protein content after butanol extraction by the BN method would appear to offer a more sensitive selection criteria on the basis of the lysine content in the dry matter. This method offers greater accuracy than either the ninhydrin color test or the prolamin turbidity test but has a lower throughput of samples; being comparable in this respect to the method of Villegas and Mertz (1971) using 2-chloro-3,5-dinitropyridine.

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Tannin Content as a Function of Grain Maturity and Drying Conditions in Several Varieties of *Sorghum bicolor* (L.) Moench

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Twelve varieties of sorghum grain were assayed for tannin content at various stages of maturity. For varieties which were found to contain tannin, maximum values per seed were obtained between 25 and 40 days after half-anthesis. Wide variability in apparent loss of tannin as the grains matured was found between varieties, with tannin contents of mature seed ranging from 3 to 93% of the maximum found in the immature seed. We suggest that varieties with maximum decreases in tannin *may* be similar to low tannin varieties in the nutritional quality of the mature grain, yet provide bird resistance during immature stages. Drying immature grain at room temperature after boiling for 3 min or freezing caused a drastic reduction in apparent tannin content over untreated controls, but these treatments had little effect on nutritional quality of the grain.

In many geographic areas otherwise well suited to growing grain sorghum, the loss of grain from depredation by birds can be substantial. Damage to the crop caused by birds in Louisiana was reported to be more serious than losses caused by insects (Tipton et al., 1970), with near total grain loss when sorghum was first commercially grown in that state. Harris (1969) observed 50% losses in Georgia and had reports of total destruction of some plantings. The problem is especially acute in parts of Africa where several billion queleas (red-beaked weaving sparrow, *Quelea quelea*) range over 20% of the continent in migrating flocks (De Grazio and Besser, 1974).

Significant protection of sorghum grain can be achieved by taking advantage of an aversion by birds for varieties containing tannin. A high negative correlation has been reported between tannin content of sorghum grain and damage by birds (Tipton et al., 1970; McMillian et al., 1972). Because of this "bird-resistance" as well as improved resistance to weathering (Harris and Burns, 1973) and preharvest germination (Harris and Burns, 1970) conferred by tannin, sorghum varieties which contain relatively high amounts of tannin are grown in many regions around the world.

The agronomic advantages of high-tannin sorghum are counterbalanced by corresponding antinutritional effects of the tannin. Tannin in animal diets causes a reduction in weight gain per unit of feed consumed and, under some conditions, in rate of growth, as well as other problems (Price and Butler, 1979). Producers whose environmental conditions enable them to grow sorghum that does not contain tannin thus gain an advantage in the export market over those who are limited to producing high tannin varieties.

Much of the bird damage occurs to the grain before maturity. For example, in the milk stage, birds were observed to crush the grain without plucking it from the head, ingesting the juice (Tipton et al., 1970). Perhaps varieties can be found which contain substantial amounts of tannin in the immature grain but in which all or part of the tannin is converted to nontoxic forms by the time the grain matures. If so, protection from birds would be provided during much of the growing season, with the nutritional problems of feeding mature, high-tannin grain reduced or eliminated. Such "disappearance" of tannin is common in many fruits upon ripening (Goldstein and Swain, 1963).

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The tannin content of sorghum grain has been reported to increase (Johari et al., 1977), remain constant (Tipton et al., 1970), and gradually rise and then remain constant or decrease depending upon variety (Davis and Hoseney, 1979) as the grain matured.

The purpose of this investigation was to measure tannin content as a function of maturity of several varieties of sorghum grain, with precautions taken to eliminate some of the uncertainty in previous studies, especially concerning the effect of different drying conditions. In particular, we sought varieties which have little or no tannin at maturity but which are high in tannin during the immature stages.

EXPERIMENTAL SECTION

The sorghum was grown at the Purdue University Agronomy Farm during the 1977 growing season. Varieties were planted in plots consisting of ten 20-ft rows. Hybrids (BR-54 and Savanna) were planted in plots of four 50-ft rows.

Individual plants were tagged when the head was at 50% anthesis, i.e., when the center of the head was first blooming. At intervals during the season, five heads were cut from the interior of the plot and placed in plastic bags. These were then frozen, within an hour, and stored until analyses could be done.

Grain was cut from an approximately 2.5-cm length at the center of each head. Two 5-g samples of grain were hand separated from spikelets and adhering glumes and bracts and the remainder returned to the freezer. Analysis showed that glumes contained relatively small amounts of tannin (e.g., 0.6% for BR-54) as determined by the vanillin assay. Because of this and the low percent by weight that glumes contribute to the grain, glumes and bracts were not completely removed from a few samples where their separation was especially difficult.

Assay of Frozen Grain. Each 5-g sample of grain was blended at room temperature for 1 min in 30 mL of methanol which was 0.01 M in ascorbic acid on a Brinkman Polytron tissue homogenizer at the maximum setting. The mixture was allowed to stand for an hour at room temperature to allow for additional extraction of tannin, then filtered with suction, after first weighing the filter paper, and washed with methanol. The filtrate was refiltered with gravity into graduated cylinders and the final volume (65–80 mL) recorded. The solid residue was allowed to dry at room temperature. One milliliter of the extract was analyzed for tannin using the vanillin assay of Burns (1971) as modified by Price et al. (1978), with a standard curve based on purified BR-54 tannin as described in the latter reference. The milligrams of tannin per gram of dry grain was calculated based on the volume of extract and weight of the residue after drying. The dried residue was ground to pass a 0.4-mm screen on a Udy Cyclone Mill (Tecator Co., Boulder, CO) equipped with a vacuum attachment, then assayed for tannin with the vanillin assay (Price et al., 1978) to detect any tannin not extracted during the blending process. On the average, the additional tannin obtained in the second extraction was approximately 10% of the first extraction. The results of the two measurements were added to give total tannin per milligram of dry grain.

Assay of Dried Grain. An additional 5 g of frozen grain was weighed out as previously described and the number of grains counted. Dry weight was obtained after drying 3 days in an 80 °C forced-air drying bin. Dried grain was ground and assayed for tannin as described for the dried residue above. The number of grains per gram of dry sorghum was used in calculations of tannin content per seed.

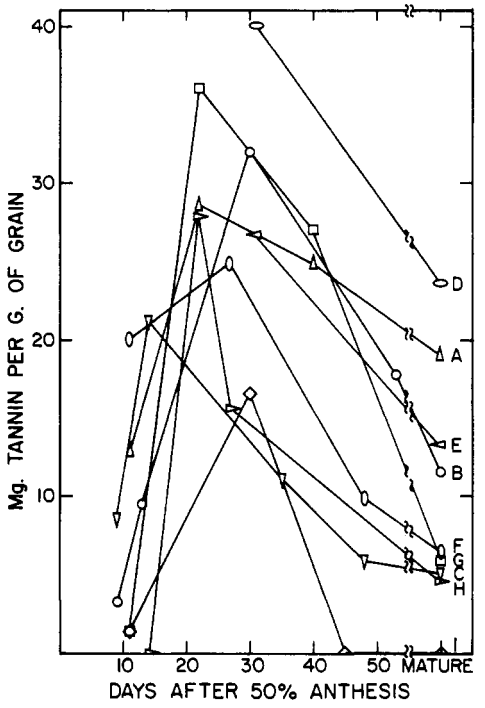

Figure 1. Milligrams of tannin per gram of grain (extracted from frozen grain and expressed on a dry weight basis) as a function of grain maturity. (A) IS-0829, (B) IS-6881, (C) IS-15067, (D) Savannah, (E) BR-54, (F) IS-8070, (G) IS-8614, (H) IS-4225, and (I) IS-3441. Data for the two commercial hybrids, BR-54 and Savannah, are limited as these were part of another study. It was decided to include them too late to obtain early samples.

Modified Vanillin Assay. Selected samples of dried grain were assayed by the modified vanillin assay of Maxson and Rooney (1972) as modified by Price et al. (1978) to identify any group II sorghum (tannin soluble in 1% HCl in methanol but not in methanol) (Price et al., 1978).

Feeding Trial with Immature Grain. Heads of IS-6881 were harvested from the 1978 crop approximately 35 days after 50% anthesis. Heads were dried in a 35–40 °C forced-air oven either immediately after harvest or after boiling in water for 3 min or being frozen for a week. A portion of the untreated, dried grain was ground and "detoxified" by moistening with 0.5 M K_2CO_3 as described by Price et al. (1978).

White weanling rats ranging from 45 to 55 g were randomly assigned to each treatment. Twelve rats, housed one rat per cage, were fed each experimental diet ad libitum for 28 days. Individual rat weight gain and feed consumption were measured. Diets contained 1.5% vitamin diet fortification mix and 4% salt mixture no. 4164 (both from Nutritional Biochemicals), 0.75% lysine hydrochloride, and 93.75% ground sorghum grain. Mean comparisons of the weight gains and feed efficiencies were made by the Neuman-Keuls multiple range test (Steel and Torrie, 1960).

RESULTS

The amount of tannin extracted from several varieties of sorghum grain as a function of maturity is shown in Figure 1. Although the data are expressed on a dry weight basis, it was obtained from samples frozen while fresh and kept frozen until extracted. Tannin content rose sharply beginning about 10 days after half anthesis and reached a maximum within 20 or 30 days. Subsequently, extractable tannin decreased substantially and in IS-3441 was undetectable by 45 days. Four varieties (IS-954114, IS-8277, IS-2042, and IS-12202) contained no more than 0.1%

Table I. Change in Tannin Content of Immature and Mature Sorghum Grain upon Drying

variety	days between time of 50% anthesis and time of max tannin content	immature grain		mature grain	
		% moisture	% change in tannin following drying	% moisture	% change in tannin following drying
IS-6881	30	49	−20	15	+70
IS-8029	40	38	+10	16	+70
IS-15067	35	39	+20	25	+190
Savanna III	31	41	−50	11	+10
BR-54	31	44	−80	20	+40
IS-8070	27	46	−80	19	+80
IS-8614	40	36	−30	18	+90
IS-4225	27	42	−50	15	+20
IS-3441	30	38	−60	16	

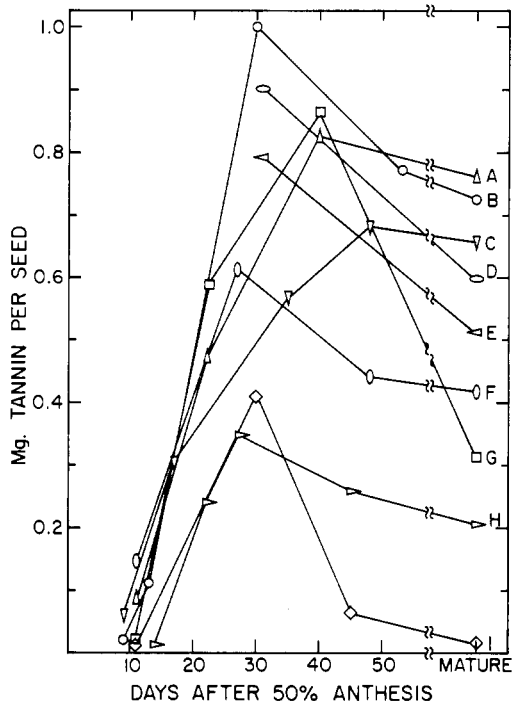

Figure 2. Milligrams of tannin per seed as a function of grain maturity. See Figure 1 legend for identity of grains. Tannin content at maturity as a percent of the maximum content during the season is as follows: (A) 93%, (B) 72%, (C) 96%, (D) 67%, (E) 65%, (F) 68%, (G) 36%, (H) 59%, and (I) 3%.

tannin at any stage of maturity and do not appear in the figure. The same data expressed on a seed rather than on a dry weight basis showed similar patterns with maximum tannin content occurring between 25 and 40 days after half anthesis.

The apparent tannin content varied not only with maturity but also with the technique used for storage and extraction of the tissue. Samples dried at 80 °C and assayed by the usual procedure for mature grain generally gave somewhat lower values for immature seeds and higher values for mature grain (Table I). Because neither technique is optimal for tannin extraction at all stages of maturation, a composite of the two sets of data, showing maximum amounts of tannin extracted per seed, is presented in Figure 2. Large differences were observed between varieties, not only in terms of tannin content of mature grain but also in the degree to which tannin that was present at immature stages diminished on maturation (see Figure 2 legend).

The apparent decrease in tannin content upon drying of immature sorghum grain was examined with three varieties under several conditions (Table II). Either boiling

Table II. Effect of Drying Temperature on Tannin Content of Immature Grain[a]

	% tannin found[b]				
	drying temperature				
	lyophilized	22 °C	40 °C	60 °C	105 °C
Savanna					
untreated		4.86	3.89	4.14	0.68
frozen	4.07	0.97	0.40	0.22	0.47
boiled		0.72	0.25	0.97	0.90
IS-6881					
untreated		4.82	4.14	3.67	0.11
frozen	1.48	0.07	0.00	0.04	0.04
boiled		0.11	0.72	0.00	0.11
IS-3441					
untreated		2.84	1.94	1.30	0.00
frozen	0.07	0.00	0.00	0.00	0.04
boiled		0.04	0.04	0.04	0.00

[a] Heads of grain at 16-21 days after half-anthesis were treated in one of three ways within an hour after cutting. Untreated: heads were subjected to no treatment other than drying at the temperature indicated. Frozen: heads were frozen and kept frozen for 5 days before drying as indicated. Boiled: heads placed in boiling water for 3 min before drying as indicated. [b] Mature Savanna later harvested from the same field had 2.05% tannin. Both IS-6881 and IS-3441 froze before reaching maturity.

for 3 min or freezing immature grain collected at a stage when tannin content was near maximal, followed by drying at temperatures from 22 to 105 °C, caused substantial reductions in the amount of assayable tannin. Boiling *mature* Savanna grain for 20 min, a much longer period, followed by drying at 24 °C caused only a 30% reduction in the amount of assayable tannin. Even lyophilizing frozen immature tissue, rather than drying, may cause decreases in the apparent tannin content. Maximal amounts of tannin, up to nearly 5% by weight, were extracted from grain dried at mild temperature (22 °C) without prior freezing or heating.

In order to determine whether the apparent reduction in tannin content, caused by freezing or briefly boiling immature grain before drying, improved the nutritional quality of the grain, feeding trials were conducted with weanling rats fed immature IS-6881 (Table III). Only small differences in weight gains and feed/gain ratios were observed, compared to the untreated control, despite a greatly lowered tannin content as a result of the treatments. Even chemical detoxification by K_2CO_3 (Price et al., 1979) caused only a small improvement, possibly because even the control diet gave weight gains much higher than would be expected for grain with such high tannin content. The amount of protein that can be precipitated per milligram of tannin from the IS-6881 grain is compa-

Table III. Effect on Nutritional Quality of Freezing or Boiling Immature High Tannin Grain (IS 6881) before Drying

		4 week rat[a]	
treatment	% tannin	wt gain	feed/gain
none[b]	3.5	$66.3_{a,b}$	4.9
freezing	1.0	70.7_{a}	4.8
boiling 3 min	1.1	57.5_{b}	5.6
K_2CO_3 detoxification	0.0	75.0_{a}	4.8

[a] Means with different subscripts are significantly different ($P < 0.05$). [b] Dried grain contained 11.25% protein.

rable to that found with mature sorghum from other varieties (Hagerman and Butler, 1978). In a subsequent feeding trial 4-week weight gains of weanling rats fed the immature IS-6881 dried at 30 °C without any other treatment averaged 67.1 g. This was significantly greater than was obtained with mature Savanna III (1.8% tannin, 49-g weight gain) and equivalent to that with mature RS-610 (0% tannin, 70.8-g weight gain). Feed to gain ratios showed the same pattern (4.8, 5.7, and 4.8, respectively).

DISCUSSION

The expression of tannin content on a per seed rather than on a weight basis (Figure 2) provides the best indication of changes in assayable tannin as the grain matures, because effects of changes in weight of the ripening grain are eliminated. Expressed in this way, tannin contents of mature grain ranged from 3 to 93% of the maximal amount found in the immature grain (Figure 2 legend). Further experiments in different locations and different years would be required to determine to what extent these varietal differences may depend on variety × environment interactions.

The basis for these decreases in tannin during maturation is of considerable interest. It must be emphasized that the apparent "disappearance" of tannin is most likely not due to an actual loss of tannin but to a change in its solubility or chemical reactivity so that it is no longer detected by the assay procedure. Decreased solubility could result from the formation of an insoluble complex between tannin and some other cellular component or, as has been suggested to occur in fruits (Goldstein and Swain, 1963), to an increase in the degree of polymerization. If vanillin reacts only with the initial catechin unit of the tannin polymer (Goldstein and Swain, 1963), an increased degree of polymerization would result not only in reduced solubility but also in less color in the vanillin assay per milligram of tannin. Thus an increase in the degree of polymerization would be interpreted as a decrease in tannin content, even if the larger polymers remained soluble. The high correlation reported by Price and Butler (1977) between tannin content of nine varieties of sorghum measured by the vanillin assay and by a redox method suggests that variation in polymer size between group III sorghum varieties (see below) may be at least uncommon.

The observation that substantial reductions in assayable tannin upon drying immature sorghum grain occur only if drying is preceded by freezing or brief boiling suggests that the disruption of cells or subcellular compartments is involved in the phenomenon. After such a disruption, complexes could form between tannin and some other cellular component, such as protein. On drying, these complexes would likely become even more tightly bound together and insoluble in methanol. Sullins and Rooney (1975) report that no cellular outlines are observed in the testa of mature sorghum grain, the cells apparently having been crushed by the expanding endosperm, so perhaps freezing or boiling disrupt protein-containing cells or vesicles surrounding the testa. The similar effect of freezing or boiling 3 min on the apparent decrease in tannin content makes the hypothesis of an enzyme-catalyzed polymerization after disruption of the subcellular vesicles appear unlikely because the boiled grain almost certainly contains no active enzymes. Nonenzymatic polymerization cannot be ruled out.

Sorghum has been divided into three groups on the basis of tannin content and solubility of mature grain (Price et al., 1978; Cummings and Axtell, 1973). Group I contains no tannin. Group III contains tannin which can be easily extracted in methanol. Tannin can be extracted from group II only with acidic methanol. All the varieties in Figures 1 and 2 except for IS-3441 belong to group III. The three which had no tannin and are not in the figure are group I. The evidence which suggests that tannin becomes undetectable if certain cells or subcellular vesicles are disrupted before the grain is dried also suggests a possible explanation for the difference between group II and III sorghum. The hypothetical vesicles could be disrupted near maturity in group II sorghum, thus causing the apparent loss of tannin. In this model, acidic methanol would be able to disrupt some of the tannin complexes which were insoluble in methanol. The vesicles may also be disrupted to varying degrees in ripening group III sorghum, accounting for the great variation observed between varieties as they matured. An objection to this is the failure to find group III varieties in which part of the tannin is soluble only in acidic methanol (Price et al., 1978), but only nine varieties were examined. The group II sorghums have been reported to be equivalent in nutritional value to the group I varieties which contain no tannin (Cummings and Axtell, 1973). This could be explained by the tannins becoming irreversibly bound to some component(s) in the pericarp which are of minimal nutritional importance. When eaten, the tannins would then be unavailable to react with the higher quality protein found in the embryo or with endogenous protein in the animal's digestive tract.

If this model is correct, then artificially disrupting these cellular compartments by freezing or briefly boiling group III grain harvested at an immature stage, followed by drying, might also overcome the deleterious nutritional effects of tannin. However, no such improvement was observed in the rat feeding trial (Table III). The results are clouded by the surprisingly large weight gains of the rats, even with the untreated grain, despite a tannin content of 3.5%. This is approximately 60% more tannin than in grain fed in an earlier trial which showed severe weight depression (Price et al., 1978), yet weight gains in this experiment are comparable to those found in earlier work with grain which contained no tannin. The superior nutritional quality of this grain was confirmed by the feeding trial comparison with *mature* high-tannin and low-tannin grain.

The observations in Figure 2 are of special importance for those interested in the problem of bird depredation of immature grain. Comparison of the tannin content of mature grain does not give an adequate picture of the tannin content during the immature stages. For example, mature IS-8614 contains only 40% as much tannin as does mature IS-0829, but at 40 days after 50% anthesis, they contained equal amounts on a per seed basis. It is possible that they were equally "bird resistant" at that stage.

The group II variety in this study, IS-3441, seems to be an especially promising candidate for combining "bird resistance" during the immature stages of growth when the tannin content is maximal, with good nutritional quality

of the harvested grain when the tannin content as measured by the vanillin assay is zero. Further studies are in progress to determine whether group II varieties are able to resist bird depredation at early stages of maturity.

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Chemical Nature of the Pigment of the Seed Coat of Guar (Cluster Bean, *Cyamopsis tetragonolobus* L. Taub)

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The pigment of the cluster bean (guar, *Cyamopsis tetragonolobus* L. Taub) seed coat was found to be a complex of ferric ions, galactose, gallic acid, and 2,3,4-trihydroxybenzoic acid.

Natural plant pigments are of diverse chemical structures (Bentley, 1960). Various phenolic compounds are constituents of plant pigments as reviewed by Singleton (1972). The presence of phenolic compounds in guar seed has previously been reported (Nagpal et al., 1971). The seed coat color of guar varies from black to dull white. Guar gum processing companies prefer seeds with a light colored seed coat. Hymowitz and Matlock (1967) studied the variations in seed coat color. They concluded that color variation is largely controlled by the environment. The chemical substances which produce the seed coat color are not known. This study was initiated to extract, purify, and identify the pigments.

MATERIALS AND METHODS

Procurement of Guar Seeds. Guar (*Cyamopsis tetragonolobus* L. Taub, var. FS 277) was grown on the farms of Panjab Agricultural University, Ludhiana, India. Seed samples were taken at 15-day intervals from flowering to maturity.

Extraction and Purification of Guar Seed Coat Pigment. The mature seeds ranging in color from light to dark brown were used for the extraction of pigment. The pigment was not extractable with organic solvents like methanol, ethanol, acetone, or ethyl acetate but was highly soluble in water. The whole guar seeds (2 kg) in small lots of 100 g each were immersed in glass-distilled water for 5 min at 15 °C. The supernatant was decanted off and filtered through Whatman No. 1 filter paper. The brown solution so obtained was lyophilized. Because it was not soluble in organic solvents it was possible to purify the water extract by several washings with methanol and acetone. The purified sample was then dried under vacuum. This purified compound was designated as G.

Extraction of Phenolic Acids. The samples (500 g) of guar seeds of various stages of maturity were extracted three–four times with 0.3 N HCl in methanol. The pH of the extract was adjusted to 8.3 with 5 N NaOH with constant stirring. A saturated solution of lead acetate was added according to the procedure of Walker (1962) and the light chocolate-grey precipitate obtained was filtered off and washed thoroughly with water until the filtrate became colorless. The precipitate was suspended in water and H_2S was passed through the suspension until the black precipitate of PbS ceased to form. The filtrate was freed from H_2S and concentrated by boiling it under reduced pressure on a water bath. The concentrate was extracted with ethyl acetate in a liquid–liquid extractor. The ethyl acetate fraction of each sample was stored in a refrigerator for chromatographic analysis.

Chromatographic and Spectral Analysis. Two-dimensional descending paper chromatography (PC) was carried out on 56 × 45 cm sheets of Whatman No. 1 filter paper (1 mm thick) employing the following solvents: (I)

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