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Major Phenolics in Apple and Their Contribution to the Total Antioxidant Capacity

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The contribution of each phytochemical to the total antioxidant capacity of apples was determined. Major phenolic phytochemicals of six apple cultivars were identified and quantified, and their contributions to total antioxidant activity of apples were determined using a 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay and expressed as vitamin C equivalent antioxidant capacity (VCEAC). Average concentrations of major phenolics and vitamin C in six apple cultivars were as follows (mg/100 g of fresh weight of apples): quercetin glycosides, 13.20; procyanidin B_2, 9.35; chlorogenic acid, 9.02; epicatechin, 8.65; phloretin glycosides, 5.59; vitamin C, 12.80. A highly linear relationship ($r^2 > 0.97$) was attained between concentrations and total antioxidant capacity of phenolics and vitamin C. Relative VCEAC values of these compounds were in the order quercetin (3.06) > epicatechin (2.67) > procyanidin B_2 (2.36) > phloretin (1.63) > vitamin C (1.00) > chlorogenic acid (0.97). Therefore, the estimated contribution of major phenolics and vitamin C to the total antioxidant capacity of 100 g of fresh apples is as follows: quercetin (40.39 VCEAC) > epicatechin (23.10) > procyanidin B_2 (22.07) > vitamin C (12.80) > phloretin (9.11) > chlorogenic acid (8.75). These results indicate that flavonoids such as quercetin, epicatechin, and procyanidin B_2 rather than vitamin C contribute significantly to the total antioxidant activity of apples.

KEYWORDS: Apples; free radical; phenolics; vitamin C equivalent antioxidant capacity (VCEAC)

INTRODUCTION

Free radicals are major molecules that cause human diseases such as cancer, heart disease, cerebrovascular disease, and aging through diverse cellular processes (*1*−*3*). Naturally occurring antioxidants have been reported to play a major role in ameliorating oxidative damage induced by free radicals. Recently, natural foods and food-derived components, such as antioxidative vitamins and phenolic phytochemicals, have received a great deal of attention because they are safe and not perceived as "medicine"; some of these are known to function as chemopreventive agents against oxidative damage. Vitamin C has been considered to be one of the most prevalent antioxidative components of fruits and vegetables and exerts substantial chemopreventive effects without apparent toxicity at a relatively high level (*4*). However, the contribution of vitamin C to the total activity of fruits was determined to be generally <15% (*5*). On the other hand, the importance of contributions of phytochemicals to the total antioxidant capacity of fruits, vegetables, grains, and tea has been suggested (*5*, *6*). Much attention has recently been paid to the possible health benefits of dietary phenolics that have antioxidant activities stronger than that of vitamin C.

The nutritional value and health-related biological activity of fruits depend not only on the concentration but also on the amount of such foods consumed daily. Apples are one of the major fruits frequently consumed by Americans. Among fresh fruits consumed in 1996, apples (8.76 kg/person/year) ranked second to bananas (12.7 kg). However, when fresh and processed products are combined, the estimated per capita consumption of apples (21.3 kg) exceeds that of bananas. Therefore, apple phenolics as antioxidant sources in the American diet may provide major protection against free radical damages in the human body (*7*).

Our previous studies showed that the antioxidative and antiproliferative activities of apples are the consequence of synergistic activities of phenolics rather than vitamin C (*6*). In particular, phenolics in apple skin showed a much higher degree of contribution to the total antioxidant and antiproliferative activities of whole apple than those in apple flesh (*6*, *8*). Furthermore, quercetin, one of the major antioxidative flavonoids in apple, exerted much stronger antioxidant and anticarcinogenic activities than vitamin C (*4*, *9*). Thus, the importance of contributions of phenolics to the total antioxidant capacity of apples has been suggested (*4*−*6*, *9*). Apples contain various antioxidative phenolics such as chlorogenic acid,

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epicatechin, procyanidin B_2, phloretin, and quercetin as well as vitamin C (*10*, *11*). In the mixture of such bioactive compounds, however, the relative contribution of each antioxidant to the total antioxidant capacity has not been clearly demonstrated. Because the contents of total phenolics or flavonoids in fruits often do not directly reflect the total antioxidant capacity, the accurate measurement of the antioxidant capacity of each bioactive compound should be warranted. In the present study, we identify major phenolics in various apple cultivars and investigate their contributions to the antioxidant activity of apples compared with that of vitamin C.

MATERIALS AND METHODS

Chemicals. Ammonium hydroxide, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as diammonium salt, ammonium phosphate monobasic ($NH_4H_2PO_4$), quercetin, epicatechin, phloretin, and chlorogenic acid were obtained from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). Procyanidin B_2 was obtained from Shimazu Co. (Kyoto, Japan). Quercetin glycosides (arabinoside, gulcoside, galactoside, and rhamnoside) and phloretin glucoside were obtained from Extrasynthese (Genay, France). Vitamin C was purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals used were of analytical or HPLC grade.

Apple Cultivars. Six apple cultivars, Golden Delicious, Cortland, Monroe, Rhode Island Greening, Empire, and NY674, were picked at commercial maturity during the 2001 harvest season at the New York State Agricultural Experiment Station orchard in Geneva, NY. Apples were stored in a 2−5 °C cold room. They were carefully cut into slices, the pits were removed, and the freeze-dried samples were ground to powder using a laboratory mill (Thomas-Willey) and then stored at −20 °C until analyzed.

Extraction of Phenolics. The phenolics were extracted by using the ultrasound-assisted method (*12*). Briefly, phenolics were extracted from 10 g of ground freeze-dried sample using 100 mL of 80% aqueous methanol. The mixture was sonicated for 20 min with a continual stream of nitrogen gas purging to prevent possible degradation of phenolics, filtered through Whatman no. 2 filter paper (Whatman International Limited, Kent, U.K.) using a chilled Büchner funnel, and rinsed with 50 mL of 100% methanol. Extraction of the residue was repeated under the same conditions. The two filtrates were combined and transferred into a 1 L evaporating flask with an additional 50 mL of 80% aqueous methanol. The solvent was evaporated using a rotary evaporator at 40 °C. The remaining phenolic concentrate was first dissolved in 50 mL of 100% methanol and diluted to a final volume of 100 mL using distilled deionized water (ddH_2O). The mixture was centrifuged at refrigerated temperature (4 °C) for 20 min and stored at −4 °C until analyses. The total extraction process was done in duplicate.

Identification of Phenolics. HPLC analysis was performed according to the method described in our previous paper (*13*). Extracted samples were filtered through a 0.45 μm poly(tetrafluoroethylene) syringe-tip filter, using a 20 μL sample loop, and were analyzed using an HPLC system (Hewlett-Packard model 1100, Palo Alto, CA) equipped with a photodiode array detector, a quaternary pump, and a vacuum degasser. A C18 reversed-phase Symmetry Analytical column (5 μm × 250 mm × 4.6 mm) was used with a Symmetry Sentry guard column of the same packing material as the analytical column (Waters Corp., Milford, MA). Three mobile phases were used: solvent A, 50 mM ammonium phosphate monobasic ($NH_4H_2PO_4$), pH 2.6 (pH adjusted with phosphoric acid); solvent B, 80:20 (v/v) acetonitrile/50 mM $NH_4H_2PO_4$, pH 2.6; and solvent C, 200 mM phosphoric acid (H_3PO_4), pH 1.5 (pH adjusted with ammonium hydroxide). The gradient for HPLC analysis was linearly changed as follows (total 60 min): 100% A at 0 min, 92% A/8% B at 4 min, 14% B/86% C at 10 min, 16.5% B/83.5% C at 22.5 min, 25% B/75% C at 27.5 min, 80% B/20% C at 50 min, 100% A at 55 min, 100% A at 60 min. Flow rate was 1.0 mL/min at constant room temperature (23 °C). Phenolic standards were used to generate characteristic UV−vis spectra and calibration curves. Individual phenolics in the sample were tentatively identified by comparison of their UV−vis spectra and retention times with spiked input of polyphenolic standard. Three replicated HPLC analyses were performed for each apple cultivar.

Quantification of Vitamin C. Ascorbic acid was determined by using the 2,6-dichloroindophenol titrimetic method, according to AOAC method 967.21 (*14*). Reference material was an ascorbic acid solution (1 mg/mL) prepared from L-ascorbic acid.

ABTS Radical Scavenging Activity. The ABTS method described earlier was used with slight modification (*9*). Briefly, 1.0 mM AAPH was mixed with 2.5 mM ABTS in phosphate-buffered saline (PBS) solution (100 mM potassium phosphate buffer containing 150 mM NaCl). The mixture was heated in a 68 °C water bath. The resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.30 ± 0.02 at 734 nm. Various doses of antioxidants (each 10 μL) were added to 190 μL of the resulting blue-green ABTS radical solution in a 96 well plate. The control consisted of 10 μL of 99% ethanol and 190 μL of ABTS radical solution. The decrease in absorbance, which resulted from the addition of test compounds, was measured at 734 nm using an ELISA reader (Emax, Molecular Devices Co., Sunnyvale, CA). ABTS radical scavenging activities of the test compounds were expressed as percent remaining ABTS radicals at each time point. The radical stock solution was prepared fresh daily.

Quantification of Total Antioxidant Capacity. A method developed by Winston et al. (*15*) was applied with slight modifications for the quantification of antioxidant value of each compound tested. The area under the kinetic curve was calculated by integration. The total antioxidant capacity (TAC) of each tested compound was then quantified according to eq 1. Percent increase in integrated area was

$$\mathrm{TAC} = 100 - \left(\int \mathrm{SA} / \int \mathrm{CA} \times 100\right) \qquad (1)$$

measured to compare each phenolic and vitamin C. Here, $\int$ SA and $\int$ CA are the integrated areas from the curve defining the sample and control reactions, respectively. The median effective dose (EC_{50}) of all samples tested was calculated from the dose−response curve. TAC of each phenolic was expressed as vitamin C equivalents (VCEAC). All tested samples were replicated six times and presented as mean value ± standard deviation.

RESULTS AND DISCUSSION

Composition and concentrations of the major phenolics of six apple cultivars studied are shown in **Table 1**. Among the apple cultivars studied, Rhode Island Greening showed the highest content in all phenolic phytochemicals analyzed. Average concentrations of the major phenolics were as follows (mg/100 g of fresh weight): quercetin glycosides, 13.20; procyanidin B_2, 9.35; chlorogenic acid, 9.02; epicatechin, 8.65; and phloretin glycosides, 5.59. Chlorogenic acid and phloretin glycosides presented lower contents compared to quercetin glycosides and procyanidin B_2. Several phenolics in apples were present as glycosides. In particular, a wide variety of quercetin glycosides were present in the apple cultivars. Galactoside was the most abundant form among the glycosides identified in most of the tested cultivars except NY674, in which rhamnoside was most abundant. In addition, xyloglucoside was an abundant form of phloretin glycoside.

There is increasing evidence that flavonoids can be absorbed into the human body in amounts that are, in principle, sufficient to exert antioxidant or other biological activities in vivo (*16*−*18*). Chlorogenic acid is absorbed with no structural change in the small intestine (*19*), whereas both epicatechin and procyanidin B_2 are absorbed as epicatechin (*20*). In general, derivatives of flavonoids and isoflavones were found to have lower biological activities in free forms compared with their parent aglycons in vitro. However, gastrointestinal hydrolase removes

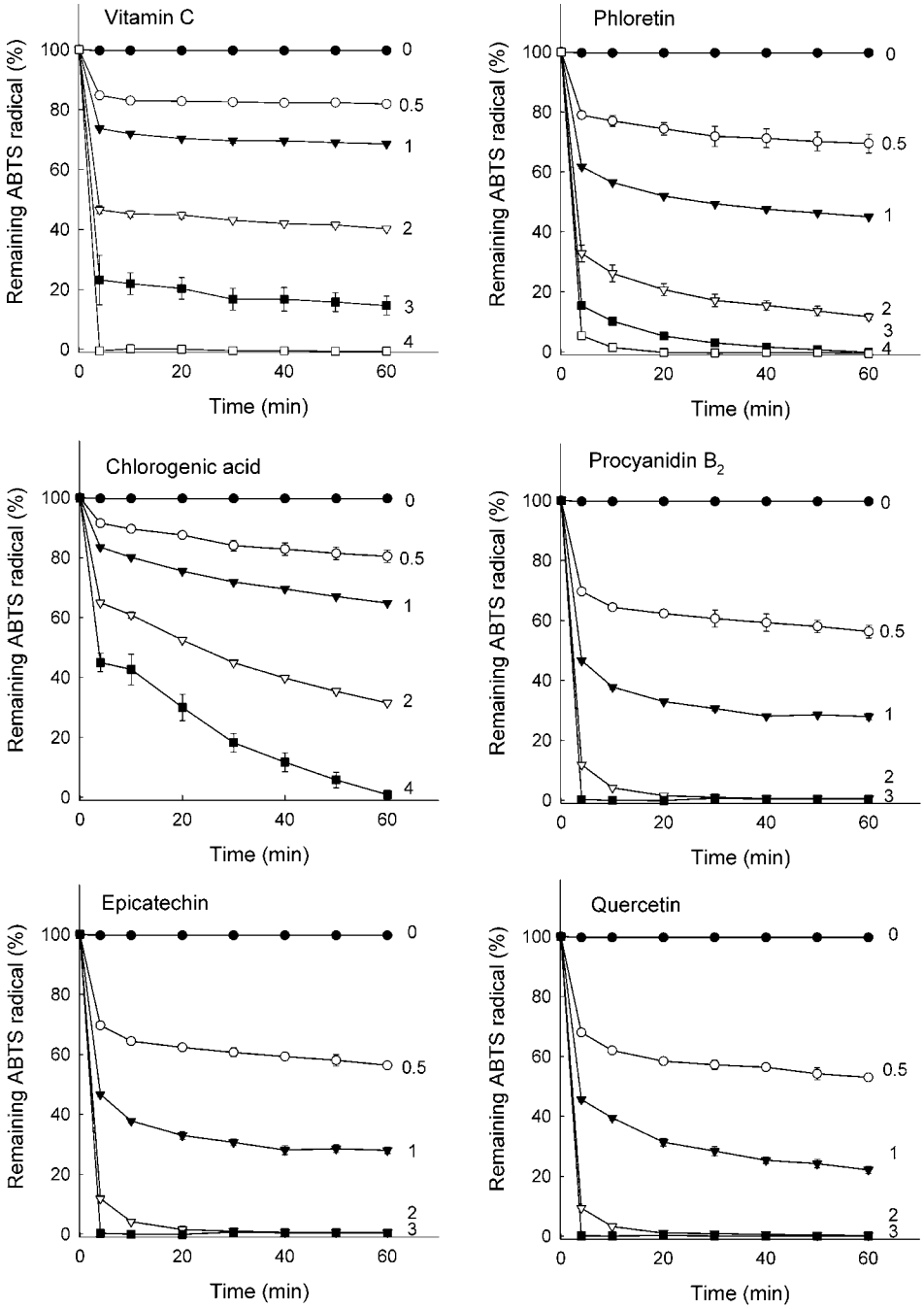

Figure 1. Kinetics of ABTS radical reactions with vitamin C, chlorogenic acid, epicatechin, phloretin, procyanidin B_2, and quercetin. Each test compound at 0, 0.5, 1, 2, 3, and 4 μg/mL was reacted with 2.5 mM ABTS radicals. Error bars represent standard deviations of each data point ($n = 3$).

the sugar moiety from flavonoid glycosides, and their aglycons are released to be absorbed in the gut (*16*). Intestinal conjugation seemed to be an important process for the absorption because only conjugated forms were detected in the mesenteric vein blood (*17*). Furthermore, when quercetin glycosides and genistin were fed to rats or humans, quercetin and genistein, their only respective aglycon forms, were detected in the urine (*17*, *18*).

Therefore, we measured herein the antioxidant activity of quercetin and phloretin instead of that of quercetin and phloretin glycosides. Scavenging rates of each tested major antioxidants in apples against the ABTS radical at different concentrations and times are shown in **Figure 1**. Vitamin C and phenolics exerted ABTS radical scavenging activity in dose- and time-dependent manners. Strong correlations ($r^2 > 0.97$) were observed between the concentrations and the TAC of vitamin C (**Figure 2**) and phenolics (**Figure 3**) in apple. The relative TAC of phenolics evaluated by the ABTS assay compared to vitamin C was as follows: quercetin (3.06) > epicatechin (2.67) > procyanidin B_2 (2.36) > phloretin (1.63) > vitamin C (1.00) > chlorogenic acid (0.97) (**Table 2**). The data show that quercetin has the lowest EC_{50} value among the major phenolics in the apple.

Although most of the phenolics are reported to have antioxidant activity, quercetin has been reported to have structural advantages as an antioxidant because the *o*-dihydroxy moiety in the B ring confers stability to the resulting free radical form (*21*). Because quercetin is mainly present in apple peel (*10*), it was suggested that consumption of apples with skins is highly desirable in order to maximize apple antioxidant activity (*22*). In parallel, quercetin showed the highest antioxidant capacity in the ABTS radical scavenging assay. Considering the amount of each compound, the estimated contribution of quercetin

Table 1. Composition and Quantification of Major Antioxidants of Six Apple Cultivars

	fresh apples with skins (mg/100 g)						
antioxidant	Golden Delicious	Cortland	Monroe	Rhode Island Greening	Empire	NY674	av
vitamin C	16.60	12.17	9.00	14.22	13.22	11.62	12.80
chlorogenic acid	8.48	5.36	10.08	14.28	11.52	4.40	9.02
epicatechin	7.12	8.32	10.72	19.16	2.28	4.32	8.65
phloretin glycosides							
glucoside	1.80	1.44	2.40	2.08	2.80	1.84	5.59
xyloglucoside	1.92	3.20	4.92	5.88	1.72	3.56	
procyanidin B_2	6.28	11.32	8.32	21.68	3.44	5.04	9.35
quercetin glycosides							
arabinoside	2.16	2.40	4.44	2.88	2.76	1.56	13.20
xyloside	1.68	1.08	2.28	1.92	2.16	1.20	
glucoside	2.40	1.56	2.40	1.20	2.40	0.36	
galactoside	4.20	3.36	4.80	4.32	4.20	1.92	
rhamnoside	3.84	2.28	3.12	4.08	3.84	2.40	
total	56.48	52.49	62.48	91.70	50.34	38.22	58.61

Table 2. Contributions of Major Antioxidants to the Total Antioxidant Activity of Apples

phytochemical	concn (mg/100 g of fresh wt)	EC_{50}	relative VCEAC value[a]	total antioxidant activity (mg of VCEAC/100 g)	relative contribution (%)
quercetin glycosides	13.20	0.56	3.06	40.39	34.7
epicatechin	8.65	0.64	2.67	23.10	19.9
procyanidin B_2	9.35	0.72	2.36	22.07	19.0
vitamin C	12.80	1.71	1.00	12.80	11.0
phloretin glycosides	5.59	1.05	1.63	9.11	7.8
chlorogenic acid	9.02	1.76	0.97	8.75	7.6
total	58.61			116.22	100.0

[a] Relative VCEAC value = VCEAC of each compound/antioxidant capacity of vitamin C.

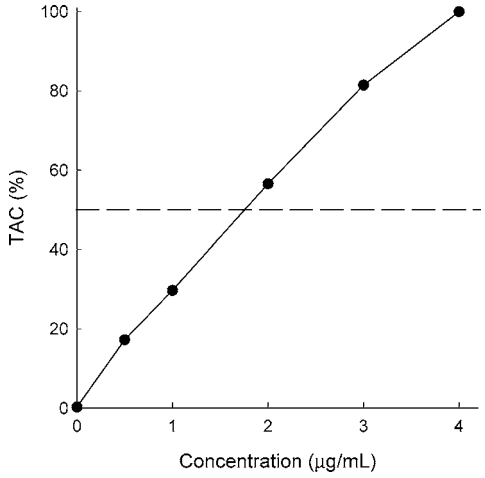

Figure 2. Total antioxidant capacity (TAC) of vitamin C.

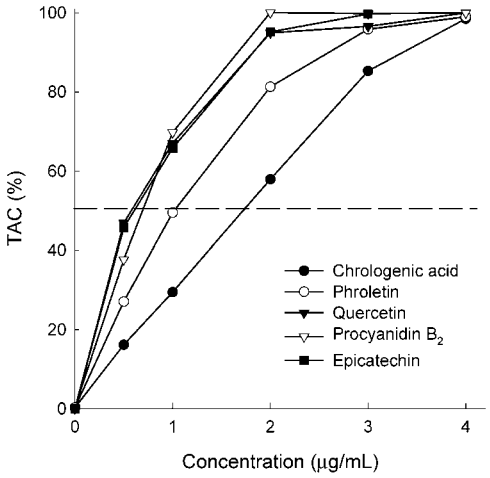

Figure 3. Total antioxidant capacity (TAC) of major phenolics in apples.

(40.39 VCEAC) to the total antioxidant capacity of apples is the highest among major phytochemicals, which was followed by epicatechin (23.10) and procyanidin B_2 (22.07), whereas chlorogenic acid (8.75) and phloretin (9.11) provide minimal contribution (**Table 2**). Moreover, vitamin C contributes only 11% of the total antioxidant capacity of apple. These results clearly indicate that flavonoids such as quercetin, epicatechin, and procyanidin B_2 rather than vitamin C contribute significantly to total antioxidant activity of apples.

Sun et al. (*23*) reported that phytochemicals in fruits including apple showed a high correlation with antioxidant capacity ($r^2 = 0.97$). On the other hand, Imeh et al. (*24*) observed a weak correlation ($r^2 = 0.58$) between the phenolic content of the fruits and the total antioxidant activity measured by ferric reducing antioxidant power assay. This was probably due to the other unquantified phenolics and/or synergism among these compounds and major phenolics. Apples, like other fruits, vary in chemical composition, even within the same variety, depending on maturity, location produced, and agricultural practices, as well as numerous other environmental factors. Indeed, significant variations in phenolic content and antioxidant activity were observed among cultivars and even among different fruits in the same cultivar (*24*).

In this study, various apple cultivars showed different levels of phenolic content and various phenolics showed different antioxidant activities. Some active phenolics such as chlorogenic acids, phloretins, epicatechins, quercetins, and procyanidin B_2 have been identified as major antioxidants in apples. The evidence shown herein in terms of the content and the capacity of antioxidants suggests that quercetin may have the highest

contribution as an antioxidant in apples. We suggest that the contribution of each nutrient or antioxidant in the daily diet should be carefully considered on the basis of its biological activity and quantitative consumption data. We also need to study the interactions among active food components and their diverse bioactivities to determine the total biological activities of the food. At present, we are studying anti-tumor promoting activities of major phenolics in apples.

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ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); VCEAC, vitamin C equivalent antioxidant capacity; TAC, total antioxidant capacity.

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