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False Positive Results in the Vanillin-HCl Assay of Tannins in Sorghum Forage[1]

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ABSTRACT

Vanillin-HCl procedures are widely used for the assay of tannins in plants. In attempts to adapt such procedures for use with sorghum [*Sorghum bicolor* (L.) Moench.] forage it was found that false positive reactions resulted, that is, red color developed in the presence of HCl with or without vanillin. Leucoanthocyanidins (monomeric proanthocyanidins) may be the constituents responsible for this red color. A "chloroform-HCl" procedure was developed for measuring leucoanthocyanidins in sorghum forage. The procedure avoids interference by chlorophyll or other chloroform-soluble constituents. With vanillin added to the solution, this procedure should also be useful for the assay of condensed proanthocyanidins (tannins).

Additional index words: Sorghum bicolor L. Moench., Leucoanthocyanidin, Proanthocyanidin, Forage quality.

As indicated by Sarkar et al. (14), tannins may be regarded as desirable (for their possible protection against bloat in grazing animals) or undesirable (for their adverse effect on digestibility) constituents of forages. Therefore, either increased or decreased tannin content might be a reasonable breeding objective, depending upon the nature and proposed use of the crop in question. Whether working toward increased or reduced levels of tannins, the breeder needs a simple, rapid, and reliable procedure for tannin assay. Vanillin-HCl procedures such as that described by Burns (2) have been developed for this purpose. In this procedure, dried and ground plant samples are extracted with methanol, and portions of the resulting extract are reacted with a solution containing vanillin and HCl in methanol. The development of a red color indicates the presence of tannins.

Vanillin-HCl and vanillin-H_2SO_4 reagents have been used extensively for the detection and assay of tannins and related substances in leguminous forage crops (2, 8, 13, 14) and in sorghum [*Sorghum bicolor* (L.) Moench] grain (1, 3, 5, 9, 11, 12). Reports of the use of vanillin-HCl procedures for the assay of tannins in sorghum forage are much less numerous. However, Cummins (4) and Paroda et al. (10) used the vanillin-HCl procedure of Burns (2), and Gourley and Lusk (6) used that of Maxson and Rooney (9) for estimates of forage sorghum tannins.

In some of the cited studies precautions were taken to avoid false positive tests for tannins. For example, Price and Butler (11) and Sarkar and Howarth (13) used controls in which vanillin was omitted from the vanillin-HCl reagent, and El Tuhami et al. (5) used extracts without either vanillin or HCl as controls. In the cited reports of tannins in sorghum forage, however, it appears that such controls were not used.

In preliminary attempts to use a vanillin-HCl procedure to detect tannins in sorghum forage, we observed a false positive reaction for some cultivars, that is, leaf extracts from these cultivars produced a red color in the presence of HCl with or without vanillin. This paper presents the results of a series of experiments in which these false positive tests were investigated.

MATERIALS AND METHODS

Blades of upper leaves of field-grown forage sorghum plants were used in these experiments. Most of the plants were at or just beyond the stage of panicle emergence when sampled. Leaves were dried at about 75 C and were then ground in a Wiley[3] mill to pass a 1-mm screen. The ground tissue was stored in tightly covered jars in the dark at room temperature until used.

The vanillin-HCl assays described by Burns (2) and Maxson and Rooney (9) served as the basis for the procedures used in this study. The Maxson and Rooney procedure differs from that of Burns in that 1% concentrated HCl in methanol rather than pure methanol is used as the extractant. Unless otherwise indicated, leaf extracts were prepared by placing ground leaf tissue in test tubes or flasks, adding methanol, capping the vessels, and shaking the mixtures occasionally over a period of 16 to 24 hours at room temperature. Filtrates or supernatant solutions from these extractions were used in the assays. Further procedural details are given in connection with specific experiments.

Reagent grade HCl and organic solvents were used in all experiments. Vanillin and D-catechin were obtained from the Sigma Chemical Co.

RESULTS AND DISCUSSION

Spectra of Methanol and Methanol-8% HCl Extracts

Samples of 'Brawley' forage sorghum leaves, which gave a positive reaction (red color) with vanillin-HCl, were ex-

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tracted with methanol or methanol-8% HCl (methanol:12 *N* HCl, 92:8, v:v) at the rate of 1 ml per 5 mg of tissue. Aliquots (1 ml) of the extracts were diluted with 5 ml of methanol, methanol-8% HCl, or vanillin-HCl [equal volumes of methanol-8% HCl and 1% (w/v) vanillin in methanol], and absorbance spectra (400 to 560 nm) were determined at 3 min and at 2.5, 7, and 24 hours after dilution. A Beckman Model DB-G spectrophotometer was used for these spectral readings; the respective diluents were used as spectrophotometer blanks.

The methanol extract was green in color and the methanol-HCl extract was red. Within 2.5 hours after dilution, all solutions had become red except the methanol extract diluted in methanol, which remained green. Except for the vanillin-containing solutions at 24 hours, each of the red solutions had an absorbance maximum near 540 nm. In the vanillin-containing solutions, the 24-hour spectral scans revealed a maximum near 500 nm and none near 540 nm.

As shown by the A_{540} values in Table 1, samples of the methanol extract diluted in methanol-HCl developed at least as much color as those diluted in vanillin-HCl. When values for the methanol-HCl-diluted samples were subtracted from values for the vanillin-HCl-diluted samples, as done by Bullard et al. (1) and Price and Butler (11) with extracts of sorghum grain, results indicated the presence of little if any tannin in the leaf extract.

Samples of the already red methanol-HCl extract, when diluted in methanol, methanol-HCl, or vanillin-HCl did not change greatly in A_{540} with time (Table 1). Use of either the methanol- or methanol-HCl-diluted sample as a control for the vanillin-HCl-diluted sample again suggested that little if any tannin was present in the Brawley leaves.

A solution of D-catechin in methanol, unlike the leaf extracts, failed to develop color in the presence of HCl only but rapidly became red in the presence of the vanillin-HCl color reagent.

Table 1. Absorbance at 540 nm at various times after addition of methanol, methanol-HCl, or vanillin-HCl to methanol and methanol-HCl extracts of leaf tissue from the sorghum cv. Brawley.

Extraction solvent	Diluent	Time after dilution			
		3 min	2.5 hours	7 hours	24 hours
		A_{540}			
Methanol	Methanol	0.008	0.008	0.008	0.036
	Methanol-HCl	0.030	0.173	0.189	0.208
	Vanillin-HCl‡	0.033	0.128	0.154	0.179
Methanol-HCl†	Methanol	0.175	0.166	0.170	0.200
	Methanol-HCl	0.194	0.213	0.223	0.258
	Vanillin-HCl	0.187	0.184	0.189	0.212

† Methanol-12 *N* HCl, 92:8, v:v.
‡ Equal volumes of methanol-HCl and 1% (w/v) vanillin in methanol.

Table 2. Absorbance at 540 nm of methanol extracts of the leaves of Brawley at three temperatures and at various times after dilution in methanol-HCl.†

Temperature (C)	Time after dilution	A_{540}
0	0	0.061
	3 hr	0.071
	20 hr	0.125
22	0	0.061
	30 min	0.124
	3 hr	0.212
50	0	0.061
	5 min	0.257
	10 min	0.312
	15 min	0.334
	20 min	0.340

† Methanol-12 *N* HCl, 92:8, v:v.

Effect of Temperature on Rate of Color Development

Aliquots (0.5 ml) of a Brawley leaf extract (1 ml methanol per 10 mg leaf tissue) were mixed with 4.5 ml of methanol-8% HCl, and the tubes were capped and held at 0 C (ice bath), 22 C (room temperature), or 50 C (water bath) for various times, after which spectra were scanned against a methanol blank. Each of the 0 and 50 C samples was quickly brought to near room temperature before its spectrum was scanned.

As shown by the rate of change in A_{540} readings (Table 2), color development was highly dependent on temperature. The A_{540} reading of the solution held at 0 C for 20 hours was similar to that of the solution held at 22 C for 30 min, and the reading of the solution kept at 22 C for 3 hours was less than that of the solution subjected to 50 C for 5 min. A period of 15 to 20 min at 50 C appeared to be sufficient for maximum color development.

Comparison of Brawley and White Collier

Dried, ground leaves (1 g portions) of Brawley and 'White Collier' were extracted with 50 ml methanol (2) or methanol-12 *N* HCl (99:1, v:v) (9) for 16 hours on a laboratory shaker, and extracts were filtered to remove tissue residue. Duplicate aliquots (0.25 ml) of the leaf extracts were then mixed with 0.25 ml of methanol and 2.5 ml of methanol-12 *N* HCl (96:4, v:v) with and without 2% (w/v) vanillin. Tubes were allowed to stand at room temperature or in a 50 C water bath for 20 min before A_{540} readings were made with a Bausch and Lomb Spectronic 20 colorimeter using methanol as the blank. For the Brawley extract, results of this experiment (Table 3) confirmed the enhancing effect of heating on color development and the nondependence of color formation upon the presence of vanillin. Readings for the White Collier extract were consistently lower than those for Brawley. The best discrimination between the two cultivars was achieved with methanol as the extractant and with color development at 50 C without vanillin (Table 3).

The Chloroform-HCl Procedure

In the foregoing comparison of methanol extracts, the solutions from White Collier were not visibly red. It seemed possible that the low but appreciable A_{540} readings obtained for White Collier might be due to chlorophyll or other constituents, elimination of which might permit improved discrimination between cultivars. Attempts were therefore made to separate chlorophyll from the constituents responsible for the development of red color in extracts of Brawley leaves. It was found that when suitable volumes of extract, HCl, and chloroform were mixed and warmed at 50 C for 20 min, red color developed in the upper (methanol-HCl) phase, and the lower (chloroform) phase remained green. Spectral scans indicated that the absorbance maximum of the red solution was near 540 nm, as was true for Brawley solutions not extracted with chloroform.

These and other observations led to the development of the following "chloroform-HCl" procedure. To 0.25 ml of methanol extract of sorghum leaves (1 ml methanol per 20 mg dry, ground leaves), 1.0 ml chloroform was added, followed by the addition of 1.75 ml of a mixture of methanol, 6 *N* HCl, and chloroform (19:12:4, v:v:v). When desired,

Table 3. Influence of incubation temperature on A_{540} readings. Methanol and methanol-1% HCl extracts of White Collier and Brawley leaves were used. Extracts were diluted in methanol-4% HCl with and without added vanillin (see text for details).

Extraction solvent	Incubation temp.	Vanillin present?	A_{540}†		
			W. Collier	Brawley	Br./W.C.
Methanol	RT‡	yes	0.099	0.161	1.6
		no	0.083	0.187	2.3
	50 C	yes	0.120	0.446	3.7
		no	0.092	0.478	5.2
Methanol-1% HCl	RT	yes	0.160	0.335	2.1
		no	0.158	0.363	2.3
	50 C	yes	0.180	0.560	3.1
		no	0.163	0.600	3.7

† A_{540} values are means of duplicates which, in all cases, differed by 5% or less.
‡ RT (room temperature) was approximately 25 C.

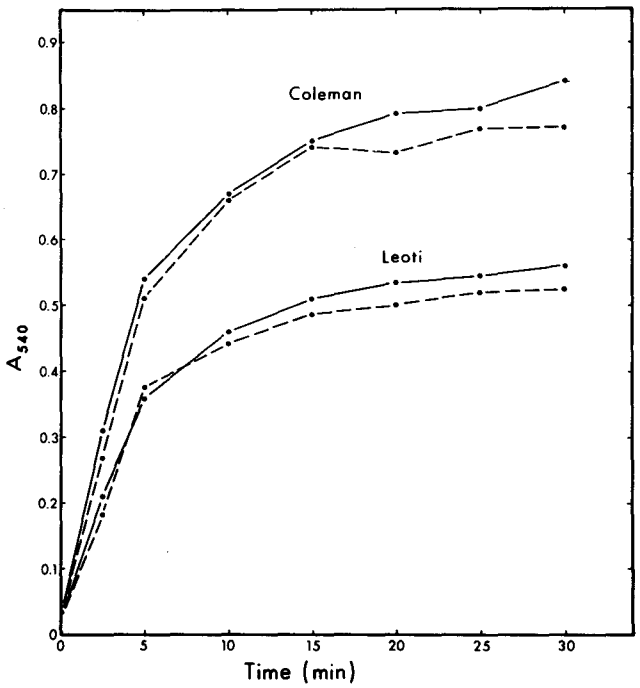

Fig. 1. Effect of duration of heating at 50 C on absorbance at 540 nm of Coleman and Leoti leaf extracts in the chloroform-HCl assay with (———) and without (------) vanillin.

vanillin (1%, w/v) was added to the methanol-HCl-chloroform solution. Tube contents were mixed on a vortex mixer, and tubes were then placed in a 50 C water bath for 20 min to permit color development and phase separation. Immediately after tubes were removed from the 50 C bath, absorbance at 540 nm was read with a Bausch and Lomb Spectronic 20 colorimeter, using methanol as the blank. Matched Bausch and Lomb colorimeter tubes were used for this procedure. With these tubes and the volumes listed, the chlorophyll-containing chloroform phase was confined to the part of the tube that was below the light path of the Spectronic 20 instrument. Thus, interference by chlorophyll was eliminated, and the A_{540} of the methanol-HCl phase was read without transferring the solution to another tube. During heating at 50 C, refluxing occasionally resulted in a non-homogeneous upper phase. This problem was readily solved by swirling the tube gently as it was removed from the 50 C bath for reading.

Rate of Color Development. Methanol extracts of the leaves of 'Coleman' and 'Leoti' forage sorghum, two cultivars that gave positive results in qualitative vanillin-HCl tests, were subjected to the chloroform-HCl procedure, with and without vanillin, with durations of heating at 50 C ranging from 0 to 30 min. During the first 15 min, A_{540} values increased rapidly (Fig. 1). Small increases in reading occurred as heating time was increased from 15 to 30 min. Twenty minutes appeared to be a suitable heating time for routine use; in subsequent experiments the duration of heating was carefully controlled at 20 min. For extracts of both Coleman and Leoti leaves, the rate of color development with vanillin was slightly greater than that without vanillin (Fig. 1).

Table 4. Absorbance at 540 nm of methanol extracts from leaves of two sorghum cultivars, and absorbance at 500 nm of D-catechin, at five concentrations of vanillin in the chloroform-HCl procedure.

Extract or solution	Incubation temp. (C)	Wavelength (nm)	Vanillin concentration (%, w/v)				
			0	0.5	1.0	1.5	2.0
			Absorbance				
Leoti	50	540	0.51	0.52	0.53	0.53	0.54
Coleman	50	540	0.75	0.80	0.80	0.79	0.85
Catechin†	50	500	0.01	0.56	0.94	1.20	1.40
Catechin	RT‡	500	0.01	0.55	0.88	1.10	1.28

† 0.25 ml of solution containing 4 mg D-catechin/ml.
‡ RT (room temperature) was approximately 25 C.

Concentration of Leaf Extract. Dilutions of the Coleman and Leoti leaf extracts were made with methanol such that 0, 0.05, 0.10, 0.15, 0.20, or 0.25 ml of original extract was present in a volume of 0.25 ml. The chloroform-HCl procedure, with and without vanillin, was used on these solutions. For the extracts of both Coleman and Leoti leaves, the response of A_{540} reading to quantity of extract was nearly linear over the range tested, and, as in previous experiments, vanillin had very little effect on the spectral readings. Regression coefficients for the relationship of A_{540} to volume of extract were as follows: Coleman—3.2 with, 3.1 without vanillin; Leoti—2.1 with, 2.0 without vanillin.

Concentration of Vanillin. The Coleman and Leoti leaf extracts, and a standard catechin solution, were used in an experiment in which the vanillin concentration in the methanol-6 *N* HCl-chloroform solution was varied from 0 to 2.0% (w/v). For both leaf extracts, varying the vanillin concentration over this range had little effect on A_{540} readings (Table 4). For catechin, absorbance values at 500 nm were read as specified in the Burns vanillin-HCl procedure (2). Readings for the catechin solutions were greatly affected by vanillin concentration, varying from near 0 at 0% vanillin to 1.40 at 2% vanillin. One set of catechin tubes was held at room temperature for 20 min rather than being heated at 50 C, and it was found that for catechin, the heat treatment had relatively little effect on A_{500} readings (Table 4). The great differences between catechin solutions and leaf extracts with respect to response to heating and vanillin concentration indicated that appreciable amounts of catechin-like substances were not present in the sorghum leaves.

Eight-cultivar Comparison. Duplicate 200-mg samples of dried, ground leaves of White Collier, 'Norkan', 'Rex', 'Rox', 'Early Sumac', Brawley, Leoti, and Coleman forage sorghum were extracted with methanol. Duplicate 0.25-ml aliquots of the resulting 16 extracts were assayed by the chloroform-HCl procedure with and without vanillin. As shown in Table 5, the chloroform-HCl procedure, with or without vanillin, separated the eight cultivars clearly into

Table 5. Mean A_{540} readings resulting from application of the chloroform-HCl procedure, with and without vanillin, to methanol extracts of the leaves of eight forage sorghum cultivars.

Cultivar	A_{540}†	
	Vanillin present $\bar{x} \pm s_{\bar{x}}$	Vanillin absent $\bar{x} \pm s_{\bar{x}}$
White Collier	0.019 ± 0.001	0.002 ± 0.001
Norkan	0.015 ± 0.002	0.005 ± 0.001
Rex	0.021 ± 0.001	0.008 ± 0.001
Rox	0.024 ± 0.002	0.010 ± 0.001
Early Sumac	0.266 ± 0.008	0.258 ± 0.004
Brawley	0.406 ± 0.007	0.386 ± 0.010
Leoti	0.568 ± 0.014	0.504 ± 0.015
Coleman	0.823 ± 0.014	0.758 ± 0.009

† Each value is the mean of four determinations (duplicate aliquots from duplicate extracts).

two groups, one with very low A_{540} readings and the other with relatively high readings. Magnitudes of means and standard errors for the low group suggested that vanillin caused a significant increase in A_{540} reading, but actual increases were small. The correlation coefficient for the relationship of the values with vanillin to those without exceeded 0.99. Vanillin obviously was not essential for color development in this experiment.

General Discussion. These experiments demonstrate that wide differences exist among forage sorghum cultivars with respect to content of leaf constituents that yield red color when heated in acid. As indicated in Haslam's excellent review on natural proanthocyanidins (7), this response to heating in acid is characteristic of leucoanthocyanidins. Haslam uses the term, leucoanthocyanidin, to refer to "monomeric proanthocyanidins such as the flavan-3,4-diols" and the term, condensed proanthocyanidin, to refer to dimers and higher oligomers of flavan-3-ols. Leucoanthocyanidins do not precipitate proteins; thus in the terminology of Haslam they are not regarded as tannins. Condensed proanthocyanidins do precipitate proteins, and they are regarded as the constituents responsible for the tannin reactions shown by many plant tissues (7). Condensed proanthocyanidins (tannins) of the type occuring in sorghum grain (1, 11, 12) give positive tests with vanillin-acid reagents after corrections are made for any color that might be developed due to the acid alone. Corrections of this type in the present work on sorghum leaves indicated that little if any condensed proanthocyanidin was present. Use of uncorrected values based on vanillin-HCl procedures would have led to the false conclusion that condensed proanthocyanidins (tannins) were abundant in leaves of some of the forage sorghum cultivars included in this study. We suggest that other reports of tannins in sorghum forage (4, 6, 10) may have been due to the presence of leucoanthocyanidins rather than condensed proanthocyanidins.

The chloroform-HCl method is proposed as an improved method for measuring leucoanthocyanidins in the forage of sorghum and possibly other plants. Also, with vanillin added to the methanol-HCl-chloroform solution, the procedure should be useful for eliminating chloroform-soluble interfering materials in measurements of condensed proanthocyanidins in various plants.

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