FEB 20, 2023

OPEN ACCESS

**DOI:**
dx.doi.org/10.17504/protocols.io.81wgbmxovpko/v1

**Protocol Citation:** Megan Nickerson, Jana M U'Ren 2023. Quantification of foliar polyphenolic concentration using a 96-well microtitre method. **protocols.io** https://dx.doi.org/10.17504/protocols.io.81wgbmxovpko/v1

**MANUSCRIPT CITATION:** Nickerson, MNN, Moore, LP, U'Ren, JM. 2023. The Impact of Polyphenolic Compounds on the In Vitro Growth of Oak-Associated Foliar Endophytic and Saprotrophic Fungi. Fungal Ecology.



**Protocol status:** Working
We use this protocol and it's working

**Created:** Jun 27, 2018

**Last Modified:** Feb 20, 2023

# Quantification of foliar polyphenolic concentration using a 96-well microtitre method

Megan Nickerson[1], Jana M U'Ren[1]

[1]University of Arizona

U'Ren lab - Ecosystem Genomics

Jana M U'Ren
University of Arizona

## ABSTRACT

Phenolics are secondary metabolites found in various fruits and vegetables. To estimate polyphenol concentrations, the Folin-Ciocalteu (F-C) reagent can be used to react with any reducing agent such as polyphenols, producing a color change that can be measured with spectrophotometry. When known concentrations of phenolics (e.g., tannic acid) are used to create a standard curve, F-C reagent enables estimation of total phenolic concentration. In this protocol, a high-throughput 96-well microtitre plate reader is used rather than a single-sample spectrophotometer. Methods are based on two publications: (1) Attard, E., 2013. "A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols". Open Life Sciences 8, 607; and (2) Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. 2000. "Use of nuclear and related techniques to develop simple tannin assays for predicting and improving the safety and efficiency of feeding ruminants on tanniniferous tree foliage".

## GUIDELINES

Folin-Ciocâlteu reagent should be stored in a brown bottle (or a foil covered bottle) and refrigerated. It should be golden in color, do not use if it has turned olive green.

**PROTOCOL integer ID:**
13409

**Keywords:** fungi, tannic acid, phenolic

## MATERIALS

MATERIALS

- Tannic acid **Sigma Aldrich Catalog #403040**
- Water, deionized **Bio Basic Inc. Catalog #WC8800.SIZE.4L**
- 70% acetone **Contributed by users**
- Folin-Ciocalteu Reagent **Contributed by users**
- Mortar and Pestels **Contributed by users**
- 96-well Plates **Contributed by users**
- Graduated Cylinder **Contributed by users**
- Lyophilizer **Contributed by users**
- 15 mL conical tubes **Contributed by users**
- Ultrasonic water bath **Contributed by users**
- Balance **Contributed by users**
- Ice **Contributed by users**
- Ice Bucket **Contributed by users**
- Micropipette **Contributed by users**
- Transfer pipette **Contributed by users**
- Multichannel Pipettor **Contributed by users**
- Vortex **Contributed by users**
- 1 M Sodium Carbonate **Contributed by users**
- Tannic Acid Dilutions **Contributed by users**
- Centrifuge Tubes **Contributed by users**

1/8 tsp metal scoops

## SAFETY WARNINGS

Acetone and Folin-Ciocâlteu reagent should be worked with in a chemical fume hood.

BEFORE START INSTRUCTIONS

-Before starting, it is advised to scan your extracts within the 600-800nm range to ensure existing compounds do not interfere with your chosen wavelength.
-This protocol has be written considering that you will be running a full 96-well plate with 24 samples run in triplicate, 6 dilutions of tannic acid run in triplicate, and 6 blanks.
-This protocol has been designed to work with lyophilized plant tissue. Before starting, it is advised that each of the samples that are being tested have been lyophilized to remove excess moisture for improved ease of grinding.
-Check that you have 1 M Sodium Carbonate.
-Prepare tannic acid dilutions by doing five 1:2 dilutions ranging from 2120 µg/ mL to 62.25 µg/mL.
-Determine total number of samples, fill out excel sheet plate map and print.

## Grinding Samples

1 Clean the tissue grinding cabinet (or other work space) with 10% bleach and 70% ethanol. Spray with 95% ethanol and allow to evaporate before using the cabinet.

2 Spray with EtOH and place your metal 1/8 tsp. scoops and any racks to be used in the cabinet. Let EtOH evaporate.

3 Label 15 mL conical tubes to match your sample IDs and record the names of samples you are grinding in your lab notebook.

4 Fill a thermos with liquid nitrogen and add enough liquid nitrogen to the dewar to submerge 15 mL conicals.

5 Gather the 50 mL tubes containing your lyophilized leaf tissue. Place them in the dewar of liquid nitrogen.

6 Unwrap a mortar and pestle carefully in the sterile cabinet. Use a new mortar and pestle for each

sample. To ensure no cross-contamination, thoroughly clean the mortar/pestles with detergent and water, rinse with DI water, submerge in a dilute bleach solution (0.5% sodium hypochlorite) for 30 minutes, rinse with DI water, wrap in aluminum foil, and autoclave for 30 minutes on dry cycle.

7 Fill the mortar with liquid nitrogen from the thermos and allow it to evaporate with the pestle submerged.

8 Fill the mortar halfway with liquid nitrogen; add lyophilized leaf tissue and push down with pestle to submerge tissue in liquid N.

9 Gently stir with a pestle until the liquid nitrogen has nearly all evaporated and then quickly grind the sample to a fine powder.

10 If necessary, add more liquid nitrogen to ensure the tissue doesn't thaw, and continue grinding until the tissue is a fine powder.

11 Add one scoop of ground tissue (with 1/8 tsp. metal scoop) for each sample to the corresponding pre-labeled 15 mL conical tubes.

12 Return the remainder of the leaf sample to its original conical tube and return to -80 freezer.

13 Clean bench space with 10% bleach and 70% ethanol in between grinding each sample.

14 The 15 mL conical with subset tissue for MTP analysis should be stored at -80°C until ready to proceed.

## Extracting Polyphenols

15 Turn on the centrifuge and set the temperature to 4°C. Get a bucket of ice.

16 Using a 5 mL pipette, add 5mL of 70% acetone to a labeled conical tube containing 1/8 tsp. scoop of ground tissue.

17 Fill the ultrasonic water bath with enough DI water to allow a plastic rack to float without touching the bottom of the water bath.

18 Loosen the cap of the conical tube and place it in the rack inside the water bath.

19 Subject the tube to ultrasonic treatment for 20 minutes.

20 Dry off the tube; centrifuge at 4° C and 3000 g for 10 minutes.

21 While the centrifuge runs, label one 5 mL tube per sample with the sample name.

22 Remove the conical tube from the centrifuge. Use a serological pipette to remove the supernatant and place it into the 5 mL tube labeled for that sample. Use a different pipette for each sample.

23 Place the tubes containing the polyphenol extract on ice.

24 Label 8-strip tubes (i.e., PCR tubes) with three tubes for each sample.

25 Using a micropipette, transfer 10 uL of extract into each of the three corresponding tubes.

26 Add 90 uL of 70% acetone to each tube and mix by pipetting.

27 Keep samples on ice.

## Preparing Stock Tannic Acid Dilutions

28 Label a 2 mL tube "Stock Tannic Acid" and the date.

29 Weigh 0.0015 g of solid tannic acid on analytical balance.

30 Add solid to the labeled "Stock Tannic Acid" tube.

31 Add 1.56 mL of nanopure water to tube and vortex to mix until tannic acid dissolves.

32 Label two 8-strip tubes with sample IDs D1-D6 and B1-B2. D1-D6 are the tannic acid dilution codes for the standard curve and B1-B2 are the two negative control blanks.

33 Add 15 µl of nanopure water to tubes D2-D6 and B1-B2.

34 Add 30 µl of stock Tannic Acid to well D1. Mix by pipetting.

35 To serially dilute Tannic Acid stock, pipette 15 µl from well D1 and add it to D2. Pipette mix.

36 Now take 15 µl from D2 and add to D3. Pipette mix.

37 Repeat serial dilutions for the remaining tubes (D4, D5, D6).

38 Store tubes in a dark place until ready to add to plate.

## Preparing Sodium Carbonate

39 Determine the volume of 1M Sodium Carbonate needed for plate:
____ samples + ____ extras x 80 uL 1M Sodium Carbonate

40 Transfer volume of 1M Sodium Carbonate determined in the previous step to new 50 mL reservoir in the chemical fume hood. You will use an 8- or 12-channel pipette to transfer to the 96 well plate.

## Preparing F-C Reagent

41 Make a 50 mL aliquot of autoclaved nanopure water.

42 Determine the total volume of 1:10 F-C Reagent needed for all samples:
____ samples + ____ extras x 100 uL total 1:10 F-C Reagent

43 Determine the volume of water and F-C Reagent for 1:10 dilution:
____uL total 1:10 F-C Reagent x 0.1 = ____uL F-C Reagent
____uL total 1:10 F-C Reagent - ____uL F-C Reagent = ____uL water

44 In the chemical fume hood, transfer the volume of water calculated above to a new 50 mL reservoir.

Note

This may be done with 5 mL pipette, which ranges from 500uL to 5000uL

45 Transfer the volume of F-C Reagent determined above to the 50 mL reservoir containing the water. Pipette mix and use immediately. You will use an 8- or 12-channel pipette to transfer to the

96 well plate.

Note

The diluted reagent should be stored in a bottle covered in tin foil and refrigerated. It should be golden in color. Do not use it if it turns olive green color.

## Preparing 96-well Plate

46 Prepare a digital platemap with your sample names and control samples D1-D6 (tannic acid serial dilutions) and B1-B2 (blank samples 1 and 2). Print out your platemap and place it in a location for easy reference (e.g., tape it to the door of the chemical fume hood).

47 To help with organization while adding reagents and samples to the plate, you can prepare your pipette tips to mimic the way reagents will be added. For example, have one pipette box with three columns only for the standard curve and blank samples; one full pipette box (12 columns) for F-C reagent; and a third full pipette box (12 columns) for Sodium Carbonate.

48 Using a 10 µl multichannel pipette, add 10 µl of the tannic acid dilutions and blanks from the pre-labeled 8-strip tubes (i.e., D1-D6 and B1-B2) and 10 µl of acetone to the wells indicated on the platemap the standard curve and blanks.

49 Using a 2-20 µl pipette, add 10 µl of sample extract into the well indicated for the corresponding sample.

50 Using 30-300 µl multichannel pipette, add 100 µl of F-C reagent into each well of the plate.

51 Using 30-300 µl multichannel pipette, add 80 µl of sodium carbonate into each well of the plate. Cover the plate with the lid.

52 Allow the plate to incubate at room temperature for 20 minutes in the dark.

53 Centrifuge the plate briefly with a tabletop centrifuge, then carefully package in Ziploc bag and place it in secondary containment (e.g., small box) that prevents light exposure.

54 Using a spectrophotometer fit for 96-well plates (e.g., BioTek Cytation5 plate reader), measure the absorbance at 630nm every 5 minutes for 60 minutes.

## Measuring Tannin Concentration

55 Add 1.0 mL of distilled water to a 2mL tube.

56 Weigh 100 mg of PVPP in a 100x12mm test tube.

57 Add 1.0 mL of the total polyphenolic extract solution.

> **Note**
>
> 100 mg of PVPP is enough to bind 2 mg of total phenols. If total phenolic content of feed is more than 10% on a dry matter basis, dilute the extract appropriately.

58 Vortex the sample.

59 Keep the tube at 4ºC for 15 minutes and vortex again.

60 Centrifuge at 3000g for 10 minutes. Collect the supernatant.

Note

This supernatant contains only simple phenolics other than tannins. The tannins have been precipitated along with the PVPP.

61 Repeat phenolic analysis outlined in the MicroMTP Method section.

Note

Take at least double the volume, preferably three times, you used for total phenol estimation, because you already diluted the extract two-fold and expect to lose tannin-phenols. Express the content of non-tannin phenols on a dry matter basis. (y%)

62 When repeating the MicroMTP Method, adding double the amount of solution will exceed the limits of the plate reader. Therefore, double the amounts in a secondary container and then transfer 190 µL of combined solution into the 96-well plate.

63 Example:
100 µl of the supernatant after PVPP treatment in the assay mixture gives 0.312 absorption = 5.75 µg tannic acid (TA) equivalent (from the standard curve).
Therefore, 1 ml supernatant = 5.75 / 0.1 = 57.54 µg TA = 0.058 mg TA.
10 mg leaf has 0.058 mg TA (since the extract is diluted 2-fold during the test) Therefore, 100 mg leaf sample has 0.058 x 10 = 0.58 mg TA
y = 0.58 / 0.95 (since dry matter of feed or seed = 95%) y = 0.611%
But total phenolics in dry matter, x = 1.04%
(x–y) is the percentage of tannins as tannic acid equivalent on a dry matter basis.
Tannins (as tannic acid equivalent) = 1.04 - 0.611 = 0.43% in the dry matter.

## Disposal of Resources Reagents

64 Leave reservoirs in chemical fume hood to evaporate with label.

65 Remove plate lid and allow the plate to evaporate.

66 Once evaporated, place reservoir in solid waste in chemical fume hood and fill out waste tag.