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Comparative analysis of strawberry total phenolics via Fast Blue BB vs. Folin–Ciocalteu: Assay interference by ascorbic acid

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ABSTRACT

Unblemished fully ripe fruit from five day-neutral strawberry cultivars were harvested on two separate dates and evaluated for ascorbic acid (AsA), fruit sugars, and phenolic composition. Individual phenolics were determined by HPLC, and total phenolics by Folin–Ciocalteu (F–C) and by a 'new' assay: Fast Blue BB (FBBB), which detects phenolics directly. FBBB reported an average 2.9-fold greater concentration of total phenolics than F–C, had a significant correlation ($r = 0.80$; $P = 0.001$) with total phenolics via HPLC and did not interact with AsA or sugars, whereas F–C, an indirect detection assay for total phenolics, appeared to under-report total phenolic concentrations, had no significant correlation ($r = 0.20$) with total phenolics via HPLC or with sugars, but had a significant correlation ($r = 0.64$; $P = 0.05$) with total AsA. Results from this study indicated that previous studies of strawberry fruit, using the standard indirect F–C assay, have greatly underestimated the total phenolics content and that this assay should be replaced in future studies by the FBBB assay.

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1. Introduction

Strawberries (*Fragaria* x *ananassa* Duch.) are an important source of phytochemicals, in particular phenolics, which strongly influence not only color (anthocyanidins) but sensorial organoleptic attributes and antioxidant value (Panico et al., 2009; Tulipani et al., 2008, 2011).

Folin–Ciocalteu (F–C) is an assay regularly used to predict total phenolics in strawberry as well as in a variety of other fruits and vegetables (Prior et al., 2005). The original F–C spectrophotometric method created to detect total phenolics in fruits and vegetables was developed by Folin and Ciocalteu (1927) and was later modified by Singleton and Rossi (1965). The modified F–C method uses molybdotungstophosphoric heteropolyanion reducing reagent which indirectly detects phenolics (Medina, 2011a), but lacks specificity (Prior et al., 2005). It has been reported by Prior et al. (2005) that the F–C assay suffers from a number of interfering substances, in particular, ascorbic acid (AsA), sugars (fructose and sucrose), aromatic amines, sulfur dioxide, organic acids, and Fe(II), and correcting for these interfering substances is essential. The list of F–C interfering substances does not stop with the aforementioned, but can include at least 50 additional organic compounds naturally found in fruits and vegetables or in the polyphenol extraction media (Prior et al., 2005). Prior et al. (2005) advised, when using the F–C assay, that the kind of phenolics measured should be considered, the steps in the analysis should rigorously follow the modified assay of Singleton and Rossi (1965), proper correction due to interfering substances should be made, and gallic acid should be the only reference standard used.

Fortunately, a new method developed by Medina (2011a) does not suffer the interfering compound fate of F–C, as this new assay utilizes Fast Blue BB diazonium salt (FBBB) where the diazonium group (–$^{+}$N=N–) specifically couples with reactive phenolic hydroxyl (–OH) groups, under alkaline conditions, to form stable azo complexes which can be measured at 420 nm. This FBBB azo-based assay has higher gallic acid equivalency values than F–C for total phenolics as demonstrated in drink samples fortified with

Abbreviations: AsA, ascorbic acid; DAsA, dehydroascorbic acid; F–C, Folin–Ciocalteu; FBBB, Fast Blue BB.

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ascorbic acid and fructose corn syrup showing total phenolic concentrations in these samples were under reported by the F–C assay (Medina, 2011b). Total phenolics in strawberry, a berry naturally abundant in ascorbic acid and fruit sugars (fructose, glucose and sucrose), likely have been underreported when assayed by the F–C due to high concentrations of a number of biological interfering compounds, particularly AsA.

The objective of this study was to compare F–C vs. FBBB assays for analysis of total phenolic concentrations in fruit from five different genotypes of strawberries, commonly grown in the USA. In the same fruit, we also measured known F–C assay interfering quality components (AsA and fruit sugars) to determine their impact, if any, on the two assays for total phenolics.

2. Materials and methods

2.1. Plant materials

Fruit, 500 g from three separate beds, were collected for each of 5 strawberry (*Fragaria* x *ananassa* Duchesne ex Rozier) cultivars: Albion, Monterey, Portola, San Andreas, and Seascape. These cultivars are "day-neutral" and were developed by the University of California. Strawberry fruit were grown at the USDA-ARS Henry A. Wallace Agricultural Research Center at Beltsville, MD, USA in a low-tunnel system. A Raised Bed Plastic Mulch Layer (Rainflow Irrigation, East Earl, PA, USA) was used to form three raised beds on 182-cm centers, with two lines of drip tape, 30 cm apart and 7 cm below two layers of plastic mulch, a layer of 0.025 mm black mulch covered by a layer of 0.025 mm "white-on-black" mulch. Plants were fertilized weekly with 2.27 kg/10,000 m^2 nitrogen. Stainless steel rods, 5 mm in diameter × 366 cm long, were pushed into the ground 15 cm from the sides of the beds, and spaced every 122 cm to act as support hoops for a layer of solid (no holes) 0.098 mm thick × 366 cm wide clear plastic sheeting (Berry Plastic Corporation, Greenville, SC, USA) 61 cm over the beds, forming a low tunnel to protect the plants from rain. Individual fruit were hand-harvested by 07.30 h Eastern Standard Time from each 6-plant plot the mornings of 22 August and 25 August, 2011, and are hereafter referred to as 1st and 2nd harvests, respectively. Only fully ripe, unblemished fruit were selected for further quality evaluations. Fruit were placed in plastic bags labeled with the plot (replication) number and chilled in an ice chest. All berries were immediately transported to the lab where they were either assayed immediately for AsA or frozen at −80 °C for subsequent phenolic and sugar analysis.

2.2. Chemicals

Phenolic standards recommended for high performance liquid chromatography (HPLC) analysis of phenolics in strawberry (Fan et al., 2012) included elagic acid, *m*-coumaric acid, *o*-coumaric acid, *p*-coumaric acid, cyanidin-3-glucoside, gallic acid, kaempherol-3-glucoside, quercetin-3-glucoside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside. All of the standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except for pelargonidin-3-rutinoside, which was obtained from Apin Chemicals (Abingdon, UK).

2.3. Ascorbic acid

One g strawberry fruit was homogenized in ice-cold 5% (w/v) *m*-phosphoric acid, centrifuged at 10,000 × *g* for 15 min at 2 °C, then the supernatant was decanted and reserved. The strawberry pellet was re-extracted 2 additional times, for a total of 15 mL, as recommend for AsA extraction of strawberry by Klopotek et al. (2005). The combined supernatant was determined for total and free AsA spectrophotometrically at 525 nm according to the procedure of Hodges et al. (2001). Total and free AsA concentrations were quantified using a previously developed standard curve in the range of 0.002–200 μg. The calibration curve was linear in the range studied with a correlation coefficient of 0.999. Total AsA equals free AsA plus dehydroascorbic acid (DAsA). Dehydroascorbic acid concentration was calculated by subtracting free AsA from total AsA.

2.4. Phenolic extraction for Folin–Ciocalteu, Fast Blue BB assay

Strawberry fruit samples were prepared by combing 2.5 g of tissue cut from the distal-half of the berry previously frozen at −80 °C with 12 mL 70% MeOH and homogenized at 12,000 rpm for 30 s using a PT10-35 GT probe (Brinkman Instruments Inc., Westbury, NY, USA) followed by dismembration for 30 s using a micro tip at 35% (ARTEK sonic dismembrator model 300 Farmingdale, NY, USA). Dismembrated homogenates were centrifuged at 6650 × *g* for 10 min at room temperature and the supernatant used to determine total phenolics.

2.5. Total phenolics via Folin–Ciocalteu, Fast Blue BB methods and HPLC

2.5.1. Folin–Ciocalteu assay

Folin–Ciocalteu (F–C) was assayed according to Medina (2011b). Fifty μL of dismembrated sample diluted 1:4 with DI H_2O, gallic acid standard, or DI H_2O for blank was added to 13 mm × 100 mm borosilicate tubes, followed by 430 μL DI H_2O, 20 μL F–C reagent, mixed, and allowed to react for 5 min before adding 50 μL 20% Na_2CO_3, 450 μL DI H_2O, mixed and allowed to stand 60 min at room temperature. Absorbance was measured at 725 nm.

2.5.2. Fast Blue BB assay

Fast Blue BB (FBBB) was assayed according to Medina (2011b). One mL of dismembrated sample diluted 1:20 with DI H_2O, gallic acid standard or DI H_2O for blank was added to 13 mm × 100 mm borosilicate tubes, followed by 0.1 mL sonicated 0.1% FBBB [4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi(-zinc chloride) salt], mixed for 30 s, followed by 0.1 mL 5% NaOH, mixed, and the resulting mixture allowed to incubate for 90 min at room temperature. Absorbance was measured at 420 nm. Both assays were evaluated with gallic acid standard dilution or a fruit sugar mixture (fructose, glucose, sucrose) standard dilution of 0, 0.01562, 0.03125, 0.0625, 0.125, 0.25, 0.50 mg/mL DI H_2O or an AsA standard dilution of 0, 0.01562, 0.03125, 0.0625, 0.125, 0.25, 0.50, 1.0 mg/mL DI H_2O. The calibration curve was linear in the range studied with a correlation coefficient of 0.999.

2.5.3. Phenolic extraction for HPLC determination

Strawberry fruit samples were prepared by combining 5.0 g of tissue cut from the distal end of the berry previously frozen at −80 °C with 25 mL 50% MeOH and homogenized at 10,000 rpm for 1 min using a PT10-35 GT probe (Brinkman Instruments Inc., Westbury, NY, USA) followed by dismembration for 2 min using a micro tip at 35% (Fisher Scientific sonic dismembrator model 300, Farmingdale, NY, USA) in an ice bath. Homogenates were centrifuged at 6650 × *g* for 10 min at 4 °C, the resulting pellet was re-extracted with 5 mL 70% MeOH, centrifuged and the supernatants were combined. Combined supernatant was placed at −80 °C for 30 min to congeal complex carbohydrates, centrifuged at 14,000 × *g* for 30 min at 4 °C and the supernatant (10 mL) was filtered through 0.45 μm filter, evaporated to dryness under a N_2 stream, then re-dissolved in 1 mL HPLC mobile phase (6% acetic acid in 2 mM Na acetate).