Evaluation of Methods for Tannin Analysis in Sorghum Grain[1]

E. D. MAXSON and L. W. ROONEY[2], Texas A&M University, College Station 77843

ABSTRACT

Several methods or variations of methods of tannin analysis for sorghum grain were evaluated. Of seven methods that appeared promising, only three were judged to have potential for use on sorghum grain. These are the Bate-Smith and Rasper, ferric ammonium sulfate, and modified vanillin-hydrochloric acid methods. The Bate-Smith and Rasper method is rapid and reproducible, but lacks a suitable standard. The modified vanillin-hydrochloric acid method is rapid but has day-to-day variation. Tannin content expressed as catechin equivalents ranged from 0.08 to 4.32 mg. per 100 mg. The method with the least variation was extraction with urea, followed by reaction with ferric ammonium sulfate to form a color complex. The tannin content of the grain analyzed ranged from 0.20 to 1.15 mg. per 100 mg. tannic acid equivalents when this method was used. None of these methods measure tannin content in clearly definable substances. Therefore, any potential method for tannin analysis in sorghum should be related to biologically significant differences in the quality of the sorghum grains.

Tannins are defined as high-molecular-weight polyphenolic compounds that have the ability to bind with protein and preserve animal hides. However, the term "tannin" is commonly used to refer to polyphenolic compounds. Tannins in this paper mean polyphenols.

Tannins in sorghum have been implicated as affecting the quality of grain for both animal and human consumption. Sorghum tannins cause a reduction in dry-matter and protein digestibility (1), and have been associated with growth retardation in chickens (2,3). However, Damron et al. (4) found no growth retardation in chicks when 50% of the corn in the diets was replaced with high-tannin bird-resistant varieties of sorghum. Tannic acid added to diets caused

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[2]Respectively, Technician, and Associate Professor. Cereal Quality Laboratory, Soil and Crop Sciences Department, Texas A&M University, College Station.

death of chicks (5) and possibly a fishy or bitter flavor in broilers (6). The factors responsible for decreased *in vitro* dry-matter digestibility of sorghum for beef cattle are located in the pericarp and testa of the kernel (1), where major portions of the tannins are located (7,8,9).

Morton (10) linked a high incidence of esophageal cancer in certain areas of the world with the use of high-tannin sorghums for human food. The hypothesis of Morton (10) was based on relatively little information; however, it illustrates the importance of tannins in sorghum.

Tannins are undesirable because they cause off-color in various products made from sorghum grain. Polyphenolic compounds migrate into the endosperm of the grain during steeping prior to wet-milling (7) and during wet weather prior to harvest, causing dark-colored starch. In addition, pigment precursors are acted upon by basic conditions and develop undesirable colors during production of tortilla products and some snacks.

Miller and Kneen (11) identified an amylase inhibitor in Leoti, Schrock, and Early Sumac sorghums. The inhibitor from Leoti was identified as a series of oligomeric condensed tannins of the leucocyanidin group (12) which precipitated gelatin.

Methods of tannin analysis are based upon precipitation of tannins, formation of colored products with tannins, oxidation of tannins, and UV spectroscopy (13). The Association of Official Agricultural Chemists (14) lists the Folin-Denis method for use on alcoholic beverages and the permanganate reducing method for tea, cloves, and allspice. The hide-powder method is used in the tanning industry and some work has been reported in which UV absorbance has been used to estimate the tannin content of wines (13), tea, and beer (15).

Several methods have been used to estimate tannins in sorghum grain, and values ranging from 0.0039 to 10.5% have been reported (Table I). These methods were not designed for use with sorghum and many of them apparently were not suited for use with sorghum grain. Statistical analyses of the data, experimental error, or reproducibility have not been included in most of the publications, which prevents any realistic comparison of values among laboratories.

An acceptable method of tannin analysis for sorghum should be relatively simple to perform, rapid, and give results that are reproducible from day to day and laboratory to laboratory. The study reported in this paper was designed to evaluate current methods of tannin analysis for sorghum grain.

MATERIALS AND METHODS

Samples and Experimental Design

Phase I. Four cultivars of *Sorghum bicolor* (L.) Moench were selected to represent a wide range in tannin content. Ga 615, a bird-resistant hybrid, had a brown testa and pericarp. The variety Martin had remnants of testa with reddish-brown pericarp. Kafir 60 had a white pericarp and no testa, while Tx 2536 had a thin, white pericarp and no testa. Tx 2536 had a yellow endosperm; all others had normal endosperm. Grain samples were produced under similar conditions at the Texas A&M University Research and Extension Center, Texas Agricultural Experiment Station, Lubbock, Tex., in 1968. All grain samples were cleaned and hand picked to remove all glumes. A representative sample of grain was ground through a 0.010-in. slotted screen with a laboratory hammer mill.

TABLE I. A SURVEY OF METHODS REPORTED FOR ANALYSIS OF TANNINS IN SORGHUM GRAIN

Method	Standard	Tannin Content, % Mean	Range	N[a]	Principle of Method	References
Vanillin-HCl	Catechin	6.6	4.6–10.5	64	Extract with methanol, react with acidified vanillin. Specific for catechins and leucoanthocyanins.	(16,17)
AOAC tannin in tea	oxalic acid	0.22	0.06–0.53	3	Reflux with water, $KMnO_4$ titration of total astringents and astringent non-tannins (tannin precipitated with gelatin).	(18,19)
AOAC Folin-Denis	tannic acid	0.52	0.14–1.65	29	Reflux with water, react with Folin-Denis reagent. Nonspecific analysis of phenolic compounds.	(20,3)
Snell Extraction plus Folin-Denis	tannic acid	0.0321	0.0039–0.1667	14	Water extraction, isolate as lead tannate, resolubilize with H_2SO_4, react with Folin-Denis reagent.	(21,22)
Snell	tannic acid	0.0404	0.0077–0.0912	5	Isolate as above and react with arsenotungstic acid.	(22)

[a]N = number of grain samples analyzed and used to determine mean and range of tannin content.

Representative samples of the ground grain were used for all analyses. On each of three different days, two replicate extractions of grain from each cultivar were made. Two subsamples from each extraction were analyzed by the method being evaluated. Standard curves were prepared for the analysis of each replicate extraction. Log (X +1) data transformation was used to minimize the tendency for the standard deviations to be proportional to the mean.

Phase II. The promising methods found in phase I were given a more complete evaluation under routine laboratory conditions.

Three sets of samples were used. Set I consisted of grain from Ga 615, a brown-colored, bird-resistant hybrid, which was produced at each of six locations for 3 years and was used to determine whether the methods could distinguish differences among high-tannin grains. Set II consisted of grain samples from cultivars which produced kernels with and without a pigmented (brown) testa and pericarp. The samples in set III were selected to provide grain with the greatest possible variation in color. This set of samples included white, lemon-yellow, red, brown, and dark-brown grain.

One extraction was made on each of three separate days. Subsamples of each extraction were analyzed. The experimental design was a randomized complete block with subsamples.

Methods of Tannin Analysis

Ferric ammonium sulfate (FAS) method. This method is based on work done by Mejbaum-Katzenellenbogen and Kudrewicz-Hubica (23). The procedure is described below. Place 2.0 g. ground grain, boiling beads, and 70 ml. distilled water into a 300-ml. T 24/40 flask. Swirl, and gelatinize the sample. Cool the sample; add 5.0 ml. Diazyme L-30 (amyloglucosidase, Marschall Division, Miles Laboratories, Inc.); and incubate at 52°C. for 45 min. After incubation, add 50.0 g. urea and antifoaming agent (2 drops Dow Corning antifoam H-10 emulsion or Dow Corning antifoam A). Reflux 24 hr., using large-bore air condensors with T 24/40 joints; cool; and make to 120 ml. Centrifuge 10 to 15 min. at 825 X g and filter through fluted filter paper. Place 2.0 ml. filtrate into each of 2 cuvettes. Add 5.0 ml. distilled water to one cuvette (blank) and 5.0 ml. ferric reagent (1 part 5% FAS + 10 parts 10% gum arabic + 89 parts 1.0M acetate buffer, pH 4.6) to the remaining cuvette. After 15 min., determine absorbance at 580 nm. for each sample and compare to tannic acid standards (0.02, 0.1, 0.2, 0.3, and 0.4 mg.) prepared daily.

This basic procedure was conducted using various extraction times and levels of urea.

Modified vanillin-hydrochloric acid (MV-HCl) method. The vanillin-hydrochloric acid procedure of Burns (24,25)) was modified in our laboratory. The samples were extracted with 1% HCl in methanol, rather than pure methanol. Sample size was modified to use 1.0 g. high-tannin grain and 2.0 g. low-tannin grain. The samples were shaken on a reciprocating shaker for 24 hr., rather than swirled occasionally.

Other methods were evaluated. Among these were the ferric ammonium citrate (24), AOAC tannin in tea (14), modified Snell (26), methanolic-HCl, Bate-Smith and Rasper (20), variations of gelatin precipitation, and the Folin-Denis method (24,27).

RESULTS AND DISCUSSION

Phase I

Data that could be analyzed statistically were obtained for seven methods or

TABLE II. MEAN SQUARE VALUES OBTAINED FOR EACH METHOD USING LOG (X + 1) TRANSFORMED DATA

Source of Variation	df	Method						
		FAS, 5 g. urea	FAS, 50 g. urea	Folin-Denis	Folin-Denis, 50 g. urea	Vanillin-HCl	Methanolic-HCl	Bate-Smith & Rasper
Total	47	...	...	...	...	...	...	...
Variety (V)	3	0.0057**	0.0771**	0.0218**	0.0288**	0.7289**	0.8626**	0.8176**
Day (D)	2	0.0001	0.0001	0.0011	0.0247*	0.0009	0.0764**	0.0067
D X V[a]	6	0.0003	0.0011	0.0006	0.0025	0.0005	0.0047**	0.0045
Replicate (R)/V/D[b]	12	0.0005**	0.0007**	0.0022**	0.0067**	0.0016**	0.0003	0.0016**
Subsample (S)/R/V/D[c]	24	0.00002	0.00003	0.0004	0.0015	0.0001	0.0002	0.0004

[a]Error term for day and variety.

[b]Error term for D X V.

[c]Error term for R/V/D.

TABLE III. TANNIN CONTENT (mg./100 mg.) AND 95% CONFIDENCE INTERVAL FOR FOUR VARIETIES OF SORGHUM GRAIN AS DETERMINED BY EACH OF SEVEN METHODS OF ANALYSIS, USING LOG (X+1) TRANSFORMED DATA (N=12)

Method of Analysis	Data Expressed as	Ga 615			B 398			B 3197			Tx 2536		
		$\bar{X}-(t_{1\,1}s_{\bar{X}})$	$\bar{X}$	$\bar{X}+(t_{1\,1}s_{\bar{X}})$	$\bar{X}-(t_{1\,1}s_{\bar{X}})$	$\bar{X}$	$\bar{X}+(t_{1\,1}s_{\bar{X}})$	$\bar{X}-(t_{1\,1}s_{\bar{X}})$	$\bar{X}$	$\bar{X}+(t_{1\,1}s_{\bar{X}})$	$\bar{X}-(t_{1\,1}s_{\bar{X}})$	$\bar{X}$	$\bar{X}+(t_{1\,1}s_{\bar{X}})$
Folin-Denis (50 g. urea)	tannic acid equivalents	7.83	8.64	9.52	7.21	8.02	8.91	5.92	6.56	7.25	6.12	6.91	7.79
Folin-Denis	tannic acid equivalents	1.43	1.61	1.81	0.48	0.53	0.59	0.34	0.37	0.40	0.32	0.36	0.40
Vanillin-HCl	catechin equivalents	2.99	3.10	3.22	0.52	0.55	0.59	0.27	0.29	0.31	0.19	0.21	0.24
Bate-Smith and Rasper	pigment-concentrate equivalents	18.73	19.78	20.90	12.62	13.15	13.74	5.12	5.56	6.03	4.63	5.03	5.46
Methanolic HCl	pigment-concentrate equivalents	6.74	7.87	9.15	2.23	2.54	2.89	1.50	1.66	1.84	1.05	1.20	1.36
FAS (5 g. urea)	tannic acid equivalents	0.12	0.16	0.19	0.04	0.07	0.09	0.03	0.04	0.05	0.03	0.04	0.05
FAS (50 g. urea)	tannic acid equivalents	0.71	0.76	0.81	0.18	0.22	0.25	0.20	0.23	0.27	0.17	0.20	0.24

variations of methods. Mean squares for each of the seven methods are reported in Table II. The mean tannin values and confidence intervals obtained by each of the seven methods are presented in Table III.

The methods that were not suitable for analysis of sorghum grain are summarized in Table IV and will not be discussed further.

MV-HCl method. This method is rapid and was reproducible when the four samples were analyzed in Phase I (Table II). Differences between varieties accounted for 99% of the variability in this method when variance-component analysis was used. However, when transformed ($\sqrt{X + \frac{1}{2}}$, and arc sine $\sqrt{X}$) and nontransformed data were used, standard deviations were proportional to the mean. This proportionality was reduced when log (X + 1) transformed data were analyzed.

Burns (25) recommended the method using pure methanol for extraction of sorghum grain. The values obtained in the present study, with the exception of Ga

TABLE IV. METHODS OF TANNIN ANALYSIS THAT WERE UNSUCCESSFUL FOR USE WITH SORGHUM GRAIN

Method	Summary of Procedure	Problems Encountered
Gelatin precipitation	Precipitate gelatin from solution with tannin and measure protein remaining in solution.	Several methods for determining protein indicated more protein remaining in solution than was present prior to precipitation with tannin.
Ferric ammonium citrate (24)	Reflux with water and react with ferric ammonium citrate.	Insensitive 30-g. samples required for extraction.
AOAC tannin in tea (14)	Boil with water, titrate with $KMnO_4$, and precipitate with gelatin.	Difficult to obtain extraction solution. Higher values obtained for astringent non-tannins than for total astringents.
Modified Snell (26)	Extract with ethanol, isolate with lead acetate, and react with arsenotungstic acid.	Time consuming. Unstable color complex. Large day-to-day variation (greatest range in values was obtained for the same sample).
Methanolic-HCl	Extract with methanol-HCl (5:1) for 24 hr. Determine absorbance at 465 nm.	Significant day-to-day variation and day X variety interaction (Table II). Inadequate standards. Differing absorption maxima for various varieties.
Folin-Denis (urea extraction)	Reflux 24 hr. with urea; react with Folin-Denis reagent and saturated Na_2CO_3 soln.	Significant day-to-day variation (Table II). Positive results for non-tannins: tyrosine, 88%; tryptophan, 34.8%; casein, 7.2%; glucose, 7.2%; starch, 6.0%.
Folin-Denis (27)	Reflux 5 hr. with water; react as above.	Absorbance poorly correlated with tannic acid concentration (r = 0.89). Nonspecific reaction (28, 29, above reactions with non-tannins). Time-consuming.
Bate-Smith and Rasper (20)	Extract with 43% H_2SO_4 in methanol. Determine absorbance at 465 nm.	Inadequate standards. High values (Table III). Differing absorption maxima for various varieties.

TABLE V. COMPARISON OF CATECHIN EQUIVALENT VALUES OBTAINED USING THE VANILLIN-HCl AND MV-HCl METHODS OF TANNIN ANALYSIS

Description of Grain		Catechin Equivalent Values (mg./100 mg.)[b]	
Color	Pigmented testa[a]	Modified	Unmodified
Brown	p	4.32	4.54
Brown	p	4.20	4.34
Brown	p	3.25	2.97
Brown	p	3.23	1.64
Brown	p	2.83	1.66
Red	pt	0.67	0.04
White	a	0.31	0.03
White	a	0.23	0.01
White	a	0.14	0.00
White	a	0.11	0.00
White	a	0.08	0.00

[a]p = present; pt = thin, does not completely surround endosperm; a = absent.

[b]Dry-weight basis. Mean values (N = 12).

615 samples, were higher when 1% HCl was used to acidify the methanol. Shaking the samples also increased the catechin equivalent values. Catechin equivalent values for both the vanillin-hydrochloric acid method and our modification of the method are presented in Table V. The catechin equivalent values obtained in this study were lower than those obtained by other workers (16,17) who used Burns' method. We cannot explain why our results are lower, but it further illustrates the need for a standardized method of tannin analysis for sorghum grain.

Phase II

The FAS and MV-HCl methods were used to measure the tannin content of three sets of samples. Analysis of variance and the means, range, and standard error of the means are presented in Tables VI and VII. The FAS method gave tannic acid equivalent values that were considerably lower than the catechin equivalent values. The FAS method did not have significant day-to-day variation.

The MV-HCl method had significant day-to-day variation in two of the three sets of samples. However, this accounted for only 0.5% of the variability in Set II as indicated by variance component analysis.

The two methods measure different substances in the grain, but the tannin values are related. The tannin values for the grain samples analyzed in phase II were highly correlated ($r = 0.92^{**}$, $N = 55$). However, correlations within each grain color were not significant for red and white grain, and the correlation for brown grain ($r = 0.38^{*}$, $N = 31$) was low.

For the standard curves of each method, tannin content was highly correlated with absorbance, but the standard curves varied from day to day. The composites of these curves were: mg. tannic acid equivalents = A + 0.0013/1.6336 ($r = 0.99^{**}$, $N = 41$) and mg. catechin equivalents = A -0.017/0.5625 ($r = 0.99^{**}$, $N = 10$) for the FAS and MV-HCl methods, respectively. Regression analysis of the standard curves of each method showed that the slopes were the same but that the intercepts were significantly different ($P<0.01$). Based on this information, standard curves should be prepared daily and would be expected to vary from one laboratory to the next.

The MV-HCl and FAS methods both appear acceptable for estimation of

TABLE VI. ANALYSIS OF VARIANCE FOR THE FAS AND MV-HCI METHODS OF TANNIN ANALYSIS USING THE THREE SETS (I, II, III) OF SORGHUM SAMPLES IN PHASE II

Source of Variation	FAS Method						MV-HCl Method					
	Degrees of freedom			Mean square			Degrees of freedom			Mean square		
	I	II	III	I	II	III	I	II	III	I	II	III
Total	107	107	79	...	...	...	107	107	119	...	...	...
Cultivars (C)	17	17	19	0.0474*	0.4499**	0.3606**	17	17	19	1.6476**	6.7697**	8.4077**
Days (D)	2	2	1	0.0096	0.0460	0.0011	2	2	2	2.5053**	0.2392*	0.1519
D X C	34	34	19	0.0197**	0.0159**	0.0078**	34	34	38	0.2239**	0.0484**	0.0625**
Subsample/D X C	54	54	40	0.0008	0.0049	0.0004	54	54	60	0.0057	0.0020	0.0031

TABLE VII. SUMMARY OF TANNIN VALUES OBTAINED FOR THE THREE SETS OF SAMPLES ANALYZED (PHASE II) BY THE FAS AND MV-HCl METHODS

	FAS Method[a]				MV-HCl Method[b]			
	N	Mean	Range	$s_{\bar{x}}$	N	Mean	Range	$s_{\bar{x}}$
Set I	18	0.89	0.77–1.10	0.06	18	3.41	2.71–4.32	0.19
Set II	17	0.64	0.27–1.05	0.05	17	1.76	0.14–2.86	0.09
Set III	20	0.51	0.28–1.15	0.04	20	1.13	0.08–3.25	0.10

[a]Expressed as tannic acid equivalents (mg./100 mg.), dry-weight basis.

[b]Expressed as catechin equivalents (mg./100 mg.), dry-weight basis.

polyphenols (tannins) in sorghum grain. The standard errors for these methods are lower than those for other methods when used for sorghum grain, with the possible exception of the vanillin-hydrochloric acid method. The FAS method is more time-consuming but gives good day-to-day reproducibility. The MV-HCl technique is faster and easier to accomplish, but has greater variability. The day-to-day variation must be considered. We do not know what biochemical compounds are being measured; each method measures different compounds. Non-polyphenolic substances that gave a high, positive test with the Folin-Denis reagent (Table IV) did not give a positive reaction for these methods, which would explain the higher values of the Folin-Denis methods when compared to the FAS method. Hopefully, the values obtained by these methods are relative measures of tannin content and can be related to differences in sorghum quality. Then, they can be standardized. Methods for analysis of tannins in sorghum should be devised or adopted to permit comparison of results among laboratories. Standardization will permit meaningful determination of the influence of tannins on sorghum quality.

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