Comparison of Common Analytical Methods for the Quantification of Total Polyphenols and Flavanols in Fruit Juices and Ciders

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Abstract: Multiple analytical methods are used for quantification of total polyphenols and total flavanols in fruit juices and beverages. Four methods were evaluated in this study: Folin-Ciocalteu (F-C), Lowenthal permanganate (L-P), 4-dimethylaminocinnamaldehyde (DMAC), and the bovine serum albumin (BSA) precipitation method. Method validation parameters, including working range, limit of detection, limit of quantitation, precision (repeatability), accuracy, and specificity, were assessed and compared. The F-C method was not specific to polyphenols, and the L-P method had the widest working range but lacked accuracy. The DMAC method was the most specific to flavanols, and the BSA method was not suitable for quantification of smaller flavanols, such as catechin and epicatechin. Quantitative performance was evaluated using commercial fruit juice samples ($n = 14$), apple juice samples of different cultivars ($n = 22$), and commercial ciders ($n = 17$). In general, the L-P titration method and DMAC method resulted in higher quantitative values than the F-C method and BSA precipitation method, respectively. However, ratios of results obtained by the L-P and F-C method ranged from 1 to 28, and ratios of results obtained by the DMAC and BSA precipitation method ranged from <1 to 280. This tremendous variation is likely due to variation in polyphenol composition and sample matrix. This information provides perspective for comparison of results obtained through these different methods, and a basis for choosing the most appropriate analytical method for quantification of polyphenols to address a specific research question when working with commercial fruit juice, apple juice from different apple cultivars, and commercial ciders.

Keywords: BSA precipitation, DMAC, flavanols, Folin-Ciocalteu, Lowenthal permanganate titration

Practical Application: This study compared results obtained when four common polyphenol quantification methods were applied to a diverse selection of fruit juices and beverages with distinct polyphenol composition and sample matrix. The matrix and polyphenol composition of the samples significantly influenced the results. Our findings can help manufacturers of fruit-based products choose the most appropriate analytical method for polyphenol quantification as part of a quality assurance program or to convey information on dietary polyphenol content to consumers. An assessment of analytical method validation parameters is provided for each of the four methods, which will help users of these methods to understand their limitations.

Introduction

Polyphenols in fruit juices and beverages

Polyphenols are secondary metabolites of plants with highly diverse chemical structures. Over 8000 polyphenols of plant origin have been characterized (Pandey & Rizvi, 2009). All polyphenols contain hydroxylated phenyl moieties, often present as glycosides (Weber, Schulze-Kaysers, & Schieber, 2014). Polyphenols are classified into flavonoids, phenolic acids, and other classes, including stilbenes and lignans based on their aglycone structures (Tsao, 2010). Raw fruits and vegetables, such as grapes, apples, and cocoa, and their products, such as wines, cider, and chocolate, are major sources of dietary polyphenols globally (Tsao, 2010). Polyphenol composition in fruits varies greatly depending on the plant species (Pandey & Rizvi, 2009). For example, anthocyanins (a subgroup of flavonoids) are the most predominant class of polyphenols in cranberry, red grape, and pomegranate. For citrus fruits, including lime, lemon, and grapefruit, flavanones (a subgroup of flavonoids) predominate. In American cranberry and prune, the predominant phenolic compounds are benzoic acid and neochlorogenic acid, respectively (both phenolic acids). Chlorogenic acid (a phenolic acid) is prevalent in most apple and blueberry cultivars. Substantial variation in polyphenol composition may also exist within different genotypes of a given species (Anastasiadi et al., 2017; Rothwell et al., 2013).

Flavanols, a subgroup of flavonoids made up of flavanol subunits, impart astringency and bitterness to fruit juices and beverages (Lea & Arnold, 1978). Bitterness and astringency can differ in wines and ciders with the same reported total polyphenol content due to variations in sensory impact among polyphenols (Brossaud, Cheynier, & Noble, 2001; Lea & Arnold, 1978). Several methods for polyphenol quantification have been used as predictors of

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bitterness and/or astringency; however, identifying the most effective method for this purpose remains a topic of current research (Boulet et al., 2016).

Consumption of dietary polyphenols is associated with positive human health outcomes, and polyphenol content is routinely reported in fruit juice and beverage marketing or point-of-sale materials (Sun-Waterhouse, 2011). Fruit producers use a range of analytical methods to quantify polyphenol content for this purpose, including those evaluated in this study (Aleixandre-Tudo, Buica, Nieuwoudt, Aleixandre, & du Toit, 2017).

Analytical methods for polyphenol quantification

Several analytical methods are routinely used to determine a "total" polyphenol value for fruit juices and beverages. The Folin-Ciocalteu (F-C), Lowenthal permanganate (L-P), 4-dimethylaminocinnamaldehyde (DMAC), and bovine serum albumin (BSA) precipitation methods are historically relevant and represent four of the most widely applied methods used in fruit juice and beverage analysis (Weber et al., 2014). Other relevant methods for polyphenol quantification include reading absorbance at 280 nm, precipitation with methyl cellulose, Prussian Blue, Bate-Smith, and Vanillin test (Aleixandre-Tudo et al., 2017). These methods are nonspecific, due to the complexity and expense of quantifying individual polyphenols (Neilson, O'Keefe, & Bolling, 2016).

The F-C method is widely used for quantification of total polyphenols in fruit juices and beverages (Everette et al., 2010). It is based on redox reactions between reducing compounds in the sample, including but not limited to polyphenols, and the F-C reagent. Several compounds, including ascorbic acid, reducing sugars, SO_2 and tyrosine, interfere with the results of the F-C method and are inadvertently quantified as polyphenols (Everette et al., 2010). Nonetheless, this method continues to be widely applied in food science, nutrition science, and horticulture likely due to its ease of use and low cost.

The L-P titration method was commonly applied in apple and cider analysis. While the L-P method has been applied occasionally with pear, peach, tea, and coffee (Barua & Roberts, 1940; Smit, Joslyn, & Lukton, 1955), it has not emerged as a preferred method for products beyond apples and ciders, perhaps due to its reported limitations such as difficulty in visually determining the titration endpoint. The method relies on oxidation of polyphenols by potassium permanganate in the presence of indigo carmine as a "redox indicator." The F-C method is generally preferred over the L-P because of greater accuracy and less interference (Singleton, Orthofer, & Lamuela-Raventós, 1999); however, this hypothesis has not been rigorously tested.

The DMAC method has been used for total flavanol quantification for decades, especially in fruit juice and beverage samples because of the sensory properties imparted by flavanols. The DMAC method relies on the reaction between the DMAC reagent and flavanols, resulting in a spectrophotometrically quantifiable color change. Degree of polymerization (DP) and flavanol linkage in fruit juices and beverages have been reported to influence quantification results (Wang et al., 2016).

The BSA precipitation method can be used to predict the intensity of astringency in wine, thus its common application in wine analysis. Astringency is a tactile sensation evoked by precipitation of proteins in the mouth by polyphenols (Brossaud et al., 2001). The BSA method is based on the assumption that the precipitation of polyphenols and protein is proportional to the concentration of polyphenols in a given sample. Application to predict astringency in other fruit juices and beverages beyond wine using this method has not been validated.

Although the strengths and weaknesses of these methods have been discussed in the literature (Aleixandre-Tudo et al., 2017; Weber et al., 2014), information on the analytical method validation parameters and the quantitative performance of four analytical methods (F-C, L-P, DMAC, and BSA) were not comprehensive. The objective of this study was to determine analytical method validation parameters and assess quantitative performance of four analytical methods (F-C, L-P, DMAC, and BSA) using a broad range of fruit juice and beverage samples to allow assessment of the influence of differences in polyphenol composition and sample matrix among fruit types (for example, berries, citrus, and apples) on quantitative results. We hypothesized that different methods would exhibit different strengths and weaknesses in terms of analytical method validation parameters and that the quantitative results of F-C would differ from L-P, and DMAC would differ from BSA. Furthermore, we expected that the magnitude of the differences would be influenced by polyphenol composition and sample matrix.

Materials and Methods

Chemicals and standards

F-C's phenol reagent, gallic acid (GA), sodium carbonate, procyanidin (PC) B2 (a PC dimer), (+)-catechin, (−)-epicatechin, chlorogenic acid, quercetin, phloretin, indigo carmine, potassium permanganate, DMAC, and sodium oxalate were purchased from Sigma-Aldrich (St. Louis, MO, USA); J. T. Baker Analyzed HPLC Ultra Gradient acetonitrile and formic acid were purchased from Avantor Performance Materials (Center Valley, PA, USA); Analytical standards PC B1 and B5 (a PC dimer), C1 (a PC trimer), and Cinnamtannin A2 (Cinn A2, a PC tetramer) were purchased from Planta Analytica (Danbury, CT, USA); L-ascorbic acid, methanol, ethyl acetate, hydrochloric acid, sulfuric acid, and acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA); BSA Fraction V was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Analytical method validation

Working range. Analytical curves for F-C, L-P, DMAC, and BSA precipitation methods were built using aqueous solutions of GA ($n = 11$; 0 to 1000 mg/L with 100 mg/L interval), aqueous solutions of GA ($n = 13$; 0 to 12 g/L with 1 g/L interval), PC B2 solutions prepared in methanol ($n = 9$; 0, 1, 5, 10, 20, 30, 40, 50, and 60 mg/L), and aqueous solutions of catechin ($n = 9$; 0, 10, 25, 50, 100, 150, 200, 250, and 300 mg/L), respectively. Each concentration of each standard was prepared in triplicate; three measurements were made per solution (Nunes, Alvarenga, de Souza Sant'Ana, Santos, & Granato, 2015). Initial concentration ranges evaluated were identified based on prior reports of linear standard curves. The upper limit of the working range was determined by evaluating increasing concentrations until either (1) the curve became nonlinear as determined through linear regression, (2) maximum solubility of the standard was reached, or (3) absorbance readings ≥1 were obtained. The lower limit of the working range was determined as limit of quantitation (LOQ).

Linear regression analysis was conducted (Microsoft Excel, Redmond, WA, USA) wherein an equation describing the linear relationship of the data and an R^2 value were obtained for each method. ANOVA of the linear regression was conducted to test the significance of the model, defined as $P < 0.05$.

LOD and LOQ. Limit of detection (LOD) and LOQ were calculated as:

$$\text{LOD} = \frac{3.3s}{b}. \quad (1)$$

$$\text{LOQ} = \frac{10s}{b}. \quad (2)$$

where s is standard deviation of the y-intercept and b is the slope of the analytical curve (Shrivastava & Gupta, 2011).

Repeatability and accuracy. Repeatability was determined as relative standard deviation (RSD). Accuracy was determined as recovery and 95% confidence interval (CI). Solutions with known concentrations (concentrations of the standard near the middle of the working range for each method) of 300 mg/L GA, 5 g/L GA, 20 mg/L of PC B2, and one cider sample were measured 10 times each with a 15-min interval on the same day under the same conditions (materials, equipment, and temperature) by the same personnel using the L-P, F-C, DMAC, and BSA precipitation methods, respectively. A cider sample expected to contain PCs of DP > 4 was evaluated in the BSA method due to the expense of purified PCs of DP > 4. RSD ($n = 10$) was calculated as:

$$\text{RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100. \quad (3)$$

Recovery was calculated for the L-P, F-C, and DMAC methods using $n = 10$ replicates:

$$\text{Recovery} = \frac{\text{Quantification result}}{\text{Known concentration of the standard}} \times 100. \quad (4)$$

The 95% ($\alpha = 0.05$, $t_{0.025}$) CI of the mean was calculated for $n = 10$ replicates:

$$\text{CI (95\% confidence)} = \text{Mean of the sample concentrations} \pm t\alpha/2\frac{\text{Standard deviation}}{\sqrt{n}}. \quad (5)$$

Selectivity. Selectivity was assessed by spiking potentially interfering compounds into solutions of polyphenol compounds. Concentrations of potentially interfering compounds ascorbic acid (1 g/L), glucose (100 g/L), and tyrosine (10 mg/L) were identified according to values present in apple juice (USDA Food Composition Database, 2019). Sodium metabisulfite concentration (0.1 g/L) was representative of cider or wine (Zoecklein, Fugelsang, Gump, & Nury, 1999).

For each method, a solution of the standard for that method was prepared in triplicate at a concentration near the middle of the working range, as determined in section "Working range." Specifically, to evaluate selectivity of the F-C method, triplicate 300 mg/L aqueous solutions of GA were made and spiked with the four potentially interfering compounds at the aforementioned concentrations. Triplicate 5 g/L aqueous solutions of GA for the L-P, 20 mg/L aqueous solutions of PC B2 for DMAC, and cider samples for BSA were spiked in the same manner, respectively. One measurement per replicate was taken for a total of three measurements for each potentially interfering compound using each method. One-way ANOVA followed by Tukey's multiple comparison test (significance where $P < 0.05$) was conducted to determine whether the presence of these compounds influenced results.

Evaluation of quantitative performance

Sample preparation. A wide range of commercially available fruit juices ($n = 14$) (not from concentrate, no added ascorbic acid) and commercially available ciders ($n = 17$, Table S1) were purchased from local grocery stores. Apples of different cultivars ($n = 22$) obtained from orchards in Virginia, USA, were pressed into juice in our laboratory (Champion Juicer, Lodi, CA, USA). The apples were not peeled nor was the core removed, consistent with cider making practice. The number of samples in each category was determined based on availability, and varied by category. All of the samples were centrifuged at 2300 × g for 10 min (with each of the samples pressed in our laboratory being centrifuged immediately following pressing), and supernatant was nitrogen flushed and stored at −80 °C until analysis. Consistent with the methods referenced in the following sections and with common application of these methods, no correction for sample color was made, although it is possible that differences in color could be one of several factors contributing to variation in quantification results by the four assays evaluated.

Quantification of total polyphenol content by F-C and L-P methods.

F-C method. Total polyphenol content was determined by F-C method, with minor modification (Spanos & Wrolstad, 1992). A six-point standard curve (0 to 500 mg/L GA in water) was used to quantify experimental samples. Samples were diluted in water to within the working range, determined in section "Working range." The F-C reagent was dissolved in water to 0.2 M. A 50 µL aliquot of each sample (diluted in water, if needed) was mixed with 450 µL of water in a cuvette (polystyrene, Fisher Scientific). A 1.25 mL aliquot of 0.2 M F-C reagent was added and mixed. A 1 mL aliquot of 75 g/L Na_2CO_3 solution was then added to the cuvette and mixed. Following incubation (2 hr, dark, room temperature), absorbance was read at 765 nm (GENESYS 10S UV-Vis spectrophotometer, Thermo Scientific, Waltham, MA, USA). This analysis, including sample dilution, was conducted in triplicate for each sample. Total polyphenol concentration was calculated using the standard curve and expressed as GA equivalents in mg/L.

L-P titration method. Total polyphenol content was determined by L-P (Lowenthal, 1877). A 0.02 N aqueous $KMnO_4$ solution was standardized against sodium oxalate (AOAC, 1995). Concentration of the standardized titrant was calculated as:

$$\text{Concentration of KMnO4 solution (in N)} = \frac{\text{mass of sodium oxalate (in g)} \times 1000}{\text{volume of KMnO4 (in mL)} \times 66.999}. \quad (6)$$

where 66.999 is the conversion factor into normality of $KMnO_4$ solution. A 1 mL aliquot of sample, 5 mL of 0.1% indigo carmine indicator (0.1% [w/v] indigo carmine in 0.92 M sulfuric acid), and 200 mL deionized water were added to a 500 mL flask, and titrated to a light green endpoint while stirring. Volume of titrant was recorded as X mL. A blank titration using 5 mL of indigo carmine indicator with 200 mL water was also conducted, with volume of titrant recorded as Y mL. Titration was conducted in triplicate for each sample and blank. Total polyphenol content expressed as "tannic acid" (TA) equivalents in mg/L was calculated

as:

$$\text{Total Polyphenol}\left(\frac{\text{mg}}{\text{L}}\text{of TA equivalents}\right) = (\text{X} - \text{Y})\,4.157\ \text{concentration of KMnO4 solution(in N)} \times 10000. \quad (7)$$

where 4.147 is the conversion factor from $KMnO_4$ equivalents to TA equivalents and 10000 is the conversion factor from percentage of tannic acid into mg/L TA equivalents.

Quantification of total flavanol content using DMAC and BSA precipitation methods.

DMAC method. Total flavanol content in the samples was determined by DMAC (Payne et al., 2010). A six-point standard curve, concentrations 0 to 50 mg/L PC B2 in methanol, was used for quantification of experimental samples. Samples were diluted in water to within the working range, determined in section "Working range." Prior work has shown that using methanol compared with water as the solvent did not impact color development at the catechin concentrations evaluated in our study (Wallace & Giusti, 2010). A 200 µL aliquot of diluted sample or standard was mixed with 1 mL of the 0.1% DMAC solution (DMAC dissolved in acidified methanol [6 mL concentrated (36%) hydrochloric acid in 54 mL methanol]) in a cuvette. Absorbance at 640 nm was recorded. This analysis, including sample dilution, was conducted in triplicate. Total flavanol concentration expressed as PC B2 equivalents in mg/L was calculated using the standard curve.

BSA precipitation method. Total flavanol content was quantified using BSA (Harbertson, Kennedy, & Adams, 2002). A six-point standard curve (0 to 150 mg/L [+]-catechin) was used for quantification of experimental samples. For standards, a stock solution of 1000 mg/L of (+)-catechin in methanol was added to Buffer 1 (5% triethanolamine [v/v] and 10% sodium dodecyl sulfate [w/v]) in cuvettes to a total volume of 875 µL. Then, 125 µL of $FeCl_3$ reagent (0.01 M $FeCl_3$ in 0.01 N HCl) was added and incubated at room temperature for 10 min. The absorbance was read at 510 nm. The BSA method involves precipitation of flavanols followed by washing, redissolution, and spectrophotometric quantification. Briefly, samples were diluted in Buffer 2 (12% ethanol in water [v/v] containing 5 g/L potassium bitartrate adjusted to pH 3.3 with HCl) to within the working range determined in section "Working range." A 1 mL aliquot of the BSA solution (1 mg/mL BSA in Buffer 3 [0.2 M acetic acid and 0.17 M NaCl adjusted to pH 4.9 with NaOH]) was then mixed with 500 µL of diluted sample, incubated at room temperature for 15 min with slow agitation, and centrifuged for 1 min (13500 × *g*). Supernatant was poured off and precipitate was dissolved in 250 µL of Buffer 3. This mixture was centrifuged for 1 min (13500 × *g*) and the supernatant poured off. The precipitate was dissolved in 875 µL of Buffer 1 and incubated at room temperature for 10 min. Absorbance at 510 nm was recorded as A_1. A 125 µL aliquot of $FeCl_3$ reagent was added and the absorbance at 510 nm after 10 min was recorded as A_2. This analysis, including sample dilution, was conducted in triplicate for each sample. The difference between A_2 and A_1 was used as the Y value to calculate flavanol content, as mg/L (+)-catechin equivalents, from the standard curve for each experimental sample.

Quantification of individual polyphenol compounds by UPLC/MS. To understand how polyphenol composition may influence results of the analytical methods evaluated, individual polyphenol compounds commonly found in apple juice and cider were quantified using the LC-MS method described by Ma et al. (2018). Separation gradient and retention times, molecular weights, and selected ion recording (SIR) channels are provided in Table S2 and S3, respectively. Due to vastly different polyphenol composition expected in commercial fruit juice samples and the expense of the wide range of analytical standards that would be required, analysis of the individual polyphenol profile of these samples was not conducted. A subset of 12 apple juices and 12 ciders expected to vary in polyphenol composition, based on prior reports of polyphenol composition (Anastasiadi et al., 2017) and on informal evaluation of their sensory attributes, was selected. Standards of PC B1, PC B2, PC B5, PC C1, Cinn A2, (+)-catechin, (−)-epicatechin, chlorogenic acid, quercetin, and phloretin were used to build five-point standard curves for the quantification of these compounds.

Statistical analysis. Results are reported as means ± Standard Error of the Mean (SEM) for three replicates. Total individual polyphenol concentration by UPLC/MS was defined as the sum of all analyzed individual polyphenols for a given sample. Un-paired *t*-tests were conducted between the quantification results to compare values for total polyphenols obtained through L-P compared with F-C, and for total flavanols obtained through DMAC compared with BSA, respectively, using GraphPad Prism v6.0e (GraphPad Software Inc., La Jolla, CA, USA). To determine whether the relationships between these values are influenced by sample composition, one-way ANOVA with Tukey's multiple comparison test was used to determine whether there were significant differences in the ratios of L-P to F-C values and ratios of DMAC to BSA values. Significant difference was defined as $P < 0.05$.

Results and Discussion

Analytical method validation

Working range. Working range for all methods is summarized in Table 1. Values of R^2 and R^2_{adjusted} near one for all analytical curves (Figure S1) show that each of the linear models explained the majority of the experimental variability. The *P* values for all analytical curves were significant ($P < 0.0001$), indicating existence of a strong relationship between responses and concentrations of standards. The upper limit of the working range for F-C found in this study (500 mg/L of GA) is in agreement with a previously suggested maximum concentration (Singleton et al., 1999). For L-P, the upper limit of the working range was 12000 mg/L of GA. The maximum solubility of GA in water is approximately 12 g/L (Budavari, 1996), representing the highest concentration evaluated in this study.

While an upper limit of the working range of 50 mg/L of PC B2 was found for DMAC in this study, standard curves with wider linear ranges were reported by others, including 0.1 to 100 mg/L of PC B2 (Payne et al., 2010) and 3.125 to 100 mg/L of PC B2 (Prior et al., 2010). Those linear ranges likely reached higher maximum concentrations because absorbance values ≥1 were allowed. For this study, we defined absorbance ≥1 as a criterion for the upper limit of the method due to increased error associated with absorbance ≥1 (Nielsen, 2010). For BSA, a standard curve linear between 0 and 100 mg/L tannic acid was previously reported (Hagerman & Butler, 1978); however, we observed a wider working range up to 150 mg/L of catechin. While tannic acid was historically used as the standard for BSA precipitation, catechin is preferred as the standard for fruit juice and beverage

Table 1–Analytical curves, LOD, and LOQ for the F-C, L-P, DMAC, and BSA precipitation methods.

Method	Analytical curve[a]	Working range	R^2	$R^2_{adjusted}$	P value[b]	LOD	LOQ
F-C[c]	$y = 0.0019x + 0.0096$	42.9 to 500	0.999	0.999	<0.0001	14.2	42.9
L-P[c]	$y = 1256.1x + 48.61$	1.47 to 12000	0.995	0.995	<0.0001	0.485	1.47
DMAC[d]	$y = 0.017x + 7E05$	5.71 to 50	0.998	0.997	<0.0001	1.71	5.71
BSA[e]	$y = 0.0067x + 0.0108$	13.7 to 150	0.999	0.998	<0.0001	4.51	13.7

[a]Regression analysis was conducted using the concentrations of standard in the *x*-axis and the measurements in the *y*-axis.
[b]P value <0.05 indicates that the slope of the linear regression is nonzero.
[c]mg/L of GA equivalents.
[d]mg/L of PC B2 equivalents.
[e]mg/L of catechin equivalents.
Note that the units differ among these values and are listed in the footnotes provided.

Table 2–Repeatability and recovery of F-C, L-P, DMAC, and BSA precipitation methods.

Method	Repeatability/(%RSD)	Recovery/%	$CI_{95\%}$
F-C	0.66	102.9 ± 0.21	307.3 to 309.9 mg/L in GA equivalents
L-P	0.70	143.5 ± 0.32	7145 to 7207 mg/L in TA equivalents
DMAC	2.2	104.1 ± 0.72	20.5 to 21.1 mg/L in PC B2 equivalents
BSA	3.6	N/A	N/A

Data were expressed as mean ± SEM.

Table 3–Selectivity of F-C, L-P, DMAC, and BSA precipitation method.

Methods	Control[a]	Ascorbic acid[b]	Potassium metabisulfite[c]	Glucose[d]	Tyrosine[e]
F-C[f]	308.6 ± 0.64 e	1053 ± 2.4 a	350 ± 0.17 c	316 ± 1.2 d	398 ± 3.3 b
L-P[g]	7176 ± 16 b	7803 ± 43 a	7238 ± 21 b	6745 ± 25 c	7685 ± 104 a
DMAC[h]	20.8 ± 0.14 a	21.5 ± 0.33 a	21.1 ± 0.043 a	20.9 ± 0.14 a	21.4 ± 0.049 a
BSA[i]	81.0 ± 0.92 a	80.2 ± 3.3 a	75.3 ± 2.0 ab	66.3 ± 2.8 b	76.4 ± 2.7 a

[a]300 mg/L of GA for F-C method, 5 g/L of GA for L-P method, 20 mg/L of PC B2 for DMAC method, and a cider sample for BSA precipitation method for $n = 10$ replicates.
[b]1 g/L ascorbic acid was spiked into the respective control for $n = 6$ replicates.
[c]0.1 g/L potassium metabisulfite was spiked into the respective control for $n = 6$ replicates.
[d]100 g/L glucose was spiked into the respective control for $n = 6$ replicates.
[e]10 mg/L tyrosine was spiked into the respective control for $n = 6$ replicates.
[f]in mg/L GA equivalents.
[g]in mg/L TA equivalents.
[h]in mg/L PC B2 equivalents.
[i]in mg/L catechin equivalents.
Data were expressed as mean ± SEM. Different lower-case letters after the value indicate significant difference ($P < 0.05$) among treatments for each method (each row, including the control), by one-way ANOVA and Tukey's HSD test.

analysis because it is found in fruit (Harbertson et al., 2002), while tannic acid is not (Food Chemicals Codex, 2019).

The limitations of this study merit consideration. The use of varying numbers of standards ($n = 11$, $n = 13$, $n = 9$, and $n = 9$) for each assay could bias the results in terms of comparing R^2 values of standard curves within working ranges. Additionally, we defined the low end of the working range as the LOQ. Although samples at 0 mg/L (reagent blank) were evaluated for each method, the number of values near 0 mg/L was limited, and 1/x weighting was not applied. This could lend disproportionate weight to samples at higher concentrations in determination of linear fit, leading to increased error at the low end of the linear ranges reported. To minimize error due to this limitation, a good practice is to concentrate or dilute samples to fit within 20% to 80% of the working range of the method (Nielsen, 2010).

LOD and LOQ. LOD and LOQ are summarized in Table 1. For F-C, LOD and LOQ were 14.2 and 42.9 mg/L GA, respectively. Others have reported lower LOD and LOQ of 0.25 and 0.82 mg/L GA, respectively (Margraf, Karnopp, Rosso, & Granato, 2015). This difference could be attributable to stronger color intensity imparted by more concentrated F-C reagent, resulting in greater slope of the analytical curve, and lower LOD and LOQ. LOD and LOQ for the L-P method have not been previously reported. For DMAC, we observed LOD and LOQ of 1.71 mg/L PC B2 and 5.71 mg/L PC B2. A comparable LOD of 1.94 mg/L and LOQ of 6.47 mg/L were reported by others using PC A2 as the standard (Feliciano et al., 2012). Our results indicate that DMAC has slightly lower LOD and LOQ compared to BSA (4.51 mg/L catechin and 13.68 mg/L catechin). No prior reports of the LOD anond LOQ of the BSA precipitation method are available for comparison.

Repeatability and accuracy. Repeatability (%RSD) and accuracy (recovery and 95% CI) are listed in Table 2. The %RSD for each method was small, indicating low variation among measurements made under the same operating conditions over short time intervals, that is, high intraday precision. Higher %RSD for F-C has been reported by others (4.98% and 6.65%), compared to 0.66% found in this study. For DMAC, we found 2.2% RSD, slightly lower than reported by others (4.0% to 9.5% RSD for intermediate products of chocolate manufacturing [Payne et al., 2010], and 2.3% to 6.1% RSD for commercial cranberry samples [Prior et al., 2010]). Differences in repeatability with DMAC could be attributable to matrix differences of food samples compared with standard solutions. For BSA, a prior report of 1.2% to 7.2% RSD for dry red wine samples is in agreement with our finding of 3.6% RSD (Mercurio & Smith, 2008).

Table 4–Total polyphenol content of commercial fruit juice samples (n = 14), apple juice samples of different cultivar (n = 22), and commercially available cider samples (n = 17) quantified by F-C and L-P methods.

Samples	F-C (mg/L GA equivalents)	L-P (mg/L TA equivalents)	P values	Ratio L-P/F-C
Commercial fruit juices				
Apple Juice	204.8 ± 0.04	1226 ± 23	<0.001	6.0 ± 0.1
Blueberry Juice	1270 ± 0.6	2108 ± 67	<0.001	1.7 ± 0.06
Blackcherry Juice	1860 ± 0.04	2163 ± 150	0.113	1.2 ± 0.08
Cranberry Juice	705.4 ± 0.3	1573 ± 149	0.004	2.2 ± 0.2
Concord Grape Juice	1161 ± 0.3	2231 ± 264	0.015	1.9 ± 0.2
Gala Apple Juice	209.3 ± 0.2	1193 ± 12	<0.001	5.7 ± 0.04
Grapefruit Juice	599.6 ± 0.2	1365 ± 3.2	<0.001	2.3 ± 0.02
Gravenstein Apple Juice	452.2 ± 0.1	1384 ± 23	<0.001	3.1 ± 0.04
Honeycrisp Apple Juice	325.8 ± 0.06	1290 ± 17	<0.001	4.0 ± 0.06
Lemon Juice	319.6 ± 0.2	1359 ± 32	<0.001	4.3 ± 0.1
Lime Juice	198.1 ± 0.2	1245 ± 41	<0.001	6.3 ± 0.2
Pomegranate Juice	3120 ± 0.5	3897 ± 51	<0.001	1.2 ± 0.01
Prune Juice	1769 ± 0.2	2354 ± 23	<0.001	1.3 ± 0.01
White Grape Juice	38.46 ± 0.05	1083 ± 34	<0.001	28 ± 1
Apple juice samples of different cultivar				
Arkansas Black	908.1 ± 0.8	1568 ± 77	<0.001	1.8 ± 0.1
Ashmead's Kernel	692.7 ± 0.2	1441 ± 41	<0.001	2.1 ± 0.05
Black Twig	421.6 ± 0.1	3501 ± 24	<0.001	8.3 ± 0.08
Cameo	277.1 ± 0.2	3045 ± 59	<0.001	11 ± 0.2
Fuji	333.7 ± 0.03	1734 ± 58	<0.001	5.2 ± 0.2
Gold Rush	383.0 ± 0.4	1770 ± 25	<0.001	4.6 ± 0.1
Golden Delicious	344.6 ± 0.5	1695 ± 18	<0.001	4.9 ± 0.1
Golden Russet	572.7 ± 0.8	1767 ± 16	<0.001	3.1 ± 0.04
Granny Smith	442.3 ± 1	1601 ± 17	<0.001	3.6 ± 0.1
Harrison 1	1169 ± 0.1	2108 ± 13	<0.001	1.8 ± 0.01
Harrison 2	1170 ± 0.01	2169 ± 18	<0.001	1.9 ± 0.02
Hewes	2180 ± 0.1	2456 ± 16	<0.001	1.1 ± 0.01
Ida Red	368.1 ± 0.1	1643 ± 190	0.003	4.5 ± 0.5
Jonagold	389.2 ± 0.9	1577 ± 19	<0.001	4.1 ± 0.1
King David	462.5 ± 0.4	1601 ± 22	<0.001	3.5 ± 0.06
Manchurian	201.4 ± 0.3	1474 ± 17	<0.001	7.3 ± 0.1
Pink Lady	360.2 ± 0.6	1625 ± 21	<0.001	4.5 ± 0.1
Red Delicious	644.0 ± 0.2	1474 ± 26	<0.001	2.3 ± 0.05
Rome	585.3 ± 0.4	1571 ± 22	<0.001	2.7 ± 0.05
Snowdrift	6607 ± 0.5	10730 ± 31	<0.001	1.6 ± 0.01
Virginia Gold	362.1 ± 0.2	1550 ± 19	<0.001	4.3 ± 0.06
York	507.6 ± 0.2	1640 ± 19	<0.001	3.2 ± 0.05
Commercial ciders				
1	205.1 ± 0.1	923.4 ± 29	<0.001	4.5 ± 0.1
2	567.0 ± 0.6	1220 ± 3.3	<0.001	2.2 ± 0.04
3	238.6 ± 0.2	966.3 ± 20	<0.001	4.0 ± 0.07
4	2187 ± 0.8	2830 ± 190	0.029	1.3 ± 0.1
5	378.3 ± 0.3	1052 ± 22	<0.001	2.8 ± 0.05
6	677.5 ± 0.6	1375 ± 5.7	<0.001	2.0 ± 0.03
7	465.1 ± 0.4	1121 ± 49	<0.001	2.4 ± 0.08
8	243.6 ± 0.2	996.0 ± 17	<0.001	4.1 ± 0.08
9	179.6 ± 0.1	986.1 ± 6.6	<0.001	5.5 ± 0.05
10	160.3 ± 0.2	926.7 ± 27	<0.001	5.8 ± 0.1
11	396.0 ± 0.9	1000 ± 6.0	<0.001	2.5 ± 0.07
12	456.9 ± 0.1	1078 ± 34	<0.001	2.4 ± 0.07
13	367.1 ± 0.1	1051 ± 33	<0.001	2.9 ± 0.1
14	638.5 ± 0.3	1356 ± 18	<0.001	2.1 ± 0.04
15	385.8 ± 0.2	1036 ± 18	<0.001	2.7 ± 0.06
16	396.9 ± 0.02	1142 ± 14	<0.001	2.9 ± 0.03
17	1318 ± 0.1	2235 ± 12	<0.001	1.7 ± 0.01

Data were expressed as mean ± SEM for n = 3 replicates. P values were reported from comparisons between the two methods by un-paired t-test.

Nearly 100% recovery for F-C and DMAC (Table 2) indicates measurements very close to reference values. Similar recovery for F-C, 90% (Blainski, Lopes, & de Mello, 2013) and 98.20% (Margraf et al., 2015) have been reported. L-P recovery was much higher, 143.5 ± 0.32%, a value beyond the accepted range of 80% to 120% (U.S. Food and Drug Administration, 2015). Though accuracy of L-P is poor compared to F-C, L-P is still used for relative comparison of polyphenol content among samples with similar polyphenol compositions, such as apples of a single cultivar, due to its high repeatability, wide linear range, and low cost.

Selectivity. Selectivity is reported in Table 3. Ascorbic acid, potassium metabisulfite, glucose, and tyrosine significantly increased total polyphenol values by F-C (Table 3, $P < 0.001$) by 241%, 13%, 2%, and 29%, respectively. Low selectivity of F-C has been observed by others (Everette et al., 2010). Reducing compounds, including but not limited to polyphenols, reduce the F-C reagent under basic conditions. Accordingly, the presence of

other nonpolyphenol reducing compounds in the sample matrix could significantly impact results. While interference from glucose may not be of practical significance (<10%), the other compounds evaluated could significantly interfere with comparisons of total polyphenol values, especially between different types of fruit.

Others hypothesized that interference by sugars in the sample matrix would be greater for L-P than for F-C because potassium permanganate is a stronger oxidizing agent compared to the F-C reagent (Singleton et al., 1999). However, our results were numerically close to the reference values (<10% difference, Table 3). Others found that 8 g/L glucose had no effect on L-P results (Celeste, Tomas, Cladera, Estela, & Cerda, 1993); however, we found that the addition of 100 g/L glucose, the concentration found in apple juice, significantly decreased results. In the L-P method, potassium permanganate does not specifically oxidize polyphenols, but all other reducing compounds that are oxidized more rapidly by potassium permanganate than indigo carmine (Smit et al., 1955). Under acidic conditions, polyphenols bind with sugars through hydrogen bonds (Bordenave, Hamaker, & Ferruzzi, 2014), decreasing polyphenol redox by making the active site (reactive hydroxyl groups) unavailable (Bors & Michel, 2002), resulting in lower apparent polyphenol concentration in the presence of sugar.

DMAC showed the greatest selectivity of all methods evaluated in this study. None of the compounds evaluated altered results compared to the control (20 mg/L of PC B2, Table 3). In the DMAC method, the reagent reacts with the C8 carbon at the terminal unit on A-ring flavanols (Wallace & Giusti, 2010). Because the four potentially interfering compounds evaluated in this study do not share this specific structure required for color development, they did not interfere with the quantitative results of the DMAC method.

For BSA, glucose was the only compound that interfered, resulting in 18% lower values for total flavanols possibly due to glucose interfering in binding between polyphenols and BSA (Bordenave et al., 2014). Other fruit juice or cider sample matrix constituents not evaluated in this project, like proteins or polysaccharides, including pectin, could also interfere with the BSA method, and merit further investigation.

Evaluation of quantitative performance

Comparison of total polyphenol quantification by F-C and L-P. Results obtained using F-C were lower for all samples compared to L-P, with the exception of black cherry juice (Table 4). This observation is consistent with prior reports on red and white wine (Celeste et al., 1993). The absolute values for F-C and L-P were expected to differ due to different principles of these methods. Perhaps more interestingly, significant differences in the ratio of L-P to F-C values for a given sample ($P < 0.0001$) were observed for all three sets of samples. These ratios range from 1.2 to 28 (Table 4). For fruit juice of lighter color (white grape, lime, apple, and lemon), the ratios were higher compared to fruit juice of darker color (blueberry juice, cranberry juice, concord grape juice, and grapefruit juice) (Figure 1A). Color in these fruits is imparted mainly by anthocyanins (Bridle & Timberlake, 1997), a class of polyphenols that may quantitatively contribute more in F-C than in L-P. Additionally, within apple juices from different cultivars, ratios range from 1.1 to 11 ($P < 0.0001$) (Figure 1B), likely due to substantial variation in polyphenol composition among apple cultivars (Anastasiadi et al., 2017) (Table 5 and 6). Ratios of L-P to F-C values for cider samples fall into a narrower range of 1.3 to 5.8

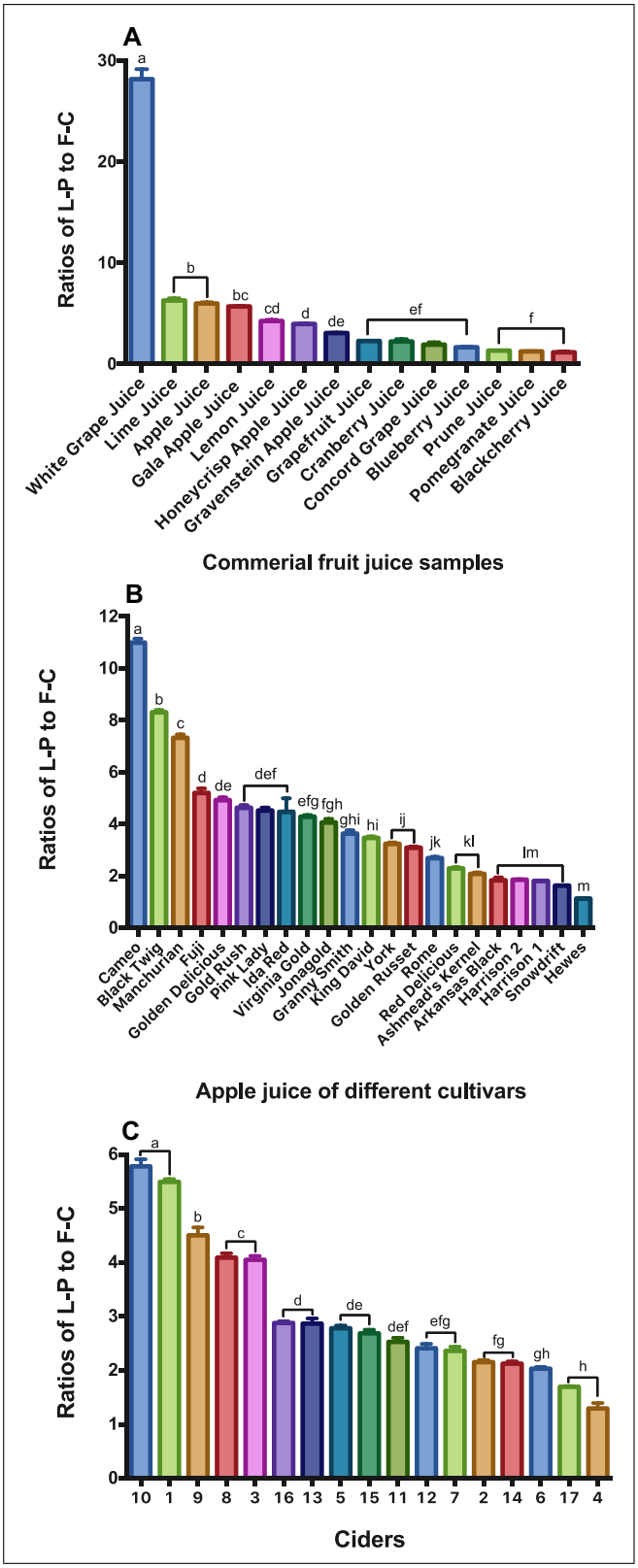

Figure 1–Multiple comparisons of ratios of L-P to F-C results among fruit juice samples (A), apple juice of different cultivars (B), and ciders (C). Bars represent the mean and error bars represent the SEM for $n = 3$ replicates. Lower case letters represent significant differences between the means. Significance was defined as $P < 0.05$.

Table 5–Concentration of individual polyphenol compounds in mg/L of apple juice from different apple cultivars (n = 12).

Cultivar	Catechin	Epicatechin	PC B1	PC B2	PC B5
Ashmead's Kernel	3.14 ± 0.15	5.49 ± 0.31	1.77 ± 0.23	3.47 ± 0.45	0.433 ± 0.033
Black Twig	0.229 ± 0.020	0.156 ± 0.014	0.0938 ± 0.012	0.0952 ± 0.012	0.074 ± 0.0072
Cameo	0.0563 ± 0.011	0.0376 ± 0.0071	0.0095 ± 0.0019	0.0100 ± 0.0018	0.0138 ± 0.0032
Fuji	0.122 ± 0.0068	0.0811 ± 0.0050	0.031 ± 0.00036	0.0331 ± 0.0011	0.0206 ± 0.00078
Gold Rush	0.416 ± 0.024	0.277 ± 0.019	1.30 ± 0.10	0.889 ± 0.038	0.476 ± 0.020
Granny Smith	0.497 ± 0.071	0.353 ± 0.043	2.65 ± 0.12	0.891 ± 0.12	0.287 ± 0.035
Harrison 1	0.398 ± 0.015	0.262 ± 0.011	0.58 ± 0.056	0.673 ± 0.056	0.140 ± 0.011
Harrison 2	5.26 ± 0.16	5.98 ± 0.44	1.97 ± 0.11	1.52 ± 0.076	0.387 ± 0.010
Hewes	1.45 ± 0.32	21.0 ± 4.6	1.67 ± 0.47	14.7 ± 3.1	1.790 ± 0.52
Manchurian	0.00447 ± 0.0025	0.132 ± 0.022	0.0023 ± 0.00060	nd	nd
Snowdrift	266 ± 8.3	158 ± 14	475 ± 30	351 ± 36	22.7 ± 1.2
York	0.124 ± 0.014	0.282 ± 0.031	0.046 ± 0.0061	0.140 ± 0.0063	0.0416 ± 0.0043

Cultivar	PC C1	Cinn A2	Chlorogenic acid	Phloretin	Quercetin	Total
Ashmead's Kernel	0.523 ± 0.044	0.286 ± 0.035	59.4 ± 3.1	0.0220 ± 0.00224	0.0435 ± 0.0014	74.6 ± 2.0
Black Twig	0.0563 ± 0.0083	0.0531 ± 0.0074	35.3 ± 1.9	0.00688 ± 0.00086	0.0262 ± 0.00051	36.1 ± 2.0
Cameo	0.00777 ± 0.0017	0.0142 ± 0.0023	19.9 ± 0.48	0.00195 ± 0.000050	0.00485 ± 0.00073	20.1 ± 0.48
Fuji	0.0132 ± 0.00054	0.0161 ± 0.0068	25.7 ± 0.93	0.00120 ± 0.00010	0.00593 ± 0.00034	26.0 ± 0.92
Gold Rush	0.649 ± 0.036	0.468 ± 0.0068	43.9 ± 2.1	0.0112 ± 0.00073	0.0260 ± 0.0010	48.4 ± 2.0
Granny Smith	0.591 ± 0.088	0.503 ± 0.044	9.17 ± 0.044	0.00478 ± 0.00065	0.0125 ± 0.0030	15.0 ± 0.25
Harrison 1	0.227 ± 0.024	0.108 ± 0.0096	318 ± 15	0.0084 ± 0.00070	0.00167 ± 0.00030	321 ± 15
Harrison 2	0.581 ± 0.012	0.210 ± 0.012	195 ± 4.4	0.0214 ± 0.0017	0.00738 ± 0.00056	212 ± 5.1
Hewes	0.830 ± 0.14	2.46 ± 0.38	58.5 ± 8.1	0.0022 ± 0.00060	0.0101 ± 0.0019	102 ± 17
Manchurian	nd	nd	2.38 ± 0.39	0.00378 ± 0.00059	0.0226 ± 0.0025	2.55 ± 0.42
Snowdrift	164 ± 11	82.0 ± 9.2	27.53 ± 1.5	0.244 ± 0.0081	0.294 ± 0.021	1550 ± 100
York	0.0694 ± 0.0029	0.0489 ± 0.0047	21.0 ± 1.9	0.00288 ± 0.00028	0.0102 ± 0.0011	21.8 ± 2.0

nd, not detected.
Data were expressed as mean ± SEM for n = 3 replicates.

Table 6–Concentration of individual polyphenol compounds in mg/L of commercial ciders (n = 12).

Sample number	Catechin	Epicatechin	PC B1	PC B2	PC B5
2	0.594 ± 0.049	0.611 ± 0.050	0.136 ± 0.013	0.521 ± 0.054	0.0408 ± 0.0031
3	0.0704 ± 0.023	0.338 ± 0.022	0.0118 ± 0.0034	0.0780 ± 0.0074	0.0176 ± 0.0010
4	9.22 ± 0.62	28.7 ± 1.7	3.80 ± 0.10	18.6 ± 2.4	2.09 ± 0.030
5	0.374 ± 0.047	1.62 ± 0.24	0.0741 ± 0.011	0.376 ± 0.040	0.105 ± 0.018
6	2.97 ± 0.074	9.32 ± 0.47	2.06 ± 0.22	6.27 ± 1.0	0.708 ± 0.020
8	0.141 ± 0.015	0.515 ± 0.057	0.0254 ± 0.0054	0.611 ± 0.14	0.0608 ± 0.0088
10	0.0769 ± 0.0061	0.153 ± 0.014	0.0117 ± 0.0021	0.0492 ± 0.0073	0.00503 ± 0.00046
12	0.472 ± 0.058	3.69 ± 0.45	0.491 ± 0.058	3.29 ± 0.80	0.351 ± 0.044
13	0.598 ± 0.039	5.23 ± 0.87	0.675 ± 0.11	4.32 ± 0.60	0.462 ± 0.0055
14	0.529 ± 0.026	2.37 ± 0.10	0.202 ± 0.043	0.660 ± 0.060	0.124 ± 0.0038
15	0.159 ± 0.022	0.375 ± 0.048	0.0145 ± 0.0031	0.141 ± 0.033	0.0325 ± 0.0049
17	3.94 ± 0.23	36.3 ± 3.4	3.60 ± 0.30	48.7 ± 5.4	4.44 ± 0.022

Sample number	PC C1	Cinn A2	Chlorogenic acid	Phloretin	Quercetin	Total
2	0.0547 ± 0.0029	nd	10.5 ± 0.36	0.0296 ± 0.00047	0.0417 ± 0.00072	12.5 ± 0.54
3	nd	nd	9.72 ± 0.060	0.0249 ± 0.00061	0.0705 ± 0.0017	10.3 ± 0.12
4	0.625 ± 0.029	1.88 ± 0.025	8.13 ± 0.19	0.277 ± 0.00075	0.573 ± 0.0066	73.9 ± 5.2
5	0.0811 ± 0.0074	nd	17.0 ± 1.8	0.766 ± 0.13	0.180 ± 0.0045	20.6 ± 2.3
6	1.03 ± 0.093	0.185 ± 0.010	43.2 ± 1.0	1.05 ± 0.011	0.0380 ± 0.00080	66.8 ± 2.9
8	0.124 ± 0.027	nd	11.1 ± 0.36	0.181 ± 0.0028	0.183 ± 0.0036	12.9 ± 0.62
10	nd	nd	1.07 ± 0.093	0.445 ± 0.0035	0.285 ± 0.0041	2.09 ± 0.13
12	0.737 ± 0.054	0.287 ± 0.020	53.6 ± 1.6	0.267 ± 0.0043	0.00440 ± 0.00022	63.2 ± 3.1
13	0.946 ± 0.097	0.324 ± 0.029	3.46 ± 0.043	0.180 ± 0.035	0.00912 ± 0.0013	16.2 ± 1.8
14	0.108 ± 0.015	nd	133 ± 9.1	0.184 ± 0.0081	0.0151 ± 0.00090	137 ± 9.3
15	nd	nd	15.0 ± 1.1	0.328 ± 0.0058	0.0696 ± 0.0013	16.2 ± 1.2
17	2.19 ± 0.16	nd	70.1 ± 3.2	0.115 ± 0.0032	0.0805 ± 0.00098	170 ± 13

nd, not detected.
Data were expressed as mean ± SEM for n = 3 replicates.

(Figure 1C), with 2 to 3 being the most prevalent ratio. Greater variation in polyphenol composition in apple juices compared to ciders made from those juices has been reported (Ewing, Peck, Ma, Neilson, & Stewart, 2019; Ma et al., 2018), and our findings are in general agreement with this.

Differences in reactivity among polyphenol compounds have been reported for F-C (Figure S2) and L-P (Smit et al., 1955), and could be expected to translate to differences in ratios of L-P to F-C results for samples with very different polyphenol composition, such as the sample set evaluated in this study. For example, for a

Table 7–Total flavanol content of commercial fruit juice samples (n = 14), apple juice samples of different cultivar (n = 22), and commercially available cider samples (n = 17) quantified by DMAC and BSA precipitation methods.

Samples	DMAC (mg/L PC B2 equivalents)	BSA (mg/L catechin equivalents)	*P* values	Ratio DMAC/BSA
Commercial fruit juices				
Apple Juice	18.23 ± 0.16	17.71 ± 0.83	0.065	1.0 ± 0.06
Blueberry Juice	195.3 ± 4.9	21.87 ± 1.9	<0.001	9.4 ± 0.8
Blackcherry Juice	143.7 ± 4.0	nd	<0.001	N/A
Cranberry Juice	221.5 ± 4.9	89.40 ± 0.50	<0.001	2.5 ± 0.06
Concord Grape Juice	416.6 ± 5.1	73.99 ± 3.3	<0.001	5.7 ± 0.2
Gala Apple Juice	12.56 ± 0.34	8.883 ± 1.6	0.112	1.4 ± 0.2
Grapefruit Juice	21.33 ± 0.62	nd	<0.001	N/A
Gravenstein Apple Juice	27.90 ± 0.21	12.17 ± 0.30	<0.001	2.3 ± 0.07
Honeycrisp Apple Juice	68.80 ± 0.34	9.922 ± 0.74	<0.001	7.0 ± 0.5
Lemon Juice	15.82 ± 0.36	nd	<0.001	N/A
Lime Juice	7.957 ± 0.23	nd	<0.001	N/A
Pomegranate Juice	154.8 ± 1.6	89307 ± 3698	<0.001	0.0017 ± 0
Prune Juice	30.58 ± 1.3	11.13 ± 0.49	<0.001	2.7 ± 0.05
White Grape Juice	2.427 ± 0.020	nd	<0.001	N/A
Apple juice samples of different cultivars				
Arkansas Black	47.29 ± 0.53	8.030 ± 0.99	<0.001	6.1 ± 0.7
Ashmead's Kernel	291.5 ± 0.52	3.110 ± 0.35	<0.001	96 ± 11
Black Twig	48.96 ± 1.3	8.030 ± 0.77	<0.001	6.2 ± 0.4
Cameo	48.90 ± 0.14	nd	<0.001	N/A
Fuji	26.39 ± 0.54	nd	<0.001	N/A
Gold Rush	30.86 ± 0.14	nd	<0.001	N/A
Golden Delicious	18.21 ± 0.078	10.18 ± 0.62	<0.001	1.8 ± 0.1
Golden Russet	22.96 ± 0.31	5.159 ± 0.67	<0.001	4.6 ± 0.5
Granny Smith	41.74 ± 0.32	nd	<0.001	N/A
Harrison 1	218.8 ± 3.8	35.83 ± 3.5	<0.001	6.2 ± 0.7
Harrison 2	212.7 ± 10	17.16 ± 2.0	<0.001	13 ± 2
Hewes	561.6 ± 31	180.8 ± 16	<0.001	3.1 ± 0.3
Ida Red	18.56 ± 0.98	nd	<0.001	N/A
Jonagold	21.09 ± 0.84	4.236 ± 0.10	<0.001	5.0 ± 0.3
King David	18.90 ± 0.54	8.851 ± 0.62	<0.001	2.2 ± 0.2
Manchurian	7.740 ± 0.052	nd	<0.001	N/A
Pink Lady	21.13 ± 0.51	nd	<0.001	N/A
Red Delicious	19.07 ± 2.1	10.18 ± 0.31	0.014	1.9 ± 0.2
Rome	40.68 ± 2.7	10.29 ± 0.88	<0.001	4.0 ± 0.1
Snowdrift	5449 ± 120	2433 ± 42	<0.001	2.2 ± 0.06
Virginia Gold	20.02 ± 0.052	nd	<0.001	N/A
York	27.94 ± 4.5	1.262 ± 0.31	0.004	25 ± 7
Commercial ciders				
1	38.51 ± 0.25	nd	<0.001	N/A
2	102.7 ± 0.43	nd	<0.001	N/A
3	39.98 ± 0.085	nd	<0.001	N/A
4	253.7 ± 5.1	152.6 ± 2.7	<0.001	1.7 ± 0.02
5	133.8 ± 0.51	nd	<0.001	N/A
6	257.6 ± 0.34	nd	<0.001	N/A
7	143.8 ± 0.78	nd	<0.001	N/A
8	41.51 ± 0.10	nd	<0.001	N/A
9	4.015 ± 0.020	nd	<0.001	N/A
10	18.45 ± 0.085	nd	<0.001	N/A
11	26.45 ± 0.29	nd	<0.001	N/A
12	33.74 ± 0.31	nd	<0.001	N/A
13	32.54 ± 0.13	nd	<0.001	N/A
14	26.39 ± 0.29	nd	<0.001	N/A
15	48.41 ± 0.52	nd	<0.001	N/A
16	24.23 ± 0.034	nd	<0.001	N/A
17	2065 ± 19	7.723 ± 1.2	<0.001	280 ± 42

nd, not detected, absorbance values of zero or below zero obtained.
Data were expressed as mean ± SEM for n = 3 replicates. *P* values were reported from between methods along the rows by un-paired *t*-test. For these methods, values that were not detected (below the LOD of each method) were treated as 0 when calculating the ratios.

solution of pure hydroquinone, the result by L-P was 2.4 times higher than the result by F-C (Hyman, Sansome-Smith, Shears, & Wood, 1985). Hydroquinone is the oxidation product of phenols, and is thus present during the reaction for both F-C and L-P. L-P results were higher compared to F-C for chlorogenic acid, catechol, and pyrogallol, while the F-C method yielded higher results for catechin, quercetin, and phenol (Smit et al., 1955). Differences in quantitative results by these two methods could also result from the distinct sample matrices. As reported in Table 3, ascorbic acid, glucose, and tyrosine, matrix constituents present at varying concentrations in many fruit juices (USDA Food Composition Database, 2019), have been reported to interfere with these methods (Everette et al., 2010), and the extent to which these interferences affect quantitative results merits further investigation. Taken together, these findings illustrate that polyphenol composition may impact both the absolute values obtained using F-C and

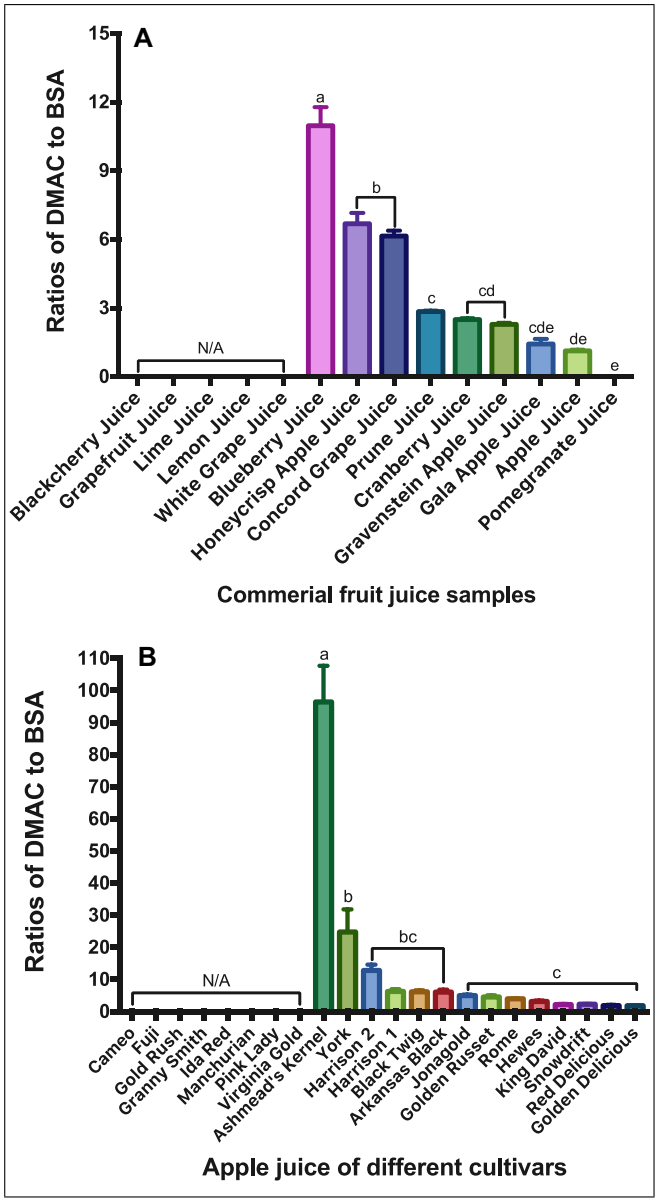

Figure 2–Multiple comparisons of ratios of DMAC to BSA results among fruit juice samples (A) and apple juice of different cultivars (B). Bars represent the mean and error bars represent the SEM for $n = 3$ replicates. Lower case letters represent significant differences among the means. Significance was defined as $P < 0.05$. Many ratios of DMAC to BSA results could not be calculated (N/A) due to the nondetectable concentrations of flavanols by the BSA method being counted as zero for the ratio calculation.

L-P methods, and also the relationship between values obtained using these two methods.

Comparison of total flavanol quantification by DMAC and BSA. For all samples except pomegranate juice, total flavanol results by DMAC were higher than those obtained using BSA (Table 7). In fact, many of the samples contained low or nondetectable concentrations of flavanols as determined by BSA. Differences in absolute values obtained using these two different methods were expected due to differences in the mechanisms of the two methods. While DMAC quantifies only flavanols sharing a common structure of a C8 carbon at the terminal unit on the A-ring (Wallace & Giusti, 2010), BSA only quantifies flavanols with DP 3 and larger due to the inability of BSA to form precipitates with flavanols monomers and dimers (Harbertson, Kilmister, Kelm, & Downey, 2014).

The ratios of DMAC to BSA results also vary significantly among samples within each category (Table 7) ($P < 0.0001$). Overall, the lowest ratio of DMAC to BSA results was found in pomegranate juice (<1) and the highest ratio in cider sample 17 (280). For pomegranate juice, the BSA result was much higher than that obtained by DMAC. Relative to other fruit juices evaluated in this study, pomegranate juice is unique due to its high content of high-molecular-weight flavanols (Akhtar, Ismail, Fraternale, & Sestili, 2015). High-molecular-weight flavanols are quantified by BSA, but give lower responses by DMAC compared to flavanols of low molecular weight (Wang et al., 2016). This is a likely explanation for the very low ratio of DMAC to BSA results for pomegranate juice. For cider sample 17, made from Hewe's Crab apples, the high ratio is likely due to the very high concentration of flavanols detected by DMAC. It is interesting to note that the apple juice made from the crab apple cultivar Snowdrift also had an extremely high concentration of flavanols detected by DMAC. However, the Snowdrift juice sample had a much lower ratio of DMAC to BSA results because it also contained a very high value of flavanols as detected by BSA precipitation (2433 mg/L catechin equivalents, the second highest value observed in this study after pomegranate), reflecting the uniquely high polyphenol content of certain crab apples.

The variation in ratios of DMAC to BSA results (Figure 2A and B) is likely due to differences in polyphenol composition among the samples. For DMAC, DP of flavanols and flavanol linkage influence the quantitative results (Wang et al., 2016) (Figure S2). For example, monomers give higher responses than oligomers by DMAC (Prior et al., 2010; Wallace & Giusti, 2010; Wang et al., 2016). For BSA, precipitates form between BSA and flavanols with DP ≥ 3. Furthermore, the ability of flavanols to precipitate BSA increases with increasing polymer size. Only 12.4% of trimers and 23.9% of tetramers are precipitated by BSA (Harbertson et al., 2014). Concentrations of 10 polyphenols with DP 1 to 4 were quantified in a subset of our samples, listed in Table 5 and 6. Monomers (catechin and epicatechin) and dimers (PC B1, B2, and B5) represent the majority of flavanols, while oligomers (PC C1 with DP 3 and Cinn A2 with DP 4) are present only in trace amounts in all samples except for Snowdrift apple juice and cider made from Hewe's Crab, both of which are genetically different from the *Malus* × *domestica* cultivars included in this study. With the majority of polyphenols detected being monomers, dimers, and nonflavanol compounds, it is not surprising that flavanols are not detectable by BSA in many cider samples. Although not conducted in this study, a thiolysis method (Neilson et al., 2016) could be used to measure the mean DP of each sample to provide more insight into the relationship of flavanol DP and the results of these methods.

Conclusion

L-P is preferable over F-C for samples of high polyphenol content with potassium metabisulfite and ascorbic acid present, despite the lack of accuracy observed for L-P. DMAC is more specific than BSA, and provides more accurate results for samples in which the majority of flavanols are monomers and dimers, even though BSA may better predict astringency. Additionally, ratios of results by L-P to F-C, and ratios of results by DMAC to BSA vary greatly, likely due to distinct polyphenol composition and sample matrix among samples. The results of this study provide a basis by which to make informed comparisons of polyphenol or flavanol

concentrations obtained using the four methods evaluated. Furthermore, our results provide a basis for selection of the most appropriate of the four methods evaluated for a given application and fruit juice or beverage sample type.

Author Contributions

Sihui Ma designed the study, collected the data, interpreted the results, and drafted the manuscript. Cathlean Kim assisted in data collection. Laura E. Griffin collected data in Supplementary Information Figure 2 and reviewed the manuscript. Andrew P. Neilson, Gregory M. Peck, Sean F. O'Keefe, and Amanda C. Stewart assisted in experimental design and revised the manuscript.

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References

Akhtar, S., Ismail, T., Fraternale, D., & Sestili, P. (2015). Pomegranate peel and peel extracts: Chemistry and food features. *Food Chemistry*, *174*, 417–425.

Aleixandre-Tudo, J. L., Buica, A., Nieuwoudt, H., Aleixandre, J. L., & du Toit, W. (2017). Spectrophotometric analysis of phenolic compounds in grapes and wines. *Journal of Agricultural and Food Chemistry*, *65*(20), 4009–4026.

Anastasiadi, M., Mohareb, F., Redfern, S. P., Berry, M., Simmonds, M. S. J., & Terry, L. A. (2017). Biochemical profile of heritage and modern apple cultivars and application of machine learning methods to predict usage, age, and harvest season. *Journal of Agricultural and Food Chemistry*, *65*(26), 5339–5356.

AOAC. (1995). Preparation and standardization of potassium permanganate solution. *AOAC Method*, *940*, 35. Association of Official Analytical Chemists (AOAC) International.

Barua, D. N., & Roberts, E. A. (1940). Methods for the volumetric estimation of tea tannin in green-leaf and black tea. A new alkaline permanganate method. *Biochemical Journal*, *34*(12), 1524–1531.

Blainski, A., Lopes, G., & de Mello, J. (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium Brasiliense* L. *Molecules*, *18*(6), 6852–6865.

Bordenave, N., Hamaker, B. R., & Ferruzzi, M. G. (2014). Nature and consequences of non-covalent interactions between flavonoids and macronutrients in foods. *Food and Function*, *5*(1), 18–34.

Bors, W., & Michel, C. (2002). Chemistry of the antioxidant effect of polyphenols. *Annals of the New York Academy of Sciences*, *957*(1), 57–69.

Boulet, J.-C., Trarieux, C., Souquet, J.-M., Ducasse, M.-A., Caillé, S., Samson, A., . . . Cheynier, V. (2016). Models based on ultraviolet spectroscopy, polyphenols, oligosaccharides and polysaccharides for prediction of wine astringency. *Food Chemistry*, *190*, 357–363.

Bridle, P., & Timberlake, C. F. (1997). Anthocyanins as natural food colours—selected aspects. *Food Chemistry*, *58*(1–2), 103–109.

Brossaud, F., Cheynier, V., & Noble, A. C. (2001). Bitterness and astringency of grape and wine polyphenols. *Australian Journal of Grape and Wine Research*, *7*(1), 33–39.

Budavari, S. (1996). *The Merck Index — An encyclopedia of chemicals, drugs, and biologicals*. Whitehouse Station, NJ: Merck and Co., Inc.

Celeste, M., Tomas, C., Cladera, A., Estela, J. M., & Cerda, V. (1993). Thermometric titration of polyhydric phenols. Application to the determination of the tannin content of wines. *Analyst*, *118*(7), 895–898.

Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). Thorough study of reactivity of various compound classes toward the Folin−Ciocalteu reagent. *Journal of Agricultural and Food Chemistry*, *58*(14), 8139–8144.

Ewing, B. L., Peck, G. M., Ma, S., Neilson, A. P., & Stewart, A. C. (2019). Management of apple maturity and postharvest storage conditions to increase polyphenols in cider. *HortScience*, *54*(1), 143–148.

Feliciano, R. P., Shea, M. P., Shanmuganayagam, D., Krueger, C. G., Howell, A. B., & Reed, J. D. (2012). Comparison of isolated cranberry (*Vaccinium macrocarpon* Ait.) proanthocyanidins to catechin and procyanidins A2 and B2 for use as standards in the 4-(dimethylamino)cinnamaldehyde assay. *Journal of Agricultural and Food Chemistry*, *60*(18), 4578–4585.

Food Chemicals Codex. (2019). *Food chemicals codex* (11th ed.). United States Pharmacopeia.

Hagerman, A. E., & Butler, L. G. (1978). Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry*, *26*(4), 809–812.

Harbertson, J. F., Kennedy, J. A., & Adams, D. O. (2002). Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *American Journal of Enology and Viticulture*, *53*(1), 54.

Harbertson, J. F., Kilmister, R. L., Kelm, M. A., & Downey, M. O. (2014). Impact of condensed tannin size as individual and mixed polymers on bovine serum albumin precipitation. *Food Chemistry*, *160*, 16–21.

Hyman, M. R., Sansome-Smith, A. W., Shears, J. H., & Wood, P. M. (1985). A kinetic study of benzene oxidation to phenol by whole cells of *Nitrosomonas europaea* and evidence for the further oxidation of phenol to hydroquinone. *Archives of Microbiology*, *143*(3), 302–306.

Lea, A. G. H., & Arnold, G. M. (1978). The phenolics of ciders: Bitterness and astringency. *Journal of the Science of Food and Agriculture*, *29*(5), 478–483.

Lowenthal. (1877). Uber die Bestimmung des Gerbstoffs. *Analytical and Bioanalytical Chemistry (Previous as Zeitschrift Für Analytische Chemie)*, *16*, 33–48.

Ma, S., Neilson, A., Lahne, J., Peck, G., O'Keefe, S., Hurley, E. K., . . . Stewart, A. (2018). Juice clarification with pectinase reduces yeast assimilable nitrogen in apple juice without affecting the polyphenol composition in cider. *Journal of Food Science*, *83*, 2772–2781.

Margraf, T., Karnopp, A. R., Rosso, N. D., & Granato, D. (2015). Comparison between Folin-Ciocalteu and Prussian Blue assays to estimate the total phenolic content of juices and teas using 96-well microplates. *Journal of Food Science*, *80*(11), C2397–C2403.

Mercurio, M. D., & Smith, P. A. (2008). Tannin quantification in red grapes and wine: Comparison of polysaccharide- and protein-based tannin precipitation techniques and their ability to model wine astringency. *Journal of Agricultural and Food Chemistry*, *56*(14), 5528–5537.

Neilson, A. P., O'Keefe, S. F., & Bolling, B. W. (2016). High-molecular-weight proanthocyanidins in foods: Overcoming analytical challenges in pursuit of novel dietary bioactive components. *Annual Review of Food Science and Technology*, *7*(1), 43–64.

Nielsen, S. S. (2010). *Food analysis* (4th ed.). Springer.

Nunes, C. A., Alvarenga, V. O., de Souza Sant'Ana, A., Santos, J. S., & Granato, D. (2015). The use of statistical software in food science and technology: Advantages, limitations and misuses. *Food Research International*, *75*, 270–280.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–278.

Payne, M. J., Hurst, W. J., Stuart, D. A., Ou, B., Fan, E., Ji, H., & Kou, Y. (2010). Determination of total procyanidins in selected chocolate and confectionery products using DMAC. *Journal of AOAC International*, *93*(1), 89–96.

Prior, R. L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M. J., & Reed, J. (2010). Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the Science of Food and Agriculture*, *90*(9), 1473–1478.

Rothwell, J. A., Perez-Jimenez, J., Neveu, V., Medina-Remón, A., M'Hiri, N., García-Lobato, P., . . . Scalbert, A. (2013). Phenol-Explorer 3.0: A major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Database, 2013, bat070–bat070. Retrieved from https://doi.org/10.1093/database/bat070

Shrivastava, A., & Gupta, V. (2011). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists*, *2*(1), 21–25.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In L. Packer (Ed.), *Methods in enzymology* (Vol. *299*, pp. 152–178). San Diego, CA: Academic Press.

Smit, C. J. B., Joslyn, M. A., & Lukton, A. (1955). Determination of tannins and related polyphenols in foods. *Analytical Chemistry*, *27*(7), 1159–1162.

Spanos, G. A., & Wrolstad, R. E. (1992). Phenolics of apple, pear, and white grape juices and their changes with processing and storage. A review. *Journal of Agricultural and Food Chemistry*, *40*(9), 1478–1487.

Sun-Waterhouse, D. (2011). The development of fruit-based functional foods targeting the health and wellness market: A review. *International Journal of Food Science & Technology*, *46*(5), 899–920.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), 1231–1246.

USDA Food Composition Database. (2019). Retrieved from https://ndb.nal.usda.gov/ndb/search/list?home=true

U.S. Food and Drug Administration. (2015). *Analytical procedures and methods validation for drugs and biologics. Guidance for Industry*. U.S. Food and Drug Administration.

Wallace, T. C., & Giusti, M. M. (2010). Evaluation of parameters that affect the 4-dimethylaminocinnamaldehyde assay for flavanols and proanthocyanidins. *Journal of Food Science*, *75*(7), C619–C625.

Wang, Y., Singh, A. P., Hurst, W. J., Glinski, J. A., Koo, H., & Vorsa, N. (2016). Influence of degree-of-polymerization and linkage on the quantification of proanthocyanidins using 4-dimethylaminocinnamaldehyde (DMAC) assay. *Journal of Agricultural and Food Chemistry*, *64*(11), 2190–2199.

Weber, F., Schulze-Kaysers, N., & Schieber, A. (2014). Chapter 15 — Characterization and quantification of polyphenols in fruits. In W. Ronald Ross (Ed.), *Polyphenols in plants* (pp. 293–304). San Diego, CA: Academic Press.

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., & Nury, F. S. (1999). Sulfur dioxide and ascorbic acid. In *Wine analysis and production* (pp. 178–191). Boston, MA: Springer US. https://link.springer.com/book/10.1007/978-1-4757-6978-4#about

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Table S1. Sample number of commercial cider samples and their corresponding names.

Supplementary Table S2. UPLC gradients for the separation of individual polyphenols.

Supplementary Table S3. Retention times, molecular weights, and SIR channels for individual polyphenol compounds.

Supplementary Figure S1. Analytical curves for (A) F-C method; (B) L-P method (not used in quantification); (C) DMAC method; and (D) BSA precipitation method. Data points represent the mean for $n = 3$ replicates and error bars represent SEM.

Supplementary Figure S2. F-C and DMAC assay response as a function of flavanol DP. PC standards were analyzed at 0.1 mg/mL for FC and at 0.01 mg/mL for DMAC. Monomers though decamers were analyzed for each assay. 1:1 mixtures of EC + PCB_1,

PCB_2 + PCC_1, PC pentamer + PC hexamer, and PC hexamer + PC heptamer were also analyzed to look at the assay responses of mDP 1.5, 2.5, 5.5, and 6.5, respectively. Each data point represents the means ($n = 3$) ± SEM for each PC standard in each assay.

Supplementary Figure S3. Example chromatograms of (A) peak for a representative polyphenol standard, PC B1 at 1.25 mg/L (in a standard mixture, identity confirmed by mass and retention time) and (B) an example showing the peak for PC B1 detected in a sample (Ashmead's Kernel Apple juice).