TITLE: Intensity of lipid oxidation and cholesterol oxidation products formation during frozen storage of raw and cooked chicken.

RUNNING TITLE: COP in raw and cooked frozen chicken.

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ABSTRACT

Raw and cooked breast chicken were stored at -18ºC during 3 months under aerobic and vacuum conditions and the intensity of the lipid oxidation process and COP (cholesterol oxidation products) formation were studied. Raw samples showed low COP levels (7.40-4.60 μg/g fat), TBARS (0.01-0.03 ppm) and peroxide values (not detected), both at aerobic and vacuum conditions. Cooked samples (grilled and roasted) showed TBARS (thiobarbituric acid reactive substances) levels around 0.36-0.99 ppm in aerobic conditions and around 0.21-0.7 ppm in vacuum conditions, and peroxides raised 38-40 and 19-23 meq O_2/kg sample in samples stored under aerobic and vacuum conditions, respectively. Total COP levels were 28.91 and 39.34 μg/g fat in grilled and roasted samples in aerobic packaging and 4.90 and 20.24 μg/g fat in vacuum packaging, respectively. Significant correlations were found between the lipid oxidation parameters and cholesterol oxidation indices. In general TBARS was better correlated with total COP than with only 7-ketocholesterol. Vacuum packaging was especially efficient to slow down the oxidation process during the frozen storage of cooked samples.

Keywords: meat, TBARS, oxysterol, freezing, vacuum packaging.

INTRODUCTION

Food storage in frozen conditions has demonstrated to be a really good technology to extend the shelf life of products with a minimal effect on the sensorial quality. The main deleterious process that takes place during frozen storage is lipid oxidation. Oxidative reactions still occur in the lipid fraction of frozen foodstuffs, although at a slower rate than at refrigeration or room temperature and tend to increase with high oxygen permeability of packaging material and higher fat content in meat [1, 2, 3] .These oxidative reactions are initiated in the highly susceptible membrane-bound phospholipids, which contain relatively large amounts of polyunsaturated fatty acids[4]. Effectively, this process affects particularly unsaturated lipids, being poultry meat one of the most susceptible species to suffer it because, according to food composition tables, the ratio between unsaturated and saturated fatty acids in chicken (1.81) is higher than in other meats: pork (1.32), beef (1.23) or mutton (0.87) [5].

An excessive oxidation of muscle lipids can produce compounds that adversely affect the quality of meat. These compounds are in some cases associated to off-flavours and off-odours, loss of color, vitamins, lower consumer acceptability and also affect the safety of meat [6] .Pikul et al.[7] working with raw breast chicken, found that TBARS (thiobarbituric acid reactive substances) values increased continuously during frozen storage finding values, after 6 months at -18ºC, 4.1-4.9 folds higher than in fresh muscle. High correlation coefficients were found between TBA and peroxide values in raw and cooked pork patties during frozen storage [8].

In other researches also the effect of anaerobic conditions during frozen storage of food has been studied. Hernández et al.[9] studying the consequences of 6 month frozen storage in vacuum packed conditions on the lipid fraction of the *longissimus dorsi*

muscle of pigs found that the TBARS showed a slight increase during the process, whereas the peroxide value and the cholesterol concentration remained constant.

Cholesterol is an unsaturated lipid susceptible to oxidation giving rise to COP (cholesterol oxidation products). Some authors have stated that the presence of a high concentration of polyunsaturated fatty acids easier enhances cholesterol oxidation susceptibility [10, 11,12] .Some of the COP are considered as transport forms of cholesterol in catabolic pathways, and they are also important as regulators of cholesterol homeostasis[13] .Many toxicological effects of COP have been reported such as: cytotoxicity, atherogenesis, mutagenesis, carcinogenesis, changes in cellular membrane properties and inhibition of 3-hydroxy-3-methylglutaril coenzyme A reductase activity [14].

There is not yet clear evidence supporting the direct implication of COP in the etiology of atherosclerosis, although it is being extensively investigated during the last years [15, 16, 17] . Leonarduzzi et al.[18] in a review about the proatherosclerotic effects of COP established that through the uptake of modified LDL, an increasing number of potentially toxic compounds are departed behind the endothelial wall, where they may exert toxicity, once properly concentrated and further metabolized. Those authors concluded that COP appear suitable candidate molecules for this kind of process. Iuliano et al.[19] studying the COP as markers of oxidant stress *in vivo* found in atherosclerotic plaque a COP level (sum of 7β-hydroxycholesterol and 7-ketocholesterol) 45 times higher than in normal vessels. Furthermore, it has been proved that COP present in the diet are absorbed, although some discrepancies exist regarding to the absorption rate[18] .Consequently, taking into account all these proved and potential health effects of COP it seems to be interesting to control their presence in the diet. There are many studies about COP levels in food products, mostly in dairy, egg, meat

and fish products and some of them show that the amount of COP in food was affected by time storage and culinary treatments [20, 21, 22, 23, 24, 25, 26,27.]

A previous work was carried out in which the oxidation process of raw, grilled and roasted chicken stored under refrigeration (6 days) was evaluated[21]. In order to complete that study samples from the same batches were immediately frozen and stored during 3 months at –18ºC. So, the objective of this paper was to evaluate the oxidation process undergone by raw chicken and chicken cooked with different technologies, stored in frozen conditions under aerobic and vacuum packaging.

EXPERIMENTAL

Sample preparation

Chicken breasts sliced 1.5cm thick were purchased from a local supermarket, where they were kept between 4-6ºC before being sold, and randomly separated into three batches: one for analyzing in raw, and the other two for analyzing after grilling and roasting, respectively. Grilling: samples were grilled in a 25cm diameter pan at 180ºC during 1.5minutes each side (internal temperature ranged between 85-90ºC) with 9 ml of sunflower oil, previously added to the pan. Roasting: after preheating the oven at 220ºC during 20minutes, samples were introduced and held at this temperature during 10minutes each side (internal temperature ranged between 95-100ºC). Internal temperature of samples was measured with a digital thermometer (51 J/K RS 614-299, Fluke, USA).

A portion of raw, grilled and roasted samples was analyzed immediately for TBA and peroxides. Other portion was divided in two groups: a) samples were put into a plastic bag in aerobic conditions (aerobic), b) samples were vacuum packed (The vacuum sealer used was Model VP-1000, Ramon, Barcelona, Spain). All bags were of polyamide/polyethylene 90 μm, Corsan, Pamplona, Spain). After 3 months at –18ºC analysis was carried out.

Chemical analysis

Moisture analysis was carried out according to the AOAC method [28]. Cholesterol analysis was done according to Kovacs et al.[29]. Fat content was obtained according to ISO-1443 [30].

TBARS value were determined according to Tarladgis et al.[31] with modifications by Zisper et al.[32], Tarladgis et al.[33], Pikul et al.[8]. Results are shown in mg malonaldehyde/Kg sample (ppm).

Extraction of lipids was made with a mixture of chloroform/methanol according to the method of Folch et al.[34]. Peroxide index values were determined using the official method of AOAC [35].

COP. Extraction of lipids and saponification.

Total lipid was extracted using chloroform/methanol (2:1,v/v) according to Folch et al. [34] procedure. Saponification, purification and derivatization of COP were made according to the method of Guardiola et al.[35]. Approximately 1g of fat was added to a flask containing 10ml of 1M KOH in methanol and 1mL of internal standard (19-hydroxycholesterol, at a concentration of 20µg/ml) and kept at room temperature during 20h to complete the cold saponification. The unsaponificable material was extracted with diethyl ether. The whole organic extract was washed with water and filtered through anhydrous sodium sulphate. Then it was recovered in a round-bottom flask, and the solvent was evaporated using a rotatory vacuum evaporator at 30ºC. A purification was made with silica cartridges (Sep-Pack Vac 6cc SPE, Waters, Millpore, Beldford MA, USA) using different proportions of hexane/diethyl ether and finally COP were recovered with acetone/methanol (60:20, v/v). A derivatization to obtain the trimethyl silyl ethers of COP was performed. Cholesterol oxides were identified and quantified by a Hewlett-Packard 6980 GC coupled to a 5973 Mass selective Detector (Wilmington, Delaware, USA).

USA). A HP-5MS column (30m X 250µm X 0.25 µm) was used and helium was the carrier gas (1ml/minute). The chromatographic conditions were as follows: initial column temperature at 80ºC, held for 1 min and programmed to 250ºC at a rate of 10ºC/min and final column temperature of 280ºC at a rate of 4ºC/min and held for 20 min. The injector temperature was 250ºC and the inlet pressure was 23.2 psig; mass range, m/z=50/550 amu (atomic mass units); solvent delay is 20 minutes. Calibration

curves were developed for seven cholesterol oxides: 7α-hydroxycholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol 7-ketocholesterol, and cholestanetriol. 19-hydroxycholesterol was used as the internal standard (IS). All these standards were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA), except for 7α-hydroxycholesterol and 19-hydroxycholesterol, purchased from Steradoids (Steraloids INC, Newport, USA). Different concentrations (80, 40, 20, 10, 5 and 2.5μg/ml) of mixtures of all the standards were analyzed in the TIC (total ion chromatogram) mode. The identification of the COP in the sample was made by checking their retention time and their mass spectra (using the HPCHEM Wiley 275 6th Edition library) with those of the standard compounds.

Statistical analysis

Four samples were analyzed from each of the different technologies used and storage conditions tested. Each parameter was determined four times in each sample.

A two way ANOVA was carried out in order to study the influence of the culinary process and the storage conditions simultaneously. Significant interactions between the two factors were detected, so an one-way analysis of variance (ANOVA) with a posteriori Tukey b test was carried out for two variables separately in order to analyze statistical differences between samples ($P \leq 0.05$). A student t test was done to analyze the statistical differences between samples in different storage conditions.

Pearson correlation test was applied to study the relationship between oxidation parameters (TBARS and peroxide value) and 7-ketocholesterol and total cholesterol oxides (total COP). Software used was SPSS 9.0 for Windows (SPSS Inc., Chicago, Ill.)

RESULTS AND DISCUSSION

The effect of cooking was detected in every analyzed sample, which showed lower moisture levels and the corresponding higher fat content. Some significant differences were found between aerobic and vacuum samples for fat and cholesterol in raw samples and for moisture and cholesterol in grilled samples. These differences are probably more related to the heterogeneity of the analyzed samples than with the variables under study.

Every analyzed cholesterol oxidation product was detected in all samples (table 2). β-epoxycholesterol was the most abundant COP in raw samples and in grilled vacuum stored samples and 7-ketocholesterol was the most abundant one in the rest of samples. Baggio et al.[36] found values of 7-ketocholesterol around 33 μg/100g in breast turkey raw samples after 16 months of frozen storage. Pie et al.[26] pointed out that the secondary oxysterols (epoxydes and cholestanetriol) showed the highest increase during frozen storage of cooked meats. In this work cholestanetriol and also 25-hydroxycholesterol, which are considered the most cytotoxic, were the less abundant in cooked samples. Amounts of 7α-hydroxycholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol and β-epoxycholesterol were lower in vacuum stored samples than in aerobically stored conditions in every sample, probably due to the lack of oxygen under vacuum, which protected cholesterol from oxidation . No differences were found for cholestanetriol in roasted samples and for 7-ketocholesterol in raw samples. Some authors have found reductions in COP levels during storage of different foods. Galvin et al.[38] analyzing the effect of dietary vitamin E supplementation on cholesterol oxidation in vacuum packaged cooked beef steaks observed that 7-ketocholesterol decreased both in some refrigerated and frozen samples. Wahle et al.[39] also reported decreases of 7-ketocholesterol levels in powdered whole egg stored for up to 18 months. Rodriguez-

Estrada et al.[40] stated that decreases in COP could be caused by their destruction or their reaction with other molecules. Total COP were 1.60, 5.90 and 1.94 folds higher in aerobic storage than in vacuum stored raw, grilled and roasted samples, respectively. In order to minimize the differences found for fat and cholesterol amounts between samples, data obtained for COP were expressed also as percentage of cholesterol oxidation. The efficiency of vacuum conditions to reduce the cholesterol oxidation during frozen storage was higher for cooked than for raw samples. Cooked samples aerobically stored showed the highest COP amounts, especially roasted samples. This fact indicates that cooking enhances cholesterol oxidation during storage.

Raw samples did not show detectable amounts of peroxides, primary oxidation products, whatever the storage conditions applied (fig 1). TBARS values (fig 2) were very low in all the analyzed raw samples (0.01-0.03 ppm) compared to values obtained in other raw meats by other authors. Novelli et al.[41] found TBARS values around 0.22-0.40 ppm for different raw meats stored at –20ºC during 3 months. All these results show that the oxidation process was not intense in the raw samples during frozen storage.

As it was expected these oxidation parameters showed higher values in frozen cooked samples. Rhee et al.[42] analyzing the lipid oxidation intensity of beef, chicken and pork samples stored raw and after cooking at 4ºC and –20ºC found that TBARS values of frozen raw samples were higher for beef and pork than for chicken. However, after cooking chicken samples showed the highest TBARS values. Those authors pointed out that this fact was due to the higher lipid oxidation potential of cooked chicken than cooked beef and pork samples. In our work peroxides raised 38-40 and 19-23 meq O_2/kg sample in samples stored under aerobic and vacuum conditions, respectively. Greater differences were found between cooked samples for TBARS, although samples

under vacuum conditions (0.21-0.7 ppm) always showed significantly lower values in relation to aerobic conditions (0.36-0.99 ppm). Kowale et al.[43] observed that the frozen storage (-10ºC) for 90 days of cooked mutton samples slightly increased TBARS. These authors found values of TBARS between 0.39-0.60 ppm for broiled and pressure cooked samples at 90 days of storage and values of 0.20-0.28 at 30 days of storage.

Data corresponding to freshly purchased raw samples and immediately cooked chicken (t=0) have also been included in those figures to be compared to frozen samples(t=3months). Raw samples did not show detectable amounts of peroxides neither before nor after freezing. TBARS values of raw frozen samples both at aerobic and vacuum conditions were low and similar to that obtained at t=0. Although samples under vacuum conditions showed even lower values than fresh raw samples. As it can be seen in figures 1 and 2 respectively, immediately cooked chicken showed levels that ranged between 0 and 4.33 meq O_2/kg sample for peroxides and 0.21 to 0.24 ppm for TBARS. Comparing these data with those obtained in frozen cooked samples, it can be observed that peroxides increased significantly during frozen storage especially under aerobic conditions in both grilled and roasted samples. In relation with TBARS similar results were found for roasted samples with significant increases especially under aerobic conditions. TBARS in grilled samples only showed significant increases in aerobic conditions. Differences between grilled and roasted samples for each storage condition were more evident for TBA than for peroxide value. It has to be pointed out that the high amounts of peroxides found for both culinary treatments at 3 months of storage made difficult their interpretation. These data clearly confirmed data obtained for COP, indicating that cooked samples show a significant degree of oxidation during their frozen storage, especially in aerobic conditions.

Table 3 shows the values obtained for the Pearsons´ coefficients of correlation between lipid oxidation parameters (TBARS, peroxides) and cholesterol oxidation intensity indices (COP, 7-ketocholesterol). The study was carried out including all the analyzed samples together (total) and also with groups of different samples: raw (aerobic and vacuum raw samples), cooked (aerobic and vacuum cooked samples), aerobic (raw and cooked samples under aerobic conditions) and vacuum (raw and cooked samples under vacuum conditions). 7-ketocholesterol has been used in many occasions, as shown in some of the cited references as an index of the intensity of cholesterol oxidation [39, 40]. So it could be interesting to study also the potential correlations between this compound and the lipid oxidation parameters. Considering all the analyzed samples, significant correlations were found between the lipid oxidation parameters and cholesterol oxidation indices, being the highest the correlation between peroxides and 7-ketocholesterol (0.919). Grau et al.[44] found significant linear correlation between COP content and TBARS values only in raw chicken meat, but not in cooked samples stored at -20ºC for 7 months**.** In this work frozen raw samples did not showed significant correlation between 7-ketocholesterol and TBARS, whereas COP was highly correlated with TBARS. Except for vacuum stored samples, the others showed higher correlations between TBARS and COP than those between TBARS and 7-ketocholesterol. However, peroxides showed in every group higher correlations with 7-ketocholesterol than with COP. This could be explained because 7-ketocholesterol is a primary COP and peroxides are also primary lipid oxidation products. Probably for this reason peroxides were also better correlated to 7-ketocholesterol than to TBARS, except for vacuum stored samples.

CONCLUSIONS

In summary, vacuum packing is an efficient way to decrease the COP formation in raw and specially cooked breast chicken during frozen storage. High correlations were found between lipid and cholesterol oxidation indices, especially between TBARS and total COP and between peroxides and 7-ketocholesterol, indicating that the measure of the intensity of lipid oxidation in foods from animal origin is also a reflect of the COP formation in this type of food.

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Table 1. Moisture, fat and cholesterol content in raw, grilled and roasted samples of chicken breast frozen under aerobic conditions or under vacuum. Values are the mean and the standard deviation, between brackets.

Culinary process	**Raw**			**Grilled**			**Roasted**		
Packaging	**Aerobic**	**Vacuum**	**LS**	**Aerobic**	**Vacuum**	**LS**	**Aerobic**	**Vacuum**	**LS**
Moisture (%)	73.34^{c} (0.9)	73.85^{2} (0.48)	0.362 n.s.	65.30^{b} (1.30)	61.83^{1} (2.01)	0.027 *	59.77^{a} (0.64)	59.71^{1} (0.28)	0.885 n.s.
Fat (%)	1.25^{a} (0.02)	1.50^{1} (0.13)	0.009 **	3.03^{c} (0.20)	2.85^{2} (0.07)	0.137 n.s.	2.36^{b} (0.12)	2.45^{2} (0.15)	0.363 n.s.
Cholesterol (mg/100g)	78.93^{b} (1.46)	83.03^{2} (0.89)	0.003 **	61.59^{a} (2.82)	75.20^{1} (3.97)	0.001 **	82.16^{b} (1.40)	80.59^{2} (2.52)	0.317 n.s.

ANOVA test: different letters denote significant differences among samples stored in aerobical conditions and different numbers denote significant differences among vacuum packed samples.

LS: level of significance: n.s. (not significant), $p>0.05$; * $p<0.05$; **$p<0.01$; *** $p<0,001$.

Table 2. Cholesterol oxidation products content (μg/g fat) in raw, grilled and roasted samples of chicken breast frozen under aerobic conditions or under vacuum. Values are the mean and the standard deviation, between brackets.

Culinary process	**Raw**			**Grilled**			**Roasted**		
Packaging	**Aerobic**	**Vacuum**	**LS**	**Aerobic**	**Vacuum**	**LS**	**Aerobic**	**Vacuum**	**LS**
7α-hydroxy cholesterol	1.31^{a} (0.06)	0.20^{1} (0.02)	0.000 ***	2.47^{a} (0.33)	0.27^{1} (0.04)	0.000 ***	6.14^{b} (1.11)	2.78^{2} (0.02)	0.009 **
7β-hydroxy cholesterol	1.49^{a} (0.07)	0.50^{2} (0.03)	0.000 ***	6.32^{b} (0.52)	0.89^{1} (0.06)	0.000 ***	9.46^{c} (1.45)	4.94^{2} (0.41)	0.006 **
β-epoxy cholesterol	2.69^{a} (0.12)	2.40^{2} (0.08)	0.01 *	3.96^{a} (0.86)	1.71^{1} (0.20)	0.011 *	6.67^{b} (1.16)	3.56^{3} (0.35)	0.002 **
α-epoxy cholesterol	0.2^{a} (0.02)	0.25^{1} (0.02)	0.031 *	2.43^{b} (0.30)	0.21^{1} (0.02)	0.001 **	2.52^{b} (0.1)	0.79^{2} (0.08)	0.000 ***
Cholestanetriol	0.92^{c} (0.05)	0.59^{1} (0.02)	0.000 ***	0.79^{b} (0.02)	0.61^{1} (0.01)	0.000 ***	0.71^{a} (0.04;)	0.74^{2} (0.05)	0.423 n.s.
25-hydroxy cholesterol	0.23^{b} (0.03)	0.12^{1} (0.01)	0.002 **	0.35^{c} (0.02)	0.21^{2} (0.02)	0.000 ***	0.14^{a} (0.01)	0.12^{1} (0.01)	0.013 *
7-keto cholesterol	0.55^{a} (0.06)	0.53^{1} (0.09)	0.790 n.s.	12.58^{b} (0.96)	1.00^{2} (0.15)	0.000 ***	13.74^{c} (0.61)	7.33^{3} (0.25)	0.000 ***.
Total COP	7.40	4.60		28.91	4.90		39.34	20.24	
% Oxidation	0.01	0.001		0.14	0.02		0.11	0.06	

ANOVA test: different letters denote significant differences among samples stored in aerobical conditions and different numbers denote significant differences among vacuum packed samples.

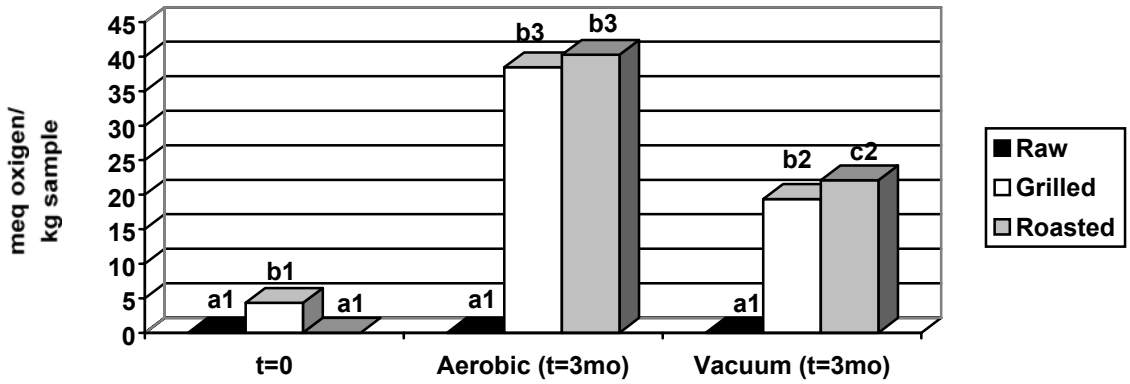
LS: level of significance: n.s. (not significant), $p>0.05$; * $p<0.05$; **$p<0.01$; *** $p<0,001$.
% Oxidation: (mg COP/100 mg cholesterol)

Table 3. Correlations between the lipid oxidation parameters and COP.

R (Pearson correlation)	Raw	Cooked	Aerobic	Vacuum	Total
TBARS/COP	0.924 **	0.781 **	0.912 **	0.255 n.s.	0.870 **
TBARS/7-Ketocholesterol	-0.023 n.s.	0.661 **	0.811 **	0.965 **	0.826 **
Peroxides /COP	___	0.894 ***	0.946 ***	0.640 *	0.878 ***
Peroxides /7-Ketocholesterol	___	0.917 ***	0.990 ***	0.760 **	0.919 ***

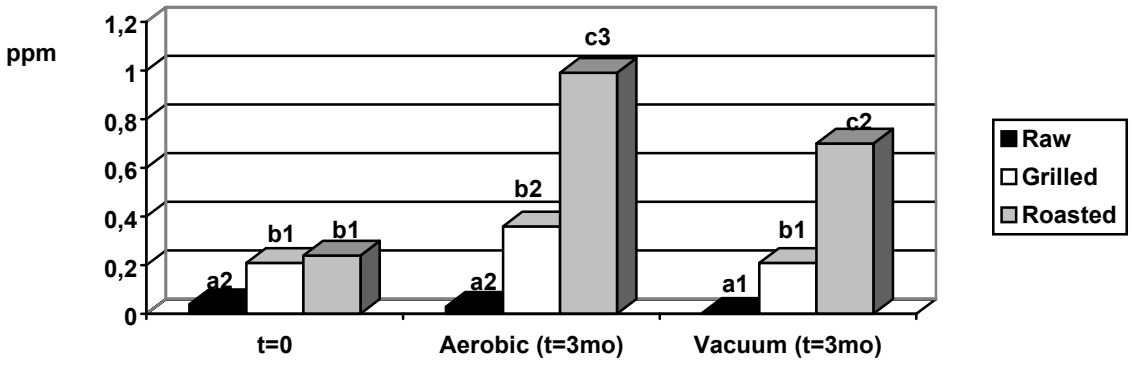
Level of significance: (***): $p<0.001$; (**): $p<0.01$; (*): $p<0.05$; ns: ($p>0.05$) no significant correlation.

Figure 1. Peroxide values (meq oxygen/kg sample) in raw, grilled and roasted samples of chicken breast at t=0 and packed in aerobic conditions or under vacuum.



ANOVA test: different letters denote significant differences ($p<0.05$) among the different culinary processes (raw, grilled and roasted) in each type of storage. Different numbers denote significant differences ($p<0.05$) among the different storage conditions (t=0, aerobic and vacuum) in each type of culinary process.

Figure 2. TBARS results (ppm) in raw, grilled and roasted samples of chicken breast at t=0 and packed in aerobic conditions or under vacuum.



ANOVA test: different letters denote significant differences ($p<0.05$) among the different culinary processes (raw, grilled and roasted) in each type of storage. Different numbers denote significant differences ($p<0.05$) among the different storage conditions (t=0, aerobic and vacuum) in each type of culinary process.