

# ***Determination of Ellagitannins***

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

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## ***Reagents***

1. 2 N H<sub>2</sub>SO<sub>4</sub>
2. Wash solution: Acetone/water/conc HCl (70/30/1, v/v/v)
3. 1 % NaNO<sub>2</sub> in water: 1.00 g NaNO<sub>2</sub> up to 100 mL with water.
4. Stock solution of ellagic acid. Ellagic acid (Sigma) 0.5 mg/ml. May be difficult to dissolve, use sonication if necessary.
5. Pyridine. You will use a lot of pyridine, which is stinky and must be used in a chemical fume hood. Pyridine should be discarded according to the advice of your safety officer, it should not be poured down the drain. Ideally, you should set up a small still and redistill the pyridine for reuse.
6. You will need a water bath (30 C) and glass cuvettes (pyridine dissolves plastic).
7. It is useful to have a filter manifold so you can filter several samples at once. We use a Radnoti filtration apparatus No 120750. You also need filter holders for 25 mm filters, and polycarbonate filters (e.g. Nucleopore SN:110607 PC MEMB 4 um) and glass fiber filters (e.g. Gelman type E).

## ***Sample preparation.***

Ellagitannins and ellagic acid are quite insoluble. Instead of analyzing extracted tannins, we analyze ellagic acid released upon hydrolysis of lyophilized plant material.

1. Weigh about 50 mg samples (record exact weight) of dry material into Pyrex test tubes which have been constricted about half way down.
2. Add 5.0 mL 2 N H<sub>2</sub>SO<sub>4</sub>, freeze in isopropanol-dry ice slush, and attach to a vacuum pump. Use a small glass blowers torch (gas/oxygen) to seal the tube at the constriction while it is under vacuum. Place the tubes in a rack to melt. As they melt, bubbles should be seen forming in the liquid. If the samples do not bubble, there is a leak in the seal and you must try again. It is difficult to learn to do this, so practice on some less important samples.

3. The melted samples are placed in a 100 C oven for hydrolysis for 10 h.  
**Caution: The oven should be clearly labeled as containing samples under pressure, and must only be opened by people wearing splash resistant chemical goggles and a lab coat. Occasionally a tube does explode during heating.**
4. After hydrolysis, allow the tubes to cool. Open the tubes, and chill them in a ice bath. Vacuum filter the sample (the ellagic acid is insoluble in ice-cold solvent, you are washing away pigments) on the filter manifold using the polycarbonate filters. Wash the insoluble material several times with ice cold wash solvent. Discard all of the washings.
5. Carefully place the membrane and all of the insoluble material into a test tube, and add 10.0 ml pyridine. Vortex to dissolve the ellagic acid and most of the filter. Insoluble plant material, and perhaps some bits of the filter, will still be present. Filter the solution with a glass fiber filter to remove the insoluble material, saving the pyridine solution.

## ***Determination of ellagic acid***

Use clean, preferably new small test tubes. Occasionally there is a residue on washed tubes that inhibits the reaction. Samples should be in a total volume of 2.1 mL pyridine. Use volumes of the ellagic acid standard to give 0-50 ug ellagic acid and an appropriate volume of your plant hydrolysate (between 0.1 and 2.1 mL, often 0.5 ml is appropriate).

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

1. Add 0.1 mL conc. HCl to samples, vortex. Place in a 30 C water bath for at least 5 min.
2. At time zero, add 0.1 ml  $\text{NaNO}_2$ , vortex, and read absorbance at 538 nm.
3. Place sample back in water bath.

Exactly 36 min after adding the  $\text{NaNO}_2$ , again read absorbance at 538 nm. The difference between the absorbances is a function of the ellagic acid concentration. Timing is critical because the color continues to change with time during the reaction. You cannot go back and read the absorbance of a sample again, but must repeat the assay if a mistake is made.