Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent

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Non-structural phenolic compounds perform a variety of functions in plants, including acting as antioxidants. We describe a microplate-adapted colorimetric total phenolics assay that utilizes Folin–Ciocalteu (F–C) reagent. The F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically at 765 nm. Although the electron transfer reaction is not specific for phenolic compounds, the extraction procedure eliminates approximately 85% of ascorbic acid and other potentially interfering compounds. This assay is performed in microcentrifuge tubes and assessed in a 96-well plate reader. At least 64 samples can be processed in 1 d.

INTRODUCTION

Plants produce an extraordinary diversity of phenolic metabolites that contain one or more acidic hydroxyl residues attached to an aromatic arene (phenyl) ring[1]. Hydroxycinnamic acids, flavonoids, anthocyanins and tannins represent the major classes of phenolics, which collectively account for approximately 40% of the organic carbon in the biosphere[1]. Although structural phenolic compounds such as lignin, suberin and other structural polymers comprise much of this carbon pool, the amazing array of non-structural phenolics have many functions in plants, including acting as antioxidants[2,3]. Phenolic compounds are excellent oxygen radical scavengers because the electron reduction potential of the phenolic radical is lower than the electron reduction potential of oxygen radicals[3,4], and also because phenoxyl radicals are generally less reactive than oxygen radicals[5]. Therefore, phenolic compounds can scavenge reactive oxygen intermediates without promoting further oxidative reactions[3]. It follows that many environmental stresses that cause oxidative stress often induce the synthesis of phenolic metabolites[3,6,7].

Many available methods of quantification of total phenolic content in food products or biological samples are based on the reaction of phenolic compounds with a colorimetric reagent, which allows measurement in the visible portion of the spectrum[8,9]. The Folin–Ciocalteu (F–C) assay is such a method[10,11] and has been proposed as a standardized method for use in the routine quality control and measurement of antioxidant capacity of food products and dietary supplements[12]. The F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 760 nm[11,13]. Although the exact chemical nature of the F–C reaction is unknown, it is believed that sequences of reversible one- or two-electron reduction reactions lead to blue species (possibly $(PMoW_{11}O_{40})^{4-}$)[14].

Major considerations in the interpretation of the F–C assay are that the chemistry is non-specific and that other oxidation substrates in a given extract sample can interfere in an inhibitory, additive or enhancing manner[11,14]. Inhibition could occur as a result of oxidants competing with the F–C reagent or air oxidation after the sample is made alkaline. For this reason, the F–C reagent is added before the alkali[11]. Additive effects occur from unanticipated phenols, aromatic amines, high sugar levels or ascorbic acid in the extract[11]. Singleton and Rossi[11] discussed the potential additive effects of various compounds and methods for correcting these factors. Ascorbic acid readily reacts with the F–C reagent and therefore must be considered. It can be measured before adding the alkali or by a more specific assay and then subtracted from the F–C value[11]. Sulfites and sulfur dioxide also react with the F–C reagent, and this has been a problem in wines, where SO_2 is a common additive[11]. Owing to the general nature of the F–C chemistry, it is indeed a measure of total phenols and other oxidation substrates. However, the F–C assay is simple and reproducible and has been widely used for studying phenolic antioxidants[14].

This protocol describes a rapid, small-scale, high-throughput assay for approximating the total phenolics and other antioxidant substrates on the basis of the improved F–C assay of Singleton and Rossi[11], using gallic acid as a standard. This protocol is provided along with an oxygen radical absorbance capacity protocol[15] and a rapid protocol for measuring ascorbate content in plant tissues[16]. Overall, these assays provide a general diagnostic tool of the antioxidant capacity of leaf tissue extracts.

MATERIALS

REAGENTS

- Methanol (Sigma, cat. no. 179337) (see REAGENT SETUP)
- F–C reagent (Sigma, cat. no. F9252)
- Sodium carbonate (Sigma, cat. no. S7795) (see REAGENT SETUP)
- Gallic acid (Sigma, cat no. 48630) (see REAGENT SETUP)

! CAUTION Irritating to eyes, respiratory system and skin.

EQUIPMENT
- Multi-detection microplate reader (Synergy HT, Bio-Tek) (see EQUIPMENT SETUP)
- Mixer mill disruptor with adaptor sets for 2-ml tubes (Qiagen tissue lyser) or mortar and pestle

REAGENT SETUP
Methanol Prepare solution of 95% (vol/vol) methanol in water.
Sodium carbonate Prepare solution of 700 mM Na_2CO_3 in water.
Gallic acid Prepare solutions of 50 μM–2.5 mM gallic acid [in 95% (vol/vol) methanol].

EQUIPMENT SETUP
Microplate reader Set up the microplate reader to run an end-point absorbance read at 765 nm (at room temperature: ~20 °C).

PROCEDURE

F–C extraction and assay

1| Harvest plant material (approximately 20 mg) in screw-capped tubes and freeze immediately in liquid nitrogen.
■ **PAUSE POINT** Samples can be stored at –80 °C for 1–2 months.

2| To homogenize the tissue, place three tungsten carbide beads and 2 ml of ice-cold 95% (vol/vol) methanol in each sample tube and insert samples into pre-cooled Teflon adaptors. Homogenize tissue for 5 min at 30 Hz. If a mixer mill is not available, tissue can be homogenized in an ice-cold mortar and pestle.

3| Remove tungsten carbide beads with a magnet and incubate the samples at room temperature for 48 h in the dark.

4| Centrifuge the samples (13,000*g* for 5 min at room temperature) and collect the supernatant in a fresh 2-ml microtube.

5| Add 100 μl of each sample supernatant, standard or 95% (vol/vol) methanol blank to duplicate 2-ml microtubes.

6| Add 200 μl 10% (vol/vol) F–C reagent and vortex thoroughly.
▲ **CRITICAL STEP** The F–C reagent should be added before the alkali to avoid the air-oxidation of phenols[13].

7| Add 800 μl 700 mM Na_2CO_3 into each tube and incubate the assay tubes at room temperature for 2 h.
▲ **CRITICAL STEP** This assay can be incubated at warmer temperatures for shorter times to obtain faster color development, but this can increase the s.e.m. of the assay[13]. For soybean leaf extracts, a 2-h incubation at room temperature resulted in low s.e.m. between duplicate samples.

8| Transfer 200 μl sample, standard or blank from the assay tube to a clear 96-well microplate and read the absorbance of each well at 765 nm.

Total phenolics calculation

9| Calculate a standard curve from the blank-corrected A_{765} of the gallic acid standards (**Fig. 1**). Calculate total phenolics as gallic acid equivalents using the regression equation between gallic acid standards and A_{765}.
? TROUBLESHOOTING

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Phenolic content varies considerably among leaf tissues and also with environmental stress[7]. In the design of experiments, we advise the use of multiple biological and technical replicates, as there can be significant variability among biological samples (**Table 1**). Different incubation times should be tested with each different species and tissue to optimize the F–C assay. Automated liquid handling systems may also significantly increase throughput and improve results[9].

Although the F–C assay has commonly been used to assay 'total phenolic content'[7,11], the assay also measures other readily oxidized substances[13,14]. When we performed the F–C assay with ascorbate or trolox, we found that 94–96% of the compounds were recovered after a spiking test, so there is potential for significant interference using these molecules. However, when we incubated ascorbate for 48 h in a way similar to that in which we extract total phenolics from the leaf samples, the recovery was reduced to approximately 15%. Further, leaves have a very high content of phenolics (15–20 mg g^{-1} DW)[15], and therefore the F–C assay is largely a measure of phenolic content. It is important to bear in mind that the F–C assay is non-specific to phenolics and should be interpreted with care.

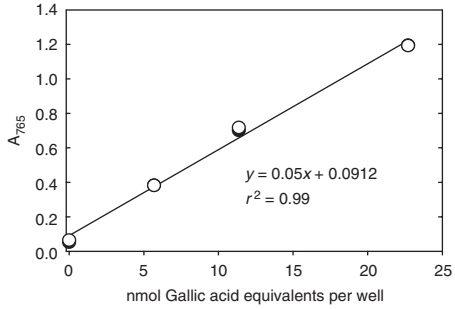

Figure 1 | Example of a gallic acid standard curve. The standard curve is used to estimate nanomoles of phenolics (gallic acid equivalents) in a 200-μl sample.

ANTICIPATED RESULTS

The F–C assay provides a convenient, rapid and simple estimation of the content of total phenolics and other

TABLE 1 | Troubleshooting table.

Problem	Potential cause	Solution
High variability among biological replicates of the same treatment	Real variability in the individual plants sampled	Increase the number of biological replicates
Variability between technical replicates of the phenolics assay	Folin–Ciocalteu (F–C) reagent not mixed with the sample properly Incubation time and temperature not ideal	Vortex the assay solution after addition of F–C and allow it to stand for 1–2 min before adding Na_2CO_3 Incubate for 2 h at room temperature or test other variations on decreased time and increased temperature

oxidation substrates in plant extracts[7]. This assay has been used widely in the past to analyze wine and other foodstuffs[13,14] but has not been as widely used for fresh plant extracts. We suggest that when it is used with other assays of total antioxidant capacity[16] or ascorbate content[17], the general antioxidant capacity of plant tissue extracts can be determined. Furthermore, these assays provide high-throughput tools for screening the response of plants to environmental stress.

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