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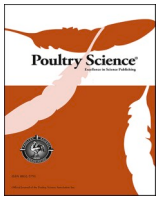

Full-Length Article

Effect of supplementing corn diet for laying hens with vitamin A and trace minerals on carotenoid content and deposition efficiency in egg yolk

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ABSTRACT

This study investigated the effects of supplementing laying hen's diet with vitamin A (5,000, 10,000 and 20,000 IU/kg) and trace minerals (Zn, Mn, Cu, Fe and Se) in inorganic and organic form on the carotenoid content and deposition efficiency in egg yolk. Hen's diet consisted of two commercial dent corn hybrids (soft- and hard-type), which differed in their carotenoid profile. The feeding trial was conducted with 252 Lohmann Brown hens allocated in 84 cages that were randomly assigned to 12 dietary treatments (2 hybrids × 3 vitamin A levels × 2 trace mineral forms). After depletion, hens were fed diets without added pigment for 7 wk. Deposition efficiency was calculated based on the carotenoid content in yolk and diets, yolk weight, egg production and diet intake. Tested hybrids significantly differed in the content of total and individual carotenoids in egg yolk, with the exception of except β-cryptoxanthin. Lutein was the main carotenoid in the soft-type hybrid and zeaxanthin in the hard-type one. Lower vitamin A levels tended to increase the yolk content of provitamin A carotenoids and the deposition efficiency of all carotenoids for both hybrid types. In addition, the highest content of lutein in egg yolk was found when the soft-type hybrid was supplemented with the lowest vitamin A level. In contrast, trace minerals showed a relatively small effect on carotenoid content and deposition efficiency. However, a significant hybrid × vitamin A × trace mineral interaction existed mainly because the hard-type hybrid had the highest deposition efficiency of all carotenoids except α-cryptoxanthin when diet was supplemented with organic trace minerals and the lowest vitamin A level. In conclusion, supplementing hen's diet with the lowest vitamin A level improved the content of provitamin A carotenoids in egg yolk for both hybrids as well as lutein content for the soft-type hybrid, whereas organic trace minerals improved the deposition efficiency of most carotenoids only for the hard-type hybrid.

Introduction

Corn grains are a main component of the diets of laying hens and are also recognized for their high content of bioactive compounds that provide desirable health benefits beyond their role as a source of energy. Yellow corn is rich in carotenoids, including xanthophylls, lutein, zeaxanthin, α-cryptoxanthin and β-cryptoxanthin, as well as carotenes, α-carotene and β-carotene, which contribute to the vibrant color of the yolk (Sheng et al., 2018; Kljak et al., 2021; Zurak et al., 2021). In addition, carotenoids exert antioxidant, anti-inflammatory, antibacterial and immunomodulatory effects that benefit both the hens and the consumers of their eggs (Nabi et al., 2020). However, to improve yolk pigmentation and the nutritional value of table eggs, the bioavailability of carotenoids must be considered. This includes release from the grain matrix, solubilization into a lipid phase and incorporation into mixed micelles so that they can be absorbed by the intestinal epithelium (Dansou et al., 2023).

In recent years, many studies have focused on understanding and improving the bioavailability of carotenoids, emphasizing the impact of various feed- and host-related factors. Among these factors, the co-digestion of carotenoids with other dietary components has been shown to affect the digestion and absorption of these compounds (Shin et al., 2015; Papadopoulos et al., 2019; Iddir et al., 2020; Dansou et al., 2023; Zurak et al., 2024a). To our knowledge, there is little data on the effects of dietary micronutrients such as vitamins and minerals on the bioavailability of corn carotenoids, as they tend to share common

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digestion steps and absorption mechanisms.

Among the various vitamins present in the diet of laying hens, vitamin A plays a vital role in maintaining optimal health and productivity of laying hens, with dietary levels usually ranging from 8,000 to 14,000 IU/kg, mainly in the form of synthetic retinyl esters. However, the intake and status of vitamin A is considered a crucial factor that could modulate the bioavailability of carotenoids due to possible competition for uptake in lipid droplets (Moreno et al., 2016; Dansou et al., 2023). It is also believed that the bioconversion potential of provitamin A carotenoids is higher in vitamin A deficiency but lower in vitamin A excess. Consistent with this, Surai et al. (1998) reported a decrease in carotenoid content in the yolk at high dietary levels of vitamin A, while Miao et al. (2023) found that at adequate dietary vitamin A status, β-carotene deposition in the yolk gradually increased with higher dietary β-carotene levels. Therefore, the metabolic fate of carotenoids at different vitamin A intakes needs to be further investigated to maximize their utilization when corn is used as the only source of pigments for laying hens.

Another dietary factor that has been shown to affect carotenoid bioaccessibility and cellular uptake in vitro is the presence of divalent minerals. Their high concentrations can interfere with digestive processes and lead to the precipitation of lipids and bile salts necessary for emulsification, which can affect the availability of fatty acids and other liposoluble microconstituents (Corte-Real and Bohn, 2018). Mineral salts such as oxides and sulfates are routinely supplied in laying hen diets; however, there is an increasing trend towards the use of organic trace minerals due to their higher bioavailability (Araújo et al., 2019; Ramos-Vidales et al., 2019). However, there are conflicting findings on the effects of different forms of minerals that warrant further investigation. For example, organic Se supplementation has been shown to improve both total carotenoid content and yolk color (Muhammad et al., 2021), while Ramos-Vidales et al. (2019) found improved yolk color in hens fed inorganic trace minerals. To our knowledge, there is little data on the response of birds to different forms of trace minerals in combination with different levels of vitamin A in the diet on the utilization of carotenoids from corn grains.

Further feeding trials are therefore required in this area to investigate the influence and possible interactions of these micronutrients on the bioavailability of carotenoids. This would allow a diet formulation that meets the trace element and vitamin A requirement of laying hens and also maximizes carotenoid absorption in the digestive tract when corn is used as the only pigment source in the diets. The aim of this study was to investigate the influence of the level of vitamin A supplementation (5,000, 10,000 and 20,000 IU/kg) and the form of trace minerals (inorganic or organic) on the content and deposition efficiency of carotenoids in the egg yolk of laying hens fed diets differing in two commercial maize hybrids.

Materials and methods

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).

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., 2021; 2024b). Both hybrids were dent types but differed in grain hardness, as determined by the Stenvert test (Zurak et al., 2024a). Based on this difference, the hybrid with the softer grains was referred to as the soft-type hybrid, while the hybrid with the harder grains was referred to as the hard-type hybrid 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 (Zurak et al., 2021, 2024a). Carotenoid content in soft-type dent hybrid was (μg/g DM): 8.45 for lutein, 13.42 for zeaxanthin, 2.08 for α-cryptoxanthin, 3.09 for β-cryptoxanthin, 0.30 for α-carotene, and 1.34 for β-carotene. Carotenoid content in hard-type dent hybrid was (μg/g DM): 5.48 for lutein, 16.20 for zeaxanthin, 1.04 for α-cryptoxanthin, 3.73 for β-cryptoxanthin, 0.24 for α-carotene, and 1.41 for β-carotene. 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 of two tested hybrids were packed in storage bags until laying hen diets were produced.

Tables 1A and 1B show the ingredient composition of diets and premixes, respectively. Experimental diets were formulated to meet the recommended nutrient requirements of commercial Lohmann Brown laying hens in the initial stage of egg production (19 to approximately 50 wk of age) according to the Lohmann Breeders GmbH (2022) guidelines. The basal mixture contained all ingredients except corn grains, which were the only sources of pigments in the diets, vitamin A and minerals and was mixed from a single batch of ingredients to reduce differences in nutrient composition. 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 vitamin A levels (5,000, 10,000 and 20,000 IU/kg; ROVIMIX A1000, DSM-Firmenich AG's, Kaiseraugst, Switzerland) and inorganic or organic trace minerals and mixed with the basal mixture, resulting in 12 dietary treatments. Bioplex (Cu 12 %, Fe

Table 1A
Ingredient and calculated nutrient composition of the basal diet.

Ingredient	Content (%)
Maize hybrid	60
Soybean meal	26.2
Sunflower oil	3
Calcium carbonate	8.8
Monocalcium phosphate	1.2
Sodium chloride	0.4
DL methionine	0.15
Vitamin premix	0.25
Calculated nutrient composition[1]	
Crude ash	12.52
Crude protein	16.43
Crude fat	5.61
Crude fibre	2.81
Calcium	3.72
Phosphorus	0.60
Phosphorus, available	0.43
Calcium/Phosphorus	6.24
Sodium	0.18
Lysine	0.87
Methionine	0.41
Methionine + cysteine	0.86
Tryptophan	0.18
Threonine	0.62
Starch	38.70
Metabolic energy (MJ/kg)	11.63

[1] 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.

Table 1B
Premixes prepared for nutritional treatments.[1].

	Dietary treatment					
Trace minerals	Inorganic			Organic		
Vitamin A, IU	5,000	10,000	20,000	5,000	10,000	20,000
Vitamin D, IU	2,500					
Vitamin E, mg	20					
Vitamin K, mg	3					
Vitamin B1, mg	1					
Vitamin B2, mg	4					
Vitamin B6, mg	3					
Vitamin B12, mg	25					
Vitamin B5, mg	10					
Vitamin B3, mg	30					
Vitamin B9, mg	0,5					
Vitamin B7, µg	50					
Choline, mg	40					
Zn, mg	80	80	80	30	30	30
Mn, mg	80	80	80	30	30	30
Cu, mg	10	10	10	5	5	5
Fe, mg	10	10	10	5	5	5
Se, mg	0.4	0.4	0.4	0.2	0.2	0.2
I, mg	1					

[1] The premixes have been formulated so that 1 kg of feed contains the amounts of vitamins and microminerals indicated in the table.

15 %, Mn 20 %, and Zn 20 %; bound to amino acids and a range of peptides; Alltech, Nicholasville, SAD) and Sel-Plex (Se 0.30 %; in the form of selenized yeast; Alltech, Nicholasville, SAD) were used as a sources of organic trace minerals. Copper sulfate, ferrous sulfate, manganese oxide, zinc oxide and sodium selenite were used as inorganic sources of these trace minerals. The dietary samples were taken at the beginning of the feeding trial for further analysis. A representative sample of diets was taken and stored at −20°C for subsequent analysis. Prior to analysis, the samples were ground in a laboratory mill (Cyclotec 1093, Foss Tocator, Hoganas, Sweden) using a 1 (nutrient compostion) or 0.3 mm (carotenoid analysis) sieve. All samples were analyzed for dry matter (DM) content by drying 3 g of each sample for 4 h at 103 ± 2 °C (ISO 6496:1999). For the nutrient composition, the crude protein (by using Kjeldahl method and multiplying N content by a factor of 6.25 according to method ISO 5983-2:2009), crude fat (by extraction with diethyl ether according to method ISO 6492:1999), ash (by ashing 1 g of the sample at 550°C for 4 h according to method ISO 5984:2002), crude fiber (by ashing the dried residue after acid and alkaline digestion according to method ISO 6865:2000), total starch [using a commercial test kit Total Starch Assay (K-TSTA; Megazyme International, Wicklow, Ireland] according to method AOAC 996.11) and sugars (using the modified Luff-Schoorl and Nelson-Somogyi methods) were determined.

Hens and housing

The experiment was conducted on 252 Lohmann Brown laying hens at 18 wk of age, in groups of 3 allocated to one cage, resulting in 84 enriched cages (Council Directive 1999/74/EC) in the experimental poultry house of the University of Zagreb Faculty of Agriculture. The cages were divided into 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 controlled automatically, 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 18 wk of age) and at the end (at 27 wk of age) of the 9-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 ingredient composition as the experimental diets containing 60 % of corn grain (Table 1A) to deplete of carotenoids from the previous diet. Laying hens were then randomly assigned to one of 12 dietary treatments in a 2 × 3 × 2 factorial design. Factors included 2 commercial corn hybrids (soft- and hard-type), one of the 3 vitamin A supplementation levels (5,000, 10,000 and 20,000 IU/kg) and mineral form (inorganic or organic). The total number of cages (replicates) per dietary treatment was 7. The experimental period lasted 63 days and was divided into a stabilization period (20 d) of the carotenoid content in the yolks and a sampling period (49 d) in which the number and weight of the eggs were recorded daily and the feed intake weekly. Based on obtained results, ADFI, egg production, egg weight, daily egg mass and FCR were calculated.

During the experimental period, one egg per cage (i.e., 7 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 20th d of the trial, the carotenoid content in the yolk had stabilized; thereafter, 3 eggs per cage (i.e., 21 eggs per treatment) were sampled once a wk for carotenoid analysis until the end of the trial. Eggs were analyzed immediately after collection; if this was not possible, eggs were stored at 4 °C until the next day. The collected eggs were cracked immediately before analysis and 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 7 samples per treatment each week.

Carotenoid analysis in the diets and egg yolks

Experimental Diets. Carotenoids from the experimental diets were extracted and quantified 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 min (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.

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, USA). 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.

Carotenoid standards were obtained from Extrasynthese (France; lutein, zeaxanthin, α- and β-cryptoxanthin; purity ≥ 98 %) and from Supelco (Bellefonte, PA; canthaxanthin, α- and β-carotene; purity ≥ 95 %). Carotenoids in the prepared extracts were identified by comparing their retention times and quantified by external standardization with calibration curves using commercially available standards ($r^2 \geq 0.99$ for all carotenoids). The total carotenoid content was calculated by summing the contents of the individual carotenoids.

Egg Yolks during the Stabilization Period (0-20 d of the Trial). The spectrophotometric method described by Surai et al. (2001) was used to determine the stabilization of carotenoid content in egg yolks. 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.

Egg Yolks during the Sampling Period (21-49 d of the Trial). Quantification of carotenoids from egg yolks collected from day 21 to the end of the experiment was performed using the reversed-phase HPLC method described previously (Section Experimental Diets) following the extraction described in Section Egg yolks according to Surai et al. (2001) with some differences; 200 mg of the combined egg yolk sample was taken for analysis. 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.

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:

Carotenoid production by egg = yolk weight (g) × yolk carotenoid content (μg/g) × egg production

Carotenoid consumption by diet = diet intake (g/d/hen) × diet carotenoid content (μg/g) based on the data obtained in the hen trial and after sample analysis.

Statistical analysis

The obtained results were analyzed using the SAS statistical software (version 9.4; SAS Institute Inc., Cary, NC). The dietary experiment was conducted as a completely randomized design with a factorial arrangement of 2 hybrids of different grain hardness, 3 levels vitamin A supplementation, and trace minerals in inorganic or organic forms 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, vitamin A level and mineral form 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 21st to 49th d of the 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

Carotenoids in the experimental diets

The analysed nutrient composition of experimental diets is shown in Table 2, while the carotenoid content is shown in Table 3. On average, the total carotenoid content of all complete feeds was 16.51 μg/g DM. α- and β- carotene were not detected, while lutein and zeaxanthin accounted for more than 80 % of the total carotenoids, with zeaxanthin being the predominant carotenoid in all experimental diets and reaching higher levels in the diets with the hard-type hybrid. Conversely, diets containing the soft-type hybrid had, on average, approximately 1.6 times higher lutein content. With a range of 1.72 to 2.46 μg/g DM, β-cryptoxanthin was the following carotenoid in all experimental diets, with higher levels in the diets with the hard-type hybrid, while the concentrations of α-cryptoxanthin were higher in the diets with the soft-type hybrid (1.16 μg/g DM vs. 0.61 μg/g DM).

Egg yolk carotenoid composition

In all treatments tested, the accumulation of carotenoids in the yolk started on d 4 after depletion, reached saturation after d 14 of the experimental period and maintained steady levels at subsequent time points (data not shown). The total carotenoid content in the yolks of laying hens fed 12 different dietary treatments ranged from 30.43 to 36.38 μg/g. Similar to the results found in the diets, both α- and β-carotene were not detected. Lutein and zeaxanthin were the predominant carotenoids and on average accounted for about 41 % and 57 % of the total carotenoids, respectively. In addition, β-cryptoxanthin was detected at about twice the level of α-cryptoxanthin, averaging 0.52 μg/g and 0.25 μg/g, respectively. The composition of both individual and total carotenoids in egg yolk was primarily affected ($P < 0.001$) by the corn hybrid used in the diet (Table 4A). Yolk samples from laying hens fed a treatment diets with the soft-type hybrid had higher lutein than zeaxanthin content, and the same treatments resulted in higher α-cryptoxanthin and total carotenoid content in the yolks. In contrast, the levels of lutein and zeaxanthin in the yolks of hens fed with the hard-type hybrid diets averaged 10.07 μg/g and 20.91 μg/g, respectively, and these yolks also contained higher levels of β-cryptoxanthin. Of the other main effects investigated, vitamin A level in the treatment diets only affected the yolk content of α-cryptoxanthin ($P = 0.002$) and β-cryptoxanthin ($P < 0.001$), with the highest yolk levels of these carotenoids found in hens fed diets containing 5,000 IU/kg of vitamin A (Tables 4A and 4B).

The hybrid × vitamin A interaction was significant for almost all individual and total carotenoids, while the hybrid × mineral form × vitamin A interaction was significant only for β-cryptoxanthin (Tables 4A). In diets with the soft-type hybrid, the highest yolk contents of lutein, α- and β-cryptoxanthin, as well as total carotenoids were found in eggs from hens fed diets supplemented with 5,000 and 10,000 IU/kg vitamin A. In contrast, the yolk contents of these carotenoids were similar in diets based on the hard-type hybrid, regardless of vitamin A supplementation level. On the other hand, the hard-type hybrid supplemented with 5,000 or 20,000 IU/kg of vitamin A resulted in the highest zeaxanthin content, while the β-cryptoxanthin content in the yolk was highest only when supplemented with 5,000 IU/kg vitamin A (Table 4B).

Efficiency of carotenoid deposition in egg yolks

The deposition efficiency of total carotenoids averaged 23.17 %. The deposition efficiency of individual carotenoids lutein and zeaxanthin in

Table 3
Carotenoid content in experimental diets differing in corn hybrid, level of vitamin A supplementation and mineral form.[1].

Corn hybrid	Vitamin A IU/kg	Mineral form	Lutein μg/kg DM	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	Total carotenoids[2]
Soft	5,000	Inorganic	6.07±0.12	6.94±0.19	1.14±0.02	1.81±0.04	15.96±0.31
	10,000		6.46±0.00	7.57±0.10	1.12±0.03	1.72±0.00	16.87±0.08
	20,000		6.47±0.10	7.59±0.27	1.22±0.02	1.83±0.01	17.11±0.39
	5,000	Organic	6.36±0.10	7.67±0.21	1.14±0.08	1.85±0.05	17.03±0.41
	10,000		5.82±0.15	7.15±0.35	1.12±0.03	1.92±0.04	16.01±0.54
	20,000		7.09±0.04	8.21±0.06	1.22±0.07	1.82±0.04	18.34±0.09
Hard	5,000	Inorganic	4.14±0.10	9.71±0.29	0.60±0.10	2.38±0.11	16.82±0.25
	10,000		3.81±0.10	8.55±0.09	0.53±0.07	2.03±0.04	14.92±0.10
	20,000		3.93±0.20	9.09±0.30	0.59±0.07	2.32±0.05	15.94±0.49
	5,000	Organic	3.55±0.37	8.15±0.42	0.58±0.04	2.10±0.01	14.37±0.77
	10,000		4.23±0.00	9.86±0.01	0.61±0.03	2.38±0.05	17.09±0.08
	20,000		4.34±0.09	10.17±0.18	0.74±0.06	2.46±0.03	17.71±0.32

[1] The composited sample of each experimental diet was used for the analysis; the values represent the mean ± SE of triplicate used in the analysis.
[2] Total carotenoid content was calculated by summarizing each individual carotenoid identified and quantified by analysis.

Table 2
Analyzed nutrient composition (%) in experimental diets differing in corn hybrid, level of vitamin A supplementation and mineral form.[1].

Maize hybrid	Vitamin A IU/kg	Mineral form	Moisture %	Crude protein	Crude fat	Ash	Crude fiber	Total starch	Sugars
Soft	5,000	Inorganic	9.3 ± 0.2	17.8 ± 0.3	5.6 ± 0.1	13.1 ± 0.1	2.9 ± 0.0	38.8 ± 0.7	3.2 ± 0.0
	10,000	Inorganic	9.0 ± 0.1	17.6 ± 0.2	5.5 ± 0.1	12.9 ± 0.0	3.1 ± 0.0	38.5 ± 0.6	3.1 ± 0.1
	20,000	Inorganic	9.0 ± 0.1	17.3 ± 0.3	5.6 ± 0.1	12.3 ± 0.1	2.6 ± 0.1	38.4 ± 0.5	3.4 ± 0.0
	5,000	Organic	9.0 ± 0.0	17.8 ± 0.3	5.7 ± 0.1	13.1 ± 0.2	2.9 ± 0.1	38.9 ± 0.4	3.5 ± 0.1
	10,000	Organic	8.7 ± 0.1	17.3 ± 0.2	5.7 ± 0.1	12.9 ± 0.1	2.8 ± 0.0	38.9 ± 0.5	3.0 ± 0.0
	20,000	Organic	8.6 ± 0.2	17.2 ± 0.1	5.7 ± 0.1	12.2 ± 0.0	3.0 ± 0.1	38.7 ± 0.5	3.4 ± 0.1
Hard	5,000	Inorganic	9.5 ± 0.0	17.7 ± 0.2	5.7 ± 0.1	12.8 ± 0.0	3.0 ± 0.0	38.5 ± 0.6	3.3 ± 0.1
	10,000	Inorganic	9.3 ± 0.1	17.8 ± 0.2	5.5 ± 0.1	12.8 ± 0.1	2.8 ± 0.1	38.6 ± 0.5	3.3 ± 0.0
	20,000	Inorganic	9.1 ± 0.2	17.6 ± 0.3	5.3 ± 0.1	13.2 ± 0.2	2.8 ± 0.0	38.6 ± 0.4	3.6 ± 0.0
	5,000	Organic	9.0 ± 0.1	17.9 ± 0.1	5.7 ± 0.1	13.0 ± 0.1	2.6 ± 0.1	38.7 ± 0.4	3.6 ± 0.1
	10,000	Organic	9.1 ± 0.2	17.4 ± 0.3	5.5 ± 0.1	13.0 ± 0.0	2.6 ± 0.0	38.4 ± 0.5	3.5 ± 0.1
	20,000	Organic	9.1 ± 0.1	17.4 ± 0.1	5.5 ± 0.1	12.9 ± 0.1	2.6 ± 0.1	38.5 ± 0.6	3.3 ± 0.0

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

Table 4A
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	Total carotenoids
			P				
Hybrid (H)			<0.001	<0.001	<0.001	<0.001	<0.001
Vitamin A (VitA)			0.183	0.517	0.002	<0.001	0.344
Mineral form (MF)			0.459	0.683	0.379	0.334	0.943
H × VitA			0.048	<0.001	0.178	0.008	0.002
H × MF			0.749	0.627	0.448	0.297	0.672
VitA × MF			0.165	0.126	0.263	0.071	0.145
H × VitA × MF			0.452	0.108	0.282	0.017	0.223
Hybrid[2]	Vitamin A level[2]	Mineral form[2]	μg/g				
Soft			17.40a	17.00b	0.32a	0.43b	35.16a
Hard			10.07b	20.91a	0.17b	0.60b	31.74b
	5,000 IU/kg		13.99	19.14	0.26a	0.54a	33.94
	10,000 IU/kg		13.70	18.72	0.25a	0.50b	33.17
	20,000 IU/kg		13.51	18.99	0.24b	0.50b	33.24
		Inorganic	13.81	18.89	0.25	0.51	33.47
		Organic	13.66	19.01	0.25	0.52	33.43

[1] n = 49 (7 replicates per treatment × 7 wk of sampling period).
[2] Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($P = 0.05$).

the yolks averaged 31.86 % and 27.53 %, respectively, which was up to 9 times higher than the deposition efficiency of α-cryptoxanthin and β-cryptoxanthin (on average 3.45 % and 3.05 %, respectively). Similar to the carotenoid content in the yolk, corn hybrid affected the deposition efficiency of zeaxanthin ($P = 0.039$), α-cryptoxanthin ($P = 0.019$) and β-cryptoxanthin ($P < 0.001$) and tended to affect the deposition efficiency of lutein ($P = 0.053$), with higher deposition efficiency observed in hens fed diets containing the hard-type corn hybrid. Of the remaining main effects, mineral form had a relatively small influence on the deposition efficiency of carotenoids, affecting only α-cryptoxanthin ($P = 0.023$), with higher values in the egg yolk of hens fed diets supplemented with inorganic trace minerals (3.56 % vs. 3.32 %). However, vitamin A had a significant effect on the deposition efficiency of all carotenoids, resulting in higher values in the egg yolks of hens fed diets containing 5,000 IU/kg vitamin A compared to other supplementation levels (Table 5A and Table 5B).

Of the interactions, corn hybrid × mineral form was significant only for α-cryptoxanthin (Table 5A). In addition, the mineral form × vitamin A interaction was significant for all individual and total carotenoids (Table 5A), with the highest deposition efficiency of lutein, zeaxanthin,

Table 4B
Effect of hybrid and vitamin A level on carotenoid content and composition in yolks of laying hens.[1,2].

Hybrid	Vitamin A IU/kg	Lutein μg/g	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	Total carotenoids
Soft	5,000	17.14a	16.63c	0.33	0.43c	34.53a
	10,000	16.67b	16.70c	0.31	0.43c	34.10ab
	20,000	16.47b	16.05c	0.30	0.40d	33.23b
Hard	5,000	9.83c	20.75a	0.17	0.61a	31.38c
	10,000	9.76c	19.97b	0.16	0.55b	31.29c
	20,000	9.68c	20.83a	0.16	0.56b	30.37c
	SEM	0.16	0.24	0.004	0.01	0.38

[1] Content od carotenoids is presented as mean ± SEM; $n = 49$ (7 replicates per treatment × 7 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 5A
Analysis of variance for carotenoid deposition efficiency in yolks of laying hens with means for the main effect of investigated factors.[1].

Source of variation			Lutein	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	Total carotenoids
			P				
Hybrid (H)			0.053	0.039	0.019	<0.001	0.632
Vitamin A (VitA)			<0.001	<0.001	<0.001	<0.001	<0.001
Mineral form (MF)			0.879	0.900	0.023	0.530	0.584
H × VitA			0.057	0.074	0.167	0.149	0.259
H × MF			0.231	0.2178	0.013	0.581	0.342
VitA × MF			<0.001	<0.001	0.005	<0.001	<0.001
H × VitA × MF			<0.001	<0.001	0.264	0.019	<0.001
Hybrid[2]	Vitamin A level[2]	Mineral form[2]	%				
Soft			32.28	26.68b	3.34b	2.82b	23.24
Hard			31.45	28.37a	3.55a	3.28a	23.10
	5,000 IU/kg		33.21a	27.55b	3.64a	3.21a	24.08a
	10,000 IU/kg		32.03b	27.50b	3.57a	3.06b	23.29b
	20,000 IU/kg		30.34c	28.75a	3.11b	2.88c	22.14c
		Inorganic	31.89	27.54	3.54a	3.06	23.26
		Organic	31.83	26.29	3.35b	3.03	23.08

[1] n = 49 (7 replicates per treatment × 7 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 5B
Effect of hybrid, mineral form and vitamin A level on carotenoid deposition efficiency in yolks of laying hens.[1,2].

Hybrid	Vitamin A IU/kg	Mineral form	Lutein %	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	Total carotenoids
Soft	5,000	Inorganic	34.66ab	27.87bcde	3.44	2.72ef	24.26b
	10,000		31.53de	26.44de	3.44	2.93bcde	23.19b
	20,000		31.51de	26.59de	3.12	2.81def	22.99bc
	5,000	Organic	33.26bcd	27.86bcde	3.70	3.11bc	24.07b
	10,000		34.11abc	28.04bcd	3.27	2.74def	24.16b
	20,000		28.59f	23.27f	2.99	2.61f	20.80d
Hard	5,000	Inorganic	29.20f	26.18de	3.72	3.18b	21.56cd
	10,000		32.35cd	29.68b	4.04	3.60a	24.31b
	20,000		32.12cde	28.54bc	3.60	3.13bc	23.23b
	5,000	Organic	35.72a	33.10a	3.69	3.81a	26.43a
	10,000		30.12ef	25.99e	3.54	2.96cde	21.50cd
	20,000		29.17f	26.77cde	2.73	2.97bcd	21.54cd
		SEM	0.74	0.69	0.11	0.09	0.54

[1] Carotenoid deposition is presented as mean ± SEM; $n = 49$ (7 replicates per treatment × 7 wk of sampling period).
[2] Means followed by the same letter in the same column do not differ statistically among themselves by Tukey test ($P = 0.05$).

β-cryptoxanthin and total carotenoids found in yolks from hens fed diets containing organic trace minerals and 5,000 IU/kg vitamin A. In contrast, the highest values for α-cryptoxanthin were found in hens fed diets containing inorganic trace minerals and 10,000 IU/kg vitamin A. For all carotenoids except α-cryptoxanthin (Table 5A), there was a significant hybrid × vitamin A × mineral form interaction, clearly indicating hybrid-specific responses. This interaction was found mainly because the hard-type hybrid had the highest deposition efficiency of all carotenoids except α-cryptoxanthin when diet was supplemented with organic trace minerals and the lowest vitamin A level (Table 5B), which was not found for the soft-type hybrid.

Discussion

The differences between the experimental diets in the carotenoid profiles were attributed to the differences in the carotenoid profiles of the commercial corn hybrids used, as these were the main source of pigment in the diets. The range of total carotenoid content was similar to the range reported for commercial diets containing 60 % of corn grain as in the present study (13.45 − 17.13 μg/g DM; Kljak et al., 2021) and higher than the values reported by Moreno et al. (2020; contining 62.06 % of corn grain; 9.2 μg/g DM) and Ortiz et al. (2021; contining 56.5 % of corn grain; 8.8 μg/g DM) for commercial yellow corn diets. The experimental diets in the present study had higher levels of zeaxanthin and

β-cryptoxanthin than those in the study by Ortiz et al. (2021) and Kljak et al. (2021), while the latter reported a similar range of lutein content. Nevertheless, diets consisting of the soft-type hybrid had higher levels of lutein and α-cryptoxanthin, i.e. α-branch carotenoids, while diets with the hard-type hybrid contained higher levels of zeaxanthin and β-cryptoxanthin, i.e. β-branch carotenoids. This aligns with previous findings suggesting an association between grain hardness and carotenoid profile (Saenz et al., 2021; Kljak et al., 2024).

The intake of carotenoids in laying hens is affected by diet intake, and in this regard, the amount of diet consumed could affect carotenoid content and deposition efficiency in the egg yolk. Therefore, the production performance of laying hens fed treatment diets was monitored in parallel with the carotenoid analysis in the egg yolk. Statistical analysis of the obtained data showed that the main factors and their interactions had a significant effect on some of the production performance parameters (data not shown). However, ADFI and egg production were similar for all dietary treatments. In addition, dietary treatments differed in egg weight and daily egg mass, but yolk weight remained similar. As ADFI, egg production and yolk weight were similar for the tested dietary treatments, the differences in yolk carotenoid content and deposition efficiency could be attributed to the differences in diet composition.

The total carotenoid content in the yolks of laying hens 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; Kljak et al., 2021; Ortiz et al., 2021; Table 4), with the yolk levels of lutein and zeaxanthin being higher than those of α-cryptoxanthin and β-cryptoxanthin throughout the experimental period. In agreement with previous findings (Liu et al., 2012; Kljak et al., 2021; Ortiz et al., 2021), the carotenoid profiles of the yolks reflected the carotenoid composition of the investigated dietary treatments, i.e. of the corn hybrids used as the basis for the experimental diets, with yolk contents of α-branch and total carotenoids being higher in hens fed the soft-type hybrid diets, while the hard-type hybrid diets resulted in higher yolk contents of β-branch carotenoids, and the levels determined were consistent with the results obtained for different corn genotypes (Moreno et al., 2016; Kljak et al., 2021; Ortiz et al., 2021). Regardless of the diet supplementation, lutein consistently showed higher deposition efficiency compared to zeaxanthin, while the deposition efficiency of α-cryptoxanthin exceeded that of β-cryptoxanthin. These current data are consistent with observations from other studies, confirming the preferential transport of more polar carotenoids from the diet into the yolk, resulting in more efficient deposition of xanthophylls compared to provitamin A carotenoids, with deposition efficiency decreasing at higher dietary carotenoid levels (Liu et al., 2012; Kljak et al., 2021; Zurak et al., 2024a). Nevertheless, deposition efficiencies were comparable to previously reported values (Kljak et al., 2021; Zurak et al., 2024a), ranging from 21.75 to 34.20 % for lutein, from 17.17 to 29.53 % for zeaxanthin, from 2.92 to 11.21 % for β-cryptoxanthin, from 4.13 to 9.88 % for α-cryptoxanthin, and from 16.11 to 24.75 % for total carotenoids.

The grain characteristics of different hybrids influence the release of carotenoids during in vitro digestibility, with the amounts of carotenoids released being higher in harder hybrids than in softer hybrids (Zurak et al., 2024b). The differences in grain porperties between the tested hybrids are the main effect that determines the amount of carotenoids available for absorption. However, whether the released carotenoids are absorbed and deposited depends on other other feed- and host-related factors. In the present study, the final carotenoid content in the yolks and the deposition efficiency depended on the dietary provision of vitamin A. The addition of 5,000 IU/kg vitamin A increased the utilization and thus the yolk content of provitamin A carotenoids and the deposition efficiency of all individual carotenoids except zeaxanthin (Tables 4A, 4B, 5A and 5B). After ingestion, provitamin A carotenoids can be directly absorbed by enterocytes or cleaved to retinal or apocarotenoids. Adequate vitamin A status (8,000 IU/kg) has been shown to limit the bioconversion efficiency of β-carotene through negative feedback regulation in the intestine, resulting in higher deposition in the body in intact form (Miao et al., 2023), which could explain the higher content and deposition of α- and β-cryptoxanthin in the yolk at a vitamin A level of 5,000 IU/kg. The results of the present study also demonstrated that an increased dietary vitamin A content reduces the bioavailability of corn carotenoids in laying hens (Table 4B), probably due to greater competition for micellar incorporation in the gastrointestinal tract. The extent to which carotenoids are absorbed in micelles depends on their polarity and fatty acid composition, chain length and saturation, which are influenced by other dietary components and the intrinsic properties of the host organism. The vitamin A content in the diet may therefore influence this equilibrium and affect the incorporation of carotenoids into lipid droplets (Moreno et al., 2016), possibly leading to reduced carotenoid absorption at higher dietary vitamin A levels. Furthermore, the vitamin A effect on yolk content was hybrid-dependent for almost all quantified carotenoids, consistent with the different response of diets with hybrids of different hardness. Although yolk contents of α- and β-cryptoxanthin were higher at the lowest dietary vitamin A level for both hybrids studied, the vitamin A supplementation level did not affect content of lutein, α-cryptoxanthin and total carotenoids in yolks from hens fed the hard-type hybrid diet (Tables 4A and 4B). This could be due to a competitive relationship between the deposition of provitamin A carotenoids and xanthophylls resulting from competition for micellar incorporation, as previously reported by Miao et al. (2023), who found that the amounts of lutein and zeaxanthin in egg yolk decreased with the deposition of β-carotene ($P < 0.05$). However, the exact mechanism requires further research.

The bioavailability of carotenoids was also influenced by the form of trace minerals in the laying hens diets (Tables 4A, 4B, 5A and 5B). However, this effect was more pronounced on the efficiency of carotenoid deposition than on the carotenoid content in the egg yolk and also depended on the corn hybrid and the vitamin A level in the diets, as indicated by the significant hybrid × vitamin *A* × mineral interaction. Our results indicate that the addition of organic trace minerals in combination with 5,000 IU/kg of dietary vitamin A can increase the bioavailability of all carotenoids except α-cryptoxanthin from corn grains of the tested hard-type hybrid (Table 5B). This could be due to less disrupted micellar incorporation at lower dietary vitamin A levels (Moreno et al., 2016) and, on the other hand, improved stability of the chelated mineral complexes, which are less affected by factors leading to precipitation of lipids and bile acids, as is the case for minerals ionized after salt solubilization (Bao and Choct, 2009). In fact, the addition of coated trace minerals to corn-soybean meal diets improved lipid metabolism in broiler chickens (Yin et al., 2022), and the results of the present study suggest that this could also lead to higher bioavailability of carotenoids as they share the same digestive pathway (Dansou et al., 2023). However, the observed higher deposition efficiency of lutein in the soft-type hybrid diets with added inorganic trace minerals indicated strong hybrid-specific responses, suggesting that differences in the fatty acid composition of the yolk resulting from variations in trace mineral sources in the diets (Hemly et al., 2024) can also influence the deposition of these carotenoids. In particular, the above authors reported higher levels of β-carotene, saturated fatty acids and polyunsaturated fatty acids in the yolks of hens fed diets supplemented with sodium selenite compared to those fed organic selenium, which had higher levels of monounsaturated fatty acids in the yolks. Nevertheless, a deeper understanding of this effect is necessary.

Conclusions

The differences in carotenoid profile between the hard- and soft-type corn hybrids had a main effect on the carotenoid content and deposition efficiency. Although recommneded supplemntation level of vitamin A in diets of Lohmann Brown laying hens is 10,000 IU/kg, the lower supplemetation level (5,000 IU/kg) resulted in the highest contents of α- and β-cryptoxanthin in egg yolk and the highest deposition efficiency for

most individual carotenoids, suggesting minimal impairment of micellization efficiency. Furthermore, these results suggest that organic trace minerals can both improve the utilization of most carotenoids from corn grains of the hard-type hybrid and reduce environmental impact, as they are added to the diets of laying hens at lower concentrations and are also more bioavailable compared to inorganic sources. However, the highest deposition efficiency of lutein and subsequently total carotenoids in the yolks of hens fed soft-type hybrid diets when inorganic trace minerals were added, warrants further investigation of factors such as dietary fatty acid profile that could influence the deposition of these carotenoids in addition to the supplementation of vitamin A and trace minerals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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