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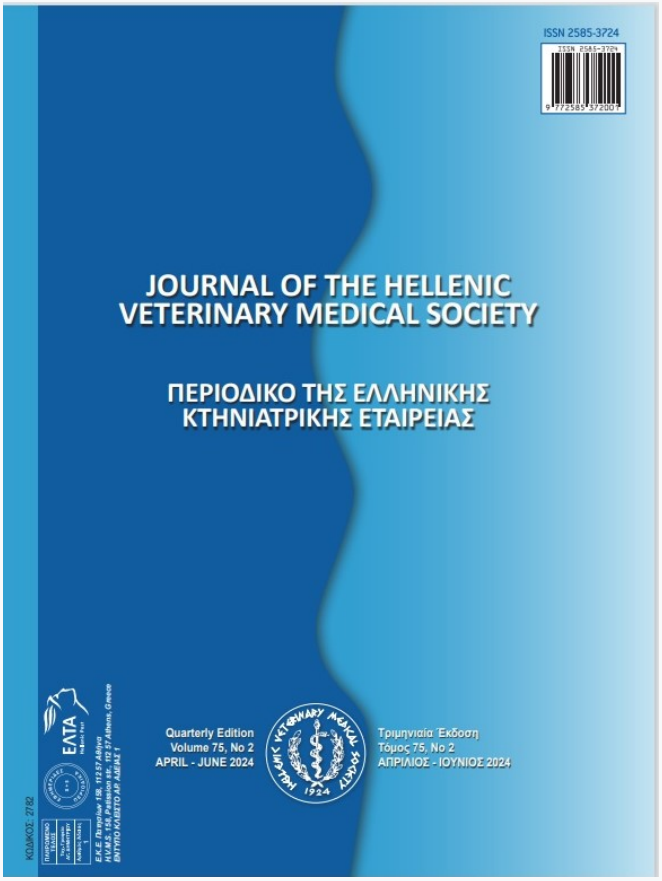

Inclusion of Lutein and Zeaxanthin in Standard or Omega-3 Fatty Acids Enriched Laying Hens Diets: Effects on Performance, Egg Quality Parameters, Fatty Acid Composition and Individual Carotenoids Concentration of Egg Yolk

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Inclusion of Lutein and Zeaxanthin in Standard or Omega-3 Fatty Acids Enriched Laying Hens Diets: Effects on Performance, Egg Quality Parameters, Fatty Acid Composition and Individual Carotenoids Concentration of Egg Yolk

H. Basmacıoğlu-Malayoğlu[1]*, B. Aktaş[1], F. Karadaş[2], P. Özdemir[3]

[1]*Department of Animal Science, Faculty of Agriculture, Ege University, 35100 Bornova, Izmir, Türkiye*

[1]*Department of Animal Science, Faculty of Agriculture, Ege University, 35100 Bornova, Izmir, Türkiye*

[2]*Department of Animal Science, Faculty of Agriculture, Van Yüzüncü Yıl University, Van, Türkiye*

[3]*International Center for Livestock Research and Training, Ankara, Türkiye*

ABSTRACT: The effects of lutein (L) and zeaxanthin (Z) inclusion to standard or omega-3 fatty acids enriched laying hens' diets, on performance, egg quality parameters, fatty acids and individual carotenoids of egg yolk were investigated. The dietary treatments were designed as: standard (S) diet, 50 mg/kg L plus 50 mg/kg Z (S+L/Z (50+50)) or 100 mg/kg lutein plus 100 mg/kg zeaxanthin (S+L/Z (100+100)) added to S diet, Omega-3 fatty acids enriched (OM3) diet, 50 mg/kg lutein plus 50 mg/kg zeaxanthin (OM3+L/Z (50+50) or 100 mg/kg lutein plus 100 mg/kg zeaxanthin (OM3+L/Z (100+100)) added to OM3 diet. The study lasted for 5 weeks. Laying hens with a mean live weight of 1525.88 ± 31.19 g were divided into six treatment groups, each group had three replicates. During the experimental period, there was no mortality rate recorded. Dietary inclusion of L/Z (50+50 or 100+100) either in the S or OM3 diets did not alter performance parameters. Eggs from hens fed an enriched diet (OM3) significantly increased ($P<0.05$) the albumen index and Haugh unit (HU). Dietary inclusion of L/Z (50+50 or 100+100) in the S or OM diet significantly improved ($P<0.05$) total carotenoid, unknown carotenoid, lutein and zeaxanthin concentrations of egg yolk compared to the S or OM diets. The yolk color a* value was significantly increased by inclusion of L/Z (50+50 or 100+100) to diets (S or OM3) ($P<0.05$) and this was correlated with increased ($P<0.05$) concentration of individual carotenoids. The major changes in egg yolk fatty acid MUFA and PUFA composition occurred when laying hens were fed with OM3 or OM3+L/Z (50+50 or 100+100) diets.

Keywords: *Egg yolk; omega-3; carotenoids; lutein; zeaxanthin*

Corresponding Author:
Basmacıoğlu-Malayoğlu H., Department of Animal Science, Faculty of Agriculture, Ege University, 35100 Bornova, Izmir, Türkiye
E-mail address: hatice.basmacioglu@ege.edu.tr

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INTRODUCTION

Consumer surveys have shown that there has been a growing concern regarding nutritional quality of eggs during the past decade. Many consumers prefer to consume eggs enriched with natural nutrients such as polyunsaturated fatty acids (omega-3), vitamins (D, E, etc.), minerals (iodine, zinc, iron, selenium, etc.), phenolics flavonoids, and carotenoids to improve human health. The three major omega-3 (n−3) PUFA, α-linolenic acid (18:3 n−3, ALA), eicosapentaenoic acid (20:5 n−3, EPA) and docosahexaenoic acid (22:6 n−3, DHA) have been acknowledged as having beneficial applications to the growth, health, and immune functions of humans and animals (Fraeye et al., 2012; Alagawany et al., 2019). Dietary manipulation with n-3 PUFA sources can improve those fatty acids in egg yolk, as declared in previous studies (Basmacıoğlu et al., 2003; Kralik et al., 2008; Wu et al., 2019).

Egg yolk colour is the most important attribute among the physical characteristics of egg quality in consumer perception (Rajput et al., 2012). Thus, the yolk colour has a higher market value. There is a variation in yolk colour due to the source of the pigmentation (natural or synthetic) and their utilization and combination between xanthophyll's, stability, availability, feed composition, genetic and physiological status of laying hens, as well as other factors (Berkhoff et al., 2020). Layer and all other animals cannot synthesize pigments in the nova, they must take them from their diet.

The natural carotenoids lutein and zeaxanthin are responsible for the orange-yellow color of the egg yolk. Egg consumption via enriched lutein and zeaxanthin (xanthophyll, carotenoids) has been epidemiologically related to specific human health benefits, including preventing macular degeneration (Mares, 2016), protecting against cardiovascular diseases (Andersen 2015), oxidative stress (Fiedor and Burda, 2014), neurodegenerative disorders (Zaheer, 2017) and cancer (Mares-Perlman et al., 2002). In the past decade, two of the most commonly used substances for enhancing the nutritional value of eggs have been omega-3 fatty acids and carotenoids, particularly lutein and zeaxanthin (Pitarque et al., 2019; Kljak et al., 2021). It is well-established that the fatty acid composition of a hen's diet and the carotenoid content directly influence the fatty acid composition of eggs and both lutein and zeaxanthin levels. Added sources of carotenoids to the diets of hens serves a dual purpose: it acts as a natural pigment to improve the color and quality of egg yolks and serves as an antioxidant to delay the oxidation of polyunsaturated fatty acids (Panaite et al., 2021). Thanks to the high-lipid composition of egg yolks, xanthophylls are present in a lipid-soluble form, making them more bioavailable compared to those derived from plant-based sources (Tudor and Pintea, 2020). Limited published information is available regarding the combination of xanthophylls with fish oil or flaxseed, which are common ingredients used in producing designer eggs (Panaite et al., 2021). In a study conducted on this subject with lutein, it was seen that chicken egg yolk was considered a rich source of lutein, and the bioavailability of lutein in chicken egg yolk was high due to its high fat content (Sawardekar, 2023).

Shi et al., (2023) reported that diets astaxanthin and lutein have insignificant effects on egg production and physical properties of the egg, but have significant effects on the color, nutrition and functionality of the egg yolk; they also stated that the two pigments could also improve laying hen antioxidant capacity and immune function.

Yunitasari et al. (2023) found that carotenoid supplementation in laying hen feed significantly improves performance (feed intake, final body weight, egg production), egg quality (egg weight, yolk colour, HU, egg yolk cholesterol, egg yolk carotenoid), and immunity (IgA).

Therefore, the main aim of this study was to investigate the effects of the inclusion of two high doses of L/Z (50+50) and L/Z (100+100) to standard or n-3 fatty acids enriched control diet on performance, egg quality parameters, fatty acid composition and individual carotenoids of egg yolk.

MATERIALS AND METHODS

According to the principles of the Ege University Animal Research Ethics Committee and national law (no. 5199), the animals used in this experiment were kept in safe conditions.

Laying hens, experimental diets and management

In an open-sided housing, 108 Super-nick laying hens were placed individually in triple-deck battery cages (41×41×50 cm). Laying hens with a mean live weight of 1525.88 ± 31.19 g were randomly distributed to six experiment groups, each group had three replicates (6 hens/each replicate). Lutein (DSM, Nutritional Products Ltd, Switzerland, 5% lutein), zea-

xanthin (OPTISHARP^{TM}, DSM, Nutritional Products Ltd, Switzerland, 5% zeaxanthin), were added to two main standard or omega-3 fatty acids enriched basal diets. The study lasted for 5 weeks. The diets were formulated according to laying hens at age of 35-40 weeks requirements reported by NRC (1994). Standard (S) basal diet contained sunflower oil (25 g/kg) and omega-3 enriched (OM3) basal diet contained flax seed (39 g/kg) and fish oil (15 g/kg) to enhance the omega-3 PUFA content of egg yolk. The nutrient composition of the diets used in this experiment is shown in Table 1. Experimental diets were also analyzed for individual carotenoids (Table 2). Each diet was freshly prepared for a week. Water and experimental feed were supplied ad libitum. The lighting schedule was 17 h light and 7 h dark. The henhouse temperature was recorded between 11-16 °C during the experimental period.

Table 1 Nutrient composition of standard and omega-3 fatty acids enriched control diets (g/kg)

Diet	S	OM3
Analysed composition (g/kg)		
Dry matter	885.0	887.0
Crude protein	171.7	173.3
Ether extract	44.20	45.20
Crude fibre	18.00	16.50
Crude ash	130.3	130.5
Starch	347.3	342.5
Sugar	55.9	58.7
Calcium	37.2	38.9
Total phosphorus	5.1	6.5
Calculated composition		
Metabolisable energy (MJ/ kg)	11.55	11.56
Fatty acid profile (%)		
ΣSFA[a]	16.84	15.82
ΣPUFA[b]	39.90	48.10
Σn-6FA[c]	39.12	45.12
Σn-3FA[d]	0.78	2.98
Σn-6FA/Σn-3FA ratio	50.15	15.14

S: Standard diet, OM3: Omega-3 fatty acids enriched diet
[a]ΣSFA (Total Saturated Fatty Acid): ΣSFA = C14:0 + C16:0 + C17:0 + C18:0+ C20:0+C21:0.
[b]ΣPUFA (Total Polyunsaturated Fatty Acid): ΣPUFA = C18:2n-6 +C18:3n-6 + C20:5n-3 + C22:6n-3.
[c]Σn-6FA (Total n-6 Fatty Acid): Σn-6FA = C18:2n-6 + C18:3n-6.
[d]Σn-3FA (Total n-3 Fatty Acid): Σn-3FA = C20:5n-3 + C22:6n-3.

Table 2 The concentration of individual and total carotenoids in experimental diets (μg/g)

	Treatments						SEM	*P*-values
Diet	S			OM3				
L/Z (mg/kg)	0	50+50	100+100	0	50+50	100+100		
Lutein	5.28^{e}	60.17^{c}	98.20^{b}	6.29^{e}	36.32^{d}	120.83^{a}	4.65	<0.001
Zeaxanthin	4.71^{c}	49.11^{b}	82.97^{a}	5.18^{c}	40.45^{b}	94.88^{a}	3.85	<0.001
β- carotene	0.25	0.24	0.15	0.17	0.20	0.55	0.12	0.274
Unknown carotenoids	0.29^{c}	0.42^{c}	0.63^{ab}	0.30^{c}	0.46^{bc}	0.80^{a}	0.04	<0.001
Total carotenoids	11.54^{e}	111.38^{c}	183.48^{b}	13.10^{e}	78.28^{d}	218.90^{a}	5.18	<0.001

S: Standard diet, S+L/Z(50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to S diet, S+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to S diet, OM3: Omega-3 fatty acids enriched diet, OM3+L/Z (50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to OM3 diet and OM3+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to OM3 diet.
SEM: Standard error of the mean
P-values: Probability of a significant effect
[a-e]Means with no common superscripts within the row of each classification are significantly ($P<0.05$) different.

Sample collection

Daily collected eggs were coded according to their treatment group. Weekly, egg production (%), feed consumption (g/hen/day) and feed conversion rate (kg feed consumed/kg eggs) were calculated. Egg quality parameters (i.e. egg weight, shape index, shell strength and shell thickness, yolk index, albumen index, Haugh unit) and yolk color were measured biweekly. The eggs were weighed (g) on a precision scale after being kept at room temperature for 24 hours. The albumen height, yolk diameter, albumen length and width of eggs were measured with a digital display caliper for to calculate yolk index and albumen index. The HU was determined by calculating with the given equation (Haugh, 1937). $HU = 100 \times \log (H + 7.5 - 1.7 \times W^{0.37})$, where H is the measurement of the albumen height (mm), W is the measurement of the egg weight (g). Shell strength (kg/cm×cm) was determined by using a machine with a spiral pressure system and shell thickness (mm) was measured using a micrometer after the shells were dried for 6-8 hours in a vacuum oven set at 60 °C. Evaluation of yolk colors was performed using Chroma meter (CR-300, Minolta, Japan) daily calibrated against a white standard instrument.

Chemical analysis

According to the procedures of AOAC (1990), the diets (S or OM3) were analyzed for dry matter (code: 930.15), crude protein (code: 976.06), crude ash (code: 942.05) and ether extract (code: 920.39). Also, according to VDLUFA method (Naumann and Bassler, 1993), the diets were analyzed for sugar, starch, phosphorus and total calcium. Metabolizable energy (ME) concentration of diets was estimated by using the equation of Carpenter and Clegg (Leeson and Summers, 2005): ME, kcal/kg= 53 + 38 [(CP, %) + (2.25 × ether extract, %) + (1.1 × (starch + sugar), %)].

Analysis of carotenoid concentrations in experimental diets and egg yolk

The carotenoid concentration evaluation of the experimental diets and egg yolk was performed by using Shimatzu Prominence HPLC. Diet samples were saponified with ethanolic potassium hydroxide in the presence of pyrogallol (Leeson and Summers, 2005). To extract the egg yolks, 200-300 mg egg yolk was weight and mixed in 0.7 ml 5% sodium chloride, 1 ml ethanol was added, samples were homogenized with 2 ml hexane twice, centrifuged, and hexane phase removed. Then the samples were evaporated under nitrogen. The final residue was re-dissolved in 1:1, v/v dichloromethane-methanol and transferred to a vial. Individual carotenoids (lutein, cis-lutein, zeaxanthin, β- carotene, unknown carotenoids) were determined by peaks identified by comparison standards (variously obtained from Sigma, Poole, UK; Hoffman-La Roche, Basel, Switzerland) (Granado et al., 1998).

Analysis of fatty acid composition of experimental diets and egg yolk

The fatty acids analysis of experimental diets and egg yolk samples extracted with a modification of the method based on Bligh and Dyer (1959) was acquired by HP (Hewlett-Packard)-Agilent/6890 GC. The conditions of chromatogram were carried out in the following orders: the oven temperature was maintained at 140 ºC for 5 minutes, after waiting at this temperature and reached 240 ºC with an increase of 4 ºC/min., and held for 20 min. at this temperature and sample volume injected 1 µl.

Statistical analysis

In SPSS 13.0 program according to 2×3 factorial design was applied for statistical analysis. Differences between the treatment groups were evaluated according to Tukey's test.

$yijl = \mu + Fi + (L/Z)j + (F \times (L/Z))ij + eijkl$where $yijl$ is the observation; μ is the overall mean; Fi the type of dietary fat source (i = 1-2); $(L/Z)j$ the equal dose of lutein and zeaxanthin (j = 1-3); $(F \times (L/Z))ij$ the interaction between the type of dietary fat source and equal dose of lutein and zeaxanthin and $eijkl$ the residual error.

RESULTS

Dietary inclusion of L/Z (50+50 or 100+100) either in S or OM3 diets did not alter performance parameters. During the 5 weeks experimental period, no mortality was recorded and the mean feed intake (FI) for laying hens fed with S and OM3 diets was117.51 (g/hen/day) and 117.80 (g/hen/day), respectively. Moreover, no variation in results of egg production (%), feed conversion ratio (FCR) and final body weight (BW) was noted as a significance of F, L/Z or their interaction F×L/Z (Table 3). The mean egg production, FCR and final BW were 95.19 (%), 1.94 (kg feed /kg egg) and 1536.82 (g), respectively. Eggs from laying hens fed an enriched diet (OM3) significantly increased (P<0.05) the albumen index and Haugh unit compared to the eggs from laying hens fed the standard (S) diet. (Table 4).

Table 3 Performance of laying hens

	Treatments							*P*-values		
Diet	S			OM3			SEM[2]	F	L/Z	F×L/Z
L/Z (mg/kg)	0	50+50	100+100	0	50+50	100+100				
Feed intake (g/hen/ day)	117.51	118.73	118.92	117.80	112.60	117.72	1.54	0.089	0.235	0.107
Egg production (%)	95.08	95.24	94.92	94.29	94.76	96.83	0.93	0.784	0.437	0.524
Feed conversion (kg feed/kg eggs)	1.98	1.91	1.92	1.97	1.89	1.95	0.04	0.983	0.177	0.781
Initial body weight (g)	1531.67	1564.67	1532.94	1566.67	1470.89	1488.44	31.19	0.179	0.425	0.173
Final body weight (g)	1534.33	1567.83	1541.22	1589.67	1494.11	1493.78	27.96	0.339	0.268	0.099

S: Standard diet, S+L/Z(50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to S diet, S+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to S diet, OM3: Omega-3 fatty acids enriched diet, OM3+L/Z (50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to OM3 diet and OM3+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to OM3 diet.
SEM: Standard error of the mean
P-values: Probability of a significant effect are due to type of dietary fat source (F), equal dose of lutein and zeaxanthin (L/Z) and their interaction (F×L/Z).

Table 4 Egg quality parameters of laying hens

	Treatments							*P*-values		
Diet	S			OM3			Pooled SEM	F	L/Z	F×L/Z
L/Z (mg/kg)	0	50+50	100+100	0	50+50	100+100				
Egg weight (g)	62.47	65.48	65.28	63.55	63.00	62.50	0.81	0.056	0.325	0.062
Shape index (%)	73.81	74.47	74.01	74.20	75.29	73.95	0.44	0.288	0.076	0.213
Shell strength (kg/cm^2)	3.08	3.29	3.18	3.26	3.16	3.22	0.13	0.761	0.910	0.838
Shell thickness (mm)	0.38	0.38	0.38	0.39	0.38	0.38	0.004	0.107	0.334	0.102
Albumen index (%)	9.30^b	9.52	9.68	10.58^a	9.91	10.29	0.34	0.010	0.711	0.086
Yolk index (%)	44.23	45.34	45.18	44.45	44.96	44.76	0.44	0.601	0.182	0.417
Haugh unit	84.88^b	85.11	86.41	88.47^a	86.55	88.06	1.28	0.040	0.545	0.242

S: Standard diet, S+L/Z(50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to S diet, S+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to S diet, OM3: Omega-3 fatty acids enriched diet, OM3+L/Z (50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to OM3 diet and OM3+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to OM3 diet.
SEM: Standard error of the mean
P-values: Probability of a significant effect are due to type of dietary fat source (F), equal dose of lutein and zeaxanthin (L/Z) and their interaction (F×L/Z).
$^{a\text{-}b}$Means with no common superscripts within the row of each classification are significantly ($P<0.05$) different.

Dietary inclusion of L/Z (50+50 or 100+100) in the S diet increased both the lutein content (by 42.84 μg/g and 49.51 μg/g) and the zeaxanthin content (by 33.85 μg/g and 38.71 μg/g) in yolks of eggs, respectively. Also, inclusion of L/Z (50+50 or 100+100) to OM3 diet increased both the lutein content (by 40.41 μg/g and 44.22 μg/g, respectively) and the zeaxanthin content (by 33.47 μg/g and 41.52 μg/g, respectively) in yolks of eggs compared to the OM3 diet. The effect of the type of dietary F and equal dose of L/Z or F×L/Z on egg yolk lutein, unknown carotenoids and total carotenoid concentration were found to be significant. However, the zeaxanthin concentration was altered by the equal dose of L/Z and the interaction between F×L/Z, regardless of the type of dietary fat source (Table 5). Similarly, the pigment concentration of lightness (L*) and redness (a*) values were significantly changed by the equal dose of L/Z and the interaction between F×L/Z. The yolk color a* value was significantly increased by inclusion of L/Z (50+50 or 100+100) to diets (S or OM3) ($P<0.05$) and this was correlated with an increased ($P<0.05$) concentration of individual carotenoids (Table 5). The changes for yolk color a* and b* values are shown in Figure 1 and 2.

The dietary treatments of S or OM3 did not vary in yolk color a* and b* over the 35-d period when compared to L/Z (50+50 or 100+100) added to these diets. Figure 1 indicated that pigment accumulation of first 5 to 7 d, after which yolk color a* produced stability when dietary L/Z (50+50 or 100+100) was added to either S or OM3 diets. After stabilization (8 to 35 d) the L/Z (100+100) added to S diet had the greatest

yolk pigmentation, followed by the L/Z (100+100) added to OM3 diet, L/Z (50+50) added to OM3 diet and L/Z (50+50) added to S diet.

Dietary inclusion of L/Z (50+50 or 100+100) in either S or OM3 diets did not alter saturated fatty acids (C14:0, C16:0, C18:0) of egg yolk. However, the F and their interaction F×L/Z on total polyunsaturated fatty acid (ΣPUFA) composition of egg yolk was significant (Table 6). The major changes in egg yolk fatty acid composition when laying hens fed with OM3 or OM3+L/Z (50+50 or 100+100) diets can be summarized as an increase of in MUFA and PUFA compared to all S diets. In fact, the egg yolk fatty acid level of C16:1 in laying hens fed with OM3 or OM3+L/Z (50+50 or 100+100) diets was determined to be higher than that of the S or S+ L/Z (50+50 or 100+100) diets. In addition, depending on the type of dietary F and their interaction with F×L/Z, the egg yolk fatty acids of C18:1, C20:1 n-9, C18:3n-3, C20:5n-3 and C22:6n-3 were increased significantly when laying hens fed with OM3 or OM3+L/Z (50+50 or 100+100) diets. Instead, the fatty acid composition of C18:2n-6 remained constant regardless of type of dietary F or F×L/Z.

Table 5 The concentration of (µg/g) individual carotenoids in the egg yolk measured by HPLC and Minolta L*, a*, b* pigment record

Results measurement by HPLC										
	Treatments							*P*-values		
Diet	S			OM3			Pooled SEM	F	L/Z	F×L/Z
L/Z (mg/kg)	0	50+50	100+100	0	50+50	100+100				
Lutein	10.35^{c}	42.84^{b}	49.51^{a}	9.26^{c}	40.41^{b}	44.22^{ab}	1.50	0.023	<0.001	<0.001
Zeaxanthin	7.43^{c}	33.85^{b}	38.71^{ab}	7.06^{c}	33.47^{b}	41.52^{a}	1.28	0.516	<0.001	<0.001
Unknown carotenoids	1.13^{c}	5.66^{ab}	6.25^{a}	0.92^{c}	4.74^{b}	5.64^{ab}	0.22	0.003	<0.001	<0.001
Total carotenoids	21.87^{d}	97.89^{bc}	111.93^{a}	19.87^{d}	90.92^{c}	105.26^{ab}	2.90	0.036	<0.001	<0.001
Results measurement by Minolta										
	Treatments[1]							*P*-values		
Diet	S			OM3			Pooled SEM	F	L/Z	F×L/Z
L/Z (mg/kg)	0	50+50	100+100	0	50+50	100+100				
L*	61.07^{a}	55.63^{b}	55.81^{b}	60.80^{a}	56.89^{b}	55.93^{b}	0.32	0.165	<0.001	<0.001
a*	-5.69^{c}	2.32^{b}	3.57^{a}	-5.40^{c}	2.49^{b}	3.61^{a}	0.16	0.224	<0.001	<0.001
b*	39.63^{ab}	41.13^{a}	41.90^{a}	35.32^{d}	37.02^{cd}	37.91^{bc}	0.53	<0.001	0.026	<0.001

S: Standard diet, S+L/Z(50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to S diet, S+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to S diet, OM3: Omega-3 fatty acids enriched diet, OM3+L/Z (50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to OM3 diet and OM3+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to OM3 diet.
SEM: Standard error of the mean
P-values: Probability of a significant effect are due to type of dietary fat source (F), equal dose of lutein and zeaxanthin (L/Z) and their interaction (F×L/Z).
[a-c]Means with no common superscripts within the row of each classification are significantly ($P<0.05$) different.

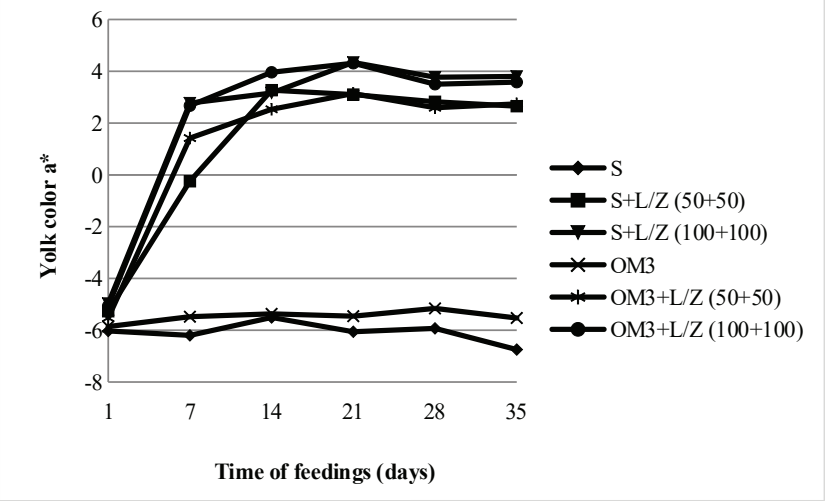

Fig. 1 Egg yolk colour a* (redness) as a function of feeding time of dietary treatments (n=3)

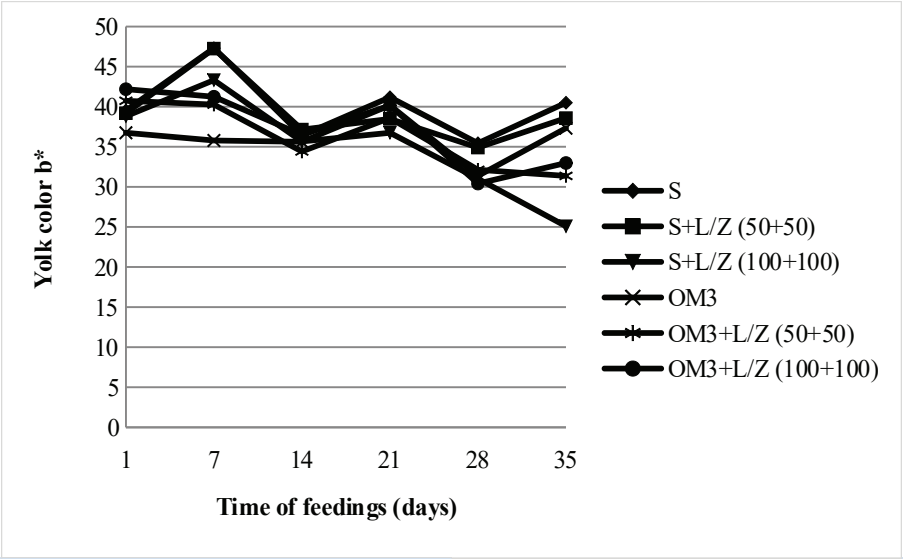

Fig. 2 Egg yolk colour b* (yellowness) as a function of feeding time of dietary treatments (n=3)

Table 6 Fatty acid composition (% of total fatty acid methyl esters) of egg yolk

	Treatments						*P*-values			
Diet	S			OM3			SEM	F	L/Z	F×(L/Z)
L/Z (mg/kg)	0	50+50	100+100	0	50+50	100+100				
C14:0 Myristic	0.34	0.36	0.34	0.36	0.38	0.39	0.01	0.081	0.651	0.388
C16:0 Palmitic	25.25	23.80	23.84	23.87	23.00	24.41	0.43	0.159	0.059	0.058
C16:1 Palmitoleic	2.84^{b}	2.84^{b}	2.90^{b}	3.30^{a}	3.36^{a}	3.43^{a}	0.23	0.019	0.912	0.255
C18:0 Stearic	6.77	6.76	6.73	7.16	7.09	7.25	0.27	0.086	0.971	0.607
C18:1 Oleic	46.16^{abc}	45.60^{bc}	44.86^{c}	47.74^{ab}	47.82^{ab}	48.30^{a}	0.45	0.000	0.163	0.001
C18:2n-6 Linoleic	18.50	18.56	18.04	18.48	18.73	18.30	0.28	0.563	0.254	0.610
C18:3n-3 Linolenic	0.58^{b}	0.60^{b}	0.71^{b}	2.31^{a}	2.44^{a}	2.49^{a}	0.12	<0.001	0.460	<0.001
C20:1n-9 Gondoic	0.32^{abc}	0.31^{bc}	0.28^{c}	0.33^{ab}	0.35^{ab}	0.36^{a}	0.01	<0.001	0.546	0.002
C20:5n-3 EPA	0.03^{b}	0.02^{b}	0.02^{b}	0.39^{a}	0.39^{a}	0.42^{a}	0.03	<0.001	0.852	<0.001
C22:6n-3 DHA	0.13^{b}	0.10^{b}	0.10^{b}	0.33^{a}	0.32^{a}	0.31^{a}	0.02	<0.001	0.234	<0.001
ΣSFA^{d}	32.36	30.92	30.91	31.40	30.46	32.04	0.50	0.820	0.097	0.138
ΣMUFA^{e}	49.32^{bc}	48.75^{c}	48.04^{c}	51.37^{ab}	51.53^{a}	52.09^{a}	0.45	<0.001	0.120	<0.001
ΣPUFA^{f}	19.24^{b}	19.28^{b}	18.87^{b}	21.51^{a}	21.88^{a}	21.52^{a}	0.76	<0.001	0.591	<0.001
Σn-6FA^{g}	18.50	18.56	18.04	18.48	18.73	18.30	0.28	0.563	0.254	0.610
Σn-3FA^{h}	0.74^{b}	0.72^{b}	0.83^{b}	3.03^{a}	3.15^{a}	3.22^{a}	0.23	<0.001	0.871	<0.001
Σn-6FA/Σn-3FA^{i}	25.00^{a}	25.78^{a}	21.73^{a}	6.10^{b}	5.95^{b}	5.68^{b}	0.79	<0.001	0.098	<0.001

S: Standard diet, S+L/Z(50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to S diet, S+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to S diet, OM3: Omega-3 fatty acids enriched diet, OM3+L/Z (50+50): 50 mg/kg lutein plus 50 mg/kg zeaxanthin added to OM3 diet and OM3+L/Z (100+100): 100 mg/kg lutein plus 100 mg/kg zeaxanthin added to OM3 diet.
SEM: Standard error of the mean
P-values: Probability of a significant effect are due to type of dietary fat source (F), equal dose of lutein and zeaxanthin (L/Z) and their interaction (F×L/Z).
[a-c]Means with no common superscripts within the row of each classification are significantly ($P<0.05$) different.
[d]ΣSFA (Total Saturated Fatty Acid): ΣSFA = C14:0 + C16:0 + C18:0
[e]ΣMUFA (Total Monounsaturated Fatty Acid): ΣMUFA= C16:1 + C18:1 + C20:1n-9.
[f]ΣPUFA (Total Polyunsaturated Fatty Acid): ΣPUFA = C18:2n-6 + C18:3n-3 + C20:5n-3 + C22:6n-3.
[g]Σn-6FA (Total n-6 Fatty Acid): Σn-6FA= C18:2n-6.
[h]Σn-3FA (Total n-3 Fatty Acid): Σn-3FA = C18:3n-3 + C20:5n-3 + C22:6n-3.
[i]Σn-6FA/Σn-3FA = Σn-6FA / Σn-3FA.

DISCUSSION

In this experiment, compared to the S or OM3 diets, the addition of pigments at high levels L/Z (50+50 or 100+100) did not have any significant impact on egg production, FI, FCR or final BW during the 5-week experimental period (P>0.05). These findings are consistent with previous studies that have reported no significant effects of pigments on FI or BW (Li et al., 2012, Karadas et al., 2016) or egg production data were not significantly affected by using different doses or types of carotenoids in laying feeds (Hasin et al., 2006; Zhang et al., 2011; Lu et al., 2013; Karadas et al., 2016). Leeson and Caston (2004) also reported that dietary 125, 250, 375, 500, 625, 750 and 1000 ppm lutein supplementation had no significant effect on egg production, which is in line with the results of this experiment. However, it has been reported that the inclusion of marigold flower extract at 150 mg/kg dose or 4 kg/ton paprika and 150 g carmoisine/ton in the diet of laying hens resulted in the highest hen-day egg production (Skřivan et al., 2015; Saleh et al., 2021) which is not in agreement with our results. The differences between studies can be attributed to factors such as the type of animals, doses or types of carotenoids, diet composition, and environmental conditions in studies.

As seen in Table 4, the mean values for albumen index and Haugh unit were parallel to each other, which were significantly increased (P<0.05) when laying hens were fed the OM3 diet. These findings are in line with previous studies (Al-Daraji et al., 2010; Promila et al., 2017) which report that higher levels of linseed or linseed oil significantly increase these values when added to layer diets. However, these finding data are not compatible with results of the studies (Scheideler et al., 1998; Grobas et al., 2001) which found no significant effect on HU and albumen index. These differences between the achieved data could be due to a strain-diet interaction, as mentioned by Scheideler et al. (1998).

The accumulation of xanthophylls (lutein/zeaxanthin) in egg yolk is notably affected by various dietary factors, including the type and concentrations of carotenoids, the type of dietary fat, the extent of processing, and non-dietary factors such as the management system and the physiological status of laying hens, including stress, diseases, age, and breed (Zaheer, 2017; Pitarque et al., 2019). Such dietary factors as the level and saturation of fat have an important role in the bioavailability of xanthophylls due to their transportation of low-density lipoprotein and high-density lipoprotein (Papadopoulos et al., 2019).

There are a series of steps that are associated with the release of lutein and zeaxanthin from the dietary matrix, the transfer of lutein and zeaxanthin to micelles, the absorption of lutein and zeaxanthin by the intestinal membrane, and the transport of lutein and zeaxanthin to the blood and egg yolk. There are several factors that affect the absorption, bioaccessibility or bioavailability of carotenoids (Goltz et al., 2012). The literature contains conflicting data regarding the impact of the fat source on carotenoids bioavailability. Some researchers (Hu et al., 2000; Gleize et al., 2013) have suggested in vitro studies that dietary fats rich in saturated fatty acids lead to higher bioavailability of lutein and zeaxanthin. On the other hand, some researchers (Failla et al., 2014; Mashurabad et al., 2016) have suggested in vitro studies that the extent of micellarization of carotenoids increases with diets rich in unsaturated fatty acids when compared to saturated fatty acids.

It is well known that there is a strong correlation between carotenoid supplementation in feed and egg yolk colour (Saleh et al., 2021). In this experiment, dietary inclusion of L/Z (50+50 or 100+100) in either S or OM3 diets significantly increased the individual carotenoid concentrations of egg yolks paralleling those in the feed. But interestingly there was significant differences were recorded for egg yolk lutein concentration between L/Z (50+50) and L/Z (100+100) added to S diet. However, the same significance was not recorded for zeaxanthin level of egg yolk. For standard egg yolk feed lutein transport to the egg yolk was more efficient compared to zeaxanthin. Parallel results were recorded with 125 ppm lutein in the diet of layers, and even with a higher level of lutein is recorded in 500 ppm lutein in the diet there was no difference in lutein concentration in egg yolk by different lutein inclusions of 375 to 1000 ppm (Leeson and Caston, 2004). Similar results were obtained by Steinberg et al. (2000) with a maximum lutein level of 120 ppm added to layer diets. Skřivan et al. (2015) reported that the addition of marigold flower extracts at a concentration of 350 mg/kg, and Pirgozliev et al. (2022) found that the inclusion of dry Stevia rebaudiana Bertoni leaves at 1% and 2% levels, which are rich in lutein and zeaxanthin, increased the lutein and zeaxanthin content of egg yolk when compared to the control diet.

These accumulations of carotenoids showed an

opposite effect when L/Z (50+50 or 100+100) was added to OM3 diet and it was seen that zeaxanthin accumulated more efficiently in egg yolks compared to lutein concentration (P<0.05). Leeson et al. (2007) indicated that when flaxseed was added to layer diets, the accumulation of lutein in the egg reduced, which is in agreement with our results. However, this concern was not observed with zeaxanthin supplemented diets. Therefore, in commercial omega-3 enriched egg production, it might be better to consider zeaxanthin supplementation in the layer diet.

In the retina, both zeaxanthin and lutein form yellow pigments that protect the eye from light (Tapiero et al., 2004). However, when it comes to enriching egg yolks with carotenoids for functional food production, we must also take into consideration the cost of feeding and the accumulation of carotenoids in the egg yolk. Factors such as appropriate doses of carotenoids, storage conditions, and the combination of dietary carotenoids influence the optimal health-promoting aspects of carotenoids (Merhan, 2017).

In this experiment, egg yolk colorimeter (L* and a*) measurement results (Table 5) showed that compared with the S or OM3 diet, the addition of pigments at high levels L/Z (50+50) and L/Z (100+100) caused significant improvement in L* and a*. This result is in agreement with report of Alay and Karadas (2017) who investigated the same dose (10 mg/kg) of carotenoids pigments (apoester, canthaxanthin, paprika oleoresin) and Aztec marigold extract pigments in addition to non-pigmented wheat-soybean based quail's diet, which improved calorimeter results for L*, a* values compared to the non-supplemented control group. However, in ISA Brown's corn-soybean basal diet supplemented with different concentrations (120, 180, and 240 ppm) of marigold extract or with 40, 60, and 80 ppm apoester, no significant effect on L* values was observed (Sirri et al., 2007), which is not consistent with our L* data. On the other hand, Hy-Line White strain egg yolk L* values decreased with higher concentrations of marigold (120 and 240 ppm) or 40, 60, and 80 ppm apoester supplementation, which showed significantly different results (Sirri et al., 2007), in line with our results. Similarly, redness (a*) results are in line with our results, as all concentrations significantly improved in both strains (Sirri et al., 2007).

Our L* and a* values are in agreement with previous reports that using red and yellow sources of pigments in hen's diets is associated with increasing a* values but a decreased L* values (Niu et al., 2008; Skřivan et al., 2015). Minolta b* values showed interesting results in our experiment, as the OM3 group showed significantly lower values compared to all other groups except OM3+L/Z (50+50) group. However, L/Z (50+50 or 100+100) added to S diet could not change b* values significantly (P>0.05), as higher concentrations of pigments shifted the color from yellowness towards redness. Similar results have been reported by (Sirri et al., 2007) using different concentrations of Marigold extract (120, 180 and 240 ppm) or 40, 60, 80 ppm apoester were added to ISA Brown's or Hy-line white strain layer corn-soybean diets did not significantly changed yellowness (b* values). It is important underline that, when compared to all standard groups b* values were significantly decreased when laying hens fed the OM3 diet. Even with the addition of L/Z (50+50) to this diet, some improvement was observed, but it only reached the level of the control group when L/Z (100+100) was added to the OM3 diet. In commercial conditions in the case of omega-3 enriched egg production, the reduction in b* values needs to be taken in consideration. Egg yolk Roche color fan score was not affected by high dose of lutein (between 250-1250 ppm) in diet of layer (Leeson and Caston, 2004) are in agreement with our results, even though we did not record Roche color fan score data for b* values. Loetscher et al. (2013) reported that lutein and zeaxanthin are highly effective as yolk colorants, which aligns with our findings.

In this experiment, the total saturated fatty acid (SFA) composition remained constant while the total PUFA and MUFA composition was higher when laying hens were fed with OM3 or OM3+L/Z (50+50 or 100+100) diets. The present results are in agreement with the findings of some other studies using different doses of fish oil, flaxseed or its oil (Galobart et al., 2001; Basmacıoğlu et al., 2003; Souza et al., 2008). Also, the absorption of MUFA especially C18:1 and C20:1n-9 seems to be encouraged by the dietary OM3, OM3+L/Z (50+50) and OM3+L/Z (100+100). Selvaraj and Cherian (2004) reported that increasing the levels of dietary fat increased the circulation of fatty acids, which are preferentially deposited into specific tissues. This could result in an increased MUFA composition of egg yolk. However, such a lutein or zeaxanthin effect has not previously been reported, so the reason for high egg yolk MUFA and PUFA composition is not due to the effect of lutein and zeaxanthin. It is important to use eggs enriched with more nutrients in human nutrition because they have a positive effect

on human health and can be called functional food. Kralik et al. (2023) reported that an adult person consuming 100 g of enriched eggs can meet approximately 86% of the daily needs for EPA and DHA.

CONCLUSION

1. The total PUFA and MUFA, as well as EPA and DHA compositions, were higher in laying hens that were fed OM3 or OM3+L/Z (50+50 or 100+100) diets compared to all S diets. The results of this experiment showed that enrichment of egg yolk with carotenoids and omega-3 are possible. It has been reported that zeaxanthin and lutein are both found in the retina to form yellow pigments to protect eye from light and retinal damage. Consumption of carotenoids enrichment egg yolk will be benefit for human health.

2. S+ L/Z (50+50 or 100+100) inclusion into layer diet significantly improved total carotenoids, unknown carotenoids, lutein and zeaxanthin concentrations of egg yolk compared to standard diets. However, lutein transfer from diet to egg yolk was efficiently than zeaxanthin. Opposite record was seen in case of omega-3 enrichment in egg yolk. Zeaxanthin accumulation was more efficiently compare to lutein. But for production of functional food as enrichment of egg yolk by omega-3 or carotenoids enrichment or both of them optimum concentration needs to be taken into account for economical purpose.

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DECLARATION OF INTEREST

The authors declare no competing interests.

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