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Modified 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Method to Measure Antioxidant Capacity of Selected Small Fruits and Comparison to Ferric Reducing Antioxidant Power (FRAP) and 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) Methods

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The measurement of antioxidant capacity in fruits differs from that of other biological samples due to their low pH and very low lipophilic antioxidant capacity. In this report, we present a modified 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method for fruits and compare its performance with the other commonly used antioxidant methods of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). The antioxidant capacity and reaction kinetics of four phenolic compounds, two antioxidant standards, and five fruits were also investigated. The modified ABTS method prepared at a pH of 4.5 with sodium acetate buffer is highly stable and easily applied to fruit samples as compared to the standard (pH 7.4) version. The measured antioxidant capacity of samples varied with the assay method used, pH, and time of reaction. Traditional antioxidant standards (trolox, ascorbic acid) displayed stable, simple reaction kinetics, which allowed end point analysis with all of assays. Of the phenolic compounds examined, chlorogenic and caffeic acids exhibited the most complex reaction kinetics and reaction rates that precluded end point analysis while gallic acid and quercetin reached stable end points. All fruit extracts exhibited complex and varied kinetics and required long reaction times to approach an end point. Because the antioxidant capacity of fruit extracts is a function of the array of individual antioxidants present, accurate comparisons among fruit samples require that reaction times be standardized and of sufficient length to reach steady state conditions and that more than one assay be used to describe the total antioxidant activity of fruit samples.

KEYWORDS: Natural antioxidants; polyphenols; phenolics; ABTS; phytonutrients; phytochemicals; oxidative stress; berries; black raspberry; red raspberry; blackberry; strawberry; grape; free radicals; antioxidant assay; dietary antioxidants

INTRODUCTION

It is well-known that diets rich in fruits and vegetables are capable of preventing or delaying the onset of certain chronic degenerative diseases of aging, including cardiovascular malfunction and common cancers (*1*, *2*). With this in mind, many scientists have investigated the phytonutrient properties of these commodities and, specifically, have measured their complements of natural antioxidants (*3*−*8*). The most thoroughly investigated natural antioxidants in fruits and vegetables are flavonoids, polyphenols, carotenoids, fiber, vitamins A, B, C, and E, tocopherols, calcium, and selenium (*9*). These compounds and elements may act independently or in combination as anticancer or cardioprotective agents by a variety of mechanisms.

Several methods have been developed to determine the antioxidant potential of food products. The trolox equivalent antioxidant capacity (TEAC) using ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) as an oxidant, the ferric reducing antioxidant power (FRAP), the DPPH (2,2′-diphenyl-1-picrylhydrazyl) free radical scavenging potential, the oxygen radical absorption capacity (ORAC), the total radical absorption potentials (TRAP), and the photochemiluminescence (PCL) assays are some of the most commonly used (*10*, *11*). Antioxidants can reduce radicals primarily by two mechanisms: single electron transfer and hydrogen atom transfer. ABTS, FRAP, and DPPH are methods that measure the former, and ORAC and TRAP represent the latter. The mechanism of PLC is still unclear (*11*).

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The initial development of many of these methods involved measuring plasma antioxidant levels (*12*−*14*). As antioxidant reactants, plasma and fruit differ in many ways. Fruit extracts are far more acidic and typically have very low lipophilic antioxidants (0.1−2% of total antioxidant capacity) (*15*). Although some comparative studies have been conducted in recent years (*10*, *16*, *17*), these investigations have focused primarily on differences in the reaction kinetics of phenolic standards among assays. As food products, and specifically fruits, typically contain mixtures of antioxidant moieties, their assay reaction kinetics may be more complex than those of standard compounds.

Single electron transfer reactions can be relatively slow and traditionally measure relative percent decrease in product rather than kinetics or total antioxidant capacity (*11*). The potential for the generation of new antioxidants through polymerization of phenolic compounds in fruit juices (*18*, *19*) may lead to the underestimation of the true antioxidant potential when reactions are not allowed to reach or near completion. The expression of this complexity among antioxidant assays has not received much attention in the antioxidant literature.

The goal of our study was to determine the total antioxidant capacity of small fruits and the most appropriate method for making these determinations. To measure the total antioxidant capacity of fruit extracts, we chose three methods (ABTS, FRAP, and DPPH) that utilize the same single electron transfer mechanism. These methods were chosen for comparison because they are commonly used to evaluate plant materials (*5*, *6*, *16*). All three methods can be used with antioxidants in an aqueous media, are relatively simple to conduct, and are cost-effective. To determine the full antioxidant potential of fruits, the experiments required stable reactants to measure the reactions for a period of time long enough to reach equilibrium. Therefore, we modified the ABTS method to increase its stability and compared the results with those given by the DPPH and FRAP assays.

MATERIALS AND METHODS

Chemicals. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tris-2,4,6-tripyridyl-2-triazine), ABTS, PBS (phosphate-buffered saline), potassium persulfate, acetic acid (glacial), acetone, sodium acetate, and ethanol were purchased from Sigma-Aldrich Chemical Co. as well as chlorogenic acid, quercetin, caffeic acid, gallic acid, and ascorbic acid. DPPH was purchased from Calbiochem Co. (San Diego, CA).

Preparation of Standards. Caffeic acid, gallic acid, quercetin, and chlorogenic acid were dissolved in ethanol at a concentration of 1 mM (*20*). A 1 mM standard stock solution of ascorbic acid was prepared in Nanopure water. Fresh standard solutions were prepared prior to each assay replication, held at −80 °C, and used within 24 h. Solutions of trolox (1 mM) were prepared in ethanol and stored at −80 °C in 1 mL Eppendorf tubes.

Preparation of Fruit Extracts. Fruit samples of Jewel black raspberry, Heritage red raspberry, Chester blackberry, Concord grape, and Honeoye strawberry were harvested from local farms, frozen immediately, and kept at −20 °C until analyzed. Samples were thawed at room temperature (≈21 °C) and homogenized in a food grade blender. The resultant slurry was centrifuged (12000*g*) for 30 min at 4 °C to separate the juice from the pulp. The freshly obtained juice materials were diluted 5× with Nanopure water, divided into multiple sample aliquots, and refrozen at −20 °C until used in antioxidant assay procedures.

Antioxidant Assay Procedures. For each antioxidant assay, a trolox aliquot was thawed and used to develop a 10−100 μmol/L standard curve. All data were then expressed as trolox equivalents (TE μmol). Assays were conducted by combining antioxidant reactants with 20 μL of individual standards or fruit juices. Reactions were allowed to progress at 28 ± 2 °C over extended periods and monitored at specific intervals. Assay results were obtained using a Beckman DU 640 spectrophotometer set at wavelengths appropriate to each assay. All assays of standards and fruit samples were performed in triplicate.

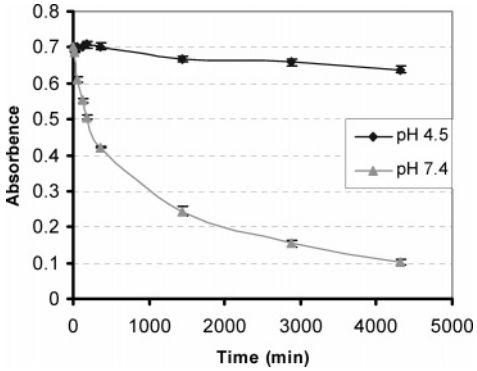

Figure 1. Absorbance over time of the ABTS•+ cation solution at pH 4.5 and pH 7.4.

For the standard TEAC assay, ABTS•+ was prepared by mixing an ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate (*23*). This mixture was allowed to stand for 12−16 h at room temperature in the dark until reaching a stable oxidative state. This reagent was stable for several weeks when stored in the dark (data not shown). The performance of the original method was ascertained using a series of phenolic standards. On the day of analysis, the ABTS•+ solution was diluted with PBS (pH 7.4) to an absorbance of 0.700 ± 0.01 at 734 nm. For the spectrophotometric assay, 3 mL of the ABTS•+ solution and 20 μL of standard or fruit extract were mixed and the absorbance was determined at 734 nm at 1−6, 10, 60, 120, 180, 360, and 1440 min after mixing. The absorbance at each time point was corrected for the absorbance of an ABTS blank.

For the modified ABTS assay, ABTS was dissolved in 20 mM acetate (pH 4.5) and prepared with potassium persulfate as described above and then diluted in acidic medium of 20 mM sodium acetate buffer (pH 4.5) to an absorbance of 0.700 ± 0.01 at 734 nm. The performance of the modified ABTS method was compared to those of the FRAP and DPPH methods using standards and fruit extracts. The antioxidant capacity of these samples was determined at 10, 30, 60, and 120 min intervals.

The FRAP method was conducted according to Benzie and Strain (*13*). A solution of 10 mM TPTZ and 20 mM ferric chloride was diluted in 300 mM sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Standards or fruit extracts (both 20 μL) were added to 3 mL of the TPTZ solution, and the absorbance at 593 nm was determined after assay samples were allowed to react for 10, 30, 60, and 120 min.

The ability of standards and fruit extracts to scavenge the DPPH radical was measured using the method of Brand-Williams et al. (*26*). DPPH (40 mg/L) was dissolved in 100% methanol. Standards and fruit extracts (both 20 μL) were added to 3 mL of the DPPH solution, and the absorbance was determined at 515 nm after 10, 30, 60, and 120 min.

RESULTS AND DISCUSSION

ABTS and pH. Oxidized ABTS (ABTS•+) has been generated by different methods (*21*−*24*). The advantages and disadvantages of these methods were discussed by Schlesier et al. (*10*). In the present study, we used the method developed by Rice-Evans et al. (*23*), in which ABTS is oxidized with potassium persulfate. **Figure 1** shows the relative stability of the ABTS•+ solution (standardized to an initial A_{734} of 0.7) at pH 4.5 and 7.4 over 72 h. Absorbance of the ABTS•+ solution prepared at pH 7.4 (without the addition of an antioxidant) decreased 1.7, 11, and 64% as compared to 0, 0.1, and 3% for the ABTS•+ solution at pH 4.5 after 10 min, 1 h, and 1 day, respectively. The data clearly demonstrate that the solution is not stable enough at pH 7.4 to allow long-term monitoring of

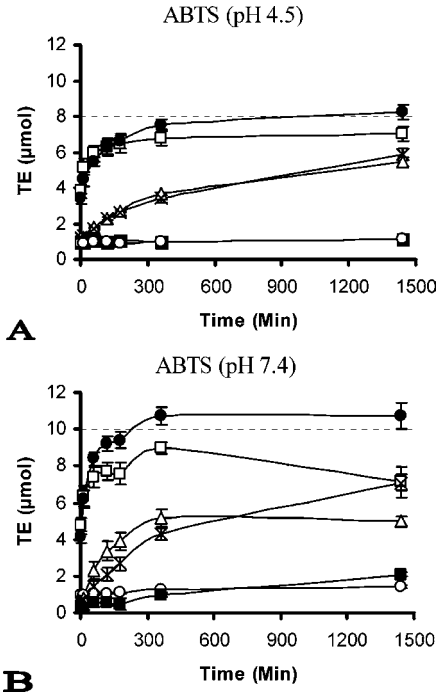

Figure 2. Antioxidant capacity expressed as TEs (μmol) of six antioxidant standards measured using the ABTS method at pH 4.5 and 7.4. Gallic acid, □; quercetin, ●; chlorogenic acid, △; caffeic acid, ×; ascorbic acid, ■; and trolox, ○.

reactions. Although frequently used at pH 7.4, the stability of $ABTS^{\bullet+}$ at this pH has been reported to be problematic (*25*, *27*) and confirmed by our work. Cano et al. (*25*) showed that $ABTS^{\bullet+}$ is stable from pH 3.0 to pH 6.5 but optimal at pH 4.5 for the purpose of using it an indicator of total antioxidant capacity.

Figure 2a,**b** shows the antioxidant capacity for individual standard compounds. When employed to characterize the oxidative behavior of phenolics and other antioxidant standards (ascorbic acid and trolox), $ABTS^{\bullet+}$ was sufficiently stable at pH 4.5 to accommodate the long reaction time necessary to reach equilibrium (**Figure 2a**). However, when conducted at pH 7.4, the relatively rapid autodegradation of $ABTS^{\bullet+}$ (**Figure 1**) precluded an accurate determination of phenolic antioxidant behavior for even 60 min (**Figure 2b**). When assayed with the modified ABTS method, quercetin and gallic acid approached equilibrium approximately 6 h after the reaction was initiated. In contrast, the oxidation of caffeic and chlorogenic acids continued for over 1440 min, failing to reach equilibrium before the assay was terminated. For these long experiments, it was essential to keep the $ABTS^{\bullet+}$/antioxidant ratio high (up to 50:1) as was shown by Cano et al. (*25*). As a practical reference, the absorbance (A_{734}) should not decrease below 50% of the initial absorbance of the assay solution. Because of the rapid decay of the $ABTS^{\bullet+}$ solution at pH 7.4 (**Figure 1**), the reliability of data from reactions with antioxidants is of concern at this pH **(Figure 2b)**.

For standard antioxidants such as trolox or ascorbic acid, $ABTS^{\bullet+}$ at pH 7.4 provided reliable end point values after 10 min (*24*). However, with standard phenolics, the results at 10 min are estimates only and do not represent equilibrium end point values based on oxidation. Also, with $ABTS^{\bullet+}$ at pH 7.4, values for the antioxidant capacity of the standard phenolics were 5−20% greater than the values determined at pH 4.5. This effect of pH on the observed antioxidant capacity has been noted

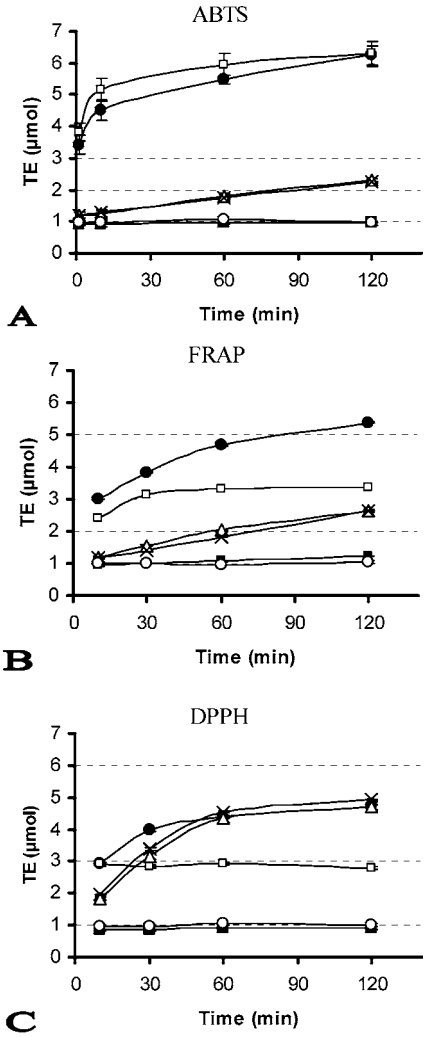

Figure 3. Antioxidant capacity expressed as TEs (μmol) of six standards measured with ABTS (at pH 4.5) (**A**), FRAP (**B**), and DPPH (**C**) assays. Gallic acid, □; quercetin, ●; chlorogenic acid, △; caffeic acid, ×; ascorbic acid, ■; and trolox, ○.

previously (*28*, *29*) and may be due to instability of $ABTS^{\bullet+}$ at the higher pH.

Comparison of DPPH, FRAP, and Modified ABTS Assays. Using the modified ABTS assay, oxidation of all standards by $ABTS^{\bullet+}$ (especially caffeic and chlorogenic acids) proceeded rapidly during the first 30 min of the reaction period and continued to increase over the 2 h assay period but at a slower rate (**Figure 3a**). The oxidation of caffeic acid and chlorogenic acid in the FRAP assay continued throughout the entire reaction period but at a more moderate rate (**Figure 3b**). In contrast, these two phenolic standards appeared to reach a stable end point after approximately 1 h of when DPPH was employed as the oxidizing (free radical) agent in the assay (**Figure 3c**). The oxidation of gallic acid and quercetin also appeared to be complete after 1 h of reaction with DPPH, but quercetin apparently did not reach equilibrium after 2 h using FRAP. Oxidation of gallic acid was complete in as little as 30 min with FRAP.

It is doubtful that differences in measured antioxidant capacity among the assays in our study could be attributed to the pH of the reaction mixture (pH 4.5 for the modified ABTS, pH 3.6 for FRAP), since the pK_a values of the oxidizeable hydroxyl groups on the standard compounds used are pH 7.0 or above

(*29*). For the DDPH assay, pH is irrelevant due to the use of methanol as the solvent.

The oxidation kinetics of the standards varied greatly with the assay used. In all three assays, quercetin had the highest antioxidant capacity and the reaction progress described a quadratic curve (**Figure 3 a−c**). Chlorogenic and caffeic acids behaved in a manner similar to each other in all three assays but showed very different oxidation profiles depending upon the assay. In the aqueous medium of the FRAP and ABTS assays, these two phenolic acids showed linear reactions over the 2 h period, never reaching equilibrium. However, with DPPH, which uses a methanol matrix, the reaction yielded a hyperbolic plot that approached an end point at 2 h. In contrast, gallic acid reached an end point almost immediately in the DPPH assay but required more than 60 min to approach the end point in the FRAP and ABTS assays.

Other researchers have equated the absolute antioxidant capacity of several phenolics to the number of available hydroxyls on a given molecule (*21*, *23*, *30*, *31*). Depending on which of the assays was utilized, the particular phenolic compound measured, and especially the length of incubation times used, our data (**Figure 3**) show that absolute antioxidant capacity cannot be predicted simply by determining the number of oxidizeable hydroxyl groups present in a given compound (*23*).

Our results also provide interesting insights into the relative effectiveness of specific phenolics as antixoidants. For those compounds exhibiting complex oxidation kinetics, the relative antioxidant capacity determined with short assay times may significantly underestimate the full antioxidant capacity of these compounds. Gallic and caffeic acids (**Figure 3**) provide good examples of these differences in response to the various protocols. In the DPPH solution, gallic acid reacts almost immediately to yield three TEs. This level of oxidation is consistent with previous reports that the antioxidant potential of phenolics is dependent upon the number of hydroxyls (gallic acid has three) and the amount and types of conjugation (*28*, *30*, *31*), and the rapidity of reaction suggests that all of the hydroxyls are open to attack by the DPPH radical in the methanol solution. In the FRAP assay, the reaction is hyperbolic and requires about 60 min for the reduction to slow with just more than three TE of Fe ultimately being reduced. However, in the modified $ABTS^{\bullet+}$ assay, the reaction at 1 min shows that there is a reduction of approximately three TEs, but at 1 h, it is over five TEs and reaches more than six TEs at 2 h.

In contrast, caffeic acid, which has only two phenolic hydroxyl groups, reacted slowly in the modified ABTS and FRAP assays showing a linear increase over time, with both measuring just less than 2 TEs after 1 h and about 2.5 after 2 h. The reaction with DPPH shows very different kinetics, with the reaction being hyperbolic starting at about 2 TEs at 10 min and proceeding to almost 5 TEs after 2 h. With both gallic and caffeic acid as well as with many of the other plant phenolic compounds, the long time periods needed for the reactions to approach equilibrium suggest that polymerization may occur during the oxidation process, which may regenerate oxidizable phenolic hydroxyl groups. Autooxidation of plant phenols including anthocyanins can result in the formation of polymers (*18*, *19*, *32*−*35*). Oxidation of the phenols by the chemical oxidants used in the antioxidant assays may also result in the formation of polymers (*36*). When chlorogenic acid reacts with $ABTS^{\bullet+}$ and the resulting mixture is purified by reverse phase and size permeation chromatography, there appears to be a shift in some of the products to higher apparent molecular weights (data not shown). These reaction products become brown in color and produce a noticeable shift in the UV/vis spectrum that is consistent with autooxidation and condensation of plant phenols observed in processed foods (*37*). The process of condensation can result in the generation of new antioxidants and account for TE values that exceed the number of available hydroxyls on the phenolic monomers (*38*). The complex oxidation kinetics of phenolics such as quercetin, caffeic acid, chlorogenic acid, and gallic acid therefore may result from their ability to polymerize after oxidation and with the ability of the new polymer to contribute new antioxidant capacity.

Fruit Extracts. Antioxidant capacity varied among the five fruit types and among antioxidant assays (**Figure 4a−d**). For instance, antioxidant values for all fruits studied were highest with FRAP, intermediate with DPPH, and lowest with modified ABTS assay, regardless of reaction time. The initial values (reaction times less than 10 min) are comparable to those reported in most studies that have used short time frames for these assays (*6*, *16*, *17*). However, sample ranking was not affected by the antioxidant assay used. The black raspberry sample had the highest antioxidant capacity in all assays followed by blackberry, red raspberry, strawberry, and red grape, respectively.

Similar to the reactions of phenolic standards, fruit extracts showed complex oxidation kinetics that required more than 2 h to reach equilibrium as compared to those of the assay standard, trolox. The reaction of some fruit extracts, most specifically, black raspberry, did not reach a steady state within 2 h using any of the assay methods employed. However, increased stability of the modified ABTS assay at pH 4.5 allowed the most accurate estimate of antioxidant capacity for all fruit samples, including black raspberry (**Figure 4a**). More remarkably, when fruit samples were assayed using the modified ABTS method for periods between 10 min to 6 h, antioxidant values for each of the fruit crops maintained their relative position to one another (**Table 1**). For instance, when the antioxidant capacity value for strawberry was used as a reference point, the value received for red raspberry would be 1.25−1.26 as large, etc. The stable ratio among fruit samples associated with the modified ABTS method (pH 4.5) would allow for the accurate comparison of the relative antioxidant value of a series of fruits samples over a broad range of reaction times.

The actual (absolute) antioxidant capacity of individual compounds and mixtures may be determined only when reactions have reached near equilibrium (steady state) conditions. Our results show that, due to the complex oxidation kinetics of plant phenolics, reactions must proceed for more than the 1−10 min as prescribed by previous studies (*5*, *12*, *21*, *22*). Reaction times of 60 min may provide relative values for individual antioxidants that are representative of the average antioxidant value, but even longer times may not produce antioxidant capacity values that represent the absolute antioxidant capacity. In our experiments, all reactions (except chlorogenic acid and caffeic acid standards in ABTS and FRAP assays) became asymptotic after 120 min, thus indicating that equilibrium had been achieved (**Figures 3** and **4**). On the basis of measurements made at 120 min, **Table 2** presents the end point antioxidant capacity of the phenolic standards and fruit samples. Using these near equilibrium values, quercetin and gallic acid exhibit the highest antioxidant capacities of the phenolic standards tested in the ABTS and FRAP assays, whereas quercetin, chlorogenic acid, and caffeic acid had the highest antioxidant capacities in the DPPH assay. In the ABTS assay, the antioxidant capacity of quercetin and gallic acid was approximately three times higher

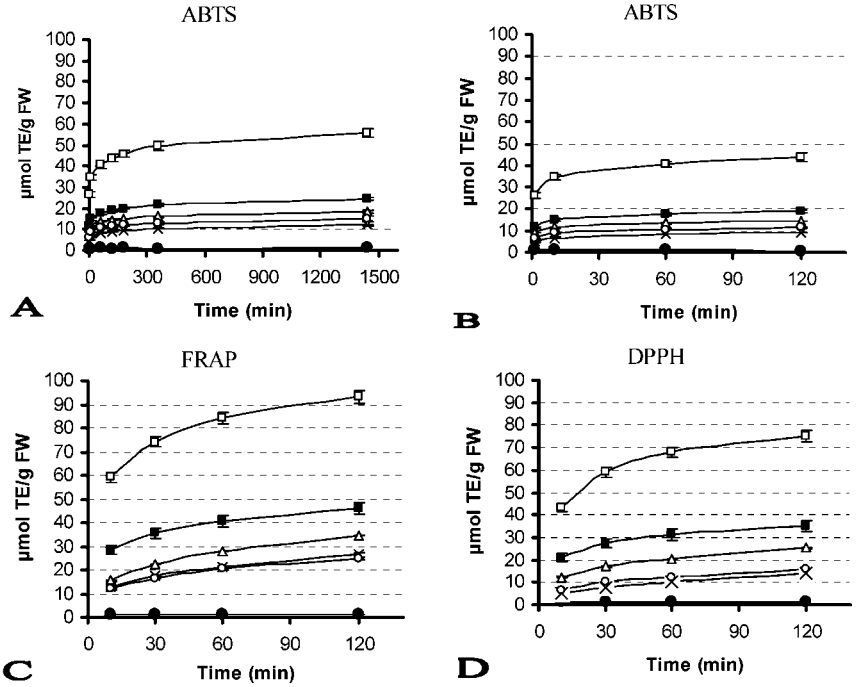

Figure 4. Antioxidant capacity expressed as TEs (μmol) of five fruits measured with ABTS (at pH 4.5) (**A** and **B**), FRAP (**C**), and DPPH (**D**) assays. Black raspberry, □; blackberry, ■; red raspberry, △; strawberry, ○; purple grape, ×; and Trolox, ●.

Table 1. Relative Ratios of Antioxidant Capacity Measured as TEs of Five Fruits Measured with ABTS, DPPH, and FRAP Assays at a Given Time

method	time (min)	strawberry	black raspberry	red raspberry	blackberry	purple grape
ABTS (pH 4.5)	1	1.00	4.19	1.46	1.82	0.79
	10	1.00	3.86	1.28	1.67	0.76
	60	1.00	3.80	1.26	1.67	0.78
	120	1.00	3.81	1.26	1.67	0.79
	180	1.00	3.82	1.25	1.67	0.80
	360	1.00	3.82	1.25	1.67	0.80
	1440	1.00	3.71	1.24	1.64	0.80
ABTS (pH 7.4)	1	1.00	4.93	1.32	2.13	0.84
	10	1.00	4.38	1.29	2.06	0.82
	60	1.00	3.85	1.35	2.00	0.84
	120	1.00	3.70	1.37	2.02	0.88
	180	1.00	3.69	1.40	2.05	0.92
	360	1.00	3.80	1.51	2.24	1.04
	1440	1.00	3.67	1.54	2.41	1.09
DPPH	10	1.00	6.82	1.93	3.28	0.79
	30	1.00	5.95	1.71	2.77	0.79
	60	1.00	5.48	1.65	2.52	0.82
	120	1.00	4.74	1.59	2.20	0.89
FRAP	10	1.00	4.92	1.29	2.36	1.06
	30	1.00	4.49	1.37	2.16	1.05
	60	1.00	4.14	1.36	1.99	1.04
	120	1.00	3.73	1.39	1.84	1.07

Table 2. Near Equilibrium (Steady State) Antioxidant Capacity Expressed as TEs (μmol) of Six Antioxidant Standards and Five Fruits Measured by ABTS (at pH 4.5), FRAP, and DPPH Assays after a 120 min Reaction Time[a]

	assay method		
	ABTS	FRAP	DPPH
antioxidant standards			
quercetin	6.3 ± 0.3	5.4 ± 0.0	4.7 ± 0.1
gallic acid	6.3 ± 0.4	3.4 ± 0.1	2.8 ± 0.0
chlorogenic acid	2.3 ± 0.1	2.7 ± 0.0	4.7 ± 0.1
caffeic acid	2.3 ± 0.1	2.6 ± 0.0	4.9 ± 0.0
ascorbic acid	1.0 ± 0.1	1.2 ± 0.0	0.9 ± 0.0
trolox	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
fruit extracts			
black raspberry	43.8 ± 1.9	93.1 ± 2.5	75.4 ± 1.5
red raspberry	14.5 ± 0.2	34.7 ± 0.1	25.3 ± 0.4
blackberry	19.2 ± 0.6	46.0 ± 2.6	35.0 ± 3.1
purple grape	9.1 ± 0.2	26.6 ± 0.9	14.2 ± 0.4
strawberry	11.5 ± 0.4	24.9 ± 0.7	15.9 ± 0.1

[a] Means are presented ± standard error.

than chlorogenic acid or caffeic acid and six times higher than ascorbic acid or trolox. These differences in antioxidant capacity among the standards were not as great in the FRAP and DPPH assays. For the fruit tested, black raspberry had the highest antioxidant capacity, regardless of assay method, and was approximately two times higher than blackberry (**Table 2**). Purple grape and strawberry antioxidant capacity was only 20−25% of that observed in black raspberry, regardless of assay method. Cano et al. (*25*) described an end point method to estimate total antioxidant capacity of ascorbic acid and citrus juices using the ABTS assay. In our experiments, ascorbic acid reached equilibrium in approximately 1 min in all assays (**Figure 3**), which is not surprising since ascorbic acid is a simple antioxidant with only one oxidizable hydroxyl group. Thus, for compounds with multiple oxidizable hydroxyl groups or with complex mixtures of antioxidants (i.e., fruit extracts), long reaction times are necessary to determine total antioxidant capacity. Furthermore, regardless of assay, the coefficient of variation decreased for individual compounds and fruit samples as the reaction time increased and equilibrium conditions were achieved (data not shown), indicating that more reproducible estimates of antioxidant capacity are possible with longer reaction times than with short reaction times, which are commonly used by other researchers (*5*, *12*, *21*, *22*).

Data in **Figure 4** and **Table 2** also demonstrate the variation in total antioxidant capacities of fruit when measured using the three assays. The modified ABTS method yields antioxidant values that are consistently lower than those determined by FRAP and DPPH. These differences are especially noticeable

when comparisons are made between the measured antioxidant capacities of dark-colored fruits (black raspberries and black berries) vs the lighter, red-colored fruits or Concord grape. The most probable explanation for these differences is that the anthocyanins in the dark-colored fruits, which make up a substantial portion of antioxidant capacity, do not react as readily with the $ABTS^{\bullet+}$ moiety as they do with those in the FRAP and DPPH assays.

Because fruit extracts contain a number of different phenolic compounds, true determination or comparison of antioxidant capacity of these samples is complicated by not only the averages of the different compounds but also by the capacity for polymerization. The three assays give very different values for the fruit in absolute terms but show the same relative ranking. The continual progress of the reactions (as shown by the lines in **Figure 4**) for all three assays suggests that for all practical purposes these assays can only provide relative antioxidant capacities (TE) for these complex mixtures of phenolics and other antioxidants with reaction times of less than 120 min. The continuous reaction with FRAP and DPPH suggests that these assays underestimate the absolute antioxidant capacity of the berries, but data taken after long reaction times (2 h) provide a better estimate of the absolute antioxidant capacity than short reaction times. We have also determined antioxidant capacity of acetone-extracted fruit samples (data not shown). These samples exhibited higher values as compared to raw fruit juice samples. However, the trends in antioxidant capacity between the fruits using the different extraction methods were the same, regardless of extraction method.

Conclusions. Measuring and reporting antioxidant capacity for fruits and other natural products requires selection of appropriate assays depending upon the hypothesis and types of potential antioxidants being tested (*39*). Variability in the hydrophilicity of the reaction mixtures and the products being tested greatly influence the data derived from the various protocols (*10*, *30*). Selectivity and stability of the various antioxidant methods currently in use have posed a serious obstacle to measuring the absolute potential of many of the plant phenolic compounds. Earlier workers have postulated an association between the antioxidant capacity and the number of available hydroxyl groups on a given molecule (*21*, *23*, *30*, *31*). However, this postulate is not supported by our data when reactions are allowed to proceed to near equilibrium.

The antioxidant protocols examined here were all developed and standardized using simple antioxidants such as trolox and ascorbic acid (*13*, *21*, *26*, *27*). These compounds show simple kinetics with all of the antioxidant assays and reach relatively stable end points quickly. Many plant phenolics, on the other hand, as well as fruit extracts, show complex kinetics and reach end points only after several hours or days, with some of the more complex compounds never seeming to reach near equilibrium (steady state) conditions within a reasonable time period. However, many reached equilibrium after 120 min, which allowed us to present near end point antioxidant capacity values (**Table 2**).

The results of this study suggest that for the ABTS assay, lowering the pH from 7.4 to 4.5 provides a stable reaction medium for measuring the antioxidant capacity of fruits and other natural products containing phenolics. A reaction pH of 4.5 allows measurement of the antioxidant potential for periods long enough to ensure a good approximation of the absolute antioxidant capacity. This modified method also offers stability of the stock solution, which we have maintained at 4 °C for several weeks with no appreciable change in absorbance. However, for samples having a physiological pH near 7 (e.g., blood plasma), neither the modified ABTS assay nor the FRAP assay described here would be suitable to determine absolute antioxidant capacity, although one could use values determined at pH 4.5 to estimate capacity near pH 7. The DPPH assay is more suited for samples with lipophilic antioxidants or those having a high lipid content.

The modified ABTS method provides for a sensitive, long-term measurement of antioxidant capacity of fruit samples. Ratios of the antioxidant capacity of fruit samples were the same at 10 min as they were after a 6 h of reaction time (**Table 1**), providing a reproducible assessment of the relative antioxidant potentials. Longer incubation periods (**Figure 4**) allow the reactions to approach near steady state values if the ratio of indicator (i.e., $ABTS^{\bullet+}$, Fe, DPPH) is kept relatively high (50:1), and we recommend reaction times of at least 1 h.

The differences in the kinetics and the TE measurements of the phenolic compounds illustrate two important facts when trying to determine the antioxidant capacity of natural plant products. First, each of the methods provides only an estimate of the capacity that is dependent upon time of reaction, method used, and the complexity of the reaction kinetics. Second, the potential for interaction/polymerization of phenolic compounds may cause antioxidant capacity to be underestimated in fruit samples and with individual compounds. Therefore, no single antioxidant assay method can provide a complete picture of the antioxidant capacity of compounds that show complex kinetics, i.e., most complex natural products. Using at least two different antioxidant methods to compare fruits samples provides the opportunity to identify variations in response that may otherwise be missed. The modified ABTS assay offers a stable method and is a good choice for combination with the FRAP or DPPH methods. DPPH can provide an advantage if the antioxidants being tested are more soluble in organic solvents. Therefore, these three protocols provide a good selection of methods to use for antioxidant measurements, which can meet the needs of most research into fruits and fruit juices.

ABBREVIATIONS USED

Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TPTZ, 2,4,6-tris-2,4,6-tripyridyl-2-triazine; ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; $ABTS^{\bullet+}$, ABTS radical; PBS, phosphate-buffered saline; DPPH, 2,2′-diphenyl-1-picrylhydrazyl; TE, trolox equivalent.

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