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Sodium Borohydride/Chloranil-Based Assay for Quantifying Total Flavonoids

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A novel sodium borohydride/chloranil-based (SBC) assay for quantifying total flavonoids, including flavones, flavonols, flavonones, flavononols, isoflavonoids, flavanols, and anthocyanins, has been developed. Flavonoids with a 4-carbonyl group were reduced to flavanols using sodium borohydride catalyzed with aluminum chloride. Then the flavan-4-ols were oxidized to anthocyanins by chloranil in an acetic acid solution. The anthocyanins were reacted with vanillin in concentrated hydrochloric acid and then quantified spectrophotometrically at 490 nm. A representative of each common flavonoid class including flavones (baicalein), flavonols (quercetin), flavonones (hesperetin), flavononols (silibinin), isoflavonoids (biochanin A), and flavanols (catechin) showed excellent linear dose–responses in the general range of 0.1−10.0 mM. For most flavonoids, the detection limit was about 0.1 mM in this assay. The recoveries of quercetin from spiked samples of apples and red peppers were 96.5 ± 1.4% (CV = 1.4%, $n = 4$) and 99.0 ± 4.2% (CV = 4.2%, $n = 4$), respectively. The recovery of catechin from spiked samples of cranberry extracts was 97.9 ± 2.0% (CV = 2.0%, $n = 4$). The total flavonoids of selected common fruits and vegetables were measured using this assay. Among the samples tested, blueberry had the highest total flavonoid content (689.5 ± 10.7 mg of catechin equiv per 100 g of sample), followed by cranberry, apple, broccoli, and red pepper. This novel SBC total flavonoid assay can be widely used to measure the total flavonoid content of fruits, vegetables, whole grains, herbal products, dietary supplements, and nutraceutical products.

KEYWORDS: Flavonoids; phytochemicals; antioxidant; phenolics; anthocyanins; fruits; vegetables; diet and cancer

INTRODUCTION

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases, such as cardiovascular diseases and cancer (*1, 2*). Phytochemicals have been suggested as being responsible for the health benefits of fruits and vegetables (*3, 4*). Flavonoids, which are one of the main classes of phytochemicals widely existing in fruits, vegetables, whole grains, and other plants, play an important role in human health (*3*). Flavonoids have been reported to have many health benefits including antioxidant activity, anti-inflammation, antibacterial, and antiviral effects, antiallergenic reaction, and antimutagenic and anticancer properties (*3–8*). Therefore, it is important to quantify the total flavonoid content in fruits, vegetables, whole grains, dietary supplements, and nutraceutical products.

Flavonoids are a class of plant secondary metabolites derived from the condensation of cinnamic acid with three malonyl-CoA groups. These compounds have a 15-carbon skeleton, which consist of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocyclic ring, or C ring (**Figure 1**). Differences in the generic structure of the heterocyclic C ring lead to their classification as flavones, flavonols, flavonones, flavononols, isoflavonoids, flavanols (catechins), and anthocyanidins (*3*) (**Figure 2**). Flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols [catechin, epicatechin, epigallocatechin, epicatechin gallate (ECG), and epigallocatechin gallate (EGCG)], flavonones (hesperetin and naringenin), anthocyanidins, and isoflavonoids (genistein and biochanin A) are common flavonoids found in the human diet (**Figure 2**). Flavonoids are most frequently found in nature as conjugates in glycosylated or esterified forms but can occur as aglycones, especially as a result of food processing (*3, 9*).

Flavonoids, which include many types of compounds, lack a unique functional group for total flavonoid analysis. At this time, there is no valid assay that can measure the total flavonoids including all of the main types described above. Currently, there are two commonly used assays for flavonoid analyses: the

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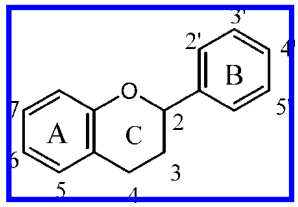


Figure 1. Generic structure of flavonoids.

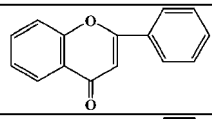
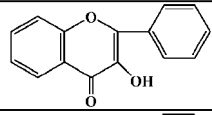
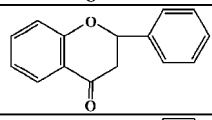
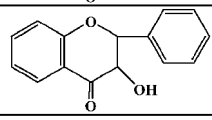
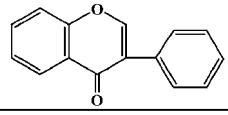
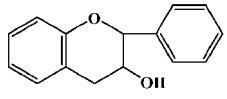
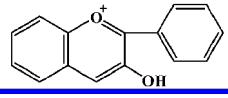
Flavonoids	Structure	Representative compounds
1. Flavone		Apigenin Luteolin Baicalein
2. Flavonol		Quercetin Kaempferol Myricetin
3. Flavonone		Hesperetin Naringenin
4. Flavononol		Silibinin
5. Isoflavonoid		Daidzein Genistein Biochanin A
6. Flavan-3-ol (Catechin)		Catechin Epicatechin ECG EGCG
7. Anthocyanin		Cyanidin Malvidin Pelargonidin

Figure 2. Chemical structures and representative compounds of the main types of flavonoids.

colorimetric Folin−Ciocalteu (F-C) assay (*10, 11*) and a high-performance liquid chromatography (HPLC) method (*12, 13*). The F-C assay is one of the most popular assays for phenolics (*10, 11*). The F-C assay is simple and widely used to determine total phenolics in fruits and vegetables (*14–20*). However, the F-C assay is not specific for flavonoids and detects compounds with active hydroxyl group(s) including phenolic acids, flavonoids, ascorbic acid, reducing sugars, some amino acids, and aromatic amines. On the other hand, the HPLC assay is specific and sensitive. However, it is limited by (1) the numbers of pure standard compounds available and (2) the separation capacity of the HPLC column. Because there are thousands of flavonoid compounds (*3*), it is impossible to determine total flavonoids in foods using the HPLC method. Therefore, there is an urgent need to develop a specific assay to determine the total flavonoid content in foods, dietary supplements, functional foods, and nutraceutical products.

Here we report a novel sodium borohydride/chloranil (SBC)-based assay for quantifying total flavonoids. This new assay is accurate, precise, and specific for flavonoids and can be widely used to determine the total flavonoid content of fruits, vegetables, whole grains, herbal products, dietary supplements, and nutraceutical products in the future.

MATERIALS AND METHODS

Chemicals. Methanol (MeOH), hydrochloric acid (HCl), acetic acid, and acetone were of analytical grade and were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Ethanol (EtOH, anhydrous, 200 proof), sodium borohydride ($NaBH_4$, reagent grade), chloranil (analytical grade), and vanillin (analytical grade) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Tetrahydrofuran (THF, analytical grade) and aluminum chloride ($AlCl_3 \cdot 6H_2O$, analytical grade) were purchased from Fisher Scientific (Fair Lawn, NJ). All flavonoids, including quercetin (≥95%), baicalein (≥98%), hesperetin (≥95%), silibinin, biochanin A, and (+)-catechin (≥98%), were purchased from Sigma-Aldrich, Inc.

Extraction of Flavonoids from Fruits and Vegetables. Red peppers, broccoli, and fresh cranberries were purchased from a local supermarket (Ithaca, NY). Apples of the Red Delicious variety were purchased from the Cornell Orchard (Cornell University, Ithaca, NY). Wild blueberries were obtained from the Wild Blueberry Association of North America (Damariscotta, ME). Phytochemicals were extracted using a modified method reported previously (*16–18*). Briefly, fresh fruits or vegetables were homogenized for 5 min with chilled 80% acetone (1:3, w/v) using a chilled Waring blender. Samples were then homogenized using a Polytron homogenizer for an additional 3 min. The homogenates were filtered and the filtrate was evaporated under vacuum at 45 °C to dryness. The samples were reconstituted using 70% methanol and stored at −40 °C until analysis.

Preparation of Reagents. The following reagents were used in this assay: (1) 50 mM sodium borohydride in ethanol; (2) 1.8% (w/v, 74.56 mM) aluminum chloride ($AlCl_3 \cdot 6H_2O$) in ethanol; (3) 20 mM chloranil in THF; (4) 0.8 M acetic acid in water; (5) 16% (w/v, 1052 mM) vanillin in methanol; and (6) 37% hydrochloric acid. Catechin standards (0.1−8.0 mM) or tested samples were dissolved in THF/EtOH (1:1, v/v).

Protocol for Determination of Total Flavonoids (SBC Total Flavonoid Assay). Samples in 70% methanol/H_2O were thawed and added into test tubes (15 × 150 mm), then dried to dryness under nitrogen gas, and reconstituted in 1 mL of THF/EtOH (1:1, v/v). Catechin standards (0.1−8.0 mM) were prepared fresh each day before use in 1 mL of THF/EtOH (1:1, v/v). Each test tube with 1 mL of sample solution or 1 mL of catechin standard solution had 0.5 mL of 50 mM $NaBH_4$ solution and 0.5 mL of 74.56 mM $AlCl_3$ solution added and then was shaken in an orbital shaker (Laboratory-Line Instruments, Inc., Melrose Park, IL) at room temperature for 30 min. Then an additional 0.5 mL of $NaBH_4$ solution was added into each test tube with continuing shaking for another 30 min at room temperature. Cold acetic acid solution (2.0 mL of 0.8 M, 4 °C) was added into each test tube, and the solutions were kept in the dark for 15 min after a thorough mix. Then 1 mL of 20 mM chloranil was added into each tube, which was heated at 100 °C with shaking for 60 min in a reciprocal shaking bath (Precision Scientific Inc., Chicago, IL). The reaction solutions were cooled using tap water, and the final volume was brought to 4 mL using methanol. Then, 1 mL of 1052 mM vanillin was added into each tube, followed by mixing. Concentrated HCl (2 mL of 12 M) was added into each tube, and the reaction solutions were kept in the dark for 15 min after a thorough mix. Aliquots of the final reaction solutions (200 μL) were added into each well of a 96-well plate, and absorbances were measured at 490 nm using a MRX Microplate Reader with Revelation work station (Dynex Technologies, Inc., Chantilly, VA). Total flavonoid content was expressed as milligrams of catechin equivalents per 100 g of sample. Data are reported as mean ± SD for at least three replicates. The recoveries of quercetin from spiked samples of apples and red peppers were 96.5 ± 1.4% (CV = 1.4%, $n = 4$) and 99.0 ± 4.2% (CV = 4.2%, $n = 4$), respectively. The recovery of catechin from spiked samples of cranberry extracts was 97.9 ± 2.0% (CV = 2.0%, $n = 4$).

Optimization of the Concentrations of Sodium Borohydride, Aluminum Chloride, Acetic Acid, Chloranil, and Vanillin. The optimizations of the concentrations of chemicals were performed using the above standard protocol with 5 mM quercetin with different concentrations of sodium borohydride, aluminum chloride, acetic acid, chloranil, and vanillin. Data are reported as mean ± SD for at least three replicates.

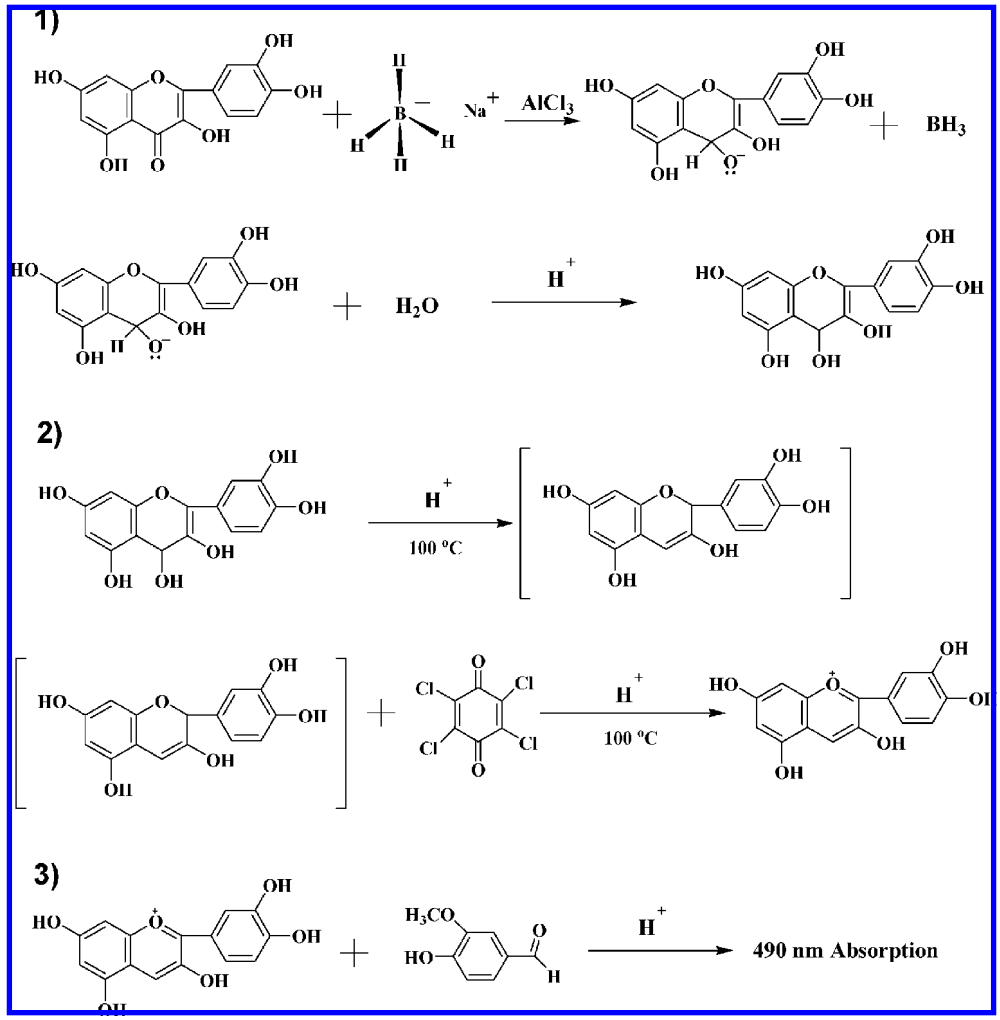


Figure 3. Main chemical reactions of the SBC total flavonoid analysis: (1) Flavonoids with 4-carbonyl group were reduced to flavan-4-ols using sodium borohydride catalyzed with aluminum chloride. (2) Flavan-4-ols were oxidized to anthocyanins by chloranil in acetic acid solution. (3) Anthocyanins were reacted with vanillin in concentrated hydrochloric acid to yield a characteristic absorbance at 490 nm.

Statistical Analysis. Data from this study were reported as the mean ± SD for at least three replicates for each sample. Statistical analysis was conducted using SigmaStat version 9.0 (Jandel Corp., San Raphael, CA). Differences among treatments were determined using a *t* test ($p < 0.05$).

RESULTS AND DISSCUSION

Principle and Main Chemical Reactions for the Total Flavonoid Analysis. Flavonoids with a 4-carbonyl group (flavones, flavonols, flavonones, flavononols, and isoflavonoids) were reduced to flavan-4-ols (catechins) using sodium borohydride (*21*). This reaction was catalyzed by the addition of aluminum chloride to achieve a high yield of flavan-4-ols. The flavanols (catechins) were oxidized to anthocyanins by chloranil in acetic acid solution (*22–24*). The anthocyanins generated in the reaction were quantified spectrophotometrically at 490 nm after the addition of vanillin and concentrated hydrochloric acid (*25, 26*). The main chemical reactions of this new assay are given in **Figure 3**.

Quercetin, a typical flavonoid existing widely in nature and participating in the reaction from the initial step, was selected for optimization of the concentrations of the key reagents, sodium borohydride, aluminum chloride, acetic acid, chloranil, and vanillin, as described below.

Effect of Sodium Borohydride Concentration on Reduction of the 4-Carbonyl Group of Flavonoids. The effect of the sodium borohydride concentration on the reduction of the carbonyl group of quercetin is given in **Figure 4a**. Catalyzed by aluminum chloride, the 4-carbonyl group of flavonoids was reduced to flavan-4-ol by sodium borohydride. This reaction occurred at room temperature. The concentration of sodium borohydride had an obvious effect on the reaction. At concentrations from ~4 to ~20 mM, sodium borohydride reduced flavonoids to flavan-4-ols in a dose-dependent manner and reached a peak at a concentration of about 20 mM; then the absorbance decreased at concentrations of 25−40 mM of sodium borohydride.

Effect of Aluminum Chloride Concentration on the Reduction of the 4-Carbonyl Group of Flavonoids. The effect of aluminum chloride concentration on the reduction of the carbonyl group of flavonoids is given in **Figure 4b**. We found that aluminum chloride had a significant catalytic effect on the reducing reaction, leading to a high yield of flavan-4-ols. The yield of flavan-4-ols increased in a dose-dependent manner with the addition of aluminum chloride, reached a peak at a concentration of 14.9 mM aluminum chloride, and then decreased at a concentration of 16.6 mM (**Figure 4b**).

Effect of Acetic Acid Concentration on the Absorbance at 490 nm. Figure 4c shows the effect of acetic acid concentration on the total flavonoid analysis. Acetic acid provided an acidic condition for the oxidation of flavan-4-ols, which was the reduced product of flavonoids with a 4-carbonyl group. The

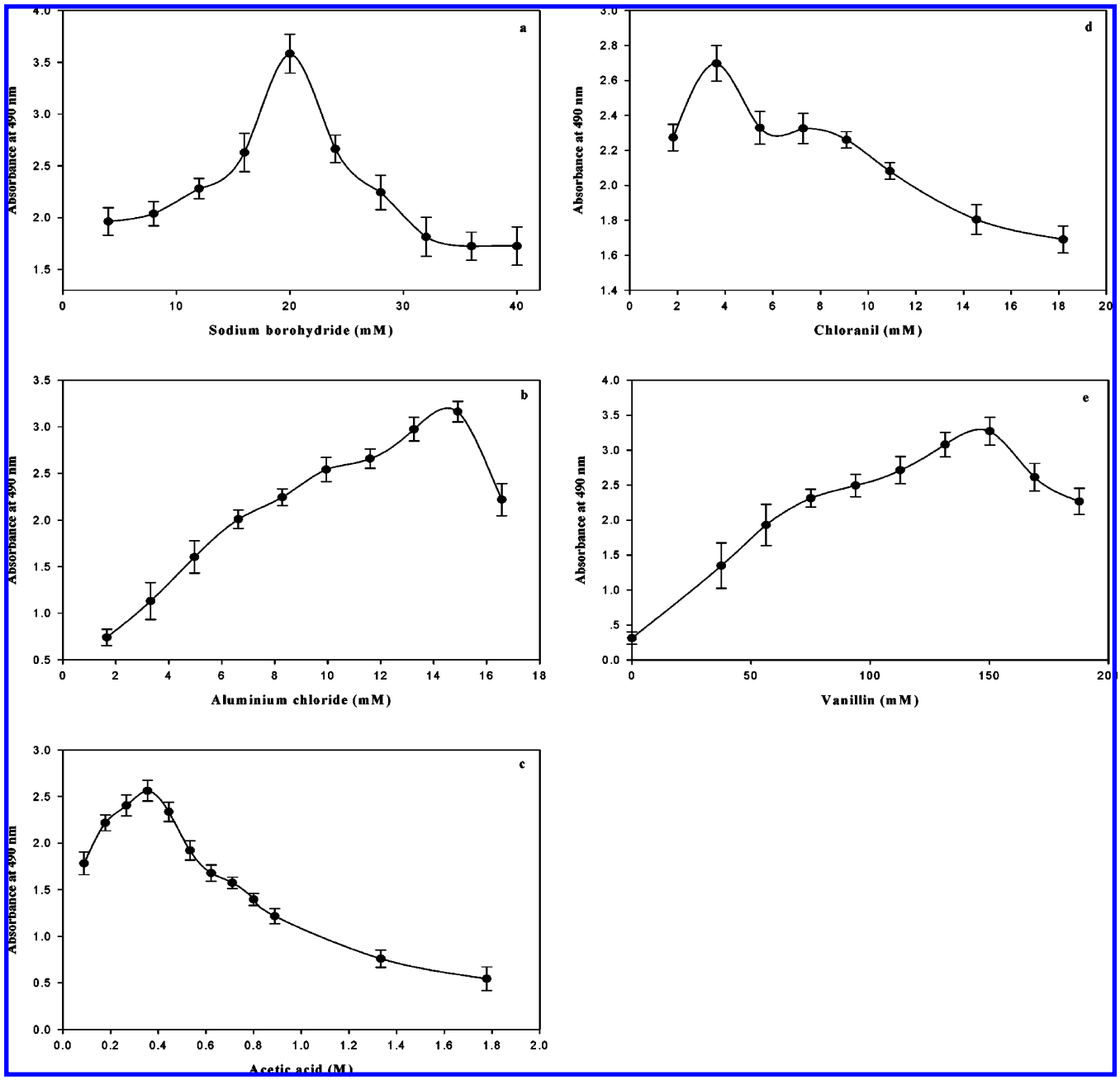


Figure 4. Optimization of the concentrations of sodium borohydride (**a**), aluminum chloride (**b**), acetic acid (**c**), chloranil (**d**), and vanillin (**e**) in the SBC total flavonoid assay. The optimizations of the concentrations of chemicals were performed using the standard protocol with 5 mM quercetin with different concentrations of sodium borohydride (4.0–40.0 mM), aluminum chloride (1.6–16.6 mM), acetic acid (0.1–1.8 M), chloranil (1.8–18.2 mM), and vanillin (0–187.8 mM). Data are reported as mean ± SD for at least three replicates.

concentration of acetic acid dramatically affected the oxidation of flavan-4-ols. The absorbance at 490 nm increased with the addition of acetic acid in a dose-dependent manner and reached a peak at a concentration of 0.36 M; then the absorbance decreased dramatically at acetic acid concentrations of >0.4 M. Therefore, the optimum concentration of acetic acid was determined as 0.36 M in this reaction.

Effect of Chloranil Concentration on the Oxidation of Flavan-4-ol. Figure 4d shows the effect of chloranil concentrations on the oxidation of flavan-4-ols. Flavan-4-ols (catechins) were oxidized to anthocyanins by chloranil under acidic condition (*21, 24*). Chloranil concentration affected the yield of anthocyanin formation. The optimum concentration of chloranil for oxidizing flavan-4-ols to anthocyanins was 3.64 mM (**Figure 4d**).

Effect of Vanillin Concentration on the Absorbance at 490 nm. Anthocyanins produced a characteristic absorbance at 490 nm after reacting with vanillin under a strong acidic condition (*25, 26*). With added vanillin, the absorbance at 490 nm was directly proportional to vanillin concentration in a dose-dependent manner up to an optimum concentration of vanillin of 150.3 mM (**Figure 4e**).

Validation of the Quantitative Method. Selected flavonoid compounds from the main classes of dietary flavonoids (*3*), including baicalein (flavone), quercetin (flavonol), hesperetin (flavonone), silibinin (flavononol), biochanin A (isoflavonoid), and catechin (flavan-3-ol), as well as two phenolic acids (gallic acid and ferulic acid) were tested using this assay. All of the flavonoids tested showed a typical dose–response curve in this assay (**Figure 5**). In the linear range of each flavonoid tested,

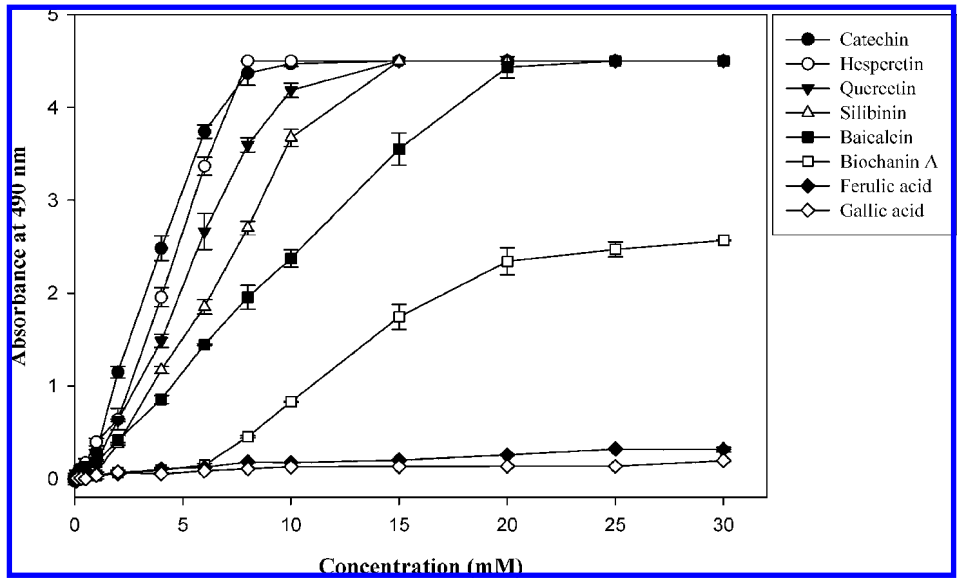


Figure 5. Dose–responses of typical flavonoid compounds (quercetin, baicalein, hesperetin, silibinin, catechin, and biochanin A) from the main classes of dietary flavonoids and of phenolic acids (gallic acid and ferulic acid) (mean ± SD, $n = 3$).

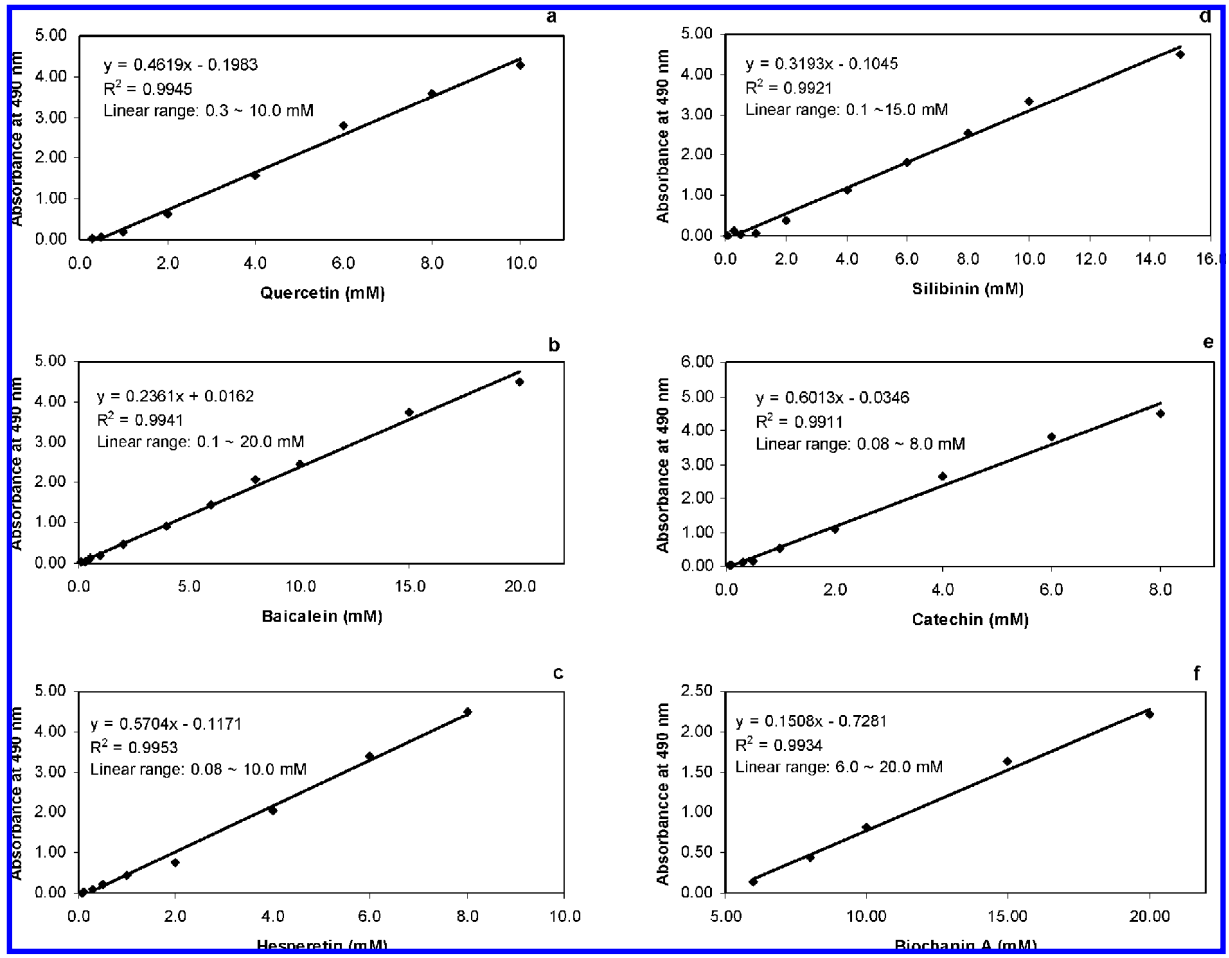


Figure 6. Standard curves for selected flavonoid compounds from the main classes of dietary flavonoids: (**a**) quercetin (flavonol); (**b**) baicalein (flavone); (**c**) hesperetin (flavonone); (**d**) silibinin (flavononol); (**e**) catechin (flavan-3-ol); (**f**) biochanin A (isoflavonoid) (mean ± SD, $n = 3$).

the absorbance at 490 nm was directly proportional to the concentration of each compound with $R^2 > 0.99$ (**Figure 6**). In addition, gallic acid and ferulic acid, two common phenolic acids in fruits, vegetables, and whole grains (*3*), were selected to test the selectivity of this new assay. These two typical phenolic acids showed negative responses to this assay (**Figure 5**), indicating this new assay exhibited high selectivity to flavonoids.

Flavonoids were converted to anthocyanins after two main chemical reactions: reduction of the 4-carbonyl group and oxidation of the C-ring of the reduction products. After being converted to anthocyanins, the vanillin−anthocyanin adducts from the different flavonoids, quercetin and catechin, had very similar UV–vis spectra at 190–800 nm (**Figure 7**), with maximal absorbances around 490 nm in the reaction systems.

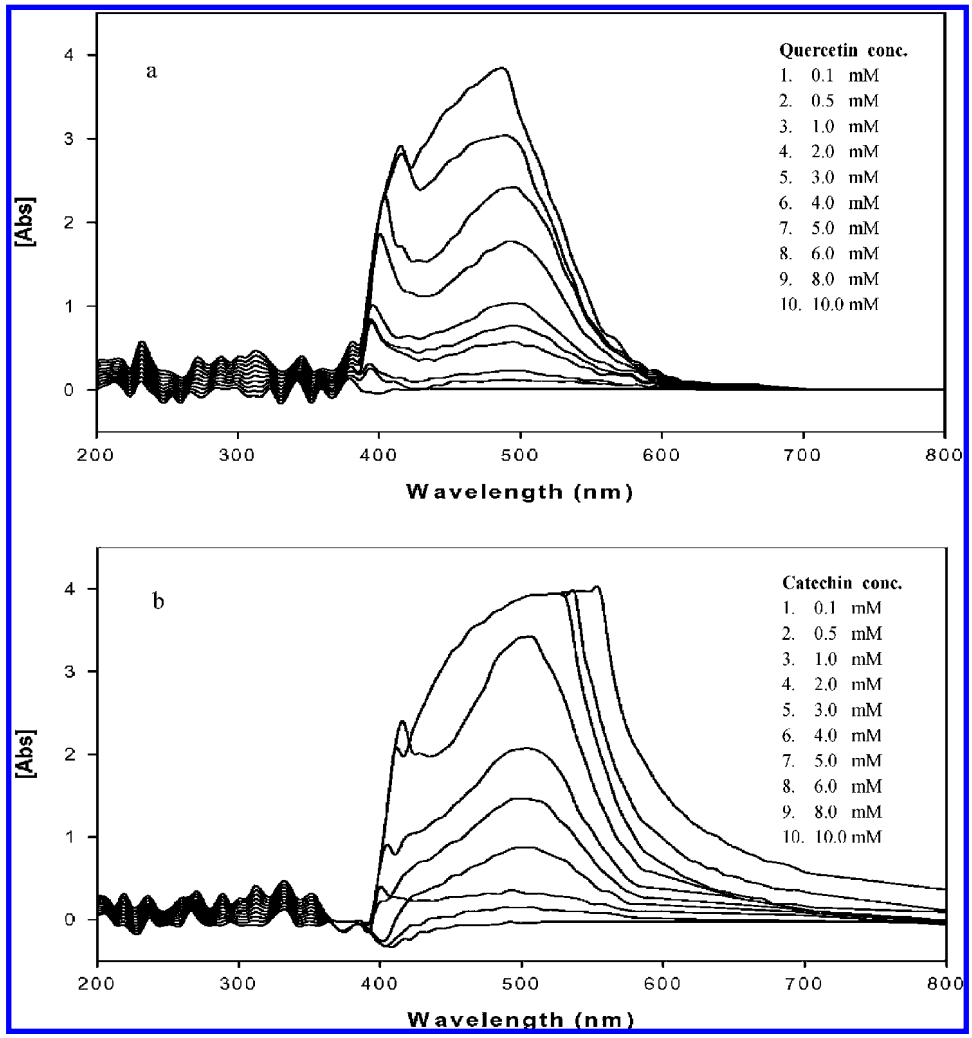


Figure 7. UV−visible spectra of the final reaction product of the SBC total flavonoid analysis using different concentrations of selected flavonoids: (**a**) quercetin (0.1−10.0 mM; quercetin participates in the reaction from the first step of the SBC assay); (**b**) catechin (0.1−10.0 mM; catechin participates in the reaction from the second step of the SBC assay).

All of the selected common flavonoid compounds from the different main types of flavonoids exhibited typical dose–responses with the SBC total flavonoid quantitative method (**Figure 5**). Their slopes in the dose–response curves were similar except for the isoflavonoids (such as biochanin A). Among the tested flavonoids using this assay, catechin showed the greatest slope. Catechin belonged to the flavanol class, so it omitted the reduction step and essentially participated directly in the assay at the second step (**Figure 3**). If there is a single carbon−carbon bond at C-2(3) of the flavonoid C ring, such as in flavonones (e.g., hesperetin) and flavononols (e.g., silibinin), the carbonyl groups at the C rings are much easier to reduce with $NaBH_4/AlCl_3$ and have a greater slope in the dose–response curves. For isoflavonoids (e.g., biochanin A), the B rings are linked at C-3 instead of at C-2. The carbonyl groups at the C rings are more difficult to reduce owing to the steric interference. Isoflavonoids showed much lower slopes when compared to the other flavonoids.

Suggested Standards for Total Flavonoid Analysis. To be able to compare the data in the literature between laboratories, the method should be standardized. We recommend catechin be used as a standard in this new assay for quantifying total flavonoids. However, for some samples rich in isoflavonoids, such as soybeans and related products, isoflavonoid should be used as the standard due to the low slope of the dose–response curve (**Figures 5** and **6f**).

Total Flavonoid Content of Selected Fruits and Vegetables. Total flavonoid contents of selected fruits and vegetables were determined using this new assay and were expressed as milligrams of catechin equivalents per 100 g of fresh weight of the edible part of the fruits or vegetables (**Figure 8**). Among the samples tested, blueberry showed the highest total flavonoid content (689.5 ± 10.7 mg of quercetin equiv/100 g of sample), followed by cranberry (341.5 ± 23.4 mg/100 g), apple (170.5 ± 7.9 mg/100 g), broccoli (116.5 ± 8.2 mg/100 g), and red pepper (73.2 ± 1.9 mg/100 g).

Advantage of the SBC Assay for Total Flavonoid Analysis. Flavonoids are a group of phenolic compounds with antioxidant activity that have been identified in fruits, vegetables, and other plant foods and have been linked to reducing the risk of major chronic diseases (*3*). They commonly have a generic structure consisting of two aromatic rings (A and B rings) linked by three carbons that are usually in an oxygenated heterocycle ring, or C ring (**Figure 1**). Differences in the generic structure of the heterocyclic C ring classify them as different types of flavonoids such as flavones, flavonols, flavonones, flavononols, isoflavonoids, flavanols (catechins), and anthocyanidins (*3*) (**Figure 2**). Although sharing the same generic structure, flavonoids from different types lack unique functional groups for total flavonoid analysis. There is no valid assay that can measure total flavonoids including all main types of flavonoids. Currently, the quantitative methods of flavonoids are based on the

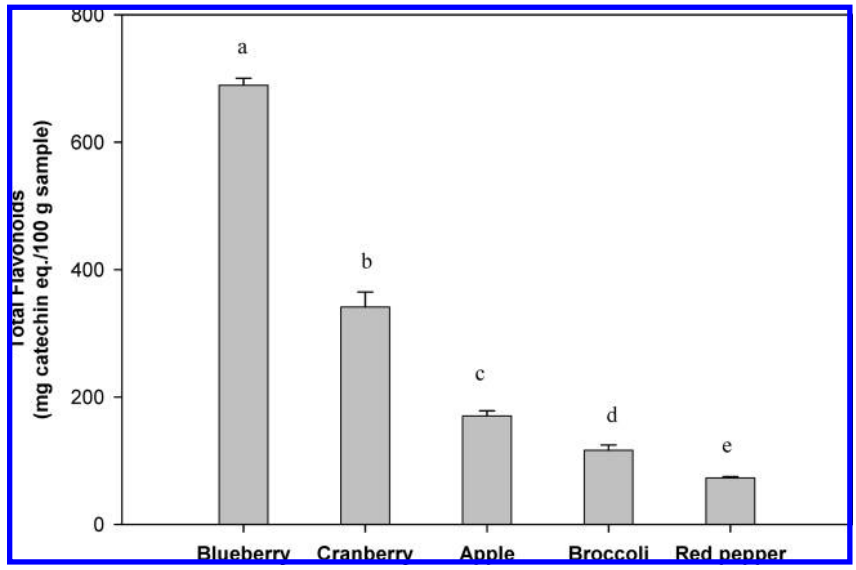


Figure 8. Total flavonoid content of selected fruits and vegetables (mean ± SD, $n = 3$). Bars with different letters are significantly different ($p < 0.05$).

colorimetric F-C assay (*10, 11*) and a HPLC method (*12, 13*). The F-C assay is one of the most popular assays for phenolic analysis (*10, 11*). The principle of the F-C assay is the reduction of the Folin−Ciocalteu reagent in the presence of phenolics resulting in the production of molybdenum−tungsten blue that is measured spectrophotometrically. The F-C assay is simple and widely used to determine total phenolics in fruits and vegetables (*15–20, 27*); however, it is not specific to flavonoids and detects compounds with active hydroxyl group(s) including phenolic acids, flavonoids, ascorbic acid, reducing sugars, some amino acids, and aromatic amines. The second commonly used assay for flavonoid analysis is HPLC. The HPLC assay is specific and sensitive. However, it is limited by the numbers of pure standard compounds available and the separation capacity of the HPLC column. In addition, there are more than 4000 flavonoids that have been identified, but a large percentage of them remain unknown (*3*). Therefore, it is impossible to determine total flavonoids in foods using the HPLC method with limited standards and the separation capacity.

The SBC assay for total flavonoids described here is very specific. This assay can detect all types of flavonoids, including flavones, flavonols, flavonones, flavononols, isoflavonoids, flavanols, and anthocyanins. The flavonoids with a 4-carbonyl group were reduced to flavan-4-ols using sodium borohydride catalyzed by aluminum chloride. Then the flavanols were oxidized to anthocyanins by chloranil in acetic acid solution. The anthocyanins were reacted with vanillin at concentrated hydrochloric acid conditions and then quantified spectrophotometrically at 490 nm. All common flavonoids, including flavones (baicalein), flavonols (quercetin), flavonones (hesperetin), flavononols (silibinin), isoflavonoids (biochanin A), and flavanols (catechin), showed excellent dose–responses in their linear ranges (**Figure 6**). The SBC flavonoid assay is accurate, precise, and specific for flavonoids and will be a powerful tool in antioxidant research. For most flavonoids, the detection limit is about 0.1 mM. This assay can be widely used to determine total flavonoid content in foods to support flavonoid-related health claims and to provide more accurate data for future epidemiological studies and human clinical trials. It would enable us to establish a link between the consumption of flavonoid-rich foods and their health benefits in chronic disease prevention.

In summary, a novel SBC assay for quantifying total flavonoids including flavones, flavonols, flavonones, flavononols, isoflavonoids, flavanols (catechins), and anthocyanins has been developed. This assay has high accuracy and precision, is specific to flavonoids, and can be widely used to determine total flavonoid content in fruits, vegetables, whole grains, dietary supplements, and nutraceutical products. The total flavonoids of some common fruits and vegetables were measured using the SBC total flavonoid assay. Among the samples tested, blueberry had the highest total flavonoid content, followed by cranberry, apples, broccoli, and red pepper. This new assay can be widely used to measure total flavonoid content in fruits, vegetables, whole grains, herbal products, dietary supplements, and nutraceutical products.

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