High-Throughput Relative DPPH Radical Scavenging Capacity Assay

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A high-throughput relative 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging capacity (RDSC) assay was developed and validated in the present study. This RDSC assay is easy to perform and has acceptable accuracy (90−110% recovery), precision [3.9−7.0% pooled relative standard deviation (RSD)], and reproducibility (2.2 and 3.5% interday and intraday RSD). This assay reports the RDSC values for antioxidant samples, which make it possible to compare the DPPH radical scavenging capacities of antioxidants determined in different laboratories. The RDSC assay may be conducted in aqueous alcohol and acetone for hydrophilic antioxidants or in the organic solvents for lipophilic antioxidants without solubilizing agents, which makes it possible to directly compare the radical scavenging capacities of hydrophilic and lipophilic antioxidants. In addition, the high-throughput RDSC assay could be utilized for EC_{50} value estimation. The high-throughput RDSC assay may be used for screening and investigating potential natural antioxidants.

KEYWORDS: Radical scavenging capacity; RDSC, DPPH radical; antioxidant activity; high-throughput; AUC

INTRODUCTION

Free radicals may attack life-important molecules such as DNA and membrane lipids and play a role in the pathology of numerous chronic diseases (*1*). Growing evidence has shown an inverse correlation between the intake of dietary antioxidants and the risk of chronic diseases such as coronary heart disease, cancer, and several other aging-associated health problems (*2−4*). This motivates the discovery and development of novel nutraceutical ingredients and functional food products rich in natural antioxidants. A number of radical scavenging capacity (RSC) assays have been established and widely used for the rapid screening and evaluation of novel antioxidant preparations using peroxyl, hydroxyl (HO•), cation ABTS (ABTS•+), per-oxide anion (O_2•−), and 2,2-diphenyl-1-picryhydrazyl (DPPH•) radicals (*5−7*). The stable DPPH• and the chemically generated ABTS•+ are still highly utilized in antioxidant research due to their simple reaction systems, which involve only the direct reaction(s) between the radical and the antioxidant(s), and have no other interference such as enzyme inhibition or the presence of multiple radicals, although they are not physiologically relevant. In contrast to the chemically generated ABTS•+, DPPH• may be utilized in aqueous and nonpolar organic solvents such as benzene and can be used to examine both hydrophilic and lipophilic antioxidants (*6, 8*). The DPPH• scavenging capacity assay performed in organic solvents may evaluate lipophilic antioxidants without any additional solubilizing agents such as

the β-cyclodextrin, required in the oxygen radical absorbing capacity (ORAC) and peroxyl radical scavenging capacity (PSC) assays (*6, 7*), which has been reported to have a strong interference in HO• scavenging capacity estimation (*5*).

The DPPH• scavenging capacity assay is considered to be a valid and easy colorimetric method for antioxidant property evaluation. This assay has been successfully utilized for investigating antioxidant properties of wheat grain and bran, vegetables, conjugated linoleic acids, herbs, edible seed oils, and flours in several different solvent systems including ethanol, aqueous acetone, methanol, aqueous alcohol, and benzene (*6, 8−11*). However, it has been hard to compare the DPPH• scavenging capacity data between different laboratories or from the same group at different times because most of the results using this radical system were reported in % DPPH• remaining or quenched, which highly depends on the reaction time and the initial concentrations of DPPH• and the antioxidant(s) in the assay mixture (*12*). Efforts have been taken to improve the RSC estimation using DPPH•. In 1998, Sanchez-Moreno and others introduced a new term, the "antiradical efficacy" (AE), to describe the DPPH• scavenging capacity (*13*):

$$AE = \frac{1}{(EC_{50}) \times (T_{EC_{50}})} \tag{1}$$

where the EC_{50} was the required concentration of a selected antioxidant to reduce the DPPH• concentration to 50% of its original in the reaction mixture, whereas $T_{EC_{50}}$ was the time required to reach the steady state for the EC_{50} (*13*). The authors

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concluded that this method took into account the reaction time and was more discriminatory than the EC_{50} alone. The authors also demonstrated that this method might be used to determine the RSC of several pure standard antioxidative compounds (*13*). However, the chemical meaning of the AE was not defined and was impossible to define. The second major drawback of this method is that the $T_{EC_{50}}$ was not experimentally determined but was obtained from the secondary plot of the "time at the steady state" against the "concentration of antioxidants" (*13*). This may lead to huge variation of $T_{EC_{50}}$ values because there is no linear relationship between the time at the steady state and the concentration of antioxidants. It is well-accepted that DPPH• scavenging capacity is highly dependent on the reaction time and the EC_{50} value is highly dependent on how the "steady state" is arbitrarily selected or DPPH• concentrations at which time point of the antioxidant−radical reaction are used. For instance, a rapid decrease of DPPH• concentration at the early reaction phase (0−30 min) was followed by a slow reduction of the DPPH• concentration later (40−1400 min) during our previous studies on conjugated linoleic acids, wheat bran, and edible seed oil extracts (*8, 11, 14*). Any time point between 40 and 1400 min could be arbitrarily selected as the steady state. Different EC_{50} values could be obtained if DPPH• concentrations at different reaction times between 40 and 1400 min were used to estimate the EC_{50} for a selected antioxidant sample. In addition, it has to be pointed out that the absolute value of either the EC_{50} or the $T_{EC_{50}}$ is highly dependent on the unit; thus, the AE value may be 1000-fold different if mg/mL or μg/mL was used for the antioxidant concentration. In conclusion, the AE method may not have adequate reproducibility and can not be used to compare the DPPH• scavenging capacity data between different laboratories, suggesting a need for such a new method.

Recently, a few high-throughput assays have been developed to rapidly examine the free radical scavenging capacities of natural antioxidants. These include but are not limited to the ORAC (*15*), hydroxyl radical scavenging capacity (HOSC) (*5*), and PSC (*7*) assays. All three assays measure the fluorescent intensity during the radical−antioxidant reactions using a microplate reader. The ORAC and HOSC assays use fluorescein as the probe and have a definite end point for the reaction, whereas the PSC assay uses dichlorofluorescin diacetate as the fluorescent probe and has no definite end point for the antioxidant−radical reactions (*5, 7, 15*). Both ORAC and PCS determine the peroxyl radical scavenging capacities of selected antioxidants, and HOSC measures the hydroxyl radical scavenging ability. All of these high-throughput assays use an area under the curve (AUC) for RSC estimation, expressed as trolox equivalents (TEs) in μmol on a per sample weight basis. These approaches take into account both the kinetic and the thermodynamic measurements of the radical−antioxidant reactions and make it possible to compare data between laboratories. However, all of these three assays generate free radicals in the reaction mixtures, and other factors such as the chemicals that may directly react with the fluorescent probes may alter the antioxidant property estimation. It is also known that the radical system may affect the antioxidant activity estimation. Therefore, more high-throughput assays involving different radicals are needed for antioxidant research.

The aim of the present study was to develop and validate a high-throughput method for relative DPPH• scavenging capacity (RDSC) estimation using a microplate reader with spectrophotometic detector. The new RDSC assay uses the AUC in calculation and reports the RDSC values in TEs per mass of testing material, just as cited in most literature (*16, 17*), which

makes it possible to compare data from different laboratories and different times and which may better meet the needs of antioxidant research. In addition, the high-throughput assay using DPPH• was demonstrated for possible application in establishing the EC_{50} values for selected antioxidant samples.

MATERIALS AND METHODS

Materials. Grain and bran samples of three hard winter wheat varieties (Jagalene, Trego, and Akron) adapted for production in Colorado and a soft winter wheat variety (Madison) were used for this study. The hard winter wheat bran and grain samples were provided by Dr. Scott Haley in the Department of Soil and Corp Sciences at Colorado State University (Fort Collins, CO), and the Madison wheat sample was a gift from the Mennel Milling Co. (Roanoke, VA). Trego is a white wheat, while Jagalene, Akron, and Madison are red wheat varieties. Chardonnay grape, pinot noir grape, and black raspberry seed flours were obtained from Botanical Oil Innovations, Inc. (Spooner, Wisconsin).

DPPH•, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), α-tocopherol, ascorbic acid, ferulic acid, caffeic acid, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water used for all experiments was prepared using an ELGA (Lowell, MA) Purelab Ultra Genetic polishing system with <5 ppb TOC and resistivity of 18.2 mΩ. All other chemicals and solvents were of the highest commercial grade and were used without further purification.

Sample Preparation. The DPPH• and all standard antioxidant compounds including trolox were dissolved in 50% acetone or ethanol. The DPPH• stock solution at a concentration of 0.625 mM was prepared monthly and kept at 4 °C in dark. The 0.208 mM fresh DPPH• working solution was made daily by further diluting the stock solution in 50% acetone or ethanol for each test. Stock solutions of trolox, α-tocopherol, ferulic acid, and ascorbic acid were prepared in 50% acetone at concentrations of 25, 5, 50, and 6.25 mM, respectively, and stored at 4 °C. A series of working solutions were made by appropriate dilutions of the above standard phenolic acid stock solutions with 50% acetone. Quality control (QC) samples for determination of accuracy at three concentrations (10, 20, and 30 μM) were also prepared by serial dilutions of the freshly prepared ferulic acid stock solution in 50% acetone and used within 48 h. In addition, stock solutions of 5 mM trolox, 10 mM gallic acid, 10 mM ferulic acid, 10 mM caffeic acid, 6.5 mM α-tocopherol, and 10 mM ascorbic acid were also prepared in absolute ethanol to validate the antioxidant activity estimation using the AUC approaches.

Extracts of the selected natural materials with known antioxidant properties including wheat bran, wheat grain, and black raspberry and grape seed flours were involved in this study to validate the new high-throughput relative DPPH• scavenging capacity RDSC assay. The wheat grain, bran, and black raspberry and grape seed flours were ground using a Bel Art micromill (Pequannock, NJ) and extracted according to the procedures previously described (*10*). In brief, 1.0 g of each sample was extracted for 15 h with 10 mL of 50% acetone under nitrogen at ambient temperature and pressure. The clear supernatants were collected by centrifugation and were kept in the dark under nitrogen at ambient temperature until further analysis.

Conventional Colorimetric Analysis. The conventional colorimetric DPPH• scavenging capacity assay was performed according to a previously described laboratory protocol (*10*). Briefly, an aliquot of 500 μL of different concentrations of sample extracts in 50% acetone was added to 500 μL of 0.208 mM DPPH• solution. The initial concentration was 0.104 mM for DPPH• in all reaction mixtures. Each mixture was vortexed for a few seconds and left to stand in the dark for 40 min at ambient temperature. The absorbance (*A*) of each reaction mixture at 515 nm was measured against a blank of 50% acetone using a UV−visible spectrometer (Thermo Spectronic, United States). The level of DPPH• remaining for each reaction was calculated as:

$$\% \text{ DPPH}^\bullet \text{ remaining} = \frac{A_{40\text{min}-\text{sample}}}{A_{40\text{min}-\text{control}}} \times 100 \qquad (2)$$

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where $A_{40\text{min}-\text{sample}}$ represented the absorbance at 515 nm for a sample after 40 min of reaction with DPPH radical and $A_{40\text{min}-\text{control}}$ represented that of the control containing no antioxidants at time 40 min. The EC_{50} value of each sample was obtained by plotting the % DPPH• remaining of each concentration of a selected antioxidant sample against the sample concentrations. The EC_{50} value is the concentration of an antioxidant to quench 50% radicals in the reaction mixture under the assay condition. Duplicate reactions were carried out for each level of every individual sample.

High-Throughput RDSC Assay. This high-throughput assay was carried out using a Victor[3] multilabel plate reader (Perkin-Elmer, Turku, Finland). The plate was covered with a lid to prevent solvent evaporation during determination. Each reaction mixture contained 100 μL of antioxidant sample at different concentrations. Five or seven different concentrations were used for each antioxidant extract and antioxidant standard in the study. Then, 100 μL of 0.208 mM DPPH• solution, which is same as that used in the conventional colorimetric method, was added into each well using an eight-channel pipetter followed by gentle shaking. The absorption at 515 nm was determined immediately after shaking, and each plate was read once per minute for 1.5 h. The total volume for each reaction mixture or in each well was 200 μL. A blank with only 200 μL of 50% acetone and a control with the mixture of 100 μL of 50% acetone and 100 μL of 0.208 mM DPPH• were also determined for absorbance (A) at 515 nm.

The percent radical remaining at 40 min was determined according to the following equation:

$$\% \text{ DPPH}^\bullet \text{ remaining} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \qquad (3)$$

where A_{sample}, A_{blank}, and A_{control} stand for the absorbance of sample, blank, and control reactions at 40 min.

To estimate the total DPPH• scavenging capacity of a selected antioxidant sample, the % DPPH• quenched was determined according to the following equation:

$$\% \text{ DPPH}^\bullet \text{ quenched} = \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}}\right) \times 100 \qquad (4)$$

where A_{sample}, A_{blank}, and A_{control} represent the absorbance of the certain concentration of a selected antioxidant, blank, and the control at 515 nm measured at the reaction time t.

The values of % DPPH• quenched at different reaction times obtained from eq 4 were plotted against the reaction time, and the AUC value can be calculated from this plot for each antioxidant and for trolox standard (**Figure 1A**). The AUC value was calculated as:

$$\text{AUC} = 0.5 f_0 + (f_1 + f_2 + f_3 + ... + f_{i-1}) + 0.5 f_i \qquad (5)$$

where f_0 is the initial DPPH• quenched reading at 0 min and f_i is the total DPPH• quenched at reaction time i. Generally, $i = 40$ min. The data were processed with a Microsoft Excel program (Microsoft, Roselle, IL) to calculate the AUC using eq 5 as shown in **Figure 1A**. The RDSCs were expressed as millimol TEs/g sample for botanical materials and millimol TE/g pure antioxidant compound in this study, with both calculations shown below:

RDSC value for botanical materials/pure compounds =

$$\frac{\text{AUC}_{\text{sample}}}{\text{AUC}_{\text{trolox}}} \times \frac{\text{molarity}_{\text{trolox}}}{\text{mass}_{\text{sample}}} \qquad (6)$$

For optimal accuracy, the RDSC value was better determined using a standard curve prepared with trolox. AUC values of at least four concentrations of trolox within the linear range of 6.4−38.4 μM were determined. The AUC values (y) were plotted against the trolox concentrations (x) (**Figure 1B**). A linear regression equation of $y = ax + b$ was obtained from the plot and was used to calculate the RDSC values (**Figure 1B**).

The EC_{50} value, which is another commonly used parameter for antioxidant activity evaluation and comparison, can also be obtained using the high-throughput DPPH• RDSC assay. On the basis of the
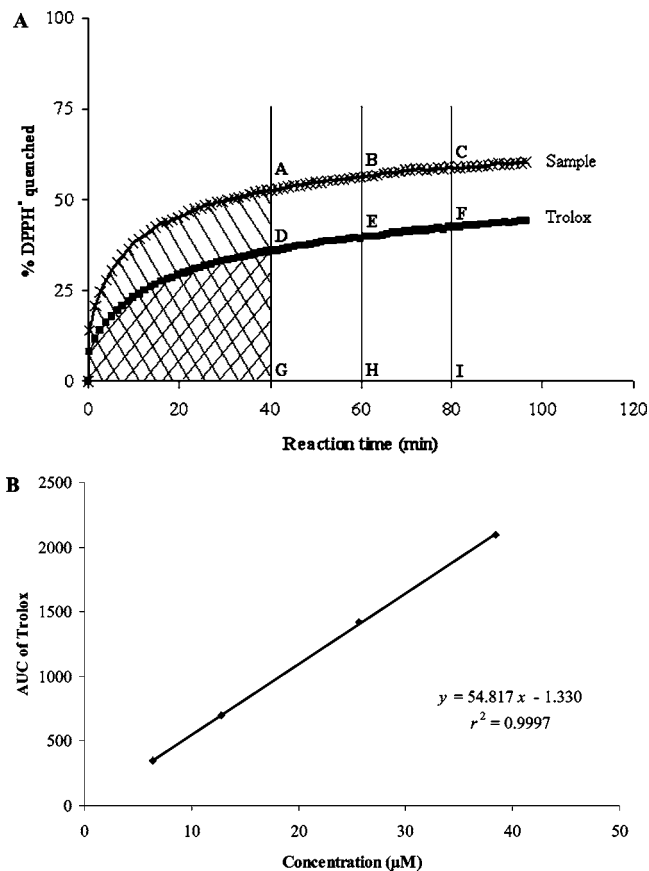

Figure 1. Determination of RDSC using the AUC. (**A**) AUC calculation using the plot of % DPPH radical quenched against DPPH•–antioxidant reaction time. The area OAGO represents the AUC for sample at 40 min of DPPH•–antioxidant reaction, whereas the area ODGO is the AUC for trolox. (**B**) Calculation of RDSC values using a standard curve prepared using trolox. The standard curve is obtained by plotting the AUCs against the corresponding trolox concentrations. At least four concentrations of trolox are required to obtain the linear regression equation, which is used to calculate RDSC values for potential antioxidant samples.

values of % DPPH• remaining at 40 min, the EC_{50} of each sample was obtained by plotting the % DPPH• remaining against the antioxidant concentrations. The EC_{50} value was the concentration of an antioxidant to quench 50% radicals in the reaction mixture under the assay condition. Duplicate reactions were carried out for each level of individual antioxidant sample.

Statistical Analysis. Statistical analysis was conducted using SPSS (version 10.0.5, 1999, SPSS Inc., Chicago, IL). Data were analyzed by analysis of variance. Comparison of means was performed using Tukey's HSD posthoc testing. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

In the present study, a simple high-throughput assay to measure the RDSC was developed using a Victor[3] multilabel plate reader with a spectrophotometric detector. The background absorbance of the 96 well plate was found to be constant among wells of the same plate and from different plates (data not shown) and thus had no effect on the antioxidant activity estimation. Unlike the conventional colorimetric method, the background absorbance of the solvents cannot be set to zero for the 96 well plate under the high-throughput assay conditions. Therefore, each plate must have a solvent blank (e.g., 50% acetone alone) containing no DPPH• or antioxidants and the control reaction containing no antioxidants. The RDSC assay
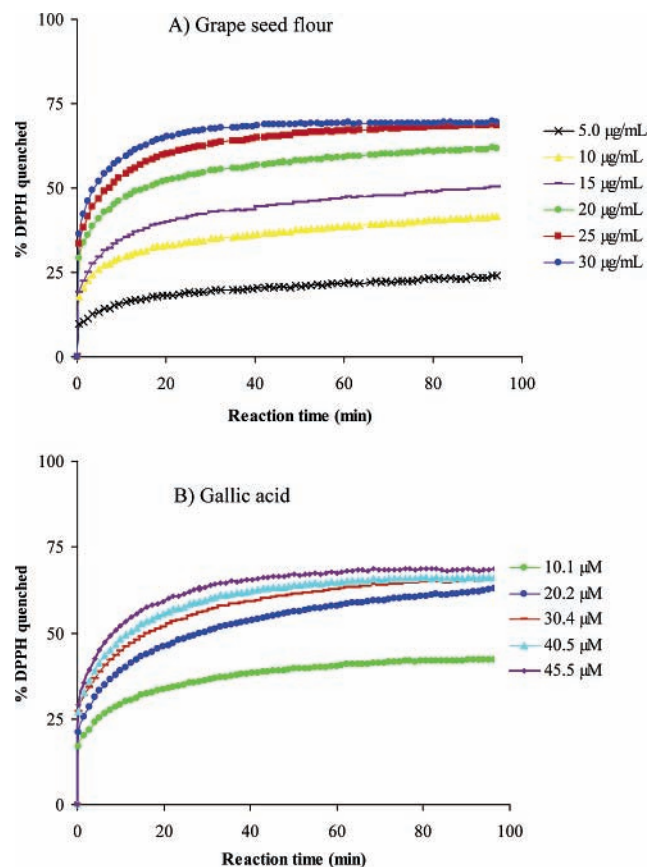
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Figure 2. Kinetics and concentration effects of antioxidant–DPPH radical reactions for (**A**) grape seed flour extract and (**B**) gallic acid. The measurements were performed using the high-throughput assay. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The initial concentrations were 5.0–30.0 μg grape seed flour equivalents per mL of the reaction mixture for grape seed flour and were 10.11–45.5 μM for gallic acid. The total reaction volume was 200 μL for all antioxidant–radical reactions. The absorbance was measured at 515 nm. The calculation was conducted according to eq 4.

uses the AUC to quantify the total RDSC and expresses the results as TEs per unit of antioxidant(s), which makes the comparison of the DPPH• scavenging capacity data between different laboratories possible. The high-throughput assay was also examined for its linearity, accuracy, determining concentration range, and precision. In addition, this assay was compared to the conventional DPPH• scavenging capacity assay and applied to evaluate the antioxidant properties of selected pure compounds and botanical extracts.

AUC for RDSC Estimation. A major drawback of the conventional DPPH• scavenging capacity assay is that the DPPH• scavenging capacity data for samples analyzed in different laboratories or at different times using this assay cannot be compared. To solve this problem, the AUC of the % DPPH• quenched against the time of antioxidant−DPPH• reaction was tested for possible quantification of DPPH• scavenging capacity using a group of pure antioxidant compounds and natural antioxidative extracts. The AUC values for 4−7 concentrations of each antioxidant compound or botanical extract were obtained from the % DPPH• quenched-reaction time plot (**Figure 2**). An excellent linear relationship ($r^2 = 0.95−0.99$) between AUCs and antioxidant concentrations for a group of pure antioxidant compounds including trolox, ferulic acid, ascorbic acid, caffeic acid, α-tocopherol, and gallic acid, as well as the selected natural botanical extracts including three wheat brans, two wheat grains,

Table 1. Linear Relationship between Antioxidant Concentrations and AUC Obtained by the High-Throughput DPPH• Scavenging Capacity Assay[a]

	slope	intercept	r^2	linearity ranges	n
trolox	54.817	−1.330	0.999	6.40–38.40 μM	4
ferulic acid	7.137	389.210	0.975	65.38–130.75 μM	5
ascorbic acid	93.936	−447.5	0.987	5.23–23.51 μM	7
caffeic acid	57.942	112.58	0.989	10.11–30.33 μM	4
gallic acid	44.894	459.21	0.954	5.06–25.30 μM	4
α-tocopherol	0.929	1.657	0.978	8.13–32.50 μM	5
JB	152.67	330.99	0.987	1.25–7.52 mg/mL	5
TB	112.28	216.11	0.973	1.88–10.02 mg/mL	5
AB	202.75	33.529	0.991	1.88–7.52 mg/mL	4
TG	80.203	237.22	0.999	2.51–15.04 mg/mL	4
MG	49.344	320.96	0.986	2.50–25.02 mg/mL	6
Ch	74.524	95.287	0.991	1.25–25.00 μg/mL	6
PN	10.436	579.24	0.998	40.00–140.00 μg/mL	6
BR	19.969	133.93	0.989	10.00–80.00 μg/mL	5

[a] Data were the means of triplicate measurements. JB, TB, and AB represent Jagalen, Trego, and Akron wheat bran, respectively, while TG and MG represent Trego and Madison wheat grain samples. Ch, PN, and BR stand for chardonnay grape, pinot noir grape, and black raspberry seed flours, respectively. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The absorbance was measured every minute at 515 nm for 40 min. The italic *n* (*n*) represents the number of the concentrations tested for each antioxidant.

two grape seed flours, and a black raspberry seed flour, is shown in **Table 1**. These data suggest that the AUC may be used to quantify the DPPH• scavenging capacity. This conclusion was supported by the previous successful application of AUC in the ORAC, HOSC, and PSC assays to measure the relative scavenging capacity of antioxidants against peroxyl and hydroxyl radicals (*5−7, 15*).

In order to compare the free radical scavenging capacities of antioxidants between different laboratories and different testing times, it is critical that the radical scavenging capacities are expressed as the relative activities of a standard antioxidant compound. Both trolox and α-tocopherol had excellent sensitivity of AUC to concentration changes with correlation coefficients (r^2) of 0.999 and 0.978 for trolox and tocopherol, respectively (**Table 1**). This suggests that either could be used as the antioxidant standards to express the DPPH• scavenging activity as trolox or α-tocopherol equivalents per unit of the other antioxidative samples, which are the RDSCs. The RDSC values can be used to compare the DPPH• scavenging activity data from different laboratories and/or data determined at different times. Trolox has been used as the antioxidant standard in several RSC estimations in aqueous systems such as the ORAC, HOSC, and cation radical ABTS•+ scavenging capacity assays (*5, 6, 14*), as well as in the PSC assay for hydrophilic antioxidants (*7*). In contrast, α-tocopherol was used as the antioxidant standard in the PSC assay to report the relative peroxyl radical scavenging activities of lipophilic antioxidants (*7*). This was because both α-tocopherol and the lipophilic antioxidants have to be released from the nonpolar core of β-cyclodextrin and diffused into the aqueous phase and be available to react with peroxyl radicals, whereas trolox is soluble in water and does not have the release and diffusion phases and is not suitable as the antioxidant standard for lipophilic antioxidants. Use of different antioxidant standards made it hard to compare the PSC between the hydrophilic and the lipophilic antioxidants obtained using the PSC assay (*7*). In the present RDSC assay, trolox can be dissolved in an aqueous system or in ethanol and can be used as the antioxidant standard to report the relative radical scavenging capacities of both hydrophilic and lipophilic antioxidants, which makes it possible to directly

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Table 2. Relative DPPH• Scavenging Capacity (RDSC) of Selected Samples Calculated Using the AUC Values at Three Different Reaction Times[a]

| | RDSC values determined at different reaction time (TE mmol/g) | | | | |
	40 min	60 min	80 min	% RSD	linear range
ferulic acid	1.00 ± 0.05	1.10 ± 0.06	1.17 ± 0.07	7.84	91.53–130.75 μM
caffeic acid	6.49 ± 0.30	6.58 ± 0.27	6.62 ± 0.26	1.10	10.11–30.33 μM
gallic acid	9.79 ± 2.27	10.23 ± 2.24	10.51 ± 2.22	3.57	5.05–25.25 μM
ascorbic acid	5.43 ± 0.28	5.40 ± 0.30	5.39 ± 0.32	0.39	13.06–23.51 μM
α-tocopherol	2.36 ± 0.21	2.37 ± 0.20	2.38 ± 0.19	0.42	8.13–32.50 μM
JB	3.16 ± 0.36	3.43 ± 0.48	3.63 ± 0.55	6.92	2.51–7.52 mg/mL
TB	1.88 ± 0.29	1.69 ± 0.90	1.95 ± 0.83	7.31	1.88–7.52 mg/mL
AB	2.24 ± 0.32	2.27 ± 0.05	2.50 ± 0.44	6.09	5.01–10.02 mg/mL
TG	1.14 ± 0.11	1.29 ± 0.16	1.35 ± 0.16	8.58	5.01–20.05 mg/mL
MG	0.92 ± 0.08	1.01 ± 0.09	1.06 ± 0.10	7.12	5.01–30.08 mg/mL
Ch	1.51 ± 0.13	2.08 ± 0.39	1.72 ± 0.19	16.29	5.0–25.0 μg/mL
PN	0.31 ± 0.03	0.34 ± 0.04	0.35 ± 0.04	6.24	60.0–120.0 μg/mL
BR	0.37 ± 0.05	0.41 ± 0.05	0.43 ± 0.04	7.57	20.0–80.0 μg/mL

[a] Data were means of triplicate measurements ± SD (n = 3). TE as millimol trolox/g material. JB, TB, and AB represent Jagalen, Trego, and Akron wheat bran, respectively, while TG and MG represent Trego and Madison wheat grain samples. Ch, PN, and BR stand for chardonnay grape, pinot noir grape, and black raspberry seed flours, respectively. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The absorbance was measured every minute at 515 nm for 80 min. The AUC was calculated according to eq 5 for 40, 60, and 80 min of antioxidant–DPPH• reactions. The trolox standard curves as shown in **Figure 1B** were prepared for 40, 60, and 80 min reaction times. RDSC values for each antioxidant sample at 40, 60, and 80 min were calculated using the corresponding trolox standard curves. The % RSD was calculated for each antioxidant.

compare the DPPH• scavenging capacities of these antioxidants. To our knowledge, little assays can directly compare free RSC of hydrophilic and lipophilic antioxidants (*17*), although several methods are able to determine the radical scavenging capacities of lipophilic samples with solubilizing agents such as β-cyclodextrin and sodium dodecyl sulfate.

Similarly to that of the PSC assay, there is no definite end point of the antioxidant–DPPH• reactions under the RDSC assay conditions (*7*). It was noted that the AUC was positively associated with progress of antioxidant–DPPH• reactions for all pure antioxidative compounds and natural botanical extracts tested in the present studies. This is demonstrated by the kinetic curves of antioxidant–DPPH• reactions for grape seed flour extract (**Figure 2A**) and gallic acid (**Figure 2B**) at a series of concentrations. Also noted from **Figures 1** and **2** was that the antioxidant–DPPH• reactions may be considered to reach their "steady state" at 40 min or anytime after that. To test the effects of the AUC determined at different reaction times on RDSC value estimation, AUC values at 40, 60, and 80 min of the antioxidant–DPPH reactions were determined and used to calculate the RDSC values for five pure antioxidant compounds and eight natural botanical extracts (**Table 2**). All tested samples, except the chardonnay grape seed flour extract, had % RSD (relative standard deviation) values less than 10% for the RDSC values calculated using the AUC data determined at 40, 60, and 80 min of the reactions, suggesting that the AUC data at 40 min of antioxidant–DPPH• reaction can be used to calculate and report the relative RDSC of potential antioxidants against DPPH•. In other words, these results indicate that the RDSC value obtained using the AUC approach is time-independent, which makes the high-throughput RSDC assay superior to the conventional DPPH radical scavenging capacity assay.

Another problem of the conventional DPPH• scavenging capacity assay is that % DPPH• remaining or quenched at a certain reaction time is dependent on the ratio of antioxidant and DPPH• in the testing reaction mixtures. In other words, the % DPPH• remaining or quenched value depends on the antioxidant concentration. This makes it hard to compare the results from different laboratories. The effect of testing concentrations on RDSC value was evaluated using the selected pure antioxidant compounds and natural botanical extracts in the present study. Four concentrations of ferulic and caffeic acids

Table 3. Effect of Testing Concentrations on the Relative DPPH• Scavenging Capacity (RDSC) Estimation Using the AUC[a]

concentration (μg/mL)	AUC	RDSC value (TE mmol/g)
	ferulic acid	
17.76	1038.07	1.07
20.29	1106.16	1.00
22.83	1205.49	0.96
25.37	1360.58	0.98
% RSD		4.57
	Chardonnay grape seed flour	
5.00	568.23	1.45
15.00	1244.83	1.44
20.00	1641.58	1.50
25.00	1882.97	1.41
% RSD		2.72
	caffeic acid	
1.82	673.09	6.76
3.64	1301.70	6.53
4.55	1643.47	6.59
5.46	1811.58	6.06
% RSD		4.65
	Trego grain	
15.04	1064.72	1.20
25.06	1619.31	1.07
30.08	1912.25	1.04
35.09	2026.50	0.94
% RSD		9.99

[a] Data were means of triplicate measurements. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The absorbance was measured every minute at 515 nm for 40 min. The AUC was calculated according to eq 5 and used for RDSC value estimation using the trolox standard curve as shown in **Figure 1B**.

and Trego wheat grain and chardonnay grape seed flour extracts were used to determine the RDSC values using trolox as the antioxidant standard. Their RDSC values are presented as molarity TEs/g of each antioxidant in **Table 3**. The % RSD was 2.72, 4.57, 4.65, and 9.99 for the RDSC values of chardonnay grape seed flour, ferulic acid, caffeic acid, and Trego grain, respectively. All % RSD values were below 10%, suggesting that the RDSC value is independent from the antioxidant concentrations. It needs to be pointed out that the RDSC value of any pure compound may also be calculated and expressed in μmol/mmol/mol of TEs per μmol/mmol/mol of

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the compound using eq 7 if necessary:

RDSC value for pure compounds =

$$\frac{AUC_{sample}}{AUC_{trolox}} \times \frac{molarity_{trolox}}{molarity_{sample}} \quad (7)$$

The RDSC assay was utilized to determine the radical scavenging capacities of a group of pure compounds and natural botanical extracts. The order of RDSC values was chardonnay grape seed flour > black raspberry seed flour > pinot noir grape seed flour on a per weight basis (**Table 2**), with higher RDSC values associated with a stronger DPPH radical scavenging capacity. This order was in agreement with that of their ORAC values and total phenolic contents previously reported from our laboratory (*18*). The order of RDSC values was gallic acid > caffeic acid > ascorbic acid > ferulic acid on a per weight basis (**Table 2**), which is similar to that of the hydro-PSC values previously reported in μmol vitamin C equivalents/μmol antioxidant (*7*). These data demonstrated that the RDSC assay using AUC is a practical approach for radical scavenging capacity estimation and comparison. It needs to be pointed out that some inherent drawbacks such as the background absorbance of colored samples still exist in this new assay.

Accuracy, Precision, and Reproducibility of the High-Throughput RDSC Assay. The accuracy of the high-throughput RDSC assay was determined by analyzing three runs of ferulic acid at three different concentrations (10, 20, and 30 μM) in triplicate. The accuracy data (expressed as percent of recovery, % REC) from the calibration standard are listed in **Table 4**. The % REC values ranged from 90.20 to 109.70% for the three testing concentrations (10, 20, and 30 μM) of ferulic acid, suggesting the excellent accuracy of the high-throughput RDSC assay (**Table 4**). The RSD values were 1.37–9.43% for the three testing concentrations (10, 20, and 30 μM) of ferulic acid (**Table 4**). The pooled % RSD was within 15%, indicating the good precision of the RDSC assay. The reproducibility is critical

Table 4. Accuracy of QCs in the High-Throughput RDSC Assay[a]

| | μm | | |
	QC 1	QC 2	QC 3
	10.00	20.00	30.00
	run 1		
mean (μM)	10.27	18.13	29.43
% REC	102.70	90.65	98.10
SD	0.96	1.04	2.04
% RSD	9.31	5.76	6.92
	run 2		
mean (μM)	9.62	18.04	30.95
% REC	96.20	90.20	103.17
SD	0.91	1.47	1.95
% RSD	9.43	8.12	6.30
	run 3		
mean (μM)	10.97	19.88	31.42
% REC	109.7	99.40	104.73
SD	0.78	0.27	1.46
% RSD	7.06	1.37	4.66
	mean of three runs		
mean of three runs	10.31	18.21	30.19
SD	0.55	1.27	1.18
% RSD	5.37	6.97	3.89

[a] Values were expressed as means of triplicate measurements. SD, standard deviation; % RSD, percent RSD; % REC, percent recovery. Ferulic acid at initial concentrations of 10, 20, and 30 μM was used for this accuracy test. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The absorbance was measured every minute at 515 nm for 40 min

Table 5. Reproducibility of the High-Throughput RDSC Assay[a]

% DPPH• remaining			
interday variability		intraday variability	
run 1	77.81 ± 0.28	day 1	74.00 ± 0.21
run 2	78.74 ± 0.11	day 2	79.34 ± 0.78
run 3	77.19 ± 6.43	day 3	81.60 ± 1.03
run 4	77.13 ± 2.28	day 4	78.70 ± 1.42
run 5	77.20 ± 0.50	day 7	75.07 ± 2.78
run 6	74.88 ± 1.02	day 8	76.03 ± 2.07
run 7	74.00 ± 0.21		
mean of seven runs	76.71 ± 1.67	mean of eight days	77.63 ± 2.68
% RSD	2.17	RSD (%)	3.46

[a] Values were expressed as means ± standard deviations ($n = 3$). Ferulic acid at a concentration of 20 μM was used for this reproducibility test. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The data represent the % DPPH• remaining at 40 min of antioxidant–radical reactions.
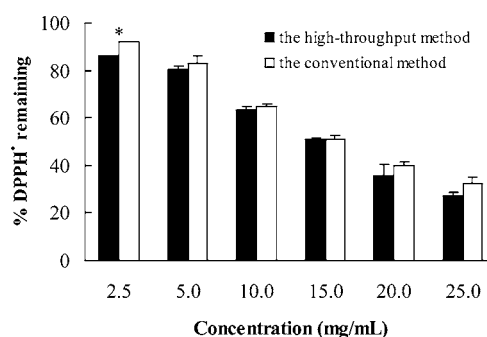

Figure 3. Comparison of the high-throughput and the conventional DPPH• scavenging capacity measurements. The RDSC was determined for six concentrations of the Madison wheat grain extract using both the high-throughput and the conventional colorimetric method. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The antioxidant concentration was expressed in mg grain equivalents per mL of the final reaction mixture. The final reaction volumes were 200 and 1000 μL for the high-throughput and the conventional methods, respectively. The absorbance at 40 min of reaction was determined for each reaction mixture. The results were expressed as % DPPH• remaining. Data were means of duplicate measurements. Vertical bars represent the standard deviations, and an asterisk (*) indicates a significant difference between these two methods at the testing concentration ($P = 0.01$).

for a high-throughput assay. The inter- and intraday variations were tested for the RDSC assay using 20 μM ferulic acid, which is a common phenolic acid present in many natural products. The DPPH radical scavenging capacity was determined in triplicate for seven times within 1 day between the entire 96 well plate for intraday reproducibility, while for day-to-day variation tests, this standard phenolic acid was examined in duplicate for 7 consecutive days using a freshly prepared standard curve each day. The RSD was 2.17% during seven runs and 3.46% for 8 days (**Table 5**). Both RSD values were below 5%, suggesting the good reproducibility of the RSDC assay for multimeasurements within 1 day and at different days. Additionally, the small % RSD values suggested that the well position within a plate had little influence on RDSC values.

Comparison of the High-Throughput Assay (RDSC) with the Conventional Colorimetric Method. The present high-throughput RDSC assay and the conventional colorimetric assay were compared using a group of samples including wheat grain and bran and black raspberry and grape seed flours. All comparisons were conducted using the % DPPH• remaining data
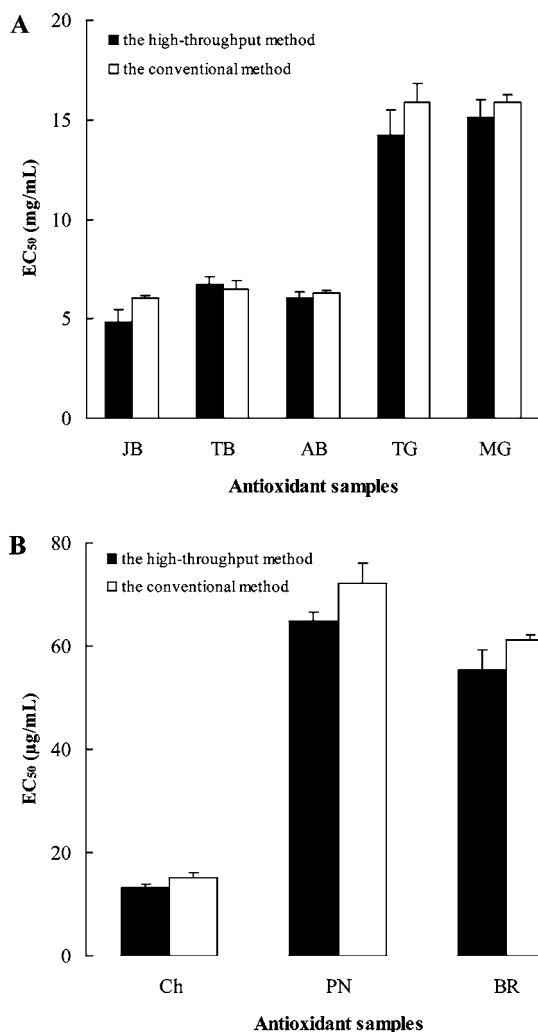
High-Throughput Radical Scavenging Capacity Assay

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Figure 4. EC_{50} values of eight selected antioxidative botanical materials determined by the high-throughput and the conventional colorimetric methods. (**A**) JB, TB, and AB represent Jagalen, Trego, and Akron wheat bran, respectively, while TG and MG represent Trego and Madison wheat grain samples; (**B**) Ch, PN, and BR stand for chardonnay grape, pinot noir grape, and black raspberry seed flours, respectively. The initial concentration was 0.104 mM for DPPH radicals in all antioxidant–radical reactions. The absorbance was measured at 515 nm after 40 min of the reaction. Data were means of duplicate measurements and expressed as mg botanical material equivalents per mL in the antioxidant–radical reaction mixtures. The vertical bars represent the standard deviation of each data points, and an asterisk (*) indicates a significant difference between these two methods at the testing concentration ($P = 0.01$).

since AUC determination using the conventional method is too time-consuming. **Figure 3** represents the % DPPH• remaining data determined for Madison wheat grain using both the high-throughput and the conventional methods. Six concentrations of the Madison wheat grain extracts were determined under the same experimental conditions except for different total antioxidant–radical reaction volumes and the detecting instruments. The results obtained by the two methods were strongly correlated to each other ($r^2 = 0.991$) and had no difference for the testing concentrations of 5, 10, 15, 20, and 25 mg/mL, suggesting the acceptability of the high-throughput assay.

To further compare the high-throughput RDSC assay with the conventional DPPH radical scavenging activity estimation method, the EC_{50} values of Trego and Madison wheat grain, bran samples of Jagalen, Trego, and Akron wheat, black raspberry seed flour, and chardonnay and pinot noir grape seed

flours were determined using both assays. EC_{50} is the required initial concentration of a selected antioxidant sample to quench 50% of the free radicals in the reaction system. No difference was observed between the EC_{50} values obtained using the high-throughput RDSC assay and the conventional method (**Figure 4A,B**). These results suggested that the high-throughput RDSC assay is acceptable for DPPH• scavenging activity evaluation and indicated that the RDSC assay using 96 well plates can be employed to establish EC_{50} values if necessary.

Kinetic Study Using the High-Throughput RDSC Assay. Antioxidants have shown different scavenging capacities in the whole reaction process with DPPH radicals, in particular the initial process (*13*). This different kinetic property may significantly affect the beneficial effects of antioxidants in vivo. For instance, Sanchez-Moreno and others reported that some antioxidants may require less time to scavenge 50% of the free radicals in the system than other antioxidants and classified them as rapid, medium, and low-acting antioxidants based on this time (*13*). Kinetic properties of antioxidant–radical reactions have been investigated in a number of previous studies (*8, 11, 13, 14*). As shown in **Figure 2A,B**, the high-throughput RDSC assay may be utilized to prepare kinetic curves of the antioxidant–DPPH• reactions. The kinetic curve obtained by this high-throughput assay is superior to that prepared using the conventional method because of a shorter testing interval time and a higher throughput.

Conclusion. The development and application of a high-throughput RDSC assay using a microplate reader with spectrophotometric detector and 96 well plates was described and validated. This assay may be conducted in aqueous alcohol and acetone for hydrophilic antioxidants or in the organic solvents for lipophilic antioxidants without solubilizing agents. The assay for evaluating lipophilic antioxidants is highly demanded. The RDSC assay also makes it possible to compare the DPPH radical scavenging capacity data between different research laboratories. This RDSC assay is easy to perform and has acceptable accuracy, precision, and reproducibility. The high-throughput RDSC assay may be used for screening and investigating the potential natural antioxidants.

Supporting Information Available: Tables of blank absorbance values. This material is available free of charge via the Internet at http://pubs.acs.org.

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