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Effect of feed on cholesterol concentration and oxidation products development of *longissimus dorsi* muscle from Iberian pigs

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The effect of dietary free-range feeding or supplementation with copper and/or vitamin E in confinement on total cholesterol, neutral and polar lipids and cholesterol oxidation of the *longissimus dorsi* muscle from Iberian pigs was studied. Free-range fed pigs had higher (P=0.001) contents of γ-tocopherol and lower concentrations of α-tocopherol in the muscle than pigs fed diets supplemented with 100 mg/kg vitamin E. The total cholesterol content of the muscle was not significantly affected by the diets. However, the cholesterol:phospholipid ratio was higher (P<0.05), and consequently the membrane fluidity was lower, in the free-range fed pigs than in the pigs fed in confinement with either copper-supplemented (P<0.05) or vitamin E-supplemented (P<0.01) diets. The proportion of saturated fatty acids in phospholipids was greater (P<0.05) in the free-range fed group, which suggests metabolic regulation to maintain membrane structure. Free-range feeding produced higher levels of free fatty acids (P<0.01), lysophosphatidylcholine (P<0.05) and phosphatidylserine (P<0.01) and lower cholesterol esters (P<0.01) and sphingomyelin (P<0.05) in the muscle than the other groups. The ratios of phosphatidylethanolamine:phosphatidylcholine and sphingomyelin:phosphatidylcholine, which are indicators of membrane fluidity, were not significantly affected in any group. Dietary α-tocopheryl acetate supplementation produced lower β-epoxide (P<0.01), 7β-OH (P<0.05), and total cholesterol oxides (P<0.01) in cooked muscle after refrigerated display than in the other groups. These results indicate that supplementation with dietary α-tocopheryl acetate is more effective in reducing cholesterol oxidation than free-range feeding in cooked muscle from

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Iberian pigs. In evaluating oxidation, the composition of the muscle and meat treatment have to be considered as well as membrane fluidity.

Keywords: α-tocopherol; cholesterol oxides; copper; Iberian pig; membrane fluidity

Introduction

Muscle foods are the major source of cholesterol in the human diet, and cholesterol has been related to coronary heart disease (CHD) development. Since saturated fatty acids are the main contributory factor for hypercholesterolaemia, many studies have focussed on substitution of saturated with monounsaturated or polyunsaturated fatty acids in animal feeding (Nielsen *et al*., 1995). Furthermore, the classes of phospholipid and composition of membranes can be altered by feeding diets that differ in fatty acid composition (Rey, López-Bote and Sanz Arias, 1997), and this may lead to changes in cell membrane fluidity. Phospholipids such as phosphatidylethanolamine and sphingomyelin increase the fluidity while cholesterol has been considered to increase rigidity (Rock, 1992). Various membrane functions such as the activity of bound enzymes, the accessibility of hormone receptors and the efficiency of transport systems are controlled by membrane fluidity (Shinitzky, 1984). *Post-mortem* changes in membrane cell fluidity affect meat quality (Puolanne *et al*., 1993) and the role of cholesterol seems to be of interest.

Cholesterol functions as an integral part of the highly unsaturated phospholipid bilayer of cell membranes and is very susceptible to oxidation. Therefore, cholesterol oxidation could be initiated by the free radicals generated during lipid oxidation (Buckley, Morrissey and Gray, 1995). Cholesterol oxidation products have been reported to produce a variety of adverse biological effects and play a role in the development of arteriosclerosis (Guardiola *et al*., 1996). A variety of cholesterol oxides have been detected in animal-derived food products (Paniangvait *et al*., 1995; Tai, Chen and Chen, 2000) including meat. Their levels increase on cooking and processing, and during refrigerated storage (Pie, Spahis and Seillan, 1991). However, many studies have shown the effectiveness of dietary α-tocopherol in controlling cholesterol oxidation (Monahan *et al*., 1992; Engeseth *et al*., 1993; Galvin, Morrissey and Buckley, 1998; Maraschiello, Esteve and García Regueiro, 1998; Zanardi *et al*., 2000; Rey *et al*., 2001). On the other hand, metal ions, such as copper and iron and haem proteins, (Apte and Morrissey, 1987) catalyse lipid oxidation *in vitro*. The effect of dietary copper on meat oxidation is not so clear (Rey and López-Bote, 2001) as it also forms an intrinsic constituent of some enzymes, e.g. superoxide dismutase, that may be involved in preventing oxidative injury (Ferns, Lamb and Taylor, 1997). Furthermore, copper supplementation is thought to play a role in lipid metabolism since it has been shown to reduce cholesterol levels in rabbits, and consequently arteriosclerosis (Lamb *et al*., 1999), and increase lipid unsaturation when incorporated into pig diets at high concentrations (>200 mg/kg) (Amer and Elliot, 1973). Due to environmental contamination, copper inclusion in pig feeding is now limited to 35 mg/kg in the late fattening phase in countries in the European Union (Anonymous, 1998).

The Iberian pig is a breed which is traditionally produced by free-range feeding in evergreen-oak forests located in south

western Europe. Its diet consists mainly of acorns and grass which are a source of copper, monounsaturated and n-3 fatty acids (Rey *et al*., 1997), and antioxidants such as α-tocopherol and γ-tocopherol (Rey *et al*., 1998). Meat products (mainly hams, shoulders and M. *longissimus dorsi*) from Iberian pigs fed under these conditions, have high quality characteristics and acceptability in markets. However, it is not always possible to produce pigs in free-range conditions and, consequently, in recent years there has been increased interest in obtaining formulated diets that imitate traditional feeds. In a previous study, the supplementation of diets with vitamin E and/or copper *v*. free-range feeding on muscle composition and the susceptibility of *longissimus dorsi* muscle to oxidation was reported (Rey and López-Bote, 2001). Lipid oxidation in microsome membranes from Iberian pigs fed free-range or vitamin E-supplemented diets has also been investigated (Rey *et al*., 1997). In both studies the trend in lipid oxidation found among treatments was different and was attributed to the presence of specific substances in the membranes from the free-range Iberian pigs. Lipid oxidation has been related to membrane fluidity. However, there is a lack of information in the literature regarding the changes caused by feed on lipid classes and total cholesterol content (as factors that may affect the fluidity of membrane, and consequently its stability) in Iberian pigs.

In this study, the effect of dietary supplementation with copper and/or vitamin E in confinement on the quantity of neutral (including cholesterol) and polar lipids classes and cholesterol oxidation of *longissimus dorsi* muscle was compared with those of pigs fed free range in the traditional way.

Materials and Methods

Animals and experimental diets

Castrated male Iberian pigs (n=125), 105 (s.d. 8.3) kg live weight, were divided into five groups (n=25) at random. One group was raised in free-range conditions according to the traditional way with freely available pasture and acorns (*Quercus ilex* and *Q. rodundifolia*) (Jerez de los Caballeros, Badajoz, Spain). The other four groups were raised in confinement and were fed a basal diet (B) containing 10 mg α-tocopheryl acetate/kg feed (Hoffman La Roche, Basel, Switzerland), or the basal diet plus 100 mg α-tocopherol/kg feed (B+E), 125 mg $CuSO_45H_2O$ (B+Cu), or 125 mg $CuSO_45H_2O$ and 100 mg α-tocopherol/kg feed (B+Cu+E). The dose of copper added to the pig diet was within the limit fixed by the European Communities (Anonymous, 1998). The experimental diets and water were provided *ad libitum*. Samples of grass and acorns were taken every 2 weeks for analysis. Determination of the compositional analysis of feeds was carried out according to AOAC procedures (AOAC, 1996). The α and γ-tocopherols and copper were extracted from feed and analysed as previously described (Rey and Lopez-Bote, 2001). Chemical and fatty acid composition of the diets is shown in Table 1.

Slaughter, sample collection and chemical analysis

After feeding with the experimental diets for 56 days (mean live weight 155 (s.d. 12) kg), the pigs were stunned and slaughtered at a local slaughter house (Mafresa, Jerez de los Caballeros, Badajoz, Spain). *Longissimus dorsi* muscle samples from the last four ribs were removed, vacuum packed and frozen at –22 °C until required for analysis.

Table 1. Composition, α- and γ-tocopherol and major fatty acid composition of acorn, grass and formulated diets

	Free-range[1]		Experimental diets (confinement)			
	Acorns	Grass	B[2]	B+Vit E	B+Cu	B+Vit E + Cu
Dry matter (DM) (g/kg)	67.1±0.52	26.4 ±0.62	89.01	88.95	90.47	90.78
Crude protein (g/kg DM)	4.71±0.08	13.7±0.24	13.62	13.56	12.19	13.39
Fat (g/kg DM)	6.34±0.252	6.26±0.724	4.47	4.75	4.84	4.24
Crude fibre (g/kg DM)	5.72±0.086	22.2±0.142	4.92	4.32	4.39	4.54
Ash (g/kg DM)	1,731±0.105	7.31±0.321	6.92	5.08	4.80	4.89
Nitrogen-free extractives (g/kg DM)	81.6±1.05	50.5±0.74	70.1	72.3	73.8	72.9
α-tocopherol (mg/kg DM)	20.2±0.35	171.0±0.17	9.5	125.4	21.6	108.0
γ-tocopherol (mg/kg DM)	63.5±0.22	16.1±0.19	3.771	2.905	3.114	5.031
Copper (mg/kg DM)	78.1±0.33	4,321±0.04	17.4	15.6	46.6	41.8
Fatty acids (g/100 g total fatty acids)						
C12:0	0.02±0.004	0.21±0.006	0.05	0.05	0.04	0.05
C14:0	0.09±0.001	0.44±0.005	0.84	0.84	0.84	0.84
C15:0	0.04±0.003	0.20±0.015	0.07	0.07	0.06	0.06
C16:0	12.59±0.581	15.57±0.477	23.72	23.93	23.51	24.03
C16:1 (n-7)	0.09±0.008	0.35±0.023	1.33	1.27	1.38	1.35
C17:0	0.10±0.003	0.24±0.003	0.20	0.21	0.19	0.20
C18:0	3.22±0.151	2.03±0.068	9.37	9.74	8.99	10.01
C18:1 (n-9)	66.06±1.171	9.35±1.550	30.31	30.18	30.44	29.55
C18:2 (n-6)	14.67±0.573	11.82±0.011	28.82	29.00	28.65	27.89
C18:3 (n-3)	1.01±0.072	44.94±0.502	3.18	3.08	3.28	2.92

[1]Mean values ± s.e.

[2]The basal diet (B) contained (g/kg DM): barley 475; wheat 400; soyabean meal 80; lard 20; calcium carbonate 8; calcium phosphate 12; sodium chloride 3; vitamin mix 2. The diet had a calculated energy content of 3190 kcal/kg DM.

The concentrations of α- and γ-tocopherol were quantified as described by Rey *et al*. (1996). Briefly, 0.8 g of sample was homogenized in 0.054 M phosphate buffer, adjusted to pH 7.0 with HCl, and mixed with absolute ethanol and hexane. The upper layer containing tocopherol was removed, evaporated and dissolved in ethanol prior to analysis by reverse phase HPLC (HP 1050, Hewlett Packard, Waldbronn, Germany), using an RP-18 column (Lichrospher 100, 5 μm) (Rey *et al*., 1996). Copper was measured as reported by Rey and Lopez-Bote (2001).

Total cholesterol was determined by a modification of the method of Fenton and Sim (1991). A weighed amount (1.5 g) of sample was saponified in 50% (w/v) KOH dissolved in an alcoholic solution (95:5 ethanol:methanol) and heated for 1 h. After cooling, toluene, KOH (1N) and water were added and after separation of the layers, the upper layer (toluene extract) was taken, evaporated and dissolved in 3 ml of N,N-dimethyl formamide. Internal standard (0.5 ml of a solution containing 0.1 mg 5-α-cholestane/ml N,N-dimethyl formamide) was added to the extract which was then analysed in a gas chromatograph (Hewlett Packard HP-5890, Avondale, PA, USA), equipped with a flame ionisation detector and a HP-1 column (methylsilicone; 5 m × 0.53 mm id and 2.65 μm film thickness). A split ratio of 1:50 was used. The injector and detector temperatures were 270 °C and the oven was maintained isothermally at 280 °C. A cholesterol standard (0.1 mg/ml) was included in the internal standard, for identification and quantification. Total cholesterol was expressed as mg/100 g meat.

Neutral and polar lipids from the muscle samples were obtained using the method of Marmer and Maxwell (1981). Lipid samples were methylated in the presence of sodium methylate (0.1N) and sulphuric acid (Sandler and Karo, 1992) to obtain fatty acid methyl esters, which were analysed by gas chromatography (HP-5890, Hewlett Packard, Avondale, PA) fusing a flame ionisation detector and a HP-Innowax capillary column (30 m × 0.32 mm id and 0.25 μm film thickness) as described previously (Rey and Lopez-Bote, 2001). Results were expressed as g/100 g fatty acids.

The separation of neutral lipids and phospholipid classes was carried out by analytical thin-layer chromatography (Kates, 1986). Phospholipids and neutral lipid extracts (0.17 mg and 1 mg, respectively) were applied by means of a syringe to 20 × 20 cm plates coated with a layer of 0.25 mm thick of silica gel G-60. The plates were placed in a tank containing the eluting solvent (chloroform, methanol, acetic acid and water in the ratio 25:15:4:1 for separating phospholipid classes, and petroleum ether, ethyl ether and acetic acid in the ratio 25:15:0.45 to separate neutral lipids). Plates were removed from the tank and sprayed with reagent, containing 0.05% $FeCl_3$ in water:acetic acid:sulphuric acid in the ratio 90:5:5 (volume basis) and afterwards dried at 100 °C for 20 min to render the lipids visible. Identification was made by comparing the migration of pure standards relative to the front (R_F) with the R_Fs of the lipids. Quantification was made using a densitometer (9001 PC, Shimadzu, Duisburg, Germany).

Colesterol oxidation products (COPs) in muscle were determined on day 3 of refrigerated (4 °C) display. Prior to analysis, the muscle was cooked at 200 °C for 20 min to an internal temperature of 70 °C. Total lipid extraction was carried out on a 2.5-g sample using the method of Folch, Lees and Sloane Stanley (1957). After addition of 50 μg of internal standard

(6-ketocholesterol/ml ethyl acetate) to the sample extract, the cholesterol oxides were separated from other muscle lipids using the sample clean up procedure proposed by Park and Addis (1985). COPs were determined by gas chromatography (Shimadzu 14A) using a RTX-1 capillary column (15 m × 0.32 id, 0.25 μm film thickness) as described previously (Rey *et al*., 2001). Identification was made by comparison with retention times of the corresponding pure standards. Results were expressed as μg of cholesterol oxides/g muscle.

Statistical analysis

The data were analysed using the General Linear Model of SAS (1989). The individual pig was the experimental unit for analysis of all data. The comparative analysis between means were determined using the following orthogonal contrasts: (1) free-range feeding *v*. confinement; (2) 10 mg α-tocopheryl acetate/kg feed *v*. 100 mg α-tocopheryl acetate/kg feed supplementation, (3) non-copper supplemented groups *v*. copper supplemented, (4) α-tocopheryl acetate × copper interaction. Data were presented as the means of each group and the standard error of the mean (s.e.) together with the significance levels of the main effects and interactions.

Results and Discussion

The composition, and α- and γ-tocopherols, copper and major fatty acid concentration of acorns, grass and formulated experimental diets are shown in Table 1. The acorns had a low concentration of protein and a relatively high concentration of fat rich in C18:1, n-9 monounsaturated fatty acids, while the grass, had relative high proportions of protein and C18:3, n-3 fatty acids. Grass also had the highest α-tocopherol concentration, higher even than the diets supplemented with α-tocopheryl acetate, while acorns had the highest γ-tocopherol concentrations. These values are within the ranges found in the literature (Lynch, 1991; Rey *et al*., 1998). The copper concentration in the diets agree with the supplementation level (Dove and Ewan, 1990). Acorns had the highest copper concentration; however, a small amount was detected in grass, in agreement with previously reported values (Cooke, 1983). The acorns and grass intake of the pigs was not measured, but can be estimated since Iberian pigs have been reported to eat a ratio of 2 to 3:1 (acorns:grass) depending on the climatic conditions (approximately 600 kg acorns and up to 300 kg grass during a 60-day free-range period; Aparicio, 1987).

The chemical composition (protein, ash, neutral and polar lipids, total pigments and copper) of the *longissimus dorsi* muscle, as affected by dietary copper and α-tocopheryl acetate, was determined in a previous study (Rey and López-Bote, 2001) and no significant differences nor interactions were detected in protein, ash, neutral and polar lipids or copper concentrations. Dietary supra-nutritional α-tocopheryl acetate (100 mg/kg feed) produced significantly ($P<0.001$) greater amounts of α-tocopherol in muscle when compared to those from pigs fed the basal diet, which contained 10 mg/kg (Table 2). Moreover, the *longisssimus dorsi* muscle from free-range pigs had higher concentrations of α-tocopherol than those from pigs fed in confinement with the basal level of α-tocopheryl acetate, but lower values than pigs fed supplemented levels in the B+E or B+E+Cu diets. The concentration of γ-tocopherol was significantly higher in muscles from the free-range fed pigs than in those fed in confinement (Table 2), which is attributed to the relative high concentration of γ-tocopherol in

Table 2. The concentration of α- and γ-tocopherol, copper and total cholesterol in *longissimus dorsi* muscle from Iberian free-range fed pigs or pigs fed in confinement on the experimental diets

	Free-range	Experimental diets[1]				s.e.	Significance of contrasts[2]			
		B	B+Vit E	B+Cu	B+Vit E+ Cu		1	2	3	4
α-tocopherol (mg/g)	2.97	2.16	3.81	2.64	3.60	0.302			***	
γ-tocopherol (mg/g)	1.50	0.004	0.010	0.004	0.003	0.104	***			
Copper (mg/g)	1.67	1.63	1.66	1.73	1.70	0.102				
Total cholesterol (mg/100 g)	37.3	36.5	37.8	39.0	35.0	1.320				
Cholesterol:phospholipid ratio	0.047	0.047	0.044	0.046	0.034	0.002	*	*	**	*

[1]B = Control (basal) diet; B+E = Control diet + 100 mg/kg α-tocopheryl acetate; B+Cu = Control diet + 125 mg/kg copper sulphate (H_2SO_4); B+E+Cu = Control diet + 100 mg/kg α-tocopheryl acetate + 125 mg/kg copper sulphate (5 H_2O).
[2]Contrast: 1 = Free-range *v*. experimental diets (confinement); 2 = Copper supplementation *v*. no copper supplementation; 3 = Vitamin E supplementation in confinement *v*. no vitamin E supplementation in confinement; 4 = interaction copper with vitamin E.

the acorns (Table 1). These data are in agreement with previous findings in which dietary acorns provide a source of γ-tocopherol, while grass is rich in α-tocopherol (Rey *et al*., 1998).

The total cholesterol concentration of *longissimus dorsi* muscle (Table 2) ranged from a minimum of 35.0 mg to a maximum of 39.0 mg/100 g of tissue. An extensive review of the literature showed no reports of the levels of total cholesterol in *longissimus dorsi* muscle from Iberian pigs; hence, comparisons cannot be made. However, in pork, average values range from 55.9 mg to 62 mg/100 g (Bohac and Rhee, 1988), which are higher than those of the present study. These low cholesterol levels could be due to the high proportion of marbling fat. Since cholesterol is an integral part of the membranes, intramuscular fat contains little cholesterol and consequently, the total cholesterol concentration decreases with an increase in lipid concentration (Tu, Powrie and Fennema, 1967). On the other hand, the cholesterol concentration was not significantly affected by the dietary treatments. The evidence on the effect of dietary lipid composition on muscle cholesterol concentration is not consistent. Some authors (Bohac and Rhee, 1988; Barowicz *et al.*, 1997; Zanardi *et al*., 2000) found no effect when using either dietary oils rich in monounsaturated or polyunsaturated fatty acids (PUFA), while others (Barowicz, Brzoska and Pietras, 2000) have reported an hypocholesteronemic influence of n-3 fatty acids. The cholesterol concentration of the muscle was not significantly affected by dietary copper at the levels added. It has been reported that copper could reduce the cholesterol content of muscle (Lamb *et al*., 1999; Engle and Spears, 2000). However, some authors only observed this effect when the dietary treatment modified the copper concentrations of the tissues (Lamb *et al*., 1999).

The cholesterol:phospholipid (cho:phos) ratio (Table 2) of muscle was calculated in order to study possible changes in membrane fluidity, which have been related to lipid oxidation (Nivsarkar, Cherian and Patel, 1998). Cholesterol decreases the fluid state of the membranes under physiological conditions, thus the cho:phos ratio can be used as an indicator of cell stability (Shinitzky, 1984). All membranes do not need the same level of fluidity for optimal function, and the membrane-stabilising effect of cholesterol molecules can be replaced by the length or the degree of unsaturation of fatty-acyl chains (Rock, 1992). Post-slaughter changes in lipid composition occur and, consequently meat quality could be affected (Puolane *et al*., 1993). In the present study, it is interesting that a significantly lower ($P<0.01$) cho:phos ratio was found in those muscle samples from pigs fed supra-nutritional vitamin-E in their diets (Table 2). An increase in membrane fluidity produced by vitamin-E supplementation has been reported previously (Pezeshk and Dalhouse, 2000). This effect may be explained by the slightly lower concentration of cholesterol (although not significant) in the groups fed in confinement on the diet supplemented with 100 mg vitamin E/kg compared to those on diets containing 10 mg/kg diet (Table 2). Agboola *et al*. (1990) found that liver cholesterol was lower in rats fed vitamin E. It was also observed that muscle from pigs fed a diet supplemented with copper had a lower ($P<0.05$) cho:phos ratio compared with those fed non-copper supplemented diets and a statistically significant interaction ($P<0.05$) between copper and vitamin E was detected. Lei *et al*. (1988) found that the fluidity of the liver plasma membranes in copper-deficient rats was significantly decreased by alteration of the phospholipid fatty

acid composition of the diet. However, other studies show a contrary effect of copper (Rock *et al*., 1995). The different results among authors might be due to interactions between membrane components which may indicate differences in cell membrane fluidity. Concerning the free-range fed group, it is of interest to note that they had a significantly higher ($P<0.05$) cho:phos ratio, which would indicate an increased membrane rigidity. This would not be expected since muscle from these pigs had a significantly greater level of γ-tococopherol and intermediate levels of α-tocopherol, which increase fluidity, than the other groups.

The free-range fed group had a higher ($P<0.05$) proportion of saturated and n-3 fatty acids in the phospholipids of their muscles than those fed in confinement. The higher n-3 fatty acid proportion can be attributed to the intake of grass, and the high saturated fatty acid proportion is likely explained by metabolic regulation. Lutz *et al*. (1998) found the highest membrane fluidity in rats fed an oil rich in n-3 fatty acids, which is contrary to the effect observed in the present study. However, saturated fatty acids enhance the rigidity of the membrane (Brasitus, Davidson and Schachter, 1985); hence, the higher concentration of these fatty acids could probably explain, in part, the results of the present study. The presence of antioxidants such as α- and γ-tocopherol, n-3 fatty acids and probably other substances provided by the particular feeding of the free-range fed pigs (Rey *et al*., 1997), interacting with the different classes of phospholipids in the muscle from these pigs, could lead to a higher incorporation of saturated fatty acids to maintain membrane organisation. Phospholipids were also separated into individual classes by thin layer chromatography (Table 3). Phosphatidylcholine (PC) was the major fraction accounting for approximately 60% of total phospholipids. The minor fractions were lysophosphatidylcholine (LPC) and phosphatidylserine (PS). Comparable results have been observed in previous researches in pigs (Christie and Moore, 1974). In the current experiments, muscle from free-range fed pigs showed significantly higher proportions of LPC ($P<0.05$) and PS ($P<0.01$) and lower sphingomyelin (SpH) concentration ($P<0.05$) compared with those groups fed in confinement. SpH is a phospholipid fluidizer (Christie and Moore, 1974), like phosphatidylethanolamine (PE), so the ratios PE:PC and SpH:PC are also used as indicators of the state of membrane fluidity. Results of the free-range fed group followed a similar trend to that observed for the cho:phos ratio even though differences in PE:PC (0.16 *v*. 0.19) and SpH:PC (0.13 *v*. 0.14) were not statistically significant. No effects of dietary copper or vitamin E were detected on the levels of the different classes of phospholipids.

When neutral lipids of pork muscle were separated into classes (Table 4), the triglyceride fraction accounted for the major portion (approximately 80%), followed by cholesterol, free fatty acids, monoglycerides and cholesterol esters. The cholesterol ester concentration was about 7% of the total cholesterol. These data are in agreement with those of Tu *et al*. (1967). It is interesting to observe that muscle from free-range fed pigs had significantly higher ($P<0.01$) proportions of free fatty acids and lower levels of ($P<0.01$) cholesterol esters; in contrast, the proportion of monoglycerides, cholesterol and triglycerides were not affected by the experimental diets. The lipid classes of Iberian pigs have not been discussed previously in the literature and there is little information on pig muscle. Free fatty

Table 3. Mean phospholipids and phospholipid classes of *M. longissimus dorsi* from either free-range fed pigs, or pigs fed in confinement on experimental diets

	Free-range	Experimental diets[1]				s.e.	Significance of contrasts[2]			
		B	B+Vit E	B+Cu	B+Vit E+Cu		1	2	3	4
				Phospholipids (g/100 g)						
Saturated	34.3	33.4	33.0	32.4	32.8	0.283	*			
Monounsaturated	25.0	23.8	24.9	26.6	24.3	0.832				
Polyunsaturated	40.7	42.8	42.1	40.9	42.9	0.929				
n-3	4.767	3.727	4.043	3.786	4.308	0.194	*			
n-6	36.0	39.1	38.0	37.2	38.6	0.963				
n-9	22.8	21.7	22.5	24.0	22.1	0.764				
UI[3]	1.40	1.44	1.44	1.42	1.45	2.118				
n-6:n-3	8.39	10.83	9.45	9.87	9.49	0.415	*			
				Phospholipid classes (g/100 g)						
Lysophosphatidylcholine	3.39	2.82	2.70	2.74	2.66	0.141	*			
Sphingomyelin (Sph)	6.56	8.85	6.98	7.44	7.80	0.263	*			
Phosphatidylcholine (PC)	53.20	57.06	55.60	55.55	56.12	0.950				
Phosphatidylserine	3.10	2.08	2.68	1.66	1.63	0.183	**	P<0.1		
Phosphatidylinositol	8.34	9.26	7.28	7.14	6.80	0.831				
Phosphatidylethanol-amine (PE)	8.41	10.09	10.92	10.14	11.14	0.833				
Cardiolipin	17.04	16.42	16.14	15.67	13.51	1.061				
PE:PC	0.16	0.18	0.20	0.19	0.21	0.016				
SpH:PC	0.13	0.16	0.13	0.13	0.13	0.004				

[1,2]See footnotes to Table 2.
[3]UI: Unsaturation index (average number of double bonds per fatty acid residue).

acids are known to be increased in blood by those processes that enhance the rate of fat utilisation, such as exercise. Goodman *et al*. (1973) previously found that trotting and conditioning increased free fatty acid levels in muscles of horses. Consequently, the higher proportion of free fatty acids of those free-range pigs was expected. Cholesterol esters are obtained from the reaction between lecithin (phosphatidylcholine) and cholesterol, catalysed by the enzyme, lecitin-cholesterol acyltransferase (LCAT) (Welch and Borlakoglu, 1992), with linoleate being the most preferred substrate for LCAT activity (Dobiassova, 1983). In the present study, feeding a free-range diet to the pigs resulted in a lower content of linoleic fatty acid in the *longissimus dorsi* muscle than the formulated diets. It has also been suggested that the increased fluidity of the phospholipid resulting from feeding PUFA could increase the rate of the LCAT reaction (Morrisett *et al*., 1977). This may explain, in part, the lower concentration of cholesterol esters in the muscle from the free-range fed pigs. More recently, Xi-Zhongsheng and Xia (1998) found lower cholesterol esters and phospholipids in liver from chicks fed diets enriched with n-6 and/or n-3 fatty acids than chicks fed palm oil, suggesting that n-3 PUFA had a greater influence in decreasing tissue lipid level compared with n-6 PUFA.

COPs levels in the muscle were also investigated (Table 5). The cholesterol concentration was not significantly affected by

Table 4. Main neutral lipids and neutral lipid classes of *M. longissimus dorsi* from free-range fed pigs or in pigs fed in confinement on experimental diets

	Free-range	Experimental diets[1]				s.e.	Significance of contrasts[2]			
		Basal	B+Vit E	B+Cu	B+Vit E+Cu		1	2	3	4
Neutral lipids (g/100 g)										
Saturated	38.06	39.1	37.83	39.68	39.63	0.829				
Monounsaturated	58.13	57.86	58.58	56.68	57.31	1.122		P<0.01		
Polyunsaturated	3.99	3.24	3.77	3.83	3.25	0.447				
n-3	0.55	0.75	0.69	0.81	0.46	0.244				
n-6	3.44	2.49	3.08	3.03	2.79	0.274	*			
n-9	51.87	51.34	52.02	50.12	49.29	0.947		*		
UI[3]	0.68	0.67	0.68	0.67	0.65	0.018				
n-6:n-3	7.43	6.45	8.55	7.87	7.47	1.757				
Neutral lipid classes (g/100 g)										
Monoglycerides	1.06	1.09	1.01	0.99	0.85	0.089				
Cholesterol	7.69	9.35	8.12	8.24	8.64	0.987				
Free fatty acids	6.91	4.94	4.52	4.22	3.65	0.803	**			
Tryglicerides	81.32	82.77	82.61	82.91	83.92	1.351				
Cholesterol esters	0.54	0.95	0.80	1.15	0.87	0.123	**			
Hidrocarbur	2.57	1.83	3.34	2.93	2.07	0.566				

[1,2]See footnotes to Table 2.
[3]UI: Unsaturation index (average number of double bonds per fatty acid residue).

dietary treatment; therefore, differences in the COPs can only be attributed to differences in oxidation. The COPs levels found in cooked muscle from Iberian pigs after 3 days of refrigerated display were principally β-epoxide, 7β-hydroxycholesterol, 25β-hydroxycholesterol and 7-ketocholesterol. The COPs concentration of Iberian pig meat has not been described previously but the levels are in agreement with previous findings in pork (Monahan *et al*., 1992; Rey *et al*., 2001). Addition of α-tocopheryl acetate to the diet consistently reduced ($P < 0.01$) total COP formation in *longissimus dorsi* muscle from Iberian pigs compared to those pigs fed diets without vitamin E supplement. However, no effect of dietary copper was found on total cholesterol oxide concentration. Vitamin E supplementation produced lower β-epoxide, 7β-hydroxycholesterol and 25β-hydroxycholesterol. On the other hand, Iberian free-range fed pigs had lower COP values than those groups supplemented with 10 mg/kg α-tocopheryl acetate but higher than those supplemented with 100 mg/kg; however, the differences were not statistically significant. The effectiveness of dietary α-tocopherol in controlling cholesterol oxidation have been extensively described (Monahan *et al*., 1992; Galvin *et al*., 1998; Zanardi *et al*., 2000; Rey *et al*., 2001). Its effects are due to its specific localisation in the cell structures (Buckley *et al*., 1995). Muscle from Iberian free-range fed pigs is known to contain antioxidant substances such as α- and γ-tocopherol (Rey *et al*., 1998), which allow maintenance of a low oxidative status of the cellular membranes (Rey *et al*., 1997). In addition, inhibition of lipid oxidation has been related to a reduction in membrane fluidity (Nivsarkar *et al*., 1998). Consequently, a low concentration of cholesterol oxides would be expected in the free-range fed group. However,

Table 5. Concentration (µg/g) of cholesterol oxidation products in cooked muscle from free-range fed Iberian pigs and from Iberian pigs fed in confinement on experimental diets

	Free-range	Experimental diets[1]				s.e.	Significance of contrasts[2]			
		Basal	B+Vit E	B+Cu	B+Vit E+ Cu		1	2	3	4
β-Epoxide (µg/g)	0.044	0.243	0.125	0.361	nd[3]	0.065	P<0.08		**	
7β-Hydroxycholesterol (µg/g)	0.362	1.076	0.664	0.793	0.205	0.184		P<0.06	*	
25-Hydroxycholesterol (µg/g)	1.252	1.267	0.811	1.378	1.191	0.156			P<0.06	
7-Ketocholesterol (µg/g)	0.635	0.990	0.454	0.824	0.667	0.208				
Total cholesterol oxidation products	2.293	3.577	2.053	3.356	2.063	0.379			**	

[1,2]See footnotes to Table 2.
[3]Not detected

cholesterol oxidation in cooked muscle did not follow the same trend as that observed for microsome oxidation in fresh muscle (Rey *et al*., 1997). It has also been reported that free-range fed pigs have a higher content of myoglobin in muscle than those fed in confinement (Rey and López-Bote, 2001). Haem proteins, such as myoglobin, can catalyse lipid oxidation (Apte and Morrissey, 1987) because they are a source of iron. Moreover, an investigation of the effects of cellular prooxidants on cholesterol oxidation in a liposomal model system showed that iron/ascorbate, copper and metmyoglobin/H_2O_2 accelerated cholesterol oxidation (Connolly *et al*., 1998), suggesting that haem proteins and metal ions could contribute to cholesterol oxidation in meats. The presence of a higher concentration of free fatty acids in the muscle from the free-range fed pigs could also act on cholesterol oxidation. However, how the *post-mortem* status of meat is affected by the level of free fatty acids of muscle is unclear. In the present study, heating could alter the membrane structure and make iron and other possible pro-oxidants more accessible in the cooked *longissimus dorsi* muscles from free-range fed pigs. The effects observed by cooking in this study may not apply to other processing treatments (e.g. salt addition) for Iberian pig meat. Therefore, further research is needed.

In conclusion, free-range feeding modified the proportion of neutral and polar lipids classes, without altering total cholesterol concentration of muscle from Iberian pigs compared with those groups supplemented with vitamin E and/or copper. Supplementation with dietary α-tocopheryl acetate is more effective in reducing cholesterol oxidation products in cooked muscle from Iberian pigs than free-range feeding. The increased membrane rigidity of muscle is not the only indicator of lipid stability, and muscle composition and meat treatment must also be considered.

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