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Effects of Pigments Extracted from the Marigold Flower on Egg Quality and Oxidative Stability of the Egg Yolk Lipids in Laying Hens

Research Article

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ABSTRACT

This research aimed to compare the effects of pigments extracted from dried petal meal of marigold flower and synthetic pigments on productive performance of laying hens, egg quality and antioxidant properties of the egg. A total of 64 laying hens, aged 75 weeks, were distributed in a completely randomized design in 4 treatments and 4 replications containing 4 hens per cages. Experimental groups contained: (1) basal diet without pigment; (2) basal diet + 25 ppm commercial synthetic pigment (red canthaxanthin); (3) basal diet + 20 ppm total carotenoids extracted from marigold flower and (4) basal diet + 40 ppm total carotenoids extracted from marigold flower. During a 8-weeks trial, egg production (number and weight), egg quality (height and diameter of the yolk, albumen height, thickness, weight of eggshell, Haugh unit), yolk color index (two methods of Roche yolk color fan and hunter lab instrument) and oxidative stability of egg yolk lipids (based on malondialdehyde value at fresh, 0 and 21th days of room temperature of storage) were measured and analyzed in a completely randomized design. There was no significant difference for egg production and its quality between experimental and control groups. However, the yolk color index in hens consumed natural or synthetic pigment was significantly higher ($P<0.01$). The oxidative stability of yolk in hens being fed marigold pigment improved significantly after 3 weeks ($P<0.05$). Consequently, although the intensity of yolk color induced by xanthophyll of marigold flower was lower than synthetic pigments, the yolk color is acceptable for Iranian consumers. In conclusion, the use of marigold as a natural colourant or incorporating its derived antioxidant components in laying hens diets should be economically evaluated.

KEY WORDS egg antioxidant properties, egg production, egg quality, marigold flower extract, pigment.

INTRODUCTION

Egg quality has different meanings, according to the perspectives of egg producers, consumers and processors. Most of the consumers are interested in shelf life, external appearance, and sensorial qualities, such as eggshell and yolk color (Faitarone *et al.* 2016). Usually, yellow corn is used in laying hens diets as the essential grain providing energy, which has xanthophyll particularly of lutein and zeaxanthin; however, this amount of xanthophyll is not enough to provide yolk color and meet the demands of market and it does not enhance the red pigmentation of yolk properly (Rowghani *et al.* 2006; Faitarone *et al.* 2016). The contents of egg components can be changed by adding specific ingredients in order to enrich the quality of yolk. Egg yolk color is known to be influenced mostly by the diet of the hens because birds are not able to synthesize pigments of yolk, but they can store the pigments obtained from the diet (Englmaierova *et al.* 2014). It is the result of the deposition and colouring capacity of oxycarotenoids, called xan-

thophylls, in the egg yolk. Therefore, the color and intensity of yolk depend on xanthophyll and its bioavailability in diet, metabolism and deposition of carotenoids in the target tissue (Breithaupt, 2007; Moeini *et al.* 2013; Englmaierova *et al.* 2014). Some of the xanthophylls such as lutein and zeaxanthin are present in common feedstuffs such corn, grains of dried distillers or alfalfa (Santos-Bocanegra *et al.* 2004). Egg yolk color is considered as a quality indicator and plays an important role in egg acceptance by the consumers (Faitarone *et al.* 2016). The preferable color intensity of yolk is different among countries. From the viewpoint of consumers, darker yolks are demanded (Englmaierova *et al.* 2014). In Iran, the orange color is more desirable (above index 10 on Roche yolk color fan, (RYCF)), which has the higher price and is famous as golden yolk egg at the market. Hence, most of the poultry producers in this country use different kinds of synthetic pigments in laying hens diet to produce orange yolk color.

Changes in yolk color can be observed when supplemental pigments (synthetic or natural) sources are added to the diet. The influence and deposition rate of synthetic pigments including Apo-8-ester are several times stronger than natural pigments for production of orange yolk color (Roche Vitamins and Fine Chemicals, 1988; Nys, 2000). However, excessive use of the permissible value of some synthetic pigments, such as canthaxanthin in poultry diets, may result in the formation of crystals in the retina of the human eye. Therefore, synthetic canthaxanthin is categorized as a potentially hazardous substance for human health in European Union (Breithaupt, 2007). Nowadays, due to more attention to the importance of feed safety and public concerns for utilization of synthetic additives, there is a demand for discovering new plant extracts for substituting natural carotenoid, delay lipid oxidation and reducing yolk cholesterol content in eggs. Beneficial activity of such natural extracted sources, like the marigold flower (*Tagetes erectus*), is related to the content of various secondary metabolites such as polyphenols, carotenoids, xanthophyll, triterpenes and essential oils (Ariana *et al.* 2011; Lokaewmanee *et al.* 2011).

One of the most important indexes of egg quality is the stability of egg yolk lipids against oxidation during the storage period. This is because lipid oxidation affects food quality, aroma, taste, and nutritional value, in addition to producing toxic compounds (Faitarone *et al.* 2016). Malondialdehyde (MDA) is a major degradation product of lipid hydroperoxides, which can be used as a marker for assessing the extent of lipid peroxidation. This component has attracted much concern since its mutagenic and carcinogenic property is shown (Botsoglou *et al.* 1994). A simple and fast method used to assess lipid oxidation in food is the thiobarbituric acid (TBA) test. This test measures the level of MDA, which is a secondary oxidation product. The reaction of MDA with 2-thiobarbituric acid, forming a coloured complex, which is measurable by spectrophotometrically at λ= 532 nm. Results are expressed as mg MDA per kg sample or frequently named as "TBA value" (Botsoglou *et al.* 1994; Osawa *et al.* 2005). It has been indicated that in addition to producing the optimal egg yolk color natural xanthophyll (such as lutein and zeaxanthin) in laying hens diet, has antioxidant properties (Kiokias and Gordon; 2004; Bou *et al.* 2009; Skrivan *et al.* 2016).

In Iran, few kinds of research were conducted on extraction and use of oleoresin or extracted pigments of marigold flower and antioxidant properties of egg yolk carotenoids in laying hens and most of them were on dried petal meal of marigold flower. So, this research aimed to compare the effects of extracted pigments of marigold flower and synthetic pigments on the production performance of laying hens, egg quality and antioxidant properties of the egg.

MATERIALS AND METHODS

Birds and management

A total of 64 Hy-Line W-36 laying hens, aged 75 weeks, were distributed in a completely randomized design with 4 treatments and 4 replications (comprised of 4 birds per replication) under standard dimension per cage. Light, temperature, nutrient requirements and the other factors were implemented according to the "Management guide of Hy-Line W-36 laying hens". From 3 weeks prior to the experiment onwards, all birds were fed the basal diet with the minimum level of carotenoids for washing out of body xanthophyll reserve.

Marigold flower pigment preparation, diets and experimental design

The pigment of marigold flower used in this research was produced from the extract of dried petal meal of marigold by hexane solvent, following saponification of extract with KOH and separation of pigment (Pratheesh *et al.* 2009). The density of total xanthophyll in extracted pigment was measured by a spectrophotometer (Strickland and Parsons, 1968). The amount of 0.157 g of pigment extracted from marigold flower was weighed and put in a crucible and soften gently. An amount of 50 mL petroleum ether was added to each sample and centrifuged at 6000 × g for 10 minutes. The supernatant was collected and transferred to a glass balloon. Quantities of balloon content were poured into the cuvette of the spectrophotometer to read the absorption rate at 474 nm. The total xanthophyll density was measured according to the formula below:

$$\text{Total xanthophyll } (\mu g/g) = A \times V\ (mL) \times 10^4 / A^{1\%}_{1cm}\ P(g)$$

Where:
A: absorption rate of the sample.
V: volume of total extract (50 mL).
P: sample weight (0.157 g).
$A_{1cm}^{1\%}$: absorption rate from the standard solution of trans Lutein equal to 2360.

The basal diet was formulated to meet the minimum amounts of total xanthophyll and the only difference of the diets was the type and the rate of the pigments. The four experimental diets were: (1) basal diet without pigment additive; (2) basal diet + 25 ppm commercial synthetic pigment (red canthaxanthin); (3) basal diet + 20 ppm total xanthophyll extracted from marigold flower and (4) basal diet + 40 ppm total xanthophyll extracted from marigold flower. Feed ingredients and chemical composition of the basal diet are demonstrated in Table 1. The total amount of carotenoids, total zanthophylls (oxycarotenoids) and lutein as the main pigment in dried petals of marigold flowers (100% dry matter) are given in the Table 2. Since, it has been proved that all xanthophylls are effective in creating egg yolk color; the total amount of xantophyll was used to determine the pigment needed for the diet.

During the 8-weeks trial feed and water were provided *ad libitum*. The data were analyzed using general linear model (GLM) procedure of SAS (2001) version 8.1 in a completely randomized design. Duncan multiple range tests ($P<0.05$) were used to compare the means.

Determination of production performance and egg quality

The numbers and weight of eggs produced by each replicate were recorded on a daily basis, and the feed intake was determined weekly. During the trial, egg quality indexes including height and diameter of the yolk, albumen height, thickness and weight of eggshell were measured once per two weeks. After breaking the eggs on a flat surface (glass plate), the height of albumen was measured at its junction site to the yolk by a micrometer. Haugh unit (HU) was calculated by the formula:

$$HU= 100 \log (H+7.57-1.7W^{0.37})$$

Where:
H: height of the egg albumen.
W: weight of the egg.

The color of yolk was assessed with two methods; (1) by use of Roche yolk color fan (Vuilleumier, 1969) and (2) by Hunter lab instrument, (color flex, USA) under the protocol of international commission d, Eclairage, which was calibrated for daylight (65D and vision angle of 10 degree).

In this measurement, three indexes of a*, b* and L* which represent redness index (green=-100 to red=100), yellowness index (blue=-100 to yellow=100) and luminosity or lightness index (black=0 to white=100) were measured and registered respectively (Grashorn and Steinberg, 2002).

Determination of oxidative stability of the egg yolk lipids

To examine the storage condition affecting the oxidation rate of egg yolk lipids, 16 eggs per replicate were collected and MDA value measured freshly at the end of the trial, as well as, after 21 days of preservation at room temperature (18 ˚C). The reaction of one mole MDA with two moles of TBA leads to a thiobarbituric acid reactive substances (TBARS), a pinkish color complex. The MDA level can be measured by a spectrophotometer (UV-visible S2100, Scinco, Korea)® at a wavelength of 532 nm (Botsoglou *et al.* 1994). Tetraethoxypropane® (1, 1, 3, 3- Tetraethoxy propane, T9889, 97%, Sigma, St. Louis, MO 63103) was used as MDA precursor in the standard curve.

RESULTS AND DISCUSSION

Table 3 demonstrates the effect of marigold flower on production performance and egg quality of laying hens. Our results revealed that marigold flower had no significant effects on production performance, including feed consumption, egg production and egg quality (eggshell weight and thickness, yolk diameter and height, albumen height and HU.

Indeed, egg characteristics including egg and eggshell weight, shell thickness, height and diameter of yolk and albumen height were not affected by the treatments.

Egg yolk color in this study changed significantly ($P<0.01$) according to the sources and concentration of pigment sources based on colored strips (RYCF) and refractory markers a*, b* and L* (Table 4). The yolk color, as well as, the yellow color (b*) and the red color indexes (a*) in hens, that consumed natural or synthetic pigment were significantly higher than the control group ($P<0.01$). The yolk color index in hens that consumed 40 ppm extracted pigment of marigolds flower was significantly lower than those using synthetic pigment. However, it was higher than in the birds that consumed 20 ppm pigment from the marigold flower ($P<0.01$). Indeed, comparison of a* and b* indices in the yolk colorimetric test with the results of the yolk color index was acceptable on the RYCF scale in our study.

Table 5 shows the effect of different experimental treatments on MDA value of egg yolk as an index of peroxidation of egg fats, which the values were increased during the storage.

Table 1 Basal diet ingredients and the chemical composition (as-fed basis)

Ingredients	%
Wheat grain	35
Yellow corn	26.31
Soybean meal	21.02
Calcium carbonate	11.90
Dicalcium phosphate	1.46
Vegetable oil	3.18
Salt	0.32
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
DL-methionine	0.21
Premix pigment	0.1
Calculated compositions	
Metabolizable energy (kcal/kg)	2700
Crude protein (%)	15.42
Linoleic acid (%)	1.36
Crude fiber (%)	3.44
Calcium (%)	4.69
Available phosphorus (%)	0.42
Lysine (%)	0.88
Methionine + cysteine (%)	0.66
Threonine (%)	0.61
Tryptophan (%)	0.21

[1] Each kg of vitamin premix contains: vitamin A: 11023 IU; vitamin E: 4600 IU; vitamin D_3: 3850 IU; vitamin K_3: 1470 mg; Thiamine: 2940 mg; Riboflavin: 5850 mg; Pantothenic acid: 20210 mg; Biotin: 550 mg; Folic acid: 1750 mg; Choline chloride: 477670 mg; vitamin B_{12}: 16500 mg; Niacin: 45930 mg and Pyridoxine: 7170 mg.
[2] Each kg of mineral premix contains: Sulfur: 27500 mg; Manganese: 150000 mg; Iron: 16800 mg; Zinc: 125500 mg; Copper: 1700 mg; Iodine: 1050 mg; Selenium: 250 mg and Molybdenum: 840 mg.

Table 2 Total amount of carotenoids, xanthophylls, and lutein in dried petal of marigold flower

Carotenoids	Carotenoids content (mg/kg)
Total carotenoids	11450
Total xanthophylls	6387
Lutein	5101

Table 3 The effects of pigments on performance and egg quality of laying hen

Item	Treatments				SE	P-value
	C^-	C^+	M1	M2		
Egg production (%)	76.47	80.77	81.56	78.42	2.17	0.09
Egg weight (g/egg)	65.33	65.00	65.38	66.97	1.60	0.88
Egg mass (g/hen/day)	49.96	52.71	53.49	51.26	1.62	0.12
Feed intake (g/day)	103.09	107.60	108.85	104.80	2.04	0.30
Feed conversion ratio	2.01	2.04	2.07	2.04	0.25	0.33
Eggshell weight (g/egg)	5.93	5.74	5.87	6.02	0.21	0.56
Eggshell thickness (mm)	0.40	0.38	0.40	0.40	0.06	0.09
Yolk diameter (cm)	4.41	4.40	4.36	4.42	0.08	0.89
Yolk height (mm)	18.61	18.75	18.64	18.63	0.18	0.95
Egg albumen height (mm)	8.64	8.85	8.72	8.23	0.42	0.32
Haugh unit	89.60	90.79	89.74	89.19	1.84	0.70

C^-: basal diet (control diet); C^+: basal diet + 25 ppm red canthaxanthin; M1: basal diet + 20 ppm pigment extracted from marigold flower and M2: basal diet + 40 ppm pigment extracted from marigold flower.
SE: standard error.

Our findings demonstrate that presence of xanthophyll in the diet and transportation to yolk causes oxidative stability of the egg yolk lipids and significantly inhibited the increasing rate of MDA after 21 days compared to the control group ($P<0.05$).

The MDA value in egg yolk of hens fed by a diet containing synthetic pigment had no significant differences with the control group and showed no protective effects against peroxidation of yolk lipids.

Similar conclusions were claimed in the previous studies that marigold flower had no significant effects on production performances (Ariana *et al.* 2011; Lokaewmanee *et al.* 2011; Moeini *et al.* 2013; Skrivan *et al.* 2015; Skrivan *et al.* 2016).

Table 4 The effects of pigments on egg yolk color indexes of laying hen

Item	Treatments				SE	P-value
	C^-	C^+	M1	M2		
Yolk color index (RYCF)	4.50[a]	13.38[d]	7.05[b]	9.05[c]	0.48	0.01
L* (lightness)	54.11[b]	50.04[a]	52.66[ab]	49.08[a]	1.39	0.02
a* (redness)	2.46[a]	11.63[c]	9.45[b]	10.11[bc]	0.88	0.01
b* (yellowness)	48.18[b]	54.97[b]	54.08[b]	55.71[b]	2.08	0.01

C^-: basal diet (control diet); C^+: basal diet + 25 ppm red canthaxanthin; M1: basal diet + 20 ppm pigment extracted from marigold flower and M2: basal diet + 40 ppm pigment extracted from marigold flower.
The means within the same row with at least one common letter, do not have significant difference (P>0.001).
SE: standard error.

Table 5 The effects of pigment extracted from the marigold flower on malondialdehyde (MDA) concentration in the yolk of fresh eggs and eggs stored for 21 days at 18 ˚C

Treatments	MDA (mg/kg)	
	The yolk of fresh eggs(0 days)	The yolk of eggs stored 21 days
C-	0.68	0.87[b]
C+	0.71	0.91[b]
M1	0.65	0.89[b]
M2	0.69	0.70[a]
SE	0.21	0.19
P-value	0.32	0.04

C^-: basal diet (control diet); C^+: basal diet + 25 ppm red canthaxanthin; M1: basal diet + 20 ppm pigment extracted from marigold flower and M2: basal diet + 40 ppm pigment extracted from marigold flower.
The means within the same column with at least one common letter, do not have significant difference (P>0.001).
SE: standard error.

Oliveira *et al.* (2017) reported that marigold flower reduced the percentage (P<0.02) and thickness (P<0.01) of the eggshell. However, in their report, there was no significant effect on the weight, specific weight, and HU and productive performance. They stated the lutein and zeaxanthin present in marigold extract could inhibit the estrogen activity in tissues and it is the reason for lower percentage and thickness of the eggshell. Skrivan *et al.* (2015) reported that addition of 150 mg/kg diet of marigold flower extract to diet increased the hen-day egg production and egg mass (g/hen/day), whereas it decreased the egg weight. By adding the dose of the marigold flower to diet, the amount of yolk lutein and zeaxanthin increased.

The natural colors of the egg yolk are the result of xanthophylls. Although marigold is also a good source of yellow xanthophylls, current results demonstrated that it is not alone sufficient for production of optimum yolk color (orange with the score more than 11 on RYCF) and / or cannot substitute the synthetic pigment.

Santos-Bocanegra *et al.* (2004) reported that hens fed by diets with 7.5 ppm of yellow xanthophylls extracted from *Tagetes* and 4.0 ppm of red xanthophylls from Capsicum had yolk eggs classified as color 11.7 ± 0.1. Indeed, synthetic carotenoids gave a yolk color that varies from 13 to 14 in the highest concentration, and from 12 to 14 at the lowest concentration. In other words, for production of orange yolk color with the score more than 11 on RYCF, there is a necessity to use simultaneously yellow and red pigment in the diet.

Hasin *et al.* (2006) suggested feeding laying pullets with 4% marigold meal to produce eggs with yolk color score 11.00. Meanwhile, Rowghani *et al.* (2006) reported adding marigold flower petal to the diet improved egg yolk color within 10-15 days after feeding and the highest RYCF was 9.15 related to adding 1.2% marigold flower to diet. Additional dietary marigold flower extract increased the yolk color score (DSM yolk color fan), and redness and yellowness of the yolks but decreased their lightness. Supplementation of this plant increased the lutein and zeaxanthin concentrations in the egg yolks from 12.34 and 5.92 mg/kg dry matter (control) to 36.33 and 25.59 mg/kg dry matter (group fed diet with 950 mg per kg) (Skrivan *et al.* 2016).

Lokaewmanee *et al.* (2010) reported the egg yolk color scores of the paprika plus marigold group (12.750) were higher than those of the paprika group in laying hen (P<0.05). Although the lightness value (42.317) decreased in paprika plus marigold group (P<0.05). It should be kept in mind that the average RYCF values achieved with natural pigments are always slightly lower than with the synthetic pigments (Santos-Bocanegra *et al.* 2004). Spasevski *et al.* (2016) added co-extruded linseed and sunflower meal into laying hens' diet and replaced the synthesized pigment with paprika and marigold flower as sources of natural pigments. They measured β-carotene and reported that the synthetic pigments can be effectively replaced by red pepper and marigold flower because the concentration of β-carotene in egg yolks increased in the experimental group after three months of the treatment.

It should be considered that pigmenting efficiency of a source depends on absorption, transport, excretion, the rate of deposition in various tissues and of conversion of the carotenoids (Nys, 2000). Since producing and using natural pigments, such as pigment extracted from marigold flower, is a costly process, an economic evaluation is required in order to use them as a colorant to obtain the desired color of egg yolk or using their derived antioxidant components (Galobart *et al.* 2004; Ariana *et al.* 2011; Englmaierova *et al.* 2014). The MDA values were increased during the storage, which is compatible with the results of other researchers reported that temperature or level and type of food supplements can significantly affect the egg quality and oxidative stability (Mohiti-Asli *et al.* 2008; Hayat *et al.* 2010; Wang *et al.* 2017). The MDA value in egg yolk of hens fed by a diet containing synthetic pigment had no significant differences with the control group and showed no protective effects against peroxidation of yolk lipids, which was in accordance with the results of Koreleski and Swiatkiewicks (2007). They indicated that synthetic carotenoids such as B-apo-8-carotenoic acid ethyl ester had no antioxidant property. Skrivan *et al.* (2016) stated that in fresh eggs only the highest amount of additional marigold flower extract (950 mg/kg diet) decreased lipid peroxidation level in comparison to the control group; however, the lipid oxidative stability in eggs stored for 28 days at 18 ˚C was significantly improved by all the additional amounts of the marigold flower extract. Foods appropriate for consumption should present lipid oxidation values below 3 mg MDA/kg of the sample, with an upper limit of 7-8 mg MDA/kg (Cadun *et al.* 2005).

CONCLUSION

This research demonstrated that utilization of extracted pigments of the marigold flower had no adverse effects on egg production and feed conversion efficiency. Improvement of egg yolk color index and oxidation protective effect of yolk lipids was the most important effect of adding 40 ppm marigold flower pigments to the diet. Although the intensity of yolk color produced by xanthophyll of marigold flower was lower than the synthetic pigments, the produced yolk color can be at an acceptable extent for Iranian consumers. In conclusion, the use of marigold as a natural colorant or incorporating its derived antioxidant components in laying hens diets should be economically evaluated.

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