Advance Analytical Course
Prof. Padma Vankar
Department of Chemistry
Indian Institute of Technology, Kanpur

Lecture No. # 03

Introduction to Chromatographic Techniques

Now, we come to the Introduction to Chromatographic Techniques. As mentioned earlier, after the extraction process, it is now important to separate the compounds. So, let us try to understand what chromatography is all about.

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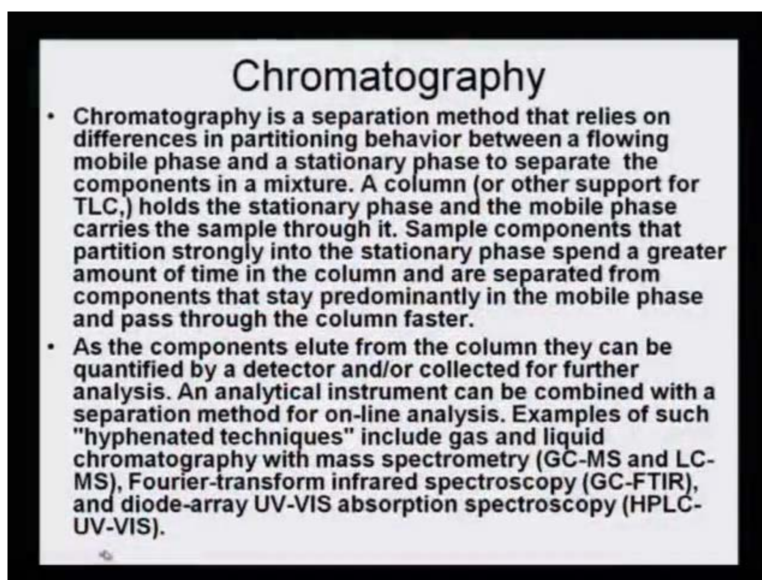

Chromatography is a separation method that relies on differences in partitioning behaviour between a flowing mobile phase and a stationary phase to separate the components in a mixture. A column or other support for TLC that holds the stationary phase and the mobile phase carries the sample through it. Sample components that partition strongly into the stationary phase spend a greater amount of time in the column and are separated from the components that stay predominantly in the mobile phase and pass through the column faster.

I will like to explain this a little bit more elaborately, that in a mixture of compounds where there is ABCD component, one may be having more affinity for the stationary phase, and the other may be having lesser affinity, and would be passing into the mobile phase. So, that is what is meant in the above paragraph.

As the components elute from the column, they can be quantified by a detector and/or collected for further analysis. An analytical instrument can be combined with a separation method for an on-line analysis. Examples of such hyphenated techniques include gas and liquid chromatography with mass spectrometry, GC-MS and LC-MS; Fourier-transform infrared spectroscopy can be hyphenated with GC and diode array UV-visible absorption spectroscopy can be hyphenated with HPLC.

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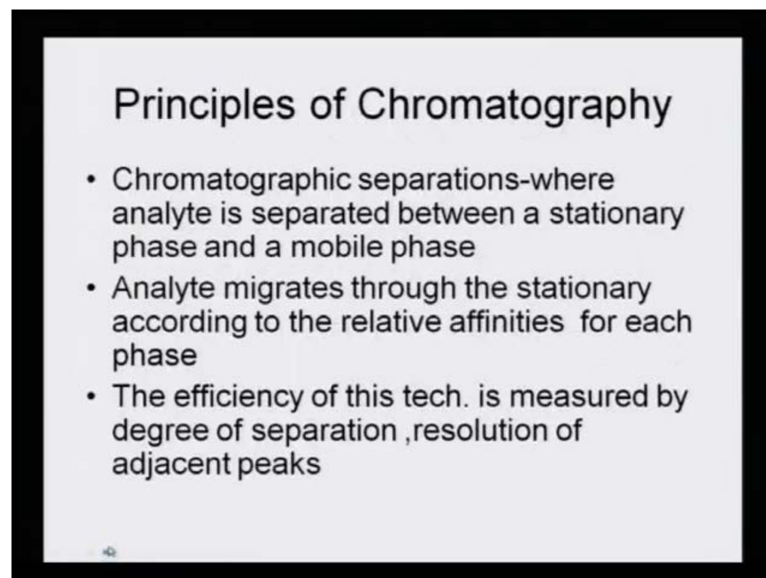

Principles of chromatography - this is the basis on which the separation actually takes place. Chromatographic separations where the analyte is separated between a stationary phase and a mobile phase. Analyte migrates through the stationary according to the relative affinities for each phase. The efficiency of this technique is measured by the degree of separation, resolution of adjacent peaks.

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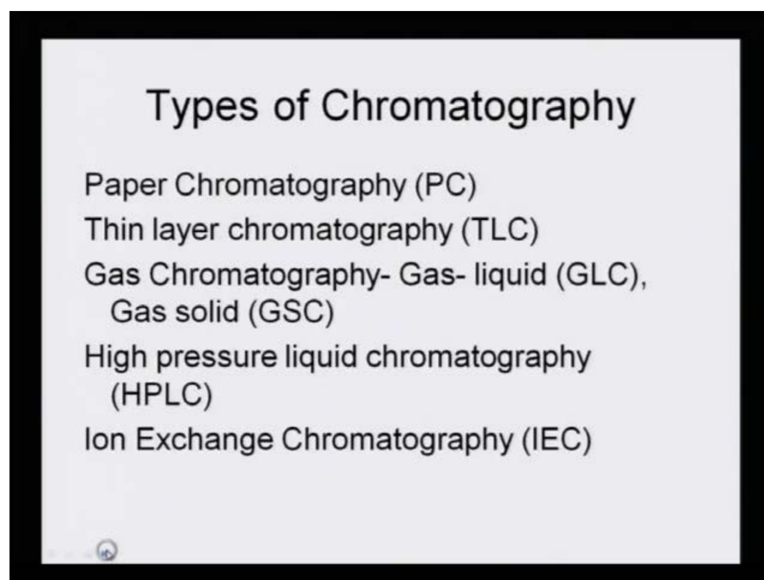

There are different types of chromatography: paper chromatography, thin layer chromatography, gas chromatography, which is gas-liquid chromatography and gas-solid chromatography, high pressure liquid chromatography, HPLC, or ion exchange chromatography, which is IEC. Paper chromatography actually is now obsolete, but it is important to mention to the students that there was the first chromatographic technique, which was invented and there was a use of paper, which was the stationary phase. However, in due course of time, newer techniques came into being and the paper chromatography became obsolete.

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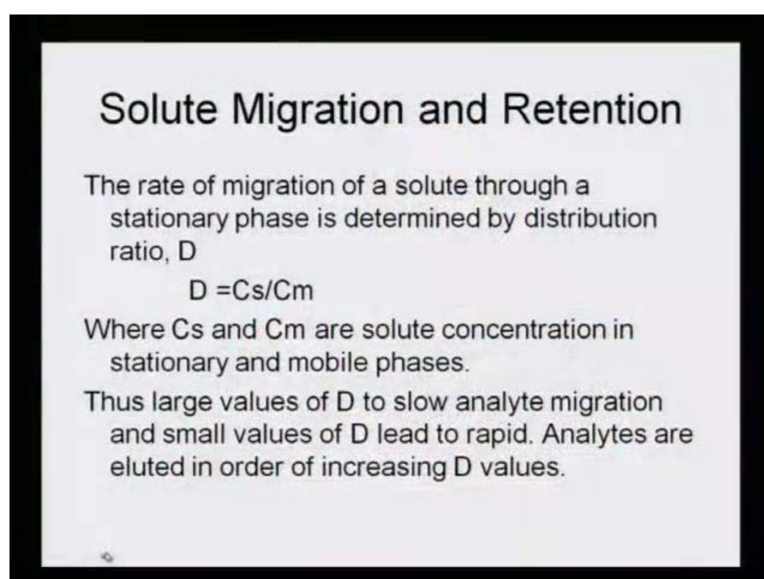

The genesis of chromatographic separation lies on the fact that there is solute migration and there is retention. The rate of migration of a solute through a stationary phase is determined by distribution ratio, D. Now, this D is actually equal to C s upon C m, where C s and C m are the solute concentrations in the stationary phase and the mobile phase. So, one has to understand that there is an analyte, whether it is remaining with the stationary phase or whether it is coming with the mobile phase, and what is its chemical reactivity towards these two phases, will determine whether it will stay back or it will flow out, and that is what is actually determined by the distribution ratio, D. Thus, large values of D to slow analyte migration and small values of D lead to rapid migration. Analytes are eluted in the order of increasing D values; as and when the D value increases, the analytes are actually coming out of the column.

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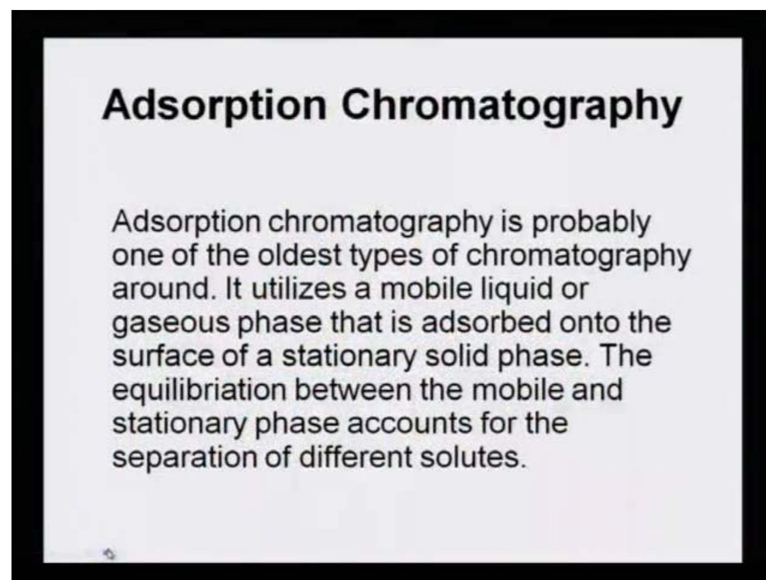

Adsorption chromatography - adsorption, as the name suggests means it is being taken up by the material; it is being adsorbed. Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of the stationary solid phase. The equilibration between the mobile and the stationary phase accounts for the separation of different solutes.

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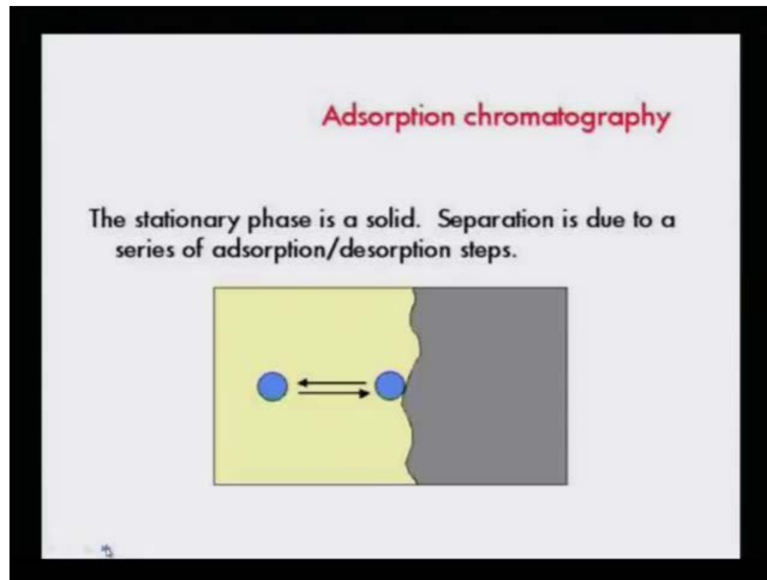

Now, a pictorial example is shown here of the adsorption chromatography. The stationary phase is a solid. Separation is due to series of adsorption and desorption steps. One has to remember one thing very clearly that wherever there is adsorption, the next step is desorption and that is how it moves on; otherwise, if it just sits there and does not move with the help of mobile phase, it will be of no consequence. So, wherever there is adsorption, the next step of that is desorption.

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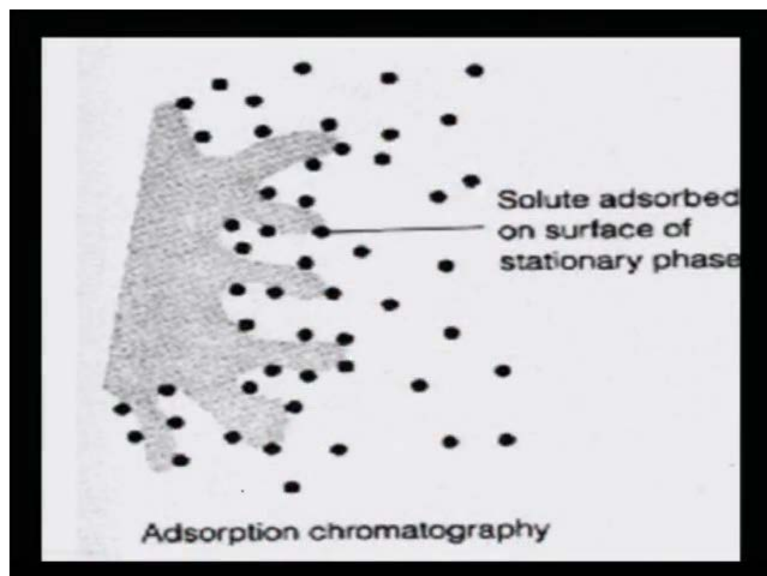

Another pictorial description of adsorption is that how the solutes are adsorbed onto the surface of the stationary phase that is being shown here.

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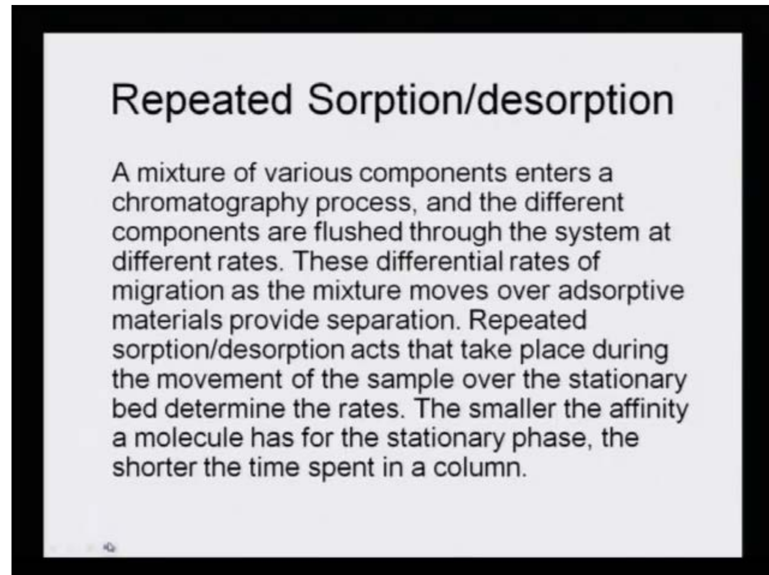

Repeated sorption and desorption. As what I mentioned a little while ago, a mixture of various components enters a chromatographic process, and the different components are flushed through the system at different rates. These differential values or rates of migration as the mixture moves over the adsorptive materials provide separation. Repeated sorption and desorption acts that take place during the movement of the sample over the stationary bed determine the rates. The smaller the affinity a molecule has for the stationary phase, the shorter time it will spend on the column and it will run out with the mobile phase. So, this is a process, which takes place again and again.

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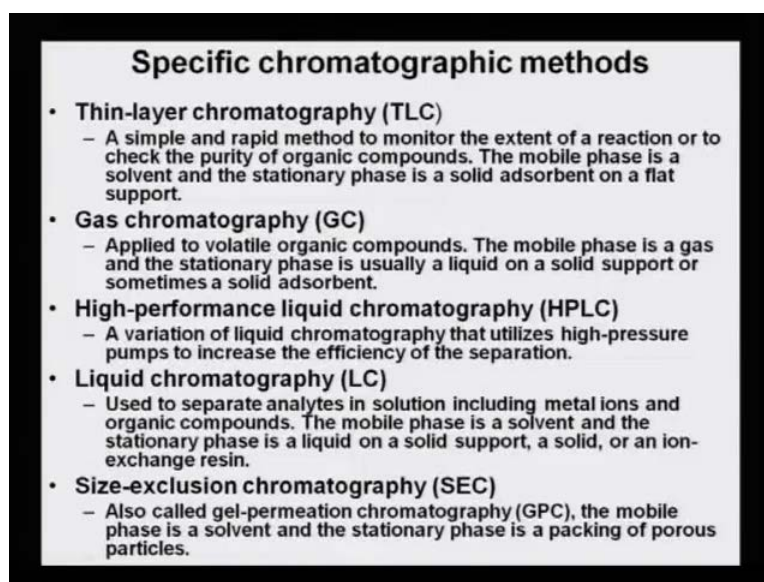

There are specific chromatographic techniques. Paper chromatography, which I said earlier, is now obsolete, but the ones which are in practise, are the following. Thin layer chromatography; that is, popularly known as TLC. Every laboratory will have these TLC jars, where TLC would be taken on a routine basis for any unknown compound. It is a simple rapid method to monitor the extent of a reaction or to check the purity of organic compounds. The mobile phase is a solvent and the stationary phase is a solid adsorbed on a flat support. You must have seen these small glass plates, which the blood sample collectors usually take at the pathological labs; it is the same glass slide and over that, there is a solid phase, which is coated.

The second type of chromatography is called gas chromatography or GC. It is applied to volatile organic compounds. The mobile phase is a gas and the stationary phase is usually a liquid on a solid support or sometimes a solid adsorbent. So, what it means that gas chromatography is meant for low boiling compounds. High-performance liquid chromatography or HPLC is the next type. A variation of liquid chromatography that utilizes high-pressure pumps to increase the efficiency of the separation; it is also meant only for compounds, which are higher boiling, which cannot be analysed or separated on the GC.

Liquid chromatography - used to separate analytes in solution including metal ions and organic compounds. The mobile phase is a solvent, and the stationary phase is a liquid on

the solid support, a solid, or an ion-exchange resin. The next type of chromatographic method is called size-exclusion chromatography or SEC; also, called as gel-permeation chromatography or GPC. The mobile phase is a solvent and the stationary phase is a packing of porous particles.

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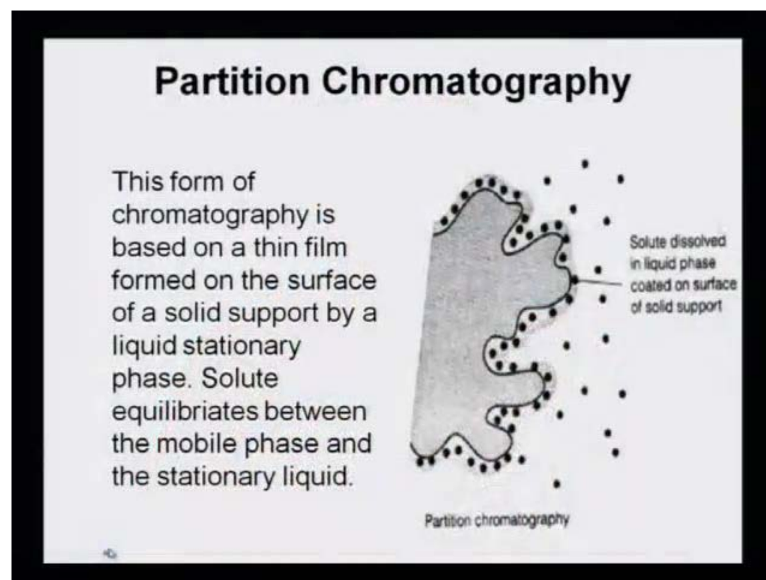

Partition chromatography - that this nomenclature partition chromatography is based on its action. This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. The solid or the solute equilibrates between the mobile phase and the stationary phase. As what the picture shows, that there is a solute dissolved in liquid phase and there is a coating on the surface of the solid support. So, this depicts the partition chromatography.

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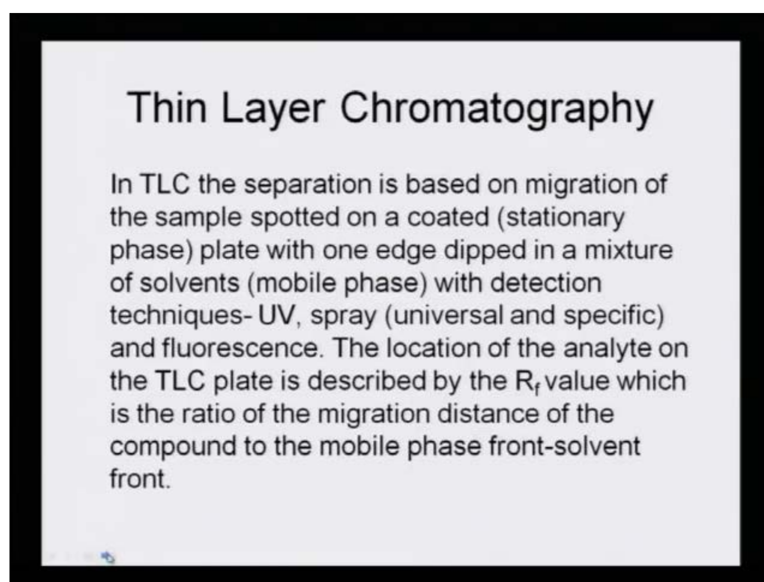

Thin layer chromatography - in TLC, the separation is based on migration of the sample spotted on the coated stationary phase or stationary plate with an edge dipped in the liquid of solvents, mobile phase with detection techniques, which can be either UV, spray - universal or specific or fluorescence; I will explain all these in a while. The location of the analyte on the TLC plate is described by the Rf value, which is the ratio of the migration distance of the compound to the mobile phase front versus the solvent front.

Now, in a TLC, which is just this long, (Refer Slide Time: 12:51) there is a coating on one side; the compounds are spotted and they are eluted with the help of the mobile phase. Now, when the compounds are moving, according to their affinity with the stationary phase, they will elute in a different speed and as a result, the analyte will show the number of components that are present in that. However, it has its own restriction.

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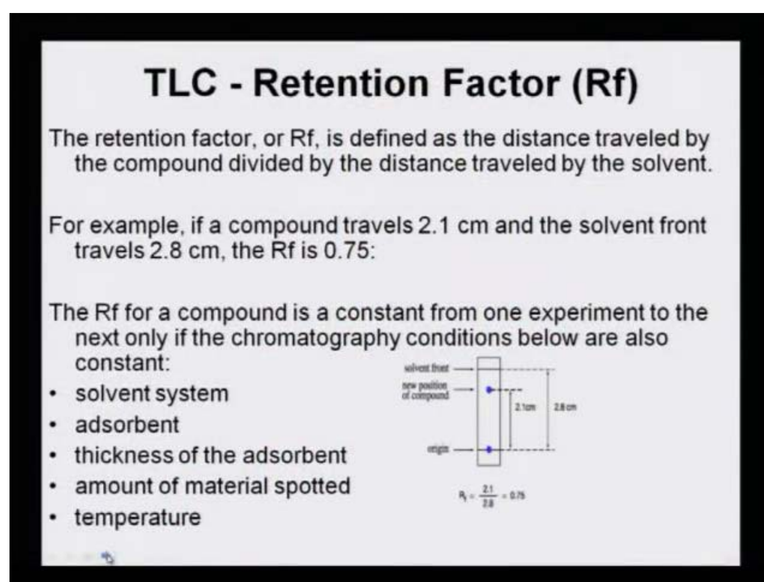

This will be a better explanation here. The retention factor, which is a very important factor of TLC is defined as the distance travelled by the compound divided... The retention factor or Rf is defined as the distance travelled by the compound divided by the distance travelled by the solvent. For example, if a compound travels 2.5 centimetres and the solvent front travels up to 2.8, the Rf is 0.075. You can see this here very clearly that this is the solvent front and the compound was originally spotted here, (Refer Slide Time: 14:24) and then it eluted to a new position and that traversing was 2.5 centimetres; whereas, the solvent actually travelled up to 2.8 centimetres. And that is how one calculates the Rf factor or the retention factor.

The Rf for a compound is a constant from one experiment to the next only if the chromatographic conditions below are also the constant; like it should have the same solvent system; it should have the same adsorbent; the thickness of the plate or the adsorbent also should be the same; amount of the material spotted also should be the same; there should not be any variation in the temperature. Then only, the retention factor will remain the same for the same compound; otherwise, it will alter.

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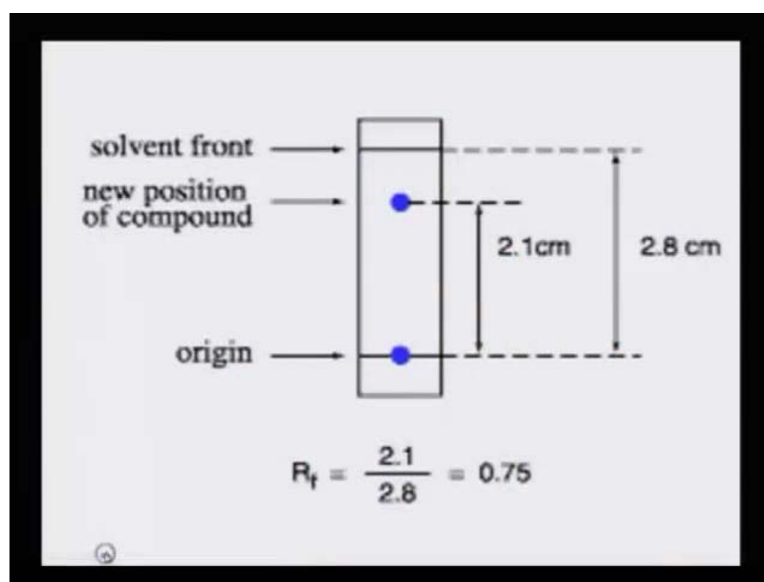

Now, this is a more elaborate pictorial description of the same slide.

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Rf for TLC

- The larger an Rf of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger Rf is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. Conversely, if you know the structures of the compounds in a mixture, you can predict that a compound of low polarity will have a larger Rf value than a polar compound run on the same plate.
- The Rf can provide corroborative evidence as to the identity of a compound. If the identity of a compound is suspected but not yet proven, an authentic sample of the compound, or standard, is spotted and run on a TLC plate side by side (or on top of each other) with the compound in question. If two substances have the same Rf value, they are likely (but not necessarily) the same compound. If they have different Rf values, they are definitely different compounds.

Coming to Rf of TLC - the larger an Rf of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger Rf is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. What I just explained to you that anything, which travels faster has less affinity for the solid phase. Conversely, if you know the structures of the compounds in a mixture, you can predict that the

compound of low polarity will have a larger Rf value than a polar compound run on the same plate.

The Rf can provide corroborative evidence as to the identity of a compound. If the identity of a compound is suspected, but not yet proven, an authentic sample of the compound; or in other words, we can call it standard is spotted and run on TLC plate is made side by side with the compound in question. If two substances have the same Rf value, they are likely, but not necessarily the same compound. If they have different Rf values, they are definitely different compounds. So, TLC gives us a basic idea of identification with a known compound and it also gives us an idea how many components are present in an analyte.

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Sorbents	Chromatographic mech.	Typical Applications
Silica gel	Adsorption	Amino acids, Vitamins hydrocarbon, alkaloids,
modified silica	Mod. Partition	HC, Non polar compounds
Cellulose powder	Partition	Amino acids , nucleotides, carbohydrate
Alumina	Adsorption	HC, alkaloids, food dyes, lipids, metal ions
Kieselguhrs	Partition	Sugar, fatty acids
Ion exch. Cellulose	Ion exchange	Nucleic acids, nucleotides, halide and metal ions
Sephadex gels	Exclusion	Polymers, proteins, metal complexes
Beta -cyclodextrins	Stereo adsorption	Mixtures of enatiomers

There are various different sorbent materials, which are used for different types of compound. This is a very important slide, where I have tried to incorporate a lot of information and you will see that what the different applications are; that means, what are the different sorbents that can be used. Of course, the most popular sorbent is silica gel, but that is not the one and only sorbent. So, there are other types of materials and these materials are used typically for specific types of compounds. So, this gives an idea if I have to use silica gel, what is the mechanism that is actually taking place, and what are the different types of compounds that can be separated by using silica gel. Silica gel - the mechanism that is carried out is adsorption and the types of compounds that can be

separated using silica gel are amino acids, vitamins, hydrocarbons, alkaloids and many more organic compounds. If we make use of modified silica, the mode of chromatography is partitioning and it is used for hydrocarbon and non-polar compounds.

If we use cellulose powder, the mode of mechanism is partitioning again. It is mainly used for amino acids, nucleotides, carbohydrates, hydrocarbons and alkaloids. Alumina is another type of chromatographic sorbent and here the mode of chromatographic mechanism is adsorption. It is used for food dyes, lipids, metal ions. Kieselguhrs is another type of sorbent, where it undertakes mechanism by partitioning, and it is specifically used for sugar and fatty acids. Ion exchange cellulose - the mechanism is ion exchange as the name suggests, and it is used for nucleic acids, nucleotides, halides and metal ions. Sephadex gel - it undertakes the chromatographic mechanism by exclusion and it is used specifically for polymers, proteins, metal complexes; that means it is used for larger molecules. Beta cyclodextrins undertake the sorbent material for the mechanism, which is stereo adsorption and it is mainly to separate the enantiomeric mixtures.

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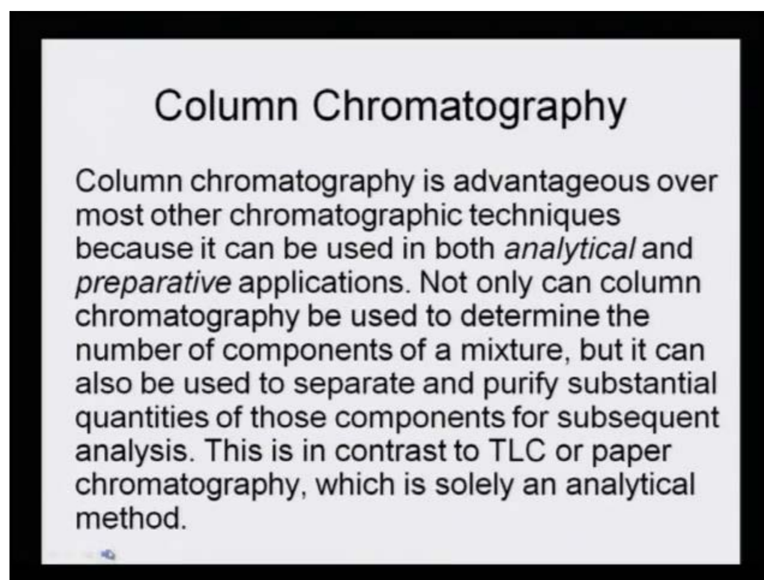

Coming to the little bigger form of thin layer chromatography, the principle remains the same, but it is more applicable method; a method in which we can actually separate the components and that is popularly known as column chromatography. Column chromatography is advantageous over most other chromatographic techniques, because it

can be used in both analytical and preparative applications. Not only can column chromatography be used to determine the number of components of a mixture, but it can also be used to separate and purify substantial quantities of those components for subsequent analysis. This is in contrast to TLC or paper chromatography, which is solely an analytical method. That means we can now get separated material to take it for spectroscopic analysis. So, this is a method, which is most popular; every chemical laboratory will have column chromatography going on all the time for the purpose of separation of components.

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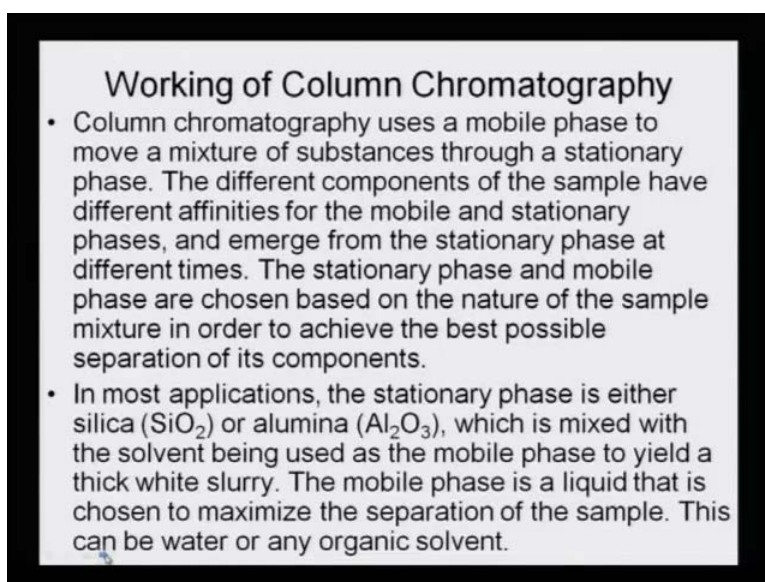

Working of column chromatography - we need to have a better understanding of what the column chromatography technique is all about. Column chromatography uses a mobile phase to move a mixture of substance through a stationary phase. The different components of sample have different affinities for the mobile and the stationary phases, and emerge from the stationary phase at different times. The stationary phase and mobile phase are chosen based on the nature of the sample mixture in order to achieve the best possible separation of it components. That is why it is important to know, which material adsorbent to use for column chromatography. If we have to use a polar compound, we cannot use keiselguhr; we will have to use keiselguhr only for sugar and fatty acids.

In most applications, the stationary phase is either silica or alumina. So, the more popular chromatographic adsorbents are silica and alumina; only under very specific cases, we

have modified cellulose and we have keiselguhr, and other types of materials, which are mixed with the solvent being used as the mobile phase to yield a thick white slurry. The mobile phase is a liquid that is chosen to maximize the separation of the sample. This can be water or any other organic solvent, but it is preferably an organic solvent.

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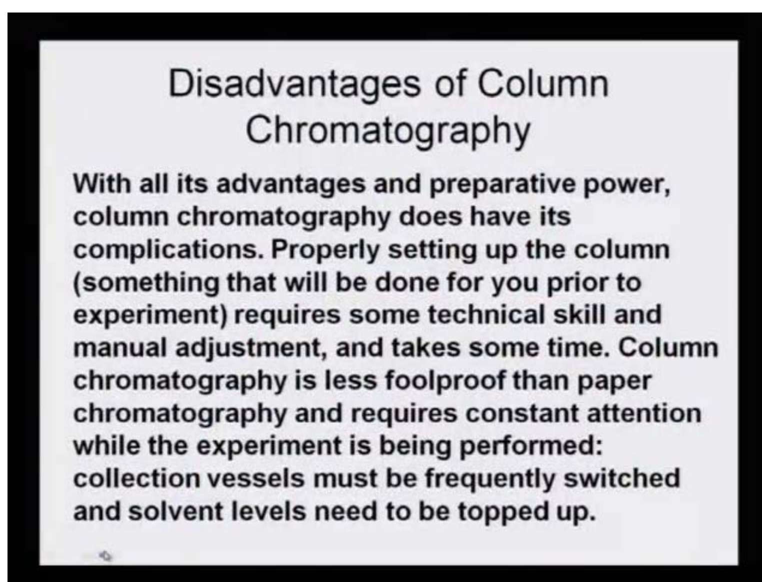

But, everything has advantages and disadvantages. So, if I do not talk about the disadvantages of column chromatography, that would not be giving a right picture of column chromatography. So, let us look at the disadvantages of column chromatography. With all its advantages and preparative power, column chromatography does have its own complications. Properly setting up column - sometimes that will be done by you prior to the experiment, requires some technical skill and manual adjustment, and takes some time. Column chromatography is less foolproof than paper chromatography and requires constant attention while the experiment is being performed: collection vessels must be frequently switched and solvent levels must need to be topped up. Column cannot go dry. If the analysis is on, one has to be careful, attentive that the fractions are collected of the same size, and that is why it only will give an efficient result; otherwise, one would land up getting all mixtures and it would be of no consequence.

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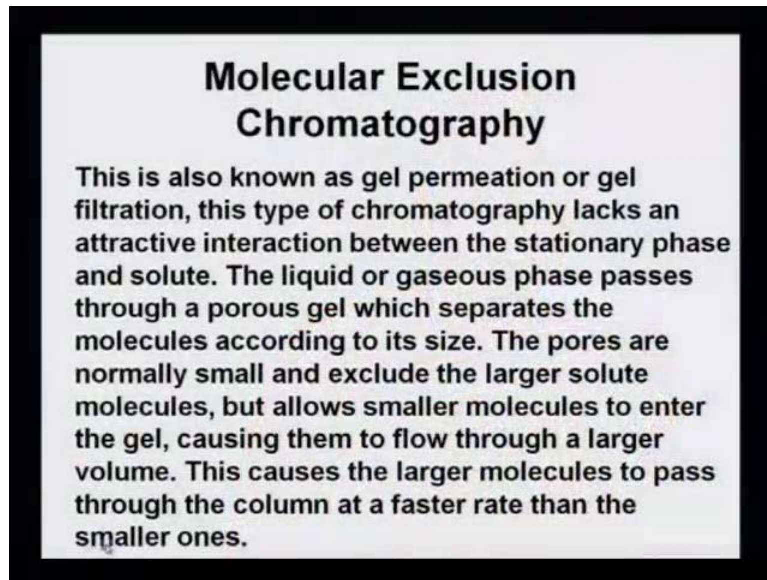

Molecular exclusion chromatography - this is also called gel permeation or gel filtration. This type of chromatography lacks an attractive interaction between the stationary and the solute phases. The liquid or gaseous phase passes through a porous gel, which separates the molecule according to its size. So, it does not take into account any affinity factor; it is only a kind of a mesh and a particular size would pass through this gel, and the others would get retained. The pores are normally small. They exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones, which are retained on the gel.

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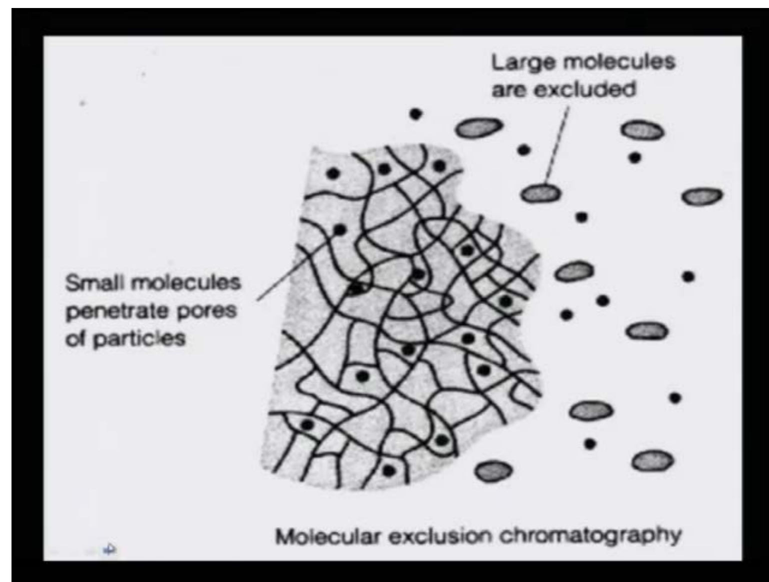

This is an example of that molecular exclusion. The larger ones are held up and the smaller ones penetrate through the pores and are separated.

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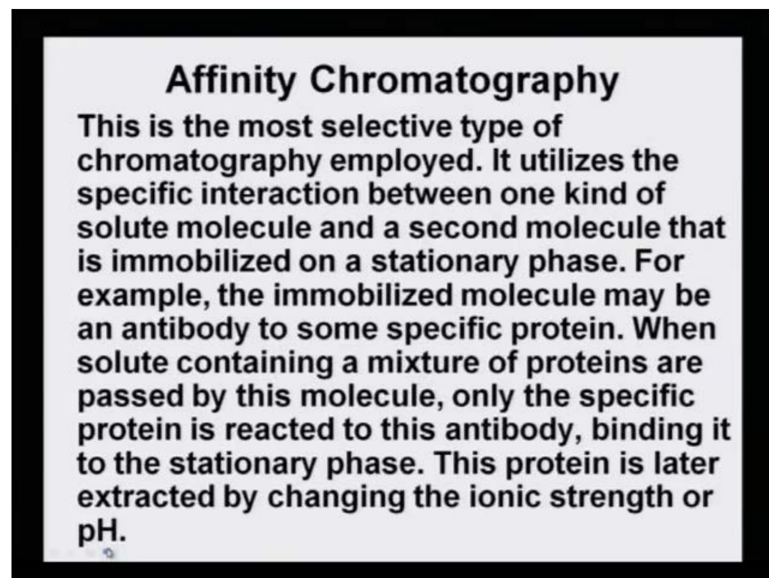

Affinity chromatography - this is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on the stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. While solute containing a mixture of proteins is passed by this molecule, only the specific protein is reacted to this

antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or the pH. So, it just solely depends on the interaction of the protein at the stationary phase. So, it has nothing else to do with the other mechanism. So, these are based on different types of mechanisms, which are again very compound specific, and that is why the choice of chromatography has to be... Again and again, I am mentioning, we made according to the type of analyte, which needs to be chromatographed.

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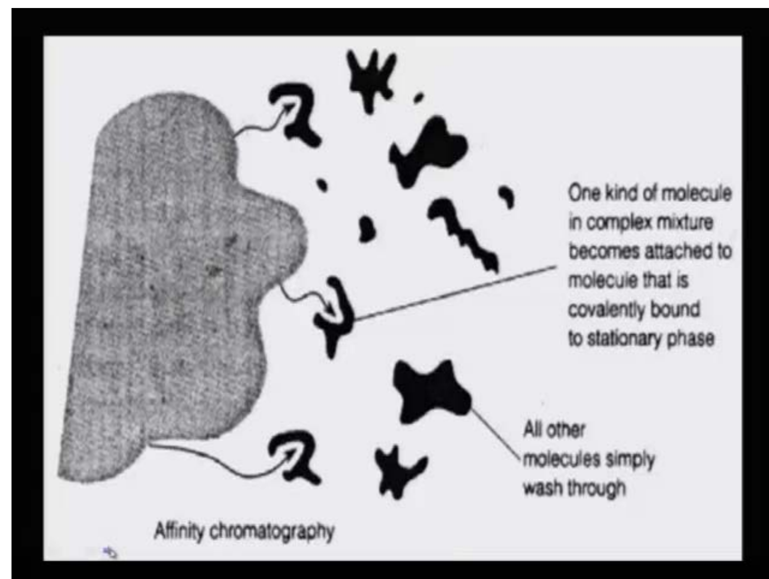

This is again another example of affinity chromatography. One kind of a molecule in complex mixture becomes attached to molecule that is covalently bound to stationary phase, and the other molecules are simply washed off.

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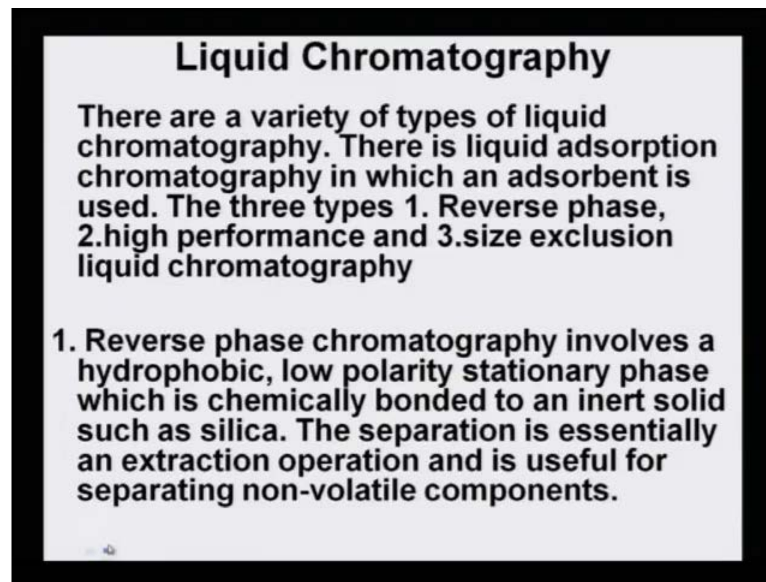

Liquid chromatography - there are a variety of types of liquid chromatography. There is liquid adsorption chromatography in which an adsorbent is used. The three different types of chromatographic techniques under this heading is - number 1, reverse phase; number 2, high performance; and the third one is size exclusion liquid chromatography.

Reverse phase chromatography as the name suggests involves a hydrophobic, low polarity stationary phase, which is chemically bounded to an inert solid such as silica. The separation is essentially an extraction operation and is useful for separating non-volatile compounds. As what I had mentioned earlier, gas chromatography is meant for volatile compounds and liquid chromatography is meant for non-volatile compounds. So, I am repeating that again. Now, reverse chromatography means that the ones which were normally retained in the column, will now come out first and the ones which normally come out first will be retained. That is why the name - reverse.

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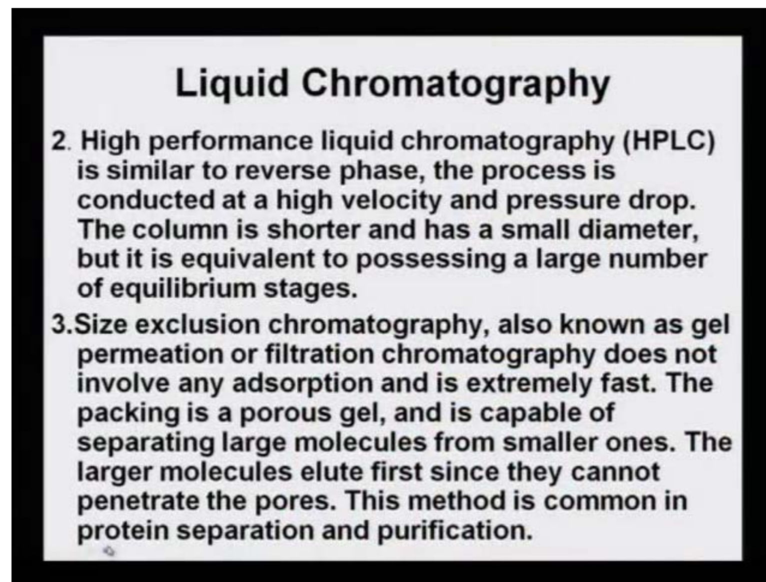

High performance liquid chromatography, that is, HPLC - it is also called high pressure liquid chromatography, where the name still remains HPLC, is similar to reverse phase. However, the process is conducted at a higher velocity and pressure drops. The column is shorter and has a small diameter, but it is equivalent to possessing a large number of equilibrium stages.

Size exclusion chromatography, also known as gel permeation or filtration chromatography does not involve any process of adsorption, and is extremely fast. It is almost like sieving a material. The packing is a porous gel, and is capable of separating large molecules from small ones. The larger molecules elute first since they cannot penetrate the pores. This method is common in protein separation and purification. So, repeatedly, I am telling you that different material require different chromatography technique based on the fact that what is the kind of chromatographic mechanism is used, the chromatographic sorbent is chosen.

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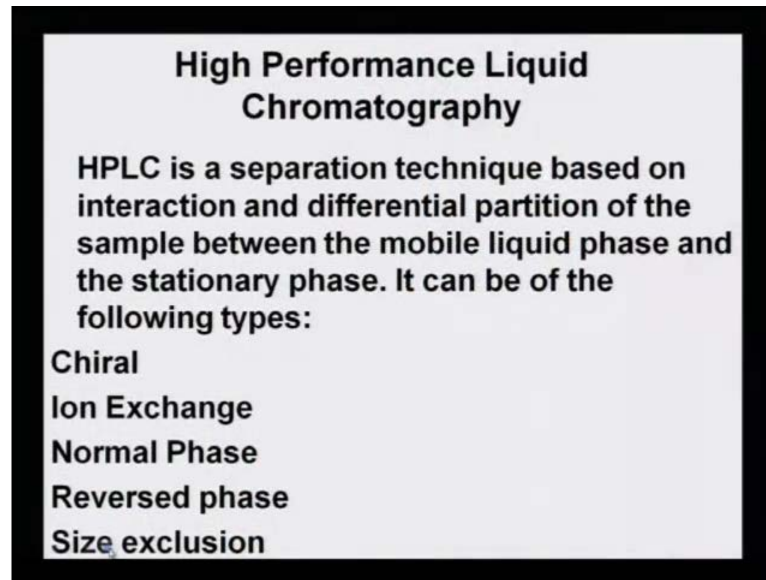

High performance liquid chromatography - HPLC is a separation technique based on interaction and differential partitioning of the sample between the mobile liquid phase and stationary phase. It can be of the following types: it can be chiral, ion exchange, normal phase, reversed phase, size exclusion. So, all these will come under the big category of HPLC.

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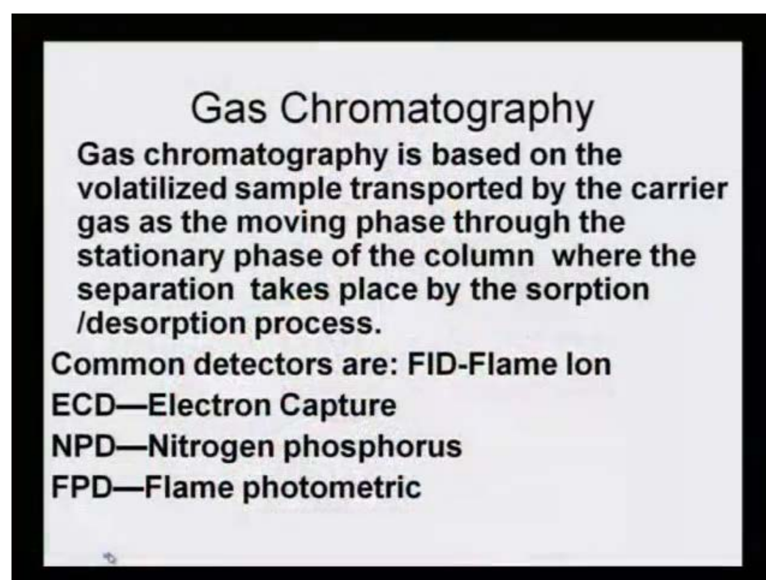

Gas chromatography however, has its own specifications. Gas chromatography is based on the volatilized sample transported by the carrier gas as the moving phase through the

stationary phase of the column where the separation takes place by the sorption and desorption processes. They have different detectors, because in the case of separation, all the compounds are colourless, then how to find out whether the separation has actually taken place. So, there needs to be a detector, and different gas chromatographies have different types of detectors. The common detectors are FID or flame ion detector, ECD - electron capture detector, NPD - nitrogen phosphorous detector, FPD - flame photometric detector and many more.

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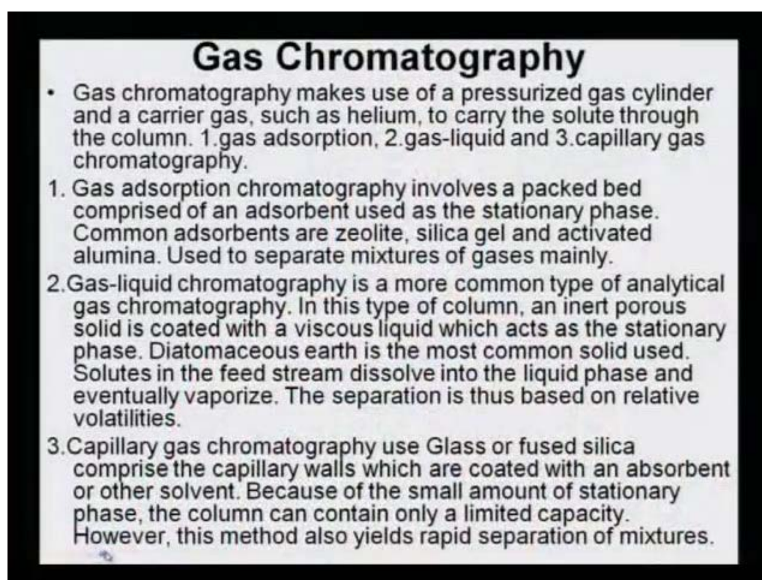

Gas chromatography makes use of a pressurized gas cylinder and a carrier gas. The mobile phase here is carrier gas, such as helium, to carry the solute through the column. Why helium? Because helium does not interact with any of the analyte components. It has three different types of gas chromatographic adsorption: one is gas adsorption, one is gas-liquid adsorption and the third one is capillary gas chromatography.

Gas adsorption chromatography involves a packed bed comprised of adsorbent used as a stationary phase. Common adsorbents here used are zeolite, silica gel and activated alumina, and it is used to separate mixtures of gaseous material mainly. As the name suggests, gas is always related to compounds, which can volatilize or which have low boiling point.

Gas-liquid chromatography is a more common type of analytical gas chromatography. In this type of column, an inert porous solid is coated with a viscous liquid, which acts as a

stationary phase. Diatomaceous earth is one of the most common solid used. Solutes in the feed stream dissolve into the liquid phase and eventually vaporize. The separation is thus based on relative volatilities or boiling points we can say.

Capillary gas chromatography uses glass or fused silica comprising of the capillary walls, which are coated with an adsorbent or some solvent. Because of the small amount of stationary phase, the column can contain only a limited capacity. However, this method yields rapid separation of mixtures. And the capillary column is actually 50 meters long, but it is not kept lengthwise as 50 meters, it is wound up and so, it appears as a small column, but it is in big circles.

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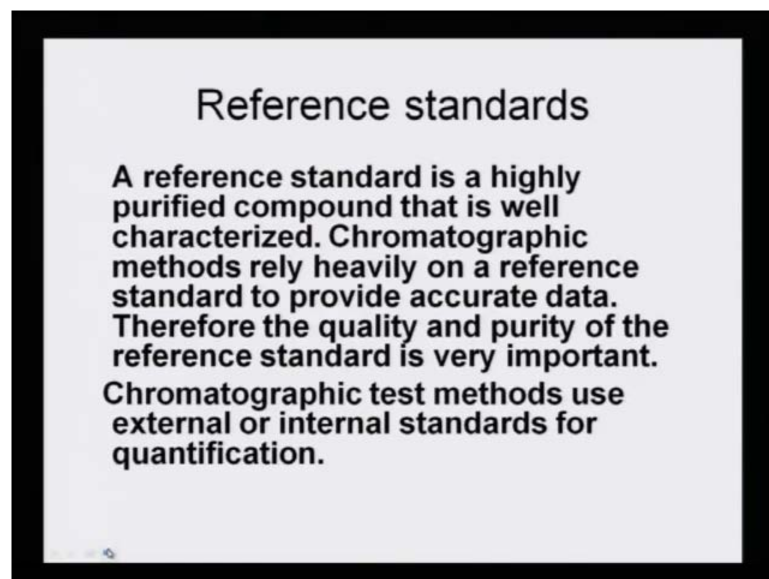

Reference standard - now, these are all calibration methods. We would need a reference point to identify whether the analyte has that same component, and that is called reference standard. A reference standard is a highly purified compound that is well characterized. Chromatographic methods rely heavily on a reference standard to provide accurate data. Therefore, the quality and purity of the reference standard is very important. Chromatographic test methods use external and internal standards for quantification.

What is the role of reference standard? Reference standard shows that if 5 ppm of a compound A shows a peak height of say 10 centimetres, then the analyte if it shows at the same retention time, a peak of 5 centimetres, it is having 2.5 ppm of that particular A

component. That is what is meant. We need to tally, we need to identify, and we need to calibrate with the help of reference standard. That is the role of reference standard.

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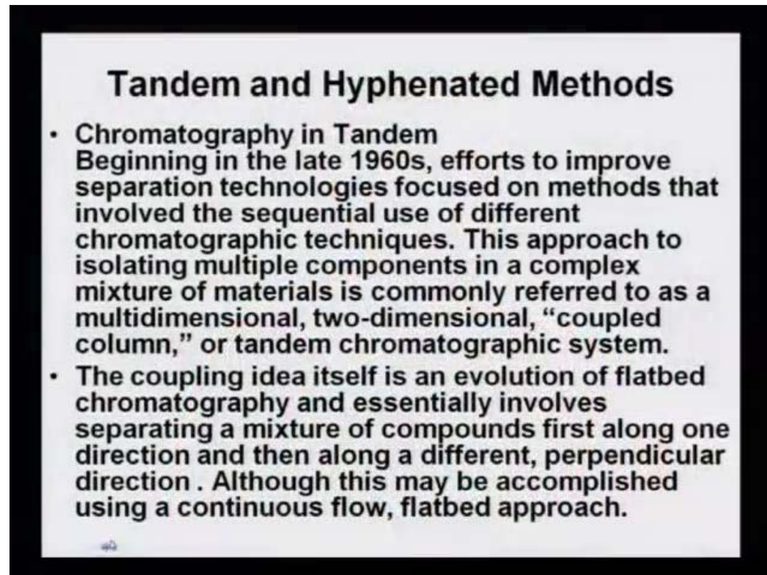

Tandem and hyphenated methods - chromatography in tandem - beginning in the late 1960s - it is very recent that these chromatographic techniques are hyphenated; efforts to improve separation technologies focused on methods that involved the sequential use of different chromatographic techniques. And that is why there is a need to couple.

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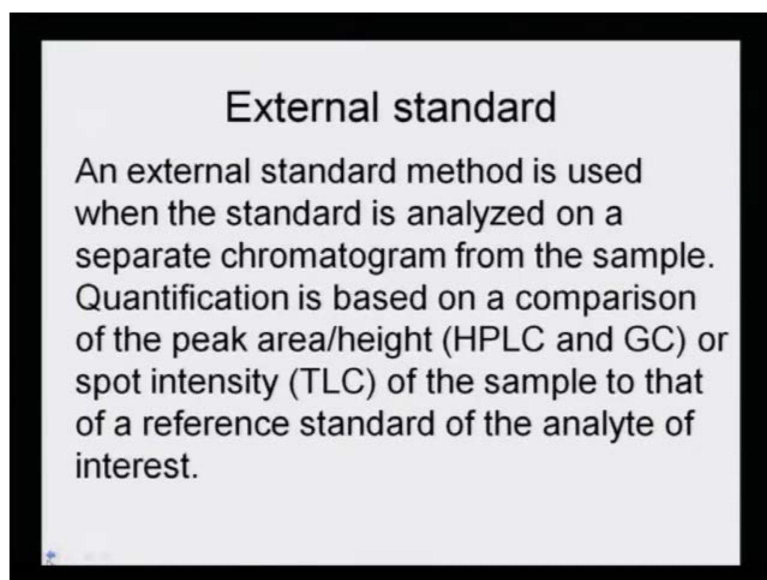

The reference standard that I was mentioning - now, it can either be used as an external or as an internal standard. An external standard method is used when the standard is analyzed on a separate chromatogram from the sample. Quantitation is done based on the comparison of the peak height and the peak area both in the case of HPLC and GC. And similarly, we have the role of external standard in the spot intensity of the TLC of the sample, so that it can act as a reference standard.

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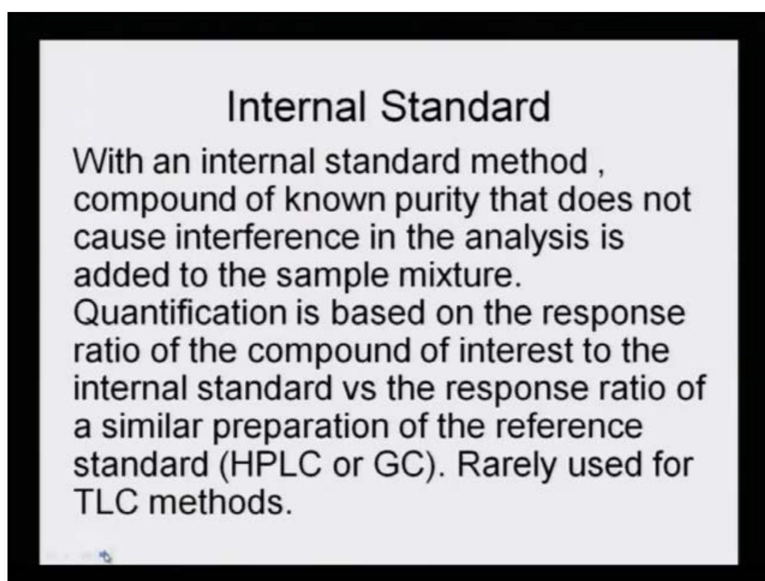

Internal standard - when an internal standard method is used, compound of known purity that does not cause interference in the analysis is added to the sample mixture. Quantitation is based on the response ratio of the compound of interest to the internal standard versus the response ratio of a similar preparation of the reference standard, and this is rarely used in the TLC method. It is more applicable in HPLC and GC. That means if the analyte is in trace quantity, we kind of magnify it by adding into the material, and the amount that is added into the material is already known to us and quantified.

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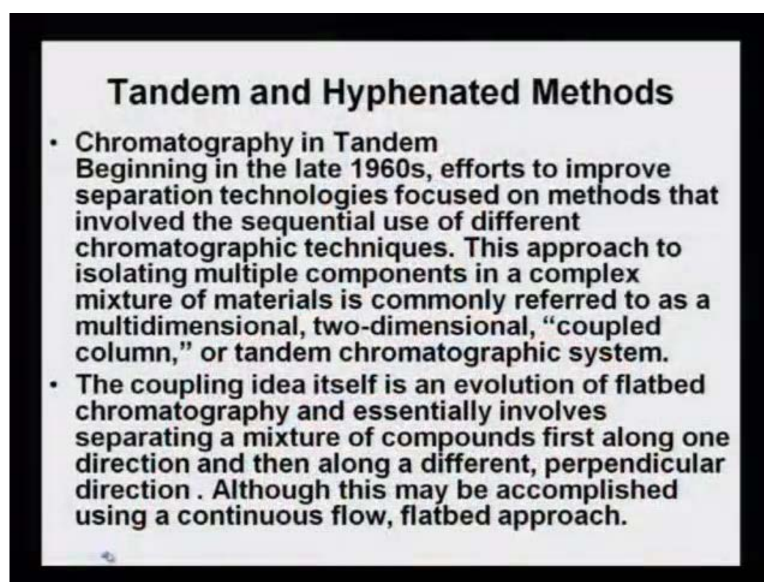

Tandem and hyphenated methods that began in 1960s are the more recent types of chromatographic techniques. This approach to isolated multiple components in a mixture of material is commonly referred as multidimensional, two-dimensional and coupled column, or tandem chromatographic techniques. So, these are different terminologies, which I need to bring to your notice, and that is why I have told you these specific terminologies.

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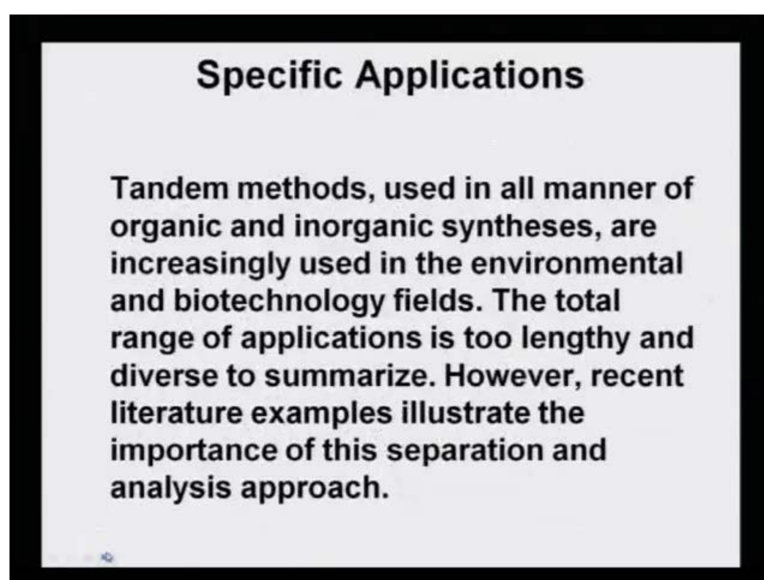

Specific applications - specific applications of tandem methods are only when we are applying it through organic or inorganic syntheses that are increasingly used in the environmental and biotechnology fields. The total range of application is too lengthy and diverse to summarize. However, I am just bringing to your notice that tandem methods are made into use as and when they are required. However, recent literature example illustrates that importance of this separation and analysis approach is very wide.