A Critical Evaluation of the Vanillin Reaction as an Assay for Tannin in Sorghum Grain

Martin L. Price, Steve Van Scoyoc, and Larry G. Butler*

Several parameters of the vanillin assay were examined to determine which must be most closely controlled to ensure accuracy and reproducibility. A 20-min extraction in methanol was found to be adequate. When corrected for background color, the modified vanillin assay was found to give nearly identical values with those obtained with the regular vanillin assay, except with group II sorghum. The reactions of tannin and catechin, the usual standard, with vanillin were found to differ markedly in reaction kinetics. Assays of purified tannin showed that use of catechin equivalents overestimates tannin content. The assay was found to be extremely temperature dependent. Revised procedures for the vanillin assay are presented which give excellent reproducibility.

The vanillin assay (Burns, 1971) is widely used for quantitative measurement of condensed tannin (or its monomeric components) in sorghum grain. The chief advantage of this method appears to be its specificity for a narrow range of flavanols and dihydrochalcones which have a single bond at the 2,3 position and free meta-oriented hydroxy groups on the B ring (Sarkar and Howarth, 1976). In contrast, redox methods, for example in the Folin-Denis (Burns, 1963) or Prussian blue (Price and Butler, 1977) assays, detect any phenol present (with varying sensitivity).

The procedures for the vanillin (methanol extraction) (Burns, 1971) and modified vanillin (1% HCl in methanol extraction) (Maxson and Rooney, 1972) assays described in the literature do not describe how the conditions were selected and which parameters must be closely controlled to ensure reproducibility between laboratories. We and others (Maxson and Rooney, 1972) find considerable variability within and between laboratories.

The work reported here was designed to examine and optimize several parameters of the vanillin assay. Factors examined were (1) the effect of extraction time, (2) the advantage, if any, of the modified vanillin assay, (3) the unusually high values for tannin content obtained from the vanillin assay compared to other assays, (4) the adequacy of catechin as a standard, (5) the effect of HCl and vanillin concentration, (6) the effect of temperature, (7) the reproducibility of the assay, and (8) the sensitivity of the results to time elapsed between grinding and analysis.

Based on these observations and revised procedures for the vanillin and modified vanillin assays, this reaction now offers a much more precise tool for studying sorghum tannin and probably tannin from other sources.

EXPERIMENTAL SECTION

Sorghum grain was provided by Dr. John Axtell from seed trials grown at the Purdue University Agronomy Farm in 1975 and 1976. Grain was hand cleaned to remove all glumes and broken grain and ground to pass a 0.4-mm sieve on a Udy cyclone sample mill (Tecator, Inc., Boulder, Col.) equipped with a vacuum attachment. Ground grain not used within a week or less was discarded. Single grains were ground for analyses for 1 min in a Wig L. Bug, Model 5 AR, amalgamator (Crescent Dental Mfg. Co., Chicago, Ill.).

Department of Biochemistry (M.L.P., L.G.B.) and Department of Agronomy (S.V.S.), Purdue University, West Lafayette, Indiana 47907.

Extracts were prepared by continuously rotating 200 mg of ground grain and 10 mL of methanol (1% concentrated HCl in methanol for the modified vanillin assay) in screw-top test tubes at room temperature for 20 min, or as specified. After centrifugation (immediate suction filtration for experiments in Figures 1 and 2), extracts were assayed as previously described (Burns, 1971), and ΔA_{500} values were calculated by subtracting the blank (Price and Butler, 1977). In later experiments, the concentration of vanillin was changed from the usual 2 to 0.5%. Fresh extracts were prepared every few days to avoid changes in tannins as the solutions aged. Most assays were performed at room temperature but, where indicated, a 30 °C water bath was used for the entire 20-min interval between mixing reagents and reading the absorbance. Absorbance at 500 nm was read in 1-cm cuvettes on a Zeiss PMQ-11 spectrophotometer (slit width, 0.03 mm) or a Beckman-24 spectrophotometer with a flow-through 1-cm cell (Figures 5 and 6 only). The effect of extraction time was studied in the same manner except that extracts were filtered in Buchner funnels and immediately analyzed.

Partially purified sorghum tannin was obtained with a modification of the published procedure (Strumeyer and Malin, 1975) by adsorbing a methanolic extract of a sorghum hybrid, BR-54, on Sephadex LH-20, washing the gel four times with methanol, eluting with 50% aqueous acetone, and removing the acetone by rotary evaporation. Lyophilization produced a fluffy, brown powder, soluble in water or methanol. Catechin was obtained from Sigma Chemical Co.

RESULTS

Extraction Time. Figure 1 shows ΔA_{500} values obtained with the vanillin assay on several varieties of ground sorghum grain, with extraction times from 1 to 60 min. Under the conditions used (100% methanol as solvent), no further increase in extracted tannin was observed after a few minutes. Values after 24 h, the recommended extraction time (Burns, 1971), had actually decreased 10–15% from the values after 1 h.

Similar data for the modified vanillin assay, in which extractions are done in 1% methanolic HCl (Maxson and Rooney, 1972), are shown in Figure 2. ΔA_{500} values decrease slowly with extraction time for most varieties. This is presumably due to destruction of tannin by HCl, as shown by a continued decrease in absorbance with time even after separation of the extract from the grain (data not shown). There are a few varieties of sorghum in the world collection for which the extraction pattern is markedly different. Three of these uncommon varieties, E, F, and perhaps H, are included in Figure 2. Two to

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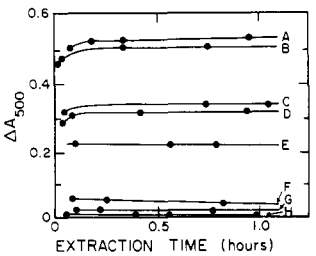

Figure 1. ΔA_{500} for the (2%) vanillin assay at room temperature vs. time of extraction of the grain (in methanol): (A) BR-54, (B) Savannah, (C) 1S-8193, (D) NK-300, (E) IS-15525, (F) 601562, (G) 601502, (H) 601530.

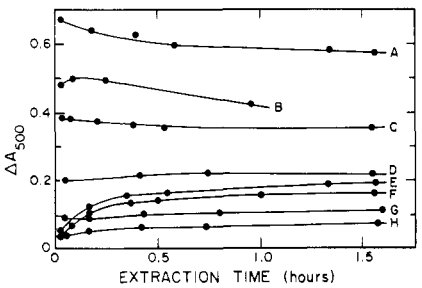

Figure 2. ΔA_{500} for the modified (2%) vanillin assay at room temperature vs. time of extraction of the grain (in 1% concentrated HCl in methanol): (A) BR-54, (B) Savannah, (C) NK-300, (D) 1S-15526, (E) IS-2319, (F) 601530, (G) 601542, (H) 601502.

three hours were required for maximum extraction of tannin with these varieties. Extractions carried out for 24 h gave 40–70% lower ΔA_{500} values for all varieties studied compared to 1-h extracts. Clearly tannin is not stable for long periods in acidic methanol even at room temperature.

An implicit assumption with the modified vanillin assay is that the higher values indicate that a greater amount of tannin is extracted in acidic methanol than in methanol itself. When a correction is made for the sometimes large amount of background color already present in the extract before the vanillin reagent was added, as described by Price and Butler (1977), the two assays gave comparable values for most grains (Figure 3). The same grains which were anomalous with regard to extraction rate were an exception here also, with tannin being detected only by the modified assay.

Criteria for Identifying Group I, II, and III Sorghum. Sorghum was first classified into three groups on the basis of differences between the results of the vanillin and modified vanillin assays (Cummings and Axtell, 1973). Grains which had catechin equivalent values (CE) less than 1 by both methods were classified as group I; those which had CE < 1 by the vanillin and > 2 by the modified vanillin assay were classified as group II; all others were group III.

A much clearer picture of the three groupings emerges when the two vanillin assays are compared after correcting for background color. Most of the grains that we have examined which have a colored testa show almost equal amounts of tannin using either assay. Values for these, the group III sorghums, fall on or near a line with a slope of 1 when values by the two assays are plotted against each other as in Figure 3. A few varieties of grain with a colored testa contain tannin that is measurable by the modified but not by the standard vanillin assay. These, the group II sorghums, fall along the x axis. Many other grains contain essentially no tannin that is detectable by either assay. These, the group I sorghums, are not plotted but would fall at or near the origin. There appears to be a clear-cut division for most varieties between groups II and

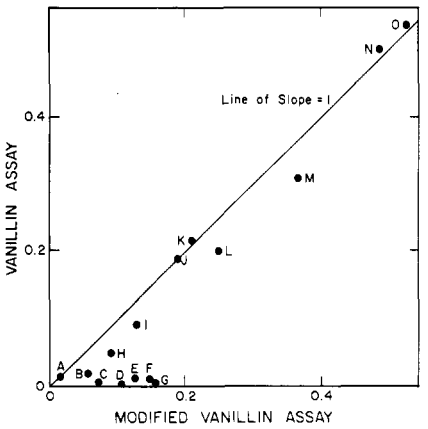

Figure 3. ΔA_{500} for the vanillin assay vs. ΔA_{500} for the modified vanillin assay at room temperature using 2% vanillin: (A) IS-1304, (B) 601502, (C) IS-9747, (D) IS-2740, (E) IS-8563, (F) 601530, (G) IS-2319, (H) 601562, (I) IS-15991, (J) IS-15612, (K) IS-15526, (L) IS-8164, (M) NK-300, (N) Savannah, (O) BR-54.

Table I. Effect of HCl Concentration on Amount of Tannin Extracted in 20 Min from a Group II Sorghum (IS-8563)

% concentrated HCl in methanol	ΔA_{500} per 200 mg of grain (corrected for blank)
0.01	0.010
0.1	0.084
1.0	0.275
4.0	0.349
8.0	0.358

III, with little or no mixing of both types of tannin in one grain. Classification of some low tannin varieties (B, H, and I) is unclear.

The group II sorghum varieties are of special interest because, in spite of the presence of a colored testa and of tannin, they are nutritionally equivalent to low tannin varieties (Cummings and Axtell, 1973). The group II tannins may be nutritionally inert because they are covalently bound to some grain component and unless released do not dissolve and therefore do not precipitate protein. The shape of the curves for extraction of group II tannin with time (Figure 2) is suggestive of the rate curve for a first-order chemical reaction. The dependence of the amount extracted in 20 min on the concentration of HCl as shown in Table I is also consistent with the extraction being dependent on an acid-catalyzed reaction to release the tannin.

To the uncertainty concerning the explanation for the unique solubility properties of group II tannin must be added the question of whether they are tannins at all. It is possible that group II "tannin" is actually monomeric flavanoids which are attached to some grain component and which give a vanillin-positive test when extracted in methanolic HCl; such monomers would have no protein precipitating power.

Adequacy of Catechin Standard. Figure 4 shows A_{500} as a function of time for the vanillin reaction with 1 mL of either 1 mg/mL of catechin or 1 mg/mL of partially purified sorghum tannin. In both cases, there is a rapid reaction in which the absorbance increases from zero to some value which could not be measured with these techniques. At the earliest times that measurements were possible, the absorbance was already diminishing. Identical kinetic behavior was observed with crude and purified tannin extracts. The catechin and the tannin exhibit markedly different reaction kinetics after the initial rapid reaction. Clearly the reactions occurring in the

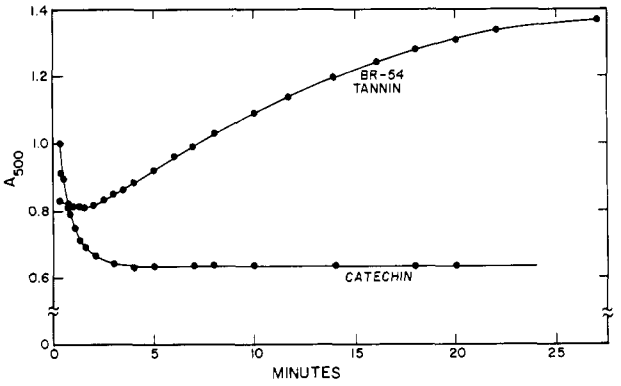

Figure 4. A_{500} for the (2%) vanillin assay of 1 mg/mL of methanolic solutions of partially purified BR-54 tannin and of catechin vs. time of reaction at room temperature.

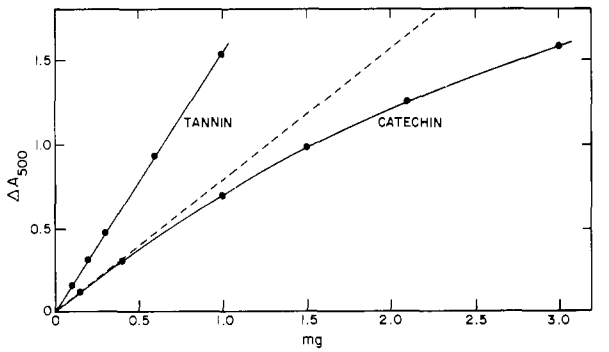

Figure 5. Standard curves for the (2%) vanillin assay: ΔA_{500} after a 20-min incubation at 30 °C vs. milligram of sample using partially purified BR-54 tannin and catechin in methanol.

vanillin assay are more complex than previously believed (Swain and Goldstein, 1963).

The kinetic data indicate that use of catechin as a standard leads to an overestimation of tannin content. This can be seen in Figure 5, which shows standard curves obtained with the vanillin assay for catechin and for the partially purified BR-54 tannin. The standard curve for catechin deviates considerably from linearity; overestimations vary from approximately twofold when ΔA_{500} is less than 0.2 to threefold at ΔA_{500} 1.4. Should an impurity be shown to be present in the tannin preparation, the overestimations would be greater.

Effect of Acid Concentration. The values of ΔA_{500} for the vanillin reaction with catechin, at times ranging from 5 to 180 min, are given in Figure 6 for HCl concentrations between 0.5 and 8%. Comparable data using a BR-54 extract are shown in Figure 7. The absorbance increased with increasing HCl concentration for both tannin and catechin. This cannot be due to a faster reaction rate with greater HCl concentration, though such a rate increase does occur, because the reactions at each concentration have reached their maximum value during the time span studied (see Figures 6 and 7). This leaves an effect on equilibrium, extinction coefficient or possibly an alteration in the sequence of reactions, as the likely explanation for the pH dependence. The reaction with tannin is not complete in 20 min at lower HCl concentrations, but is complete in 5 min with catechin. This is not surprising in view of the reaction kinetics presented in Figure 4.

Effect of Temperature. An increase in temperature would be expected to increase the reaction rate of both catechin and tannin with vanillin. This would have a minimal effect on the 20-min absorbance for catechin, since the reaction is complete even at the lower temperature (see

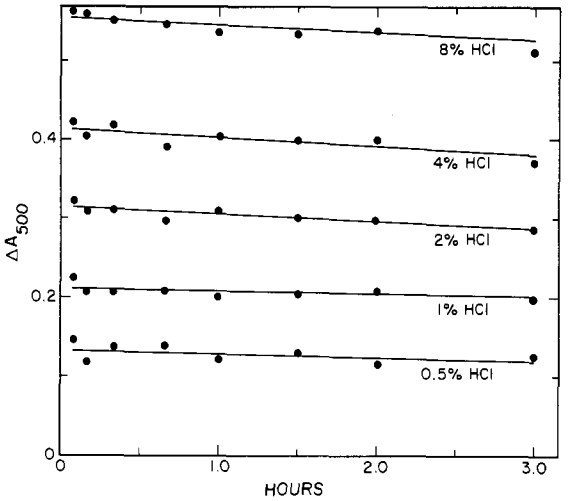

Figure 6. ΔA_{500} for the (2%) vanillin assay of catechin at room temperature vs. time for different concentrations of HCl in the vanillin reagent.

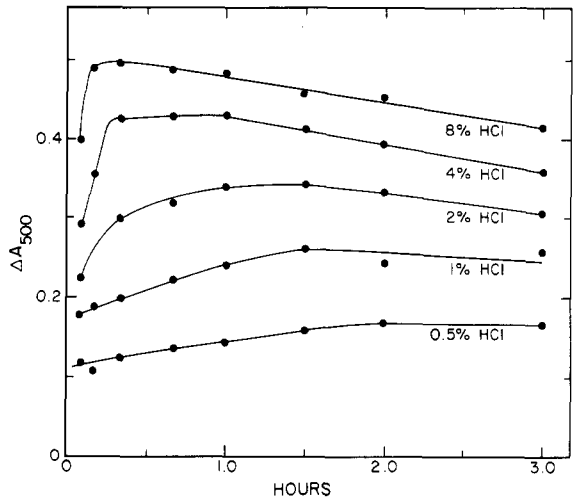

Figure 7. ΔA_{500} for the (2%) vanillin assay of a methanolic BR-54 extract at room temperature vs. time for different concentrations of HCl in the vanillin reagent.

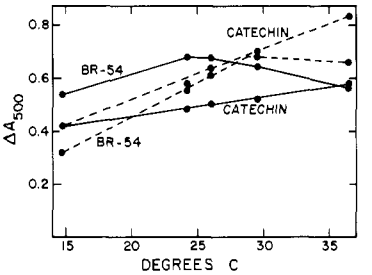

Figure 8. ΔA_{500} for the vanillin assay vs. temperature: (---) 2% vanillin, (—) 0.5% vanillin. The BR-54 data for the two vanillin concentrations were obtained on the same extract.

Figure 4), but it would raise the absorbance of the tannin sample. Figure 8 shows the effect of temperature on the vanillin assay of catechin and of a Br-54 extract with concentrations of vanillin in the reagent of 2% (broken lines) and 0.5% (solid lines). A change in room temperature from 24.2 to 26 °C, for example, caused an 11% increase in absorbance of the Br-54 extract when the usual 2% vanillin reagent was used. In a non-airconditioned laboratory in a warm climate, readings could vary over 30% between morning and afternoon assays.

Effect of Vanillin Concentration. The temperature dependence of ΔA_{500} varies with the vanillin concentration.

Table II. Reproducibility of Vanillin Assay[a] and Effect of Time Elapsed between Grinding and Analysis of BR-54

	ΔA_{500} at various times after grinding			
	day 1	day 4	day 8	day 18
sample I[b]	0.676	0.643	0.642	0.574
	0.676	0.646	0.636	0.574
		0.640	0.639	0.567
mean ± av dev.	0.676 ± 0.000	0.643 ± 0.002	0.639 ± 0.002	0.572 ± 0.003
sample II[b]	0.730	0.692	0.687	0.623
	0.710	0.694	0.684	0.625
		0.697	0.679	0.614
mean ± av dev.	0.720 ± 0.010	0.694 ± 0.002	0.683 ± 0.003	0.621 ± 0.004

[a] Results reported as ΔA_{500} per 200 mg of grain, after subtracting the blank. Each result is for a separate extraction, with 1 mL of methanol extract and 5 mL of 2% vanillin in 4% concentrated HCl in methanol incubated in a 30 °C water bath 20 min before reading. [b] Twelve grams of cleaned BR-54 was divided into a 4-g (I) and an 8-g (II) sample and ground separately.

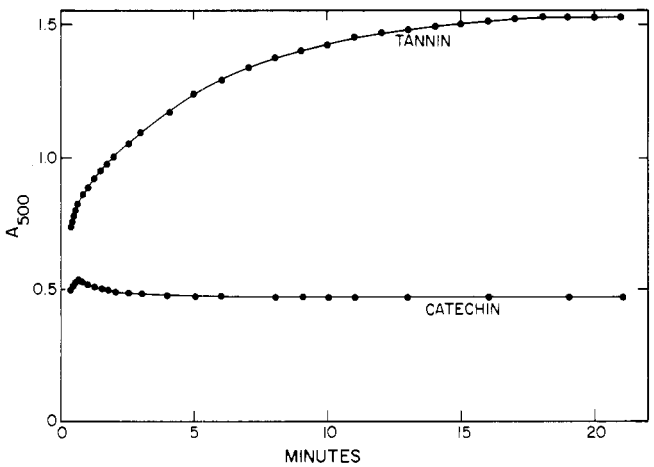

Figure 9. A_{500} for the (0.5%) vanillin assay of 1 mg/mL of methanolic solutions of partially purified BR-54 tannin and of catechin vs. time of reaction at room temperature.

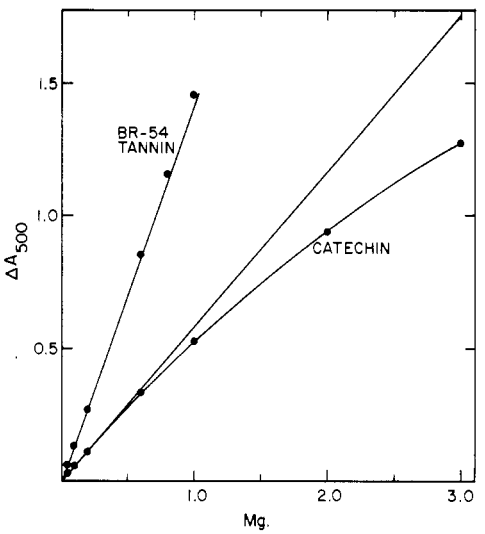

Figure 10. Standard curves for the (0.5%) vanillin assay: ΔA_{500} after a 20-min incubation at 30 °C vs. milligram of sample using partially purified BR-54 tannin and catechin in methanol.

As can be seen in Figure 8, a change in vanillin concentration may result in either an increase or a decrease in ΔA_{500} depending upon the temperature. The data suggest that less variation would be caused by temperature fluctuations if a 0.5% vanillin reagent were used.

Figure 9 shows that the kinetics of the reactions of BR-54 tannin and of catechin with 0.5% vanillin are quite different from that found with 2% vanillin. Standard curves prepared with 0.5% vanillin (Figure 10) show the expected changes in the slopes (compare with Figure 5).

Reproducibility. Use of the Udy cyclone mill and fine particle size enabled excellent reproducibility to be obtained when 200-mg samples were taken from one batch of ground grain (Table II), providing that assays were done in a 30 °C water bath.

A slow but steady decrease in results of the vanillin assay occurred with time after the grain was ground. Part of a sample of BR-54 which had been ground and mixed was placed in a tightly sealed plastic bag and the remainder stored in a paper envelope. Both were left at room temperature for 27 days, then assayed using the vanillin reagent. The ΔA_{500} value of the grain stored in the plastic bag was 0.590 ± 0.007; that for the portion stored in the paper envelope was 0.547 ± 0.006. This suggests that oxidation may be the cause of the decreases with time observed in Table II.

Values between batches of grain ground independently showed considerably more variation than did replicate assays within one batch of ground grain. The difference between samples I and II in Table II is typical of this observation. Twelve grams of cleaned BR-54 grain was divided into a 4-g (I) and an 8-g (II) portion, and these were then ground separately. This variation could be caused by irregular loss of some grain component rich in tannin during grinding or by a large variation in tannin content of the individual grains.

Stratification of white and colored material could be seen in the collection bottle, the former having two times the tannin content of the latter. This is presumably caused by the testa breaking up with the lighter endosperm rather than with the darker pericarp. So local enrichment of tannin content is possible during grinding as well as a theoretically possible selective removal of lighter tannin-rich particles by the vacuum. Contents were mixed thoroughly before emptying the bottle into an envelope for storage, and the envelope was rotated to mix contents before sampling.

Selective removal of a high tannin fraction was ruled out by assaying five independently ground 5-g samples of BR-54, ground on a Laboratory Construction Co. grinder, five 2-g samples ground for 1 min in a hand-held Krups 75 coffee grinder (grain could be quantitatively recovered from both grinders), and five 5-g samples on the Udy mill. The means and standard deviations were 0.630 ± 0.035, 0.616 ± 0.042, and 0.615 ± 0.036, respectively. Replicate 200-mg samples within a batch of ground grain were comparable in variability to values seen in Table II.

It was desired to determine whether there could be sufficient variability between grains to cause the observed variation between independently ground samples. Fifteen individual grains from a 5-g sample were selected with weights ranging from 6.8 to 37.8 mg and averaging 26.5 mg. Representative colors and shapes were included. Tannin

content varied widely; based on the standard curve in Figure 10, tannin content ranged from 0.0021 to 0.0345 mg/mg of grain, with a mean of 0.0135 ± 0.009.

DISCUSSION

Our results suggest several changes in the conditions employed for the vanillin assay which will improve its sensitivity, reproducibility, accuracy, and convenience. The extraction time in the vanillin assay can be greatly reduced, thus allowing several analytical runs to be completed in 1 day. This is consistent with results obtained with the Prussian blue assay using water extraction reported earlier (Price and Butler, 1977). It is essential, however, that each laboratory determine the appropriate extraction time for the grinding and extraction technique employed there. We suggest that a 20-min extraction with rotation will be adequate in most cases.

The use of blanks considerably sharpens the definition of and distinction between groups I, II, and III sorghum and shows that there is no appreciable difference in results when the vanillin assay is compared to the modified vanillin assay for most sorghum varieties. The "tannin" in group II sorghums, however, is measurable only by the modified vanillin assay.

The main sources of variability within and between laboratories are failure to use blanks, temperature differences, nonlinearity of the standard curve, and inherent variability in tannin content between samples. The latter appears to be considerably greater than variability of replicate assays when the procedure recommended here is followed.

The slit width selected and instrument characteristics determine the width of wavelengths passing through the cuvette and hence the amount of absorbance due to background color. (We have found that the wavelength of maximum background absorbance varies from 465 to 540 nm depending upon the variety.) This results in variation between laboratories unless blanks are subtracted.

We obtained excellent reproducibility using 2% vanillin in the vanillin reagent as long as a 30 °C water bath was used. However, because of the lessened temperature dependence, the improved linearity of the standard curve and the less complex reaction kinetics that result, we recommend that future assays be done with the more dilute reagent (0.5%). A 30 °C water bath should still be used, though if that is impossible, the error due to temperature fluctuations will not be as great as when 2% vanillin is used.

Use of the catechin standard curve, which deviates considerably from linearity, to measure a substance which shows good linearity can itself be a cause of variability. For example, a tannin extract twice as concentrated as another would have twice the ΔA_{500} but would appear to have more than twice as much tannin based on the catechin standard curve. It would be more satisfactory, if pure tannin is not available, to use an extrapolation of the catechin curve based on the initial points which at least approximate linearity.

In spite of the similarity between the calculations of catechin equivalents and percent tannin, it is essential that they not be considered in any way equivalent. The true percent by weight of (extractable) tannin is 42% of the catechin equivalent values or milligrams of catechin based on the standard curve in Figure 10. Once tannin has been purified from a sufficient variety of sorghums, it may be possible to multiply catechin equivalents by a constant to obtain percent tannin. Based only on tannin purified from BR-54 this factor is, as already mentioned, 0.42 when 0.5% vanillin is used at 30 °C. Using this figure, our BR-54 grain contains approximately 2% tannin by weight. Should future research show that there is still a contaminant in the tannin purified by present techniques, this would accentuate the overestimation by the catechin standard.

We suggest the following procedure for vanillin assays. Grain ground to pass a 0.4-mm seive (approximately 200 mg) is extracted within 1 day after grinding with 10 mL of methanol in capped, rotating test tubes for 20 min, and centrifuged in a desk top centrifuge. Assays are performed on the supernatant at 30 °C with reagents previously warmed to this temperature. Vanillin reagent prepared fresh daily by mixing equal volumes of 1% vanillin in methanol and 8% concentrated HCl in methanol is added (5 mL) at 1-min intervals to 1-mL aliquots of the samples. Five milliliters of 4% concentrated HCl in methanol is added to a second 1-mL aliquot (the blank) also at 1-min intervals. A_{500} is read after 20 min (the tubes remaining in the water bath until sample must be removed for reading), and the absorbance of the blank is subtracted from the absorbance with vanillin. Small differences in original weight are corrected by multiplying ΔA_{500} by 200/(sample weight in milligram). Samples will give comparable results when analyzed any time within a day and somewhat lower values if analyzed later. The modified method is similar, except that the extraction is done in 1% concentrated HCl in methanol and the extracts must be analyzed promptly.

A standard curve should be constructed using catechin concentrations up to 0.3 mg/mL and extrapolating to higher concentrations.

If temperature cannot be controlled and fluctuations are limited to between 22 and 30 °C, no more than a 10% variation should be introduced. The standard curve should always be run at the same temperature, and temperatures should be reported for all assays.

NOTE ADDED IN PROOF

A recent article has reported a reversible temperature dependence of the A_{500} for the vanillin reaction with catechin after initial incubation at 22 °C and recommends that the assay be run at 30 °C [Dalby, A., Shuman, A. C., *Anal. Biochem.* **85**, 325–327 (1978)].

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