Chapter 12

Methods for Determination of Condensed and Hydrolyzable Tannins

Ann E. Hagerman, Yan Zhao, and Sarah Johnson

Department of Chemistry, Miami University, Oxford OH 45056

Tannins are natural products which are characterized by their phenolic nature and their ability to precipitate protein. Their diverse biological effects, and their common occurrence in plants used to make foods, beverages, herbal medicines, and animal feeds, have created widespread interest in methods for the analysis of tannins. However, their chemical complexity and diversity impose constraints upon the application of many of the analytical methods that have been developed. After briefly reviewing the chemistry of tannins, we describe some of the methods available for analysis of tannins, and outline the limitations of those methods. The discussion of each method emphasizes the utility of the method for analysis of mixtures of tannin, like those found in many foods. A procedure for preparative scale purification of a standard gallotannin, pentagalloyl glucose, is included. A new method for simultaneous determination of condensed and hydrolyzable tannins is summarized.

Distribution and Roles of Tannins

The natural products known as tannins are found in many plants, including some used as foods, herbal medicines or in the production of beverages (*1*,*2*,*3*,*4*,*5*). Many plants consumed by wild or domestic animals contain tannin (*6*,*7*,*8*). The taxonomic distribution of tannins in certain plant families has been surveyed (*9*,*10*,*11*,*12*). Tannins are common in the roots, flowers, leaves and wood of various Gymnosperms and Dicots. Although at one time tannins were not thought to be found in the vegetative tissues of Monocots (*13*), recent reports have demonstrated their presence in leaves of grasses (*14*). Up to 2% by weight of the cereal grains sorghum and barley may be tannin (*15*). Marine brown algae contain compounds known as phlorotannins, which have reactivities similar to those of the tannins of higher plants (*16*). The widespread occurrence of tannins, and their

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pronounced biological effects, have led some investigators to postulate that they serve to protect plants against herbivores and pathogens (*17,18,19*).

Tannins contribute to the taste of foods and beverages by providing astringency, a sensation of dryness that is especially pronounced in some red wine, tea, and unripe fruit. The sensation apparently results from the interaction between tannins and proteins of the saliva and the mucous tissues of the mouth (*20,21*). Beyond taste, consequences of tannin consumption often include apparent depression in protein utilization (*17,22,23*). A variety of other effects have been reported in some experimental systems (*24,25,26*). Variability in the reported effects of tannins can be attributed in part to the variety of sources and types of tannin used in feeding experiments and the variable responses of different experimental animals (*27,28,29,30,31,32*). Amount of protein in a diet, or steps in diet preparation such as heating (*33*) influence the effects of tannin.

Tannins may contribute to the therapeutic effects of certain herbal medicines (*2,3*). *In vitro* effects of some tannins on some microbes have been noted (*34,35,36*). Consumption of certain tannin-rich beverages, including red wine and tea, has been associated with reduced incidence of heart disease or cancer. Like some smaller phenolics, tannins may serve as dietary antioxidants, and may thus have beneficial effects (*37*).

The common occurrence of tannins in foods, beverages and feeds, and the diverse biological effects of ingested tannin, make qualitative and quantitative analysis of tannins important. The structural chemistry of the tannins, and widely used methods for their analysis, are reviewed here. Before attempting analysis of tannins, it is necessary to develop appropriate techniques for tissue collection, preservation and extraction (*38,39,40,41,42,43*).

Chemistry of Tannins

The term "tannin" was historically used to describe a chemically heterogeneous group of compounds which precipitate proteins. The name derives from the tradition of "tanning" animal hides with infusions of oak or chestnut bark to make leather (*44*). The term tannin was somewhat more rigorously defined by Bate-Smith in 1962 when he stated that tannins are "water-soluble phenolic compounds having molecular weights between 500 and 3,000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins" (*45*). Modern methods of separation chemistry and spectroscopic structure determination have advanced our knowledge of tannins substantially. It is now realized that several groups of compounds have in common the general properties of tannins as defined by Bate-Smith, but that these groups are quite distinct from one another in their detailed structural chemistry (*44,46*).

Proanthocyanidins. The condensed tannins, or proanthocyanidins (*12,44,47,48*), are flavanol-based compounds. Condensed tannins react in alcoholic solutions of strong mineral acid to release the corresponding anthocyanidin, which has a

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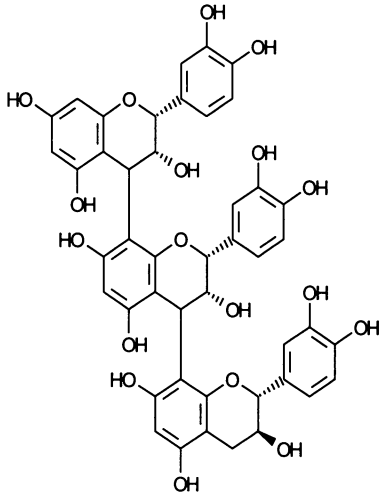

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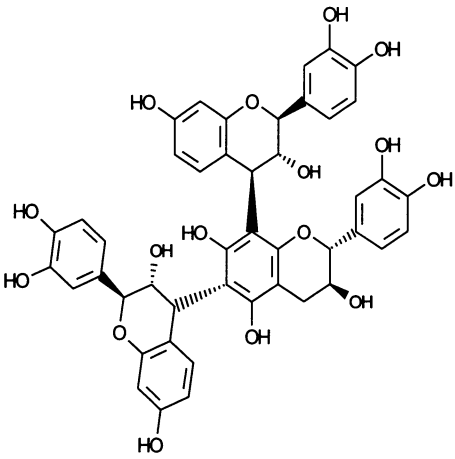

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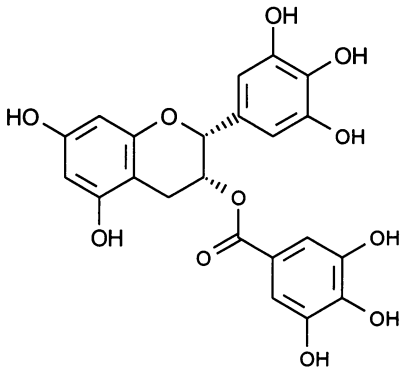
characteristic color. Structural diversity in the proanthocyanidins is a consequence of the substitution patterns and stereochemistry of the flavanol subunits. Structural complexity also results from the diversity of positions for interflavan bond formation, and from the stereochemical variation in the interflavan bond. The procyanidin (**1**) found in sorghum grain (*49*) is representative of the simplest condensed tannins. It is a linear (4→8) polymer of epicatechin with catechin terminal units. The only commercially available condensed tannin is the profisetinidin (**2**) found in preparations of Quebracho tannin. Quebracho tannin, a crude extract of the bark of *Schinopsis spp.*, contains a complex mixture of phenolics including a branched chain condensed tannin comprised of (4→6)- and (4→8)-linked monomers of the 5-deoxyflavan-3-ol fisetinidol (*50*).



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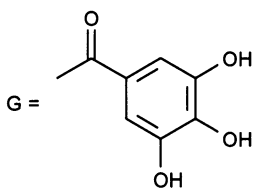
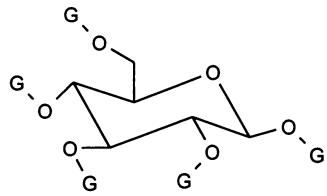
The galloylated catechins are related to the condensed tannins. In these compounds, the 3-hydroxyl group of the flavanol subunit is esterified by gallic acid. Addition of this galloyl group significantly alters reactivity; for example, epigallocatechin does not precipitate protein, but epigallocatechin gallate (**3**) is the principle astringent component of green tea (*51*). Higher molecular weight galloylated catechins have not been characterized.

Hydrolyzable Tannins. The hydrolyzable tannins are comprised of phenolic acids, such as gallic acid or hexahydroxydiphenic acid (HHDP), esterified to a polyol such as glucose. The simple gallotannins, comprised only of galloyl esters of glucose or quinic acid, are relatively rare in nature but are the principal constituents of commercial tannic acid (*52*,*53*). In the simple gallotannins, the core (e.g. pentagalloyl glucose, **4**) has been further elaborated by addition of galloyl groups



gallic acid



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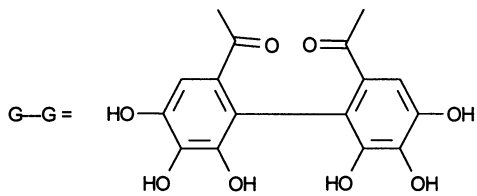
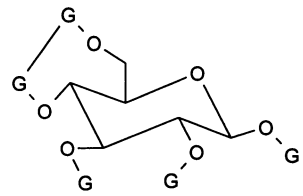
as "depsides", or esters of phenolic hydroxyls, yielding polyesters containing up to 12 galloyl groups. The ellagitannins such as tellimagrandin II (**5**) are biosynthetically derived from the gallotannins by oxidative coupling of adjacent galloyl residues to yield HHDP esters (*54*). *In vitro* hydrolysis of the ellagitannins



hexahydroxydiphenic acid



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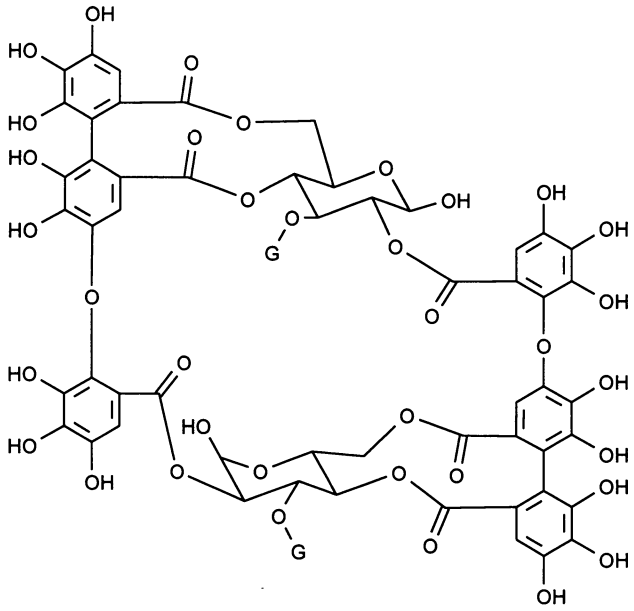
releases HHDP, which spontaneously lactonizes to yield ellagic acid. Structural diversity in the gallotannins and ellagitannins is a consequence of various degrees and patterns of esterification, and of various patterns of oxidative coupling (*44*,*54*).

Oligomeric hydrolyzable tannins such as oenothein B (**6**) were unknown before 1982 but may be the most common type of hydrolyzable tannin (*55*). The oligomeric hydrolyzable tannins have at least two ester subunits comprised of polyols esterified with HHDP and/or gallic acid. The ester subunits are linked by

oxidative coupling between a phenolic hydroxyl group from one subunit and an aromatic ring carbon from another subunit. The order of linkage of the units, and the stereochemistry of the crosslinking bond contribute to significant structural diversity within this class of tannins.



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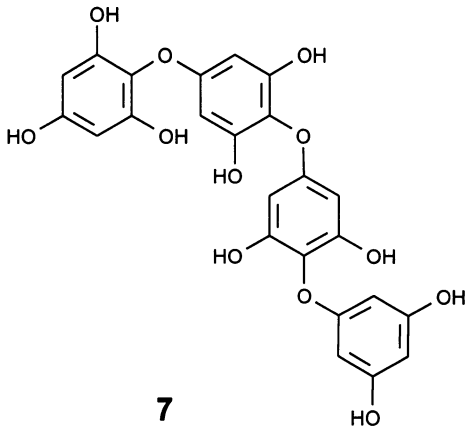
Phlorotannins. A final class of compounds which have typical characteristics of tannins are the phlorotannins. These compounds, comprised of aryl-aryl or ether-linked phloroglucinol subunits (e.g. **7**) , are found only in marine brown algae. Like the more familiar terrestrial tannins, they precipitate proteins, and may contribute to both the taste and nutritional quality of brown algae for marine herbivores. Details of their chemistry, analysis and reactions with protein (*16,56*) will not be discussed further here.



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Nontannin phenolics. In addition to tannins, plants contain a wide variety of nontannin phenolics. These phenolics, which are usually relatively low molecular weight (<500), are distinguished from the tannins by their inability to precipitate proteins. Dietary nontannin phenolics have different metabolic fates from tannins because of their different reactivity.

Analytical Approaches

Numerous methods for quantitative analysis of tannins have been described (*43*,*46*,*57*,*58*,*59*). In planning an analysis, it is important to realize that each type of tannin responds differently in each of these assays, consistent with the diversity of structural chemistry of the tannins. This variability in response makes it impossible to use any single method to accurately assesses the "tannin" content of a complex mixture. Instead, several methods must be employed to obtain a qualitative and quantitative picture of the tannins present in the mixture. Complex mixtures are of particular importance since typical higher plants, including those used as foods, beverages and herbal medicines, contain one or more types of tannins and a variety of nontannin phenolics. For example, strawberries (*Fragaria ananassa*) contain both condensed and hydrolyzable tannins (*5*).

In general, the methods used for tannin analysis are based on either general phenolic reactions; protein precipitation; functional group reactions; or HPLC. Several of the most widely used methods in analysis of tannins are discussed below, with emphasis on the utility of the methods for analyzing tannins in complex mixtures. For each method of analysis, proper standards must be selected to allow meaningful interpretation of the data (*59*).

Total Phenolics Methods. The Prussian blue method (*60*) or the Folin-Denis method (*61*) are used to measure both tannin and nontannin phenolics in plant extracts. In both methods, the phenolic analyte is oxidized and the reagent is reduced to form a chromophore. Easily oxidized nonphenolics such as ascorbic acid interfere with the methods. The Folin method is plagued by formation of precipitates which interfere with spectrophotometric determination, and many variations on the original method have been developed in attempts to minimize this problem (*43*). Interferences are rare with the Prussian blue method, and a recent modification to diminish time dependence of color formation simplifies the method (*62*).

Since the pattern of substitution affects the redox chemistry of phenolics (*60*), significant differences in response in these total phenolic methods must be expected for different tannins. For example, with the Prussian blue assay, the color yield for epicatechin is only 70% of the color yield for gallic acid. Furthermore, the redox methods provide neither a means to distinguish tannins (protein precipitating phenolics) from nontannin phenolics, nor a means to identify specific types of tannins in a mixture.

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Methods based on formation of colored phenolic-metal ion complexes are also useful for measuring total phenolics. Several methods dependent on formation of the ferric-phenolate complex at high pH have been described (*63*,*64*). These methods are useful for any phenolic containing *ortho*-substituted phenolics, and interference from nonphenolics is unlikely. Nontannin phenolics cannot be distinguished from tannin with this method, and specific types of tannin cannot be identified. It has been suggested that condensed tannin can be distinguished from hydrolyzable tannin by formation of different colored ferric ion complexes in neutral solution (*64*) but that idea has not been thoroughly evaluated, and must be used with caution (*65*).

Precipitation Methods. The defining reaction of tannins is their ability to precipitate protein (*66*,*67*), and many methods for determining tannin have been developed based on reactions with protein (*59*,*68*). Among the simplest of these methods is one in which the phenolic is determined after precipitation by a standard protein. The precipitated phenolic is redissolved in basic solution, and reacted with ferric ion to form a colored complex (*63*). This method discriminates tannin from nontannin phenolics, but does not allow selective determination of various types of tannin.

Several methods for determining the amount of protein precipitated by tannins have been described. Since tannin and protein are very similar in their reactivity to many reagents, it is essential to either use protein that is labeled in some fashion or to isolate the protein after precipitation. Proteins can be labeled with a radioisotope (*69*) or with a chromophore (*70*,*71*). Separation achieved chromatographically (*72*) or with a membrane (*73*) can be followed by conventional analysis of the protein. None of these methods allow selective determination of various types of tannin.

All precipitation methods are sensitive to reaction conditions. For example, the amount of protein precipitated by a given amount of tannin may depend on the pH (*63*), the presence of organic solvent (*74*), the ionic strength (*73*), and the time allowed for precipitation (*74*). The ratio of tannin to protein influences the precipitation reaction, with soluble complexes forming preferentially when protein is in excess and precipitable complexes forming when tannin is in excess (*74*). The interaction between tannin and protein is quite specific (*75*,*76*), so that structural variations in either tannin or in protein may alter the stoichiometry and solubility of the complex.

Although the ability of tannins to precipitate proteins is widely recognized, under some conditions soluble complexes between tannin and protein may form. Attempts to analyze soluble complexes electrophoretically (*28*) are limited by their qualitative nature. More success has been obtained using competitive binding assays (*75*).

Functional Group Methods. These methods depend on the specific chemical reactivity of the functional group characteristic of a given type of tannin. As a

result, these methods are often very specific and provide both qualitative and quantitative information on the tannins found in a plant sample.

Condensed Tannins. The vanillin assay (*77*) is a method for determining proanthocyanidins. Aromatic aldehydes such as vanillin react with *meta*-substituted phenolics to yield highly conjugated colored products. The method has been used to determine condensed tannin because many proanthocyanidins have an appropriate *meta*-substitution on the "A" ring (**1**) and thus react with vanillin. However, interpretation of the results is complicated by the fact that vanillin reacts with nontannin flavanols such as catechin, but does not react with proanthocyanidins based on 5-deoxyflavanols, such as the profisetinidin in Quebracho tannin (**2**). Additional problems stemming from the complex kinetics of the reaction with vanillin, and from the sensitivity of the reaction to trace amounts of water, limit the utility of the method.

The acid butanol method is a simple, specific method for determining proanthocyanidins (*78*). The method depends on the oxidative cleavage of the interflavan bond in alcoholic solutions of hot mineral acid to yield the colored anthocyanidin. Reaction conditions must be carefully controlled; for example, traces of iron catalyze the reaction while water inhibits the reaction (*78*). The response is dependent on the structure of the tannin, with profisetinidins yielding less color because of the increased stability of the (4→6) interflavan bond typical of 5-deoxyflavanols (*58*). A method based on similar chemistry but utilizing sulfuric acid instead of HCl/butanol (*79*) is apparently less sensitive to the presence of water.

The acid butanol method is satisfactory for selectively determining condensed tannins in the presence of hydrolyzable tannins. Most nontannin phenolics do not interfere with the method, although anthocyanidins and other pigments can interfere. A modification of the acid butanol method for determining proanthocyanidins in the presence of chlorophyll has been described (*80*). That method can also be used to determine the unusual 3-deoxy proanthocyanidins (*12*) and the nontannin leucoanthocyanidins (flavan-3,4-diols and flavan-4-ols).

None of these methods provide unambiguous information on the composition or size of the condensed tannins. Although NMR and mass spectroscopy are powerful tools for analysis of proanthocyanidins (*46,81*), the spectra of polymeric condensed tannins are difficult to interpret. Chemical methods for evaluating composition and estimating molecular weight are more accessible. The degree of polymerization of procyanidins can be estimated by comparing the results from the vanillin/glacial acetic acid assay (in which only terminal units react to form chromophore) with the results from the acid butanol assay (in which only extender units react to form chromophore) (*82,83*). The method can only be used to compare chemically similar tannins, since reactivity in the acid butanol assay is a function of the reactivity of the interflavan bond. Degree of polymerization can also be determined by gel permeation chromatography of acetylated condensed tannins (*84*). Proanthocyanidins can be oxidatively cleaved in the presence of a nucleophile such as phloroglucinol to yield the underivitized terminal flavanol and the phloroglucinol

adducts of the extender flavanols. These products can be separated and quantitated by HPLC to yield information on the composition and degree of polymerization of the parent tannin (*85*).

Hydrolyzable Tannins. The iodate assay (*86*) relies on the reaction of potassium iodate with gallate esters to yield a chromophore. The chemistry of the color-producing reaction is poorly understood. The method cannot be employed with samples containing complex mixtures of tannins, because samples turn brown instead of the characteristic pink (*87*). A method for determining intact ellagitannins relies on color changes in the presence of nitrous acid (*88*).

The rhodanine assay and nitrous acid assays were devised to allow estimation of gallotannins or ellagitannins in mixtures (*89*,*90*). The basis for each assay is selective determination of the phenolic acid released by hydrolysis of the parent hydrolyzable tannin. Successful hydrolysis is critical to the methods. The acid hydrolysis must be carried out in the absence of oxygen, since gallic acid and HHDP are sensitive to oxygen. Following hydrolysis, gallic acid can be reacted with rhodanine to form a chromophore. Ellagic acid can be isolated using pyridine and then reacted with nitrous acid to form a chromophore. Each of the methods is very selective, allowing determination of the esterified forms of the specific acid of interest. Unfortunately, sample hydrolysis is difficult and inconvenient for large numbers of samples. In addition, the results do not provide information on the degree of esterification or structure of the parent tannin. Degree of esterification can be a critical determinant of biological activity of hydrolyzable tannins (*29*).

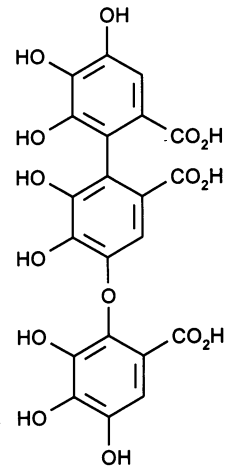
The degree of esterification of an unknown mixture of gallotannins can be determined using either normal phase (*29*,*52*) or reversed phase HPLC (*91*). In each of these chromatographic systems, the log of the retention time is a linear function of the degree of esterification. The methods can be standardized using commercially available methyl gallate and pentagalloyl glucose.

Pentagalloyl glucose is prepared from commerical tannic acid using a method modified from Yoshizawa *et al.* (*92*). Tannic acid from *Rhus semialata* galls ("Chinese" tannin) is a suitable starting material since it is comprised of higher esters of pentagalloyl glucose (*54*). However, "Turkish" or "Aleppo" tannins, from *Quercus infectoria* galls, are based on a tetragalloyl glucose core and do not yield pentagalloyl glucose (*52*,*53*). The tannic acid (0.5 g) is dissolved in 10 mL of 70% methanol/30% acetate buffer (0.1 M acetate, pH 5.0) and is incubated at 65°C for 15 h. The solution is then stirred while adjusting the pH to 6.0 with 0.25 M NaOH, and is evaporated under reduced pressure while maintaining the temperature at <30°C. As the methanol is removed, water is added to maintain the volume. The aqueous solution is extracted three times with diethyl ether, and is then extracted three times with ethyl acetate. The ethyl acetate fractions are combined and evaporated under reduced pressure. Water is added to maintain the volume as the ethyl acetate is removed. When all the organic solvent has been removed, the aqueous suspension is freeze dried to yield pure pentagalloyl glucose. Proton NMR is used to confirm the identity of the purified material [from TMS: Glucose C-1,

6.3 ppm (d, 1H); glucose C-2, C-4, 5.6 ppm (q, 2H); glucose C-3, 6.0 ppm (t, 1H); glucose C-5, 4.5 ppm (d, 1H); glucose C-6, 4.4 ppm (dd, 1H); galloyl group, between 6.9-7.2 ppm, 5 singlets (2H)].

Many of the smaller ellagitannins are easily crystallized to yield pure compounds which can be identified by NMR and MS (*57*). Methods for HPLC of ellagitannins have been described (*93*,*94*,*95*) but are not useful for identification of unknowns.

None of the functional group methods are satisfactory for the oligomeric hydrolyzable tannins. Although gallic acid and ellagic acid may be released from oligomeric hydrolyzable tannins, other complex phenolic acids are major products of these compounds. For example, valoneic acid (**8**), a hydrolysis product of oenothein B (**6**), is unreactive in all these functional group assays. The oligomeric hydrolyzable tannins can be separated using the HPLC methods described for gallotannins and ellagitannins, but the results are not useful for identification of unknowns. NMR and MS can be used to establish structures of purified complex tannins (*57*).



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Evaluation of Mixtures of Tannins. It is possible to use the array of chemical and spectroscopic methods described here to qualitatively and quantitatively describe the tannins in an extract. In many instances, assessment of the gross composition of the tannin is of more importance than detailed chemical evaluation. The acid butanol method provides a quick, reliable method for determining proanthocyanidins in extracts. Because it is more difficult to perform the functional group assays necessary for hydrolyzable tannins, this group of tannins is often neglected (*96*).

In an effort to simplify screening of samples for hydrolyzable tannins, we have developed a new method for rapid assessment of crude tannin extracts. The extracts are analyzed with the radial diffusion assay (*97*), a simple protein precipitation method in which the plant extract is allowed to diffuse through a protein-containing gel. Tannins in the extract precipitate the protein in the gel to form a visible ring. The diameter of the ring is proportional to the amount of tannin in the extract. This method as originally developed is not useful for discriminating condensed tannins from hydrolyzable tannins, since both types of tannin form rings of precipitate.

Hydrolyzable tannins, like other esters, are susceptible to cleavage under mild conditions with hydroxylamine hydrochloride (*98*). Upon reaction, the hydrolyzable tannins yield the core polyol and the hydroxamic acid of the phenolic residues. Simple gallotannins are completely decomposed by reaction with hydroxylamine hydrochloride under mild conditions (pH 5.5, 70°C, 48h) while proanthocyanidins are unchanged (*99*).

To screen samples for the presence of hydrolyzable tannins, the radial diffusion method is performed twice for each extract. One sample of the extract is assayed directly, and a second sample of the extract is assayed after treatment with hydroxylamine hydrochloride. The precipitation ring obtained with the first sample represents all of the tannin in the extract. If a smaller precipitation ring is obtained with the hydroxylamine-treated extract, it suggests that the extract contained some hydrolyzable tannins which were destroyed by the hydroxylamine hydrochloride. If the size of the ring is not changed by the hydroxylamine hydrochloride treatment, the sample did not contain hydrolyzable tannins.

Although this method is not a substitute for complete chemical characterization of tannins, it does provide a simple method for screening for hydrolyzable tannins. Availability of such a simple method should ensure that this important group of tannins receives more attention in future studies.

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