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CHOOSING APPROPRIATE METHODS AND STANDARDS FOR ASSAYING TANNIN

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Abstract—Tannins are chemically diverse polyphenolics that have multiple biological activities. Attempts to establish the ecological significance of tannins have been hindered by the complexities of tannin analysis. A multitude of analytical procedures for tannins has been described, but it is difficult for the nonspecialist to select appropriate methods. We have classified the most common procedures for determining tannin as either chemical assays, appropriate for determining the amount and the chemical nature of the tannin in a sample, or as protein-binding assays, suitable for determining the potential biological activity of the tannin in a sample. We have recommended procedures that are particularly reliable and straightforward for general use. We have also considered the problems encountered in selecting appropriate standards for tannin analysis and have recommended standards that are readily available.

Key Words—Tannin, proanthocyanidin, gallotannin, ellagitannin, protein precipitation, Folin assay, phenolic analysis.

INTRODUCTION

Investigations of the ecological or nutritional significance of tannins are often flawed by inappropriate choices of tannin assays and standards, leading to erroneous conclusions (Mole et al., 1989; Wisdom et al., 1987; Mole and Waterman, 1987a,b). A more complete understanding of the chemical structure of tannins and their chemistry will improve the quality of these studies. We present here a brief summary of the major considerations involved in choosing and interpreting methods of tannin analysis.

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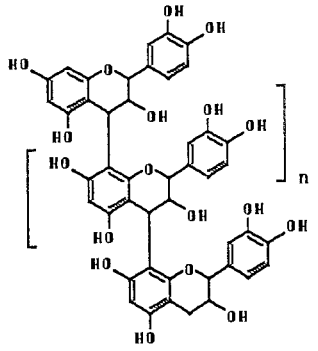

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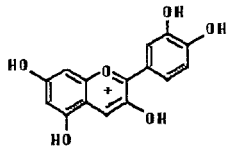

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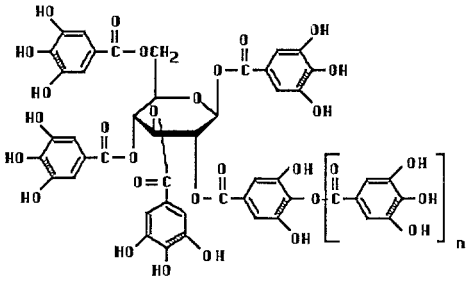

n = 1, 2, 3

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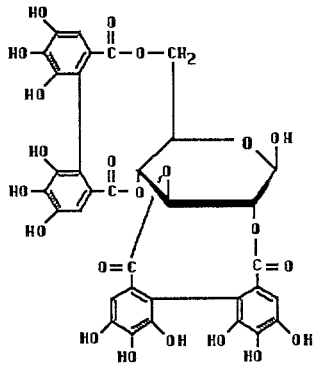
FIG. 1. Representative structures of tannins and tannin subunits: **1** is the condensed tannin (proanthocyanidin) from *Sorghum*, with polymer length of 6–7 (Butler et al., 1982); **2** is cyanidin, the anthocyanidin produced from *Sorghum* condensed tannin during the acid butanol assay; **3** is Chinese gallotannin, from one to three galloyl groups are linked as depsides (phenol–carboxylic acid esters) to the gallic acid ester on carbon 2 of the glucose in this tannin (Haslam, 1981); **4** is a simple ellagitannin, pendunculagin; **5** is gallic acid; **6** is hexahydroxydiphenic acid; and **7** is the lactone ellagic acid, which spontaneously forms from **6.**

Tannins are water-soluble polymers, rich in phenolic groups, capable of binding and/or precipitating water-soluble proteins (Bate-Smith and Swain, 1962). The two major types of tannin are chemically quite different (Figure 1). Condensed tannins (proanthocyanidins) (**1**) are flavonoid polymers, with car-



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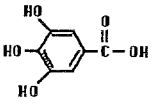

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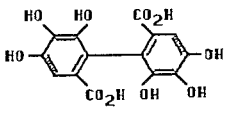

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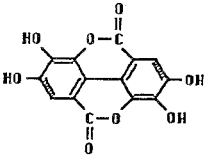

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FIG. 1. Continued

bon–carbon bonds joining the individual flavonoid monomers. Condensed tannins are not susceptible to hydrolysis but can be oxidatively degraded in strong acid to yield anthocyanidins (**2**) (Porter et al., 1986). Hydrolyzable tannins (**3**, **4**) are gallic or hexahydroxydiphenic acid esters of glucose or other polyols (Haslam, 1979). The ester bonds are acid, base, and enzyme labile (Haslam, 1979), and the hydrolyzable tannins are easily broken down to gallic acid (**5**) or hexahydroxydiphenic acid (**6**) subunits and the core polyol. Free hexahydroxydiphenic acid spontaneously forms the internal ester ellagic acid (**7**). A few examples of more complex materials, chemically related to condensed and/or hydrolyzable tannins, have been described but remain poorly characterized (Swain, 1965, 1979). Water-insoluble resins that may bind protein have been

detected (Bryant, 1981), but the structures of these materials have not been elucidated. At the present time, it is not appropriate to classify these water-insoluble materials as tannins.

Bate-Smith surveyed a large number of plants for condensed and hydrolyzable tannins and found that fungi, algae, mosses, and liverworts do not contain tannin (Haslam, 1979). More recently, tannins have been reported in some algae (Nishizawa et al., 1985). The presence of condensed or hydrolyzable tannins has been correlated with the woodiness of the plant (Swain, 1965) or with the phylogenetic rank of the plant (Haslam, 1979; Haddock et al., 1982). Results of other surveys do not suggest any taxonomic or morphological correlation with the presence of tannin (Jung et al., 1979). Conflicting conclusions in taxonomic studies of tannins are due, at least in part, to differences in assay choice and interpretation of results.

Tannins are present in the leaves, wood, flowers, or seeds of plants. Often tannins are found in only a single tissue of a plant; for example, the vegetative tissue of grasses does not contain tannin (Haslam, 1979; Watterson and Butler, 1983), but the seeds of several grasses including *Sorghum* and barley may be tannin-rich (Eggum and Christensen, 1975; Strumeyer and Malin, 1975).

Both condensed and hydrolyzable tannins interact with proteins to form soluble or insoluble complexes (Haslam, 1979). Under mild conditions, the interaction with proteins is based on noncovalent, hydrogen and hydrophobic bonds (Hagerman and Butler, 1978, 1981; McManus et al., 1981). The complexes formed can be dissociated by detergents, which disrupt hydrophobic interactions, or by high pH, which ionizes the phenolic hydroxyl and thus destroys its hydrogen-bonding ability (Hagerman and Butler, 1978, 1981). Both condensed and hydrolyzable tannins are susceptible to oxidation at high pH. The oxidation products can form covalent bonds with nucleophiles including the amino or sulfhydryl groups of proteins (Pierpoint, 1969; Leathan et al., 1980; Beart et al., 1985).

The chemical reactivity and protein-binding capacity of the tannins, their oxidation products, and, in some cases, the nontannin phenolics associated with tannins are responsible for the broad range of biological systems affected by tannins. Ecologists are interested in the role of tannins in diet selection by herbivores (Feeny, 1968; Schultz and Baldwin, 1982; Batzli, 1983; Waterman et al., 1984) and in range management (Provenza and Malechek, 1984; Owen Smith and Cooper, 1987; Robbins et al., 1987). Nutritionists need to know the effect of tannin on feed consumption, rate of weight gain, and efficiency of feed utilization by livestock (Glick and Joslyn, 1970a,b; Jambunathan and Mertz, 1973; Schaffert et al., 1974; Kumar and Singh, 1984; Mitaru et al., 1984). Agronomists investigate the effect of tannins on crop resistance to pathogens,

molds, birds, and preharvest germination (Tipton et al., 1970; Harris and Burns, 1970; Mishra et al., 1980).

In all of these fields, the major question is usually whether a particular biological characteristic is significantly correlated with the amount of tannin present. There are a variety of published methods available for analyzing tannins, and new methods appear in the literature regularly (Wisdom et al., 1987; Armory and Schubert, 1987; Makkar et al., 1987; Inoue and Hagerman, 1988). Choosing an appropriate method may seem overwhelming to the nonspecialist. None of the available methods are universally useful; for example, measurements of total phenolics correlate with biological data in some studies (e.g., McKey et al., 1978), but measurements based on protein precipitation correlate with biological value in other studies (e.g., Robbins et al., 1987). However, understanding the fundamental principles of tannin analysis will enable the investigator to select an appropriate method from a small group of well-established methods. One critical parameter to consider is whether a chemical assay or a protein-binding assay is more appropriate. Chemical assays are particularly useful for determining the amount of tannin in a sample and for elucidating the structure of the tannin. Protein-binding assays are more useful for determining the potential biological activity of tannin in a sample. The analyst must also decide whether a commercial or a noncommercial tannin standard is appropriate. Methods of tissue preservation and tannin extraction have been compared in an earlier paper (Hagerman, 1988).

Specific instructions for performing the methods discussed below, including directions for preparing the reagents and for ensuring that the methods are giving reliable data, are available in IBM-compatible form from A.E. Hagerman.

CHEMICAL ASSAYS FOR TANNIN

There are two types of chemical assays for tannin and other phenolics: General phenolic assays and specific functional group assays. These assays can be used to determine the amount of tannin and the chemical nature of the tannin in a sample. When interpreting data from chemical assays for tannin, it is essential to fully understand the specificity of each assay. Several of these assays have been reviewed (Tempel, 1982; Mole and Waterman, 1987a,b).

Redox Assays. The general phenolic assays measure properties of phenols, such as their redox potential or their ability to complex various metals. Redox assays such as the Folin (Folin and Denis, 1915; Swain and Hillis, 1959) or Prussian blue (Price and Butler, 1977) assays are used to determine total phe-

nolics. It must be recognized that these methods do not discriminate between tannin and nontannin phenolics or between phenolics and other easily oxidized material such as ascorbic acid. The two assays are similar in sensitivity; both require timed addition of several reagents, and both are susceptible to interferences from redox active compounds including ascorbic acid and thiol reagents. The nonprotein imino acid *N*-methyl-*trans*-4-hydroxyl-L-proline gives a large response with the Folin assay (Figliuolo et al., 1987), as do some common purine bases (Ikawa et al., 1988). The Folin assay was modified by Lowry (Pederson, 1979) to enhance its sensitivity to protein; both proteins and phenolics, including tannin, respond in the Lowry assay. Protein does not give a significant response in the Prussian blue assay, although purine bases do interfere (Hagerman and van Pottelsberghe, unpublished). We recommend that the Prussian blue assay be used for general phenol analysis because it is less susceptible to interference from proteins than is the Folin assay.

Metal Complexing. Assays based on the formation of colored phenolic–metal ion complexes are more specific than the redox assays since the color of the complex depends on the pattern of substitution on the phenolic ring (Wesp and Brode, 1934). Although nonphenolics such as protein do not react in these assays, nontannin phenolics respond positively. Ferric chloride reacts with phenolic compounds in alkali to form complexes with the general formula $Fe(OR)_6^{3-}$, where (OR^-) represents the ionized phenol (Wesp and Brode, 1934). Various phenolics, including condensed and hydrolyzable tannins, form violet complexes with ferric ion in solutions containing sodium dodecyl sulfate and triethanolamine (Mole and Waterman, 1987a). This method is appropriate for determining total phenolics in samples containing materials that interfere with the redox methods.

In neutral solution, condensed and hydrolyzable tannins form ferric ion complexes with distinctive colors. Mole and Waterman (1987a) found that condensed tannin yields green complexes with ferric ion, while hydrolyzable tannin yields blue complexes. However, Grove and Pople (1979) point out that the color of phenolic–ferric ion complexes does not provide an unambiguous means of discriminating substitution patterns of various phenolics, so this method must be used with caution. We recommend that the more specific functional group assays described below be used to distinguish condensed from hydrolyzable tannins.

Methods for assaying and for isolating phenols based on the formation of phenol–metal ion complexes have been suggested but are not suitable for general use. A gravimetric method for total phenols based on precipitation of the phenolic by ytterbium has been described (Reed et al., 1985), but it has not been widely adopted. Although it has been suggested that lead selectively pre-

cipitates tannin, other phenols form complexes with lead (Robinson, 1980), and therefore we do not recommend that precipitation by lead be used as a criterion for distinguishing tannin from other phenols.

Functional Group Assays. The functional group assays detect and measure specific molecular structures. With functional group assays, the structural differences between condensed and hydrolyzable tannin are exploited to obtain selective methods of analysis. Although chromatographic methods have been used to detect and differentiate condensed and hydrolyzable tannins (Howard, 1987), the methods described here provide more convincing evidence for the presence of specific types of tannin.

The acid butanol assay is specific for proanthocyanidins (condensed tannins) if the optimized reaction conditions described by Porter et al. (1986) are used. This method is generally the best assay for selective determination of condensed tannins. In this assay, the flavonoid subunits of the condensed tannin polymer are oxidatively cleaved to yield the anthocyanidin; the reaction does not involve hydrolysis, and color development is decreased by water. Because color yields are dependent on the solvent, standards must be dissolved in the solvent to be used for the final analysis. Acid butanol can also be used to detect leucoanthocyanidins (flavan-3,4-diols, flavan-4-ols) by using different reaction conditions (Watterson and Butler, 1983).

The vanillin assay (Price et al., 1978) is specific for flavanols (Sakar and Howarth, 1976) and thus can be used to selectively determine condensed tannin in the presence of hydrolyzable tannin or other phenolics. Widely distributed flavanols such as catechin and epicatechin respond to this assay and could compromise the results unless an independent assay is used for confirmation. Although analogs of vanillin can be used in the assay to increase sensitivity (Putnam and Butler, 1985), the assay as described by Price et al. (1978) is adequately sensitive for most applications. The correction for "blank" color (Price et al., 1978) is essential to this method.

The vanillin and proanthocyanidin assays can be combined to provide a measure of the degree of polymerization of condensed tannin (Butler et al., 1982). This approach to determining the molecular weight of condensed tannin is feasible in situations where methods (Foo et al., 1982) relying on sophisticated spectroscopic techniques are impossible. It is particularly useful for comparing the condensed tannin from materials at different stages of plant maturity (Butler, 1982).

The recently developed rhodanine assay (Inoue and Hagerman, 1988) is specific for gallic acid esters and can be used to determine gallotannin-type hydrolyzable tannins. This assay is not subject to the ambiguities of the iodate assay previously used to determine hydrolyzable tannin (Bate-Smith, 1977; Mole

and Waterman, 1987a), and we recommend that it be adopted instead of the iodate assay. The method is not specific for tannin, since any esterified gallic acid, including low-molecular-weight, nontannin species, will react.

An appropriate assay for ellagitannin-type hydrolyzable tannins has not been devised (Bate-Smith, 1972; Mole and Waterman, 1987a). Ellagic acid can be chromatographically identified (Haddock et al., 1982).

PROTEIN-BINDING ASSAYS

Protein-binding assays can be used either to determine the amount of tannin in a sample or to determine the biological activity of the tannin. To quantitate tannin via protein binding, the amount of tannin precipitated by a standard protein is measured. For biological activity assays, either the amount of protein precipitated by the tannin in a sample is determined, or the nature of the complexes formed between tannin and protein is established. Some authors suggest use of an alkaloid, such as caffeine, instead of protein as the precipitating agent (Wall et al., 1969), but protein binding methods have been better characterized and are preferable. Several protein binding methods have been reviewed by Mole and Waterman (1987b).

Each of the protein-binding assays gives rather different responses with different preparations of tannin. The tendency of tannin to form insoluble complexes with proteins is a complex function of factors including features of the tannin (molecular weight, structure heterogeneity) and the protein (degree of glycosylation, amino acid composition, molecular weight) and the reaction conditions (pH, temperature, reaction time, relative concentrations of reactants). The complexity of the reaction between tannin and protein means that results of protein precipitation assays can be compared directly only to results obtained under virtually identical conditions. In addition, the results of those assays must be interpreted cautiously.

Tannin precipitated by protein can be measured directly in a reaction that depends on the formation of the ferric ion–phenol complex in alkaline, detergent-containing solution (Hagerman and Butler, 1978). This assay is simple to perform, gives excellent results with either condensed or hydrolyzable tannin, and is recommended as a reliable assay for most situations. However, if the procedure is modified so that a large excess of protein is used, soluble tannin–protein complexes form and complicate the data treatment (Hagerman and Robbins, 1987).

The radial diffusion method (Hagerman, 1987) is a very simple assay suitable for determining insoluble tannin–protein complexes. The amount of precipitated complex is proportional to the amount of tannin in the sample. Both

condensed and hydrolyzable tannin can be determined with the method. The radial diffusion assay is particularly suitable if a large number of samples are to be analyzed or if laboratory facilities are limited.

The amount of protein precipitated by tannin-containing samples can be used to estimate the biological activity of the tannin (Robbins et al., 1987). Protein precipitated by tannin can be measured radiochemically (Hagerman and Butler, 1980a) or colorimetrically (Bate-Smith, 1973; Schultz et al., 1981; Martin and Martin, 1983; Asquith and Butler, 1985). Of these methods, the blue BSA method (Asquith and Butler, 1985) is probably the easiest to perform, but it is not very sensitive. If colorimetric methods such as the Lowry assay (Pederson, 1979) or Bradford (Coomassie blue) assay (Martin and Martin, 1983) are used to determine protein in mixtures of tannin and protein, special effort must be made to ensure that all tannin is removed from the mixture before it is assayed, since these assays respond positively to tannin as well as to protein. The radiochemical assay is the most sensitive and least susceptible to interference but requires sophisticated instrumentation and techniques.

The amount of protein precipitated is proportional to the amount of tannin present in the sample if the tannin-to-protein ratio has been set properly to control the formation of soluble tannin–protein complexes (Calderon et al., 1968; Mole and Waterman, 1987b; Hagerman and Robbins, 1987). Excess protein in the reaction mixture decreases the amount of precipitable complex that forms, resulting in an apparent decrease in the amount of tannin present. Methods like the blue BSA (Asquith and Butler, 1985) or hemoglobin precipitation assay (Schultz et al., 1981) can be used to determine the amount of tannin in a sample only under carefully controlled conditions.

The biological activity of tannin is not dependent on the formation of insoluble tannin–protein complexes. Assays that detect both soluble and insoluble complexes are therefore necessary for estimation of biological activity of tannin. Formation of soluble or insoluble tannin–protein complexes can be detected using competitive binding assays (Hagerman and Butler, 1981; Asquith and Butler, 1985), immobilized tannin (Oh et al., 1980; Austin et al., 1988), electrophoresis (Austin et al., 1988), or enzyme inhibition (Goldstein and Swain, 1965). The competitive binding assays are the most generally applicable methods for detecting soluble complexes.

The biological activity of tannin is mediated by the affinity of tannin for proteins; tannin has a high affinity for some proteins and lower affinity for other proteins. Competitive binding methods can be used to determine the affinity of protein for tannin (Hagerman and Butler, 1981; Asquith and Butler, 1985). These assays can be used to determine affinity both in systems in which soluble complexes form and in systems in which insoluble complexes form. The procedure used by Asquith and Butler (1985) is simple and recommended for most

situations. Other methods of determining binding constants include equilibrium dialysis (Barbeau and Kinsella, 1983) or ultrafiltration (Artz et al., 1987); however, it is difficult to completely eliminate possible artifacts arising from interaction between the phenolic material and the dialysis or ultrafiltration membrane. Calorimetry has been used to investigate soluble tannin–protein complexes, but it is applicable only to conditions quite unlike physiological conditions (McManus et al., 1981).

COMBINED ASSAYS

By combining chemical and protein-binding assay data, diverse tannin samples can be compared. For example, tannin-specific activity (Hagerman and Butler, 1980b) has been defined as the amount of protein precipitated by a tannin sample (Hagerman and Butler, 1980a) divided by the total phenol content of that sample (Price and Butler, 1977). That parameter can be used to compare the contamination of tannin preparations, since the tannin-specific activity increases, as nontannin phenolics are removed from the preparation (Hagerman and Butler, 1980b). The tannin-specific activity can also be used to compare preparations from different plants (Mole and Waterman, 1988).

A somewhat different parameter, the tannin–phenol ratio, has been devised to allow comparison of various tannin samples. This ratio is obtained by dividing the amount of protein precipitable phenols (Hagerman and Butler, 1978) by total phenols (Price and Butler, 1977). This ratio has been used (Butler, 1982) to establish the amount of nontannin phenolics in crude plant extracts, but was incorrectly called tannin-specific activity in that paper.

For condensed tannins, the precipitation–size ratio, or amount of protein precipitated (Hagerman and Butler, 1980a) divided by the flavan-3-ol end units (Butler et al., 1982), can be used to determine how activity changes as the molecular weight of the tannin changes (Table 1). It is generally believed that moderate-sized condensed tannin polymers precipitate protein more efficiently than either very small or very large polymers (Haslam, 1979).

TANNIN STANDARDS

It is a practical impossibility to devise a simple extraction procedure that yields chemically pure tannin, so the analyst must devise a way to determine tannin in crude extracts that contain tannin, tannin-complexed proteins, and nontannin phenolics. The crude extracts must be compared with standard tannins in order to make estimates of the tannin content of the samples, expressed in terms of the chosen standard. Selection of a suitable standard is critical if meaningful

TABLE 1. PROTEIN PRECIPITATED PER FLAVANOL END GROUP[a]

Tannin	Chain length	BSA ppt/ flavanol end group[b] (mg/A520)
Pinto bean	2.9	257
Sorghum	4.2	672
Quebracho	5.1	733
Wattle	5.9	553

[a] Calculated from Asquith and Butler (1985).
[b] Flavanol end groups measured as absorbance at 520 nm with the vanillin assay in HOAc.

results are to be obtained from the analyses. Two types of tannin standards can be used: absolute standards and relative standards.

Suitable tannin standards for determining the *absolute* amount of tannin in a given plant could be obtained only by purifying the standard from the plant of interest, a procedure recommended by Wisdom et al. (1987). However, that approach is not simple. Most plants contain several chemically distinct types of tannin, often including both condensed and hydrolyzable tannins (Haslam, 1979). Not only may the ratio of the chemical species change with environmental, physiological, and seasonal changes, but also the extractability of the various molecular forms may change (Hatano et al., 1986; Baldwin et al., 1987; Hagerman, 1988). Adequate characterization of the pure tannin obtained is necessary to ensure that it is truly pure and to identify all the chemical species present. Even in simple systems containing only condensed tannin (e.g., *Sorghum* grain) obtaining pure tannin is laborious (Hagerman and Butler, 1980b). For more complex plant systems, obtaining a representative pure tannin standard for the absolute determination of tannin may well be impossible.

Using commercially available standards, relative amounts of tannin can easily be determined. The assay is standardized with the commercial standard, and the tannin in the plant extracts is then reported in terms of the weight of the selected standard. Nontannin phenolics are sometimes adequate standards. For example, the rhodanine assay and the various total phenol assays can be standardized with reagent-grade gallic acid, and the vanillin assay can be standardized with commercial catechin. The acid butanol assay can be standardized with commercial cyanidin, although that standardization would be most appropriate for procyanidin, not for prodelphinidins or other proanthocyanidins.

Tannic acid is probably the best commercially available tannin standard,

but ideally the commercial material should be purified. A simple purification using Sephadex LH20 (Hagerman and Klucher, 1986) yields an appropriate standard for the rhodanine assay, for any of the redox assays, or for any precipitation assay. Tannic acid cannot be used to calibrate the acid butanol assay. Unpurified commercial preparations of tannic acid contain variable amounts of low-molecular-weight galloyl esters and of nontannin material (Table 2). Each of these preparations is a unique mixture of galloyl esters, and each has a different ability to precipitate protein (Weerasuriya, Wilson and Hagerman, unpublished). If unpurified commercial tannic acid is used as a standard, its source must be indicated clearly because the preparations are so variable in composition.

Although tannic acid is an appropriate standard for analysis of tannins, it may be an inappropriate material to use for in vivo tests of the biological activity of tannin. Recent work (Robbins and Hagerman, unpublished) suggests that tannic acid does not affect nitrogen utilization in ruminants, whereas other hydrolyzable tannins diminish nitrogen utilization. It is not surprising that a material might be appropriate as a chemical standard but quite inappropriate for bioassay.

Sorghum tannin is an appropriate standard condensed tannin, but it is not available commercially and is rather tedious to purify (Hagerman and Butler, 1980b). Quebracho tannin is the only readily available condensed tannin. It must be purified (Asquith and Butler, 1985) because it contains large amounts of nontannin materials. There are probably substantial differences among the materials provided by various suppliers and even among various lots from a single supplier. Since a single commercial standard is not available, we suggest that all workers start to use purified quebracho standard prepared from a single

TABLE 2. GALLIC ACID IN VARIOUS PREPARATIONS OF TANNIC ACID[a]

Tannic acid source	Total GA (mg/mg TA)	Ester GA (mg/mg TA)
Sigma (pfs)[b]	0.633	0.546
MCB reagent	0.689	0.598
Mallinckrodt technical[c]	0.691	0.610
Mallinckrodt reagent	0.449	0.413
Mallinckrodt purified[d]	0.809	0.766

[a] Total gallic acid and free (unesterified) gallic acid were determined with the rhodanine assay (Inoue and Hagerman, 1988) in triplicate for each sample of tannic acid, and esterified gallic acid was calculated by difference. TA = tannic acid, GA = gallic acid.
[b] From Ken Albrecht, University of Wisconsin.
[c] From Charles Robbins, Washington State University.
[d] By the method of Hagerman and Klucher (1986) (fraction 3).

lot of the crude commercial mixture. A.E. Hagerman will provide the crude material and instructions (Asquith and Butler, 1985) for preparation of the purified standard to any interested laboratories. Use of a single standard should significantly reduce confusion about analysis of condensed tannin.

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