Use of Lycopene, an Antioxidant Carotinoid, in Laying Hens for Egg Yolk Pigmentation

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ABSTRACT : The possibility of lycopene affecting egg yolk pigmentation was studied with lycopene diets containing 0, 4, 8, and 12 μg/g meal, respectively. The addition of lycopene above 4 μg/g meal significantly improved yolk color after four days of supplementation. The transfer of lycopene into egg yolk was confirmed by thin layer chromatography, and high-performance liquid chromatography-mass spectrometry (HPLC-MS). The deposition rate of lycopene into egg yolk was approximately 2%, which was quantitatively determined using a HPLC with a UV detector. The result indicates that lycopene is a good candidate for egg yolk pigmentation and for making functional eggs. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 12 : 1799-1803)***

Key Words : Lycopene, HPLC-MS, Egg Yolk, Pigmentation, Deposition Rate

INTRODUCTION

Egg yolk pigmentation is a major marketing concern of the poultry industry. The pigments responsible for egg yolk pigmentation are principally carotinoids. Many studies on egg yolk pigmentation have been reported (Janky et al., 1982; Damron et al., 1984; Schaeffer et al., 1987; Hencken, 1992; Balnave and Bird, 1996; Sikder et al., 1998; Park et al., 2002). Among many kinds of pigments, canthaxanthin is the most common red carotinoid used for egg yolk pigmentation and fish coloring.

Lycopene, the red pigment that is abundant in tomatoes, is a C_{40}, open-chain hydrocarbon carotinoid (Boileau et al., 2002). Several studies have reported the antiproliferative effects of lycopene against cancer cells in culture (Levy et al., 1995; Stahl et al., 1996; Gerster, 1997). In addition, lycopene is known to be effective in the prevention and treatment of several cancers, such as prostate cancer (Giovannucci et al., 1995; Pastori et al., 1998), bladder cancer (Helzlsouer et al., 1989), digestive tract cancer (Franceschi et al., 1994). Many of the putative biological effects and health benefits of lycopene are hypothesized to occur via protection against oxidative damage (Bohm et al., 1995; Stahl and Sies, 1996; Gerster, 1997), because lycopene has very strong antioxidant activity among carotinoids (DiMascio et al., 1989; Halliwell, 1994)

Animals, including poultry, cannot synthesize lycopene *de novo*. (Balnave and Bird, 1996). Ollilainen et al. (1989) reported that only a trace of lycopene was present in egg yolk. As far as we know, no report has been published on the lycopene transfer into egg yolk through dietary feed.

The purposes of this study were to confirm egg yolk pigmentation through a lycopene diet, and to determine lycopene in egg yolk from laying hens quantitatively and qualitatively using high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), and high-performance liquid chromatography-mass spectrometry (HPLC-MS).

MATERIALS AND METHODS

Husbandry

This study was conducted with 25-week-old ISA-Brown layers receiving 15 h of light per day. The experimental diets contained 0, 4, 8, and 12 μg lycopene per g meal, respectively (Table 1), and each diet was fed to a group of 50 layers. The layers were individually caged (45×30×35 cm), and each layer was restricted to 110 g of feed per day. The layers had free access to water at all times. The basal diet was fed to caged laying hens for a minimum of 14 days before their eggs were collected for evaluation of yolk pigmentation. The diets containing lycopene were stored under dark and cool conditions for no longer than 7 days. Eggs used for determining yolk pigmentation were collected during a 16 day period. Strict hygienic measures were taken and the house temperature was maintained between 18 to 22°C during the experiment period.

Reagents

Lycopene (10%, WS), which was purchased from Roche (Switzerland), was used for the feeding experiments. Standard lycopene was purchased from Sigma (St. Louis, MO, USA). All other reagents such as hexane, ethanol, methanol, chloroform, acetone, acetonitrile, potassium hydroxide and sodium chloride were purchased from Merck (Darmstadt, Germany). All solvents used for HPLC were HPLC grade and were degassed and filtered through a 0.45

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Table 1. Composition of basal diet

Ingredient	Percentage
Corn	53.98
Soybean meal (46% protein)	22.00
Corn gluten meal (60% protein)	3.80
Tallow	2.00
Wheat bran	8.10
DL-methionine	0.12
DCP	1.30
Limestone	8.20
Salt	0.30
Choline chloride (50%)	0.05
Vitamin premix[1]	0.05
Trace mineral premix[2]	0.10
Total	100.00
Calculated value:	
ME (kcal/kg)	2,751.79
Crude protein, %	18.26
Lysine, %	0.88
Methionine, %	0.44
Methionine+cystine, %	0.76
Calcium, %	3.55
Phosphorus (available), %	0.35

[1] Supplied per kg diet: vitamin A 12,000 IU; cholecalciferol 3,600 IU; vitamin E 50 IU; vitamin K 2.25 mg; cobalamin 0.023 mg; thiamin 1.5 mg; riboflavin 7.5 mg; folic acid 0.75 mg; biotin 0.12 mg; pantothenic acid 12 mg; niacin 28 mg; pyridoxine 7.5 mg.

[2] Supplied per kg diet: manganese 90 mg; copper 125 mg; zinc 80 mg; iodine 0.4 mg; selenium 0.3 mg; iron 80 mg.

μm PTFE filter (Millipore, USA) before use. Approximately 1 mg of lycopene (Sigma) was weighed in a 50 ml volumetric flask and dissolved in acetone (0.037 mM lycopene stock standard solution). This solution was kept at -20°C and its spectrometric intensity was unchanged at 472 nm for 8 weeks.

Color scoring

The degree of pigmentation in egg yolk was determined using a BASF color fan with the range of 1 to 15, as the color standard.

Sample preparation for instrumental analysis

Eggs laid every day were stored overnight at 18°C and 75% relative humidity before analysis as described (Schaeffer et al., 1987). Briefly, the yolks were passed through a conventional household yolk separator on rolled, moistened filter paper (Whatman paper No. 2) to remove any remaining adherent albumin. After homogenization, lycopene was extracted by adding 100 ml of 4% KOH in an ethanol solution and incubating for 2 h at room temperature. Then, 75 ml of n-hexane was added and mixed vigorously ; the solution was allowed to stand for 1 h before being filtered with filter paper (Whatman paper No. 2). The remnant was mixed with 150 ml of n-hexane-ethanol (4:3) and filtered again. The filtered portion was mixed with 100 ml of 10% NaCl solution and then the water layer was removed. This step was repeated two or three times, using distilled water. After removing the water layer and filtering with Whatman paper No. 2, the extracted sample was evaporated under N_2.

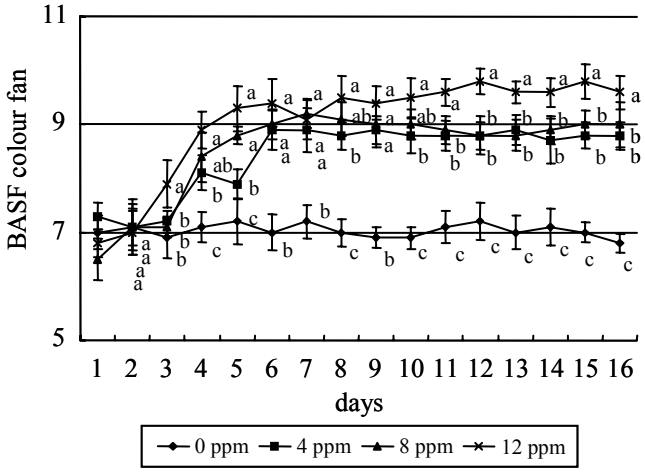

Figure 1. Effect of lycopene on egg yolk pigmentation. Mean yolk color scores were derived from 30 eggs for each point.

TLC analysis

The lycopene extracted from egg yolk was analyzed on a silica gel TLC plate (Silica gel 60, F254, Merck, USA). For separation, petroleum ether-acetone (80:20, v/v) was used as the mobile solvent.

HPLC analysis

Reversed phase HPLC with absorbance detection (at 472 nm) based on the modified method of Tzouganaki et al. (2002) was used for analyzing lycopene. HPLC separation was carried out using a Jasco (Japan) 1580 pump system and a UV-absorbance detector (UV-1575). A Luna C_{18} column (4.5 μm i.d, 4.6×250 mm, Supelco, USA) was used with methanol-acetonitrile-chloroform (47:47:6, v/v) as an isocratic eluent. Each sample extract was dissolved first in 1 ml chloroform, and methanol-acetonitrile-chloroform (47:47:6, v/v) was added to bring the total volume in the flask to 5 ml. Quantification of the eluted lycopene was accomplished by the peak area method using the calibration range of 0.93-18.63 μM lycopene (Sigma) as external standards.

Mass spectrometry

HPLC peaks corresponding to lycopene and its isomer in the extract were identified using HPLC-MS. A Quattro triple quadruple mass spectrometer (Micromass, Manchester, UK) equipped with an atmospheric pressure chemical ionization (APCI) source was used to obtain the mass spectra of the components separated by HPLC. The

Figure 2. TLC results. (a) the Roche lycopene (10%, WS) used for the feeding experiments (b) standard lycopene (Sigma) (c) the extracted sample of an egg yolk from a layer fed on the Roche lycopene (10%, WS).

APCI conditions were set as follows: corona voltage, 3.62 kV; cone voltage, 35 V; extractor voltage, 3 V; source block temperature, 150°C; desolvation temperature, 400°C.

Statistical analysis

Data were analyzed by ANOVA to identify the variance between groups with a general linear model using SAS software. Significant differences were then compared using Duncan's multiple range test ($p<0.05$) (SAS, 1998).

RESULTS AND DISCUSSION

Color scoring

To achieve pigmentation of egg yolks to an acceptable level for the consumer is very important. Figure 1 plots the yolk color values from the feeding of various lycopene concentrations over a 16 day period. In comparison with the control diet, lycopene feeding above 4 μg/g meal showed a significant increase in yolk pigmentation from the third to fourth day of supplementation. The mean BASF color score for egg yolks from layers fed on a diet containing 12 μg lycopene per gram of meal was significantly higher than that of the groups fed on a diet containing 4 and 8 μg lycopene per gram of meal after 11 days of supplementation ($p<0.05$). However, the difference in yolk color scores between the groups fed on diets containing 4 and 8 μg lycopene per gram of meal was not significant after 6 days of supplementation. The mean BASF color scores of egg yolks from laying hens fed on diets containing 4, 8, 12 μg lycopene per gram of meal after 16 days of supplementation were 8.8, 9.0 and 9.6, respectively. These values were 2.0, 2.2 and 2.8 units greater than the corresponding control value. The yolk color significantly increased with increasing dietary levels of lycopene ($p<0.05$).

Thin layer chromatography (TLC)

The extracted samples of egg yolk, as described in the Materials and Methods, were analyzed by TLC. Figure 2 shows the thin layer chromatograms of the 0.037 mM lycopene stock standard solution (Sigma), the Roche lycopene (10%, WS) used for the feeding experiments, and the extracted sample of an egg yolk from a layer fed on the Roche lycopene (10%, WS), respectively. The R_f value of the major spot in the extracted sample (Figure 2c) was the same as that of the standard lycopene (Figure 2b) and the Roche lycopene (Figure 2a), indicating that the major spot in the extract corresponds to lycopene.

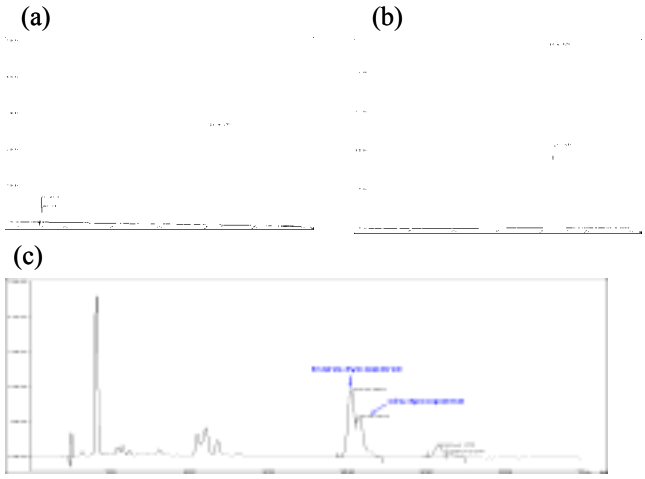

Figure 3. High-performance liquid chromatography analysis. (a) external standard lycopene (Sigma), (b) the Roche lycopene (10%, WS) used for the feeding experiments (c) the extracted sample of an egg yolk from a layer fed on the Roche lycopene (10%, WS).

HPLC analysis

The extracted samples of egg yolk were subjected into HPLC. Peak identification was based on a comparison of retention time with the lycopene standard. Figure 3a shows the chromatogram for the lycopene standard, which is *trans*-lycopene. As shown in Figure 3b and 3c, the chromatogram for the Roche lycopene and the extract shows doublets at the retention time of lycopene. The doublets are due to *trans*- and *cis*-isomer mixture because the *cis*-isomers of lycopene elute just after *trans*-isomers (Craft et al., 1992). Although about 90% of the lycopene in dietary sources is found in a linear, all-*trans* conformation, human tissues contain both *trans*- and *cis*-isomers (Boileau et al., 2002). As shown in Figure 3b and 3c, lycopene is successfully transferred from the diet to the egg yolks. The limit of quantification for lycopene was found to be 0.16 μg/g egg yolk. The recovery of lycopene (spiked in egg yolk, n=5) was 90.2%.

HPLC-MS

To clearly identify the components observed in the HPLC chromatogram, HPLC-MS analysis was carried out,

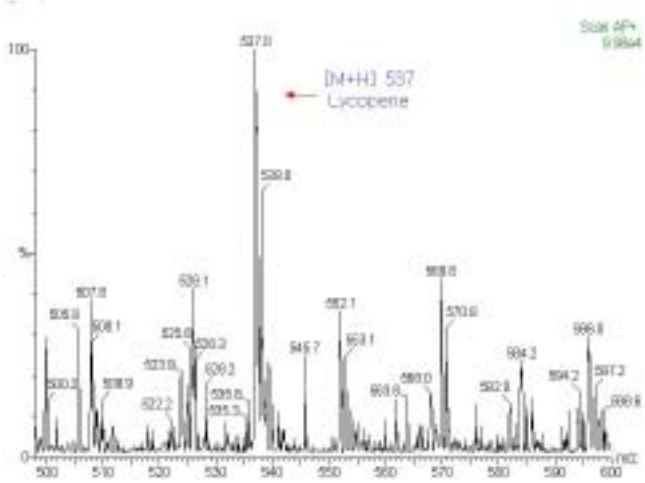

Figure 4. Positive ion APCI mass spectrum with protonated molecule of lycopene at m/z 537 after chromatographic separation of the extracted sample.

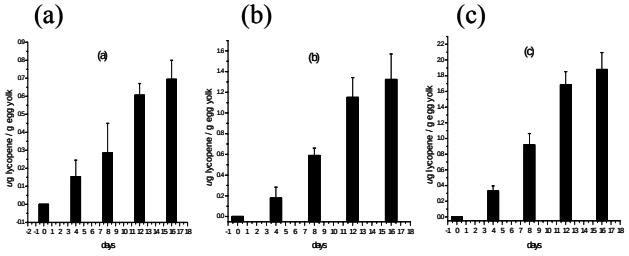

Figure 5. Comparison of lycopene content per gram of egg yolk from laying hens fed on a diet containing 4, 8, 12 μg lycopene per gram of meal, respectively (a) lycopene 4 μg/g meal (b) lycopene 8 μg/g meal (c) lycopene 12 μg/g meal.

as shown in Figure 4. The APCI-MS spectra observed for the two HPLC peaks, which correspond to *trans*- and *cis*-lycopene, showed the same protonated molecule at m/z 537 as in the case of the lycopene standard.

Deposition rate of lycopene in egg yolk

The relative degree of lycopene deposition is very important for an economical means of yolk pigmentation. Quantification of the eluted lycopene was accomplished by the peak area method using lycopene (Sigma) as an external standard. The deposition rate for the lycopene was calculated as a percentage of consumed lycopene and deposited lycopene in the egg yolk. Individual yolk weights and an average feed intake of 134 g for each egg produced were used in this calculation. The results are shown in Figure 5. When the layers were fed 12 μg lycopene per gram of meal for 16 days, the accumulation of the lycopene averaged 1.88 μg per yolk gram, which comes to an average of 31.1 μg per egg. The lycopene content per gram of egg

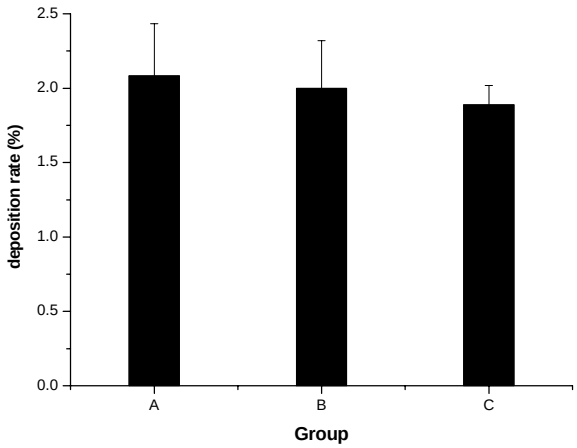

Figure 6. Deposition rate of lycopene in egg yolk after 16 days of supplementation. A group, 4 μg/g meal; B group, 8 μg/g meal; C group, 12 μg/g meal.

yolk was significantly different after 8 days of supplementation for each of the groups fed on a diet containing 4, 8, and 12 μg lycopene per gram of meal ($p<0.05$).

Figure 6 shows that lycopene was deposited at a rate of approximately 2%. The efficiency of depositing lycopene was reduced slightly with a higher concentration of lycopene in the diet. For canthaxanthin, the most common carotinoid, the rate of deposition into the egg yolk is reportedly 38% of that consumed when 5.5 μg per g meal is supplied in the diet (Balnave and Bird, 1996). The relative efficiency of lycopene deposition into the egg yolk is much lower than other carotinoids. This variation is probably due to differences in the nature of carotinoids (Sikder et al., 1998), meal composition (Erdman et al., 1986), and so on. Adding a combination of lycopene and other carotinoids to the diet may increase the deposition rate of lycopene (Erdman et al., 1986; Balnave and Bird, 1996). In addition, checking the level of lycopene in the tissue and plasma of laying hens fed on a diet containing lycopene is necessary. Further efforts are also necessary to increase the bioavailability of lycopene.

Over the years, investigators have used an assortment of analytical techniques to study yolk pigmentation. Although column chromatography is a good tool for the quantification of major yolk carotinoids, the possibility that a mixture of two carotinoids can elute at the same time or that the elution time of the same carotinoid may differ according to the degree of saponification cannot be excluded (Schaeffer et al., 1987). The HPLC-MS technique, used for the determination of lycopene in this study is a powerful tool that enables excellent resolution and sensitive detection of lycopene and other carotinoids.

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