Advance Analytical Course

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Lecture No. # 01

Separation of Compounds

The first lecture that I begin today is related to Separation of Compounds. Advance Analytical Course - the first lecture that I begin today is related to Separation of Compounds.

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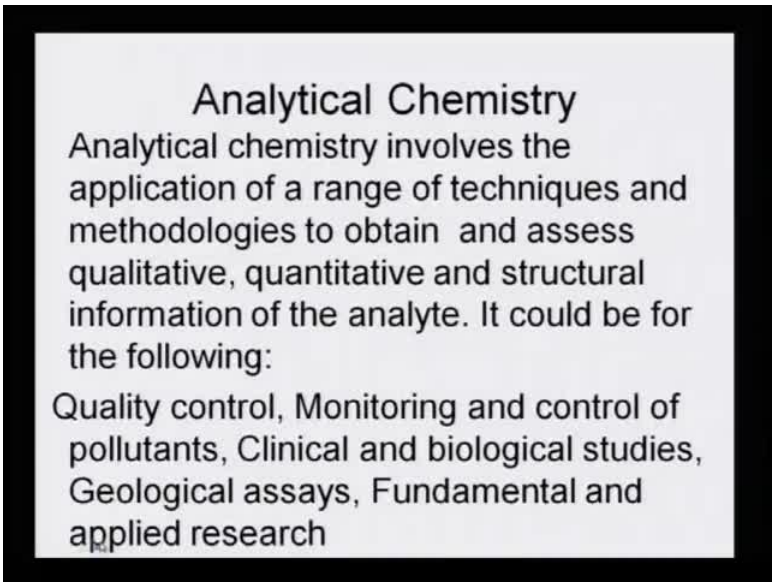

Analytical Chemistry, as you know, involves the application of a wide range of techniques and methodologies to obtain and assess qualitative, quantitative and structural information of the analyte. It could be for the following reasons: quality control, monitoring and quality of pollutants, clinical and biological studies, geological assays, fundamental and applied research.

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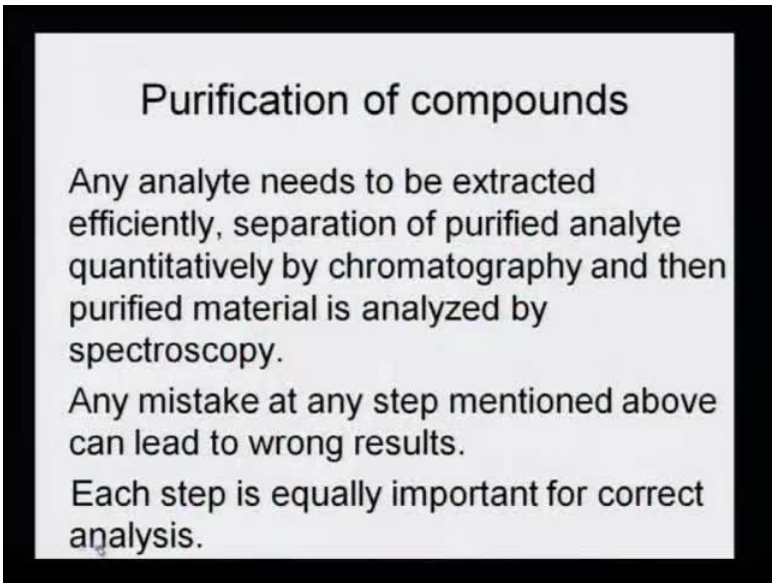

I would like to draw your attention to the word 'analyte'. Analyte is the substance of which we do the analysis. Further on, purification of compounds is related to the fact that any analyte needs to be extracted efficiently, separation of purified analyte takes place quantitatively by chromatography, and then, the purified material is analyzed by spectroscopic method. So, if we see, there are three main steps: one is the extraction, the second step is the chromatographic separation and the third step is the spectroscopic analysis. Any mistake, in any step mentioned above, can lead to wrong results. Each step is equally important for correct analysis.

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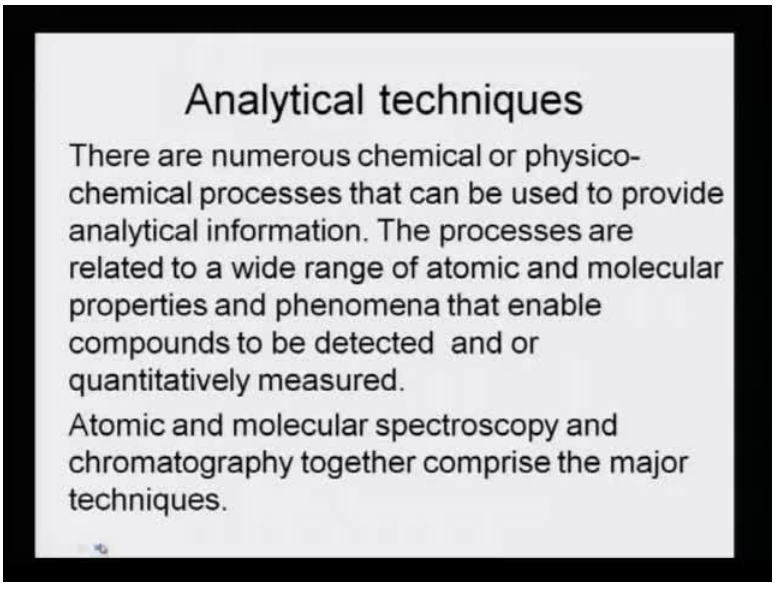

Analytical techniques - there are numerous chemical and physicochemical processes, that can be used to provide analytical information. The processes are related to a wide range of atomic and molecular properties and phenomena that enable compounds to be deducted and/or quantitatively measured. Atomic and molecular spectroscopy and chromatography together comprise the major technique of analytical chemistry.

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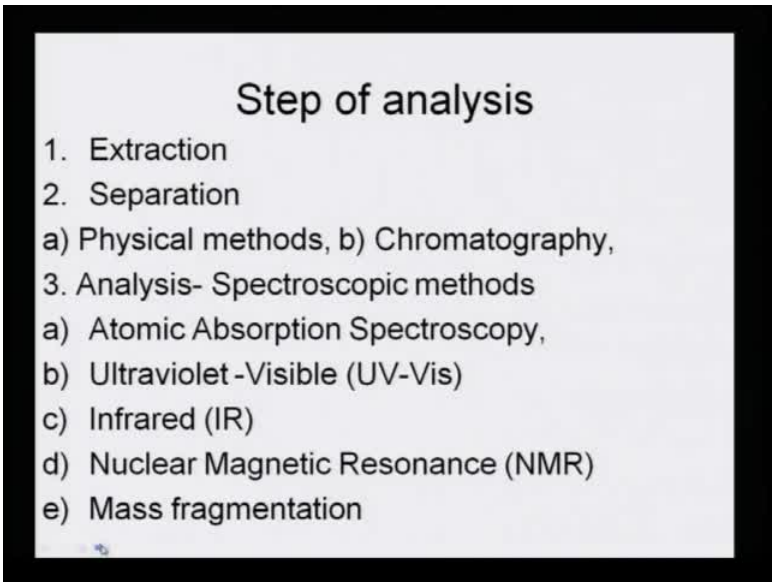

Steps of analysis - if we try to look in are just three of them, as what I mentioned a little while ago. First step is extraction, where we take out the analyte into a medium from where it can be analyzed. The second step is the process of separation or separation; now this could be a physical method or by chromatography. And the third step related to analysis, is related to spectroscopic methods; it could be by atomic absorption spectroscopy; or by ultraviolet, that is, UV-visible spectroscopy; infrared or IR spectroscopy; nuclear magnetic resonance - NMR spectroscopy; or mass fragmentation called the mass spectroscopy; either of them or all of them. So, one looks at it, and any mistake, I again repeat that any mistake in any one of these steps, can lead to wrong results.

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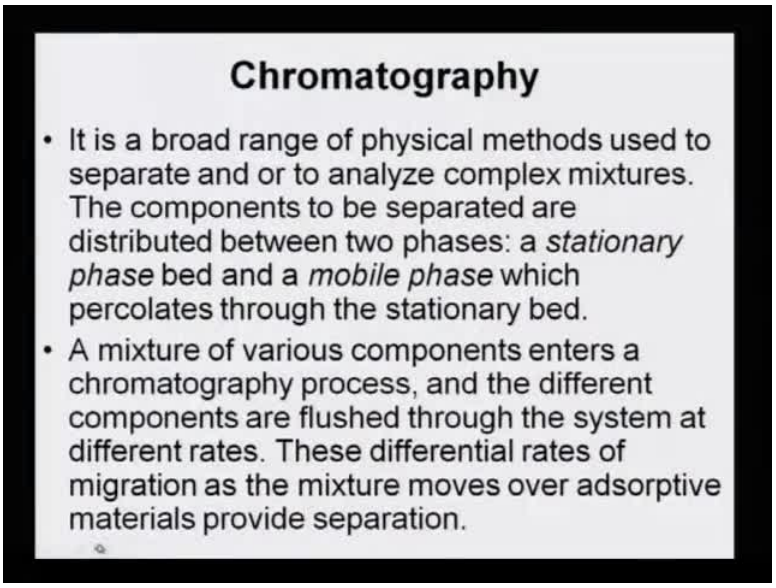

Chromatography - it is a broad range of physical methods used to separate or to analyze complex mixtures. The components to be separated are distributed between two phases: a stationary phase, which is called the bed or a mobile phase, which percolates through the stationary bed. Now, this should be understood very clearly, that there has to be two phases and the analyte has to partially go into one of the phases to get separated.

A mixture of various components enters a chromatographic process and the different components are flushed through the system at different rates. These differential rates of migration, in the mixture moves over to the absorptive material, provides the actual separation. If I have to explain it in simpler words, there is a stationary bed, and there is a mobile bed, and the mobile then percolates. And according to the nature of the analyte or the components of the analyte, the separation takes place in these two phases and they start moving at a different phase. That is how the phase widens and the separation actually takes place.

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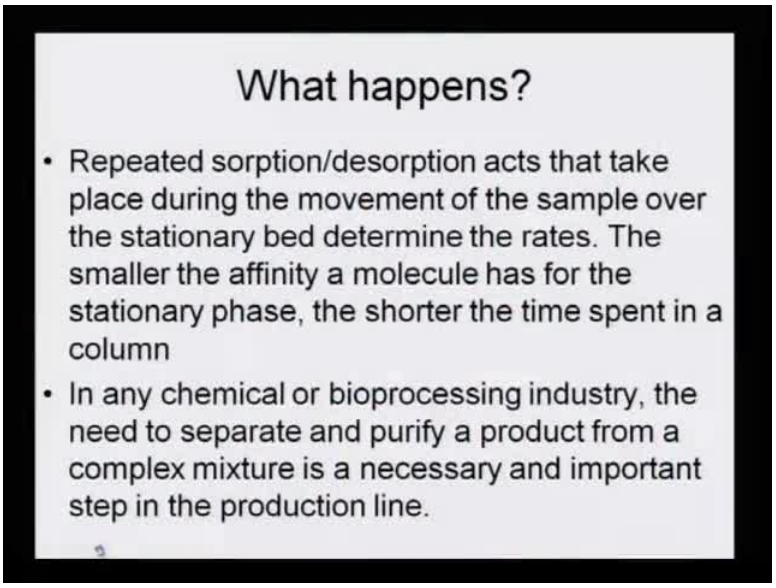

What happens? Repeated sorption and desorption acts that take place during the movement of the sample, over the stationary bed, determines the rates. The smaller the affinity the molecule has for the stationary phase, the shorter the time spent in the column. Now, if we try to look at the whole process, if we imagine that a column is made up of several layers, then there is an adsorption in the first layer; desorption-adsorption-desorption. And this process, for an analyte makes it get separated from the other components of the analyte. And the lesser the affinity it has for the stationary bed, it would move out of it faster. So, one analyte may have a different rate of affinity and the other analyte may have a different rate. And that is how, due to these differences in rate of affinity and rate of traversing, the analytes are separated.

In any chemical or bioprocessing industry, the need to separate and purify a product from a complex mixture is necessary and important step in the production line, because these are continuous processes and one needs to check whether the right kind of product is getting to the next step.

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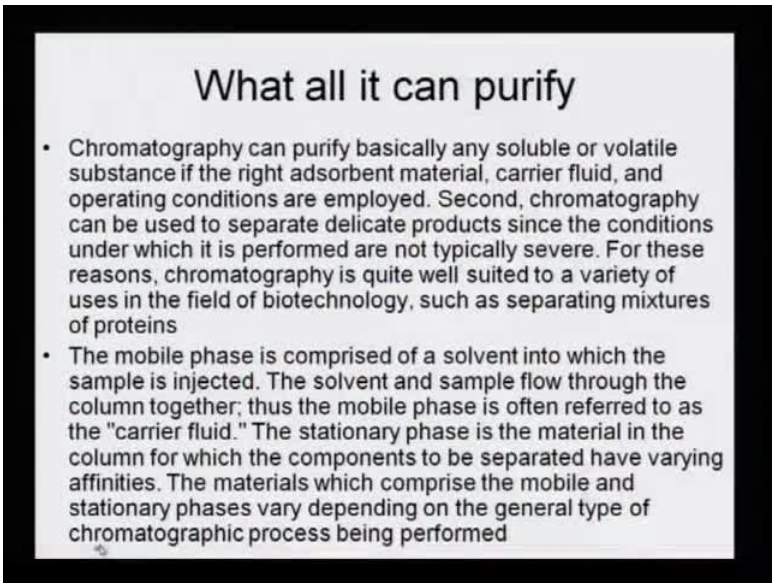

What all can the purifying process do? Chromatography can purify basically any soluble or volatile substance if the right adsorbent material is used, if the right carrier fluid is used and the operating conditions are rightly employed. Second, chromatography can be used to separate delicate products since the production and the conditions under which it is performed are not typically severe. For these reasons, chromatography is quite well suited to a variety of uses in the field of biotechnology, such as separating mixtures of proteins. Now, you know that proteins are very sensitive material; they cannot be subjected to very high heating or very cold weather or very adverse conditions. And chromatography conditions are very nominal and they can be used for the separation of proteins very efficiently. Therefore, chromatography is widely used in biotechnology.

The mobile phase is comprised of a solvent into which the sample is injected. The solvent and the sample flow through the column together; thus, the mobile phase is often referred as carrier fluid. The stationary phase is the material in the column for which the components to be separated have varying affinities. The materials which comprise the mobile and the stationary phases vary depending on the general type of chromatographic processes being performed. So, if I have to sum up, I would say that chromatographic separations involve only two phases: one is the mobile phase and the other one is the stationary phase. And the analyte, then has different rates of movement and absorption, desorption helps it to get separated on this column, which is the stationary phase.

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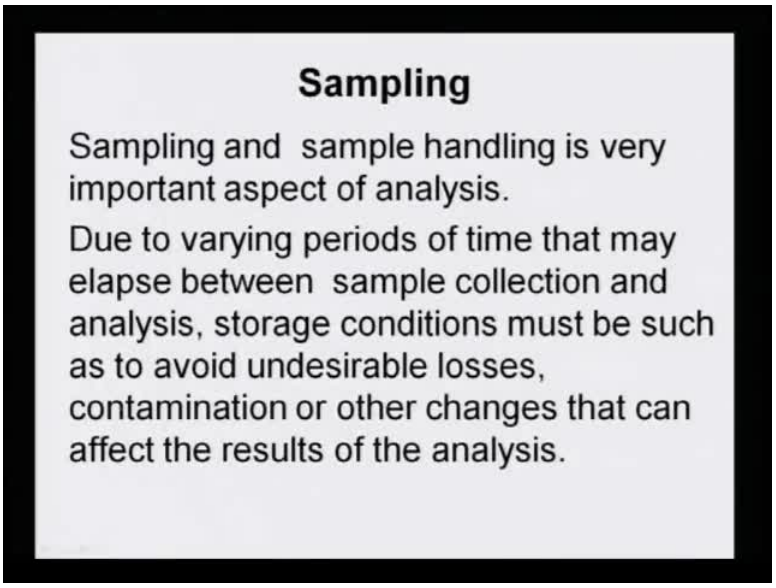

Sampling - sampling and sample handling is very important aspect of analysis. Due to varying periods of time that may elapse between the sample collection and analysis, storage conditions must be such as to avoid undesirable losses, contamination or other changes that can affect the results of the analysis. I would like to draw your attention to this very fact, that any and every method of sampling is not allowed in analytical chemistry. There is a protocol and the protocol must be met in order to save the sample from any kind of contamination or any kind of undesirable losses.

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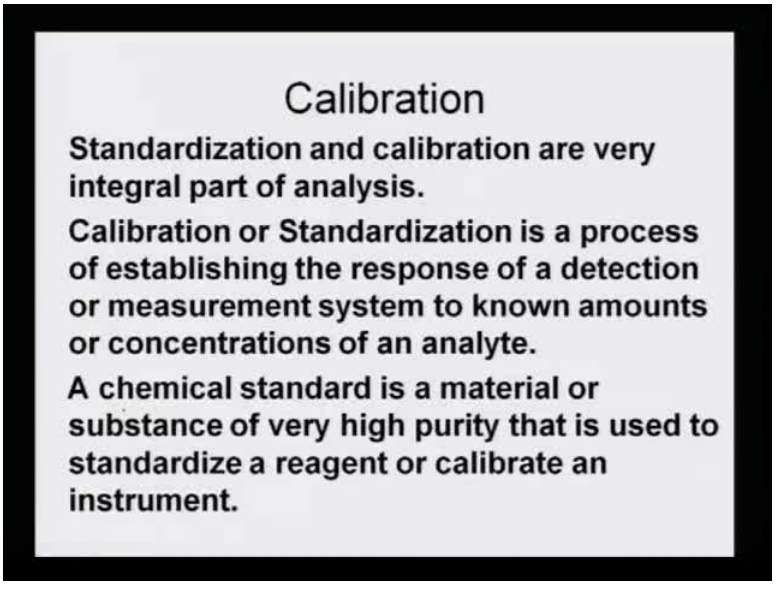

Calibration - standardization and calibrations are very integral part of any analysis. Calibration or standardization is a process of establishing the response of a detection or measurement system to known amounts or concentrations of an analyte. A chemical standard is a material or substance of a very high purity that is used to standardize a reagent or calibrate an instrument.

Now, I would like to draw your attention to the fact that calibration and standardization play a very vital role in identifying the analyte. If we have an unknown pesticide sample and we want to find out which pesticide is this, we would have to evaluate it against many of these standard materials of pesticides, and then take a look at which peak matches with the unknown analyte. So, that is why calibration and standardization play a very vital role in analytical chemistry.

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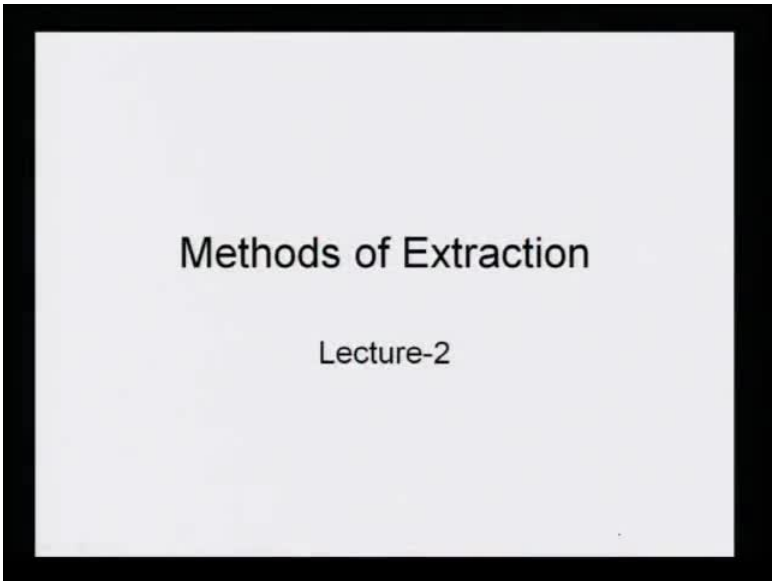

As I mentioned earlier that the first step for analysis is extraction. Now, we try to look at different methods of extraction in the lecture number 2.

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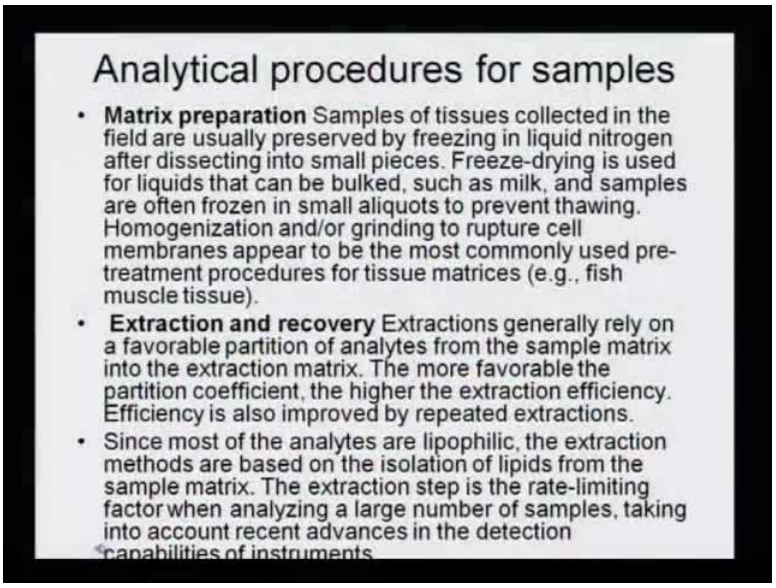

Analytical procedures for samples - matrix preparation: samples of tissues collected in the field are generally preserved by freezing in liquid nitrogen after dissecting into small pieces. Freeze-drying is used for liquids that can be bulked, such as milk, and samples are often frozen in small aliquots to prevent thawing. Homogenization and/or grinding to rupture cell membranes appear to be the most commonly used pretreatment procedures for tissue matrices; that is, for example, fish or muscle tissues. Now, I would like to draw your attention that sampling we talked about a little while ago, and similarly, when the sample is collected and if it is not to be analyzed immediately, needs to be preserved properly. And the best method to do that is by freeze-drying. Thus, we have to then take the matrix out from the tissues and take the analyte for analysis.

Extraction then comes into picture. Extraction and recovery: extractions are generally relying on a favorable partitioning of analytes from the sample matrix into the extraction matrix. Always there will be two situations and from one it will come to another one. Obviously, the analyte will come to the matrix where it has more affinity. The more favorable the partitioning coefficient, the higher the extraction efficiency. Efficiency is also improved by repeated extractions. I like to draw your attention here, that when we try to do one time extraction with 100 ml of a solvent versus three times with 50 ml of the solvent, it is always that in the latter case, we have better extraction of the analyte. Now, this happens, because partitioning coefficient in every cycle sums up, and in three

cycles, it is always more than the partitioning coefficient in one cycle. That is what makes the more number of repeated analyses or extractions as the best method.

Since most of the analytes are lipophilic, the extraction methods are based on the isolation of lipids from the sample matrix. The extraction step is the rate-limiting step. And therefore, when analysis is done on a large number of samples, this is the only step, which goes very slow. Rate determining step means, that it is the entire extraction depends on this process, where the coefficient must be high for partitioning, so that the analyte comes out and is separated properly. And this depends on the capability of the instrument.

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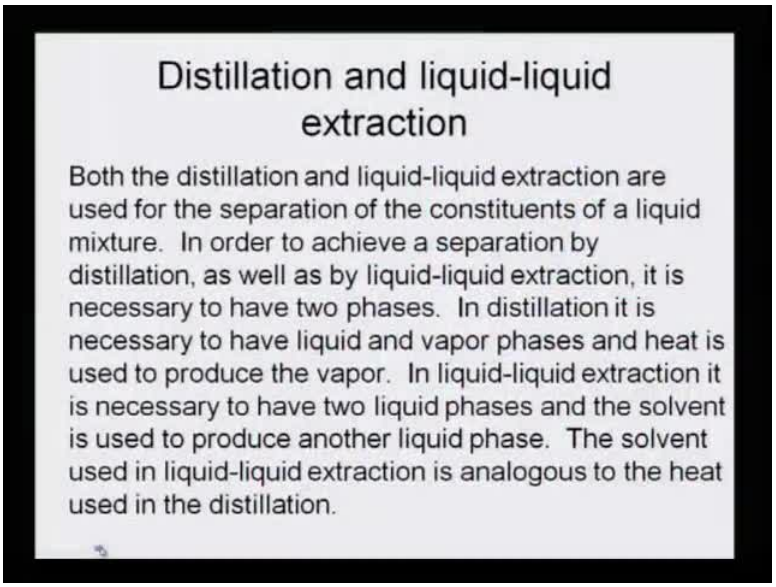

The most common extraction method for liquid-liquid extraction is distillation. Both the distillation and the liquid-liquid extraction are used for separation of the constituents of a liquid mixture. In order to achieve a separation by distillation as well as by liquid-liquid extraction, it is necessary to have two phases. In distillation, it is necessary to have a liquid and a vapor phase, and heat is used to produce the vapors. In liquid-liquid extraction, it is necessary to have two liquid phases and the solvent is used to produce another liquid phase. The solvent used in liquid-liquid extraction is analogous to the heat used in the distillation.

So, in a way, distillation and liquid-liquid extraction are quite similar, but in another way, they are also different. One analyte has to be decided, which one should be taken

for distillation and which one should be separated by liquid-liquid extraction. It is not a universal rule that every compound must be distilled, because there are some heat sensitive compounds that cannot be distilled. And so, they should be separated by liquid-liquid extraction.

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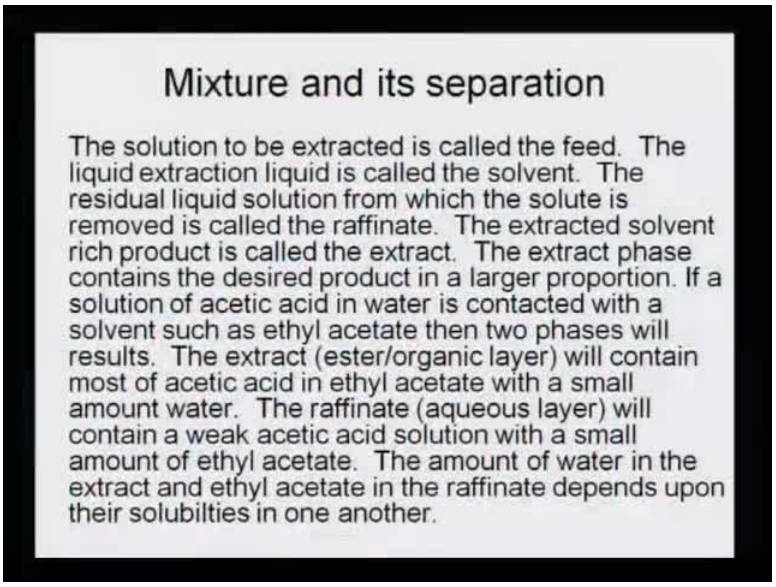

Mixture and its separation - because normally, any sample from the environment, any sample from the chemical reactions, is never pure, and so, it is in the form of a mixture. Now, this mixture has to be separated. And so, how do we go about doing the separation? The solution to be extracted is called the feed. The liquid extraction liquid is called the solvent. The residual liquid solution from which the solute is removed is called the raffinate. The extracted solvent rich product is called the extract. So, these are different terminologies: we have the feed, we have the solvent, we have raffinate and extract. All are very important in their respective roles.

The extract phase contains the desired product in a larger proportion. If a solution of acetic acid in water is contacted with the solvent such as ethyl acetate, then the two phases will result. The extract, that is, the ester and the organic layer, will contain most of the acetic acid in ethyl acetate with a very small amount of water. The raffinate, that is, the aqueous layer, will contain a weak acetic acid solution with a small amount of ethyl acetate. The amount of water in the extract, and ethyl acetate in the raffinate, depends upon their solubilities in one another. So, what does it mean? That between the

two solvents - water and ethyl acetate - the acetic acid was having more affinity for ethyl acetate than water. That is why it is found more in the extract and less in the raffinate. That is what gives the different idea.

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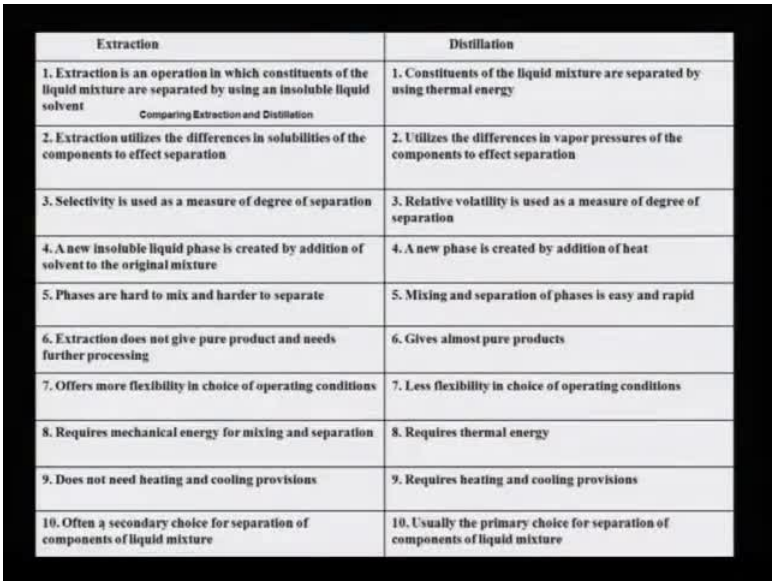

Extraction	Distillation
1. Extraction is an operation in which constituents of the liquid mixture are separated by using an insoluble liquid solvent	1. Constituents of the liquid mixture are separated by using thermal energy
2. Extraction utilizes the differences in solubilities of the components to effect separation	2. Utilizes the differences in vapor pressures of the components to effect separation
3. Selectivity is used as a measure of degree of separation	3. Relative volatility is used as a measure of degree of separation
4. A new insoluble liquid phase is created by addition of solvent to the original mixture	4. A new phase is created by addition of heat
5. Phases are hard to mix and harder to separate	5. Mixing and separation of phases is easy and rapid
6. Extraction does not give pure product and needs further processing	6. Gives almost pure products
7. Offers more flexibility in choice of operating conditions	7. Less flexibility in choice of operating conditions
8. Requires mechanical energy for mixing and separation	8. Requires thermal energy
9. Does not need heating and cooling provisions	9. Requires heating and cooling provisions
10. Often a secondary choice for separation of components of liquid mixture	10. Usually the primary choice for separation of components of liquid mixture

Comparing Extraction and Distillation

Now, if we try to make a comparison of the two methods - one is the extraction and the distillation method - extraction is an operation in which constituents of the liquid mixtures are separated by using an insoluble liquid solvent. So, there has to be another solvent, which will help for the extraction of the analyte; whereas, in distillation, the constituents of the liquid mixtures are separated using only heat or thermal energy; nothing else is required, no other solvent is required.

Second point of difference - extraction utilizes the differences in solubilities of the components to effect the separation; whereas, in distillation, it utilizes the differences in vapor pressures of the components to effect separation. So, there are minor differences, yet they are very significant differences, and as what I mentioned earlier, that the two processes cannot be used everywhere. The analyte and the analyst, have to decide which analyte will be used for extraction, and which will be used for distillation purposes, for the method of separation.

Selectivity is used as a measure of degree of separation; relative volatility is used as a measure of degree of separation. So, again there are very discrete differences between the two processes. Phases are hard to mix and harder to separate; mixing and separation

of phases is easy and rapid. Phases are harder to mix and separate; mixing and separation of phases is easy and rapid. Extraction does not give pure product and needs further processing; gives mostly very pure products. So, you see that distillation has a little advantage on one side and it has a disadvantage on the other side. So, that is why I said that the two processes must be chosen very carefully.

Offers more flexibility in choice of operating conditions and it offers less flexibility in choice of operating conditions. Requires mechanical energy for mixing and separation and distillation requires thermal energy. Does not need heating or cooling provisions in the case of extraction; as I said, even the heat sensitive compounds can be easily extracted; whereas, in distillation, it is always using the thermal energy. So, it is at an elevated temperature; does not need heating or cooling provisions as mentioned. And it does need heating and cooling provisions, because otherwise, the distillation vapor pressure, the thermal energies will not come into action in the case of distillation. Often a secondary choice for separation of the liquid-liquid mixture is required; usually, this is the primary choice of separation, where the liquid components can be separated very easily.

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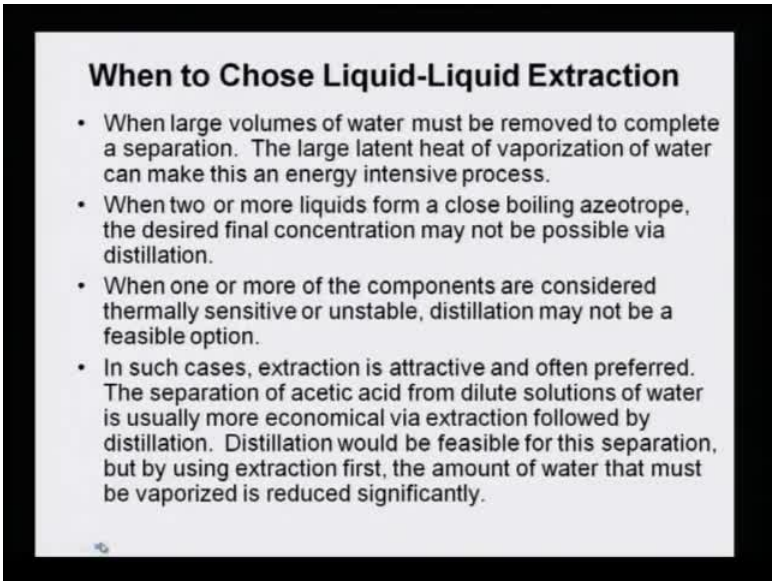

When to choose liquid-liquid extraction? When large volumes of water must be removed to complete a separation. The large latent heat of vaporization of water can make this an energy intensive process. Now, what happens is that an analyst has to make a choice -

whether to do an extraction process or to do a distillation process. When the analyte has lots and lots of water component, in that case, it is better to go for a liquid-liquid extraction. When two or more liquids form a close boiling azeotrope, the desired final concentration may not be possible via distillation and that is where the liquid-liquid extraction process must come into picture.

When one or more of the components are considered thermally sensitive or unstable, distillation may not be a feasible option. That means, that if one of them is heat sensitive, it will definitely get decomposed when distillation is done by the mode of thermal energy and in such a case, liquid-liquid extraction is what is required.

In another situation, extraction is attractive and often preferred. The separation of acetic acid from dilute solutions of water is usually more economical via extraction followed by distillation. Distillation would be feasible for this separation, but by using extraction first, the amount of water must be removed or reduced significantly, and then distillation of the acetic acid can be carried out.

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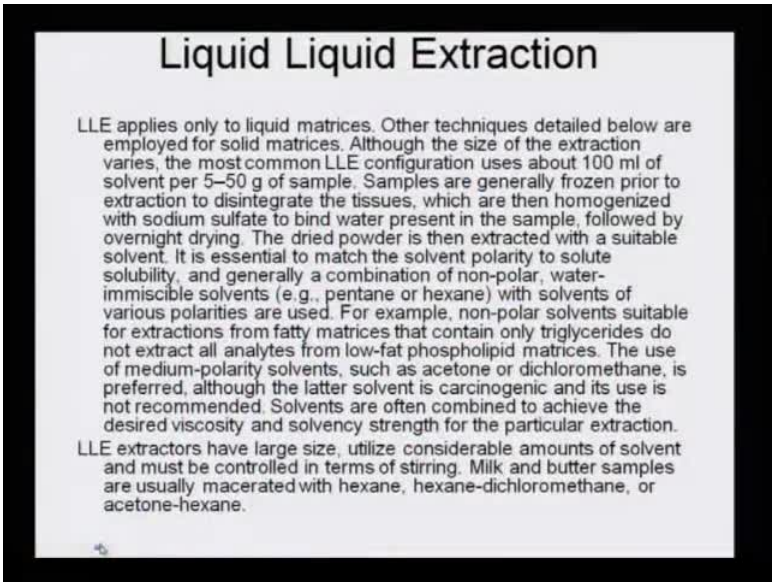

Liquid-liquid extraction - I am going to talk more in details about this process, because it is supposed to be one of the most commonly used extraction methods in every laboratory, whether it is an organic laboratory, whether it is an inorganic laboratory or an analytical laboratory. Liquid-liquid extraction or in short form we call it LLE, applies only to liquid matrices. Other techniques detailed below are employed for solid matrices.

Although the size of extraction varies, the most common liquid-liquid extraction configuration uses about 100 ml of the solvent per 5 to 50 gram of solvent or sample. Samples are generally frozen prior to extraction to disintegrate the tissues, which are then homogenized with sodium sulphate to bind water present in the sample, followed by overnight drying. The dried powder is then extracted with a suitable solvent. It is essential to match the solvent polarity to solute solubility, and generally, a combination of non-polar, water immiscible solvents, that is, the use of pentane or hexane with solvents of various polarities are used.

For example, non-polar solvents suitable for extractions from fatty matrices that contain only triglycerides do not extract all analytes from low-fat phospholipid matrices. The use of medium-polarity solvents, such as acetone or dichloromethane, is preferred, although the latter solvent is carcinogenic and its use is not recommended. But this is only under the circumstance, where the analyte is not coming very well into the solvent material; only then, acetone and dichloromethane type of solvents must be used. Solvents are often combined to achieve the desired viscosity and solvency and through the proper strength of the particular extraction. So, that is why one has to make the right choice of solvent and go ahead with liquid-liquid extraction.

Liquid-liquid extractors are large in size, utilize considerable amounts of solvent and must be controlled in terms of stirring. Milk and butter samples are usually macerated with hexane or hexane dichloromethane or acetone hexane. So, sometimes combinations of solvents are also required for liquid-liquid extraction.

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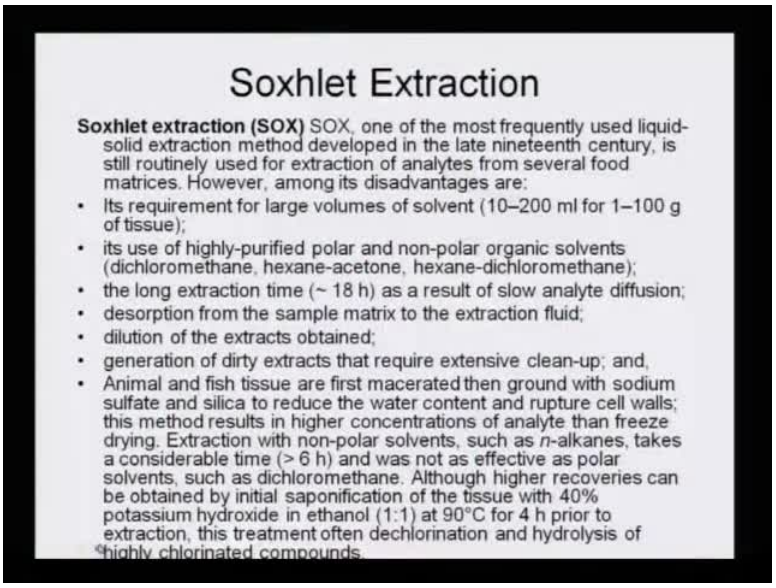

The first type of extraction is the Soxhlet extraction. Soxhlet extraction or SOX is one of the most frequently used liquid-solid extraction method developed in the late 19th century and is still routinely practiced in every laboratory. It is used for extraction of analytes from several food matrices or any other situation. However, among its disadvantages are that it requires large volumes of solvent; that means, almost 10 to 200 ml for 1 to 100 grams of the tissues. So, the amount of solvent that must be used in Soxhlet extractor is very large. It is used for highly polar and non-polar solvents, such as dichloromethane, hexane-acetone, hexane-dichloromethane - are also recommended, which are not very easily available and have a carcinogenic effect also. So, they are avoided, but sometimes, Soxhlet extractors have to use this kind of combination of solvents. The long extraction hours, that means, it may take almost 8 to 18 hours as a result of slow analyte diffusion, is also a good point to be noted in terms of its disadvantages.

Desorption from the sample matrix to the extraction fluid also takes place very slowly. Dilution of the extracts are always there, because large volumes of solvents are used. Generation of dirty extracts that require extensive clean up and animal and fish tissues are first macerated, then ground with sodium sulphate and silica to reduce water content and rupture the cell walls. This method results in high concentrations of analyte than freeze-drying. Extraction with non-polar solvents, such as n-alkane, takes a considerable time, as long as 6 hours, and was not as effective as polar solvents are, such as

dichloromethane. Although, higher recoveries can be obtained by initially doing a saponification of the tissues with 40 percent of potassium hydroxide in ethanol - 1 is to 1, at 90 degrees, for 4 hours prior to extraction. However, this treatment often dechlorinates and hydrolysis some of the chlorinated products. So, one has to make this difference in the mind, whether this soxhlet extraction should be carried out with polar solvents or non-polar solvents or with mixtures.

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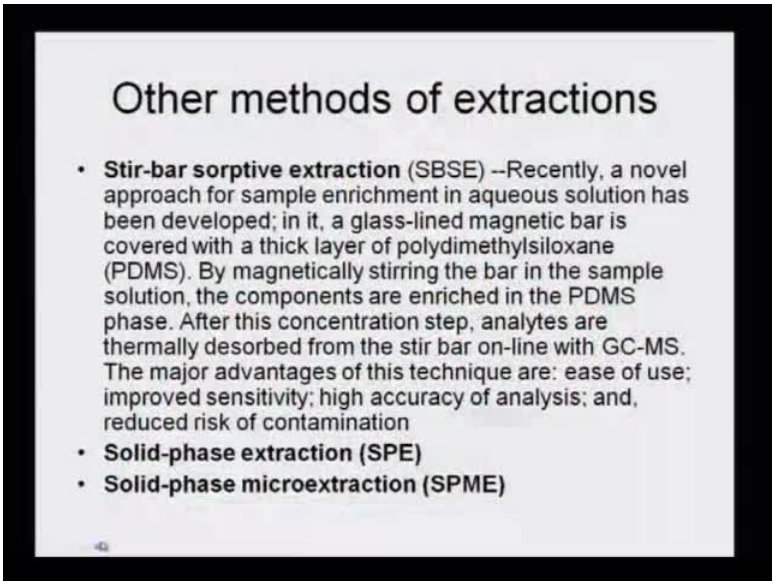

Other methods of extractions - I am trying to draw your attention to the various other techniques in the extraction methodology. The second one in the line is called Stir-bar sorptive extraction or in abbreviation, we call it SBSE. Recently, a novel approach for sample enrichment in aqueous solution has been developed; it is a glass-lined magnetic bar and is covered with a thick layer of polydimethylsiloxane - PDMS. By magnetically stirring the bar in the sample solution, the components are enriched in this PDMS, which is revolving in that or stirring in that phase. After this concentration step, the analytes are thermally desorbed from the stir bar on-line with the GC-MS. The major advantages of this technique are that it is very easy to use; it has improved sensitivity; it has high accuracy of analysis and has reduced rate of contamination.

I would like to slightly elaborate, that there is a stirring bar, and the analyte is in a round bottom flask, and the stirring bar is actually covered with polydimethylsiloxane - PDMS. It is this polydimethylsiloxane, which absorbs the analyte. So, that is how it is called

Stir-bar sorptive extraction. Similarly, we have solid-phase extraction, that is, SPE. We have solid-phase microextraction, which is SPME.

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The third in line is called Supercritical fluid extraction or we call it in abbreviation SFE. SFE has attracted intense interest during the past 20 years, mainly for extraction of solid samples, because it offers short extraction times and minimum use of organic solvents. A SFE is defined as an element or compound above its critical pressure and temperature, resulting in a fluid that is more penetrative and of high solvating power. Carbon dioxide has been popular because of it is low cost, availability and safety, and its suitable critical temperature is 31.2 degree Celsius and pressure 72.8 atm.

I would like to slightly elaborate on this SFE method, where one would try to understand that all you know about carbon dioxide that it is a gas, but actually, this carbon dioxide gas is compressed and made into liquefied carbon dioxide. This liquefied carbon dioxide then has more penetrating power and solvating power to take away the analyte, and do the extraction process. The beauty of this process is that from the same material, keeping this critical temperatures and pressures at a different values, different compounds can be extracted in phases; so, nothing goes wasted.

Extraction of food, particularly fish and seafood, by SFE to determine different contaminants; organic contaminants dissolve easily in carbon dioxide; it is the liquefied carbon dioxide and are co-extracted with lipids. Fat retainers are usually introduced into

the extraction thimble to achieve a fat-free extract; the most commonly used fat-free retainers are basic alumina, neutral alumina, florisil and silica; they absorb the fat only. An option for reducing fat co-extraction was the use of milder extraction conditions; however, these conditions often result in incomplete extraction of poly chlorinated biphenyls. So, one has to make a very good judgment of what to use as a co-extractant.