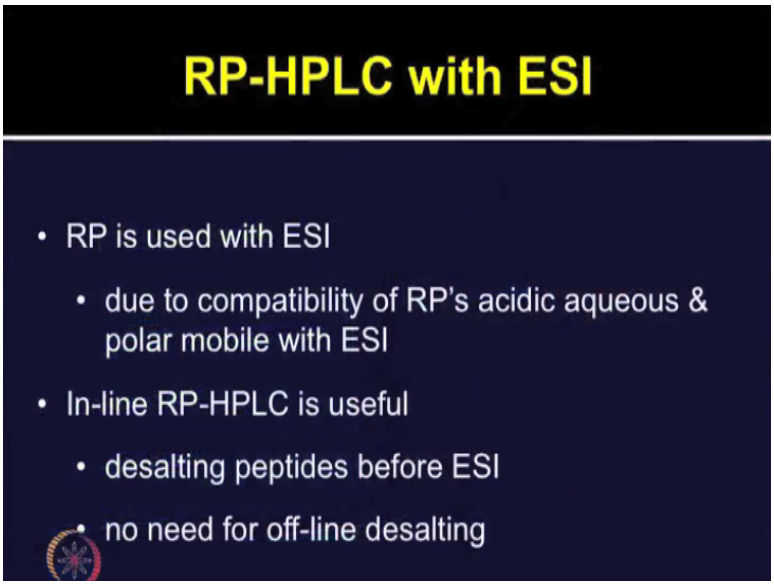

As I mentioned in the proteomic one can use ESI and liquid chromatography in-line, so directly samples can be pre-fractionated and further analyze using Mass specter. The In-line reversed-phase, HPLC very useful because it can do the desalting of peptides prior to ionization in ESI. There is no need for doing separate offline desalting and pre-fractionation. It can focus peptides from the dilute samples into the narrow chromatography bands and it also enhances the sensitivity.

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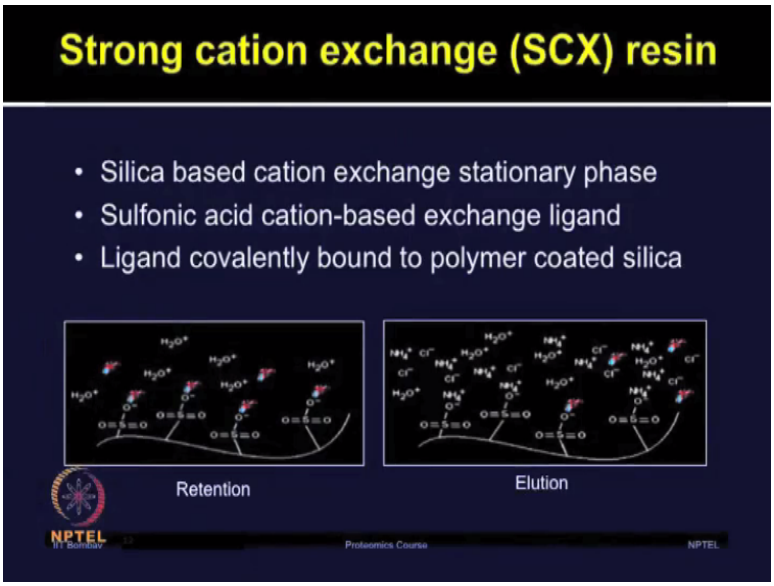

Let us now talk about another separation technique which is also commonly employed in proteomics which is this Strong cation exchange or SCX and SCX. So in SCX resin this silica based cation exchange use in the stationary phase. The Sulfonic acid cation-based exchange

ligands are used. These ligands are covalently bound to the polymer coated silica. Now as I have shown in this slide the two important phenomenon here one is Retention other is Elution.

In retention, that is based on the electrostatic attraction between the negatively charged Sulfonic acid and positively charged peptides. The Elution can be performed by an exchange of peptides for cation of mobile phase additive, the ammonium ions. And this is a reaction to the high concentration of cations. Now HPLC can be Microcapillary, it can be Nano LC or it can also be different type of chip-based chromatography.

Now for proteomic various type of Microcapillary, Nano LC and different type of chip technologies are currently used.

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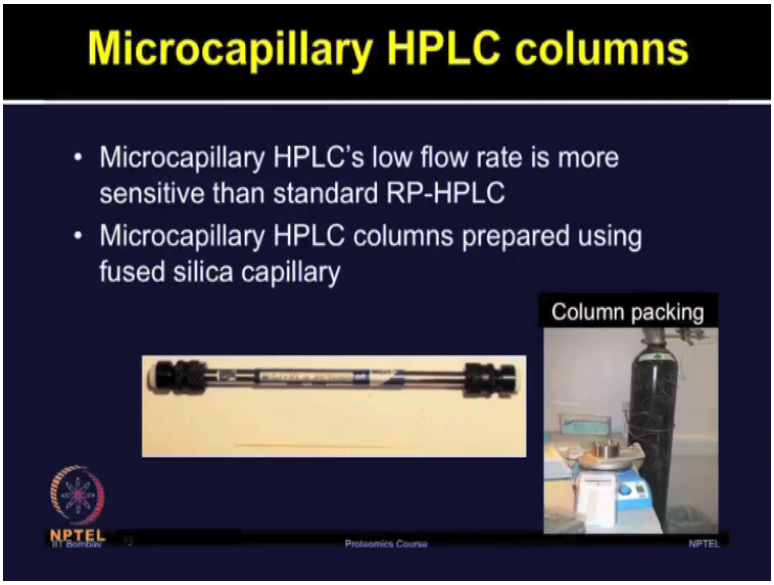

In the Microcapillary HPLC the low flow rate which is < one microliter per minute is more sensitive as compare to the standard Reversed-Phase – HPLC which is around 50 microliter per minute. The Microcapillary HPLC is required for the analysis of (()) (08:18) amount of the peptide. One can prepare the Microcapillary HPLC by using fused silica capillaries and then pack that with the Reversed-Phase packing material.

For pre-fractionation multi-dimensional separations are used. The different types of principles are involved for separating these peptides.

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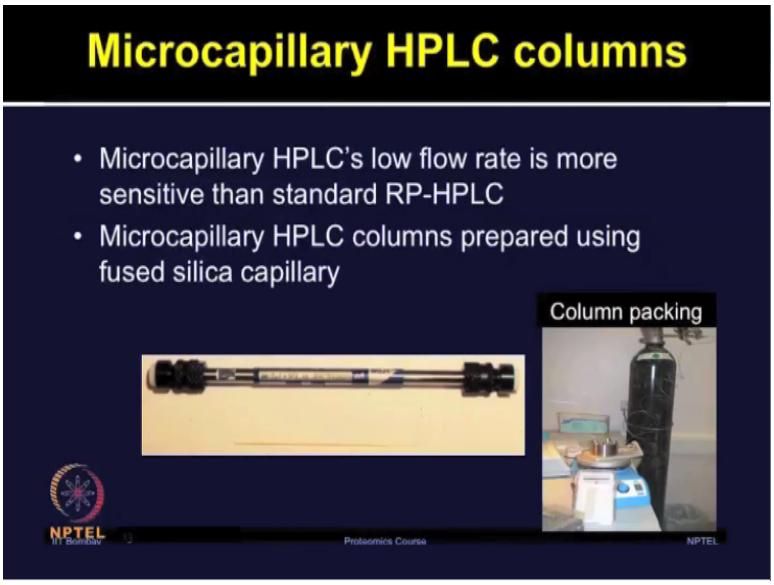

One can use Size exclusion chromatography which separate based on the molecular weight or the size. Ion exchange chromatography based on the charge. Capillary electrophoresis based on charge. Reversed-phase based on the hydrophobic. Affinity chromatography which is based on the biological interactions.

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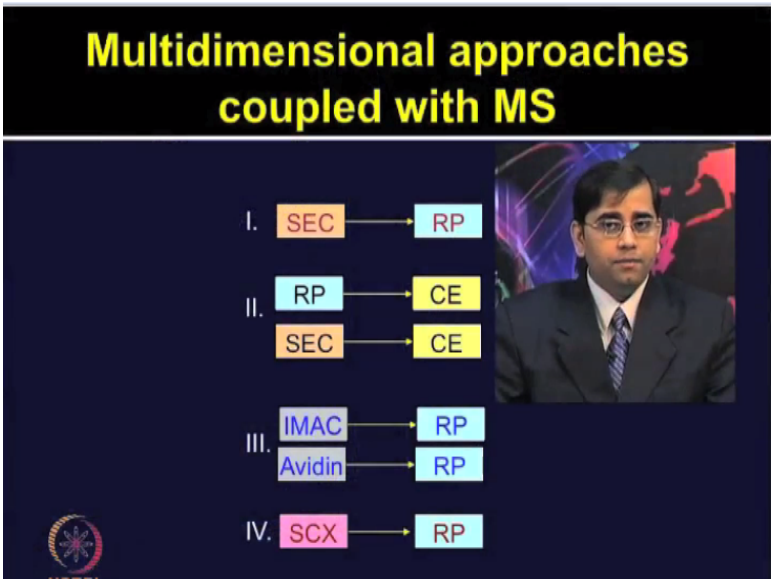

Now Multi-dimensional approaches can be coupled with the Mass spectrometry. One can use various type of liquid chromatography method in (()) (09:26) and then do the multi-dimension separation. Different type of approaches have been tried for example, Size exclusion

chromatography followed by Reversed-phase chromatography but it has resulted into the poor resolution of peptide and size exclusion chromatography, so that is not very widely used.

Other approaches include Reversed-phase chromatography followed by the capillary electrophoreses or Size exclusion chromatography followed by the capillary electrophorus. Due to the limited loading capacity and low loading volume of the capillary electrophoreses again it is not very popular. The Affinity chromatography based separation such as IMAC or Avidin followed by the Reversed-phase chromatography those are commonly used.

And its Strong cation exchange followed by the Reversed- phase is most commonly used. So this slide gives you the overview of various type of methods liquid chromatography separation which can be employed prior to injection into the ionization source.

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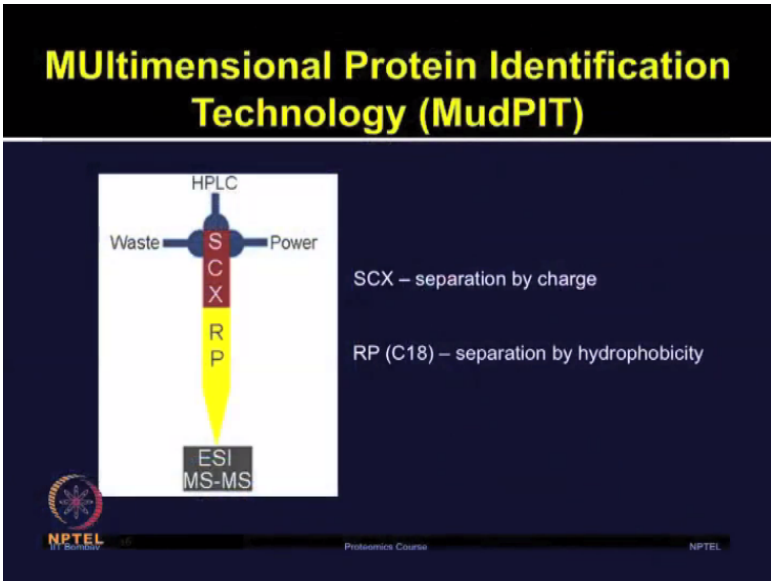

As I mentioned this Strong cation exchange as well as Reversed-phase chromatography together can be employed for various proteomic application, which have been demonstrated in the multi-dimensional protein identification technology or MudPit. In this technique the strong cation exchange separates by the charge, which provide low resolution fractionation in the beginning and then Reversed-phase C18 column which separates peptide based on the hydrophobicity and it provides high resolution gradient.

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Let me describe some of the concepts involved in the liquids chromatography in following animation. I will also discuss MudPit and some of the chip-based approaches which are integrated for proteomic application with the liquid chromatography. So let us discuss these concepts in following animation.

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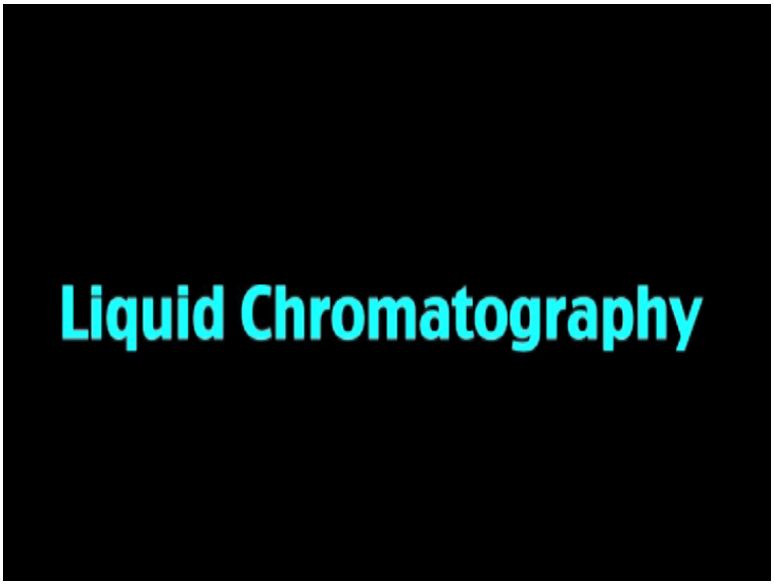

A typical liquid chromatography setup consists of solvent bottles, (()) (11:51), sample injector, column and detectors.

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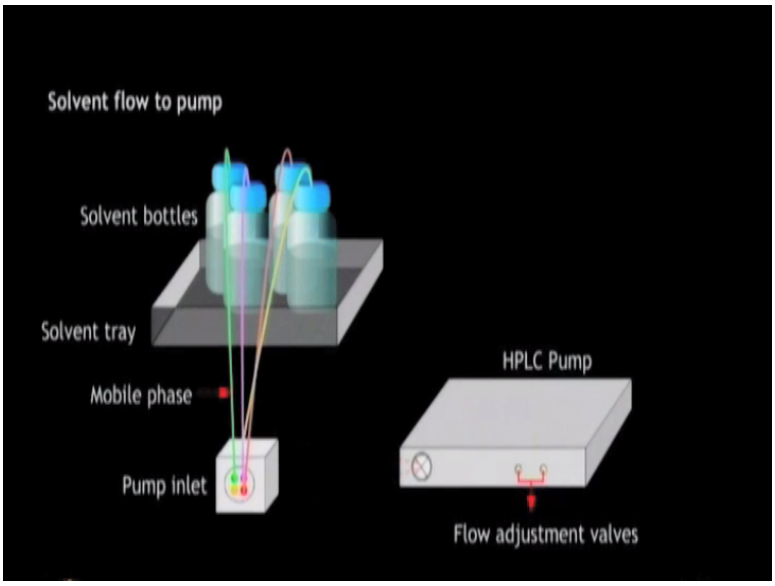

Here you can see the various components which are involved in performing the liquid chromatography. Different solvents can be placed in the solvent bottles depending upon the purification requirements. These solvents are mixed in the desired ratio and pumped into the column.

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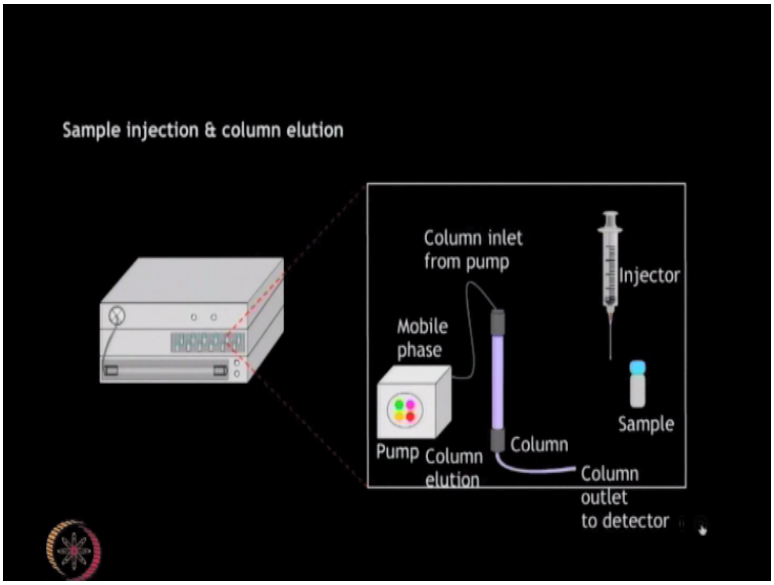

During elution after removal of any tray there inside by means of the big amplifier. The sample injection system maybe automatic or manual. The automatic sample uses a syringe to inject the sample which is placed in a vial directly into the column. Once the sample is injected mobile phase flows into the column through the pump, the column consists of stationery metric that

preferentially bind certain analyst. The outlet from the column enters the flow cell where it can be detected.

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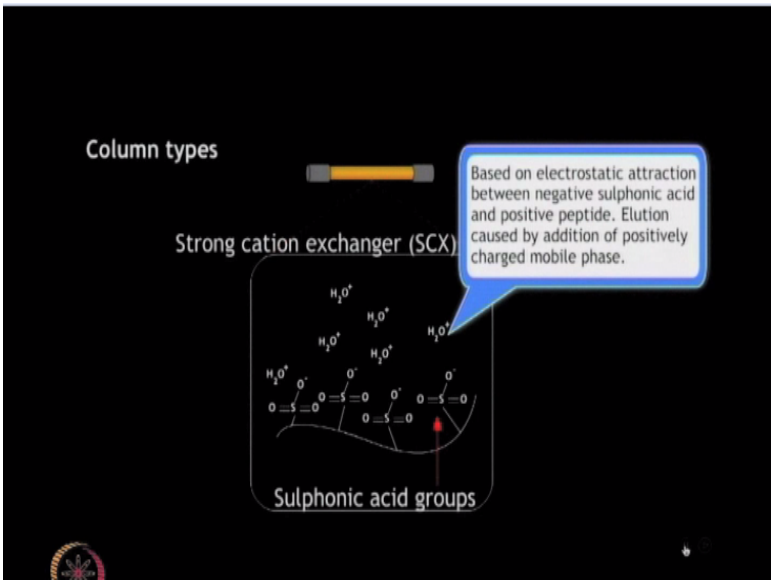

The various stationary phase matrices are available that separate the components of the mixture based on different principle where of the most commonly used matrices include the Strong cation exchanger or SCX which separates charged peptide based on their electrostatic interactions with negatively charged Sulfonic acid group on the resin surface. Now elution can be caused by the addition of positively charged mobile phase.

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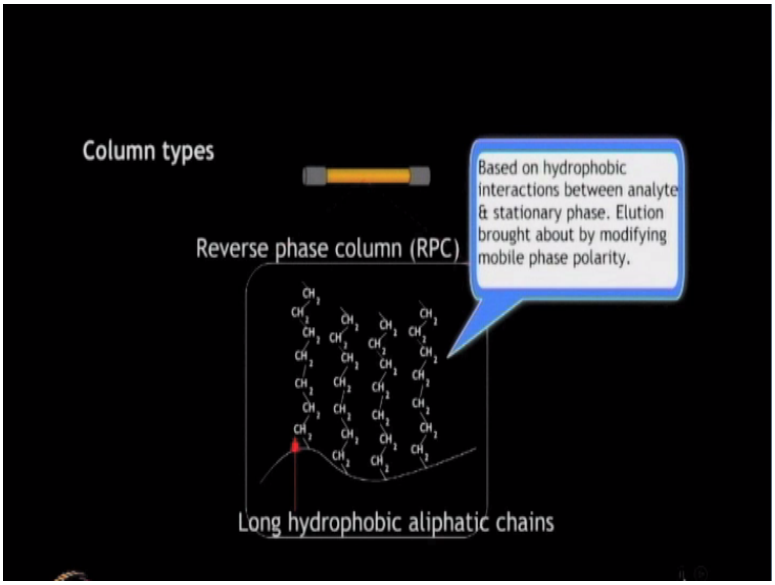

The Reversed-phase chromatography is another commonly use tool which uses a hydrophobic metrics consisting of long aliphatic carbon chains. These resin analyze separate on the basis of hydrophobic interactions and be eluted by changing the polarity of the solvent.

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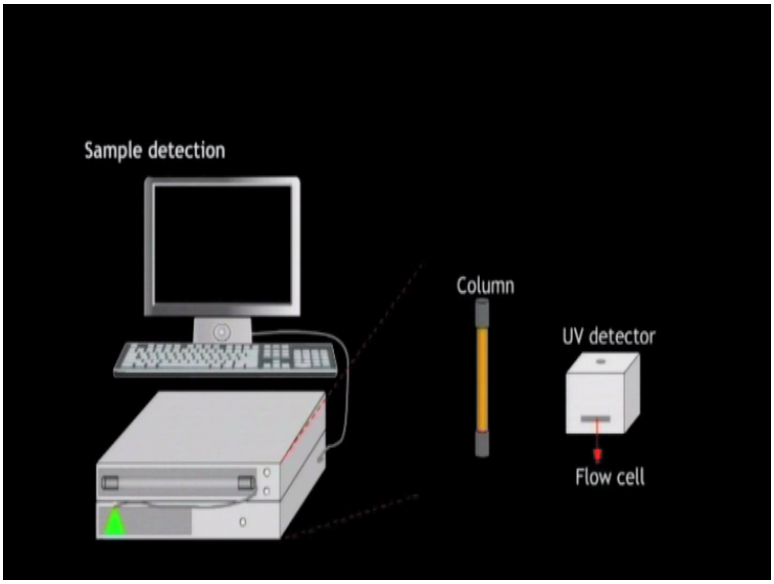

The Nano liquid chromatography, which make use of C18 Capillary columns has gained popularity for the proteomic studies due to their ability to achieve finite separation. Now these separated components pass from the column outlet into the flow cell which is present in the detector. The most commonly used detector for protein analysis UV detector which analysis the protein observance at 280 nanometer and plot a graph of retention time against intensity.

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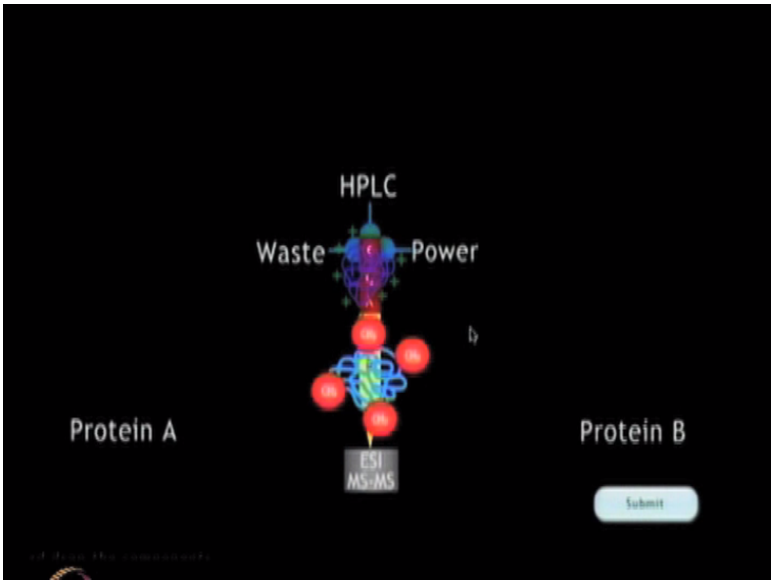

Multi-dimensional protein identification technology or MudPit is a widely adopted strategy that carries out two consecutive protein separation based on different principle as shown in the animation here. Shown either side is a protein but different properties. Earlier have talked about how one can make use of different properties of Strong cation exchange and Reversed-phase chromatography to separate out peptides.

By use those basic concept and drag and drop the protein that will interact with the SCX and RP regions of the columns respectively. So your answer is correct. You can use the protein properties and separate those in the multi-dimensional protein identification technology. MudPit is a (()) (16:55) technique to separate and identify individual components of complex protein and peptide mixtures of a proteome.

It has been shown that MudPit have potential to be used as a substitute of traditional to dimensional inject electrophoreses since it can separate peptides in the 2D liquid chromatography. MudPit techniques allow greater separation of peptides which can directly be interfaced with Mass Spectrometry ionization source. It also avoids band broadening which is one of the drawbacks of many chromatographic methods.

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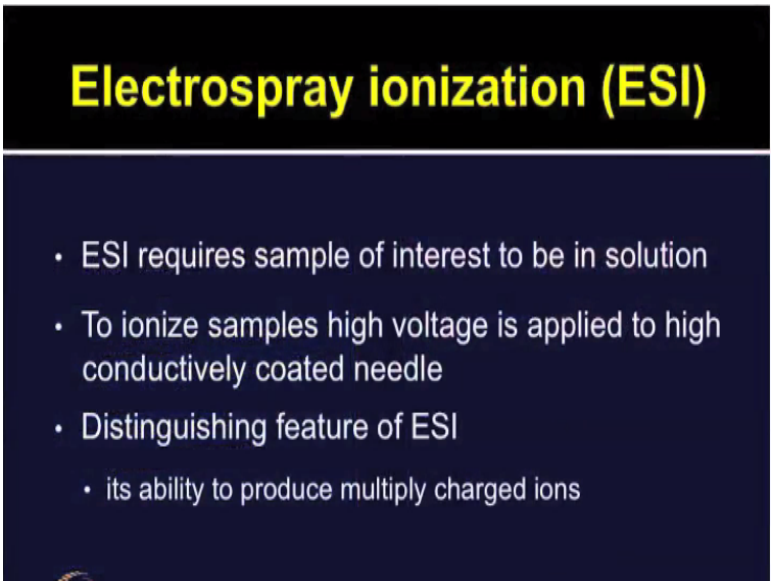

So ESI it requires sample of interest to be in solution mentioned that we can use the in-line separation along with the liquid chromatography. To ionize the sample high voltages are applied

to high conductively coated middle, so this voltage results into the sample becoming charged either positive or negative. The positive ions are primarily used for the analysis of proteins.

The distinguish features of Electrospray ionization includes its ability to produce multiple charged ions. The number of charges that can be accepted by a particular molecule depends on its necessity and its size.

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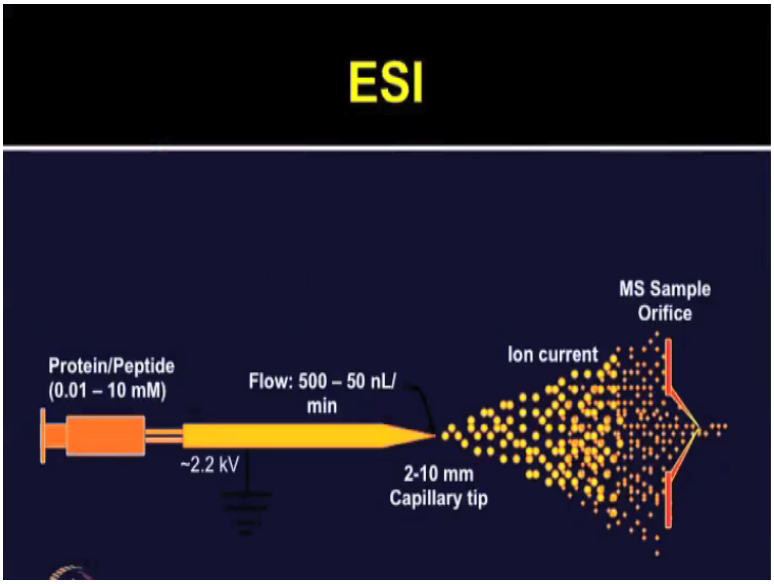

Now here you can get an overview of the process involved in the Electrospray ionization. The small droplets of solutions are generated by the Taylor cone which contain the peptide analogue, from the acidic solution provides droplet the positive charge so that it can move from the needle to the negatively charges instrument.

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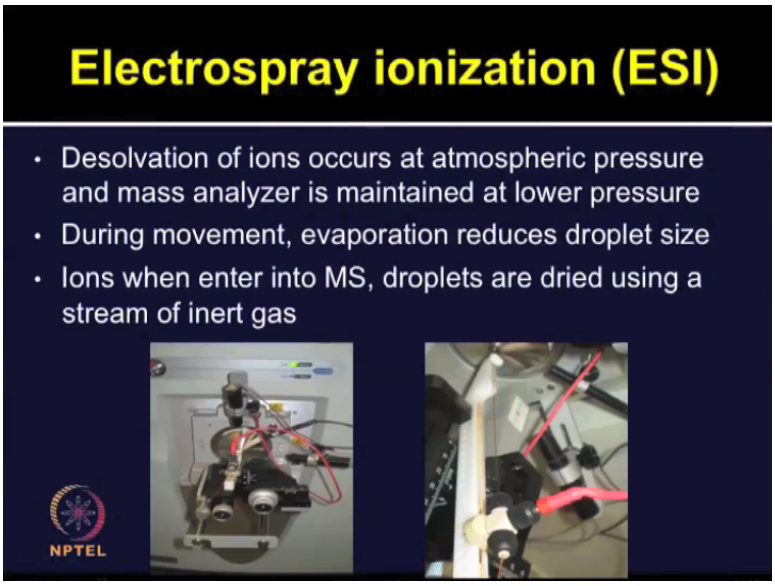

In ESI, the desolvation of ions occur at the atmospheric pressure and mass analyzer is maintained at a lower pressure, so that ions can be drawn into the MS based on the pressure differential. During movement, the evaporation reduces the size of droplets and then it splits into the small charge droplets. Ions when internecine the mass spectrometer the droplets are dried using a vacuum of the inner gas which results into a gas phase ion acceleration through analyzer towards the detector.

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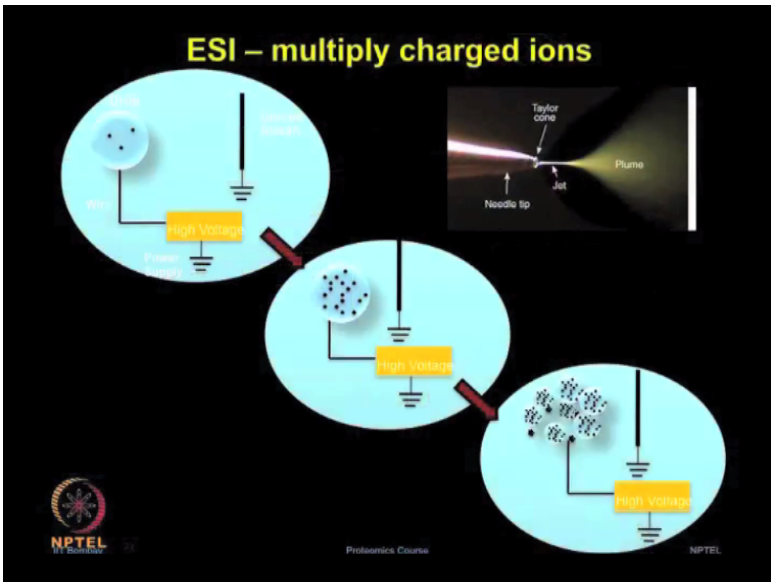

You can see this process in much, with much clarity here in this slide. The top panel is showing the Taylor cone generation and the center it is shown that production of multiple charged ions, usually it is coupled to the MS via real-time liquid separation.

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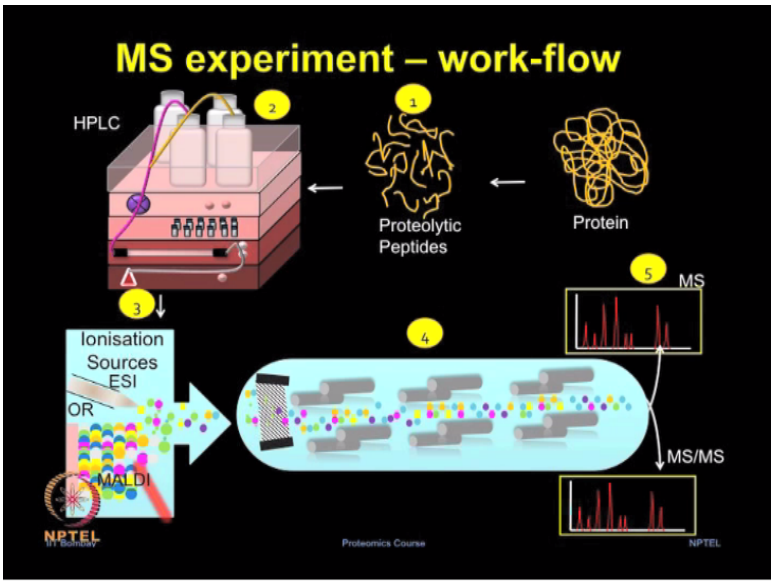

So we can see in the slide in the MS experiment – work-flow the multiple steps are involved. I have numbered those from 1 to 5. So protein samples are digested using chips and peptide mixtures are fractionated by using liquid chromatography or LC. These fractions are subjected to an electrical potential which results into spray formation. In ESI or Electrospray Ionization it leads to desolvation and ionization of peptides.

The Mass (()) (21:00) are ratio are measured in Mass analyzer which is shown as step 4 especially coins are randomly selected in the (()) (21:10) and then based on the collusion the resulting fragment ion are further measured in the second mass analyzer. The MS (()) (21:21) ion intensity can be use for peptide quantification and MS/MS ion fragmentation can be use for the sequence information and protein identification.

So this workflow all these steps are equally important starting from the first step the (()) (21:42) digestion, second pre-fractionation strategies using liquid chromatography then ionization sources, mass analyzer and then spectrum generation MS or MS/MS.

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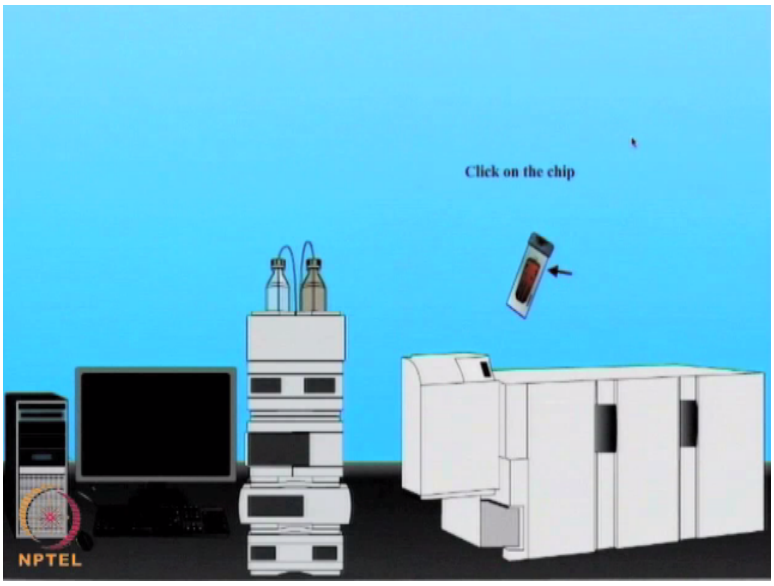

We have discussed different type of liquid chromatography systems. In a traditional Nano flow, LC/MS system there is several fittings and connections are required which is one of the major limitation of using those traditional system. The certain commercial advancement available to improve these type of limitations, HPLC technology is a Microfluidic device which carries out nano flow high performance liquid chromatography and reduces limitations of several fitting and connections.

The Microfluidic device contains circuits of tiny close channels and valve are (()) (22:55) onto a glass or plastic micro chip. Different forces such as pressure or electrokinetic can push a small volume of fluids in a defined manner. This technology integrates functional components onto a reusable bio-compatible chip which integrates sample enrichment and analytical nano columns, nano spray emitter, fittings, and connections capillaries on a reusable bio-compatible polymer chip.

The chip-based technology reduces the possibility of lead and dried volumes. It improves sensitivity and reliability during the liquid chromatography steps. By making the integrated system it avoids several Pit falls which are associated with separate tubing and fitting. Another important component of this technology is the HPLC chip MS interface. A chip is inserted into the interface, which mount on a Mass spectrometer.

So this design configuration ensures that the electrospray is in the optimal position for mass analysis when the chip is inserted in the mass spectrometer. So compare to the conventional nano spray technique this technology can achieve maximum sensitivity with minimum sample size by integrating sample preparation, separation and electrospray on the single chip technology.

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