Advance Analytical Course

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Lecture No. # 13

Enzymatic digestion and ultrasonification - a powerful combination in analytical chemistry.

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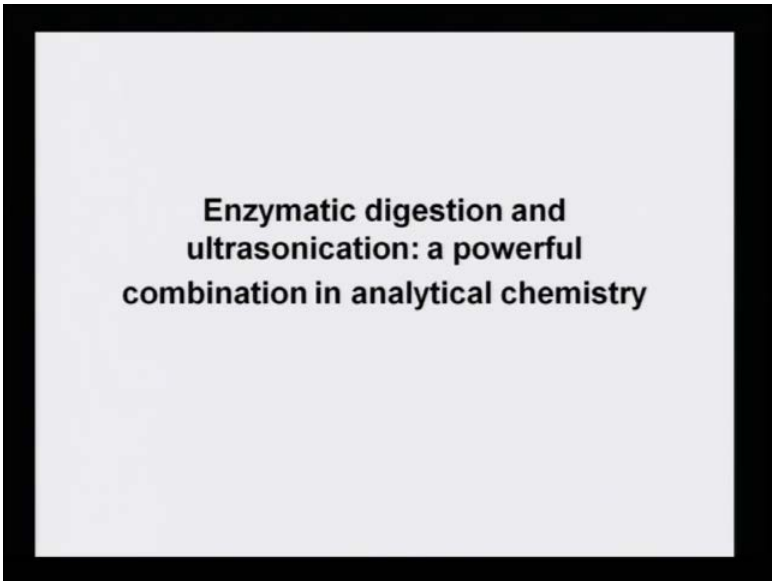

Enzymatic digestion and ultrasonication - a powerful combination in analytical chemistry, as you would all know that it is important to get the extract of the analyte carefully out of the matrix and that is why different methods have been adapted for the extraction of the analyte from the matrix.

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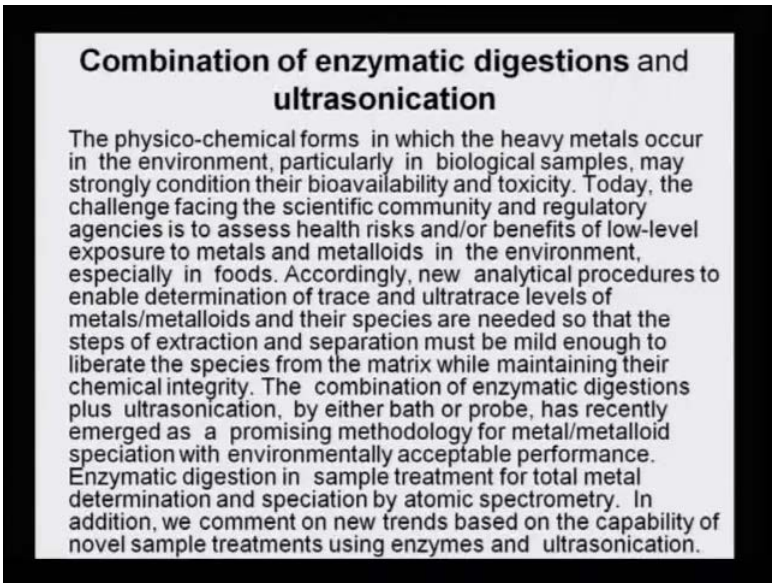

Combination of enzymatic digestion and ultrasonication has brought about a lot of changes and advancement in the process of extraction of the analyte. The physico-chemical forms in which the heavy metals occur in the environment, particularly in biological samples, may strongly condition their bioavailability and toxicity. Yesterday, we were talking about the bioavailabilities of POPs. Similarly, metals need to be extracted properly so that they are into the mat from the matrix into the analyte. Today, the challenge facing the scientific community and regulatory agencies is to assess health risk and benefits of low-level exposure to metals and metalloids of low level exposure to metals and metalloids in the environment, especially in foods.

Accordingly, new analytical procedures to enable determination of trace and ultra-trace levels of metals and metalloids and their species are needed, so that the steps of extraction and separation must be mild enough to liberate the species from the matrix, while maintaining their chemical integrity. The combination of enzymatic digestion plus ultrasonification, by either bath or probe, has recently emerged as a promising methodology for metal metalloid speciation with environmentally acceptable performance; enzymatic digestion in sample treatment for total metal determination and speciation by atomic spectrometry. In addition, we recommend and we also comment on new trends based on the capability of the novel sample treatments using enzymes and soni ultrasonification.

Now, it is important to understand, why we are talking about these two things; they are quite different, but they are used together, or sometimes separately, to be able to get the metal or the metalloid out of the matrix. Unless and until the metal comes out completely from the matrix, the results or the data derived from the atomic spectrometry will not be correct. So, this particular field and this particular lecture, I have dedicated for enzymatic digestion and ultrasonification because it is a very modern technique which is being acceptive and practised by the analysts all over the world.

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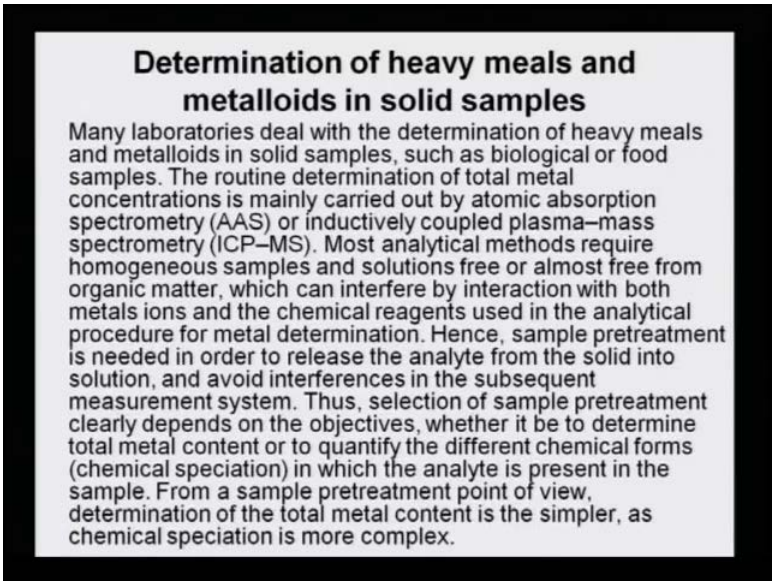

Determination of heavy metals and metalloids in solid samples - many laboratories deal with the determination of heavy metals and metalloids in solid samples, such as biological or food samples. The routine determination of total concentration is mainly carried out by atomic absorption spectrometry or AAS.

Now, here, today's lecture is completely dedicated to how metals can be extracted and analysed, and the metal extraction is carried out successfully with the help of enzymatic digestion and ultrasonification. Another thing that is important is that, for metal analysis, the machine that is used for the identification and characterization, quantification and qualification, is atomic absorption spectrometry or it is inductively coupled plasma mass spectrometry that is ICP-MS.

Most analytical methods require homogenous samples and solution, free or almost free from the organic matter, which can interfere by interaction with both metal ions and the

chemical reagents used in the analytical procedure for metal determination. Hence, sample pre-treatment is needed in order to release the analyte from the solid into solution and avoid interferences in the subsequent measurement system. Thus, selection of sample pre-treatment clearly depends on the objectives, whether it be to determine total metal content or to quantify the different chemical forms that is the chemical speciation, in which the analyte is present in the sample. From a sample pre-treatment point of view, determination of total metal content is the simpler, as chemical speciation is more complex.

Now, whether it is a food sample, whether it is a biological sample, that means if it is from any tissues or fish or any kind of biological sample, how to be able to extract the metal is a big challenge because the better the extraction, the more correctness comes in the result.

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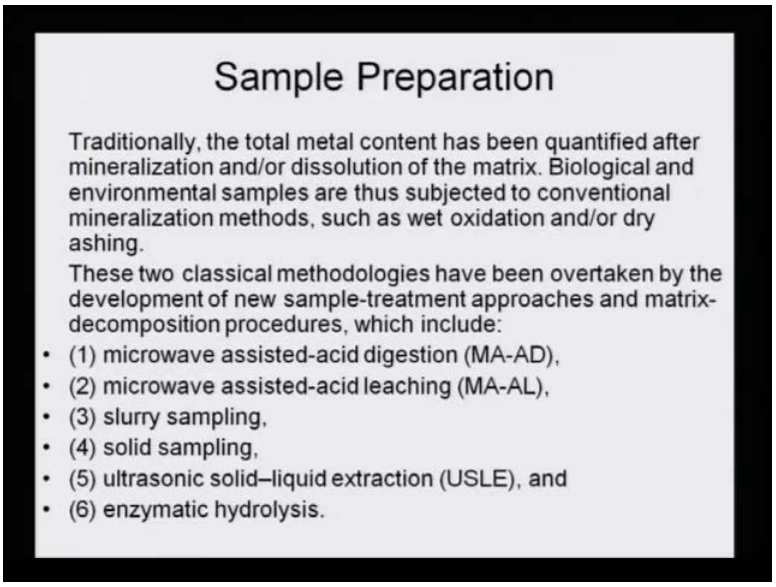

Sample preparation, therefore, becomes a very important factor. Why? because it is the prime and the crux of the whole and analysis. If the extraction or the sample preparation is not done correctly, the data derived from the analysis will never be correct. So, time and again, I have again and again repeatedly told that, for an analyst, sample preparation is the most crucial step; however, precaution has to be carried out throughout analysis.

Sample preparation – traditionally, the total metal content has been quantified after minera mineralization and/or dissolution of the matrix. Biological and environmental

samples are thus subjected to convention conventional mineralization methods, such as wet oxidation and/or dry ashing. So, one can do it by digesting it with wet acids like dilute Nitric acid or dilute Sulphuric acid or concentrated Nitric acid or concentrated Sulphuric acid which are wet oxidation process, or by simply ashing the material, that is charring the material in a at a high temperature in an oven.

These two classical methodologies have been overtaken by the development of new sample-treatment approaches and matrix-decomposition procedures which include: microwave acid digestion which will be called as MA-AD, microwave assisted-acid leaching which will be called as MA-AL, slurry sampling, solid sampling, ultrasonic solid-liquid extraction (USLE), and enzymatic hydrolysis.

So, now, you see that, from the classical methods of just simple digestion, or simply wet oxidation and ashing, now, 5 new or 6 new sample preparation methods have come up in the recent trend; microwave acid digestion, microwave assisted-acid leaching, slurry sampling, solid sampling, ultrasonic solid-liquid extraction, and enzymatic hydrolysis.

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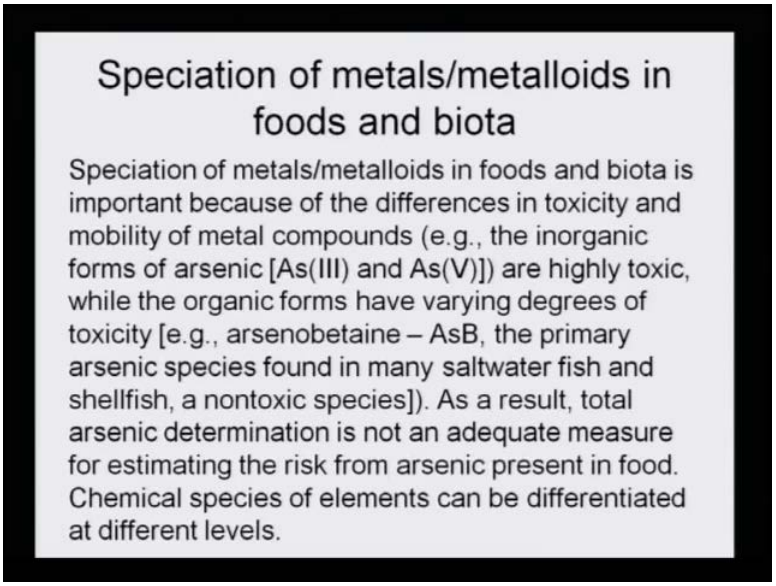

Speciation of metal/metalloids from food items and biotic material - speciation of metal and metalloids in food and biological sample is very important because of the differences in the toxicity and mobility of the metal compounds. For example, the inorganic forms of arsenic, that is the arsenic 3 and the arsenic 5, are highly toxic, while the organic forms have varying degree of toxicity that is arsenobetaine, the primary arsenic species found

in many saltwater fish fishes and shellfish are nontoxic species. So, the same arsenic compound, when it is in its inorganic form is highly highly toxic, but when it becomes an organometallic compound like arsenobetaine, it is not toxic because it is found in fishes and shellfishes. As a result, total arsenic determination is not an adequate measure for estimating the risk from arsenic present in food. Chemical species of elements can be differentiated at different level.

So, you see, whether it is in its inorganic form, or its organic form, will actually make it possible to know, how much it is available and what is the risk factor that is associated with it.

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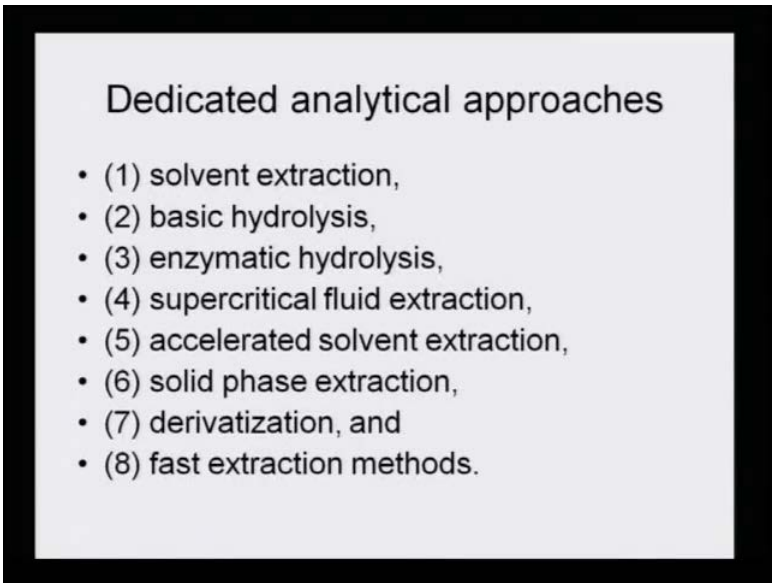

Dedicated analytical approaches are that, for such a metal/metalloid analysis, solvent extraction, basic hydrolysis, enzymatic hydrolysis, supercritical fluid extraction, accelerated solvent extraction, solid phase extraction, derivatization, and fast extraction methods are required to be approached. It is not that, all of them have to be used together; the procedure can be selective; for a particular metal, one of these methods can be selected.

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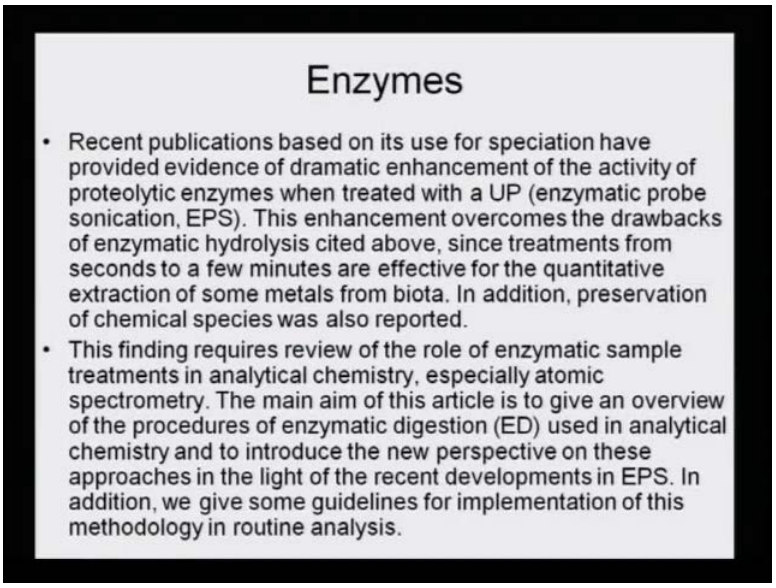

Enzymes – now, enzymatic digestion - what role does it play and why is it necessary for using it in the biotic sample or the food sample? Recent publications, based on its use for speciation have provided evidence of dramatic enhancement of the activity of proteolytic enzymes when treated with the UP that is enzymatic probe sonification. This enhancement overcomes the drawbacks of enzymatic hydrolysis cited above, since treatments from the seconds to a few minutes are effective for the quantitative extraction of some metals from biota. In addition, preservation of chemical species are also reported.

Now, what is an advantage over wet oxidation with very harsh acid vis-à-vis the enzymes? The enzymes are much milder and when it is used in combination with sonification, ultrasonification, what happens is that, at a very short period of time, the metalloid or the metal is extracted without disturbing or without interfering the other chemical species that are present in the biota. This finding requires review of the role of enzymatic sample treatments in analytical chemistry, especially atomic spectrometry.

The main aim of this particular lecture is to give an overview of the procedures of enzymatic digestion, which I will be referring as ED, used in analytical chemistry and to introduce the new perspective on these approaches in the light of the recent developments in the enzymatic digestion process. In addition, we give some guidelines for implementation of this methodology in routine analysis also. Time and again, I have

taken different types of examples because I feel that it is important for you to know that where all and why all you should use this enzymatic digestion or a particular methodology, so that you know the discrimination between the two - what to use? Where?

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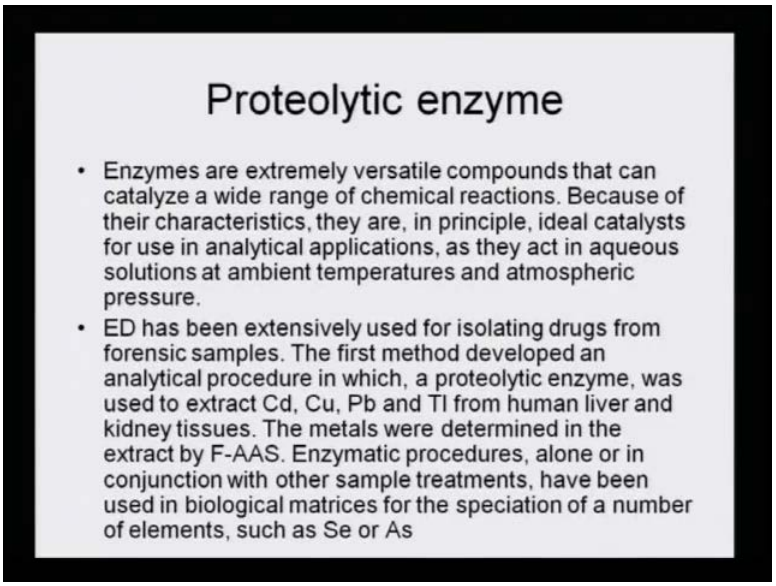

Proteolytic enzyme - enzymes are extremely versatile compounds that can catalyse a wide range of chemical reactions. Because of their characteristics, they are, in principle, ideal catalysts for use in analytical applications, as they act in aqueous solutions at ambient temperatures and atmospheric pressures. So, you see, they work at a very ambient temperature and pressure. No high pressure temperatures are required and they are more or less like a catalyst in their behaviour.

Enzymatic digestion (ED) has been extensively used for isolation of drugs from the forensic samples. The first method developed an analytical procedure in which, a proteolytic enzyme, was used to extract Cadmium, Copper, Lead, and Tellurium from human liver and kidney tissues. The metals were determined in the extract by the flame AAS; the flame AAS is referred as F-AAS. Enzymatic procedures, alone or in conjunction with other sample treatments, have been used in biological matrices for the speciation of a number of elements, such as Selenium and/or Arsenic.

Now, here, I would like to draw your attention because it is an important area of analysis where metal and metalloids have to be analysed, and if a milder method can be adapted

for the extraction of metal from the matrix, it is always better than using very harsh or acids or ashing because that destructs many other chemical species which need to be analysed at a later stage.

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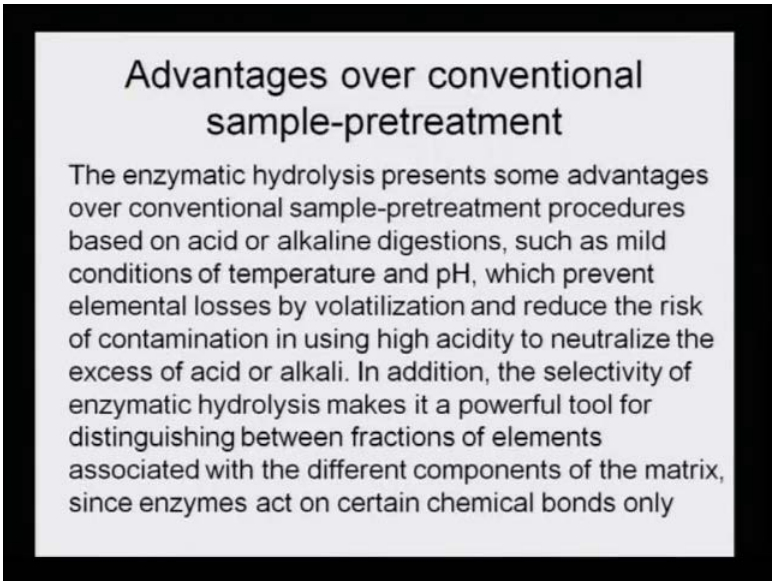

Advantages over conventional sample preparation or sample pre-treatment - the enzymatic hydrolysis presents some advantages over conventional sample pre-treatment procedures based on acid or alkaline digestions, such as mild conditions of temperature and pH, which prevent elemental losses by volatilization and reduce the risk of contamination in using high acidity to neutralize the excess of acid or alkali. In addition, the selectivity of the enzymatic hydrolysis makes it a powerful tool for distinguishing between fractions of elements associated with the different components of the matrix, since enzyme act as a certain chemical bonds only.

So, you see that enzymes are milder as compared to these acids or alkali digestions and they work at a very ambient and low temperature, and at a very normal pH; they do not work on very harsh conditions and that is why there is an advantage over the conventional sample treatment methods.

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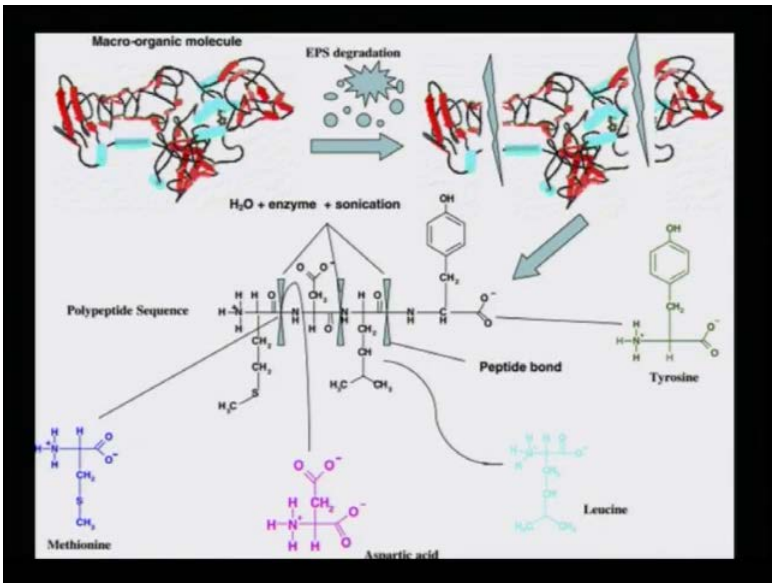

Now, this is a very very pictorial description of a macro organic molecule where the enzymatic proteolytic enzyme digestion, how does it help to break down the peptide linkages, and then the amino acids are released in a very mild manner.

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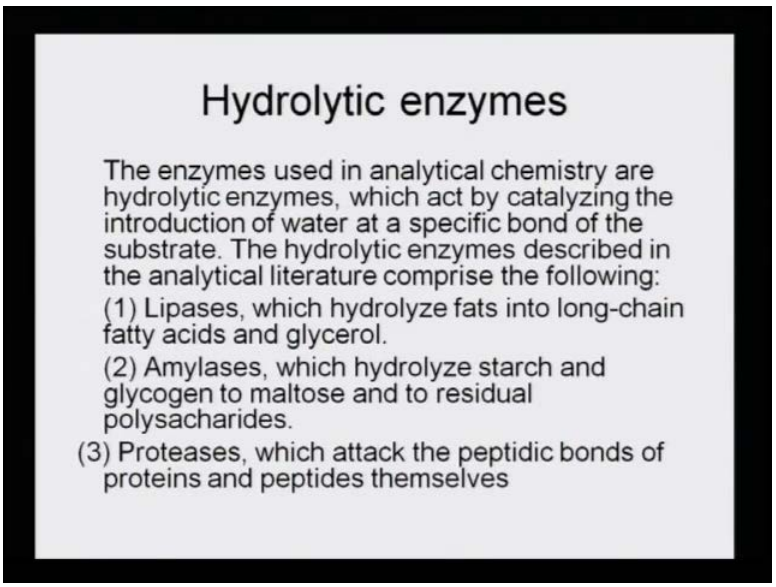

Hydrolytic enzymes - the enzymes used in analytical chemistry are hydrolytic enzymes, which act by catalysing the introduction of water at a specific bond of the substrate. Hydrolysis - the word itself means that breaking a bond with the help of water. Hydrolytic enzyme means, an enzyme which is making use of water and breaking the

chemical bond in the macro molecule. The hydrolytic enzyme described in the analytical literature comprise of the following: it is either the class which is called lipases which hydrolyse fat into long-chain fatty acid and glycerol, or it could be amylases which hydrolyse starch and glycogen to maltose and to residual polysaccharides, or the third class could be proteases, which attack the peptide bonds of the proteins and peptides themselves and break them into amino acids.

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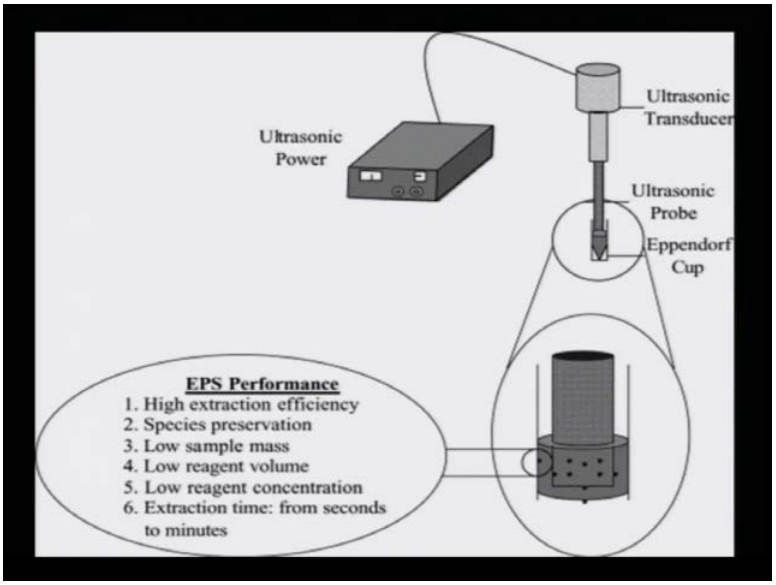

This is how the ultrasonic machine looks like; high it the enzymatic performance has certain advantages: it has high extraction efficiency, species preservations, low sample mass, low reagent volume, low reagent concentration, and the extraction time is only a few seconds to minutes. So, that is the beauty that, if we use enzymatic digestion in an ultrasonic probe. What happens is that, the ultra-sonication helps this whole reaction to take place very fast; just from time that is required is few seconds to a few minutes and there is no long drawn process. And this is how, there is an ultrasonic transducer and there is a probe and there is a cup where the analyte is kept, and the digestion takes place in a very efficient manner.

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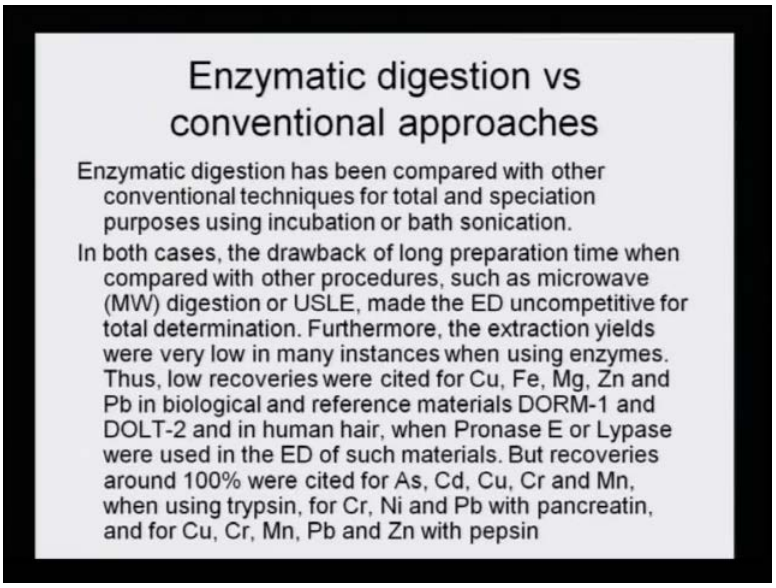

Enzymatic digestion - if it has to be compared with conventional approaches, what would be the outcome? Enzymatic digestion has been compared with other conventional techniques for total and speciation purposes using incubation or bath soni sonication. In both cases, the drawback of long preparation time when compared with other procedures, such as microwave digestion or USLE, made the enzymatic digestion uncompetitive for total determination. Furthermore, the extraction yields were very low in many instances when using enzyme. Thus, low recoveries were cited for Copper, Iron, Magnesium, Zinc, and Lead in biological and reference materials. And so, from the samples of human hair that is DORM-1 and DOLT-2, when Pronase E and Lypase were used in the enzymatic digestion of such materials. But recoveries around 100 percent were cited for Arsenic, Cadmium, Copper, Chromium, and Manganese, when using trypsin, for Chromium, Nickel and Lead with pancreatin, and for Copper, Chromium, Manganese, Lead and Zinc with pepsin.

So, now you see that, there is a very clear cut idea that these enzymes are very very sensitive, not only to the digestion process and efficiency for certain metals, and therefore there is a demarcated compatibility between the metal and the enzyme. You saw that different biological samples when treated with different kind of enzyme, for the similar type of metals, showed their preferences in a different manner. Therefore, there is a compatibility factor which is very important.

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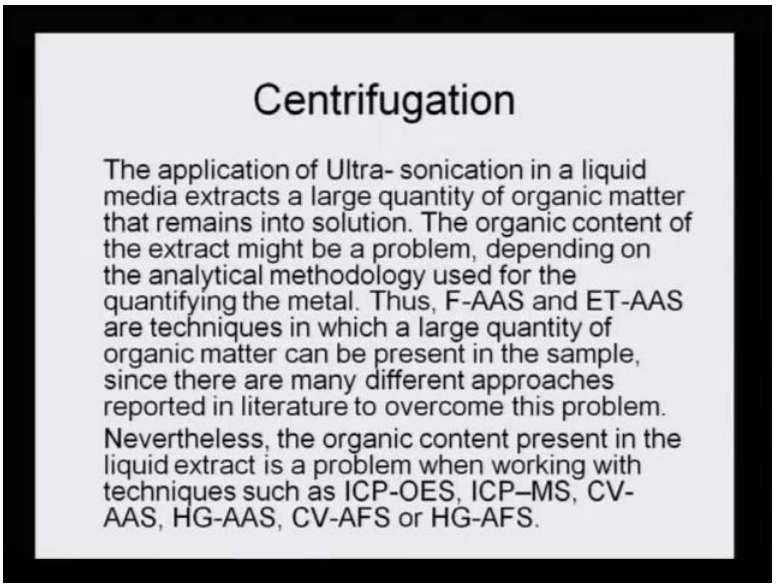

Centrifugation – now, the better the agitation, the better is the result because more and more analyte, more and more metal or metalloid will come into analyte. The application of ultra-sonication in a liquid media extracts a large quantity of organic matter that remains into solution. The organic content of the extract might be a problem, depending on the analytical methodology used for the quantifying the metal. Thus, flame AAS and ET-AAS are the techniques in which, a large quantity of organic matter can be present in the sample, since there are many different approaches reported in the literature to overcome this problem.

Nevertheless, the organic content present in the liquid extract is a problem when working with techniques such as ICP-OES, ICP-MS, CV-AAS, HG-AAS, CV-AFS and HG-AFS. These are different types of atomic spectrometry or inductively couple plasma spectrometry where different techniques have been applied, but the problem is that, when metal has to be extracted from organic material or biotic material, the extraction process becomes very challenging, and therefore, a proper combination of either an enzyme or sonication or both, and milder conditions should be adapted.

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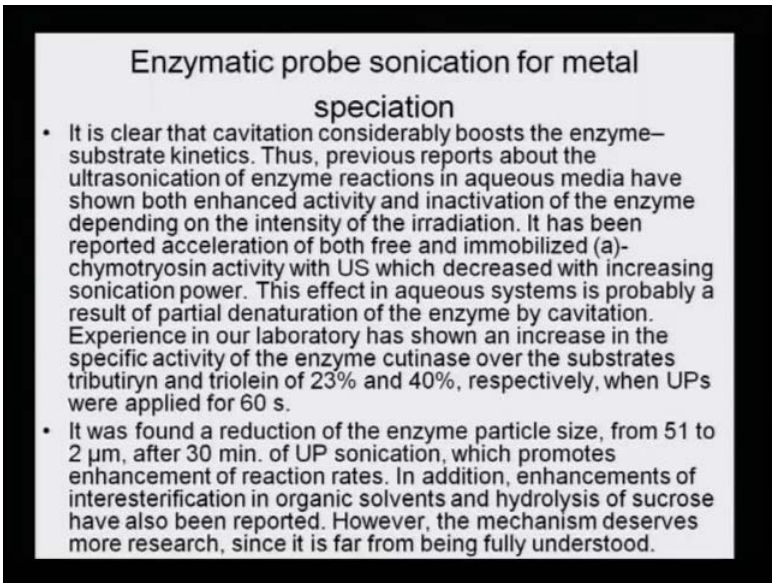

Enzymatic probe sonification for metal speciation - it is clear that cavitation considerably boosts the enzyme-substrate kinetics. Thus, previous reports about the ultra-sonication of enzyme reactions in aqueous media have shown both enhanced activity and inactivation of enzyme depending on the intensity of irradiation. It has been reported acceleration of both free and immobilized chymotrypsin activity with the ultra-sonication which decreased and increasing with the increasing power of sonication power. This effect in aqueous system is probably a result of partial denaturisation of the enzyme by cavitation. So, there could be some problem even while using very mild conditions.

Experience in our laboratory have shown an increase in the specific activity of the enzyme cutinase over the substrates tributiryn and triolein of 23 and 40 percent, respectively, with the ultra-sonification were applied for just 60 seconds; it was found a reduction of enzyme particle size from 51 to 2 micrometre, about in 30 minutes of the ultrasonification, which promotes the enhancement of the rate of reaction. In addition, enhancement of interest interesterification in organic solvents and hydrolysis of sucrose have also been reported. However, the mechanism deserves more research, and it is far from being fully understood.

So, now, lot of things keep happening because enzyme also then starts taking part in other chemical reactions. So, there is always a fear of what is going to happen and what

will be the kinetics? Which will be extracted faster? And that is why a lot of research is still going on when using enzymatic probe sonification.

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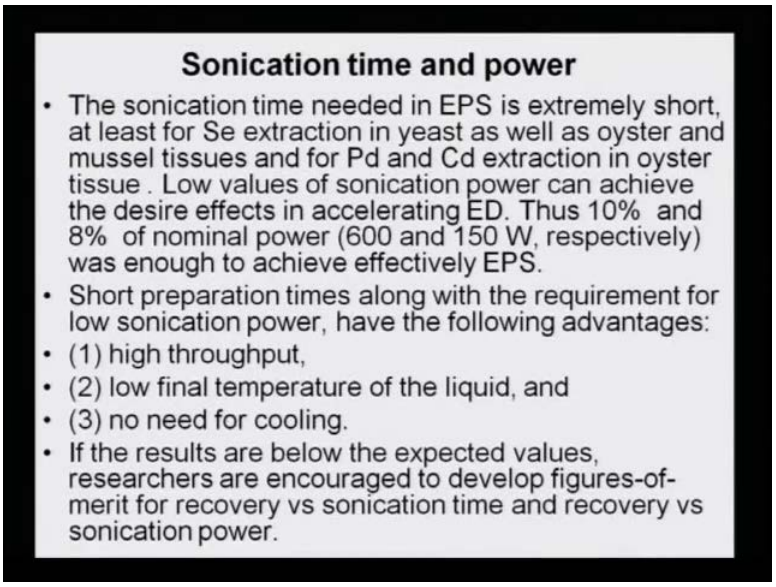

Sonication time and power - the sonication time is very important and it needs to be understood very carefully in EPS, and it is extremely short, at least for selenium extraction in yeast as well as oyster and mussel tissues and for palladium and cadmium extraction in oyster tissue. Low values of sonication power can achieve the desired effects in accelerating ED. Thus, 10 percent and 8 percent of nominal power which is 600 or 150 Watt, respectively, was enough to achieve the effective EPS.

Short preparation times along with the requirement for low sonication power, have the following advantages: it has high throughput, low final temperature of the liquid, and no need for cooling because when we are agitating for only a short while, it will not generate very big heat at the point of the reaction.

Let me explain you, what exactly happens when the sonication takes place. When sonication is taking place, there is a generation of micro bubbles. These micro bubbles then come together and implode. When the implosion takes place, there is an instant rise in temperature and rise in pressure. Now, that is what is helping in the digestion process, but when? All this is happening at a slightly higher temperature than the room temperature. So, the temperatures are not very very high; it is not at boiling temperature and that is why there is no need to cool, and the final temperature of the liquid need not

to be manipulated or cooled down, or there should not be an ice bath to manoeuvre it. And the total result of this implosion is so high and it digests so fast at that particular point of implosion. that It is the most efficient process that can be adapted for metal metalloid analysis.

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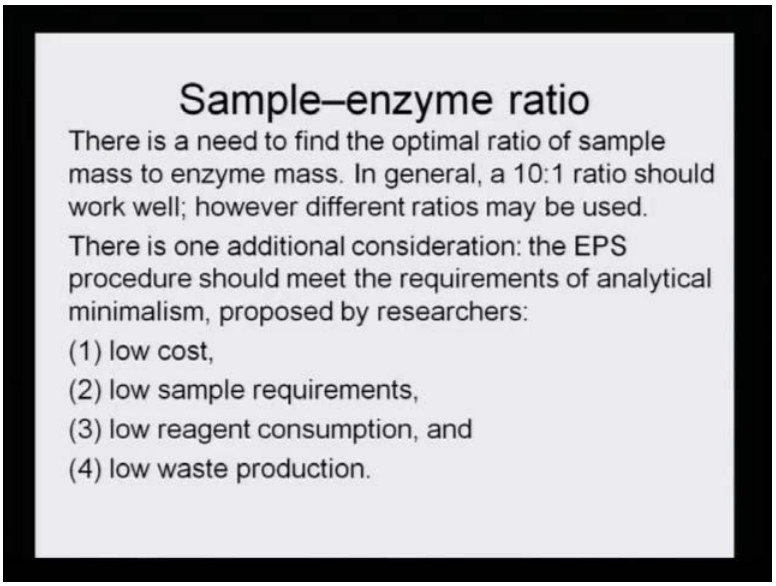

Sample-enzyme ratio – now, this is very critical; if we use very large amount of enzyme, enzymes will start getting into reaction with the other part of the biological material. Therefore, there is need to find the optimal ratio of sample mass to enzyme mass; in general, 10 is to 1 ratio should work well; however, different ratios may be used. There is no additional consideration. The EPS procedure should meet the requirement of analytical minimization, proposed by the researchers. It should have a low cost, low sample requirements, low reagent consumption and low waste production. It is not that bulk of enzyme is required; otherwise, the procedure will not be low cost and it will not be feasible for an analyst to use enzymes.

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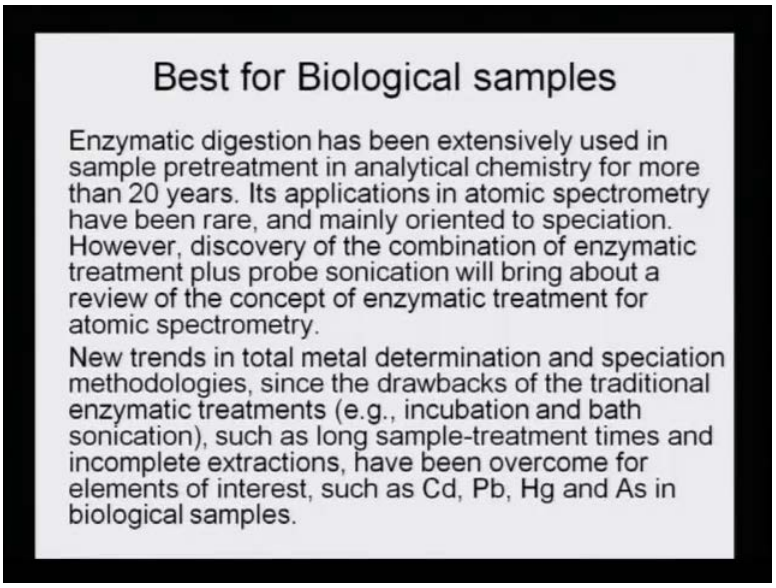

Best for biological samples – why enzymes are better than harsh acid? Because they are compatible with the biotic material. Enzymatic digestion has been extensively used in sample pre-treatment in analytical chemistry for more than 20 years. Its application in atomic spectrometry has been rare, and mainly oriented to speciation. However, discovery of the combination of enzymatic pre-treatment does however discovery of the combination of enzymatic treatment plus probe sonication will bring about a review of the concept of enzymatic treatment for atomic spectrometry. So, far it was not a very prevalent method. However, this particular step in the advancement of use of enzyme for atomic spectrometry sample preparation is a very new trend.

New trends in total metal determination and speciation methodologies, since the drawbacks of the traditional enzymatic treatments (incubation and bath sonication), such as long sample-treatment times and incomplete extractions, have been overcome for the elements of interest, such as Cadmium, Lead, Mercury and Arsenic in biological samples.

Why are these analyses so important? Because, all these metals Cadmium, Lead, Mercury, Arsenic, Copper, Chromium, Zinc, Nickel - these are all heavy metals and they have been labelled as very notorious toxic metals for the biological system. So, that is why, methods have to developed, and very accurate methods have to be developed, so

that it can be a used for especially for biological sample and the extraction of metal and metalloids from biological samples.

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Now, we come to another topic which is also related to this; focused-microwave-assisted strategies for sample preparation. So, you see that, sample preparation is such a crucial step that, at every stage, we need to take care, and from sample to sample new modification and new methods have to be adapted, and that is why this lecture is dedicated to focused-microwave-assisted strategies for sample preparation. You had seen in the previous lecture that microwave assisted digestion was adapted for sample preparation for biotic samples as well as food samples. You also saw that microwave acid leaching methodology was also adapted.

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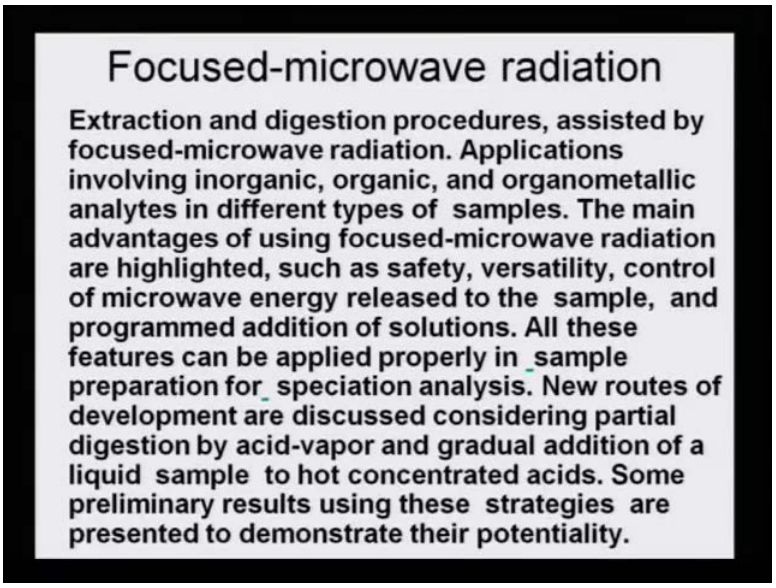

Focused-microwave radiation - extraction and digestion procedures, assisted by focused-microwave radiation; applications involving inorganic, organic, organometallic analytes in different types of samples. So, it is not just restricted to biological sample or food sample; it can be used for inorganic sample, it can be used for organic sample, and it can be used for organometallic samples of all types.

The main advantages of using focused-microwave radiation are highlighted, such as safety, versatility, control of microwave energy released to the sample, the programmed addition of the solution - all these features can be applied properly in sample preparation for speciation analysis. New routes of developments are discussed considering partial digestion by acid-vapour and gradual addition of a liquid sample to hot concentrated acids are no more required to be done. Some preliminary results using these strategies are presented to demonstrate their potentiality.

Now, you see that, if I am talking about a method and if I do not give you examples where all it can be used or where all the researchers have used these, it will not be fair and it will not give you a proper idea, as to what to use? where?

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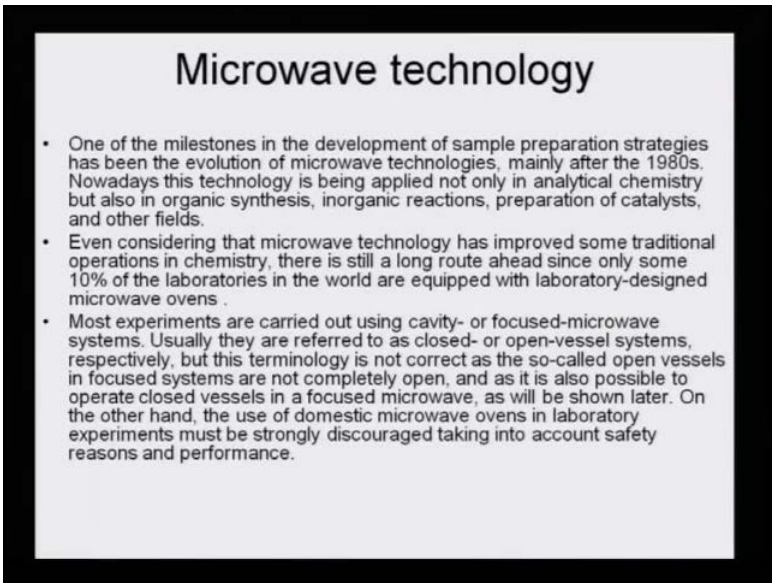

Microwave technology - one of the milestones in the development of sample preparation strategies have been the evolution of microwave technologies, mainly after 1980s. Nowadays this technology is being applied not only in analytical chemistry, but also in organic synthesis, inorganic reactions, preparation of catalysts, and other fields. Even considering that microwave technology has improved from traditional operations in chemistry, there is still a long route ahead since only some 10 percent of the laboratories in the world are equipped with laboratory-designed microwave ovens.

You may have seen microwaves at home. These are smaller microwave which are just used for either heating the food or making some bakery items at home, but about 10 percent of the laboratories in all over the world have these laboratory microwaves, where it can be used for analysis purposes.

Most experiments are carried out using cavity or focused-microwave-systems. As the name suggests, cavity means there is a vessel and there is microwave which is hitting the vessels. So, it is quite focused. So, the name cavity or the focused-microwave; usually they are referred as closed or open vessel system, respectively. But this terminology is not correct, as the so-called open-vessel in focused system are not completely open, and as it is also possible to operate closed-vessels in a focused microwave, as this can be shown later. On the other hand, the use of domestic microwave ovens in laboratory experiments must be strongly discouraged, taking into account the safety reasons and

performances. Now, there is a warning that these domestic microwaves should not be utilized in the laboratory. Why? Because in the laboratory, we are analysing or extracting or digesting very harsh and toxic chemicals. Now, they should not be carried out in the domestic microwaves.

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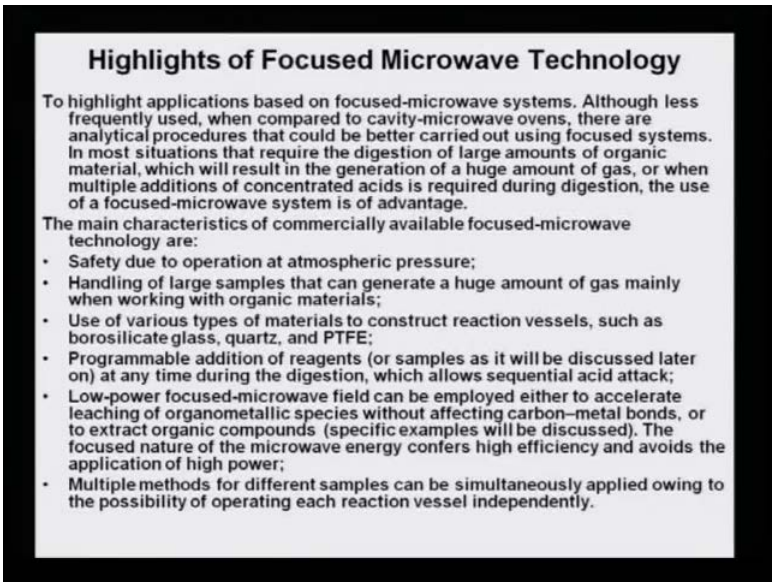

Highlights of focused microwave technology - to highlight application based on focused-microwave systems, although less frequently used, when compared to cavity-microwave ovens; there are analytical procedures that could be better carried out using focused systems. In most situations that requires the digestion of large amounts of organic material, which will result in the generation huge amount of gas, or when multiple additions of concentrated acid is required during digestion, the use of focused-microwave system is of advantage. I will come to it a little later. Let me just first give you the salient features.

The main characteristic of commercial available focused-microwave technologies are: its safety due operation at atmospheric pressure; handling of large samples that can generate a huge amount of gas mainly when working with organic materials; use of various types of material to construct reaction vessels, such as borosilicate glass, quartz, or PTFE. Programmable addition of reagents is possible at any time during the digestion which allows sequential acid attack; low-power focused-microwave field can be employed either to accelerate leaching or organometallic species without affecting carbon-metal

bonds, or to extract organic compounds. The focused nature of the microwave energy confers high efficiency and avoids the application of high power. Multiple methods for different samples can be simultaneously applied owing to the possibility of operating each reaction vessel independently.