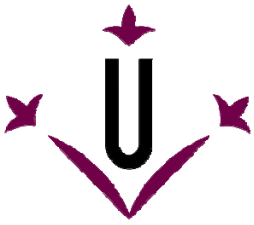

# Provitamin A carotenoids from an engineered high-carotenoid maize are bioavailable and zeaxanthin does not compromise β-carotene absorption in poultry

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## Abstract

High-carotenoid (HC) maize, a biofortified staple crop which accumulates β-carotene, β-cryptoxanthin, lutein and zeaxanthin, was used as a feed component in a chicken feeding trial to assess the bioavailability of provitamin A (PVA) carotenoids in the kernel matrix compared to the synthetic and natural color additives routinely used in the poultry industry. We found that the PVA carotenoids in HC maize were not metabolized in the same manner: β-carotene was preferentially converted into retinol in the intestine whereas β-cryptoxanthin accumulated in the liver. We also considered the effect of zeaxanthin on the absorption of PVA carotenoids because zeaxanthin is the major carotenoid component of HC maize. We found that chickens fed on diets with low levels of zeaxanthin accumulated higher levels of retinol in the liver, suggesting that zeaxanthin might interfere with the absorption of β-carotene, although this observation was not statistically significant. Our results show that HC maize provides bioavailable carotenoids, including PVA carotenoids, and is suitable for use as a feed component.

**Keywords:** *chicken, bioavailability, metabolic engineering, pigments, β-carotene*

## Introduction

Carotenoids are isoprenoids classified as carotenes (hydrocarbons) or xanthophylls (oxygenated derivatives) (Namitha and Negi 2010). Some carotenoids possess provitamin A (PVA) activity (e.g., α-carotene, β-carotene and β-cryptoxanthin) whereas others act as antioxidants but cannot be converted into vitamin A (e.g., zeaxanthin and lutein) (Farré et al. 2010). Humans acquire dietary carotenoids predominantly from plant-based foods. PVA carotenoids are important nutrients that are required to prevent

vitamin A deficiency (VAD), whereas other carotenoids provide more general health-promoting antioxidant activity (Institute of Medicine 2001; Bramley 2003).

Staple crops with enhanced carotenoid levels have been developed through conventional breeding (e.g., potato, maize, cassava and pumpkin) or genetic engineering (e.g., rice, maize, potato and tomato) and these could help to maintain the health of populations at risk of VAD without the need to fortify processed foods or provide supplements (Gómez-Galera et al. 2010; Bai et al. 2011; Saltzman et al. 2013; Zhu et al. 2013). High-carotenoid (HC) maize, which was generated through transformation of the South African white maize inbred line M37W, accumulates large amounts of β-carotene, β-cryptoxanthin, lutein and zeaxanthin (Zhu et al. 2008).

In the human body, vitamin A is absorbed as preformed vitamin A from meat and dairy products or as PVA carotenoids from plants (Sanahuja et al. 2013). Animal models are useful to evaluate carotenoid absorption and metabolism because target tissues such as the liver can be tested (Lee et al. 1999). Chickens are a suitable model to study vitamin A absorption and availability because vitamin A and carotenoid metabolism is similar to humans, hence chickens are also susceptible to VAD (NRC 1994; Pretorius and Schönfeldt 2013). Like most other animals, chickens obtain carotenoids from their diet (Breithaupt 2007; Moreno et al. 2016). Carotenoids supplements are commonly used in poultry nutrition to achieve the skin pigmentation desired by many consumers and vitamin A is added as retinyl ester to ensure normal growth and development (Castañeda et al. 2005; Yuan et al. 2014).

Previous studies have established HC maize as an alternative to color additives and vitamin A supplements in poultry feed formulations for laying hens (Moreno et al. 2016) and broilers (Nogareda et al. 2016). It is important to focus on nutrient

bioavailability rather than on the total amount of nutrient provided, because the bioavailability (defined as the amount of an ingested nutrient that is available for utilization or storage) is influenced by dietary and physiological factors (Sanahuja et al. 2013; Carbonell-Capella et al. 2014). Furthermore, carotenoid absorption and metabolism are affected by interaction with other carotenoids (Furr and Clark 1997; Yeum and Russell 2002). For example, in chicks, the ingestion of more zeaxanthin inhibits the accumulation of lutein in plasma and non-retinal tissues (and vice versa) whereas the ingestion of more β-carotene inhibits the accumulation of zeaxanthin and lutein in plasma and most tissues (Wang et al. 2010). The competition between lutein and β-carotene has been widely studied, but the role of zeaxanthin has not been investigated in detail (Yeum and Russell 2002; Mamatha and Baskaran 2011).

We therefore investigated the bioavailability of the PVA carotenoids β-carotene and β-cryptoxanthin supplied as intrinsic components of the HC maize compared to the same carotenoids provided by a near isogenic maize line supplemented with synthetic or natural color additives. We also considered whether the absorption of PVA carotenoids is affected by zeaxanthin, the most abundant carotenoid in HC maize.

## Materials and methods

### *Diet preparation*

The South African white maize inbred M37W and HC maize (Zhu et al. 2008) were grown in an experimental field at the University of Lleida, Northeast Spain. After harvest, maize cobs were dried at 35°C for 24 h in a drying chamber before milling (Ras® Mill, Romer® Labs Inc., Union, MO, USA). The diets were formulated and prepared at the Mas de Bover Research Center (IRTA, Reus, Spain) according to National Research Council recommendations (NRC 1994). The feed composition was

58% maize, 18% soybean meal, 16% soybean hull and 4% soybean oil (Supplementary Table S1). Vitamin A was included in the vitamin-mineral premix at a concentration of 8,000 IU/kg (2.4 mg retinol/kg) to ensure normal growth and development (Yuan et al. 2014). The M37W control diet was prepared before the HC diet to avoid cross-contamination. Humidity, protein, fat, fiber and ash contents were measured following European Union (EU) regulations (European Commission 2009). Mycotoxin levels were determined using enzyme immunoassay kits (Ridascreen®, R-Biopharm AG, Darmstadt, Germany).

Four experimental diets were prepared: (a) M37W control diet supplemented with synthetic color additives (zeaxanthin, lutein and β-carotene); (b) M37W control diet supplemented with synthetic color additives (lutein and β-carotene but not zeaxanthin); (c) M37W control diet supplemented with natural color additives (marigold flowers and red paprika extracts); (d) HC diet, based on high-carotenoid maize, with no color additives. The synthetic and natural color additives were introduced using a planetary mixer (Sammic, Azkoitia, Spain). Fortification was carried out in three batches of 4 kg to avoid carotenoid degradation, and each batch was used immediately after preparation.

The carotenoid content of the HC diet has been analyzed (Díaz-Gómez et al. *in press*), and similar levels of PVA carotenoids as found in the HC diet were added to the control diets as synthetic (3.6 mg β-carotene/kg feed) or natural (1 g red xantophylls/kg feed and 0.3 g yellow xanthophylls/kg feed) color additives using a balance with a precision of 0.1 mg (Gram Precision, Barcelona, Spain). The purity of each synthetic compound and the marigold and red pepper extract compositions were taken into account in the calculations. We did not add β-cryptoxanthin to the feed formulation when synthetic additives were included, but its activity as a PVA carotenoid was taken into consideration in the calculations: 24 μg of β-cryptoxanthin is required to produce 1 μg

retinol activity equivalent (RAE) (Institute of Medicine 2001). The corresponding quantity was added as β-carotene (12 μg of β-carotene is required to produce 1 μg of RAE) (Institute of Medicine 2001). The PVA carotenoid content of the diets is shown in Table 1. Different levels of zeaxanthin were introduced to determine whether zeaxanthin affects the absorption of PVA carotenoids. The zeaxanthin and lutein content of the diets is shown in Table 2. Yellow xanthophylls (marigold flower extract, Capsantal EBS-40-NT) and red xanthophylls (red paprika extract, Capsantal FS-20-NT) were provided by Industrial Técnica Pecuaria S.A. (Barcelona, Spain) and synthetic additives (zeaxanthin, lutein and β-carotene) were provided by Cymit Química S.L. (Barcelona, Spain).

*Experimental design and growth performance*

Thirty-six 7-day-old male broiler chickens of the Ross 308 strain were obtained from a commercial hatchery, where they were fed on a wheat-barley-soy diet. The animals were weighed, wing-banded for identification and placed in a temperature-controlled room in the University of Lleida Animal Research Center. All chickens were fed on a basal diet with a low carotenoid content (based on M37W maize with no color additives) for the first 7 days to deplete and equalize the quantity of carotenoids in the animals. After this depletion period, four chickens were slaughtered to determine baseline serum and liver carotenoid concentrations. The remaining chickens (n = 32) were then randomly allocated to individual cages (40 cm width, 40 cm depth and 45 cm height, with a floor slope of 8%), eight cages per treatment. From day 14 until slaughter on day 28, the animals were fed on one of the four prepared diets described above. During the first week on the basal diet, feed and tap water were provided *ad libitum* through the same feeders and water dispensers, whereas individual feeders and water dispensers were used for each experimental treatment during the remaining 2 weeks. No

143 medication was administered during the feeding trial. Environmental conditions
144 (temperature, humidity, lighting and ventilation) were monitored with a mini data
145 logger (Testo, Lenzkirch, Germany) and changed according to the age of the birds,
146 following National Research Council guidelines (NRC 2011).

147 Body weights were measured each week of the experimental period and feed intake was
148 measured every time the feeders were filled in accordance with standard industry
149 methods, using a balance with a precision of 0.01 g (Kern, Balingen, Germany). After 2
150 weeks on the experimental diets, all the chickens were slaughtered on day 28 for gross
151 necropsy. Pre-chilled whole liver, spleen and bursa of Fabricius were collected and
152 weighed. Serum and freeze-dried (fd) liver samples were stored at –20°C for carotenoid
153 analysis.

154 The experimental procedure was approved by the Ethics Committee for Animal
155 Experimentation of the University of Lleida and the Catalan Government (reference
156 number DAAM 7692), following EU regulations (European Commission 2007;
157 European Parliament 2010) as well as best practices recommended by the International
158 Life Sciences Institute (ILSI 2007).

159 *Carotenoid analysis*

160 The HC diet was analyzed before the feeding trial to calculate the quantities of PVA
161 carotenoids to be added to the control diets. Because the synthetic and natural color
162 additives were introduced into the control diets in three batches, each batch was
163 randomly sampled, and the samples from each batch were combined to obtain an
164 aggregate sample for carotenoid analysis. Total carotenoids were extracted from freeze-
165 dried samples in 20 mL methanol containing 12% KOH at 65°C for 1 h. Lipophilic
166 compounds were partitioned into 30% diethyl ether in petroleum ether, the upper phase

was collected and the solvent was evaporated under a stream of nitrogen gas at 37°C. For HPLC separation, samples were re-dissolved in 100 µL methanol/dichloromethane (50:50, v/v) and a 20-µL aliquot was injected immediately. Compounds were separated on a 15-cm Nucleosil C18 3-µm column with an acetonitrile, methanol and 2-propanol mobile phase (85:15:5, v/v/v) at 20°C. Samples were monitored with a Kontron 440 photodiode array detector with online registration of the spectra, and were identified by comparison with authentic reference compounds (Sandmann 2002).

*Colorimetric analysis*

A compact portable spectrophotometer CM-700d (Konica Minolta, Tokyo, Japan) was used to measure the color according to the CIELab trichromatic system based on lightness (L*), redness (a*) and yellowness (b*) (CIE 2004). Coordinate L* represents lightness ranging from 0 (black) to 100 (white), a* indicates the red/green component and b* indicates the yellow/blue component. The range of both chromatic components is between −128 and 128 (Sharifzadeh et al. 2014). The illuminant D65, a viewing angle of 10° and an 8-mm mask were used for all measurements. Color evolution during the trial was measured twice per week in the footpad, which was cleaned before analysis. After slaughter, the color was measured in pre-chilled footpad, breast skin and breast and thigh muscles.

*Statistical analysis*

Analysis of variance (ANOVA) statistical tests and Tukey's honest significant difference (HSD) test were used for comparison of means (JMP® Pro 12 SAS institute, 2015). Differences among means were regarded as significant at $P < 0.05$. A Grubbs' test was used to detect significant outliers ($P < 0.05$). Variables expressed in percentages were normalized using the arcsine of the square root of the probability.

## Results

### *Compositional analysis*

The diets were substantially equivalent except for carotenoid levels according to the standard compositional analysis (Supplementary Table S1). Mycotoxin analysis detected only fumonisins (FBs) and zearalenone (ZEA), at 0.32 and 0.18 mg FBs/kg feed and 5.16 and 1.83 µg ZEA/kg feed, in the control and HC diets, respectively. The mycotoxin levels were therefore below the thresholds set by EU regulations (European Commission 2002, 2003, 2006, 2013).

### *Growth performance*

There were no significant differences ($P > 0.05$) in productivity parameters (Table 3) or in organ weight (Supplementary Table S2) among chickens fed on the different diets.

### *Carotenoid and retinol levels*

The β-carotene levels were quite similar in all the diets, although they were slightly lower in the control diet with natural additives (Table 1). Only the HC and control diet with natural additives contained β-cryptoxanthin, and there was no significant difference between the levels in each diet ($P > 0.05$). As expected, there were significant differences ($P < 0.05$) in zeaxanthin and lutein levels among the diets, given that different amounts of zeaxanthin and lutein were deliberately added to the control diet (Table 2). The HC diet contained the highest amount of zeaxanthin whereas the control diet with natural additives contained the highest amount of lutein. Violaxanthin was only detected in the HC diet (0.11 ± 0.01 µg/g fd feed).

The differences in zeaxanthin and lutein levels in the feed were also observed in the serum and liver samples (Table 2). The highest zeaxanthin levels were detected in the

serum and liver of chickens fed on the HC diet, whereas the highest lutein levels were found in the serum and liver of chickens fed on the control diet with natural additives. Violaxanthin was not detected in the serum from chickens in any of the diet groups, but violaxanthin and/or similar polar epoxides were detected in the liver. Chickens fed on the HC diet had significantly higher levels of polar epoxides in the liver compared to the other diets (0.94 ± 0.13 µg/g fd liver; $P < 0.05$). The control diets with natural or synthetic additives contained similar levels of these compounds (0.32–0.50 µg/g fd liver). We detected β-cryptoxanthin only in the serum and liver from chickens fed on the HC diet (0.16 ± 0.02 µg/mL serum, 5.69 ± 1.21 µg/g fd liver). We did not detect β-carotene in the serum or liver of any chickens in any of the diet groups. However, β-carotene epoxides were detected in the liver samples from chickens fed on the HC diet (1.33 ± 0.39 µg/g fd liver). There were no significant differences in retinol levels (Figure 1) in the serum or liver among chickens fed on the different diets ($P > 0.05$). Nevertheless, chickens fed on the control diet with synthetic additives lacking zeaxanthin had the highest retinol levels in the liver. Principal component analysis (PCA) revealed a trend towards higher retinol levels in liver when PVA carotenoids were supplied by diets containing low levels of zeaxanthin (Supplementary Figure S1).

*Color parameters*

Significant differences ($P < 0.001$) in redness and yellowness among chickens fed on the different diets were observed 3 days into the feeding trial (day 17), whereas significant differences ($P < 0.001$) in lightness were not found until a week (day 21) (Figure 2). The lowest redness and yellowness were found in chickens fed on the control diet with synthetic additives lacking zeaxanthin.

The differences in color parameters found during the trial were corroborated when color was measured after slaughter in pre-chilled footpad, breast skin, breast muscle and thigh muscle (Supplementary Table S3). There were no significant differences in lightness ($P > 0.05$), except for footpad lightness ($P < 0.001$), with the highest values found in chickens fed on the control diet with synthetic additives lacking zeaxanthin. There were significant differences in redness and yellowness in breast skin and in breast and thigh muscles ($P < 0.05$). Chickens fed on the HC diet had the highest yellowness score whereas chickens fed on the control diet with natural additives had the highest redness score. This difference was more evident in the breast skin and breast muscle yellowness score. The lowest redness and yellowness scores in breast and thigh muscles were observed in chickens fed on the control diet with synthetic additives lacking zeaxanthin.

## Discussion

The xanthophylls lutein and zeaxanthin are found in yellow maize, whereas PVA carotenoids such as β-carotene are not present at significant levels (Davis et al. 2008). Novel crops with enhanced carotenoid levels, such as HC maize, offer an alternative to the fortification of processed food products or the provision of feed additives (Gómez-Galera et al. 2010; Nogareda et al. 2016). Nevertheless, carotenoid bioavailability in novel crops must be assessed because the efficiency of nutrient uptake from foods is influenced by many factors, including the food matrix, food processing and gastrointestinal absorption (Sanahuja et al. 2013). We therefore tested the bioavailability of the PVA carotenoids in HC maize compared to the same carotenoids provided by its near isogenic line (M37W) (Zhu et al. 2008) supplemented with synthetic or natural color additives. We also investigated whether zeaxanthin, a major carotenoid in HC maize, affects the absorption of PVA carotenoids.

HC maize as a feed component did not adversely affect any of the criteria we evaluated (e.g., production parameters and organ weight), which confirms the results of earlier poultry nutrition experiments with this variety (Moreno et al. 2016; Nogareda et al. 2016). The performance of the control diet supplemented with synthetic and natural color additives also agree with earlier trials, confirming that the additives did not affect body weight or feed consumption (Pérez-Vendrell et al. 2001; Liu et al. 2008). We added synthetic β-carotene to the M37W control diets to achieve the same levels present in the HC diet, but there was some loss during feed preparation and storage despite the preparation of diets in three batches to avoid carotenoid degradation (Jintasataporn and Yuangsoi 2012). Similar losses of vitamin A have been reported in supplemented poultry diets, although the losses were not statistically significant (Pretorius and Schönfeldt 2013). As previously reported for other biofortified crops (e.g., maize and pumpkin), the carotenoids in HC maize are likely to be protected from degradation by interactions with the food matrix (Díaz-Gómez et al. 2017).

Zeaxanthin and lutein are the major carotenoids in HC maize and marigold flowers, respectively, and this explains why those carotenoids were present at the highest levels in the HC diet and the control diet with natural additives, respectively (Breithaupt 2007; Naqvi et al. 2009). The small amount of zeaxanthin found in the control diet supplemented with synthetic carotenoids lacking zeaxanthin came from the M37W maize, which contains traces of carotenoids, primarily lutein and zeaxanthin (Naqvi et al. 2009). As expected, xanthophyll levels in the serum and liver were primarily determined by feed composition: chickens fed on the HC diet and control diet with natural additives accumulated the highest serum/liver zeaxanthin and lutein levels, respectively. Violaxanthin and/or similar polar epoxides were found in livers from chickens fed on all four diets despite only being detectable in the HC diet formulation,

286 which may reflect the metabolic conversion of zeaxanthin to violaxanthin. Nutrients
287 integrated in the food matrix are released more slowly and absorbed over a longer
288 period for more efficient assimilation (Moreno et al. 2016), and for that reason
289 violaxanthin levels in livers from chickens fed on the HC diet were 8.5–fold more
290 concentrated than the levels in the HC diet itself. This efficient transfer was previously
291 observed in eggs laid by hens fed on a diet based on BKT maize, a biofortified maize
292 rich in ketocarotenoids (Moreno et al. 2016).

293 The PVA carotenoids in HC maize (β-carotene and β-cryptoxanthin) were bioavailable
294 and contributed to the storage of vitamin A in the liver as retinol (Moreno et al. 2016).
295 Consequently, β-carotene was not detected in the serum or liver samples from any of the
296 diet groups because it was converted efficiently into retinol. However, in chickens fed
297 on the HC diet, β-carotene epoxides were detected in liver, and these could have a
298 protective role due to their antioxidant activity (Gurak et al. 2014). In contrast, β-
299 cryptoxanthin was detected in liver samples but only from chickens fed on the HC diet,
300 and the concentration of this PVA carotenoid was higher than originally present in the
301 feed. The higher β-cryptoxanthin levels in the liver may reflect the fact that β-carotene
302 is a better substrate for β-carotene 15,15'-dioxygenase (BCDO1) (Kim and Oh 2009;
303 Kim and Oh 2010). These results support our earlier study in chickens in which the food
304 matrix was shown to play an important role in the bioavailability of carotenoids, given
305 that β-cryptoxanthin in HC maize is provided as an intrinsic component (Díaz-Gómez et
306 al. *in press*). Regarding vitamin A absorption, it would have been ideal to study β-
307 carotene and β-cryptoxanthin conversion to retinol separately. However, this is not
308 practical as PVA carotenoids in HC maize are provided within the kernel matrix in
309 which zeaxanthin and lutein are also present.

The efficiency of β-carotene transfer from the feed to liver was highest in the control diet with synthetic additives lacking zeaxanthin, followed by the control diet with natural additives, the control diet with synthetic additives including zeaxanthin, and finally the HC diet. Zeaxanthin appears to attenuate the bioaccessibility of β-carotene, and the underlying mechanism may be competition for solubilization in mixed micelles and absorption in the intestine (Furr and Clark 1997; Yeum and Russell 2002). Bioaccessibility is the amount of an ingested nutrient that is released from the food matrix in the gastrointestinal tract and becomes available for absorption, whereas bioavailability is the amount of an ingested nutrient that is available for utilization or for storage (Carbonell-Capella et al. 2014; Díaz-Gómez et al. 2017). Carotenoids can interact with each other during absorption, metabolism and transport (Yeum and Russell 2002; Bohn 2008). Xanthophylls such as zeaxanthin and lutein are absorbed more efficiently than β-carotene in chickens and humans because they are more polar (Furr and Clark 1997; Na et al. 2004). The location of carotenoids in the lipid droplet also affects its absorption because oxygenated carotenoids (xanthophylls) are located near the surface whereas hydrocarbon carotenoids (carotenes) are located within the core (Borel et al. 1996; Yeum and Russell 2002). However, the bioaccessibility of PVA carotenoids is minimally affected by maize xanthophylls, but their uptake by cells is inhibited by lutein in a dose-dependent manner (Thakkar and Failla 2008). Different concentrations of maize xanthophylls did not have any effect on the bioefficacy (retinol/β-carotene total) (Tanumihardjo 2002) of PVA carotenoids in Mongolian gerbils (Davis et al. 2008), but these animals are not a good model for xanthophyll absorption because they only absorb and store small quantities of these molecules (Molldrem and Tanumihardjo 2004; Escaron and Tanumihardjo 2006). Carotenoids

may compete for specific transporters (e.g., class B type I scavenger receptors) at the level of individual enterocytes (Bohn 2008; Reboul 2013).

Interactions among carotenoids in mammals are also observed when PVA carotenoids are cleaved by an oxygenase to produce retinal (Lietz et al. 2010). The enzyme BCDO1 preferentially cleaves PVA carotenoids whereas β-carotene-9',10'-dioxygenase (BCDO2) also metabolizes non-PVA carotenoids such as zeaxanthin (Palczewski et al. 2014). Zeaxanthin can interact with the BCDO1, as shown by the limited conversion of intestinal β-carotene to vitamin A *in vitro* (Grolier et al. 1997). Nevertheless, the supplementation of poultry diets with xanthophylls (20 or 40 mg/kg) did not have any effect on *BCDO1* mRNA expression in the liver or small intestine (Gao et al. 2016). Therefore, zeaxanthin appears to interact with β-carotene at the levels of solubilization and absorption rather than competing for enzymatic cleavage.

We found that higher lutein levels were present in the control diet with natural additives compared to the other diets, but the HC diet resulted in tissues with higher yellowness scores. This may reflect the high zeaxanthin content of the HC diet. Higher levels of yellow rather than red xanthophylls are routinely used in the poultry industry, but in our trial we used more red than yellow xanthophylls to achieve PVA carotenoid levels similar to those present in the HC maize. The higher yellowness scores in chickens fed on the HC diet show that xanthophylls from HC maize can achieve the golden skin color desired by many consumers, so HC maize could replace xanthophylls commonly used in the poultry industry. Redness was slightly higher in chickens fed on the control diet with natural additives than in chickens fed on the HC diet due to the higher proportion of red xanthophylls we used to achieve PVA carotenoid levels similar to those present in the HC diet.

We established that PVA carotenoids supplied as intrinsic components of HC maize are bioavailable at least to the same extent as synthetic compounds and natural extracts, but the metabolism was not the same for all types of molecules. Specifically, β-carotene was preferentially converted into retinol in the intestine whereas β-cryptoxanthin was accumulated in the liver. Lutein, zeaxanthin and β-carotene were bioavailable to the extent that was predicted based on their levels in the HC feed, whereas β-cryptoxanthin and violaxanthin were found at higher levels than originally present in the feed, highlighting the need to evaluate individual nutrient bioavailability rather than the total amount of nutrient provided. Despite the high zeaxanthin content in the HC diet, which interferes with the absorption of β-carotene, the retinol levels in chicken liver remained high. Furthermore, chickens fed on the HC diet had higher yellowness scores for breast and thigh muscles. We therefore conclude that HC maize provides bioavailable carotenoids, including PVA carotenoids, and is suitable for development as a feed component in the poultry industry.

## Associated content

Supplementary information accompanies this paper.


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## Notes

The authors declare no competing financial interest.

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545 **Figure and table captions**

546 **Figure 1.** Retinol levels in **(a)** serum and **(b)** liver of chickens fed on four different
547 diets. Values represent the mean and standard error (n = 4). Zea: zeaxanthin; lut: lutein;
548 β-car: β-carotene.

549 **Figure 2.** Color evolution in the footpad of chickens fed on the different diets for 2
550 weeks, using the CIELab trichromatic system to score **(a)** lightness (L*), **(b)** redness
551 (a*) and **(c)** yellowness (b*). Values represent the mean and standard error (n = 8 in all
552 diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-
553 carotene). Zea: zeaxanthin; lut: lutein; β-car: β-carotene.

554 **Table 1.** Provitamin A carotenoid composition (µg/g freeze-dried feed) of the diets.
555 Values represent the mean and standard error (n = 3). Means within a column with no
556 superscript in common are significantly different ($P < 0.05$). ND: not detected; zea:
557 zeaxanthin; lut: lutein; β-car: β-carotene; β-cry: β-cryptoxanthin.

558 **Table 2.** Zeaxanthin and lutein levels in feed (µg/g freeze-dried), serum (µg/mL) and
559 liver (µg/g freeze-dried) in chickens fed on four different diets. Values represent the
560 mean and standard error (n = 3 for feed; n = 4 for serum and liver). Means within a row
561 with no superscript in common are significantly different ($P < 0.05$). Zea: zeaxanthin;
562 lut: lutein; β-car: β-carotene.

563 **Table 3.** Broiler production parameters. Values represent the mean and standard error (n
564 = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and
565 β-carotene). Means within a column with no superscript in common are significantly
566 different ($P < 0.05$). FCR: feed conversion ratio; zea: zeaxanthin; lut: lutein; β-car: β-
567 carotene.

**Table 1.** Provitamin A carotenoid composition (µg/g freeze-dried feed) of the diets. Values represent the mean and standard error (n = 3). Means within a column with no superscript in common are significantly different ($P < 0.05$). ND: not detected; zea: zeaxanthin; lut: lutein; β-car: β-carotene; β-cry: β-cryptoxanthin.

| | β-cry | β-car |
|---|---|---|
| High-carotenoid diet | 1.04 ± 0.05 [a] | 3.06 ± 0.17 [a] |
| Control diet + zea + lut + β-car | ND | 2.72 ± 0.2 [ab] |
| Control diet + lut + β-car | ND | 2.41 ± 0.11 [ab] |
| Control diet + natural additives | 0.91 ± 0.05 [a] | 2.24 ± 0.08 [b] |

**Table 2.** Zeaxanthin and lutein levels in feed (µg/g freeze-dried), serum (µg/mL) and liver (µg/g freeze-dried) in chickens fed on four different diets. Values represent the mean and standard error (n = 3 for feed; n = 4 for serum and liver). Means within a row with no superscript in common are significantly different ($P < 0.05$). Zea: zeaxanthin; lut: lutein; β-car: β-carotene.

| | | High-carotenoid diet | Control diet + zea + lut + β-car | Control diet + lut + β-car | Control diet+ natural additives |
|---|---|---|---|---|---|
| Lut | Feed | 3.45 ± 0.12 $^{b}$ | 1.45 ± 0.09 $^{c}$ | 0.95 ± 0.05 $^{d}$ | 5.76 ± 0.11 $^{a}$ |
| | Serum | 1.09 ± 0.11 $^{b}$ | 0.43 ± 0.02 $^{c}$ | 0.33 ± 0.02 $^{c}$ | 2.61 ± 0.17 $^{a}$ |
| | Liver | 1.83 ± 0.47 $^{b}$ | 0.38 ± 0.05 $^{c}$ | 1.18 ± 0.29 $^{bc}$ | 5.11 ± 0.31 $^{a}$ |
| Zea | Feed | 9.80 ± 0.58 $^{a}$ | 3.46 ± 0.36 $^{b}$ | 0.83 ± 0.04 $^{c}$ | 2.01 ± 0.06 $^{bc}$ |
| | Serum | 3.05 ± 0.32 $^{a}$ | 0.92 ± 0.05 $^{b}$ | 0.19 ± 0.00 $^{c}$ | 0.72 ± 0.04 $^{bc}$ |
| | Liver | 7.85 ± 1.77 $^{a}$ | 1.62 ± 0.18 $^{b}$ | 0.44 ± 0.10 $^{b}$ | 0.56 ± 0.04 $^{b}$ |

**Table 3.** Broiler production parameters. Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Means within a column with no superscript in common are significantly different ($P < 0.05$). FCR: feed conversion ratio; zea: zeaxanthin; lut: lutein; β-car: β-carotene.

| | Body weight (g) | Feed intake (g) | FCR |
|---|---|---|---|
| High-carotenoid diet | 1242 ± 43 [a] | 1325 ± 30 [a] | 1.52 ± 0.05 [a] |
| Control diet + zea + lut + β-car | 1206 ± 18 [a] | 1368 ± 40 [a] | 1.64 ± 0.03 [a] |
| Control diet + lut + β-car | 1210 ± 26 [a] | 1313 ± 31 [a] | 1.55 ± 0.02 [a] |
| Control diet + natural additives | 1202 ± 48 [a] | 1300 ± 45 [a] | 1.55 ± 0.04 [a] |

**Figure 1.** Retinol levels in **(a)** serum and **(b)** liver of chickens fed on four different diets. Values represent the mean and standard error (n = 4). Zea: zeaxanthin; lut: lutein; β-car: β-carotene.

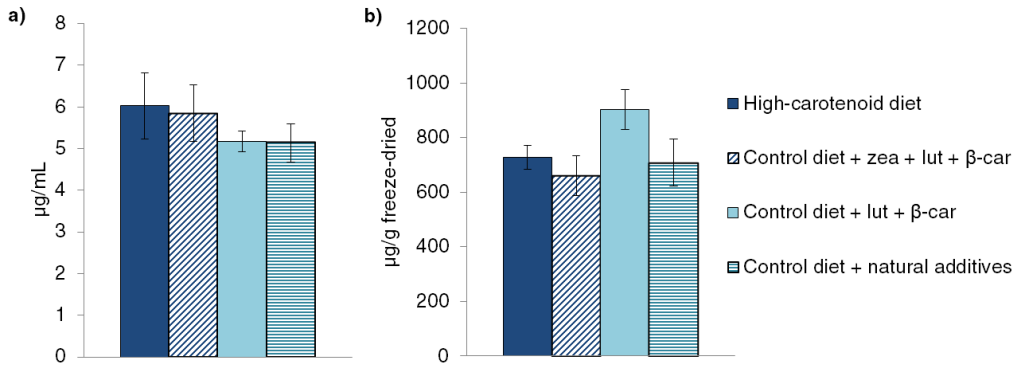
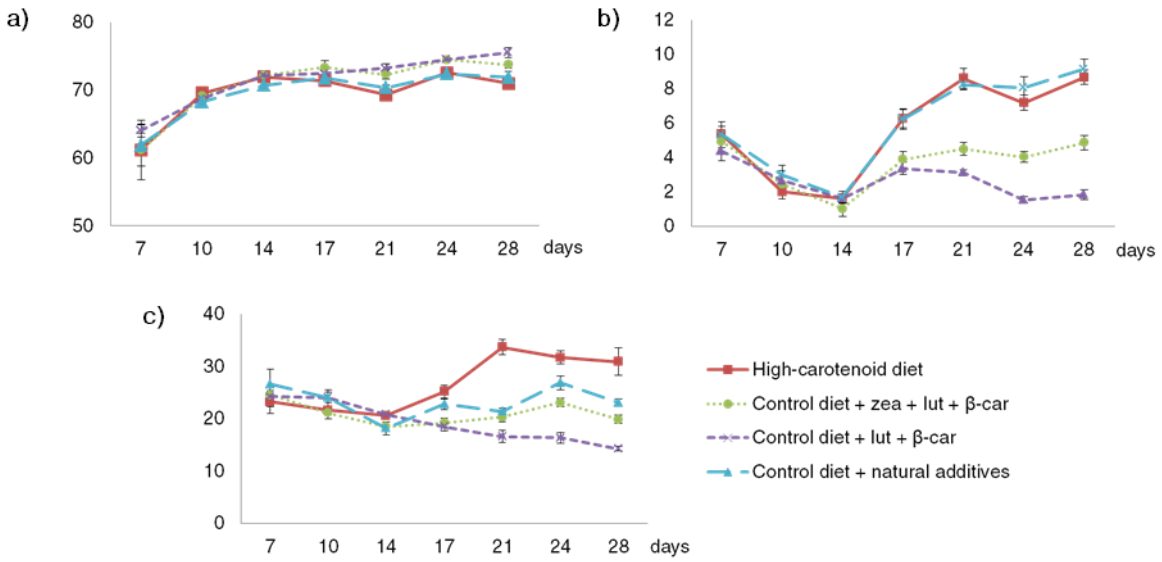


**Figure 2.** Color evolution in the footpad of chickens fed on the different diets for 2 weeks, using the CIELab trichromatic system to score **(a)** lightness (L*), **(b)** redness (a*) and **(c)** yellowness (b*). Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Zea: zeaxanthin; lut: lutein; β-car: β-carotene.



**Supplementary Table S1**. Diet composition.

| **Ingredient (g/kg)** | Control diet | High-carotenoid diet |
|---|---|---|
| Control maize (M37W) | 582.00 | 0 |
| High-carotenoid maize | 0 | 582.00 |
| Soybean meal 47.5% | 178.50 | 178.50 |
| Soy hulls | 158.50 | 158.50 |
| Soybean oil | 42.00 | 42.00 |
| Monocalcium phosphate | 12.28 | 12.28 |
| Calcium carbonate | 10.97 | 10.97 |
| Sodium chloride | 3.17 | 3.17 |
| Sodium bicarbonate | 1.19 | 1.19 |
| Choline chloride 60% | 0.42 | 0.42 |
| L-Lysine chloride 79% | 2.77 | 2.77 |
| DL-Methionine 99% | 3.33 | 3.33 |
| L-Threonine 98% | 0.47 | 0.47 |
| Vitamin-mineral premix [1] | 3.03 | 3.03 |
| Vitamin premix grower [2] | 1.04 | 1.04 |
| Coccidiostat salinomycin 12% | 0.52 | 0.52 |

| **Chemical analysis (g/kg)** | Control diet | High-carotenoid diet |
|---|---|---|
| Crude protein | 233.60 | 222.90 |
| Crude fiber | 40.80 | 26.70 |
| Crude fat | 112.40 | 111.30 |
| Ash | 43.30 | 47.40 |
| Moisture | 145.40 | 110.30 |

[1] Vitamin-mineral premix: vitamin $D_3$ 1700 IU/kg; vitamin $B_1$ 2 mg/kg; vitamin $B_2$ 6.4 mg/kg; vitamin $B_6$ 3 mg/kg; vitamin $B_{12}$ 0.02 mg/kg; vitamin E 50 mg/kg; vitamin K 3 mg/kg; folic acid 1 mg/kg; nicotinic acid 40 mg/kg; panthotenic acid 11.7 mg/kg; biotin 0.1 mg/kg; cooper 6 mg/kg; zinc 54 mg/kg; iron 40 mg/kg; manganese 77 mg/kg; selenium 0.45 mg/kg; iodine 2.28 mg/kg; BHT antioxidant 125 mg/kg.

[2] Vitamin premix grower: vitamin A 8,000 IU/kg; vitamin $D_3$ 300 IU/kg; vitamin $B_2$ 1.6 mg/kg; vitamin E 20 mg/kg.

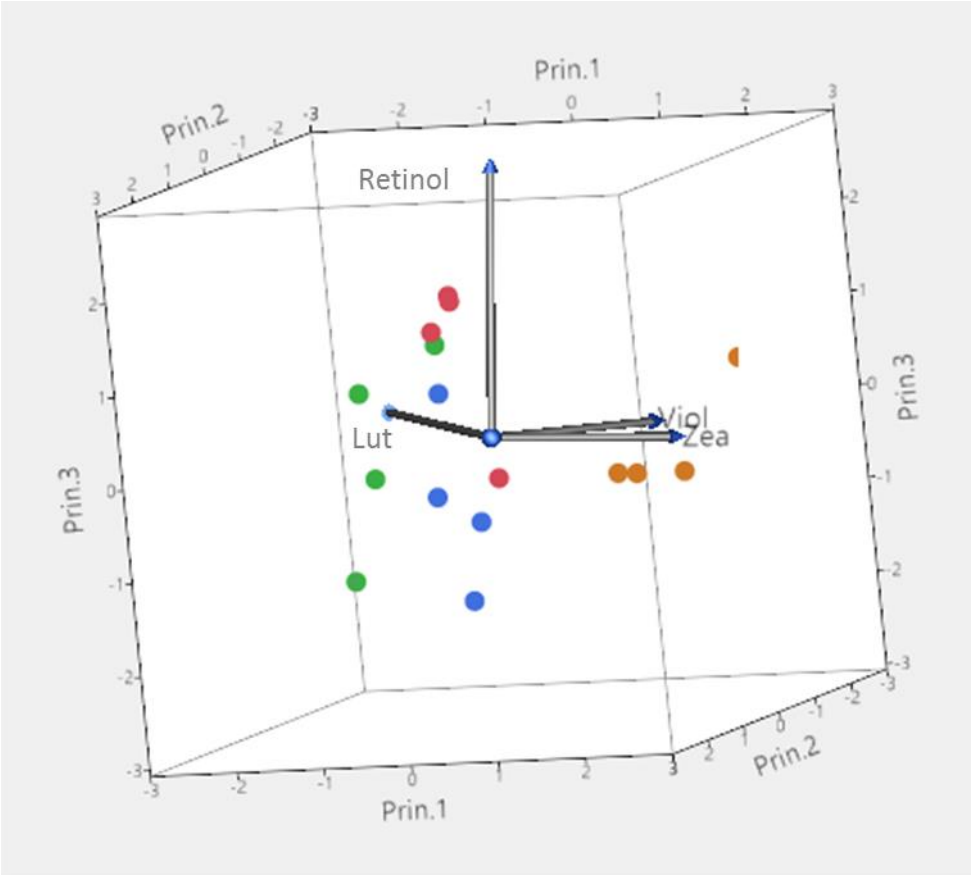
**Supplementary Table S2.** Effects of diets on the relative organ weight of chickens (expressed as % relative to whole live animal weight). Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Means within a row with no superscript in common are significantly different ($P < 0.05$). Zea: zeaxanthin; lut: lutein; β-car: β-carotene.

| | Liver | Spleen | Bursa of Fabricius |
|---|---|---|---|
| High-carotenoid diet | 2.12 ± 0.10 [a] | 0.09 ± 0.01 [a] | 0.20 ± 0.01 [a] |
| Control diet + zea + lut + β-car | 1.96 ± 0.06 [a] | 0.08 ± 0.00 [a] | 0.24 ± 0.01 [a] |
| Control diet + lut + β-car | 1.92 ± 0.03 [a] | 0.08 ± 0.01 [a] | 0.25 ± 0.01 [a] |
| Control diet + natural additives | 1.98 ± 0.08 [a] | 0.08 ± 0.01 [a] | 0.21 ± 0.02 [a] |

**Supplementary Table S3.** Color determination in footpad, breast skin, breast muscle and thigh muscle of chickens fed on the different diets after slaughter, using the CIELab trichromatic system to score lightness (L*), redness (a*) and yellowness (b*). Values represent the mean and standard error (n = 8 in all diets, except n = 7 in the control diet supplemented with zeaxanthin, lutein and β-carotene). Means within a row with no superscript in common are significantly different ($P < 0.05$). Zea: zeaxanthin; lut: lutein; β-car: β-carotene.

| | | High-carotenoid diet | Control diet + zea + lut + β-car | Control diet + lut + β-car | Control diet + natural additives |
|---|---|---|---|---|---|
| Footpad | L* | 70.98 ± 0.65 $^{c}$ | 73.74 ± 0.49 $^{ab}$ | 75.54 ± 0.72 $^{a}$ | 71.91 ± 0.84 $^{bc}$ |
| | a* | 8.68 ± 0.43 $^{a}$ | 4.87 ± 0.43 $^{b}$ | 1.85 ± 0.29 $^{c}$ | 9.17 ± 0.57 $^{a}$ |
| | b* | 30.86 ± 2.64 $^{a}$ | 19.87 ± 0.80 $^{bc}$ | 14.23 ± 0.42 $^{c}$ | 23.09 ± 0.71 $^{b}$ |
| Breast skin | L* | 66.20 ± 1.60 | 66.24 ± 1.00 | 68.92 ± 1.16 | 65.19 ± 0.68 |
| | a* | 2.50 ± 0.45 | 1.24 ± 0.29 | 1.28 ± 0.65 | 3.47 ± 0.77 |
| | b* | 12.99 ± 1.55 $^{a}$ | 6.05 ± 0.89 $^{b}$ | 3.97 ± 1.18 $^{b}$ | 7.04 ± 0.74 $^{b}$ |
| Breast muscle | L* | 48.41 ± 0.89 | 48.91 ± 0.73 | 49.43 ± 1.00 | 48.12 ± 0.81 |
| | a* | 2.45 ± 0.25 $^{a}$ | 1.00 ± 0.15 $^{b}$ | 0.47 ± 0.22 $^{b}$ | 2.91 ± 0.28 $^{a}$ |
| | b* | 7.42 ± 0.41 $^{a}$ | 3.91 ± 0.54 $^{bc}$ | 2.74 ± 0.39 $^{c}$ | 5.38 ± 0.63 $^{b}$ |
| Thigh muscle | L* | 51.99 ± 0.71 | 51.40 ± 0.37 | 53.29 ± 0.69 | 51.67 ± 0.82 |
| | a* | 4.76 ± 0.47 $^{ab}$ | 3.49 ± 0.17 $^{bc}$ | 2.19 ± 0.34 $^{c}$ | 5.50 ± 0.29 $^{a}$ |
| | b* | 6.80 ± 1.02 $^{a}$ | 4.18 ± 0.81 $^{ab}$ | 2.26 ± 0.78 $^{b}$ | 5.14 ± 1.35 $^{ab}$ |

**Supplementary Figure S1.** Principal component analysis of carotenoid and retinol levels in the livers of chickens fed on four different diets. Zea: zeaxanthin; lut: lutein; β-car: β-carotene; viol: violaxanthin.




- High-carotenoid diet
- Control diet + zea + lut + β-car
- Control diet + lut + β-car
- Control diet + natural additives