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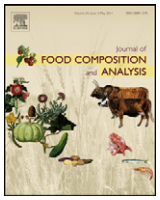

Original research article

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).

Table 1
Mean fructose, glucose and sucrose (g/100 g fresh weight) and soluble solids concentration (SSC %) of strawberry fruit at two harvests.

Cultivar	22 August, 2011				25 August, 2011			
	Fructose	Glucose	Sucrose	SSC %	Fructose	Glucose	Sucrose	SSC %
Albion	1.44Ba	1.24Aa	1.29Ba	9.0Aa	1.30Ba	1.07Aa	1.10Ba	8.5Aa
Monterey	1.85A	1.49Aa	1.58Aa	11.0Aa	1.74Ab	132Aa	1.38Ab	8.9Aa
Portola	1.13Ba	1.25Aa	1.33ABa	8.1Aa	1.31Ba	1.3.0Aa	1.37Aa	8.0Aa
San Andreas	1.47Bb	1.38Aa	1.48ABa	9.4Aa	1.73Aa	1.32Aa	1.43Aa	8.3Ab
Seascape	1.49Ba	1.28Aa	1.34ABa	9.5Aa	1.40Ba	1.23Aa	1.25ABb	8.6Aa

Upper-case letters within a column indicate significant difference (LSMEANS 0.05) among cultivars within a harvest period. Lower-case letters across a row indicate significant differences (LSMEANS 0.05) within a cultivar over harvest periods. $N = 3$.

Individual phenolic compounds of strawberries were analyzed by HPLC using a modified procedure of Fan et al. (2012) and Tsao and Yang (2003) using an Agilent (Agilent Technologies, Santa Clara, CA, USA) 1260 HPLC system equipped with a binary pump and coupled with a photodiode array detector. Twenty μL samples were injected and phenolic sample analytes were separated at room temperature with a Luna C18(2) column (250 mm × 2 mm; 5 μm particle size; Phenomenex, Torrence, CA, USA) using a mobile phase made of solvents: A (2 mM sodium acetate, pH 2.55) and B (acetonitrile) at a flow rate of 1.0 mL/min The mobile phase was 100% A for 40 min, lowered to 85% A over 5 min, to 70% A over 2 min, to 50% A over 3 min, and to 0% A over 1 min, then raised to 100% A over 1 min with a 5 min hold. The detector was set at 255, 320, 350 and 520 nm for simultaneous monitoring of the different phenolic groups. Data were collected and analyzed by an Agilent ChemStation version B.04.02 SP1. (Agilent Technologies, Santa Clara, CA, USA). Total phenolic compounds were divided into five groups and quantified as follows: ellagic acid (255 nm); benzoic acid using gallic acid; hydroxycinnamic acids using *p*-, *m*- and *o*-coumaric acids (320 nm); flavonol using quercetin-3-glucoside and kaempferol-3-glucoside (350 nm); and anthocyanins using cyanidin-3-glucoside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside (520 nm). The compounds were identified by comparing their retention times and UV spectra at each specific wavelength with those for the external standards. The results were expressed as mg/100 g fresh weight. The quantification of phenolic compounds was performed using the calibration curves of their respective standards in the range of 0.01–1.0 μg/20 μL injection. The calibration curves were linear in the range studied, with a correlation coefficient of 0.999.

2.6. Fruit sugar determination

Fruit sugars (fructose, glucose and sucrose) were extracted from 0.3 g of lyophilized tissue with EtOH (80%) at 80 °C and quantified by HPLC as previously described for fruit tissues by Lester (2008). Refractive index detection of sugars was quantified using a previously developed standard curve in the range of 0.1–80 μg/20 μL injection. The calibration curve was linear in the range studied, with a correlation coefficient of 0.999.

2.7. Soluble solids concentration

Soluble solids concentration (SSC) was determined on 5-mm thick sections of berry tissue cut from the distal-half of the berry; sections from each berry per replicate were combined. Tissue sections were frozen, thawed and squeezed using a hand-held garlic press and SSC of the expressed juice was determined using a temperature corrected, digital refractometer (Reichert Scientific Instruments, Buffalo, NY, USA).

2.8. Repeatability and precision

The repeatability of all phytonutrients was checked by conducting two injections or spectrophotometric readings of each replicate ($N = 3$) of each sample from each harvest. The precision and sample stability were evaluated by running (daily) either an external standard curve comparison or internal and external standards with each sample and external standards with each set throughout the analysis.

2.9. Statistical analyses

Analysis of variance of the randomized complete block design was done with the general linear model procedure (SAS, ver. 9.1; SAS Institute, Cary, NC, USA). Mean comparisons were made using protected LSMEANS with significant differences reported at $P \leq 0.05$. Correlation analysis was carried out between phenolic detection assays F–C and FBBB and HPLC-determined total phenolics using the LSMEANS procedure (SAS, ver. 9.1; SAS Institute, Cary, NC, USA). Replication $N = 3$.

3. Results and discussion

Strawberry fruit sugars (fructose, glucose and sucrose) and soluble solids concentration (SSC) were abundant in the cultivars assayed, with significance among them for fructose and sucrose (Table 1). Sugars and SSC were slightly higher in all cultivars from the 1st vs. 2nd harvests, but the difference was insignificant due to berries from both harvests having received nearly equal amounts of solar radiation 48–120 h prior to harvest (meteorological data not shown). Glucose, the biochemical precursor to AsA (Loewus

Table 2
Mean ascorbic acid (mg/100 g fresh weight) and total phenolics determined via Folin–Ciocalteu (F–C) or Fast Blue BB (Fast BBB) (gallic acid equivalents, g/100 g fresh weight basis) of strawberry fruit at two harvests.

Cultivar	22 August, 2011					25 August, 2011				
	Total AsA	Free AsA	DAsA	F–C	Fast BBB	Total AsA	Free AsA	DAsA	F–C	Fast BBB
Albion	160Aa	109Ba	51Aa	0.46Ba	0.79Ba	126Ab	95Aa	31Ab	0.20Ab	0.65Ab
Monterey	182Aa	131Aa	52Aa	0.29Aa	1.02Ba	148Ab	111Ab	36Ab	0.23Ab	0.62Aa
Portola	114Ba	83Ba	31Ba	0.41Aa	0.79Ba	123Aa	103Aa	20Ab	0.20Ab	0.43Bb
San Andreas	148Aa	105Aa	43Ba	0.54Aa	0.99Aa	126Aa	101Aa	26Ab	0.21Ab	0.56Bb
Seascape	136Ba	100Ba	36Ba	0.58Aa	0.90Aa	134Aa	106Aa	28Ab	0.24Ab	0.52Bb

AsA, ascorbic acid; DAsA, dehydroascorbic acid. Upper-case letters within a column indicate significant difference (LSMEANS 0.05) among cultivars within a harvest period. Lower-case letters across a row indicate significant differences (LSMEANS 0.05) within a cultivar over harvest periods. $N = 3$.

Table 3
Mean phenolic composition (mg/100 g fresh weight) of strawberry fruit from five cultivars at two harvests.

Cultivar	Ellagic acid (255 nm)	Gallic acid (320 nm)	Total hydroxy-cinnamic acid (320 nm)	Total flavonoids (350 nm)	Total anthocyanins (520 nm)	Total phenolics[a]
22 August, 2011						
Albion	2.56Ca	2.11Aa	3.91Ca	0.29Ca	14.88Ba	23.76Ba
Monterey	6.00Aa	1.66Ba	5.44Ba	0.85Ba	18.77Aa	32.72Aa
Portola	4.52Ba	0.83Ca	3.87Ca	1.48Aa	14.26Ba	24.96Ba
San Andreas	4.06Ba	1.07Ca	6.98Aa	0.64Ba	15.52Ba	28.27Ba
Seascape	4.19Ba	2.28Aa	6.76Aa	0.84Ba	16.03Ba	30.10ABa
25 August, 2011						
Albion	2.97Ca	2.19Aa	2.98Ca	0.23Ca	1.77Aa	22.14ABa
Monterey	5.17Aa	1.79Ba	4.89Ba	0.45BCb	13.71Ab	26.01Ab
Portola	4.00Ba	0.63Ca	4.19Ba	0.82Ab	8.64Bb	18.28Bb
San Andreas	3.53BCa	0.91Ca	6.32Aa	0.51Ba	9.60Bb	20.87Bb
Seascape	3.97BCa	2.23Aa	5.74Aa	0.68ABa	9.32Bb	21.94Bb

Upper-case letters within a column and within harvest date indicate significant difference (LSMEANS 0.05) among cultivars. Lower-case letters within a column and across harvest dates indicate significant differences (LSMEANS 0.05) within a cultivar over harvest periods. Total phenolics were quantified as: ellagic acid, gallic acid, hydroxycinnamic acids (*p*-coumaric, *m*-coumaric, *o*-coumaric), flavonoids (kaempferol-3-glucoside and quercetin-3-glucoside), and anthocyanins (cyanidin-3-glucoside, pelargondin-3-glucoside, pelargonidin-3-rutinoside). $N = 3$.

[a] Total phenolics is the sum of the five classes of phenolics.

and Jang, 1958) was slightly higher in most of the 1st harvest berries, which coincided with slightly higher total AsA concentrations compared to 2nd harvest berries (Table 2). Although 1st harvest strawberries generally had higher total AsA concentrations than the 2nd harvest berries, 1st harvest berries also had significantly higher DAsA concentrations (Table 2). Not only were DAsA concentrations higher in the 1st vs. 2nd harvest berries, the ratio of free AsA:DAsA was 2.5:1 vs. 3.8:1 for 1st vs. 2nd harvest

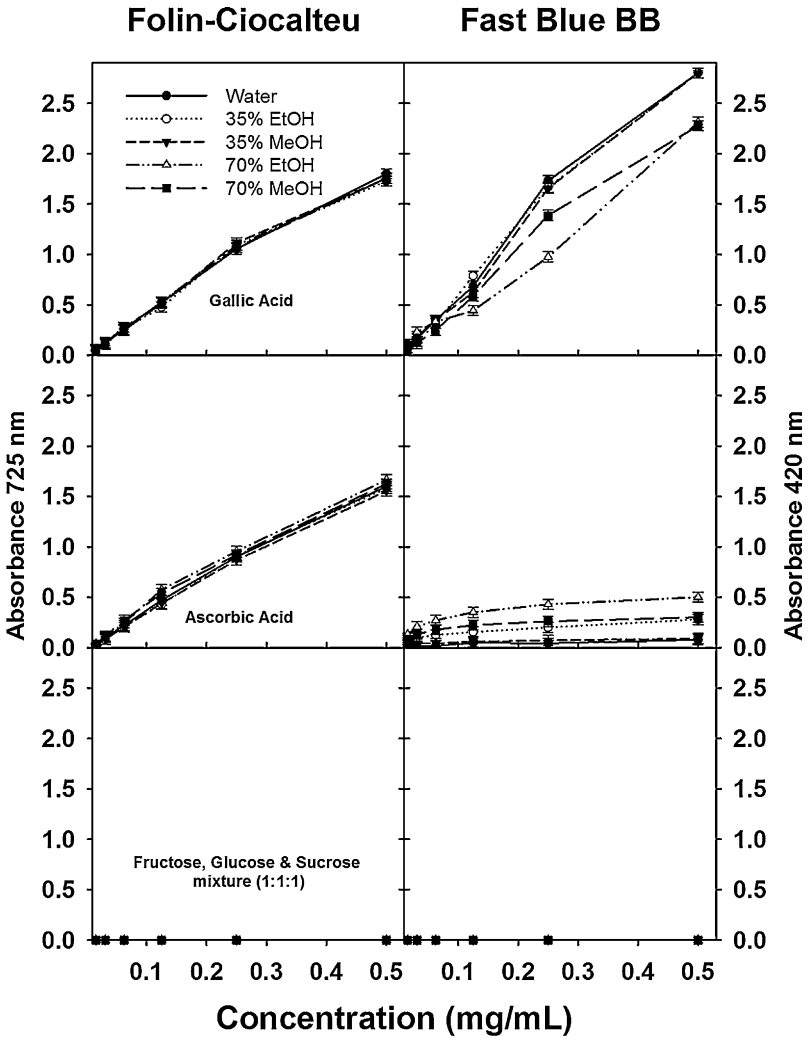

Fig. 1. Folin–Ciocalteu and Fast Blue BB detection of gallic acid, and of total phenolic-assay interfering substances: ascorbic acid and solutions of fruit sugars (fructose, glucose and sucrose, 1:1:1 mg/mL) in water, 35% and 70% methanol (MeOH) and 35% and 70% ethanol (EtOH).

berries, respectively. Relatively high DAsA concentrations are an indicator of stress (Lester et al., 2010) and the higher DAsA concentrations preceding the 1st vs. 2nd harvests are likely due to the higher temperatures and humidities preceding the 1st vs. 2nd harvests (meteorological data not shown).

Stress is also a causal factor in heightened total phenolic concentrations in plant tissues (Reyes et al., 2004). Total phenolic concentrations in strawberries determined by HPLC were higher in the 1st vs. 2nd harvest berries (Table 3). The FBBB assay, which directly detects phenolic substances (Medina, 2011a, b), proved to be a more accurate measure of strawberry fruit total phenolics than the F–C assay. Although F–C total phenolic concentrations were similar to those reported previously for strawberry fruits (Aaby et al., 2005; Klopotek et al., 2005; Medina, 2011a; Panico et al., 2009; Shin et al., 2007; Tulipani et al., 2008), these values were lower in the same fruit when compared to total phenolics assayed via FBBB assay. When FBBB total phenolics were correlated with total phenolics via HPLC the factor ($r = 0.80$) was significant ($P \leq 0.001$) whereas F–C was not significantly correlated ($r = 0.22$) with total phenolic via HPLC. It is unclear what total phenolic data via F–C means as this method had a significant positive linear response to ascorbic acid ($r = 0.98$) and gallic acid ($r = 0.99$) standards whereas FBBB only gave a liner response to gallic acid ($r = 0.99$) (Fig. 1).

Ascorbic acid is a reducing compound (non-phenolic antioxidant), and a natural component of almost all fruits, especially strawberries (Table 2), and vegetables and it reduces the F–C reagent (polyphosphotungstate-molybdate) to form a blue color in alkaline pH (Singleton et al., 1999). As a result the F–C assay had a significant correlation ($r = 0.64$; $P = 0.05$) with strawberry fruit total AsA; whereas FBBB had no correlation with berry total AsA. However, the FBBB method responded to specific, non phenolic alcohol moieties as it reacted to 35% and 70% EtOH and to 70% MeOH extraction solvents, all having higher absorbencies than corresponding 35% MeOH, which responded the same as water (Fig. 1).

Cicco et al. (2009) and Cicco and Lattanzio (2011) were the first to describe the interference of alcohol in F–C reaction mixtures. They recommended that final reaction mixtures not exceed 4% alcohol by volume, although Singleton et al. (1999) suggested the F–C reaction mixture not exceed 1% alcohol by volume. Cicco and Lattanzio (2011) determined that as alcohol concentration rises beyond 4%, the degree of saturation of the solute in the reaction mixtures decreases reducing the medium dielectric property affecting the development of color. FBBB appeared to be more affected by alcohol interference than F–C at 70% EtOH and MeOH and in some cases at 35% EtOH, but not at 35% MeOH, as shown by absorbance differences compared to water (Fig. 1).

The FBBB reaction with substrate gallic acid was highly linear, and was not affected in reaction mixtures of water, 35% EtOH or MeOH, but was affected by 70% EtOH and MeOH. However in the presences of fruit sugars, no alcohol or fruit sugar substrate interaction occurred. Neither F–C nor FBBB gave a response to sugar (fructose, glucose and sucrose) standards; which is not surprising as sugars are reported to interfere with the F–C method only when heated (Slinkard and Singleton, 1977). From our comparison results, it would appear that the aforementioned total phenolic findings for strawberry via the indirect detection F–C assay underestimated the concentration by as much as 2.9 fold vs. the direct detection FBBB assay (Table 2).

4. Conclusion

Our results indicate that the FBBB assay provides a higher and more accurate estimate of total phenolics due to its direct reaction with phenolics in strawberry fruits, than the current indirect total phenolics F–C assay. Previous studies of strawberry fruit, using the F–C assay, have greatly underestimated the total phenolic concentration, and this assay should be replaced in future studies by the Fast Blue BB assay.

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