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Trace Mineral Sources and Rosemary Oil in the Diet of Brown Laying Hens: Egg Quality and Lipid Stability

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ABSTRACT

Two experiments were carried out to evaluate the effects of rosemary oil (RO) and trace mineral sources (MS) on the internal quality and lipid stability of brown layer eggs. The treatments consisted of diets supplemented with two trace mineral sources (inorganic or organic) and three levels (0, 100, or 200 mg kg^{-1}) of rosemary oil (RO), and three egg storage times. Eggs were stored at a controlled temperature (CT; 25.0ºC) in Experiment I and under refrigeration (RT; 5.0ºC) in Experiment II. The following parameters were analyzed on days 0 (fresh), 15 and 30 of storage: malonaldehyde level (MDA), egg weight (EW), Haugh unit (HU), yolk index (YI), albumen and yolk pH, raw yolk color (RYC), and egg weight loss. Data were analyzed according to completely randomized design in a 2x3x3 factorial arrangement (MS x RO x storage time). In Experiment I, there was an interaction between treatments for EW, HU and ALBp. Dietary OTM inclusion improved the results for all analyzed variables. The addition of 200 mg kg^{-1} RO reduced MDA and increased HU, YI and RYC. In experiment II, 200 mg kg^{-1} of RO in the diet improved HU. The internal quality of eggs stored both at CT and under RT is adversely affected by increasing storage periods, but this effect can be minimized by the dietary supplementation of OTM and 200 mg kg^{-1} rosemary oil. The lipid stability of eggs stored at CT improves with the supplementation of OTM and 200 mg kg^{-1} rosemary oil, but not of eggs stored under refrigeration.

INTRODUCTION

Regarded as an excellent source of essential fatty acids, the lipid fraction of eggs is located in the yolk, and consists mainly of unsaturated fatty acids, which are susceptible to oxidation, particularly during storage (Hayat *et al*., 2010).

Because lipid oxidation is a spontaneous and inevitable phenomenon that causes food flavor, aroma, and nutritional quality losses, various methods have been studied to delay its onset. In particular, studies on possible antioxidants found in natural products are promising (Theron *et al*., 2003, Coimbra *et al*., 2007; Abramovič *et al*., 2012). These products are free from chemical residues and are perceived by consumers as safe, making them quite popular in the food industry (Brenes & Roura, 2010).

Rosemary essential oil has emerged as an alternative to synthetic antioxidants and antimicrobials in feeds due to its ability to delay lipid oxidation, to inhibit microbial growth, and to improve nutrient digestibility (Hernández *et al*., 2004; Jang *et al*., 2008; Klancnik *et al*., 2009). Moreover, the antioxidant compounds found in rosemary can be transferred to eggs through the hen's diet, as detected by Botsoglou *et al*. (2005), who evaluated the inclusion of rosemary (5g kg^{-1}), saffron

(20 mg kg^{-1}), and oregano (5 g kg^{-1}) extracts in the diets of laying hens.

It was demonstrated that essential oils added to animal feeds are able to chelate dietary trace minerals, favoring their absorption and improving mineral utilization by the hens (Stef & Gergen, 2012). This likely results in greater ability to activate antioxidant enzymes in the hen's body, while also enhancing their transfer to the eggs, given that trace minerals are directly involved in the ability to activate those enzymes, including glutathione peroxidase (GSH) by selenium (Se), catalase (CA) by iron (Fe), and superoxide dismutase (SOD) by zinc (Zn), copper (Cu), and manganese (Mn) (Andrade & Marreiro, 2011).

Trace minerals are supplemented in layer diets to supply one of the main nutritional limitations of this animal category, as practical layers feeds are commonly based on corn and soybean meal, which have low concentrations of trace minerals.

The partial or full replacement of inorganic trace minerals, which are commonly supplemented in layer feeds, by organic trace mineral sources has been evaluated. Organic sources present higher bioavailability, and improve production performance (Klecker *et al*., 2002; Richards *et al*., 2010), and eggshell quality (Gravena *et al*., 2011), in addition of contributing to reduce trace mineral excretion in the environment, and consequently, environmental contamination (Osman *et al*., 2010).

The objective of the study was to evaluate the effect of the inclusion of rosemary oil and different trace mineral sources in the diet of brown layers on the internal quality and oxidative stability of eggs stored under controlled temperature (25.0ºC) or refrigeration (5.0ºC).

MATERIAL AND METHODS

The study was performed at the Poultry Sector and the Animal Product Quality Laboratory of the State University of Mato Grosso do Sul, Aquidauana University Unit. The experimental procedures were approved by the Ethics Committee of that institution under protocol n. 005/2013.

Two experiments were carried out, varying only as to storage temperature, which was 25.0ºC in experiment I and 5.0ºC in experiment II.

In total, 288 Hy Line Brown laying hens, with initial age of 30 weeks, were evaluated during four 28-day cycles. Each treatment comprised six replicates of with eight layers per cage. Hens were housed in pairs inside mesh wire cages, in a standard egg layer shed. Feeding, supplied in trough-type feeders, occurred twice a day and water was supplied from nipple-type drinkers. The light schedule adopted was 17 hours a day (natural + artificial lighting).

The experimental feeds were based on corn and soybean meal, and were formulated to contain equal energy and nutrient levels (Table 1) and to meet the nutritional requirements of the hens, according to the genetic line management guide (Hy Line, 2014) and to Rostagno *et al*. (2011).

The treatments consisted of six experimental diets with two trace mineral sources (inorganic or organic), three levels (0, 100, or 200 mg kg^{-1}) of rosemary oil (RO), and three egg storage periods. The following diets were fed: D1 – diet supplemented with inorganic trace minerals (ITM) and no RO inclusion (conventional diet); D2 – diet supplemented with ITM and RO at 100 mg kg^{-1}; D3 – diet supplemented with ITM and RO at 200 mg kg^{-1}; D4 – diet supplemented with organic trace minerals (OTM) and no RO inclusion; D5 – diet supplemented with OTM and RO at 100 mg kg^{-1}; and D6 – diet supplemented with ITM and RO at 200 mg kg^{-1} (Table 1)

Rosemary oil was added to the feeds in a powdered form, together with the mineral and vitamin supplements. The OTM supplement consisted of metal-amino acid complexes of Cu, Fe, Mn, Zn, and Se as selenium yeast. The different inclusion levels between the ITM and the OTM supplements are due to differences in their trace mineral levels. The ITM source contained copper sulfate (Cu, 25%), iron sulfate (Fe, 28%), manganese sulfate (Mn, 31%), sodium selenite (Se, 45%), and zinc sulfate (Zn, 35%), and the OTM source contained copper chelate (Cu, 10%), iron chelate (Fe, 10%), manganese chelate (Mn, 16%), selenium proteinate (Se, 0.2%), and zinc chelate (Zn, 16%).

The experimental diets were supplied for 112 days. All eggs produced were collected during the last four days, out of which 20 eggs/treatment/day were selected based on the absence of cracks, spots, and stains in the eggshell. Eggs were identified per treatment, placed in a paper egg carton, stored for different periods (0, 15, or 30 days) at controlled room temperature (25.0ºC and 63.03% RH) in Experiment I and under refrigeration (5.0ºC and 41.32% RH) in Experiment II.

Eggs were evaluated according to a completely randomized design in a 2x3x3 factorial arrangement (trace mineral source x rosemary oil levels x storage period), with measurements repeated over time. Two eggs/treatment/day were selected per storage period

Table 1 – Ingredients and calculated compositions of the experimental feeds.

Ingredients	Inorganic trace minerals	Organic trace minerals				
		Rosemary oil (mg kg^{-1})				
	0	100	200	0	100	200
Corn, grain	62.08	62.08	62.08	62.08	62.08	62.08
Soybean meal, 45%	25.34	25.34	25.34	25.34	25.34	25.34
Soybean oil	0.45	0.45	0.45	0.45	0.45	0.45
Limestone	9.97	9.97	9.97	9.97	9.97	9.97
Dicalcium phosphate	1.09	1.09	1.09	1.09	1.09	1.09
L-lysine HCl	0.01	0.01	0.01	0.01	0.01	0.01
DL-methionine	0.22	0.22	0.22	0.22	0.22	0.22
Salt	0.49	0.49	0.49	0.49	0.49	0.49
Mineral and vitamin supplement*	0.15	0.15	0.15	0.35	0.35	0.35
Inert	0.20	0.20	0.20	0.00	0.00	0.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated values						
Metabolizable energy (kcal kg^{-1})	2.750	2.750	2.750	2.750	2.750	2.750
Crude protein (%)	17.00	17.00	17.00	17.00	17.00	17.00
Digestible methionine + cystine (%)	0.704	0.704	0.704	0.704	0.704	0.704
Digestible lysine (%)	0.774	0.774	0.774	0.774	0.774	0.774
Calcium (%)	4.20	4.20	4.20	4.20	4.20	4.20
Available phosphorus (%)	0.30	0.30	0.30	0.30	0.30	0.30
Linoleic acid (%)	1.60	1.60	1.60	1.60	1.60	1.60

*Composition per kg of feed: Vitamin A, 7,500 IU; Vitamin D_3, 2,000 IU; Vitamin E, 10 IU; Vitamin K_3, 1.8 mg; Vitamin B_1, 1.5 mg; Vitamin B_2, 4.0 mg; Nicotinic acid, 25 mg; Pantothenic acid, 10 mg; Vitamin B6, 1.7 mg; Vitamin B12, 0.013 mg; Folic acid, 0.5 mg; Biotin, 0.05 mg; Choline, 220 mg; Cu, 11 mg; Fe, 55 mg; I, 1.1 mg; Mn, 77 mg; Se, 0.33 mg; Zn, 72 mg.

*Composition of inorganic trace mineral supplement (per kg): Vitamin A, 10 g; Vitamin D_3, 2.67 g; Vitamin E, 6.67 g; Vitamin K_3, 2.33 g; Vitamin B_1, 1.02 g; Vitamin B_2, 3.33 g; Nicotinic acid, 16.87 g; Pantothenic acid, 6.67 g; Vitamin B_6, 1.40 g; Vitamin B_{12}, 8.67 g; Folic acid, 0.40 g; Biotin, 1.67 g; Choline, 244.47 g; Cu, 29.33 g; Fe, 130.93 g; I, 1.20 g; Mn, 165.53 g; Se, 0.47 g; Zn, 137.13 g; Vehicle, 229.8 g.

*Composition of organic trace mineral supplement (per kg): Vitamin A, 4.29 g; Vitamin D_3, 1.14 g; Vitamin E, 2.86 g; Vitamin K_3, 1.0 g; Vitamin B_1, 0.44 g; Vitamin B_2, 1.43 g; Nicotinic acid, 7.23 g; Pantothenic acid, 2.86 g; Vitamin B_6, 0.60 g; Vitamin B_{12}, 3.71 g; Folic acid, 0.17 g; Biotin, 0.71 g; Choline, 104.77 g; Cu, 31.43 g; Fe, 157.14 g; I, 0.51 g; Mn, 137.51 g; Se, 47.14 g; Zn, 128.57 g, Vehicle, 366.48 g.

to evaluate internal quality, as determined by Haugh units, yolk index, yolk color, and albumen and yolk pH.

Lipid oxidation (TBARS method) was measured by adapting the methodology described by Ramanathan & Das (1992), in three eggs/treatment/day per storage period.

For internal quality analysis, the collected eggs were individually weighed in a semi-analytical scale (± 0.001g), and then cracked on a flat and smooth glass surface. pH was measured using a workbench pH meter by directly introducing the electrode into the yolk and the albumen.

Albumen and yolk heights were measured using a digital pachymeter, and expressed in millimeters (mm). Haugh units were calculated based on albumen height (mm) and egg weight (g), according to the equation described by Silversides & Budgell (2004): $HU= 100\log(H+7.75-1.7W^{0.37})$; In which, H = albumen height (mm) and W = egg weight (g). Horizontal yolk diameter was measured using a digital pachymeter (± 0.05 mm). The yolk index (mm) was then calculated based average yolk height/diameter values.

Raw yolk color was determined using a DSM color fan (Yolk Color Fan®, DSM, Germany), on 1-15 scale, ranging from light yellow to dark yellow (orange).

All data were subjected to the Shapiro-Wilk normality test. Analysis of variance was applied on those variables which residuals showed a normal distribution, and means were compared using Tukey's test (at $p<0.01$ and $p<0.05$). A generalized linear model was used for all other variables that did not show normal distribution, assuming a gamma distribution with inverse function, and means were compared by the Student t-test ($p<0.01$ and $p<0.05$). The data were analyzed using the statistical program R®, version 3.0.2 (R Core Team, 2013).

RESULTS AND DISCUSSION

Experiment I – Storage at controlled temperature

A significant interaction ($p<0.01$) between trace mineral sources and rosemary oil levels was observed for egg weight when eggs were stored at controlled

temperature (25.0 °C), independently of the storage period (Table 2). Layers fed the OTM diet laid heavier eggs compared with those fed ITM when the diet was not supplemented with RO, whereas the lightest eggs were obtained when layers were fed the diet supplemented with OTM and 100 mg kg^{-1} RO (Table 3). The higher egg weight obtained with OTM supplementation may be a reflex of increased yolk and albumen weights, as a result of better improved nutrient utilization, as observed by Nunes *et al*. (2013) feeding OTM to laying hens. Evaluating replacement levels (33%, 66% and 100%) of inorganic with organic trace mineral sources in the diet of laying hens, Figueiredo Júnior *et al*. (2013) observed higher egg weight at the 33% replacement level.

Likewise, the inclusion of rosemary oil in the diets containing the ITM source may have favored the absorption of nutrients by the laying hens (Hernández *et al*., 2004; Al-Kassie *et al*., 2011). In addition of its antioxidant effect, rosemary extract may present antimicrobial action against *E. coli* and *S. aureus*, by damaging the integrity of the lipid membrane of these microorganisms, thereby controlling the growth of pathogens in the intestine, as observed Lima *et al.* (2014), who evaluated the antimicrobial activity *in vitro* of using crude rosemary extract (40 and 20 mg/mL). However, the antimicrobial active ingredients and their concentration present in rosemary extracts may vary according to extraction methodology, region the plant is grown, and part of the plant to be used.

Table 3 – Egg weight laid brown-egg layers fed different trace mineral sources and rosemary oil levels, and stored up to 30 days at controlled temperature (25.0ºC)

Trace mineral source	Rosemary oil (mg $kg^{-1}$of feed)		
	0	100	200
Inorganic	59.52b	59.80	60.35
Organic	62.34aA	57.93B	59.45AB

Means followed by different lowercase letters in the same column and uppercase letters in the same row are significantly different at 1% probability level.

Relative to storage period, it was detected that egg weight decreased (p<0.01) until 15 days of storage, and remained similar up to 30 days of storage. Egg weight loss is correlated with albumen water loss through the eggshell pores during the gas exchange process (exit

Table 2 – Concentration of malonaldehyde (MDA), egg weight (EW), Haugh unit (HU), yolk index (YI), albumen pH and yolk, raw yolk color (RYC), and weight loss (WL) of eggs stored for different periods (SP) at controlled temperature (25.0ºC) laid by brown layers fed different trace mineral sources (TMS) and rosemary oil levels (RO)

Variables	MDA (mg kg^{-1})	EW (g)	HU	YI (mm)	pH	WL (%)	RYC	
					Yolk	Albumen		
TMS								
Inorganic	0.243a	59.89	54.46	0.299	6.48a	8.92	2.86a	4.93
Organic	0.226b	59.90	69.13	0.327	6.23b	8.57	2.55b	4.98
RO (mg kg^{-1} of feed)								
0	0.242a	60.93	58.52	0.303b	6.35	8.75	2.66	4.87b
100	0.235ab	58.86	61.94	0.311b	6.37	8.76	2.68	4.93ab
200	0.225b	59.90	64.93	0.324a	6.34	8.73	2.78	5.06a
SP (days)								
Fresh	0.196a	61.58a	101.65	0.446	5.88c	7.50	0.00c	5.06a
15	0.229b	59.18b	47.76	0.268	6.42b	9.31	2.83b	5.00a
30	0.278c	58.93b	35.98	0.225	6.75a	9.43	5.28a	4.81b
Normality*	0.045	0.555	0.006	<0.01	<0.01	<0.01	<0.01	<0.01
Mean	0.234	59.89	61.79	0.313	6.35	8.74	2.70	4.95
SEM[1]	0.00	0.39	2.55	0.01	0.04	0.08	0.16	0.03
p value								
MS	<0.01	0.984	<0.01	<0.01	<0.01	<0.01	0.05	0.250
RO	<0.01	0.071	0.054	<0.01	0.925	0.686	0.735	0.01
SP	<0.01	0.005	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
MS x RO	0.391	0.023	0.755	0.519	0.717	0.771	0.625	0.558
MS x SP	0.061	0.955	<0.01	<0.01	0.454	<0.01	0.172	0.532
RO x SP	0.497	0.318	0.056	0.138	0.289	0.174	0.771	0.087
MS x RO x SP	0.945	0.120	0.298	0.303	0.231	0.751	0.753	0.400

[1]Standard error of the mean; *Values with p>0.05 present normal distribution by the Shapiro-Wilk test

Means followed by different letters in the same column are significantly different at 1% and 5% probability level.

of moisture and carbon dioxide), which continuously occurs after lay and may be accelerated by inadequate storage conditions, especially at high temperatures (Freitas *et al*., 2011). In a study evaluating eggs stored for up to 35 days, Barbosa *et al*. (2008) observed an average 9.20% reduction in the weight of eggs stored in a high temperature and moisture environment. Similar findings were reported by Figueiredo *et al*. (2011), who verified greater egg weight loss, from 0.65g to 1.03g, in eggs stored at 21ºC for 5 and 10 days, respectively.

Haugh units were affected by the interaction (p<0.01) between trace mineral source and storage period. Eggs stored for 30 days presented higher Haugh units values when laid by hens fed OTM diets compared with those fed the ITM diets. However, regardless of the trace mineral source, Haugh unit values were reduced with storage time (Table 4).

Table 4 – Haugh units of eggs laid by brown layers fed different trace mineral sources and rosemary oil levels, and stored up to 30 days at controlled temperature (25.0ºC).

Trace mineral source	Storage period (days)		
	Fresh	15	30
Inorganic	97.22bA	40.77bB	25.39bC
Organic	106.08aA	54.7 bA	46.57aC

Means followed by different lowercase letters in the same column and uppercase letters in the same row are significantly different at 1% probability level.

Reductions in Haugh units were also reported by other authors, such as Alleoni & Antunes (2001), who observed a 53.5% reduction in eggs stored at 25.0ºC for seven days compared with fresh eggs. Similar results were also found in studies with eggs stored without refrigeration for 16 (Garcia et al., 2010) and 21 days (Santos *et al*., 2009; Freitas *et al*., 2011).

The supplementation of organic trace minerals in layer diets may enhance the production of egg internal components. The dietary of organic selenium inclusion promoted egg gland dilation and the preservation of the ciliary epithelium of the magnum, isthmus, and shell gland, resulting in greater albumen height, Haugh unit, eggshell thickness, and yolk pigmentation index (Attia *et al*., 2010; Santos, 2010). Positive effects on albumen deposition were also reported by Nunes *et al*. (2013), supplementing laying hen diets with organic trace minerals.

However, Haugh unit reduction during storage is inevitable. During storage, carbonic acid (H_2CO_3) – one of the components of the albumen buffer system – dissolves, yielding water and CO_2. Carbon dioxide (CO_2) and moisture losses to the environment through the eggshell pores increase albumen pH (from 6.5 to 9.5), and result in the hydrolysis of the amino-acid chains of albumen protein system, which consists of ovomucin fibers and globular proteins (Stadelman *et al*., 1996). When O-glycosidic bonds of the ovomucin polypeptide chains are broken, gelling properties are partially lost, increasing the fluidization and reducing the viscosity of the thicker albumen, resulting in a reduction of Haugh unit values (Sgarbieri, 1996).

The interaction observed (p<0.01) between the trace mineral source and storage period for albumen pH reinforces the observed positive effects of OTM on Haugh units. Albumen pH increased until the end of the storage only when the hens were fed ITM, whereas, it increased only up to day 15 of storage, and remained stable until the end of the storage period in the eggs of OTM-fed hens (Table 5).

Table 5 – Albumen pH of eggs laid by brown layers fed different trace mineral sources and rosemary oil levels, and stored up to 30 days at controlled temperature (25.0ºC).

Trace mineral source	Storage period (days)		
	Fresh	15	30
Inorganic	7.64aC	9.40aB	9.73aA
Organic	7.37bB	9.22bA	9.12bA

Means followed by different lowercase letters in the same column and upper case letters in the same row are significantly different at 1% probability level.

Eggs with high amounts of dense albumen show lower moisture loss during stocking (Brake *et al*., 1997), thereby minimizing the sharp changes in albumen and yolk pH, as well as egg weight loss throughout the storage period.

An interaction was observed (p<0.01) between trace mineral source and storage period for yolk index, with higher values observed during all storage periods in the eggs of the OTM-fed hens. However, yolk index decreased as storage period increased, independently of trace mineral source (Table 6).

Table 6 – Yolk index of eggs laid by brown layers fed different trace mineral sources and rosemary oil levels, and stored up to 30 days at controlled temperature (25.0ºC).

Trace mineral source	Storage period (days)		
	Fresh	15	30
Inorganic	0.417bA	0.246bB	0.207cB
Organic	0.438aA	0.278aB	0.234aC

Means followed by different lowercase letters in the same column and uppercase letters in the same row are significantly different at 1% probability level.

The highest yolk index values found for eggs in the treatment with organic trace minerals are likely linked to increased yolk production due to improved nutrient use during digestion (Nunes *et al*., 2013). As such, larger yolks have less significant losses as a result of the storage period. The water released as a result

of albumen protein denaturation is transferred to the yolk by osmosis, increasing its content, but making it flaccid and flat and weakening the yolk membrane, which makes it highly susceptible to breaking during handling (Garcia *et al.*, 2010). The effects of storage time on yolk index observed in the present study are in agreement with the findings of Garcia *et al*. (2010) observed a 43.48% reduction in the yolk index of eggs stored for up to 16 days.

The yolk index was also influenced (p<0.01) by the dietary addition of rosemary oil (Table 2). The inclusion of 200 mg kg^{-1} of RO promoted the highest values for this variable. The beneficial effects of rosemary oil on nutrient absorption (Al-Kassie *et al.*, 2011) may have increased yolk weight. Moreover, the inclusion of rosemary oil may have reduced fatty-acid oxidation in the hens' metabolism, particularly of linoleic acid, which stimulates protein secretion in the oviduct (Whitehead *et* al., 1993), thus increasing yolk weight.

Trace mineral sources, rosemary oil levels, and storage period showed individually influenced (p<0.01) yolk malonaldehyde values (Table 2). The results demonstrate that lipid oxidation was reduced when the diets were supplemented with organic trace minerals or 200 mg kg^{-1} rosemary oil. However, malonaldehyde values steadily increased with storage period, evidencing the negative effect of storage time on egg lipid stability.

Malonaldehyde is one of the main products of the decomposition of hydroperoxides resulting from the oxidation of polyunsaturated fatty acids. Its concentration is used to estimate lipid peroxidation in food items and biological systems by the TBARS (thiobarbituric acid reactive substances) test. At high concentrations, this compound can adversely affect the flavor and aroma of food items, making them inedible (Osawa *et al.*, 2005).

The inclusion of organic trace minerals in the experimental diets possibly led to a higher activation of antioxidant enzymes, such as glutathione peroxidase, inside the eggs, as these enzymes are mineral-dependent (Andrade & Marreiro, 2011) and capable of slowing the rates of hydroperoxide decomposition and malonaldehyde production, which are secondary products of oxidation (Ferreira & Matsubara, 1997). Likewise, the active compounds of rosemary (such as carnosol and carnosic acid) are capable of terminating free-radical reactions and scavenging reactive oxygen species (Sánchez-Escalante *et al.*, 2001), preventing the oxidation of yolk fatty acids, as these antioxidant compounds can be transferred from the hen's diet to the egg (Botsoglou *et al.*, 2005).

Trace mineral source and storage period had isolated effects (p<0.01) on yolk pH and egg weight loss (Table 2). The eggs of hens fed OTM presented reduced yolk pH and lower weight loss compared with those fed ITM. However, yolk pH values and egg weight loss continuously increased with storage period. This was probably caused the exposure of due to esous (CO_2 and water) exchanges between the egg and the environment, which inevitably occurs after laying and reduces egg internal quality after long periods of storage, especially when there is no refrigeration.

Raw yolk color was influenced by RO level and storage time (p<0.01). The dietary inclusion of 200 mg kg^{-1} rosemary oil intensified egg yolk color. Conversely, yolk color values decreased (p<0.01) with storage time.

It was shown that the phenolic compounds present in essential oils are able to chelate metallic ions, such as iron and copper, and that the absorption of trace minerals and their accumulation in the hen's liver increases with polyphenol levels (Stef & Gergen, 2012). Therefore, the dietary inclusion of 200 mg kg^{-1} of RO in the present experiment possibly promoted higher iron absorption, as this mineral is responsible for the intense yellow color of the yolk (Paik *et al.*, 2009).

However, iron is transferred from the yolk to the albumen during storage, rapidly reducing the intensity of yolk pigmentation, as mentioned by Santos *et al*. (2009). Moreover, the reduction in yolk pigmentation may also be related to lipid oxidation during egg storage, as free radicals may oxidize carotenoids, resulting in the whitish appearance of food items (Woodall *et al.*, 1997).

Experiment II – Storage under refrigeration

No interaction (p>0.05) was observed between trace mineral sources and rosemary oil for the main egg internal quality parameters evaluated (Table 7). Egg yolk malonaldehyde levels constantly increased (p<0.05) with storage period, possibly due to the self-oxidation of lipids by the spontaneous reaction between oxygen and the unsaturated fatty acids present in the yolk (Ramalho & Jorge, 2006).

Egg weight decreased (p<0.01) as storage period increased, with fresh eggs presenting higher values compared with those stored for 15 and 30 days. These results are similar to those determined eggs stored at controlled temperature (Experiment I), demonstrating the negative effect of long storage periods on egg weight.

Table 7 – Malonaldehyde level (MDA), egg weight (EW), Haugh unit (HU), yolk index (YI), albumen and yolk pH, raw yolk color (RYC), and egg weight loss (WL) of eggs laid by brown layers fed different trace mineral sources and rosemary oil levels, and stored for different storage periods (SP) under refrigeration (5.0ºC).

Variables	MDA (mg kg^{-1})	EW (g)	HU	YI (mm)	pH Yolk	WL Albumen	RYC	(%)
(MS)								
Inorganic	0.218	57.93	86.95b	0.411b	6.37	8.63a	5.07	1.73
Organic	0.217	58.28	94.41a	0.443a	6.26	8.37b	5.15	1.80
(RO mg kg^{-1} of feed)								
0	0.226	59.06	88.71b	0.422	6.30	8.52	5.04	1.76
100	0.215	57.28	90.48b	0.428	6.33	8.50	5.10	1.68
200	0.212	57.99	92.84a	0.431	6.32	8.49	5.18	1.87
SP (days)								
Fresh	0.195c	60.90a	101.65a	0.445a	5.88b	7.50b	5.14	0.00c
15	0.215b	57.24b	89.05b	0.418b	6.52a	8.98a	5.14	1.75b
30	0.242a	56.17b	81.34c	0.417b	6.55a	9.03a	5.04	3.56a
Mean	0.217	58.10	90.68	0.427	6.31	8.50	5.11	1.76
SEM[1]	0.00	0.41	0.86	0.00	0.04	0.06	0.03	0.10
Normality*	<0.01	0.512	0.004	<0.01	<0.01	<0.01	<0.01	<0.01
p value								
MS	0.107	0.621	<0.01	<0.01	0.061	<0.01	0.097	0.347
RO	0.888	0.130	<0.01	0.163	0.923	0.797	0.059	0.151
SP	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.146	<0.01
MS x RO	0.423	0.159	0.407	0.507	0.549	0.754	0.218	0.405
MS x SP	0.343	0.471	0.797	0.104	0.875	0.503	0.808	0.420
RO x SP	0.208	0.137	0.386	0.455	0.787	0.790	0.433	0.064
MS x RO x SP	0.452	0.125	0.654	0.442	0.169	0.782	0.330	0.070

[1]Standard error of the mean; Values with $p>0.05$ present normal distribution by the Shapiro-Wilk test

Means followed by different letters in the same column are significantly different at 1% and 5% probability level.

The different trace mineral sources, rosemary oil levels and storage period had isolated effects ($p<0.01$) on Haugh unit. Higher Haugh unit values were determined in the eggs laid by hens fed OTM compared with ITM, and 200 mg kg^{-1} RO compared with 0 and 100 mg kg^{-1} levels; however, HU values were gradually reduced as storage time increased.

Due to their higher bioavailability, organic trace mineral sources may have favored the synthesis of albumen proteins, such as selenocysteine (Pan *et al*., 2010). Furthermore, the dietary inclusion of 200 mg kg^{-1} rosemary oil may have improved the nutrient digestibility and absorption, resulting in greater deposition in the albumen and yolk.

Egg yolk index was influenced ($p<0.01$) by trace mineral source and storage time. Higher egg yolk index ($p<0.01$) was determined in the eggs of OTM-fed hens increased compared with ITM. Yolk index values were reduced from day 0 to day 15 of storage, and remained constant until day 30 (Table 7). Similar results were obtained for albumen pH: lower values obtained in the eggs of OTM-fed hens compared with ITM ($p<0.01$), and increased between days 0 and 15 of storage ($p<0.01$), but was not different between days 15 and 30. These results consistent with those obtained when eggs were stored at controlled temperature (Experiment 1), where the OTM diets promoted an increase in inner egg content and improved albumen quality, hindering gas exchanges.

Yolk pH and egg weight loss were influenced ($p<0.01$) by storage period. As with albumen pH, yolk pH values increased until day 15 of storage, and remained constant until day 30. Egg weight loss increased with storage period.

The constant egg quality loss observed during the storage period is inevitable, especially due to the physical-chemical changes in albumen and yolk. However, they can be less severe when eggs are kept at refrigerated temperatures. This may explain the stabilization of the yolk index and albumen and yolk pH values from day 15 of storage. Oliveira *et al*. (2009) observed greater losses in egg internal when eggs were stored at 25ºC compared with 6ºC for 30 and 50 days, respectively. Similar results were obtained by Figueiredo *et al*. (2011) when storing eggs for up to 15 days at different temperatures (2.6ºC±0.9ºC and 25.6ºC±1.7ºC).

There was no effect (p>0.05) of the evaluated treatments on raw yolk color.

Thus, it is concluded that, in brown layers, the internal quality of eggs stored both at controlled temperature (25.0ºC) and under refrigeration (5.0ºC) is adversely affected by increasing storage periods, but this effect can be minimized by the dietary supplementation of organic trace minerals and 200 mg kg^{-1} rosemary oil. The lipid stability of eggs stored at controlled temperature (25.0ºC) increases with the supplementation of organic trace minerals and 200 mg kg^{-1} rosemary oil, but not of eggs stored under refrigeration.

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