

Gallotannin Determination with Rhodanine

From Inoue and Hagerman, *Anal. Biochem.* 169: 363-369 (1988) A functional group method for the determination of gallic acid released by hydrolysis of gallotannins permits estimation of gallotannins.

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Reagents

- Rhodanine (Sigma). 0.667 % (w/v) in methanol (0.667 g in 100 mL methanol). Store in refrigerator, stable for at least two weeks.
- Gallic acid stock. 0.10 mg/ml in 0.2 N H₂SO₄. Stable 2 weeks at room temperature. Once a bottle of solid gallic acid has been opened, the top surface starts to oxidize. Store the bottle tightly capped in the dark to minimize degradation, and mix the solid before taking samples to prepare solutions. If the material has oxidized, color yields will be low.
- KOH. 0.5 N KOH in water (2.8 g in 100 mL). Stable for several weeks if kept tightly capped to prevent absorption of CO₂.
- 4 N H₂SO₄. In water. Stable indefinitely.

Determination of gallic acid

Samples should be in 0.2 N H₂SO₄. Sample volume is 1.0 mL. 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 0.2 N H₂SO₄.

Sample hydrolysis

Gallic acid is present in two forms in plants: as esters, with the higher mol wt esters (e.g. pentagalloyl glucose) acting as tannins, and as the free acid. To estimate gallotannins, the amount of total (ester plus free) gallic acid and the amount of free gallic acid are determined. Ester gallic acid is then calculated by difference. The rhodanine assay detects only free gallic acid, so esters must be hydrolyzed before determination. The hydrolysis must be done anaerobically to prevent destructive oxidation; we have found evacuated hydrolysis tubes the simplest, most reliable method for anaerobic hydrolysis.

Extraction

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

Hydrolysis

The hydrolysis is done in 2 N H_2SO_4 , mixing plant extract and 4 N acid in proportions to make the final concentration 2 N.

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

When thawed, the tubes are placed in a 100 C oven for hydrolysis for 26 h.

CAUTION: The oven should be clearly labeled as having samples under pressure, and should only be opened by people wearing splash resistant chemical goggles and a lab coat. Occasionally a tube does explode during heating.

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

Free gallic acid

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