REVIEW

Protein Precipitation Methods for Quantitation of Tannins: A Review

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The methods available for quantitation of tannins can be divided into two classes based on (i) their chemical properties and (ii) their operational properties, i.e., precipitation of proteins. The advantage of protein precipitation methods is that they measure the biological activity of tannins in food and feed. This paper compiles and discusses various methods available for quantitation of tannins based on their property of precipitating proteins.

Tannins are widely distributed in higher plants and occur at high levels in various plant tissues utilized as human food or animal feed. The presence of tannins has been associated with lower nutritive value and lower biological availability of macromolecules like proteins and carbohydrates, amino acids, vitamins, and minerals (Deshpande and Cheryan, 1985; Makkar et al., 1987a). On the other hand, tannins defend plants from herbivores (Swain, 1979), control bloat, and improve protein utilization in ruminants (Broadhurst and Jones, 1978). The transformation of animal hide into leather and making it resistant to putrefaction by tannins has long been recognized.

Various methods have been used for analysis of tannins (Table I). Colorimetric methods are widely used mainly due to their simplicity and high sensitivity. These include Folin–Ciocalteu, Folin–Denis, and Prussian Blue methods for total phenols, vanillin–HCl assay for catechins, and butanol–HCl assay for proanthocyanidins. However, these are not specific and do not distinguish low molecular weight phenols (which generally do not adversely affect the nutritional quality) from polyphenols of nutritional concern (Deshpande et al., 1986). Lately, attention has been focused on quantitation of tannins based on their property of binding/precipitating proteins, as both the ecological and biological roles of tannins are attributed to the complexation of tannins with proteins (Bate-Smith, 1973; Hagerman and Butler, 1978, 1980; McManus et al., 1981; Martin and Martin, 1982; Deshpande et al., 1986; Mole and Waterman, 1987). Moreover, protein precipitation assays of tannins are highly correlated with the biological value of tannin-rich food and feed (Hahn et al., 1984; Deshpande and Cheryan, 1987).

An excellent review is available on the analysis of tannins in food products (Deshpande et al., 1986); however, it does not deal in depth with protein precipitation assays of tannins. In addition, since the publication of the review, many advances have been made in the development of assay procedures for tannins based on their property of binding/precipitating the proteins. Moreover, the literature on this aspect is scattered. Because of the immense importance of these assays, this paper attempts to compile and discuss the methods.

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PROTEIN BINDING/PRECIPITATION METHODS

A number of methods are available for determination of protein precipitation capacity of tannins (Table I). The most common methods used are those of Bate-Smith (1973), Hagerman and Butler (1978), and the AOAC (1965). The method of Bate-Smith (1973) is based on the reaction of tannins with the protein of hemolyzed blood and colorimetric determination of residual hemoglobin. This method requires freshly drawn blood; the commercial preparations of hemoglobin are unsatisfactory (Asquith and Butler, 1985). Briefly, the freshly drawn blood is diluted 50-fold with distilled water. To 1 mL of this hemolyzed blood is added 1 mL of the extract containing the equivalent of 0.3–0.8 mg of tannic acid. The precipitated protein is centrifuged. The color of supernatant is measured at 578 nm and compared with that of the control. The results are expressed as relative astringency, i.e., the ratio of the concentration of the tannic acid to that of the tannin producing the same extent of precipitation. The relationship between absorbance of hemoglobin remaining in the supernatant and tannin concentration is linear over a wide range of tannin concentrations and for several tannins (Schultz et al., 1981). Besides the requirement of freshly drawn blood, which makes it inconvenient for routine use, other limitations of the method are as follows: (i) A number of moieties such as anthocyanidins present in the plant extract interfere with the assay as they absorb at similar wavelengths as hemoglobin (Asquith and Butler, 1985). (ii) It does not give satisfactory results on high-tannin sorghum (Bullard et al., 1981). (iii) Saponins and other plant metabolites also interfere with the assay (Bate-Smith, 1977).

The method of Hagerman and Butler (1978) involves formation of a protein–tannin complex between the tannin-containing solution and the protein, bovine serum albumin (BSA). The complete precipitation of protein by tannins could take 15 min to 24 h depending upon the nature and quantity of tannins (Hagerman and Robbins, 1987; Makkar et al., 1988a). The protein–tannin complex is dissolved in a detergent system consisting of 1% sodium dodecyl sulfate (SDS) and 5% (v/v) triethanolamine in distilled water. The triethanolamine maintains high alkalinity and helps in dissolution of the complex. The tannins/phenolics present in the dissolved complex are measured spectrophotometrically at 510 nm by the addition of ferric chloride (Figure 1). The results are expressed as A_{510nm}/g of the material or relative to tannic acid

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Table I. Some Methods for Determination of Tannins

	tannins measd	remarks	references
		(A) Colorimetric Methods	
Folin–Denis (FD), Folin–Ciocalteu (FC), formaldehyde–HCl, Prussian Blue (PB)	total phenols	nonspecific; reducing compounds interfere in these methods	Singleton and Rossi (1965), Price and Butler (1977), Julkunen-Tiitto (1985), Deshpande et al. (1985)
titanium method	total phenols	like above methods, phenols of varying hydroxylation pattern yield different molar absorptivities	Eskin et al. (1978)
ferric ammonium citrate	total phenols	more specific than FD or HCHO–HCl methods, not very sensitive	Burns (1963), Deshpande et al. (1986)
vanillin–HCl	leucoanthocyanidins, proanthocyanidins	reacts with monomeric as well as polymeric flavonoids, lacks reproducibility, reproducible under strict assay conditions	Broadhurst and Jones (1978), Dalby and Shuman (1978), Butler (1982)
proanthocyanidin assay	proanthocyanidin	like vanillin–HCl method, measure of condensed tannins and not hydrolyzable tannins, also measures non-tannin monomeric flavonoids along with polymeric flavonoids	Bate-Smith (1977)
		(B) Protein Precipitation	
hemoglobin precipitation		requires fresh blood, interference by plant pigments, saponins etc.	Bate-Smith (1973), Schultz et al. (1981)
AOAC		accuracy ±10%, not suitable for sorghum grains, suffers from disadvantages associated with FD, FC, PB methods	AOAC (1965)
β-glucosidase inhibition		relationship between enzyme activity and insoluble complex formation not known, cumbersome expensive	Goldstein and Swain (1965)
immobilized protein method		time-consuming, expensive, difficult to handle large number of samples at a time	Hoff and Singleton (1977)
dye-labeled BSA precipitation		simple but rather insensitive, preferentially forms soluble complexes	Asquith and Butler (1985)
radial diffusion		insensitive to acetone, simple but involves an element of subjectivity	Hagerman (1987)
BSA precipitation		simple method, measures phenolics in the complex, nonspecific binding of phenols to the complex can introduce an error, not suitable for comparing tannins from different sources	Hagerman and Butler (1978)
		indirect method, protein estimations by Lowry or Bradford assays, takes longer time, less sensitive	Martin and Martin (1982, 1983)
		indirect method, protein determination by Kjeldahl method, less sensitive	Amory and Schubert (1987)
		indirect method, protein determination by HPLC, more sensitive compared to above indirect methods	Verzele et al. (1986)
		ninhydrin method used to measure protein in complex after its alkaline hydrolysis, takes only 20 min for the hydrolysis, assay a bit messy because of ninhydrin reagent	Makkar et al. (1987b)
		same as above in the method of Makkar et al. (1987b) except that hydrolysis of the complex is under acidic condition and takes longer (22 h)	Marks et al. (1987)
		measures both tannins and protein	Makkar et al. (1988b)
		highly sensitive, requires microquantity of tannins, allows quantitation of both hydrolyzable and condensed tannins in a plant extract	Dawra et al. (1988)
		(C) Other Methods	
UV spectrophotometry	phenolic compounds	suitability of the method depends on the material to be analyzed, useful for identification of phenols	Deshpande et al. (1986)
gravimetry (using trivalent ytterbium)	total phenols	sensitivity expected to be less than for colorimetric methods	Reed et al. (1985)
gas–liquid chromatography, high-performance liquid chromatography, nuclear magnetic resonance	phenolic compounds	highly specific and sensitive, require special equipment	Julkunen-Tiitto (1985), Torres et al. (1987), Mueller-Harvey et al. (1987), Czochanska et al. (1980), Nonaka et al. (1985)

equivalents. In fact, the values obtained are protein-precipitable phenolics and not the protein precipitation capacity of tannins, as tannins and not the proteins are measured in the method. The data obtained are a function not only of the amount of tannins precipitated but also of their structure. The method therefore is satisfactory for tannins that are comparable among themselves, but not for comparing tannins from different sources, because of different structures of tannins present (Martin and Martin, 1982). In addition, nonspecific binding of phenols of the complex could introduce a large error in the method (Hagerman and Butler, 1978).

Later, Hagerman and Butler (1980) modified their method, which enables measurement of most of the protein in the tannin–protein complex. But this method requires special equipment and some degree of expertise and is hazardous and expensive, because the method is based on radioactive measurements. Tannin-containing extract is added to 2 mL of buffer containing up to 12 mg of iodine-125-labeled BSA. The precipitate is separated by

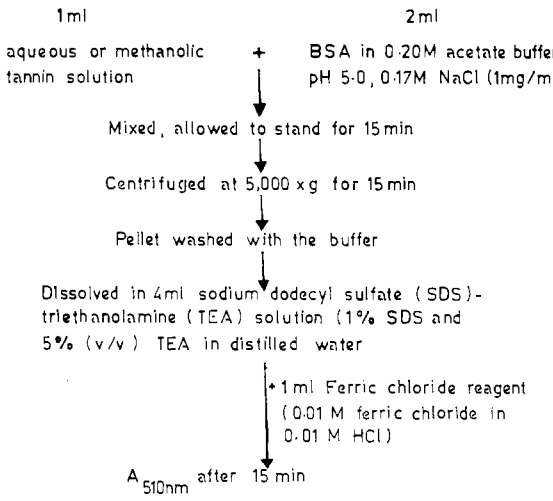

Figure 1. Procedure in brief for determination of protein-precipitable phenolics (Hagerman and Butler, 1978).

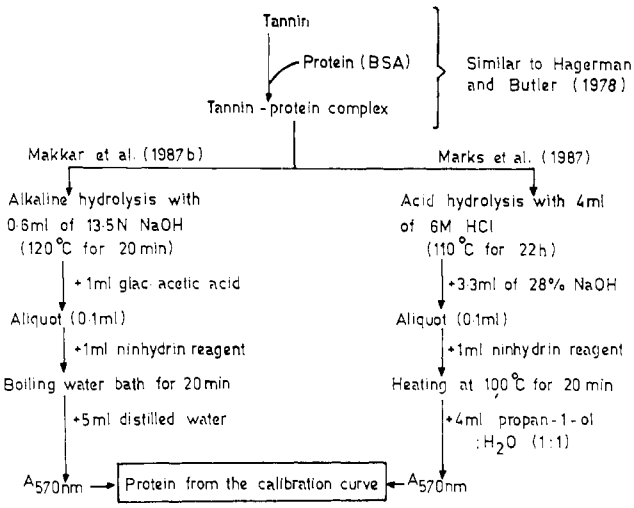

Figure 2. Methods in brief of Makkar et al. (1987b) and Marks et al. (1987).

centrifugation and counted in a γ-counter. The amount of protein precipitated is calculated from a standard curve relating counts per minute to milligrams of BSA.

The official AOAC method (1965) is quite popular among researchers. This method is based on the dual principles of precipitation of tannins by gelatin, hide powder, or kaoline and oxidation of tannins in acid solutions by potassium permanganate in the presence of indigocarmine. The indigocarmine acts as a limiter of oxidation, as it is less oxidizable than tannins and also enables determination of the end point accurately. However, a major limitation of this method is that different phenols at the same concentration do not reduce the same amount of the permanganate. The accuracy of this method has been reported to be ±10% (Deshpande et al., 1986). This method has been found to be of little value for tannin determination of sorghum grains (Maxson and Rooney, 1972). The use of hide powder for precipitation of tannins has been criticized because of its higher affinity with low molecular weight phenols/tannins (Verzele et al., 1986).

In the method of Martin and Martin (1982) the amount of protein in the tannin–protein complex is measured after the protein remaining in the supernatant following addition of tannins is subtracted from the protein concentration of the original solution. They overcame the problem of interference of phenols in the estimation of protein in the supernatant by passing the supernatant containing tannins and proteins from a gel filtration column, thereby separating tannins from proteins. Mainly because of this step, this method is cumbersome and takes more time and only a limited number of samples can be handled at a time. This method is an indirect method and can reduce a large error especially at low levels of tannins, and the method appears to be less sensitive. Moreover, controls to eliminate the effects of interfering materials are laborious (Asquith and Butler, 1985). Amory and Schubert (1987) in their indirect method measured protein in the original solution and unprecipitated proteins in the supernatant by the Kjeldahl method. Although it obviates the use of column for separation of protein from tannins in the supernatant, it suffers from almost all the other disadvantages of Martin and Martin (1982) method. Another indirect method has been put forward by Verzele et al. (1986). In this method high-performance liquid chromatography (HPLC) has been used to measure BSA in the original solution and the supernatant. Because of the use of HPLC, this method is expected to be more sensitive and would take less time compared to those of Martin and

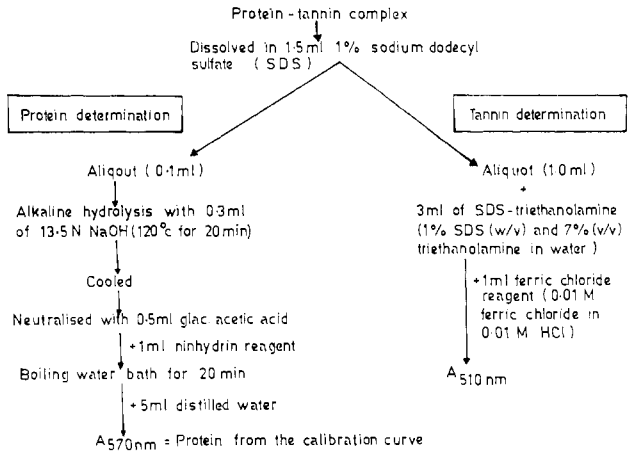

Figure 3. Method in brief for estimation of both protein and tannin in a tannin–protein complex (Makkar et al., 1988b).

Martin (1982) and Amory and Schubert (1987).

Recently, two methods (Marks et al., 1987; Makkar et al., 1987b) based on the same principle appeared almost simultaneously. The protein in the tannin–protein complex is measured by the ninhydrin assay of amino acids released by hydrolysis of the complex. The phenolics do not interfere in the ninhydrin method. In the method of Marks et al. (1987), the complex is subjected to acid hydrolysis whereas that of Makkar et al. (1987b) uses alkali hydrolysis. The procedures in brief are presented in Figure 2. It is evident from this figure that the method of Makkar et al. (1987b) takes much less time. Unlike the methods of Martin and Martin (1982, 1983) these methods do not require prior separation of tannins and proteins.

Subsequently we made simple modifications (Figure 3) of the methods of Hagerman and Butler (1978) and Makkar et al. (1987b), enabling measurements of both tannin and protein in a tannin–protein complex (Makkar et al., 1988b). The ratio of protein to tannins in the complex (specific activity) could also provide valuable information regarding changes in the nature of tannins, vis-à-vis their protein-binding capacity associated with, for example, the process of development and maturation (Makkar et al., 1988a) and postharvest storage of feeds and foods. Besides, it gives insight into the bird- and mold-resistant and -susceptible varieties. One way of obtaining the protein to tannin ratio in the complex is to measure protein in a complex (Makkar et al., 1987b) and tannins (Hagerman and Butler, 1978) in another complex formed under

Table II. Protein Precipitation Capacity of Mature Leaves of *Quercus incana* and *Quercus semecarpifolia*

	mg BSA[a] bound/g dry wt leaves	
	Makkar et al. (1987b)	Dawra et al. (1988)
Quercus incana	79.51	67.3
Quercus semecarpifolia	304.32	273.9

[a] Bovine serum albumin.

identical conditions. This takes more time and labor, requires more chemicals, and extracts of the samples containing tannins. Besides alleviating the above-mentioned problems associated with separate measurements of tannin and protein, a large number of samples can be handled and the variation in the protein to tannin ratio is smaller by the modified method (Makkar et al., 1988b).

The methods mentioned above are unable to estimate protein-binding capacity if the quantity of tannin available is low. We devised another method (Dawra et al., 1988) requiring microquantities of the sample and having much higher sensitivity compared to other available methods. Furthermore, it allows the determination of protein-binding capacity of both hydrolyzable and condensed tannins. In this method, tannins or other phenolics are applied on chromatography paper and reacted with BSA and unbound BSA is washed. The protein in the tannin–protein complex is measured spectrophotometrically after being stained with Ponceau S. Other potential applications of this method could be monitoring of eluants from column chromatography in terms of their protein-binding capacity and determination of protein-binding capacity of tannins separated by paper chromatography without elution. The results obtained by the methods developed in my laboratory (Makkar et al., 1987b; Dawra et al., 1988) were comparable (Table II). The adaptation of this technique on thin-layer chromatography should be attempted as thin-layer chromatography has advantages over paper chromatography. The former technique is quicker and requires less chemicals.

A simple method using blue dye labeled BSA has been developed by Asquith and Butler (1985). The dye, Remazol brilliant blue R, is covalently bound to BSA. This dye was chosen as it reacts with BSA under mild conditions without apparent denaturation, and its maximum absorption is at longer wavelengths than the plant pigments like anthocyanidins, which minimizes interference with the assay. The sample (generally 1.0 mL) containing tannin is added to 4 mL of acetate buffer (0.2 M, pH 4.8) containing about 8 mg of dye-labeled BSA. After centrifugation, the precipitate obtained is dissolved in 3.5 mL of 1% (w/v) sodium dodecyl sulfate–5% (v/v) triethanolamine–20% (v/v) 2-propanol (reagent A), and the absorbance is read at 590 nm. The A_{590nm} is converted to milligrams of BSA with the calibration curve drawn by diluting aliquots of the dye-labeled BSA to 3.5 mL of reagent A. Each new batch of dye-labeled BSA needs standardization. The protein concentration of dye-labeled BSA is determined by the Lowry assay (Peterson, 1983).

Hagerman and Robbins (1987) compared three methods: i.e., radiolabeled (^{125}I) protein precipitation assay (Hagerman and Butler, 1980), Martin and Martin (1982) method, and dye-labeled protein precipitation assay (Asquith and Butler, 1987). Under identical conditions, the methods of Martin and Martin (1982) and ^{125}I-labeled BSA gave comparable results. However, the values obtained by the method employing dye-labeled protein were significantly lower, suggesting that the dye-labeled protein precipitation method is rather insensitive. It has also been observed that the dye-labeled BSA preferentially forms soluble rather than insoluble complexes (Hagerman and Robbins, 1987). It appears that covalent binding of the dye to BSA changes the nature of the protein, thereby affecting its interactions with tannins.

In all the above methods, the first step is the formation of a tannin–protein complex. The solvents used for extraction of tannins can affect the complex formation. It has also been observed that even a small amount of acetone inhibits the formation of the complex. Therefore, acetone must be removed from acetone-containing extracts by evaporation under vacuum before these methods, probably with the exception of the method of Dawra et al. (1988), are employed. Hagerman (1987) described a method insensitive to a number of solvents generally used for preparation of extracts including acetone. In this assay, tannins placed in a well diffuse into the agar gel containing protein and form a disk-shaped tannin–protein precipitate. The area of the ring is linearly related to the amount of tannin in the extract. Although this method is simple and is not affected by solvents used for the extraction, it involves an element of subjectivity in measurement of diameters of rings formed.

Other methods based on the ability of tannins to inhibit β-glucosidase and α-amylase activities (Goldstein and Swain, 1965; Davis and Hoseney, 1979) employing immobilized protein (BSA on Sepharose matrix), which binds specifically tannins, and then estimation of tannins after their release from the column using dimethylformamide (Hoff and Singleton, 1977) have not become popular. These methods are cumbersome, time-consuming, and expensive and do not allow handling of a large number of samples at a time. In addition, in methods employing enzymes the relationship between enzyme activity and insoluble tannin–protein complex formation is not fully known (Hagerman and Butler, 1978).

CONCLUSIONS

Generally the facilities available and the number of samples to be analyzed determine the method used. The literature shows that the most widely used methods for determination of protein precipitation capacity of tannin are those of Hagerman and Butler (1978), Bate-Smith (1973), and the AOAC (1965). However, they suffer one or more disadvantages. These have become popular mainly because they can be carried out with the equipment routinely available in the laboratory. The methods recently put forward (Marks et al., 1987; Makkar et al., 1987b, 1988b; Dawra et al., 1988) have several advantages over the above-mentioned three methods and do not require any sophisticated equipment and, therefore, could well become the methods of preference. The method of Hagerman and Butler (1980) is quite simple and could be the method of choice for those laboratories having accessibility to ^{125}I-labeled BSA and γ-counter to measure it. The indirect methods (Martin and Martin, 1982, 1983; Verzele et al., 1986; Amory and Schubert, 1987) should be avoided when tannin content in the sample is low. At low concentrations the method of Dawra et al. (1988) is advantageous, as it allows determination of protein-binding/precipitation capacity of microquantities of tannins. Protein-binding capacity of tannic acid as low as 5 μg could be determined by this method. Moreover, this is the only method available in the literature by which protein-binding capacity of both hydrolyzable and condensed tannins can be determined in a plant extract. The dye-labeled protein precipitation assay is simple and does not require special equipment or reagents but is less sensitive.

In order to ensure reproducibility within and between laboratories, the conditions and parameters of precipitation must be chosen with care. The extracts are generally prepared in aqueous acetone or methanol. Acetone must be removed before the precipitation methods are used. If it is not possible to remove acetone or other interfering moieties from the extract, the estimations can be done by the radial diffusion method of Hagerman (1987), although it is not possible to use this method to understand mechanisms of tannin–protein complex formation in the homogeneous systems.

The time of complete formation of tannin–protein complex is equally important. Time varying from 15 min to 24 h has been recommended in the literature. This parameter should be standardized in each laboratory, as it depends upon the nature and quantity of tannin under investigation.

Another factor influencing the degree of protein precipitation is the protein content in the assay mixture. To reduce the variability and to have a meaningful comparison of the results from different laboratories, it is suggested to use 2 mg of BSA in the reaction mixture of volume 3 mL. The pH and ionic strength also influence the precipitation reaction, and it is further suggested to use 0.2 M acetate buffer, pH 4.9–5.0, containing 0.17 M NaCl. These are the conditions initially employed by Hagerman and Butler (1978), and subsequently most of these have been used by various workers (Hagerman and Butler, 1980; Martin and Martin, 1982; Asquith and Butler, 1985; Marks et al., 1987; Makkar et al., 1987b, 1988b; Dawra et al., 1988) while devising their methods. The protein precipitation capacity of tannins is also a function of the protein used for the assay. Different proteins are precipitated to different extents (Martin and Martin, 1982). BSA has the advantage of being inexpensive, easily available in most of the laboratories, and soluble.

The recent developments in the methodology of tannin quantitation, enabling determination of both tannin and protein in the tannin–protein complex, would provide a better understanding of the protein–tannin interactions under various conditions and nutritional, physiological, and ecological significance of tannins.

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