Spectranomics Protocol: Total Phenol and Tannin Determination

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PROTOCOL: Total Phenol and Tannin Determination in Fresh Leaves
[modified from Ainsworth and Gillespie (2007) for Phenols; Toth and Pavia (2001) and Makaar (2007) for Tannins]

(i) Equipment

- Sartorius balance (0.0001 g)
- Talboys high-throughput homogenizer
- Mini centrifuge
- Vortex mixer
- Orbital shaker
- Microplate reader

(ii) Consumable Materials

- Gallic Acid Monohydrate
- Methanol NF-grade, 99.5%
- Polyvinylpyrrolidone (PVP)
- Folin-Ciocalteau Phenol TS (F-C reagent)
- Anhydrous Sodium Carbonate
- 5/8" stainless steel threaded lugs ¼" width and 1/8" stainless steel coneballs
- 2 ml threaded and snap-cap microcentrifuge tubes
- 96-well polystyrene clear flat bottom plates
- 15 ml centrifuge tubes

(iii) Extract Preparation

- Weigh one frozen leaf disc (0.385 cm^2) and transfer into a threaded centrifuge tube with ball bearings and lugs.
- Add 0.75ml 95% methanol in water (vol/vol) and homogenize for two minutes at high intensity.
- Add 0.75ml 95% MeOH to sample and shake extract.
- Aliquot exactly 0.75 ml extract to a new 2 ml snap-cap tube containing another 0.75ml 95% MeOH and shake.
- Aliquot 0.5 ml portion of this extract to a new 2 ml tube for tannin analysis (detailed below).
- Dilute remaining extract with 0.5 ml 95% MeOH to a total volume of 1.5 ml. This is the 'Phenol Extract.'
- Place the phenol extract on an orbital shaker, cover, and incubate at room temperature for 48 hrs before phenol analysis (detailed below)

(iv) Tannin Precipitation and Analysis

- Add 0.5 ml methanol to 2 ml tubes containing 10 mg PVP and place on ice.
- For each sample, transfer 0.5 ml of each extract (referenced above) to a tube with PVP and methanol, vortex for ten seconds, and incubate on ice for 30 min.

- Prepare two test blanks with 1 ml methanol and 10 mg PVP. These blanks are essential for removing PVP contribution to the absorbance values.
- Centrifuge samples and test blanks for 2 min. Keep tubes on ice.
- Transfer 0.75 ml supernatant into a new tube with 10 mg PVP only for a second precipitation step. Repeat this step for PVP blanks.
- Vortex for 10 seconds and incubate on ice for 30 min.
- Centrifuge samples and, for each sample, transfer exactly 0.2 ml supernatant into new 2 ml tube for 'Folin-Ciocalteau Assay' (detailed below).

(v) **Phenol Preparation and Analysis**

- After a 48-hour incubation period, remove phenol extract tubes from orbital shaker.
- Centrifuge samples and, for each sample, transfer exactly 0.1 ml supernatant into new 2 ml tube for 'Folin-Ciocalteau Assay' (detailed below).

(vi) **Folin-Ciocalteau Assay**

- Prepare Standard Assay tubes with 0.1ml of previously prepared working standards (refer to 'Standard Preparation Procedure' below). These standards are always assayed with the samples.
- Add 0.2 ml 10% Folin-Ciocalteau Phenol reagent in water (vol/vol) to each standard and sample tube and vortex for 10 sec.
- Cover and incubate samples for exactly 30 min at room temperature.
- Add 0.8 ml of aqueous 700 mM sodium carbonate solution and vortex. Cover and incubate at room temperature for 2 hours.
- Centrifuge samples for 2 min.
- Transfer exactly 0.25 ml of assayed sample (in triplicates) into 96 well plates. Make sure to plate each standard and blanks once on each plate. The estimated values for the standard curve will be based on the average absorbances from all three wells.
- Immediately scan in SAFIRE at a fixed wavelength of 735 nm.

(vii) **Measurement Procedure**

- Measure absorbance at 735 nm of triplicate samples on a microplate reader (Tecan SAFIRE). This measurement includes 2x2 multiple reads per well to reduce potential variability caused by the well.
- Transfer all absorbance values to a central phenol and tannin determination sheet where estimates of both secondary metabolites are expressed as Gallic Acid Equivalents (GAE mg/L) relative to the standard curve (refer to 'Data Preparation and Finalization' below).

(viii) **Standard Preparation Procedure**

- Prepare 5 mg/ml stock solution of Gallic Acid in 95% methanol solution in a 100ml volumetric flask every month. Cover flask with parafilm and store in the freezer at -20°C.

- Prepare 8 working standards (25-200mg/L with of 25 mg/L) from the Gallic Acid stock solution every week using 10ml volumetric flasks. Assay working standards with each batch of samples for phenols and tannins, cover, and store at 4°C

Working Standard Concentration		**Stock Volume (ml) Gallic Acid (5mg/ml)**
V_{25}	25 mg/L	0.05 ml
V_{50}	50 mg/L	0.100 ml
V_{75}	75 mg/L	0.150 ml
V_{100}	100 mg/L	0.200 ml
V_{125}	125 mg/L	0.250 ml
V_{150}	150 mg/L	0.300 ml
V_{175}	175 mg/L	0.350 ml
V_{200}	200 mg/L	0.400 ml

(ix) **Data Preparation and Finalization**

- Phenol and tannin concentrations are calculated in terms of gallic acid equivalents (GAE) mass basis and corrected for standardization blanks, PVP test blanks, and dilution factor using the following equations:

- Phenol Concentration in solution after Folin-Ciocalteau Assay (GAE mg/L)

  = [(A735sample-A735blank)-Intercept GA standard curve]/slope GA standard curve

- Total Phenols (GAE mg/L) = Phenol Conc in the solution that was incubated for 48 hrs.

- Tannins = Total Phenols – Phenol Conc in the solution analysed after PVP precipitation.

- GAE (mg) = GAE (mg/L) x Total extract volume (ml)/1000 (ml/L)

- GAE mg/g = GAE (mg) / Disk dry weight (g)

- Disk dry weight is calculated as [Disk wet weight (g) - (disk wet weight (g) *%H_2O from total leaf)]