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Effect of high hydrostatic pressure on the oxidation of washed muscle with added chicken hemoglobin

Ricard Bou[a,*], Mar Llauger[a], Rachel Joosse[a,b], José Antonio García-Regueiro[a]

[a] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Food Technology, Monells, Finca Camps i Armet s/n, 17121, Spain
[b] HAS University of Applied Sciences, Onderwijsboulevard 221, 5223 DE's-Hertogenbosch, The Netherlands

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ABSTRACT

The role of chicken hemoglobin in lipid oxidation of washed chicken muscle exposed to high hydrostatic pressure (0, 200, 400 and 600 MPa) was examined. The observed decrease in redness was higher with elevated pressures (5.0 *vs* 3.7). During storage, redness decreased in samples exposed to 400 and 600 MPa. This decrease was concomitant with the progression of oxidation and the 2.5 fold decrease of soluble heme. An additional experiment was conducted to examine the effect of the hemoglobin mode of addition into pressurized muscle. The exposure at 600 MPa led to washed muscle oxidation even in the absence of hemoglobin, thus indicating that pressure treatments triggered lipid oxidation. However, the presence of native or pressurized hemoglobin into pressurized washed muscle caused more hexanal than the pressurized control without hemoglobin. Overall, results suggest that membrane disruption and the release of hemin are crucial for the onset of oxidation.

1. Introduction

The demand for healthy and convenient foods has gained popularity due to busier lifestyles (Geeroms, Verbeke, & Van Kenhove, 2008). In this context, high pressure processing (HPP) is the most successful non-thermal food preservation method that can be applied for the production of a great number of ready-to-eat meals and minimally processed foods (Huang, Wu, Lu, Shyu, & Wang, 2017). This fact explains why this technology continues to grow in popularity across North America, European countries and Asia.

In meat products, the use of HPP can improve texture, water-holding capacity and perception of saltiness (Clariana et al., 2011; Inguglia, Zhang, Tiwari, Kerry, & Burgess, 2017). Therefore, this technology offers interesting possibilities and allows the development of different products, such as those based on poultry meat that are very popular, possess a healthy image, and are commercialized in a great variety of products (Barbut, 2012). Despite this use, HPP is currently mainly used to increase shelf-life and to improve food safety of ready-to-eat products, as a novel post-packaging decontamination technology (Bajovic, Bolumar, & Heinz, 2012). To inactivate microorganisms efficiently, it is necessary to pressurize above 400 MPa. However, at these pressures, the initiation of lipid oxidation seems to be critical and thus represents the main limitation of the meat's shelf-life (Bajovic et al., 2012; Guyon, Meynier, & de Lamballerie, 2016; Ma & Ledward, 2013). The mechanisms by which HPP induces lipid oxidation are not fully understood but membrane disruption and increased accessibility for iron from hemoproteins have been reported to be responsible for the increased susceptibility to oxidation.

The primary hemoproteins in muscle foods are myoglobin (Mb) and hemoglobin (Hb), which are also responsible for the red color of meat and certain fish species. In mammalian species, Mb is the main pigment (Baron & Andersen, 2002; Bou, Hanquet, Codony, Guardiola, & Decker, 2010). In broilers, however, the only extractable pigment in white muscles is Hb, whereas in thigh muscle it accounts for the 86% of the total content in hemoproteins (Kranen et al., 1999). In fish species such as mackerel and trout, Hb accounts for more than 50% and 96% of the total hemoproteins, respectively (Richards, Dettmann, & Grunwald, 2005; Richards & Hultin, 2002). In general, heme compounds act as potent catalysts of lipid oxidation in different raw and cooked muscle systems (Carlsen, Moller, & Skibsted, 2005; Maqsood, Benjakul, & Kamal-Eldin, 2012). However, their pro-oxidant activities differ and, for instance, Hb from fish and poultry promotes oxidation in washed muscles more rapidly than beef Hb (Richards, Modra, & Li, 2002). Ferric bovine Hb (MetHb) promoted lipid oxidation more effectively than ferric bovine Mb (MetMb), and this difference is in part explained by the increased release of hemin (ferric protoporphyrin) from MetHb (Cai, Tatiyaborworntham, Yin, & Richards, 2016; Thiansilakul, Benjakul, Park, & Richards, 2012).

* Corresponding author.
E-mail address: ricard.bou@irta.cat (R. Bou).

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HPP treatments lead to the disruption of non-covalent interactions and result in unfolding of the protein, which facilitates the formation of new intra- and inter-molecular bonds. These changes may cause protein coagulation, aggregation or gelation (Grossi et al., 2016; Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). Hb is a tetramer and thus expected to be particularly sensitive to pressure because the quaternary structure of proteins is mainly held by hydrophobic interactions (Rastogi et al., 2007). In fact, its exposure to different pressurization treatments has been reported to cause spectral shifts as in other heme proteins (Gibson & Carey, 1977). The occurrence of such changes may explain the enhanced enzymatic hydrolysis of Hb by trypsin when the former has been submitted to HPP (Toldrà, Parés, Saguer, & Carretero, 2011). However, the color of red blood cells has been reported to be relatively stable after treatment at 400 MPa (Toldrà, Elias, Parés, Saguer, & Carretero, 2004). Conversely, Mb denatures and turns brown at pressures of 400 MPa and above (Bajovic et al., 2012). Accordingly, those changes in the reddish color of pressurized meat products have been related to the denaturation of the globin, the modification or disruption of the porphyrin ring and the heme moiety redox chemistry (Bolumar, Andersen, & Orlien, 2014; Carlez, Veciana-Nogues, & Cheftel, 1995). The reported protective effect of chelators on various meat systems exposed to HPP support the idea that transition metals are released and act as major catalysts of lipid oxidation (Beltran, Pla, Yuste, & Mor-Mur, 2004; Cheah & Ledward, 1997; Ma, Ledward, Zamri, Frazier, & Zhou, 2007). However, the content in non-heme iron in chicken breast muscle was found to be unaffected by HPP treatments (Orlien, Hansen, & Skibsted, 2000). In fact, it was found that chicken breast muscle was, in general, more stable towards oxidation than beef *longissimus dorsi* when exposed to different treatments combining pressure and temperature (Ma et al., 2007).

Regarding pressure treatments, Mb is more stable in pure solutions than in meat systems, which is likely due to its coprecipitation with other food components (Ma & Ledward, 2013). This finding can be in part attributed to membrane disruption that facilitates the contact of membrane lipids with relatively lipophilic heme moieties and other pro-oxidants (Bajovic et al., 2012; Ma & Ledward, 2013). Indeed, the application of HPP and thermal treatments can result in a different exposure of the catalytic heme group and different amino acid residues with antioxidant properties (Bou et al., 2010; Carlez et al., 1995; Cheah & Ledward, 1997). In this connection, the denaturation of Hb as a consequence of thermal treatment and its loss of solubility have been associated with decreased pro-oxidant activity (Bou et al., 2010). Thus, complex interactions may occur with the different components of a food matrix upon pressurization and affect the catalytic effect of heme compounds. Therefore, the susceptibility towards oxidation in muscle foods can be affected by a number of factors. This fact explains why cooked chicken, with a lower content in heme pigments, is more prone to lipid oxidation than cooked beef or pork muscles (Rhee, Anderson, & Sams, 1996).

Overall, lipid oxidation in meat products can be accentuated by HPP and the presence of Hb, which is often disregarded. We hypothesized that the pro-oxidant activity of Hb is relevant and can be enhanced as a consequence of HPP. This fact could be related to Hb redox chemistry, solubility and hemin loss, which may also explain its interaction with other food components. Therefore, the aim of this work was to gain a better understanding of the effect of Hb on the lipid oxidation of meats exposed to different pressurization treatments and thereafter stored at refrigeration temperatures. To that aim, two different studies employing chicken Hb and washed chicken breast muscle were designed.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade with the exception of methanol and chloroform used in the determination of lipid hydroperoxides, which were of HPLC grade. Cumene hydroperoxide, 1,1,3,3-tetraethoxypropane and hexanal were purchased from Sigma-Aldrich (Madrid, Spain). Deionized water was used in all analysis.

2.2. Preparation of washed chicken breast muscle

Whole chicken breasts without skin from several animals were purchased in a local market. Breasts were trimmed to remove all remaining adipose tissue, cut into small pieces and ground using a grinding apparatus (4 mm plate diameter). The homogeneous minced muscle was washed twice with cold distilled water with a mince/water ratio of 1:3 (*w/v*) and stirred with a glass rod for 2 min. Subsequently, the mixture was allowed to stand for 15 min before dewatering with a strainer. The retentate was mixed with cold 50 mM sodium phosphate buffer (pH 6.2) with a mince/buffer ration of 1:1 (*w/v*) and homogenized for 3 min at speed of 13,000 rpm. The pH of the muscle slurry was checked and adjusted to 6.2 if necessary, and after standing for 15 min, the mixture was centrifuged at 15,000 g for 25 min. The pellet was collected. All processes were performed at 4 °C. Washed mince was measured for moisture content as described elsewhere (AOAC, 2005), packaged in aluminum bags, vacuum-sealed and stored at −80 °C until use.

2.3. Preparation of hemoglobin

Blood was removed from the jugular vein of stunned laying hens according to commercial procedures. Approximately 10 mL of blood were collected into tubes containing sodium heparin (17 U heparin per mL). Hb was prepared as described elsewhere (Fyhn et al., 1979; Thiansilakul et al., 2012). Four volumes of cold 1.7% NaCl in 1 mM Tris buffer (pH 8.0) were added to heparinized blood. The mixture was centrifuged at 700 g for 10 min at 4 °C. After the plasma was removed, the red blood cells were washed by suspending them three times in 10 volumes of the same buffer. Red blood cells were lysed in 3 volumes of 1 mM Tris (pH 8.0) for 1 h. A 1/10 vol of 1 M NaCl was then added to aid in stromal removal before ultracentrifugation at 28,000 *g* for 15 min at 4 °C. Low molecular mass components (< 10 kDa) were removed from Hb using a prepacked PD-10 column (column volume: 13.5 mL, medium volume: 8.3 mL of Sephadex G-25 medium; GE Healthcare Europe GmbH, Barcelona, Spain) eluted with 50 mM Tris buffer (pH 8.5) with gravity flow.

The amount of Hb was calculated by measuring the absorbance at 523 nm and methemoglobin (MetHb) was reduced by mixing the Hb solution with sodium hydrosulfite (ratio 1:0.4 *w/w*), as described in Bou et al. (2010). The mixture was vortexed at 4 °C, and thereafter the excess of reductant was removed by passing the Hb solution through a PD-10 column. The freshly prepared desalted Hb was adjusted to pH 6.2, and the amount of Hb was calculated again before its addition into washed mince. The percentages of OxyHb, DeoxyHb and MetHb were calculated as described elsewhere (Benesch, Benesch, & Yung, 1973).

2.4. Experimental design: Preparation of washed muscle with Hb

In all samples, an appropriate volume of the Hb solution and phosphate buffer (50 mM, pH 6.2) were added to obtain a final concentration of 12 μmol Hb per kg of washed chicken muscle (48 μmol/kg on a heme basis) with the exception of controls. This level of Hb addition was selected since it is in agreement with the Hb levels in chicken meat (Kranen et al., 1999). The pH was checked and adjusted to 6.2 when necessary before the application of the treatments described below. In all cases, streptomycin sulfate (200 g/kg washed chicken breast muscle) was added to inhibit microbial growth and homogenized using an UltraTurrax T25 model at 9000 rpm for 1 min before submitting the samples to the corresponding treatments with an adjusted final moisture content of 90%. This fact is in good agreement with the

proximate composition of samples (87.0 ± 1.3%, protein 12.0 ± 1.4%, fat 0.3 ± 0.1%) determined according the AOAC procedures (AOAC, 2005).

In experiment 1, the effect of HPP on lipid oxidation of samples containing Hb was studied. To that aim, samples containing Hb were pressurized at 0, 200, 400 and 600 MPa for 6 min with water at 10 °C as pressure-transmitting medium (Hyperbaric Wave 6500/120; N.C. Hyperbaric, S.A., Burgos, Spain). The pressurization rate was 135 MPa/min and the time for decompression was ≤10 s. The control treatment without Hb consisting of washed mince plus the appropriate volume of buffer solution was not pressurized (control NP). Sample aliquots of 60 g were vacuum-sealed (800 Pa) in polyamide/polyethylene bags (90 μm, 150 × 200 mm, oxygen transmission rate 50 mL/m^2 day) and stored at 4 °C up to 3 weeks. In parallel, 2.5 mL aliquots of 100 μM Hb in phosphate buffer (pH 6.2, 50 mM) were vacuum-packed and exposed to the same experimental conditions to observe Hb spectral changes. The solubility of native Hb and pressurized Hb was determined after centrifugation of samples at 14,000 g for 15 min and then determining the protein content of the supernatant fraction (Bou et al., 2010).

In experiment 2, the effect of pressurizing the washed chicken breast mince with and without the presence of Hb was studied. To do so, all treatments included the presence of washed mince, which was vacuum-packaged and subjected to a typical commercial pressurization of 600 MPa for 6 min at 10 °C. These treatments include the control sample that did not contain Hb (control 600) and 3 other treatments that contained 12 μmol Hb/kg but differing in the Hb mode of addition (Supplementary Figure). The first of these treatments involved the addition of the appropriate volume of a concentrated native Hb solution (approximately 100 μM of reduced Hb dissolved in 50 mM phosphate buffer, pH 6.2) into the pressurized washed mince (referred to as 600-N treatment). The second involved the addition of the same volume of the concentrated Hb solution into pressurized washed mince but after being separately pressurized (600 MPa, 6 min, 10 °C) under the same conditions (separately pressurized, 600-S). Finally, the same volume of the concentrated Hb solution was mixed together with the washed mince prior to its pressurization (pressurized together; 600-T). In 600-N and 600-S treatments, it was necessary to open the sample bags to add Hb after their exposure to HPP. Therefore, the remaining bags corresponding to the control 600 and 600-T treatments were also opened in order to mimic the same conditions. All treatments were then subsequently vacuum-sealed and stored at 4 °C for up to 3 weeks.

2.5. Instrumental color

Washed chicken muscle with different treatments was opened, poured into Rodac petri dishes, and the CIE-Lab redness (a*) values were immediately determined by using a colorimeter (Minolta, Japan). D65 and a 10° observer angle were used. The reflectance spectra between 400 and 700 nm in 10 nm intervals were also obtained. A white calibration plate was used to calibrate the instrument.

2.6. Heme iron determinations

Ferric protoporphyrin IX (hemin) was determined spectrophotometrically based on Hornsey's method (Lee, Tatiyaborworntham, Grunwald, & Richards, 2015). The heme content (present as native Hb, free hemin, and other heme compounds) was measured in the whole washed chicken breast muscle (hereafter referred to as total heme) and in the supernatant fraction after centrifugation (5 g) at 15,500 g for 10 min at 4 °C. Given that volume of the supernatants was similar regardless of the pressurization treatment, it allows an estimation of still soluble heme compounds (hereafter referred to as aqueous soluble heme). To measure the total heme content, washed chicken breast samples (2 g) were weighed and extracted with 5 mL of acid acetone reagent (acetone:H_2O:12 M aqueous HCl, 90:8:2, *v/v/v*). Similarly, an aliquot (0.5 mL) of the supernatant was collected and extracted with 5 mL of the same acid acetone to measure the aqueous soluble heme. In both cases, the mixtures containing the acid acetone were immediately vortexed for 30 s and then incubated at room temperature in the absence of light for 1 h. After incubation, the tubes were centrifuged at 2000 g for 10 min. The acetone phase was scanned from 350 to 700 nm. The difference between the absorbance values at 640 and 700 nm against the control treatment was used for calculating the concentration of hemin in the samples using the extinction coefficient (4.80 mM^{-1} cm^{-1}).

2.7. Determination of non-heme iron content

Non-heme iron was analyzed by the ferrozine method as described elsewhere (Bou et al., 2010). Two hundred microliters of sodium nitrite (0.16% w/v) and 3 mL of extraction solution (6 N HCl plus 40% TCA in equal volumes) were added to screw-cap tubes containing 2 g of sample. The tubes were closed, mixed and incubated in a water bath at 65 °C for 20 h. After cooling, the mixtures were centrifuged at 1750 g for 10 min and the supernatants were passed through 0.45-μm filters. To 1 mL of each filtrate, 1 mL of 0.8% ascorbic acid was added and the samples were allowed to stand for 15 min. Then, 1 mL of 16% ammonium acetate and 1 mL of 0.8 mM ferrozine were added and the absorbance at 562 nm was measured after 10 min. Concentrations were obtained using a standard curve from 0 to 2 mg of iron/L produced from ferric chloride.

2.8. Content in lipid hydroperoxides

The determination of lipid hydroperoxides was performed as described elsewhere (Grunwald & Richards, 2006) A 7.5-mL volume of cold chloroform:methanol (1:1) was added to a 600–700 mg muscle sample. Then, the sample was homogenized in the solvent for 30 s using a small disperser probe (Ultra-Turrax T25 model). The probe was then rinsed for 20 s with another 7.5 mL of the solvent, which was also added to the centrifuge tube. A 4.62-mL volume of cold 0.5% NaCl was added to the centrifuge tube and vortexed. The mixture in the centrifuge tube was centrifuged at 1800 *g* for 6 min at 4 °C. By using a glass syringe, a 1-mL volume of the lower chloroform layer was removed from the centrifuged sample and transferred to a glass tube. Then, 1 mL of methanol (HPLC quality) was added to 1 mL of extract. Next, 25 μL of 3.94 M ammonium thiocyanate and 25 μL of 18 mM iron (II) chloride were added to the tube and vortexed after each addition. The sample was then incubated at room temperature for 20 min, and the absorbance was read at 500 nm. A standard curve was constructed using cumene hydroperoxide, and the concentration of lipid hydroperoxides in the sample was expressed as μmol lipid hydroperoxides/kg muscle.

2.9. Measurement of thiobarbituric acid reactive substances (TBARS)

Lipid oxidation was monitored as described elsewhere (Bou et al., 2010). A standard curve was prepared using 1,1,3,3-tetraethoxypropane. TBARS were calculated as micromoles of malondialdehyde per kg sample.

2.10. Hexanal content

Samples were weighed (1 g) into 10-mL vials and placed into a CombiPAL injector autosampler (CTC Analytics, Zwingen, Switzerland) suitable for headspace solid-phase microextraction (HS–SPME) analysis. The sample vial was incubated at 60 °C for 10 min. A 75 μm Carboxen™/polydimethylsiloxane (CAR/PDMS) Stable Flex SPME fiber (Supelco, Bellefonte, PA) was inserted through the septum into the vial and exposed to the headspace. Vial penetration depth was set at 22 mm and, after 20 min of extraction, the SPME fiber was inserted into the injection port of the Agilent GC (Agilent, Santa Clara, CA) model 6850 coupled to a mass spectrometer model 5975C VL MSD. The injection

penetration depth was set at 54 mm. The SPME fiber was desorbed at 240 °C for 1.5 min in split mode. The chromatographic separation was performed on an Agilent J&W DB-5 MS column (30 m, i.d. 0.25 mm, film thickness 1 μm). The oven was held 3 min at 40 °C and programmed to heat up to 200 °C at 5 °C/min and then held for 10 min. Helium was used as carrier gas at a flow of 0.8 mL/min; the split ratio was 1:15. The EI source was at 70 eV and the mass range was m/z 40 to 200. Hexanal identification was carried out by injection of commercial standard and spectral comparison using the NIST 05 Mass Spectral Library and Mass Hunter software. The abundance of m/z 56 was used for semiquantitative determination. The results were expressed in area units (AU) $\times 10^{-5}\, g^{-1}$.

2.11. Statistical analysis

The experiments were performed in triplicate. A one-way analysis of variance (ANOVA) was carried out to examine the effect of the different treatments on the studied variables at each storage period. Similarly, the effect of the storage time on the studied variables was examined for each treatment. Tukey's HSD test was performed at a 95% confidence level ($p \leq 0.05$) to identify significant differences among samples.

3. Results

3.1. Effect of high pressure on the absorption spectra of chicken Hb

Typically, fully DeoxyHb shows a single peak near 560 nm whereas OxyHb presents a characteristic valley at 560 nm and maxima at 540 and 580 nm (Richards & Dettmann, 2003). Fig. 1 shows the absorbance spectra and overall appearance of chicken Hb in phosphate buffer after its preparation at the typical post-mortem pH. The maximum difference in absorbance between MetHb and OxyHb occurs at 580 nm and thus the level of Hb oxygenation can be compared by measuring the absorbance difference between 580 and 560 nm, where a deeper valley indicates a higher affinity for oxygen (Richards & Dettmann, 2003; Richards et al., 2002). According to Benesch et al. (1973), the estimated percentages of chicken OxyHb and DeoxyHb after its preparation at a pH of 6.2 were 53% and 26%, respectively. In agreement with our findings, the DeoxyHb content of chicken Hb at a pH of 6.3 was found to be between that of beef and trout (56 and 6%, respectively) (Richards & Dettmann, 2003; Richards et al., 2002). This low oxygen affinity can be attributed to the Root effect for which the level of Hb oxygenation decreases with acidic pH (Manning, Dumoulin, Li, & Manning, 1998).

Upon pressurization, the overall appearance of Hb in aqueous solutions did not change markedly, but in those samples exposed to 600 MPa, it is possible to observe some cloudiness that is likely to be caused by protein aggregation (Fig. 1 inset) leading to 15% solubility loss. This cloudiness is in agreement with the increased absorbance. However, in Fig. 1 it is also possible to observe some changes in the Q-band region (500–600 nm). The shallower valley at 560 nm and higher absorbance at longer wavelengths suggests that chicken Hb is partially oxidized into MetHb and less oxygenated upon pressurization.

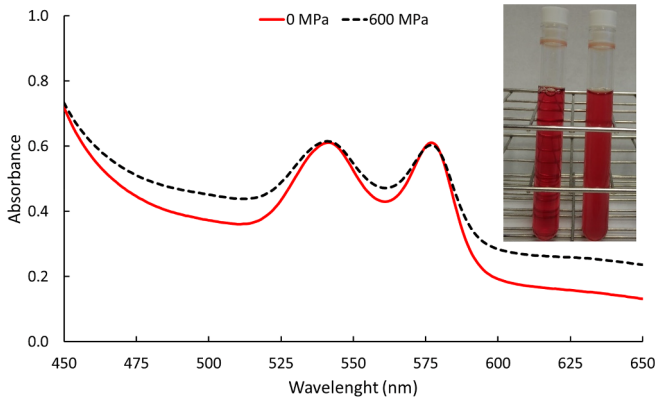

Fig. 1. Average spectra of chicken hemoglobin (14 μM in 50 mM phosphate buffer, pH 6.2) before and after its exposure to 600 MPa for 6 min. Inset: visual aspect of chicken hemoglobin before (left) and after (right) the pressurization treatment.

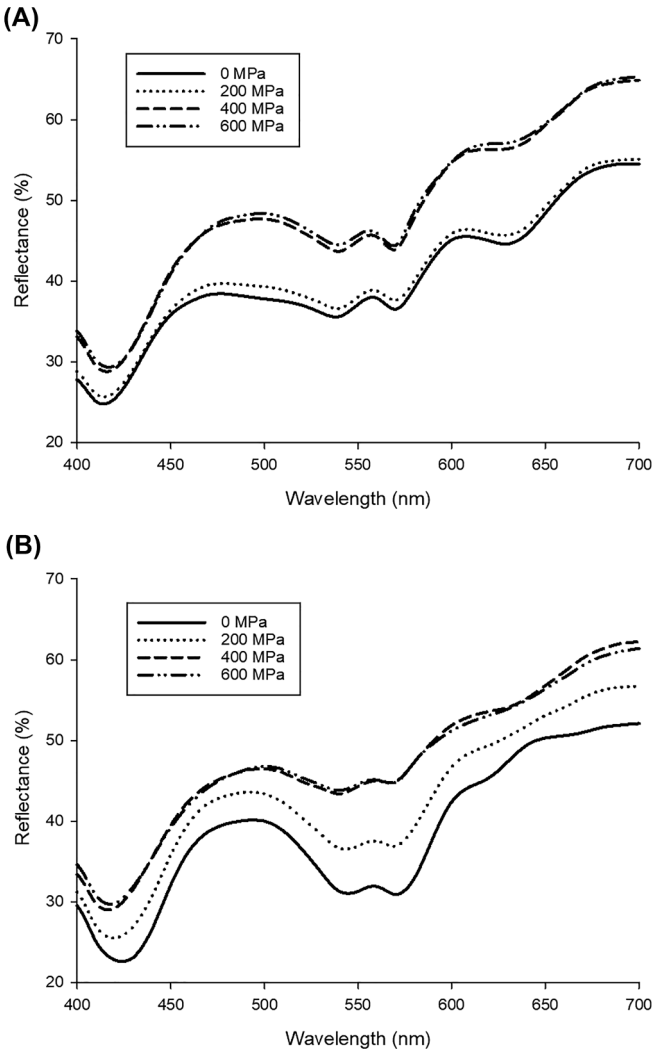

Fig. 2. Average reflectance spectrum of vacuum-packed washed chicken breast muscle containing hemoglobin after their exposure to 0, 200, 400 and 600 MPa for 6 min at 10 °C and subsequent storage under vacuum at 4 °C for 1 day (A) and 21 days (B).

3.2. Experiment 1: Effect of applying different pressures to washed chicken muscle with Hb stored at refrigeration temperatures

After 1 day of vacuum storage, the spectral shape of all samples was similar, but they differed in reflectance level (Fig. 2A). In all samples, the reflectance curves display marked minima at 540 and 570 nm, followed by maxima at 610 nm and minima at 630 nm. Therefore, a shift from 580 to 570 nm occurs upon Hb incorporation into washed chicken mince (Figs. 1 and 2A). In the low wavelength region, there is also a minimum shift from 410 nm to 420 nm with higher pressures. At the end of storage, the unpressurized treatment showed more pronounced minima at 540 and 570 nm than those at the initial time, whereas the minimum at approximately 630 nm was almost imperceptible (Fig. 2B). In addition, a maximum occurred at approximately 490 nm in washed chicken muscle exposed to 0 and 200 MPa. Those samples exposed to 400 and 600 MPa showed similar spectra

Table 1
Effect of different high hydrostatic pressures (0, 200, 400 and 600 MPa) on instrumental redness and content in non-heme iron, total heme compounds, heme compounds in the supernatant, lipid hydroperoxides, TBARS values and hexanal during the storage (4 °C) of vacuum-packed washed chicken muscle with added hemoglobin.

	Treatment	1 day	7 days	14 days	21 days
Redness (a*)	Control NP	1.0 ± 0.08 a,A	1.0 ± 0.07 a,A	1.2 ± 0.12 a,A	1.0 ± 0.08 a,A
	0 MPa	5.0 ± 0.38 d,A	6.0 ± 0.16 e,A	6.2 ± 0.65 c,A	6.0 ± 0.37 d,A
	200 MPa	4.5 ± 0.40 cd,AB	3.8 ± 0.25 d,A	5.4 ± 0.57 c,B	5.0 ± 0.36 cd,B
	400 MPa	4.2 ± 0.23 bc,B	4.0 ± 0.13 c,AB	3.8 ± 0.44 b,AB	3.2 ± 0.18 bc,A
	600 MPa	3.7 ± 0.06 b,B	3.0 ± 0.08 b,A	2.8 ± 0.27 b,A	2.5 ± 0.61 ab,A
Non-heme iron (μmol/kg)	Control NP	23 ± 2.5 a,A	30 ± 12.1 a,A	25 ± 1.6 a,A	32 ± 15.2 a,A
	0 MPa	27 ± 7.9 a,A	26 ± 1.8 a,A	33 ± 3.0 ab,A	36 ± 4.5 a,A
	200 MPa	41 ± 9.9 a,A	30 ± 0.3 a,A	30 ± 3.0 a,A	34 ± 2.7 a,A
	400 MPa	37 ± 8.2 a,A	31 ± 4.5 a,A	33 ± 2.0 ab,A	42 ± 9.5 a,A
	600 MPa	31 ± 3.8 a,A	32 ± 4.7 a,A	39 ± 4.9 b,AB	44 ± 3.2 a,B
Total heme (μmol/kg)	Control NP	–	–	–	–
	0 MPa	34 ± 5.1 a,A	36 ± 7.2 a,A	32 ± 5.1 a,A	29 ± 8.6 a,A
	200 MPa	31 ± 8.0 a,A	38 ± 2.0 a,A	34 ± 1.5 a,A	34 ± 3.6 a,A
	400 MPa	33 ± 4.4 a,A	40 ± 1.7 a,A	32 ± 4.7 a,A	34 ± 9.4 a,A
	600 MPa	39 ± 3.1 a,B	34 ± 3.1 a,AB	28 ± 4.0 a,AB	23 ± 6.5 a,A
Aqueous heme (μmol/L)	Control NP	–	–	–	–
	0 MPa	8 ± 3.0 a,A	10 ± 2.2 ab,A	19 ± 2.8 a,B	20 ± 4.5b,B
	200 MPa	9 ± 1.0 ab,AB	4 ± 1.2 a,A	13 ± 3.2 a,B	12 ± 2.2 ab,B
	400 MPa	22 ± 5.3c,A	18 ± 7.5b,A	16 ± 7.4 a,A	12 ± 5.3 ab,A
	600 MPa	17 ± 3.1 bc,B	12 ± 3.0 ab,AB	10 ± 2.6 a,AB	8 ± 2.4 a,A
Hydroperoxides (μmol/kg)	Control NP	3 ± 2 a,A	4 ± 3 a,A	2 ± 1 a,A	66 ± 8 a,B
	0 MPa	1 ± 2 a,A	21 ± 12 a,A	4 ± 7 a,A	69 ± 15 a,B
	200 MPa	1 ± 1 a,A	44 ± 41 a,A	44 ± 51 a,A	51 ± 13 a,A
	400 MPa	1 ± 1 a,A	17 ± 10 a,A	220 ± 168 ab,AB	627 ± 319 ab,B
	600 MPa	2 ± 2 a,A	156 ± 54 b,AB	503 ± 278 b,AB	903 ± 316 b,B
TBARS (μmol/kg)	Control NP	0.2 ± 0.16 a,A	0.5 ± 0.19 a,A	1.0 ± 0.19 a,B	1.2 ± 0.12 a,B
	0 MPa	n.d.	0.9 ± 0.23 a,B	1.3 ± 0.40 a,B	1.5 ± 0.15 a,B
	200 MPa	0.1 ± 0.11 a,A	1.5 ± 0.58 a,B	2.0 ± 0.35 ab,BC	2.6 ± 0.22 ab,C
	400 MPa	0.3 ± 0.01 ab,A	0.7 ± 0.30 a,AB	3.9 ± 0.96 b,BC	6.4 ± 2.26 bcC
	600 MPa	0.5 ± 0.10 b,A	3.5 ± 1.13 b,AB	7.4 ± 1.36 c,BC	10.2 ± 3.17 c,C
Hexanal ($AU \times 10^{-5}\,g^{-1}$)	Control NP	63 ± 20b,B	19 ± 6 a,A	22 ± 8 a,A	11 ± 2 a,A
	0 MPa	11 ± 3 a,A	24 ± 4 a,A	28 ± 7 a,A	21 ± 21 ab,A
	200 MPa	4 ± 2 a,A	42 ± 11 a,B	35 ± 10 a,AB	22 ± 17 ab,AB
	400 MPa	5 ± 3 a,A	22 ± 11 a,A	90 ± 41 a,AB	128 ± 53 b,B
	600 MPa	21 ± 7 a,A	168 ± 18 b,AB	285 ± 83 b,B	324 ± 73 c,B

Values are the mean ± SD ($n = 3$). Treatments involve a control consisting of washed chicken breast muscle without the presence of hemogoblin (control NP) and a mixture of washed chicken plus hemoglobin and thereafter pressurized at different pressures (0, 200, 400 and 600 MPa for 6 min at 10 °C). Different letters in the same column (a–d) or in the same row (A–C) indicate significant differences ($p \leq 0.05$). n.d. – not detected.

after 21 days of storage, and the minima at 540 and 570 nm were less marked than those at the initial time. At the end of storage, the reflectance spectra of washed chicken muscle exposed to 200 MPa were intermediate compared to the other samples.

The loss of red color in washed muscle with added Hb can be used to estimate the conversion of ferrous Hb to ferric Hb (MetHb). At the initial time, all samples containing Hb recorded higher values in redness (a*) than the control (Table 1). The unpressurized samples with Hb (0 MPa) recorded the highest redness values but similar to those pressurized at 200 MPa. The treatment involving 600 MPa recorded the lowest redness values among those samples containing Hb. These results are in line with the reported reduction in a* values at pressures higher than 350 MPa (Bak, Bolumar, Karlsson, Lindahl, & Orlien, 2017; Carlez et al., 1995). The overall appearance (not shown) and instrumental redness tendency from relatively reddish to more brownish colors with higher pressures can be attributed to the formation of MetHb and denatured ferrihemochromes.

During storage, the unpressurized samples (0 MPa) recorded similar redness values (Table 1). Those samples treated at 200 MPa remained fairly unchanged and, at the end of storage, they were not different from the non-pressurized samples (0 MPa) or those submitted to 400 MPa. Conversely, those samples that received the higher pressurization treatments (400 and 600 MPa) showed a tendency for lower redness values over the course of storage (Table 1). The decrease in redness values during storage of meat products is associated with the formation of oxidized forms of heme iron (Carlez et al., 1995; Faustman, Sun, Mancini, & Suman, 2010; Maqsood et al., 2012).

In our conditions, the non-heme and heme contents remained unaffected after the application of the different pressurization treatments (Table 1). In addition, no differences between treatments were observed for both contents at the end of the storage period. However, the heme content decreased during the storage of those samples treated at 600 MPa, whereas the opposite trend was observed for non-heme content. With regards to the amount of heme iron that remains soluble after centrifugation, an initial higher content was recorded in those samples pressurized at 400 and 600 MPa when compared with those that were not pressurized (0 MPa) or pressurized at 200 MPa, which, in turn, were similar (Table 1). Over the course of storage, the heme content in the supernatant of the treatment at 600 MPa showed a decrease with longer storage times and, at the end of storage, this content was lower than that of the unpressurized samples containing Hb (Table 1). Conversely, the aqueous soluble heme content in this latter treatment (0 MPa) increased with longer storage periods. Those samples that received 200 and 400 MPa recorded intermediate contents in aqueous soluble heme during the course of storage; at the end of storage, no differences could be established when compared with the other treatments.

The content in lipid hydroperoxides at the initial time was very low, regardless of the treatment (Table 1). TBARS values and hexanal

content were also assessed with regards to the formation of secondary products of lipid oxidation. As shown in Table 1, TBARS values were low at the beginning of storage. However, the treatment receiving the highest pressure resulted in higher oxidation values than the controls with and without Hb (0 MPa and control NP, respectively). For the hexanal content, the control without Hb recorded a higher initial content than the other treatments, which were similar (Table 1).

In general, the amount of lipid hydroperoxides increased progressively over the course of vacuum storage (Table 1). This can be due to the instability of lipid hydroperoxides and to distinct lag periods in the initiation of lipid oxidation of the replicates. Despite that, at the end of storage, the highest content was recorded in those samples exposed to 600 MPa followed by those subjected to 400 MPa, which were not different from those samples receiving 200 MPa or no pressurization. Similarly, TBARS values increased with longer storage periods, and those samples that received the highest pressurization treatment recorded elevated values. The hexanal content followed a similar pattern, and thus higher oxidation was recorded with longer storage periods and higher pressure intensities. However, the non-pressurized control (control NP) displayed the highest content in hexanal at the initial time and then remained low over the course of storage (Table 1).

3.3. Experiment 2: Effect of the presence of native and pressurized Hb on pressurized washed chicken muscle stored at refrigeration temperatures

In agreement with the preceding experiment, the content of lipid hydroperoxides in the muscle was at trace levels after its pressurization at 600 MPa regardless of the presence of Hb and its method of addition (Table 2). Likewise, lipid oxidation increased progressively by following a similar trend for all treatments. This contributed to the fact that, after 14 days of storage, there were no differences between treatments, including that involving the omission of Hb (control 600). However, at the end of storage, it was possible to observe some differences between the different treatments. In this storage period, the pressurized control recorded the lowest oxidation values, and all treatments containing Hb had higher contents of hydroperoxides. The exposure of heme compounds to membrane lipids can explain the increased progression of oxidation during the course of storage (Carlsen et al., 2005). However, only the treatment involving the addition of native Hb (600-N) and that in which Hb was pressurized together with washed meat (600-T) were significantly different from the control at the end of storage.

With regards to secondary oxidation products, very low values of TBARS were observed in all treatments at the initial time (Table 2). In line with the content of lipid hydroperoxides, the oxidation progressed with storage time, and all treatments followed a similar pattern through 14 days. Thereafter, the treatment involving the addition of native Hb had greater values than the pressurized control without Hb and showed no differences between the treatments that combined the addition of Hb

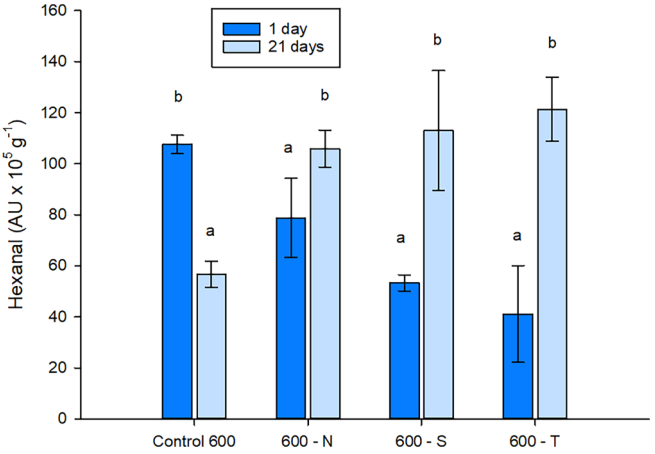

Fig. 3. Hexanal content in vacuum-packed washed chicken breast muscle (pH 6.2) after its pressurization at 600 MPa for 6 min and further storage at 4 °C for 1 and 21 days. The following treatments consisted of: a control in which hemoglobin was not added (control 600); the addition of native chicken hemoglobin (12 μM) into pressurized washed chicken muscle (600-N); the addition of chicken hemoglobin (12 μM) after its exposure to the same pressurization treatment as washed chicken muscle (600-S); the addition of native chicken hemoglobin (12 μM) before washed chicken muscle pressurization (600-T). For each storage time, treatments with different letters differ significantly. Error bars represent the SD.

exposed at 600 MPa into pressurized washed meat (600-S) and that in which Hb and washed meat were pressurized together at 600 MPa (600-T). The initial and final content of hexanal in these samples is depicted in Fig. 3. The pressurized control had a higher hexanal content at the initial time. On the contrary, the control recorded the lowest content at the end of the storage.

4. Discussion

4.1. Effect of pressure intensity in washed chicken muscle containing Hb

The different mechanisms by which Mb and Hb promote oxidation have been reviewed elsewhere (Baron & Andersen, 2002; Carlsen et al., 2005; Maqsood et al., 2012). Hb is a tetrameric molecule made up of four heme groups per globin, whereas Mb possesses one single heme group surrounding a globin. Their resemblance explains their similar stability and redox chemistry. With regard to Hb, the level of oxygenation at post-mortem pH and under an air atmosphere depends on the animal species, which has been related to differing abilities to promote oxidation (Richards et al., 2002). For instance, beef Hb is more oxygenated and less pro-oxidant than fish and poultry Hb, which are relatively deoxygenated because of the Root effect (Manning et al., 1998;

Table 2
Effect on lipid hydroperoxide content and TBARS values of the addition of native and pressurized hemoglobin before or after exposing washed chicken muscle to high pressure processing at 600 MPa and during its storage under vacuum at 4 °C.

	Treatment	1 day	7 days	14 days	21 days
Hydroperoxides (μmol/kg)	Control 600	2 ± 1 a,A	148 ± 10 bc,B	186 ± 6 a,C	202 ± 13 a,C
	600-N	2 ± 3 a,A	178 ± 8 c,B	371 ± 35 a,C	506 ± 18 b,D
	600-S	1 ± 1 a,A	116 ± 18 ab,B	248 ± 36 a,C	338 ± 37 ab,D
	600-T	n.d.	92 ± 32 a,A	218 ± 96 a,A	500 ± 179 b,B
TBARS (μmol/kg)	Control 600	0.2 ± 0.07 ab,A	1.3 ± 0.63 a,A	4.4 ± 0.60 a,B	4.1 ± 0.43 a,B
	600-N	0.1 ± 0.03 a,A	0.9 ± 0.57 a,A	6.0 ± 0.23 a,B	9.3 ± 1.76 b,C
	600-S	0.2 ± 0.02 ab,A	2.1 ± 0.45 a,B	6.3 ± 0.16 a,C	6.9 ± 1.07 ab,C
	600-T	0.3 ± 0.01 b,A	0.8 ± 0.89 a,A	4.8 ± 1.66 a,B	5.8 ± 1.83 ab,B

Values are the mean ± SD (n = 3). Treatments involve a control consisting of washed chicken breast muscle pressurized at 600 MPa without hemoglobin (control 600) and a mixture of pressurized washed chicken plus hemoglobin (see Supplementary Figure). Different letters in the same column (a–c) or in the same row (A–D) indicate significant differences ($p \leq 0.05$). n.d. – not detected.

Park, Undeland, Sannaveerappa, & Richards, 2013; Richards et al., 2002). Immediately after its preparation, chicken Hb is mainly present as OxyHb (Fig. 1). However, pressure modifies the water structure and leads to higher water densities with increased pressure, which has implications for the denaturation and reactivity of proteins (Cho, Urquidi, Singh, Park, & Robinson, 2002). This fact can explain the observed changes in the solubility and visible spectra of Hb (Fig. 1). The exposure of chicken Hb to HPP may have caused increased content in MetHb and/or other denatured heme species at the expense of OxyHb. The transformation of reduced chicken Hb into oxidized heme compounds is in line with the reported change of OxyMb into MetMb and other denatured ferric forms, ferrihemochromes, in pressurized samples (Bak et al., 2017).

However, the study of the effect of Hb and HPP in meat systems is relevant because the stability of Mb in meat systems is less stable than in pure solutions (Bak et al., 2017). The observed increase in the reflectance spectra with higher pressures is in line with those reported in porcine meat (Bak, Lindahl, Karlsson, & Orlien, 2012). These increases in reflectance levels most likely reflect the denaturation of myofibrillar proteins (Fig. 2). The observed minima at 540 and 570 nm and the maxima at 610 nm in all samples suggest that Hb is, in part, present as OxyHb, whereas the shoulder close to 630 nm may indicate the presence of both DeoxyHb and MetHb (Fernández-López, Pérez-Alavarez, Sayas-Barberá, & Aranda-Catalá, 2000). The shift in the Soret band from 410 to 420 nm in samples exposed to 400 and 600 MPa can be attributed to changes in the meat matrix and in the Hb itself.

These findings in reflectance spectra are in line with the minimal changes in redness values of washed meat exposed to 200 MPa (Table 1). Ros-Polski et al. (2015) also reported that ground white chicken pressurized up to 300 MPa had similar redness values than samples that were not pressurized. Conversely, redness was found to be increased in chicken breast fillets treated at 300, 450 and 600 MPa, when compared to a non-pressurized control (Kruk et al., 2011). Despite that, the treatment at 300 MPa led to higher a* values when compared with the other pressurized treatments. As can be seen in Table 1, there is a decrease in redness in samples exposed to 400 and 600 MPa. The loss of redness with higher pressures and the appearance of brownish colors in meat samples has been mainly attributed to the transformation of ferrous Mb into MetMb, although the denaturation of the globin and/or the modification of the porphyrin ring have also been reported to contribute to these color changes (Bak et al., 2017).

Various authors reported that the induced color changes in meat samples as a result of HPP can be explained by iron release from the heme moiety (Carlez et al., 1995; Cheftel & Culioli, 1997). Heme and non-heme contents were unaffected after sample pressurization (Table 1). Therefore, the iron ion is not released from the porphyrin ring after the exposure to HPP but pressures above 200 MPa seem to cause a higher content of ferrihemochromes (i.e. decreased redness). This finding is in agreement with the lack of effect of various HPP treatments on the content of non-heme iron in chicken breast muscle (Orlien et al., 2000). However, at Day 1 the content of heme in the supernatants was lowered in those samples exposed to 0 and 200 MPa. This finding could be due to the ability of Hb and Mb to bind to different compounds, including actomyosin (Chaijan, Benjakul, Visessanguan, Lee, & Faustman, 2008; Faustman et al., 2010; Park et al., 2013). Park et al. (2013) reported that during their storage at refrigeration temperatures, the concentration of Hb rapidly decreased (< 1 day) in the supernatants of washed muscles in which perch Hb was added, whereas the addition of pig Hb in washed muscles only resulted in a slight decrease. As reported by the authors, it is unclear if those findings are due to the different binding ability of Hb to insoluble components of washed muscle or to a different hemin loss. Indeed, the application of pressures leads to protein unfolding and subsequent formation of new interactions within and between proteins that usually lead to a loss of protein solubility (Ma & Ledward, 2013; Rastogi et al., 2007). This is the case for myosin and actin, which lose their native solubility above 400 MPa; however, other myofibrillar proteins are less modified by pressure and remain soluble (Grossi et al., 2016; Ma & Ledward, 2013). These molecular changes may affect the above-mentioned binding of Hb with washed chicken muscle and help to explain the observed initial higher content of aqueous heme in those samples exposed to 400 MPa and 600 MPa (Table 1).

The formation of free radicals during HPP, particularly at pressures ≥500 MPa, can generate different oxidation compounds (Bolumar et al., 2014). However, the hexanal content was higher in the non-pressurized control, whereas no differences were observed in those samples containing Hb (Table 1). This fact suggests that some preliminary oxidation occurred during the preparation of samples and washed chicken mince. Hexanal and TBARS are usually well correlated, although they offer complementary information, as different interactions between oxidation compounds and food components may occur (Faustman et al., 2010; Pignoli, Bou, Rodriguez-Estrada, & Decker, 2009).

4.2. Effect of the vacuum storage of washed chicken muscle containing Hb exposed to different pressures

After 21 days of storage, the reflectance spectrum of the unpressurized washed meat showed marked peaks at 540 and 570 nm, which is in agreement with redness values (Fig. 2B and Table 1). However, there is a tendency to higher redness with longer storage periods in 0 and 200 MPa treatments. Various authors reported the ability of meat to bloom at moderate pressures, which could be explained by the endogenous enzymatic systems related to meat oxygen consumption and reducing activity (Bak et al., 2017; Bak et al., 2012). In washed meat, this effect can also be caused by the presence of various Hb forms (Oxy, Deoxy and Met) with complex redox chemistry and relative pressure stability (Bak et al., 2017; Baron & Andersen, 2002; Carlsen et al., 2005; Maqsood et al., 2012). The reflectance increases at approximately 490 nm, and the observed changes above 600 nm after 21 days of storage in 0 and 200 MPa samples may indicate increased DeoxyHb (Fig. 2B). Conversely, the exposure to higher pressures resulted in smoother shapes and thus suggested the formation of MetHb and maybe denatured ferrihemochromes during storage (Bak et al., 2012; Fernández-López et al., 2000).

Lipid oxidation can be initiated by the presence of reduced Hb and as a consequence of the formation of the superoxide anion radical from the autoxidation of Hb (OxyHb), which can dismutate to form hydrogen peroxide; this process in turn generates hypervalent Hb species from the autoxidized MetHb (Baron & Andersen, 2002; Maqsood et al., 2012). Moreover, at low pH, the autoxidation of Hb, its dissociation into dimers, the release of hemin, and the solubility of free iron are enhanced. All of these factors are known to favor lipid oxidation (Maqsood et al., 2012). In addition, hexanal and various aldehydes have been reported to enhance MetMb formation from OxyMb and, specifically, hydroxynonenal accelerates OxyMb oxidation by binding covalently to specific histidine residues in the protein's primary sequence (Faustman et al., 2010). However, the reported effect of aldehydes on decreasing OxyMb redox stability was less pronounced at a pH of 5.6 than at 7.4 and this was attributed to greater autoxidation and potentially decreased nucleophilicity of candidate histidine residues at this pH. This fact could explain why the storage of those samples exposed to 0 and 200 MPa resulted in redness minimal changes and decreased susceptibility towards oxidation. Chaijan et al. (2008) reported that the amount of Mb bound to actomyosin was concomitant with the transformation of OxyMb into MetMb. However, the ability of Hb to bind to insoluble washed muscle components was found to be inconsistent with its pro-oxidative effect in washed cod mince (Sannaveerappa, Cai, Richards, & Undeland, 2014). Interestingly, these authors reported that the rapid hemin release from the anionic Hb appeared to be a primary facilitator of lipid oxidation, overshadowing the greater binding ability of the cationic Hb.

The heme content in the supernatants of 0 and 200 MPa samples remained unchanged or increased (Table 1). This finding suggests that the binding of Hb to washed meat compounds is likely a reversible electrostatic interaction. As can be observed, the interaction between heme compounds and insoluble components of washed chicken seems to be dependent on the applied pressure and increased with longer storage periods. In unpressurized samples, the ratio between the heme content in the aqueous phase and the total heme content was increased. However, in those samples exposed to 600 MPa, this ratio remained almost unchanged (data not shown). It is unclear if the heme group remains bound to Hb to some extent or dissociated from the protein (hemin loss). However, the observed lower redness values and elevated oxidative status with higher pressures support the idea that Hb is likely oxidized to MetHb, and hemin is subsequently released as a consequence of the reported reduced affinity of the latter for the heme group (Park et al., 2013). The release of hemin will readily decompose preformed lipid hydroperoxides, producing lipophilic free radicals that stimulate lipid oxidation. In addition, the hydrophobic nature of the heme moiety and its likely interaction with phospholipids may explain the decreased content of heme in the supernatants.

As shown in Table 1, a certain amount of iron can be released from Hb during its storage when exposed to elevated pressurization treatments, which also contribute to oxidation. Given that the increased accessibility for iron is a driving factor in initiating lipid oxidation, its release is in good agreement with the reported protective effect of chelators in HPP meats (Ma & Ledward, 2013). However, it should be noted that, in complex meat systems, this protective effect may also be consequence of the released iron from other iron complexes (e.g., ferritin, transferrin) rather than Hb. In muscle microsomes, the release of iron from the porphyrin moiety as a consequence of thermal treatments was found to be relatively limited (Bou et al., 2010). Despite that, it is worth noting that when bovine MetHb and OxyHb were heated at 45 °C and added to microsomes, the ability of EDTA to inhibit TBARS was higher when adding the former, which suggests that iron was released more easily from MetHb (Bou et al., 2010). In such conditions, however, non-chelatable iron was the most important pro-oxidant in the promotion of microsomal oxidation. The same statement may be valid for pressurized samples, given that the denaturation of proteins due to thermal and pressure treatments are to some extent similar.

Overall, various mechanisms seem to contribute to the progression of oxidation during the storage of pressurized samples containing Hb. The increased content in insoluble ferric forms was dependent on the applied pressure and concomitant with the studied oxidation parameters, which indicates that membrane disruption and the likely interaction between heme compounds and lipids are determining the onset of oxidation. The loss of redness can be attributed to the formation of MetHb and subsequent hemin release could explain the decrease of aqueous heme.

4.3. Effect of the form of addition of Hb into pressurized washed chicken muscle

Various authors reported that in meat systems exposed to HPP, lipid oxidation is triggered by membrane disruption (Bajovic et al., 2012; Ma & Ledward, 2013). In the preceding experiment, it was shown that the initiation and progression of oxidation can be affected by the combination of pressure treatments and the presence of ferrihemochromes that have been estimated by the loss of redness. Therefore, a second experiment was aimed at gaining a better understanding of the role of Hb and its interaction with food components on the onset of the oxidation in meats exposed to HPP. To that aim, a control of washed chicken muscle without Hb was exposed to 600 MPa and was compared with several treatments combining high pressure with the addition of Hb to determine its pro-oxidant effect. The differences in these latter treatments involved the mode of Hb addition (Supplementary Figure).

At the initial time, the pressurized control (control 600) of this additional experiment recorded higher hexanal levels than the unpressurized control (control NP) in the previous one (Table 1 and Fig. 3). This finding, therefore, confirms that HPP contributes to the formation of oxidation compounds, most likely due to membrane disruption. It is also worth noting that at the initial time, the pressurized control without Hb (control 600) recorded higher levels of hexanal than those treatments containing Hb, whereas the opposite is observed after 21 days of vacuum storage (Fig. 3). These findings are in agreement with those of the preceding experiment and can be attributed to this and other aldehydes interaction with various matrix compounds (Faustman et al., 2010; Pignoli et al., 2009).

The extent to which secondary products of lipid oxidation contribute to meat color and hemoproteins catalytic activity is relatively unknown. However, the initial lower content of hexanal in those samples containing Hb can be attributed to its reaction with heme compounds and can explain the observed pro-oxidant effect (Fig. 3). In agreement with this finding, the content in lipid hydroperoxides at the end of the storage period was higher in the 600-T and 600-N treatments than the pressurized control (Table 2). Given that a similar content of aldehydes may be formed as a consequence of pressurization at 600 MPa, this may explain why, in all treatments containing Hb, the progression of lipid hydroperoxides and TBARS during vacuum storage were not different, regardless of the mode of addition.

In general, there were no differences in lipid oxidation between samples at up to 15 days of storage when compared with the control. This fact seems to confirm that membrane disruption plays a crucial role in triggering oxidation in meats exposed to HPP. Despite that, at the end of storage, the content of lipid hydroperoxides and hexanal was higher in the 600-T than the pressurized control. The enhanced pro-oxidant activity can be attributed to the interaction between pressurized Hb and matrix compounds. Under the studied conditions, it is reasonable to think that the heme group can be shuttled to lipids as suggested elsewhere (Grunwald & Richards, 2006). This reasoning is also in good agreement with the lowered heme content in the supernatant with longer storage periods (Table 1). Nevertheless, the addition of native Hb into pressurized washed meat also had higher primary oxidation, TBARS values and hexanal content than the control at the end of the storage period. The increased susceptibility to oxidation of the 600-N treatment compared to the pressurized control can be attributed to a number of factors related to increased mobility and reactivity. In addition to the previously discussed interaction with hexanal (Fig. 3), it is worth noting that under vacuum, native Hb can be transformed into DeoxyHb, which can be easily converted into MetHb and subsequently release hemin. This mechanism explains why those Hb types with low oxygen affinity are potent catalysts of lipid oxidation (Park et al., 2013; Richards et al., 2002; Sannaveerappa et al., 2014). In contrast, the Hb solubility loss in 600-S samples together with the likely impeded interaction between the heme groups and membrane lipids during pressurization may explain why there were no differences in the progression of oxidation when compared with the pressurized control. Similarly, the exposure of beef Hb aqueous solutions to thermal treatments has been reported to cause its precipitation and hinder the catalytically active heme moiety within protein aggregates (Bou et al., 2010).

Collectively, these studies suggest that HPP mainly contributes to the oxidation of washed chicken muscle due to membrane disruption. However, in those samples containing Hb, the progression of oxidation can be enhanced by the heme pro-oxidant activity and its exposure to membrane lipids. In pressurized meat samples, the formation of MetHb and other ferrihemochromes may favor the release of hemin during storage, which in turn favors oxidation. Future work should corroborate these mechanisms by studying lipid and protein oxidation in more depth and examining strategies to inhibit Hb-mediated oxidation in this and other muscle food systems containing hemoproteins. In those cases in which the presence of chicken Hb may be relevant, the inhibitory effect may be in part accomplished by maintaining the hemoprotein as

OxyHb and ultimately minimizing the interaction between released hemin and membrane lipids.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.04.067.

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