Research Article

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Extraction and characterization of type I collagen from scales of Mexican Biajaiba fish

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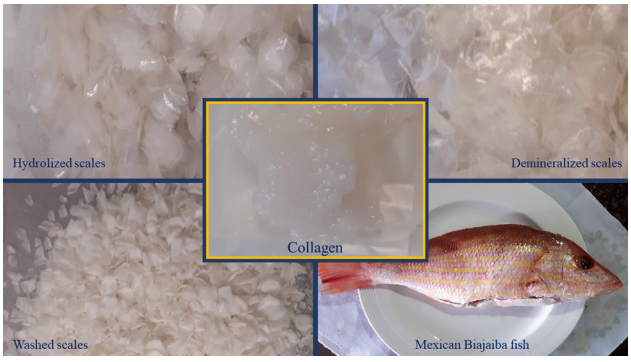
Abstract: Type I collagen is a high-value polymer found naturally in animal species and with many applications in the biomedical field. Collagen is frequently obtained from bovine tendons, but this source presents the risk of disease transmission, thus marine collagen is becoming an alternative source of this valuable material. In the present work, we report the successful extraction and characterization of the natural collagen found in the scales of Biajaiba, a highly consumed fish native to the Gulf of Mexico. We obtained acid- and pepsin-soluble type I collagens with high denaturation temperatures, high hydroxyproline contents, and yields in the ranges reported for other fish scales. Our work proposes a useful alternative for transforming the huge quantities of discarded fish scales in our country to extract a high-value product for biomedical applications.

Keywords: type I collagen, hydroxyproline, Biajaiba fish, fish scales



Graphical abstract

1 Introduction

Collagen is the most abundant protein in animal bodies being present in bones, skin, hair, and tendons. Its main function is to provide tissue flexibility and the capability to stretch. The collagen required by industries and health research is usually obtained from bovines, rats, avians, porcines, amphibians, goats, and marine species, and all of them pose risks, difficulties, and advantages, as reviewed in the study by Naomi et al. [1]. However, marine collagen is becoming more popular since it avoids the transmission of mammalian diseases and, furthermore, represents a new alternative to transform organic wastes into materials with added value to be used in healthcare [2].

There are about 28 collagen types, all exhibiting helices, formed by triple chains of polypeptides linked by hydrogen bonds. Among them, type I collagen forms fibers made of repetitive triple-helical structures of glycine, proline, and hydroxyproline (HDP) [1]. This particular type of collagen is found in connective tissues such as bones, skin, and tendons in the human body, which is why its recovery becomes a challenging aim for biomaterials researchers.

Biajaiba fish (*Lutjanus synagris*) is one of the species of the *Lutjanidae* (commonly known as *Lutjanus*) family. There are about 100 species in this family, all of which are native to tropical and subtropical waters such as the Gulf of Mexico. The Mexican Biajaiba fish has a typical length of 25 cm on average, and its normal weight is around 600 g [3]. The area in the Gulf of Mexico where it is commonly found (Campeche and Yucatán) has a range of temperatures between 25 and 27°C. Mexico produces annually 5,000 tons on average of the *Lutjanus* family fishes, leading to about 50 tons of scales that are totally discarded [4]. Despite this huge quantity of organic residues, there are no reports about the use of Biajaiba scales for any recovery process leading to high-value products in our country. There have also been previous investigations on collagen extraction from the *Lutjanus* family, but they have focused on

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processing the fish skin in other countries [5,6], and none of them have used the *Lutjanus synagris* species reported here.

Fish scales mainly consist of calcium phosphates, collagen, and keratin. Their composition and biocompatibility make them a very promising biomaterial. Some works have reported the extraction of collagen from fish scales of different species, such as Tilapia [7], *Ctenopharyngodon idella* [8], grass carp [9], Lubina [10,11], loach [12,13], and Mediterranean fishes [14]. It is worth noting that most of these works only discuss the recovery of pepsin-soluble collagen (PSC), while some of them deal with both acid-soluble collagen (ASC) and PSC obtained from the same fish species [11–14]. Moreover, not all of them reported yield values, and thus the comparison of yields is not direct and needs to consider the details of the extraction method as well as the particularities of each species.

In the present work, we present the successful extraction of type I collagen, a high-value product for biomedical applications, following two different routes that produce ASC and PSC from the scales of a native, highly consumed Mexican fish. We tried double extraction on the processed scales and found that this process can give extra yields without compromising the quality of the material.

2 Methods

2.1 Collagen extraction

The experimental methods to extract the collagen included a pre-treatment stage, hydrolysis, demineralization, extraction, precipitation, dialysis, and lyophilization, as shown in the flow diagram in Figure 1(a). All steps were done in a small system that kept the temperature between 4 and 8°C, and all solutions were moderately stirred with magnets. Between each step, scales were always washed with cold distilled water.

Scales from Biajaiba fishes were obtained weekly from fresh fishes at a market in Mexico City. Once collected, scales were washed with cold tap water, dried, and kept in a freezer until they were processed. Three batches of 25 g (named A, B, and C) of scales were separated and marked. The batches were washed with cold distilled water and dried at room temperature before they were treated (Figure 1(b)). The treatment stage was based on the methodology described in [8], with some modifications that will be detailed below.

The treatment stage consisted of three steps: (1) the hydrolyzation with NaOH 0.1 M for 36 h, keeping a pH between 7 and 9, and changing the solution every 12 h; (2) the demineralization using EDTA 0.5 M at a ratio of 1:10 (w/v) for 72 h, keeping the same pH, and changing the solution every 24 h; and (3) the extraction of collagen using acetic acid with or without pepsin. Figure 1(c) shows the demineralized scales just before the extraction step.

During collagen extraction, for batch A, we exactly followed the method described in [8] to obtain only PSC, while for batches B and C, we slightly modified the extraction process. We obtained a first extraction with 0.1% pepsin for batch B and with only acetic acid for batch C. Then, for both batches, a second extraction was done using 1% pepsin instead of 0.1%. As reported previously, type I collagen extracted from fish has a high solubility in acid in contrast to mammalian collagen [1].

The first extraction was accomplished by soaking scales for 72 h with moderate stirring in 0.5 M acetic acid with 0.1% pepsin to produce PSC (for batches A and B) or without pepsin to produce ASC (batch C). The solid residues of the first extraction were washed, and then extraction was repeated using acetic acid containing 1% pepsin to obtain a second yield of PSC for batches B and C. Suspensions were centrifuged for 20 min with 10,000 *g* at 4°C, and then we salted out the floating material with 2 M NaCl, leaving the solution for 12 h. Precipitated collagen was collected by centrifugation at 2,095 *g* for 40 min. The salting-out step was then repeated. Collagen gels were dialyzed in cold distilled water using a dialysis membrane of 12–14 kDa. Finally, gels were lyophilized, so we obtained dry flakes of collagen.

2.2 Characterization of samples

All extracted collagens were characterized by ultraviolet (UV) spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, electrophoresis (SDS–PAGE), atomic force microscopy (AFM), and thermogravimetric analysis (TGA). We used commercial collagen from bovine Achilles tendon (Sigma Aldrich) as a standard (STD) reference for characterizations.

UV absorbance spectrophotometry was done using a NanoDrop 2000, UV–Vis spectrophotometer. The samples were obtained by solving 5 mg of collagen in 0.5 M acetic acid to have a concentration of 1 mg/ml. Spectra were recorded between 200 and 400 nm.

FTIR spectroscopy was performed with a Varian 3600 FTIR Excalibur Series spectrophotometer, reading from 4,000 to 500 cm^{-1}, and using the lyophilized collagens.

Electrophoresis (SDS–PAGE) was done using a Bio-Rad Mini-PROTEAN electrophoresis system. We have followed the method of Laemmli [15] with 8% of separating gel and 4% of concentrating gel. Collagen samples of 13 µl, solved in acetic acid 0.05 M with a concentration of 1 mg/ml, were

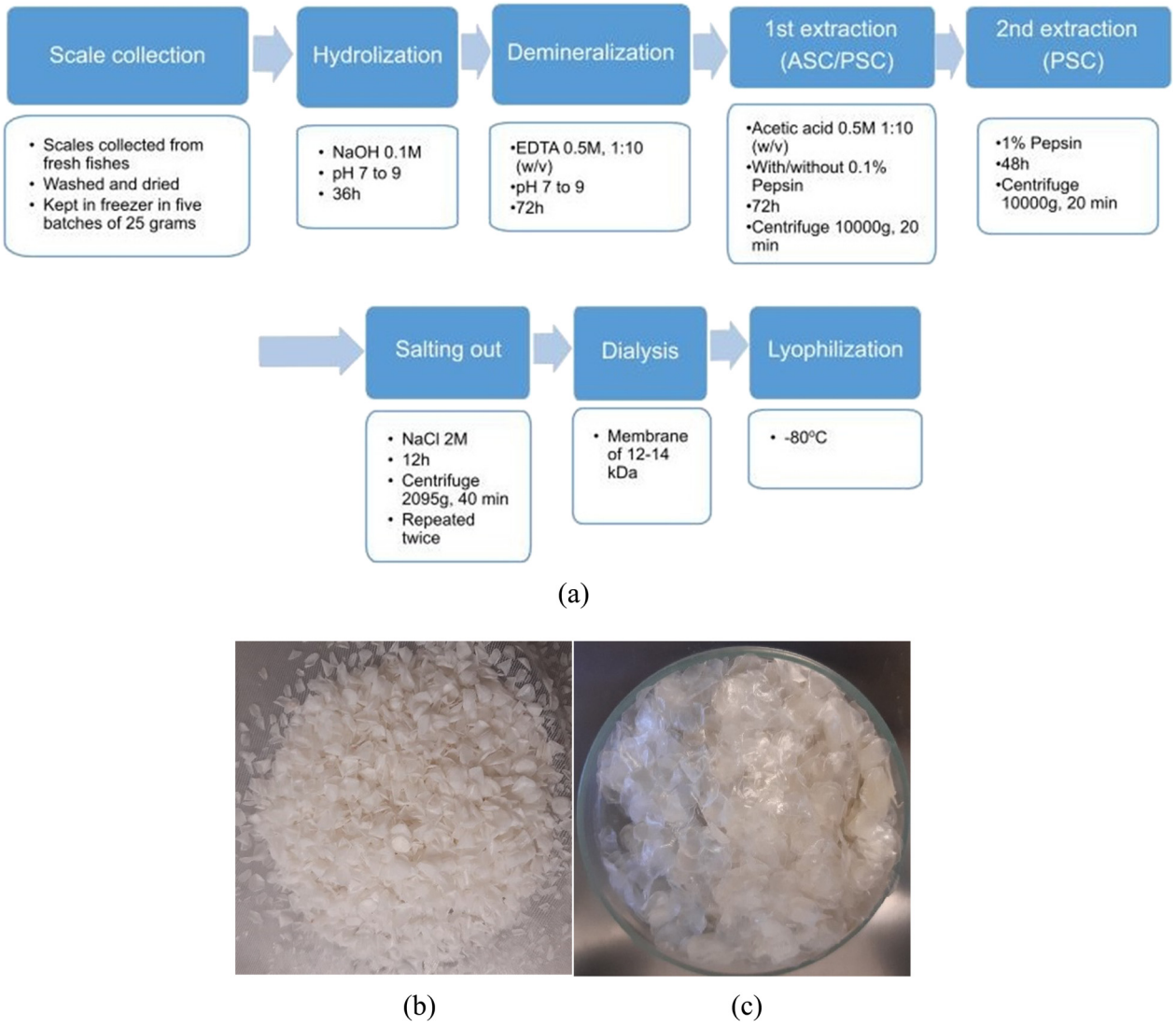


Figure 1: Experimental details. (a) Flow diagram of the extraction process. Images of Biajaiba scales, (b) after being washed and dried, just before hydrolyzation, and (c) after demineralization, just before the extraction steps.

mixed with a buffer solution of Tris–HCl (0.5 M, pH 6.8), glycerol, SDS 10%, 2-mercaptoethanol, and bromophenol blue 1%. These mixtures were microwaved for 30 s. Electrophoresis was performed at 180 V for 45 min, then the gel was stained with a Coomassie blue solution. Patterns of our samples were compared to a STD collagen of bovine Achilles tendon (Sigma Aldrich).

TGA was performed with a Thermo SDT Q600 in air atmosphere, in a range of temperatures between 25 and 650°C with a heating rate of 10°C/min.

AFM images were taken in an Agilent 5500 microscope in tapping mode with soft tapping tips from Budget Sensor. A drop of the collagen solution (dilution 1/50) was deposited on a mica surface and allowed to dry. Images were taken in air after thoroughly rinsing the sample with distilled water.

The quantification of HDP was done following the Woessner method [12]. An STD solution was prepared with 1 mg HDP in 100 ml HCl 0.001 N, and this solution was diluted with deionized water to obtain the calibration curve in the range of 0–7 µg. Preparations of 1 mg of collagen hydrolyzed with 1 ml of HCl 6 N were heated at 130°C for 3 h. The quantity of HDP was determined by reading the absorbance twice for each 0.5 ml of sample. Absorbance spectra were obtained at 557 nm using a Shimadzu UV-1800 UV/Visible scanning spectrophotometer.

Collagen (ASC and PSC) yields were calculated as follows: Yield (%) = (Dry collagen weight/Dry scales weight) ×

100. In the case of collagens obtained in a first extraction, the weight of scales used for this calculation was 25 g (the whole batch), while for collagens from the second extraction, the value used was the weight of the solid residues after the first extraction.

3 Results

In this section, we organize our results by dividing them into two parts: first, the characterizations of the extracted collagens to confirm the presence of type I collagen, and then the quantification of HDP and the collagen yields. We present the characterization of five samples of collagen obtained from three batches of scales, as explained in Section 2. Samples were named A, B, B′, C, and C′. We use the apostrophe to point out that B′ and C′ are the samples for the second extraction of PSC from samples B and C. In particular, C was our sample for the extraction of ASC during the first stage.

3.1 Characterization of extracted collagen

We obtained five collagens from the scales, four PSCs and one ASC. All of them were characterized by various techniques, as described in Section 2. First, we used three characterization techniques to confirm that we obtained type I collagen: UV spectrophotometry, electrophoresis, and FTIR spectroscopy. The UV absorption spectra of our five extracted collagens and the STD collagen are shown in Figure 2. All of them show an absorbance peak between 211 and 222 nm. These wavelengths are in good agreement with the values reported for ASC and PSC from fish skins (218 [12], and 232 nm [16]) and for the scales of other fish species (230–232 nm [14]). The fact that our spectra do not exhibit other peaks in the studied range can be understood as a low contamination of aromatic amino acids in our collagens.

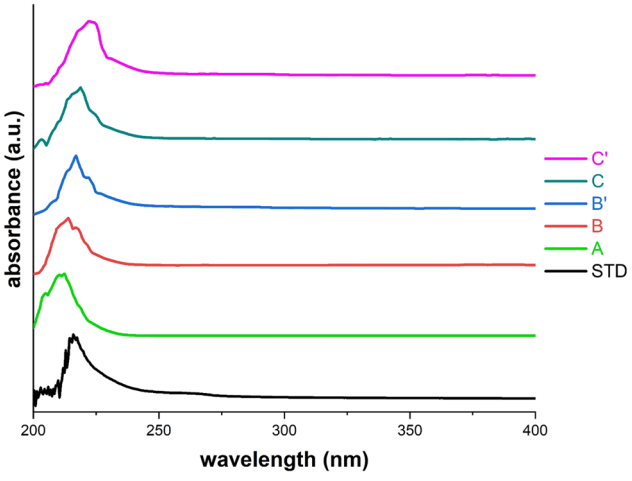


Figure 2: UV absorption spectra of the five extracted collagens and the STD collagen.

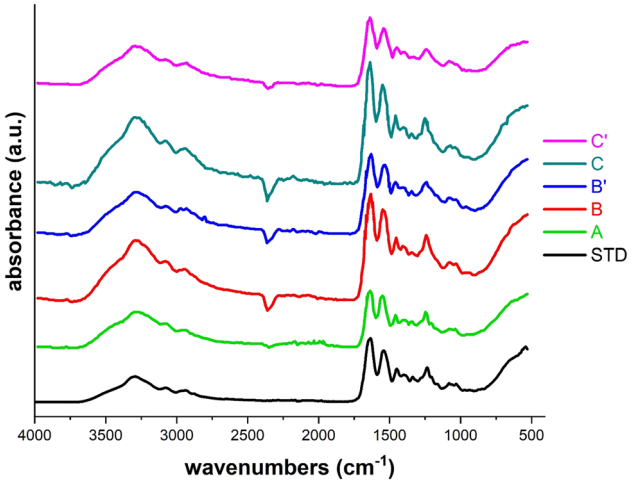


Figure 3: FTIR spectra of the five extracted collagens and the STD collagen. The position and assignment of absorbance peaks are detailed in Table 1.

The FTIR spectrum for each sample was obtained to look for the characteristic peaks of collagen. In Figure 3, we show the infrared spectra of our five samples and the STD collagen. As can be seen, the spectra of our extracted collagens are in good agreement with the STD collagen. The patterns exhibit all characteristic peaks for the five amide bonds (Table 1), as reported for ASC and PSC in other fishes [8,9]. We also observe that absorbance peaks for both extracted collagens (PSC and ASC) are in almost the same positions, supporting the idea that enzymatic treatment does not affect the structure of molecules, as reported in previous experiments [14].

Table 1: Position and assignment of absorbance peaks from the FTIR spectra of all collagens

	Wavenumber (cm^{-1})					
Bond	**C′**	**C**	**B′**	**B**	**A**	**STD**
Amide A	3,284	3,286	3,280	3,278	3,290	3,293
Amide B	2,927	2,929	2,929	2,935	2,935	2,952
Amide I	1,629	1,629	1,629	1,629	1,629	1,660
Amide II	1,544	1,546	1,535	1,546	1,546	1,540
Amide III	1,236	1,236	1,232	1,236	1,238	1,219

The presence of the amide III peak is associated with the integrity of the triple helical structure [17,18], and the low frequency observed for amide A peaks (below 3,400 cm^{-1}) can be attributed to the presence of hydrogen bonds with carbonyl groups, as in other fish collagens [17]. Amide I is considered very useful to know more about the secondary structure of proteins, and in the case of our collagens, it may correspond to imide residues [18]. Amide II corresponds to N–H bonds. About amides A and B, we observe that they appear somehow merged. As discussed in [18], this observation indicates that these two bands tend to merge when carboxylic acid groups are in stable intermolecular crosslinks.

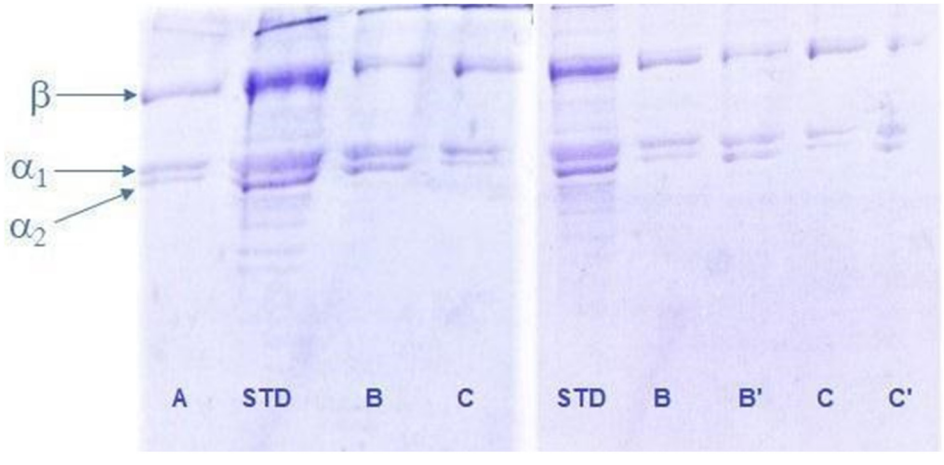
Electrophoresis was performed on our five samples along with the STD collagen. The results are shown in Figure 4. First, STD was compared to samples A, B, and C, which are collagens obtained during the first extraction process (Figure 4a). They correspond to PSC for samples A and B and to ASC for sample C. As can be noticed, all three samples exhibit the β, α1, and α2 bands, confirming the presence of type I collagen [17]. We also obtained electrophoretic patterns for samples B, B′, C, and C′ (Figure 4b), and all of them also exhibit the β, α1, and α2 bands. All results support the fact that we obtained type I collagens during the first and second extractions.

Two more characterizations were performed on our collagens, AFM and TGA, to learn more about their textural and thermal properties. We selected two of our samples to be observed with these techniques to compare ASC (sample C) and PSC (sample C′) characteristics, which also correspond to the first and second extractions of the same batch.

AFM images corresponding to ASC and PSC obtained in the first and second extractions of batch C are shown in Figure 5. The aim was to know more about the topological organization of both materials and to see textural differences in both types of collagen. We observe the formation of thin collagen fibers for both materials, although they are better defined for the sample obtained during the first extraction (Figure 5a).

Collagen fiber diameters can range between 20 and 500 nm, depending on the species, tissue, or developmental stage [19]. We can estimate from the fiber widths shown in Figure 6 that the diameters of the fibers extracted are small, around 20 nm, but that they can associate into thicker filaments of around 60 nm in diameter. Collagen fibers obtained in the first and second extractions are then compatible with microfibrils that cross-link to form long fibers in type I collagen [20]. As can be observed in Figures 5 and 6, the fibers present in ASC are thinner and better defined than those found in PSC, which are immersed in a poorly defined mesh. In both cases, collagens are made of thin fibers running in different directions, building a network that could be useful to support cell proliferation, as reported for collagens from other fishes [21]. Our findings are also in agreement with the observation of a smooth surface with thin fibrils in a network, reported for ASC from Mediterranean fishes [14].

Finally, we performed thermal analysis on the same two samples (C and C′, corresponding to ASC and PSC, respectively) and on the STD collagen (Figure 7). In all these collagens we found two main weight losses, below 100°C and around 350°C (indicated with dashed lines in Figure 7).



(a) (b)

Figure 4: Electrophoresis analysis patterns. (a) Collagens obtained in the first extraction of batches A, B, and C. (b) Comparison of collagens obtained in the first and second extractions of batches B and C (the apostrophe indicates second extraction). STD corresponds to the STD of bovine tendon collagen used to mark bands α1, α2, and β.

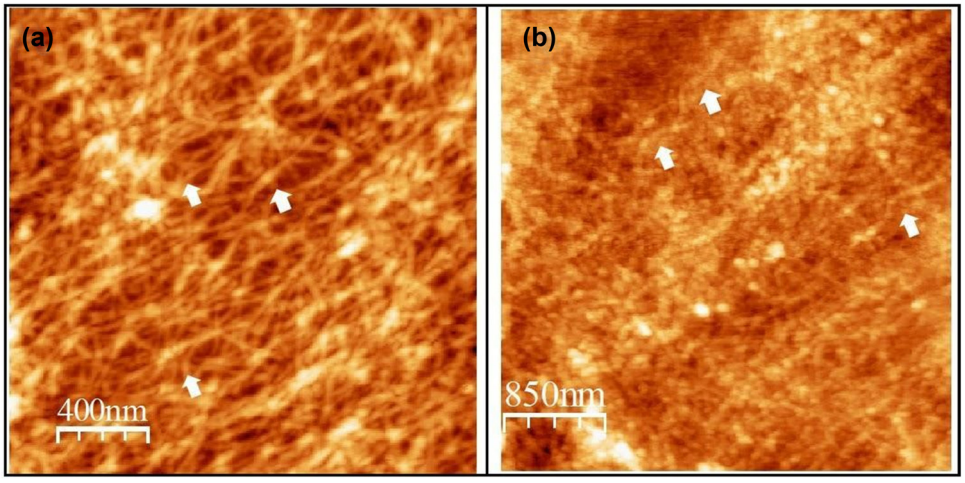


Figure 5: Topographic images of a mesh of collagen fibers air-dried on mica obtained by AFM. Fibers in ASC (a) and PSC (b) extractions from batch C. The white arrows point toward individual collagen fibers. Fibers in (b) are less defined, most likely due to the presence of some additional material present in between them.

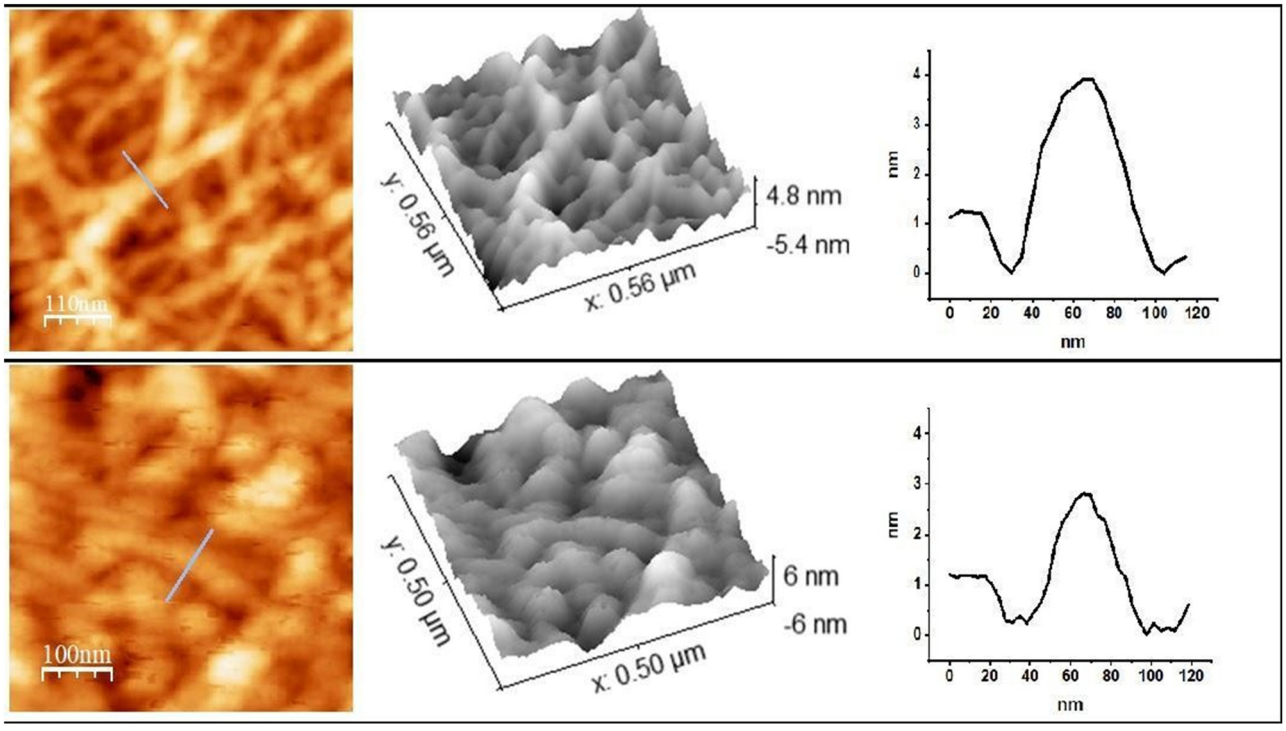


Figure 6: AFM profiles and surface images of collagen fibers obtained in the first (top, ASC) and second (bottom, PSC) extractions from batch C. Left, two-dimensional representations. Center, three-dimensional representation of the same area. Right, height profile of the lines shown across the fibers.

These weight losses were found based on the curve derivative calculated by the equipment. The first one is mainly due to water loss from the protein, which reduced the weight by 3.1 and 3.6% for PSC (sample C') and ASC (sample C), respectively, and 3.4% for the STD collagen. These values are in the order of reductions of ASC (4.3–6.1%) and PSC (4.4–5.5%) obtained from the scales of some Mediterranean fishes [14]. In our extracted collagens, this initial weight loss peaks around 38 and 42°C. We assume that these values point out the denaturation temperatures of our collagens, even though these temperatures are higher than those reported for collagens extracted from the skins of other species (between 30 and 34.5°C) [22,23], or from some fish scales (between 29 and 35°C) [13,24], but not so different

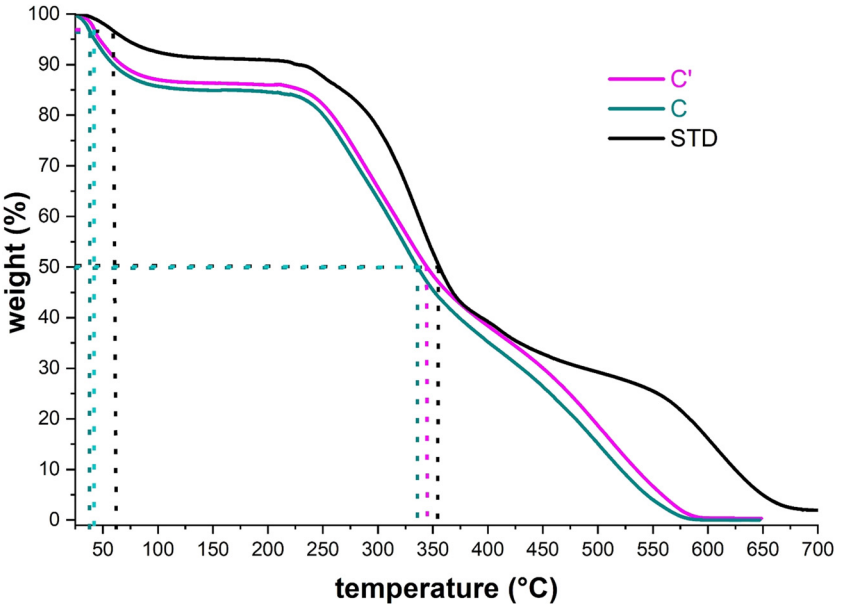


Figure 7: Thermogravimetric curves obtained for samples C (ASC) and C′ (PSC), and the STD collagen. Two main weight losses are marked in the curves: elimination of water (below 100°C) and the major degradation temperatures (around 350°C).

from the scales of other fishes (38–39°C) [11]. Higher denaturation temperatures are linked to the thermal stability of collagen, which has been related to the temperature of the living environment, as discussed in [9]. In our case, Biajaiba fishes prefer warm sea waters such as the Gulf of Mexico, which has a superficial temperature between 25 and 27°C [4].

The second main weight loss for all collagens was observed between 336 and 356°C, which indicates the main decomposition of collagen. These temperatures are similar to those measured for the scales of other fishes (303–323°C) [14]. We also observed that above 550°C, there is a final rapid weight loss, and near 600°C, there is almost no remaining material in our extracted collagens, denoting that our scales were adequately demineralized and have no presence of inorganic elements commonly found in fish scales (mainly calcium and phosphate) [23].

3.2 Quantification of HDP and collagen yields

Quantification of HDP was done using spectrophotometry, as described in Section 2. We measured between 6.10 and 6.63 µg of HDP in 0.5 ml of collagen sample (average 6.3 µg), as shown in Figure 8. Then, in our collagen samples, HDP represents, on average, 12.60 ± 0.53% which, although

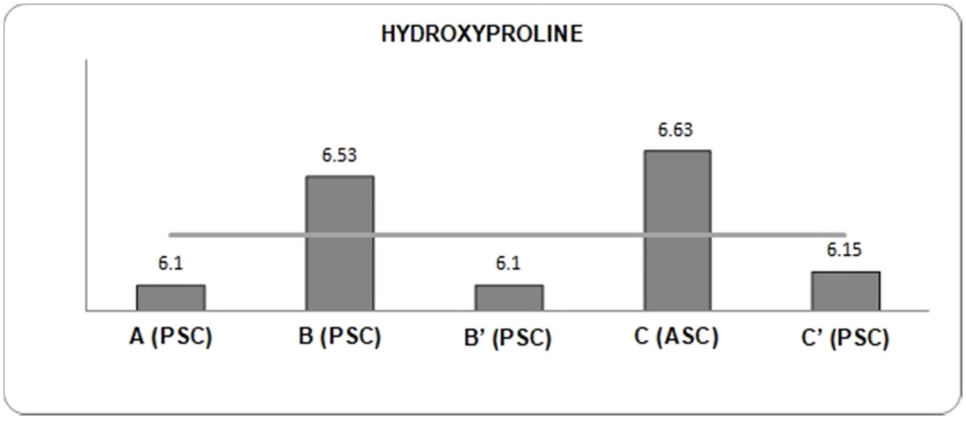
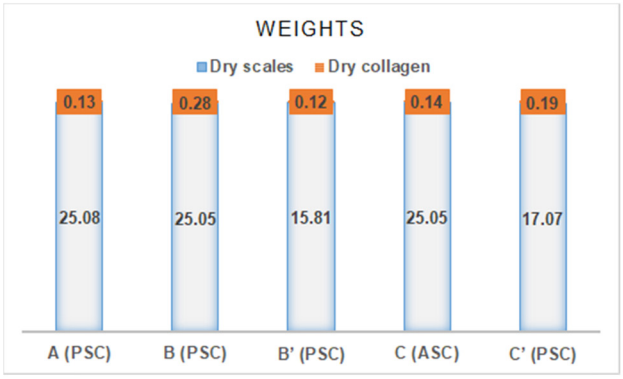
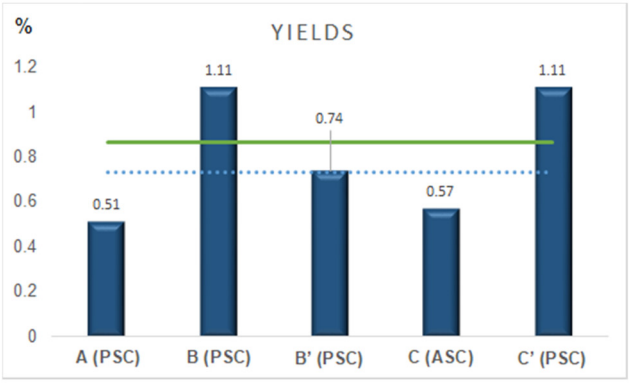


Figure 8: HDP contents of extracted collagens. Quantification was done using spectrophotometry, as described in Section 2. Values are given in µg per each 0.5 ml of sample. The mean value (6.3) is indicated with a continuous line.



(a)



(b)

Figure 9: Yields of collagens extracted from the five batches of scales. (a) Dry weights (in grams) of collagens extracted from the indicated weight of scales. (b) Collagen yields of the five batches calculated as (Dry collagen weight/Dry scales weight) × 100. The mean value of first extractions (A, B, and C) is 0.73% indicated with a blue dashed line, and the mean value of only PSC samples is 0.8675% indicated with a green continuous line.

higher than quantities found for some fishes and sharks [2,17,25], is in coincidence with the HDP content in human and calf type I collagens (12.8–13%) [26]. We also note a slightly higher HDP content in our sample of ASC than in the PSC samples, as reported in a previous work [12]. On the other hand, this high content of HDP could explain the higher denaturation temperatures of our samples, as discussed in [24]. Our results support the idea that the thermal stability of collagen for fishes living in warm environments is higher than that for fishes from cold waters [2], since Biajaiba fishes prefer warm sea waters such as the Gulf of Mexico, which has a superficial temperature between 25 and 27°C [4].

Finally, Figure 9 summarizes the yields obtained for both collagen types, PSC and ASC. The bars in Figure 9a indicate the weights of dry collagen extracted from each batch of dry scales. Note that for second extractions (batches B′ and C′), the weights are lower than for first extractions since we are taking the weight of the remaining solid residues after the first extraction. As can be seen in Figure 9b, the sum of the first and second extractions of collagen from batches B and C gave similar yields, 1.85 and 1.68%, respectively (dry weight). The average yield of first extractions from the three batches was 0.73%, while the mean value of PSCs was 0.8675%. Yields of extracted collagen from our Biajaiba scales are in accordance with values reported for ASC from fish scales (between 0.4 and 7%) but are lower than some reports for PSC (38–51%) [2]. However, a recent work done on Mediterranean fish scales obtained yields between 0.39 and 1.13% for ASC and between 1.04 and 2.03% for PSC (dry weight) [14]. Furthermore, one study on the collagen extraction from the *Lutjanus vitta* fish skin reported 9% of ASC and 4.7% of PSC [5], and they concluded that it is a particularity of this fish skin to have more ASC than PSC. In our work with the *Lutjanus synagris* scales, both types of collagen seem to be in similar proportion, thus differences in yields could be due to habitat or extraction procedures. During first extractions, we obtained more and less PSC (batches B and A) than ASC (batch C), but in the second extraction of PSC, batch C gave more yield than batch B. This behavior agrees with other works reporting a higher yield for PSC than for ASC [17,27].

4 Conclusion

Fish scales are commonly discarded after human consumption. However, they constitute a good raw material to obtain a high-value product such as type I collagen. The Biajaiba fish, a species of the *Lutjanus* family native to and greatly consumed in Mexico, has not been studied for this purpose before the present work, as far as we know. As the amount of discarded fish scales in our country is huge, our proposal for collagen extraction emerges as a good option to reuse these organic wastes and to open its application for biomedical purposes.

Acronyms

ASC	Acid-soluble collagen
AFM	Atomic force microscopy
CSIC	Consejo Superior de Investigaciones Científicas
FTIR	Fourier Transform Infrared Spectroscopy
HDP	Hydroxyproline
ICP	Instituto de Catálisis y Petroleoquímica
PSC	Pepsin-soluble collagen

STD Standard
TGA Thermogravimetric analysis
UAM-A Universidad Autónoma Metropolitana campus Azcapotzalco

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Conflict of interest: Authors state that there is no conflict of interest.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: All data generated or analyzed during this study are included in this published article.

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