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Effects of Different Rearing and Feeding Systems on Lipid Oxidation and Antioxidant Capacity of Freeze-Dried Egg Yolks

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Lipid oxidation and antioxidant capacity of freeze-dried egg yolks produced with two rearing systems (battery cages and free-range) and two types of feedings (conventional and organic) were studied. Nine fresh egg yolks of each crossed treatment were pooled, frozen for a month, freeze-dried, vacuum-packed, and kept at −18 °C until analysis. No significant differences were observed in the lipid (58.0−62.1%) and total sterol contents (33.0−35.5 g/kg of lipids) of the freeze-dried egg yolks. Free rearing and conventional feeding systems resulted in significantly higher total tocopherol, α-tocopherol, and lutein contents, as compared to the battery cage and the organic feed, respectively. However, no significant differences were found in lipid oxidation (peroxide value = 0.7−0.9 mequiv of O_2/kg of fat; thiobarbituric reactive substances = 1.0−1.3 mg of malonylaldehyde/kg of sample) and cholesterol oxidation (28.8−43.5 mg of cholesterol oxidation products/kg of lipids; 0.08−0.12% oxidized cholesterol) of freeze-dried egg yolks except for 7α-hydroxycholesterol, which was significantly lower in samples obtained with organic feed.

KEYWORDS: Freeze-dried egg yolks; lipid oxidation; antioxidant capacity; rearing; feeding; organic; conventional

INTRODUCTION

Eggs are characterized by a complex and unique composition of proteins and lipids, which makes them suitable as food ingredients for different technological applications (*1*). The great consumption of industrialized egg-containing foods, such as bakery products and pasta, has imposed the need to transform eggs into egg powders (*2*), in order to increase the microbiological safety and to reduce the volume with respect to that of unshelled or liquid eggs (*3*). Freeze-dried eggs are widely used in food preparations, due to their microbial stability (*3*) and their long shelf life. Despite its relatively high cost, freeze-drying supplies the best ingredient quality, because the dried egg is easily reconstituted and its deterioration is negligible during dehydration process. However, it is known that the quality of the lipid fraction of egg powder can be largely affected by processing and storage conditions (*4*, *5*). In fact, one of the most critical chemical modifications that can occur is lipid oxidation, due to the egg powders' large surface area. Wahle et al. (*6*) reported marked changes in the concentration of lipid peroxidation products and fatty acid oxidation in spray-dried egg powder stored at room temperature and exposed to air and light for 18 months.

Lipid oxidation is not limited to only fatty acids; it can also affect other molecules that have double bonds in their chemical structures, such as cholesterol, giving rise to the formation of undesirable compounds, such as cholesterol oxidation products (COPs). These compounds have been studied in depth because they are likely to be involved in lipid metabolism, various chronic and degenerative diseases (such as cancer, aging, and human atherosclerosis), and disturbance of cell functionality (*7*).

Galobart and Guardiola (*8*) have recently reviewed the formation of COPs in eggs and egg products, obtained under different processing and technological conditions. Elevated amounts of COPs have been found in egg powders (up to 166 mg/kg of sample) (*8*), due to their high cholesterol content (∼17 mg/g of egg powder) (*9*), and, as aforementioned, to their elevated surface to volume ratio exposed to air. Furthermore, plasma COP concentrations in humans have been found to increase with consumption of oxidized egg powders containing approximately 230 mg of COPs/kg of sample (*10*). The presence and quantity of COPs in egg powders seem to markedly vary according to the processing and storage conditions. In freeze-dried egg yolks, several authors have reported the absence or trace levels of COPs (*8*). In fact, Obara et al. (*11*) found that the total amount of COPs was higher in spray-dried egg powder than in freeze-dried ones and that the water content of egg powder had a highly significant influence on oxysterol accumulation during storage. Caboni et al. (*5*) observed that COP concentration in spray-dried egg powders reached 167 mg/kg of fat after 12 months of storage at 20 °C, which corresponded to 0.6% of cholesterol oxidation ratio. In a recent

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study, Mazalli and Bragagnolo (*2*) reported that the COP content in spray-dried whole egg powders could largely vary (24−227 mg/kg in dry weight basis), depending on the product brand, batch, and time of storage. Van de Bovenkamp et al. (*12*) found a very high level of COPs in dried egg yolk powder (4151 mg/kg of sample), after 1 year of storage followed by irradiation with UV light for 3 weeks (*8*). Boselli et al. (*13*) observed that COP content in egg yolk powder ranged between 21.3 and 35 mg/kg of lipids, depending on the lipid extraction method used.

To partially contrast the effects of processing technology and storage, an alternative strategy that has recently been followed is to modify egg lipid stability by changing the feed composition (*4*, *8*). In fact, the amount of antioxidants present in eggs (such as tocopherols and carotenoids) can be altered by diet supplementation (*4*, *11*, *14*). Another parameter that seems to influence the quality of egg lipids is the type of rearing, which is related to animal welfare (*15*). All of these strategies are addressed to face the challenge that arises from the need to promote the production of added value food products that rely on alternative farming systems, such as the organic one, as these approaches are considered to be more respectful toward the environment and farm animals.

The aim of this study was to compare the effects of two rearing systems (traditional cages and free-range) and two types of feedings (conventional and organic) on the extent of lipid and cholesterol oxidation, as well as the total natural antioxidant content, of freeze-dried egg yolks obtained under those conditions. A crossed-testing design was used to evaluate those effects. Peroxide value (POV), thiobarbituric acid reactive substances (TBARs), COPs, fatty acid composition, and lipid, sterol, tocopherol, and carotenoid contents of freeze-dried egg yolks were determined.

MATERIALS AND METHODS

Materials and Reagents. Methanol, chloroform, hydrochloric acid, barium chloride dihydrate, ammonium thiocyanate, iron(II) sulfate, iron(III) chloride, L-(+)-ascorbic acid, pyrogallol, sodium dihydrogen phosphate, and trisodium phosphate were supplied by Carlo Erba (Milan, Italy), whereas propan-2-ol was purchased from Prolabo (Paris, France). 2-Thiobarbituric acid (TBA), anhydrous monobasic sodium phosphate, and 1,1,3,3-tetramethoxypropane were supplied by Sigma-Aldrich (St. Louis, MO). Trichloroacetic acid (TCA) was purchased from Acros Organics (Morris Plains, NJ). Ethylenediaminetetraacetic acid (EDTA) disodium salt was supplied by Curtin Matheson Scientific Inc. (Houston, TX), whereas potassium hydroxide, ethanol, and sodium chloride were purchased from Merck (Darmstadt, Germany).

High-performance liquid chromatography (HPLC) grade solvents (acetone, *n*-hexane, ethyl acetate, acetic acid, and water) were supplied by Carlo Erba; they were vacuum filtered through nylon filtration membranes (0.45 μm; Micron Separation, Westboro, MA) and degassed before analysis.

Tocopherol standards (α-, β-, γ-, and δ-tocopherols) were purchased from Merck. The standard mixture of fatty acid methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN). Cholest-5-ene-3β,19-diol (19-hydroxycholesterol, 19-HC) (purity = 99%) and cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol, 7α-HC) (purity = 99%) were purchased from Steraloids (Newport, RI). Tridecanoic acid methyl ester, cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol, 7β-HC) (purity = 90%), 5α,6α-epoxycholestan-3β-ol (α-epoxycholesterol, α-CE) (purity = 87%), 5β,6β-epoxycholestan-3β-ol (β-epoxycholesterol, β-CE) (purity = 80%), cholestane-3β,5α,6β-triol (cholestanetriol, triol) (purity = 99%), cholest-5-en-3β-ol-7-one (7-ketocholesterol, 7-KC) (purity = 99%), cholest-5-en-3β-ol (cholesterol) (purity = 99%), (24*R*)-ethylcholest-5-en-3β-ol (β-sitosterol) (purity = 60% β-sitosterol, 40% campesterol), and lup-20(29)-ene-3β,28-diol (betulin) (purity = 98%) were purchased from Sigma (St. Louis, MO). The purity of the standards was controlled in our laboratory, by gas chromatography (GC).

Table 1. Ingredients and Composition of the Conventional and Organic Diets

ingredient	conventional (%)	organic (%)
corn meal	56.1	55.5
soybean meal	19.0	
full-fat soybean		18
calcium carbonate	8.1	8.0
corn gluten meal	4.0	5.0
wheat sharps	4.0	
wheat flour middling		6.0
sunflower meal	2.5	
sunflower seed oil meal		2.5
dehydrated alfalfa meal	2.0	2.0
soybean oil	2.0	
dicalcium phosphate	1.3	1.5
brewers' yeast		0.5
vitamin and mineral premix	0.5[a]	0.5[b]
sodium chloride	0.4	0.4
DL-methionine	0.1	0.1
chemical analysis		
dry matter	91.5	92.6
crude protein	16.4	16.4
crude fat	5.4	5.4
crude fiber	4.5	4.1
ash	9.7	9.2
calcium	2.9	3.2
phosphorus	0.6	0.7
metabolizable energy (MJ/kg)	12.31	12.22

[a] Vitamins and minerals provided the following per kilogram of premix: vitamin A, 2,600,000 IU; vitamin D_3, 600,000 IU; vitamin E, 4000 mg; vitamin B_1, 400 mg; vitamin B_2, 1000 mg; vitamin B_6, 600 mg; vitamin B_{12}, 4 mg; vitamin K_3, 400 mg; vitamin PP, 6000 mg; vitamin H, 20 mg; folic acid, 100 mg; vitamin B_5, 2000 mg; choline, 100,000 mg; zinc (zinc oxide), 10000 mg; iron (iron carbonate), 10,000 mg; manganese (manganese oxide), 12,000 mg; copper (copper sulfate), 1000 mg; iodine (potassium iodide), 200 mg; cobalt (cobalt sulfate), 100 mg; 40 mg; BHT, 1000 mg; pigments and xanthophylls, 6.000 mg. [b] Vitamins and minerals provided the following per kilogram of premix: vitamin A (cod liver oil), 1,000,000 IU; vitamin D_2 (cod liver oil), 400,000 IU; vitamin E (wheat germ oil), 130 mg; vitamin B_1 (yeast), 130 mg; vitamin B_2 (yeast), 50 mg; vitamin B_6 (yeast), 2 mg; vitamin PP (yeast), 4000 mg; zinc (marine algae), 1.5 mg; manganese (marine algae), 48 mg; copper (marine algae), 1.5 mg; iodine (marine algae), 120 mg; cobalt (marine algae), 0.15 mg; selenium (sodium selenite); β-carotene (alfalfa meal), 500 mg/kg; carotenoids (corn gluten meal), 100 mg/kg.

NH_2 solid-phase extraction (SPE) cartridges (Strata NH_2, 500 mg/3 mL) from Phenomenex (Torrence, CA) were used for COP purification. The silylation mixture was prepared with dried pyridine, hexamethyldisilazane, and trimethylchlorosilane (all from Sigma) at a ratio of 5:2:1 by volume.

Rearing Conditions and Feed Composition of Laying Hens. After one pretrial period of adaptation to the environment and diet, 108 Warren-Isa Brown laying hens, aged 8.5−9 months, were divided into 4 groups of 27 hens each. The hens were allocated so as to obtain groups with homogeneous characteristics in terms of weight and egg production.

Two groups were reared in 18 battery cages (9 cages per group, housing 3 hens each); the cage sizes (50 cm × 50 cm × 50 cm) fell within the limits established by Italian Law Decree 267 of July 29, 2003 (*16*). During the trial, temperature (22−26 °C) and humidity (75%) were automatically controlled within the housing facility.

The other two groups were allocated in six outdoor areas of 40 m^2 each (three areas per group, with nine hens each); these areas were furnished with henhouses containing nests, feed troughs, and nipple drinkers.

Feed and water were provided *ad libitum* for both treatments. Two types of feedings were administered to animals: conventional (C) or organic (O). **Table 1** reports the ingredients and compositions of the diets. In the organic treatment, the integral soybean was used instead of the soybean meal, because the latter is not allowed in organic feed. The diets were isoenergetic and isonitrogenous, and drinking water was always available. A representative sample of each feeding was kept at −18 °C for analysis of the composition parameters.

Considering the two rearing systems [battery cages (B) and free-range (F)] and two types of feedings [conventional (C) and organic (O)], a total of four crossed treatments were obtained: BC, reared in battery cages on conventional feeding; BO, reared in battery cages on organic feeding; FO, free-range and organic feeding; FC, free-range and conventional feeding.

The trial lasted 4 months; feed intake and egg production were calculated over the entire trial period. At the end of the trial period, 1 egg was collected per cage for each treatment (BC and BO) during three consecutive days, generating a 9-egg pool per day for each treatment; this resulted in three 9-egg pools per treatment, which corresponded to a total cage sampling of 54 eggs. For the outdoor areas, 3 eggs were collected per area for each treatment (FC and FO) during three consecutive days, generating a 9-egg pool per day for each treatment; this gave rise to three 9-egg pools per treatment, which corresponded to a total free-range sampling of 54 eggs.

Preparation of Freeze-Dried Egg Yolks. Egg white was separated from egg yolks, and three sets of 9-egg yolk pools per treatment were prepared. All egg yolk pools were separately frozen for 1 month and then freeze-dried at −45 °C for 72 h (Olsa, Milan, Italy). Samples were vacuum-packed in polyethylene/polyamide bags (140 μm thickness; 1.6 g/m^2/24 h and 40 mL/m^2/24 h of water and oxygen permeability, respectively), covered with aluminum foil, and kept at −18 °C until analysis.

Lipid Extraction. Lipids were extracted according to a modified version of the method described by Folch et al. (*17*). The extraction procedure was the same as that recently reported by Boselli et al. (*18*), except that the solvent volumes were 2.5 time larger. About 2 g of egg yolk powder or 15 g of feed was homogenized with 500 mL of a chloroform/methanol solution (1:1, v/v) in a glass bottle with a screw-cap. The bottle was kept at 60 °C for 20 min before 250 mL of chloroform was added. After 3 min of homogenization, the content of the bottle was filtered through filter paper. The filtrate was mixed thoroughly with a 1 M KCl solution and left overnight at 4 °C to obtain phase separation. The lower phase was collected and dried with a rotary evaporator. The fat content was determined gravimetrically. Three extractions were performed per treatment.

GC-FID Determination of Total Fatty Acid Methyl Esters (FAME). About 20 mg of lipid extract was methylated with 200 μL of diazomethane (*19*), 1.9 mg of tridecanoic acid methyl ester was added (as internal standard), and the mixture was transmethylated with 40 μL of 2 N KOH in methanol (*20*), vortexed for 1 min, left standing for 5 min, and centrifuged at 1620*g* for 5 min. Supernatant was transferred to a vial before being injected into the gas chromatograph−flame ionization detector (GC-FID).

The GC-FID instrument was a GC8000 series (Fisons Instruments, Milan, Italy) with a flame ionization detector (FID) and interfaced with a computerized system for data acquisition (Chromcard Data System, ver. 2.3.1, Fisons Instruments). A J&W HP88 fused-silica column (100 m × 0.25 mm × 0.2 μm film thickness) (Agilent Technologies, Santa Clara, CA) coated with 88% cyanopropyl aryl siloxane was used. Oven temperature was programmed from 100 to 180 °C at a rate of 3 °C/min, kept at 180 °C for 10 min, and then taken to 240 °C at a rate of 3 °C/min; the final temperature was kept for 30 min. The injector and detector temperatures were both set at 250 °C. Helium was used as carrier gas at a constant flow of 0.7 mL/min. The split ratio was 1:70.

Tridecanoic acid methyl ester was used as internal standard for FA quantification, and peak identification was carried out by comparing the peak retention times with those of the GLC 463 FAME standard mixture. The GC response factor of each fatty acid was calculated by using the GLC 463 FAME standard mixture and the internal standard (C13:0). The limit of detection (LOD) of FAMEs was 0.006 mg, whereas the limit of quantification (LOQ) was 0.01 mg. LOD and LOQ were calculated as a signal-to-noise ratios equal to 3:1 and 10:1, respectively.

HPLC-FD Determination of Tocopherols. A normal phase method was utilized for the HPLC separation and quantification of tocopherols, as suggested by Panfili et al. (*21*). The HPLC instrument was an HP1050 series (Agilent Technologies, Palo Alto, CA), which was coupled to a 20 μL loop injector (Rheodyne, Cotati, CA), a 5 μm Luna Silica (2) (250 mm × 4.6 mm i.d.; Phenomenex, Torrance, CA), and an HP1100 fluorescence detector (FD) (Agilent Technologies, Palo Alto, CA). Data were acquired with Chemstation for LC3D software (Agilent Technologies, Palo Alto, CA). The analyses were run under isocratic conditions, using *n*-hexane/ethyl acetate/acetic acid (97.3:1.8:0.9, v/v/v) as mobile phase and a flow rate of 1.6 mL/min. The detection wavelength was set at 290−330 nm. The samples were filtered through 0.45 μm nylon filtration membranes before being injected into the HPLC. Three replicates were run per sample lipid extract.

Tocopherols were quantified by using external standard calibration curves with concentration ranges of 0−35, 0−6, 0−6, and 0−6 μg/mL for α-, β-, γ-, and δ-tocopherols, respectively. Linear regression equations and correlation coefficients were calculated for α-tocopherol ($y = 16.728x - 6.6299$; $r^2 = 0.9983$), β-tocopherol ($y = 8.6795x - 0.0721$; $r^2 = 0.9996$), γ-tocopherol ($y = 18.786x - 0.2826$; $r^2 = 0.9964$), and δ-tocopherol ($y = 9.4645x + 0.371$; $r^2 = 0.9989$); five concentration points were used, and three injections were performed per concentration point. The LODs were 0.45, 0.05, 0.32, and 0.03 μg/mL for α-, β-, γ-, and δ-tocopherols, respectively, whereas their LOQs were 1.49, 0.17, 1.06, and 0.11 μg/mL. LOD and LOQ were calculated as 3- and 10-fold the standard deviation of the intercept of the calibration curve (*22*). Tocopherol content of the samples was expressed as milligrams per kilogram of sample.

HPLC-DAD Determination of Carotenoids. A reverse phase method was utilized for the HPLC separation and quantification of carotenoids, as suggested by Mìnguez-Mosquera et al. (*23*). The HPLC instrument was an HP1050 series (Agilent Technologies, Palo Alto, CA), which was coupled to a 20 μL loop injector (Rheodyne), a 5 μm Sphereclone ODS (2) column (250 mm × 4.6 mm i.d.; Phenomenex), and an HP1050 diode array (DAD) UV−vis detector (Agilent Technologies, Palo Alto, CA). Data were acquired with Chemstation for LC3D software (Agilent Technologies, Palo Alto, CA).

The gradient elution was performed by using acetone and water as mobile phases A and B, respectively, according to the following gradient: from 0 to 15 min, 75% A; from 15 to 20 min, increased to 95% A; from 20 to 27 min, held at 95% A; from 27 to 32 min, increased to 100% A; and from 32 to 37 min, decreased to 75% A.

A 10 min conditioning stage prior to the injection and a postanalysis cleaning stage were necessary to achieve the right elution conditions. A flow rate of 1.5 mL/min was used. The detection wavelength was set at 450 nm. The samples were filtered through 0.45 μm nylon filtration membranes before being injected into the HPLC. Three replicates were run per sample lipid extract.

Because only lutein was detected in the freeze-dried egg yolk samples, an external standard calibration curve with a concentration range of 0−5 μg/mL was utilized for the quantification of this carotenoid. Linear regression equation and correlation coefficient were calculated ($y = 117.74x - 75.122$; $r^2 = 0.9908$); five concentration points were used, and three injections were performed per concentration point. LOD and LOQ of lutein were 0.097 and 0.323 μg/mL, respectively. LOD and LOQ were calculated as 3- and 10-fold the standard deviation of the intercept of the calibration curve (*22*). Lutein content of the samples was expressed as milligrams per kilogram of sample.

Spectrophotometric Determination of Peroxide Value (POV). POV was determined in 50 mg of lipid extract, as suggested by Shantha and Decker (*24*). This method is based on the ability of peroxides to oxidize ferrous ions to ferric ions. Ammonium thiocyanate reacts with ferric ions, resulting in a colored complex that can be measured spectrophotometrically. POV was evaluated at 500 nm with a double-beam UV−visible spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan), and it was calculated from the absorbance. For the quantitative determination of POV, a Fe(III) standard calibration curve was used with a concentration range of 0.1−5 μg/mL ($y = 0.0282x - 0.0003$; $r^2 = 0.999$). POV was expressed as milliequivalents of O_2 per kilogram of fat. Three replicates were run per sample.

Spectrophotometric Determination of Thiobarbituric Acid Reactive Substances (TBARs). TBARs value was determined in 2 g of egg powder sample according to a modified method of Witte et al. (*25*). This method is based on the reaction between the thiobarbituric acid with aldehydes that derive from secondary oxidation of lipids present in the sample, resulting in a colored complex that can be measured spectrophotometrically. TBARs were evaluated at 530 nm with a double-beam UV−visible spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan), and they were calculated from the absorbance. For the quantitative determination of TBARs, a 1,1,3,3-tetramethoxypropane

Table 2. Fatty Acid Composition of Feeds and Freeze-Dried Egg Yolks As Related to Feeding Systems and Rearing−Feeding Conditions, Respectively[a]

	fatty acid composition of feeds (%)																
factor	C14:0	C16:0	C16:1 n-7	C18:0	C18:1 n-9	C18:2 n-6	C20:0	C18:3 n-3	C20:4 n-6	C22:6 n-3	SFA	MUFA	PUFA n-3	PUFA n-6	total PUFA	UFA/SFA	n-6/n-3
feeding																	
C	0.13b	12.93a	0.16b	3.41	22.34b	54.71	0.38a	4.23b	nd	nd	17.64a	23.00b	4.23b	55.13	59.36b	4.67b	13.05a
O	0.15a	11.98b	0.21a	3.41	22.91a	54.69	0.33b	4.81a	nd	nd	16.52b	23.67a	4.82 a	54.99	59.81a	5.06a	11.42b
stat signif	**	**	**	ns	**	ns	**	**			**	**	**	ns	**	**	**

	fatty acid composition of egg yolks (%)																
factor	C14:0	C16:0	C16:1 n-7	C18:0	C18:1 n-9	C18:2 n-6	C20:0	C18:3 n-3	C20:4 n-6	C22:6 n-3	SFA	MUFA	PUFA n-3	PUFA n-6	total PUFA	UFA/SFA	(n-6)/(n-3)
rearing																	
B	0.35a	24.73	1.64	8.82	31.89b	25.63a	0.16a	1.05	1.93	1.10	34.55a	34.96b	2.28a	28.21a	30.49a	1.90b	12.42
F	0.29b	24.15	1.68	8.70	34.70a	23.90b	0.13b	0.97	1.83	0.96	33.71b	37.91a	2.06b	26.31b	28.37b	1.97a	12.76
stat signif	**	ns	ns	ns	**	*	*	ns	ns	ns	*	**	**	**	**	*	ns
feeding																	
C	0.35a	25.10a	1.78	8.50	33.01	24.70	0.14	0.99	1.84	0.94b	34.55a	36.61	2.07b	27.17	29.24	1.90b	13.16a
O	0.29b	23.78b	1.55	9.02	33.58	24.84	0.14	1.03	1.92	1.11a	33.71b	36.66	2.28a	27.34	29.63	1.97a	12.02b
stat signif	**	**	ns	ns	ns	ns	ns	ns	ns	*	*	ns	**	ns	ns	*	*
rearing × feeding																	
BO	0.32	23.82	1.43	9.23	31.99	25.96	0.16	1.08	2.03	1.23	34.01	34.91	2.45a	28.62	31.08	1.94	11.68
BC	0.38	25.64	1.86	8.42	31.79	25.30	0.16	1.02	1.83	0.96	35.08	35.01	2.12b	27.79	29.91	1.85	13.16
FO	0.27	23.75	1.66	8.82	35.16	23.71	0.13	0.98	1.81	1.00	33.42	38.41	2.11b	26.06	28.17	1.99	12.36
FC	0.31	24.55	1.71	8.58	34.23	24.09	0.13	0.97	1.85	0.92	34.01	37.42	2.02b	26.56	28.58	1.94	13.16
stat signif	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns

[a] Each value is the average of three replicates. Abbreviations: B, battery cages, BO, reared in battery cages on organic feed; BC, reared in battery cages on conventional feed; C, conventional feed; F, free-range; FO, free-range and organic feed; FC, free-range and conventional feed; MUFA, monounsaturated fatty acids; O, organic feed; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; UFA, unsaturated fatty acids; stat signif, statistical significance [ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; a, b statistically different means (Tukey's test; $P \leq 0.05$)].

Table 3. Effects of Feeding Systems on Tocopherol and Sterol Compositions of Feeds[a]

	parameters				
factor	α-tocopherol (mg/kg of lipids)	β-tocopherol (mg/kg of lipids)	γ-tocopherol (mg/kg of lipids)	δ-tocopherol (mg/kg of lipids)	total tocopherols (mg/kg of lipids)
feeding					
C	292.1a	18.8b	102.4b	98.1b	511.4a
O	58.4b	28.5a	142.6a	145.8a	375.3b
stat signif	**	**	**	**	**

	parameters			
factor	campesterol (mg/kg of lipids)	stigmasterol (mg/kg of lipids)	β-sitosterol (mg/kg of lipids)	total sterols (mg/kg of lipids)
feeding				
C	1676.6b	885.8	6040.8	8603.1
O	1854.1a	874.1	6023.0	8751.2
stat signif	**	ns	ns	ns

[a] Each value is the average of three replicates. Abbreviations: C, conventional feed; O, organic feed; stat signif, statistical significance [ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; a, b, statistically different means (Tukey's test; $P \leq 0.05$)].

standard calibration curve was used with a concentration range of 0.045−0.113 μg/mL ($y = 0.0087x - 0.0051$; $r^2 = 0.999$). TBARs value was expressed as milligrams of malonylaldehyde (MDA) per kilogram of sample. Three replicates were run per sample.

GC-FID and GC-MS Determination of Total Sterols and Cholesterol Oxides. A 250 mg lipid subfraction of the Folch extract was added with known amounts of the internal standard solution (0.08 g of betulin and 0.02 g of 19-hydroxycholesterol for the determination of total sterols and COPs, respectively). Subsequently, the sample was dried under nitrogen and treated with 10 mL of 1 N KOH solution in methanol to perform a saponification at room temperature for 18 h (*26*). For the extraction of the unsaponifiable matter, 10 mL of water and 10 mL of diethyl ether were added to the samples, which were shaken, and the diethyl ether fraction was separated; the extraction with 10 mL of diethyl ether was repeated twice. The three portions of diethyl ether were pooled, treated with 5 mL of a 0.5 N KOH solution, and extracted. The resulting ethereal extract was washed twice with 5 mL of water. The ether solution was finally evaporated in a rotary evaporator, after elimination of excess water by addition of anhydrous sodium sulfate. One-tenth of the unsaponifiable matter was used for the determination of total sterols, whereas the remaining part was utilized for COP analysis.

The determination of total sterols (sum of free and esterified) was achieved by GC-FID after silylation (*27*). The GC instrument was an HRGC 5300 model (Carlo Erba), which was equipped with a split−splitless injector and a flame ionization detector. A CP-SIL 5CB Low Bleeds/MS (30 m × 0.32 mm i.d. × 0.25 μm film thickness) (Varian Chrompack, Middelburg, The Netherlands) was used. The oven temperature was programmed from 265 to 280 °C at 0.5 °C/min and then from 280 to 325 °C at 4 °C/min; the injector and detector temperatures were both set at 325 °C. Helium was used as carrier gas at a flow of 2.9 mL/min; the split ratio was 1:15.

COPs were purified by NH_2 SPE of $^9/_{10}$ of the unsaponifiable matter, according to the method of Rose-Sallin et al. (*28*). After silylation, COPs were injected into the GC under the same conditions as reported for the determination of total sterols.

The identification of sterols and COPs was confirmed by comparing their retention times and mass spectra with those of sterols and COP standards. GC−mass spectrometry (MS) identification of sterols and COPs was performed with a GC Hewlett-Packard 6890 coupled to a 5973 mass selective detector (Agilent Technologies, Palo Alto, CA). The system was fitted with a capillary SPB-5 column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Supelco, Bellefonte, PA), and helium was used as the carrier gas (1 mL/min). The oven temperature was programmed from 250 to 310 °C at 0.8 °C/min. The injector and transfer line temperatures were set at 310 and 280 °C, respectively. Manual injection of 1 μL of a solution of derivatized COP standards, a solution of derivatized sterol standards and the silylated purified COPs from the freeze-dried egg yolk samples, was performed in the split mode, at a 1:10 split ratio. The filament emission current was 70 eV. A mass range from m/z 40 to 650 was scanned at a rate of 1500 amu/s.

Sterols and COPs were quantified with the internal standard method, using betulin and 19-hydroxycholesterol, respectively. Their response factors were evaluated with respect to the corresponding internal standards. GC-FID LOD and LOQ of sterols were 1.47 and 4.9 μg, respectively, whereas those of COPs were 0.08 and 0.28 μg, respectively. LOD and LOQ were calculated as a signal-to-noise ratios equal to 3:1 and 10:1, respectively.

Statistical Analysis. The data are reported as mean values of three independent replicates ($n = 3$) of each analytical determination (**Tables 2**−**6**). One-way-analysis of variance (ANOVA) was used for feeding samples. Tukey's test was performed at a 95% confidence level ($P \leq 0.05$) to identify significant differences among samples within the evaluated parameters (FA composition, tocopherols, and sterols contents) of the different feedings.

Factorial ANOVA was performed for data from the crossed treatments to study the influence of the different rearing and feeding systems, as well as their interaction, on several quality parameters (lipid content, FA composition, α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, total tocopherols, lutein, cholesterol, campesterol, β-sitosterol, total sterols, POV, TBARs, 7α-HC, 7β-HC, β-CE, 7-KC, total COPs, oxidized cholesterol). Tukey's honest significance test was carried out to separate means of statistically different parameters and interactions ($P \leq 0.05$).

Spearman correlation coefficients were used to examine possible relationships between quality parameters of feedings and those of the freeze-dried egg yolks, as well as within the lipid composition and oxidation parameters of freeze-dried egg yolks over the whole data set ($n = 12$). Statistical analysis of the data was performed by Statistical 6.0 software (2001, StatSoft, Tulsa, OK).

RESULTS AND DISCUSSION

Lipid Composition of Feeds. Table 2 reports the fatty acid composition (expressed as percent) of feeds. GC-FID analysis of feed FAME evinced the presence of 22 fatty acids (FA), but only the main FA were included in the table. The most abundant FA in both feeds was linoleic acid (∼55% of total FA), followed by oleic (∼22−23%), palmitic (∼12−13%), linolenic (∼4−5%), and stearic acids (∼3%). Saturated FA (SFA), monounsaturated FA (MUFA), and polyunsaturated FA (PUFA) accounted for 17−18, 23−24, and 59−60% of total FA, respectively. The FA profile of conventional feed was significantly more saturated than the organic one, which can be mainly ascribed to the palmitic acid content. On the other hand, the higher unsaturation degree of the organic feed was mainly due to the oleic and linolenic acid contents. In addition, a significantly higher content of PUFA n-3 was detected in the organic feed, which can be attributed to the ingredients (cod liver oil, wheat germ oil, and marine algae) used for the preparation of the vitamin and mineral premix of the

Table 4. Effects of Rearing and Feeding Systems on the Average Lipid Content, Single and Total Tocopherol Contents, and Lutein Content of Freeze-Dried Egg Yolks[a]

	parameters						
factor	lipids (%)	α-tocopherol (mg/kg of lipids)	β-tocopherol (mg/kg of lipids)	γ-tocopherol (mg/kg of lipids)	δ-tocopherol (mg/kg of lipids)	total tocopherols (mg/kg of lipids)	lutein (mg/kg of lipids)
rearing							
B	58.7b	158.1	5.8b	55.0	11.1	230.0b	59.5b
F	61.5a	170.5	6.7a	62.3	12.5	252.0a	66.4a
stat signif	*	ns	**	ns	ns	*	*
feeding							
C	60.8	211.1a	6.4	58.2	11.0	286.7a	67.9a
O	59.4	117.5b	6.1	59.1	12.6	195.3b	58.1b
stat signif	ns	**	ns	ns	ns	**	**
rearing × feeding							
BO	58.0	103.5	5.3	54.3	11.4	174.8	50.7b
BC	59.5	212.6	5.8	55.8	10.9	285.1	68.4a
FO	60.8	131.4	6.5	64.0	13.8	215.8	65.5a
FC	62.1	209.5	6.9	60.7	11.1	288.3	67.3a
stat signif	ns	ns	ns	ns	ns	ns	**

[a] Each value is the average of three replicates. Abbreviations: B, battery cages, BO, reared in battery cages on organic feed; BC, reared in battery cages on conventional feed; C, conventional feed; F, free-range; FO, free-range and organic feed; FC, free-range and conventional feed; O, organic feed; stat signif, statistical significance [ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; a, b, statistically different means (Tukey's test; $P \leq 0.05$)].

Table 5. Effects of Rearing and Feeding Systems on the Average Cholesterol, Campesterol, β-Sitosterol, and Total Sterol Contents of Freeze-Dried Egg Yolks[a]

	parameters			
factor	cholesterol (mg/kg of lipids)	campesterol (mg/kg of lipids)	β-sitosterol (mg/kg of lipids)	total sterols (mg/kg of lipids)
rearing				
B	36105.0	0.2b	0.2b	36105.4
F	34204.3	0.6a	0.4a	34205.3
stat signif	ns	**	**	ns
feeding				
C	35490.7	0.3b	0.3	35472.2
O	34837.6	0.5a	0.3	34838.4
stat signif	ns	*	ns	ns
rearing × feeding				
BO	36719.3	0.3	0.2	36719.7
BC	35490.7	0.2	0.2	35419.0
FO	32955.9	0.8	0.4	32957.1
FC	35452.7	0.4	0.4	35453.5
stat signif	ns	ns	ns	ns

[a] Each value is the average of three replicates. Abbreviations: B, battery cages, BO, reared in battery cages on organic feed; BC, reared in battery cages on conventional feed; C, conventional feed; F, free-range; FO, free-range and organic feed; FC, free-range and conventional feed; O, organic feed; stat signif, statistical significance [ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; a, b, statistically different means (Tukey's test; $P \leq 0.05$)].

organic feed, because they are good sources of such long-chain PUFA. The differences in the PUFA profile resulted in a significantly higher n-6/n-3 ratio in the conventional feed with respect to the organic one. It must be pointed out, though, that the significant differences observed in the single FA and FA classes did not exceed 1%.

Table 3 reports the effects of feeding systems on tocopherol and sterol composition (expressed as mg/kg of lipids) in feeds. Total tocopherol content of conventional feed (~511 mg/kg of lipids) was significantly higher than that of organic feed (~375 mg/kg of lipids), which can be attributed to the higher α-tocopherol content in the conventional feed. The tocopherol profile of the feeds was completely different; in fact, the most abundant tocopherol in the conventional feed was α-tocopherol, followed by γ-, δ-, and β-tocopherols, whereas the most representative tocopherol in the organic feed was δ-tocopherol, followed by γ-, α-, and β-tocopherols. Moreover, the organic feed had significantly higher contents of δ-, γ-, and β-tocopherols as compared to the conventional feed. The quali-quantitative differences on the tocopherol profile can be directly related to the feeding composition. In fact, besides diet supplementation with vitamin E (as such or as wheat germ oil), tocopherols are naturally present in some of the ingredients, such as corn and soybean oil; in fact, these ingredients are rich in α- and γ-tocopherols and α-, β-, γ-, and δ-tocopherols, respectively (*29*).

Total sterol content in feeds ranged from 8603 to 8751 mg/kg of lipids (**Table 3**), and no significant differences were observed. The most abundant sterol was β-sitosterol (70% of total sterols), followed by campesterol (20%) and stigmasterol (10%). These results are related to dietary oil sources here used (such as corn and soybean oil), whereas β-sitosterol is about 2−4 times higher than campesterol (*29*). A significantly higher content of campesterol was found in organic feed with respect to the conventional one, though.

Lipid Content of Freeze-Dried Egg Yolks. Table 4 reports the effects of rearing and feeding systems on the average lipid content of the freeze-dried egg yolks, which varied from 58.0 to 62.1%; these values are in agreement with those found in the literature (*13*). The factorial analysis shows that F resulted in a significantly higher lipid content, but no significant differences could be ascribed to both the feeding systems and the crossed treatment interaction. Although F rearing favors animal movement, traditional cage rearing causes more stress, which leads to a higher lipid consumption (*30*) and, therefore, to a lower lipid content of eggs.

Fatty Acid Composition of Freeze-Dried Egg Yolks. Table 2 also reports the effects of rearing and feeding systems on the fatty acid composition (expressed as percent) of the freeze-dried egg yolks. In general, the most abundant FA was oleic acid (~32−34% of total FA), followed by linoleic (~24−26%), palmitic (~24−26%), and stearic acids (~9%). Among long-chain PUFA, arachidonic and docosahexaenoic (DHA) acids were the most abundant, whereas eicosapentaenoic acid was not detected. Hidalgo et al. (*31*) reported the FA composition of commercial eggs from four housing systems (i.e., cage, barn, free-range, and organic), which was similar to the one observed for the freeze-dried egg yolks, except for the palmitic (25−26%) and linoleic (17−20%) acids contents; in fact, the latter were higher and lower than those of the present study, respectively. These

Table 6. Effects of Rearing and Feeding Systems on the Average Peroxides Value, TBARs, Single and Total COP Contents, and Cholesterol Oxidation Ratio (OR) of Freeze-Dried Egg Yolks[a]

	parameters							
factor	POV (mequiv of O_2/kg of lipids)	TBARs (mg of MDA/kg of sample)	7α-HC (mg/kg of lipids)	7β-HC (mg/kg of lipids)	β-CE (mg/kg of lipids)	7-KC (mg/kg of lipids)	total COPs (mg/kg of lipids)	cholesterol OR (%)
rearing								
B	0.8	1.2	10.4	7.1	10.0	8.7	36.2	0.10
F	0.7	1.1	9.5	6.2	11.5	5.7	32.9	0.10
stat signif	ns	ns	ns	ns	ns	ns	ns	ns
feeding								
C	0.8	1.0	10.9	5.1b	8.4	5.2	29.6	0.08
O	0.7	1.2	9.0	8.2a	13.1	9.2	39.5	0.12
stat signif	ns	ns	ns	*	ns	ns	ns	ns
rearing × feeding								
BO	0.8	1.3	10.6ab	9.3	11.2	12.4	43.5	0.12
BC	0.9	1.1	10.1ab	4.9	8.8	5.3	28.8	0.08
FO	0.7	1.2	7.3b	7.1	15.0	6.1	35.5	0.11
FC	0.7	1.0	11.8a	5.3	7.9	5.3	30.4	0.09
stat signif	ns	ns	*	ns	ns	ns	ns	ns

[a] Each value is the average of three replicates. Abbreviations: B, battery cages, BO, reared in battery cages on organic feed; BC, reared in battery cages on conventional feed; C, conventional feed; F, free-range; FO, free-range and organic feed; FC, free-range and conventional feed; O, organic feed; stat signif, statistical significance [ns, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; a, b, statistically different means (Tukey's test; $P \leq 0.05$)].

differences can be ascribed to the influence of hen feed formulation on FA composition of egg yolk (*32*).

The factorial analysis of the single FA content shows that myristic, linoleic, and arachidonic acids were significantly lower in F rearing with respect to B, whereas a significantly higher amount of oleic acid was found in the F system. Feeding had significant effects on the amount of myristic, palmitic, and DHA acids, the first two FA being higher in the C feeding, whereas DHA was more abundant in O feeding. All single FA did not display significant crossed treatment interactions.

SFA, MUFA, and PUFA accounted for 33−35, 35−38, and 30−31% of total FA, respectively. Samman et al. (*33*) evaluated the FA composition of certified organic, conventional, and omega-3 fresh egg yolks, purchased from supermarkets and organic food outlets in Sydney (Australia). Their results were different from those obtained in the present study, which might be due to diverse diet supplementation, because the MUFA level was twice that found in the present study; however, no information is provided about the diets utilized (*33*).

The factorial analysis of the FA classes shows that B rearing resulted in significantly higher SFA, PUFA, PUFA n-3, and PUFA n-6 levels, as compared to the F rearing system; the latter, however, displayed a significantly higher MUFA content and UFA/SFA ratio. With regard to the feeding factor, C feeding had a significantly higher SFA content and n-6/n-3 ratio as compared to O feeding, whereas PUFA n-3 content and UFA/SFA ratio were significantly higher in O feeding. However, only one significant difference was found in the crossed treatment interactions, where BO exhibited a significantly higher level of PUFA n-3 with respect to the other three undifferentiated treatments.

FA composition of the freeze-dried egg yolks was not similar to those of the feedings, which could be ascribed to several factors. Although the ingredients of the free-range and cage diets were the same, it must be pointed out that hens grown with the free-range rearing had access to grassland areas, so they could have eaten grass, insects, and worms. This aspect might partly explain some differences between the FA composition of feedings and those of the freeze-dried egg yolks. In addition, essential FA (linoleic and linolenic acids) are precursors of the long-chain PUFA n-6 and n-3 series so, besides being absorbed, essential FA are elongated and desaturated to give rise to long-chain PUFA n-6 and n-3 (such as DHA). In fact, DHA was not detected in either feeding, but it was found in all crossed treatments (∼1%). Some authors suggest that PUFA are protected from oxidation by tocopherol supplementation or that tocopherols enhance the synthesis of a number of PUFA through the Δ^6 desaturase pathway (*34*).

Tocopherol Content of Freeze-Dried Egg Yolks. Table 4 shows the effects of rearing and feeding systems on the average amount of α-, β-, γ-, and δ-tocopherols, as well as the average content of total tocopherols, of the freeze-dried egg yolks. Total tocopherol content ranged from 174.8 to 288.3 mg/kg of lipids (∼101.3−171.1 mg/kg of sample); these data are similar to those previously reported by Qi and Sim (*35*), which are directly related to the diet composition. The factorial analysis shows that F rearing and C feeding systems resulted in significantly higher total tocopherol content as compared to B and O, respectively; in fact, conventional feeding already contained a higher amount of tocopherols, provided as vitamin E and soybean oil. However, no significant differences were found in the crossed treatment interaction, meaning that the two factors had additive effects on the parameters.

The most abundant tocopherol was α-tocopherol, followed by γ-, δ-, and β-tocopherols. In the case of α-tocopherol, it varied from 103.5 to 212.6 mg/kg of lipids (∼60.0−130.1 mg/kg of sample), being significantly lower in samples obtained with O feeding only; this is directly related to the feeding composition. With regard to β-tocopherol (5.3−6.9 mg/kg of lipids, ∼3.3−4.3 mg/kg of sample) and γ-tocopherol (54.3−64.0 mg/kg of lipids, ∼31.5−39.0 mg/kg of sample), significant differences were just found with respect to the rearing system, being higher in the F one. Feeding and rearing had no significant effects on the amount of δ-tocopherol (10.9−13.8 mg/kg of lipids, ∼6.5−8.4 mg/kg of sample). All single tocopherols did not display significant crossed treatment interactions.

Carotenoid Content of Freeze-Dried Egg Yolks. The average amount and type of carotenoids present in yolk of the newly laid egg depend to a large extent on the carotenoid content and composition present in the diet of the parent hen (*36*); in addition, xanthophylls are known to impart desirable yellow color to skin and egg yolk of poultry. Despite feedings having added pigments, xanthophyll- and carotenoid-containing natural ingredients (such as corn, dehydrated alfalfa meal, and corn gluten), the freeze-dried egg yolks here analyzed contained only lutein; this might be partly due to oxidation of the other carotenoids.

Xanthophylls from corn (mainly zeaxanthin) have been reported to be better utilized by the chick for skin deposition of pigments than those from dehydrated alfalfa meal and corn gluten (*37*).

Table 4 reports the effects of rearing and feeding systems on the average lutein content of the freeze-dried egg yolks, which varied from 50.7 to 68.4 mg/kg of lipids (~29.3−41.7 mg/kg of sample); these values are slightly higher than those found in the literature (*38*). The factorial analysis shows that F rearing and C feeding systems resulted in significantly higher lutein content with respect to B and O, respectively. In fact, the highly significant interaction evinced that only BO was statistically lower than the rest of the crossed treatments; this means that the feeding systems had no effect on lutein content of free-rearing samples, whereas O feeding decreased it with respect to C in freeze-dried egg yolks obtained from caged animals.

Sterol Content of Freeze-Dried Egg Yolks. Table 5 shows the effects of rearing and feeding systems on the average amount of cholesterol, β-sitosterol, and campesterol of the freeze-dried egg yolks, as well as the average content of total sterols. Total sterol content ranged from 32957 to 36719 mg/kg of lipids (~20028−21971 mg/kg of sample), and no significant effects of rearing, feeding, and crossed treatment interaction were observed.

The most abundant sterol was cholesterol, followed by campesterol and β-sitosterol. Cholesterol constituted about 99% of total sterols, and it was not significantly affected by any of the parameters here considered. The levels of cholesterol detected in the freeze-dried egg yolks are similar to those reported in the literature (*39*).

Significant effects of both rearing and feeding systems were observed on campesterol content (0.2−0.8 mg/kg of lipids, ~0.1−0.5 mg/kg of sample), being higher in F and O, respectively. With regard to β-sitosterol (0.2−0.4 mg/kg of lipids, ~0.1−0.3 mg/kg of sample), significant differences were found only with respect to the rearing system, being higher in F. All single sterols did not display significant crossed treatment interactions.

It must be noted that the relative presence of the single phytosterols in the freeze-dried egg yolks did not correspond to the sterol composition of the dietary oil sources (corn and soybean oil), where β-sitosterol was about 2−4 times higher than campesterol (*29*). It might be possible that phytosterols have been selectively absorbed in different amount and/or have been partially metabolized/degraded by the laying hen, converting them into other compounds.

Lipid Oxidation of Freeze-Dried Egg Yolks. Table 6 shows the effects of rearing and feeding systems on the POV and TBARs of freeze-dried egg yolks. In general, a low lipid oxidation level was observed in all samples, which was confirmed by both primary and secondary oxidation product parameters; these data are in agreement with those reported in the literature (*40*). In fact, POV varied from 0.7 to 0.9 mequiv of O_2/kg of fat, whereas TBARs ranged from 1.0 to 1.3 mg of MDA/kg of sample. The factorial analysis shows no significant differences due to rearing, feeding, and crossed treatment interaction. Therefore, the unsaturation degree of the lipids originally present in the conventional and organic diets did not have a significant effect on the oxidation level of freeze-dried egg yolks.

Cholesterol Oxidation of Freeze-Dried Egg Yolks. Table 6 shows the effects of rearing and feeding systems on the single and total COP contents of freeze-dried egg yolks, as well as on the cholesterol oxidation rate. Total COP content ranged from 28.8 to 43.5 mg/kg of lipids (~17.2−25.8 mg/kg of sample), which is higher than those found in the literature for freeze-dried egg yolks (*8*, *11*). This might be related to several factors: type and quality of the feed ingredients, storage time in the freezer before actual freeze-drying, use of compressed air during freeze-drying, and/or incomplete air removal during vacuum packaging due to the porous nature of the sample. Moreover, because of the high surface activity of sterols, they tend to migrate to the oil−water interface, where oxidative stress is high (*41*); this behavior would indicate that cholesterol locates at the interface before water removal during freeze-drying, remaining at the surface level of the powder and being thus directly exposed to air and consequent oxidation.

With regard to the factorial analysis of the total COP content, no significant differences were observed due to rearing, feeding, and crossed treatment interaction. COP levels of egg yolk powders have been found to vary from 8 to 311 mg/kg (*42*), which depend on the type of drying process, as well as on the drying and storage conditions (such as temperature, oxygen partial pressure). As aforementioned, spray-dried egg yolks display a more elevated COP amount than freeze-dried ones.

The most abundant COPs were 7α-HC and β-CE, followed by 7β-HC and 7-KC; no α-CE and triol were detected. It must be noted that, under the analytical conditions here used, 7β-HC partially overlapped with campesterol. In the case of 7α-HC, it varied from 7.3 to 11.8 mg/kg of lipids (~4.4−7.3 mg/kg of sample), being significantly lower in samples obtained with O feeding; in addition, the significant interaction shows that, in free-rearing samples, organic feeding (FO) was significantly lower than the conventional one, whereas the two feedings were at an intermediate, undifferentiated level in freeze-dried egg yolks obtained from caged hens. With regard to β-CE (7.9−15.0 mg/kg of lipids, ~4.9−12.3 mg/kg of sample), 7β-HC (4.9−9.3 mg/kg of lipids, ~2.9−5.6 mg/kg of sample) and 7-KC (5.3−12.4 mg/kg of lipids, ~3.0−4.6 mg/kg of sample), no significant differences were observed due to rearing, feeding, and crossed treatment interaction. Several studies report the presence of α-CE and triol in spray-dried egg yolks (*8*, *13*), where processing conditions are more severe with respect to freeze-drying, thus favoring the bimolecular interaction between cholesterol and the hydroperoxyl radical that leads to the formation of epoxides that are, in turn, converted into triol.

The cholesterol oxidation rate varied from 0.08 to 0.12%, which is higher than those reported in the literature for freeze-dried egg yolks (*8*); this might be related to the factors discussed in the previous paragraphs. The factorial analysis of the cholesterol oxidation rate showed no significant differences due to rearing, feeding, and crossed treatment interaction. The cholesterol oxidation rate of egg yolk powders has been found to vary from 0.52 to 1.46% (*43*), being higher in spray-dried egg yolks as compared to freeze-dried ones.

The low amounts of COPs found in the freeze-dried egg yolks should not represent a health risk, because negative biological effects of COPs seem to manifest at relatively high concentrations in comparison with the daily intake that these foods would represent (*7*, *8*).

Correlations between Lipid Composition of Feed and Composition and Oxidation Parameters of Freeze-Dried Egg Yolks. A correlation study (Spearman test, $\alpha = 0.05$) was performed on the results obtained for lipid composition of feeding and for the four crossed treatments deriving from the two types of rearing and feeding systems. For better data comprehension, only significant correlations are here discussed.

FA composition of freeze-dried egg yolks was affected by feed FA composition. In fact, a positive, linear correlation was found between palmitic acid contents of feeds and egg yolks ($r = 0.7066$, $P = 0.010$*), between SFA of feeds and palmitic content in the eggs ($r = 0.7066$, $P = 0.010$*), and between linolenic acid content of feeds and DHA level of egg products ($r = 0.5795$, $P = 0.048$*).

The latter confirms the role of essential FA (linolenic acid) as precursor of the long-chain PUFA n-3 series.

The n-6/n-3 ratio of feeds was linearly correlated to the n-6/n-3 ratio of egg yolks ($r = 0.7492$, $P = 0.005$**), whereas it was inversely correlated to DHA ($r = -0.5937$, $P = 0.042$*) and PUFA n-3 of egg products ($r = -0.5937$, $P = 0.042$*). On the other hand, the n-6/n-3 ratio of egg yolks displayed negative, linear correlations with linolenic acid ($r = -0.7633$, $P = 0.004$**), total PUFA ($r = -0.6502$, $P = 0.022$*), and PUFA n-3 contents of feeds ($r = -0.7209$, $P = 0.008$**).

Some correlations were also observed between the FA composition of the feedings and the lipid and cholesterol oxidative parameters determined in the freeze-dried egg yolks. MUFA content of the feeds was linearly correlated to TBARs of egg yolks ($r = 0.6230$, $P = 0.030$*). The palmitic acid content of the feed was inversely correlated to 7β-HC ($r = -0.6361$, $P = 0.026$*), 7-KC ($r = -0.6078$, $P = 0.036$*) and total COP contents of egg powders ($r = -0.6219$, $P = 0.031$*); a similar trend was observed for the SFA content of feed and the level of the aforementioned COPs in the egg yolks. On the contrary, oleic acid content of the feed was directly correlated to 7β-HC in the egg yolk ($r = 0.6502$, $P = 0.022$*). Linolenic acid content of the feed was also directly correlated to 7β-HC ($r = 0.6078$, $P = 0.036$*), 7-KC ($r = 0.6078$, $P = 0.036$*), and total COP contents of egg powders ($r = 0.6926$, $P = 0.013$*). Positive, linear correlations were found between total PUFA of the feed and 7β-HC ($r = 0.6361$, $P = 0.026$*), 7-KC ($r = 0.6078$, $P = 0.036$*), and total COP contents of egg yolks ($r = 0.6219$, $P = 0.031$*). All of these correlations confirm the importance of the FA composition of the dietary fat on the cholesterol oxidation of freeze-dried egg yolks, because FA unsaturation is known to favor such degradation processes.

A positive, linear correlation was found between total tocopherol contents of feeds and egg yolks ($r = 0.8057$, $P = 0.002$**), as well as between their α-tocopherol levels ($r = 0.7492$, $P = 0.005$**). Tocopherols' presence in the diet influenced the antioxidant capacity of the freeze-dried egg yolks, preventing PUFA from oxidation and delaying both lipid and cholesterol degradations. In fact, a positive, linear correlation was found between β-tocopherol of feeds and PUFA n-3 in egg yolks ($r = 0.5795$, $P = 0.048$*), as well as between γ-tocopherol of feeds and DHA in egg products ($r = 0.6219$, $P = 0.031$*). In addition, α-tocopherol and total tocopherol contents of feeds were inversely correlated to TBARs of egg yolks ($r = -0.6018$, $P = 0.038$* and $r = -0.6938$, $P = 0.012$*, respectively). With regard to cholesterol oxidation, α-tocopherol content of feeds was inversely correlated to 7β-HC ($r = -0.6219$, $P = 0.031$*), β-CE ($r = -0.5937$, $P = 0.042$*), 7-KC ($r = -0.5795$, $P = 0.048$*), and total COPs contents of egg powders ($r = -0.6643$, $P = 0.018$*). The latter trend was also confirmed by the cholesterol oxidation rate of freeze-dried egg yolks, which showed a negative, linear correlation with α-tocopherol ($r = -0.6643$, $P = 0.018$*) and total tocopherol contents of the feeds ($r = -0.5937$, $P = 0.042$*).

Correlations between Composition and Oxidation Parameters of Freeze-Dried Egg Yolks. As for the previous paragraph, only significant correlations are here discussed. Positive, linear correlations were found between the lipid content and the α-tocopherol ($r = 0.5961$, $P = 0.041$*), β-tocopherol ($r = 0.8642$, $P < 0.001$**), γ-tocopherol ($r = 0.7655$, $P = 0.004$**), total tocopherols ($r = 0.5750$, $P = 0.050$*), campesterol ($r = 0.6879$, $P = 0.013$*), and β-sitosterol contents ($r = 0.6808$, $P = 0.015$*), which confirms that the liposoluble component concentration is directly related to the amount of lipids of egg yolk.

Palmitic acid was inversely correlated to 7β-HC ($r = -0.7273$, $P = 0.007$**) and total COP contents ($r = -0.6504$, $P = 0.022$*), whereas DHA and PUFA n-3 were linearly correlated to 7-KC ($r = 0.6294$, $P = 0.028$* and $r = 0.6014$, $P = 0.038$*, respectively). These trends evince the important role of FA unsaturation degree on the extent of cholesterol oxidation.

Negative, linear correlations were noted between total tocopherol content and two lipid oxidation parameters [TBARs ($r = -0.6410$, $P = 0.025$*) and 7-KC ($r = -0.6294$, $P = 0.028$*)], which confirms the importance of tocopherols as antioxidants in animal products. Laying hens fed tocopherol-enriched diets have shown a lower TBARs content (*35*).

α-Tocopherol was also linearly correlated to total tocopherol ($r = 0.9860$, $P < 0.001$**) and lutein contents ($r = 0.6014$, $P = 0.039$*), which, in the case of total tocopherols, reflects the chemical composition of the feed ingredients. α-Tocopherol was the most abundant tocopherol in the freeze-dried egg yolks and, therefore, it directly influenced the total tocopherol level. On the other hand, negative, linear correlations were observed between α-tocopherol and two lipid oxidation parameters [TBARs ($r = -0.7040$, $P = 0.011$*) and 7-KC ($r = -0.6084$, $P = 0.036$*)], as observed for the total tocopherol content.

With regard to lutein, negative, linear correlations were observed with two lipid oxidation parameters [TBARs ($r = -0.8511$, $P < 0.001$**) and 7β-HC ($r = -0.5804$, $P = 0.048$*)], which is in agreement with the antioxidant role of carotenoids.

Cholesterol was linearly correlated to the total sterol content ($r = 1.000$, $P < 0.001$**), because cholesterol was by far the most abundant sterol in the freeze-dried egg yolks (99.9%).

β-Sitosterol was linearly correlated to the campesterol content ($r = 0.9650$, $P < 0.001$**), despite the fact that their relative contents in the freeze-dried egg yolks do not correspond to the sterol composition of the feed ingredients.

Positive, linear correlations were found between total COPs and the most abundant COPs [7β-HC ($r = 0.8741$, $P < 0.001$**), β-CE ($r = 0.6154$, $P = 0.033$*), and 7-KC ($r = 0.8462$, $P = 0.001$**)], as well as with the cholesterol oxidation rate ($r = 0.9580$, $P < 0.001$**). The latter was also linearly correlated with the same COPs [7β-HC ($r = 0.8531$, $P < 0.001$**), β-CE ($r = 0.6434$, $P = 0.024$*), and 7-KC ($r = 0.7902$, $P = 0.002$**)], as previously observed. 7-KC is one of the major COPs in food products and has often been used as a marker of the extent of cholesterol oxidation (*42*).

7β-HC was also linearly correlated to 7-KC ($r = 0.6294$, $P = 0.028$*), which might be due to the fact that they are generated following the same cholesterol oxidation pathway.

Although 7-KC and β-CE originate from the same precursors following different oxidation pathways, they were linearly correlated ($r = 0.6503$, $P = 0.022$*). In fact, 7-KC derives from dismutation of 7-hydroxy derivatives, whereas β-CE is formed from the bimolecular interaction between a cholesterol molecule and a hydroperoxyl radical.

In conclusion, this study evinces a low lipid and cholesterol oxidation level in freeze-dried egg yolks produced with two rearing systems (battery cages and free-range) and two types of feedings (conventional and organic). Although rearing and feeding systems seem to significantly affect the total tocopherols, α-tocopherol, and lutein contents, this did not lead to significant differences in the overall oxidative quality of these food products. The extent to which the type of rearing and feeding are able to influence the quality of freeze-dried egg yolks remains a major point of controversy in the discussion surrounding organic versus conventional animal products.

ABBREVIATIONS USED

7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; 7-KC, 7-ketocholesterol; 19-HC, 19-hydroxycholesterol;

C, traditional cages; α-CE, α-epoxycholesterol; β-CE, β-epoxycholesterol; BC, reared in battery cages on conventional feed; BO, reared in battery cages on organic feed; C, conventional; cholesterol OR, cholesterol oxidation ratio; COPs, cholesterol oxidation products; EDTA, ethylenediaminetetraacetic acid; F, free-range; FA, fatty acids; FC, free-range and conventional feed; FO, free-range and organic feed; GC, gas chromatography; GC-FID, gas chromatography−flame ionization detector; GC-MS, gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; HPLC-DAD, high-performance liquid chromatography−diode array detector; HPLC-FD, high-performance liquid chromatography−fluorescence detector; IS, internal standard; LOD, limit of detection; LOQ, limit of quantitation; MDA, malonylaldehyde; MUFA, monounsaturated fatty acids; O, organic; POV, peroxide value; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SPE, solid-phase extraction; triol, cholestanetriol; TBA, 2-thiobarbituric acid; TBARs, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; UFA, unsaturated fatty acids.

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