Selective Determination of Condensed or Hydrolyzable Tannins with Radial Diffusion

Ann E. Hagerman (c) 2002

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Introduction

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

The method takes advantage of the structural differences between condensed and hydrolyzable tannins. The characteristic ester bonds in hydrolyzable tannins are susceptible to hydroxylaminolysis. Treatment of simple hydrolyzable tannins with hydroxylamine hydrochloride at slightly acidic pH decomposes the tannin to the core polyol and the hydroxamates of the phenolic acids (gallic acid and ellagic acid). These phenolics do not precipitate protein and are not detected in the radial diffusion assay. Condensed tannins do not react with hydroxylamine hydrochloride. Crude plant extracts (in alcohol; acetone interferes with the hydroxylaminolysis) are prepared, and total tannins are assessed with the radial diffusion assay. The extract is then reacted with the hydroxylamine, and tannin remaining in the extract after destruction of esters is measured with the radial diffusion assay. For samples containing only condensed tannin, the radial diffusion measurement is the same before and after hydroxylaminolysis. For samples containing only simple hydrolyzable tannins, no ring forms when radial diffusion is performed after hydroxylaminolysis. For samples containing both types of tannin, a larger ring is obtained with the crude extract and a smaller ring with the hydroxylamine-reacted extract.

Although the method shows promise as an analytical tool to solve the difficult problem of analysis of both types of tannin in a single extract, it has not been adequately verified by use with a variety of types of plants. Additional work is needed to make this method into a robust and useful method that could be widely used.

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Reagents

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

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

Plant extracts

1. Weigh about 10 mg dried leaf tissue, recording the weight exactly.
2. Extract two times for 30 min with 100 uL 70% acetone, sonicating at 4oC to maximize extraction.
3. Centrifuge plant debris away after each extraction and combine the two extracts (final volume about 150 uL).
4. Evaporate each sample under nitrogen to about 25 uL to remove all acetone.
5. Add distilled water to bring volume to 60 uL. We use an electronic micropipette (Rainin, EDP digital micropipette) which can be set to measure unknown volumes in this step.

Hydroxylamine reaction

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

Control sample

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

Analysis

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

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

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

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difference to estimate total tannin in consistent units, using a factor of 2.9 to relate rings obtained with condensed tannins to those obtained with simple hydrolyzable tannins:

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

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

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

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