Carotenoid deposition in yolks of laying hens fed with corn diets differing in grain hardness and supplemented with rapeseed oil and emulsifier

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Carotenoid deposition in yolks of laying hens fed with corn diets differing in grain hardness and supplemented with rapeseed oil and emulsifier

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ABSTRACT This study investigated the effects of supplementing diets consisting of two dent corn hybrids (soft- and hard-type) with different amounts of rapeseed oil (2, 3, and 4%) and with (0.05%) or without emulsifier (Lysoforte Extended, Kemin) on the content and deposition of carotenoids in egg yolk. The feeding trial was conducted with 216 Lohmann Brown laying hens which were by 3 located in 72 cages. The cages were randomly assigned to 12 dietary treatments (2 hybrids × 3 rapeseed oil levels × 2 emulsifier levels), resulting in 6 cages (replicates) per each dietary treatment. After depletion, hens were fed treatment diets without added pigment for 7 wk. After stabilization of the carotenoid profile (lutein, zeaxanthin, α- and β-cryptoxanthin and β-carotene and total carotenoids), eggs were collected once a week until the end of the experiment and deposition efficiency was calculated based on carotenoid content in yolk and diets, yolk weight, egg production and diet intake. Corn hybrid and rapeseed oil affected ($P < 0.05$) the yolk content and deposition efficiency of most carotenoids. Moreover, a significant ($P < 0.05$) hybrid × rapeseed oil level interaction for all carotenoids indicated hybrid-specific responses to rapeseed oil supplementation. In the soft-type hybrid, the addition of 3% rapeseed oil enhanced the carotenoid content compared to 2% of rapeseed oil, whereas for the hard-type hybrid, 2 and 3% of rapeseed oil resulted in similar contents. Supplementation of 4% rapeseed oil reduced the content regardless of the hybrid. Emulsifier addition positively affected ($P < 0.05$) the deposition efficiency of all carotenoids except β-carotene. In conclusion, supplementing corn diets with rapeseed oil and emulsifier affected carotenoid utilization and these responses varied in hybrids differing in grain hardness, which should be considered when using corn as the sole source of carotenoids in hen diets.

Key words: corn hybrid, rapeseed oil, emulsifier, carotenoid, laying hen

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INTRODUCTION

Egg yolk is an important carrier of lipid-soluble bioactive compounds − carotenoids, which are beneficial for their coloration, antioxidant and vitamin A status in animals and humans. It is known that the composition and content of carotenoids and in the yolk as well as its color intensity are influenced by the carotenoid profile of the diets, as laying hens are able to transfer pigments from the ingested feed into the yolk (Nabi et al., 2020). Yellow corn grains are not only used as a primary source of energy in feed for laying hens, but also exhibit a natural variation in carotenoid content characterized by a predominance of lutein and zeaxanthin and a relatively lower content of provitamin A carotenoids (Kurilich and Juvik, 1999; Saenz et al., 2021; Zurak et al., 2021). Although the grains of different corn genotypes may contain considerable concentrations of carotenoids, the proportion that is absorbed and available for physiological function or deposition in tissues (i.e. carotenoid bioavailability) is variable, as shown by the wide range of carotenoids (1.81−57.50 μg/g) in egg yolk when corn was used as the only source of pigment in feed for laying hens (Moreno et al., 2020; Ortiz et al., 2021; 2022). As lipid-soluble compounds, carotenoids undergo the same digestive fate, which involves their release from the grain matrix, solubilization into a lipid phase, incorporation into mixed micelles, absorption through the intestinal epithelium and biodistribution (Dansou et al., 2023).

During the different phases of digestion, several feed- and host-related factors, jointly known by the abbreviation SLAMENGHI, influence the bioavailability of carotenoids. These include the species of carotenoids, the

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molecular linkage, the amount of carotenoids consumed in a meal, the matrix into which the carotenoid is incorporated, the effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors and mathematical interactions (Castenmiller and West, 1998). Regarding feed-related factors, different carotenoid sources have different bioavailability of carotenoids in laying hens, which is reflected in differences in yolk carotenoid content and pigmentation efficiency (Karadas et al., 2006; Hammershøj et al., 2010; Panaite et al., 2021; Kljak et al., 2021a). Nevertheless, the presence of lipids is considered to be an important factor in enhancing the absorption and bioavailability of carotenoids from different dietary matrices. The lipids exert their effect by simulating the gallbladder for the production and release of bile into the small intestine, creating a hydrophobic environment in which the carotenoids can solubilize and promote micelle formation (Priyadarshani, 2017). The importance of the type and amount of fats contained in the feed for the carotenoid content of the yolk was demonstrated in the study by Papadopoulos et al. (2019), whose results showed that the content of lutein, zeaxanthin, cis-lutein and total carotenoids was higher in eggs from a low-energy diet with a higher ratio of unsaturated to saturated fatty acids (79.5, 7.9, 19.6 and 118.6 μg/g, respectively) compared to eggs from a low-energy diet with a lower ratio of unsaturated to saturated fatty acids (60.8, 6.2, 15.8 and 92.4 μg/g, respectively). Similarly, eggs from laying hens fed a high-oleic peanut diet had higher yolk color scores and β-carotene levels than eggs from laying hens fed a conventional corn-soybean meal diet (Toomer et al., 2019). Considering that vegetable oils are the most commonly used energy sources in laying hen diets, including appropriate dietary levels could present a practical approach to improve the digestibility and utilization of carotenoids from corn grains and thus improve their yolk content.

On the other hand, the improvement of lipid digestion could favor the bioavailability of carotenoids in laying hens and their final yolk content, as both processes are interdependent. Exogenous emulsifiers in poultry feed have been reported to improve fat digestibility by increasing the active surface area of lipase. This enzymatic action facilitates the breakdown of large fat globules into smaller fat droplets and promotes the incorporation of free fatty acids into micelles, thereby increasing the absorption of dietary lipids and improving the utilization of ingested energy (Oliveira et al., 2021; Ferreira et al., 2022; Oketch et al., 2023; Ullah et al., 2023). Moreover, literature data show that supplementing exogenous emulsifiers increases the pigmentation of the yolk, suggesting that their addition favors the absorption of carotenoids. For example, Souza et al. (2019) observed improved pigmentation by adding 2% of soy gum to a corn-soybean meal diet, with the most intense yolk coloration achieved at a gum concentration of 3%. Similarly, Ferreira et al. (2022) reported increased yolk color values in hens fed a corn-soybean meal diet supplemented with a 0.01% emulsifier. Taken together, the addition of exogenous emulsifiers in laying hen diets could be one of the strategies to improve the pigmentation potential of corn grains. This improvement could be due to the efficient formation and stabilization of emulsions, which increases the enzymatic digestion of carotenoids, their micelle incorporation and consequently the yolk content (Oketch et al., 2023).

While the presence and quantity of carotenoids in egg yolk have been extensively studied, the feed-related factors that influence their digestion and utilization from corn grains and subsequent deposition and content in the yolks warrant further investigation. As the above studies show, supplementation of lipids and exogenous emulsifiers improved both the carotenoid content and the coloration of the yolk, suggesting increased excretion of bile salts and improved emulsification. This highlights the potential benefits associated with the inclusion of these components in the diet of laying hens to enhance the release of carotenoids from the corn grain matrix during digestion and, subsequently, their absorption. However, it should be noted that current research data provide limited data on the simultaneous inclusion of lipids and exogenous emulsifiers in the diet of laying hens where corn grains serve as the primary pigment source. The aim of this study was therefore to compare the effects of supplementing two maize-based diets for laying hens with different levels of rapeseed oil (2, 3, and 4%) and with (0.05%) or without emulsifier on the content and deposition of carotenoids in the egg yolk.

MATERIALS AND METHODS

Grain Production and Treatment Diets

Two commercial corn hybrids were used in the study that were selected from 103 commercial hybrids based on physicochemical properties, in vitro analyzes of carotenoid bioaccessibility and trial with hens (Zurak et al., 2024a; Zurak et al., 2024b). Both hybrids were dent types but differed in grain hardness as determined by the Stenvert test (Zurak et al., 2024b); based on this difference, the hybrid with the softer grains was labeled soft, while the hybrid with the harder grains was labeled hard in the present study. In addition, two tested hybrids had similar carotenoid content but differed in carotenoid profile, in vitro bioaccessibility and deposition efficiency into egg yolk in the laying hen trial. Seeds of two tested corn hybrids were obtained from a commercial supplier. The corn hybrids were grown on the same test field in central Croatia near Zagreb in the growing season of 2022. Each hybrid was planted on a 70 m wide and 50 m long plot under the same agroclimatic conditions, following the recommendations of seed companies for seeding density and grown under an intensive production system. At harvest, the corn crop was mechanically harvested and dried at 85°C until the moisture content reached about 120 g/kg. After drying, the corn grains for 2 tested hybrids were packed in storage bags until laying hen diets were produced.

Table 1. Ingredient and calculated nutrient composition of the experimental diets.

	Rapeseed oil			Rapeseed oil + emulsifier		
	2%	3%	4%	2 + 0.05%	3 + 0.05%	4 + 0.05%
Ingredient[1]	proportion (%)					
Maize hybrid	61	60	59	61	60	59
Soybean meal	26.2	26.2	26.2	26.2	26.2	26.2
Rapeseed oil	2	3	4	2	3	4
Calcium carbonate	8.8	8.8	8.8	8.8	8.8	8.8
Monocalcium phosphate	1.2	1.2	1.2	1.2	1.2	1.2
Sodium chloride	0.4	0.4	0.4	0.4	0.4	0.4
DL methionine	0.15	0.15	0.15	0.15	0.15	0.15
Premix[1] + emulsifier	0	0	0	0.25	0.25	0.25
Premix[1]	0.25	0.25	0.25	0	0	0
Calculated nutrient composition[2]						
	%					
Ash	12.54	12.52	12.51	12.54	12.52	12.51
Crude protein	16.5	16.43	16.35	16.5	16.43	16.35
Crude fat	4.64	5.61	6.57	4.64	5.61	6.57
Crude fiber	2.83	2.81	2.79	2.83	2.81	2.79
Calcium	3.72	3.72	3.72	3.72	3.72	3.72
Phosphorus	0.6	0.6	0.59	0.6	0.6	0.59
Phosphorus, available	0.44	0.43	0.43	0.44	0.43	0.43
Sodium	0.18	0.18	0.18	0.18	0.18	0.18
Lysine	0.87	0.87	0.87	0.87	0.87	0.87
Methionine	0.42	0.41	0.41	0.42	0.41	0.41
Methionine + cysteine	0.86	0.86	0.85	0.86	0.86	0.85
Tryptophan	0.18	0.18	0.18	0.18	0.18	0.18
Threonine	0.62	0.62	0.62	0.62	0.62	0.62
Starch	39.35	38.7	38.1	39.35	38.7	38.1
Metabolic energy (MJ/kg)	1.14	1.16	1.19	1.14	1.16	1.19

[1]The premix provided per kg of diet: vitamin A 10,000 IU, vitamin D3 2500 IU, vitamin E 200 IU, vitamin K3 3 mg, vitamin B1 1 mg, vitamin B2 45 mg, vitamin B3 30 mg, vitamin B5 10 mg, vitamin B6 3 mg, vitamin B7 50 mg, vitamin B9 0.5 mg, vitamin B12 25 mg, choline 400 mg, antioxidant (BHA, EQ) 50 mg, I 1 mg, Fe 5 mg, Cu 5 mg, Mn 30 mg, Zn 30 mg, Se 0.2 mg.

[2]The calculated nutrient composition of the diets was calculated on the basis of the table values for the composition of the feeds used for diets.

Tables 1 and 2 provide diet formulation, calculated and analyzed nutrient content. Experimental diets were formulated to meet or exceed the recommended nutrient requirements of commercial Lohmann Brown laying hens in the initial stage of egg production according to National Research Council (NRC, 1994) and Lohmann Breeders GmbH (2022) guidelines. The basal mixture contained all ingredients except corn grains, which were the only sources of pigments in the diets, rapeseed oil and emulsifier, and it was mixed from a single batch of ingredients to reduce differences in nutritional composition. The addition of corn grain, rapeseed, and emulsifier was adjusted to obtain experimental diets with similar nutrient compositions. Immediately before the start of the feeding trial, the grains of both hybrids were transported to a feed mill near Zagreb, Croatia, and ground through a 6 mm sieve. Each corn hybrid was then assigned to one of the three rapeseed oil levels (2, 3, and 4 %) with (0.05%) or without a commercial emulsifier (Lysoforte Extended Kemin, Des Moines, IA) and mixed with the basal mixture and adequate premix and rapeseed oil amount, resulting in 12 dietary treatments. The primary active ingredient in the emulsifier is lysolecithin, while it also contains monoglycerides and emulsifiers (Kemin, 2023). The dietary samples were taken at the beginning of the feeding trial for further analysis. Three representative samples of each diets were taken and stored at -20°C for subsequent analysis. Prior to analysis, the samples were

Table 2. Analyzed nutrient composition (%) in experimental diets differing in corn hybrid, level of rapeseed oil supplementation and addition of emulsifier.[1]

Maize hybrid	Rapeseed oil / %	Emulsifier / %	Moisture	Ash	Crude protein	Crude fat	Neutral detergent fiber	Starch	Sugar	Calcium
Soft	2	0	9.7	13.6	16.8	4.8	9.6	36.9	4.3	4.3
	3	0	9.3	13.9	16.7	5.3	9.0	37.4	3.8	4.5
	4	0	8.7	13.4	16.9	6.5	9.4	36.5	4.4	4.1
	2	0.05	8.6	13.1	16.8	5.0	9.5	37.9	4.2	4.1
	3	0.05	8.9	13.2	16.9	5.9	9.2	37.5	4.3	3.5
	4	0.05	9.0	13.5	16.4	6.8	9.4	37.0	3.9	3.8
Hard	2	0	9.8	13.8	16.8	5.0	8.3	37.1	4.5	4.0
	3	0	9.5	13.3	16.8	5.9	8.7	37.0	4.5	3.8
	4	0	9.4	13.0	17.0	6.5	8.1	37.4	4.1	3.9
	2	0.05	9.6	13.7	16.2	4.8	8.8	38.6	4.3	3.9
	3	0.05	9.1	13.8	16.4	5.8	8.4	38.0	4.4	3.8
	4	0.05	8.9	13.9	16.6	6.7	8.8	36.7	4.4	3.8

[1]The composited sample of each experimental diet was used for the analysis; the values represent the mean of triplicate used in the analysis.

ground in a laboratory mill (Cyclotec 1093, Foss Tocator, Hoganas, Sweden) using a 0.3 mm sieve. All samples were analyzed for dry matter (**DM**) content by drying 3 g of each sample for 4 h at 103 ± 2°C.

Hens and Housing

The animal experiment was conducted in accordance with the Croatian directives (Animal Protection Act, OG 102/17, and Regulation on the Protection of Animals Used for Scientific Purposes, NN 55/13; NN 39/17), which correspond to the European guidelines for the care and use of animals used for scientific purposes. The animal procedures used in this study were approved by the Ethics Committee for the protection of animals used in scientific research within the Ministry of Agriculture of the Republic of Croatia (EP 349/2022).

The experiment was conducted on 216 Lohmann Brown laying hens at 30 wk of age, allocated by three to one cage, resulting in 72 enriched cages (Council Directive 1999/74/EC) in the experimental poultry house of the University of Zagreb Faculty of Agriculture. The cages were organized in four free-standing laying batteries providing 1,269 cm^2 per hen. Each cage was equipped with a feeder on the outside of the cage front (minimum 12 cm/bird), two nipple drinkers, a perch (at least 15 cm per hen), and a claw-shortening device. The eggs laid by the hens were collected in a wired egg cradle placed parallel to and below the feeder. The environmental conditions of the experimental house were automatically controlled, with temperature sensors placed throughout the house to monitor conditions and adjust ventilation accordingly. The room temperature was maintained at 18 ± 2°C throughout the experiment. The light period consisted of 16 h of light per day, while diet and water were provided ad libitum to the laying hens. The laying hens were weighed at the beginning (at 30 wk of age) and at the end (at 41 wk of age) of the 7-wk trial period.

Experimental Design

Prior to the feeding trial, the laying hens were fed a white maize-based diet without added pigments for 4 wk with the same calculated ingredient composition as the experimental diets containing 60% of corn grain (Table 1) to deplete of carotenoids from the previous diet. Cages were then randomly assigned to one of 12 dietary treatments in a 2 × 3 × 2 factorial design. Factors included corn hybrid (soft- and hard-type), rapeseed oil level (2, 3, or 4%) and addition of commercial emulsifier (without addition and 0.05%). The total number of cages (replicates) per dietary treatment was 6. The experimental period lasted 49 d and was divided into a stabilization period (14 d) of the carotenoid content in the yolks and a sampling period (35 d) in which the number and weight of the eggs were recorded daily and the feed intake weekly.

During the experimental period, one egg per cage (i.e., 6 eggs per treatment) was sampled every 3 d to quantify the total carotenoid content in the yolk and to monitor the stabilization of carotenoid levels. After the second week, the carotenoid content in the yolk had stabilized; after that, three eggs per cage (i.e., 18 eggs per treatment) were sampled once a week for carotenoid analysis until the end of the trial. The eggs were analyzed in the shortest possible time and, if necessary, stored at 4°C. The collected eggs were cracked immediately before analysis, the yolks were separated from the whites and dried on a paper napkin. After the yolks were separated, their weight was recorded. To determine the stabilization of carotenoid content, each yolk was analyzed individually. During the sampling period, 3 yolks from each cage were combined for carotenoid analysis, resulting in 6 samples per treatment each week.

Carotenoid Analysis in Egg Yolk and Experimental Diets

The spectrophotometric method described by Surai et al. (2001) was used to determine the stabilization of carotenoid content in egg yolks (collected from 0 to 14 d of the trial). Yolk samples (200−500 mg) were homogenized with 2 mL of a 1:1 (v/v) mixture of 5% sodium chloride solution and ethanol, followed by the addition of 3 mL of hexane and further homogenization for 3 min. After centrifugation (5 min, 1,200 *g*), the extract was collected in a 10 mL volumetric flask, and the extraction was repeated until the upper layer was colorless. The combined extracts were then diluted to volume with hexane and their spectrum was measured between 400 and 500 nm (Helios γ, Thermo Electron Corporation, UK). The absorbance at the maximum was used and the total carotenoid content was calculated as β-carotene equivalents (μg/g) using the β-carotene calibration curve with concentrations between 0.2 and 2.5 mg/L.

Quantification of carotenoids from egg yolks collected from d 15 to the end of the trail was performed using the reversed-phase HPLC method following the extraction described above (Surai et al. 2001) with some differences; 200 mg of the combined egg yolk sample was taken for analysis using β-apo-carotenal as an internal standard (100 μL). After the extraction procedure, the combined hexane extracts were evaporated using a rotary vacuum concentrator (RVC 2-25CD plus, Martin Christ) and reconstituted in 300 μL acetonitrile:dichloromethane: methanol (45:20:35, v/v/v) containing 0.1% BHT.

Carotenoids from the experimental diets were extracted as described by Kurilich and Juvik (1999) using β-apo-carotenal as an internal standard (100 μL). Each sample was analyzed in triplicate, and the mean value was taken as the result. The grinned samples of 600 mg were homogenized with 6 mL ethanol containing 0.1% butylated hydroxytoluene (**BHT**) and then precipitated in a water bath at 85°C (5 min) before

saponification with 120 μL 80% potassium hydroxide for 10 min. All samples were vortexed once during saponification. Upon removal, the test tubes were cooled in an ice bath with the addition of deionized water (3 mL). Then, 3 mL of hexane was added to each sample; they were vortexed and centrifuged at 1,200 g for 10 minutes (Centric 322A, Tehtnica, Železniki, Slovenia). The upper hexane layer was then pipetted into a separate tube and the extraction procedure was repeated until the upper hexane layer was colorless (about 4 extractions). The collected supernatants were evaporated using a rotary vacuum concentrator (RVC 2-25CD plus, Martin Christ, Osterode am Harz, Germany) and dissolved in 200 μL acetonitrile:dichloromethane:methanol (45:20:35, v/v/v) containing 0.1% BHT.

Lutein, zeaxanthin, α- and β-cryptoxanthin, and β-carotene in the extracts were quantified using the reversed-phase HPLC method described by Kurilich and Juvik (1999). Carotenoids were separated and quantified using a SpectraSystem HPLC instrument (Thermo Separation Products, Inc., Waltham, MA) equipped with a quaternary gradient pump, an autosampler and a UV-vis detector. Compounds were separated on two sequentially connected C18 reversed-phase columns Vydac 201TP54 column (5 μm, 4.6 × 150 mm; Hichrom, Reading, UK), followed by a Zorbax RX-C18 column (5 μm, 4.6 × 150 mm; Agilent Technologies, Santa Clara, CA). The separation columns were protected by a Supelguard Discovery C18 guard column (5 μm, 4 × 20 mm; Supelco, Bellefonte, PA). The mobile phase consisted of acetonitrile:methanol:dichloromethane (75:25:5, v/v/v) containing 0.1% BHT and 0.05% triethylamine. An aliquot of 30 μL was injected, and the flow rate was 1.8 mL/min. The separations were performed at room temperature, and carotenoids were monitored at 450 nm.

Carotenoids [lutein (purity 99%), zeaxanthin (purity 99%), α- and β- cryptoxanthin (purity of both 99%), and β-carotene (purity 98%)] were identified by comparing their retention times and quantified by external standardization with calibration curves using commercially available standards (Extrasynthese, France; $r^2 \geq 0.99$ for all carotenoids). The total carotenoid content was calculated by summing the contents of the individual carotenoids.

Carotenoid Deposition Efficiency

The carotenoid deposition efficiency for each cage within the dietary treatment and for each week of sampling period was calculated using the following equation (Karadas et al., 2006):

$$\text{Carotenoid deposition efficiency (\%)} = \frac{\text{Carotenoid production by egg}}{\text{Carotenoid consumption by diet}} \times 100$$

where carotenoid production by eggs and consumption by diet were calculated using the following equations:

$$\begin{aligned}\text{Carotenoid Production by Egg} &= \text{Yolk Weight (g)} \\ &\times \text{Yolk Carotenoid Content } (\mu\text{g/g}) \\ &\times \text{Egg Production}\end{aligned}$$

$$\begin{aligned}\text{Carotenoid consumption by diet} &= \text{diet intake (g/d/hen)} \\ &\times \text{diet carotenoid content } (\mu\text{g/g})\end{aligned}$$

based on the data obtained in the hen trial and after sample analysis.

Statistical Analysis

The obtained results were analyzed using SAS statistical software (version 9.4; SAS Institute Inc., Cary, NC). The dietary trial was conducted as a completely randomized design with a factorial arrangement of 2 hybrids of different grain hardness, 3 levels of rapeseed oil supplementation, and with or without the addition of an emulsifier with 12 dietary treatments, defining a cage with three hens as the experimental unit. Differences between the treatment diets were subjected to an analysis of variance using the MIXED procedure, with corn hybrid, rapeseed oil level and emulsifier addition as fixed effects. The same procedure was used to analyze differences between treatments in yolk carotenoid content and carotenoid deposition efficiency using repeated measurements ANOVA, with results obtained from the third week and until the end of the dietary trial. Mean values were defined by the least squares means statement and compared using the PDIFF option. The threshold for statistical significance was defined as $P < 0.05$.

RESULTS

Carotenoid Profile of Experimental Diets

The total carotenoid content in the experimental diets averaged 22.48 μg/g DM (Table 3). The diets with hard hybrid had a higher zeaxanthin content than those with soft hybrid (on average 12.58 and 9.00 μg/g DM, respectively), which in turn had higher lutein contents (on average 7.90 and 5.34 μg/g DM, respectively). Of the remaining three carotenoids, β-cryptoxanthin was the following carotenoid in all experimental diets, averaging 3.06 μg/g DM. The experimental diets consisting of hard hybrid had a higher content of β-carotene than of α-cryptoxanthin (on average 1.11 and 0.40 μg/g DM, respectively), while the opposite was found for the diets containing soft hybrid (on average 0.99 and 1.09 μg/g DM, respectively).

Table 3. Carotenoid content (μg/kg DM) in experimental diets differing in corn hybrid, level of rapeseed oil supplementation and addition of emulsifier.[1]

Maize hybrid	Rapeseed oil / %	Emulsifier / %	Lutein	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	β-carotene	Total carotenoids[2]
Soft	2	0	7.98 ± 0.03	9.08 ± 0.32	1.03 ± 0.03	2.34 ± 0.06	1.06 ± 0.03	21.50 ± 0.21
	3	0	7.93 ± 0.25	8.87 ± 0.40	1.07 ± 0.02	2.36 ± 0.04	1.00 ± 0.04	21.23 ± 0.19
	4	0	8.26 ± 0.21	9.00 ± 0.49	1.17 ± 0.02	2.51 ± 0.09	1.01 ± 0.03	21.95 ± 0.15
	2	0.05	7.89 ± 0.33	9.09 ± 0.20	1.12 ± 0.03	2.48 ± 0.01	1.00 ± 0.01	21.58 ± 0.48
	3	0.05	7.72 ± 0.03	8.70 ± 0.06	1.08 ± 0.01	2.39 ± 0.04	0.92 ± 0.01	20.81 ± 0.07
	4	0.05	7.67 ± 0.18	9.25 ± 0.15	1.07 ± 0.01	2.51 ± 0.03	0.92 ± 0.01	21.42 ± 0.27
Hard	2	0	5.50 ± 0.24	12.74 ± 0.23	0.44 ± 0.01	3.80 ± 0.14	1.07 ± 0.07	23.55 ± 0.05
	3	0	5.71 ± 0.22	12.57 ± 0.12	0.41 ± 0.01	3.72 ± 0.07	1.06 ± 0.01	23.47 ± 0.44
	4	0	5.36 ± 0.07	13.11 ± 0.10	0.39 ± 0.01	3.76 ± 0.04	1.11 ± 0.03	23.74 ± 0.23
	2	0.05	5.34 ± 0.06	12.44 ± 0.32	0.37 ± 0.01	3.61 ± 0.14	1.10 ± 0.01	22.86 ± 0.47
	3	0.05	5.05 ± 0.15	12.12 ± 0.21	0.38 ± 0.01	3.72 ± 0.08	1.17 ± 0.01	22.44 ± 0.21
	4	0.05	5.06 ± 0.13	12.47 ± 0.47	0.40 ± 0.01	3.79 ± 0.11	1.12 ± 0.00	22.84 ± 0.46

[1]Content od carotenoids is presented as mean ± SEM; n = 3.
[2]Total carotenoid content was calculated by summarizing each individual carotenoid identified and quantified by analysis.

Carotenoid Content of Egg Yolks

The total content of carotenoids in the egg yolks from hens fed dietary treatments averaged 34.50 μg/g. On average, zeaxanthin was the major carotenoid in all yolk samples, followed by lutein, which accounted for 54.87% and 40.82% of the total carotenoids, respectively. β-cryptoxanthin, α-cryptoxanthin and β-carotene in the yolks accounted for an average of 2.47, 1.32, and 0.42% of the total carotenoids, respectively.

With the exception of β-carotene, the composition of all individual and total carotenoids in egg yolk was primarily affected by the corn hybrid, as shown in Table 4. Yolk samples from laying hens fed a treatment diets containing hard grain hybrid had 2-fold higher zeaxanthin than lutein content, and the same treatments resulted in higher β-cryptoxanthin content in the yolk. In contrast, the contents of lutein and zeaxanthin in the yolks of hens fed a soft hybrid diet averaged 17.67 and 16.12 μg/g, respectively, and these yolks also contained higher α-cryptoxanthin content. The addition of rapeseed oil affected the yolk content of lutein (P = 0.016), zeaxanthin (P = 0.007) and total carotenoids (P = 0.013), with the highest yolk lutein content found for diets supplemented with 3% of rapeseed oil. The emulsifier affected only α-cryptoxanthin content (P = 0.044), and the higher content was detected in yolks from hens fed diets with addition of emulsifier compared to without addition.

Interaction between corn hybrid and rapeseed oil level were observed for the yolk content of all individual (P < 0.05) and total carotenoids (P < 0.01; Table 4). As shown in Table 5, in diets with soft hybrid, the highest contents of lutein, zeaxanthin, β-carotene and total carotenoids were found in the yolks of hens whose diet was supplemented with 3% of rapeseed oil and the lowest in the diets supplemented with 2% of rapeseed oil, while the contents of α- and β-cryptoxanthin increased with increasing levels. In contrast, the levels of all and total carotenoids in diets with hard hybrid were similar for diets supplemented with 2 and 3% of rapeseed oil, while the lowest values were found in diets supplemented with 4% of rapeseed oil. Interaction between the hybrid

Table 4. Analysis of variance for carotenoid content in yolks of laying hens with means for the main effect of investigated factors.[1]

Source of variation			Lutein	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	β-carotene	Total carotenoids
					P			
Hybrid (H)			<0.001	<0.001	<0.001	<0.001	0.630	0.005
Rapeseed oil level (RO)			0.016	0.007	0.140	0.201	0.122	0.013
Emulsifier (E)			0.197	0.512	0.044	0.290	0.156	0.751
H × RO			0.029	0.007	0.001	<0.001	0.043	0.004
H × E			0.001	0.644	0.237	<0.001	0.904	0.075
RO × E			0.306	0.404	0.028	0.539	0.132	0.486
H × RO × E			0.199	0.735	0.005	0.025	0.665	0.317
Corn hybrid[2]	Rapeseed oil level[2]	Emulsifier[2]				μg/g		
Soft			17.67a	16.12a	0.55a	0.73b	0.15	35.24a
Hard			10.49b	21.74b	0.36b	0.97a	0.14	33.75b
	2%		13.73b	19.05a	0.44	0.83	0.49	34.24b
	3%		14.62a	19.42a	0.46	0.86	0.15	35.54a
	4%		13.89b	18.33b	0.46	0.86	0.14	33.71b
		0	13.91	19.02	0.46a	0.86	0.14	34.42
		0.05%	14.25	18.84	0.45b	0.84	0.15	34.58

[1]n = 30 (6 replicates per treatment × 5 wk of sampling period).
[2]Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test (P = 0.05).

Table 5. Effect of hybrid and rapeseed oil on carotenoid content (μg/g) and composition in the yolks of laying hens.[1,2]

Corn hybrid	Rapeseed oil level	Lutein	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	β-carotene	Total carotenoids
Soft	2%	16.81 ± 0.32b	15.64 ± 0.33b	0.52 ± 0.01b	0.66 ± 0.02c	0.14 ± 0.29b	33.77 ± 0.62b
	3%	18.40 ± 0.32a	16.70 ± 0.32a	0.56 ± 0.01ab	0.73 ± 0.02b	0.16 ± 0.28a	36.58 ± 0.60a
	4%	17.81 ± 0.33ab	16.02 ± 0.34ab	0.59 ± 0.01a	0.79 ± 0.02a	0.14 ± 0.29b	35.36 ± 0.63ab
Hard	2%	10.65 ± 0.32a	22.46 ± 0.33a	0.36 ± 0.01a	1.00 ± 0.02	0.83 ± 0.29	34.71 ± 0.62a
	3%	10.85 ± 0.32a	22.13 ± 0.33a	0.37 ± 0.01a	0.99 ± 0.02	0.15 ± 0.28	34.52 ± 0.61a
	4%	9.98 ± 0.33b	20.64 ± 0.34b	0.34 ± 0.01b	0.94 ± 0.02	0.14 ± 0.29	32.08 ± 0.63b

[1]Values represent means ± SEM; n = 30 (6 replicates per treatment × 5 wk of sampling period).
[2]Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($P = 0.05$).

and the addition of emulsifier was significant for lutein, where the addition of emulsifier in diets based on a soft hybrid decreased the content, while in diets based on a hard hybrid, it increased the content, and for β-cryptoxanthin, where an opposite trend was observed. A significant interaction between the rapeseed oil level and the addition of emulsifier was determined only for α-cryptoxanthin, while the interaction between all tested effects was significant only for α- and β-cryptoxanthin.

Efficiency of Carotenoid Deposition in Egg Yolks

The deposition efficiency of total carotenoids in the yolks of the tested treatments averaged 16.88%, although there were slight fluctuations during the experimental period (Figure 1). The deposition efficiency of lutein and zeaxanthin was up to eight times higher than that of α-cryptoxanthin, β-cryptoxanthin and β-carotene. Similar to the yolk carotenoid content, corn hybrid affected ($P < 0.01$) the carotenoid deposition efficiency; however, this effect was not determined for zeaxanthin (Table 5). On average, laying hens fed soft hybrid diets had higher deposition of lutein, β-cryptoxanthin and total carotenoids, while those fed hard grain hybrid diets had a higher deposition efficiency of α-cryptoxanthin and β-carotene. In agreement with the results for carotenoid content, the addition of rapeseed oil affected the deposition efficiency of lutein, zeaxanthin and total carotenoids ($P < 0.05$), with the highest levels found in the yolks of hens fed a treatment diet with 3% of rapeseed oil and an emulsifier. Furthermore, addition of emulsifier increased the deposition efficiency of lutein, zeaxanthin, α- and β-cryptoxanthin and total carotenoids ($P < 0.05$). From interactions, the significant was only the interaction between rapeseed oil level and emulsifier addition for lutein, where addition of emulsifier increased deposition efficiency in diets supplemented with 3 and 4% of rapeseed oil while it was similar in diets supplemented with 2% of rapeseed oil.

DISCUSSION

The experimental diets in the present study had a wider range of total carotenoids compared to the range reported for the experimental diet based on commercial corn hybrids (5.7−17.13 μg/g DM; Moreno et al., 2020; Ortiz et al., 2021; Kljak et al., 2021b; Table 1), Give that the corn hybrids were the primary source of pigment for laying hens, the primary carotenoids present in the grains, namely, lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin and β-carotene, were quantified in the diets. Consistent with previous findings on the carotenoid composition of treatment diets from commercial corn hybrids (Moreno et al., 2020; Ortiz et al., 2021; Kljak et al., 2021b), xanthophylls lutein and zeaxanthin were predominant compared to provitamin A carotenoids (α-cryptoxanthin, β-cryptoxanthin and β-carotene). However, the prevalence of zeaxanthin over other carotenoids in all diets studied is in contrast to the findings reported by Ortiz et al. (2021) and Kljak et al.

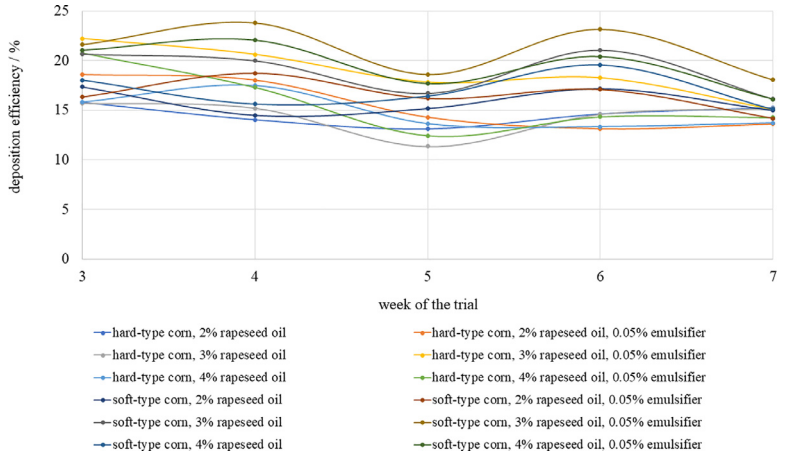

Figure 1. Changes in the deposition efficiency of total carotenoids in the yolk of eggs laid by hens fed 12 dietary treatments during the sampling period.

(2021b), where lutein was the most abundant carotenoid. The experimental diets reflected the carotenoid profile of the corn hybrids used, with diets of the soft hybrids containing a higher proportion and concentration of α-branch carotenoids (lutein and α-cryptoxanthin), while diets of the harder hybrids contained higher levels of β-branch carotenoids (zeaxanthin, β-cryptoxanthin and β-carotene). This was consistent with the study by Saenz et al. (2021), whose results show that genotypes with different grain hardness have contrasting carotenoid profiles.

In all treatments tested, the accumulation of carotenoids in the yolk started on the 4th d after depletion and reached saturation after 14 d of the experimental period. This was consistent with the previously reported duration of the accumulation and saturation phase for biofortified and commercial corn (Moreno et al., 2020; Ortiz et al., 2021; Kljak et al., 2021b; 2022). During the experimental period, yolk levels of lutein and zeaxanthin were higher than that of α-cryptoxanthin, β-cryptoxanthin and β-carotene. The content of all individual carotenoids remained stable until the 6th wk of the experiment, after which a slight decrease in their content was observed. Thereafter, the carotenoid content in the yolks remained constant until the end of the experiment. The determined decrease in carotenoid content observed in the yolks could indicate a possible degradation and carotenoid losses during storage of diets with milled corn grain, considering that all experimental diets were prepared on the same day (Gunjević et al., 2024).

However, the less pronounced fluctuations in the total carotenoid content in the yolk indicate that the commercial corn hybrids investigated serve as a stable source of carotenoids in laying hen diets (data not shown).

The total carotenoid content in the yolks of the studied treatments was higher than the ranges reported for biofortified and commercial corn diets (21.97−26.18 μg/g; Moreno et al., 2020; Ortiz et al., 2021; Kljak et al., 2021b; Table 4). The carotenoid profile of the yolk samples reflected the carotenoid composition of the treatments, i.e. the commercial corn hybrid used for the diet preparation, with the hard hybrid-based diets resulting in higher contents of zeaxanthin and β-cryptoxanthin in the yolk and the soft hybrid-based diets resulting in higher contents of lutein and α-cryptoxanthin in the yolk. Regardless of the differences in the diet concentration, the β-carotene levels in the yolk did not differ in any of the treatments tested, similar to the findings of Liu et al. (2012). Nevertheless, compared to the findings of previous studies with different corn genotypes (Moreno et al., 2019; Ortiz et al., 2021; Kljak et al., 2021b), the diets with soft grain hybrids resulted in higher yolk concentrations of lutein and α-cryptoxanthin, while the diets with hard grain hybrids resulted in higher yolk concentrations of zeaxanthin. The concentrations of other individual carotenoids in the yolks were comparable to the results reported in the aforementioned studies.

The efficiency of carotenoid deposition showed slight fluctuations during the experimental period, as shown in Figure 1 for total carotenoids. These fluctuations are consistent with the fluctuations in total carotenoid content in the yolks, however, the deposition efficiency could also be influenced by weekly fluctuations in diet intake. Overall, the efficiency of carotenoid deposition varied depending on the differences in the type and content of carotenoids in the diet. In all treatments tested, lutein showed higher deposition efficiency compared to zeaxanthin, while the deposition efficiency of α-cryptoxanthin exceeded that of β-cryptoxanthin and β-carotene (Table 6). The observed preferential transport of xanthophylls from the diet into the yolk and the decreasing deposition efficiency with higher carotenoid content in the diet was in agreement with the findings of other studies (Kljak et al., 2021b; Ortiz et al., 2022). Kljak et al. (2021b) investigated the use of commercial corn hybrids as the sole pigment source in hen diets and reported a range of 21.75−28.46% for the deposition efficiency of lutein, 20.87−29.53% for

Table 6. Analysis of variance for carotenoid deposition efficiency in yolks of laying hens with means (%) for the main effect of investigated factors.

Source of variation[1]			Lutein	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	β-carotene	Total carotenoids
					P			
Hybrid (H)			0.002	0.274	<0.001	0.002	<0.001	0.002
Rapeseed oil level (RO)			0.025	0.003	0.100	0.060	0.172	0.011
Emulsifier (E)			0.001	0.001	0.008	0.044	0.273	0.009
H × RO			0.721	0.141	0.745	0.356	0.241	0.417
H × E			0.281	0.433	0.253	0.169	0.246	0.963
RO × E			0.041	0.592	0.285	0.738	0.924	0.367
H × RO × E			0.231	0.409	0.220	0.656	0.507	0.442
Corn hybrid[2]	Rapeseed oil level[2]	Emulsifier[2]				μg/g		
Soft			24.80a	19.58	5.56b	3.30a	1.49b	17.99a
Hard			21.50b	18.68	9.70a	2.81b	4.02a	15.60b
	2%		21.73b	17.35b	7.17b	2.83	2.53	15.55c
	3%		25.04a	20.98a	8.19a	3.27	2.92	18.28a
	4%		22.69b	19.06ab	7.52b	3.08	2.81	16.54b
		0	24.83a	20.51a	8.16a	3.21a	2.85	17.78a
		0.05%	21.48b	17.75b	7.10b	2.91b	2.66	15.80b

[1]n = 30 (6 replicates per treatment × 5 wk of sampling period).
[2]Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($P = 0.05$).

zeaxanthin, 6.90−11.21% for β-cryptoxanthin, 2.94 −8.95% for β-carotene and 19.31−24.75% for total carotenoids. From the above, it can be seen that the deposition efficiency of almost all individual carotenoids in this study falls within the reported ranges, while the deposition efficiency of β-carotene falls within the lower values of the range.

In the present study, the lutein, zeaxanthin and total carotenoid content of the yolk and their deposition efficiency depended on the level of rapeseed oil supplementation in the diet. A rapeseed oil supplementation of 3% increased the utilization and thus the yolk content and the deposition of the individual and total carotenoids compared to supplementation of 2 and 4%. These results are consistent with previous findings that oils increase the utilization of carotenoids by improving the efficiency of micellization, resulting in increased content in the yolk when oils are supplemented in hen diets (Mutsokoti et al., 2017; Papadopoulos et al., 2019). Although rapeseed oil contains high concentrations of unsaturated fatty acids, for which several authors have reported negative effects on the yolk content of lutein, zeaxanthin and β-carotene (Papadopoulos et al., 2019; Toomer et al., 2019; Yuan et al., 2019), the results of the present study clearly showed that supplementation of 3% of rapeseed oil increased carotenoid utilization from corn grains. The supplemental oil probably led to an increase in the secretion of bile salts and the efficiency of micellization and, consequently, the content and efficiency of carotenoid deposition in egg yolk (Dieudonné et al., 2023). However, an increase in supplementation from 3 to 46% resulted in a decrease in carotenoid content and deposition efficiency, suggesting that at higher supplementation levels of rapeseed oil, the high concentrations of unsaturated fatty acids impair carotenoid utilization. Although the deposition efficiency was similar, the effect of rapeseed oil level in the diets depended on the hardness of the corn hybrids. In hard hybrid-based diets, supplementation of 2 and 3% resulted in similar contents, while in soft hybrid-based diets, 2% of rapeseed oil was not sufficient for maximum carotenoid utilization. It can be suggested that rapeseed oil levels up to 3% are sufficient for increased carotenoid utilization of hard hybrids as they generally remain longer in the digestive system and are digested more slowly, while higher oil levels simulate the excretion of larger amounts of bile salts and thus increase the carotenoid utilization of highly digestible soft hybrids (Zhao et al., 2016; Singh and Ravindran, 2019). Furthermore, the presence of monoglycerides did not contribute to the effect of the rapeseed oil level, as no significant interaction was found between the rapeseed oil level and the emulsifier addition.

Although emulsifiers are primarily added to laying hen diets to increase the energy efficiency of the diets, the results of the present study have shown that their addition can also lead to a higher bioavailability of carotenoids in addition to the positive effect on digestion and lipid absorption. However, this effect was more pronounced for the efficiency of carotenoid deposition than for the carotenoid content in the egg yolk. The data obtained are difficult to compare, as the studies using emulsifiers in laying hen diets mainly provide information on egg quality parameters (i.e., yolk color) and not on the carotenoid content in the yolk (Klementavičiūtė et al., 2016; Ferreira et al., 2022). However, the results obtained indicate that the addition of rapeseed oil and emulsifier can increase the utilization of carotenoids from corn grains due to increased secretion of bile salts and, on the other hand, improved emulsification, which increases the digestibility and micellization of carotenoids and, consequently, their content and deposition in the yolk. Considering that the effect was hybrid-dependent only for lutein and β-cryptoxanthin, the addition of an emulsifier could increase the content of xanthophylls in the yolk of hens fed diets containing a grain of harder hybrids as the primary source of carotenoids. It is possible that the emulsifier facilitated the solubilization of carotenoids and their transfer to the micelles in hard hybrid diet due to a longer retention in the digestive system and slower digestion (Fernández-García et al., 2008; Zhao et al., 2016; Singh and Ravindran, 2019). On the other hand, it is possible that in soft hybrids, which are highly digestible (Zhao et al., 2016; Singh and Ravindran, 2019), the addition of the emulsifier containing monoglycerides contributed to the competition between xanthophylls and fats for incorporation into micelles (Bohn, 2008), resulting in lower contents in yolks.

CONCLUSIONS

Corn hybrid, rapeseed oil level and addition of emulsifier affected the content and deposition efficiency of carotenoids in the yolk. The supplementation of 3% of rapeseed oil resulted in the highest content and the highest deposition efficiency among the tested levels. However, the grain hardness of the corn hybrid used in the diet should be taken into account, and if a harder hybrid is used, a lower level of supplementation could be sufficient. Levels above 3% should not be supplemented regardless of the grain hardness of the hybrid. Furthermore, the addition of an emulsifier increased the carotenoid deposition efficiency, but it should be considered that it may impar carotenoid content in diets based on the grain of softer corn hybrids. The combined addition of rapeseed oil and an emulsifier could be a promising strategy to maximize carotenoid utilization from corn grains in layer diets.

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DISCLOSURES

The authors declare no conflicts of interest.

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