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Concentrations of cholesterol oxidation products in raw, heat-processed and frozen-stored meat of broiler chickens fed diets differing in the type of fat and vitamin E concentrations

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The present study was performed to investigate the effect of dietary fat and vitamin E on concentrations of cholesterol oxidation products (COP) in broiler muscle. A total of 144 1-d-old broiler chicks were fed diets with either palm oil, soyabean oil or linseed oil and vitamin E concentrations of 20, 40 or 200 mg/kg for 35 d. COP concentrations were analysed in raw, heat-processed (180°C, 20 min) and frozen-stored (−20°C, 6 months) breast and thigh muscles. COP concentrations were influenced by dietary vitamin E concentration, dietary fat, treatment and type of muscle ($P<0{\cdot}001$). Increasing the dietary vitamin E concentration generally reduced the concentration of COP. This effect was strongest in broilers fed linseed oil and weakest in broilers fed palm oil; the effect of vitamin E was also stronger in heated muscles than in raw or frozen-stored muscles. Moreover, the concentration of COP in thigh muscle was more strongly influenced by dietary vitamin E than that in breast muscle. COP concentrations in muscles were on average highest in broilers fed linseed oil and lowest in broilers fed palm oil, but the effect of the dietary fat also depended on the vitamin E concentration, the treatment and the type of muscle. In conclusion, our study shows that dietary fat and vitamin E influence the concentrations of total COP in broiler muscle. However, the effects of these factors were not only influenced by interactions between each other, but also depended on the treatment of the muscle and the type of muscle.

Broiler: Dietary fat: Vitamin E: Cholesterol oxidation products: Muscle

Cholesterol, an important constituent of cellular membranes in animals, is a molecule that is susceptible to oxidation (Smith, 1996). Several cholesterol oxidation products (COP) are generated during the non-enzymatic oxidation of cholesterol. As constituents of foodstuffs, COP are highly relevant from a pathophysiological viewpoint. In man, they are absorbed in the intestine, enter the liver, are incorporated into lipoproteins and reach all tissues of the body (Emanuel *et al.* 1991; Linseisen & Wolfram, 1998). In cells, they are predominantly incorporated into membranes, where they affect important membrane properties such as their structure and fluidity and the activities of membrane-bound enzymes (Stubbs & Smith, 1984). COP also promote the development of several diseases such as CHD (Chisolm *et al.* 1994; Brown & Jessup, 1999) and cancer (Morin & Peng, 1992).

Animal products contain COP, which are generated from cholesterol during storage or heat-processing (Paniangvait *et al.* 1995; Maraschiello *et al.* 1998). Most COP are formed in the course of autoxidation of PUFA (Terao *et al.* 1985; Sevanian & McLeod, 1987). The meat of broilers contains relatively high quantities of PUFA and is therefore susceptible to oxidation, particularly during storage or heat-processing. The autoxidation of PUFA during the storage or heat-processing of broiler meat can be modified by dietary fats or vitamin E (Jensen *et al.* 1995; Galvin *et al.* 1997; O'Neill *et al.* 1998; Ruiz *et al.* 1999; Sanz *et al.* 1999; Grau *et al.* 2001). It has been shown that dietary vitamin E reduces the concentrations of COP in raw or cooked chicken meat (Li *et al.* 1996; Galvin *et al.* 1997; Lopez-Bote *et al.* 1998; Grau *et al.* 2001).

The effects of dietary fats on the formation of COP in broiler meat are, however, less clear because only a few studies have been conducted, which have yielded controversial results. In the study of Maraschiello *et al.* (1998), the formation of COP during cooking was lower in the meat of chickens fed olive oil than in the meat of chickens fed sunflower oil or lard. The beneficial effect of olive oil in this respect might, however, have been at least partially due to its high amount of native antioxidants, which might have reduced the formation of COP. In the study of Grau *et al.* (2001), the replacement of beef tallow by linseed oil, a highly unsaturated fat, even reduced concentrations of COP in cooked chicken meat, although it strongly increased the concentrations of thiobarbituric acid-reactive substances (TBARS). This study therefore did not show a correlation between the oxidation of PUFA and the oxidation of cholesterol. Therefore, the effects of dietary fats on the formation of COP require further elucidation.

With this aim, we conducted a study with growing chickens fed diets with either palm oil, soyabean oil or linseed oil. Palm oil was used as a fat source with a low concentration of PUFA, whereas soyabean oil and linseed oil were used as fat sources

Abbreviations: COP, cholesterol oxidation product; LS, least squares ; TBARS, thiobarbituric acid-reactive substances.

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with a high concentration of PUFA. Our hypothesis was that an enrichment of chicken muscle with PUFA would enhance the formation of COP as a result of an increased rate of lipid peroxidation. If this hypothesis were shown to be true, an interaction between dietary PUFA and dietary vitamin E would be expected. In order to study such an interaction, we used diets with low (20 mg/kg), adequate (40 mg/kg) and high (200 mg/kg) concentrations of vitamin E. To discover a possible relationship between the rate of PUFA autoxidation and the rate of cholesterol oxidation, we calculated correlations between concentrations of PUFA oxidation products (TBARS and hydroperoxides) and the concentrations of COP in the chicken meat. The formation of COP in chicken meat is stimulated by heating or storage (Maraschiello *et al.* 1998; Grau *et al.* 2001; Lee *et al.* 2001). Therefore, we expected that dietary PUFA and vitamin E might have stronger effects on the COP concentrations in heat-processed or stored meat than in raw meat, in which the rate of lipid peroxidation might be low. To test this, we determined the concentrations of COP in raw, heat-processed and stored chicken thigh and breast muscles. Thigh and breast muscles were chosen because they differ in their resistance to the oxidation of lipids (Galvin *et al.* 1997). We expected that dietary fat and vitamin E might have a stronger effect on the formation of COP in thigh muscle than in breast muscle. It has been shown that different types of COP, i.e. 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol-epoxides, exert different effects in the human body (Morel & Lin, 1996). We therefore considered the formation of various COP that were detectable in broiler meat.

Materials and methods

Animals and diets

A total of 144 1-d-old broiler chicks (Ross) were used. These were assigned to nine groups of sixteen animals each. They were allocated to floor pens on wood shavings with eight birds per pen in an animal house at the Universities Research Centre. Permanent artificial illumination was applied, and room temperature decreased in the following steps: day 1, 34°C; day 2, 33°C; days 3–5, 32°C; days 6 and 7, 31·5°C; days 8–15, 30°C; thereafter the temperature was continuously reduced and maintained at 22°C. From day 2 to day 36, the chickens were fed a nutritionally adequate basal diet according to recommendations from the German Nutrition Society (GfE 1999; Table 1). Either palm oil (groups 1, 2 and 3), soyabean oil (groups 4, 5 and 6) or linseed oil (groups 7, 8 and 9) was used as the source of fat.

The tocopherol concentrations of the oils were analysed. Concentrations of α-tocopherol equivalents in the oils were calculated assuming that 1 mg γ-tocopherol is equivalent to 0·1 mg α-tocopherol, and 1 mg δ-tocopherol is equivalent to 0·03 mg α-tocopherol (Horwitt, 1976); β-tocopherol was not detected in any oil. The concentrations of α-tocopherol equivalents of palm oil, soyabean oil and linseed oil were 165, 146 and 46 mg/kg, respectively. The basal diet without fat supplement contained 9·0 mg α-tocopherol equivalents/kg. The diets were individually supplemented with DL-α-tocopherol acetate (VWR International, Darmstadt, Germany) to adjust the vitamin E content to either 20 (groups 1, 4 and 7), 40 (groups 2, 5 and 8) or 200 mg (groups 3, 6 and 9) α-tocopherol equivalents/kg, considering that 1·49 mg DL-α-tocopherol acetate has an activity of 1 mg α-tocopherol equivalent (Weiser & Vecchi, 1982). The fatty acid composition of total lipids in the diets containing palm oil, soyabean oil and linseed oil is shown in Table 2.

Table 1. Composition of the basal experimental diet*

Ingredient	Amount (g/kg)
Wheat	500
Soyabean meal	250
Corn	110
Fat†	60
Rapeseed meal	40
Mineral and vitamin premix	10
$CaHPO_4$	17·6
$CaCO_3$	8·0
NaCl	3·2
DL-Methionine	1·2
Analysis (g/kg)	
Crude protein	219
Crude fat	84
Crude fibre	35
Crude ash	62
Energy (MJ metabolisable energy/kg), calculated‡	13·4

* For details of diets and procedure, see p. 634 and Table 2.
† Calculated according to data provided by Jahrbuch für die Geflügelwirtschaft (2000).

Feed was offered *ad libitum* from one trough per pen, and water was continuously available from nipple drinkers with attached cups. Body weight was measured at the beginning and the end of the trial. Feed intake was determined per pen. All experimental procedures described were approved by the council of Saxony-Anhalt.

Sample preparation

On day 35, the broilers were stunned, slaughtered and bled at a local slaughterhouse using standard industry procedures. Twenty-four hours after slaughter, the right breast and leg muscles were separated. Skin and adhering tissue were carefully removed. The breast and thigh muscles of two broilers from each diet group were pooled. The pooled meat was homogenised (Homogenisator 2094; Foss GmbH, Hamburg, Germany). Half of the meat was vacuum-packed and stored at −20°C for 6 months before use. Aliquots of approximately 50 g were formed into meatballs of 12 mm height and heated in an oven at 180°C for 20 min. The remaining meat was used immediately for analysis of the raw meat.

Analyses

Concentrations of crude nutrients in the diets were analysed according to the official Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten methodology (Bassler &

Table 2. Fatty acid composition (mol/100 mol total fatty acids) of total lipids of the diets containing either palm oil, soyabean oil or linseed oil*
(Mean values of three separate analyses per diet)

Dietary fat	Palm oil	Soyabean oil	Linseed oil
14 : 0	1·4	0·3	0·2
16 : 0	37·9	13·3	10·0
18 : 0	4·9	4·1	5·1
18 : 1	36·3	26·4	24·0
18 : 2*n*-6	16·7	46·9	18·4
18 : 3*n*-3	0·6	6·7	40·4

* For details of diets and procedure, see p. 634.

Buchholz, 1993). Lipids were extracted from muscle samples with a mixture of n-hexane and isopropanol (3 : 2, v/v) at room temperature for a period of 18 h (Hara & Radin, 1978). The fatty acid composition of the total lipids from the muscles and diets was analysed by GC. Fats were methylated with trimethylsulfonium hydroxide (Butte, 1983). Fatty acid methyl esters were separated by GC using a system (HP 5890; Hewlett Packard GmbH, Waldbronn, Germany) equipped with an automatic on-column injector, a polar capillary column (30 m FFAP, 0·53 mm internal diameter; Macherey and Nagel, Düren, Germany) and a flame ionisation detector (Eder & Brandsch, 2002). The carrier gas was He with a flow rate of 5·4 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards. The unsaturation index of the fatty acids has been calculated as the average number of double bonds per mol fatty acids.

Concentrations of tocopherols in muscle and diets were determined by HPLC (HP 1100; Hewlett Packard) (Balz *et al.* 1993). Samples of approximately 50 mg were mixed with 1 ml 0·1 g/l pyrogallol solution (ethanol, absolute) and 150 μl saturated NaOH solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with n-hexane. Individual tocopherols of the extracts were separated isocratically by HPLC using a mixture of n-hexane and 1,4 dioxane (94 : 6, v/v) as mobile phase and a LiChrospher Si-60 column (5 μm particle size, 250 mm length, 4 mm internal diameter; Hewlett Packard) and detected by fluorescence (excitation wavelength 295 nm, emission wavelength 330 nm). The total tocopherols were calculated as the sum of α-, γ-, and δ-tocopherols. β-Tocopherol was not detected in any sample.

The concentrations of TBARS were measured in an aqueous extract of muscle tissue. Muscle tissue (1·5 g) was homogenised with a Potter Elvehjem homogeniser (Potter® 5;B. Braum Bintech International, Melsungen, Germany) in 4 ml phosphate buffer (0·05 M, pH 7·0) on ice. The homogenates were centrifuged (1·000 **g**, 10 min, 4°C), and the supernatants were used for a modified version of the TBARS assay (Sidwell *et al.* 1954). Sample aliquots were mixed with thiobarbituric acid reagent (8 g/l thiobarbituric acid with 7 % perchloric acid; 2 : 1, v/v) and heated for 60 min at 95°C. TBARS were extracted with n-butanol, and absorption was measured at 532 nm. Concentrations were calculated via a standard curve with 1,1,3,3,-tetraethoxypropane.

Concentrations of lipid hydroperoxides were measured in a methanolic aqueous extract of muscle tissue: 1·0 g muscle tissue was homogenised with an Ultraturrax (T25; IKA, Stauffen, Germay) in 5 ml methanol on ice. The homogenates were centrifuged (1·000 **g**, 10 min, 4°C), and the supernatants were analysed using the ferrous oxidation xylenol orange method (Hermes-Lima *et al.* 1995).

Concentrations of COP were determined in lipid extracts by Gc/ms in selected ion-monitoring mode according to the method of Mori *et al.* (1996) with modifications. Aliquots of the extracts were mixed with 20 μg internal standard 5α-cholestane. The samples were dried under N_2 gas, mixed with 2 ml 1 mol/l methanolic NaOH solution and incubated for 18 h at room temperature. The cholesterol fraction of the extract was isolated by HPLC according to the method of Nourooz-Zadeh & Appelqvist (1988) and collected by a fraction collector. The solvent was evaporated under low pressure and the dried fraction dissolved in 100 μl pyridine. After derivatisation of the oxides with 100 μl 0·1 g/l trimethylchlorosiane solution (bis(trimethylsilyl)-trifluoroacetamide), samples were separated using a non-polar capillary column (DB-5, 30 m × 0·25 mm internal diameter; J&W Scientific, Folsom, CA, USA) and detected by single-ion monitoring. The carrier gas was He with a flow of 1·0 ml/min. The COP 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide (β-epoxycholesterol), cholestanetriol and 25-hydroxycholesterol were identified by comparing their retention times with those of authentic standards and quantified against the internal standard.

Statistics

The statistical analysis was performed with the SAS package (version 9.1, procedure mixed, procedure corr; SAS Institute, Cary, NC, USA). For analysis of the COP, hydroperoxides and TBARS concentrations, we used a linear model with fifteen fixed effects (four main effects – fat, vitamin E, treatment and muscle – and all corresponding two-way, three-way and four-way interactions). To analyse the total tocopherol concentrations, we included seven fixed effects (three main effects – fat, vitamin E and muscle – and all corresponding two-way and three-way interactions). To take into account the partly differing magnitudes of the parameters, the error variances of COP, hydroperoxides and TBARS were calculated within the vitamin E × treatment × muscle level, and the error variances of total tocopherols within the vitamin E × muscle level.

Since the data from different treatments and muscles are observed for the same animal, a random animal effect was included. Because we have a mixed linear model with heterogeneous variances and unbalanced data, we used the restricted maximum likelihood method for variance component estimation and the Kenward–Roger method to calculate the standard errors and degrees of freedom (Kenward & Roger 1997; Spilke *et al.* 2005). To control the type I statistical error on a per experiment basis, we used the Tukey–Kramer test for least square means comparison (Dufner *et al.* 2002). For analysis of correlation, the Pearson product moment correlation coefficient was calculated using the Minitab statistical software (Release 13; Minitab Inc., State College, PA, USA).

Results

Growth performance of the broilers

Initial and final body weights, feed intake and body weight gains did not differ between the nine groups of broilers. The initial body weights of the broilers were (means, *n* 144) 38 (SE 1) g, average daily feed intake was 56 (SE 1) g, average daily body weight gain was 39·6 (SE 0·4) g, and the final body weights of the broilers at day 35 were 1425 (SE 13) g.

Fatty acid composition of breast and thigh muscle total lipids

The fatty acid composition of the total lipids from the breast and thigh muscles was dependent on the dietary fat but independent of the dietary vitamin E concentration. Breast and thigh muscles from broilers fed palm oil had higher amounts of saturated fatty acids and MUFA but lower amounts of PUFA in total lipids than did muscles from broilers fed soyabean or linseed oil. The breast and thigh muscles from broilers fed soyabean oil had higher amounts of *n*-6 PUFA (18 : 2, 20 : 4 and 22 : 4) and lower amounts of *n*-3 PUFA (18 : 3, 20 : 5, 22 : 5 and 22 : 6) than the

breast muscle from broilers fed linseed oil (data not shown). The unsaturation index of the fatty acids in both muscles was highest in broilers fed linseed oil and lowest in broilers fed palm oil (breast muscle: palm oil 1·08 ± 0·01, soyabean oil 1·38 ± 0·01, linseed oil 1·46 ± 0·01; thigh muscle: palm oil 0·91 ± 0·01, soyabean oil 1·25 ± 0·02, linseed oil 1·41 ± 0·01).

Concentrations of total tocopherols in breast and thigh muscle

Concentrations of total tocopherols were determined in raw muscles (Table 3). There were significant effects of dietary fat, vitamin E and muscle on the concentration of total tocopherols. Thigh muscle had higher concentrations of total tocopherols than breast muscle (least squares (LS) means, *n* 72; 211 (SE 4) *v.* 110 (SE 3) nmol/g DM), in average of dietary treatment (fat and vitamin E). Tocopherol concentrations in muscles increased with increasing vitamin E concentrations of the diets. Tocopherol concentrations in broilers fed diets with 20, 40 and 200 mg vitamin E/kg were (LS means, *n* 48; 38 (SE 1), 105 (SE 3) and 339 (SE 6) nmol/g DM, respectively.

There were significant interactions between dietary vitamin E and fat and muscle. Increasing the dietary vitamin E concentration from 20 to 200 mg/kg increased the tocopherol concentrations in broilers fed palm oil to a greater extent than in broilers fed linseed or soyabean oil. Moreover, increasing the dietary vitamin E concentration from 20 to 200 mg/kg increased the tocopherol concentrations in thigh muscle to a greater extent than in breast muscle. On average for both muscles and dietary vitamin E concentrations, tocopherol concentrations were highest in broilers fed palm oil and lowest in broilers fed linseed oil (LS means, *n* 48; palm oil 185 (SE 4), soyabean oil 161 (SE 4) and linseed oil 135 (SE 4) nmol/g dry matter, respectively).

Concentrations of thiobarbituric acid-reactive substances in broiler muscles

Concentrations of TBARS in broiler muscles are shown in Table 4. There were significant effects of dietary fat, vitamin E, treatment and muscle on concentrations of TBARS. Thigh muscle had higher concentrations of TBARS than breast muscle (LS means, *n* 7277; SE 4) *v.* 25 (SE 1) nmol/g DM), on average of dietary treatment (fat and vitamin E) and treatment of muscles. Moreover, TBARS concentrations were highest in heated muscles and lowest in raw muscles (LS means, *n* 48; raw 38 (SE 4), heated 67 (SE 4), frozen-stored 49 (SE 2) nmol/g DM). On average of both muscles, dietary fat and treatment of muscles, TBARS concentrations in muscles decreased with increasing vitamin E concentrations of the diets. TBARS concentration in broilers fed diets with 20, 40 and 200 mg vitamin E/kg were (LS means, *n* 48; 94 (SE 5), 42 (SE 3) and 18 (SE 1) nmol/g DM, respectively).

There were significant interactions between dietary vitamin E and dietary fat, treatment and muscle. The effect of vitamin E on the concentration of TBARS was strongest in broilers fed linseed oil and weakest in broilers fed palm oil. The effect of dietary vitamin E on TBARS concentrations was stronger in heated muscles than in raw or frozen-stored muscles. Finally, increasing the dietary vitamin E concentration decreased TBARS concentrations in thigh muscle to a greater extent than in breast muscle. On average of both muscles, all three treatments and all three dietary vitamin E concentrations, TBARS concentrations were highest in broilers fed linseed oil and lowest in broilers fed palm oil (LS means, *n* 48; palm oil 21 (SE 3), soyabean oil 35 (SE 3), linseed oil 97 (SE 3) nmol/g DM, respectively).

Significant interactions were noted between dietary fat and vitamin E and muscle. The effect of dietary fat on TBARS concentrations was strongest at the lowest vitamin E concentration (20 mg/kg). At the highest vitamin E concentration (200 mg/kg), no differences were observed in TBARS concentration between broilers fed linseed oil and those fed palm oil or soyabean oil. Moreover, the effect of the dietary fat on TBARS concentration was stronger in thigh muscle than in breast muscle. The absence of an interaction between dietary fat and treatment of muscle shows that the effect of dietary fat on TBARS concentration was similar in raw, heated or frozen-stored muscles.

Table 3. Concentrations of total tocopherols (nmol/g DM) in raw breast and thigh muscle of broilers fed diets containing either palm oil, soyabean oil or linseed oil with various vitamin E concentrations*
(Least squares (LS) mean values with their standard erros for eight broilers per group)

Dietary fat	Dietary Vitamin E (mg/kg)	Breast muscle LS means	Breast muscle SE	Thigh muscle LS means	Thigh muscle SE
Palm oil	20	33[d]	2	61[d]	3
Palm oil	40	78[c]	5	146[c]	8
Palm oil	200	280[a]	12	512[a]	16
Soyabean oil	20	34[d]	2	80[d]	3
Soyabean oil	40	79[c]	5	153[c]	8
Soyabean oil	200	213[b]	12	409[b]	16
Linseed oil	20	5[e]	2	13[d]	3
Linseed oil	40	58[c]	5	115[c]	8
Linseed oil	200	211[b]	12	409[b]	16
		Test of fixed effects (*P*)			
Main effects		Interactions			
Fat	< 0·001	Fat × vitamin E		< 0·001	
Vitamin E	< 0·001	Fat × muscle		NS	
Muscle	< 0·001	Vitamin E × muscle		< 0·001	
		Fat × vitamin E × muscle		NS	

[a,b,c,d,e]Mean values within a column with unlike superscript letters were significantly different ($P < 0·05$).
* For details of diets and procedure, see p. 634.

Table 4. Concentrations of thiobarbituric acid-reactive substances (nmol/g dry matter) in raw, heated or frozen-stored breast and thigh muscle of broilers fed diets containing palm oil, soyabean oil or linseed oil with various vitamin E concentrations* DM
(Least squares (LS) mean values with their standard erros for eight broilers per group)

		Breast muscle						Thigh muscle					
		Raw		Heated		Frozen-stored		Raw		Heated		Frozen-stored	
Dietary fat	Dietary Vitamin E (mg/kg)	LS means	SE	LS means	SE	LS means	SE	LS means	SE	LS means	SE	LS means	SE
Palm oil	20	12·6^{b}	5·2	19·6^{bc}	8·6	9·6^{bc}	12·2	18·7^{bc}	36·1	89·2^{bc}	28·3	39·9^{bc}	14·7
Palm oil	40	9·7^{b}	3·0	6·6^{c}	5·7	5·9^{c}	2·1	25·5^{bc}	5·7	29·7^{c}	26·5	37·5^{c}	3·4
Palm oil	200	9·5^{b}	1·6	4·4^{c}	1·1	3·6^{c}	1·1	15·7^{c}	3·1	12·4^{c}	2·5	31·7^{c}	2·5
Soyabean oil	20	13·9^{b}	5·2	58·2^{b}	8·6	23·1^{b}	12·2	33·4^{bc}	36·1	133·8^{bc}	28·3	59·4^{bc}	14·7
Soyabean oil	40	11·4^{b}	3·2	29·8^{bc}	5·7	13·5^{b}	2·3	29·9^{bc}	5·7	71·7^{bc}	26·5	51·6^{b}	3·4
Soyabean oil	200	7·7^{b}	1·6	7·2^{c}	1·1	7·7^{bc}	1·1	20·2^{c}	3·1	17·3^{c}	2·9	37·2^{c}	2·5
Linseed oil	20	53·4^{a}	5·2	113·9^{a}	9·2	112·4^{a}	12·2	290·3^{a}	36·1	329·2^{a}	28·3	276·7^{a}	15·7
Linseed oil	40	19·7^{b}	3·0	49·0^{b}	6·1	32·9^{b}	2·5	59·3^{b}	5·6	197·5^{ab}	26·5	73·8^{b}	3·4
Linseed oil	200	15·2^{b}	1·6	8·6^{c}	1·2	15·9^{c}	1·1	30·0^{c}	3·1	30·0^{c}	2·5	50·2^{bc}	2·5

Test of fixed effects (P)

Main effects		Interactions			
Fat	<0·001	Fat × vitamin E	<0·001	Fat × vitamin E × treatment	<0·05
Vitamin E	<0·001	Fat × treatment	NS	Fat × vitamin E × muscle	<0·001
Treatment	<0·001	Fat × muscle	<0·001	Vitamin E × treatment × muscle	<0·005
Muscle	<0·001	Vitamin E × treatment	<0·001	Fat × treatment × muscle	NS
		Vitamin E × muscle	<0·001	Fat × vitamin E × treatment × muscle	NS
		Treatment × muscle	<0·05		

[a,b,c]Mean values within a column with unlike superscript letters were significantly different (P<0·05).

* For details of diets and procedure, see p. 634.

There was also a significant interaction between treatment (heated *v.* raw *v.* frozen-stored) and type of muscle. The effect of treatment on TBARS concentration was greater in thigh muscle than in breast muscle. Significant three-way interactions were also recorded between fat, vitamin E and treatment, between fat, vitamin E and muscle, and between vitamin E, treatment and muscle on the concentrations of TBARS.

Concentrations of hydroperoxides in broiler muscles

The concentrations of hydroperoxides in broiler muscles are shown in Table 5. The concentrations of hydroperoxides in breast and thigh muscle, on average of dietary treatment (fat and vitamin E) and treatment of muscles, did not differ (LS means, *n* 72; breast muscle 484 (SE 15), thigh muscle 504 (SE 29) nmol/g DM). Heated muscles had higher concentrations of hydroperoxides than raw or frozen-stored muscles (LS means, *n* 48; raw 344 (SE 8), heated 783 (SE 47), frozen-stored 357 (SE 11) nmol/g DM). Moreover, the muscles of broilers fed diets with 20 mg vitamin E/kg had the highest concentrations of hydroperoxides, and those of broilers fed diets with 200 mg vitamin E/kg had the lowest concentrations. Hydroperoxide concentration in broilers fed diets with 20, 40 and 200 mg vitamin E/kg (LS means, *n* 48) were 814 (SE 36), 400 (SE 31) and 269 (SE 10) nmol/g DM, respectively.

Significant interactions were noted between dietary vitamin E and fat, treatment and muscle. The effect of vitamin E on the concentration of hydroperoxides was strongest in broilers fed linseed oil and weakest in broilers fed palm oil. The effect of dietary vitamin E on the concentration of hydroperoxides was also stronger in heated muscles than in raw or frozen-stored muscles. Finally, an increase in dietary vitamin E concentration from 20 to 40 or 200 mg/kg decreased the concentration of hydroperoxides in thigh muscle to a greater extent than in breast muscle. On average of both muscles, all three treatments and all three dietary vitamin E concentrations, concentrations of hydroperoxides were highest in broilers fed linseed oil and lowest in those fed palm oil (LS means, *n* 8; palm oil 281 (SE 29), soyabean oil 387 (SE 28), linseed oil 816 (SE 28) nmol/g DM, respectively).

There were significant interactions between dietary fat and vitamin E, muscle and treatment of muscle. The effect of dietary fat on the concentration of hydroperoxides in muscles was strongest at the lowest vitamin E concentration (20 mg/kg) and weakest at the highest vitamin E concentration. At the highest vitamin E concentration (200 mg/kg), the muscles of broilers fed linseed oil did not differ in their concentrations of hydroperoxides from the muscles of broilers fed palm oil or soyabean oil. The effect of the dietary fat on hydroperoxide concentration was stronger for thigh muscle than for breast muscle. Moreover, the dietary fat had a stronger effect for heated muscles than for raw or frozen-stored muscles. There was no interaction between treatment (raw *v.* heated *v.* frozen-stored) and type of muscle, which means that the effect of the treatment on the concentration of hydroperoxides was similar in both muscles. There were significant three-way interactions between fat, vitamin E and treatment, between fat, vitamin E and muscle, and between vitamin E, treatment and muscle on the concentrations of hydroperoxides, and there was even an interaction between the four factors.

Concentrations of cholesterol oxidation products in broiler muscles

In raw and frozen-stored breast and thigh muscles, 7β-hydroxycholesterol and 7-ketocholesterol were the only COP that could be detected. In heat-processed meat, β-epoxycholesterol and cholestantriol were additionally detected. The sum of the concentrations of these COP ('total COP') in raw, heat-processed and frozen-stored breast and thigh muscles is shown in Table 6. The concentration of total COP on average of dietary treatment (fat and vitamin E) and treatment of muscles was higher in thigh muscle than in breast muscle (LS means, *n* 72; 28·7 (SE 3·6) *v.* 9·0 (SE 0·4) nmol/g DM). Heated muscles had much higher concentrations of total cop than raw or frozen-stored muscles (LS means, *n* 48; raw 4·0 (SE 0·4), heated 49·5 (SE 5·4), frozen-stored 3·0 (SE 0·2) nmol/g DM). Moreover, the muscles of broilers fed diets with 20 mg vitamin E/kg had higher concentrations of total COP than the muscles of broilers fed diets with 40 or 200 mg vitamin E/kg. Concentrations of total COP in the muscles of broilers fed diets with 20, 40 and 200 mg vitamin E/kg were (LS means, *n* 48) 36·5 (SE 5·2), 14·1 (SE 1·2) and 5·9 (SE 0·3) nmol/g DM, respectively.

There were also significant interactions between dietary vitamin E and fat, treatment and muscle. The effect of vitamin E on concentration of total COP was strongest in broilers fed linseed oil and weakest in broilers fed palm oil. The effect of dietary vitamin E on concentrations of total COP was also stronger in heated muscles than in raw or frozen-stored muscles. The effect of dietary vitamin E on COP concentration was also greater in thigh muscle than in breast muscle. On average of dietary treatment (fat, vitamin E) and both muscles, concentrations of total COP were higher in broilers fed linseed oil than in broilers fed palm oil or soyabean oil (LS means, *n* 48; palm oil 10·3 (SE 3·5), soyabean oil 13·6 (SE 3·0), linseed oil 32·6 (SE 2·8) nmol/g DM, respectively).

There were significant interactions between dietary fat and vitamin E, muscle and treatment of muscle. The effect of dietary fat on the concentration of total COP in muscles was strongest for the lowest vitamin E concentration (20 mg/kg) and weakest for the highest vitamin E concentration. At the lowest vitamin E concentration, broilers fed linseed oil had higher concentrations of total oxysterols than those fed palm oil or soyabean oil. At the highest vitamin E concentration (200 mg/kg), concentrations of total COP in muscles did not differ between broilers fed linseed oil and those fed soyabean oil or palm oil. The effect of the dietary fat on the concentration of total COP was stronger in thigh muscle than in breast muscle. Moreover, dietary fat had a stronger effect in heated muscles than in raw or frozen-stored muscles. There was also a significant interaction between treatment (heated *v.* raw *v.* frozen-stored) and type of muscle. The effect of treatment on COP concentration was greater in thigh muscle than in breast muscle.

In addition, there were significant three-way interactions between fat, vitamin E and treatment, between fat, vitamin E and muscle, between vitamin E, treatment and muscle, and between fat, treatment and muscle on the concentrations of total cop, and there was also an interaction between the four factors.

The concentrations of individual COP in broiler muscles responded in a similar way to total COP concentration to the experimental factors dietary fat and dietary vitamin E. Data for the individual COP are therefore not shown.

Table 5. Concentrations of hydroperoxides (nmol/g DM) in raw, heated or frozen-stored breast and thigh muscle of broilers fed diets containing either palm oil, soyabean oil or linseed oil with various vitamin E concentrations*
(Least squares (LS) mean values and SE for eight broilers per group)

		Breast muscle						Thigh muscle					
		Raw		Heated		Frozen-stored		Raw		Heated		Frozen-stored	
Dietary fat	Dietary Vitamin E (mg/kg)	LS mean	SE	LS mean	SE	LS mean	SE	LS mean	SE	LS mean	SE	LS mean	SE
Palm oil	20	205[b]	52	541[b]	170	367[ab]	69	153[b]	31	200[b]	333	304[b]	64
Palm oil	40	206[b]	29	547[b]	89	340[ab]	44	213[b]	41	124[b]	300	313[b]	37
Palm oil	200	203[b]	30	519[b]	65	273[b]	27	120[b]	18	176[b]	65	247[b]	29
Soyabean oil	20	231[b]	48	930[b]	170	402[ab]	69	163[b]	31	1437[ab]	311	296[b]	56
Soyabean oil	40	266[b]	29	620[b]	89	327[ab]	44	220[b]	41	157[b]	299	303[b]	37
Soyabean oil	200	213[b]	30	497[b]	65	306[b]	27	152[b]	16	152[b]	59	296[b]	27
Linseed oil	20	1210[a]	56	1782[a]	170	638[a]	74	1815[a]	33	3280[a]	311	701[a]	59
Linseed oil	40	275[b]	31	779[b]	83	352[ab]	44	180[b]	44	1635[a]	299	342[b]	37
Linseed oil	200	226[b]	30	526[b]	65	301[b]	27	136[b]	17	186[b]	55	316[b]	27

Test of fixed effects (P)

Main effects		Interactions			
Fat	$<0{\cdot}001$	Fat × vitamin E	$<0{\cdot}001$	Fat × vitamin E × treatment	$<0{\cdot}001$
Vitamin E	$<0{\cdot}001$	Fat × treatment	$<0{\cdot}001$	Fat × vitamin E × muscle	$<0{\cdot}001$
Treatment	$<0{\cdot}001$	Fat × muscle	$<0{\cdot}001$	Vitamin E × treatment × muscle	$<0{\cdot}001$
Muscle	NS	Vitamin E × treatment	$<0{\cdot}001$	Fat × treatment × muscle	$<0{\cdot}001$
		Vitamin E × muscle	$<0{\cdot}01$	Fat × vitamin E × treatment × muscle	$<0{\cdot}001$
		Treatment × muscle	NS		

[a,b]Mean values within a column with unlike superscript letters were significantly different ($P<0{\cdot}05$).
* For details of diets and procedure, see p. 643.

Table 6. Concentrations of total cholesterol oxidation products (nmol/g DM) in raw, heated or frozen-stored breast and thigh muscle of broilers fed diets containing either palm oil, soyabean oil or linseed oil with various vitamin E concentrations†
(Least squares (LS) mean values with their standard erros for eight broilers per group)

		Breast muscle						Thigh muscle					
		Raw		Heated		Frozen-stored		Raw		Heated		Frozen-stored	
Dietary fat	Dietary Vitamin E (mg/kg)	LS mean	SE	LS mean	SE	LS mean	SE	LS mean	SE	LS mean	SE	LS mean	SE
Palm oil	20	1·08^{b}	0·46	17·9^{bc}	6·6	1·54^{b}	1·40	2·21^{bc}	4·41	76·3^{b}	61·2	2·01^{ab}	1·03
Palm oil	40	1·07^{b}	0·14	10·5^{c}	3·0	1·80^{b}	0·41	1·65^{bc}	0·47	32·7^{b}	14·2	2·54^{ab}	0·32
Palm oil	200	1·05^{b}	0·20	9·2^{c}	0·8	1·39^{b}	0·27	1·56^{c}	0·27	18·5^{bc}	3·0	2·06^{ab}	0·27
Soyabean oil	20	0·76^{b}	0·51	35·5^{b}	5·7	1·95^{b}	1·50	2·32^{bc}	4·41	87·3^{b}	51·7	3·12^{ab}	1·10
Soyabean oil	40	0·93^{b}	0·14	21·7^{bc}	2·6	2·13^{b}	0·39	2·27^{bc}	0·44	49·8^{b}	11·6	2·83^{ab}	0·28
Soyabean oil	200	0·91^{b}	0·20	9·6^{c}	0·8	1·72^{b}	0·25	2·26^{bc}	0·29	17·6^{b}	2·3	2·83^{ab}	0·27
Linseed oil	20	5·03^{a}	0·51	62·6^{a}	5·7	10·54^{a}	1·40	39·06^{a}	4·77	299·5^{a}	48·4	8·03^{a}	1·03
Linseed oil	40	1·01^{b}	0·14	26·5^{b}	2·4	2·00^{b}	0·39	3·88^{b}	0·47	86·2^{b}	11·6	3·73^{ab}	0·32
Linseed oil	200	1·03^{b}	0·20	11·3^{c}	0·8	1·55^{b}	0·25	3·64^{b}	0·34	18·6^{c}	2·1	2·28^{b}	0·29

Test of fixed effects (P)

Main effects		Interactions			
Fat	<0·001	Fat × vitamin E	<0·001	Fat × vitamin E × treatment	<0·001
Vitamin E	<0·001	Fat × treatment	<0·001	Fat × vitamin E × muscle	<0·05
Treatment	<0·001	Fat × muscle	<0·005	Vitamin E × treatment × muscle	<0·001
Muscle	<0·001	Vitamin E × treatment	<0·001	Fat × treatment × muscle	<0·001
		Vitamin E × muscle	<0·001	Fat × Vitamin E × treatment × muscle	<0·001
		Treatment × muscle	<0·001		

[a,b,c]Mean values within a column with unlike superscript letters were significantly different (P<0·05).

* For details of diets and procedure, see p. 634.

Correlation between concentrations of oxidation products of PUFA (thiobarbituric acid-reactive substances, hydroperoxides) and concentrations of cholesterol oxidation products

There were significant linear correlations between the concentrations of TBARS or hydroperoxides and those of individual and total COP in raw, heated and frozen-stored breast and thigh muscle (Table 7).

Discussion

The aim of the present study was to explore interactions between dietary fats and vitamin E on the concentrations of COP in the muscle of broilers. We therefore determined the concentrations of various COP in raw, heated and frozen-stored breast and thigh muscles of broilers fed diets varying in their type of fat and vitamin E concentration. Oxidation can occur in the A and B cholesterol rings and also in the cholesterol side chain (Smith, 1987). We found that B-ring cholesterol oxides, i.e. 7β-hydroxycholesterol, 7-ketocholesterol, β-epoxycholesterol and cholestantriol, were quantitatively the most important oxysterols in broiler meat. This finding is in agreement with studies by Maraschiello *et al.* (1998) and Lee *et al.* (2001), which identified 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol and β-epoxycholesterol as the principal oxysterols in chicken meat. In the study of Grau *et al.* (2001), very small concentrations of 25-hydroxycholesterol were additionally found in raw and cooked chicken meat.

Recent studies have already shown that dietary vitamin E reduces the formation of COP in meat of broilers (Li *et al.* 1996; Galvin *et al.* 1997; Lopez-Bote *et al.* 1998; Grau *et al.* 2001). The present study shows for the first time that the effects of vitamin E on COP concentrations depend largely on the dietary fat, treatment of muscle and type of muscle. Vitamin E exerted a stronger effect in broilers fed linseed oil than in broilers fed soyabean or palm oil; moreover, it had a stronger effect on COP concentrations in heated muscle than in raw or frozen-stored muscle, and it had a stronger effect on thigh muscle than on breast muscle. The finding of significant linear correlations between the concentrations of autoxidation products of PUFA (TBARS and hydroperoxides) and the concentrations of all the COP identified (7β-hydroxycholesterol, 7-ketocholesterol, β-epoxycholesterol and cholestantriol) indicates that the formation of all of these COP was closely linked to the oxidation of PUFA. De Vore (1988) also observed a linear relationship between TBARS and 7-ketocholesterol concentrations in beef, and Monahan *et al.* (1992) reported a similar relationship between total COP and fatty acid oxidation in pork. It has been suggested that cholesterol oxidation in meat is initiated by fatty acid acyl radicals of neighbouring fatty acids in the phospholipid membrane (Park & Addis, 1987). This hypothesis is supported by the close relationship between the oxidation of PUFA and the oxidation of cholesterol in broiler muscle observed in this study.

The present study shows that dietary fats rich in PUFA such as linseed oil cause a dramatic increase in COP concentration in broiler muscle, particularly during heating, if the dietary vitamin E concentration is low. The effect of linseed oil in this respect might be due to the incorporation of (*n*-3) PUFA into membrane phospholipids, which makes them more susceptible to oxidation, particularly if tissue vitamin E concentrations are low. It was shown, however, that a dietary vitamin E concentration of 40 mg/kg is sufficient to completely suppress COP formation by linseed oil in raw or frozen-stored muscle. If palm oil or soyabean oil was used as the dietary fat, even a low dietary vitamin E concentration of 20 mg/kg was sufficient to prevent the formation of COP in raw or frozen-stored muscles. The finding that such low vitamin E concentrations were sufficient to suppress the oxidation of cholesterol might result from a low rate of PUFA oxidation in raw or frozen-stored muscles.

Recent studies have already found that heating broiler muscle increases its concentrations of COP (Maraschiello *et al.* 1998; Grau *et al.* 2001). An increase in COP production during heating is probably the consequence of accelerated lipid peroxidation (Engeseth & Gray, 1994). Furthermore, the present study shows for the first time that the formation of COP in broiler muscle during heating is strongly dependent on the dietary fat and the dietary vitamin E concentration. At a low dietary vitamin E concentration, heating increased the COP concentration in the muscle of broilers fed linseed oil to a greater extent than in the muscle of broilers fed soyabean or palm oil. At a high dietary vitamin E concentration, the COP concentration increased much less during heating than at a low vitamin E concentration; the increase was similar in broilers fed all three types of fat. This means that a

Table 7. Correlation between concentrations of thiobarbituric acid-reactive substances (TBARS) or hydroperoxides and those of cholesterol oxidation products in raw, heated or frozen-stored breast and thigh muscle of broilers†

	Breast muscle			Thigh muscle		
	Raw	Heated	Frozen-stored	Raw	Heated	Frozen-stored
TBARS *v.*						
7β-Hydroxycholesterol	0·76***	0·85***	0·92***	0·66***	0·68***	0·89***
7-Ketocholesterol	0·69***	0·82***	0·87***	0·56***	0·69***	0·76***
β-Epoxycholesterol	–	0·80***	–	–	0·67***	–
Cholestantriol	–	0·73***	–	–	0·65***	–
Total cholesterol oxidation products	0·73***	0·82***	0·89***	0·60***	0·70***	0·82***
Hydroperoxides *v.*						
7β-Hydroxycholesterol	0·81***	0·73***	0·70***	0·86***	0·64***	0·53***
7-Ketocholesterol	0·80***	0·73***	0·68***	0·81***	0·66***	0·38**
β-Epoxycholesterol	–	0·73***	–	–	0·62***	–
Cholestantriol	–	0·69***	–	–	0·67***	–
Total cholesterol oxidation products	0·81***	0·73***	0·69***	0·83***	0·63***	0·44***

**$P<0·01$, ***$P<0·001$.
† Pearson product moment correlation coefficients.

high dietary vitamin E supplementation completely suppressed the adverse effect of linseed oil on COP formation during heating. The effect of vitamin E might be mediated by its ability to scavenge fatty acyl peroxide radicals. Vitamin E is deposited in membranes in positions adjacent to the PUFA moiety of the phospholipids (Fukuzawa & Fujii, 1992). It is likely that the protection of neighbouring fatty acids by vitamin E, and the subsequent reduction in fatty acyl radical formation, in turn reduces the extent of cholesterol oxidation. The lack of effect of vitamin E in raw or frozen-stored muscles of broilers fed palm oil or linseed oil was not unexpected due to the low rate of cholesterol oxidation that had occurred.

It has been shown that broiler thigh muscle has higher concentrations of COP than broiler breast muscle (Galvin *et al.* 1997). The present study, moreover, shows that COP concentrations in thigh muscle are more strongly influenced by dietary fat, vitamin E concentration and treatment of the muscle than COP concentrations in breast muscle. This might be mainly due to the fact that lipids in thigh muscle are more susceptible to lipid oxidation than are lipids in breast muscle, because of a higher content of total lipids, phospholipids (and therefore unsaturated fatty acids) and Fe. Moreover, thigh muscle has a higher concentration of cholesterol than breast muscle (Galvin *et al.* 1997).

In conclusion, the present study shows that dietary fat and vitamin E concentration influence COP concentrations in broiler muscles. The effects of dietary fat and vitamin E in this respect are, however, stronger in heated muscle than in raw or frozen-stored muscle, and stronger in thigh muscle than in breast muscle. At low dietary vitamin E concentrations, dietary PUFA accelerated the formation of COP, particularly during heating. This adverse effect of dietary PUFA could be completely avoided by increasing the dietary vitamin E concentration. The protection of COP formation in broiler muscle by the choice of a favourable combination of dietary fat and vitamin E concentration is undoubtedly beneficial because COP in human diets play a role in the development of important diseases such as cancer or CHD.

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