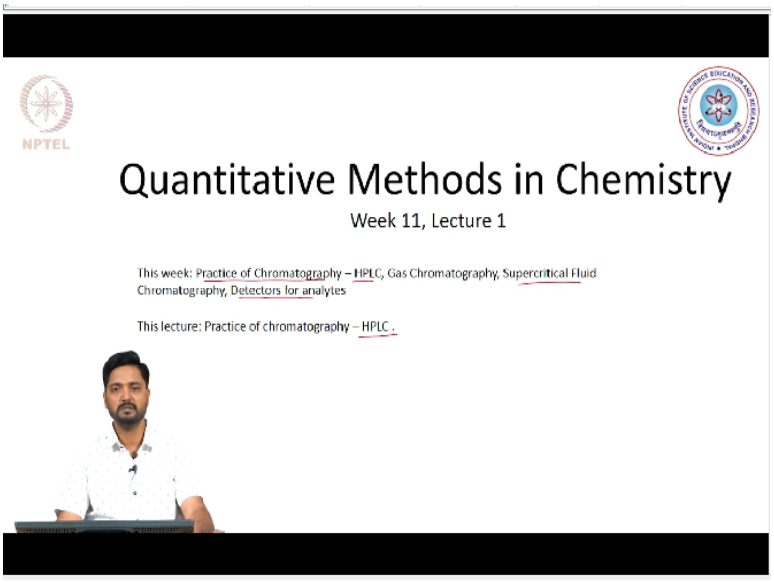
Quantitative Methods in Chemistry
Prof. Dr. Aasheesh Srivastava, Dr. Bharathwaj Sathyamoorthy
Department of Chemistry
Indian Institute of Science Education and Research-Bhopal

Lecture-43
Practice of Chromatography-HPLC

(Refer Slide Time: 00:29)



Hello and welcome back to this course title quantitative methods in chemistry being offered on the platform of NPTEL. We have reached the week 11 for this course, and this is lecture 1 for week 11. So, in the last week, our emphasis was on obtaining a detailed theoretical understanding of how chromatographic separations happen. We got introduced 2 terms like the plane height, plate count.

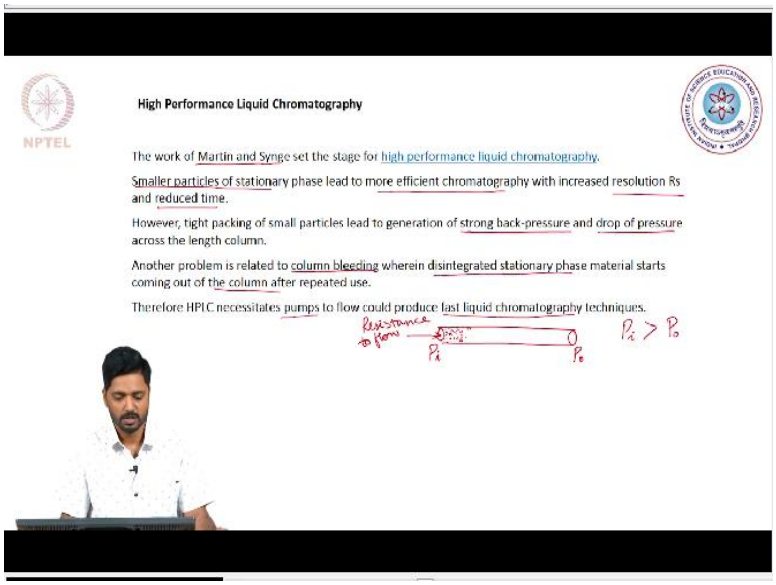
And we also got to know the van Deemter equation, which takes into consideration how the solute partitions between the solid and mobile phase and that concept is used to explain how the solute moves inside the chromatographic column. So, that theoretical understanding has now created a good platform for us to start exploring the practice of chromatography and that would be the key theme for this week's lecture.

So, we will be talking to you about the practice of chromatography in terms of how HPLC is performed, what is gas chromatography the principles and the practice of it and we will also get introduced to a very interesting form of chromatography which utilizes supercritical fluids as the mobile phase and our emphasis or understanding will also be developed in terms of what detectors are used to detect the analytes that are eluting out of these chromatographic columns.

So, we will gain an understanding of what are the characteristics that are required for undertaking chromatography of different compounds, what do we need with regards to the detectors and what are the properties of the mobile phase that dictate how the solute moves inside the chromatography column in HPLC, as well as gas chromatography and supercritical fluid chromatography.

So, let us begin lecture 1 by understanding how high performance liquid chromatography works in real practice.

(Refer Slide Time: 03:06)



And as has been already indicated in the previous lecture, the work of Martin and Synge is considered to be important in terms of developing the high performance liquid chromatography and we already mentioned that in improving the performance of a liquid chromatography process, we require smaller particles for the stationary phase because these small particles lead to more efficient chromatographic separations.

And as a result, the resolution of the peak separations is improved while the elution times are reduced. So, of course a key consideration when we move from a normal column chromatography that is conducted day to day in synthetic organic chemistry laboratories to a high performance liquid chromatography, the key change that is happening is in the quality and size of the stationary phase that is being implied.

For high performance liquid chromatography, we emphasize on the use of smaller particles for our stationary phase. Now, what we also understand is that we need to pack these particles quite well within the chromatographic column. So, if I have a column I would want to ensure that my stationary material particles are very nicely packed with inside these columns, so that efficient separation can be achieved.

Now, that ensues or brings about another level of challenges with regards to the actual practice of liquid chromatography and that is the generation of strong back pressure and drop of the pressure across the column length. Let us try to understand this strong back pressure means that when the fluid flows through this column which contains now very nicely packed stationary particles which are also small in size.

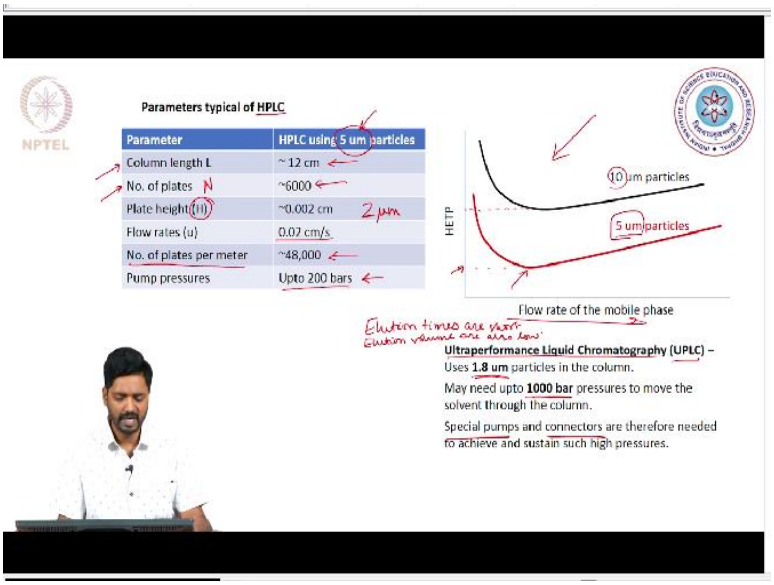
So, then the fluid will experience a lot of resistance to its flow. So, a resistance to flow with be experienced by the fluid or the mobile phase that is passing through the column, this resistance will generate a pressure back on to the pump that is pumping the liquid into the chromatographic column. Similarly, this concept also extend that the pressure initial at the beginning of the column will be very different from the pressure final or the output pressure at the end of the column.

And usually, when strong pressure drop happens, the initial pressure at the beginning of the column will be much higher than the pressure that comes out of the column. Of course, we also mentioned that another significant problem with regards to using very small particle sizes for achieving an efficient separation is that of column bleeding. And this relates to the disintegrated stationary particle phase particles moving out of the column.

And clogging the various tubing's and pipes that are used to connect the column to the detectors. And it also results in compromised performance of the column that is being used repeatedly. So, to pump the liquid through the chromatographic column, we need special pumps that will push the liquid into the chromatographic column to produce the fast liquid chromatography techniques and these pumps become the mainstay of the high performance liquid chromatography systems.

And based on the particle size that is being used in the chromatographic column, the pump performance has to be carefully chosen.

(Refer Slide Time: 08:19)



So, in the next slide here, I have listed some of the parameters that we have already encountered in our previous classes. And what I am trying to portray is what are these parameters in regards to the high performance liquid chromatography. So, the column length is typically about 10 to 12 centimeters, we can use small column lengths because the separation is more efficient here and we are having a high resolution for peak separations inside the HPLC columns.

Of course, the number of plates N or the plate count is decently high and it is in the range of about 6000 for this small length of the column. As a consequence, we can calculate the plate height, which now comes out to be about 2 microns. The flow rates that are typical of high

performance liquid chromatography are not very high. That is as mentioned previously, because very high flow rates will result in reduced efficiencies.

And will also result in increased column bleeding. So, the flow rates here are of the order of 0.02 centimeters per second, and these are sufficient enough for us to achieve the chromatographic separations that are to be undertaken through high performance liquid chromatography. Now, an important parameter that can be derived from the number of plates and the length of the column is the number of plates per meter of the column that can be used.

And this number is very, very high for high performance liquid chromatography systems and it can be in the order of about 50,000 for typical HPLC columns and, of course, the pump pressures that needs to be sustained, can reach up to 200 bars or 200 atmospheres, please note that these numbers that I am talking about are related to the HPLC column that is using 5 micron particles.

And this is a typical number or size of the particle that is often used in high performance liquid chromatography, because it allows us to use pumps which do not have to generate very high pressures, we still need to generate decently high pressures, but, as we reduced the column particle diameter, the pump pressures that would be required would be much higher. Now, on the right hand side, this curve here indicates how the height equivalent of theoretical plate changes as we change the particle size of the stationary phase.

And what we notice is that the for similar rate of flow of the mobile phase, the 5 micron particles will consistently result in much smaller HTTP values compared to particles, which are larger and in this case, which are about 10 micron average particle diameter, and this we understand comes due to the van Deemter equation, where both the longitudinal diffusion as well as the Eddy diffusion terms have very clear correlation with the particle size.

And so does the resistance to the mass transfer. So, all of these terms can be minimized by using smaller size particles that are efficiently packed inside the HPLC column and that results in significantly lower edge values for the 5 micron stationary phase particles. A sort of extreme to

this is what is known as the ultra performance liquid chromatography and this is now picking up quite rapidly and it is abbreviated as UPLC.

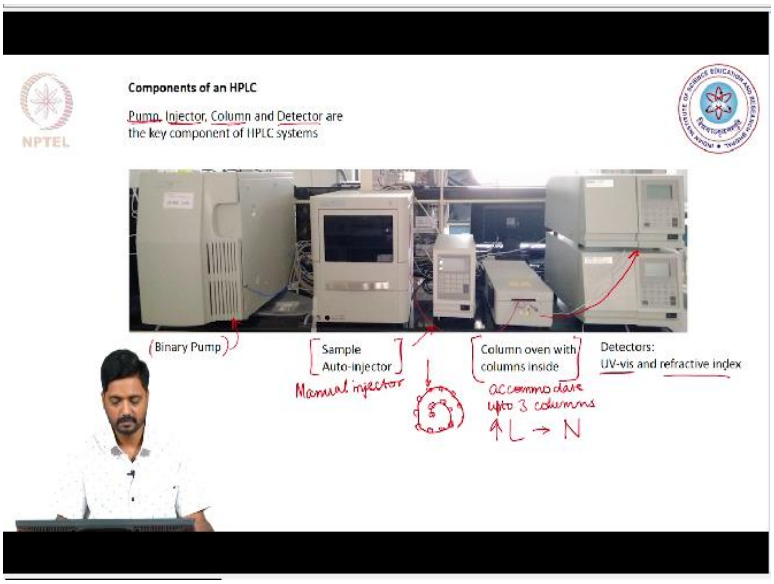
And here you can see that the particle dimensions have been reduced considerably and we use a typical particle size of 1.8 micron particles in the chromatography column. So, we pack our UPLC columns with particles that have the average diameter of 1.8 microns. However, as can be expected, this comes with an important cost and that is with regards to the pumping pressure, when we are using particles which are really tiny for making up the stationary phase for the chromatographic column.

We need to pump at a much higher pressure of up to 1000 bars. This is not trivial, because, now the pump has to be of the appropriate rating so that it can actually consistently pump at say 1000 bar pressures and you also require special connectors that will connect the tubing's beings and the column with the detector and sample injector. So, these will increase the costs for the system considerably.

And this aspect needs to be kept in mind when choosing whether to go for an HPLC or UPLC. Now, of course, an advantage with regards to the UPLC is that the election times are very short. And another consequential advantage is the elution volume which are also low. So, we can achieve the same separation or elution of our analytes at much shorter time periods. So, we can increase the throughput of our instrument plus the waste solvent that is coming out from the UPLC is much lower in volume.

So, this has advantage in terms of environmental considerations. However, this comes at the cost of the instrument, which is considerably higher than the high performance liquid chromatography system. Now, let us go back to the HPLC system.

(Refer Slide Time: 16:53)



And see what are the components that are involved in the making up of our HPLC system, so, you we have a pump which will pump the liquid into the chromatographic system. We have a sample injector and then the actual column for the HPLC. And finally, we will have a set of detectors to see the analyte or to detect the analyte that is coming out of the chromatographic column.

Now, regarding the pump, this is an HPLC system, which is present in my group. So, we have an HPLC that has a binary pump. That means we can simultaneously inject 2 solvents or a mixture of 2 solvents into our chromatographic system because of the throughput of the samples that we have, we have used auto injector to automatically inject the samples into the chromatographic system.

Now, this auto injector is a sort of a robotic system, where we have the vials arranged in a spiral format and each vial will have its position fixed. So, this is the kind of spiral system in which the vials will be arranged and there will be a syringe that will come and pick up the sample from a particular while present at a particular position. So, this auto injector as the name suggests injects the sample automatically into the HPLC system.

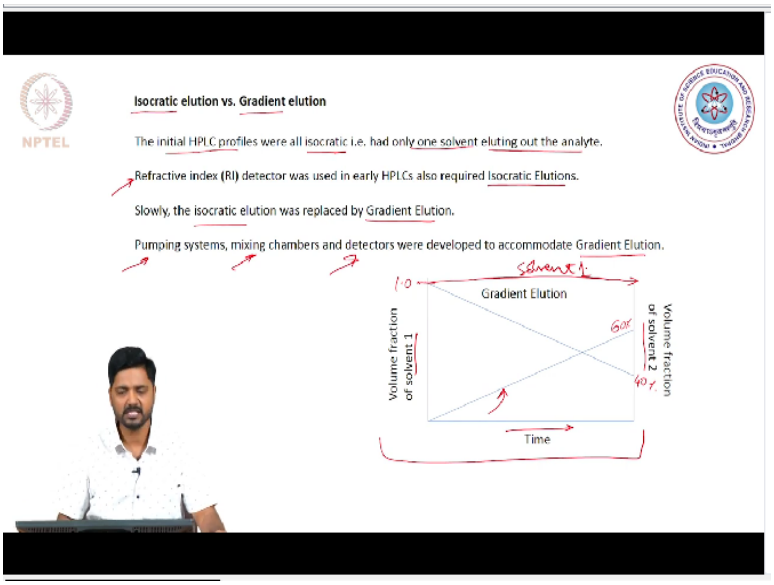
One can also have what is known as the manual injector where the injection of the sample is done manually by using a low volume syringe, once the sample is injected into the HPLC

system, it goes through the various tubings, some of which may be visible here and also at the back. So, we have a column of one within which we keep the columns because, as we understood in the previous classes, that temperature has a very clear influence on the various parameters in the van Deemter equation, especially the long longitudinal diffusion, as well as the resistance to the mass transfer terms.

So, we would want to keep our columns at a very controlled temperature environments and for that, we use a column oven which can be set at a particular temperature. Now this particular column oven can accommodate up to 3 chromatographic columns inside it and these columns can be interconnected together to increase the total length of the columns that are available and consequently, the number of plates that will be available for achieving the purification.

Now, finally, as the solute or the analyte moves out of this column, it will be passing through the detectors, which are shown here. And these detectors in our case are the UV-vis detector and refractive index detectors. And finally, after passing through the detectors, the detector responses noted and the element now moves out into a waste portal where it is collected.

(Refer Slide Time: 21:48)



So, while undertaking elution of the analyte we would want to consider a few things, we would want to consider whether the elution is to be an isocratic elation or we would want a gradient elution and what was originally done in the early HPLCs and we are talking again about times,

which are about 50 years already ago. So, in those cases, the HPLC were all isocratic that means, they had only one solvent that was used for as the mobile phase and used to elute out the analyte or analytes of interest.

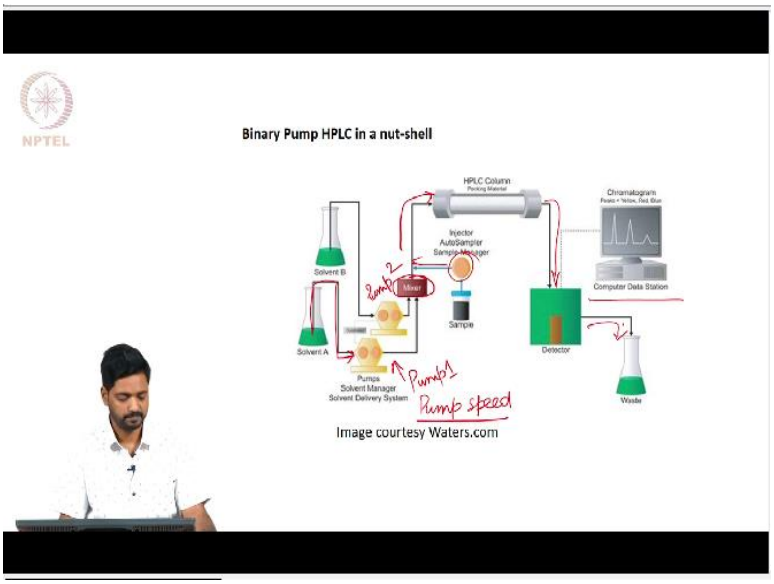
Obviously, this had its limitations, but the previous detectors are also limited in terms of handling, only isocratic elution. For example, the reflective index detector that was used in the early HPLCs could respond only to isocratic elutions. And if there were solvent that were makes, that will result in generation of spurious changes in the refractive index could not be used. And of course, this was not the most helpful condition.

So, people slowly realize that isocratic elution has to be replaced by gradient elution. And to achieve that the pumping systems, the mixing chambers and the detectors were all sort of revised or developed to accommodate the gradient elution. So here in this profile, I have shown you what the gradient elution would look like for 2 solvents solvent 1 and solvent 2 that are mixed before injecting into the chromatographic system.

So, here what I have tried to show is that at the beginning, we have the volume fraction of 1 for solvent 1 that means, the solvent that is being injected initially is only composed of solvent 1, but, as the time progresses, we would want to continuously keep adding more and more of solvent 2 and increase its volume fraction. And finally, we may have a condition where the solvent 1 contributes to about 40% of the volume fraction while the solvent 2 which may be more polar will now contribute about 60% of the volume fraction.

And this implies that we will be able to achieve elution of compounds, which would have otherwise not eluted out if an isocratic elution was used. The isocratic elution profile would be very straightforward it will be simply a straight line where the solvent 1 will only be contributing all across the chromatographic time.

(Refer Slide Time: 26:00)

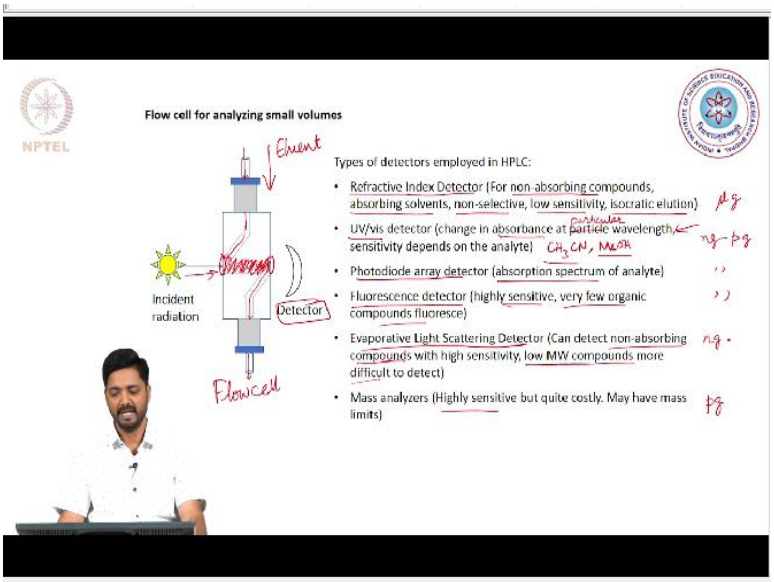


In the next slide let us look at how the binary pump HPLC works and this image has been taken from the website of waters.com and what we have are 2 pumps in a binary system. So, this will be pump 1 for solvent A and we also have another pump 2 for solvent B and these 2 pumps are being input through either the solvent A or the solvent B and the rate at which these pumps pump the solvent will be utilized to control the volume fraction of the solvent that goes into our HPLC column.

So, we have a mixing chamber which is indicated here where the solvent A and the solvent B will mix before they are injected into the chromatographic column. And as already mentioned, we control the pump speed to control the volume fraction of the solvent into the mixture and the sample is injected using either an auto sampler or a sample manager after the mixing of the 2 solvents.

So, the solute is injected at this point, it goes through the column and comes out to the detector of our interest and the response of the detector is then collected using computer data station and of course, the waste from the detector is also collected to be discarded.

(Refer Slide Time: 28:11)



Now, I have also listed to you the unique design of the flow cell that can be used for analyzing small volumes of eluents that are coming out of the chromatographic column. And often the detectors are continuously monitoring the eluted solvent system to check for the presence of the analyte in the eluted solvent. So, this is a typical flow cell that can be utilized for detecting the presence of the analyte using say UV visible detector.

So, we have the eluent coming in from the top, it moves through this compartment and goes through here and finally fills up this region and after filling up, it comes out of the flow cell. So, because this region is now full of the eluent, it can be used to detect the presence of analyte. For that, we simply have cords windows at the two ends of this capillary and we can pass the incident radiation from one side.

And detect the change in its absorption using a detector on the other side. Now, this is a design that can be utilized for multiple detectors. Let us also look at what are the various detectors that are available to us in the high performance liquid chromatography. The first and foremost detector that was used was, of course, the refractive index detector. And in fact, indeed, even now, people are using RA detector.

Because it is a very, very versatile detector. It can work well for even compounds that do not observe the UV or visible light radiation, it can even work if our solvent is absorbing the

radiation because it is uninfluenced by the absorption profile of either the compound or the solute or the solvent. It is of course, non selective, but is having 2 considerable drawbacks in terms that it has a low sensitivity it can detect in micromolar ranges.

And requires an isocratic elution as has already been mentioned. Now, the other detector, which you saw in the system that I had was a UV- vis detector. Now, this detector is simply different directing the change in absorbance at a particular wavelength and of course, it requires that our molecule or the solute be the absorbing system in the mixture of eluents that are coming out. The UV-vis detector cannot be used with solvents that are inherently absorbing.

So, solvents such as acetonitrile, methanol etc. will work much better than a solvent say like a ethyl acetate because ethyl acetate will have strong UV base absorption profile and the same will be true for aromatic solvent such as benzene or toluene. So, we would be restricted in terms of what solvents can be used, when we can we are using a UV-vis detector. Finally, a revised version of or an improved version of the UV or visible detector is the photodiode array detector where an array of photodiode diodes is arranged.

So, that we can create the complete absorption profile of the analyte that is eluting out of the chromatography column and this is where the key difference between the UV-vis detector and the photodiode array detector is in the photodiode array detector we get the complete absorption profile while in the UV-vis detector we are only observing the changes in the absorption at a particular wavelength.

Another similar detector that work on the principle of absorption or emission of photon is the fluorescence detector, which is utilized if the compound that we are dealing with or the analyte that we are dealing with can fluoresce or can emit radiation at a different wavelength. Now, of course, this detector is highly sensitive. It is also selective for compounds, which flores, but it has to be remembered that this detector has somewhat limited utility only for compounds that can emit fluorescence.

And there are very few organic compounds that actually fluoresce. A sort of different principle detector is the evaporative light scattering detector or ELSD, which has the advantage that it can detect non absorbing compounds with very high sensitivity. It can also detect compounds mainly by allowing the eluent to evaporate out and this evaporation will result in analyte particles being formed inside the ELSD.

And these particles of the analyte will then scatter the light radiation and the scattering intensity can be plotted with respect to time to generate a elution profile or a chromatogram. So, in the evaporative light scattering detector as the name suggests, there is evaporation of the mobile phase and generation of the solute particles which scatter light and are hence detected. Now, as can be considered that, this detector will work best for particles that scattered well.

And such particles will be composed of compounds or polymers that have high molecular weight. So, this detector has a limitation that it finds the detection of low molecular weight compounds are difficult. Now, the final detector that routinely used is the various mass analyzers which detect the mass of the analyte that is coming out of the chromatography column and these detectors of course, again have very high sensitivity.

So, that they can detect in picogram levels, but are quite costly and may have mass limits or they can detect only up to a certain number of mass of the analyte that is coming out. So, if I were to list out the sensitivity of these detectors, the refractive index can sense in the range of microgram UV-vis detector will sense in the range from nanogram to picogram depending on how absorbing our analyte is.

And the same will be true for the photodiode array detector and the fluorescence detector. The evaporative light scattering detector again will have a wide range in the similar manner of a few nanogram regime, the mass analyzers can definitely go up to picogram level of detection. So, what we see in this lecture is that we can use a wide variety of detectors based on what analyte we are eliciting out of our chromatography column.

So, if we have a good idea of what may be the characteristics of the analyte and the mobile phase that we are using, we will be able to choose the appropriate detector for our purpose. So, with this we come to an end to this lecture 1 of week 11 for this course. In the subsequent lectures of this week our efforts would be directed towards gaining understanding on how gas chromatography works, how supercritical fluid chromatography works and what are the detectors that are available for these techniques. So, at this point, I stopped my lecture 1 of week 11. Thank you.