Mass Spectrometry Based Proteomics
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Mod 02 Lecture Number 07

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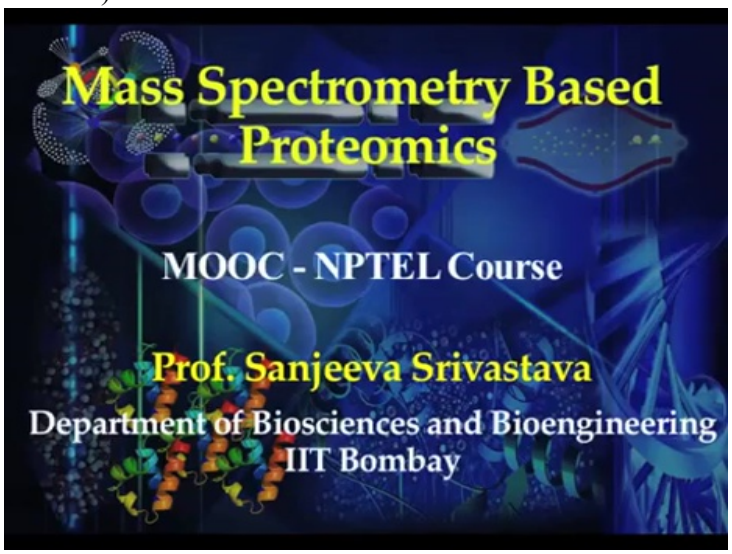

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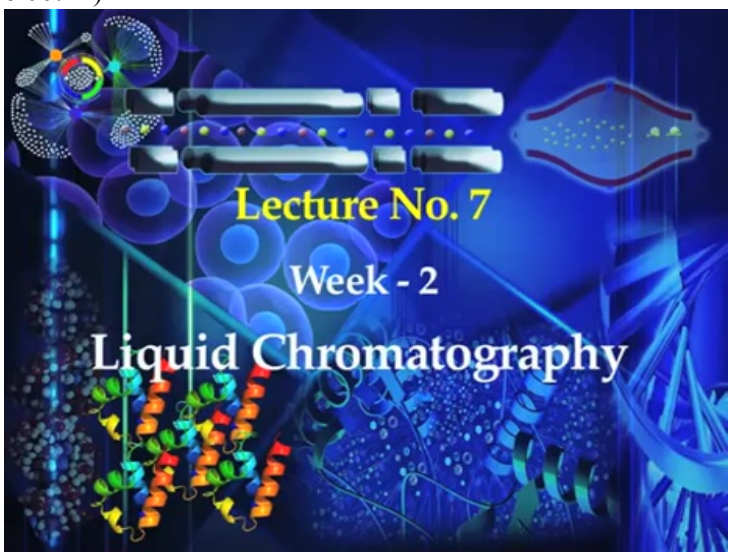

Today we will talk about liquid chromatography mass spectrometry or LC-MS/MS.

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MS is based on production of ions which are subsequently separated according to their m by z ratio. The resulting mass spectrum provides a plot of relative abundance generated as a function of the m by z.

MS provides the most versatile platform and comprehensive analytical technique useful for the proteomic scientists for wide variety of applications.

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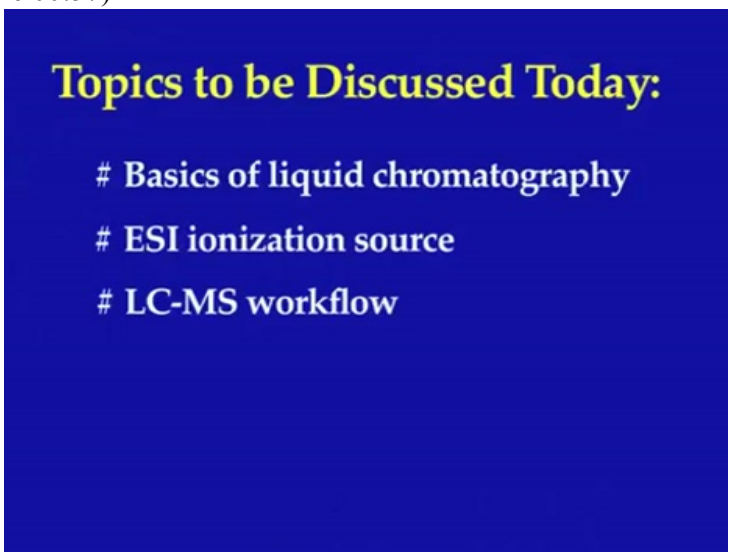

So in today's lecture I will first give you an overview of mass spectrometry workflow. We will then talk about individual components in some more detail such as liquid chromatography, ionization sources.

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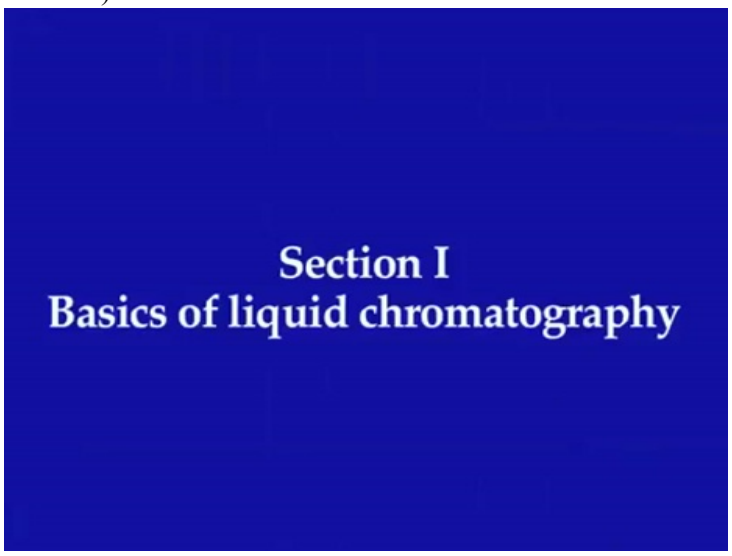

After doing in-gel digestion, now let's move to …

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… the second part, the separation technology, liquid chromatography or LC

So, chromatography in general is a physical separation method in which components for separation are selectively distributed amongst two immiscible phases, a mobile phase flowing through the stationary phase.

Now depending upon the mobile phase the technique is termed as either liquid chromatography or gas chromatography etc.

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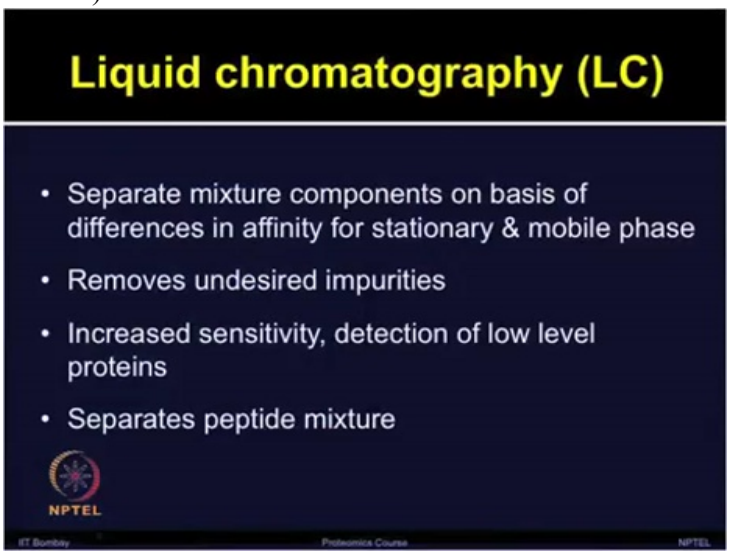

So what is Liquid chromatography?

The peptide mixtures can be fractionated in-line with the instrument prior to the introduction into mass spectrometer. That is, I think, one of the advantages for doing the proteomic applications. So in LC, it can separate mixtures or components on the basis of differences in the affinity for the stationary and mobile phase.

Liquid chromatography is also useful for removing the undesirable impurities.

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

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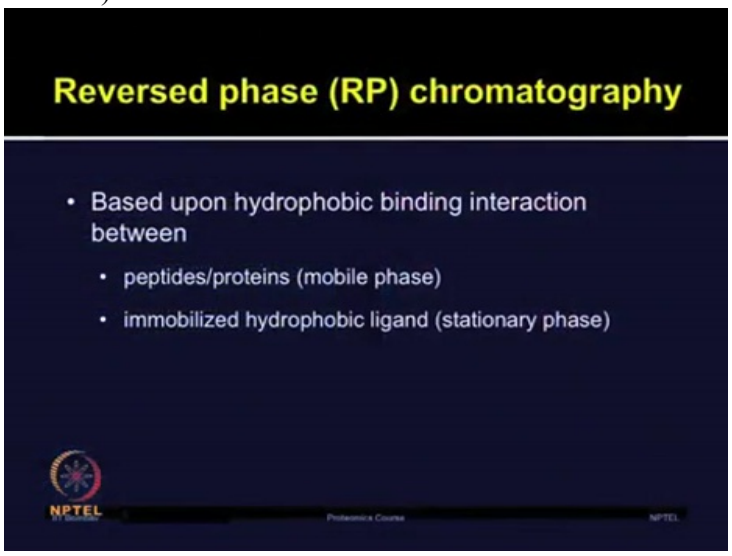

There are various types of chromatography one can use for different applications.

In proteomics, the most commonly used method for peptide fractionation is Reversed phase liquid chromatography or RPLC which separates peptides based upon the hydrophobic binding or interaction between the peptides or proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase.

By utilizing this hydrophobicity, one can separate the peptides. However, if your proteome mixture or the peptides are very complex then one can further use another type of chromatographic method such as strong cationic exchange as well as different types of multidimensional separation can be employed.

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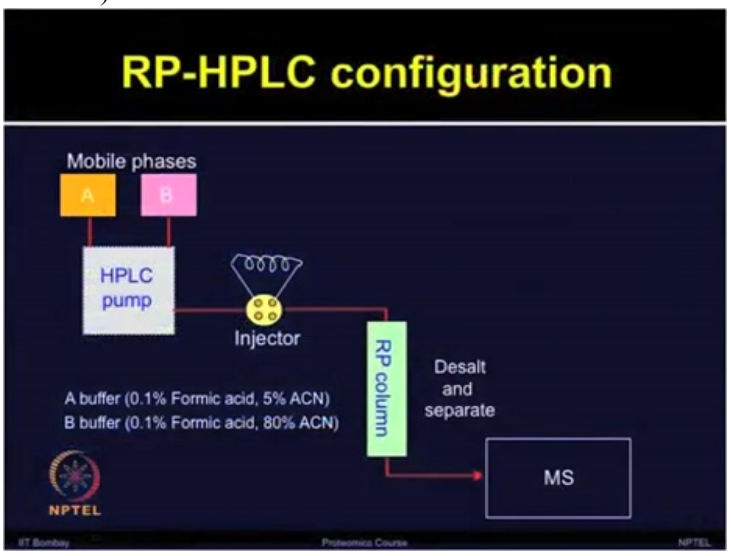

In this slide is shown the configuration for Reversed Phase HPLC. As you can see, the two components in the mobile phase, A and B are linked with the HPLC pump. "A" buffer can provide between the range of 0.1% formic acid to 5% of acetonitrile while the "B" buffer 0.1% to 80% of acetonitrile.

So first of all, equilibrate the system in buffer "A", then load the peptides and wash those.

Then run the gradient of increasing mobile phase "B".

Now wash the reverse phase with buffer "B" and then re-equilibrate in" A".

In this way the peptides can be separated and prior to MS analysis it can be desalted so that there is no interference of salts.

Now Reversed phase is most commonly employed with the electrospray ionization because of its compatibility of Reversed phase acidic, aqueous and polar mobile with electrospray ionization.

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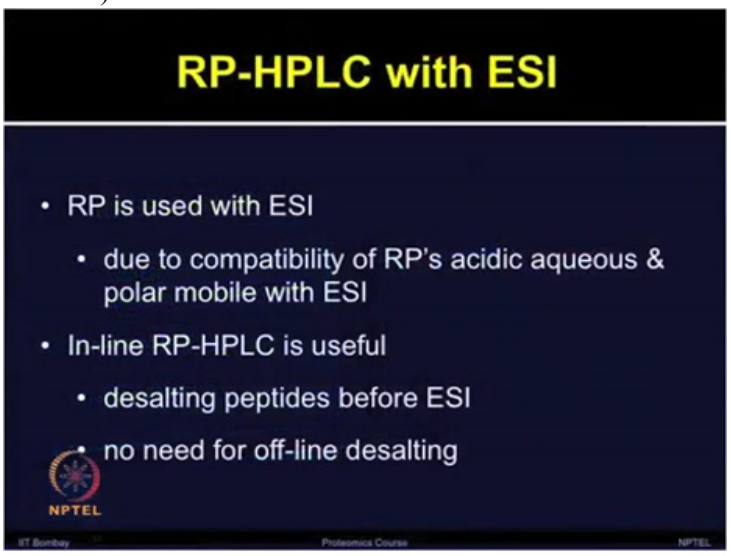

As I mentioned, in the proteomics one can use ESI and Liquid chromatography inline. So directly samples can be prefractionated and further analyzed during mass spec.

The inline Reversed Phase HPLC is very useful because it can do the desalting of peptides prior to ionization in ESI. There is no need of doing separate offline desalting and defractionation. It can focus peptides from the dilute samples into the narrow chromatographic bands and it also enhances the sensitivity.

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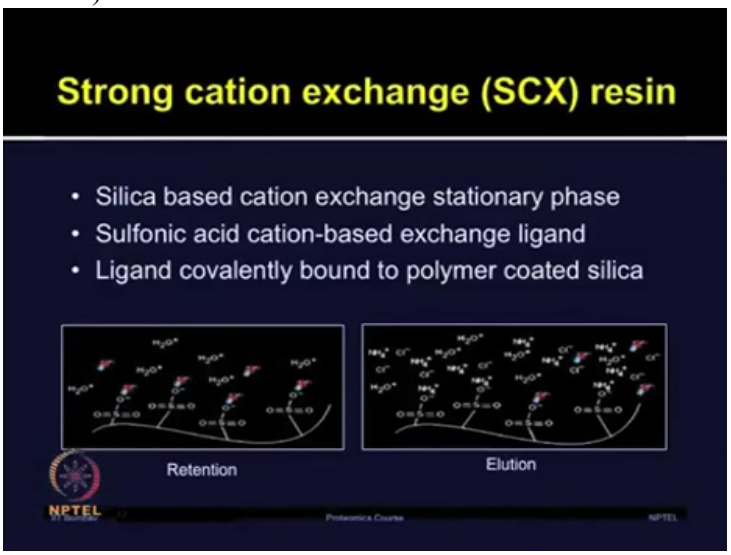

Let us now talk about another separation technique which is also commonly employed in proteomics, Strong Cation eXchange or SCX.

In the SCX resin, the silica based cation exchange is used in the stationary phase. Sulfonic acid cation-based exchange ligands used. These ligands are covalently bound to the polymeric coated silica.

Now, as I have shown in the slide, there are two important phenomena 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 cations of the mobile phase additive, the ammonium ions. And this is a reaction to the high concentration of cations.

Now HPLC can be a microcapillary, it can be nano-LC or it can also be different type of chip-based chromatography separation. Now, for proteomics, various types of microcapillary, nano-LC…

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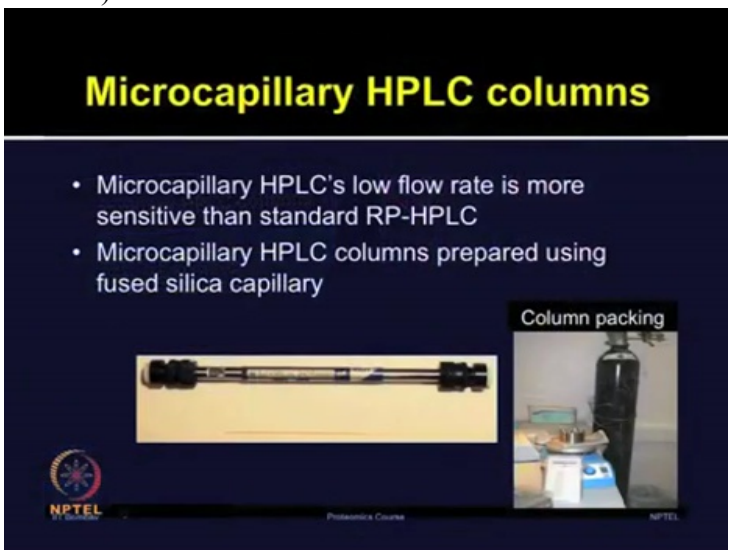

… and different types of chip technologies are currently used. In the microcapillary HPLC, the low flow rate, which is less than 1 micro liter per minute, is more sensitive as compared to the standard Reversed Phase HPLC, which is around 50 micro liters per minute.

The microcapillary HPLC is required for the analysis of low femtomole amount of the peptides. One can prepare the microcapillary HPLC by using fused silica capillaries and then pack that with the Reversed phase packing material.

For pre-fractionation multidimensional separations are used. There are different types of principles are involved for separating these peptides.

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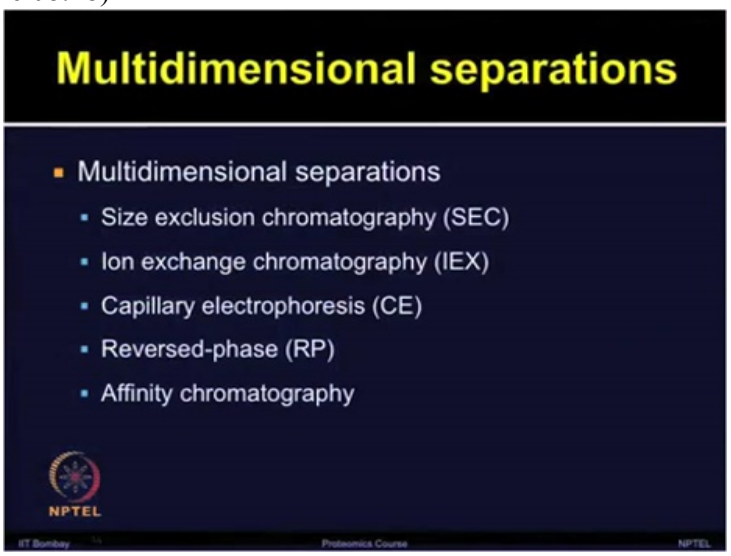

One can use Size Exclusion Chromatography which separates based on the molecular weight or the size, Ion EXchange chromatography based on the charge, Capillary Electrophoresis based on charge, Reversed-Phase (RP) based on hydrophobicity, Affinity Chromatography which is based on the biological interactions.

Now multidimensional approaches can be coupled with the mass spectrometry. So one can use various types of liquid chromatography methods in tandem and then do the multidimensional separation.

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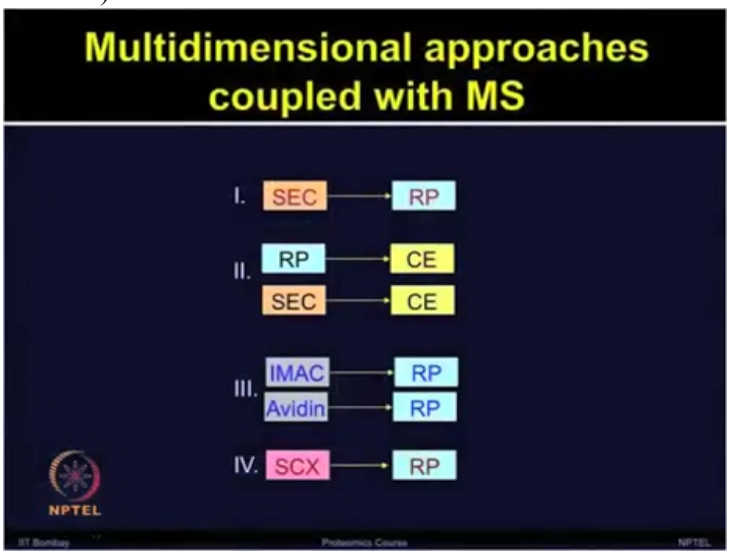

Different types of approaches have been tried. For example Size Exclusion Chromatography followed by Reversed-Phase Chromatography, but it has resulted in the poor resolution of peptides in Size Exclusion Chromatography. So this was not very widely used.

Other approaches include Reversed-Phase Chromatography followed by Capillary Electrophoresis or Size Exclusion Chromatography followed by Capillary Electrophoresis. Due to the limited loading capacity and the low loading volume of the Capillary Electrophoresis, 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 Strong Cation eXchange followed by the Reversed-Phase is most commonly used.

So this slide gives you an overview of various types of methods of liquid chromatography separation which can be employed prior to the injection into the ionization source.

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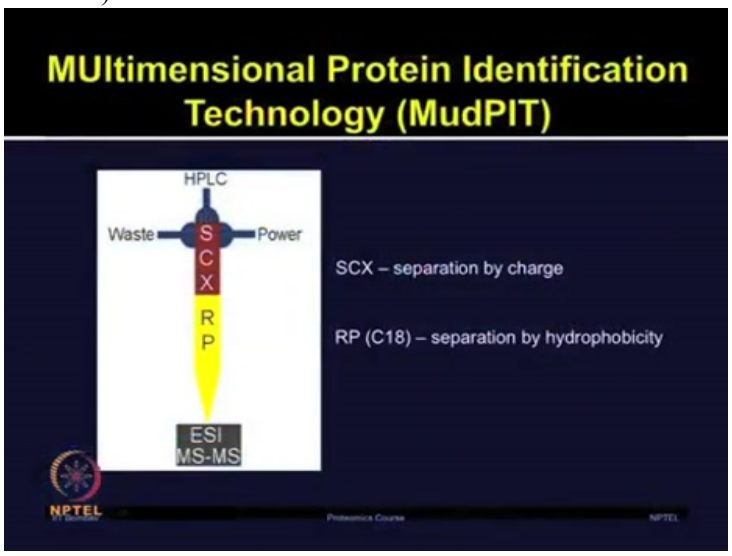

As I mentioned, Strong Cation eXchange as well as Reversed-Phase chromatography together can be employed for various proteomic applications which have been demonstrated in the Multidimensional Protein Identification Technology or MudPIT.

In this technique, Strong Cation eXchange separates by the charge which provides low resolution fractionation in the beginning and then the Reversed-Phase C-18 column which separates peptides based on hydrophobicity and it provides a high resolution gradient.

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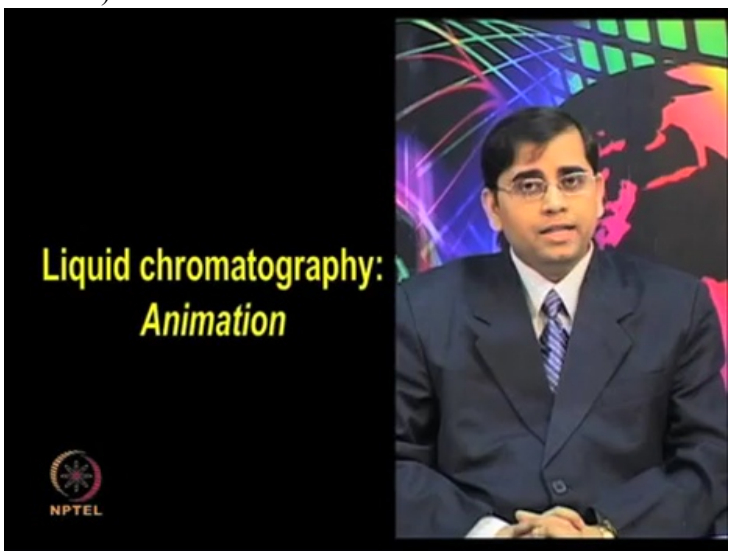

Let me describe some of the concepts involved in the liquid chromatography in the following animation. I will also discuss MudPIT and some of the chip-based approaches which are integrated for proteomic application with the liquid chromatography

Let’s discuss these concepts in following animation.

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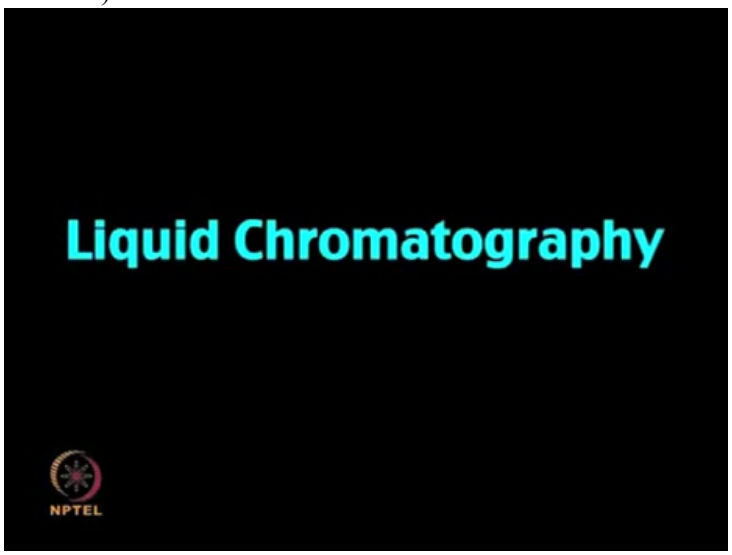

A typical liquid chromatography setup consists of the solvent bottles, degassifier, dual or quaternary pumps, sample injector, column and detectors.

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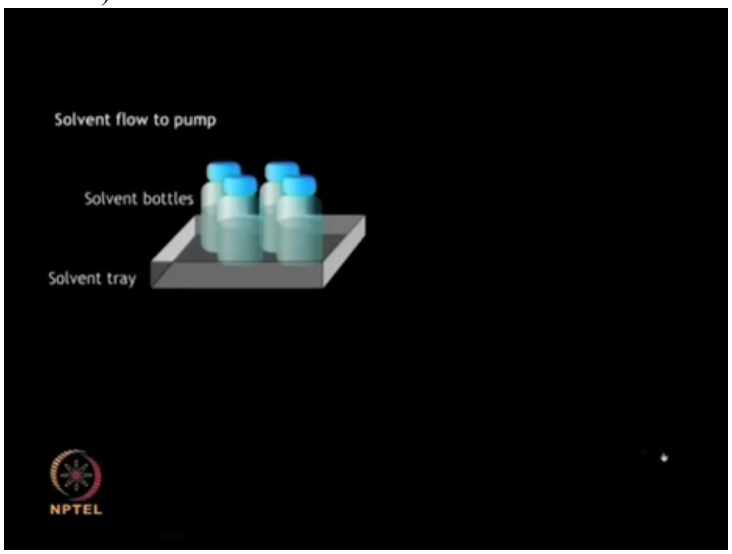

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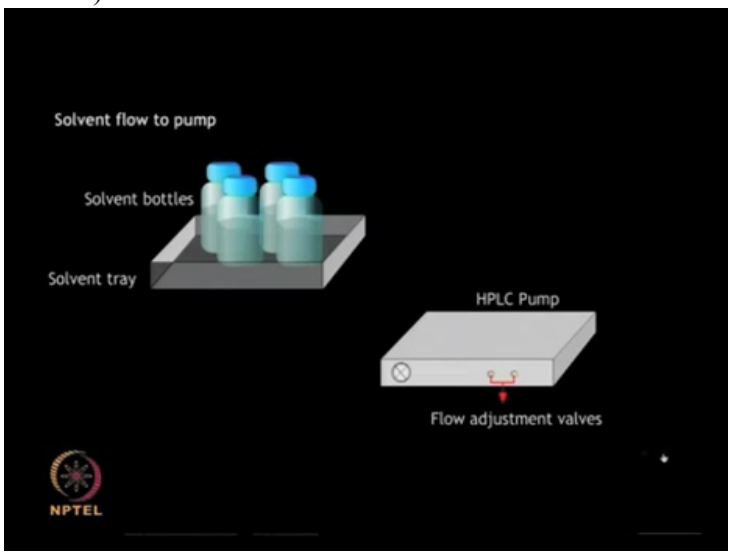

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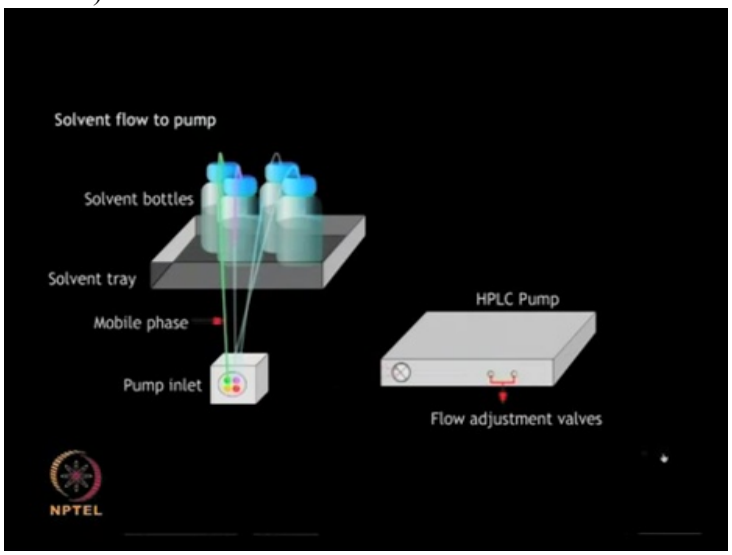

Here we can see the various components which are involved in performing the liquid chromatography. Different solvents can be placed in the solvent bottles depending upon the purification requirement.

These solvents are mixed in the desired ratio and pumped into the column during elution…

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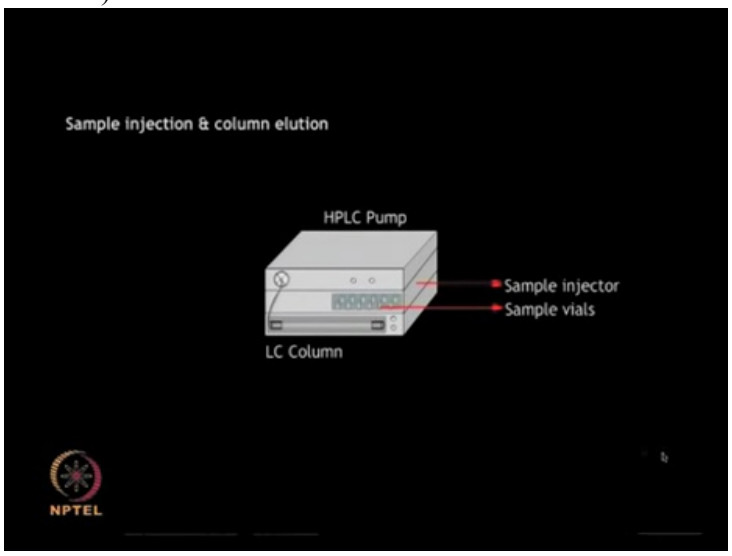

…after removal of any trapped air inside it by means of the de-gassifier

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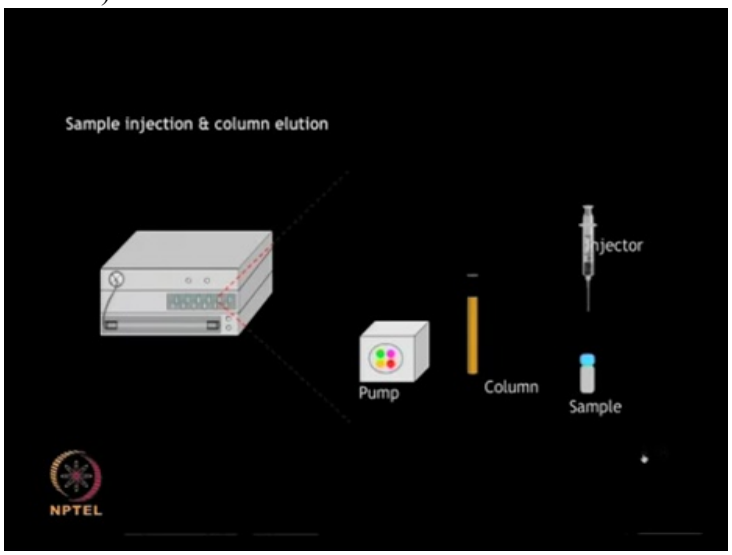

The sample injector system may be automatic or manual. The automatic sampler uses a syringe to inject the sample which is placed…

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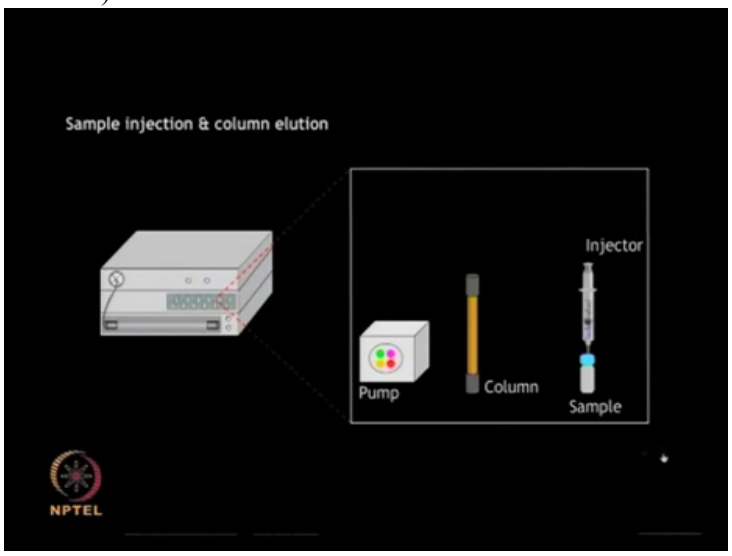

…in a vial directly below the column

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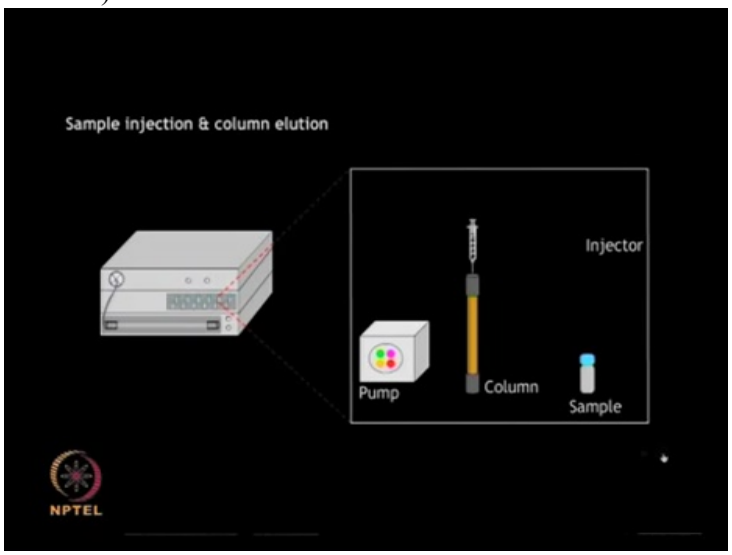

Once the sample is injected, the mobile phase flows into the column

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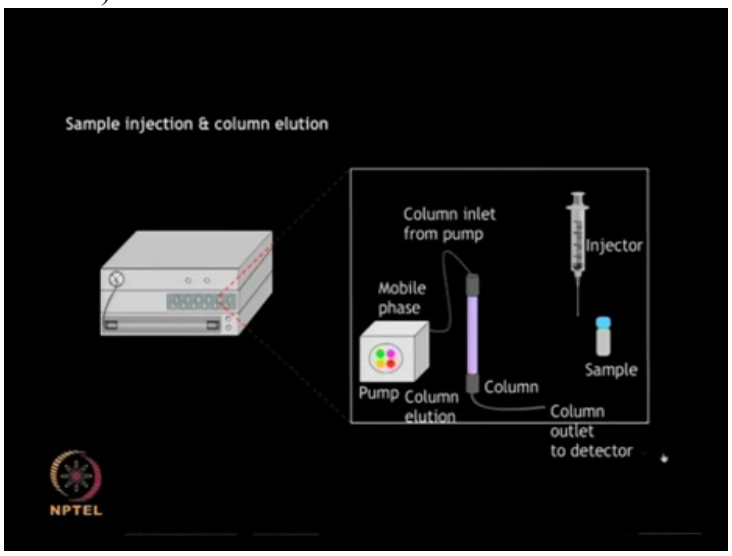

...through the pump The column consists of a stationary matrix that preferentially binds certain analytes. The outlet from the column enters …

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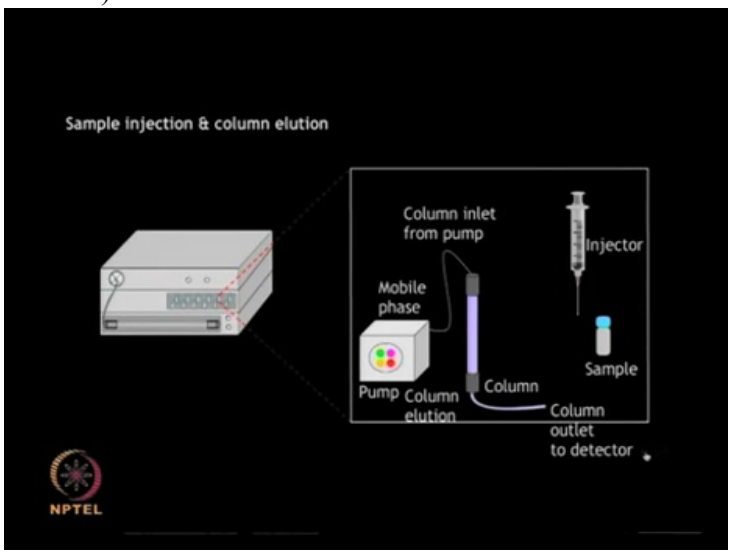

… the flow cell where it can be detected

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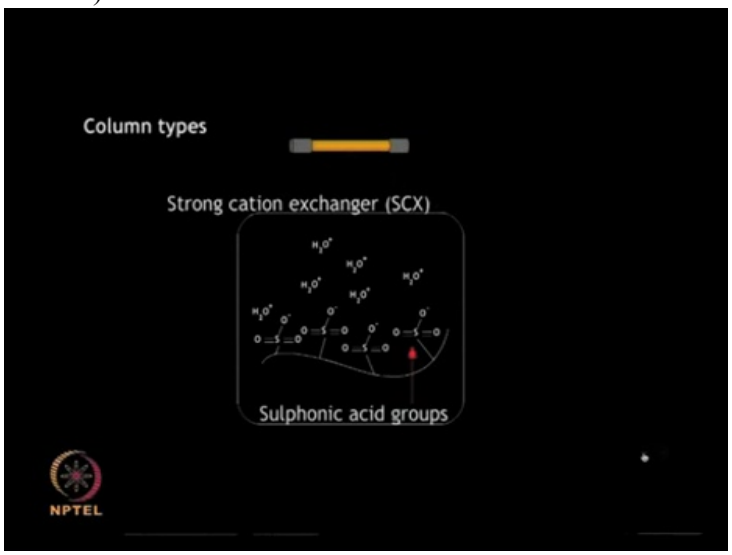

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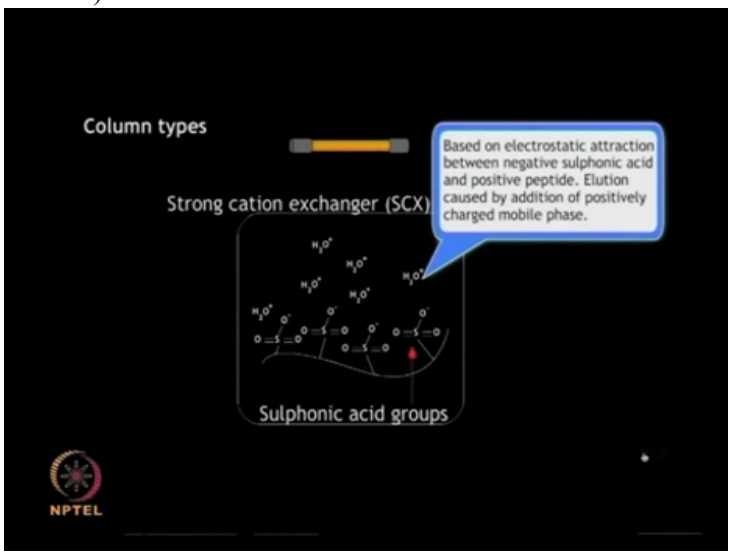

There are various stationary phase matrices are available that separate the components of the mixture based on different principles.

One of the most commonly used matrices include the Strong Cation eXchanger or SCX which separates charged peptides based on their electrostatic interactions with negatively charged sulphonic acid groups on the resin surface.

Now elution can be caused by the addition of the positively charged mobile phase.

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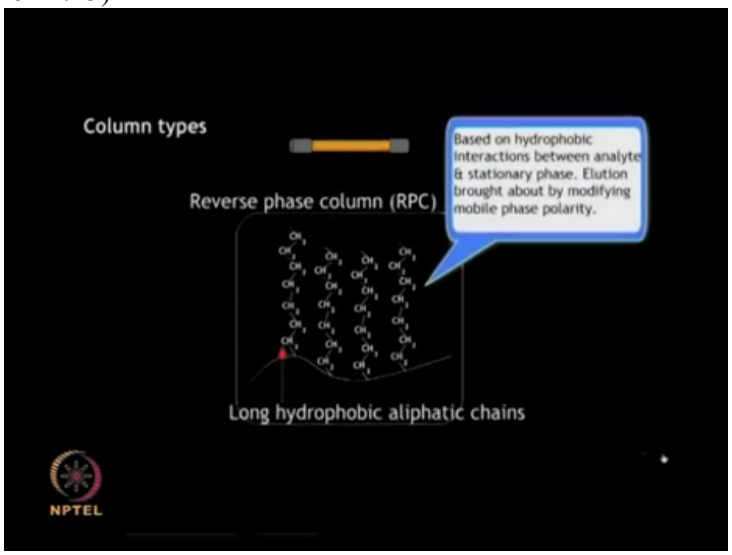

The Reverse phase chromatography is another commonly used tool, which uses a hydrophobic matrix consisting of long aliphatic carbon chains. These resin analytes separate

on the basis of their hydrophobic interactions and can be eluted by changing the polarity of the solvent.

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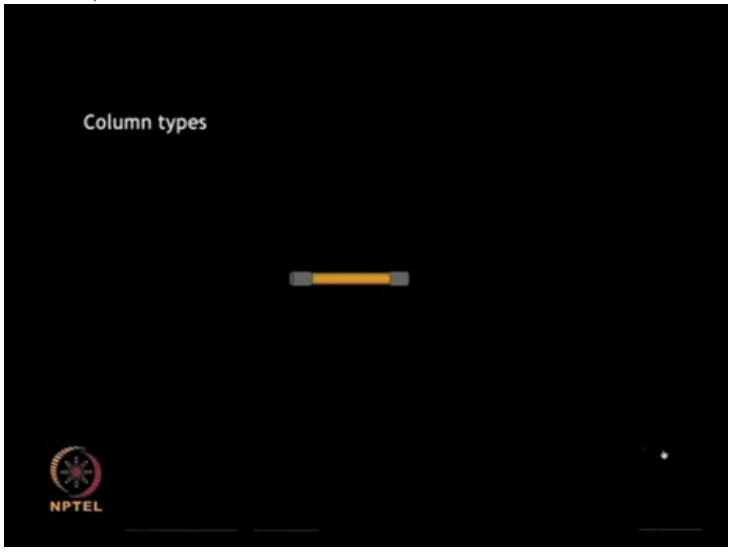

Nano-liquid chromatography, which makes use of C-18 capillary columns…

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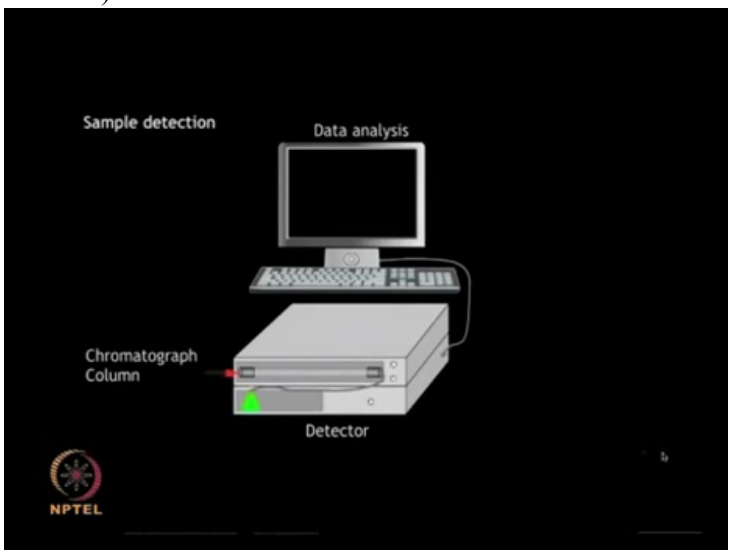

… has gained popularity for proteomic studies due to their ability to achieve finer separation

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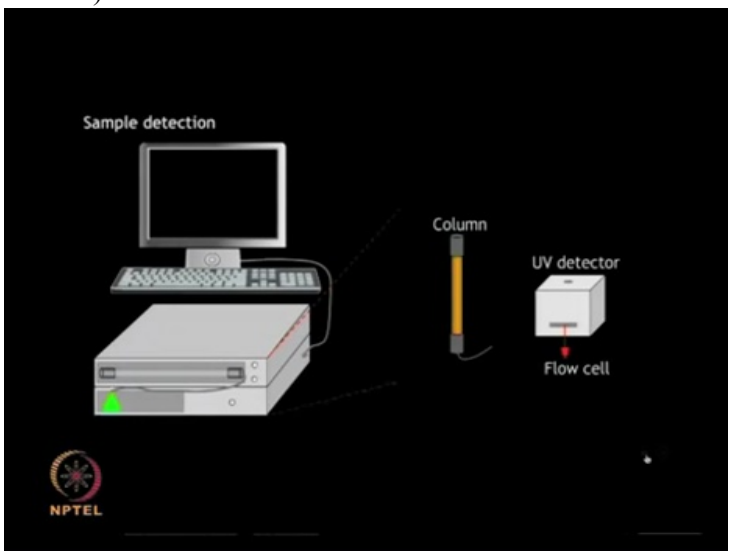

Now these separated components pass from the column outlet into the flow cell which is present in the detector. The most commonly used detector for protein analysis …

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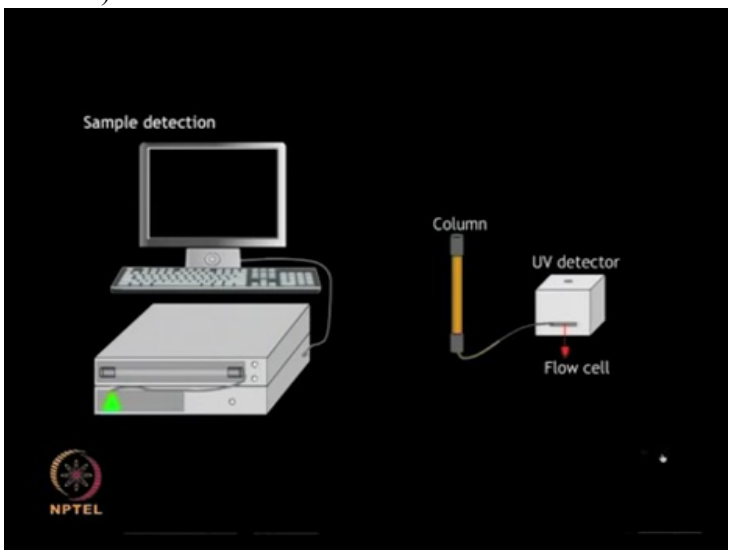

… is the UV detector which analyzes the protein absorbance…

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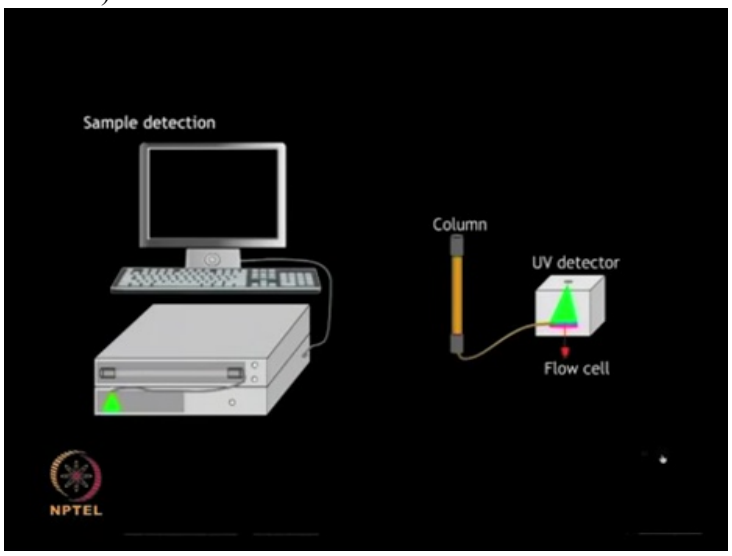

…at 280 nanometer and plots a graph of retention time against intensity.

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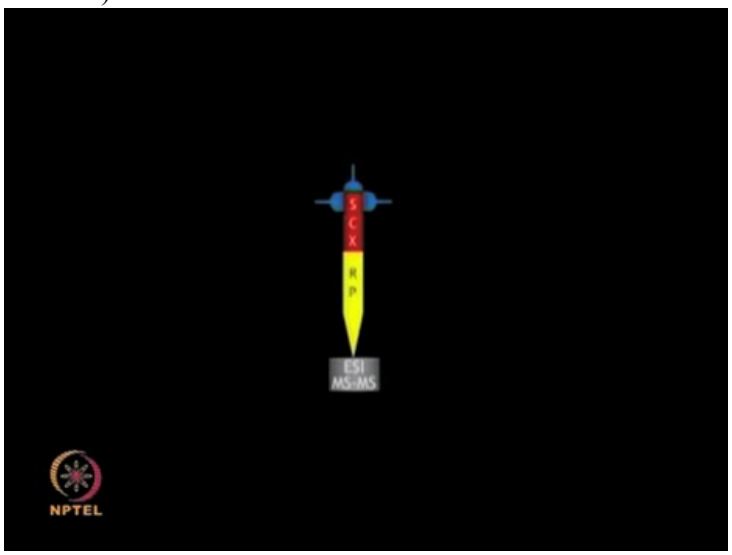

Multidimensional Protein Identification Technology or MudPIT is a widely adopted strategy that carries out…

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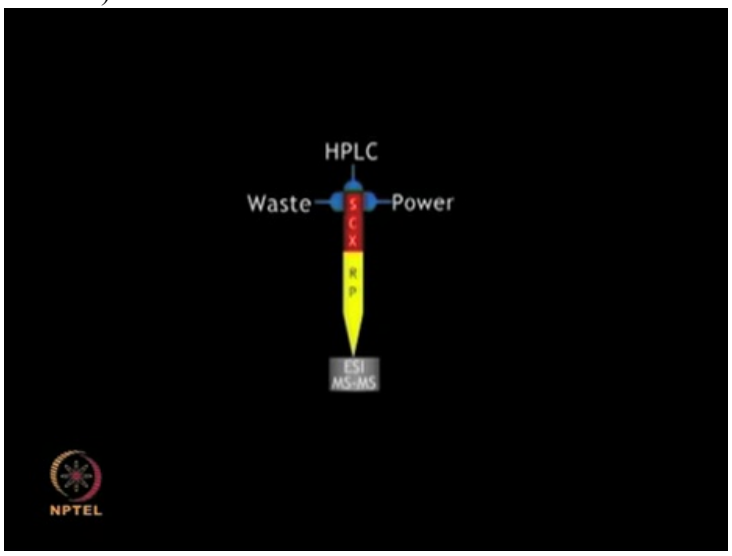

… two consecutive …

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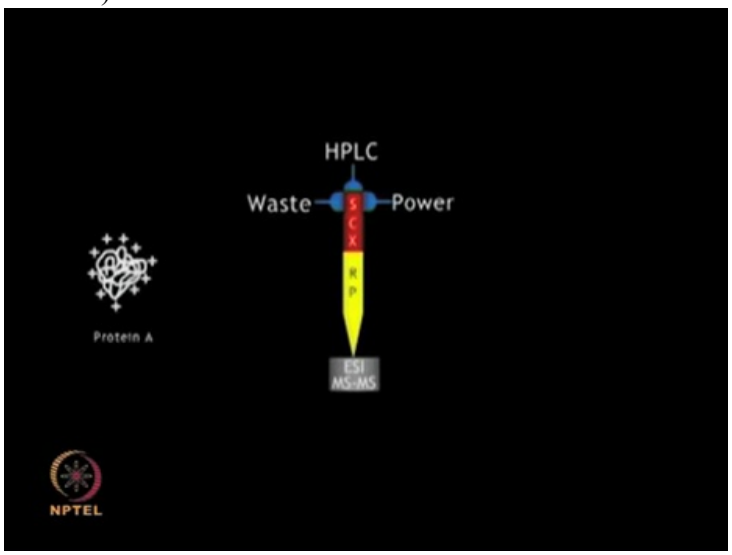

…protein …

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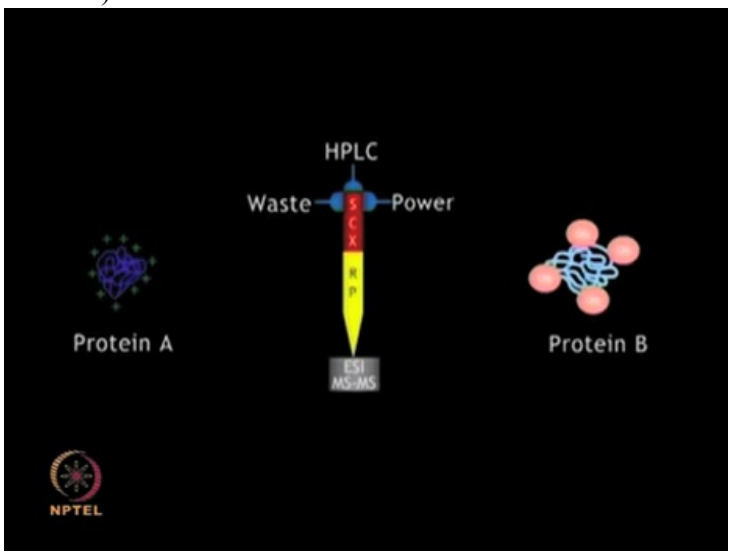

…. separations …

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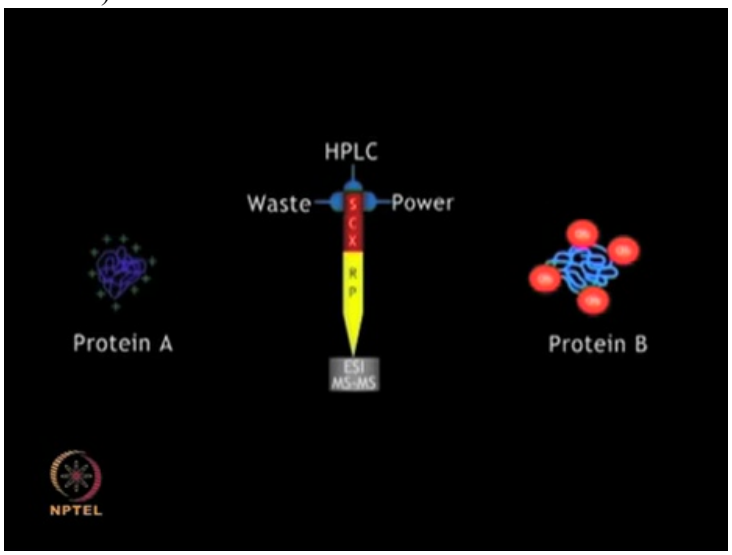

… based on different principles as shown in the animation below; shown either side is a protein with different properties.

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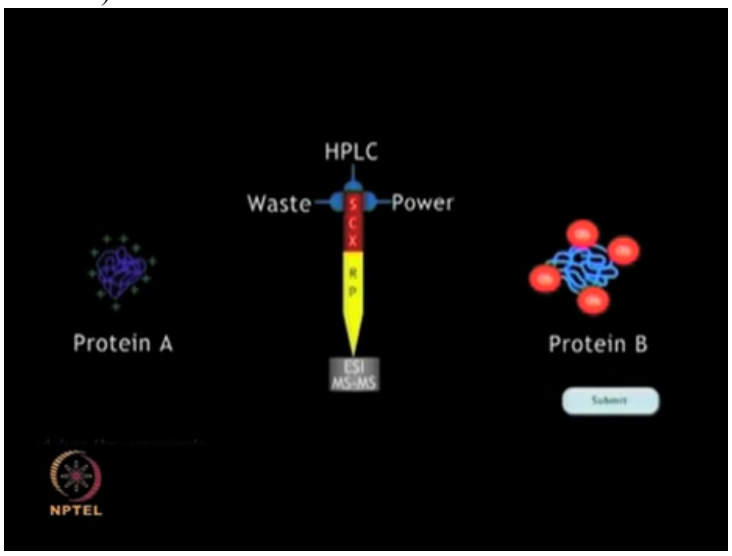

Earlier we had talked about how we can make use of different properties of Strong Cation eXchange and the Reversed-Phase Chromatography to separate out peptides. Now use those basic concepts and drag and drop the proteins that will interact with the SCX and RP regions of the columns respectively.

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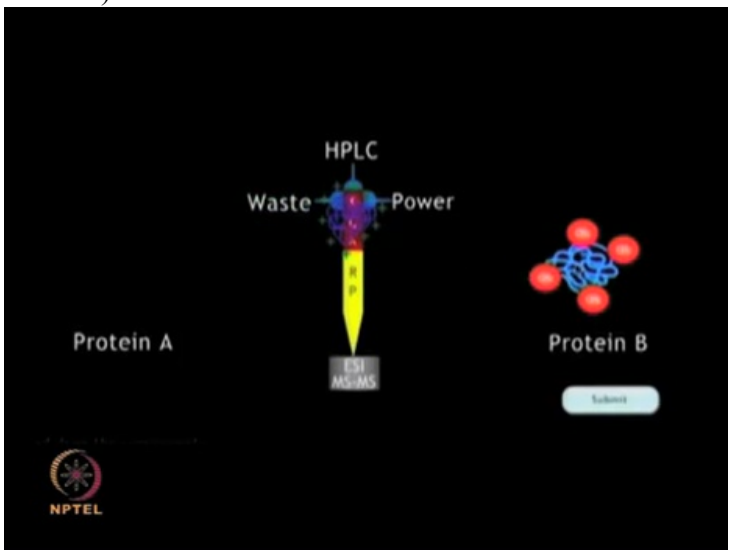

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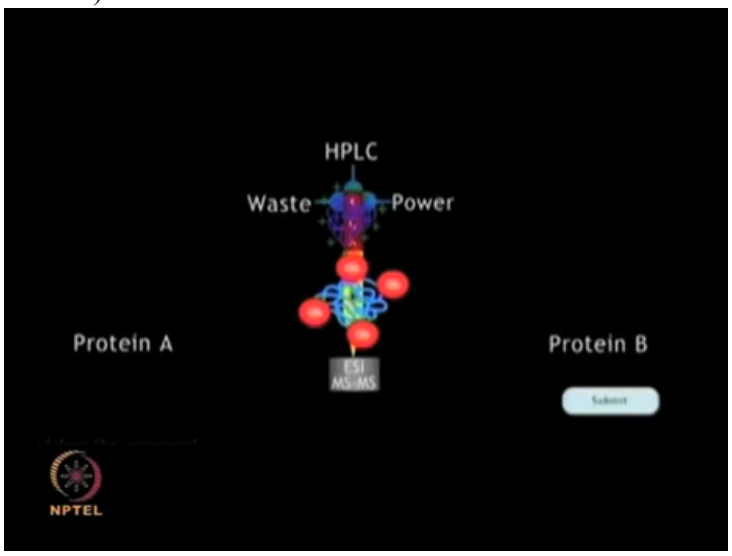

So your answer is correct, you can use the protein properties and separate those in the multi-dimensional protein identification technology.

MudPIT is a non-gel technique to separate and identify individual components of complex proteins and peptide mixtures of a proteome.

It has been shown that MudPIT has the potential to be used as a substitute of traditional two-dimensional gel electrophoresis since it can separate peptides in the 2D liquid chromatography.

MudPIT technique allows 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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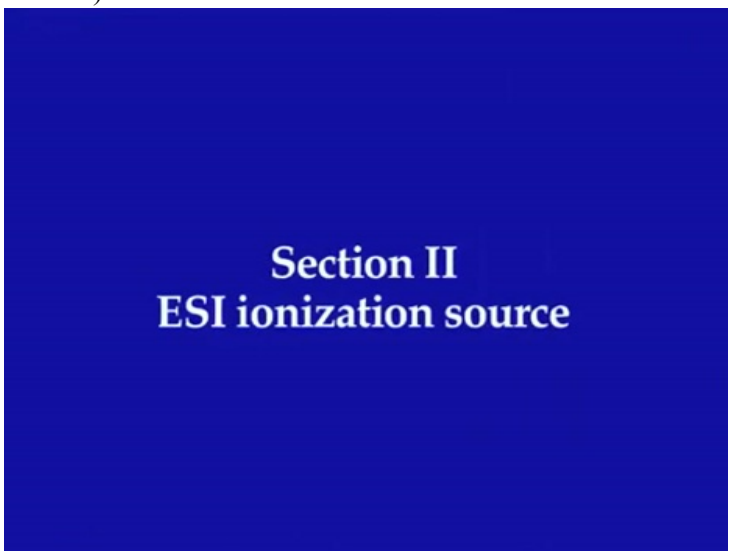

So ESI, it requires the sample of interest to be in solution and that's why we mentioned that we can use in-line separation along with the liquid chromatography.

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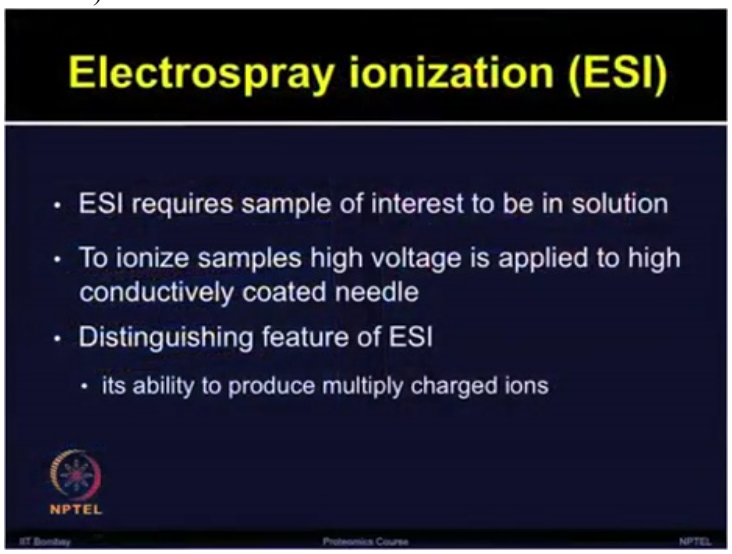

To ionize the sample, high voltage is applied to high conductively coated needle. So this voltage results into the sample becoming charged either positive or negative. The positive ions are primarily used for the analysis of proteins. The distinguishing features of ESI include its ability to produce multiple charged ions.

The number of charges that can be accepted by a particular molecule depends on its basicity and size.

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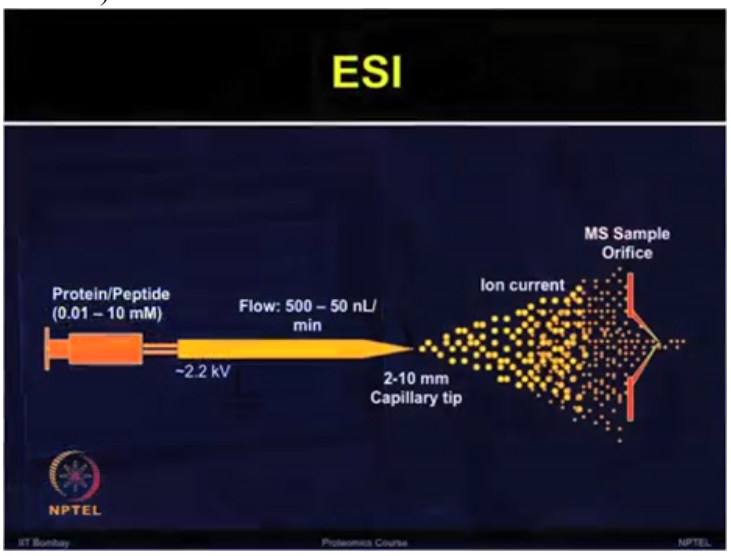

Here you can get an overview of the process involved in ElectroSpray Ionization. The small droplets of solution are generated by the Taylor cone which contains the peptide analyte. Protons from the acidic solution provide the droplet with positive charge so that it can move from the needle to the negatively charged instrument.

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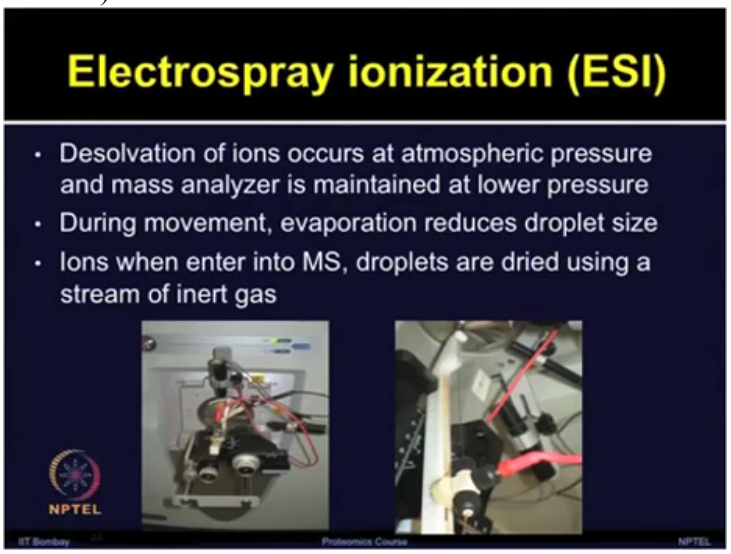

In ESI the desolvation of ions occur at atmospheric pressure and the mass analyzer is maintained at a lower pressure so that the ions can be drawn in to the MS based on the pressure differential.

During movement, the evaporation reduces the sizes of the droplets and then it spreads into the small charged droplets.

Ions when enter the mass spectrometer, the droplets are dried using the vacuum of the inert gas which results into gas phase ion acceleration through analyzer towards the detector.

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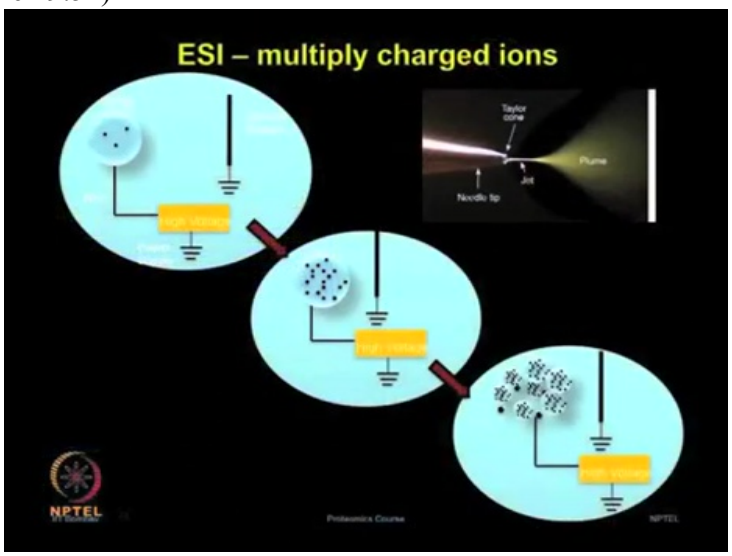

You can see the process in much clarity here in the slide.

The top panel showing the Taylor cone generation, and the center, it is shown that there are production of multiple charged ions. Usually it is coupled to MS via real-time liquid separation.

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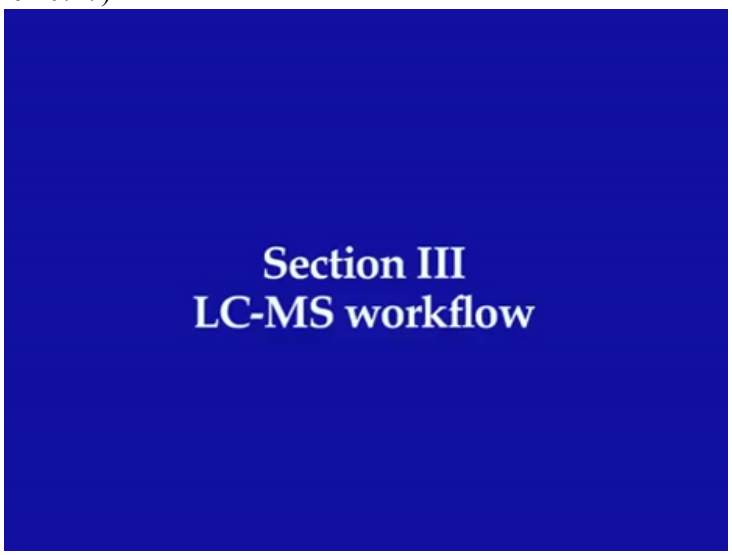

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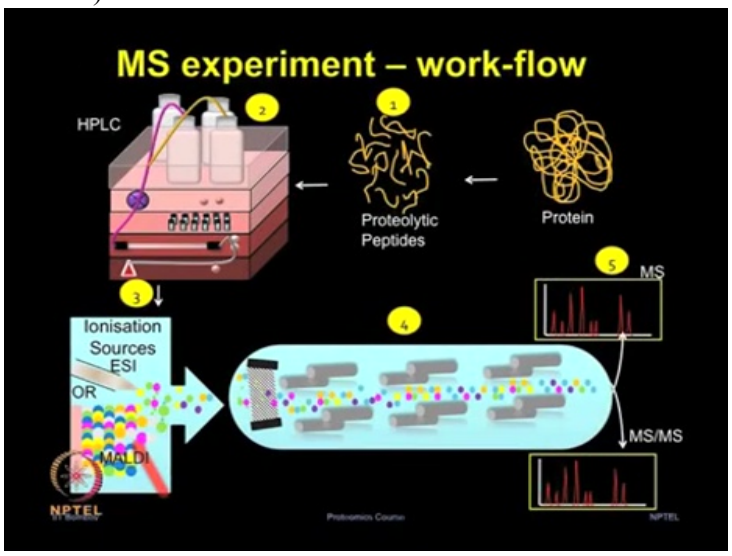

So we can see in the slide, in the MS experiment workflow, multiple steps are involved. I have numbered those from 1 to 5. So protein samples are digested using Trypsin and peptide mixtures are fractionated by using liquid chromatography or LC.

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 to charge ratio are measured in mass analyzer which is shown in step 4.

Specific ions are randomly selected in the collision cell and then based on the collision induced dissociation, the resulting fragment ions are further measured in the second mass analyzer.

The MS precursor ions intensity can be used for peptide quantification and MS/MS ion fragmentation can be used for the sequence information and protein identification.

So in this workflow all the steps are equally important. Starting from first step, the peptic digestion, second the prefraction strategies using liquid chromatography, then ionization sources, then mass analyzer and then spectrum generation MS or MS/MS.

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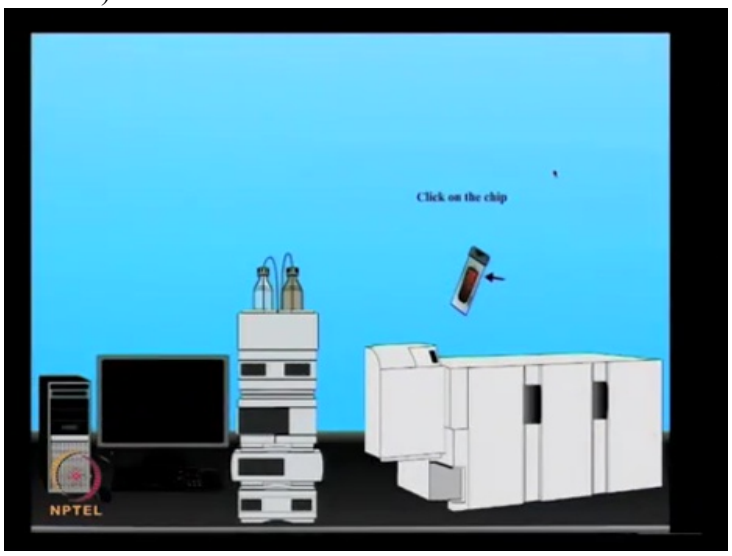

We have discussed different types of liquid chromatography systems. In a traditional nanoflow LC_MS system, several fittings and connections are required which is one of the major limitations of using those traditional systems.

There are certain commercial advancements available to improve these types of limitations. Agilent's HPLC-Chip technology is a micro-fluidic device which carries out nanoflow high performance liquid chromatography and reduces limitations of several fittings and connections.

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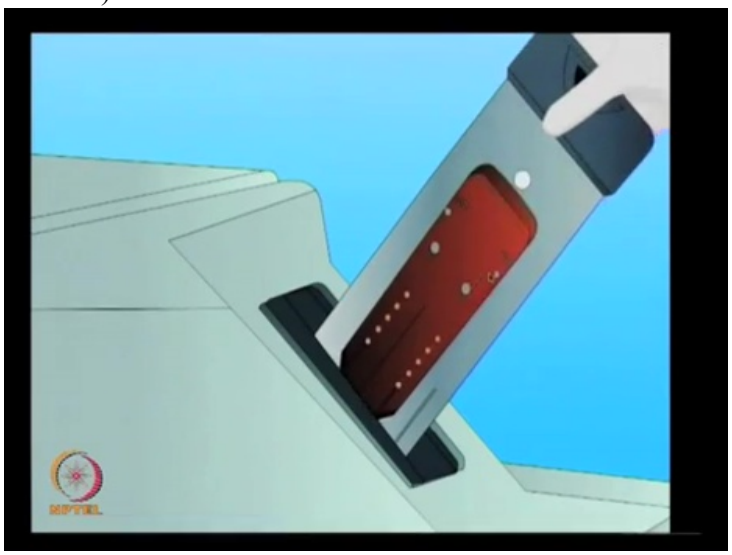

The micro-fluidic device contains circuits of tiny closed channels and wells, which are etched onto a glass or …

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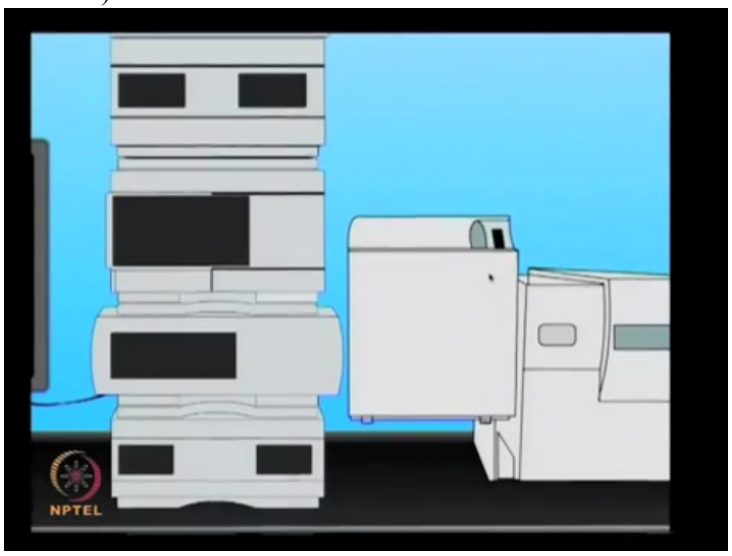

… plastic microchip…

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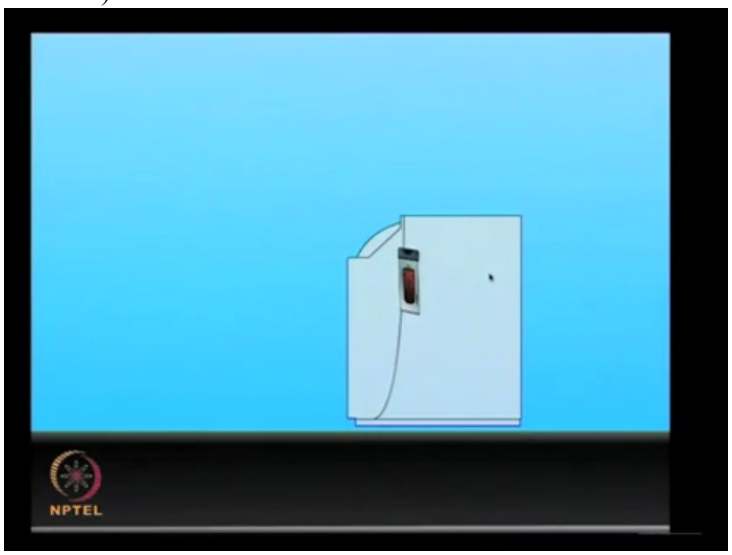

Different forces such as pressure or electro-kinetic force can push small volume

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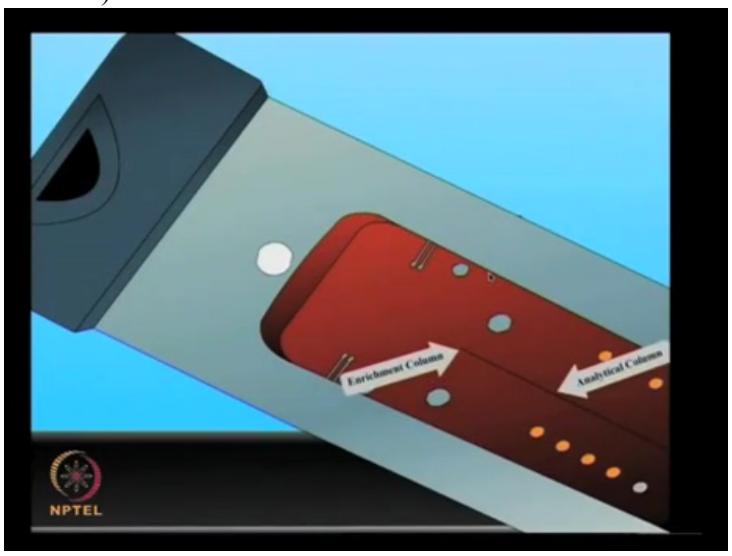

… of fluids in a defined manner

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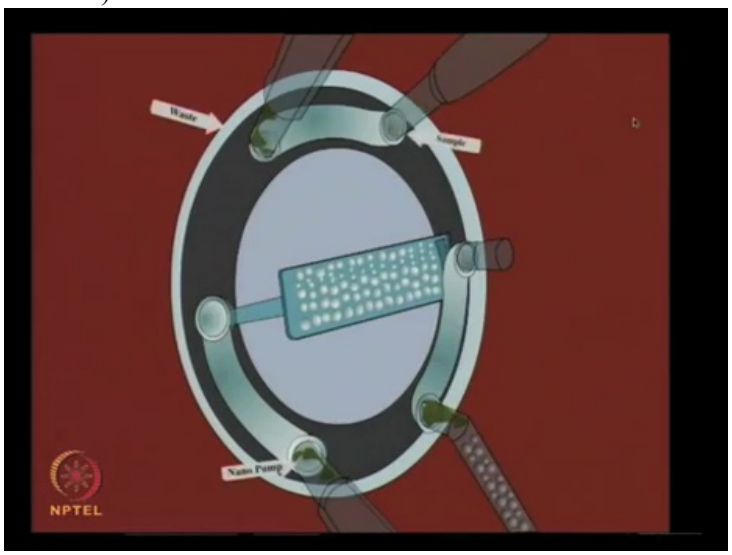

This technology integrates functional components onto a reusable, biocompatible chip, which integrates…

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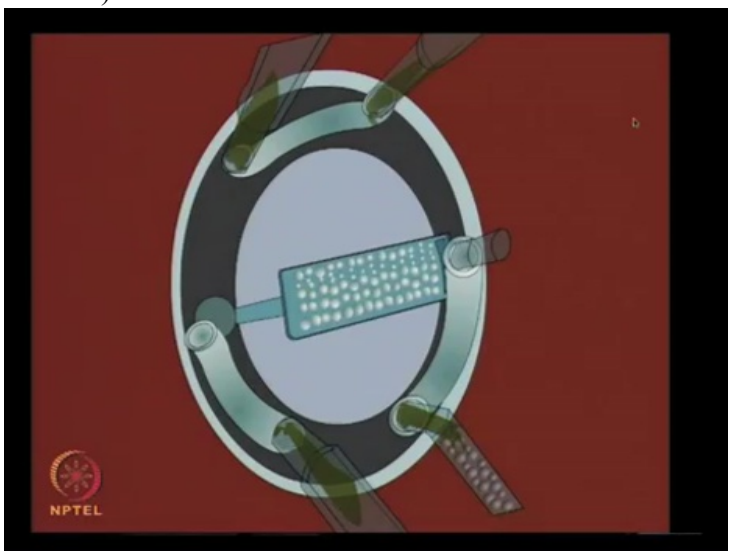

… sample enrichment and analytical nanocolumns …

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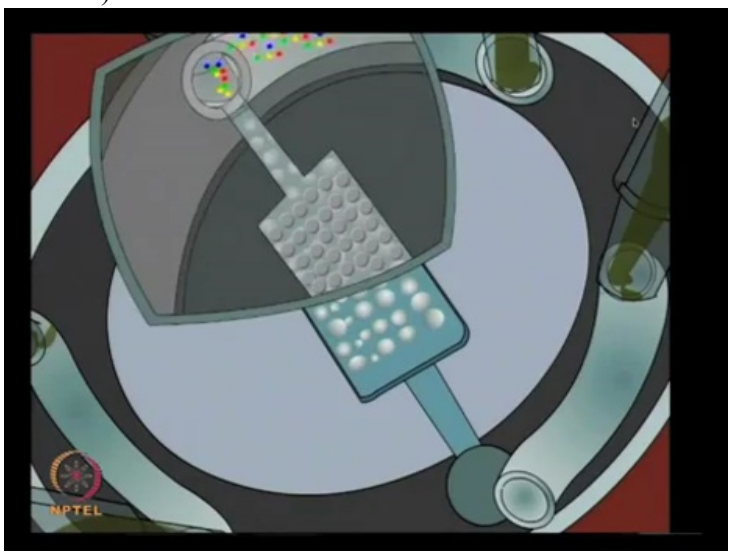

…nanospray emitter…

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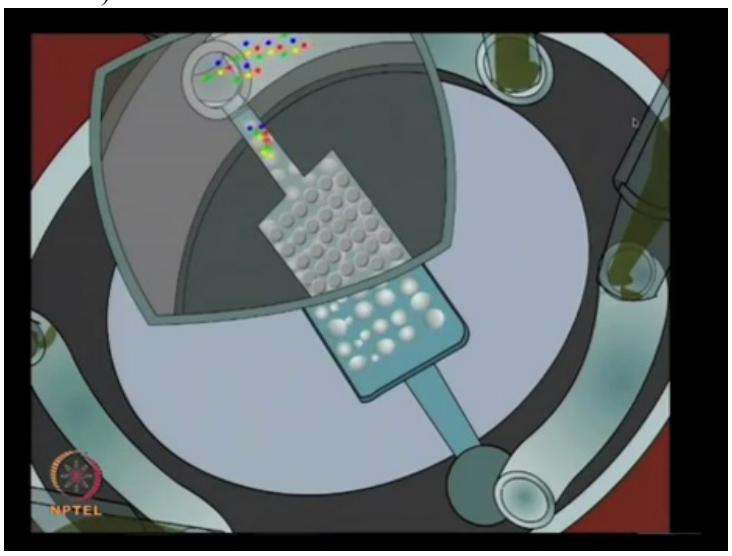

… fittings and connection capillaries on a reusable …

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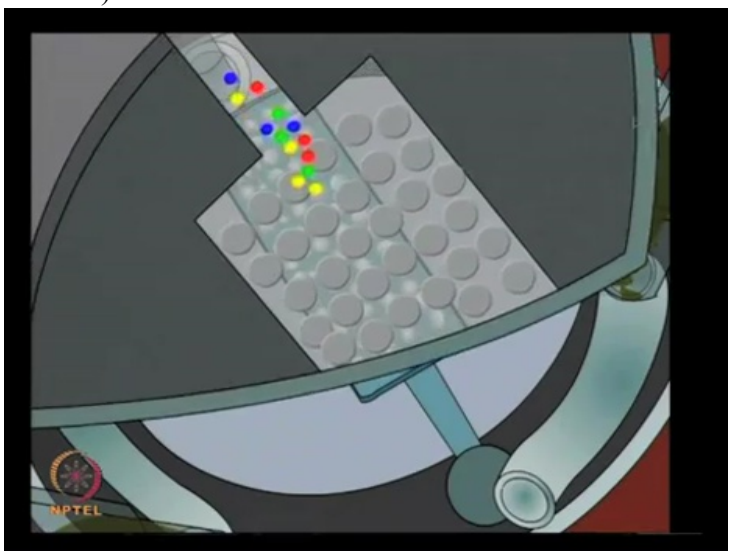

… biocompatible …

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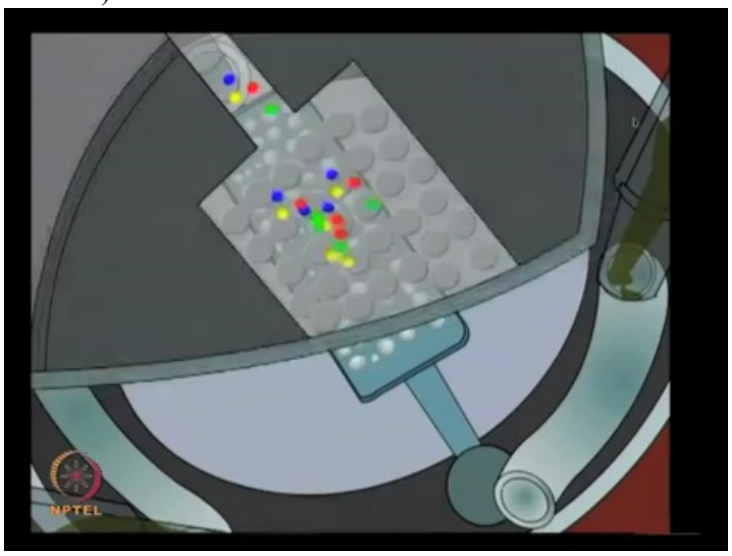
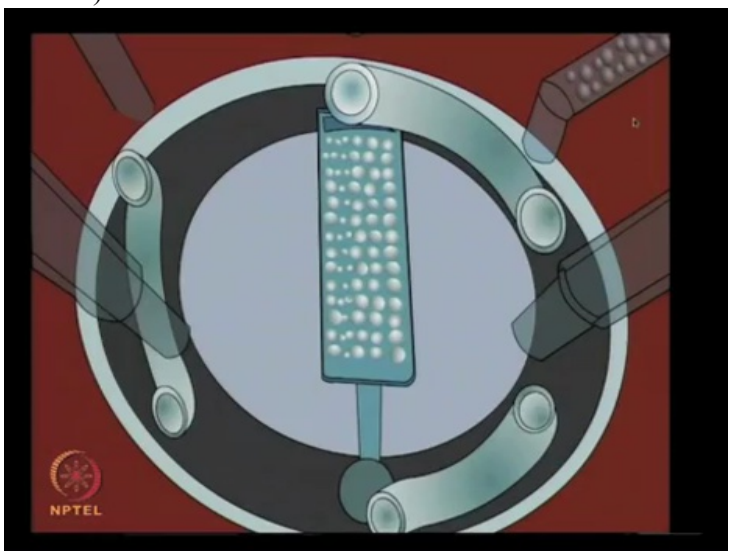


… polymer chip

(Refer Slide Time 23:44)



The chip-based technology reduces the possibility of leaks and dead volumes.

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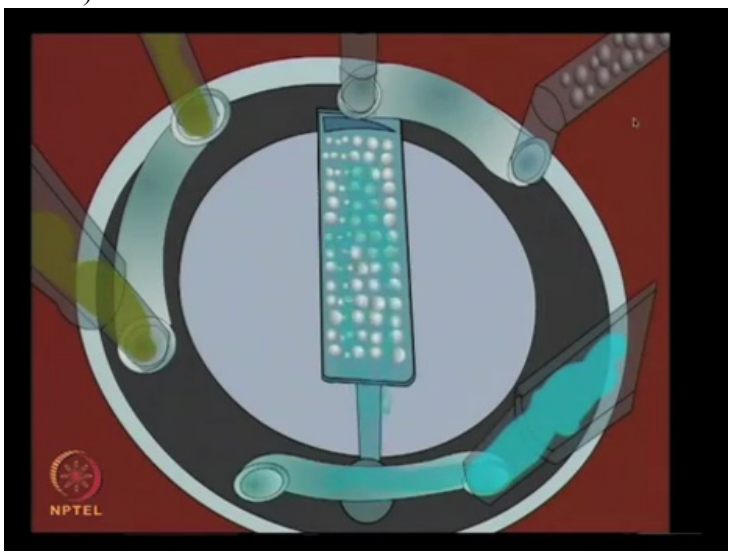

It improves sensitivity and reliability during the …

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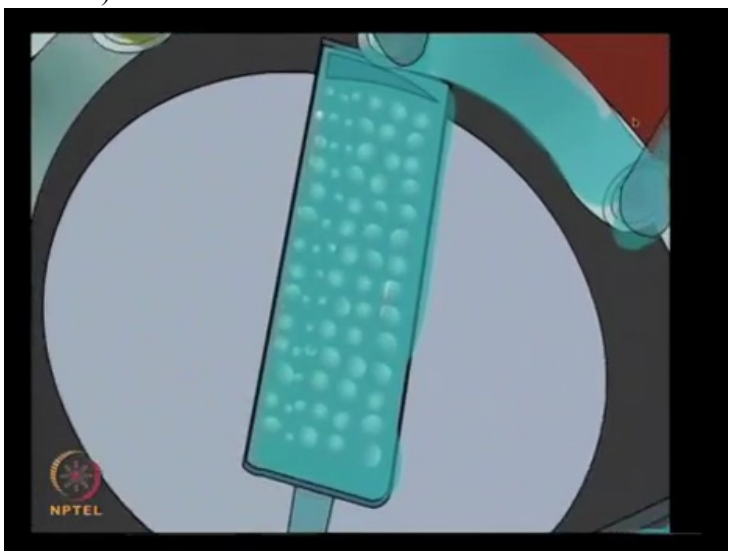

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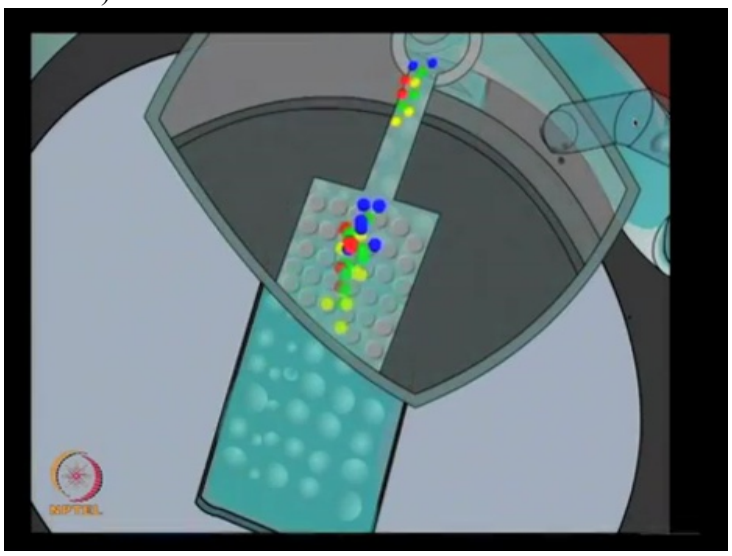

… liquid chromatography …

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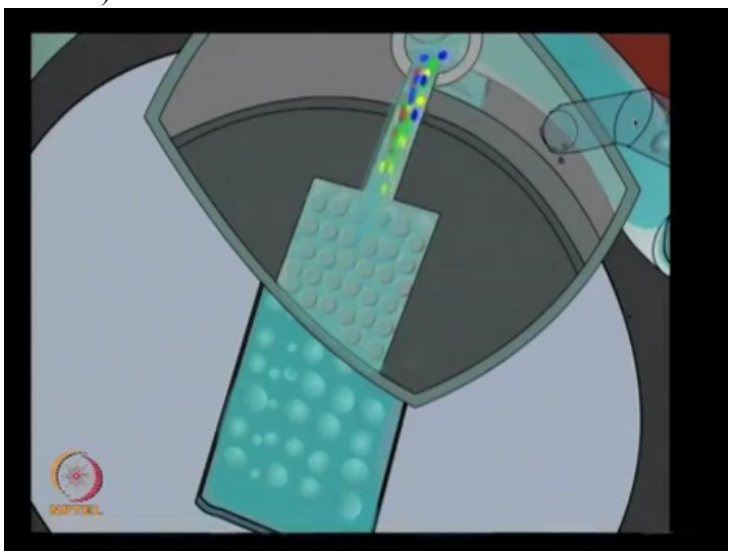

… steps

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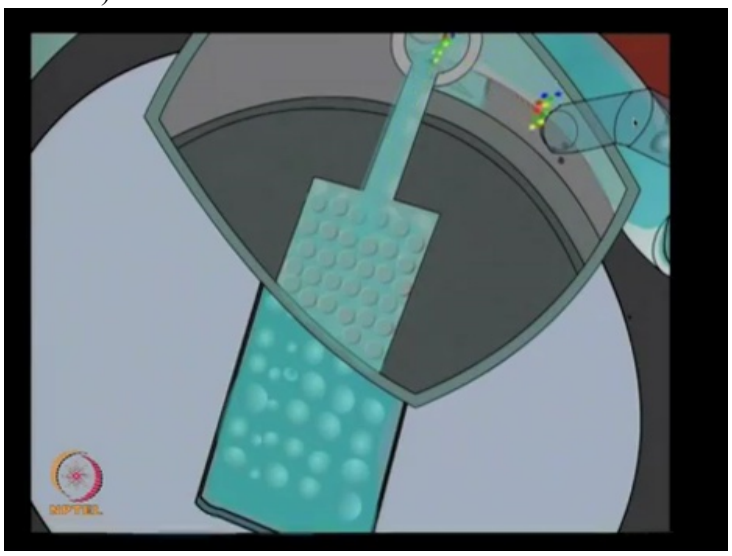

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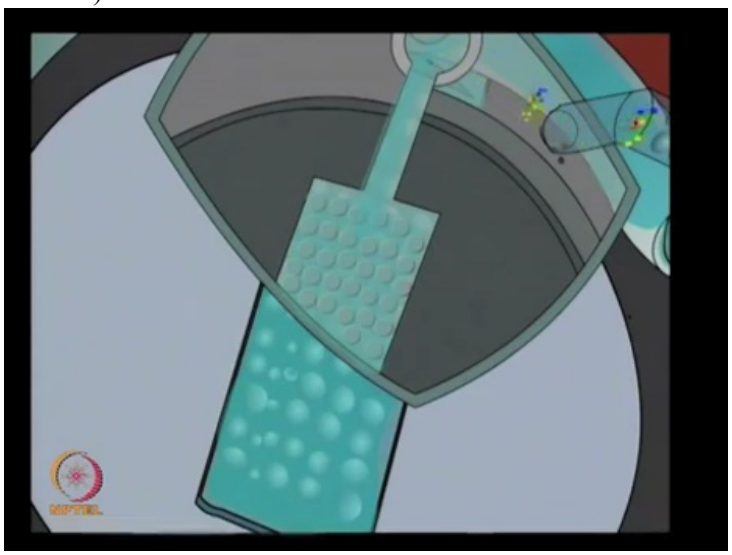

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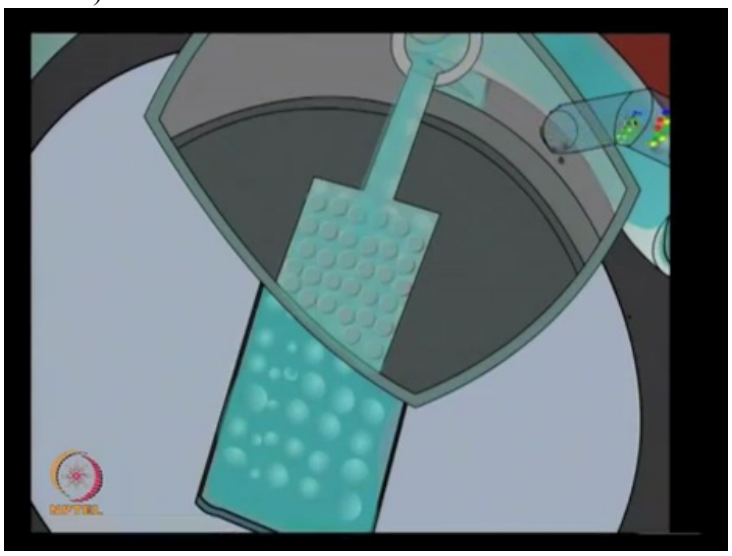

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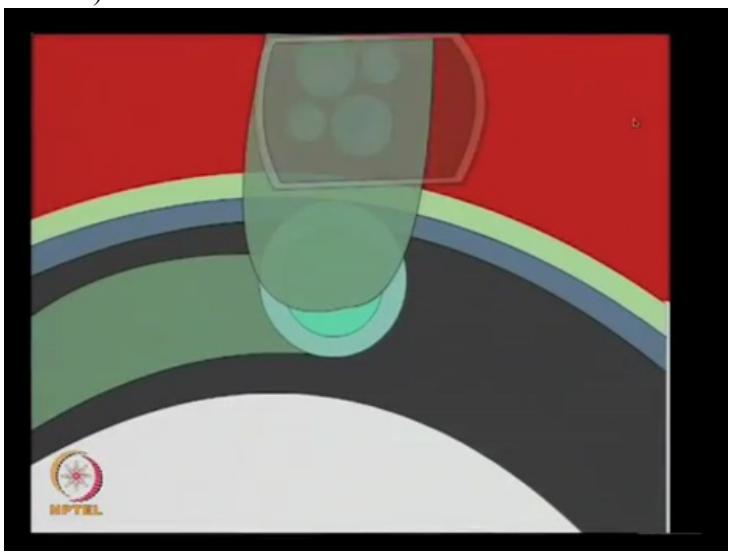

While making the integrated system it avoids several pitfalls which are associated with …

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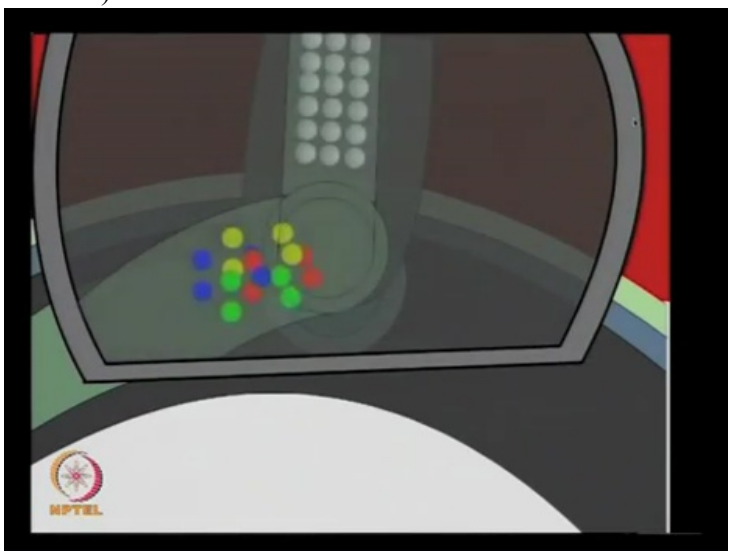

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…separate tubings and fittings

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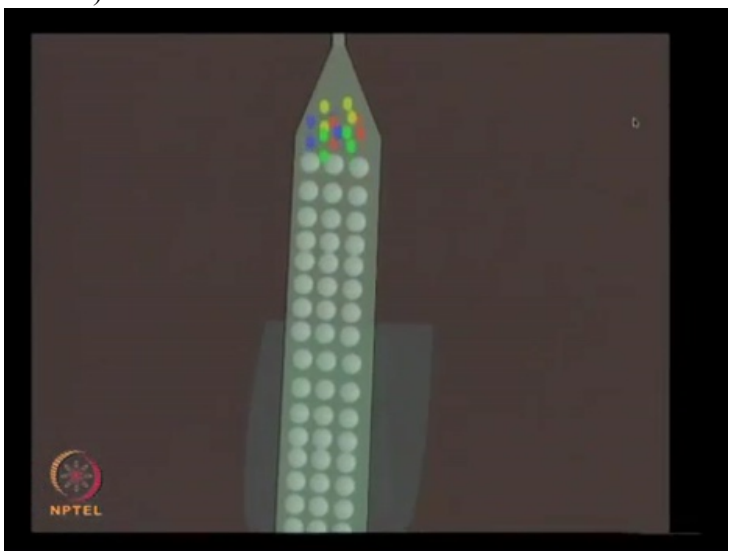

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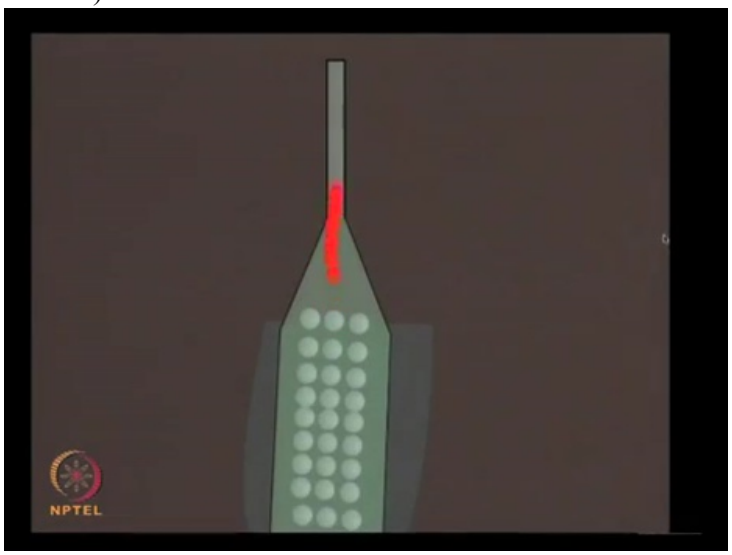

Another important component of this technology is …

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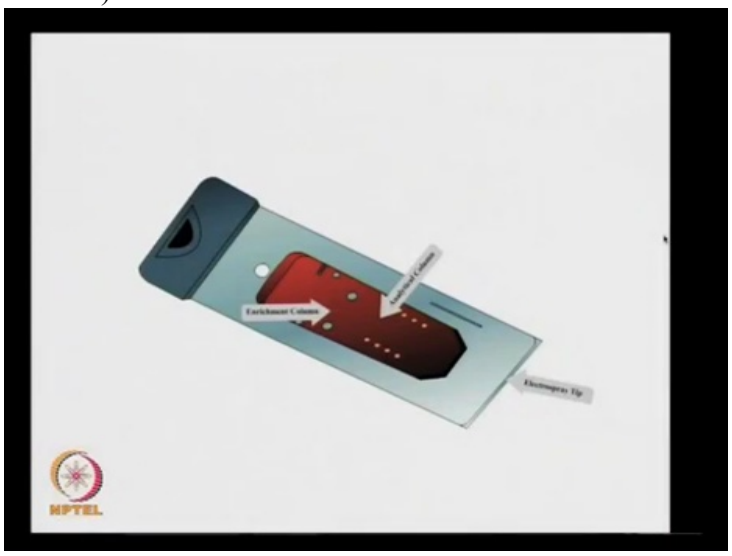

…the HPLC-Chip/MS interface. A chip is inserted into the interface

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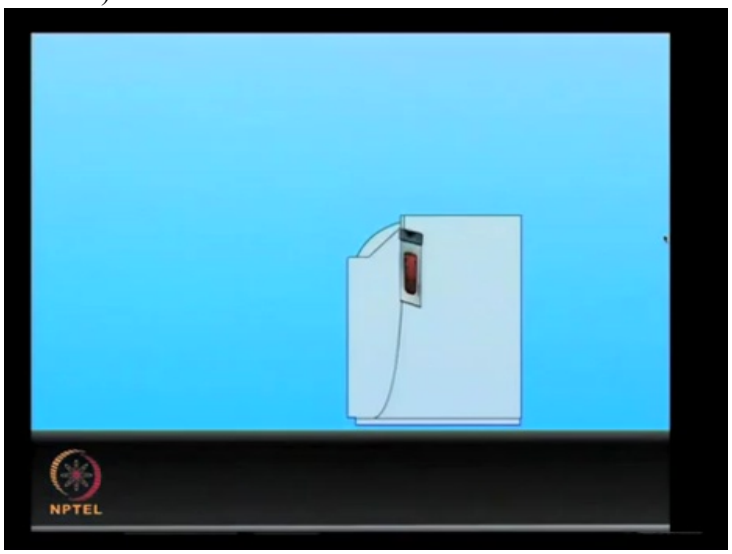

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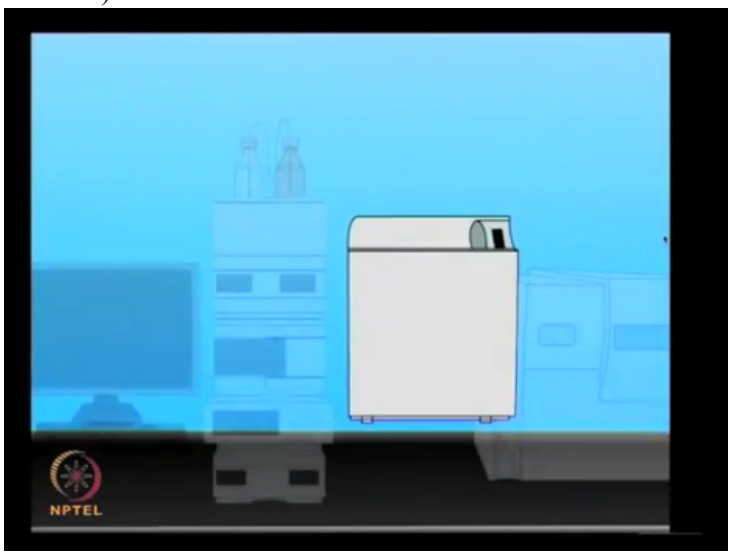

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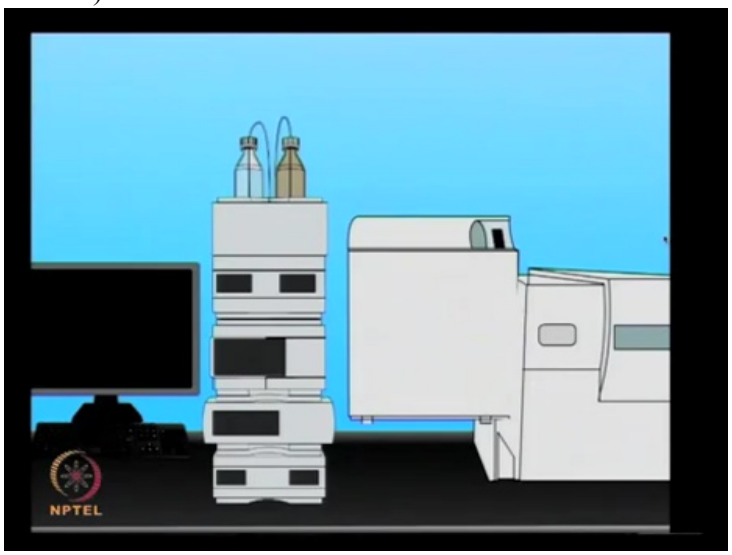

… which mounts on a mass spectrometer

So this design configuration ensures that the electrospray tip is in the optimal position for mass analysis when the chip is inserted in the mass spectrometer.

So compared to the conventional nanospray techniques, this technology achieves maximum sensitivity with minimal sample sizes by integrating sample preparation, separation, and electrospray tip on a single chip technology.

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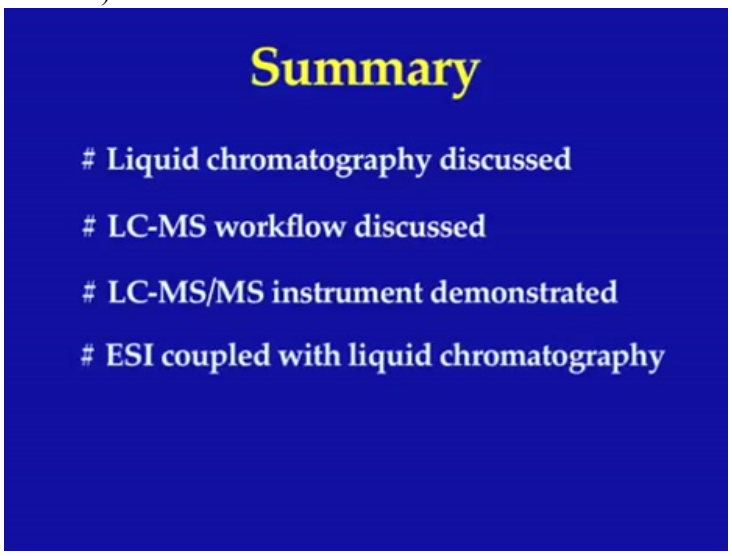

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