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Response surface methodology optimization of demineralization from sardine (*Sardina pilchardus)* scale

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Abstract
Sardine scales can serve as an additional source of proteins, especially of collagen. To obtain native collagen previous deproteinization and demineralization of scales are necessary. Therefore, the aim of this work is to determine optimal parameters for demineralization of sardine scales by complexing metal ions with ethylenediaminetetraacetic acid (EDTA). In this study, the response surface methodology is used to build a model to predict and optimize the demineralization process. The optimal conditions are X1=0,3M (EDTA concentration) X2=18h (EDTA treatment time). Under this optimal condition, the experimental values agreed with the predicted values, using analysis of variance, indicating a high goodness of fit of the model used and the success of response surface methodology for modeling demineralization extraction of scale sardine.

Keywords: *Sardina pilchardus*, Sardine scales, Optimization, Demineralization, RSM

1. Introduction
Fish by-product such as skins, bones and scales which are generated during fish processing industry can serve as potential source of proteins, especially of collagen. Collagen is an important material which is used in various fields such as cosmetic and food products in the form of gelatin. Generally, collagen extracted from fish scale has a less malodorous smell than fish skin and bones. Fish scales are biocomposites of highly ordered type I collagen fibers and hydroxyapatite [1,2]. However, utilization of fish scales for collagen extraction has only been reported from the scales of carp [3-7], sardine scales [1,8], Cololabis saira, sea bream and red tilapia [9-11], the subtropical fish black drum and sheepshead scales [12] deep-sea redfish scales [13] and scales of spotted golden goatfish [14].
Sardina pilchardus scales presented an ash content as high as 50%, therefore pre-treatment removal of calcium from fish scale was needed to increase the purity of extracted collagen [15].This raw material is effectively decalcified by using ethylenediaminetetraacetic acid (EDTA). The most important property of ethylenediaminetetraacetic acid (EDTA) is its ability to chelate or complex metal ion. The decalcification can be also achieved by using inorganic acid, especially hydrochloric acid.
In the present study, the response surface methodology (RSM) [16] is used to optimize the demineralization condition of sardine fish scales. The basic principle of RSM is to determinate model equations that describe interrelations between the independent variables and the dependent variables [17]. The RSM is 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 objective of this work is to apply factorial design methodology to determine the optimal values for demineralization from Sardina pilchardus scale in ethylenediaminetetraacetic acid (EDTA), simultaneously without losing collagen content.

2. Material and Methods
2.1 Raw material
Sardine (Sardina pilchardus) caught in January 2014 from the south of Atlantic sea in Morocco, stored in ice and transported to the 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.

2.2 Removal of non-collagenous protein

Non-collagenous protein was removed from minced sardine scales according to procedures described by Bellali *et al* (2013). The samples were soaked in 0.1 M NaOH with scale/solution ratio of 1:8 w/v with continous stirring for 4h. The solution was changed every 2h. Then, the alkali-treated scales were washed with cold distilled water until neutral pH was reached.

The dry weight, ash, total protein and hydroxyproline in the deproteinized sardine scales were determined.

2.3 Demineralization process

The deproteinized samples were demineralized using ethylenediaminetetraacetic acid (EDTA) (pH 7.5) at concentrations of 0.2, 0.3 and 0.4M, 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. The treated scales were filtered using cheesecloth. The residue was washed three times with distilled water.

All preparation procedure was carried out at 4°C. The ash content in the residue, the protein content and hydroxyproline content in all supernatants were determined.

2.4 Ash content

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

2.5 Protein content

The protein content in the each treatment solutions was determined by the Bradford's method [19].

2.6 Hydroxyproline content

The hydroxyproline content was determined by the method of Bergman and Loxly [20].

2.7 Experimental design

A tree-level Factorial Design of Experiment, with three replicates of the central point, applied to investigate the interactive effects of various variables and then used to determine the optimum condition in order to achieve maximum demineralization, simultaneously, without losing of collagen content. The effect of two factors such as acid concentration (X_1) and incubation time (X_2) was evaluated. Each variables was coded at three levels as -1, 0 and 1 for upper, central and lower, respectively. Independent variables and their levels are shown in Table 1.

Table1: Experimental values and coded levels of the independent variables for factorial design.

Factors	Range and levels		
	Lower (-1)	Central (0)	Upper (1)
Concentration of EDTA, X_1 (M)	0.2	0.3	0.4
Treatment time, X_2 (h)	12	18	24

The yields of demineralization (Y_1), protein content (Y_2) and hydroxyproline content (Y_3) were selected as the dependent variables for the combination of the independent variables are shown in Table 2.

The model generated as a function of these variables the predicted response is first-order polynomial and is represented as Equation 1:

$$Y=\beta_o+\sum_{i=1}^{K}\beta_i X_j+\sum_{j<i}\sum\beta_{ij}X_iX_j+\varepsilon \quad (1)$$

Where β_0, β_i and β_{ij} are the regression coefficients for the intercept, linear and interaction coefficients, respectively, y is the dependent variable or the response, x_i and x_j are the independent variables in coded units, and ε is the error term.

Table 2: Factorial design and responses of dependent variable for demineralization of sardine scale to independent variables.

Run N°	Independent Variables		Responses		
	X_1	X_2	Y_1	Y_2	Y_3
1	-	-	55.64	8.17	0.052
2	+	-	93.62	15.32	0.052
3	-	+	97.76	16.34	0.052
4	+	+	98.08	31.66	0.068
5	0	0	94.50	13.27	0.060
6	0	0	95.56	14.3	0.064
7	0	0	94.80	13.27	0.060

Y_1: Yield of demineralization, Y_2: Protein content (%), Y_3: Hydroxyproline content (%), X_1: Concentration of EDTA (M), X_2: Treatment time (h).

2.8 Analysis of data

The analysis of results was performed with statistical and graphical analysis software (Minitab 16). This software was used for regression analysis of the data obtained and to estimate the coefficient of regression equation. ANOVA (analysis of variance) which is statistical testing of the model in the form of linear term and interaction term was also utilized to test the significance of each term in the equation and goodness of fit of the regression model obtained. Effects and interactions were considered significant at $p<0.05$.

3. Results and Discussion

Demineralization of sardine scales aims to remove the calcium and other inorganic substance. The presence of these ions affects functional properties such as clarity of collagen solutions in industrial applications. The inorganic substances in sardine scales can be removed by treatment with dilute acids such as ethylenediaminetetraacetic acid (EDTA).

Therefore optimization of demineralization of the sardine scales is important to establish a working range of ethylenediaminetetraacetic acid (EDTA) concentration with corresponding suitable treatment time. Factorial design was used in experiments to determine the region in which the demineralization was as complete as possible. Experimental results of the two factors with tree-level factorial design are presented in Table 2.

3.1 Main effects and interactions plots

The main effects of the factors and their interactions were experimentally analyzed. Figure 1 represented the graph which analyzed the effect of the parameters involved: influence of EDTA concentration (X_1) and treatment time (X_2) on yield of demineralization (y_1), protein content (Y_2) and hydroxyproline content (Y_3). The steep effect lines obtained for the main effects of X_1 and X_2 denote that they affected yield of demineralization, protein content and hydroxyproline content significantly. The yield of demineralization, protein content and hydroxyproline content showed tendencies to increase linearly as X_1 and X_2 were increasing.

The increases in these 2 variables favors leaded to increase the yield of demineralization, which indicate that salts present in the inorganic phase of the sardine scales have been eliminated.

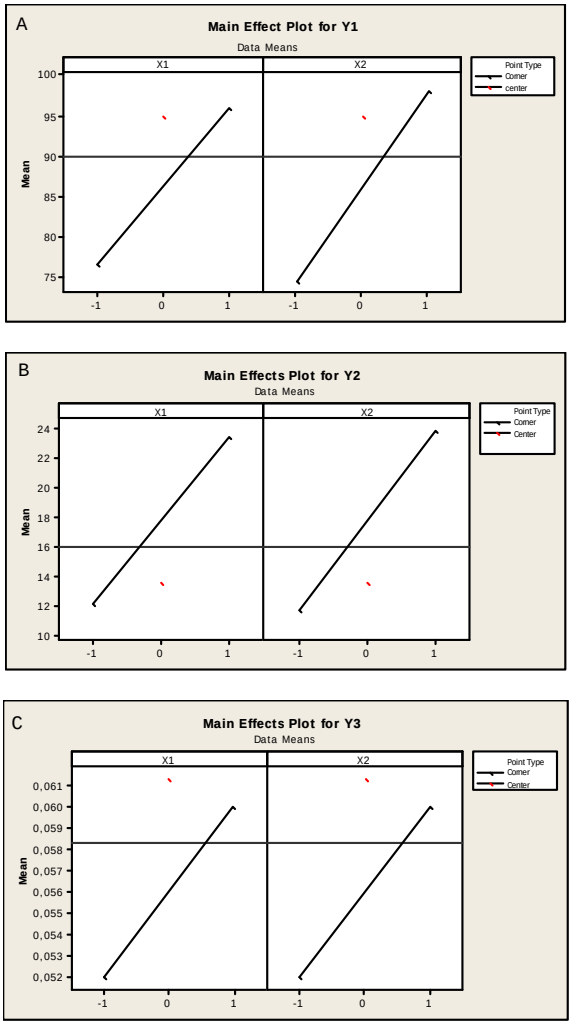
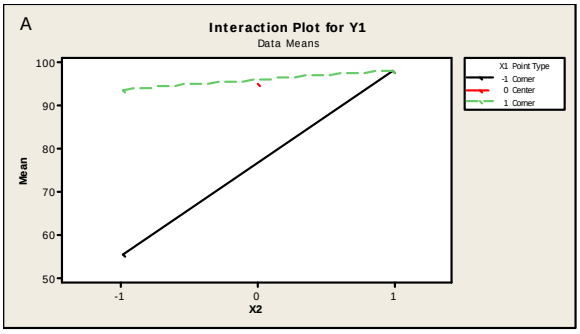


Fig 1: Plot of main effects at various levels of factors for: a- yield of demineralization (Y_1); b-protein content (Y_2); c- hydroxyproline content (Y_3) on demineralization sardine scale

Figure 2 shows the interaction effect of ethylenediaminetetraacetic acid (EDTA) concentration and treatment time on the response. These plots show that the interaction between the two variables showed the concentration of ethylenediaminetetraacetic acid (EDTA) (X_1) and the treatment time (X_2) tended to be affected by the yield of demineralization due to the appearance of an intersection on the graph. Fig 2B illustrates the other two-factors interaction are not noticeable on the protein content, because the lines on the plots are almost parallel to each other for different levels of two factors, this mean there are not interactions between these factors.



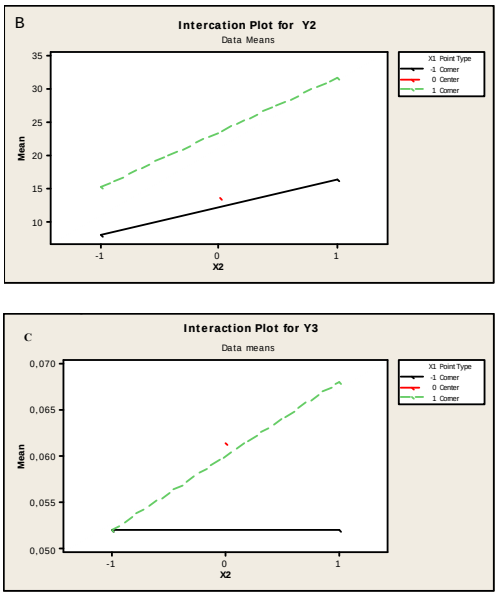
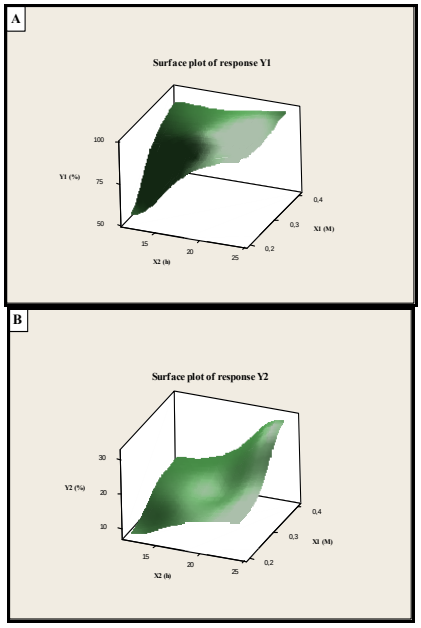


Fig 2: Graphical representation of interactions of influence factor for yield of demineralization (Y_1), protein content (Y_2) and hydroxyproline content (Y_3) on demineralization of sardine scale at different levels of factor

Figures 3 show the response plot for sardine scales. Each surface plot shows showed a increase in yield of demineralization on increasing both the ethylenediaminetetraacetic acid (EDTA) concentration and the treatment time. Moreover, when the ethylenediaminetetraacetic acid (EDTA) concentration was ≥ 0.3 M the higher level of yield of demineralization, 90 to 100%, was obtained with an incubation time in the range of 12 - 24h. Nomura *et al*, 1996, reported a removal of 97% of the calcium present in fish scaled with 0.5% M Analysis of variance (EDTA) during demineralization. In samples pagrus major, oreochromis niloticus, carp, rohu and catla, demineralization takes 2 days [9, 13].

The protein content in demineralization solution ranged from 8% to16% and smaller amounts of hydroxyproline was observed. The absence of Hydroxyproline suggested that most of the protein in the ethylenediaminetetraacetic acid (EDTA) solution were noncollagenous proteins.



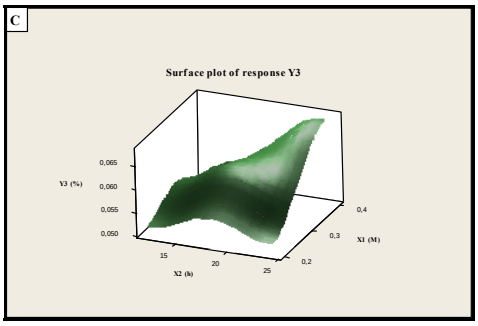


Fig. 3: Response surfaces showing the effect of EDTA concentration and treatment time on the yield of demineralization (Y_1), protein content (Y_2) and hydroxyproline content (Y_3)

3.2 Fitting the models

Regression is employed to fit a polynomial equation to the experimental data. The significance of the coefficient of all the variables of linear (X_1, X_2) and interactions are determined by student's t-test and p values. The regression coefficients estimated for the response models in terms of coded units are presented in Table 3.

Table 3: Estimates coefficient of the fitted polynomial equation for different response based on t-statistic.

Response	Term	Coefficient	T-value	P-value
Y_1	Constant	86.275	315.80	0.000
	X_1	9.575	35.05	0.001
	X_2	11.645	42.63	0.001
	X_1X_2	-9.415	-34.46	0.001
Y_2	Constant	17.872	60.11	0.000
	X_1	5.618	18.89	0.003
	X_2	6.128	20.61	0.002
	X_1X_2	2.043	6.87	0.021
Y3	Constant	0.056	48.50	0.000
	X_1	0.004	3.46	0.074
	X_2	0.004	3.46	0.074
	X_1X_2	0.004	3.02	0.074

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

According to Table 3 all the coefficients of linear (X_1, X_2) and interaction (X_1X_2) are highly significant at P<0.05, in all models.

The regression models for demineralization with ethylenediaminetetraacetic acid (EDTA) using the coefficient of the coded variables are presented as:

$$Y_1 = 86.27+9.57X_1+11.64X_2-9.41X_1X_2 \quad (3)$$
$$Y_2 = 17.87+5.61X_1+6.12X_2+2.04X_1X_2 \quad (4)$$
$$Y_3 = 0.056+0.004X_1+0.004X_2+0.004X_1X_2 \quad (5)$$

According to Equation 3, 4 and 5 the positive sign of coefficients for main effects X_1 and X_2 indicate their synergistic effects on demineralization while the negative sign of coefficient for X_1X_2 interaction denotes an antagonistic effect. This means that concentration of acid solution (X_1) and incubation time (X_2) tends to increase the yield of demineralization while their interaction term, that is [acid] x time (X_1X_2), tends to decrease it.

3.3 Adequacy of the Refined Models

The adequacy of fit of the first order models for demineralization (Eq.3, Eq 4 and 5) is analyzed by Analysis of variance (ANOVA) at 5% significance level (Table 4). Table 4 shows ANOVA for the models that explains the response of the dependent variables (Y_1, Y_2 and Y_3).

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

The determination coefficient (R^2) of the models obtained for demineralization with EDTA is 0.9999 (99.99%) indicates the models are well fitted with the experimental data. The value of the adjusted determination coefficients (Adj R^2) is also satisfactory for confirming the significance of the models.

Table 4: Analysis of variance (ANOVA) for polynomial model obtained from experimental design

Responses	Source	DF	SS	MS	F-value	P-value
Y_1	Main Effects	2	909.147	454.573	1522.69	0.001
	X_1	1	366.722	366.722	1228.41	0.001
	X_2	1	542.424	542.424	1816.96	0.001
	X_1X_2	1	354.569	354.569	1187.70	0.001
	Residual	2	0.597	0.299	-	-
	Total	6	-	-	-	-
	R^2 = 99.96%Adj R^2 =99.87%					
	Source	DF	SS	MS	F-value	P-value
Y_2	Main Effects	2	276.41	138.205	390.81	0.003
	X_1	1	126.22	126.225	356.94	0.003
	X_2	1	150.18	150.185	424.69	0.002
	X_1X_2	1	16.86	16.687	87.49	0.021
	Residual	2	0.70	0.345	-	-
	Total	6	-	-	-	-
	R^2 = 99.78%Adj R^2 =99.35%					
	Source	DF	SS	MS	F-value	P-value
Y_3	Main Effects	2	0.000	0.000	12.00	0.077
	X_1	1	0.000	0.000	12.00	0.074
	X_2	1	0.000	0.000	12.00	0.074
	X_1X_2	1	0.000	0.000	12.00	0.074
	Residual	2		0.000	-	-
	Total	6	-	-	-	-
	R^2 = 95.76%Adj R^2 =87.27%					

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

3.4 Optimization using desirability function approach

As it's shown in figure 4, the individual desirability values, the overall desirability D and predicted value are calculated by Minitab. The factors obtained at the maximum point of Y1 (target: 94%) and the minimum points of Y_2 and Y_3 (target: 13.6% and 0.06% respectively) are calculated as X_1=0.3M and X_2=18h which are known as estimated condition (Fig3).

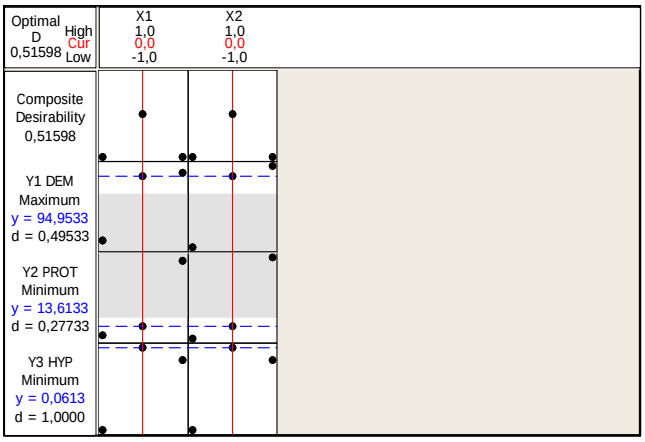


Fig. 4 Optimization plot

3.5 Verification of optimal parameters

Validation experiments are applied at the estimated condition. The results are in agreement with the predicted value, it's also confirmed that the model used in this experiment is appropriate (Table 5).

Table 5: Test results for verification of the results of demineralization with EDTA

Optimal Condition		Predicted values			Verification experiment		
X_1 (M)	X_2 (h)	Y_1 (%)	Y_2 (%)	Y_3 (%)	Y_1 (%)	Y_2 (%)	Y_3 (%)
0.3	18	94.9	13.61	0.06	95.00±0.26	12.9±1.2	0.07±0.08

4. Conclusion

Demineralization is required to remove salts present in the inorganic phase of the sardine scales. The presence of these ions affects functional properties such as clarity of collagen solutions in industrial applications. Demineralization can be achieved with dilute acids such as EDTA. Excessive acid concentrations or/and extremely long treatment time can result in loss of yield and collagen quality through hydrolysis of the collagen. Therefore optimization of demineralization of the sardine scale was important to establish a working range of ethylenediaminetetraacetic acid (EDTA) concentration with corresponding suitable treatment time. The factorial design methodology is important tool that allows following of the optimization of processes from an appropriate experimental design of a limited number of experiments. The demineralization of sardine scale is significantly influenced by the treatment time and the ethylenediaminetetraacetic acid (EDTA) concentration. Linear effect of these two variables affect yield of demineralization and protein content. The optimum condition that was predicted verified the constraints values which were found in agreement with the experimental results. At the end of this work, our results showed that the optimization of demineralization with ethylenediaminetetraacetic acid (EDTA) have used low reactant concentration values with a relatively short processing time. The utility of our findings in research and industry could help competitiveness of enterprises which need to reconcile the good yields with lower production cost.

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