JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phenolic Compounds and Fatty Acids from Acorns (*Quercus* spp.), the Main Dietary Constituent of Free-Ranged Iberian Pigs

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The aim of the present work was to identify and quantify the phenolic compounds and fatty acids in acorns from *Quercus ilex*, *Quercus rotundifolia*, and *Quercus suber*. The concentration of oleic acid was >63% of total fatty acids in all cases, followed by palmitic and linoleic acids at similar concentrations (12−20%). The concentrations of α-tocopherol in *Q. rotundifolia*, *Q. ilex*, and *Q. suber* were 19, 31, and 38 mg/kg of dry matter (DM), respectively, whereas the concentrations of γ-tocopherol were 113, 66, and 74 mg/kg of DM, respectively. Thirty-two different phenolic compounds were distinguished. All of them were gallic acid derivatives, in the form of either galloyl esters of glucose, combinations of galloyl and hexahydroxydiphenoyl esters of glucose, tergallic *O*- or *C*-glucosides, or ellagic acid derivatives. Several tergallic acid *C*-glucosides were also present in the extracts obtained from *Q. suber*. Acorns from *Q. ilex* and *Q. rotundifolia* showed similar polyphenol patterns mainly with gallic acid-like spectra. Chromatograms of *Q. suber* showed mainly polyphenols with ellagic acid-like spectra. Valoneic acid dilactone was especially abundant in *Q. suber* skin. The contribution of skin to the total phenolics of the acorn was relatively small in *Q. rotundifolia* and *Q. ilex* but relatively high in *Q. suber*. Skin extracts from *Q. suber*, *Q. rotundifolia*, and *Q. ilex* showed 1.3, 1.4, and 1.0 antioxidant efficiencies, respectively (compared to that of butylhydroxyanisole). Endosperm extracts showed lower capacity to prevent lipid peroxidation than skin extracts.

KEYWORDS: Acorn; antioxidant; tocopherol; fatty acid; phenolics; ellagitannins; Iberian pig; *Quercus* spp.

INTRODUCTION

Acorns from *Quercus* spp. are usually used as a feed source for free-ranging wild animals. In southwestern continental Europe, acorns mainly come from *Q. ilex*, *Q. rotundifolia*, and *Q. suber* and are frequently used by domestic animals under free-range conditions, especially the Iberian pigs, an autochthonous breed from the Iberian Peninsula. The main feature of acorns and Iberian pigs is related to dry meat products. Meat products from Iberian pigs fed extensively on acorns of the above three species and fresh pasture during the fattening phase are considered to be of superior quality to those from pigs fed with mixed diets (*1*).

The pigs find the acorns palatable and can consume a great quantity daily (7−10 kg) (*2*). In addition, visual observation of the Iberian pigs suggests that they also consume grass during the whole fattening period (*3*), achieving higher concentrations of both α- and γ-tocopherol in muscle and microsomes than pigs given mixed diets (formulated grain-based mixture of ingredients to provide a complete and balanced source of nutrients) with a basal level of α-tocopheryl acetate (*2*, *3*). Moreover, a lower susceptibility to the oxidation of microsome extract from Iberian pigs fed on acorn and grass compared with those from pigs fed on mixed diets supplemented with α-tocopheryl acetate suggests that other dietary constituents from the diet that the pigs receive when fed under free-range conditions may play a role in the stabilization of microsomal lipids (*2*).

In meat and meat products from Iberian pigs fed on acorn and grass, oleic acid accounts for >55% of the total fatty acids, which implies a condition very favorable for the progress of the autoxidation phenomena because of the very long maturation process of >2 years. However, a limited lipid oxidation is produced, which is mainly located in the ham external layer, but many volatile substances are accumulated inside the meat,

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10.1021/jf030216v CCC: $25.00
Published on Web 09/10/2003

giving rise to a very palatable and appreciated product. It has been shown that tocopherol ingested by pigs during the fattening phase may play a role in the modulation of lipid oxidation during the aging process (*4*). However, to the best of our knowledge nothing is known on the phenolic content of acorns, which may also participate in the control of the lipid oxidation phenomena. Therefore, the aim of the present work was to study the acorn fatty acid composition and the identification and quantification of their phenolic compounds belonging to three *Quercus* species that are widely consumed by Iberian pigs. In addition, a set of in vitro assays was made to assess the antioxidant capacity of these phenolic compounds.

MATERIALS AND METHODS

Reagents. 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), ellagic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, linoleic acid, manganese dioxide (MnO_2), and 3-*tert*-butyl-4-hydroxyanisole (BHA) were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade and supplied by Merck. Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Acorns. Acorns from *Q. suber* L., *Q. rotundifolia* Lam., and *Q. ilex* L. (Fagaceae) were collected in Extremadura (Spain) in February 2002. Plant species were identified by the Department of Botany, University of Extremadura. Acorns were collected from trees having reached the mature stage. Ten trees of each species were sampled, and ~100 g was harvested per tree. Acorns were vacuum packed and frozen at −40 °C until analysis. This was carried out within 3 weeks in all cases.

Fatty Acid Analysis. For each analysis three replicates of 100 g of acorns (8−20 acorns) were extracted and analyzed. The relative weight of skin and endosperm was calculated by weighing samples of 15 acorns. Lipids from endosperm were extracted with chloroform/MeOH (1:2 v/v) using an Ultraturrax T-25 (Janke and Kunkel, Ika-Labortechnick, Germany) at room temperature for 5 min at 10000 rpm. The extract was taken to dryness under reduced pressure (40 °C), and this was weighed for "total fat" evaluation. Lipid samples (300 mg) were methylated in the presence of 3 mL of 0.1 M sodium metal in MeOH and 3 mL of 5% sulfuric acid in anhydrous MeOH to obtain the fatty acid methyl esters (*3*). Then, 1 μL was analyzed using a Hewlett-Packard HP-5890 (Avondale, PA) gas chromatograph equipped with a flame ionization detector and a 30 m × 0.32 mm i.d. and 0.25 μm HP-Innowax capillary column. Helium at 2 mL/min was used as the carrier gas, and the split/splitless injector was used with a split/splitless ratio of 10:1. The temperature program was as follows: injector and detector temperature, 250 °C; initial column temperature, 200 °C; maintained for 2 min; raised from 200 to 245 °C at 3.5 °C/min; held for 7 min. Fatty acid methyl esters were identified by comparison with standards run previously and quantified on a dry matter basis.

Tocopherol Analysis. Concentrations of α- and γ-tocopherol were quantified as previously described by Rey et al. (*3*). Samples of endosperm were homogenized in a 54 mM dibasic sodium phosphate buffer adjusted to pH 7.0 with HCl. After the samples had been mixed with absolute ethanol and hexane, the upper layer containing tocopherols was evaporated and dissolved in ethanol prior to analysis by reverse-phase HPLC on an HP 1050 instrument (Hewlett-Packard, Waldbronn, Germany) equipped for separation with a (5 μm particle size, 25 × 0.4 cm) RP-18 reverse-phase column (Hewlett-Packard). The mobile phase was MeOH/water (97:3 v/v) at a flow rate of 2 mL/min, and the detector (HPIB 10, Hewlett-Packard) was fixed at 292 nm.

Extraction of Phenolic Compounds. Skin and endosperm from acorns were separated with the help of a sharp knife. Skin represented approximately 7, 3, and 5% of the total weight of acorn for *Q. suber*, *Q. rotundifolia*, and *Q. ilex*, respectively (this was calculated producing three samples including 15 acorns each). One gram of air-died sample (three replicates of 1 g obtained from 8−20 acorns, either skin or endosperm) was homogenized in an Ultraturrax T-25 (Janke and Kunkel, Ika-Labortechnick) at 24000 rpm for 1 min after the addition of 10 mL of a solution of MeOH/water (80:20, v/v) containing 0.8 mM NaF to prevent sample oxidation. The extracts were centrifuged at 5000*g* for 5 min in a Centromix centrifuge (Selecta, Barcelona, Spain), concentrated 3-fold in MeOH by using Sep-Pak cartridges (Millipore Corp.), further filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corp.), and analyzed by HPLC-DAD.

Phenolic Quantification by HPLC-DAD. A sample of 50 μL of the above extract was analyzed using a Merck-Hitachi HPLC system with an L-7100 pump model and a model 7455 diode array detector. Separations were achieved on a 12 × 0.4 cm, 5 μm, RP-18 Licrochart column (Merck, Darmstadt, Germany). The mobile phase consisted of water with 5% formic acid (solvent A) and HPLC grade MeOH (solvent B) at a flow rate of 1 mL/min. Elution was performed with a gradient starting with 3% B to reach 25% B at 6 min, 35% B at 25 min, and 90% B at 35 min and then isocratic for 5 min. HPLC experiments were repeated three times. Phenolic compounds were quantified at 280 and 255 nm according to their gallic acid-like and ellagic acid-like UV spectra, respectively. Phenolic compound content was expressed as milligrams per kilogram of fresh acorn weight.

HPLC-MS/MS. Chromatographic separation was carried out on a 25 × 0.4 cm, particle size 5 μm reverse-phase C_{18} Lichrocart column using water/formic acid (95:5, v/v) (A) and MeOH (B) as the mobile phases. Elution was performed using the HPLC analysis conditions detailed above. The HPLC system equipped with a DAD and mass detector in series consisted of an HPLC binary pump, an autosampler, a degasser, and a photodiode array detector controlled by software from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer equipped with an electrospray ionization system and controlled by software. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS/MS) spectra were measured from *m*/*z* 100 to 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode for both gallic and ellagic acid derivatives. UV chromatograms were recorded at 280 and 255 nm.

Evaluation of the Antioxidant Activity. The free radical scavenging activity versus both DPPH• and $ABTS^{•+}$ of skin and endosperm acorn extracts was compared to that of Trolox (a synthetic analogue of vitamin E). In addition, the capacity to inhibit lipid peroxidation (ferric thiocyanate method, FTC) of these acorn extracts was also compared to that of Trolox and the food antioxidant BHA. To simplify, the term "antioxidant" will be used henceforward to describe both the antiradical (free radical scavenging activity) and antioxidant (activity to prevent lipid peroxidation) activities. Antioxidant activity was expressed as milligrams of Trolox equivalent antioxidant capacity (TEAC) following the nomenclature of Rice-Evans and Miller (*5*). The coefficient of variation [CV = (SD/mean) × 100] in the determination of the antioxidant activity was always <10% in the three assays (DPPH•, $ABTS^{•+}$, and FTC). Antioxidant assays carried out by using the three methods were repeated at least three times.

DPPH• Assay. Antioxidant activity using the free radical DPPH• (*6*) was evaluated by measuring the variation in absorbance at 515 nm after 1 h of reaction in parafilm-sealed glass cuvettes, to avoid MeOH evaporation, at 25 °C (*7*). DPPH• was dissolved in MeOH to achieve an initial absorbance of 1. Final assay volume was 1 mL. The extracts previously obtained for the analysis of phenolic compounds were diluted 4-fold with MeOH, and 5 μL of this solution was assayed. This sample volume contained 1.8, 1.9, and 2.3 μg of phenolics from *Q. suber*, *Q. ilex*, and *Q. rotundifolia*, respectively, in the case of skin extracts and 0.6, 3.5, and 3.55 μg of phenolics in the case of endosperm extracts.

$ABTS^{•+}$ Assay. The radical cation $ABTS^{•+}$ was chemically generated with MnO_2 from ABTS (*8*). The antioxidant activity of acorn extracts was evaluated by measuring the disappearance of absorbance (initial absorbance was adjusted to the value 1) at 414 nm after 1 h of reaction at 25 °C (*8*). The final assay volume was 1 mL. In this case, the samples were diluted 10-fold with MeOH, and 5 μL of this solution was assayed as sample volume. The sample volume contained 0.73, 0.76, and 0.91 μg of phenolics from *Q. suber*, *Q. ilex*, and *Q. rotundifolia*, respectively, in the case of skin extracts and 0.23, 1.4, and 1.42 μg of phenolics in the case of endosperm extracts.

Table 1. Selected Parameters, Fat, Tocopherols, and Major Fatty Acid (FA) Composition of Acorns[a]

	Q. rotundifolia	*Q. ilex*	*Q. suber*
fatty acid of endosperm			
C-14:0 (g/100 g of total FA)	0.25	0.06	0.11
C-16:0 (g/100 g of total FA)	12.52	13.40	10.54
C-16:1 (g/100 g of total FA)	0.18	0.04	0.17
C-18:0 (g/100 g of total FA)	4.15	1.60	1.69
C-18:1*n*–9 (g/100 g of total FA)	66.74	67.00	63.13
C-18:2*n*–6 (g/100 g of total FA)	14.29	16.12	21.32
C-18:3*n*–3 (g/100 g of total FA)	0.85	0.68	1.15
C-20:0 (g/100 g of total FA)	0.67	0.26	071
C-20:1*n*–9 (g/100 g of total FA)	0.34	0.74	0.11
C-22:1*n*–9 (g/100 g of total FA)	0.41	Tr[b]	0.08
weight (15 acorns) (g)	68.11	178.78	134.78
endosperm (15 acorns) (g)	45.90	142.80	96.33
skin (from 15 acorns) (g)	6,19	8.36	5.88
shell (from 15 acorns) (g)	16.02	27.62	32.57
fat (g/100 g of endosperm DM)	2.91	2.24	2.71
tocopherols (mg/kg of endosperm DM)			
α-tocopherol	19	31	38
γ-tocopherol	113	66	74

[a] Results are the mean of three determinations. [b] Traces.

FTC Assay. Inhibition of lipid oxidation was determined by using the FTC method according to the procedure of Larrosa et al. (*9*). Briefly, the assay mixture in a final incubation volume of 1.275 mL was an oil-in-water emulsion consisting of 2.5% linoleic acid in 50 mM, pH 7, sodium phosphate buffer with AAPH to accelerate lipid peroxidation (*9*) and 50 μL of acorn (either skin or endosperm) extracts. This sample volume contained 73.3, 75.6, and 90.5 μg of phenolics from *Q. suber*, *Q. ilex*, and *Q. rotundifolia*, respectively, in the case of skin extracts and 22.8, 141, and 142 μg of phenolics in the case of endosperm extracts. Trolox (135 μg) and BHA (16 μg) were assayed as antioxidant standards. Linoleic acid peroxidation was determined by measuring hydroperoxide accumulation as the increase in absorbance at 500 nm. One hundred percent oxidation was taken as the maximum absorbance reached by the control sample without antioxidant after 10 h of reaction. Peroxidation inhibition (percent) was expressed as $100 - (A_{sample}/A_{control} \times 100)$ (*9*).

The variations of absorbance were recorded, in the three assay methods, with a UV-1603 Shimadzu spectrophotometer (Tokyo, Japan). Temperature was controlled at 25 °C with a temperature controller (CPS 240 Shimadzu), using a precision of ±0.1 °C.

RESULTS AND DISCUSSION

Fat and Fatty Acids of Endosperm. Data on selected characteristics and fatty acids profiles of acorns are given in **Table 1**. The endosperm is the main part consumed by the animals. The fatty acid composition (**Table 1**) was very similar to that reported previously (*3*). It reflects a very high content of oleic acid, >63% of total fatty acids, followed by palmitic and linoleic acids at similar concentrations (12−20%). A number of studies have been published that demonstrated the degree to which dietary fat and/or oil influence the fatty acid content of pork fat (*10*−*13*). The high content of C:18:1*n*−9 of acorns is probably the main reason for the high concentration of this fatty acid in adipose (*14*, *15*), hepatic (*16*), and muscle (*17*, *18*) tissues and even in dry hams (*19*) from Iberian pigs raised extensively. The content of C18:1*n*−9 in tissues from pigs fed on acorns is always >55% and that of C18:2*n*−6 is ∼5%. This peculiar composition is used to discriminate between the pork and meat products coming from pigs fed on acorns under free range conditions and those from pigs fed mixed feeds.

Tocopherol of Endosperm. The α- and γ-tocopherol contents are shown in **Table 1**. The α- and γ-tocopherol levels of grass available during the fattening phase have been previously measured by Rey et al. (*2*), providing between 226−276 and 63−66 mg/kg of dry matter (DM), respectively. This means that the acorns are relatively richer than grass in γ-tocopherol, which may be a relevant feature because tocopherol uptake is accumulated in the tissues, as it has been demonstrated in brain and muscles of rats (*20*). It may therefore contribute to the oxidative stability of meat (*21*). In addition, γ-tocopherol has a higher antioxidant capacity than the α-isomer (*22*). Therefore, the tocopherols from both grass and acorns may contribute to modulation of the susceptibility to oxidation in vivo and postmortem. Moreover, the antioxidant effect of tocopherol is extended in tissues after slaughter; it has been demonstrated that the lipid peroxidation susceptibility is inhibited by tocopherol in liver (*23*), back fat (*24*), and muscle tissue (*3*), and also a similar effect has been observed in dry fermented sausages (*25*). This is probably the reason for the modulation role attributed to these antioxidants during the very long aging period of Iberian dry ham (*4*).

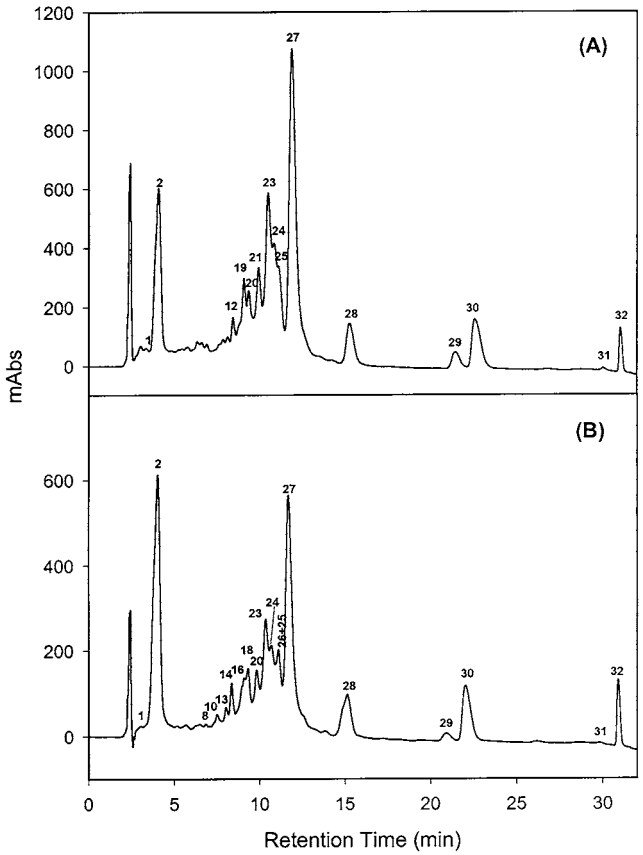

Figure 1. HPLC chromatograms of *Q. rotundifolia* (A) and *Q. ilex* (B) skin extracts (255 nm). For compound identification see **Table 3**.

Identification of Phenolic Compounds in the Skin and Endosperm of *Quercus* spp. Acorns. As a general rule, the phenolic profiles observed for the skin and endosperm extracts were quite similar, especially in the case of *Q. rotundifolia* and *Q. ilex*, which were nearly identical (**Figure 1**). Only in *Q. suber* were some significant quantitative differences observed between skin and endosperm extracts as compounds **12** and **22** were especially present in the endosperm, whereas compounds **3**, **5**, and **6** were mainly present in the skin (**Figure 2**). The UV spectra of the different phenolic compounds present in these extracts, recorded with a DAD, showed that they could be arranged into two groups—those showing a characteristic spectrum of ellagic acid (**Figure 3A**) and those showing spectra with a single maximum similar to that of gallic acid (**Figure**

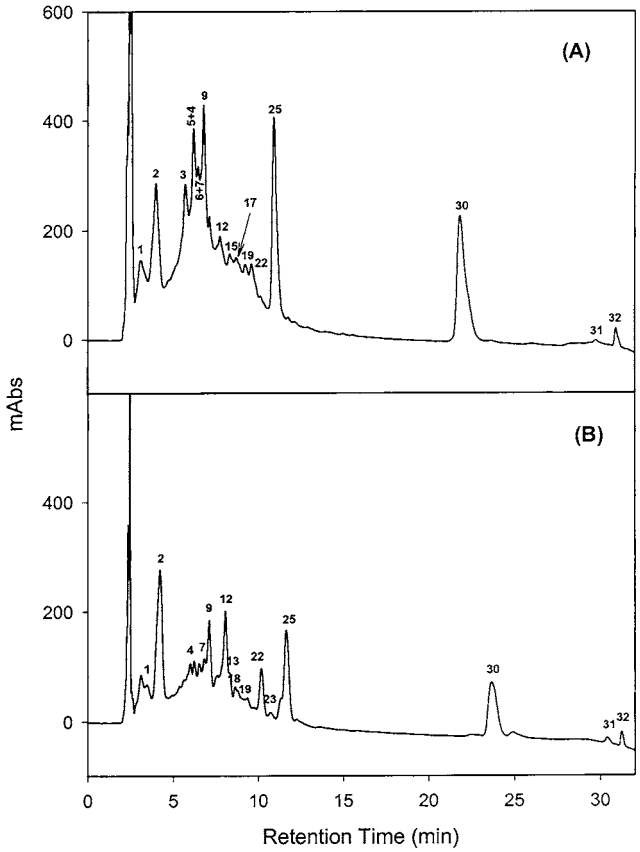

Figure 2. HPLC chromatograms of *Q. suber* skin (A) and endosperm (B) extracts (255 nm). For compound identification see **Table 3**.

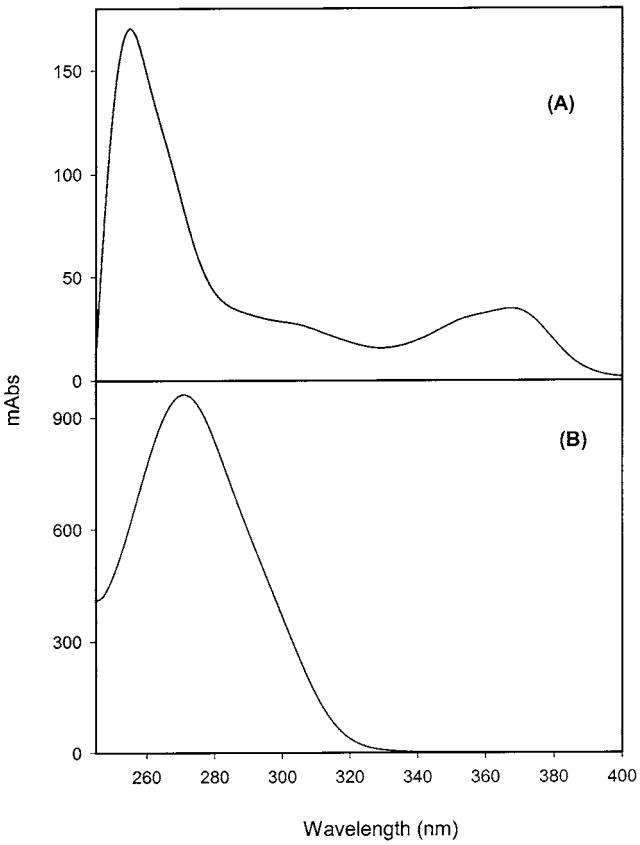

Figure 3. UV spectra of main *Quercus* phenolics: (A) ellagic acid type; (B) gallic acid and hexahydroxydiphenic acid type.

3B). The first group includes all of the compounds that present an ellagic residue in their molecular structure, and the second group includes all of the galloyl and hexahydroxydiphenoyl derivatives. The hexahydroxydiphenoyl derivatives give rise to ellagic acid after hydrolysis, and they are considered to be ellagitannins. These compounds are easily identified in the MS/MS analysis as they produce a daughter ion at *m*/*z* 301 corresponding to ellagic acid (**Table 2**). The chromatograms of *Q. ilex* and *Q. rotundifolia* appeared to be quite similar (**Figure 1**) and characterized by polyphenols with gallic acid-like spectra, especially for those peaks eluting during the first 15 min. However, the chromatogram of *Q. suber* showed mainly polyphenols with ellagic acid-like spectra (**Figure 2**).

Thirty-two different phenolic compounds (**1**−**32**) were distinguished in the methanolic extracts obtained from the acorns of the three *Quercus* species (**Table 2**). All of them were gallic acid derivatives, in the form of either galloyl esters of glucose, the combinations of galloyl and hexahydroxydiphenoyl esters of glucose, tergallagic *O*- or *C*-glucosides, or ellagic acid derivatives. Some of them were as simple as gallic acid (**2**) (*m*/*z* 169 $[M - H]^-$) and ellagic acid (**30**) (*m*/*z* 301 $[M - H]^-$). The series of galloyl glucose esters was also detected as different isomers of galloyl glucose (**1**), digalloyl glucose (**8**, **10**, **13**, and **14**), trigalloyl glucose (**11**, **16**, **18**, **20**, and **24**), tetragalloyl glucose (**26**), and pentagalloyl glucose (**28**). Different isomeric combinations of gallic acid and hexahydroxydiphenic acid with glucose were also detected as digalloyl-hexahydroxydiphenoyl-glucose (**4**, **7**, and **12**) and trigalloyl-hexahydroxydiphenoyl-glucose (**21**−**23** and **27**). All of these compounds had a characteristic gallic acid-like UV spectrum, with a single maximum at 275−277 nm (**Figure 3B**). In addition, several compounds with ellagic acid-like UV spectrum were detected (**Figure 3A**). These were tentatively identified as tergallic *C*-glucoside (**3**), dehydrated tergallic *C*-glucoside (**5** and **6**), tergallic-*O*-glucosides (**15**, **17**, and **19**), valoneic acid dilactone (**25**), ellagic acid pentoside (**29**), valoneic acid dilactone with loss of CO_2 (**31**), and an ellagic acid dimer with loss of H_2O (**32**). The general structures of these types of compounds are shown in **Figures 4** and **5**. Some of these compounds are hydrolyzable tannins as they can be hydrolyzed to yield gallic acid and/or ellagic acid. They were tentatively identified by their HPLC-MS and MS/MS analyses (**Table 2**) and by chromatographic comparisons with authentic markers when these were available (ellagic and gallic dilactone).

The trigalloyl-hexahydroxydiphenoyl-glucose (**21**−**23** and **27**, $[M - H]^-$ at *m*/*z* 937) and digalloyl-hexahydroxydiphenoyl-glucose (**4**, **7**, and **12**, $[M - H]^-$ at *m*/*z* 785) isomers always showed M − H ions that suffered the loss of a galloyl residue (M − H − 152 to give daughter ions at *m*/*z* 785 and 633, respectively) and the loss of a hexahydroxydiphenoyl residue (M − H − 302 to give daughter ions at *m*/*z* 635 and 483, respectively). Sequential losses of galloyl residues (−152 mass units) were also observed to yield characteristic fragments at *m*/*z* 617 and 465, as well as a fragment at *m*/*z* 301 corresponding to ellagic acid (**Table 3**).

Several tergallic acid *C*-glucosides were also present in the extracts obtained from *Q. suber*. They were similar to those previously reported in the wood of other *Quercus* species known as vescalagin and castalagin or the corresponding vescalin and castalin (*26*). The main difference is that in this case the acorn compounds have an ellagic acid residue in the molecule, providing this compound with the characteristic ellagic acid-like UV spectrum (**Figure 3A**). Two similar types of compounds were detected—one at *m*/*z* 631 and the other one with a loss of

Table 2. UV and HPLC-MS/MS Analyses of Acorn Polyphenols (UV Spectra as in **Figure 3**)

no.	t_R (min)	structure	UV spectrum type	HPLC-MS $(M-H)^-$ *m/z*	MS/MS *m/z*
1	3.5	galloyl glucose	B	331	271, 169
2	4.0	gallic acid	B	169	125
3	5.7	tergallagic *C*-glucoside	A	631	613, 493, 301
4	6.0	digalloyl-hexahydroxydiphenoyl-glucose	B	785	633, 483, 419, 301
5	6.2	dehydrated tergallagic *C*-glucoside	A	613	595, 523, 493, 301
6	6.4	dehydrated tergallagic *C*-glucoside	A	613	595, 523, 493, 301
7	6.5	digalloyl-hexahydroxydiphenoyl-glucose	B	785	633, 483, 419, 301
8	6.6	digalloyl glucose	B	483	423, 313, 271, 193, 169
9	6.8	dehydrated tergallagic *C*-glucoside	A	613	493, 301
10	6.9	digalloyl glucose	B	483	423, 313, 271, 193, 169
11	7.5	trigalloyl glucose	B	635	483, 465, 313
12	8.0	digalloyl-hexahydroxydiphenoyl-glucose	B	785	633, 483, 419, 301
13	8.1	digalloyl glucose	B	483	423, 313, 271, 193, 169
14	8.4	digalloyl glucose	B	483	423, 313, 271, 193, 169
15	8.7	tergallagic *O*-glucoside	A	631	451, 301
16	8.9	trigalloyl glucose	B	635	465, 423, 313, 271, 193
17	9.2	tergallagic *O*-glucoside	A	631	451, 301
18	9.3	trigalloyl glucose	B	635	465, 423, 313, 271
19	9.6	tergallagic *O*-glucoside	A	631	451, 301
20	9.7	trigalloyl glucose	B	635	465
21	9.8	trigalloyl-hexahydrodiphenoyl-glucose	B	937	862, 785, 301
22	10.2	trigalloyl-hexahydrodiphenoyl-glucose	B	937	785, 767, 635, 465, 419, 301
23	10.4	trigalloyl-hexahydrodiphenoyl-glucose	B	937	862, 785, 617, 301
24	10.7	trigalloyl glucose	B	635	483, 425, 423, 313
25	11.1	valoneic acid dilactone	A	469	426, 301
26	11.2	tetragalloyl glucose	B	787	635, 617, 465, 313
27	11.9	trigalloyl-hexahydrodiphenoyl-glucose	B	937	917, 852, 769, 617, 465, 301
28	15.3	pentagalloylglucose	B	939	787, 769, 635, 617, 431
29	21.1	ellagic acid pentoside	A	433	301
30	22.2	ellagic acid	A	301	
31	30.0	valoneic acid dilactone $-$ CO_2	A	425	301
32	30.9	ellagic acid dimer $-$ H_2O	A	585	415, 301

Table 3. Contents of Phenolic Compounds in (A) *Q. rotundifolia*, (B) *Q. ilex*, and (C) *Q. suber*[a]

(A) *Q. rotundifolia*

	2	other G	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**27**	**28**	**29**	**30**	**31**	**32**	TPh
skin	3.6	8.7	4.2	2.5	1.4	Tr	4.0	3.5	1.7	12.1	1.4	0.5	5.3	0.07	0.8	49.8
	(0.7)	(1.5)	(1.3)	(0.7)	(0.4)		(0.8)	(0.7)	(0.6)	(3.5)	(0.4)	(0.2)	(1.3)	(0.0)	(0.2)	(0.6)
endosperm	108.6	87.6	96.9	60.5	47.9	163.7	136.9	Tr	40.4	495.6	51.9	22.4	95.5	1.1	29.2	1438.2
	(45.7)	(21.3)	(11.3)	(3.7)	(1.5)	(20.6)	(14.7)		(2.2)	(35.5)	(2.3)	(3.1)	(13.4)	(0.5)	(4.1)	(60.9)

(B) *Q. ilex*

	2	other G	**18**	**20**	**23**	**24**	**25 + 26**	**27**	**28**	**29**	**30**	**31**	**32**	TPh
skin	12.7	11.0	7.7	5.7	6.5	5.9	3.7	13.8	1.9	0.2	11.3	0.2	1.1	81.7
	(1.9)	(1.5)	(1.8)	(1.3)	(1.5)	(1.5)	(0.2)	(2.4)	(0.4)	(0.03)	(2.2)	(0.06)	(0.3)	(3.4)
endosperm	417.5	105.9	114.8	150.2	240.8	153.9	90.5	574.7	73.9	36.5	151.3	2.4	63.0	2175.4
	(49)	(18.3)	(11.1)	(21.0)	(31.3)	(6.9)	(5.7)	(47.4)	(16.3)	(1.8)	(12.0)	(0.8)	(2.2)	(83.3)

(C) *Q. suber*

	2	**3**	other G	**5**	**9**	**12**	**15**	**25**	**30**	**31**	**32**	TG	TE	TPh
skin	9.0	5.9	19.0	11.3	6.0	Tr	3.34	19.9	31.2	0.4	1.0	28.0	79.0	107.0
	(1.9)	(1.7)	(0.4)	(4.0)	(3.0)		(1.0)	(7.3)	(5.4)	(0.2)	(0.3)	(1.9)	(25.8)	(25.9)
endosperm	68.3	Tr	98.6	Tr	Tr	48.6	Tr	31.6	45.9	2.6	3.0	215.5	83.1	298.6
	(7.0)		(8.9)			(0.8)		(5.3)	(5.5)	(0.9)	(0.2)	(15.1)	(10.8)	(18.6)

[a] Values are expressed as micrograms per gram of acorn. Standard deviations are reported in parentheses ($n = 3$). The numbers correspond to the identified compounds by HPLC-DAD-MS/MS (**Table 3**). Other G indicates gallic acid type compounds not separable for identification; TE, total ellagic type compounds; TG, total gallic type compounds; TPh, total phenolics. Tr, trace amounts.

water at *m/z* 613. This water loss can be attributed to an internal ester formation between the carboxyl residue and one hydroxyl of the glucose moiety (**Figure 5**). The MS/MS spectrum of the tergallic *C*-glucosides (**3**, **5**, **6**, and **9**) always showed a loss of water (M − H − 18) and the losses of 90 and 120 mass units, in agreement with the fragment losses observed in the case of flavone-*C*-glucosides (*27*), corresponding to the fragmentation of the *C*-glucose residue. This type of *C*-glucoside has already been reported in oak tissues (*26*, *28*). Three tergallic *O*-glucosides were also detected (**15**, **17**, and **19**). In this case, the UV spectrum was similar to that of the *C*-glucosides, but the MS/MS analysis clearly showed a fragment for tergallagic acid at *m/z* 451 and a fragment of ellagic acid at *m/z* 301 (**Table 2**).

The MS/MS spectra of the different galloyl glucose com-

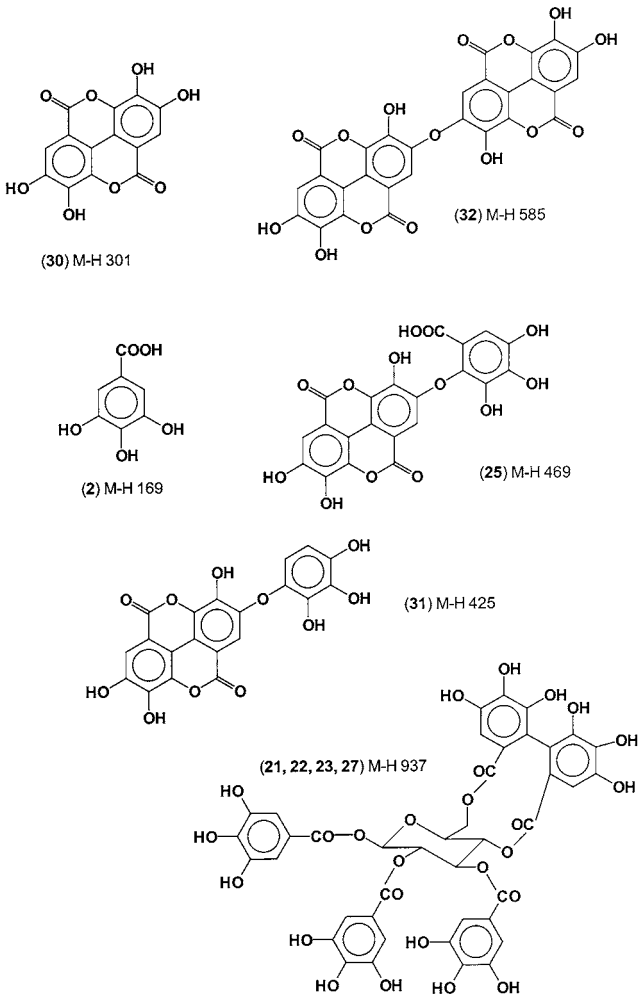

Figure 4. Acorn phenolic structures and M − H values.

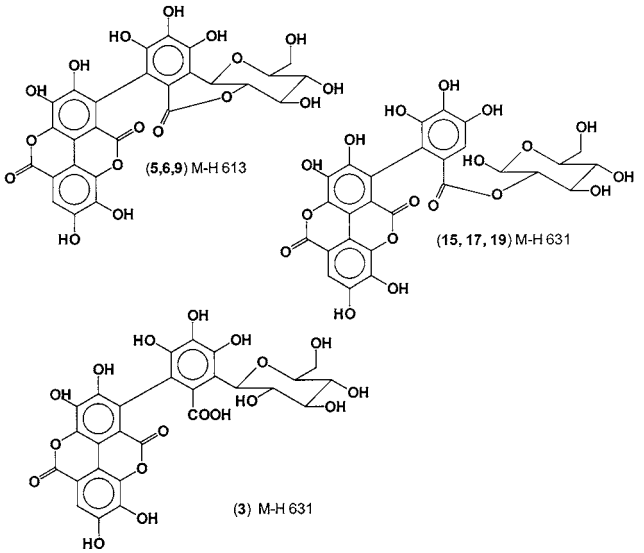

Figure 5. Acorn phenolic structures and M − H values.

pounds, from monogalloyl glucose to pentagalloyl glucose, were always characterized by the sequential loss of gallic acid residues. Therefore, in pentagalloyl glucose (**28**) the breakdown of the molecular ion M − H at *m*/*z* 939 produced a first loss of a galloyl residue (M − H − 152) to give a fragment at *m*/*z* 787, and in addition the loss of gallic acid was also observed (M − H − 170) at *m*/*z* 769 as was the sequential loss of another galloyl residue (769 − 152) to give a fragment at *m*/*z* 617 (**Table 2**). The same behavior was observed in the case of tetragalloyl glucose (**26**) in which a first loss of a galloyl residue (M − H − 152) to give a fragment at *m*/*z* 635, and in addition the loss of gallic acid was also observed (M − H − 170) at *m*/*z* 617 as was the sequential loss of another galloyl residue (617−152) to give a fragment at *m*/*z* 465. In digalloyl glucose (**8**, **10**, **13**, and **14**) a first loss of gallic acid was observed (M − H − 170) to give a daughter ion at *m*/*z* 313 as well as other fragments that are common to galloyl glucose and trigalloyl glucose (fragments at *m*/*z* 271, 193, and 169) (**Table 2**).

In addition, valoneic acid dilactone (**25**) was also detected. Its MS/MS fragments (*m*/*z* 426 and 301) were in agreement with those previously reported for this compound (*24*). An ellagic acid pentoside (**29**) was also identified in trace amounts (M − H at *m*/*z* 433) as was a dehydrated ellagic acid dimer (**32**) with an M − H ion at *m*/*z* 585 and MS/MS daughter fragments at *m*/*z* 415 and 301. A valoneic acid dilactone with loss of a CO_2 molecule (**31**) was also detected with M − H at *m*/*z* 425 and MS/MS fragment at 301.

Phenolic Compound Content from *Quercus* spp. Acorns. The phenolic contents of acorns from the three species are shown in **Table 3**. The results show that the contribution of skin to the total phenolics of the acorn is relatively small in *Q. rotundifolia* and *Q. ilex* (skin/endosperm ratios of 1:20 and 1:200, respectively), whereas it is relatively high in *Q. suber* (ratio of about 1:3). This fact may be related with the greater bitter taste of acorn from the latter species because the skin is the first part introduced into the mouth. It is interesting to note that in forests with mixed *Quercus* spp., Iberian pigs have a preferential intake of acorns from *Q. rotundifolia* and *Q. ilex*, whereas acorns from *Q. suber* are consumed only when the others have been eaten. The richest source of phenolics is *Q. ilex*, which is the main acorn used for pig feeding, providing an amount of >2 g of phenolics/kg of acorn ingested. Taking into account the fact that a pig can eat between 7 and 10 kg of acorns per day (*2*), a pig can ingest between 14 and 20 g of polyphenols from the acorns daily. The amount is smaller in the case of *Q. rotundifolia* acorns that provide ∼1.5 g/kg of acorns and even smaller in the case of *Q. suber*, being <0.5 g of polyphenols/kg of acorns. This dietary intake is much higher than that of tocopherols from oak acorn that provides some 25−50 mg/kg of fresh acorn (*2*, *3*).

Antioxidant Activity of Acorns Extracts. Although the main purpose of the present study was to characterize both the phenolic and fatty acid compositions of *Quercus* spp. acorns, the antioxidant capacity of the acorn extracts was also evaluated. With all of the caution called for in drawing conclusions from in vitro antioxidant experiments regarding the involvement of acorn phenolics in the lipid oxidation rate of Iberian pig, the dietary *Quercus* spp. acorns could be the "other dietary constituents" from the diet characteristically used to feed Iberian pig that might play a role in the stabilization of microsomal lipids (*2*), and it may be extended even during the dry ham aging (*4*).

When the antioxidant activity of the different acorn extracts was evaluated with the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ methods, no direct correlation between the polyphenol content and the antioxidant capacity became evident (data not shown). This could indicate that not only the polyphenol amount but also the polyphenol type were involved in the scavenging activity against these radicals. In general, the antioxidant capacity evaluated using the DPPH$^{\bullet}$ method was higher than that observed against the ABTS$^{\bullet+}$ (**Table 4**), which agrees with previous reports (*29*). This can be explained by the higher solubility of these

Table 4. Antioxidant Capacity per Gram of Corresponding Tissue

	Q. suber		*Q. rotundifolia*		*Q. ilex*	
	DPPH	ABTS	DPPH	ABTS	DPPH	ABTS
skin (mg of TEAC/g of skin)	3.15	1.57	1.96	1.27	1.54	1.31
endosperm (mg of TEAC/g of endosperm)	1.16	0.81	2.49	0.81	2.42	1.23
total phenols (μg of phenol/g of acorn)	406		1488		2257	

[a] Conditions for DPPH and ABTS assays are specified under Materials and Methods. Coefficient of variation was always <10%.

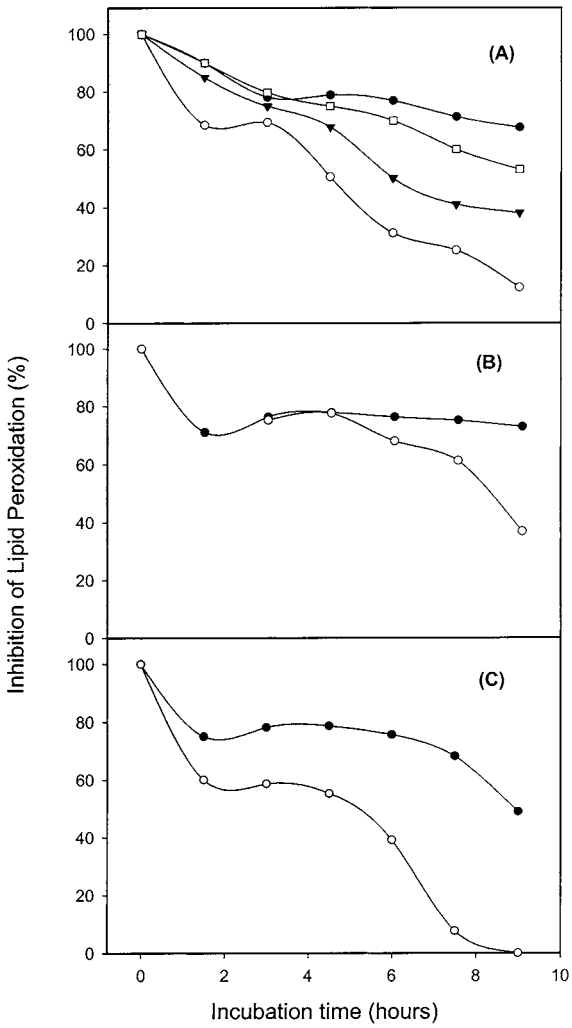

Figure 6. Evaluation of the ability to delay the oxidation of linoleic acid by different acorn extracts (thiocyanate method): (A) *Q. suber*; (B) *Q. rotundifolia*; (C) *Q. ilex*; (●) skin extracts; (○) endosperm extracts. (A) also includes antioxidant standards: (□) 16 μg of BHA; (▼) 135 μg of Trolox. The assay mixture included 50 μL of acorn extract (either skin or endosperm).

polyphenols in MeOH, the solvent used in the DPPH assay, than in water, which is the solvent used for the ABTS assay, as well as for the different stoichiometries previously reported for the reaction between phenolics and free radicals (*9*).

The highest antioxidant capacity was detected in the case of the skin extracts from *Q. suber* acorns using the DPPH• method (**Table 4**). For instance, as a comparative example, this value of 3.15 mg of TEAC/g of skin (**Table 4**) in the case of *Q. suber* is quite similar to that previously reported for methanolic extracts of raw artichoke byproducts with 3.9 mg of TEAC/g of fresh byproduct (*29*), which, however, contained almost 2-fold higher phenolic content than skin extracts from *Q. suber* acorn. In the case of the endosperm extract from *Q. suber*, the value of 1.16 mg of TEAC/g of endosperm is ~3-fold lower than that of artichoke extract, but the phenolic content in the endosperm extract was almost 7-fold lower than in artichoke. In addition, TEAC values of *Q. suber* were 10-fold higher than those reported previously for table grapes (results not shown), a fruit considered to be rich in antioxidant phenolics (*30*).

The FTC assay revealed that acorn extracts were able to delay linoleic acid oxidation (**Figure 6**). After 9 h of incubation, the skin extracts from *Q. suber*, *Q. rotundifolia*, and *Q. ilex* acorns (73.3, 90.5, and 75.6 μg of phenolics, respectively, in the assay) showed different efficiencies to prevent lipid peroxidation when compared to that of Trolox and BHA. Skin extracts from *Q. suber*, *Q. rotundifolia*, and *Q. ilex* showed 1.3-fold higher, 1.4-fold higher, and equal efficiencies, respectively, compared with 16 μg of BHA. The capacities to prevent lipid peroxidation after 9 h of the same skin extracts were 1.8-, 1.9-, and 1.3-fold higher than that of 135 μg Trolox. Endosperm extracts showed lower capacity to prevent lipid peroxidation than skin extracts. Among endosperm extracts, the highest capacity was observed in *Q. rotundifolia*, which was 1.4-fold lower than that of BHA and similar to that observed for Trolox (**Figure 6**).

These results suggest that dietary acorn polyphenols, and/or some derived antioxidant metabolites, could also be responsible for the reduced lipid oxidation observed in free-ranged Iberian pigs. However, this assumption deserves further studies concerning the metabolism of these polyphenols, types of possibly absorbed metabolites, and their accumulation in the different tissues of the pig.

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Received for review March 25, 2003. Revised manuscript received July 14, 2003. Accepted July 18, 2003. E.C. is holder of a grant from the Movilidad de Investigadores y Tecnólogos program under the action "Mit-Becas; Modalidad F2". This work has been partially supported by CICYT-AGL2001-1162 and AGL2003-05803.

JF030216V