Analytical Technologies in Biotechnology
Prof. Dr. Ashwani K. Sharma
Department of Biotechnology
Indian Institute of Technology, Roorkee

Module - 3
Chromatographic Method
Lecture - 1
Introduction and Basic Concepts in Chromatography

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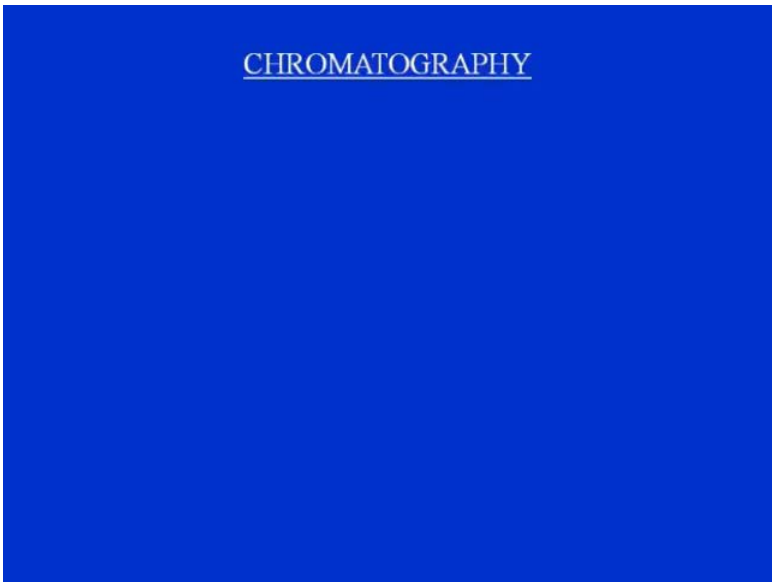

In coming lectures, we are going to start a new topic chromatography. Now, in chromatography, we are going to discuss about basic concepts of chromatography as well as some techniques particularly the techniques of column chromatography. Now, as we all know that there are lots of bio chemical processes, which are going on inside the cell. Now, to understand the bio chemical processes it is very important to understand the function and physical and chemical properties of the constituents or the active participants of these bio chemical process.

And to understand their function or their physical or chemical nature, it is important to get these constituents in pure form. So, that we can understand the particular function of an analyte or a particular molecule inside a cell. So, if we analyze our understanding of bio chemical processes have gone in parallel with our ability to isolate, and purify biological molecules which includes macro molecules such as proteins and nucleic acids and also lot of other biological molecules.

Now, most bio chemical investigation requires that material may be purified so that that is free of contaminants and this biological material then can be utilized for characterization in terms of its physical and chemical nature. A typical cell contains thousands of different kinds of molecules and many of them will be closely resembling in physical and chemical properties. So, it becomes a challenging task to obtain a homogenous solution of a particular substance. So, if you see many times these molecules of interest which needs to be investigated they adjust in very small amount that it is they might be like 0.1or may be less than of the total cell content.

And another problem which comes in is that they may be unstable actually. So, when a substance is present in a very small amount and this has to be brought to purity level of almost above or around ninety eight percent. And you have to have a substantial quantity for analysis or characterization. So, the task of purifying biological molecules is quite challenging. And in modern times it relies heavily on chromatographic methods.

Now, these chromatographic methods started with paper chromatography. So, these include paper chromatography then thin layer chromatography came. And in now a days column chromatography is widely used. Now, column chromatography particularly is the most prevalent method in bio chemistry for purification and also in certain like clinical applications and other areas of bio technology.

It is a continuous separation process where separated components can be collected into separate fractions for analysis. Now, high performance liquid chromatography has been revolutionized the technique where a high degree of purity could be achieved. So, column chromatography is one where we can use it for getting a good amount of pure compound to bit be analyzed. Now, let us introduce little chromatography and then get into bit of history of chromatography. Now, chromatography it comes from Greek where chroma means color and graphy means to write, so it is like color writing. So, it is usually introduced as a technique for separating and identifying or we can say or identifying the components of a mixture.

Now, chromatography literally meaning color writing was first employed by Russian scientist Michael Sweat in 1900 and he continued to work with chromatography in the first decade of 20^{th} century. And primarily for the separation of plant pigment such as chlorophyll, carotenes, xanthophylls, and so on. So, that is how the chromatography

started. Chromatographic techniques then developed substantially as the result of the work of two scientist that is Martin and Cinch during the 1940's and 1950's. They established the principles and basic techniques of partition chromatography and their work encouraged the rapid development of several chromatography methods.

Some of them we are going to discuss in this section. Now, the basic principle of chromatography is that the components in a mixture have different tendencies to adsorb in a surface or dissolve in a solvent. Now, in all forms of chromatography methods there will be a stationary phase. Now, this stationary phase could be solid gel or liquid. And this is immobilized then there will be a mobile phase that is it could be liquid or it could be gaseous and it will. And this mobile phase flows over or through the stationary force phase.

So, what you have essentially is an immobilized stationary phase and a mobile or moving mobile phase which moves through the stationary phase. Now, separation of various components of a mixture is based on differential partitioning or we can say distribution behavior between these two phases that is stationary phase and mobile phase. So, depends on how a particular component divides or distributes itself into the two phases. So, chromatography may be preparative or analytical.

Now, the purpose of preparative chromoto chromatography is certainly to separate the component and get the substantial amount in the form of purification. And in analytical chromatography is done normally with a smaller amount of material and is for measuring the relative proportions of analyte in a mixture. And two are both are used extensively and they are not mutually exclusive. Now, let us get into little bit the general principle of chromatography the basis of all forms of chromatography is partition or distribution coefficient as we were talking about.

And it is represented by kd that is distribution coefficient. Now, this distribution coefficient describes the way in which a compound distributes or partition itself in between two immiscible phases. Now, the various components of a mixtures will get separated due to varying affinities or we can say distribution behavior of those components for the stationary phase and the mobile phase. So, if we consider two immiscible phases a and b that could be stationary or mobile phase.

Now, value for distribution co efficient will be a constant at a given temperature and is given by the expression if you see on your screen that is the concentration in phase a and divided by concentration in phase b equals the distribution co efficient or kd value. So, this will give you a sense of how a particular analyte distributes itself between the two immiscible phases. Now, there is another term which is known as effective distribution coefficient and it is defined as the total amount rather than the concentrations of compounds present in one phase divided by the present in a phase.

Now this will be like so here the concentration of one compound in one phase divided by the total amount present in other phase. So, it is the distribution co efficient multiplied by the ratio of the volumes of the two phases present. So, what that does that mean rather that you are not talking about the concentration as such in effective distribution co efficient the total amount is being taken.

So, the distribution co efficient of a compound say for example, between the two phases is 1, that is it is equally distributed then, but if you have volumes between 5 ml and 1 ml then the concentration in two phases will be the same, but the total amount present in the two phases will be different that is one phase will contain 5 times more than the other phase. So, these two terms kind of one signifies distribution in terms of concentration. And another signifies distribution in terms of amount of substance present. Now, there could be different combinations of stationary and mobile phases.

They could be selected on the basis of a particular compound to be separated and it should be selected so that if you are taking an analyte it could separated. And it has a varying distribution co efficient from other analyte, so different combinations could be and we will be discussing these in detail as we go along. And also we are going to discuss certain techniques here particular techniques in column chromatography based on these combinations. So, one combination could be based on equilibrium or partition equilibration.

Here, in partition equilibrium occurs between a stationary liquid phase and a mobile liquid phase or a gas phase. In this case like for example, there could be partition chromatography there could be reversed phase chromatography there could be gas liquid chromatography. These are the examples of partition chromatography. There could be a

absorption equilibrium, so an absorption equilibrium occurs between a stationary solid and a liquid mobile phase.

An example of these particular type of these chromatography is absorption chromatography then there could be ion exchange equilibration. In an ion exchange equilibrium it occurs between a stationary ion exchanger and a mobile electrolyte phase. So, the typical example of this is ion exchange chromatography, then an equilibrium could occur between a liquid phase trapped inside the pores of a stationary porous structure and a mobile liquid phase.

And typical example is exclusion chromatography or gel chromatography. Another combination could be equilibration between a stationary immobilize ligand and the mobile liquid phase. So, we are what has been done is a ligand is immobilized on to the stationary phase or it acts as a stationary phase and the mobile liquid phase passes on through it. And typical examples of this type of chromatographic techniques include affinity chromatography techniques different types of affinity chromatographic techniques. So, you see there could be lot of different combinations of stationary and mobile phase and these will be discussed in detail.

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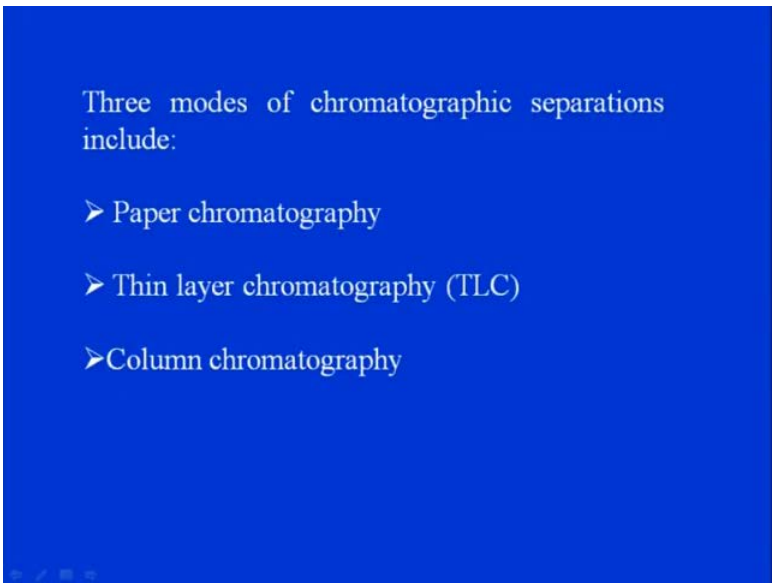

Now, there are three modes or rather we can say usually two modes of chromatographic separations. Now, here three modes where paper chromatography could be one mode it has been kind of replaced by thin layer chromatography. Paper chromatography and thin

layer chromatography comes under plainer chromatography as the bed you can say the chromatographic material or bed where this separation is present on a plainer surface. So, paper chromatography and thin layer chromatography bed is present on a plainer surface. As far as column chromatography is concerned the column chromatography the stationary phase is packed in a tube or in a column. So, that is why it is called a column chromatography. So, let us little bit discuss about paper chromatography and thin layer chromatography. And then we will move on to column chromatography in particular.

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Paper chromatography is a analytical technique essentially and it is utilized for separation and identification of mixtures that may or may not be colored like earlier it started with color like we were discussing about pigments from the plants. And other colored substances, but you can also use substances which are not colored, but could be stained by or could be analyzed by different methods.

Now, in this technique stationary phase is supported by the cellulose fibers of a paper sheet and hydrated cellulose fibers or we can say absorbed water in fibers acts a stationary phase. The mobile phase relatively less polar solvent system will pass along the paper sheet either by gravity feet or by capillary action. So, if you see here that stationary phase is a paper which is cellulose fiber of a paper sheet. And this cellulose fibers could be watt man paper there could be modifications.

In this paper where you can change its property in terms of ionic imparting ionic properties or other properties to the paper could be an option. Likewise the water in fibers acts as a particularly acts as a stationary phase here. So, you can say that hydrated cellulose fibers acts as a stationary phase. Now, mobile phase which is relatively less polar as compared water will pass along the paper sheet and will carry the mixture of substances which are present on the paper. So, what is done is the mixture of substances distributed between stationary and mobile phase, as per their affinities for two phases.

So, substance having higher affinity for mobile phase will certainly move rapidly as compared to those having higher affinity for hydrophilic stationary phase. So, those compounds which are relatively non polar will move in the mobile phase, but those which are not will be sticking to the stationary phase. So, this is how the separation in paper chromatography will take place.

Now, here the sample or more than one sample could be placed on to the paper as a small spot or line of sample solution on a chromatography paper. I will show you in a little while the whole procedure here. Now, ones these spots are placed spots, one has to be careful that how much sample is being put you should not put too less or too high amount of sample. And it should be on a clear spot should be placed on to the paper if more than one sample is there they are in a pro in a single line.

Once you have spotted and dried the paper then it will be placed in a jar containing a shallow layer of mobile phase. Remember spots should not be dipping in the mobile phase they should be above the mobile phase. And the jar is sealed actually, as the mobile phase rises above the stationary phase and encounters sample mixture it carries sample with it. Now, the different components of the sample will travel different distances according to how strongly they absorb on to the stationary phase as compared to dissolve in the mobile phase as we have discussed earlier.

Now, the compounds within the mixture will travel farther if they are non polar as compared to polar components which will interact with hydrophobic stationary phase or surface. So, this is how a chromatogram will run as far as paper chromatography is concerned. Once it has been run, you can take it out and then detection of the resolved components has to be performed. So, the detection of the resolved components may to be

performed either visually if they are colored or by physical and chemical detection for colorless compounds.

So, the detection methods may include that could be staining with certain dyes or other reagents it could be auto radiography if you are utilizing radio isotopes, it could be fluorescents or other physical methods could be utilized. Now, after detection the resolved compounds may be identified based on their retardation or retention factor that is Rf value. Rf value is defined as the ratio of the distance travelled by the analyte to the distance travelled by the solvent front and from a definite point actually.

Rf value will be affected by type of mobile phase that is solvent like say few are utilizing different types of solvent certainly Rf value will change the temperature and the different temperatures you will have different Rf values, nature of the sample and paper will also affect Rf value. Now, let us little bit get into like let me show you on your screen the whole procedure here. Now, in paper chromatography what is done is like we were discussing first thing is we have to take the proper paper that is the cellulose paper it could be watt man paper.

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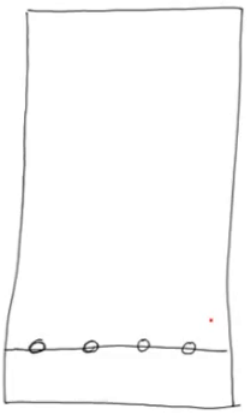

Now, this paper now this paper like I said first thing is you have to spot your samples on this paper. This paper could be simple paper or it could be derivatized for other applications so what you can do is a very thin line could be drawn or you can do it visually. And here you have to prep spot your samples and these spots have to be

appropriate or accurate. Now, spots more than one spot could be placed on line here on one side of the paper. So, once you have spotted this then this could be dried and the paper could be put in a jar like I said jar containing a shallow a jar containing a shallow mobile phase.

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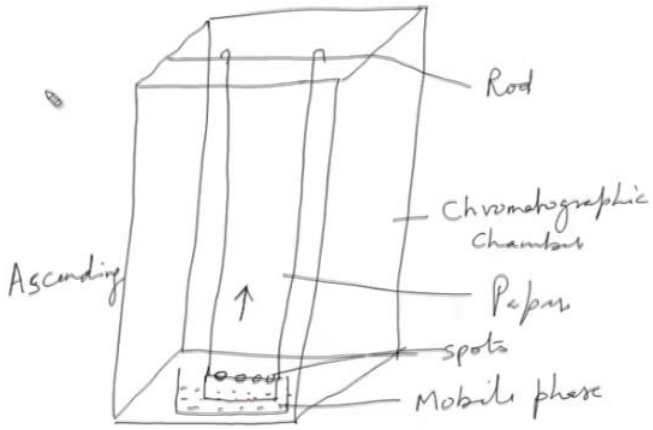

Now, let us see what does that mean. So, what you have is that you have particular kind of container or a jar. It is a very simple way to make a container, so you have a container here and this container what you have to have a paper here if I can have a rod to support the paper this is a rod to support the paper. Now, what is done is you have a paper which is hanging sort of or it is supported paper which can remain straight in here. The main aim to put a rod and this jar has to be sealed completely, so the main aim to have a rod or a support system is to keep the paper straight.

Now, this paper here as you can see as there will be spots in here as we have spotted there and they will be a mobile phase here. Now, remember the spots are not submerged here in this one so what you have is you have this is chromatographic chamber. This is chromatographic chamber then this is chromatographic paper which is you are utilizing here, this is mobile phase which could be here. Now, a mobile phase mostly is like I said is relatively non polar as compared to water.

So, this one contains what you call it could be say organic solvent water combination and other additives could be utilized like say it could be phenol water, it could contain

methanol lot of different types of combinations are available. So, what you have is that once you have placed this then automatically there will be and this is your spots here which are many spots are being put in here. So, once you have placed this and this remember the spots are not dipping in here the water will move or the mobile phase will move up. So, this is called ascending order or you can say it is being run in ascending fashion there could be another way that is it could be the paper could be upside down.

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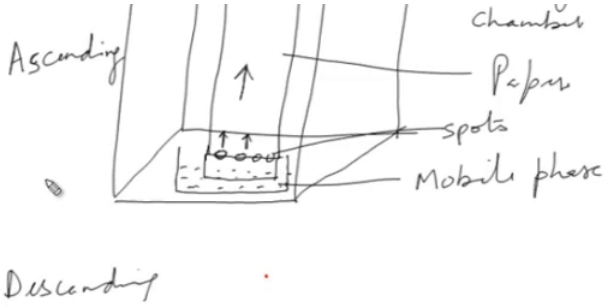

Descending

That will be called descending manner where it could run that particular chromatogram also paper chromatogram also. So, once these is has run these spots or the samples will be carried up in here as per the affinities for the mobile or stationary phase. And what you are going to have finally, you will be developing a chromatogram. So, finally, what we will have a paper which could be taken out from a jar.

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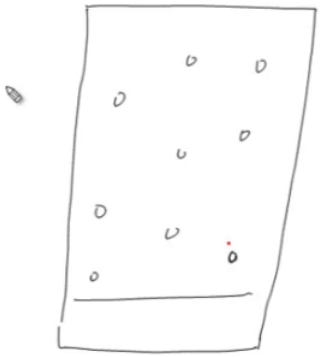

And what you will see is you will see various spots in here as per how many spots you have loaded. And these spots could be then either if they are colored you can see them visually or if they are not colored you can see them while staining them with different agents or say like for example, in hydrogen is neutralized to see amino acids. It could be lot of other different re agents or you can use fluorescence or auto radiography to see this. So, this is about a little bit about the paper chromatography. Now, let us move continue our discussion here. So, hope you have been able to understand the simple methodology of paper chromatography, here the next one that plainer chromatography system is thin layer chromatography. Now, thin layer chromatography this technique has almost replaced the paper chromatography with the advantages of speed that is it could be run fast better separations due to high resolving powers.

So, you have a much higher resolution here it is much faster and you can have a choice between different kinds of stationary phases which could be used. Now, this could be used for both analytical as well as preparative purpose. So, thin layer chromatography could be both analytical as well as preparative. Now, as we have discussed in paper chromatography lot of things are same so we are not going to repeat them, but a few things about thin layer chromatography. Now, the stationary phase or the absorbent material is mostly like silica gel or aluminum oxide or cellulose paper or and other agents are also other absorbents materials are also utilized.

So, they will be coated thinly on to a glass or a plastic or a metal foil plate and the mobile liquid phase will pass across the thin layer plate held either horizontally or vertically by capillary action. Now, the process is similar to paper chromatography. Now, the these plates are thin layer plates as we say how these are prepared. So, for preparation of these plates layers of powdered solid in the form of slurry can be applied to a clean glass plate with a binder. And then they could be dried and activated by heating now tlc plates or thin layer chromatography plates are commercially available with standard particle size ranges for reproducibility.

You have to have standard particle size so that you can reproduce your result. They are prepared by mixing adsorbents say silica gel or alumina with a small amount of inert binding agent for example, calcium sulphate or gypsum could be used with water. The mixture is spread as a thick slurry on an inert carrier sheet and then, like I said it could be glass alumina or plastic then plate is dried and activated by heating in an oven at say 100 to 120 degree celsius particularly. Mostly 110 degree celsius for 30 minutes.

Now, plates of uniform thickness are opted here for analytical purpose the plates of 0.1 to 0.5 millimeter. And for preparative purpose 0.5 to 2 millimeter plates could be prepared. So, like I said it could commercially you can obtain them without any problem as per your requirement you can use them. Now, tlc can be used for lot of different application like paper chromatography and tlc almost have a similar applications.

Like in paper chromatography it could be used for analysis of lot of different samples right from nucleio tides, amino acids peptides, drugs, pesticides. And in cosmetic industry food industry, whole lot of things whole lot of areas it could be utilized likewise tlc can also be utilized for lot of different applications. Now, before we go into the applications I will just like to show you how it is done. Like I said it is almost similar to what you call it is almost similar to paper chromatography.

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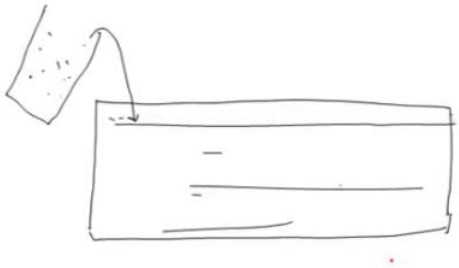

Now, here other than a paper what it is there is a glass or say certain plate which could be plastic or thin foil. Now, on this thin foil you will have say a slurry which is prepared in here and this will be poured on this glass plate and spread evenly which will be put in whole place. And it is like this slurry here acts as if it has certain thickness. And this slurry will be utilized or will be acting as the stationary phase, which could be like as silica gel or alumina gel or others. So, all other things are as are as the paper chromatography except for the stationary material, but it like I said it has lot of advantages over paper chromatography.

So, tlc could be used for monitoring the progress of reactions for example, it could be used for monitoring of organic chemistry reactions. Say at different stages the compounds which are made could be identified by their Rf values. Then identifying the compounds in a mixture or to assist the purity of the compound that is also very important application of both paper and thin layer chromatography where you have a pure compound and you can assist the purity of that compound. You can use it for separating lot of different kinds of substances compounds.

For example, you can use it to separate liquids steroids, alkaloids, sugars, phenols, nucleotides, amino acids and peptides. And many others actually it could be used for detection of pesticides and insecticides in food and water, it could be utilized for identification of constituents of a medicinal plant, it could be utilized in for scenic

science, it could be utilized to assist the radio chemical property of radio pharmaceuticals and so on.

So, there are a whole lot of different applications of thin layer chromatography. Now, practical advantage of thin layer chromatography or paper chromatography over column chromatography is that large number of samples can be studied simultaneously here. Rather than in column chromatography, you can use at a time one mixture one sample mixture could be analyzed. So, we have discussed about two plainer chromatography techniques one paper another is thin layer chromatography.

Now let us discuss about column chromatography and which is the main topic here. Now, in column chromatography this is the separation technique in which stationary phase or stationary phase on a suitable supported matrix is packed in a column or a tube made of it could be made of glass, metal or poly propylene that is plastic. Now, the mobile phase passes through the column either by gravity feet or pumping system, as we will discuss in coming lectures. Now, the method has been become the most commonly used mode of chromatography.

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So, let me show you the how column looks like actually. Now, if you see the column the column is a simple tube like column structure which is closed on one side, it will be closed on both sides while running, but to support the matrix there is a filter a disc support with a filter the material will be filled with in this here, which is your matrix the

stationary phase or matrix is put in here. The mobile phase will be put in from the top through gravity or pumping system as I will show you in the coming lectures drops which are which comes out and as per the affinity to the stationary mobile phase analyte will be separated.

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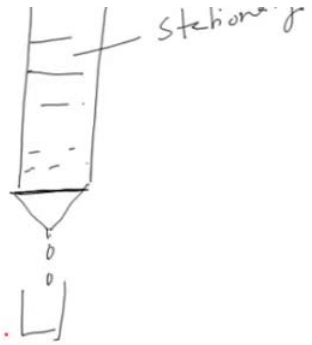

And these fractions can be collected separately. So, this is simple depiction of column chromatography and we are going to see it more as we go along. All right, so let us move on this lecture. So, what we have done is in this lecture what we have seen is you have like different combinations of stationary phase and mobile phase, and there is a distribution co efficient a term known as distribution co efficient, which defines how a particular analyte will separate between the two phases that is a stationary phase and a mobile phase. Now, that depends on the affinity of that particular analyte two phases. And the separation is based the of different analytes in a mixture.

In that they have different affinities for the two phases. There are different modes of chromatography systems and there could be different combinations of stationary. Mobile phase as we have seen absorption equilibration or ion exchange equilibration, or partition equilibration and there are a whole lot of combinations could be there. And the modes of chromatography there are paper chromatography and thin layer chromatography, which are plainer chromatography techniques. Then there is column chromatography where you have stationary phase being packed into a column. Now, before we end this lecture.

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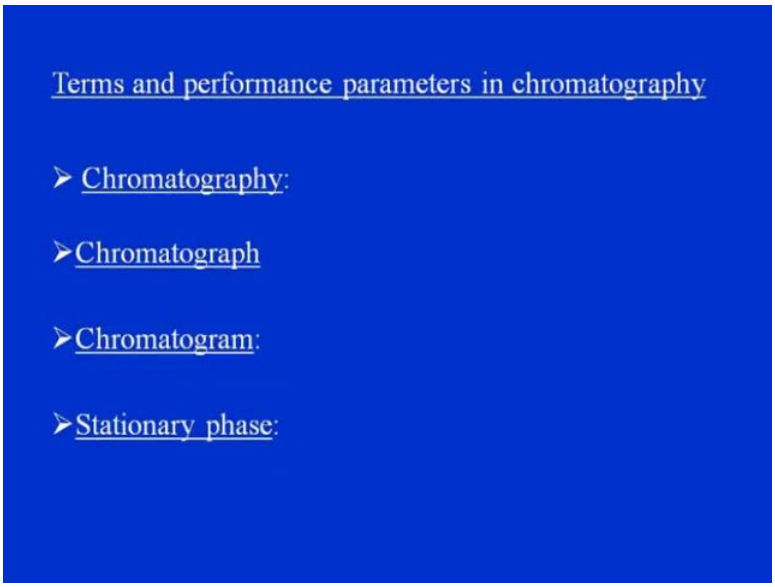

Let us see as we are going to discuss more about column chromatography few terms and will continue to discuss these terms in the next section, but few terms which are important. Let us discuss them here one is chromatography as we are listening this term, what is chromatography means? Here now chromatography is a physical method of separation, where components to be separated are distributed between the two phases that is stationary and mobile phase that is what chromatography means.

And second term is chromatograph, now here if you see chromatograph is an equipment that enables the separation in chromatography. So, chromatography is the technique whereas, chromatograph is the equipment used or the instrument that enables the separation in chromatography and that or it enables to do the chromatography. Now, there is another term called chromatogram. Now, chromatogram is the visual output of the chromatograph in the form of different peaks, or pattern like say when you are separating different analytes then as they come out of the column then they will be coming out in a particular pattern. And these are represented as peaks as will be showing you that in a little while in the next lecture.

So, chromatogram is another important term here, then stationary and mobile phase that is the substance, which is fixed in place for the chromatography method. And it is immobilized on support or stationary phase could also be separate and could be immobilized on a support particle and can be packed it could be solid gel or liquid. Then

there could be mobile phase which is the phase which moves on to the stationary phase or through the stationary phase. And then it carries the sample as per the affinities with it so that the separation could take place.

So, like here you have different ways you can like we said different combinations of stationary and mobile phase. Now, mobile phase and also there are two more terms one is called eluent. Eluent is the carrier portion of the mobile phase and it moves the analyte in the chromatography or chromatograph that is instrument. So, eluent is the solvent that is part of the mobile phase, but it is the carrier portion and elute another term is elute which is the mobile phase leaving the column. And it consists of both the carriers and the analyte material.

So, these two terms are important one is eluent and another is elute elute is which is coming out of the column eluent is the carrier portion of the mobile phase. Then analyte analyte is the substance to be separated during chromatography. So, analyte could be if your are accessing the purity it could be single analyte. Analyte could be present in a mixture or a sample containing lot of simple analytes, which needs to be purified say one analyte could be purified from a mixture or there may be more than one analyte could be purified from the sample. So, these are few terms which are related to the to the chromatography where we have discussed about chromatography, chromatograph, chromatogram stationary phase mobile phase and analyte. So, in the in the next lecture we are going to discuss certain terms which define the performance of chromatography systems. Like for example...

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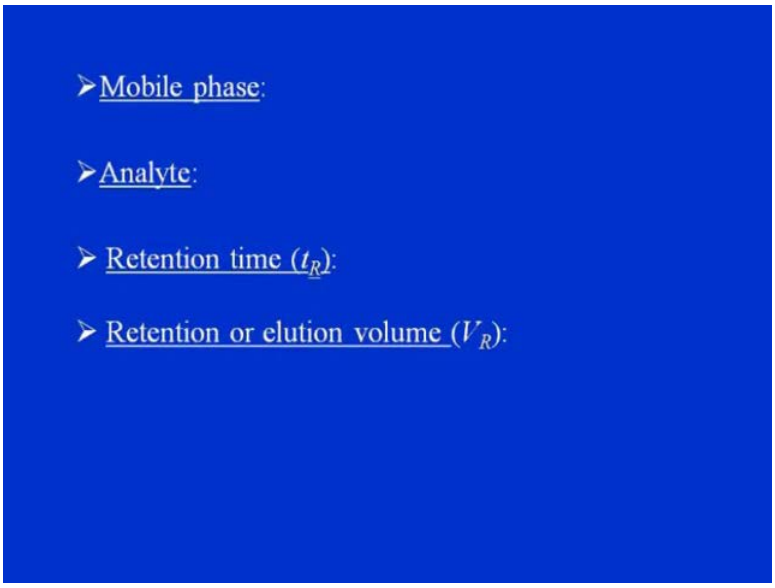

Retention time, retention volume.

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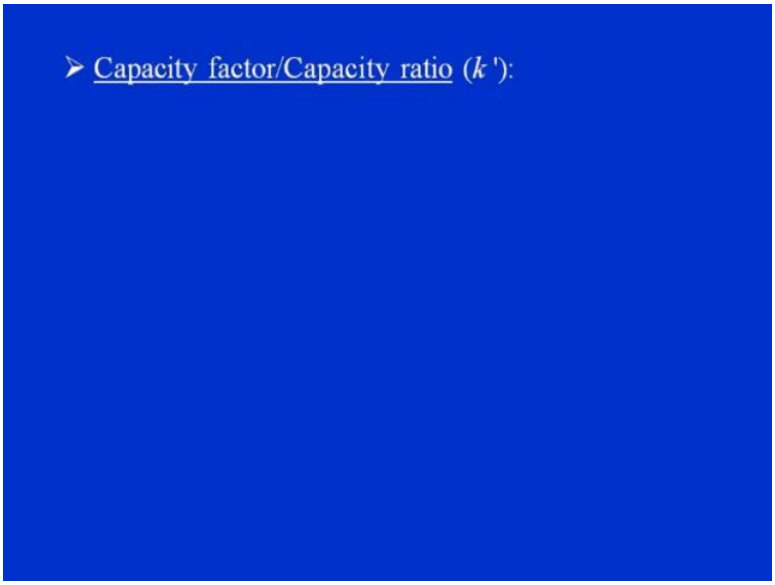

Capacity factors.

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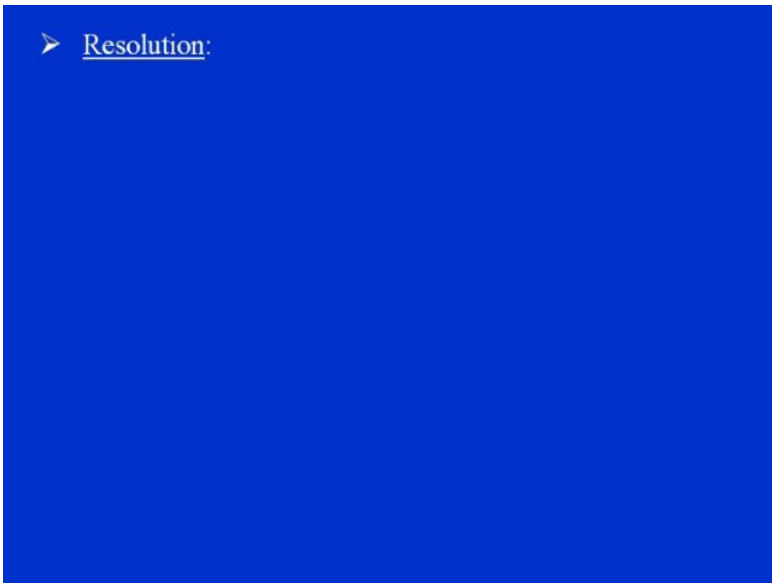

Resolutions and all different kinds of terms we are going to discuss. To summarize this lecture, what we have learnt today is in this lecture is that there is one the chromatography separation occurs due to the varying affinities of a particular analyte in the two immiscible phases. That is phase a and phase b and their distribution is measured by distribution coefficient that if say the distribution coefficient is one. That means they have distributed themselves equally.

Then there is a effective coefficient distribution that is the amount in the two phases rather than the concentration in the two phases. There could be lot of different kinds of combinations and a stationary phase is mostly solid or liquid, whereas, mobile phase is either liquid or gas. There could be different modes of chromatographic systems like paper thin layer and column chromatography. And our discussion in the next lecture is going to be on basic concepts relating to the performance of the chromatographic system and various techniques about the column chromatography.

Thank you.