

# **PHARMACOGNOSY AND PHYTOCHEMISTRY**

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**Week 11**

**Lecture 52**

## **Quantitative Evaluation of Herbal Drugs Using Chromatographic Methods (Part 1)**

Hello everyone, and welcome to the next session on quality control methods for herbal drug evaluation. So, in this particular week, we have seen different methods of evaluation, and more particularly, chemical methods of evaluation. Right now, we have finished organoleptic, macroscopic, microscopic, and physical methods of evaluation. When coming to chemical methods of evaluation, if you recollect,

we saw that the chemical methods of evaluation were broadly classified into two categories: qualitative methods as well as quantitative methods. So, when we discuss the qualitative methods, we did something called phytochemical tests. Microchemical or histochemical evaluation, limit test as well as thin layer chromatography as ways of qualitative analysis to determine whether a particular compound is present or whether a drug is genuine.

We then moved on to quantitative methods, wherein we started quantifying certain things, especially the phytochemicals. So, we saw last time the UV method. We did see last time a UV method as well as a fluorometric method for the quantification of certain compounds like quinine and flavonoids such as quercetin. So, in this particular session, we will delve into the next topic: understanding how we can

perform quality control of herbal drugs using chromatographic techniques. Regarding chromatographic techniques, there are different methods involved. This includes HPTLC, HPLC, and gas chromatography. Numerous other chromatographic techniques exist, but

we will focus our interest on these three techniques. We will start with the first technique of evaluation, which is HPTLC.

HPTLC stands for high-performance thin-layer chromatography. So if you just recollect from your previous session where we did qualitative analysis, we saw that you can run a TLC plate, and depending upon the polarity of the compound, you get what is called the RF or retention factor. Now, in this particular session,

we will try to understand whether the same plate can be used for quantification of compounds and how. So, the quantification of compounds can be done by HPTLC if you follow a particular technique and if you have a standard with you. So, if you have a standard to compare, you can definitely measure how much compound is there in your extract. So, for it, a quick step-wise sequence would be: the first step is you take your material of interest.

Now, this material of interest can be a herbal drug. It could be a herbal preparation, a herbal medicine, a polyherbal formulation, or in some cases, even substances which people claim as herbal or natural. Say, for example, your herbal shampoos. Wherein the amount of actives or herbals added is very less,

but still, are they being added or not, and how much they've been added, we can still quantify them with the help of HPTLC. So, you start with any sample of your interest. The next step involved is sample preparation. Now, this sample preparation from your sample matrix, be it plant, formulation, or any preparation. So, from that particular matrix, you are going to extract the compound of your interest

or the compound that you are going to analyze. These are your simple marker compounds, what they are referred to as your phytochemical reference standards, if you recollect. They are very unique for a particular plant and that can be used as your marker compounds. So this phytochemicals which are unique to a particular plant

which are often referred to as marker compounds are used as standards for analysis. Say for example, I am analyzing ashwagandha preparation. A good standard or a good marker for me to analyze ashwagandha preparation would be withaferin A which is more unique

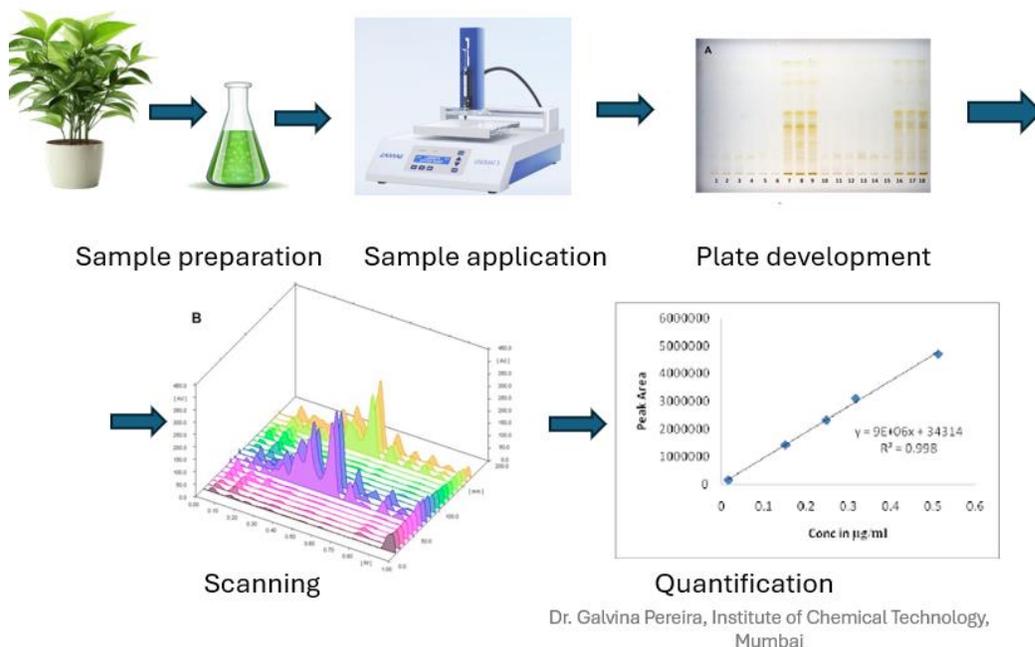
to ashwagandha rather than having something like your gallic ellagic acid which is seen in different or numerous other plants.

So if I pick up unique marker that will kind of tell me that yes this preparation is showing me ashwagandha spot for withaferin and since it is showing withaferin definitely it has ashwagandha. So I will prepare my sample in a way that I can take withaferin out or I can extract withaferin out. From my ashwagandha raw material or from any ashwagandha kada, syrup or tablet. I should be able to do that using suitable solvent.

Now once the solution that is your sample is ready. That is your ashwagandha extract is ready more specifically. It is then applied on a TLC plate. Now when you do a normal TLC we generally apply with hand because it's qualitative. We are not interested in the quantity.

In this case, we are interested in how much is applied. And we just have to apply a very specific calculated amount because we are going to take the readings from it. So this is done in a machine-controlled manner called an applicator. So then you have an applicator, a machine which applies the spot in a measured quantity at a measured volume. Then you run your plates like you run your TLC plate.

Then, an important step. What differs in your TLC versus HPTLC is that you are going to use this plate for quantification by scanning it. What do you scan in the process? You scan the intensity of the spots. So you can see here, I can see some spots which are very intense here, and then the spots here as well as below are very light.



So when I am scanning the intensity, do you see this? So there is a corresponding peak here. So these two peaks, peak number 1 and 2, correspond to these two spots. This is 1, this is 2. So you can have these two peaks here and these two peaks here.

You know they are more intense. So here you can see the peak height as well as the peak area, and the curve is also higher. OK, so if this happens to be my area or this happens to be my compound of interest, I am going to measure the area and the curve of this particular substance. Then I'm going to plot the peak area versus concentration.

How much did I apply? And then what I get is a very linear curve, very similar to that of UV. So in that manner, I can carry out my quantification. But yes, there are a few critical things that we need to understand. So let's try to understand them one by one.

So the first thing, or the first and most important step, is sample preparation. So from the plant, say ashwagandha, from your tablets, from your preparations, from your thick viscous syrups, how are you going to prepare it? The key considerations I should keep in mind are: in my plant, in my tablet, or in my syrup, what is the concentration of my phytoconstituent that I'm trying to determine?

You might have a liter of a formulation having just 1 mg of withaferin, or you might have a kg of plant material having a very minuscule or microgram level of a given compound. So if I have to quantify that, how much sample do I take to extract that particular compound? That will be the deciding factor. So if the concentration is less, you will have to take a large volume of samples. On the other side, if the concentration is very high, say 2%, 5%, in that case,

even if I take 1 gram of sample for my analysis, it's perfectly fine. But if my 1 mg is there in about, you know, a kilo of substance, I'll have to extract a kilo of that formulation or a liter of that formulation in order to get 1 mg of my analyte compound in my extract. So, you all see the concentration first, and depending upon the concentration, you have to take a call.

How much sample do you need to take? And that will be decided by the concentration, which you have to find out by literature or based on the sample. what is called as the label of a formulation so if the label claims your ashwagandha extract is one percent do a little back-calculation: how much ashwagandha is going to be there in the extract, and how much ashwagandha is going to be there in the final formulation. So, once I do that, it becomes much easier for me to take an estimate of what quantity of sample I need to extract. The next thing that determines the sample extraction is the solubility. How much is the solubility in the given solvent? So, we do use what is called 'like dissolves like.'

So, if it is a polar compound such as tannin, I use alcohol. If it is a non-polar compound like your carotenoid, lycopene, we just saw. They are extensively lipophilic. You can use petroleum ether, chloroform, benzene, and so on. The next thing is, when I am preparing the sample, is my compound stable?

In some cases, when we saw your alkaloids, we said that basifying it could extract them better in organic solvent. But are they stable to bases? We will also have to check that. So, you also have to check the stability of your compound. Certain compounds like anthocyanins cannot be heated.

For other processes, you might use heat to get better extraction, as heating increases solubility. But certain compounds, like anthocyanins, will brown, turn dull, or deteriorate if heated. In that case, you will have to perform a cold extraction. So you have to consider both the physical and chemical properties of the compound you are analyzing.

Now, different methods are involved, such as maceration or percolation. But a very effective method to extract a substance or formulation completely is the Soxhlet method. The Soxhlet method uses a Soxhlet assembly, but we will not go deep into extraction here. But the Soxhlet assembly works in a way that the sample is always exposed to fresh solvent, resulting in complete extraction of your component.

You need not worry about the volume of solvent. The assembly will recycle the same solvent and extract more of your compound. So, in most analytical methods where your compound is heat-stable, sample preparation is done using a Soxhlet assembly with a suitable solvent. Then you also have to consider the nature of impurities or other substances present.

Imagine you are trying to extract something water-soluble, but your substance, especially your drug, is lipophilic—it's oily. So what will happen is that the oily nature of the drug will hinder the extraction of your polar compounds. So first you might require to defat it. In some cases where the pigments are very much intense so much so that your actual compound will not be seen

unless and until you remove the pigments out like chlorophyll or in beetroot there are your betalains. So this deep color pigments often hinder with extraction and sometimes even with detection. So in that cases, it's always good to kind of get rid of them using the solvent, which will selectively remove your pigments, fats or other impurities. And thereafter, what you're left is with a simple solvent like alcohol to extract your analyte of interest.

So you can choose a solvent, say, for example, if you are extracting tannins. from your triphala churna. Now you know harada, baida, amla all of them have gallic, ellagic acid and they all have good solubility in alcohol. So I can just take the whole triphala churna, mount it in my or load it in the thimble of my Soxhlet assembly assembly and thereafter extract it completely till no more tannins are coming out.

Then I concentrate it and I can use this extract for my HPTLC analysis. So this is with the sample preparation. Now we go to sample application. Now sample application is done with a, if you can imagine this, this is more like your printer. So it's like your inkjet printer.

So what you can see here is a syringe. So this syringe is filled with your extract and then this syringe is controlled. This is kind of a 100 microliter syringe and you can control the rate. That is on the plate I just want to spray 1 microliter. On the next spot I want to spray 2 microliter.

So I can effectively control the volume that I apply on the plate which doesn't happen with hand. So this kind of assembly uses nitrogen or inert gases to create the fine mist of the extract and then sprays or kind of prints your extract on the TLC plate. So you can see here there is a TLC plate and this is the syringe nozzle. So syringe nozzle is also laced.

You can see this tubing. This tubing is of nitrogen gas. So this nitrogen gas helps kind of nebulize it into a tiny droplets and this tiny droplets are applied here. This plate or this steel plate moves and as a result the application is more effective here. Once you decided to apply it, you will have to take few key considerations in the mind.

First thing is the extract concentration. If the extract is too much loaded, you know it causes tailing. So I should have or use my extract optimally. In many of the cases, people often apply extracts in three different concentrations. Like 5 microliters, 10 microliters and 15 microliters.

Just to be sure that at least one of them will be running very smoothly. Then you have to plot the standard curve. This is quantification. So you have to also apply along with your extract your standards in a particular concentration like 2 microgram, 4 microgram, 6 microgram and so on.

So those spots are also placed simultaneously on the plate. Then you have to see that because this is a syringe and it's very fine creates a very fine mix your extracts. If you see your herbal extracts they are sticky and they often precipitate compounds which will often tend to block the needle of your applicator. So always filter your extracts and there should be no particles for a very seamless application.

Otherwise what is going to happen is if there is a particle it will obstruct the flow and create a blob later on if it is kind of blocking your syringe. Once your sample is applied you go for a plate development. The plate development is very much similar to that you saw in your TLC. You remember there is what is called as your TLC chamber.

Now, this TLC chamber is covered to prevent the evaporation of your mobile phase. The mobile phase is often placed below. In some cases, you have a single trough. In some cases, you might have something like this, called a twin trough, wherein you can save or store the solvent in a particular layer as well.

Generally, they are equilibrated, and what is done to achieve chamber saturation is your filter paper is applied. Now, what does the filter paper do? The filter paper will absorb all the mobile phase and then easily diffuse it in the chamber, causing instantaneous chamber saturation. So your chamber will get saturated, and once the chamber is saturated, you run your plate.

So you can place your TLC plate here, on which you have applied the spot, and then they will run. Now, if this is your normal phase TLC plate like a silica plate, of your nonpolar compounds will be on top, and your polar compounds will be at the bottom because your stationary phase is polar and your mobile phase is nonpolar. Now, there are a few additional things to consider when herbal extracts are taken into account or applied.

Now, you might see that apart from silica, there are other stationary phases, such as alumina. You can even have cellulose-based ones. These are used to separate different types of constituents. Not only that, you can change your mobile phase depending on the compounds. So say for example I have alkaloids.

A good or more universal mobile phase for alkaloids is toluene ethyl acetate diethylamine in a ratio 7 is to 2 is to 1. Now the reason why you use diethylamine is something that we discussed earlier. So if you see alkaloids, these alkaloids are present as salts. So if a compound is present as salt, imagine something like your NaCl, what is going to happen is the salt form is extensively polar. So it will tend to stay here.

So what do you do is you basify it. Now when you basify your alkaloid the alkaloid will no longer exist in the salt form. It will exist as a free base form and free base form is more nonpolar. So instead of binding here when I am using a mobile phase using diethylamine it will basify the alkaloid and as a result cause it to move because it increases its unionized nature.

Similarly, if you talk about flavonoids or if you talk about tannins, what happens is these are acidic substances. So if I am just using it as such, again because of their polarity, they will tend to bind in this region. But imagine if I have a mobile phase which has a little bit of acid. glacial acetic acid or formic acid, you will often come across this in those mobile phase. So what happens?

Now, these flavonoids OH and tannic acids or tannin COH are all in unionized nature. If they are unionized, they will run well. That's why most alkaloidal mobile phases contain amines, while flavonoid- and tannin-based mobile phases contain acids. the flavonoid and tannins based mobile phase have acids in them. Now apart from that if you have more neutral substances like volatile oil you can have a extensively lipophilic mobile phase

toluene-ethyl acetate, where toluene is as high as 93% and only 7% ethyl acetate is added. An important consideration for a good TLC plate is proper chamber saturation at a constant temperature, preferably lower, to prevent solvent volatilization and escape from the plate. the reason being your solvent should not volatilize and escape from the plate. Once your plate is developed, the next step is visualization.

So, how do you see the plates or the spots? In many cases where compounds are colored—take this, for example— I can visually see the compounds because they are colored. Even placing it under a white lamp will help. But imagine if the compound is not colored.

For example, paracetamol, aspirin, or alkaloids like quinine— They are not colored. So when I run the plate, it will appear entirely white. I don't see any color on them. So in that case, I need to have what is called as derivatization.

So Either I do a detection using UV vis or I do a derivatization. Now, what is this detection using UV vis? Now, under visible light, certain times the compounds are not visible. But when you put it under UV light,

especially there are two UV lamps in your TLC viewing chamber, that is one is at 254 nanometres. And another one is at 365 nanometers. So this two lamps there is a reason many a times your TLC plate contains a fluorescent substance which fluoresces at 254 nanometers and then what happens is your aromatic substances quench on them.

We subsequently see the example of that whereas the 365 nm is more of a purple or a violet UV region which is more you know which gives you more of a fluorescent substance or a fluorescent glow in some substances. Not only that there are compounds such as aflatoxin which will fluoresce when you put it under UV 365 nanometers. Now despite this a better way to see it under normal light or white light is derivatizing. So there are certain reagents which will react with your phytochemicals

to give you different different colors and these colors can be seen visibly. Say for example, your alkaloids, you remember we use Dragendorff's test. In the same manner, the same Dragendorff's reagent, if I dip my TLC, will give you orange color. For polyphenols, you remember you had your ferric chloride test for tannins. Similar plate, if you dip it in, all your tannin bands will become bluish-green in color.

Volatile oils, you all can treat it with what is called anisaldehyde sulfuric acid or vanillin sulfuric acid reagent. However, These reagents are not reactive at room temperature. So you might have to heat the plate for about 5 minutes at a temperature exceeding 100. So they will gradually get derivatized, and you will see different colors of terpenaceous compounds.

Flavonoids, you can derivatize them with a natural product reagent. If you are not sure about what compound there are and if you just want to detect non-specifically, you can keep it in the iodine chamber wherein iodine vapors react with any compound that is unsaturated. That is, the double bonds, and then those bands become yellowish in color. Not only that, even if the compound is saturated, you can still detect it by charring it with sulfuric acid reagent.

So let's see a few examples here. So this is a UV plate of black pepper fruits. So when we run this black pepper, especially in toluene, ethyl acetate, what happens here is you can see at one spot I have marked my standard piperine, and at two I have my black pepper alcoholic extract. So you can see standard piperine is just a single band, and this black pepper fruit contains numerous compounds.

Now this I am seeing under UV 254. So my plate has a fluorescent indicator that fluoresces the whole plate. But the moment there is any aromatic compound on top of it, it's going to quench, and that quenching is going to result in dark-colored spots. That means in that region, the fluorescence cannot reach my eye because these compounds are going to absorb it.

But now if I see the same TLC under UV 365, what happens here is you can see the same spots which earlier quenched now produce a faint glow or fluorescence. Take the same plate and then dip it in Dragendorff reagent and slightly wash it with water. What happens is your Dragendorff reagent is a little orange. So it renders an orange color.

The moment you wash it with water, the orange color remains only on places where alkaloids are present. So when you see so many bands, what is happening is you are definitely seeing so many compounds. Here you can see even more. So in the UV, there are more in the visible region also. If you see the tiny amounts, some of them have not run in.

So UV region shows you so many bands but the moment I derivatize and I wash it and I see it under visible region I see is a faint piperine standard which is orange, and this is the piperine extract. What does it mean? The remaining other compounds are not alkaloids. Similarly I can do a TLC and check for the presence of aflatoxins.

So these are aflatoxins standard B1, B2, G1, G2 and if you see different samples you can say that they have been contaminated with aflatoxins because they show fluorescence at a very same Rf. Now once we do the visualization the next step is scanning the scanning is often referred to as densitometric scanning because it measures the density of the spot being there now just take example now this is your solanum

and in your Solanum you have different different alkaloids such as your solasodine solasonin and solmargin now in that case what is done is I am using mixed standards of different concentration So I am going to prepare a standard curve for say for example solasodine. I am going to see here area under curve and I am going to write here as concentration. So this is the concentration of 2 microliter, 4 microliter, 6 microliter, 10 microliter something like that.

Now what happens is when I prepare a standard curve I might get something called as a You know, good linear observation. So at higher concentration, I'll have higher area under curve. Now, what I do is I spot my plant extracts. You can see a 24- 25 plant extracts.

Now, when I see that, I measure the intensity here, the intensity here. It's something like your area and the curve of this particular chart. Now if I see this area and the curve and I plot it I get the concentration in a very similar manner. So I can do or I can quantify my compounds using HPTLC but the only thing that I require is I'll require my standard of solasodine to quantify solasodine.

Similarly if I have solasonine standard I can quantify that as well. The thing is it can all happen simultaneously. What one more parameter I need to check is the values which is required for validation and that is LOD, LOQ, linearity. I am just emphasizing on this but there is ruggedness, robustness and so on. So what is this is whenever you try to quantify there is something called as you know slope of this curve.

Now if you take what is called as you know standard deviation of this values then Whatever you are getting in your standard curve. So if it is like 3.3 times standard deviation. If you divide it by slope of this is what is called as your LOD. That is limit of detection.

That much quantity you can detect. And LOQ. is a value which is often given by 10 times standard deviation of this values divided by the slope. So you should see that when you are quantifying them, your quantity of the extract. So this spot should be higher than the LOQ.

If it is not, you have to reapply and do the measurement one more time. Now here are a few examples. So you can see here your chlorogenic acid. You can see this is the standard LOQ. And that can be visualized using different reagents.

So you can visualize it under UV. You can visualize now what has happened here. Here you have applied an NP or natural product reagent. And this is under normal daylight. So you can see it's a very faint thing under normal daylight.

But if you apply a derivatizing agent, you can see the color intensity much better. And for quantification, you would definitely prefer this plate over this plate. Similarly, I can detect curcuminoids if I have standard curcuminoids with me, and then I can apply different turmeric samples. I can see them under UV 254.

I can see them under visible that is normal light or I can derivatize them. You know your haldi that is turmeric when you add any alkali. They become red in color. So you can use alkali or you can even derivatize it with something like your anisaldehyde sulfuric acid to get a colored. So this is after derivatization.

So you can quantify this plate at this wavelength, at this wavelength or even at this wavelength. In some cases it can be quantified and then you see where the intensity of those peak is more. Wherever the intensity of that peak is more, always use that wavelength for measurement. Not only that, in certain cases, spices, we just saw ASTA values. Sometimes people deliberately add inks or dyes to it.

So you can see here. They have spiked it with different different colorants and your HPTLC is able to make out even the most dilute version of the spiked colorants. So, you know, it's just 2 ppm which is spiked in that colorant. So, still you are able to identify versus your natural sample.

So, now going on to few more applications, your HPTLC based on the fingerprint application. So if I know that this is going to be my fingerprint of Solanum, I can just use this fingerprint and analyze other plants. If other plants are showing the same pattern, there's a good chance it is Solanum, but if it is showing slightly different bands, what does that mean? Is it another plant or another species? Then you can quantify the phytoconstituents we just saw.

You can validate and standardize the raw material. There's a lot of variation happening in the raw material, even if you take it from different geographical places or even if you take

it from different seasons. Your HPTLC will help you quantify the actives and tell you how much variability is happening. Not only that, we just saw it with colors.

So you can detect adulterations, you can detect other plant species, you can detect, for example, your methyl salicylate—whether it's a natural methyl salicylate or a synthetic one. synthetic one will have more of your salicylic acid as an impurity impurity, whereas your methyl salicylate—that is, your wintergreen oil—will show more of your monoterpene bands in that, so you can know which oil is natural and which oil is synthetic. Not only that, you can detect your aflatoxins.

And yes, you can quantify this not only from the plant matrices but also from the herbal materials. So here are a few more references if you wish to understand more about HPTLC as a method of quality control. And thank you, everyone, for your patient listening. Thank you.