Tannin Chemistry

This site contains information on the plant secondary metabolites known as tannins. The Hagerman laboratory methods originally in the "Tannin Handbook" are now available through this site, linked to information about tannins. Hyperlinks are indicated by blue text. Turn on Acrobat bookmarks to see titles of the pages.

- Condensed Tannin Structural Chemistry
- Hydrolyzable Tannin Structural Chemistry
- Purification and Identification
- Quantitative Analysis
- Biological Activities
- Biosynthesis

The "Tannin Handbook" can no longer be obtained in hard copy. Instead, print the pages you need from this site.

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Hagerman vita

Hagerman publication list

WHAT IS A TANNIN?

Plants accumulate a wide variety of "secondary" compounds, including alkaloids, terpenes and phenolics. Although these compounds apparently do not function in "primary" metabolism such as biosynthesis, biodegradation and other energy conversions of intermediary metabolism, they do have diverse biological activities ranging from toxicity to hormonal mimicry, and may play a role in protecting plants from herbivory and disease.

Phenolic metabolism in plants is complex, and yields a wide array of compounds ranging from the familiar flower pigments (anthocyanidins) to the complex phenolics of the plant cell wall (lignin). However, the group of phenolic compounds known as tannins is clearly distinguished from other plant secondary phenolics in their chemical reactivities & biological activities.

Tradition use of tannins as agents for converting animal hides to leather ("tanning") is one manifestation of the most obvious activity of the tannins: their ability to interact with and precipitate proteins, including the proteins found in animal skin. The term "tannin" comes from the ancient Celtic word for oak, a typical source for tannins for leather making.

Bate-Smith defined tannins as "water-soluble phenolic compounds having molecular weights between 500 and 3000...[giving] the usual phenolic reactions...[and having] special properties such as the ability to precipiate alkaloids, gelatin and other proteins".

Haslam has more recently substituted the term "polyphenol" for "tannin", in an attempt to emphasize the multiplicity of phenolic groups characteristic of these compounds. He notes that molecular weights as high as 20,000 have been reported, and that tannins complex not only with proteins and alkaloids but also with certain polysaccharides. I prefer to use the term tannin, which emphasizes the character which sets tannins apart from all other phenolics: the ability to precipitate proteins.

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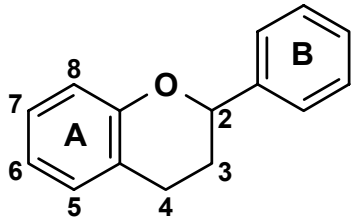
CONDENSED TANNIN STRUCTURAL CHEMISTRY

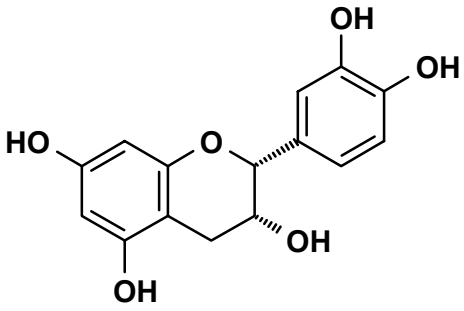
Ann E. Hagerman

Proanthocyanidins (condensed tannins) are polymeric flavanoids. The flavanoids are a diverse group of metabolites based on a heterocyclic ring system derived from phenylalanine (B) and polyketide biosynthesis (A). Although the biosynthetic pathways for flavanoid synthesis are

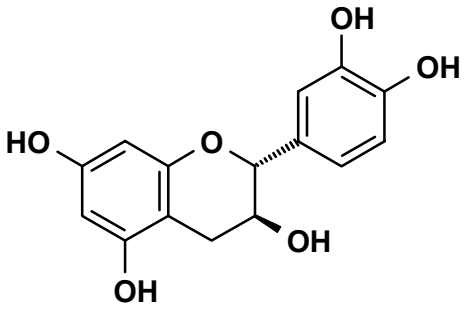


well understood, the steps leading to condensation and polymerization have not been elucidated. The flavanoid skeleton, the standard letters to identify the rings, and the numbering system are shown here.

The most widely studied condensed tannins are based on the flavan-3-ols (-)-epicatechin and (+)-catechin.



epicatechin

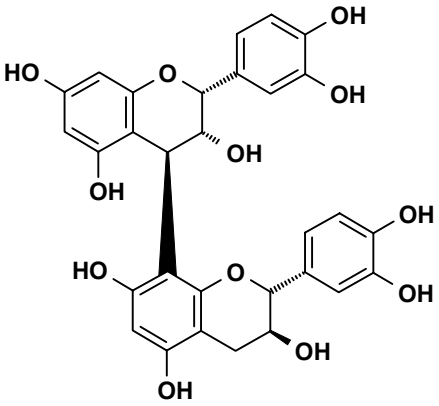


catechin

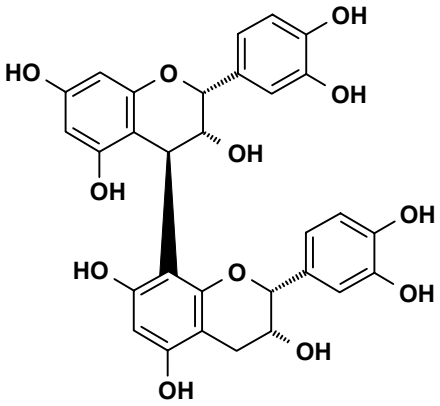
Flavan-3-ols

Addition of a third phenolic group on the B ring yields epigallocatechin and gallocatechin. Much less common are flavan-3-ols with only a single phenolic group on the B ring, *para* to C-2 (epiafzelechin, afzelechin with stereochemistry corresponding to epicatechin, catechin respectively).

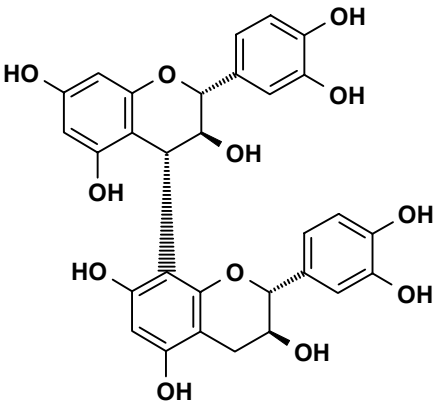
The best characterized condensed tannins are linked via a carbon-carbon bond between C8 of the terminal unit and C4 of the extender. The four common modes of coupling are illustrated by the dimers isolated by Haslam, and originally named B-1, B-2, B-3 and B-4. The more complete names specify the position and stereochemistry of the interflavan bond completely. In addition to these dimers, related dimers linked by C6 of the terminal unit and C4 of the extender have been isolated.



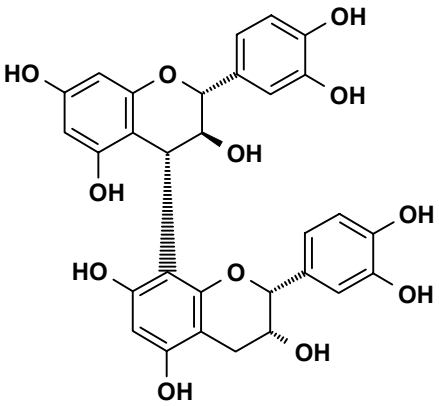
B-1
epicatechin-(4β->8)-catechin



B-2
epicatechin-(4β->8)-epicatechin

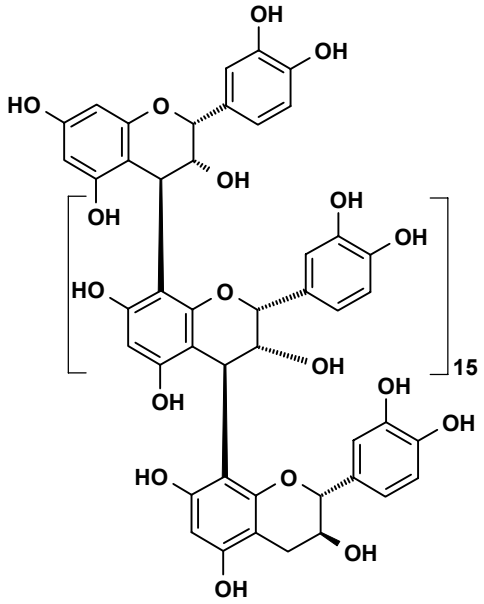


B-3
catechin-(4α->8)-catechin



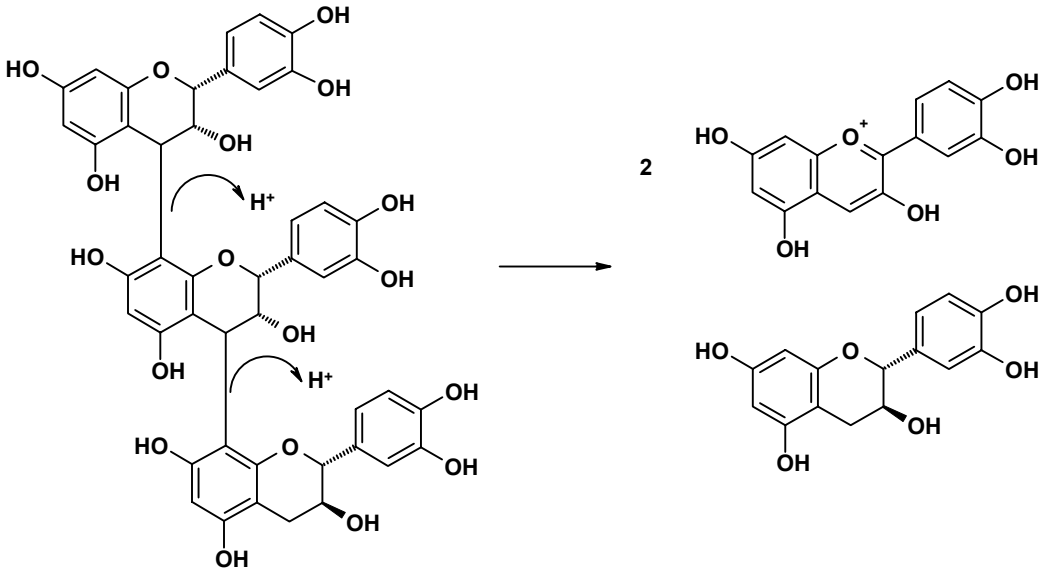
B-4
catechin-(4α->8)-epicatechin

Further polymerization can yield the linear 4,8 polymers such as the Sorghum procyanidin. Linear polymers based on 4,6 dimers; and branched dimers containing both 4,6 and 4,8 linkages are less common.



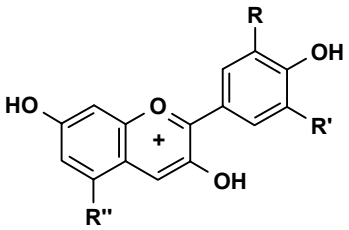
Sorghum procyanidin
epicatechin-[(4β->8)-epicatechin]$_{15}$-(4β->8)-catechin

Although the term condensed tannins is still widely used to describe these flavonoid-based polyphenolics, the chemically more descriptive term "proanthocyanidin" is gaining acceptance. Proanthocyanidins are compounds that yield anthocyanidin pigments upon oxidative cleavage (NOT hydrolysis) in hot alcohols, e.g.via acid butanol chemistry.



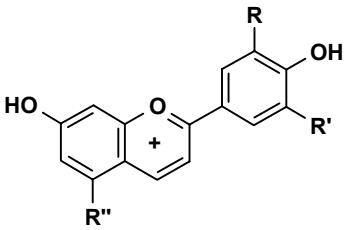
procyanidin
epicatechin$_2$ 4β−−>8 catechin

2 cyanidin + catechin
(extender) end group



R'' = OH
R = R' = H, pelargonidin
R = H, R' = OH, cyanidin
R = R' = OH, delphinidin

R'' = H
R = R' = H, guibourtinidin
R = H, R' = OH, fisetinidin
R = R' = OH, robinetinidin

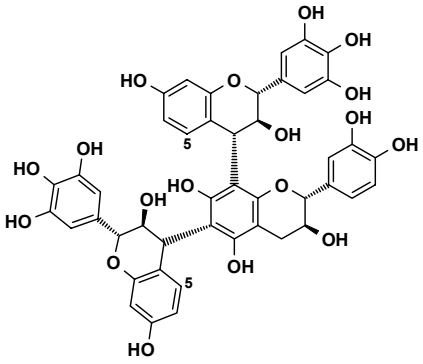


R'' = OH
R = R' = H, apigeninidin
R = H, R' = OH, luteolinidin

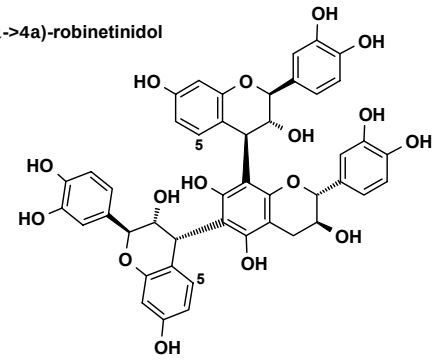
Anthocyanidins

The products of the acid butanol reaction are an unmodified terminal unit, and the colored anthocyanidins produced by the extender units. Catechin- and epicatechin-based polymers produce cyanidin, and thus are known as procyanidins. Gallocatechin and epigallocatechin-based polymers yield delphinidin, and the rare mono-substituted flavan-3-ol based polymers yield pelargonidin.

An important group of condensed tannins are 5-deoxy-flavan-3-ols polymers. Branching is common in these tannins, because of the reactivity of the 5-deoxy A ring. Profisetinidins and



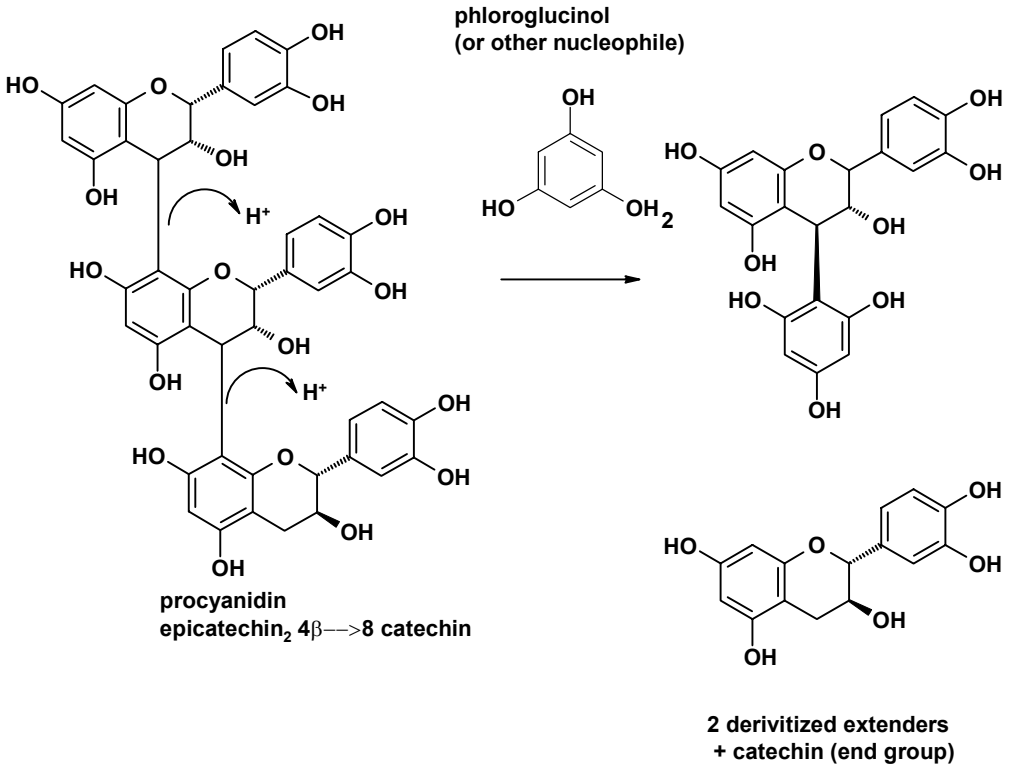
robinetinidol-(4α–>8)-catechin-(6α->4a)-robinetinidol



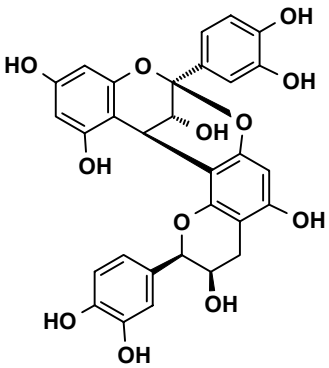
profisetinidin

prorobinetinidins comprise the major tannins found in quebracho and acacia tannin preparations. Acid butanol reaction yields the 5-deoxy anthocyanidins fisetinidn and robinetinidin.

The acid butanol reaction can be carried out with a nucleophilic trapping agent to produce the terminal unit plus derivitized extender units. These can usually be separated and quantitated by HPLC to give composition and average molecular weight estimates for the parent tannin. Thiols such as toluene-α-thiol are often used in this reaction, but phloroglucinol is more convenient. The efficiency of the reaction with branched condensed tannins, especially the 5-deoxy-flavanol-based tannins, has not been established.



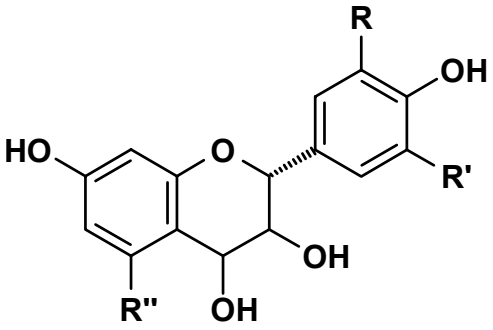
Another type of linkage that has been described but not studied extensively involves oxidative C-O coupling between the flavonoid rings to yield A2 and related proanthocyanidins.



epicatechin-(2β-->7,4β-->8)-epicatechin

proanthocyanidin A-2

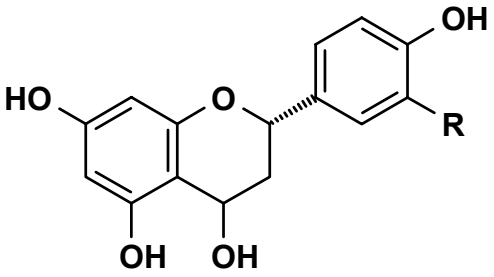
The flavan-3,4-diols, or luecoanthocyanidins, are sometimes confused with proanthocyanidins. The flavan-3,4-diols are monomeric flavonoids that yield the anthocyanidins upon treatment with heat and acid. They thus have reactive chemistry similar to that of the condensed tannins, but they do not interact with protein to form precipitable complexes.



Flavan-3,4-diols

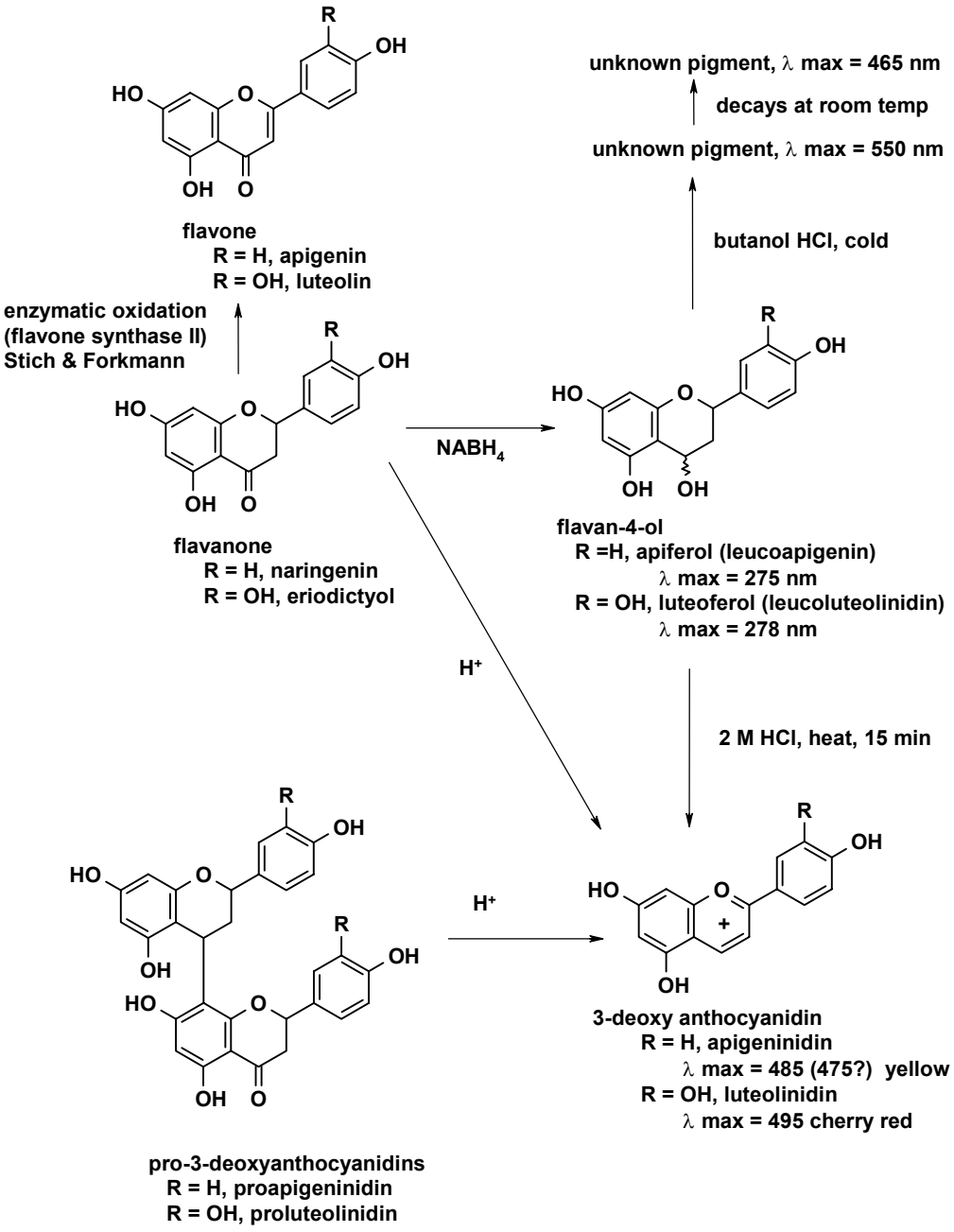
R" = H (stable)
R = H, R' = OH, leucofisetinidin
R" = OH (unstable)
R = R' = H, leucopelargonidin
R = H, R' = OH, leucocyanidin
R = R' = OH, leucodelphinidin

The flavan-4-ols are also luecoanthocyanidins, but are unique in their lability. They yield the anthocyanidins upon treatment with alcoholic acid at room temperature.



Flavan-4-ols

R = H, apiferol (leucoapigeninidin)
R = OH, luteoferol (leucoluteolinidin)



Stafford has suggested that pro-3-deoxyanthocyanidins might exist in a few plants. Evidence to date is limited to spectroscopy and some chemical conversions that are consistent with the chemistry shown here. (Stafford, H.A. *Flavonoid Metabolism;* CRC Press: Boca Raton, FL, 1990, pages 65-83).

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HYDROLYZABLE TANNIN STRUCTURAL CHEMISTRY

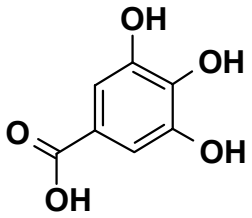
Ann E. Hagerman

Hydrolyzable tannins are derivatives of gallic acid (3, 4, 5-trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins.

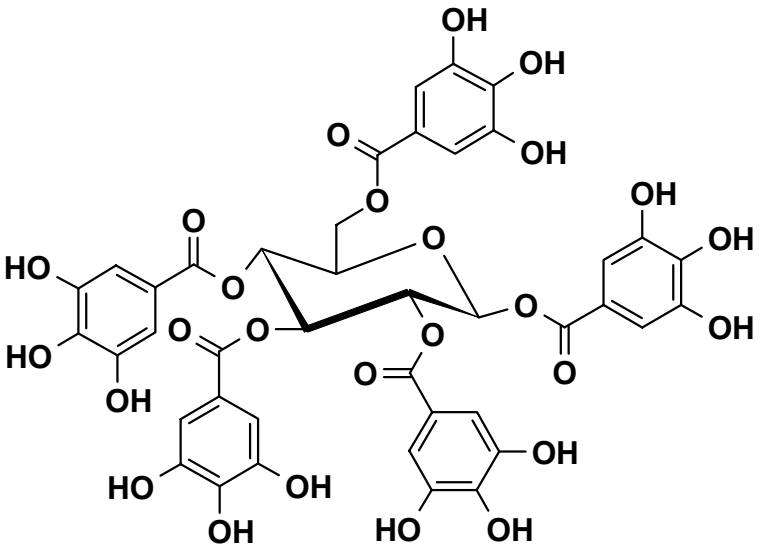
Early work on hydrolyzable tannins included Haslam's significant elucidations of the structures of the simple gallotannins (Haslam, E. *Plant polyphenols. Vegetable tannins revisited*, ed.; Cambridge University Press: Cambridge, U. K., 1989). More recently, Okuda et al.(Okuda, T.; Yoshida, T.; Hatano, T. Hydrolyzable tannins and related polyphenols. *Progress in the Chemistry of Organic Natural Products* **1995**, *66*, 1-117) have been particularly active in characterization and classification of complex hydrolyzable tannins. Feldman's synthetic work (Feldman KS, Lawlor MD, and Sahasrabudhe K Ellagitannin chemistry. Evolution of a three-component coupling strategy for the synthesis of the dimeric ellagitannin coriariin A and a dimeric gallotannin analogue. 2000; 8011-9) has provided useful insights into likely biosynthetic routes for the complex hydrolyzable tannins. A limited survey of structures and their relationships is provided here.

Gallotannins.

The simplest hydrolyzable tannins, the gallotannins, are simple polygalloyl esters of glucose. The prototypical gallotannin is pentagalloyl glucose (β-1,2,3,4,6-Pentagalloyl-*O*-D-Glucopyranose). Pentagalloyl glucose, or PGG, has five identical ester linkages that involve aliphatic hydroxyl groups of the core sugar. The alpha anomer is not common in nature.

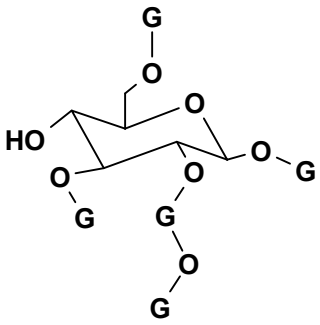


gallic acid

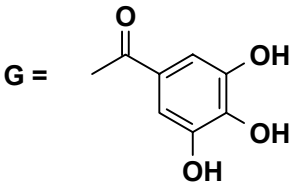


β-1,2,3,4,6-pentagalloyl-O-D-glucose

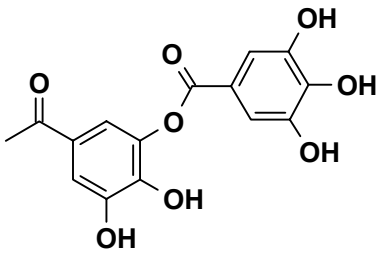
Like all of the gallotannins, PGG has many isomers. The molecular weights of all the isomers of PGG are the same (940 g/mol), but chemical properties such as susceptibility to hydrolysis and chromatographic behavior; and biochemical properties such as ability to precipitate protein; are structure-dependent.



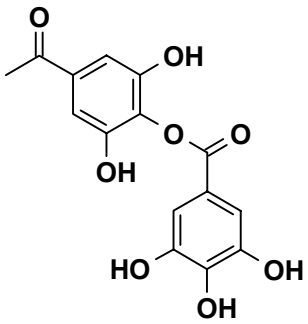
β-1,2,2,3,6-Pentagalloyl-O-D-Glucose



galloyl ester



GOG = digalloyl ester, *meta*-depside bond

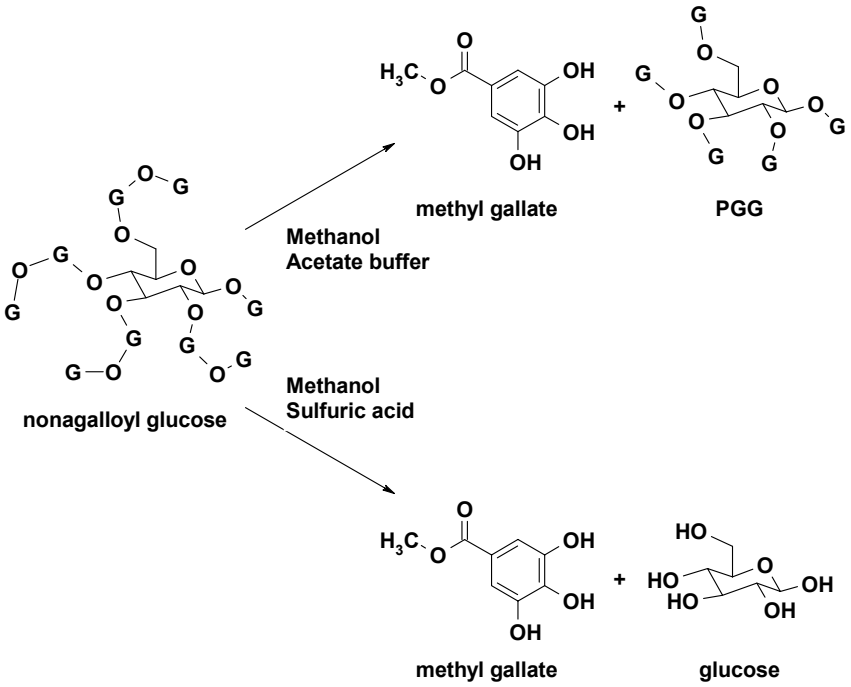


GOG = digalloyl ester, *para*-depside bond

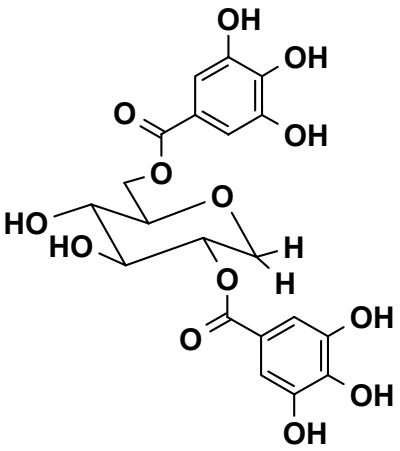
The polygalloyl ester chains found in gallotannins are formed by either *meta*- or *para*-depside bonds, involving a phenolic hydroxyl rather than an aliphatic hydroxyl group. The depside bond is more easily hydrolyzed than an aliphatic ester bond. Methanolysis in weak acid in methanol breaks depside but not ester bonds. Thus the core polyol with its esterified galloyl groups can be produced from complex mixtures of polygalloyl esters by methanolysis with acetate buffer. Strong mineral acid, heat and methanol can be used to methanolzye both despide and ester bonds yielding the core polyol and methyl gallate. Hydrolysis with strong acid converts gallotannins to gallic acid and the core polyol.

Simple gallotannins with up to 12 esterifed galloyl groups and a core glucose are routinely found in tannins from sumac or oak galls. Commercial tannic acid is comprised of mixtures of gallotannins from sumac (*Rhus semialata*) galls (Chinese gallotannin); Aleppo oak (*Quercus infectoria*) galls (Turkish gallotannin); or sumac (*R. coriaria, R. typhina*) leaves (sumac gallotannin). Although commercial sources provide a nominal molecular weight for tannic acid (1294 g/mol), the preparations are heterogeneous and variable mixtures of galloyl esters. Tannic acid is not an appropriate standard for any tannin analysis because of its poorly defined composition.

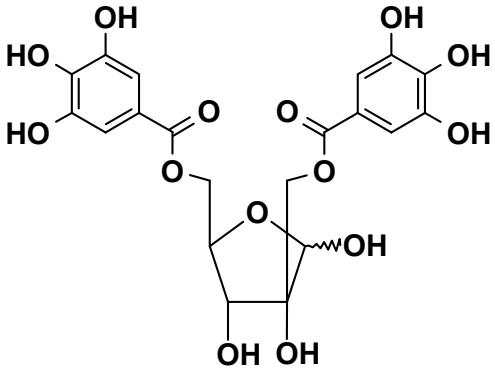
PGG can be prepared from some commercial tannic acids by methanolysis in acetate buffer. For the preparation to be successful, the tannic acid must have PGG as its core ester, most likely in preparations of Chinese or sumac gallotannin. Turkish gallotannin is comprised of esters of 1,2,3,6-tetragalloyl glucose; or 1,3,4,6-tetragalloyl glucose.



Although for many gallotannins glucose is the alcohol, other polyols including glucitol; hammamelose; shikimic acid; quinic acid; and quercitol; have been reported as constitutents of gallotannins from a few species. For example, aceritannin is found in leaves of several species



aceritannin
2,6-di-O-galloyl-1,5-anhydro-D-glucitol

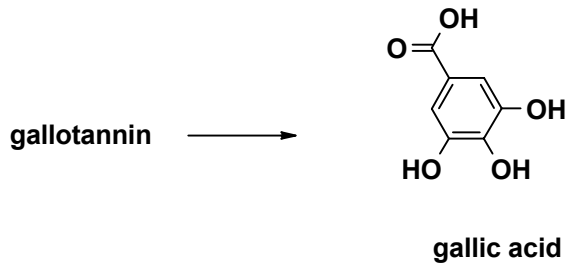


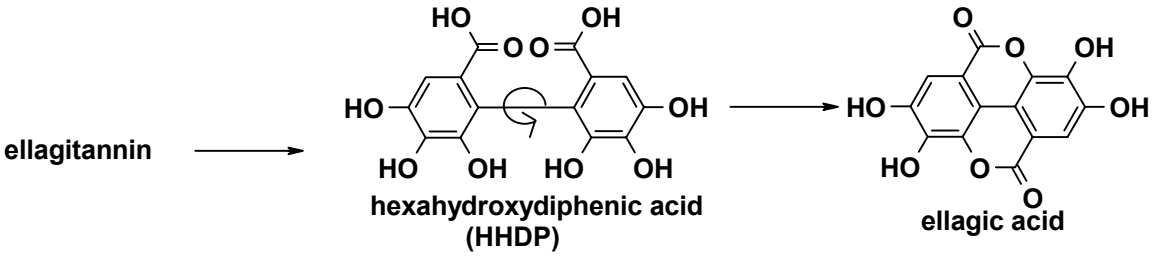
hamamellitannin

of maple (Acer), and hamamelitannin is found in bark of witch hazel (*Hamamelis virginiana)*, oak (*Quercus rubra)*, and several chestnut species (*Castanea* sp.).

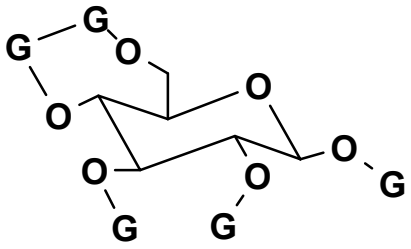
Ellagitannins.

Oxidative coupling of galloyl groups converts gallotannins to the related ellagitannins. The simple ellagitannins are esters of hexahydroxydiphenic acid (HHDP). HHDP spontaneously lactonizes to ellagic acid in aqueous solution.

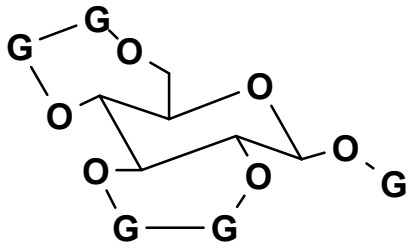




Intramolecular carbon-carbon coupling to form HHDP is most common between C-4/C-6 (e.g. eugeniin); and C-2/C-3 (e.g. casuarictin, also has C-4/C-6), as would be expected for polygalloyl glucoses in the more stable $^{4}C_{1}$ conformation. However, in a few plants intramolecular coupling

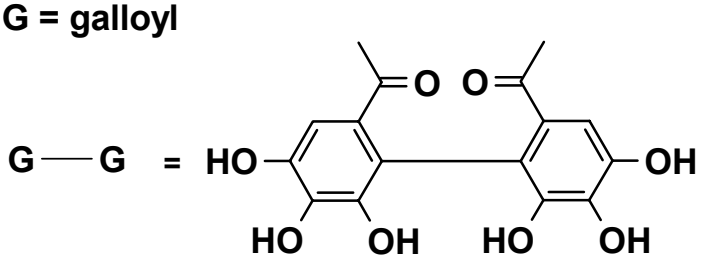


eugeniin

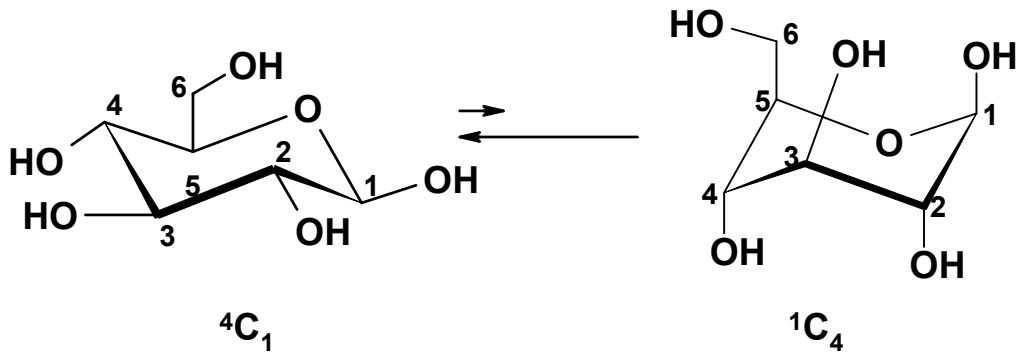


casuarictin

G = galloyl

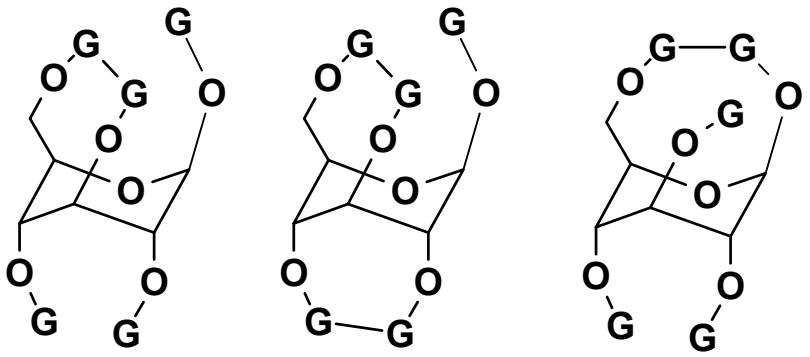


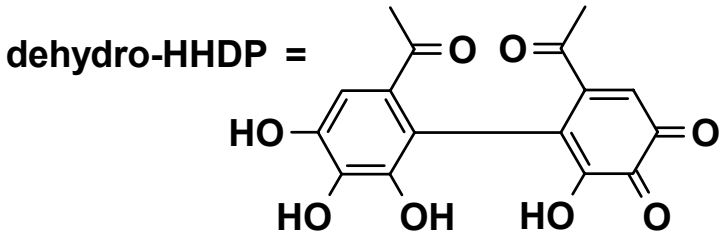
occurs at C-3/C-6 (e.g. corilagin), C-2/C-4 (e.g. geraniin, also has C-3/C-6), or C-1/C-6 (e.g. davidiin), suggesting the polygalloyl glucose starting material was in the less stable $^{1}C_{4}$



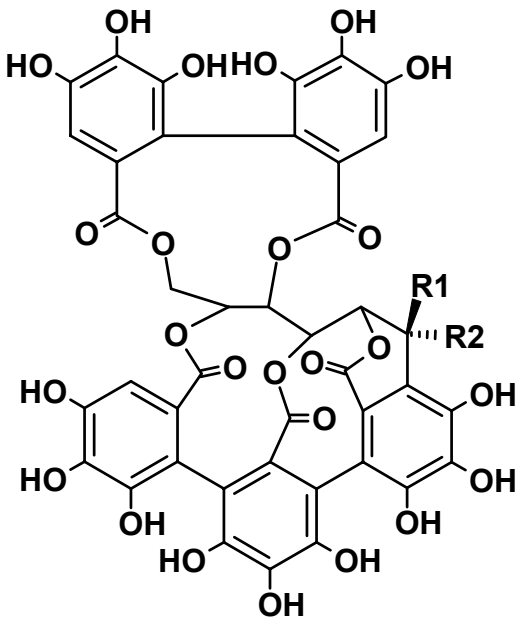
β-D-glucose

conformation. Geraniin is further characterized by partial oxidation of the C-2/C-4 HHDP to dehydro-HHDP, and in aqueous solution several forms of dehydro-HHDP can be detected in geraniin by nmr.



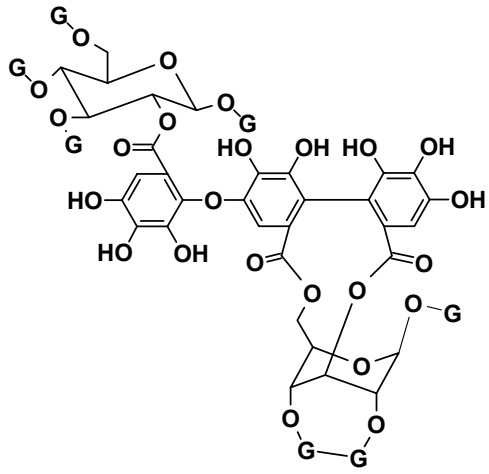


In some plants including oak and chestnut the ellagitannins are further elaborated via ring opening. Thus after conversion of casuarictin to pedunculagin, the pyranose ring of the glucose opens and the family of compounds including casuariin, casuarinin, castalagin, and castlin; stachyurin, vescalagin and vescalin forms.

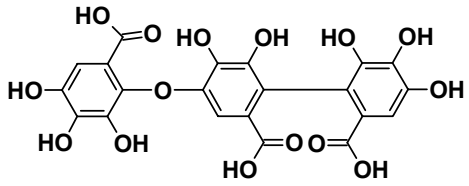


R1 = H, R2 = OH castalagin
R1 = OH, R2 = H vescalagin

The ellagitannins can undergo intermolecular oxidative coupling with other hydrolysable tannins to yield dimers. For example, in several euforbs (e.g. *Euphorbia watanabei*) geraniin oxidatively condenses with PGG to yield various euphrobins, characterized by the valoneoyl group.

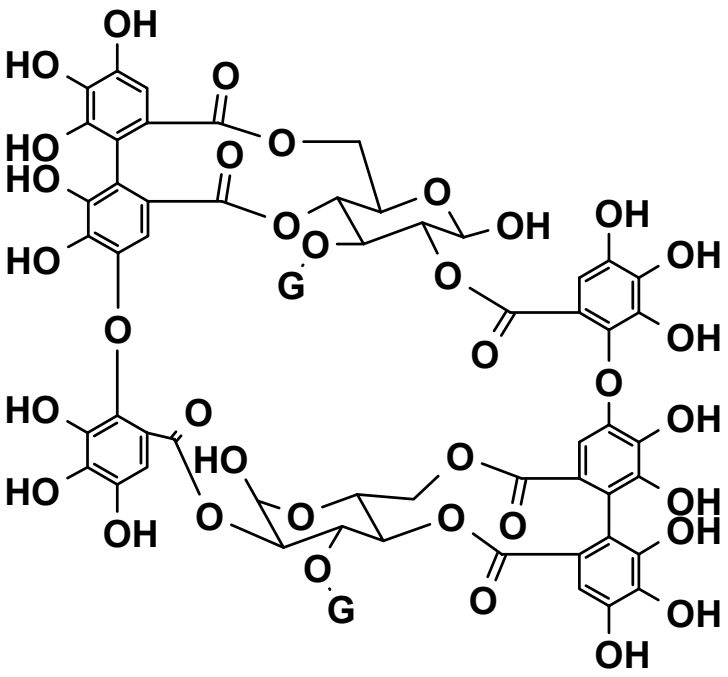


euphorbin A
G--G is a partially oxidized HHDP



valoneic acid

Oenothein B, Woodfordin C, Cuphiin D_1 and Eugeniflorin D_1 are macrocyclic dimers linked by two valoneoyl groups, and the nobotanins are macrocyclic trimers.



oenethein B

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BIOLOGICAL ACTIVITIES OF TANNINS

Tannins have diverse effects on biological systems because they are potential metal ion chelators, protein precipitating agents, and biological antioxidants. Because tannins can play such varied biological roles, and because of the enormous structural variation among tannins, it has been difficult to develop models which allow accurate prediction of the effects of tannins in any system. An important goal of future work on the biological activities of tannins is the development of structure/activity relationships so that biological activities can be predicted.

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TANNINS AS METAL ION CHELATORS

Phenolics can affect the biological availability or activity of metal ions by chelating the metal (McDonald, M.; Mila, I.; Scalbert, A. J. Agric. Food Chem. 1996, 44, 599). Chelation requires appropriate patterns of substitution and a pH above the pKa of the phenolic group.
Bacterial siderophores with multiple phenolic groups and very high affinities for essential metals such as iron have been characterized (Harris, W.R.; Carrano, C.J.; Cooper, S.R.; Sofen, S.R.; Avdeef, A.E.; McArdle, J.V.; Raymond, K.N. J. Am. Chem. Soc. 1979, 101, 6097). The similarity between siderophore ortho-dihydroxy substitution pattern and the substitution patterns on condensed and hydrolyzable tannins suggests that tannins may also have very high affinities for metals.

Phenolic-metal ion complexes are often colored, and it has been suggested that characteristic colors can be used to identify specific arrangements of phenolic groups (Mole, S.; Waterman, P.G., Oecologia 1987, 72, 137-147). However, these methods have not been adequately tested and are not recommended.

It is widely believed that tannin-chelated metal ions are not bioavailable. For example, consumption of large quantities of tea or other tannin-rich foods is sometimes associated with deficiency diseases such as anemia (Baynes, R.D. and Bothwell, T.H. Ann Rev. Nutr. 1990 10, 133). In many ecosystems, the slow decomposition of tannin-rich leaves has been attributed in part to the low levels of biologically available metal ions (Vituosek, P.M.; Turner, D.R.; Parton, W.J. and Sanford, R.L. Ecology 1994, 75, 418). The populations of microfauna essential to leaf decomposition and soil formation are unable to grow when metals are unavailable.

Metal ion chelation can alter the redox potential of the metal, or prevent its participation in redox reactions. Thus metal ion chelators can be inhibitors or enhancers of Fenton-driven oxidative reactions.

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DEOXYRIBOSE ASSAY

The deoxyribose assay is used to determine the reactivity of tannins toward hydroxyl radicals. The method sounds simple and straightforward, but it is technically difficult to get good results, and interpretation of the data is ambiguous especially for phenolics (which can participate not only as hydroxyl radical scavengers, but also as "pro-oxidants" and as metal ion chelators). I do not recommend this method for general use. Instead, the metmyoglobin (Randox) method is simple and yields better results.

Results we have obtained for tannins are described in Hagerman, A.E.; Riedl, K.M.; Jones, G.A.; Sovik, K.N.; Ritchard, N.T.; Hartzfeld, P.W.; Riechel, T.L. J. Agric. Food. Chem. 1998, 46, 1887-1892.

Original descriptions can be found in:

1. Aruoma, O. (1994) Methods in Enzymology. 233, 57-66
2. Halliwell, B., Gutteridge, J.M.C., and Aruoma, O. (1987) Analytical Biochemistry. 165, 215-219.

Reagents

Deaerated water. Heat an appropriate amount of distilled water at a hard boil for 5 to 10 minutes and allow to come to room temperature. Alternatively, bubble nitrogen through the water for approximately 10 minutes.

33.6 mM Deoxyribose. (450.7 mg deoxyribose in 100 mL of distilled water). Allow the deoxyribose solid to come to room temperature before weighing. Refrigerate the solution. The 2-Deoxy-D-Ribose is purchased from Sigma (D-2751). (Final concentration in the assay is 2.8 mM).

0.1 M $FeCl_3$/0.1 M HCl (16.221 g of solid ferric chloride in 50 mL of 2N HCl and bring final volume to 1000 mL with deaerated water). Store indefinitely.

300 µM $FeCl_3$ (150 µL of 0.1 M solution to a final volume of 50 mL of deaerated water). Prepare immediately before use. (Final concentration in the assay is 25 µM).

1.2 mM EDTA (35.1 mg disodium salt in 100 mL water). Store indefinitely. (Final concentration in the assay is 100 µM).

120 mM Phosphate Buffer (1.6330 g potassium phosphate monobasic in 100 mL water). Adjust pH to 7.4 with 25% KOH. Store indefinitely. (Final concentration in the assay is 10 mM).

1.2 mM Ascorbate (21.1 mg L-ascorbic acid in 100 mL deaerated water). Prepare immediately before use. (Final concentration in the assay is 100 µM).

50 mM sodium hydroxide (200 mg sodium hydroxide dissolved in 100 mL distilled water). Stable for one week.

33.6 mM Hydrogen Peroxide (335 µL stock 30 % w/v solution to 100 mL distilled water). Prepare immediately before use. (Final concentration in the assay is 2.8 mM).

1 % w/v TBA (0.25 g thiobarbituric acid in 25 mL of 50 mM sodium hydroxide). Stir for approximately 1 hour and prepare daily.

2.8 % w/v TCA (1.4 mL 100% w/v trichloroacetic acid solution diluted with 50 mL water). Prepare daily.

Tannin (approximately 10 mg tannin in 1 mL deaerated water). Make sure to record the actual concentration of tannin used in a laboratory book. If not all the tannin dissolves, vortex the solution and sonicate for about 1 minute. Remove the tube from the sonicator, vortex, and centrifuge. Transfer the solution to a new tube. Repeat the centrifugation as needed.

A 37 C water bath and a boiling water bath will be needed in this experiment.

Method

Start by preparing a spreadsheet of all the reagent and sample amounts. Include blanks for each set of samples and the various reagent conditions.

Obtain and label an appropriate number of 15 mL polypropylene screw-top clinical centrifuge tubes for each set of samples to be tested in triplicate.

Add 35 µL each of the buffer and the deoxyribose solutionm to the 15 mL tubes. Add the appropriate amount of water listed on the spreadsheet. Vortex the tubes.

Add 35 µL of H2O2 and vortex again.

Add 35 µL each of the EDTA and FeCl3 solutions. Vortex the tubes.

Finally, add the tannin and 35 µL of the ascorbate. Vortex and cap the tubes.

The final volume of the mixture in each tube should be 420 µL. Centrifuge @ 3200 rpm (~ 1900 g-force) for 1 minute to ensure that the entire sample is at the bottom of the tube. Vortex each tube lightly.

Place the tubes in the 37 C water bath for 1 hour.

Allow the tubes to cool for 5 minutes. Vortex and shake the tubes. Centrifuge @ 3200 rpm for 1 minute.

Add 350 µL each of the TBA and the TCA solutions, in that order. The volume in each tube is 1120 µL.. Loosely cap and vortex the tubes. Place them in the boiling water bath for exactly 20 minutes.

Carefully remove the tubes with a pair of tongs to avoid splashing the boiling water and cool for 20 minutes. The pink color will form.

Vortex and shake the tubes. Centrifuge @ 3200 rpm for 1 minute.

Add 1120 µL 1-butanol and shake gently to mix the two layers. Centrifuge @ 3200 rpm for 6 minutes to separate the layers.

Using a solvent-resistant (glass or quartz) microcuvette, blank the spectrophotometer at 532 nm with 1-butanol. Take at least 900 µL of the upper layer of each sample and record the absorbance.

Subtract the blank absorbance from each appropriate sample set reading.

The typical absorbance for the control (no tannin) is 1.02 (although this seems to vary substantially).

Modifications

Several modifications were made to the Aruoma method in Reference #1.

The volume of the overall reaction was reduced by 35%. This allows less tannin to be used in each sample.

The temperature of the first water bath needs to be exactly 37 C. The second water bath was originally described as an 80 C bath, but a boiling water bath (~ 100 C) gives better reproducibility.

When the ascorbate and/or the EDTA are omitted, water is used to replace the missing volume. The volume before the TBA and the TCA are added needs to be 420 µL.

The order of addition of the reagents given in the Aruoma method is changed for our experiments. In the Aruoma method, the order of addition is $FeCl_3$, EDTA, buffer, water, H_2O_2, tannin, dOR, and Asc.

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TANNINS AS ANTIOXIDANTS

Although dietary tannins are often perceived as detremental because of their potential to affect protein digestibility or metal ion availability, it is also possible that tannins are beneficial. It is likely, based on our knowledge of tannin chemistry, that tannins are potential biological antioxidants.

Antioxidants are widely believed to be an important line of defense against oxidative damage, which has been implicated in a range of diseases including cancer, cardiovascular disease, arthritis, and aging (Kehrer, J.P. Crit. Rev. Toxicol. 1993, 23, 21). Biological antioxidants are generally divided into three groups: enzymes, such as superoxide dismutase; inhibitors of radical formation, such as Fenton reaction inhibitors; and free radical quenching agents, such as alpha-tocopherol (Vitmain E). Phenolics are good candidates as antioxidants because of their favorable redox potentials and the relative stability of the aryloxy radical (Simic, M.G. and Jovanovic, S.V. 1994. In: Ho, C-T., Osawa, T., Huand, M-T., & Rosen, R.T. (Editors), Food Phytochemicals for Cancer Prevention II. Teas, Spices and Herbs. Page 20, American Chemical Society: Washington DC).

Many low molecular weight, naturally occurring phenolics scavenge radicals as effectively as the antioxidant vitamins E and A when tested in vitro (Rice-Evans, C.A.; Miller, N.J.; Paganga, G. Free Rad. Biol. Med. 1996, 20, 933). Our recent work suggests that free or protein-complexed condensed and hydrolyzable tannins are more effective than small phenolics

Some low molecular weight phenolics are pro-oxidants in Fenton-driven systems, apparently because the phenolic is able to redox cycle the metal ion required for radical formation (Aruoma, O.I.; Murcia, A.M.; Butler, J.; Halliwell, B. J. Agric. Food Chem. 1993, 41, 1880). The tannins we have tested do not act as pro-oxidants in Fenton systems, and in fact react very rapidly to quench the hydroxyl radical.

We have used the deoxyribose method and the metmyoglobin (Randox) method to characterize the antioxidant capabilities of the tannins (Hagerman, A.E.; Riedl, K.M.; Jones, G.A.; Sovik, K.N.; Ritchard, N.T.; Hartzfeld, P.W.; Riechel, T.L. J. Agric. Food. Chem. 1998, 46, 1887-1892). We have also

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PROTEIN DIGESTIBILITY

Although high levels of dietary tannin can interfere with protein utilization [Salunkhe, D.K.; Chavan, J.K.; Kadam, S.S. 1990. Dietary tannins: Consequences and remedies; CRC Press: Boca Raton, 1990], there is little evidence that tannins consumed in moderate amounts are detrimental to health. There is some evidence that low levels of dietary tannins are beneficial to ruminants [Lees, G.L. 1992. In: Hemingway, R.W. & Laks, P.E. (Editors), Plant Polyphenols. Synthesis, Properties, Significance, Page 915, Plenum Press: New York] and perhaps to humans [e.g., Jankun, J.; Selman, S.H.; Swiercz, R.; Skrzypczak-Jankun, E. Nature 1997, 387, 561], and some mammals have developed mechanisms for accomodating even rather high levels of dietary tannins [McArthur, C.; Hagerman, A.; Robbins, C.T. 1991. In: Palo, R.T. & Robbins, C.T. (Editors), Plant Defenses against Mammalian Herbivory. Page 103, CRC Press: Boca Raton]. Reports of tannin toxicity are generally linked to ingestion of large amounts of tannin or introduction by routes other than oral ingestion . Chemical modification of the tannin, which may occur during food preparation or cooking, may increase or decrease the toxicity of the tannin to certain animals

A major family of proteins secreted by the salivary glands of some animals constitutes the best characterized of the "defense mechanisms" against the possible toxic effects of dietary tannins . The parotid and submandibular savliary glands of some mammals synthesize a group of proteins which are unusually high in proline, the so-called salivary proline-rich proteins (PRPs) . The PRPs are characterized by four general regions: a signal peptide, a transition region, a repeat region, and a carboxyl-terminal region [Carlson, D.M.; Zhou, J.; Wright, P.S. Prog. Nucl. Acid Res. Mol. Biol. 1991, 41, 1]. These unusual proteins undergo various post-translational modifications including proteolysis, phosphorylation, and glycosylation. PRPs collectively constitute about 70% of the proteins in human saliva, and several functions for these proteins have been proposed, including calcium binding, inhibition of hydroxylapatite formation, and formation of the dental-acquired pellicle . Recent evidence suggests that a primary role for these proteins may be protection against dietary tannins.

PRPs are constitutive in human saliva, but are induced by treatment with the beta-agonist isoproterenol in parotid and submandibular glands of rats , mice, or hamsters . In rats, dietary tannins induce the same biochemical and morphological changes and polyploidy events in the parotid glands (but not the submandibular glands) as does isoproterenol treatment . When young rats are fed a high tannin diet (2-4% tannin) they lose weight during the first three days, but after induction of the PRPs on the third day of the diet the animals start to gain weight at about the same rate as those on the control diet [Mehanso, H.; Hagerman, A.; Clements, S.; Butler, L. Rogler, J.; Carlson, D.M. Proc. Natl. Acad. Sci. (USA), 1983, 80, 3948]. The logical conclusion is that the PRPs are induced by dietary tannins to "neutralize" the detrimental effects of the tannins. Further evidence for the ability of PRPs to neutralize tannins is provided by observations with hamsters. Dietary tannins do not induce PRP synthesis in hamsters, and tannins have pronounced detrimental effects on hamsters . If weanling hamsters are fed a diet containing 2% tannin the animals fail to gain weight, and increasing the tannin level to 4% causes most of the animals to die within three days.

Salivary tannin-binding proteins have been found in some wild herbivorous mammals which consume tannin-containing plants. For example, mule deer saliva contains a protein which has high affinity for tannin ; mule deer are generalist herbivores and can accommodate tannin in their diets [Hagerman, A.E.; Robbins, C.T.; Weerasuriya, Y.; Wilson, T.C.; McArthur, C. J. Range Manag. 1992, 45, 57]. Herbivores such as sheep, which do not produce salivary tannin-binding proteins, prefer to consume tannin-free forages [Austin, P.J.; Suchar, L.A.; Robbins, C.T.; Hagerman, A.E. J. Chem. Ecol. 1989, 15, 1335] and are unable to accommodate dietary tannin [Hagerman, A.E.; Robbins, C.T.; Weerasuriya, Y.; Wilson, T.C.; McArthur, C. J. Range Manag. 1992, 45, 57]. The affinity of salivary tannin-binding proteins for specific types of tannin may be related to the feeding preferences of herbivores. Moose and beaver are specialist herbivores, and have very selective tannin-binding proteins . The tannin-binding proteins of generalist herbivores such as mule deer and bear show little selectivity for binding specific tannins [Hagerman, A. E.; Robbins, C.T. Can. J. Zool. 1993, 71, 628]. Although the amino acid sequences have not been reported for the salivary tannin-binding proteins from any wild mammals, the protein found in mule deer saliva is proline-rich.

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METMYOGLOBIN ASSAY

The metmyoglobin assay is a rapid method which provides the degree of antioxidant protection possessed by an individual species. Original descriptions of the method can be found in:

1. Halliwell, B. (1987) FASEB J. 1, 358-364

2. Southorn, P.A. (1988) Mayo Clin. Proc. 63, 390-408

3. Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V., and Milner, A. (1993) Clin. Sci. 84, 407-412

Our application to tannins is described in:

4. Hagerman, A.E.; Riedl, K.M.; Jones, G.A.; Sovik, K.N.; Ritchard, N.T.; Hartzfeld, P.W.; Riechel, T.L. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agric. Food Chem. (1998) in press.

The reagents for the metmyoglobin method can be purchased as a kit from Randox. It is much less expensive to prepare them yourself as follows:

Reagents

PBS Buffer (0.005 M Phosphate, 0.145 M NaCl)

A: Dissolve 0.68 g KH_2PO_4 and 8.5 g NaCl in 1 L of distilled water
B: Dissolve 1.14 g $K_2HPO_4 \times H_2O$ and 8.5 g NaCl in 1 L of distilled water

Mix the two solutions together to give a pH of 7.4 (approximately 770 mL of A and 2 L of B). (Final concentration in assay is 5 mM).

Myoglobin (initial concentration between 1 mg myoglobin/mL PBS to 5 mg/mL). The initial concentration doesn't matter since the solution will be diluted as it runs through the column. The myoglobin is purchased from Sigma (# M-0630).

$K_3Fe(CN)_6$ (0.24 mg $K_3Fe(CN)_6$ in 1 mL water)

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (0.8 mg ABTS in 1 mL PBS buffer). The ABTS is purchased form Sigma (# 1888). (Final concentration in the assay is 610 µM).

Trolox Standard (0.1564 g Trolox in 250 mL PBS buffer). The Trolox is purchased from Aldrich (# 23,881-3). If the solid does not dissolve, sonicate gently. (Final concentration in the assay is 2.5 mM).

Tannin Sample (1 mg tannin/mL of water). Make sure the actual concentration used is recorded in the laboratory notebook for the calculations. Prepare 1:10 dilutions.

Hydrogen Peroxide Dilute the 30 % w/v stock solution as follows:
#1 1 part (H_2O_2) plus 9 parts water
#2 1 part (#1) plus 9 parts water
#3 1 part (#2) plus 9 parts water
#4 1.7 parts (#3) plus 8.3 parts PBS buffer
(Final concentration in the assay is 250 µM).

Sephadex G-10-120 column (Height of 20 cm and Diameter of 1 cm). Equilibrate the column with the PBS buffer before using. This column can be prepared and reused indefinately, stored at room temperature.

Metmtoglobin. Mix equal volumes of myoglobin and $K_3Fe(CN)_6$ solutions. Run sample through column and collect the second fraction where the brown color starts to come off the column. The first fraction is just buffer and the third fraction is yellow containing the $K_3Fe(CN)_6$. Read the Abs @ 490 nm of the second fraction. Adjust the solution with buffer to give an absorbance reading of 0.147 so that the final concentration of metmyoglobin in the assay will be 6.1 µM). The equations used to calculate the amounts of the various forms of the myoglobin are found in Reference #3.

Procedure

Set up an appropriate number of 0.65 mL microfuge tubes to run each sample in duplicate or triplicate.

Add the 20 µL of the sample (water, standard, or tannin), 250 µL of metmyoglobin and 250 µL of ABTS to the tubes. Vortex the tubes.

Using a microcuvette (1 mL), blank the spectrophotometer at 600 nm with water.

Read the absorbance of each sample and record as Abs1.

Add 100 µL of the hydrogen peroxide substrate to the tubes. After exactly 3 minutes, read the absorbance and record as Abs2. (Hint: Add the substrate to four tubes at 30 second intervals. This provides enough time to read each sample at exactly three minutes).

Subtract Abs1 from Abs2 for each sample. The typical change in absorbance for the control (water as sample) is 0.296.

Calculations

The amount of putative antioxidant required to supress absorbance of the ABTS radical cation by 50% is compared to the amount of Trolox required for 50% supression in order to compare potency of various antioxidants. A Trolox standard curve is run with each set of samples because there is substantial day-to-day variability in the assay.

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Chemical preparation of ABTS radical cation

Described by Re et al.(Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C Antioxidant activity applying an improved ABTS radical cation decolorization assay Free Rad Biol Med 1999; 26(9-10):1231-7).

ABTS is prepared in the desired buffer at 3.84 mg/mL (7.01 mM). That solution (15 mL) is mixed with 1 mL of $K_2S_2O_8$ (10.6 mg/mL, 39.2 mM) prepared in the same buffer. The mixture is incubated at room temperature in the dark for 16 hours, and is then diluted with buffer to obtain the working solution of radical cation. This method has fewer side reactions and is much

The λmax of the ABTS radical cation is 734 nm, and there is a linear relationship between radical cation concentration and absorbance through at least an absorbance of 2.0. The extinction coefficient E is 12867 $M^{-1}cm^{-1}$ and is independent of pH at pH values 3-7.4.

ABTS radical cation quenching by tannin-protein complexes
ABTS radical cation decolorization capacity

As described in Riedl, K.M.; Hagerman, A.E. Tannin-protein complexes as radical scavengers and radical sinks. *Journal of Agricultural and Food Chemistry* **2001** 49, 4917-4923.

Quenching: Protein and procyanidin are mixed in 1.5 mL cuvettes by combining 450 μL protein solution (0-180 μg/mL) with 450 μL of PC solution (4-8 μg/mL). The solution was inverted to mix, and incubated for 10 min at room temp before zeroing the spectrophotometer at 734 nm. The decolorization reaction was initiated by adding 100 μL of 65 μM $ABTS^{+\bullet}$, and immediately mixing by inversion and placing the mixture in the spectrophotometer. Under these conditions, the A_{734} at the beginning of the reaction was about 0.7.

Capacity: To maintain a large excess of radical, 163 nmoles (150 μL of 1.09 mM $ABTS^{+\bullet}$) is added to 900 μL of solution containing 0.203 nmoles (1 μg) of PC at the desired pH. The starting A_{734} is about 2.0. The absorbances of both tannin-free control, and of PC-containing samples, are monitored during the reaction to account for the spontaneous decolorization of $ABTS^{+\bullet}$, which increases as pH is increased. The amount of $ABTS^{+\bullet}$ scavenged at any time corresponds to the difference in absorbance between the control and the PC sample. The extinction coefficient of the $ABTS^{+\bullet}$ can be used to calculate the number of moles of radical scavenged per mole of PC.

PROTEIN PRECIPITATION BY TANNINS

Although the ability to precipitate protein is the defining characteristic of tannins, the detailed chemistry of the interaction is still only partly understood. It is now clear that both the type of interaction and the strength of interaction are dictated by both the chmeistry of the tannin and the chemistry of the protein. In addition, the interaction is influenced by reaction conditions including temperature, pH, solvent compostion, and tannin:protein ratio.

Review articles which summarize the current knowledge of tannin-protein interactions have been published by Hagerman; Haslam; and Butler. Recent comparisons of condensed to hydrolyzable tannins are useful (Hagerman, A.E.; Rice, M.E.; Ritchard, N.T. J. Agric. Food Chem. 1998, in press).

Numerous methods for determining tannins which take advantage of the interaction between tannin and protein have been devised. The radial diffusion method is convenient; the protein-precipitable phenolics method is robust and gives excellent results with many types of tannins; the radiochemical method is very sensitive but requires specialized equipment; a similar but less sensitive method can be done with blue dye-labeled protein. Qualitative assessments of binding can be made with electrophoretic methods. A method for assessing phlorotannin-protein interactions has also been described.

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Tannin Bibliography--A very brief list of useful references from the primary literature.

Bate-Smith, E.C. & Swain, T. Flavonoid Compounds, pp. 705-809 in Comparative Biochemistry vol 3A (Mason and Florkin, eds), Academic Press 1962.

Hagerman, A.E.; Butler, L.G. *J. Chem. Ecol*. 1989, 15, 1795.

Hagerman, A.E.; Butler, L.G. Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. *Meth Enz* 1994**,** *234*, 429-437.

Hagerman, A.E.; Zhao, Y.; Johnson, S. 1997. In: Shahadi, F. (Editor), Antinutrients and Phytochemicals in Foods, Page 209, American Chemical Society: Washington DC.

Hagerman, A.E.; Carlson, D.M. Biological responses to tannins and other polyphenols. In *Recent Research Developments in Agricultural and Food Chemistry*. 1998 2, 689-704.

Hagerman, A.E.; Rice, M.E.; Ritchard, N.T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$(4->8)catechin (procyanidin). *Journal of Agricultural and Food Chemistry* 1998 46, 2590-2595.

Haslam, E., Plant Polyphenols. Vegetable Tannins Revisited. Cambridge University Press 1989.

Haslam, E. Practical polyphenolics: from structure to molecular recognition and physiological function, Cambridge University Press: Cambridge, U.K., 1998.

Harborne, J.B. 1991. In: Palo, R.T. & Robbins, C.T. (Editors), Plant Defenses against Mammalian Herbivory, Page 45, CRC Press: Boca Raton.

Hemingway, R.W.; Karchesy, J.J. (Editors); Chemistry and Significance of Condensed Tannins; Plenum Press: New York, 1989.

Hemingway, R.W.; Laks, P.E. (Editors); Plant Polyphenols: Synthesis, Properties, Significance, Plenum Press: New York, 1992.

Hemingway, R.W.; Gross, G.G.; Yoshida, T., (Editors); Plant Polyphenols: Chemistry and Biology; Plenum Press: New York, 1999.

Okuda, T.; Yoshida, T.; Hatano, T. *Progress in the Chemistry of Organic Natural Products* 1995, *66*, 1-117.

Stafford, H.A. 1990. Flavonoid Metabolism. CRC Press: Boca Raton.

Yoshida T.; Hatano T.; and Ito H. *Biofactors* 2000; 121-5.

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TANNIN HANDBOOK
May 11, 1998; May 6, 2002

This handbook was originally published for use in the Hagerman laboratory. The original printed Tannin Handbook is no longer available, please print pages that you need from this site.

All of the methods have been published in the primary literature, and it is strongly recommended that each individual attempting to perform these methods first refer to the original literature to obtain a better understanding of the utility and limitations of the methods. The results of these analyses are only meaningful when they are interpreted with a full understanding of the chemistry underlying the analysis.

Major limitations on all methods of tannin analysis are the different responses given by different phenolics; and the difficulty of procuring an appropriate standard.

Differential response means that "tannin level" or "phenolic level" of a sample cannot be adequately expressed as a single value. Differential response prevents use of any single commercially available compound as a convenient standard, since the relative response of the standard and the analyte in the assay are not known.

To overcome these difficulties, several methods based on different chemistries should be employed to obtain a qualitative and quantitative picture of the tannins present in the mixture. Either the tannin of interest should be purified for use as a standard, or a well characterized standard should be prepared and used with a good understanding of the limitations.

Methods used for quantitative analysis of tannins can be classified as follows:

- General phenolic methods
- Functional group methods
- HPLC
- Protein precipitation methods

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TANNIN PURIFICATION

Methods for purification of either hydrolyzable or condensed tannins are now widely available, so that an increasing amount of work has been done with purified compounds rather than with poorly characterized mixtures. The sucess of any attempt to purifiy tannins from plant tissues depends in large part on the methods used for tissue preservation, grinding and extraction.

Purity of the products can be assessed either by TLC, or by HPLC. Structures can be established either by degradative functional group methods or by spectroscopic methods.

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PURIFICATION OF GALLOTANNINS

Gallotannins can be purified to serve as chromatographic standards or as standards in various assays. Tannic acid, a commercially available gallotannin is the most convenient starting point for purification of gallotannins. Commercial tannic acid can be fractionated chromatographically to yield specific galloyl esters or can be methanolyzed to yield homogeneous pentagalloyl glucose.

Commercial preparations of tannic acid vary significantly in their composition, with some having only very small galloyl esters (mono-tetra galloyl glucose) and others having much larger esters. The larger esters precipitate protein more effectively than the small esters. The molecular weight reported by the manufacurer is usually a theoretical mol wt based on a presumed composition. The reported purity indicates how much material other than gallotannin is present. Neither value can be reliably used to determine the composition of the commercial preparation.

Preparative scale HPLC or column chromatography can be used to fractionate commercial tannic acid to yield specific galloyl esters or a mixture enriched in the esters of interest.

Methanolysis can be used to produce pentagalloyl glucose from some preparations of tannic acid. Recall that gallotannins such as tannic acid consist of a glucose (or similar polyol) core which is esterified to up to five gallic acid groups. These core gallic acid groups may be linked via "depside" bonds (ester bonds involving a phenolic OH instead of alcoholic OH) to additional gallic acids. The ester bonds are somewhat more difficult to hydrolyze than the depside bonds. Treatment of the gallotannin under mildly acidic conditions in the presence of methanol selectively methanolyzes only the depside bonds. Products of methanolysis are the core galloyl glucose and methyl gallate. If each of the available groups on the glucose is esterified to a gallic acid, the core galloyl glucose is pentagalloyl glucose (PGG).

Methanolysis does not always yield PGG. For example, a some preparations of commercial tannic acid are based on a tetragalloyl glucose core (e.g. 1,2,4,6 galloyl glucose). PGG, hexa galloyl glucose, hepta galloyl glucose etc. in these preparations methanolyze to yield tetragalloyl glucose and methyl gallate.

It is essential to use an independent technique, such as nmr, to confirm the identity of standards used to calibrate your HPLC.

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HPLC OF GALLOTANNINS

Hydrolyzable tannins can be fractionated by HPLC to provide both qualitative information on the homogeneity of a particular preparation; estimation of molecular weight; and quantitative information on specific compounds. Either normal phase or reversed phase HPLC can be used for hydrolyzable tannins. There are not as many methods for polymeric condensed tannins on HPLC.

Detection:

Gallotannins can be detected at 254 nm if you have a fixed wavelength UV detector, or at 280 nm if you have a variable wavelength detector. More sensitivity can be obtained by detection at 220 nm, although many organic compounds absorb at low wavelengths so some selectivity is lost. It is convenient to have an integrator set up so that you can determine peak size for each peak. Using a detector set at 280 nm, we set the output at 0.1 AU full scale and inject 20 uL of tannic acid samples made up to between 100 and 500 ug/ml.

Standards:

Gallic acid and methyl gallate are commercially available. Defined galloyl glucoses are not commercially available, but can be prepared from tannic acid.

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Condensed tannin HPLC

HPLC of condensed tannins has proved more difficult than HPLC of hydrolysable tannins. Good separations of monomeric flavonoids are easily achieved. Oligomeric proanthocyanidins (dimers-pentamers) can be resolved. However, separation of the high molecular weight polymeric procyanidins has not been successful.

A few references to HPLC methods are given below, but we are not experienced in using these methods and cannot specifically recommend any particular one.

Cheynier V, Labarbe B, and Moutounet M. Estimation of procyanidin chain length. *Methods Enzymol* 335, 82-94. 2001.

Kennedy JA and Waterhouse AL. Analysis of pigmented high-molecular-mass grape phenolics using ion-pair, normal-phase high-performance liquid chromatography. *J Chromatogr A* 866(1), 25-34. 2000.

Lazarus SA, Hammerstone JF, Adamson GE, and Schmitz HH. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in food and beverages. *Methods Enzymol* 335, 46-57. 2001.

Natsume M., Osakabe N., Yamagishi M., Takizawa T., Nakamura T., Miyatake H., Hatano T., and Yoshida T. Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal-phase and reversed-phase HPLC. *Biosci Biotechnol Biochem* 64(12), 2581-7. 2000.

Prior RL, Lazarus SA, Cao G, Muccitelli H, and Hammerstone JF. Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium spp.) using high-performance liquid chromatography/mass spectrometry. *J Agric Food Chem* 49(3), 1270-6. 2001.

Rigaud, J., Escribano-Bailon, M.T., Prieur, C., Souquet, J.M., & Cheynier, V. (1993) *Journal of Chromatography 654*, 255-260.

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NORMAL PHASE HPLC OF GALLOTANNINS

A method particularly useful for separating the constitutents of tannic acid in a simple isocratic system. Better resolution can be obtained with reversed phase systems, especially if gradient HPLC is available.

Normal phase system described in Hagerman, A. E.; Robbins, C. T.; Weerasuriya, Y.; Wilson, T. C. and McArthur, C. J. Range Manag. 45: 57-62 (1992).

Detection:

Gallotannins are conveniently detected with UV detectors.

Column:

Silica (Alltech Econosphere), 150 mm x 4.6 mm, 5 um particles (Alltech, Deerfield IL)

Precolumn containing Perisorb A (Anspec Co., Ann Arbor, MI).

Store the pre-column/column overnight or weekends in isopropanol. Re-equilibrate in 10 volumes of mobile phase before using. Wash in absolute methanol, then equilibrate in isopropanol after using.

Mobile phase:

A mixture of two solvents, hexan and Solvent A, is used to acheive isocratic separation of the various galloyl esters. The proportion of these two solvents can be varied to alter retention times. A good starting point is 58% hexane to 42% Solvent A.

Hexane. We usually use HPLC grade hexane but analytical grade is often adequate.

Solvent A. Solvent A contains reagent grade methanol/tetrahydrofuran, 3/1 (v/v) and reagent grade trifluoroacetic acid (0.01 %, by volume). (Original method used citric acid, TFA is a volatile acid and is easier to remove from samples, also eliminates solubility problems).

Sample run:

Run ioscratically at 1.0 ml/min. Typical run takes 30 min to ensure elution of all peaks, although longer runs could be necessary if a sample had very high molecular weight tannins.

Dissolve the samples in the mobile phase to run. Some samples are not completely soluble in the mobile phase, and insolubles must be removed before chromatography to avoid damaging the equipment. Use a syringe or centrifugal filter for all samples.

If the samples contain even a trace of water, they will cause the mobile phase to separate into two layers when you are trying to dissolve the samples. Hexane and water are immiscible. If this happens, dry the sample (under nitrogen is convenient) and try again.

Some samples are less soluble than the commercial gallotannins. In that case it can be helpful to first dissolve the material in methanol, and then add the hexane, THF and TFA. Less polar samples might be dissolved first in hexane, and then the other solvents added. If the sample is dissolved in a solvent other than the mobile phase, its elution time will be altered by the other solvent. Accurate estimates of molecular weight can only be obtained if the system is recalibrated with standards dissolved in that other solvent.

Results:

The galloyl esters are separated according to polarity, with the least polar eluting first. The number of galloyl groups seems to dictate polarity; more galloyl groups makes the molecule more polar, since it has more hydroxyl groups. Methyl gallate elutes first, then gallic acid, monogalloyl glucose, digalloyl glucose, trigalloyl glucose etc. The log of the retention time is a linear function of the number of galloyl groups in the ester (methyl gallate represents no galloyl groups; gallic acid is omitted from the analysis). Isomers of a given ester may elute at slightly different times, so for example hexagalloyl glucose may be represented by a major peak for the main isomer and several "shoulders" representing other isomers.

Representative chromatograms for several commercial preparations of tannic acid are shown in the paper by Hagerman et al. (1992).

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REVERSED PHASE HPLC OF GALLOTANNINS

Excellent resolution of gallotannins can be achieved by gradient elution on reversed phase systems as described in Kawamoto, H.; Nakayama, M.; Murakami, K. Phytochemistry 1996, 41, 1427.

In some cases, adequate resolution may be obtained with isocratic separations on reversed phase systems. Separation with normal phase HPLC is also possible.

Column:

C-18 (ODS) such as Beckman Ultrasphere 4.6 mm x 25 cm, 5 um particles with similar C-18 precolumn.

Elution:

0.1% aqueous trifluoroacetic acid

0.1% trifluoroacetic acid in HPLC grade acetonitrile

gradient, 1 mL/min, 4:1 aqueous to 3:2 aqueous over 7 min (retention time, pentagalloyl glucose, 5.9 min)

Detection:

Gallotannins are conveniently detected with UV detectors. We use UV detection at 220 nm, and can easily detect ng levels of gallotannins.

Sample preparation:

Gallotannins can be dissolved in methanol or acetone (gives large solvent peak). Gallotannin-bovine serum albumin precipitates can be dissolved in 1% aqueous sodium dodecyl sulfate and injected directly onto the HPLC (Hagerman, A.E.; Rice, M.E.; Ritchard, N.T. J. Agric. Food Chem. 1998 46, 1409-1421).

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ISOCRATIC REVERSED PHASE HPLC OF GALLOTANNINS

This simple isocratic system is described in Barbehenn and Martin, J. Insect Physiol. 38, 973-980 (1992). Resolution is far better if gradient HPLC is used instead.

Detection:

Gallotannins are conveniently detected with UV detectors.

Column:

Ultrasphere 5 RP-18 column (4.6 mm x 25 cm) (Beckman) with a 4mm x 4 mm precolumn with same packing

Mobile phase:

1% acetic acid:acetonitrile, 80:20 (v/v). (Better results might be obtained by substituting 0.1% trifluoroacetic acid for the acetic acid).

Samples and elution:

Isocratic run at 1 mL/min, for at least 15 min.

Samples dissolved in the mobile phase.

Results:

Elution is dependent upon polarity, with the most polar molecules eluting first. The most polar compounds are gallic acid, followed by monogalloyl glucose, digalloyl glucose, trigalloyl glucose etc. In this system, additional galloyl groups appear to make the molecule less polar, presumably because of the additional aromatic rings.

It is typical for the order of elution from a RP column to be opposite to the order from a normal phase column, but for the gallotannins the order is the same in both systems. The complex chemical nature of the tannins is reflected in this unusual chromatographic behavior.

CONDENSED TANNIN METHODS

Condensed tannins (proanthocyanidins) are flavanoid-based tannins.

Functional group methods can be used to detect characteristic flavanoid monomers of condensed tannins. In the acid butanol method the interflavanoid bond is oxidatively cleaved by acidic alcohol and the anthocyanidin which is released is quantitated. Several modificiations of this method have been described to overcome problems with interference by pigments or chlorophyll.

An alternative method for condensed tannins, the vanillin method, is difficult to perform reproducibly and to standardize properly.

Modifications of the vanillin method and of the acid butanol method have been used to estimate molecular weights of condensed tannins. If acid butanol reactions are carried out in the presence of a nucleophilic agent such as phloroglucinol then reaction products useful for qualitative and quantitative characterization of the tannin are obtained.

HPLC, mass spec, nmr and other instrumental methods are less well developed for condensed tannins than for hydrolyzable tannins. Instead, chemical methods have been devised for providing sturctural information. For example, in the phloroglucinol method for determining condensed tannin composition and molecular weight, degradation products from condensed tannin are separated by HPLC.

Alternative methods for separating condensed tannin include centrigual partition chromatography (Okuda, T.; Yoshida. T.; Hatano, T. 1994. In: Foucault, A.P. (Editor) Centrifugal Partition Chromatography. Vol 68 in Chromatographic Science Series, Page 99, Marcel Dekker: New York) or chromatography of acetylated tannins (Williams, V.M.; Porter, L.J.; Hemingway, R.W. Phytochemistry 1983, 22, 569).

Appropriate standards for condensed tannin methods include purified Sorghum tannin, purified quebracho tannin, and commercially available anthocyanidins such as cyanidin. In some cases the flavan-3-ol catechin may be useful.

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FUNCTIONAL GROUP METHODS

These methods are dependent on the chemical reactivity of the functional groups characteristic of a given type of tannin. These methods are much more selective than the general phenolic methods, and provide both quantitative and qualitative information about tannins in an extract. Coupling these methods with methods which estimate biological activity is recommended.

The specificity of these methods can lead to problems with interpretation, since subtle differences in structural chemistry can lead to substantial differences in reactivity of the functional groups.

Functional group methods have been described for:

- Condensed tannins
- Hydrolyzable tannins, including
    - Gallotannins
    - Ellagitannins
- Phlorotannins

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GENERAL PHENOLIC METHODS

Phenolic comounds undergo characteristic reactions due to the chemistry of the phenolic functional group. Among the most useful of these reactions for analytical purposes is the tendency for the phenolic group to be oxidized. If oxidation is coupled to reduction of an appropriate reagent then quantitative analysis is achieved. Widely used methods based on redox chemistry include the Prusssian blue method and the Folin method (Swain, T. &.Hillis, W.E. J. Sci. Food Agric. 1959, 10, 63). Other general phenolic methods are based on formation of colored phenolic-metal ion complexes, but these methods are not recomended (Hagerman, Zhao & Johnson, 1997).

Differences in the redox potential and stoichiometry for different phenolics leads to differential responses with redox assays. Although these assays are often standardized with simple phenolics such as gallic acid, complex polyphenolics may have a very different response on a molar or mass basis than the simple standard. Thus the results must be expressed as "gallic acid equivalents" rather than as absolute weight percent.

General phenolic methods can be useful for screening plants for the presence of phenolics. Use of the more selective functional group methods and of methods used to assess biological activity is strongly recommended.

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MODIFIED PRUSSIAN BLUE ASSAY FOR TOTAL PHENOLS

The Price and Butler method as modified by H. D. Graham [J. Agric Food Chem. 40, 801-805 (1992)] to give greater color stability. The volumes are also scaled down in this method. We are now using this method.

Reagents:

0.02 M $FeCl_3$ in 0.10 M HCl

Dilute concentrated HCl to 0.10 M by bringing 8.3 mL of the concentrated acid to 1 L with distilled water.

Make the ferric chloride by dissolving 3.24 g of anhydrous ferric chloride in 1 L of the 0.10 M HCl. This will make a pale yellow solution.

0.016 M $K_3Fe(CN)_6$

Dissolve 5.26 g of potassium ferricyanide in 1 L of distilled water. This will make a yellow solution.

Stabilizer (Stable for 1 week in refrigerator). You need 5 mL/sample.

30 mL distilled water, 10 mL 85% H_3PO_4, 10 mL 1% gum arabic.

85% H_3PO_4 is the typical, commercially available phosphoric acid.

1% gum arabic is prepared by suspending 1.0 g gum arabic (or gum acacia; Sigma G-9752 or equivalent) in about 80 mL distilled water and boiling the suspension for 25 min. Vacuum filter the mixture with #1 paper and bring the volume of the filtrate up to 100 mL with distilled water. Refrigerate the solution.

Method:

Dispense 0.10 mL sample (or a smaller appropriate volume of sample made up to 0.10 mL with sample solvent) into a test tube.

Add 3.00 mL distilled (deionized) water and vortex the mixture. Poor quality water, especially iron-containing water, will give high blanks and unacceptable results.

To each sample, add 1.00 mL $K_3Fe(CN)_6$ followed immediately by 1.00 mL $FeCl_3$ and immediately

vortex the mixture. The interval between handling each sample should be approximately 1 min, although exact timing is not as critical as with the Price and Butler method.

Fifteen min after adding the reagents to a sample, add 5.00 mL stabilizer to the sample and vortex. Each sample should have a reaction time of 15 min before the stabilizer is added.

Read the absorbance at 700 nm; after the addition of stabilizer the colors are stable, so timing is not critical. Include solvent-only blanks, and either "blank" the spec with them or subtract the absorbance of the blank from the absorbance obtained for each sample.

Standardize the assay against an appropriate phenolic, for example 0.001 M gallic acid (0.019 g gallic acid monohydrate dissolved in 100.0 mL methanol).

The standards must be dissolved in the same solvent that the samples are dissolved in. The values obtained with this method are similar to those obtained with the Price and Butler method, but not identical, probably due to the complexity of the reactions involved in phenolic oxidation and in formation of Prussian blue.

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PRUSSIAN BLUE ASSAY FOR TOTAL PHENOLS

This is the original method described by Price and Butler [J. Agric. Food Chem. 25, 1268-1273 (1977)]. The major difference between the method described here and the method as published is the use of ferric ammonium sulfate instead of ferric chloride as the first reagent. Solubility problems are common with ferric chloride, but are eliminated by using ferric ammonium sulfate. We have recently started to used a modified Prussian blue method.

Reagents:

0.10 M $FeNH_4(SO_4)_2$ in 0.10 M HCl

Dilute concentrated HCl to 0.10 M by bringing 8.3 mL of the concentrated acid to 1 L with distilled water.

Make the ferric ammonium sulfate by dissolving 48.2 g of the dodecahydrate salt ($FeNH_4(SO_4)_2$ .12 H_2O in 1 L of the 0.10 M HCl. This will make a pale yellow solution.

0.008 M $K_3Fe(CN)_6$

Dissolve 2.63 g of potassium ferricyanide in 1 L of distilled water. This will make a yellow solution.

Method:

Dispense 0.10 mL sample (or a smaller appropriate volume of sample made up to 0.10 mL with sample solvent) into a 125 mL Erlenmeyer flask. Add 50.0 ml distilled (deionized) water. Poor quality water, especially iron-containing water, will give high blanks and unacceptable results.

Add 3.0 ml $FeNH_4(SO_4)_2$ and swirl. Additions should be timed; 1 min intervals are convenient.

Exactly 20 min after the addition of the ferric ammonium sulfate, start timed (1 min intervals) additions of 3.0 ml $K_3Fe(CN)_6$. Swirl.

Exactly 20 min after the addition of the ferricyanide, read Absorbance at 720 nm, making readings at 1 min intervals

Include solvent-only blanks. Subtract the absorbance of the blank from the absorbance obtained for each sample. Standardize against 0.01 M gallic acid (0.094 g gallic acid monohydrate per 50 mL methanol.

ACID BUTANOL ASSAY FOR PROANTHOCYANIDINS

This assay is widely used to determine condensed tannins (proanthocyanidins). Although the assay is simple and gives good indication of the presence of condensed tannins, chemical characteristics of the tannins such as position of the interflavan bond and oxygenation pattern affect color yield significantly. For example, color yield with quebracho tannin is much lower than color yield with procyanidins such as Sorghum tannin because the interflavan bond in quebracho is not readily broken (Hemingway in The Chemistry and Significance of Condensed Tannins (R. W. Hemingway and J. J. Karchesy, eds; Plenum Press) page 98 (1989).

The improvements described by Porter, Hrstich, and Chan, Phytochemistry 25, 223-230 (1986) are used here.

Reagents:

Acid butanol: Mix 950 mL of n-butanol with 50 mL concentrated HCl

Iron reagent: 2% Ferric ammonium sulfate in 2 N HCl. Bring 16.6 mL of concentrated HCl up to 100 mL with distilled water to make 2 N HCl. Dissolve 0.5 g $FeNH_4(SO_4)_2$ x 12 H_2O in 25 mL of 2 N HCl. Store in a dark bottle.

Method:

In a 13x100 mm screw cap culture tube add 6.0 mL of the acid butanol reagent to a 1.0 mL aliquot of the sample (or a smaller volume of the sample made up to 1.0 mL with the sample solvent).

Add 0.2 mL of the iron reagent, and vortex the sample. Cap the tube loosely, and put in a boiling water bath for 50 min.

Cool the tube and read the absorbance at 550 nm. Subtract the absorbance of a blank containing only sample solvent, acid butanol and iron from the sample absorbance.

Because water decreases the color yield in this reaction, the reaction must be standardized with standard dissolved in exactly the solvent that the samples are to be dissolved in. Nonaqueous samples will give the greatest color, but the assay is sensitive enough to give adequate results even with substantial amounts of water present.

Standardize the assay with an appropriate proanthocyanidin, for example 1 mg/mL Sorghum tannin.

Modification for pigments:

If the samples are colored before heating (due to flower pigments or chlorophyll) you can read the color before heating and subtract that value from the color after heating [Watterson and Butler, J. Agric. Food Chem. 31, 41-45 (1983)].

For chlorophyll, this correction is only approximate because some of the chlorophyll is destroyed upon heating, so the subtracted blank is larger than the actual contribution due to the chlorophyll. Better results can be obtained with the assay incorporating PVP ,which also allows determination of leucoanthocyanidins

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PURIFICATION OF QUEBRACHO TANNIN

Purification of crude quebracho tannin to yield a condensed tannin (5-deoxyproanthocyanidin)-enriched fraction. Based on Asquith and Butler J. Chem. Ecol. 11: 1535-1544 (1985) and modified by Hagerman, 10/1/84.

DAY 1

Suspend 1 g of quebracho in 10 mL of 80% ethanol (80 mL absolute etOH/20 mL water). Stir until solid no longer adheres to the bottom of the flask, then allow it to settle overnight without stirring in a refrigerator.

The Sephadex LH 20 is prepared in 80% ethanol instead of the usual 95-100% ethanol. Swell about 25 g of Sephadex in 80% ethanol to make 100 mL of slurry. Stir the beads gently with a stirring rod to facilitate moistening, then allow them to settle and decant off the supernatant and fines. Repeat this process three times. Store tightly capped in the refrigerator. This can be reused indefinitely if it is regenerated by completely washing away the acetone with 80% ethanol at the end of the purification process and storing tightly capped in the refrigerator. Before reusing old Sephadex LH20, several washes should be carried out with 80% ethanol as described above to remove fines and residual adsorbed materials.

DAY 2

Filter the quebracho suspension through Whatman #40 (medium) filter paper, using a Buchner funnel and gentle vacuum if necessary. Add about 100 ml of a slurry of Sephadex LH20 in 80% ethanol to the filtrate, stir for 3 min with a stirring rod, then filter through a coarse sintered glass funnel. If the tannin is allowed to mix longer with the LH 20, brown tar which is difficult to remove from the Sephadex forms. Wash the LH20 with 95% ethanol until the absorbance at 280 nm starts to approach zero. It is best to wash fairly slowly, allowing the LH20 to equilibrate with the fresh ethanol at each step. Slow gravity filtration is better than vacuum filtration. The ethanol washes can be discarded. The final eluate should be colorless, and the Sephadex should be brown.

Wash the Sephadex with 50% aqueous acetone (acetone:water, 1:1, v/v), combining and saving these washes. Continue to wash until the Sephadex is white and the eluate is nearly colorless. Refrigerate overnight (or continue with the next step if you have time to complete it).

DAY 3

Rotary evaporate (evaporate under reduced pressure) the acetone washes to completely remove the acetone. It may also be desirable to reduce the volume of the aqueous sample, but do not bring the sample to dryness or concentrate it to the extent that material starts to precipitate. The temperature should

be maintained at less than 30 C during the evaporation.

The aqueous sample should be extracted three times with an equal volume of ethyl acetate, each time discarding the organic (upper) phase. After the third extraction, traces of ethyl acetate should be removed by rotary evaporation, again maintaining the temperature below 30C.

The aqueous sample should be frozen and then lyophilized to yield a fluffy brown powder, which should be stored desiccated in a freezer. Yields are disappointingly small with this process, but the purified material is a much better standard than the crude preparation.

Commercial suppliers of quebracho (large quantities)

Trask Chemical Corporation
3200 W. Somerset Court
Marietta, Georgia 30067
(404)955-9190
[Cabeza de Caballo is on the bag we obtained]
(material from L. Butler is from Trask)

Tannin Corporation
60 Pulaski St.
P.O. Box 606
Peabody, MA 01960
(617)532-4010
(Charlie Robbins obtained quebracho from Tannin Corp.)

Small quantities of crude quebracho tannin can be obtained from

Dr. A. E. Hagerman
Department of Chemistry & Biochemistry
Miami University
Oxford, OH 45056

or send me e-mail at hagermae@muohio.edu

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SEPHADEX LH 20

Sephadex LH 20 sorbs tannins in alcohol, and releases them in aqueous acetone. Chromatography on Sephadex is very useful for separating tannin from nontannin phenolics, or for fractionating hydrolyzable tannins. Condensed tannins (Sorghum or quebracho) can be prepared using Sephadex LH-20. Size-based separation is NOT achieved on any Sephadex for tannins, since phenols sorb to the packing.

Strumeyer and Malin J. Agric. Food Chem. 23, 909-914 (1975). first reported on purifications of tannins with Sephadex.

Sephadex LH-20 can be obtained from Pharmacia 17-00-0-01, 25-100 micron or Sigma. Separations can be performed in columns or batchwise in a large sintered glass funnel (medium or coarse sintered glass). If columns are used, they must have plastic connectors and tubing that are resistant to alcohol and acetone.

Equilibrate the Sephadex according to manufacturers directions in ethanol (absolute or 80%, depending on purification you are following; do not use denatured alcohol). If you plan to use a column, wash with several bed volumes of ethanol after packing the column.

Phenolics are applied to the Sephadex in ethanol, and ultimately tannins are eluted with acetone. If a column is used, you must reequilibrate the column in ethanol before using it again. Because the Sephadex beads swell to different volumes in the alcohol and aqueous solvents, it is necessary to use mixtures of acetone/water and ethanol to slowly bring the column back to ethanol. Direct change from aqueous acetone to ethanol will cause cracks to form in the bed of Sephadex and make repacking the column essential.

The Sephadex may be stored indefinitely in the refrigerator (not freezer) in either aqueous acetone or alcohol.

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SEPARATION OF TANNIN FROM NONTANNIN PHENOLICS

Tannins adsorb to Sephadex LH 20 in alcohol while small phenolics elute from the material. Tannins can then be eluted with aqueous acetone. We have used this method, coupled with Prussian blue assays, to estimate tannin vs. nontannin phenolics in crude plant extracts.

95% ethanol

70% acetone (70:30 acetone:water).

Sephadex LH-20 Pharmacia 17-00-0-01, 25-100 micron

Column1.5 cm x about 8 cm.

Fraction collector.

Continuous UV monitor--useful for continuous monitoring of the eluate, but not essential.

Method:

Plant extracts are applied to the column after removing all acetone from the sample, and reducing the volume as much as possible. Add ethanol to keep the sample in solution. Centrifuge before applying to the column to remove any insoluble materials (you can redissolve this insoluble fraction and assay with Prussian blue; we find this fraction accounts for less than 10% of the total phenolics). About the equivalent of 90 umol equivalents of gallic acid (Prussian blue assay) can be loaded onto the column described here.

Apply the sample to the column and elute with ethanol (1 mL/min). Elute until the absorbance at 280 is no longer changing and is near baseline. The ethanol eluate can be combined, rotary evaporated to reduce the volume, and assayed with the Prussian blue assay to determine nontannin phenolics. It can also be dried to constant weight and nontannin phenolics determined gravimetrically (Zhao, M.S. Thesis, Miami University 1995).

Elute the column with 70% acetone; tannins are usually visible as a brown band of pigments. This eluate cannot be monitored in the UV because of the strong UV absorbance of acetone. The Prussian blue spot test can be used to establish when all of the tannin has been eluted. Combine the tannin fractions and use the Prussian blue to determine tannin. The tannin can also be determined gravimetrically after drying to constant weight.

We find that total recovery is routinely greater than 75%. Most of the unrecovered material is tannins which irreversibly sorb to the column.

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EXTRACTION OF PHENOLICS FROM PLANTS

The method used to extract phenolics must be planned with care since all results of the analyses are affected by the extraction step. Solvent and method of homogenization affect both recovery of extraction and selectivity of extraction. Some solvents are incompatible with some methods of analyses. In particular, acetone inhibits all protein precipitation assays except the radial diffusion assay. Crude plant extracts are not chemically stable, and can change rapidly during aging.

Phenolics can be extracted from fresh, frozen, dried or lyophilized plant samples. Sample treatment does affect phenolic extractibility, so all samples should be treated similarly [Hagerman, J. Chem. Ecol. 14, 453-461 (1988); Cork and Krockenberger, J. Chem. Ecol. 17, 123-134 (1991); Orians, C.M. J. Chem. Ecol. 21, 1235 (1995); Lindroth, R.L.; Koss, P.A. J. Chem. Ecol. 22, 765 (1996).

Lyophilizing tissue

Dry tissue is easy to handle, but phenolics are frequently not stable to sun drying or drying in an oven. To conveniently lyophilize, or freeze dry, plant tissue, cut the fresh tissue into small pieces with scissors, and place it in a mortar with liquid nitrogen. Grind with the pestle to yield a fine powder, keeping it frozen with liquid nitrogen throughout. Put the sample in a bag made of Miracloth, and lyophilize immediately. (When samples are lyophilized, or freeze dried, water is removed by sublimation. A variety of types of lyophilizers can be obtained from makers of refrigeration or vacuum equipment).

Extraction with a sonicator

A sonicator uses high energy sound waves to force solvent to penetrate "unwettable" material. In a sonicating bath, multiple samples can easily be handled at once.

Use about 2 g dry weight or 8 g wet weight of the plant tissue. If using fresh or frozen tissue, grind it first with liquid nitrogen with a mortar and pestle.

Transfer the sample to a 50 mL screw cap plastic centrifuge tube and record the sample weight. Add 20.0 mL 70% acetone (acetone:water, 70:30) and sonicate 30 min at 4 C.

Centrifuge 10 min at 2500 x g. Remove supernatant and record exact volume. Save supernatant at 4 C.

Repeat the extraction three more times. We then usually combine the first two extractions (about 75 % of the total phenolics) and the second two extractions (about 20 % of the total phenolics) for analyses.

Extraction with a homogenizer

Torti et al. [J. Chem. Ecol. 21, 117-125 (1995)] showed that for some samples phenolics can be extracted

efficiently and reproducibly using a Polytron-type homogenizer. Care must be taken not to cross-contaminate samples with the homogenizer blade, which must be cleaned throughly to remove traces of plant debris. "Stringy" samples are difficult to homogenize, and the volume of liquid must be fairly large to accommodate the blade.

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RADIAL DIFFUSION ASSAY FOR TANNINS

The radial diffusion method (Hagerman J. Chem. Ecol. 13, 437-449,1987) is a particularly simple protein precipitation method and is appropriate for situations wehere laboratory facilities are limied or numerous samples must be analyzed. Unlike other protein precipitation methods, acetone does not inhibit the preciptation reaction so acetone-containing extracts can be conveniently assayed with this method. A more sensitive modification of the method has been developed, and a modification for assessing extract composition has been proposed.

Differential response is pronounced in this assay, and results must be interpreted in light of this differential response.

Reagents:

Buffer: 0.05 M acetate containing 60 uM ascorbic acid; pH 5.0. For one liter, dilute 2.85 mL glacial acetic acid to about 800 mL, add 10.6 mg ascorbic acid, and adjust pH to 5.0 with 2N NaOH. Then bring final volume to 1.00 L with water.

Agarose: Type I, low EEO, gel point 36 C (Sigma A-6013

Bovine Serum Albumin: Fraction V powder, 96-99 % albumin (Sigma A-3350)

Well punch: 4.0 mm (Biorad 170-4029)

Disposable Petri dishes (nominally 10 cm, actual diameter about 8.5 cm). Can be washed and reused numerous times. We use Fisher catalog # 8-757-13, 100 x 15 mm dishes.

Parafilm

Water bath, hot plate/stirrer, refrigerator, 30 C incubator

Preparation of plates:

To make 8-9 plates (32-36 samples), add 1.0 g agarose to 100.0 mL of buffer in a 150 mL glass beaker. Heat with continuous stirring until the agarose dissolves. The solution will boil, and must not be allowed to boil over.

Put the hot solution into the water bath set at 45o C. Allow the agarose solution to cool, with occasionally stirring, to 45o C. If the solution is not adequately cooled, the BSA will denature when it is added. If the solution is cooled too much, the agarose will set and the mixture must be remelted. After the

BSA has been added the mixture cannot be remelted because the protein will be denatured.

When the solution has reached 45o C, add 0.10 g BSA with stirring. The BSA should be completely dissolved without allowing the solution to cool.

Using a 10.0 mL serological pipette with a large tip opening, dispense 9.5 mL of solution per plate. Dispense the solution carefully into the Petri dishes. Do not allow the solution to bubble, and be sure that the surface of each dish is covered by solution. Allow the solution to harden while the dishes are on a level surface.

Cover each dish and seal with a strip of Parafilm. Store at 4 C for up to several days before use. The layer of agarose in the dish should be of uniform thickness and free of bubbles or other imperfections.

Assay:

Punch wells in each plate. Arrange the wells so there are four per plate, as far apart as possible. Use only gentle suction to remove the plugs of agarose, since you want uniform wells.

Using a 10 uL glass syringe (Hamilton) or a micropipette, apply the sample in 8 uL aliquots to the wells. The sample can be dissolved in any solvent that is convenient. Aqueous solutions evaporate and penetrate the wells very slowly and are less convenient than aqueous organic mixtures. The surface tension of pure organic solvents is low making it difficult to dispense the solutions. We find 50% methanol to be easiest to handle, but also frequently use 70% acetone.

If the tannin is very dilute, 8 uL may not be sufficient for a response. You can add larger volumes by dispensing repetitive 8 uL samples. You must not allow the well to become completely dry between successive aliquots that are to be added.

After applying the samples, cover the plates and again seal with Parafilm. Place the plates in a level incubator at 30o C. Allow the rings to form for at least 96 h. Remove the plates, uncover, and use a plastic ruler to estimate the diameter of the ring that has formed. The square of the diameter is proportional to the amount of tannin in the sample.

The plates can be stored after development at 4o C for several weeks. They should be covered, sealed with Parafilm, and stored at 4oC.

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MODIFIED RADIAL DIFFUSION FOR INCREASED SENSITIVITY

In the radial diffusion assay, each molecule of tannin migrates through the gel until it encounters free protein and precipitates. The sensitivity of the method can be increased by decreasing the amount of protein in the gel, since if there is less protein in the gel some molecules of tannin will have to migrate further before encountering free protein and precipitating. Although the ring size is increased by decreasing protein concentrations, the ring intensity is diminished so with this modified method it is usually necessary to stain the gel to make the rings visible.

Method:

Use the same protocol as in the normal radial diffusion assay but use only 0.01 g BSA for 100 mL of agar solution. The remainder of the method is exactly as for the normal radial diffusion assay.

Staining:

After the plates have incubated at 30oC for 96 h, remove them from the incubator. The staining steps include a wash step which removes unprecipitated protein from the gel and a Prussian blue staining step.

1. Mark the gels so that the samples can be identified after removing the gels from the Petri dishes. We mark the gels using a small gauge needle and India ink. Simply dip the needle in the ink, then pierce the gel with the needle. A small black mark will appear in the gel. We mark near sample well #1 with one mark and near sample well #2 with two marks, so the orientation of the gel is unambiguous.

2. Remove the gels from the Petri dishes, using filter paper to support the gel so it doesn't break during handling. Take a piece of dry filter paper that just fits into the dishes (we cut 9 cm paper down to size) and place it over the gel. With your fingers, flatten the paper so it contacts the entire gel. The loosen the gel from the edge with a spatula, and pry the gel and paper out of the dish. Place the gel into a larger dish for staining, and remove and discard the paper.

3. Wash each gel with 40 mL of 0.3 M NaCl (35.2 g/2L water) for 20 min, shaking continuously. Discard the saline and rinse the gel twice with 10 mL of saline. Repeat the entire wash procedure 4 times. This removes unprecipitated protein from the gel so that the tannin-protein complexes can be visualized.

4. Stain each gel with 20 mL of Prussian blue reagent. Make the reagent fresh daily by mixing equal volumes of 0.10 M $FeNH_4(SO_4)_2$ in 0.10 M HCl and 0.008 M $K_3Fe(CN)_6$. Stain for several minutes; the tannin-containing rings of precipitate will stain dark blue or blue-green. Pour off the excess stain, and rinse the gel with 0.1 N HCl. Measure the rings immediately; the entire gel eventually turns blue.

SELECTIVE DETERMINATION OF CONDENSED OR HYDROLYZABLE TANNINS WITH RADIAL DIFFUSION

This modification of the radial diffusion method provides a way to distinguish condensed tannins from hydrolyzable tannins in the radial diffusion assay. The method was described in Hagerman, A.E.; Zhao, Y.; Johnson, S. Methods for determination of condensed and hydrolyzable tannins. In Antinutrients and Phytochemicals in Foods (Shahadi, F., ed) American Chemical Society, Washington, DC, 1997 pp. 209-222.

The method takes advantage of the structural differences between condensed and hydrolyzable tannins. The characteristic ester bonds in hydrolyzable tannins are susceptible to hydroxylaminolysis. Treatment of simple hydrolyzable tannins with hydroxylamine hydrochloride at slightly acidic pH decomposes the tannin to the core polyol and the hydroxamates of the phenolic acids (gallic acid and ellagic acid). These phenolics do not precipitate protein and are not detected in the radial diffusion assay. Condensed tannins do not react with hydroxylamine hydrochloride.

Crude plant extracts (in alcohol; acetone interferes with the hydroxylaminolysis) are prepared, and total tannins are assessed with the radial diffusion assay. The extract is then reacted with the hydroxylamine, and tannin remaining in the extract after destruction of esters is measured with the radial diffusion assay. For samples containing only condensed tannin, the radial diffusion measurement is the same before and after hydroxylaminolysis. For samples containing only simple hydrolyzable tannins, no ring forms when radial diffusion is performed after hydroxylaminolysis. For samples containing both types of tannin, a larger ring is obtained with the crude extract and a smaller ring with the hydroxylamine-reacted extract.

Reagents:

Hydroxylamine hydrochloride reagent. Prepare fresh daily. The reagent contains 2 M hydroxylamine hydrochloride in ethanol:water (48/52, v/v). The solution is adjusted to pH 5.5 with 10 N NaOH before use. We prepare the reagent by dissolving 0.25 g hydroxylamine hydrochloride in 0.8 mL 95% ethanol and adding 1.0 mL distilled water. After vortexing to ensure that the hydroxylamine is dissolved, we adjust the pH to 5.5 with 10 N NaOH.

Radial diffusion plates and stains made for the increased sensitivity method.

Plant extracts:

Weigh about 10 mg dried leaf tissue, recording the weight exactly. Extract two times for 30 min with 100 uL 70% acetone, sonicating at 4oC to maximize extraction. Centrifuge plant debris away after each extraction and combine the two extracts (final volume about 150 uL). Evaporate each sample under

nitrogen to about 25 uL to remove all acetone. Add distilled water to bring volume to 60 uL. We use an electronic micropipette (Rainin, EDP digital micropipette) which can be set to measure unknown volumes in this step.

Hydroxylamine reaction:

Place 25 uL of the extract into a microfuge tube (with tightly sealing cap, e.g. Fisher type 05-664-35) and add 300 uL hydroxylamine reagent. Incubate at 70oC for 48 h. Apply 50 uL of the reaction product to a radial diffusion plate.

Control sample:

Place 25 uL of the extract into a microfuge tube and add 300 uL distilled water. Immediately apply 50 uL of this sample to a radial diffusion plate.

Analysis:

Each of the radial diffusion plates must be sealed and incubated at 30oC for 96 h to allow the ring size to come to equilibrium. The plates can then be washed and stained (as in the modified radial diffusion assay) and the rings measured.

The difference between the area of the ring obtained before hydroxylaminolysis and the area obtained after the reaction represents the area due to simple hydrolyzable tannins. The area of the ring obtained after hydroxylaminolysis represents the area due to condensed tannins (and complex phenolics resistant to degradation).

Ring size in the radial diffusion assay is dependent on the chemistry of the tannin, and its ability to react with protein. For model compounds (Sorghum tannin and tannic acid) the difference in slope for the two tannins is about 2.9 [see Figure 4 in Hagerman J. Chem. Ecol. 13: 437-449 (1987)]. We have used that difference to estimate total tannin in consistent units, using a factor of 2.9 to relate rings obtained with condensed tannins to those obtained with simple hydrolyzable tannins.

Modified area = area due to simple gallotannins + 2.9*(area due to condensed tannins)

We do not have sufficient data to evaluate whether using the ratio 2.9 is appropriate for all samples, although it worked well for a limited set of plant samples which we tested it on.

Complex hydrolyzable tannins, such as dimeric tannins [Hatano et al. J. Chem. Soc. Perk. Trans. I: 2735-2743 (1990)] can complicate the analysis. These tannins are hydrolyzable, but may contain hydroxylamine-resistant phenolic groups which are large enough to precipitate protein and thus react in the radial diffusion assay. Chemical analyses such as the acid butanol assay, rhodanine assay and ellagic

acid assay are useful complements to this hydroxylaminolysis method for determining the exact composition of plant tannins.

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FRACTIONATION OF TANNIC ACID

From Hagerman and Klucher in Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure Activity Relationships (V. Cody, E. Middleton, and J. Harborne, eds. New York: Alan R. Liss, Inc.). pp. 67-76 (1986).

Tannic acid can be purified without preliminary methanolysis to yield selectively enriched mixtures of galloyl esters. Sephadex LH20 selectively adsorbs high molecular weight polyphenols in alcohol and releases them in aqueous acetone. The product of purification can be characterized by HPLC. Preparative scale normal phase HPLC can also be used to purify specific galloyl esters.

It is crucial to use a preparation of tannic acid which is contains a significant proportion of the desired components. Preliminary HPLC analysis will indicate whether a given preparation is suitable.

Purification on Sephadex LH 20

Tannic acid should be dissolved in absolute ethanol and applied to Sephadex LH 20. The gel is washed with ethanol until the eluate is free of phenolics as detected by spot tests with the Prussian blue reagent. The tannin can then be eluted from the gel with 50% aqueous acetone containing 0.001 M ascorbic acid to prevent oxidation. The tannin quickly becomes discolored due to oxidation in the absence of the ascorbic acid. The acetone is removed by evaporation under reduced pressure at temperatures less than 30 C. The aqueous solution is then lyophilized to yield a white powder.

The constitutents of the material can be characterized by HPLC. Ascorbic acid is a minor component of the product .

Purification by preparative scale HPLC

You can use the normal phase system. or the reversed phase system.

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HYDROLYZABLE TANNIN METHODS

Functional group methods can be used to detect specific phenolic acids characteristic of the various hydrolyzable tannins. Gallotannins can be hydrolyzed to release gallic acid, which can be quantitatively determined with either the rhodanine assay or the potassium iodate method. Ellagic acid, released from ellagitannins, can be determined with a nitrous acid method. The more complex phenolic acids characteristic of the oligomeric hydrolyzable tannins do not react in these simple color tests but can be determined with HPLC. HPLC also provides useful qualitative and quantitative information for the various hydrolyzable tannins.

Appropriate standards for hydrolyzable tannin methods include gallic acid, methyl gallate, and ellagic acid; pentagalloyl glucose or well characterized tannic acid.

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GALLOTANNIN DETERMINATION WITH RHODANINE

From Inoue and Hagerman, Anal. Biochem. 169: 363-369 (1988)

A functional group method for the determination of gallic acid released by hydrolysis of gallotannins permits estimation of gallotannins.

Reagents

Rhodanine (Sigma). 0.667 % (w/v) in methanol (0.667 g in 100 mL methanol). Store in refrigerator, stable for at least two weeks.

Gallic acid stock. 0.10 mg/ml in 0.2 N H_2SO_4. Stable 2 weeks at room temperature. Once a bottle of solid gallic acid has been opened, the top surface starts to oxidize . Store the bottle tightly capped in the dark to minimize degradation, and mix the solid before taking samples to prepare solutions. If the material has oxidized, color yields will be low.

KOH. 0.5 N KOH in water (2.8 g in 100 mL). Stable for several weeks if kept tightly capped to prevent absorption of CO2.

4 N H_2SO_4. In water. Stable indefinitely.

Determination of gallic acid.

Samples should be in 0.2 N H_2SO_4. Sample volume is 1.0 mL. The samples from the hydrolysis method described below are adjusted to the correct sulfuric acid concentration.

Add 1.5 mL of rhodanine to the sample in a graduated test tube (or graduated cylinder). After at least 5 min, add 1.0 mL of KOH to the sample. After at least 2.5 min, dilute to 25.0 mL with distilled water. 5 to 10 min later read the absorbance at 520 nm.

Standardize with the gallic acid stock. Use 0.02-0.1 mg gallic acid (0.2 mL-1.0 mL of gallic acid stock) made up to 1.0 mL with 0.2 N H_2SO_4.

Sample hydrolysis

Gallic acid is present in two forms in plants: as esters, with the higher mol wt esters (e.g. pentagalloyl glucose) acting as tannins, and as the free acid. To estimate gallotannins, the amount of total (ester plus free) gallic acid and the amount of free gallic acid are determined. Ester gallic acid is then calculated by

difference. The rhodanine assay detects only free gallic acid, so esters must be hydrolyzed before determination. The hydrolysis must be done anaerobically to prevent destructive oxidation; we have found evacuated hydrolysis tubes the simplest, most reliable method for anaerobic hydrolysis.

Extraction.

The plant extracts, normally made with aqueous acetone, should be evaporated to remove the acetone. Its volatility makes high temperature hydrolysis in evacuated tubes dangerous.

Hydrolysis.

The hydrolysis is done in 2 N H_2SO_4, mixing plant extract and 4 N acid in proportions to make the final conc 2 N. The plant extract, usually 1.0 mL, is placed in a Pyrex test tube which has been constricted about half way down. Add 1.0 mL of 4 N H_2SO_4, mix, and freeze the solution in a dry ice isopropanol bath. Put the sample on a vacuum pump, and evacuate the tube, then use a small glass blowing torch (gas/oxygen) to seal the tube at the constriction while it is still on the pump. Place the tube in a rack to melt. During melting, you should be able to see bubbling in the sample. If there is no bubbling, there was a leak and the tube is not evacuated. Learning to do this step is difficult, so practice is recommended before important samples are done.

When thawed, the tubes are placed in a 100 C oven for hydrolysis for 26 h. The oven should be clearly labeled as having samples under pressure, and should only be opened by people wearing splash resistant chemical goggles and a lab coat. Occasionally a tube does explode during heating.

Samples are removed from the oven and cooled. They can be stored without opening for several days before analysis. When cool, the tubes are opened by carefully breaking off the top. All of the sample is transferred to a graduated container, and the sample is diluted with water to a final sulfuric acid concentration of 0.2 N. (1 ml sample plus 1 ml of 4 N H_2SO_4 should be diluted to a final volume of 10 mL). Some of the water used to dilute the sample should also be used to rinse the hydrolysis tube to ensure complete recovery. The diluted sample (0.2 N H_2SO_4) is then used for the gallic acid determination.

Free gallic acid.

Free gallic acid is determined by adding sulfuric acid to the plant extract to make it 0.2 N H_2SO_4. The extract is then used for determination without hydrolysis. For the samples described above, after removing the acetone 1.0 mL of the sample is mixed with 1.0 mL of 4 N H_2SO_4, and the mixture is diluted to 10 mL with water. The diluted sample is used for gallic acid determination.

DETERMINATION OF HYDROLYZABLE TANNINS (GALLOTANNINS AND ELLAGITANNINS) AFTER REACTION WITH POTASSIUM IODATE

from Hartzfeld, Forkner, Hunter and Hagerman, J. Agric. Food Chem. 50: 1785-1790 (2002)

This functional group method for determining gallic acid esters was originally described by Haslam (Haslam, E. Phytochemistry 1965, 4, 495-498). The method was modified slightly by Bate-Smith (Bate-Smith, E.C., Phytochemistry 1977, 16, 1421-1426) and more recently was examined by Willis and Allen (Willis, R.B.; Allen, P.R. The Analyst 1998, *123*, 435-439). The KIO_3 method has been employed in some studies of oaks and maples, species rich in hydrolyzable tannins, to provide an estimate of those compounds in crude plant extracts (Schultz, J.C.; Baldwin, I.T. Science 1982, *217*, 149-151; Hunter, M.D.; Forkner, R.E. Ecology 1999, 80, 2676-2682).

The well known limitations of the method are consequences of the instability of the red oxidation product of the reaction between methyl gallate and KIO_3 Furthermore, the color yield is quite different for structurally distinct hydrolyzable tannins, and the dependence of product accumulation on reaction solvent, pH and temperature is poorly understood.

We modified the potassium iodate method so that the hydrolyzable tannins are converted to methyl gallate via methanolysis. The methyl gallate is reacted with KIO_3 and yields a chromophore. The protocols that can be implemented in simple laboratory settings with limited instrumental capabilities.

Reagents

Anhydrous methanol, reagent grade or better
Concentrated sulfuric acid, 18 M
Ethanolamine (a liquid, commercial preparation is 100% ethanolamine)
3.7 M ammonium acetate (281.3 g up to 1 liter with distilled water). Comes as a hygroscopic crystal, so after the bottle has been opened the crystals may be quite wet. Approximate the mass needed as accurately as possible, and keep the container tightly closed when not in use.
5.00 mg/mL methyl gallate (Sigma-Aldrich) dissolved in methanol
0.3 N HCl
5% (w/v) KIO_3, potassium iodate (5 g KIO_3 up to 100 mL with distilled water)

Methanolysis and pH adjustment.

To achieve consistent results with structurally diverse hydrolyzable tannins it is necessary to generate a common structural element from the various potential analytes. The common structural moiety for most of the hydrolyzable tannins is gallic acid, which can

be produced by acid hydrolysis of most gallotannins and ellagitannins. However, gallic acid is oxidized by KIO_3 to form a nonspecific yellow compound that is not useful for selective determination. We found that methyl gallate reacts with KIO_3 to form a unique red chromophore so conversion of hydrolyzable tannins to methyl gallate has been optimized using HPLC to assess acid strength, temperature and time required to maximize yield of methyl gallate. Acid strength, acid concentration, temperature, time and methanol purity were critical to efficient conversion. It was essential to use anhydrous methanol for the methanolysis, because even traces of water caused production of a mixture of gallic acid and methyl gallate. If the methanolysis tubes were not tightly capped during methanolysis, significant evaporation of the methanol during the 20 hours reaction caused problems in quantitation. Evaporation and other irreproducible results during methanolysis were minimized by using a heating block that held the temperature constant for the entire period with minimal cycling to higher or lower temperatures. The method was simplified by carrying out the methanolysis *in situ* in the plant tissue.

1. Weigh approximately 20 mg of dried, ground plant tissue into 16 x 125 mm (20 mL) pyrex screw top tube with Teflon cap liners; the top part of tube acts as a condenser during the methanolysis, so smaller tubes cannot be substituted. Record the exact mass of each sample.
2. Add 2.0 mL methanol and 200 μL of concentrated sulfuric acid (18 M). Tighten caps finger tight so that solvent will not evaporate during heating. Place in heating block previously brought to 85°C and allow to react for 20 h at 85°C.
3. Centrifuging briefly in a table top centrifuge (3000 *g*). Quantitatively transfer the supernatant to a 5.0 mL graduated cylinder by washing the methanolysis tube three times with minimum volumes of distilled water, and recentrifuging as necessary. Adjust the volume is adjusted to 3.0 mL with distilled water.
4. Add four 50 μL aliquots of ethanolamine (commercial preparation, 100% ethanolamine) with gentle swirling between each addition. The heat of neutralization is quite high so the ethanolamine must be added carefully and in small aliquots.
5. To buffer the sample, add 500 μL of 3.7 M ammonium acetate and adjust the pH to 5.5 ± 0.1 using a pH meter and small volumes of dilute ethanolamine or dilute sulfuric acid.
6. Bring the sample to a final volume of 4.0 mL with distilled water, and mix throughly. Store tightly capped at 4°C for up to 48 hours. A precipitate sometimes formed during storage; in that case the solution is recentrifuged briefly to remove the precipitate before analysis.

Standards and controls.

The method is standardized with methyl gallate. To control for any losses during methanolysis, 2.0 mL of 5.00 mg/mL methyl gallate dissolved in methanol is methanolyzed and pH-adjusted as described above for the plant samples.

A phenolic-free, pH 5.5, blank reagent is prepared by treating a mixture of 2.0 mL methanol and 200 μL concentrated sulfuric acid exactly as described above for the

samples. This blank solution did not contain any plant sample or standard polyphenolic compound, and is used to dilute the standards when preparing the standard curve.

Reaction with KIO_3

The pH 5.5 $\pm$ 0.1 samples described above are analyzed by reaction with potassium iodate, which formed a characteristic pigment with λ_{max} 525 nm. Some flavanoids and other plant consitutents reacted under these conditions form brownish pigments that absorbed weakly at 525 nm. To correct background color, each sample is analyzed in parallel with a reagent mixture and with a background mixture at low pH as described below.

1. Up to 100 µL of sample (methanolyzed and pH-adjusted sample comprised of plant tissue, purified tannin, or standard methyl gallate) is dispensed into a 2.0 mL microcentrifuge tube. The pH 5.5 blank reagent is added as necessary to bring the sample volume to 100 µL. For methanolysis products of most plants, a 100 µL aliquot of the methanolysis solution is used. For standards, the volume of pH-adjusted methyl gallate solution that is added to the microfuge tubes is varied from zero to 100 µL.
2. Water (350 µL) and methanol (1000 µL) are added and the samples are vortexed.
3. The tubes are tightly capped and placed in a 30 ^{o}C water bath.
4. A background mixture is prepared for each sample. The background samples are exactly like the reaction samples except that the 350 µL of water is replaced by 350 µL of 0.3 N HCl.
5. Add the KIO_3 is added at timed intervals. Exactly 40 µL of 5% (w/v) KIO_3 is added to each sample, the sample is capped, vortexed and returned to the 30^{o}C water bath.
6. Exactly 50 min after adding the KIO_3 to the sample its absorbance at 525 nm is recorded (vs. water).

Under these conditions, a linear relationship between methyl gallate and absorbance at 525 nm is obtained. A typical relationship with our spectrophotometer is:

$$\text{Abs} = 0.0132 * \text{ug} + 0.0701$$

R^2 = 0.996, standard error of Y = 0.0303, standard error of slope = 0.00019.

The limits of detection by this method are 1.5 µg methyl gallate, and the method is linear through at least 120 µg. There are no interferences when the method is used to determine methyl gallate after methanolysis of purified hydrolyzable tannins including tannic acid, epigallocatechin gallate, and oenothein B.

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DETERMINATION OF ELLAGITANNINS

A functional group method for ellagitannins based on Wilson and Hagerman, J. Agric Food Chem. 38, 1678-1683 (1990). Ellagitannins in the plant are hydrolyzed to release ellagic acid, which forms a colored product upon reaction with nitrous acid.

Reagents

2 N H_2SO_4

Wash solution: Acetone/water/conc HCl (70/30/1, v/v/v)

1 % $NaNO_2$ in water: 1.00 g $NaNO_2$ up to 100 mL with water.

Stock solution of ellagic acid. Ellagic acid (Sigma) 0.5 mg/ml. May be difficult to dissolve, use sonication if necessary.

Pyridine. You will use a lot of pyridine, which is stinky and must be used in a chemical fume hood. Pyridine should be discarded according to the advice of your safety officer, it should not be poured down the drain. Ideally, you should set up a small still and redistill the pyridine for reuse.

You will need a water bath (30 C) and glass cuvettes (pyridine dissolves plastic).

It is useful to have a filter manifold so you can filter several samples at once. We use a Radnoti filtration apparatus No 120750. You also need filter holders for 25 mm filters, and polycarbonate filters (e.g. Nucleopore SN:110607 PC MEMB 4 um) and glass fiber filters (e.g. Gelman type E).

Sample preparation.

Ellagitannins and ellagic acid are quite insoluble. Instead of analyzing extracted tannins, we analyze ellagic acid released upon hydrolysis of lyophilized plant material. Weigh about 50 mg samples (record exact weight) of dry material into Pyrex test tubes which have been constricted about half way down. Add 5.0 mL 2 N H_2SO_4, freeze in isopropanol-dry ice slush, and attach to a vacuum pump. Use a small glass blowers torch (gas/oxygen) to seal the tube at the constriction while it is under vacuum. Place the tubes in a rack to melt. As they melt, bubbles should be seen forming in the liquid. If the samples do not bubble, there is a leak in the seal and you must try again. It is difficult to learn to do this, so practice on some less important samples.

The melted samples are placed in a 100 C oven for hydrolysis for 10 h. The oven should be clearly labeled as containing samples under pressure, and must only be opened be people wearing splash resistant chemical goggles and a lab coat. Occasionally a tube does explode during heating.

After hydrolysis, allow the tubes to cool. Open the tubes, and chill them in a ice bath. Vacuum filter the sample (the ellagic acid is insoluble in ice-cold solvent, you are washing away pigments) on the filter manifold using the polycarbonate filters. Wash the insoluble material several times with ice cold wash solvent. Discard all of the washings.

Carefully place the membrane and all of the insoluble material into a test tube, and add 10.0 ml pyridine. Vortex to dissolve the ellagic acid and most of the filter. Insoluble plant material, and perhaps some bits of the filter, will still be present.

Filter the solution with a glass fiber filter to remove the insoluble material, saving the pyridine solution.

Determination of ellagic acid.

Use clean, preferably new small test tubes. Occasionally there is a residue on washed tubes that inhibits the reaction.

Samples should be in a total volume of 2.1 mL pyridine. Use volumes of the ellagic acid standard to give 0-50 ug ellagic acid and an appropriate volume of your plant hydrolysate (between 0.1 and 2.1 mL, often 0.5 ml is appropriate).

INCUBATIONS AND VORTEXING MUST BE DONE IN A CHEMICAL FUME HOOD

Add 0.1 mL conc HCl to samples, vortex. Place in a 30 C water bath for at least 5 min. At time zero, add 0.1 ml $NaNO_2$, vortex, and read absorbance at 538 nm. Place sample back in water bath. Exactly 36 min after adding the $NaNO_2$, again read absorbance at 538 nm.

The difference between the absorbances is a function of the ellagic acid concentration.

Timing is critical because the color continues to change with time during the reaction. You cannot go back and read the absorbance of a sample again, but must repeat the assay if a mistake is made.

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PENTAGALLOYL GLUCOSE

Pentagalloyl glucose is easily prepared from appropriate preparations of tannic acid. The method described here is from Hagerman, A.E.; Zhao, Y.; Johanson, S. Methods for Determination of Condensed and Hydrolyzable Tannins. In Antinutrients and Phytochemicals in Foods (Shahadi, F., ed.). American Chemical Society, Washington DC, 1997; pp. 209-222.

Reagents:

Acetate buffer (0.1 M) pH 5. Add 0.57 mL glacial acetic acid to 80 mL distilled water, titrate to pH 5 with NaOH solution, then bring volume to 100 mL with distilled water.

Methanolysis solution: Mix 70 mL methanol with 30 mL of the acetate buffer.

Ethyl acetate

Diethyl ether (peroxide-free)

Method:

In a test tube, dissolve 0.5 g tannic acid in 10 mL of the methanolysis solution. Cover, and place in 65oC water bath for 15 h.

Immediately raise pH to 6, while stirring, with 0.25 M NaOH. Evaporate under reduced pressure (rotary evaporate) at temperatures below 30oC to remove methanol. As the methanol is removed, add water to maintain volume. Do not let the sample go to dryness.

After removing all of the methanol, extract the aqueous solution three times with diethyl ether (in a small separatory funnel). The PGG should be in the aqueous (bottom) layer.

Extract the aqueous layer three times with ethyl acetate. Combine the ethyl acetate extractions and rotary evaporate to reduce the volume. Add water to maintain the volume, and continue to evaporate to remove all of the organic solvent. The aqueous suspension should be centrifuged and suspended in 2% methanol /98% water, and re-centrifuged three times. Methanol is removed by rotary evaporation and the aqueous

Analysis:

The starting material, and the various fractions at each step of the procedure, should be monitored by normal phase HPLC to verify that methanolysis occurred (loss of late eluting peaks, large increases in methyl gallate and PGG). The final product purity should be assessed by HPLC and by proton nmr in

deuterated acetone. The assignment of peaks in the nmr will confirm that the product is PGG, possibly contaminated with a trace of methyl gallate and gallic acid.

Proton nmr (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).

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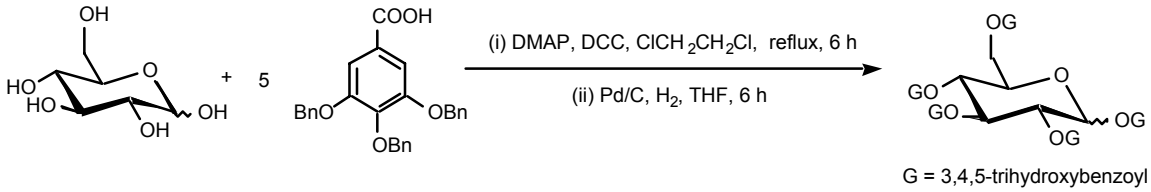
Preparation of 1,2,3,4,6-Penta-O-galloyl-[U-^{14}C]-D-glucopyranose

Chen, Y.; Hagerman, A.E.; Minto, R.E., *J. Radiolabeled Compounds* submitted May, 2002

Summary

The synthesis of 1,2,3,4,6-penta-*O*-galloyl-[U-^{14}C]-D-glucopyranose is described. [U-^{14}C]glucopyranose was reacted with tri-*O*-benzylgallic acid forming 1,2,3,4,6-penta(tri-*O*-benzylgalloyl)-[U-^{14}C]-D-glucopyranose as chromatograpically separable anomers. Removal of the benzyl group by catalytic hydrogenation afforded 1,2,3,4,6-penta-*O*-galloyl-[U-^{14}C]-D-glucopyranose in 54% overall yield.



Introduction

Hydrolyzable tannins are plant secondary metabolites that are synthesized by a wide variety of plants [1]. Their building blocks are gallic acid and polyols, typically glucose, with ester bonds formed between the gallic acid and polyol residues. Hydrolyzable tannins are found in human foods, animal feeds, and medical herbs [1-4]. The fate of hydrolyzable tannin after ingestion is still elusive. The central compound in the biosynthetic pathway for hydrolyzable tannins is 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (PGG), and all the other hydrolyzable tannins with more than five galloyl moieties are derived from it.[1, 5] PGG can bind proteins, inhibit enzymes, and has substantial antioxidant activity.[1-4, 6] Availability of radiolabeled PGG would facilitate studies of protein-binding, enzyme inhibition, and antioxidant activities, and would provide a probe for tracing the fate of hydrolyzable tannins after ingestion. *In vivo* incorporation of $^{14}CO_2$ using staghorn sumac leaves has been previously reported for the biosynthesis of [U-^{14}C]-PGG.[7] We now report a chemical synthesis of PGG uniformly [^{14}C]-labeled at the hexose. With chemical synthesis, a higher proportion of the radiolabeled precursor is incorporated into the desired product. Furthermore, modification of our procedure will allow synthesis of structural variants of PGG that are not biosynthetically accessible.

Results and Discussion

In the synthesis of 1,2,3,4,6-penta-*O*-(tri-*O*-benzylgalloyl)-[U-^{14}C]-D-glucopyranose (BnPGG, **1**), 1,2-dichloroethane was used as solvent instead of dichloromethane as had previously been reported.[8] This modification reduced the reaction time from 36 h to 6 h. Centrifugal thin-layer chromatography (TLC), using a Chromatotron, was an improvement over column chromatography for the purification. Clean separation of the impurities from the products was easily achieved, and less [^{14}C]-contaminated solvent waste was produced. The separation of protected PGG anomers could also be achieved using centrifugal TLC with a 2 mm silica plate. In order to do this, the crude sample was first purified and a 20 mg aliquot of the anomeric mixture of **1** was applied to the Chromatotron for resolution of the anomers. After removing the benzyl groups, pure α- and β-[U-^{14}C]PGG were obtained (Fig. 1). The mass yield and radioactivity recovery from this synthesis were 60% and 55%, respectively.

No purification was required after the catalytic hydrogenation **ii**. Toluene, the byproduct from the cleavage of the benzyl groups by hydrogenation, was removed under reduced pressure. A purity of at least 95%, as determined by HPLC, was routinely achieved for the final product **2** (Fig. 1). Mass yield and radioactivity recoveries were 90% and 93%, respectively. As unlabeled glucose was added at the outset of the synthesis as a diluent, the specific radioactivity of the final product was 1.19 mCi/mmol, starting with 1 mCi of D-[U-^{14}C]glucopyranose (specific activity 4.68 mCi/mmol).

The overall mass yield and radioactivity recovery were 54% and 51%, respectively, providing a direct convenient route to the radiolabelled hydrolyzable tannin **2**.

Experimental

[U-^{14}C]-D-Glucopyranose in a ethanol/H_2O solution (0.1 mCi/mL, 4.68 mCi/mmol) was purchased from PerkinElmer Life Science, Inc. (Boston, MA). All other reagents (ACS reagent grade) were purchased from Aldrich (St. Louis, MO). Before use, 1,2-dichloroethane was distilled. All other chemicals were used without further purification.

Radioactivity was quantified with a Wallac 1409 liquid scintillation analyzer (Turku, Finland). UV spectra were taken in acetonitrile solution with an Agilent 8453 UV-visible spectrometer (Agilent Technologies, Palo Alto, CA). The Chromatotron (Harrison Research, CA Model 8924) was equipped with a pump (Model RHSY, Fluid Metering Inc., NY). A Hewlett Packard 1050 HPLC system with two pumps, an autosampler and UV-detector was equipped with a C-18 column (Adsorbosphere XL C18 90 Å, 3 μm, 100 × 4.6 mm, Alltech, Deerfield, IL). Mobile phase A was water containing 0.1% acetic acid (v/v) and mobile phase B was acetonitrile containing 0.1% acetic acid (v/v). The flow rate was 1 mL/min. The UV detector was set at 220 nm. The gradient started at 5% B, increased to 100% B in 3 min, then returned to 5% B in 3 min, and the column was re-equilibrated for 7 min.

The reaction conditions were optimized using unlabeled reagents. Identities and purities of natural abundance intermediates and products were determined by ^{1}H and $^{13}C\{^{1}H\}$ NMR spectroscopy (Bruker 200 MHz Avance spectrometer), electrospray mass spectrometry (Campus Chemical Instrumentation Laboratory, Dept. of Chemistry, The Ohio State University), and UV-visible spectra (Fig. 2), and were in agreement with previously reported data [9]. The identities of the [^{14}C]-labeled intermediate and final products were established by HPLC co-elution with authentic unlabelled compounds.

1,2,3,4,6-Penta-*O*-(tri-*O*-benzylgalloyl)-[U-^{14}C]-D-glucopyranose

[U-^{14}C]-D-Glucopyranose (0.5 mCi) was transferred into a 50-mL 3-necked round-bottom flask. The solvent was removed under reduced pressure using a rotary evaporator and benzene was added to azeotropically eliminate residual H_2O. Anhydrous D-(+)-glucose (31 mg) was added to provide a total of 50 mg glucose (0.28 mmol, 1.0 eq). Tri-*O*-benzylgallic acid (0.915 g, 2.08 mmol, 7.5 eq),[10] 1,3-dicyclohexylcarbodimide (DCC, 0.46 g, 2.22 mmol, 8 eq), and 4-(*N*,*N*-dimethylamino)pyridine (DMAP, 0.270 g, 2.22 mmol, 8 eq) were added. After the addition of 1,2-dichloroethane (15 mL), the reactants were stirred and refluxed for 6 h under a N_2 atmosphere. The reaction mixture was cooled to room temperature, diethyl ether (15 mL) was added, and the mixture was incubated in ice-bath for 1 h. The suspension was vacuum filtered through a Celite pad, and the filtrate was washed with water (2 × 30 mL), saturated $NaHCO_3$ solution (3 × 30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4 and the solvent was removed using a rotary evaporator under reduced pressure. The resulting residue was dissolved in CH_2Cl_2 (8 mL) and insoluble impurities were removed by filtering through a sintered glass funnel. The filtrate was loaded onto the Chromatotron (2 mm plate) and eluted with CH_2Cl_2 (7 mL/min). When the first short-wavelength UV-active band eluted, the eluent composition was shifted to CH_2Cl_2:acetone (200:1). The second and third bands were collected. Following solvent removal, 0.40 g (60%) of the anomeric mixture of **1** was obtained as an off-white foam. Separation of 20 mg of the anomeric mixture was routinely achieved by a second Chromatotron separation using the same conditions. After purification, washing the Chromatotron plate with 200 mL methanol removed all impurities on the plate.

1,2,3,4,6-Penta-O-D-[U-^{14}C]-Glucopyranose

In a 50-mL round-bottom flask, **1** (anomeric mixture, 0.40 g, 0.23 mmol) and 10% Pd/C (1.04 g) were suspended in dry THF (10 mL) and the apparatus was flushed with N_2 thrice. A H_2 atmosphere, provided with a balloon, was attached to the flask. After 6 h, the reaction mixture was flushed with N_2 and acetone (5 mL) was added. After vacuum filtration through a Celite pad, evaporation of solvent with a rotary evaporator yielded an anomeric mixture of 1,2,3,4,6-pentagalloyl-*O*-D-[U-^{14}C]glucopyranose (0.36 g, 90%. 1.19 mCi/mmol). An identical procedure was successful with the individual isomers.

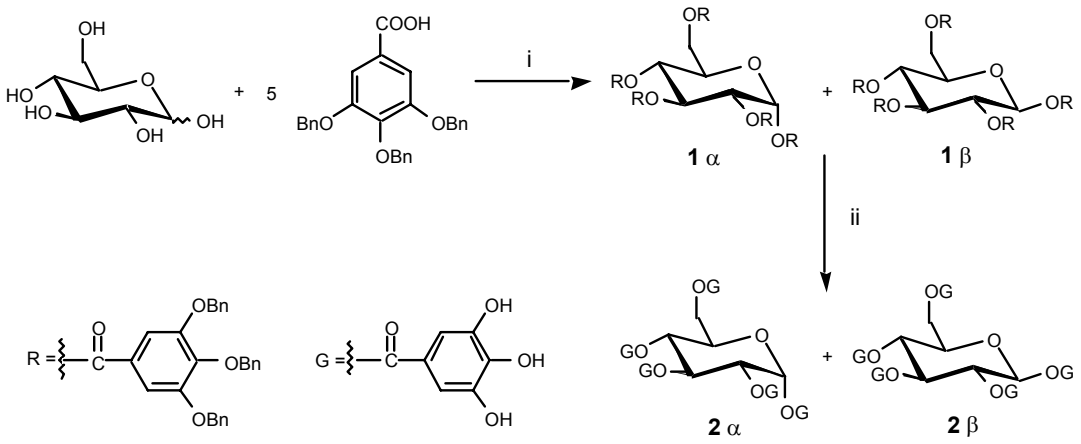
References

1. Haslam, E, Lilley, TH, Cai, Y, Martin, R, Magnolato, D. *Planta Medica* **1989**; 55: 1-8.

2. Zhu M, Phillipson D, Greengrass PM, Bowery N, Cai Y. *Phytochemistry* **1997**; 44: 441-447.

3. Hagerman, AE, Riedl, KM, Jones, GA, Sovik, KN, Ritchard, NT, Hartzfeld, PW, Riechel, TL. *J Agric Food Chem* **1998**; 46: 1887-1892.

4. Hagerman AE, Carlson DM. *Recent Res Devel Agric Food Chem* **1998;** 2: 689-704.
5. Hofmann, AS, Gross, GG. *Arch Biochem Biophys* **1990**; 283: 530-532.
6. Feldman KS, Sahasrabudhe K, Lawlor MD, Wilson SL, Lang CH, Scheuchenzuber WJ. *Bioorg Med Chem Lett* **2001**; 11: 1813-1815.
7. Rausch H, Gross GG. *Z Naturforsch, C: Biosci* **1996**; 51: 473-476.
8. Khanbabaee K, Lotzerich K. *Tetrahedron* **1997**; 53: 10725-10732.
9. Polya GM, Wang BH, Foo LY. *Phytochemistry* **1995**; 38: 307-314.
10. Morris SG, Riemenschneider RW. *J Am Chem Soc* **1946**; 68: 500-501.



Scheme 1. Reagents and conditions: (i) DMAP, DCC, $ClCH_2CH_2Cl$, reflux, 6 h; (ii) Pd/C, H_2, THF, 6

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EXTRACTION AND PURIFICATION OF SORGHUM TANNIN

This method for purifying tannin from Sorghum grain yields a very high quality procyanidin. The original method (Hagerman and Butler J. Agric. Food Chem. 28, 947-952,1980) included a phenol extraction step to ensure that the product was essentially protein free. However, the phenol extraction step has been omitted from these instruction since it is rarely essential. These instructions can be modified as needed to purify tannin in large quantity from virtually any plant source.

Because Sorghum tannin is not commercially available, some workers prefer to purify commercial quebracho tannin for use as an analytical standard.

Reagents:

Absolute ethanol containing 10 mM ascorbic acid. Dissolve 1.76 g ascorbic acid in 1000 mL ethanol.

Methanol containing 10 mM ascorbic acid. Dissolve 1.76 g ascorbic acid in 1000 mL methanol. Prepare fresh, because ascorbic acid rapidly oxidizes in solution. It is functioning as an antioxidant during extraction steps.

0.05 M acetate pH 4. Dilute 2.85 mL glacial acetic acid with about 800 mL distilled water. Adjust the pH to 4.0 with a concentrated solution of NaOH. Bring the final volume to 1 liter with distilled water.

50% acetone. Mix equal volumes of acetone and distilled water.

Method:

In cold room, grind 200 g dry, mature high tannin (bird resistant) Sorghum grain in Waring blender. Add 600 mL absolute ethanol containing 10 mM ascorbic acid. Stir for 45 min with a stirrer from above; centrifuge (or filter) and discard this extract (contains low molecular weight phenolics).

Extract the ground grain 4x with 150 mL methanol containing 10 mM ascorbic acid. Each extraction should be for about 45 min-1 h, in cold room, stirring from above. After each extraction, the samples are centrifuged and the tannin-containing supernatant is saved. The extract should be filtered to clarify if necessary. An equal volume of 0.05 M acetate pH 4 is added to the extract, yielding a cloudy orange solution.

The methanol is completely removed by rotary evaporation at 30C. After extractions are complete, and all methanol has been removed, the pooled extract is purified.

The tannin-containing solution is extracted 3x with 300 mL ethyl acetate. For each extraction, the sample is shaken and the lower (aqueous) phase is saved. The upper layer and the interface are discarded.

The sample is rotary evaporated at 30C to a total volume of about 20 mL, and absolute ethanol is then added to make the final sample solvent 80% ethanol.

The sample is applied to about 4 volumes of Sephadex LH20 slurry in a coarse sintered glass funnel. The LH20 should be equilibrated in 80% ethanol. Use very gentle suction to pull the liquid through the beads; stir the beads gently with a glass rod. You want the tannin to have maximum opportunity to contact and adsorb to the beads. Most of the brown color (tannin) will adsorb to the beads.

Wash the beads with absolute or 95% ethanol, gently mixing and then filtering, several times until the eluate no longer absorbs light in the UV. Discard all of this eluate. It may take a fairly large volume of ethanol.

Wash the beads with 50% acetone, saving the washes. The washing should again be done slowly and gently. As the tannin is washed off the beads in the acetone, start to rotary evaporate at 30oC to remove acetone from the solution. Combine the washes from the beads. When the washes are no longer dark brown, the amount of tannin being recovered is quite low, and can be discarded. The beads should eventually be restored to pure white; they can be stored overnight in 50% acetone to wash the remainder of the tannin off of them. The beads should then be re-equilibrated in 80% ethanol and stored tightly covered in the refrigerator until next time they are needed.

After removing all of the acetone from the tannin, the tannin should be freeze dried and stored in glass vials in a freezer. It can be characterized spectroscopically or by degradative methods.

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DETERMINATION OF DEGREE OF POLYMERIZATION--HPLC OF PHLOROGLUCINOL DERIVATIVES

Modified from Foo and Karchesy, Phytochemistry 30, 667-670 (1991); Koupai-Abyazani et al. J. Chrom. 594, 117-123 (1992).

Quantitative application for molecular weight determination in Schofield, Hagerman & Harold, J. Chem. Ecol. August 1998.

Proanthocyanidins are incubated with phloroglucinol in acidic ethanol. The interflavan bonds break and the extender units react to form the phloroglucinol adduct. The adduct, and unreacted terminal flavanols, are extracted into ethyl acetate and analyzed by reverse phase HPLC.

For (4->8) or (4-> 6) procyanidins the adducts are catechin or epicatechin, substituted at position 4 by phloroglucinol. The terminal unit is unmodified catechin or epicatechin. It is believed that (4-> 6) interflavan bonds are less easily cleaved than the (4 ->8) interflavan bonds.

Reagents:

Acidic ethanol. 1% HCl in ethanol--make 1.0 mL conc HCl up to 100 mL with absolute ethanol

Phloroglucinol solution. 5 mg/mL phloroglucinol in the acidic ethanol. Prepare fresh daily.

70% aqueous methanol

Reaction with pure proanthocyanidin standards:

Pure proanthocyanidin (1 mg) is dissolved in 150 uL of the phloroglucinol solution and allowed to react at room temperature overnight. The solvent is then evaporated under nitrogen, and the residue dissolved in 50 uL distilled water. This solution is extracted three times with ethyl acetate (150 uL per extraction). The three ethyl acetate fractions are combined and evaporated under nitrogen. The residue is dissolved in 100 uL of 70% aqueous methanol and then injected onto the HPLC.

A parallel sample is run substituting acidic ethanol for the phloroglucinol solution.

Purified catechin, epicatechin and phloroglucinol can be used to identify those peaks on the chromatograms. The usual order of elution is phloroglucinol; epicatechin phloroglucinol, catechin phloroglucinol (as a pair); catechin; epicatechin. A purified proanthocyanidin of known composition is

used to confirm the identity of the phloroglucinol adducts. For example, Sorghum proanthocyanidin is comprised of catechin terminal units and predominantly epicatechin extender units.

Purified proanthocyanidin does not have any low molecular weight, ethyl acetate soluble constituents. Because high molecular weight procyanidins are difficult to resolve on HPLC, the samples in which acidic alcohol is substituted for the phloroglucinol should not show any peaks on the HPLC.

Average chain length for the purified proanthocyanidin can be determined by comparing the production of the phloroglucinol-derivitized extenders and of the terminal units. A standard curve must be prepared to convert peak areas to molar concentrations, using pure authentic samples of monomers and adducts.

HPLC system.

Solvent A: 1% aqueous acetic acid (1.0 mL glacial acetic acid up to 100 mL with distilled & purified water).

Solvent B: Methanol:Solvent A, 60:40, v/v

Column: Ultrasphere 5 RP-18 (4.6 mm x 25 cm) (Beckman)

Guard column: 4 x 4 mm, RP-18 packing

Runs: Ambient temperature, 1 mL/min, 20 uL injection loop, 280 nm detection

Linear solvent gradient: t = 0, 100% solvent A; t = 60 min, 40% solvent A, 60% solvent B; t = 65 min, 100% solvent B

Assay of crude extracts.

Sample preparation: The aqueous acetone extract from leaves or other plant tissues is evaporated under nitrogen. The residue is dissolved in 150 uL of phloroglucinol solution and incubated at room temperature overnight. The sample is evaporated with nitrogen, the residue dissolved in 100 uL distilled water, and extracted three times with ethyl acetate (300 uL per extraction). The ethyl acetate extracts are combined, evaporated under nitrogen, and dissolved in 75 uL of 70% methanol for separation by HPLC.

For each extract, a control sample is run, substituting acidic ethanol for the phloroglucinol solution.

The terminal units and phloroglucinol-derivitized extender units are identified by comparison to the standards. The chromatograms are far more complex than for the purified proanthocyanidins, because crude plant extracts contain many ethyl acetate soluble components such as small, nontannin phenolics. The chromatograms of the control samples (not reacted with phloroglucinol) will provide quantitative

information about those peaks.

Comparison of peak areas can be used as above for determination of an average chain length for the proanthocyanidins.

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ACID BUTANOL ASSAY MODIFIED WITH PVP

Watterson and Butler J. Agric. Food Chem. 31, 41-45 (1983)

This is a method for preventing interference from chlorophyll, which absorbs at 550 nm, from the acid butanol assay. The method is tedious, so should be used only if interference seems to be substantial.

The method allows simultaneous determination of leucoanthocyanidins (flavan-3,4-diols and flavan-4-ols), proanthocyanidins and 3-deoxy proanthocyanidins. The absorbance in the acid butanol assay is read before and after heating so both the heat stable anthocyanidins and heat labile 3-deoxy anthocyanidins are measured.

Reagents.

Insoluble polyvinylpyrrolidone (Polyclar AT) (e.g. Sigma P-6755). Boil the PVP for 10 min in 10% HCl, then decant the fines. Filter and dry the PVP.

Acid butanol: Mix 950 mL of n-butanol with 50 mL concentrated HCl

Iron reagent: 2% Ferric ammonium sulfate in 2 N HCl. Bring 16.6 mL of concentrated HCl up to 100 mL with distilled water to make 2 N HCl. Dissolve 0.5 g $FeNH_4(SO_4)_2$ x 12 H_2O in 25 mL of 2 N HCl. Store in a dark bottle.

Method:

Place about 0.2 g PVP in a 13x100 mm screw cap culture tube.

Add 1.0 mL of chlorophyll-containing plant extract.

Add 5.0 mL of reagent grade methanol and mix for 5 min. Spin to pellet the PVP (10 min, 2000 x g). Discard the chlorophyll containing supernatant. Repeat this step until the supernatant is colorless--usually three washings of the PVP.

Add 7.0 mL of butanol-HCl to the PVP. Add 0.2 mL of the iron reagent.

Agitate on a Lab-Quake mixer at room temperature for 1 hour. Centrifuge.

Read A550 of the supernatant. Then return the supernatant to the PVP-containing sample tubes (any absorbance at this stage of the assay is due to flavan-4-ols and/or 3-deoxy proanthocyanidins).

Heat the tubes at 95 C for 1.5 h with caps loosely screwed on. Cool to room temperature and centrifuge to pellet the PVP.

Read A550 of the supernatant. Subtract the absorbance obtained before heating, and compare the absorbances to those of standards.

The standards must be run according to this procedure even if they do not contain chlorophyll, because adsorption to PVP alters the color yield of the reaction.

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VANILLIN ASSAY

Price, van Scoyoc, and Butler, J. Agric. Food Chem. 26, 1214-1218 (1978)

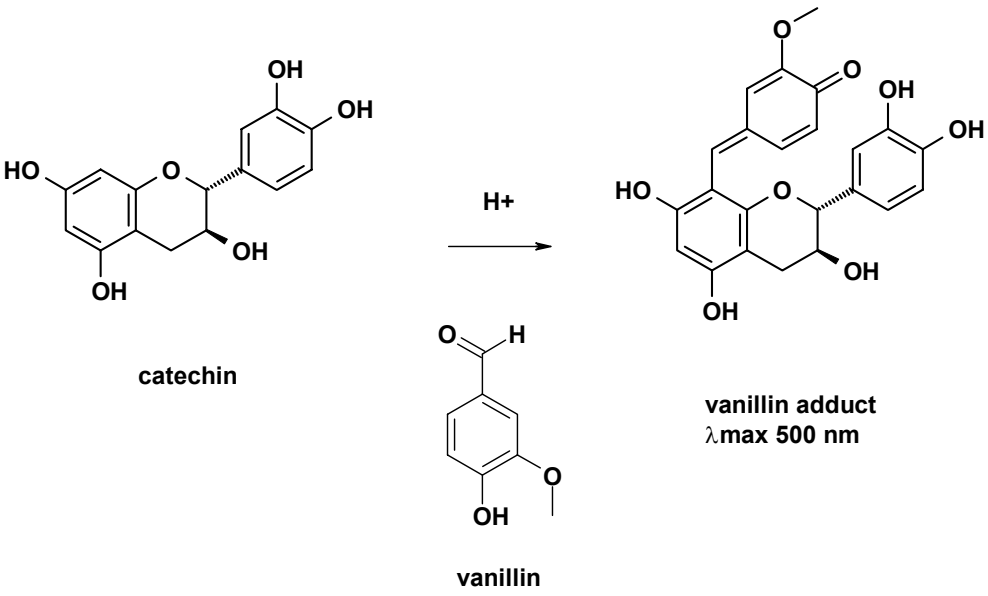
This functional group method for condensed tannins is especially widely used by agronomists. There are significant difficulties in interpretation of the method. We rarely use this method in my lab, since I find the acid butanol method simpler and more reliable.

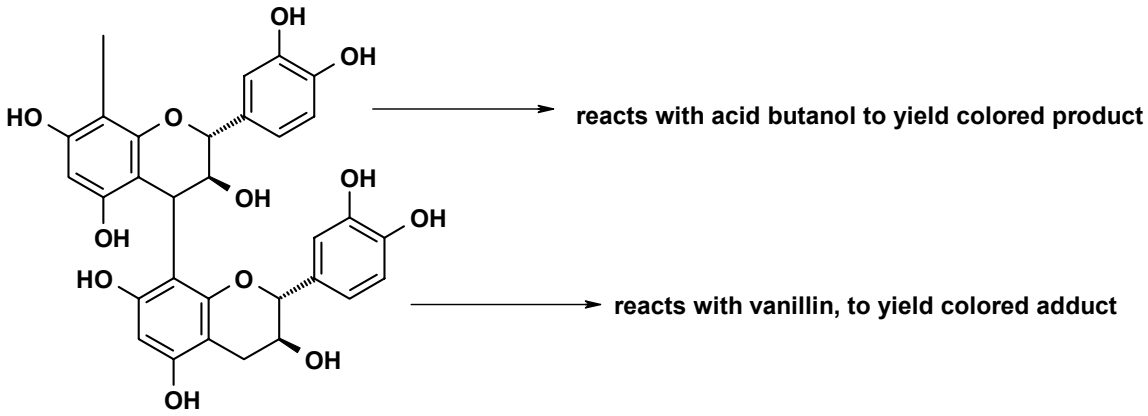
Understanding the structural chemistry of condensed tannins and flavanoids is essential to proper use of the vanillin assay. The vanillin reaction involves reaction of an aromatic aldehyde, vanillin, with the meta-substituted ring of flavanols to yield a red adduct. Although the vanillin reaction has been widely used to estimate condensed tannin (proanthocyanidin), the reaction is not specific for condensed tannins. Any appropriately substituted flavanol reacts in the assay. Thus the formal "monomer" of the condensed tannins, catechin, also reacts to yield a red colored adduct. Furthermore, because the vanillin reacts only with meta-substituted flavanoids, the 5-deoxy proanthocyanidins (e.g. quebracho) do not produce much color with vanillin. Reactivity with vanillin is not sufficient evidence for the presence of condensed tannins.

Catechin is commonly used to standardize the vanillin reaction, but there are problems with interpreting the meaning of "catechin equivalents". Under the normal conditions for the vanillin assay (methanol solvent), tannins (proanthocyanidins) and catechin both react with vanillin, but the rates of reaction of the polymer and the monomer are quite different. In general, the color yield is lower for the monomer than for the polymer. Although the absorbances obtained from running the vanillin reaction in methanol on an unknown tannin-containing sample can be converted to "catechin equivalents" the complexities of the system make it difficult to interpret the meaning of those equivalents on the molecular level. The modified vanillin method was developed to overcome those problems, but proves to be more useful for estimating molecular weight of condesned tannin than for quantitative analysis.

The vanillin method described here was developed for analysis of condensed tannin in Sorghum grain; modification of the methods for sample preparation would probably be necessary to use the method with other tissues. The method described here uses 0.5% vanillin rather than the 2% originally recommended by Burns. By decreasing the vanillin concentration, the dependence of the reaction on temperature is minimized.

The vanillin reaction is very sensitive to the presence of water. Even a small amount of water in the reaction mixture will substantially quench color yield. All standards should be prepared in anhydrous organic solvents (usually methanol). If water must be present in the samples to be analyzed, the same amount of water should be added to the standards.





proanthocyanidin

Vanillin reacts only with free flavan-3-ols, or with the terminal unit of the proanthocyanidin. The vanillin method combined with the acid butanol method provides an estimate of degree of polymerization.

Vanillin method **Reagents:**

1% vanillin in methanol (1.0 g vanillin up to 100 mL with absolute methanol). Store in a dark bottle at 4C

8% concentrated HCl in methanol (8.0 mL concentrated HCl brought to 100 mL with absolute methanol).

4% concentrated HCl in methanol (4.0 mL concentrated HCl brought to 100 mL with absolute methanol).

Constant temperature water bath set at 30C. (If this is not available, there will be temperature-dependent variation in the data).

0.3 mg/mL catechin (3.0 mg catechin brought to 10.0 mL with absolute methanol). Store in a dark bottle at 4oC for up to three days.

Preparation of Working Reagents:

The working vanillin reagent must be prepared daily from the solutions described above. One part of the 1% vanillin solution is mixed with one part of the 8% HCl solution.

The working vanillin reagent and the 4% HCl solution are brought to 30oC in the water bath before starting the analysis each day.

Extraction:

For best results, the extraction and analysis should be carried out on a single day. The grain should be ground no more than one day in advance of analysis.

About 200 mg ground Sorghum grain is weighed exactly, and then extracted with 10.0 mL absolute methanol for 20 min in rotating (Labquake rotator) screw cap culture tubes (13x100 mm). The mixture is centrifuged for 10 min at 3000 x g, and the supernatant is used in the analysis.

Analysis of Standards:

0 to 1.0 mL aliquots of the catechin standard are dispensed into two sets of culture tubes and each sample is brought to 1.0 mL by the addition of absolute methanol. Tubes are incubated in the water bath.

5.0 mL of the working vanillin reagent is added at 1.0 min intervals to one set of standards, and 5.0 mL of the 4% HCl solution is added at 1.0 min intervals to the second set of standards.

The samples are left in the water bath for exactly 20.0 min, and are then removed and the absorbance at 500 nm is read. Because the color continues to develop as time passes, you cannot go back and re-read any sample. You must maintain the strict 1.0 min intervals for reading that you used in the addition of reagents.

The absorbance of the blank (vanillin reagent with no catechin) is subtracted from the absorbance of the corresponding vanillin-containing sample. A standard curve is constructed (Abs vs. mg catechin) and the linear portion of the curve is extrapolated to produce the standard curve.

Analysis of Sorghum extracts:

1.0 mL aliquots of each extract are dispensed into culture tubes. Tubes are incubated in the water bath. Duplicates of each sample are required so that the sample and a blank can be run for each sample.

5.0 mL of the vanillin reagent is added at 1.0 min intervals to one set of samples and 5.0 mL of the 4% HCl solution is added at 1.0 min intervals to the second set of samples (the blanks).

The samples are left in the water bath for exactly 20.0 min, and are then removed and the absorbance at 500 nm is read. Because the color continues to develop as time passes, you cannot go back and re-read any sample. You must maintain the strict 1.0 min intervals for reading that you used in the addition of reagents.

The absorbance of the blank is subtracted from the absorbance of the corresponding vanillin-containing sample. The blank can be substantial for tissues that contain large amounts of pigments. The value obtained is compared to the standard curve to obtain "catechin equivalents".

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MODIFIED VANILLIN ASSAY (FOR MOLECULAR WEIGHT ESTIMATION)

One major problem with the vanillin assay (methanol) is the different kinetics of the reaction of catechin and tannin, which makes it difficult to use catechin as a valid standard for determining tannin (Butler, Price and Brotherton J. Agric. Food Chem., 30, 1087-1089,1982). It is well known that changing the solvent for a reaction can dramatically change rates of the reaction, and the modified vanillin assay takes advantage of this effect of solvent on kinetics.

When the vanillin reaction is run with glacial acetic acid as the solvent, tannin (proanthocyanidin) and catechin react with similar kinetics. The similar kinetics are probably the result of the specificity of the reaction between vanillin and condensed tannin in glacial acetic acid. Only the terminal units of the tannin react with vanillin in glacial acetic acid.

The site-specificity of the reaction can be exploited to estimate the degree of polymerization of purified condensed tannins. The absorbance obtained in the vanillin (glacial acetic acid) assay for equal weights of tannin and of tannin monomer is directly compared to estimate degree of polymerization of the tannin. As with the vanillin assay itself, this method cannot reliably be used with 5-deoxy proanthocyanidins (e.g. quebracho), since a meta-substituted ring is required for reaction with vanillin.

Methanol diminishes the color yield of the reaction. If the tannin monomer and polymer solutions do not dissolve in glacial acetic acid, they can be dissolved in a minimal volume of methanol and then diluted with glacial acetic acid. For example, 1 part methanol to 4 parts glacial acetic acid can be used as the solvent. The amount of methanol in all samples that are to be compared must be the same.

It is not recommended that the vanillin assay (glacial acetic acid) be used to quantitate tannin in crude extracts. The vanillin assay (methanol) is preferable for quantitation for two reasons: First, the vanillin assay (methanol) is not sensitive to methanol in the extracts. Second, monomers which may be present in crude plant extracts yield less color in the vanillin (methanol) assay than do polymers, and so cause less interference.

Reagents.

1% vanillin in glacial acetic acid (1.0 g vanillin brought to 100 mL with glacial acetic acid). Store in a dark bottle at 4oC.

8% concentrated HCl in glacial acetic acid (8.0 mL concentrated HCl brought to 100 mL with glacial acetic acid).

Constant temperature water bath set at 30oC. (If this is not available, there will be temperature-dependent

variation in the data).

0.05 mg/mL tannin monomer (catechin, for the common procyanidins) (0.5 mg catechin dissolved in the minimum volume of methanol, and then brought to 10.0 mL with glacial acetic acid). Store in a dark bottle at 4oC for up to three days.

0.1 mg/mL tannin (1 mg purified tannin dissolved in the minimum volume of methanol and then brought to 10.0 mL with glacial acetic acid). Prepare fresh each day.

Preparation of Working Reagents:

The working vanillin reagent must be prepared daily from the solutions described above. One part of the 1% vanillin solution is mixed with one part of the 8% HCl solution. The working vanillin reagent is brought to 30oC in the water bath before starting the analysis.

Method:

0 to 1.0 mL aliquots of the samples (catechin and condensed tannin) are dispensed into culture tubes and each sample is brought to 1.0 mL by the addition of glacial acetic acid. Tubes are incubated in the water bath.

5.0 mL of the working vanillin reagent is added at 1.0 min intervals to the samples.

The samples are left in the water bath for exactly 20.0 min, and are then removed and the absorbance at 510 nm is read. Because the color continues to develop as time passes, you cannot go back and re-read any sample. You must maintain the strict 1.0 min intervals for reading that you used in the addition of reagents.

The absorbance of the blank (reagent with no tannin) is subtracted from the absorbance of the corresponding vanillin-containing sample. Two standard curves are constructed, and each is checked for linearity and for zero intercept. The absorbance value obtained with a certain weight of catechin is divided by the absorbance value obtained with the same weight of condensed tannin to estimate the absolute degree of polymerization. Butler, Price and Brotherton J. Agric. Food Chem., 30, 1087-1089,1982 provides data which can be used to validate the assay.

This method has not been validated for branched chain condensed tannins. It is likely that all the terminal units of a branched chain condensed tannin will react with vanillin, so the apparent molecular weight of these polymers will be low. However, further investigations into the chemistry of the reaction are required to confirm this assumption.

VANILLIN/ACID BUTANOL RATIO FOR DEGREE OF POLYMERIZATION

Without pure proanthocyanidin, the modified vanillin method cannot be used to directly estimate degree of polymerization. In that case, the relative degree of polymerization for the crude extracts can be estimated by comparing the absorbance obtained with the modified vanillin assay (only terminal units react) with the absorbance obtained with the acid butanol assay (all units except the terminal unit react).The best application of this method is probably monitoring changes in tannins in a single species. Butler (J. Agric. Food Chem. 1982, 30, 1090-1094) described that type of study, following tannins during maturation of Sorghum grain.

This method is only useful for comparisons of structurally identical tannins, since the reactivity in the acid butanol assay is a function of the reactivity of the interflavan bond. The assumption made for this method is that all of the flavanoid units are released and form anthocyanidin pigments in the acid butanol assay; if some interflavan bonds are not broken, then the color yield in the acid butanol assay will be low and the apparent degree of polymerization will be underestimated. The stability of the interflavan bond depends on the pattern of substitution of the flavanoids; for example 5-deoxy-proanthocyanidins give low color yields in the acid butanol assay (Hemingway in The Chemistry and Significance of Condensed Tannins (R. W. Hemingway and J. J. Karchesy, eds; Plenum Press) page 98 (1989).

The crude extracts are assayed with the acid butanol assay and with the modified vanillin (glacial acetic acid) assay. For each extract, the ratio of the absorbances (acid butanol assay/vanillin assay) describes the relative degree of polymerization. The absolute degree of polymerization cannot be determined using crude extracts.

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CHEMICAL MODIFICATION OF TANNINS

Tannins can be modified to change their solubility properties or to eliminate the reactive phenolic functional groups. The modified tannins do not retain the characteristic chemical or biological reactivities of native tannins.

Acetylation

Puts an acetyl group on each hydroxyl group of the starting material. Polarity of the tannin is diminished, and it is insoluble in aqueous solvents.

Slowly drip a mixture of 5 mL pyridine and 5 mL fresh acetic anhydride into a flask containing 2 g tannic acid. Pour the solution into water; a solid should form. The solid is washed with dilute acetic acid (to remove the pyridine) and then with water. It can be freeze dried. Its IR spectrum shows loss of the phenolic OH group.

Methylation

Converts each hydroxyl group to its methyl ester. Polarity of the tannin is diminished and its solubility altered. We have not attempted this procedure.

To methylate tannin, mix it with excess methyl iodide, reagent acetone and solid potassium carbonate. Reflux the mixture overnight and purify the product.

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DMBA ASSAY FOR PHLOROTANNIN

Phlorotannins are found in brown algae. They are based on phloroglucinol-type monomers. In a reaction similar to the reaction of vanillin with the meta-substituted ring of flavanoids, phlorotannins react with 2,4 dimethoxy benzaldehyde in glacial acetic acid to form a red conjugate. The method given here is the original method (Stern J. L.; Hagerman, A. E.; Steinberg, P. D.; Winter, F. C.; Estes, J. A. A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. J. Chem. Ecol. 1996, 22, 1273-1293) and also a microscale method.

This reaction can be used to determine phlorotannin in phlorotannin-protein precipitates.

Because of the acetic acid fumes, as many steps as possible should be done in a chemical fume hood. Tubes and water baths should be covered to minimize release of fumes into the surroundings.

Reagents

Standard tannin dissolved in methanol at 10 mg/mL. Prepare fresh daily. (Or use methanol extract of algae of interest).

16% HCl in glacial acetic acid: 16 mL conc HCl made up to 100 mL with glacial acetic acid. Stable at room temperature.

DMBA: 2.0 g of 2,4-dimethoxy benzaldehyde (Sigma D 3269) made up to 100 mL with glacial acetic acid. Prepare fresh daily. Prepare only the amount needed. Will require 1.25 mL DMBA per sample (original method) or 700 uL per sample (microscale method).

Bring water bath to 30 C.

Original Method

Dispense samples (0-20 uL of standard tannin solution or plant extract) into conical 15 mL glass tubes. Add methanol to make volume to 20 uL.

Add 10 uL N,N-dimethyl formamide (DMF) to each sample.

Add 1.25 mL 16% HCl to each sample. Mix.

At exactly 1 min intervals, add 1.25 mL of DMBA reagent to each sample, mix, and place sample in 30 C bath. Cover samples.

Exactly 60 min after addition to first sample, start reading absorbance at 510 nm at 1 min intervals. Spec should be zeroed on 16% HCl reagent.

Microscale Method

This method has the advantage of using less reagent and sample, and is done in the snap-top microfuge tubes so that fumes are contained very well. It is linear to about 70 ug of Ecklonia tannin.

Dispense samples (0-10 uL of 5 ug/uL tannin solution or plant extract) into 1.5 mL microfuge tubes. Add methanol to make volume to 10 uL.

Add 10 uL DMF to each sample.

Add 700 uL 16% HCl to each sample. Mix.

At exactly 1 min intervals, add 700 uL DMBA reagent to each sample, cap, vortex vigorously, and place sample in 30oC water bath.

Exactly 60 min after addition to first sample, start reading absorbance at 510 nm at 1 min intervals, using microcuvette. Spec should be zeroed with 16% HCl solution.

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PRECIPITATION OF PHLOROTANNIN BY PROTEIN

Because phlorotannins do not have the ortho phenolic groups typical of condensed or hydrolyzable tannins, the protein precipitable phenolics method cannot be used to determine phlorotannin in precpitates. This method was devised to take advantage of the reaction between phlorotannins and dimethoxybenzaldehyde. The phlorotannin and protein are reacted in a buffer solution to form a precipitate. The precipitate is dissolved in N,N-dimethylformamide and the amount of phlorotannin in the precipitate is measured using 2,4 DMBA The protein and tannin are reacted in a buffer containing a reducing agent (DTT) because of the sensitivity of phlorotannin-protein interactions to oxidation. Both the original method described in Stern, J. L.; Hagerman, A. E.; Steinberg, P. D.; Mason, P. K. Phlorotannin-protein interactions. J. Chem. Ecol. 22, 1877-1899 1996, and a microscale method are given here.

The method can be coupled with the radiolabeled protein precipitation assay to allow determination of protein in the precipitates.

Reagents:

Buffer J: 0.1 M acetate, 0.43 M NaCl.

> Mix 5.7 mL glacial acetic acid with about 800 mL water and add 25.16 g NaCl. Adjust to pH 5.0 with conc NaOH. Bring volume to 1 L with water. Store refrigerated.

1 M dithiothreitol (DTT): Bring 1.54 g DTT up to 10.0 mL with water. Divide into 150 uL aliquots and store frozen.

Buffer J with DTT: Because DTT is unstable, the DTT is added to buffer J on the day of the assay.

> Bring 150 uL of 1 M DTT up to 10.0 mL with buffer J (final DTT concentration is 15 mM).

Saline: 6.5 g NaCl up to 1 L with water.

Protein: Prepare the protein (standard is BSA, others can be used) at 10 mg/mL in saline. Keep refrigerated, prepare fresh frequently. (For Ribulose bisphosphate carboxylate prepare 2.5 mg/mL and centrifuge to remove insoluble materials. Use 80 uL instead of 20 uL in assay and reduce the buffer to 120 uL).

Tannin: 10 mg/mL in methanol

16% HCl in glacial acetic acid: 16 mL conc HCl made up to 100 mL with glacial acetic acid. Stable at

room temperature.

DMBA: 2.0 g of 2,4-dimethoxy benzaldehyde (Sigma D 3269) made up to 100 mL with glacial acetic acid. Prepare fresh daily. Prepare only the amount needed. Will require 1.25 mL DMBA per sample (original method) or 700 uL per sample (microscale method).

Bring water bath to 30 C.

Original method:

1. Dispense buffer (180 uL) into 15 mL conical centrifuge tubes. Add tannin (10 uL), mix, add protein (20 uL). Mix. Incubate 30 min at room temp.

2. Need to prepare at the same time "no protein" blanks, each has 210 uL buffer and 10 uL tannin.

3. Centrifuge all tubes 15 min at 3200 rpm, then aspirate off supernatant. Wash ppt by gently adding 100 uL J+DTT to each tube, and centrifuging 5 min at 3200 rpm (maximum speed on a clinical centrifuge).

4. Aspirate off wash and immediately add 10 uL of DMF to each tube. Vortex.

5. Add 1.25 mL of 16% HCl to each sample.

6. Prepare the tubes for two types of controls by rinsing with 200 uL J+DTT. Total phlorotannin controls contain 10 uL phlorotannin, 10 uL DMF, 1.25 mL 16% HCl. Reagent controls contain 1.25 mL 16% HCl.

7. At timed 1 min intervals, add 1.25 mL of DMBA reagent to all tubes. Mix, cover tubes, and place in 30 C water bath. After exactly 60 min, start timed reading of absorbance at 510 nm. Note any tubes that contain precipitate at time of reading. Spec should be zeroed with 16% HCl.

Microscale method.

The advantage is use of less reagent and snap cap microfuge tubes which contain the fumes very well.

Reagents are as for original method, but tannin is at 5 mg/mL and protein is at 5 mg/mL.

Buffer (180 uL), tannin (up to 10 uL) and methanol (up to 10 uL, to make final volume after protein addition up to 210 uL) are vortexed in a 1.5 mL microfuge tube. Protein (20 uL) is added with immediate vortexing. Steps 2-4 from the original method (above) are followed, except that samples can be

centrifuged in a microfuge at about 4000 rpm. High speed centrifugation is not recommended, since it may make the precipitate difficult to dissolve.

Steps 5-7 are followed as in original procedure, except that 700 uL of the HCl reagent and 700 uL of the DMBA reagent are used.

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RADIOLABELED BSA PRECIPITATION METHOD

This method provides the most sensitive method for determining amounts of protein precipiated by a tannin. It is also useful for running competitive binding assays to characterize tannin-protein interactions. The method has been used extensively in studies of mechanism and specificity of tannin-protein interactions. The method described here (Hagerman, A.E.; Rice, M.E.; Ritchard, N.T. J. Agric. Food Chem. 1998, in press) has been scaled down from original method (Hagerman and Butler, J. Biol. Chem. 256, 4494-4497, 1981).

This method can be conveniently coupled with HPLC to determine gallotannins such as pentagalloyl glucose in the precipitates; with the protein-precipitable phenolics method to determine condensed tannins such as Sorghum tannin in the precipitates; or with the protein precipitable phlorotannin method to determine phlorotannin in the precipitates.

CAUTION: PROPER HANDLING OF ISOTOPES IS REQUIRED FOR THIS METHOD. YOU MUST BE APPROVED FOR ISOTOPE USE BY THE APPROPRIATE COMMITTEE OF YOUR INSTITUTION. If isotope use is impossible, the amount of protein precipitated can be determined with the blue BSA method.

Reagents:

pH 4.9 acetate buffer (Buffer A)

> 0.20 M acetic acid, 0.17 M NaCl, pH adjusted to 4.9 with NaOH (11.4 ml glacial acetic acid, 9.86 g NaCl dissolved in about 800 ml water, then adjust to pH 4.9 with a solution of NaOH, then bring to a final volume of 1 liter)

To make large volumes of Buffer A conveniently, prepare the following two solutions:

> 2 M acetic acid, 1.7 M NaCl. Add 114 mL glacial acetic acid to about 800 mL distilled water, add 99.4 g NaCl, and bring to 1 L with distilled water. Store in refrigerator.
>
> 2 M sodium acetate, 1.7 M NaCl. Add 164.1 g sodium acetate to about 800 mL distilled water. Add 99.4 g NaCl and bring to 1 L with distilled water. Store in refrigerator.
>
> Buffer A: Mix 40 mL of the acetic acid solution with 60 mL of the sodium acetate solution and bring to 1 L. Check the pH; it should be 4.9.

Radioiodinated bovine serum albumin (see method of preparation below) in Buffer A

Dialyze and then dilute the hot protein with cold BSA to a final protein concentration of about 0.5 mg/mL and about 30,000 cpm in 50 uL. The protein concentration should be checked immediately before each assay by measuring the optical density at 280 nm (E1%=6.6).

Tannin dissolved in water (or methanol, although in some cases methanol supresses the precipitation; see Hagerman, A.E.; Rice, M.E.; Ritchard, N.T. J. Agric. Food Chem. 1998, in press) dissolve just before you are ready to use the solution. For example, use

Sorghum procyanidin: 0.05 mg/mL in water or methanol

Pentagalloyl glucose: 0.5 mg/mL in water

100% trichloroacetic acid; commercially purchased solution (Sigma) is convenient, or dissolve

100 g of solid trichloroacetic acid up to 100 mL with distilled water. This solution is stable indefinitely. More dilute solutions of TCA cannot be used reliably after prolonged storage.

Method for Precipitation Assays: (amount of BSA precipitated by purified tannin or plant extracts).

The assay is run in 0.5 mL microfuge tubes. Buffer A and labeled protein, are added to the tubes, which are then capped and vortexed. The tannin is then added with immediate vortexing. The samples are incubated at an appropriate temperature (see Hagerman, A.E.; Rice, M.E.; Ritchard, N.T. J. Agric. Food Chem. 1998, in press) for 30 min, and are then centrifuged for 5 min in a microfuge (13,000 x g) at an appropriate temperature. The supernatants are carefully aspirated without disrupting the pellets. The pellets are gently washed with 100 uL buffer, and the solution aspirated away. The pellets are then counted in a gamma counter directly, without transferring them.

Assay tubes contain:

50 uL labeled protein

250 uL buffer A (making the total aqueous volume 300 uL)

100 uL tannin solution

For each assay, the following controls are run:

total counts used per aliquot labeled protein.

background: substitute methanol or water, as appropriate, for the tannin solution. This value is subtracted from all values for counts precipitated. We usually find that the background is less than 10% of the counts added.

TCA precipitable counts: 50 uL labeled protein plus 250 uL unlabeled BSA (~5 mg/mL) mixed with 60 uL of 100% TCA and incubated for 15 min at 4C. The counts precipitated by the TCA are taken as total counts, since presumably counts not precipitated by TCA are not associated with protein. It is desirable to use labeled protein in which >90 % of the counts are TCA-precipitable.

Calculations:

The amount of protein precipitated is calculated directly from the counts in the precipitate, after correcting for nonspecific precipitation. The initial concentration of the labeled BSA solution is used for the conversion.

Method for Competitive Binding:

This method provides a measure of the relative affinity of tannin for various proteins. When a new tannin is to be examined using this assay, preliminary experiments must be run to set the protein and tannin concentrations correctly. You need to set the two concentrations so that about 50% of the counts are precipitated in the absence of competitor, and so that the amount of protein precipitated is relatively independent of protein concentration (region of saturation). The concentrations described here are appropriate for 0.05 mg/mL purified procyanidin from Sorghum grain.

Competing proteins:

Prepare unlabeled competing proteins at a concentration of about 1 mg/mL in Buffer A. Exact concentration of each competitor should be determined spectrophotometrically, if the extinction coefficient is known, or by protein assay; we find the Pierce BCA assay simple and reliable.

Calculations:

"100% bound" is the amount of labeled protein (counts) precipitated by tannin in the absence of any competitor. It is desirable to have about half of the TCA-precipitable counts precipitate in this sample, since that ensures that the labeled protein is present in excess during the assay.

"% bound" is the amount of labeled protein (counts) precipitated in the presence of competitor taken as a percentage of 100% bound (counts precipitated in the absence of competitor). Background precipitation is subtracted from all values before calculations.

% bound is plotted as a function of the log of mass (or moles) of competing protein. Sigmoidal curves are expected.

For preliminary assessment of a potential tannin-binding substance, it is best to test competitor concentration over several orders of magnitude. The concentrations required for detailed assessment of relative affinity can then be selected.

If large excesses of competitor are used, the amount of labeled protein precipitated is sometimes larger than the amount precipitated in the absence of competitor. This is a result of nonspecific binding when large, bulky precipitates form.

Assessing tannin-binding proteins in saliva with competitive binding assay.

Saliva can be used as the competitor. For small monogastric animals, saliva can be used directly, because very small volumes of saliva (<10 uL) effectively compete. For larger animals, or for ruminants, large volumes of saliva (>200 uL) may be required to observe competition. In that case, the pH of the saliva must be adjusted so that it does not shift the pH of the assay mixture away from pH 4.9. Some animal saliva is quite high pH and strongly buffered, and can have substantial effect on the assay pH.

Try to adjust the pH of the saliva by adding concentrated HCl with mixing to bring the pH to somewhere between 2.3-5.1.

Prepare Buffer B (a more concentrated acetate buffer; dilution of 50 uL of buffer B with 200 uL unbuffered saliva/water gives a solution with acetate concentration similar to buffer A). Buffer B is 1 M acetic acid, 0.85 M NaCl, pH 4.9. To prepare 1 L, add 57 mL glacial acetic acid to 800 mL distilled water, adjust to pH 4.9 with NaOH solution. Add 49.7 g NaCl and bring final volume to 1 L. Refrigerate.

Sample tubes should contain 50 uL hot protein, 50 uL buffer B, up to 200 uL saliva, and water to bring the total volume to 300 uL. 100 uL of tannin solution is used as above. Conduct the assay and the controls as described above.

Radioiodination of bovine serum albumin.

This is the Chloramine T method, described by Greenwood, Hunter and Glover [Biochem. J 89, 114-123, (1963)]. We have also had good success with BioRad "Enzymobeads" as a convenient method of radioiodinating BSA.

The potential problems of the Chloramine T method are the possible damage to the protein when using a strong oxidizing agent like Chloramine T, and the potential release of volatile radioactive I_2 during the reaction.

Reagents:

We usually buy 1 mCi of NaI and iodinate on two successive days, using half the radioactivity each day. This ensures at least one good preparation. This yields sufficient labeled protein to last us for about 6 months.

Buffer stock A (0.1 M Phosphate): 6.9 g Na_2HPO_4 x H_2O up to 500 mL distilled water

Buffer stock B (0.1 M Phosphate): 7.1 g NaH_2PO_4 up to 500 mL distilled water

0.1 M Buffer, pH 7.4: Mix A and B to give a pH 7.4 solution.

0.05 M Buffer, pH 7.4: Dilute 1 part of the 0.1 M pH 7.4 buffer with 1 part distilled water and check the pH.

NaI: 0.10 g dissolved in 10 mL 0.05 M Buffer

Sodium metabisulfite: 24 mg dissolved in 10 mL 0.05 M buffer

Bovine serum albumin: 20 mg dissolved in 10 mL 0.1 M buffer (refrigerate this solution) (Sigma Fraction V, Fatty Acid Free).

10 mL Sephadex G25 column in disposable column. Equilibrate the Sephadex in 0.1 M buffer, then just before use wash with the unlabeled BSA solution, and then once more with the 0.1 M buffer. The BSA wash saturates nonspecific binding sites on the column. This is quite important for recovery of the labeled protein.

Just before iodination, prepare 4 mg/mL Chloramine T in 0.05 M buffer.

Set up a hand held survey meter so isotope use can be monitored during reaction. In a functioning chemical fume hood, behind a barricade of lead bricks, set up a stir motor with a 1.5 mL microfuge tube mounted so it is immobile on the stir box. Put a stir flea in the microfuge tube, and add the following reagents while mixing:

25 uL BSA
5 uL I-125 as NaI (0.5 mCi)
25 uL Chloramine T

Immediately add 100 uL sodium metabisulfite to quench the reaction, and add 200 uL cold sodium iodide.

Put the entire reaction mixture on the Sephadex column, and wash the reaction vessel with 400 uL cold sodium iodide and apply that wash to the column. Elute the column with the 0.1 M buffer, adding the buffer in 400 uL aliquots and collecting 400 uL fractions. Monitor the fractions with the survey meter. The labeled protein should elute in fractions 7-10. Leave the remaining material, including the free iodide, on the column for convenient disposal. Dispose of all contaminated materials properly.

Mix the protein fraction with 20 mL of 1 mg/mL cold BSA in Buffer A (used for competitive binding assays). Freeze in 1 mL aliquots. In our hands, 10 uL of this diluted material contains about 300,000 counts immediately after preparation. The half life of I-125 is 60 days, so there is rather rapid loss of counts. The iodinated protein slowly decomposes during storage, so that a larger and larger percentage of the counts are associated with peptides and other non-protein components. This is detected as a loss of TCA-precipitability of the counts. The labeled protein should be diluted to the desired activity for the assay, and then dialyzed against several changes of acetate buffer used in the precipitation assay to remove the non-protein counts from the preparation before using the protein in competitive binding assays.

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PROTEIN PRECIPITABLE PHENOLICS

This method (Hagerman and Butler, J. Agric. Food Chem. 26, 809-812, 1978) measures the amount of condensed or hydrolyzable tannin precipitated by a standard protein, bovine serum albumin. The precipitate is dissolved at high pH in the presence of a detergent, and the colored iron-phenolate complex is determined spectrophotometrically. The method is robust and works well with virtually all plant extracts, although the exact nature of the interaction between protein and tannin affect the assay for each unique plant extract.

Both the standard and microscale methods are described here.

Even traces of acetone inhibit precipitation of protein by phenolics, so you must remove all acetone from plant extracts before attempting the method.

Reagents:

Buffer A: 0.20 M acetic acid, 0.17 M NaCl, pH adjusted to 4.9 with NaOH (11.4 ml glacial acetic acid, 9.86 g NaCl dissolved in about 800 ml water, then adjust to pH 4.9 with a solution of NaOH, then bring to a final volume of 1 liter).

BSA: 1 mg/ml bovine serum albumin (Sigma A-6003) in buffer A

SDS/TEA: 5% (v/v) triethanolamine, 1% (w/v) SDS (50 ml triethanolamine, 10 g SDS brought up to 1 liter with water).

$FeCl_3$: 0.01 M $FeCl_3$ in 0.01 M HCl. To make 0.01 M HCl, dilute 0.83 mL conc HCl up to 1.00 L with water. Dissolve 1.62 g ferric chloride in 1 L of the acid solution, allow it to sit for several hours. Gravity filter through #1 paper.

Original Method:

Dispense 2.00 ml BSA into 15 ml centrifuge tubes or culture tubes that can be centrifuged in a desk top centrifuge.

Add 1 ml of alcoholic or aqueous tannin solution or plant extract. The tannin cannot contain any acetone, since even traces of acetone inhibit the precipitation reaction.

Mix immediately, and allow sample to sit for 15 min at room temperature (purified tannin) or for 24 h at 4o C (plant extracts).

Centrifuge 15 min at 3000 x g (high speed in a desk top centrifuge), pour off supernatant.

Redissolve pellet in 4.00 ml SDS/TEA.

Add 1.00 ml $FeCl_3$, vortex immediately.

After about 15 min read absorbance at 510 nm. Subtract an appropriate blank (ferric chloride in SDS/TEA).

Standardize with purified tannin from the plant of interest (best) or with purified quebracho tannin or tannic acid.

Scaled down method.

Use microfuge tubes and 1 mL cuvettes.

Prepare the SDS/TEA and ferric chloride reagents as described above. Prepare the BSA solution in buffer A as above, but make it at 5 mg/mL.

Prepare the tannin solution at about 0.5 mg/mL in methanol (for Sorghum procyanidin).

Mix 50 uL BSA with 250 uL buffer A. Add 100 uL tannin and vortex immediately. Allow to incubate at room temperature for 30 min, then centrifuge for 5 min at 13,000 rpm (max speed on microfuge). Aspirate off the supernatant, then redissolve the pellet in 800 uL of SDS/TEA. The precipitate must be completely redissolved--sometimes the high speed of the microfuge makes the pellets hard to dissolve. Add 200 uL ferric chloride and after 15 min read the absorbance at 510 nm.

If the pellets cannot be redissolved after microfuging, then do the assay on this scale but centrifuge in a clinical centrifuge at 3000-5000 rpm to obtain softer pellets.

Ultramicroscale method.

This is useful for determining precipitated procyanidin in the radiolabeled protein precipitation method. The precipitate is carefully dissolved in 100 uL of the SDS/triethanolamine solution and then reacted with 50 uL of the $FeCl_3$ reagent. The absorbances are read in a nanoliter scale cuvette.

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BLUE BSA METHOD FOR DETERMINING PROTEIN PRECIPITATED BY TANNIN

A standard protein, bovine serum albumin, is labeled with a blue dye so that it can be selectively measured in tannin-protein precipitates (Asquith and Butler, J. Chem. Ecol. 11, 1535-1544, 1985). The method is simple but less sensitive than the radiolabeled BSA precipitation method.

Extracts must not contain acetone, which inhibits protein precipitation by tannins.

The method involves:

making the blue BSA

standardizing the blue BSA (allows conversion of blue dye color to mg protein)

using the blue BSA in precipitation assays

Making the blue BSA

Prepare 100 mL of 1 % $NaHCO_3$ by dissolving 1.0 g of sodium bicarbonate in 100 mL water.

Dissolve 2.0 g bovine serum albumin (Sigma A 6003) in 40 mL of the NaHCO3. Add 150 mg of Remazol brilliant blue dye (Sigma R 8001) to the protein solution. Let it stir gently for 30 min at room temperature.

Prepare 3 L of acetate buffer by diluting 34.2 mL of glacial acetic acid with about 1800 mL of water, and then adjusting the pH to 4.8 by adding 2 N NaOH (80 g NaOH dissolved in 1 L of water) drop by drop and monitoring the pH continuously at a pH meter. After the pH has been adjusted, add water to make the final volume 3 L. Store this in the cold.

Put the BSA and dye mixture into a dialysis bag made with 12-14,000 MW cutoff dialysis tubing, and dialyze against 1 L of the acetate buffer overnight in the cold (4 C). Discard the acetate buffer and dialyze again overnight with fresh buffer. Discard the buffer again.

Dilute the 40 mL of dialyzed blue BSA to 1 L with acetate buffer. Store this diluted sample in the cold.

Standardizing the blue BSA:

(using the Lowry assay as modified by Peterson, Meth Enz 91, 95-119).

Reagents:

CTC Dissolve in 100 ml water:

0.1 g copper sulfate ($CuSO4 \times 5 H2O$)
0.2 g sodium potassium tartrate ($KNaC_4H_4O_6 \times 4 H_2O$)
10 g sodium carbonate (Na_2CO_3)

SDS Dissolve in 100 mL water:

5 g sodium dodecyl sulfate (sodium lauryl sulfate, SDS)

NaOH Dissolve in 100 mL water:

3.2 g NaOH

Just before running the assay, prepare:

Reagent A:
1 part CTC
2 parts SDS
1 part NaOH

Reagent B:
1 part commercial Folin's reagent (stored at 4o C)
5 parts water

Lowry Assay (determining concentration of blue BSA):

Run the Lowry assay on replicate samples of the standard protein and the unknown (blue BSA). Use 10-100 uL of the standard, making each sample to 1 mL with water. Use 10-50 uL of the blue BSA, making each sample to 1 mL with water.

To 1 mL of sample, add 1 mL of reagent A. Mix.

About 10 min later add 0.5 mL of reagent B. Mix.

About 30 min later read absorbance at 750 nm in a 1 cm cell.

Known protein solution for standardizing the method:

Prepare approximately 1 mg/ml BSA (Sigma) by dissolving 10 mg BSA in 10 mL acetate buffer. Determine the exact concentration of this standard spectrophotometrically by placing a sample in a cuvette, pathlength 1 cm, and determining the absorbance at 280 nm. Calculate the concentration from the known extinction coefficient of BSA (extinction coefficient, 280 nm, 1% (w/v) solution = 6.6).

A = (extinction coeff)*(path length)*(concentration)

So for an absorbance of 0.599 the calculated concentration of the standard would be 0.91 mg/mL.

Converting blue color to mg protein:

To calculate amounts of protein precipitated by tannins, the blue color in the precipitate must be converted into ug blue BSA precipitated, and a standard curve is needed for that conversion. The absorbance properties of the dye are dependent on the solvent composition, so this calibration is done in the isopropanol/SDS/TEA solution that is used to dissolve the precipitated protein in the assay (see below).

Aliquots of the blue BSA solution are brought to a final volume of 3 mL with the isopropanol/SDS/TEA solution, and the absorbance at 590 nm is determined.

Blue BSA precipitation by plant extracts or other tannin preparations:

Blue BSA is precipitated by tannin, and the precipitate is redissolved and color determined.

Isopropanol/SDS/TEA Reagent.

Add to a one liter graduated cylinder:

50 mL triethanolamine (2,2',2''-nitrilotriethanol)
200 mL isopropanol
10 g SDS (sodium dodecyl sulfate, also called sodium lauryl sulfate)

Bring to 1 L with water.

Precipitation:

Put a volume of blue BSA solution equivalent to 4 mg of blue BSA plus enough acetate buffer to make the volume of protein 4.0 mL into a screw cap tube. Add tannin containing sample (1 mL extract, or extract diluted to 1 mL). Vortex. Allow the mixture to incubate 2 h in the cold (4 C). Centrifuge 15 min at 3000 x g (high speed in a clinical or table top centrifuge). Carefully pour off the supernatant, without disturbing the blue precipitate. Add 3.0 mL of isopropanol/SDS/TEA to the precipitate, and vortex vigorously to completely redissolve the precipitate. Read the absorbance at 590 nm, and calculate the

amount of protein precipitated from the calibration curve.

Sometimes plant extracts have pigments which interfere with the blue color. If the color of the redissolved precipitate is different than the color of just blue BSA, set up a sample-only blank containing the sample plus isopropanol/SDS/TEA and measure its absorbance. Subtract this value, and note the unusual presence of these interfering pigments.

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TLC OF TANNIN

For assessing purity of tannins

For hydrolyzable tannins:

from Lea J. Sci. Food Agric. 29, 471 (1978)

Mobile phase: Toluene:acetone formic acid 60:60:10 (v/v/v)
Stationary phase: Silica plates

Tannin stays at the origin. Other phenolics migrate. Tannic acid often gives multiple spots corresponding to the various degrees of esterification.

For identifying various procyanidin dimers and trimers:

from Porter in Methods in Plant Biochemistry Vol. 1 (J. B. Harborne, ed., Academic Press 1989) pages 389-419.

Mobile phase 1: tert-butanol:acetic acid:water, 3:1:1 (v/v/v)

Mobile phase 2: 6% acetic acid

Stationary phase: Cellulose plates

A diagram showing the positions of various procyanidins is given in Porter's chapter.

Spray for phenolics on TLC:

A useful TLC spray can be made by mixing equal volumes of the two Price and Butler Prussian blue reagents and spraying onto dry plates. Phenolics give bright blue spots. A blue background eventually develops. The spray mixture must be made fresh, and should be brown in color. It should be discarded if it turns blue.

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TANNIN-BINDING PROTEINS DETECTED BY ELECTROPHORESIS ON NATIVE GELS

This method allows detection of proteins in saliva or other samples which selectively bind to tannins. Either precipitable or nonprecipitable complexes can be detected with this method, as with gel shift assays for other protein ligands. The method described here was specifically developed for assessing saliva, and is modified from Austin et al., J. Chem. Ecol. 15, 1335-1347 (1989).

Saliva:

Should be frozen immediately after collection. Add PMSF (phenyl methyl sulfonyl fluoride) at a final concentration of about 40-50 ug/mL saliva to prevent proteolysis during storage. PMSF can be prepared as a stock solution at 10 mg/mL in isopropyl alcohol and stored at room temperature; it is unstable in aqueous solution. PMSF is very toxic.

Incubation of saliva with tannin:

These amounts are based on ruminant saliva--adjustments to compensate for the concentration of proteins in other samples may be necessary.

Prepare a tannin solution containing antioxidant by diluting 10 uL of 100 mM EDTA in 1 mL 50% methanol/50% water. Add 0.009 g ascorbic acid and mix to dissolve the acid. A bit may remain undissolved.

Add tannin to make a 20 ug/uL stock solution (condensed tannin) or a 5 ug/uL stock solution (gallotannin). I make about 75 uL of stock solution at a time. (The solution cannot be saved as the tannin oxidizes too readily). Prepare a 1:10 dilution of the stock tannin solution.

Mix the saliva with tannin: Dispense 30 uL samples of saliva into a series of microfuge tubes. To each, add a total of 10 uL of the 50% methanol solution containing 0-200 ug condensed tannin or 0-50 ug gallotannin. Mix the solutions and incubate overnight at 4C.

Prepare the samples to go on the gel. Mix each sample with 10 uL of bromophenol blue/ glycerol/buffer. Centrifuge each sample 3 min at about 3000 rpm.

Prepare the gels:

Make native gels, using the Laemli recipes without SDS. Use 12% acrylamide. I use a Hoefer minigel system 0.75 mm thick gels about 5 x 8 cm, and a sample comb with 10 lanes.

Apply 10 ul sample (supernatant--there may be substantial precipitate, or may be none) to each lane. Run the gels. Fix and stain with silver stain .

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SILVER STAIN FOR ACRYLAMIDE GELS

Silver staining is a redox-based staining method for proteins on electrophoresis gels. We use this method to assess interactions between salivary protein and tannin after native gel electrophoresis, or to assess composition of crude saliva after SDS gel electrophoresis.

This method is more sensitive than Coomassie staining, but is also more expensive. Proteins may be stained either as dark bands on the gel or as light bands against a faint background. Tannin enhances the overall sensitivity of the stain. However, tannin also reacts with the stain to give dark brown smears which can interfere with interpretation of the gel. The fixing step described here minimizes this interference, and also ensures that proline-rich proteins are properly fixed in the gel. The method given here is modified from Hochstrasser, Patchornik, and Merril, Anal. Biochem. 173: 412-423 (1988).

These amounts and times are for small, 0.75 mm thick gels. For larger gels you would need larger volumes of the same solutions; for thicker gels you would need longer times to allow complete diffusion of the solutions into the gel.

Solutions

6% perchloric acid: 86 mL of reagent (70%) perchloric acid, $HClO_4$, up to 1 liter with water. Perchlorate salts are contact explosives, and perchloric acid is a strong oxidizing acid. Be careful.

Fix: Prepare a stock solution of 257 mL ethanol plus 600 mL water. Just before use, mix 130 mL of the stock with 22 mL reagent (37%) formaldehyde. If the formaldehyde solution is cloudy, do not use it--it will make it impossible to properly develop the stain later.

SDS wash: 200 mL ethanol, 100 mL glacial acetic acid, 1700 mL water. (Used to remove SDS from SDS gels).

Stain: Prepare 20% silver nitrate just before use (4 g $AgNO_3$ up to 8 mL distilled water; chloride in the water will give you a cloudy solution that cannot be used). Just before staining, mix 1.5 mL concentrated ammonia, 200 uL of 10 N NaOH, and 20 mL distilled water. Then add, dropwise with constant stirring, the silver nitrate solution. As you add the silver, masses of brown precipitate will form (silver hydroxide) and then disappear (ammoniacal silver). The solution should be clear and colorless when you are done. Then bring the final volume to 100 mL with distilled water and mix.

Developer: Prepare a stock solution of citric acid (4 g citric acid up to 200 mL with distilled water). This must be refrigerated because microorganisms love to grow in citric acid solutions. Just before using, mix 100 mL water, 500 uL citric acid stock, and 100 uL reagent formaldehyde (37%).

Stop: 1 mL glacial acetic up to 100 mL with water.

Method

1. Acid fix the gel for exactly 10 min in the perchloric acid solution. Agitate while fixing.

2. Wash the gel for exactly 5 min with distilled water. Agitate.

3. Fix the gel overnight in the ethanol/water/formaldehyde fix solution.

4. At this point, I transfer the gels into individual plastic trays--they sometimes stick to glass trays in the next steps, which causes poor staining or tearing.

5. Wash SDS gels 4 times, 10 min each wash, with the SDS wash solution, agitating while washing. Skip this step for native gels.

6. Wash with water 3 times, 10 min each wash, then 1 time 30 min. Agitate. You can be pretty flexible on the times in this step, but be sure to wash long enough.

7. Stain for 10 min with agitation. At this point, some brown streaks (tannin) may appear on the gels, but bands of protein will not be visible yet.

8. Wash 3 times with water, 5 min each time, with agitation.

9. Develop, on a light box to monitor bands. Stop when development is satisfactory. Store gels temporarily in stop, then transfer to water and dry between sheets of cellophane.

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COOMASSIE STAIN FOR PRPS ON SDS GELS

Proline-rich proteins have unusual solubility and staining characteristics. This method of staining takes advantage of those characteristics to stain proline-rich proteins pink or violet; other proteins stain blue. The method is not as sensitive as silver stain, and is not a method for evaluating whether proteins bind tannin, because it is used in SDS gels. We have found that some tannin-binding proteins (deer) do not stain distinctively with this method. We have used the method most successfully with rat saliva. The method given here was adapted from Beeley et al. Electrophoresis 12, 1032-1041 (1991).

Separate the proteins on 12% SDS gels, 0.5 or 1.5 mm thick, in Hoeffer minigel apparatus as usual.

Stain:

0.5 g Coomassie blue R-250
200 mL absolute ethanol (or 210 mL 95% ethanol)
50 mL glacial acetic acid
up to 500 mL with water

Stain the gels for 2 h in this stain, and destain in 10% acetic acid (10 mL glacial acetic acid up to 100 mL with water). Normal proteins stain blue or violet, while (some) salivary proline-rich proteins eventually destain to form pink bands. It make take 4 days for the pink color to show up.

A useful contrast is to run an identical gel and stain with same solution, but destain with 10% acetic acid/10% ethanol (10 mL glacial acetic acid plus 10 mL ethanol up to 100 mL with water). The proline-rich proteins are destained to almost colorless bands, so their absence contrasts nicely with their pink color in the acid destain.

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Biosynthesis—some references

Gallotannins

Hagenah, S.; Gross, G.G. Biosynthesis of 1,2,3,6-Tetra-O-Galloyl-beta-D-Glucose. *Phytochemistry* **1993**, *32*, 637-641.

Niemetz, R.; Gross, G. Gallotannin biosynthesis: β-glucogallin: hexagalloyl 3-O-galloyltransferase from Rhus typhina leaves . *Phytochemistry* **2001**, *58*.

Proanthocyanidins

Stafford, H.A. Enzymic regulation of procyanidin biosynthesis; Lack of a flav-3-en-3-ol intermediate. *Phytochemistry* **1983**, *22*, 2643-2646.

Stafford, H.A.; Lester, H.H. Flavan-3-ol biosynthesis. The conversion of (+)-dihydroquercetin and flavan-3,4-diol (leucocyanidin) to (+)-catechin by reductases extracted from cell suspension cultures of Douglas fir. *Plant Physiology* **1984**, *76*, 184-186.

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