DOI:10.1021/jf1034306

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Phenolic Compounds and Antioxidant Activity of Kernels and Shells of Mexican Pecan (*Carya illinoinensis*)

Laura A. de la Rosa,[†] Emilio Alvarez-Parrilla,[†] and Fereidoon Shahidi*,[§]

[†]Departamento de Ciencias Químico-Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Anillo Envolvente del PRONAF y Estocolmo s/n, Ciudad Juárez, Chihuahua, CP 32310, Mexico, and [§]Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3X9

The phenolic composition and antioxidant activity of pecan kernels and shells cultivated in three regions of the state of Chihuahua, Mexico, were analyzed. High concentrations of total extractable phenolics, flavonoids, and proanthocyanidins were found in kernels, and 5−20-fold higher concentrations were found in shells. Their concentrations were significantly affected by the growing region. Antioxidant activity was evaluated by ORAC, DPPH$^{\bullet}$, HO$^{\bullet}$, and ABTS$^{\bullet-}$ scavenging (TAC) methods. Antioxidant activity was strongly correlated with the concentrations of phenolic compounds. A strong correlation existed among the results obtained using these four methods. Five individual phenolic compounds were positively identified and quantified in kernels: ellagic, gallic, protocatechuic, and *p*-hydroxybenzoic acids and catechin. Only ellagic and gallic acids could be identified in shells. Seven phenolic compounds were tentatively identified in kernels by means of MS and UV spectral comparison, namely, protocatechuic aldehyde, (epi)gallocatechin, one gallic acid−glucose conjugate, three ellagic acid derivatives, and valoneic acid dilactone.

KEYWORDS: Pecan; polyphenolics; flavonoids; condensed tannins; hydrolyzable tannins; environmental conditions

INTRODUCTION

In recent years, nuts have gained recognition as healthy foods, because epidemiological studies and intervention trials have consistently demonstrated that daily consumption of 1.5 oz (42 g) of nuts may lower the risk of cardiovascular disease (*1*). In addition to being rich in several vitamins and minerals, unsaturated fatty acids, and fiber, nuts contain numerous phytochemicals, including phenolic compounds (many of them still not fully identified and characterized), that may contribute to health promotion and risk reduction of chronic disease (*2*). Numerous studies have also found an inverse correlation between the consumption of foods rich in phenolic compounds and the occurrence of cardiovascular disease (*3*). Therefore, there is a growing interest in characterizing and quantifying phenolic compounds in the edible parts of different nut species. Moreover, inedible plant parts may supply the raw material for obtaining purified phenolic fractions (*4*), and nut byproducts are rich in phenolic compounds with high antioxidant activity (*5*), so their characterization is also of great interest.

Pecan is among the most preferred of all nuts and an economically important crop in the United States and Mexico. Pecan ranks third in U.S. production of nuts but is tied with the walnut as the second most frequently consumed tree nut in the United States after almonds (*6*). However, compared to walnut and almond, very few studies have been published on the phytochemicals and health benefits of pecan. A clinical study by Rajaram et al. (*7*) showed that a pecan-enriched cholesterol-lowering diet improved lipid profiles (reduction of triacylglycerides, total, and LDL cholesterol and increase of HDL cholesterol) beyond the pecan-free diet. Pecan has been ranked among the foods with the highest phenolic content and antioxidant activity (*8*), and the classes of phenolic compounds found in pecan kernels are flavan-3-ols, anthocyanidins (*9*), proanthocyanidins (*10*), phenolic acids (*11*), and, more recently, ellagic acid (*12*). However, genetic and environmental factors regulate the concentration of phenolic phytochemicals in plant tissues, and more studies are needed to provide current data on the composition and concentration of phenolic constituents in different varieties of pecan, grown in different geographical areas and with different agronomic practices (*6*).

In the present study, the phenolic composition and antioxidant activity of pecan kernels and shells cultivated in the state of Chihuahua, Mexico, were analyzed. Pecans grown in the state of Chihuahua are derived from a blend of Wichita and Western cultivars, and to the best of our knowledge no information has been published on the phenolic composition of this variety. Moreover, HPLC-MS was used to attempt a more detailed characterization of the individual phenolic compounds that can be found in pecans.

MATERIALS AND METHODS

Chemicals. The compounds 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS^{2-}), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin−Ciocalteu phenol reagent, sodium carbonate, monobasic sodium phosphate, dibasic sodium phosphate, gallic acid,

*Corresponding author [phone (709) 737-8552; fax (709) 737-4000; e-mail fshahidi@mun.ca or fshahidi@gmail.com].

 Published on Web 12/07/2010

Table 1. Phenolic Compounds, Flavonoids, and Proanthocyanidins of Pecans Grown in the State of Chihuahua, Mexico[a]

	phenolic compounds (mg GAE/g FW)		flavonoids (mg CE/g FW)		proanthocyanidins (mg CE/g FW)	
growing area	kernel	shell	kernel	shell	kernel	shell
CG	11.7 ± 0.3 b	86.4 ± 7.1 a	5.9 ± 0.7 a	33.1 ± 1.8 a	20.6 ± 1.7 b	396.0 ± 30.2 a
Del	12.5 ± 0.2 a	65.3 ± 6.9 b	6.4 ± 0.8 a	26.3 ± 2.6 b	26.7 ± 4.5 a	316.1 ± 17.3 b
Jim	11.9 ± 0.3a,b	92.5 ± 9.0 a	5.8 ± 0.8 a	36.1 ± 1.8 a	20.3 ± 0.5 b	464.4 ± 38.0 a

[a] Different letters in the same column indicate significant difference between samples ($n = 3$, Tukey $P < 0.05$).

catechin, epicatechin, protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, ellagic acid, ferric chloride, fluorescein, ferrous chloride, oxygen peroxide, sodium bicarbonate, and vanillin were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON, Canada). All other chemicals and solvents were purchased from Fisher Scientific (Nepean, ON, Canada) and were of ACS grade or better.

Pecan Samples. Pecan nuts grown in Chihuahua, derived from a successful blend of Wichita and Western varieties, were kindly donated by local producers from three regions of the state of Chihuahua, Mexico (Casas Grandes, Delicias, and Jimenez). Nuts were harvested in 2008 and stored at 4 °C until the time of analysis (November 2009−June 2010). At that time, nuts were manually cracked, and the kernels (containing the testa or pellicle attached to them) and shells were separated and stored in vacuum-sealed plastic containers at −20 °C until used.

Extraction of Phenolic Compounds. Pecan kernels were finely ground in a coffee grinder (Black and Decker Canada Inc., Brockville, ON, Canada) and then defatted by blending with hexane (1:10, w/v, 3 min, three times) in a Waring blender (model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at room temperature. Defatted kernels (10 g) were placed in a flat-bottom flask with a screw cap, 100 mL of 80% acetone was added, and the phenolic compounds were extracted by mixing at 50 °C for 30 min. Afterward, the extract was centrifuged (3000*g*, 10 min, room temperature) and the pellet re-extracted with the same solvent. Both supernatants were combined, and the solvent was removed under vacuum at 40 °C. The concentrated slurries were freeze-dried for 72 h at −45 °C (Labconco 6 freezone, Labconco Corp., Kansas City, MO). Pecan shells were first ground manually in a mortar and then finely ground in a coffee grinder. Extraction was carried out as for the kernels, using 15 g of the shell powder and 150 mL of 80% acetone. Dried extracts were stored in tightly sealed glass vials at −20 °C.

Determination of Total Phenolic Content. Kernel and shell extracts were dissolved in methanol (0.5 and 0.25 mg/mL, respectively), and 0.5 mL was mixed with 2.5 mL of 10% Folin−Ciocalteu's reagent (v/v) and incubated at room temperature for 2 min. Next, 2 mL of 7.5% (w/v) sodium carbonate was added, and the mixture was incubated at 50 °C for 15 min in the dark and cooled to room temperature, and then the absorbance was read at 760 nm using a diode array spectrophotometer (8452A, Agilent Technologies, Mississauga, ON, Canada). Gallic acid was used as a standard, and results were expressed as milligrams gallic acid equivalents (GAE) per gram of fresh sample weight (FW).

Determination of Total Flavonoid Content. Total flavonoids were determined according to the method of Zhishen et al. (*13*), with slight modifications. The extracts of kernels or shells (0.5 mL) dissolved in methanol (0.5 and 0.25 mg/mL, respectively) were mixed with 2 mL of water and 150 μL of 5% $NaNO_2$. After 5 min, 150 μL of 10% $AlCl_3$ was added to the mixture, which was allowed to stand for 3 min. Then 2 mL of 0.5 M NaOH was added, the mixture was incubated at room temperature for 30 min in the dark, and the absorbance was read at 510 nm using a diode array spectrophotometer Catechin was used as a standard, and the results were expressed as milligram catechin equivalents (CE) per gram of FW.

Determination of Condensed Tannins. Condensed tannins (proanthocyanidins) were determined by the vanillin assay, according to the method of Chavan et al. (*14*). The kernel or shell extracts were dissolved in methanol (0.5 and 0.25 mg/mL, respectively), and 0.5 mL was mixed with 2.5 mL of 0.5% vanillin in acidified methanol (4% HCl, v/v) and incubated for 20 min at room temperature in the dark. Catechin was used as a standard, and results were expressed as milligram catechin equivalents (CE) per gram of FW. For every sample and standard, blanks were prepared for background subtraction by mixing 0.5 mL of sample (or standard) with 2.5 mL of acidified methanol (4% HCl, v/v). Absorbance of the blanks and samples was measured at 500 nm using a diode array spectrophotometer.

Identification and Quantification of Phenolic Compounds by HPLC-ESI-MS. Analysis of phenolic compounds by HPLC coupled to electrospray ionization mass spectrometry (ESI-MS) was carried out in crude acetone extracts and in acid-hydrolyzed extracts. Hydrolysis was carried out similarly to the procedure described by Li et al. (*15*). The dried kernel or shell extracts (300 mg) were suspended in 24 mL of 2 M HCl and heated to 95 °C over 4 h; the mixture was then cooled and its pH adjusted to 2 with 10 M NaOH. Phenolic compounds were extracted with 35 mL of diethyl ether three times, and the ether extracts were then combined and evaporated to dryness at 30 °C under vacuum; the residue was freeze-dried for 24 h at −45 °C. Dried extracts were redissolved in HPLC-grade methanol to a final concentration of 10 (crude extracts) or 5 (hydrolyzed extracts) mg/mL, filtered through a 0.45 μm syringe filter, and stored (for < 1 month) in tightly sealed glass vials at −20 °C before subsequent HPLC-ESI-MS analysis.

An Agilent 1100 HPLC system (Agilent Technologies, Mississauga, ON, Canada) with a quaternary pump, degasser, autosampler, and UV diode array detector (UV-DAD) coupled to an Agilent 1100 SL LC/MSD ion trap mass spectrometry system via an ESI interface was used. Separation was achieved in a Supercosil LC-18 reverse-phase column (5 μm particle size, 25 cm × 4.6 mm i.d., Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) at room temperature with a method modified from that of John and Shahidi (*5*). A binary mobile phase was used (solvent A, acetonitrile 5% in methanol; solvent B, formic acid 1% in water), using a gradient program as follows: 0 min, 5% A; 5 min, 15% A; 10 min, 30% A; 15 min, 32% A; 19 min, 33% A; 24 min, 50% A; 34 min, 95% A; isocratic for 10 min at 95% A. Injection volume was 15 μL; flux rate was 0.8 mL/min; detection wavelengths were set at 254 and 280 nm, and spectral data were collected from 240 to 550 nm. MS ESI analyses were performed in the negative ion detection mode with gas temperature set at 350 °C, nebulizer pressure at 70 psi, and drying gas flow of 10 L/min. MS spectra were recorded in the range of m/z 100−1000.

Quantification of phenolic compounds for which standards were available was carried out using appropiate calibration curves. Standards were dissolved in methanol at a concentration of 1 mg/mL, except ellagic acid, which was dissolved at a concentration of 0.38 mg/mL.

Antioxidant Activity. *Total Antioxidant Capacity (TAC) Determined as ABTS$^{\bullet-}$ Scavenging Activity*. TAC was determined as described by Siriwardhana and Shahidi (*16*) with slight modifications. ABTS$^{\bullet-}$ was prepared in 100 mM saline phosphate buffer (PBS, pH 7.4, 0.15 M NaCl) by mixing 100 mL of 2.5 mM AAPH (in PBS) with 100 mL of 2 mM $ABTS^{2-}$ (in PBS); this solution was protected from light, heated at 60 °C for 30 min, and then cooled to room temperature. This solution was filtered several times during the experiment through a no. 1 filter. The kernel or shell extracts were dissolved in 80% methanol (0.1 and 0.05 mg/mL, respectively), and 40 μL of samples, Trolox standards, or 80% methanol (control) was mixed with 1960 μL of ABTS$^{\bullet-}$ solution. The mixture was incubated for 6 min in the dark, and then the absorbance at 734 nm was read. Because the absorbance of the control decayed over the experimental period (≤2 h), a control was used for each sample or standard. The percent radical scavenging capacity (% RSC) was calculated using eq 1.

$$\%\ \mathrm{RSC} = 100 - \frac{(\mathrm{Abs}_{\mathrm{sample}})}{(\mathrm{Abs}_{\mathrm{control}})} \times 100 \qquad (1)$$

Trolox (50−400 μM) was used as a standard, and the results were expressed as micromoles of Trolox equivalents (TE) per gram of sample FW.

DPPH• Scavenging Activity. The DPPH• scavenging activity assay was performed using electron paramagnetic resonance (EPR) spectroscopy, according to the method of Madhujith and Shahidi (*17*) with slight modifications. EPR spectra were recorded on a food analyzer Bruker E-scan (Bruker Biospin Co., Billercia, MA), using the following parameters: 5.02 × 102 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.258 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. Samples were prepared by mixing 2 mL of 190 μM DPPH (in methanol) with 500 μL of kernel or shell extracts (0.1 and 0.05 mg/mL, respectively, in methanol). After 10 min, the samples were injected into the EPR spectrometer, and the height of the second positive peak was recorded. The % RSC was then calculated according to eq 2:

$$\%\ \mathrm{RSC} = 100 - \frac{(\text{EPR signal of the sample})}{(\text{EPR signal of the control})} \times 100 \qquad (2)$$

Trolox (31.25−250 μM in methanol) was used as a standard, and the results were reported as micromoles of TE per gram of sample FW.

Hydroxyl Radical (HO•) Scavenging Activity. The hydroxyl radical scavenging activity method was performed using EPR spectroscopy following the method of Madhujith and Shahidi (*17*) with slight modifications. EPR spectra were recorded under the same experimental conditions as those in the DPPH assay. Kernel and shell extracts were dissolved in deionized water (0.2 mg/mL) by vortexing and sonicating for approximately 10 min. Samples (100 μL) were mixed with 100 μL of 10 mM H_2O_2, 200 μL of DMPO, and 100 μL of 100 μM $FeSO_4$ (dissolved in deoxygenated water). After 1 min, EPR spectra were recorded and % RSC was calculated using eq 2. Gallic acid (0.625−10 mM) was used as a standard, and results were reported as millimoles of GAE per gram of sample FW.

Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay was performed according to the method of Liyana-Pathirana et al. (*18*), using a FLUOstar OPTIMA microplate reader (BMG Labtechnologies GmbH, Offenberg, Germany) equipped with FLUOstar OPTIMA evaluation software version 1.30−0 and black, polystyrene, nontreated 96-well microplates (Costar Corning Inc., Corning, NY). Only the internal wells of the microplate were used. Measuring solutions (in triplicate) were prepared directly in a microplate by mixing 20 μL of kernel or shell extracts dissolved in phosphate buffer [4 and 2 μg/mL, respectively, in 75 mM phosphate buffer (PB), pH 7.4] or Trolox calibration standards (0−50 μM, dissolved in PB) with 120 μL of fluoresceine (96 nM dissolved in PB) and kept at 37 °C for 20 min. Then 60 μL of AAPH (12 mM final concentration) was automatically injected into each well, and fluorescence was measured every 2 min for 120 min, with excitation and emission filters of 485/20 and 528/25, respectively. A gain adjustment was performed by pipetting 200 μL of fluorescein onto a designated well before starting the program to optimize signal amplification. Values of antioxidant capacity were calculated from the differences in the area under the fluorescence decay curves between blank and samples and reported as micromoles of TE per gram of sample FW.

Statistical Analysis. Two extractions of each sample were carried out to calculate extraction yields. The dried extracts were combined, and all other analyses were performed at least in triplicate. Differences between samples were determined by ANOVA and Tukey's post hoc test, and correlation between variables was evaluated with Pearson's (*P*) coefficient, with a significance level of $P < 0.05$. All statistical analyses were performed using SPSS (18.0) software.

RESULTS AND DISCUSSION

Phenolic Compounds, Flavonoids, and Proanthocyanidins. Total phenolic compounds, flavonoids, and proanthocyanidins were determined in pecan kernels and shells grown in three geographical areas of the state of Chihuahua, Mexico, namely, Casas Grandes (CG) in the north, Delicias (Del) in the central area, and Jimenez (Jim) in the south. Defatted matter constituted 23−25% of whole kernels independent of the geographical origin of the samples. Yields for the extraction of phenolic compounds (g of extract obtained per 100 g of deffated kernel or whole shell) in 80% acetone were 25−26% for all defatted kernels, 12% for the Del shells, 14% for the CG shells, and 16% for the Jim shells. Aqueous acetone was chosen as the extraction solvent because it has been shown to be the most effective medium for samples suspected to have high tannin content. **Table 1** shows the concentration of phenolic compounds (total acetone-extractable phenolics), flavonoids, and proanthocyanidins (condensed tannins) found in all samples, expressed as milligrams of GAE for phenolic compounds and milligrams of CE for flavonoids and proanthocyanidins. The three compound classes showed the same trends: (i) concentrations were higher in shells than in kernels (5−8 times for phenolic compounds and flavonoids, 10−20 times for proanthocyanidins); and (ii) samples with higher phenolics in kernel had lower phenolics in shell. Pecan samples grown in Del showed the highest concentrations of all polyphenol classes in kernels and the lowest in shells. In fact, the concentrations of total acetone-extractable phenolic compounds and proanthocyanidins in pecan kernels were statistically ($P < 0.05$) affected by the growing location. The phenolic constituents of the shells were even more affected by their geographical origin.

To the best of our knowledge, the concentration of total acetone-extractable phenolic compounds for pecan kernels and shells of a Wichita−Western blend has not previously been analyzed; however, our values (11.7−11.9 mg GAE/g kernel) are similar to those previously reported (*11*, *23*) for pecan kernels from unknown cultivars sold in the United States and at the European Food Markets. Total extractable phenolics in shells (65.3−92.5 mg GAE/g shell) were also similar (somewhat lower) to the values reported by Villarreal-Lozoya et al. (*12*) for other pecan cultivars grown in Texas, using a 0.6 conversion factor for changing data from chlorogenic acid equivalents to gallic acid equivalents.

The concentration of total flavonoids in pecan samples was determined by means of their complexation with Al(III). Total flavonoids in kernels were approximately 6 mg CE/g kernel, about half the concentration of total extractable phenolics; in shells, flavonoid concentrations ranged from 26.3 to 36.1 mg CE/g shell (about one-third of the total extractable phenolics), with significantly lower concentrations in shells from Del-grown samples. These values are considerably higher than total flavonoid concentrations determined for some fruits and vegetables with a similar method (*19*).

Proanthocyanidins or condensed tannins (oligomeric and polymeric forms of flavan-3-ols) are usually quantified by means of the vanillin assay, which is fairly specific for monomeric and polymeric flavan-3-ols. Using this technique we found values ranging from 20.6 to 26.7 mg CE/g kernel and from 316.1 to 464.4 mg CE/g shell, which are high (suggesting that up to 46% of the pecan shell may be made up of proanthocyanidins), but comparable to those found in previous studies (*12*, *20*). However, these results should be examined with some caution because the use of catechin as a standard in samples with high content of tannins may over- or underestimate their concentration, because the reactivity of vanillin with catechin is different from that of vanillin with proanthocyanidins (*21*). Gu et al. (*10*) assayed proanthocyanidins in several foods by means of HPLC-MS and found that pecan nuts contained an average of 4.9 mg of proanthocyanidin/g of sample, a value 4−5 times lower than those in our study reported here. Despite a possible overestimation, it seems clear that both kernels and shells of pecan nuts, but especially the latter, are good natural sources of proanthocyanidins.

Identification and Quantification of Individual Phenolic Compounds. Several phenolic compounds were identified and quantified by comparison of retention times (t_R) and UV and mass spectra to those of authentic standards. Others have been tentatively identified on the basis of their mass and UV spectra,

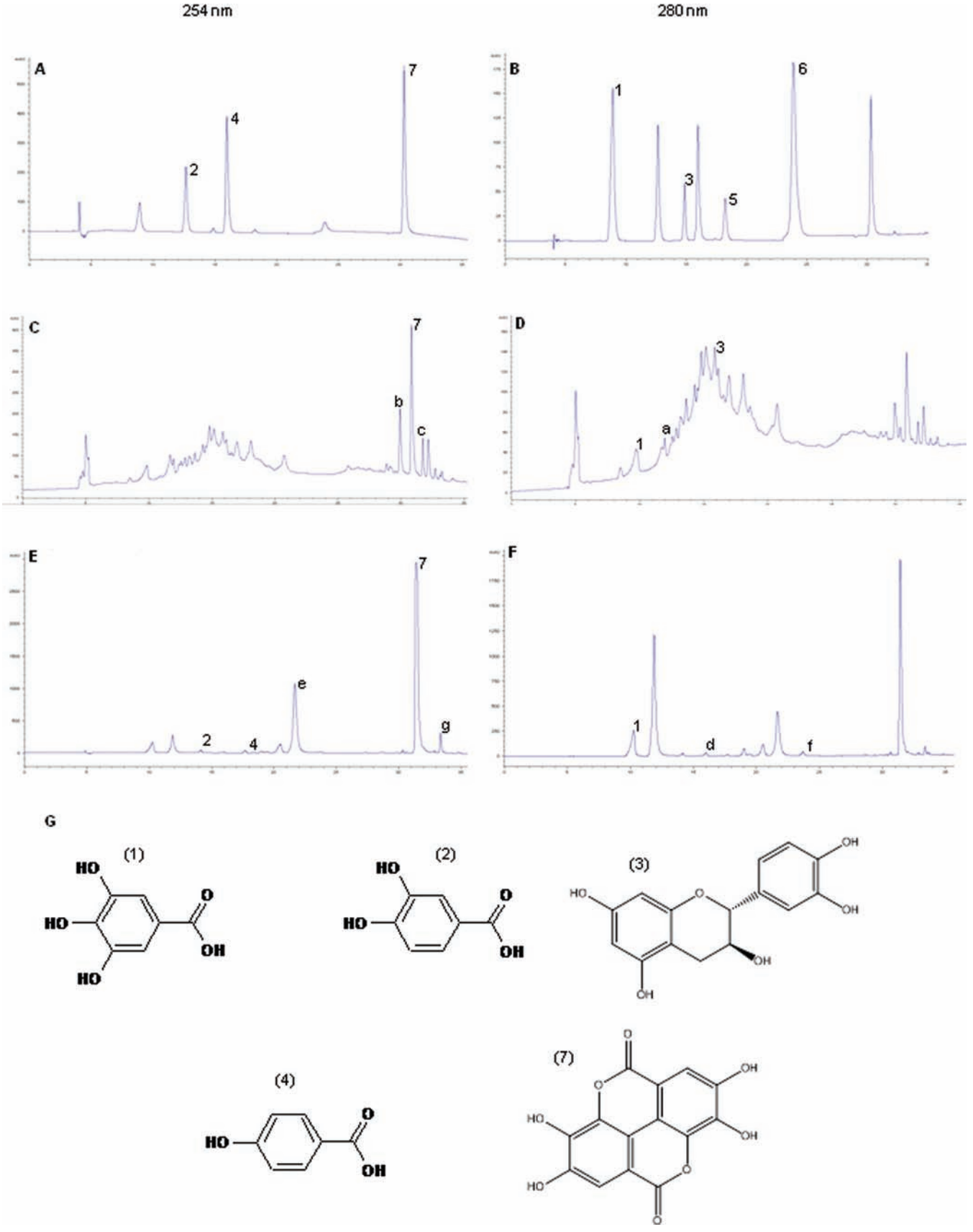

Figure 1. HPLC chromatograms with UV detection at 254 (**A**, **C**, **E**) and 280 (**B**, **D**, **F**) nm of (**A**, **B**) pure standards of (**1**) gallic acid, (**2**) protocatechuic acid, (**3**) catechin, (**4**) *p*-hydroxybenzoic acid, (**5**) epicatechin, (**6**) *p*-coumaric acid, and (**7**) ellagic acid; (**C**, **D**) crude extract (10 mg/mL in methanol) of pecan kernels grown in CG; and (**E**, **F**) acid-hydrolyzed (HCl 2 N, 95 °C, 4 h) extract of pecan kernels grown in Jim (5 mg/mL in methanol). Tentative identification of peaks marked a−g is described in the text. HPLC conditions are also described in the text, under Materials and Methods. (**G**) Chemical structures of phenolic compounds that were positively identified in pecan kernels and shells (standards **1**−**4** and **7**).

Table 2. Concentration of Individual Phenolic Compounds Found in Pecans Grown in Different Locations of the State of Chihuahua, Mexico ($n = 2$)

	ellagic acid (mg/g)		gallic acid (μg/g)		*p*-hydroxybenzoic acid (μg/g)		protocatechuic acid (μg/g)	
sample	free	total	free	total	free	total	free	total
CG	1.4 ± 0.1	5.0 ± 1.0	64.3 ± 4.2	196.9 ± 8.9	nd[a]	90.1 ± 4.2	nd	13.1 ± 9.5
Del	1.7 ± 0.0	4.6 ± 1.4	80.7 ± 3.6	274.5 ± 26.7	nd	29.0 ± 4.4	nd	30.5 ± 4.1
Jim	1.5 ± 0.0	5.5 ± 1.0	69.3 ± 4.0	189.0 ± 9.1	nd	46.7 ± 4.4	nd	19.0 ± 7.8

[a] nd, not detected.

similar to other compounds that have previously been described in fruits, seeds, and nuts. **Figure 1** shows typical HPLC chromatograms of authentic standards (**A** and **B**), a crude pecan kernel extract (**C** and **D**), and an acid-hydrolyzed kernel extract (**E** and **F**).

Ellagic (**7**) and gallic (**1**) acids were the major polyphenols found in kernel and shell extracts, and they were quantified in free and hydrolyzed kernel extracts (**Table 2**). Catechin (**3**) was identified on the basis of t_R, UV, and m/z comparison, in crude kernel extracts from the three growing locations; however, due to the close elution of many other unidentified compounds, it could not be quantified. Protocatechuic (**2**) and *p*-hydroxybenzoic (**4**) acids were identified and quantified in hydrolyzed kernel extracts. **Table 2** shows the concentrations of these phenolic acids, in pecan samples from different locations, expressed on a fresh weight basis. Phenolics quantified in crude extracts are considered to be in the free form, whereas those quantified in the hydrolyzed kernel extracts are labeled as "total", although it should be noted that these total polyphenols are actually "total acetone-soluble polyphenols" because bound phenolics were not liberated, and some authors have shown that different nut species contain considerable amounts of phenolic compounds in the bound form (*5*, *22*).

Ellagic acid (EA) concentrations in pecan kernels were 1.4−1.7 mg of free EA/g (FW) and 4.6−5.5 mg of total EA/g (FW), which are higher than those reported for walnuts, heartnuts (0.09−1.62 mg/g), strawberries (0.68−0.85 mg/g), and other berries (0.01−3.3 mg/g) (*15*, *23*), indicating that pecan nuts are good sources of ellagic acid. The presence of EA in pecan kernels was recently reported in pecans grown in Texas (*12*); its quantification was carried out after basic and acid hydrolysis, with values ranging from 0.75 to 1.41 mg/g FW (considering 70% lipid content), which are comparable to the values of free EA found in the pecan samples grown in the state of Chihuahua, Mexico. In the present study we found some ellagic acid derivatives that can be considered further evidence that pecans contain ellagitannins; moreover, we showed that EA concentration increases around 3-fold after acid hydrolysis, which liberates EA from ellagitannins (*23*) or those in other bound forms. Further isolation and characterization of pecan ellagitannins, as well as their biological activities, should be carried out.

Gallic acid (GA) is also a major component of hydrolyzable tannins. Total GA concentration in Mexican pecan kernels (189.0−274.5 μg/g FW) was in the range of GA concentration in Texas pecans from different cultivars (*12*). Similar to EA, GA concentration increased around 3-fold after acid hydrolysis. Protocatechuic (PA) and *p*-hydroxybenzoic (HBA) acids were also identified and quantified in hydrolyzed kernel extracts. Their concentrations (13.1−30.5 μg of PA and 29.0−90.1 μg of HBA/g pecan kernel) were lower than those for GA and EA, but higher than those found previously by Senter et al. (*11*). However, the order of abundance of the phenolic acids in kernels in the present study was similar to that found by Senter, that is, GA > HBA > PA (EA was not identified in the older study). GA and EA were the only major polyphenols identified in crude extracts of pecan shells; however, their quantification was not attempted.

The identification of some of the major chromatographic peaks (a−g in **Figure 1**) was attempted, based on their UV and mass spectra. Peak a had a negative molecular ion $[M - H]^-$ at m/z 333.1, a fragment at m/z 169.3, corresponding to gallic acid, and a UV spectrum similar to that of gallic acid ($\lambda_{max} = 268$) and therefore was tentatively identified as monogalloylglucose (*24*). Peak b displayed a $[M - H]^-$ at m/z 433.1 and a UV spectrum similar to that of ellagic acid ($\lambda_{max} = 256, 363$) and therefore was tentatively identified as an EA−pentose conjugate (*24*). Peak c showed a $[M - H]^-$ at m/z 447.2, a fragment at m/z 301.3, corresponding to EA, and a UV spectrum similar to that of EA; therefore, it was tentatively identified as EA−rhamnoside (*25*). Peaks a−c were found in crude kernel extracts from all growing locations.

Peak d had a $[M - H]^-$ at m/z 137.0, and its UV spectrum was similar to that of protocatechuic acid ($\lambda_{max} = 276, 312$) and that of protocatechuic aldehyde; therefore, it was tentatively identified as protocatechuic aldehyde. The mass spectrum of peak e (**Figure 2B**) showed fragments at m/z 469, 425, and 301, and the UV spectrum (**Figure 2A**) showed absorption maxima at 255 and 365 nm, similar to that of EA (**Figure 2E**). An EA derivative was described in walnuts with a similar fragmentation pattern and UV spectrum, where the fragment at m/z 469 corresponded to the negative molecular ion of valoneic acid dilactone (**Figure 2G**), the fragment at 425 corresponded to the neutral loss of a carboxyl moiety $[M - 44 - H]^-$, and the one at m/z 301 indicated the presence of an EA moiety in the molecule (*15*). Therefore, it may be concluded that compound **e** is probably the same compound as that identified in walnuts, which is probably valoneic acid dilactone. An unidentified EA derivative was earlier reported in hydrolyzed pecan kernel extracts (*12*), and other authors have found that, besides ellagic acid, two ellagic acid derivatives are detected whenever acid hydrolysis is used for determining ellagitannins (*23*). It would be interesting to analyze if valoneic acid lactone (if its identity is confirmed) is always present as an ellagitannin hydrolysis product.

Peak f had a UV spectrum (**Figure 2C**) similar to that of catechin ($\lambda_{max} = 281$ nm) and a mass spectrum (**Figure 2D**) with $[M - H]^-$ at m/z 305.1, corresponding to the negative molecular ion of (epi)gallocatechin, and a fragment at m/z 261.3, which has also been identified as a major fragment of gallocatechin and epigallocatechin in ESI-MS experiments (*26*). Therefore, peak f was tentatively identified as gallocatechin or epigallocatechin. Epigallocatechin and catechin have been identified as the major flavan-3-ols of pecan kernels (*9*). Peak g had a $[M - H]^-$ at m/z 315 and a UV spectrum similar to that of EA; therefore, it was tentatively identified as methylellagic acid. **Table 3** summarizes data of UV and m/z spectra of all tentatively identified phenolic compounds.

It should be mentioned that, whereas peaks a−e and g were identified in all kernel samples, peak f [(epi)gallocatechin] was not found in Del-grown pecans, suggesting that growing region may affect not only the phytochemical concentration but, to a certain degree, also the phytochemical profile of pecans. This is also confirmed by the fact that Del samples showed a distinctive

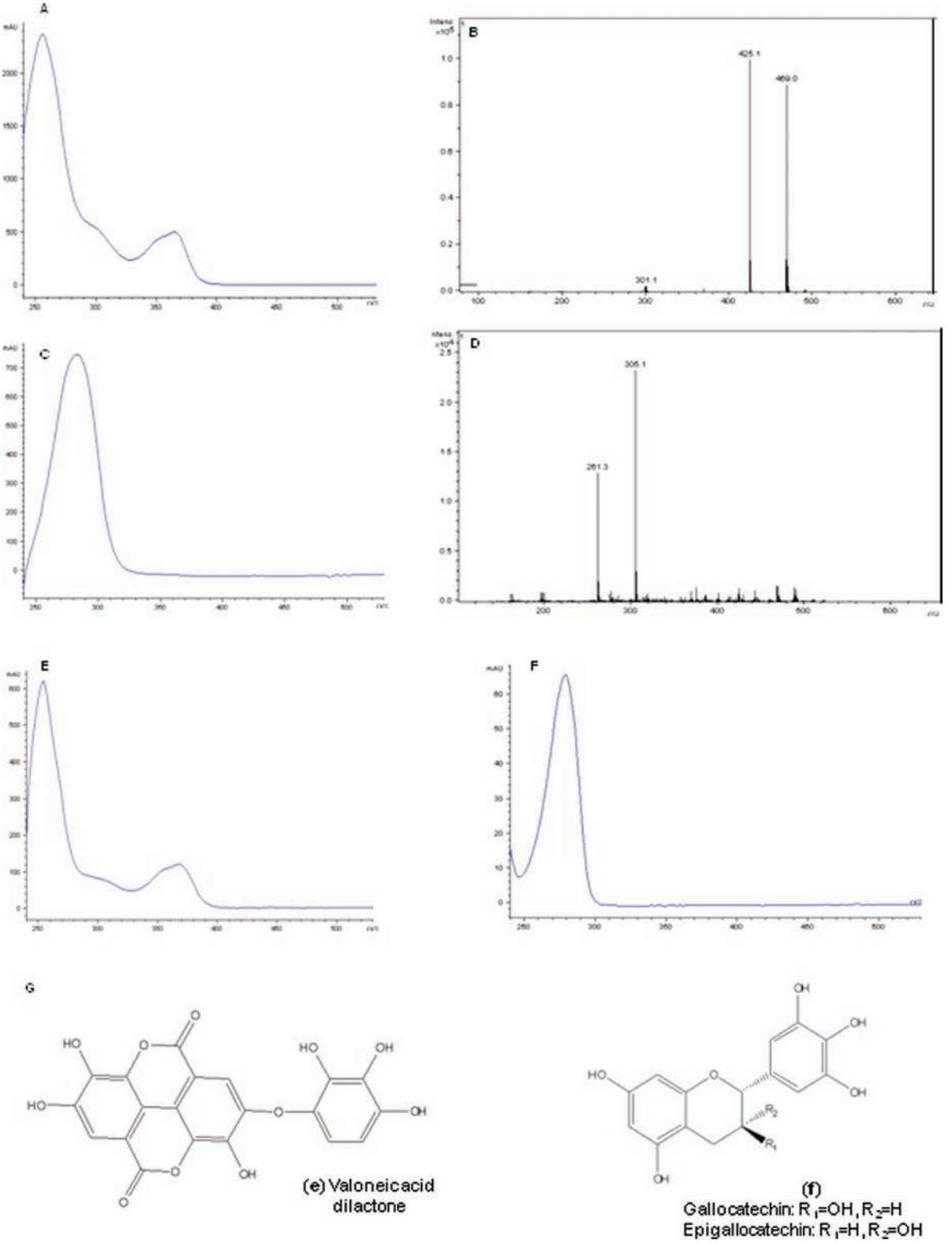

Figure 2. (**A**) UV spectrum of compound **e**. (**B**) Mass spectrum of compound **e**. (**C**) UV spectrum of compound **f**. (**D**) Mass spectrum of compound **f**. (**E**) UV spectrum of ellagic acid. (**F**) UV spectrum of catechin. (**G**) Possible chemical structures of compounds **e** and **f**.

phenolic profile with a low EA/GA ratio (21 for free EA/GA in Del samples vs 22 in CG and Jim samples; and 17 for total EA/GA in Del samples vs 25−29 in CG and Jim samples), but highest total phenolics and proanthocyanidins. Many studies have highlighted the importance of environmental conditions, agricultural practices, and postharvest treatments on the concentration of phytochemicals in different crops. Synthesis of phenolic compounds is usually stimulated by biotic and abiotic stress (*4*), and therefore their concentrations must be highly dependent on environmental factors. Schwartz et al. (*27*) found that anthocyanins and hydrolyzable tannins in pomegranate peels and arils were significantly and inversely affected by the growing region and concluded that the main environmental factors that modulated their concentrations could be temperature and sun

Table 3. Chromatographic, UV, and Mass Spectra Information Used for the Tentative Identification of Phenolic Compounds in Pecan Kernel Extracts

peak[a]	t_R (min)	$[M - H]^-$ (m/z)	fragment ions (m/z)	λ_{max} (nm)	compound
a	12.3	333.1	169.3	258.0	monogalloylglucose
b	30.1	433.1		265.0, 363.0	ellagic acid−pentose conjugate
c	32.6	447.2	301.3	265.0, 364.0	ellagic acid−rhamnoside
d	15.9	137.0		276.0, 312.0	protocatechuic aldehyde
e	21.8	469.0	425.0, 301.0	255.0, 365.0	valoneic acid dilactone
f	23.7	305.1	261.3	281.0	(epi)gallocatechin
g	33.5	315.0		256.0, 363.0	methylellagic acid

[a] Peaks a−c were identified in crude extracts. Peaks d−g were identified in acid-hydrolyzed extracts.

irradiation. Genetic factors also modulate the concentration of phenolic compounds in different crops; this has been shown for various pecan cultivars grown in the state of Texas (*12*). In our study we have found evidence for the modulation of pecan shell and kernel phenolic compounds by environmental conditions in a Wichita−Western cultivar; however, identification of the specific factors involved awaits further studies. Moreover, the effect of postharvest storage must also be considered since the samples used in the present study were stored by producers for nearly 1 year at 4 °C.

Despite the high concentration of proanthocyanidins found with the vanillin assay, our HPLC-MS method was not well suited to the detection of these compounds. Difficulty in analyzing proanthocyanidins with reverse phase HPLC methods with diode array detection has been documented, and their analysis is best achieved by normal phase HPLC separation coupled to atmospheric pressure ionization electrospray (API-ES) mass spectrometry, fluorescence detection, and/or analysis of proanthocyanidin cleavage products (*28*). Further studies should be carried out to characterize pecan proanthocyanidins.

In summary, phenolic compounds identified in pecan kernels included phenolic acids (only hydroxybenzoic acid derivatives), ellagic acid, ellagic and gallic acid derivatives, and monomeric flavan-3-ols. Only ellagic and gallic acids were identified in shells.

Antioxidant and Radical Scavenging Activity. Many methods have been used to evaluate the antioxidant activity, or capacity, of phenolic compounds and phenolic-rich extracts. Authors have classified methods according to the mechanism of radical deactivation (hydrogen or electron transfer), according to the physiological relevance of the free radical, or according to the competitive or direct approach of the reaction (*29*). Acetone extracts of pecan have been previously analyzed by the ORAC and DPPH methods (*8*, *12*). In the present study several additional methods were used to analyze the antioxidant activity of pecan kernel and shell extracts. The antioxidant activity determined with all of these methods depends ultimately on the ability of the extracts to scavenge different free radicals. To simplify the comparison between methods, and with previously published results, we attempted to use a single reference compound as a standard in all methods and express antioxidant activity in terms of molar equivalents of the reference compound. Trolox, a water-soluble analogue of vitamin E, is regularly used as a reference standard with the ORAC and TAC ($ABTS^{\bullet-}$ scavenging) methods; therefore it was also chosen as a reference to evaluate $DPPH^{\bullet}$ scavenging activity.

Scavenging of the DPPH radical is generally evaluated in organic media by monitoring the absorbance decrease at 515 nm; however, these spectrophotometric measurements can be affected by compounds that absorb at the same wavelength (*29*). These pitfalls may be avoided by the use of EPR. EPR signals of $DPPH^{\bullet}$ in the presence and absence of Trolox, pecan polyphenols, and extracts are shown in **Figure 3**. Trolox, in a concentration range of 31.5−250 μM, effectively scavenged the DPPH radical, as evidenced by a concentration-dependent decrease in the intensity of the EPR signal. The height of the second peak was used to calculate the RSC of each Trolox concentration as a percentage of the control (eq 2), and a linear calibration curve was built and used to calculate the antioxidant activity of the samples in terms of micromoles of TE per gram of nut (FW). Kernel and shell extracts were highly effective $DPPH^{\bullet}$ scavengers, the latter being almost 3 times higher than the former. At concentrations of 0.1 mg of kernel extract/mL and 0.05 mg of shell extract/mL, kernel extracts scavenged almost 40% of free radicals and shell extracts > 50% (**Figure 2C**,**E**), in 10 min. The $DPPH^{\bullet}$ scavenging activity of the main phenolic compounds present in pecan extracts was also analyzed and compared to that of Trolox (**Figures 2D**,**F**). Both catechin and EA, found in kernel extracts, showed higher scavenging activity than an equimolar Trolox concentration; gallic acid, also abundant in kernel and shell extracts, had an activity similar to that of EA (data not shown). Therefore, it is reasonable to assume that the high $DPPH^{\bullet}$ scavenging activity of pecan kernel and shell extracts is due to its main phenolic constituents; nevertheless, the extracts contain these compounds in their free forms (as monomers) and as polymerized forms, with yet unknown structures. The RSC of the polymeric forms is probably different from that of the monomers. Several authors have found that antioxidant activity, as determined by different methods, is higher in high molecular weight fractions of phenolic extracts, as compared to low molecular weight fractions of the same extracts (*30*), indicating that polymeric polyphenols are better at scavenging various free radicals.

Table 4 shows the antioxidant activity of kernel and shell extracts cultivated in different areas, evaluated as their ability to scavenge different free radicals and expressed as micro- or millimoles equivalents of a reference compound per gram of nut on a fresh weight basis. Antioxidant activity was consistently higher in shells than in kernels, in agreement with many authors who have found that nut byproducts possess higher antioxidant activity than nut kernels (*5*, *12*) and with higher levels of phenolic compounds in shells. Values of antioxidant activity in kernels, determined by the ORAC (227.0−261.5 μmol TE/g FW) and DPPH (102.6−108.7 μmol TE/g FW) methods, are similar to those reported by Wu et al. (*8*) and Villarreal-Lozoya et al. (*12*), although ORAC values are slightly higher; in contrast, the antioxidant activity of shells (538−720 μmol TE/g FW) was lower than that reported by Villarreal-Lozoya et al. (*12*) using the DPPH method (1900 μmol TE/g FW). All of these data consistently show that pecan nuts and their byproduct (shells) are rich sources of phytochemicals with high antioxidant capacity, and the same is confirmed using $ABTS^{\bullet-}$ and $HO^{\bullet}$ scavenging methods of determination of antioxidant activity.

The determination of the $ABTS^{\bullet-}$ scavenging activity of a sample is generally referred to, in the scientific literature, as TEAC (Trolox equivalent antioxidant capacity) or TAC (total antioxidant capacity) assay. When evaluated by this method, the antioxidant activity of pecan kernels was between 75.9 and 83.4 μmol TE/g FW. These values are lower than those reported

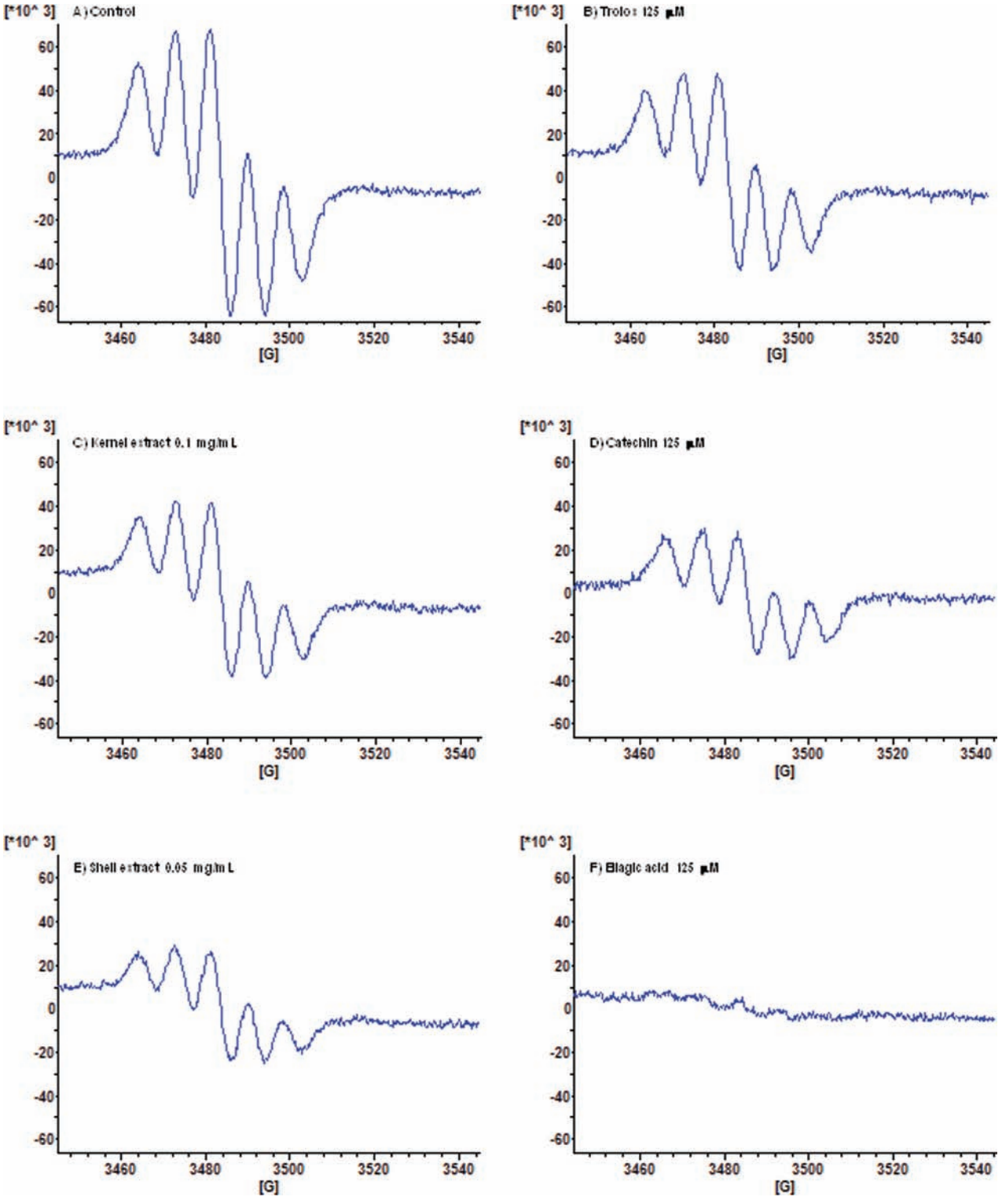

Figure 3. EPR spectra of DPPH$^{\bullet}$ recorded on a food analyzer Bruker E-scan after a 10 min incubation in the presence or absence of phenolic compounds or pecan extracts: (**A**) DPPH$^{\bullet}$ 190 μM in methanol; (**B**) DPPH$^{\bullet}$ + Trolox 125 μM; (**C**) DPPH$^{\bullet}$ + kernel extract (0.1 mg/mL); (**D**) DPPH$^{\bullet}$ + catechin 125 μM; (**E**) DPPH$^{\bullet}$ + shell extract (0.05 mg/mL); (**F**) DPPH$^{\bullet}$ + ellagic acid 125 μM.

for walnuts (120 μmol TE/g FW) but higher than those reported for other nut species such as pine nut (2.1 μmol TE/g FW), pistachio (15−37 μmol TE/g FW), and Brazil nut (11.2 μmol TE/g FW, considering 69% lipid content), among others (*5*, *22*). In fact, pecan kernel ABTS$^{\bullet-}$ scavenging activity found in the present study was higher than those of most other food products analyzed by Pellegrini et al. (*22*). Antioxidant activity in shells was 6−8 times higher than that in kernels, and its values were comparable to those obtained in peanuts and hazelnut byproducts (roasted skins), close to 4 mmol TE/g extract (*30*).

The HO$^{\bullet}$ scavenging activity of pecan extracts was assayed with an EPR apparatus. Hydroxyl radical was generated via Fe(II)-catalyzed Fenton reaction, spin-trapped with DMPO, and the resultant DMPO−OH adduct was detected, giving a characteristic 1:2:2:1 quartet. Trolox could not be used as a reference standard because its highest concentration soluble in distilled

Table 4. Radical Scavenging Activity of Pecans Grown in the State of Chihuahua, Mexico[a]

	ORAC (ROO• scavenging) (μmol TE/g FW)		DPPH• scavenging (μmol TE/g FW)		ABTS•⁻ scavenging (μmol TE/g FW)		HO• scavenging (mmol GAE/g FW)	
growing area	kernel	shell	kernel	shell	kernel	shell	kernel	shell
CG	231.2 ± 15.0 a	859.5 ± 180.8 b	104.4 ± 8.3 a	655.1 ± 49.9 a	83.4 ± 1.2 a	594.5 ± 83.6 a	12.8 ± 1.6 a	37.0 ± 3.1 ab
Del	261.5 ± 37.6 a	680.3 ± 66.8 b	108.7 ± 9.0 a	537.8 ± 33.8 b	81.8 ± 3.0 a	518.4 ± 80.7 a	11.9 ± 0.5 a	30.2 ± 2.2 b
Jim	227.0 ± 50.1 a	1350.3 ± 85.9 a	102.6 ± 9.3 a	720.3 ± 50.2 a	75.9 ± 11.8 a	644.2 ± 62.2 a	13.0 ± 1.9 a	41.7 ± 5.8 a

[a] Different letters in the same column indicate significant difference between samples ($n = 4$, Tukey $P < 0.05$).

water (125 μM) did not decrease the DMPO−OH signal; in fact, it increased it slightly, indicating a prooxidant effect. Gallic acid, which is highly water-soluble, used at a concentration range of 0.625−10 mM, induced a concentration-dependent decrease of the EPR signal. The percentage RSC (eq 2) linearly correlated with the GA concentration, and this compound was used as a reference HO• scavenger; the antioxidant activity of samples was calculated as millimoles GAE/g nut. Nobuchi and Uchikura (*31*) found gallic acid to be a better HO• scavenger than Trolox when assayed with a chemiluminiscence-based method. Pecan samples also showed good HO• scavenging properties, and their antioxidant activity expressed in terms of GAE was 11.9−13.0 mmol GAE/g kernel and 30.2−41.7 mmol GAE/g shell. Shell extracts showed a higher antioxidant activity than kernel extracts, but the difference was not as high as that found with the other methods. The radical scavenging activity of pecan kernels and shells was higher than that reported for barley, using a similar assay, by Madhujith and Shahidi (*17*), who found EC_{50} values of 0.5−3.75 mg extract/mL, higher than those that may be estimated for pecan shells (0.17 mg extract/mL) and kernels (0.22−0.23 mg extract/mL) in the present study.

The growing location influenced antioxidant activity of pecan kernels and shells, similar to that of affected phenolic compounds; that is, the kernel samples grown in Del (central region of the state of Chihuahua) showed higher antioxidant activity, whereas Del shells showed generally the lowest values. However, the differences were not statistically significant for any of the kernel samples and were significant for shells when assayed by all methods except ABTS•⁻ scavenging (**Table 4**). This suggests that, despite differences in polyphenolic composition, pecan kernels remain high in antioxidant capacity, and probably the same will be true for other biological actions; however, a clear tendency was observed in which antioxidant activity, evaluated with the four methods employed here, was directly correlated with the concentrations of all classes of phenolic compounds that were quantified (**Table 5**), except with *p*-hydroxybenxoic and protocatechuic acids (data not shown). In kernels, antioxidant activity correlated best with total gallic acid, when evaluated with the ORAC and DPPH methods, and negative correlations (although not significant) were found with total ellagic acid. However, when the HO• scavenging activity was analyzed, negative correlations were found with all individual phenolics except total ellagic acid. Correlations between methods of evaluating antioxidant activity were very strong and highly significant. Each of the methods tested in the present study evaluated the extracts' scavenging capacity against a different free radical, all methods were based on different reaction mechanisms between the free radical and the antioxidants (*29*), and the total antioxidant capacity was less affected by environmental conditions than the concentrations of phenolic compounds. In consequence, it may be concluded that antioxidant activity of pecan nuts is due to their content of phenolic compounds with powerful radical-scavenging properties and that the combination of several types of phenolic compounds allows for a strong antioxidant activity against many types of free radicals through a combination of mechanisms of action.

Table 5. Correlation (Pearson Coefficient) between Antioxidant Activity and Concentration of Phenolic Compounds and between Different Methods of Antioxidant Activity in Pecans

variables		*P* coefficient[a]
ORAC and	total extractable phenolics	0.945**
	flavonoids	0.948**
	proanthocyanidins	0.956**
	free ellagic acid (kernels)	0.906
	total ellagic acid (kernels)	−0.886
	free gallic acid (kernels)	0.923
	total gallic acid (kernels)	1.000*
	DPPH•	0.944**
	ABTS•⁻	0.930**
	HO•	0.966**
DPPH• scavenging and	total extractable phenolics	0.999**
	flavonoids	1.000**
	proanthocyanidins	0.999**
	free ellagic acid (kernels)	0.882
	total ellagic acid (kernels)	−0.908
	free gallic acid (kernels)	0.902
	total gallic acid (kernels)	0.997*
	ABTS•⁻	0.999**
	HO•	0.995**
ABTS•⁻ scavenging and	total extractable phenolics	0.995**
	flavonoids	0.997**
	proanthocyanidins	0.996**
	free ellagic acid (kernels)	0.058
	total ellagic acid (kernels)	−0.830
	free gallic acid (kernels)	0.101
	total gallic acid (kernels)	0.459
	HO•	0.990**
HO• scavenging and	total extractable phenolics	0.997**
	flavonoids	0.997**
	proanthocyanidins	0.998**
	free ellagic acid (kernels)	−0.875
	total ellagic acid (kernels)	0.915
	free gallic acid (kernels)	−0.875
	total gallic acid (kernels)	−0.996*

[a] *, significant at $P < 0.05$; **, significant at $P < 0.01$.

The antioxidant activity of shells was far more affected by environmental conditions than that of kernels, which is not unexpected because outer shells are much more exposed to severe environmental conditions (for example, light exposure) that influence polyphenol synthesis, degradation, or polymerization in plant tissues (*4*, *27*). Polyphenol contents and antioxidant activity of pecan shells are also significantly affected by cultivar (*12*). It is worth mentioning that, in the present study, the main reason for the difference among values of antioxidant activity in the pecan shells was the different extraction yields. If the data were analyzed in terms of antioxidant activity per gram of extract, no significant differences were apparent. We may conclude that the concentration of total acetone-extractable phenolics in pecan shells of a Wichita−Western cultivar was the main variable affected by environmental growing and/or postharvest conditions

and that the amount of total extractable phenolics, composed mainly of condensed and, to a lesser extent, hydrolyzable tannins, determines the antioxidant activity of pecan shells. It would be interesting to analyze if some bound forms of phenolic compounds are also present in pecan shells, because these may be the source of an even higher antioxidant potential of this byproduct, as they are in cereals (*32*), nuts, and several other plant foods (*22*).

It may be concluded that pecans are rich in phenolic compounds, mainly condensed and hydrolyzable tannins, but also monomeric flavonoids and hydroxybenzoic acids, all of which confer pecan nuts with a high radical-scavenging activity against several physiological and nonphysiological free radicals. The concentration of phenolic compounds and antioxidant activity were several-fold higher in shells than in kernels, and they were influenced by the region in which nuts, of the same cultivar, were grown.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate); GAE, gallic acid equivalents; CatE, catechin equivalents; TE, Trolox equivalents; FW, fresh weight; TAC, total antioxidant capacity; ORAC, oxygen radical absorbance capacity; RSC, radical scavenging capacity; EPR, electron paramagnetic resonance; PB, phosphate buffer; CG, Casas Grandes; Del, Delicias; Jim, Jimenez; EA, ellagic acid; GA, gallic acid; PA, protocatechuic acid; HBA, *p*-hydroxybenzoic acid.

ACKNOWLEDGMENT

We are grateful to Daniel Marquez from UACJ and Francisco Ortega from "Industria Pecanera SPR de RL" for providing the pecan samples. E.A. thanks Conacyt, Mexico, for granting him a sabbatical leave.

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Received for review September 3, 2010. Revised manuscript received November 18, 2010. Accepted November 18, 2010.