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Fatty acids profile and oxidative stability of eggs from laying hens fed diets containing hemp seed or hempseed cake

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Abstract

High levels of polyunsaturated fatty acids (PUFAs) are desirable in eggs for its nutritional quality, but render them vulnerable to oxidation. The aim of this trial was to assess the effects of dietary intake of hemp (seeds or cake) on the fatty acid (FA) profile and oxidative stability of eggs. The control diet (C), which was composed of corn, soybean meal and sunflower oil (2.5%), was compared with two experimental diets that were designed to replace sunflower oil with fat from hemp seed (HS diet) or hempseed cake (HC diet). One hundred and twenty Tetra-SL LL laying hens (24-week old) were used in a 10-week trial. Each treatment was replicated five times with eight birds each. Average hen-day egg production was not affected by feeding either the HS or the HC diet. The α-linolenic acid (ALA) concentration in eggs was increased by substituting the HS- or HC-based diets fed to the hens with dietary ALA. Similar deposition profiles were exhibited by eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA) in yolks in response to increasing the dietary ALA supply. The HS group showed a greater concentration of egg yolk ALA and EPA than the HC group, which had a higher concentration of linoleic acid (LA). These alterations in yolk composition resulted in n−6 : n−3 FA ratio values as low as 2.98 and 4.15 for HS and HC, respectively, compared to 11.07 for the control diet. The atherogenicity index and cholesterol level were not affected by hemp (seed or cake) inclusion, while the thrombogenicity index decreased when compared to the control diet. On days 0, 15 and 30 of storage (4 °C), two eggs were selected randomly from each replicate (totalling 10 eggs per treatment) and analyzed. The PUFAs were not affected by storage. An exception occurred in the HC group, in which eggs had lower n-6 FA content. Egg storage for 30 d led to a reduction in egg α-tocopherol and an increase of malondialdehyde (MDA) concentration, an indicator of lipid peroxidation. The HS treatment resulted in the lowest MDA (0.22 mg MDA/kg yolk for fresh eggs and 0.35 mg for eggs in 30-day storage). The study demonstrates that the level and type of PUFAs, level of α-tocopherol and duration of egg storage significantly affected the oxidative stability of eggs. The results obtained suggest that the inclusion of hemp seed appears to be more effective in maintaining the oxidative stability of egg lipids than hempseed cake.

Keywords: egg enrichment, α-tocopherol, malondialdehyde, omega-3, storage time
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Introduction

Eggs are a food source for human consumption and contain most of the nutrients needed by humans. However, egg consumption should be limited due to the high content of saturated fatty acids (SFA) and cholesterol that increase the risk of developing many diseases (Attia *et al*., 2015). By contrast, the omega-3 fatty acids, especially α-linolenic acid (ALA, C18:3n-3), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), have positive effects on human health (Simopoulos, 2002). Therefore, maximizing the three fatty acid (FA) levels in eggs would be beneficial to human health.

A large number of studies have concluded that the FA composition of eggs is dependent on the FA composition of the feed of the laying hens, hence enriching eggs is done by enriching the feed of the laying hens with a source of n-3 PUFAs that will be assimilated in the eggs (Hammershøj & Johansen, 2016; Nyberg, 2017). To increase the concentration of polyunsaturated fatty acids (PUFAs) in eggs, sources such as fish oil, linseed oil, corn oil, rapeseed oil, soybean oil and sunflower oil can be included in the diet of laying hens.

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Hemp (*Cannabis sativa* L.) was initially grown for its fibre content and seed production in the Nordic countries, but was prohibited because of its high narcotic element (delta-9-tetrahydrocannabinol - THC) content. In 2003, hemp varieties with low concentrations of THC were allowed to be grown in the European Union (EU Council Directive, 2003) for fibre and oil production. Currently in the EU, only cultivars with less than 0.2% THC, as described in Council Regulation (EC) No 1420/98, are permitted. In 2012, the hemp cultivar, Armanca, was found in Romania to be a variety that could be utilized for oil seed production.

The increased production of hemp and the availability of hemp seed and hempseed products have created opportunities for use in livestock rations. Whole hemp seeds contain approximately 25% crude protein (CP), 33% to 35% oil and 34% carbohydrate, in addition to a broad range of vitamins and minerals (Gakhar *et al.*, 2012). In a recent study, the inclusion of hemp seed and hempseed oil as a source of PUFAs, especially ALA, in the diet of laying hens was effective in improving the egg yolk FA profile by increasing the n-3 fatty acid concentration of eggs (Gakhar *et al*., 2012).

Chain length and the number of double bonds compromise n-3 FA oxidative stability. The hazard to lipid oxidation is higher in eggs enriched in EPA and DHA rather than ALA (González-Esquerra & Leeson, 2000). The oxidative damage of yolk lipids and overall reduction in egg quality is aggravated during storage (Hayat *et al*., 2010). Therefore, the control of lipid oxidation in such products is required to prevent the loss of nutritional value and to prevent the production of potentially toxic compounds. To maintain a high quality, the concentration of antioxidants should therefore be elevated. In egg yolks the concentration of α-tocopherol and carotenoids as antioxidants is believed to be important for oxidative stability (Criste *et al*., 2018). Dietary vitamin E supplementation has been shown to alleviate this problem with eggs (Hayat *et al*., 2010).

One potential opportunity for the use of hemp seed as a feed ingredient relates to the relatively high level of ALA (17% - 19%) (Parker *et al*., 2003) compared to other vegetable oils (<9%), though flax being a notable exception. Because flaxseed has been used successfully in the diets of laying hens to produce n-3 enriched eggs (Basmacioglu *et al*., 2003; Souza *et al*., 2008; Hayat *et al*., 2010; Petrovic *et al*., 2012) the level of ALA in hemp seeds or hempcake might provide additional options to improving egg quality. Hitherto, only a limited number of scientific reports can be found on hemp and its derived products in poultry diets and its nutritive and antioxidant efficacy in poultry (Gakhar *et al*., 2012; Konca *et al*., 2014). Consequently, the current study was designed to assess the effect of dietary intake of hemp seeds or hempcake on laying hen performance and egg yolk FA profile. As stated, high levels of PUFAs in eggs are desirable for its nutritional quality but this renders it vulnerable to oxidation. Accordingly, the secondary objective of the study was to examine the oxidative stability of yolk lipids in relation to the FA profile and tocopherols concentration of eggs throughout a 30-day refrigerated storage period, when the diet included a source rich in PUFAs.

Materials and Methods

One hundred and twenty, 24-week-old Tetra-SL LL laying hens were assigned to three dietary treatments with five replicates of eight hens each (40 laying hens/treatment), in a completely randomized design. The control diet (C), which contained corn, soybean meal and sunflower oil (2.5%) as basic ingredients, was compared with two experimental diets that were designed to replace sunflower oil and part of the soybean meal in the control diet with fat from hemp seed (HS diet) or hempseed cake (HC diet).

The hens were fed a balanced commercial diet as their daily requirement for two weeks prior to commencement of the study to allow them to adapt and reach a standard level of egg production. Before starting the trial, the egg production of the hens was measured individually. Hens with equal egg production were blocked and within block were placed in each replicate. Each replicate of eight birds was housed in a cage of 1.0 m×1.5 m. A bird occupied an area of approximately 1875 cm^2/hen. Feed and water were offered ad libitum during the experiment. Environmental temperature was set at 22 °C. A regimen of 14 h constant lighting (15 lux) and continuous ventilation were provided. All the birds were kept under uniform management conditions throughout the experimental period. The experiment lasted 10 weeks, with the hens reaching the age of 34 weeks.

The hemp seeds (*Cannabis sativa* L., cv. Armanca variety for oil) were cold-pressed by a commercial oil producer (RosbroAvicom SRL), to obtain hempseed cake. The composition of the hemp seed and hempseed cake used for the formulation of diets was based on analysis realized in the laboratory. Samples were analyzed for dry matter (DM) (ISO, 1999), neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Van Soest *et al*., 1991) on a fibre analyser (Ankom Technology, Fairport, NY), crude fat (Soxhlet method, AOAC, 1996) and crude protein (CP) (Kjeldahl technique) (Vapodest Distillation Systems - Gerhardt; AOAC, 1996) (Table 1). Hemp seed and hempseed cake were supplied in quantities calculated to provide the same amounts of fat, that is, 25 g/kg feed.

Eggs were collected twice daily at 9:30 am and 18:30. Feed intake was measured weekly for each replicate. Eggs collected over two days at the end of each week were weighed. All birds were weighed at 24 and 34 weeks of age. Eggs were collected for a period of eight consecutive days from all of the pens (two

eggs per replicate) within each of the three treatments (a total of 240 eggs; 80 eggs/treatment; 16 eggs/ replicate) during weeks 9 and 10. Samples of the hemp seed, hempseed cake and experimental diet samples (n = 3) were collected and stored at -20 °C until analyzed for fatty acids.

On days 0, 15 and 30 of storage, two eggs were selected randomly from each replicate (totalling 10 eggs per treatment). The eggs were stored in a cold room at 4 °C. Lengths of storage times of 15 and 30 days were considered similar to the duration of consumer storage of shell eggs. Egg yolks were separated, and an aliquot was taken for FA profile, tocopherols concentration and lipid oxidation products, measured as TBA reactive substances (TBARS - thiobarbituric acid reactive substances).

The FA composition of the hemp seed, hempseed cake and dietary and yolk samples was determined using standard gas chromatographic techniques of fatty acid methyl esters (FAME) (AOAC, 1996). Fatty acids were extracted with a chloroform/methanol mixture according to Folch *et al*. (1957). At the first stage fat was saponified, using a 0.5N KOH methanol solution at 70 °C. Esterification with methanol was conducted in the presence of sulphuric acid as a catalyst.

Chromatographic analysis was performed using a Shimadzu GC-2010 gas chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with a DB-23 column (60 m x 0.25 mm i.d. and 0.25 µm film thickness). Column and detector temperatures were 190 and 240 °C, respectively. Carrier gas was helium at 1.0 mL/min ratio. The temperature of injection port was 230 ºC with the split ratio of 1 : 80; temperature programme was 80ºC/5 min, 200 ºC/30 min and 230 ºC/15 min (Criste *et al*., 2018). The FAME were identified using external standards (Supelco 37 Component FAME mix; Supelco Bellefonte, PA, USA), and the FA concentrations were calculated from the peak area of the corresponding FAs in relation to the total area of all peaks.

Egg yolks were also analyzed for total cholesterol content by the Washburn & Nix (1974) method, and calculated and expressed as milligrams per egg. Cholesterol was separated from fat after saponification with KOH and extracted with ethyl ether. The sample was subjected to chromatographic analysis under the following conditions: the length of a glass column, 1 m; internal diameter, 4 mm; film thickness, 0.25 µm; temperature of detector, 300 °C; temperature of injector, 290 °C; temperature of column, 260 °C; carrier gas, argon, flow rate, 50 cm^3/min (Criste *et al*., 2018).

Extraction of tocopherols was done with a mixture of diethyl ether and petroleum ether (1 : 1), in aliquots of 20 mL. The ether phase, after separation, was saponified with methanolic KOH solution (10%), after which tocopherols were extracted in hexane, washed with water in a separating funnel, and evaporated to dryness. After evaporation of the hexane phase, separation of the tocopherols was by high performance liquid chromatography (HPLC) using the Parkin-Elmer LC-295 system, equipped with the Alltech C18 column (length 15 cm, internal diameter 4.6 mm and particle size 3 µm). The mobile phase for the various tocopherols was acetonitrile : methanol (85 : 15)/isopropanol 90/10. The content of tocopherol in the samples of hemp (seed and cake) and egg yolks were calculated according to external standards (Sigma-Aldrich, USA) through a linear regression from known standards (Criste *et al*., 2018).

Malondialdehyde (MDA) arising from the oxidation of PUFAs was chosen as a marker of secondary products of lipid oxidation. The MDA concentration was determined based the 2-thiobarbituric acid (TBA), as described by Salih *et al*. (1987) with some modifications by Cherian *et al*. (1996). Egg yolk samples (2 g) were weighed into test tubes, and perchloric acid added. The samples were homogenized for 15 s at high speed. Butylated hydroxytoluene (BHT) was added to each sample during homogenization to control lipid oxidation. The homogenate was filtered through filter paper. The filtrate (2 mL) was mixed with 2 mL of 20 mM TBA in distilled water and incubated in a boiling water bath for 30 min. Absorbance was determined at 531 nm. The oxidative stability was expressed in milligrams of MDA per kg of egg yolk.

Health lipid quality was assessed by calculating the atherogenic and thrombogenic indices as well as the ratio between the hypocholesterolemic and hypercholesterolemic, using the following equations:

- Atherogenicity index (AI) = [(4 x C14:0) + C16:0 + C18:0] /MUFA + PUFA n-6 + PUFA n-3 (Ulbricht & Southgate, 1991);
- Thrombogenicity index (TI) = (12:0 + 16:0 + 18:0)/[(0.5×MUFA)+(0.5×n-6 PUFA)+(3×n-3 PUFA)+(n-3 PUFA/n-6 PUFA)] (Ulbricht & Southgate, 1991);
- Hypocholesterolemic/hypercholesterolemic (h/H) = (C18:1 + PUFA)/(C12:0 + C14:0 + C16:0), (Fernandez *et al*. (2007).

The results of the analysis were compared to the analysis of variance (ANOVA) with the diet as principal effect (SAS, Software Version 8.2. SAS Institute, Inc., Cary, NC). The ANOVA for fatty acid level, α-tocopherol and MDA of yolk included the main effect of the diet, storage day and the interaction between these two main effects. In cases of significant differences, Duncan's multiple-range test was employed to compare differences among means obtained during different treatments. In addition, Pearson correlations were used to evaluate the relationships between egg yolk oxidative stability (MDA values) and α-tocopherol, PUFAs, and storage time. The level of significance to detect statistical differences was set at $P < 0.05$ for all analyses.

Results and Discussion

Hemp seeds contained 24.7% CP and 31.2% crude fat (Table 1). The cold-pressing process removes 60.4% of the fat, and increased the CP level to 31.2%. These results are in line with those observed by Silversides & Lefrancois (2005), Halle & Schone (2013) and Neijat *et al*. (2014), who concluded that hemp products may serve as potentially valuable feed ingredients for laying hens.

Table 1 Chemical composition, major fatty acid content of the lipid and tocopherol content of hemp seed and hempseed cake

	Hemp seed	Hemp seed cake
Analyzed nutrients (%)		
Dry matter	91.1 ± 1.97	90.3 ± 2.05
Crude protein	24.68 ± 1.25	31.22 ± 1.83
Crude fat	31.19 ± 2.29	12.35 ± 1.74
Crude ash	4.72 ± 0.37	6.81 ± 0.55
Crude fibre	16.06 ± 0.67	25.14 ± 1.34
Neutral detergent fibre	35.41 ± 2.57	44.17 ± 3.78
Acid detergent fibre	24.35 ± 1.39	29.72 ± 1.27
AMEn, kcal/kg[1]	3186	1849
Fatty acid (FA) (% of FAME)		
C16:0 (palmitic acid)	6.54 ± 0.28	8.65 ± 0.32
C18:0 (stearic acid)	2.59 ± 0.09	3.70 ± 0.12
C18:1 n-9 (oleic acid)	9.07 ± 0.26	12.25 ± 0.19
C18:2 n-6 (linoleic acid)	56.5 ± 1.67	53.5 ± 1.98
C18:3 n-3 (α-linolenic acid)	21.46 ± 1.15	18.75 ± 1.37
C18:3 n-6 (γ-linolenic acid)	3.81 ± 0.11	3.14 ± 0.18
Saturated FA	9.13 ± 1.21	12.35 ± 0.97
Monounsaturated FA	9.07 ± 1.45	12.25 ± 0.91
Polyunsaturated FA	81.8 ± 2.96	75.4 ± 2.45
n-6 : n-3 FA	2.81 ± 0.39	3.02 ± 0.28
Tocopherols (mg/100 g lipid)		
α-tocopherol	4.66 ± 0.19	3.16 ± 0.22
γ- tocopherol	62.7 ± 1.28	37.8 ± 1.53
δ- tocopherol	3.79 ± 0.14	2.09 ±0.18
Total tocopherols	71.2 ± 2.18	43.1 ± 1.79

Values are means ± SD, analyzed individually in four replications
[1]AMEn: apparent metabolizable energy, nitrogen-corrected (calculated values according to the equation of Sibbald (1980))
FAME: fatty acid methyl esters

Hempseed oil contained 21.4% linolenic acid, a lower concentration compared to flaxseed oil, which contains about 50% linolenic acid (Hayat *et al*., 2010). Hempseed oil also contains 56.5% linoleic acid, a higher concentration compared to flaxseed oil (15%) and rapeseed (21%). Finally, hempseed oil is richer in PUFA (81.8%) compared with the other oilseeds (camelina seed, 73%; flaxseed, 69%; sunflower, 66%; soybean, 60%; and rapeseed, 31%) (Hurtaud & Peyraud, 2007). Hemp seeds showed a high content of tocopherols (Table 1) which can ensure high oxidative stability of egg yolks.

All diets were balanced for energy, CP, crude fat, essential amino acids and minerals as required for laying hens (TETRA SL LL guidelines) (Table 2). The α-linolenic acid constituted 4.8% and 15.3% -12.9% of total FAs, and the linoleic acid (LA) : ALA ratios of the diets were 7.9 and 2.6 - 3.0 for the control and hemp diets, respectively.

Table 2 Formulation and nutrient composition of diets containing hemp seed or hempseed cake compared with the control diet

Item	Control	Hemp seed	Hemp seed cake
Ingredient (%)			
Corn	57.0	58.2	56.3
Soybean meal	26.8	15.5	3.0
Corn gluten meal	2.5	7.0	9.0
Sunflower oil	2.50	-	-
Hemp seed	-	8.04	-
Hempseed cake	-	-	20.32
Natuzyme P_{50}	0.02	0.02	0.02
Lysine-HCl	-	0.10	0.27
DL-Methionine	0.12	0.07	0.03
Limestone	8.9	8.9	8.9
Monocalcium phosphate	1.5	1.5	1.5
Salt	0.2	0.2	0.2
Vitamin-mineral premix*	0.5	0.5	0.5
Calculated nutrient content			
AMEn (kcal/kg)	2810	2808	2803
Crude protein (%)	18.1	18.1	18.0
Crude fat (%)	5.25	5.26	5.22
Crude fibre (%)	2.79	3.52	5.98
Lysine (%)	0.85	0.85	0.85
Methionine (%)	0.42	0.42	0.42
Calcium (%)	3.76	3.75	3.75
Available phosphorus (%)	0.40	0.40	0.40
Analyzed nutrient content			
Dry matter (%)	91.6	91.8	91.6
Crude protein (%)	17.83	17.89	18.02
Crude fat (%)	5.37	5.25	5.18
Crude fibre (%)	3.12	3.71	6.20
Linoleic acid (LA, % of FAME)	38.8	40.1	39.6
α-Linolenic acid (ALA, % of FAME)	4.86	15.33	12.93
Ratio LA to ALA	7.98	2.68	3.06

* Providing per kg of diet: Mn 80 mg; Zn 50 mg; Fe 60 mg; Cu 7 mg; Co 0.4 mg; I 2 mg; Se 0.10 mg; choline chloride 230 mg; vitamin A 11000 IU; vitamin D_3 2500 IU; vitamin E 50 mg; vitamin K_3 3.3 mg; vitamin B_1 3 mg; vitamin B_2 5 mg; niacin 25 mg; calcium pantothenate 11 mg; vitamin B_6 6 mg; vitamin B_{12} 0.08 mg; folic acid 1.7 mg
AMEn: apparent metabolizable energy, nitrogen-corrected; FAME: fatty acid methyl esters

For hens consuming diets containing hemp seeds (HS group), egg weights and egg mass (g/hen/day) were significantly higher (P <0.05) than those observed in both the control and the HC diets (Table 3). With respect to other measures of production performance, the inclusion of hemp seeds or hempseed cake in laying hen diets did not affect feed intake, layer weight, feed conversion efficiency or hen-day egg production significantly. These results correspond with those of a previous study (Gakhar *et al*., 2012) which reported that the addition of hemp seed increased egg weight and did not lead to significant differences in feed intake, final bird weights and average hen-day egg production. On the other hand, Scheideler & Froning (1996) reported a positive effect on egg weight after feeding flaxseed at 5%, 10% and 15% levels for six weeks.

Table 3 Performance and egg quality of hens consuming diets containing hemp seed or hempseed cake

Item	Dietary treatment			SEM[1]	*P*-values
	Control	Hemp seed	Hemp seed cake		D[2]
Feed intake (g/hen/d)	106.5	109.1	102.6	1.012	n.s.
Egg weight (g)	58.8[b]	61.7[a]	58.3[b]	0.937	*
Laying rate (%)	96.4	96.6	95.7	0.872	n.s.
Egg mass (g/hen/d)	56.6[b]	59.6[a]	55.8[b]	0.443	*
Feed conversion ratio (g of feed/g of egg)	1.87	1.83	1.83	0.037	n.s.
Layer's weight (kg) - initial	1.61	1.58	1.60	0.066	n.s.
Layer's weight (kg) - final	1.86	1.85	1.87	0.054	n.s.
Yolk weight (g)	13.7	13.9	13.7	0.227	n.s.
Egg yolk fat content (%)	29.54	30.12	29.76	0.314	n.s.

Means within a row with different superscripts are different (*P* <0.05)
[1] SEM: standard error of the mean
[2] D: Effect of experimental diet; **P* <0.05; ns: *P* >0.05

In this study no effects of the hemp diet were recorded in yolk weight or total lipid content. This agrees with the findings by Johansson (2010) where laying hens were fed diets containing hempseed cake. However, it has been reported that the increased levels of n-3 PUFAs in the diet affected yolk weight (Cabrera *et al*., 2006). Gonzalez-Esquerra & Leeson (2000) suggested that n-3 fatty acids in fish oil leads to a decrease in circulating triacylglycerols in blood plasma which may have a limiting effect on the availability of lipids for yolk formation

The fatty acid composition of the egg yolks is presented in Table 4. There was no significant difference between the SFAs in all the egg yolk samples. The C group had a lower PUFA concentration compared to the other groups, which was largely because of the low n-3 FAs.

Palmitic acid (C16:0), oleic acid (C18:1n-9) and linoleic acid (C18:2n-6) were the fatty acids with the highest concentrations in all egg yolk samples. Palmitic acid did not differ between the treatments whereas the oleic acid concentration was higher in C group compared to the HS and HC groups. Palmitic acid, oleic acid and linoleic acid were the most abundant fatty acids found in the egg yolks. This has also been shown in other studies (Omidi *et al*., 2015; Nyberg, 2017).

The total SFA concentration in egg yolks in this study ranged from 34.1% to 35.2%, and in the studies that were used for comparison, the SFA ranged from 30.8% to 38.8%, which shows that SFA is maintained within a very narrow range (Petrovic *et al*., 2012; Omidi *et al*., 2015; Nyberg, 2017). Cabrera *et al*. (2006) related this finding to the generally lower absorption of SFA, and the fact that hens tend to maintain an optimal saturated : unsaturated FA ratio for growth and hatchability of chicks. PUFAs (n-6 and n-3 series) in the diet are more effective in reducing MUFA than SFA in eggs through the inhibition of Δ-9 desaturase enzyme activity on the production of C18:1n-9 (Mazalli *et al*., 2004).

The HS and HC eggs did not show any difference in MUFA and PUFA concentration, but the egg yolks of hens in the HS group had a significantly higher amount of n-3 PUFA than the egg yolks of hens in the HC group; these having had a significantly higher amount of LA (C18:2n-6) in egg yolks. It is also important to note that the HS egg weight was higher compared to that of the HC eggs, which could also have an impact on fatty acid composition, but that was not investigated in this experiment.

The hemp product inclusion in the diet (HS and HC) led to an increase in the concentration of ALA in the laying hen diet and led to increases in the individual n-3 fatty acids (ALA, EPA and DHA) and the total n-3 fatty acid concentration in eggs yolks. The overall increase in the total n-3 content of the egg yolks occurred at the expense of the level of oleic acid, which decreased at inclusion of HS or HC in the laying hen diet. The ALA in the eggs from the hens fed the HS and HC diets were 5.8 and 4.7 times, respectively, higher than in the control eggs. Gakhar *et al*. (2012) and Souza *et al*. (2008) reported similar results, where higher levels of this FA in the eggs from the hens fed hemp seed and flaxseed or its oil were observed compared to those with corn, soybean, canola and sunflower oils. The LA concentration was significantly lower (*P* <0.01) in the eggs produced by the hens fed diets of hemp seed and the control diet.

Table 4 Effect of hemp seed or hempseed cake on the fatty acid profile and cholesterol concentrations of egg yolks in laying hens

	Dietary treatment			SEM[1]	*P*-values
	Control	Hemp seed	Hemp seed cake		D[2]
Fatty acid (FA) (% of FAME):					
Lauric (C12:0)	0.06	0.06	0.05	0.001	n.s.
Myristic (C14:0)	0.34	0.30	0.32	0.018	n.s.
Palmitic (C16:0)	25.82	24.74	24.45	0.327	n.s.
Stearic (C18:0)	8.98	9.05	9.42	0.181	n.s.
Palmitoleic (C16:1)	3.25	3.96	3.47	0.108	n.s.
Oleic (C18:1 n-9)	39.79^{a}	36.29^{b}	35.69^{b}	0.912	**
Linoleic (C18:2 n-6) (LA)	16.69^{b}	16.47^{b}	18.62^{a}	0.673	**
α-Linolenic (C18:3 n-3) (ALA)	0.60^{c}	3.51^{a}	2.87^{b}	0.171	***
γ-Linolenic (C18:3 n-6) (GLA)	0.06^{b}	0.18^{a}	0.16^{a}	0.014	**
Arachidonic (C20:4 n-6) (AA)	1.90^{a}	1.66^{b}	1.65^{b}	0.060	*
Eicosapentaenoic C20:5 n-3 (EPA)	0.41^{c}	1.48^{a}	0.93^{b}	0.094	***
Docosahexaenoic C22:6 n-3 (DHA)	0.68^{b}	1.15^{a}	1.12^{a}	0.087	***
Others FA	1.36	1.16	1.25	0.024	n.s.
∑ Saturated FA (SFA)	35.20	34.15	34.24	0.325	n.s.
∑ Monounsaturated FA (MUFA)	43.04^{a}	40.25^{b}	39.16^{b}	0.419	**
∑ Polyunsaturated FA (PUFA)	20.40^{b}	24.44^{a}	25.35^{a}	0.475	**
∑ n-6 FA	18.71^{b}	18.30^{b}	20.43^{a}	0.518	*
∑ n-3 FA	1.69^{c}	6.14^{a}	4.92^{b}	0.212	***
n-6 : n-3 FA	11.07^{a}	2.98^{c}	4.15^{b}	0.193	***
LA : ALA	27.81^{a}	4.69^{c}	6.49^{b}	0.572	***
PUFA : SFA	0.58^{b}	0.72^{a}	0.74^{a}	0.002	**
Hypercholesterolemic FA[3]	26.22	25.10	24.82	0.429	n.s.
Hypocholesterolemic FA[4]	60.19	60.73	61.04	0.718	n.s.
h/H	2.29	2.42	2.46	0.210	n.s.
AI	0.57	0.54	0.54	0.023	n.s.
TI	0.97^{a}	0.70^{b}	0.76^{b}	0.018	***
Total cholesterol:					
- mg/g of yolks	13.65	12.98	13.59	0.372	n.s.
- mg/egg	187.0	180.4	186.2	2.137	n.s.

Means within a row with different superscripts are different significantly (*P* <0.05);
[1] SEM: standard error of the mean;
[2] D: effect of experimental diet (ns: *P* >0.05; **P* <0.05; ***P* <0.01; ****P* <0.001);
[3] Hypercholesterolaemic FA: (C12:0 + C14:0 + C16:0);
[4] Hypocholesterolemic FA: (C18:1 + polyunsaturated FA)
h/H: hypocholesterolemic/Hypercholesterolemic ratio; AI: atherogenicity index; TI: thrombogenicity index
FAME: fatty acid methyl esters

The inclusion of hemp seed or hempseed cake dramatically reduced the LA : ALA ratio in egg yolks from 27.8 : 1 in the control diet to 4.6 - 6.4 : 1 in the HS and HC diets (see Table 4). This is due to the high amount of ALA in hemp seed and hempseed cake, consistent with the findings of previous studies which showed a reduced LA : ALA ratio in eggs from hens consuming diets containing hemp seed (Gakhar *et al*., 2012; Raza *et al*., 2016) or hempseed oil (Neijat *et al*., 2016).

Hemp seed has a high concentration of ALA (>21% - Table 1), which makes it a good ingredient for n-3 fatty acid enrichment in egg yolk, but, unfortunately, hemp seed lacks the n-3 FAs long-chain of EPA and DHA. The present study has shown that hemp seed and hempseed cake significantly increased both EPA and DHA in egg yolk. This is because hens have a high ability to convert ALA to n-3 FA long-chain (Howe *et al*., 2002). Hempseed cake is less efficient than whole raw seed in increasing eggs yolk ALA and EPA content. Van Elswyk (1997), comparing different sources of n-3 PUFA enrichment in eggs, showed that hens fed ground or crushed flaxseed showed higher levels of EPA and DHA in the yolk. The intake of ALA appeared to be the primary determinant of the total n-3 fatty acid content of egg yolks (Gakhar *et al*., 2012). Conversion of ALA to EPA and DHA occurs through the sequential actions of Δ-6 desaturase, elongase and Δ-5 desaturase (Gakhar *et al*., 2012).

The amount of AA (C20:4n-6) in egg yolk decreases in HS and HC diets. The results obtained by Petrovic *et al*. (2012) show a significant decrease in AA content which was in correlation with the amount of n-3 PUFA added to the feed (r = -0.958). The increased ingestion of ALA in a diet results in a significant decrease in the formation of AA (da Silva *et al*., 2009) due to the greater utilization of Δ-6 desaturase in the n-3 FA with respect to the n-6 pathway. Thus, the high level of ALA limits the synthesis of AA from LA, because ALA competes with LA by the same Δ-6 desaturase enzymes (Cabrera *et al*., 2006; Ceylan *et al*., 2011).

Our results show that enrichment of laying hens' diets with n-3 PUFA does not affect the level of cholesterol in eggs, which is in accordance with the results of Ceylan *et al*. (2011) who demonstrated that different dietary oil sources rich in n-3 PUFA (fish oil, linseed oil, rapeseed oil) at two levels of inclusion (1.5% and 3.0%) in laying hen diets, affected FA composition of the egg yolk, while cholesterol content was not influenced. It has been hypothesized that the inability to markedly reduce egg cholesterol levels is due to a physiological control mechanism that ultimately causes the cessation of egg production when yolk cholesterol deposition is inadequate for embryo survival (Ceylan *et al*., 2011). In contrast to our finding, the study conducted by Shahid *et al*. (2015) showed that the yolk cholesterol level of laying hens whose diet had been treated with hemp seed at a rate of 15% - 25% was significantly lower (P <0.05), compared to the control group. The results of a study by Johansson (2010) suggest that hemp seed may lower the cholesterol levels in egg yolk.

The PUFA/SFA and n-6/n-3 PUFA ratios, atherogenity index (AI) and thrombogenity index (TI) are commonly used to assess the nutritional value of animal fat on consumer health. In general, a ratio of PUFA to SFA above 0.45 and a ratio of n-6/n-3 below 4.0 are required in human diets to combat 'lifestyle diseases' such as coronary heart disease and cancer (Simopoulos, 2002). In the present study, the PUFA/SFA ratios (0.57 - 0.74 : 1) were higher than the recommended values for all groups, whereas the n-6/n-3 FA ratios were within the recommended levels for the HS and HC groups (2.98 - 4.15 : 1), and considerably higher than the recommended values for C group (11.07:1) (Table 4). Basmacioglu *et al*. (2003) reported a decrease in the n-6/n-3 FA ratio from the usual value in commercial eggs (12:1) to a value below 3 : 1 using feed enriched with fish oil, linseed oil, or a combination of both, results which are in line with those of our study. The introduction of hemp seeds in laying hen feed (HS group) provided the most beneficial ratios of PUFA/SFA (0.71) and n-6/n-3 PUFA (2.98) in egg yolk. The high levels of ALA, EPA and DHA in the egg yolk of HS and HC groups are partly countered by the contents of MUFA, in particular oleic acid.

As shown in Table 4, the inclusion of hemp seed and hempseed cake in the diet decreased the value of TI, but did not alter the AI value and the hipocholesterolemic /hipercholesterolemic FA ratio in egg yolk.

The values of n-3 FA, ALA and EPA, higher, and n-6/n-3 FA ratio lower in HS than HC (P <0.05), suggest that the inclusion of hemp seed, rather than hempseed cake, appears to be more effective in ameliorating these indices in egg yolk. Storage had no effect on the concentration of SFA in eggs from the HS and HC groups (Table 5). However, on day 15 and day 30, a reduction in SFA and an increase in MUFA were observed in the control group. During storage (day 20 through to day 60), an increase (>4% to 9%) in MUFA content, mainly on the basis of oleic acid, in egg yolks, was observed by Hayat *et al*. (2010).

PUFAs were not affected by storage. An exception occurred in the HC group, where the day 15 and day 30 eggs showed a lower n-6 FA content than day 0 eggs (P <0.05). The n-3 FA in egg was stable during storage in all treatments. The reduction in n-6 FA in HC eggs during storage suggests that hempseed cake tocopherols were not effective in minimizing the degradation of PUFAs in eggs stored.

The changes in n-6 : n-3 fatty acid ratios upon storage occurred in eggs from hens fed the control diet, when compared with the HS and HC groups, suggesting the role of α-tocopherol in modulating the desaturase and elongating the pathway in a favourable way.

Maximum α-tocopherol concentration was observed in the eggs from hens fed hemp seed (Table 5). Storage led to a reduction in egg α-tocopherol content in all groups. This reduction was increased between 15 day and 30 day of storage. At day 30 of storage, cumulative decreases in egg α-tocopherol of 30.7%,

Table 5 Effect of feeding hemp seed or hempseed cake on egg fatty acids profile and content of α-tocopherol and thiobarbituric acid reactive substances (TBARS) during storage

Concentration of yolk	Storage time (days)	Dietary treatment		
		Control	Hemp seed	Hemp seed cake
SFA (% of FAME)	0	35.20^{x}	34.15	34.24
	15	33.53^{y}	34.02	33.60
	30	33.83^{y}	34.22	33.68
MUFA (% of FAME)	0	$43.04^{a,y}$	40.25^{b}	$39.16^{b,y}$
	15	$45.18^{a,x}$	40.83^{b}	$41.57^{b,x}$
	30	$45.37^{a,x}$	40.88^{b}	$42.14^{b,x}$
PUFA (% of FAME)	0	20.40^{b}	24.44^{a}	$25.35^{a,x}$
	15	19.73^{b}	24.06^{a}	$23.55^{a,y}$
	30	19.42^{b}	23.61^{a}	$23.00^{a,y}$
n-6 FA (% of FAME)	0	18.71^{b}	18.30^{b}	$20.43^{a,x}$
	15	18.20	18.39	18.87^{y}
	30	17.92	18.20	18.23^{y}
n-3 FA (% of FAME)	0	1.69^{c}	6.14^{a}	4.92^{b}
	15	1.53^{c}	5.67^{a}	4.68^{b}
	30	1.50^{c}	5.41^{a}	4.77^{b}
n-6/n-3	0	$11.07^{a,y}$	2.98^{c}	4.15^{b}
	15	$11.89^{a,x}$	3.24^{c}	4.03^{b}
	30	$11.95^{a,x}$	3.36^{b}	3.82^{b}
α-tocopherol (µg/g of yolk)	0	$24.1^{c,x}$	$38.6^{a,x}$	$27.2^{b,x}$
	15	$22.4^{b,x}$	$36.8^{a,x}$	$23.4^{b,x}$
	30	$16.7^{c,y}$	$28.7^{a,y}$	$19.5^{b,y}$
TBARS (mg MDA /kg of egg yolk)	0	$0.29^{a,y}$	$0.22^{b,y}$	$0.27^{a,y}$
	15	$0.32^{a,y}$	$0.24^{b,y}$	$0.29^{a,y}$
	30	$0.49^{a,x}$	$0.35^{b,x}$	$0.48^{a,x}$

P-value	Diet	Storage	Diet x Storage	SEM[1]
SFA	n.s.	*	n.s.	0.412
MUFA	**	*	*	0.378
PUFA	**	*	n.s.	0.475
n-6 FA	*	*	n.s.	0.324
n-3 FA	***	n.s.	n.s.	0.172
n-6/n-3	***	*	n.s.	0.124
α-tocopherol	**	**	n.s.	1.093
TBARS	**	***	n.s.	0.037

[a-c]Means within a row with no common superscript differ (P <0.05);
[x-y]Means within a column with no common superscript differ (P <0.05);
[1] SEM: standard error of the mean; ns: P >0.05; *P <0.05; **P <0.01; ***P <0.001
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; FAME: fatty acid methyl esters; FA: fatty acid; TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde

25.6% and 28.3% for eggs from hens from the C, HS and HC groups, respectively, were observed. These findings corroborate previous results of Hayat *et al*. (2010), who noticed a decline of 28% - 32% in

α-tocopherol concentration of eggs after 40 days of storage at 4 °C. The difference in the extent of α-tocopherol decrease may be due to the FA composition of the eggs.

The alpha isomer of tocopherol (vitamin E) has a high value in animal nutrition; yet, is relatively poor as an antioxidant (Helm, 2006). In contrast, the beta-, gamma- and delta-tocopherols are not nearly as effective as nutrients, but are 130, 200 and 500 times more powerful, respectively, in their antioxidant capacities (Helm, 2006). The primary tocopherol in hemp seed and hempseed cake is the gamma isomer (88.1% and 87.8% respectively, of total tocopherols) as its major antioxidant, with minor amounts of α-tocopherol (Table 1).

Malondialdehyde (MDA) is the main product of oxidation of PUFAs and it is widely used as a marker of lipid peroxidation in food. The MDA values of lipids in storage eggs were higher than those in fresh eggs. The highest MDA values related to storage were found in eggs from the control diet. The extent of lipid oxidation differed among the dietary treatments only in 30-day stored eggs, and revealed the lowest lipid oxidation in group HS. The lower than expected oxidation in relation to the n-3 FA content noted in the HS eggs may be attributed to the higher concentration of α-tocopherol in the yolk. The high MDA in the HC eggs may be attributed to the lower concentration of α-tocopherol in the yolk, possibly due to the lower amounts of total tocopherols in the hempseed cake (hempseed cake contributes 10.8 mg to the total tocopherols/kg of diet compared to hemp seed which contributes 17.7 mg to the total tocopherols/kg of the diet). Supplementing the diets of laying hens with α-tocopheryl acetate increases, in a dose dependent manner, the content of α-tocopherol in eggs (16.6, 49.8, 78.9 and 132.3 µg/g of egg for 0, 50, 100 and 200 mg/kg α-tocopheryl acetate respectively, in the diet) and significantly reduces lipid oxidation (Galobart *et al*., 2001).

These studies demonstrate that the level and type of PUFA, the level of α-tocopherol and duration of egg storage significantly affected the oxidative stability of eggs. There is an inverse relationship between MDA content, an indicator of egg yolk lipid peroxidation, and α-tocopherol level in eggs. Ren *et al*. (2013) also found that the MDA content in yolk increases significantly when stored at 4 °C for 28 days, and storage also results in a reducing the proportion of PUFAs and increases that of MUFA in egg yolks.

The correlation analysis showed the potential relationship between PUFAs and tocopherol content and lipid oxidation of storage eggs (Table 6). The most important observation is the presence of positive relationships of storage time and n-3 FA level, with MDA, indicating that an increase of storage time and n-3 FA level accompanies an increase in MDA, which is consistent with our results. In addition, there was an inverse correlation between yolk fat MDA concentrations and yolk α-tocopherol levels ($r = -0.348$, $P < 0.01$) indicating that tocopherols from hemp products have positive potential as a natural antioxidant and could contribute to preventing lipid oxidation in egg yolks.

Table 6 Correlation between oxidative stability (concentration of malondialdehyde(MDA), storage time and content of PUFAs and α-tocopherol content in egg yolks

Correlation		Pearson's (r)	*P**
MDA	- storage time	0.154	0.164
	- PUFAs	0.471	< 0.01
	- FA n-6	0.144	0.662
	- FA n-3	0.312	< 0.01
	- α-tocopherol	-0.348	< 0.01

MDA: malondialdehyde; PUFAs: polyunsaturated fatty acids; FA: fatty acids
**P*-value indicates the significance of the correlation

Conclusions

The inclusion of HS and HC in the diets of laying hens does not have an adverse effect on the performance of laying hens, and these products can be included in the diets of laying hens to serve as alternative sources of ALA for the production of eggs with higher levels of total n-3 content. The diets do not significantly affect cholesterol content in yolk. Egg storage for 30 days led to a reduction in egg α-tocopherol and an increase in MDA content. The oxidative stability of lipids measured as MDA content in egg yolks was improved by hemp seed supplementation. The results of this study (the increase egg weight, the values of ALA, EPA, total n-3 FA higher and n-6/n-3 FA ratio, and MDA content lower in HS than HC) suggest that the

inclusion of hemp seed in the diet of the hens appears to be more effective in ameliorating nutritional quality of eggs than hempseed cake.

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Conflict of Interest Declaration

The author declares that there was no conflict of interest.

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