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Antioxidant Activity of Commercial Wild Rice and Identification of Flavonoid Compounds in Active Fractions

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The health benefits of whole grain consumption have been attributed to their content of complex carbohydrates, vitamins, minerals, and other phytochemical constituents. Wild rice is a whole grain finding applications in gourmet foods due to its nutritional value and unique taste. However, little is known about its antioxidant properties and phytochemical components. The objectives of this study were to evaluate the antioxidant properties of wild rice. Eleven commercial wild rice samples (raw, mixed, and processed) were extracted with acetone and fractionated using a Sephadex LH-20 column. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging activity, oxygen radical absorbance capacity (ORAC), and total phenolic content were evaluated to determine the antioxidant properties of wild rice. The antioxidant activity of wild rice was found to be 30 times greater than that of the control white rice. Significant differences ($p < 0.05$) in antioxidant activities were found among raw, mixed, and processed samples. For raw samples, DPPH$^{\bullet}$ radical scavenging activities and ORAC values ranged from 611 to 917 μmol of Trolox equivalent (TE)/100 g and from 4069 to 6064 μmol of TE/100 g, respectively. For mixed and processed wild rice, DPPH$^{\bullet}$ radical scavenging activities were 373 and 441 μmol of TE/100 g, respectively. The corresponding ORAC values were 2284 and 2557 μmol of TE/100 g. Total phenolic content (TPC) of raw wild rice varied from 2472 to 4072 mg of ferulic acid equivalent (FAE)/kg, higher than that of the mixed sample (1460 mg of FAE/kg) and processed sample (2076 mg of FAE/kg). TPC was highly correlated with total antioxidant activity of wild rice ($r = 0.92$). Tandem mass spectrometric techniques revealed the antioxidants identified in wild rice to be flavonoid glycosides (diglucosyl apigenin, glucosyl-arabinosyl apigenin, and diarabinosyl apigenin) in factions 2 and 3 and flavan-3-ols (catechin, epicatechin, and oligomeric procyanidin) in fractions 4 and 5.

KEYWORDS: Wild rice; antioxidant activity; DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl radical); ORAC (oxygen radical absorbance capacity); TE (Trolox equivalent); tandem mass spectrometry; flavonoid glycosides; procyanidins

INTRODUCTION

Wild rice (*Zizaniae palustris* and *Zizaniae aquatica*) indigenous to the northern United States and southern Canada was historically consumed by Native Americans as a staple food (*1*, *2*). In the late 20th century, wild rice was commercially cultivated to meet increased demand (*3*). Due to its unique flavor, wild rice is currently used in a wide range of gourmet food products including soups, salads, and desserts. Most wild rice is sold in the raw form, but mixed wild rice and processed wild rice including quick cooking wild rice are also available on the market.

With regard to the nutritional components, wild rice is high in proteins and starch and low in fat (*4*). In 2006, wild rice was recognized as a whole grain by the U.S. Food and Drug Administration (FDA), an agency of the U.S. Department of Health and Human Services. It is universally accepted that regular consumption of whole grains is beneficial in human health, resulting in reduced incidences of chronic diseases (*5*). Phytochemicals have been suggested to be the key contributors to these health benefits due to their antioxidant properties (*5*). The antioxidant properties of wild rice have received limited attention in the literature. Few studies have focused on the role of wild rice in lipid peroxidation. When incorporated into meat products, crude or cooked wild rice was able to retard lipid oxidation (*6*−*8*). Methanol extracts of wild rice grain and wild rice hull also enhanced the stability of ground beef, implying they can be effective antioxidants (*9*). However, the specific antioxidants in wild rice have not been well studied with the exception of phytic acids (*9*).

The primary aim of this study was to evaluate the antioxidant properties of commercial wild rice and identify specific compounds responsible for antioxidant activity. A secondary objective of the investigation was to compare the antioxidant properties of raw, mixed, and processed wild rice samples.

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MATERIALS AND METHODS

Samples. Eleven commercial wild rice samples were involved in this study. The dehulled samples were divided into three categories as follows: (1) one mixed sample (a three-blend mix wild rice consisting of wild rice, white rice, and white basmati) was obtained from Kagiwiosa Manomin Inc. (Dinorwic, ON, Canada); (2) one processed sample (a quick-cooking wild rice, already precooked and dehydrated) was obtained from Gourmet House of Riviana Food Inc. (Houston, TX); and (3) nine raw samples (three samples, Manomin, large size, and small size wild rice) were purchased from Kagiwiosa Manomin Inc. and six others (A black, B black, Canadian, C scarified, hand harvested, and Minnesota cultivated wild rice) were kindly donated by Gourmet House of Riviana Food Inc..

White rice purchased from Superstore (Winnipeg, MB, Canada) was used as a control sample. Each sample was ground into fine powder (<0.5 mm) using a cyclone mill (Model 3010−018, Udy Corporation, Fort Collins, CO, USA) and stored at −20 °C before extraction.

Chemicals. Folin−Ciocalteu reagent, DPPH, and ferulic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). HPLC grade acetone and methanol were used in the extraction and fractionation. Procyanidin standards catechin, epicatechin, B1, and B2 were purchased from Sigma-Aldrich Chemical Co. MS grade water, acetonitrile, and acetic acid were used in LC-MS analysis. All of the HPLC grade and MS grade solvents were purchased from Sigma-Aldrich Chemical Co.

Extraction. To ground rice (2.0 g) was added 40 mL of acetone/water/acetic acid (70:29:1, v/v/v) prior to sonication for 1 h at room temperature. The mixture was centrifuged at 5000 rpm (model 2C5C, MANDEL, Guelph, ON, Canada) and 20 °C for 20 min. The supernatant was removed and used as crude extract to determine the antioxidant activity and total phenolic content. The crude extract was stored at −20 °C for further fractionation.

Fractionation. To discover procyanidins in wild rice, crude extracts was further purified by fractionation to avoid the contamination of sugars and other impurities. The fractionation method previously described by Gu et al. (*10*) was used with some modifications.

Sample Preparation. Hexanes (3 × 100 mL) were added into the crude extract to remove lipids. The organic solvent was evaporated under vacuum at 35 °C. The residue was redissolved with 5 mL of 50% methanol to get concentrated extract.

Column Preparation. Sephadex LH-20 was hydrated with 20% methanol for 2 h and then manually packed into a glass column (50 × 1.5 cm). The Sephadex LH-20 column was conditioned using 250 mL of 20% methanol.

After loading the concentrated extract (5 mL), the column was eluted with 100 mL of 20% methanol, 150 mL of 60% methanol, and 100 mL of 70% acetone in that sequence. The eluents were collected separately. The 20% methanol eluent was collected as fraction 1 (F1). The 50 mL eluents of 60% methanol gave three fractions, F2, F3, and F4. Lastly, the 70% acetone eluent produced F5. Each fraction was dried by a rotary evaporator (model RE-51, Yamato Scientific America Inc., Santa Clara, CA) at 35 °C and then redissolved in 2 mL of 100% methanol. The concentrated fractions were kept at −20 °C for further analysis.

Measurement of Total Phenolic Content (TPC). The TPC of crude extracts was evaluated by using modifications of the Folin−Ciocalteu method (*11*). Briefly, 200 μL of the appropriate dilutions of crude extracts was reacted with 1.8 mL of 10-fold diluted Folin−Ciocalteu reagent, which was freshly made. The mixture was then neutralized with 1.8 mL of sodium carbonate (60 g/L). The absorbance was measured at 725 nm after 90 min of reaction at room temperature. Ferulic acid was used as the standard. Results were expressed as milligrams of ferulic acid equivalents (FAE) per kilogram of rice (dry weight basis).

Determination of DPPH Radical Scavenging Activity. This assay was based on the method of Brand-William (*12*) as modified by Li et al. (*13*). Briefly, 200 μL of crude extract (or fraction) was added to 3.8 mL of 60 μM DPPH radical solution, which was freshly made. After 60 min of incubation at room temperature, the absorbance at 515 nm was measured. DPPH free radical scavenging activities of crude extracts were expressed as micromoles of Trolox equivalents (TE) per 100 g of rice (dry weight basis) using a standard curve of Trolox.

Evaluation of Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay was based on the method described by Huang et al. (*14*) and modified by Li et al. (*13*). A Precision 2000 automated microplate pipetting system (BIO-TEK Instruments, Inc., Winooski, VT) was used for plate-to-plate transfer of solutions. An FL×800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by software KC4 3.0 (version 29) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm.

First, 120 μL of fluorescence working solution was automatically transferred to a 96-well flat-bottom polystyrene microplate (Corning Inc., Corning, NY) and used as the substrate. Then 20 μL each of buffer solution (blank), Trolox (standard control), appropriately diluted samples, and catechin (sample control) were added to the designated wells, respectively. After 20 min of incubation at 37 °C, 60 μL of freshly made AAPH solution was added to each well to generate a peroxyl radical. The total reaction time was 50 min. The fluorescence of the reaction mixture was recorded every minute.

The area under the fluorescence decay curve (AUC) was calculated according to the equation

$$\mathrm{AUC} = 0.5 + f_1/f_0 + f_i/f_0 + \ldots + f_{49}/f_0 + 0.5(f_{50}/f_0)$$

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i min. Final ORAC values were calculated as follows and expressed as micromoles of TE per 100 g of rice (dry weight basis):

$$\text{ORAC value} = [(\mathrm{AUC}_{\mathrm{sample}} - AUC_{\mathrm{blank}})/(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}_{\mathrm{blank}})] \times \text{dilution factor}$$

HPLC-MS/MS Analysis. An HPLC (Waters 2695) equipped with a photodiode array detector (PDA) (Waters 996) and autosampler (717 plus, Waters) coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA) was employed for HPLC and mass spectometric analyses (LC-MS/MS). A 150 mm × 4.6 mm, 5 μm RP 18 column (Gemini, Phenomenex, Torrance, CA) was used for separation. During the LC-MS analysis, 10 μL of sample was loaded and injected by an autosampler and eluted through the column with a gradient mobile phase consisting of A (water containing 0.1% acetic acid) and B (acetonitrile containing 0.1% acetic acid) with the flow rate of 0.5 mL/min, prior to introduction into the Q-TOF MS. A 50 min linear gradient was programmed as follows: 0−5 min, 5−10% B; 5−15 min, 10−15% B; 15−20 min, 15−20% B; 20−30 min, 20−25% B; 30−40 min, 25−40% B; 40−45 min, 40−10% B; 45−50 min, 10% B. The Q-TOF MS was calibrated using sodium iodide for the negative mode through the mass range of 100−2000. A resolution of 5000 was achieved. The quantification of proanthocyanidin compounds was based on the area of the peak at a wavelength of 280 nm by using (+)-catechin as external standard and expressed as milligrams of catechin equivalent (CE) per 100 g of dry weight. Full mass spectra were recorded in negative mode by using a capillary voltage of 1.2 kV and a cone voltage of 45 V. The flow rates of desolvation gas (N_2) and cone gas (He) were 900 and 50 L/h, respectively. The desolvation gas temperature and the ion source temperature were set at 350 and 150 °C, respectively. The MS/MS spectra were acquired by using a collision energy of 30 V.

In the above assays, all of the samples were extracted and analyzed in triplicate.

Statistical Analysis. The results were reported as mean ± standard deviation (SD). Data were analyzed by a one-way analysis of variance (ANOVA) test using SAS version 9.1 (SAS Institute Inc., Cary, NC). Least significant differences (LSD) at $p < 0.05$ were tested to assess significant differences in antioxidant properties among samples.

RESULTS AND DISCUSSION

Total Phenolic Content. Phenolic compounds are considered to be the major antioxidants occurring in fruits, vegetables, and cereals; therefore, the measurement of TPC tends to be important when their contribution to antioxidant activity is assessed. **Table 1** shows the TPC of crude, aqueous acetone extracts from white rice (control) and wild rice. Raw wild rice (range = 2472−4072 mg/kg) contained 10−15 times more TPC than white rice (279 mg/kg). The TPC of mixed wild rice ranked between that of white rice and

Table 1. Total Phenolic Content (TPC) in Acetone Extracts from White Rice Control and Wild Rice

class	no.	sample	TPC (FAE[a], mg/ kg)
control	A	white rice	279 ± 13 i
mixed	B	three-blend mix	1460 ± 29 h
processed	C	quick cooking	2076 ± 43 g
raw	D	Manomin	4072 ± 113 a
	E	C scarified	3796 ± 43 b
	F	A black	3575 ± 34 c
	G	Minnesota cultivated	3376 ± 22 d
	H	B black	3342 ± 29 d
	I	large size	2760 ± 68 e
	J	small size	2743 ± 32 e
	K	hand harvested	2498 ± 26 f
	L	Canadian	2472 ± 39 f

[a] Ferulic acid equivalent. Least significant difference level of probability ($p < 0.05$). Sample means with the same letter are not significantly different.

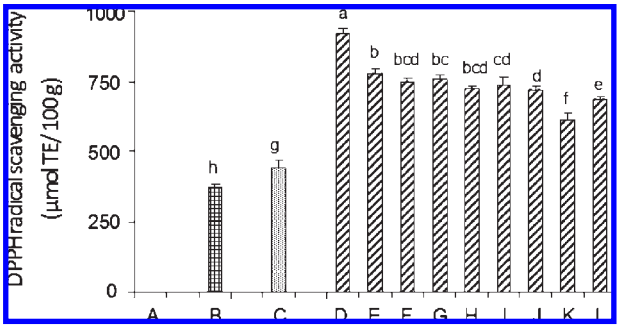

Figure 1. Free radical scavenging activity of acetone extracts from white and wild rice on DPPH radical (at 60 min): A, white rice; B, three-blend mix; C, quick cooking; D, Manomin; E, C scarified ; F, A black; G, Minnesota cultivated; H, B black; I, large size; J, small size; K, Canadian; L, hand harvested.

raw wild rice due to the dilution of the endogenous phenolics of wild rice by white rice and white basmati. Significant differences in TPC were found between raw and processed wild rice samples. Manomin and C scarified had the highest TPC among the raw samples; however, quick-cooking wild rice displayed significantly lower TPC (2076 mg/kg) than uncooked ones. Thus, the process of producing the quick-cooking product substantially lowers the phenolic levels found in wild rice. The general procedure for production of quick-cooking wild rice involves soaking, cooking, and drying, conditions that result in loss of phytochemicals due to leaching in the liquid used or destruction and/or transformation of chemical structures present in raw grain (*13*). The loss of water-soluble and free antioxidants during rice cooking is also responsible for reduced levels of phenolics (*15*).

DPPH Radical Scavenging Activity. The DPPH• photometric method is widely employed for evaluating the free radical scavenging activity of hydrogen-donating antioxidants in plant extracts (*12*). The DPPH• scavenging activities of crude acetone extracts presented in **Figure 1** are expressed as micromoles of Trolox equivalents (TE) per 100 g of rice on a dry weight basis. Because of the very low levels, the DPPH• scavenging activity of the white rice control could not be estimated by using the Trolox standard curve. The DPPH• scavenging activities of raw samples (717−917 μmol of TE/100 g) were significantly higher ($p < 0.05$) than that of the mixed wild rice (373 μmol/100 g). The highest and lowest values were observed in Manomin and hand-harvested wild rice, respectively. The DPPH• scavenging activities were not significantly different between A and B black wild rice or between large- and small-sized wild rice ($p > 0.05$). The grade and the size

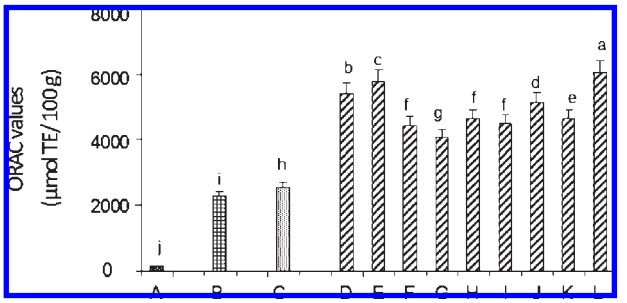

Figure 2. ORAC values of acetone extracts from white and wild rice: A, white rice; B, three-blend mix; C, quick cooking; D, Manomin; E, C scarified; F, A black; G, Minnesota cultivated ; H, B black; I, large size; J, small size; K, Canadian; L, hand harvested.

of wild rice did not affect antioxidant activity among the samples examined. Significant differences in DPPH• scavenging activity were found between raw and processed samples. The average DPPH• scavenging activity of raw wild rice (741 μmol of TE/100 g) was 40% higher than that of quick-cooking wild rice. These results are consistent with the reduction in TPC observed in quick-cooking wild rice.

Oxygen Radical Absorbance Capacity. In the ORAC assay, antioxidant activity is determined by using fluorescein as the fluorescent probe and 2,2′-azobis(2-amidinopropane) dihydrochloride (APPH) as the peroxyl radical generator. Due to its biological relevance to in vivo antioxidant efficacy, it has been widely used to investigate the antioxidant activity of foods including fruits, vegetables, nuts, cereals, and spices (*16*). Wu et al. (*16*) reported that hydrophilic ORAC (H-ORAC) values are much higher than lipophilic ORAC (L-ORAC) values, contributing to 95% of the total antioxidant capacity for most foods. Finocchiaro et al. (*15*) also pointed out that the antioxidant capacities of red and white rice were not due to the lipophilic compounds. Therefore, only the H-ORAC assay was carried out in our study.

H-ORAC values of white rice control and wild rice are shown in **Figure 2**, expressed as micromoles of TE per 100 g of rice on a dry weight basis. For the control, the H-ORAC value was 153 μmol of TE/100 g. Contrary to the control, raw wild rice had significantly higher H-ORAC values ranging from 4069 to 6064 μmol of TE/100 g for Canadian and Minnesota cultivated samples, respectively. High ORAC values were also observed in C scarified (5799 μmol of TE/100 g) and Manomin (5421 μmol of TE/100 g) wild rice. Although lower than raw wild rice samples (average = 4970 μmol of TE/100 g), the H-ORAC value of mixed wild rice (2284 μmol of TE/100 g) was 15 times higher than that of the control white rice. Mixing white rice with wild rice can therefore improve the peroxyl radical scavenging activity of white rice. Consistent with DPPH scavenging activity results, processed wild rice had a significantly lower value of H-ORAC than raw samples. The H-ORAC value of quick-cooking wild rice was 2557 μmol of TE/100 g, about half the average value of the raw samples.

Significant differences in antioxidant activities among some raw wild rice samples can be attributed to several complex factors including cultivar, growing environment, and harvesting conditions. According to Mitchell et al. (*17*), organic food products have higher antioxidant activity than inorganic or conventional food products, which may explain the higher antioxidant activity of Manomin wild rice than of Minnesota cultivated wild rice. Manomin wild rice is organically grown at Wabigoon Lake by Ojibway aboriginal people in northwestern Ontario, Canada, whereas Minnesota cultivated wild rice is grown in a man-made

Table 2. DPPH Scavenging Activity (Percent) of Each Fraction Eluted from the Sephadex LH-20 Column[a]

class	sample name	F1	F2	F3	F4	F5
control	white rice	nd[b]	nd	nd	nd	nd
mixed	three-blend mix	1.50 ± 0.04	11.32 ± 0.82	9.49 ± 0.50	5.02 ± 0.21	7.75 ± 0.41
processed	quick cooking	1.77 ± 0.04	13.52 ± 0.74	11.89 ± 0.28	4.65 ± 0.06	6.15 ± 0.07
raw	Manomin	4.26 ± 0.20	19.69 ± 0.23	16.80 ± 0.43	6.21 ± 0.09	11.97 ± 0.18
	C scarified	4.39 ± 0.20	17.01 ± 0.75	15.87 ± 0.59	7.64 ± 0.08	11.56 ± 0.39
	A black	3.70 ± 0.18	17.99 ± 0.18	16.58 ± 0.24	5.65 ± 0.30	9.66 ± 0.41
	Minnesota cultivated	3.56 ± 0.15	16.71 ± 0.82	15.85 ± 0.37	5.12 ± 0.10	13.92 ± 0.40
	B black	2.33 ± 0.09	15.86 ± 0.21	14.10 ± 0.66	3.45 ± 0.11	10.87 ± 0.56
	large size	3.28 ± 0.18	16.85 ± 0.40	13.93 ± 0.77	4.85 ± 0.4	11.51 ± 0.32
	small size	3.22 ± 0.05	15.81 ± 0.66	14.30 ± 0.35	7.58 ± 0.34	11.68 ± 0.16
	hand harvested	2.47 ± 0.23	15.59 ± 0.27	12.61 ± 0.80	4.47 ± 0.07	10.40 ± 0.44
	Canadian	3.91 ± 0.07	15.32 ± 0.48	14.95 ± 0.37	5.55 ± 0.32	9.69 ± 0.25

[a] The fractions used for analyzing the DPPH radical scavenging activity were obtained from one fractionation. [b] Not detectable.

paddy instead of a natural lake or river. Environmental variations such as growing location and temperature also considerably affect the content of antioxidants and the antioxidant activity of cereals (*18*).

Antioxidant Activity of Fractions F1−F5. The crude extract was fractionated with methanol and acetone on a Sephadex LH-20 column. The antioxidant activities of each fraction were evaluated by measurement of their DPPH• scavenging ability and expressed as percent DPPH• quenched at 60 min. The results are summarized in **Table 2**. For raw samples, the DPPH• scavenging activities ranged from 2.33 to 4.39% for F1, from 15.01 to 19.69% for F2, from 12.61 to 16.80% for F3, from 3.45 to 7.64% for F4, and from 9.66 to 11.97% for F5. A decline in DPPH• scavenging activities was found in the following order: F2 > F3 > F5 > F4 > F1. The antioxidants were, therefore, more abundantly distributed in F2, F3, and F5 than in other fractions. As suggested by Gu et al. (*10*), the compounds in the first fraction (F1) eluted with 20% methanol are mainly sugars; the compounds eluted with 60% methanol (F2, F3, and F4) are simple phenolic acids and other low molecular weight phenolics; and the 70% acetone eluent (F5) may consist of some polymerized phenolic acids and other high molecular weight polyphenol constituents. To identify and characterize the antioxidants in wild rice, the active fractions (F2−F5) were analyzed by LC-MS and MS-MS analysis.

Identification of Flavonoid Glycosides in F2 and F3. Flavonoids have received considerable attention due to their antioxidant activity (*19*). As the secondary metabolites in plants, in most cases, flavonoids are conjugated with sugars and generally occurring as flavonoid *O*- or *C*-glycosides. The unconjugated flavonoids without a sugar moiety are called aglycones, among which flavones, flavonols, and flavanones are the most commonly encountered. As reported by van Acker et al. (*20*), the antioxidant activity of flavonoids is related to their structural aspects, with better scavenging activity being associated with the presence of the catechol moiety in ring B. Thus, HPLC-MS/MS was applied in our study to characterize flavonoids in wild rice and investigate their chemical structures.

Panels **a** and **b** of **Figure 3** are the HPLC chromatograms (330 nm) of F2 and F3 eluted from Manomin wild rice acetone extract. The major peaks were numbered according to their elution time. As seen in **Figure 3a**, peak 2 (t_R = 25.58 min) and peak 3 (t_R = 28.57 min) together with a smaller peak (peak 1, t_R = 23.12 min) were the major peaks detected in F2. In comparison to F2, F3 contained more peaks with longer retention that abundantly occurred between 25 and 32 min (**Figure 3b**). Due to the similarity of the retention times (**Figure 3d**), peak 2′ (t_R = 25.67 min) and peak 3′ (t_R = 28.68 min) observed in F3 presumably contained the same compounds as peaks 2 and 3 in F2. The UV spectra of all the numbered peaks were characterized by two major absorption bands: I around λ_{max} 330 nm and II at λ_{max} 270 nm. As a representative, the UV spectrum of peak 3 is shown in **Figure 3c**.

In mass analysis, negative ion mode was selected because it provided extensive structural information via collision-induced dissociation. To facilitate discussion on mass fragmentations, the nomenclature of product ions introduced by Domon and Costello (*21*) was employed here with slight modifications. The letter A represents the aglycone. Fragment ions from deprotonated molecules containing the aglycone part are denoted X^- and Y^-. The X^- ions are formed by cleavage within the glycosidic rings and usually labeled ${}^{k,l}X_j^-$. The subscript j represents the number of the interglycosidic bond counting from the aglycone with the glycosidic bond linking to the aglycone being numbered 0. The superscripts k and l indicate the ring bonds that have been broken in the sugar residues. In the case of diglycosides, ${}^{(k,l),(k,l)'}X_j^-$ is employed for the ions formed by simultaneous losses from both sugars. The Y^- ions are the characteristic ions for *O*-glycosides produced by cleavage at glycosidic *O*-linkages, which leads to the complete loss of one or more sugar moieties. In the case of diglycosides, Y_1^- is formed after the loss of one sugar residue, and Y_0^- corresponds to the loss of two sugar residues.

Peak 1 (t_R = 23.12 min): MS, $[M - H]^-$ 593; MS/MS, $[M - H - 18]^-$ 575, $[M - H - 90]^-$ 503, $[M - H - -120]^-$ 473, $[M - H - 210]^-$ 383, $[M - H - 240]^-$ 353, $[M - H - 254]^-$ 339. The presence of ions at m/z 353 (A + 83) and 383 (A + 113) indicated that the alycone was apigenin (4′,5,7-trihydroxyflavone) (*22*). The ions at m/z 503 and 473 corresponding to the loss of 90 and 120 Da from the deprotonated molecule $[M - H]^-$ were formed by cross-ring cleavages in a hexose residue, thus labeled ${}^{0,3}X_0^-$ and ${}^{0,2}X_0^-$ in **Figure 5a**. The ions at m/z 413 denoted ${}^{(0,3)(0,3)'}X^-$ revealed the simultaneous losses of 90 Da from both hexose residues. The water loss was revealed by the detection of the ions at m/z 575. The above characteristic losses (18, 90, and 120 Da), which are usually considered diagnostic for *C*-glycosides, indicated that peak 1 was one of them. In contrast to *C*-glycosides, the typical sugar losses for *O*-glycosides are 162 (hexose), 146 (deoxyhexose), and 132 (pentose), and there is no water loss detected. The high intensity of $[M - H]^-$ (relative intensity > 85%) and the absence of Y_0^- ($[M - H - 162 - 162]^-$ m/z 269) and Y_1^- ($[M - H - 162]^-$ m/z 431) also suggested a *C*-glycosylation. To date, *C*-glycosylation is only found at C-6 and C-8 positions of the flavonoid aglycone (*23*). Therefore, peak 1 was designated 6,8-di-*C*-hexosyl apigenin (M_r = 594), comprising apigenin (270) and two hexoses (162 + 162). According to the previous reports on the product ions from $[M - H]^-$ 593 or $[M + H]^+$ 595 (*22*, *24*, *25*), the most probable flavonoid glycoside corresponding to peak 1 is 6,8-di-*C*-glucosyl apigenin (vicenin-2).

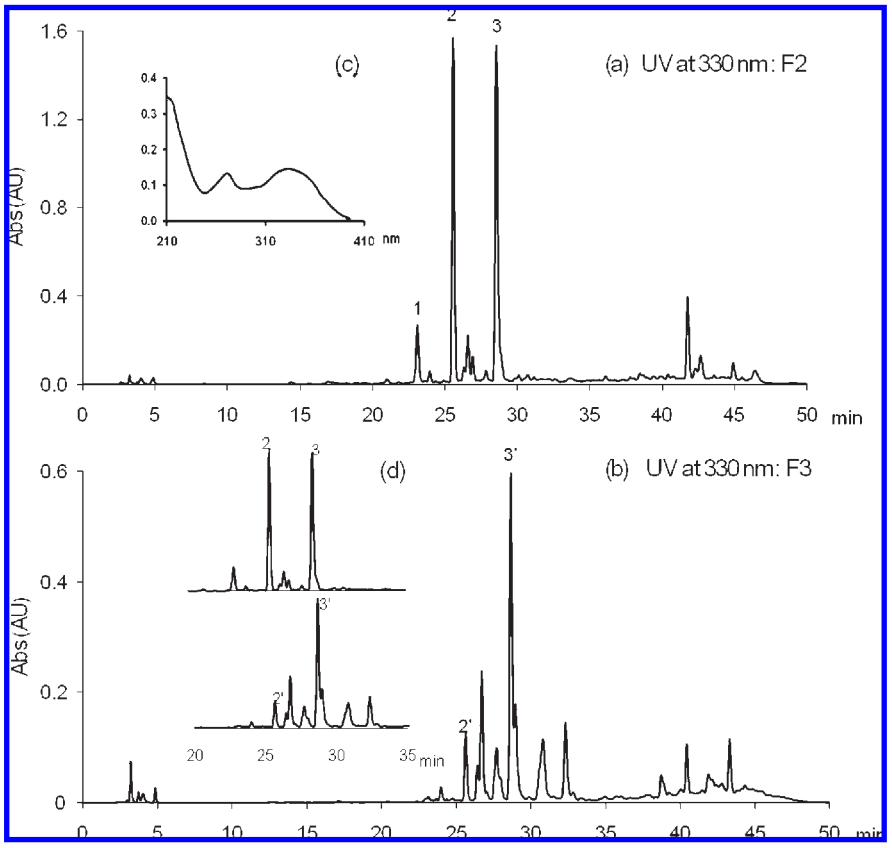

Figure 3. (**a**) Full LC chromatograms (0−50 min) recorded at 330 nm of F2 isolated from Manomin wild rice; (**b**) full LC chromatograms (0−50 min) recorded at 330 nm of F3 isolated from Manomin wild rice; (**c**) full UV spectra of peak 3; (**d**) highlights of LC chromatograms (20−35 min) for F2 and F3.

Table 3. Retention Time, Maximum UV Absorption, Deprotonated Molecular Mass, Fragmentation Pattern of Ion Loss, and Relative Intensity of Product Ions of Peaks 1−3

				relative intensity of product ions (%)					
peak	t_R (min)	λ_{max} (min)	$[M-H]^-$ m/z	$-H_2O$	-60	-90	-120	A + 83[a]	A + 113
1	23.13	270, 334	593	5	0	28	83	100	73
2	25.58	271, 328	563	3	4	55	85	100	70
3	28.57	270, 331	533	2	38	71	0	100	85

[a] Base peak.

Peak 2 (t_R = 25.58 min): MS, $[M-H]^-$ 563; MS/MS, $[M-H-18]^-$ 545, $[M-H-90]^-$ 473, $[M-H-120]^-$ 443, $[M-H-150]^-$ 413, $[M-H-180]^-$ 383, $[M-H-210]^-$ 353, $[M-H-238]^-$ 325. The same alycone moiety (apigenin) was assumed for peak 2 due to the occurrence of A + 83 (m/z 353) and A + 113 (m/z 383). The ions at m/z 473 and 443 exhibited the characteristic sugar losses of *C*-glycosides (90 and 120 Da). The ions at m/z 413 were likely formed after the loss of 60 Da from pentose and the further loss of 90 Da from hexose. The ions at m/z 545 formed by the loss of water further pointed to the C-glycosylation. Thus, peak 2 was assigned as hexosyl-pentosyl apigenin (M_r = 564, apigenin 270 + hexose 162 + pentose 132). 6-*C*-Glucosyl-8-*C*-arabinosyl apigenin and 6-*C*-arabinosyl-8-*C*-glucosyl apigenin are in the greatest possibility. As reported by Ferreres et al. (*21*), 6-*C*-glucosyl-8-*C*-arabinosyl apigenin gave rise to $[M-H-120]^-$, whereas 6-*C*-arabinosyl-8-*C*-glucosyl apigenin was pronounced for $[M-H-90]^-$. Thus, the former is preferred here because the higher relative intensity of $[M-H-120]^-$ was observed in peak 2 (**Table 3**).

Peak 3 (t_R = 28.57 min): MS, $[M-H]^-$ 533; MS/MS, $[M-H-18]^-$ 515, MS/MS, $[M-H-60]^-$ 473, $[M-H-90]^-$ 443, $[M-H-150]^-$ 383, $[M-H-180]^-$ 353, $[M-H-222]^-$ 311. As seen in **Table 3**, the ions at m/z 383 (A + 113) were still of the highest intensity as the base peak with the second most abundant ions observed in A + 83 at m/z 353, indicating the aglycone of apigenin. However, the relative intensity of the precursor ion $[M-H]^-$ went down when peak 1 was compared with peak 2. A water loss was observed by the presence of the ions at m/z 515. The most distinctive loss observed in peak 3 from the previous two peaks was the abundant loss of 60 Da originating from pentoses. As reported by previous authors (*23*), the losses of 60 and 90 Da were the characteristic sugar losses for *C*-glycosides, formed by cross-cleavages within pentose residues. Thus, peak 3 was designated *C*-dipentosyl apigenin containing apigenin and two pentose residues.

The general terms hexose and pentose were used for sugar moieties due to the difficulty in detecting stereochemical structures of carbohydrate residues. Glucose is the most commonly encountered sugar; rhamnose, xylose, and arabinose are less common, whereas mannose and fructose are rare (*23*). The glycosylation positions are also challenging to determine or identify. In the case of *C*-glycosides, C-6 and C-8 are the only two positions, and the differentiation can be made by high-energy collision-induced dissociation (CID) according to the abundance of water loss (preferable at the C-6 position). **Figure 4** illustrates the major fragmentation pathways in peaks 1, 2, and 3 when they are designated 6,8-di-*C*-glucosyl apigenin, 6-*C*-glucosyl-8-*C*-arabinosyl apigenin, and 6,8-*C*-diarabinosyl apigenin, respectively.

Peaks 2′ and 3′ observed in F3 giving the same MS and MS-MS spectra as peaks 2 and 3 confirmed the previous assumption that they comprised the same compounds. In addition to peaks 2′ and 3′, the other peaks detected in F3 with retention times between 20 and 35 min were found as the isomers of peaks 1−3, when plotting

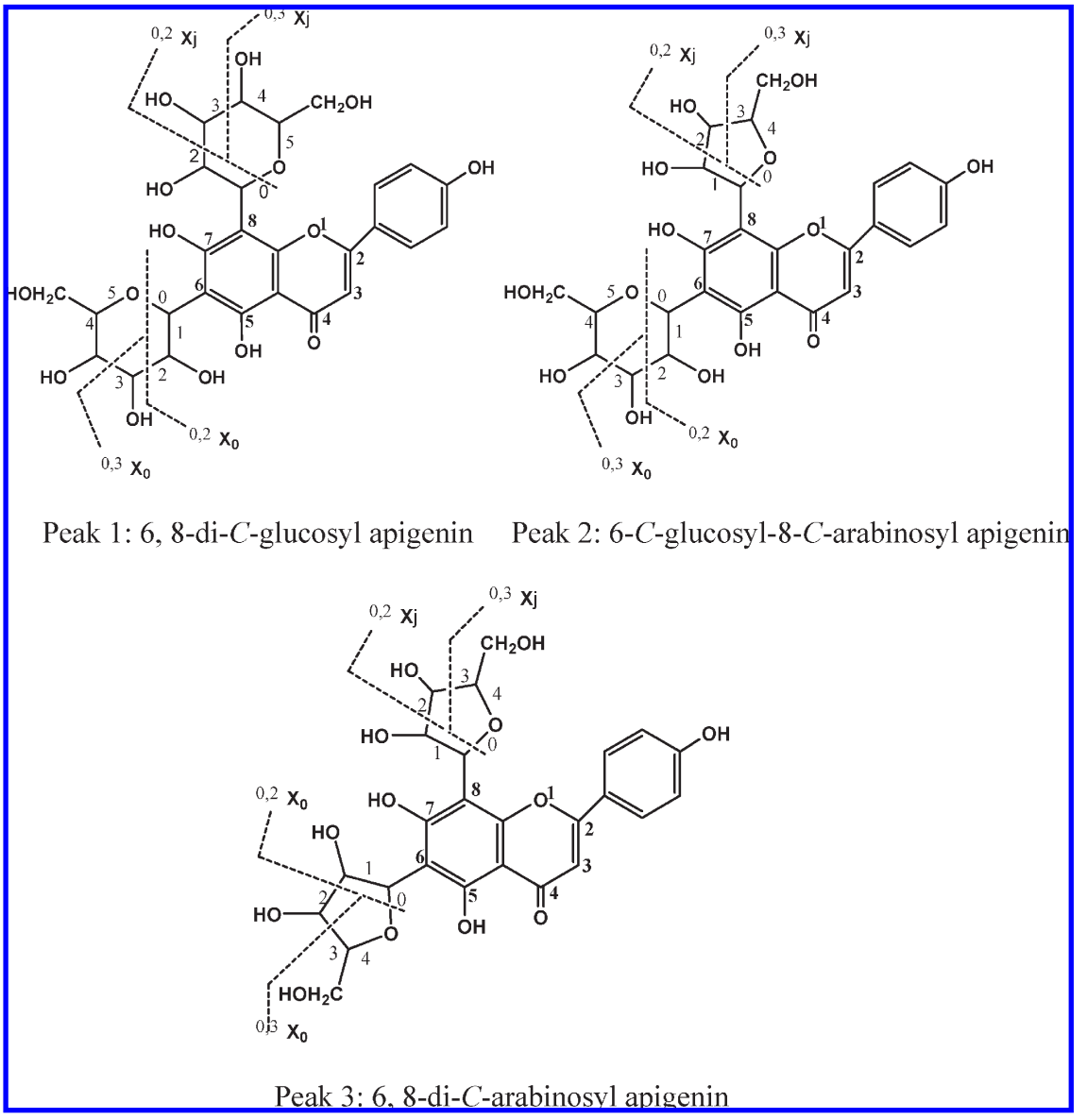

Figure 4. Fragmentation pathways assumed in peaks 1 (6,8-di-*C*-glucosyl apigenin), 2 (6-*C*-glucosyl-8-*C*-arabinosyl apigenin), and 3 (6,8-di-*C*-arabinosyl apigenin).

the extraction ion chromatograms of ions at *m*/*z* 593, 563, and 533 (**Figure 5**) . Thus, flavones glycosides were likely the predominant antioxidant compounds detected in F2 and F3.

Identification of Monomeric and Oligomeric Flavan-3-ols in F4 and F5. Flavan-3-ols, namely, catechins and procyanidins, are a subclass of flavonoids commonly found in plants and have been demonstrated to exhibit radical scavenging activity (*19*). Cereal procyanidins have been well characterized in barley and sorghum (*26*, *27*). However, only limited literature can be found on rice in general. Red rice only has been reported to contain oligomeric procyanidins with degrees of polymerization (DP) ranging from 1 to 8 (*15*). To complement this knowledge, procyanidins in wild rice were investigated in our study. In agreement with Taylor et al. (*28*), the fractionation of crude acetone extracts prior to LC-MS analysis greatly improved the separation and detection of procyanidins, especially for those with low molecular weight. Nevertheless, the oligomers with DP ≥ 6 and the polymeric procyanidins were not detected in wild rice extracts by LC-MS.

Figure 6a is the HPLC chromatogram (at 280 nm) of the standards consisting of (+)-catechin, (−)-epicatechin, B1, and B2. **Figure 6b** shows the LC chromatogram of F4 isolated from Manomin wild rice. By comparison with retention times of standards, the peaks at 19.22 min (peak 4) and at 23.50 min (peak 5) were respectively designated (+)-catechin and (−)-epicatechin. The UV spectra of these two peaks recorded by a PAD showed a maximum absorption at 279.68 nm, the same as the standards. To further confirm the identification, analyses were conducted using Q-TOF MS and MS-MS. The total ion chromatogram (TIC) of F4 in negative ion mode is shown in **Figure 6c**. By plotting the extracted ion chromatogram (EIC) of a single ion at *m*/*z* 289 from **Figure 6c**, it can been seen that peaks 4 and 5 originated from the ions at *m*/*z* 289. Two peaks were observed in **Figure 6d**: the first and most abundant one was detected at 19.22 min, corresponding to (+)-catechin; the second and smaller peak was present at 23.49 min, corresponding to (−)-epicatechin. The intensity of (+)-catechin was found to be 4 times higher than that of (−)-epicatechin. To further confirm the identification of catechin monomers in F4, the fragmentation patterns of peaks 4 and 5 were recorded by tandem mass spectrometry and characterized by the abundant ions $[M - H - 44]^-$ at *m*/*z* 245, which were in concordance with the

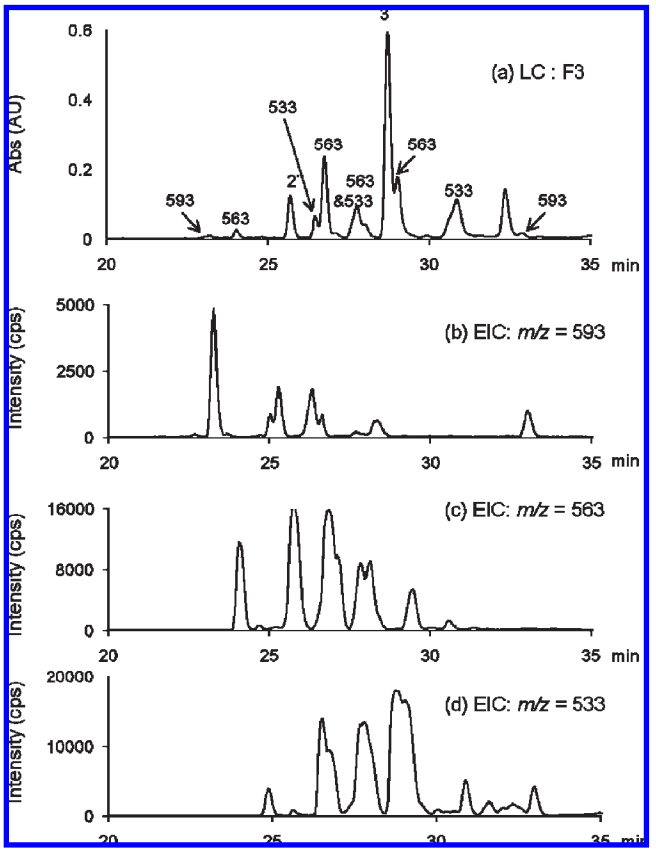

Figure 5. (**a**) Highlights of LC chromatograms (20−35 min) for F3 from Manomin wild rice recorded at 330 nm; extracted ion chromatogram (EIC) of ions at (**b**) *m*/*z* 593, (**c**) *m*/*z* 563, and (**d**) *m*/*z* 533.

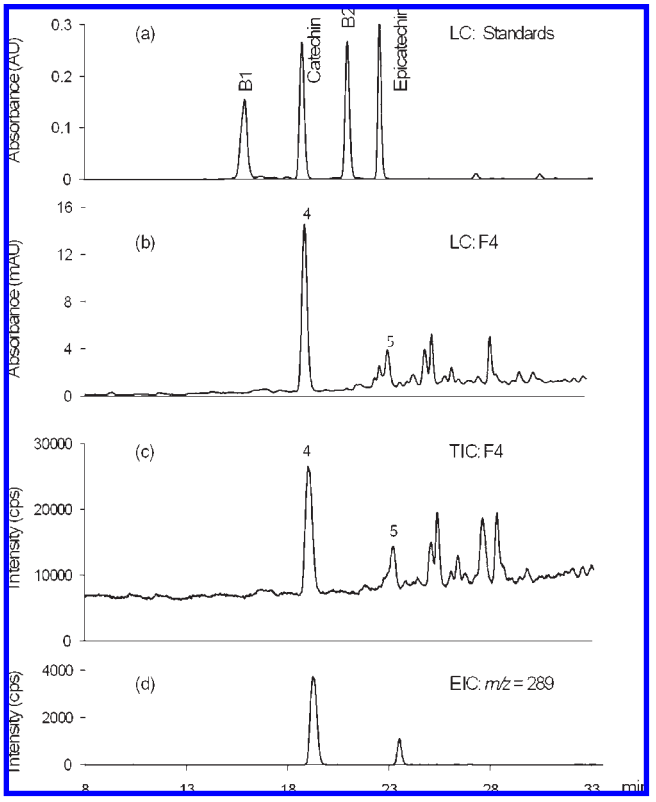

Figure 6. (**a**) LC chromatogram (at 280 nm) of standards; (**b**) LC chromatogram (at 280 nm) of F4 extracted from Manomin wild rice; (**c**) total ion chromatogram (TIC) of F4; (**d**) extracted ion chromatogram (EIC) of a single ion at *m*/*z* 289.

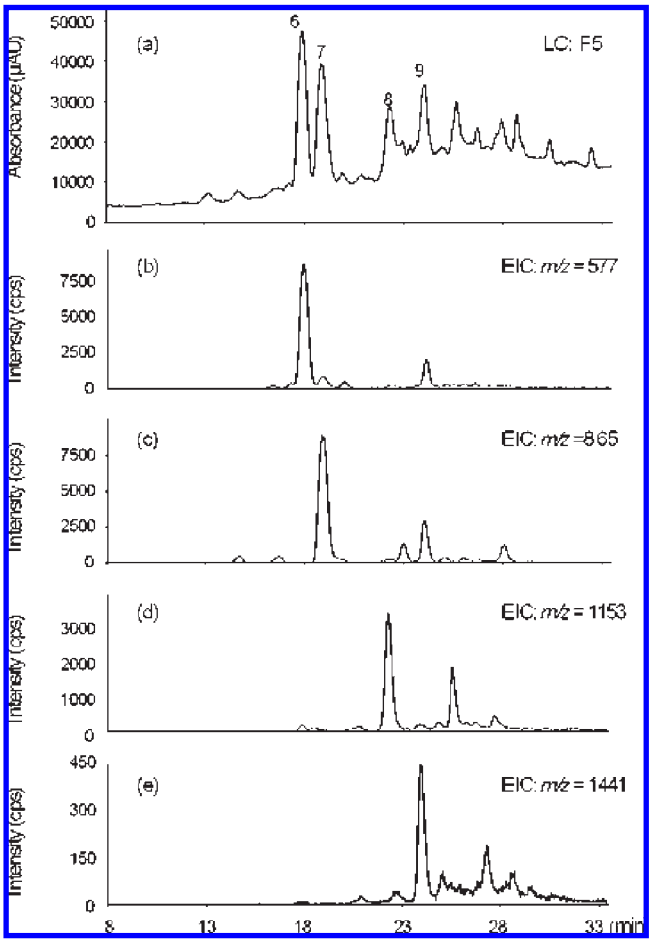

Figure 7. (**a**) LC chromatogram at 280 nm of F5 for Manomin wild rice; (**b**−**e**) extracted ion chromatograms (EIC) of deprotonated molecules with (**b**) *m*/*z* 577, (**c**) *m*/*z* 865, (**d**) *m*/*z* 1153, and (**e**) *m*/*z* 1441.

Table 4. Identification of Catechins and Procyanidin Oligomers in F4 and F5 by LC-MS/MS in Negative Ion Mode

peak	compound assignation	fraction	t_R (min)	$[M - H]^-$ *m*/*z*	product ion *m*/*z*
4	(+)-catechin	F4	19.22	289	245
5	(−)-epicatechin	F4	23.50	289	245
6	dimer	F5	17.88	577	407, 289
7	trimer	F5	18.82	865	577, 407, 289
8	tetramer	F5	22.26	1153	865, 407, 289
9	pentamer	F5	23.96	1441	1153, 865, 577

molecular masses of catechin and epicatechin standards. Thus, it became evident that peak 4 was (+)-catechin and peak 5 was (−)-epicatechin. As suggested by Flamini (*29*), these fragment ions were formed by the loss of a $-CH_2-CHOH-$ group from the precursor molecular ion $[M - H]^-$ at *m*/*z* 289.

Figure 7a is the UV chromatogram (at 280 nm) of F5 for Manomin wild rice. The extracted ion chromatograms of deprotonated molecular ions $[M - H]^-$ at *m*/*z* 577, 865, 1153, and 1441 (**Figure 7b**−**e**) indicated the presence of dimer, trimer, tetramer, and pentamer of procyanidins, respectively. The peaks shown in **Figure 7a** at the same retention times as the ions *m*/*z* 577, 865, 1153, and 1441 were numbered as peaks 6, 7, 8 and 9, presumed to be the corresponding procyanidin oligomers. The identification of these peaks was further completed by using tandem mass analysis. As shown in **Table 4**, the fragmentation characteristics of the numbered peaks were consistent with the previous reports on procyanidin oligomers (*30*). Hence, the previous assumption has been confirmed.

Table 5. Content of Procyanidin Monomers and Oligomers in Wild Rice[a]

		procyanidin oligomers (μg/g)					
group	sample name	mono-[b]	di-	tri-	tetra-	penta-	total
control	white rice	nd[c]	nd	nd	nd	nd	nd
mixed	three-blend mix	6.64	11.99	17.31	nd	nd	35.93
processed	quick cooking	12.43	11.91	nd	nd	nd	24.34
raw	Manomin	13.74	42.42	69.18	52.67	61.62	239.22
	C scarified	18.15	17.42	tr[d]	nd	nd	35.57
	A black	13.46	23.87	49.41	31.47	30.96	149.17
	Minnesota cultivated	12.46	25.26	55.03	29.71	32.23	154.70
	B black	13.56	23.88	52.18	34.12	37.37	161.10
	large size	9.27	22.88	35.85	32.98	43.43	144.41
	small size	11.17	24.47	34.45	34.08	57.74	161.91
	hand harvested	7.16	tr	nd	nd	nd	7.16
	Canadian	11.42	12.42	tr	nd	nd	23.84

[a] Data are the averages of duplicate tests. [b] The monomer content comprises catechin and epicatechin. [c] Not detected. [d] Trace.

Peaks 6, 7, 8, and 9 are identified as dimer, trimer, tetramer, and pentamer of procyanidins, respectively.

In contrast to wild rice, no procyanidins were detected in the white rice control. The absence of such constituents may explain the low antioxidant activity of the control.

Quantification of Catechins and Oligomeric Procyanidins in F4 and F5. Catechin monomers (catechin and epicatechin) were commonly found in all of the wild rice samples, whereas the presence of procyanidin oligomers varied among samples. To quantify these flavan-3-ols in wild rice, (+)-catechin was used as the standard for all of the peaks due to the lack of oligomeric procyanidin standards. The contents of procyanidins in wild rice samples are shown in **Table 5** and expressed as micrograms of catechin equivalents per gram. The term "trace" (tr) used in **Table 5** represents the compounds that can be detected by mass analysis, but fail in quantification due to no signals in the UV chromatograms.

Among raw samples, Manomin wild rice contained the highest procyanidin content (239 μg/g), followed by small-sized wild rice (162 μg/g) and B black wild rice (162 μg/g). The lowest total content of procyanidins (7 μg/g) was found in hand-harvested wild rice because only monomers were detected in this wild rice, with the presence of minor dimers. Procyanidin oligomers (DP > 3) were also absent in C scarified and Canadian wild rice, which resulted in their low procyanidin contents (36 and 24 μg/g, respectively). The other raw samples were found to contain oligomeric procyanidins up to pentamer, among which trimers were the most abundant. Compared with raw samples, the processed sample, quick-cooking wild rice, contained a relatively low procyanidin content of 24 μg/g due to the absence of trimer, tetramer, and pentamer. The mixed sample was also found to be low in procyanidin content (36 μg/g).

Correlation between Antioxidant Activity and TPC in Wild Rice Crude Extracts. To assess the contribution of TPC to total antioxidant activity of wild rice, the relationship among TPC, DPPH free radical scavenging activity, and ORAC was investigated (**Table 4**). A high correlation ($r = 0.92$, $P < 0.0001$) was found between TPC and DPPH$^{\bullet}$ scavenging activity. TPC and ORAC were also correlated ($r = 0.64$, $P < 0.05$). Therefore, the higher antioxidant activity of wild rice with respect to white rice is attributed to the larger amount of total phenolics in the former.

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