Advance Analytical Course

Prof. Padma Vankar

Department of Chemistry

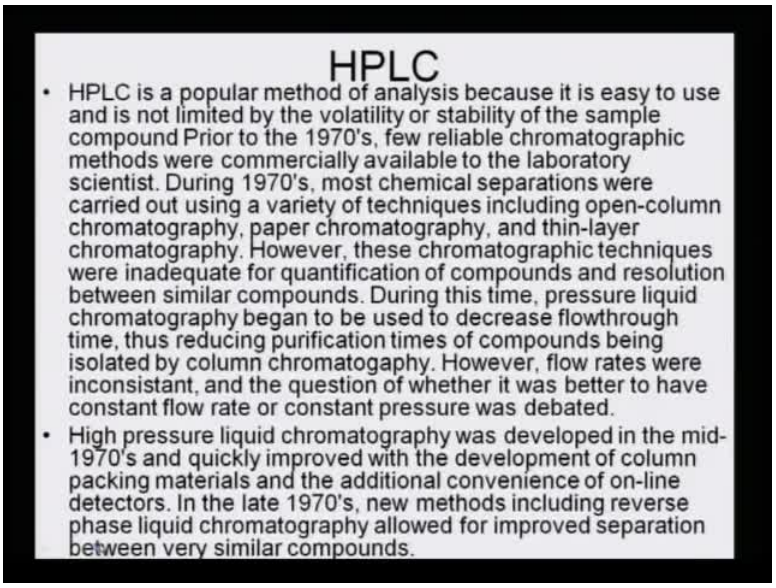
Indian Institute of Technology, Kanpur

Lecture No. # 05

(Refer Slide Time: 00:31)

Having understood the gas chromatographic technique, now we come to the second different type of technique, under the chromatographic technique, called High Performance Liquid Chromatography or High Pressure Liquid Chromatography.

(Refer Slide Time: 00:36)

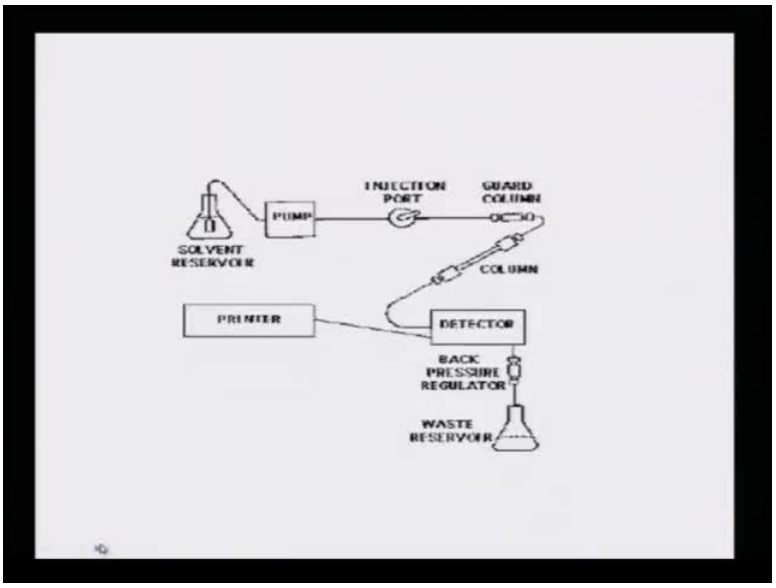


HPLC is the abbreviation for it. HPLC is a popular method of analysis, because it is easy to use and is not limited by the volatility or stability of the sample compound. Prior to the year 1970, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970s, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, thin-layer chromatography.

However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flowthrough time, thus reducing purification times of the compounds being isolated by column chromatography. However, flow rates were inconsistent and the question of whether it was better to have constant flow rate or constant pressure was a debated issue.

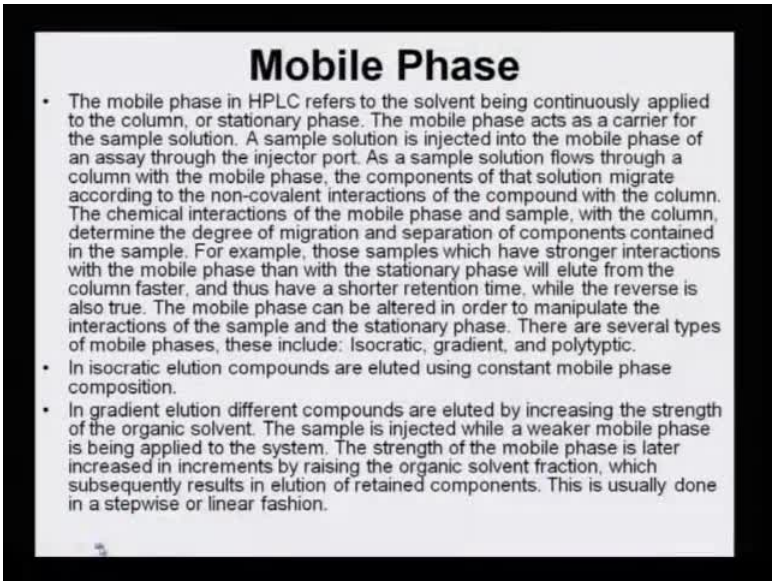
Now, there was, as I said, from time-to-time as the science advanced, there were issues of improving the technique, and in order to improve the separation technique, modifications were made one after the other. High pressure liquid chromatography was developed in the mid-1970s and quickly improved with the development of column packing materials and the additional convenience of online detectors. In the late 1970s, new methods, including reverse phase liquid chromatography, allowed for improved separation between very similar compounds.

(Refer Slide Time: 02:44)



Now, this is what the machine ideally looks like. There is a solvent reservoir, there is a pump, there is an injector port, there is a guard column, there is a small column, and then there is the outlet of the column goes into the detector, and then, the detector then identifies and puts it in the waste reservoir, and the detector is also connected to the printer; this is the entire machine and the components are added in this manner; they are attached to each other in this manner. There is a very important mobile phase, which is very, very important in the case of any chromatographic technique.

(Refer Slide Time: 03:37)



The mobile phase in the HPLC refers to the solvent being continuously applied to the column or stationary phase. The mobile phase acts as a carrier for the sample solution; that is what helps the sample to move further.

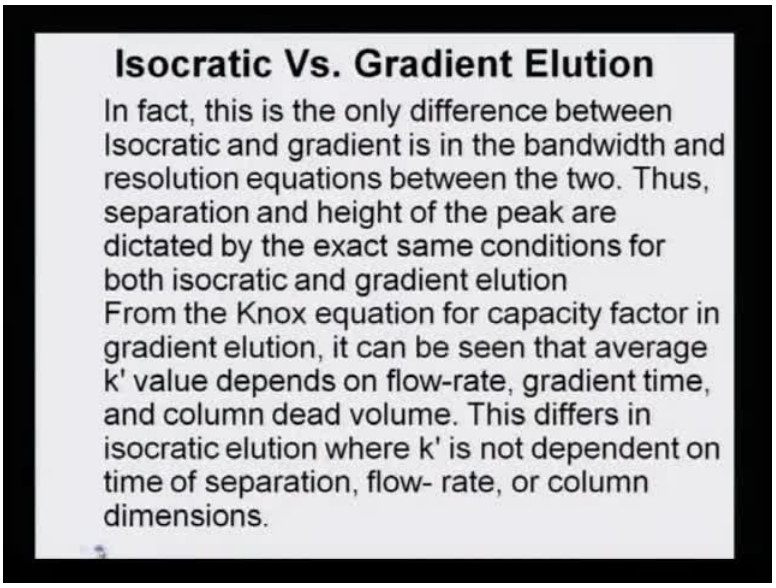
A sample solution is injected into the mobile phase of an assay, through the injector port. So, as the name suggests, the injection is done at the injector port. As the sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column.

The chemical interactions of the mobile phase and sample with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase, will elute from the column faster and thus have a shorter retention time, while the reverse is also true. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase. There several types of mobile phases; these include: isocratic, gradient, polytyptic.

The isocratic elution compounds are eluted using constant mobile phase composition; that means, that we do not alter the percentage of any of the components; it starts with one percent and it ends in that same percent.

In gradient elution, different components are eluted by increasing the strength of the organic solvent. The sample is injected, while a weak mobile phase is being applied to the system. The strength of the mobile phase is later increased in increments, by raising the organic solvent fraction, which subsequently results in elution of the retained compounds. This is usually done in a stepwise or a liner fashion, and never, never abruptly. Because, if we change the composition or the polarity of the solvent abruptly, the separation will get disturbed, and the whole idea of carrying out HPLC analysis is to have an efficient separation, and if we disturb this, that means, the separation process will go haywire.

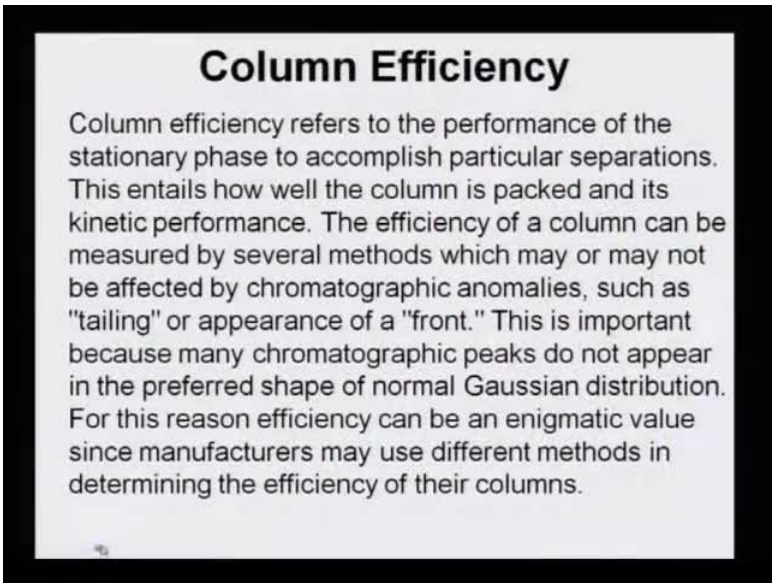
(Refer Slide Time: 06:36)



Isocratic versus Gradient Elution - now, when to use isocratic and when to use gradient, has to be the judgment of the analyst. In fact, this is only difference between isocratic and gradient, is in the bandwidth and the resolution equations between the two. Thus, separation and height of the peak are dictated by the exact same conditions for both isocratic and gradient elution.

From the Knox equation for capacity factor in gradient elution, it can be seen that average k prime value depends on flow rate, gradient time and column dead volume. This differs in isocratic elution, where the k prime is not dependent on the time of separation, flow rate or column dimensions. So, there is a very hairline kind of a difference, whether we should use gradient or whether we should use isocratic, but it is heavily dependent on the type of analyte, that has to be separated. And the mixtures, if they are very close, a gradient will always be better than isocratic.

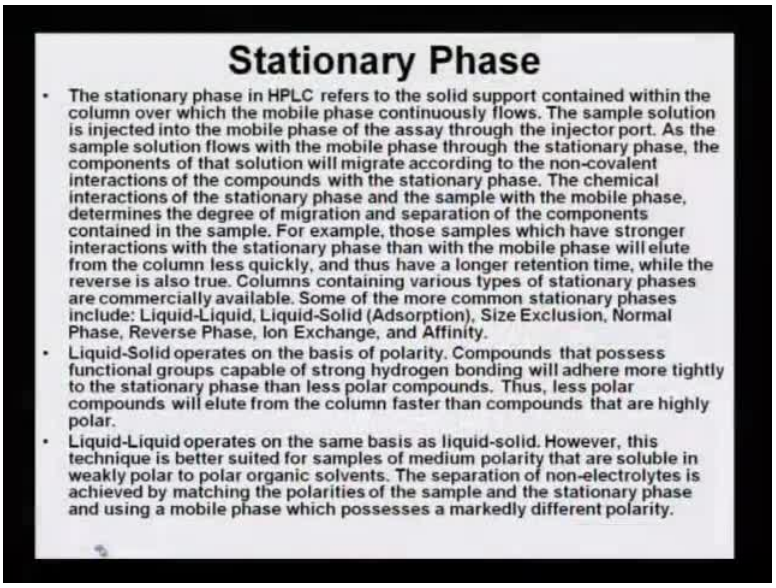
(Refer Slide Time: 08:04)



Column Efficiency - column efficiency refers to the performance of the stationary phase to accomplish particular separations. This entails how well the column is packed and its kinetic performance. The efficiency of a column can be measured by several methods, which may or may not be affected by chromatographic anomalies, such as tailing or appearance of a front. This is important, because many chromatographic peaks do not appear in the preferred shape of the normal Gaussian distribution. For this reason, efficiency can be an enigmatic value, since manufactures may use different methods in determining the efficiency of their columns.

Now, what it means is that, after all column is where the separation is being carried out and if the column does not have a good packing or has some kind of anomalous behavior, it is going to truly reflect on the analytical data.

(Refer Slide Time: 09:16)



Stationary Phase - stationary phase or column is synonymous. The stationary phase of HPLC, refers to the solid support contained within the column over which the mobile phase continuously flows. The sample solution is injected into the mobile phase of the assay through the injector port. As the sample solution flows with the mobile phase through the stationary phase, the component of that solution may migrate according to the non-covalent interactions of the compound with the stationary phase. So, there is no chemical reaction that is actually occurring.

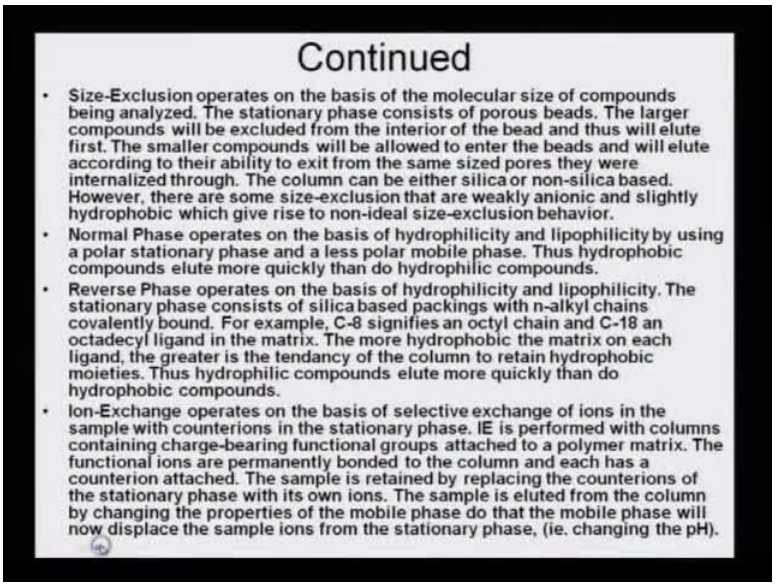
It is a non-covalent kind of interaction between the mobile phase and the stationary phase. The chemical interactions of the stationary phase and the sample with mobile phase, determines the degree of migration and separation of the components contained in the sample. For example, those samples which have stronger interactions with the stationary phase than the mobile phase will elute from the column less quickly, and thus, have a longer retention time, while the reverse also true. Columns containing various types of stationary phases are commercially now available. Some of the more common stationary phases include: liquid-liquid, liquid-solid, size exclusion, normal phase, reverse phase, ion exchange and affinity.

Columns - liquid-solid operates on the basis of its polarity. Compounds that possess functional groups, capable of strong hydrogen bonding, will adhere more tightly to the stationary phase than less polar compounds. Thus, less polar compounds will elute from

the column faster than compounds that are highly polar. So, these are certain things, which one has to understand from time-to-time, in order to able to educate oneself, that in an HPLC machine, what are the parameters that one has to keep in mind, while designing the experiment.

Liquid-liquid operates on the same basis as liquid-solid. However, this technique is better suited for samples of medium polarity than that are solvable in weaker polar to polar organic solvents. The separation of non-electrolytes is achieved by matching the polarities of the sample and the stationary phase and using a mobile phase which possess markedly different polarity. So, one has to make an evaluation as to what is the stationary phase that is optimal for a particular kind of separation process in HPLC.

(Refer Slide Time: 12:44)



Size exclusion depends or operates on the basis of molecular size. And we have seen in the previous few lectures, that we have dealt this in a more fleeting way, but now we are coming to a more concrete use, and more examples are being given in order to make you understand properly, what size inclusion means and how does it operate.

The stationary phase consists of porous beads. The larger compounds will be excluded from the interior of the bead and thus will elude first. The smaller compounds will be allowed to enter the beads, and will elute accordingly to their ability to exit from the same sized pores they were internalized through. The column can be either silica or non-

silica based. However, there some size exclusion that are weakly ionic and slightly hydrophobic, which give rise to non-ideal size exclusion behavior.

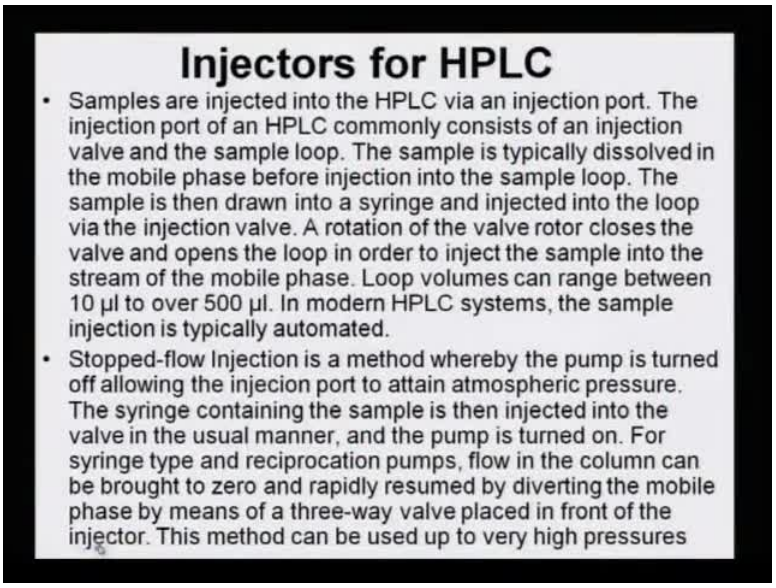
Normal Phase HPLC - now, one has to understand that this is the most popular HPLC system and that is why we call it normal phase or NPLC. Operates on the basis of hydrophilicity and lipophilicity by using a polar stationary phase and a less polar mobile phase; thus, hydrophobic compounds elute more quickly than do the hydrophilic compounds.

Reverse phase operates on the basis of again hydrophilicity and lipophilicity, but in the reverse order. The stationary phase consists of silica-based packing with an n-alkyl chains covalently bound. For example, C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. These are two very popular: C-8 and C-18; if these columns are being used, that means it is a reverse phase analysis that is being carried out on the HPLC machine and we popularly call it as RPLC. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain the hydrophobic moieties. Thus, hydrophilic compounds elute more quickly than do hydrophobic compounds.

Now, I will like to draw your attention here to analysis of some of these phenolic compounds, natural compounds; it is better to analyse them on the RPLC rather than on NPLC, because the compound of choice is actually very polared. So, if we use RPLC, it will elute much faster and normal phase will retain it for a longer time.

Ion-exchange operates on the basis of selected exchange of ions in the sample with counter ions in the stationary phase. The ion exchange is performed with the columns containing large charge-bearing functional groups attached to a polymer matrix. The functional ions are permanently bonded to the column and each has a counter ion attached. The sample is retained by replacing the counter ions of the stationary phase with it is own ions. The samples is eluted from the column by changing the properties of the mobile phase and they do not have… The mobile phase will now be displacing the sample ions from the stationary phase, by the change of the ph; so that is what happens in an ion exchange.

(Refer Slide Time: 17:16)



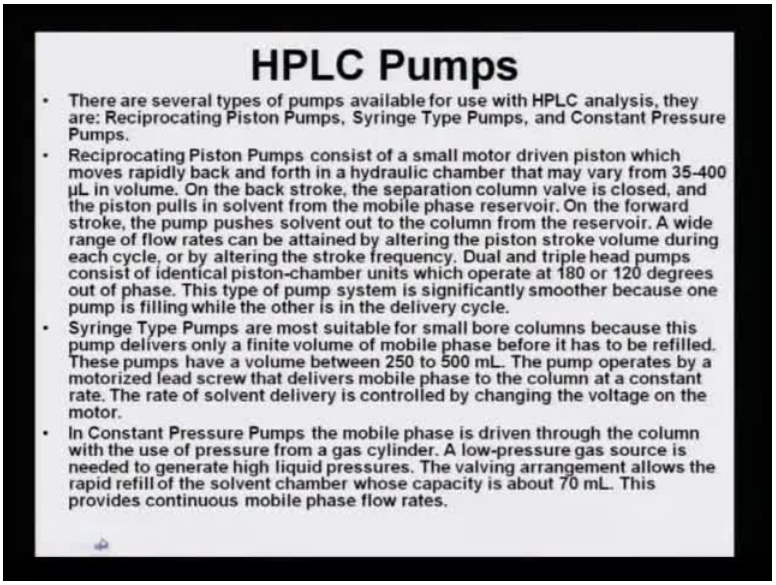
What are the different types of injectors in an HPLC machine? As what we had a different set of injectors or injection mode for GC, similarly, there are options of injectors in HPLC too.

Samples are injected into the HPLC via an injection port. The injection port of an HPLC, commonly consists of an injection valve and a sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 micro liters to 50 micro liters depending on the size of the column and the size of the HPLC system. In modern HPLC system, the sample injection is typically automated. So, what it means, that there is a loop, and there is valve, and there is an injector. So, once it is injected, it is lying in the loop, but it is not allowed to go into the column till it is ready for the process.

Stopped-flow injection is a method whereby the pump is turned off, allowing the injector port to attain atmospheric pressure. The syringe containing the sample is then injected into the valve in the usual manner, and the pump is then turned on; the pump is then turned on. For the syringe type and reciprocation pumps, flow in the column can be brought to 0 and typically resumed by diverting the mobile phase by means of a three-way valve placed in front of the injector. This method can be used to very high pressures.

Remember we are talking about HPLC - high pressure liquid chromatography or high performance liquid chromatography. So the pressures in these and the mobile phases pressure is much, much higher and must be controlled properly.

(Refer Slide Time: 19:38)



HPLC mobile phase are controlled by pumps. There are several types of pumps available for use with HPLC analysis. They are: reciprocating piston pumps, syringe type pumps and constant pressure pumps.

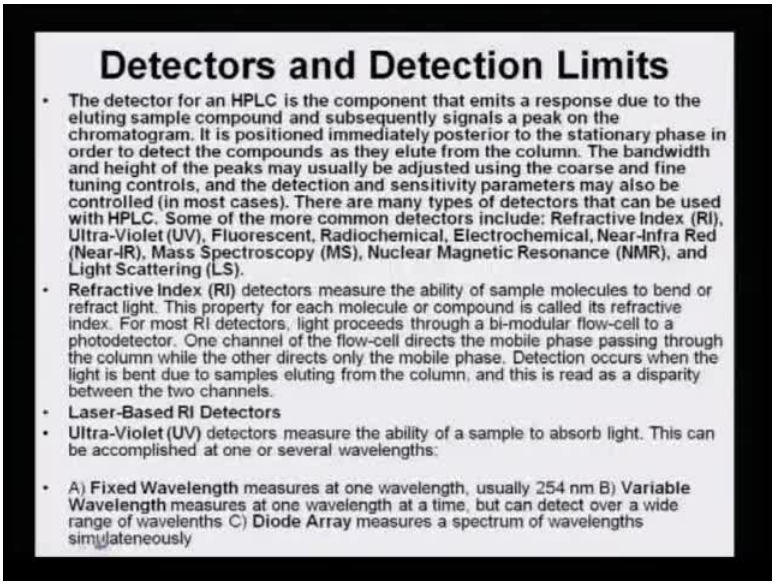
Reciprocating piston pumps consist of a small motor driven piston, which moves rapidly back and forth in hydraulic chamber, that may vary from 35 to 400 microliter in volume. On back stroke, the separation column valve is closed and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the reservoir. A wide range of flow rates can be attained by altering the piston stroke volume during each cycle or by altering the stroke frequency. Dual and triple head pumps consist of identical piston-chamber units, which operate at 180 to 120 degrees out of phase. This type of pump system is significantly smoother, because one pump is filling, while the other is in delivery cycle. So, one can have pumps in, in you know, in series; and so, when one is taking these strokes, the other can be functional, and when the second one is taking the strokes, the third one can be functional.

Syringe type pumps are most suitable for small bore columns, because this pump delivers only a finite volume of mobile phase, before it can be refilled. These pumps

have volume between 250 to 500 microliters. The pump operates by motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor. Now, all along, the pressure and the rate of the flow of the mobile phase must be maintained; otherwise, the analysis will go wrong, will go haywire, and the analyte which was a mixture in the beginning, would be remaining a mixture at the end of the analysis.

In constant pressure pumps, the mobile phase is driven through the column with the use of pressure from the gas cylinder. A low-pressure gas source is needed to generate high liquid pressures. The valving arrangement allows the rapid refill of the solvent chamber, which has a capacity of about 70 ml - microliter; this provides continuous mobile phase flow rates.

(Refer Slide Time: 22:55)



Detectors and Detection Limits - if this machine did not have a detector, the analysis would not be detected, even though separation process must have been taken place, but who would be able to tell whether the separation has taken place or it has not taken place, unless and until there is a device that can detect, and such a device is called detector. And obviously, every detector will have its detection limit; it cannot detect everything on this earth. So, that is called a detection limit.

The detector of an HPLC, is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned

immediately posterior to the stationary phase in the order to detect the compound as they elute from the column. So, one-by-one as they get separated and elute into the column, they are able to get to the end of the detector and get noticed, and they appear as a peak on the chromatogram. The bandwidth and the height of the peak may usually be adjusted using the coarse and fine-tuning control, and the detection and sensitivity parameters, may also be controlled in most of the cases.

There are many types of detectors that can be used with HPLC. Some of the most common detectors include: refractive index or RI detector, ultra-violet or UV detector, fluorescent and radiochemical detector, electrochemical detector, near-infrared or near-IR detector, mass spectroscopy detector or MS detector, NMR or nuclear magnetic resonance detector, and light scattering or LS detector.

Refractive Index - RI - detectors, measure the ability of sample molecules to bend or refract light. This property for each molecule or compound is called it is refractive index. For most RI detectors, light proceeds through a bi-modular flow cell to a photo detector. One channel of the flow cell directs the mobile phase passing through the column, while the other directs only the mobile phase. Detection occurs when the light is bent due to samples eluting from column and this is read as a disparity between the two channels. So, that means, that there are two different medium and the light is travelling through that, and when the light is, you know, flow of the light is altered, that time, it is understood that the medium has some compound which is interfering.

Laser based RI detectors are also there.

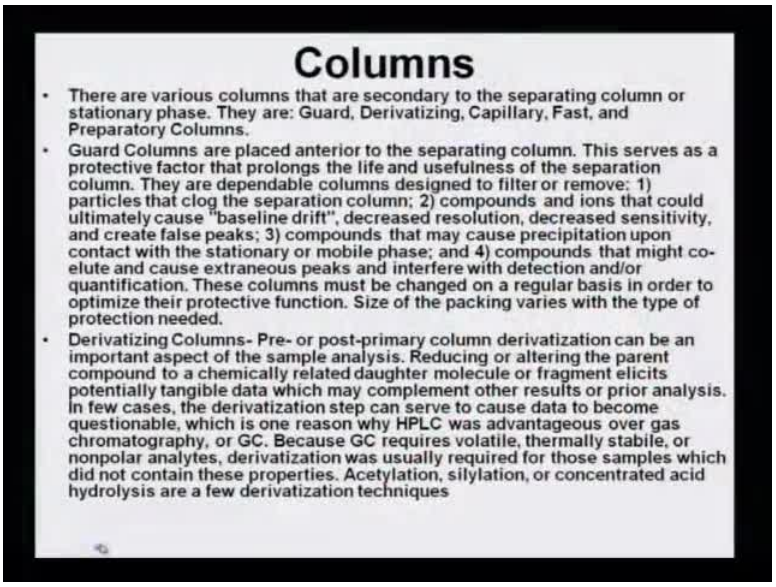
UV detectors - these are most popularly used detectors of HPLC. UV detectors measure the ability of a sample to absorb light. This can be accomplished at one or several wavelengths.

There are different types of UV detectors: one is fixed wavelengths, which measures at a, at just one wavelength; that is, one cannot make any alteration with the wavelength; one wavelength is setup on the machine and that is 254 nanometers.

The other one is variable wavelength, which measures one wavelength at a time, but there may be two or three options that can take place. And the third one is diode array -

measures a spectrum of wavelengths simultaneously; that means, throughout the analysis it is scanning all the wavelengths that are possible in the UV visible region.

(Refer Slide Time: 27:20)



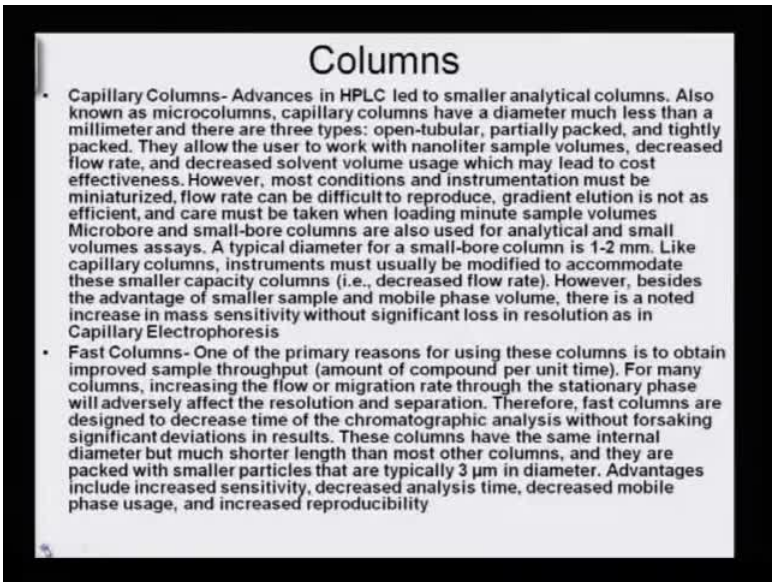
Columns - there are various columns that are secondary to the separating column or stationary column. They are called: guard column, derivatizing column, capillary column, fast and preparatory column. Now these are varieties of columns, which are used at different instances.

Guard column, as the name suggests, is actually meant to guard the main column, because the column is very costly material and if it is not guarded, with impurities it is likely to get blocked, and once it is blocked, the cost is so high that the number of analysis that it would have ideally carried out, would not be possible. Guard column are placed anterior to the separating column; this serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove: number one - particles that clog the separation column; number 2 - compounds and ions, that could ultimately cause baseline drift, decreased resolution, decreased sensitivity and create false peaks; and number 3 - the compounds that may cause precipitation upon contact with the stationary or mobile phase; and number 4 - compounds that might co-elute and cause extraneous peaks, and interfere with detection and/or quantification.

So, they have major role in trying to protect the main column, in trying to remove all the impurities, in trying to resolve all the precipitation mechanism that may or may not occur. So, they have a role to play and they are at the sacrificing end, because the main column is then protected.

Derivatization column - pre- or post-primary column derivatization can be possible and is an important aspect of the sample analysis. Sometimes, some compounds do not show on the detector and they do not show any response on the detector. As a result, they need to be chemically derivatized, so that the new derivative is then having a detector response peak showing capacity. Reducing or altering the parent compound to a chemically relative daughter molecule or fragment elicits potentially tangible data, which may complement other results or prior analysis. In few cases, the derivatization step can serve to cause data to become questionable, which is one reason why HPLC was advantageous over gas chromatography or GC, because GC requires volatile thermally stable and nonpolar analytes, derivatization was usually required for those samples, which did not contain these properties. Acetylation, silylation or concentrated acid hydrolysis are few derivatization techniques that are possible to be done on HPLC, but not on GC.

(Refer Slide Time: 31:00)

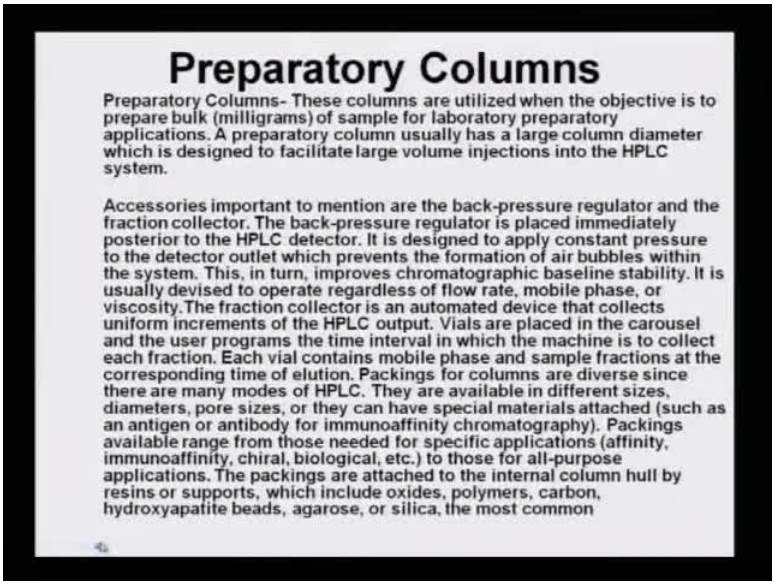


Columns are of the capillary type, which are also being used, because everywhere micro analysis is becoming very, very important. So, just like we have capillary columns in

GC, we also have capillary columns for doing smaller analysis or micro analysis using capillary columns.

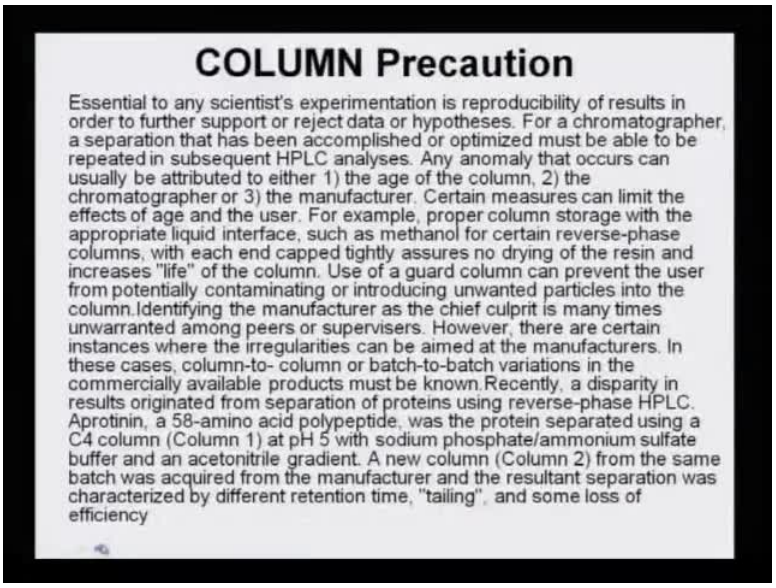
And we have fast columns; one of the primary reasons for using these columns is to obtain improved sample throughput amount of compound per unit time. For many columns, increasing the flow or migration rate through the stationary phase, will adversely affect the resolution and separation. Therefore, fast columns are designed to decrease the time of that chromatographic analysis without forsaking significant deviation in results. These columns have the same internal diameter, but much shorter length than most of the other columns, and they are packed with smaller particles, that are typically 2 micrometer in diameter. Advantages include increased sensitivity, decreased analysis time, decreased mobile phase usage and increased reproducibility.

(Refer Slide Time: 32:27)



Preparatory columns are of course for bulk separation. These columns are utilized when the objective is to prepare bulk or milligrams of the sample for the laboratory purpose application. The ideal example for preparative column usage is in pharmaceuticals; you know, when the intermediate compounds have to be separated, and further on more reaction have to be done, it has to be done on a little larger scale. So, columns could be analytical type or they could be preparative type.

(Refer Slide Time: 33:08)



Column precaution - this is very, very important, because one has to keep in mind, that if the column is not preserved properly, if it is not used properly, then the number of analysis that can be carried out on these columns would reduce and the cost of these columns is very high - almost of the order of 50,000 rupees per column.

Essential to any scientist’s experimentation is reproducibility of results in order to further support or reject data or hypotheses. For a chromatographer, a separation that has been accomplished or optimized must be able to repeated in subsequent HPLC analyses. Any anomaly that occur, can usually be attributed to three reasons: either the age of the column, the chromatographer’s ability to separate or the manufacturer who has not given a proper column.

Certain measures can limit the effect of age and the user. For example, proper column storage with appropriate liquid interface, such as methanol, such as methanol for certain reverse phase columns are very, very essential and the end cap should be tightly assures no drying of the resin, definitely increases the life of the column. Use of a guard column can prevent the user from potentially contaminating or introducing unwanted particles into the column.

Identifying the manufacturers as the chief culprit is many-a-times unwarranted among peers or supervisors, because you see, they have fully checked while manufacturing; so one should not blame the manufacturer without understanding the mistake of the column.

However, there are certain instances, where the irregularities can be aimed at the manufacturers also. In these cases, column-to-column or batch-to-batch variation in the results originated from separation of proteins; so, there are a few cases, of course, where the manufacturer can be blamed, but not always.

(Refer Slide Time: 35:40)

Future trends - HPLC will continue to be the most important laboratory separation techniques for analytical as well as preparative purposes. Biotechnology and the life sciences is where HPLC will be a crucial procedure, and the most predominantly used procedure. Fast and microbore columns will be important in analytical and mass scale preparative applications for biological and heterologously samples expressed in gene products are very, very important. Affinity and immunoaffinity techniques will be utilized more frequently for the production of biotechnological pharmaceuticals, because of the need for the ultra-purification in order to remove all unwanted material from the host cell. So, you see that how important it is not only for the chemist, but also for the biotechnologist and the analytical chemist. HPLC will continue to be the most important mainstay in purification and separation of proteins, peptides and nucleotides.

Smaller analytical column will be needed to map the human genome. The need for more accurate and higher capacity chiral separation columns will be needed by the pharmaceutical companies in order to optimize efficiency in mass production of active enantiomeric compound. Specialization in detection and identification methods will

increase in importance. This would involve the monitoring of environmental pollutants using special chelate packing to detect metal contaminants in water, developing fast separations to monitor fermentation and developing rapid methods in identification of viruses and bacteria.

So, if I have to sum up: HPLC-MS will be the most important tool in today's world of analysis.