Increase of Cholesterol Oxidation and Decrease of PUFA as a Result of Thermal Processing and Storage in Eggs Enriched with n-3 Fatty Acids

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In this work, cholesterol oxide formation and alteration of fatty acid composition were analyzed in n-3 enriched eggs under different storage periods and two temperatures. The eggs enriched with n-3 fatty acids were stored at 5 or 25 °C for 45 days and subsequently boiled or fried. For each treatment, 12 yolks were analyzed every 15 days including time zero. The concentrations of the cholesterol oxides 7-ketocholesterol, 7β-hydroxycholesterol, and 7α-hydroxycholesterol increased during the storage period and were higher in fried eggs. Only the 7-ketocholesterol was affected by the storage temperature, and its concentration was highest in eggs stored at 25 °C. There was no significant difference in the contents of cholesterol and vitamin E at the different storage periods; however, the concentration of vitamin E decreased with thermal treatment. In addition, the n-3 polyunsaturated fatty acids, especially 18:3, 20:5, and 22:6, were reduced throughout the storage at 5 and 25 °C.

KEYWORDS: Cholesterol oxides; cholesterol; n-3 enriched eggs

INTRODUCTION

Currently, there is an increasing interest on the consumption of foods that have higher content of substances beneficial to human health, such as the n-3 polyunsaturated fatty acids (n-3 PUFAs), specifically α-linolenic acid (LNA, 18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6). Studies have demonstrated that these polyunsaturated fatty acids act on serum lipid levels and have antithrombolytic activity, which makes them important in preventing cardiovascular diseases (*1*, *2*). However, the enzymes that originate n-3 PUFAs by desaturation and elongation processes are the same as those that originate n-6 PUFAs. Consequently, there is competition in the metabolism of the n-3 and n-6 series, and the excess of n-6 in the diet limits the synthesis of n-3 PUFAs (*3*). Thus, the PUFA benefits for human health depend on the balanced ingestion of these acids, and the generally accepted ideal n-6/n-3 ratio is around 4:1 (*4*).

The poultry industry has been aware of the n-3 PUFA beneficial effects on human health, expanding the offer of eggs enriched with n-3 PUFA. The increase of n-3 PUFA content in eggs has been accomplished by supplementation of hen diets with fish oil, vegetable seeds, or oils rich in n-3 PUFA (*5*, *6*). However, the increase in the unsaturation of fatty acids in eggs can lead to the increase of lipid susceptibility to oxidation; therefore, it is usual to also add antioxidants, such as vitamin E, to a poultry diet for oxidative stabilization of PUFA.

Lipid oxidation is one of the main reactions that occur during the heating of foods at high temperatures in the presence of metals, photosensitizers, and oxygen. Hence, fatty acid alterations may occur during food heating and storage, resulting in a considerable loss of its nutritional value and in the production of free radicals that accelerate the formation of the cholesterol oxides.

Cholesterol oxides in foods are produced by autoxidation or by enzymatic oxidation. Autoxidation occurs through complex chain reactions based on free radical formation through oxygen triplets (*7*, *8*). The main compounds are originated from the oxidation of carbon 7: 7α-hydroxycholesterol, 7β-hydroxycholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, 7-ketocholesterol, and cholestanetriol (*9*). The cholesterol oxides are considered atherogenic, cytotoxic, mutagenic, and carcinogenic (*10*, *11*). Further studies are needed to improve the understanding of changes in the content of fatty acids, cholesterol, and cholesterol oxides during food storage and thermal treatments.

The objective of this work was to evaluate the effects of storage time and thermal processing on cholesterol oxide formation and on the changes of fatty acid composition in eggs enriched with n-3 fatty acids.

MATERIALS AND METHODS

Materials. Cholesterol and cholesterol oxide standards, including 19-hydroxycholesterol (19-OH), 20α-hydroxycholesterol (20α-OH), 22R-hydroxycholesterol, (22R-OH), 22S-hydroxycholesterol (22S-OH), 25-hydroxycholesterol (25-OH), 7-ketocholesterol (7-keto), 7β-hydroxycholesterol (7β-OH), 5,6α-epoxycholesterol (5,6α-epoxy),

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5,6β-epoxycholesterol (5,6β-epoxy), and colestan-3β-5α-6β-triol (triol) were obtained from Sigma (Miliford, MA, USA). The other oxides standards, 24S-hydroxycholesterol (24S-OH) and 7α-hydroxycholesterol (7α-OH), were obtained from Steraloids (Newport, RI, USA). A total of 37 saturated, monounsaturated, and polyunsaturated fatty acid standards (Supelco FAME Mix 18919, Bellefonte, PA, USA) were used. Internal standards for quantification consisted of methyl esters (99% pure, Sigma, St. Louis, MO, USA) of tricosanoic acid (23:0) and tridecanoic acid (13:0). The α-tocopherol standard was obtained from Merck (Darmstadt, Germany). The accuracy of the cholesterol and fatty acid analyses was verified by using the reference standard of whole egg powder (SRM 1846 and SEM 8415) from NIST (Gaithersburg, MD, USA).

Samples. Two batches of eggs enriched with n-3 fatty acids and referred to as batch 1 and 2 were acquired directly from a farm (average temperature 28 °C and RH 75%) in Sao Paulo, Brazil, on the same day of eggs-laying and were analyzed on the following day. The poultry were Hy-Line W-36 commercial laying hens, 35 weeks of age, housed in cages (6 hens per cage of 50 × 45 cm), in an open-sided house, and diets were given ad libitum (**Table 1**). The eggs, of medium size, were washed and disinfected, and the shells were not oiled. Eggs weighed on average 51.5 and 50.5 g in batches 1 and 2, respectively; the yolks weighed 15.4 g. Each batch consisted of 30 dozen eggs distributed as follows: 15 dozen were stored at 25 ± 2 °C, 75% RH, in the dark, and 15 dozen were kept in the refrigerator at 5 ± 1 °C. Samples were analyzed at time zero and every 15 days up to 45 days. In each time (0, 15, 30, and 45 days) and in each storage temperature (25 and 5 °C), 36 eggs were separated and divided in 3 batches of 12 eggs. One batch was analyzed as raw eggs, and the other two were submitted to thermal treatments, which consisted of shell boiling and frying. The eggs with shell were boiled for 5 min at 97 °C. In frying, the eggs were submerged in 200 mL of soy oil at 190 °C, for 4 min, in a Master Grill (Britania, Brazil). The yolks did not remain intact during frying and were separated from the whites and then passed on absorbing paper. The yolks of the raw eggs and the ones submitted to boiling also were separated, homogenized, and analyzed in triplicate.

Lipid Determination. The lipids were extracted from the yolk according to the method of Folch et al. (*12*). Aliquots of 10 mL were taken and the total lipids determined gravimetrically.

Determination of Moisture Content. Aliquots of 1 ± 0.5 g of yolks were dried to constant weight in an oven at 100 °C, according to the AOAC methodology (*13*).

Determination of Fatty Acids. Approximately 25 mg of the oil extracted from eggs were methylated according to Joseph and Ackman (*14*). The fatty acid methyl esters were separated in a gas chromatograph (Varian 3400 CX, Walnut Creek, CA, USA), equipped with a flame ionization detector, a split injector (1:50), a CP-SIL 88 column (100 m, 0.25 mm, 0.20 μm; Chrompack, Netherlands), and a workstation star. The chromatographic conditions followed the ones described by Mazalli and Bragagnolo (*15*). The fatty acids were identified by comparing the retention times of the standards with those of the samples, and quantification was performed by internal standardization using the methyl esters of tricosanoic and tridecanoic acids as the internal standards. The results were calculated in milligrams per 100 g of sample according to the AOCS method (*16*). The method was validated using the certified reference egg powder standard (SRM 8415, NIST, USA). The results obtained were similar to those specified for the reference material.

Simultaneous Determination of Cholesterol and Cholesterol Oxides by HPLC-UV-RI. The chromatographic methods of separation and quantification of the cholesterol oxides were performed according to Mazalli et al. (*17*). A Shimadzu liquid chromatograph (Kuoto, Japan) equipped with UV (SPD-10AV$_{vp}$) and refractive index (RID-10A) detectors was used. A Nova Pack CN HP, 300 mm × 3.9 mm × 4 μm, column (Waters, Millford, MA, USA) was used with a temperature of 32 °C. Quantification was done by external standardization with concentrations varying from 1 to 100 μg/mL for the oxides and from 2 to 2.5 mg/mL for cholesterol. The identity of cholesterol and cholesterol oxides was confirmed by using the liquid chromatograph interfaced with an atmospheric pressure chemical ionization source (APCI) and mass detection (MS) (*17*).

Cholesterol recovery varied from 92 to 102%, while cholesterol oxides ranged from 93 to 96%. The detection limit found for cholesterol was 0.026 μg/g, and 7-keto, 7α-OH, and 7β-OH were 0.002, 0.01, and

Table 1. Diet Composition

ingredient	(%)
yellow corn	56.48
soybean meal (46%)	26.33
dicalcium phosphate (18% P, 21% C)	1.96
limestone	8.85
salt	0.34
flaxseed oil	3.00
choline chloride (60%)	0.05
DL-metionine	0.13
sand	2.61
vitamin—mineral premix[a]	0.25
metabolizable energy (Mcal/kg)	2.80
protein	17.00
calcium	3.90
sodium	0.17
available phosphorus	0.45
metionine	0.40
metionine + cystine	0.69
lysine	0.89

[a] Supplied per kilogram of diet: vitamin A, 6.250 IU; vitamin D, 2.500 IU; vitamin E, 100 IU; vitamin K, 0.04 mg; tiamin, 0.25 mg; riboflavin, 34 mg; pyridoxine, vitamin B^{12}, 20 μg; pantothenic acid, 3.8 mg; niacin, 9.9 mg; biotin, 0.1 mg; folic acid, 0.25 mg; copper, 6 mg; iron, 52.5 mg; iodine, 0.33 mg; selenium, 0.21 mg; manganese, 48 mg; zinc, 60.23 mg; ethoxiquin, 0.313 mg.

0.025 μg/g, respectively. The quantification limit for cholesterol was 0.088 μg/g, and 7-keto, 7α-OH, and 7β-OH were 0.006, 0.062, and 0.079 μg/g, respectively. Both detection limits were calculated according to Long et al. (*18*). The cholesterol content obtained for the certified reference material (SRM 1846, NIST, USA) was 19.0 ± 0.2 mg/g of yolk, equal to the value of the certified reference material.

Determination of α-Tocopherol. Vitamin E was determined according to the AOCS method (*19*) by a liquid chromatograph using a Perkin-Elmer 250 apparatus equipped with a fluorescence detector (Shimadzu RF-10 AXL), with excitation at 290 and 330 nm of emission. A LiChrosorb Si 60, 250 × 4 mm, 5 μm (Merck, Darmstadt, Germany) column, n-hexane/isopropyl alcohol (99:1, vol/vol) as mobile phase, and a flow rate of 1.1 mL/min were used. The quantification of vitamin E was carried out by an external standard curve (concentrations of α-tocopherol varying from 1 to 20 μg/mL).

Statistical Analysis. The analysis of variance was applied using a factorial arrangement with three factors being thermal processing (raw, boiled, and fried), storage temperature (5 and 25 °C), and storage time (0, 15, 30, and 45 days). The data of total lipids, moisture, cholesterol, cholesterol oxides, and fatty acids were analyzed using the software Statistic 5.5 (St Clara, CA). The average values were compared by the Tukey test at the 5% significance level.

RESULTS AND DISCUSSION

Total Lipid and Moisture Content in Eggs Enriched with n-3 Fatty Acids. Results show that time and temperature of storage as well as the interactions among factors were not significant. Thermal processing was significant only at fried temperature; as the yolks did not remain intact during frying, probably some lipids were transferred from yolks to oil. Lowest moistures were found in the fried, boiled and raw yolks in crescent order (**Table 2**). The results of lipids were lower in raw yolk and similar in boiled and fried yolks, and the moisture values of raw yolks were similar to the values reported by the USDA (*20*).

Fatty Acids in Eggs Enriched with n-3 Fatty Acids. The main fatty acids identified were 16:0, 18:0, 16:1 n-7, 18:1 n-9, 18:2 n-6, 20:4 n-6, 18:3 n-3, and 22:6 n-3 (**Tables 3** and **4**). In the analysis of total fatty acids, no significant interaction was found among the experimental factors. The storage time and the procedures of thermal processing modified the concentration of some fatty acids. However, the storage temperature did not affect the total fatty acid content.

Table 2. Total Lipids (g/100g Yolk, Dry Basis) and Moisture (g/100g Yolk) in Eggs Enriched with n-3 Fatty Acids Stored during 45 Days at 25 and 5 ± 2 °C

		Lipids				
temperature	yolk	0 day	15 days	30 days	45 days	means
	raw	31.70	31.46	30.94	30.92	31.26 A
25 °C	boiled	31.44	30.35	31.94	30.65	31.10 A
	fried	29.66	30.82	29.27	28.61	29.59 B
	means[a]	30.93[a]	30.88[a]	30.72[a]	30.06[a]	30.65 A
	raw	31.70	31.34	31.12	32.15	31.58 A
5 °C	boiled	31.44	31.00	31.48	31.28	31.30 A
	fried	29.66	30.33	29.56	29.45	29.75 B
	means[a]	30.93[a]	30.89[a]	30.72[a]	30.96[a]	30.88 A
		moisture				
	yolk	0 day	15 days	30 days	45 days	means
	raw	51.16	50.37	49.50	49.56	50.15 A
25 °C	boiled	47.90	47.12	49.93	47.94	48.22 B
	fried	44.95	46.26	45.58	44.49	45.32 C
	means[a]	48.00[a]	47.91[a]	48.34[a]	47.33[a]	47.90 A
	raw	51.16	50.94	49.99	50.68	50.69 A
5 °C	boiled	47.90	48.55	47.78	47.28	47.88 B
	fried	44.95	44.67	45.57	45.45	45.16 A
	means[a]	48.00[a]	48.05[a]	47.78[a]	47.80[a]	47.91 A

[a] Means of the six analyses (two batches in triplicate). Means in the same row with the same letter do not differ significantly (*p* > 0.05). Means in the same column with same letter do not differ significantly (*p* > 0.05).

Table 3. Fatty Acids (g/100g of Yolk, Dry Basis) in Eggs Enriched with n-3 Fatty Acids Storage During 45 Days at 25 °C[a]

	zero time				15 days				30 days				45 days				means[f]			
fatty acids	R[b]	B[c]	F[d]	means[e]	R[b]	B[c]	F[d]	means[e]	R[b]	B[c]	F[d]	means[e]	R[b]	B[c]	F[d]	means[e]	R[b]	B[c]	F[d]	means[g]
14:0	0.55	0.53	0.55	0.54 a	0.54	0.55	0.50	0.53 a	0.53	0.52	0.52	0.52 a	0.50	0.54	0.54	0.53 a	0.53 a	0.54 a	0.53 a	0.53 a
15:0	0.08	0.08	0.07	0.08 a	0.07	0.08	0.07	0.07 a	0.07	0.06	0.07	0.07 a	0.07	0.06	0.07	0.07 a	0.07 a	0.07 a	0.07 a	0.07 a
16:0	3.97	3.98	3.96	3.97 a	3.95	3.95	3.90	3.93 a	3.92	3.93	3.93	3.93 a	3.94	3.93	3.93	3.93 a	3.95 a	3.95 a	3.93 a	3.94 a
17:0	0.26	0.24	0.25	0.25 a	0.26	0.24	0.25	0.25 a	0.23	0.25	0.24	0.24 a	0.23	0.25	0.23	0.24 a	0.25 a	0.25 a	0.24 a	0.24 a
18:0	1.50	1.50	1.49	1.50 a	1.45	1.46	1.48	1.46 a	1.46	1.47	1.45	1.46 a	1.45	1.46	1.45	1.45 a	1.47 a	1.47 a	1.47 a	1.47 a
20:0	0.10	0.08	0.09	0.09 a	0.09	0.08	0.08	0.08 a	0.08	0.07	0.08	0.08 a	0.09	0.07	0.07	0.08 a	0.09 a	0.08 a	0.08 a	0.08 a
22:0	0.10	0.10	0.10	0.10 a	0.09	0.08	0.10	0.09 a	0.09	0.08	0.10	0.09 a	0.10	0.08	0.08	0.09 a	0.10 a	0.09 a	0.10 a	0.09 a
24:0	0.02	0.02	0.01	0.01 a	0.01	0.01	0.02	0.01 a	0.02	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 a	0.01 a	0.01 a	0.01 a	0.01 a
14:1n-9	0.11	0.10	0.09	0.10 a	0.10	0.10	0.08	0.09 ab	0.11	0.10	0.08	0.09 ab	0.09	0.10	0.06	0.08 b	0.10 a	0.10 a	0.08 b	0.09 a
16:1n7	5.25	4.71	4.92	4.96 a	4.99	4.35	4.58	4.64 ab	4.27	3.96	4.19	4.14 b	3.90	3.17	3.82	3.63 b	4.60 a	4.05 a	4.38 a	4.34 a
17:1n7	0.24	0.17	0.20	0.20 a	0.17	0.15	0.16	0.16 b	0.14	0.12	0.15	0.14 b	0.10	0.12	0.10	0.11 c	0.16 a	0.14 b	0.15 ab	0.15 a
18:1n-9	6.64	5.97	6.09	6.23 a	6.06	5.05	5.99	5.70 b	5.74	4.54	5.59	5.29 c	5.40	4.37	5.10	4.96 d	5.96 a	4.98 c	5.69 b	5.54 a
20:1n-9	0.38	0.34	0.37	0.36 a	0.35	0.33	0.35	0.34 ab	0.35	0.30	0.33	0.30 bc	0.31	0.29	0.30	0.30 c	0.35 a	0.31 b	0.33 ab	0.33 a
18:2n-6	5.31	5.13	5.05	5.16 a	4.94	4.43	4.64	4.67 b	4.69	4.08	4.60	4.46 bc	4.50	4.43	4.34	4.43 c	4.86 a	4.51 b	4.66 ab	4.68 b
18:3n-3	1.82	0.83	1.59	1.41 a	1.69	0.71	1.36	1.25 ab	1.65	0.53	1.24	1.14 b	1.56	0.57	1.15	1.09 b	1.68 a	0.66 c	1.33 bc	1.22 a
18:3n-6	0.02	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 b	0.01 a	0.01 b	0.01 ab	0.01 a
20:2n-6	0.29	0.25	0.28	0.27 a	0.28	0.25	0.25	0.26 a	0.26	0.22	0.23	0.24 ab	0.25	0.20	0.22	0.22 b	0.27 a	0.23 b	0.25 b	0.25 a
20:3n-6	0.03	0.02	0.02	0.02 a	0.02	0.02	0.02	0.02 ab	0.02	0.01	0.02	0.02 ab	0.02	0.01	0.02	0.02 b	0.02 a	0.02 b	0.02 a	0.02 a
20:4n-6	0.20	0.13	0.15	0.16 a	0.17	0.10	0.14	0.14 ab	0.14	0.09	0.14	0.12 b	0.10	0.07	0.13	0.10 c	0.15 a	0.10 b	0.14 ab	0.13 a
20:5n-3	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 b	0.01	0.00	0.01	0.01 c	0.01 a	0.01 b	0.01 b	0.01 a
22:6n-3	0.26	0.14	0.22	0.21 a	0.24	0.13	0.21	0.19 ab	0.23	0.13	0.15	0.17 b	0.19	0.12	0.12	0.14 c	0.23 a	0.13 c	0.17 b	0.18 a
18:1 n-9*t*	0.03	0.02	0.02	0.03 a	0.03	0.02	0.02	0.02 ab	0.02	0.02	0.02	0.02 ab	0.02	0.02	0.02	0.02 b	0.03 a	0.02 a	0.02 a	0.02 a
18:2 n-6*t*	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.011 ab	0.01	0.01	0.01	0.01 ab	0.01	0.01	0.01	0.01 b	0.01 a	0.01 a	0.01 a	0.01 a

[a] Means of the six analyses (two batches in triplicate). Means in the same row with the same letter do not differ significantly (*p* > 0.05). Means in the same column with the same letter do not differ significantly (*p* > 0.05). [b] Raw. [c] Boiled. [d] Fried. [e] Means of days. [f] Means of each treatments. [g] Means stored at 25 °C. 21:0, 17:1 n-7, 15:1 n-9; 22:1 n-9, 24:1 n-9, and 20:3 n-3 were detected for traces (less than 1 mg/100 g).

As for the saturated fatty acids, there were no significant differences between the raw yolk samples and the ones submitted to the thermal treatments, and no reduction occurred during the evaluated storage periods.

The unsaturated fatty acids 18:1 n-9, 18:3 n-3, 18:3 n-6, 20:2 n-6, and 20:5 n-3 were mostly reduced in the boiled and fried

eggs; 14:1 n-9, 20:1 n-9, 20:3 n-6, 22:6 n-3, and 20:4 n-6 in the fried eggs; 17:1 n7 and 18:2 n-6 only in the boiled eggs. In the storage periods, the reduction of the fatty acid 14:1 n-9, 16:1 n7, 20:3 n-6, and 18:3 n-3 occurred only during the first 15 days of storage, while for the fatty acids 18:2 n-6 and 20:2 n-6, it was reduced up to the 30th day; the 17:1 n7, 18:1 n-9, 20:1 n-9, 18:3

Table 4. Fatty Acids (g/100g of Yolk, Dry Basis) in Eggs Enriched with n-3 Fatty Acids during 45 Days of Storage at 5 °C[a]

fatty acids	zero time R[b]	B[c]	F[d]	means[e]	15 days R[b]	B[c]	F[d]	means[e]	30 days R[b]	B[c]	F[d]	means[e]	45 days R[b]	B[c]	F[d]	means[e]	Means R[b]	B[c]	F[d]	means[g]
14:0	0.55	0.53	0.55	0.54 a	0.50	0.51	0.50	0.50 a	0.49	0.52	0.50	0.50 a	0.50	0.50	0.49	0.50 a	0.51 a	0.52 a	0.51 a	0.51 a
15:0	0.08	0.08	0.07	0.08 a	0.07	0.08	0.08	0.08 a	0.08	0.06	0.07	0.07 a	0.07	0.07	0.07	0.07 a	0.08 a	0.07 a	0.07 a	0.07 a
16:0	3.97	3.98	3.96	3.97 a	3.94	3.93	3.91	3.93 a	3.93	3.93	3.94	3.93 a	3.92	3.94	3.94	3.93 a	3.94 a	3.95 a	3.94 a	3.94 a
17:0	0.26	0.24	0.25	0.25 a	0.24	0.23	0.25	0.24 a	0.25	0.25	0.24	0.25 a	0.23	0.24	0.23	0.23 a	0.25 a	0.24 a	0.24 a	0.24 a
18:0	1.50	1.50	1.49	1.50 a	1.48	1.45	1.46	1.46 a	1.44	1.48	1.50	1.47 a	1.47	1.45	1.45	1.46 a	1.47 a	1.47 a	1.48 a	1.47 a
20:0	0.10	0.08	0.09	0.09 a	0.07	0.08	0.08	0.08 a	0.08	0.08	0.07	0.08 a	0.08	0.07	0.08	0.08 a	0.08 a	0.08 a	0.08 a	0.08 a
22:0	0.10	0.10	0.10	0.10 a	0.09	0.08	0.09	0.09 a	0.09	0.09	0.08	0.09 a	0.09	0.08	0.09	0.09 a	0.09 a	0.09 a	0.09 a	0.09 a
24:0	0.02	0.02	0.01	0.01 a	0.02	0.01	0.02	0.01 a	0.01	0.01	0.02	0.01 a	0.01	0.01	0.01	0.01 a	0.01 a	0.01 a	0.01 a	0.01 a
14:1n-9	0.11	0.10	0.09	0.10 a	0.09	0.10	0.08	0.09 ab	0.09	0.10	0.08	0.09 ab	0.09	0.09	0.06	0.09 b	0.10 a	0.10 a	0.08 b	0.09 a
16:1n7	5.25	4.71	4.92	4.96 a	4.93	4.51	4.19	4.54 ab	3.95	3.96	4.37	4.09 b	3.27	3.98	3.30	3.52 b	4.35 a	4.29 a	4.19 a	4.28 a
17:1n7	0.24	0.17	0.20	0.20 a	0.16	0.13	0.16	0.15 b	0.13	0.10	0.12	0.12 c	0.08	0.09	0.09	0.09 d	0.15 a	0.12 b	0.14 ab	0.14 a
18:1n-9	6.64	5.97	6.09	6.23 a	6.10	4.87	5.96	5.64 b	5.76	4.63	5.51	5.30 c	5.26	4.12	4.88	4.75 d	5.94 a	4.90 c	5.61 b	5.48 a
20:1n-9	0.38	0.34	0.37	0.36 a	0.38	0.32	0.36	0.35 ab	0.32	0.31	0.34	0.32 bc	0.31	0.28	0.27	0.29 c	0.35 a	0.31 a	0.33 ab	0.33 a
18:2n-6	5.31	5.13	5.05	5.16 a	4.64	4.81	4.75	4.76 b	4.60	4.43	4.34	4.46 bc	4.34	2.92	3.86	3.71 c	4.72 a	4.32 b	4.50 a	4.51 b
18:3n-3	1.82	0.83	1.59	1.41 a	1.67	1.01	1.33	1.34 ab	1.60	0.93	1.27	1.27 b	1.16	0.99	1.10	1.08 b	1.56 a	0.94 c	1.32 bc	1.27 a
18:3n-6	0.02	0.01	0.01	0.01 a	0.02	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 b	0.01 a	0.01 b	0.01 b	0.01 a
20:2n-6	0.29	0.25	0.28	0.27 a	0.26	0.24	0.25	0.25 a	0.22	0.23	0.22	0.22 ab	0.21	0.19	0.21	0.20 b	0.24 a	0.23 b	0.24 ab	0.24 a
20:3n-6	0.03	0.02	0.02	0.02 a	0.02	0.02	0.02	0.02 ab	0.02	0.01	0.02	0.02 ab	0.01	0.01	0.02	0.02 b	0.02 a	0.02 b	0.02 a	0.02 a
20:4n-6	0.20	0.13	0.15	0.16 a	0.12	0.10	0.14	0.12 ab	0.10	0.10	0.13	0.11 b	0.10	0.08	0.10	0.09 c	0.13 a	0.10 b	0.13 a	0.12 a
20:5n-3	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 ab	0.01	0.01	0.01	0.01 b	0.01	0.00	0.01	0.01 c	0.01 a	0.01 b	0.01 b	0.01 a
22:6n-3	0.26	0.14	0.22	0.21 a	0.21	0.12	0.21	0.18 ab	0.20	0.12	0.14	0.15 bc	0.20	0.11	0.12	0.15 c	0.22 a	0.12 c	0.17 b	0.17 a
18:1 n-9t	0.03	0.02	0.02	0.03 a	0.03	0.02	0.02	0.02 ab	0.02	0.02	0.02	0.02 ab	0.02	0.02	0.02	0.02 b	0.03 a	0.02 a	0.02 a	0.02 a
18:2 n-6t	0.01	0.01	0.01	0.01 a	0.01	0.01	0.01	0.01 ab	0.01	0.01	0.01	0.01 ab	0.01	0.01	0.01	0.01 b	0.01 a	0.01 a	0.01 a	0.01 a

[a] Means of the six analyses (two batches in triplicate). Means in the same row with the same letter do not differ significantly ($p > 0.05$). Means in the same column with the same letter do not differ significantly ($p > 0.05$). [b] Raw. [c] Boiled. [d] Fried. [e] Means of days. [f] Means of each treatments. [g] Means stored at 5 °C. 21:0, 17:1 n-7, 15:1 n-9; 22:1 n-9, 24:1 n-9, and 20:3 n-3 were detected for traces (less than 1 mg/100 g).

n-6, 20:4 n-6, 20:5 n-3, and 22:6 n-3 were gradually reduced until the final storage time.

As for the acids *trans*-18:1 n-9 and 18:2, there was no difference in their contents among the raw, boiled, and fried samples. The 18:1 n-9t was reduced after 15 days of storage and the 18:2 n-6t until the end of the storage period.

The thermal processing and the time of storage reduced the concentration of monounsaturated and polyunsaturated fatty acids, as was verified in the average fatty acid concentrations of the raw, boiled, and fried yolks. The relationship n-6/n-3 varied from 3.4 to 7.3 in the samples (**Table 5**), which is considered acceptable according to the literature (*3*). Nahtigal et al. (*21*) described significant loss of saturated, monounsaturated, and polyunsaturated fatty acids in both boiled and fried eggs. Koo et al. (*22*) reported that in eggs enriched with DHA and stored at 4 °C for 3 weeks, the total content of fatty acids was higher during the first two weeks and decreased in the third week.

Vitamin E in Eggs Enriched with n-3 Fatty Acids. The content of vitamin E in the n-3 enriched eggs was determined considering that hens are usually fed with diets rich in polyunsaturated fatty acids, which are supplemented with antioxidants. Vitamin E is an important additive for increasing the oxidative stability of these acids, and therefore, it is necessary to know the vitamin E levels in the eggs. The lowest content of vitamin E was observed in fried eggs, and there was no reduction during storage time. Meluzzi et al. (*23*) also observed that the concentration of vitamin E was not altered in eggs enriched with n-3 and vitamin E that was stored for 28 days at room temperature (20 to 25 °C).

The average content of vitamin E was 11.50 mg/100 g of raw yolk (**Table 6**), a value similar to that obtained by Mazalli et al. (*24*) in eggs of hens fed with different sources of fatty acids (fish oil, flaxseed oil, canola oil, sunflower, and ground flaxseed) and vitamin E (50 and 100 IU/kg of feed). The content of vitamin E found in the present work was indeed high, varying from 5.00 to

Table 5. Saturated, Monounsaturated, Polyunsaturated and *trans*-Fatty Acids in Eggs Enriched with n-3 Fatty Acids, Analyzed at Time Zero (T0) and After 45 Days (Tf)[a]

g/100 g (dry basis)		raw means ± RDS[a]	boiled means ± RDS[a]	fried means ± RDS[a]
saturated (SAT)	T0	6.58 ± 1.36 Aa	6.53 ± 1.37 Aa	6.52 ± 1.36 Aa
	Tf	6.38 ± 0.01 Aa	6.38 ± 0.03 Aa	6.37 ± 0.01 Aa
monounsaturated	T0	12.83 ± 1.14 Aa	11.58 ± 0.78 Ab	12.15 ± 1.77 Ab
(MONO)	Tf	9.97 ± 0.67 Ba	8.07 ± 0.01 Bc	8.89 ± 0.01 Bb
n-6 PUFA	T0	6.14 ± 0.42 Aa	5.65 ± 0.16 Ac	5.94 ± 0.61 Ab
	Tf	5.13 ± 0.50 Ba	3.96 ± 0.01 Bc	4.46 ± 0.01 Bb
n-3 PUFA	T0	2.04 ± 0.32 Aa	1.77 ± 0.50 Ac	1.21 ± 0.38 Ab
	Tf	1.54 ± 0.38 Ba	0.62 ± 0.01 Bb	0.61 ± 0.01 Bb
polyunsaturated	T0	8.17 ± 0.74 Aa	7.43 ± 0.63 Ab	7.15 ± 1.00 Ab
(PUFA)	Tf	6.67 ± 0.89 Ba	4.58 ± 0.01 Bc	5.06 ± 0.01 Bb
trans-fatty acids	T0	0.02 ± 0.01 Ac	0.04 ± 0.01 Ab	0.06 ± 0.01 Aa
	Tf	0.04 ± 0.00 Ba	0.03 ± 0.00 Bb	0.03 ± 0.01 Bb
unsaturated	T0	21.00 ± 1.87 Aa	19.00 ± 1.41 Aa	19.30 ± 2.76 Aa
	Tf	16.64 ± 1.56 Ba	12.65 ± 0.01 Bb	13.96 ± 0.01 Bc
		Ratio		
n-6 PUFA/n-3 PUFA-	T0	3.04 ± 0.27 Bb	3.29 ± 0.79 Bb	5.08 ± 1.10 Ba
	Tf	3.39 ± 0.51 Ac	6.44 ± 0.07 Ab	7.31 ± 0.18 Aa
PUFA/SAT	T0	1.15 ± 0.12 Aa	1.12 ± 0.08 Ab	1.06 ± 0.11 Ac
	Tf	1.11 ± 0.15 Aa	0.91 ± 0.00 Bc	0.93 ± 0.00 Bb

[a] Means and relative standard deviation (DRS) of six analyses (two batches in triplicate). Means in the same row with the same letter do not differ significantly ($p > 0.05$). Means in the same column with the same letter do not differ significantly ($p > 0.05$).

5.53 mg per egg. Since according to the USDA (*20*) the natural vitamin E content must be 0.525 mg per egg, in both cases the eggs weighed 50 g.

Cholesterol and Cholesterol Oxides in Eggs Enriched with n-3 Fatty Acids. The cholesterol content in both batches remained the same during eggs storage, and no significant interaction was

detected among the evaluated factors; however, in batch 1 the cholesterol content was lower than that in batch 2. The cholesterol content in raw samples was higher than that in fried and boiled eggs, although the values obtained were significantly different (**Table 7**) only in the fried eggs. In a previous work, Bragagnolo and Rodriguez-Amaya (*25*) also did not find significant differences (*p* > 0.05) in the cholesterol content of raw and boiled eggs. Similar results of cholesterol in raw eggs were reported in other studies (*26*, *27*). The average values of cholesterol in the raw samples were inferior to the values referred by the USDA (*20*).

The cholesterol oxides 7-keto, 7α-OH, and 7β-OH varied substantially between the two batches of eggs; therefore, the results were analyzed with reference to each batch. The highest oxide contents were observed in the fried samples of batch 2 (**Table 8**). There was significant interaction among thermal treatments, time, and temperature of storage. The storage conditions did not affect the 7β-OH and 7α-OH oxides, but the 7-keto content of batch 1 was higher in samples stored at 25 °C.

In both experimental batches, there was an increase of the 7-keto and 7α-OH oxides in raw and thermal-treated samples, as assayed from time zero until 30 days of storage at 25 and 5 °C (**Table 8**). However, the 7β-OH content in boiled and raw samples of batch 1 was reduced in the first 15 days of storage at 25 and 5 °C. In fried samples, 7β-OH oxide increased with storage time, while in raw samples of batch 2, there was a reduction of this cholesterol oxide throughout storage.

The decrease of cholesterol in time zero in raw eggs was 8% in batch 1 and 4% in batch 2, compared to the eggs analyzed after frying; the average of cholesterol oxides 7-keto, 7β-OH, and 7α-OH in fried eggs increased, respectively, 316, 448, and 110% when compared that of to raw eggs. Sarantinos et al. (*27*) reported a cholesterol decrease of 14% in boiled eggs, but the cholesterol oxides increased 75%. The reduction of cholesterol in thermal-treated eggs can be attributed to the loss of this substance together with the fat released from food during thermal

Table 6. Vitamin E (mg/100g Yolk) in Eggs Enriched with n-3 Fatty Acids, Stored at 25 and 5 ± 2 °C, and Analyzed at the Time Zero (T0) and after 45 Days (Tf)[a]

		Vitamin E		
	yolk	T0	Tf	means[a]
25 °C	raw	11.62	11.25	11.43 A
	boiled	10.77	11.50	11.13 A
	fried	10.32	9.99	10.15 B
	means[a]	10.90 a	10.91 a	10.91 A
5 °C	raw	11.62	11.39	11.50 A
	boiled	10.77	10.87	10.82 B
	fried	10.32	10.70	10.51 B
	means[a]	10.90 a	10.99 a	10.94 A

[a] Means of the six analyses (two batches in triplicate). Means in the same row with the same letter do not differ significantly (*p* > 0.05). Means in the same column with the same letter do not differ significantly (*p* > 0.05).

Table 7. Cholesterol (mg/100g Yolk, Dry Basis) in Eggs Enriched with n-3 Fatty Acids during 45 Days of Storage at 25 and 5 ± 2 °C[a]

Cholesterol (Batch 1)					
yolk/days	zero	15	30	45	means
25 °C					
raw	2401	2426	2423	2434	2421 A
boiled	2350	2349	2341	2344	2346 B
fried	2213	2190	2198	2211	2203 C
means[a]	2321 a	2322 a	2321 a	2330 a	2323 B
5 °C					
raw	2401	2430	2417	2441	2423 A
boiled	2350	2342	2331	2347	2342 B
fried	2213	2214	2210	2213	2212 C
means[a]	2321 a	2328 a	2319 a	2334 a	2326 B
Cholesterol (Batch 2)					
yolk/days	zero	15	30	45	means
25 °C					
raw	2546	2536	2522	2512	2529 A
boiled	2502	2499	2501	2510	2503 A
fried	2444	2437	2429	2455	2441 B
means[a]	2497 a	2490 a	2484 a	2492 a	2491 A
5 °C					
raw	2546	2540	2510	2521	2529 A
boiled	2502	2504	2490	2515	2503 A
fried	2444	2428	2439	2440	2438 B
means[a]	2497 a	2491 a	2480 a	2492 a	2490 A

[a] Means in triplicate. Means in the same row with the same letter do not differ significantly (*p* > 0.05). Means in the same column with the same letter do not differ significantly (*p* > 0.05).

Table 8. Cholesterol Oxides (μg/g Yolk, Dry Basis) in Eggs Enriched with n-3 Fatty Acids during 45 Days of Storage at 25 and 5 $\pm$ 2 °C [a]

Batch 1

yolk/days	7-keto					7β-OH					7α-OH				
	zero	15	30	45	means	zero	15	30	45	means	zero	15	30	45	means
25 °C															
raw	0.00 Bc	0.86 Bb	0.98 Bab	1.49 Ba	0.83C	3.02 Ba	0.61 Bbc	0.86 Bb	0.58 Bc	1.27 C	3.88 Bc	4.17 Bbc	4.31 Cb	8.98 Ba	5.34 B
boiled	0.16 Ac	0.39 Bc	1.66 Bb	3.78 Aa	1.57 B	5.50 Aa	0.92 Bc	1.31 Bbc	1.50 Bb	2.30 B	4.09 Bb	4.38 Bb	7.08 Ba	8.47 Ba	6.01 B
fried	0.44 Ac	1.94 Ab	3.15 Aa	3.61 Aa	2.21 A	6.55 Ac	7.44 Abc	8.96 Ab	13.28 Aa	9.05 A	8.80 Ac	9.72 Ab	14.55 Aa	16.92 Aa	12.50 A
means[a]	0.20 d	1.06 c	1.93 b	2.96 a	1.54 A	5.03a	2.99 b	3.71 b	5.11 B	4.21 B	5.59 c	6.09 c	8.65 b	11.46a	7.95 B
5 °C															
raw	0.00 Bd	0.13 Bc	0.23 Bb	0.33 Ca	0.17 B	3.02 Ba	0.87 Bc	1.04 Bb	2.44 Ba	1.84 C	3.88 Bc	4.15 Bbc	6.98 Bb	11.21 Ba	6.55 B
boiled	0.16 Bc	0.23 Bb	0.29 Bb	0.53 Ba	0.30 B	5.50 Aa	1.05 Bc	1.45 Bb	1.36 Bb	2.34 B	4.09 Bc	4.93 Bbc	6.19 Bb	9.94 Ba	6.29 B
fried	0.44 Ac	0.44 Bc	1.86 Ab	5.06 Aa	1.95 A	6.55 Abc	7.22 Ab	6.50 Ac	11.29 Aa	7.90 A	8.80 Ac	11.7 Ab	12.83 Ab	16.50 Aa	12.47 A
means[a]	0.20 c	0.27 c	0.79 b	1.97 a	0.81 B	5.03 a	3.05 b	3.00 b	5.03 B	4.03 B	5.59 c	6.95 c	8.66 b	12.55 a	8.43 B

Batch 2

yolk/days	7-keto					7β-OH					7α-OH				
	zero	15	30	45	means	zero	15	30	45	means	zero	15	30	45	means
25 °C															
raw	0.00 Bc	0.39 Cb	0.41 Cab	0.68 Ca	0.37 C	4.31 Ba	1.88 Bb	0.64 Cc	0.68 Cc	1.88 C	4.08 Bb	4.25 Bb	6.33 Ba	7.96 Ba	5.65 B
boiled	0.87 Ac	1.02 Bb	1.10 Bab	1.25 Ba	1.06 B	2.12 Cb	1.59 Bc	2.15 Bb	4.97 Ba	2.71 B	3.98 Bc	4.12 Bc	8.25 Bb	11.21 Aa	6.89 B
fried	1.23 Ac	2.23 Ab	2.78 Ab	4.56 Aa	2.70 A	8.25 Ac	8.41 Abc	9.02 Ab	16.65 Aa	10.58 A	10.23 Ab	10.69 Ab	13.58 Aa	17.01 Aa	12.87 A
means[a]	0.70 d	1.21 c	1.43 b	2.16 a	1.37 A	4.89 b	2.71 c	3.94 b	7.43 A	5.02 A	6.09 c	6.35 c	9.39 b	12.06 a	8.47 A
5 °C															
raw	0.00 Bc	0.41 Cb	0.54 Cb	0.95 Ca	0.47 C	4.31 Ba	2.11 Bb	0.42 Cc	0.21 Cd	1.76 C	4.08 Bc	5.01 Bbc	6.28 Cb	12.05 Ba	6.85 B
boiled	0.87 Ab	0.98 Bb	1.26 Ba	1.52 Ba	1.16 B	2.12 Cb	1.33 Cc	2.69 Bb	5.02 Ba	2.79 B	3.98 Bb	3.96 Bb	9.51 Ba	12.84 Ba	7.57 B
fried	1.23 Ac	2.21 Ab	2.84 Ab	5.20 Aa	2.87 A	8.25 Ac	9.01 Ab	9.32 Ab	16.41 Aa	10.74 A	10.23 Ab	10.56 Ab	14.37 Aa	18.42 Aa	13.38 A
means[a]	0.70 d	1.20 c	1.55 b	2.56 a	1.50 A	4.89 b	4.15 c	4.14 c	7.21 A	5.10 A	6.09 c	6.51 c	10.03 b	14.44 a	9.26 A

[a] Means in triplicate. Means in the same row with the same letter do not differ significantly ($p > 0.05$). Means in the same column with the same letter do not differ significantly ($p > 0.05$).

processing, through thermal degradation, association with other molecules, or probably by the combination of these factors (*28*).

The monounsaturated and polyunsaturated fatty acid content decreased during egg storage as well as in both thermal treatments (i.e., in fried and boiled eggs). These fatty acids are susceptible to oxidation, and consequently, free radicals and peroxides are produced, accelerating cholesterol oxide formation. According to Smith (*7*), the fatty acid oxidation products attack the cholesteril portion of the cholesterol ester molecules. Moreover, the thermal processing at high temperatures accelerates lipid reactions with molecular oxygen (*29, 30*).

In a previous work, Sarantinos et al. (*27*) reported 4 to 8 μg/g 7α-OH and 34 to 59 μg/g 7β-OH in fried and boiled eggs, respectively; the authors did not find 7-keto. In powdered eggs, the oxides 5,6α and 5,6β-epoxides are usually found at significant concentrations, varying from 1.0 to 12 μg/g (5,6α-epoxy) and 4.1 to 27 μg/g (5,6β-epoxy) due to the spray-drying treatment at approximately 180 °C (*31−33*). However, in our work no epoxides were detected in eggs fried at approximately 180 °C. In addition, in the spray-dried egg treatment, the contact surface with molecular oxygen is much greater than that in the frying process; besides, the water activity in eggs is much higher than that in powdered eggs, protecting them from oxidation. According to Obara et al. (*34*), the highest accumulation of oxysterols was found in egg powder with the lowest water content.

In the present work, cholesterol oxides were already present at time zero, and their content increased significantly during egg storage. The presence of these oxides at time zero suggests that either lipid oxidation had already occurred or these products are metabolites originated enzymatically by hen metabolism. Moreover, they could be derived from poultry feed containing cholesterol oxides. According to Galobart et al. (*35*), fresh eggs of hens fed with sunflower oil were rich in hydroperoxides (324.34 μg/g of egg) and substances reactive to thiobarbituric acid (30 a 50 ng malonaldehyde/g of egg), which can accelerate cholesterol oxidation (*7*). During eggs storage, the eggshell could have prevented this lipid oxidation (*36*). Considering the increase of cholesterol oxides during egg storage, penetration of molecular oxygen through the eggshell possibly occurred. Vitamin E was not reduced during egg storage, and there was a simultaneous increase of cholesterol oxides as well as a decrease of unsaturated fatty acids. Therefore, vitamin E did not have an effective antioxidant effect in these egg samples, probably due to the presence of water in the egg yolks (50 g/100 g), which prevented its action. According to Gordon (*37*), the mechanism of metal chelation by phenolic antioxidants, such as vitamin E, is more effective in oils than in food containing water. In addition, a gradual vitamin E loss was observed, accompanied by a simultaneous increase in fatty acid oxidation and cholesterol oxide formation, in eggs dehydrated by spray-drying and stored for 18 months at room temperature, exposed to air and light (*38*).

On the basis of the results obtained in the present work, it can be concluded that thermal processing, especially the frying process, reduces the total lipid content of eggs, cholesterol, and fatty acids and raises the levels of cholesterol oxides. The 45-day storage period at 5 and 25 °C did not modify the content of total lipids and cholesterol but considerably reduced the level of unsaturated fatty acids, especially that of the polyunsaturated ones. At the same time, there was an increase in the content of 7-keto, 7α-OH, and 7β-OH oxides in the n-3 enriched eggs. In the evaluated storage conditions, 7-keto oxide was increased in eggs stored at 25 °C, demonstrating the importance of storing the eggs under refrigeration in order to avoid the formation of this oxide.

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