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ORIGINAL PAPER

Preservative Effect of a Previous High-Pressure Treatment on the Chemical Changes Related to Quality Loss in Frozen Hake (*Merluccius merluccius*)

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Abstract The objective of this study was the quality loss inhibition of hake (*Merluccius merluccius*) during the frozen storage. For it, the effect of a previous high-pressure (HP) treatment (150–450 MPa for 2 min) was analysed throughout a 5-month storage at − 10 °C. Quality changes were monitored by complementary chemical analyses. Inhibition ($p < 0.05$) of dimethylamine (DMA), free fatty acid (FFA), formaldehyde (FA), trimethylamine, total volatile amine and fluorescent compound (tertiary lipid oxidation compound) formation was concluded by previous pressure treatment according to the one-way ANOVA analysis. On the contrary, no effect ($p > 0.05$) on the *K* value, polyene index and formation of peroxides and thiobarbituric acid reactive substances was achieved. Additionally, a multifactor ANOVA test (pressure and frozen storage time effects; i.e. comparison among HP treatments) showed an inhibitory effect ($p < 0.015$) on DMA and FFA formation, this effect increasing with pressure level applied. This inhibitory effect on the formation of such molecules related to quality loss can be explained on the basis of the damage caused to different kinds of enzymes such as trimethylamine oxide demethylase, lipases and phospholipases, so that their activity during the subsequent frozen storage would decrease. The work here presented provides for the first time information concerning the employment of HP technology to inhibit the DMA, FA and FFA formation during the frozen storage of hake. Further research focussed on commercial frozen conditions (− 18 °C) and including sensory and nutritional aspects is foreseen.

Keywords High pressure · *Merluccius merluccius* · Frozen storage · Formaldehyde · Dimethylamine · Free fatty acids

Introduction

Gadiforms is a large group of fish species such as cod, hake, pollack, haddock, whiting and saithe, which represents an important percentage of the overall fish catching and consumption in most European countries (FAO 2007). Thus, in addition to being commercialised in the fresh condition, most frozen fishery products like fillets, surimis and fish fingers are made from whole or minced muscle of these fish. However, fish species are recognised as developing fast and easy deterioration during the post-mortem period as a result of undergoing different kinds of damage pathways such as microbial attack, autolysis and lipid oxidation (Özogul 2010). Moreover, large quantities of trimethylamine oxide demethylase (TMAOase) are included in different body parts of Gadiform species, such as the kidney, spleen and microsomal fraction of the muscle. This enzyme has been found responsible for the breakdown of trimethylamine oxide into different kinds of deteriorative molecules such as trimethylamine (TMA), formaldehyde (FA) and dimethylamine (DMA) (Leelapongwattana et al. 2005; Malinowska-Pańczyk and Kołodziejska 2016).

Frozen storage has widely been employed to maintain fish properties before consumption or further use in other

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technological processes. In the case of frozen Gadiform species, protein denaturation has shown to be a major problem during the commercialisation of such kinds of products, leading to texture losses and off-odour development (Aubourg et al. 2007). Different pathways have been proposed to explain this degradation process (Sotelo and Rehbein 2000): (i) partial dehydration of proteins during freezing due to the formation of ice crystals, (ii) interaction of proteins with FA and with oxidised and hydrolysed lipids and (iii) modification of the protein micro-environment caused by the concentration of salts in the liquid phase surrounding proteins. In agreement with this high susceptibility to quality loss, substantial efforts are needed to meet the increasing consumer demand for high-quality and safe products.

Among advanced food processing technologies, high hydrostatic pressure technology has proved to inactivate microbial populations and endogenous enzymes while retaining the sensory and nutritional properties of foods (Campus 2010; Tabilo-Munizaga et al. 2016). Concerning high-pressure (HP) application to seafood, previous research accounts for its practical and beneficial effect when employed prior to a subsequent processing or storage. This strategy has successfully been checked in the cases of further chilled (Hurtado et al. 2001), cold-smoked (Lakshmanan et al. 2003) or semi-dried (Gou et al. 2012) storage. Related to freezing, frozen storage and thawing processes, pressure-shift technology has also been applied (Chevalier et al. 2000; Tironi et al. 2010); as a result of its employment, different kinds of advantages in protein properties have been demonstrated such as a lower denaturation and toughening extent and an increased water-holding capacity. In the case of a further frozen storage, research has been focussed on fatty fish species. Thus, a previous optimised HP treatment led to a quality enhancement of chemical properties of frozen mackerel (Vázquez et al. 2013), horse mackerel (Torres et al. 2013) and sardine (Méndez et al. 2017).

European hake (*Merluccius merluccius*) is an abundant Gadiform species of great market demand because of its excellent organoleptic features. Although frozen storage has shown to partially retain its quality and shelf life time, marked chemical changes have proved to be produced and lead to substantial quality losses by increasing the frozen storage time and temperature (Aubourg and Medina 1999). Previous research concerning HP treatment of European hake is scarce. This accounts for its positive effect on physicochemical properties of isolated sarcoplasmic (Villamonte et al. 2016) and myofibrillar (Cando et al. 2014) proteins and for the inhibition of *Anisakis simplex* L3 in hake muscle (Vidacek et al. 2009). The present research focusses on the development of chemical changes related to quality loss during the frozen storage of hake. In it, the effect of a previous treatment at 0–300 MPa for 2 min on the lipid oxidation and hydrolysis development, volatile amine and FA formation and *K* value was studied.

Materials and Methods

HP Conditions, Raw Fish, Processing, Storage and Sampling

HP treatments at 150, 169.27, 300, 430.73 and 450 MPa were performed in a 55-L high-pressure unit (Hiperbaric 55, Hiperbaric NL, Burgos, Spain) according to the experimental design described below; in all cases, a pressure holding time of 2 min was employed. Water applied as the pressurising medium at 3 MPa s^{-1} yielded 54, 63, 100, 115 and 121 s as the come up times for the 150, 169.27, 300, 430.73 and 450 MPa treatments, respectively, while decompression time was less than 3 s. Cold pressurising water was used to maintain temperature conditions during HP treatment at 20 °C.

A single set of European hake (102 individuals) was caught in November 2015 near the Galician Atlantic coast (North-Western Spain) and transported under ice to the laboratory. The length and weight of the specimens ranged 27.5–29.5 cm and 180–205 g, respectively. Whole hake individuals were placed in flexible polyethylene bags (three individuals per bag) and vacuum sealed at 150 mbar (Albipack-Packaging Solutions, Águeda, Portugal).

Fish individuals were distributed into 17 batches (6 individuals in each). The distribution of batches into pressure level and frozen storage time conditions was as follows (Table 1): (i) one batch was pressurised at 150 MPa and stored for 2.50 months (batch T-7); (ii) two batches were pressurised at 169.27 MPa and stored for 0.32 and 4.68 months (batches T-4 and T-13, respectively); (iii) five batches were pressurised at 300 MPa and stored for 0.00, 2.50, 2.50, 2.50 and 5.00 months (batches T-2, T-8, T-9, T-10 and T-16, respectively); (iv) two batches were pressurised at 430.73 MPa and stored for 0.32 and 4.68 months (batches T-5 and T-14, respectively); (v) one batch was pressurised at 450 MPa and stored for 2.50 months (batch T-11); (vi) five batches were not pressurised but were stored for 0.00, 0.32, 2.50, 4.68 and 5.00 months (unpressurised controls; batches T-1, T-3, T-6, T-12 and T-15, respectively); (vii) one batch was maintained as fresh fish (not pressurised and not frozen; batch T-0).

After HP treatments, hake individuals were kept at − 20 °C for 48 h and then stored at − 10 °C. The same procedure was carried out for unpressurised controls. Fresh fish was analysed immediately. For each batch, three replicates (n = 3) were analysed independently. Each analysis was based on the corresponding extract from the fish white muscle pooled from two individual fish. A frozen storage temperature (− 10 °C) higher than commercial practice (− 18 °C) was chosen as an accelerated test condition to estimate the extent of the pressure level and frozen storage effects on the chemical quality indices of frozen hake. All solvents and chemical reagents used were of reagent grade (Merck, Darmstadt, Germany).

Table 1 Lipid oxidation assessment in frozen hake muscle previously processed under different pressure levels

Batch number[a]	Pressure (MPa)	Storage time (months)	Quality index			
			PV	TBA-i	FR	PI
T-0	Starting fresh fish		0.73 aA	0.32 aAB	0.58 aA	2.67 B
			(0.47)	(0.12)	(0.03)	(0.06)
T-1	0.10	0.00	1.93 bB	0.63 bC	0.98 bC	2.83 BCD
			(0.10)	(0.16)	(0.20)	(0.13)
T-2	300	0.00	0.97 aA	0.50 bB	0.79 abD	2.76
			(0.28)	(0.02)	(0.18)	(0.05)
T-3	0.10	0.32	1.62 abAB	0.80 C	0.80 BC	2.88 CD
			(0.81)	(0.10)	(0.23)	(0.08)
T-4	169.27	0.32	0.59 aA	0.87 B	0.66	2.74
			(0.26)	(0.15)	(0.10)	(0.05)
T-5	430.73	0.32	1.46 bA	0.61	0.57	2.86
			(0.22)	(0.23)	(0.08)	(0.10)
T-6	0.10	2.50	11.45 dC	0.35 abB	0.87 dBC	2.76 BC
			(3.97)	(0.04)	(0.16)	(0.06)
T-7	150	2.50	2.50 a	0.25 a	0.54 bc	2.86
			(0.14)	(0.06)	(0.07)	(0.15)
T-8	300	2.50	3.68 bcB	0.50 bB	0.61 cCD	2.74
			(0.51)	(0.17)	(0.04)	(0.17)
T-9	300	2.50	4.75 bcB	0.35 abAB	0.38 aA	2.66
			(0.84)	(0.07)	(0.02)	(0.11)
T-10	300	2.50	3.50 abB	0.39 bB	0.42 abAB	2.79
			(0.95)	(0.03)	(0.10)	(0.04)
T-11	450	2.50	4.52 bc	0.30 ab	0.60 abcd	2.69
			(1.32)	(0.06)	(0.21)	(0.04)
T-12	0.10	4.68	7.65 abC	0.17 abA	0.75 abBC	2.51 aA
			(2.42)	(0.07)	(0.24)	(0.05)
T-13	169.27	4.68	5.33 aB	0.10 aA	0.48 a	2.75 b
			(0.81)	(0.02)	(0.08)	(0.10)
T-14	430.73	4.68	8.34 bB	0.37 b	0.61 b	2.80 b
			(1.47)	(0.15)	(0.03)	(0.05)
T-15	0.10	5.00	3.08 B	0.15 aA	0.73 bB	3.00 bD
			(1.68)	(0.04)	(0.01)	(0.07)
T-16	300	5.00	2.61 AB	0.28 bA	0.53 aBC	2.73 a
			(1.45)	(0.02)	(0.01)	(0.11)

Lipid oxidation indices: PV (peroxide value; meq active oxygen kg^{-1} lipids), TBA-i (thiobarbituric acid index; mg malondialdehyde kg^{-1} muscle), FR (fluorescence ratio) and PI (polyene index). For each quality index and for each frozen storage time, mean values followed by different lower-case letters denote significant ($p < 0.05$) differences. For each quality index and for each high-pressure level, mean values followed by different capital letters denote significant ($p < 0.05$) differences. No letters are indicated when significant differences are not found ($p > 0.05$)

[a] Seventeen batches (from T-0 to T-16) were considered in agreement with the statistical experiment design described in the experimental section. Pressure level (0.1, 150, 169.27, 300, 430.73 and, 450 MPa) and frozen storage time (0.00, 0.32, 2.50, 4.68 and 5.00 months) values for each batch are described in the second and third columns, respectively

Experimental Design and Statistical Analysis

The statistical experiment design was formulated using the Design Expert® 7.1.1 software (Stat-Ease, Inc., Minneapolis, MN, USA). The models were validated through a multifactor ANOVA test in agreement with the Box and Behnken (1960) design. Thus, the set of batches (T-0 to T-16) followed a Box-Wilson central composite design (CCD), which had three

groups of design points: (a) two-level factorial or fractional factorial design points, (b) axial points (also called "star" points) and (c) centre points. The present design has five levels of independent variables with desirable statistical properties. This kind of procedure creates designs with desirable statistical properties but with a reduced number of experiments required when using a five-level factorial design.

Data (n = 3) obtained from the different chemical quality indices were subjected to the multifactor ANOVA test to explore differences as a result of pressure level and frozen storage time (i.e. comparison among pressure treatments). Furthermore, data (n = 3) obtained from the different chemical quality indices were subjected to one-way ANOVA method ($p < 0.05$) to explore differences as a result of the freezing step, frozen storage time and pressure level. Comparison of means was performed using a least-squares difference (LSD) method (Statsoft, Statistica, version 6.0, Tulsa, OK, USA).

Lipid Oxidation Assessment

Lipids were extracted by the Bligh and Dyer (1959) method employing a 1:1 chloroform-methanol mixture for the single-phase solubilisation of lipids in the fish muscle. Quantification results were expressed as g lipid kg^{-1} muscle.

The peroxide value (PV) in the lipid extract was determined by peroxide reduction with ferric thiocyanate, according to the Chapman and McKay (1949) method. Results were expressed as meq active oxygen kg^{-1} lipids.

The thiobarbituric acid index (TBA-i) was determined as described by Vyncke (1970) and based on the reaction between a 5% trichloracetic acid extract of the fish muscle and thiobarbituric acid. Content on thiobarbituric acid reactive substances (TBARS) was spectrophotometrically measured at 532 nm and results were expressed as mg malondialdehyde kg^{-1} muscle.

The formation of fluorescent compounds was determined in the aqueous phase obtained during the lipid extraction by measurements at 393/463 and 327/415 nm (Aubourg and Medina 1999). Relative fluorescence (RF) was defined as the F/F_{st} ratio where F is the fluorescence measured at each excitation and emission maximum, and F_{st} is the fluorescence intensity of a 1 μg mL^{-1} quinine sulphate solution in 0.05 M H_2SO_4 at the corresponding wavelength. A fluorescence ratio (FR) was calculated as the ratio between the two RF values, i.e. $FR = RF_{393/463\ nm} / RF_{327/415\ nm}$.

Lipid extracts were converted into fatty acid methyl esters (FAME) by reaction with acetyl chloride and then analysed using a Perkin-Elmer 8700 gas chromatograph equipped with a fused silica capillary column SP-2330 (0.25 mm i.d. × 30 m, 0.20 μm film, Supelco Inc., Bellefonte, PA, USA) and using nitrogen at 68.95 kPa as carrier gas (linear flow rate of 1.0 mL min^{-1}), a flame ionisation detector at 250 °C, and C 19:0 fatty acid as internal standard for quantitative analysis (Torres et al. 2013). Peaks corresponding to fatty acids were identified by comparison of the retention times of two standards mixtures (Qualmix Fish, Larodan, Malmo, Sweden; FAME Mix, Supelco Inc., Bellefonte, PA, USA). Peak areas were automatically integrated. The polyene index (PI) was calculated as the following fatty acids ratio: C20:5ω3 + C22:6ω3/C 16:0.

Determination of Volatile Amines

Total volatile base-nitrogen (TVB-N) values were measured by the Antonacopoulos (1960) method with some modifications. Briefly, 10 g fish muscle were extracted with 60 g L^{-1} perchloric acid in 30 mL water and brought up to 50 mL. An aliquot of the acid extracts was rendered alkaline to pH 13 with 200 g L^{-1} aqueous NaOH and then steam-distilled. Finally, the TVB-N content was determined by titration of the distillate with 10 mM HCl. Results were expressed as mg TVB-N kg^{-1} muscle.

Trimethylamine-nitrogen (TMA-N) values were determined by the picrate method, as previously described by Tozawa et al. (1971). This involves the preparation of a 5% trichloroacetic acid extract of fish muscle (10 g/25 mL). Results were expressed as mg TMA-N kg^{-1} muscle.

Dimethylamine-nitrogen (DMA-N) content was determined in the 5% TCA extracts using the spectrophotometric method of Dowden (1938). This method is based on the specific complex formation from secondary amines in the presence of a copper salt and carbon sulphur in basic medium. Results were expressed as mg DMA-N kg^{-1} muscle.

Other Chemical Assessments

Free fatty acids (FFA) content in the lipid extract was determined by the Lowry and Tinsley (1976) method based on complex formation with cupric acetate-pyridine followed by spectrophotometric assessment at 715 nm (Beckman Coulter DU 640, London, UK). Results were expressed as g FFA kg^{-1} lipids.

Extraction of free formaldehyde (FA) from fish muscle was carried out according to Rey-Mansilla et al. (2001). FA content was determined by the spectrophotometric (Beckman Coulter DU 640 spectrophotometer) method of Nash (1953) and expressed as mg kg^{-1} muscle.

Nucleotide extracts were prepared and analysed by HPLC according to Aubourg et al. (2005). Standard curves for adenosine 5′-triphosphate (ATP) and each compound involved in its degradation pathway, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (INO) and hypoxanthine (HX) were constructed in the 0–1 mM range. Results were

expressed as the *K* value (%) that was calculated according to the following concentrations ratio:

$$K\ value\ (\%) = 100 \times \frac{ATP + ADP + AMP + IMP + INO + HX}{INO + HX}$$

Results and Discussion

Lipid Oxidation Assessment

Concerning peroxides assessment (Table 1), a slight formation ($p < 0.05$) could be observed as a result of the freezing process according to the comparison between T-0 and T-1 batches. Meantime, samples without previous HP treatment showed a marked peroxide formation at month 2.50 (11.45 ± 3.97 meq active oxygen kg^{-1} lipids), followed by a progressive decrease till the end of the experiment (3.08 ± 1.68 meq active oxygen kg^{-1} lipids). Taking into account samples corresponding to each pressure applied (i.e. 169.27, 300 and 430.73 MPa), a PV increase with frozen storage time could be observed in most cases. When the effect of the previous pressure is evaluated, some significant differences ($p < 0.05$) could be observed at each frozen storage time; however, a general trend could not be concluded. Thus, a multifactor ANOVA analysis did not provide a significant ($p > 0.05$) effect of pressure applied on the peroxide formation.

For the secondary oxidation compounds (TBA-i; Table 1), a significant increase was detected as a result of the freezing step in agreement with the comparison between T-0 and T-1 batches. As for the peroxide detection, comparison among samples without previous pressure treatment did not provide a definite trend of TBARS formation throughout the frozen storage time. Thus, a marked increase was obtained till month 0.32 (0.80 ± 0.10 mg malondialdehyde kg^{-1} muscle), followed by a progressive decrease till the end of the study (0.15 ± 0.04 mg malondialdehyde kg^{-1} muscle). A decreasing TBA-i was observed with frozen storage time in HP-treated samples corresponding to 169.27, 300 and 430.73 MPa. Regarding to the effect of the previous pressure applied, a definite trend could not be concluded when comparing the different kinds of samples at each frozen storage time. As for the PV, a multifactor ANOVA analysis did not supply a significant ($p > 0.05$) effect of pressure applied to the TBARS formation.

Tertiary lipid oxidation compounds measured by fluorescence detection showed a significant formation as a result of the freezing step (Table 1). However, comparison among samples without previous pressure treatment did not show ($p > 0.05$) any changes throughout the frozen storage time; furthermore, a general trend about the effect of the frozen storage time could not be obtained for samples corresponding to 169.27, 300 and 430.73 MPa treatments. However, at each frozen storage time, FR mean values were higher in control samples (i.e. 0.10-MPa batches), although significant ($p < 0.05$) differences were only obtained at months 2.50 and 5.00. Therefore, a partial inhibition of tertiary oxidation compounds formation could be concluded as a result of the previous pressure applied. However, a multifactor ANOVA analysis did not provide a significant ($p > 0.05$) effect of pressure applied on the FR value.

Finally, the PI score remained quite stable throughout the study for all kinds of samples, being included in the 2.51–3.00 range (Table 1). Thus, no effect ($p > 0.05$) of the freezing step, frozen storage or the previous pressure applied could be concluded on this quality index.

In order to explain and justify lipid changes produced in the present research, two different and opposite mechanisms ought to be taken into account. On one side, the inhibitory effect of pressure applied on the endogenous enzymes (lipoxygenases, peroxidases, etc.) activity should produce a decrease of lipid oxidation development during the further frozen storage period (Campus 2010; Tabilo-Munizaga et al. 2016). On the other side, iron-bound protein denaturation during HP treatment has been reported to facilitate the increase of free metal ion content, which would lead to a lipid oxidation increase in fish meat after HP treatment (Gudmundsson and Hafsteinsson 2002; Lakshmanan et al. 2003).

Previous related research accounts for contradictory results when the effect of HP treatment on lipid oxidation development in frozen fish is evaluated. Thus, Ohshima et al. (1992) found a TBARS formation increase in frozen cod (*Gadus morhua*) and mackerel (*Scomber scombrus*) muscle that had been subjected to HP treatment at 200–600 MPa for 15–30 min. Later on, the use of pressure-shift freezing at 140 MPa was compared with air-blast freezing at − 20 °C in turbot (*Scophthalmus maximus*) fillets (Chevalier et al. 2000); analysis of TBARS formation during subsequent storage at − 20 °C for 75 days did not provide a definite effect of the previous HP processing. Closely related to the present research, a marked inhibition of tertiary lipid oxidation compounds formation in frozen mackerel (Vázquez et al. 2013) and horse mackerel (Torres et al. 2013) by previous HP treatment at 150–450 MPa for 0.0–5.0 min was obtained; this effect was greater when increasing the pressure level or the pressure holding time.

Concerning PUFA damage, present results agree with previous related research. Thus, Ohshima et al. (1992) did not find differences in saturated, monounsaturated and polyunsaturated fatty acids content in cod (*Gadus morhua*) and mackerel (*Scomber scombrus*) muscle after 6 days of storage at − 2 °C when previously treated under 200–600 MPa for 15 min. Recently, no significant changes in polyene content were obtained in frozen mackerel (Vázquez et al. 2013) and

horse mackerel (Torres et al. 2013) that was previously treated under 150–450 MPa for 0.0–5.0 min.

Volatile Amines Formation

Formation of TVB can be considered as relatively low, being all values included in the 194.3–254.6 mg TVB-N kg^{-1} muscle range (Table 2). The freezing step led to a higher mean value (comparison of T-0 and T-1 batches), although differences were not found significant ($p > 0.05$). Comparison among samples without previous HP treatment did not provide a general trend with the storage time, being obtained the two highest scores in fish corresponding to 2.50 and 5.00 months; a general trend about the effect of the frozen storage time could not be observed for samples corresponding to 169.27, 300 and 430.73 MPa treatments. Concerning the pressure effect, a higher mean score was obtained at most frozen storage times in hake belonging to the 0.10-MPa batch, showing significant differences ($p < 0.05$) at months 0.00 and 2.50. Accordingly, an inhibition of TVB formation could be concluded at such storage times as a result of the previous pressure applied. However, if multifactor ANOVA analysis (pressure and frozen storage time effects) is checked (i.e. comparison among pressure treatments), a significant ($p > 0.05$) effect on the total volatile amines formation was not obtained.

TMA formation revealed a significant increase ($p < 0.05$) as a result of the freezing step (Table 2). Furthermore, a relevant formation was observed with frozen storage time when comparing the different samples corresponding to batches without previous HP treatment, so that a 9.70 ± 2.82 mg TMA-N kg^{-1} muscle value was reached at the end of the experiment; a general trend concerning the effect of frozen storage time on TMA formation in each of the pressures applied (i.e. 169.27, 300 and 430.73 MPa) could not be reached. When the previous pressure effect is analysed, lower TMA-N values ($p < 0.05$) were detected in HP-treated fish at months 0.00, 2.50 and 5.00. Thus, an inhibition of the TMA-N value could be concluded at those times as a result of the previous pressure applied. However, when a multifactor ANOVA analysis (pressure and frozen storage time effects) was taken into account (namely, comparison among pressure treatments), a significant ($p > 0.05$) effect on the TMA formation was not implied.

A marked DMA formation was detected as a result of the freezing step (Table 2). A progressive formation of DMA could be observed in 0.10-MPa samples with frozen storage time for the 0.00–2.50-month period, being then the highest mean value reached at the end of the experiment. An increasing DMA content could also be observed with frozen storage time for samples corresponding to 169.27, 300 and 430.73 MPa. When considering the previous pressure effect, a higher mean DMA-N score was obtained at all frozen storage times in samples corresponding to fish without previous pressure treatment; differences were significant ($p < 0.05$) after 2.50 and 5.00 months. This effect was found to increase with the previous pressure applied. Accordingly, a relevant inhibitory effect on the formation of this degradative compound was implied by previous pressure treatment.

Furthermore, the multifactor ANOVA analysis (pressure and frozen storage time effects) indicated an important effect on DMA formation, yielding a significant ($p < 0.0002$) model with an F value of 27.99. The evaluation of the F values for the two independent variables confirmed their individual effects. Thus, DMA formation was highly affected by frozen storage time (F value = 51.66; p value probability > F was $p < 0.0001$); meantime, an important effect of pressure level was also observed (F value of 4.33; p value probability > F was $p < 0.0710$). The correlation value of the model was $r^2 = 0.8750$ with adjusted and predicted r^2 values of 0.8437 and 0.7405, respectively, and a signal/noise ratio of 13.78. These statistical parameters confirmed that an empirical coded equation (Eq. 1) could be used to model the effect of previous pressure applied and frozen storage time on the DMA formation.

$$Y = 1.8426–0.2657x_1 + 0.9177x_2 \quad (1)$$

In this equation, Y is the DMA-N value (mg kg^{-1} muscle), x_1 is the coded variable for pressure level (MPa) and x_2 is the coded variable for frozen storage time (months). Figure 1 shows the graph that represents the prediction of the model for the DMA formation.

In the present research, TMA and DMA, well-known for their characteristic off-flavours, revealed an important formation as a result of the freezing step, as well as throughout the frozen storage time. Since microbial activity is not likely to occur under the actual experimental conditions, TMA formation can be explained on the basis of protein damage (Méndez et al. 2017). Concerning DMA, its formation during the current study has shown to be the most important event concerning volatile amines formation. According to the general behaviour of most Gadiform species under frozen conditions, its formation can be explained on the basis of the TMAO breakdown catalysed by an endogenous enzyme widely present in such fish species (i.e. TMAOase) and be produced equimolecularly with FA. Differently to TMA results, a marked inhibitory effect of previous pressure treatment on DMA formation was proved in the current study, this effect being increased with the pressure level applied. This result can be attributed to the TMAOase damage resulting from the previous pressure treatment, this leading to an inhibitory effect of its activity. In agreement with the strong impact on the quality loss of frozen Gadiform species, this inhibitory effect should lead to an increased quality and accordingly, to an increased added value. To our knowledge, no previous research accounts for the inhibitory effect of HP treatment on DMA formation during a further frozen storage of hake.

Table 2 Volatile amines formation in frozen hake muscle previously processed under different pressure levels

Batch number[a]	Pressure (MPa)	Storage time (months)	Quality index		
			TVB-N	TMA-N	DMA-N
T-0	Starting fresh fish		201.62 abA	0.20 aA	1.83 aA
			(3.90)	(0.05)	(0.41)
T-1	0.10	0.00	211.36 bAB	1.11 cB	5.29 bB
			(5.80)	(0.33)	(1.54)
T-2	300	0.00	196.66 aA	0.43 bA	3.83 bA
			(4.80)	(0.00)	(1.10)
T-3	0.10	0.32	217.64 aB	1.06 aB	11.20 C
			(4.95)	(0.07)	(2.35)
T-4	169.27	0.32	229.42 b	7.66 bB	10.90 A
			(5.28)	(1.81)	(4.82)
T-5	430.73	0.32	213.78 a	1.43 a	9.46 A
			(9.78)	(0.20)	(2.85)
T-6	0.10	2.50	254.60 cC	5.92 cC	46.74 dDE
			(12.83)	(0.54)	(12.20)
T-7	150	2.50	214.98 b	2.05 b	19.59 abc
			(6.83)	(0.29)	(1.72)
T-8	300	2.50	207.37 abAB	1.79 bB	19.79 bcB
			(11.06)	(0.44)	(0.53)
T-9	300	2.50	203.38 abAB	3.27 bBC	18.61 abB
			(8.09)	(2.31)	(1.71)
T-10	300	2.50	211.38 bB	3.08 bBC	22.68 cC
			(2.55)	(1.06)	(2.02)
T-11	450	2.50	194.38 a	0.91 a	15.91 a
			(10.28)	(0.14)	(3.11)
T-12	0.10	4.68	220.94 B	4.62 bC	35.15 bD
			(13.97)	(1.33)	(7.51)
T-13	169.27	4.68	206.63	4.00 bA	34.31 bB
			(15.30)	(0.83)	(6.88)
T-14	430.73	4.68	198.71	1.60 a	22.34 aB
			(4.78)	(0.29)	(1.84)
T-15	0.10	5.00	252.23 C	9.70 bD	54.82 bE
			(10.86)	(2.82)	(6.14)
T-16	300	5.00	235.51 C	3.95 aC	25.25 aC
			(7.74)	(0.76)	(4.44)

Volatile amine indices: TVB-N (total volatile amines-nitrogen; mg kg^{-1} muscle), TMA-N (trimethylamine-nitrogen; mg TMA-N kg^{-1} muscle) and DMA-N (dimethylamine-nitrogen; mg DMA-N kg^{-1} muscle). For each quality index and for each frozen storage time, mean values followed by different lower-case letters denote significant ($p < 0.05$) differences. For each quality index and for each high-pressure level, mean values followed by different capital letters denote significant ($p < 0.05$) differences. No letters are indicated when significant differences are not found ($p > 0.05$)

[a] Description of batches as expressed in Table 1

Previous research related to volatile amines formation and TMAOase activity inhibition by HP treatment can be considered scarce. Thus, Malinowska-Pańczyk and Kołodziejska (2016) showed that formation of DMA in frozen minced cod (*Gadus morhua*) meat after 40 days at − 5 °C was strongly reduced if previously HP-treated at 193 MPa. Additionally, Gou et al. (2010) reduced the TMAOase activity and TMA and DMA formation in refrigerated squid (*Todarodes pacificus*) by previous treatment at 300 MPa for 20 min, while Malinowska-Pańczyk and Kołodziejska (2016) showed that

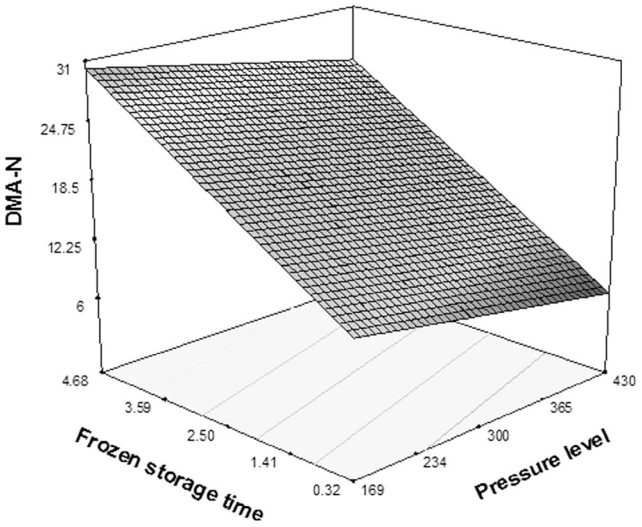

Fig. 1 Prediction model obtained for the effect of pressure level (MPa) and frozen storage time (months) on the dimethylamine-nitrogen (DMA-N) content (mg kg^{-1} muscle) in frozen hake muscle previously processed under high-pressure conditions

formation of TMA in refrigerated minced cod (*Gadus morhua*) meat was strongly reduced if previously HP-treated at 193 MPa. Finally, Gou et al. (2012) reduced the formation of TMA and DMA in semi-dried squid (*T. pacificus*) by previous treatment at 500 MPa for 0–10 min. However, when a pelagic fatty fish species was concerned (sardine; Méndez et al. 2017), a progressive TMA content was obtained throughout a 9-month frozen storage at − 18 °C, but no inhibitory effect of a previous treatment at 125 MPa was implied.

Assessment of Other Chemical Indices

Comparison between fish corresponding to T-0 and T-1 batches proved a great formation ($p < 0.05$) of FFA as a result of the freezing step (Table 3). FFA values obtained for samples corresponding to 0.10-MPa batches revealed a significant ($p < 0.05$) increase with the frozen storage time throughout the whole experiment. An increasing FFA content could also be observed with frozen storage time for samples corresponding to 169.27, 300 and 430.73 MPa. Concerning the pressure effect, at each frozen storage time, a lower FFA value could be obtained by increasing the previous pressure applied, being the differences significant ($p < 0.05$) in most cases. Consequently, an inhibitory effect on FFA formation could be concluded by previous HP treatment. Present results concerning the inhibition of FFA formation in frozen hake can be explained on the basis of the lipases and phospholipases inactivation produced during the previous HP treatment, so that a lower activity of such enzymes would be developed during the frozen storage.

Furthermore, the multifactor ANOVA analysis (pressure and frozen storage time effects) yielded a significant ($p < 0.0001$) model with an F value of 36.33. The evaluation of the F values for the two independent variables confirmed their individual effects. Thus, FFA formation was highly affected by frozen storage time (F value = 63.10; p value probability > F was $p < 0.0001$); meantime, an important effect of pressure level could be observed (F value of 9.55; p value probability > F was $p < 0.0149$). The correlation value of the model was $r^2 = 0.9008$ with adjusted and predicted r^2 values of 0.8760 and 0.8061, respectively, and a signal/noise ratio of 16.41. These statistical parameters confirmed that an empirical coded equation (Eq. 2) could be used to model the effect of previous pressure applied and frozen storage time on the FFA formation.

$$Y = 10.2267 – 0.0148 x_1 + 2.2885 x_2 \quad (2)$$

In this equation, Y is the FFA value (g kg^{-1} lipids), x_1 is the coded variable for pressure level (MPa) and x_2 is the coded variable for frozen storage time (months). Figure 2 shows the graph that represents the prediction of the model for the FFA formation.

Reported research concerning the effect on FFA formation after HP treatment followed by a frozen storage is scarce. Thus, Ohshima et al. (1992) found that enzymatic degradation of phospholipids (i.e. phospholipases action) in cod (*Gadus morhua*) muscle was successfully inhibited during storage at − 2 °C for 6 days when previously treated at pressures above 400 MPa for 15 and 30 min; however, no effect was observed when applying a pressure of 200 MPa. Later on, freezing by pressure-shift freezing at 140 MPa was compared with air-blast freezing at − 20 °C in turbot (*Scophthalmus maximus*) fillets (Chevalier et al. 2000); as a result, FFA formation during subsequent storage at − 20 °C for 75 days did not provide differences. Finally, Atlantic mackerel (*Scomber scombrus*; Vázquez et al. 2013) and horse mackerel (*Trachurus trachurus*; Torres et al. 2013) were subjected to HP treatment at 150–450 MPa for 0.0–5.0 min prior to frozen storage at − 10 °C for 3 months; the results revealed a marked reduction of FFA content for all HP pre-treated samples, with the reduction effect increasing with the previous pressure level and the pressure holding time employed.

No FA formation could be observed as a result of the freezing step (Table 3). However, evaluation of frozen hake without previous HP treatment led to a progressive FA formation with storage time; thus, a marked FA formation was reached at month 2.50, while the highest mean value was obtained at the end of the experiment. An increasing FA content could also be observed with frozen storage time for samples corresponding to 169.27, 300 and 430.73 MPa treatments. Concerning the effect of the applied pressure, higher mean FA values were obtained in fish corresponding to batches without previous pressure treatment (0.32–5.00-month period), showing significant differences ($p < 0.05$) at months 2.50 and 5.00. Thus, a marked inhibition of FA formation was obtained in frozen hake with

Table 3 Assessment of free fatty acid (FFA; g kg^{-1} lipids) formation, formaldehyde (FA; mg kg^{-1} muscle) content and *K* value (%) in frozen hake muscle previously processed under different pressure levels

Batch number[a]	Pressure (MPa)	Storage time (months)	Quality index		
			FFA	FA	*K* value
T-0	Starting fresh fish		25.70 aA	0.83 A	6.98 aA
			(1.31)	(0.05)	(3.61)
T-1	0.10	0.00	47.48 cB	0.70 A	16.77 abBC
			(5.39)	(0.07)	(6.31)
T-2	300	0.00	35.56 bA	0.86 A	17.67 b
			(1.70)	(0.08)	(1.86)
T-3	0.10	0.32	88.35 bC	0.91 bA	16.44 B
			(13.25)	(0.10)	(2.25)
T-4	169.27	0.32	74.20 bA	0.72 aA	14.70
			(5.62)	(0.06)	(2.23)
T-5	430.73	0.32	52.92 aA	0.77 abA	16.07
			(2.85)	(0.08)	(6.04)
T-6	0.10	2.50	266.00 eD	6.60 dBC	26.48 D
			(23.01)	(2.80)	(5.94)
T-7	150	2.50	155.94 d	1.66 c	20.00
			(13.46)	(0.15)	(4.62)
T-8	300	2.50	121.87 cC	1.47 bC	20.70
			(2.76)	(0.05)	(2.62)
T-9	300	2.50	112.32 bB	1.17 aB	21.06
			(3.41)	(0.15)	(4.66)
T-10	300	2.50	138.70 cdC	1.67 cD	20.83
			(21.41)	(0.05)	(2.85)
T-11	450	2.50	99.83 a	1.45 ab	22.65
			(2.19)	(0.25)	(8.04)
T-12	0.10	4.68	327.98 cE	4.01 bB	22.12 CD
			(11.75)	(1.24)	(1.16)
T-13	169.27	4.68	171.97 bB	4.06 bB	21.05
			(4.24)	(0.89)	(4.95)
T-14	430.73	4.68	128.96 aB	2.05 aB	25.27
			(8.77)	(0.27)	(2.13)
T-15	0.10	5.00	338.77 bE	7.00 bC	29.61 D
			(12.92)	(1.20)	(2.50)
T-16	300	5.00	172.33 aD	2.46 aD	21.56
			(16.49)	(0.82)	(4.32)

For each quality index and for each frozen storage time, mean values followed by different lower-case letters denote significant ($p < 0.05$) differences. For each quality index and for each high-pressure level, mean values followed by different capital letters denote significant ($p < 0.05$) differences. No letters are indicated when significant differences are not found ($p > 0.05$)

[a] Description of batches as expressed in Table 1

previous applied pressure. In agreement with previous comments on inhibition of DMA formation, the lower FA content found in HP-treated fish can be explained on the basis that previous pressure treatment has led to a TMAOase damage, so that an inhibitory effect on the activity of such enzyme was produced during the frozen storage.

Furthermore, the multifactor ANOVA analysis (pressure and frozen storage time effects) yielded a significant ($p < 0.0001$) model with an *F* value of 41.42. The evaluation of the *F* values for the two independent variables showed that FA formation was highly affected by frozen storage time (*F* value = 81.77; *p* value probability > *F* was $p < 0.0001$); meantime, a not significant effect of pressure could be observed (*F* value 0.62; *p* value probability > *F* was $p < 0.4564$). The correlation value of the model was $r^2 = 0.9221$ with adjusted and predicted r^2 values of 0.8998 and 0.8623, respectively,

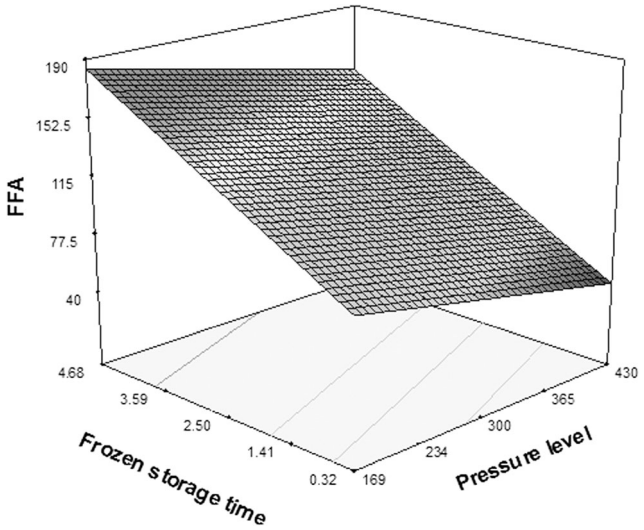

Fig. 2 Prediction model obtained for the effect of pressure level (MPa) and frozen storage time (months) on the free fatty acids (FFA) content (g kg^{-1} lipids) in frozen hake muscle previously processed under high-pressure conditions

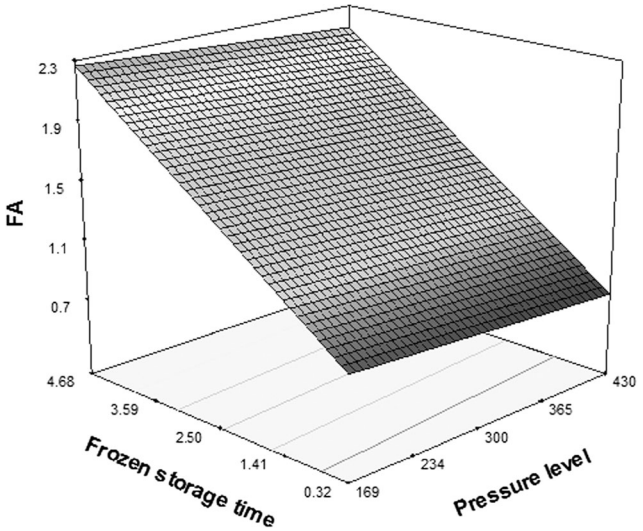

Fig. 3 Prediction model obtained for the effect of pressure level (MPa) and frozen storage time (months) on the formaldehyde (FA) content (mg kg^{-1} muscle) in frozen hake muscle previously processed under high-pressure conditions

and a signal/noise ratio of 16.44. These statistical parameters confirmed that an empirical coded equation (Eq. 3) could be used to model the effect of previous pressure applied and frozen storage time on the FA formation.

$$Y = 150.3200–6.1268x_1 + 70.2936x_2 \quad (3)$$

In this equation, Y is the FA content (mg kg^{-1} muscle), x_1 is the coded variable for pressure level (MPa) and x_2 is the coded variable for frozen storage time (months). Figure 3 shows the graph that represents the prediction of the model for the FA formation.

Previous research concerning the effect of HP treatment on FA formation can be considered very scarce. Thus, Malinowska-Pańczyk and Kołodziejska (2016) showed that formation of FA and inhibition of TMAOase activity in frozen minced cod (*Gadus morhua*) meat were strongly reduced if previously treated at 193 MPa at − 20 °C.

Mean K value showed a marked increase as a result of the freezing step (Table 3), although differences were not significant ($p > 0.05$). Evaluation of samples without previous HP treatment showed some increasing tendency with frozen storage time, leading to a 29.61 ± 2.50 score at the end of the storage; similarly, an increasing trend with frozen storage time was implied in samples corresponding to 169.27, 300 and 430.73 MPa. When analysing the effect of the previous pressure applied, significant differences were not obtained at any of the frozen storage times considered, so that it could be concluded that the previous HP treatment did not affect the K value in frozen hake. Accordingly, a multifactor ANOVA analysis did not provide a significant ($p > 0.05$) model about the effect of the two variables of the process, i.e. pressure level and frozen storage time.

Previous research accounts for a K value decrease in refrigerated and chilled fish by means of inactivation of enzymes that catalyse ATP degradation by previous HP treatment. Such results concern tilapia (*Oreochromis niloticus*) fillets (Ko and Hsu 2001), yellowfin tuna (*Thunnus albacares*) chunks (Kamalakanth et al. 2011) and white prawn (*Fenneropenaeus indicus*) (Ginson et al. 2013). However, no information is available to our knowledge concerning the HP effect on the K value in frozen fish.

Conclusions

The effect of a previous HP treatment at 150–450 MPa for 2 min on frozen hake quality throughout a 5-month storage at − 10 °C was studied. Inhibition ($p < 0.05$) of DMA, FFA, FA, TMA, TVB-N and fluorescent compounds formation was obtained by previous pressure treatment according to a one-way ANOVA test. On the contrary, no effect ($p > 0.05$) on the K value, PI and formation of peroxides and TBARS was observed in the present study. Additionally, a multifactor ANOVA test (pressure and frozen storage time effects; i.e. comparison among HP treatments) showed an inhibitory effect ($p < 0.015$) on DMA and FFA formation as a result of the previous HP treatment, this effect increasing with pressure level applied. This inhibitory effect on the formation of such molecules related to quality loss can be explained on the basis of the damage caused to different kinds of enzymes such as TMAOase, lipases and phospholipases, so that their activity during the subsequent frozen storage would decrease.

The frozen storage of most Gadiform species is known to be strongly limited by the TMAO breakdown into DMA and

FA, two highly deteriorative compounds. The work here presented provides for the first time with information concerning the employment of the HP technology to inhibit the formation of DMA, FA and FFA during the frozen storage of hake. Further research focussed on commercial frozen conditions (− 18 °C) and including sensory and nutritional aspects is foreseen.

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