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Oxidative Stability of Omega-3 Polyunsaturated Fatty Acids Enriched Eggs

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ABSTRACT: Omega-3 polyunsaturated fatty acids (n-3 PUFA) enriched eggs have a growing market share in the egg industry. This study examined the stability of n-3 PUFA enriched eggs fortified with antioxidants (vitamin E or organic Selenium [Sel-Plex] or both) following cooking and storage. The total fat content was not affected by cooking or simulated retail storage conditions, whereas, n-3 fatty acids were reduced. The content of n-3 fatty acids in boiled eggs was higher than in fried eggs. Lipid oxidation was significantly affected by the different cooking methods. Fried eggs contained higher levels of malondialdehyde (MDA, 2.02 μg/kg) and cholesterol oxidation products (COPs, 13.58 μg/g) compared to boiled (1.44 and 10.15 μg/kg) and raw eggs (0.95 and 9.03 μg/kg, respectively, for MDA and COPs). Supplementation of antioxidants reduced the formation of MDA by 40% and COPs by 12% in fried eggs. Although the content of MDA was significantly increased after 28 days of storage, COPs were not affected by storage. Our study indicated that the n-3 PUFA in enriched eggs was relatively stable during storage and home cooking in the presence of antioxidants.

KEYWORDS: *omega-3 eggs, oxidative stability, antioxidants, cholesterol oxidation products (COP), TBARs*

INTRODUCTION

Omega-3 polyunsaturated fatty acids (n-3 PUFA), mainly eicosapentaenoic acid (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3) and alpha-linolenic acid (ALA, 18:3 n-3), are suggested to reduce the risk of cardiovascular diseases and certain forms of cancer, as well as to improve brain development and function.[1,2] Due to compelling evidence supporting the health benefits of n-3 PUFA consumption, various organizations and governments recommend dietary intake of 1.4−2.5 g/d total n-3 PUFA, with EPA plus DHA ranging from 140 to 600 mg/d to decrease the risk of diseases and improve overall health.[3] Although marine fish is the richest dietary source of DHA and EPA, many people consume little or no fish.[4] Eggs are widely consumed globally as a nutritious and convenient food commodity. Therefore, food enrichment is emerging as perhaps the best long-term solution to encourage n-3 PUFA intake without significantly dietary changes, especially n-3 enriched eggs.[5] In addition to being highly digestible for humans, eggs are well-known as an efficient "carrier" for nutrients. Enriched egg products continue to expand market share globally.

However, the relatively high concentration of unsaturated fatty acids in enriched egg products make these products susceptible to oxidative damage during cooking preparations and storage. Lipid oxidation yields free radicals, which can cause more oxidation damage as chain reactions and trigger secondary oxidation reactions. Some secondary oxidation products of lipid are toxic chemicals, including malonaldehyde (MA) and other dicarbonyl compounds.[6] Oxidation of n-3 PUFA (particularly susceptible to oxidative damage during cooking) not only produces genotoxic compounds (causing cell and DNA damage), but also accelerates the formation of cholesterol oxidation products (COPs), which have been shown to be responsible for the proatherogenic action of cholesterol.[7] Therefore, vitamin E has been traditionally added to diets to help stabilize n-3 PUFA. Organic selenium (selenomethionine) is an essential part of a variety of selenoproteins, such as glutothione peroxidase (GSH-Px). Selenomethionine increased activity of GSH-Px, an important antioxidant complex.[8] Vitamin E works synergistically with GSH-Px, because GSH-Px continues the work of vitamin E by detoxifying hydroperoxides.[9] The combination of vitamin E and organic Se could therefore be an effective antioxidant for n-3 PUFA in enriched eggs and egg products during storage. However, little work has been undertaken to test their effects on the stability of omega-3 fatty acids under different cooking methods and during storage. Therefore, this study focused on combined effect of cooking vs storage and vitamin E vs selenium on the stability of n-3 PUFA enriched eggs and the formation of potential hazardous compounds associated with lipid oxidation.

In the current study, the stability of n-3 PUFA enriched eggs fortified with antioxidants (vitamin E or organic Selenium [Sel-Plex] or both) was tested. The objectives of the current study were to determine the effect of dietary vitamin E or Sel-Plex or both on (1) the stability and fate of n-3 PUFA in n-3 PUFA enriched eggs under simulated retail storage conditions and varied cooking methods, (2) the cholesterol-oxidized genotoxic compounds formed during storage and cooking.

MATERIALS AND METHODS

Animals and Dietary Treatments. A total of 120 laying hens (White Leghorn, 37 weeks old) were randomly divided into four

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groups of 30 (housed in pairs). Omega-3 polyunsaturated fatty acids enriched diets were fed to all hens (18.7% Crude Protein; 3000 kcal/kg), and included 17% LinPRO (50:50 extruded mixed flaxseed and field peas, O & T Farm, Regina, SA, Canada). There were two antioxidants (vitamin E and selenomethionine source [Sel-Plex]), both of which were provided at a low (50 IU/kg vitamin E or 0.1 mg/kg Se as sodium selenite) and high (200 IU/kg vitamin E or 0.3 mg/kg Sel-Plex) levels, in a 2 × 2 factorial arrangement. The four diets were as follows: (1) control diet: base diet only, which contained 50 IU/kg Vitamin E and 0.1 mg/kg Se as sodium selenite (low vitamin E+ low selenium, LE × LSe); 2) vitamin E diet: base diet +200 IU/kg vitamin E supplement (high vitamin E+ low selenium, HE × LSe); 3) selenium diet: base diet +0.3 mg/kg Sel-Plex, (low vitamin E + high selenium, LE × HSe); 4) vitamin E + selenium: base diet +200 IU/kg Vit E and 0.3 mg/kg Sel-Plex, (high vitamin E + high selenium, HE × HSe).

The diets were presented in Table 1. Following 28 days of feeding the experimental diets, 238 eggs were collected and equally divided into four pools per treatment. Following 0 or 28 days of simulated retail storage (4 °C, in shell inside Styrofoam cartons), half of the eggs from each pool were cooked using home-style cooking methods (raw/boiled/fried), then frozen at −20 °C until analysis.

Egg Traits. At the start and end of the trial, total egg and component weights were measured. The analyzed egg traits included egg weight, specific gravity, yolk weight, albumen height, shell think and shell weight. Daily egg production was recorded throughout the study.

Sample Preparation. To prepare boiled eggs, eggs were placed in single layer in a saucepan, with at least one inch of cold water over tops of shells. After boiling 10 min, eggs were placed under running water (room temperature) for 5 min. Then, yolks were separated from albumen and packaged using a vacuum packager (Food Saver Vac 1200, 110 V). To prepare fried eggs, a nonstick frying pan (Multicuisine SK200 nonstick frying pan, Black & Decker, MD) was preheated for 30 min (177 °C/350 °F) and approximately 15 g of yolk were oil-free fried for 80 s (40 s each side) and vacuum packaged. To prepare raw yolk samples, yolks were separated from albumen and vacuum packaged. All egg samples were freeze-dried and stored in sealed container at −20 °C pending further analysis.

Lipid Extraction. Total lipids were extracted from the fine powder of raw, boiled and fried yolk samples by direct chloroform extraction method.[10] Around 0.5 g of powder sample was weighed, and 10 mL of chloroform (Fisher Scientific, Ottawa, ON) was added to dissolve the yolk lipid completely, sample placed in chloroform for more than 16 h. Then, 10 mL of hexane (Fisher Scientific, Ottawa, ON) was added; after centrifugation at 3000*g* for 5 min, 10 mL of supernatant was transferred into a pre weighed scintillation vial, and dried under nitrogen (2h, 55 °C).

Analysis of Fatty Acid Composition. Dry yolk lipid was redissolved in a known quantity of chloroform to a final lipid concentration of 0.2 g/mL. Fifty μL of lipid-chloroform solution was then divertised using 2 mL of methylating reagent (Methanolic HCl, 1N, Sigma, Oakvilly, ON) in a water bath at 60 °C for 60 min. After cooling to room temperature, 100 μL of water, a known amount of internal standard (heptadecanoic acid, 17:0, Sigma, Oakvilly, ON) and 5 mL of hexane was added, mixed thoroughly, and centrifuged at 1500*g* for 3 min. The top hexane layer was transferred to a clean test tube containing a pinch of anhydrous sodium hydroxide in order to remove water from hexane completely. After the third centrifugation (3 min, 1500*g*), 1 mL of hexane solution was transferred to gas chromatograph (GC) vial and 1 μL of hexane was injected to GC for analysis. Fatty acid composition was determined with a gas chromatograph (model 3400, Varian, Palo Alto, CA), equipped with a flame ionization detector and a SGE BP20 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Scientific Instrument Services Inc., Ringoes, NJ). Operating conditions for the GC were the following: held 0.2 min at 50 °C, then increased to 120 °C at a rate of 20 °C/min for 5 min. The temperature continued increasing at a rate of 10 °C/min until the final temperature (230 °C) was reached, then maintained at 230 °C till the end of total running time (30.2 min). A cool-on-column injection method was used, with an initial and final injector temperature (CO_2) of 60 °C (0.2 min) and 230 °C (28 min) respectively, increasing at a rate of 150 °C/min. The temperature of the detector was 240 °C and the column head pressure of the carrier gas (helium) was 25 PSI. The fatty acid peak integration was performed using the Galaxie Chromatography Data System (Varian). Samples were analyzed in duplicate.

Table 1. Diet Composition (% of Total Ingredients) and Nutrient Content of Base Diet, High Vitamin E Diet, High Selenium Diet and High Vitamin E Plus Selenium Diet for White Leghorn

treatments	LE × LSe[d]	HE × LSe[e]	LE × HSe[f]	HE × HSe[g]
Ingredient Name				
soybean meal deh - plant[a]	16.62	16.63	16.63	16.63
wheat, hard, grain	50.87	50.88	50.88	50.88
calcium carbonate	9.46	9.46	9.46	9.46
dicalcium phosphate	1.61	1.60	1.61	1.61
salt, plain (NaCl)	0.35	0.35	0.35	0.35
D,L-methionine	0.13	0.13	0.13	0.13
L-threonine	0.0002	0.0002	0.0002	0.0002
linpro	17	17	17	17
layer vit/min premix[a]	0.5	0.5	0.5	0.5
choline chloride premix[b]	0.5	0.5	0.5	0.5
generic enzyme[c]	0.05	0.05	0.05	0.05
canola oil	2.90	2.90	2.90	2.90
vitamin E (IU/kg)		200		200
Sel-Plex 600 (g/kg)			0.5	0.5
Nutrient Analysis:				
Nutrient Name				
M.E. (kcal/kg)	3000	3000	3000	3000
crude protein, %	18.7	18.7	18.7	18.7
crude fat, %	7.48	7.48	7.48	7.48
crude fiber, %	2.94	2.94	2.94	2.94
total phosphorus, %	0.64	0.64	0.64	0.64
available phosphorus, %	0.4	0.4	0.4	0.4
Met + Cys, %	0.76	0.76	0.76	0.76
methionine, %	0.42	0.42	0.42	0.42
lysine, %	0.90	0.90	0.90	0.90
tryptophan, %	0.26	0.26	0.26	0.26
arginine, %	0.94	0.94	0.94	0.94

[a]Layer premix provided per kilogram of diet: vitamin A (retinyl acetate), 12 000 IU; cholecalciferol, 3000 IU; vitamin E (DL-α-tocopheryl acetate), 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; Mn, 75 mg; Cu, 15 mg; Zn, 80 mg, Se, 0.1 mg; and Fe, 100 mg. [b]Provided choline chloride in the diet at a level of 100 mg/kg. [c]Generic enzyme: Avizyme 1302, xylanase enzyme, Danisco Animal Nutrion, Marlborough, Wiltshire, UK. [d]LE × LSe = low vitamin E and low selenium treatment. [e]HE × LSe = high vitamin E and low selenium treatment. [f]LE × HSe = low vitamin E and high selenium treatment. [g]HE × HSe = high vitamin E and high selenium treatment.

Extraction and Purification of Cholesterol Oxidation Products (COPs). Extraction of COPs was performed according to the method described by Guardiola et al.[11] with slight modifications. COPs extraction and purification procedure included four steps: lipid extraction,[12] cold saponification, cartridge purification, and silanization. About 0.5 g of dried yolk was weighed into a 50 mL screwed test tube,

Table 2. Fatty Acids Composition (g/100 g of Total Fatty Acids) of Omega-3 Polyunsaturated Fatty Acids (n-3 PUFA) Enriched Eggs Affected by the Main Factors: Cooking, Storage, Vitamin E and Selenium[a]

		fatty acid profile										
effects	n^b	C18:2 n-6	C18:3 n-3	C20:4 n-6	C20:5 n-3	C22:5 n-3	C22:6 n-3	MUFA[c]	SFA[d]	total n-6	total n-3	n-6: n-3
Cooking (Mean)												
raw	16	14.42b	5.72b	1.68	0.24a	0.25a	2.28a	42.20	32.94	16.38b	8.49ab	1.93b
boiled	16	14.87a	5.92a	1.65	0.22b	0.24a	2.29a	41.96	32.57	16.80a	8.67a	1.94b
fried	16	14.77a	5.78ab	1.64	0.21b	0.21b	2.19b	41.54	33.37	16.69ab	8.40b	1.99a
SEM[e]		0.094	0.05	0.027	0.006	0.003	0.029	0.306	0.29	0.114	0.068	0.005
Storage (Mean)												
0 days	24	14.62	5.78	1.70a	0.23a	0.25a	2.36a	41.70	33.07	16.61	8.63a	1.93b
28 days	24	14.76	5.83	1.62b	0.21b	0.22b	2.14b	42.10	32.85	16.64	8.41b	1.99a
SEM		0.077	0.041	0.022	0.005	0.002	0.024	0.25	0.237	0.093	0.056	0.004
Vitamin E (Mean)												
high	24	14.75	5.82	1.62b	0.22	0.23b	2.24	41.88	32.97	16.64	8.51	1.96
low	24	14.62	5.80	1.69a	0.23	0.24a	2.27	41.92	32.95	16.60	8.53	1.95
SEM		0.077	0.041	0.022	0.005	0.002	0.024	0.25	0.237	0.093	0.056	0.004
Selenium (Mean)												
high	24	14.62	5.78	1.63	0.22	0.23b	2.24	41.89	33.13	16.52	8.46	1.96
low	24	14.75	5.83	1.68	0.23	0.24a	2.27	41.91	32.80	16.72	8.57	1.95
SEM		0.077	0.041	0.022	0.005	0.002	0.024	0.25	0.237	0.093	0.056	0.004
source of variation		probability										
cooking (C)		0.0066	0.0300	0.5866	0.0240	<.0001	0.0408	0.3245	0.1685	0.0411	0.0278	<.0001
storage (S)		0.2049	0.3550	0.0181	0.0046	<.0001	<.0001	0.2654	0.5151	0.7965	0.0113	<.0001
vitamin E (E)		0.2614	0.6669	0.0399	0.3639	0.0126	0.3416	0.9216	0.9592	0.7608	0.7758	0.1002
selenium (Se)		0.2475	0.4057	0.1212	0.1187	0.0002	0.3077	0.9542	0.3339	0.1426	0.1768	0.5583
C×S		0.1832	0.0002	0.0049	0.4732	0.0249	0.0005	0.0197	0.0112	0.3812	0.2926	<.0001
E×Se		0.0011	<.0001	0.0244	0.1700	0.3955	0.0055	0.4812	0.1577	0.0197	<.0001	<.0001
E×C		0.5958	0.0028	0.0189	0.9181	0.2046	0.0963	0.1270	0.1026	0.3242	0.1902	<.0001
Se×C		0.1927	0.0717	0.2840	0.0460	0.0403	0.8613	0.6881	0.5025	0.3170	0.3011	0.3884
E×S		0.0834	0.0003	0.3421	0.6971	0.3413	0.0185	0.0829	0.0082	0.2132	0.0580	0.0046
Se×S		0.0304	0.1764	0.0371	0.7177	0.1192	0.0287	0.4288	0.0295	0.0215	0.0458	0.8893
E×C×S		0.3738	0.0431	0.3276	0.8479	0.4438	0.0950	0.2244	0.2999	0.5131	0.6639	<.0001
Se×C×S		0.0186	0.0004	0.2783	0.0222	<.0001	0.0129	0.1284	0.7310	0.0736	0.0003	<.0001
E×Se×C		0.4197	0.0066	0.0100	0.7196	0.0069	0.0527	0.1036	0.1319	0.1733	0.3860	<.0001
E×Se×S		0.1014	0.0002	0.4338	0.8470	0.7067	0.6144	0.0394	0.3251	0.2177	0.0024	<.0001
E×Se×C×S		0.6281	0.0011	0.3989	0.3174	0.0115	0.0834	0.9394	0.7360	0.6404	0.0141	<.0001

[a]a−c Means within a column in each treatment with no common superscripts differ ($P < 0.05$). [b]n = sample number, each sample calculation of eight eggs, (pool of eight eggs) = (sample) [c]MUFA = monounsaturated fatty acid. (16:1 + 18:1 n-7 + 18:1 n-9) [d]SFA = saturated fatty acid. (14:0 + 16:0 + 18:0) [e]SEM = standard error of the means

where 50 μg of 19-hydroxycholesterol (19-OH) (Steraloids, Inc., Newport, RI) was added as internal standard. Then, 16 mL of folch solution was added and kept the samples in dark for overnight at 21 °C to extract lipid and COPs. After filtering through Whatman No. 1 filter paper, the residue in the test tube was extracted for the second time using 8 mL of folch solution. Combining the filtrates, sodium chloride solution (0.88%, w/v) was mixed and centrifuged at 2200*g* for 15 min. The bottom layer (organic layer) was transferred into a scintillation vial and dried in nitrogen. Cold saponification was done by following the procedures described by Guardiola et al.[11] The organic extract obtained from the saponification was redissolved in 5 mL of hexane and applied to the silica cartridge (Sep-pack, Vac 6 cm^3, 1g, SPE, Waters, Millpore, Bedford MA). In the cartridge purification, gradually increasing polarity solvent mixtures (10 mL of 95/5 hexane/ether, 30 mL of 90/10 hexane/ether and 10 mL of 80/20 hexane/ether) were applied to remove the interfering compounds and finally 10 mL of acetone/methanol (60/20) was used to obtain the COPs portion. Then, acetone/methanol elute (60/20) was collected and dried under Nitrogen (1 h, 40 °C). The final residue was redissolved in 50 μL of anhydrous pyridine and 50 μL of Sylon BTZ (Supelco Inc., Bellefonte, PA) to complete the silanization reaction for 20 min at 21

°C. Silanization reaction is done to produce silyl derivatives which are stable at −20 °C for several days.[11]

GC-MS and GC Analysis of COPs. Identification of 7 major COPs in sample was performed according to the method of Lee et al.[13] by gas chromatography mass spectroscopy (GC-MS) (PerkinElmer, Clarus 600, Waltham). The seven COPs include 7-α-hydroxycholesterol (7α-OH), 7-β-hydroxycholesterol (7β-OH), β-cholestanetriol (β-CE), α-cholestanetriol (α-CE), 25-hydroxycholesterol (25-OH), cholestanetriol (CT) and 7-keto cholesterol (7-KC) as well as internal standard 19-OH. Quantification of COPs was done using a DB5-column (Agilent J&W GC Columns, 300 Laurier Blvd, Brockville, Ontario K6 V 5W1, Canada) coupled with a gas chromatograph (model 3400, Varian, 3120 Hansen Way, Palo Alto, CA). A flame ionized detector was used for COP quantification. Helium was used as a carrier gas, and carbon dioxide as a cooling gas. The initial column temperature was 70 °C holding for 12 s, then temperature was increasing at an rate of 20 °C/min until the final temperature of 250 °C was reached and hold for 2 min. Temperature continued increasing at a rate of 15 °C/min until the final temperature of 280 °C was reached and then held for 17 min. Injector and detector temperatures were 90 and 300 °C, respectively. The head pressure of the column was set at 22−23 PSI.

The Thiobarbituric Acid Reactive Substances (TBARs). The TBARs test was performed according to the method of Slater and Sawyer.[15] Two gram of yolk samples were weighed into screw tap tubes, 4 mL of 1.15% KCl was immediately added and the mixture was homogenized for 30s using a polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario), and then 1 mL of 80 mM Tris/maleate buffer solution and 4 mL of TCA-TBA-HCl (15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25N hydrochloric acid; this solution was heated to completely dissolve the TBA) were added, mixed rapidly, and filtered through #1 Whatman Filter Paper. The filtrate was incubated in the dark from 15 to 17 h at 21 °C. After incubation, absorbance at 532 nm was recorded spectrophotometrically (UV/vis spectophotometer). An extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$ was used to estimate the malondialdehyde (MDA) values.[14]

Statistical Analysis. All data were subjected to a 2 × 2 × 2 × 3 factorial ANOVA to determine the significant differences between the two vitamin E levels, two Sel-Plex levels, two storage periods, and three cooking methods. The mixed models procedure of SAS (SAS System, 2002, Cary, NC) was used. Means were separated using Tukey adjustment procedure of SAS. To study relationships among the content of vitamin E, Se, fatty acids, COPs and MDA, the data were submitted to correlation analysis in SAS (SAS System, 2002, Cary, NC). Unless otherwise stated, significance was assessed at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Effects of Diet, Cooking and Storage on Fatty Acid Composition. Egg production as well as egg weight, specific gravity, yolk weight, albumen height, shell thickness, and shell weight were not affected by the dietary treatments (*Data not shown*). The mean egg weight was 61.3 g and yolk represented 28.2% of egg weight. The lack of dietary effect on egg traits ($p > 0.1$) indicates that any treatment-based differences were due to diet rather than the egg size or weight.

The total fat content was not affected by cooking ($p = 0.46$) or simulated retail storage ($p = 0.73$) (mean = 31.2% of yolk weight). Egg yolks contained predominantly oleic acid (18:1 n-9, 39%) followed by linoleic acid (LA, 18:2 n-6, 15%, (*data not shown*)). The content of n-3 PUFA and n-6 PUFA were not affected by vitamin E and Sel-Plex (Table 2), which was consistent with the results of Qi and Sim[15] who reported that vitamin E (tocopherol) supplementation did not affect the n-3 PUFA level in n-3 PUFA enriched eggs. Although similar results were reported by Galobart et al.[16] at a supplemental level of 50 mg vitamin E/kg, they observed a decrease in LNA, EPA, DPA, DHA, and total n-3 PUFA content at the supplemental level of 200 mg vitamin E/kg. In the current study, supplemental vitamin E did not affect the contents of LNA and LA, whereas the long chain PUFA (EPA, DPA, DHA, and AA) decreased at the higher level of vitamin E (Table 2). Earlier research reported that vitamin E at higher doses can decrease the content of n-3 PUFA in eggs by interfering in the intestinal absorption and transportation of long-chain n-3 PUFA.[17]

The contents of the two essential fatty acids (EFA), LNA (approximately 65% of total n-3 PUFA) and LA (approximately 90% of total n-6 PUFA) were not affected by 28 days of storage (Table 2). Similarly, Ahn et al.[18] reported that LNA and LA in eggs were stable over 49 days of storage at 4 °C; however, long chain PUFA (EPA, DPA, DHA, and arachidonic acid [**AA**, 20:4 n-6]) were decreased by 8.74%, 12.0%, 6.74%, and 4.72%, respectively. The loss of n-3 PUFA was relatively more than the loss of n-6 PUFA, therefore, the n-6 to n-3 ratio increased from 1.93 to 1.99 after 28 days of storage (Table 2).

Saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) levels were very consistent during cooking, whereas the content of n-6 PUFA was significantly increased in boiled eggs (Table 2). The mean value of n-6 to n-3 ratio was higher (1.99) in the fried eggs than those of in boiled eggs and raw eggs (1.94 and 1.93, respectively), which are close to the recommended ratio of 2/1.[19] The typical n-6 to n-3 ratio of 15/1 to 16.7/1 was reported in western diet.[20] The LNA was not affected in most treatments. Boiling had less effect on the composition of the fatty acids than that of frying. This was probably because the yolk temperature in boiling is lower than frying. The presence of albumen and shell surrounds the yolk, which might prevent the interaction of oxygen and light with the lipids during boiling, whereas a direct contact with oxygen and light is inevitable in frying. Therefore, level of n-3 PUFA was higher in boiled eggs than in the fried eggs. Similarly, Cortinas et al.[21] reported that boiling did not significantly affect fatty acids composition, whereas the total n-3 PUFA in scrambled eggs was reduced compared with fresh eggs.

Effects of Diet, Storage and Cooking on Thiobarbituric Reactive Acid Substances. Adding vitamin E in hen's diet significantly reduced the formation of MDA (Table 3). Several studies indicated that adding vitamin E and Sel-Plex in poultry diet would protect lipids from oxidation damage, leading to a better oxidative stability of eggs during cooking and storage.[22,23] Vitamin E is the major chain break antioxidant, which can break the oxidation at propagation phase; selenium is an important part to activate GSH-Px, the major antioxidant to remove the reactive oxygen species (ROS) in the initiation phase.

Storage can cause oxidative damage as well. After 28 days of storage, the content of MDA was significantly increased (1.23 vs 1.44 μg MDA/kg sample, for 0 days and 28 days storage, respectively; Table 3). Similar result were reported by Cherian et al.[23] and Mohiti-Asli et al.[22] who observed that the content of TBARs was increased during storage, especially at elevated temperature. Fried eggs generated the highest content of MDA (2.02 μg/kg) compared to boiled (1.44 μg/kg) and raw eggs (0.56 μg/kg; Table 4). Cortinas et al.[21] reported that boiling and scrambling increased the TBARS value by 2- and 9-fold, respectively. It is well established that oxidation increases with heating temperature, duration of heating and light.[24] The elevated TBARs level in fried eggs compared with boiled eggs

Table 3. Mean Values of MDA Content (TBARs) in Omega-3 Polyunsaturated Fatty Acids (n-3 PUFA) Enriched Eggs Affected by the Main Factors: Cooking, Storage, Vitamin E and Selenium[a]

effects	n[b]	MDA
Cooking (Mean)		
raw	16	0.56c
boil	16	1.44b
fry	16	2.02a
SEM[c]	0.077	
Storage (Mean)		
0	24	1.23b
28	24	1.44a
SEM	0.062	
Vitamin E (Mean)		
high	24	1.23b
low	24	1.45a
SEM	0.062	
Selenium (Mean)		
high	24	1.31
low	24	1.37
SEM		0.062

source of variation	probability
cooking methods (C)	<.0001
storage (S)	0.0256
vitamin E (E)	0.0239
selenium (Se)	0.4454
C×S	0.1918
E×Se	0.0027
E×C	0.1460
Se×C	0.0156
E×S	0.7203
Se×S	0.8337
E×C×S	0.7829
Se×C×S	0.7168
E×Se×C	0.0449
E×Se×S	0.2010
E×Se×C×S	0.4456

[a]Mean values of the TBARs, is the μg MDA/kg of sample. a−c Means within a column in each treatment with no common superscripts differ ($P < 0.05$). [b]n = sample number, each sample calculation of eight eggs, (pool of eight eggs) = (sample) [c]SEM = standard error of the means;

Table 4. Mean Value of Cholesterol Oxidation Products (COPs) Content in Omega-3 Polyunsaturated Fatty Acids (n-3 PUFA) Enriched Eggs Affected by the Main Factors: Cooking, Storage, Vitamin E and Selenium (dwb[b])[a]

effects	n[c]	7-KC	7α-OH	7β-OH	total
Cooking (Mean)					
raw	16	1.50	5.90c	1.62b	9.03b
boiled	16	1.93	6.73b	1.96b	10.15b
fried	16	1.86	7.74a	3.98a	13.58a
SEM[d]		0.152	0.270	0.248	0.435
Storage (Mean)					
0	24	1.67	7.46a	2.65	11.46
28	24	1.85	6.12b	2.40	10.37
SEM		0.124	0.220	0.202	0.355
Vitamin E (Mean)					
high	24	1.53b	6.19b	2.60	10.32b
low	24	1.99a	7.38a	2.45	11.51a
sem		0.124	0.220	0.202	0.355
Selenium (Mean)					
high	24	1.71	6.70	1.98b	10.23b
low	24	1.82	6.88	3.07a	11.61a
SEM		0.124	0.220	0.202	0.355

source of variation	probability			
cooking methods (C)	0.1282	0.0003	<.0001	<.0001
storage (S)	0.3039	0.0002	0.3969	0.0400
vitamin E (E)	0.0150	0.0008	0.6076	0.0268
selenium (Se)	0.5202	0.5735	0.0008	0.0112
C×S	0.0944	0.0036	0.2545	0.0051
E×Se	<.0001	0.0087	0.3811	<.0001
E×C	0.1306	0.0011	0.0157	0.0006
Se×C	0.1892	0.2611	0.0748	0.4173
E×S	0.0642	0.3334	0.4723	0.8861
Se×S	0.0005	0.0374	0.0204	0.1810
E×C×S	0.6406	0.7031	0.5387	0.6144
Se×C×S	0.0691	0.0001	0.2080	0.0366
E×Se×C	0.0014	0.1412	0.0675	0.0040
E×Se×S	0.0064	0.0178	0.0920	0.6524
E×Se×C×S	0.3776	0.2700	0.2394	0.8619

[a]Mean values of the COPs μg/g dry sample. a-c Means within a column in each treatment with no common superscripts differ ($P < 0.05$). [b]dwb = dry weight basis. [c]n = sample number, each sample calculation of eight eggs, (pool of eight eggs) = (sample). [d]SEM = standard error of the means.

was due to the exposure to a more severe cooking condition. The surface temperature of the frying pan was 177 °C compared with approximately 98 °C during boiling (temperature lower than 100 °C due to the elevation of research site). Tai et al.[25] indicated that frying could stimulate n-3 PUFA oxidation due to the presence of light, heat and oxygen through photo-oxidation and auto-oxidation. The interaction between cooking methods and dietary treatments on TBARs was presented in Figure 2. The content of MDA in both raw and boiled samples was not affected by antioxidants; however, in fried eggs, a greater than 40% reduction was observed for the antioxidant groups. The combination of vitamin E and Sel-Plex did not have additional benefits in terms of reducing the formation of MDA compared with supplying them individually (Figure 2).

When oxidation occurred in yolk, lipids such as DPA are oxidized into active radicals, such as lipid oxygen radicals (L*) and peroxyl radicals (LOO), leading to other secondary oxidation reactions. The LOO*, a highly reactive radical, can attack available peroxidizable material producing hydroperoxide (LOOH). Therefore, the amount of MDA (secondary oxidation products) increased while DPA content decreased.[26] The content of n-3 PUFA such as DPA was negatively correlated with MDA content ($r = -0.4733$, $P = 0.0195$). On the other hand, the content of vitamin E was negatively correlated with the amount of MDA ($r = -0.7857$, $P < 0.0001$), indicating a protective role of vitamin E against oxidation of fatty acids in eggs.

Effect of Diet, Storage and Cooking on Cholesterol Oxidation Products. Oxidation of n-3 PUFA (particularly susceptible to oxidative damage during cooking) not only produces genotoxic compounds (causing cell and DNA damage), but also accelerate the formation of COPs, which are responsible for the proatherogenic action of cholesterol.[7] Eggs are a rich source of cholesterol. The presence of a high concentration of PUFA in enriched eggs can enhance the susceptibility of cholesterol oxidation through the process called "co-oxidation".[27] Larkeson et al.[28] pointed out that the major COPs in foods are 7α-OH, 7β-OH, β-CE, α-CE, 25-OH, CT, and 7-KC. Cholesterol oxidation products are consistently found within walls of major arteries, mainly in characteristic lesions of atherosclerosis.[29] Among the COPs, 7-KC is the most potent inhibitor of cellular proliferation, and 25-OH was an effective inducer for apoptosis (programmed cell death).[30] Lizard et al.[31] reported that the toxic concentration of 7-KC and 7β-OH are >40 and >20 μM, respectively, based on human smooth muscle cells. In another study, the concentration of 25-OH or 7β-OH causing apoptosis was >20 μM on human monocytes.[32] The presence of three COPs in the egg samples, 7α-OH, 7β-OH, and 7-KC, was identified by GC-MS and quantified by GC. In the current study, the amount of 7-KC was 0.028 and 0.037 μM in raw and cooked eggs, respectively. The amount of 7β-OH was 0.030 and 0.073 μM in raw and fried eggs, respectively. The concentration of COPs in egg is far below the toxic level, even though the egg contains a high level of cholesterol.

Compared with the eggs from the control diet, eggs additionally fortified with vitamin E and Sel-Plex reduced the total COPs formation by 12% and 13%, respectively (Table 4 and Figure 1). The results demonstrated an antioxidant effect

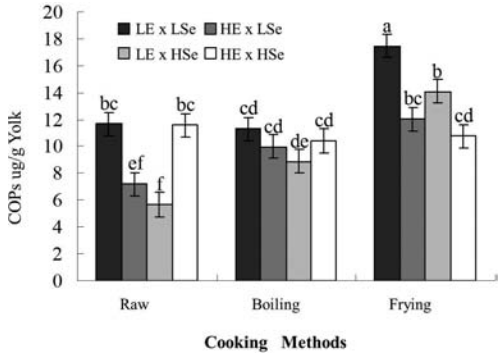

Figure 1. The content of cholesterol oxidation products (COPs) in omega-3 polyunsaturated fatty acids (n-3 PUFA) enriched eggs were affected by the interaction between dietary treatments and different cooking methods. LE × LSe = low vitamin E and low selenium treatment. HE × LSe = high vitamin E and low selenium treatment. LE × HSe = low vitamin E and high selenium treatment. HE × HSe = high vitamin E and high selenium treatment. a–f Means with no common letters differ ($P < 0.05$).

of vitamin E and Sel-Plex in preventing cholesterol oxidation. Storage, however, did not increase COPs levels, in fact there was a significant decrease in 7α-OH after 28 days of storage (Table 4). Mazalli and Bragagnodo[33] reported that the COPs content of spay-dried egg powder was not affected by 1 month of storage at 25 °C. They also found that the content of 7-KC, 7α-OH, and 7β-OH increased rapidly at the third month of storage and stayed consistent from the third month to the sixth month at 25 °C. Our results suggested that simulated retail storage conditions (28 days in fridge at 4 °C) did not affect cholesterol oxidation.

The total content of COPs was affected by cooking methods. The fried samples had the highest total COPs compared with the boiled and raw samples (13.58, 10.15, and 9.03 μg/g dry yolk, respectively). The level of 7-KC was not affected by cooking, but that of 7α-OH was increased in both boiled and fried samples compared with the control ($P < 0.05$; Table 4). The dehydrated reaction of cholesterol hydroperoxide produces 7-KC, 7α-OH, and 7β-OH. It was reported that more 7α-OH and 7β-OH could be produced than 7-KC during a short period of heating; however, 7-KC increased rapidly at prolonged (more than 12 h) heat treatment. Hence, a heat treatment extended over 24 h accelerates oxidation of cholesterol. This phenomenon may be due to the reduction reaction proceeding faster than the dehydration reaction during the initial heating period.[13]

A significant interaction was observed between dietary treatment and cooking methods. The highest amount of 7-KC was found in the fried egg yolk samples obtained from control (LE × LSe) compared to the other dietary treatments (2.754 vs 0.995, 1.863, and 1.807 μg/g, for HE × LSe, LE × HSe, and HE × HSe dietary treatment, respectively). Likewise, the highest amount of total COPs was detected in the control group (LE × LSe) (17.47 vs 10.01, 14.076, and 10.74 μg/g dry yolk, for HE × LSe, LE × HSe, and HE × HSe treatments, respectively). According to the levels of COPs and MDA, the addition of antioxidants was more effective in fried eggs than in boiled eggs (Figures 1 and 2). Egg yolk contains natural

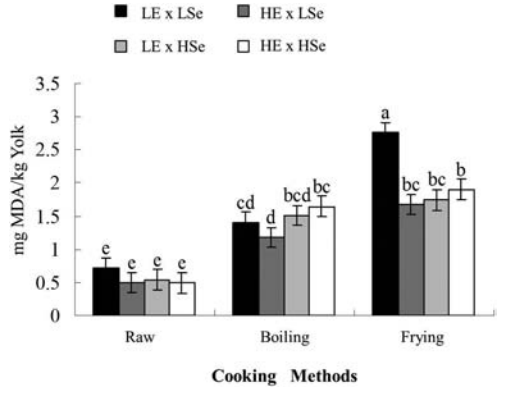

Figure 2. The amount of malondialdehyde (MDA) in omega-3 polyunsaturated fatty acids (n-3 PUFA) enriched eggs were affected by the interaction between dietary treatments and different cooking methods. LE × LSe = low vitamin E and low selenium treatment. HE × LSe = high vitamin E and low selenium treatment. LE × HSe = low vitamin E and high selenium treatment. HE × HSe = high vitamin E and high selenium treatment. a–e Means with no common letters differ ($P < 0.05$).

antioxidants such as tocopherols, carotenoids and metal ion chelators (phosvitin). Those natural antioxidants could protect lipid and cholesterol, which are located in yolk. Furthermore, yolk is surrounded by albumen and shell, which provide a physical buffer against atmospheric oxygen and light during boiling.[24] Cooking increased oxidation of n-3 PUFA and cholesterol in yolk. The amount of long chain n-3 PUFA also decreased after cooking, especially frying. Refrigerated storage of shell eggs had no effect on COPs after 28 days, but the amount of MDA increased after storage. Four weeks of storage

also led to a reduction of long chain n-3 PUFA in yolk sample. Antioxidants reduced yolk fatty acids from oxidative damage during cooking and storage. Our study indicated that the n-3 PUFA in enriched eggs was relatively stable during storage and home cooking in hens fed antioxidants.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

n-3 PUFA,Omega-3 polyunsaturated fatty acids; EPA,eicosapentaenoic acid; DPA,docosapentaenoic acid; DHA,docosahexaenoic acid; ALA,alpha-linolenic acid; n-6 PUFA,omega-6 polyunsaturated fatty acids; MA,malonaldehyde; COPs,cholesterol oxidation products; GSH-Px,glutothione peroxidase; 7α-OH,7-α-hydroxycholesterol; 7-β-OH,7-β-hydroxycholesterol; α-CE,α-cholestanetriol; β-CE,β-cholestanetriol; 25-OH,25-hydroxycholesterol; CT,cholestanetriol; 7-KC,7-keto cholesterol; SFA,Saturated fatty acids; MUFA,monounsaturated fatty acids; EFA,essential fatty acids; AA,arachidonic acid; L*,lipid oxygen radicals; LOO,peroxyl radicals; LOOH,hydroperoxide

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