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Rapid Peroxyl Radical Scavenging Capacity (PSC) Assay for Assessing both Hydrophilic and Lipophilic Antioxidants

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This paper reports a simple, rapid, and sensitive assay for assessing peroxyl radical scavenging capacity (PSC) of both hydrophilic and lipophilic antioxidant compounds and food extracts. The assay is based on the degree of inhibition of dichlorofluorescin oxidation by antioxidants that scavenge peroxyl radicals, generated from thermal degradation of 2,2′-azobis(amidinopropane). For hydrophilic antioxidant activity, the dose required to cause a 50% inhibition of the reaction (EC_{50}) ranged from 2.41 ± 0.02 (EGCG) to 21.26 ± 0.38 μM (ferulic acid). EC_{50} values for the hydrophilic antioxidant activity of food extracts ranged from 309.2 ± 3.63 (apple) to 3345.1 ± 151.5 μmol of vitamin C equiv/100 g for wheat bran. The EC_{50} values for lipophilic antioxidant activity were 1.58 ± 0.11 (Trolox), 4.35 ± 0.43 (α-tocopherol), 18.94 ± 0.38 (BHA), and 182.69 ± 13.7 μM (BHT). Whole grain lipophilic antioxidant activity ranged from 3.49 ± 0.57 (wheat) to 8.79 ± 1.98 μmol of α-tocopherol equiv/100 g of rice. Hydrophilic antioxidant activity contributed >98% of the total antioxidant activity (hydrophilic plus lipophilic) of whole grains tested. The PSC assay was accurate (86−108% recovery), precise (0.12−11% CV), and reproducible (12% RSD) and produced results comparable to those of similar published assays. The PSC assay can be routinely used to analyze or screen both hydrophilic and lipophilic antioxidants or food extracts and will be a valuable alternative biomarker for future epidemiological studies of chronic diseases.

KEYWORDS: Antioxidant activity; hydrophilic; lipophilic; peroxyl radical; whole grain; cyclodextrin

INTRODUCTION

The destructive effects of free radicals have been linked to the etiology of many chronic diseases and aging. This is because reactive free radicals can attack important biomolecules such as proteins, DNA, and lipids and lead to chronic disease initiation and progression (*1*−*4*). The human body has several mechanisms to combat reactive free radicals to maintain a balance between oxidants and antioxidants. Under certain conditions, the body's antioxidant defense mechanism may be overwhelmed. The resulting imbalance in favor of free radials leads to oxidative stress. Therefore, the body needs sufficient external sources of antioxidants to keep this balance and to prevent oxidative stress and the accompanying chronic diseases (*5*). The reduced risk of chronic diseases associated with dietary antioxidant intakes have been reported (*6*, *7*).

Techniques that measure total antioxidant activities of foods can be valuable in studying the relationship between dietary factors and chronic disease prevention, as well as providing vital information for selecting good dietary sources of antioxidants. This has led to the development of diverse new or improved methods applied to all kinds of plant-based foods (*8*−*14*). Most of the data presented in the literature have been on antioxidant activities of water-soluble food extracts. Of additional interest, however, is the antioxidant activity of lipid-soluble components of foods, which are becoming very important in combating specific types of radicals and diseases. Lipophilic antioxidants play unique roles in the body (*15*−*19*). Therefore, measurement of their contributions to the total antioxidant activity of foods can assist in elucidating the role these compounds play in disease prevention and health promotion. Information on total antioxidant capacity (both lipophilic and hydrophilic) would therefore give a more complete spectrum of protection provided by foods and their contribution toward disease prevention. Most current antioxidant capacity assays are performed in aqueous solutions and are not suitable for lipophilic antioxidant capacity measurements.

Recent developments to measure antioxidant capacities of lipophilic compounds are mere modifications of methods for hydrophilic compounds with major emphasis on increasing the solubility of lipophilic compounds in the aqueous environments used (*8*−*12*). Cyclodextrin has exhibited great potential for incorporating lipophilic compounds into aqueous environments (*20*, *21*) and was used in lipophilic antioxidant activity assays. Various derivatives of cyclodextrin are available, and their solubilizing power is dependent on the derivative type, the

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compound to be solubilized, and the medium (*21*). Randomly methylated β-cyclodextrin was used to increase the solubility of lipophilic antioxidants in aqueous solution in the modified $ORAC_{FL}$ (*12*).

Another major improvement in measuring antioxidant capacity has been the introduction of fluorescein dyes as fluorescent probes for monitoring the reaction (*12*−*14*). Incorporation of fluorescent probes and cyclodextrin into antioxidant capacity assays have extended the application and usefulness of data generated from such experiments. For example, the methods of Ou et al. (*13*) and Huang et al. (*12*) were recently adopted by Wu et al. (*22*) to measure the hydrophilic and lipophilic antioxidant capacities of over 100 common foods in the U.S. market.

The overall objective of this study was to develop a rapid and sensitive peroxyl radical scavenging capacity (PSC) assay for accurately assessing both hydrophilic and lipophilic antioxidants. Our specific objectives were (1) to develop a hydrophilic peroxyl radical scavenging capacity (hydro-PSC) assay incorporating dichlorofluorescin diacetate (DCFH-DA) as a fluorescent probe to monitor the reaction; (2) to further modify the hydro-PSC assay as a lipophilic peroxyl radical scavenging capacity (lipo-PSC) assay, using randomly methylated β-cyclodextrin (RMCD) to increase the solubility of lipophilic compounds and extracts in aqueous solution; and (3) to test the accuracy, precision, and reproducibility of the two assays as the methods for assessing both hydrophilic and lipophilic antioxidants or food extracts.

MATERIALS AND METHODS

Chemicals. Ascorbic acid, catechin, butylated hydroxytoluene (BHT), DCFH-DA, epigallocatechin (EGCG), chlorogenic acid, caffeic acid, quercetin, Trolox, and ferulic acid were purchased from Sigma-Aldrich (St. Louis, MO). RMCD was from Cyclodextrin Technologies (High Springs, FL). Gallic acid and α-tocopherol were purchased from ICN Biomedicals, Inc. (Aurora, OH). 2,2′-Azobis(amidinopropane) (ABAP) was purchased from Wako Chemicals (Richmond, VA).

Sample Description and Preparation. Pure compounds for hydrophilic antioxidant activity analyses were dissolved in 12.5% acetone/water mixtures, whereas pure compounds for lipophilic antioxidant activity analyses were dissolved in acetone. All stock solutions were further appropriately diluted with 75 mM phosphate buffer (pH 7.4) for hydrophilic antioxidant activity analyses or 12% RMCD, prepared in 50% acetone in water, for lipophilic antioxidant activity analyses (*12*). Fresh apple, grape, and cranberry fruits were obtained from the local market. Wheat was obtained from the Cornell Small Grains Program in the Department of Plant Breeding at Cornell University (Ithaca, NY). Corn, oats, and rice were obtained from General Mills (Golden Valley, MN). All grain samples were milled into whole grain flour using 60-mesh size screen and thoroughly mixed. Also, the wheat sample was milled into two fractions: endosperm and bran/germ flour fractions by the U.S. Department of Agriculture Soft Wheat Quality Laboratory in Wooster, OH. Each grain sample was stored at −20 °C until used. White wine and red wine samples were provided by New York State Wine and Grape Foundation (Penn Yan, NY). Plasma samples were obtained in our laboratory from rats fed with and without cranberry extracts.

Extraction of Phytochemicals. Phenolic compounds of fruits were extracted using a previously reported method (*23*). Briefly, 200 g of fresh fruit was extracted with 80% acetone in water. The solvent was evaporated at 45 °C, and the extracts were dissolved in water. Water-soluble phytochemicals from wheat bran were obtained by alkaline digestion with 2 M NaOH followed by extraction of the acidified digest with ethyl acetate (*24*, *25*). Hydrophilic phytochemicals of whole grain flours of corn, oats, wheat, and rice were extracted using modified versions of methods previously described (*24*, *25*). Whole grain flour (0.7 g) was extracted with 3 × 2 mL of methanol/tetrahydrofuran (1:1, v/v) at 75 °C for 5 min, and the extracts were pooled. The solid residue was kept for further analysis. The pooled extracts were diluted with 2 mL of water and extracted twice with 5 mL of hexane. Free hydrophilic phytochemicals were then extracted from the acidified aqueous phase using ethyl acetate. The ethyl acetate fraction was evaporated to dryness under nitrogen gas at 35 °C, and the extracts were redissolved in 12.5% acetone in water for hydrophilic antioxidant capacity analysis (free hydrophilic). The remaining whole grain solid residue was digested with 3 mL of 2 M NaOH, and the acidified digest was extracted with ethyl acetate. Similarly, ethyl acetate fractions were pooled and evaporated, and the extracts were redissolved in 12.5% acetone in water for bound hydrophilic antioxidant capacity analyses. Lipophilic phytochemicals in whole grain flour (5 g) were extracted by sonication using 3 × 15 mL of hexane/dichloromethane (1:1, v/v) for 5 min at room temperature. The organic solvent was evaporated under nitrogen gas stream at 35 °C, and residues were redissolved in 300 μL of acetone and further diluted with 2.7 mL of 12% RMCD for lipophilic antioxidant capacity analyses. Wine samples were centrifuged at 13000*g* and appropriately diluted with buffer for analysis.

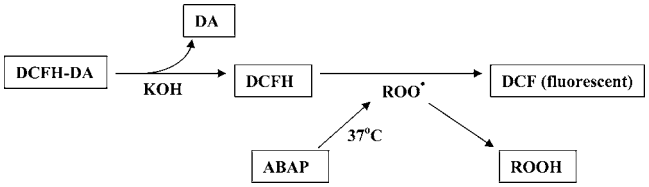

Figure 1. Proposed reaction mechanism of peroxyl radical scavenging capacity assay.

Hydrophilic Peroxyl Radical Scavenging Capacity Assay. The underlying reaction mechanism for this assay is shown in **Figure 1**. Thermal degradation of ABAP produces peroxyl radicals (ROO•) (*26*, *27*), which oxidize nonfluorescent dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF). The degree of inhibition of DCFH oxidation, by antioxidants that scavenge peroxyl radicals, was used as the basis for calculating antioxidant activity. Just prior to use in the reaction, 80 μL of 2.48 mM DCFH-DA was hydrolyzed with 900 μL of 1.0 mM KOH for 3−5 min in a vial to remove the diacetate (DA) moiety and then diluted to 6 mL total volume with 75 mM phosphate buffer (pH 7.4). DCFH-DA was stable to oxidation, whereas DCFH was very slowly oxidized at ambient conditions without ABAP. ABAP (200 mM) was prepared fresh in buffer, and each batch was kept at 4 °C between runs and discarded after 6 h. In a run, 100 μL of pure compounds or food extracts appropriately diluted in 75 mM phosphate buffer (pH 7.4) was transferred into reaction cells on a 96-well plate, and 100 μL of DCFH was added. The 96-well plate was loaded into the plate holder for the Fluoroskan Ascent fluorescent spectrophotometer (Thermo Labsystems, Franklin, MA), and the solution in each cell was mixed by shaking at 1200 rpm for 20 s. The reaction was then initiated by adding 50 μL of ABAP from the autodispenser on the equipment. The autodispenser was emptied and rinsed with fresh ABAP before each run. Each set of dilutions for a replicate and control was analyzed three times in adjacent columns. The reaction was carried out at 37 °C, and fluorescence was monitored at 485 nm excitation and 538 nm emission with the fluorescent spectrophotometer. The buffer was used for control reactions. Reaction conditions were all optimized from preliminary studies for the control reaction to be completed (to reach maximum fluorescence) in 40 min. Data were acquired with Ascent software, version 2.6 (Thermo Labsystems, Franklin, MA) running on a PC. Fluorescence values were averaged across columns for each set of dilutions. The areas under the average fluorescence−reaction time kinetic curve (AUC) for both control and samples (up to 36 min) were integrated and used as the basis for calculating antioxidant activity according to eq 1

$$\text{PSC unit} = 1 - (\text{SA}/\text{CA}) \quad (1)$$

where SA is AUC for sample or standard dilution and CA is AUC for the control reaction using only buffer. Compounds inhibiting the oxidation of DCFH produced smaller SA and higher PSC units. The median effective concentration (EC_{50}) was defined as the dose required

to cause a 50% inhibition (PSC unit = 0.5) for each pure compound or sample extract and was used as the basis for comparing different compounds or samples. More powerful antioxidants would have lower EC_{50} values. Results obtained for sample extract antioxidant activities were expressed as micromoles of vitamin C equivalents per 100 g or 100 mL of sample ± standard deviation (SD) for triplicate analyses.

Lipophilic Peroxyl Radical Scavenging Capacity Assay. A modified version of the method described above was adopted for measuring the antioxidant activity of lipophilic compounds. The focus here was to increase the solubility of nonpolar compounds to enable their reaction in an otherwise aqueous environment. Solubility of lipophilic compounds was ensured by dissolving them in 12% RMCD prepared in 50% acetone in water (*12*). The fluorescent dye was prepared by hydrolyzing 11 μL of 2.48 mM DCFH-DA with 1.0 mL of 1.0 mM KOH and then diluted to a total volume of 8 mL with 75 mM phosphate buffer (pH 7.4). The reaction mix of the lipo-PSC assay contained 100 μL of appropriate dilutions of pure compounds or extracts in 12% RMCD, 100 μL of DCFH, and 50 μL of 200 mM ABAP. Control reactions used 12% RMCD. The reaction conditions, data acquisition, and processing were as described above for the hydro-PSC assay. The control reaction of the lipo-PSC assay was completed (to reach maximum fluorescence) in 45 min; therefore, AUC was calculated up to 40 min as described previously (*27*). Results obtained for lipophilic antioxidant activity for sample extracts were expressed as micromoles of α-tocopherol equivalent per 100 g of sample ± SD for triplicate analyses.

Method Validation. *Hydro-PSC Assay—Interactions with DCFH, Accuracy, Precision, and Reproducibility.* To investigate the possible interactions of samples with DCFH, sample concentrations that generated PSC units ≥0.5 were analyzed and the reaction was carried out until control and sample reactions were completed and their maximum fluorescence values compared. Quality control standards of gallic acid (54 μg/mL) were prepared in 12.5% acetone in water, and 4 mL aliquots were stored at −40 °C. These were analyzed over time to test the accuracy, precision, and reproducibility of the hydro-PSC assay.

Lipo-PSC Assay—Interactions with DCFH and RMCD, Accuracy, Precision, and Reproducibility. Possible interaction of pure lipophilic standards and sample extracts with DCFH was investigated by analyzing concentrations that generated PSC units ≥0.5. Analysis with and without RMCD were also done to test interactions between DCFH and RMCD. Similarly, all reactions were carried out to completion and their maximum fluorescence values compared. Quality control standards of α-tocopherol (3 mg/mL) were prepared in acetone, and 0.5 mL aliquots of this solution were stored at −40 °C. These were analyzed over time to test the accuracy, precision, and reproducibility of the lipo-PSC assay.

Statistical Analysis. Data from this study were reported as mean ± SD for at least three replicates for each antioxidant or sample extract. Results were subjected to ANOVA and differences among means determined using Fisher's pairwise comparison tests run on Minitab release 12 software (State College, PA).

RESULTS

Hydro-PSC Assay. The kinetics of DCFH oxidation by peroxyl radicals monitored as fluorescence generation is shown in **Figure 2** for ascorbic acid, gallic acid, and catechin. The results indicate that peroxyl radicals ($ROO^{\bullet}$) generated from thermal degradation of ABAP oxidize DCFH into fluorescent products over time (control reaction) and that antioxidants, such as ascorbic acid, gallic acid, and catechin, could scavenge peroxyl radicals and inhibit the oxidation reaction in a dose-dependent manner. The dose−response curve and median effect plot are shown in **Figure 3** for gallic acid. The concentration range within which various antioxidants inhibit DCFH oxidation and the concentration of each antioxidant required to cause a 50% inhibition of the reaction (EC_{50} or PSC unit = 0.5) are presented in **Table 1**.

Interactions with DCFH, Accuracy, Precision, and Reproducibility. Both control and sample reactions achieved similar maximum fluorescence values when reactions were completed. The precision and accuracy results of the hydro-PSC assay are presented in **Table 2**. The accuracy of measurement was determined for low to high concentrations of gallic acid. The percent recovery (accuracy) ranged from 86 to 107% within batches and from 96 to 101% among all batches. The precision of the hydro-PSC assay was expressed as the coefficient of variation (% CV) and was 0.12−8.7% within and 1.2−9.7% between batches. To test assay ruggedness, gallic acid quality control standards were assayed over time with at least 2 days between analyses. The results show the assay is consistent in producing reproducible results over time, and the overall relative standard deviation (% RSD) between analyses was 11% (n = 24).

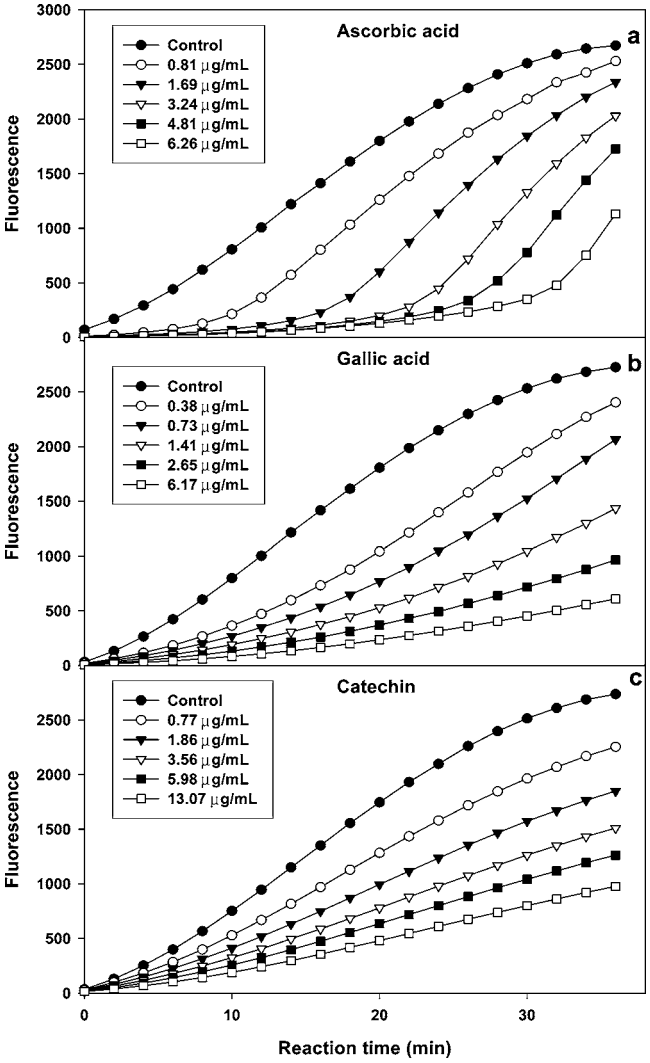

Figure 2. Time kinetics and dose–response of hydrophilic antioxidant activities of ascorbic acid, gallic acid, and catechin. Reaction was at 37 °C in the presence of 25 mM phosphate buffer, pH 7.4.

Pure Compounds and Sample Analyses. Results from analyzing several pure antioxidants and sample extracts are summarized in **Tables 1** and **3**. They show concentration ranges for inhibiting DCFH oxidation, R^2 of dose−response and median effect plots, and EC_{50} values. The concentration of pure antioxidants required to cause 50% inhibition of DCFH oxidation under our experimental condition ranges from 2.41 μM for EGCG to 21.26 μM for ferulic acid. The results expressed in reference to the antioxidant power of ascorbic acid correspondingly ranged from 3.89 μmol of Vit C equiv/μmol for EGCG

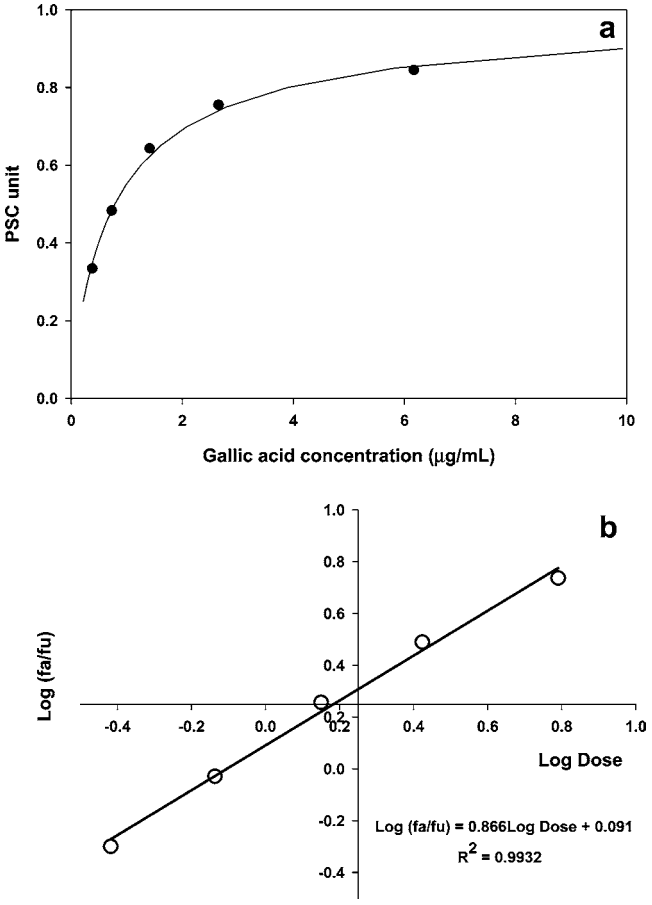

Figure 3. Dose response (**a**) and median effect (**b**) plots of gallic acid inhibition of DCFH oxidation by ABAP.

Table 1. Hydrophilic Antioxidant Activity of Pure Compounds

compound	concn range (μg/mL)	dose curve R^2	EC_{50} (μM) (mean ± SD)	hydro-PSC value[a] (μmol of Vit C equiv/μmol)
EGCG	0.27–5.32	0.9978	2.41 ± 0.02	3.89
chlorogenic acid	0.20–4.0	0.9990	3.33 ± 0.21	2.81
caffeic acid	0.21–4.17	0.9968	3.41 ± 0.14	2.75
quercetin	0.39–8.90	0.9995	3.42 ± 0.30	2.74
gallic acid	0.38–6.17	0.9986	4.64 ± 0.02	2.02
ascorbic acid	0.823–6.39	0.9985	9.36 ± 0.65	1.00
Trolox	0.40–11.43	0.9984	10.98 ± 0.87	0.85
catechin	0.78–13.33	0.996	12.23 ± 0.19	0.77
ferulic acid	0.78–17.78	0.9968	21.26 ± 0.38	0.44

[a] PSC value is the EC_{50} expressed as micromoles of ascorbic acid equivalents per micromole of antioxidant.

to 0.44 μmol of Vit C equiv/μmol for ferulic acid. The developed method was then applied to measuring the hydrophilic antioxidant activity of fruit, whole grain, wine, and plasma samples. The results show significant differences ($p < 0.05$) in antioxidant activities of fruit extracts with the following order: grape > cranberry > apple (**Table 3**). There were significant differences in antioxidant activity between the different types of whole grains; corn, wheat, oats, and rice. The red wine sample had significantly higher ($p < 0.05$) antioxidant activities than the white wine sample. Plasma from rats on a cranberry diet had higher ($p < 0.05$) antioxidant capacity than plasma from rats on a normal diet (**Table 3**).

Lipo-PSC Assay. *Optimizing RMCD Concentration and Equilibration Time.* To be effective in the reaction, lipophilic compounds had to be solubilized in an otherwise aqueous environment. Increased solubility was achieved using RMCD and also by dissolving the pure compounds and sample extracts in acetone. More α-tocopherol was dissolved at higher RMCD concentrations with an accompanying increase in antioxidant power (lower EC_{50} value). Increasing RMCD concentrations significantly decreased ($p < 0.05$) α-tocopherol's EC_{50} from 547 μg/mL at 3% RMCD to 12.96 μg/mL at 11−15% RMCD. α-Tocopherol was completely solubilized at 11% RMCD, and its antioxidant activity was not significantly different from that using 15% RMCD ($p > 0.05$). On the basis of these results, 12% RMCD was selected for all subsequent analyses and also to accommodate dilutions and future solubility problems with new compounds or sample extracts. For equilibration studies α-tocopherol (22.4 μg/mL) was equilibrated in 12% RMCD and analyzed at 15 min time intervals. Longer equilibration times resulted in lower PSC units, although mean values at 0 and 60 min were not significantly different ($p > 0.05$).

Table 2. Accuracy and Precision of Hydro-PSC Assay

	av gallic acid concn		
	0.75 μg/mL	2.40 μg/mL	5.78 μg/mL
recovery run 1			
% recovery	107.98	100.70	101.63
% CV	8.70	0.12	0.13
N	3	3	3
recovery run 2			
% recovery	91.68	98.95	99.58
% CV	2.86	1.35	1.56
N	3	3	3
recovery run 3			
% recovery	86.31	100.40	99.64
% CV	7.18	0.41	1.65
N	3	3	3
recovery run 4			
% recovery	98.44	103.13	98.87
%CV	5.05	0.84	1.27
N	3	3	3
pooled runs			
% recovery	96.10	100.79	99.93
% CV	9.72	1.72	1.19
N	12	12	12

Interactions with DCFH, Accuracy, Precision, and Reproducibility. No sample or standard interference with DCFH was observed as both control and sample/standard reactions achieved similar maximum fluorescence when reactions were completed. The accuracy of measurement was determined for low to high concentration ranges using α-tocopherol as quality control standard (**Table 4**). The percent recovery (accuracy) ranged from 91 to 100% within batches and from 92 to 99% among all batches. The precision of the lipo-PSC assay expressed as % CV and was 0.31−11% within and 0.4−4% between batches. Reproducibility results for the lipo-PSC assay were obtained using α-tocopherol as quality control standard assayed over time with at least 2 days between analyses. The assay produced reproducible results over time with an overall % RSD between analyses of 12% ($n = 17$).

Pure Compounds and Sample Analyses. **Table 5** shows for various lipophilic antioxidants the concentration range for inhibiting DCFH oxidation, the R^2 of dose−response and median effect plots, and EC_{50} values. The antioxidant activity of α-tocopherol was 4 and 42 times higher ($p < 0.01$) than that of BHA and BHT, respectively. Trolox was a more powerful antioxidant ($p < 0.05$) than α-tocopherol.

Table 3. Antioxidant Activity of Samples

Hydrophilic Antioxidant Activity				
sample	concn range (mg/mL)	dose curve R^2	EC_{50} (mg/mL) (mean ± SD)	hydro-PSC value[a] (μmol of Vit C equiv/100 g)
apple	1.02–14.91	0.9995	3.03 ± 0.04	309.2 ± 3.63
cranberry	0.38–5.58	0.9975	0.92 ± 0.10	1019.9 ± 104.4
grape	0.12–1.81	0.9991	0.45 ± 0.03	2108.9 ± 148.8
wheat bran	0.23–4.10	0.9972	0.28 ± 0.01	3345.1 ± 151.5
whole corn, free	32.9–168.0	0.9981	49.92 ± 0.33	18.74 ± 0.13
bound	0.55–5.60	0.9979	1.43 ± 0.08	655.8 ± 36.11
whole wheat, free	32.94–168.0	0.9969	90.44 ± 0.69	10.35 ± 0.08
bound	1.71–10.77	0.9989	3.25 ± 0.22	288.6 ± 19.75
whole oats, free	32.94–168.0	0.9949	54.48 ± 5.44	17.26 ± 1.72
bound	2.55–11.20	0.9983	3.50 ± 0.08	267.654 ± 6.38
whole rice, free	32.94–168.0	0.9864	113.06 ± 15.24	8.35 ± 1.13
bound	1.33–14.0	0.9996	4.29 ± 0.46	219.70 ± 22.41
sample	concn range (μL/mL)	dose curve R^2	EC_{50} (μL/mL) (mean ± SD)	hydro-PSC value[a] (μmol of Vit C equiv/100 mL)
red wine	0.40–9.23	0.9968	1.24 ± 0.07	757.5 ± 41.02
white wine	2.82–54.42	0.9943	8.13 ± 0.05	115.2 ± 0.70
plasma (normal diet)	2.06–27.27	0.9986	10.30 ± 0.32	90.87 ± 2.74
plasma (cranberry diet)	2.06–27.27	0.9959	8.73 ± 0.22	107.2 ± 2.70
Lipophilic Antioxidant Activity				
sample	concn range (mg/mL)	dose curve R^2	EC_{50} (mg/mL) (mean ± SD)	lipo-PSC value[b] (μmol of Vit E equiv/100 g)
whole corn	124.9–1967.6	0.9990	96.09 ± 15.03	4.60 ± 0.69
whole wheat	124.9–1967.6	0.9998	126.9 ± 20.81	3.49 ± 0.57
whole oats	88.18–1388.9	0.9985	82.78 ± 9.09	5.30 ± 0.59
whole rice	88.18–1388.9	0.9989	51.45 ± 13.23	8.79 ± 1.98

[a] PSC value is the EC_{50} expressed as micromoles of ascorbic acid equivalents per 100 g or 100 mL of sample. [b] PSC value is the EC_{50} expressed as micromoles of α-tocopherol equivalents per 100 g of flour.

Table 4. Accuracy and Precision of Lipo-PSC Assay

	av α-tocopherol concn		
	1.88 μg/mL	4.45 μg/mL	9.49 μg/mL
recovery run 1			
% recovery	100.36	91.24	99.03
% CV	3.35	3.26	1.15
N	3	3	3
recovery run 2			
% recovery	90.96	94.15	98.47
% CV	10.88	0.62	2.69
N	3	3	3
recovery run 3			
% recovery	95.89	91.04	99.33
% CV	0.56	1.75	0.31
N	3	3	3
recovery run 4			
% recovery	94.45	92.46	98.60
% CV	7.21	5.95	0.48
N	3	3	3
pooled runs			
% recovery	95.41	92.22	98.86
% CV	4.08	1.55	0.40
N	12	12	12

Table 5. Lipophilic Antioxidant Activity of Pure Compounds

compound	concn range (μg/mL)	dose curve R^2	EC_{50} (μM) (mean ± SD)	lipo-PSC value[a] (μmol of Vit E equiv/μmol)
Trolox	0.121–1.742	0.9974	1.58 ± 0.11	2.754
α-tocopherol	1.01–9.51	0.9983	4.35 ± 0.43	1.000
BHA	1.87–16.53	0.9964	18.94 ± 0.38	0.230
BHT	7.5–114.0	0.9905	182.69 ± 13.76	0.024

[a] PSC value is the EC_{50} values expressed as micromoles of α-tocopherol equivalents per micromole of antioxidant.

DISCUSSION

Free radicals are involved in the etiology of many chronic diseases. Dietary antioxidants are very important in combating the destructive actions of these free radicals in the body (*28*). Hence, it is necessary to assess dietary intakes of antioxidants. Several methods are available for assessing the antioxidant capacity of foods. More recent methods were modifications of older methods to make them more reliable, stable, faster, and simpler to run. In this study, we developed a rapid PSC assay for assessing both hydrophilic and lipophilic antioxidants.

EC_{50} Calculations. There are several methods available for calculating antioxidant activities. Most are based on the relationship between the doses of antioxidants and the effects (AUC, lag times, amount of substance produced or consumed, etc.) they produce under the given sets of experimental conditions. These relations often follow typical dose−response relationships. Therefore, these effects should be quantified by taking into consideration the overall effects of antioxidants over a wide concentration range, characterized by the dose−response curves or median effect plots that are unique to each antioxidant under the given experimental conditions. In the literature, however, results have often been obtained using only the linear sections of the dose−response curve, probably on the basis of assumptions of the need for linearity in analytical measurements. There is a possibility to overestimate or underestimate antioxidant capacities because dose−effect relationships may appear to be linear with close data points.

In this assay, we adopt the method proposed by Winston et al. (*27*) and later modified by Eberhardt et al. (*29*) as presented in eq 1. Basically, the PSC unit represents the extent of inhibition of the control reaction by antioxidants. A plot of PSC unit

against antioxidant concentration gives a typical dose−response curve that depicts the true and characteristic inhibition profile of the antioxidant under the given experimental condition (**Figure 3a**). EC_{50} values extrapolated from such a curve become unique and characteristic values for the particular antioxidant. EC_{50} is the concentration of antioxidant that causes a 50% inhibition of the control reaction and therefore produce a PSC unit of 0.5 according to eq 1. Because the EC_{50} may not necessarily occur in the linear range of PSC unit versus antioxidant concentration curve, we decided to use antioxidant concentration ranges that include the EC_{50} and fit the points to the curves depicted in **Figure 3**. This fit represents the typical dose−response curve and shows the true nature of inhibition or antioxidant effect over all concentration ranges, in this case, for gallic acid. Therefore, it may not be necessary to find the linear range of antioxidant concentration for the analysis. For accuracy and reproducibility, the concentration range that includes the EC_{50} can be used for analyses using this approach.

Hydro-PSC assay. *Method Development and Validation.* Results from tests of the ruggedness of the hydro-PSC assay (11% RSD) show the assay can produce fairly consistent results over time comparable to previous data (*13*, *14*, *30*). This assay therefore meets the necessary criteria for antioxidant measurements. Also, the assay demonstrated a high degree of accuracy (% recovery) and precision (% CV) (**Table 2**). No significant interference between DCFH and all samples was noted, as both control and sample finally attained similar maximum fluorescence values at the end of their respective reactions. Similar effects have been demonstrated for fluorescein (*13*). For very powerful antioxidants, a higher concentration of ABAP (600 mM) was required to complete the reaction within a reasonable time. In this case, interference results were the same as that when 200 mM ABAP was used.

Autoxidation of DCFH at 37 °C in the absence of ABAP was insignificant when compared with the control with ABAP present. The ability of pure antioxidants and sample extracts to cause oxidation of DCFH in the absence of ABAP was also tested. Only ascorbic acid was noted to cause low but significant oxidation of DCFH in a dose-dependent manner. We suspected this was due to the formation of hydroxyl radicals from ascorbic acid reaction with transition metals in the solution (*31*). The problem was resolved by incorporating EDTA at low levels to chelate trace metals from reagents such as acetone, KOH, and phosphate buffer. It was also noted that EDTA addition was most important for the initial dissolution of ascorbic acid in 12.5% acetone in water. Therefore, ascorbic acid was dissolved in 12.5% acetone in water containing 50 mg/L EDTA. Subsequent analyses showed EDTA had no effect ($p > 0.05$) on EC_{50} values for gallic acid and Trolox and that its application was necessary for only ascorbic acid.

Pure Antioxidants. Hydrophilic antioxidants inhibited the oxidation of DCFH by scavenging peroxyl radicals generated from the thermal degradation of ABAP (**Figure 2**). The rate and extent of inhibition were different for all compounds analyzed (**Table 1**). Both similarities and differences may be observed when our results are compared with previously published results, but, in general, the order of antioxidant activities of pure compounds was similar. Thus, differences could be attributed to only specific experimental conditions used, as well as methods for calculating antioxidant capacities. For example, relative $ORAC_{FL}$ values obtained for ascorbic acid and catechin were 0.95 and 6.76 Trolox equiv, respectively, indicating ascorbic acid was 7 times less potent than catechin (*13*). Our results show ascorbic acid was 1.3 times more potent than catechin (**Table 1**). However, our results were in agreement with studies showing ascorbic acid ($ORAC_{FL} = 0.95$) was as powerful as Trolox (*13*). Gallic acid was approximately 2 times more potent than ascorbic acid ($p < 0.01$) and 3 times more potent than catechin ($p < 0.01$). We attribute the discrepancies to different experimental conditions and methods used for calculating antioxidant activity from the data. **Figure 2** also shows the rate of reaction of antioxidants with free peroxyl radicals, allowing the antioxidants to be classified as fast acting or slow acting (*26*, *27*). These results suggest that ascorbic acid is a fast-acting antioxidant that rapidly reacts with peroxyl radicals until the ascorbic acid present is exhausted. The kinetic curves show well-defined lag times or induction periods, which increase with increasing concentrations of ascorbic acid. In contrast, catechin reacts more slowly with peroxyl radicals and thus offers a more constant protection during the reaction. The kinetic curves of catechin do not show a well-defined lag phase or induction period. The kinetic curves for gallic acid show an intermediate reaction rate pattern between rates for ascorbic acid and catechin (**Figure 2**). The rate at which antioxidants compete for or react with free radicals, fast or slow, may partly contribute to their effectiveness in protecting the body during acute or chronic free radical influxes (*27*).

Sample Extracts. Hydrophilic extract antioxidant activities of grape, cranberry, and apple were significantly different ($p < 0.05$) (**Table 3**). These results contrast those reported elsewhere showing the order of hydrophilic antioxidant activity as cranberry ≫ apple > grape (*22*). These differences may be mainly attributed to varietal effects or different extraction procedures used in the two separate studies. Fruits samples were first extracted with a hexane/dichloromethane mixture before hydrophilic antioxidants were extracted with an acetone/water mixture (*22*). On the other hand, extracts in this study were obtained using only an acetone/water extraction solution. Significant differences in antioxidant activity of whole grains (**Table 3**) have previously been reported (*24*, *25*, *32*). We previously reported the same order and similar number-of-fold difference in total hydrophilic antioxidant activities for corn, wheat, oats, and rice (*24*), although mean values are different from current results. Wine samples were significantly different ($p < 0.05$) in antioxidant activities (**Table 3**), similar to previously reported data (*33*, *34*). Inclusion of grape skin in red wine production adds more phenolic compounds that result in higher antioxidant activities for red wines (*33*). Cranberries contribute more antioxidant activity to blood plasma when included in the diet (**Table 3**). This effect was also documented in human subjects who consumed blueberries (*35*).

Lipo-PSC Assay. *Method Development and Validation.* We modified the hydro-PSC assay for measuring the antioxidant activity of lipophilic compounds by incorporating RMCD as a solubility enhancer (*12*). In this study, the EC_{50} for α-tocopherol decreased with increasing RMCD levels to a minimum at 11% RMCD and remained constant up to 15% RMCD (data not shown). On the basis of these results, the reaction would be less sensitive to slight variations in RMCD content between 11 and 15% RMCD. These results also demonstrate that, at higher RMCD concentrations, lipophilic antioxidants such as α-tocopherol tend to be more soluble in an otherwise aqueous environment and hence more effective in inhibiting the oxidation of DCFH. Similar effects of RMCD on α-tocopherol antioxidant capacity have been reported (*12*). RMCD did not contribute antioxidant activity and also did not interfere with the antioxidant activity of compounds with which it complexes (*12*). We confirmed this by running control reactions with and without

Table 6. Percentage Contributions to the Total Antioxidant Activity

sample	hydro-PSC value (Trolox equiv/100 g)	lipo-PSC value (Trolox equiv/100 g)	% hydro-PSC contribution[a]
corn	873.5	1.67	99.81
wheat	387.1	1.55	99.67
oats	368.8	1.92	99.48
rice	295.3	3.19	98.93

[a] Percentage contribution of hydrophilic antioxidant activity to the total antioxidant activity.

RMCD. Maximum fluorescence values at the end of the reaction were similar with or without RMCD. However, RMCD significantly lowered initial fluorescence values, probably from physical protection of DCFH from peroxyl radicals (*21*). Therefore, a longer reaction time was necessary to complete the reaction. The time needed to solubilize lipophilic antioxidants in 12% RMCD was also investigated in this work. Longer equilibration times resulted in lower ($p > 0.05$) antioxidant activity for α-tocopherol and BHT (data not shown). This could be attributed to autoxidation of antioxidants over time. This may have significant implications with regard to the final relative capacities of different antioxidants because their autoxidations may proceed at different rates. For example, the modified $ORAC_{FL}$ assay for lipophilic antioxidants requires standards to be dissolved in 7% RMCD and shaken for 1 h before analysis (*12*). The final antioxidant activity of the solutions may be different after 1 h. In the lipo-PSC assay, samples were dissolved in acetone and then further diluted in 12% RMCD, vortexed, and used immediately. This provides for high sample throughput while maintaining sample integrity. The lipo-PSC assay demonstrated good accuracy, precision (**Table 4**), and reproducibility comparable to other published results (*12*, *22*, *35*).

Pure Antioxidants. The order of antioxidant activity for lipophilic compounds was Trolox > α-tocopherol > BHA > BHT (**Table 5**) and was similar to previous reports using 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as peroxyl radical source (*12*). These results were different from reports that α-tocopherol (ORAC = 0.5) was ~3 times more powerful than BHT (ORAC = 0.16) in the $ORAC_{FL-lipo}$ assay (*12*). On the contrary, our results are in agreement with previous reports that Trolox was approximately 2 times as powerful as α-tocopherol (*12*). Differences in these results could be attributed to the different experimental conditions and different methods used to calculate antioxidant activity from the data. The structure−activity relationship that explains the order of antioxidant activity among these lipophilic compounds is discussed by Huang et al. (*12*).

Sample Extracts. The lipo-PSC assay was applied to measuring the lipophilic antioxidant activity of lipophilic extracts of whole corn, wheat, oats, and rice (**Table 3**). Significant differences ($p < 0.05$) in lipophilic antioxidant activities were observed among the four whole grains tested, with whole rice flour having the highest lipo-PSC value of 8.8 μmol of α-tocopherol equiv/100 g of flour. Lipophilic compounds in whole grain flour that may contribute to antioxidant activity include carotenoids, tocopherols, and tocotrienols (*25*, *36*, *37*).

To find the percentage contributions of both hydrophilic and lipophilic antioxidant activities to the total antioxidant activities of food extracts (**Table 6**), the whole grain samples were selected for both hydrophilic and lipophilic PSC assay analyses. Results obtained in this study were also expressed as Trolox equivalents per 100 g of flour and used to calculate the percentage contributions. Our results showed that the hydrophilic antioxidant activity contribution to total antioxidant activity of whole grain flours was >98% and was consistent with the reported values (*22*).

We expressed hydrophilic and lipophilic antioxidant activities of analyzed food extracts as vitamin C equivalents per 100 g and α-tocopherol equivalents per 100 g, respectively (**Table 3**). The objective was to demonstrate how physiologically important antioxidants combat physiologically important peroxyl radicals. For practical purposes, this should give a more practical and consumer-friendly interpretation of the antioxidant capacity of foods to combat radicals that cause chronic diseases. However, in order to compare contributions from hydrophilic and lipophilic antioxidant activities, the results had to be expressed in terms of Trolox equivalents. This is because Trolox conveniently performs well in both the hydro-PSC and lipo-PSC assays. It should be noted that DCFH was relatively stable in both hydro-PSC and lipo-PSC assays, with only 2 and 3% loss in fluorescence, respectively, 30 min after maximum fluorescence was achieved. Discrepancies in results obtained by the PSC assay compared to other methods could be attributed to type of radical source used, the ratio of ABAP to DCFH, the concentrations of ABAP and DCFH relative to sample concentrations, and other experimental conditions. For the PSC assay, we observed that the antioxidants were slightly less effective in inhibiting DCFH oxidation when either ABAP or DFCH concentrations were increased with the other held constant. This pattern was consistent with competitive reactions and has also been noted by other researchers (*35*). The concentrations of ABAP and DCFH used were optimized for reactions to be completed in ~40 min.

In this study, we developed a rapid, simple, and reliable approach for measuring both hydrophilic and lipophilic antioxidant capacity of pure antioxidant compounds, food extracts, and biological fluids toward biologically relevant radicals, based on the oxidation of DCFH by peroxyl radicals. The partial inhibition of DCFH oxidation is the basis for the PSC assay, in that the protection offered by an antioxidant is a function of the ratio of AUC for sample versus control reactions. The PSC assay requires a maximum 40 min run when compared to 60−120 min required for a TOSC assay for a range of antioxidant concentrations (*27*, *29*). By using a 96-well plate, four samples in triplicate can be analyzed in 40 min, allowing for high sample throughput. In comparison, the TOSC assay can analyze only one replicate of a sample each hour. Incorporation of the fluorescence probe DCFH allows for less interference and variation in the data due to the specificity of the measurement. Using RMCD simplifies the preparations steps and most importantly ensures enhanced solubility of lipophilic compounds without any interfering side reactions, because RMCD is chemically inert in this reaction. Use of both hydro-PSC and lipo-PSC assays extends the applicability of this method to include both hydrophilic and lipophilic antioxidant capacities of foods. Results from such analyses should give more complete information on the antioxidant protection offered by a large diversity of foods. The PSC assay also generates results comparable to similar more modern assays using fluorescein derivatives as probes and may even have certain additional advantages. For instance, with the hydroxyl radical averting capacity (HORAC) assay, it was observed that the intercept of the linear standard curves keep changing with each run, suggesting that, to eliminate the uncertainty of the intercept, it may be necessary to obtain standard curves within the run to compute final values (*14*). The PSC assay does not require a

linear standard curve, and for practical purposes using a concentration range that includes the EC_{50} produces accurate and reproducible results. The PSC assay is also very sensitive, being able to run in the low micromolar range. Adapting the PSC assay to run in a 96-well plate and also using a fluorescent dye allows the assay to be performed on simple fluorescent spectrophotometers, which are more readily available to other researchers. The method can be easily automated as has been done for the ORAC assay (*30*). During initial method development, dilutions were done in glass test tubes and samples transferred to reaction cells on a 96-well plate. In subsequent analyses, dilutions carried out in 96-well plates with 650 μL cell volumes achieved similar results. In using EC_{50} values to compare antioxidant activities of unknown samples, we recommend that the original sample concentration should give a PSC unit of >0.5 to allow adequate dilutions. We believe this assay is sensitive enough to accommodate even low concentrations of most food extracts. The use of CA in eq 1 corrects for minor variations in experimental conditions and ensures reproducible results.

In this paper, we present a new approach for analyzing both hydrophilic and lipophilic antioxidant activities of pure compounds and food extracts. The new method is simple, reliable, robust, sensitive, and precise and can produce acceptable results comparable to those obtained with similar published assays. Further work would analyze a wider range of pure compounds, foods, and biological fluids.

LITERATURE CITED

(1) Fraga, C. G.; Motchnik, P. A.; Shigenaga, M. K.; Helbock, H. J.; Jacob, R. A.; Ames, B. N. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11003−11006.

(2) Wagner, J. R.; Hu, C.; Ames, B. N. Endogenous oxidative damage of deoxycytidine in DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3380−3384.

(3) Halliwell, B.; Murcia, M. A.; Chirico, S.; Aruoma, O. I. Free radicals and antioxidants in food and in vivo: what they do and how they work. *Crit. Rev. Food Sci. Nutr.* **1995**, *35* (1−2), 7−20.

(4) Duthie, S. J.; Ma, A.; Ross, M. A.; Collins, A. R. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Res*. **1996**, *56*, 1291−1295.

(5) Liu, R. H. Health benefits of fruits and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr*. **2003**, *78*, 517S−520S.

(6) Serafini, M.; Bellocco, R.; Wolk, A.; Ekstrom, A. M. Total antioxidant potential of fruit and vegetables and risk of gastric cancer. *Gastroenterology* **2002**, *123*, 85−99.

(7) Giugliano, D. Dietary antioxidants for cardiovascular prevention. *Nutr. Metab. Cardiovasc. Dis.* **2000**, *10* (1), 38−44.

(8) Pulido, R.; Bravo, L.; Saura-Calixo, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem*. **2000**, *48*, 3396−3402.

(9) Naguib, Y. M. A. Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.* **2000**, *48*, 1150−1154.

(10) Arnao, M. B.; Cano, A.; Acosta, M. The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chem.* **2001**, *73*, 239−244.

(11) Aldini, G.; Yeum, K. J.; Russell, R. M.; Krinsky, N. I. A method to measure the oxidizability of both the aqueous and lipid compartments of plasma. *Free Radical Biol. Med.* **2001**, *31*, 1043−1050.

(12) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **2002**, *50*, 1815−1821.

(13) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−4626.

(14) Ou, B.; Hampsch-Woodill, M.; Flanagan, J.; Deemer, E. K.; Prior, R. L.; Huang, D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J. Agric. Food Chem.* **2002**, *50*, 2772−2777.

(15) Bernstein, P. S.; Khachik, F.; Carvalho, L. S.; Muir, G. J.; Zhao, D. Y.; Katz, N. B. Identification and quantitation of carotenoids and their metabolites in the tissues of the human eye. *Exp Eye Res.* **2001**, *72* (3), 215−223.

(16) Snodderly, D. M. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am. J. Clin. Nutr.* **1995**, *62* (6), 1448S−1461S.

(17) Beatty, S.; Koh, H.; Phil, M.; Henson, D.; Boulton, M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv. Ophthalmol.* **2000**, *45* (2), 115−134.

(18) Landrum, J. T.; Bone, R. A. Lutein, zeaxanthin, and the macular pigment. *Arch. Biochem. Biophys.* **2001**, *385* (1), 28−40.

(19) Burton, G. W.; Ingold, K. U. Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.* **1986**, *19*, 194−201.

(20) Pfitzner, I.; Francz, P. I.; Biesalski, H. K. Carotenoid: methyl β-cyclodextrin formulations: an improved method for supplementation of cultured cells. *Biochim. Biophys. Acta* **2000**, *1474*, 163−168.

(21) Szente, L.; Mikuni, K.; Hashimoto, H.; Szejtli, J. Stabilization and solubilization of lipophilic natural colorants with cyclodextrins. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1998**, *32*, 81−89.

(22) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026−4037.

(23) Sun, J.; Chu, Y.-F.; Wu, X.; Liu, R. H. Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* **2002**, *50*, 7449−7454.

(24) Adom, K. K.; Liu, R. H. Antioxidant activity of grains. *J. Agric. Food Chem.* **2002**, *50*, 6182−6187.

(25) Adom, K. K.; Sorrells, M. E.; Liu, R. H. Phytochemical profiles and antioxidant activity of wheat varieties. *J. Agric. Food Chem.* **2003**, *51*, 7825−7834.

(26) Pryor, W. A.; Cornicelli, J. A.; Devall, L. J.; Tait, B.; Trivedi, B. K.; Witiak, D. T.; Wu, M. A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. *J. Org. Chem*. **1993**, *58*, 3521−3532.

(27) Winston, G. W.; Regoli, F.; Duga, A. J., Jr.; Fong, J. H.; Blanchard, K. A. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Radical Biol. Med.* **1998**, *24* (3), 480−493.

(28) Liu, R. H. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.* **2004**, *134* (12), 3479S−3485S.

(29) Eberhardt, M. V.; Lee, C. Y.; Liu, R. H. Antioxidant activity of fresh apples. *Nature* **2000**, *405*, 903−904.

(30) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem*. **2002**, *50*, 4437−4444.

(31) Buettner, G. R.; Jurkiewicz, B. A. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat. Res.* **1996**, *145*, 532−541.

(32) Yu, L.; Haley, S.; Perret, J.; Harris, M. Comparison of wheat flours grown at different locations for their antioxidant properties. *Food Chem*. **2004**, *86* (1), 11−16.

(33) Beer, D. D.; Joubert, E.; Gelderblom, W. C. A.; Manley, M. Antioxidant activity of South African red and white cultivar

wines: free radical scavenging. *J. Agric. Food Chem.* **2003**, *51*, 902−909.

(34) Zafrilla, P.; Morillas, J.; Mulero, J.; Cayuela, J. M.; Martiänez-Cachaa, A.; Pardo, F.; Nicolas, J. M. L. Changes during storage in conventional and ecological wine: phenolic content and antioxidant activity. *J. Agric. Food Chem*. **2003**, *51*, 4694−4700.

(35) Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$) of plasma and other biological and food samples. *J. Agric. Food Chem.* **2003**, *51*, 3273−3279.

(36) Lloyd, B. J.; Siebenmorgen, T. J.; Beers, K. W. Effect of commercial processing on antioxidants in rice bran. *Cereal Chem*. **2000**, *77* (5), 551−555.

(37) Hentschel, V.; Kranl, K.; Hollmann, J.; Lindhauer, M. G.; Bohm, V.; Bitsch, R. Spectrophotometric determination of yellow pigment and evaluation of carotenoids by high-performance chromatography in durum wheat grain. *J. Agric. Food Chem.* **2002**, *50*, 6663−6668.

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