JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2005, 53, 4989−4995 4989

Which Polyphenolic Compounds Contribute to the Total Antioxidant Activities of Apple?

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The antioxidant activities of eight apple cultivars were studied by using the ferric reducing/antioxidant power (FRAP), the β-carotene−linoleic acid model system (β-CLAMS), and the photochemiluminescent (PCL) assays. The antioxidant activity of apples is highly correlated to the total phenolic content (TPC) measured by the Folin−Ciocalteu method and the total polyphenolic index (TPI) obtained by HPLC. Extracts of the peel and flesh were analyzed and assayed separately. The FRAP activities of both peel and flesh extracts correlate well with the TPC ($r = 0.95$ and 0.99, respectively) and the TPI ($r = 0.82$ and 0.99, respectively). Similar results were found in the β-CLAMS activities, showing correlation coefficients of $r = 0.90$ and 0.91 with the TPC for the peel and flesh extracts and of $r = 0.90$ and 0.84 with the TPI for the peel and flesh extracts, respectively. The antioxidant activity measured by the PCL assay was not correlated with TPC or TPI due to the lack of integratable lag phase in this method with the flavan-3-ols/procyanidins. Among the five major polyphenolic groups, flavan-3-ols/procyanidins had the highest positive correlation with the FRAP and β-CLAMS activities: $r = 0.84$ and 0.88 for the peel extracts, respectively; and $r = 0.98$ and 0.87 for the flesh extracts, respectively. At individual compound level, epicatechin and procyanidin B2 were the major contributors to the antioxidant activity of apple. Hydroxycinnamic acids may have a significant role in the flesh.

KEYWORDS: Apple; cultivar; polyphenolic, phenolic; flavonoid; procyanidins; quercetin glycosides; cyanidin-3-galactoside; hydroxycinnamic acid; dihydrochalcones; antioxidant activity; FRAP; β-CLAMS; PCL

INTRODUCTION

An apple a day keeps the doctor away. This saying has encouraged many researchers to search for the “magic” ingredient of the apple that may reveal its validity. Recent epidemiological and *in vitro* experimental studies indeed have shown the linkage between the consumption of apple or related products and many chronic human diseases. Most noticeably, apples have been associated with lowered risks of cancer, particularly prostate, liver, colon, and lung cancers (*1*−*4*), and cardiovascular diseases (*5*, *6*). These chronic diseases are considered to be caused by oxidative processes, especially those involving excess free radicals and reactive oxygen species (ROS). Phytochemicals such as polyphenolics have been found to be the major source of antioxidants in apple, rather than essential vitamins such as vitamin C (*2*, *6*, *7*−*13*).

The mechanisms of the antioxidant activity of polyphenolics have been studied extensively. They usually have high redox potentials, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (*14*). In addition, they have a metal-chelating potential (*15*). The antioxidant activity of individual polyphenolic compounds, however, may vary significantly depending on the chemical characteristics and the mechanisms of assays used.

There are five major groups of polyphenolic compounds in apple, including hydroxycinnamic acids, flavan-3-ols, anthocyanidins, flavonols, and dihydrochalcones. The phenolic profile, however, is much more complicated because of polymerization of flavan-3-ols and glycosylation of other polyphenolics. More specifically, flavan-3-ols can be found in their monomers, oligomers, and polymers (procyanidins); flavonols are often associated with sugar moieties. The predominant sugars involved in glycosylation are galactose, glucose, rhamnose, arabinose, and xylose; and the disaccharide, rutinose, has also been found in apple. Dihydrochalcones are mainly associated with glucose and xyloglucose, and the oxidization of the 3′-position in dihydrochalcones further complicates the profiles (*16*−*19*). The complexity of the chemical profile and the variations caused by growth period, growing season, geographic location, and,

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10.1021/jf048289h CCC: $30.25 Published 2005 by the American Chemical Society
Published on Web 05/14/2005

most importantly, genetic variation (*16*, *18*, *19*) predispose the variation in apple antioxidant activity.

Only one of the major apple flavonols, quercetin and its derivatives, has been used in the various *in vivo* experiments. Quercetin was found to inhibit human prostate and lung cancer cell growth (*3*, *4*) and to reduce the incidence of cardiovascular diseases (*20*). However, investigating one particular component may not represent the whole picture and can be misleading. Quercetin derivatives are not the major polyphenolic component of apple, and they are not necessarily the strongest antioxidants among apple polyphenolics either (*7*). Other compounds, such as the procyanidins, showed even greater antioxidant activity (*7*, *21*). This suggests that the roles of the individual compounds are important in the total antioxidant activity of apple, and they must be investigated thoroughly.

In this study, we used three different assays, the ferric reducing/antioxidant power (FRAP) assay, the β-carotene–linoleic acid model system (β-CLAMS), and the photochemiluminescent (PCL) assay, to evaluate the antioxidant activities of the extracts of eight apple cultivars and the major individual polyphenolics. Correlations between the antioxidant activity and the total and individual phenolic concentrations were examined.

MATERIALS AND METHODS

Chemicals and Solvents. Phenolic standards were purchased from various manufacturers. Gallic acid, chlorogenic acid, *p*-coumaric acid, catechin, epicatechin, sodium carbonate (Na_2CO_3), and the Folin–Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO); quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Fluka Chemie GmbH (Buchs, Switzerland); quercetin-3-arabinoside and quercetin-3-xyloside were from Apin Chemicals Ltd. (Abingdon, U.K.); procyanidins B1 and B2, phloridzin, and cyanidin-3-galactoside were from Indofine Chemical Co. (Hillsborough, NJ). Water used for HPLC analysis was purified in-house from distilled and deionized water using a NanoPure system (Dubuque, IA). All other solvents were of HPLC grade and were purchased from Caledon Laboratories Ltd. (Georgetown, ON).

Sample Preparation. Sample preparation for the eight apple cultivars (10 apples each), Golden Delicious, Red Delicious, McIntosh, Empire, Ida Red, Northern Spy, Mutsu, and Cortland, followed the same procedures as described in our previous paper (*19*). Peel and flesh samples were separately extracted with 70% aqueous methanol at 1:1 (w/v) using a Polytron blender (Brinkmann Instruments, Westbury, NY), filtered, and stored at −20 °C before being analyzed and assayed (within 30 days).

Total Phenolic Content (TPC). The Folin–Ciocalteu (FC) reagent method (*22*) was modified and followed as described in our previous paper (*19*). TPCs were expressed as micrograms of gallic acid equivalents (GAE) per milliliter of solution. All samples were prepared and analyzed in duplicate.

Identification and Quantification of Polyphenols by HPLC Method. The method of identification and quantification of polyphenols in apples has been reported previously (*19*). Briefly, the separation was carried out in a C18 column with a binary mobile phase consisting of 6% acetic acid in 2 mM sodium acetate buffer and acetonitrile and a diode array detector. The total polyphenolic index (TPI) was calculated according to the method given in our previous paper (*23*). TPI is the sum of concentrations of all polyphenolic compounds separated and detected by HPLC. All samples were prepared and analyzed in duplicate.

Ferric Reducing/Antioxidant Power (FRAP) Assay. The FRAP assay used was a modified version of that of Benzie and Strain (*24*). The assay was based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ), which increases the absorption at 593 nm. In this study, the above method was modified for the 96-well microplate reader. Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). All standards were prepared at 500 μM in water or methanol, and samples were diluted to ensure that the readings were in the linear range of the standard curve. The reagent (300 μL) and the standard or sample solutions (10 μL) were added to each well and mixed thoroughly. The absorbance was taken at 593 nm immediately after and at 1-min intervals up to 4 min using a UV–vis microplate kinetics reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT). The plate was incubated at 37 °C for the duration of the reaction. All treatments were run in triplicate. The FRAP value of the samples was calculated on the basis of 500 μM ascorbic acid (equivalent to 1000 μM FRAP values).

β-Carotene–Linoleic Acid Model System (β-CLAMS). The β-CLAMS (*25*, *26*) method was based on the decoloration of β-carotene by the peroxides generated during the oxidation of linoleic acid (a free radical chain reaction) at elevated temperature. In this study, the β-CLAMS was modified for the 96-well microplate reader. In brief, β-carotene (0.5 mg) was dissolved in ~2 mL of $CHCl_3$ in a 200-mL round-bottom flask, to which 25 μL of linoleic acid and 200 mg of Tween 40 were added. $CHCl_3$ was removed using a rotary evaporator. Oxygenated HPLC grade water (100 mL) was added, and the flask was shaken vigorously until all materials dissolved. This test mixture was prepared fresh and used immediately. To each well were added 250 μL of the test mixture and 35 μL of sample solution or solvent (blank). The plate was incubated at 45 °C. Readings were taken at 490 nm immediately after and at 120 min using the same microplate kinetics reader as stated above. All antioxidant standards and samples were prepared at 200 ppm, and all treatments were run in triplicate. The results were expressed as percent inhibition of the degradation of β-carotene at 120 min:

$$(\%)\ \text{inhibition} = [(120\ \text{min}\ A_{490\text{nm}})/(0\ \text{min}\ A_{490\text{nm}})] \times 100$$

Photochemiluminescent (PCL) Assay. The principle of PCL was based on an ~1000-fold acceleration of the oxidative reaction *in vitro* compared to normal conditions. This effect was achieved by optical excitation of a suitable photosensitizer, which exclusively results in the generation of the superoxide radical $O_2^{\bullet -}$. The radicals were visualized with a chemiluminescent detection reagent. A synthetic fluorescent compound, luminol, was used in this assay. This compound plays a dual role, acting as both the photosensitizer and the radical reaction agent (*27*). A commercial PCL instrument, the Photochem (Berlin, Germany) system was used in this study. This system is very flexible and can be used for both water-soluble and lipid-soluble antioxidative substances. The company supplied the complete kits for both. For water-soluble substances, the assay mixture contained 1 mL of reagent 1 (sample solvent), 1.5 mL of reagent 2 (reaction buffer), 25 μL of diluted reagent 3 (luminol), and 10 μL of reagent 4 (ascorbic acid) for the calibration curve or 10 μL of sample solution for the antioxidant activity. For water-soluble antioxidants, the evaluation of the activity was based on the lag phase in seconds (*27*). When necessary, samples were diluted so that the PCL curves fell within the linear range of the standard, ascorbic acid (0.05–0.3 mM).

Statistical Analysis. Statistical analysis was conducted using Unitstat version 5.5 (London, U.K.). Tests for an association between the concentration of a compound (or the total phenolic concentration) and the antioxidant activity measured by three different methods, or between two different antioxidant methods, were run using standard Pearson correlation.

RESULTS AND DISCUSSION

Major Polyphenolics in Apple. The polyphenolic profiles and concentrations of major groups in the peel and flesh of the eight most popular apple cultivars grown in Ontario and the TPIs and TPCs were reported in our earlier paper (*19*). The major polyphenolic groups were hydroxycinnamic acids (chlorogenic acid and *p*-coumaroylquinic acid), cyanidin-3-galactoside, flavan-3-ols/procyanidins (mainly catechin, epicatechin,

Table 1. Total Phenolic Concentrations and Antioxidant Activities of the Apple Peel and Flesh Extracts

apple	TPC[a]	TPI[b]	FRAP[c]	PCL[d]	β-CLAMS[e]
			Peel		
Empire	782 ± 4	1017 ± 11	2736 ± 57	3800 ± 4	17 ± 2
Mutsu	1017 ± 26	1089 ± 25	6820 ± 55	4643 ± 24	20 ± 3
McIntosh	1163 ± 17	1636 ± 22	6436 ± 46	5531 ± 23	31 ± 3
Golden Delicious	1265 ± 22	1249 ± 6	9616 ± 66	5223 ± 11	22 ± 0
Cortland	1323 ± 21	1659 ± 28	11908 ± 42	4133 ± 16	32 ± 2
Ida Red	1479 ± 21	1763 ± 16	12083 ± 37	5958 ± 17	26 ± 1
Northern Spy	1548 ± 24	2073 ± 12	10044 ± 20	6112 ± 14	36 ± 1
Red Delicious	2012 ± 20	2350 ± 29	17851 ± 42	4112 ± 13	59 ± 1
			Flesh		
Empire	164 ± 4	177 ± 1	550 ± 12	1457 ± 3	8 ± 0
Mutsu	198 ± 10	313 ± 13	1584 ± 20	1895 ± 14	39 ± 1
Ida Red	237 ± 2	489 ± 1	2749 ± 41	3069 ± 0	51 ± 1
MacIntosh	255 ± 3	488 ± 10	2785 ± 19	2967 ± 1	58 ± 1
Golden Delicious	260 ± 1	417 ± 15	2036 ± 20	2192 ± 4	45 ± 2
Red Delicious	358 ± 12	534 ± 6	3215 ± 27	2192 ± 4	49 ± 2
Cortland	364 ± 5	498 ± 11	3660 ± 23	1629 ± 1	65 ± 1
Northern Spy	561 ± 7	934 ± 7	6425 ± 6	5049 ± 1	71 ± 2

[a] Total phenolic content (TPC) measured by Folin–Ciocalteu method in micrograms of gallic acid equivalent per gram of fresh weight (data were taken from ref *19*). [b] Total polyphenolic index (TPI) calculated according to the HPLC method in ref *23*; data were taken from ref *19*. [c] FRAP values in micromolar. [d] Ascorbic acid equivalent in micromolar. Values are for extracts obtained from 1:1 fresh sample/70% methanol (w/v) extraction (see ref *19*). [e] Relative inhibition values (percent) at 120 min; apple peel samples were diluted by a factor of 4 before being assayed.

Table 2. Antioxidant Activity of Polyphenol Standards

compound	FRAP[a]	β-CLAMS[b]	PCL[c]
phloridzin	37 ± 3	24 ± 1	ND[d]
quercetin-3-xyloside	934 ± 5	58 ± 2	305 ± 6
quercetin-3-galactoside	1017 ± 24	68 ± 1	296 ± 15
chlorogenic acid	1023 ± 13	40 ± 0	590 ± 2
catechin	1042 ± 70	56 ± 0	NO[e]
quercetin-3-glucoside	1049 ± 12	46 ± 2	241 ± 6
epicatechin	1209 ± 35	47 ± 3	NO
quercetin-3-rhamnoside	1382 ± 8	66 ± 1	457 ± 5
procyanidin B1	2338 ± 11	56 ± 3	NO
procyanidin B2	2645 ± 18	53 ± 2	NO
cyanidin-3- galactoside	3232 ± 43	81 ± 1	485 ± 1

[a] All standards were measured at 500 μM; FRAP values in micromolar. [b] All standards were at 200 ppm; the relative inhibition values (percent) were at 120 min. [c] Ascorbic acid equivalent in micromolar. [d] Not detectable. [e] Not obtainable.

and procyanidins B1 and B2), flavonols (quercetin galactoside, glucoside, rhamnoside, arabinoside, and xyloside), and dihydrochalcones (mainly phloridzin and phloretin-2′-xyloglucoside). No anthocyanins were detected in the flesh (*19*). The TPCs and TPIs were extracted from our previous paper and are listed in **Table 1** together with the antioxidant activities obtained in this study. Among all of the polyphenolics, the concentration of total procyanidins was the greatest, representing more than half of the total phenolics in both peel (60%) and flesh (56%) (*19*). The antioxidant activity of procyanidins therefore will significantly affect the total antioxidant activity of apple.

FRAP Assay of Polyphenolic Standards and Apple Extracts. Cyanidin-3-galactoside showed the highest activity, whereas phloridzin showed the lowest (**Table 2**). For procyanidins (B1 and B2), the activities of dimers were ∼2 times greater than those of the monomers, possibly due to the fact that there were two monomeric units in the dimers. The antioxidant activity (FRAP) of epicatechin was greater than that of catechin, and that of procyanidin B2 was greater than that of B1. This implied that the *cis*-hydroxyl group on C-3 of the C-ring is more effective than the trans configuration in making a compound a strong reducing agent. Quercetin glycosides as a group had only moderate FRAP activity, and the hydroxycinnamic acid derivative, chlorogenic acid, had the second lowest activity. On average, the antioxidant activities of the five polyphenolic groups of apple were in the following decreasing order: cyanidin-3-galactoside > procyanidins > quercetin glycosides > chlorogenic acid > phloridzin.

For apple samples, peel extracts had much greater antioxidant activities than the flesh extracts (**Table 1**). Red Delicious peel extract had the highest antioxidant activity, whereas the Empire peel extract had the lowest; this result was consistent with the total polyphenolic concentration in Red Delicious and Empire peels. The FRAP activity of the peels from different cultivars positively correlated with both TPC and TPI of the samples ($r = 0.95$ and 0.82, respectively) (**Table 3**). When calculated against the major groups of polyphenols, the FRAP values of the peel extracts were found to have the best positive linear correlation with the total flavan-3-ols/procyanidins ($r = 0.84$), suggesting that the procyanidins are a major contributor to the FRAP antioxidant activity of the peel. This result was not surprising considering that flava-3-nols/procyanidins accounted for 60% of the total phenolics in the peel and were strong individual antioxidants (*19*; **Table 2**). Cyanidin-3-galactoside had the highest antioxidant activity among all tested standards; however, it accounted for only 5.4% of the total polyphenolics of the peels (*19*), and its concentration did not correlate with the FRAP activity ($r = 0.10$; **Table 3**). Cyanidins were therefore considered to have limited contribution to the total antioxidant activity of apple. Similar observations were found in flesh extracts; the FRAP activity had even better linear correlations with the TPC and TPI ($r = 0.99$ and 0.99, respectively). Once again, the antioxidant activity correlated well with only the flavan-3-ols in the flesh ($r = 0.98$; **Table 3**). Procyanidins were also the major antioxidants in the flesh, which again was supported by the high percentage (56%) in the TPC and TPI of the flesh (*19*) and the high antioxidant activity of individual flavan-3-ols/procyanidins. The FRAP assay clearly showed that the flavan-3-ols, including monomers, dimers, and oligomers, were the most important antioxidants in both apple peel and flesh.

***β*-CLAMS of Polyphenolic Standards and Apple Extracts.** Although the mechanism of the β-CLAMS assay was different from that of the FRAP assay, among the individual polyphenolic standards, cyanidin-3-galactoside again showed the highest activity and phloridzin the lowest (**Table 2**). In the flavan-3-ol group, catechin was a better antioxidant than epicatechin, and procyanidin B1 was stronger than procyanidin B2. In this assay, the *trans*-hydroxyl group of C-3 of the C-ring seemed to be more effective than the *cis* configuration. Unlike the FRAP assay where values can be stoichiometrically calculated, results from the β-CLAMS assay were difficult to quantify. This was perhaps why procyanidin dimers did not show greater activity than the monomers. Procyanidins as a group had lower activity than quercetin glycosides in this assay. Overall, the average activities of the five groups of apple polyphenolics (decreasing order) were cyanidin-3-galactoside > quercetin glycosides > procyanidins > chlorogenic acid > phloridzin.

Despite the differences between the antioxidant activities of the individual standards measured by the above two methods, in the actual apple samples the β-CLAMS activities followed trends similar to those observed in the FRAP assay. In the peels, the β-CLAMS values were closely correlated to the TPC and

Table 3. Correlation Coefficients (*r*) between Total or Individual Polyphenolic Concentrations and the Antioxidant Activities Measured with Three Different Methods and between Two Antioxidant Methods

method/concentration[a]	peel			flesh		
	FRAP	β-CLAMS[d]	PCL	FRAP	β-CLAMS[d]	PCL
FRAP	1.00			1.00		
β-CLAMS	0.83	1.00		0.85	1.00	
PCL	0.04	−0.08	1.00	0.82	0.62	1.00
chlorogenic acid	−0.48	−0.47	0.67	0.61	0.37	0.93
p-coumaroylquinic acid	−0.22	0.01	0.42	0.55	0.77	0.29
total hydroxycinnamics[b]	−0.50	−0.47	0.71	0.67	0.46	0.95
catechin	0.49	0.52		0.94	0.81	
epicatechin	0.88	0.95		0.87	0.85	
procyanidin B1	0.49	0.58		0.92	0.70	
procyanidin B2	0.81	0.80		0.91	0.87	
other procyanidins	0.86	0.87				
total flavan-3-ols[b]	0.84	0.88		0.98	0.87	
cyanidin-3-galactoside	0.10	0.18	−0.65			
total anthocyanins[b]	0.10	0.18	−0.65			
quercetin-3-galactoside	0.34	−0.01	−0.43			
quercetin-3-glucoside	−0.49	−0.35	−0.54			
quercetin-3-xyloside	−0.06	0.12	−0.16			
quercetin-3-arabinoside	0.04	0.08	0.26			
quercetin-3-rhamnoside	−0.57	−0.41	0.46	−0.14	−0.05	−0.19
total flavonols[b]	−0.46	−0.37	−0.27	−0.14	−0.05	−0.19
3-hydroxyphloretin-2′-xyloglucoside	−0.75	−0.64				
3-hydroxyphloretin-2′-glucoside	0.38	0.53				
phloretin-2′-xyloglucoside	0.07	0.10		0.25	0.35	
phloridzin	0.64	0.74		0.13	−0.02	
total dihydrochalcones[b]	0.51	0.62		0.22	0.10	
TPI (HPLC)[c]	0.82	0.90	0.27	0.99	0.84	0.89
TPC (FC)[c]	0.95	0.90	0.19	0.99	0.91	0.78

[a] Average of concentrations of each compound found in all eight apple varieties, in micrograms per gram of fresh weight. [b] Sum of all compounds in the respective categories. Total flavan-3-ols included all monomeric and oligomeric flavan-3-ols. [c] Data were taken from ref *19*. [d] Correlation with the percent inhibition at 120 min.

TPI ($r = 0.90$ and 0.90, respectively) (**Table 3**). Although pure flavan-3-ols were of moderate strength in terms of antioxidant activity in the β-CLAMS assay, the flavan-3-ols/procyanidins as a group again had the best positive correlation with the β-CLAMS activity ($r = 0.88$). Very similar correlations were observed in the flesh samples; $r = 0.91$ and 0.84 with TPC and TPI, respectively, and $r = 0.87$ with the total flavan-3-ols. Among the flavan-3-ols, epicatechin and procyanidin B2 again had the best linear correlation with the β-CLAMS values ($r = 0.95$ and 0.80, respectively). The β-CLAMS results led to a similar conclusion; that is, the flavan-3-ols/procyanidins are likely to be the major antioxidants in apple, and epicatechin and procyanidin B2 are the two most important flavan-3-ols.

PCL Assay of Apple Polyphenolic Standards and Extracts. According to the manufacturer, PCL can be used to assess nearly all water-soluble and fat-soluble phytochemical antioxidants; however, we found that due to the way the activity was calculated, that is, according to the retarded lag phase (*27*), PCL could not be used to evaluate the antioxidant activity of flavan-3-ols/procyanidins. These compounds did not have suitably retarded lag phases necessary for our calculation. The lack of a proper lag phase may not be caused by the concentration, as we found that neither dilution nor concentration generated integratable lag phases. Unlike weak antioxidants such as phloridzin, which had a very short integratable lag phase, procyanidins seemed to strongly inhibit the generation of superoxide as demonstrated by catechin in **Figure 1**. The lag phase appeared to be formed and the curve was very flat, but the shape could not be integrated properly by the software (**Figure 1**). Using the kit for fat-soluble antioxidants did not improve the calculation of the antioxidant activity of the flavan-3-ols/procyanidins. Without proper quantitative data, it was therefore difficult to compare the antioxidant activity of the flavan-3-ols with other compounds. In all of the tested standards except for the flavan-3-ols, chlorogenic acid turned out to have the greatest activity and phloridzin remained the least. The overall PCL activity in decreasing order was chlorogenic acid > cyanidin-3-galactoside > quercetin glycosides > phloridzin (**Table 1**).

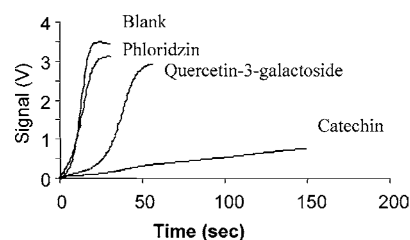

Figure 1. Typical time courses of antioxidants in the photochemiluminescent assays using the Photochem system: blank, without addition of any antioxidants; phloridzin, quercetin-3-galactoside, and catechin were measured at the same concentration (20 μM).

For the same reason mentioned above, when PCL was performed for actual apple samples, no correlation was observed between the peel PCL activity and the peel TPC or TPI ($r = 0.19$ and 0.27, respectively) (**Table 3**). The lack of correlation, in turn, may be indirect proof that the flavan-3-ols/procyanidins were the major contributors to the total antioxidant activity of the peel extracts, because these were the major components in the peel. In flesh samples, however, the correlation was relatively high; $r = 0.78$ and 0.89 with TPC and TPI, respectively. This was probably because of the high percentage of total hydroxycinnamic acids in the total polyphenolic content (40% including *p*-coumaroylquinic acid) and the strong PCL activity of chlo-

Table 4. Comparison of Measured and Calculated FRAP Values

apple	peel			flesh		
	measured	calculated[a]	%[b]	measured	calculated[a]	%[b]
Empire	2736 ± 57	3403 ± 10	124 ± 0	550 ± 12	459 ± 19	83 ± 5
McIntosh	6436 ± 46	5699 ± 20	89 ± 2	2785 ± 19	1593 ± 7	57 ± 1
Mustu	6820 ± 55	3545 ± 33	52 ± 2	1584 ± 20	1050 ± 37	66 ± 4
Golden Delicious	9616 ± 66	4069 ± 35	42 ± 2	2037 ± 20	1407 ± 19	69 ± 1
Northern Spy	10044 ± 20	7737 ± 41	77 ± 2	6425 ± 6	3353 ± 30	52 ± 1
Cortland	11908 ± 42	6448 ± 51	54 ± 4	3660 ± 23	1799 ± 28	49 ± 1
Ida Red	12083 ± 37	6473 ± 38	54 ± 0	2749 ± 41	1647 ± 19	60 ± 1
Red Delicious	17851 ± 42	8858 ± 14	50 ± 0	3215 ± 27	1912 ± 36	60 ± 2
average	9687 ± 19	5779 ± 23	50 ± 0	2876 ± 1	1653 ± 1	60 ± 1

[a] See text for calculation. [b] Percent calculated FRAP value versus measured FRAP value.

rogenic acid (**Table 2**). The high correlation ($r = 0.95$) between the total hydroxycinnamic acids and the PCL activity suggests that hydroxycinnamic acids may be another group contributing to the antioxidant activity of apple flesh.

Comparison of the Three Antioxidant Assays. Among the three assay methods, FRAP and PCL are more quantitative than β-CLAMS. PCL, which detects the antioxidants in the nanomole range, is more sensitive than the other two; however, as our data have shown, it may not provide quantitative data for certain groups of phytochemical antioxidants. Therefore, when samples are rich in certain compounds, for example, flavan-3-ols, PCL will not reflect the true antioxidant activity of these samples. The FRAP assay provides quantitative values of the reducing power of a compound (it is simple and of low cost). The β-CLAMS assay provides an alternative mechanism by measuring the capability of a compound to resist peroxidation and free radical chain reaction; however, the quantification is arbitrary; in our study, we took the readings at 120 min, and most β-CLAMS protocols in the literature took measurements anywhere from 15 to 300 min (*28*, *29*). Such "arbitrary quantification" can sometimes lead to different conclusions.

The complexity of the chemical profiles in apple makes it difficult to evaluate the activity based on any one single assay method. Some compounds, such as phloridzin in this study, may display low activity in all assays, whereas others such as cyanidin-3-galactoside always show high activity. Conclusions based on these compounds were readily made; however, not all phytochemicals were like these two extreme examples. For instance, chlorogenic acid showed very low activity in the FRAP (the second lowest) and the β-CLAMS (the third lowest), but the highest in PCL; on the other hand, quercetin-3-galactoside was one of the strongest in the β-CLAMS but one of the weakest in the FRAP and of moderate strength in the PCL assay. This inconsistency reconfirms that it is necessary to have at least two methods with different modes of actions in one study to accurately evaluate the antioxidant activity (*30*).

Comparison of Results with Previous Studies. The positive correlation between the antioxidant activity and the total polyphenolic content that was observed in this study has also been found by numerous others (*7*, *21*). The antioxidant activity of apple was not only correlated to the total polyphenolic concentration but was also dependent on the polyphenolic composition. Our results clearly indicate that the flavan-3-ols/procyanidins contributed the most to the total antioxidant activities of both apple peel and flesh. This conclusion was consistent with a number of previous studies using different assay methods (*21*, *31*, *32*), but not all (*10*). By using the DPPH radical scavenging assay, Chinnici and co-workers (*21*) found that the antioxidant activity of individual compounds followed the order of quercetin glycosides > procyanidins > chlorogenic > phloridzin; however, in the actual apple samples, procyanidins (including monomeric flavonols) contributed the most to the total antioxidant activity ($r = 0.94$). The results were similar to our β-CLAMS assay, which was essentially based on the free radical chain reaction of fatty acids. Other studies (*31*, *32*) also found that flavan-3-ols were the top contributor, followed by quercetin glycosides, chlorogenic acid, cyanidin-3-galactoside, and phloridzin. In a study using the ABTS radical scavenging activity assay, however, Lee and co-workers (*10*) found that quercetin glycosides were the dominating contributor to the antioxidant activity of apple. It should be noted that the concentrations of flavan-3-ols in their study were underestimated (only epicatechin and procyanidin B2 were quantified); second, because of the underestimation of the flavan-3-ols, the assumption that quercetin derivatives were the major contributor to the antioxidant activity of apple due to their high activity as pure quercetin glycosides may be unconvincing; third, the high antioxidant activity of individual quercetin glycosides may not lead to good correlation between these compounds and the antioxidant activity of actual apple samples, as shown in this study and others (*21*).

Measured versus Calculated Antioxidant Activity. Due to the above-discussed shortcomings of the β-CLAM (lack of definite quantification) and PCL (lack of lag phase for the procyanidins) assays, we attempted to use only the FRAP values and the concentrations of the polyphenolics (HPLC data) for the "calculated antioxidant activity". The total calculated FRAP values were a sum of all calculated FRAP values of the detected polyphenols in each sample; the calculated FRAP value of a compound was obtained as follows:

$$\text{calculated FRAP value} = \mathrm{C} \times \mathrm{FRAP}_{\mathrm{std}}/C_{\mathrm{std}}$$

C is the molar concentration of the individual compound, $\mathrm{FRAP}_{\mathrm{std}}$ is the FRAP value of the corresponding standard, and C_{std} is the molar concentration at which the $\mathrm{FRAP}_{\mathrm{std}}$ was obtained. For polyphenols without commercially available standards, we used chlorogenic acid, procyanidin B2, quercetin-3-galactoside, and phloridzin for their respective groups. It should be noted that the total calculated antioxidant activity does not reflect any synergistic, antagonistic, or other effects of the polyphenols in the samples. As shown in **Table 4**, on average, in the peels, 68% (between 42 and 124%) of the measured antioxidant activity was explained by the calculated FRAP values; in the flesh, 62% (between 49 and 83%) was attributed to the detected polyphenols. Except for the Empire peel sample, all calculated results were smaller than those of the measured values, suggesting possible synergism among the polyphenols

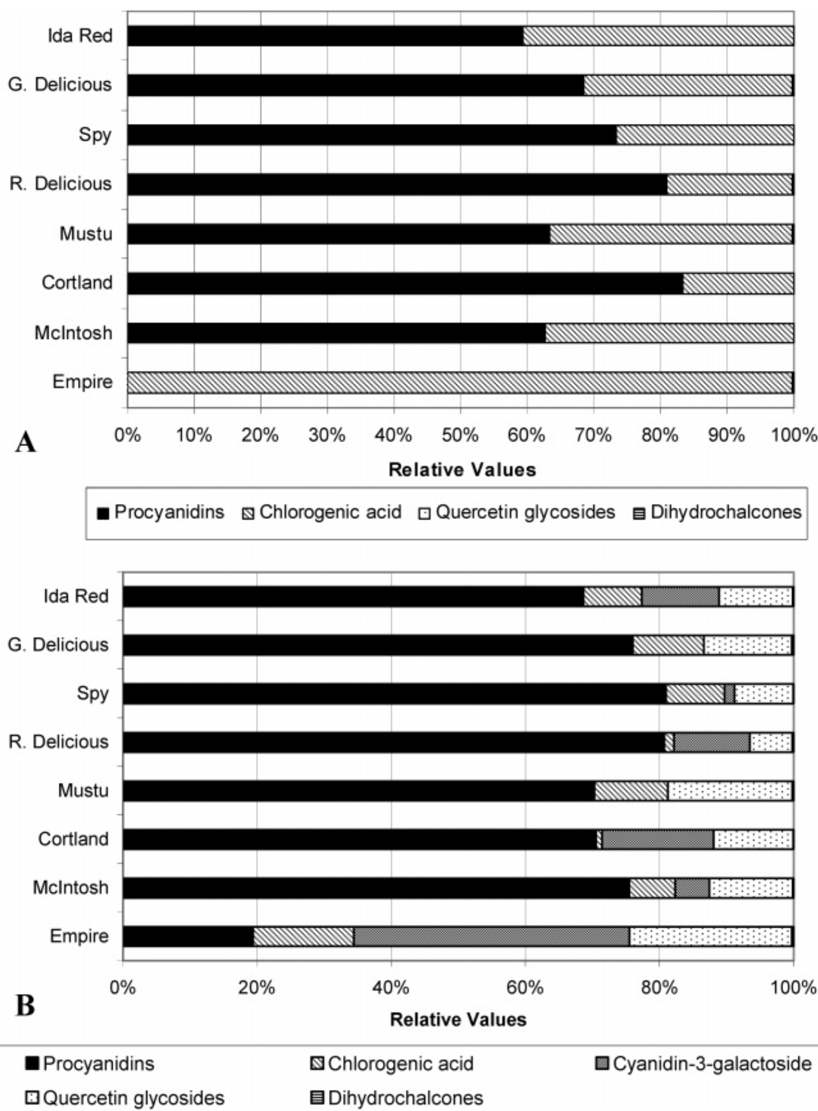

Figure 2. Contribution of different polyphenol groups to the calculated FRAP values (see text for calculation): (**A**) flesh samples; (**B**) peel samples. The relative contribution of each group was calculated by dividing the FRAP value of each group by the total calculated FRAP values and multiplying by 100.

and the effect of other antioxidants such as ascorbic acid. Our calculated numbers were higher than the 35−50% reported by Van der Sluis et al. (*31*, *32*) and lower than the 85% reported by Chinnici et al. (*21*). The variation may be due to the different assay methods, apple cultivars, and methods for quantification of polyphenolics. At group levels, in apple flesh (**Figure 2A**), an average of 69% of the calculated antioxidant activity was attributed to the flavan-3-ols, 31% to the hydroxycinnamic acids, and the rest (<0.1%) to the quercetin glycosides and dihydrochalcones; in the peels, an average of 71% of the calculated antioxidant activity derived from the total polyphenolic content can be ascribed to the flavan-3-ols (**Figure 2B**), followed by quercetin glycosides (12%), cyanidin-3-galactoside (10%), hydroxycinnamic acids (7%), and dihydrochalcones (0.1%). These results were consistent with the correlation results (**Table 3**).

Conclusion. The antioxidant activity of apple was positively correlated with the total polyphenolic concentrations of both TPC and TPI, the concentration of flavan-3-ols/procyanidins at the group level, and procyanidin B2 and epicatechin at the individual compound level. We therefore conclude that the flavan-3-ols/procyanidins are the most important contributors to the *in vitro* antioxidant activity of apple and that procyanidin B2 and epicatechin are the most important individual antioxidants in apple. Hydroxycinnamic acids may have a significant role in the flesh.

ACKNOWLEDGMENT

We thank Jason McCallum for technical assistance and the McCallum farm (Woodstock, ON) for supplying fresh apples.

LITERATURE CITED

(1) Willett, W. C. Diet, nutrition, and avoidable cancer. *Environ. Health Perspect*. **1995**, *103*, 165−170.

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

(3) Le-Marchand, L.; Murphy, S. P.; Hankin, J. H.; Wilkens, L. R.; Kolonel, L. N. Intake of flavonoids and lung cancer. *J. Natl. Cancer Inst.* **2000**, *92*, 154−160.

(4) Xing, N.; Chen, Y.; Mitchell, S. H.; Young, C. Y. F. Quercetin inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. *Carcinogenesis* **2001**, *22*, 409−414.

(5) Knekt, P.; Jarvinen, R.; Reunanen, A.; Maatela, J. Flavonoid intake and coronary mortality in Finland: a cohort study. *Br. Med. J.* **1996**, *312*, 478−81.

(6) Hyson, D.; Studebaker-Hallman, D.; Davis, P. A.; Gershwin, M. E. Apple juice consumption reduces plasma low-density lipoprotein oxidation in healthy men and women. *J. Med. Food* **2000**, *3*, 159−166.

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

(8) Hanasaki, Y.; Ogawa, S.; Fukui, S. The correlation between active oxygen scavenging and antioxidative effects of flavonoids. *Free Radical Biol. Med*. **1994**, *16*, 845−850.

(9) Wang, H.; Cao, G.; Prior, R. L. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996**, *44*, 701−705.

(10) Lee, K. W.; Kim, Y. J., Kim, D.-O.; Lee, H. J.; Lee, C. Y. Major phenolics in apple and their contribution to the total antioxidant capacity. *J. Agric. Food Chem.* **2003**, *51*, 6516−6520.

(11) Guo, C.; Cao, G.; Sofic, E.; Prior, R. L. High-performance liquid chromatography coupled with coulometric array detection of electroactive components in fruits and vegetables: relationship to oxygen radical absorbance capacity. *J. Agric. Food Chem.* **1997**, *45*, 1787−1796.

(12) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem.* **1996**, *44*, 3426−3431.

(13) Cao, G.; Russell, M.; Lischner, N.; Prior, R. L. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J. Nutr.* **1998**, *128*, 2383−2390.

(14) Kahkonen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J. P.; Pihlaja, K.; Kujala, T. S.; Heinonen, M. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **1999**, *47*, 3954−3962.

(15) Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* **1995**, *22*, 375−383.

(16) McRae, K. B.; Lidster, P. D.; de Marco, A. C.; Dick, A. J. Comparison of the polyphenol profiles of the apple fruit cultivars by correspondence analysis. *J. Sci. Food Agric.* **1990**, *50*, 329−342.

(17) Guyot, S.; Marnet, N.; Laraba, D.; Sanonerm P.; Drilleau, J. F. Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a French cider apple variety (*Malus domestica* var. Kermerrien). *J. Agric. Food Chem.* **1998**, *46*, 1698−1705.

(18) Awad, M. A.; de Jager, A.; van Westing, L. M. Flavonoid and chlorogenic acid levels in apple fruit: characterization of variation. *Sci. Hortic. (Amsterdam)* **2000**, *83*, 249−263.

(19) Tsao, R.; Yang, R.; Young, J. C.; Zhu, H. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). *J. Agric. Food Chem.* **2003**, *51*, 6347−6353.

(20) Knekt, P.; Isotupa, S.; Rissanen, H.; Heliovaara, M.; Jarvinen, R.; Hakkinen, S.; Aromaa, A.; Reunanen, A. Quercetin intake and the incidence of cerebrovascular disease. *Eur. J. Clin. Nutr.* **2000**, *54*, 415−417.

(21) Chinnici, F.; Bendini, A.; Gaiani, A.; Riponi, C. Radical scavenging activities of peels and pulps from cv. Golden Delicious apples as related to their phenolic composition. *J. Agric. Food Chem*. **2004**, *52*, 4684−4689.

(22) Slinkard, K.; Singleton, V. L. Total phenol analysis: automation and comparison with manual methods. *Am. J. Enol. Vitic.* **1977**, *28*, 49−55.

(23) Tsao, R.; Yang, R. Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. *J. Chromatogr. A* **2003**, *1018*, 29−40.

(24) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem*. **1996**, *239*, 70−76.

(25) Miller, H. E. A simplified method for the evaluation of antioxidants. *J. Am. Oil Chem. Soc*. **1971**, *48*, 91−95.

(26) Taga, M. S.; Miller, E. E.; Pratt, D. E. Chia seeds as a source of natural lipid antioxidants. *J. Am. Oil Chem. Soc.* **1984**, *61*, 928−931.

(27) Popov, I.; Lewin, G. Antioxidative homeostasis: characterization by means of chemiluminescent technique. In *Methods in Enzymology, Vol. 300, Oxidants and Antioxidants, Part B*; Packer, L, Ed.; Academic Press: San Diego, CA, 1999; pp 437−456.

(28) Shih, F. F.; Daigle, K. W. Antioxidant properties of milled-rice co-products and their effect on lipid oxidation in ground beef. *J. Food Sci*. **2003**, *68*, 2672−2675.

(29) Birch, A. E.; Fenner, G. P.; Watkins, R.; Boyd, L. C. Antioxidant properties of evening primrose seed extracts. *J. Agric. Food Chem*. **2001**, *49*, 4502−4507.

(30) Verhagen, H.; Aruoma, O. I.; van Delft, J. H. M.; Dragsted, L. O.; Ferguson, L. R.; Knasmüller, S.; Pool-Zobel, B. L.; Poulsen, H. E.; Williamson, G.; Yannai, S. The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in *in vitro* experiments and animal studies *in vivo*. *Food Chem. Toxicol*. **2003**, *41,* 603−610.

(31) Van der Sluis, A. A.; Dekker, M.; Skrede, G.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *J. Agric. Food Chem*. **2002**, *50*, 7211−7219.

(32) Van der Sluis, A. A.; Dekker, M.; Jongen, W. M. F. Polyphenolic antioxidants in apple. Effect of storage conditions on four cultivars. *Acta Hortic*. **2003**, *600*, 533−540.

Received for review October 14, 2004. Revised manuscript received March 2, 2005. Accepted March 14, 2005. This is scientific publication S204 of the Food Research Program, Agriculture and Agri-Food Canada, Guelph.

JF048289H