PHARMACOGNOSY AND PHYTOCHEMISTRY

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Week 10

Lecture50

Week 10: Lecture 50: Chemical Methods of Quality Control of Herbal Drugs

Hello everyone, and welcome back to the NPTEL course in pharmacognosy and phytochemistry. We are in week 10, where we are exploring different quality control methods for the evaluation of herbal drugs. So far, we have covered microscopic methods, macroscopic methods, organoleptic methods, and physical methods of analysis for quality control. Today, we are going to delve into a different set: the chemical methods of quality control. Now, chemical methods of quality control, as we previously saw, can be broadly divided into two categories.

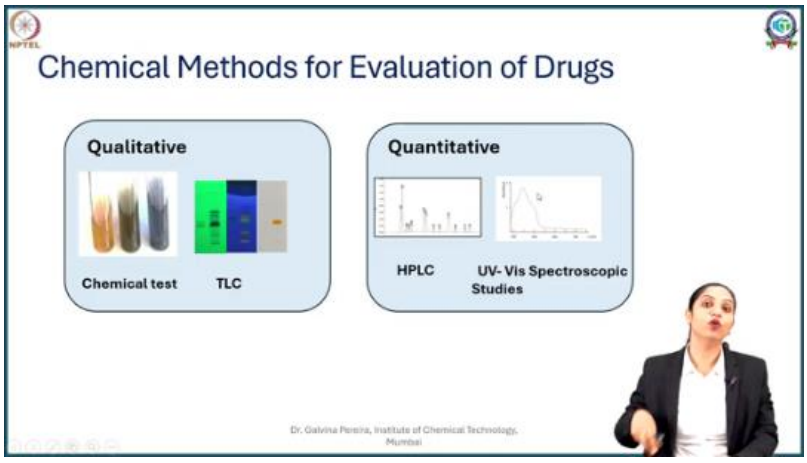
The qualitative method. Qualitative methods are those that tell us whether the compounds—especially the phytochemicals or the chemicals representative of the plant—are present or not in the given sample. This sample can be plant material, a plant extract, or a herbal formulation. When we do this, we can determine whether a compound—especially a flavonoid or a triterpenoidal compound—is present or not. Recalling our chapter on tannins, where you saw these three test tubes—if you recall, this was your ferric chloride test.

We said that when your tannin extract is treated with ferric chloride and the color of the solution changes to green, we conclude that they are condensed tannins. But if the color of the solution changes to blue, we conclude they are hydrolyzable tannins. Through this test, we confirm that our extract contains tannins. Through this test, we also determine what type of tannins are present. So, qualitative methods provide that kind of data.

Similarly, another example where you can perform a qualitative test is thin-layer chromatography. Here, you can see that you can mark a standard compound or a set of standard compounds and check if the compounds in the extract correspond to the same Rf. So, if the corresponding band is always seen in the extract, we can be sure that our marker compound is present. So, is this present? Is it not?

And what type of compound is present is what you can determine from the qualitative analysis. Now, coming to quantitative analysis, in this case, you are specifically going to quantify. That means determining how much of these compounds are present. So, if you see this graph, perhaps under UV light. There is something we call the absorbance value.

So, the more the compound present, the higher the absorbance value will be. Whereas, if you see your HPLC again, the position of the peak will tell you whether the compound is there or not. But if you see this peak. The area and the curve of this peak will tell us how much quantity of the compound is present. So, chemical methods of evaluation are much more accurate.



They give us what types of compounds are present. They tell us how much of the compound is present. So let's see different chemical methods of evaluation. So when we are seeing or discussing our methods of evaluation today, we are going to discuss qualitative methods, which include phytochemical tests or identification tests. It includes microchemical tests or histochemical tests, which are done under a microscope.

This might also include your limit tests for heavy metals. See, whenever you are processing an herb, there are impurities that might come in, and some of these impurities include heavy metals. Heavy metal poisoning causes a lot of acute disorders, and as a result, the limit to the amount of heavy metals that should be present in the sample has been specified by different pharmacopoeias. So, we will be seeing limit tests as well as TLC as a method. So, in this particular session, we will be seeing phytochemical evaluation, microchemical tests, limit tests, as well as thin-layer chromatography.

In the subsequent sessions, we'll also be discussing a few quantitative tests, including quantitative tests for different classes of phytochemicals. Specific determination of compounds using chromatographic techniques such as HPLC, HPTLC, UPLC, and gas chromatography. Then we will be analyzing our pesticidal residues, determining mycotoxins such as aflatoxins, and during the process of extraction, you know, we use solvents. Are there any residual solvents remaining in our sample? This will be discussed in the subsequent session.

Now, seeing the depth of qualitative methods of analysis, let's start with phytochemical methods. Phytochemical methods, as I can say, summarize the previous nine weeks that we have discussed. In the nine weeks that we discussed, we covered different sets of phytochemicals. We examined their properties, and at the same time, we studied their chemical properties. What does it mean?

We observed their chemical reactions. What is the shape or structure of the compound? Specifically, what does the compound react with? What colored reactions do they produce? These are called phytochemical tests.

Each chapter provided a brief overview of evaluating those classes of compounds through phytochemical tests. So, let's quickly summarize them here. If you recall, in the flavonoids, when you have your benzogamma pyrone or, more specifically, phenyl benzogamma pyrone nucleus, it responded to what is called the Shinoda test. Do you remember what it is? It is essentially a test used to reduce the flavonoid nucleus, performed using magnesium turnings and concentrated HCl, which reduced the flavonoids to anthocyanins, producing a cherry-red coloration in pure form and a slight orange-pink coloration in extract form.

Similarly, whenever they were treated with alkali, you got what is called an alkaline reagent test, which gave a nice, vivid yellow coloration with flavonoids. Now, similarly, recollecting your Unit 4, that is your tannins—if you remember, tannins have the ability to tan the skin. In this test, or particularly in this Goldbeater's test, we checked its ability to tan the Ox intestine. Similarly, the ability of tannins to precipitate proteins was also evaluated by the gelatin test. Then, maybe in your Unit 5 or Week 5, we saw alkaloids, and alkaloids, because of their nitrogen content, gave you a positive Dragendorff's test, which was potassium bismuth iodide reagent. And gave you an orange-colored precipitate.

Whereas with Mayer's test, which is potassium mercuric iodide, you got a whitish-colored precipitate. Similarly, when you see anthraquinones, anthraquinone drugs. Can you all recollect?

We did two anthraquinone-containing drugs. Specifically, your aloes as well as your senna. At the same time, we also saw a colorant called cochineal. So all of these anthraquinones follow a test called Bornträger's test, which, in the presence of an alkaline pH, your free anthraquinones gave you a pink or cherry-pink coloration. This was true test.

That is, the Bontrager's test is true for O-glycosides. But when you want free anthraquinones from C-glycosides, so that is when you want your free anthraquinones from your C-glycosides. What you do in this case? you use little harsher reagent and that harsher reagent was your ferric chloride. So you're going to take ferric chloride and hydrochloric acid. You're going to hydrolyze the C-glycoside and then kind of partition in chloroform and then you kind of made it alkaline either by caustic alkalis or by ammonia that test was your modified Bontragers test similarly for your coumarins they have their inherent ability to produce fluorescence and this fluorescence can be seen more enhanced in alkaline and in some cases even in acidic pH so you saw fluorescence test for your coumarins

Volatile oils, we saw non-specifically you pour volatile oil on any filter paper it will evaporate. So initially it will stain and that stain will be transient because the oil is evaporating. At the same time because of the terpeneceous content it gives what is called as your vanillin sulfuric acid test. So when your volatile oil containing chloroform extract

is treated with vanillin sulfuric acid reagent in that case you get a red to reddish brown coloration. Not only that, we also discussed steroids and triterpenoidal compounds.

Do you all recollect this test? This test is more specifically called as your Liberman-Burchard test in which you took anhydrous chloroform extract of your drug and in that you carefully added acetic anhydride and slowly a small quantity of concentrated sulfuric acid. What happens is you get a greenish coloration. Similarly, you did the Salkowski test but without the acetic anhydride.

And a simple test were your foam test for saponins where you just had to take the aqueous extract and shake it well. It generates a copious foam which is stable even after 15 minutes. Similarly, saponins have the ability to hemolyze the RBCs, and this hemolysis test forms the evaluation of saponins. So if you're given a drug, a herbal formulation, or a herbal extract, what we have done so far is discuss drugs category-wise. When we mentioned orange peel, we discussed flavonoids, We didn't discuss pectin or essential oil. But if you take this orange peel and perform the phytochemical test, there is a good chance that the essential oil test will be positive because orange contains volatile oil.

It will give a positive test for pectin. It will give a positive test for flavonoids. Similarly, if you take licorice as a drug, licorice contains flavonoids, tannins, and even saponins. So a herbal drug, depending on the constituents it contains, will give a set of positive tests. This is what you call phytochemical evaluation.

So whenever you are given a new drug to assess, you carry out a series of tests to determine which phyto-constituents are present. Then you check the literature. Do they correspond to that? If you observe that in tannin-containing drugs, such as nutgalls, The tannins test, that is, the goldbeater's test, is negative.

What does that mean? Either that gall sample has been exhausted, meaning the tannins have already been removed, or it is a sample other than nut gall. So, there is a good chance of adulteration or substitution happening there. Now, in certain cases, it is not a class of phytoconstituent that is detected. Sometimes you check for specific phytochemicals.

So, say for example, I want to check whether the sesame oil given to me is genuine or not. In that case, I will perform my Baudouins test, which is more specific for sesame oil rather than a test for lipids. If you remember, your test for lipids was the saponification test. That is not going to tell me whether it is sesame oil or castor oil.

Sometimes I need to be more specific and move drug-wise. So, some drugs respond to some specific tests. That specific test will help me ascertain whether the sample given to me is genuine or not. Here are a few examples. So, sesame oil gives you your Baudouins test.

If you recollect, in this, what is done is you take a little bit of sucrose and react it with hydrochloric acid, shake it for a while, keep it for five minutes, and you should get a reddish coloration.

Similarly, if you recollect aloes. Now, the anthraquinone test will be given by aloes, it will be given by senna, and it will be given by most of the drugs containing anthraquinone. So, Borntrager's test is going to be non-specific. If I have to specifically check if the sample given to me is aloes or not, what I can do is a Borax test.

So, if you recollect, the Borax test was a test in which aloes was initially boiled in water, filtered, and to that filtrate, a little bit of borax was added and heated. So later on, when you put this solution under UV, you got a greenish color fluorescence. Now, this test is not going to be answered by all anthraquinones. So this is a more specific test for aloes.

Remember, we also did a fluorescence test for asafoetida, but this time the fluorescence was blue in color. So asafoetida contains umbellic acid, and you could convert it into umbelliferone first by hydrolyzing the ester and then cyclizing it. So initially, you treat it with hydrochloric acid and later on treat it with ammonia to get a nice blue color fluorescence. Now, not all your oleo-gum-resins will give you a fluorescence test, but definitely all oleo-gum-resins will give you an emulsification test. So the emulsification test will tell me it's an oleogum resin, but your fluorescence test will tell me specifically if it is asafoetida or not.

Giving you few more examples from the carbohydrates when we did acacia we said acacia contains an enzyme especially the fresh sample called as peroxidase. So if you take this and if you take little bit of benzidine. And hydrogen peroxide, this peroxidase enzyme will help in oxidizing it and your benzidane will form a blue color compound. So the presence of blue coloration will indicate its acacia. When I compare or if I am doubting whether the sample given to me is acacia or is it going to be tragacanth, maybe in some cases I have mixed sample up. So there's going to be trigacanth and I'm not sure what it is. Rather than doing the test for gums, I will carry out more specific test for acacia.

Now another specific test is your Vitali - Morin test which is true for tropane alkaloids especially if you see your atropine. So in this case you are going to extract your alkaloids in a suitable solvent and dry it and to that dry residue you initially treat it with nitric acid and later on with a potassium hydroxide to get a purplish coloration or a residue. Now the last example I am giving in this case is colophony.

So colophony again is an example of resin. Now resins again are non-specific. So I know colophony contains abetic acid. So making that abetic acid react with copper acetate or cupric acetate more specifically. So what will it do? It will convert that cupric acetate to a nice emerald green coloration. That will help me ascertain that it's colophony and not plastic masses.

So previously, in the previous slide, what we saw here is that you have classes of compounds. So I can take a polyherbal formulation. I can take a herb. I can take a sample and evaluate whether this class of compounds is present or not. Whereas if I see the subsequent slide, in this slide, the specific test tells me whether this herb is particularly present, whether this phytoconstituent is specifically present in the plant or not. So in some cases where the drug is exhausted, you will predominantly know if the compound is there or not using specific phytochemical tests. In some cases, what is done is the chemical test will tell you if the impurities are present. So forget the classes of compounds, forget the compounds.

What if you can specifically check? If some impurities are there. I'll give you an example. Say, for example, when we discuss lipids, we said lipids are esters of fatty acids. Now compare that to paraffin.

Paraffin is just a hydrocarbon. Now, when you do a saponification test, your fatty acids will saponify, whereas your paraffin will not saponify. So, a saponification test will tell me whether it is a triglyceride or a fat or a wax, or if it is really paraffin that does not saponify at all. On saponification, your soap will dissolve in water, but paraffin cannot dissolve in water because it will not saponify. Similarly, In some cases, what happens is your acacia or agar is a little expensive.

So, people often tend to dilute them with starch because starch is very cheap and easily available. So, you know that acacia and agar do not give you blue coloration with iodine, but starch does. So, when you take your acacia or agar sample and you treat it with iodine—especially iodine solution, which is iodine and potassium iodide—if they show you blue coloration, that means there is a good chance that your acacia or agar has been adulterated with starches. Similarly, cottonseed oil is a very cheap oil and is not considered to be good or properly edible. So, in some cases, this being a cheap oil is often used as an adulterant for other oils.

So, I can easily check. What I need to do is... I need to dissolve that oil in carbon disulfide and then add a little bit of sulfur in amyl alcohol. If I get a reddish coloration, that means that sample of your fixed oil contains what is called cottonseed oil. We also saw adulteration of honey, and in that case, we checked what is called Fiehe's test.

So Fiehe’s test is basically a test where you can check the quantity or amount of hydroxymethyl furfural. Now, this hydroxymethyl furfural is naturally present in your honey, but it is present in a very minute quantity. But when you are dealing with synthetic invert sugars, the quantity of hydroxymethyl furfural becomes very high. So in that case, if I just take this honey and treat it or extract it with ether, and this ether layer, if I treat it with resorcinol and hydrochloric acid, if I get a reddish coloration, which is persistent. In that case, I say that my Fiehe’s test is positive. That means my honey is adulterated with artificial invert syrup.

Similarly, in some cases, you will find that there are natural medicines, or people claim that those are natural medicines, and then they add certain steroids to them for getting an enhanced effect, what I can do in this case is check for those steroids. Now, mind you, in some cases, what will happen is the plant may also contain some natural steroids. Those cannot be evaluated if the plant material contains steroids. But if the plant material is not a rich source of steroids and contains steroidal compounds in a very minuscule amount, so that they give this test almost negative. In that case, suppose with the formulation, you get this test positive.

That means it indicates that your given preparation contains steroids. Now, this can be evaluated by the same tests, that is, your Liebermann-Burchard test as well as the Salkowski test. Now, moving on to the next set of evaluation tests. Now, this set of evaluation tests uses a microscope. Now imagine chemical tests done under a microscope or chemical tests done in a quantity so minuscule that you will have to use a microscope.

Yes, and this happens when you use something called a microchemical or histochemical test. Now, microchemical or histochemical tests are as simple as adding iodine on a section. So, when you do a transverse section, if you add iodine, you know your iodine is going to stain your starch. So, you can see here this is a turmeric sample transverse section. So, when I carry out the transverse section of this rhizome, I mounted it. It's going to be colorless with some yellow specks.

And these yellow specks resemble curcuminoids. Now, if I have to check the presence of starch, you can see some blue specks here. What I have done is I have just added iodine. So, if a drug naturally contains starch and it turns blue, that is good because it's a genuine drug. But if a drug naturally does not contain starch and still stains blue, that means some other plant part has been added to it.

Similarly, we saw that there are certain plants which contain mucilage. A good example of that is isabgol. You can check whether it is genuine isabgol or not by staining it with ruthenium red and seeing it under a microscope. So, if it is genuine mucilage, it is swelling. It will acquire a reddish coloration with the ruthenium red.

But in some cases, you know, if it is swelling or if it is forming a slimy consistency because of carbohydrates, which are not mucilaginous. Say, for example, gums. In that case, you will not get your red coloration. Similarly, you can check for the presence of lipids, that is, the occurrence of fats, waxes, or even oils. More specifically, your fixed oils and volatile oils by staining with a lipophilic stain.

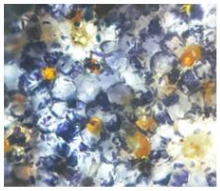
Now, these lipophilic stains are your Sudan red and tincture alkana. So, they have a good affinity for lipids. So, what is going to happen? Because of their affinity, your lipid is going to absorb that stain.

So, your whole section will be colorless, but wherever the lipids are present, you will get a nice pink to reddish coloration, whether you use your Sudan red or your tincture alkana. Now, not only that, you might have seen that in certain transverse sections, say, for example, Rauvolfia that we discussed previously, just addition of nitric acid and seeing that coloration will tell us whether Rauwolfia contains reserpine or not. Is it there, or is it an exhausted drug? Now, similarly, you can do it for Alkanna. Now, Alkanna, you know, contains your naphtha-zerine pigments, and these naphtha-zerine pigments turn blue.

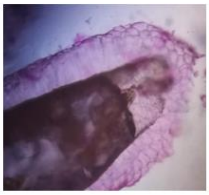
When they are treated with an alkali. So take those alkanna sections and just add a drop of your base. If the section or part of the section turns blue, in that case, you can tell that it's definitely a genuine alkanna drug. Similarly, for clove, clove contains volatile oil, and a chief constituent of that volatile oil is eugenol. So if you have a clove thick section and you drop in your potassium hydroxide, within those oil cavities, you should see crystals of potassium eugenate.

If you do not see it, that means the clove has been deprived of the oil. Similarly, you can also check simple features such as suberin and lignin. A good example in this section is the section of ephedra, and you can see here So this is the brownish matter, which is pith. But you can see the xylem.

Microchemical / Histochemical tests



T.S. of Curcuma rhizome



Isapghol treated Ruthenium red

Transverse Section of Ephedra stem

- **Mucilage** : Rhuthenium Red
- **Lignin:** Phloroglucinol- Hydrochloric acids
- **Lipids(Oils):** Sudan Red, Tincture alkanna
- **Starch :** Iodine reagent
- **Reserpine:** Concentrated Nitric acid
- **Alkanna:** Potassium hydroxide reagent
- **Clove :** Potassium Eugenate test

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It acquires a slightly pink coloration with your Phluoroglucinol HCl, and that indicates your xylem here is lignified, as are your pericyclic fibers. So certain staining reagents also help in identifying the different tests.

Moving on to the next set of qualitative evaluations. Now, this is not clearly qualitative; this is a kind of semi-quantitative evaluation. Now, semi-quantitative evaluation—I'll give you an example, and that's your limit test. So whenever I want to lay a benchmark or a limit test, On the quantity of substance that can be used, I refer to the pharmacopoeia and use what is called the limit test. Now this includes your limit test for chlorides, heavy metals, and other substances. So let's see the limit test for heavy metals, as more and more monographs, especially the pharmacopoeia monographs of herbal drugs, specify that you should carry out the limit test for herbal drugs. Now, in order to do that, what you need to do is

First, if you take the herbal drug, it is colored and maybe odd in consistency. There are drugs which are organized, and there are drugs which are unorganized, and that creates a lot of problems. So the raw material, whenever you want to do a limit test, is the ash. So you are going to take a gram of a drug substance and completely incinerate it. Now, with the previous sessions, you know that once you ash it, your organic content is gone, and what is left is your inorganic content.

Now, this inorganic content is kind of suspended or dissolved. And you use what are called Nessler cylinders because we want comparison. So in this case, for example, when I am doing the test or the limit test for heavy metals, what I do is use a standard lead solution to compare. And this standard lead solution is about 20 ppm. So the solution should not exceed 20 ppm per ml of lead.

Now I will take my ash. This ash, I will also keep it, and I want the same thing. So how do I do this? I will use my standard lead solution. I will use my sample ash.

Now, in both cases, I will treat them with H2S gas. Now, H2S gas—how do you do it? You create an H2S solution by bubbling H2S gas through water and then pour it in. Now, there are different methods. If you check the Indian Pharmacopoeia, they use H2S gas in methods 1 and 2 and sodium sulfite in method 3. So pharmacopoeias also specify different methods. So what is going to happen?

Say, for example, your lead metal is there. It will react with H2S, and what you will get is PbS. Now, PbS is actually a black-colored precipitate. So, as a result, this whole solution, when I make up the volume, will appear as a blackish-brown solution. Now, this blackish-brown solution, when I compare it with the standard— Now, the standard, because I know it already has 20 ppm, will acquire a certain brown tint.

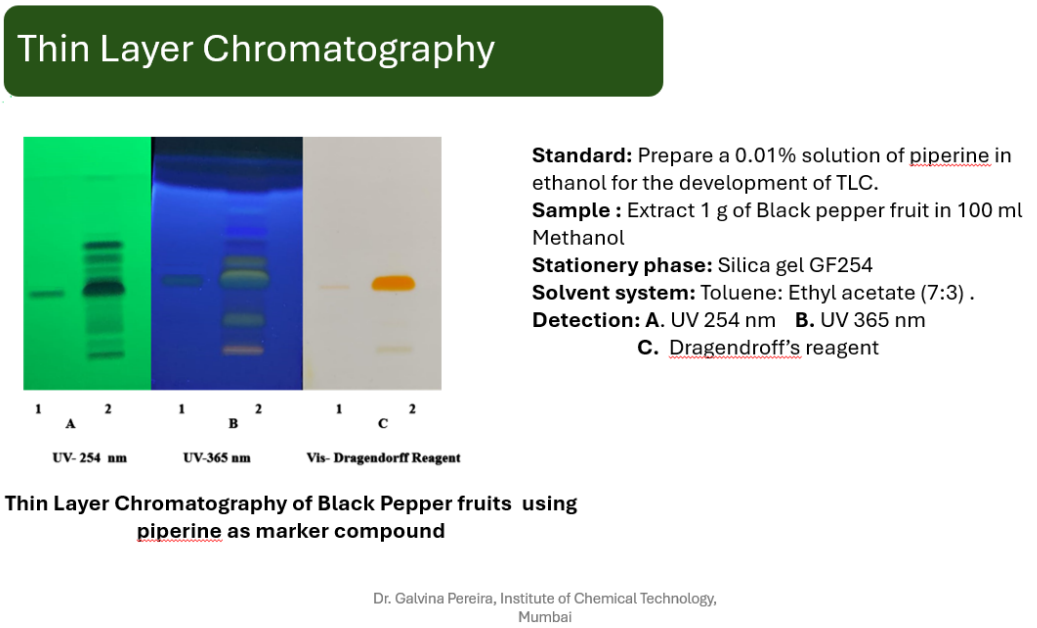
If the brown tint of my sample is darker than that, what does it mean? That means my lead concentration is higher than 20 ppm. In that case, my sample fails, but if the color of my sample is lighter than the standard 20 ppm lead, then I can confidently say that yes, my sample might have heavy metals, but they are below the limits. So that is how you do the limit test for metals.

Another qualitative test, which we have also seen previously, is thin-layer chromatography.So, thin-layer chromatography, if you recollect, uses a very polar stationary phase. It uses a very non-polar mobile phase. In this case, toluene ethyl acetate. And then you spot your sample and make it run. Now, in a developing chamber.

Now, when you run your sample in a developing chamber, as the sample passes, Because of its lipophilicity and hydrophilicity, There is a pull and push, and eventually, it stops at

a particular point. We call this your retention factor or Rf. So, depending upon how polar or non-polar it is, your extremely non-polar compounds will be seen at the top, whereas extremely polar compounds will be seen at the bottom.

And depending upon how polar or non-polar other compounds are, say for example, I can say of all the compounds this is the most non-polar compound which is present in my black pepper whereas this is the most polar compound which hasn't even run in the given mobile phase. Now I might have different visualizing agents also. The first case the plate is just seen under UV 254. The reason is I am using a fluorescent plate so it already has a fluorescent so whenever an organic compound will run on it the fluorescence is not able to reach my eye because this organic compound is quenching it. So this quenching leads to formation of black color spots.



In the second case, I observe a little bit of faint coloration and that is because these compounds produce a slight glow and a fluorescent. So under UV365, these compounds are fluorescent. But in some cases, if I don't see the compound at all in these two wavelengths, what I can use is I can use a derivatizing agent. These are the same reagents which we use for chemical tests. So remember for black pepper, it's piperine.

So, piperine is an alkaloid. So, I am using Dragondorf reagent as a derivatizing agent. And as a result, I can see instead of a orange brown precipitate, the spot where my piperine is present has become orange brown. So, these are few chemical quantitative, these are few chemical qualitative tests for evaluation of herbal drugs. Here are few references if you wish to dwell more into this.

And thank you, everyone, for your patient listening. Thank you.