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Antioxidant Constituents of Almond [*Prunus dulcis* (Mill.) D.A. Webb] Hulls

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Almond hulls (Nonpareil variety) were extracted with methanol and analyzed by reversed phase HPLC with diode array detection. The extract contained 5-*O*-caffeoylquinic acid (chlorogenic acid), 4-*O*-caffeoylquinic acid (cryptochlorogenic acid), and 3-*O*-caffeoylquinic acid (neochlorogenic acid) in the ratio 79.5:14.8:5.7. The chlorogenic acid concentration of almond hulls was 42.52 ± 4.50 mg/100 g of fresh weight ($n = 4$; moisture content = 11.39%). Extracts were tested for their ability to inhibit the oxidation of methyl linoleate at 40 °C. At an equivalent concentration (10 μg/1 g of methyl linoleate) almond hull extracts had higher antioxidant activity than α-tocopherol. At higher concentrations (50 μg/1 g of methyl linoleate) almond hull extracts showed increased antioxidant activity that was similar to chlorogenic acid and morin [2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one] standards (at the same concentrations). These data indicate that almond hulls are a potential source of these dietary antioxidants. The sterols (3β,22*E*)-stigmasta-5,22-dien-3-ol (stigmasterol) and (3β)-stigmast-5-en-3-ol (β-sitosterol) (18.9 mg and 16.0 mg/100 g of almond hull, respectively) were identified by GC-MS of the silylated almond hull extract.

KEYWORDS: Almond hulls; antioxidant; HPLC

INTRODUCTION

Autoxidation of polyunsaturated fatty acids not only lowers the nutritional value of food (*1*) but is also associated with membrane damage, aging, heart disease, and cancer in living organisms (*2*). The addition of antioxidants has become popular as a means of increasing the shelf life of food products and to reduce wastage and nutritional losses by inhibiting and delaying oxidation (*3*). Synthetic antioxidants such as 2+3-*tert*-butyl-4-methoxyphenol (BHA) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) are widely used in the food industry. However, there are serious concerns about carcinogenic potential of these substances (*4*−*7*). Therefore, there is great interest in replacing them with natural antioxidants. Natural antioxidants are primarily plant polyphenolic compounds that may be obtained from plant parts such as oilseeds, grains, beans, vegetables, fruits, leaf waxes, bark, and roots. Plant phenolics are multifunctional and can act as reducing agents (free radical terminators), metal chelators, and singlet oxygen quenchers. Epidemiological studies have shown that consumption of foods and beverages rich in phenolic content is correlated with reduced incidences of heart disease (*8*). Other studies have shown that phenolic compounds contained in fruits are potent oxidation inhibitors of low-density lipoprotein (LDL) both in vitro (*9*−*14*) and in vivo (*15, 16*). The most common plant phenolic antioxidants include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols, and polyfunctional organic acids (*17*).

The antioxidant activity of plant hulls from buckwheat (*18*), navy bean (*19*), mung bean (*20*), peanut (*21*), rice (*22, 23*), and sunflower (*24*) have been studied. The almond [Prunus dulcis (Mill.) D.A. Webb] is one of the most versatile nuts in the world, and California is the only place in North America where almonds are grown commercially. In the past, almond hulls, a byproduct of the almond industry, were removed from the almonds after harvesting and used as supplemental livestock feed. Almond hulls have been shown to be a rich source of three triterpenoids (about 1% of the hulls), betulinic acid, oleanolic acid, and ursolic acid, which have reported antiinflammatory, anti-HIV, and anticancer activities (*25*). Sang and co-workers (*26*) identified 3-prenyl-4-O-β-glucopyranosyloxy-4-hydroxylbenzoic acid, catechin, protocatechuic acid, and ursolic acid in almond hulls. These constituents have beneficial biological activities. Recently, there is interest in using almond hulls as a natural source for sweetener concentrate and dietary fiber. The aim of this study was to identify the phenolic consituents in almond hulls and evaluate their potential antioxidant activity.

EXPERIMENTAL PROCEDURE

Materials. The almond hulls (variety: Nonpareil) were supplied by the Northern Merced Hulling Association (Ballico, CA). The dried hulls were separated from the shells and ground in a Wiley mill to pass a 6.4 mm screen.

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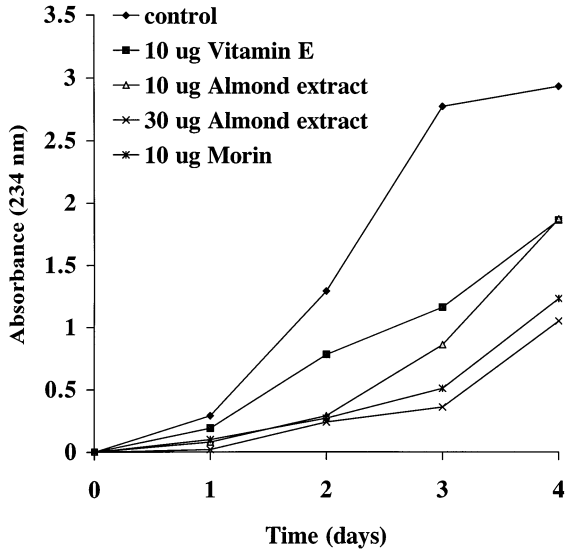

Figure 1. Antioxidant activity of Nonpareil almond hull extract (methanol), vitamin E, and morin.

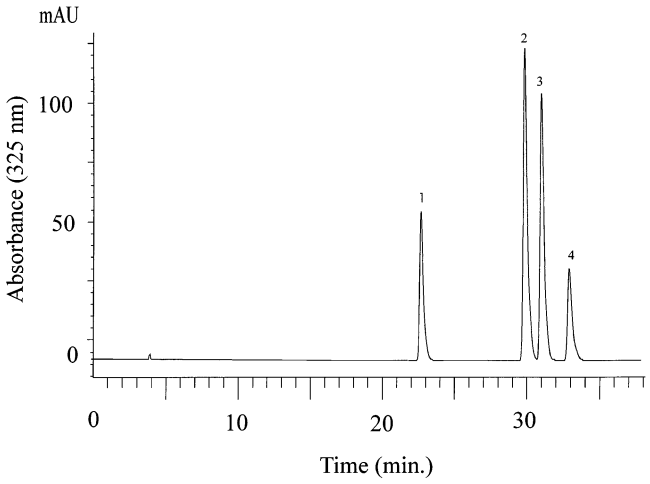

Figure 2. HPLC chromatogram of chlorogenic acid and its isomers. Peaks: (1) 3-*O*-caffeoylquinic acid; (2) 5-*O*-caffeoylquinic acid; (3) 4-*O*-caffeoylquinic acid; (4) caffeic acid.

Chemicals. Methyl linoleate (MeLo) was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Morin, stigmasterol, 5α-cholestane, and *β*-sitosterol were supplied by Sigma Co. (St. Louis, MO). Chlorogenic acid and α-tocopherol (vitamin E, 97%) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Solvents were of HPLC spectroquality grade unless otherwise stated.

Extraction. The ground almond hulls were sequentially extracted with freshly distilled diethyl ether containing ca. 0.001% Ethyl antioxidant 330 [(1,3,5-trimethyl-2,4,6-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)benzene] followed by methanol in a Soxhlet extractor under subdued light. The methanol extract was evaporated to dryness using a rotary evaporator and stored in the dark at 4 °C until used.

Thin-Layer chromatography (TLC). Preparative thin-layer chromatography was performed on 0.25 mm thick, 20 × 20 cm silica gel precoated plates (Alltech Associates, Deerfield, IL). A 50 μL volume of methanol extract was applied as a single band along with chlorogenic acid standard. The plate was developed with ethyl acetate−formic acid−acetic acid−water (100:11:11:27, v/v) until the solvent front reached 13 cm. The plate was then air-dried and sprayed with natural product reagent (1% methanolic diphenylboric acid ethylamino ester) followed by 5% ethanolic poly(ethylene glycol) 4000 to visualize the spots under UV light (*27*).

High-Performance Liquid Chromatography (HPLC). The HPLC system was used as previously described (*28*). The extract was dissolved

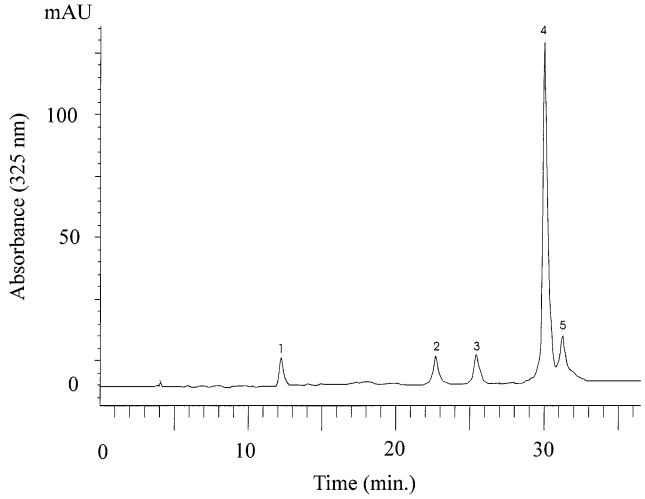

Figure 3. HPLC chromatogram of Nonpareil almond hull extract (methanol). Peaks: (1) unknown; (2) 3-*O*-caffeoylquinic acid; (3) unknown; (4) 5-*O*-caffeoylquinic acid; (5) 4-*O*-caffeoylquinic acid.

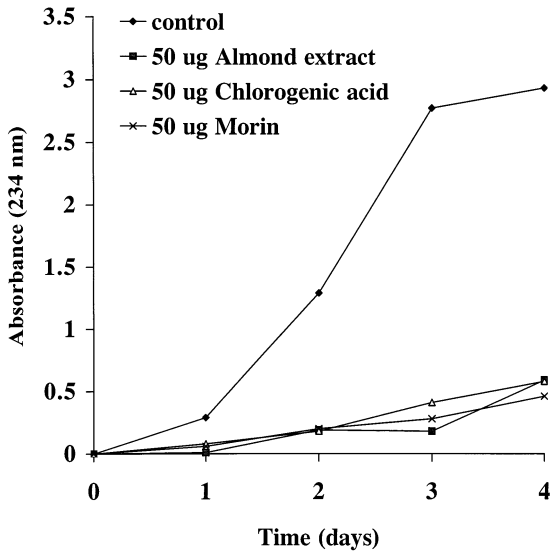

Figure 4. Antioxidant activity of almond hull methanol extract, chlorogenic acid, and morin.

in water and passed through a C_{18} solid-phase extraction cartridge (Accubond, J&W Scientific, Inc., Folsom, CA), prewetted with methanol and water. The cartridge was washed three times with 2 mL aliquots of water and then eluted with 2 mL of methanol and stored in the dark at 4 °C until used. The methanol eluent (0.5 mL) was diluted with an equal volume of 0.07 M KH_2PO_4 solution (adjusted to pH = 2.5 with H_3PO_4) before HPLC injection. The remaining methanol eluent was saved for other studies. The solvent system used was a linear gradient of 2% to 40% methanol in 0.07 M KH_2PO_4 (pH = 2.5) over 40 min with a flow rate of 1 mL/min. The sample loop volume was 20 μL, and the diode array detector was monitored at 325 nm.

Derivatization. 5α-Cholestane ($C_{27}H_{48}$) was employed as the internal standard. An aliquot (50 μL of a 1 mg/mL solution of 5α-cholestane in pentane) was added to a 1.0 mL Wheaton V-vial equipped with an open top black phenolic screw cap with Teflon-faced silicone liner (Wheaton Science Products, Millville, NJ). The pentane was removed under a stream of nitrogen. About 20 mg of almond hull extract was added to the vial along with 200 μL of Sylon BFT (BSTFA + TMCS, 99:1; Supelco, Bellefonte, PA). Derivatized standards of chlorogenic acid, stigmasterol, and *β*-sitosterol were prepared by adding a few milligrams of each to V-vials along with 100 μL of Sylon BFT. The samples were sonicated for 10 min in an ultrasonic bath and then heated at 65 °C for 1 h.

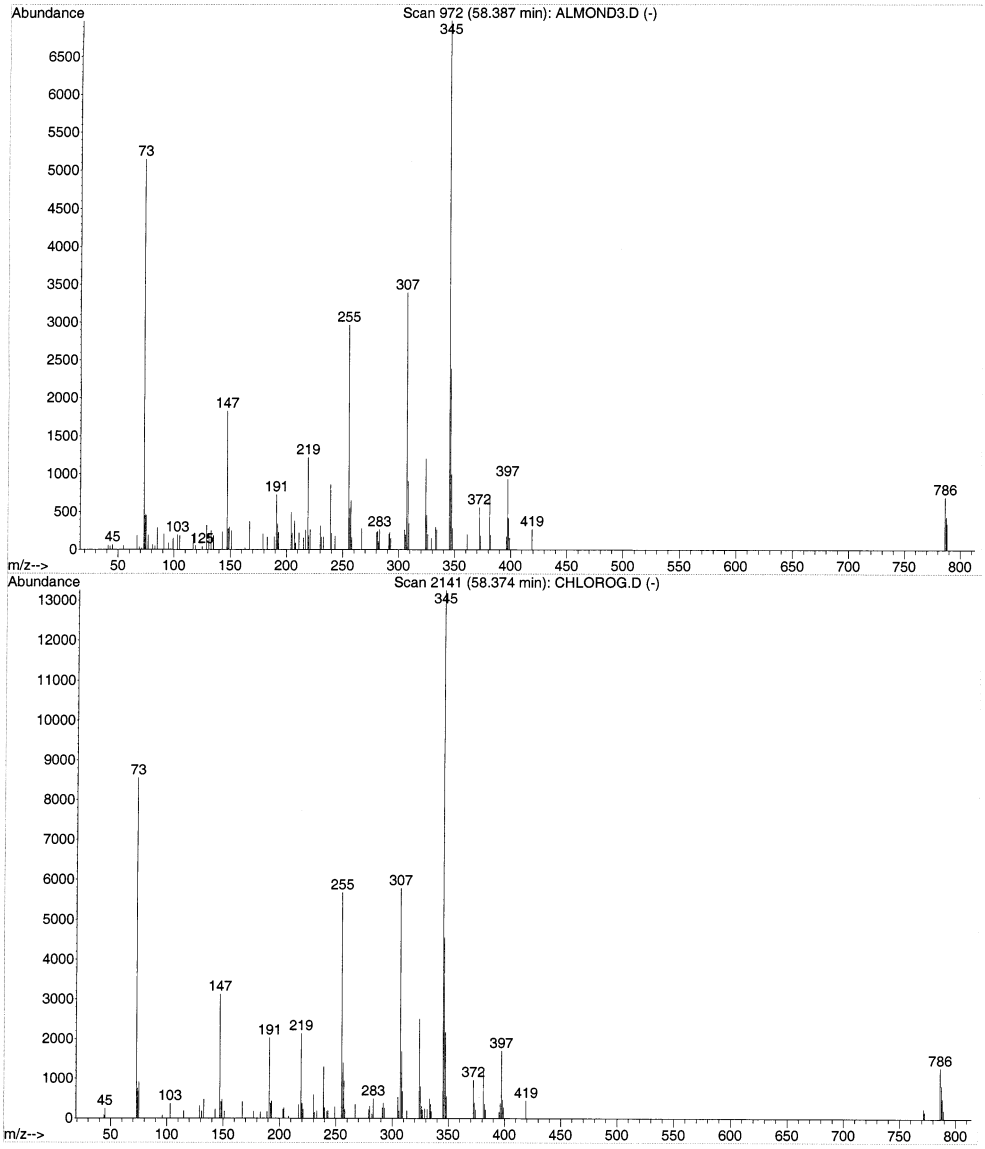

Figure 5. Mass spectra of the TMS derivatives of unknown from silylated almond hull extract (top) and chlorogenic acid standard (bottom).

Gas Chromatography (GC). Silylated almond hull extracts were analyzed with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (FID). A 30 × 0.25 mm i.d. DB-1 HT ($d_f = 0.1$ μm) fused silica capillary column (J&W Scientific, Folsom, CA) was employed. The oven temperature was held at 150 °C for 4 min and then programmed at 2 °C/min to 280 °C (final hold 20 min). The helium carrier gas linear velocity was 31.6 cm/s (150 °C). The injector and detector temperatures were 250 and 300 °C, respectively. The split ratio of the injector was 1:20.

Gas Chromatography−Mass Spectrometry (GC-MS). A Hewlett-Packard 5973 mass selective detector (MSD) was directly coupled to a Hewlett-Packard 6890 gas chromatograph. The column and oven temperature programming conditions were the same as described in the previous section.

Determination of Chlorogenic Acid Content. Chlorogenic acid content in almond hulls was determined by HPLC. Methanol extract (0.5 g) was dissolved in 5 mL of water with stirring. The sample was prepared in a similar manner as described in the HPLC section above. The diluted extract (1 mL) was passed through a C_{18} SPE cartridge prewetted with methanol and water. The cartridge was washed three times with 2 mL aliquots of water, dried under a stream of nitrogen, and eluted with 1 mL of methanol. The methanol eluent was treated and analyzed as described in the HPLC section. Chlorogenic acid content was quantified on the basis of an external standard curve of chlorogenic acid standard.

Measurement of Antioxidant Activity. The antioxidant activity experiments were performed according to a published procedure (*29*) with some modification. Briefly, the methanolic extracts were added to 1 g of methyl linoleate (MeLo), and methanol was evaporated under a stream of nitrogen. Oxidation of MeLo was carried out in the dark at 40 °C. Sample aliquots (10 mg) were taken at regular intervals (24 h) and dissolved in 5 mL of 2,2,4-trimethylpentane (isooctane) for spectrophotometric measurements (Hewlett-Packard 8453 UV−vis spectrophotometer) of conjugated diene absorption at 234 nm. Isooctane was used as the blank. The antioxidant activity was determined by the UV absorption maximum at 234 nm (conjugated dienes). All analyses were carried out in duplicate.

Chlorogenic Acid Isomers. The chlorogenic acid isomers (1-, 3-, and 4-*O*-caffeoylquinic acids) were synthesized from chlorogenic acid (5-*O*-caffeoylquinic acid) according to the procedure reported by Nagels et al. (*30*). Chlorogenic acid was dissolved in a saturated solution of $NaHCO_3$, and the temperature was raised to 90 °C. Heating was stopped after 30 min, and the pH was adjusted to 3 with H_2SO_4. The obtained mixture of isomeric caffeoylquinic acids was extracted with ethyl acetate. The ethyl acetate phase was concentrated to dryness, and the residue was dissolved in a mixture of methanol and 0.07 M KH_2PO_4 (50:50 v/v) with the solution filtered through a 0.45 mm disposable filter membrane before HPLC analysis. The 3- and 4-isomers were obtained in this way. The 1-isomer was present in only negligible amounts (*30*).

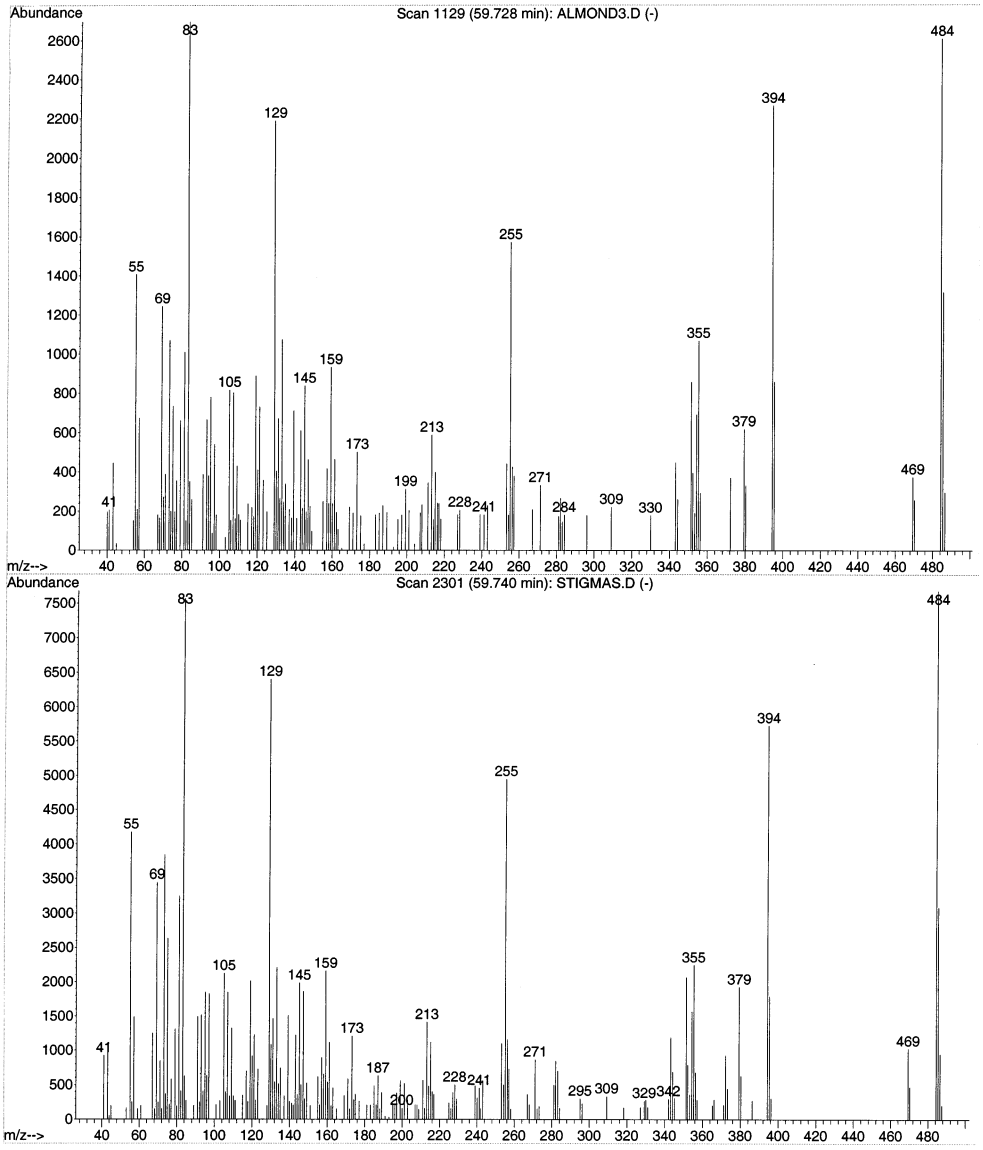

Figure 6. Mass spectra of the TMS derivatives of unknown from silylated almond hull extract (top) and stigmasterol standard (bottom).

RESULTS AND DISCUSSION

The methanol extract comprised 45.03 ± 0.80% (n = 3) of the fresh weight of the Nonpareil almond hulls (moisture content = 11.39%). Antioxidant activity of the methanol extract is shown in **Figure 1**. The methanol extract slowed the formation of conjugated dienes (234 nm) during the oxidation of methyl linoleate. Oxidation was strongly inhibited for the first 3 days with 10 μg of almond hull extract compared with 10 μg of Vitamin E. However, after 4 days, the antioxidant activity of 10 μg of almond hull extract was similar to that of vitamin E. Antioxidant activity increased as the level increased to 30 μg (**Figure 1**). This activity was even higher than 10 μg of morin. Morin was reported as a flavonoid having greater antioxidant activity than catechin, quercetin, rutin, luteolin, and kaempferol (*31*). Additional study showed that vitamin E was a prooxidant when its concentration was higher than 10 μg/1 g of MeLo (data not shown).

Thin-layer chromatography of almond hull extract (methanol) revealed a strong yellow fluorescent band under UV light with an R_f = 0.57 (same R_f value as chlorogenic acid standard) along with several weak fluorescent bands. This result suggested that the major phenolic compound present in almond hulls was chlorogenic acid. High-performance liquid chromatography (HPLC) of chlorogenic acid isomers and caffeic acid is shown in **Figure 2**. Caffeic acid and the three chlorogenic acid isomers gave four well-separated peaks. The 3-*O*-caffeoylquinic acid eluted first at 22.7 min (isomer with the caffeoyl moiety axial (*30*), followed by 5-*O*-caffeoylquinic acid (chlorogenic acid) at 30 min (peak 2) and then 4-*O*-caffeoylquinic acid (isomer with the caffeoyl moiety equatorial) with a retention time of 31.2 min (peak 3), and the last eluted peak at 33.1 min (peak 4) is caffeic acid (hydrolysis product of chlorogenic acid). The HPLC chromatogram of methanolic almond hull extract showed five major compounds (**Figure 3**). Cochromatography of almond hull extract and chlorogenic acid isomers also showed 5 separated peaks in which the UV absorbance intensities of peaks 2, 4, and 5 were increased. Their on-line UV−vis spectra and elution times were the same as those of chlorogenic acid and its isomers. The presence of chlorogenic acid was additionally confirmed by GC-MS of silylated almond hull extract. Almond hulls contained 5-*O*-caffeoylquinic acid (chlorogenic acid), 4-*O*-caffeoylquinic acid (cryptochlorogenic acid), and 3-*O*-caffeoylquinic acid (neochlorogenic acid) in the ratio 79.5:14.8:5.7. The total percentage of chlorogenic acid and

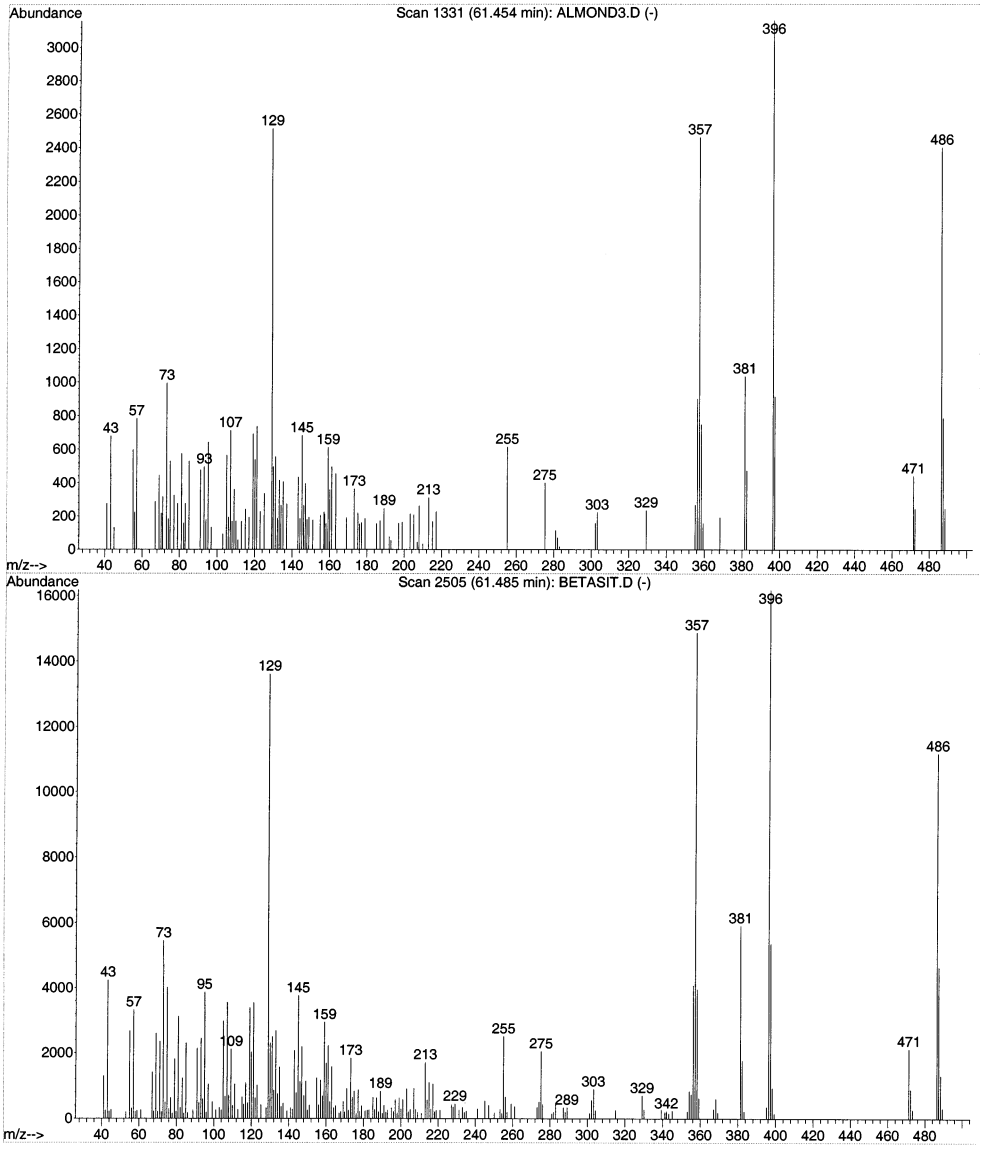

Figure 7. Mass spectra of the TMS derivatives of unknown from silylated almond hull extract (top) and β-sitosterol standard (bottom).

its isomers detected (at 325 nm) in almond hulls was 88% compared to 80% in sweet potatoes (*32*). Peaks 1 and 3 which comprised the remaining 12% have not yet been identified. These percentage values should be considered as only approximate since the extinction coefficients of peaks 1 and 3 are unknown and may be very different from those of the chlorogenic acid isomers. The chlorogenic acid content of almond hulls was 42.52 $\pm$ 4.50 mg/100 g of fresh weight (n = 4). The concentrations of the other isomers were determined using the chlorogenic acid standard curve. The approximate levels of 4-*O*-caffeoylquinic acid and 3-*O*-caffeoylquinic acid were 7.90 mg and 3.04 mg/100 g of fresh weight almond hulls, respectively.

Since chlorogenic acid and its isomers are the major phenolic compounds in almond hulls, we decided to compare the antioxidant activity of almond hull extract with chlorogenic acid standard. Chlorogenic acid was found to be the most abundant phenolic acid in various plant extracts and also the most active antioxidant constituent (*13*, *17*, *32*−*36*). It has been shown that the antioxidant activities of 3-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid are almost the same as chlorogenic acid when assayed for scavenging activity on superoxide anion radicals and inhibitory effect against oxidation of methyl linoleate (*37*). The antioxidant activities of almond hull extract, chlorogenic acid, and morin are shown in **Figure 4**. At 50 μg/1 g of MeLo, morin, chlorogenic acid, and almond hull extract all showed similar strong antioxidant activity after 4 days of MeLo oxidation. Again, this illustrated that the antioxidant activity of phenolic compounds in almond hulls increased with increasing concentration (in comparison to the 10 and 30 μg levels shown in **Figure 1**).

Almond hull extract was silylated with BSTFA-TMCS (99:1) to form trimethylsilyl (TMS) ether derivatives. The derivatized extract was analyzed by GC and GC-MS. The majority of the sample consisted of carbohydrates. Three peaks were identified as chlorogenic acid (I^{DB-1} = 3186), stigmasterol (I^{DB-1} = 3235), and β-sitosterol (I^{DB-1} = 3295) by comparing retention indices and mass spectra (**Figures 5**−**7**) to those of authentic standards. Semiquantitiative analysis was performed using 5α-cholestane as the internal standard. The concentrations of chlorogenic acid, stigmasterol, and β-sitosterol were 27.8, 18.9, and 16.0 mg/100 g of almond hull (fresh weight), respectively (n = 2). These values should be considered approximate since response factors were not determined. The chlorogenic acid concentration determined by HPLC is probably more accurate. Stigmasterol and β-sitosterol, common

plant constituents (*38*), were identified for the first time in almond hulls. The latter compound was recently found in almond nuts (*39*).

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