PHARMACOGNOSY AND PHYTOCHEMISTRY

Dr. Galvina Pereira

Department Of Pharmaceutical Science and Technology

Institute Of Chemical Technology Mumbai

Week 11

Lecture 54

Quantitative Evaluation of Herbal Drugs Using Gas Chromatography Methods

Hello everyone, and welcome back to the fourth session of week 11 of the NPTEL course in pharmacognosy and phytochemistry. So far, we have learned many methods of evaluating herbal drugs, and this week, we are particularly focusing on the chemical methods of analysis. When we discussed chemical methods of analysis, we divided them into two parts. The qualitative methods included phytochemical tests, identification tests, microchemical tests, and histochemical tests. We also saw tests for heavy metals, as well as thin-layer chromatography to

identify the compounds present in herbs. Now in this week what we are dwelling more are we are seeing different different quantitative methods have already seen HPTLC as a method of analysis, and we have also explored HPLC methods of analysis. In today's session, we will study in a little more detail how gas chromatographic techniques can be used.

Previously, in the HPLC method, we also saw that HPLC could be used for the determination of pesticides. It could also be used for the determination of aflatoxins. Not only that, it could be used for the quantification of the majority of phytochemicals. Now, in today's session, we will explore the gas chromatographic method. So, what is this gas chromatographic method?

The gas chromatographic method, as the name indicates, uses gas as a mobile phase, and the chromatographic separation is carried out on a stationary phase, which might be a liquid or a solid. If the stationary phase is solid, it is referred to as gas-solid chromatography. And

if this stationary phase is a liquid, in that case, it is referred to as gas-liquid chromatography. So why do we use gas here?

So basically, gas chromatography is a technique where your substances, especially when you take samples from plants, have a low boiling point. Or when you heat them, they do not deteriorate. So they are thermally very stable compounds which do not decompose on heating, and they have a varied low range of boiling points.

So the changes in their boiling point and their affinity to the stationary phase can be used and can be modified by using different combinations of gases as well as stationary phases. So you can change the mobile phase, you can change the stationary phase, and achieve a good separation. Now this separation can be better in some cases, especially for volatile compounds, as compared to HPLC. In such cases, your gas chromatography is used.

So what is this gas chromatography? So initially, what we'll do is we have a sample here. Now this sample could be a plant, say for example, a plant containing volatile oil. Take for example your eucalyptus. So now your eucalyptus oil is to be extracted, and I want to see what volatiles are present there.

your eucalyptus so if you take this eucalyptus I will extract it in a solvent in which my volatiles are more soluble in your HPLC, where I wanted other compounds which were non-volatile, in this case, since my intention is to analyze the volatiles present in my eucalyptus leaf, I will choose a non-polar solvent because most of the volatile oils or volatile ingredients are lipophilic in nature. So I may take a solvent like your chloroform or your hexane or a dichloromethane to dissolve and extract the solvent.

This solvent once extracted will be injected here. You can see here this is kind of an injection point and in this injection point what is happening is you also see a gas cylinder here. So from this gas cylinder like I said This is your mobile phase for your gas chromatography.

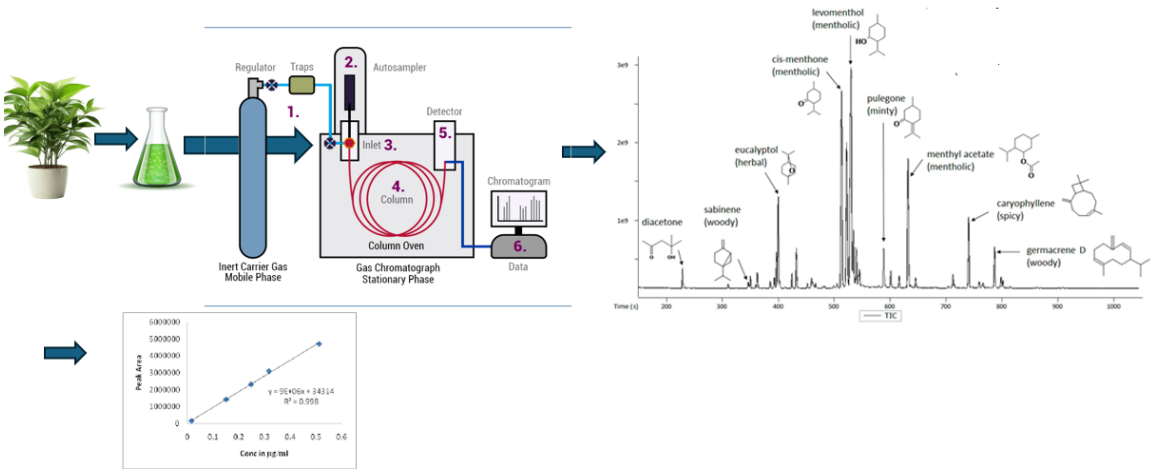
So, a inert gas kind of moves through this column. So, what is column? We will discuss it shortly. But right now, just imagine it something to be imagined earlier like your HPLC column where your compounds are going to resolve.

So, from this column, your gas is moving. Your eucalyptus is injected and at the same time it is being heated. Now, what is going to happen is Every component of eucalyptus, say for example, your eucalyptol or pugilone or your menthol, all of them have a slightly different boiling point.

So they will kind of volatilize and depending upon their boiling point, they will evaporate and come into vapor phase. Once they come into vapor phase, they will start moving along with the carrier gas. As they move in the carrier gas, they further get resolved in this column based on their affinity either to the stationary phase or the mobile phase, and then they are eluted. Once they are eluted, they are detected by the detector, and this is how you see the chromatograph.

So, you can see from this eucalyptus leaf, I could nicely separate compounds such as sabinine, eucalyptol, menthone, levomenthone, pugilone, and so many others. So, all of these volatiles are then separated based on their boiling point and their affinity. Usually, the low-boiling compounds come out first, whereas the high-boiling, less volatile compounds come out at the end in gas chromatography.

Now, similar to HPLC, what you can do is analyze these peaks for the area under the curve. So if you have a standard concentration being injected and you can prepare a standard curve on that, you can then inject an unknown concentration, extrapolate it on the graph, and determine how much eucalyptol is present in your eucalyptus sample. So, I can do the qualitative analysis, what all compounds are present, is eucalyptol present or not,



https://blog.perkinelmer.com/posts/gas-chromatography-explained-what-it-is-and-how-it-works/
https://www.azom.com/article.aspx?ArticleID=20833

as quantitative analysis by injecting or analyzing it with a standard concentration of eucalyptol. So, how do we go about this? Step by step, the first and most important step for gas chromatographic analysis is sample preparation. The sample is very vital here because the requisites for the sample are that it should be low-boiling or volatile. The second requisite is that it should be thermostable.

The reason is that in gas chromatography, we steadily increase the temperature. That's when your compounds start evaporating based on their boiling point. So, you have to convert your compounds into what are called volatile derivatives. So this sample preparation for volatile oils is not at all a problem because the compounds as such are volatile. But imagine there are compounds which are not volatile.

So is it that my gas chromatographic techniques cannot analyze them? They can somehow be analyzed if I can derivatize them. So the first thing I have to do is see how much volatile compounds are present in the sample. If needed, concentrate the extract to get a good fraction of it. Now once I concentrate, I should take into consideration what my analyte is.

Is it a volatile compound or a non-volatile compound? For example, when I was talking about terpenes, eucalyptol, and menthol, they are volatile. I need not do any derivatization of them. But take, for example, your fatty acids, your lipids, or your cooking oils. Your cooking oils are not low boiling.

They are not volatile. In that case, you have to modify them to make them more volatile, and only then can they be analyzed. The good part is that both of these are thermally stable. So there are some derivatization techniques which are often used. One thing that is used is silylation.

So you convert your compounds into what are called trimethylsilyl derivatives. So, say, for example, your fatty acid is RO, R-C-O-O-H. So, I can convert it into R-C-O-O-silyl, and to this silyl, I can have trimethyl. Now, you will see that comparatively, when it is converted into esters, this can even happen with alcohol.

I can convert ROH into trimethylsilyl derivatives and so on. So the first thing you can do is silylation. Converting it into silyl derivatives will definitely make it more volatile. Another approach is, if you have an alcohol, you can do acetylation. Now, this acetylation can be done with the help of compounds such as pentafluoropropionyl anhydride.

or pentafluoropropionyl alcohol. Now, that will add a group to it, that is, your pentafluoropropyl group, and that will make your compounds more volatile. Similarly, you can also do alkylation. It could be a simple alkylation using dimethylformamide, or you could alkylate using boron trifluoride.

So, this boron trifluoride in the presence of alcohol. Say, for example, methanol will convert it into a methyl ester. So, you can use methanol, you can use propanol, or you can use butanol in the presence of any acid or BF3, which will convert it into a corresponding alkyl derivative. You can also use tetrabutylammonium hydroxide.

That is also one reagent which is an alkylating agent. Now, in some cases where your compounds are enantiomeric, and you still wish to separate them, there are specific derivatizing agents which can cause or help in chiral separations. This includes your Mosher's reagent as well as N-trifluoropropyl chloride. So, there are a few derivatizing agents.

So, you can take your sample, allow it to react with this derivatizing agent. Once this reaction proceeds, you will get these compounds there. Kind of prep up this reaction, get your sample and this sample you analyze by your GC, you will have much much better

results compounds are more volatile, and some of these derivatizing techniques also convert your compounds into more stable derivatives.

Now, without going deep into this, there are different columns which can be used in gas chromatography. Sometimes these columns can be entirely packed columns. So, you know, it's like a circle or a hollow column, which is long or large in length. And this is a completely filled column. With a porous solid, that porous solid will allow the gas to pass through.

But because it is completely filled, this is called a packed column. But sometimes you have what is called a tubular column. So what it is, is you have a tubular structure in there. So in this tubular structure, sometimes what happens is your stationary phase is coated on this column. So this is what is called a wall-coated column.

In some cases, you might have certain support like porous silica. So this is called a support, and this support can be coated with your analyte. So imagine the support beads. Now they are coated with one more layer of your stationary phase. This is what is called support-coated.

Then you have your multi-capillary. Then you have your microchip. Multi-capillary is something you can imagine. You have a whole column which is filled with numerous tiny capillaries. Inside each capillary is your stationary phase.

And from that, your capillaries. Gas is moving. This increases your rate of mass transfer and improves your efficiency. Whereas if you look at the microchip one, the microchip can be in various forms. So you can imagine, you know, it's kind of fabricated.

Now imagine inside this, through this, your gas is passing. So again, you have a good amount of area. This could be circular, trapezoidal, quadrangular, or any shape depending on how convenient it is to fabricate. Not only that, you can adjust temperatures for the analysis of different compounds. For example, your volatile compounds.

Your volatile compounds are generally heated. Between 150 to 300 degrees Celsius for their analysis. If you want to analyze steroids, they are derivatized, and post-derivatization,

they are heated to about 250 degrees Celsius. Pesticides are very resilient. You require slightly higher temperatures with them.

So for pesticides, many of the methods even go to temperatures as high as 400 degrees Celsius. Now here, the different carrier gases are also important. The Most efficient ones are hydrogen and helium. But the problem with hydrogen is its combustibility.

So we prefer helium over it for its inertness. The other gas is nitrogen, but nitrogen slightly decreases the efficiency. Still, you can use air also if you want a cheaper substitute. And in some cases, it is also blended with oxygen. Now, once the compound is separated, it goes to the detector unit.

There are different types of detectors used in GC. So, the different types of detectors used in the analysis of gas chromatography are thermal conductivity. Here what is done is your thermal conductivity of your helium gas which is normally passing through is measured and when your analyte comes out what is happening the thermal conductivity is changing. So that change in thermal conductivity will give you a signal.

Same way normal helium you check how much that gas can ionize your say for example mobile phase. Now compare it with your analyte moving through that column. The degree of ionization changes when you have a compound coming out. How much is that change that is detected by a peak? Same way you can see the intensity.

You know certain times you know certain metals produce light of a different intensity. So that is where you measure your light intensity. So in a similar manner, you can see the flame color change in intensity and analyze it. And that is what is called as flame photometric determination. Apart from that, you also have electron capture detector where you have kind of, you know, a small radioactive element

or a element or a mechanism where electrons are completely bombarded on a detector surface. And whenever your analyte passes through it, What is going to happen is there is going to be some change in the electron flow. That change is kind of perceived as a signal. Sometimes, some compounds are made to glow.

We call it chemiluminescence. This is used for the detection of sulfur as well as nitrogenous compounds. So, for our importance, let us see a few examples here. So, chemiluminescence. This is an example of the analysis of a volatile oil, and you can see here that during the volatile oil analysis, especially this oil is your peppermint essential oil. So, when you are analyzing peppermint essential oil, we want to separate out the different compounds in it. So, essential oil is not just one or two compounds. It might contain somewhere between 20 to even 100 compounds, depending upon that. And in some cases, they are blended.

So, take for example, in this gas chromatographic method, they have used a phenyl-based SLB column. which is containing almost 5% phenyl substitution, the length of this column, the GC column length is generally more because transfer and the separation to take place in the gas-liquid phase or in the gas-solid phase takes time. So, you kind of buy more time and more separation by increasing the column length. And this is the internal diameter, which is just 0.25 mm.

And if you see that inner coating, which is discussed, this is the support, and your stationary phase is kind of coated. The thickness of this coating is just 0.25 micrometers. Now, what you are going to do is inject the sample. In some cases where the analyte is very low, they use what is called splitless injection. But when the analyte is in high concentration, you can also define a split ratio.

So, your sample is going to get divided and then analyzed. So, after splitting, Mostly for volatile oils, splitting is desired. So after splitting, you start heating to a temperature of 200 degrees Celsius. So that's your injection temperature, and there most of your compounds will volatilize.

Now, if you see your column oven, your column is located inside the oven, and your starting temperature is 50 degrees Celsius. So all your compounds will start getting heated and mobilizing from this point. And it is continuously being heated at a rate of 3 degrees Celsius per minute. So after one minute, it will be 53. The second minute will be 56.

This goes on until you reach a temperature of 300 degrees Celsius. And if you see the detector, the detector temperature is set at the highest point. That is 300 degrees Celsius

with a carrier gas that's helium with a flow rate of 30 centimeter per second. so when you kind of put this whole thing in a program you will get a kind of chromatograph which looks like this and then if you subject it to a mass spectroscopy this mass spectroscopy many a times is coupled with library search this includes your NIST what is called as the NIST library you have different in your lcms you have the MELTINs library now you have your m/z mind library There are many, many multiple libraries available. Now, with machine learning, they even have a fragmentation-based plant metabolomic library. So even you can use that, you can use something like your software to analyze this.

And based on those fragmentation patterns, based on those retention time, your output, your computer will tell you, you know what, these are all the compounds. So at number one, you have diethyl tetrahydrofurane. The peak what you're seeing as number two is thujene and so on. So not only will it tell you which peak is what, it will also tell you the relative concentration based on the area under curve.

So this is one which can be used for quantification of essential oils. You can also quantify total amount of lipids, fats, sterols by either alkylation or acetylation. Similarly, when you see, if you remember your HPLC, we did what is called as pesticidal residue analysis. Now, this is very vital and it is also or it can be also analyzed using gas chromatography. So we use the very same method.

Quechers method and here what you're going to do is the same sample extraction if you recollect in the Quechers method it was your sample which was pulverized then acetonitrile was added then water was added and slowly slowly you added salt like a magnesium sulfate what happens is the more salt you add the acetonitrile is pushed out this acetonitrile was taken pass through more of what is called as solid phase extractors, cleaned up, centrifuged and analyzed.

Now, this analysis again can be done on a very hydrophobic GC columns, such as your HP columns. And in this case, you can observe different, different pesticidal residues being there. Now, this method has also been used Notified by AOAC. Here what they do is they kind of use a very standardized method.

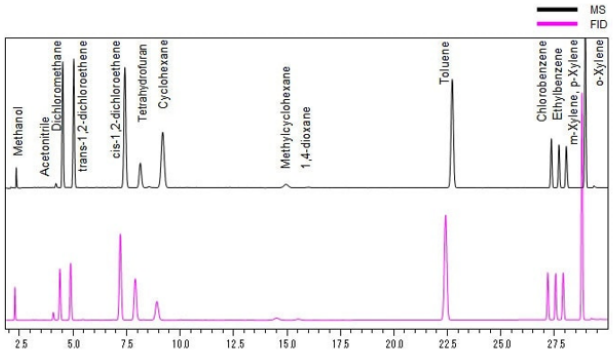
So this standardized methods of GC are also there in terms of how you should prepare your sample for GC analysis of pesticides and what program should be set. The good part of this is nowadays most of the equipments, especially if you go to see makes such as Agilent or Shimazu, these companies already have a preset software for pesticides. So if you already have a program which is there in your machine, you just run your sample, not only will they analyze your sample,

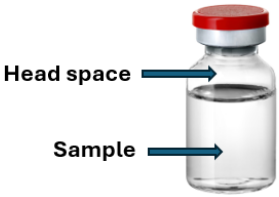
The programs are so accurately set that based on their retention time, they can tell you which pesticide is there and at what concentration. So, you know, in their software's data of 300 to 400 such pesticides are already locked. So the moment you analyze it using their methods, you can see that the machine will tell you that this many pesticides with this many quantity are present.

Now the next part of our analysis is solvent analysis. Now many a times what happens is when you are preparing herbal extracts, we often use a solvent. So this herbal extracts you can extract it in alcohol, chloroform, petithur, so on. Now sometimes the solvents are very inert but many a times the solvent are undesired and we do not want them in our preparations or medicines.

And as a result there is always a limit to solvent given in every extract. So how do you do the solvent analysis? Now solvent analysis can be much much accurately done by what is called as headspace analysis. So now what is this headspace analysis? Take for example this is my sample.

Determination of Solvent Residues





Headspace sampling:

- The gas layer, or the headspace above the sample in a vial, is analyzed
- Compounds of interest should have high volatility, and the sample should be non volatile
- Temperature controlled oven heats up the sample before analysis begins to achieve equilibrium

Dr. Galvina Pereira, Institute of Chemical Technology, Mumbai

10

Now this is where your extract is. So I put my extract here, and my extract is kind of very viscous. It cannot be volatilized. Imagine a consistency like your chawanprash. So it's non-volatile.

It's definitely going to clog my machine, my syringes or whichever I'm going to use. And as a result, it's not recommended that I inject this extract in the machine. So what is done is this extract or sample are carefully heated in the vial and this vial are nicely sealed. So when I am heating this gradually what is happening is as the temperature increases it reaches to this boiling point of the solvent.

So methanol you can say at about 60 or 70 degree Celsius will start boiling. You will have chloroform. These are all low boiling. So if my temperature is crossing 100 degree Celsius for this sample most of my solvents will start volatalizing. Then what I do is I incubate it at a particular temperature and this incubation will help in equilibration.

So I will not say my entire solvent goes out in the headspace but what I say is my molecules of solvent equilibrate between air as well as the sample phase. So once this equilibration is achieved, depending upon their partition coefficient, I will know how much is going to be there in my extract and how much is going to be there in that. The formula for that is aptly there.

Now what is going to do is your headspace analyzer will come in. It will prick the needle in this while it will kind of take in. all the head space. So it will kind of use vacuum to pull in all this vapors which are generated and this vacuum will now take in your ethanol, methanol, chloroform, whatever is there.

Now because of this, everything that is there in head space is now pulled out and that volume is injected into your gas chromatography. So what will happen depending upon your boiling point, you can see different solvents that are all present will be seen. So whether it's alcohol, whether it's even toluene or chlorobenzene, whatever has gone into that extract can be analyzed using my solvent residual analysis by headspace capturing.

In some cases, they also use what are called adsorbents. So in that case, what is happening is this is used in the perfumery industry. So there is a probe here. And what the probe does is, you know, when you spray perfume, there is what is called a top note, which is made up of more volatile compounds.

But after an hour or so, the volatile compounds vanish, and you get what is called the middle note and later on the base note. To see what changes happen, they kind of put that perfume or spray that perfume in this. Okay, then what you see is the first note is being captured by this adsorbent, and this adsorbent phase is then used for gas chromatography. So it will analyze the first note; after one hour, again, a next probe of adsorbent is pulled in.

So it's just kind of a modification of headspace where, instead of vacuum, the whole thing is kind of adsorbed for a certain period of time and then analyzed. So definitely you could use GC for compounds which are more volatile like your pesticides, like your solvents, like your volatile compounds, and you could use it for non-volatile compounds which can be converted into volatile derivatives and are thermostable.

But all in all, if you see in terms of versatility over HPTLC and GC, your HPLC methods are still preferred. So This headspace analysis is going to use or going to give you a more versatile or more user-friendly approach, even with the sticky residual samples. It's not necessary here that the sample should be volatile.

So the temperature oven can help you regulate whatever volatiles are present, and this can be done even on a mL scale. So there might be vials that are as small as 2 mL. There might be vials that are as big as 100 mL for headspace analysis. This is all sucked in and put into your GC, and that can help you analyze the solvent. So, here are a few references if you wish to read more about gas chromatographic analysis, and thank you everyone for your patient listening. Thank you.