Introduction to Proteomics
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Lecture - 39
Lab session - Targeted Proteomics using Triple Quadrupole Mass Spectrometry

Okay, very good afternoon to all of you, I am Shalindrani, Assistant manager for LC LCMS department from Shimadzu. Today, we are going to discuss about the basic principles of LCMS and LCMS MS system. First of all, what we will do? We will try to understand, what is the triple quadrupole? What is LCMS? What is the basic function of LCMS? How it works exactly? And after this we can exactly see, how we can inject the sample? How we can get the data. And how this type of the instrument is helpful for the targeted proteomics experiment.

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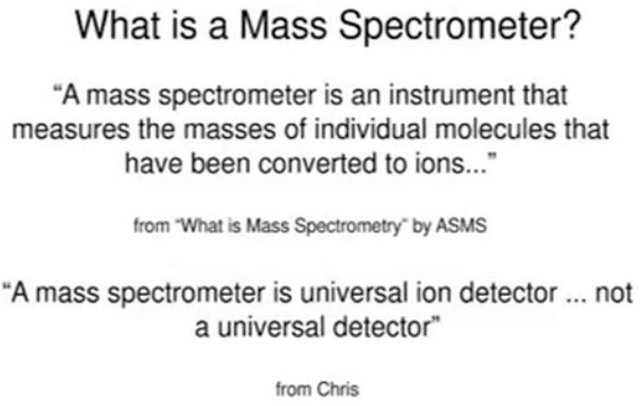

What is a Mass Spectrometer? Mass spectrometer is an instrument that measures the masses of individual molecules that have been converted to ion. A mass spectrometer is universal ion detector that means, what is the basic requirement for mass spectrometer? You should convert the compound into ionic form. And mass spectrometer is not a universal detector, it is a universal ion detector.

Therefore, you have to make a condition feasible for the ionization of your compound, it does not mean that you can ionize each and every compounds which are present in the sample, you

have to make sure all this compound at least a targeted compound should ionized okay and this ionized compound you can separate based on mass to charge ratio, and you can do the analysis, qualitative as well as quantitative analysis.

How to ionize your compound? How to separate this compound? That we are going to discuss later. But basically, what is the mass spectrometer? Mass spectrometer is a universal ion detector.
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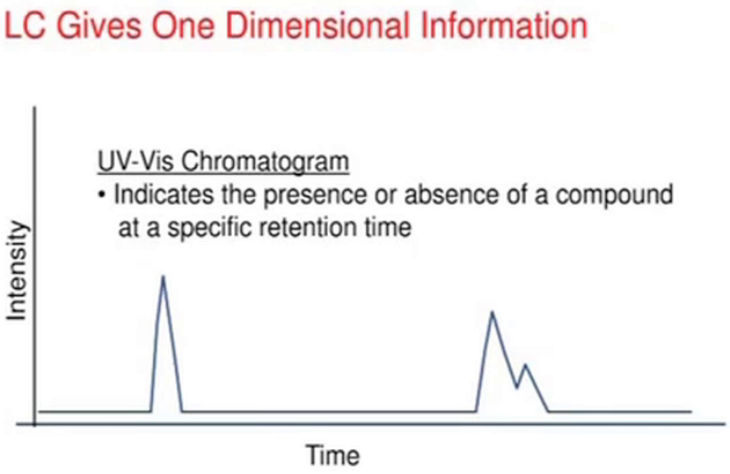

LC gives one dimensional information to feed the sample on the mass spectrometer either we can use a LC as a frontend or we can directly insert the sample into the system, based on the property of compound you can select any type of detector. You can only give the information about the time and the intensity that means if you want to identify a compound on the same condition you have to run a standard, you have to match retention time.

Retention time means the time from the injection of the sample to get a peak consumer, the time is known as retention time. By matching the retention time of the compound under identical condition you can identify a compound and based on the response or intensity of this compound, you can quantitative compound okay. But there is a possibility that for example if you are trying to do the sample analysis from the complex matrix single peak does not make only one compound.

There is a possibility two or three compound make correlate, you cannot distinguish by only LC right.

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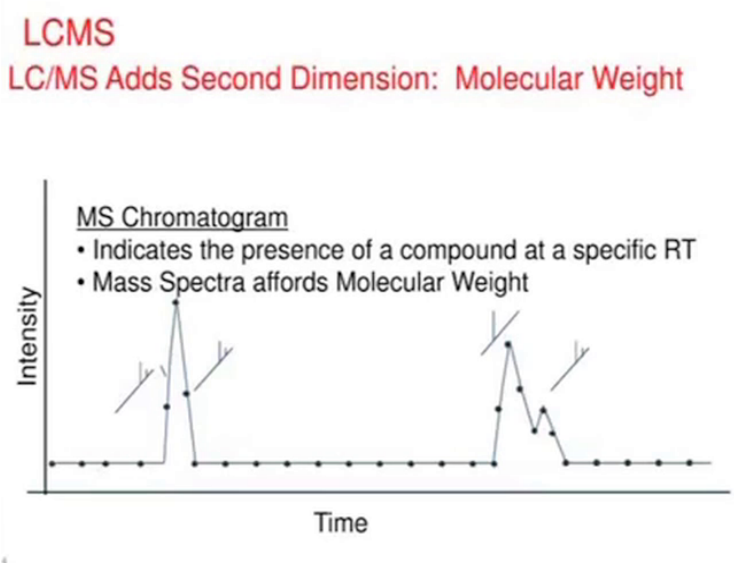

In that case you can use a MS as a one of the detector, MS what is the information you are getting not only the retention time but based on the mass spectra, mass spectra like mass to charge ratio you can do the confirmation. There are also possibility two or more compounds which are eluted at the same retention time may have same molecular weight okay. How we can confirm the peak or compound which is eluted at this retention time is pure or not right.

For example, you are doing the analysis of the small molecule from the matrix cell plasma, the molecular weight of compound is 490 okay, you are getting the particular retention time for example 2 minute. You are getting the m/z ratio 491 or 489 okay, 491 in positive and 489 in negative based on the protonation and deprotonation okay, but there is a possibility when you deal with the analysis of such compound from the matrix.

Few of the compounds for example proteins, carbohydrates or any other biological compound may have the same molecular weight, it can elute at the same retention time, how we can confirm it is only single compound or multiple compound.

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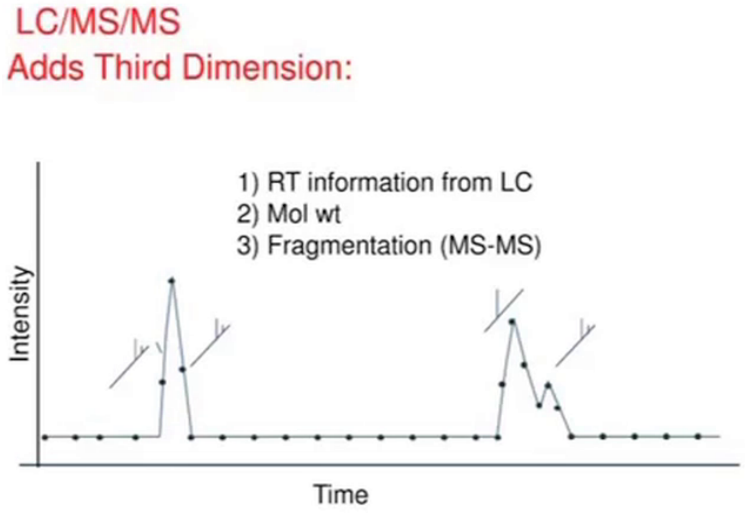

In that case you should add one more dimension that is the LC MS MS, in LC MS MS what are the information you are getting? The retention time from the LC, the molecular weight from the first MS and fragmentation pattern from MS MS. Let us take the same example the molecular weight of your compound is 490 and the interface molecular weight also having the molecular weight of 490, you are getting a m/z ratio 491.

But when you do the fragmentation the product ion, which formed the compound of interest and interface molecule will be different okay. Therefore, what you have to do? You have to focus that product ion that specific for your component of interest, in that case you can avoid the detection of interface molecule and you can quantitate your compound in presence of different biological matrix or interface molecules.

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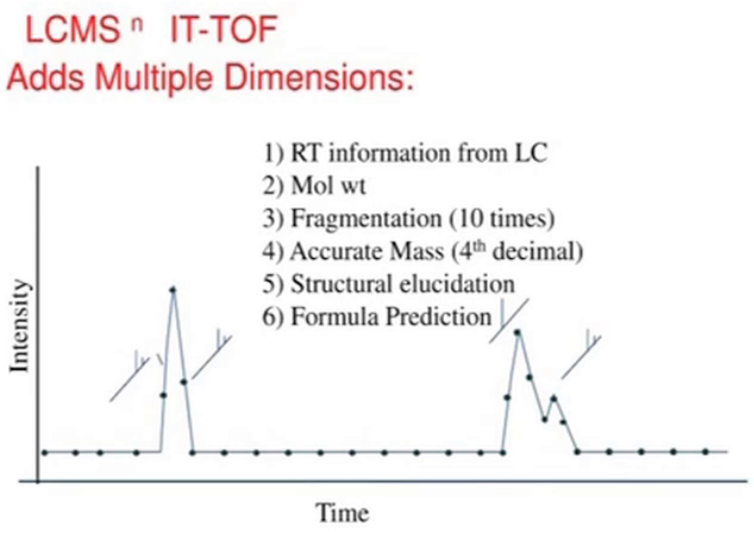

You can also did the LCMS MS MS analysis by using the IT-TOF Ion-Trap with the Time-of-Flight, this type of analysis may help for the structure elucidation of a compound, where we can get the accurate mass up to the four decimal, you can do the structural elucidation and formula prediction.

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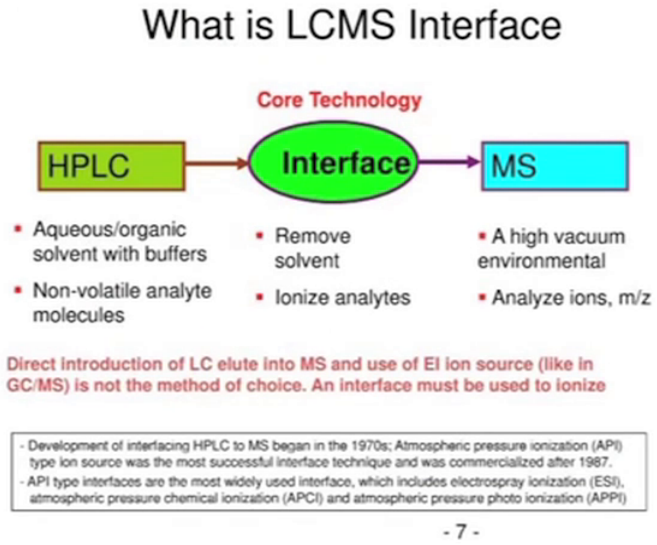

The interface are present between HPLC and MS, HPLC we are using aqueous or organic solvent with buffers, in HPLC you have to use the volatile buffer why? Because if we are using the nonvolatile buffer in case when you are using in the MS what happen? It gets precipitated it forms the salt formation and MS get contaminated. Therefore, in the case of the MS when you

deal with analysis for HPLC you have to use volatile buffer or volatile organic solvent. The MS you have to use a high vacuum environment and analyze based on mass to charge ratio.

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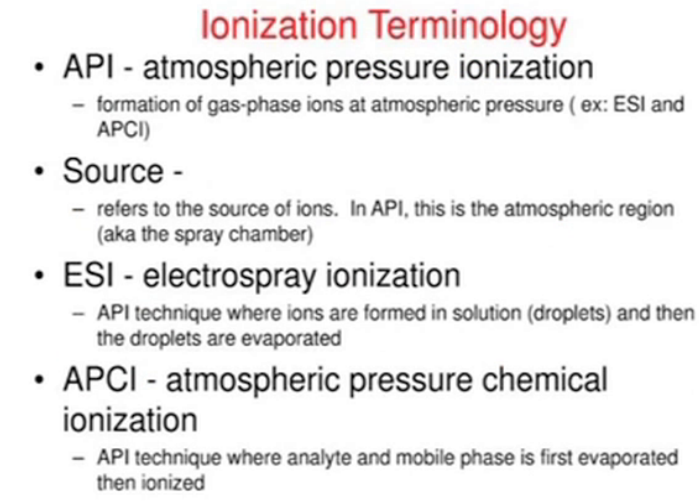

What are the different ionization terminologies are there, API is the atmospheric pressure ionization, under API there are two technique one is the ESI and APCI. ESI is a electrospray ionization technique. APCI is the atmospheric pressure chemical ionization. The one more technique is there that is known as APPI that is atmospheric pressure photoionization. Different techniques have different application based on polarity of your compound you can select any one of the technique for the ionization of your compound.

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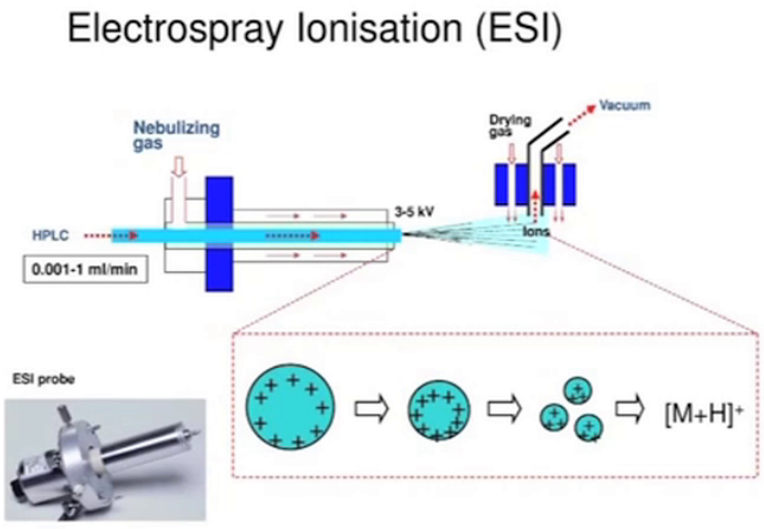

Let us see ESI that is electrospray ionization technique, in this case there are 2 concentrated capillaries are there, from the middle capillary there is a flow of the liquid or mobile phase coming from the HPLC, from the outer capillary there is a flow of the nebulizing gas, here we are using the nitrogen as a nebulizing gas, at the tip of the capillary we are giving high voltage between 3 to 5 kilovolt.

What happened? Because of the nebulizing gas nebulized into the fine droplet, and because of the high voltage it forms the charge either positive or negative, it forms the single type of the charges. Then what happened due to the high temperature the solvent start evaporating. There are two forces which are acting on the solvent droplet, the one is the columbic force of repulsion between the same charges, the second is the surface tension.

The surface tension try to put the all the ions together, but the columbic force of the repulsion they try to apart from each other, and once the columbic force of repulsion overcome the surface tension that is now relic limit, now what happen? The ions get separated okay it comes out. And finally it enters into the mass spectrometer through the desolvation okay, this is the phenomena which offers in the electrospray ionization.

Generally, electrospray ionization used for the polar type of the compounds and for the protein analysis most of the cases, this is the only technique which are used for the ionization ESI technique okay.

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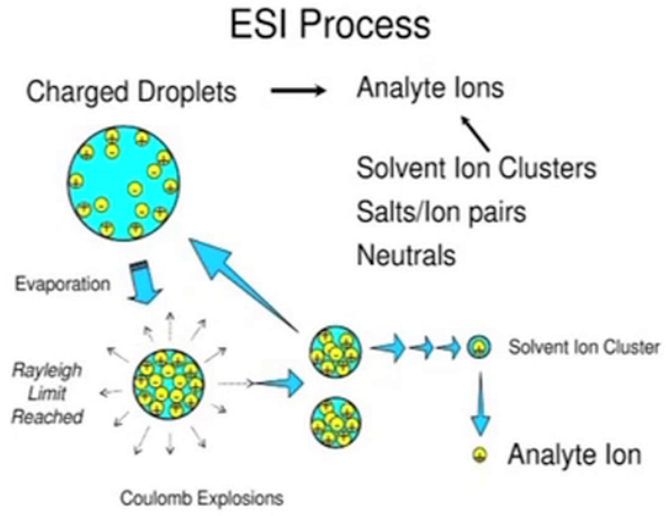

This is the process which I already explained, how compound get ionized and enters into the mass spectrometer.

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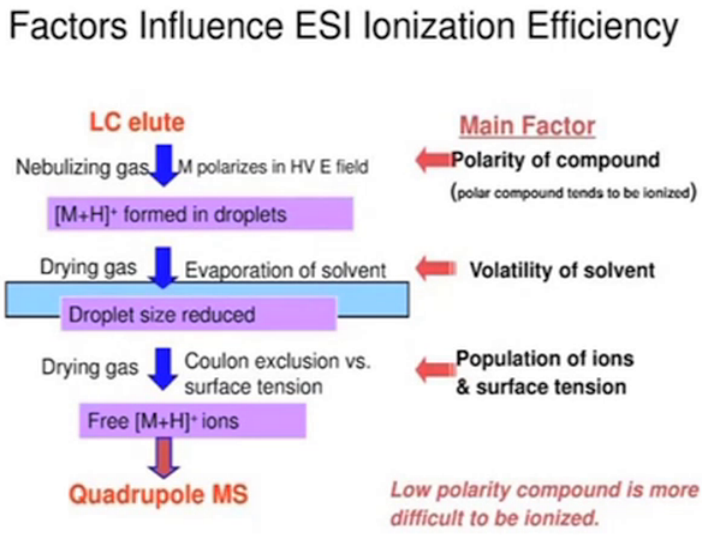

The factor influencing the ESI ionization efficiency are mentioned here, the air we are using the LC elute, the nebulizing gas this polarized into the high voltage it form the molecular weight +1 that is protonation or deprotonation. Protonation means addition of the H+ ion, deprotonation means removal of the H+ ion. Therefore, always you are getting if the protonation occurs you are getting molecular weight +1.

For example, molecular weight 390 you are getting the spectra of 391, in case of the negative mode you are getting 389 okay that is due to the protonation and deprotonation of your molecule. Then the driving gas that is used for the evaporation of the solvent, because of that what will happen the droplet size would reduce, and then when the competition between the columbic force are the exclusion or the surface tension. And then there is a formation of free ions and that enters into the mass spectrometer.

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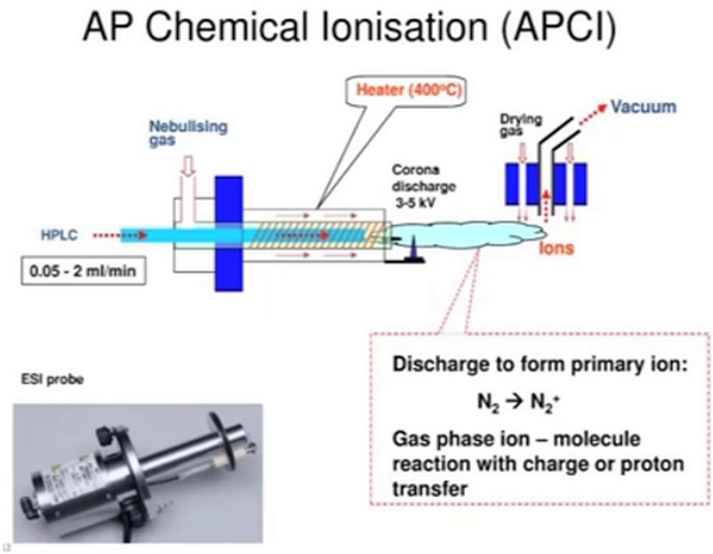

The next technique is the APCI, the APCI is the atmospheric pressure chemical ionization techniques, ESI used for the polar component, APCI used for the mid polar compounds. How it works? Here also there are 2 concentrated capillaries are there okay, and there is a heater plates are there, what happened? Here also we are using nebulizing gas it nebulize between this heater plates that is maintained at the higher temperature okay.

And what happens it forms the small, small droplets okay, the compounds are not yet ionized, there is a corona discharge needles is there that is maintained at a 3 to 5 kilovolt. When the compounds in the vapour form this right to the corona discharge model, this is where the initiation takes place, nitrogen get a ionized, and when its strike to your molecules what happened that the charge get transferred and compound get ionized.

This is a process of the chemical ionization okay, and then finally the compound which are ionized get enters into the vacuum in the mass spectrometer, generally this type of the technique used for the analysis of the mid polar compounds or compounds which are difficult to ionized. The main difference in the ESI the compounds ionization takes place in liquid form, and in case of the APCI the compound get ionized in the gaseous form okay.

For example, steroids or vitamin D2, D3 this type of the compounds we can use this type of the technique for the ionization.

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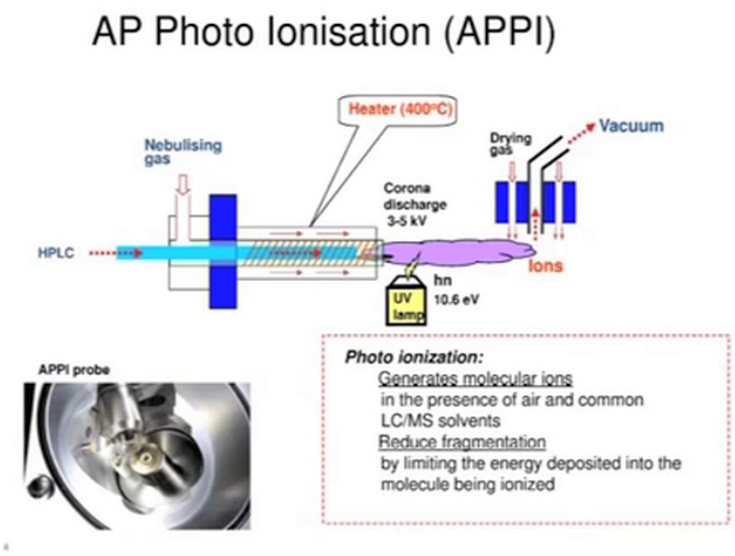

Then APPI atmospheric pressure photoionization technique, in this case the technique is almost same for instead of the corona discharge needle we are using the UV lamp at the particular frequency for the ionization of your compound okay, it is under the molecule as in the presence of the air and the common LCMS solvent the reduced defragmentation by limiting the energy deposited into the molecule being ionized. Generally, this type of technique used for the ionization of nonpolar compounds.

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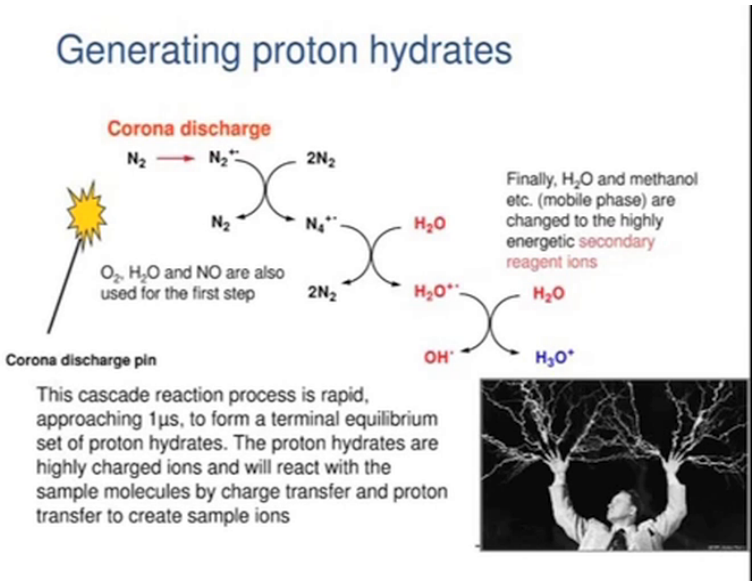

This is the process how the compound get ionized and the charge get transferred to molecules, I am not going to discuss in detail okay.

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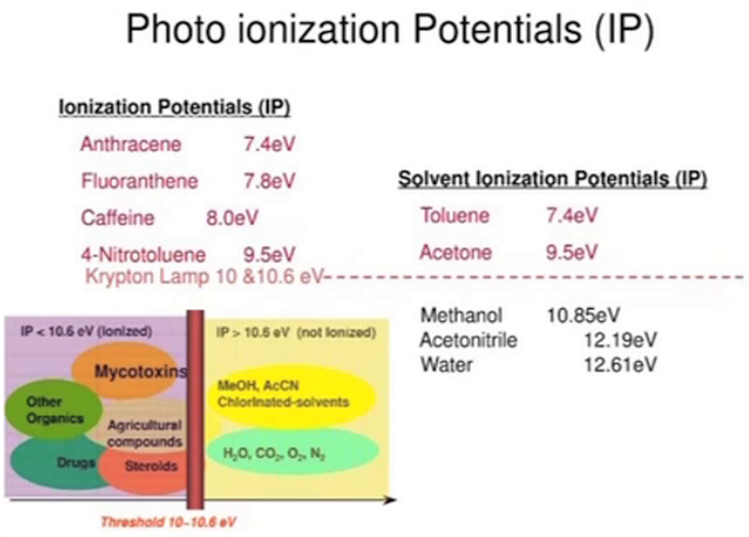

The initiation potentials these are the different types of compounds, which can ionized or you can analyses by APPI, these are different types of the potentials which you can apply to that the laser beam, so that the compound get ionized okay.

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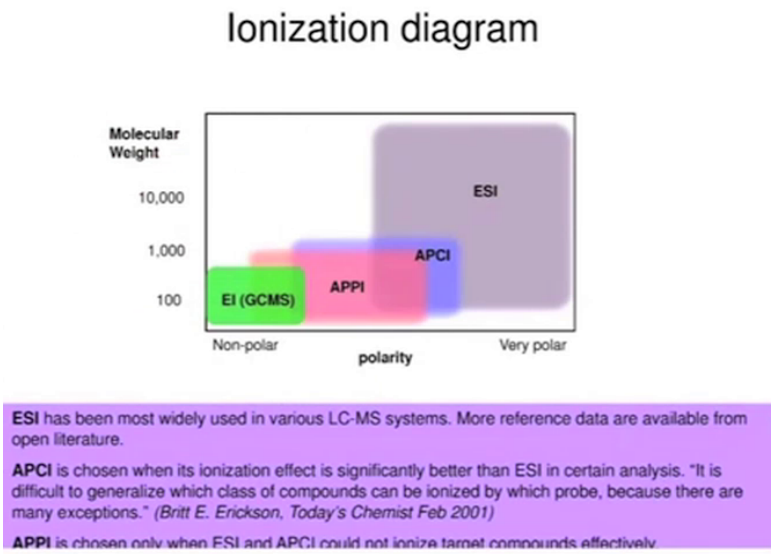

This chart will help you based on the molecular size and the different polarity which technique is suitable, ESI you can use for the very polar compound to mid polar compound which having the high molecular weight compound also you can utilize. APCI used for the mid polar to nonpolar. APPI is also similar way. And EI, which is used in the GCMS is specifically used for the small molecular non-polar molecular compounds.

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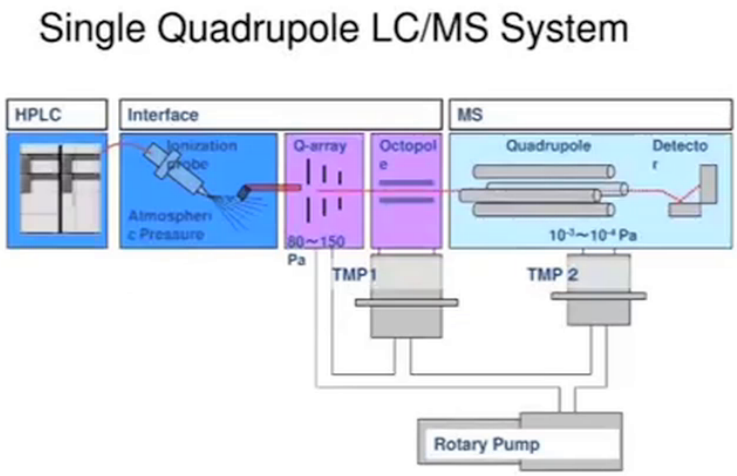

The single quadrupole this is the basic configuration of the single quadrupole that we are going to discuss, in any LCMS system what it contains? It contains the interface, it contains the ion guides and then mass analyzer and detectors okay. The interface as I told you are used for the

ionization of your compound and to remove the solvent. The ion guides, here the ion guides are the key variant octopols which are focused the ion in the same direction.

And then the mass analyzer, the different type of the mass analyzer you can utilize for the separation of the ions based on mass to charge ratio, here we are using the quadrupole as a mass analyzer okay. How it works? It contains the 4 rods the opposite rods having the similar type of charges, we are providing the DC and RF voltage, the RF voltage are used for the acceleration of the ions in the forward direction, and DC voltage are used for the selection of the ions.

What happens generally, when a compound gets ionized and then it enters into this is a desolvation line where compound get enters into the mass spectrometer, all these things which are in the under vacuum condition, where giving primary secondary and tertiary type of vacuum. Generally, for the low vacuum we are using the rotary pump, and for high vacuum are using the turbo-molecular pump which can use the vacuum up to 10 to the power -4 Pascal.

Then ions enters into the ion guides, the ion guides what is the main function? To focus the ions okay, in the same direction it avoids the loss of the ions, if the ion get lost in this what happened the total intensity will decrease or sensitivity will decrease. Therefore, in most of the vendor they are focusing for a development of this ion guides, so more and more ions get focused, there is no loss of the ions okay.

And for the ion guides we are providing the RF frequency, why RF frequency? Because as I told you the RF frequency helps for the focusing of the ions, they go to the quadrupole, quadrupole along with the RF frequency also giving the DC voltage. The DC voltage are used for the selection of the particular ions, the RF voltage for the movement of the ions in the forward direction.

For example, if you want to analyze the particular ions okay of particular m/z ratio the instrument is tuned in such a way that based on molecular weight the DC to RF voltage tuned, because this LCMS or any other any LCMS before doing the analysis you have to do the

calibration of the LCMS. In the calibration we are using the different molecule size of the compound okay whose molecule size is known to us.

And it is recorded by the software and based on the molecular weight of that compound or that ions, it tuned the DC to RF voltage, and the information gets stored into the instrument. When you do the analysis of a compound of interest, for example I want to do the analysis of the compound having the molecular of 390, then what happened 390 what is the DC to RF voltage is there the information already stored the quadrupole maintained that type of the voltage only that ion get directed into the quadrupole or selected into the quadrupole.

The rest of the ions get destabilized and goes to the vacuum, and finally its goes to the detector. The detector is the electron multiplier with conversion diagnose, where ion strike is generated the electrons and the electrons get multiplied and we are getting the results, okay you are getting the chromatograms okay. Just now we discussed about the basic model that is single quadrupole okay, what is the working of the single quadrupole? Now we are going to focus on the triple quadrupole right.

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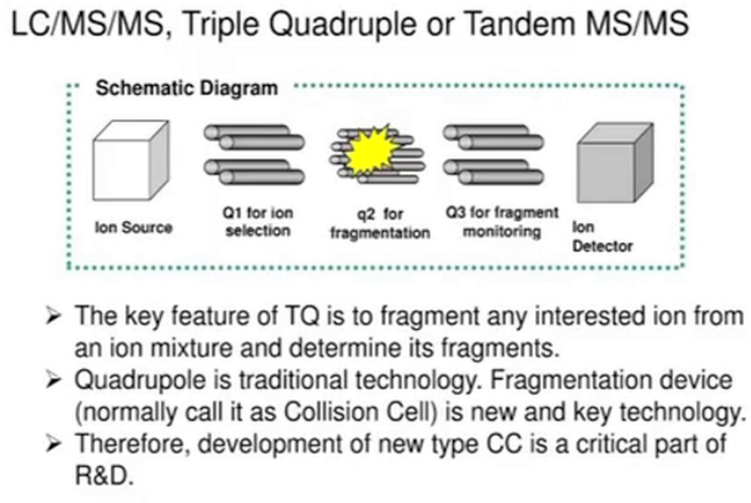

In triple quadrupole how it works? It contains the three quadrupole Q1, Q2 and Q3. Filtration of the ions takes place in the Q1 and Q3, the second quadrupole only used for the fragmentation okay. How exactly it works? Here, we can select a particular ion of the particular interest in the

first quadrupole it passed to the second quadrupole pole, the second quadrupole it contains some collision gas that is known as argon or nitrogen okay.

And what happened? The ions are having some kinetic energy, you are providing some energy are some potential to the argon gas it gets vibrate when it is strike to your compound of interest it gets fragmented and form the product ion in the q2, this product ion get accelerated into Q 3, it gets separated and getting the more information. Therefore, to get the more information about your compound you can use this type of the technique.

This not only the one technique is possible, there different type of the modes are possible like the MRM multiple reaction monitoring, the precursor ion scan, Newton law's scan okay, these are different types of the modes which are possible and different modes having different type of the applications okay that we are going to discuss in subsequent slide, but what is in short what is the difference between single quadrupole and triple quadrupole?

Triple quadrupole you can use, you can do the fragmentation of a compound of interest you can generate more information and by selecting the particular fragments ions okay you can do the quantification of your compound of interest, okay.

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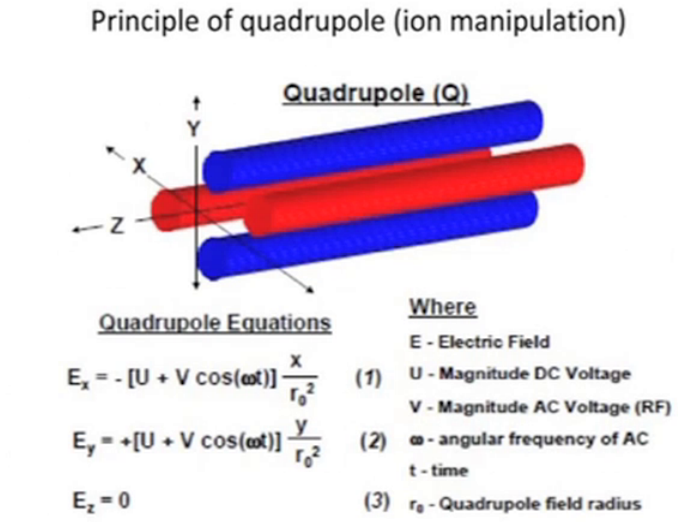

The quadrupole we already discussed what is the principle of the quadrupole it contains the 4 molybdenum rods okay, and the opposite rods having the similar type of the charges okay, and you are providing the RF and DC voltage okay, but that is voltages are constantly gets fluctuating. What happens when the ions gets introduced into the quadrupole okay, for example if the ions forms the negative charge okay attract to the positive charge.

But when it goes to that type of quadrupole that rods what happens the charge gets changed to next rods, then ions move to the next rods when it is going to restore that rod the charge again gets changed on the rods it comes it, like that way there is a motion of the ions between this rods okay. And then what happen? It moves due to the RF voltage it moves on the forward direction okay, and those ions which get destabilized it moves out okay and then it goes to the vacuum.

They were like that way the ions are moving between these quadruples and by providing the particular DC voltage you are stabilizing the particular m/z ratio mass ion among these quadrupole and rest of the ions get stabilized into the vacuum, and like that way you can select the particular compound of interest.

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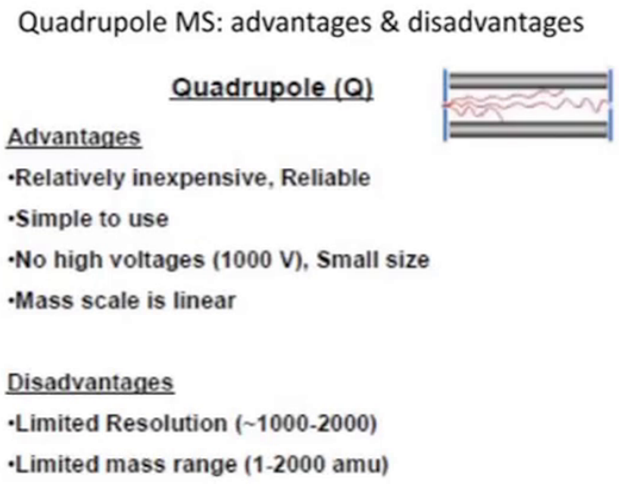

What is the advantage? Relatively inexpensive, reliable, it is simple to use, no high voltage are required we are not applying any voltages here, mass scale is linear. The limit resolution up to 1000 to 2000, limited mass range up to 2,000 amu. Generally, the quadrupole we are getting the

unit resolution okay, suppose there are 2compound having the molecular weight of 400 and 400.5 or 400.51 this compound cannot be distinguished okay.

Because here it is the unique resolution mass spectrometer okay that means it can difference between the 400 and 401, it cannot distinguish from 400.51 or 400.5 okay, because it will not have the so much of the mass accuracy like time of flight okay.

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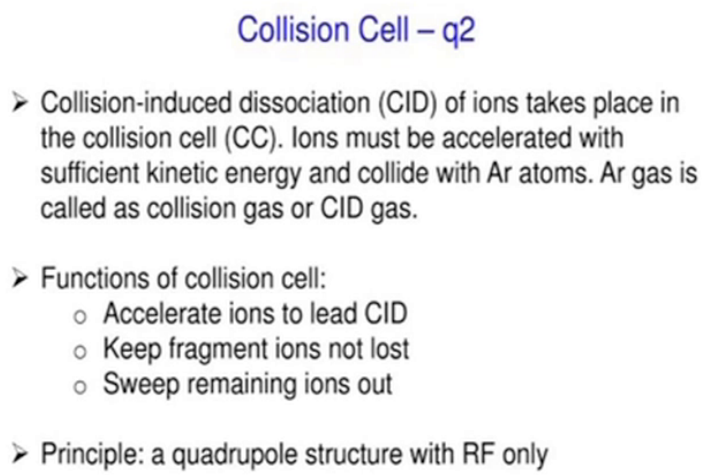

The collision induced dissociation of ion takes place in the collision cell okay, ions must be accelerated with sufficient kinetic energy, and collide with argon atoms argon gas is called as the collision gas. As I already explained you once the ions gets selected in the first quadrupole it goes to the second quadrupole it collides with the argon gas of the particular energy and based on the collision energy the compound gets fragmented. And then this product ion gets scanned in the third quadrupole, okay.

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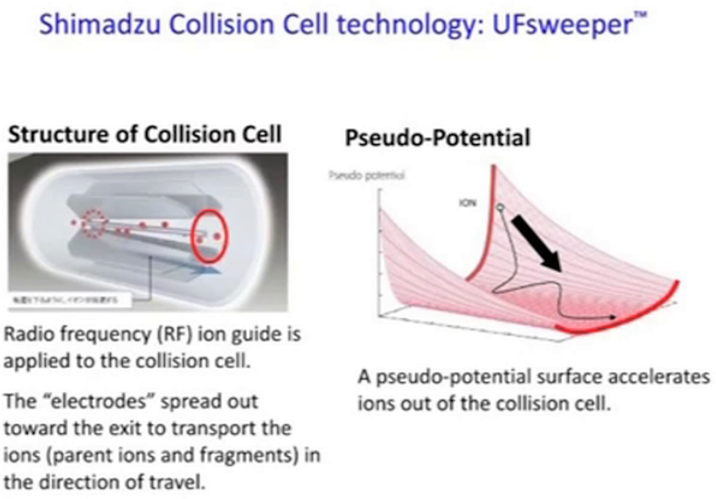

This is the one of the technique which are utilized by the Shimadzu instrument that is the ultra-fast sweeper technique, what happens when you use the multiple type of the event okay what happens the ions coming from the first quadrupole okay. It strikes with the argon gas it form the product ions, during that process it will lost some of the energy okay, the product ions which are formed in the q2 okay it lost the some momentum.

Therefore, it moves slowly towards the third quadrupole, if the next event will start okay, it contains the similar type of the quadrupled you may get the crosstalk effect to avoid that we have provided with this type of technique that is known as the UF sweeper. In UF sweeper this type of alignment of the collision cell, what happens? It provides the pseudo-potential, in pseudo-potential what will happen?

Generally, when the ions get fragmented in the second quadrupole ions are moves like this way okay it moves slowly it follow the longest path way. When you apply the pseudo-potential the ion swipe very easily from the second quadrupole, what will happen? You will now get the crosstalk effect okay. This type of the technique helpful to reduce the cross talk effect when you deal with the multiple MRM events, okay.

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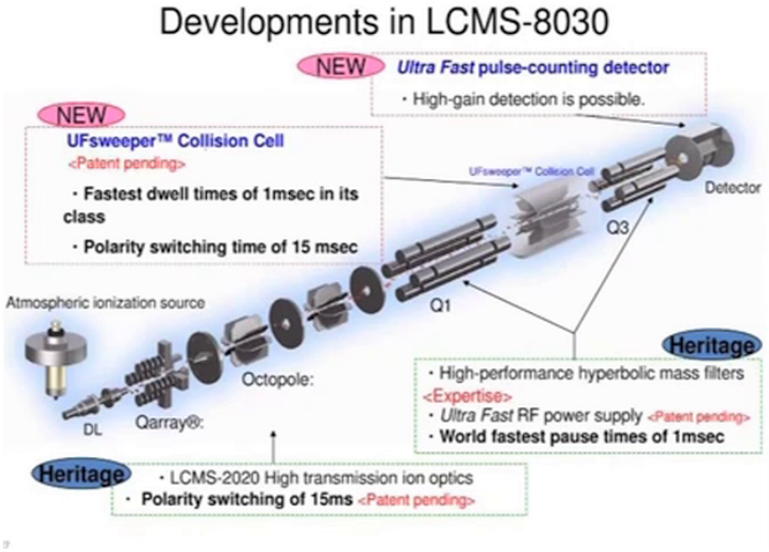

This is inside of the triple quadrupole, this is interface you can use the ESI or APCI, this is DL desolvation line, you can give the temperature for the desolvation line, so that it removes the solvent that you are associated with other molecules ions. The ions get enters into the queue array on the octopoles, these are act as a ion guides that focused the ions in the same direction, and then these are the schemer.

The schemer is used to maintain the vacuum difference, it enters into the quadrupole where the selection of the ion gets the takes place based on the mass to charge ratio, then this is collision cell where the fragmentation takes place. Third quadrupole where the product ion gets scanned or detected and there is a detector.

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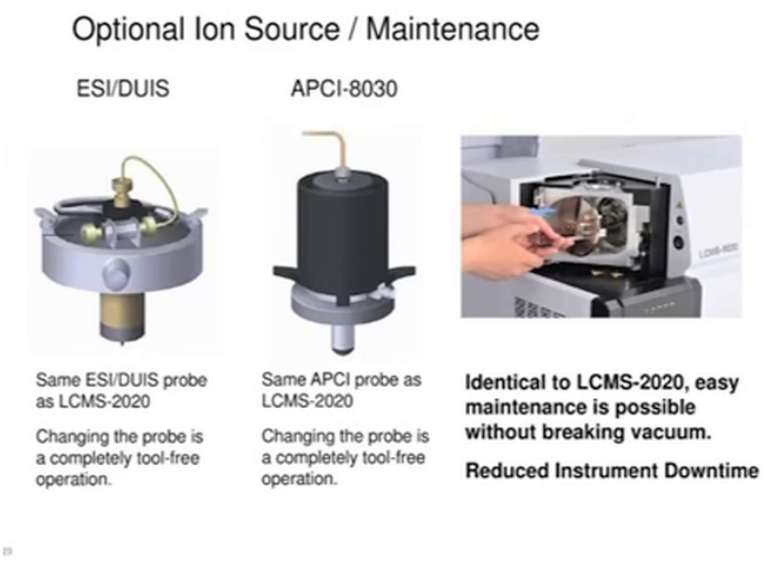

Generally, as I said there are three main interfaces are there ESI, APCI and APPI okay, most of the time we are using ESI and APCI okay, the one more also there that is a DUIS mode. DUIS mode means dual ionization interface, in that case what happens? ESI and APCI you can do simultaneously okay, then the ESI technique used for the ionization polar compounds, APCI for the mid polar to nonpolar compounds.

Suppose you have the mixture of the samples, suppose you have the sample it contains the mixture of the polar and nonpolar compounds okay in that case what you have to do? You have to first run on the ESI, you have to change the interface, you have to run on the APCI and then you have to match the data. But when you have the DUIS mode, in DUIS mode we are using the ESI probe, but along with that we are using the corona discharge needle.

What happens due to was you can analyze both the type of the polar and mid polar compound by using the type of technique I can generate the data.

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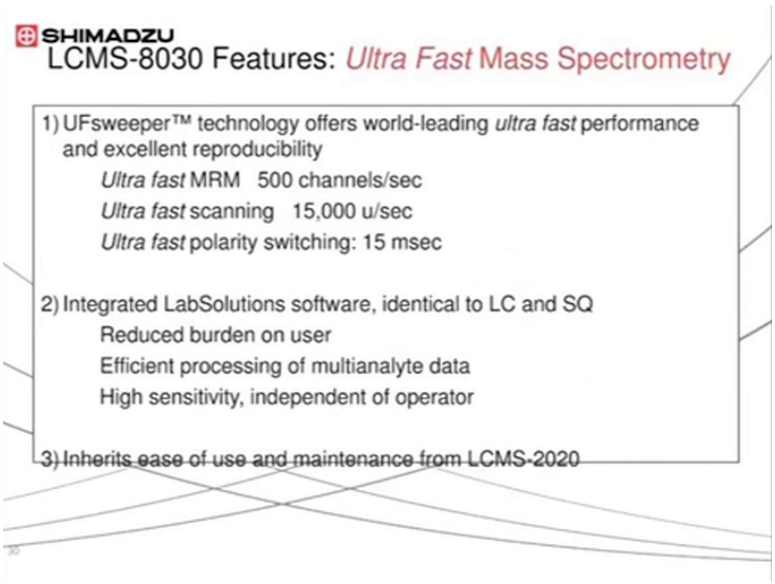

These are the some of the features of the instrument and you can.

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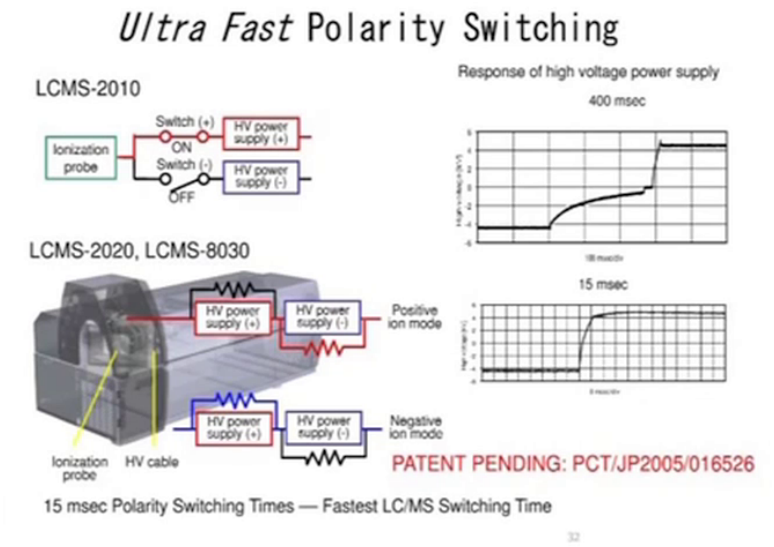

Polarity switching this is also one of the important functions when you deals with the different type of the ions having different polarity okay, when the compound get ionized some of the compound get ionized in the positive form, some of them ionized to get negative form. When you analyze the positive ions all the voltage from the starting to end detected can converted into negative, so the all the positive ions that separated okay.

When you want to ionize the negative compound, the voltage switchover from negative to positive, the switch over time is known as the polarity switching time. If the polarity switching

time is very slow what happens? During the switching time most of the ions get lost and because of that the quality of the data gets reduced, okay. Therefore, you required the polarity switching times should be very, very fast.

So that you can analyze both the positive and negative time simultaneously and you can detect the data.

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Qualitative methods on TQ (tandem MS/MS)

- TQ is a tool of choice for qualitative analysis in various operation modes and for various targets.

Operation mode	Q1	q2 (Collision Cell)	Q3	Application
Q1 Scan	Scan	All ions pass	All ions pass	Full spectrum like SQ
Q3 Scan	Pass all ions	All ions pass	Scan	Full spectrum like SQ
Product ion scan	Selected ions pass	Ions are fragment	Scan	Detect all fragments of a targeted compound
Precursor ion scan	Scan	All ions are fragment	Monitor interested fragments	Monitor a targeted compound by detecting its fragments
Neutral Loss scan	Scan	Selected ions are fragmented	Scan	Monitor interested neutral loss from mixed sample
Automatic MS/MS	Scan	All ions are fragmented	Scan	Detect all fragments of every compounds

The scan Q1 scan means you are scanning the first quadrupole all the masses, second quadrupole and third quadrupole act as the ion guides that means it is on the RF voltage that means here the triple quadrupole works as the single quadrupole, only you have to detect all the ions generated by the interface. Similar, for the Q3 the product ion scan, in the product ion scan you have to select the particular precursor in the first quadrupole, it passed through the second quadrupole it do the fragmentation.

And what are the fragment we are getting? What are the products you are getting you have to scan, this is we are getting the qualitative information. The 2 more scans are there precursor ion scan and neutral loss scan, these two types of the scanning which are used for the screening purpose. When you want to screen the particular group of the compound you can use this type of scan and I am not going to discuss in detail.

Most important is this one that is the MS MS or MRM, MRM is a multiple reaction monitoring okay, what you have to do? Here, the first quadrupole in the SIM mode, you are selecting the particular precursor ion it passed to the second quadrupole, it gets fragmented from the different product ion among all the product ion you are selecting that product ion that is specific your compound of interest and the intensity is also good that means third quadrupole in the SIM mode.

And this from the precursor to product that is known as the MRM transitions, and this is used for the quantitative analysis of the compound from the different matrix okay.

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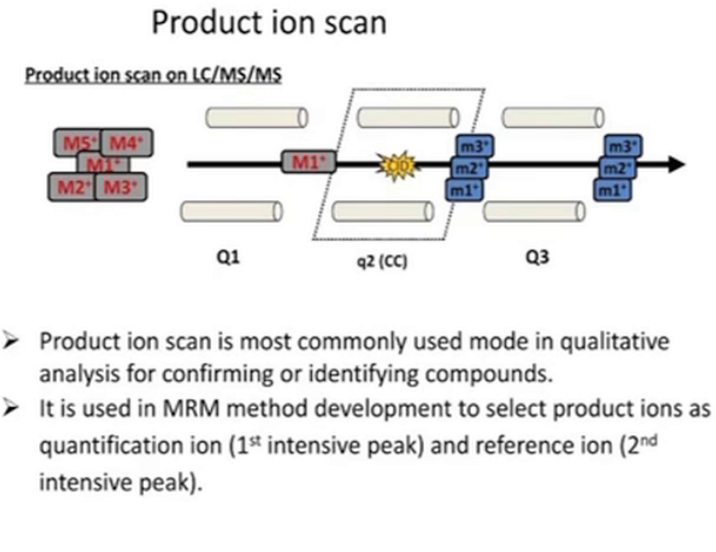

This is the product ion scan as I explained already, there are different types of the ions are formed only you are selecting the particular ions of interest okay that goes to the second quadrupole, it gets fragmented it forms the different types of the product ions I was scanning all the products ions that gives the good information of your compound of interest and it is very good qualitative tool.

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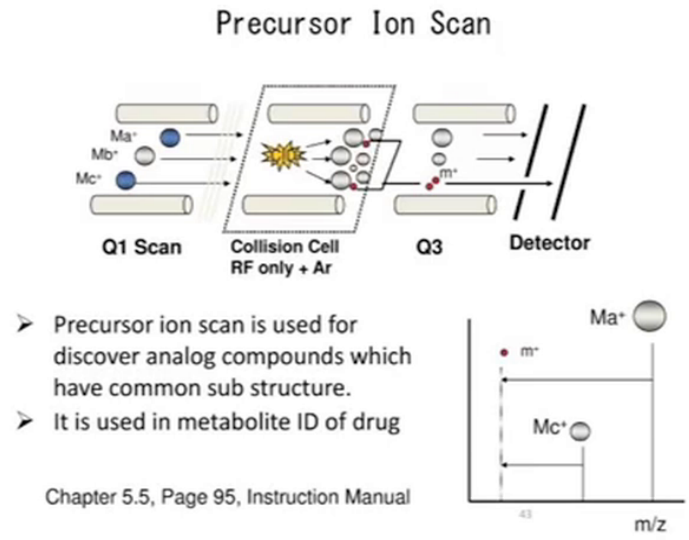

Then the precursor ion scan, okay, in the precursor ion scan what you are doing? Q1 in the scan mode and Q3 in the SIM mode that is opposite to the product ion, you have to look for the group of the compound will having the common function group or common precursor. First step of the screening purpose you are using the precursor ion scan.

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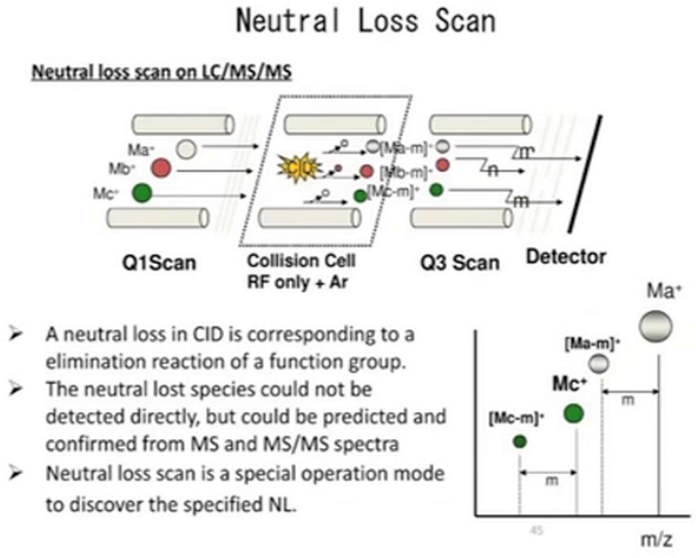

Generally, what happens when the compound gets fragmented is not always form the charge compound, sometime it may formed of neutral compound and sometime it may formed the neutral +charge, suppose you want to monitor the group of the compound having the common neutral, how you can identify? You cannot monitor the neutral by any technique because it is not ionized and this is a ion detector, then what you have to do?

You have to monitor the difference, for example the neutral molecule weight is around 100 okay the precursor molecular weight is around 500 okay. If there is a neutral loss you will get the product ion of the 400, and 500-100 that is the 400 that means if your compound giving the product ion of the 400 that means you can say that this gives the neutral of the 100 okay like that way you can identify how many compound in the sample use the neutral loss of 100, and that also one of the tool for the screening of the compound okay.

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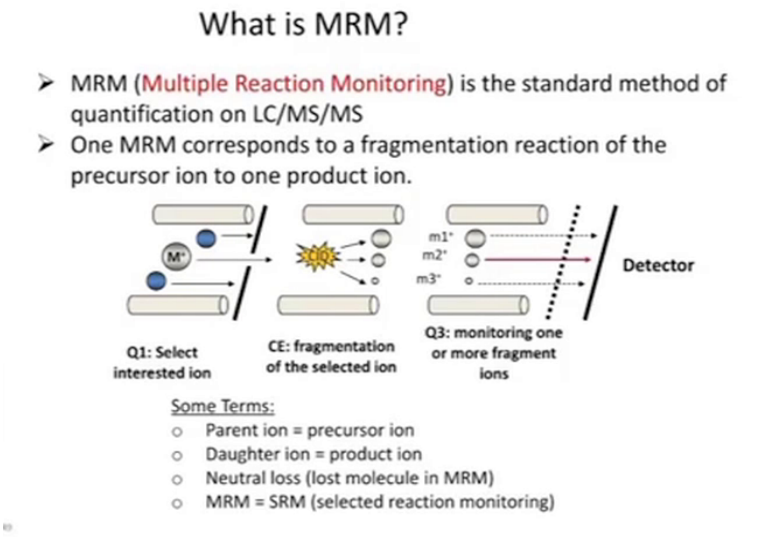

This is very, very important that you should understand what is MRM? The MRM is a multiple reaction monitoring, it is the standard method for quantification on LCMS MS okay, how it acts as in the MRM you have to select the particular compound or ions or particular peptides on the first quadrupole, it goes to the second quadrupole it from the it undergoes the fragmentation and you have to select only that product ions okay that having the highest intensity okay that means here the Q1 and Q3 both are in the SIM mode okay.

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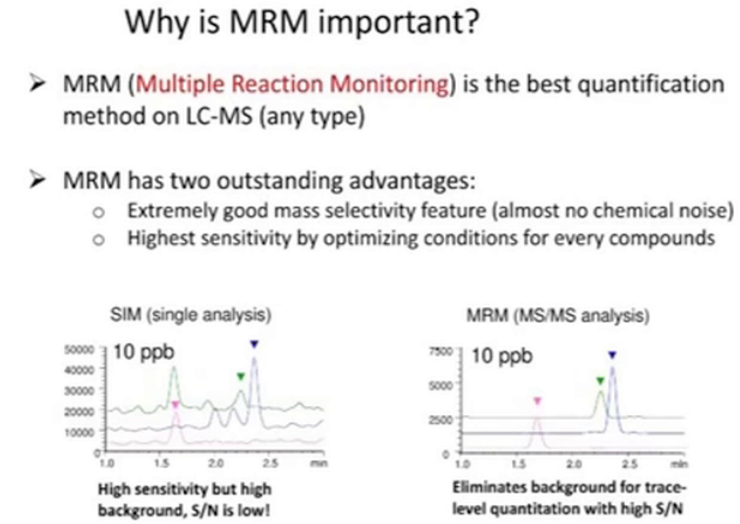

Why the MRM is important? Because MRM has two outstanding advantage, it extremely good mass selectivity future, suppose you can take this example there is a SIM analysis single and monitoring an MRM, in single and monitoring means you are only monitoring only one ion of interest. Suppose your molecular weight of compound is 500 okay, you are only monitoring the 500 only.

But when you deal with the analysis from the complex matrix the different molecules having the similar type of the molecular weight and the baseline will not be stable you are getting some unwanted peak okay. Suppose same molecule if you tried to monitor by MRM, in MRM what you are doing the final molecule you are doing the fragmentation okay by fragmentation you are getting the product ion of 200, 300 or 400.

You are selecting the 300 as product ion, because it is giving the high intensity. Now the translation is the 500 gives the 300, now what will happen because of this specific transition we are getting the good selectivity we are getting very less noise and signal noise ratio will improve. Therefore, the MRM is a good quantitative tools okay for the quantification of your compound like peptides from the complex matrix.

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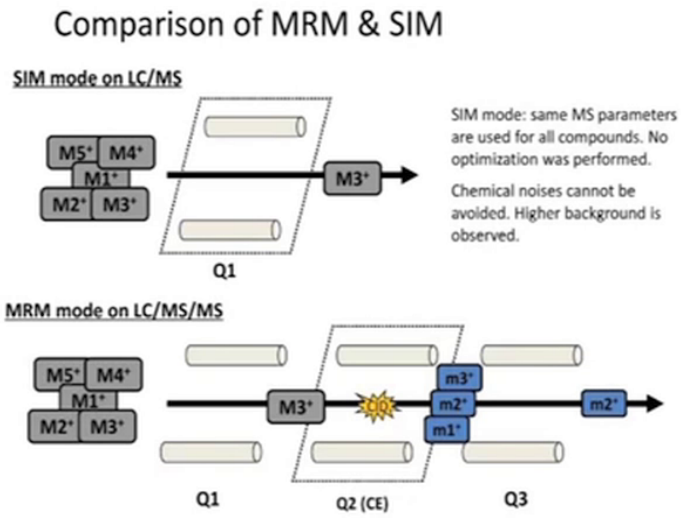

This is the comparison of MRM and SIM, SIM only single line we are monitoring in MRM okay you are monitoring the transition precursor to product. **(30:03 - 51:39: Voice not clear")**

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So let us start from the HPLC first, so any HPLC which is connected through MOSFET is called as frontend system okay, frontend HPLC. So there are different types of HPLC is available in the market like conventional HPLC, fast HPLC, Nano HPLC like now what we have seen here is new HPLC system. It is a fast HPLC system, HPLC stands for Ultra High Performance Liquid Chromatogram okay.

So the name of this system is Nexera, Nexera stands for next era okay, so fine once I explaining the flow path of the HPLC system so these are reservoirs port A and port B is there and okay. There are two types of configuration of HPLC available one is called as high pressure binary gradient system, and one is called as low pressure quaternary gradient system. So high pressure binary gradient system means, if you have a 2 mobile phase ports then there will be two pumping units one for each of the mobile phase.

So that will become high pressure binary, binary why? Because there are two, so it becomes high pressure binary. Other will be low pressure quaternary, where there will be only one mobile phase pumps pumping unit and 4 reservoir and one pump is controlling all the four right okay. So these 2 have pumping units, so from mobile phase here it goes through the unit mobile phase will come to this pumping system.

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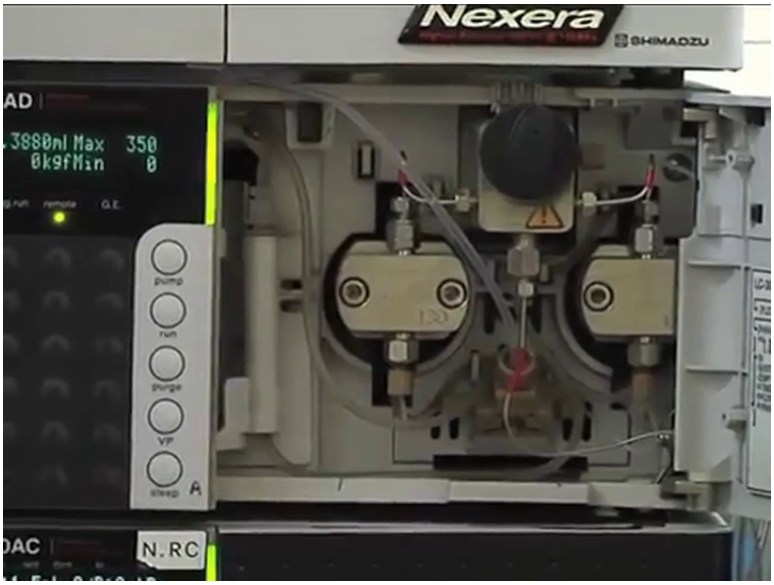

So suppose this is pumping from the laser unit this coming to this okay, this pumping unit is called dual plunger reciprocating type of pump. **"Professor - student conversation starts"** So tell me what characteristic HPLC pumping unit should have? It should have high pressure; I mean its pressure with standing capacity should be high okay. **"Professor - student conversation ends."** So pressure withstanding capacity should be high.

Second, it should give us wide range of flow rates right, and then flow should be pulseless, so what do you mean by pulseless flow, pulseless output continuous output, because from the column you are analyzed will pass through the detector only when there is a flow right, if then flow stops then analyze will be standing on the column, it will not go ahead, so you will not get analyze to continuously pass through the detector, so your outlet output should be pulseless output.

Now here if you see these are plunger reciprocating type of a pump, see this is the plunger unit where mobile phase will get stored, these two are check valves these to have long return cable check valves that means when this valve open this will be closed, and then this is opened this will be closed, this ensures that flow of the mobile phase will be uni-directional okay. If both are opened at the same time half of the flow will go this way and half of the flow will go this way, we will not get the scenario.

So initially what will happen this valve will be open mobile phase get free in this valve okay, then this will get closed now plunger will move it will pass mobile phase through the column that this column. Now after the mobile phase in this particular plunger unit it has to do refilling action right, so that will take hardly milliseconds, because of the capacity of the plunger is 10 micrometer. So this will take hardly milliseconds.

But then millisecond also there is more flow in the column, and do not want this scenario to happen, so instead of using only one reciprocating plungers we use 2 plunges, so when this is passing mobile phase to the column this will get and vice versa okay, so and back pressure withstanding capacity of this particular model is 98% okay 130 megapascal.

So you can use higher fluids as well you can use smaller particles size column, smaller tiny columns which normally tend to generate higher back pressure, but still those can be used on this particular system okay. After this is clear after pumping unit I need to mix A and B right, in particular combination.

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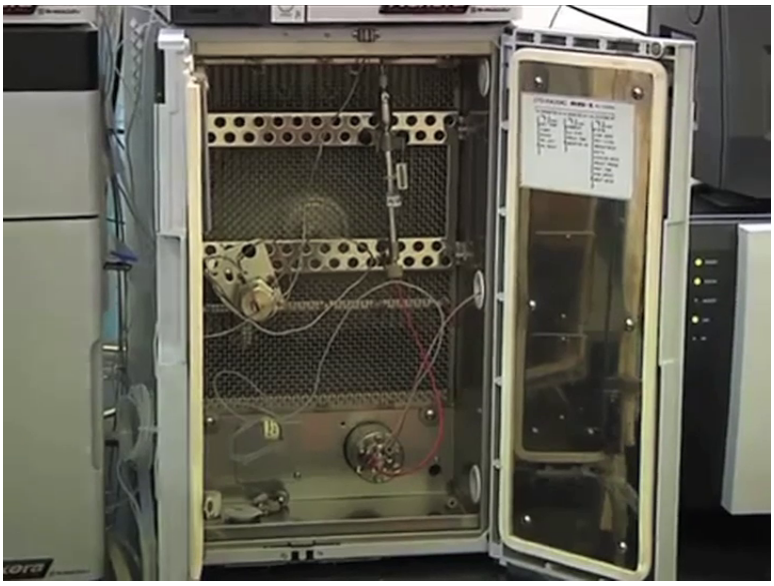

So there comes the mixture, so here whatever mixer we are using, so this is the mixer unit, I think micro reactor technology, so okay generally this is the fast HPLC system. So fast HPLC system main uses reduction in the analysis time, so if my analysis time is only 5 minutes or 10 minutes and in that I want to do radiant analysis that is my A to B concentration continuously changing and mixer should be so efficient that it in that small amount of time it would give me proper mixing.

So this mixer is specifically designed with micro reactor technology so mixing efficiency and missing accuracy is very good okay, there are different types of mixer available 20 microliters, 40 microliter, so depending upon the environment you can select which mixer is good for your analysis, of course higher the volume better will be the mixing, but increase in the system volume, so your peaks will slightly lit.

So you will have to strike balance whether I want to finish analysis very fast or I want better mix, so depending upon that you will be selecting your mixer so after mixer your flow will go through auto sampler okay.

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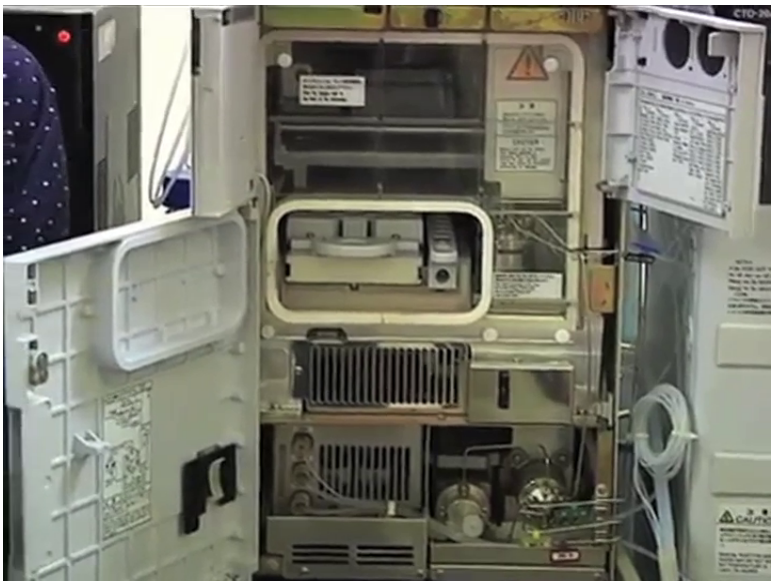

In the auto sampler unit, you will see it has three parts okay, one is high pressure valve, one is low pressure valve and one is mixing pump or metric pump. So what is the function of the metric pump whenever you will give command that okay inject 10 micrometer of sample that is done by this metric pump okay, so it will inject the micrometer depending upon.

Now high pressure valve, your mobile phase will always pass through high pressure valve and there will be central loop attached to the high pressure valve, so whenever you do injection mobile phase will switch and it may take it will flow from the sample loop it will connect the center and then it will pass on to the valve. So mobile phase is always passing through this high pressure valve.

Now what is the function of low pressure valve, suppose you have injector okay, when you inject two different samples in between the samples you perform mixing of the syringe, so that there will not be cross right, so when we are using auto sampler that function also needs to be done automatically and that is the purpose of this low pressure valve. So in between analysis whatever mixing that happens because of low pressure okay.

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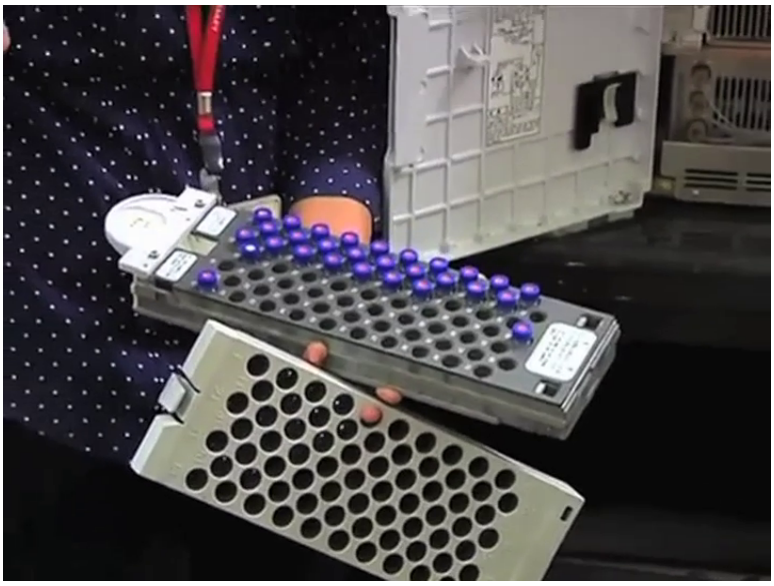

What is the feature of this auto sampler is it gives you very low carryover, what is carryover? It is almost near carry, so what do you mean by carry over so you have to understand how injection happens. So there is a needle right, now in the needle you will put your samples in this auto sampler vials like this okay, and you will filling the vial and needle will enter to this and take the sample, so outer surface of the needle is coming in contact with sampler.

Now if the central needle goes there to inject the hole and injects the sample there is a possibility that whatever is stuck to outer surface you will get deposited from the inside pro when you perform next analysis injective sample if you are injecting only organic solvent only auto then those residues pass and give you this and that is called as carry over. Now increase of this auto sampler this carryover is avoided by certain means.

Firstly, these needle is coated with special type of material, polymeric material which does not allow anything to get absorb on its surface okay, so that is one way you are not allowing carry over to occur okay. Secondly, another port which is given for rinsing, so there is a option of rinsing your needles outer surface, so if we performs the injection. So injected port there are given options one option is called as before and after aspiration.

What does it mean is injected when you give command of injection injector port will go through outer surface then it will come to vial aspirate the sample it will again go to rising port outer

surface will get it then inject the sample, so this is the way how you can reduce the carryover. Thirdly, when there is a contact between your needle and injector port if I remove reduced this contact area then there is a less chance of sample getting deposited on the injector port?

So in this particular auto sampler injector port design of injector and design is such a way that there is minimum contact area between these two and that also does not allow carry over to take place right, also this is a fast auto sampler it takes only 10 seconds to inject 10 microliters, so this particular function is very important when you perform fast HPLC kind of analysis, because suppose this is generally for small molecule as you are if you are come to one particular compound for your sample okay.

And your run time is as short as 1 minute or 2 minute, pure auto sampler is taking if 25 seconds to inject one sample you definitely losing, in that case you have auto sampler which have efficiently and fastly inject the sample right. So that is why this can be coupled to the fast HPLC this is the first HPLC system up to this part is clear fine. We will go to the column after this is a column oven fairly large area so that you can accommodate smaller as well as bigger length problems okay.

And there are two kinds of column ovens available one is called as post and circulation which is this type okay, and other only is called as heat blocked column oven, heat block type of column oven there will be heat type block this site and goes aluminium block and then transfer heat to your column, there is one slight disadvantages is there, the part which is coming tightly contact with heat block migrate some extra heat as compared to other part okay.

So heat budges migrate from, to avoid this scenario post air circulation type of column oven you see, so here column is not any hot surface okay this entire chamber will get heated up and there is fan rotating behind which will circulate this hot air uniformly throughout the chamber, so uniform heat distribution is there and there will not be any heat budges okay. So this part is called as flow control path FCV.

What is the use of this path? If you want to minimize the contamination of MS, you can use FCV, for example I know that up to 10 minutes all my mixes are coming out of column and from 10 minutes to remaining 5 minutes more than 10 minutes whatever is coming is the unwanted from the tissue like that you do not want to set MOSFET because it is might contaminate the MOSFET. So I can give commands okay here you can see the column outlets is coming to this particular valve.

So there is another valve which is going through the base and another valve which is going to the MS okay, so I can give command line from 1 to 10 minutes faster flow to headers and direct the flow to base, so it will not contaminate my MOSFET okay. There is one small nothing but small cartridge which is filled exactly same material as your column using CFL column purpose of that is it will remove the unwanted, so that life of your column will be increase okay.

Now we will move on to okay observe whatever columns we are using for this analysis fast HPLC are fast columns okay, UFHPLC columns so if different from your conventional analytical column, so conventional analytical column you have 15 to 250 mm, 25 centimeter in length, 4.6 mm it and 5 Micron particles, and this columns are the IDs are also low and microns size is 2.2 Micron or 1.6 Micron.

So whenever you are performing the analysis to this kind of columns the back pressure generated by these columns are very high. So here what will happen if on conventional HPLC system if your run times are like 60 minutes or 70 minutes on this columns, your run time will be 10 minutes or 15 minutes or lesser than that.

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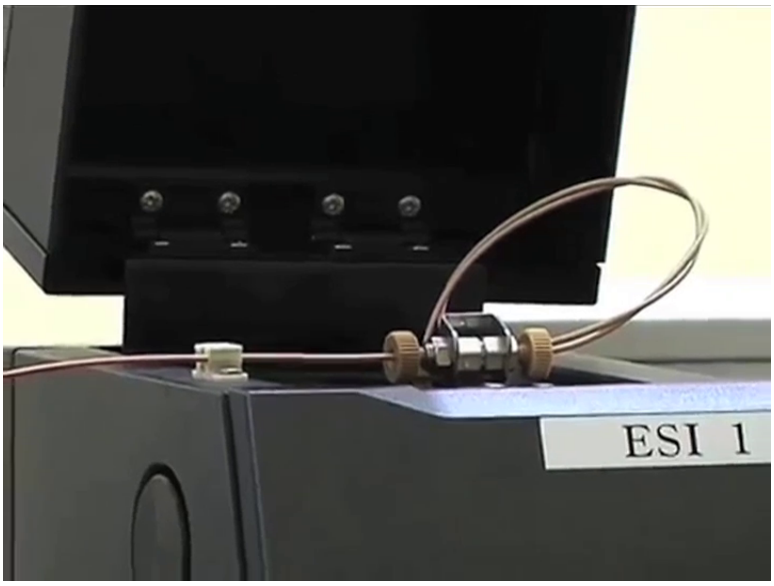

Now if your peaks are very fast from that column you are mobile phase along with your analyze is passing through this capillary okay, now this ESI is wireless ESI, so you will not see any voltage wire or nebulizing gas attachment nothing you will see, this is just you can remove the probe and directly put the probe, so you do not have to adjust putting any wires nothing, all this made internal connections okay.

So pass through this capillary and surrounding that capillary there will be a flow of nitrogen gas as well as this is the heated ESI probe there will be again heated air passing through the surrounding the capillary okay, nebulizing gas is nitrogen and the heating gas is here zero air. So to this capillary we also apply high voltages 3 to 5 kilovolt okay, so what will happen in this chamber this is liquid flow surrounding is the gas flow.

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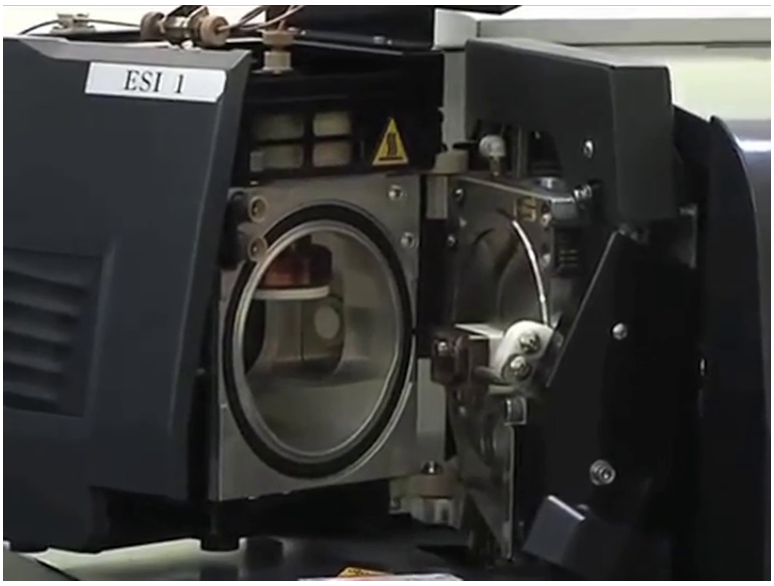

So it will get spray in the form of droplets right and those droplets will have charge, now if you see here in this chamber also we provide heat okay, so slowly, slowly you are losing in case of LCMS generally volatile mobile phases like even if you are adding some volatile, so whatever temperature apply to this chamber in this form 200 to 500 degree centigrade, so everything will be start evaporating, so droplet will shrink in size.

So what will happen whatever charges are present on the reservoir, they will start coming closer and closer and those charges are same charges positive, positive or negative, negative, so slowly, slowly repulsive forces will increase and where repulsive forces exceed the surface and droplet around it will goes into smaller droplet. So this process will continue till the end is analyzation takes place in case of ESI mode.

Then if you see here this spraying position is like this, and it will entry to the MOSFET analyzer is 90 degrees, so all your unwanted mobile phase is going to the base and only your is taken into the MOSFET, of course smaller amount of mobile phase also enter and for to remove that there is desolvation like so desolvation line also we apply some sort of heat and that will evaporate remaining phases.

And after unless there will be first quadrupole pole then there will be collision cell which is nothing but your second quadrupole, and then there is a third quadrupole and finally the detector,

detector is your emu okay. Here, one more thing was the special feature of this condition energy collision cell is sweeper that explain like I explained right like I explained you carry over effect same carry over effect can come in your MOSFET as well right.

So suppose one particular transition so that will apply inside the collision cell it generated some fragments okay and I detected fragments, but if all the fragments are not getting emptied from that collision cell, then I started monitoring by second peptide, some of the peptides fragments are common between these two condition then I can get enhanced signal because of the presence of previous transition, so that is called as cross talk right.

And I want to avoid the crosstalk, so how can I avoid the cross talk is I have to empty my fragments very fast from that collision cell, so here design of collision cell is it is not like straight collision cell, it is slightly angled collision cell towards the end it is slightly angled like this manner okay, and you apply pseudo-potential okay. So your potential increases from this point to this point and so ions will travel very fast in this manner and get emptied from the collision cell very fast and that is how you can reduce the crosstalk.

And secondly we are see concentration of 1 gas present in your collision cell that also you can control, so there is a variable levels of electron search are argon 270 kpa, but if you feel that okay that is smaller amount of ultra and this is not giving me good fragmentation efficiency I can increase the amount of argon up to 450 kpa as well okay.