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Conditions optimization for demineralization of sardine scales with hydrolic acid using factorial experimental design

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Abstract

A 3^2 full factorial design was used to optimize the experimental conditions of demineralization of sardine scales without loss collagen in hydrolic acid. Critical parameters such as hydrolic acid concentration (X_1,M) and reaction time (X_2,h) were studied to evaluate their effect on yield of demineralization (Y_1,%) and hydroxyproline content (Y_2,%). Two regression models of these variables (X_1 and X_2) were adopted with the response values (Y_1 and Y_2). The results obtained showed that the optimum demineralization conditions were: hydrolic acid concentration of 0.1M and reaction time of 24h. These conditions led to 99.26% of demineralization and 0.1% of hydroxyproline content.

Keywords: S*ardina pilchardus*, fish scales, demineralization, collagen, full factorial design

1. Introduction

Sardina pilchardus (Walbaum, 1792) is one of the most popular sea fish sources in Morocco. The total amount of sardine production in year 2015 was 850 000 tons [1]. Most of the discards are composed of heads, skin, bones and scales. These waste are discarded without recovery and may cause environmental issues. Whereas, unused waste generated by seafood processing industries could become potential precious bio-resources, if they are processed/ by modern biotechnology to make a highly value-added products [2]. Sardine scale waste constitute approximately 2% of the fish weigh and it is rich source of protein [3]. In addition, it has an interesting raw material for collagen or gelatin production. Nomura et al (1996) reported on the preparation of type I collagen extracted from sardine scales and suggested its utilization as a food material [4]. Since the presence of minerals in fish scale impairs the physicochemical properties of collagen preparation, it is possible to obtain pure collagen from fish scale only after removing minerals [5]. Therefore, one of the most important steps to prepare collagen for a successful collagen extraction from fish scale is demineralization. Recently, many studies has been reported about the optimization of demineralization process [6,7,8]. Hydrolic acid (HCl) as a demineralizing agent has been more popularly used by most researchers [9,10,4] compared to ethylenediaminetetraacetic acid (EDTA) because it is cheaper than EDTA and can accomplish complete elimination of inorganic salts. In general, demineralization efficiency of a compound is influenced by multiple parameters, such as concentration of demineralizing agent and time. Response surface methodology (RSM) [14] was used in different research experiment because it provides the best information regarding the effects of independent variables and their interaction on model parameters with the minimal number of runs [15]. Therefore, the aim of this study was to determine the optimum conditions for demineralization of *Sardina pilchardus* scale in hydrolic acid solution using response surface methodology. A full factorial design is

adopted to study the effects of different variables and their interaction on demineralization and validate the models with the test values.

2. Material and Methods

2.1. Raw material

Sardine (*Sardina pilchardus*) was obtained from the south of Atlantic sea in Morocco. The raw materials were transported to the laboratory under ice. Upon arrival, the scales were manually separated from fresh fish and were cleaned by tap water. Finally, the samples were frozen at -25°C until their use for no longer than 2 months. All the chemicals used were of analytical grade.

2.2. Deproteinization of sardine scales

NaOH solution was used to remove non-collagenous protein from sardine scales according to procedures described by Bellali and al [15]. After treatment, deproteinized sardine scales were collected by filtration, washed to neutrality with cold distilled water.

2.3. Demineralization of deproteinized sardine scales

The deproteinized samples were demineralized using hydrochloric acid (HCl) at concentration of 0.1, 0.2 and 0.3M (Table 2). The ratio of demineralized scale for each treatment was 1:10 (w/v). The demineralising solutions were stirred continuously with a magnetic stirrer at 4°C for 12-24h and changed of the solution every 12h. After treatment, the demineralized sardine scales were collected by filtration using cheesecloth, washed to neutrality with distilled water. All preparation procedure was carried out at 4°C. The ash content in the residue and hydroxyproline content in all supernatants were determined.

The yield of the demineralization was calculated using the following equation [17]:

$Y (\%) = (A-B)/Ax100$

Where: Y: the yield of demineralization of sardine scales (%).

A: concentration of ash in the raw material (%).

B: concentration of ash in the demineralized sample (%).

2.4. Ash content

The ash content was determined according to AOAC methods [18].

2.5. Hydroxyproline content

The hydroxyproline content was determined by the method of Bergman and Loxly [19] with L-hydroxyproline (Sigma-Aldrich, Inc.) as the standard.

2.6. Collagen loss

Collagen loss (%) was determined indirect method as the ratio of hydroxyproline extracted with HCl solutions to their initial concentration in the raw material.

The established conversion factor used for calculating of the collagen loss in the treatment solution from hydroxyproline content was 8.6 [20].

2.7. Experimental design and statistical analysis

Hydrolic acid solutions were used to remove mineral salts from sardine scales. The concentrations of hydrolic acid and reaction time are important factors affecting the demineralization of sardine scales [17]. Factorial design is used for studying the effect of several factors influencing the response by varying them simultaneously leading to a limited number of experiments. In addition, response surface methodology (RSM) was applied to determine the mutual interactions among the identified variables and their corresponding optimum levels. The experimental design applied to this study was a full 3^2 factorial design using a three-level with three replicates of the central point. Three levels were coded to three different ranges of -1, 0 and 1. The experimental matrix is given in Table 1.

Table 1: High and low levels of factors

Factors	Range and levels		
	Lower (-1)	Central (0)	Upper (1)
Concentration of HCl, X_1 (M)	0.1	0.2	0.3
Reaction time, X_2 (h)	12	18	24

For demineralization, the concentration of HCl (factor X_1, M) and reaction time (factor X_2, h) were chosen as the independent variables. The yields of deminearlization (Y_1, %) and hydroxyproline content (Y_2, %) were selected as the dependent variable, for the combination of independent variables as shown in table 2. The two main effects and their interaction were analyzed by Analysis of Variance (ANOVA). All data were treated with the aid of Minitab 16.0 Statistical Software.Ink.

3. Results and discussion

Results for yield of demineralization (Y_1) and hydroxyproline content (Y_2) uptake are shown in Table 2.

Table 2: Experimental factorial design results for yield of demineralization (Y_1) and hydroxyproline content (Y_2) uptake

Run N°	Factors				Responses	
	X_1	X_2	[HCl] (M)	Time (h)	Y_1 (%)	Y_2 (%)
1	-	-	0.1	12	**33,94**	**0,07**
2	+	-	0.3	24	**41,96**	**0,12**
3	-	+	0.1	12	**98,72**	**0,15**
4	+	+	0.3	24	**99,78**	**0,18**
5	0	0	0.2	18	**20,86**	**0,17**
6	0	0	0.2	18	**20,04**	**0,17**
7	0	0	0.2	18	**20,92**	**0,17**

X_1 (concentration of HCl, %), X_2 (reaction time, h), Y_1 (yield of demineralization, %) and Y_2 (hydroxyproline content, %)

3.1.Development of response surface model and data analysis

The results showed in Tables 3 and 4. Main, interaction effect, coefficients of the model, standard deviation of each coefficient, and probability for the full 3^2 factorial designs were presented in Table 4.

Table 3: Results of regression analysis for yield of demineralization (Y_1) and hydroxyproline content (Y_2)

Term	Coefficient	t-value	p-value
Yield of demineralization (Y_1)			
Constant	68.60	279.05	0.000
X_1	2.27	9.23	0.012
X_2	30.65	124.68	0.000
X_1X_2	-1.74	-7.08	0.019
Hydroxyproline content (Y_2)			
Constant	0.031	54.13	0.000
X_1	0.004	7.36	0.018
X_2	0.018	32.48	0.001
X_1X_2	0.005	9.96	0.010

Not significant at P < 95%. All other coefficients were significant at P < 95%,
X_1 (concentration of HCl, M), X_2 (reaction time, h).

The codified mathematical model employed for the full 3^2 factorial designs was:

$$\mathbf{Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j} \quad [1].$$

Where Y represented the estimated response β_0, β_i and β_{ij} are the regression coefficients for the intercept, linear and interaction coefficients, respectively, X_i and X_j are the independent variables in coded units.
The mathematical models representing yield of demineralization (Y_1) and hydroxyproline content (Y_2) in the experimental region studied can be expressed by Eqs. (2) and (3), respectively:

$$\mathbf{Y_1 = 68{,}60 + 2{,}27X_1 + 30{,}65X_2 - 1{,}74X_1X_2} \quad [2].$$
$$\mathbf{Y_2 = 0{,}128 + 0{,}019X_1 + 0{,}037X_2 - 0{,}007X_1X_2} \quad [3].$$

Table 4 shows the analysis of variance for the models used to investigate the dependence of demineralization yield (Y_1) and hydroxyproline content (Y_2) on the independent factors.

Table 4: Regression analysis for Y_1 and Y_2 by first-order model fitting (ANOVA)

Source	DF	SS	MS	F-value	p-value
Yield of demineralization (Y_1)					
Model	2	3778.30	1889.15	7815.02	0.000
Residual	2	0.48	0.24	-	0.000
Total	6	-	-	-	
Hydroxyproline content (Y_2)					
Model	2	0.0007	0.0035	662,72	0.002
Residual	2	0.0000	0.0000	-	
Total	6	-	-	-	

FD: degree of freedom, SS: sum of Square, MS: Mean square

The regressions are all statistically significant at the 95% confidence level. The models representing yield of demineralization (Y_1) and hydroxyproline content (Y_2) presented high determination coefficients (R^2 = 0.999, 0.998) explaining 99.9 and 99.8% of the variability in the response, respectively.This proof that the models were adequates for prediction within the range of experimental variables. The factorial design results show that reaction time (X_2) had the strongest effect on yield of demineralization (Y_1) and hydroxyproline content (Y_2). Increasing hydrolic acid concentration (X_1) increases yield of demineralization (Y_1) and hydroxyproline content (Y_2). The β_2 coefficient is the largest positive coefficient for all the model equations (see Eqs. (2) and (3)) (Table 3). It is known that is larger coefficient is the effect of related parameter. The positive sign also shows that there is a direct relation between the parameter and dependent variable. Eqs. (2) and (3) are also reveal that two-variable interactions (X_1X_2) are significant but has a negative influence. Similar results were reported by Feng et al [21].

3.2. Main and interaction effect plots

The main effects and interaction between factors were determined. Figure 1(a) and 1(b) show the main effect plots of the two factors on demineralization yield (Y_1) and hydroxyproline content (Y_2), respectively. The main effects represent deviations of the average between the high and low levels for each factor. When the effects of a factor is positive, demineralization yield (Y_1) and hydroxyproline content (Y_2) increases as the factor changes from low to high levels. From Figure 1, it is inferred that the larger the vertical line, the larger the change in Y_1 and Y_2 when changing from level−1 to level +1. It should be pointed out that the statistical significance of a factor is directly related to the length of the vertical line. The effects of X_1 and X_2 factors are positive, that is an increase of Y_1 and Y_2 observed when the factor changes from low to high. X_2 and X_1 factors result in a higher mean Y_1 at their high level, compared to that at the low level. In addition, X_2 had a greater effect on Y_1 and Y_2, as is evident by the longer vertical line.

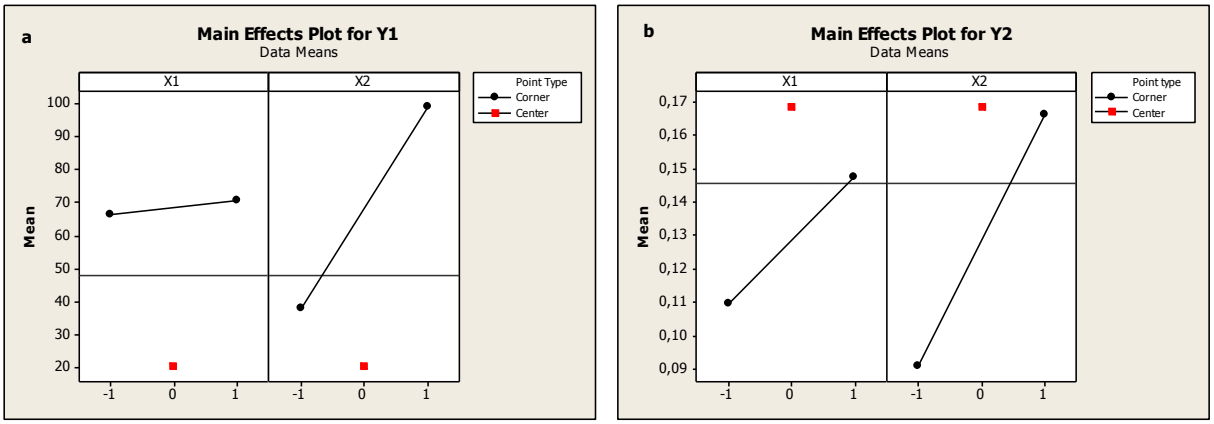

Figure 1: Main effects plot for: a- yield of demineralization (Y_1) and b- hydroxyproline content (Y_2).

Interaction plot of effects is shown in Figure 2. An interaction (Fig. 2) is effective when the change in the response from low to high levels of a factor is dependent on the level of a second factor, i.e. when the lines do not run parallel. The unparallel effect line for the X_1X_2 interaction implies that there was a rather strong two-way interaction between the main effects of X_1 and X_2 .This had shown that the yield of demineralization (Y_1) tended to have higher values when the hydrolic acid concentration (X_1) was increasing in the interactions with treatment time (X_2).

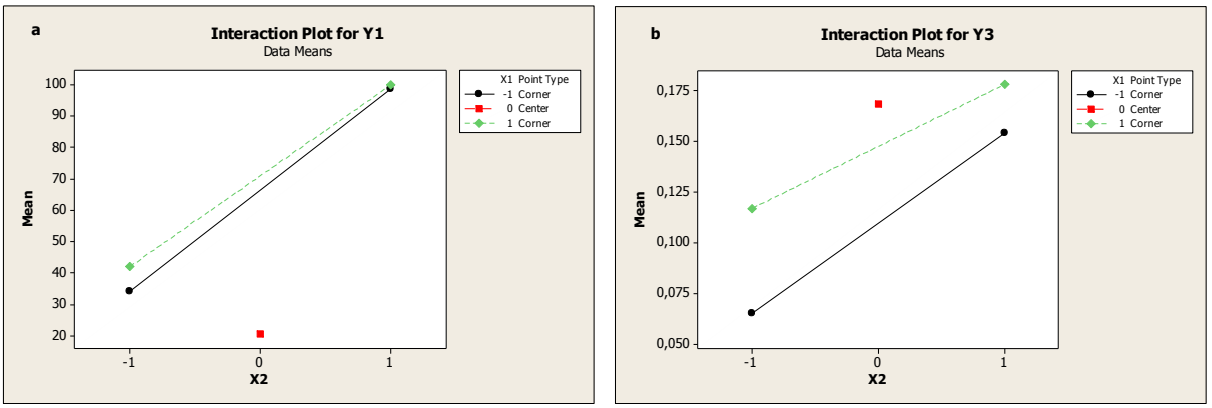

Figure 2: Interaction plot for: a- yield of demineralization (Y_1) and b- hydroxyproline content (Y_2).

3.3. Response Surface Plots

Figures 3 and 4, respectively, shows the response plot and the contour plot for yield of demineralization (Y_1) and hydroxyproline content (Y_2) versus hydrolic acid concentration (X_1) and reaction time (X_2). Figure 3 show that in general the amount of dissolved minerals increased with the increase of hydrolic acid concentration within a studied range. It also showed that low hydrolic acid concentrations require longer reaction times for significant reduction in the mineral content of the sardine scales. The solubility of minerals after 24 h of treatment was amounted about 98.7% in 0.1 M HCl and 99.7% in 0.3 M HCl, respectively. Larger efficiency of removing of mineral salts was achieved in 0.1 M solution of HCl (about 98% after 24h). The extension of the hydrolic acid concentration of demineralization to 0.3M brought only small changes in the content of ash in sardine scales. Previous studies showed that the demineralization yield of fish (sardine) scales was to come up about 90% with concentration of hydrolic acid 1.0M [4]. Fahmi et al [10] also demonstrated that the demineralization of sea bream scales by 0.6 M HCl after 24h have removed 90% of the inorganic matter. In response surface methodology (RSM) study of demineralization of fish scales, reported that the demineralization yield of bream scale can reach 79.18 % with 0.43M HCl solution [7].

However, the 99.78 % yield of demineralization of sardine scales was slightly higher than 87.62 % yield reported by Wu et al [8], processing time was largely reduced.

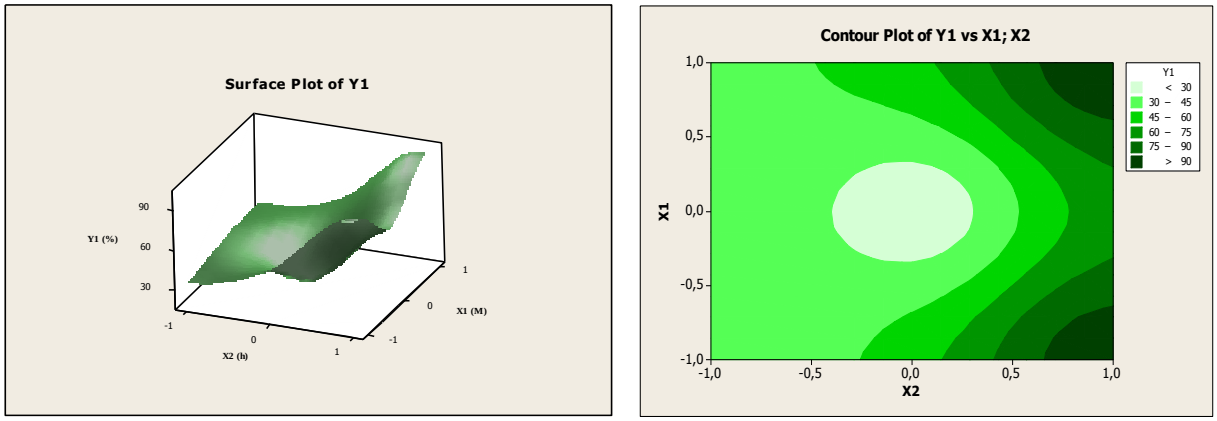

Figure 3: Response surface (a) and contour plot (b) for yield of demineralization (Y_1) as function of hydrolic acid concentration (X_1) and reaction time (X_2)

During the demineralization, the part of collagen contained in sardine scales was solubilized. The solubility of collagen depended both on the concentration of hydrolic acid solution and time of the process [17]. Figure 4 shows that the hydroxyproline content increased lowly to an optimum value by rising in concentration of hydrolic acid and reaction time. Smaller amount of hydroxyproline was observed after demineralization for 24h in 0.1M and 0.3M HCl solutions and it is ranged from 0.07% and 0.18%, respectively. According to the report by Wang and Regenstein [22], the hydroxyproline content after demineralization of silver carp scale in 0.06M and 0.4M HCl respectively amounted to about 4% and 5%.

In such acidic small (Figure 4) collagen swells and this facilitates its solubility. The collagen was considerably less soluble in 0.1M HCl solution. The loss of collagen amounted to only from 0.6%
to 1%. This observation demonstrates that a concentration of hydrolic acid as low as 0.1M HCI was effective for demineralization, this condition may be economical and may prevent deterioration of the native collagen chain.

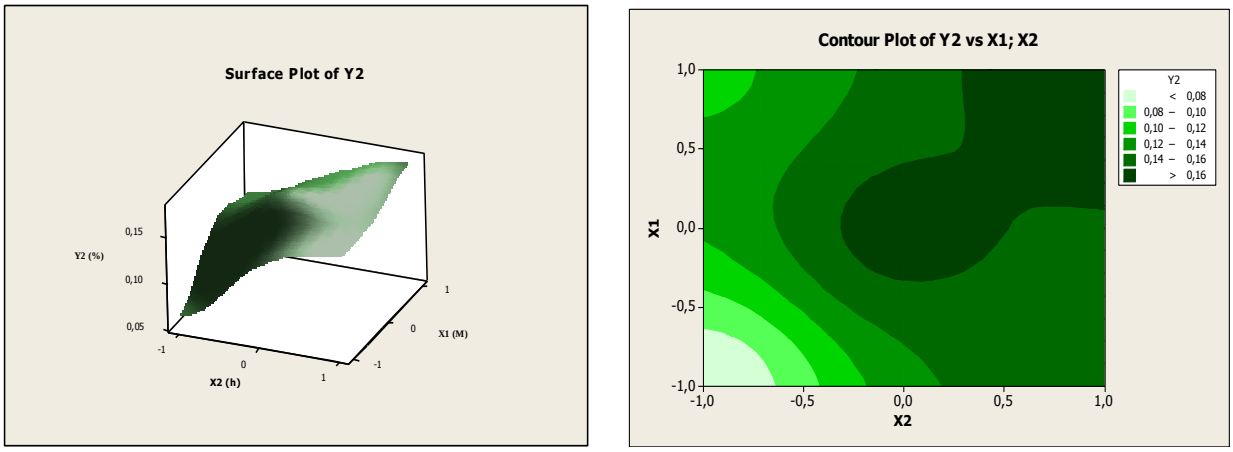

Figure 4: Response surface (a) and contour plot (b) for hydroxyproline content (Y_2) as function of hydrolic acid concentration (X_1) and reaction time (X_2)

3.4. Conditions for optimum response

The effect of concentration of hydrolic acid (X_1) and reaction time (X_2) on the yield of demineralization (Y_1) and hydroxyproline content (Y_2) was determined using response surface methodology (RSM). Desirability function approach was employed to optimize the process of demineralization without considering the loss of collagen. Concentration of hydrolic acid (X_1) and reaction time (X_2) were set in arranged ranges, while

dependent variable for Y_1 was fixed at maximum and Y_2 at minimum. The results of optimization and predicted responses by desirability function of MINITAB statistical software are given in Figure 5.
The optimum conditions were concentration of hydrolic acid (0.1M) and reaction time (24 h). The predicted values of multiple response optimal conditions were Y_1=98.72 % and Y_2=0.15% (Table 5).

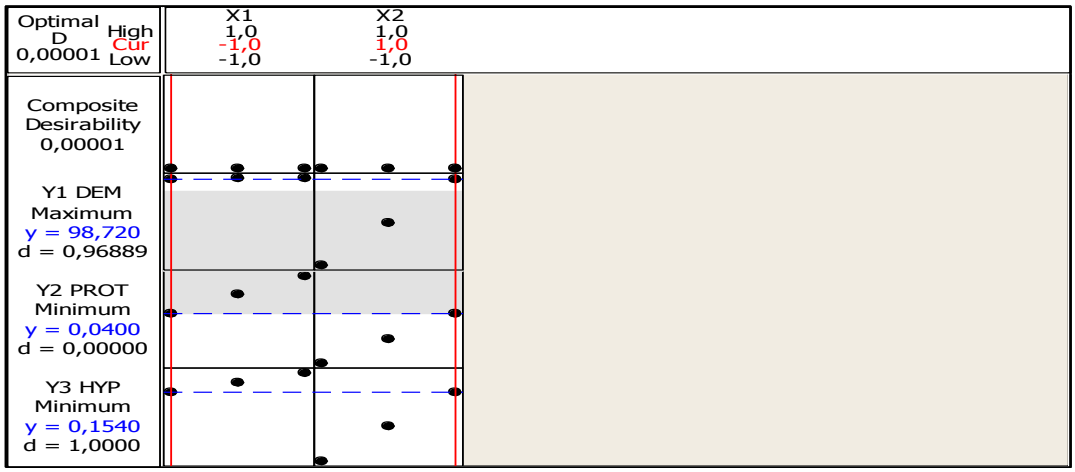

Figure 5: Optimization plot

Verification experiment was conducted under optimal conditions to compare predicted value and actual value of dependent variable. The actual values repeated three times were Y_1=99.26±1.10 % and Y_2=0.10±0.12%, which were agreed well with the predicted values (Table 5). In these optimized conditions, a demineralization degree of 99.26% was obtained larger than values generally given in literature [21,7,8].

Table 5: Test results for verification of the results of demineralization with hydrolic acid

Optimal solution		Predicted response	Experimental value	
X_1(M)	X_2 (h)		Y_1 (%)	Y_2 (%)
0.1	24	Predicted value individual desirability composite desirability	98.72	0.154
		Experimental value	99.26±1,10	0.10±0.12

Y_1 : yield of demineralization, Y_2 : hydroxyproline content , X_1: concentration of HCl, X_2 : reaction time.

Conclusion

A full 3^2 factorial experimental design has been used the demineralization process of sardine scales in order to reduce the number and cost of experiments. The statistical analysis showed that hydrolic acid concentration (X_1) and reaction time (X_2) as well as their interaction, have the significant effects on yield of demineralization (Y_1) and hydroxyproline content (Y_2). The models have been found to describe the experimental range studied adequately. An optimum condition was formulated according to the optimization results, which is comprised of: hydrolic acid concentration (0.1M) and reaction time (24h). Under these conditions would be useful to obtain >98% demineralization of sardine scales, simultaneously without losing collagen. This study suggest that demineralization process of sardine scale using hydrolic acid could be considered as an effective pretreatment to produce a high-quality collagen.

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