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MONITORING LIPID PEROXIDATION IN EGGS ENRICHED IN OMEGA 3 POLYUNSATURATED FATTY ACIDS (Ω – 3 PUFA)

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Abstract

The 6-week experiment used 168 Tetra SL (26 weeks) layers assigned to 4 groups. Compared to the conventional diet formulation used for the control group (C), in order to enrich the eggs in ω – 3 PUFA, the diet formulations used for the experimental groups (E1, E2, E3) included 7% flaxseed meal. Diet E1 used vitamin E (100 mg/kg feed) as antioxidant, while the other diets used 3% (E2) and 1.5% (E3) grape seeds powder. Egg samples were collected throughout the experimental period: initially, in the beginning of the experiment, in weeks II, IV and VI and when the experiment ended. The collected eggs were used to form egg yolk samples, which were assayed for fatty acids, peroxide value (PV), concentration of conjugated dienes (CD) and concentration of conjugated trienes (CT) and total antioxidant capacity. The yolks of the eggs from the experimental groups had a significantly (P≤0.05) higher concentration of ω – 3 PUFA compared to those from the control group. The lowest PV was determined in the yolk from E2 groups (3% grape seeds powder). The lowest CD concentrations were traced in the eggs collected on week II from groups E1 (6.278±1.931 µmol/g) and E2 (6.254±0.196 µmol/g). The yolks from groups E2 and E3 collected on weeks II and VI had the lowest CT absorbance. The antioxidant capacity was higher in the yolk from groups E1 (100 mg vitamin E/kg CF) and E2 (3 % grape seeds powder). The yolks from E3 (1.5% grape seeds powder) eggs collected in the end of the experiment had a significantly (P≤0.05) lower antioxidant capacity than E1 and E2.

Key words**:** *antioxidant capacity, dienes, eggs, peroxidation, PUFA ω – 3, trienes.*

INTRODUCTION

The interest for foods high in polyunsaturated fatty acids (ω – 3 PUFA) increased a lot over the past two decades (Siro et al., 2008). However, the susceptibility of autoxidation, major reaction of lipid degradation, is a major concern both for the food industry and for the consumers. The oxidation of the fatty acids from feeds and animal foods increases with their level of unsaturation and affects drastically the nutrients, the flavour and safety of the product, its storage and the economic efficiency (Labuza, 1971; Frankel, 1980). Once started, oxidation progresses throughout a chain mechanism with free radicals (Labuza, 1971). Within this context, lipid peroxidation must be assessed by tracing the primary lipid peroxidation products, the hydroperoxides (Nouroozzadeh J. et al., 1994), the conjugated dienes (Iversen SA et al., 1985), or some secondary products such as malondialdehyde (Draper et al., 1993), alkanes (Burk and Ludden, 1989).

The content of hydroperoxides can be determined by several methods such as iodometric titration (Gray, 1978); spectrometry (Griffiths et al., 2000; Bou R. et al., 2008); chemiluminiscence (Miyazawa et al., 1987; Shahidi et al., 2002); chromatography (Dobarganes and Velasco., 2002). Monitoring the amount of hydroperoxides function of time shows whether the lipids are in the stage of increase or decrease of concentration. This information can be used to consider the acceptability of a food in terms of the extent of deterioration.

The lipids which contain methyl dienes or polyenes undergo a change in the position of the double bond during oxidation due to the isomerization and conjugation (Logani and Davies, 1980). The resulting conjugated dienes and trienes are quantified by spectrophotometry

(Papuc et al., 2012). Lipid oxidation in foods and other biological systems is often determined by spectrophotometry with 2-thiobarbituric acid (TBA) and reading at 530-532 nm wavelengths (Tarladgis et al., 1964). Shahidi et al. (1986) developed an alternative spectrophotometric method to monitor lipid oxidation, measuring the total volatile carbonyl compounds that were formed through hydroperoxides degradation, based on the absorbance of the quinoidal ion, derivative of aldehydes and ketones.

The production of eggs enriched in PUFA ω-3, food with functional properties, can be achieved by using flax in layer diets under different forms: seeds (Bean and Leeson, 2003; Criste et al., 2009); oil (Milinsk et al., 2003; Souza et al., 2008); flaxseed meal (Aziza et al., 2013; Panaite et al., 2016). The presence of a material rich in PUFA (flaxseed meal, for instance) in layer diets requires the presence of an antioxidant. This is any substance which delays significantly or inhibits the oxidation of a substrate (Semb, 2012). The dietary antioxidants can minimize lipid oxidation, therefore preserving the quality of the eggs enriched in ω – 3 PUFA (Qi and Sim, 1998; Galobart et al., 2001). Despite the efficiency, constant quality and the rather low cost of the synthetic antioxidants, there is a recent worldwide trend to use natural antioxidants (Frankel, 2005, Pokorný, 1991). Many scientists reported that the winery by-products are rich in polyphenols (Katalinić et al., 2010) and flavonoids (Yilmaz and Toledo, 2004). Antioxidant capacity can be assessed by determining their antioxidant capacity. Burits and Bucar (2000) evaluated the antioxidant capacity by the capacity to annihilate radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), as shown by the discoloration of the DPPH solution. Another method to determine the antioxidant capacity is the ABTS spectro-photometric assay (Paulova et al., 2004).

The purpose of this experiment was to monitor lipid peroxidation in the egg enriched in ω – 3 PUFA during an experiment in which the layers were fed diets high in ω – 3 PUFA. The following determinations were performed on the eggs collected at different moments during the feeding trial: peroxide value (PV), concentration of conjugated dienes (CD) and concentration of conjugated trienes (CT), total antioxidant capacity.

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MATERIALS AND METHODS

A 6-week feeding trial was conducted to obtain eggs enriched in ω – 3 PUFA. The experiment used 168 TETRA SL layers (aged 26 weeks), assigned to 4 groups of 42 layers each. The birds were housed in an experimental hall under controlled environmental conditions (average temperature 21.94±1.96°C; air humidity 56.83±6.38 %), in agreement with TETRA SL management guide. The light regimen (16 h light/24 h), in agreement with TETRA SL management guide, was provided by light bulbs. The layers had free access to the feed and water.

The compound feeds for the 4 groups had the same basic formulation (Table 1). Compared to the conventional formulation, given to the control group (C), the diets of the experimental groups (E1, E2, E3) included 7% flax seeds meal, to enrich the eggs in ω – 3 PUFA. Diet E1 used vitamin E (100 mg/kg feed) as antioxidant, while the other diets used 3% (E2) and 1.5% (E3) grape seeds powder, mechanically degreased.

Samples were collected from each batch of compound feed and assayed for the basic chemical composition and fatty acids concentration. The methods from Regulation (CE) 152/2009 (Methods of sampling and analysis for the official control of feed) have been used: the gravimetric method for dry matter (DM) and ash; the Kjeldahl method for crude protein (CP); extraction in organic solvents for ether extractives (EE); successive hydrolysis in alkali and acid environment for crude fibre (CF). The fatty acids concentration has been determined by gas chromatography according to standards SR CEN ISO/TS 17764-1/ 2008 (Feeds. Determination of the fatty acids content. 1. Preparation of the fatty acids methyl esters) and SR CEN ISO/TS 17764-2/ 2008 (Feeds. Determination of the fatty acids content. 2. Method of gas chromatography) (Panaite et. al., 2016).

Table 1. Diet formulations

Ingredients	Control group (C)	Experimental group 1 (E1)	Experimental group 2 (E2)	Experimental group 3 (E3)
Corn, %	20	20	20	20
Wheat, %	28.25	26.6	24.18	24.47
Rice bran, %	10	10	10	10
Soybean meal, %	18.72	13.28	15.32	13.36
Rapeseeds meal, %	8	8	4.68	8
Oil, %	3.53	3.29	3.9	3.84
Flaxseeds meal, %	-	*7*	*7*	*7*
Grape seems powder, %	-	-	*3*	*1.5*
Methionine, %	0.08	0.16	0.18	0.16
Lysine, %	-	0.17	0.16	0.16
Carbonate, %	8.72	8.74	8.78	8.74
Monocalcium phosphate, %	1.27	1.32	1.36	1.33
Salt, %	0.38	0.39	0.39	0.39
Choline, %	0.05	0.05	0.05	0.05
Premix A6 (IBNA) *, %	1	-	1	1
A6 (100 mg vit.E/kg CF) , %	-	1	-	-
Total	100	100	100	100

*1kg premix IBNA (A6) contains: = 1.350.000 IU/kg vit. A; 300.000 IU/kg vit. D3; 2700 IU/kg vit. E; 200 mg/kg Vit. K; 200 mg/kg Vit. B1; 480 mg/kg Vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg Vit. B6; 4 mg/kg Vit. B7; 100 mg/kg Vit. B9; 1.8 mg/kg Vit. B12; 2500 mg/kg Vit. C; 7190 mg/kg manganese; 6000 mg/kg iron; 600 mg/kg copper; 6000 mg/kg zinc; 50 mg/kg cobalt; 114 mg/kg iodine; 18 mg/kg selenium;

To determine the fatty acids concentration in the egg yolks, 18 eggs per group were collected in the beginning of the trial, and on weeks 2, 4 and 6. Six samples per group (3 egg yolks/sample) were formed and dried in a drying cabinet at 65^0C, after which the fatty acids profile was determined by gas chromatography. For the preparation of the fatty acids methyl esters in agreement with the standard ISO 5508:2002, we weighed a sample of about 1 g of fat extracted from the dried yolk (65^oC). The analysis of the methyl esters was done according to standard SR EN ISO 5509:2002 (Panaite et al., 2016).

To monitor lipid peroxidation in the eggs enriched in omega 3 polyunsaturated fatty acids (ω – 3 PUFA), we collected eggs in the first experimental weeks, on weeks 2 and 4, and in the final week of the experiment (week 6). In the initial sampling we collected 30 eggs, used to form 10 egg yolk samples. To evaluate lipid degradation, in the experimental weeks 2, 4 and 6, we collected a total of 72 eggs (18 eggs/group), and six samples per group (3 egg yolks/sample) were formed. The yolk samples were stored in 15 mL plastic tubes and frozen. Before analysis, the yolk samples were thawed, and assayed for the peroxide value, concentration of conjugated dienes and trienes and for the total antioxidant capacity.

To determine the peroxidation products of the lipids, the lipids from the yolk samples were extracted according to the method described by Folch et al. (1957). We homogenized 2 g yolk sample with 10 mL methanol and 20 mL de chloroform, and stirred for one hour. The resulting solution was filtered through a separation funnel, to which 7.5 mL 0.88% KCl solution was added, and the phases were thus separated. The lower layer was collected in a 100 mL Berzelius beaker and left to evaporate at room temperature until the next day. The beakers with the sample were weighed and the amount of fat extracted from the sample was determined by the difference from the initial beaker weight.

Peroxide value determination relied on peroxide capacity to oxidize the ferrous ion at

low pH, using the ferrous oxidation-xylenol orange (FOX) assay. A blue-violet compound is formed with the ferrous ion, whose concentration is determined spectrophotometrically (at 560 nm). Between 0.01- 0.05 g of the fat extracted from the yolk samples were put into a glass tube, to which 9.9 mL chloroform-methanol mixture (7:3, v/v) was added and stirred. After the addition of 50 µL xylenol orange solution, 10 mM, and of 50 µL $FeCl_2$ (1000 mg/kg) solution, the mixture was homogenized for five minutes and the absorbance was read at 560 nm, using a JASCO V-530 spectrophotometer. The standard curve was obtained using a $FeCl_3$ (10 mg/kg) solution. The peroxide value was expressed in mEq O_2/ kg fat.

The conjugated dienes and trienes were determined by molecular absorption spectrometry in UV, as described by Papuc et al. (2012). The aliquots from the fat collected in Berzelius beakers after lipid extraction, as described above, was mixed with 10 mL isooctane to be dissolved. If the fat didn't dissolve, more isooctane was gradually added until a maximum volume of 50 mL. For the limpid solutions, we read the absorbance at 233 nm (conjugated diene) and at 268 nm (conjugated trienes), using a JASCO V-530 spectrophotometer. The concentration of conjugated dienes was expressed in µmol/g fat, while the concentration of conjugated trienes was expressed in absorbance units (A_{268} nm).

The total antioxidant capacity was determined using the spectrophotometric method, as described by Prieto et al. (1999). The method relies on the reduction of Mo (VI) to Mo (V), by the sample analytes and the subsequent formation of a green phosphate/ Mo (V) complex, at acid pH. We weighed 1 g egg yolk, in 30-50 mL centrifuge tubes. We added 10 mL 80% methanol solution, homogenized and stirred for one hour in darkness. We centrifuged at 10,000 rpm, for 15 minutes, then collected the supernatant. In 15 mL tubes we pipetted 0.2 mL sample solution, added 4 mL ammonium phosphomolybdate reagent and incubated for 90 minutes at 95°C. The samples were left to cool down and the absorbance was read at 695 nm, compared to the blank, using a JASCO V-530 spectrophotometer. The results were expressed in mM ascorbic acid equivalent.

Statistical analysis

The analytic data were compared by variance analysis (ANOVA) using Stat View for WINDOWS (SAS, version 6.0).

The differrences of the means were considered significant for $P \leq 0.05$. The results were expressed as mean± SD for all measurements.

RESULTS AND DISCUSSIONS

The analysis of the 4 compound feeds (Table 2) has showed that the crude fat percentage increased in the feeds for the experimental groups, compared to the control, due to the dietary flaxseeds meal.

The same ingredient also increased significantly ($P \leq 0.05$) the α-linolenic acid (ω – 3 PUFA acid) concentration n the feeds for the experimental groups, compared to the control group (Table 2).

Table 2.Chemical characterization of the dietary compound feeds for layers

Item	Control group (C)	Experimental group 1 (E1)	Experimental group 2 (E2)	Experimental group 3 (E3)
Dry matter, %	89.85±0.081	90.27±0.208	90.27±0.129	90.38 ± 0.067
Crude protein, %	19.147±0.765	19.29±0.856	19.33±0.637	19.02±0.667
Fat, %	7.037±0.117^{cd}	8.03±0.07^{cd}	8.133±0.188^{ab}	8.05±0.243^{ab}
Crude fibre, %	5.283±0.27 ^{bcd}	6.05±0.218 ^{a}	6.567±0.257 ^{a}	6.547±0.598 ^{a}
Ash, %	13.72±0.805	13.51±1.245	14.583±0.211	14.133±0.775
Linoleic acid C 18:2n6	48.485±0.403^{bd}	45.315±0.912^{a}	47.32±0.17	46.425±1.336^{a}
α-linolenic acid C 18:3n3	1.195±0.177 ^{bcd}	6.865±0.163^{a}	6.42±0.297^{a}	6.43±0.523^{a}

*Where: a,b,c,d, significant differences ($P \leq 0.05$) compared to C, E1, E2, E3;

The concentration of linolenic acid was, in average, 5.5 times higher than in the control feeds. The literature shows that the flaxseeds meal is rich in polyunsaturated fatty acids,

which account for 73% of the total fatty acids; it has a moderate content of monounsaturated fats (18%) and a low amount of saturated fats (9%) (Cunnane et al., 1993; Dubois et al., 2007).

Because the concentration of polyunsaturated fatty acids was rather similar in the eggs collected on weeks 2, 4 and 6, table 3 shows the mean of the determinations conducted during the experiment.

The supplemental flaxseed meal given to the experimental groups increased significantly ($P \leq 0.05$) ω – 3 PUFA in the egg yolk, compared to the control group (Table 3). Regarding ω – 6 PUFA, its concentration was higher ($P \leq 0.05$) in the yolk of the eggs from group C, compared to the experimental groups (Table 3). ω – 6/ ω – 3 ratio was significantly higher ($P \leq 0.05$), by about 3 times, in the yolk of the eggs collected from the control group, compared to those from the experimental groups (Table 3). Our data confirm the literature findings, which show that the use of flax, under different forms, in layer diets, increased PUFA concentration in the eggs (Leeson et al., 1998; Aziza et al., 2013).

Table 3. Fatty acids concentration (g% total fatty acids) in egg yolk, depending on their level of saturation

Item	Initial	Mean of the determinations performed during the experiment			
		Control group (C)	Experimental group 1 (E1)	Experimental group 2 (E2)	Experimental group 3 (E3)
ΣSFA	34.60±1.112^{b}	34.43±0.814^{a,e}	33.91±1.025	33.61±0.923	32.85±0.916^{b}
ΣUFA	64.62±0.850cde	65.45±0.838^{e}	65.93±1.041^{a}	66.22±0.8^{a}	66.99±0.906^{ab}
ΣMUFA	56.62±0.668^{bcde}	33.96±0.944^{a}	33.94±1.156^{a}	33.29±0.903^{ae}	34.44±0.914^{ad}
ΣPUFA Of which:	7.99±0.338^{bcde}	31.49±0.303 ^{acde}	31.99±0.300^{abde}	32.92 ±0.255^{abc}	32.55±0.512^{abc}
-ΣPUFA ω - 3	1.71±0.07^{cde}	1.56±0.19^{cde}	4.58±0.21^{abe}	4.39±0.24^{abe}	4.13±0.20^{abcd}
-ΣPUFA ω - 3	28.62±0.45 ^{bc}	29.93±0.3 ^{acde}	27.41±0.18^{abde}	28.54±0.30^{bc}	28.42±0.49^{bc}
-PUFA ω – 6/ ω - 3	16.75±0.55 ^{bcde}	19.23±2.39^{acde}	5.99±0.27^{ab}	6.50±0.41^{ab}	6.88±0.35^{ab}

Where: SFA = saturated fatty acids; UFA = unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; a,b,c,d,e= significant differences ($P \leq 0.05$) compared to initial, C, E1, E2, E3.

The evaluation of lipid degradation level in the eggs collected during the experiment started with the determination of the peroxide value (PV).

Frankel (2005), showed that if no antioxidant is used, the higher is the proportion of unsaturated fatty acids in the fat, the higher is the peroxide value. PV decreased (Figure 1) in the yolk of the eggs collected from groups E1 and E2, unlike the control group and E3 (increasing trend).

Throughout the experimental period, irrespective of the sampling week, the yolk from E2 eggs had the lowest PV (Figure 1), with the lowest one being in the eggs collected in the end of the experiment (week 6).

This shows a higher oxidative stability of the yolk from E2 eggs (3% grape seeds powder), compared to groups C, E1 (100 mg vitamin E/kg CF) and E3 (1.5% grape seeds powder).

Compared to the initial sampling, yolk PV decreased in all experimental groups (Figure 1).

Significant ($P \leq 0.05$) differences were noticed, in the concentration of conjugated dienes (CD) andtrienes (CT), secondary products of lipid oxidation, between the experimental groups (E1, E2, E3) and the control group, C, for each week of sampling and between these intervals.

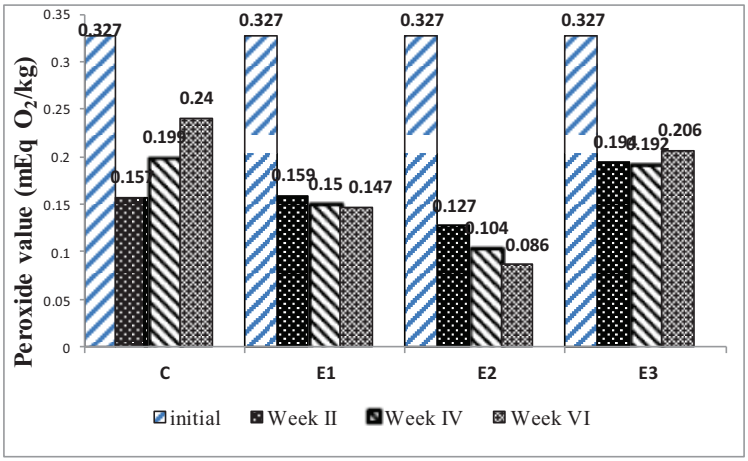

Figure 1.Evolution of yolk PV

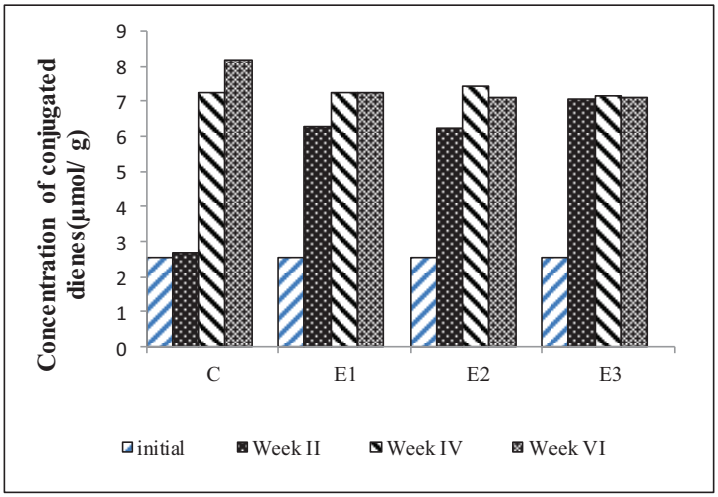

Figure 2. Evolution of yolk concentration of conjugated dienes (CD)

Compared to the initial sampling, CD concentration increased in the yolk from all groups (C, E1, E2, E3), throughout the experiment (Figure 2).

Comparable concentrations were noticed, during the last week of experiment, in the yolk of the eggs from groups E2 and E3, which shows the beneficial effect of the supplemental grape seeds powder (diet formulations E2 and E3) on the oxidative stability of the lipids in the yolks enriched in ω – 3 PUFA.

Figure 3 shows the results of CT (A_{268} nm) absorbance from the yolk samples, and the chemical changes of the lipids determined during the successive sampling periods (weeks 2, 4 and 6), compared to the initial sampling.

Conjugated CT absorbance displays a decreasing trend in all groups (C, E1, E2, E3). The yolk of the eggs collected from groups E2 and E3 were rather similar, and were the lowest in weeks 2 and 6.

Corroborating Figures 2 and 3, one may observe that the yolk of the eggs collected from groups E2 and E3 displayed the highest stability against lipid peroxidation.

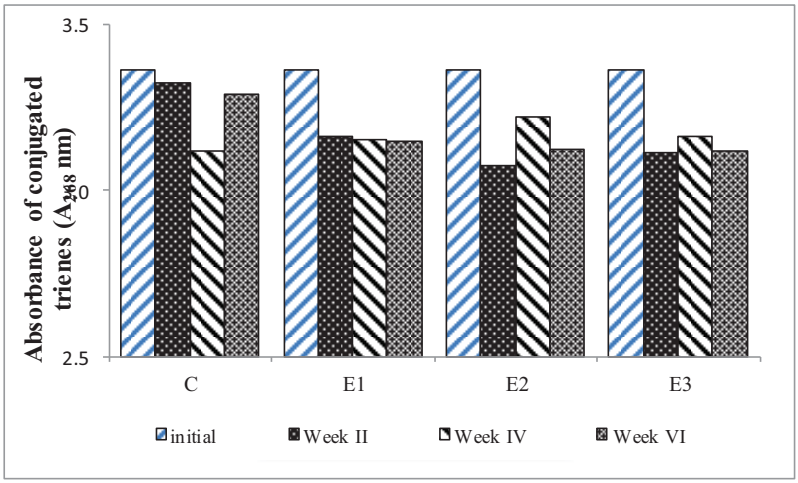

Figure 3. Evolution of conjugated trienes (A_{CT268} nm) formation in the yolk

Throughout the experimental period, the yolk from groups E1 and E2 displayed a higher antioxidant capacity (Table 4). The yolk of E3 eggs collected in week 6, had a significantly ($P \leq 0.05$) lower antioxidant capacity than the other experimental groups (Table 4).

This result, corroborated with the peroxide values (Figure 1), shows that the supplemental 1.5% grape seeds powder was too low to inhibit yolk lipids peroxidation. The yolk of the eggs collected in week 6 from group E2 had a higher antioxidant capacity (8.672 mM ascorbic acid equivalent) compared to the other periods of sampling. This is due to the antioxidant activity of the grape seeds powder (E2).

Papuc et al., (2008) have shown that the oxidation of the unsaturated fatty acids from the foods can be slowed down by the addition of plants rich in compounds with antioxidant activity.

Table 4. Antioxidant capacity of the yolk depending on the period of sample collection

Item	Initial	Week II	Week IV	Week VI
	mM ascorbic acid equivalent	mM ascorbic acid equivalent	mM ascorbic acid equivalent	mM ascorbic acid equivalent
Initial	4.78 ± 1.644^{abcde}	-	-	-
Group C	-	5.037 ± 1.67^{cd}	5.557 ± 0.68	5.795 ± 1.78^{ae}
Group E1	-	6.543 ± 1.79^{abde}	5.866 ± 3.57	7.866 ± 0.71^{ae}
Group E2	-	7.806 ± 1.17^{abce}	7.079 ± 1.89^{a}	8.672 ± 3.67^{ae}
Group E3	-	5.199 ± 0.67^{cd}	6.465 ± 0.75^{a}	6.103 ± 0.36^{abcd}

* Where: a,b,c,d,e, significant differences ($P \leq 0.05$) compared to initial, C, E1, E2, E3.

CONCLUSIONS

The lowest PV of the yolk lipids was determined in group E2. This shows the beneficial action of the 3% dietary grape seeds powder to delay the onset of lipid peroxidation in the yolks, compared to the lower concentration (1.5%) of dietary grape seeds powder, as noticed in group E3. The concentration of conjugated dienes, secondary product of lipid degradation, was lower in the yolk from the experimental groups, compared to the control group, irrespective of the sampling period. The highest antioxidant capacity was determined in the yolk of the eggs from the experimental groups E1 (100 mg vitamin E/kg CF) and E2 (3 % grape seeds powder).

The experimental results show the opportunity of using natural antioxidants in the compound feeds rich in omega 3 polyunsaturated fatty acids. If they are added in a proper

concentration, they have a constant effect of preventing lipid peroxidation.

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