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Identification and Quantification of Cholesterol and Cholesterol Oxidation Products in Different Types of Iberian Hams

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Cholesterol and cholesterol oxidation products (COPs) were determined in four different groups of dry-cured Iberian hams, based on the feeding received by pigs and their degree of crossbreeding. After lipid extraction, GC-FID for cholesterol determination and GC-MS to analyze COPs were used. Cholesterol content ranged from 30 to 34 mg/100 g of muscle. Some of the COPs analyzed, such as 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol, were detected in all of the samples. The major cholesterol oxide was 7-ketocholesterol; its concentration ranged from 57 to 71 μg/100 g of muscle. The content of cholesterol and cholesterol oxides in intramuscular lipids of hams was not affected by diet or crossbreeding of Iberian pigs.

KEYWORDS: Cholesterol; lipid oxidation; cholesterol oxides; Iberian ham

INTRODUCTION

The Iberian dry-cured ham is a meat product of high sensory quality with a first-rate consumer acceptance. This high quality seems to be related to the special characteristics of the raw material obtained from the Iberian pig and the prolonged dry-cured processing of Iberian hams (18−24 months). Iberian ham is characterized by a high intramuscular fat content (*1*), an elevated percentage of monounsaturated fatty acids (60%), and lower concentrations of saturated and polyunsaturated fatty acids (*2*) than other hams from white pigs (*3*). Iberian ham pieces have a wide variety of prices in the market depending on the rearing system and the degree of crossbreeding of the pigs. Consumers prefer hams elaborated from pure Iberian pigs raised exclusively with acorns and pasture ("montanera") during a period of time prior to slaughtering (~3 months), which are considered to have the highest quality. Many changes occur in lipids during the processing of Iberian ham, such as lipolysis (*2*, *4*) and oxidation reactions (*1*, *5*, *6*). These reactions give rise to numerous volatile compounds, which play a definitive role in the sensory quality of the final product (*7*−*10*). However, the changes related to cholesterol during processing have not been described in Iberian dry-cured ham so far.

Cholesterol is one of the constituents of the unsaponifiable fraction of lipids. At this moment, consumers show a great interest in this compound, its content in foods, and the possible consequences of cholesterol and its oxidation products (COPs) on cardiovascular disease incidence. Cholesterol is a molecule prone to oxidation. In addition, lipid radicals formed during the processing and storage of foods can accelerate the formation of COPs (*11*, *12*). Generally, heat, pH, light, oxygen, water activity, and the presence of unsaturated fatty acids are the major factors that influence COP formation during processing or storage (*13*). COPs have received considerable attention in recent years because of their biological activities associated with human health disorders. Studies on human health and COPs have demonstrated that the quantity of oxidized lipids in the diet was directly related to the concentrations of oxidized lipids in serum. Oxidized cholesterol in the diet could be directly absorbed into circulation, and thus high intakes of cholesterol oxides can increase plasma COP concentration (*14*).

Many food products have been analyzed to determine their contents of cholesterol and COPs. Eggs and egg-containing products have been studied extensively (*15*−*17*). Milk and milk products (*11*, *17*−*19*), meat and meat products (*19*−*24*), and other processed foods including seafood (*13*, *25*−*31*) has also been studied.

On the other hand, the effect of breeding and feeding on the Iberian ham characteristics has been previously studied by several authors (*5*, *8*, *32*−*34*). The present work deals with the effect of crossbreeding and the amount of cholesterol and cholesterol oxides in the feed of different types of Iberian hams (Iberian pure pigs and Iberian × Duroc crossbreed pigs fed on acorns and pasture or with concentrated feed).

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MATERIALS AND METHODS

Samples. A total of 34 Iberian dry-cured hams from pure Iberian pigs and Iberian × Duroc (50%) crossbreed pigs were used in this study. The hams were divided into four groups following a 2 × 2 factorial design according to crossbreeding (purebred and crossbred, Iberian and Iberian × Duroc) and the types of feeding during the fattening period (55 days) prior to slaughter: montanera (fed on acorns and pasture) and intensive system (fed with concentrated feed). The hams studied were divided into four groups: Iberian pigs fed on montanera ($n = 10$), Iberian pigs fed with concentrated feed ($n = 10$), Iberian × Duroc pigs fed on montanera ($n = 7$), and Iberian × Duroc pigs fed with concentrated feed ($n = 7$).

Hams were processed following a traditional method including two defined steps: salting/postsalting and ripening. During the first period (6 months), salting and postsalting were combined with low temperature (0−3 °C) and high relative humidity (80−90%). Then, the hams were aged for 15 months in a cellar at temperatures ranging from 10 to 27 °C with relative humidities of 58−80%.

After processing, the biceps femoris muscle was dissected from hams and immediately was vacuum packaged and stored at −80 °C until analysis.

Reagents and Chemicals. The solvents used were of the following origin and quality: PRS (extra pure) grade supplied by Panreac (Barcelona, Spain). Cholesterol standard (5-cholesten-3β-ol) was supplied by Merck (Darmstadt, Germany), and 5α-cholestane was obtained from Supelco (Bellefonte, PA). The standards of cholesterol oxides (20α-hydroxycholesterol; 7-ketocholesterol; 7α-hydroxycholesterol; 7β-hydroxycholesterol; 25-hydroxycholesterol; cholestanetriol) were supplied by Cromlab (Barcelona, Spain).

Lipid Extraction. Intramuscular fat was extracted with a chloroform/methanol solution (1:2 v/v) following the procedure of Bligh and Dyer (*35*). Then, the organic phase was evaporated, first in a rotary evaporator at 35 °C temperature and finally under nitrogen.

Determination of the Cholesterol Content. The method proposed by Guardiola et al. (*36*) was followed, with some modifications, according to the procedure described as follows. About 100 mg of intramuscular fat was weighed, and then 10 mL of 1 M KOH was added for cold saponification. Then, the mixture was kept at room temperature for 20 h to complete saponification. The blend was then transferred to a separating funnel, and 10 mL of diethyl ether and 10 mL of distilled water were added. After the separation of phases, the organic phase containing the unsaponifiable fraction was transferred to a second separating funnel, and the aqueous phase was again re-extracted twice with 10 mL of diethyl ether. The new organic phases obtained were also transferred to the second funnel. For washing the organic phase, 5 mL of 0.5 M aqueous KOH solution and two portions of 5 mL of distilled water were successively added to the second funnel. The washed organic extract was filtered through anhydrous sodium sulfate, and then, the solvent was evaporated to dryness under nitrogen stream. Afterward, the nonsaponifiable extract in addition to 2.5 mg of internal standard (5α-cholestane) was redissolved in 5 mL of pyridine. A quantity of 50 μL from this extract was silanized with 50 μL of bis-(trimethylsilyl) trifluoroacetamide (BFSTA) (supplied by Sigma, St. Louis, MO). The mixture was kept at room temperature for 20 min to complete the silanization reaction.

Cholesterol was identified using 5-cholesten-3β-ol as cholesterol standard and determined by GC, as described below.

GC was performed using a Hewlett-Packard HP-5890-II chromatograph, equipped with a flame ionization detector and a fused silica capillary column (12 m × 0.2 mm i.d.) with a film thickness of 0.33 μm, stationary phase of methyl silicone. Helium was used as carrier gas at a flow rate of 14.9 mL/min.

The oven temperature program was from 210 to 264 °C at 2 °C/min, from 264 to 290 °C at 5 °C/min, and 2 min at 290 °C. The injector and detector temperatures were 280 and 290 °C, respectively. The split ratio was 1:30. The inlet pressure was 14 psi, and the sample volume injected was 2 μL.

Determination of the Cholesterol Oxide Composition. Six COPs were identified according to the method of Ulberth and Rössler (*37*), with some modifications, according to the procedure described below. Each sample (10 mg of intramuscular fat) and 5 mL of hexane/diethyl ether (95:5) were added to a silica-SPE cartridge (Sep-Pak, Waters, Barcelona, Spain) and washed successively with three mixtures of hexane/diethyl ether in the following quantities and proportions: 10 mL of hexane/diethyl ether (95:5), 25 mL of hexane/diethyl ether (90:10), and 15 mL of hexane/diethyl ether (80:20). This procedure allowed recovery of both apolar lipids and cholesterol fraction. After that, COPs were extracted with 10 mL of acetone. Then, acetone was removed under vacuum at 25 °C on a rotary evaporator. To reduce contaminants (traces of cholesterol and/or partial glycerides) to a further degree, the COP-containing residue of the silica-SPE method was taken up in 3 mL of hexane/ethyl acetate (9:1) and applied to an NH_2-SPE cartridge (provided from Varian), which was eluted with a further 16 mL of the same mobile phase (*38*). Purified COPs were eluted with 10 mL of acetone. Then, acetone was removed under vacuum at 25 °C on a rotary evaporator.

Afterward, the COP residue was silanized with 50 μL of Sylon HTP hexamethyldisilazane (HMDS) plus trimethylchlorosilane (TMSC) plus pyridine, 3:1:9 (Supelco, Bellefonte, PA). The mixture was kept at 60 °C for 1 h and then cooled until room temperature. COPs silanized were evaporated to dryness under nitrogen and were then redissolved with 50 μL of chloroform.

Identification and Quantitative Determination of COPs. COPs were analyzed by gas chromatography−mass spectrometry (GC-MS) on a Hewlett-Packard HP-6890II chromatograph, equipped with a Hewlett-Packard 5973A mass selective detector and a fused silica capillary column (30 m × 0.25 mm i.d.) with a film thickness of 0.25 mm, stationary phase of methyl silicone. Helium was used as carrier gas at a flow rate of 0.54 mL/min.

The oven temperature program was 230 °C for 2 min, from 230 to 290 °C at 5 °C/min, and 10 min at 290 °C. The injector and detector temperatures were 270 and 280 °C, respectively. The inlet pressure was 12 psi, and the sample volume injected was 1 μL.

The injection was performed in splitless mode. The MS detector was run in full-scan mode. Cholesterol oxides were identified in the mass range (*m*/*z*) from 100 to 500. All samples were analyzed in selected ion monitoring (SIM) mode for quantification purposes of the compounds, in which the ions of *m*/*z* 7α-hydroxycholesterol (*m*/*z* 456.3), 7β-hydroxycholesterol (*m*/*z* 456.3), 20α-hydroxycholesterol (*m*/*z* 201.2), cholestanetriol (*m*/*z* 403.2), 25-hydroxycholesterol (*m*/*z* 131.1), and 7-ketocholesterol (*m*/*z* 472.3) were selected as the most characteristic of the oxides of cholesterol determined.

The content of each COP was calculated using an external standard procedure: 1 μL of an external standard mixture of 7α-hydroxycholesterol, 7β-hydroxycholesterol, 20α-hydroxycholesterol, cholestanetriol, 25-hydroxycholesterol, and 7-ketocholesterol (20 ng/μL) was analyzed separately from the sample under identical conditions. It was used to facilitate the qualitative identification and quantitative determination of the sample components.

Data Analysis. A two-way analysis of variance was performed to compare means for intramuscular fat, cholesterol, and COP data. The effects of feeding system (montanera or concentrated feed) and crossbreeding (Iberian pure or Iberian × Duroc) were analyzed using the General Linear Model. When a significant effect ($p < 0.05$) was detected, the comparative analyses between means were conducted using the Tukey test. Data are presented in the tables as the means of each group and standard error together with the significance levels of the main effects and interactions. Statistical analyses were performed by using SPSS version 10.0.

RESULTS AND DISCUSSION

Cholesterol. Four groups of hams were analyzed to research the differences in cholesterol and COP contents. The results of the cholesterol quantitative analysis and intramuscular lipid content are reported in **Table 1**. Hams from pigs fed on montanera showed a greater intramuscular lipid content than did hams from pigs fed the concentrate feed ($p < 0.05$), but no significant differences existed in the cholesterol concentrations among the different groups analyzed.

Table 1. Intramuscular Lipid Content (Grams per Kilogram of Muscle) and Cholesterol Content (Milligrams per 100 g of Muscle) in Dry-Cured Hams (Biceps Femoris Muscle) from Iberian and Iberian × Duroc Pigs Fed on Montanera or Concentrate Feed (Mean ± Standard Error)

	Iberian		Iberian × Duroc		*p*		
	montanera	concentrate feed	montanera	concentrate feed	diet	crossbreeding	interaction
lipid content	112.8 ± 10.7a	104.3 ± 6.8ab	107.2 ± 8.8ab	75.3 ± 3.7b	0.03	0.059	0.174
cholesterol	32.62 ± 1.45a	31.84 ± 1.66a	34.03 ± 2.71a	29.89 ± 1.50a	0.219	0.890	0.399

[a] Values in the same column with the same letters do not present significant differences at the 5% level.

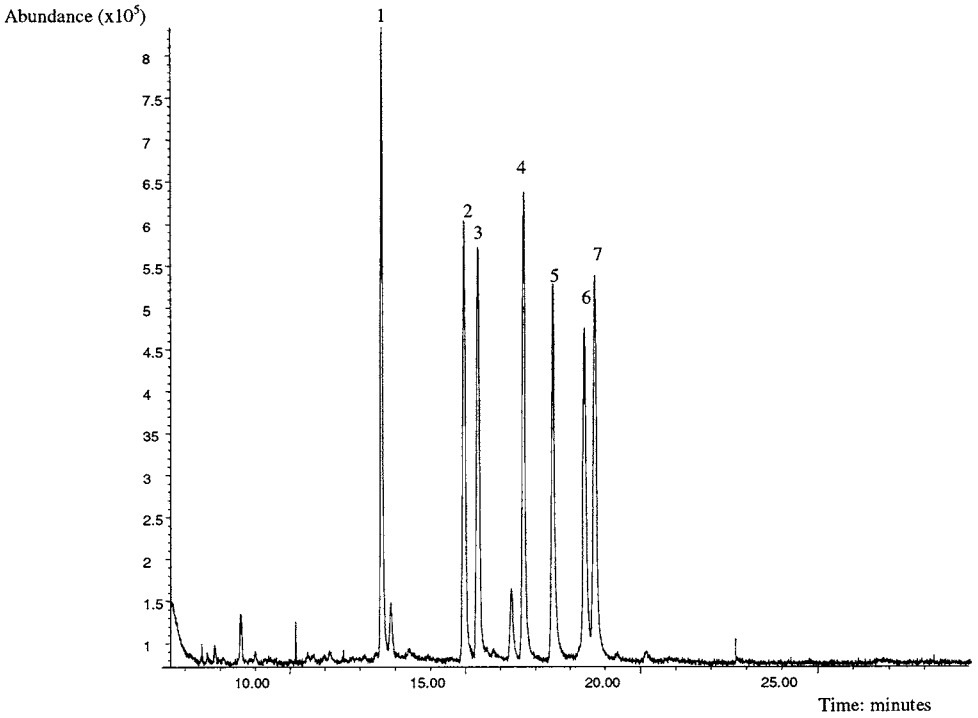

Figure 1. Chromatogram of cholesterol oxides standards: 1, 7α-hydroxycholesterol; 2, cholesterol; 3, 7β-hydroxycholesterol; 4, 20α-hydroxycholesterol; 5, cholestanetriol; 6, 25-hydroxycholesterol; 7, 7-ketocholesterol.

Cholesterol content ranged from 30 to 34 mg/100 g of muscle, and these values are lower than those reported for fresh meat from different animal species. Concentrations of cholesterol from 37 (*39*) to 70 mg/100 g of muscle (*40*) have been described in pigs fed with concentrate feed. Values from raw beef range from 36 (*41*) to 114 mg/100 g of meat (*42*). The different quantities described are partially due to the variety of samples used (*43*). However, examination of the cholesterol assay methods used in the literature revealed that differences in cholesterol content might be attributable, to a great extent, to differences in the analytical procedure used. Tejeda (*44*) analyzed cholesterol in fresh ham describing a higher content (41−58 mg/100 g of muscle) than in our samples. Considering that exactly the same analytical procedure was used for both studies and that they were the same batches of hams (fresh in Tejeda study and after maturing in the present work), these differences in cholesterol content could be related to the changes that take place during the maturation process on the lipid fraction, such as lipolysis and oxidation (*2*, *6*, *45*, *46*).

Considering the prolonged traditional processing method, which requires between 18 and 24 months of ripening, the content of cholesterol may be reduced with regard to fresh meat, and this decrease may be due to oxidation reactions during ripening. Cholesterol contents reported for different meat products are higher than that found in the present study for Iberian dry-cured ham. Thus, Zanardi et al. (*24*) described cholesterol contents in Salami Milano (95−110 mg/100 g), Coppa (110−118 mg/100 g), and Parma ham (61−67 mg/100 g).

In the present work, intramuscular lipid content found in our samples ranged from 75.3 to 112.8 g/kg of muscle, quantities that are in concordance with previous data from Iberian dry-cured ham (*1*). It is well-known that intramuscular fat contents from Iberian breed pigs are greater than those found in white pigs, due to the adipogenic character of the Iberian pigs. In the same sense greater values in intramuscular fat would be found in Iberian dry-cured hams than in hams from white pigs, independent of the dry-curing process: Díaz (*3*) described values of intramuscular fat of 35 g/kg in *biceps femoris* of hams from the Large-White breed. Although Iberian dry-cured ham is characterized by a high intramuscular fat content, an elevated content in cholesterol is not found, as is reflected in the values obtained in the samples analyzed in the present work that have been described previously (30−34 mg/100 g of muscle). Also, it is remarkable that hams from Iberian × Duroc pigs fed in an intensive system showed significantly lower concentrations of intramuscular fat than those from Iberian pigs fed in montanera ($p < 0.05$), whereas all groups had the same concentrations of cholesterol. In addition, the overall correlation coefficient was calculated between intramuscular fat content and cholesterol content of biceps femoris muscle. Low correlation between intramuscular fat and cholesterol was found ($R^2 = 0.0159$).

Table 2. Oxides of Cholesterol Content (Micrograms per 100 g of Muscle) in Dry-Cured Hams (Biceps Femoris Muscle) from Iberian and Iberian × Duroc Pigs Fed on Montanera or Concentrate Feed (Mean ± Standard Error)

	Iberian		Iberian × Duroc		*p*		
	montanera	concentrate feed	montanera	concentrate feed	diet	crossbreeding	interaction
7α-hydroxycholesterol	42.91 ± 3.26	36.25 ± 2.43	37.28 ± 4.07	44.27 ± 4.56	0.675	0.826	0.739
7β-hydroxycholesterol	27.40 ± 2.84	17.47± 2.34	15.04 ± 0.75	22.64 ± 1.13	0.362	0.968	0.559
7-ketocholesterol	71.21 ± 3.20	61.94 ± 3.49	56.61 ± 1.59	62.40 ± 5.65	0.621	0.707	0.880
cholestanetriol	trace	trace	trace	trace			
20α-hydroxycholesterol	trace	ND[a]	ND	ND			
25-hydroxycholesterol	trace	trace	ND	ND			

[a] Not detected.

These results are in agreement with previous studies in beef and pork meat, in which it has been reported that cholesterol content not only does not increase but even decreases when the intramuscular fat content of the products is greater (*47*, *48*). On the other hand, Dorado et al. (*40*) found in pork meat samples with low intramuscular fat content that the higher the intramuscular fat content, the higher the cholesterol content. This fact could be explained due to the particular distribution of cholesterol in muscle tissue. A study about the subcellular distribution of cholesterol in raw beef muscle showed cholesterol belongs mainly to membrane lipids; however, adipocytes are the cellular structures implicated in the increase of fat in muscle tissue (*49*).

Cholesterol Oxides. Figure 1 shows a chromatogram of the standards of COPs analyzed (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 20α-hydroxycholesterol, cholestanetriol, and 25-hydroxycholesterol).

Three cholesterol oxides were found in all of the samples analyzed: 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. Nonsignificant differences existed in the concentrations of these cholesterol oxides among the samples of hams from Iberian and Iberian × Duroc pigs fed montanera and concentrated feed (**Table 2**). The rest of the cholesterol oxides analyzed (20α-hydroxycholesterol, cholestanetriol, and 25-hydroxycholesterol) can be assumed to be minimal for total cholesterol oxide concentration; some of them were practically absent (**Table 2**). The most abundant cholesterol oxide was 7-ketocholesterol in the four groups studied, with values ranging from 57 to 71 μg/100 g of ham. 7α-Hydroxycholesterol ranged from 36 to 43 μg/100 g of ham, and the lowest values were for 7β-hydroxycholesterol (from 15 to 27 μg/100 g of ham).

In meat products, different authors support the findings obtained in the present study for dry-cured ham, showing low percentages of COPs. Thus, Zanardi et al. (*24*) describe concentrations of 7-ketocholesterol of 52 μg/100 g in salame Milano, 116 μg/100 g in coppa, and 32 μg/100 g in Parma ham and concentrations of 7β-hydroxycholesterol of 56 μg/100 g in salame Milano, 5 μg/100 g in coppa, and 21 μg/100 g in Parma ham. Regarding cooked chicken meat, Maraschiello et al. (*22*) described a higher concentration of COPs compared to fresh meat. Also, Du et al. (*29*) indicated that a large amount of COPs could be formed in cooked meat in turkey, pork, and beef, but these results could be explained by the effect of temperature during cooking (*22*). The exposure of food containing cholesterol to heat increase the production of COPs (*11*).

Works reporting cholesterol oxides do not describe any presence of 20α-hydroxycholesterol, cholestanetriol, or 25-hydroxycholesterol (*24*, *50*, *51*). Deviations observed in different products could be due to processing conditions or analytical techniques.

Particular attention must be paid to the detected values of 7-ketocholesterol. In meat products, this cholesterol oxide has been described as the most predominant one, not only in the present work but in previous studies as well (*24*, *30*). Moreover, in numerous works, this oxide has been considered a useful marker of the total oxidative process (*52*−*56*).

In this work has been described for the first time cholesterol and COP contents in dry-cured Iberian ham. It can be concluded that Iberian ham, which presents a high content of intramuscular fat and a long maturation process, is a dry-cured product with low amounts of cholesterol and COPs.

On the other hand, the content of cholesterol and cholesterol oxides in intramuscular lipids is not affected by diet or crossbreeding of Iberian pigs ($p < 0.05$).

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