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Polyphenolic Profiles and Antioxidant Activities of Heartnut (*Juglans ailanthifolia* Var. *cordiformis*) and Persian Walnut (*Juglans regia* L.)

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The polyphenolic compositions of three heartnut (*Juglans ailanthifolia* var. *cordiformis*) varieties (Imshu, Campbell CW1, and Campbell CW3) were examined and compared with those of two Persian walnut (*Juglans regia* L.) varieties (Combe and Lake). The nuts were defatted, extracted, and separated into three different fractions, the free phenolic acid (FPA), acid-hydrolyzable phenolic acid (AHPA), and bound phenolic acid (BPA) fractions. The total phenolic contents (TPCs) in both FPA and AHPA of the Persian walnuts were significantly higher ($P < 0.001$) than those of the heartnuts, but not in the BPA ($P = 0.20$). LC-ESI-MS^n studies revealed that except for the FPA fraction, the major polyphenolics in both heartnut and Persian walnut were ellagic acid and valoneic acid dilactone. Persian walnuts contained an average of 0.29 and 1.31 mg of ellagic acid/g nut in the 80% methanol extractable fractions FPA and AHPA, respectively. Heartnuts contained an average of 0.16 and 0.60 mg of ellagic acid/g nut in the respective fractions. Bound ellagic acid in the residue was 0.93 and 0.70 mg/g of nut in the Persian walnut and in the heartnut, respectively. Valoneic acid dilactone was tentatively identified and quantified as milligrams of ellagic acid equivalent per gram of nut. These components were found to contribute to the strong total antioxidant activities measured using ferric reducing antioxidant power and photochemiluminescence methods.

KEYWORDS: Walnut; heartnut; polyphenols; ellagic acid; antioxidant activities; *Juglans ailanthifolia* var. *cordiformis; Juglans regia* L.; LC-ESI-MS

INTRODUCTION

The seed of Persian walnut (*Juglans regia* L.) has been found to be a rich source of essential unsaturated fatty acids, tocopherols (*1*−*3*), and the hormone and strong antioxidant melatonin (*4*). Walnut seed also contains several groups of polyphenolic phytochemicals, which are also known to contribute significantly to the total antioxidant activities of this tree nut (*5*−*8*). Among the polyphenols found in walnut, ellagitannins, which are also referred to as hydrolyzable tannins, have been reported to dominate the phenolic profile of the seed of *J. regia* L. (*5*−*7*). Ellagitannins can be hydrolyzed with mineral acids to yield ellagic acid and its derivatives, and due to the lack of standards the quantification of total ellagitannins in different plants has been based on ellagic acid equivalent concentrations (*7*, *9*, *10*).

Several studies have demonstrated that dietary ellagitannins from different plant sources including walnut are not directly absorbed in humans, but they are converted to ellagic acid in vivo as a result of digestion, and it is the ellagic acid that has been absorbed and further metabolized by the human colonic microflora to yield the bioavailable 3,8-dihydroxy-6*H*-dibenzo-[*b*,*d*]pyran-6-one (durolithin AT) derivatives (*7*, *11*). Larrosa et al. (*12*) suggested that the anticarcinogenic effect of dietary ellagitannins could be mainly due to their hydrolysis product, ellagic acid, which induced apoptosis via the mitochondrial pathway in colon cancer Caco-2 cells but not in normal colon cells. Ellagitannins and ellagic acid from different plant sources have also been studied for their antioxidant properties in vitro (*5*, *6*, *13*−*15*). Fukuda et al. (*6*) isolated and identified 15 ellagitannins from the aqueous ethanol extract of walnut seeds and found that these compounds had a superoxide dismutase (SOD)-like activity with an EC_{50} of 21.4−190 mM and a remarkable radical scavenging effect against 1,1-diphenyl-2-picrylhydrazyl (DPPH) ($EC_{50} = 0.34$−4.72 mM). Their study indicated that ellagitannins with a galloyl group plus a hexahydroxydiphenoyl (HHDP) group or valoneoyl and its isomeric group tend to exhibit a more potent radical scavenging effect against DPPH than those with only a HHDP group. Anderson et al. also found that ellagic acid (1.0 μmol/L) and walnut (*J. regia* L.) extract significantly inhibited 2,2′-azobis(2-amidino-

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propane) hydrochloride (AAPH)-induced low-density lipoprotein (LDL) oxidation (*5*). Similarly, copper-mediated LDL oxidation was also inhibited by ellagic acid and walnut extract, indicating that ellagic acid may be completely or partly responsible for the observed in vitro effect on maintaining LDL α-tocopherol (*5*). Individual polyphenols such as ellagic acid monomers, polymeric ellagitannins, and other phenolics were identified on the basis of data obtained from high-performance liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS). However, identification of these compounds and their hydrolysis products in walnut by LC-ESI-MS provided no detailed information on fragments and fragmentation patterns (*5*, *7*).

In the meantime, most of the research has been focused on the common walnut (*J. regia* L.), and virtually no scientific studies have been carried out with heartnut (*J. ailanthifolia* var. *cordiformis*), a naturally occurring genetic oddity of the more common Japanese walnut. To the best of our knowledge, there has been no peer-reviewed publication on this special tree nut and its nutritional content. Trees of this heart-shaped and generally hardier nut are grown mostly in the Great Lakes region in Ontario, Canada, and in recent years, growing interests have been found among both nut growers and consumers for its sweeter taste (Ernie Grimo, personal communication, http://www.grimonut.com/section1.htm). In this paper, we report for the first time on the polyphenolic profiles of three heartnut varieties using LC-ESI-MS^n and the antioxidant activities of the extracts and the hydrolysis products using chemical models. The polyphenolic composition and antioxidant activity were compared with those of the Persian walnuts.

MATERIALS AND METHODS

Chemicals, Solvents, and Nut Samples. The three heartnut varieties (Imshu, Campbell CW1, and Campbell CW3) and two Persian walnut varieties (Combe and Lake) were harvested in September 2005 and kindly provided by Ernie Grimo (Niagara-on-the-Lake, ON, Canada). Ascorbic acid, 2,4,6-tripyridyl-*s*-triazine (TPTZ), Tween 40, and the Folin−Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Ellagic acid was purchased from Sigma-Aldrich (Oakville, ON, Canada). Ferric chloride ($FeCl_3$) and sodium acetate were from Aldrich Chemical Co. (Milwaukee, WI). Regents for the photochemiluminescence (PCL) assay were purchased from Analytik Jena AG (Berlin, Germany). All solvents were of HPLC grade and purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada).

Extraction and Fractionation. Each of the samples was manually cracked and shelled, and the seed was immediately put into liquid nitrogen and ground in a coffee blender. For each sample, 5 g of finely ground walnut was accurately weighed in a 100 mL screw-capped flask and extracted twice each with 50 mL of hexane at room temperature in the dark for 2 h. Each time, the extract was filtered through a Whatman no.1 filter paper. The residue was then extracted twice, each with 50 mL of 80% methanol for 2 h and filtered through a Whatman no.1 filter paper. The combined filtrate was concentrated to 20 mL in vacuo at <40 °C, acidified to pH 2 with 2 N HCl, and then partitioned with 40 mL of ethyl acetate (EtOAc) three times. The combined EtOAc fraction contained mainly free phenolic acids (FPA) and was evaporated to dryness at <40 °C and redissolved in 4 mL of methanol. The resulting aqueous phase from the partitioning was neutralized to pH 7 with 2 N NaOH and dried using a vacuum evaporator. The residue was dissolved in 20 mL of 2 N HCl and heated at 95 °C for 4 h, adjusted to pH 2, and then partitioned with 40 mL of EtOAc three times. The EtOAc layers were combined, evaporated to dryness at <40 °C, and redissolved in 4 mL of methanol. This fraction contained mainly 80% methanol extractable acid-hydrolyzable phenolic acids (AHPA).

The residue resulting from the 80% methanol extraction was suspended in 20 mL of 2 N HCl and heated at 95 °C for 8 h, adjusted to pH 2, and then partitioned with 40 mL of EtOAc three times. The combined EtOAc layer was dried and reconstituted in 4 mL of methanol. This fraction contained mainly methanol-unextractable bound phenolic acid (BPA). All fractions (in methanol) were filtered through a 0.45 μm syringe filter, sealed, and stored at −20 °C before subsequent analysis of the total phenolic content, separation and identification by HPLC-ESI-MS^n, and measurement of the antioxidant activities.

Determination of Total Phenolic Contents (TPCs). The Folin−Ciocalteu method following similar procedures published earlier (*16*) was used for the determination of TPCs of the samples. Briefly, each fraction (0.2 mL) was mixed with 1 mL of the Folin−Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate solution. The mixture was allowed to stand at room temperature for 30 min, and then the absorbance was measured at 765 nm in a Varian Cary 3C spectrophotometer (Varian, Palo Alto, CA). A standard curve was generated with gallic acid ($r^2 = 0.9960$, concentration range is from 50 to 250 μg/mL), from which TPCs in the various fractions were calculated and expressed as milligrams of gallic acid equivalent (GAE) per gram of fresh nut. All samples were tested in duplicate.

HPLC Analysis. An Agilent Technology 1100 series HPLC system equipped with a quaternary pump, a degasser, a thermostatic autosampler, and a photodiode array detector (DAD) was used for the analysis of phenolic compounds in different fractions of heartnut and walnut. The separation was carried out in a Phenomenex ODS-C_{18} column (250 × 4.6 mm, 5 μm) with a C_{18} guard column. The binary mobile phase consisted of acetonitrile (solvent A) and water containing 2% acetic acid (solvent B). The flow rate was kept constant at 1.0 mL/min for a total run time of 30 min. The system was run with a gradient program: 95% B to 60% B in 20 min, 60% B to 45% B in 5 min, and 45% B to 95% B in 5 min. The sample injection volume was 10 μL. Peaks of interest were monitored at 280 nm, but spectral data from 210 to 600 nm were collected at 1 nm interval for identification purposes using DAD.

LC-ESI-MS^n. LC-ESI-MS^n experiments were carried out using a Finnigan LCQ DECA ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with an electrospray ionization (ESI) source. To reach its optimum performance, the sheath gas and auxiliary flow rates were set at 82 and 45 (arbitrary units), respectively. The capillary voltage was set at −4 kV, and its temperature was controlled at 350 °C. The entrance lens voltage was fixed at 12 V, and the multipole RF amplitude was set at 730 V. The ESI needle voltage was controlled at 4.5 kV. The tube lens offset was −29 V, the multipole lens 1 offset was 8.10 V, and the multipole lens 2 offset was 11.5 V. The electron multiplier voltage was set at −1010 V for ion detection. The collision-induced dissociation (CID) was performed directly in the octapole region, where the relative collision energy was 70%. LC conditions were the same as described above for the HPLC.

Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay is based on the reduction of ferric ion (Fe^{3+}) to the ferrous form (Fe^{2+}) at low pH, which has an intense blue color (593 nm) when in complex with TPTZ (Fe^{2+}/TPTZ) (*17*). The FRAP assay followed similar procedures published earlier (*16*). Briefly, the FRAP reagent was prepared freshly by mixing acetate buffer (300 mM, pH 3.6), 10 mM TPTZ in 40 mL of HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). L-Ascorbic acid and ellagic acid were prepared at the same concentration of 500 μM in methanol. Ten microliters of standard or sample solution was pipetted into the wells of the microplate separately and then mixed with 300 μL of FRAP reagent per well. All fraction samples were diluted 20 times before FRAP analysis. The plate was kept at 37 °C, and the absorbance was taken at 593 nm immediately after and at 4 min intervals by using a visible−UV microplate kinetics reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT). All samples were tested in triplicate. The final FRAP value of the samples was calculated on the basis of 500 μM ascorbic acid (equivalent to 1000 μM FRAP values).

Photochemiluminesence (PCL) Assay. The PCL assay also followed the procedures reported by us before (*16*). The PCL assay is based on a photoinduced, chemiluminescence-accompanied, and antioxidant-inhibitable autoxidation of luminal (*18*). The measurement of chemiluminescence was carried out in a commercial PCL instrument, the Photochem (Berlin, Germany). In our experiment, we used the

Table 1. Total Phenolic Contents in Different Fractions of Persian Walnuts and Heartnuts[a]

sample	extractable phenolic acids		
	FPA[b]	AHPA[c]	BPA[d]
Combe Persian walnut	10.42	3.54	3.73
Lake Persian walnut	9.61	3.78	2.75
Campbell CW1 heartnut	1.91	1.65	2.75
Campbell CW3 heartnut	1.48	1.40	1.94
Imshu heartnut	2.48	1.39	2.63

[a] Values are average of duplicate samples in gallic acid equivalents (mg/g of nut). [b] Free phenolic acids. [c] Acid-hydrolyzable phenolic acids. [d] Bound phenolic acids.

ACW-Kit (for integral measurement of water-soluble antioxidants), which was commercially available from the company. Luminol in this assay had a dual function: being a photosensitizer and a chemiluminogenic probe for free radicals. Briefly, the mixture contained 1.0 mL of reaction buffer containing 0.1 M sodium carbonate (pH 10.5) and 0.1 mM Na_2-EDTA, 1.5 mL of ACW-diluent (water), 25 μL of 1 mM luminol, and 10 μL of a standard or sample solution. When necessary, samples were diluted so that the PCL curves fell within the linear range of the standard, ascorbic acid (0.25−3 nmol). All samples were tested in duplicate. The antioxidant capacity of the sample was expressed in equivalent units of the standard (L-ascorbic acid).

Statistical Analysis. Statistical analysis was performed by using linear correlation and *t* test (two-sample assuming equal variances) functions of Microsoft Excel (v. 10.5815.4219 SP-2).

RESULTS AND DISCUSSION

Total Phenolic Contents. The TPCs of the three heartnut varieties (Imshu, Campbell CW1, and Campbell CW3) and the two Persian varieties (Combe and Lake) are shown in **Table 1**. In general, TPC in both free phenolic acid (FPA) and acid-hydrolyzable phenolic acid (AHPA) fractions of the heartnut were significantly lower ($P < 0.001$) than those of the walnut; however, no significant difference was found in bound phenolic acids (BPA) between the two nuts ($P = 0.20$).

The lower concentration of phenolics in heartnut is perhaps what makes it sweeter as a higher phenolic content is considered to contribute to the bitterness. The highest average TPC was found in the FPA fraction of the Persian walnuts at 10.02 mg of GAE/g of walnut, which was >5-fold the average TPC in heartnuts ($P < 0.001$). TPCs in each FPA fractions of the two Persian walnuts were similar to what was reported by Kornsteiner et al. (*1*), who found that the TPCs of walnuts were between 1020 and 2052 mg of GAE/100 g of fresh walnut. TPCs in AHPA and BPA were significantly lower than that of the FPA ($P < 0.01$), and there was no significant difference between AHPA and BPA ($P = 0.50$) for the Persian walnuts. In heartnuts, significant difference was found only between AHPA and BPA ($P < 0.05$) (**Table 1**). The BPA fraction contained the highest TPC at 2.44 mg of GAE/g of heartnut, followed by the TPCs in FPA (1.96 mg of GAE/g of heartnut) and AHPA (1.48 mg of GAE/g of heartnut) (**Table 1**).

Separation, Identification, and Quantification of Phenolic Acids by HPLC and LC-ESI-MS^n. As mentioned before, the presence of free ellagic acid often suggests the occurrence of its bound forms, the ellagitannins, in walnuts (*6*, *7*, *11*). Ellagitannins can be hydrolyzed with acids or bases to yield ellagic acid; however, only acid hydrolysis was performed on both extract and residue in the present study. Preliminary time course studies were carried out to obtain optimal conditions for the AHPA and BPA fractions by monitoring the release of free ellagic acid. Hydrolysis at 95 °C for 4 h and at 95 °C for 8 h gave the highest concentrations of ellagic acid in these two fractions, respectively. Ellagic acid was identified and quantified in different fractions of nut samples using LC-ESI-MS and by comparing the retention time and UV spectral data with the standard. Valoneic acid dilactone was also tentatively identified, as described below.

Typical HPLC-DAD chromatograms of the AHPA fractions of heartnut and Persian walnut are given in **Figure 1**. A good separation was achieved within 30 min. Although the polyphenolic profiles of heartnut and Persian walnut were different quantitatively, the compositions of major peaks were highly identical (**Figure 1**). Peaks 1 and 2 were the major peaks detected in heartnut; thus, our focus was placed on these two compounds. Peak 2 was identified as ellagic acid by congruent retention time and UV spectrum with those of the authentic standard. Peak 1 had UV spectrum similar to that of ellagic acid, as shown in **Figure 1**.

Structures of peaks 1 and 2 were identified using LC-ESI-MS and source collision-induced dissociation (CID) experiments. Peaks 1 and 2 in **Figure 1** exhibited intense deprotonated molecular ions $[M - H]^-$ at *m*/*z* 469 and 301 in the negative mode (**Table 2**), from which the molecular weights of peaks 1 and 2 were confirmed to be 470 and 302, respectively. The LC-

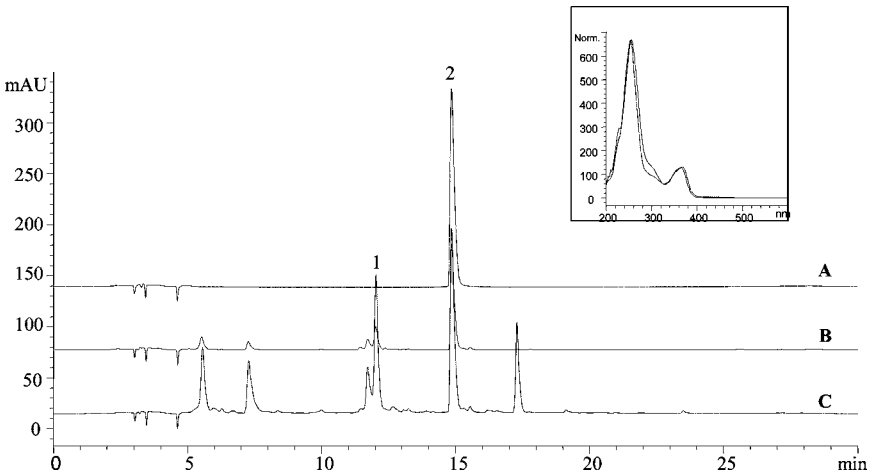

Figure 1. HPLC profiles and UV spectra of standard ellagic acid (**A**) and AHPA profiles of Campbell CW1 heartnut (**B**) and Combe Persian walnut (**C**). Compounds related to peaks 1 and 2 were identified as valoneic acid dilactone and ellagic acid, respectively. The UV spectrum with dotted line is ellagic acid; the solid line is valoneic acid dilactone.

Table 2. LC-ESI-MS^n Data Obtained from the [M − H]$^-$ Ions of Phenolic Compounds in the Extract of Walnuts and Heartnuts

	[M-H]$^-$	MS^2	MS^3	MS^4
Ellagic acid and ion at m/z 301	301	-30 → 271 -44 → 257 -72 → 229		
ion at m/z 469	469	-44 → 425 -168 → 301 -170 → 299	425: -124 → 301; -126 → 299 301: -30 → 271; -44 → 257; -72 → 229	

ESI-MS^n of peak 2 is shown in **Figure 2**. The deprotonated molecular ion [M − H]$^-$ (*m*/*z* 301) with high abundance was the only ion observed in the negative mode (**Figure 2A**). Further investigation in the MS−MS experiment of the *m*/*z* 301 ion ([M − H]$^-$) yielded three main fragment ions at *m*/*z* 271, 257, and 229 (**Figure 2B**), all of which were produced directly from the parent ion *m*/*z* 301 (**Table 2**). The ion at *m*/*z* 257 [M − 44 − H]$^-$ is considered to be from the neutral loss of the carboxyl moiety from the parent ion. The ion at *m*/*z* 301 can also lose a carboxyl unit and a CO unit together to produce the *m*/*z* 229 ion as [M − 44 − 28 − H]$^-$. The ions at *m*/*z* 257 and 229 obtained in our study are consistent with the literature reports on ellagic acid from different plant sources (*19*, *20*). However, the ion at *m*/*z* 271, which we observed in both standard ellagic acid and peak 2 of all samples, was not observed in these reported studies (**Figure 2B**; **Table 2**). We concluded that on the basis of these ESI-MS^2 data and the matching retention time and UV spectrum, peak 2 was in fact ellagic acid.

The LC-ESI-MS of peak 1 is shown in **Figure 3**. A deprotonated molecular ion [M − H]$^-$ (*m*/*z* 469) was the only ion found in the negative ESI-MS (**Figure 3A**). During the CID experiment, the ion at *m*/*z* 469 formed three fragment ions: *m*/*z* 425, 301, and 299 (**Figure 3B**; **Table 2**). This fragmentation pattern was similar to that of an ellagic acid derivative identified by others (*19*−*22*). The ion at *m*/*z* 425 was produced directly from the parent ion of *m*/*z* 469, corresponding to the neutral loss of a carboxyl moiety [M − 44 − H]$^-$ (**Table 2**), which is supposed to be an external carboxyl unit in the gallic acid. CID of the ion at *m*/*z* 425 produced ions at *m*/*z* 301 and 299 (**Figure 3C**), which is perhaps an indication of the presence of an ellagic acid moiety in the molecule (*21*). The ion at *m*/*z* 301 comes from the neutral loss of the gallic acid unit [M − 168 − H]$^-$ from the parent ion. The ion at *m*/*z* 301 in the CID spectrum (**Figure 3D**) followed the same fragmentation patterns as that of ellagic acid as discussed above (**Figure 2B**). The ions at *m*/*z* 257 and 229 in the MS^3 analysis confirmed that an ellagic acid was associated with this molecule according to the literature reports (*19*, *20*, *22*). On the basis of the CID fragment patterns of peak 1, the structural information of ellagic acid (peak 2), and the ESI-MS data obtained in other studies (*19*−*22*), peak 1 was found to be highly related to valoneic acid dilactone. Although there was no standard available and the exact structure of this compound needs to be confirmed by NMR spectroscopy and other analytical techniques in future work, a tentative identity was given to peak 1 as valoneic acid dilactone in the present study. The proposed structure of valoneic acid dilactone, together with the structure of ellagic acid, is shown in **Figure 4**.

Quantification was consequently performed for these two identified ellagic acid derivatives. Hydrolysis of both the

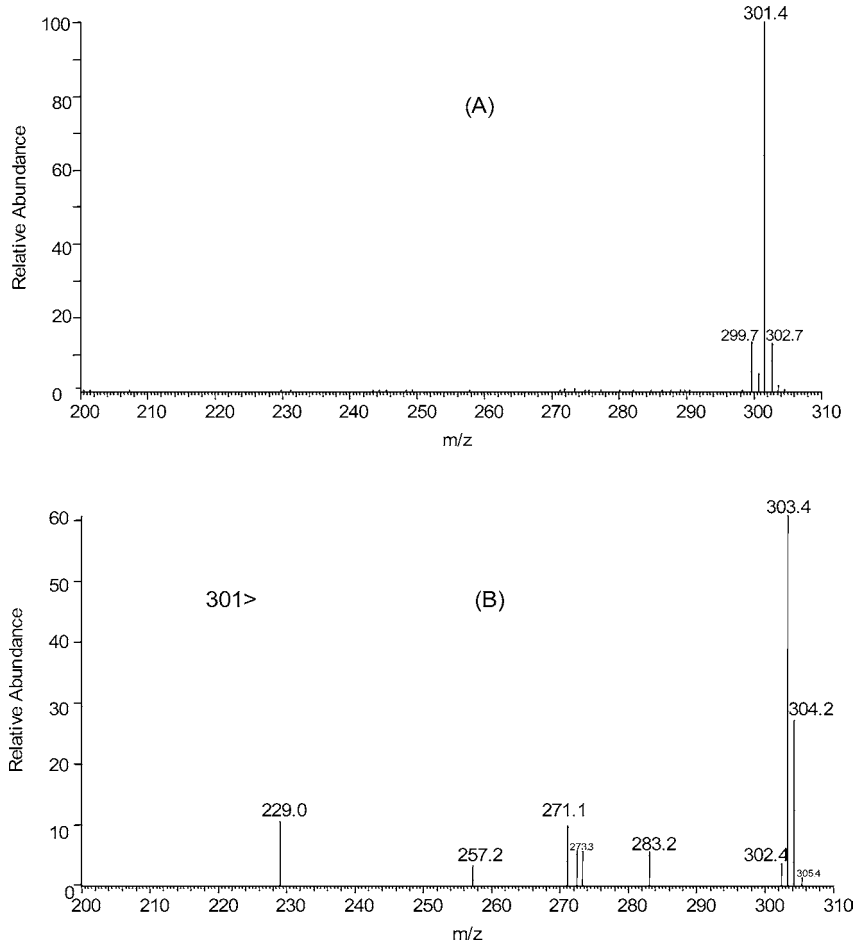

Figure 2. (**A**) LC-ESI-MS of peak 2 obtained in the negative ion mode; (**B**) CID spectrum of the parent ion *m*/*z* 301 of peak 2.

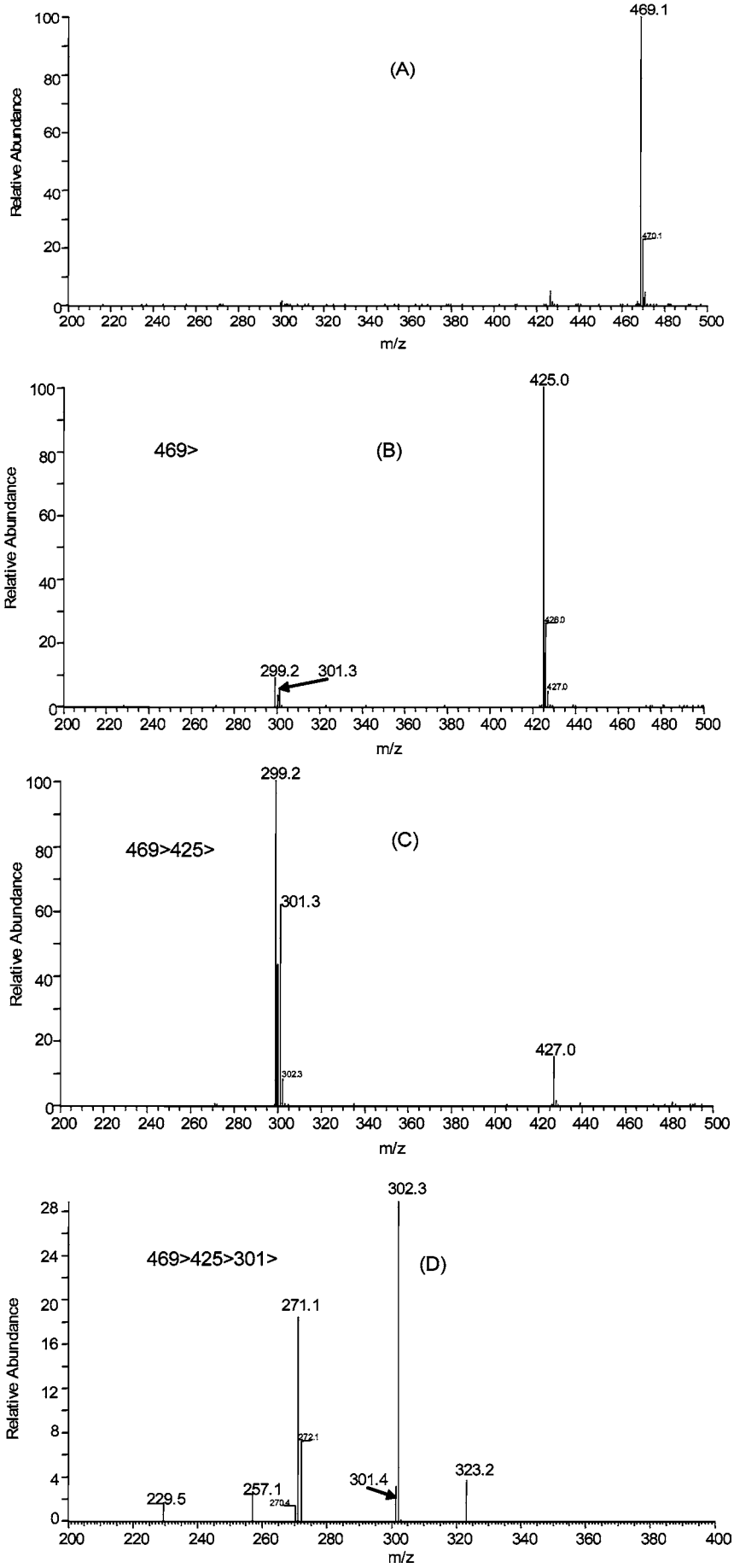

Figure 3. (**A**) LC-ESI-MS spectrum of peak 1 obtained in the negative ion mode; (**B**) the MS^2 spectrum of the parent ion *m*/*z* 469; (**C**) MS^3 spectrum of the parent ion *m*/*z* 469; (**D**) MS^4 spectrum of the parent ion *m*/*z* 469.

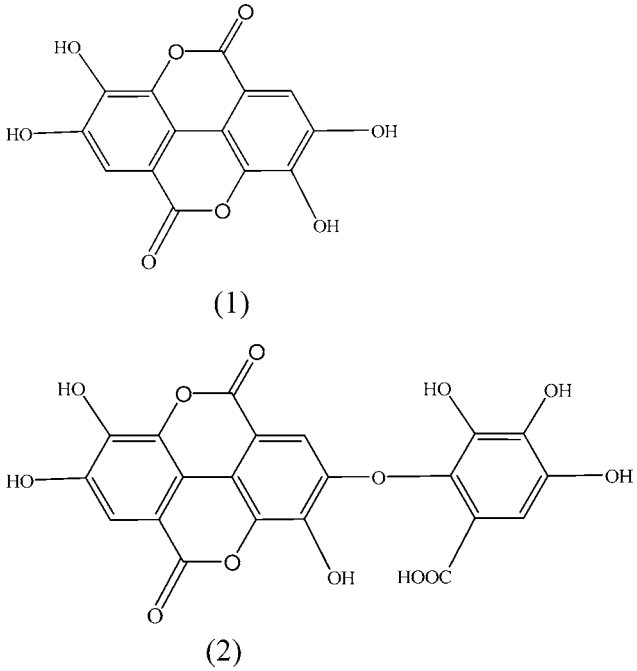

Figure 4. Structures of (**1**) ellagic acid and (**2**) valoneic acid dilactone.

Table 3. Phenolic Acids in Different Fractions of Persian Walnuts and Heartnuts

	ellagic acid (mg/g of nut)			valoneic acid dilactone (ellagic acid equivalent) (mg/g of nut)		
sample	FPA[b]	AHPA[c]	BPA[d]	FPA[b]	AHPA[c]	BPA[d]
Combe Persian walnut	0.32	1.30	1.21	ND[e]	0.68	0.70
Lake Persian walnut	0.25	1.33	0.64	ND	0.71	0.44
Campbell CW1 heartnut	0.14	0.66	0.70	ND	0.14	0.62
Campbell CW3 heartnut	0.09	0.56	0.55	ND	0.12	0.34
Imshu heartnut	0.24	0.58	0.85	ND	0.10	0.41

[a] Values are average of duplicate samples expressed in mg/g of nut. [b] Free phenolic acids. [c] Acid-hydrolyzable phenolic acids. [d] Bound phenolic acids. [e] Not detectable.

aqueous methanolic extract and the residue showed that ellagic acid was the major bound phenolic acid in all nuts. As shown in **Table 3**, the Persian walnuts contained an average of 0.29, 1.31, and 0.93 mg of ellagic acid/g of nut in the FPA, AHPA, and BPA fractions, respectively. Averages of 0.16, 0.60, and 0.70 mg of ellagic acid/g of nut were found in the FPA, AHPA, and BPA fractions of heartnuts, respectively. The highest concentration of ellagic acid was found in the AHPA fraction of the Persian walnut, whereas for heartnuts it was in the BPA fraction (**Table 3**). FPA fractions contained the lowest concentration of ellagic acid in both heartnut and Persian walnut, indicating that most of the ellagic acid in these nuts was in bound forms, and they are released only upon acid hydrolysis. These results are in agreement with the literature reports on walnuts (*6*, *7*, *11*). The lower concentration of free ellagic acid as compared to the hydrolyzable and bound ellagic acid in both heartnut and Persian walnut and the high TPC found in the FPA fraction of walnut (**Table 1**) also indicate the possible existence of other phenolic compounds in these fractions. This also suggests that the FPA fraction may contain the most of such phenolics, which could include chlorogenic, caffeic, *p*-coumaric, ferulic, sinapic, and syringic acid, as found in the pellicle of walnut by Colaric et al. (*8*). Quantification of valoneic acid dilactone was based on ellagic acid and the concentration was

Table 4. Antioxidant Activities of Different Fractions of Persian Walnuts and Heartnuts Measured by the FRAP Assay[a]

	FRAP value (μM)		
sample	FPA[b]	AHPA[c]	BPA[d]
Combe Persian walnut	3127.32 ± 0.07	1775.85 ± 0.04	2534.37 ± 0.44
Lake Persian walnut	3454.27 ± 0.27	1738.79 ± 0.06	1956.37 ± 0.28
Campbell CW1 heartnut	1082.49 ± 0.05	770.47 ± 0.05	1641.36 ± 0.18
Campbell CW3 heartnut	871.49 ± 0.04	757.32 ± 0.00	1218.77 ± 0.17
Imshu heartnut	1649.13 ± 0.07	744.77 ± 0.12	1490.74 ± 0.37
ellagic acid (500 μM)		1453.08 ± 0.02	

[a] The FRAP value of the samples ($n = 3$) was calculated on the basis of 500 μM ascorbic acid (equivalent to 1000 μM FRAP values). [b] Free phenolic acids. [c] Acid-hydrolyzable phenolic acids. [d] Bound phenolic acids.

in milligrams of ellagic acid equivalent per gram of nut (**Table 3**). No valoneic acid dilactone was detected in the FPA fraction of either heartnut or the Persian walnut (**Table 3**), but, upon acid hydrolysis, a significant amount of valoneic acid dilactone was released (0.68−0.71 and 0.44−0.70 mg of ellagic acid equivalent/g in the AHPA and BPA fractions of Persian walnut, respectively; and 0.1−0.14 and 0.34−0.62 mg of ellagic acid equivalent/g in the AHPA and BPA fractions of heartnut, respectively) (**Table 3**).

Antioxidant Activities. Phenolic compounds in walnut seeds have an important antioxidative role in protecting fatty acids from oxidation (*23*). As well, many health beneficial effects of walnut phenolics have been described, including the antioxidant activity (*5*, *6*, *23*, *24*). The hydrolysis products of walnut are an excellent source of ellagic acid, which has been found to have good antioxidant activity. Although phenolics extracted from the Persian walnuts have been examined for their antioxidant activities using free radical scavenging methods (*5*, *6*, *15*), antioxidant activities measured using other models with different mechanisms such as FRAP and PCL used in this study have not been reported, for either the extract or the hydrolysis products. Antioxidant activities have not been attempted for heartnut.

In the present study, the FRAP assay was developed to determine the ferric reducing ability of the different fractions of nuts. The antioxidant capacity of the samples was estimated from their ability to reduce the TPTZ−Fe^{3+} complex to the TPTZ−Fe^{2+} complex (*17*). Calculation of FRAP values was based on ascorbic acid as described above. FRAP values were found to be significantly higher in all fractions of the Persian walnut than in heartnuts (**Table 4**). For the Persian walnut, the highest FRAP value was found in the FPA fraction, followed by BPA and AHPA (**Table 4**), whereas, for heartnut, in general the highest FRAP value seemed to be in the BPA fraction, followed closely by FPA and AHPA (**Table 4**). Considering that these extracts were diluted 20 times before the FRAP assay (see Materials and Methods), they are very strong antioxidants. For example, the FRAP values of such a dilution of the BPA fraction of heartnuts (average = 1451 μM) were as good as that of 500 μM ellagic acid (1453 μM) (**Table 4**). The total antioxidant activity of nut fractions by FRAP, namely, FPA, AHPA, and BPA, correlated well with their total phenolic content with correlation coefficients of $r^2 = 0.95$, 0.99, and 0.91, respectively, indicating that phenolic acids are the components responsible for the antioxidant effect. Ellagic acid and valoneic acid dilactone, as the major individual compounds in the fractions, therefore may contribute significantly to the total antioxidant activity as the major phenolic acids found in the extracts, although the role of other unknown phenolics cannot be excluded (*8*). In addition, our present study showed that

Table 5. Antioxidant Activities of Different Fractions of Persian Walnuts and Heartnuts Measured by the PCL-ACW Assay[a]

sample	equivalent units of L-ascorbic acid (nmol/mg of nut)		
	FPA[b]	AHPA[c]	BPA[d]
Combe Persian walnut	61.43	49.15	53.24
Lake Persian walnut	76.39	48.90	28.68
Campbell CW1 heartnut	19.87	14.505	29.32
Campbell CW3 heartnut	18.84	12.61	21.07
Imshu heartnut	35.71	14.32	25.48
ellagic acid		4.52[e]	

[a] Values are average of duplicate samples. [b] Free phenolic acids. [c] Acid-hydrolyzable phenolic acids. [d] Bound phenolic acids. [e] Nanomoles of ascorbic acid equivalent.

although both TPC and FRAP of heartnut fractions were low compared with the Persian walnut, different varieties of heartnut showed different degrees of antioxidant activity, which corresponded to their respective TPC (**Tables 1** and **4**).

The antioxidant activity of different nut fractions was further evaluated using the PCL-ACW method. The sample solutions have to be prepared so that their lag phase falls within the linear range of the standard curve, which was generated by measuring the PCL of ascorbic acid prepared at 0, 0.5, 1, 2, and 3 nmol. As a result, the antioxidant activities of the extracts and their hydrolysis products are shown in **Table 5**. The antioxidant activities measured by PCL-ACW followed a pattern similar to those found in the FRAP assay. The PCL activities of all Persian walnut fractions were higher than those of their respective fractions of heartnut (**Table 5**). In general, the FPA of Persian walnuts showed the strongest antioxidant activity, followed by AHPA and BPA, whereas BPA of the heartnuts had stronger activity than FPA and AHPA. The PCL-ACW activity also showed strong correlation coefficients of $r^2 = 0.89$, 0.99, and 0.90 with the TPC for the FPA, AHPA, and BPA fractions, respectively, indicating that phenolic acids are the components responsible for the antioxidant effects. Again, ellagic acid is considered to be one of the major contributors to the total antioxidant activity as it exhibited a 4.5-fold stronger activity than ascorbic acid (**Table 5**).

Due to the different mechanisms involved in the antioxidative processes, it has been strongly recommended that at least two methods be used in a study (*25*). The PCL measures the reduction of preformed superoxide radicals, whereas the FRAP assay measures the reducing power against the ferric ion. PCL is a highly sensitive method as it can measure the activity of an analyte at the nanomolar level. Although the PCL and FRAP methods used in this study differed in antioxidant mechanism, results from the two assays agreed well with one another. A strong correlation was observed for the total phenolic content and the antioxidant activities in both assays.

Conclusion. Although three heartnut varieties contained lower phenolic contents and showed lower antioxidant activities in the two assays conducted than the Persian walnut in the present study, as a specialty tree nut with a sweeter taste and a nearly perfect heart shape, it presents a good commercial potential. In addition, heartnuts also have essential fatty acid and tocopherol contents similar to those of the Persian walnut (data not shown); these essential nutrients are also known as antioxidants. Consumption of walnuts has been known to reduce many health risks; thus, further study on this special tree nut can potentially lead to the development of a larger market that meets the current and future consumer demand.

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