The Tannin Handbook

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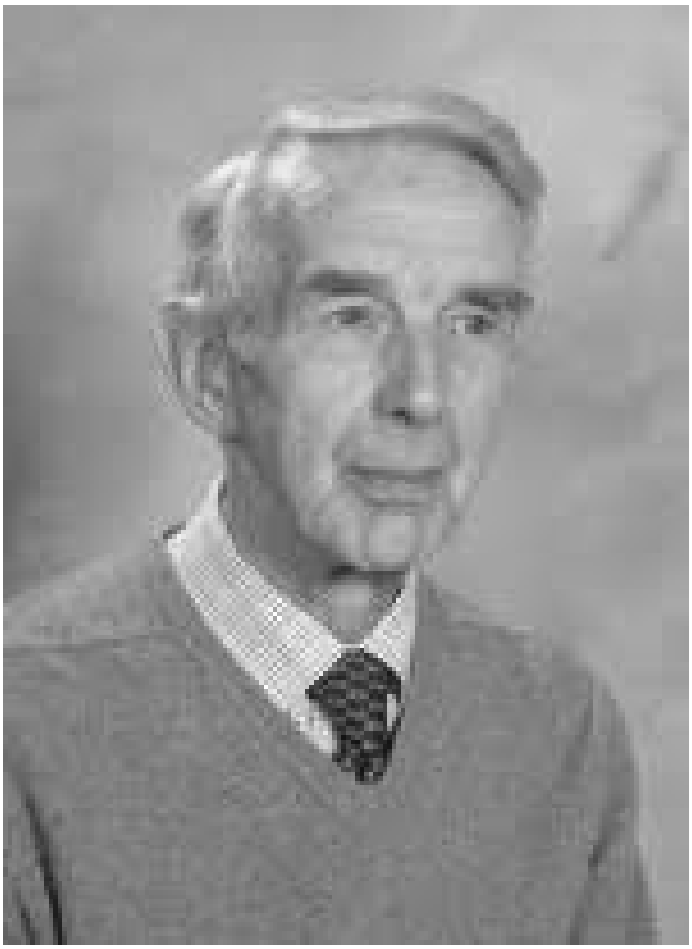
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Volume One: What is a Tannin?

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Professor Edwin Haslam (1932-2013). University of Sheffield, United Kingdom. Haslam was the first to use modern chemical methods to characterize both condensed and hydrolyzable tannins. He coined the phrase "plant polyphenols" to more clearly express the relationship between tannins and related plant secondary metabolites.

Introduction to the Tannin Handbook

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This handbook was originally published for use in the Hagerman laboratory. The original printed Tannin Handbook is no longer available. There were several on-line versions published on my group website from the late 1990s through the early 2000s. This 2025 edition is the final version of the book. It has been reorganized as described below. Please cite this version and its web address as well as the original literature when you use these methods.

There are several rogue versions of the handbook available on predatory publishing sites that re-use authors material without permission. Those sources are not reliable. They have not been checked by any knowledgeable person and should not be used or cited. The only authentic version is available from the Miami University Library Scholarly Commons.

All of the methods in this handbook 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 procedures are only meaningful when they are interpreted with a full understanding of the chemistry underlying the method.

For the analytical methods, major limitations 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.

The biological activities of tannins are best understood in the context of clearly defined compounds with confirmed structural chemistry. Many early studies attributed activities to the “tannins” found in whole tissues or crude extracts, but science has moved beyond that approach now. Activity directed fractionation is a powerful tool especially when the fractionation is accompanied by chemical characterization of active fractions. The simple tools in this book, and the more advanced instrumental tools that are now available offer the potential to truly understand the diverse, multifunctional group of natural products that we call “tannins”.

What Is a Tannin?

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Plants accumulate a wide variety of "secondary" compounds, including alkaloids, terpenes and phenols. 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 highly methoxylated phenols that strengthen and stabilize plant cell walls (lignin). Many plant phenols meet the definition of polyphenol, a shikimate pathway product comprising more than one phenolic ring and lacking nitrogen. The subgroup of polyphenols known as tannins is clearly distinguished from other plant secondary phenols in their chemical reactivities and biological activities.

The quintessential activity of the tannins is their ability to interact with and precipitate proteins. Tannin from bark or wood extracts has been used for centuries to crosslink proteins in animal skin yielding a stable, impervious material with a multitude of uses. The term "tannin" comes from the ancient Celtic word for oak, a typical source for tannins for leather making (“tanning” hide to make leather).

The chemistry of the plant compounds responsible for protein precipitation was quite obscure until the 1950s when Edgar C. Bate-Smith began to do careful chemical analysis of tannin-containing extracts. He 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 precipitate alkaloids, gelatin and other proteins". His work served as the basis for Edwin Haslam’s extensive investigations that provided tools and terminology to differentiate the many structural subclasses of tannins. Bioactivities including protein binding, antioxidant potential and metal binding were linked to chemical characteristics by my group, prompting many innovative studies of tannins in the environment, in foods and beverages, and in material science in recent years.

The current literature is filled with papers about tannins and polyphenols—surprisingly, those new papers often re-describe phenomena that were first noted years ago. Unfortunately, modern papers often use polyphenol preparations that are not accurately described or named, so care needs to be taken when reading the literature. The following list of classic papers might be useful to individuals that want to more deeply understand the fundamentals of tannin chemistry.

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

Hagerman, A.E. & Butler, L.G. (1981) The specificity of proanthocyanidin-protein interactions. *Journal of Biological Chemistry* 256, 4494-4497

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Mehansho, H. Hagerman, A.; Clements, S.; Butler, L.; Rogler, J.; Carlson, D.M.(1983) Modulation of protein-rich protein biosynthesis in rat parotid glands by Sorghums with high tannin levels. *Proceedings of the National Academy of Sciences of the U.S.A*. 80, 3948-3952.

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Scalbert, A.; Mila, I.; Expert, D.; Marmolle, F. Albrecht, A-M.; Hurrell, R.; Huneau, JH-F.; Tome, D. (1999). Polyphenols, metal ion complexation and biological consequences. In: Gross, G.G., Hemingway, R.W., Yoshida, T., Branham, S.J. (eds) Plant Polyphenols 2. Basic Life Sciences, vol 66. Springer: Boston, MA.

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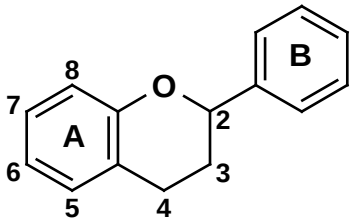
Condensed Tannin Structural Chemistry

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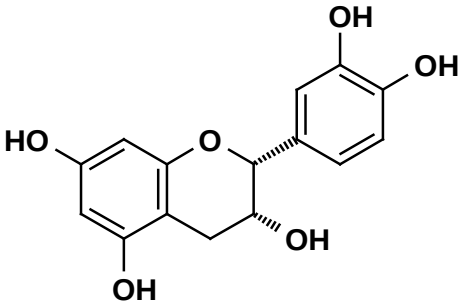
Condensed tannins are widely distributed in the plant kingdom. It is easy to detect condensed tannins and to estimate their concentration in plant tissues using the acid butanol assay. This analytical accessibility means that many publications conflate the general term "tannin" with the easily detected condensed tannins, completely ignoring the hydrolyzable tannin subcategory of tannins/polyphenols.

The structural chemistry of the condensed tannins was elucidated largely through the efforts of Haslam's group (1). More modern information is summarized by Quideau and Salminen and their respective research groups (2,3)

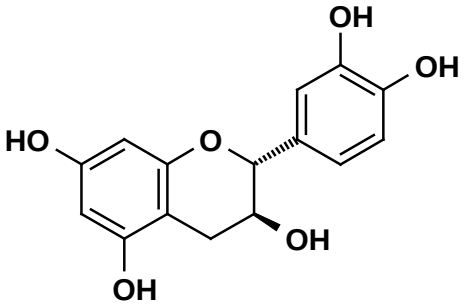
Proanthocyanidins (condensed tannins) are polymeric flavanoids. The flavanoids are a diverse group of metabolites based on a heterocyclic ring system derived from phenylalanine (B) and polyketide biosynthesis (A). Although the biosynthetic pathways for flavanoid synthesis are well understood, the steps 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. Their epimers (+)-epicatechin and (-)-catechin are rare in nature.

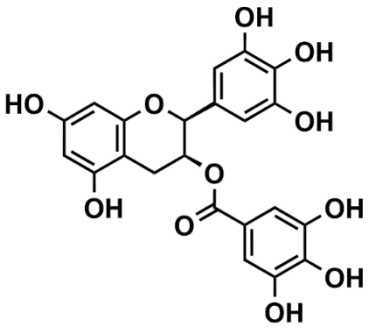


epicatechin



catechin

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).

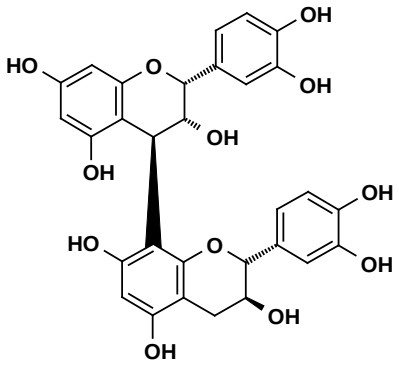


Epigallocatechin gallate (EGCg)

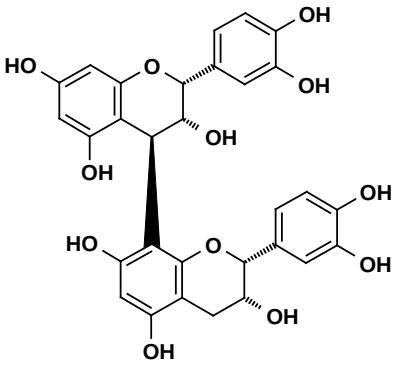
Many plants produce esters of the flavan-3-ols. Typically gallic acid is added at C3 of the parent flavan-3-ol to produce galloylated compounds like epigallocatechin gallate, EGCg. EGCg is the most important polyphenol in fresh leaves of *Camellia sinensis*, and is widely known as the green tea tannin.

Any of the flavan-3-ols can be condensed by metabolic reaction that have not been clearly elucidated. In some plants the polymers are quite simple, typically with a catechin terminal unit and epicatechin extender units. Other plants produce more complex condensed tannins by incorporating gallocatechin, epigallocatechin, and/or galloylated flavan-3-ols into the polymer. Glycosylation is rarely observed.

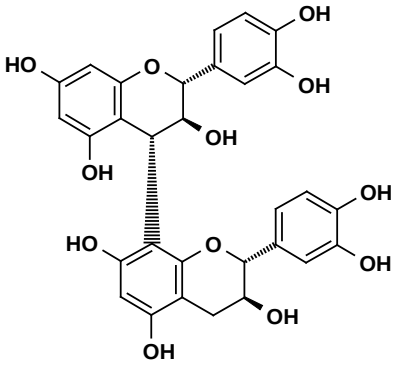
The best characterized condensed tannins are linked via a carbon-carbon bond between C8 of the terminal unit and C4 of the extender. The four common modes of coupling are illustrated by the dimers isolated by Haslam, and originally named B-1, B-2, B-3 and B-4. The more complete names specify the



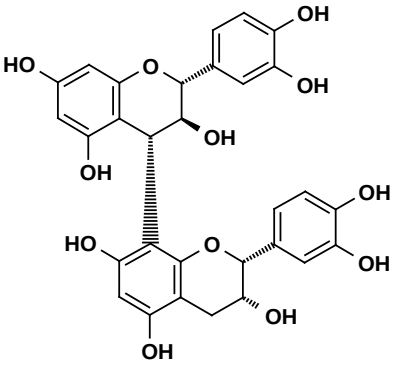
B-1
epicatechin-(4β->8)-catechin



B-2
epicatechin-(4β->8)-epicatechin



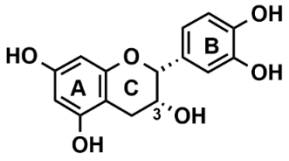
B-3
catechin-(4α->8)-catechin



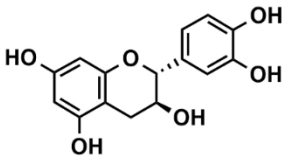
B-4
catechin-(4α->8)-epicatechin

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.

Further polymerization can yield the linear 4,8 polymers such as the Sorghum procyanidin. The chemically correct name for the polymer specifies both the subunits and the position/stereochemistry of the linkage. For example, the Sorghum condensed tannin shown in the figure is correctly named ***epicatechin-[(4β->8)-epicatechin]$_{15}$-(4β->8)-catechin***. Linear polymers based on 4,6 dimers; and branched dimers containing both 4,6 and 4,8 linkages are less common. 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. 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. Each of the anthocyanidin products has a unique visible spectrum with characteristic λ_{max}. See the Acid Butanol Handbook pages for details.

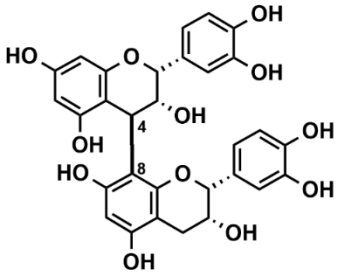


epicatechin

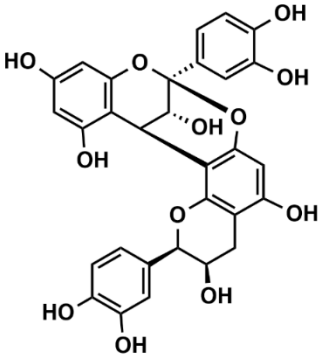


catechin

Monomers

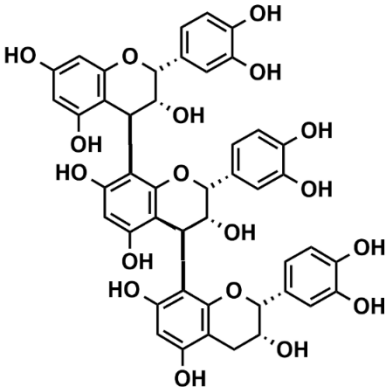


B2 dimer

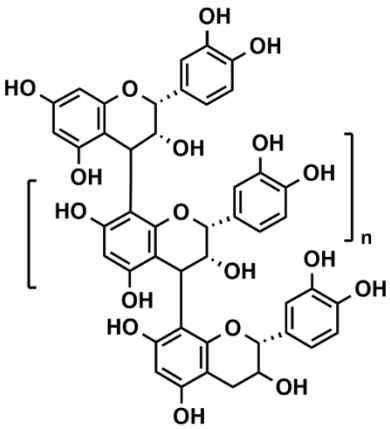


A2 dimer

Dimers



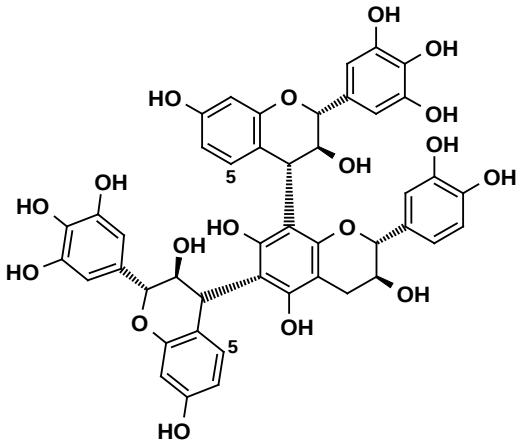
C1 trimer



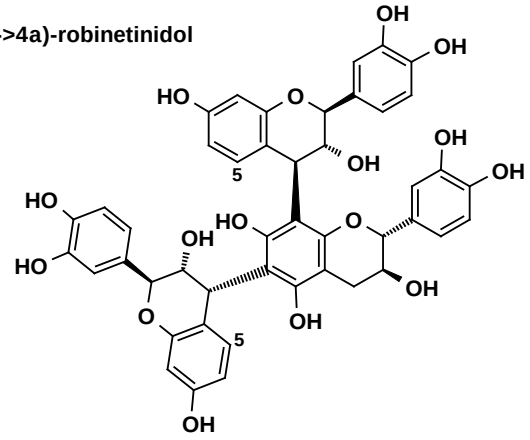
n = 4 cocoa PC
n = 14 sorghum PC

Oligomers

An important group of condensed tannins are 5-deoxy-flavan-3-ol polymers. Branching is common in these tannins, because of the relatively low nucleophilicity of the 5-deoxy A ring (resorcinol-like) compared to the phloroglucinol-like B-ring of e.g. epicatechin. Instead of extending the chain by adding subunits at position 8 of the low reactivity robinetinidol, extension takes place by addition at position 6 of the core catechin. These condensed tannins are important because profisetinidins and prorobinetinidins comprise the major tannins found in quebracho and acacia tannin preparations. Acid butanol reaction yields the 5-deoxy anthocyanidins fisetinidin and robinetinidin.



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

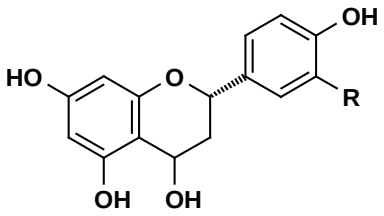


profisetinidin

For all of the condensed tannins, subunits can be identified using thiolysis chemistry. An acid butanol-type reaction is 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 some authors prefer to use phloroglucinol. Further details can be found in the Handbook pages on Thiolysis.

The flavan-3,4-diols, or leucoanthocyanidins, are sometimes confused with proanthocyanidins. The flavan-3,4-diols are monomeric flavonoids that yield the anthocyanidins upon treatment with heat and

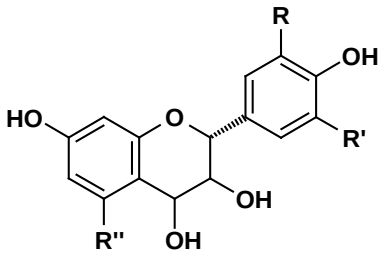
acid. They thus have reactive chemistry similar to that of the condensed tannins, but they do not interact with protein to form precipitable complexes.



Flavan-4-ols

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

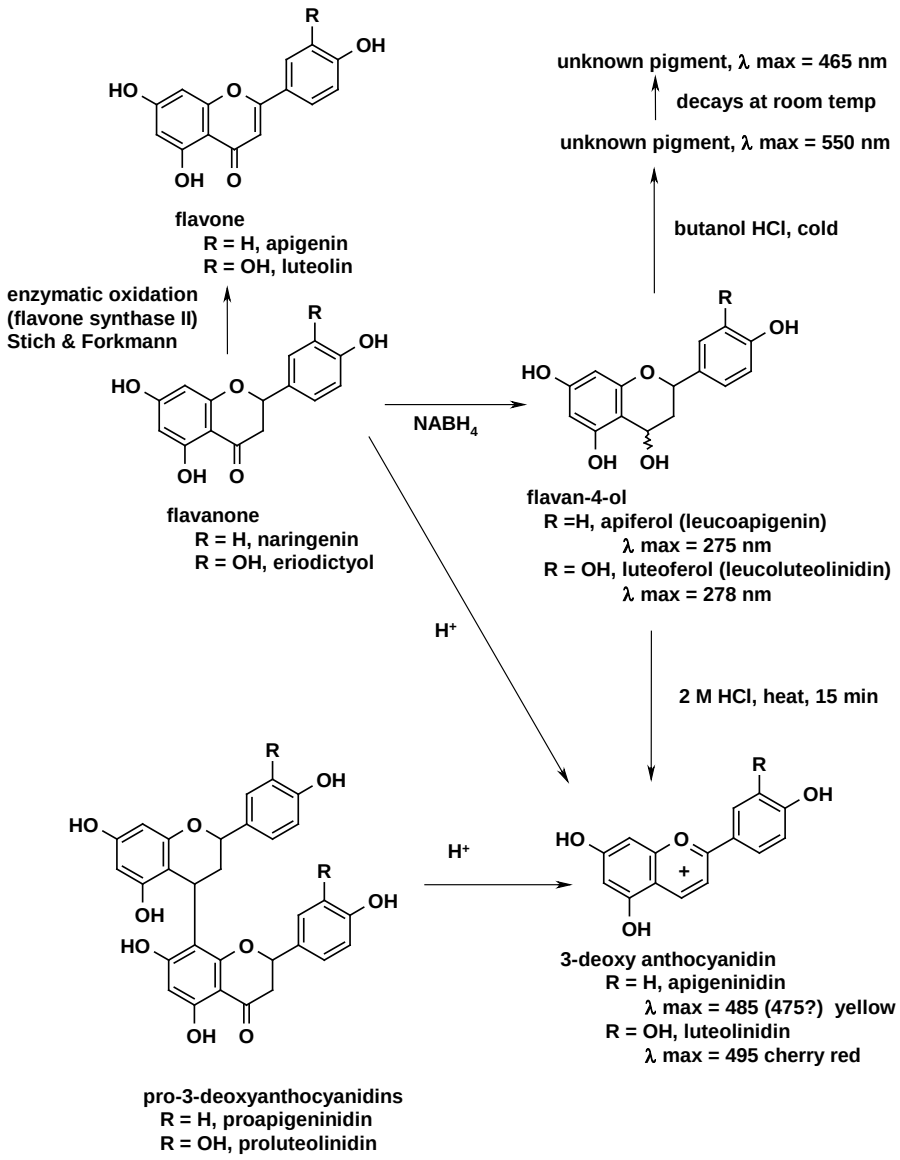
The flavan-4-ols are also leucoanthocyanidins, but are unique in their lability. They yield the anthocyanidins upon treatment with alcoholic acid at room temperature. The condensed tannins require extended reaction at high temperature to produce the anthocyanidin.



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

Stafford suggested that pro-3-deoxyanthocyanidins might exist in a few plants (4) Evidence to date is limited to spectroscopy and some chemical conversions that are consistent with the chemistry shown on the next page.



Citations

1. Haslam, E. (1989) *Plant polyphenols. Vegetable tannins revisited*, Cambridge University Press, Cambridge, U. K.
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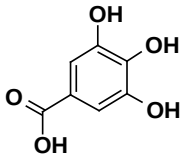
Hydrolyzable Tannin Structural Chemistry

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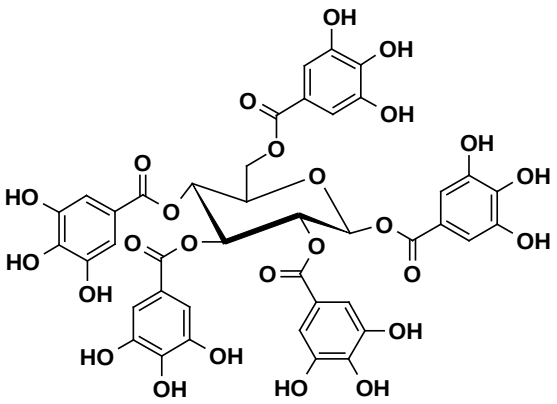
Hydrolyzable tannins are derivatives of gallic acid (3, 4, 5-trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolyzable tannins.

Early work on hydrolyzable tannins included Haslam's significant elucidations of the structures of the simple gallotannins (1). Okuda and co-workers have been particularly active in characterization and classification of complex hydrolyzable tannins (2). Early synthetic work on ellagitannins was undertaken by Feldman (3). Quideau extended that work and has extended it to encompass elegant studies of structure-activity relationships for many tannins (4).

Gallotannins



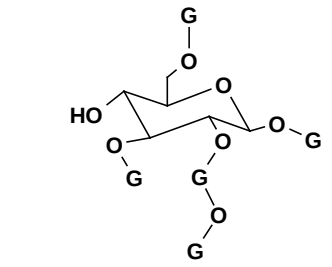
gallic acid



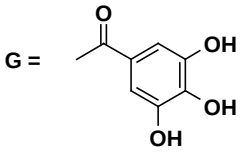
β-1,2,3,4,6-pentagalloyl-O-D-glucose

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.

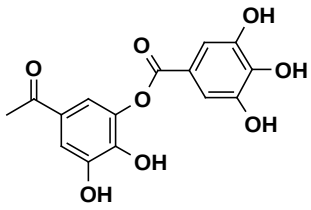
Like all of the gallotannins, PGG has many isomers. The molecular weights of all the isomers of PGG are the same (940 g/mol), but chemical properties such as susceptibility to hydrolysis and chromatographic behavior; and biochemical properties such as ability to precipitate protein; are structure-dependent.



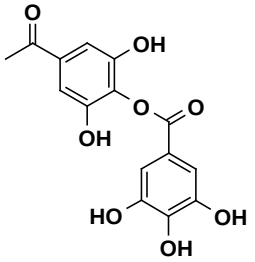
β-1,2,2,3,6-Pentagalloyl-O-D-Glucose



galloyl ester

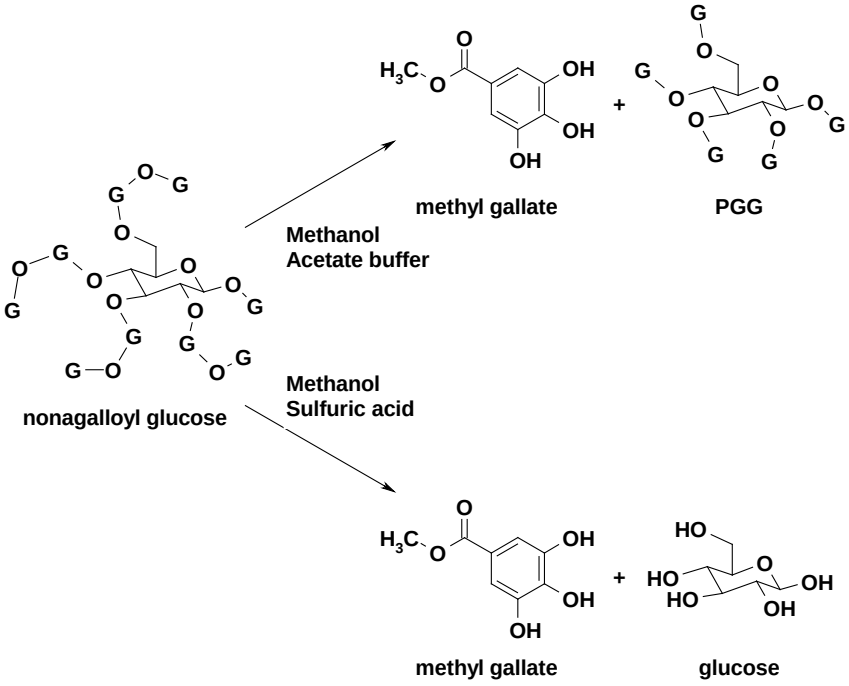


GOG = digalloyl ester, *meta*-depside bond



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

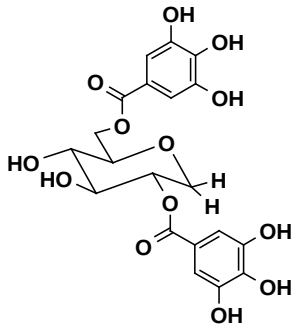


methanol can be used to methanolzye both despide and ester bonds yielding the core polyol and methyl gallate. Hydrolysis with strong acid converts gallotannins to gallic acid and the core polyol.

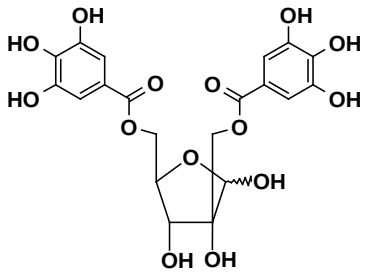
Simple gallotannins with up to 12 esterifed galloyl groups and a core glucose are routinely found in tannins from sumac or oak galls. Commercial tannic acid is comprised of mixtures of gallotannins from sumac (*Rhus semialata*) galls (Chinese gallotannin); Aleppo oak (*Quercus infectoria*) galls (Turkish 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.

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



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

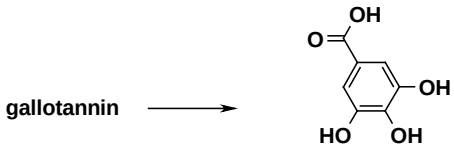


hamamellitannin

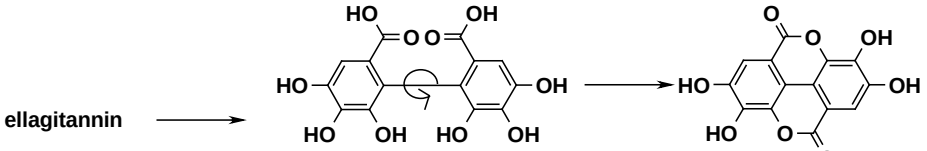
Examples of tannic acid heterogeneity revealed by HPLC analysis can be found in several papers: (5-8). It is not sufficient to rely on published data since a given manufacturer may use different sources for tannic acid for different lots. Unfortunately, this information is not usually provided by suppliers of tannic acid, and is not well described in public databases such as PubChem.

Ellagitannins

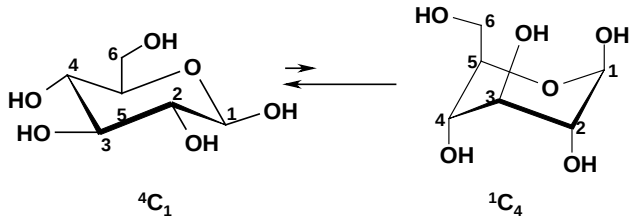
Oxidative coupling of adjacent galloyl groups converts gallotannins to the related ellagitannins. The simple ellagitannins are esters of hexahydroxydiphenic acid (HHDP). HHDP spontaneously lactonizes to ellagic acid in aqueous solution.



gallic acid



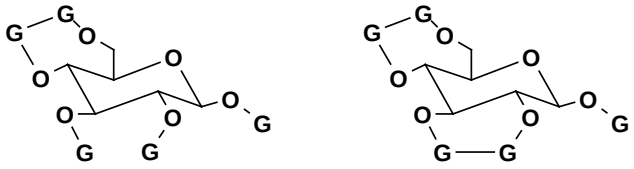
Glucose is more stable in the $^4C_1$ conformation compared to the $^1C_4$ conformation due to steric crowding.



$^4C_1$ $^1C_4$

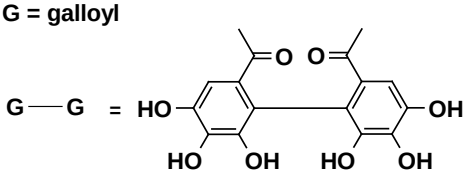
β-D-glucose

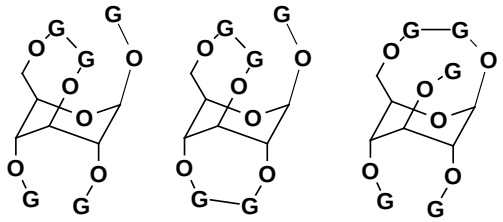
As expected, intramolecular carbon-carbon coupling to form HHDP is most common between C-4/C-6 (e.g. eugeniin); and C-2/C-3 (e.g. casuarictin, also has C-4/C-6).



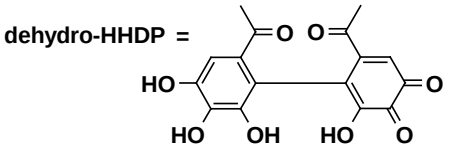
eugeniin casuarictin

G = galloyl





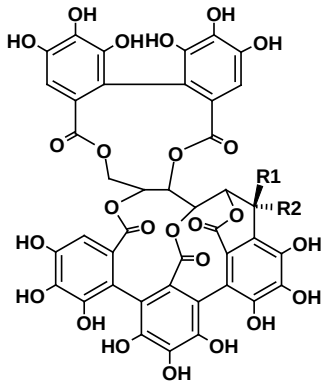
corilagin **geraniin** **davidiin**



However, in a few plants intramolecular coupling 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). In these cased the polygalloyl glucose starting material was presumably in the $^{1}C_{4}$ conformation.

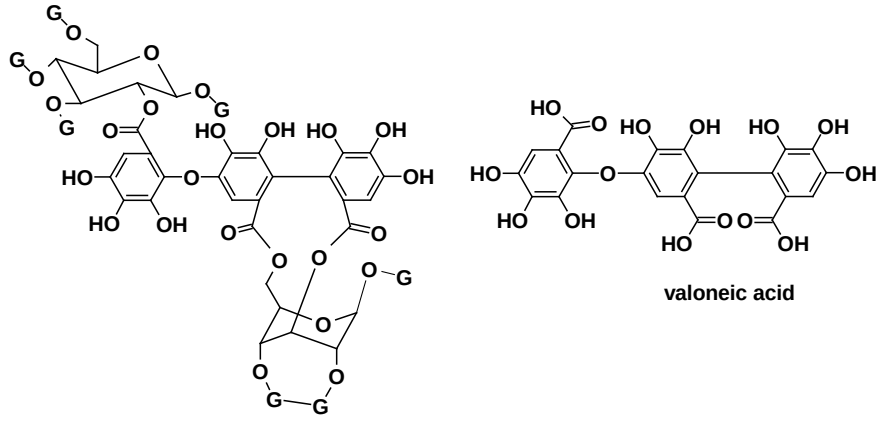
Geraniin is further characterized by partial oxidation of the C-2/C-4 HHDP to dehydro-HHDP. In aqueous solution several forms of dehydro-HHDP can be detected in geraniin by nmr.

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

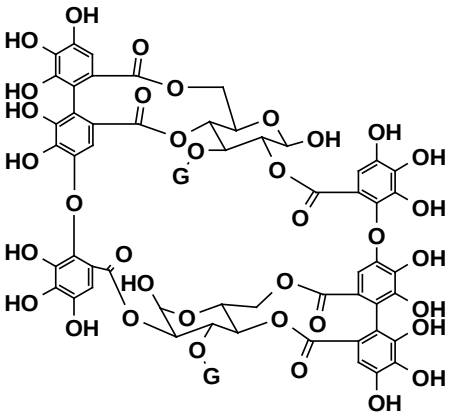
The ellagitannins can undergo intermolecular oxidative coupling with other hydrolyzable tannins to yield dimers. For example, in several euforbs (e.g. *Euphorbia watanabei*) geraniin oxidatively condenses with PGG to yield various euphrobins, characterized by the valoneoyl group.



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

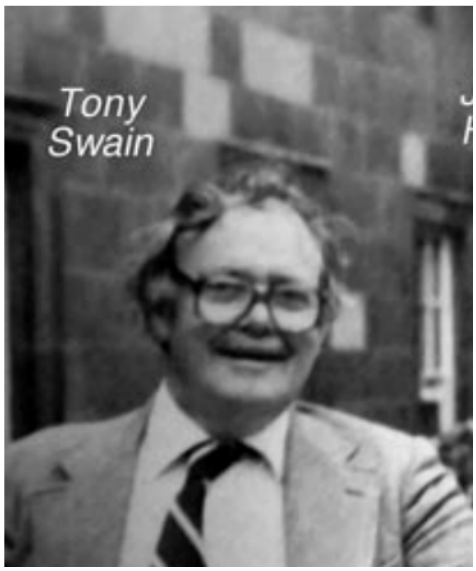
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Volume Two: Extraction & Purification

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Professor Tony Swain (1922-1987). University of Cambridge, Boston University, Kew Gardens. Swain enthusiastically used paper chromatography, a novelty in the 1940s, to characterize the tannins and other phenolics in a wide range of plants. He developed the principles of chemotaxonomy based on his examination of similarities and differences in phenolic profiles of plants. In addition to his scientific accomplishments he was a leader in developing the societies, books and journals that help distribute knowledge of polyphenols to the scientific community.

Extraction and Purification of Plant Polyphenols

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The methods used to extract and purify plant polyphenols must be planned with care since all results of the subsequent experiments are affected by these steps. The tissue treatment during and after collection; the extraction solvent; and the method of homogenization affect both the amount obtained in an extraction and the selectivity of an extraction. A number of older publications systematically explored extraction methods (1-4). The general principles from the older literature hold true, but newer papers provide information on specific plants or tissues, such as blueberries (5) or industrial scaling (6)(.

If the crude extract will be analyzed, consider whether the extraction solvent interferes with the method of analysis. In particular, even traces of acetone inhibit all protein precipitation assays except the radial diffusion assay.

Crude plant extracts are not chemically stable, and can change rapidly during aging. Rotary evaporation to dryness can create "tar" comprising altered compounds that often cannot be re-dissolved. Best practice is to freeze dry the extract or partially purified product for storage when purification cannot be completed in one continuous process.

During purification, product yield and product purity work against one another. As you add steps to increase purity, you incur losses that decrease yield. It is important to evaluate each step to determine if it is helping you achieve the necessary yield and purity for your work. Eliminating steps that do not work towards the desired goal will save time and energy in the often tedious purification process.

Tissue

Phenolics can be extracted from fresh, frozen, dried or lyophilized plant samples. Sample treatment does affect phenolic extractability, so all samples within one experimental design should be treated similarly. Methods that work well for some species may not work at all for others—you should test your methods before collecting a large number of samples to make sure your tissues will be useful.

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 (Sigma-Aldrich), 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).

It may not be possible to freeze dry tissue in some field studies. Tissue can be temporarily stored on ice, dry ice, or immersed in liquid nitrogen. Some field conditions make it necessary to store the samples in

alcohol for transportation to the lab site. As noted above, sun or air drying can change plant chemistry, but in some field situations carefully controlled drying may be the only option.

The dried tissue should be ground before attempting to extract polyphenols. Use a sieve to get uniform defined particle size. A ball mill or a Waring blender can be used for larger samples, or a simple coffee grinder for smaller samples. Keep the grinder clean to prevent cross-contamination.

Fresh or frozen tissue can be ground with liquid nitrogen with a mortar and pestle and the ground material added directly to the extraction solvent.

Solvent

Extraction takes advantage of the polarity of the target molecule. If the extraction matrix can be matched in polarity to the molecule of interest, efficient and somewhat selective extraction can be achieved. In general, polyphenol polarity increases as the number of phenolic groups on the compound increases; at the same time, hydrophobicity increases as the amount of exposed aromatic surface increases. For complex molecules, polarity can be measured as the octanol-water partition coefficient, K_{ow}. More polar molecules have a smaller K_{ow} consistent with their tendency to remain in the aqueous layer in the partitioning experiment. The range of K_{ow} for polyphenols is quite large, with the nearly water-insoluble pentagalloyl glucose (PGG) K_{ow} = 160 compared to the very water-soluble Sorghum procyanidin K_{ow} = 0.002. Muller-Harvey compiled a useful table of K_{ow} for polyphenols (7).

Tannins can be extracted with alcohol or acetone, absolute or aqueous. The most widely used solvent for extracting tannins is 70% acetone (70:30, acetone: water). The acetone can be removed by evaporation under reduced pressure (rotary evaporation) while maintaining the temperature <30 ^{o}C.

Extraction

Samples can be extracted in a beaker or Falcon tube with a stir bar or in a rotator to provide some agitation, or you can use more vigorous methods such a sonication or a homogenizer as described below. After the extraction step, filtration or centrifugation can be used to separate the extract from the tissue. Several rounds of extraction are usually necessary to obtain all the polyphenols from the tissue. These crude extracts contain a variety of polyphenols, contaminated with carbohydrate, chlorophyll and other pigments, and small amounts of protein.

Sonicator

A sonicator uses high energy sound waves to force solvent to penetrate "unwettable" material. For example, we often use about 2 g dry weight or 8 g wet weight of the plant tissue. Place the sample in 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 ^{o}C. Centrifuge 10 min at 2500 x g. Remove supernatant and record exact volume. Repeat the extraction three more times. The first two extractions comprise about 75 % of the total phenolics and the second two extractions account for about 20 % of the total phenolics.

Homogenizer

Torti et al. showed that for some samples phenolics can be extracted efficiently and reproducibly using a Polytron-type homogenizer (8). Care must be taken not to cross contaminate samples with the homogenizer blade, which must be cleaned thoroughly 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. Very soft samples can be homogenized with a Dounce-type homogenizer.

Removing Pigments and Non-Tannin Phenols

Partitioning in a sep funnel

After removing the organic solvent from the crude extract, the aqueous material can be partitioned against a nonpolar solvent such as ethyl acetate or methylene chloride to remove pigments such as chlorophylls, lipids and small phenols. The interface between the aqueous and organic layers may be an emulsion that is difficult to break. You may wait as long as overnight for layers to separate, or you may decide to include the emulsion with the non-tannin (organic) layer. Be consistent in your preparatory steps since small changes can have big impacts on your product.

Sephadex LH-20

Sephadex LH-20 equilibrated in alcohol or aqueous alcohol adsorbs tannins. After removing nontannin contaminants by washing the Sephadex with the alcohol, the tannin can be released with aqueous acetone. Adsorption on Sephadex is very useful for separating tannin from non-tannin phenolics, or for fractionating tannins. Strumeyer and Malin first reported on purifications of tannins with Sephadex LH-20 (9). There are many additional reports in the older and modern literature that can be used to develop a Sephadex LH-20 method for your extract.

Although Sephadex resins (G-series) are widely used in protein and carbohydrate biochemistry to achieve size-based separations, Sephadex LH-20 does not separate tannins by size. The polyphenols are adsorbed by the resin, and adsorption is contrary to the whole principle of size exclusion. Separations on Sephadex LH-20 depend on analyte polarity. There is no general principle to relate tannin polarity to its molecular size.

Sephadex LH-20 (Sigma-Aldrich) is stable and can be used once or washed extensively and re-used. With use, the resin can become brown-colored indicating it should be replaced. The adsorption and eluting steps can be performed in columns or batch-wise. If columns are used, they must have plastic connectors and tubing that are resistant to alcohol and acetone. The methods are the same, it is largely convenience to choose batch vs. column mode. If the batch mode is used, it is effective to mix the resin with the eluting solvent on a rotary mixer at each step to ensure good contact of solvent with the resin. After each mixing step, remove the eluting solvent from the beads in a large sintered glass funnel with paper filter over the glass and gentle vacuum. Keep the medium or coarse sintered glass clean and well-rinsed to prevent cross contamination by material trapped in the pores.

Equilibrate the Sephadex according to manufacturer's directions in ethanol or aqueous ethanol. Do not use denatured alcohol because the denaturant will contaminate your preparation. If you plan to use a column, wash with several bed volumes of ethanol after packing the column. The polyphenol extract is applied to the top of the column in minimal volume. The column is washed exhaustively with aqueous ethanol, and ultimately tannins are eluted with acetone.

If you doing small scale preparations you might simply use the dry Sephadex instead of pre-swelling it. In this case, the dry Sephadex powder is added directly to the aqueous alcohol extract, in small increments, with stirring between each step. When the mixture is the consistency of wet sand, stop adding Sephadex and proceed to batch washing and eluting steps.

The sample should be dissolved in the same aqueous ethanol mixture that the Sephadex is equilibrated in. Solubility of the sample will dictate this. If you cannot completely dissolve the sample, you should centrifuge and discard the solid before proceeding to Sephadex. As you elute with aqueous alcohol, you can monitor the eluate at 280 nm. Absorbance at 280 indicates that aromatic materials, presumably low molecular weight compounds, are eluting—ideally you will wash with aqueous ethanol until A280 approaches zero.

Acetone has a very high absorbance at 280 nm (A280), so when you switch to the aqueous acetone you cannot monitor UV. Instead, you can use the Prussian blue spot test described below to establish when all the polyphenols have been eluted.

Because the Sephadex beads swell to different volumes in the alcohol and aqueous solvents, it sometimes advantageous to step through various aqueous acetone mixtures for elution and re-equilibration. For example, 95% ethanol might be followed by 30% acetone, 50% acetone, 70% acetone to purify tannin. It is useful to evaluate each eluate to check efficacy of purification and recovery. Direct changes in solvent without steps will cracks to form in the bed of Sephadex. The cracks decrease efficiency of the separation since the material can flow through cracks without encountering the resin and thus not achieving the adsorptive step.

The Sephadex can be washed in aqueous ethanol and stored indefinitely in the refrigerator (not freezer) or at room temperature. Make sure the packing does not dry out during storage—you must discard dried out resin. If doing small samples, you may choose to discard the Sephadex and start with fresh dry packing for each sample.

Rotary evaporation

Rotary evaporation is essential to remove organic solvents such as acetone, alcohols, ethyl acetate. You must maintain a good vacuum/condensation system for the large volumes you will work with. Set the water bath for the boiling pot to 30 ^{o}C or slightly lower to keep your sample intact.

Prussian Blue Spot Test

- 0.10 M $FeNH_4(SO_4)_2$ in 0.10 M HCl
  1. Dilute concentrated HCl to 0.10 M by bringing 0.83 mL of the concentrated acid to 100 mL with distilled water. Store in a dark bottle at room temperature for months.
  2. Make the ferric ammonium sulfate by dissolving 4.82 g of the dodecahydrate salt ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$) in 100 mL of the 0.10 M HCl. This will make a pale yellow solution.
- 0.008 M $K_3Fe(CN)_6$ Dissolve 0.263 g of potassium ferricyanide in 100 mL of distilled water. This will make a yellow solution. Store at room temperature for months.
- On the day you will test samples, mix equal volumes of the two reagents. For each test, put one drop of the mixture (pale yellow) into a white background test plate and add one drop of the column eluate. Polyphenol samples will immediately turn green to blue depending on the amount of polyphenol. The color will very slowly change to greenish on samples with no polyphenol. Use some solvent blanks and positive controls to learn to interpret the test. Discard the mixture at the end of the day.

Extraction/Purification Examples

In the following sections, methods that we have employed for extracting and purifying tannins from several sources are described in detail. There are many other methods detailed in the literature. The methods described here can be used as templates to develop the best method for your own samples.

Extraction and Purification of Sorghum Tannin

This method for purifying tannin from Sorghum grain yields a relatively large amount of very high quality procyanidin (about 0.5 g purified tannin from 300 g grain). The original method included a phenol extraction step to ensure that the product was essentially protein free (10). However, the phenol extraction step has been omitted from these instructions since it is rarely required.

Reagents

Prepare these solutions on the day they will be used. Ascorbic acid rapidly oxidizes in solution. It serves as an antioxidant during extraction steps.

- 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.
- Buffer (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.
- Sephadex LH20 equilibrated in 95% ethanol

Method

1. In a cold room, grind 300 g dry, mature high tannin (bird resistant) Sorghum grain in a Waring blender.
2. Transfer the ground grain to a beaker and add 500 mL absolute ethanol containing 10 mM ascorbic acid.
3. Sonicate (75% power, 5 min) and allow the extract to settle. Pour the liquid layer into 50 mL falcon tubes and centrifuge on a tabletop centrifuge at about 3000 xg. Discard this ethanol extract that is rich in low molecular weight phenolics.
4. Return any grain from the Falcon tubes to the beaker, and extract the ground grain with 500 mL methanol containing 10 mM ascorbic acid using the sonicator as above. After the extraction, the samples are centrifuged as above. The tannin-containing methanol extract (supernatant) should be saved. Repeat this step 4 times, combing the extracts.
5. As you collect the methanol extracts, start to rotary evaporate to remove the solvent. Add the buffer (0.05 M acetate pH 4) as the methanol is removed. The buffer will make the solution change from clear brown to cloudy orange. Do not allow the sample to evaporate to dryness. Add enough acetate buffer to maintain solubility. A very large volume of very dilute extract will be difficult to handle in subsequent steps.
6. The methanol must be completely removed during the rotary evaporation. You can check its status by taking a small subsample and mixing in a glass test tube with some ethyl acetate. If the phases separate, the sample is methanol-free.
7. Transfer the sample to a separatory funnel and extract 3x with about 0.5 volume of ethyl acetate. Shake the funnel thoroughly, allow the phases to separate, and save the lower

(aqueous) layer. The upper layer and the interface are discarded—nontannin phenolics and pigments.

8. Rotary evaporate the sample to remove all of the ethyl acetate.
9. Freeze dry the aqueous sample. The freeze-dried crude sample can be saved in the freezer for several weeks if necessary.
10. Re-dissolve the dry sample in 80% ethanol (ethanol: water, 80:20). Centrifuge to remove any undissolved material.
11. Apply the sample to Sephadex LH 20 equilibrated in 95% ethanol in a large sintered glass funnel. 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.
12. Wash the beads with 95% ethanol, gently mixing and then filtering, several times until the A280 of the eluate is low and stable. Discard all of this eluate. It may take a fairly large volume of ethanol.
13. Wash the beads with 50% acetone, saving and combing the washes. The washing should again be done slowly and gently.
14. As the tannin is washed off the beads in the acetone, start to rotary evaporate to remove acetone from the solution. Monitor the washes with Prussian blue spot test to decide how many washes are needed to recover as much of the tannin as possible.
15. After removing all of the acetone from the tannin, freeze dry the sample. Store it in a -20 ^{o}C freezer or colder, tightly capped and in the dark.

Purification of Quebracho Tannin

There are no commercial sources of purified condensed tannins. Small amounts of dimers or trimers can be purchased for hplc standards. The quantity/expense of these standards makes them impractical for biological experiments e.g. feeding studies. Some investigators have used commercial quebracho tannin (*Schinopsis spp.)* as a source of condensed tannin. However, the material must be used cautiously. Unlike the tannins from many other plants, quebraco extracts are predominantly 5-deoxy proanthocyanidins. As a result, quebracho tannin has different chemical and biological characteristics than the more common 5, 7-oxy proanthocyanidins. Furthermore, commercial quebracho is highly contaminated with low molecular weight polyphenols. Butler's groups developed a purification method that can be used to obtain purified quebracho tannin (11). The yield is poor, so large scale biological trials with purified quebracho are not feasible. The material can be used as an analytical standard.

Reagents

- 80% methanol (methanol:water, 80:20)
- Sephadex LH 20 equilibrated in 80% ethanol. Start with 25 g of dry Sephadex
- 50% aqueous acetone (acetone:water, 1:1)

Method

Day 1

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

Day 2

1. Filter the quebracho suspension through Whatman #40 (medium) filter paper, using a Buchner funnel and gentle vacuum if necessary.
2. Add the slurry of Sephadex LH-20 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 Sephadex LH-20, brown tar forms and the preparation must be discarded.
3. Wash the Sephadex LH-20 with 95% ethanol until the absorbance at 280 nm starts to approach zero. It is best to wash fairly slowly, allowing the Sephadex to equilibrate with the fresh ethanol at each step. Discard the ethanol washes.
4. Wash the Sephadex LH-20 with 50% aqueous acetone. Save and combined these washes. Continue to wash until the eluate is nearly colorless. Refrigerate the eluate overnight.

Day 3

1. Rotary evaporate the eluate to completely remove the acetone. Do not concentrate the solution to the extent that material starts to precipitate.
2. Extract the concentrated aqueous sample 3x with an equal volume of ethyl acetate, each time discarding the organic (upper) phase.
3. Remove traces of ethyl acetate from the aqueous phase by rotary evaporation
4. Freeze dry the aqueous sample and store it in a -20 ^{o}C freezer tightly capped and in the dark.

Microscale Extraction of Willow Leaf Tissue

Freeze dried willow leaf tissue is a convenient source of condensed tannin. Willow also accumulates substantial salicylic glycosides (low molecular weight polyphenols) that are easily removed with Sephadex LH-20. Different species of willow have distinct compositions as summarized in the literature.

Brown, Mueller-Harvey and Zeller et al. carefully developed several purification methods (12). Their Method 4 is convenient for microscale purification of condensed tannin from freeze dried leaf tissue and is summarized here. The two-step Sephadex LH-20 purification is a simplification of the original method to get a single sample of condensed tannin instead of samples with slightly different degrees of polymerization.

This method yields about 10-15 mg purified tannin from 0.5 g willow leaf tissue.

Reagents

- Aqueous acetone:
    - 70% acetone (acetone:water, 7:3)
    - 50% acetone (acetone: water, 1:1)
    - 30% acetone (acetone: water, 3:7)
- Ethyl acetate
- Sephadex LH-20 (dry, not pre-equilibrated)
- 50% methanol (methanol:water, 1:1)

Method

1. Use a coffee mill to grind 0.5 g of dry leaves to <1 mm particle size.
2. In a 50 mL beaker extract the leaves with 5 mL of 70% acetone. Stir 30-40 min at room temp with a magnetic stirrer.
3. After extraction, use #1 filter paper in a Buchner funnel with a slight vacuum to remove the extract from the leaf residue. Transfer the liquid to a 50 mL rotovap flask. Return the leaves to the beaker and re-extract two more times (total of three extractions).
4. Rotary evaporate the pooled extracts to remove the acetone. Start evaporating with the first extract and add the others as you collect them. Make sure you remove all of the acetone but do not over-concentrate the aqueous solution.
5. Partition the aqueous sample against 10 mL ethyl acetate four times in a separatory funnel. (The original method used dichloromethane, which works very well but is on many “do not use” safety lists).
6. Save the lower phase (aqueous phase). (With dichloromethane the aqueous is the top layer).
7. Remove traces of organic solvent on rotary evaporator. Transfer the aqueous sample to a 50 mL Falcon tube.
8. Freeze-dry and store in the Falcon tube at -20 °C. You can save this dry crude material until you are ready to go on.
9. In the Falcon tube, dissolve the extract in 3.5 mL of 50% methanol.

10. Add 1.5 g Sephadex LH-20 to achieve the consistency of wet sand. Mix with 5 mL more 50% MeOH. Screw the lid on tight and put the sample on a rotary mixer for 10 minutes at room temp.
11. Transfer the sample to a mini-sintered-glass filter funnel with a filter paper frit over the bottom to minimize clogging. Use gentle suction to filter the sample.
12. Discard the 50% methanol eluate (Fo in the original publication)—it is nontannin phenolics.
13. Return the Sephadex to the Falcon tube. Repeat the elution with 50% methanol three more times, discarding the eluate each time.
14. At this point you can do either step-wise or two-step elution.
15. Stepwise elutions: Use 5 mL solvent each time. As you extract, pool the extracts from each step in the round bottom flask and continue to evaporate. The fractions F1-F3 contain condensed tannins with different DP. F1 often also contains some low MW polyphenols.
    a. Do a total of four elutions consisting of resin mixing/filtering/pooling/rotovap with 30% acetone (F1).
    b. Repeat 4 times with 50% acetone (F2).
    c. Repeat 4 times with 70% acetone (F3).
16. Two step elution: Use 5 mL solvent each time. As you extract, pool the extracts from each step in the round bottom flask and continue to evaporate. The fraction F4 contains the mixture of all condensed tannins.
    a. Continue mixing/filtering with 50% methanol until the eluate has A280 = 0. This ensures removal of all low molecular weight phenolics.
    b. Do a total of four elutions (resin washing/filtering/pooling/rotovap) with 70% acetone (F4).
17. For either elution method, as each pooled sample finishes on the rotovap, move it to the N2 manifold to finalize solvent removal.
18. Freeze dry each fraction in 50 mL falcon tube. Store samples at -20 °C in the dark.

Purification of Cocoa Tannin

This method for purifying tannin from commercial cocoa powder yields a product useful for biological studies. The yield is about 100-300 mg tannin from 10 g cocoa powder. The method is derived in part from Sorghum tannin purification methods (10) but additional steps were added to deal with the high fat content of cocoa powder (13).

Theobromine is a major constituent of cocoa powder and it is important to remove it from the purified tannin. Check the product for theobromine contamination by HPLC or NMR.

Reagents

- Commercial cocoa powder. Hershey's Natural Unsweetened Cocoa is suitable. This is not alkalized, and it does not contain any sweetener or milk products.
- Large Soxhlet extractor and cellulose thimbles
- Hexane
- 95% ethanol
- 80% ethanol (ethanol: water, 8:2)
- 70% acetone (acetone: water, 7:3)
- Sephadex LH-20 equilibrated with 95% ethanol in a suitable column

Method

1. Place about 10 g of cocoa powder in the Soxhlet extractor thimble. Do not fill thimble more than half full with the solid.
2. In a fume hood, place about 300 mL of hexane in a 1 L round bottom flask. Adjust the heating mantle so the hexane is gently boiling (about 67 ^{o}C). Attach the extractor and cold water condenser. Chill the water entering the condenser by running the tubing through an ice bath. Extract for about 2 hours.
3. Remove the thimble from the apparatus. Place it in a clean beaker in the hood to drain & dry overnight. Discard the hexane/lipid mix from the round bottom with organic waste.
4. Transfer the dry defatted powder from the thimble into a 500 mL beaker, making sure to mix and break up the powder thoroughly. Add 100 mL of 70% acetone to the beaker.
5. Mix the slurry and divide it among 4 Falcon tubes (50 mL). Tightly cap each tube and rotate for 20 min at room temperature.
6. Centrifuge the samples in a table top centrifuge at about 3000 x g for 6 min.
7. Remove the supernatant and pool in a beaker. Repeat the extraction two more times in the Falcon tubes, using 100 mL total of the 70% acetone each time. Use a spatula to stir up the solid when you add the fresh extraction solvent. Add these supernatants to the pool.
8. The ~300 mL pooled fraction is rotary evaporated to remove all of the acetone. As you evaporate, add small portions of 100% ethanol to the flask, about 10-15 mL each time. Check for complete removal of the acetone by carefully smelling the extract. If the sample is

cloudy, centrifuge and decant the clear brown liquid. You can store this extract overnight in the refrigerator if necessary.

9. If the sample was stored overnight, centrifuge once more before applying to the column to prevent any solids from entering the column. Apply the solution to a Sephadex LH-20 column equilibrated in 95% ethanol. Use about 10 volumes of Sephadex LH-20 relative to the sample (80 mL sample onto 800 mL bed volume). After the sample has entirely entered the column, carefully apply 95% ethanol and build up a comfortable head.
10. Wash the column with 95% ethanol, following the absorbance at 280 periodically. You should see a brown band near the top of the column that moves quite slowly—that is the desired product. Wash with 95% ethanol, noting that the first part of the eluate is a white suspension rather than a solution.
11. Continue to wash with the 95% ethanol until the A280 has started to plateau and you have obtained at least 1200 mL of clear, light brown liquid. It is important to remove and discard as much of the non-tannin material as possible since you are removing unwanted theobromine and sugars. You can check the ethanol washes for theobromine with HPLC as you proceed.
12. Switch the elution solvent to 70% acetone. Discard the first fractions, but save the acetone eluate once the dark band from the top of the column starts to come off—this is your condensed tannin. Continue to wash the column until you can no longer clearly see the brown band. The column should be uniformly light colored and the eluate should be colorless.
13. Remove the acetone by rotary evaporation. Freeze dry the aqueous solution. Store the material at -20 ^{o}C in the dark.

Purification of Apple Dimer

The method was developed by Li et al. to provide a source of dimer condensed tannins for analytical standards (14). The product is predominant dimer B-2 (epicatechin-(4β->8)-epicatechin) but may contain a trace of dimer B-1 (epicatechin-(4β->8)-catechin). Preparative HPLC can separate these two dimers. The original method describes how to use the dimer for standardization of the acid butanol assay. Dimers and trimers can be obtained commercially, although the cost for 50 mg (easily obtained from 1.5 kg apple) is prohibitive.

Phytochemical composition can be cultivar specific, so use Granny Smith apples to obtain the B-2 dimer.

Reagents

- Granny Smith apples from grocery store or market. You will need 7-8 apples
- Methanol
- Amberlite XAD-7HP resin washed and equilibrated with water to yield 600 mL thick slurry
- Ethanol
- Ethyl acetate
- Toyopearl TSK HW-50F equilibrated in a 3 x 50 cm column with methanol

Method

1. The preparation of the crude extract is done on one apple (200 g). The crude extract is ultimately pooled for the final chromatographic step to yield the pure dimer.
2. Wash an apple and cut it into small cubes. The tissue browns rapidly, so cut the apple up immediately before starting the purification.
3. Transfer the cut apple into a 1 L round bottom flask and add four volumes of methanol (about 800 mL).
4. Reflux on a heating mantle for 1.5 h, using an ordinary cold water condenser to return the solvent to the flask as it is heated.
5. Filter the extract through #1 Whatman paper.
6. Put about 300 mL of the filtered extract into a 1 L rotary evaporation flask and start to evaporate. Store the rest of the extract at 4 ^{o}C, adding extract as necessary to maintain the volume in the rotary evaporation flask.
7. Repeat the reflux extraction two more times, pooling all of the extracts.
8. Reduce the volume of the total pooled extract (about 2400 mL) to about 250 mL with the rotary extraction.
9. Transfer the concentrated extract to a 1 L beaker containing the XAD resin.
10. Periodically swirl the resin to mix the slurry during a 45 min incubation.
11. Allow the resin to settle and decant the liquid. Discard liquid, save the resin.
12. Add 200 mL water and incubate 45 min as above.
13. Allow the resin to settle and decant the liquid. Discard liquid, save the resin and repeat this water wash.
14. Add 200 mL ethanol to the resin and incubate 45 min as above.
15. Filter the suspension through a course fritted glass funnel using filter paper on the frit to prevent blockage. Keep the ethanol filtrate.
16. Rotary evaporate the ethanol to dryness.
17. Redissolve the sample in 50 mL water.

18. In a separatory funnel partition the sample against and equal volume of ethyl acetate. Transfer the ethyl acetate (top) layer to a rotary evaporator flask.
19. Rotary evaporate the sample, adding water to ensure that the sample does not go to dryness.
20. Re-extract the aqueous layer with ethyl acetate three times. Discard the water layer after the third extraction.
21. Pool the ethyl acetate fractions and rotary evaporate to remove all the organic solvent.
22. Freeze dry the sample to obtain about 30 mg of dry crude extract.
23. After extracting 7-8 apples, you should have about 200 mg of crude dry extract. Dissolve it in 2 mL methanol.
24. Apply the sample to a Toyopearl column equilibrated in methanol.
25. Wash with methanol, collecting 5 mL samples. Check the absorbance of each fraction at 280 nm, pool peaks, and confirm peak identities by HPLC-MS.

Fractionation of Tannic Acid

Gallotannins can be purified to serve as chromatographic standards or as standards in various assays. Commercially available tannic acid is the most convenient starting point for purification of specific gallotannins. Unlike many commercial chemicals, tannic acid has a variable composition dependening on manufacturer and lot number.

Recall that gallotannins such as tannic acid consist of a glucose (or similar polyol) core which is esterified with up to five gallic acid groups. These core gallic acid groups may be further linked via "depside" bonds (ester bonds involving a phenolic OH instead of alcoholic OH) to additional gallic acids. The exact mixture of esters (families comprising the various isomers of monogalloyl glucose through dodecagalloyl glucose) depends on the plant source and method of extraction/purification used by the manufacturer. For all commercial preparations of tannic acid, the molecular weight reported by the manufacturer is a theoretical mol weight 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.

Material from some suppliers/lots comprise mainly mono- through tetragalloyl glucose. Other preparations contain hepta- through dodecagalloyl glucose. HPLC can be used to evaluate various starting materials. Examples of tannic acid heterogeneity revealed by HPLC analysis can be found in several papers: (15-18).

Methods provided here either fractionate the tannic acid into individual families (for example, octagalloyl glucose isomers) or purify the single defined compound pentagalloyl glucose (1,2,3,4,6-penta-O-galloyl-D-glucopyranose). Be aware that it is essential to use an independent technique, such as HPLC-MS or NMR, to confirm the identity of the fractions that you obtain with these methods.

Purification of Galloyl Ester Families (Trigalloyl Glucose, 4GG, etc).

Tannic acid can be fractionated without preliminary methanolysis to yield enriched mixtures of the various families of galloyl esters (19). For the initial purification, Sephadex LH-20 is used to fractionate the tannic acid. 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. For each of the galloyl esters, there are many possible positional isomers. Chromatography including prep scale HPLC can be used to identify and if necessary to fractionate the isomers after the preliminary fractionation described here.

Reagents

- Aqueous acetone:
    - 70% acetone (acetone:water, 7:3)
    - 50% acetone (acetone: water, 1:1)
    - 30% acetone (acetone: water, 3:7)
- Ethanol
- Sephadex LH-20 equilibrated in ethanol, in a suitable column

Method

1. Dissolve tannic acid in absolute ethanol.
2. Apply the sample to the Sephadex LH-20 column.
3. Wash the column successively with ethanol, then 30% acetone; 50% acetone; 70% acetone to release fractions of galloyl glucoses. The eluates need to be monitored with the quick Prussian blue test to evaluate when to change to the next stronger eluting solution.
4. Rotary evaporate each eluted fraction to remove the organic solvent and freeze dry the material.
5. Evaluate the fractions with HPLC or HPLC-MS to identify families (trigalloyl glucoses, tetragalloyl glucoses, etc.)
6. The fractionation can be refined by introducing aqueous ethanol solutions or by using intermediate aqueous acetone solutions to achieve the desired fractionation.

Purification of Pentagalloyl Glucose (PGG) from Tannic Acid

Ester bonds are somewhat more stable than the depside bonds. Treatment of tannic acid in mildly acidic 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 PGG.

Methanolysis does not always yield PGG. For example, some preparations of commercial tannic acid are based on a tetragalloyl glucose core (e.g. 1,2,4,6 galloyl glucose). Higher esters in those mixtures yield tetragalloyl glucose and methyl gallate under methanolysis conditions.

The method described here yields about 0.2 g of PGG is obtained from 0.5 g of tannic acid (20). The method can be scaled up to start with 25 g.

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.
- 2% methanol (methanol: water, 2:98)
- Methanolysis solution. Mix 70 mL methanol with 30 mL of the acetate buffer.
- 0.25 M NaOH
- Ethyl acetate
- Diethyl ether (peroxide-free)

Method

7. In a test tube, dissolve 0.5 g tannic acid in 10 mL of the methanolysis solution. Cover, and place in 65°C water bath for 15 h.
8. After the 15 h incubation, increase the pH of the mixture to 6, while stirring, with 0.25 M NaOH.
9. Rotary evaporate at temperatures below 30°C to remove methanol. As the methanol is removed, add water to maintain volume. Do not let the sample go to dryness.
10. 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. Discard the top layer after each extraction, using appropriate methods to discard ether.
11. Extract the aqueous layer three times with ethyl acetate, saving the ethyl acetate (top) layer.
12. Combine the ethyl acetate extracts and rotary evaporate to reduce the volume. Add water to maintain the volume, and continue to evaporate to remove all of the organic solvent.
13. Centrifuge the aqueous suspension.
14. Collect the precipitate and redissolve it by gentle heating in 10 mL of 2% methanol.
15. Slowly cool the solution to room temperature, and collect the precipitated PGG by centrifugation.
16. Wash the precipitate twice with 10 mL of ice-cold 2% methanol
17. Wash the precipitate once with 10 mL of ice cold water.
18. Re-suspend the material in water, and freeze dry the suspension to yield pure white PGG.

Purification of Oenothein B (fireweed tannin)

Fireweed (*Epilobium angustifolium*) is a convenient source for the ellagitannin Oenothein B. We use freeze-dried leaf tissue as the starting material.

Reagents

- 70% acetone (70:30, acetone:water)
- Methanol
- 95% ethanol
- XAD-7 resin pre-equilibrated in water
- Sephadex LH-20 equilibrated in methanol

Method

1. Suspend 10 g of freeze-dried tissue in 200 mL of 70% acetone in the cold. Divide the suspension among 50 mL Falcon tubes, tightly cap, and rotate for 30 min in the cold. Allow the samples to settle and decant and save the extract. Repeat a total of three times, pooling the extracts.
2. Filter the extracts through #1 filter paper.
3. Rotary evaporate the sample to remove all of the acetone. Do not allow the sample to evaporate to dryness.
4. In a sintered glass funnel with a paper filter to prevent clogging, mix the aqueous sample with the XAD-7. Use gentle suction to wash the water through the filter, and discard (sugars, nontannin phenolics). Wash with at least 4 volumes of water.
5. Elute the tannin from the XAD-7 with acetonitrile, pooling the eluates. Use the Prussian blue spot test to confirm that all the tannin is recovered.
6. Rotary the evaporate the sample just to dryness and re-dissolve immediately in methanol.
7. In a sintered glass funnel with a paper filter to prevent clogging, mix the methanol sample with Sephadex LH-20 equilibrated in methanol. Use gentle suction to wash the extract through the filter, and discard the methanol (nontannin phenolics).
8. Mix the resin with 95% ethanol and elute until the A280 of the eluate is low and stable. Discard this eluate, it is nontannin phenolics.
9. Mix the resin with 70% acetone and elute until the Prussian blue spot text is negative (light green-yellow).
10. As you collect the eluate, rotary evaporate to remove the acetone. Freeze dry the aqueous liquid and check purity by HPLC.
11. Re-equilibrate resins with exhaustive washing.

Isolation of (-)Epigallocatechin from Green Tea Extract

Introduction

There are many published methods for extracting polyphenols from green tea, but in general these do not produced high levels of EGC. In this method, the yield is maximized by using tannase to hydrolyze the abundant polyphenol EGCg in the tea extract (21).

Reagents

- Tannase (25 mL, 0.1mg protein/mL)
- 3% aqueous sodium alginate (67 mL)
- 2% $CaCl_2$ (20 g $CaCl_2$ dissolved in 1 L distilled water)
- 0.025% glutaraldehyde. Commercial solutions of 25% glutaraldehyde are available. Dilute 1 mL of the 25% solution into 1 L of distilled water to make 0.025% glutaraldehyde
- 50 mM sodium acetate buffer pH 5.0
- Crude green tea extract obtained as a powder from SilvaTeam (100 mg)
- Ethanol
- Dichloromethane:ethanol, 1:1 (v/v) (You will need a chlorinated solvent waste disposal system to use this solvent)
- Silica gel column (2 cm i.d. x 25 cm) pre-equilibrated by washing with 300 mL dichloromethane : ethanol (85:15, v/v). Discard the solvent appropriately.
- 80% ethanol containing 0.2% acetic acid (ethanol:water:acetic acid 80:19.8:0.2, v/v/v)
- Sephadex LH-20 equilibrated in the ethanol:water:acetic acid mixture. This can be reused as long as it is washed thoroughly with the ethanol:water:acetic acid mixture after each purification.

Methods

Immobilized tannase preparation:

1. Mix tannase and aqueous sodium alginate by magnetic stirring for 30 minutes.
2. Use a syringe to drip the solution into 1 L of 2% $CaCl_2$. Beads of sodium alginate will form.
3. Wait for 1h for hardening.
4. Cross-link by adding 1.0 L of 0.025% glutaraldehyde (1 L) and stirring gently for 2.5 hours.
5. After incubation, rinse tannase beads twice with sodium acetate buffer.
6. Store the beads suspended in the buffer at 4 °C.

Preparation of the tea polyphenols:

1. Mix 100 mg of tea extract with 5 mL of ethanol
2. Mix on a rotary mixer for 10 minutes.
3. Centrifuge the mixture for 5 minutes at 800 x g.
4. Remove the supernatant to pool with two successive extractions.
5. Re-extract the pellet with ethanol two times for a total of 15 mL of pooled extract.
6. Rotary evaporate the pooled supernatant to dryness (do not over-dry).

7. Redissolve the residue in 10 mL dichloromethane : ethanol.
8. Store the crude extract at -20°C until it is convenient to purify the sample.

Purification of EGCg:

1. Load the sample carefully on top of the silica gel
2. Elute the column with 200 mL of the dichloromethane:ethanol (85:15) mixture. Discard this eluate as chlorinated solvent waste
3. Wash the column with 100 mL of ethanol. Check this eluate with HPLC before discarding.
4. Wash the column with 100 mL of ethanol. Check this eluate with HPLC to confirm that it is EGCg-rich.
5. Rotary evaporate the EGCg-containing eluate to dryness.
6. Redissolve the residue in water and filter the suspension through a double layer of filter paper.
7. Freeze dry the eluate and store at -20 °C until it is convenient to perform the tannase-catalyzed hydrolysis.

Tannase-catalyzed hydrolysis:

1. Dissolve the lyophilized solid (100 mg) in 20 mL of 50 mM sodium acetate buffer (pH 5.0).
2. Mix the solution with about 10 g tannase beads (about 5 mL) and add enough acetate buffer to achieve a 50 mL reaction volume.
3. Allow the mixture to sit at room temperature for 30 min.
4. Remove the tannase beads from the solution by filtering the suspension. Discard the beads.
5. Extract the filtrate with ethyl acetate (100 mL) and save the ethyl acetate (upper) layer.
6. Rotary evaporate the ethyl acetate to dryness.
7. Redissolve the material in water.
8. Filter the water solution and freeze dry the material.
9. Store the lyophilized hydrolysis products at -20°C for isolation and purification with Sephadex LH-20 chromatography.
10. Use HPLC before and after the tannase treatment to confirm hydrolysis of the EGCg to EGC.

Isolation and purification with Sephadex LH-20 chromatography:

1. Dissolve the lyophilized hydrolysis product (100 mg) in 7 mL of 80% ethanol containing 0.2% acetic acid.
2. Filter the product with a 0.22 µm nitrocellulose filter.
3. Prepare the Sephadex LH-20 in a suitable column (i.d. 2cm x 27 cm) and equilibrate with 80% ethanol containing 0.2% acetic acid.
4. Apply the hydrolysis product to the column and elute with 80% ethanol containing 0.2% acetic acid. Collect 50 fractions of eluate (8.5 min/fraction) and record the absorbance of each fraction at 220 nm and 289 nm. Pool the fractions that are rich in EGC.
5. Rotary evaporate the pooled fractions to dryness.
6. Redissolve the product in water, freeze dry the sample, and store the material at -20 °C.

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Condensed Tannin HPLC

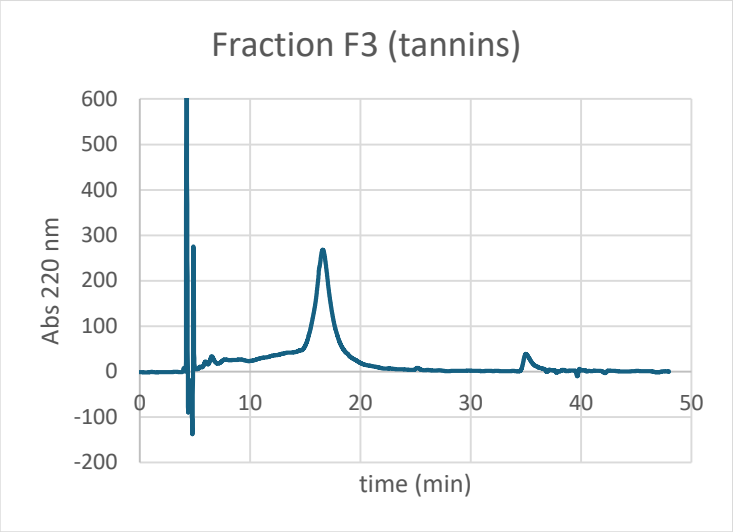
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HPLC of condensed tannins has proved more difficult than HPLC of hydrolysable tannins. Good separations of monomeric flavonoids are easily achieved. Oligomeric proanthocyanidins (dimers-pentamers) can be resolved. However, separation of the high molecular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

Gradient reversed phase separations usually yield a "condensed tannin hump" once a moderate amount of organic solvent is reached. Often the hump can be optimized (almost peak-like) by adding a fairly steep change in solvent composition—the elution seems dependent on the rate of change of the solvent, not a specific % organic solvent. For example, the solvent gradient we use to analyze thiolysis samples has a steep change in solvent between 8-10 min. The tannin "hump" in unthiolyzed samples run with this gradient is usually centered at about 16 min. Lower molecular weight phenolics normally elute before the hump and thiolysis products elute later. The example shown below has a main tannin hump, with traces of acetone exaggerating the injection peak and a small peak towards the end of the chromatogram corresponding to solvent re-equilibration.

Flow rate: 0.5 mL/min
Aqueous Solvent: 0.13%TFA in water
Organic Solvent: 0.1%TFA in acetonitrile
Temperature: 27.0 °C
Detection: 220 nm DAD

Time [min]	% Aqueous	%Organic
0	85.00	15.00
3	85.00	15.00
8	80.00	20.00
10	70.00	30.00
28	70.00	30.00
32	30.00	70.00
37	30.00	70.00
40	85.00	15.00
48	85.00	15.00



Condensed tannin 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 integrate the peaks, and with diode array detection you can obtain the UV-visible spectrum of the peaks which can assist you with peak identification.

Flavan-3-ol monomers are commercially available. Some dimers and trimers can be purchased. Other standards need to be prepared in-house based on your own plants and natural product profile.

Some very effective HPLC-MS tools have been developed by the Salminen group in Turku. Reference to the Natural Chemistry Research Group web pages will introduce you to these sophisticated tools for separating and characterizing tannins.

The HPLC-related papers provided below could provide inspiration for researchers willing to tackle this difficult analytical problem.

Citations

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Kelm, M., J. Johnson, R. Robbins, J. Hammerstone and H. Schmitz (2006). High-performance liquid chromatography separation and purification of cacao (Theobroma cacao L.) procyanidins according to degree of polymerization using a diol stationary phase. *J Agric Food Chem* **54**: 1571-1576.

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

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Hydrolyzable Tannin HPLC

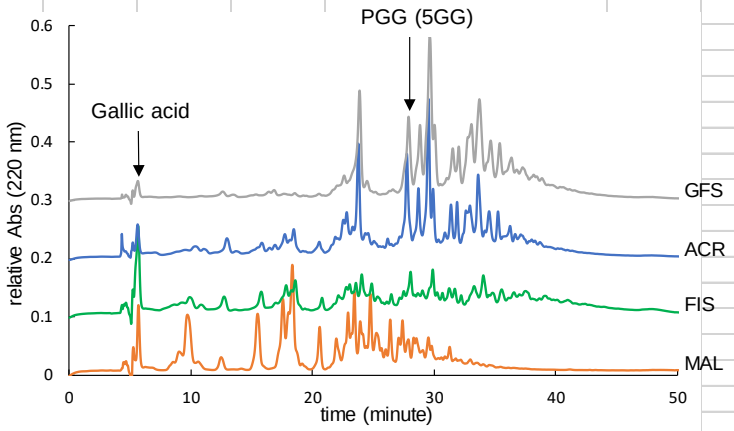
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Hydrolyzable tannins can be fractionated by HPLC to provide both qualitative information on the homogeneity of a particular preparation; estimation of molecular weight; and quantitative information on specific compounds.

Either normal phase or reversed phase HPLC can be used for hydrolyzable tannins. Reversed phase is recommended since the solvents are generally easier to work with.

For tannic acid, we typically use a simple gradient system. With a C-8 column, we use a water/acetonitrile gradient with trifluoroacetic acid as modifier. The chromatogram below shows the variable composition of tannic acid preparations from four suppliers (Acros, Mallinckrodt, GFS Chemicals and Fisher Scientific). The arrows indicate elution times of commercial gallic acid and in-house purified PGG. The peaks are sharp and well-separated, but the preparations are clearly complex mixtures. A similar gradient can be used for more complex hydrolyzable tannins including ellagitannins.

Tannic Acid			
Stoptime		**60**	**min**
Solvent	**A**	**0.13% TFA H2O**	
Solvent	**B**	**0.1% TFA MeCN**	
temperature		**27**	**oC**
time	**%A**	**% B**	**rate**
min	**aqueous**	**organic**	**mL/min**
0	90	10	0.5
3	90	10	0.5
40	70	30	0.5
50	20	80	0.5
55	90	10	0.5
60	90	10	0.5



Typical chromatograms can be found in some older papers (1,2). The separation shown here is from (3).

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 integrate the peaks, and with diode array detection you can obtain the UV-visible spectrum of the peaks which can assist you with peak.

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

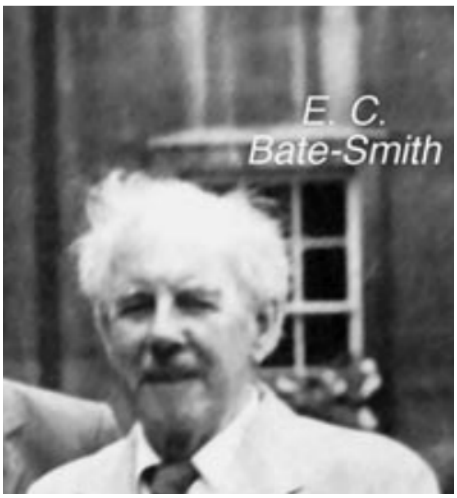
Citations

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Volume Three: Chemical Methods

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Professor Edgar Charles Bate-Smith (1900-1989) was a chemist at the Low Temperature Research Station in Cambridge, UK. He contributed to a modern understanding of tannin chemistry and bioactivity with his careful development of analytical tools. His Acid Butanol Method for detecting and quantitating condensed tannins is still a key method for polyphenol chemistry. He developed an early protein precipitation assay using hemoglobin as the protein partner to the tannin, and that method served as the basis for development of many modern methods for assessing protein precipitation and binding by tannins.

Introduction to Chemical Methods

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Diverse chemistry has been developed to exploit the reactivity of either the phenolic functional group or phenol-derivatized aromatic rings. These methods can be used to quantitatively and qualitatively evaluate plant extracts. The only instrumentation required for most of these methods is a simple UV-visible spectrophotometer and standard laboratory glassware. The methods are robust as evidenced by their frequent citation in the natural products literature. HPLC and mass spectrometry provide more details but these simple functional group methods are invaluable tools for tannin determination.

The literature citations that describe the approaches summarized below are found on the detailed Methods sheet for each method.

General Phenolic Methods

Phenolic compounds 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.

Differences in the redox potential and reaction 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.

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(1). However, these methods have not been adequately tested and are not recommended.

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.

Condensed Tannins (Proanthocyanidins)

Condensed tannins 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 cleaved by acidic alcohol and heat. The reaction is frequently called a “hydrolysis” in the literature, but its correct chemical description is oxidative cleavage. The anthocyanidins that are released are quantitated by visible spectroscopy. Several modifications 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 provide additional structural details for condensed tannins.

Appropriate standards for condensed tannin methods include purified Sorghum tannin, purified quebracho tannin, and various preparations of grapeskin or grapeseed tannins. Simple dimers and trimers of catechin and epicatechin are commercially available. Flavan-3-ols (catechin, epicatechin) and their derivatives are commercially available, and the various anthocyanidins (e.g. cyanidin, delphinidin) can be purchased.

Hydrolyzable Tannins

Hydrolyzable tannins comprise phenolic acids esterified to a carbohydrdate core. Functional group methods can be used to detect the phenolic acids released by hydrolysis. characteristic of the various hydrolyzable tannins.

Gallic acid is released in large quantity when gallotannins are hydrolyzed, and in smaller quantity from ellagitannins. Gallic acid can be quantitatively determined with either the rhodanine assay or the potassium iodate method. Care must be taken during the hydrolysis to avoid oxidative destruction of the gallic acid.

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.

Phlorotannins

Phloroglucinol, the basic subunit of the phlorotannins, reacts similarly to the meta-hydroxylated A ring of the condensed tannins. Chemistry similar to vanillin reaction chemistry can be used to determine phlorotannins. Instead of vanillin, dimethoxybenzaldehyde is used and the reaction conditions are optimized for the phlorotannins. The method has not been optimized to reveal the chemical diversity of the phlorotannins.

Citations

1. Mole, S., and Waterman, P. G. (1987) A critical analysis of techniques for measuring tannins in ecological studies. I. Techniques for chemically defining tannins. *Oecologia* **72**, 137-147

Determination of Total Phenols with Prussian Blue

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Introduction

The Prussian blue assay was first described by Price and Butler in 1977 (1). Like the more widely used Folin assay, the Prussian blue assay is a redox method that detects all compounds with a suitable reducing potential. Neither the Folin assay nor the Prussian blue assay is specific for polyphenols. Thiols (e.g. cysteine), ascorbic acid, and protein are common interfering compounds in plant extracts.

In the Prussian blue assay, a redox reaction between iron (III) and the analyte yields iron (II) which is converted to a blue-green chromophore by reaction with potassium ferricyanide. Some problems with formation of insoluble side products are minimized with the modified Prussian blue assay developed by H. D. Graham (2). Both methods require several reagents and careful timing. Instructions for both the original method and the modified method are provided. The original method was a macro-scale reaction but the modified method is done on a more convenient micro-scale.

One significant difference between the methods described here and the method as originally published is the use of ferric ammonium sulfate instead of ferric chloride as the first reagent. Problems frequently encountered when attempting to prepare the ferric chloride reagent are eliminated by using ferric ammonium sulfate. For both methods, poor quality water, especially iron-containing water, will give high blanks and unacceptable results

Original 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 ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$) 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.
- Both reagents can be stored for long periods at room temperature in brown bottles. The reagents should be prepared fresh if precipitate forms or the color changes

Method

1. 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.
2. Add 3.0 ml $FeNH_4(SO_4)_2$ and swirl. Additions should be timed; 1 min intervals are convenient.
3. 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.

4. Exactly 20 min after the addition of the ferricyanide, read Absorbance at 720 nm, making readings at 1 minute intervals.
5. 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.

Modified Prussian Blue Assay

Reagents

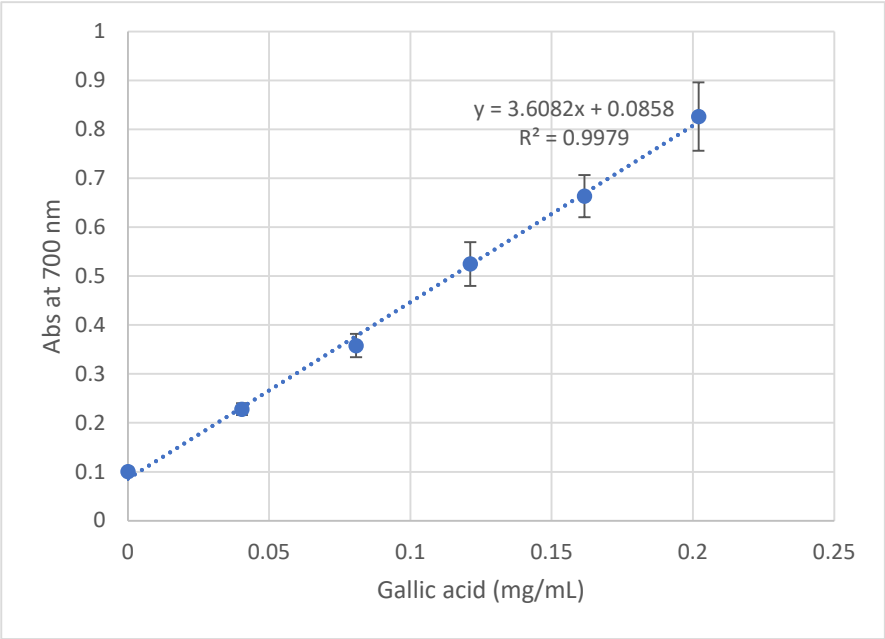
- 0.02 M $FeNH_4(SO_4)_2$ in 0.10 M HCl
  1. Dilute concentrated HCl to 0.10 M by bringing 8.3 mL of the concentrated acid to 1 L with distilled water.
  2. Make the ferric ammonium sulfate by dissolving 9.64 g of ($FeNH_4(SO_4)2$ x 12 H_2O) (dodecahydrate salt of ferric ammonium sulfate) in 1 L of the 0.10 M HCl..
- 0.016 M $K_3Fe(CN)_6$ Dissolve 5.26 g of potassium ferricyanide in 1 L of distilled water.
- Both iron reagents can be stored for long periods at room temperature in brown bottles. The reagents should be prepared fresh if precipitate forms or the color changes
- Stabilizer (Stable for 1 week in refrigerator). You need 5 mL per assay sample. 30 mL distilled water, 10 mL 85% H_3PO_4, 10 mL 1% gum arabic.
  1. 85% H_3PO_4 is the typical, commercially available phosphoric acid.
  2. 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.
  3. 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

1. 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.
2. Add 3.00 mL distilled (deionized) water and vortex the mixture.
3. To each sample, add 1.00 mL $K_3Fe(CN)_6$ followed immediately by 1.00 mL $FeNH_4(SO_4)_2$ 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 original method.
4. 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.
5. 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.
6. 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).
7. 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.

Typical data for the modified Prussian blue assay

1. You need to prepare your own standard curve with your samples. This is a typical curve with our instruments and samples.
2. Other standards can be used, but the slope of the response curve will be different for each polyphenol depending on its redox potential and number of hydroxyl groups.



Citations

1. Price, M. P., and Butler, L. G. (1977) Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* **25**, 1268-1273
2. Graham, H. D. (1992) Stabilization of the prussian blue color in the determination of polyphenols. *J. Agric. Food Chem.* **40**, 801-805

Determination of Total Phenols with the Folin Assay

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Introduction

The Folin Assay is a redox-based spectrophotometric method for determining total phenolics in some plant extract, soil extract, or other type of sample. The method given here is modified from a microscale method (1).

The Folin Reagent turns from yellow to blue (measured at 750 nm) upon reduction by polyphenols. However, any other reducing agents in the sample, for example ascorbic acid, will also reduce the Folin Reagent. Therefore, this is a non-specific, but quick and convenient assay for measurement of total reducing agents. The value is often used as an estimate of total polyphenols.

A similar method for total polyphenols is the Prussian blue method. The Prussian blue method has similar shortcomings to the Folin assay. The Lowry assay is a modified Folin assay with increased sensitivity for protein. The Lowry method does work for polyphenols but has no advantages compared to the Folin method.

Reagents

- Approximately 0.2 mg/mL methyl gallate standard: Weigh ~1 mg methyl gallate into a test tube. Add 200 uL methanol and vortex. Add 5 mL water and vortex. The exact concentration of this standard is determined spectrophotometrically after completing the analysis
- 20% sodium carbonate, Na_2CO_3: Weigh out 20 g anhydrous Na_2CO_3. Bring up to 100 mL with distilled H_2O
- Folin reagent (commercial, e.g. Sigma F9252)
- High quality distilled water
- Standard 96 well plates, plate reader with visible light capacity, plate shaker

Method

1. Dispense five standards in triplicate into the desired wells on the plate. The wells should contain about 0, 1, 2, 3, and 5 ug of methyl gallate. Assuming a standard concentration of 0.2 mg/mL, this is 0, 5, 10, 15, and 25 uL. This amount of methyl gallate gives a linear response while keeping absorbance readings below 2. Make up each well to 25 uL with water.
2. Prepare unknowns in triplicate. Use 25 uL of unknown for analysis.
3. Add 50 uL Folin Reagent to each well. A multichannel pipette greatly increases the efficiency of this step.
4. Thoroughly mix on a plate shaker. Mix carefully at the lowest speed to ensure none of the reaction mixture sloshes out of the wells.
5. Add 20 uL of 20% Sodium Carbonate to each well. Watch wells carefully as they may start to vigorously fizz upon this addition.

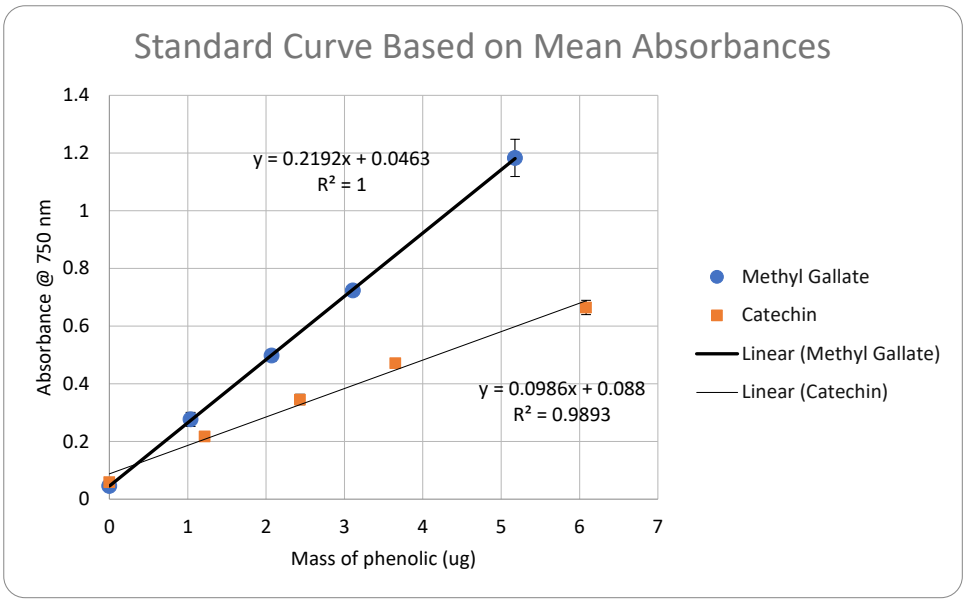
6. Thoroughly mix on a plate shaker. Mix extremely carefully here, since well plates will fizz more as they are mixed. To make sure the wells do not fizz over, mix on the lowest speed for 3 seconds maximum, then stop and wait for the fizzing to go down, about 10 seconds. Repeat at least 5 times to ensure thorough mixing.
7. Incubate the well plate in the dark at room temperature for 20 minutes.
8. Bubbles or foam in the wells will interfere with spectrophotometric readings, so use a micropipette to remove foam if necessary.
9. Read the plate at 750 nm.

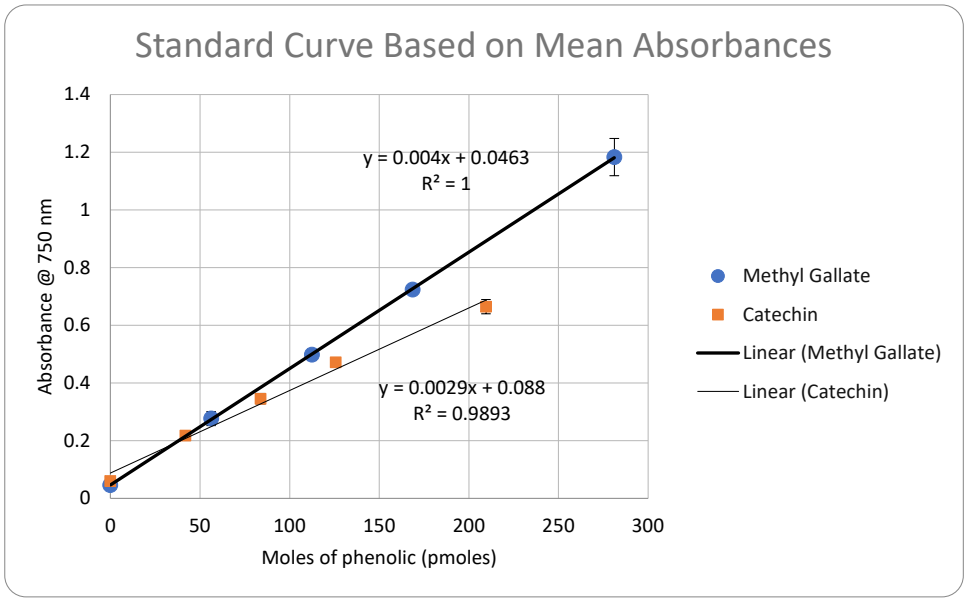
Setting the Standard Curve

1. Prepare 0.01x dilution of the methyl gallate standard that you prepared for the assay by diluting 100 uL standard with 900 uL water to provide 0.1x standard. Vortex this dilution before using it. In another microfuge tube, dilute 100 uL 0.1x with 900 uL water to yield the 0.01x standard. Vortex this dilution before using it.
2. Take the absorbance of the 0.01x standard at 274 nm using a standard UV spec and quartz cuvette.
3. Determine the molar concentration of methyl gallate in the standard using the A_{274} and the molar extinction coefficient of methyl gallate (11.81 mM^{-1} cm^{-1}).
4. Prepare standard curve from the microplate reader data. Convert the molar concentration of methyl gallate to mass concentration using its molar mass (184.15 g/mol). Use the volume of standard in each well of the microplate and the mass concentration of the standard to determine the mass of methyl gallate in each well. Plot the absorbance at 750 nm vs. mass of methyl gallate for each well. You should obtain a linear response.
5. Use the standard curve to interpolate the mass of polyphenols in 25 uL of each sample. Apply dilution factors and mass of biological tissue extracted as needed to obtain ug methyl gallate equivalents per mass of biological sample.

Typical Data and Troubleshooting

1. Our typical standard curve is shown below for methyl gallate. We sometimes use catechin as a standard, and its standard curve is also shown. The exact slope and intercept will depend on the geometry of the wells, the optics of the reader, and the exact length of the reaction time. You cannot use these standard curves, but must run your own standard curve in every plate.
2. If the absorbance of any unknown is greater than the highest absorbance within the linear standard curve, that sample must be re-analyzed. In the re-analysis, use either a more dilute sample of that unknown or a smaller volume of that unknown (maintaining total volume of the analytical sample when changing the volume of analyte).
3. We use the aqueous solution of methyl gallate as a standard. Organic solvent in extracts (e.g. 70% acetone) does not affect color yield but can cause problems with carbonate solubility. Wells with white precipitate cannot be used for reliable data.
4. Fizzing and bubble formation is the largest problem with the plate reader version of the Folin assay. The carbonate is neutralized during the reaction, and can bubble out of the wells or form bubbles that interfere with good spectrophotometric reading. If samples or standards bubble out of the wells, you should use a slightly more dilute sodium carbonate reagent or decrease the volume of sodium carbonate slightly. You should maintain the total volume of the analytical sample if changing the volume of carbonate. Bubbles in the wells can be removed by carefully stirring the wells with a pipette tip before reading the plate.





Citations

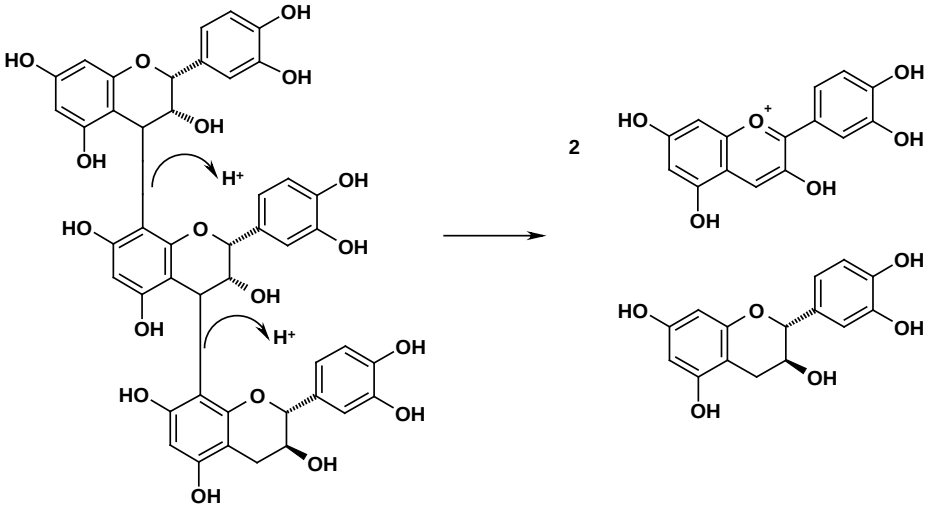
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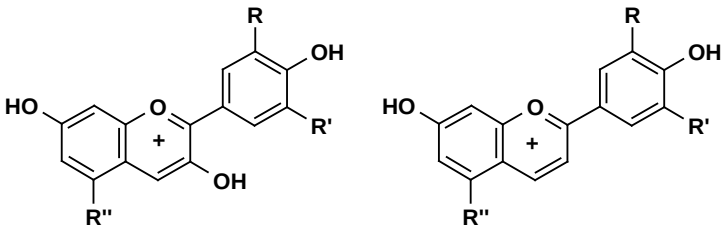
Determination of Proanthocyanidins with Acid Butanol

Ann E. Hagerman © 2025

Introduction

This assay is widely used to determine condensed tannins (proanthocyanidins). The chemical basis for the reaction is oxidative cleavage of the proanthocyanidin in hot acidic alcohol. The oxidation step is controlled by addition of a trace of iron (III). The products of the reaction are the unmodified termal unit and the colored anthocyanidins produced by the extender units. Catechin and epicatechin based polymers produce cyanidin while gallocatechin and epigallocatechin-based polymers yield delphinidin, and the rare mono-substituted flavan-3-ol based polymers yield pelargonidin. Proanthocyanidins can be called procyanidins or prodelphinidins if the structure is predominantly (epi)catechin-type or (epi)gallocatechin-type subunits, respectively.





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

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 (1)). The improvements described by Porter's group solved many of those problems and are incorporated here (2).

Four variations on the method are described in this document. First, a **Macroscale Method** is described. This procedure is easy to carry out in any lab with a conventional UV-visible spectrophotometer and 1 or 3 mL cuvettes (1 cm path length). The second method included here is a **Microscale Method** that uses the same reagents and setup as the macroscale version, but with volumes adjusted to accommodate limited sample. A standard UV-vis with 1 mL cuvettes (1 cm path length) is used to read the absorbances. We use this method frequently.

For some samples, insoluble condensed tannins are not completely released by the Porter acid butanol method. The **Heterogeneous Sample Method** describes a modified solvent to increase color yield from insoluble samples (3). The third method included here describes using the Grabber method for samples from microbiological cell cultures.

It can be difficult to analyze highly colored samples with the acid butanol method. The fourth method **Acid Butanol with PVP** uses polyvinylpyrrolidone to allow analysis in samples containing substantial chlorophyll (4). The method is tedious, so should be used only if interference seems to be substantial. If only low levels of pigment are present, it may be possible to subtract the absorbance of the colored non-tannin components. That approach is described in the Macroscale Method.

The PVP 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.

All of the methods include a heating step in acid butanol. This does not work well in multiwell plates. If desired, the final reaction product from any acid butanol method can be transferred to multiwell plates to read in a plate reader UV-vis.

Macroscale Method

Reagents

- Acid butanol: Mix 950 mL of n-butanol with 50 mL concentrated HCl
- Iron reagent: 2% Ferric ammonium sulfate in 2 M HCl. Bring 16.6 mL of concentrated HCl up to 100 mL with distilled water to make 2 M HCl. Dissolve 0.5 g $FeNH_4(SO_4)_2$ x 12 H_2O in 25 mL of 2 M HCl. Store in a dark bottle.

Method

1. 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).
2. Add 0.2 mL of the iron reagent, and vortex the sample. Cap the tube loosely, and put in a 95 oC water bath for 50 min.
3. 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.
4. 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.
5. 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.

Microscale Method

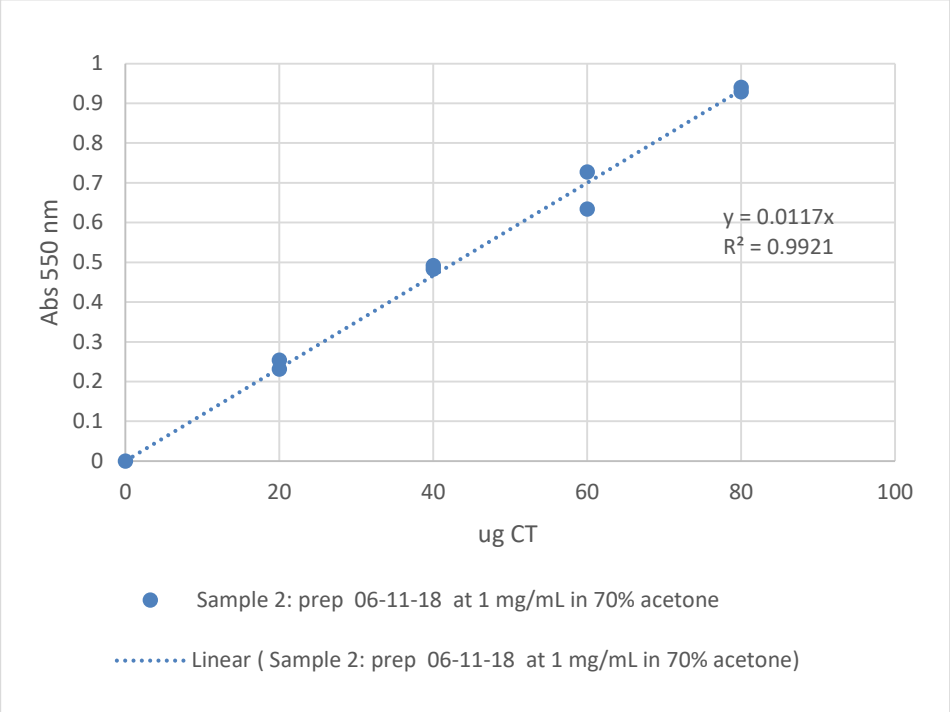
Reagents

- Acid butanol: Mix 950 mL of n-butanol with 50 mL concentrated HCl
- Iron reagent: 2% Ferric ammonium sulfate in 2 M HCl. Bring 16.6 mL of concentrated HCl up to 100 mL with distilled water to make 2 M HCl. Dissolve 0.5 g $FeNH_4(SO_4)_2$ x 12 H_2O in 25 mL of 2 M HCl. Store in a dark bottle.

Method

1. In a 13x100 mm screw cap culture tube add 3.0 mL of the acid butanol reagent to a 100 uL aliquot of the sample (or a smaller volume of the sample made up to 100 uL with the sample solvent).
2. Add 100 uL of the iron reagent, and vortex the samples. Cap the tubes loosely, and put in a 95 oC water bath for 50 min.
3. Cool the tubes 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.
4. 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.

5. Standardize the assay with an appropriate proanthocyanidin. A typical standard curve for Sorghum procyanidin (CT) dissolved in 70% acetone is provided. Remember that the slope of the standard curves depends on many variables, you must make your own calibration plot to accurately determine tannin levels.



Heterogeneous Sample Method

Reagents

The reagent is a mixture of n-butanol, acetone (increases release from insoluble condensed tannins) and iron (III) (facilitates oxidation of released residues to the colored anthocyanidin). In this version of the assay, the iron solution is prepared and stored in a dark bottle. The iron reagent, butanol and acetone are mixed on the day of the analysis to create the working reagent.

For each sample, you need 1 mL of working reagent to run the assay. You then need working reagent for the five-fold dilution step required to bring the absorbance to a useful scale. If you plan to use a 1 mL cuvette, you need a final volume of 1 mL of diluted sample to read. If you plan to use a plate reader, you need 200 uL of diluted sample for each well.

1. Stock Iron reagent solution: 40 mg Ammonium Iron(III) Sulfate Dodecahydrate mixed with 3.3 mL H_2O. Stir for 30 minutes. Add 5.0mL of 12 M HCl (commercial concentrated HCl is 12 M). Store this iron reagent in a dark bottle at room temperature.
2. Working reagent: Mix n-butanol:acetone:stock iron reagent at volume proportions of 5:6:1 on the day of the analysis.

3. Standard: 5 mg/mL Sorghum tannin in the appropriate solution (same as the unknown samples—for example culture medium buffer samples of suspended microbial cells. Prepare the standard fresh on day of analysis.

Method

1. Dispense 100 uL samples into 2.0 mL screw cap microfuge tubes. For example, you can use Fisher 21-403-200 Thermo Scientific™ Screw Cap Micro Tubes™.
2. For standards, use various volumes of the standard brought to 100 uL with the appropriate dilution solution.
3. Add 1.0 mL of the acid butanol reagent to each sample.
4. Cap the tubes tightly, vortex thoroughly, and put in a hot water bath at 70°C for 2.5 hours. If the samples have a lot of particulate matter, vortex them periodically during the incubation stage.
5. Cool the samples, vortex, and dilute five times with the working reagent as described below. (Save the samples just in case).
    - For a conventional UV-Vis, dilute 200 uL with 800 uL of working reagent. Vortex after dilution and read the absorbance at 550 nm after blanking the UV-Vis with working reagent.
    - For a plate reader, dilute 50 uL with 200 uL of working reagent in the plate. Mix on the jitterbug and read absorbance at 550 nm with samples of working reagent as the blank samples.
6. If results are not satisfactory (too high or too low absorbance) go back to the samples and standards and prepare new dilutions. You can re-read the samples or diluted samples any time within the day, but discard all samples at the end of the day.

Acid Butanol with PVP

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 M HCl. Bring 16.6 mL of concentrated HCl up to 100 mL with distilled water to make 2 M HCl. Dissolve 0.5 g $FeNH_4(SO_4)_2$ x 12 H_2O in 25 mL of 2 M HCl. Store in a dark bottle.

Method

1. 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.
2. 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.
3. 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.

4. Centrifuge.
5. 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).
6. Heat the tubes at 95 C for 1.5 h with caps loosely screwed on.
7. Cool to room temperature and centrifuge to pellet the PVP.
8. 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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Degree of Polymerization-Thiolysis or Phloroglucinolysis

Ann E. Hagerman © 2025

Introduction

Proanthocyanidins are incubated with a nucleophile in acidic alcohol. The interflavan bonds break and the extender units react to form the adduct. The terminal units are released as the unmodified flavan-3-ols. The adduct, and unreacted terminal flavanols, are analyzed by reverse phase HPLC. For (4->8) or (4-> 6) procyanidins the adducts are catechin or epicatechin, substituted at position 4 by the nucleophile. 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.

Convenient nucleophiles are tolulene α-thiol (benzyl mercaptan) or phloroglucinol. There are slight differences in the procedures. The adducts have completely different retention times and UV properties. Each research group should choose one method and develop the standards and calibration data for that method. We prefer thiolysis because the reaction workup is easier (1,2). We have confirmed suitability of thiolysis by careful comparisons to NMR and HPLC-MS of condensed tannins (3).

Thiolysis is simple to perform but the thiol is smelly. The reaction must be performed in a chemical fume hood and the HPLC must have odor control for the waste container. All tips, tubes, gloves must be contained to minimize odors in the lab. Thiol odors are neutralized by hypochlorite bleach (Clorox).

Phloroglucinol is odorless. The adduct must be extracted into ethyl acetate, dried and redissolved before HPLC.

Thiolysis methods have been described by many authors. Our methods are described in (1-3).

Phloroglucinolysis methods are found in (4-6).

Thiolysis

Reagents—should be prepared fresh about every 3 months

- 32% HCl in methanol (v/v). Add 32 mL HCl (concentrated) to a grad cylinder containing about 50 mL of methanol. Mix and bring to 100 mL with methanol.
- 5% Toluene-α-thiol in methanol (v/v). Add 1 mL T-α-T to a grad cylinder containing 15 mL methanol. Mix and bring to 20 mL. (Rinse all containers with Clorox-water to remove smell).
- Standard CT with diverse composition. *Lespedeza stuevei* from Harley Naumann (University of Missouri) is convenient but other plants can be used.

Thiolysis

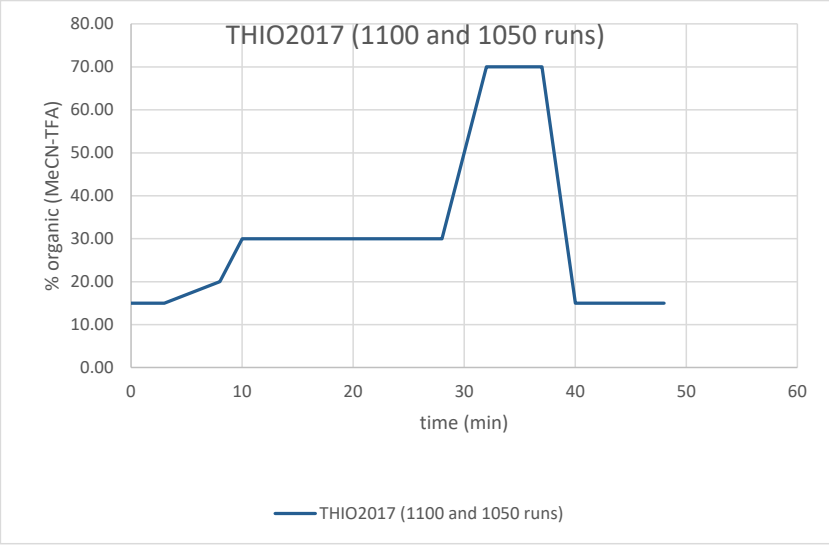
1. Take a tiny amount of the PC on a pipette tip and dissolve it in 200 uL of methanol in a microfuge tube (less than 1 mg (one red scoop) of the PC)
2. Put 100 uL of each sample into a 2nd tube. Label one "thiolysis" and the other "control"
3. For each thiolysis tube, add 10 uL of the HCl/MeOH and 24 uL of the toluene-α-thiol reagent. Vortex each sample after each addition.
4. For each control tube, add 34 uL methanol. You can run these on HPLC while the reactions incubate
5. Incubate reaction mixtures at 40 °C for 30 min, then place samples at -20 °C for at least 5 min.
6. You may store samples at -80 °C until analysis but must conduct all analysis within 24 hours of completing the reactions
7. Run the samples on HPLC using method Thio2017 with 1-10 uL injection. MeOH for the blank run. See next section for Thio2017 info.
8. Check the peak height for the largest thiol derivative (usually ECT). Should be less than 500 to keep peaks in scale.

HPLC information

We use a C8 column with matching pre-column and control the temperature at 27 °C. Monitor at 220 nm for maximum sensitivity. You must determine retention times and response factors on your own instrument, do not use literature values to identify peaks.

Flow rate: 0.5 mL/min
Aqueous Solvent: 0.13%TFA in water
Organic Solvent: 0.1%TFA in acetonitrile
Temperature: 27.0 °C
Detection: 220 nm DAD

Time [min]	% Aqueous	%Organic
0	85.00	15.00
3	85.00	15.00
8	80.00	20.00
10	70.00	30.00
28	70.00	30.00
32	30.00	70.00
37	30.00	70.00
40	85.00	15.00
48	85.00	15.00



Phloroglucinolysis

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

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

Assay of crude extracts

1. The aqueous acetone extract from leaves or other plant tissues is evaporated under nitrogen.
2. The residue is dissolved in 150 uL of phloroglucinol solution and incubated at room temperature overnight.
3. 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).
4. The ethyl acetate extracts are combined, evaporated under nitrogen, and dissolved in 75 uL of 70% methanol for separation by HPLC.

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

The terminal units and phloroglucinol-derivatized extender units are identified by comparison to the standards. The chromatograms are far more complex than for the purified proanthocyanidins, because crude plant extracts may 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.

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

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Determination of Hydrolyzable Tannins with KIO_3

Ann E. Hagerman©2025

Introduction

This functional group method for determining gallic acid esters was originally described by Haslam (1). The method was modified slightly by Bate-Smith (2) and re- examined by Willis and Allen (3). The method described here was optimized to make the method more reliable and useful for characterizing plants (4).

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 (5,6).

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 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 that is analytically useful.

We use controlled methanolysis to convert the hydrolyzable tannins in the sample to methyl gallate. We used 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. Centrifuge 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 re-centrifuging 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 $\pm$ 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 thoroughly. Store tightly capped at 4°C for up to 48 hours. A precipitate sometimes forms during storage; in that case the solution is re-centrifuged 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 does 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 samples described above are analyzed by reaction with potassium iodate, which formed a characteristic pigment with λmax 525 nm. Some flavanoids and other plant constituents reacted under these conditions form brownish pigments that absorb 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 microfuge 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 tubes are tightly capped.
3. Each sample is vortexed and placed in a 30 °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 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 °C water bath.
6. Exactly 50 min after adding the KIO_3 to the sample the absorbance at 525 nm is recorded (vs. water). Under these conditions, a linear relationship between methyl gallate and absorbance at 525 nm is obtained.
7. A linear calibration plot is made using the methyl gallate standards. The amount of methyl gallate in a plant sample is calculated based on interpolation from the calibration plot and corrections for dilution factor and sample mass to establish mg methyl gallate per g tissue.

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Determination of Gallotannins with Rhodanine

Ann E. Hagerman©

Introduction

A functional group method for the determination of gallotannins (1). Gallic acid reacts with rhodamine to form a red colored complex. The reaction is quite specific. Neither gallate esters nor ellagic acid react with rhodanine. Other phenolic compounds including quinones and hydroquinones form green complexes instead of the characteristic cherry red complex formed with gallic acid.

In plant extracts, most gallic acid is esterified, but a small amount of free gallic acid is also usually present. 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. It can be challenging to learn the hydrolysis step but with practice the method described below works well.

The instructions here describe the rhodanine reaction first, followed by the detailed instructions for sample hydrolysis for plant analysis.

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 0.1 M 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.
- 0.5 M KOH in water (2.8 g in 100 mL). Stable for several weeks if kept tightly capped to prevent absorption of CO_2.
- 2 M H_2SO_4. In water. Stable indefinitely. (In older literature, this is described as 4 N sulfuric acid). Add 11.2 mL concentrated sulfuric acid to about 70 mL water, stir, and then bring volume to 100 mL.

Determination of Gallic Acid

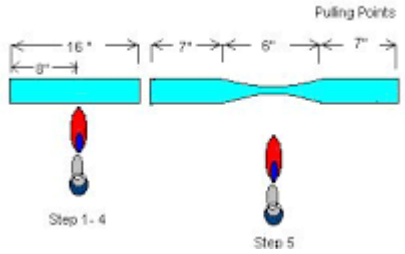
Samples should be in 0.1 M H_2SO_4. Sample volume is 1.0 mL. The sample can either be the stock solution of gallic acid as listed in the reagents list, or samples from hydrolysis. The samples from the hydrolysis method described below are at the correct sulfuric acid concentration.

1. Add 1.5 mL of rhodanine to the sample in a graduated test tube (or graduated cylinder).
2. After at least 5 min, add 1.0 mL of KOH to the sample.
3. After at least 2.5 min, dilute to 25.0 mL with distilled water.
4. 5 to 10 min later read the absorbance at 520 nm.

5. 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 0.1 M H_2SO_4.

Sample Hydrolysis

The hydrolysis must be done anaerobically to prevent destructive oxidation; we have found evacuated hydrolysis tubes the simplest, most reliable method for anaerobic hydrolysis. We use a constricted tube that is sealed with a glass blowing torch. The image shows constriction of an open glass tube. We start with a 6 inch test tube and constrict it in about the middle. After putting the sample in the constricted tube, we seal the open end under vacuum. You could use hydrolysis tubes obtained commercially from e.g. Pierce glassware as shown in the image on the right.





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

The hydrolysis is done in 1 M H_2SO_4, mixing the plant extract and 2 M acid 1:1 to make the final concentration 1 M.

1. Put the plant extract, usually 1.0 mL, in a Pyrex test tube that has been constricted about half way down.
2. Add 1.0 mL of 2 M H_2SO_4, mix, and freeze the solution in a dry ice isopropanol bath.
3. 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.
4. 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.
5. When thawed, the tubes are placed in a 100 C oven for hydrolysis for 26 h.

CAUTION: ***The oven should be labeled "Samples under pressure", and should only be opened by people wearing splash resistant chemical goggles and a lab coat. Tubes may explode during heating.***

6. Remove samples from the oven and place them in a rack to cool. They can be stored without opening for several days before analysis. When cool, the tubes are opened by carefully breaking off the top.
7. All of the sample is transferred to a graduated container, and the sample is diluted with water to a final sulfuric acid concentration of 0.1 M. (1 ml sample plus 1 ml of 2 M 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 of the sample.
8. The diluted sample (0.1 M 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 acetone-free, unhydrolyzed plant extract to make it 1 M H_2SO_4. The extract is then used as the sample in the rhodanine reaction. For the samples described above, after removing the acetone 1.0 mL of the sample is mixed with 1.0 mL of 2 M H_2SO_4, and the mixture is diluted to 10 mL with water. The diluted sample is used for gallic acid determination.

Citations

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Determination of Ellagitannins with Nitrous Acid

Ann E. Hagerman © 2025

Introduction

The nitrous acid method is a functional group method for ellagitannins (1). Ellagitannins in the plant are hydrolyzed to release ellagic acid, which forms a colored product upon reaction with nitrous acid. This method is technically challenging, but does provide quantitative information about the amount of esterified ellagic acid in plant samples. Direct HPLC analysis of ellagitannins might provide more useful information in many cases.

Hazards

The method uses pyridine as a solvent for the virtually insoluble ellagic acid. Pyridine has a strong, unpleasant odor and the fumes are hazardous. You must use pyridine 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.

The method requires you to seal tubes under vacuum. The sealed tubes are then heated in a 100 ^{o}C oven for hydrolysis. The tubes do occasionally break due to pressure build up during heating. Discuss with your safety office where the oven should be located. You must wear gloves, goggles and a lab coat for this part of the work.

Reagents & Equipment

1. 1 M H_2SO_4 Purchase this, or prepare it by diluting concentrated sulfuric acid (18.3 M) with distilled water
2. Wash solution: Acetone/water/conc HCl (70/30/1, v/v/v)
3. 1 % $NaNO_2$ in water: 1.00 g $NaNO_2$ up to 100 mL with water.
4. Pyridine
5. Concentrated HCl
6. Stock solution of ellagic acid. Ellagic acid (Sigma) 0.5 mg/ml in pyridine May be difficult to dissolve, use sonication if necessary.
7. Hydrolysis tubes. We use Pyrex glass tubes that we constrict about half way down using a glass blowing torch. The tubes are sealed as described below. You could also use reusable hydrolysis tubes (e.g. Wilmad tubes) but cleaning them could be difficult.
8. Vacuum pump to evacuate tubes.
9. Oven (100 ^{o}C) for hydrolysis. You cannot substitute with a heating block or other open heating system; the tubes must be contained in an oven to reduce hazards.
10. 20 mL glass tubes (for samples); new 5 mL glass tubes (for assay)
11. Water bath (30 C).
12. Glass cuvettes (pyridine dissolves plastic).

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

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.

1. Weigh about 50 mg samples (record exact weight) of dry material into hydrolysis tubes. Make sure the sample is all below the constriction of your tubes, or below the seal of commercial tubes.
2. Add 5.0 mL 1M H_2SO_4.
3. Freeze the suspension in isopropanol-dry ice slush, and attach to a vacuum pump.
    a. For a constricted tube, use a small glass blowers torch (gas/oxygen) to seal the tube at the constriction while it is under vacuum.
    b. For a commercial tube, close the seal while the sample is under vacuum.
4. 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.
5. The melted samples are placed in a 100 ^oC oven for hydrolysis for 10 h.
6. After hydrolysis, allow the tubes to cool. Carefully 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.
7. Carefully place the membrane and all of the insoluble material into a 20 mL glass 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 in the 20 mL glass tube.
8. The 10 mL sample is sufficient for multiple determinations. You can use up to 2.1 mL of this solution in the assay, depending on the concentration of ellagic acid in the sample.

Determination of Ellagic Acid

Use clean, preferably new 5 mL 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. Use an appropriate volume of your plant hydrolysate (between 0.1 and 2.1 mL, often 0.5 ml is appropriate).

Caution: Incubations and vortexing must be done in a chemical fume hood

1. Add pyridine to bring each sample to 2.1 mL.
2. Add 0.1 mL conc. HCl to samples, vortex. Place in a 30 C water bath for at least 5 min.
3. At time zero, add 0.1 ml $NaNO_2$, vortex, and read absorbance at 538 nm.
4. Place sample back in water bath.
5. Exactly 36 min after adding the $NaNO_2$, again read absorbance at 538 nm.

6. The difference between Abs(36 min) and Abs(0 min) 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.
7. Prepare a linear calibration curve from the ellagic acid samples. Interpolate the data from the unknown samples and apply appropriate dilution and mass factors to report the ellagic acid as mg/g dry tissue.

Citations

1. Wilson, T. C., and Hagerman, A. E. (1990) Quantitative determination of ellagic acid. *J. Agric. Food Chem.* **38**, 1678-1683

Determination of Phlorotannins with DMBA

Ann E. Hagerman © 2025

Introduction

Phlorotannins are found in brown algae. They are based on phloroglucinol-type monomers. Their structural chemistry is quite complex, with many subgroups based on structural moieties. A review encompassing biology and chemistry is available at (1).

Phlorotannins react with 2,4 dimethoxy benzaldehyde in glacial acetic acid to form a red conjugate. The reaction is reaction similar to the reaction of vanillin with the meta-substituted ring of flavonoids. The method was optimized using several types of phlorotannin. The original method and a microscale version of the method are provided here (2).

This reaction can be used to determine phlorotannin in phlorotannin-protein precipitates as described elsewhere in this Handbook.

Hazards: 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 purified phlorotannin dissolved in methanol at 10 mg/mL. Prepare fresh daily.
- Plant samples—a methanol extract of the algae of interest works well.
- N,N-dimethyl formamide (DMF)
- 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).

Original Method

1. Bring water bath to 30 °C.
2. 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.
3. Add 10 uL N,N-dimethyl formamide (DMF) to each sample.
4. Add 1.25 mL 16% HCl to each sample. Mix.
5. At exactly 1 min intervals, add 1.25 mL of DMBA reagent to each sample, mix, and place sample in 30 °C bath. Cap samples loosely with screw cap or marble.
6. Exactly 60 min after addition to first sample, read the absorbance of the first sample at 510 nm. Continue to read the absorbances at 1 min intervals. The reaction is very time-dependent so the absorbance of each sample must be recorded at exactly at 60 min.
7. Spec should be zeroed on 16% HCl reagent.

8. A linear standard curve can be made with the purified phlorotannin of interest, and the amount of phlorotannin in the sample established by interpolation on the curve and correction for dilution factors and mass of the sample.

Microscale Method

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

1. Dispense samples (0-10 uL of 5 ug/uL tannin solution or plant extract) into 1.5 mL microfuge tubes.
2. Add methanol to make volume to 10 uL.
3. Add 10 uL DMF to each sample.
4. Add 700 uL 16% HCl to each sample. Close the cap and vortex the sample.
5. At exactly 1 min intervals, add 700 uL DMBA reagent to each sample, cap, vortex vigorously, and place sample in 30 °C water bath.
6. Exactly 60 min after addition to first sample, read the absorbance of the first sample at 510 nm using a microcuvetter for 700 uL samples. Continue to read the absorbances at 1 min intervals. The reaction is very time-dependent so the absorbance of each sample must be recorded at exactly at 60 min.
7. Spec should be zeroed with 16% HCl solution.
8. A linear standard curve can be made with the purified phlorotannin of interest, and the amount of phlorotannin in the sample established by interpolation on the curve and correction for dilution factors and mass of the sample.

Citations

1. Amsler, C., and Fairhead, V. (2006) Defensive and sensory chemical ecology of brown algae. *Adv. Bot. Res.* **43**, 1-91
2. Stern, J. L., Hagerman, A. E., Steinberg, P., Winter, F. C., and Estes, J. A. (1996) A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *J. Chem. Ecol.* **22**, 1273-1293

Methanolysis to Obtain Anthocyanidins from Condensed Tannin

Ann E. Hagerman © 2025

Introduction

Methanolysis oxidatively degrades condensed tannins to their constituent anthocyanidins by reaction with strong acid at elevated temperature. There are many variations on the process in the literature. The spectral characteristics of the anthocyanidins are slightly different depending on the alcohol used as seen in the table provided here.

Reagents

- 6.25% HCl in Methanol (625 uL conc HCl up to 10 mL with methanol)
- HPLC Solvent B: 0.1% Trifluoroacetic acid in acetonitrile
- HPLC Solvent C: 0.13% Trifluoroacetic acid in water

Method

1. Bring heating block to 70 °C
2. Put < 1 mg of tannin into 1.5 mL microfuge tube
3. Add 200 uL of the HCl-methanol
4. Incubate at 70 °C for 2 hours
5. Store at -20 overnight if necessary
6. Add 400 uL of MeOH
7. Transfer to an appropriate microcuvette and obtain the absorbance spectrum
8. Or inject 10 uL of the product to a C-8 HPLC column and use the following gradient for separation

Flow rate: 0.5 mL/min
Aqueous Solvent: 0.13%TFA in water
Organic Solvent: 0.1%TFA in acetonitrile
Temperature: 27.0 °C
Detection: 220 nm DAD

Time [min]	% Aqueous	%Organic
0	85.00	15.00
30	70.00	30.00
32	85.00	15.00
37	85.00	15.00

Results

HPLC retention times are dependent on the exact column characteristics and the volume of the system. The data here indicate the order of elution but cannot be used to identify compounds by retention time.

Compound	Retention time (min) HPLC	Lambda max (nm) HPLC	Lambda max (nm) Ethanol-HCl (1)	Lambda max (nm) MeOH-HCl (2)
Delphinidin	10.72	530	557	548
Cyanidin	15.26	525	545	538
Pelargonidin	20.38	510	530	523
Robetinidin				532
Fisetinidin				520
Guibortinidin				504

Citations

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2. https://www.extrasynthese.com/

Determination of Proanthocyanidins with Vanillin

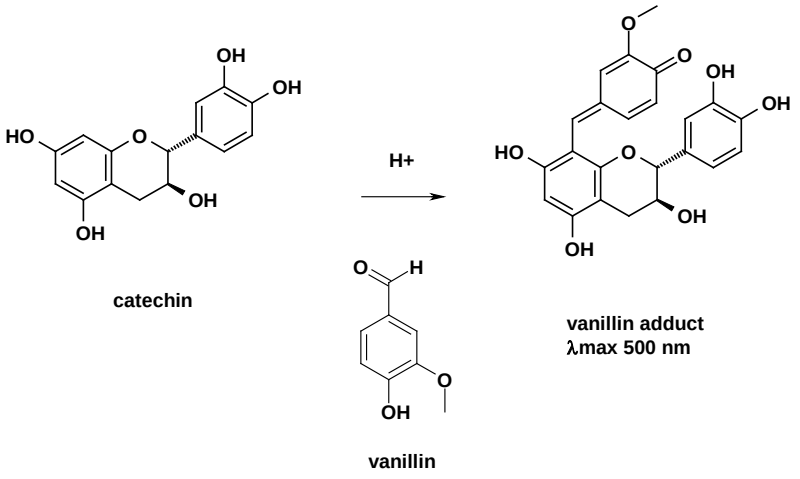
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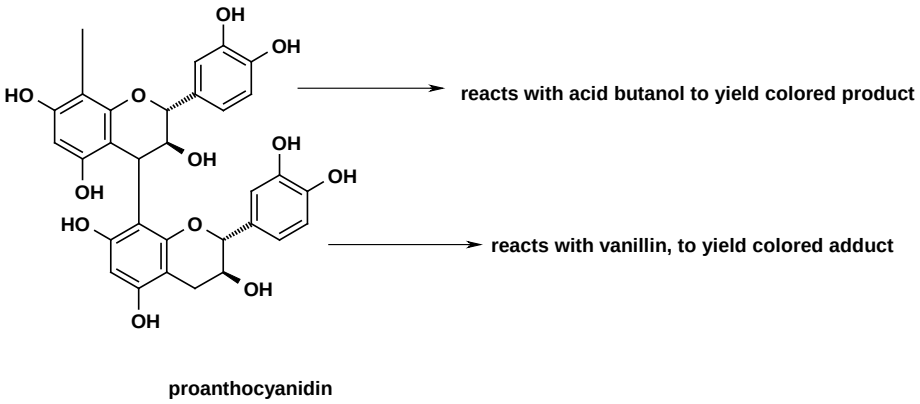
Introduction

The vanillin method and several related methods have been used to quantify condensed tannins. I rarely use these methods in my lab, since the acid butanol method is simpler and more reliable. The following pages describe three variations on the method:

1. The original vanillin method optimized for analysis of Sorghum grain (1).
2. The modified (kinetic) vanillin method that was proposed to overcome problems with catechin standards for the method (2).
3. The vanillin-acid butanol ratio method that was proposed to estimate the degree of polymerization of condensed tannins (3).

Understanding the structural chemistry of condensed tannins and flavonoids 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 as shown in the first reaction in the image.





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 flavonoids, the 5-deoxy proanthocyanidins (e.g. quebracho) do not produce much color with vanillin.

The same type of reaction takes place if 2,4-dimethoxy benzaldehyde is used instead of vanillin. The group of brown algal tannins known as phlorotannins give strong reactions with DMBA. Determination of phlorotannins with DMBA is described elsewhere in this book. A few authors have suggested that 4-dimethylaminocinnamaldehyde (DMACA) can be substituted for vanillin to determine condensed tannin, but the reaction suffers from the same shortcomings as the original vanillin method.

Reactivity with vanillin or its analogs is not sufficient evidence for the presence of condensed tannins. The acid butanol method is a better approach to establishing if a plant contains condensed tannin.

Catechin is commonly used to standardize the vanillin reaction, but there are problems with interpreting the meaning of "catechin equivalents". Under the conditions for the original 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 by taking advantage of the kinetics of the reaction. The method uses glacial acetic acid as a solvent so appropriate safety measures must be implemented The modified method can be combined with the acid butanol reaction to selectively determine terminal units (vanillin) and extender units (acid butanol) as shown in the 2nd reaction In the image. The ratio between the two measurements may indicate the degree of polymerization of the proanthocyanidin, although it can be difficult to find suitable standards. Thiolysis is generally a more reliable method for establishing degree of polymerization.

Original Vanillin Method

The method described here uses 0.5% vanillin rather than the 2% originally recommended (4). 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. The vanillin method described here was developed for analysis of condensed tannin in Sorghum grain; modification of the methods for sample extraction would probably be necessary to use the method with other tissues.

Reagents

- 1% vanillin in methanol (1.0 g vanillin up to 100 mL with absolute methanol). Store in a dark bottle at 4 ^{o}C.
- 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 4 °C 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 30 °C 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

1. 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.
    a. One set of tubes is for the reaction
    b. The second set of tubes is for the blanks
2. Both sets of tubes are incubated in the water bath.
3. Reaction tubes: 5.0 mL of the working vanillin reagent is added at 1.0 min intervals to the set of standards designated as “reaction”.
4. Blank tubes: 5.0 mL of the 4% HCl solution is added at 1.0 min intervals to the set of standards designated as “blank”.
5. Both sets of tubes are left in the water bath for exactly 20.0 min
6. The absorbances of the tubes are read at 500 nm at 1 min intervals.
7. 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.
8. The absorbance of the blank is subtracted from the absorbance of the corresponding vanillin-containing sample.
9. A standard curve is constructed (Abs vs. mg catechin). A linear fit is expected.

Analysis of Sorghum extracts

1. For each sample, duplicate 1.0 mL aliquots are dispensed into culture tubes.
    a. One of the duplicates is for the reaction
    b. The other duplicate is the blank
2. Tubes are incubated in the water bath.

3. Reaction tubes: 5.0 mL of the vanillin reagent is added at 1.0 min intervals to the samples designated as “reaction”.
4. Blank tubes: 5.0 mL of the 4% HCl solution is added at 1.0 min intervals to the samples designated as “blank”.
5. The samples are left in the water bath for exactly 20.0 min,
6. The absorbances of the tubes are read at 500 nm at 1 min intervals.
7. 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.
8. 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.
9. “Catechin equivalent” for each sample is extrapolated using the standard curve for catechin.

Modified (Kinetic) Vanillin Method

One major problem with the original vanillin assay 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 (2). 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.

Methanol diminishes the color yield of the modified vanillin 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 modified vanillin assay be used to quantitate tannin in crude extracts. The original vanillin assay is preferable for crude extracts for two reasons: First, the original vanillin assay is not sensitive to methanol in the extracts. Second, monomers that may be present in crude plant extracts yield less color in the original vanillin 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 4°C.
- 8% concentrated HCl in glacial acetic acid (8.0 mL concentrated HCl brought to 100 mL with glacial acetic acid).
- 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 condensed 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.
- Constant temperature water bath set at 30 °C.

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 30 ^{o}C in the water bath before starting the analysis.

Method

1. Dispense 0 to 1.0 mL aliquots of the samples (catechin and condensed tannin) into test tubes.
2. Bring each sample to 1.0 mL by the addition of glacial acetic acid.
3. Incubate the samples in the water bath for a brief period to bring them to temperature equilibrium.
4. At exactly 1.0 minute intervals, add 5.0 mL of the working vanillin reagent to each sample.
5. Leave the samples in the water bath for exactly 20.0 min.
6. At 1.0 min intervals, read the absorbance of each sample at 510 nm. 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.
7. The absorbance of the blank (reagent with no tannin) is subtracted from the absorbance of the corresponding vanillin-containing sample.

Vanillin-acid Butanol Ratio Method

The relative degree of polymerization of condensed tannins can be estimated by comparing the absorbance obtained with the modified vanillin assay (only terminal units react with vanillin) with the absorbance obtained with the acid butanol assay (only extender units produce colored anthocyanidins).

This method is particularly useful for monitoring changes in degree of polymerization with treatment. For example, Butler examined the relative degree of polymerization of the tannins extracted from Sorghum grain at various stages during maturation of the grain (3).

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 method is based on the assumption that all of the extender 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 (5).

Furthermore, the method is based on the assumption that only the terminal unit of the proanthocyanidin reacts with vanillin in the modified vanillin assay. Branched chain tannins would presumably have relatively more terminal units than linear tannins. The relative degree of polymerization of a branched chain tannin would be lower than that of a linear chain tannin.

Method

The plant extracts are assayed with the acid butanol assay and with the modified vanillin 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 this method. The ratio can only be used under tight constraints of a single specifies of plant in a single study with identical analytical methods for all samples.

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Chemical Derivatization of Tannins

Ann E. Hagerman © 2025

Introduction

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 the acetylated product is insoluble in aqueous solvents. In a chemical fume hood, 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.

Volume Four: Bioactivity

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Professor Jeffrey Harborne (1928-2002). University of Reading, United Kingdom. Harborne used chemical methods to qualitatively and quantitatively describe the phytochemistry of diverse plants. He founded the field of chemical ecology with his careful descriptions of chemical composition and biological interactions throughout the plant kingdom. He was an active author and editor of key publications in phytochemistry, especially the phytochemistry of the polyphenols.

Biological Activities of Tannins & Prospects for Future Research

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Tannins have received attention from researchers in a wide variety of applied and basic sciences. As polymers that are highly decorated with phenolic functional groups, tannins have three principle bioactivities: they interact with proteins, they are redox active, and they chelate metals. These three reactivities can work independently or synergistically to give polyphenols diverse roles across many scientific domains.

Proteins

Protein precipitation is the defining characteristic of tannins, and the phenomenon has been studied for decades (1). None-the-less, the detailed chemistry of the tannin-protein interaction is still only partly understood. Both the type of interaction and the strength of interaction are dictated by the chemistry of the tannin and the chemistry of the protein. In addition, the interaction is influenced by reaction conditions including temperature, pH, solvent composition, and tannin:protein ratio. Earlier methods relied heavily on precipitation reactions, and were often optimized to provide quantitative information about the efficacy of precipitation. Although precipitation is easy to detect and facilitates analysis, tannins can also form soluble complexes with protein. More modern approaches have used chemical and spectroscopic probes to develop models of interaction independent of precipitation.

Interactions with proteins have long been associated with nutritional quality and taste of polyphenol-containing foods and feeds. Although historically dietary tannin was strongly associated with reduced protein digestibility and depressed nitrogen utilization, that model is now considered simplistic (2). For insects, tannins do not appear to affect nitrogen availability. For most mammals, tannins consumed in moderate amounts are not detrimental to health unless the diet is very protein deficient. There is some evidence that low levels of dietary tannins are beneficial to ruminants (3). Some mammals have developed mechanisms for accommodating even rather high levels of dietary tannins (4).

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 salivary glands of some mammals synthesize a group of proteins that 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 (5). 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 hydroxyapatite 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 (6).

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 (7). 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 that 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 (8). Herbivores such as sheep, which do not produce salivary tannin-binding proteins, prefer to consume tannin-free forages (9) and are unable to accommodate dietary tannin. 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 (10). 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.

At a molecular level, the interaction between tannins and proteins involves both polar and nonpolar interactions (11). Hydrogen bonding between the donor phenolic OH group and the acceptor peptide carbonyl results in crosslinking and precipitation of the protein at favorable tannin:protein ratios. The particularly strong hydrogen bonding of the X-Pro peptide bond contributes to the effective protein binding by proline-rich proteins. Polyphenols can also coat the protein via the hydrophobic effect, reducing solubility by disrupting the folded structure and adding a nonpolar layer to the outside of the protein. Some small polyphenols including the small tannin EGCg (epigallocatechin gallate, green tea tannin) may have specific binding sites on the protein (12). Some recent efforts have been made to use computational modeling to visual tannin-protein interactions, but there is not sufficient information about the three-dimensional structure of any tannins in solution to develop a valid model.

Antioxidants

Polyphenols have redox potentials similar to biological redox couples, positioning tannins to serve as biological reducing agents (antioxidants). The redox chemistry of phenolic compounds depends on structure although structure-function relationships have not been carefully characterized. The reaction conditions such as pH and solvent composition influence the redox potential, with the potential generally decreasing as the pH is increased. The large number of oxidizable sites on polyphenols increases the capacity for electron transfers.

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. Despite the many papers published making claims for the benefits of dietary antioxidants, there is limited concrete evidence for their efficacy (13,14). In most organisms, the intrinsic *in vivo* antioxidant system generally has sufficient capacity to deal with natural oxidative stress. In fact, dietary antioxidants may not reach target organs due to poor uptake or inactivation in the gastrointestinal tract (15).

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 (Vitamin E). Free radical quenching can occur via single electron transfer (SET) reactions or hydrogen atom transfer (HAT) reactions. Polyphenols participate in Fenton chemistry and as free radical quenchers (16).

Phenolics may inhibit Fenton reactions by chelating the metal to prevent its redox participation. Alternatively, the hydroxyl radicals produced in the Fenton reaction can be quenched, preventing Fenton damage to the system. The tannins we have tested react very rapidly to quench the hydroxyl radical (17).

Phenolics are good scavengers of hydroxyl radicals and other biological reactive oxygen species (ROS) because of their favorable redox potentials and the relative stability of the aryloxy radical (18). *In vitro*, many low molecular weight, naturally occurring phenolics scavenge organic radicals as effectively as the antioxidant vitamins E and A (19). Free or protein-complexed condensed and hydrolyzable tannins have even higher radical scavenging activity than small phenolics (20).

Metal Chelation

The ortho dihydroxy phenolic moiety characteristic of tannins chelates metals ranging from aluminum through lead. Chelation can affect the biological availability or activity of metal ions (21). Chelation only occurs at pH values above the pKa of the phenolic group. That pKa is typically around pH 9, but metal ions can decrease the pKa as much as 5 pH units (22). Bacterial siderophores with multiple phenolic groups and very high affinities for essential metals such as iron have been characterized (23). 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, and that has been confirmed in a few cases (24).

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 (25). Tannins may protect plants from toxic metals (26), and polyphenols may be responsible for metal accumulation by some plants (27). In some ecosystems, the slow decomposition of tannin-rich leaves has been attributed in part to the low levels of biologically available metal ions (28). The populations of microfauna essential to leaf decomposition and soil formation are unable to grow when metals are unavailable. It is not known whether the diversity of tannins composition of different species of plants is related to specific interactions controlling metal ion availability in the plant's milieu.

Higher Level Effects

In biological systems, polyphenols can simultaneously participate in reactions with proteins and/or oxidizing/reducing agents and/or metals. There are many knowledge gaps to address in these ternary systems.

For example, metal ion chelation by a polyphenol can promote Fenton reactions in a way that is pro-oxidative instead of antioxidant. Redox cycling the metal in a pro-oxidative mode has been reported for some low molecular weight polyphenols (29). Pro-oxidant activities of tannins have largely been ignored.

Interactions between tannins and metalloproteins can involve metal centers in addition to the protein backbone (30). In that study, the tannin chelated a copper center in the protein and reduced the metal, thus changing the metalloprotein redox reactivity. There are great opportunities to study other metalloprotein-polyphenol interactions using spectroscopic tools to obtain molecular-level information.

The methods described here are generally the traditional methods that can provide a knowledge base for further studies. Careful examination of the current literature is recommended to identify suitable methods for addressing the many deficits in our understanding of the biological activities of tannins.

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Radial Diffusion Assay for Tannins

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Introduction

The radial diffusion method is a particularly simple protein precipitation method and is appropriate for situations where laboratory facilities are limited or numerous samples must be analyzed (1). Unlike other protein precipitation methods, acetone does not inhibit the precipitation reaction so acetone-containing extracts can be conveniently assayed with this method. Mixtures of tannins can be analyzed, making the method suitable for ranking crude plant extracts for protein precipitation activity.

In the radial diffusion assay, each molecule of tannin migrates through the gel until it encounters free protein and precipitates. The original method used a high concentration of protein in the gel, resulting in intense rings of precipitation easily visible without staining. The sensitivity of the method can be increased by decreasing the amount of protein in the gel. If the protein concentration in the gel is low, the distance traveled by the tannin before it encounters free protein and forms a precipitate is maximized. 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.

Differential response by different tannins is pronounced in this assay, and results must be interpreted in light of this differential response. Both the slope of the response curve and the appearance of the rings varies for different types of tannin. Some tannins produce simple rings with sharp, distinct borders. Other tannins produce diffuse rings that are difficult to measure accurately. Some preparations yield concentric circles. It is not clear what characteristics of the tannin dictate ring appearance and response slope.

The response is sensitive to the exact depth of the agar and the diameter of the punched hole so uniform plate preparation is quite important.

Reagents for Original and Modified Assay

- 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 2M 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
- Hot plate/stirrer or microwave
- Water bath at 45 ^{o}C
- Incubator at 30 ^{o}C

Preparation of Plates for Original and Modified Assay

1. 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.
2. Heat with continuous stirring until the agarose fully melts. The solution will boil, and must not be allowed to boil over. The mixture can be heated in a microwave, but needs to be checked and stirred frequently.
3. Put the hot solution into the water bath set at 45°C. Allow the agarose solution to cool, with occasionally stirring, to 45°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 re-melted. After the BSA has been added the mixture cannot be re-melted because the protein will be denatured when the agarose is melted.
4. When the solution has reached 45°C, add 0.10 g BSA while stirring with a glass rod.
    a. Original assay: add 0.10 g BSA to the 100 mL warm agarose solution
    b. Modified assay: add 0.01 g BSA to the 100 mL warm agarose solution
5. The BSA should be completely dissolved without allowing the solution to cool.
6. 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.
7. 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.

Sample Application for Original and Modified Assay

1. 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. Mark the plate with an indelible marker for identification of individual samples in wells.
2. Using a micropipette with a gel-loading tip apply the sample in 8 uL aliquots to the wells.
3. 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.
4. 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.
5. After applying the samples, cover the plates and again seal with Parafilm. Place the plates in a level incubator at 30° C. Allow the rings to form for at least 96 h. Remove the plates, uncover, and use a 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.

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

Staining the Plates for Modified Assay

Reagents

The staining solutions are the same solutions used for the Prussian blue assay.

- India ink and a small gauge needle for sample identification
- Whatman #1 filter paper cut to the same diameter as the gels
- 0.3 M NaCl (35.2 g dissolved in 2 L water)
- 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 ($FeNH_4(SO_4)_2 \cdot 12\ H_2O$) 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

After the plates have incubated at 30°C for 96 h, remove them from the incubator. The next 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. Then 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.

5. Use a 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.

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Determining Tannin Precipitated by BSA

Ann E. Hagerman © 2025

Introduction

This method measures the amount of condensed or hydrolyzable tannin precipitated by a standard protein, bovine serum albumin (1). 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. The original method and two microscale methods are described here.

Reagents for Original and Microscale Methods

- 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.
- BSA: (Sigma A-6003) in buffer A as specified in the method of choice
- SDS/TEA: 5% (v/v) triethanolamine, 1% (w/v) SDS (50 ml triethanolamine, 10 g SDS brought up to 1 liter with water). Triethanolamine becomes yellow with age. You can use it when it is light yellow but if it becomes brown you should discard and purchase a fresh bottle. Use electrophoresis grade SDS (sodium dodecyl sulfate).
- $FeCl_3$: 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

1. Protein reagent for the original method: 1 mg/mL BSA in buffer A
2. Dispense 2.00 ml BSA into 15 ml centrifuge tubes or culture tubes that can be centrifuged in a desk top centrifuge.
3. 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.
4. Vortex immediately
5. Allow samples to incubate at room temperature for 15 min (purified tannin) or for 24 h at 4 ^{o}C (plant extracts).
6. Centrifuge 15 min at 3000 x g (high speed in a desk top centrifuge)

7. Remove the supernatant with a transfer pipette or by aspiration. Discard the supernatant.
8. Redissolve the pellet in 4.00 ml SDS/TEA. Use a transfer pipette and vigorous voretxing to help break up the precipitate. The sample should be completely redissolved with no visible solid particles or haze.
9. Add 1.00 ml $FeCl_3$ and vortex each sample immediately.
10. After about 15 min read absorbance at 510 nm.
11. Use an appropriate blank (ferric chloride in SDS/TEA).
12. Standardize with purified tannin from the plant of interest (best) or with another purified standard tannin.

Microscale Method

1. Use microfuge tubes and 1 mL cuvettes.
2. Protein reagent for the microscale method: 5 mg/mL BSA in buffer A
3. In microfuge tubes, mix 50 uL BSA with 250 uL buffer A.
4. Add 100 uL tannin and vortex immediately.
5. Allow samples to incubate at room temperature for 30 min.
6. Centrifuge 5 min at 13,000 rpm (max speed on microfuge).
7. Remove the supernatant by aspiration with a fine tip aspirator. Discard the supernatant.
13. Redissolve the pellet in 800 uL of SDS/TEA. Use vigorous voretxing to help break up the precipitate. The sample should be completely redissolved with no visible solid particles or haze.
8. Add 200 uL $FeCl_3$ and vortex each sample immediately.
9. After about 15 min read the absorbance at 510 nm.
10. Use an appropriate blank (ferric chloride in SDS/TEA).
11. Standardize with purified tannin from the plant of interest (best) or with another purified standard tannin.
12. sIf 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 tannin in conjunction with the radiolabeled protein precipitation method.

1. Follow the microscale method to form and spin down the precipitate,
2. After aspirating the supernatant, carefully dissolve the precipitate in 100 uL of the SDS/triethanolamine solution
3. Add 50 uL of the $FeCl_3$ reagent.
4. After about 15 min read the absorbance at 510 nm in a nanoliter size cuvette.

Citations

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Determining Protein Precipitation with Radiolabeled BSA

Ann E. Hagerman © 2025

Introduction

This method provides the most sensitive method for determining amounts of protein precipitated 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 (1) is a modification of the original method (2). The modifications make the method more sensitive and minimize the amount of radioactivity used.

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. The method can be used in a competitive binding mode to compare the affinity of a tannin for various proteins (3).

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 assay, although that method is not as sensitive or as reliable.

For each tannin preparations you should optimize he solvent carrier for the tannin. Precipitation is inhibited by even traces of acetone. Alcohol or aqueous alcohol can be good solvents for dissolving the tannin, but may inhibit precipitation for some types of tannin. Water is an ideal carrier. Temperature can also affect the precipitation reaction, so both the temperature for incubation and for centrifugation should be selected based on the behavior of your system. The pH of the method is set to optimize precipitation of bovine serum albumin. If a different labeled protein is used for the assay, conditions such as reaction pH should be correctly set for that protein.

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.
    - To make 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 ($E_{1\%}$ = 6.6).

- Tannin dissolved in water, aqueous alcohol or alcohol. Even traces of acetone inhibit precipitation. 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

1. The assay is run in 0.5 mL microfuge tubes. Add 250 uL Buffer A and 50 uL labeled protein to the tubes. Tightly cap each tube and vortex
2. Add 100 uL tannin solution, cap the tube, and immediately vortex.
3. Incubate the samples at room temperature for 30 min.
4. Centrifuge for 5 min in a microfuge (13,000 x g, maximum speed) at an appropriate temperature.
5. Carefully aspirate the supernatants without disrupting the pellets.
6. Wash the pellets with 100 uL Buffer A, and recentrifuged if necessary before aspiration of the supernatant.
7. Count the pellets in a gamma counter directly in the reaction assay tube, using adaptors if necessary to fit the microfuge tubes into the counter trays.

For each assay, the following controls are run:

1. Total counts used per 50 uL aliquot labeled protein.
2. Background: substitute the appropriate carrier for the tannin solution. This value represents counts that adhere to the microfuge tube. It is subtracted from all values for counts precipitated. We usually find that the background is less than 10% of the counts added.
3. TCA precipitable counts:
    a. Dispense 50 uL labeled protein plus 250 uL unlabeled BSA (~5 mg/mL) into a microfuge tube.
    b. Add 60 uL of 100% TCA.
    c. Cap the tube and vortex.
    d. Incubate the mixture for 15 min at 4C.
    e. Aspirate the supernatant and count the pellet directly in the gamma counter
    f. 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 for Precipitation Assay

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 (determined by absorbance at 280 nm) is used for the conversion.

Method for Competitive Binding Assay

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 tannin and protein 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.

1. Prepare unlabeled competing proteins at a concentration of about 1 mg/mL in Buffer A. The 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.
2. In microfuge tubes, dispense the labeled protein (50 uL) plus various volumes (0 to 250 uL) of the competing protein.
3. Add Buffer A to each tube to bring the total volume to 300 uL. Tightly cap and vortex the samples.
4. Add tannin to each tube, and proceed with the steps described above for the precipitation assay.

Calculations for Competitive Binding Assay

"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.

Radioiodination of Bovine Serum Albumin.

Radioiodinated protein can be purchased, but the price may be prohibitive for routine use. It is not difficult to radioiodinate bovine serum albumin if you have experience and permission to use radioisotopes.

We use the Chloramine T method (4). 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 I2 during the reaction.

You cannot label the protein with Bolton-Hunter reagent for tannin-precipitation assays. The ligand carrying the iodine interferes with the precipitation.

Reagents

We usually buy 1 mCi of sodium iodide (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 H2O 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.
- Unlabeled sodium iodide: 0.10 g NaI dissolved in 10 mL 0.05 M Buffer
- Sodium metabisulfite: 24 mg $Na_2S_2O_5$ dissolved in 10 mL 0.05 M buffer
- Unlabeled 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 (commercial). 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.
- Chloramine T. This reagent must be stored in dry conditions. You should check that the reagent is still good before attempting your iodination. Make a small amount of Chloramine T solution and add a crystal of NaI. If the Chloramine T is good, the solution will turn brown (it oxidizes the I- to I2). The reagent solution of Chloramine T should be prepared just before iodination. Dissolve 4 mg/mL Chloramine T in 0.05 M buffer.

Procedure

1. Set up a hand held survey meter so isotope use can be monitored during reaction.
2. 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.
3. 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
4. Immediately add 100 uL sodium metabisulfite to quench the reaction, and add 200 uL cold sodium iodide.
5. 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.
6. Elute the column with the 0.1 M buffer, adding the buffer in 400 uL aliquots and collecting 400 uL fractions.
7. 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.
8. Dispose of all contaminated materials properly.

9. Mix the protein fraction with 20 mL of 1 mg/mL cold BSA in Buffer A (used for competitive binding assays).
10. 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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Determining Protein Precipitation with Blue BSA

Ann E. Hagerman © 2025

Introduction

A standard protein, bovine serum albumin, is labeled with a blue dye so that it can be selectively measured in tannin-protein precipitates (1). 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 absorbance to mg protein); using the blue BSA in precipitation assays

Making the Blue BSA

Reagents

- Sodium bicarbonate (1%): Dissolve 1.0 g of $NaHCO_3$ in 100 mL water.
- BSA: Dissolve 2.0 g bovine serum albumin (Sigma A 6003) in 40 mL of the $NaHCO_3$.
- Remazol brilliant blue dye (Sigma R 8001): need 150 mg per preparation of blue BSA
- Acetate buffer: Dilute 34.2 mL of glacial acetic acid with about 1800 mL of water, and then adjust the pH to 4.8 by adding 2 M 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 bring the final volume to 3 L. Store this in the cold.
- 12-14,000 MW cutoff dialysis tubing hydrated and rinsed before use.

Method

1. Add 150 mg of Remazol brilliant blue to the protein solution.
2. Let it stir gently for 30 min at room temperature.
3. After the pH has been adjusted, add water to make the final volume 3 L. Store this in the cold.
4. Put the BSA-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.
5. Dilute the 40 mL of dialyzed blue BSA to 1 L with acetate buffer. Store this diluted sample in the cold.

Color Yield for the Blue BSA

The amount of blue dye conjugated to the protein varies from one batch to the next, so the color yield must be established for each preparation of blue BSA.

Reagents

- BSA protein standard: Commercial protein standard, 1 mg/mL BSA in saline or phosphate buffer. (It may be useful to know the E_{280} for a 1% (w/v) [1 mg/mL] solution of BSA: E_{280} = 6.6 mL/cm·mg)
- CTC Dissolve in 100 ml water:

  - 0.1 g copper sulfate ($CuSO_4$ x 5 H_2O)
  - 0.1 g sodium potassium tartrate ($KNaC_4H_4O_6$ x 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 Lowry assay, prepare Reagent A
  - 1 part CTC 2 parts SDS 1 part NaOH
- Just before running the Lowry assay, prepare Reagent B
  - 1 part commercial Folin's reagent (stored at 4 °C) 5 parts water

Lowry Assay (determining concentration of blue BSA)

Run the Lowry assay as described by Peterson, ***Meth Enz*** 91, 95-119. Use replicate samples of the standard protein and the unknown (blue BSA).

1. Use 10- 100 uL of the standard, making each sample to 1 mL with water.
2. Use 10-50 uL of the blue BSA, making each sample to 1 mL with water.
3. To 1 mL of sample, add 1 mL of reagent A. Mix.
4. About 10 min later add 0.5 mL of reagent B. Mix.
5. About 30 min later read absorbance at 750 nm in a 1 cm cell.

Color yield (A_{590} per mg blue BSA)

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. The absorbance of the blue solution is plotted as a function of the protein concentration (determined from the Lowry assay, above) to yield a calibration curve to convert absorbance at 590 to mg protein.

Blue BSA Precipitation by Plant Extracts or Tannin Preparations

Blue BSA is precipitated by tannin, and the precipitate is redissolved for spectrophotometric measurement of A_{590}. The calibration plot is used to calculate mg protein precipitated by the tannin sample.

Reagents

- Pellet redissolving solution. Add to a one liter graduated cylinder:
  - 50 mL triethanolamine (2,2',2"-nitrilotriethanol) (should be colorless or very light yellow—if it is brown then it is too old, must be correctly discarded and replaced).
  - 200 mL isopropanol
  - 10 g SDS (sodium dodecyl sulfate, also called sodium lauryl sulfate)
  - Bring to 1 L with water

- Plant extract or tannin solution. All samples should be dissolved in the same solution. Methanol, ethanol, or water are all compatible with the method. Acetone inhibits protein precipitation by tannin so samples cannot contain any acetone.

Precipitation

1. 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 glass test tube.
2. Add tannin containing sample (1 mL extract, or extract diluted to 1 mL).
3. Vortex. Allow the mixture to incubate 2 h in the cold (4 C).
4. Centrifuge 15 min at 3000 x g (high speed in a clinical or table top centrifuge).
5. Carefully pour off the supernatant, without disturbing the blue precipitate.
6. Add 3.0 mL of isopropanol/SDS/TEA to the precipitate, and vortex vigorously to completely redissolve the precipitate.
7. Read the absorbance at 590 nm, and calculate the amount of protein precipitated from the calibration curve.
8. Sometimes plant extracts have pigments that 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.

Citations

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Determining Phlorotannin Precipitation with DMBA

Ann E. Hagerman © 2025

Introduction

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. Instead, the reaction between phlorotannins and dimethoxybenzaldehyde can be used to determine precipitated phlorotannins. 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 and a microscale method are given here (1). The method can be coupled with the radiolabeled protein precipitation assay to allow determination of both protein and phlorotannin in the precipitates.

Reagents for Original and Microscale Method

- 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 in the refrigerator.
- 1 M dithiothreitol (DTT): Bring 1.54 g DTT up to 10.0 mL with water. Divide into 150 uL aliquots and store at -20 ^{o}C.
- 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 bovine serum albumin in saline. Keep refrigerated, prepare fresh every few days.
    - For the Original Method, use 10 mg/mL BSA
    - For the Microscale Method, use 5 mg/mL BSA
- Tannin should be fully dissolved in methanol
    - For the Original Method, use 10 mg/mL phlorotannin in methanol
    - For the Microscale Method, use 5 mg/mL phlorotannin 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).

Original Method

A precipitation reaction and a no-protein blank reaction are run in tandem for each analytical sample.

1. Bring water bath to 30 C.

2. For the analytical samples, dispense buffer J with DTT (180 uL) into 15 mL conical centrifuge tubes.
3. Add tannin (10 uL, 10 mg/mL) and vortex the tubes.
4. Add protein (20 uL, 10 mg/mL) to the precipitation reaction tubes and 20 uL buffer to the no-protein blank tubes.
5. Vortex all the tubes.
6. Incubate the tubes 30 min at room temp.
7. Centrifuge the tubes 15 min at 3200 rpm (high speed, table top centrifuge)
8. Remove the supernatant with a transfer pipette or by aspiration.
9. Wash each sample by gently adding 100 uL Buffer J with DTT
10. Centrifuge for 5 min at 3200 rpm.
11. Remove the supernatant by aspirating.
12. Immediately add 10 uL of DMF to each tube, and immediately vortex each tube.
13. Add 1.25 mL of 16% HCl to each sample.
14. Prepare the controls: a total phlorotannin control and a reagent control.
    a. The controls are set up in 15 mL conical centrifuge tubes that are pre-rinsed with 200 uL J+DTT.
    b. To the total phlorotannin control tube, add 10 uL phlorotannin, 10 uL DMF and 1.25 mL 16% HCl.
    c. To the reagent control tube, add 1.25 mL 16% HCl.
15. Include all the sample tubes, the total phlorotannin control, and the reagent control in the following steps:
    a. 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.
    b. After exactly 60 min, start timed reading of absorbance at 510 nm, with the spectrometer zeroed with 16% HCl.
    c. Note any tubes that contain precipitate at time of reading.
16. The absorbance of the reagent control should be subtracted from all other absorbances.
17. The absorbance of the total phlorotannin control tube can be used to calculate the % of added phlorotannin that was precipitated.

Microscale Method

The microscale method uses less reagent. It is carried out in snap cap microfuge tubes which contain the DTT and HCl fumes.

1. Add Buffer J with DTT (180 uL) to microfuge tubes.s
2. Add tannin (10 uL, 5 mg/mL) and cap and vortex the samples.
3. Add protein (20 uL, 5 mg/mL) and immediately cap and vortex the tubes.
4. Incubate the tubes 30 min at room temp
5. Centrifuge the tubes in a microfuge at 4000 rpm—much lower than maximum speed. High speed centrifugation is not recommended, since it may make the precipitate difficult to dissolve.
6. Remove the supernatant with a transfer pipette or by aspiration.
7. Wash each sample by gently adding 100 uL Buffer J with DTT
8. Centrifuge for 5 min at 4000 rpm.
9. Remove the supernatant by aspirating.
10. Immediately add 10 uL of DMF to each tube, and immediately vortex each tube.
11. Add 700 uL of 16% HCl to each sample.

12. At timed 1 min intervals, add 700 uL of DMBA reagent to all tubes. Mix, cover tubes, and place in 30 C water bath.
13. After exactly 60 min, start timed reading of absorbance at 510 nm, with the spectrometer zeroed with 16% HCl.
14. Note any tubes that contain precipitate at time of reading

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Assessing Radical Quenching with ABTS

Ann E. Hagerman © 2025

Introduction

In the ABTS assay, the colored radical $ABTS^{+}$ is decolorized by radical quenching agents. Biological antioxidants such as ascorbic acid or the water soluble Vitamin D analog called Trolox are excellent radical quenching agents. Polyphenolics are highly effective radical quenching agents (1).

The assay provides comparative data on radical quenching, but does not reveal the fate of the newly formed radical. Redox cycles to eliminate ascorbic acid or vitamin D radicals are found in cellular systems. Pathways for eliminating phenolic radicals have not been identified, leading to the potential for polyphenols to serve as pro-oxidants instead of antioxidants in some systems.

The first step in the ABTS assay is generation of the ABTS radical. The original method used metmyoglobin to form the radical (2,3). More recent methods use a chemical generation system (4). Chemical generation of the ABTS radical and its use for evaluating radical quenching by tannins is described here.

A broad review of radical quenching assays including the ABTS method can be found at (5).

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 x H_2O and 8.5 g NaCl in 1 L of distilled water
    - Mix the two solutions together while monitoring pH, to obtain a final pH of 7.4 (approximately 77 mL of A and 200 mL of B).
- 2,2'-Azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (15 mL of 3.84 mg/mL (7 mM) in PBS buffer). The ABTS is purchased from Sigma (# 1888).
- Potassium persulfate ($K_2S_2O_8$) (1 mL of 10.6 mg/mL (39.2 mM) in PBS buffer).
- 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.
- Tannin sample (1 mg tannin/mL of water). Prepare several dilutions of this solution in order to check linearity of the quenching activity.

Methods

Chemical preparation of ABTS radical cation

1. Mix the 15 mL of ABTS with the 1 mL of $K_2S_2O_8$ to obtain a 6.5 mM solution of ABTS
2. Incubate the mixture at room temperature in the dark for 16 hours to yield a dark blue solution (6.5 mM) of radical cation (ABTS+•)

3. The radical cation is stable for two days if stored in the dark at room temperature. The starting absorbance will be slightly lower in an older solution.

The λ_{max} 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.

The same method can be used to prepare the radical cation in other buffers. Both the ABTS and the potassium persulfate should be dissolved in the same buffer to prepare the radical.

Procedure for quenching by tannin or other polyphenolics

1. Dilute the desired volume of radical cation with PBS to obtain the working solution of 65 µM ABTS+•. Incubate the solution at 30 °C
2. Using a microcuvette (1 mL), blank the spectrophotometer at 734 nm with PBS.
3. Dispense 10 uL of tannin stock and dilutions into a series of microfuge tubes and incubate these tubes at 30 °C.
4. At the spectrophotometer, add1000 uL of the radical cation solution to the first sample.
5. Vortex and return to the 30 °C incubator.
6. Exactly 1 min later, record the Absorbance at 734 nm of the sample.
7. The blank (solution with no quenching agent) should give an absorbance of about 0.7

Calculations

The amount of putative antioxidant required to suppress absorbance of the ABTS radical cation by 50% is compared to the amount of Trolox required for 50% suppression in order to compare potency of various antioxidants. A Trolox standard curve is run with each set of samples because there is substantial day-today variability in the assay. The reaction time is important, especially for tannins that often have a complex kinetic profile for quenching.

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Assessing Radical Quenching with Deoxyribose

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Introduction

The deoxyribose assay is used to determine the reactivity of tannins or other antioxidants 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. The ABTS method, described elsewhere in this Handbook, is simple and yields better results.

We have obtained useful data with the deoxyribose method using tannins as the quenching agent (1). To use the method with tannins, we slightly modified the original method (2,3). The changes are as follows:

- 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 ^{o}C. The second water bath was originally described as an 80 ^{o}C bath, but a boiling water bath 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, H2O2, tannin, dOR, and Asc.

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 in the dark at room temp.
    - Immediately before the assay, dilute the stock ferric chloride to 300 µM $FeCl_3$ by mixing 150 µL of the 0.1 M solution to a final volume of 50 mL of deaerated water. (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 at room temperature.
- 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. Prepare fresh daily.
- 2.8 % w/v TCA (1.4 mL 100% w/v trichloroacetic acid solution diluted with 50 mL water). Prepare fresh daily.
- 1-butanol (also called n-butanol). Cannot substitute with tert-butanol.
- Tannin (approximately 10 mg tannin in 1 mL deaerated water). Make sure to record the actual concentration of tannin used.
    - 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 ^{o}C water bath and a boiling water bath will be needed in this experiment. Each sample is prepared in a 15 mL polypropylene screw-top clinical centrifuge tube (Falcon tube).
    - The sample composition is complicated, work from a spreadsheet to ensure each sample is prepared correctly.

Method

1. Add 35 µL of phosphate buffer and 35 µL of the deoxyribose solution to each of the 15 mL tubes. Add the appropriate amount of water listed on the spreadsheet. Vortex the tubes.
2. Add 35 µL of the hydrogen peroxide solution and vortex again.
3. Add 35 µL of the EDTA and 35 µL of the $FeCl_3$ solutions. Vortex the tubes.
4. Add the tannin. Typically several concentrations of tannin will be tested (triplicate for each concentration).
5. Add 35 µL of the ascorbate. Vortex and cap the tubes.
6. 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.
7. Place the tubes in the 37 C water bath for 1 hour.
8. Allow the tubes to cool for 5 minutes. Vortex and shake the tubes. Centrifuge @ 3200 rpm for 1 minute.
9. Add 350 µL of the TBA solution, and then 35 µL of the TCA solution. The volume in each tube should be 1120 µL.
10. Loosely cap and vortex the tubes. Place them in the boiling water bath for exactly 20 min.
11. Carefully remove the tubes and cool for 20minutes. The pink color will form.
12. Vortex and shake the tubes. Centrifuge @ 3200 rpm for 1 minute.
13. Add 1120 µL 1-butanol and shake gently to mix the two layers. Centrifuge @ 3200 rpm for 6 minutes to separate the layers.
14. 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.
15. 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)

Citations

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3. Halliwell, B., Gutteridge, J. M. C., and Aruoma, O. I. (1987) The deoxyribose method: A simple 'test tube' assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **165**, 215-219

Detecting Tannin-binding Proteins with Native PAGE

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Introduction

This method allows detection of salivary or other proteins that selectively bind to tannins. Either precipitable or nonprecipitable complexes can be detected with this method (1).

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. To maintain stability PMSF is prepared as a stock solution at 10 mg/mL in isopropyl alcohol and stored at room temperature.

Caution: PMSF is very toxic.

Reagents

- EDTA (100 mM): Disodium EDTA dissolved in 10 mL water
- Antioxidant solution. Prepare fresh daily: Dilute 10 uL of 100 mM EDTA with 1 mL of 50% methanol. Add 9 mg ascorbic acid and mix.
- 50% methanol: equal volumes of methanol and water

Forming and Detecting the Complexes

These amounts are based on ruminant saliva.

1. Prepare tannin in the antioxidant solution on the day of use.
    a. 20 ug/uL stock solution (condensed tannin)
    b. 5 ug/uL stock solution (gallotannin).
2. Prepare a 1:10 dilution of the stock tannin solution in the antioxidant solution.
3. 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.
4. Mix the solutions and incubate overnight at 4C.
5. 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.
6. Prepare 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.
7. Apply 10 ul sample to each lane. Use only the supernatant—there may be substantial precipitate from the saliva. Run the gels. Fix and stain with silver stain.

Citations

1. Austin, P. J., Suchar, L. A., Robbins, C. T., and Hagerman, A. E. (1989) Tannin binding proteins in the saliva of deer and their absence in the saliva of sheep and cattle. *J. Chem. Ecol.* **15**, 1335-1347

Silver Stain for All Proteins on PAGE

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Introduction

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 (1). These amounts and times are for small, 0.75 mm thick gels e.g. Hoefer Mighty Small. 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.

Commercial silver staining kits are available. Modifications to the kit systems might be necessary for tannin-containing samples

Reagents

- 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 (0.8 g $AgNO_3$ up to 4 mL distilled water). This should give a clear solution, if your solution is cloudy then your water contains chloride. A cloudy solution will not work. 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. 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 while washing.
3. Fix the gel overnight in the ethanol/water/formaldehyde fix solution.
4. At this point, 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 for 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. Put the gel in its tray on a light box to monitor bands. Add the developer solution and watch the bands. Add the stop when development is satisfactory, then pour off the solution and add new stop. Store gels temporarily in stop.
10. If you like, you can transfer the gels to water and dry between sheets of cellophane.

Citations

1. Hochstrasser, D. F., Patchornik, A., and Merril, C. R. (1988) Development of polyacrylamide gels that improve the separation of proteins and their detection by silver staining. *Anal. Biochem.* **173**, 412-423

Coomassie Stain for Proline-rich Proteins on SDS-PAGE

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Introduction

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; under the same conditions, other proteins stain blue (1). 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.

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

Stain

1. Dissolve 0.5 g Coomassie blue R-250 in 200 mL absolute ethanol (or 210 mL 95% ethanol). Add this to a solution of 50 mL glacial acetic acid and bring the final volume to 500 mL with water. You can store this solution and reuse it repeatedly as long as it remains clear and free of particulates.
2. 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).
3. Normal proteins stain blue or violet, while (some) salivary proline-rich proteins eventually destain to form pink bands.
4. It may take 4 days for the pink color to show up.
5. 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.

Citations

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