Advance Analytical Course

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

Lecture No. # 08

In continuation with our lecture, this particular lecture I have designed based on phytoanalysis - a challenge for analyst. Why I am calling it a challenge, is because it is not easy to analyze each and every component in plants; because, in plants the compounds are in huge number, and a variety of compounds are present, even among say for example, dyes, it would not be that a plant will have only one dye, it will have a number of dyes of similar kinds and a number of dyes of different kinds.

Similarly, polyphenolic compounds could be of different origin and different structural details, and along with that, there would be essential oils, there would be terpenoids, there would be many other compounds; so, it is a huge challenge to be able to analyze plant extract.

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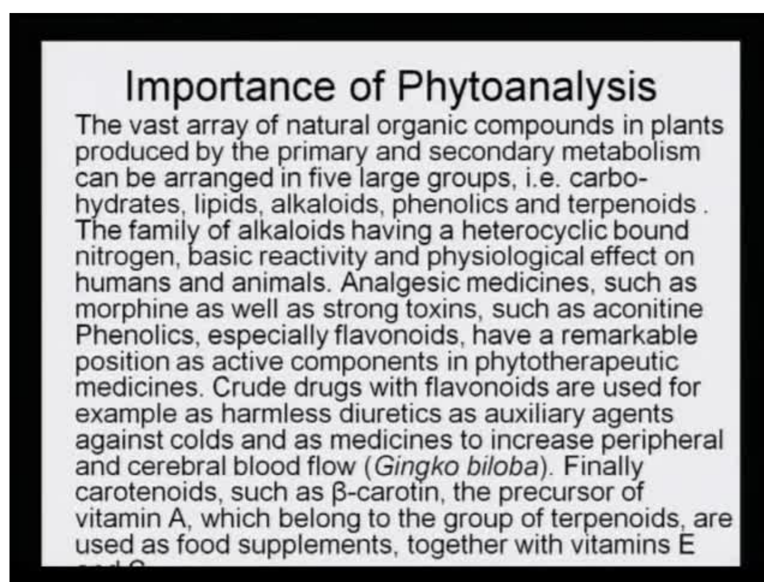

Importance of phytoanalysis - a vast array of natural organic compounds in plants produced by the primary and secondary metabolism can be arranged in five large groups:

carbohydrates, lipids, alkaloids, phenolics and terpenoids. The family of alkaloids having a heterocyclic bound nitrogen, basic reactivity and physiological effect on the human and animals. Analgesic medicines, such as morphine, as well as strong toxins, such as aconitine, phenolics, especially flavonoids, have a remarkable position as active components in phytotherapeutic medicines. Crude drugs with flavonoids are used for medicines.

For example, harmless diuretics as auxiliary agents against cold, and as medicines to increase peripheral and cerebral blood flow, the Gingko biloba are common compounds derived from this biological plant. Finally, carotenoids such as beta-carotin - the precursor of vitamin A - which belongs to the group of the terpenoids are used as food supplements, together with vitamin E and vitamin C. Now, you understand that these are all present in the plants and they have their specific role in our human body.

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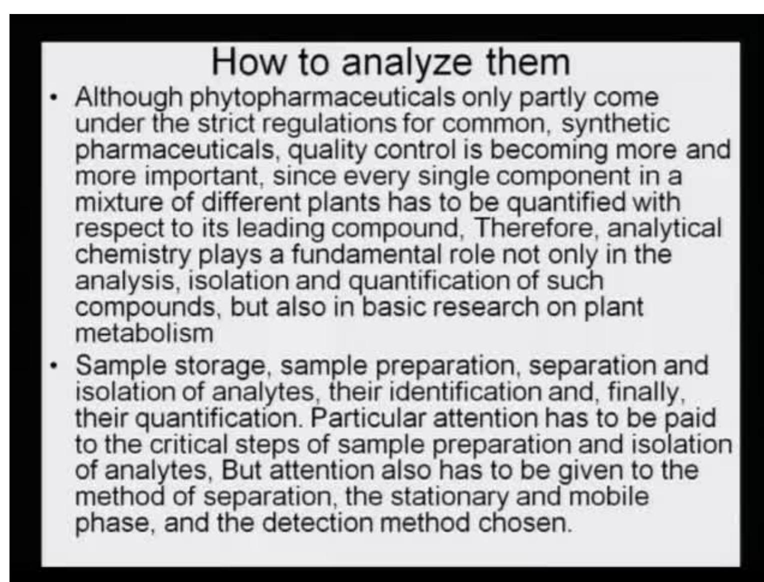

How to analyze them – although phytopharmaceuticals only partly come under the strict regulations for common synthetic phytopharmaceuticals, quality control is becoming more and more important, since every single component in a mixture of different plant has to be quantified with respect to its leading compound. Therefore, analytical chemistry, which plays a fundamental role, not only in the analysis, isolation and quantification of such compounds, but also in basic research on plant metabolism. So, you know, that it is not easy to do this phytopharmaceuticals analysis.

Sample storage, sample preparation and isolation of analytes, their identification, and finally, their quantification is very, very important. Particular attention has to be paid to the critical steps of sample preparation and isolation of the analytes. But attention also has to be given to the method of separation, the stationary and mobile phase, and the detection method chosen.

We have just learned about the various chromatographic techniques; and which one to use where, is what is the crucial decision of the analyst, and if he makes a wrong decision, he gets wrong results. So, it is very important to understand the chemistry of these phytochemicals, and then, based on their structure, their isolation, preparation of sample, and analysis needs to be planned out.

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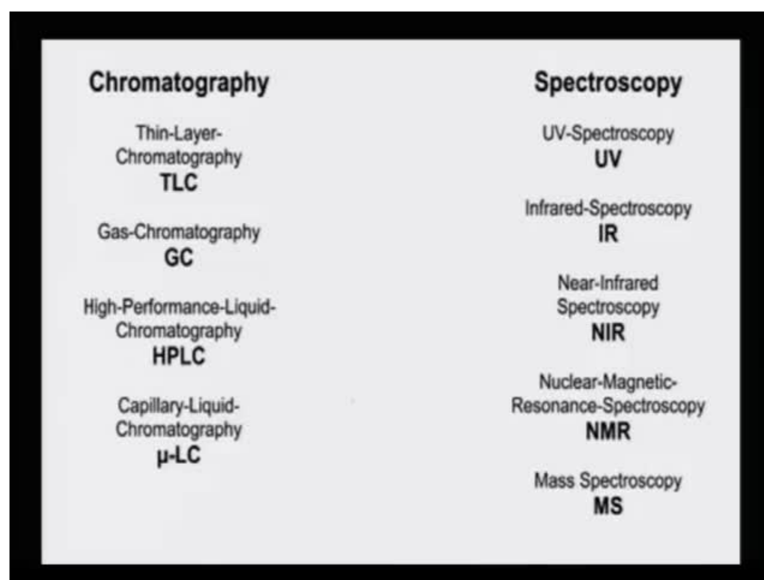

If we look at a phytochemical analysis and the methodology, one can first look at the chromatographic techniques. The first technique that one would like to get an idea of the phytochemicals, is by means of thin layer chromatography. In order to recapitulate what we had done in the past few lectures, I will briefly say that thin-layer chromatography is a small plate on which the compound is potted, and it is eluted with the solvent, and by the capillary action of the solvent, the solvent front rises, and in that coated plate, the silica gel acts as the bound material, the solid material, and the mobile phase, helps the compounds to get separated. And by taking a thin layer chromatographic plate, we understand how many components are present in the phytochemical mixture.

Then, based on the fact that whether we want to analyze the volatiles or whether we want to analyze the non-volatiles, it is either put on gas chromatography or on high performance liquid chromatography or HPLC.

Similarly, depending on the trace quantity analysis, it can also be put on the capillary liquid chromatography, which analyzes micrograms of the components in the phytochemical extract. So, this is more or less the separation part, and which one to use, how to use - the basic information is provided by thin-layer chromatography; and then, the more intricate information about the volatiles is provided by the gas chromatography; and if it has more condensed molecular structures, which have higher molecular weight and higher boiling point, they need to be separated by high performance or high pressure liquid chromatography, that is HPLC.

Once the compounds are separated, they need to be analyzed by spectroscopic methods, and there are no single methods, which can give full information about the molecular structure. We need to depend on various spectroscopic methods to be able to analyze the chemical structure of a compound. For example, UV visible spectroscopy is one such mode, which uses the presence of, or which identifies the presence of the unsaturated functional groups in a molecule.

Infrared spectroscopy gives a part information about the functional groups that are present in a molecule. Similarly, we have near infrared spectroscopy, also a method which identifies only the functional group presence and it also shows how many different types of functional groups are present in a molecule.

The fourth spectroscopic method is called the nuclear-magnetic-resonance-spectroscopy - NMR - which shows two different types of NMR spectroscopy; one is the proton NMR and the other one is the C 13 or the carbon 13 NMR. Now, proton NMR, gives us an idea about the different types of protons that are present around the carbon chain and their immediate interactions with each other; whereas, C 13 NMR gives us the idea of the carbon backbone, which is present in the molecule.

And mass spectroscopy is what gives the total and final information about the fragmentation pattern of a molecule. When we were learning GCMS, I had briefly mentioned about mass spectroscopy, where I had mentioned that mass spectroscopy is important, because it gives the molecular ion peak, which is equivalent to the molecular

weight. And then, the molecule then fragments into different daughter nuclei, and further, it breaks down into smaller fragments, and this breaking down is always similar, and the more labile the bond is, that bound will break faster.

So, with this background that which chromatographic technique we should adapt and what are the different spectroscopic methods that we need to use for analyzing phytochemicals, we proceed further.

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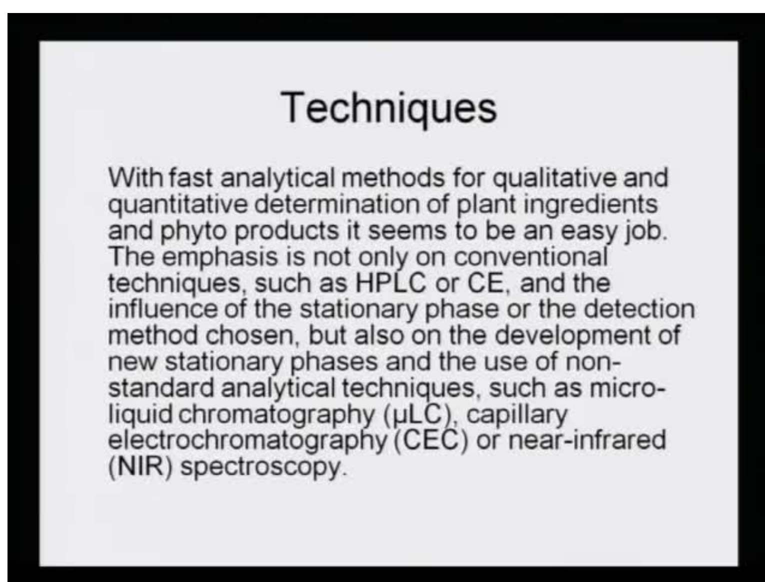

The techniques that are usually applied for phytochemical analysis are: with fast analytical methods for qualitative and quantitative determination of plant ingredients and phyto product, it seems to be an easy job. The emphasis is not only on conventional technique, such as HP or capillary electrophoresis (CE) and the influence of the stationary phase or the detection method chosen, but also on the development of new stationary phases and the use of nonstandard analytical techniques, such as micro-liquid chromatography, capillary electrochromatography or near infrared spectroscopy. So these latter three names that I uttered - micro-liquid chromatography, capillary electrochromatography, near infrared spectroscopy - are very, very typical for plant extract analysis.

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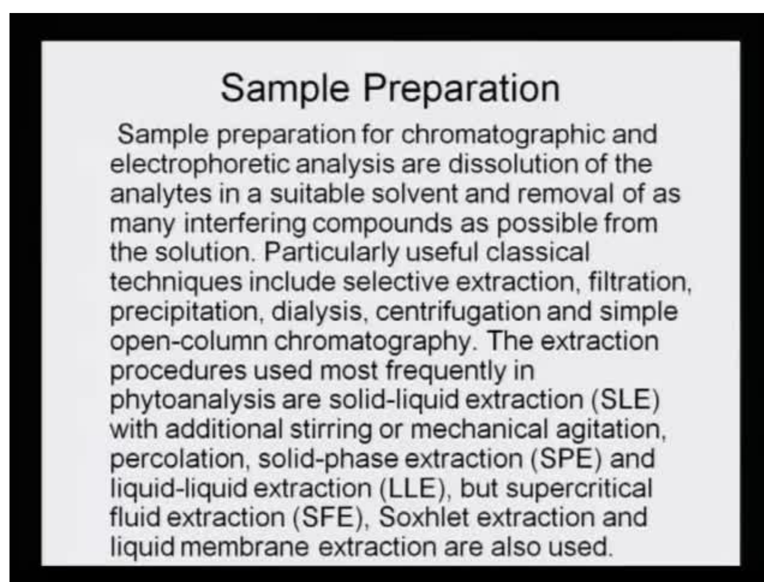

Sample preparation - as I have time and again mentioned, that sample preparation is very crucial; it would be very, very important to understand, that every sample needs to be prepared in a typical manner. There is no standard one method by which the sample can be prepared. Depending on what is the matrix, depending on what is the substance, the sample preparation has to be modified. The better the sample preparation, the better would be the result.

Sample preparation for chromatographic and electrophoretic analysis, are dissolution of the analytes in a suitable solvent and removal of as many interfering compounds as possible from the solution. So, all the interfering compounds must be removed, only the total extract or the electrophoretic compound or the chromatographic suitable compound should come into the medium.

Particularly useful classical techniques include: selective extraction, filtration, precipitation, dialysis, centrifugation and simple open-column chromatography. The extraction procedures used most frequently in phytoanalysis are solid-liquid extraction. The extraction procedures used more frequently in phytoanalysis are solid-liquid extraction or SLE with additional stirring or mechanical agitation, percolation, solid-phase extraction (SPE) and liquid-liquid extraction, which I have repeatedly talked about LLE, but supercritical fluid extraction is also picking up and is very important (the SFE), the Soxhlet extraction and the liquid membrane extraction are also used and are useful.

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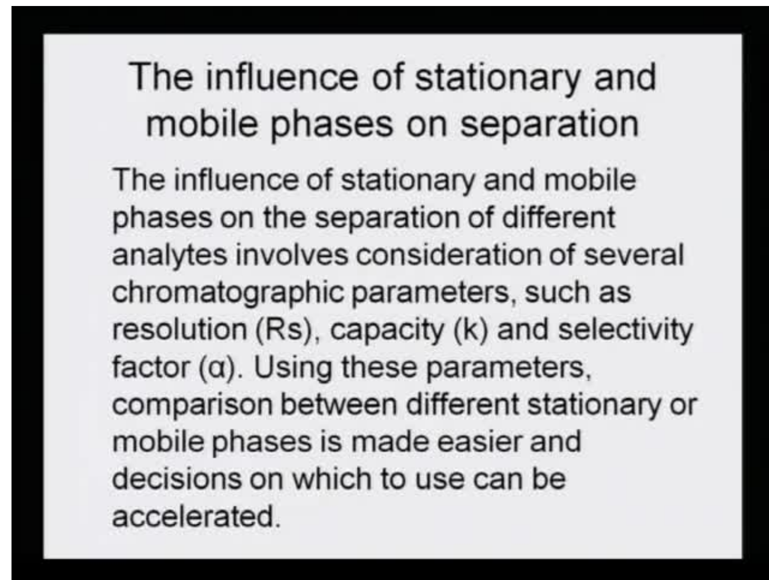

The influence of stationary and mobile phase on separation - the entire chemistry of separation relies on the fact, that how do the stationary phase and the mobile phase react towards the extract. The better the extraction, the better the reaction between the extract and the mobile phase and stationary phase, the better will be the separation.

The influence of stationary and mobile phases on the separation of different analytes involves consideration of several chromatographic parameters, such as resolution, capacity and selectivity factor. See, it has to be understood very, very clearly, that there is a compound a, and there is a compound b in an extract. Let us just take a system which has only two components - a and b; their reactivity towards the mobile phase and their reactivity towards the solid phase will be different; a will react differently and b will react differently and that causes separation. The better their reactivity, the better will be the separation and that is what is the crux of chromatography technique. Using these parameters, comparison between different stationary or mobile phases is made easier and decisions on which to use can be accelerated.

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Quantitative analysis of carotenoids

Some importance attaches to analytical techniques for the quantitative determination of carotenoids, such as lutein, zeaxanthin, β-cryptoxanthin, and α- and β-carotene in complex sample matrices. Although most common separation systems concentrate on the use of silica C18 stationary phases, Column length, particle size and end-capping of free silanol groups can affect retention, but the mobile phase, column temperature and flow rate are also important.

Qualitative and quantitative analysis of carotenoids - some importance attaches to analytical techniques for the quantitative determination of carotenoids, such as lutein, zeaxanthin, beta-cryptoxanthin, and alpha- and beta-carotene, in a complex sample matrices is not easy. And there has to be a lot of permutation-combination that needs to be done and adaptation that needs to be done.

Although, most common separation system concentrate on the use of silica C 18 stationary phase - which is the reverse column. Column length, particle size and end capping of the free silanol group can affect retention, but the mobile phase, column temperature and flow rate are also important. So, when we are analyzing carotenoids, we have to keep all these points in mind, and by using C 18 column - which is a reverse phase column - one can do this analysis having all these different types of carotenoids and they are able to get separated on C 18 column.

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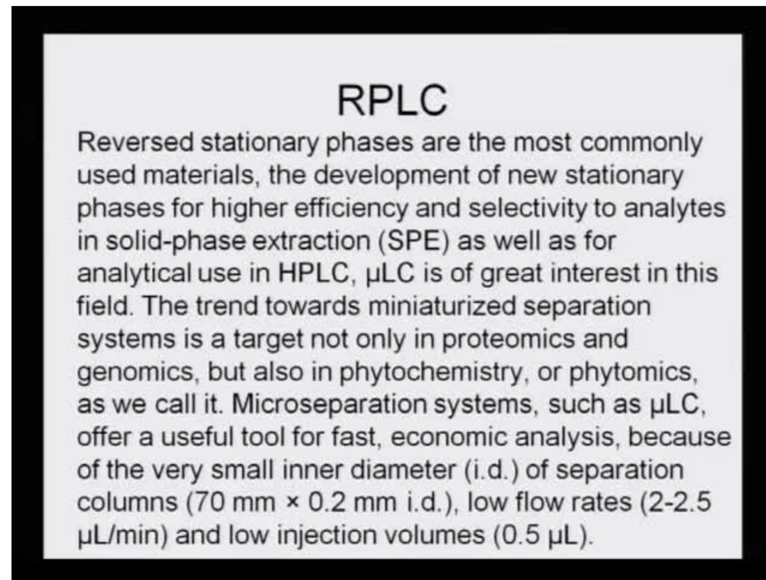

RPLC - reverse phase liquid chromatography - the reversed stationary phases are the most commonly used materials, particularly in phytochemical analysis. The development of new stationary phases for higher efficiency and selectivity to analytes in solid phase extraction, as well as for analytical use in HPLC, microLC is of great interest in this field.

The trend towards miniaturization separation system is a target not only in proteomics and genomics, but also in phytochemical and phytomics. I will repeat this - the trend towards miniaturization separation systems is a target not only in proteomics and genomics, but also in phytochemitry or phytomics, as we call it. Microsepartion systems, such as microLC, offer a useful tool for fast economic analysis, because of the very small inner diameter - the i.d, that is the inner diameter of the column - of separation column, which is almost 70 mm into 0.2 mm i.d, low flow rates which is in the range of 2 to 2.5 micro liter per minute, and low injection volumes, that is 0.5 micro liter injection are just the ideal kind of parameters, that need to be used while analyzing phytochemicals.

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Quantitative analysis of tocopherols

- Vitamin E is a generic term for a combination of tocopherols (α, β, γ, and δ), trienols, α-tocopherylacetate and nicotinate. Since the discovery of vitamin E in 1922 , several analytical techniques have been applied for the qualitative and quantitative analysis: TLC, GC, normal phase (NP)-HPLC and RP-HPLC using UV-absorbance and MS detection have been known.
- A comparison between NP-HPLC and RP-HPLC systems, the influence of the stationary phase on the separation of the vitamin E analytes, the difference is that the order of elution is reversed.

Quantitative analysis of tocopherol - tocopherol is one type of vitamin E. Vitamin E is a generic term for a combination of tocopherols, that is, alpha, beta, gamma and delta trienols; alphatocopherylacetate and nicotinate - these are different types of compounds, but coming under the generic name of vitamin E. Since the discovery of vitamin E in 1922, several analytical techniques have been applied for the qualitative and quantitative analysis – TLC, GC, normal phase HPLC, RPHPLC using UV-based absorbance and mass detector, have been known and have been practiced.

A comparison between normal phase HPLC and RPLC system - the influence of the stationary phase on the separation of vitamin E analytes, the difference is that the order of illusion is reversed.

Now, I would like to draw your attention, that when we used normal phase HPLC and when we use RPLC or RPHPLC, it means the same, but there is a difference in these two methods. In one, the column will elute according to the increasing order of the polarity of the compounds and that one we call as normal HPLC; whereas, in reverse phase column, what happens is that, the more polar compounds will come first, and the least polar compound will adhere to the column, and would elute much later. So, most of the phytochemicals are very polar compounds. So, if we use RPLC, definitely, we save on time, on solvent and it is a more efficient method for highly polar compounds of the phytochemical extract.

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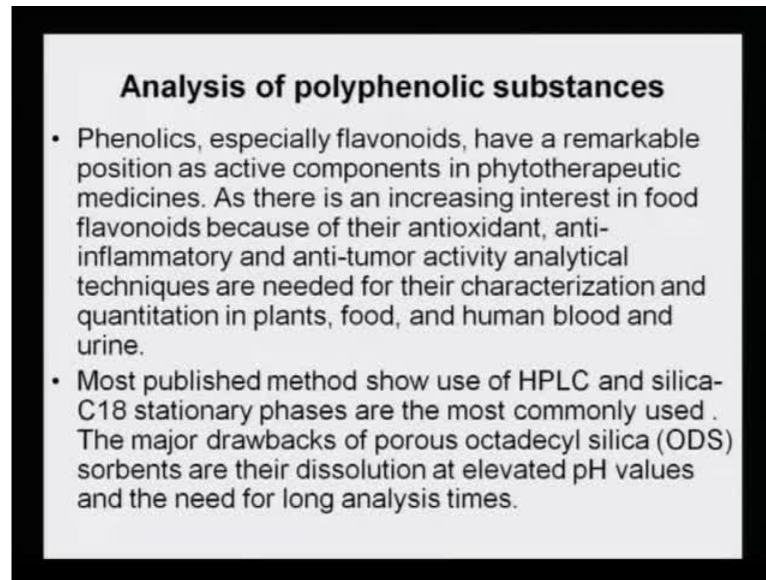

Analysis of polyphenolic substances - phenolics, especially flavonoids, have a remarkable position as active components in phytotherapeutic medicines. As there is an increasing interest in food flavonoids, because of their antioxidant, anti-inflammatory, anti-tumor activity, analytical techniques are needed for their characterization and quantitation in plants, food, and human blood, and urine.

Most published method show use of HPLC and silica based C 18 stationary phases - that is the RPLC - are most commonly used. The major drawbacks of porous octadecyl silica (ODS) sorbents are their dissolution at elevated pH values and the need for long analysis times.

So, if we use C 18 column, what happens is that the more polar compound elutes faster, and the polyphenol, being very polar compound gives good result in shorter period of time; and, but one thing is sure, that at elevated pH, the results are not obtained in the desired manner. So, one has to check the pH, before one starts the C 18 analysis.

So, with this, we have come to an end of one particular type of use of the chromatographic techniques that are used in phytochemical analysis.

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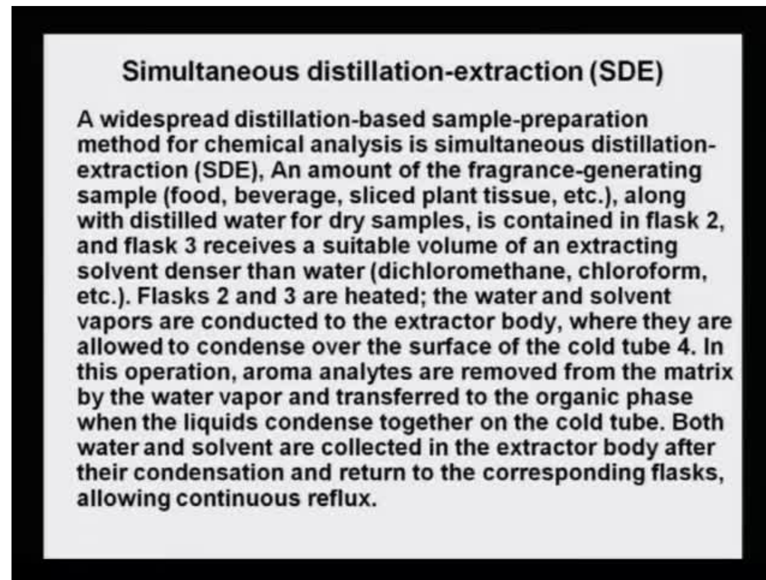

Simultaneous distillation and extraction (SDE) - this is also one very special technique, which is used in flavor and aroma compounds. A wide spread distillation-based sample-preparation method for chemical analysis is simultaneous distillation extraction; that means, we are distilling and extracting. An amount of the fragment-generating sample - food, beverage, sliced plants tissues - along with distilled water, for dry sample is contained in flask. And then, it receives a suitable volume of an extracting solvent, denser than water, particularly dichloromethane, chloroform, etcetera. They are put in flasks 2 and 3 and they are heated.

The water and solvent vapors are conducted to the extractor body, where they are allowed to condense over the surface of the cold tube, which is numbered as 4. In this operation, aroma analytes are removed from the matrix by water vapor, and transferred to the organic phase, when the liquid condense together on the cold tube. Both, water and solvents, are collected in the extractor body, after their condensation, and return to the corresponding flask allowing continuous reflux.

So, what happens is, in this particular type, that there is water, distillation, helping in the distillation; then, there is an extractor in the organic medium, and both the vapors, are then being transferred to a cold tube. So, the vapor actually condenses in the cold tube; whereas, the carrier water solvent and the organic solvent, get condensed in their respective flask.

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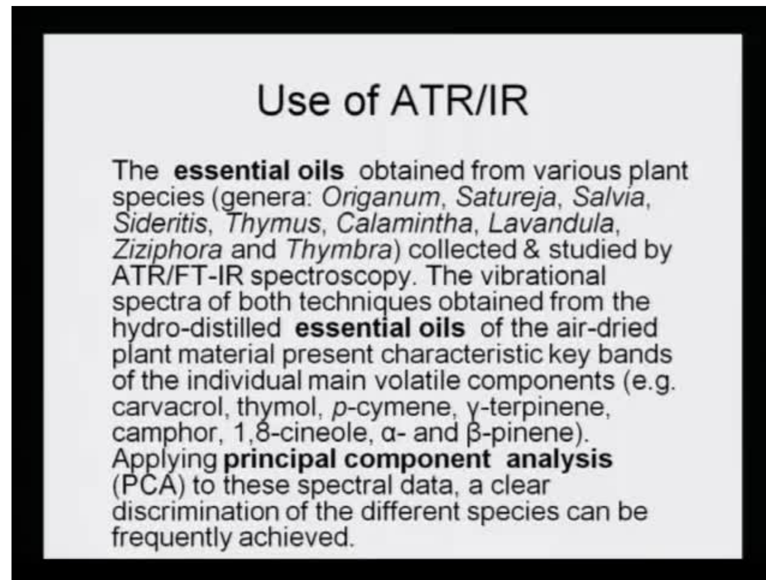

Another very special technique which is used in the flavor and aroma analysis is described - the use of ATR and the IR spectroscopy. The essential oils obtained from various plants species, particularly all those which are used for herbs, are collected and studied by ATR FTIR spectroscopy; and these are Origanum, Satureja, Salvia, Sideritis, Thymus, Calamintha, Lavandula, Ziziphora and Thymbra. The vibration spectra of both techniques obtained from the hydro-distilled essential oils of the air-dried plant material, present characteristic key bands of the individual main volatile components, such as carvacrol, thymol, cymene, gamma-terpinene, camphor, 1 8-cineole, alpha-beta-pinene. Applying principal component analysis, that is the PCA, to these spectral data, a clear discrimination of the different species can be frequently achieved.

Now ATR-IR is a very special technique, which is a modification of the FT-IR. When I will be covering spectroscopic method, I will get into these details of the methodology.

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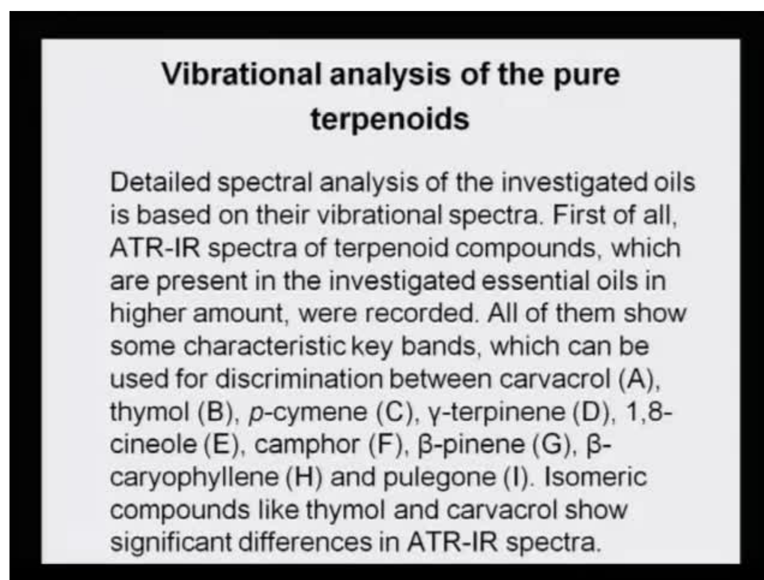

Vibrational analysis of the pure terpenoids - detailed spectral analysis of the investigated oils is based on their vibrational spectra. First of all, ATR-IR spectra of the terpenoid compounds, which are present in the investigated essential oils, in higher amount, were recorded. All of them shows some characteristic key bands, which can be used for the discrimination between the carvacrol, thymol, cymene, terpinene, and camphor; and then, the isomeric compounds, like thymol and carvacrol, show significant differences in their ATR-IR spectra.

So, that is where the discrimination and identification of different components can take place. And this particular… Because normally an FT-IR is used only to give the functional groups, but ATR-FT-IR gives more information, and that is why it is being used in this particular case.

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Characteristic Bands

All of them show some characteristic key bands, which can be used for discrimination between carvacrol (A), thymol (B), *p*-cymene (C), γ-terpinene (D), 1,8-cineole (E), camphor (F), β-pinene (G), β-caryophyllene (H) and pulegone (I). Isomeric compounds like thymol and carvacrol show significant differences in ATR-IR spectra.

Characteristic bands - all of them shows some characteristic key bands, which can be used for discrimination between the carvacrol, thymol, cymene, terpinene, 1 8 cineole, camphor and beta pinene. Isomeric compounds, like thymol and carvacrol, show significant differences in their ATR-IR spectra. So there, because the issue of characteristic different bands, they are able to be identified; otherwise, if they were showing similar bands, it would not have been possible to identify these different compounds in the flavor and aroma compounds.

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Normal Analysis of the oils

- GC analyses are carried out on a gas chromatograph equipped with a FID and a DB-5 capillary column (30 m × 0.25 mm; 0.25 μm film thickness). The oven temperature was held at 50 °C for 0.5 min then programmed at 2.5 °C/min to 265 °C. Other operating conditions were as follows: carrier gas, N2 with a flow rate of 1.5 ml/min; injector temperature, 250; detector temperature, 300 °C; split ratio, 1:10.
- GC/MS analyses were performed on a GC coupled with Mass system and a DB-1 capillary column (30 m × 0.25 mm; 0.25 μm film thickness). The operating conditions were the same conditions as described above but the carrier gas was He. Mass spectra were taken at 70 eV. Mass range was from *m/z* 35–375 amu.

Normal analysis of the oil - GC analysis are carried out on gas chromatography equipped FID, and a column which is DB 5 capillary column, having dimension of 30 meters 0.25 mm; point 0.25 micrometer film thickness is used. The oven temperature was held at 50 degrees for 0.5 minutes, then programmed to 2.5 minutes per 2.5 degrees per minute to 265 degree Centigrade. Other operating conditions were as follows: carrier gas, Nitrogen with the flow rate of 1.5 ml per minute, injected temperature 250, detected temperature 300, split ratio 1 is to 10; this is the parameter that was followed for the flavor and aroma compound analysis

GC/MS were performed on the GC coupled with mass system and a DB 1 column was used and the operating conditions were also varied. And this gave a very good mass spectra of mass range from 35 to 375 amu. So, you just try to understand that the GC programming is also a very essential part; it is not that, one can do any analysis, on any system and still get the best result. To be able to get best result, one needs to identify and to be able to find out which is the best optimum condition for the analysis.

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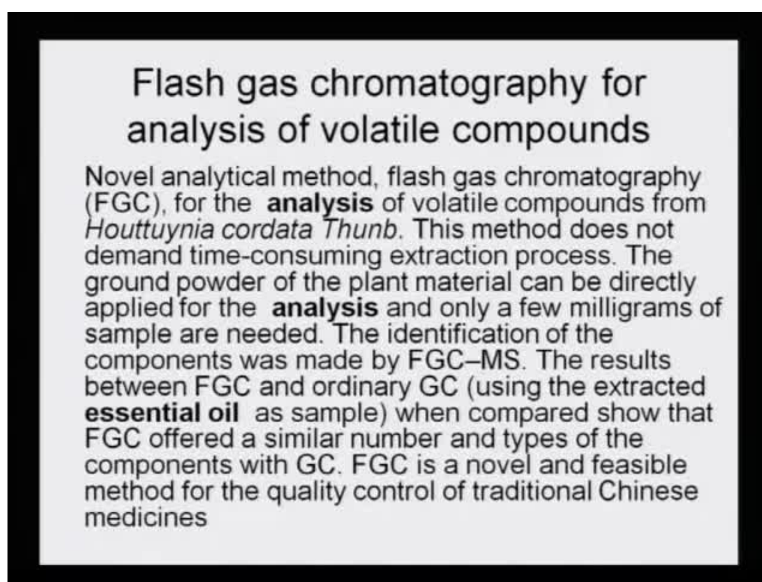

Flash gas chromatography for analysis of volatile compounds - novel analytical method like flash gas chromatography (FGC) for the analysis of volatile compounds from Houttuynia cordata - this is a biological name of a plant. This method does not demand time consuming extraction process. The ground powder of the plant material can be directly applied for the analysis and only a few milligrams of the samples are needed.

The identification of the component was made by flash gas chromatography technique with the mass detector. The results between the FGC and the ordinary GC, using the extracted essential oil as sample, when compared, show that FGC offered a similar number of and types of components, as what the GC showed. However, FGC, is a novel and feasible method for the quality control of traditional Chinese medicine.

Now, what happens when there are several harvest products available, and the analyst has to find out which harvest or which farm has provided the best type of plant material for analysis? A flash gas chromatography can give very quick results.

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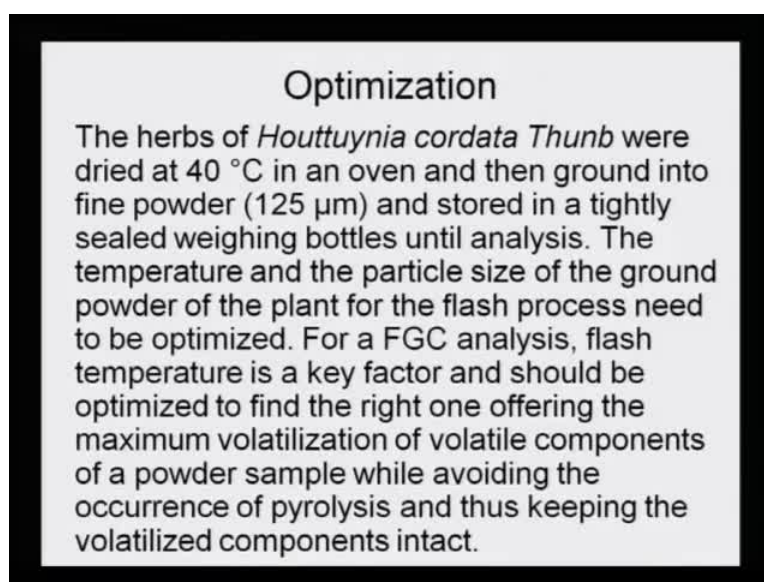

Optimization - the herb of Houttuynia were dried at 40 degrees in an oven, and then ground into fine powder of 125 micrometer particle size, and stored in tightly sealed weighing bottles until analysis. The temperature and the particle size of the ground powder of the plant, for the flash processes, need to be optimized. For a FGC analysis, flash temperature is a key factor and should be optimized to find the right one offering the maximum volatilization or volatile components of a powder sample, while avoiding the occurrence of pyrolysis and thus keeping the volatized component intact.

See, there is a very discrete difference between pyrolysis which breaks down the compound completely, so the flash temperature should be such that, it should be just optimum to keep the compound alive and the compound should not get hampered.

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Advantages

Flash gas chromatography is a novel and efficient method for the analysis of volatile compounds from Traditional Chinese Medicines (TCMs). Using this method, the demanding extraction process is avoided. The ground powder can be directly applied for the analysis of volatile compounds and only a few milligrams is needed. Flash gas chromatography is a feasible analytical method and can be used for the quality control of TCMs.

Advantages of flash gas chromatography, is that it is a novel and efficient method for the analysis of volatile compounds from traditional Chinese medicines. Using this method, the demanding extraction processes is avoided. The ground powder can be directly applied for the analysis of volatile compounds and only a few milligrams are needed. Flash gas chromatography is a feasible analytical method and can be used for the quality control of the traditional Chinese medicines.

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Application of comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry in the analysis of volatile oil

Comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC × GC–TOF MS) **analysis** of *Pogostemon cablin Benth* (*Cablin Patchouli*) volatile **oil.** The suitable column system and operation conditions were chosen on the basis of the properties of composition of the volatile **oil.** One-dimensional gas chromatography (1D-GC) and GC × GC, GC–MS and GC × GC–TOF MS were compared under appropriate conditions, and the enhanced sensitivity and superior resolution of GC × GC were demonstrated. 394 components were tentatively identified by GC × GC–TOF MS.

Application of comprehensive two-dimensional gas chromatography time-of-flight spectrometry in the analysis of volatile oil - comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry; that is, GC is coupled with another GC which has a time-of-flight mass spectrometry as its ion. I was talking about quadrupole, I was talking about ion trap, but here the analyzer is a time-of-flight mass spectrometry analysis of Pogostemon; it is an essential oil of patchouli. The suitable column system and operation conditions were chosen on the basis of the properties of composition of the volatile oil.

One-dimensional gas chromatography and compared with GC and GC-MS with GC-TOF were compared according to the appropriate conditions. And the enhanced sensitivity and superior resolution of the GC, coupled with GC were demonstrated. 394 components were tentatively identified by this combination of one GC and a GC with a time-of-flight mass spectrometry.

I will explain this, because the separation that took place in one GC, was not enough to be able to separate these components, and when it was put into another GC, these separated compounds further separated, and 394 components could be identified.

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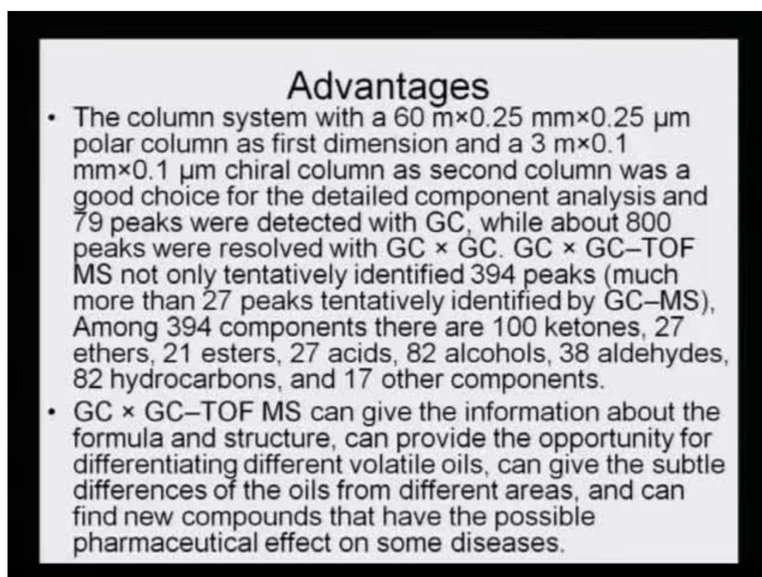

Advantages - the column system with 60 minutes meters length, 0.25 mm diameter and 0.25 micrometer coating as first dimension was used. And in the second one, the parameter was slightly altered and that showed that it tentatively identified 394 peaks.

Among 394 components, there are 100 key tones, 27 ethers, 21 esters, 27 acids and 82 alcohols, 38 aldehydes, and 82 hydrocarbons.

So, you see there are huge variety of compounds that are present and that had been ideally identified through this. The GC and the GC-TOF MS can give the information about the formula and structure, can provide the opportunity for differentiating different volatile oils, can give the subtle differences of the oils from different areas and can find new compounds that have the possible pharmaceutical effect on some diseases. So, you can see that it has a huge range of opportunities.

One thing that should be born in mind is that one GC alone can do smaller separation; when it is attached to another GC, it further separates, and when it further separates, the components which were coming together now come as individual components. And when they come as individual components, as many as 394 could be identified, and they were compounds of different nature; they were key tones, they were aldehydes, they were different types of compounds that made together 394 components. So, that is what I wanted to talk about, that different compounds need different adaptability on the same kind of GC machine.