Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Lecture 27 : Proteomic Techniques- High performance liquid chromatography (HPLC)

. Hello students, welcome back to your lecture series on comprehensive molecular diagnostics and advanced gene expression analysis. We are in module 6 of proteomics and today we will be discussing one very important technique of proteomics that is HPLC. We discussed about the overview of proteomics, what is the workflow and we discussed that it can be done in I mean the very one of the three basic steps that is protein identification characterization can be done in many methods one is gel based and one is chromatography based. We discussed the gel based method in details and we also discussed about the various principles of chromatographic methods. And today we will be continuing this discussion where we will be discussing a very very very important method that is often used in high throughput proteomics that is HPLC alright.

So, we will be covering this lecture under the heading of the principle of chromatography and HPLC, the components of the equipment the components of the HPLC apparatus what are the types of HPLCs basically there are many two phases of HPLC as well as the application of HPLC of the liquid chromatography in proteomics alright. And HPLC as you know as discussed in earlier class stands for high performance liquid chromatography alright. So, let us dive in where we will clear all of these concepts and we will be very much familiar with what this topic is all about ok. Now, to start with let us go back and revise from the last lecture what is chromatography and as you already might be aware it is a technique what that is used to separate components of a mixture.

How the separation is done because of interaction of the molecules or the mixture of the sample various components of the sample with various phases alright as they move through different phases they are separated ok. Now, we discussed in last class that is column chromatography. Now, this high performance liquid chromatography as you can see there is the word liquid chromatography already engraved in the term let us let me tell you this is a actually form of column chromatography. So, for HPLC you need column alright. This is done to quantify the compounds mainly to quantify, but this also

a very very very important compound where we can also use it mainly to separate as well as identify the proteins alright.

Now, this I told you in the last lecture that in modern times liquid chromatography has been evolved to HPLC. So, what do we meant by modern times? See it has been there since 1960s and early 70s ok. So, it is not much modern if you consider that we are in 2023, but it is much modern considering that liquid chromatography has already in existence since early 20th century. So, almost after 60 to 70 years almost after 7 decades that we modified or improved the traditional liquid chromatography right. Why why that improvement was done? Because we needed better we needed better speed, we needed better efficiency, we needed better resolution, we needed better picture the final outcome.

So, that actually arched you always know necessity is the mother of invention. So, that is why this urge of development, this urge for need, this urge for outcome just like you read in last module how the need of more and more sequencing help sequencing to gain technologies leaps and bounds. Similarly liquid chromatography that was very running the traditional way for the 60 years developed into high performance liquid chromatography or HPLC. Anyway so, again touching of the basics a chromatography system consists of two phases all right. One is mobile phase and one is stationary phase.

So, mobile phase is actually either it can be liquid phase or gaseous phase and why it is mobile because it is moving it flows ok. And it flows through something it flows through some immobile components and that immobile components is actually the stationary phase all right. So, mobile phase moves through stationary phase and this stationary phase even it is immobile it is solid generally, but it can be liquid or it can be mixture of both all right. So, depending on the design both the solid stationary phase and mobile phase are the chemicals are designed in such way the nature is designed in such a way. So, that the analyte this is a term which we use for any sample that needs to be separated sample that needs to be separated all right.

So, analyte is dissolved in mobile phase it moves through the stationary phase all right and then it comes out. The material that comes out is known as effluent EFFLUNT effluent. So, analyte goes in effluent comes out ok. These are the few basic terms of chromatography that you need to be familiar with. Now, coming to high performance liquid chromatography as I already discussed in the last two lectures I mean last lecture as well as in this lecture in the very few minutes preceding this slide that it has been an extension it has evolved from traditional or conventional liquid chromatography.

So, liquid chromatography as discussed in the last class means the mobile phase is actually liquid it has to be liquid ok. And it is very powerful tool I am repeating this over

and over again and you will realize this as we go into the next lecture and the upcoming lecture of proteomics that HPLC is a invariable is an invariable tool that we all most need every time all right in order to undergo various steps very powerful tool. Now, how it has evolved how does it differ from traditional liquid chromatography? See in traditional liquid chromatography it is a column chromatography the columns were made of glass ok. And the material that is the solid phase or the stationary phase was packed in the column in a very loose manner right. And we used to pour the mobile phase from the top and it took some time a lot of time to percolate slowly.

So, that the compounds could interact all right we already saw how liquid chromatography or what are the interactions I again repeat from the last class and I will again repeat again I will again repeat size exclusion chromatography. So, separation of particles based on size affinity or adsorption chromatography based on the affinity with the immobilized substrate as well as ion exchange. So, based on the interaction of charge. So, all of these phenomena were in place since early 19th century right, but it was driven by gravity. So, drip by drip depending on the gravity the fluid slowly percolated down, but it was not efficient ok.

Nowadays so, what happened? Now this method of delivery or moving of the mobile phase through the stationary phase is has been sped up we use high speed we use a pump to push the liquid through the stationary phase and that is why to withstand the pressure the columns are very tightly packed and they are made up of metals ok. We will be again discussing that again in details when we go on to the various components of HPLC. So, what happens the components are packed in very I mean very compactly and it has got a very small granular materials ok. What happens is due to very small particle size the more the particle the more the surface area all right you have the concept if there is a big ball of a compound a big crystal the surface area is only the outer part and the whole thing is embedded inside. If you break it into smaller chunks the surface area exposed surface area is much more and we need much more surface area all right because one of the important principles of based on which the compounds are separated is adsorption right.

It is not absorption, but I hope if you are watching this course you already know the difference between absorption and adsorption. Absorption is a type of interaction in which the two molecules one is attracted adhere to the surface of another molecule that is known as adsorption. Whereas, in case of absorption the suppose a sponge will absorb water the whole sponge will get wet right, but in case of adsorption only the surface will be clung with the compound all right. So, we need more and more surface area and the interaction with the stationary phase and mobile phase while the mobile phase is flowing through high speed through the columns right and this much better surface area means much more interaction much more time to separate all the components gives a very good

much better separation compared to the earlier methods of traditional liquid chromatography. Talking of high pressure often high performance liquid chromatography in some articles and some cases also referred to as high pressure liquid chromatography HPLC right because there is high pressure, pressure almost 400 to 500 times of the atmospheric pressure 500 psi all right it is so huge all right.

So, instead of dripping through gravity we are pumping through high pressure that is why in traditional liquid chromatography it took hours and even days to separate compounds on a long glass column. Now, the whole thing can be done in 5 to 10 minutes due to high pressure and the design of the columns all right. So, this has spread up. So, this is why now the techniques are high throughput means you can achieve much bigger result much better result in much less time ok. So, component of HPLC component of HPLC you can see from the diagram let these are more or less the important components that you need to know about.

This is the storage unit for mobile phase. So, what actually happens the mobile phase is stored in a bottle or a container all right this is called a storage units we need to make sure everything is air tight all right. So, the end of the tube here should go below the level of the mobile phase that is in the storage container. Next with the help of a pump this storage this mobile phase is pumped into an HPLC column which is a tightly packed small column metallic column all right. Somewhere in between we inject the sample with the help of injector ok.

This injector injects the sample and this is pushed into the column ok and during the interaction with the column as it passes through it is separated into various components. These components are detected by a detector all right there is a computer that is also paired up all right and this will give us the chromatogram ok. And after we have detected the final there is also waste collector in which the final output is collected all right. Here all it is also important regarding the degasser this is a mechanism by which the whole solution is made bubble free. We will get into all of these components in details, but this is the basic workflow and this is the basic principle of HPLC instead of gravity you are using a force and these are all the components.

So, let us look briefly at all the components one by one. So, this is the pump is actually at the top ok. This is mainly the main development of traditional liquid chromatography to HPLC is due to the pump generation of so much high pressure all right. What it does? It allows flow of the mobile phase into the solid phase stationary phase also after the separation is done it allows flow of the final effluent to the waste collector via the detector all right. This is how a pumps looks like and how it is done? You see the requirement of the pump is to maintain a constant pressure.

The pack columns are very tightly packed. So, unless we give such a high pressure at a consistent rate at a constant rate it is not possible to maintain the flow all right. Nowadays most modern pumps uses the current which can move the flow back and forth by the motion ok. So, basically it is not a single continued motion we have consistent pressure, but the flow of the piston in motion occurs by pulses. So, in one pulse some amount of fluid is pushed to again another pulse amount will push so like that.

So, thus the pump is the main force generator of the HPLC system which drives the sample as well as the mobile phase into the next component ok. Now while the mobile phase is moving from the container to the column somewhere in between we have to inject the sample ok. Now this injection the injector this is the injector how it looks like all right. You can see is in the diagram there are two position one is the load position and one is inject position definitely and this valve can be rotated just like this. So, from one position we can rotate.

Now you can see what happens actually with the help of you follow this write up is the same the diagram is changing. So, this way when we are first injecting the sample the valve needs to be put in the injector position. So, now, the valve is in injector position. So, after injecting this is a very narrow board syringe known as Hamilton syringe. Hamilton syringe with the help of Hamilton syringe we can push it after pushing it we can we need to change the valve to the load phase.

And then again there are instruction there are standard operating procedures and then we need to start the pump there are multiple instruction that we need to do step by step and then the flow will happen. So, why it is actually done cannot we inject the thing just like that definitely not because all of this has a specific mechanism since everything is connected in a single pipeline we need to make sure that the sample is not going back towards the you can see. We need to make sure the sample is not travelling back towards the mobile phase in spite of there is a pump the pump is not on. So, how it is achieved it is actually achieved by sample loops. So, this is the manual injection procedure ok, where we are injecting it manually and we are changing from load to inject.

There are also automated sample collection where you can just put the sample it will be automatically injected this is how the assembly is done it is connected to a further downstream process. Now, you can see the a tube is coming from the sample this yellow knob is the sample knob we can actually put the sample elsewhere and via this automatically the capillary the sample will be pulled in the HPLC machine. Now, this is the mechanism of the injector believe me you do not need to know in much details, but since we are trying to clear concepts here at your level you should just by knowing we need to put it in a injector position inject and then turn the knob to load position. What it does it actually during the injector phase it make sure that the mobile phase is not

connected ok. I mean the mobile phase the valve is cutting off so that it the backflow does not occur.

Only when after the entire thing has been injected there is a sample loop ok, which is a basically a pipe where the entire thing is collected the sample is collected in excess. So, we are actually not injecting the whole thing directly in the circuit the pipeline, but we are injecting the sample in the sample loop it is in excess there is also waste indicator. I mean whenever the sample is being we are loading the sample in the sample loop injecting sample in sample loop and when you are putting to load position then it can easily go. Now, depending on the operations manual there are there can be some deviation some equipment may ask you to load the position in the loop. So, you need to inject in the load position and thereafter you need to take it out into seal the hole and then you switch into inject and then you switch on the pumps.

So, depending on the instruction manual you can go ahead alright. Next we need to know about the column. So, this is only valid if you are doing the things hands on and I believe you must be exposed to HPLC if you are interested in proteomic experiments. The aim of this course is to make you theoretically sound so that when you are operating the equipment everything is very crystal clear to you how it is happening inside alright. Now, as you know it is a form of column chromatography the columns are made up of stainless steel they are resistant to high pressure.

Generally the packing material is silica or other polymer gels we will be discussing what are the most in commonly used packing materials in the column. In earlier days loosely packed calcium carbonate was used not now alright. Now, the eluent that is the final component that we are adding in this case the mobile phase in case of HPLC which actually separates the mixture after they have reacted with the column it can be either polar or non polar acidic to basic depending on what we are separating right. As discussed most columns are made up of stainless steel because not only they are pressure resistant due to the high pressure of the pump they are also chemically inert and resistance resistant to a large variety of solvents. Now, columns can be mostly there are small thin columns, but in industrial grade HPLC high thick columns can also be used for example, this you will not find this column in a standard laboratory in your any practical laboratory where you are doing your dissertation or any research work, but definitely if you are a part of industrial large scale separation or high throughput proteomics is happening in the factory definitely you will encounter this type of column if you are in an industrial job.

Next the detector. So, what does a detector do? So, you understand that the analytes are actually separated, but we cannot see the job the detector is to make us realize that the components have been separated. So, the composition of the eluent is consistent alright.

So, a detector it is actually a there are mechanism this is actually a chemical process or there are multiple modes I will discuss what do we what are the various mechanisms of detection. So, whenever there is a changes in composition of the eluent the detector will measure the change in composition or the change in chemical characteristic and how the change in chemical characteristic will happen whenever there is an analyte present alright. So, detector helps us to detect the difference is monitored in form of electronic signal ok.

They will not directly tell us suppose we mixture 3 amino acids or 3 different length of a proteins peptides they will not directly tell us what we suppose this is the 18 carbon peptide. We will not get that information directly, but it will be a just a tick in electric signal we need to further modify that electric signal to our understanding ok. So, there are so, this is basically the job of the detector is to detect what components are being separated how it is reacting with the eluent and the stationary phase. Now, one important thing for you to know that there are various detection methods in HPLC to name a few these are the most commonly used once. So, there can be a spectrophotometric method detection, fluorometric method, electrochemical method, mass spectrometric method, refractive index detector there can be many.

I have got a comparison comparative chart for you to understand or at least to follow what changes I mean how the methods differ for example, in spectrophotometric method we the detection limit is less than 1 nanogram right. The ability to observe UV or visible light of various analytes is how we detect them alright. So, here it measures absorbance of light in case of fluorometric measures fluorescence and because of electrochemical detector we can actually there is a we design the column in such a way. So, that the analytes are oxidized or reduced and then the changes or the we all know oxidation reduction are basically reactions of electrons either it donates electron or accepts an electron and that is detected by analytes. For example, very important MCQ electrochemical detectors are very important for catecholamines ok.

That is outside I mean that is also a part of very much a part of proteomics because catecholamines you should know is a part of metabolic disorder which are excreted in urine ok in case of defect in tyrosine metabolism. Anyway you can to clear these concepts you can also enroll in our another course that is overview and integration of cellular metabolism where we have covered those metabolic disorders and how to detect them in detail. So, that means, that you can see the spectrophotometric method the sensitivity is much less it is less than 1 nanogram whereas, it is picogram to nanogram it can detect much smaller and then again it is less expensive these methods are more expensive. Now, femtogram to nanogram again alright much more sensitive mass spectrometric method what does it detect it is ions separated by mass to charge ratio ok. And for that analyte must be converted to ionized form we will be detecting I mean

discussing mass spectrometry in detail alright.

And refractometric method it detects change in refractive index the sensitivity is much lower this is one of the cheapest ok, but for initial screening this can be used to detect many compounds ok the sensitivity detection limit is 1 microgram. So, much much much less than mass spectrometer. So, if we I mean if you get a question what is the best method for detecting an analyte using HPLC I mean best detecting method using an HPLC in case of proteomics of course, the answer will be mass spectrometry. So, HPLC liquid chromatography coupled with the mass spectrometry detector gives the best result and that is the thing that we do in high throughput proteomic experiments. And hence that of that many important techniques that we will be learning in this module one HPLC and one mass spectrometry is very basic and it is must know for high throughput proteomic experiment.

So, if in an ideal world what are the properties of a detector ideal HPLC detector it should be highly sensitive it should be able to detect compounds which are very low in concentration. Linearity the amount of detection of signal should vary linearly with the change in concentration. So, suppose it gives a signal of 1 when the measurement of the analyte is 1 it should give a signal of 2 when the measurement is 2 right it should not vary ideally. Selectivity it should be we should be able to design the detector so, it is very much selective for a specific compound alright. Stability it should be very stable after all this reaction it should not ideally I mean it should not degrade chemically alright.

Low noise there can be multiple contaminants the detector should be I mean detection method should be such that it should not give a very much background noise alright. What do you mean by stability is even after so, much reaction it should give consistent readings alright. Low noise we discussed wide dynamic range it should be able to detect high concentration to low concentration of analyte, rapid response time it should be fast it should be compatible with multiple mobile phases alright. So, if you are selecting one mobile phase we should need to make sure the detector depending on various mobile phases the ideal detector should be able to detect everything it should be very easy to use it should be durable and reliable the machine alright it should be versatile. So, for wide range of analytes one detector should be preferable.

Again it cannot be very huge because we are coupling that with the machine right it should be compact in size and again it should be very much compatible with not only the mobile phase, but also the chromatography columns and it should be cost effective right. So, in as you know in biological system in ideal world there are no such detector detection method that will satisfy all the entire characteristic, but with modern development mass spectrometry comes very very very close to take almost all the

required components when it comes to proteomics ok. So, mind it if you need to choose one method of detection mass spectrometry will be the default method of choice. So, next the recorder so, after it is detected after the signal is recorded.

So, some device or mechanism must be in place. So, it records the signal in older days what happened there was a pen and paper based chart recorder which used to record the chart this is an example of a traditional old chart that was used in older days. Nowadays it has been replaced by computer based software computer based software which actually automatically detects the chart. So, not only there is an ill built printer that will give you a colored images or black and white images depending on the model that you choose, but it is almost always automated. And not only that it does not only does the data acquisition data acquisition is the phase where it captures the data it not only does the signaling, but there are multiple features like peak fitting baseline correction if there are noise the curve will automatically correct them if there is a pre mentioned standard it will automatically calculate the concentration it will determine the molecular weight. So, much can be detect done now within very short time and that is why the high performance not only in technology, but also in analysis has reduced the time into very narrow 10 to 15 minutes is all we need to separate even complex mixtures.

So, degasser as I told you if even if we are taking all the precautions to load to inject to make sure the whole thing is bubble free, but still there are some dissolved air which is not visible to our eyes and that dissolved air in signal analysis can give us this jitteriness in baseline which is known as noise all right. Yes the machine can correct the noise right, but imagine if there is a small peak which is happening like this it will be very difficult to understand whether suppose the amount of peak can curve the width of the peak as well as the height of the peak can correspond to the amount and nature of the analyte. So, it may so happen that the analyte is giving a peak which is smaller in size for example, this in size right, but if there is so much noise it will be very difficult to visualize whether or understand whether it is a noise or a legitimate peak for higher peaks it is fine. So, even if we correct the baseline using software if there is a lot of noise it is of no use. So, in to make sure it is actually noise free to start with that is why degasser plays a very important role it provides a very stable baseline if the gas are already I mean if the eluent that is going into the mobile phase that is going into the as well as the sample that is going into the pump is properly degassed.

How does it do with helps of a membrane based polymer which I mean while flowing through the tube there are multiple vacuum and there are multiple mechanisms we do not need to understand the I mean physics of how it is going on, but you should know that with the help of a degasser that is also built in I mean one component of the HPLC system the liquid is made bubble free air free dissolved air free before it goes into the pump right. So, this was all most important components of HPLC we did not discuss the

storage for mobile phase and the waste because I hope you know all those what are those all right. So, now we will be discussing the types of HPLC. So, depending on the relative polarity very important how we can classify HPLC relative polarity of the solvent and the stationary phase. So, whether they are polar or nonpolar there are two phases right one is first is normal phase and the one is reversed phase ok.

So, you just remember one combination and therefore, the other combination will be very easy to remember why do I say combination you see normal phase the column is polar very important you just remember in normal phase has got polar columns you can remember in any way you like one example is silica. So, when the column or the stationary phase is polar the mobile phase has to be nonpolar all right. So, mobile for example, nonpolar solvents like hexane isopile alkalizes much less polar compared to methanol or ethanol. So, it is actually ideally used for separating nonpolar substances it is a tridrofuran very important.

So, you can remember one example that is hexane. So, polar column nonpolar mobile phase. So, what will happen the polar substance actually it mix with the polar adsorbent ok. So, polar substance will be attracted to the column right and nonpolar substance will quickly pass through the column because they are not interacting and thereafter we can elute them and thus we can separate a mixture of polar and nonpolar compound for example, amino acid. So, what how specially it is used to separate water sensitive compounds geometric isomer cis trans isomer chiral compounds all those things if we need to separate this type of normal phase HPLC is preferred. So, from this slide only you can easily guess what are the components of reversed phase HPLC just the opposite.

In case of reversed phase HPLC the column the stationary phase is nonpolar for example, C 18 this is the capital C ok. C 18 I will tell you what is C 18 it is basically a polymer that has got 18 carbon molecule all right and the mobile phase is polar for example, any water and any other miscible solvent for example, methanol right. So, what will happen in this case the polar molecules will travel through the column more quickly because polar molecules are clinging to the polar solvent they are dissolved in water whereas, the nonpolar molecules will be attached to the column and they will pass very slowly. So, what I mean it can be used for to separate polar nonpolar ionizable and relate and ionic samples mind it reversed phase HPLC is most commonly used for HPLC in proteomic techniques very very very important. So, again HPLC method of choice compared to if I there are many techniques of protein separation if you are thinking HPLC you should mark, but if reversed phase HPLC is mentioned that will be the specific method of choice ok be very cautious about that.

So, we prefer this reversed phase HPLC. So, since we are discussing proteomics we need to know a detail about the chemical nature of the columns and the mobile phases let

us discuss about the column first. So, octadecile silane or octylsilane or butylsilane. So, C 18 C 8 C 4 these are the nonpolar columns these are hydrocarbons nonpolar mind it. Next there are also hydrophobic interaction liquid chromatography columns, Heilig columns ok basically Heilig columns are made up of these ok. Next again ion exchange columns size exclusion columns you can see ion exchange columns can also be classified into I mean the sub variety are strong cation exchange columns strong ion exchange columns.

So, you should be familiar with all these names. So, any name if it is coming up you should keep in mind the question is asking about reversed phase HPLC very very very important ok. Use of mobile phases acetonitrile water, methanol water, formic acid, trifluoroacetic acid any solvent, if you see water right polar solvent reversed phase HPLC ok. Next we need to know the concept of retention time, what is retention time is the time taken for a particular compound to travel through the column to the detector ok. So, in the column the components will be separated now they are travelling to the detector and that is the time taken that is known as retention time. So, from the time at which the sample is injected the point the display shows the maximum height for the compound.

You will see that there is a small peak ok and then it takes some time to reach ok. Generally this small peak is referred to as a void volume or the time taken up to I mean from the initial injection to reach the column. This the first peak is actually ignored when you are analysing this is again a practical part it will vary from chemical to chemical from your experiment to experiment, but one thing you should know regarding the resolution of the peak means when you are trying to visualise a separate two compounds if the peaks are overlapping then it is not a very good resolution it is low resolution. We need to refine the experiment standards we need to refine the mobile phase may be column we can change the experiment condition may be change the temperature. So, that the columns are separated then we refer it to a good resolution where we can visualise the two peaks separately they are not overlapping ok.

So, lastly what are the application of HPLC in proteomics the applications are huge protein separation you any two different proteins with different chemical nature you put it you design the column you put a detector it will be separate they will give very good looking chromatogram. Peptide separation peptide you know when proteins are fragmented into small amino acid chains they are known as peptides and that is one of the most common thing that is done in a proteomic experiment. So, what happens proteins are digested with the help of peptidase enzyme there are multiple compounds which were long peptide chain can be digested into short fragments just as in DNA sequencing in shotgun sequencing what we did we broke a big peptide into small fragments and then we analysed ok similar thing can be done. So, instead of separating

two proteins we can actually fragment one protein to multiple compound multiple peptides and HPLC can separate that peptide also ok very important. So, identification of protein so, we can identify the nature not only we can separate a mixture we can separate and identify as well ok specially when coupled with mass spectrometry detector this is the D method of choice I will put 4 ticks ok.

Next quantitative proteomics what is the amount of protein that is present very well it can be commented using isotope labeling or label free methods we will be discussing them in this module in our follow up class following classes. Next purification can we purify definitely we can purify specially if you use a size exclusion column all the unwanted things will be eluted and we can get our protein a much purified form. It has got very high sensitivity we can design it to a very high resolution and with modern techniques that has got high throughput means we can do much in a very short time. So, if we want to summarize HPLC is actually very versatile indispensable tool in proteomics without HPLC without liquid chromatography mind it whenever you are seeing the word LC in any proteomic experiment it refers to HPLC LC in today's date is high performance liquid chromatography there is these 2 terms are synonymous ok. So, we can see I you know while discussing method and workflow in proteomics we discuss sample preparation separation of protein identification quantification of protein in reality HPLC can do all the 3 specially when coupled with mass spectrometry will be discussing mass spectrometry in detail this is a very important method.

So, when these 2 are coupled anything is achievable you can design an entire proteomic experiment concerning these separation identification quantification of protein only these 2 equipment ok. So, any complex peptide any complex biological models can be analyzed and as I told you when it is coupled with mass spectrometry many many many multidimensional workflow can be done and complex proteomic analysis can be achieved ok. So, with this I finish today's lecture we have discussed the principles of chromatography liquid chromatography HPLC how liquid chromatography evolved from HPLC what are the various components of HPLC apparatus we discuss the normal reverse phase HPLC and in short we have also discussed the application of liquid chromatography in proteomics. So, these are the references from today's for today's lecture and I thank you for your kind attention and patient hearing.