

## Sources and levels of selenium on breast meat quality of broilers

### Fontes e níveis de selênio sobre a qualidade da carne do peito de frangos de corte

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#### ABSTRACT

*Qualitative characteristics of breast meat of broilers fed diets supplemented with different concentrations (0; 0.3 and 0.5mg kg<sup>-1</sup>) of selenium in the form of selenomethionine and sodium selenite were analyzed. A total of 1050 one-day-old male Cobb broiler chicks were arranged factorially at random to five treatments (two concentrations x two sources + control diet without addition of selenium) with 7 replications of thirty birds each and received an isonitrogenous and isocaloric diets in all phases according to their ages (1-21, 22-35, and 36-42 days). At 42 days of age, TBARS (thiobarbituric acid reactive substances) after storage at 4°C for one, seven and 15 days and also after 30 days under freezing temperatures (-15°C), color (CIELab), water holding capacity, cooking loss, shear force, pH and selenium concentration were determined in slaughtered birds breast meat. Results indicated that the use of selenomethionine provides less lightness and lower oxidation in chicken breast meat stored up to 15 days at 4°C. There was a positive effect of dietary different sources and levels of selenium on breast meat quality of broilers. It was observed a linear effect of dietary selenium levels on the amount of selenium deposited in the muscle, and the organic source (selenomethionine) is more effective than inorganic one (sodium selenite) for broiler meat conservation.*

**Key words:** broiler, meat quality, pectoralis major, selenomethionine, sodium selenite.

#### RESUMO

*Analisaram-se características qualitativas da carne do peito de frangos de corte alimentados com rações suplementadas com diferentes concentrações (0; 0,3 e 0,5mg kg<sup>-1</sup>) de selênio nas formas de selenometionina e selenito de sódio. Foram utilizados 1050 pintainhos machos de um dia de idade da linhagem Cobb,*

*que foram distribuídos em delineamento inteiramente casualizado em esquema fatorial em 5 tratamentos (duas concentrações x duas fontes + tratamento controle, sem adição de selênio) e sete repetições de trinta aves cada e receberam rações isoproteicas e isoenergéticas em todas as fases de criação (1 a 21, 22 a 35 e 36 a 42 dias). Aos 42 dias de idade, TBARS (substâncias reativas ao ácido tiobarbitúrico) após armazenamento a 4°C por um, sete e 15 dias e 30 dias sob congelamento (15°C), coloração (CIELab), capacidade de retenção de água, perdas de peso por cocção, força de cisalhamento, pH e concentração de selênio foram determinadas na carne do peito das aves abatidas. Os resultados indicaram que o uso da selenometionina ocasiona queda da oxidação da carne e luminosidade aos 15 dias de armazenamento a 4°C. Existiu um efeito positivo da suplementação da ração com as diferentes fontes e concentrações de selênio sobre a qualidade da carne do peito das aves. Houve efeito linear dos níveis dietéticos de selênio na quantidade de selênio depositado no músculo, e a fonte orgânica (selenometionina) foi mais efetiva que a inorgânica (selenito de sódio) na conservação da carne de frango.*

**Palavras-chave:** frango de corte, qualidade da carne, pectoralis major, selenito de sódio, selenometionina.

#### INTRODUCTION

Selenium (Se) is a trace mineral that performs many functions in the body, it is important for good animal performance and for cell regulation of the anti-oxidant system (CHOCT & NAYLOR, 2004). It is a part of the enzyme glutathione peroxidase (GSH-Px) as selenocysteine, and it is readily ionizable at physiological pH. It protects the cell against free

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radicals in mammalian species that use the oxidative metabolism, and therefore it is crucial for cell survival by reducing hydrogen peroxide and lipid hydroperoxides. This trace element is also involved in biochemical processes such as reproductive and immune functions, and the production of thyroid hormones (KOHRL et al., 2000).

KRSTIĆ et al. (2012), reported that the concentration of selenium in the soil in many parts of the globe is insufficient to ensure the minimal trace element in foods, causing its deficiency in humans and consequently decrease the activity of several selenoenzymes necessary for physiological homeostasis of the organism. CROMWELL et al. (1999) noted that the concentration of selenium in corn and soybean meal of 15 American states, ranged between 0.02 to 0.29mg kg<sup>-1</sup> and 0.08 to 0.95mg kg<sup>-1</sup>, respectively.

CLOSE (1998) and KRSTIĆ et al. (2012) reported that selenium is currently added to the poultry feed at concentrations ranging from 0.15 to 0.5mg kg<sup>-1</sup>. The form of sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>) is usually used as the main source of selenium, followed by selenomethionine (an organic source with greater availability and higher efficiency).

JUNIPER et al. (2011) evaluated the effect of using selenomethionine concentrations of 0.3 and 0.5mg Se/kg diet comparing to 0.3mg Se kg<sup>-1</sup> diet in the form of sodium selenite. These authors analyzed concentration of selenium and glutathione peroxidase activity in meat (chest and leg) and blood of turkeys slaughtered at 84 days of age, as well as meat qualitative traits. They found higher concentrations of selenium and glutathione peroxidase activity in blood and flesh when using selenomethionine, being the effect greater when using 0.5mg kg<sup>-1</sup> of selenium. These authors also found that, despite the higher selenoenzyme glutathione peroxidase activity, there was no significant effect on meat quality. On the other hand, HOOGE (2007) reported a reduction in weight loss by dripping and higher concentrations of selenium in the breast meat of broilers fed diets supplemented with selenomethionine (from 0.1 to 0.3mg kg<sup>-1</sup> of selenium). Therefore, this study was conducted to evaluate the effect of dietary different concentrations and sources of selenium on qualitative traits of breast meat (pectoralis major) in broiler chickens.

## MATERIALS AND METHODS

A total of 1050 one-day-old male Cobb chicks were raised up to 42 days in an experimental open poultry house. Chicks were divided at random into 35 equal groups, and allocated in pens

(2.5mx1.0m). Feed and water were provided *ad libitum*. The experimental diets containing different supplementations of selenium (0; 0.3 and 0.5mg kg<sup>-1</sup> of feed) in the form of selenomethionine and sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>) were produced in a horizontal ribbon mixer with a capacity of 75 to 500kg (Table 1). Experimental diets were formulated to meet or exceed the nutritional requirements for broilers according to the Brazilian Tables for Poultry (ROSTAGNO et al., 2011). The inorganic and organic sources of selenium were sodium selenite and selenomethionine (minimum of 1.000mg of Se kg<sup>-1</sup> of the product), respectively. The premix of vitamins and mineral sources did not contain selenium, and they were pre-diluted and added separately.

At the end of the experiment two birds from each pen (14 birds per each treatment) were slaughtered after eight hours of feed withdrawal in a commercial slaughterhouse. Chicken breasts were deboned approximately four hours after slaughter at *rigor mortis* to promote pH stabilization and to perform the TBARS (TMP mg kg<sup>-1</sup> of sample), determined by quantification of substances reactive to thiobarbituric acid (PIKUL et al., 1989) in meat packaged under vacuum and stored four hours after slaughter (day one); 7 and 15 days in the refrigerator at 4°C; and 30 days in the deep freezer at -15°C. Meat color was determined by the colorimeter Minolta Chrome Meter, adopting to the CIELAB system, which determines L\* (lightness), a\* (redness) and b\* (yellow intensity). Also water holding capacity (%) were measured in approximately 2.0g of each deboned breast according to HAMM (1960). Cooking loss (%) was determined according to HONIKEL (1998). Shear force (kgf cm<sup>-2</sup>) were measured in cooked breast meat and were cut after reaching room temperature into strips of 1.5cm wide, placed with its muscle fibers oriented perpendicularly to the sensing device strip Texture Analyzer by Stable Micro Systems - Godalming, Surrey, United Kingdom (TA-XT2i coupled to the Warner-Bratzler device), according to LYON et al. (1998). The pH was measured in triplicate samples by direct introduction of the pH electrode in the muscle pectoralis major. Selenium concentration in the meat (µg kg<sup>-1</sup> of dry matter) was determined by atomic absorption spectrophotometry using the technique of hydride generation and oxidation of organic matter (VIDAL, 1984). The experimental design was completely randomized with five treatments arranged factorially (2x2+1) as two concentrations x two sources of selenium + control diet without selenium supplementation (7 replicates each of 30 birds). After verifying the homogeneity of variance by Bartlett's

Table 1 - Ingredient and nutrient composition of the basal diets.

Ingredients (%)	Starter 1 -21 days	Grower 22-35 days	Finisher 36-42 days
Corn	57.20	63.99	65.24
Soybean meal, 45% CP	36.94	30.26	27.86
Soybean oil	1.81	2.47	3.87
Dicalcium phosphate	1.83	1.63	1.38
Limestone	1.30	0.85	0.95
Salt (sodium chloride)	0.30	0.30	0.30
Vit. and min. premix *	0.50	0.40	0.30
DL-methionine (98%)	0.12	0.10	0.10
Calculated composition			
Crude protein (%)	21.50	19.00	18.00
Met. energy (kcal kg <sup>-1</sup> )	3000	3130	3235
Available phosphorus (%)	0.45	0.40	0.35
Calcium (%)	0.95	0.84	0.80
Methionine + cystine (%)	0.85	0.78	0.75
Methionine (%)	0.50	0.46	0.45
Lysine (%)	1.20	1.06	1.00
Analyzed selenium (mg kg <sup>-1</sup> )**	0.020	0.019	0.011

\*Product composition (kg): vit. A 7.000.000IU, vit. D3 4.000.000IU, vit. E 5000mg, vit. K 1200mg, vit. B1 360mg, vit. B2 2000mg, vit. B6 700mg, vit. B12 7000mcg, niacin 7500mg, biotin 30mg, pantothenic acid 6000mg, folic acid 300mg, choline 200mg, Fe 11.000mg, Cu 3000mg, Mn 18.000mg, Zn 12.000mg, I 240mg, Mg 50g, S 40g, antioxidant 1000mg, vehicle (q.s.p.) 1000g. \*\* According to VIDAL (1984).

test, averages were compared by Tukey test at 5% significance level. The F test was also performed to determine the orthogonal contrasts among treatments and to estimate the linear effect of selenium levels.

## RESULTS AND DISCUSSION

An interaction between sources and concentrations of selenium ( $P < 0.05$ ) on the 15<sup>th</sup> day of meat storage was observed (Table 2) for the values of thiobarbituric acid reactive substances (TBARS). Breast meat from birds fed diets without selenium supplementation showed similar oxidation levels to those fed diets containing the mineral in all periods of storage ( $P > 0.05$ ). These results indicated that selenium concentrations equivalent to 0.020, 0.019, and 0.011 mg kg<sup>-1</sup> at the initial, growth, and final phases, respectively, in the control group were sufficient to maintain the concentration of TBARS in compare to treated groups. RONEUS and LINDHOLM (1983) reported that a concentration of selenium around 0.1 mg kg<sup>-1</sup> diet is needed to maintain the normal activity of the enzyme glutathione peroxidase. This value is higher than those found in samples from the control group in this study.

The orthogonal contrast test showed that the meat oxidation on breasts from birds fed diets supplemented with 0.3 mg of Se kg<sup>-1</sup> diet in the form of sodium selenite was significantly higher than in the

control group at 7 days of storage (0.885 vs 0.471 mg TMP kg<sup>-1</sup>, respectively). This result is not in agreement with the literature, because instead of been minimized, the meat oxidation increased when the inorganic source of selenium was tested.

There was a positive effect on the prevention of oxidation by the organic source, providing on the 7<sup>th</sup> day of storage significant decrease ( $P < 0.05$ ) in TBARS values in compare to breasts of birds fed diets supplemented with sodium selenite, which probably indicates that the organic source of selenium improved the action of the glutathione peroxidase in the process of detoxification of peroxides. At the 1<sup>st</sup> and 15<sup>th</sup> days of storage, there was no difference ( $P > 0.05$ ) for oxidation of the meat of birds fed with different sources of selenium, and such a result can be explained by the short period of exposure to oxidizing agents and low storage temperature (-15°C) at different times, respectively.

There was no significant difference between the two concentrations of selenium evaluated regarding TBARS results. These findings are in disagreement with those reported by HOOGE (2007), who studied the effect of different concentrations (0, 0.15, and 0.30 mg kg<sup>-1</sup>) and sources of selenium (selenomethionine and sodium selenite) on the concentration of TBARS in breast chicken meat. This author reported that broilers fed diets without selenium supplementation had high levels of

Table 2 - Effect of dietary different sources and concentrations of selenium on thiobarbituric acid reactive substances - TBARS (mg TMP kg<sup>-1</sup>) of raw meat samples from broilers slaughtered at 42 days of age and stored in different conditions for different periods.

Item	1 Day (4°C)	7 Days (4°C)	15 Days 4°C)	30 Days (-15°C)
Control vs all others				
Control	0.12	0.47	1.77	0.25
Others	0.11	0.46	1.52	0.28
LSD	0.03	0.22	0.24	0.05
Selenium Source (S)				
Sodium selenite (SS)	0.10	0.94 a	1.73	0.29
Selenomethionine (MS)	0.12	0.59 b	1.30	0.28
LSD	0.03	0.20	0.24	0.05
Selenium Concentration (C)				
0.3mg kg <sup>-1</sup>	0.11	0.71	1.53	0.28
0.5mg kg <sup>-1</sup>	0.11	0.82	1.51	0.29
LSD	0.03	0.20	0.22	0.05
CV (%)	31.07	36.22	18.14	22.73
General model				
Contrasts (F test)				
Control vs all others	0.29 <sup>NS</sup>	0.57 <sup>NS</sup>	1.22 <sup>NS</sup>	1.66 <sup>NS</sup>
Selenium source (S)	1.44 <sup>NS</sup>	12.43 *	16.30 *	0.22 <sup>NS</sup>
Selenium concentration (C)	0.28 <sup>NS</sup>	1.29 <sup>NS</sup>	0.02 <sup>NS</sup>	0.44 <sup>NS</sup>
S x C	0.37 <sup>NS</sup>	0.001 <sup>NS</sup>	6.55 *	0.001 <sup>NS</sup>
Cont. vs SS (0.3mg kg <sup>-1</sup> )	0.06 <sup>NS</sup>	10.63*	1.28 <sup>NS</sup>	2.61 <sup>NS</sup>
Cont. vs SS (0.5mg kg <sup>-1</sup> )	1.20 <sup>NS</sup>	0.25 <sup>NS</sup>	2.22 <sup>NS</sup>	0.04 <sup>NS</sup>
Cont. vs MS (0.3mg kg <sup>-1</sup> )	5.45 <sup>NS</sup>	3.37 <sup>NS</sup>	0.10 <sup>NS</sup>	2.33 <sup>NS</sup>
Cont. vs MS (0.5mg kg <sup>-1</sup> )	1.54 <sup>NS</sup>	4.16 <sup>NS</sup>	1.99 <sup>NS</sup>	1.59 <sup>NS</sup>
-----Breakdown of the interaction between S X C - 15 days-----				
C (mg/kg)	-----S-----		P value	
	Sodium selenite	Selenomethionine		
0.3	1.87 A	1.16 B	< 0.001	
0.5	1.59	1.43	0.83	
P value	0.33	0.44		

Means followed by different lowercase (columns) or uppercase (rows) letters indicated significant differences by Tukey test at 5% significance level. NS = Not significant. \*(P<0.05). CV = Coefficient of variation. LSD = least significant difference.

lipid oxidation in meat (P<0.05); the inorganic source was only effective when given at concentrations of 0.15mg kg<sup>-1</sup> and that TBARS values were similar to the control group when birds received diets supplemented with 0.30mg kg<sup>-1</sup> of selenium. In the present study, no difference for lipid oxidation of the stored meat (15 days) were noted between the different concentrations of both sources (Table 2). The organic source was significantly (P<0,05) better than the inorganic ones in preventing oxidation of the meat when broilers fed diets containing 0.3mg of selenium per kg of feed, but this effect was not observed in the second concentration, whereas no difference (P>0.05) between the two sources were observed. The way of absorption of the dietary different selenium sources may be the explanation for this result, probably the concentration of 0.5mg Se kg<sup>-1</sup> diet exceeds the

necessities of selenium to improve de glutathione peroxidase activity when offered in the organic form.

Table 3 presents the values for meat color. No significant interaction between the factors in any of the parameters evaluated was observed (Table 3). A significant decrease in brightness was noted when organic source of selenium was used. These results are consistent with MAHAN et al. (1999), who reported a reduction in pig meat brightness in response to dietary organic selenium comparing to dietary inorganic selenium. These authors attributed this result to the relation between muscle light and water holding capacity, as the inorganic source causes greater water loss and consequently, better muscle brightness. This explanation does not apply in this study, as there was no significant difference between treatments for water holding capacity (Table 4). Physical differences

Table 3 - Effect of dietary different sources and concentrations of selenium on meat color L (brightness), a \*(redness) and b \*(yellow intensity) of broilers slaughtered at 42 days of age.

Item	L	a*	b*
Control vs all others			
Control	47.64	5.90	3.30
Others	47.48	5.48	3.25
LSD	2.03	0.74	0.95
Selenium Source (S)			
Sodium selenite (SS)	48.78 a	5.51	3.54
Selenomethionine (MS)	46.18 b	5.46	2.96
LSD	2.01	0.70	0.93
Selenium Concentration (C)			
0.3mg kg <sup>-1</sup>	47.57	5.59	3.09
0.5mg kg <sup>-1</sup>	47.39	5.37	3.41
LSD	2.01	0.70	0.93
CV (%)	4.96	15.46	33.74
General model			
Contrasts (F test)			
Control vs all others	0.03 <sup>NS</sup>	1.30 <sup>NS</sup>	0.01 <sup>NS</sup>
Selenium source (S)	8.53 *	0.03 <sup>NS</sup>	1.97 <sup>NS</sup>
Selenium concentration (C)	0.04 <sup>NS</sup>	0.45 <sup>NS</sup>	0.58 <sup>NS</sup>
S x C	0.54 <sup>NS</sup>	0.26 <sup>NS</sup>	0.48 <sup>NS</sup>
Cont. vs SS (0.3mg kg <sup>-1</sup> )	0.66 <sup>NS</sup>	0.11 <sup>NS</sup>	0.16 <sup>NS</sup>
Cont. vs SS (0.5mg kg <sup>-1</sup> )	0.71 <sup>NS</sup>	0.85 <sup>NS</sup>	0.15 <sup>NS</sup>
Cont. vs MS (0.3mg kg <sup>-1</sup> )	0.66 <sup>NS</sup>	0.52 <sup>NS</sup>	1.60 <sup>NS</sup>
Cont. vs MS (0.5mg kg <sup>-1</sup> )	1.69 <sup>NS</sup>	3.25 <sup>NS</sup>	0.001 <sup>NS</sup>

In the same column, means followed by different letters indicated significant differences by Tukey test at 5% significance level. NS = Not significant. \*(P<0.05). CV = Coefficient of variation. LSD = least significant difference.

between broiler and pig meat can be an explanation for this result. According to LENGGERKEN et al. (2002) the pig meat is composed of red (13%), intermediate (17%) and white (70%) fibers. In contrast, fibers of the Pectoralis muscle from chickens are almost exclusively white.

Red and yellow intensities in broiler meat were not affected (P>0.05) by dietary different selenium sources (Table 3), which disagreement of what was reported by CAO et al. (2001), who found significant differences in these parameters with increasing intensity of red and decreasing intensity of yellow of broiler breasts that fed diets containing organic source of the trace mineral. In the same experiment, these authors found that such rise could also increase red intensity. In addition, they suggested that this increased intensity of red can be a result of reduced fat and myoglobin oxidation.

There was no effect (P>0.05) of selenium supplementation on the water holding capacity, cooking loss, shear strength and pH. However, an interaction (P<0.05) between sources and concentrations of selenium was observed for shear force. A larger pH value

(P<0.05) of the meat was found when selenomethionine was used, probably due to a depletion in the synthesis of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), that is catalyzed by enzyme glutathione peroxidase in H<sub>2</sub>O and O<sub>2</sub>.

A higher concentration of selenium was observed in the meat of broilers fed diets supplemented with selenium, being more effective in the meat of the birds that received the organic source (Table 4). This result is consistent with that obtained by PAYNE and SOUTHERN (2005), who noted a significant increase in the deposition of selenium in the muscle according to higher concentration of organic and inorganic selenium in broiler diets. These results show that both sources respond to the increase of selenium concentration, but the inorganic form is less efficient than the organic ones. These findings may be explained by the presence of selenium in the biochemical structure of selenomethionine. Thus, it is used by the body more efficiently, being absorbed as an amino acid.

The organic source caused lower shear force (P<0.05) when offered at a concentration of 0.5mg Se kg<sup>-1</sup> diet (Table 4). This result could be

Table 4 - Effect of dietary different sources and concentrations of selenium on water holding capacity (WHC), cooking loss (CL), shear force (SF), pH and concentration of selenium in meat of broilers slaughtered at 42 days of age.

Item	WHC (%)	CL (%)	SF (kgf cm <sup>2</sup> )	pH	Selenium (µg kg <sup>-1</sup> )
Control vs all others					
Control	69.11	11.91	1544	5.94	17.21 b
Others	67.53	11.32	1670	5.98	81.97 a
LSD	3.27	2.62	421.06	0.11	12.70
Selenium Source (S)					
Sodium selenite (SS)	67.54	11.44	1649	5.93 b	37.54 b
Selenomethionine (MS)	67.52	11.20	1690	6.04 a	95.21 a
LSD	3.19	2.54	415.10	0.10	14.85
Selenium Concentration (C)					
0.3mg kg <sup>-1</sup>	67.69	12.35	1843	5.96	41.08 b
0.5mg kg <sup>-1</sup>	67.37	10.30	1497	6.01	81.12 a
LSD	3.19	2.54	415.10	0.10	10.54
CV (%)	5.58	26.52	29.65	2.16	21.10
General model					
Contrasts (F test)					
Control vs all others	0.97 <sup>NS</sup>	0.21 <sup>NS</sup>	0.37 <sup>NS</sup>	0.56 <sup>NS</sup>	110.70**
Selenium source (S)	0.001 <sup>NS</sup>	0.04 <sup>NS</sup>	0.05 <sup>NS</sup>	5.38 *	148.12**
Selenium concentration (C)	0.05 <sup>NS</sup>	3.18 <sup>NS</sup>	3.53 <sup>NS</sup>	1.03 <sup>NS</sup>	83.24**
S x C	1.40 <sup>NS</sup>	1.64 <sup>NS</sup>	5.54 *	0.04 <sup>NS</sup>	1.11 <sup>NS</sup>
Cont. vs SS (0.3mg/kg <sup>-1</sup> )	1.02 <sup>NS</sup>	0.35 <sup>NS</sup>	0.12 <sup>NS</sup>	0.22 <sup>NS</sup>	171.45**
Cont. vs SS (0.5mg kg <sup>-1</sup> )	0.17 <sup>NS</sup>	1.25 <sup>NS</sup>	0.32 <sup>NS</sup>	0.01 <sup>NS</sup>	152.51**
Cont. vs MS (0.3mg kg <sup>-1</sup> )	0.13 <sup>NS</sup>	0.10 <sup>NS</sup>	3.16 <sup>NS</sup>	0.56 <sup>NS</sup>	166.56**
Cont. vs MS (0.5mg kg <sup>-1</sup> )	1.50 <sup>NS</sup>	0.39 <sup>NS</sup>	2.03 <sup>NS</sup>	1.64 <sup>NS</sup>	151.85**
-----Breakdown of the interaction between S X C for SF-----					
C (mg kg <sup>-1</sup> )	-----S-----				P value
	Sodium selenite	Selenomethionina			
0.3	1606	2080 a			0.381
0.5	1693	1300 b			0.566
P value	0.997	0.041			

In the same column, means followed by different letters indicate significant differences by Tukey test at 5% significance level. NS = Not significant. \* (P<0.05). \*\* (P<0.01) CV = coefficient of variation. LSD = least significant difference.

related to lower muscle brightness observed when the organic source was used (Table 3). Under high brightness, the cell fluid loss is high in the muscle surface, thereby reducing succulence and increasing the shear strength. However, there were no differences between treatments for water holding capacity, a parameter which is closely related to muscle cell integrity. There was no significant difference in shear force regardless the source or the concentration of selenium used.

## CONCLUSION

The use of selenomethionine provided less lightness and lower oxidation in chicken breast meat stored up to 15 days at 4°C. There was a positive

effect of dietary different sources and levels of selenium on breast meat quality of broilers. It was observed a linear effect of dietary selenium levels on the amount of selenium deposited in the muscle, and the organic source (selenomethionine) is more effective than inorganic one (sodium selenite) for broiler meat conservation.

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## ETHICAL STANDARDS

The experimental protocol number 002134 / 2012 were approved by the Animal Ethics Committee of Agriculture and Veterinary Sciences College - FCAV/UNESP (Jaboticabal, São Paulo, Brazil), also were in accordance with the guidelines on animal welfare.

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