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Received for review October 31, 1977. Accepted April 7, 1978. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

# Protein Precipitation Method for the Quantitative Determination of Tannins

Ann E. Hagerman and Larry G. Butler*

The tannin content of crude plant extracts or of purified preparations was determined by adding the sample to a standard solution of protein, isolating the insoluble tannin–protein complex, dissolving it in alkaline solution, and measuring the absorbance at 510 nm after adding ferric chloride. Plots of absorbance as a function of the amount of tannin are linear for tannic acid and partially purified sorghum tannins for amounts of tannin ranging from 0.20 to 1.0 mg. Nontannin components of crude methanolic extracts of sorghum and cowpeas do not interfere with the assay. The results of the precipitation method are qualitatively similar to results obtained with the vanillin assay. The precipitation assay can be used to study the effects of pH and other parameters on tannin–protein interactions.

Tannins are polyphenolic compounds which form insoluble complexes with proteins (Swain, 1965). They are present in a wide variety of plants used for foods and feeds including sorghum (Bate-Smith and Rasper, 1969), beans (Martin-Tanguy et al., 1977), barley (Bate-Smith and Rasper, 1969), millet (Ramachandra et al., 1977), and some legume forage species (Jones et al., 1976). The interactions of tannins with proteins may play a role in the antinutritional effects of tannin-containing feeds which have been observed in nonruminants (Tamir and Alumot, 1970; Jambunathan and Mertz, 1973; Schaffert et al., 1974; Martin-Tanguy et al., 1977); tannin-containing forages may be useful in the control of bloat in ruminants (Driedger and Hatfield, 1972).

Tannin content is usually determined by assays such as the vanillin test (Burns, 1971), the Prussian Blue test (Price and Butler, 1977), and the Folin-Denis test (Burns, 1963), which are based on the chemical characteristics of tannins. An assay for tannins based on their ability to precipitate proteins might provide useful information about the nutritional value of foods and feeds which contain tannin. Existing precipitation methods are of limited value. The official method of the Association of Agricultural Chemists (1965) for the determination of tannins in tea, based on the precipitation of gelatin, has been reported to be of little value for the determination of tannin in sorghum grain (Maxson and Rooney, 1972). Bate-Smith (1973) has suggested an assay based on the precipitation of hemoglobin by tannins. This method is inconvenient because freshly drawn blood is used, and saponins and other plant metabolites interfere with the assay (Bate-Smith, 1977). Goldstein and Swain (1965) have suggested a method based on the ability of tannins to inhibit the enzymatic activity of $\beta$-glucosidase, but the results of the assay are difficult to interpret, because the relationship between enzymatic activity and the formation of insoluble complexes is not fully understood.

The precipitation method described here does not suffer from the above disadvantages. It is rapid, reproducible and can be used with either condensed or hydrolyzable tannins. Purified extracts containing only polyphenolic components or crude extracts containing phenolic and nonphenolic components can be analyzed with this technique.

## MATERIALS AND METHODS

**Materials.** Reagent grade chemicals were used throughout. The standard protein solution, 1.0 mg/mL

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907.

0021-8561/78/1426-0809$01.00/0 

of bovine serum albumin (Sigma Fraction V), was prepared in 0.20 M acetate buffer, pH 5.0, containing 0.17 M sodium chloride. The sodium dodecyl sulfate (SDS)–triethanolamine solution contained 1% SDS and 5% (v/v) triethanolamine in distilled water. The ferric chloride reagent contained 0.01 M ferric chloride in 0.01 N hydrochloric acid. All reagents were stable for months.

Solutions of the following proteins (2 mg/mL) were prepared in distilled water: bovine serum albumin (Sigma Fraction V), pancreatic trypsin (Calbiochem), ovalbumin (Sigma), lysozyme (Worthington), pepsin (Difco). A series of buffers with pH values ranging from 1.7 to 12.1 were prepared by mixing 0.01 M trisodium phosphate and 0.01 M phosphoric acid in amounts to produce solutions of the desired pH.

Grain was provided by Dr. John Axtell from crops grown on the Purdue University Agronomy Farm. Grain was ground on a Udy Cyclone mill to pass a 0.4-mm screen.

Partially purified tannins were prepared from methanolic extracts of ground *Sorghum bicolor* (L.) Moench (BR 54, a high tannin variety). The ground grain was defatted by three 1-h extractions with ethyl ether (3 mL of ether/g of ground grain), then extracted three times with methanol (3 mL of methanol/g of ground grain). Extractions were done at 4 °C; the grain was stirred throughout the process. The combined methanolic extracts were mixed with Sephadex LH-20 which had been previously equilibrated with methanol (3 mL of Sephadex LH-20 slurry/mL of extract). The gel was washed with 5 volumes of methanol, and the tannins were then eluted with 50% aqueous acetone (Strumeyer and Malin, 1975). The acetone was removed under reduced pressure on a rotary evaporator at temperatures below 30 °C; the sample was then lyophilized. The fluffy brown powder was stored at 4 °C in a desiccator. It was soluble in water or methanol.

Crude extracts of several varieties of sorghum and cowpeas (*Vigna unguiculata* L.) were prepared from ground samples. Approximately 200 mg of ground grain was extracted in screw-top test tubes rotated on a Lab Quake (Lab Industries). The grain was defatted with 5 mL of ethyl ether for 15 min and the ether extract was discarded. The grain was then extracted for 15 min with 5 mL of methanol. After centrifugation to remove the grain, the extract was assayed for tannin. All assays were done within 8 h of the extraction.

**Analytical Procedures.** The aqueous or methanolic tannin solution (up to 1.0 mL of a 1.0 mg/mL of solution or 1.0 mL of the crude extract) was added to 2.0 mL of the standard protein solution (1.0 mg/mL) in a 15-mL glass centrifuge tube. The solutions were mixed and allowed to stand at room temperature for about 15 min and were then centrifuged for 15 min [IEC Clinical Centrifuge, maximum speed (5000 g)]. The supernatant was discarded, and the surface of the pellet and the walls of the tube were washed with buffer without disturbing the pellet. The precipitate was dissolved in 4 mL of SDS–triethanolamine solution. One milliliter of the ferric chloride reagent was added, and the solutions were mixed immediately. Approximately 15 to 30 min after the addition of the ferric chloride, the absorbance at 510 nm was read on a Zeiss Spectrophotometer (PMQII) (slit width 0.03 mm; path length 1.0 cm; zeroed against air). The average $A_{510}$ of triplicate samples of SDS–triethanolamine solution plus ferric chloride reagent was subtracted from the $A_{510}$ of each sample to correct for background absorbance.

It was experimentally shown that quantitative incorporation of the tannins into the precipitate is assured by using at least twice as much protein as tannin (by weight). Excess protein does not interfere with the assay. The protein solution can contain up to 50% methanol before precipitation of the protein interferes with the assay.

## RESULTS AND DISCUSSION

Ferric chloride reacts with phenolic compounds in solution to form complexes with the general formula $Fe(OR)_6^{-3}$, where $-OR^-$ represents the ionized phenol (Wesp and Brody, 1934). The $\lambda_{max}$ of the complex is dependent upon the nature of the phenol and the solvent; the complex formed between condensed tannins and ferric chloride in alkaline solution is violet ($\lambda_{max}$ 510 nm). Yatzidis (1977) suggested the use of ferric chloride for a protein assay; he found that the amount of tannin in tannin–protein complexes was proportional to the amount of protein in the sample. He used a reagent which contained 0.01 M ferric chloride in 50% triethanolamine. Our experiments with ferric chloride reagents containing from 2.5 to 50% triethanolamine showed that the sensitivity of the reagent increases as the triethanolamine concentration decreases. Our experiments also showed that the stability of the reagent was increased by dissolving the ferric chloride in dilute hydrochloric acid to prevent the formation of iron hydroxide. The triethanolamine, necessary for the maintenance of high pH, was incorporated into the detergent (SDS) solution used to dissolve the tannin–protein complex.

It has been suggested that the tannin–protein complexes might be more stable at low temperatures (Jones and Mangan, 1977). Our experiments showed that there was no measurable difference in the amount of tannin found in the precipitate when sorghum tannins were incubated with the standard protein at 4 °C and at 22 °C. Samples were routinely assayed at room temperature.

The amount of sorghum tannin found in the tannin–protein complex was independent of the ionic strength when the protein was incubated with the tannin in solutions with sodium chloride concentrations ranging from 0.05 to 5.0 M. However, Goldstein and Swain (1965) reported that the inhibition of $\beta$-glucosidase by tannic acid is decreased when the ionic strength is low (phosphate concentration less than 10 mM). These results indicate that the interactions of hydrolyzable tannins (tannic acid) with proteins may be more sensitive to ionic strength than are the interactions of proteins with the condensed tannins present in sorghum.

A plot of absorbance vs. amount of tannin is linear for commercial tannic acid and partially purified sorghum tannins for amounts of tannin ranging from 0.2 to 1.0 mg (Figure 1). The assay is about 50% more sensitive for tannic acid than for sorghum tannins. Although the standard curve for sorghum tannins passes through the origin, for tannic acid the $y$ intercept is not zero. This indicates that there may be a threshold level of hydrolyzable tannins required for protein precipitation.

Figure 2 shows the amount of sorghum tannin found in the tannin–protein complex as a function of pH for several proteins. The isoelectric point (pI) of each protein is indicated. Maximum amounts of tannin were precipitated when the pH of the incubation mixture was within one pH unit of the pI of the protein. It has been reported (Loomis and Battaile, 1966) that the ability of condensed tannins to precipitate protein is essentially independent of pH at pH values less than 8.0, and decreases sharply at pH 8.0. It has been suggested that tannins may not bind proteins at high pH because the phenolic groups of the tannins are ionized and are unavailable for hydrogen bonding (Loomis and Battaile, 1966). However, the results obtained here indicate that the maximum precipitation of trypsin and

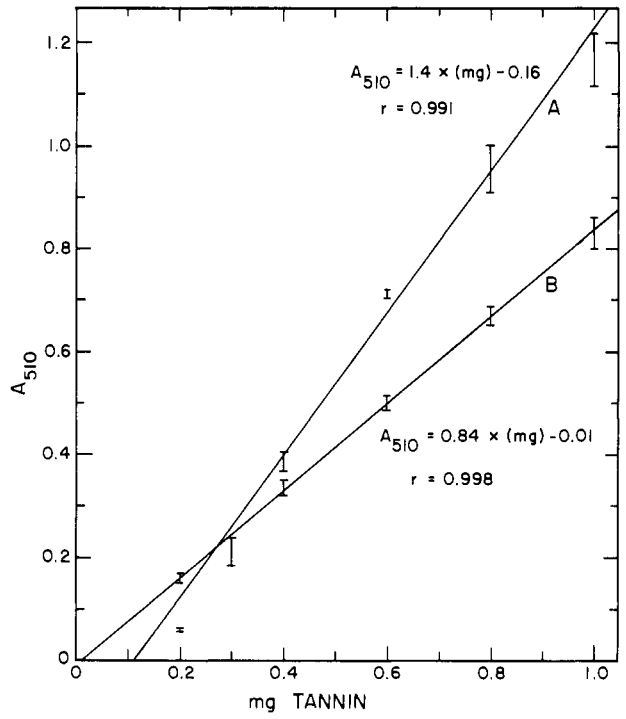


**Figure 1.** Standard curve for tannic acid (A) and partially purified sorghum tannin (B). Assay procedure as described in text. Points are the average values of three or four determinations; bar indicates the range of values. Lines are drawn according to the method of least squares, and $r$ represents the correlation coefficient (Selby and Girling, 1965).

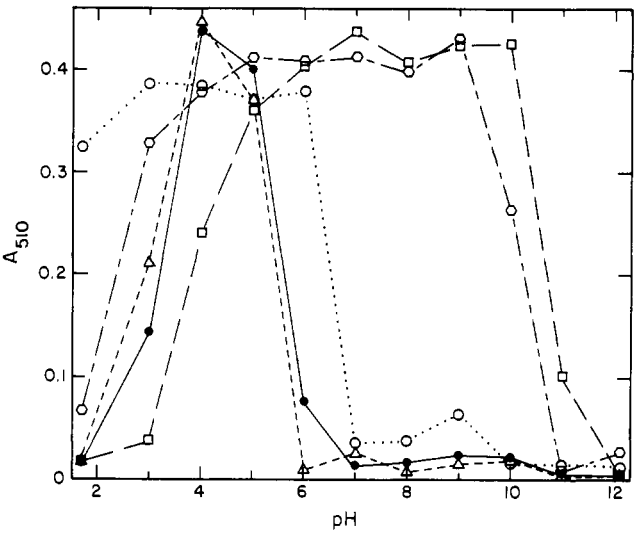


**Figure 2.** Precipitation of several proteins as a function of pH; details are described in text: (●) bovine serum albumin, pI 4.9; (○) pepsin, pI 1.0; (○) trypsin, pI 10.1; (□) lysozyme, pI 11.0; (△) ovalbumin, pI 4.6.

lysozyme occurs at pH values greater than 8.0. Bovine serum albumin and ovalbumin are significantly precipitated by condensed tannins only when the pH of the incubation mixture is between 3.0 and 5.0. It can be concluded that for the proteins tested here precipitation is not entirely dependent on hydrogen bond formation with unionized phenolic groups.

Catechin is a monomeric unit of many condensed tannins, including those of sorghum (Fletcher et al., 1977). Although catechin reacts with ferric chloride in alkaline solution to form a violet complex ($\lambda_{max}$ 510 nm), catechin does not precipitate protein so it is not detected by the precipitation test. However, if 2.0 mg of catechin is added to 1.0 mg of partially purified sorghum tannins, there is an apparent increase of 10% in the amount of tannin detected in the precipitate. The error is presumably due to the nonspecific binding of the catechin to the protein–tannin complex. Such errors may be especially important in the determination of tannin in crude extracts which contain both tannins and low molecular weight phenolics.

**Table I. Comparison of Regular Vanillin and Precipitation Test for Several Varieties of Sorghum and Cowpeas**

| Grain | Vanillin,[a] $A_{495}$/g | Precipitation,[b] $A_{510}$/g |
|---|---|---|
| Sorghum | | |
| RS 610 | 0.116 | 0.232 |
| IS 2319 | 0.420 | 0.243 |
| NK 300 | 1.76 | 2.84 |
| BR 54 | 5.86 | 4.92 |
| Cowpeas | | |
| B 45 | 0.120 | 0.082 |
| B 43 | 0.190 | 0.265 |
| B 53 | 0.322 | 0.467 |
| B 49 | 0.468 | 0.595 |
| B 44 | 2.12 | 1.85 |

[a] Five milliliters of 4% vanillin in methanol containing 4% HCl was added to 0.5 mL of the crude methanolic extract. After 20 min the absorbance at 495 nm was determined. The recorded absorbance values have been corrected for background color as described by Price and Butler (1977). Results are expressed in terms of absorbance units per gram of extracted grain. [b] Assayed as described in text. Results are expressed in terms of absorbance units per gram of extracted grain.

Crude extracts of several strains of sorghum and cowpeas were assayed with the vanillin test (Burns, 1971) and the precipitation test. The results are shown in Table I. The precipitation method agrees qualitatively with the vanillin test. Both tests result in the same order when the grains are ranked according to tannin content. However, a quantitative comparison reveals differences between the two tests. For example, BR 54 contains 50 times as much tannin as RS 610 according to the vanillin test, but only 20 times as much according to the precipitation method. Such differences are to be expected because the two tests detect tannins on the basis of different properties. The vanillin reagent reacts with a specific functional group of the monomers of condensed tannin (Sakar and Howarth, 1976). The precipitation assay is based on the capacity of tannins to interact with protein in a manner that results in precipitation. Degree of polymerization of the phenolics and the presence of low molecular weight phenolics in crude preparations probably affect the methods in different ways, resulting in discrepancies. Work is presently being done in our laboratory (Price and Butler, unpublished results) to improve the reliability and sensitivity of the vanillin assay.

The precipitation test described is convenient and reproducible. The assay may be used to provide new information about the biological activity of tannins and the nutritional value of tannin-containing foods and feeds. The assay, based on the ability of tannins to bind to proteins, provides information which cannot be obtained with chemical assays.

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Received for review December 5, 1977. Accepted March 13, 1978. This work was supported by a National Science Foundation Graduate Fellowship (No. 76-22874) to A.E.H. Paper No. 6989, Agriculture Experiment Station, Purdue University.

# Separation and Quantification of Red Beet Betacyanins and Betaxanthins by High-Performance Liquid Chromatography

Kent R. Vincent* and Robert G. Scholz

A high-pressure liquid chromatographic method has been developed for betacyanin and betaxanthin pigments in red beet extracts and liquid and powdered products. The method employs a reverse-phase microparticular $C_{18}$ column, ion pairing, and a methanol–water mobile phase. Column chromatographic data indicates that configurational isomers of the principal betacyanins, betanin and betanidin, can be resolved through solvent gradient programming. The quantitative method for betanin, using prepared standards, has shown good linearity of response over a concentration range of 0.005 to 0.030%. Concentrations as low as 10–4% w/v are detectable. Recoveries of betanin were typically in the 95–99% range with relative standard deviations of 2% for replicate determinations.

The synthetic food colorants have come under considerable scrutiny lately by regulatory agencies as a result of the well-publicized studies questioning the safety of some of them. Red beet color extracts are currently under development as alternatives to certain of the FD&C red food color dyes for application in food systems. A number of processing modifications including conversion of beet juice to powders and concentrates and fermentation procedures for concentrating the colorants have enhanced the potency and applicability of beet extract as a food colorant (Adams and von Elbe, 1976). To assist in this development program, qualitative and quantitative methods for monitoring dyestuff composition in raw and finished products were sought.

Methods to date for separating and/or quantifying the major pigments or dyestuffs in beets have involved electrophoretic techniques (Nilsson, 1970; von Elbe et al., 1972), mainly for qualitative work, and spectrophotometry at multiple wavelengths, mainly for quantitative work (Nilsson, 1970). The high loading capacities available with large packed polyamide and polyacrylamide gel columns have made them particularly attractive for preparative separation of beet colorants; quantitative analysis with gel columns has been carried out successfully as well (Adams and von Elbe, 1977). High-performance liquid chromatography (LC) was investigated as a means of improving the resolution of beet color components, speed of analysis, and the quality of data presentation over other techniques.

Beatrice Foods Co., Research Center, Chicago, Illinois 60605.

## MATERIALS AND METHODS

**Sample Preparation.** Beet juice was expressed from raw milled beets and filtered and diluted as necessary. Red beet powder and yellow beet powder derived from fermented beet juice (J. H. von Elbe, University of Wisconsin, Madison, Wis.) were dissolved in water. All samples were filtered through a 0.45-$\mu$m MF-Millipore filter (Millipore Corp., Bedford, Mass.) prior to chromatography.

Partially purified crystalline betanin was prepared by the method of Adams and von Elbe (1977) and dissolved in water for quantitative analysis calibration standards.

**Method and Apparatus.** High-pressure liquid chromatography was conducted with two Model M6000 pumps and a Model 660 solvent programmer from Waters Associates (Milford, Mass.), together with a Model 7120 syringe loading sample injector (Rheodyne, Berkeley, Calif.), a Vari-Chrom UV-visible variable wavelength detector (Varian Instruments, Palo Alto, Calif.), and a 100-mV single channel strip recorder (Sargent-Welch Scientific Co., Skokie, Ill.). The bonded reverse-phase column was a $\mu$-Bondapak/$C_{18}$ column (Waters Associates, Milford, Mass.).

Detailed conditions for the qualitative solvent gradient procedure are shown in Table I along with conditions for the quantitative isocratic procedure.

It was discovered that the 90:10 water–methanol mobile phase containing PIC A reagent, premixed and delivered through one pump (Table I), is not strictly comparable to a 90:10 mixture delivered through a mixing chamber from

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