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RESPONSE SURFACE METHODOLOGY OPTIMIZATION OF DEPROTEINIZATION FROM SARDINE (*SARDINA PILCHARDUS*) SCALE OF MOROCCAN COAST

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ABSTRACT

The present study was designed to determine the optimum conditions for the deproteinization of Moroccan sardine scale (Sardina pilchardus) by using a central composite design of response surface methodology.A central composite design was consisted of eight experimental points and five replications at the center point, which was used to determine the effects of two independent variables (NaOH concentration and treatment time) on hydroxyproline content. A second-order polynomial model was used for predicting the response. Until now, a very few researchers has focused on deproteinization of scale by using a surface response methodology. The results showed the optimal conditions for lowest value of response when the concentration of NaOH was 0.5% and the treatment time was 4h. The experimental value for the hydroxyproline content was 2.46mg/l. That result was in agreement with the predicted value, which indicates that the model used was adequate for deproteinization step.

Keywords: *Sardina pilchardus*, Sardine scales, Optimization, Deproteinization, Surface response methodology.

1. INTRODUCTION

Sardina pilchardus is one of the major species produced in Morocco; this fish can be commercially purchased fresh, frozen or canned. Moreover, Morocco is known as the leader

supplier of exporting canned Sardine to European Market compared to Africa, Middle East and United States.

During fish processing industry, an important quantity of by-products such as skins, bones, scales, heads and guts are generated. They are usually used as animal's food. Moreover a great amount of fish scales are produced representing 2 % of fish weight (Zall, 2004). According to the first inquiries of the Moroccan seafood processing companies realized by the National Institute of Halieutic Research in the region of Agadir (South Atlantic of Morocco), results showed that these processing generate a huge quantity of scales.

Fish scale is a potential source to isolate collagen that is used in food industry, cosmetic and biomedical (Nagai *et al*., 2004). Recently, many studies have been carried out to obtain collagen from fish scale of different fish species (Kimura *et al*., 1991; Nomura *et al*., 1996; Ikoma *et al*., 2003; Nagai *et al*., 2004; Ogawa *et al*., 2004; Sankar *et al*., 2008; Wang *et al*., 2008; Duan *et al*., 2009; Pati *et al*., 2010; Matmaroh *et al*., 2011; Mori *et al*., 2013).

Scientists have proved that the mammalian collagen may cause a serious disease, thus the existence of bovines infected with Bovine Spongiform Encephalopathy (BSE) has been reported in Japan (Yamauchi, 2002). Porcine collagen and other collagen from animals are also known to be forbidden and not acceptable to some ethnic groups and religions such as Muslims and Hindus. It matters to look for an alternative collagen extraction from fish species that will reduce the environmental pollution and increase the economic value of the collagen from fish scale is communally extracted with acid and/or pepsin-soluble collagen process, but this extraction might be pretreated before. The first pretreatment is deprotenization of fish scale that involves alkaline or acidic pretreatment in order to remove non-collagen protein (Zhou and Regenstein, 2005). The second one is demineralization of fish scale; it is used to remove the calcium by using acid solution (Gómez-Guillén *et al*., 2011).

The removal of non-collagenous materials on collagen has a mutual precedent for both collagen isolation and extraction of gelatin (Herpandi and Adzitey, 2011). Zhou and Regenstein (Zhou and Regenstein, 2005)confirmed that the role of alkaline pretreatment is not only removing non-collagenous protein with minimum collagen loss but also excluding the effect of endogenous protease on collagen, which might be in high gelatin yield. Furthermore, the type of alkalis does not make a significant difference during the alkaline pretreatment, but the concentration of alkalis, time and temperature are critical (Zhou and Regenstein, 2005; Benjakul *et al*., 2012). Several authors performed the removal of non-collagenous protein and pigment of fish scale with 0.4% NaOH at 4°C (Nagai *et al*., 2004; Duan *et al*., 2009; Matmaroh *et al*., 2011; Duan *et al*., 2012; Liu *et al*., 2012).

In our study the application of response surface methodology (RSM) (Box and Wilson, 1951) was used to optimize the deproteinization condition of sardine fish scales. This pretreatment should be able to remove non-collagenous protein effectively, but must generate the lowest hydroxyproline loss. The basic principle of RSM is to determinate model equations that describe interrelations between the independent variables and the dependent variables (Edwards and

Jutan, 1997). The application of RSM aimed to reduce the number and cost of experiments, to evaluate the interactions among the variables, to generate the mathematical model and optimize the process levels.

Therefore, the aim of this work is to apply Central Composite Design (CCD) based on Response Surface Methodology (RSM) to determine the optimal values for deproteinization from sardine pilchardus scale, simultaneously without losing collagen content.

2. MATERIAL 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

Sardina pilchardus caught in winter from the south of Atlantic sea in Morocco, stored in ice and transported to our laboratory. The scales ware removed manually and washed with chilled tap water (to remove the impurities adhering to the surface), then placed in polyethylene bags and stored at -25°C until analysis.

2.3. Physico-Chemical Analysis

The dry weight and ash were determined according to AOAC methods (AOAC, 1990), total lipids were performed according to the method of Bligt and Dyer (Bligh and Dyer, 1959) and total crude protein was determined by estimating its total nitrogen content (Kjeldahl, 1883). A conversion factor of 6.25 was used to convert the nitrogen value to protein (Crooke and Simpson, 1971). All measurements were performed in triplicate.

2.4. Deproteinization Assay

This experiment was performed according to the central composite design (CCD) matrix presented in table 2 and the independent variables and their levels were selected based on previous studies.

In order to remove non-collagenous proteins, scales were treated with various concentration of NaOH: 0.26%, 0.3%, 0.4% and 0.54%, (1:10 (w/v)) under stirring for different times (3.18 h, 4 h, 6 h, 8 h and 8.82 h) at 4°C. The solution was changed every 2 hours. Then deproteinized samples were washed with distilled water at 4°C and filtered with two layers of cheesecloth. The filtrate was collected and ready for hydroxyproline content determination.

2.5. Hydroxyproline Content

Hydroxyproline content was determined after hydrolysis of the filtrate in 6N HCl for 24h at 110°C, using the calorimetric method (described by the rapid procedure) of Bergman and Loxley (Bergman and Loxley, 1963).

The hydrolysate was well mixed with 200µl of isopropanol, 100µl of oxidant solution (mixture of Chloramine T and acetate citrate) was added and mixed thoroughly. After 4min, 1300µl of Ehrlich's reagent solution (p-dimethylaminobenzaldehyde in isopropanol) was added and then the chromophore was developed by incubating samples at 60°C for 25 min. After that, the solution was diluted with isopropanol. Absorbance of each sample was read at 558nm. A hydroxyproline standard solution, with concentration ranging from 0 to 60 ppm, was also included. Hydroxyproline content was calculated from a standard curve of hydroxyproline and expressed mg/l.

2.6. Collagen Content

Hydroxyproline content was measured for using as index of collagen content. Therefore, the conversion factor used for calculating the collagen content from hydroxyproline of sardine scale was 8.6 (Nagai *et al.*, 2004).

Collagen content (mg/g) was estimated using the following relationship:

Collagen content (mg/g) = hydroxyproline content (mg/ml)x8.6 (conversion factor).

2.7. Optimization Experimental Design

The preliminary results proved the relationship between two independent variables and the response that was not linear but modeled as a second order polynomial using central composite design (CCD).

A central composite design of response surface methodology (RSM) (Box and Wilson, 1951) was used for the optimization of deproteinization from sardine scale. Table 1 shows the experimental design for deproteinization. The latter was optimized using five levels and two factors. The CCD in the experimental design consisted of 22 factorial point, four axial points and three replicates of the central point.

Table- 1. Experimental values and coded levels of the independent variables for central composite design (CCD).

Independent variables	**Symbol**	**Range and Levels**				
		-1,414	**-1**	**0**	**+1**	**+1,414**
Concentration of NaOH (%)	X_1	0.26	0.3	0.4	0.5	0.54
Treatment time (h)	X_2	3.18	4	6	8	8.82

For deproteinization, the concentration of NaOH (%,X1) and treatment time (h,X2) were chosen as the independent variables. Hydroxyproline content (mg/l,Y) was selected as the dependent variable, for the combination of independent variables as shown in table 2.

Table- 2. Central composite design and responses of dependent variable for deproteinization of sardine scale to independent variables

Run N°	Code level of variable		Response
	X_1	X_2	Y(mg/l)
1	-1	-1	2.03
2	1	-1	2.78
3	-1	1	4.74
4	1	1	5.13
5	-1.414	0	1.95
6	+1.414	0	2.43
7	0	-1.414	3.42
8	0	+1.414	8.20
9	0	0	5.15
10	0	0	5.06
11	0	0	5.14

Y: Hydroxyproline content (mg/l), X_1: concentration of NaOH (%), X_2: treatment time (h).

2.8. Analysis of Data

The second order model was fitted to the data by multiple regression analysis. This model can be expressed with the coded variables (X_1, X_2) with following equation.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_{21} + \beta_{22} x_{22} + \beta_{12} x_1 x_2 + \varepsilon$$

Where Y represented the estimated response, β_0 represented the equation parameters for the constant term, β_1 and β_2 represented the linear terms, β_{11} and β_{22} represented the quadratic terms for a single variable, β_{12} represented the interaction terms and ε represented the random error. Experimental data from different treatment was statistically analyzed using Minitab 16 Statistical Software.

3. RESULTS AND DISCUSSION

3.1. Proximate Composition of *Sardina Pilchardus* Scales

According to table 3 *Sardina pilchardus* scales contained a high protein and ash but a few lipids content. However, compare to some other marine species, the protein content is higher for instance: 56.9% in deep-sea redfish (Wang *et al*., 2008), 38.9% in lizardfish (Wangtueai and Noomhorm, 2009) and 34.46 in spotted golden goatfish (Matmaroh *et al*., 2011). The high content of ash might be due to the presence of calcium phosphate compounds such as hydroxyapatite (Sankar *et al*., 2008). The collagen content of scales was 34.22 mg/g sample.

Table- 3. The chemical composition of *sardina pilchardus* scales

Sample	Proximate compositions (% dry weight)				Collagen (mg/g sample)
	Moisture	Ash	Lipid	Protein	
Sardine fish scales	59.51±0.69 2	50.23±0.473	2.35±0.002	32.25±2.30	34.22±0.276

Data presented as means ± standard deviation of triplicate determinations

3.2. Development of Response Surface Model

All the 11 experimental points were evaluated and the dependent variable results (Y) for each point are shown in Table 2. The response surface regression (RSREG) procedure for Minitab software was used to fit the quadratic polynomial equation to the experimental data. The coefficients of all the variables of linear (X_1, X_2), quadratic (X_{12}, X_{22}) and interactions were calculated for significant by using t-statistic test. The regression coefficients estimated for the 2nd response surface model in terms of coded units are presented in Table 4.

The linear coefficient, X_2 (P=0,000) has a significant effect on the level of P<0.05, while the quadratic coefficient, X_1X_1 (P=0,000) was highly significant at P<0.05, but X_2X_2 (P=0.132) was not significant at P>0.05. On the other hand, the coefficient for interaction terms was not significant at P>0.05.

Table- 4. Estimated coefficient of the fitted quadratic polynomial equation for different response based on t-statistic.

Term	Coefficient	t-value	P-value
Constant	5.11667	25.363	0.000
X1	0.22735	1.840	0.125
X2	1.47749	11.960	0.000
X1.X1	-1.54583	-10.513	0.000
X2.X2	0.26417	1.797	0.132
X1.X2	-0.09000	-0.515	0.628

Not significant at P < 95%. All other coefficients were significant at P < 95%

Y (hydroxyproline content, mg/l), X1 (concentration of NaOH, %), X2 (treatment time, h)

The regression equation obtained for the second degree model in terms of coded factors is as follow:

$$Y= 5.11+ 0.22X_1+1.47X_2-1.54X_1X_1+0.26X_2X_2-0.09X_1X_2$$

Where Y, X_1 and X_2 are the hydroxyproline content (mg/l), NaOH concentration (%) and treatment time (h), respectively.

The determination coefficient (R^2) for the regression model on hydroxyproline content (%) was 98.27%. This value indicated that the model is well fitted with the experimental data. The predicted determination coefficient (Pred R^2=0.877) is in reasonable agreement with the adjusted determination coefficient (Adj R^2=0.965), which is also satisfactory for confirming the significance of the model.

3.3. Analysis of Variance

The analysis of variance (ANOVA) for the hydroxyproline content response was used to evaluate the significance of the quadratic polynomial model equation. Table 5 shows ANOVA for the model that explains the response of the dependent variables (Y) (hydroxyproline content).

In the ANOVA result for the regression model was highly significant (P<0.05) at 95% probability, which indicate that the terms in the model have a significant effect on the response.

The linear terms and quadratic terms contributed significantly to the model ($P<0.05$), whereas the interaction term (X_1X_2, $P>0.05$) was considered insignificant at 95% probability level. However, the dependent variable (Y) in the lack-of-fit analysis was significant at the 95% probability level.

Figure 1 shows residual plot for the dependent variable, Y (hydroxyproline content, mg/l) in the model. It shows that the residual distribution for the response follow a fitted normal distribution, because in the opposite case, the points would have been scattered in the graphic space instead of showing a normal probability distribution. Therefore, those results affirmed that the model was adequate.

Figure- 1. Normal Probability Plot of the Residuals

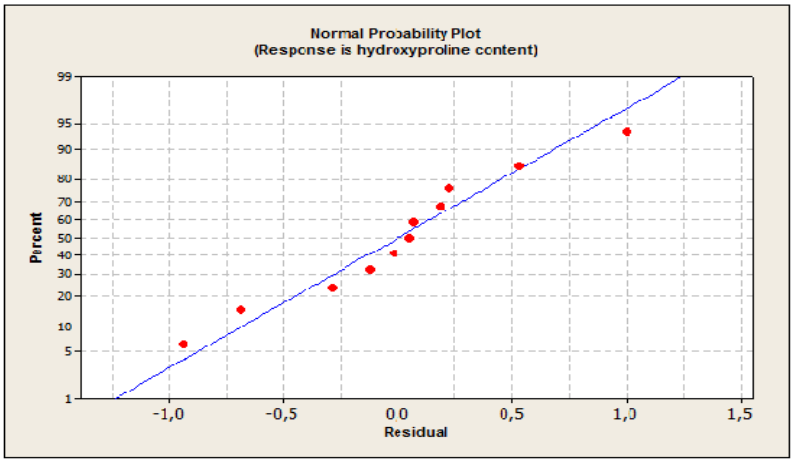


Table- 5. Analysis of variance (ANOVA) for the response surface model

Source	DF	SS	MS	F-value	P-value
Regression	5	34.5982	6.9196	56.67	0.000
Linear	2	17.8774	8.9387	73.21	0.000
X1	1	0.4135	0.4135	3.39	0.125
X2	1	17.4639	17.4639	143.03	0.000
Square	2	16.6884	8.3442	68.34	0.000
X1X1	1	16.2943	13.4942	110.52	0.000
X2X2	1	0.3941	0.3941	3.23	0.132
Interaction	1	0.0324	0.0324	0.27	0.628
Residual Error	5	0.6105	0.1221	-	-
Lack-of-Fit	3	0.6056	0.2019	82.96	0.012
Pure Error	2	0.0049	0.0024	-	-
Total	10	35.2087	-	-	-

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

3.4. Conditions for Optimum Responses

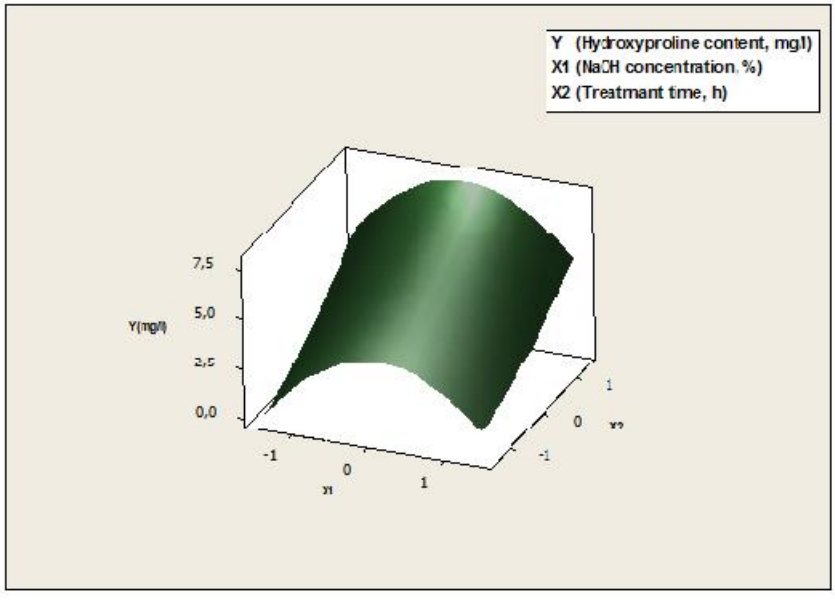
The optimal conditions to obtain the lowest hydroxyproline content loss were estimated at NaOH concentration of 0.5% (X_1) and treatment time of 4h (X_2). From the model equation derived from optimal condition, the predicted value of hydroxyproline content was 2.67mg/l. In

order, to verify this prediction, the experimental value repeated three times of 2.46±0.34 mg/l was obtained. That result was in agreement with the predicted value, it also confirmed that the model used in this experiment is appropriate.

3.5. Response Surface Plots

The three-dimentional response surface plots of dependent variable (Y) plotted two independent variables (X_1,X_2) was drawn with Minitab software and shown in Figure 2.

Figure- 2. Response surfaces showing effect of NaOH concentration and treatment time on Hydroxyproline content



Surface plot depicts the effect of NaOH concentration and treatment time on the hydroxyproline content in deproteinizing solution. When the coded values of the X_1 and X_2 independent variables were close to zero, hydroxyproline content increased, means that hydroxyproline loss is increase. It is mainly due to the increase of time incubation and NaOH concentration. It shows that treatment time affect the hydroxyproline content more significantly compared to the level of NaOH concentration. Plus, the loss of the collagen was monitored by hydroxypoline content in deproteinization solution. Moreover, the use of maximal concentration of NaOH for deproteinization is benifical, because the highest amount of NAOH will not only dissolve the non-collagenous protein, however it will also modified the collagen polypeptide chains. In this case, it's certain that we will lose an important amount of collagen in the solution (Sato *et al*., 1987; Zhou and Regenstein, 2005).

The previous studies have shown that the pretreatment of NaOH from 0.04 to 0.4 % have removed a smaller amount of non collagen protein and the yield of protein extraction did not incearse(Sato *et al*., 1987; Zhou and Regenstein, 2005).

4. CONCLUSION

The central composite design has been used to determine the optimum conditions for deproteinization. Hence, the model was developed and the analysis of variance provided a statistical suitable models. On one hand, the optimal conditions to obtain loss hydroxyproline has shown that in 0.5% NaOH for 4h of treatment has given the following response Y= 2.46mg/l. On the other hand, the prediction of hydroxyproline value was subsequently confirmed by verification experiments.

This study is performed on the optimization of sardine scale deproteinization by using RSM aimed to provide fish industry a new source of fish collagen to food and pharmaceutical applications.

On the industrial scale, the NaOH optimization will provide a considerable saving of time, reagent and energy. Indeed, short treatment time with minimum NaOH concentrations of deproteinization will certainly decrease the cost of whole collagen extraction process.

In further studies, those results performed on deproteinization by using RSM could be easily extrapolated to sardine scale demineralization and collagen extraction stage.

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