Small-Scale Determination of Total Phenols, Tannins, and Flavonoids from Foliar Tissue Using Colorimetric Assays

Angel E. Bravo-Monzón (✉ abravomonzon@gmail.com)

Laboratorio de Ecología Funcional de Comunidades. Escuela Nacional de Estudios Superiores Unidad Mérida. Universidad Nacional Autónoma de México, Mérida, Yucatán, México. <https://orcid.org/0000-0001-7172-0302>

Cristina Montiel-González

Laboratorio de Ecología Funcional de Comunidades. Escuela Nacional de Estudios Superiores Unidad Mérida. Universidad Nacional Autónoma de México, Mérida, Yucatán, México. <https://orcid.org/0000-0002-5832-8215>

Luis Daniel Avila-Cabadilla

Laboratorio de Ecología Funcional de Comunidades. Escuela Nacional de Estudios Superiores Unidad Mérida. Universidad Nacional Autónoma de México, Mérida, Yucatán, México. <https://orcid.org/0000-0003-2501-5690>

Mariana Y. Alvarez-Añorve

Laboratorio de Ecología Funcional de Comunidades. Escuela Nacional de Estudios Superiores Unidad Mérida. Universidad Nacional Autónoma de México, Mérida, Yucatán, México. <https://orcid.org/0000-0003-1288-992X>

Method Article

Keywords: Secondary metabolites, phenolics, leaf defensive traits, trees, miniaturized

Posted Date: March 29th, 2023

DOI: <https://doi.org/10.21203/rs.3.pex-1234/v2>

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Abstract

The quantification of plant secondary metabolites at levels higher than the population (i.e. community and ecosystem) requires the implementation of efficient, low-cost, and small-scale assays. We propose a modified protocol based on the Folin-Ciocalteu colorimetric assay that integrates the simultaneous quantitative determination of total phenols and total tannins from 40 μ L of methanolic extract, and a modified aluminum chloride assay to quantify total flavonoids from 20 μ L of methanolic extract. These miniaturized microplate colorimetric assays were tested and adjusted to work with as little as 50 mg of foliar tissue and to encompass the variation of secondary metabolite concentrations present in trees of 77 species, 58 genera, and 27 families from conserved and secondary tropical dry forest. At present, these new protocols are of great relevance to study ecological processes in highly diverse and strongly threatened ecosystems.

Introduction

The present manuscript reports on an extension to a protocol previously published by Ainsworth & Gillespie (2007) designed to quantify total phenolics in plant tissues using the Folin-Ciocalteu assay. We have modified the existing protocol to expand its applications by combining it with two other microplate-adapted assays that quantify total tannins (Makkar et al. 2007), total flavonoids (Marinova et al. 2005).

The resulting protocol was implemented in a study aimed to describe the variation of foliar secondary metabolites in trees growing in a tropical dry forest ecosystem and to explore the ecological and environmental factors associated with such variation. In that study, we quantified the total phenols, total tannins, and total flavonoids from 1753 individuals belonging to 77 species growing in contrasting habitats (Bravo-Monzón et al. 2020).

Our objective is to facilitate the characterization of the vegetation through the quantification of hard traits – rare in literature due to the difficulty to quantify them –. Although complex to measure, such informative traits allow us to understand important aspects of their response to natural and anthropogenic disturbance such as the assembly and disassembly of plant communities, the mechanisms underlying the distribution of species in anthropic landscapes, the relationship between functional diversity and ecosystem services and the degree of resilience of ecosystems

We believe that our enhanced protocol fulfills the current requirements for fast, accurate, inexpensive, small-scale, low waste assays for quantifying plant secondary metabolites required in the ever-growing number of phytochemical studies at the community or ecosystem levels.

Reagents

Aluminum chloride (Sigma-Aldrich 206911)

(+)-Catechin hydrate ((Sigma-Aldrich C1251)

Folin & Ciocalteu's phenol reagent (Sigma-Aldrich F9252)

Gallic acid monohydrate (Sigma-Aldrich 398225)

Methanol (Sigma-Aldrich 179337)

Polyvinylpyrrolidone (PVP) (Sigma-Aldrich PVP40)

Sodium carbonate (Sigma-Aldrich S7795)

Sodium hydroxide (Sigma-Aldrich S5881)

Sodium nitrite (Sigma-Aldrich S2252)

Water deionized and distilled (dd H₂O)

Reagent Setup

Aluminum chloride: Prepare solution of 10% in methanol.

Folin-Ciocalteu reagent: Prepare solution of 10% (vol/vol) F&C in water.

Methanol: Prepare solution of 95% (vol/vol) methanol in water.

Sodium carbonate: Prepare solution of 700mM Na₂CO₃ in water.

Sodium hydroxide: Prepare solution of 1M NaOH in water.

Sodium nitrite: Prepare solution of 5% NaNO₂ in water.

Equipment

Analytical Balance.

Microcentrifuge (reaching a minimum of 5,000 rpm).

Vortex mixer.

Microplate Spectrophotometer, UV-Visible.

Transfer pipettes from 20 µL to 1 mL and plastic tips.

Mortar and pestle (300 mL).

Microcentrifuge tubes with hinged lid, 2 mL.

Flat bottom 96-well clear polystyrene microplates (non-treated, 400 µL total volume).

Procedure

Sample Preparation

- 1.- Homogenize 50 mg of dry foliar tissue using an ice-cold mortar and pestle, and 2 mL of cold methanol (95%).
- 2.- Place the sample in a 2 mL microtube and vortex for 10 seconds.
- 3.- Incubate at room temperature for 48 h in the dark.
- 4.- Vortex the sample for 10 seconds and centrifuge it at 5000 rpm for 4 minutes.

Total Phenols Analysis

- 5.- Transfer 40 µL of supernatant to a new microtube and add 960 µL of methanol (95%).
- 6.- Vortex for 10 seconds and transfer 100 µL to a new microtube for Folin-Ciocalteu analysis.

Folin-Ciocalteu Colorimetric Assay

- 7.- Add 200 µL of F-C 10% and vortex for 10 seconds.
- 8.- Cover and incubate the sample for 30 minutes at ambient temperature.
- 9.- Add 800 µL of Na₂CO₃ (700 mM).
- 10.- Cover and incubate for 2 hours at ambient temperature.
- 11.- Centrifuge the sample at 5000 rpm for 4 minutes.
- 12.- Transfer 300 µL to a well in a microplate and read absorbance at 735nm.

Total Tannins Analysis

- 13.- Transfer the 900 µL of supernatant from step 5 to a new microtube containing 20 mg of PVP.

- 14.- Vortex for 10 seconds and incubate in freezer (-4°C) for 30 minutes.
- 15.- Prepare a blank microtube with 1 mL of methanol (95%) and 20 mg of PVP.
- 16.- Centrifuge at 5000 rpm for 10 minutes. Keep cold.
- 17.- Transfer 100 µL to a new microtube and proceed with the Folin-Ciocalteu analysis (step 7).

Total Flavonoids Analysis

- 18.- Transfer 20 µL of supernatant (step 4) to a new microtube and add 80 µL of dd H₂O.
- 19.- Add 30 µL of NaNO₂ (5%) and wait for 5 minutes.
- 20.- Add 30 µL of AlCl₃ (10%) and wait for 1 minute.
- 21.- Add 200 µL of NaOH (1M).
- 22.- Add 640 µL of dd H₂O.
- 23.- Vortex for 10 seconds and transfer 300 µL to read absorbance at 510 nm.

Total Concentrations Calculations

- 24.- Calculate a standard curve from the blank-corrected at 765 nm of the gallic acid standards (Fig. 1). Calculate total phenols and total tannins concentrations as gallic acid equivalents using the regression equation of gallic acid standards.
- 25.- Calculate a standard curve from the blank-corrected at 510 nm of the (+)-catechin standards (Fig. 2). Calculate total flavonoids concentrations as catechin equivalents using the regression equation of catechin standards.

Troubleshooting

Results from the F-C assay should be interpreted with caution, as this method is known to be affected by many interfering substances, in particular, ascorbic acid, fructose, sucrose, aromatic amines, sulfur dioxide, and Fe(II) (Lester et al. 2012).

Troubleshooting advice can be found in Table 1.

Time Taken

Step 1: 2-20 min depending on the hardness of foliar tissue; Step 2: 10 sec; Step 3: 2 days; Step 4: 4.5 min; Step 5-6: 2 min; Step 7-12: 3 h; Step 13-17: 1 h; Step 18-23: 10 min.

Anticipated Results

The development of efficient, accurate, inexpensive protocols that quantify plant traits with adaptive value (e.g. secondary metabolites) on large scales is urgent to understand the mechanisms that shape the community structure and dynamics. The miniaturized protocols presented here are an accessible alternative to estimate the total concentration of phenols, tannins, and flavonoids using the same methanolic leaf extract.

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Acknowledgements

A DGAPA-UNAM Postdoctoral Fellowship to A.E.B-M. supported this research. We thank Dra. Joanna Ortiz-Alcántara and M.C. Korhyntia Lopez-Aguilar for their technical assistance in the lab, and Virginia Santana for her help during the chemical analyses.

Figures

Figure 1

Example of a gallic acid standard curve. This standard curve is used to estimate the concentration of total phenols and tannins.

Figure 2

Example of a catechin standard curve. This standard curve is used to estimate the concentration of total flavonoids.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.pdf](#)