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# Tuna skin gelatin production: optimization of extraction steps and process scale-up

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**ABSTRACT**

Advantages of fish gelatin production include reduction of environmental impacts from waste, improved functionality, and fewer socio-cultural constraints. The extraction of gelatin from Yellowfin tuna (*Thunnus albacares*) skin was optimized using response surface methodology. Conditions for acid pretreatment (acetic acid concentration and treatment time) had a significant effect ($p < 0.05$) on the response variables skin hydration, gelatin extract, gel strength, and gel pH. Conditions for the extraction step (treatment temperature and treatment time) had a significant effect ($p < 0.05$) on the response variables gelling temperature, melting temperature, and gel strength. Process conditions affected the gel properties, and some effects were confirmed by evaluating the molecular weight profile of extracted proteins obtained through SDS-PAGE. The feasibility of the general extraction process scale-up was evaluated by comparing process yields and characteristics of gelatins, obtained at laboratory and pilot plant scales (scale-up ratio 80:1).

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Fish gelatin; tuna skin; acid pretreatment; extraction process; gel properties

## Producción de gelatina de piel de atún: optimización de las etapas de extracción y escalamiento del proceso

**RESUMEN**

Las ventajas de la producción de gelatina de pescado incluyen la reducción de los impactos ambientales producto de los desechos, una funcionalidad mejorada y menos restricciones socio-culturales. La extracción de gelatina de la piel de atún aleta amarilla (*Thunnus albacares*) se optimizó utilizando la metodología de superficie de respuesta. Las condiciones del pretratamiento ácido (concentración de ácido acético y tiempo de tratamiento) tuvieron un efecto significativo ($p < 0.05$) sobre las variables respuesta hidratación de la piel, extracto de gelatina, fuerza del gel y pH del gel. Las condiciones de la etapa de extracción (temperatura de tratamiento y tiempo de tratamiento) tuvieron un efecto significativo ($p < 0.05$) sobre las variables respuesta temperatura de gelificación, temperatura de fusión y fuerza del gel. Las condiciones de proceso afectaron las propiedades de gelificación, y algunos efectos se confirmaron al evaluar los perfiles de peso molecular de las proteínas extraídas, obtenidos por SDS-PAGE. La factibilidad del escalamiento del proceso general de extracción se evaluó comparando los rendimientos del proceso y las características de las gelatinas obtenidas a escala de laboratorio y de planta piloto (relación del escalamiento 80:1).

**PALABRAS CLAVE**
Gelatina de pescado; piel de atún; pretratamiento ácido; proceso de extracción; propiedades del gel

## 1. Introduction

Collagen, a glycoprotein, is the main component of connective tissue. It is composed of tropocollagen monomers comprised of three polypeptide α chains that may be identical or different (Asghar & Henrickson, 1982). Gelatin is obtained from collagen. Controlled acid or alkaline hydrolysis is applied to convert collagen into a form suitable for extraction and to remove other organic substances that occur naturally in the raw materials, including proteoglycan, blood, mucins, and sugars. Pretreated collagen is converted into gelatin by employing a five-step process: washing, extraction, purification, concentration, and drying. This process is optimized to obtain the maximum yields of gelatin with the required physical and chemical properties (Johnston-Banks, 1990).

Although interest in gelatin in the food, photographic, cosmetic, and pharmaceutical industries is mostly based on its gel-forming and viscoelastic properties, new applications have been found for gelatin. These include uses as emulsifiers (Tan et al., 2020), foaming agents (Casanova et al., 2020), biodegradable packaging materials (Loo & Sarbon, 2020), and micro-encapsulating agents (García-Saldaña et al., 2016). Also, gelatin is a source of biologically active peptides, some of which exhibit promising antimicrobial, antioxidant, and other functional properties (Gómez-Guillén et al., 2011). The most common raw materials for gelatin extraction are skins or hides, bones, tendons, and cartilage. Materials from fish and poultry have received attention, but their limited production makes them less competitive than gelatin from other sources (Gómez-Guillén et al., 2011).

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The supplemental data for this article can be accessed here.

Other challenges associated with fish gelatin compared to bovine and porcine products include inferior rheological properties (Choi & Regenstein, 2000), insufficient availability of raw materials, variable gelatin quality (Karim & Bhat, 2009), and intrinsic quality factors such as odor, color, and stability (Ferraro et al., 2010). On the other hand, the use of fish gelatin is not restricted by socio-cultural and health-related concerns such as the avoidance of consumption of pork-related products (Judaism and Islam), avoidance of consumption of cow-related products (Hinduism), adherence to vegetarian diets and lifestyles, and concerns regarding transmission of pathogenic agents (such as prions) (Karim & Bhat, 2009; Lv et al., 2019). For some applications, fish skin gelatins may provide better functionality than mammalian gelatins given their lower gel strengths and melting temperatures. In addition, the possibility of obtaining added-value products and reducing waste from the fishery industry is encouraging the search for sustainable and productive uses of fish by-products (Boran & Regenstein, 2010).

In order to ensure an efficient and effective gelatin extraction process, conditions should be optimized to obtain the best possible product by minimizing the effects of extraction treatments that can damage collagen molecules. On the other hand, the raw materials used in gelatin manufacture have obvious effects on gelatin quality, primarily due to differences in the amino acid composition of the collagen of the raw material (Boran & Regenstein, 2010). Fish by-products are perishable, due in part to proteases and highly unsaturated lipids that are prone to oxidative deterioration. Knowledge of autolytic activities and lipid variations for each species, season, and fishing ground is also necessary and must be taken into account by gelatin manufacturers in order to extract gelatin with desirable properties from fish processing by-products (Karayannakidis & Zotos, 2016). Therefore, process conditions should respond to the characteristics of the raw materials and optimization efforts must be designed accordingly.

The aims of this study were to optimize the operating conditions for acid pretreatment (acetic acid concentration and treatment time) and the extraction step (treatment temperature and treatment time) for production of gelatin from Yellowfin tuna (*T. albacares*) skin, and to evaluate the feasibility of the general extraction process scale-up.

## 2. Materials and methods

### 2.1. Materials

Two independent lots of Yellowfin tuna (*T. albacares*) skin provided by TUNATUN S.A. (Alajuela, Costa Rica) were mixed to obtain a single, homogeneous, 76 kg lot which was used throughout the study. Tuna skin was chopped (approximately 1.5 $cm^2$ pieces), mixed, packed in bags, and kept frozen (–20ºC) until used. Four samples were obtained for physicochemical analyses (pH, moisture, protein, fat, and ash).

### 2.2. General extraction process

The following process was carried out on a laboratory scale for the two optimization experiments, and on both the laboratory scale and a pilot plant scale for the scale-up experiment. Frozen, chopped tuna skin was thawed at 4ºC (150 g of skin, 24 h for laboratory scale; 12 kg of skin, 72 h for pilot plant scale), and mixed. An acid pretreatment was carried out by mixing the skin with an aqueous acetic acid solution and heating the mix with constant stirring (600 g solution, 21 ± 1ºC, glass beaker on a stirring hot plate (Corning PC-420D, Reynosa, Mexico) for laboratory scale; 48 kg solution, 25 ± 3ºC, manually stirred steam kettle for pilot plant scale). For the acid pretreatment optimization experiment, acetic acid concentration and treatment time varied according to the corresponding experimental design. For the extraction step optimization experiment and the scale-up experiment, acid concentration was 0.225 M and treatment time was 2.5 h. The mix was then filtered with a sieve to remove most of the spent acid solution. Three consecutive washes using distilled water and the sieve (400 g water for laboratory scale; 32 kg water for pilot plant scale) were carried out and the spent wash water was discarded. The extraction step was carried out using distilled water and heating the mix with constant stirring (550 g water, glass beaker on a stirring hot plate (Corning PC-420D, Reynosa, Mexico) for laboratory scale; 44 kg water, manually stirred steam kettle for pilot plant scale). For the acid pretreatment optimization experiment, treatment temperature was 62ºC and treatment time was 2.5 h. For the extraction step optimization experiment, treatment temperature and time varied according to the corresponding experimental design. For the scale-up experiment, treatment temperature was 54ºC and treatment time was 0.38 h. The mix was then filtered with a sieve to remove the solid residue. The remaining solution was centrifuged (Labnet Hermle Z 300, Wehingen, Germany, 3900 g, 20 min, 30 ± 1ºC for laboratory scale; Westfalia Separator AG, GEA, Oelde, Germany, 3800 g, 60 ± 2ºC for pilot plant scale) and the sediment was discarded. The supernatant liquid (gelatin extract) was kept at 4ºC for at least 12 h to produce a firm gel. The gel was finely cut and freeze-dried using a Sublimator 2x3x3 (Zirbus Technology GmbH, Bad Grund, Germany) to obtain dried gelatin.

### 2.3. Optimization experiment 1: acid pretreatment

Response surface methodology was used to optimize the acid pretreatment, in order to increase the efficiency of gelatin extraction and improve gel characteristics. A central composite rotatable design was used with two independent variables: acetic acid concentration (0.000–0.346 M) and treatment time (0.38–4.62 h). The range of values for each variable was determined in preliminary experiments. Five levels of each independent variable were coded as: –1.414, –1, 0, 1, 1.414. The experimental design consisted of 11 experimental points (trials), including three replicates of the central point. Trial order was completely randomized. Seven response (dependent) variables were measured in each trial: skin hydration (%), gelatin extract (%), protein extraction yield (%), gelling temperature (ºC), melting temperature (ºC), gel strength (g), and gel pH. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed to determine the molecular weight profile of extracted proteins.

A second-order polynomial equation [1] was used to evaluate the relationship between dependent and independent variables, where $Y$ corresponds to each response (dependent) variable, $\beta_0$, $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$, correspond to

regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and $X_i$ and $X_j$ correspond to coded experimental levels of the independent variables.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad [1]$$

Analysis of variance (ANOVA) was performed using JMP Pro 13.0 (SAS Institute Inc., Cary, NC) to determine the regression coefficients for each model. The statistical significance of models and regression coefficients was assessed by computing the F values; $p$ values below 0.05 were considered significant. Contour and line plots were created using DataGraph 4.4 (Visual Data Tools, Inc., Chapel Hill, NC). The resulting models were verified by conducting a confirmation trial (performed in triplicate) using the experimental conditions that maximized selected response variables (according to JMP Pro 13.0 *Maximize Desirability* function), and comparing predicted and observed results.

### 2.4. Optimization experiment 2: extraction step

The same methodology and design described in the previous section were used to optimize the extraction step with two independent variables: treatment temperature (37–87°C) and treatment time (0.38–4.62 h). Four response (dependent) variables were measured in each trial: protein extraction yield (%), gelling temperature (°C), melting temperature (°C), and gel strength (g). SDS-PAGE was performed to determine the molecular weight profile of extracted proteins. A confirmation trial was also performed as described in the previous section.

### 2.5. Scale-up experiment

The general extraction process (section 2.2) was carried out on a laboratory scale and on a pilot plant scale. The scale-up ratio was 80:1. Both processes used the experimental conditions determined in the optimization experiments (acid pretreatment and extraction step). Trials were carried out in triplicate. For each condition (laboratory and pilot plant scale), yield was calculated on a wet basis, dividing the mass of the resulting product by the mass of initial tuna skin, after each of the following unit operations: acid pretreatment, wash 1, wash 2, wash 3, extraction, and centrifugation. The pH and moisture, protein, fat, and ash contents were determined for each raw material, product, and by-product obtained in the process: tuna skin, spent acid solution, spent wash water 1, spent wash water 2, spent wash water 3, solid residue, sediment, and gelatin extract. Gelling temperature, melting temperature, and gel strength were also determined for the final product. SDS-PAGE was performed to determine the molecular weight profile of the extracted proteins. Student's t-tests were performed using JMP Pro 13.0 (SAS Institute Inc., Cary, NC) for means comparisons and to determine the effect of scale (laboratory and pilot plant) on the measured responses ($p$ values below 0.05 were considered significant).

### 2.6. Physicochemical analyses

Moisture (oven-drying method), protein (total Kjeldahl nitrogen method), and ash (muffle furnace method) contents, and pH (using a pH meter equipped with an electrode) were determined using standard AOAC methods (AOAC, 2012). Gel pH was determined on a 6.67% gelatin and distilled water gel. Fat content was determined as previously described (Carpenter et al., 1993) by using the Goldfish method.

Gel strength was determined by the method described by Gómez-Guillén et al. (2002) with minor modifications: the solution was cooled in a refrigerator at 4°C (maturation temperature), and gel strength was measured at 4°C in samples with a 4.3 cm diameter and 4.5 cm height on a texturometer TA.XT plus (Stable Micro Systems, Surrey, England) equipped with a cylindrical polyoxymethylene plunger (1.27 cm diameter). Gel strength was expressed as the maximum force (g) measured when the plunger penetrated 4 mm into the gel. Skin hydration and process yields were calculated as mass of hydrated skin or product divided by the initial mass of tuna skin and expressed as percentages (%). The gelatin extract was determined after the centrifugation step by weighing the supernatant liquid with a balance, and calculated by dividing its mass by the initial mass of tuna skin and expressed as percentage (%). The protein extraction yield was determined after the freeze-drying step by weighing the dried gelatin with a balance, and measuring the moisture content. Gelling and melting temperatures (°C) were determined following the method described by Gómez-Guillén et al. (2002) with some minor modifications: a Physica MCR51 rotary rheometer (Anton Paar GmbH, Ostfildern, Germany) was used with a cone plate angle of 1° and a heating and cooling scan rate of 1°C/min. Gelling and melting temperatures were measured when a phase angle of 45° was reached during cooling and heating, respectively. The molecular weight profile of extracted proteins was determined by SDS-PAGE as described by Laemmli (1970), with some modifications. A 4 mg/mL gelatin solution was prepared with distilled water. The solution was heated to 60°C for 20 min with continuous agitation and then centrifuged. The supernatant was mixed at a 3:1 ratio with a buffer that consisted of 0.5 M Tris-HCl, pH 6.8, glycerol, 4% SDS, and 5% 2-mercaptoethanol. The samples were heated to 100°C for 5 min. Electrophoresis was performed using either Novex 8–16% Tris-Glycine Mini Gels (ThermoFisher Scientific) or 4–15%

Table 1. Physicochemical characterization of Yellowfin tuna skin. Results shown are mean values ± 95% confidence intervals ($n$ = 4).
Tabla 1. Caracterización fisicoquímica de la piel de atún aleta amarilla. Los resultados mostrados son los valores promedios ± intervalos de confianza al 95% ($n$ = 4).

| Analysis (units) | Result |
|---|---|
| Moisture (g/100 g) | 59 ± 2 |
| Protein (g/100 g) | 28 ± 3 |
| Fat (g/100 g) | 6 ± 1 |
| Ash (g/100 g) | 4.2 ± 0.5 |
| pH | 6.0 ± 0.2 |

Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad). Gels were stained with Coomassie Brilliant Blue R-250 dye (ThermoFischer Scientific). A Precision Plus Protein (Bio-Rad) standard was used as a molecular weight marker.

## 3. Results and discussion

### 3.1. Characterization of raw material

The physicochemical characterization of Yellowfin tuna skin is shown in Table 1. Results were in accordance with previous reports (Cho et al., 2005; Rahman et al., 2008; Sousa et al., 2017). Minor variations in the proximate composition of tuna fish muscle and skin were expected due to dietary and environmental factors, such as location and water temperature, as well as intrinsic factors, such as life stage, sex, and weight (Huss, 1995; Nakamura et al., 2007).

### 3.2. Optimization experiment 1: acid pretreatment

During the gelatin manufacturing process, raw material is pretreated with acid or alkaline solutions to cause collagen swelling and increase the efficiency of gelatin extraction during thermal hydrolysis (Ahmad et al., 2017). The low pH of the acid pretreatment favors the access of water to collagen fibers. The acid concentration influences the swelling properties and solubilization of the collagen, which leads to variations in the molecular weight distribution and gelling properties of the resulting gelatins (Giménez et al., 2005). A previous study to optimize extraction of tuna skin gelatin employed the alkaline pretreatment method (Cho et al., 2005). Other reports in the literature concerning process evaluation and characterization of tuna skin gelatin have employed an alkaline pretreatment followed by acidic extraction conditions (Karayannakidis et al., 2014; Karayannakidis & Zotos, 2015; Rahman et al., 2008). The resulting response surface methodology models for four of the seven response variables were deemed adequate. Models for response variables protein extraction yield, gelling temperature, and melting temperature were not significant ($p$ = 0.5137, 0.2986, 0.3082, respectively). Table S1 (supplementary material) shows the experimental design for optimization of acid pretreatment and experimental data for all response variables. Table 2 shows the regression coefficients and analysis of fitted models for response variables skin hydration, gelatin extract, gel strength, and gel pH ($R^2$, adjusted $R^2$, $p$ values of model and lack of fit, and mean squares of pure error and lack of fit). The linear effects on gel strength of both acetic acid concentration and treatment time were significant. Effects are depicted in a contour plot in Figure 1a. Nikoo et al. (2014) obtained similar results for the effect of acid concentration on physicochemical properties of Amur sturgeon skin gelatin, but contrasting results for treatment time. These authors reported decreased gel strength with increasing acid concentrations (0.05–0.20 M) and pretreatment times (3–6 h). Likewise, Díaz-Calderón et al. (2017) reported significantly decreased gel strength of salmon skin gelatin at higher acid concentrations (pH 3–5) and longer extraction times (2–5 h); gel strength was influenced more by extraction pH than extraction time. In contrast, gel strength of gelatin extracted from Megrim skins was not improved when acid concentration was increased (0.05–0.50 M) (Gómez-Guillén & Montero, 2001).

The linear and quadratic effects of acetic acid concentration on skin hydration and gelatin extract were significant. Figure 1b and 1c, respectively, show line plots depicting the effects. Two mechanisms of collagen hydration have been described: hydration due to ionic groups and their charges in acid or base ("osmotic swelling") and hydration caused by the interaction of ions of neutral salts or non-ionic reagents with non-ionic bonds of collagen ("lyotropic hydration"). Acetic acid produces both types of swelling, but lyotropic hydration predominates (Asghar & Henrickson, 1982). Compared to other organic acids used for the extraction of gelatin from Megrim skins, acetic acid causes the highest swelling capacity of collagen, which in turn favors extraction and solubilization (Gómez-Guillén & Montero, 2001). The association between response variables skin hydration and gelatin extract can be quantified using the Pearson correlation coefficient, which for the 11 trials was 0.98 ($p \leq 0.001$). Apparently, when a highly hydrated skin is subjected to extraction, the water content of the gelatin extract is higher, which increases the mass of the extract.

The linear effect of acetic acid concentration on gel pH was significant and is shown as a line plot in Figure 1d. Lower pH values of the extracting collagen favor extraction rates, but negatively affect physical properties such as viscosity (Johnston-Banks, 1990) and gel strength (Díaz-Calderón et al., 2017). The solubility and extractability of collagen are determined by the pH of the extraction medium, which regulates the charge density of the collagen and thus affects the electrostatic interaction and collagen structure (Liu et al., 2015).

**Table 2.** Regression coefficients and analysis of fitted models for selected response variables, from acid pretreatment optimization trials.
**Tabla 2.** Coeficientes de regresión y análisis de los modelos ajustados para variables respuesta seleccionadas, a partir de los ensayos de optimización del pretratamiento ácido.

| | Predicted values | | | |
|---|---|---|---|---|
| Regression coefficients and analysis of fitted models | Skin hydration (%) | Gelatin extract (%) | Gel strength (g) | Gel pH |
| $\beta_0$ (intercept) | 34.17 *** | 269.12 *** | 514.49 *** | 5.45 *** |
| $\beta_1$ (acetic acid concentration) | 1154.64 ** | 653.14 ** | − 321.03 *** | − 1.64 *** |
| $\beta_2$ (treatment time) | - ns | - ns | 9.02 * | - ns |
| $\beta_{11}$ (acetic acid concentration)$^2$ | − 2464.60 ** | − 1377.60 ** | - ns | - ns |
| $\beta_{22}$ (treatment time)$^2$ | - ns | - ns | - ns | - ns |
| $\beta_{12}$ (concentration × time) | - ns | - ns | - ns | - ns |
| $R^2$ | 0.8086 | 0.7763 | 0.8922 | 0.7723 |
| Adjusted $R^2$ | 0.7607 | 0.7204 | 0.8652 | 0.7470 |
| $p$-Value of model | 0.0013 | 0.0025 | 0.0001 | 0.0004 |
| $p$-Value of lack of fit | 0.1850 | 0.2885 | 0.8031 | 0.3754 |
| Mean square of pure error | 435.33 | 202.11 | 349.18 | 0.01 |
| Mean square of lack of fit | 986.09 | 311.29 | 161.92 | 0.01 |

$^{ns}p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

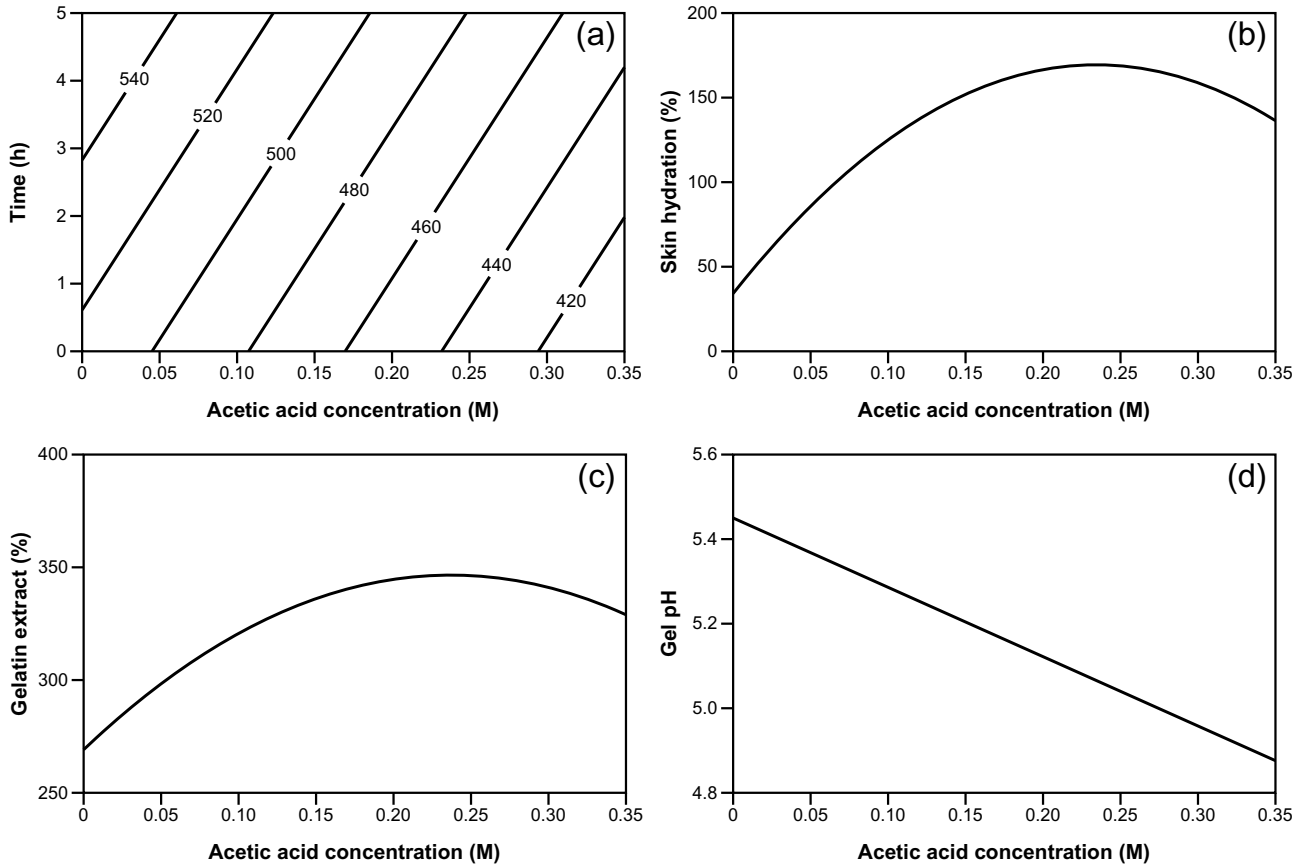


Figure 1. Contour plot depicting the effects of acetic acid concentration and treatment time on gel strength (a), and line plots depicting the effects of acetic acid concentration on skin hydration (b), gelatin extract (c), and gel pH (d).
Figura 1. Gráfico de contorno que muestra los efectos de la concentración de ácido acético y del tiempo de tratamiento sobre la fuerza del gel (a), y gráficos de lineales que muestran los efectos de la concentración de ácido acético sobre la hidratación de la piel (b), el extracto de gelatina (c) y el pH del gel (d).

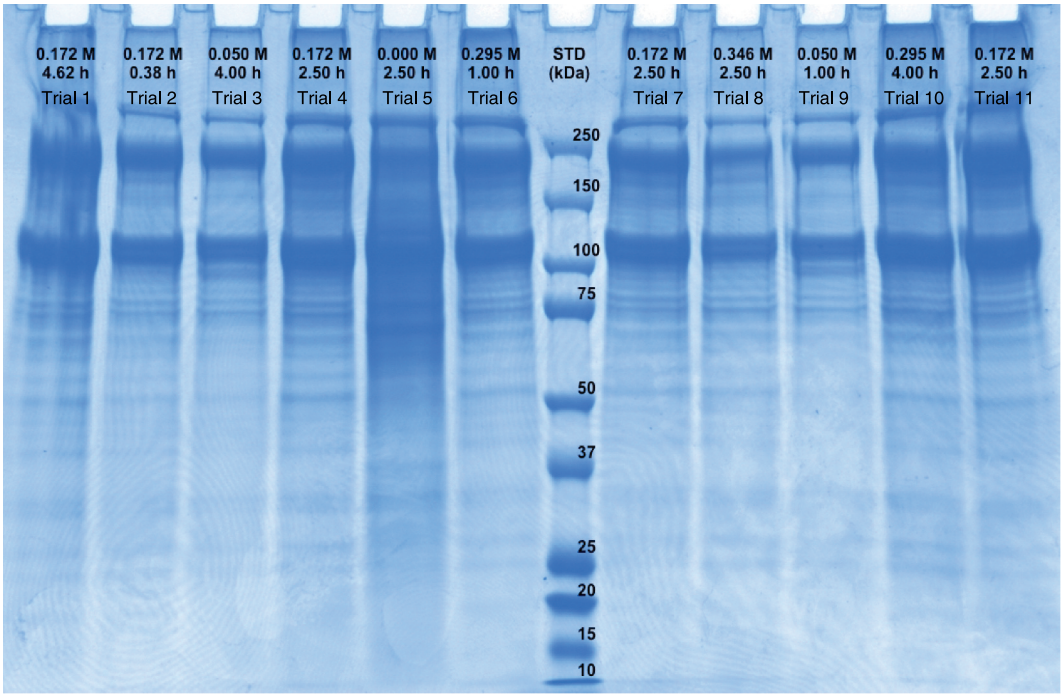


Figure 2. Molecular weight profile of extracted proteins obtained through SDS-PAGE from optimization of acid pretreatment trials, with standards (STD) showing molecular weights (kDa).
Figura 2. Perfiles de peso molecular de las proteínas extraídas en los ensayos de optimización del pretratamiento ácido, obtenidos por SDS-PAGE, con los estándares (STD) mostrando los pesos moleculares (kDa).

Figure 2 shows the molecular weight profiles of extracted proteins from the acid pretreatment optimization trials. With the exception of the markedly different profile from trial 5 (0.000 M, 2.50 h), no clear dissimilarities between the treatments were apparent. Gel strength is related to the α and β chain components (Johnston-Banks, 1990) and is affected by

their degradation (Ahmad et al., 2017). Furthermore, the molecular weight of the gelatin chains directly affects gel strength (Díaz-Calderón et al., 2017). However, other factors may have affected the observed results. Gudmundsson and Hafsteinsson (1997) noted that gel strength may be dependent on the isoelectric point; when the pH approaches this value, the charges of the gel polymers are closer to neutral and stronger gels can form. The models for response variables skin hydration, gelatin extract, gel strength, and gel pH were verified in a confirmation trial using an acetic acid concentration of 0.225 M and treatment time of 2.5 h. Other response variables were not considered, given the inadequacy of the corresponding models. The value for acetic acid concentration was selected from a range that maximized response variables skin hydration and gelatin extract, obtained from maximization of the desirability function. Treatment time was selected as the central point of the experimental design since it did not significantly affect response variables skin hydration and gelatin extract. Figure S1 (supplementary material) shows the contour plot of overall desirability. Table 3 shows predicted (from models) and observed (from confirmation trials) response variables, as well as the percent error for value comparison.

### 3.3. Optimization experiment 2: extraction step

After an acid or alkaline pretreatment, the collagen should be sufficiently hydrated and suitable for extraction by thermal hydrolysis. During the extraction step, the hydrogen and covalent bonds that stabilize the collagen helices are broken and the molecules adopt a disordered conformation, resulting in the formation of gelatin (Djabourov et al., 1993).

The resulting response surface methodology models were adequate for three of the four response variables. The model for response variable protein extraction yield was not significant ($p$ = 0.2955). Table S2 (supplementary material) shows the experimental design for optimization of the extraction step and experimental data for all response variables. Table 4 shows the regression coefficients and analysis of fitted models for response variables gelling temperature, melting temperature, and gel strength ($R^2$, adjusted $R^2$, $p$ values of model and lack of fit, and mean squares of pure error and lack of fit). Both the linear and quadratic effects of treatment temperature and the linear effect of treatment time on gelling temperature were significant. Effects are shown in a contour plot in Figure 3a. For the response variable melting temperature, the linear and quadratic effects of treatment temperature and the linear effect of treatment time were significant, but the interaction term was also significant. Figure 3b depicts the corresponding contour plot. Overall, shorter extraction times increased both gelling and melting temperatures at higher treatment temperatures, and a range of treatment temperatures (between 45–65ºC) increased both response variables. It has been reported that changes in gelling temperatures of pollock skin gelatin are caused by differences in the molecular weight of gelatin samples (gelling temperature increases with increasing weight average molecular weight), and that increasing extraction temperature decreases weight average molecular weight, due to increasing hydrolysis of the gelatin (Eysturskarð et al., 2009). A similar effect of extraction temperature on gelling temperature has also been reported for Yellowfin tuna (Sousa et al., 2017). A significant and inverse effect of extraction time (but not of extraction temperature) on melting temperatures of

Table 3. Predicted (from models) and observed (from confirmation trial) response variables at optimized acid pretreatment conditions (acetic acid concentration 0.225 M and treatment time 2.5 h).
Tabla 3. Variables respuesta predichas (según los modelos) y observadas (según el ensayo de confirmación) en las condiciones óptimas del pretratamiento ácido (concentración de ácido acético 0.225 M y tiempo de tratamiento 2.5 h).

| Response variable | Predicted value [a] | Observed value [b] | Error (%) |
|---|---|---|---|
| Skin hydration (%) | 169 ± 22 | 145 ± 6 | 16.7% |
| Gelatin extract (%) | 346 ± 14 | 369 ± 3 | 6.2% |
| Gel strength (g) | 465 ± 11 | 579 ± 17 | 19.7% |
| Gel pH | 5.08 ± 0.08 | 5.19 ± 0.02 | 2.0% |

[a]Predicted value ± 95% confidence interval.
[b]Means of observed results ± 95% confidence interval ($n$ = 3).
[a]Valor predicho ± intervalo de confianza al 95%.
[b]Promedios de los resultados observados ± intervalo de confianza al 95% (n = 3).

Table 4. Regression coefficients and analysis of fitted models for selected response variables, from extraction step optimization trials.
Tabla 4. Coeficientes de regresión y análisis de los modelos ajustados para variables respuesta seleccionadas, a partir de los ensayos de optimización de la etapa de extracción.

| | Predicted values | | |
|---|---|---|---|
| Regression coefficients and analysis of fitted models | Gelling temperature (ºC) | Melting temperature (ºC) | Gel strength (g) |
| $\beta_0$ (intercept) | 5.65 *** | 12.30 *** | 266.48 *** |
| $\beta_1$ (treatment temperature) | 0.55 *** | 0.48 *** | 11.22 *** |
| $\beta_2$ (treatment time) | − 0.38 * | 0.81 ** | 82.44 $^{ns}$ |
| $\beta_{11}$ (treatment temperature)$^2$ | − 0.005 *** | − 0.004 *** | − 0.096 * |
| $\beta_{22}$ (treatment time)$^2$ | - $^{ns}$ | - $^{ns}$ | - $^{ns}$ |
| $\beta_{12}$ (temperature × time) | - $^{ns}$ | − 0.02 * | − 1.30 * |
| $R^2$ | 0.9225 | 0.9609 | 0.9366 |
| Adjusted $R^2$ | 0.8892 | 0.9348 | 0.8943 |
| $p$-Value of model | 0.0003 | 0.0002 | 0.0010 |
| $p$-Value of lack of fit | 0.4678 | 0.3090 | 0.8414 |
| Mean square of pure error | 0.31 | 0.09 | 1029.24 |
| Mean square of lack of fit | 0.43 | 0.22 | 340.60 |

$^{ns}p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

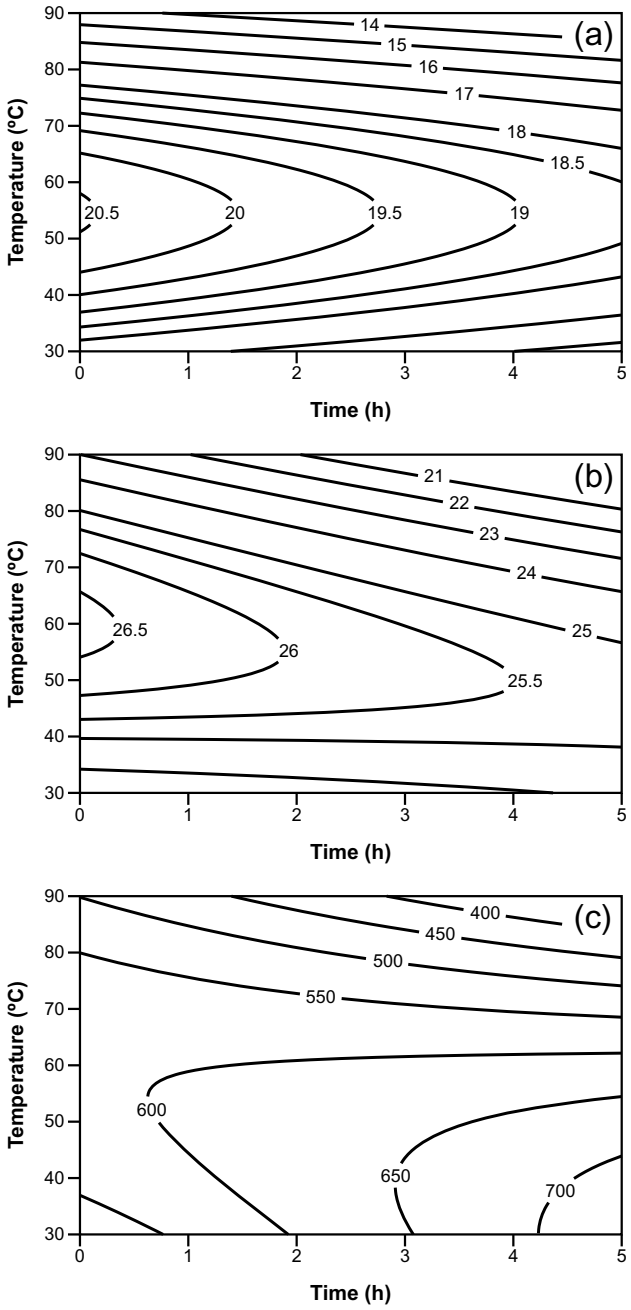


Figure 3. Contour plots depicting the effects of treatment temperature and treatment time on gelling temperature (a), melting temperature (b), and gel strength (c).
Figura 3. Gráficos de contorno que muestran los efectos de la temperatura de tratamiento y del tiempo de tratamiento sobre la temperatura de gelificación (a), la temperatura de fusión (b) y la fuerza del gel (c).

Yellowfin tuna skin gelatin has been reported (Karayannakidis et al., 2014). Authors have mentioned that increased degradation of gelatin as time increases produce lower melting points. On the other hand, Eysturskarð et al. (2009) reported that melting temperature is also dependent on the weight average molecular weight of the fish gelatin, which in turn is affected by extraction temperature (and to a lesser extent, extraction time).

Gel strength was significantly affected by the linear and quadratic effects of treatment temperature, as well as the interaction between variables. Gel strength was maximized when longer process times and lower process temperatures were used during the extraction step (Figure 3c). It has been reported that gelling power decreases with increasing extraction severity (which in turn is a function of temperature and time) (Normand et al., 2000), and that gel strength decreases with an increase in extraction temperature due to protein degradation, and production of protein fragments which lowers gelling ability (Cho et al., 2005).

Figure 4 shows the molecular weight profiles of extracted proteins obtained in the extraction step optimization trials. Overall, with the exception of trials 2, 4, and 9, in which the highest extraction temperatures were employed, the SDS-PAGE patterns were similar among treatments. Trials 2 and 4, which used an extraction temperature of 80°C, showed a higher proportion of degraded peptides or proteins within the molecular weight range of 37 kDa to 100 kDa. Trial 9, which used the highest extraction temperature (87°C), showed no band at 250 kDa, decreased band intensity at 100 kDa, and a moderate proportion of lower molecular weight protein bands in the 37 kDa to 100 kDa range. This result is likely due to the severe extraction conditions,

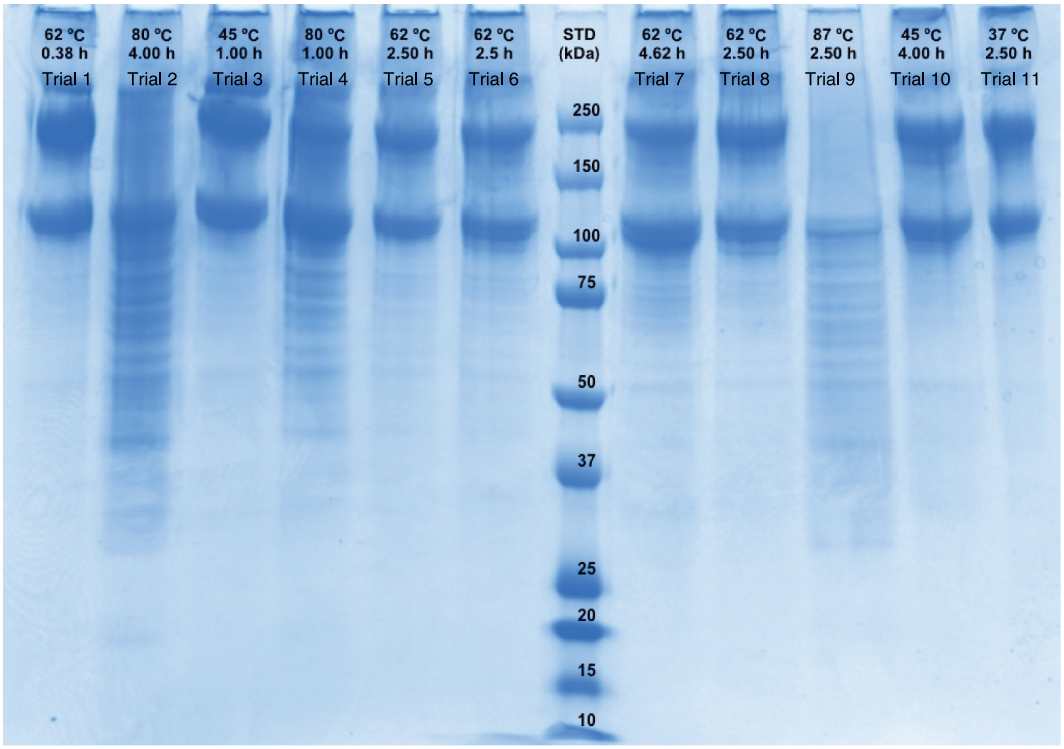


Figure 4. Molecular weight profile of extracted proteins obtained through SDS-PAGE from optimization of extraction step trials, with standards (STD) showing molecular weights (kDa).
Figura 4. Perfiles de peso molecular de las proteínas extraídas en los ensayos de optimización de la etapa de extracción, obtenidos por SDS-PAGE, con los estándares (STD) mostrando los pesos moleculares (kDa).

particularly the higher treatment temperatures, which caused protein hydrolysis and resulted in more proteins with lower molecular weights or peptides (Kittiphattanabawon et al., 2010; Normand et al., 2000). Accordingly, as seen in Table S2 (supplementary material), trial 9 presented the lowest gelling and melting temperatures, and the lowest gel strength. The second- and third-lowest values for these response variables were obtained in trials 2 and 4. These results are expected, since extraction conditions (particularly temperature) affect protein degradation, which in turn affects characteristics of gels (Cho et al., 2005; Eysturskarð et al., 2009). Models for response variables gelling temperature, melting temperature, and gel strength were verified by conducting a confirmation trial using a treatment temperature of 54.0°C and treatment time 0.38 h. Protein extraction yield was not considered, given the inadequacy of the model for this response variable. The value for treatment temperature was selected from a range that maximized response variables gelling and melting temperatures while the value for treatment time maximized both response variables. Values were obtained from maximization of the desirability function. Figure S2 (supplementary material) shows the contour plot of overall desirability. Table 5 shows predicted (from models) and observed (from confirmation trials) response variables, as well as percent error for comparing values.

### 3.4. Scale-up experiment

In order to confirm that results from the laboratory scale optimization experiments would produce similar results at a pilot plant scale, the extraction process was carried out under both conditions. Ideally, laboratory settings and results should be successfully replicated at a pilot plant scale and eventually at a production scale. Alternatively, key production aspects or conditions that were not successfully scaled-up should be identified and adjusted accordingly. Normally, the aim of the processing scale-up is to determine the optimum production process for product quality, product yield, process control, and costs (Earle & Earle, 2009); however, in this study only yield and product composition throughout the process, and gel characteristics of the final product were evaluated (no cost or process control analyses were performed).

Table 6 shows calculated yield values after each of the main unit operations in the extraction processes under laboratory and pilot plant conditions. Significantly different results ($p \leq 0.05$) between processing conditions were observed for the third wash and the centrifugation step. In both cases, laboratory conditions resulted in higher yields. Differences at the centrifugation step were likely due to the equipment used and the operating conditions. Although yields were about 20% higher at the laboratory scale than at the pilot plant scale, this result was likely due to the larger volume of water present in the gelatin extract, which was eventually eliminated through freeze-drying to obtain dried gelatin.

Table 7 shows the pH and moisture, protein, fat, and ash contents of raw materials, products, and by-products obtained from extraction processes under laboratory and pilot plant conditions. Significantly different results ($p \leq 0.05$) between by-products produced under the two conditions were found only in the pH values of the second and third spent wash waters, and the solid residue from the extraction step. In all three cases, pH values were higher in the pilot plant trials. The only additional significant difference ($p \leq 0.05$) was in the protein content of the solid residue, which was higher at the pilot plant scale.

Table 5. Predicted (from models) and observed (from confirmation trial) response variables, at optimized extraction conditions (treatment temperature 54.0°C and treatment time 0.38 h).
Tabla 5. Variables respuesta predichas (según los modelos) y observadas (según el ensayo de confirmación) en las condiciones óptimas de la etapa de extracción (temperatura de tratamiento 54.0 °C y tiempo de tratamiento 0.38 h).

| Response variable | Predicted value [a] | Observed value [b] | Error (%) |
|---|---|---|---|
| Gelling temperature (°C) | 20 ± 1 | 19.0 ± 0.4 | 7.2% |
| Melting temperature (°C) | 26.4 ± 0.7 | 25.6 ± 0.3 | 3.0% |
| Gel strength (g) | 596 ± 42 | 558 ± 42 | 6.9% |

[a]Predicted value ± 95% confidence interval.
[b]Means of observed results ± 95% confidence interval (n = 3).
[a]Valor predicho ± intervalo de confianza al 95%.
[b]Promedios de los resultados observados ± intervalo de confianza al 95% (*n* = 3).

Table 6. Calculated yield values (%) after main unit operations in the extraction processes under laboratory and pilot plant conditions.[a]
Tabla 6. Valores de rendimiento (%) calculados después de las principales operaciones unitarias de los procesos de extracción, bajo condiciones de laboratorio y de planta piloto.[a]

| Process | Acid pretreatment | Wash 1 | Wash 2 | Wash 3 | Extraction | Centrifugation |
|---|---|---|---|---|---|---|
| Laboratory scale | 175 ± 10 [a] | 174 ± 10 [a] | 183 ± 11 [a] | 196 ± 15 [a] | 447 ± 33 [a] | 434 ± 35 [a] |
| Pilot plant scale | 164 ± 10 [a] | 161 ± 7 [a] | 170 ± 1 [a] | 168 ± 10 [b] | 409 ± 14 [a] | 361 ± 9 [b] |

[a]Means of observed results ± 95% confidence interval (n = 3). Values in the same column with different letters are significantly different (p ≤ 0.05).
[a]Promedios de los resultados observados ± intervalos de confianza al 95% (n = 3), los valores en la misma columna con letras diferentes son significativamente diferentes (p ≤ 0.05).

Table 7. Results of physicochemical analyses performed on raw materials, products, and by-products obtained from extraction processes under laboratory (L) and pilot plant (PP) conditions.[a]
Tabla 7. Resultados de los análisis fisicoquímicos realizados en materias primas, productos y subproductos obtenidos de los procesos de extracción bajo condiciones de laboratorio (L) y de planta piloto (PP).[a]

| | pH | | Moisture (g/100 g) | | Protein (g/100 g) | | Fat (g/100 g) | | Ash (g/100 g) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Material | L | PP | L | PP | L | PP | L | PP | L | PP |
| Tuna skin | 5.9 ± 0.1 | 5.9 ± 0.1 | 58.0 ± 3.0 | 60.8 ± 0.7 | 28.0 ± 5.0 | 25.8 ± 0.9 | 6.0 ± 1.0 | 5.0 ± 1.0 | 3.5 ± 0.5 | 3.5 ± 0.7 |
| Spent acid solution | 4.5 ± 0.1 | 4.6 ± 0.2 | 99.0 ± 0.5 | 99.6 ± 0.1 | 0.6 ± 0.2 | 0.7 ± 0.2 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| Spent wash water 1 | 4.6 ± 0.1 | 4.6 ± 0.1 | 99.6 ± 0.1 | 99.6 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 |
| Spent wash water 2 | 4.6 ± 0.1 b | 4.8 ± 0.1 a | 99.9 ± 0.1 | 99.9 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 |
| Spent wash water 3 | 4.6 ± 0.1 b | 4.8 ± 0.1 a | 99.9 ± 0.1 | 99.9 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 |
| Solid residue | 5.5 ± 0.1 b | 5.7 ± 0.1 a | 73.0 ± 5.0 | 67.0 ± 2.0 | 15.0 ± 4.0 b | 23.0 ± 3.0 a | 3.0 ± 1.0 | 5.0 ± 1.0 | 3.0 ± 2.0 | 2.5 ± 0.7 |
| Sediment | - | 5.0 ± 0.1 | – | 98.0 ± 2.0 | – | 2.0 ± 2.0 | – | 0.7 ± 0.7 | – | 0.1 ± 0.1 |
| Gelatin extract | 4.9 ± 0.2 | 5.1 ± 0.2 | 99.0 ± 1.0 | 99.0 ± 1.0 | 1.0 ± 1.0 | 3.0 ± 2.0 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 |

[a]Means of observed results ± 95% confidence interval (n = 3), values in the same row, for each analysis, with different letters are significantly different(p ≤ 0.05).
[a]Promedios de los resultados observados ± intervalos de confianza al 95% (*n* = 3), los valores en la misma fila, para cada análisis, con letras diferentes son significativamente diferentes (*p* ≤ 0.05).

Determinations were not performed on the sediment (by-product from the centrifugation step) at laboratory conditions, since the amount produced was insufficient for analyses. Comparison of the protein content of the gelatin extracts showed no significant differences ($p > 0.05$), which indicated that the difference in yield obtained after the centrifugation step (Table 6) was due to the difference in water content. Figure 5 shows the gelling temperature, melting temperature, and gel strength of gels obtained from extraction processes under laboratory and pilot plant conditions. The gelling temperature was significantly higher ($p \leq 0.05$) in the gel produced at the laboratory scale; the difference in gelling temperatures was 0.82°C. Figure 6 shows the molecular weight profile of proteins extracted under laboratory and pilot plant conditions. The SDS-PAGE patterns of proteins extracted under pilot plant conditions showed a higher proportion of lower molecular weight protein bands (37 kDa to 100 kDa range). This was likely due to the longer extraction times at the pilot plant scale. Longer exposure to higher temperatures induces hydrolysis and results in larger amounts of lower molecular weight proteins (Kittiphattanabawon et al., 2010; Normand et al., 2000). This consequence could affect gel characteristics of the final product; however, as discussed (Figure 5), only the gelling temperature was significantly affected ($p \leq 0.05$).

## 4. Conclusions

In order to improve the general extraction process of gelatin from Yellowfin tuna (*Thunnus albacares*) skin, this study aimed to optimize the operating conditions for acid pretreatment. Response variables skin hydration, gelatin extract, gel strength, and gel pH were successfully modelled. Acetic acid concentration of 0.225 M and treatment time of 2.5 h maximized skin hydration and gelatin extract. Operating conditions for the extraction step were also optimized. Response variables gelling temperature, melting temperature, and gel strength were successfully modelled. Treatment temperature of 54°C and treatment time of 0.38 h maximized response variables. In general, process conditions affected the gel properties, and some effects were confirmed by evaluating the molecular weight profile of extracted proteins obtained through SDS-PAGE. The feasibility of the general extraction process scale-up was confirmed by comparing process yields and characteristics of gelatins, obtained at laboratory and pilot plant scales (scale-up ratio 80:1). However, further trials are needed to determine the optimum production conditions, particularly those related to process control and

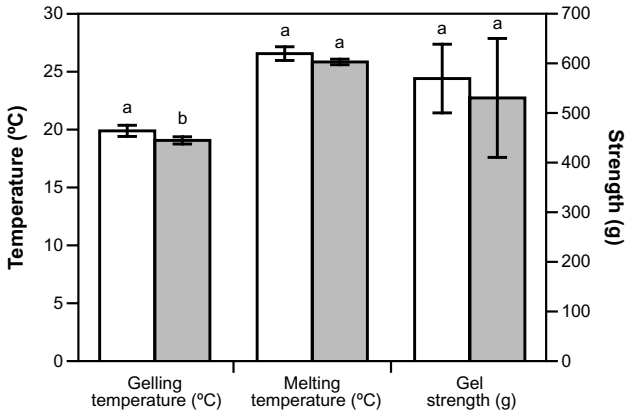


**Figure 5.** Gelling temperature, melting temperature, and gel strength of gels obtained under laboratory (white bars) and pilot plant (grey bars) conditions.[a]
**Figura 5**. Temperatura de gelificación, temperatura de fusión y fuerza del gel de los geles obtenidos bajo condiciones de laboratorio (barras blancas) y de planta piloto (barras grises).[a]

[a]Means of observed results, error bars represent 95% confidence interval ($n$ = 3). Columns in the same analysis, with different letters are significantly different ($p \leq 0.05$).
[a]Means of observed results, error bars represent 95% confidence interval (n = 3), Columns in the same analysis, with different letters are significantly different (p ≤ 0.05).
[a]Promedios de los resultados observados, las barras de error representan intervalos de confianza al 95% (n = 3), columnas en el mismo análisis, con diferentes letras son significativamente diferentes (p ≤ 0.05).

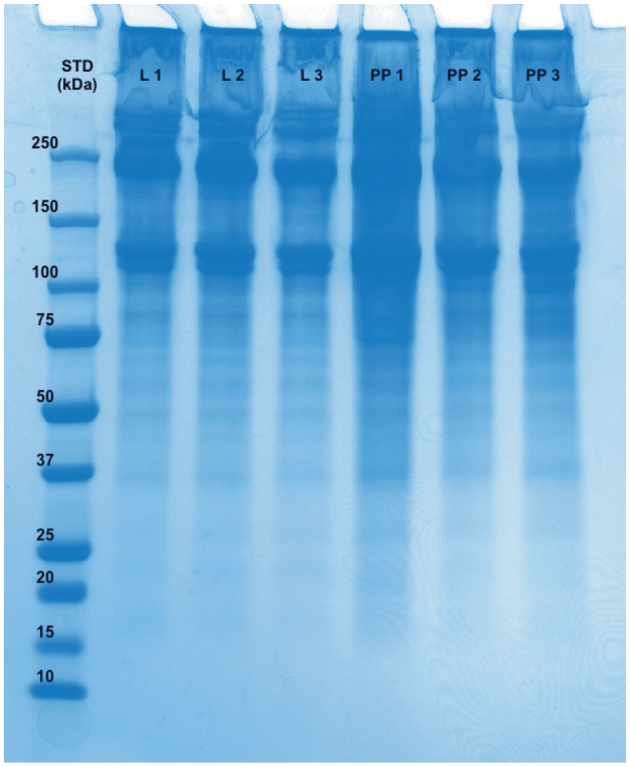


**Figure 6.** Molecular weight profile of extracted proteins obtained through SDS-PAGE from extraction processes under laboratory (L) and pilot plant (PP) conditions, with standards (STD) showing molecular weights (kDa).
**Figura 6**. Perfiles de peso molecular de las proteínas extraídas en los procesos de extracción bajo condiciones de laboratorio (L) y de planta piloto (PP), obtenidos por SDS-PAGE, con los estándares (STD) mostrando los pesos moleculares (kDa).

costs. Attention should be given to the protein extraction yields, in order to determine if they are affected by inadequate collagen swelling, which affects extraction efficiency, or gelatin loss during the wash steps.

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## Disclosure statement

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