Research Article

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Effects of dietary marigold extract on lutein content, yolk color and fatty acid profile of omega-3 eggs

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Abstract

BACKGROUND: Lutein is a plant pigment very important for eye health in humans. Its bioavailability in humans is better from egg yolk than from dietary supplements. The aim of this research was to determine the influence of lutein (marigold extract powder) supplemented to laying hens' feed rich in omega-3 fatty acids on the content of lutein, fatty acid profile in egg yolks and yolk color. The diets that contained 5% of oils as a source of omega-3 fatty acids were supplemented with 0, 1 and 2 g kg^{-1} of marigold powder.

RESULTS: The best enrichment of eggs with lutein was achieved by supplementing 2 g marigold kg^{-1} of feed. Yolk color was significantly intensified ($P < 0.001$) by supplementing 1 g marigold kg^{-1} of feed. The content of total saturated fatty acids, monounsaturated fatty acids and n-6 polyunsaturated fatty acids (PUFA) remained unchanged, whereas the content of total n-3 PUFA ($P = 0.017$) and docosahexaenoic acid ($P < 0.001$) was higher in the group with 2 g marigold kg^{-1} of feed. This group also had the most favorable ratio of n-6:n-3 PUFA.

CONCLUSION: Results of this research showed that addition of marigold powder to laying hens' feed significantly increased egg lutein content and yolk color, and altered the fatty acid profile in yolk. Eggs with increased lutein content and a favorable profile of fatty acids are a good source of these ingredients in human nutrition.

Keywords: eggs; lutein; omega-3 fatty acids; yolk color

INTRODUCTION

Lutein is a plant pigment contained in green leafy vegetables, and it is often studied by scientists because of its protective effect on human eyes, skin, blood vessels and the nervous system. Research has confirmed that lutein is an antioxidant,[1] an efficient filter of high-energy blue light,[2] and a catcher of free radicals and singlet oxygen.[3] Besides zeaxanthin, lutein is the most common pigment in the eye tissue of humans, macula lutea and eye lens.[4] Lutein absorbs blue light selectively, as its peak absorption is in the spectrum at 446 nm. Blue light causes 100 times more damage compared to orange light, depending on the exposure duration. Because of its filtration capabilities, lutein is effective in preventing photoreceptor damage caused by blue light.[2] The protective influence of lutein is proved in slowing down of age-related macular degeneration (AMD). In developed countries, AMD is the leading cause of blindness in people over 65 years of age. Seddon *et al.*[5] were the first to prove a direct link between lutein intake and AMD risk reduction. It was found that individuals who took less lutein are at greater risk of developing AMD. Photoreceptor damage caused by high-energy blue light and free radicals present in the eye leads to the loss of central vision and visual acuity.[2] The research of Curran-Celentano *et al.*[6] showed a positive correlation between lutein level in serum or food and macular pigment density, suggesting that dietary sources of lutein can affect the lutein content in eye tissues or the protective role of lutein from food against AMD. Because of its antioxidant properties, lutein reduces the phototoxic damage of protein and DNA of the lens,[7] and it reduces lipid peroxidation in the eye lens of diabetics.[8] Moreover, there are also research into the role of lutein in maintaining cognitive function in adults,[9] in development of the nervous system in children,[10] in skin health protection[11] and in slowing down the development of atherosclerosis.[12] In the poultry industry, lutein has been mostly used as a coloring agent for egg yolk and poultry skin.

By defining specific feeding treatment of poultry, producers can influence the composition of eggs and the content of certain active ingredients in eggs. Addition of vegetable or fish oil

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can increase desirable n-3 polyunsaturated fatty acids (PUFA) in the egg yolk. Addition of various antioxidants, such as vitamin E, selenium and lutein, enhance the stability of fatty acids but also increase the content of those ingredients in the egg.[13] Supplementation of increased amounts of lutein to the feed affects the increase in lutein concentration in egg yolk. Although egg yolk is not the best nutritional source of lutein, its bioavailability in humans is better from egg yolk than from plant sources or from dietary supplements.[14] In egg yolk, lutein is present in a soluble lipid matrix, consisting of cholesterol, triacylglycerol and phospholipids, as well as some micronutrients, such as vitamins A, D and E.[15] It is believed that the content of cholesterol, as well as the composition of fatty acids in eggs, affect the improved utilization and increased lutein content in human serum.[14] Eggs enriched with omega-3 fatty acids and lutein are a foodstuff with high-quality nutritional ingredients that have positive effects on human health and prevention of certain diseases.

The aim of this research was to find out how lutein, when added to laying hens' feed rich in omega-3 fatty acids, influences the content of lutein and fatty acid profile in eggs.

MATERIAL AND METHODS

Animals and feeding mixtures

The research was carried out on a total of 300 31-week-old Tetra SL hybrid laying hens. Hens were randomly assigned to three experimental groups and each group was further divided into five replicates of 20 hens each (five hens per cage, four cages per replicate). Prior to starting the experiment with the addition of marigold extract to hens' mixtures, the hens were fed mixtures that contained 5% of oils as a source of omega-3 fatty acids (i.e. soybean oil 1.25%, rapeseed oil 2.00%, linseed oil 1.00% and fish oil 0.75%) for 4 weeks, to accustom them to the feed and to eliminate differences between groups at the beginning of the experiment with the addition of marigold extract to the mixtures. After the initial 4 weeks the marigold extract was added to mixtures as follows: O_0, without marigold extract; O_1, 1 g marigold extract kg^{-1} of feed; and O_2, 2 g marigold extract kg^{-1} of feed. The experimental period lasted for 5 weeks. Feed and water were offered for *ad libitum* consumption. Marigold extract (*Calendula officinalis* flowers) was purchased from Phyto Nutraceutical Inc. (Changsha, Hunan, China) and contained lutein (20%) as active ingredient. Accordingly, the calculated amount of lutein in mixtures for hens was 0, 200 and 400 mg kg^{-1}. The composition of the feeding mixtures is shown in the Table 1.

Table 1. Composition of the mixtures for laying hens

Ingredients (g kg^{-1})	O_0	O_1	O_2
Corn	463.4	462.4	461.4
Soybean meal	216.0	216.0	216.0
Toasted soybean	40.0	40.0	40.0
Sunflower meal	60.0	60.0	60.0
Alfalfa	25.0	25.0	25.0
Yeast	10.0	10.0	10.0
Limestone	103.4	103.4	103.4
Monocalcium phosphate	15.0	15.0	15.0
Salt	3.32	3.32	3.32
Sal CURB®[a]	3.32	3.32	3.32
Mineral Detox[b]	2.40	2.40	2.40
Probio P-500[c]	0.52	0.52	0.52
Lysoforte®[d]	0.32	0.32	0.32
Synthetic methionine	1.52	1.52	1.52
Marigold extract	0.0	1.0	2.0
Oil mixture[e]	50.0	50.0	50.0
Premix[f]	5.8	5.8	5.8
Total	1000	1000	1000
Crude protein (%)		18.00	
ME (MJ kg^{-1})		11.50	

[a] Antimicrobial (KEMIN®, Herentals, Belgium).
[b] Natural zeolite (Mineral Promet, Velika Gorica, Croatia).
[c] Probiotic (4b1841, *Enterococcus faecium* DSM 7134) 10×10^9 cfu (Lactosan, Kapfenberg, Austria).
[d] Absorption enhancer (KEMIN®, Herentals, Belgium).
[e] Oil mixture consisted of 2.00% rapeseed oil, 1.25% soybean oil, 1.00 flaxseed oil and 0.75% fish oil.
[f] Premix (Schaumann Agri, Brunn am Gebrige, Austria), content per kg diet: vitamin A 11.600 IU, vitamin D_3 2.900 IU, vitamin E 116 mg, vitamin K_3 2.32 mg, vitamin B_1 2.436 mg, vitamin B_2 5.22 mg, vitamin B_6 3.132 mg, vitamin B_{12} 13.34 mg, folic acid 0.986 mg, pantothenic acid 8.12 mg, niacin (nicotinic acid) 29 mg, biotin 98.6 µg, choline chloride 464 mg, vitamin C 26.1 mg, iodine 1.044 mg, manganese 81.2 mg, zinc 72.5 mg, iron 34.8 mg, copper 5.8 mg, organic selenium 0.464 mg, BHT 19.72 mg, propyl gallate 8.12 mg, canthaxanthin 3.48 mg, beta-apo-beta-carotenoic acid 1.16 mg.

Content of lutein in feeding mixtures and in egg yolks

Analysis of the lutein content in feed fed to laying hens was performed on six samples of each feeding mixture (on a total of 18 samples). Samples of analyzed feeding mixtures were prepared according to the method of Leeson *et al.*[16] Content of lutein in feeding mixtures is shown in the Table 2.

Table 2. Content of lutein in experimental mixtures ($\bar{x} \pm s$; $n = 6$ per group)

	Content of lutein in feeding mixtures (mg kg^{-1})	
Group	Supplemented	Analyzed
O_0	0	18.77 ± 2.29
O_1	200	191.33 ± 14.50
O_2	400	388.70 ± 25.87

$\bar{x}$, arithmetic mean; s, standard deviation; O_0, without marigold extract; O_1, 1 g marigold extract kg^{-1} of feed; O_2, 2 g marigold extract kg^{-1} of feed.

Content of lutein in egg yolks was analyzed on two occasions: first on eggs collected in the middle of the experiment (day 21 of research), and then at the end of the experiment (day 35 of research). On these two occasions, ten eggs were randomly selected from each group (a total of 60 eggs) and prepared for analysis of lutein content. Samples of egg yolks were prepared for the analysis according to the method of Leeson and Caston.[17] Prepared samples were analyzed on a high-performance liquid chromatographic system (Shimadzu Corporation, Kyoto, Japan) equipped with auto injector, UV–visible detector and Viva C18 column (5 µm, 250 × 4.6 mm; RESTEK Corporation, Bellefonte, PA, USA). The mobile phase consisted of a mixture of methanol and tetrahydrofuran (THF) (9:1, v/v). Mobile phase flow rate was 1 mL min^{-1}, the analysis lasted for 20 min and the wavelength of measurement was 450 nm. The volume of injected sample was 20 µL. A standard curve of lutein was prepared by using the lutein standard purchased from ChromaDex® (Irvine, CA, USA).

Table 3. Average content of fatty acids in mixtures fed to laying hens (g 100 g^{-1} of total fatty acids; $n = 6$; $\bar{x} \pm s$)

Fatty acid	Feed
Lauric acid C12:0	0.026 ± 0.008
Myristic acid C14:0	0.630 ± 0.031
Pentadecanoic acid C15:0	0.100 ± 0.009
Palmitic acid C16:0	11.601 ± 0.440
Heptadecanoic acid C17:0	0.140 ± 0.007
Stearinic acid C18:0	3.925 ± 0.156
Arachidic acid C20:0	0.384 ± 0.007
Behenic acid C22:0	0.233 ± 0.019
Tricosanoic acid C23:0	0.031 ± 0.006
Lignoceric acid C24:0	0.105 ± 0.011
SFA	**17.174 ± 0.607**
Palmitoleic acid C16:1	0.557 ± 0.025
Elaidic acid C18:1n9t	0.234 ± 0.036
Oleinic acid C18:1n9c	29.921 ± 0.419
Octadecenoic acid isomer C18:1 (cis)	1.750 ± 0.067
Octadecenoic acid isomer C18:1 (trans)	1.433 ± 0.133
Eicosenoic acid C20:1n9	0.464 ± 0.017
Erucic acid C22:1n9	0.031 ± 0.003
MUFA	**34.391 ± 0.393**
Linoleic acid C18:2n6	35.460 ± 0.290
γ-Linolenic acid C18:3n6	0.065 ± 0.011
Eicosadienoic acid C20:2	0.056 ± 0.004
Arachidonic acid C20:4n6	0.091 ± 0.007
Octadecadienoic acid isomer (A) C18:2	0.977 ± 0.126
Octadecadienoic acid isomer (B) C18:2	0.720 ± 0.086
Octadecadienoic acid isomer (C) C18:2	0.623 ± 0.063
Octadecadienoic acid isomer (D) C18:2	0.576 ± 0.054
n-6 PUFA	**38.658 ± 0.151**
α-Linolenic acid C18:3n3	7.966 ± 0.400
Eicosatrienoic acid C20:3n3	0.031 ± 0.003
Eicosapentaenoic acid C20:5n3	0.834 ± 0.072
Docosapentaenoic acid C22:5n3	0.186 ± 0.014
Docosahexaenoic acid C22:6n3	1.430 ± 0.119
n-3 PUFA	**10.447 ± 0.571**
n-6:n-3 PUFA	**3.700 ± 0.210**

$\bar{x}$, arithmetic mean; s, standard deviation.

Fatty acids in feeding mixtures and in egg yolks

On the last experimental day, six eggs were randomly collected from each group (a total of 18 eggs) and the yolks analyzed for fatty acid profile. The profile of fatty acids was also determined in samples of feeding mixtures and is presented in Table 3.

Analysis of samples was performed according to the method of Csapó *et al.*[18] Fatty acid methyl esters were separated in a WCOT (wall coated open tubular) column that contained CP-SIL 88 (FAME) stationary phase, and the content was determined using a flame ionization detector at 270 °C. The temperature of the split injector was 270 °C, and the carrier gas was helium with a pressure of 235 kPa. The temperature of the oven was set to 140 °C (10 min), increasing by 10 °C min^{-1} up to 235 °C (26 min). The analysis was performed using a gas chromatograph (CP 9000, Chrompack BV, Middleburg, Netherlands). Specific fatty acids contained in feed and in egg yolks are presented as g FA 100 g^{-1} total fatty acids and mg FA g^{-1}yolk, respectively.

Table 4. Content of lutein in egg yolks ($\bar{x}$; $n = 10$ per group)

Group	Time of analysis (days)	Lutein content (μg g^{-1} yolk)	Lutein content (mg per 60 g egg)[e]
O_0	21	20.11d	0,31d
	35	22.57d	0,35d
O_1	21	107.41bc	1,65bc
	35	103.74c	1,59c
O_2	21	118.86a	1,82a
	35	113.31ab	1,74ab
SEM		3.171	0,049
Sources of variation			
Treatment (T)		<0.001	
Time of analysis (TA)		0.388	
T x TA		0.422	

$\bar{x}$, arithmetic mean; SEM, standard error of the mean; a,b,c,d values within a column with different letter differ significantly at $P < 0.05$; O_0, without marigold extract; O_1,1 g marigold extract kg^{-1} of feed; O_2, 2 g marigold extract kg^{-1} of feed.
[e]Edible part.

Color of egg yolks

Egg yolk color was analyzed on fresh eggs (1 day after collection on the farm) and stored eggs (after 28 days of storage in a refrigerator at 4 °C). Each time, yolk color was measured with two different instruments: an egg multi-tester EMT-5200 device (Robotmation Co. Ltd, Japan) ($n = 30$ eggs per group) and a CR-300 chroma meter (Konica Minolta, Osaka, Japan) ($n = 10$ eggs per group). The color of samples measured by the egg multi-tester is represented by numbers 1–15 and with the chroma meter by three values: CIE L^*, a^* and b^*.[19] The value L^* refers to the degree of lightness, from dark to light (0–100). The value a^* represents redness and indicates degree of red–green color, in which a higher positive value refers to a redder color. The value b^* (yellowness) refers to the degree of yellow–blue color, in which a higher positive value refers to a yellower color. The white calibration plate with specifications $Y = 93.0$, $x = 0.3159$ and $y = 0.3324$ was used for device calibration. The optical lens diameter was 8 mm, illumination D65 and standard observation 10°.

Statistical analysis

Research results were processed using Statistica for Windows, v.13.0.[20] Statistical parameters were arithmetic mean ($\bar{x}$), standard error of the mean (SEM) and standard deviation (s). Testing of significance of differences within a group and between groups was done by using the GLM procedure of one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA, 3 × 2). The calculated F-value was compared to the critical theoretical F-value at a significance level of 5%. Significance of differences between mean values was determined by Fisher's LSD test.

RESULTS

Lutein content in egg yolks

Supplementation of marigold extract to laying hens' feed had a significant influence ($P < 0.001$) on the content of lutein in egg yolks, achieving the highest values in group O_2. The time of analysis did not affect the content of lutein in egg yolks ($P > 0.5$). The results of lutein content in egg yolks are shown in Table 4.

Results of analysis performed on day 21 of the experiment proved that the lutein content in egg yolks increased from 20.11 to

Table 5. Profile of fatty acids in egg yolks (mg FA g^{-1} yolk; $\bar{x} \pm s$; $n = 6$ per group)

Fatty acid	O_0	O_1	O_2	*P*-value
Myristic C14:0	0.610 ± 0.04	0.690 ± 0.02	0.610 ± 0.03	0.142
Pentadecanoic C15:0	0.211 ± 0.00	0.210 ± 0.02	0.187 ± 0.01	0.195
Palmitic C16:0	60.565 ± 1.35	64.996 ± 2.90	61.131 ± 1.60	0.275
Heptadecanoic C17:0	0.529 ± 0.03	0.534 ± 0.03	0.529 ± 0.02	0.988
Stearic C18:0	17.306 ± 0.68	17.592 ± 0.32	18.402 ± 0.55	0.345
Arachidic C20:0	0.048 ± 0.00	0.049 ± 0.00	0.048 ± 0.00	0.162
Heneicosanoic C21:0	0.024 ± 0.00	0.024 ± 0.00	0.024 ± 0.00	0.191
Behenic C22:0	0.024 ± 0.00	0.024 ± 0.00	0.024 ± 0.00	0.191
Tricosanoic C23:0	0.024 ± 0.00	0.024 ± 0.00	0.024 ± 0.00	0.194
Lignoceric C24:0	0.024 ± 0.00	0.024 ± 0.00	0.024 ± 0.00	0.205
SFA	**79.367 ± 1.05**	**84.169 ± 2.70**	**81.005 ± 1.41**	0.203
Myristoleinic C14:1	0.091 ± 0.01	0.100 ± 0.00	0.100 ± 0.01	0.754
Palmitoleic C16:1	5.665 ± 0.52	5.887 ± 0.28	5.516 ± 0.38	0.804
Elaidic C18:1n9t	0.371 ± 0.01	0.333 ± 0.02	0.405 ± 0.04	0.159
Oleinic C18:1n9c	91.632 ± 0.74	89.688 ± 2.96	91.333 ± 1.27 3.549 ± 0.16	0.737
Octadecenoic isomer C18:1 (*cis*)	3.481 ± 0.10	2.904 ± 0.28		0.057
Octadecenoic isomer C18:1 (*trans*)	0.661 ± 0.04	0.685 ± 0.06	0.993 ± 0.24	0.221
Eicosenoic C20:1n9	0.353 ± 0.01	0.320 ± 0.03	0.368 ± 0.01	0.191
MUFA	**102.254 ± 0.87**	**99.917 ± 3.36**	**102.265 ± 1.13**	0.661
Linoleic C18:2n6	47.105 ± 1.09	44.944 ± 1.36	43.881 ± 0.89	0.148
Octadecadienoic isomer (A) C18:2	0.154 ± 0.01	0.140 ± 0.01	0.241 ± 0.05	0.058
Octadecadienoic isomer (B) C18:2	0.154b ± 0.01	0.130b ± 0.01	0.284a ± 0.05	0.006
Octadecadienoic isomer (C) C18:2	0.087b ± 0.01	0.082b ± 0.01	0.207a ± 0.03	<0.001
Octadecadienoic isomer (D) C18:2	0.120 ± 0.03	0.063 ± 0.01	0.125 ± 0.02	0.067
γ-Linolenic C18:3n6	0.231 ± 0.02	0.227 ± 0.02	0.207 ± 0.01	0.542
c9,t11-CLA	0.044 ± 0.00	0.044 ± 0.01	0.044 ± 0.00	0.999
Eicosadienoic C20:2	0.281 ± 0.01	0.247 ± 0.02	0.271 ± 0.01	0.143
Eicosatrienoic C20:3n6	0.363 ± 0.01	0.339 ± 0.02	0.421 ± 0.04	0.092
Arachidonic C20:4n6	2.269 ± 0.18	2.035 ± 0.03	2.197 ± 0.05	0.334
Docosatetraenoic C22:4n6	0.175 ± 0.03	0.175 ± 0.01	0.189 ± 0.01	0.832
n-6 PUFA	**50.983 ± 1.33**	**48.425 ± 1.44**	**48.068 ± 1.05**	0.236
α-Linolenic C18:3n3	4.878 ± 0.31	4.887 ± 0.36	4.937 ± 0.27	0.990
Eicosatrienoic C20:3n3	0.053ab ± 0.00	0.044b ± 0.01	0.068a ± 0.00	0.020
Eicosapentaenoic C20:5n3	0.145 ± 0.01	0.155 ± 0.01	0.155 ± 0.01	0.680
Docosapentaenoic C22:5n3	0.252b ± 0.02	0.355ab ± 0.05	0.466a ± 0.05	0.005
Docosahexaenoic C22:6n3	2.933b ± 0.06	2.914b ± 0.08	3.904a ± 0.11	<0.001
n-3 PUFA	**8.262b ± 0.28**	**8.355b ± 0.36**	**9.529a ± 0.28**	0.017
n-6/n-3 PUFA	**6.17a ± 0.76**	**5.80ab ± 0.75**	**5.04b ± 0.42**	0.025

$\bar{x}$, arithmetic mean; *s*, standard deviation; a,b values within a row with different letter differ significantly at $P < 0.05$; O_0, without marigold extract; O_1, 1 g marigold extract kg^{-1} of feed; O_2, 2 g marigold extract kg^{-1} of feed.

107.41 μg g^{-1} in group O_1, and to 118.86 μg g^{-1} in group O_2, which was the highest recorded value and significantly ($P < 0.001$) different from the value of the O_1 group. Results of analysis performed on day 35 of the experiment showed a slightly lower content of lutein in egg yolks than on day 21, but the differences were not statistically significant.

Fatty acids in egg yolks

The profile of fatty acids in egg yolks is presented in the Table 5. Supplementation of marigold extract to the feeding mixtures did not influence the content of individual or total saturated (SFA) or monounsaturated (MUFA) fatty acids. There was slightly higher content of total SFA and slightly lower content of total MUFA in the O_1 group than in other two groups, but the differences were not statistically significant. Total n-6 PUFA exhibited a decreasing trend with increase in marigold extract content supplemented to the feed: 21.17% > 20.10% > 19.96%; but there was no statistically significant difference. In n-3 PUFA, marigold extract had a significant influence on the content of total n-3 PUFA ($P = 0.017$), and on the content of docosapentaenoic acid (DPA) (22:5n3) ($P = 0.005$) and docosahexaenoic acid (DHA) fatty acid (22:6n3) ($P < 0.001$).

Content of the stated fatty acids in the experimental groups was higher than in the control, due to which the content of total n-3 PUFA was also higher in the experimental groups. Therefore, the ratio of n-6:n-3 PUFA was more favorable in groups fed mixtures supplemented with marigold extract ($P = 0.025$). The narrowest and the most favorable ratio of 5.04 was determined in group O_2,

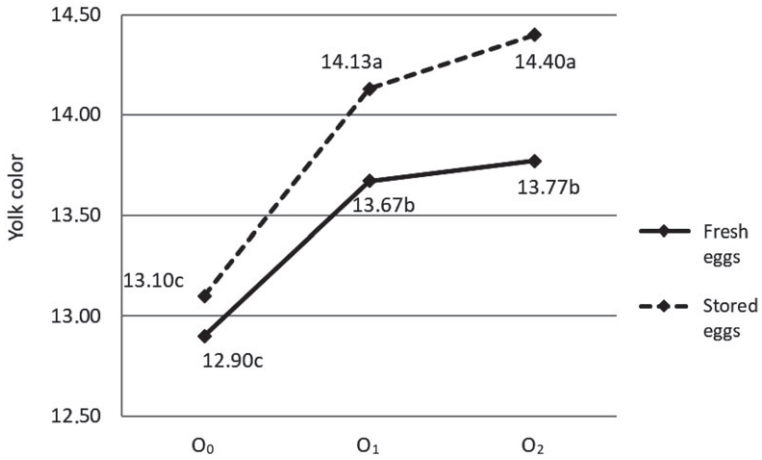

Figure 1. Color of egg yolks of experimental groups. O_0, without marigold extract; O_1, 1 g marigold extract kg^{-1} of feed; O_2, 2 g marigold extract kg^{-1} of feed; each group consisted of 100 31-week-old Tetra SL hybrid laying hens which were fed experimental diets for 5 weeks; $n = 30$ eggs per group; eggs were analyzed as fresh (1 day after collection on the farm) and stored (after 28 days of storage in a refrigerator at 4 °C); a,b,c values with different superscripts differ significantly at $P < 0.05$.

followed by 5.80 in group O_1, and then by a ratio of 6.17 in the control group.

Color of egg yolks

Dietary supplementation of marigold extract and storage duration of eggs had a statistically significant influence ($P < 0.001$) on the egg yolk color. Results of the determination of egg yolk color are presented on the Fig. 1. The highest value of egg yolk color was recorded in the stored eggs of the O_2 group, being 14.40. This group also exhibited the highest value of color for fresh eggs, which was 13.77. These values were expected, since this group had been fed a diet supplemented with 2 g kg^{-1} marigold extract. The value of egg yolk color of the control group with no supplemented marigold extract was 12.90 for fresh and 13.10 for stored eggs. When compared to the O_0 group, supplementation of 1 g kg^{-1} marigold extract to the group O_1 caused a significant increase in color values, both of fresh and stored eggs. Although there was a further increase in color intensity with the addition of 2 g kg^{-1} marigold extract, there was no significant difference determined with respect to the O_1 group. The storage duration did not affect the value of egg yolk color of the control group, while experimental groups had significantly higher values of egg yolk color of stored eggs than of fresh eggs.

Yolk color measured by the CR-300 chroma meter and expressed by the CIE *Lab* values indicated the significant ($P < 0.001$) influence of marigold extract and of storage duration only for the a^* value (degree of redness) (Table 6). Supplementation of marigold extract influenced the increase of value from 10.74 in the control to 15.94 in the O_2 group, as did the storage duration in all experimental groups. Fresh eggs did not exhibit differences between O_0 and O_1 groups, but a significant difference was determined in the O_2 group when compared to the other two groups. Eggs stored for 28 days in a refrigerator exhibited a significant difference with supplementation of 1 g kg^{-1} marigold extract, while there was no difference between O_1 and O_2 group. Supplementation of marigold extract and storage duration did not influence the L^* (degree of lightness) and b^* (degree of yellowness) values, but it was still noted that lightness and yellowness of yolk were decreased by an increase in the amount of supplemented marigold extract. Storage duration led to a decrease in the L^* value and a decrease in the b^* value of egg yolk.

Table 6. Color of egg yolks depending on the supplemented marigold extract and storage duration ($\bar{x}$; $n = 10$ per treatment)

Treatment	Storage duration (days)	Yolk color CIE		
		L^*	a^*	b^*
O_0	1	61.24	10.74e	55.54
	28	60.57	12.79cd	57.51
O_1	1	60.00	11.72de	53.21
	28	59.41	15.18ab	55.34
O_2	1	59.70	14.14bc	53.86
	28	59.18	15.94a	55.63
SEM		0.734	0.527	1.359
Sources of variation				
Treatment (T)		0.113	<0.001	0.229
Storage duration (SD)		0.329	<0.001	0.083
T x SD		0.995	0.244	0.991

$\bar{x}$, arithmetic mean; SEM, standard error of the mean; a,b,c,d,e values within a column with different letter differ significantly at $P < 0.05$; O_0, without marigold extract; O_1, 1 g marigold extract kg^{-1} of feed; O_2, 2 g marigold extract kg^{-1} of feed.

DISCUSSION

Lutein content in egg yolks

The best results after enriching egg yolks were obtained with supplementation of 2 g kg^{-1} marigold extract after 21 days of feeding. In their research, Leeson and Caston[17] supplemented corn- and soybean-based feeding mixture with 8% ground linseed as a source of omega-3 fatty acids, and 0, 125, 250 and 500 mg kg^{-1} lutein. Control mixture was based on corn and soybean, without supplements. When compared to the control group, analysis of results showed worse deposition of lutein in eggs laid by hens that were fed mixtures supplemented with linseed. Supplementation of higher amounts of lutein to feed influenced the increase in lutein content in egg yolks from 0.24 mg per 60 g egg to 1.40 mg per 60 g egg (by supplementing 250 mg kg^{-1} lutein). These results are comparable with those in our research, but in our research content of lutein in the control group was slightly

higher (0.33 mg per 60 g egg), and by adding 1 g kg^{-1} marigold extract to feed (200 mg kg^{-1} lutein) average content of lutein increased to 1.62 mg per 60 g egg, which is 15.7% higher than the results of the mentioned authors, when adding 250 mg kg^{-1} lutein. Moreover, Leeson *et al.*[16] supplemented laying hens' feed with 10% ground linseed and 0, 125 and 250 mg kg^{-1} lutein. Content of lutein in egg yolks of the control group was 0.11 mg per 60 g egg, and in groups fed 125 and 250 mg kg^{-1} lutein it was 1.39 and 1.73 mg per 60 g egg, respectively. Higher content of lutein in egg yolks was achieved with a lower amount of supplemented lutein (250 mg kg^{-1}) than in our O_2 group, where the supplemented lutein at an amount of 400 mg kg^{-1} achieved on average 1.78 mg per 60 g egg of egg. Nain[21] fed laying hens a mixture with 10% linseed and 500 mg kg^{-1} lutein and monitored the increase in lutein content in egg yolks over 56 days of treatment. When comparing the results with the group that was fed a mixture with only linseed (10%), the best enrichment of egg yolk was achieved after 28 days of feeding treatment, as the lutein content in the experimental group was 34.59 μg g^{-1} of egg yolk, and 8.64 μg g^{-1} of egg yolk in the group fed with linseed supplement only. At the beginning of the research, content of lutein in the experimental group was 9.38 μg g^{-1} of egg yolk, and in the group with linseed it was 9.70 μg g^{-1} of egg yolk. There was a confirmed trend of egg yolk enrichment as in our research, although the values are far lower than ours. In our research lower lutein values were found on day 35 of analysis and similar results were reported by Islam *et al.*[22] and Nain,[21] in whose research the content of lutein in egg yolks was lower on the 21st day than on the 14th day, i.e. on the 56th than on the 28th day of experiments, respectively. However, those differences were also statistically not significant. A limitation of this study is the fact that we do not have data on the content of lutein in eggs from all three treatments on day 0, but since hens were fed mixtures of the same composition 4 weeks before addition of marigold extract to mixtures, and because there were no differences in lutein content in eggs of the control group on the 21st and 35th days of the experiment, we consider that the differences did not exist even on day 0 of the research.

Fatty acids in egg yolk

Many authors have investigated the influence of dietary supplementation of various vegetable seeds and oils, and oils of marine organisms, on the profile of fatty acids in egg yolk. Sultan *et al.*[23] determined that the addition of 10% flaxseed in feed for laying hens caused a significant ($P < 0.05$) reduction in content of total SFA, MUFA and n-6:n-3 PUFA and an increase ($P < 0.05$) in content of n-3 PUFA and total PUFA. Inclusion of 3.4% of linseed oil in diets of laying hens has a similar effect on FA content like flaxseed, causing decrease in content of total SFA, MUFA and *trans*-FA ($P < 0.05$) while simultaneously increasing n-3 PUFA and total PUFA ($P < 0.05$).[24] Enrichment of eggs with n-3 PUFA using flaxseed or linseed oil in hens' diet is mainly due to an increase in content of ALA (C18:3n-3). In order to increase the content of long-chain PUFAs (eicosapentaenoic acid (EPA; C20:5n-3) and DHA (C22:6n-3)) in eggs, it is necessary to include sources of those fatty acids, such as fish oil, to laying hens' diet. In the study of Ceylan *et al.*[25] it was found that eggs from hens fed with the addition of fish oil to the diet contained significantly higher content of DHA than the eggs of hens that had received sunflower, linseed or rapeseed oil in the mixtures. Supplementation of various combinations of fish, linseed, rapeseed and soybean oils[26–29] affects the increase in content of total and individual n-3 PUFA, by simultaneous lowering n-6 PUFA, thus resulting in a more favorable, narrower ratio of n-6:n-3 fatty acids. However, there are not many studies that have explored the influence of dietary supplementation with a combination of four oils as used in our research. Moreover, the available literature offers not many data on the influence of lutein as dietary supplement on the profile of fatty acids in egg yolks enriched with omega-3 fatty acids. Nain[21] studied the effects of supplementation of 10% linseed and 500 mg kg^{-1} lutein to laying hens' feed on the profile of fatty acids in egg yolks. The control group was fed with a mixture containing 10% linseed. The author did not determine any major differences between the two groups. Thus the content of SFA, total PUFA and n-6 PUFA was slightly higher in the group fed with supplemented lutein, but the differences were not statistically significant. Content of MUFA and n-3 PUFA was similar in both groups. The ratio of n-6:n-3 increased slightly in the group with lutein supplementation. Except for the content of SFA and MUFA, which was also similar in our research, other results do not correspond to those published by that author. In our research, the content of n-6 PUFA decreased with the addition of marigold extract, although it was not significant ($P = 0.236$), while the content of n-3 PUFA was significantly higher in experimental groups than in the control ($P = 0.017$). These changes caused the ratio of n-6:n-3 to improve from 6.17 in the control group to 5.04 in the group that had 2 g kg^{-1} marigold extract ($P = 0.025$). Higher content of total n-3 PUFA, and especially DHA, in the group with 2 g kg^{-1} marigold extract (O_2) could be explained by the protective action of lutein. Lutein is an effective antioxidant because of its ability to quench singlet oxygen and scavenge peroxy radicals.[30] Surai *et al.*[31] have shown that lutein in combination with vitamin E, in the presence of a high content of the highly unsaturated DHA in designer eggs, significantly decreased malondialdehyde formation as a result of Fe-stimulated lipid peroxidation. The findings of Mohn *et al.*[32] suggest that lutein may preserve DHA concentrations and may contribute to inhibiting its oxidation in certain brain regions of rhesus monkeys, which were supplemented with lutein and zeaxhantin, by reducing oxidative stress. Based on these results we suppose that an increased level of lutein in yolks of eggs from the O_2 experimental group had an influence on the preservation of DHA in egg yolk lipids, while in the control group, due a to lower level of lutein, oxidative processes were more pronounced, which affected DHA damage, and therefore its level in the control group was lower. We assume that lutein actually 'keeps' DHA from oxidation and thus maintains higher levels of DHA in the experimental group. Further studies are required to determine the combined effect that different oils and lutein in laying hens' dietary supplements have on the fatty acid profile in egg yolks.

Color of egg yolks

Along with the egg freshness and eggshell quality, color of egg yolk is one of the indicators of egg quality that is very important to consumers. Although yolk color preferences varies among countries and regions of the world, European consumers usually prefer a more intense egg yolk color.[33] Because of this, different pigments, often synthetic, are added to the hens' mixtures in order to increase the intensity of egg yolk color. More recently, however, the tendency has been to add natural pigments, such as lutein from plant sources. Leeson and Caston[17] supplemented lutein to hens' feed in amounts from 0 to 1000 mg kg^{-1} and studied its influence on the egg yolk color. They determined a significant increase in yolk color value after only 7 days of feeding from 6/7 to 12/13.

Color of egg yolk was stabilized at 13/14, and supplementation of lutein in portions higher than 250 mg kg^{-1} did not affect further increase in color values. In their research, Leeson *et al.*[16] used a combination of lutein and 10% linseed as a supplement to laying hens' feed, and achieved enrichment of eggs with omega-3 fatty acids and lutein. The authors determined that supplementation of 125 mg kg^{-1} lutein effected a significant ($P < 0.001$) increase in egg yolk color, from 6.7 as of the control, to 13.7. Further lutein supplementation of 250 mg kg^{-1} resulted in just a slight increase in egg yolk color to 13.9. Englmaierová *et al.*[34] reported an increase in egg yolk color from 6.4 to 13.1 ($P < 0,001$) by supplementation of 250 mg kg^{-1} lutein to laying hens' feed, while Englmaierová and Skřivan[35] supplemented 100 mg kg^{-1} lutein to hens' feed and achieved an increase in egg yolk color from 7.7 in the control to 8.4 ($P < 0.001$). Jang *et al.*[36] confirmed that supplementation of even small amounts of lutein (40 mg kg^{-1}) affected egg yolk color ($P < 0.05$). Our research confirmed the positive influence of dietary supplementation of lutein on the intensity of egg yolk color, so our results are in accordance with the results of the cited authors. The difference occurs in the egg yolk color of the control group, which is more intensive than the egg yolk colors reported by the authors mentioned. Results obtained in our research showed a significantly ($P < 0.001$) higher intensity of yolk color of eggs stored in a refrigerator for 28 days than of fresh eggs in all three groups. The difference between two occasions of measurement was not significant in the control group, but the significance of differences was determined for the O_1 and O_2 groups. There are not many data in the available literature on the changes in color intensity in eggs enriched with omega-3 fatty acids and lutein that occur during storage. Therefore, a comparison is made of the results of research performed by Kralik *et al.*[37] referring to changes in color of egg yolks of omega-3-enriched eggs during storage. In that research, storage of eggs for a period of 14 days in a refrigerator affected the reduction of egg yolk color from 13.24 to 12.76 in the group that consumed 3.5% fish and 1.5% rapeseed oils, and from 13.24 to 13.00 in the group that consumed 1.5% fish and 3.5% rapeseed oils, but those differences were not significant ($P > 0.05$). Barbosa *et al.*[38] did not determine an influence of storage duration on changes in yolk color of eggs enriched with omega-3 fatty acids stored in a refrigerator for 35 days, whereas eggs stored at room temperature exhibited a significant ($P < 0.05$) decrease in color value from 11.50 to 10.38.

Studies on the effects of lutein on the CIE *Lab* color indicators, as available in the literature, were performed mostly on conventionally produced fresh eggs. Lokaewmanee *et al.*[39] supplemented various amounts of lutein (10–40 mg kg^{-1}) to hens' feed and confirmed a significant increase ($P < 0.05$) in a^* color value, as in our research. They confirmed the decrease in L^* value and increase in b^* value, but the differences were not statistically significant. Sirri *et al.*[40] reported that the increased amount of lutein supplemented to feeding mixtures (80–160 mg kg^{-1}) significantly ($P < 0.001$) influenced the increase in redness degree (a^*), whereas values of lightness and yellowness were not significantly different between experimental groups, although L^* and b^* values decreased, which is in accordance with our results. Englmaierová *et al.*[34] also conducted research into the effects of lutein supplemented in an amount of 250 mg kg^{-1} of hens' feed on the CIE *Lab* indicators of yolk color. Unlike the results of our research, their results showed a significant influence ($P < 0.001$) of lutein supplementation on all color indicators. Dietary supplementation of lutein effected a decrease in L^* value and an increase in a^* and b^* values of yolk color.

CONCLUSIONS

Based on the research results, we can conclude that dietary marigold extract is very effective in the enriching of eggs with lutein as well as increasing egg yolk color intensity. It can also be noted that it has an effect on increasing the content of the desired n-3 PUFA and reducing the ratio of n-6:n-3 PUFA. Further studies are needed to determine the influence of different sources and concentrations of lutein on fatty acid composition in egg yolks. Eggs simultaneously enriched with lutein and omega-3 fatty acids are a good source of these ingredients in human nutrition.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Sindhu ER, Preethi KC and Kuttan R, Antioxidant activity of carotenoid lutein in vitro and in vivo. *Indian J Exp Biol* **48**:843–848 (2010).
2 Nilsson SEG, Sundelin SP, Wihlmark U and Brunk UT, Aging of cultured retinal pigment epithelial cells: oxidative reactions, lipofuscin formation and blue light damage. *Doc Ophthalmol* **106**:13–16 (2003).
3 Landrum J, Bone R, Mendez V, Valenciaga A and Babino D, Comparison of dietary supplementation with lutein diacetate and lutein: a pilot study of the effects on serum and macular pigment. *Acta Biochim Pol* **59**:167–169 (2012).
4 Gao S, Qin T, Liu Z, Caceres MA, Ronchi CF, C-YO C *et al.*, Lutein and zeaxanthin supplementation reduces H_2O_2-induced oxidative damage in human lens epithelial cells. *Mol Vis* **17**:3180–3190 (2011).
5 Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC *et al.*, Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. *JAMA* **272**:1413–1420 (1994).
6 Curran-Celentano J, Hammond JBR, Ciulla TA, Cooper DA, Pratt LM and Danis RB, Relation between dietary intake, serum concentrations, and retinal concentrations of lutein and zeaxanthin in adults in a Midwest population. *Am J Clin Nutr* **74**:796–802 (2001).
7 Roberts JE, Wielgus AR, Boyes WK, Andley U and Chignell CF, Phototoxicity and cytotoxicity of fullerol in human lens epithelial cells. *Toxicol Appl Pharmacol* **228**:49–58 (2008).
8 Barker F, Dietary supplementation: effects on visual performance and occurrence of AMD and cataracts. *Curr Med Res Opin* **26**:2011–2023 (2010).
9 Johnson EJ, Vishwanathan R, Johnson MA, Hausman DB, Davey A, Scott TM *et al.*, Relationship between serum and brain carotenoids, α-tocopherol, and retinol concentrations and cognitive performance in the oldest old from the Georgia centenarian study. *J Aging Res* **2013**:13 (2013).
10 Vishwanathan R, Kuchan MJ, Sen S and Johnson EJ, Lutein and preterm infants with decreased concentrations of brain carotenoids. *J Pediatr Gastroenterol Nutr* **59**:659–665 (2014).
11 Palombo P, Fabrizi G, Ruocco V, Ruocco E, Fluhr J, Roberts R *et al.*, Beneficial long-term effects of combined oral/topical antioxidant treatment with the carotenoids lutein and zeaxanthin on human skin: a double-blind, placebo-controlled study. *Skin Pharmacol Physiol* **20**:199–210 (2007).
12 Wang M-X, Jiao J-H, Li Z-Y, Liu R-R, Shi Q and Ma L, Lutein supplementation reduces plasma lipid peroxidation and C-reactive protein in healthy nonsmokers. *Atherosclerosis* **227**:380–385 (2013).
13 Grčević M, Kralik Z, Kralik G, Galović D and Pavić M, The effect of lutein additives on biochemical parameters in blood of laying hens. *Poljoprivreda/Agriculture* **22**:34–38 (2016).
14 Chung H-Y, Rasmussen HM and Johnson EJ, Lutein bioavailability is higher from lutein-enriched eggs than from supplements and spinach in men. *J Nutr* **134**:1887–1893 (2004).

15 Golzar Adabi SH, Kamali MA, Davoudi J, Cooper RG and Hajbabaei A, Quantification of lutein in egg following feeding hens with a lutein supplement and quantification of lutein in human plasma after consumption of lutein enriched eggs. *Arch Gefluegelk* **74**:158–163 (2010).
16 Leeson S, Caston L and Namkung H, Effect of dietary lutein and flax on performance , egg composition and liver status of laying hens. *Can J Anim Sci* **87**:365–372 (2007).
17 Leeson S and Caston L, Enrichment of eggs with lutein. *Poult Sci* **83**:1709–1712 (2004).
18 Csapó J, Sugar L, Horn A and Csapo-Kiss Z, Chemical composition of milk from red deer, roe and fallow deer kept in captivity. *Acta Agron Hung* **36**:359–372 (1987).
19 CIE, Colorimetry – Part 4: CIE 1976 L*a*b* colour space. [Online]. Available: http://www.cie.co.at/publications/colorimetry-part-4-cie-1976-lab-colour-space [8 November 2018].
20 TIBCO Software Inc. (2018). Statistica (data analysis software system), version 13. http://tibco.com [2 July 2018].
21 Nain S, Improving the effectiveness of laying hens for use in value-added egg production. MSc thesis, Department of Agricultural, Food and Nutritional Science, University of Alberta (2011).
22 Islam KMS, Khalil M, Männer K, Raila J, Rawel H, Zentek J *et al.*, Lutein specific relationships among some spectrophotometric and colorimetric parameters of chicken egg yolk. *J Poult Sci* **54**:271–277 (2017).
23 Sultan A, Obaid H, Khan S, Ur Rehman I, Shah MK and Khan RU, Nutritional effect of flaxseeds on cholesterol profile and fatty acid composition in egg yolk. *Cereal Chem* **92**:50–53 (2015).
24 Oliveira DD, Baião NC, Cançado SV, Grimaldi R, Souza MR, Lara LJC *et al.*, Effects of lipid sources in the diet of laying hens on the fatty acid profiles of egg yolks. *Poult Sci* **89**:2484–2490 (2010).
25 Ceylan N, Ciftçi I, Mızrak C, Kahraman Z and Efil H, Influence of different dietary oil sources on performance and fatty acid profile of egg yolk in laying hens. *J Anim Feed Sci* **20**:71–83 (2011).
26 Farrell DJ, Enrichment of hen eggs with n-3 long-chain fatty acids and evaluation of enriched eggs in humans. *Am J Clin Nutr* **68**:538–544 (1998).
27 Škrtić Z, Kralik G, Gajčević Z, Bogut I and Hanžek D, The increase of the n-3 PUFA content in eggs. *Poljoprivreda/Agriculture* **13**:47–52 (2007).
28 Kralik G, Škrtić Z, Suchý P, Straková E and Gajčević Z, Feeding fish oil and linseed oil to laying hens to increase the n-3 PUFA of egg yolk. *Acta Vet Brno* **77**:561–568 (2008).
29 Škrtić Z, Kralik G, Gajčević Z, Hanžek D and Bogut I, Effect of different source of oils on fatty acid profile and organoleptic traits of eggs. *Acta Agric Slov Supplement* **2**:129–134 (2008).
30 Wang M, Tsao R, Zhang S, Dong Z, Yang R, Gong J *et al.*, Antioxidant activity, mutagenicity/anti-mutagenicity, and clastogenicity/anti-clastogenicity of lutein from marigold flowers. *Food Chem Toxicol* **44**:1522–1529 (2006).
31 Surai PF, MacPherson A, Speake BK and Sparks NHC, Designer egg evaluation in a controlled trial. *Eur J Clin Nutr* **54**:298–305 (2000).
32 Mohn ES, Erdman JW, Kuchan MJ, Neuringer M and Johnson EJ, Lutein accumulates in subcellular membranes of brain regions in adult rhesus macaques: relationship to DHA oxidation products. *PLoS ONE* **12**:1–18 (2017).
33 Englmaierová M, Bubancová I and Skřivan M, Carotenoids and egg quality. *Acta Fytotech Zootech* **17**:55–57 (2014).
34 Englmaierová M, Skřivan M and Bubancová I, A comparison of lutein, spray-dried chlorella, and synthetic carotenoids effects on yolk colour, oxidative stability, and reproductive performance of laying hens. *Czech J Anim Sci* **58**:412–419 (2013).
35 Englmaierová M and Skřivan M, Effect of synthetic carotenoids, lutein, and mustard on the performance and egg quality. *Sci Agric Bohem* **44**:138–143 (2013).
36 Jang I, Ko Y, Kang S, Kim S, Song M, Cho K *et al.*, Effects of dietary lutein sources on lutein-enriched egg production and hepatic antioxidant system in laying hens. *J Poult Sci* **51**:58–65 (2014).
37 Kralik G, Škrtić Z, Gajčević Z and Hanžek D, Utjecaj različitih ulja u hrani za nesilice na kakvoću jaja i sadržaj masnih kiselina u žumanjku jajeta [Effect of different oils supplemented to laying hens' diets on the quality of eggs and the fatty acids content in egg yolk]. *Krmiva* **49**:115–125 (2007).
38 Barbosa VC, Gaspar A, Calixto LFL and Agostinho TSP, Stability of the pigmentation of egg yolks enriched with omega-3 and carophyll stored at room temperature and under refrigeration. *Rev Bras Zootec* **40**:1540–1544 (2011).
39 Lokaewmanee K, Yamauchi K, Komori T and Saito K, Enhancement of yolk color in raw and boiled egg yolk with lutein from marigold flower meal and marigold flower extract. *J Poult Sci* **48**:25–32 (2011).
40 Sirri F, Iaffaldano N, Minelli G, Meluzzi A, Rosato MP and Franchini A, Comparative pigmentation efficiency of high dietary levels of apo-ester and marigold extract on quality traits of whole liquid egg of two strains of laying hens. *J Appl Poultry Res* **16**:429–437 (2007).