JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Supplementation of Laying-Hen Feed with Palm Tocos and Algae Astaxanthin for Egg Yolk Nutrient Enrichment

Laurie A. Walker,[†] Tong Wang,*[,†] Hongwei Xin,[‡] and David Dolde[§]

[†]Department of Food Science and Human Nutrition and [‡]Department of Agricultural and Biosystems Engineering and Animal Science, Iowa State University, Ames, Iowa 50011-1061, United States

[§]Pioneer Hi-Bred International, Johnston, Iowa 50131-0184, United States

ABSTRACT: Adding supplements to hen feed can increase egg nutritional value. Astaxanthin, tocotrienols, and tocopherols are potent antioxidants that provide health benefits to humans. We hypothesized that the addition of these nutrients to hen feed would result in an increased nutrient content in egg yolk with minimum changes in functional properties. Laying hens (Hy-Line W-36 breed) were fed four diets with different supplementation levels of palm toco concentrate and algae biomass containing astaxanthin for 8 weeks. Egg yolks were analyzed for physical, chemical, and functional properties. The feed with the highest nutrient concentration was also studied for stability of these antioxidants using the Arrhenius approach. No significant differences were observed in functional properties except for emulsification capacity and sensory characteristics among eggs from different diet treatments. Changes in egg yolk color reached the maximum values at day 8. Incorporation of tocopherols and tocotrienols increased until day 8, astaxanthin incorporation increased until day 10, and all decreased thereafter. Feed nutrients resulted in a dose−response relationship of these compounds in the egg yolk. The transfer efficiency ranged from 0 to 9.9% for tocotrienols and tocopherols and from 7.6 to 14.9% for astaxanthin at their peak values. Results of the Arrhenius accelerated stability study showed significant differences in the shelf life of various nutrients, and these results can be used to properly formulate and store the feed materials.

KEYWORDS: *astaxanthin, egg quality, nutrient transfer, tocopherols, tocotrienols*

INTRODUCTION

Hen eggs are regarded as one of the nature's most wholesome foods because they contain high-quality protein and lipids as well as essential and nonessential minerals and vitamins. Egg composition can be altered by heredity of genes, diet, and a hen's age.[1] Egg yolk contains natural carotenoids, and its yellow color is attributed to the presence of β-carotene, zeaxanthin, crypotoxanthin, and lutein naturally found in commercial feed.[2]

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a dark-red pigment and a member of the carotenoid family. It is the main carotenoid found in algae, such as *Haematococcus pluvialis*; *Phaffia rhodozyma* yeast;[3] aquatic animals including salmon, trout, and shrimp; and birds, such as flamingo and quail.[4] Astaxanthin has a strong antioxidant activity, 10 times higher than β-carotene and 300 times more effective than α-tocopherol.[5,6] Astaxanthin from *H. pluvialis* is an excellent compound not only for pigmentation but also for protecting against lipid oxidation, UV photoxidation, inflammation, cardiovascular disease, and cancer.[7] Tocotrienols and tocopherols (i.e., tocos) are fat-soluble vitamins and are generically regarded as vitamin E, for which the antioxidant properties are well studied. Vitamin E is thought to prevent atherosclerosis, protect against coronary disease, and prevent neurons from oxidative stress and the development of Alzheimer's disease and cancers.[8,9]

Because of the health benefits of tocos and astaxanthin and possibly good transfers of these compounds from the diet to eggs, we proposed to enhance the levels of these nutrients in eggs through hen feeding. Currently, there is a gap in the literature about a dose−response relationship, especially for astaxanthin. Previously, astaxanthin has been used successfully in adding pigmentation to egg yolks, salmon, trout,[10,11] and chicken meat.[12] Studies by Akiba et al. and Dike et al.[13,14] examined the effects of feeding a low concentration of astaxanthin-containing yeast, *P. rhodozyma*, to increase the color of egg yolk. Therefore, the majority of the past research with astaxanthin is for pigmentation, not for nutrient enrichment.

Although there have been several feeding studies on vitamin E-enriched feed for the nutrient to transfer to eggs, current literature is not comprehensive on transfer evaluation among the different vitamin E analogues. Flachowsky et al.[15] examined the effect of α-tocopherol supplementation on its concentration in foods of poultry origin and found that eggs had the highest concentration of α-tocopherol. Franchini et al.[16] successfully used DL-α-tocopheryl acetate to increase its level in eggs. Galobart et al.[17] used α-tocopheryl acetate in a base diet containing linseed oil to determine its transfer to eggs, and they showed a dose-dependent enrichment with a range of 16−132 μg/g in whole egg. Grobas et al.[18] also successfully used α-tocopheryl acetate and vitamin A to supplement their levels in egg yolk. All of these feeding studies were focused on α-tocopherol transfer to eggs, not the tocotrienols, which have health benefits that are different from those offered by tocopherols.[9] Because tocotrienols possess powerful neuroprotective, anticancer, and cholesterol-lowering properties that

Received: November 20, 2011
Revised: January 23, 2012
Accepted: January 26, 2012
Published: January 26, 2012

ACS Publications © 2012 American Chemical Society

are not often shown by tocopherols,[19] we intended to examine their transfers to egg yolk by feeding.

It is expected that successful incorporations of both astaxanthin and tocos in eggs will ultimately further improve egg nutrition and human health. We proposed to test how well these nutrients are transferred to eggs and hypothesized that such enrichment would result in egg yolks with increased nutrient content but with minimum changes in other physical and functional properties.

MATERIALS AND METHODS

Feeding Experiments. An 8 week feeding experiment was performed with 72 laying hens (Hy-Line W-36) of 35 weeks old at the start of the experiment that was approved by the Iowa State University Institutional Animal Care and Use Committee. The hens were allowed to adjust to the new surroundings and feeding system for 1 week. An environmentaly controlled room with 24 cages (8 stacks × 3 tiers per stack), three birds per cage (as the experimental unit, EU), was used to house the hens. Slightly slanted cage floors allowed the eggs to roll for easy collection. Eighteen hens were assigned to each of the three dietary treatments (i.e., six replicates for each treatment) for a total of three treatments and 18 hens for a control diet. Daily lighting program conditions were controlled to 16 h of light and 8 h of dark, as used in commercial operations. Thermoneutral conditions (temperature of 22−25 °C and relative humidity of 40−65%) were maintained throughout the experimental period.

The base diet formulation met the hen's NRC[20] nutrient recommendations. A vertical feed mixer combined the base diet (Table 1) for 30 min. From the base diet ingredients, some corn and animal-rendered oil were held back to mix in the nutrients, Tocomin 50% (Carotech Inc., Edison, NJ) (Table 2) and BioAstin, the dry algae flake/powder (containing 1.35% natural astaxanthin, Cyanotech Corp., Kailua-Kona, Hawaii) (Table 3), in a Hobart mixer (model H-600; Hobart, Troy, OH). The held-back portion was then added back to the base diet. The *Haematococcus* algae product was designed to be used for human, fish, and animal consumption. The oil in this algae contained about 40% polyunsaturated fatty acids (PUFA), including 21% 18:2, 13% 18:3, 2% 18:4, and 4% long chain PUFA. Tocomin contained naturally occurring tocotrienols and tocopherols extracted from crude palm oil.

The base diet was divided into four equal parts for each treatment. To make diet A, the control, the withheld corn and fat were added back to the base diet. For the lowest diet enrichment concentration, diet B, 0.49% algae (as-is basis) and 0.012% Tocomin were added. For diet C, 1.47% algae and 0.036% Tocomin were added. For diet D, 2.94% algae and 0.072% Tocomin were added. Feeds were stored at 4 °C when not used. Approximately a 6 day supply quantity was brought to the experimental site when the feed levels became low. The birds were fed and watered ad libitum. Daily measurements included feed intake, egg production, and egg weight. The weekly hen body weight was also measured. There was no mortality during the experimental period.

Egg Physical Properties. *Egg Production Measurements.* Scheduled daily egg collection resulted in the previous 24 h production data. The numbers of eggs and feed comsumption from 1 week's collection were combined to calculate the egg production rate and daily feed intake. Once the study began following the 1 week acclimation period, eggs from days 0, 2, 4, 6, 8, 10, 12, 14, 16, 17, 21, 28, 35, 42, 49, and 56 were collected for egg yolk total lipid extraction, color analysis, and measurement of egg moisture content from each cage. For each

Table 1. Composition of Base Diet for Laying Hens[a]

ingredient	composition (%)
corn	60.79
soybean meal	24.56
coarse calcium carbonate	5.87
fine calcium carbonate	3.92
dicalcium phosphate	2.03
animal rendered oil	1.58
vitamin and trace mineral premix	0.68
sodium chloride	0.38
DL-methionine	0.18
crude protein	16.05
metabolizable energy (kcal/kg)	2,825
crude fat	3.94
linoleic acid	1.82
calcium	4.20
phosphorus (nonphytate)	0.48
sodium chloride	0.18
chloride	0.26
lysine (digestible)	0.77
methionine (digestible)	0.41
methionine + cystine (digestible)	0.63

[a]Premix includes (per kilogram of diet): vitamin A, 9000 IU; vitamin D_3, 3000 IU; vitamin E, 20 IU; cobalamine, 13 μg; riboflavin, 6 mg; niacin, 45 mg; pantothenic acid, 12 mg; choline, 487 mg; menadione, 1.2 mg; folic acid, 1.5 mg; pyridoxine, 1.2 mg; thiamine, 1.5 mg; biotin, 45 μg; magnesium, 136 mg; manganese, 136 mg; zinc, 136 mg; iron, 140 mg; copper, 14 mg; and selenium, 0.27 mg.

Table 2. Composition of Tocomin 50%[a]

analogues	composition (mg/g)
D-α-tocotrienol	115
D-β-tocotrienol	15
D-γ-tocotrienol	210
D-δ-tocotrienol	55
D-α-tocopherol	115
D-β,γ,δ-tocopherol	3
total tocotrienol/tocopherol	513

[a]Composition of Tocomin 50% from Carotech and information adapted from Carotech product specification sheet.

Table 3. Composition of BioAstin[a]

components	composition (%)	components	composition (%)
natural astaxanthin	1.35	dietary fibers	18
total fat	20−25	moisture	<9
protein	20−35	ash	<17
carbohydrates	30−55		
other carotenoids (relative to astaxanthin)			
β-carotene	2.22	lutein	2.96
canthaxanthin	1.48		
natural astaxanthin[b]			
free astaxanthin	1.9	diester	13.9
monoester	84.3		
astaxanthin isomers			
di-*cis*-astaxanthin	2.22	13-*cis*-astaxanthin	8.15
trans-astaxanthin	74.07	15-*cis*-astaxanthin	0.74
9-*cis*-astaxanthin	6.66		

[a]All other information is from the Cyanotech certificate of analysis and Cyanotech analysis report. Additional information on *Haematococcus* algae can be found at http://www.cyanotech.com/pdfs/bioastin/axbul60.pdf. This algae oil contains about 40% polyunsaturated fatty acids (PUFA), including 21% 18:2, 13% 18:3, 2% 18:4, and 3.7% long chain PUFA. [b]Information adapted from the ALGAtechnologies specification sheet.

three-hen cage, eggs were combined as one EU. Throughout the experiment, whole egg and egg yolk weights were recorded daily. Eggs collected from different days after reaching steady-state color enrichment were used for different tests to compare diet effect, because it was not possible to perform all analyses with the small number of eggs produced from 1 day.

Color Analysis. Separation of egg yolk from albumen was completed with a small strainer and a spatula. Manual stirring homogenized the sample of the combined three-hen cage eggs. A LabScan XE spectrophotometer with a Hunter Lab system (Hunter Associate Laboratory Inc., VA) was used for color analysis. The system conditions were set to D65 (daylight 65), 10° standard observer, 2.00 in. port size, and 1.75 in. diameter area view conditions. The CIE $L^*a^*b^*$ scale was used for measurements where high L^* value represented the lightness, positive a^* represented red, and positive b^* represented yellow. Approximately 10 g of fresh yolk sample was placed into a plastic Petri dish (60 mm × 15 mm) with a white tape covered cardboard sleeve to avoid light escaping the system.

HunterLab color analysis was also performed with hard-boiled yolk samples from collection days 39, 40, and 41. Approximately 7 g of cooked yolk was used because this quantity matched the height from 10 g of fresh yolk samples. Cooking was done by boiling 8 eggs for 8 min. Egg yolks were carefully separated and mixed together for each cage to make a homogeneous mixture. Samples were gently pressed into the Petri dish to decrease surface roughness and light scattering. All samples, fresh or hard-boiled, were measured once.

Haugh Unit (HU). Eggs collected on days 51, 52, and 53 were used for HU measurement. Eggs were analyzed after a 24 h equilibrium period in an 18 °C cooler. A standard U.S. Department of Agriculture method[21] was followed. Each egg was weighed and broken onto a glass top over a reflective mirror. Thick albumin measurements were taken immediately after the breaking using a micrometer. The HU was calculated using egg weight and thick albumin height, namely, HU = $100 \log (H - 1.7 \times W^{0.37} + 7.6)$, where H is the observed height of the albumen (mm) and W is the weight of egg (g). Each egg was measured once and then averaged within the cage for the EU value.

Egg Yolk Functional Properties. *Raw Egg Yolk Viscosity Measurement.* The viscosity measurement was completed with eggs collected on day 44 using a Haake RS 150 Rheometer (ThermoOrion, Karlsruhe, Germany). Careful separation of egg yolk from the white was done to not break the yolk membrane. The yolk was rolled in a filter paper to remove any remaining albumen. The yolks from the same cage were combined and mixed. Approximately 10 drops of egg yolk was used for each measurement. Samples were placed between a plate (diameter of 35 mm) and attachment (PP35T) with a gap of 1.0 mm. The rheometer was maintained at 25 °C. Samples were examined with two viscosity tests. The first test involved changing the shear rate linearly from 0.10/s to 60.00/s for 30 s, and the results were fitted to the following equation:[22] $\tau = \kappa \times (\gamma/\gamma_o)^n$, where τ is the shear stress (Pa), γ is the shear rate (s^{-1}), γ_o is the constant shear rate (s^{-1}), κ is the consistency index (Pa), and n is the flow index. The second test used a constant shear rate at 60.00/s for 60 s to give the apparent viscosity measure. Each sample was subsampled in duplicate for each test.

Egg Yolk Texture Measurement. The egg yolk texture profile was measured on eggs collected on days 38 and 45. A TA-XT2i texture analyzer (Texture Technologies, Scarsdale, NY) was used to evaluate the hard-boiled egg yolk texture profile. The whole yolk was measured using a modified two-bite (TPA) method of Juliano et al.[23] The system was fitted with probe TA-4 (3.8 cm diameter acrylic cylinder of 20 mm tall), pretest speed of 2.0 mm/s, test speed of 1.0 mm/s, posttest speed of 2.0 mm/s, and distance of 15.0 mm. Each egg was measured once and then averaged within the cage for the EU value.

Characteristics measured include fracturability, hardness, cohesiveness, adhesiveness, springiness, and chewiness. Fracturability (g) was determined as the force at the first significant break in the curve. Hardness (g) was calculated by measuring the peak force obtained during the first compression cycle. Cohesiveness (dimensionless) was determined by the ratio of the positive force area during the second compression to that of the first compression. Adhesiveness (g × s) was determined by the first bite's negative area, representing the work necessary to pull the probe away from the sample. Springiness (mm) was defined as the height the yolk recovers during the time lapse between the first and second bites. Chewiness (g) was calculated by the product of hardness, cohesiveness, and springiness.[24]

Egg Yolk Emulsification Properties. The method was adapted from that reported by Wang and Wang.[25] Egg yolk of 1.5% dwb (dry weight basis) suspension in 25 mL of water was mixed with dyed soybean oil (Sudan Red 2B at about 4 ppm) at a constant rate of oil addition, 5 mL/min. A Bamix brand hand-held blender (Switzerland) continuously mixed the oil and protein dispersion at the "low" speed until apparent phase inversion was observed, at which point the oil-in-water emulsion system lost viscosity. The amount of oil added until phase inversion was used to calculate the emulsification capacity (EC, g oil/g yolk).

For emulsion stability (ES) measurement, the same dyed vegetable oil of 8 mL was added into 32 mL of 1.5% yolk dwb water suspension and mixed with the same hand-held blender for 1 min on the "high" speed setting. From the resulting emulsion, 10 mL was transferred into a 15 mL plastic centrifuge tube with 0.5 mL marked intervals. The ES (%) was calculated by dividing nonseparated volume by total volume after 1 day of standing at ambient temperature.

Sensory Evaluation. Thirteen panelists (seven male and six female college students) were trained for the sensory evaluation. For the training, characteristic standard definitions were defined, and standard samples were placed along an unstructured 15 cm line scale. The training session included egg yolks from a local grocery store, diet A and diet D fresh eggs, and 2 week old diet D eggs stored at 4 °C. The panelists agreed on the standard sample placement on the line during training. Three test replicates (three separate sessions) were completed in a standard sensory test facility under red light. The characteristics measured include flavor profile (sulfur and fishy) and texture profile (hardness, astringent, lumpy, and dry) modified from Juliano et al.[26] A general preference (likeness) test was also conducted using a rating scale of overall quality (10 being excellent to 1 being very poor) and overall flavor intensity (10 being bland to 1 being extreme).[27] Eggs were cut in half exposing the yolk, and one-half from each of the diets, A, B, C, and D, was given to the panelists. Samples were placed in a random order. A three-digit random number identified egg samples with the key only known to the researchers.

Egg Chemical Properties. *Egg Yolk Lipid Extraction.* The egg yolk was separated and mixed as described before. Total lipids were extracted by using Folch extraction method.[28] Briefly, 6 g of fresh yolk was extracted three times with 2:1 chloroform:methanol. DI water was then added to the separatory funnel with a final volume ratio of 8:4:3 of chloroform:methanol:water, and time was allowed for phase separation. The organic layer was rotoevaporated, and the resulting oil was weighed and transferred with hexanes into 22 mL glass vials. Vials were stored at 4 °C in the dark until further analysis. Each sample was extracted once.

Egg Yolk Lipid GC Fatty Acid Composition Analysis. Oil samples of eggs collected on day 10 were used for fatty acid methyl ester (FAME) composition determination. One milliliter of 1 M sodium methoxide was used, and the reaction was allowed for 5 h at ambient temperature. Three milliliters of DI water and 2 mL of hexanes were added to stop the reaction and separate the FAME from the solution. The FAMEs were analyzed by GC (Hewlett-Packard 5890 Series II) with a flame ionization detector and a silica capillary column (15 m long, 0.25 mm internal dia., 0.2 μm film thickness, a SP-2330 column from Supelco, Bellefonte, PA). The chromatographic parameters were as follows: injector and detector temperature of 230 °C, oven temperature program was programmed from 140 to 200 at 10 °C/min heating rate with no holding time. The carrier gas (He) was set at

5.4 mL/min, the auxiliary gas (He) was set at 19.4 mL/min, H_2 was at 13.9 mL/min, and air was at 426 mL/min. The split ratio was 24:1. A standard FAME mixture was used to identify all FA peaks.

^{31}P NMR Phospholipid Quantification. Concentrated egg oil samples (80−90 mg/mL) from day 10 were used for the analysis. The internal standard, triphenol phosphate (TPP), was added to the oil [approximately 10 mg was dissolved in 1 mL of chloroform-*d*, 1 mL of methanol, and 1 mL of Cs-EDTA (0.2 N, pH 8.5)]. After vigorous shaking, the samples were centrifuged at 1250*g* for 2.5 min. The lower phase was transferred to a 5 mm NMR tube (Kimble/Kontes, Vineland, NJ). A Varian VXR-400 spectrometer (Varian, Inc., Palo Alto, CA) with a Bruker Magnet (Bruker BioSpin, Billerica, MA) operating at 162 MHz was used to obtain ^{31}P NMR spectra. The samples were analyzed with an inverse-gated decoupling pulse sequence to suppress any nuclear Overhauser effect. The NMR scan conditions were as follows: pulse width of 22 μs, sweep width of 9718 Hz, acquisition time of 1.2 s, relaxation delay of 10 s, and 256 scans. All chemical shifts were recorded relative to TPP (δ −17.8). The phospholipid content in the extracted egg oil was calculated as follows: phospholipid content (%) = 100 × [phospholipids (g) in the sample/starting oil weight (g)].[29]

Tocotrienols and Tocopherols High-Performance Liquid Chromatography (HPLC) Quantification. Normal-phase HPLC (Waters Alliance 2695, Waters Corp., Milford, MA) was used to determine tocotrienol and tocopherol concentrations of all oil samples dissolved in 1 mL of hexanes. The detector (Waters 474 Fluorescence, Waters Corp.) was set to an excitation wavelength of 292 nm and an emission wavelength of 335 nm. The injection volume was 5 μL, and the run time was 22 min. The column (Phenomenex, Luna 3 μ NH_2 100 Å, 150 mm long × 3.0 mm internal diameter) was set to 30 °C. Tocopherol standards (Matreya, Pleasant Gap, PA) and tocotrienol standards (Davos Life Science, Singapore) with concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2.5, and 5 ppm had 0.995 or greater linearity coefficient (R^2) for the calibration curves. The mobile phases were HPLC grade 100% isopropanol (solvent A) and 100% hexanes (solvent B). The HPLC ran 2% solvent A and 98% solvent B isocratically.

Astaxanthin HPLC Quantification. Reverse-phase HPLC (Beckman Coulter Gold HPLC, Beckman Coulter Inc.) was performed to determine the astaxanthin level in all extracted egg yolk oil samples dissolved in 1 mL of 50:50 ethyl acetate:ethanol. The UV/vis detector (System Gold 16 Detector, Beckman Coulter Inc.) was set to 450 nm wavelength. The injection volume was 20 μL, and the run time of 50 min was at a flow rate of 1 mL/min. The column (YMC carotenoid, Waters, 3 μ particle size, 250 mm long × 4.6 mm internal diameter, Waters Corp.) was set to 25 °C. A linear gradient mobile phase of 81:15:4 methanol:methyl *t*-butyl ether:water (solvent A) and 6:90:4 methanol:methyl *t*-butyl ether:water (solvent B) was used for lipid separation. The gradient program adapted from Sander et al.[30] was used as follows: linear decrease from 100 to 77.8% A in 20 min, linear increase to 100% B from time 20 to 23 min, then holding at 100% B until 35 min. For preparation for the next sample, a linear decrease from 100 to 0% B was completed in 5 min and 10 min conditioning at 100% A.

Because of the lack of astaxanthin standard, β-carotene standard was used for quantification. A standard curve was generated using 50−7000 ppm β-carotene solutions. The astaxanthin area was converted to β-carotene equivalents using an extinction coefficient correction. Astaxanthin's extinction coefficient was reported as $E_{1cm}^{1\%}$ = 2100 for free, monester, and diester forms, while β-carotene's extinction coefficient was $E_{1cm}^{1\%}$ = 2600.[13]

Astaxanthin Identification by Liquid Chromatography−Mass Spectroscopy. Negative ion mode APCI mass spectrometer (6540 UHD Accurate-Mass Q TOF LC-MS, Agilent Technologies, Schaumburg, IL) with a diode array LC detector (450 nm) was used for egg oil and algae biomass extract astaxanthin identification with a method modified from Holtin et al.[31] The same separation conditions were used as described for the HPLC astaxanthin quantification. The LC system (Agilent 1200 Series) delivered the solvent at a flow rate of 1 mL/min and 1 μL sample injection. The MS was tuned and calibrated using the Agilent APCI tune mix. Agilent MassHunter Software (Version B.0.3) was used to identify the five astaxanthin isomers.

Nutrient Transfer Efficiency into Egg Yolk. The transfer efficiency for each nutrient additive was calculated as the ratio of the amount of the additive transferred in the eggs (mg) both at the peak value and at days 35 and 56 of feeding to the amount of additive consumed (mg) during the same period. The amount of additive consumed was calculated as the nutrient concentration in the feed times the amount of feed consumed.

Shelf-Life Study of the Feeding Material Using the Arrhenius Approach. The highest enrichment diet was used to more readily detect the changes in concentration. Approximately 100 g samples of diet D feed were placed in four ovens set at 50, 60, 70, and 80 °C. Samples were taken every 7 days for the 50 °C sample, 5 days for the 60 °C sample, 3 days for the 70 °C sample, and 2 days for the 80 °C sample. The Arrhenius equation is as follows: $k = Ae^{-Ea/RT}$, where k is the degradation rate coefficient, A is a constant, E_a is the activation energy, R is the universal gas constant, and T is the temperature (in Kelvin). By linearly correlating $\ln(k)$ with $1/T$, the E_a can be estimated, and the shelf life of the nutrient at any temperature can be estimated using the Arrhenius equation.

Feed Lipid Extraction. The diet D feed total lipid was extracted using the same Folch method mentioned for egg yolk oil extraction with a minor modification. A 9 g sample was extracted using 50 mL of 2:1 chloroform:methanol for 1 h. Mixtures were then filtered, and extraction was repeated two more times. Solvent extracts were combined for Folch wash, and the total lipid was collected as before for toco and astaxanthin quantification.

HPLC Quantification of Astaxanthin in Feed Lipid. Reverse-phase HPLC was also performed on the feed lipid samples for astaxanthin degradation rate determination. The same HPLC systems, software, column, and solvents were used. The gradient used[30] was different from egg yolk astaxanthin quantification. Briefly, 100% solvent A changed to 100% solvent B linearly over 90 min. Ten minutes of 100% solvent A followed each sample run to condition the column for the next run. Astaxanthin in feed and algae biomass were mainly in the monoester forms. The total area of all of the ester peaks was used for quantification.

Statistical Analysis. Statistical analysis was performed using SAS (version 9.2, SAS Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) was used for mean comparisons, and Fisher's least significant differences were calculated at $P < 0.05$ ($LSD_{0.05}$). All treatments had six replicates.

RESULTS AND DISCUSSION

Visual Observation. After mixed in the feed, the astaxanthin induced a color change in the feed from a pale yellow to a burnt red. The color was directly related to the amount of astaxanthin-containing algae biomass added to the diet; the higher biomass added, the more red the feed became.

Visual observations were made of the hen's appearance. A red color was observed in the combs and feet of the hens receiving the nutrient-enriched diets. This astaxanthin-induced pigmentation paralleled that as seen in broiler feeding trials that showed birds fed diets with higher levels of astaxanthin had more red coloration in the skin and muscle tissues.[14,32] The fecal matter of hens also showed a darkened color from the treatment diets, a result of incomplete absorption of the astaxanthin. Hencken[33] reported that 70% of astaxanthin fed was excreted.

Egg Physical Properties. *Hen Performance and Egg Quality.* The production performance of the hens and physical quality parameters of the eggs are shown in Table 4. The laying rate (i.e., hen-day egg production), hen weight, egg yolk weight, moisture content, and HU for all of the diets had no significant difference among the treatments at P = 5%. The mean laying rate was above 90% for all of the diets. The percentage of laying

Table 4. Laying Performance and Egg Quality Parameters with Different Levels of Supplementation[a]

diet[b]	laying rate (%) (n = 8)	hen weight (kg/hen) (n = 12)	daily feed intake (g/day/hen) (n = 8)	whole egg weight (g) (n = 348)	egg yolk weight (g) (n = 168)	HU (n = 18)	moisture content (%) (n = 15)
A	91.2 ± 4.0	1.46 ± 0.10	104.2 ± 10.2	60.46 ± 3.29 a	16.08 ± 1.57	88.89 ± 3.80	50.3 ± 1.40
B	93.1 ± 2.0	1.50 ± 0.09	106.2 ± 9.7	60.64 ± 3.80 a	16.05 ± 1.25	88.72 ± 1.73	50.1 ± 1.50
C	92.7 ± 3.2	1.43 ± 0.08	105.1 ± 10.1	59.36 ± 2.83 b	16.20 ± 2.16	89.07 ± 3.45	50.4 ± 1.82
D	93.0 ± 2.7	1.46 ± 0.06	108.4 ± 9.9	60.77 ± 2.40 a	16.05 ± 0.96	89.26 ± 3.33	50.1 ± 1.44
P value[c]	NS	NS	NS	**	NS	NS	NS

[a]Values are means ± standard deviations. [b]Diets are A, no nutrient supplement; B, enriched with 0.012% Tocomin and 0.49% algae biomass; C, enriched with 0.036% Tocomin and 1.47% algae biomass; and D, enriched with 0.072% Tocomin and 2.94% algae biomass. Different letters in the same column indicate significant differences at the 95% confidence interval. [c]**P value ≤0.01; NS, not significant at 5%.

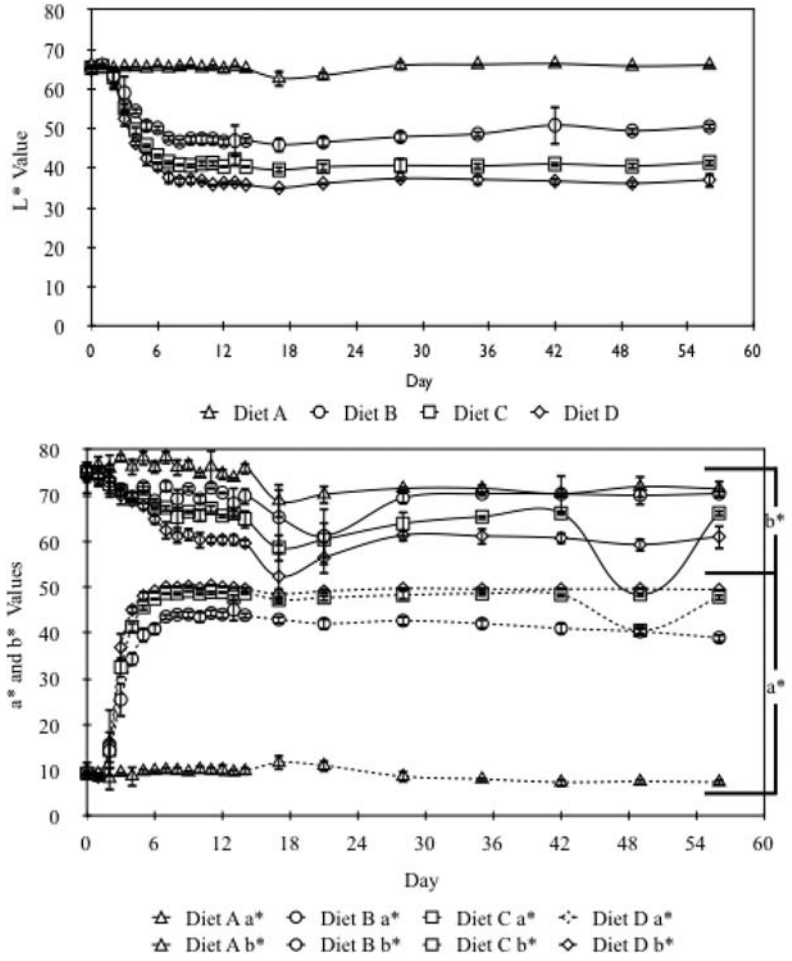

Figure 1. Color change of egg yolk from day 0 to day 56 for fresh egg yolk samples, with means and standard deviations. Diet abbreviations are as seen in the Table 4 footnote.

rate was calculated as the daily number of eggs produced/the number of hens × 100. The whole egg weight was not statistically significant among diets A, B, and D, but diet C tended to yield slightly lighter eggs than those of the other diets. The egg yolk weight for all of the diets was not significantly different, averaging 16 g/egg yolk. The yolk moisture content for all of the diets was about 50%, which matches the value found in the literature.[2] HU values were 88−89 for all of the diets, and these values are comparable to the values reported by Franchini et al.[16] The feed intake was similar for diets A−C, ranging from 104 to 106 g/day, but was slightly (not statistically significant) higher (mean of 108 g/day) for diet D. Prayitno et al.[34] reported that a higher intensity of red color encouraged the hens to consume more feed, and this might explain the somewhat higher feed consumption for diet D that had the highest intensity of red color.

Yolk Color Enrichment. All fresh egg yolk samples changed in L^*, a^*, or b^* values, but they became relatively constant after day 8. A similar observation was made by Fredriksson et al.[1] The color changes became noticeable by day 3. Samples decreased in L^* value to day 8, where each diet plateaued at different values, with the highest concentration diet being the darkest (Figure 1). The a^* values of yolk increased drastically when the samples became more red with increasing feeding time and concentration, with diet D having the highest value of redness. The b^* values exhibited smaller and slower changes as

Table 5. Color Analysis of Fresh and Hard-Boiled Egg Yolk Samples with Different Levels of Supplementation[a]

	fresh yolk[c] (n = 84)			boiled yolk[d] (n = 18)		
diet[b]	L^* value	a^* value	b^* value	L^* value	a^* value	b^* value
A	64.62 ± 7.26 a	9.34 ± 1.94 c	72.36 ± 8.65 a	72.57 ± 3.42 a	2.81 ± 1.09 d	40.85 ± 5.91 b
B	47.86 ± 2.23 b	42.75 ± 1.91 b	69.27 ± 3.67 b	62.67 ± 3.95 b	31.26 ± 4.85 c	46.74 ± 4.06 a
C	40.86 ± 1.18 c	48.42 ± 0.87 a	64.85 ± 2.96 c	56.43 ± 4.82 c	39.08 ± 2.82 b	48.78 ± 3.84 a
D	35.26 ± 6.93 d	47.96 ± 9.30 a	58.45 ± 9.73 d	53.71 ± 3.64 c	41.78 ± 3.22 a	50.34 ± 2.24 a
P value[e]	**	**	**	**	**	*

[a]Values are means ± standard deviations. Different letters in the same column indicate significant differences at the 95% confidence interval. [b]Diets are as seen in the Table 4 footnote. [c]Means and standard deviations include days 8−56, once steady-state incorporation has been reached. [d]Means and standard deviations are from days 39, 40, and 41. [e]*P value ≤0.05. **P value ≤0.01.

Table 6. Viscosity and Emulsification Egg Yolk Samples with Different Levels of Nutrient Supplementation[a]

diet[b]	viscosity (kg/s m) (n = 12)	consistency index (Pa) (n = 6)	flow index (n = 6)	emulsification capacity (average g oil/g yolk) (n = 6)	emulsion stability (n = 6) (%)
A	0.88 ± 0.31	4.26 ± 1.68	0.66 ± 0.08	62.00 ± 3.14 a	62.23 ± 6.71
B	0.75 ± 0.28	2.90 ± 1.35	0.64 ± 0.07	56.35 ± 2.16 b	61.00 ± 9.45
C	0.93 ± 0.25	8.08 ± 6.15	0.55 ± 0.12	53.98 ± 5.61 c	63.50 ± 7.18
D	0.82 ± 0.26	4.21 ± 4.76	0.64 ± 0.13	51.53 ± 1.21 c	58.17 ± 10.07
P value[c]	NS	NS	NS	*	NS

[a]Values are means ± standard deviations. Different letters in the same column indicate significant differences at the 95% confidence interval. [b]Diets are as seen in the Table 4 footnote. [c]*P value ≤0.05; NS, not significant at 5%.

Table 7. Sensory Panel Evaluation of Egg Yolks with Different Levels of Nutrient Supplementation[a]

diet[b]	sulfur	fishy	hard	astringent	lumpy	dry	overall quality	overall intensity
A	8.7 ± 4.8 a	2.5 ± 3.2 b	6.8 ± 4.0	6.7 ± 4.0 a	5.6 ± 4.5 b	8.1 ± 4.3	8 ± 2 a	2 ± 1
B	7.6 ± 4.7 a	3.0 ± 4.3 b	7.1 ± 4.6	5.1 ± 3.7 b	7.0 ± 5.2 ab	6.5 ± 3.7	8 ± 1 a	2 ± 1
C	7.8 ± 4.5 a	3.3 ± 3.6 b	7.6 ± 4.3	7.4 ± 4.2 a	7.4 ± 4.5 ab	7.7 ± 4.4	7 ± 2 ab	2 ± 1
D	5.6 ± 4.1 b	5.9 ± 4.2 a	8.0 ± 3.9	7.4 ± 4.1 a	8.6 ± 4.1 a	6.7 ± 4.4	7 ± 1 b	2 ± 0
P value[c]	*	**	NS	*	**	NS	*	NS

[a]Values are means ± standard deviations; N = 39. Measurements (cm) are with a 15 cm long line scale; the higher the value is, the higher the intensity is. The overall quality is 10 for excellent and 1 for very poor, and the overall flavor intensity is 10 for bland and 1 for extreme. Different letters in the same column indicate significant differences at the 95% confidence interval. [b]Diets are as seen in the Table 4 footnote. [c]*P value ≤0.05. **P value ≤0.01; NS, not significant at 5%.

compared to the L^* and a^* values. Hard-boiled yolk samples showed the same trends in color analysis except that the values were lower for a^* and b^* and higher for L^* (Table 5), possibly due to the change in yolk matrices induced by cooking and protein denaturation. Another reason for the difference could be light scattering from the surface of solid samples that was not optically flat. In the future, we need to design a practical and effective means to create such a nonscattering surface for color measurement.

Egg Yolk Functional Properties. *Effect of Diet Enrichment on Yolk Viscosity.* The viscosity of raw egg yolks illustrated a shear thinning profile, as reported by Ibarz and Sintes.[35] The apparent viscosity showed no significant difference among the diets (Table 6). The viscosity coefficients or consistency indices were similar for diets A, B, and D, whereas diet C showed a seemingly higher value for unknown reason(s), as eggs from all diets shared similar oil and moisture contents. The viscosity coefficient is defined as the viscosity at a shear rate or stress of 1 s^{-1}. From the power law model, the exponent, flow index, had no significant difference among the diets. The flow index is a measure of how non-Newtonian the fluid is. If the index is 1, the fluid is Newtonian; if the index is between 0 and 1, the fluid is shear-thinning; and if the index is greater than 1, the fluid is shear-thickening.

Effect of Diet Enrichment on Emulsification Properties. Emulsification capacity and ES measure, respectively, the amount of oil that the protein suspension can emulsify and the stability of the emulsion formed with a specific amount of oil. There were no significant differences in ES among the diets (Table 6). The capacity did exhibit differences among the diets in that diet A—the control—had the highest emulsification capacity, while diets C and D had the lowest levels. This decrease cannot be explained with certainty. The possibility of the nutrient incorporation causing a different interaction between the oil and the yolk protein would be low. A possibility might exist that the diets changed the proportion of various types of yolk protein. However, the focus of this study was only on lipid components and how the yolk as a whole may have different properties. Future studies may investigate the potential changes in protein type, proportion, and/or functionality as caused by nutiriet additives in hen's diets.

Effect of Diet Enrichment on Egg Yolk Texture. The texture measurements obtained were fracturability, 416 g; hardness, 797 g; adhesiveness, −34.8 g × s; cohesiveness, 0.6; springiness, 6.2 mm; and chewiness, 3340 g. These values are the averages of the four dietrary regimens because there was no treatment difference. Kassis et al.[36] also reported no significant differences

Table 8. Phospholipid Composition (Weight %) of Egg Yolk Lipid as Determined by ^{31}P NMR[a]

	diet[b]				
phospholipids[c]	A	B	C	D	*P* value[d]
CL	1.22 ± 1.36	0.36 ± 0.88	1.32 ± 1.46	0.83 ± 1.29	NS
PE	19.86 ± 0.73	19.09 ± 1.22	19.62 ± 1.58	18.37 ± 1.62	NS
SM	1.75 ± 0.27	1.57 ± 0.28	1.56 ± 0.53	1.72 ± 0.23	NS
LPC	0.04 ± 0.09	0.04 ± 0.11	0.08 ± 0.20	0.00 ± 0.00	NS
PI	1.73 ± 0.17	0.84 ± 0.95	1.66 ± 0.25	1.39 ± 0.70	NS
PC	75.40 ± 0.60	78.10 ± 2.11	75.75 ± 2.20	77.69 ± 1.83	NS
total in oil	31.18 ± 0.79	29.15 ± 4.19	28.30 ± 4.94	30.61 ± 2.32	NS

[a]Values are means ± standard deviations. [b]Diets are as seen in the Table 4 footnote. [c]CL, cardiolipin; PE, phosphatidylethanolamine; SM, sphingomyelin; LPC, lysophophatidylcholine; PI, phosphatidylinositol; and PC, phosphatidylcholine. [d]*P* value; NS, not significant at 5%.

in these textural properties for eggs from a krill oil astaxanthin enrichment study.

Sensory Evaluation. Data from the sensory evaluation are shown in Table 7. Perceived sulfur levels were noticeably lower with an increase of fishy flavor for diet D. The change in normal egg flavor with this diet was attributable to the high algae biomass concentration in the feed and the addition of the highly unsaturated fatty acids contained in the algae (about 40% polyunsaturated fatty acid in algae oil). Algae biomass has a fishy characteristic; therefore, the egg flavor may have been affected by the astaxanthin addition. No observed differences were found among diets for hardness, which correlates with the texture analysis performed. Astringency was significantly less for diet B as compared to the other diets, although the reason was unknown. Eggs from diet A had lower lumpy characteristics than eggs from other diets, while eggs from diet D were the lumpiest and cottage cheeselike. There was no significant difference in dryness among the diets. The last two parameters in Table 7 were not from trained panel evaluation but were given as preliminary indicators of consumer acceptance. The overall quality was significantly higher for diet A and diet B than for diet D, but there was no significant difference in overall flavor intensity. These differences were small and, hence, may not lead to practical distinctions. A consumer level sensory evaluation should be performed to assess acceptability.

Egg Chemical Properties. *Fatty Acid Composition.* All egg yolk oil compositions were not significantly different, and the fatty acid percentages were similar to the reported values from the U.S. Department of Agriculture nutrient database, with 24.7, 9.3, 39.6, and 14.0% of 16:0, 18:0, 18:1, and 18:2, respectively, as the main fatty acids.

Diet Effect on Egg Yolk Phospholipids. ^{31}P NMR phospholipids analysis showed good separation of various polar lipid classes as presented by Yao and Jung.[29] Cardiolipin, phosphatidylethanolamine, sphingomyelin, lysophophatidylcholine, phosphatidylinositol, and phosphatidylcholine were identified. Table 8 illustrates the phospholipid class composition and total phospholipid content relative to total oil from the day 10 eggs. These phospholipid compositions are not significantly different among the diet treatments.

Astaxanthin Identification. The monoesters and free astaxanthins from algae lipid extract and egg oil were well separated. These chromatograms were similar to those reported by Holtin et al.[31] and Miao et al.,[37] showing that astaxanthin in algae is mainly monoesters. However, hens are able to convert the esters to free astaxanthin and deposit the free forms in eggs because only free forms were detected in eggs. The five isomers are due to the *cis* and *trans* double bond configuration difference and the hydroxyl group orientation.[31] All astaxanthin peaks were confirmed by LC-MS. The five isomers of astaxanthin were all-*trans*-3*S*,3′*S*-astaxanthin, all-*trans*-3*R*,3′*R*-astaxanthin, all *trans*-3*S*,3′*R*-astaxanthin, 9-*cis*-astaxanthin, and 13-*cis*-astaxanthin. The sum of areas of all five peaks was used to calculate astaxanthin amount in egg yolk lipid extract.

Diet Effect on Nutrient Concentration in Egg Yolk. HPLC quantification was completed for tocotrienols, tocopherols, and astaxanthin. All toco analogues were well separated, as well as the major isomers of the astaxanthin. The additives' concentration in yolk lipid increased as their concentration in feed increased (Figures 2 and 3) except for γ- and δ-tocopherols. The increase in concentration continued until day 8 for diets B−D for all tocotrienols. However, tocopherol enrichment showed different profiles. At the end of 56 days of feeding, many tocopherol concentrations tended to be lower than those at the start of the trial, especially for δ- and γ-tocopherols, which showed a steady reduction, and for α- and β-tocopherol from the control and diet B. The reason could be that in the palm toco concentrate (Table 2), there was a negligible amount of β-, δ-, and γ-tocopherols (a total of 3 mg/g, 0.6% of total tocos). However, the feed itself had its own natural tocopherols from the natural ingredients and the vitamin and mineral premix (Table 1). With the added tocotrienols and α-tocopherol, the absorption of the δ- and γ-tocopherols in the feed itself was greatly suppressed. Because tocotrienols are not typically found in the natural ingredients in feeds, their feed enrichment led to a steady enrichment in eggs and in a dose−response relationship. There seems to be a feedback or competitive inhibition for all of the nutrients. With α-tocopherol, there was a decrease from the peak value of 30−65% depending on the diet. Other tocos exhibited a more dramatic decrease, with δ-tocopherol having a 75% reduction as compared to the initial value. Franchini et al.[16] saw a reduction of α-tocopherol similar to the current study. Differences among various studies could be due to several factors, such as genetics, laying parameters, type and concentration of tocopherol used, experimental conditions, basal diets, and the presence of other antioxidants.

Astaxanthin increased in concentration up to day 10 for diets B−D, and there were slight reductions thereafter (Figure 3). Astaxanthin had smaller decreases of 38−43% relative to peak values from diet D to diet B. This may indicate that the feedback inhibition for this antioxidant is not as strong as for tocopherols.

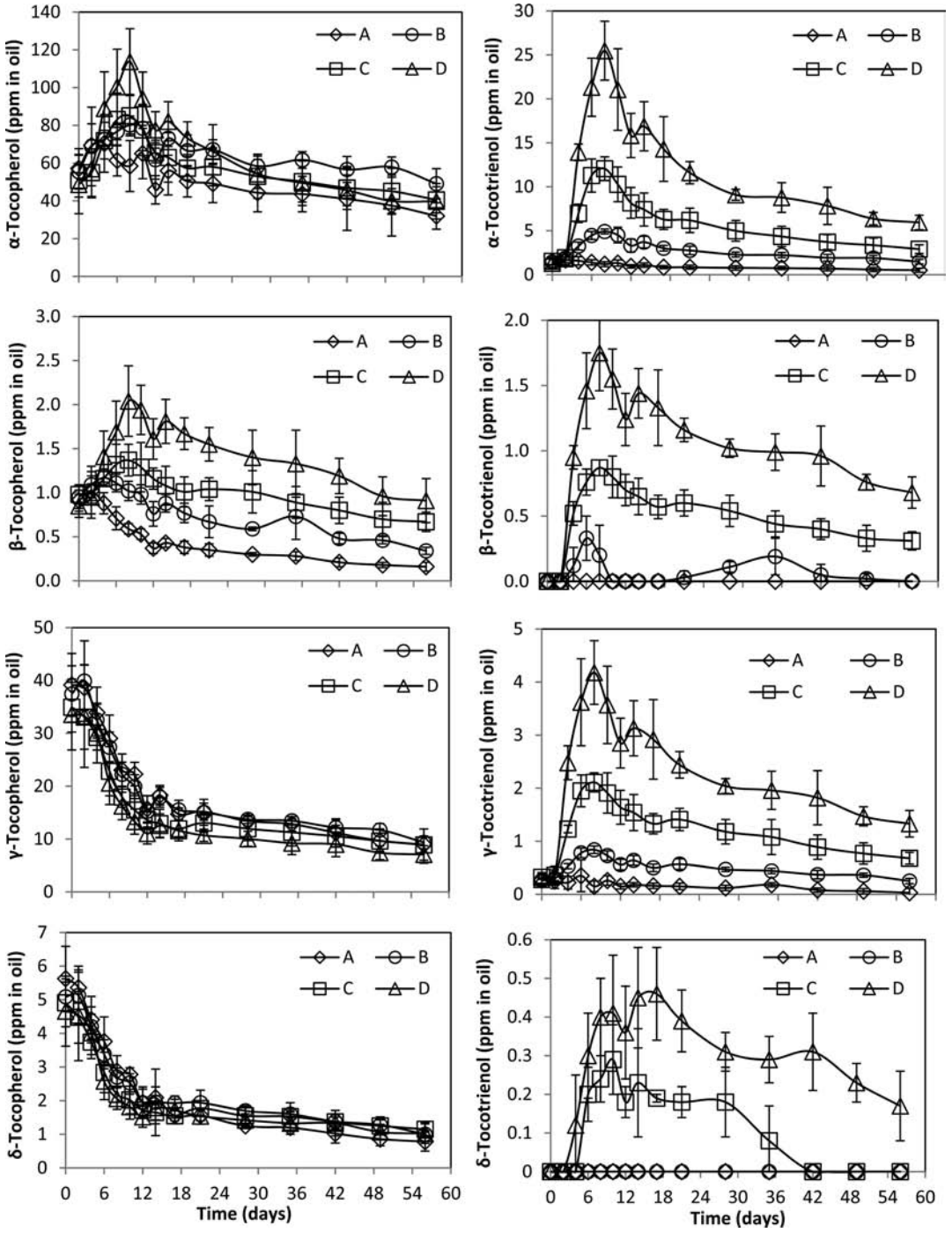

Figure 2. HPLC quantification of tocotrienols and tocopherols over the 56 day feeding period, showing means and standard deviations from six replicates. Diet abbreviations are as seen in the Table 4 footnote.

Nutrient Transfer Efficiency into Egg Yolk. From HPLC quantification, transfer efficiency can be calculated. Because days 8 and 10 of the study exhibited the highest nutrient concentration for tocos and astaxanthin in yolk lipid, respectively, these data were used for the calculation of the highest efficiency. Another two times were selected to show the lower efficiencies, and the results are shown in Table 9. The highest transfer efficiency occurred at the lowest feed concentration for most of the compounds. Efficiency differences for different compounds can be attributed to the difference of bioavailability and biochemical processes. A protein, α-tocopherol transfer protein (αTTP), has been identified to bind and transfer this compound between membranes in the liver cytosol of animals. Hosomi et al.[38] examined the affinities of αTTP toward different tocos and calculated relative affinity taking α-tocopherol as 100%: β-tocopherol, 38%; γ-tocopherol, 9%; δ-tocopherol, 2%; and α-tocotrienol, 12%. Packer et al.[39] also concluded that the liver preferentially enriches VLDL with α-tocopherol, discriminating other tocos. The present study confirmed this trend, with the highest vitamin E analogue transferred being the α-tocopherol. This transfer protein's specificity could be the cause for this study failing to enrich the egg yolk sufficiently with tocotrienols. The low affinity of the transfer protein for α-tocotrienol in the presence of α-tocopherol resulted in a small

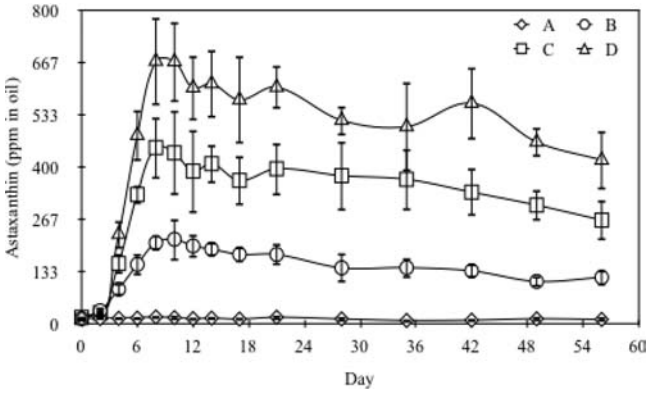

Figure 3. HPLC of astaxanthin quantification over the 56 day feeding period, showing means and standard deviations from six replicates. Diet abbreviations are as seen in the Table 4 footnote.

amount of α-tocotrienol transferred from the feed to egg. The other tocotrienols transferred were even lower (<1%). However, there is an interesting observation comparing the relative transfer efficiency of α-tocotrienol to α-tocopherols at different feed concentrations: 17, 36, and 41% for diets B, C, and D, respectively. Apparently, at higher concentrations, more tocotrienols may be forced into the circulation system. These values were higher than the 12% as reported by Hosomi et al.[38]

The α-tocopherol exhibited the highest transfer efficiency at 9.9% in diet B to 3.4% in diet D. Galobart et al.[17] also observed a decrease from 41.8% with 50 mg/kg in the diet down to 26.7% with 200 mg/kg. In our study, the amount of added total tocos was 60 mg/kg in diet B, 180 mg/kg in diet C, and 360 mg/kg in diet D. However, because α-tocopherol was only 22.5% of the total tocos, the added α-tocopherol in the three diets were 13.5, 40.5, and 81 mg/kg, respectively. Therefore, our feed enrichment level was similar to that of Galobart et al.[17] but with much lower transfer efficiency. A similar trend was also observed in another vitamin E supplementation study.[15]

Astaxanthin transfer efficiency was determined to be as high as 14.9% at the low enrichment level. Its transfer efficiency reduced to 7.6% when the enrichment was increased 6-fold in the diet. Although the total concentration in yolk was increased by the increase in enrichment, the efficiency was greatly reduced. Akiba et al.[10] observed 3.6% transfer efficiency from their study using *P. rhodozyma* as a source of astaxanthin. A study by Johnson et al.[40] using the same yeast also observed a 3−4% efficiency in laying quails. Our data showed much higher transfer efficiency from algae biomass as compared to red yeast.

It should be noted that we used the peak concentration of the nutrients to discuss the maximum transfer efficiencies. In reality, a steady-state concentration, if observed, should have been used for such calculation. Because our concentrations showed steady reductions, and no plateaus were observed after 56 days, especially for the high enrichment concentrations, we decided to use the peak values for comparing the differences among different dietary regimens. Efficiencies from days 35 and 56 are also presented in Table 9, and they are much lower than the peak values. The negative values reflect the absorption inhibition.

Shelf-Life Study of the Feeding Material Using the Arrhenius Approach. A shelf-life study of diet D was completed using the Arrhenius approach. Figure 4 shows an example of such a shelf-life study. The slopes from Arrhenius plots were used for activation energy determination. A summary of the kinetics is shown in Table 10. The half-lives of the nutrient compounds show that certain compounds had very short half-lives, such as γ-tocopherol. The longest half-lives were exhibited by astaxanthin and δ-tocopherol. The low R^2 values may be attributed to the 70 °C oven temperature fluctuation, causing irregular degradation trend.

This study shows that astaxanthin and tocos can be incorporated into egg yolk with different degrees of success by adding the ingredients into the feed. The nutrient

Table 9. Nutrient Transfer Efficiency to Egg Yolk (%)[a]

		diet[b]			
day	component	B	C	D	*P* value[c]
8 or 10	α-tocopherol	9.90 ± 1.85 a	3.83 ± 1.30 ab	3.43 ± 0.95 b	*
	α-tocotrienol	1.72 ± 0.20 a	1.39 ± 0.12 b	1.41 ± 0.15 b	*
	β-tocotrienol	0.52 ± 0.60 b	0.75 ± 0.05 a	0.72 ± 0.10 a	**
	γ-tocotrienol	0.16 ± 0.02 a	0.13 ± 0.01 ab	0.13 ± 0.02 b	*
	δ-tocotrienol	0.00 ± 0.00 c	0.06 ± 0.02 a	0.05 ± 0.01 b	**
	astaxanthin	14.89 ± 2.20 a	10.59 ± 1.83 b	7.60 ± 1.26 c	*
35	α-tocopherol	3.95 ± 2.18 a	−0.27 ± 1.30 b	−0.19 ± 0.76 b	**
	α-tocotrienol	0.89 ± 0.13 a	0.45 ± 0.24 b	0.54 ± 0.08 b	**
	β-tocotrienol	0.54 ± 0.33	0.34 ± 0.17	0.46 ± 0.06	NS
	γ-tocotrienol	0.09 ± 0.01 a	0.06 ± 0.03 b	0.07 ± 0.01 b	**
	δ-tocotrienol	0.00 ± 0.00 b	0.01 ± 0.02 b	0.04 ± 0.01 a	**
	astaxanthin	11.95 ± 4.02 a	9.46 ± 2.25 ab	6.31 ± 1.16 b	**
56	α-tocopherol	−1.11 ± 3.03	−1.48 ± 0.85	−0.74 ± 0.59	NS
	α-tocotrienol	0.56 ± 0.08 a	0.38 ± 0.07 b	0.36 ± 0.73 b	**
	β-tocotrienol	0.00 ± 0.00 b	0.30 ± 0.07 a	0.31 ± 0.07 a	**
	γ-tocotrienol	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	NS
	δ-tocotrienol	0.00 ± 0.00 b	0.00 ± 0.00 b	0.02 ± 0.01 a	**
	astaxanthin	9.26 ± 1.77 a	7.08 ± 1.64 b	5.12 ± 1.02 c	**

[a]Values are means ± standard deviations. The day 8 values are for all tocos, and those of day 10 are for astaxanthin. [b]Diets are as seen in the Table 4 footnote. [c]*P value ≤0.05. **P value ≤0.01. NS, not significant at 5%.

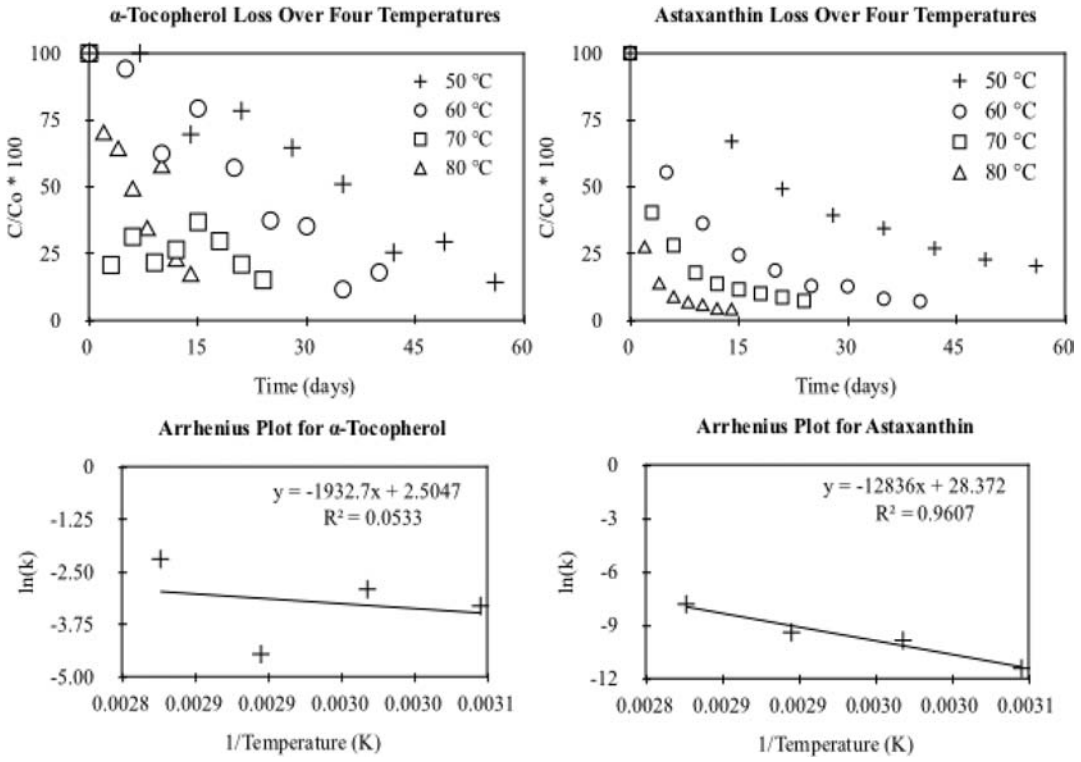

Figure 4. Arrhenius shelf-life study of diet D showing the loss of α-tocopherol and astaxanthin over time at four elevated storage temperatures.

Table 10. Degradaiton Kinetics of Hen Feed Using the Arrhenius Approach

component	activation energy (kJ/mol)	R^2	k (day^{-1}) at 25 °C	$t_{1/2}$ (days) at 25 °C
α-tocopherol	28.8	0.05	1.0×10^{-2}	67.5
α-tocotrienol	307.6	0.01	1.3×10^{-2}	53.9
β-tocopherol	48.5	0.06	4.8×10^{-4}	72.8
β-tocotrienol	29.5	0.40	4.5×10^{-4}	23.1
γ-tocopherol	36.7	0.43	5.0×10^{-1}	0.3
γ-tocotrienol	49.0	0.19	5.9×10^{-5}	39.5
δ-tocopherol	48.8	0.30	2.7×10^{-3}	254.6
δ-tocotrienol	50.9	0.05	3.6×10^{-5}	56.5
astaxanthin	106.7	0.96	4.2×10^{-7}	307.1

concentrations in the egg yolk and in the feed are positively related, although transfer efficiency tends to be inversely related to the dietary nutriet level. Even though α-tocotrienol is a nutrient with unique health benefits, it cannot be as effectively transferred into eggs as α-tocopherol. The highest concentrations of astaxanthin, α-, β-, γ-, δ-tocopherols, and their corresponding tocotrienols reached were 670, 114, 2, 34, 5, 25, 2, 4, and 0 ppm relative to the total yolk lipid.

AUTHOR INFORMATION

Corresponding Author

*Tel: 515-294-5448. Fax: 515-294-8181. E-mail: tongwang@iastate.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We sincerely appreciate the assistance of Dr. Christjan Bregendahl of Sparboe Farms for feed formulation, Dr. Hui Wang for obtaining the algae biomass, Cyanotech Corporation (Kailua-Kona, Hawaii) for the generous donation of the algae biomass, and Dr. Linxing Yao for assistance in ^{31}P NMR phospholipid quantification and rheological measurement. We also thank the Iowa Egg Council for funding this research.

REFERENCES

(1) Fredriksson, S.; Elwinger, K; Pickova, J. Fatty acid and carotenoid composition of egg yolk as an effect of microalgae addition to feed formula for laying hens. *Food Chem.* **2006**, *99*, 530−537.

(2) Anton, M. Composition and Structure of Hen Egg Yolk. In *Bioactive Egg Compounds*; Huopalahti, R., López-Fandiño, R., Anton, M., Schade, R., Eds.; Springer: Berlin, New York, 2007; pp 1−5.

(3) Domínguez-Bocanegra, A. R.; Guerrero Legarreta, I.; Martinez Jeronimo, F.; Tomasini Campocosio, A. Influence of environmental and nutritional factors in the production of astaxanthin from *Haematococcus pluvialis*. *Bioresour. Technol.* **2004**, *92*, 209−214.

(4) Johnson, E. A.; An, G. Astaxanthin from Microbial Sources. *Crit. Rev. Biotechnol.* **1991**, *11*, 297−326.

(5) Rao, R.; Sarada, A. R.; Baskaran, C.; Ravishankar, G. A. Identification of carotenoids from green alga *Haematococcus pluvialis* by HPLC and LC-MS (APCI) and their antioxidant properties. *J. Microbiol. Biotechnol.* **2009**, *19*, 1333−1341.

(6) Higuera-Ciapara, I.; Félix-Valenzuela, L.; Goycoolea, F. M. Astaxanthin: A review of its chemistry and applications. *Crit. Rev. Food Sci.* **2006**, *46*, 185−196.

(7) Guerin, M.; Huntley, M. E.; Olaizola, M. Haematococcus astaxanthin: Applications for human heath and nutrition. *Trends Biotechnol.* **2003**, *21*, 210−216.

(8) Li, Y.; Liu, L.; Barger, S. W.; Mrak, R. E.; Griffin, S. T. Vitamin E suppression of microglial activation is neuroprotective. *J. Neurosci. Res.* **2001**, *66*, 163−170.

(9) Aggarwal, B. B.; Sundaran, C.; Prasad, S.; Kannappan, R. Tocotrienols, the vitamin E of the 21st century: Its potential against cancer and other chronic diseases. *Biochem. Pharmacol.* **2010**, *80*, 1613−1631.

(10) Gobantes, I.; Choubert, G.; Gómez, R. Quality of pigmented (astaxanthin and canthaxanthin) rainbow trout (*Oncorhynchus mykiss*)

fillets stored under vacuum packaging during chilled storage. *J. Agric. Food Chem.* **1998**, *46*, 4358−4362.
(11) Barbosa, M. J.; Morais, R.; Choubert, G. Effect of carotenoid source and dietary lipid content on blood astaxanthin concentration in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **1999**, *176*, 331−341.
(12) Takahashi, K.; Watanabe, M.; Takimoto, T.; Akiba, Y. Uptake and distribution of astaxanthin in several tissues and plasma lipoproteins in male broiler chickens fed a yeast (*Phaffia rhodozyma*) with a high concentration of astaxanthin. *Br. Poult. Sci.* **2004**, *45*, 133−138.
(13) Akiba, Y.; Sato, K.; Takahashi, K.; Takahashi, Y.; Akemi, F.; Konashi, S.; Nishida, H.; Tsunekawa, H.; Hayasaka, Y.; Nagao, H. Pigmentation of egg yolk with yeast *Phaffia rhodozyma* containing high concentration of astaxanthin in laying hens fed on a low-carotenoid diet. *Jpn. Poult. Sci.* **2000**, *37*, 77−85.
(14) Dike, A. O.; Lettnew, F.; Zollitsch, W. The supplementation of layers feed with the yeast-*Phaffia rhodozyma*-as pigment carrier. *Archiev Geflugelkunde* **1992**, *56*, 205−209.
(15) Flachowsky, G.; Engelman, D.; Sünder, A.; Halle, I.; Sallman, H. P. Eggs and poultry meat as tocopherol sources independence on tocopherol supplementation of poultry diets. *Food Res. Int.* **2002**, *35*, 239−243.
(16) Franchini, A.; Sirri, F.; Tallarico, N.; Minelli, G.; Iaffaldano, N.; Meluzzi, A. Oxidative stability and sensory and functional properties of eggs from laying hens fed supranutritional doses of vitamins E and C. *Poult. Sci.* **2002**, *81*, 1744−1750.
(17) Galobart, J.; Barroeta, A. C.; Baucells, M. D.; Cortinas, L.; Guardiola, F. α-Tocopherol transfer efficiency and lipid oxidation in free and spray-dried eggs enriched with ω3-polyunsaturated fatty acid. *Poult. Sci.* **2001**, *80*, 1496−1505.
(18) Grobas, S.; Méndez, J.; Lopez Bote, C.; Blas, C.; Mateos, G. G. Effect of vitamin E and A supplementation on egg yolk α-tocopherol concentration. *Poult. Sci.* **2002**, *81*, 376−381.
(19) Sen, C. K.; Khanna, S.; Roy, S. Tocotreinols: Vitamin E beyond tocopherols. *Life Sci.* **2006**, *78*, 2088−2098.
(20) NRC, National Research Council. *Nutrient Requirements of Poultry*, 9th ed; National Academy Press: Washington, DC, 1994.
(21) U.S. Department of Agriculture. *Egg-Grading Manual*; U.S. Department of Agriculture: Washington, DC, **2000**; Vol. 75, pp 34−35.
(22) Buxmann, W.; Bindrich, U.; Heinz, V.; Knorr, D.; Franke, K. Influencing emulsifying properties of egg yolk by enzymatic modification by phospholipase D from *Streptomyces chromofuscus* part 1: Technological properties of incubated egg yolk. *Colloids Surf., B* **2010**, *76*, 186−191.
(23) Juliano, P.; Clark, S.; Koutchma, T.; Ouattara, M.; Mathew, J. W.; Dunne, C. P.; Barbosa-Cánovas, G. V. Consumer and trained panel evaluation of high pressure thermally treated scrambled egg patties. *J. Food Qual.* **2007**, *30*, 57−80.
(24) Bourne, M. C. Texture profile analysis. *Food Technol.* **1978**, *32*, 62−66.
(25) Wang, G.; Wang, T. Egg yolk protein modification by controlled enzymatic hydrolysis for improved functionalities. *Int. J. Food Sci. Technol.* **2009**, *44*, 763−769.
(26) Juliano, P.; Li, B.; Clark, S.; Mathew, J. W.; Dunne, P. C.; Barbosa-Cánovas, G. V. Descriptive analysis of precooked egg products after high-pressure processing combined with low and high temperatures. *J. Food Qual.* **2006**, *29*, 505−530.
(27) AOCS. *Official Methods and Recommended Practices of the AOCS*, 4th ed; AOCS: Champaign, IL, additions and revisions 1993; Method Cg 2−83.
(28) Folch, J.; Lees, M.; Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497−509.
(29) Yao, L.; Jung, S. ^{31}P NMR profiling of soybean emulsion recovered from aqueous extraction. *J. Agric. Food Chem.* **2010**, *58*, 4866−4872.
(30) Sander, L. C.; Sharpless, K. E.; Craft, N. E.; Wise, S. A. Development of engineered stationary phases for the separation of carotenoid isomers. *Anal. Chem.* **1994**, *66*, 1667−1674.
(31) Holtin, K.; Keuhnle, M.; Rehbein, J.; Schuler, P.; Nicholson, G.; Albert, K. Determination of astaxanthin and astaxanthin esters in microalgae *Haematococcus pluvialis* by LC-(APCI)MS and characterization of predominant carotenoid isomers by NMR spectroscopy. *Anal. Bioanal. Chem.* **2009**, *395*, 1613−1622.
(32) Akiba, Y.; Sato, K; Takahashi, K. Meat color modification in broiler chickens by feeding yeast *Phaffia rhodozyma* containing high concentrations of astaxanthin. *J. Appl. Poult. Res.* **2001**, *10*, 154−161.
(33) Hencken, H. Chemical and physiological behavior of feed carotenoids and their effects on pigmentation. *Poult. Sci.* **1992**, *71*, 711−717.
(34) Prayitno, D. S.; Phillips, C. J. C.; Stokes, D. K. The effects of color and intensity of light on behavior and leg disorders in broiler chickens. *Poult. Sci.* **1997**, *76*, 1674−1681.
(35) Ibarz, A.; Sintes, J. Rheology of egg yolk. *J. Texture Stud.* **1989**, *20*, 161−167.
(36) Kassis, N.; Drake, S. R.; Beamer, K.; Matak, K. E.; Jaczynski, J. Development of nutraceutical egg products with omega-3-rich oils. *Food Sci. Technol.* **2010**, *43*, 777−783.
(37) Miao, F.; Lu, D.; Li, Y.; Zeng, M. Characterization of astaxanthin esters in *Haematococcus pluvialis* by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Anal. Biochem.* **2006**, *352*, 176−181.
(38) Hosomi, A.; Arita, M.; Sato, Y.; Kiyose, C.; Ueda, T.; Igarashi, O.; Arai, H.; Inoue, K. Affinity for α-tocopherol transfer protein as a determinant of biological activities of vitamin E analogs. *FEBS Lett.* **1997**, *409*, 105−108.
(39) Packer, L.; Weber, S. U.; Rimbach, G. Molecular aspects of α-tocotrienol antioxidant action and cell signaling. *J. Nutr.* **2001**, *131*, 369S−373S.
(40) Johnson, E. A.; Lewis, M. J.; Grau, C. R. Pigmentation of egg yolks with astaxanthin from the yeast *Phaffia rhodozyma*. *Poult. Sci.* **1980**, *59*, 1777−1782.