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Optimize gelatin extraction from yellow-fin tuna (Thunnus albacares) fish skin based on yield, collagen content, and color.

Amirreza Shaebani Darejazi (amirreza.shaabani.m051@gmail.com)
Mahsa Abedi (mahsa.abedi.k@gmail.com)
Reza Roostaazad* (roosta@sharif.edu)
Chemical and Petrochemical Engineering Department, Sharif university of Technology, Tehran, Iran

Abstract

Gelatin was extracted from the skin of the fish; yellowfin tuna (Thunnus albacares) and process parameters including yield, collagen content (CC), and CIE L*a*b* Color Difference (ΔE°) were optimized by Box-behnken method in RSM. Independent variables were H_2O_2 pretreatment; concentration (cH_2O_2) (at 0, 5, and 10 (v/v%)) and pH (pH_2O_2) (at 6, 8, and 10) and extraction temperature (ET) (40,50,60 (°C)) as X_1, X_2 and X_3 respectively. Desirability 53.9% were selected and the independent variables were as follows: H_2O_2 pretreatment (10 (v/v%), pH 10) and extraction treatment (temperature 51.098 (°C)) with the response yield 7.274, CC 67.66, ΔE° 19.068. Analysis of Variance (ANOVA) test showed the parameters were significant. AT the optimum point, the kinetic and solvent/solution ratio effects on the mentioned responses were evaluated and 12 hr was suggested as the best extraction time. ET is highly affected by the responses. Although ET has a positive effect on yield, but it has negative on CC and ΔE°. Moreover, cH_2O_2 and pH_2O_2 have a positive effect on yield and CC, although mostly the effects are not significant.

1. Introduction

Gelatin is a denatured fibrous protein derived from nature collagen by partially thermal hydrolysis. Gelatin has wide applications in the food industry, with major uses as jellies, meat products, and confectionary. Moreover, it uses in pharmaceutical industry especially as soft and hard capsules. [1]
Traditionally, gelatin was produced from cattle and pigs as bovine and pig gelatins respectively [1]. These gelatins have some disadvantages such as risk of diseases, occurrence of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD) [2] [3]. Moreover, Muslims and Jews are not capable to use porcine and bovine gelatin unless the animal is religiously slaughtered [4]. consumption of this product is banned by Hindus as well. Therefore, attention has recently increased to alternative sources, especially fish waste. Fish wastes including skin, bones, fins, and scales are mostly structured by gelatin and they are free of infections such as BSE, TSE and FMD [3] .
Canned tuna industry is developing in Asian countries such as Thailand [5], Korea, Japan [6] and Iran [7]. One of the most important types of commercial fish for tuna industry is yellowfin tuna and is one of the largest species of tuna found in the Persian Gulf and the Sea of Oman. Currently, there are a lot

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of canning factories in Iran that use this type of fish as a raw material. [7] The fish industry often produces as much as two-third of total fish weight as wastes including skin, scale, fins, and skeletons that could be considered as a rich source of gelatin [8]. Producing byproduct environmentally decreases pollution and economically generate capital. [6]

Important specifications of commercial gelatin include yield, CC [9], color [10]. Light color is preferred for customers because gelatin can mix with various foods and beverages without obvious color transfer [11].As far as production procedure is concerned, process yield, and CC are the most important characteristics to achieve. Yield value expresses how much dry extract is produced from the provided substrate, while CC expresses the amount of gelatin in the dry extract and evaluates the relative extraction efficiencies relative to specific target material [12]. Different protocols influence yield and CC including pretreatment and extraction methods and materials. Neutral NaCl solution and H_2O_2 pretreatment were shown to affect the color of gelatin. Hydrogen peroxide is an environmentally friendly oxidizing agent that is mainly used for bleaching.
The aim of this project is the evaluation and optimization of gelatin extraction based on yield, CC, and ΔE°.

2. Material and methods

2.1. Materials

NaCl, Sodium hydroxide, Polysorbate 80, H_2O_2, $MgSO_4$, $CaCl_2$ were obtained from Neutron (Pharmachemical Co., Iran)
Yellow fin tuna (Thunnus albacares) skin with scales was collected from the local market and immediately transported to the laboratory under chilled conditions on ice. Skin was cut into pieces (20 cm × 20 cm) and was stored at −20 °C until pretreatment with gelatin. Before H2O2 pretreatment, the skins were cut into small pieces (2 cm × 2 cm) According to Figure 1B.

Thorough Mixing was carried out using a 100 L polyethylene mixer equipped with stainless blades procured from the local market.

Gentle mixing was carried out using magnetic stirrer (85-2 A, Sile Instrument Co., Ltd., Shanghai, China).

Filter was carried out using Büchner funnel procured from local market and Whatman no. 4 filter papers (Whatman, Maidenstone, England) were used as filter paper.

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2.2. Methods

Pretreatment procedure was carried out by gentle mixing of the skin sample at 30 ^{0}C in multiple steps and the sample was thoroughly washed with cold distilled water between each step. All skin samples were 25 g in weight. To remove scale myo-fibrillar proteins, partial pigments, and partial fat, the skin was soaked in 1.5 M NaCl solution for 24 hr. (skin to solution ratio 1:20 (w/v). Then, the non-collagen proteins were removed by mixing with 0.1M NaOH for 24 hr. (skin to solution ratio 1:30 (w/v), 24 h). To remove skin fat, the product was soaked in nonionic Tween 80 detergent for 24 hr. (detergent to solution ratio 0.5% (v/v), Skin to solution ratio 1:15). The product was then subjected to the following H_2O_2 pretreatment.

Pretreated skin was bleached by H_2O_2 solution for 24 hr. (Skin to solution ratio 1:20). After H_2O_2 pretreatment, the bleached skin was washed with cold distilled water until reaching a neutral or faintly basic pH.

Extraction was carried out using hot water so that the temperature was fixed by a water bath. Skin to solution ratio of all of the samples at this stage was 1:10 and the extraction time was 24 h. Gelatin extracted solutions were filtered and finally the clear filtrate was dried in oven at 45°C. Yield of gelatin was calculated based on the weight of the dried gelatin film (W_1) by the wet weight of the original (wet) skin waste as received (W_2) according to Eq.(1)

$$\text{Yield } (\%) = \frac{W1}{W2} \times 100 \quad (1)$$

CC was evaluated by measuring the hydroxyproline content using the Kiazist commercial kit. CC was calculated as follows:

$$\text{CC} = (\text{HypCnt } \times C.F) \times 100\ \% \quad (2)$$

Where:

C.F is a conversion factor for type I collagen, that is, a 10 number

HypCnt is the hydroxyconcentration in 1 mg gelatin.

Gelatin color was determined with a Spectrophotometer (CHN spec, CS-810, China) at an illumination condition of the light source (C) with a 2°C standard observer. Results were expressed as L*, a*, and b*, Where:

L* is the measure of brightness from black (0) to white (100)

a* indicates the degree of redness (+a*) to greenness (-a*)

and

b* is the measure of yellowness (+b*) to blueness (-b*).

Another important parameter of CIE76 color difference (ΔE°)is calculated as:

$\Delta E^{\circ} = \sqrt{((L* - L_0^*)^2 + (a* - \alpha_0^*)^2 + (b* - b_0^*)^2)}$

(3)

Where:

L_0^* , α_0^* and b_0^* are ideal lightness, redness, and yellowness, respectively. Double distilled water (DDW) was considered as the ideal solution.

Box Behnken Design (BBD) was employed for statistical optimization of gelatin extraction. Four variables at three levels (−1, 0, +1) were set in these experiments. (0) was considered as the central point, (−1) as low level (below the central point), and (+1) as high level (above the central point). Box-Behnken design (BBD) experimental design is one of the 3 varieties of the RSM statistical method of experimentation (Central Composite Design (CCD), Box-Behnken design (BBD) and D-optimal design) and has excellent predictability.

Independent variables and their adopted levels based on the results obtained from the initial experiments are shown in Table 1. 17 experiments were performed in triplicate and proper independent variables X_1 (concentration of H_2O_2 pretreatment), X_2 (pH of H_2O_2 pretreatment) and X_3 (extraction temperature) were set.

Table 1. Independent variables in Box-Behnken design

Independent variables	Symbol	Range and levels		
		-1	0	+1
pH H2O2 pretreatment	X1	6	8	10
Concentration H2O2 pretreatment to solution (v/v%)	X2	0	5	10
Temperature extraction(°C)	X3	40	50	60

A model of a full quadratic equation was developed to fit the data and finally predict the optimal point according to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (5)$$

Where:

Y represents the response variables

β_0 is a constant

β_i, βii, and β_{ij} are the linear, quadratic, and cross-product coefficients, respectively

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x_i and xj are the levels of the independent variables.
The efficiency of the model and the statistical significance was analyzed using F-test and the R-test. The effect of an individual variables was analyzed by performing detailed analysis of variance (ANOVA) on the coded level of variables. RSM and design of the experiments were done using Design-Expert.

3.Results and discussions

As mentioned before, fish skin waste was procured from the local market and pretreatment was applied to this waste. Figure 1 illustrates the initial condition of the waste and pretreatment setup.

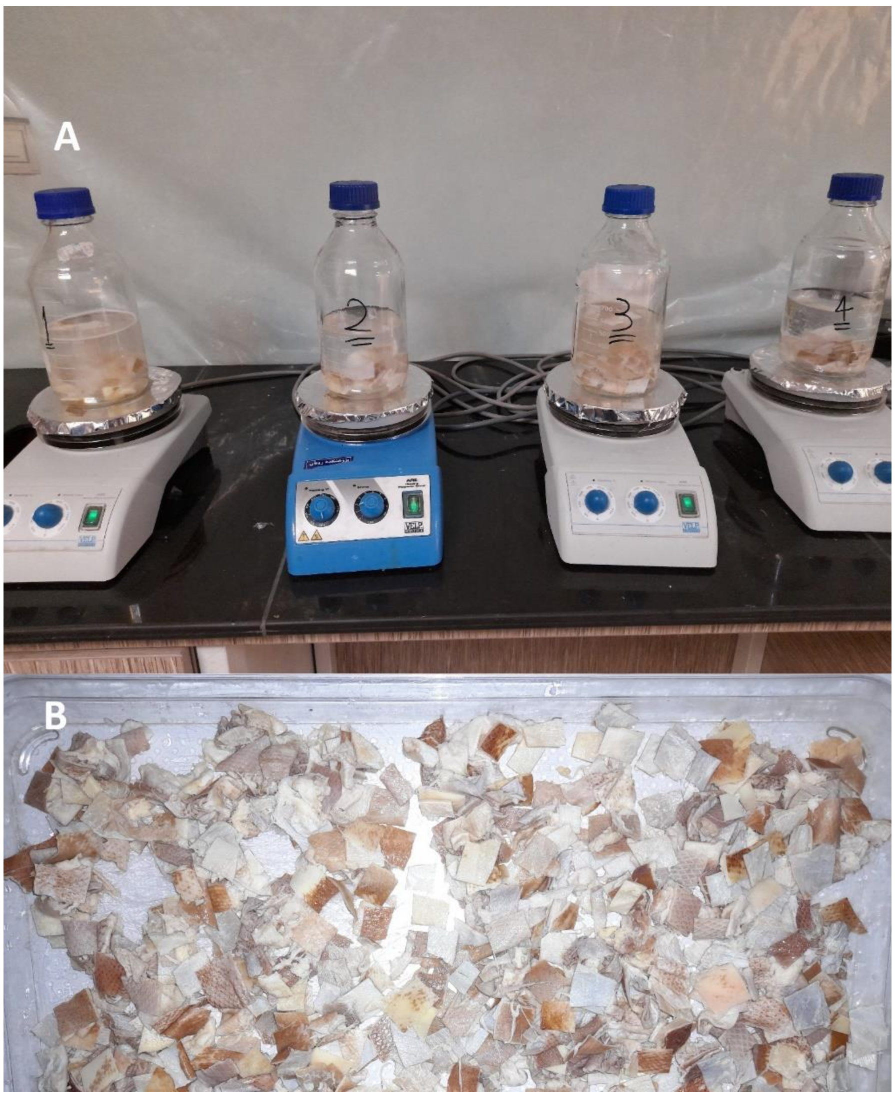

Figure 1. A: pretreating skin and B: yellowfin tuna (Thunnus albacares) skin waste

After pretreatment, extraction was carried out as elaborated in M&M section. Table presents details of the experiment run in this section.

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Table 2. Box-Behnken design matrix with uncoded and coded values of the extraction conditions and observed responses.

Run No	Coded level of variables			Gelatin responses		
	X1	X2	X3	Yield	CC	ΔE°
1	0	0	0	8.4	23.35	46.6
2	+1	0	+1	13.32	24.24	32.52
3	0	-1	-1	3.1	79.53	1.14
4	-1	-1	0	8.24	33.19	40.52
5	0	+1	+1	6.64	32.82	48.49
6	-1	+1	0	5.84	98.07	49.19
7	-1	0	+1	14.84	32.72	54.89
8	0	0	0	8	32.92	35.09
9	-1	0	-1	2.98	56.16	32.59
10	+1	-1	0	8.28	20.46	24.97
11	0	0	0	7.6	28.51	28.76
12	+1	0	-1	3.96	86.72	2.4
13	0	+1	-1	2.4	81.19	0.41
14	0	0	0	9.92	59.7	34.08
15	+1	+1	0	8	98.12	9.66
16	0	0	0	9.51	52.24	37.33
17	0	-1	+1	14.64	40.3	40.76

Experimental results of the Box-Behnken design are also presented in Table . Quadratic polynomial equation to the experimental data is suggested by the software design expert to fit the experimental data. To develop the fitted response surface model equations, all insignificant terms ($P \geq 0.05$) were eliminated. Fitted equations are also shown in Table . Linear vs Mean model is suggested and selected by Design-expert 13 for Y_1, Y_2, Y_3 responses.

Statistical significance of the quadratic polynomial model equation was evaluated by the analysis of variance ANOVA. Analysis of variance for the quadratic polynomial models is presented in Table . Table shows the ANOVA for the models that explain the response of the dependent variables. However, for all responses, only one term (selected model hr) of all linear and quadratic terms contributed significantly to the models ($p \leq 0.05$) and were significant but P-values for the lack-of-fit test were large and show that the models are adequate for predicting the gelatin production conditions. Overall, three responses can be used to navigate the design space.

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Table 3. Final equation obtained in terms of coded factors for three responses

Responses	Quadratic polynomial model	P-value	R^2
Y_1	Y_1= -9.66191 +0.041500 X_1 -0.711250 X_2 +0.462500 X_3	< 0.0001	0.8195
Y_2	Y_2= +90.69382 +0.235000 X_1 +8.54500 X_2 -2.16900 X_3	0.0210	0.5147
Y_3	Y_3= -43.74706 -2.69100 X_1 +0.022500 X_2 +1.75150 X_3	< 0.0001	0.8052

Table 4. Summary of statistical analysis of the model

Responses	Source	Sum of squares	df	Mean Square	F-value	p-value
Y1	Mean vs Total	1082.73	1	1082.73		
	Linear vs Mean	**187.66**	**3**	**62.55**	**19.67**	**< 0.0001**
	2FI vs Linear	16.01	3	5.34	2.11	0.1630
	Quadratic vs 2FI	12.23	3	4.08	2.18	0.1785
	Cubic vs Quadratic	9.17	3	3.06	3.11	0.1510
	Residual	3.93	4	0.9834		
	Total	1311.73	17	77.16		

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Y2	Mean vs Total	45577.79	1	45577.79		
	Linear vs Mean	**6111.24**	**3**	**2037.08**	**4.60**	**0.0210**
	2FI vs Linear	442.75	3	147.58	0.2775	0.8404
	Quadratic vs 2FI	1421.26	3	473.75	0.8508	0.5089
	Cubic vs Quadratic	2902.72	3	967.57	3.89	0.1113
	Residual	995.12	4	248.78		
	Total	57450.88	17	3379.46		
Y3	Mean vs Total	15869.20	1	15869.20		
	Linear vs Mean	**3902.51**	**3**	**1300.84**	**17.91**	**< 0.0001**
	2FI vs Linear	176.94	3	58.98	0.7688	0.5372
	Quadratic vs 2FI	417.12	3	139.04	2.78	0.1197
	Cubic vs Quadratic	179.63	3	59.88	1.41	0.3639
	Residual	170.37	4	42.59		
	Total	20715.78	17	1218.58		

Based on the experimental results and the statistical analysis afterwards, the effect of independent variables on various dependent variables may be discussed as follows.

3.1.Effect of yield

The effect of the independent variables on yield is shown in Figure . The results show the effect of ET on yield is highly significant ($p \leq 0.0001$) and pH_2O_2 is statistically significant ($p = 0.0419$) with a threshold 0.05 While the effect of cH_2O_2 is insignificant.

ET has a direct relation to yield. This result agreed with Benjakul et al. Higher temperatures provide higher energy for destroying hydrogen bonds stabilizing the protein localized in the skin.[1]

When the pH_2O_2 was decreased from 10 to 6, the yield of gelatin increased. This result is in contradictory with Liu et al. that determined on collagen and resulted in higher pH_2O_2, the skin becomes much looser, and the penetration of acid and pepsin becomes easier. [13]

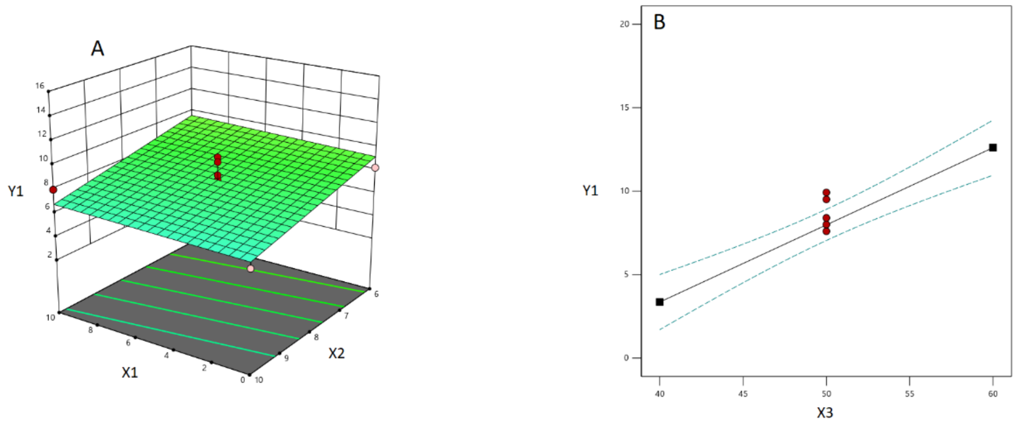

Figure 2. Effects of A: X_1 and X_2 B: X_3 on yield.

3.2.Effect on CC

The effect of the independent variables on CC is shown in Figure . The results show the effects of pH_2O_2 and ET were significant ($p = 0.0389$ and 0.0121 respectively) while cH_2O_2 is insignificant.

pH_2O_2 has a direct relation with CC. This is due to as the pH increases, the skin swells better and the collagen dissolves better in solution. [13]

ET has an inverse relation with CC. These results show, however, the yield increases with increasing extraction temperature, but the gelatin solution content dissolved higher non-collagen substances. Therefore, CC decreases.

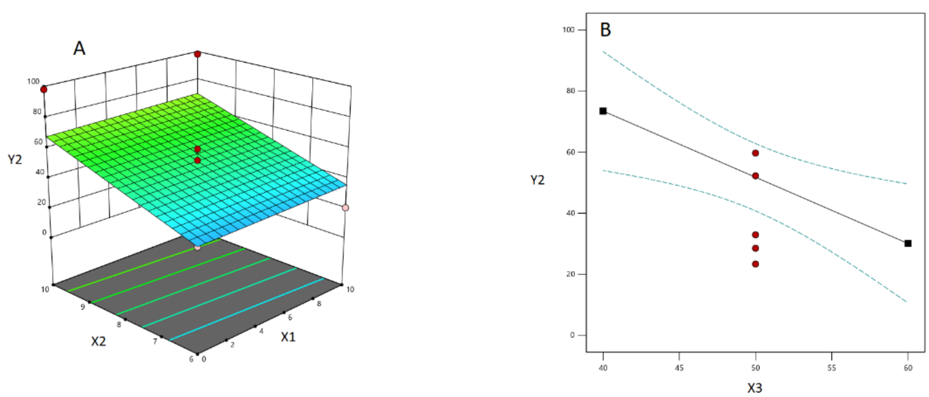

Figure 3. Effects of A: X_1 and X_2 B: X_3 to CC.

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3.3 Effect on color ($\Delta E°$) of solution

The effect of the independent variables on ΔE° is shown in Figure . The results show the effect of ET and cH_2O_2 on ΔE° is highly significant ($p \leq 0.0001$ and $p = 0.0006$ respectively) while pH_2O_2 is insignificant on ΔE°.

ET has direct relation to ΔE°.Whenever extraction temperature increases, non-collagen substances including pigments better are dissolved in solution.

cH2O2 has inverse relation to ΔE°. This result is in accordance with Liu et al. and it happens because bleaching decreases the yellowness (a*) and redness (b*) of the skin [13].

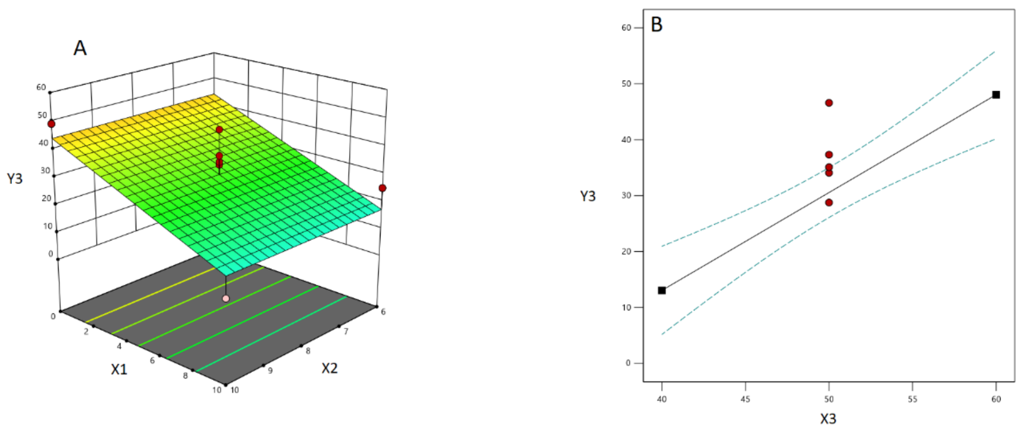

Figure 4. Effects of A: X_1 and X_2 B: X_3 on color

Error! Reference source not found. shows the real difference of gelatin powder when undergoing different operating variables.

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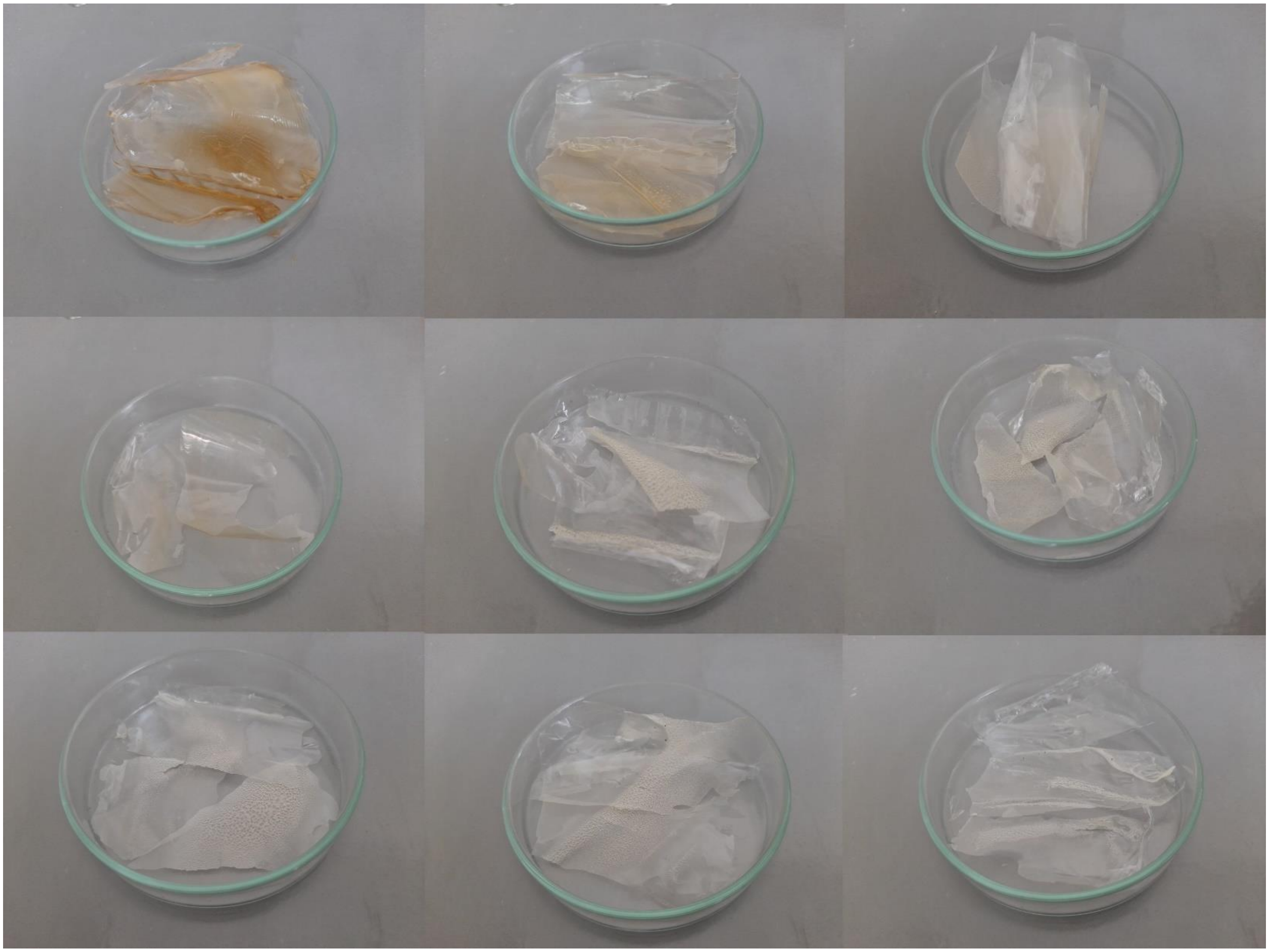

Figure 5. Gelatin film color produced by different independent variables.

3.4. Conditions for optimum response

The desirability function has been used for optimizing multiple responses. [14]. It is based on maximizing yield and CC and minimizing ΔE°. Figure illustrates desirability function for the optimization of gelatin extraction. The results show that sample (X_1=10, X_2=10, X_3=51.104) is the best optimized point. The best desirability is 53.9% and the responses are yield 7.278, CC 67.638, and ΔE° 19.085.

The reason for the lack of higher desirability is the growth trend of response variables is in contradictory condition of independent variables. At the optimized point, gelatin was extracted from the skin of tuna.

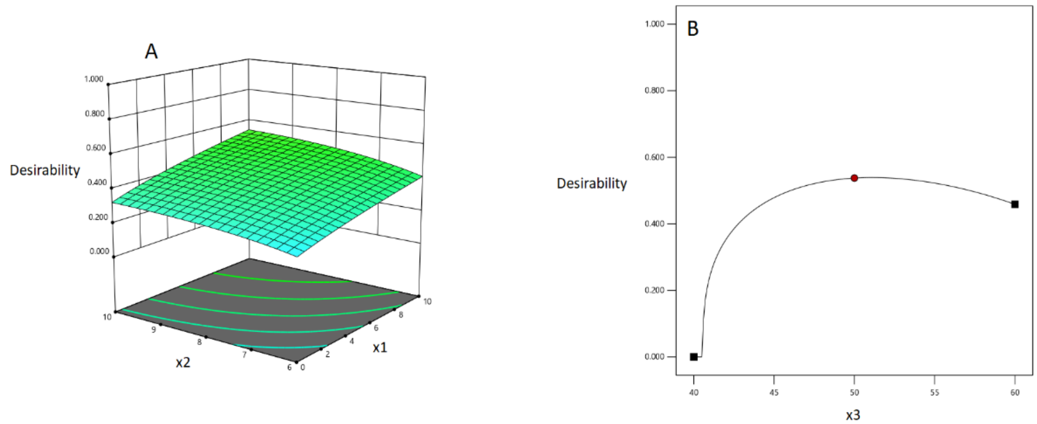

Figure 6. Optimization based on total response using desirability

3.5. Validation:

At the optimum condition, collagen was extracted three times and the results were validated with computational responses. Validation was done by linear regression and the results showed that the relations were significant. (P-value<0.02). (Table).

Table 5. Experimental and computational response values at optimum condition

Process\Responses	Yield(%)	Collagen content(%)	ΔE°
Experimental	7.278	67.638	19.085
Computational	7.02	64.58	18.29

3.6. *Kinetic effect*

Optimization of extraction kinetic was performed by extending the time of contact between solvent and fish skin for 6 to 48. Other variables were at optimum conditions determined in the previous steps. [15]. (**Error! Reference source not found.**)

Yield increases with time. At CC plot and ΔE° plot there are a peak and a minimum point at 12 hr, respectively. The trend illustrates after that 12 hr non-collagen substance were dissolved in solution.

Overall, at 12 hr is suggested as the best point although the yield is below desirability point but CC and ΔE° are improved.

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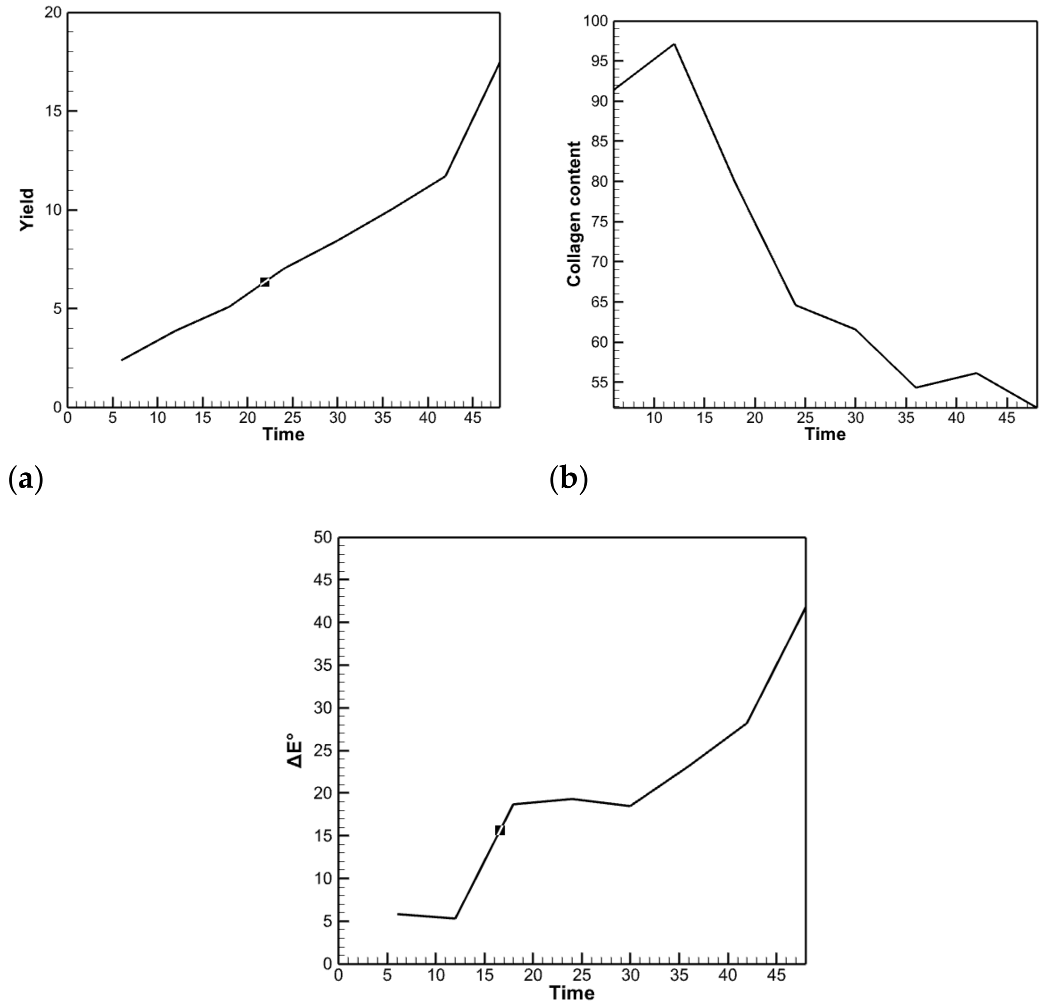

(**a**) (**b**)

(c)

Figure 7 Kinetics effect on: (a) yield; (b) CC; (c) ΔE°

3.7. Solvent/solid ratio

Solvent to solid ratio was optimized by performing a collagen extraction process within the range of 2.5 to 15 ml/g of fish skin. Yield and ΔE° have the same trend and as the solvent to solution ratio increases, so do yield and ΔE° but CC has the inverse trend. The results show by increasing the solvent solid ratio, more non-collagenous materials are dissolved in the solution and have a negative effect on CC and ΔE°. (Figure 8)

Choosing the best point based on the ratio of solvent to solid is difficult because the response variables behave differently from each other, so if we give equal weight to the response variables, we will not have the desired result.

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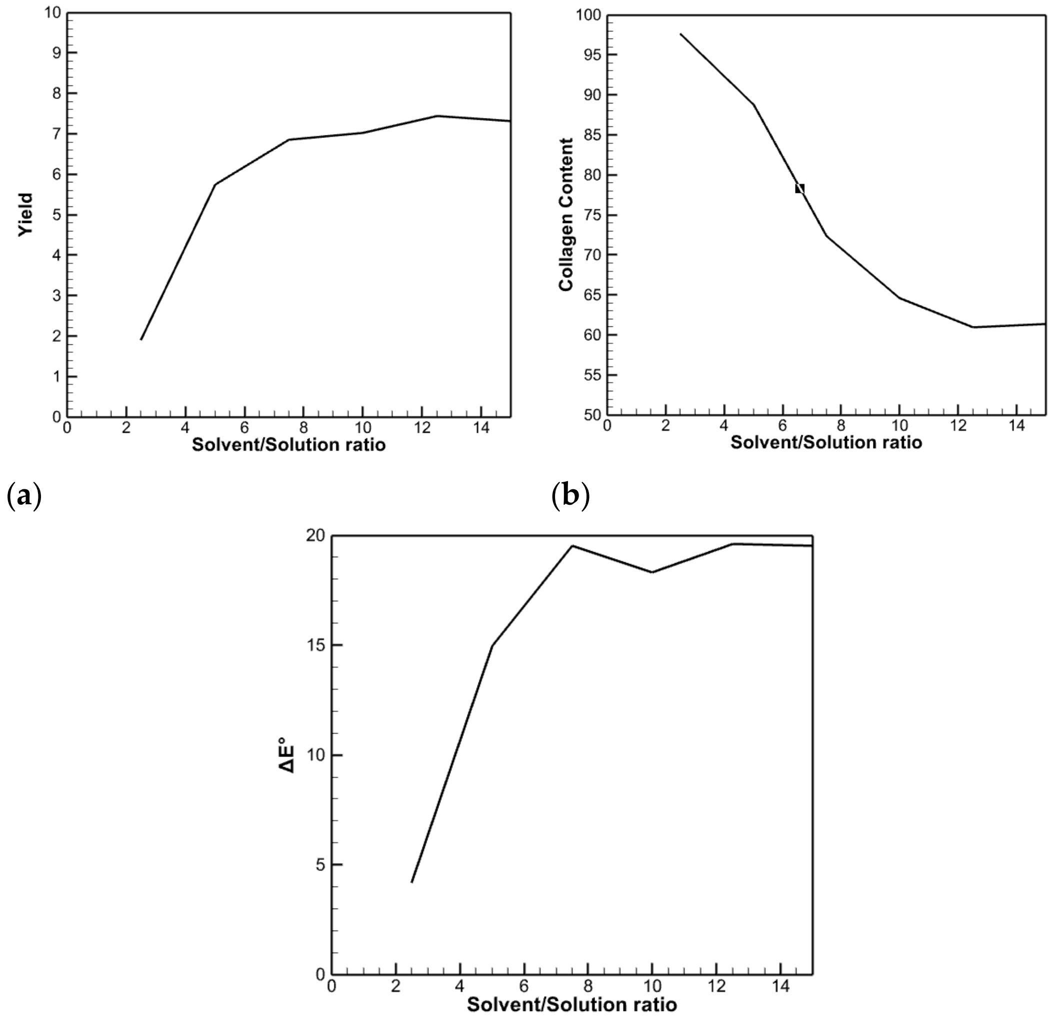

(**a**) (**b**)

(c)

Figure 8 Solvent/solution effect in extraction treatment on: (a) yield; (b) collagen content; (c) ΔE°

4.Conclusion

The results show that the concentration of hydrogen peroxide in gelatin has a similar effect to collagen and the higher pH_2O_2 has a positive effect on the desirability function, but in cH_2O_2 gelatin has a contradictory trend with collagen and it has a positive effect on gelatin extraction.

Although increasing the ET has a direct effect on the yield, it has a negative effect on the collagen content and color. Therefore, in the desirability function it has a moderate temperature range.

Examination of the time and ratio of solvent to solid at the optimum point shows that these two parameters have a highly effect on the response variables.

Although the optimal point of the previous step can be improved by examining the time and solvent-to-solid ratio, but the possibility of independent variables being interdependent means that we

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cannot conclude confidently from this study to obtain the best point and to get the optimal points we need to examine all effective parameters.

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