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Rapid Visual Estimation and Spectrophotometric Determination of Tannin Content of Sorghum Grain

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A new technique was developed to quickly distinguish between zero, low, intermediate, and high tannin varieties of sorghum by the development of shades of yellow, green, and blue colors. A spectrophotometric method was developed which detects low concentrations of tannin and other polyphenolics by the formation of the Prussian blue complex. This method plus a rapid extraction procedure enables polyphenol content of grains to be quantitatively compared in 20 min. The vanillin test was shown to give misleading results unless a suggested modification is included in the procedure; of 35 varieties of grain studied, 20 that had been considered low in tannin have no tannin detectable by this test. Some phenolics which are extracted in water are not extracted in 0.2 M NaCl. This suggests a method which may distinguish between tannins and nontannin polyphenols.

In recent years it has been recognized that the tannins present in many varieties of sorghum diminish the nutritional quality of the grain. Several investigators have reported lower weight gains in young rats and chickens fed high tannin grain compared with those fed low tannin varieties. Typically the weight gains are 30–50% less for the high tannin varieties (Armstrong et al., 1973; Jambunathan and Mertz, 1973), though actual weight *loss* has been reported in rats fed one high tannin variety (Jambunathan and Mertz, 1973). Similar results have been reported comparing the effects of high and low tannin beans on weight gain in rats (Ronnenkamp, 1977) and on in vitro dry matter disappearance trials (Bond, 1976). The increasing concern over the nutritionally harmful effects of tannins in sorghum is creating strong pressures for the sorghum industry to provide grain of low tannin content.

However, there are economic incentives for the producers to grow high tannin varieties of sorghum. The presence of tannin makes grain less desirable to depredatory birds, so "bird resistant" (high tannin) varieties can be of great importance in some regions. Estimated losses of 50% in Georgia (Harris, 1969) and 48–72% in Arizona (Voight, 1966) have been reported from bird depredation. Tannin also apparently is associated with decreased susceptibility of the grain to preharvest germination (Harris and Burns, 1970) and seed molding (Harris and Burns, 1973).

This conflict between the benefits of tannin to the grower and the deleterious effects of tannin for the consumer should lead to a reflection of tannin content in the price paid for the grain. However, analytical procedures to quickly and accurately determine tannin content have not been available. The current method of tannin "analysis" used by grain elevators consists of soaking the grain in bleach and alkali to remove the pericarp, so that the testa (if present and colored) becomes visible. If a testa is seen, the grain is assumed to contain high amounts of tannin. The method is subject to error because of interference in some varieties by plant pigments, the color of which may persist through the bleach test and make identification of a testa ambiguous, and because there is not an absolute correlation between the presence of a colored testa and the deleterious nutritional effects ascribed to tannins. For example, the grain of IS 2319 has a clearly identifiable testa, yet results of a feeding trial showed it to be superior to three low tannin varieties without a testa (Oswalt, 1975). The method also is unsatisfactory because it is not quantitative.

The method of quantitative analysis for tannins that has become most widely used for sorghum grain in the laboratory is the vanillin test (Burns, 1971). This test is not convenient for use at the grain elevator because it involves an overnight extraction and at least minimal laboratory facilities.

In this paper, a new analytical procedure is described which can be used, without instrumentation, to provide a rapid and convenient visual estimation of the quantity of tannin present in sorghum grain. In variations of this test, which can be completed in 1–10 min, the yellow color changes to shades of green and blue with increasing tannin content. For more precise determination of tannin content by those without a laboratory, a simplified procedure requiring only a fixed wavelength colorimeter, some basic glassware, a grinder, and reagents has been devised. Several samples can be determined in an hour. An even more precise spectrophotometric procedure is described which requires about 20 min for analysis in the laboratory. A new method is suggested to differentiate between large polymeric tannins and simple flavanoids, anthocyanidins, and small polymers. An essential modification of the

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Table I. Visual and Colorimetric Estimation of Tannin Content of Sorghum Grain

Grain	Estimation methods: Visual (variation I) Color	Rank	Tannin content	Colorimetric: Water (A_{720})	Colorimetric: minus NaCl blank (A_{720})	Standard spectrophotometric methods: Corrected vanillin, C.E.	Prussian blue (water extract), C.E.
Br-54	Deep blue	1	High	0.71	0.58	3.2	0.58
NK-300	Blue	2	High	0.60	0.48	2.2	0.56
IS-8164	Turquoise	3	Moderately high	0.50	0.35	1.4	0.36
IS-15612	Dark green	4	Moderately high	0.40	0.25	1.0	0.33
IS-15991	Green	5	Intermediate	0.15	0.09	0.4	0.04
RS-610	Lime green	6	Low	0.04	0.00	0.0	0.04
IS-954063	Lime green	6	Low	0.04	0.00	0.00	0.00

vanillin test is also suggested.

EXPERIMENTAL SECTION

Reagents. All solutions were prepared using deionized or distilled water. The 0.10 M $FeCl_3$ was prepared in 0.1 N HCl and the solution filtered. D-Catechin was purchased from Sigma Inc. The label indicates 2.5 mol of water of hydration was present. Reagent grade ferrous ammonium sulfate was used for the iron(II) standard curves. Reagent grade catechol, phenol, gallic acid, quercetin dihydrate, and cyanidin chloride were used without further purification.

Sorghum Grain. Sorghum grain was obtained through the courtesy of Dr. John Axtell from the 1975 crop grown in seed trials at Purdue University. Grain for the visual or colorimetric estimations was ground 1 min in an electric, hand-held Krups 75 coffee grinder. All data in Table I was obtained within 3 days of grinding. For spectrophotometric studies grain was ground to pass a 0.4-mm seive in a cyclone mill. The ground grain was stored in paper envelopes at room temperature. Data were collected during the second and third months after grinding (except for data in Table I). Collection of data for graphs showing comparisons was completed within a few days whenever possible to minimize any changes in tannin content with time.

Visual Estimation of Tannin Content. *Variation I.* Ground grain, loosely scooped up in a 2-mL measuring spoon with excess scraped off flat, was added to a 125-mL flask containing 50 mL of water. Contents were swirled frequently for 3 min. At 4 min 1-mL aliquots from each flask were mixed with 1 mL of 0.008 M $FeCl_3$ in 0.008 N HCl, followed by 1 mL of 0.003 M $K_3Fe(CN)_6$. All volumes were measured with Pasteur pipets calibrated to a 1-mL volume. Color developed immediately, but observations were recorded after 1 min to allow color to become more stable.

Variation II. Two milliliters of ground grain was measured as in variation I and placed in a 250-mL flask, followed by 200 mL of 0.0004 M $K_3Fe(CN)_6$ and 10 mL of 0.008 M $FeCl_3$ in 0.008 N HCl. Color developed within seconds, then deepened slowly over the next few minutes as more tannin was extracted.

Variation III. Three grains were split in two longitudinally and placed in a small test tube, followed by 0.5 mL of 0.0015 M $K_3Fe(CN)_6$ and 0.008 M $FeCL_3$ in 0.008 N HCl. Tubes were swirled occasionally, then the color noted after 10 min.

Colorimetric Estimation. The flasks prepared in variation I were swirled occasionally for 20 min. After settling for 10 min, 1-mL aliquots were removed. To this was added 2 mL of 0.008 M $FeCl_3$ in 0.008 N HCl and 10 mL of 0.0015 M $K_3Fe(CN)_6$. Absorbance at 720 nm was read 30 s after adding the final reagent. NaCl blanks, if desired, were prepared in the same manner, except extraction was in 50 mL of 0.2 M NaCl, and subtracted from the first reading.

Spectrophotometric Measurement. Ground grain (60 mg) was shaken constantly for 60 s with 3 mL of methanol in a test tube, then poured into a Buchner funnel with the suction already turned on. The tube was quickly rinsed with an additional 3 mL of methanol and the contents poured at once into the funnel. The filtrate was mixed with 50 mL of water and analyzed within an hour. For aqueous extractions, 5 mL of water was used for the extraction and for the rinse, and the filtrate was added to 50 mL of water. Other ratios of water and extract can be used as convenient, as long as appropriate standard curves are prepared.

Three milliliters of 0.1 M $FeCl_3$ in 0.1 N HCl was added to the extract, followed immediately by timed addition of 3 mL of 0.008 M $K_3Fe(CN)_6$. When methanol was present, the $FeCl_3$ had to be added at the same timed intervals because of a small increase in OD with time of exposure of $FeCl_3$ to methanol. The optical density was read after 10 min in 1-cm glass cells at 720 nm on a Zeiss PMQ II spectrophotometer which had been zeroed with water. Ten minutes for color development was chosen because the rate of reaction was considerably slower after that time and because a precipitate often formed after 15–20 min. A blank of identical composition, but omitting the sorghum extract, was analyzed and subtracted from all other readings.

Results were expressed as catechin equivalents using standard curves prepared daily for the conditions used in the analysis, from fresh solutions of commercial D-catechin. Catechin equivalents are milligrams of "catechin"/100 mg of sorghum grain that would be required to give the observed absorbance. Standard curves relating A_{720} with moles of ferrous ion produced during the oxidation of extracted tannin were prepared, using reagent grade ferrous sulfate.

General Aspects of the Prussian Blue Test. Glassware and cuvettes are stained blue after a few determinations, especially if not rinsed immediately at the conclusion of each experiment. Cuvettes must be scrubbed with a cotton swab after each set of experiments before the stain dries. Once the stain has dried on glassware, it can be easily scrubbed off after soaking overnight in an aqueous oxalic acid solution.

A large excess of $FeCl_3$ is added in all variations of the test. This is to ensure rapid and complete reaction. The

blank is kept low by using low $K_3Fe(CN)_6$ concentrations. The absorbance increases with time. Formation of the complex appears to be rate limiting, the redox reaction apparently reaching completion in seconds.

Because of inherent instability of dilute solutions of $FeCl_3$, this reagent is not added until just before the solutions are ready to be analyzed. The results do not seem to change noticeably when $FeCl_3$ is added up to half an hour before analysis of the samples, as long as no organic solvents are present. A solution containing 6 mL of methanol in 50 mL of water, however, showed a 30% increase in absorbance of the blank when the iron was added 30 min early. Although the absorbance due to tannin was not affected, this increase in background requires that ferric chloride be added at timed intervals when organic solvents are present. All dilutions were made from 0.1 M $FeCl_3$ in 0.1 N HCl (added to increase stability). This stock solution is stable for months.

The A_{720} readings are extremely sensitive to the slit width of the spectrophotometer. Widening the slit width from 0.08 mm to near maximum (0.2 mm) reduced the absorbance by 46%.

Vanillin Test. The vanillin test was performed as described by Burns (1971). Ground grain (200 mg) was extracted with 10 mL of methanol in screw-capped test tubes which were continuously rotated for 18 h. For the "corrected" vanillin test (see Results) separate blanks were read for each sample and substracted from the results of the regular vanillin test. The blanks were run under conditions identical with the regular vanillin test except that vanillin was omitted from the 4% HCl in methanol.

RESULTS

Visual Estimation of Tannin Content. This test is based on the reduction by tannin and other polyphenols of ferric ion to ferrous ion, followed by the formation of a ferricyanide–ferrous ion complex. The colored product (commonly known as Prussian blue) absorbs maximally at 720 nm. The same reagents in more concentrated form are often used to visualize phenolic compounds on paper chromatograms. Initially the solution is yellow, the color of the reagents. Increasing amounts of tannin result in the production of increasing amounts of the blue pigment, which absorbs the red end of the spectrum. The solution, however, appears green because the blue end of the spectrum is still masked by unreacted ferricyanide. If the initial ferricyanide concentration is sufficiently low, it will become noticeably depleted with higher amounts of tannin. The result is a deepening of the green color, followed by a change to turquoise and blue. It should be emphasized that because these colors vary with conditions used, they only reflect relative tannin contents. A few grains of known tannin content should always be run for comparison. However, once these precautions are taken, grains can be ranked as high, intermediate, and low in tannin with reasonable certainty.

The sensitivity of the visual estimation can be adjusted to maximally distinguish differences between high tannin varieties of grain by using more water in the extraction step or by adding water to the aliquot taken for developing the color. Adding more ferricyanide can serve somewhat the same purpose by requiring more tannin to be present before enough ferricyanide has been used up to allow the solution to appear blue. If the amount of ferricyanide is increased without also adding more water, however, the green color becomes so intense that it is difficult to distinguish changes in shade of color.

If many samples are to be analyzed, the $FeCl_3$ and $K_3Fe(CN)_6$ solutions can be combined into a single color reagent. However, this mixture is stable for only a few hours unless kept in the dark or in a brown bottle. If the visual test is used in the field it must be done in the shade. Direct sunlight causes color to develop within minutes once reagents are mixed.

Table I shows the results of a typical determination of tannin content for seven varieties of sorghum grain, using variation I of the visual estimation. The colors obtained, the relative ranking of the grains according to tannin content, and estimated tannin content are presented. The amount of "tannin" present as determined by two different spectrophotometric methods are shown for comparison.

If rapid completion of the analysis is more crucial than precision, reagents can be added directly to the ground grain, as in variation II. Within a few seconds low, medium, and high tannin varieties give shades of yellow, green, and blue. The main problem with this approach is that the amount of extracted tannin is changing rapidly during the first minute or two. After a few minutes even low tannin varieties become dark green. Such change with time is not a problem in the procedure involving a 4-min extraction, since over 90% of the tannin has been extracted by that time (see next section).

In many cases it may be desirable to obtain an estimate of tannin content where grinding is not feasible, such as when only one or a few grains must be tested, or for work in the field, or when speed is of prime importance. In such cases one–three grains can be split with a razor blade and a small amount of the color reagents added (variation III). Assuming roughly equal sized grains, the tannin extracted in a given time will be proportional to the total tannin content. High, medium, and low tannin grains can easily be distinguished in this way after a few minutes. Changes in color over several minutes are most pronounced for this variation of the visual estimation method. The two high tannin grains in Table I were blue and clearly identifiable as high in tannin. The others could be distinguished but with less certainty. IS-15991 became light blue, probably because it was a much larger grain than the others.

The colorimetric estimation is designed for situations where greater precision is desired than can be obtained by visual estimation, but a laboratory is not available. A simple colorimeter with a fixed wavelength above 700 nm, flasks, test tubes, medicine droppers, an inexpensive coffee grinder, and reagents could perhaps be sold as a package. Values obtained by this method are included in Table I. Extraction times from 30–180 min gave essentially equivalent values.

Six determinations were made on Br-54 using this procedure. A mean A_{720} of 0.79 and a standard deviation of 0.06 was obtained.

As will be discussed more fully in the following sections, these methods make no distinction between tannin and other polyphenols. But none of the sorghum varieties we have studied seem to have enough nontannin phenolics to cause significant error. A low value by the visual estimation method is nearly certain to mean the grain is low in tannin, and a high value that it is high in tannin. This may be more of a problem in other seeds which have larger proportions of nontannin polyphenols. For example, we found in a related project that one variety of cowpea gave high readings, but contained little tannin.

This problem could be eliminated in the colorimetric estimation method (at the expense of doubling the work) by doing a duplicate extraction in 0.2 M NaCl. Supposedly only nontannins are extracted in this "NaCl blank" (see next section). Values after such a correction are also included in Table I.

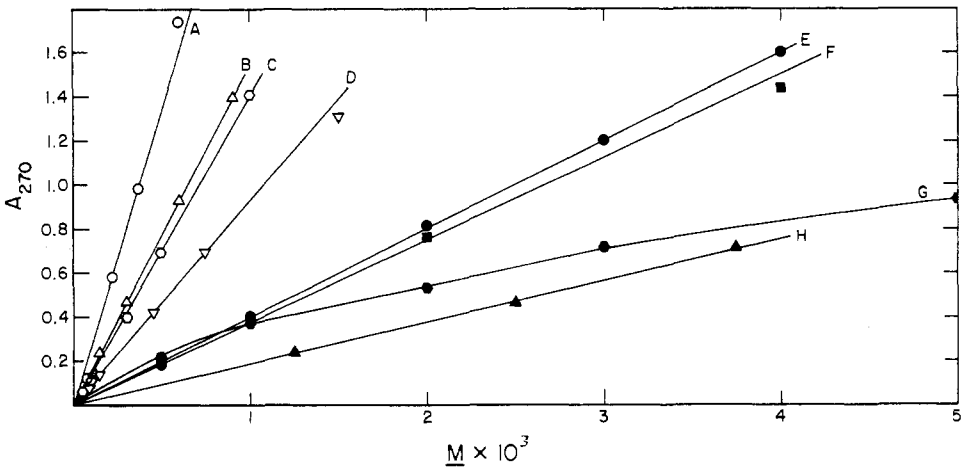

Figure 1. A_{720} for Prussian blue method vs. concentration of Fe^{2+} and various phenols: quercetin (A), catechin (B), gallic acid (C), cyanidin chloride (D), catechol (E), hydroquinone (F), phenol (G), and $Fe(NH_4)_2(SO_4)_2$ (H). Slopes relative to (H), except for phenol, are in order: 12.4, 7.3, 6.7, 4.3, 1.9, 1.8.

SPECTROPHOTOMETRIC MEASUREMENT

Method. The $FeCl_3/K_3Fe(CN)_6$ system provides a sensitive method for quantitative determination of dilute concentrations of polyphenolics in any of several solvents. Figure 1 shows standard curves for A_{720} vs. concentration of F^{2+}, catechin, catechol, gallic acid, *p*-dihydroquinone, phenol, quercetin, and cyanidin. As can be seen, excellent linearity is obtained well above one OD unit for each compound except phenol. The sensitivity of the test toward flavanoid compounds is sufficient to determine concentrations less than 10^{-4} M. This can easily be increased fivefold by reducing the volume of H_2O added to each sample to 10 mL or less.

An advantage to this method is that comparison of the observed slopes to the $Fe(NH_4)_2(SO_4)_2$ slope provides a measure of how many moles of Fe^{3+} are reduced per mole of phenolic. The ratios of these slopes to $Fe(NH_4)_2(SO_4)_2$ are given in Figure 1. The reason that some of these are not integers may be due to uncertainty as to the degree of hydration of some polyphenolics and to use of compounds without further purification. We are investigating the usefulness of this technique for determining the degree of oxidation of purified tannin fractions.

The wide variation in the degree to which each molecule is oxidized (e.g., a sevenfold difference between quercetin and hydroquinone) should serve to emphasize the importance of using caution in interpreting results when *mixtures* of polyphenols are analyzed by *any* redox method.

The Prussian blue method should give good estimates of relative polyphenol content for mixtures of anthocyanidins, since they are not likely to differ greatly in the extent to which they are oxidized by Fe^{3+}. Likewise, different condensed tannins might be estimated fairly accurately, depending upon just how great the differences are between them. These are the two most likely polyphenols present in the sorghum extracts studied here (Strumeyer and Malin, 1975). It is presently not possible to determine whether the same number of Fe^{3+} ions are reduced per molecule of proanthocyanidin as per "subunit" of tannin.

Justification of 1-Min Extraction. A requirement for rapid analysis is that the amount of tannin extracted in a few minutes be proportional to the total amount of tannin in the grain. Figure 2 shows the catechin equivalents (C.E.) extracted by methanol in 1 min from 25 varieties of sorghum plotted against the catechin equivalents extracted in 18 h. The points fit a line with a slope

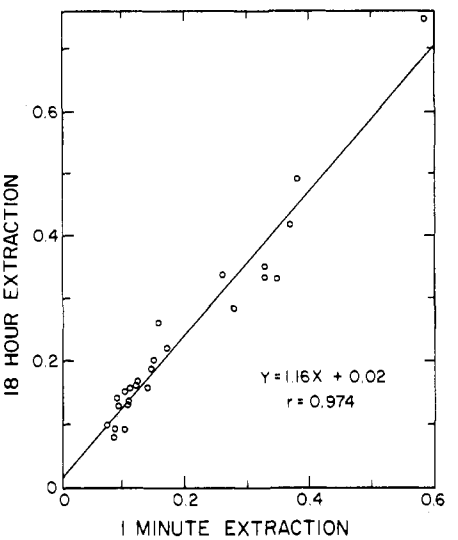

Figure 2. A_{720} for 18 h extraction of "tannin" from 25 varieties of sorghum compared to A_{720} for 1-min extraction, measured by Prussian blue formation.

of 1.16 with a correlation coefficient of 0.974. Catechin equivalents were determined spectrophotometrically by measuring the formation of Prussian blue. The slope of the line indicates that approximately 86% of the tannin that can be extracted in 18 h has already been extracted in 1 min. Results of the 1-min determination can be compared directly with the 18-h determination by multiplying by a factor of 1.16.

To check reproducibility of this method, five determinations were made, each using 50 mg of Br-54. A mean A_{720} of 1.084 and a standard deviation of 0.015 resulted. If more coarsely ground grain were used, the percent of tannin extracted in 1 min and reproducibility would be lowered. Sampling might also then become a problem unless more grain were used.

Comparison with the Vanillin Test. The prescribed method for the vanillin test (Burns, 1971) is apparently based on the assumption that the background color in the absence of vanillin is too low to require the subtraction of a blank for each sample. This assumption is clearly not valid; the solvent extracts colored materials which absorb at the same wavelength. Table II lists C.E. values obtained by the regular method and by correcting for the blank. Evaluated this way, over half of the grain contained no tannin at all. Therefore, several varieties of sorghum that

Table II. Regular and Corrected Results for the Vanillin Test

Grain	C.E.	C.E. after subtracting the blank
Br-64	3.42	2.45
IS-8193	2.68	2.11
IS-8164	1.99	1.74
IS-15526	1.83	1.34
NK-300	1.71	1.15
IS-15612	1.50	1.22
IS-15526	1.42	1.01
IS-15346	1.11	0.70
IS-6881	1.10	0.81
19-8687	0.70	0.02
IS-15991	0.60	0.39
IS-2279	0.58	0.05
IS-10486	0.57	0.00
IS-8544	0.51	0.00
IS-9950	0.50	0.09
IS-9954	0.49	0.00
121142	0.42	0.00
IS-0339	0.36	0.00
131161	0.35	0.00
IS-9528	0.35	0.10
19-0158	0.33	0.00
121168	0.33	0.00
IS-9180	0.32	0.00
121199	0.27	0.00
IS-0114	0.26	0.00
IS-1031	0.26	0.00
IS-10594	0.21	0.00
IS-10562	0.20	0.00
IS-12279	0.19	0.01
19-2057	0.17	0.00
IS-10523	0.16	0.00
10493	0.16	0.00
IS-2042	0.15	0.00
IS-12317	0.15	0.00
121180	0.13	0.00

have been considered to be low in tannin actually contain no tannin that is detectable by the vanillin test. We have found that errors caused by not subtracting blanks in the modified vanillin test (Maxson and Rooney, 1972) are considerably greater. Acid used in the vanillin test will itself cause an increase in absorbance with time, but this does not appreciably affect the blank in 20 min. For example a Br-54 extract gave A_{500} of 0.82 in the vanillin test and a blank of 0.072. The blank was already 0.051 at 15 s.

Without further modification the Prussian blue test cannot be compared to the vanillin test, even after the latter is corrected for background color. This is bacause redox methods, as we have just seen, measure the total of tannins and other polyphenols.

Extraction with Aqueous Salt Solutions. It is desirable to measure only the (polymeric) tannins by the spectrophotometric Prussian blue method, despite the presence of anthocyanidins or other low molecular weight phenols which respond in the test. Because salt has long been used to precipitate condensed tannins (Quesnel, 1968), it seems likely that salt would even more effectively prevent their extraction. Anthocyanidins can be extracted quantitatively with aqueous salt solutions (Goto et al., 1976). Thus the difference in solubility in water and in salt solutions might provide the basis of a Prussian blue test which is specific for condensed tannins.

Figure 3 shows the Prussian blue color per 50 mg of grain measured after extracting 1 min with aqueous solutions of increasing salt concentration. Before the color was developed, all solutions were adjusted to the same salt concentration. The great sensitivity of some varieties to quite low salt concentrations is striking. Some sorghum

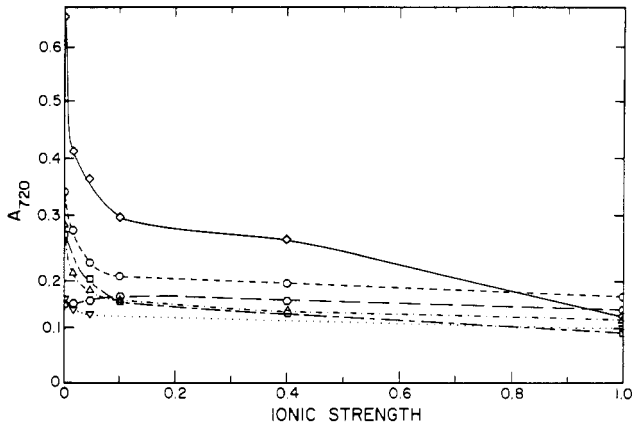

Figure 3. A_{720} for 1-min aqueous extraction as a function of ionic strength of the NaCl solution, measured by Prussian blue formation. Sorghum grains extracted: IS-8164 (◊), IS-2279 (○), IS-9180 (□), IS-8687 (▽), IS-2319 (○), IS-15991 (△).

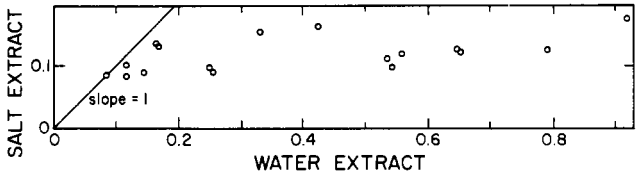

Figure 4. A_{720} per 50 mg of sorghum extracted in 1.0 M NaCl vs. A_{720} per 50 mg of extracted in H_2O, measured by Prussian blue formation, for 17 varieties of sorghum grain.

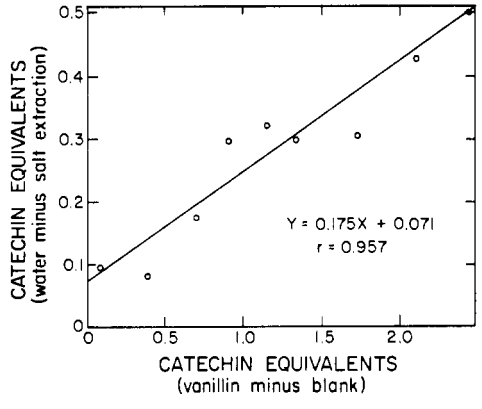

Figure 5. Prussian blue C.E. values for 1-min extraction of nine varieties of sorghum in H_2O minus the value from 1-min extraction in 1.0 M NaCl vs. C.E. values for the corrected vanillin test.

varieties appear to contain water soluble components, presumably tannin, which are not extracted in 0.2 M NaCl. All sorghum varieties tested contain other components, presumably anthocyanidins, whose extractability is unaffected by up to 1.0 M sodium chloride.

Figure 4 compares extraction with water and 1.0 M NaCl. All the C.E. values in salt solution are low in comparison to C.E. values for *high-tannin* grains extracted in water. The salt-extracted polyphenolics apparently vary only within a low range, their concentration never approaching the concentrations of polyphenolics in true high tannin lines. If this were not true, the visual tests described earlier, variations I–III, would not be valid. The procedure described in variation I of the visual estimation method has sufficient water and $K_3Fe(CN)_6$ added to cause minimal color change by the relatively low amounts of nontannins that are usually present.

Figure 5 shows the C.E. values for the 1-min aqueous extraction of ten varieties of grain corrected for "nontannins" by subtracting the values of a 1-min extraction

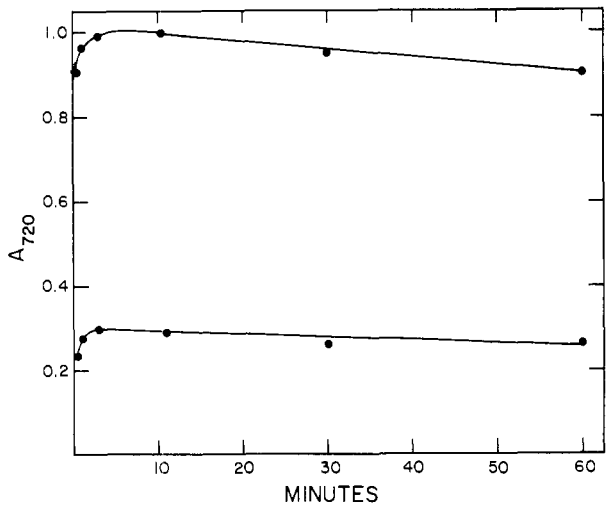

Figure 6. Quantity of polyphenols extracted in water and in 1.0 M aqueous NaCl, measured by A_{720} in the Prussian blue test, vs. time of extraction. Upper curve, H_2O extraction; lower curve, NaCl extraction.

in 1.0 M NaCl, plotted against the C.E. values for the vanillin test corrected for "nontannins" by subtracting the background color.

When a larger quantity of grain was extracted with the same volume of salt solution, a nearly proportional increase in A_{720} was found. This ruled out the possibility that the salt simply reduces the total solubility of all polyphenols.

The salt could reduce the rate at which the polyphenols dissolved, so that 1-min extraction times were not comparable with similar data from water extracts. A comparison of the amount of phenols extracted by water and by 1.0 M NaCl as a function of time is shown in Figure 6. In both solvents, maximum extraction is obtained at about 10 min and then declines, presumably due to air oxidation of the extracted material. Differences in completeness of extraction after 1 min in the two solvents can account for no more than a 4% difference between them. Clearly the large differences between the curves in Figure 6 are not caused by differences in rate of extraction in water and NaCl solution.

After dialysis of a 1 M NaCl extract for 24 h against 1 M NaCl, the outside concentration of polyphenol, assayed by the Prussian blue method, was 35% as high as the inside concentration. The comparable figure for a water extract dialyzed against water was 11%. This is consistent with a relatively smaller proportion of high molecular weight polymeric tannins extractable by salt solutions.

Extent of Oxidation of Tannins. The C.E. values obtained by the Prussian blue method are always lower than those obtained by the corrected vanillin test, as can be seen by the slope of only 0.175 in Figure 4. This is due in part to an inherent overestimation when catechin is used as a standard for the vanillin test. Rate studies (Price and VanScoyoc, unpublished data) show that the absorbance with catechin decreases to a fraction of its peak in the 20 min allowed for color development, whereas the absorbance is still increasing for sorghum extracts. The Prussian blue test may underestimate when using a catechin standard if tannin is more oxidized or less reactive than monomers.

DISCUSSION

The advantages of the Prussian blue method for visual estimation of tannin content in sorghum are: (1) The series of colors produced can be much more easily distinguished visually than would be the case if only one color of varying intensity resulted. The latter would be the case, e.g., if the vanillin reagent were used. (2) The results are a direct measure of soluble polyphenol content. (3) The test is so sensitive that no interfering color is present at the dilutions used. (4) The rapidity and simplicity of the test makes it ideal for use at the grain elevator.

The formation of the Prussian blue complex offers a sensitive, versatile method for spectrophotometric determination of total polyphenols. The main disadvantage of this or any other redox method is that no distinction is made between tannins and other phenols. A measure of actual tannins present in the grain is more accurately measured by the aqueous Prussian blue test when corrected by the salt extraction method or by the vanillin test as corrected in this paper.

Even these modifications cannot be said to definitely measure only tannins. Monomeric proanthocyanidins will give a positive vanillin test, and it is not known what effect polymerization will have on the extent of the reaction and the extinction coefficient of the product. The Prussian blue method always yields a complex with the same extinction coefficient, but some error is introduced when polyphenolics with varying hydroxylation patterns, degrees of polymerization, etc., are mixed in unknown proportions. It cannot be considered proven that the salt extraction is removing only anthocyanidins or that it removes them completely.

It must be remembered in all methods used that only polyphenolics extractable under the given conditions are being measured. Changing solvents after exhaustive extraction with another solvent has been known to bring out additional tannin (Hillis and Swain, 1959).

The long-known chemistry of the formation of the Prussian blue pigment can thus be adapted to determination of tannins in sorghum and probably other plant products, employing either precise laboratory work or simple, visual estimations in the laboratory, the field, or at the grain elevator.

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Note Added in Proof: Bleach Test: Five grams KOH, 1 tablespoon of grain, and 0.25 cup of house-hold bleach are shaken in a jar until the KOH dissolves. After 20 min, contents are placed in a tea strainer and rinsed with running water, then spread on a paper towel and examined. The pericarp is removed by the process and the testa, if present, will be exposed and appear dark in color. Otherwise the seed will appear bleached, either white or yellow, depending on the genetic constitution of the endosperm. [Based on Weak, E. D., Miller, G. D., Farrell, E. P., Watson, C. A., *Cereal Chem.* **49**, 653–663 (1972)].