**Advance Analytical Course**

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**Module No. # 01**

**Lecture No. # 04**

Continuing with what we are doing in previous lectures, the today's lecture deals with chromatographic techniques, more in detail, because we now have to understand each one of them more elaborately. Planar separation: both in plate chromatography as well as thin layer chromatography, separations are halted before the mobile phase has travelled completely across the surface, which means that there is a limiting factor both in terms of the paper. There is a limiting size and in case of TLC there is the limiting size of the plate. Analyte or solute is characterized by the distance it has migrated relative to the solvent front. Solute retardation factor or R f is the distance travel by the solute and the distance moved by the solvent.

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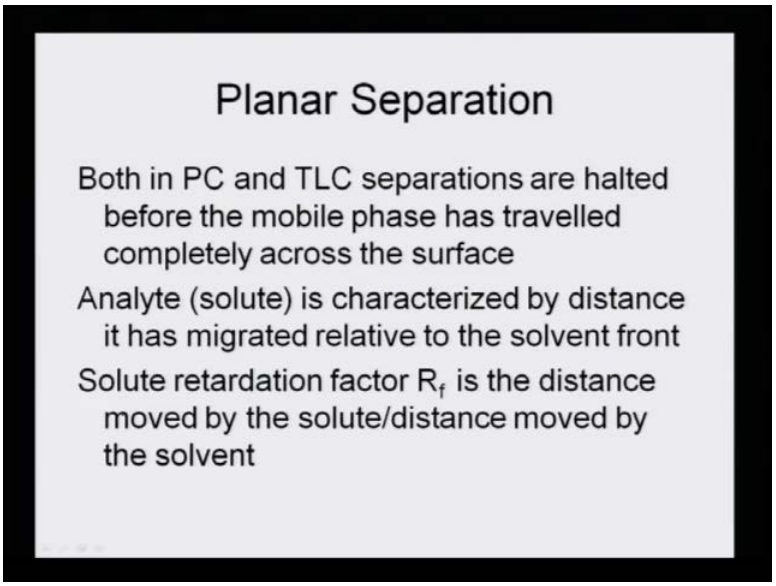

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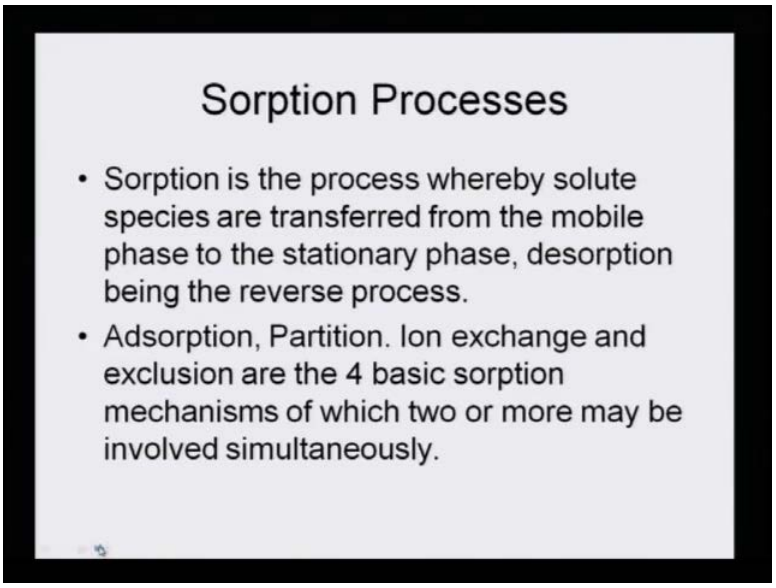


In this whole process, what is the sorption process? The sorption process is the process whereby solute species are transferred from the mobile phase to the stationary phase and then by the more of desorption being the reverse process. Adsorption, partition, ion exchange and exclusion are the 4 basic sorption mechanisms of which 2 or more may be involved simultaneously. So, do not get confused by the different nomenclatures because of the nomenclature, depends either of the mechanism or on its mode of actions.

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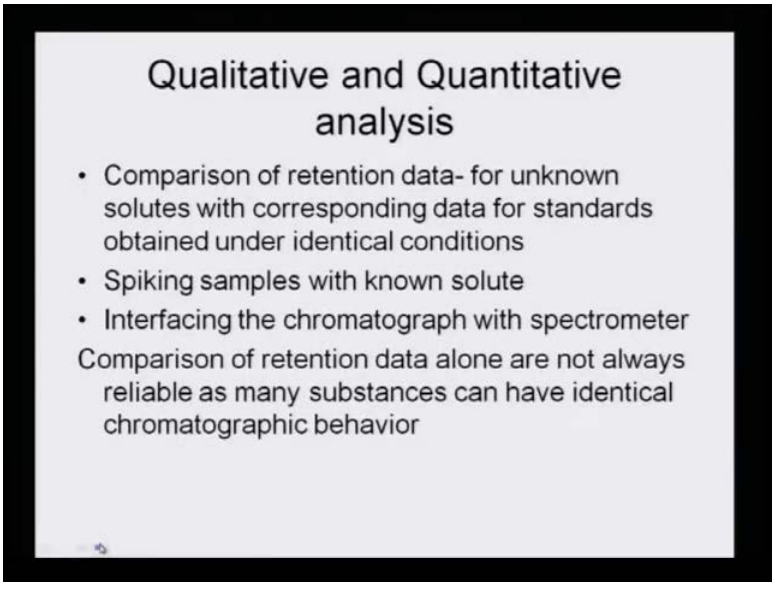

Qualitative and quantitative analysis: how this chromatographic technique helps us in qualitative and quantitative analysis? We will try to learn this in the following few slides. Comparison of retention time for unknown solutes with corresponding data for standards obtained under identical conditions. That means, that we first run a standard - a standard means a compound which has a purity of 99 percentages with respect to the unknown and if the unknown is the same compound then, the retention time will tally under the conditions that they both have been run under the same machine system.

If we alter any parameter, it would change the retention time; so, one has to remember that under identical condition the standard and the unknown must be run in order to make a qualitative analysis. Spiking samples with known solute in case of trace analysis where the peak may be very small. In that case, an internal standard is added to the sample in order to increase the concentration of the compound per say and that spiking is done in a quantitative manner which means that suppose if the analyte is in trace quantity and we add a 5 ppm solution to it a 1 micro liter of that then, it should show a 5 ppm plus a few digits after the decimal. Interfacing the chromatograph with spectrometer: that also helps us in understanding the quantitative analysis of any separation technique. Comparison of retention data alone are not always reliable, as many substances can have identical chromatographic behavior.

Now, when we say that it is the method of calibration or it is the method of validation, it means that we are only making a comparative data between the standard and the sample but, it need not be that all samples which have the same retention time are the same molecules because, they could be molecules coming at the same retention time. So, one has to remember that this is just calibration method and identification method but not a fool-proof method.

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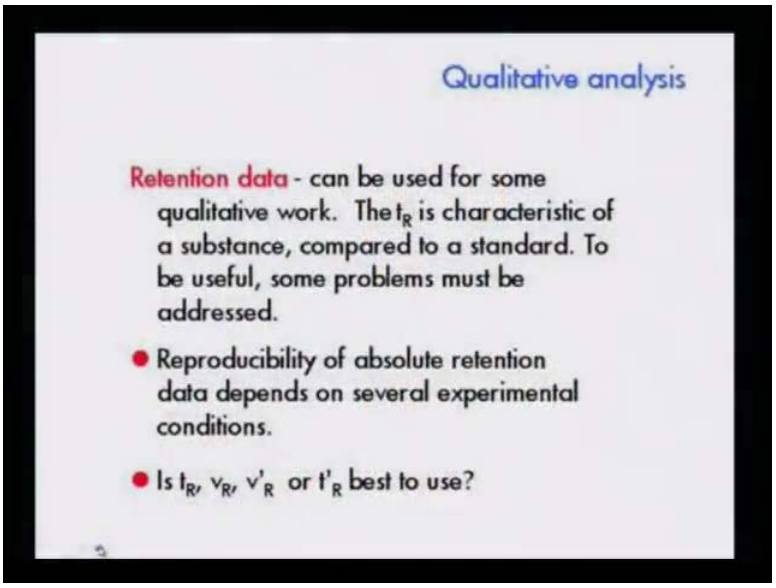


Now, when we make qualitative analysis, retention data is of absolute importance but it is not an absolute value; it is not a fixed value. Retention data can be used for some qualitative work; the retention data is characteristic of a substance compared to a standard. To be useful, some problems must be addressed. I will take a comparative analysis to show you that if the standard will use the retention time 10.2 minutes and the sample also shows a retention time while eluting same operational conditions at 10.2 minutes, which means they may be identical because they are eluting at the same time; so that is how make a comparison between the retention data.

Reproducibility of absolute retention data depends on several experimental conditions. Now, it is also true that under the same operational condition the retention data or the retention time must be the same. A little bit change in the decimal can be from time to time allowed, but not otherwise.

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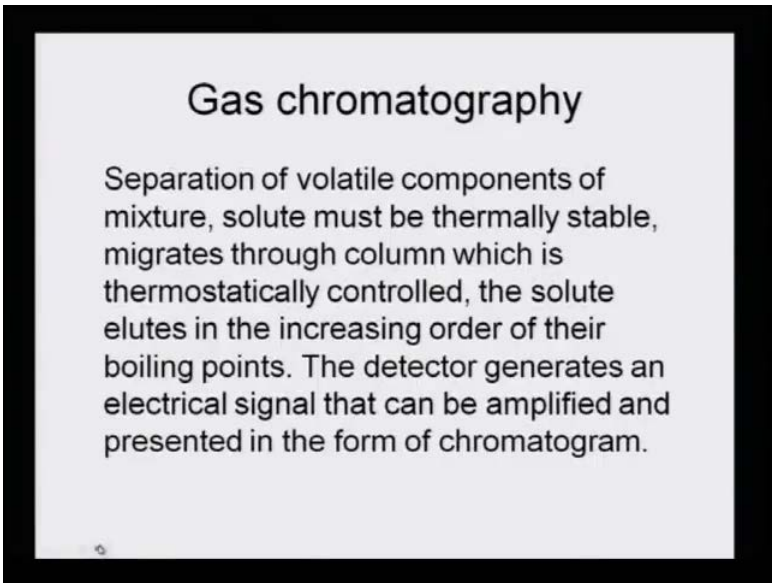


Now, coming to gas chromatography we have just now understood the general phenomena of chromatographic techniques. But,we must also now understand machine by machine; how they work; what is the principle behind it and what are the substances that can be purify by gas chromatography and so on.

Separation of volatile components of mixture, solute must be thermally stable; migrates through column which is thermostatically controlled. The solute elutes in the increasing order of their boiling points. Now, there are coupling thing it needs to be explained here. It is gas chromatography is meant for low boiling, which means volatile compounds and when a mixture of such low boiling compounds are there obviously, there will be difference in their boiling points. Even if the difference is by just 5 degrees, the differences still exists and they will elute according to the rate of their boiling points.

The solute must be thermally stable; that means, that the analyte which is being injected in the gas chromatographic machine must not disintegrate; must not break down; must not decompose; otherwise, what will happen? That molecule which we will get after the separation will not be the same what we have injected. So, that must be kept in mind that the solute must be thermally stable. Migration through the column which is thermostatically controlled that means, there is a temperature control throughout the column length and through the column length the compound should not disintegrate or decompose. The solute then, when it elutes will elute in the increasing order of the

boiling point that means the low boiling will come first and the higher boiling will come later. The detector generates an electrical signal that can be amplified and presented in a form of graph for a chromatogram. Now, the data that is generated from a chromatographic machine is called chromatogram. These terms must be very clear; we are talking in terms of chemistry in terms of analysis.

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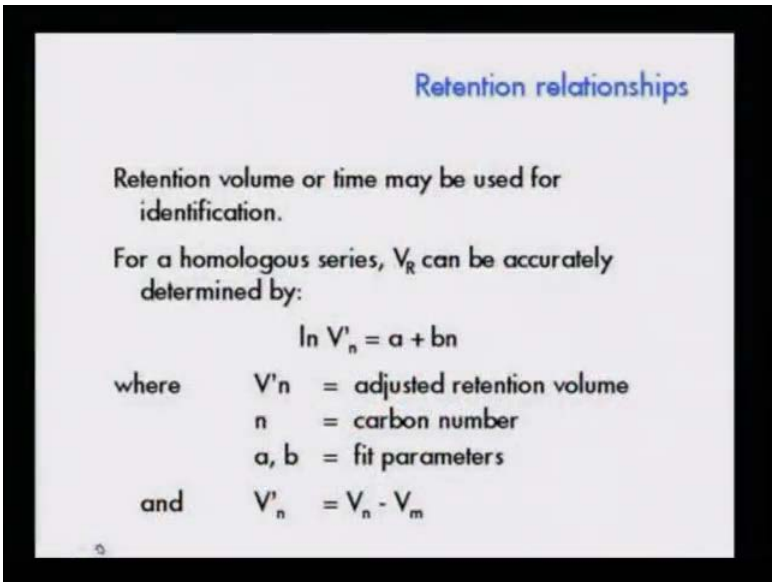


Retention relationship: retention volume or time may be used for identification for a homologous series V R can be accurately determined and thus, we can say that if the peaks are coming one after the other, peaks are actually belonging to its homologous series. That is, they are different by a C H 2 in their molecule structure as what we know as the boiling point of butane, pentane, hexane, is slightly increasing one by one. Similarly, the molecular size is also increasing by a factor of C H 2 and such a series you know we call as homologous series. So, the same way there is a retention time relationship between its appearance on the retention time of the chromatograph; the bigger the molecule the later it will appear on the chromatogram.

Now, when we make an evaluation of any peak in the gas chromatogram, one thing that has to be kept in mind is that the peak height is actually related to the amount of quantities that is present; either we take the peak area or the peak height. But, peak height is more easy to evaluate. In some cases, assume peak height is proportional to

concentration and that is what is made use of it when we trying to do a quantitative analysis through gas chromatograph.

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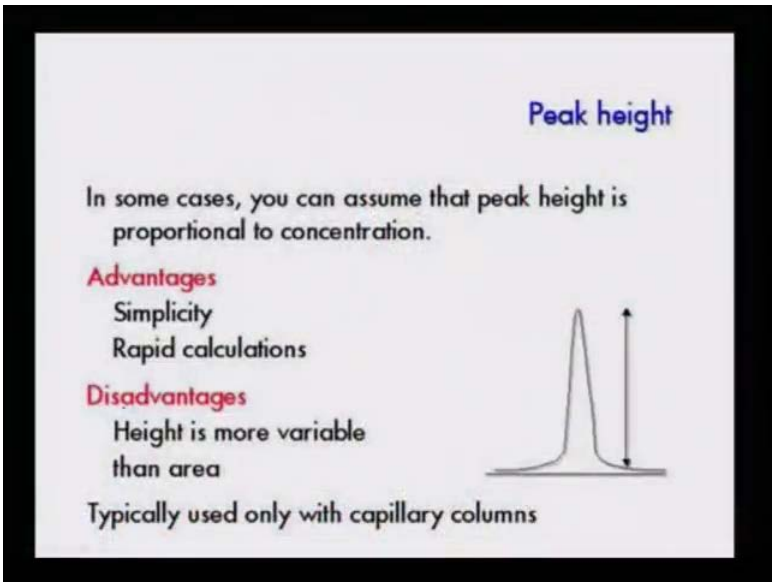


The advantages are that it is very simple to handle; it is very simple to understand and to evaluate and it is very rapid to make calculation because, you just have to measure with the help of the scale what is the peak of the standard and what is the peak height of the sample. But, there are disadvantages also; height is more variable then area. Sometimes, a peak can be broad and they can be short; so they are, there is a deception and they are the result not be accurate. So, that is why sometimes, some people take into account the entire peak area; typically used for capillary column. But, when we are using capillary column, peak height is the one which is more commonly used.

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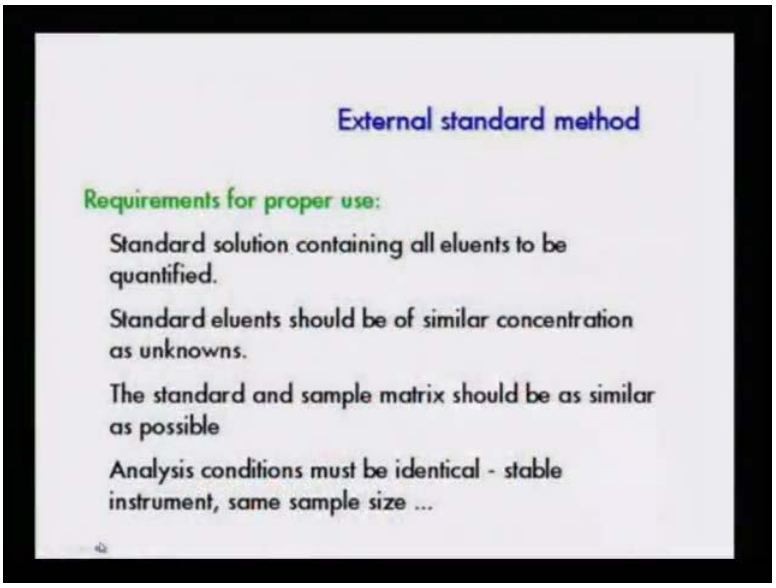


Now, external standard methods I have just explained a while ago; but, I shall repeat again for you to understand because, it is important to understand what is the calibration method. As what I said in a given chromatographic condition, in the gas chromatographic condition, one tries to set certain parameters. Under those parameters the machine is operated and the same parameters is applied to the pure compound which is referred as standard and under the same condition, the sample is analyzed. Now, this kind of method is called external standard method. The standard is first run in the machine and after a while, the sample is run. The requirements of proper use are: that is standard solution containing all eluents to be quantified. The standard must have all those components; the standard eluents should be similar concentration as the unknown.

We have to understand that if, we are expecting the unknown in the range of 5 ppm, we cannot take a standard of 100 ppm; the difference will be too large. So, we have to take in the range of 2 to 10 ppm in order to analyze our unknown which is in the order of 5 ppm. So, that kind of range one must bear in mind because, its analysts should know, should not go beyond the range of the standards.

The standard and sample matrix should be as similar as possible. Analysis condition must be identical; stable instruments, same sample size and only then, we can make a comparison. If the sample sizes are different, if the conditions are different, time - the retention time will be different. So, what will we compare with? We have to keep the

operational conditions same. The sample size is same; that means, we are injecting 1 micro liter in a capillary column standard. We will have to inject one micro liter of the sample in the capillary column to be able to analyze, evaluate and compare.

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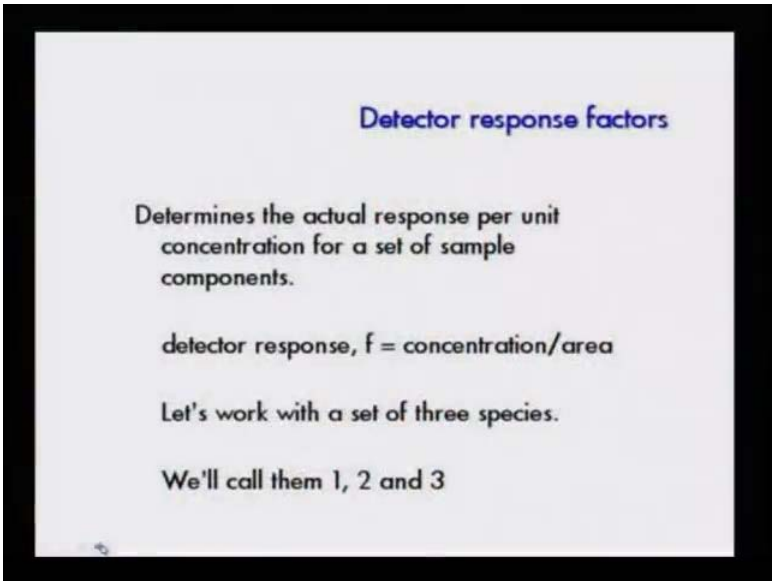


Detector response factors: it is a very important factor. Why? Because, detector is the heart of the machine; that is what actually the diagnosis; whether the compound is present or absent, determines the actual response per unit concentration for a set of sample components and that is why for a machine to be working in a proper condition, it is important to check the detector response which means that concentration over area.

Let us work with the few species; we call them 1, 2, 3 and now, if we have different detector responses, it will never show the same peak height, which means that the detector response is directly related to the reproducibility of the result. That is why it is important from time to time calibrate the machine to validate the machine; in order to get the detector response in the proper manner.

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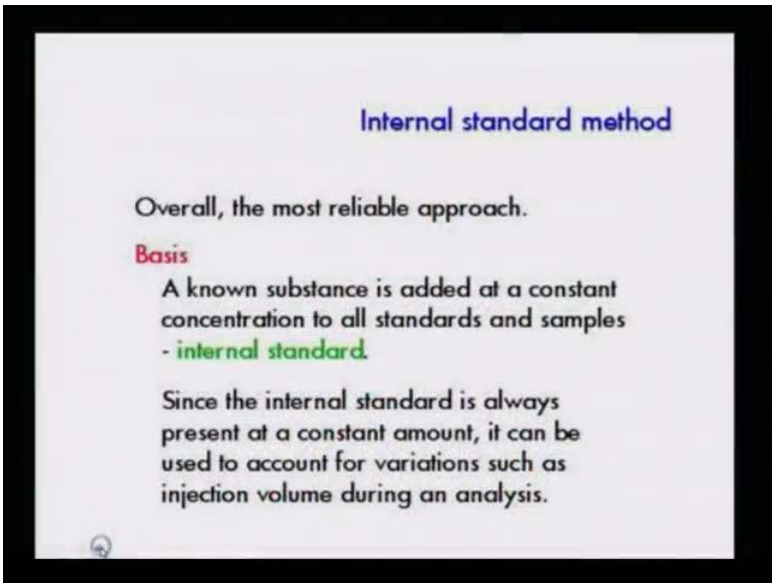


Internal standard method: overall the most reliable approach. The basis is that, a known substance or a standard is added at a constant concentration to all standards and samples and that is why it is called internal standard.

Now, we will carry out only one run; no two runs are required. Not that we are running the standard first and sample first in the sample itself standard but of a known quantity is added and then, it is evaluated 1 time now that extra peak which the standard has made an enhancement is actually the amount which is present in the sample. Since the internal standard is always present at a constant amount it can be used to account for variations such as injection volume during an analysis.

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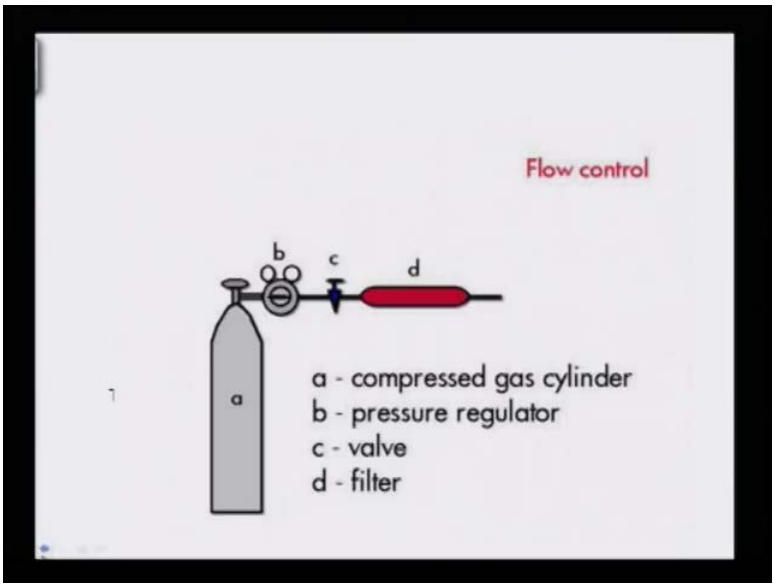


Now, if I have to then explain to you, what are the different methods of looking at the gas chromatographic machine, first part which is very important is a gas flow. And obviously, the gas cylinder must have a good regulator to control the gas flow. If the gas flow is very fast, all the components will be flushed out of the column and there would not be any separation process at all. If the gas flow is very slow, then the components will take hours to reach the detector. So, the gas flow must be very optimum and that is what is very important and this is the construction of the cylinder and you see that compressed gas is filled here. That is called a career gas; there is a regulator which place the very white in roll; there is a valve which allows the gas flow or stop the gas to flow and then there is a filter because, all these must be very purified gas in order to able to analysis properly.

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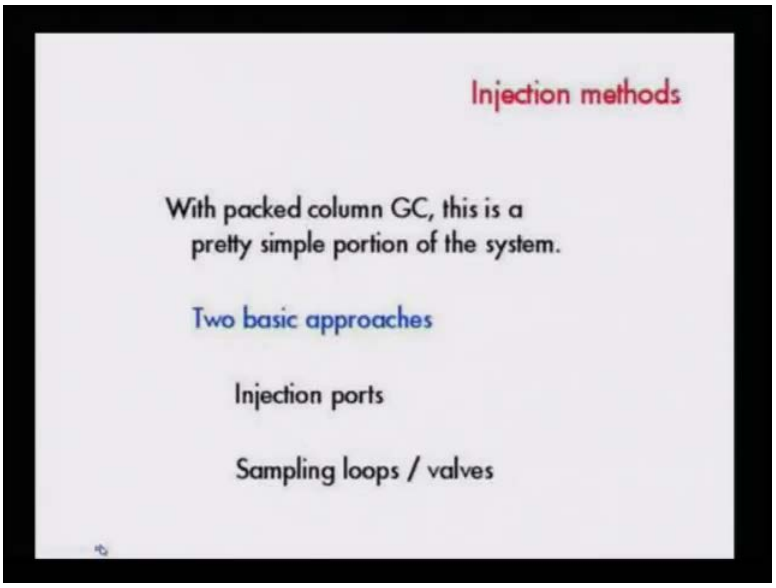


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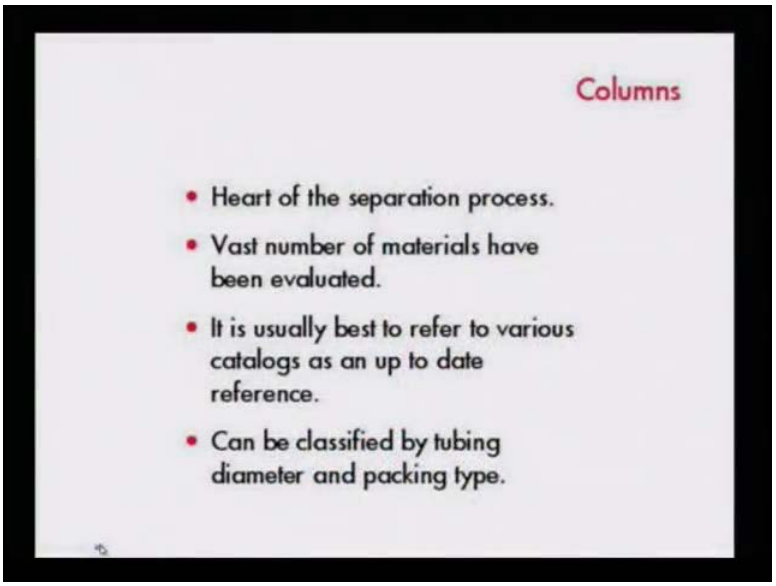


Now, injection method: in this there are several modes by which the injection into the gas column can be done with pack columns of GC this is a pretty simple portion of the system. Two basic approaches are there. There is an injection port and there is a sampling loop and there is a valve. That means, first the injection is done and then the valve is closed. Once the gas starts flowing and slowly, the valve is released but, what is, when we are using broader columns or packed columns. But, in the case of capillary columns, there is the mode, is only injector mode column which is the heart of the separation process.

Because this is where process actually takes place; a mix analyte is converted into purifier samples. Vast number of material have been evaluated; it is usually best to refer to various catalogs as an up to date reference because, now there are tailor-made columns. Specific analysis once look at the catalog choose the right kind of column for typical analysis and it can be classified by tubing diameter and packing type. That is, columns could be packed type which is broader and could be capillary columns which are almost of the hair size width.

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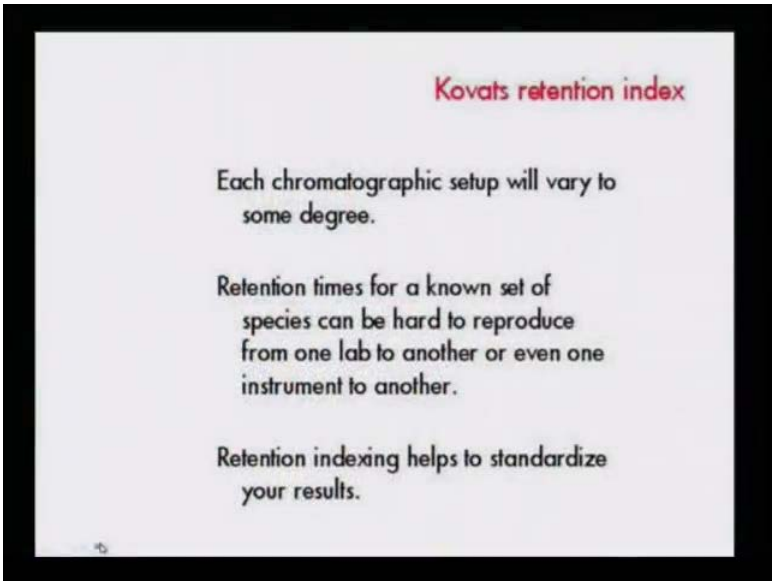


Kovats retention index: this is also just like the retardation time or the retention time. Each chromatographic setup will vary to some degree from time to time; from machine to machine, is a factor which is called the Kovats retention index. Retention times for a known set of species can be hard to produce or reproduce from one lab to another or even from one instrument to another. Retention indexing helps to standardize our results. That is how we try to look at these retention differences in retention data but, nevertheless, the retention time on a given machine will not vary very widely.

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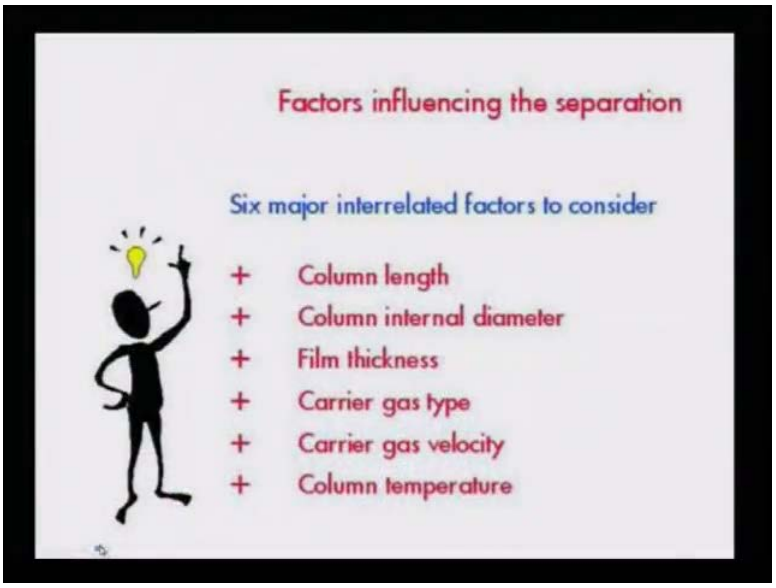


There are certain important influencing factors in the separation mode and this light particularly, is very important. There are six major interrelated factors to be considered when one is analyzing or when one is acting as an analyst. It is important to remember what is the column length, what is the column, internal diameter, what is the film thickness in that internal diameter and the carrier gas type. The carrier gas velocity which is the speed of the flow of the gas column temperature; if we do not take these into account, one tends to make lot of error while doing gas chromatography. That is why it is important to understand that there is factor where we can do temperature programming.

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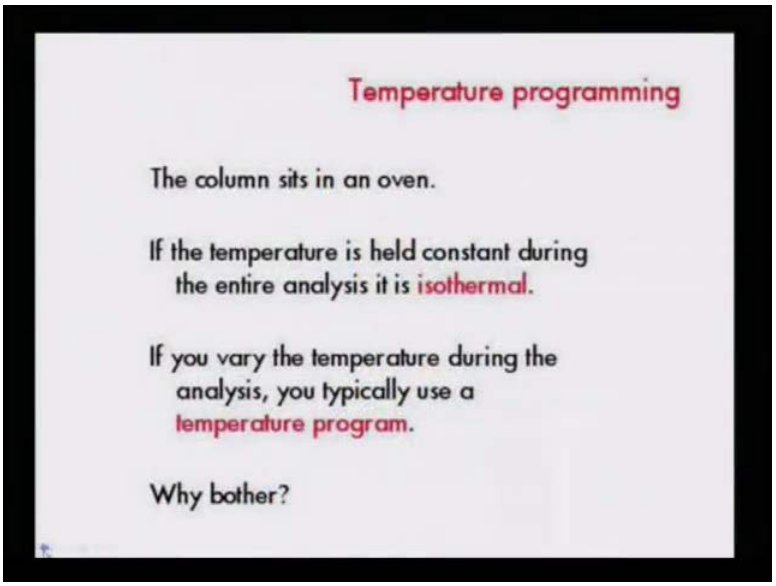

Sometimes, if we have only two components in the analyte and we know the they have the difference, large difference in their boiling point, one need not have a ram or a gradual increase in temperature. one can have a breath change in a temperature scale but, when one is trying to analyze components which have lower difference in their boiling point, one needs to do programming of temperature. The column sits in an oven; if the temperature is held constant during the entire analysis it is called isothermal; but, if you vary the temperature during the analysis, you typically use a temperature program. So, this is up to the analysis to understand whether there is need to have a ramp or a temperature programming or whether, one can carry out the analysis keeping a constant temperature at 180 degrees. But, that is a little risky if you have both low boiling and high boiling components, one cannot jump into those very easily.

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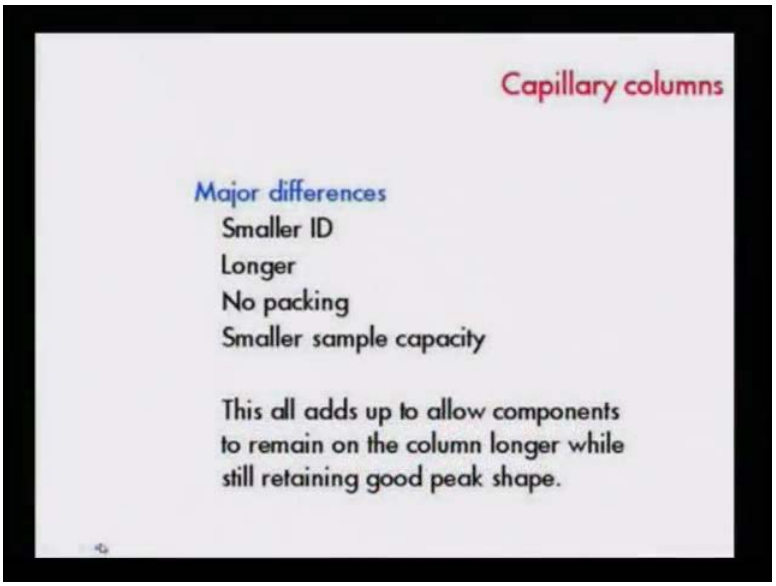


Capillary columns more emphasis has been made on capillary column because, in today's experimental conditions more and more use of capillary columns are being noticed because packed column meant for larger volume of analysis. So, the capillary column needs a special retention. Major differences in the capillary column and the packed column are that they are smaller in their internal diameter id; they are much longer in size. I said they are almost like 50 meters and they have been rolled up.

No packing sometime material is used of sometime very thin packing material is used; smaller sample capacity because, they are capillary columns one cannot inject larger

value micro liters of samples can be injected and micro liters only can be analyzed. This all adds up to allow components to remain on the column longer while still retaining good peak shape.

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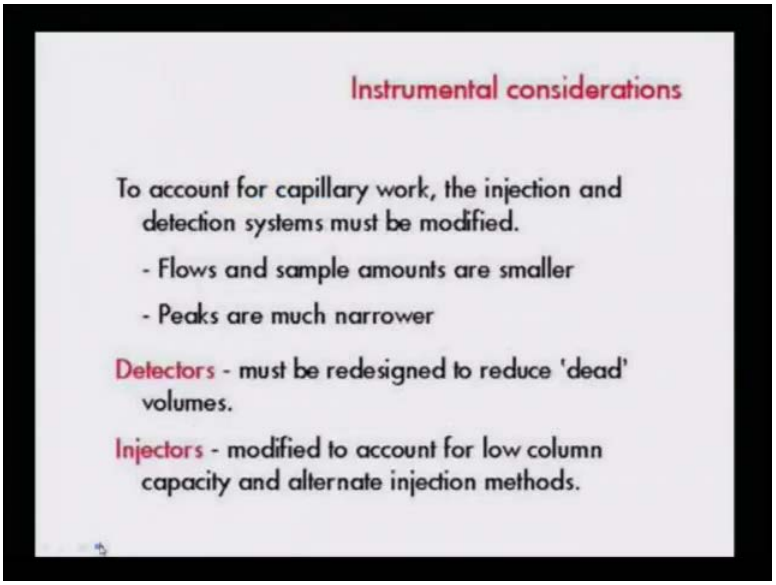


Instrumental consideration: to account for capillary work the injection and the detection systems must be modified. Obviously, we cannot have the same detector and the same injector for packed column and capillary column. So, there has to be certain modification allowed for capillary column to be inside the machine and therefore, flow and sample amounts are much smaller in the case of capillary conditions. Peaks are much narrower, sharper; detector must be redesigned to reduce dead volumes injectors modified to account for low column capacity alternate injection methods.

Now, here I would like to emphasis that many times when even 1 micro liters is injected, only 10 percent is allowed into the capillary column while 90 percent is rejected and this kind of injected mode is called split method of injection. That means, injection was 1 micro liter but, in the 1 micro liter also only 10 percent has gone into the column and 90 percent has been stopped from going into the column. That is why call it split method; there are split-less method also where, the entire volume goes through the column.

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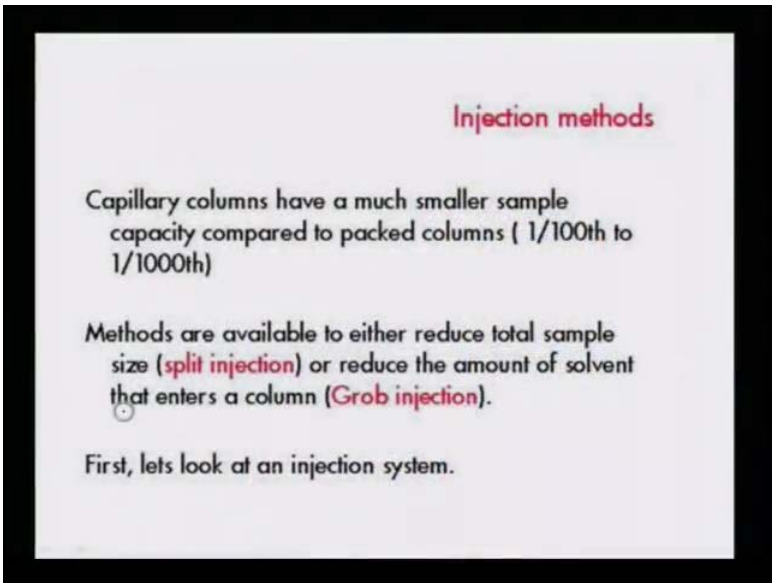


So, even that precision is possible in capillary column injection method. Capillary column have a much smaller sample capacity compared to packed column almost like one hundredth or sometimes even one thousandth volume can be accommodated in a capillary column whereas, packed column can take 1000 times or even 100 times of the same amount. Methods are available to either reduce the total sample size by split injection or reduce the amount of solvent column by Grob injection; that means, solvent is taken up, only the compound is allowed to enter. These are certain, very specific technique but nevertheless, I think it is important to mention these so that, when you are reading from the books you are able to comprehend what these terms mean.

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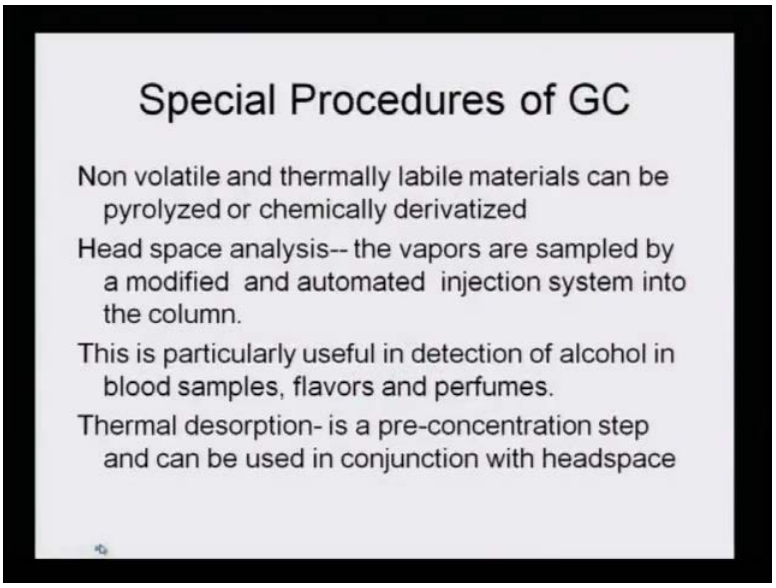


First, let us look at the injection system special procedures of GC nonvolatile and thermally labile materials can be pyrolyzed or chemically derivatized. There are now, the machine now getting into more and more specific kind of analysis and sometimes, some materials which are nonvolatile and which are also at times thermally labial which means, that the decompose at high temperature can be pyrolized or they can be turned into a derivative - we call it chemically derivatized.

Head space analysis vapors are sampled by a modified and automated injection system into the column; this is particularly useful in injection in alcohol, in blood samples, flavors and perfumes.

Thermal desorption it is a preconditioning or preconcentration step and can be used in conjunction with the headspace now when there are samples because how to inject that sample into the gas chromatography for the separation. For the detection, these are head space thermal desorption; these are modification to inject the gracious samples gas chromatograph (Refer Slide Time: 29:50).

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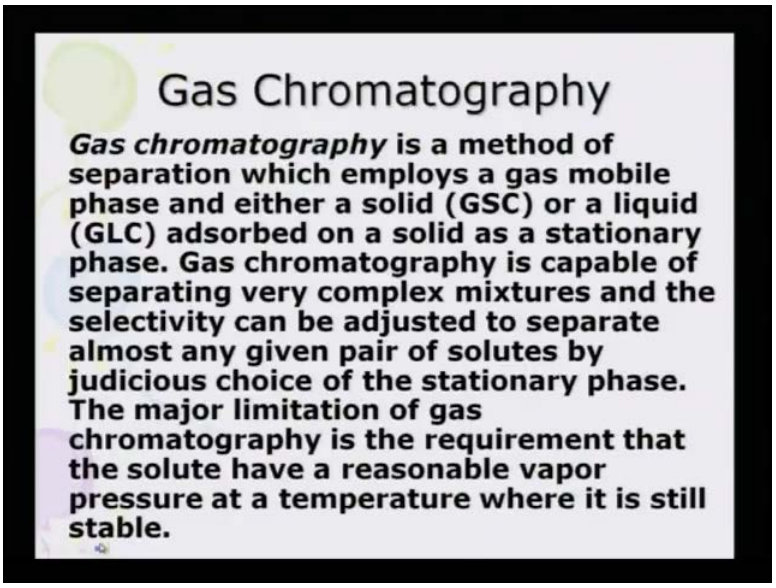


Now, when we start understanding gas chromatography HPLC comes automatically because it is meant for higher boiling compounds but before proceeding for HPLC, I would now like to go back again to some more modification of GC continuing with gas chromatographic techniques. I would now like to reevaluate what we have talked so far. Gas chromatography is a method separation which employs a gas mobile phase and either a solid or a liquid absorbed on a solid as a stationary phase. Gas chromatography is capable of separating very complex mixtures and the selectivity can be adjusted to separate almost any given pair of solutes by judicious choice of the stationary phase.

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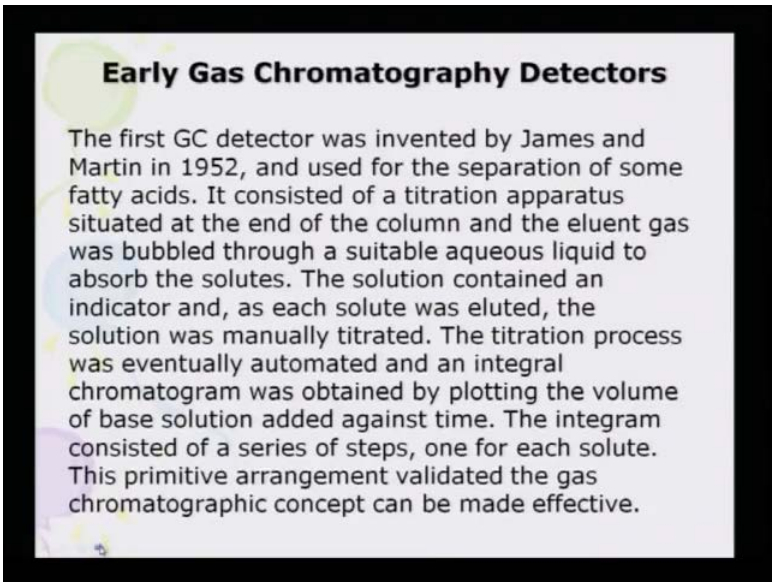

The major limitation of gas chromatography is the requirement that the solute have a reasonable vapor pressure at a temperature where it is still stable. So, that is a mandatory factor; we are just trying to reevaluate and we understand what we have studied so far.

The first GC detector was invented by James and Martin in 1952 and used for the separation of some fatty acids. It consists of a titration apparatus situated at the end of the column and the eluent gas was bubbled through a suitable aqueous liquid to absorb the solutes. The solution contained an indicator and as each solute was eluted, the solution was manually titrated. The titration process was eventually automated and an integral chromatogram was obtained by plotting the volume of the base solution added against time.

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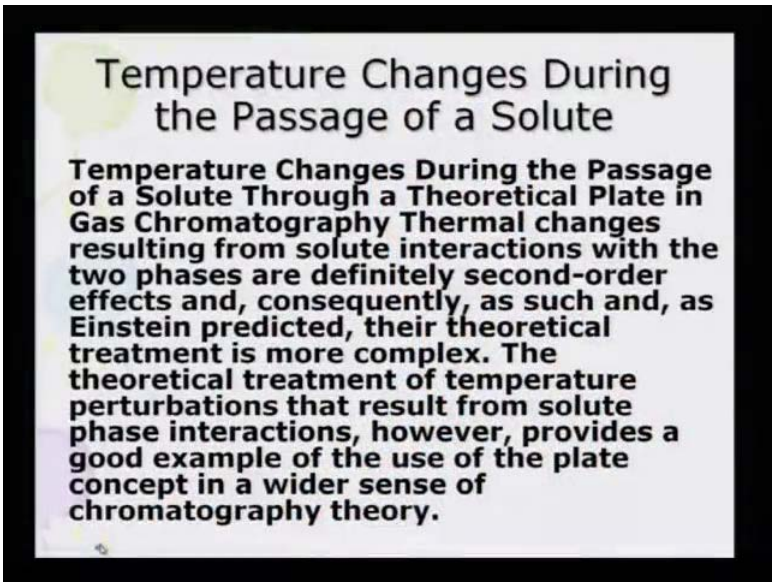


The integram consisted of a series of steps; one of each solute. This is the primitive arrangement. However, this is how chromatographic started; so, you know you see over a period of time with the invention of new techniques with more innovation into the machine, lot of things have got automotive. Temperature changes during the passage of a solute through a theoretical plate in gas chromatography. Thermal changes resulting from solute interactions with the two phases are definitely second order effects and consequently, as such, as Einstein predicted, theoretical treatment is much complex.

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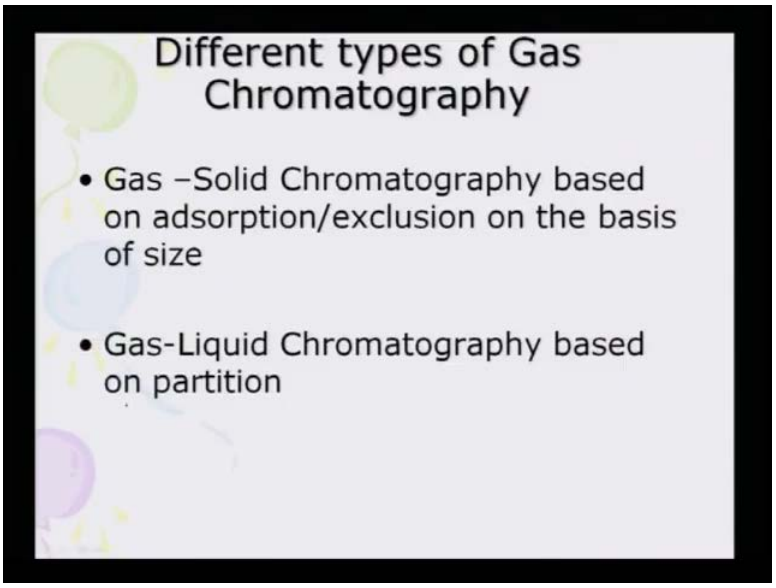


It is imagined that it is a plate theory; that it is going from one plate to another plate but, then it is much more complicated. The theoretical treatment of temperature perturbations that result from solute phase interaction however, provide a good example of the use of the plate concept in a wider sense of chromatography theory. Different chromatograph: let us just have a recap of what we have studied so far. Gas solid chromatography based on the absorption or the exclusion principle on the basis of the size; gas liquid chromatography based on the partition mode.

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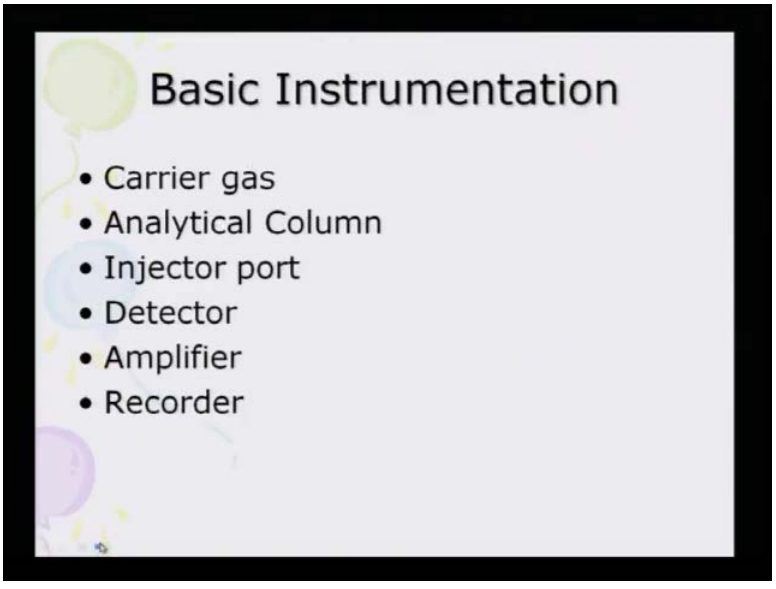

Now, these are different mode basic instrument contains carrier gas analytical column injector detector amplifier and recorder.

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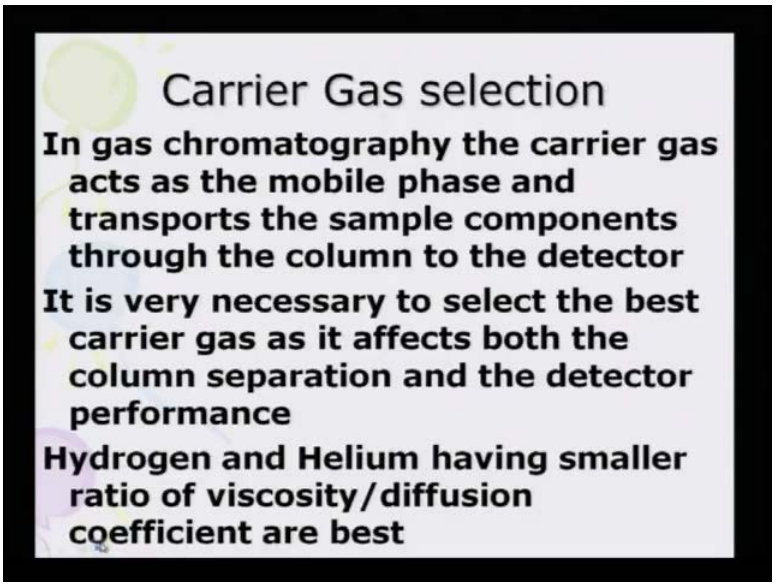


Carrier gas selection: it is a very important and a crucial decision in gas chromatography. The carrier gas acts as a mobile phase and the transports the sample components through the column to the detector. It is the main part which is you know, allowing it to move; it is very necessary to select the best carrier gas as it affects both the column separation and the detector performance.

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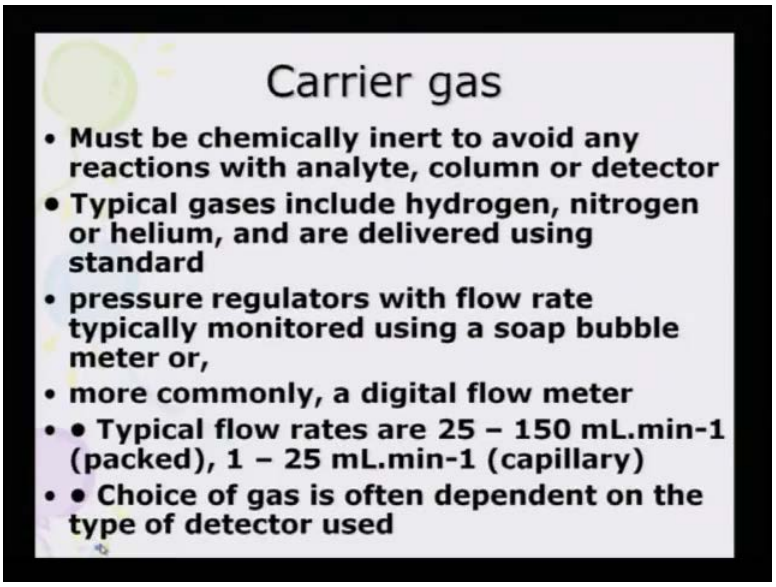

Hydrogen and helium having small ratio of viscosity diffusion coefficient are the best carrier gases which are most commonly used. Carrier gas must be chemically inert to avoid any reactions with analyte column or detector. That means, the choice of carrier gas should be such that it should not chemically interact with any of the three, either the analyte column or the detector. Typically, it should be inert; typical gas include hydrogen, nitrogen or helium are delivered using standard pressure regulators with flow rate typically monitored using a soap bubble meter or more commonly, a digital flow meter. Now a day, the digital flow meter is the one which is used. Typical flow rates are 25 to 150 mL per minute for packed columns and 1 to 25 mL per minute capillary columns. Choice of gas is often dependent on the type of detector that is to be used.

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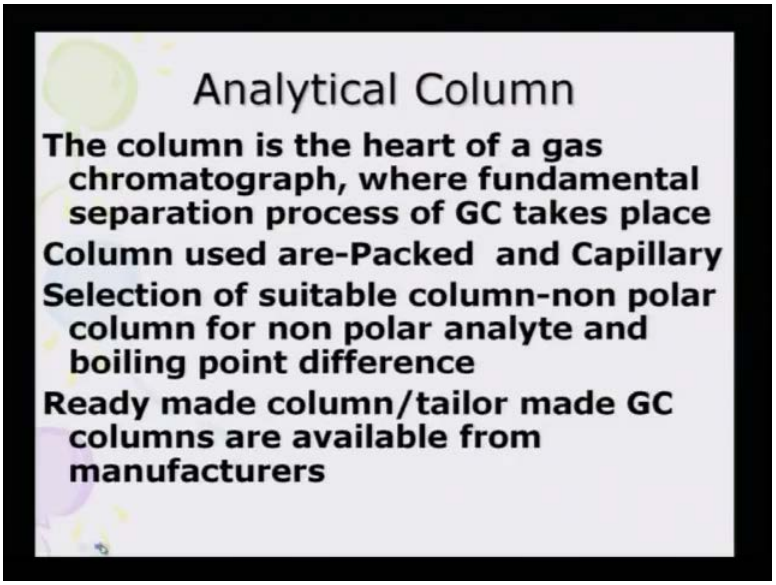


Analytical column: the column is the heart of the gas chromatography where fundamental separation process of GC takes place, used could be of the packed type or the capillary type. Selection of suitable column for non-polar columns: we use non-polar analyte; uses non-polar column and the boiling point difference should be much different. Readymade column tailor-made column as I mentioned earlier, must be used from the catalogs that are already available. So that one can design the analysis and do it accordingly.

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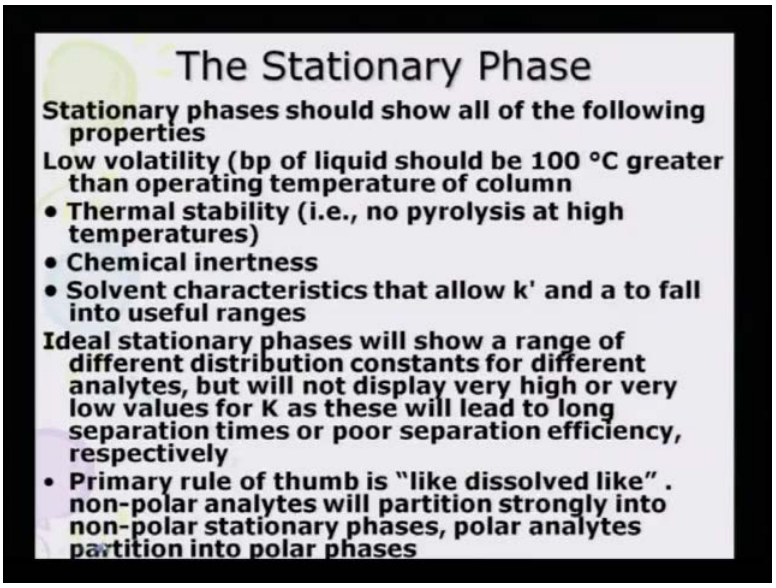


The stationary phase: stationary phase should show all of the following properties. Low volatility: that means, the boiling point should not be 100 degrees greater than operating temperature. Thermal stability: no pyrolysis should take place of the stationary phase. Chemical inertness; solvent characteristics: that allows k prime value to fall into the useful ranges. Ideal stationary phase will show a range of different distribution constants for different analyte but, will not display very high or very low values of k as these will lead to long separation times.

So, what it means is that we have to make an optimum choice of everything in order to have an optimal analysis. Primarily, rule of thumb rule is, “like dissolves like”. Non-polar analytes will partition strongly into the non-polar stationary phases, while polar analyte partition very well into the polar phases.

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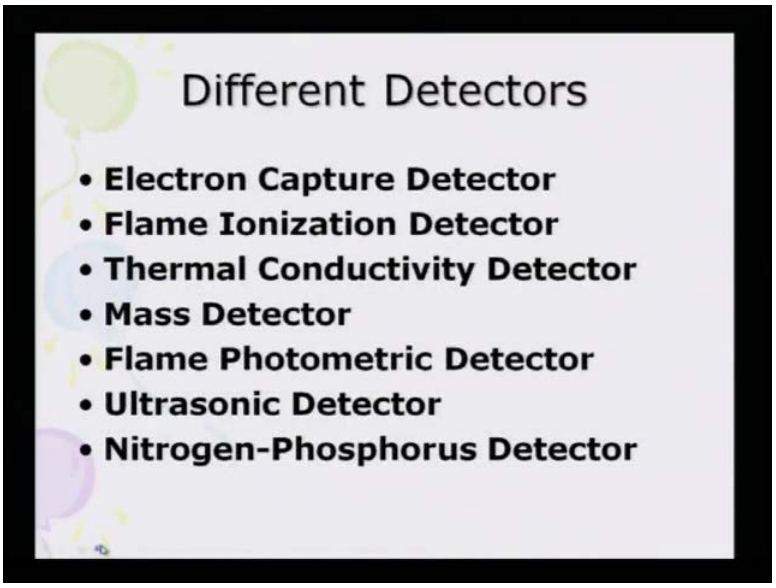


Different detectors - I had mentioned earlier also; but, this is just like a recap. So, we are trying to look at all the GC components; all important features one by one electron capture detector flame ionization detector, thermal conductivity detector, mass detector, flame photometric detector, ultrasonic detector, nitrogen phosphorus detector and many more.

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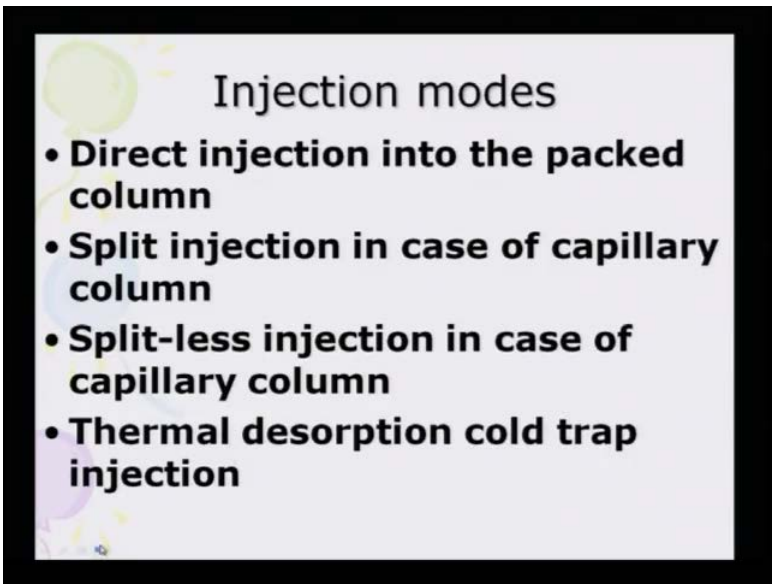

Injection mode could be direction injection into the packed columns. Split injection in the case of capillary column; split-less injection also in the case of capillary column. Thermal desorption cold trap injection which are very specific for gaseous samples.

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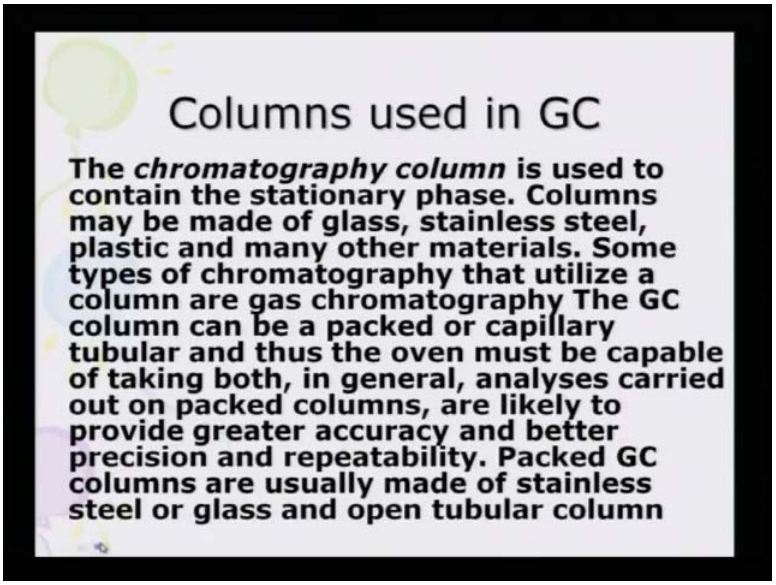


Column used in GC: the chromatography column is used to contain the stationary phase columns may be made of glass, stainless steel, plastic and many other materials. Some types of chromatography that utilize a column are gas chromatography. The GC column can be packed or capillary tubular and thus the oven must be capable of taking all the different types of columns that can be seated in that oven.

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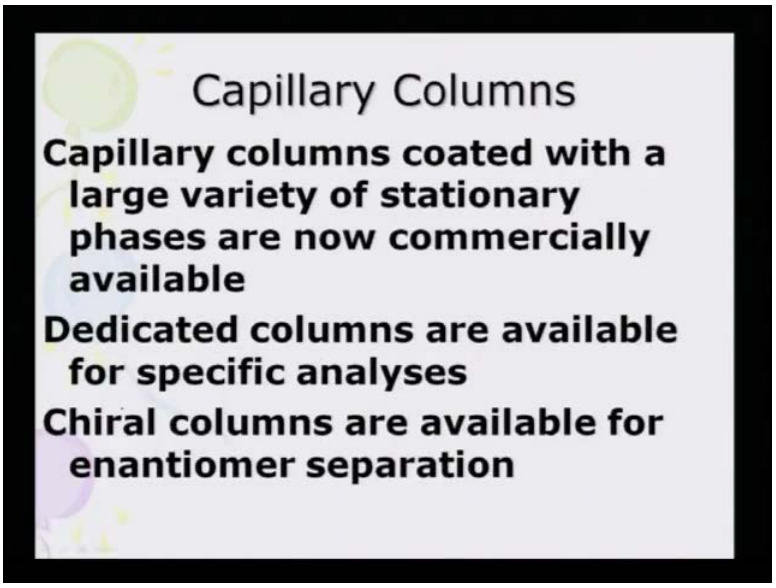


In general, the analysis carried out on packed columns are likely to provide greater accuracy and better precision and repeatability packed GC columns are usually made up of stainless steel or glass and are open tubular columns but, as what I mentioned that capillary columns are the order of the day. Capillary columns coated with the large variety of stationary phases are now commercially available. Dedicated columns are available for specific analysis chiral columns are also available for enantiomer separation.

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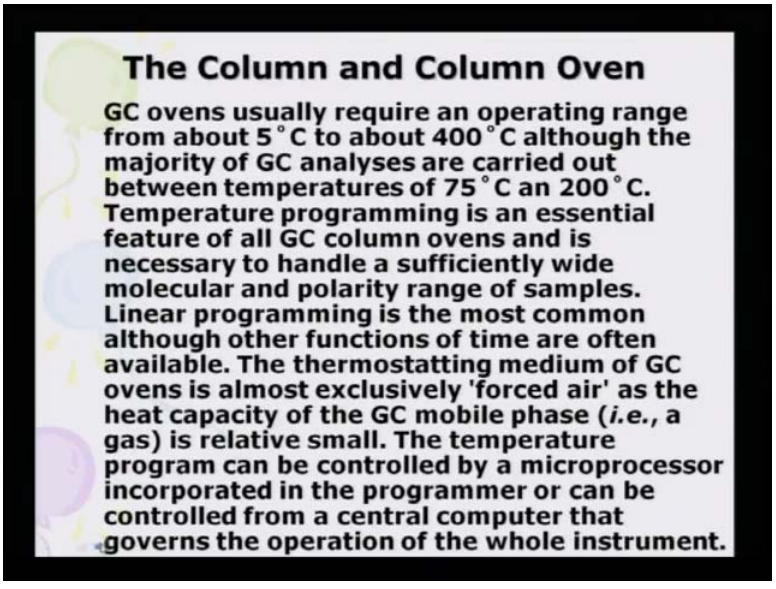

Column and column oven because, as I said the column is seated in an oven GC - ovens usually require an operating range of 5 degrees to 400 degrees, although, majority of the GC analyses are carried out between temperatures of 75to 200 degrees. Temperature programming is an essential feature of all GC column ovens and is necessary to handle a sufficiently wide molecular and polarity range of samples. Linear programming is the most common although other functions of time are also available. The thermostatic medium of GC oven is almost exclusive by forced air as the heat capacity of the GC mobile phase is relatively small. The temperature program can be controlled by a microprocessor incorporated in the programmer or can be controlled from a central computer that governs the operation of the whole instrument.

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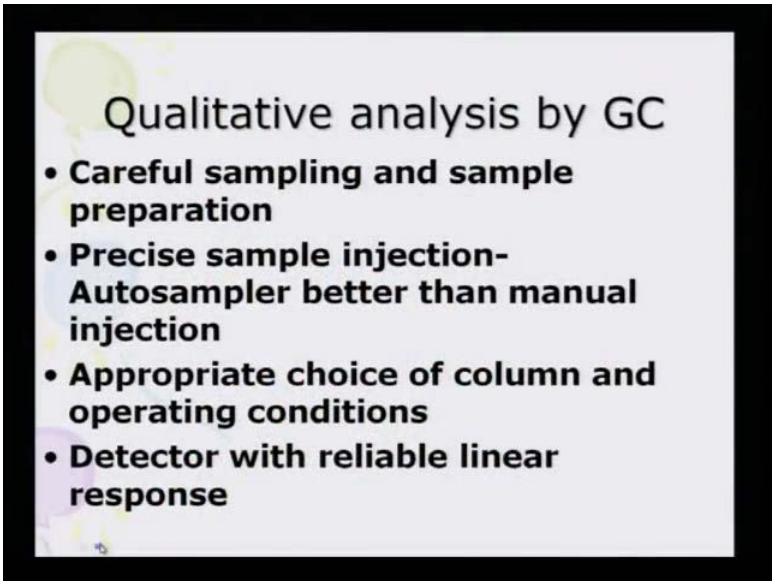


Now, as I said the temperature ramp can be given or isothermal conditions can also be carried out. Qualitative analysis by GC careful sample and careful preparation is mandatory. Precise sample injection autosampler better than manual injection; appropriate choice of column and operating conditions are also very necessary. Detector with reliable linear response have to be taken into account.