

Vanillin Assay

Ann E. Hagerman © 2002

Contents

Introduction	1
Reagents.....	3
Preparation of Working Reagents.....	3
Extraction	3
Analysis of Standards	3
Analysis of Sorghum extracts.....	3

Introduction

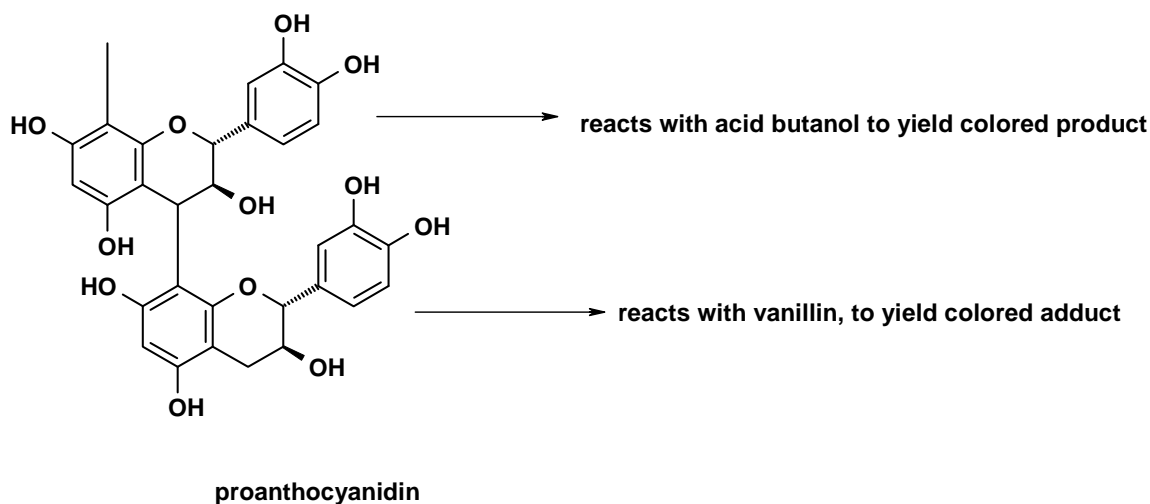
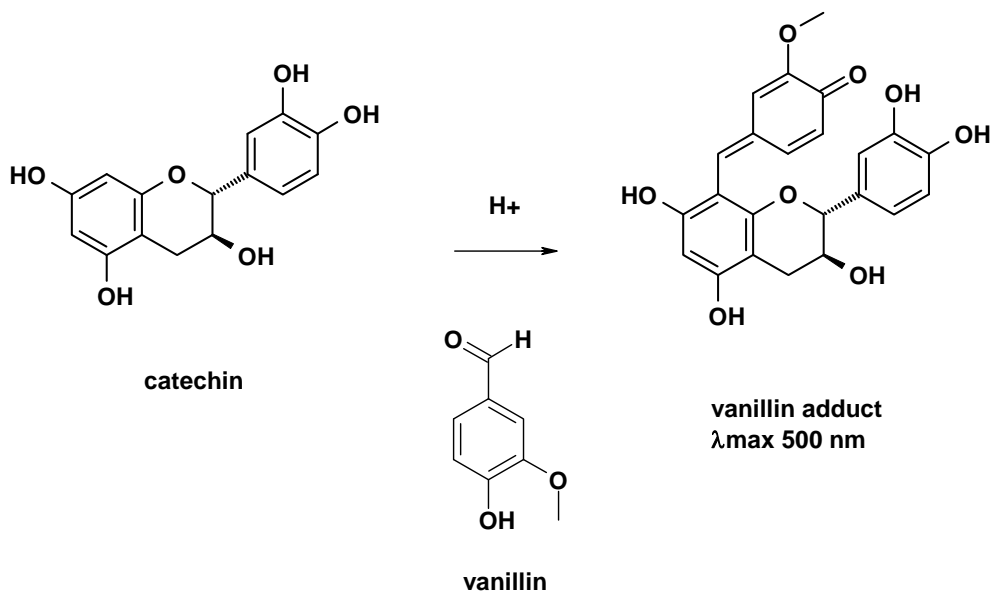
From Price, van Scoyoc, and Butler, *J. Agric. Food Chem.* 26, 1214-1218 (1978)

This functional group method for condensed tannins is especially widely used by agronomists. There are significant difficulties in interpretation of the method. We rarely use this method in my lab, since I find the acid butanol method simpler and more reliable.

Understanding the structural chemistry of condensed tannins and flavanoids is essential to proper use of the vanillin assay. The vanillin reaction involves reaction of an aromatic aldehyde, vanillin, with the metasubstituted ring of flavanols to yield a red adduct. Although the vanillin reaction has been widely used to estimate condensed tannin (proanthocyanidin), the reaction is not specific for condensed tannins. Any appropriately substituted flavanol reacts in the assay. Thus the formal "monomer" of the condensed tannins, catechin, also reacts to yield a red colored adduct. Furthermore, because the vanillin reacts only with meta-substituted flavanoids, the 5-deoxy proanthocyanidins (e.g. quebracho) do not produce much color with vanillin. Reactivity with vanillin is not sufficient evidence for the presence of condensed tannins.

Catechin is commonly used to standardize the vanillin reaction, but there are problems with interpreting the meaning of "catechin equivalents". Under the normal conditions for the vanillin assay (methanol solvent), tannins (proanthocyanidins) and catechin both react with vanillin, but the rates of reaction of the polymer and the monomer are quite different. In general, the color yield is lower for the monomer than for the polymer. Although the absorbances obtained from running the vanillin reaction in methanol on an unknown tannin-containing sample can be converted to "catechin equivalents" the complexities of the system make it difficult to interpret the meaning of those equivalents on the molecular level.

The modified vanillin method was developed to overcome those problems, but proves to be more useful for estimating molecular weight of condensed tannin than for quantitative analysis. The vanillin method described here was developed for analysis of condensed tannin in Sorghum grain; modification of the



methods for sample preparation would probably be necessary to use the method with other tissues.

The method described here uses 0.5% vanillin rather than the 2% originally recommended by Burns. By decreasing the vanillin concentration, the dependence of the reaction on temperature is minimized. The vanillin reaction is very sensitive to the presence of water. Even a small amount of water in the reaction mixture will substantially quench color yield. All standards should be prepared in anhydrous organic solvents (usually methanol). If water must be present in the samples to be analyzed, the same amount of water should be added to the standards. Vanillin reacts only with free flavan-3-ols, or with the terminal unit of the proanthocyanidin. The vanillin method combined with the acid butanol method provides an estimate of degree of polymerization.

Reagents

- 1% vanillin in methanol (1.0 g vanillin up to 100 mL with absolute methanol). Store in a dark bottle at 4°C
- 8% concentrated HCl in methanol (8.0 mL concentrated HCl brought to 100 mL with absolute methanol).
- 4% concentrated HCl in methanol (4.0 mL concentrated HCl brought to 100 mL with absolute methanol).
- Constant temperature water bath set at 30°C. (If this is not available, there will be temperature-dependent variation in the data).
- 0.3 mg/mL catechin (3.0 mg catechin brought to 10.0 mL with absolute methanol). Store in a dark bottle at 4°C for up to three days.

Preparation of Working Reagents

The working vanillin reagent must be prepared daily from the solutions described above. One part of the 1% vanillin solution is mixed with one part of the 8% HCl solution. The working vanillin reagent and the 4% HCl solution are brought to 30°C in the water bath before starting the analysis each day.

Extraction

For best results, the extraction and analysis should be carried out on a single day. The grain should be ground no more than one day in advance of analysis. About 200 mg ground Sorghum grain is weighed exactly, and then extracted with 10.0 mL absolute methanol for 20 min in rotating (Labquake rotator) screw cap culture tubes (13x100 mm). The mixture is centrifuged for 10 min at 3000 x g, and the supernatant is used in the analysis.

Analysis of Standards

1. 0 to 1.0 mL aliquots of the catechin standard are dispensed into two sets of culture tubes and each sample is brought to 1.0 mL by the addition of absolute methanol. Tubes are incubated in the water bath.
2. 5.0 mL of the working vanillin reagent is added at 1.0 min intervals to one set of standards, and 5.0 mL of the 4% HCl solution is added at 1.0 min intervals to the second set of standards.
3. The samples are left in the water bath for exactly 20.0 min, and are then removed and the absorbance at 500 nm is read.
4. Because the color continues to develop as time passes, you cannot go back and re-read any sample. You must maintain the strict 1.0 min intervals for reading that you used in the addition of reagents.

The absorbance of the blank (vanillin reagent with no catechin) is subtracted from the absorbance of the corresponding vanillin-containing sample. A standard curve is constructed (Abs vs. mg catechin) and the linear portion of the curve is extrapolated to produce the standard curve.

Analysis of Sorghum extracts

1. 1.0 mL aliquots of each extract are dispensed into culture tubes. Duplicates of each sample are required so that the sample and a blank can be run for each sample.

2. Tubes are incubated in the water bath.
3. 5.0 mL of the vanillin reagent is added at 1.0 min intervals to one set of samples and 5.0 mL of the 4% HCl solution is added at 1.0 min intervals to the second set of samples (the blanks).
4. The samples are left in the water bath for exactly 20.0 min,
5. The absorbance at 500 nm is read.

Because the color continues to develop as time passes, you cannot go back and re-read any sample. You must maintain the strict 1.0 min intervals for reading that you used in the addition of reagents.

The absorbance of the blank is subtracted from the absorbance of the corresponding vanillin-containing sample. The blank can be substantial for tissues that contain large amounts of pigments.

The value obtained is compared to the standard curve to obtain "catechin equivalents".