

# ***Radial Diffusion Assay for Tannins***

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## ***Introduction***

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

## ***Materials***

- Buffer: 0.05 M acetate containing 60  $\mu$ M ascorbic acid; pH 5.0. For one liter, dilute 2.85 mL glacial acetic acid to about 800 mL, add 10.6 mg ascorbic acid, and adjust pH to 5.0 with 2N NaOH. Then bring final volume to 1.00 L with water.
- Agarose: Type I, low EEO, gel point 36 C (Sigma A-6013)
- Bovine Serum Albumin: Fraction V powder, 96-99 % albumin (Sigma A-3350)
- Well punch: 4.0 mm (Biorad 170-4029)
- Disposable Petri dishes (nominally 10 cm, actual diameter about 8.5 cm). Can be washed and reused numerous times. We use Fisher catalog # 8-757-13, 100 x 15 mm dishes.
- Parafilm
- Water bath, hot plate/stirrer, refrigerator, 30 C incubator

## ***Preparation of plates***

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 dissolves. The solution will boil, and must not be allowed to boil over.
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.

4. When the solution has reached 45°C, add 0.10 g BSA with stirring. The BSA should be completely dissolved without allowing the solution to cool.
5. 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.
6. Cover each dish and seal with a strip of Parafilm. Store at 4 C for up to several days before use. The layer of agarose in the dish should be of uniform thickness and free of bubbles or other imperfections.

## ***Assay***

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.
2. Using a 10 uL glass syringe (Hamilton) or a micropipette, 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 plastic ruler to estimate the diameter of the ring that has formed. The square of the diameter is proportional to the amount of tannin in the sample.

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