https://doi.org/10.17221/116/2018-CJAS

Alfalfa meal as a source of carotenoids in combination with ascorbic acid in the diet of laying hens

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Citation: Englmaierová M., Skřivan M., Vít T. (2019): Alfalfa meal as a source of carotenoids in combination with ascorbic acid in the diet of laying hens. Czech J. Anim. Sci., 64: 17–25.

Abstract: The purpose of the experiment was to determine how the addition of dehydrated alfalfa (DA) and ascorbic acid (AA) into the diet of hens influences the performance characteristics and egg quality characterised by physical parameters, yolk carotenoid content, and the oxidative stability of yolks. Two hundred and forty Hisex Brown hens were divided into 6 treatment groups according to the level of DA (0 and 40 g/kg) and concentration of AA (0, 100, and 200 mg/kg) in the diet. Hen-day egg production and egg mass production were reduced by the supplement of DA ($P < 0.001$) and AA ($P = 0.017$). The addition of DA itself increased the feed intake per egg ($P < 0.001$) and the feed conversion ratio ($P < 0.001$). The heaviest eggs ($P < 0.001$) were laid by hens fed a diet with 100 mg/kg of AA (67.6 g). The AA supplement improved egg content quality, i.e., Haugh units ($P < 0.001$), albumen index ($P < 0.001$), and yolk index ($P < 0.001$), whereas the addition of DA increased values of yolk colour ($P < 0.001$) and had a negative effect on shell quality. The interaction of DA and AA was detected in the zeaxanthin concentration in the yolks ($P = 0.002$). The highest concentration was found in eggs from hens fed a diet with a DA supplement and 100 or 200 mg/kg of AA (17.33 or 17.25 mg/kg dry matter of yolk). In addition, 40 g/kg of DA significantly increased the concentration of other carotenoids, lutein ($P < 0.001$) and β-carotene ($P < 0.001$) in the yolks and increased the oxidative stability of fresh eggs ($P < 0.001$). The results suggest that adding DA in the amount of 40 g/kg to the hen diet provides a good source of carotenoids and, in combination with AA, a better deposition of carotenoids in egg yolks. On the other hand, DA deteriorates performance and shell quality.

Keywords: antioxidants; dehydrated alfalfa; feed intake; physical parameters of egg quality; vitamins

Egg quality is one of the major themes of poultry husbandry and is influenced by several factors, including nutrition. A good source of n-3 fatty acids, vitamins, carotenoids, and minerals, which is reflected in the meat and eggs of poultry, is alfalfa (*Medicago sativa* L.) (Grela et al. 2014). Alfalfa, as a natural source of carotenoids, increases concentrations of lutein, zeaxanthin, and β-carotene in egg yolks (Karadas et al. 2006). Additionally, the possibility of grazing (Skrivanova et al. 2017a) or marigold extract additive can increase carotenoids content in poultry products (Skrivanova et al. 2017b). Lutein-enriched eggs have greater lutein bioavailability for humans than do other supplements (Chung et al. 2004). Therefore, increased consumption of lutein-enriched eggs is advantageous for human health, e.g., as a protection against age-related macular degeneration (Granado et al. 2003). Alfalfa also contains high amounts of saponins (2–3% of dry matter) that have been shown to have hypocholesterolaemic, anticarcinogenic, anti-inflammatory, and antioxidant activities

Supported by the Ministry of Agriculture of the Czech Republic (Project No. MZeRO0719).

https://doi.org/10.17221/116/2018-CJAS

(Ponte et al. 2004). Alfalfa meal is a commercially available feedstuff that is rich in protein (17.5%) with a well-balanced profile of amino acids, but with a high fibre content (24.1%) and a relatively low value of metabolizable energy (5024 kJ/kg) (Jiang et al. 2012). Due to the high fibre and low energy content, dehydrated alfalfa (DA) meal is used at very low levels in poultry feeding. This problem could be overcome by reducing the fibre level of alfalfa, e.g., by the application of sieving air classification (Laudadio et al. 2014).

An antioxidant that is widely used in poultry feed is ascorbic acid (AA). AA is a water-soluble vitamin and many animals can synthesise ascorbate in the liver and/or kidney (Dutta Gupta et al. 1973). Poultry require AA to maintain normal metabolic activities, metabolise amino acids and minerals, and synthesise hormones (McDowell 1989). The addition of AA to the hen diet is essential for optimal growth, egg production, and fertility. In addition, AA is an anti-stress factor and is therefore important during periods of high temperatures. The disadvantage is the relatively low stability of this vitamin, which is highly sensitive to oxidation initiated or catalysed by oxygen, light, heat, humidity or metal ions, such as copper and iron. Currently, this problem is resolved by offering stabilised forms of AA.

The separate effects of dietary supplementation with alfalfa or AA on the hen performance and egg quality were tested, but information about the combined effect of these two factors is not available. Therefore, the aim of the study was to evaluate the effect of the addition of DA and AA, both alone and in combination, on the performance characteristics of the hen, the physical parameters of egg quality, the carotenoids content in yolks, and the oxidative stability of yolks.

MATERIAL AND METHODS

Hens, husbandry, and diets. Two hundred and forty 39-week-old Hisex Brown hens were randomly assigned to 6 dietary treatments groups with 4 replicate cages at 10 hens per cage. The hens were housed in three-floor enriched cages in the same air-conditioned facility. The cage area was 7560 cm^2. A nest box, a feeder (120 cm), and 3 nipple water dispensers were included in each cage. Additionally, the cages were equipped with a perch (150 cm), a dust bath, and an equipment for claw abrasion, which conformed to the European Council Directive 1999/74 EC. Room temperature was maintained at 20–22°C, and the light cycle was 16 h of light and 8 h of darkness. The light intensity was approximately 10 lx in the central storey. A completely randomised experimental design with a 2 × 3 factorial arrangement of treatments was employed: 2 levels of DA (0 and 40 g/kg) and 3 concentrations of AA (0, 100, and 200 mg/kg) in mixed feed. The protected form of AA (Lutavit® C Monophosphate 35), which was produced by Orffa Nederland Feed B.V. (Giessen, the Netherlands) and provided by Trouw Nutrition Biofaktory s.r.o. (Prague, Czech Republic), was

Table 1. Ingredients and chemical composition of the diets[1]

Ingredient (g/kg)	Diet without alfalfa	Diet with alfalfa
Wheat	310	300
Maize	251.2	253.5
Soybean meal	265	250
Dried alfalfa	0	40
Cellulose	17	0
Rapeseed oil	40	40
Limestone	95	95
Monocalcium phosphate	10.3	10
Sodium chloride	3	3
L-Lysine hydrochloride	2	2
DL-Methionine	1.5	1.5
Vitamin-mineral premix[2]	5	5
Analysed nutrient content (g/kg)		
Dry matter	888.5	892.6
AME_N by calculation (MJ/kg)	10.97	10.46
Crude protein	162.5	164.1
Calcium	35.6	35.9
Available phosphorus	3.6	3.4

[1]other experimental diets were supplemented with 100 or 200 mg/kg ascorbic acid

[2]vitamin-mineral premix provided per kg diet: retinylacetate 3 mg, vitamin D3 3000 IU, vitamin E 30 mg, niacin 25 mg, Ca pantothenate 8 mg, thiamine 2 mg, riboflavin 5 mg, pyridoxine 4 mg, folic acid 0.5 mg, biotin 0.075 mg, cobalamin 0.01 mg, choline Cl 250 mg, menadione 2 mg, betain 100 mg, butylated hydroxytoluene 7.5 mg, ethoxyquin 5.6 mg, butylhydroxyanisole 1 mg, DL-methionine 0.7 g, Mn 70 mg, Zn 50 mg, Fe 40 mg, Cu 6 mg, I 1 mg, Co 0.3 mg, Se 0.2 mg

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Table 2. Content of vitamins, carotenoids, and tannins in diets and supplements

Dried alfalfa (DA) (g/kg)	0			40			DA	AA
Ascorbic acid (AA) (mg/kg)	0	100	200	0	100	200		
Ascorbic acid (mg/kg)	1.50	67.40	142.51	7.45	69.41	146.85	77.60	671 151
Zeaxanthin (mg/kg)	0.69	0.71	0.65	2.60	3.09	3.18	90.30	
Lutein (mg/kg)	0.88	0.91	0.81	2.91	3.40	3.35	86.30	
β-Carotene (mg/kg)	0.103	0.097	0.089	2.344	2.675	2.348	24.80	
Retinol (mg/kg)	1.06	3.01	2.26	1.57	1.36	1.52		
Tannins (g/kg)							7.42	

used in the experiment. The protected Lutavit® contains at least 350 g/kg of AA; the active ingredient is L-ascorbic acid-2-monophosphate-calcium sodium salt × $3H_2O$; the molar mass is 319.1 g/mol; the bulk density is 0.6–0.8 g/cm^3; and the particle size is 98% < 0.5 mm. AA was supplied in a mixture as part of the vitamin–mineral premix. The composition of the diets is shown in Table 1, and the contents of vitamins, carotenoids, and tannins in the diets and supplements are stated in Table 2. Feed and fresh water were supplied *ad libitum*. The experiment lasted 14 weeks. The Ethical Committee of the Institute of Animal Science, Prague-Uhříněves, approved the study protocol.

The numbers of eggs and hens and their health status were monitored daily. The hen-day egg production and feed intake were calculated weekly on a per-cage basis. The egg weights were determined once per week.

Physical parameters of egg quality. For the physical parameter determination, eggs were collected in the 43rd, 47th, and 51st weeks of the hens' age. A whole day of egg production was analysed in each collection period. A total of 620 eggs were analysed. The yolk and albumen height were measured using a digital micrometre head IP54 (Swiss Precision Instruments, Inc., USA).

The yolk index (YI) was calculated as follows:

$$YI = (\text{yolk height}/\text{yolk diameter}) \times 100$$

The albumen index (AI) was determined using the formula:

$$AI = \{\text{albumen height}/[(\text{long diameter of albumen} + \text{short diameter of albumen})/2]\} \times 100$$

The Haugh units (HU) were calculated according to the formula:

$$HU = 100 \times \log(\text{albumen height} - 1.7 \times \text{egg weight}^{0.37} + 7.6)$$

The shell breaking strength was determined on the vertical axis using an Instron 3360 apparatus (Instron, USA). The shell thickness (values were measured at the sharp and blunt ends and the equator, and the three obtained values were averaged) was measured using a micrometre after removing the shell membranes. The yolk colour was determined using the DSM Yolk Colour Fan (DSM Nutritional Products, Switzerland) and Minolta CR-300 colorimeter (Konica Minolta, Japan). The L*, a*, b* parameters correspond to the lightness (0 = black, 100 = white), redness (–100 = green, 100 = red), and yellowness (–100 = blue, 100 = yellow), respectively.

Laboratory analyses. One hundred and forty-four eggs were evaluated for the retinol and carotenoid content in their yolks during the experiment (46th week of the hens' age; 3 eggs per sample; n = 8). The content of lutein and zeaxanthin in the yolks was measured by high-performance liquid chromatography (HPLC) according to a modified method mentioned in the article of Froeschеis et al. (2000) with the use of an HPLC instrument (VP series; Shimadzu, Japan) equipped with a diode-array detector. The modification consisted in that the extracts were evaporated at 50°C and methanol/tetrahydrofurane was used instead of hexan; dichloromethane was used to dissolve residues in a vacuum evaporator. An aliquot of 60 µl was subjected to HPLC (VP series) analysis. A Kinetex C18 column (100 × 4.6 mm; 2.6 µm) (Phenomenex, USA) was used. A gradient system was established with acetonitrile : water : ethyl acetate (88 : 10 : 2) as eluent A and acetonitrile : water : ethyl acetate (88 : 0 : 15) as eluent B. The retinol and β-carotene contents in the yolks were determined in accordance with the European standards EN 12823-1 (2000) and EN 12823-2 (2000), respectively, by a Shimadzu HPLC system (VP series) equipped with a diode-array detector.

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The lipid peroxidation levels in the yolks of fresh eggs (63rd week of the hens' age; n = 12) and eggs stored on paper trays for 28 days at temperature 18°C and relative humidity 55–60% (63rd week of the hens' age; n = 12) were assayed using the modified method mentioned in the article of Konieczka et al. (2014). Half a gram of a sample (homogenised egg yolk) was saponified with 5 ml of 1 M KOH and 50 µl of 0.02 M 2,6-di-*tert*-butyl-*p*-cresol BHT in methanol. The mixture was placed in a plastic tube and then in a water bath at 60°C for 1 h with continuous shaking in the dark. At the end of this procedure, the resulting solution was allowed to cool down and then was acidified with concentrated HCl to approximately pH 2. Then, the acidified hydrolysate solution was centrifuged at 10 000 *g* for 10 min at ~5°C. To 500 µl of the supernatant, 50 µl of the 2,4-dinitrophenylhydrazine (DNPH) solution was added. The resulting mixture was vigorously mixed and kept at 50°C for 1 h in the dark. The clear solution was transferred to a vial and then 40 µl were injected onto the column for chromatographic analysis (Shimadzu HPLC system (VP series)) equipped with a diode-array detector). The column utilised was a Phenomenex C18 column (Synergi 2.5 µm, Hydro-RP, 100 Å, 100 mm × 3 mm) (Phenomenex). The samples were analysed using a linear binary gradient of acetonitrile in water. Solvent A consisted of water-acetonitrile (95 : 5, v/v), and solvent B consisted of acetonitrile. The thiobarbituric acid reactive substances (TBARS) content was expressed as mg of malondialdehyde (MDA) per kg of egg.

The feed and yolk dry matter was determined by drying in an oven (Memmert ULM 500; Memmert, Germany) at 105°C to a constant weight, and the feed crude protein content was measured using a Kjeltec Auto 1030 instrument (Tecator, Sweden). Analyses of the P and Ca content in the diets were conducted. Dry homogenised diets were ashed in a muffle furnace (LAC Ht40 AL; LAC, Ltd., Czech Republic) at 550°C, and the ash was dissolved in 3 M hydrochloric acid and quantitatively transferred into a volumetric flask. The total P in the solution was determined using a vanadate-molybdate reagent (method No. 965.17; AOAC 2005), and the Ca concentration in the hydrochloric acid extract was measured by atomic absorption spectrometry using a Solaar M6 instrument (TJA Solutions, UK). A modified European Standard EN 14130 (2004) was used to determine the AA (namely, AA and its oxidised form dehydroascorbic acid) content in the feed and supplements. The samples were homogenised, and AA was extracted using 2% metaphosphoric acid (MPA). The samples were filtered through a 0.22-µm membrane filter and then analysed by HPLC using the Synergi 4 µ Fusion-RP 80A column (Phenomenex) and a gradient elution (25 mM KH_2PO_4 : acetonitrile). The pH of the 25 mM KH_2PO_4 was adjusted to 3 using 20% MPA. The method for determination of tannins is based on the determination of total phenolics and tannins using the Folin–Ciocalteu method described by Makkar (2003). The second step includes removal of tannins from the tannin-containing extract and determination of phenols without tannins. From these two measurements, it is possible to calculate the content of tannins in the sample. The method includes a reaction with the Folin–Ciocalteu reagent in the presence of sodium carbonate and a measurement of absorbance at 725 nm. The part of removal of tannins includes a reaction with polyvinyl pyrrolidone, which binds tannins. The concentrations of retinol and carotenoids in the feed and supplements were determined using the methods described previously.

Statistical analyses. The data were analysed using two-way analysis of variance (ANOVA) with the General Linear Models (GLM) procedure in the SAS software (Statistical Analysis System, Version 9.3, 2003). The main effects were the level of dehydrated alfalfa in diet (DA), the content of ascorbic acid in diet (AA), and the interaction between these two factors (DA × AA). All the differences were considered to be significant at $P < 0.05$. The results in the tables are presented as the mean and standard error of the mean (SEM).

RESULTS

The performance characteristics are shown in Table 3. A significant ($P < 0.001$) interaction of DA and AA was ascertained in egg weight. The heaviest eggs were laid by hens fed the diet supplemented with 100 mg/kg of AA (67.6 g), and the lightest eggs were laid by hens fed the treatment with 100 mg/kg of AA and 40 g/kg of DA (65.3 g). Both DA and AA decreased hen-day egg production ($P < 0.001$ and $P = 0.017$) and egg mass production ($P < 0.001$ and $P = 0.017$). In addition, the DA supplement

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Table 3. Effect of dried alfalfa and ascorbic acid on performance

Dried alfalfa (DA) (g/kg)	0			40			SEM	Probability		
Ascorbic acid (AA) (mg/kg)	0	100	200	0	100	200		AA	DA	AA × DA
Egg weight (g)	66.3^{bc}	67.6^{a}	66.3^{bc}	67.1^{ab}	65.3^{c}	66.6^{ab}	0.19	ns	ns	< 0.001
Hen-day egg production (%)	88.30	86.88	85.27	85.16	81.74	82.89	0.441	0.017	< 0.001	ns
Egg mass (g/day/hen)	51.48	50.65	49.71	49.65	47.66	48.32	0.257	0.017	< 0.001	ns
Feed intake (g/day/hen)	127.00	127.32	126.78	129.70	125.31	125.86	0.443	ns	ns	ns
Feed intake (g/egg)	144.25	147.00	149.41	152.48	148.00	152.81	0.765	ns	< 0.001	ns
Feed conversion ratio (g/g)	2.47	2.52	2.56	2.61	2.63	2.62	0.131	ns	< 0.001	ns
Mortality (pcs)	0	0	0	0	0	1				

SEM = standard error of the means, ns = non-significant

$^{a–c}$means in the same row with different superscripts differ significantly

increased the feed intake per egg ($P < 0.001$) and the feed conversion ratio ($P < 0.001$).

The positive effect of AA was ascertained in the egg content quality (Table 4). The values of Haugh units ($P < 0.001$), albumen index ($P < 0.001$), and yolk index ($P < 0.001$) increased with increasing AA concentration in the diet, whereas the eggshell quality was negatively influenced by DA addition in the amount of 40 g per kg of diet. The values of shell breaking strength ($P = 0.003$) and shell thickness ($P = 0.002$) were lower in groups with DA addition compared to eggs from hens fed the diets without DA supplement. On the other hand, DA in the diet increased the yolk colour, which was measured by a subjective method using a DSM yolk colour fan ($P < 0.001$). This finding corresponded with the results of decreasing the value of lightness in these treatments ($P < 0.001$). Both DA and AA increased the values of redness ($P < 0.001$ and $P < 0.001$) and yellowness ($P < 0.001$ and $P = 0.006$) of the yolks.

As is evident from Table 5, the combined effect of DA and AA was found in zeaxanthin concentration in yolks ($P = 0.002$). The highest values were detected in eggs laid by hens fed the diet with the DA supplement and 100 or 200 mg/kg of AA (17.33 or 17.25 mg/kg dry matter of yolk). In addition, the hen diet supplemented with DA significantly increased the concentration of other carotenoids, lutein ($P < 0.001$) and β-carotene ($P < 0.001$) in yolks. Moreover, AA in the diet positively influenced lutein content in the yolk

Table 4. Effect of dried alfalfa and ascorbic acid on physical parameters of egg quality

Dried alfalfa (DA) (g/kg)	0			40			SEM	Probability		
Ascorbic acid (AA) (mg/kg)	0	100	200	0	100	200		AA	DA	AA × DA
Sample size (*n*)	108	106	106	97	103	100				
Haugh units	82.9	83.9	85.5	83.3	82.8	85.3	0.26	< 0.001	ns	ns
Albumen index (%)	8.63	8.85	9.34	8.68	8.68	9.40	0.065	< 0.001	ns	ns
Yolk index (%)	41.7	42.4	42.5	41.0	42.5	43.4	0.12	< 0.001	ns	ns
Yolk colour										
DSM Yolk Colour Fan	6.29	6.18	6.21	8.18	8.36	8.40	0.073	ns	< 0.001	ns
Lightness (L*)	64.09^{a}	63.97^{a}	63.17^{b}	60.85^{c}	61.14^{c}	61.18^{c}	0.109	ns	< 0.001	0.024
Redness (a*)	4.09	4.62	4.70	8.42	8.87	8.70	0.096	< 0.001	< 0.001	ns
Yellowness (b*)	43.19	44.48	43.59	50.92	51.62	50.98	0.217	0.006	< 0.001	ns
Shell thickness (μm)	366	366	360	357	356	359	1.1	ns	0.002	ns
Shell breaking strength (N/cm^2)	46.27	45.45	46.67	42.96	45.27	44.48	0.315	ns	0.003	ns

SEM = standard error of the means, ns = non-significant

$^{a–c}$means in the same row with different superscripts differ significantly

https://doi.org/10.17221/116/2018-CJAS

Table 5. Effects of dried alfalfa and ascorbic acid on carotenoids and retinol content in yolks and the oxidative stability of yolks (MDA)

Dried alfalfa (DA) (g/kg)	0			40			SEM	Probability		
Ascorbic acid (AA) (mg/kg)	0	100	200	0	100	200		AA	DA	AA × DA
Zeaxanthin (mg/kg DM)	4.57^{c}	5.40^{c}	5.90^{c}	14.25^{b}	17.33^{a}	17.25^{a}	0.825	< 0.001	< 0.001	0.002
Lutein (mg/kg DM)	9.54	11.68	12.53	26.49	26.95	27.23	1.176	0.021	< 0.001	ns
β-Carotene (mg/kg DM)	< 0.01	< 0.01	< 0.01	0.122	0.140	0.120	0.0094	ns	< 0.001	ns
Retinol (mg/kg DM)	8.61^{e}	9.60^{d}	10.16^{cd}	12.01^{a}	11.82^{ab}	11.05^{bc}	0.219	ns	< 0.001	0.002
MDA 0 (mg/kg)	0.326	0.323	0.305	0.281	0.288	0.230	0.0054	ns	< 0.001	ns
MDA 28 (mg/kg)	0.358	0.340	0.295	0.361	0.354	0.348	0.0067	ns	ns	ns

SEM = standard error of the means, ns = non-significant, DM = dry matter, MDA 0 = malondialdehyde content (mg/kg) in yolks of fresh eggs, MDA 28 = malondialdehyde content (mg/kg) in eggs stored at 18°C for 28 days

sample size for carotenoids and retinol content in yolks n = 8 and for the oxidative stability of yolks n = 12

$^{a–e}$means in the same row with different superscripts differ significantly

(P = 0.021). Another significant interaction was ascertained by the retinol content in yolk (P = 0.002). A separate addition of DA increased the retinol concentration in yolk (12.01 mg/kg dry matter of yolk) compared to the control group (8.61 mg/kg dry matter of yolk). In fresh eggs, 40 g/kg of DA in the diet significantly decreased the lipid peroxidation level (P < 0.001). There was no observed treatment effect on the lipid oxidative stability of eggs stored for 28 days at 18°C.

DISCUSSION

The performance of laying hens was especially influenced by DA addition. The dose of 40 g/kg deteriorated hen-day egg production, egg mass production, feed intake per egg, and feed conversion ratio. On the other hand, there are several studies in which alfalfa meal (Al-shami et al. 2011; Laudadio et al. 2014) or extract (Deng et al. 2012) did not influence the hen performance. The negative effect of DA on hen performance could be caused by tannins contained in alfalfa. The concentration of tannins in DA was 7.42 g/kg. It follows that 1 kg of mixed feed contained 300 mg of tannins from DA. Small controlled doses of these polyphenolic compounds, recognised as regulators and stabilisers of microflora populations in the intestine, are applied in the treatment of digestive disturbances in animals and humans (Jamroz et al. 2009). Schiavone et al. (2008) showed that tannins from chestnut wood have a positive influence on growth performance if included in the diet up to 0.2%. However, tannins can negatively affect the protein digestibility in monogastric animals (Garcia et al. 2004). Tannins applied at a dose of 1000 mg per kg caused negative changes in jejunal wall histology, disadvantageous microbial status of jejunum content, and a slight decrease in performance parameters in chickens (Jamroz et al. 2009). In our previous research (Skrivan et al. 2018), diets supplemented with freeze-dried pasture herbage at 2% and 4% reduced the digestibility of amino acids and fatty acids in broiler chickens. The concentration of tannins in these diets was 0.63 and 0.88 g/kg, respectively. Consistent with the result of the present study, Sell et al. (1983) showed that a diet rich in tannins significantly reduced egg production and feed efficiency. Egg weight was not affected by tannin content. In addition, Ebadi et al. (2005) showed that if tannin intake was less than 1.7%, it had no negative effects on laying hen performance.

In the present study, AA had adverse effects on egg production and egg mass production. On the other hand, Keshavarz (1996) and Saki et al. (2010) did not find the effect of AA on performance characteristics of hens. Njoku and Nwazota (1989) showed that the beneficial effect of AA added to

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feeds for laying hens on egg production was particularly evident when the birds were exposed to stress caused by high air temperature or increased stocking rate of birds per unit area. In addition, the length of feeding AA affects the result. In chickens that were fattened for a short time (35 days), the AA was more effective (Skrivan et al. 2012) than in hens with a long-term intake (24 weeks) (Skrivan et al. 2013). A lower egg production in the present study can be explained by the fact that AA has a strong acidic reaction, acidifies feed mixture and can cause calcium excretion from the organism (Buclin et al. 2001).

AA had positive effect on the physical parameters of yolk and albumen quality. Consistent with our results, de Souza et al. (2001) showed that low doses of AA (100, 150, and 200 mg/kg) increased ($P < 0.05$) the value of Haugh units. The yolk index was not influenced by the AA addition. On the other hand, Haugh units and albumen index in the study of Saki et al. (2010) were increased ($P < 0.05$) only at the highest dose of AA (750 mg/kg) in eggs from hens at the age of 28 weeks. In older hens (35 weeks of age), there was no effect on the quality of the albumen. The age of hens used in the present experiment was from 39 to 52 weeks. Additionally, Keshavarz (1996) detected the increase of Haugh units ($P < 0.05$) at a high dose of AA (1 g/kg). From the review, it is evident that the results are inconsistent, and are influenced by multiple factors, such as age or genotype.

In the present study, DA positively influenced yolk colour and decreased values of shell quality characteristics. On the other hand, aqueous alfalfa extract (0.1 and 0.15%) in the study by Deng et al. (2012) increased shell strength. Moreover, shell thickness was higher at 2, 5, and 7% of alfalfa leaf meal in the diet (Al-shami et al. 2011). Eggshell quality was not influenced in the experiment of Laudadio et al. (2014). Al-shami et al. (2011) and Laudadio et al. (2014) also found that alfalfa meal in the diet significantly increased yolk colour. The yolk colour relates with carotenoid content in the diet. The carotenoid profile is much more important for yolk colouration than is the quantity of total carotenoids in the egg yolk. The extract of alfalfa was a significant source of β-carotene in comparison with tomato powder or marigold extract. Total carotenoid concentration in the egg yolk was increased by approximately 10 times as a result of dietary inclusion of the alfalfa concentrate product (Karadas et al. 2006). A double increase in the β-carotene in egg yolks of hens fed alfalfa meal is also evident from the experiment of Laudadio et al. (2014). In our study, AA positively influenced the storage of zeaxanthin and lutein contained in DA into yolks. This effect can be explained by the antioxidant action of AA on carotenoids, which increased zeaxanthin and lutein concentration in the yolks.

Alfalfa meal is a rich source of carotenoids. A higher content of carotenoids (antioxidants) in yolks treated with DA in the diet reduces the susceptibility of these yolks to oxidation, which is evident in the fresh eggs from our study. Higher oxidative stability may be related to a higher concentration of retinol in these yolks. In addition, Skrivan et al. (2013) showed that supplementation of the basal diet with AA significantly reduced the oxidative stability of yolk lipids, indicating that AA acted as a pro-oxidant. AA, which is a known antioxidant, can in some circumstances act as pro-oxidant, particularly when animals have adequate vitamin E stores and AA is supplemented in large doses (Chen 1989). In the present experiment, no negative effect of AA on the oxidative stability of yolk lipids was noted.

A positive interaction of AA and DA was found in the retinol content of the yolk. A higher content of retinol in yolks from hens fed a diet with DA is caused by the fact that DA is a source of β-carotene, which is a precursor of retinol. Conversely, Karadas et al. (2006) showed that a deposition of retinol in the egg yolk was not influenced by the extract of alfalfa. In the study by Yigit et al. (2002), serum AA and retinol concentrations increased with increasing amounts of AA in the diet. AA protected retinol against oxidation and increased serum retinol levels in parallel with increased serum AA levels.

CONCLUSION

The addition of DA into the hen diet decreased egg production and shell quality. This reduction was probably caused by anti-nutritional factors, such as tannins, which are present in DA. Despite this fact, the combined effects of DA and AA increased the content of carotenoids and vitamins in the eggs, which increased the internal quality of the eggs. DA in the amount of 40 g/kg added to

https://doi.org/10.17221/116/2018-CJAS

the hen diet is a good source of carotenoids, which are deposited in yolks. Moreover, this deposition can be positively influenced by AA addition.

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Received: 2018–06–08
Accepted: 2018–09–13