Obtaining reduced-salt restructured white tuna (*Thunnus alalunga*) mediated by microbial transglutaminase

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ABSTRACT

White tuna (*Thunnus alalunga*) is a blue fish with nutritional significance and high benefits to health. The aim of this work was to develop white tuna restructured products with reduced-salt (15 g/kg) using transglutaminase. Textural profile analysis, expressible water, dry matter, water activity and colour parameters were measured. In a first study, the temperature effect of the setting phenomenon was evaluated at three conditions: 4 ºC/12 h, 25 ºC/2 h or 40 ºC/20 min. The results showed that texture parameters were improved using transglutaminase at 4 ºC/12 h. Considering the results, in a second study the setting at 4ºC was selected and the transglutaminase concentration (150, 300, 450 U/kg) and setting time (6, 12, 18 h) were optimized. The better setting treatment was at 4ºC for 18 h using 450 U/kg of transglutaminase. Restructured products were obtained under these conditions and in a third study the microbial load was evaluated during the storage at 4ºC for 12 days. Different microbiological groups (psychrotrophs, aerobic mesophiles, anaerobes, proteolytic bacteria and Enterobacteriaceae) were measured. The results showed that the manipulation after the cooking step is critical. Overall, it was demonstrated that the restructuring of white tuna is feasible.

Keywords: restructured fish products; transglutaminase; texture; *Thunnus alalunga*, setting

1. Introduction

White tuna (*Thunnus alalunga*) is mainly commercialized fresh or in canned preserves. It is a blue fish with mild flavour. Its protein content has large nutritional significance with high benefits to health. As functional food, its flesh has high levels of polyunsaturated fatty acids (PUFA) with anticholesterol, antithrombotic activity, anti-inflammatory, antiarrhythmic and vasodilatory activity (Moreno, Carballo, & Borderías, 2008). Moreover, the protein digestibility and high nutritive value of "ready-to-eat" meals prepared from this fish are appreciated in several countries (Wang et al., 2013; Hu et al., 2014).

Current knowledge on fish muscle gelation processes (initially developed for surimi-based products) allows to obtain novel foods resembling cooked ham or turkey breast using fish muscle (Martelo-Vidal, Mesas, & Vazquez, 2012). The application of new ingredients allows new mechanical properties or appearance in products with low salt or low cholesterol and fats (Andrés-Bello, García-Segovia, Ramírez, & Martínez-Monzó, 2011; Herranz, Tovar, Borderias, & Moreno, 2013).

Restructuring technology allows to use several commercial and non-commercial fish species as well as trimmings from filleting. The application of restructuring technology to high-value mince of white tuna provides a high-quality meal with high nutritive value (Ramirez, Uresti, Velazquez, & Vazquez, 2011).

To obtain fish restructured products, the process needs the following steps: i) extraction and solubilisation of myofibrillar proteins with salt; ii) setting phenomenon at 0-40 ºC; ii) denaturation of protein structure applying heat (90 ºC for at least 40 min) allowing aggregation of proteins in organized structure matrix (Ramirez, Uresti, Velazquez, & Vazquez, 2011; Yin, Reed, & Park, 2014).

After being mixed with salt, sols prepared below 40°C from most fish species have a unique ability to form a highly deformable gel. This process is called setting. A stronger gel results by heating at higher temperatures than by cooking directly (Ramirez, Rodriguez-Sosa, Morales, & Vazquez, 2003). Setting phenomenon improves functional and mechanical properties of gels and it is dependent of habitat temperature of fish species (Martínez, Robledo, Velazquez, Ramírez, Vázquez, & Uresti, 2014). Furthermore, adding microbial transglutaminase (protein–glutamine gamma-glutamyltransferase, EC 2.3.2.13, MTG) to mince improve several functional properties of the gels obtained (Martínez, Robledo, Velazquez, Ramírez, Vázquez, & Uresti, 2014; Song & Zhao, 2014), such as mechanicals properties, heat stability, gelation and emulsifying (Hinz, Huppertz, Kulozik, & Kelly, 2007). The use of MTG in fish restructured products was firstly used in surimi industry and quickly applied in other protein foods (Cortez-Vega, Fonseca, Feisther, Silva, & Prentice, 2013; Castro-Briones, Calderon, Velazquez, Rubio, Vazquez, & Ramirez, 2009).

Transglutaminase is a family of enzymes that encourage polymerization of the cross-linking of proteins (Guerra-Rodríguez & Vázquez, 2014; Kudre & Benjakul, 2014). The endogenous transglutaminase is calcium-dependent (Dluzewska, Marciniak-Lukasiak, & Kurek, 2015). When calcium is added to fish mince and the setting phenomenon occurs at the range 4-

40ºC, good gels can be obtained after cooking at 90ºC (Ramirez, Rodriguez-Sosa, Morales, & Vazquez, 2003). MTG is different from fish transglutaminase because MTG is calcium independent (Tellez Luis, Ramirez, & Vazquez, 2004).

Activity of MTG is highly dependent on temperature (Yao & Zhao, 2015). Optimal activity is at 60 ºC. On the other hand, the effect of transglutaminase is promptly inhibited by completely denatured myofibrillar protein aggregates (Martínez, Robledo, Velazquez, Ramírez, Vázquez, & Uresti, 2014).

The fish mince is sterile but the skin, gills, guts and mucus contain high levels of microorganisms (Ananou, Zentar, Martínez-Bueno, Gálvez, Maqueda, & Valdivia, 2014). Fish and fish products have high water content, about 65 to 80 %, large amount of non-protein nitrogen and pH post mortem of 6-7 (Cosansu, Mol, Alakavuk, & Ozturan, 2013). Consequently, the fish preservation is difficult due to microbial growth and physicochemical degradations (enzymatic and chemicals degradations). Microbial contamination causes changes in appearance, textural characteristics and mainly the quality of these foods. Therefore, it is important to determine and to study the bacterial load as well as textural profile guarantying quality of fish products (Atanassova et al., 2014; Carrascosa et al., 2014).

The aim of this work was to develop white tuna (*Thunnus alalunga*) restructured products and to optimize the setting phenomenon of the process. The effect of setting conditions using MTG and a reduced-salt content on mechanical properties were evaluated. Microbial load on the optimal restructure product was also determined.

2. Materials and methods

2.1. *Raw Materials*

White tuna (*Thunnus alalunga*) was caught near the Galician coast in Northwest Spain at the Celeiro harbour (Lugo, Spain) and transported into ice in polystyrene boxes to the laboratory (Lugo, Spain) within 8 h after the harbour discharge. Skin and bones were removed manually. MTG was manufacture in our laboratory following the process described in our Spanish patent (Vázquez & Guerra-Rodriguez, 2012). The enzyme activity was determined by the colorimetric procedure (Grossowicz, Wainfan, Borek, & Waelsch, 1950).

2.2. *Methods*

2.2.1. Production of white tuna restructured products

Fish muscle was chopped and washed with distilled water (ratio muscle:water, 1:3) at temperature lower than 15 ºC for 5 min (Martelo-Vidal, Mesas, & Vazquez, 2012). Then it was drained and the tuna paste was mixed with NaCl and MTG for 5 min. It was stuffed in stainless steel molds. The filled molds were immersed in water bath to allow the setting phenomenon. After the setting phenomenon, they were heated in water bath at 90 ºC for 45 min, cooled in ice-water bath for 5-7 min and stored in fridge at 4ºC for 12 h.

The effect of the setting phenomenon was assayed at three conditions: 4ºC for 12 h, 25 ºC for 2 h or 40 ºC for 20 min. The experiments were performed using 15 g/kg NaCl and 300 U of MTG per kg of muscle. The MTG concentration was fixed on the basis of previous literature (Martelo-Vidal, Mesas, & Vazquez, 2012). Controls at the same setting conditions without MTG were assayed. After selection the best setting temperature, an experimental design was followed to evaluate the optimal MTG concentration and time of the setting phenomenon (see below).

2.2.2. Mechanical properties

Samples of restructured white tuna were equilibrated at room temperature for 30 min in plastic bags to avoid dehydration. Then restructured products were cut into cube of 2 x 2 x 2 cm. Mechanical properties were determined using a TA-XTplus (Stable Micro System, Viena Court, UK). Texture Profile Analysis (TPA) was carried on using cylindrical aluminium probe (P/50) of 50 mm of diameter. Samples were compressed to 75% of their original height using a compression speed of 60 mm/min (Andrés-Bello, García-Segovia, Ramírez, & Martínez-Monzó, 2011). Hardness, adhesiveness, springiness, cohesiveness and chewiness were determined for each treatment and ten samples were analysed for each treatment (Aubourg, Torres, Saraiva, Guerra-Rodríguez, & Vázquez, 2013; Torres, Saraiva, Guerra-Rodríguez, Aubourg, & Vázquez, 2014).

2.2.3. Expressible water, water activity and dry matter

Expressible water (Ew) for each treatment was analysed. Samples were weighed (2 ± 0.2 g) and put in two layers of filter paper disk. Then they were placed in 10 mL centrifuge tubes and centrifuged 1000 g at 4ºC for 15 min. After centrifugation, wet disk filter papers were removed and sample were weighed. The percentage of expressible water was calculated as equation (1):

$$Ew = \frac{G_0 - G}{G_0} \cdot 100 \quad (1)$$

Where Ew is the percentage of expressible water, G_0 is the initial weight (g) and G is the final weight. Four samples were analysed for each treatment (Martelo-Vidal, Mesas, & Vazquez, 2012).

Furthermore, water activity (A_w) values were determined for each treatment using AquaLab meter (Pullman, USA). Three replicates were analysed for each treatment.

Percentage of dry matter was determined by triplicate for each treatment. Samples were weighed (5 ± 0.2 g) and put into a crucible preweighed. Then samples were drayed for 24 h in 90 ºC. Following, samples were reweighed and then dry matter were determined as equation (2)

$$DM = \frac{P_d}{P_w} \cdot 100 \quad (2)$$

Where DM is the percentage of dry matter, P_d is the dry weigh (g) and P_w is the wet weight.

2.2.4. Colour determinations

Colour of samples was determined using a ColorStriker meter (Mathai, Hannover, Alemania) (Uresti, Lopez-Arias, Ramirez, & Vazquez, 2003). Values of L* (Lightness), a*(redness) and b*(yellowness) were calculated based on illuminant C and the 2º standard observer. Eight samples were measured for each treatment.

2.2.5. Experimental design and statistical analysis

Duncan's multiple-range test at a significance level of 0.05 was used for the comparison of the mean values between treatments.

An experimental design to evaluate the MTG concentration and time for the setting were performed. Results were analysed by response surface using Design Expert 7.1.1 software (Stat-Ease Inc., Minneapolis, Minnesota, USA). A derived Box-Behnken (Box & Behnken, 1960) design was performed (Martelo-Vidal, Mesas, & Vazquez, 2012; Aubourg, Torres, Saraiva, Guerra-Rodríguez, & Vázquez, 2013).

All experiments were performed at 4 ºC as setting temperature. The variables studied were MTG (TG: 150, 300 and 450 U/kg) and setting time (t: 6, 12 and 18 h). The normalized dimensionless variables were expressed as functions of real variables in the following form (Guerra-Rodríguez, Portilla-Rivera, Ramírez, & Vázquez, 2012):

$$X_1 = \frac{TG - 300}{150} \quad (3)$$

$$X_2 = \frac{t - 12}{6} \quad (4)$$

The interrelationship between the studied variables, the independent and dependent variables were established through a model that includes linear terms, interaction and second order terms. The mathematical model used as a first approximation can be summarized in the equation (5)

$$V = a_0 + \sum a_i x_i + \sum b_i x_i^2 + c x_1 x_2 \quad (5)$$

Where V is the dependent variable in every case (hardness, adhesiveness, springiness, cohesiveness, chewiness, Ew, Aw, DM, L*, a* and b*), x_i is the independent variable normalized (x_1 or x_2), a_0, a_i, b_i and c are the coefficients of the regression calculated with the experimental values by multiple linear regressions. Model terms were selected based on the p-value with 95% confidence level. Partial models of the quadratic model were also obtained and analysed by ANOVA (Guerra-Rodríguez & Vázquez, 2013).

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194 *2.2.6. Bacteriological analysis and pH*
195 Bacterial load was determined at 0, 5 and 12 days after processing. Samples of
196 restructured products were weighed (25 g) and put in sterile bag (Nasco, Fort Atkinson, Wis)
197 with 225 mL of 0.1% peptone water (BPW, Fluka). Samples were homogenised and from these
198 suspensions were prepared nine decimal dilutions in peptone water by mixing 1 mL of the
199 previous dilution with 9 mL of sterile peptone water.
200 Total viable counts of aerobic mesophiles and psychrotrophic bacteria were determined
201 using Plate Count Agar (PCA, Fluka) (Carrascosa et al., 2014). Anaerobic bacteria were also
202 determined using PCA and plates were placed at anaerobic atmosphere inside anaerobiosis
203 jars. Plates used to aerobic and anaerobic bacteria were incubated at 30 ºC ± 1ºC for 48 h and
204 plates used to count psychrotrophic bacteria were incubated at 4ºC for 10 days. Enterobacteriae
205 were determined using Violet Red Bile Glucose (Fluka) incubated 30 ºC ± 1 ºC for 24 h (García-
206 Soto, Fernández-No, Barros-Velázquez, & Aubourg, 2014). Proteolytic bacteria were
207 determined in casein agar medium incubated at 30 ºC ± 1 ºC for 48 h (Ben-Gigirey, Vieites
208 Baptista de Sousa,Juan M., Villa, & Barros-Velazquez, 2000).
209 Results of bacteria counts were transformed into log of the number of colony-forming
210 units per gram (log ufc/g) of product (Muela, Alonso, Morago, Calanche, Roncales, & Beltran,
211 2014). All microbiological analyses were performed in duplicate and using a blank to discard
212 external contaminations.
213 The pH was measured using a portable pH meter (PCE-PH22, PCE Ibérica, Albacete,
214 Spain) equipped with a penetration probe.
215
216 **3. Results and discussion**
217
218 *3.1. Selection of setting temperature*
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220 Cold-water fishes need lower temperature to denature the muscle proteins than hot-
221 water fishes (Ramirez, Uresti, Velazquez, & Vazquez, 2011). Therefore to obtain good gels
222 using cold-water fishes a setting phenomenon at 4 ºC for 12-24 h or 25 ºC for 2 h is
223 recommended meanwhile for warn-water fishes a setting phenomenon at 40 ºC for 20 min is
224 recommended (Morales, Ramirez, Vivanco, & Vazquez, 2001). White tuna is a cold-water fish
225 but it can be found also in warn-water areas. For that reason, in a first study the effect of the
226 setting temperature was evaluated at three levels: 4 ºC for 12 h, 25 ºC for 2 h or 40 ºC for 20
227 min.
228
229 *3.1.1. Texture Parameters*
230 Table 1 shows the results for the textural parameters (hardness, adhesiveness,
231 springiness, cohesiveness and chewiness) of the restructured products obtained with or without
232 MTG (control).

Comparing the values of the textural parameters obtained without MTG, the setting at 25ºC/2 h showed the best mechanical properties with a significant high value for the chewiness. This occurs probably because at this temperature endogenous transglutaminase was active and the endogenous proteases were less active than at 40 ºC. However, the adhesiveness showed a significant low value. It means the significant highest adhesiveness.

When MTG was added, the textural parameters were improved for all time/temperature setting conditions. The setting treatment at 4 ºC/12 h using MTG showed the significant highest values for hardness (20981.22 g), springiness (0.793) and cohesiveness (0.416). It was also obtained the product with the significant least adhesiveness (-2.15 g·s) and the highest chewiness (6930.35 g).

Overall, the mechanical properties of restructured products obtained with 1.5% NaCl and 300 U of MTG/kg of muscle were better than those of the controls obtained using 1.5% NaCl without MTG.

3.1.2. Expressible water, water activity and dry matter

The expressible water (Ew) is associated with water holding capacity. A low value of expressible water means a high water holding capacity (Ramirez, Rodriguez-Sosa, Morales, & Vazquez, 2003; Morales, Ramirez, Vivanco, & Vazquez, 2001). Ew affects product juiciness. Therefore restructuring process should have a positive effect on Ew parameter to preserve an acceptable sensory quality of final products (Aubourg, Torres, Saraiva, Guerra-Rodríguez, & Vázquez, 2013).

Figure 1a showed the results of Ew. This parameter varied from 36.37 % to 43.71 % for restructured product without MTG and from 32.77 % to 37.00 % for restructured product with MTG. When the setting was performed at 4 ºC for 12 h (with or without MTG), Ew values were lower than the obtained for the other setting conditions. Adding MTG improved the value of Ew and the juiciness of the product. The best Ew was the lowest value (32.77 %) obtained using MTG at 4ºC for 12 h. This value compares very well with those of commercial products of turkey breast (Martelo-Vidal, Mesas, & Vazquez, 2012).

Water activity (Aw) is shown in Figure 1b. It varied in the range 0.970-0.980 for the products without MTG and in the range 0.969-0.980 for product with MTG. It is interesting to obtain products with low Aw because it improves the self-life of the product, reducing the rate of enzyme reactions, chemical reactions and microbial growth.

When setting treatment was performed at 4 ºC for 12 h without MTG, water activity showed high values (0.980 ± 0.004). However, at the same temperature using MTG, the water activity decreased up to 0.969 ± 0.005.

Results of Dry matter (DM) are shown in Figure 1c. It was observed a clear increase in the DM of the products obtained using MTG. This suggests that MTG made bonds that retain proteins in the structure of the product, increasing the DM, consequently the yield of the process.

3.1.3. Colour determinations

Colour parameters of the products are shown in Figure 2. Values of L* varied from 66.04 to 69.82 (Figure 2a). When setting was performed at 4 ºC for 12 h, L* values were lower than those obtained using higher setting temperatures (25 ºC for 2 h or 40 ºC for 20 min). This fact was observed in both groups, with or without MTG.

The values of the a* attribute varied from 3.32 to 4.42 (Figure 2b). The lower value was determined in restructured product without MTG and setting at 25 ºC for 2 h. The b* parameter (Figure 2c) varied in the narrow range from 11.02 to 12.82. These results suggest that temperature and time of setting have a scarce effect on colour parameters. In the same way, the use of MTG did not show an effect on the parameter a*.

3.2. Optimization of setting phenomenon at 4ºC

Considering the overall results, the setting at 4ºC was selected as the best for products obtained from white tuna (*Thunnus alalunga*). Although a time of 12 h is usually used for the setting at this temperature, it is interesting to evaluate the optimal time and the optimal MTG concentration for the setting at 4ºC.

Table 2 shows the variables involved in the experimental design of optimization. The independent variables were transglutaminase concentration and time of incubation at 4ºC. It was evaluated 3 levels of each variable: 150, 300, 450 U/kg for TG and 6, 12 or 18h for incubation time. Table 3 shows the experiments essayed including the dimensionless variables obtained from the real variables.

3.2.1. Mechanical Properties

Table 4 shows the results on textural parameters. Hardness increased from 15989 g using 150 U/kg of MTG and 6 h of setting treatment to 23978 g using 450 U/kg of MTG and 18 h of setting treatment. Using 450 U/kg of MTG during 6 h, the adhesiveness was lower than using 150 U/kg of MTG and 18 h (-1.89 g·s and -12.13 g·s, respectively). Springiness varied from 0.632 of restructured with 150 U/kg MTG and 18 h of setting to 0.802 of restructured with 450 U/kg of MTG and 12 h of setting. Cohesiveness were less affected, it was varying from 0.352 using 150 U/kg MTG and 18 h of setting and 0.416 with 300 U/kg of MTG and 12 h of setting treatment. Chewiness increased from 4167.17 g of restructured with 150 U/kg of MTG and 18 h of setting to 7308.36 g using 450 U/kg of MTG and 6 h of setting.

All texture parameters were submitted to multifactor ANOVA test. The F-values of 1.11 (hardness), 3.26 (adhesiveness), 3.40 (springiness), 3.82 (cohesiveness) and 3.78 (chewiness) implied that all mathematical models were not significant. The values of r^2 were 0.65 (hardness), 0.85 (adhesiveness), 0.56 (springiness), 0.56 (cohesiveness) and 0.56 (chewiness).

Therefore texture parameters were affected by MTG concentration and setting time in the range of study but it was not possible to model their effects being the means values the best predictors.

313 *3.2.2. Expressible water, water activity and dry matter*

314 Table 5 shows the results on expressible water, water activity and dry matter.
315 Expressible water of restructured varied from 27.69 % to 36.16 %. These values correspond
316 with 150 U/kg and 6 h of setting and 450 U/kg and 18 h of setting, respectively. Expressible
317 water of restructured products was affected by MTG concentration and time of setting treatment
318 but the model obtained was not significant (F-value was 1.99 and r^2 was 0.77).

319 Water activity varied from 0.962 to 0.982. Furthermore, DM varied from 24.46 % to
320 27.02 %. The effect of MTG concentration and setting time on Aw and DM were evaluated by
321 multifactor ANOVA showing F-values of 6.61 and 15.03, respectively. This implying that both
322 models were significant. The r^2 were 0.80 for Aw and 0.96 for DM, both of them were considered
323 good. The statistical parameter “adequate precision" measures the signal to noise ratio. A ratio
324 greater than 4 is desirable. It was 8.35 for Aw and 4.38 for DM. Then the models fitted well to
325 experimental data.

326 Figure 3a shows the graph that represents the prediction of the model for Aw. Values of
327 Aw decrease when increase MTG concentration and setting time. The lowest Aw was obtained
328 using 450 U/kg of MTG and 18 h setting time. When Aw is lower than 0.95, products can
329 preserve itself at fridge temperatures better than other products with higher Aw.

330 Figure 3b shows the graph that represents the prediction of the model for DM. The
331 model predicted the highest DM using 450 U/kg of MTG and 6 h of setting time. The model
332 obtained is interesting for economical evaluations.

333

334 *3.2.3. Colour determinations*

335 The values of colour determinations on restructured products of white tuna varied from
336 63.73 to 70.34 for L*, 2.38 to 4.68 for a* and 10.98 to 12.36 for b*. Effect of MTG concentration
337 and setting on L*, a* and b* were also evaluated by multifactor ANOVA getting F-values of 2.64
338 for L* parameter, 22.85 for a* and 26.89 for b*, implying that the models for a* and b* were
339 significant and model for L* was not significant. Values of r^2=0.974 and r^2=0.978 for a* and b*
340 (respectively) were considered good. Figure 4 shows the prediction of the models for a* and b*
341 parameters. For both parameters the effect of incubation time is more important that the effect
342 of MTG concentration used.

343 Table 6 summarizes the models obtained and the statistical indexes. Overall, the
344 optimal setting was considered when it was used 450 U/kg of MTG and 18 h of setting at 4ºC. It
345 allows to obtain tuna restructured products with the lowest Aw, high value of DM and low values
346 of colour attributes. The use of molds allowed to obtain any shape for the products. As example,
347 Figure 5 shows some of the products of reduced-salt restructured white tuna obtained with
348 several shape (including the shape of chicken).

349

350 *3.3. Bacteriological analysis and pH*

351 The restructured product obtained using optimal conditions for setting were
352 manufactured and Table 7 shows the results of bacteriological analyses (log ufc/g) and pH

353 measures at several times (0, 5 or 12 days) of fridge storage (4ºC). Results are expressed as
354 mean values for each time. Microbial load increased gradually throughout the storage period
355 from day 0, except in the case of proteolytic bacteria, which began growing from day 5. The
356 statistical analysis showed significant differences with the time for all variables except for the pH
357 values.
358 Psychrotrophic and mesophilic bacteria on day 0 were 5.34 and 2.50 log ufc/g,
359 respectively. They increased during fridge storage reaching up to 5.63 and 5.40 log ufc/g on day
360 5, respectively.
361 The initial value of mesophilic bacteria is lower than that considered as maximum
362 acceptable limit (5 log ufc/g) for fish baked products by European Commission Regulations No
363 2074/2005 (Cai, Wu, Li, Zhong, Li, & Li, 2014). On day 12, the psychrotrophic and mesophilic
364 bacteria increased reaching 9.53 and 10.47 log ufc/g, respectively. Fish is normally stored at
365 refrigeration or freezing temperatures, and then psychrotrophs are more representative bacteria
366 than mesophilic bacteria. There are no legal limits for psychrotrophic bacteria. But for
367 mesophilic bacteria is 5 log ufc/g. Then it could be considered acceptable the same criteria for
368 psychrotrophic bacteria. Considering the growth for mesophilic bacteria, psychrotrophic bacteria
369 followed a similar trend.
370 Furthermore, the initial Enterobacteriaceae counts and total number of anaerobic
371 bacteria at time 0 were lower (1.48 and 2.74 log ufc/g), indicating acceptable quality. But it was
372 followed by an increased at day 5 (4.13 and 5.25 log ufc/g) that in case of Enterobacteriaceae
373 was higher than limits for fish baked products (3 log ufc/g). Moreover, growth of these bacteria
374 was lower than other bacteria, maybe Enterobacteriaceae has tendency to grow slowly at
375 refrigeration temperatures (Cai, Wu, Li, Zhong, Li, & Li, 2014).
376 The initial proteolytic bacteria were undetectable on time 0 followed by a prominent
377 increased after 5 and 12 days of storage (4.73 and 10.36 log ufc/g).
378 Considering that in the production of fish restructured products there is a step of heat
379 (90-95ºC for 50 min), it can be suppose that these bacteria proceed from a bad manipulation of
380 operators after heating. This can be considered as a critical point in this kind of products.
381 The pH values of restructured products was increased slightly with time varying from
382 6.15 on 0 time to 6.28 on 12 days. These values were higher than values determined by other
383 authors in other species of tuna like *Thunnus obesus* (Ruiz-Capillas & Moral, 2005) and similar
384 to that determine on mince tuna (6.60) (Takahashi et al., 2012). Usually, pH value is increasing
385 quickly with fish products degradation but in our products the increase was slow.
386
387 **4. Conclusions**
388
389 The results showed that is feasible to obtain reduced-salt restructured fish products
390 from white tuna using transglutaminase and the setting phenomenon at low temperature (4ºC).
391 Parameters of texture, L* and Ew of the restructured obtained showed to be independent of
392 setting temperature and transglutaminase concentrations in the range of study. However, the

variations of transglutaminase concentration and setting affected the a* and b* colour parameters, water activity and dry matter of restructured products, and this dependence was modelled. The better setting treatment was at 4ºC for 18 h using 450 U/kg of transglutaminase. Microbial study showed that the manipulation after the cooking step is critical.

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Table 1
Textural parameters of white tuna restructured products performed using different temperature/time for setting conditions (TG is transglutaminase)*.

Setting Conditions	Hardness (g)	Adhesiveness (g·s)	Springiness	Cohesiveness	Chewiness (g)
4ºC/12 h (No TG)	13122.23^{b}	-39.878^{b}	0.519^{b}	0.299^{ab}	2085.81^{a}
25ºC/2 h(No TG)	13842.93^{b}	-22.787^{b}	0.593^{bc}	0.334^{b}	2846.26^{b}
40ºC/20 min(No TG)	11485.18^{a}	-67.29^{c}	0.443^{a}	0.285^{a}	1480.28^{a}
4ºC/12 h (TG)	20981.22^{c}	-2.15^{a}	0.793^{d}	0.416^{c}	6930.35^{c}
25ºC/2 h (TG)	14270.80^{b}	-16.983^{b}	0.559^{b}	0.335^{b}	2695.01^{b}
40ºC/20 min (TG)	14664.42^{b}	-2.786^{a}	0.647^{c}	0.364^{b}	3485.47^{b}

Note: *Different letters for the same parameter indicate a significant difference ($p < 0.05$).

Table 2

Variables involved in the experimental design for the study of the setting at 4ºC in the process for restructuring white tuna and their nomenclature, units and values or ranges.

	Nomenclature	Units	Values or ranges
(a) Fixed variables			
NaCl concentration		g/kg	15
Ratio mince/water for washing			1/3
Time of stage of washing		min	5
Stage of mixing		min	5
Cooking Temperature	Tc	ºC	90
Cooking Time	tc	min	50
Cooling Temperature	Te	ºC	4
Cooling Time	te	min	30
Time of waiting until analysis	ta	h	12
Temperature of waiting until analysis	Ta	ºC	4
(b) Independent variables			
Concentration of transglutaminase	TG	U/kg	(150, 300, 450)
Time of incubation	t	h	(6, 12, 18)
(c) Independent normalized variables			
Temperature of incubation	x_1		(-1, 0, 1)
Time of incubation	x_2		(-1, 0, 1)
(D) Dependent variables			
Hardness		g	
Adhesiveness		g·s	
Springiness			
Cohesiveness			
Chewiness		g	
Expressible water	*EW*	%	
Water activity	Aw		
Dry matter	*DM*	%	
Colour parameters	*L, a*, b**		

Table 3. Operational conditions assayed with the experimental design for the setting at 4ºC.

Experiment	*TG*	*t*	Dimensionless	
	U/kg	*h*	x_1	x_2
1	150	6	-1	-1
2	300	6	0	-1
3	450	6	1	-1
4	150	12	-1	0
5	300	12	0	0
6	450	12	1	0
7	150	18	-1	1
8	300	18	0	1
9	450	18	1	1

Table 4

Experimental results of textural properties achieved for the treatments of the experimental design for the setting at 4ºC.

Experiment	Hardness (g)	Adhesiveness (g·s)	Springiness	Cohesiveness	Chewiness (g)
1	15989	-8.79	0.728	0.410	4781,66
2	19256	-3.77	0.713	0.385	5400,19
3	23782	-1.89	0.751	0.406	7308,36
4	17743	-4.38	0.653	0.364	4256,29
5	20981	-2.15	0.793	0.416	6930,35
6	18760	-4.70	0.802	0.410	6168,61
7	18835	-12.13	0.632	0.352	4167,17
8	22999	-3.01	0.717	0.359	6022,53
9	23978	-2.65	0.713	0.386	6638,41

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550 **Table 5.**

551 Experimental results of expressible water (Ew), water activity (Aw), dry matter (DM) and colour
552 parameters achieved for the treatments of the experimental design for the setting at 4ºC.

Experiment	Ew	Aw	DM	L*	a*	b*
1	36.16	0.977	25.53	66.22	3.16	10.98
2	35.31	0.981	26.84	68.89	2.44	11.15
3	32.81	0.982	27.02	70.10	2.38	11.29
4	34.98	0.974	24.46	65.53	4.47	12.05
5	32.77	0.969	24.95	66.09	4.37	12.02
6	35.02	0.977	25.15	63.73	4.68	12.36
7	35.69	0.978	25.43	67.74	3.18	11.17
8	31.02	0.971	26.09	70.34	2.88	11.50
9	27.69	0.962	26.67	70.21	3.05	11.52

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555 **Table 6**. Models obtained and statistical indexes.

Model	r^2	Adjusted r^2	Predicted r^2	Adequate precision
$Aw = 0.996 + 0.001 * TG - 0.0010 \cdot t$	0.799	0.678	0.454	8.349
$DM = 28.663 + 0.011 * TG - 0.950 \cdot t + 0.039 t^2$	0.962	0.898	0.540	11.90
$a^* = -0.751 - 0.010 \cdot TG + 1.080 \cdot t - 0,046 \cdot t^2$	0.974	0.933	0.690	11.95
$b^* = 8.11 + 0.001 \cdot TG + 0.601 \cdot t - 0.024 \cdot t^2$	0.978	0.942	0.819	14.00

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Table 7.

Bacteriological analysis and pH measured on restructured products of white tuna on three times (0, 5 and 12 days) stored on refrigeration*.

Time (day)	Psychrotrophs (log ufc/g)	Mesophiles (log ufc/g)	Anaerobes (log ufc/g)	Proteolytic bacteria (log ufc/g)	Enterobacteriaceae (log ufc/g)	pH
0	5.341[a]	2.498[a]	2.740[a]	0.000[a]	1.477[a]	6.145[a]
5	5.628[a]	5.402[b]	5.248[b]	4.732[b]	4.130[b]	6.235[a]
12	9.531[b]	10.473[c]	10.323[c]	10.359[c]	7.924[c]	6.280[a]

Note: *Different letters for the same parameter indicate a significant difference ($p < 0.05$).

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568 **FIGURE CAPTIONS**

569 **Fig 1.** Changes of expressible water (Ew), water activity (Aw) and dry matter (DM) of
570 restructures fish products with MTG (white bars) and controls without MTG (black bars). Lines
571 show standard deviations.

572 **Fig 2.** Changes in colour attributes (L*, a* and b*) of restructured fish products restructures fish
573 products with MTG (white bars) and controls without MTG (black bars). Lines show standard
574 deviations.

575 **Fig 3.** Prediction model for dependence of water activity (Aw) and dry matter (DM) on
576 transglutaminase concentration and time of setting phenomenon at 4ºC.

577 **Fig 4.** Prediction model for dependence of a* and b* on transglutaminase concentration and
578 time of setting phenomenon at 4ºC.

579 **Fig 5.** Low-salt restructured white tuna (*Thunnus alalunga*) obtained with several shapes.

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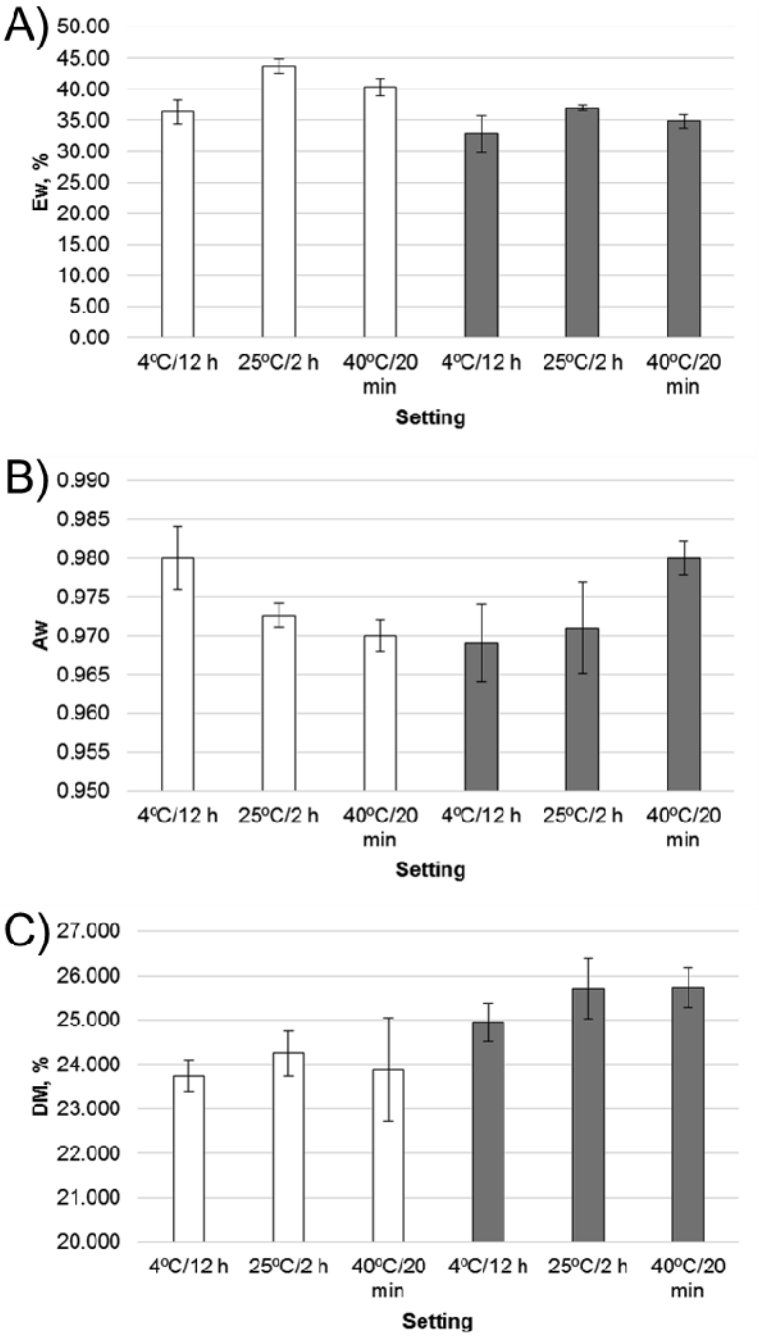

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585 **Figure 1**

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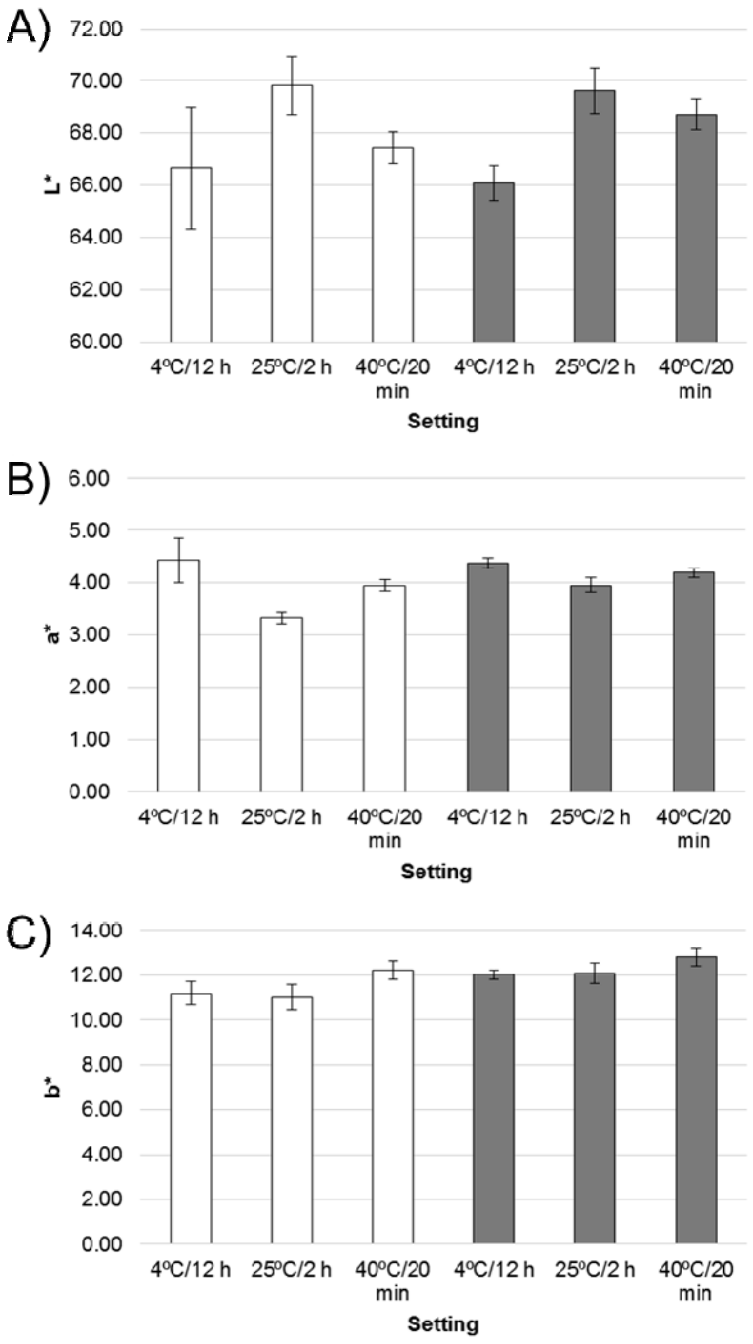

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591 **Figure 2**

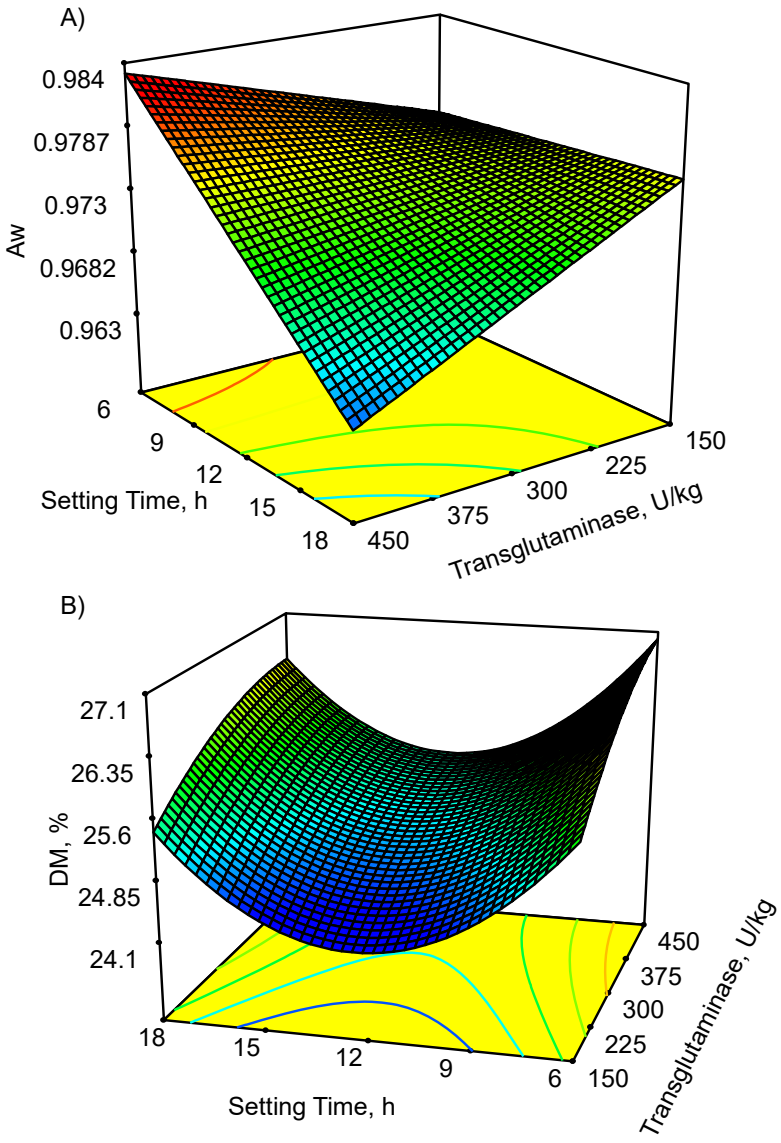
A)
0.984
0.9787
0.973
0.9682
0.963
Aw
6
9
12
15
18
Setting Time, h
450
375
300
225
150
Transglutaminase, U/kg
B)
27.1
26.35
25.6
24.85
24.1
DM, %
18
15
12
9
6
Setting Time, h
450
375
300
225
150
Transglutaminase, U/kg

Figure 3

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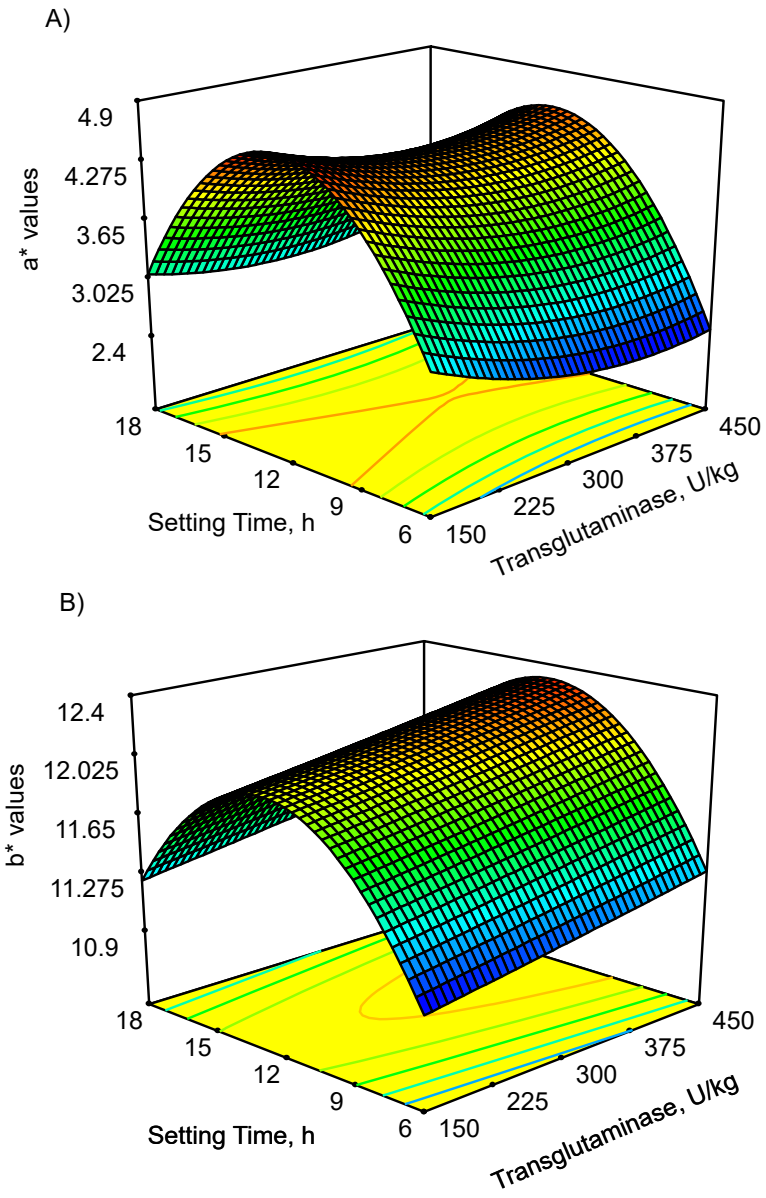

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603 **Figure 4**

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Figure 5