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Fatty acid and carotenoid composition of egg yolk as an effect of microalgae addition to feed formula for laying hens

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Abstract

Hens were fed five diets based on rapeseed/corn oils with or without addition of the marine microalgae, *Nannochloropsis oculata.* Colours of egg yolk, fatty acid profiles in phospholipid (PL) and triacylglycerol (TAG) fractions and carotenoid contents were analysed. The major effects on yolk fatty acid composition were observed in the PL fraction with changes in 18:2$n-6$, 18:3$n-3$, 20:4$n-6$, 20:5$n-3$ and 22:6$n-3$. The highest amounts of eicosapentaenoic acid and docosahexaenoic acid were obtained from the diet containing 20% *N. oculata*, whereas the highest arachidonic acid content in yolk was from the corn oil diet. In addition, colour (a^* value) and carotenoid content increased when *N. oculata* was included in the diet. Long chained fatty acids were almost exclusively present in the PL fraction of yolk. This study shows that fatty acid composition and carotenoid content of egg yolk are improved by addition of *N. oculata* in laying hen diet.
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1. Introduction

Eggs have been collected and eaten for centuries before the domestication of hens and other birds (Rose, 1997). They are both an inexpensive food and a food ingredient and are consumed worldwide and accepted in all cultures. An egg contains all essential nutrients, such as lipids, amino acids and vitamins, required for a new life, i.e., the chicken (Farrell, 1997). Egg composition is suggested to be altered by heredity, diet and age of the hen; the largest fraction of yolk lipids is triacylglycerols (TAG, 66%), followed by phospholipids (PL, 30%) and cholesterol (CL, 5%) (Rose, 1997).

The human diet changed dramatically 10,000 years ago and cereals became a staple food. Cereal grains are rich in $n-6$ polyunsaturated fatty acids (PUFA) but low in $n-3$ PUFA. At present, the dietary intakes of $n-3$ PUFA and antioxidants are decreasing whereas the amounts of total fat, saturated fat and $n-6$ PUFA are increasing in human nutrition. Before the diet changed, the $n-6/n-3$ ratio used to be approximately 1:1, but, in the western diet today, the ratio is 15:1 (Simopoulos, 2002). The essential linoleic acid (LA, 18:2$n-6$) and α-linolenic acid (ALA, 18:3$n-3$), as well as their longer chain derivates, are important components of cell membranes (Stauffer, 1996). Arachidonic (AA, 20:4 $n-6$) and eicosapentaenoic acid (EPA, 20:5$n-3$) are precursors of eicosanoids, which are important for various biological functions (Horrocks & Yeo, 1999). In addition, EPA and docosahexaenoic acid (DHA, 22:6$n-3$) are important for brain and nervous development (Stauffer, 1996). The enzymes required for the desaturation (Δ6 and Δ5-desaturases) of 18 carbon $n-6$ and $n-3$ PUFA are the limiting factor, since these FA compete for the same enzymes. This underlines the importance of a balance in the dietary intake of these two fatty acids (Sargent, Bell, Bell, Hendersson, & Tocher, 1995). It is known that a diet rich in LA tends to inhibit the formation of EPA from ALA (Cordain, 1999). Due to the imbalanced $n-6/n-3$ PUFA ratio of the western diet

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today, dietary saturated FA and $n-6$ PUFA are promoters of chronic diseases, such as arteriosclerosis, essential hypertension, obesity, diabetes and possibly some forms of cancers, as suggested by Simopoulos (2002). Thus, lipids, and especially FA composition of diet are currently gaining increased attention. There is evidence that $n-3$ FA-enriched eggs in the human diet have positive health effects, such as decreased plasma TG, and blood pressure and lower platelet aggregation (Lewis, Seburg, & Flanagan, 2000; Oh, Ryue, Hsieh, & Bell, 1991). The aim of this study was to investigate how the FA composition and the carotenoid content in egg yolk could be altered by diet. The concentrated microalga, *Nannochloropsis oculata*, was used as a feed supplement in two poultry feeds, one with rapeseed oil and one with rapeseed/corn oil to evaluate how the FA and carotenoid compositions and the colour of the egg yolk are affected. Furthermore, the FA compositions in TAG and PL fraction were studied to investigate into which fraction the long-chained polyunsaturated fatty acids (LCPUFA) were incorporated. Most fatty acids in *N. oculata* are present in form of PL, including approximately 37% EPA, and therefore, we expected some of the EPA to be found in the PL fraction of egg yolk.

2. Materials and methods

2.1. Animals and diets

A total of 30 Hy-line W-98 hens, 55 weeks of age were used in this study. The hens were fed 5 diets with different contents of $n-3$ and $n-6$ PUFA. Feed (120 g dry matter (DM)/hen/day) was given pen-wise in a trough once a day. The control diet, which was based on rapeseed oil (RO) was the ordinary diet used in the stable (Table 1).

A concentrated semi-dry microalga, CCMP525 *N. oculata* (Reed Mariculture Inc. CA, USA), was used as a source of $n-3$ long chained PUFA and carotenoids. The 5 diets were: RO feed (11.2 ME MJ/kg, 151 g protein/kg and 68 g fat/kg), rapeseed oil/corn oil (RO/CO) feed (3:7), RO feed supplied with 10% (on DM basis) of *N. oculata,* RO/CO (7:3) feed with 10% (on DM basis) of *N. oculata* and RO feed with 20% (on DM basis) *N. oculata.* All diets were administered during 4 weeks to duplicate groups of 5 hens. The group fed RO/CO feed received 10% *N. oculata* after 4 weeks, the group fed RO feed, mixed with 10% alga *N. oculata,* got increased supplement of *N. oculata* to 20% after 4 weeks and the control group stayed on the RO diet for 8 weeks.

Table 1
Proximate composition of diet

Components	Diet
Wheat	14.3
Barley	22.0
Oat	15.0
Triticale	10.0
Lucerne meal	3.6
Soy meal	19.0
Oils[a]	4.7
Calcium	8.5
Salt	0.3
Dicalciumphosphate	1.4
Vitamins + trace elements	1.0
DL-methionine	0.16
L-lysine–HCl	0.08

[a] The oils consist of either rapeseed oil (RO) or corn/rapeseed oil (RO/CO (3:7)).

Dry matter, crude fat content, lipid class and FA composition of RO diet, RO/CO diet and algae mixed diets are shown in Table 2. Dry matter of algae, control and CO feed were determined gravimetrically after drying (16–18 h at 105 °C) and fat content was analyzed (Soxtec System H+ equipment, Foss-Tecator AB, Höganäs, Sweden).

2.2. Sampling and storage of eggs

Feed intake and egg production were recorded daily. Eggs were collected daily and stored at 4 °C for 48–72 h before sampling, weighing and colour measurements. Eggs from each treatment were analysed after four weeks of feeding.

2.3. Lipid and fatty acid analysis

2.3.1. Sample preparation

Feed samples (3 g) were homogenized (2×30 s) in duplicates and extracted in 54 ml HIP (hexane: isopropanol 3:2, v/v) by using an Ultra-turrax macerator (Janke & Kunkel, IKA Werke, Germany). Next, 21 ml 6.67% of Na_2S0_4 salt solution were added to remove non lipids (Hara & Radin, 1978). The samples were shaken vigorously and centrifuged for 5 min at 17,000*g* and 18 °C (Sorvall Super T21, Sorvall Pruducts L.P., Newton, Connecticut, USA). The lipid extract was transferred to a pear-shaped flask and evaporated with a BÜCHI rotavapor R-205, equipped with a BÜCHI heating bath B490 at 28 °C and a vacuum pump (VACUUBRAND MZ 2C). The dry sample was solved in 1 ml of chloroform. One gramme of algae suspension, in duplicate was extracted for the egg yolk lipids below.

2.3.2. Thin layer chromatography (TLC) analysis of lipid classes in feed

Algae, control and corn feed lipids were analysed by TLC to investigate composition of different lipid classes. As a stationary phase, glass plates pre-coated with silica gel TLC plates (20×10 cm; Silica gel 60; 0.20 mm layer, Merck, Darmstadt, Germany) were used. The analysis was performed according to Olsen and Hendersen (1989) with slight modifications. Prior to use, the plates were pre-developed to full length with hexane:diethyl ether:acetic acid (85:15:1, v/v/v) as mobile phase and dried for 5 min in 110 °C. The upper 1 cm of the silica gel was removed and the plates were activated in 110 °C for 1 h and stored in a vacuum desiccator prior to further use. The samples were diluted in hexane to a concentration of

Table 2
Dry matter (DM) and lipid composition in the five diets and algae

%	RO	RO/CO	RO + 10% algae	RO + 20% algae	RO/CO + 10% algae	Algae
DM	94.6	94.7	87.2	79.7	87.3	20.0
Lipids	9.8	9.8	9.2	8.6	9.2	3.6
Triacylglycerides	48.1	61.0	44.2	40.3	55.9	9.2
Phospholipids	11.0	15.4	12.9	14.7	16.8	29.7
Free fatty acids	19.4	9.9	21.9	24.6	13.4	45.2
Sterols	2.7	5.4	3.7	4.8	6.2	12.9
Sterol esters	3.9	4.7	3.8	3.4	4.3	1.6
Fatty acid composition						
14:0	0.1	0.1	0.2	0.4	0.2	3.5
16:0	8.9	11.0	9.2	9.6	11.2	17.9
16:1$n-7$ trans	0.2	0.2	0.5	0.8	0.4	7.2
16:1	Trace	Trace	0.5	1.0	0.5	11.8
18:0	1.6	1.7	1.6	1.6	1.7	0.7
18:1$n-9$	45.9	34.0	44.3	42.5	32.9	5.6
18:1$n-7$	2.9	1.5	2.7	2.6	1.5	Trace
18:2$n-6$	29.4	45.1	28.6	27.6	43.7	7.4
18:3$n-3$	8.4	4.7	8.3	8.2	4.8	6.7
20:1	0.7	0.4	0.7	0.7	0.3	Trace
20:4$n-6$	–	–	0.1	0.3	0.1	3.7
20:3$n-3$	–	–	Trace	Trace	Trace	0.4
22:1	0.6	0.14	0.6	0.5	0.1	–
20:5$n-3$	Trace	Trace	1.5	3.1	1.5	37.1
24:1	0.2	0.1	0.2	0.1	0.1	Trace
$n-3$	8.4	4.7	9.8	11.4	6.3	44.1
$n-6$	29.6	45.2	28.9	28.0	43.9	11.4
$n-6/n-3$	3.5	9.6	3.4	3.2	9.2	0.3

1 μg/μl. Samples were applied on a HPTLC plate with a CAMAG TLC sampler 4 (Camag, Switzerland) in 2-mm bonds with an application speed of 250 nl/s. Nitrogen was used as a spray gas. The samples were applied in triplicates on the plates. The distance between the bands was 10 mm. The lipids were then separated in a Twin Through Chamber 20×20 (Camag, Switzerland) using 25 ml hexane:diethyl ether:acetic acid (85:15:1, v/v/v) as mobile phase; the chamber saturation was increased by placing a piece of filter paper in the chamber. The plates were removed from the chamber when they had developed 6.9 cm from the base line, air-dried at room temperature and sprayed with a solution of 3% cupric acetate in 8% phosphoric acid and then charred for 20 min at 160 °C. Plates were scanned with Camag TLC scanner 3 (Camag, Switzerland) to identify lipid classes. The scanning was performed at a speed of 20 mm/s and a data resolution of 100 μm/step with a slit dimension of 6×45 mm at a wavelength of 350 nm. Lipid classes were identified by comparing to an external standard (TLC 18-4A, Nu-Chek Prep, Elysian, USA). For data filtering, the mode Savatisky-Golay 7 and manual baseline correlation were used.

2.3.3. Egg yolk

One gramme of egg yolk (pooled from 2 eggs) was homogenized (2×30 s) in duplicate and extracted with 18 ml HIP (hexane:isopropanol 3:2, v/v) by using an Ultra-turrax macerator (Janke & Kunkel, IKA Werke, Germany). Next, 14 ml of 6.67% Na_2S0_4 were added to remove non-lipids. Subsequent analytical steps were similar to those described above.

2.3.4. Separation of lipids

The total egg lipids were fractionated on TLC silica-coated plates 20×20 cm 60 F 254 (MERCK, Darmstadt, Germany). First, 2 mg of lipids were applied on the TLC plate. Each sample was placed on a 2 cm band. The lipids were separated by placing the plates in hexane:diethylether:acetic acid (85:15:1, v/v/v) solvent for 1 h. TAG and PL were then scraped off for further analyses. PL was extracted sequentially in 3 ml chloroform:methanol (1:1, v/v), 2 ml chloroform:methanol (2:1, v/v) and 2 ml chloroform. TAG fractions were extracted three times in chloroform (3, 2 and 2 ml, respectively). The lipid extracts were transferred to new tubes and the solvents were evaporated under nitrogen gas until dry. The dried PL and TAG samples were dissolved in 0.5 ml hexane (Dutta & Appelqvist, 1989).

2.3.5. Preparation of fatty acids methyl esters (FAME)

Total lipids of feed and algae were methylated according to Appelqvist (1968). First, 2 ml of 0.01 M NaOH in dry methanol were added to 10 mg of fat. The samples were shaken and heated for 10 min on a heating block at 60 °C. Next, 3 ml BF_3 reagent (boron trifluoride–methanol complex) were added and the samples were reheated for 10 min. The samples were cooled under running water and 2 ml 20% NaCI and 2 ml hexane were added. Test

tubes were shaken vigorously and centrifuged for 5 min at 440*g* at 18 °C. The FAME were transferred to new test tubes and evaporated under nitrogen gas. The dry samples were dissolved in hexane, vortexed and stored in a freezer at −20 °C prior to GC analyses. Both PL and TAG fractions from eggs fatty acids were methylated according to Appelqvist (1968). First, 2 ml of 0.01 M NaOH in dry methanol were added to the samples. The samples were shaken vigorously and heated for 30 min on a heating block at 60 °C. Then, 2 ml of 50% $NaHS0_4$:25% NaCl solution in H_2O and 3 ml distilled H_2O were added and the samples were cooled under water. FAMES were extracted with 2 ml hexane. The samples were shaken vigorously and centrifuged for 5 min at 440 g at 18 °C. The FAMES were evaporated under nitrogen gas. Hexane was added to the samples, which were then stored at −20 °C prior to GC analyses.

2.3.6. Gas chromatography

FAMEs were analysed in a gas chromatograph CP9001 (Chrompack, Middelburg, Netherlands) equipped with flame ionization detector and split injector and fitted with a fused silica capillary column BPX 70 (SGE, Austin, Texas, USA), length 50 m i.d. 0.22 mm, 0.25 μm film thickness. The samples were injected by a CP9050 auto sampler, split mode. The split ratio 1:30 was used. Column temperature was programmed at 2 °C/min from 158 to 220 °C. Injector and detector temperatures were 230 and 250 °C, respectively. Fatty acids were identified and their responses were determined by comparing the peaks to those of standard sample GLC-68 A (Nu-Chek Prep, Inc, Elysian, Minnesota, USA). Peak areas were integrated using a maestro 2 version 2.4 integrator (Chrompack, Middelburg, Netherlands). The carrier gas was helium (22 cm/s, flow rate 0.8 ml/min). Make up gas was nitrogen.

2.4. Colour, carotenoids, weight and fat content analyses of egg yolk

Colour was measured by using the colour scale and with a Minolta Chroma Meter CR-300 (Japan) to detect the $L^*a^*b^*$-values. The L^* value represents lightness (negative towards black, positive towards white), the a^* value red–greenness (negative towards green, positive towards red) and the b^* value the blue–yellow colour scale (negative towards blue, positive towards yellow). The instrument was calibrated using a Minolta calibration plate CR-22/CR-300 (Japan). To analyse the yolk colour, the tip of the measuring head was placed against the surface of the yolk. In addition, colour was also identified with the Hoffman–LaRoche 15-point colour fan. Fresh yolks were weighed and lipid percentage was calculated on a weekly basis.

Total carotenoid content in egg yolk was analyzed by extracting egg yolk for the lipids; the extracts were solved in hexane and the absorbance were measured at a wavelength of 450 nm using a spectrophotometer (UV-2401 (PC) CE, Shimadzu). Carotenoid content was calculated by using the extinction coefficients: concentration = absorbance * 10,000/2550. For identification of the different carotenoids, egg yolks were further analysed by high performance liquid chromatography (HPLC). Two duplicate samples per dietary treatment were analysed.

Extractions were performed with acetone and the samples were dried under nitrogen gas and dissolved in chloroform:methanol (1:1 v/v). Samples were filtered through a 0.45 μm hydrophobic membrane filter to remove particulate residues before injection. The HPLC (Merck-Hitachi) was equipped with a ReproSilPur 120 18 °C column (250 × 4.0 mm 5 pm), an L6200A intelligent pump, D-2000 injector and an L4200 Vis/UV-detector set at 474 mm with 0.1 absorbance units at full scale. A linear elution gradient was adopted with methanol:water:ethyl acetate at start 82:8:10 (v/v/v) and after 16 min 29:1:70 (v/v/v). Standards (astaxanthin, canthaxanthin and lutein, SIGMA ALDRICH, St Louis Missouri, USA) were used to check the recovery of carotenoids during extraction and reproducibility of the analysis method applied. Solvents used for HPLC analyses were of HPLC grade.

All solvents and chemicals used were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

2.5. Statistical analyses

The SAS analysis programme was used for statistical analysis. Procedure MIXED (SAS Institute Inc., Cary,

Table 3
Colour values and weights of the egg yolks measured after 4 weeks of feeding

Colour	RO, $n = 8$	RO + 10% algae, $n = 8$	RO + 20% algae, $n = 8$	RO/CO, $n = 8$	RO/CO + 10% Algae, $n = 8$
Weight (g)	20.3 ± 1.3	19.2 ± 1.5	18.7 ± 1.2	19.6 ± 1.4	19.5 ± 0.9
L^*	55.9^a ± 0.7	46.2^b ± 2.5	42.6^c ± 1.2	55.6^a ± 2.0	44.5^b ± 2.0
a^*	−4.7^a ± 0.2	12.1^b ± 2.7	16.1^c ± 0.6	−5.12^a ± 0.6	12.0^b ± 1.1
b^*	36.7 ± 2.6	39.9 ± 2.8	35.8 ± 2.9	39.3 ± 8.9	37.5 ± 2.2
Colour scale	6.0^b ± 0.0	14.0^a ± 0.0	14.0^a ± 0.0	6.0^c ± 0.0	13.0^a ± 0.0

Data presented as mean values ± SD of eight samples (each treatment).
$^{a–d}$ Mean values in the same row with different letters differ significantly ($P < 0.05$).
L^* = lightness (negative towards black, positive towards white), a^* = red–greenness (negative towards green, positive towards red) and b^* = blue–yellowness (negative towards blue, positive towards yellow).

Table 4
The carotenoid content in eggs after 4 weeks of feeding

Diet	Total carotenoids (mg/kg) $n = 2$	Lut/Zea (mg/kg) $n = 2$	Canthax (mg/kg) $n = 2$	β-Carotene (mg/kg) $n = 2$
Algae 20%	37.0	22.0	7.7	1.3
Control	9.7	8.0	n.d	n.d

Lut/Zea = Lutein/Zeaxanthin, Cantax = Canthaxanthin, n.d., not detected ($P < 0.02$).

N.C., USA, versions 8.2) was used to compare the different feeding regimes for fatty acid composition and colours. The statistical model used contained the fixed effect of feed.

3. Results

3.1. Yolk weight and fat content

Egg yolk weight and fat content are shown in Tables 3 and 5, respectively. Yolk weight and fat content did not differ significantly due to feeding regime.

3.2. Egg yolk colour and carotenoids

Yolk colour measurements, after 4 weeks of feeding, are shown in Tables 3. Colour changes were registered after

Table 5
Egg yolk total lipid, total carotenoid content and phospholipid and triacylglycerol fatty acid composition

	RO, $n = 6$	RO + 10% algae, $n = 4$	RO + 20% algae, $n = 4$	RO/CO, $n = 4$	RO/CO + 10% algae, $n = 4$
Lipid %	22.2 ± 0.6	23.5 ± 0.7	24.5 ± 1.5	23.4 ± 1.1	23.5 ± 0.9
Total carotenoids mg/kg	9.1^{a} ± 1.1	35.5^{b} ± 3.5	42.1^{c} ± 5.5	6.9^{a} ± 0.7	30.2^{d} ± 5.7
Phospholipids					
14:0	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.06	0.2 ± 0.02	0.2 ± 0.05
16:0	22.3^{a} ± 1.12	23.8^{bc} ± 1.07	23.2^{bc} ± 1.28	22.9^{ab} ± 1.19	24.9^{c} ± 0.78
16:1 $n-7$	0.3^{a} ± 0.04	0.3^{c} ± 0.02	0.3^{c} ± 0.03	0.2^{b} ± 0.02	0.2^{b} ± 0.02
16:1	0.5^{a} ± 0.07	0.6^{b} ± 0.06	0.6^{b} ± 0.08	0.5^{a} ± 0.03	0.5^{ab} ± 0.08
18:0	18.6 ± 0.90	17.6 ± 0.65	17.7 ± 1.32	18.6 ± 0.38	18.0 ± 0.99
18:1 $n-9$	24.7^{a} ± 1.12	25.1^{a} ± 0.75	25.2^{a} ± 1.27	22.3^{b} ± 0.49	21.8^{b} ± 0.64
18:1 $n-7$	1.3^{a} ± 0.10	1.4^{a} ± 0.02	1.4^{a} ± 0.05	1.0^{b} ± 0.06	1.0^{b} ± 0.06
18:2 $n-6$	13.7^{a} ± 0.85	13.7^{a} ± 0.22	13.2^{a} ± 0.53	17.0^{b} ± 0.60	16.8^{b} ± 1.00
18:3 $n-3$	0.5^{a} ± 0.06	0.4^{a} ± 0.05	0.4^{a} ± 0.04	0.3^{b} ± 0.05	0.2^{b} ± 0.02
20:1 $n-9$	0.3^{a} ± 0.03	0.3^{a} ± 0.03	0.2^{c} ± 0.02	0.2^{bc} ± 0.01	0.2^{b} ± 0.01
20:2 $n-6$	0.2^{a} ± 0.08	0.2^{ac} ± 0.02	0.2^{ad} ± 0.01	0.3^{b} ± 0.03	0.3^{bcd} ± 0.02
20:3 $n-6$	0.4^{a} ± 0.04	0.5^{b} ± 0.02	0.4^{ab} ± 0.06	0.5^{ab} ± 0.03	0.4^{a} ± 0.06
20:4 $n-6$	7.5^{a} ± 0.70	6.6^{ab} ± 0.62	6.4^{b} ± 0.87	8.3^{a} ± 0.55	7.6^{a} ± 0.62
20:5 $n-3$	0.1^{a} ± 0.02	0.3^{b} ± 0.02	0.5^{c} ± 0.04	Nd	0.2^{d} ± 0.02
22:4 $n-6$	0.4^{a} ± 0.05	0.3^{bc} ± 0.06	0.3^{b} ± 0.06	0.5^{a} ± 0.06	0.4^{ac} ± 0.07
22:5 $n-6$	0.3^{a} ± 0.03	0.1^{ac} ± 0.10	0.2^{ad} ± 0.04	0.7^{b} ± 0.06	0.5^{bcd} ± 0.04
22:5 $n-3$	0.5^{a} ± 0.10	0.6^{a} ± 0.05	0.6^{a} ± 0.06	0.3^{c} ± 0.04	0.4^{d} ± 0.06
22:6 $n-3$	6.5^{a} ± 0.25	6.9^{ac} ± 0.34	7.4^{c} ± 0.59	5.1^{b} ± 0.54	5.8^{d} ± 0.42
$n-3$	7.6^{a} ± 0.32	8.3^{b} ± 0.37	8.8^{b} ± 0.65	5.7^{c} ± 0.58	6.7^{d} ± 0.45
$n-6$	22.6^{a} ± 0.47	21.5^{b} ± 0.59	20.7^{c} ± 0.55	27.4^{d} ± 0.16	25.9^{e} ± 0.44
$n-6/n-3$	3.0^{a} ± 0.18	2.6^{b} ± 0.09	2.4^{b} ± 0.11	4.8^{c} ± 0.51	3.9^{d} ± 0.31
Triacylglycerols					
14:0	0.3 ± 0.03	0.3 ± 0.04	0.3 ± 0.03	0.3 ± 0.04	0.3 ± 0.03
16:0	20.1^{a} ± 0.47	19.8^{a} ± 0.40	19.6^{a} ± 0.76	21.1^{b} ± 0.75	21.0^{b} ± 0.40
16:1 $n-7$	1.5^{a} ± 0.16	1.4^{ab} ± 0.11	1.3^{b} ± 0.09	1.1^{c} ± 0.11	1.1^{c} ± 0.14
16:1	2.0^{ab} ± 0.12	2.2^{a} ± 0.03	2.2^{a} ± 0.15	1.7^{b} ± 0.23	2.0^{ab} ± 0.24
18:0	4.4^{a} ± 0.51	4.3^{a} ± 0.37	4.5^{a} ± 0.36	5.1 ± 0.24	5.5 ± 0.55
18:1 $n-9$	50.5^{a} ± 1.57	50.4^{a} ± 0.51	50.9^{a} ± 0.96	45.3^{b} ± 0.96	45.1^{b} ± 0.74
18:1 $n-7$	2.4^{a} ± 0.09	2.5^{b} ± 0.05	2.6^{b} ± 0.04	1.9^{c} ± 0.09	1.9^{c} ± 0.10
18:2 $n-6$	14.5^{a} ± 1.60	14.9^{a} ± 0.61	14.6^{a} ± 0.29	20.1^{b} ± 0.87	19.9^{b} ± 0.87
18:3 $n-3$	2.1^{a} ± 0.14	2.4^{b} ± 0.08	2.5^{b} ± 0.14	1.4^{c} ± 0.10	1.4^{c} ± 0.07
20:1 $n-9$	0.3 ± 0.03	0.3 ± 0.03	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.02
20:2 $n-6$	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.03	0.2 ± 0.01	0.1 ± 0.02
20:4 $n-6$	0.3^{a} ± 0.03	0.2^{a} ± 0.04	0.2^{a} ± 0.05	0.3^{b} ± 0.02	0.3^{b} ± 0.02
20:5 $n-3$	0.1^{a} ± 0.06	0.1^{a} ± 0.07	0.1^{a} ± 0.06	0.02^{b} ± 0.04	0.06^{ab} ± 0.07
22:6 $n-3$	0.3 ± 0.03	0.3 ± 0.09	0.3 ± 0.16	0.2 ± 0.04	0.2 ± 0.04
$n-3$	2.5^{a} ± 0.23	2.8^{ac} ± 0.22	3.0^{c} ± 0.31	1.6^{b} ± 0.14	1.7^{b} ± 0.06
$n-6$	15.1^{a} ± 1.64	15.4^{a} ± 0.69	15.0^{a} ± 0.42	20.8^{b} ± 0.85	20.6^{b} ± 0.86
$n-6/n-3$	6.0^{a} ± 0.40	5.6^{ab} ± 0.24	5.1^{b} ± 0.44	13.4^{c} ± 1.20	12.3^{d} ± 0.75

Results expressed as percentage of the total fatty acids. Data presented as mean values ± standard deviation (SD) of four samples (each treatment).
[a–d] Mean values in the same row with different letter differ significantly ($P < 0.05$).
n.d., Not detected.

one week of feeding. When 10% algae was included in the diet, the a^* value significantly increased ($P < 0.001$), whereas the L^* value was significantly lower ($P < 0.001$). When the amount of algae was increased to 20%, the a^* value further increased ($P = 0.001$) and the L^* value decreased even more ($P = 0.05$).

Adding algae to the diet significantly increased the content of total carotenoids in egg yolk ($P < 0.001$) (Table 5). When 20% algae was included in the diet, the content of total carotenoids increased significantly. Lutein/zeaxanthin and β-carotene increased dramatically and canthaxanthin increased from 0 to 7.7 mg/kg (Table 4).

3.3. Fatty acid composition

Table 5 shows composition of FA in PL and TAG of egg yolk. The LCPUFA were mostly found in the PL fraction of egg yolk. Yolk PL level of 18:2 $n-6$ differed significantly ($P < 0.001$) when RO/CO diets were compared with the RO diet, and RO diet with addition of 10 or 20% algae. PL of RO/CO yolk contained significantly more 20:4$n-6$ than eggs by RO + 10% algae ($P < 0.002$) and RO + 20% algae ($P < 0.001$) diets. DHA in PL was significantly higher ($P < 0.001$) in egg yolk from the RO diet, the RO + 10% algae and the RO + 20% algae than in the CO diet. Moreover, the RO + 20% algae diet significantly increased the proportion of DHA in PL compared with the RO diet ($P < 0.001$). EPA levels in PL differed among all diets ($P < 0.003$). Total proportion of $n-3$ fatty acids in yolk PL increased as the total proportion of $n-6$ FA decreased ($R^2 = 0.899$). Addition of algae significantly lowered ($P < 0.001$) the $n-6/n-3$ ratio in egg yolk PL. The ratio was 2.35 when feeding the RO + 20% algae diet, 2.92 when feeding only RO diet, 5.72 when feeding RO/CO and 3.91 when using the RO/CO + 10% algae feed (Table 5).

In the TAG fraction of egg yolk, the FAs 18:1$n-9$, 18:1$n-7$ and 18:3$n-3$ were present in significantly higher amounts and 18:2$n-6$ and 20:4$n-6$ were present in significantly lower amounts ($P < 0.001$) when RO diets (with or without algae) were compared to diet based on CO (Table 5).

4. Discussion

The microalga, *N. oculata*, is rich in the $n-3$ PUFA EPA and carotenoids. *N. oculata* has a high level of PL in contrast to fish oil, which has often been used to enrich poultry feed to produce $n-3$ PUFA-enriched eggs (Hammershoj, 1995). Microalgae provide naturally-encapsulated $n-3$ PUFA and antioxidants for feed supplementation, which protect it from oxidative deterioration (Herber-McNeill & Van Elswyk, 1998). *N. oculata* has been used as a supplement in hen diet as freeze–dried biomass (Nitsan, Mokady, & Sukenik, 1999).

Due to the unique fatty acid composition of *N. oculata*, high in EPA and ALA (the total amount of $n-3$ fatty acids is 44%), we could study the effect of desaturation ability of the hen towards DHA. Hens fed high amounts of EPA had low concentrations of EPA but increased levels of DHA in both the PL and TAG fractions of egg yolk. This indicates an elongation of EPA to DHA and probably also a catabolism of EPA in the liver, as suggested by Nitsan et al. (1999). In the RO and the CO feed in the present study, the longest fatty acid was ALA. The RO feed supplied the hen with more ALA than did the CO feed. More ALA in the feed gave an egg yolk with significantly more DHA, which indicates an elongation and desaturation of ALA towards DHA. Diets rich in $n-3$ PUFA resulted in a decreased ratio between $n-6$ and $n-3$ PUFA in yolk.

The relatively high amounts of the EPA and DHA obtained in this study, compared to results of others, may be a result of combined effects of age and strain, together with the dietary regime applied. The age and strain importance have been shown to have effects on FA composition, as suggested by Scheideler, Froning, and Jaroni (re. age, 1998) and Milinsk, Murakami, Gomes, Matsushita, and Souza (re. strain, 2003). Scheideler et al. (1998) suggested that an older hen has a larger liver and thereby can more effectively elongate ALA into DHA. The authors conclude that older hens produce larger yolks, caused by the larger liver size and its increased ability to absorb lipids. Milinsk et al. (2003) showed that strain of bird affects FA composition. On the other hand, Sim, Jaroni, and Froning (1995) reported that strain does not affect FA composition in the egg yolk. Nielsen (1998) showed that the percentage of AA and DHA is higher in eggs produced by young hens, suggesting an underlying rationale that they have smaller yolks and lower frequencies of laying and thereby compensatory amounts of LCPUFA.

Addition of LCPUFA, via algal enrichment in the feed, did not much affect the incorporation of $n-3$ LCPUFA into TAG. The $n-3$ LCPUFA were mainly identified in the PL fraction, which indicates a selective incorporation of $n-3$ LCPUFA into PL. Jiang, Ahn, and Sim (1991) concluded that LCPUFA, synthesized from ALA, are almost exclusively found in the PL, which is in accordance with our results. Similar results are detected when supplying hens with seal blubber oil in the diet. Schreiner, Hulan, Razzazi-Fazeli, Bohm, and Iben (2004) observed an increase of $n-3$ LCPUFA when the diet was supplied >1.25% seal blubber oil. This finding indicates a maximum incorporation capacity of $n-3$ LCPUFA in PL. It was also concluded that the incorporation of $n-3$ LCPUFA in TAG was less effective than that in PL (Schreiner et al., 2004). Our results are similar, even though $n-3$ LCPUFAs of *N. oculata* were ingested as PL. This indicates that the ingestion of $n-3$ LCPUFA, from TAG and PL origin, respectively, does not give a different result when it concerns incorporation in the yolk PL and TAG.

The yolk weights in our study tend to decrease with increasing amount of $n-3$ PUFA in the diet (Table 5). Addition of $n-3$ PUFA from fish oil has been shown to be associated with reduced egg weight (Elwinger & Inborr, 1999; Van Elswyk, 1997). Gonzalez-Esquerra and Leeson

(2000) suggest that the decrease in egg weight is due to the intake of $n-3$ PUFA, which are known to decrease circulating TAG and thereby lipids available for yolk formation. It has been found that hen diet rich in $n-3$ FA also decreases the serum cholesterol level of the hen (Basmacioglu et al., 2003). Whitehead, Bowman, and Griffin (1993) suggest that lower egg weight is a result of decreased oestrogen metabolism, caused by the dietary $n-3$ LCPUFA.

Colour changes were observed after including *N. oculata* in the diet. High amounts of several carotenoids, including canthaxanthin, were found in eggs from hens supplied with algae. Lutein/zeaxanthin were the dominant carotenoids in egg yolk in the RO and the RO/CO diets. These carotenoids originate from lucern meal, which was included in the RO and CO diet. Our results are in agreement with Herber-McNeill and Van Elswyk (1998) who reported that canthaxanthin efficiently deposits in egg yolk if added to poultry feeding regimes.

The food matrix in which the carotenoids are present affects their bioavailability. Carotenoids in egg yolk are associated with the matrix of the yolk lipids, which make them a highly bioavailable source of antioxidants, since lipid-soluble antioxidants, such as carotenoids, are taken up by diffusion of micelles and transported to the liver in blood (Handelman, 1999).

It is well known that many diseases, such as coronary disorders and metabolic syndrome, can be related to a diet with insufficient $n-3$ LCPUFA. There is a need to increase the intake of $n-3$ LCPUFA in the daily diet rather than as supplements (Simopoulos, 1999). A laying hen fed a diet rich in $n-3$ LCPUFA can produce eggs with a tailored fatty acid composition. In the present study, both the RO diet and the combination of RO diet and algae result in eggs with a high DHA content. From an economical point of view, the RO feed would be preferable for production of $n-3$-enriched eggs. Eggs, being a common ingredient in human food, are an excellent source of $n-3$ LCPUFA when enriched. Besides direct consumption, eggs have various applications; for instance, they are consumed as liquid mixtures, frozen and dried egg products for use in instant soups, cake mixtures and meat products. Also, dry egg yolk powder is frequently used in nutritional formulas, medical care and neonate foods.

5. Conclusions

Despite the high content of $n-3$ LCPUFA in the form of EPA in the algae supplement of the feed, no substantial increase of EPA was observed in yolk PL. Diets rich in $n-3$ PUFA, such as the RO diet and the RO diet with addition of marine algae, gave an egg yolk rich in $n-3$ PUFA. The algae carotenoids also contributed to the carotenoid content of egg yolk. RO diet and algae enhance the nutritional value of egg yolk in terms of antioxidants and fatty acids. Eggs with tailored nutritional composition might become a requested food and food ingredient in the future.

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