

# ***Determination of Hydrolyzable Tannins (Gallotannins and Ellagitannins) after Reaction with Potassium Iodate***

Ann E. Hagerman © 2002

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## ***Introduction***

Taken from Hartzfeld, Forkner, Hunter and Hagerman, *J. Agric. Food Chem.* 50: 1785-1790 (2002)

This functional group method for determining gallic acid esters was originally described by Haslam (Haslam, E. *Phytochemistry* 1965, 4, 495-498). The method was modified slightly by Bate-Smith (Bate-Smith, E.C., *Phytochemistry* 1977, 16, 1421-1426) and more recently was examined by Willis and Allen (Willis, R.B.; Allen, P.R. *The Analyst* 1998, 123, 435-439).

The KIO<sub>3</sub> 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 (Schultz, J.C.; Baldwin, I.T. *Science* 1982, 217, 149-151; Hunter, M.D.; Forkner, R.E. *Ecology* 1999, 80, 2676-2682).

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<sub>3</sub>. 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<sub>3</sub> 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<sub>3</sub>, potassium iodate (5 g KIO<sub>3</sub> 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<sub>3</sub> to form a nonspecific yellow compound that is not useful for selective determination. We found that methyl gallate reacts with KIO<sub>3</sub> to form a unique red chromophore so conversion of hydrolyzable tannins to methyl gallate has been optimized using 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  $\mu$ L 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. Centrifuging 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  $\mu$ L 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  $\mu$ L of 3.7 M ammonium acetate and adjust the pH to 5.5 + 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 formed 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 methanolized 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  $\mu$ L concentrated sulfuric acid exactly as described above for the samples. This blank solution did not contain any plant sample or standard polyphenolic compound, and is used to dilute the standards when preparing the standard curve.

## ***Reaction with KIO<sub>3</sub>***

The pH 5.5 + 0.1 samples described above are analyzed by reaction with potassium iodate, which formed a characteristic pigment with  $\lambda_{\text{max}}$  525 nm. Some flavanoids and other plant constituents reacted under these conditions form brownish pigments that absorbed 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  $\mu$ L of sample (methanolized and pH-adjusted sample comprised of plant tissue, purified tannin, or standard methyl gallate) is dispensed into a 2.0 mL microcentrifuge tube. The pH 5.5 blank reagent is added as necessary to bring the sample volume to 100  $\mu$ L. For methanolysis products of most plants, a 100  $\mu$ L 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  $\mu$ L.
2. Water (350  $\mu$ L) and methanol (1000  $\mu$ L) are added and the samples are vortexed.
3. The tubes are tightly capped 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  $\mu$ L of water is replaced by 350  $\mu$ L of 0.3 N HCl.
5. Add the KIO<sub>3</sub> is added at timed intervals. Exactly 40  $\mu$ L of 5% (w/v) KIO<sub>3</sub> 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<sub>3</sub> to the sample its absorbance at 525 nm is recorded (vs. water). Under these conditions, a linear relationship between methyl gallate and absorbance at 525 nm is obtained.

A typical relationship with our spectrophotometer is:

$$\text{Abs} = 0.0132 * \mu\text{g} + 0.0701$$

$$R^2 = 0.996, \text{ standard error of Y} = 0.0303, \text{ standard error of slope} = 0.00019.$$

The limits of detection by this method are 1.5  $\mu$ g methyl gallate, and the method is linear through at least 120  $\mu$ g. There are no interferences when the method is used to determine methyl gallate after methanolysis of purified hydrolyzable tannins including tannic acid, epigallocatechin gallate, and oenothien B.