

Deproteinization of Moroccan Sardine Waste (*Sardina pilchardus*): A Pilot-Scale Study

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Abstract: The scale-up remains a complicated operation that affects the transition to an industrial scale. The main objective of this study was to examine the pilot-scale deproteinization of *Sardina pilchardus* scales waste by alkali treatment. Initially, a scale laboratory experiment was carried out to determine the optimal deproteinization condition of Sardine waste. The alkali treatment was found to be effective in the deproteinization of sardine wastes. Deproteinization optimum condition (4 ± 1.5 mg/g sample) was recorded with a material-solution ratio of 1:10. This optimal condition was then used to process the sardine scales waste at a pilot scale. The lab-scale and pilot-scale reactors investigation has been conducted on batch processes. The deproteinization of fish waste was then demonstrated on a pilot-scale (2Kg scales), resulting in protein content of 2 ± 0.10 mg/g sample and hydroxyproline content of 0.21 mg/g sample. Those results indicated that the pilot-scale experiment showed similar performance deproteinization to the lab-scale one. We can conclude that the deproteinization operation is validated at the pilot scale since it can be reproduced without quality and yield losses. The fish scales waste could then be potentially useful as raw substrates for value-added products such as collagen, gelatin, and chitosan.

Keywords: deproteinization; fish scale waste; *Sardina pilchardus*; lab scale; pilot scale.

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1. Introduction

In Morocco, the fish processing industry is an important source of income for a large number of by-products, including skins, bones, heads, guts, and scales. Those underutilized resources, particularly scales, contain many proteins, calcium, chitin, and chitosan [1-4]. Fish Scales resulting from the transformation operation can be used as raw material for collagen or gelatin production [5-7]. In Morocco, Uncollected scales cause serious problems for manufacturers by waterlogging. Beyond these drawbacks in factories, the scales are dumped directly into the public networks. This practice has led to serious environmental pollution problems near production sites. Considering this strong expectation of the regional fish industry scales, sardine upgrading is well justified. In addition, political and societal demands for sustainability and environment-friendly industrial production systems, coupled with the depletion of fish resources, drive this trend forward. Therefore, the fish scale used as a potential source to isolate collagen and gelatin has many applications in the food, cosmetic and

biomedical industries [6]. The main commercial process for collagen extraction from fish scales is based upon deproteinization by alkali treatment and demineralization by acid pre-treatment.

The transition from lab-scale to pilot-scale is a critical stage in technological development. The overall purpose of our research was to investigate the optimal deproteinization conditions of sardine scales waste to obtain a high collagen quality, which could be transferred into a pilot or industrial scale. The first aim of this work was to determine the best material-solution ratio for deproteinization and compare the non-collagens protein removal efficiency by 0.5% NaOH from sardine scales. The second aim was to evaluate the feasibility of a pilot-scale deproteinization from *Sardina pilchardus* scales waste by alkali treatment. A pilot-scale study was conducted at plant site conditions of the Specialized Center of Valorization and Technology of Sea Products in Agadir, Morocco. The optimal condition for the deproteinization, which was validated at the laboratory scale, was employed in the pilot-scale procedure.

2. Materials and Methods

2.1. Chemicals.

All reagents were of analytical grade. Chloramine-T, p-dimethylaminobenzaldehyde, and trans-4-Hydroxy-L-proline (trans-L-4-hydroxypyrrolidine-2-carboxylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Raw material and preparation.

The raw materials used in this work were the scale waste of sardine (*Sardina pilchardus*). All the scales waste with an average weight range of 3.0–3.2 kg. Fish scales were obtained in fresh conditions from fish processing industries located at Agadir. The scales waste was immediately washed with tap water at room temperature (28 ± 2 °C) in order to remove protein, organic matter, and other impurities attached to the surface (Figure 1). A portion of the washed sardine scale was dried in a forced-air drying oven (D.H.MASTER, KD-21A, NAGANUMA/KUCHO, capacity: 50 ~ 80 kg/butch) at 30 ± 2 °C for 12 h (Figure 2), then was wrapped in polyethylene packaging and kept at -20°C for use in further experiments.



Figure 1. Shows the careful cleaning, triage, and drying of the fish at the pilot scale.



Figure 2. Master drying.

2.3. Lab-scale alkali treatment of sardine scales waste.

Alkali solution (NaOH) removed non-collagenous protein from sardine scales. For all experiments, reactions were carried out in an Erlenmeyer flask (250 ml capacity), each having 10 g of wet sardine scales. The preliminary experiment was to select the appropriate material ratio to the solution. With the deproteinization time of 4h in 0.5% of NaOH obtained from the previous study [8], scales were deproteinized under various material ratios to solution ranging from 1:2 to 1:12. The solution was changed every 2h. After alkali treatment, deproteinized sardine scales were collected by filtration, washed to neutrality with distilled water. In order, the protein content and hydroxyproline content in the residue and in all supernatants were determined.

2.4. Pilot-scale alkali treatment of sardine scales waste.

A pilot-scale study was conducted at plant site conditions of the Specialized Center of Valorization and Technology of Sea Products National Institute of Fisheries Research in Agadir, Morocco. The system comprises a mixer (MIXER MD-20 YANAGIYA MACHINERY/TAKEUCHI FOOD MACHINERY, Standard capacity: 20Kg and Tank capacity: 57L) (Figure 3) to carry out the deproteinization process. The sardine scales were deproteinized, similar to those for lab-scale deproteinization. This deproteinization was scaled-up in the mixer (with a capacity of 20Kg), each having 2kg dry or 25 g wet of sardine scales with 20L 0.5% NaOH followed by incubation for 4h (Figure 4). After treatment, deproteinized sardine scales were collected by centrifugation (CENTRIFUGAL HYDRO-EXTRACTOR DT2-18, Basket capacity: ϕ 457 x D220mm, 20 L, Maximum rotation speed: 1,300 rpm) (Figure 5) washed to neutrality with cold distilled water.

The pre-treatment solutions were collected to determine the non-collagenous proteins removed and the collagen lost during the pre-treatment processes.



Figure 3. The mixer.



Figure 4. Process plant during deproteinization of sardine scales.



Figure 5. Centrifugal hydro-extractor.

2.5. Analytic methods.

The dry weight and ash were determined according to AOAC methods [9], total lipids were performed according to the method of Bligt and Dyer [10], and total crude protein was determined by estimating its total nitrogen content [11]. A conversion factor of 6.25 was used to convert the nitrogen value to protein [12]. The protein content in each pre-treatment solution was determined by Bradford's method [13]. The yield of collagen was measured by determining hydroxyproline in the residue and extracting solutions. The hydroxyproline content was determined after hydrolysis of the filtrate in 6N HCl for 24h at 110°C, using Bergman's calorimetric method (described by the rapid procedure) Loxley [14].

2.6. Statistical analysis of results.

All experiments were completed in triplicate. The results are presented as mean \pm standard deviation, and the data obtained were statistically analyzed by the Analysis of Variance (MINITAB Release 16.00, Minitab), and the means were separated according to Tukey's test. The significance level was determined at the 95% probability level.

3. Results and Discussion

To determine the ratio of the scales to the raw material, we carried out a mass balance of different by-products (heads, viscera, scales, etc.) from the sardine processing (Figure 6).

Figure 7 shows the yield of raw material and by-products compared to whole fish. Furthermore, the results obtained have shown that the mass yield from the raw material relating to the whole sardine, namely fillets, skins, bones, heads, viscera, and scales, represents respectively 69%, 15%, 14%, and 2%.



Figure 6. Photographs of the different parts obtained after sardine filament (*Sardina pilchardus*): fillet, heads, viscera, and scales.

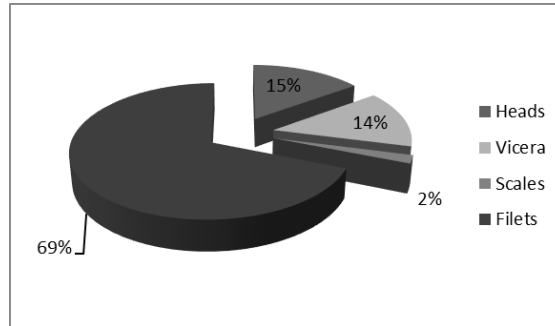


Figure 7. *Sardina pilchardus* by-product fractions as a percentage of the total wet weight.

Figure 7 presented *Sardina pilchardus* by-product fractions as a percentage of the total wet weight. The sardine scales waste used in the study contained moisture (59.51 ± 0.69 %) as a major component, followed by protein (36.25 ± 2.3 %) and ash (50.23 ± 0.47 %), respectively.

In this study, the influence of crude sardine scales on the basic solution ratio (1:8, 1:10, and 1:12) in the alkali treatment solutions was investigated. For this purpose, protein and hydroxyproline content assays with various material-solution ratios were carried out. The deproteinization solutions were collected to determine the non-collagenous proteins removed and the collagen lost during pre-treatment processes at 2h, 4h, 6h, and 8h. Figure 8 and Figure 9 show the accumulative protein and hydroxyproline contents in deproteinizing solutions. The results showed a difference significant in accumulative protein and hydroxyproline contents of the deproteinizing solution when material-to-solution ratios were increased from 1:8 to 1:12. From the results, the optimum condition for deproteinization of fish waste was 1:10 for 4h due to the mildest condition and shortest time.

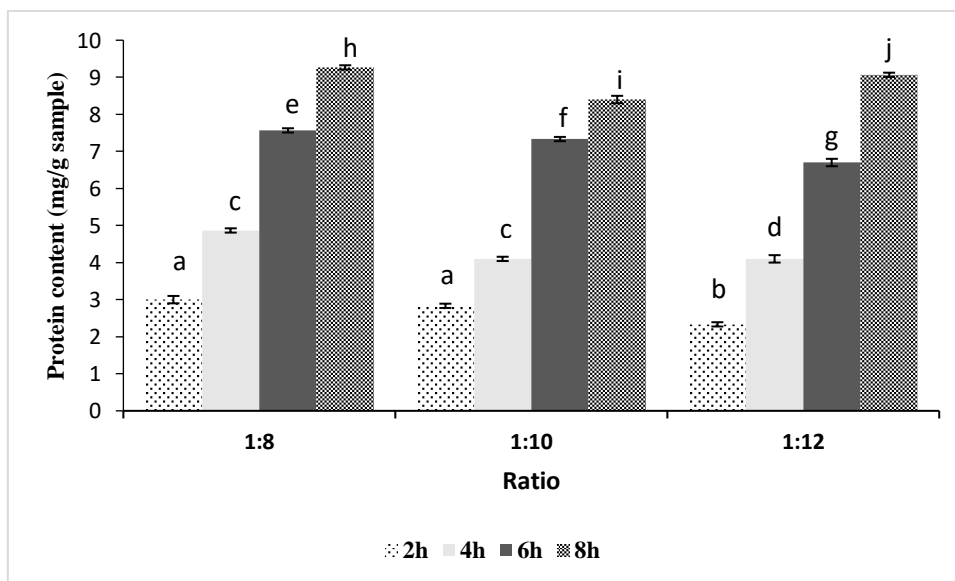


Figure 8. Accumulative protein content in deproteinizing solution of sardine scales. Data are expressed as mean \pm SEM. According to Tukey's multiple comparisons test, different letters indicate significant differences ($p < 0.05$) between material-solution ratios within each alkali treatment time.

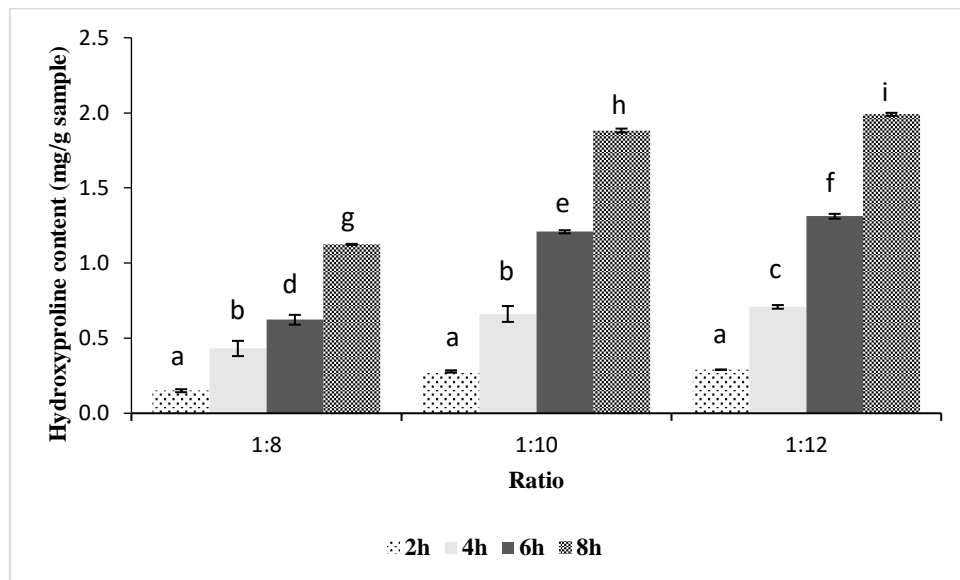


Figure 9. Accumulative hydroxyproline content in deproteinizing solution of sardine scales.

Data are expressed as mean ± SEM. According to Tukey's multiple comparisons test, different letters indicate significant differences ($p < 0.05$) between material-solution ratios within each alkali treatment time.

Table 1. Biochemical composition of sardine scales after deproteinization at both laboratory and pilot scales.

	Filtrates		Residues		
	Proteien content (m/g sample)	Hydroxyproline content (mg/g sample)	Protein content (%)	Hydroxyproline content (%)	Collagen content (%)
Deproteinization at laboratory-scale	3.2±0.15	0.26±0.037	32.4±0.30	8.2±0.10	70.5±0.22
Deproteinization at pilot-scale	2±0.10	0.21±0.10	72.7±0.37	8.3±0.17	71.4±0.20
Signification	p<0.05	ns	p<0.05	ns	ns

All results are expressed as mean ± SD from three experiments (n=3). Values are significantly different ($P \leq 0.05$) by Student's t test.

Table 1 summarizes the average protein content and the matrix in hydroxyproline identified in the filtrate and residue matrixes after deproteinizing sardine scales waste in the laboratory and pilot scales. In the pilot-scale experiment, after deproteinization of the sardine waste, the total protein content and the hydroxyproline content of deproteinizing solution were 2 mg /g sample and 0.21 mg/g sample.

The ratio of the scales to the raw material was determined from a mass balance of different by-products (heads, viscera, scales, etc.) from the sardine processing. Furthermore, the results obtained have shown that those values are almost identical to those obtained by Dumay [15] where the sardine heads and the viscera respectively represent 20% and 13% and that the ratio of the scales which represent 2% is similar to that which is found by Zall [16]. The sardine scales had a higher ash content. This was mainly due to the presence of calcium-deficient hydroxyapatite localized in two distinct regions; an upper osseous layer and a lower fibrillar plate of fish scale [17]. To obtain the collagen with the lower ash content in the scales, the appropriate demineralization of *Sardina pilchardus* scales should be accomplished before collagen extraction [18]. The hydroxyproline content of sardine scales waste was 47.86 mg/l. Thus, the scales of *Sardina pichardus* be a valuable source of collagen. The crisis caused by the outbreak of bovine spongiform encephalopathy increased the demand for fish collagen, which can be a good substitute for collagen obtained from mammalian sources.

It is possible to obtain native, pure collagen from scales only after removing non-collagenous protein and minerals [5]. Deproteinization and demineralization are two possible

pre-treatments that may be applied to sardine scales [8,18]. An earlier study showed that the alkali treatment before collagen extraction was critical for the yield of fish sardine collagen [19]. Based on the studies of deproteinization conditions from sardine scales using RSM [8], several possible effects of these pre-treatments, such as sodium hydroxide concentration and reaction time, were investigated: removing unwanted material such as non-collagenous proteins with minimum collagen loss, excluding the effect of the ratio for deproteinization.

The sardine scales waste is used for alkali treatment without drying on an industrial scale. First, it would not be economical to fully dry the sardine scales and then add sodium hydroxide solution for pre-treatment. This is contrary to most of the optimization studies in the labs, which generally take dried starting material for collagen extraction [20].

The apparent protein concentration of the moist sardine scales was thus lower than that of the dried shells. Therefore, the sodium hydroxide solution requirement for non-collagenous protein removal was lower for moist sardine scales than for dried scales (data not presented). However, sodium hydroxide solution may be diluted for effective deproteinization if excess water or moisture are present in the sardine scales. Therefore, it was necessary to press off excess water before sodium hydroxide solution addition carefully.

The protein content and yield of hydroxyproline in the pre-treatment solutions are measured, and it suggests that small amounts of proteins could be extracted from sardine scales by alkali treatments. However, no significant amount of hydroxyproline was observed when more sodium hydroxide solution was added. However, the use of more sodium hydroxide increases the costs for pre-treatment. Since the non-collagenous protein removal was only improved from 3 to 9 mg/g sample for an increase of the material-solution ratio from 1:8 to 1:12. A ratio of 1:10 was chosen as optimum concerning the minimal loss of collagen for further deproteinization studies. The lowest hydroxyproline content in the deproteinizing solution was obtained under this condition, suggesting the great portion of collagen retained and effective removal of non-collagenous proteins. This condition might be reducing the large waste alkali volumes and energy for fish scales pre-treatment. In most reports, an s:a ratio of 1:8 to 1:10 was applied to deproteinize different fish scales with 0.1M NaOH [21-23]. This pre-treatment with sodium hydroxide solution should be able to remove non-collagenous protein with minimum collagen loss and decrease the degradation of collagen extract by proteases [19].

The purpose of this study was to provide experimental responses about the feasibility of implementing such a process deproteinization on a pilot scale. The optimum conditions for deproteinization, determined beforehand, were chosen to test the feasibility of the process in the pilot-scale, to be precise: 0.5% sodium hydroxide solution during 4 hours. In selected deproteinization operating conditions for the pilot after deproteinization of the sardine waste, the total protein content (2 mg /g sample) and the hydroxyproline content (0.21mg/g sample) of deproteinizing solution are consistent with the values predicted by the study of the response surfaces [8]. Therefore, using second-degree polynomial models to predict the results (Table 1) is justified.

Otherwise stated, the deproteinization of sardine scales in a pilot mixer while maintaining an effective stirring at 4°C has eliminated a significant amount of non-collagenous proteins (20.29 %) with minimal loss of collagen. In addition, if we look closely at the average protein content in the residue recorded at the pilot scale, we can see that it is doubled compared to its analog noted during laboratory experimentation (32.4% versus 72.7 %). The reason for this significant increase ($p \leq 0.05$) in deproteinization yield could be found in the effectiveness

of homogenization achieved by the 5 blades of the mixer at the pilot scale. In another light, the lower yield of deproteinization in the laboratory could be explained because the agitation was carried out by magnetic stirrers only. As shown in Table 1, the test results at the laboratory scale are consistent with the test results at the pilot-scale, thereby demonstrating the feasibility of deproteinizing sardine scales on a pilot scale. These results also show that after the pilot-scale deproteinization, the sardine scales have low non-collagenous protein content, relatively constant ash content, and a very low loss of collagen; this step is necessary to initialize the economic optimization of this process concept on an industrial scale.

4. Conclusions

The data presented herewith highlights the excellent reproducibility of deproteinization from the laboratory scale to the pilot scale. Following this increase in scale, we have demonstrated that the proposed sardine scale deproteinization, with optimized conditions, is time-saving and reagent and energy saving. Indeed, a short deproteinization time would significantly impact the whole collagen extraction process. Meanwhile, it would be wise to do further experimentation to explore the effect of the method of agitation on the deproteinization yield and to take this into account both in the modeling and the optimization of the deproteinization process.

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Conflicts of Interest

The authors declare no conflict of interest.

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