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Original Article

Effects of The Dietary Supplementation of Layer Diets with Natural and Synthetic Antioxidant Additives on Yolk Lipid Peroxidation and Fatty Acid Composition of Eggs Stored at Different Temperatures and Duration

■Author(s)

Cimrin T[I] https://orcid.org/0000-0002-5868-4148
Avsaroglu MD[II] https://orcid.org/0000-0002-3106-9209
Ivgin Tunca R[III] https://orcid.org/0000-0003-0745-6732
Kandir S[IV] https://orcid.org/0000-0002-8404-7994
Ayasan T[V] https://orcid.org/0000-0001-7397-6483

[I] Hatay Mustafa Kemal University, Agriculture Faculty, Department of Animal Science 31034, Hatay, Turkey.
[II] Department of Animal Science, Faculty of Agriculture, Ahi Evran University, 40100, Kırsehir, Turkey.
[III] Plant and Breeding Department Ula Ali Koçman Vocational School, Mugla Sıtkı Kocman University, 48640, Mugla, Turkey.
[IV] Cukurova University Ceyhan Faculty of Veterinary Medicine Department of Physiology, Adana, Turkey.
[V] East Mediterranean Agricultural Research Institute, Adana, Turkey.

■Mail Address

Corresponding author e-mail address
Tulay Cimrin
Hatay Mustafa Kemal University, Agriculture Faculty, Department of Animal Science 31034, Hatay, Turkey.
Phone: +90-326-2455845 (113 44)
Email: tcimrin@hotmail.com

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Fatty acids, malondialdehyde, rosemary, thyme, vitamin E.

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ABSTRACT

In this study, the effects of the supplementation of natural and synthetic antioxidant additives in layer diets on egg weight loss, yolk lipid peroxidation (TBARS values) and fatty acid composition of eggs stored at different temperatures and duration were evaluated. In total, 112 48- weeks-old Bovans White layers were randomly allotted to four dietary treatments with four replicates of seven birds each. The treatments consisted of a control diet, containing no additives, and diets with the inclusion of 200 mg synthetic vitamin E/kg, 1000 mg thyme extract/kg and 1000 mg rosemary extract/kg. Dietary treatments did not influence (p>0.05) relative weight loss of eggs stored for 14, 28, and 56 days, except for those from rosemary-fed hens stored at room temperature on d 42, which were significantly lighter than the eggs from vitamin E- and thyme-fed hens (p<0.001). Relative egg weight loss was significantly higher (p<0.001) when stored at room temperature than under refrigeration, independently of storage time. In eggs stored at room temperature, yolk TBARS values were significantly lower (p<0.001) in the eggs of vitamin E-fed hens, whereas no influence (p>0.05) of dietary treatment on yolk TBARS values were detected in refrigerated eggs. The inclusion of the synthetic and both natural antioxidants in layer diets significantly reduced stearic acid (C18: 0) level in the egg yolk. In addition, only natural antioxidants significantly increased yolk levels of palmitoleic acid (C16: 1) and vaccenic acid (C18: 1n7). The results of the present study showed that adequate storage temperature was more effective in improving egg shelf life than feeding layers synthetic or natural antioxidant additives. However, the positive effects of the evaluated natural antioxidants on yolk fatty acid composition suggest their supplementation to layer diets may provide health benefits to the consumer.

INTRODUCTION

The function of antioxidants is to protect the cells and lipids in the cell membranes against peroxidation damage (Tengerdy, 1990). In some plants, naturally occurring antioxidants have been shown to be anti-carcinogenic, but synthetic antioxidants may have co-carcinogenic effects on precancerous lesions and cause cancer (Sitich, 1991). These developments related to synthetic antioxidants have led to the restriction or ban of the use of these products and have started the search for natural antioxidants as alternatives. In this process, a large number of studies have been conducted on the antioxidant capacity of some plants.

Thymol is used as a natural antioxidant additive to improve the oxidative stability poultry feed and meat (Luna *et al.*, 2017). Thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.)

have antibacterial, antifungal, anti-inflammatory, antitumoral, analgesic, antipyretic, cardiac tonic, antiasthmatic, and antioxidant effects (Hameed & Mohammed, 2017). It is stated that essential oils obtained from thyme, rosemary, and laurel can be used as natural antimicrobial and antioxidant agents in food industry (Tural & Turhan, 2017). Eleven different oils, including thyme and rosemary oils, have been reported to be natural antioxidants and antimicrobial agents (Sacchetti *et al.*, 2005). The main components of thyme are thymol and carvacrol, which have antioxidant capacity (Ruberto & Baratta, 2000). The antioxidant capacity of the rosemary plant is given mainly to the components carnosol and carnosic acid, and alpha-pinene, bornyl acetate, camphor and 1,8-cineole account for rosemary antimicrobial activity (Moghtader & Afzali, 2009). Thymol showed similar effects as synthetic butylated hydroxytoluene (BHT) to protect poultry feed against lipid oxidation (Luna *et al.*, 2017). In different studies with poultry, the addition of rosemary to layer feeds decreased egg yolk lipid oxidation in and addition of 100 mg rosemary essential oil/kg diet increased the yolk content of oleic acid (Cimrin & Demirel, 2016b; Batista *et al.*, 2017), which is one of the main fatty acids present in oil seeds (Duru & Konuskan, 2014). Malondialdehyde levels in eggs stored for 60 days from layers fed thyme remained at the same levels as in fresh eggs (Botsoglou *et al.*, 1997). On the other hand, both natural (D-α-tocopherol) and synthetic forms (DL-α-tocopherol acetate) of vitamin E are frequently used as feed additives, and many studies have shown they have potent antioxidant activity (Traber & Atkinson, 2007; Altiner *et al.*, 2017; Cheng *et al.*, 2017), reducing the levels of thiobarbituric acid reactive substances (TBARS) in eggs during storage (Kaya & Turgut, 2012; Cimrin & Demirel, 2016a; Asadi *et al.*, 2017).

This study was carried out to investigate the effects of different egg storage conditions and times on yolk fatty acid composition and TBARS levels of eggs from layer fed diets with the inclusion of vitamin E, and thyme and rosemary extracts.

MATERIAL AND METHODS

This study was conducted at Etaş Afyon poultry company, Kırşehir-Turkey. A total of 112 Bowans White 48-weeks-old layers were used in this study. Hens were randomly distributed into four dietary treatments, with four replicates of seven hens each. The treatments consisted of a control diet, containing no additives, and diets with the inclusion of 200 mg synthetic vitamin E/kg, 1000 mg thyme extract/kg or 1000 mg rosemary extract/kg.Thyme and rosemary extracts was obtained from a commercial company (Agromiks Feed Additives and Livestock Industry and Trade Limited Company), and their active ingredients and levels in the plant extracts are given in Table 1. Synthetic vitamin E (DL-α-tocopherol acetate) was provided by a commercial company (Vimar Food, Agriculture and Livestock Joint-Stock Company) under the commercial brand TOCOMIX 500.

Hens were housed in cages (30cmx44cmx44cm) in a poultry house. A 16/8 h light/dark lighting regime was applied. Feed as mash and water were provided *ad libitum* during the 60-d experimental period.

Table 1 – Active ingredients of the evaluated plant extracts and % values*.

Thyme (*Thymbra spicata*)	Rosemary (*Rosemarinus officinalis* L.)
Carvacrol: 87.81%	1.8 cineole: 34.08%
Thymol: 9.58%	Camphor: 27.95%
L-Linalool: 0.86%	Alpha-pinene: 14.50%
Borneol: 0.74%	Borneol:8.65%
	Alpha-terpineol:7.39%
	Alpha-Thujone: 1.09%
	Camphene: 0.55%

*The values were informed by the manufacturer.

Eighty eggs per treatment (16 eggs per dietary treatment x five storage times = 80 eggs stored; 40 of these eggs were stored at room temperature and 40 were stored in a refrigerator) were collected during in the last five days of the experiment. In total, 320 eggs (80 eggs x four treatments) were stored for 1, 14, 28, 42, and 56 days to determine egg weight and thiobarbituric reactive substance (TBARS) values. Eggs were individually weighed on a digital scale to 0.01g and the weights recorded. Eggs were then stored at room temperature (22-34°C) or under refrigeration (4°C) for 1, 14, 28, 42, and 56 days during the summer (July-August) of the Central Anatolia Region. Egg weight loss was determined as the percentage of egg weight measured on each storage day relative to initial egg weight (d1). Egg yolk protein amount of was defined according to Biuret Protein Analysis Method by Layne (1957). Malondialdehyde (MDA) analysis in yolk eggs were made according to Buege and Aust (1978). The TBARS values were calculated as malondialdehyde. [Malondialdehyde (MDA) = OD x sample volume /1.56 x 10^5 x total volume x protein (mg / ml)], where OD = absorbance value.

At the end of the study, egg yolk fatty acid levels were determined in 16 eggs per treatment according to ISO standard 12966:2 (ISO, 2017).

The obtained data were submitted to one-way analysis of variance using the GLM procedure of SPSS 21.0 statistical package (IBM, Chicago, IL). Means were compared by Duncan's multiple comparison test. Differences were considered significant when $p<0.05$.

RESULTS

Relative weight loss values of eggs stored at different storage conditions for 14, 28, 42 and 56 days are given in Table 2. The dietary addition of natural and synthetic antioxidant additives did not influence ($p>0.05$) relative weight loss of eggs stored for 14, 28, and 56 days under the same storage temperature, except on d 42, when eggs from the rosemary group lost significantly more weight than those from the vitamin E and thyme groups ($p<0.001$). However, eggs stored under refrigeration lost significantly less weight ($p<0.001$) than those stored at room temperature, independently of dietary treatment.

Table 2 – Relative egg weight loss (%) as a function of storage temperature and duration, and layer dietary treatment.

Storage temperature	Dietary treatment	Storage time (days)			
		14	28	42	56
Room (22-34 °C)	Control	3.303 a	6.507 a	11.097 ab	13.400 a
	Vitamin E	3.238 a	6.550 a	10.332 b	13.376 a
	Thyme	3.128 a	7.328 a	10.153 b	13.078 a
	Rosemary	3.510 a	6.791 a	11.686 a	14.562 a
Refrigeration (4 °C)	Control	0.827 b	1.748 b	2.885 c	4.240 b
	Vitamin E	0.536 b	1.758 b	3.190 c	4.352 b
	Thyme	0.663 b	1.752 b	2.506 c	4.621 b
	Rosemary	0.636 b	1.766 b	2.763 c	4.400 b
F test		81.972	59.963	109.809	68.936
p value		0.000	0.000	0.000	0.000
SEM		0.055	0.123	0.145	0.210

[a, b, c]: Means in the same column with different letters significantly differ ($p<0.05$).

Yolk TBARS values of egg stored for 1, 14, 28, 42 and 56 days at room temperature or under refrigeration according to dietary treatment are given in Table 3. There was no influence of dietary treatment or storage conditions ($p>0.05$) on TBARS values of fresh eggs (d 0 of storage). However, when eggs were stored at room temperature for 14, 28, 42 and 56 days, significantly lower TBARS levels ($p<0.001$) were determined in eggs from layers fed synthetic vitamin E compared with the other dietary treatments. No significant TBARS differences among treatments ($p>0.05$) were determined in egg stored under refrigeration for 14, 28, 42 and 56 days were found non-significant. TBARS values in the eggs of hens fed synthetic vitamin E were not influenced by storage temperature ($p>0.05$).

The effect of the addition of thyme and rosemary extracts and synthetic vitamin E to the basal diet of layers on egg yolk fatty acid levels is given in Table 4. Myristic acid, palmitic acid, oleic acid, linoleic acid, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA) and total polyunsaturated fatty acid (PUFA) levels were not different among treatments

Table 3 – Mean egg yolk TBARS values (MDA, nmol/mg) as a function of storage temperature and duration, and layer dietary treatment.

Storage temperature	Dietary treatment	Storage time (days)				
		1	14	28	42	56
Room (22-34 °C)	Control	0.215	0.610 a	0.693 a	0.791 a	0.896 a
	Vitamin E	0.200	0.237 b	0.307 b	0.306 b	0.365 b
	Thyme	0.205	0.492 a	0.580 a	0.611 a	0.782 a
	Rosemary	0.202	0.502 a	0.575 a	0.606 a	0.824 a
Refrigerator (4 °C)	Control	0.226	0.295 b	0.362 b	0.376 b	0.536 b
	Vitamin E	0.189	0.205 b	0.235 b	0.264 b	0.331 b
	Thyme	0.207	0.279 b	0.326 b	0.331 b	0.531 b
	Rosemary	0.203	0.271 b	0.316 b	0.324 b	0.530 b
F test		0.816	4.839	5.570	6.265	9.087
p value		0.578	0.000	0.000	0.000	0.000
SEM		0.004	0.024	0.025	0.027	0.025

[a.b] Means in the same column with different letters significantly differ ($p<0.05$)

(p>0.05). Stearic acid was significantly higher in the control group than in the other groups (p<0.001). Palmitoleic acid was significantly higher in the rosemary group than in the other groups (p<0.05). Vaccenic acid, which is the isomer of oleic acid, was significantly higher in the thyme and rosemary groups compared to the control group (p<0.05).

DISCUSSION

There effect of storage temperature on egg weight loss was significant (Table 2), with higher weight loss determined in the eggs stored in the room compared to the eggs stored under refrigeration. The weight loss measured on d 14 in the eggs stored at room temperature was be greater than that determined on d 42 in the eggs stored under refrigeration (Table 2). When maintained at room temperature, weight loss of the eggs of the rosemary group was significantly higher those of the vitamin E and thyme group, which may be due to an unnoticed eggshell problem during storage of the eggs of the rosemary group. While storage conditions and duration had stronger effects on egg weight loss, dietary additives may influence eggshell quality, and therefore, egg weight loss during storage. Asadi *et al.* (2017) reported that the addition of organic selenium to layer diets reduced weight loss of eggs stored at 4 °C for 14 days. However, in the present study, the dietary treatments influenced egg weight loss only in one out of the four storage periods evaluated and storage at room temperature, suggesting a possible eggshell defect that was not noticed during storage. In a similar study, a significant interaction between storage time and temperature on egg weight loss was detected, with higher weight losses observed in without shrink film eggs stored at room temperature for longer periods (Petek *et al.*, 2014). Furthermore, the internal quality parameters of the eggs stored at room temperature (18-20 °C) gradually deteriorated as storage period increased compared to the eggs stored under refrigeration (Petek *et al.*, 2014). During the summer (33 °C), Yenilmez *et al.* (2017) determined that the weight loss in the eggs stored under wholesaler and consumer conditions increased significantly with storage period.

Table 4 – Egg yolk fatty acid composition values (%) according to layer dietary treatment.

Fatty acids	Dietary treatments						
	Control	Vitamin E	Thyme	Rosemary	F test	P value	SEM
Myristic acid (C14:0)	0.327	0.420	0.352	0.372	1.888	0.185	0.014
Palmitic acid (C16:0)	26.89	27.23	26.77	28.43	2.959	0.075	0.220
Stearic acid (C18:0)	15.46a	13.72b	14.18b	13.06b	8.004	0.003	0.180
Palmitoleic acid (C16:1)	2.03b	2.39b	2.29b	3.00a	4.688	0.022	0.095
Oleic acid (C18:1n9c)	44.33	43.85	43.85	42.89	1.006	0.424	0.300
Vaccenic acid (C18:1n7)	1.39b	1.57ab	1.66a	1.78a	4.744	0.021	0.038
Linoleic acid (C18:2n6)	9.65	10.81	10.88	10.47	2.226	0.138	0.189
SFA (C14:0+ C16:0+ C18:0)	42.68	41.37	41.30	41.86	1.950	0.175	0.228
MUFA (C16:1+C18:1n9c+ C18:1n7)	47.75	47.82	47.82	47.67	0.017	0.997	0.267
PUFA(C18:2n6)	9.65	10.81	10.88	10.47	2.226	0.138	0.189

[a.b] Means in the same column with different letters significantly differ (p<0.05).

The results of this study are consistent with the findings of previous studies indicating that egg weight loss increases with storage temperature and duration, with consequent negative results on egg internal quality (Jin *et al.*, 2011; Chung & Lee, 2014; Yenilmez *et al.*, 2017). The eggs laid by hens fed vitamin E presented lower TBARS values (p<0.001) on d 14, 28, 42, and 56 compared with the other treatments when stored at room temperature, but not under refrigeration (Table 3). Studies have shown that poultry feed additives are transferred to the egg. For example, Florou-Paneri *et al.* (2005) reported that the antioxidant properties of alpha-tocopheryl and oregano essential oils included in layer diets were transferred to egg yolk and prevented lipid oxidation. Cimrin & Demirel (2016a,b) fed laying hens with 200 mg vitamin E/kg and found that a significant amount of vitamin E was transferred to the eggs, and reduced egg yolk MDA values during 1-56 days of storage. Radwan Nadia *et al.* (2008) determined that the addition of 100 and 200 mg vitamin E/kg diet reduced egg yolk MDA values compared with the addition of thyme, rosemary and turmeric, and reported that the oxidative stability of eggs stored at room temperature increased. Many studies related to this subject found that egg yolk TBARS values decreased and oxidative stability increased during storage with the dietary addition of vitamin E (Botsoglou *et al.*, 2012; Kaya & Turgut, 2012; Botsoglou *et al.*, 2013; Cimrin & Demirel, 2016a; Asadi *et al.*, 2017).

The thyme and rosemary extracts used in the present study reduced numerically, but not statistically, egg yolk TBARS values compared to the control group. The antioxidant effects of these extracts remained low compared to the synthetic vitamin E, which is consistent with published studies (Florou-Paneri *et al.* 2005; Cimrin & Demirel, 2016a). In contrast, Kaya & Turgut (2012) found that the dietary supplementation of herbal extracts had similar effect as vitamin E in preventing lipid oxidation, but significant differences were detected among 0, 150, 300 ppm levels of sage, thyme, and mint extracts and vitamin E. Luna *et al.* (2018) reported that dietary thymol supplementation improved meat and egg oxidative stability during storage. It is important to use appropriate doses in herbal extracts. Botsoglou *et al.* (1997) reported that thyme components were transferred from the layer feed into egg yolk and that the presence of 278 mg/g thymol components in the egg yolk prevented lipid oxidation. In a later study, Botsoglou *et al.* (2005) observed decreased yolk lipid oxidation in the eggs of laying hens fed rosemary, thyme, saffron and vitamin E; however, yolk lipid oxidation was not influenced by storage time. Batista *et al.* (2017) found that the addition of 200 mg rosemary oil/kg diet reduced egg yolk MDA levels, indicating better lipid stability of eggs stored at 25 ºC, but not at 5 ºC.

Kayahan (2003) stated that, except for unsaturated fatty acids, which is the main factor influencing lipid oxidation, storage conditions, such as temperature and humidity, light in the environment, wavelength, partial oxygen pressure, contamination with polyvalent metals, the surface area of the lipid in contact with oxygen, pro- and antioxidant activity and level affect the onset and acceleration of oxidation reactions. Furthermore, Osborn & Akoh (2003) reported that phenolic compounds influence oxidation and that their antioxidant activity changes according to food composition, affecting the rate of lipid oxidation by different mechanisms. In this study, as mentioned above, the addition of antioxidants to the diets helped to slow lipid oxidation in egg yolk. The findings of the present study showed lipid oxidation rate varies depending on the phenolic compounds present in the three different antioxidants used, as well as egg storage temperatures and duration.

The dietary addition of thyme and rosemary extracts and synthetic vitamin E significantly reduced yolk content of stearic acid, a saturated fatty acid, compared to the control group (p<0.001; Table 4). On the other hand, dietary thyme and rosemary extracts significantly increased yolk content of vaccenic acid, which is the isomer of the mono-unsaturated fatty acid oleic acid (p<0.05). In addition, rosemary extract increased the amount of palmitoleic acid relative to the other treatments. This is an important step in terms of the quality of eggs and functional egg production, as the egg is a food that is easily accessible to all people in the world. Tasan & Daglioglu (2005) reported that trans isomers of palmitoleic acid, oleic acid and polyunsaturated fatty acids are found in the leaves and seeds of various plants, but the amount of oil in the leaves of the plants is low. Yi *et al.* (2014) reported that egg fatty acid composition can be influenced by the feeding strategies of laying hens. In the present study, the increased yolk fatty palmitoleic acid and vaccenic acid contents may be related to their contents in the plants, as pointed out by Tasan & Daglioglu (2005). In the study of Gerzilov *et al.* (2015), the addition of a mixture containing rosemary and thyme oils increased two-fold the linolenic acid content of egg yolk phospholipids, and the authors emphasized its importance for poultry performance and health. In a study with laying hens, Çimrin & Demirel (2016b) showed that 100 mg rosemary essential oil/kg diet increased MUFA oleic acid content in the egg yolk. Hashemipour *et al.* (2013) reported that the dietary addition of thymol and carvacrol reduced serum SFA levels, but increased PUFA levels in broilers. Luna *et al.* (2018) observed an increase in PUFA levels in the meat and eggs of poultry fed thymol. In the present study, despite the observed variations in the yolk contents of some fatty acids (stearic acid, palmitoleic acid and vaccenic acid), total SFA and MUFA contents were not different among dietary treatments. This may be related to the levels of additives used. In the present study, the results clearly show that egg fatty acid content was positively be influenced by the evaluated additives.

It has long been known that poultry feed additives are transferred to the egg. Therefore, it is very important to know the purpose, the content, the metabolism, the appropriate amount and the different substances involved in the interactions when supplying feed additives. Moreover, it is also possible that the same additives give different results in different studies. In particular, natural additives are generally available as commercial products, and the conditions under which the plants were harvested, processed and stored are not known. In the current study, dietary synthetic vitamin E significantly inhibited yolk lipid oxidation of eggs stored at room temperature, whereas in eggs stored under refrigeration, its effect was not significant.

The results of the present study showed that suitable storage temperature was more effective in improving yolk lipid oxidative stability than the dietary supplementation of layer diets with synthetic or natural antioxidant additives. However, taking into consideration of the positive effects of the evaluated thyme and rosemary extracts on egg yolk fatty acid composition, it is suggested that these extracts may be supplemented in layer diets as a natural antioxidant source.

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