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Simple and Rapid Method for the Analysis of Phenolic Compounds in Beverages and Grains

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Supporting Information

ABSTRACT: A new method for the detection of phenolics in food systems was developed. This method is based on interactions of phenolics with Fast Blue BB diazonium salt in alkali pH, forming azo complexes, with the absorbance measured at 420 nm after 60 min. The linear regression correlations (R^2) of gallic acid calibration standards were >0.99. The phenolic content (gallic acid equivalent) of samples analyzed yielded higher ratios (1.7−6.6) of the total phenolics by Fast Blue BB to Folin−Ciocalteu methods in most beverages and grain samples, but in flaxseed and some juice blends, the ratios were <1. The lower ratios suggest the presence of non-phenolic reducing constituents measured with the Folin−Ciocalteu method as "total phenolics". This method is simple and inexpensive and can be used to rapidly assess the total phenolics of foods and beverages.

KEYWORDS: Total phenols, polyphenols, diazonium, Fast Blue BB, Folin−Ciocalteu, beverages, tea, grains

INTRODUCTION

Polyphenolic compounds are ubiquitous in the plant kingdom. These compounds contribute to antioxidant properties of food, juices, and beverages, where these phytochemicals have been found to have some preventive and disease-fighting properties. The phyto-polyphenols include the flavonoids (flavones, flavonols, flavans, flavanones, isoflavones, catechins, chalcones, anthocyanins, and anthocyanidins), chlorogenic acid isomers, cinnamic and benzoic families, phenolic acids, coumarins, stilbenes, lignans and lignins, tannins, and tocopherols and tocotrienols. These classes and their structures have been previously described.[1−3] These compounds have been identified in fruits, vegetables, grains, nuts, oil seeds, herbs, and other plant materials.

The Folin−Ciocalteu method has been used to measure total phenolics in natural products using heteropolyphosphotungstate−molybdate for determination of protein content through its tyrosine group.[4] This method was improved by adding a higher proportion of molybdate and lithium sulfate to prevent precipitation, and this modification yielded higher sensitivity and reproducibility.[5] The basic mechanism is an oxidation/reduction interaction contributed by the reducing properties of phenols, other non-phenolic reducing agents, and possibly metal chelators. The quantification basis of this method is the oxidizability of the phenolic compounds. The oxygen uptake by the phenolate ion is rapid near or above the pK of phenolate (around pH 10). Thus, the reaction at alkaline pH was used for the total phenol assay.[6] Typically Na_2CO_3 is added to the Folin−Ciocalteu assay, where a blue color is produced with the reducing compounds and the optical density (OD) was measured at 725−765 nm. The concentration of each sample was determined from the linear regression curve of gallic acid (GA) or other phenolic standards. The interferences with the "total phenols" measurement were contributed by non-phenolic antioxidants and reducing substances, such as ascorbic acid, glucose, fructose, and sulfites, that are common food additives or are naturally present in juices, fruits, and vegetables. Aromatic amino acids (tyrosine and tryptophan) and proteins containing these amino acids also formed a blue color with the phosphomolybdic−phosphotungstic reagent. The Folin phenol reagent has been used for the determination of tyrosine and tryptopohan[5] and for quantitative analysis of proteins.[7−9]

Other methods for total phenol determination were the Prussian Blue assay based on ferrous colorimetry[10] and the vanillin assay[11] for tannins. Permanganate was also used to determine the phenolic antioxidant additives in edible oils[12] and ultraviolet (UV) absorbance. The UV absorbance method resulted in interferences from other compounds with the same wavelength as the phenolic compounds, which also displayed varying absorbance maxima.[6] Various methods of analysis of phenolic compounds were reviewed.[13] Numerous chromatographic methods have been developed for isolation, separation, and identification of the phenolic compounds.

There is a need for a simple, rapid, and direct detection of phenolics or polyphenols in foods, beverages, and agricultural byproducts. The existing standard method (Folin−Ciocalteu) indirectly measures the "total phenolics" through the reducing capacity of components of food or beverage samples. In contrast, the proposed method in this study is based on the coupling of phenolic compounds in food and beverage samples with diazonium salts, resulting in the formation of azo complexes. Previous studies showed that the Fast Dark Blue-R-salt diazonium compound was used to detect growth-promoting hormones.[14−17] In our laboratory, we developed a solid-phase cleanup and thin-layer chromatographic (TLC) method for detection of veterinary drugs, estradiol, diethylstibesterol (DES), and zeranol.[18,19] These phenolic estrogens were detected with Fast Corinth V at a basic pH by exposing the TLC surface to ammonia vapor

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followed by heating of the TLC plates. Fast Violet B salt was also used as a spray to detect zearalenone mycotoxin.[20] Other applications included detection of phenolics in cannabis with azo-forming dyes[21] and detection of phenolic constituents of plant cell walls.[22]

The objectives of this research were to evaluate the optical properties of the Fast Blue BB interaction with GA, optimize the type and concentration of the alkali used, optimize the time to complete the reaction, and compare the results of the total polyphenol analysis with the Folin−Ciocalteu method[6,25−27] in beverages and grain samples.

MATERIALS AND METHODS

Reagents and Equipment. Fast Blue BB (4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi[zinc chloride] salt), Fast Corinth V (4-methyl-6-nitro-4-[(3-methyl-6-methoxyphenyl)azo]-benzenediazonium hemi[zinc chloride] salt), Fast Corinth V salt (Azoic Diazo Component 39), Folin−Ciocalteu's phenol reagent (2 N), and GA were from Sigma-Aldrich (St. Louis, MO). Borosilicate culture tubes (12 × 75 mm) and IKA Ultra-Turrax T25 homogenizer and Sorvall Legend RT centrifuge were from Thermo-Fisher Scientific (Pittsburgh, PA). The Biotek Synergy HT Multilabel Plate Reader was from Biotek (Winooski, VT). The Tomy MTX50 centrifuge was from Peninsula Laboratories, Inc. (Belmont, CA), and the Titer Plate Shaker was from Lab-line Instruments, Inc. (Melrose Park, IL).

Total Phenolic Assay by the Folin−Ciocalteu Method. The procedures for measuring total phenolics[6,25−27] were evaluated. The more recent procedures[26,27] were adapted and modified by reducing the reagent volumes. The miniaturized assays were designated as Folin−Ciocalteu-A (FC-A)[26] and Folin−Ciocalteu-B (FC-B).[27] These procedures were evaluated with GA at 0, 10, 25, 50, 100, 200, 250, and 500 μg/mL (ppm). In the FC-A procedure, 0.9 mL of deionized water (DI H_2O) was transferred to 12 × 75 mm borosilicate tubes, followed by 100 μL of GA standard, samples, and DI water for blank, and mixed. The Folin−Ciocalteu reagent (100 μL) was added, mixed, and allowed to react for 5 min before adding 1 mL of 7% Na_2CO_3, followed by the addition of 400 μL of DI H_2O. The mixture was allowed to react for 90 min at room temperature, and 200 μL aliquots were transferred to microtiter plates. The absorbance was measured by the Biotek microplate reader at 725, 750, and 760 nm. The FC-B miniaturized procedure was derived using $^1/_{10}$ of the original reagent and sample volumes. This modified FC-B procedure was comprised of transferring 50 μL of GA standard or sample into the borosilicate tubes, followed by the addition of 430 μL of DI H_2O and 20 μL of FC reagent, and mixed well. Saturated Na_2CO_3 (50 μL) was added and mixed, and finally, 450 μL of DI H_2O was added. The mixtures were allowed to react for 1 h, and 200 μL aliquots were transferred to microtiter plates. The ODs were measured at 725, 750, and 765 nm.

Fast Blue BB Method A. Using a 100 ppm (μg/mL) GA concentration, the spectrophotometric scan (200−800 nm) of the Fast Blue BB and GA interaction was determined. GA (0.1 mL of 100 ppm) was transferred to borosilicate tubes, followed by the addition of 0.1 mL of Fast Blue BB (0.05 or 0.025%), and mixed for 1 min. Saturated Na_2CO_3 (0.1 mL) was added, and the absorbance was scanned at 0, 10, 15, and 30 min reaction times. The mixtures were also acidified or neutralized with 0.1 mL of 0.1 N HCl, and its effects were compared to the basic mixture.

Fast Blue BB Method B. GA standards (1 mL) with concentrations of 0, 10, 50, 100, 200, 250, and 500 ppm were transferred to borosilicate tubes and 0.1 mL of 0.01% Fast Blue BB. The samples were mixed for 1 min, followed by the addition of 0.1 mL of 7% Na_2CO_3, and the OD was measured at 0, 10, 15, 20, 30, and 60 min. The absorbance was measured at 310 and 420 nm. A total of 100 μL of 0.1 M HCl was added to neutralize the mixtures, followed by heating at 5 min (60 °C). The method was evaluated with 1 mL of juice (1:1 dilution) and coffee (1:10 dilution) and with the addition of 100 μL of Na_2CO_3 (7 and 10% and saturated solution). The pH of the mixture and the OD were determined at 300 and 420 nm.

Fast Blue BB Method C. A total of 1 mL of GA standards (0, 10, 50, 100, 250, and 500 ppm) were transferred to borosilicate tubes, and a total of 0.1 mL of 0.1% Fast Blue BB dissolved in water or ethanol was added to determine the solvent effects. Samples were mixed, and 0.1 mL of 20% Na_2CO_3 was added. The samples were kept at room temperature, and the OD was measured at 420, 500, and 520 nm at 0, 30, and 60 min.

Beverage and Juice Sample Analysis. Tea samples were prepared by immersing one tea bag in 200 mL of boiling water for 5 min. The coffee samples were brewed according to package directions. The juices with haze or solid suspension were homogenized with the Ultraturrax, followed by either filtration through a polypropylene wool or centrifuged at 6000 rpm (2790 rcf) for 10 min. The purpose of the analysis is to compare the total phenolic results of the Folin−Ciocalteu and the new Fast Blue BB diazonium methods. The samples were analyzed with appropriate dilutions from 1:1 to 1:50. The optimum dilutions selected for the samples were those that resulted within the range of 50−400 ppm GA equivalent (GAE). The total phenolics were measured with the Folin−Ciocalteu method (method B) and the Fast Blue BB diazonium method (method C), using 20% Na_2CO_3 and 5% NaOH, respectively. The OD was measured at 750 nm with the FC-B method and 420 nm with the Fast Blue BB method.

Intra- and interassay reproducibility was determined by analyzing 1 mL of brewed black tea, Ceylon tea, and herbal tea. To 1 mL samples, 0.1% Fast Blue BB (diluted in dH_2O) and 0.1 mL of NaOH were mixed and the reaction was allowed to complete at room temperature. Aliquots of 200 μL were transferred to microwells, and the OD was measured after 60 min at 420 nm. The intra-assay variability was determined in five replicate samples, and the interassay variability was determined in three trials.

Grain Samples. Barley, wheat, and buckwheat samples were pulverized using a coffee grinder. The metal cup was filled half full, and the samples were ground intermittently for 45−60 s to a fine powder. The powder was then transferred to sealed containers and stored at room temperature and in −80 °C for longer storage. Aliquots of 10 g were transferred to a flask with a ground glass top and extracted with 100 mL of 70% ethanol. Samples were extracted for 1 and 24 h over a rotary shaker, and aliquots of the extracts were centrifuged at 4000 rpm (2500 rcf). The phenolics in the supernatant were measured with the Fast Blue BB method (method C) and the Folin−Ciocalteu method (method B).

Additional grains (white rice, black rice, and quinoa) and flaxseed were also analyzed for total phenols by the Folin−Ciocalteu method (method B) and the Fast Blue BB method (method C). Quinoa is from a species of goosefoot (*Chenopodium*). Black rice is a variety of *Oryza sativa*, and flaxseed is *Linum usitatissimum*. The grains and flaxseed were pulverized in a coffee grinder, as described in a previous paragraph. A 10 g aliquot was extracted with 100 mL of 70% ethanol using the mircoplate shaker. Aliquots (25 mL) were taken out at 30 and 60 min and 24 and 48 h. The sample mixtures were centrifuged at 4000 rpm (3700 rcf), and the supernatants were analyzed at 1:2, 1:5, or 1:10 dilutions with dH_2O. The phenolics in the supernatants were quantified with the Fast Blue BB method (method C) and the Folin−Ciocalteu method (method B), and the ODs were measured after a 60 min reaction time at room temperature.

In both grain samples, total phenolics were expressed as mg of GAE/100 g and were determined from the GA calibration standards (0 and 10−500 ppm). The GAE values of the diluted samples were multiplied with the dilution factor, and the values derived were μg/mL original extract. GAE from samples were corrected with values of the blank. The correction with blank samples, particularly with beverages, can eliminate non-phenolic constituents that may have an OD at or near 420 nm. Each 1 mL extract was equivalent to 0.1 g of grain sample. Therefore, μg/mL extract × 10 = μg/g of sample, and then, divided by 10 = mg of GAE/100 g.

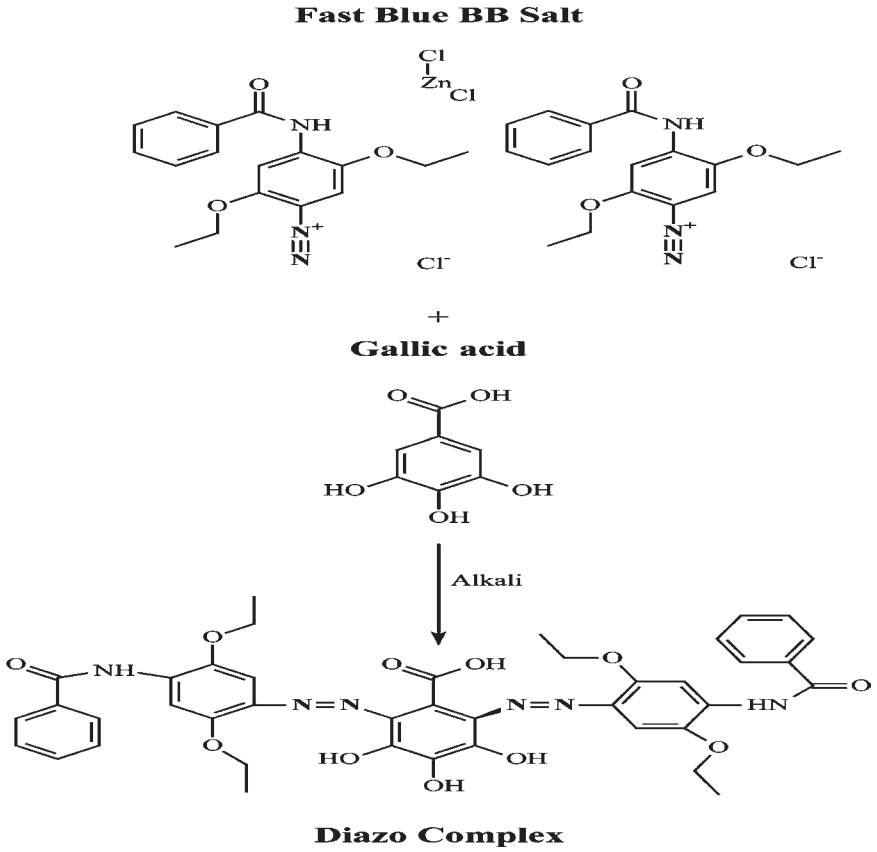

Figure 1. Proposed interaction of GA and Fast Blue BB diazonium salt.

RESULTS AND DISCUSSION

The Folin−Ciocalteu procedures[26,27] were miniaturized for total phenols analysis in this study. The concentration versus absorbance had linear responses from three trials of the FC-A and FC-B miniaturized procedures. The mean regression correlations were R^2 = 0.9981 [0.0015 standard deviation (SD)] for FC-A and R^2 = 0.9973 (0.0009 SD) for FC-B when measured at 725, 750, and 765 nm. The results indicated no difference in OD in these wavelengths. The tea and coffee samples were analyzed with these procedures, and results showed that total phenolics (GAE) were higher in method A than method B but the percent relative standard deviation (% RSD) values in both methods were 0.1−1%. In the FC-A procedure, the reaction was completed in 90 min, as compared to 60 min with the FC-B procedure. The FC-B procedure and 750 nm wavelength were adapted for the subsequent sample analysis.

Fast Blue BB Diazonium Method. In developing an assay for detection and quantification of total phenolics in food and beverages, the proposed chemical method used the coupling property of a diazonium salt with phenolic compounds forming azo compounds. The absorbance of the resulting azo compounds was measured with a spectrophotometer. Aromatic diazonium ions normally couple with active substrates, such as phenols.[23,24] The diazonium (−N_2^+−) compound attacks the electron reactive group (−OH) of the phenols, releasing a proton, resulting in an electrophilic aromatic substitution. The nitrogen of the diazonium group is retained during this coupling. Phenols are coupled in mildly alkaline solution, where they are converted to the more active phenoxide ions. Coupling primarily occurs *para* to the phenolic activating group; however, if the *para* position is already occupied, the substitution occurs in the *ortho* position to the activating −OH group. In this study, the diazonium group of the Fast Blue BB couples with the −OH of the phenolic groups of GA, resulting in the formation of the gallo−diazo complex. The proposed mechanism for the interaction of GA and Fast Blue BB is shown in Figure 1. Similarly, the proposed mechanism for the interaction of Fast Corinth V and the phenolic group of zeranol, a resorcylic compound, was reported.[19]

Screening of diazonium compounds. Several compounds were tested for their interactions with GA (results not presented). Fast Blue BB and Fast Corinth V were selected for optimization of the diazonium interaction with GA. Fast Blue BB yielded better results than Fast Corinth V (results not presented).

Optimum Wavelength Selection. Using Fast Blue BB method A, the wavelength maxima of the Fast Blue BB with GA were observed at the UV range of 300 and 320 nm, while secondary peaks were also observed at 370 and 420 nm. Heating the mixtures for 2 and 5 min yielded the same results, but heating of samples were not applied in subsequent analysis. The absorbance values at zero time showed the highest OD, and the results showed a nonlinear response at zero time (R^2 = 0.8702); however, the absorbance values decreased with a longer reaction time. Linearity improved in 10, 15, 20, and 30 min with R^2 = 0.9459, 0.9758, 0.9837 and 0.9888, respectively. With the addition of 0.1 mL of 0.1 N HCl, linearity improved from 0, 10, 15, 20, and 30 min at R^2 = 0.9152, 0.9940, 0.9983, and 0.9995, respectively. With half of the concentration (50 μL) of HCl, R^2 was lower at 0.8381, 0.9806, 0.9860, 0.9923, and 0.9977. Use of Na_2CO_3 alone yielded higher absorbance values than with the addition of HCl, suggesting that the reaction favored alkaline conditions.

Table 1. Total Phenolics (μg/mL GA) by Fast Blue BB (Method B) in Coffee and Fruit Juice[a]

samples	10 min	15 min	20 min	30 min	60 min
coffee	3100	2470	2180	1730	1120
	2856		2130	1830	na[b]
juice	207	154	110	125	120
R^2 GA standard	0.9634	0.9817	0.9875	0.9948	0.9977

[a] Procedure: 1 mL samples of 0.1 mL of 0.01% Fast Blue BB and 0.1 mL of 7% Na_2CO_3. Coffee was analyzed at 1:10 dilution, and cranberry/grape juice was analyzed at 1:1 dilution. [b] na = not analyzed.

The OD of all samples was also measured at 300, 310, 420, and 450 nm. Absorbance at 300 and 310 nm generated a high OD, but the background signals with the blank and zero samples were also high, resulting in nearly 10% of the OD of the maximum dose of 500 ppm. The OD values at 420 were 50% greater than at 450 nm. Using the Fast Blue BB method A, the phenolic values at different reaction times are shown in Table 1. Linearity improved from 0 to 60 min reaction time, and lower linearity (R^2) indicated an incomplete reaction between GA and the Fast Blue BB diazonium compound.

Various concentrations of Na_2CO_3 (0, 7, 10, 15, and 20% and saturated solutions) were added to juice samples (1 mL) using the Fast Blue BB method B. The resulting pH values were 2.6, 9.38, 10.08, 10.31, 10.44, and 10.47, respectively. The saturated sodium carbonate compared to 7 and 10% yielded the highest pH (10.5), and the highest absorbance was also obtained at 420 nm. A 20% sodium carbonate was used in subsequent analysis because of effects of room temperature in the preparation of the saturated sodium carbonate. The carbonate concentration can vary at differing room temperatures. At lower room temperature, a salting out may occur, while a higher concentration is soluble at a higher temperature.

To further refine the method, a higher concentration of Fast Blue BB was studied. In panels A and B of Figure 2, the interaction of 0.1% Fast Blue BB in ethanol or water (0.1 mL) and 20% Na_2CO_3 were further compared at 420, 500, and 520 nm. ODs were higher at 420 nm at 30 and 60 min, with regression correlations (R^2) greater than 0.99 achieved in both water and ethanol dilutions and with RSD values below 10%. The ODs at 0 and 10 ppm were similar at 500 or 520 nm, and the phenolics at 10 ppm were not detectable, indicating lower detection sensitivity at these wavelengths.

The interactions of Fast Blue BB with various concentrations of Na_2CO_3 and NaOH (5, 10, and 20%) were studied with the absorbance measured at 420, 500, and 520 nm at 30 and 60 min. The use of 20% NaOH and 20% Na_2CO_3 was compared to Fast Blue BB dissolved in water, and the use of NaOH resulted in high regression correlations ($R^2 > 0.99$). OD values at 500 and 520 nm were similar, but the slopes were lower than at 420 nm. The values at zero had background interference, but 10 ppm GA was detectable at 500 and 520 nm. Fast Blue BB dissolved in ethanol yielded slightly better linearity (R^2) in all wavelengths, but the OD of the zero concentration was near the absorbance values of 10 ppm GA. The regression correlations (R^2) of the interactions between the Fast Blue BB and the GA standards were determined from the concentrations of 0, 10, 50, 100, 250, and 500 ppm. The results showed an improvement of the R^2 values from 30 to 60 min reaction times, except for 20% Na_2CO_3, where the increase in the R^2 values was shown with GA dissolved in ethanol. However, these differences may not be significant because the range of R^2 at 1 SD was 0.9903−0.9979 at 420 nm and 0.9948−0.9989 at 520 nm. The confidence limit (at 1 SD) of the calibration lines at 420 nm and 60 min was

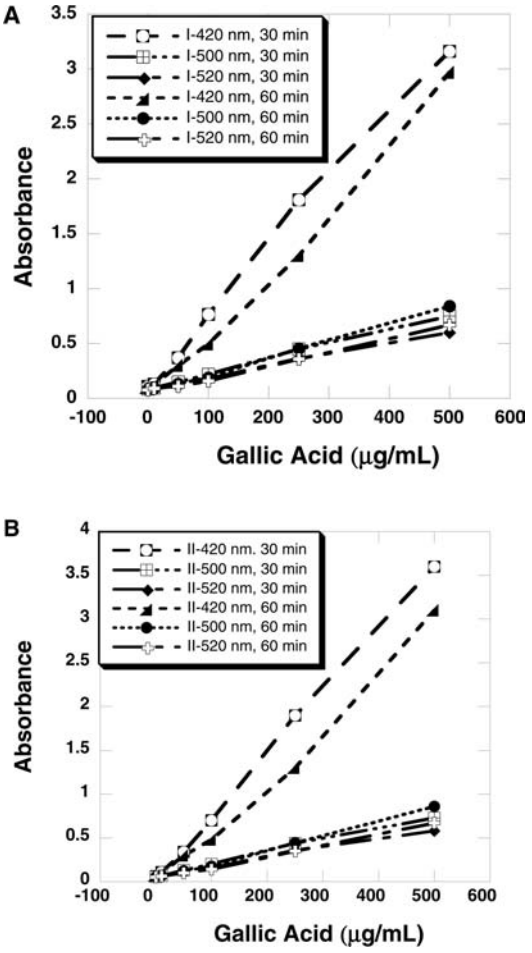

Figure 2. Absorbance of the Fast Blue BB interaction with 0, 10, 50, 100, 250, and 500 μg/mL GA. A 0.1% Fast Blue BB in (A) DI water and (B) ethanol was used, and the OD was measured at 420, 500, and 520 nm wavelength in 30 and 60 min. The top lines are absorbance values at 420 nm wavelength.

0.9927−0.9996. All regression correlations for all concentrations of NaOH were within the confidence limits. The highest R^2 values were obtained with 5% NaOH at 420 nm. Because the results with Fast Blue BB dissolved in water and ethanol were similar, either ethanol or water was used as a solvent in subsequent sample analysis. Either 5% NaOH or 20% Na_2CO_3 with an absorbance reading at 420 nm was used for subsequent sample analysis. However, better linearity was obtained with 5% NaOH at 60 min, while equal linearity was obtained at 90 min with Na_2CO_3.

Sample Analysis. The results from the analysis of various beverages are shown in Table 2. The tea samples indicated about 2−3 times higher phenolic content with the Fast Blue BB method than with the Folin−Ciocalteu method. The herbal teas had lower phenolic values than the black, oolong, and green teas, but the ratios of Fast Blue BB to Folin−Ciocalteu methods were 1.8−2.6. The phenolic values of the brewed tea were not normalized for the weight of tea in the tea bags, but each tea bag was infused in 200 mL of boiling water. The ready to drink (RTD) teas had slightly higher phenolic values than the brewed teas, yielding 6 times higher ratios of Fast Blue BB phenolics to Folin−Ciocalteu total phenolics. Freshly brewed coffee contained higher phenolics than brewed tea samples, with a total phenolic ratio (Fast Blue BB/FC-B) of 2.6. Because other antioxidant vitamins are natu-

Table 2. Total Polyphenolic Content of Beverages Measured by Fast Blue BB (FBBB) and Folin-Ciocalteu (FC-B) Methods[a]

beverage	FBBB method (mg of GAE/100 mL) (*n*)	% RSD	FC-B method (mg of GAE/100 mL) (*n*)	% RSD	ratio of FBBB/FC-B
		Black Teas			
sample 1	185 (4)	6	62.25 (4)	6	3.0
sample 2	185 (2)	2	89.00 (2)	14	2.0
		Green Teas			
green (y)	152 (4)	7	55.68 (5)	7	2.7
jasmine green (y)	180 (4)	8	72.16 (5)	6	2.5
		Oolong Teas			
oolong (y)	176 (4)	8	70.84 (5)	12	2.5
		Herbal Teas			
chamomile	12.5 (2)	10	6.00 (4)	3	2.1
ginger spice	16.5 (2)	5	12.55 (4)	11	1.8
lemon zinger	58.2 (2)	5	30.00 (2)	5	1.9
pomegranate	135 (5)	16	50.76 (5)	13	2.6
diabetic	62.6 (2)	3	27.50 (2)	1	2.3
		Coffee			
coffee (g)	389 (4)	0.06	147 (4)	2.8	2.6
coffee (s)	365 (4)	6.6	142 (4)	4.6	2.6
		Ready To Drink Teas			
iced tea	197 (3)	4.7	34 (3)	4.1	5.8
white tea	210 (3)	4.5	35 (3)	5.3	6
green tea	273 (3)	5.9	46 (3)	4.1	5.9
		Juices			
blueberry/kiwi	34 (6)	5	30 (6)	4	1.1
cranberry (v)	45 (4)	8	73 (4)	3	0.6
cranberry/grape	149 (6)	4	83 (6)	3	1.8
goji punch	13 (6)	12	8 (6)	5	1.6
orange, natural	20 (2)	13	70 (3)	14	0.29
pomegranate, organic	240 (4)	4	85 (4)	3	2.8
pomegranate/blueberry	637 (4)	4	156 (4)	4	4.0
pomegranate/blueberry, organic	227 (3)	9.2	77 (3)	5	2.9
pomegranate/cherry	419 (2)	1	194 (2)	8	2.2
malt	86 (2)	2.3	80 (2)	9	1.1
tamarind nectar	29 (4)	16	14 (4)	8	2.1
vitamin water, energy	6.9 (4)	2	42 (4)	5	0.16
vitamin water	5.3 (4)	12.5	38 (4)	1.1	0.14
white cranberry peach	10 (4)	1.7	25 (4)	1.5	0.4

[a] Polyphenols measured as GAE per 100 mL. Each value is the mean of multiple analyses (*n*).

rally present or added to juices, the Folin−Ciocalteu phenolic values were higher than the Fast Blue BB values, wherein this method detects only the polyphenolic compounds. Reducing sugars naturally present or added to juice mixes also contribute to higher Folin−Ciocalteu values. Pomegranate juice mixtures had a 3−4 ratio, but some juice or juice blends and fortified water had <1 GAE ratio. Ratios below 1 indicate the presence of a higher concentration of non-phenolic reducing constituents, as detected by the Folin−Ciocalteu method.

Grain Sample Analysis. To determine the application of the Fast Blue BB method with solid samples, barley, wheat, and buckwheat samples were analyzed. Grain extraction for 24 h had higher results than with 1 h of extraction, and results with 24 h of extraction are shown in Table 3. The buckwheat samples yielded the highest polyphenolic content (GAE) as analyzed by Fast Blue BB and FC-B methods. The GAE values of the grain polyphenols had approximately 1.7−2.4 ratios with Fast Blue BB/FC-B methods. Because of higher polyphenol levels with the Fast Blue BB method, the number of reported analyses (*n*) were at times lower than the number of analyses with the FC-B method. At varying sample dilutions, the total phenolic values were outside the reliable concentration (<50 and >500 ppm GA) of the standard curve. These grain samples were from different sources, and therefore, the processing effects on wheat and barley may not be compared in this analysis.

Total phenolic analysis of white rice, black rice, white quinoa, and flaxseed with Fast Blue BB and FC-B methods are shown in Table 4. Extraction efficiency indicates higher phenolic content

(mg of GAE/100 g of grain samples) at 24 and 48 h of extraction versus 30 and 60 min. White rice had no detectable phenolics, while black rice had the highest total phenolics at 1596 and 1568 mg/100 g at 24 and 48 h of extraction, respectively. Flaxseed had lower Fast Blue BB phenolics than FC-B reducing phenolic values, resulting in a Fast Blue BB/FC-B ratio of <1. These results suggest that flaxseed had higher non-phenolic antioxidant constituents than quinoa and black rice. Black rice had a Fast Blue BB/FC-B ratio of 4−4.8 in 1, 24, and 48 h extracts. The quinoa had a Fast Blue BB phenolic content of 439−476 mg/100 g, but the FC-B GAE values had high variability, particularly at levels below 100 mg/100 g of sample. This suggests that the non-phenolic antioxidants (reducing properties) in quinoa were labile. The highest Fast Blue BB phenolic levels in grains and flaxseeds were at 24 and 48 h of extraction. High % RSD values were also observed at 30 min of extraction. These results suggest that a 1 or 24 h extraction can be used.

This method is a three-step procedure, transferring the samples to tubes, adding the Fast Blue BB diazonium compound and alkali (NaOH or Na_2CO_3), and measuring the absorbance at 420 nm after 60 min with NaOH and 90 min with Na_2CO_3. This method was used to measure the polyphenols in beverages and grains. The results showed that the Fast Blue BB diazonium assay resulted in higher GAE values than the standard Fast Blue BB for "total phenolics". The total phenolic content ratio (Fast Blue BB/FC-B) was higher in brewed tea and coffee at a 2−3 ratio, but in RTD tea, the ratio was around 6. The total phenolic ratios ranged from 0.1 to 4 in juices and juice drinks, from 1.7 to 2.4 in barley, wheat, and buckwheat, 4.8 in black rice, 5.8 in quinoa, and 0.94 in

Table 3. Total Phenolics in Grains Analyzed with Folin-Ciocalteu (FC-B) and Fast Blue BB (FBBB) azo Method[a]

sample	FBBB method (mg of GAE/100 g) (*n*)	% RSD	FC-B (mg of GAE/100 g) (*n*)	% RSD	ratio of FBBB/FC-B[b]
hulless barley	359 (2)	11	174 (3)	8.7	2
whole barley	348 (2)	13.6	147 (3)	11	2.4
peeled barley	199 (2)	10	83 (3)	17	2.4
hulled wheat	95 (2)	5.2	48 (3)	8	2
peeled wheat	202 (2)	12	126 (3)	4	1.7
buckwheat kernel	699 (5)	2.5	345 (3)	4.1	2

[a] Samples were extracted for 24 h. Total phenolics expressed as GAE values were determined at 60 min reaction times with Fast Blue BB and FC-B methods. [b] Ratios of GAE values by Fast Blue BB/FC-B methods.

Table 4. Total Phenolics (mg/100 g) in Grains Extracted at 30 and 60 min and 24 and 48 h Measured by Fast Blue BB (FBBB) Method C and Folin-Ciocalteu (FC-B)[a]

samples	30 min (*n* = 3)	% RSD	60 min (*n* = 3)	% RSD	24 h (*n* = 2)	% RSD	48 h (*n* = 2)	% RSD
				White Rice				
FBBB	0		0		0		0	0
FC	0		0		0		0	
				Black Rice				
FBBB 1:5	1015	1.9	1328	3.8	1372	2.8	1375	2.9
FBBB 1:10	1067	0.5	1530	6.8	1820	4.3	1760	8.0
mean FBBB	1041		1429		1596		1568	
FC-B 1:5	222	8.1	368	5.7	375	1.3	392	5.9
FC-B 1:10	120	0	345	13	285	5.3	320	1.6
mean FC	171		356		330		356	
FBBB/FC ratio	6.1		4.0		4.8		4.4	
				Quinoa				
FBBB 1:2	338	16.3	433	2.5	443	8.3	470	2.1
FBBB 1:5	380	1	445	1.1	470	6.4	482	3.7
mean FBBB	359		439		456		476	
FC-B 1:2	92	13	107	11.2	115	15	95	1.1
FC-B 1:5	55	12	92.5	13.5	40	2.5	50	10
mean FC	74		100		78		72.5	
FBBB/FC ratio	2.3		4.4		5.8		6.6	
				Flaxseed				
FBBB 1:2	134	6	138	16.9	238	9.2	243	7
FBBB 1:5	185	4.4	205	12.7	268	3.1	240	14.6
mean FBBB	160		172		253		242	
FC-B 1:2	191	11	289	7.6	274	3.5	325	2.1
FC-B 1:5	142	18	272	4.8	265	7.8	325	7.7
mean FC	166		280		270		325	
FBBB/FC ratio	0.96		0.61		0.94		0.74	

[a] % RSD represents inter-assay variability. With both methods, the ODs were measured after a 60 min reaction at room temperature. GA phenolic values below 100 tend to have high variability because these concentrations are near the lower limits of the calibration standards. Each value represents μg/mL extract = mg of GA/100 g of sample. Each 1 mL extract was equivalent to 0.1 g of grain sample.

flaxseed samples extracted for 24 h. The FC-B method detects other non-phenolic reducing compounds, and their presence reduced the Fast Blue BB/FC-B ratios. The Fast Blue BB values may be useful for measuring "total phenolics" in raw and processed foods and may be used for labeling the phenolic content of products. This novel method uses an inexpensive and stable reagent, costing less than 1 cent per test. An assay of 20 samples may be completed in 2 h. The Fast Blue BB method may also be automated for high-throughput analysis. Future studies will include identification of the phenolic−azo reaction products, analysis of other food systems, effects of processing, and storage on polyphenols.

■ ASSOCIATED CONTENT

Supporting Information. Effects of pH and the Na_2CO_3 concentration on absorbance values of Fast Blue BB (Table S1) and regression correlation values (R^2) of the Fast Blue BB interaction with GA in various alkali concentrations at 30 and 60 min (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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