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Reduction of Mercury from Mackerel Fillet Using Combined Solution of Cysteine, EDTA, and Sodium Chloride

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ABSTRACT: An acidic solution containing mercury chelating agents to eliminate mercury in raw fish (mackerel) fillet was developed. The solution contained hydrochloric acid, sodium hydroxide, cysteine, EDTA, and NaCl. The optimum conditions for mercury reduction were achieved using response surface methodology (RSM) at cysteine concentration of 1.25%, EDTA of 275 mg/L, NaCl of 0.5%, pH of 3.75, and exposure time of 18 min. The optimized conditions produced a solution which can remove up to 91% mercury from raw fish fillet. Cysteine and EDTA were identified as potential chelating agents with the greatest potential for use. The solution can be employed in fish industries to reduce mercury in highly contaminated fish.

KEYWORDS: *mercury reduction, fish, food safety, acidic solution*

INTRODUCTION

Mercury contamination of aquatic ecosystems is a global environmental concern.[1] Any contaminated fish has the ability to transmit dangerous toxins through ingestion, and the risk is believed to increase as the quantity consumed increases. Marine fish, being at the top of the food chain, have been shown to contain high quantities of mercury.[2] Methylmercury toxicity is better characterized than other organic mercury compounds.[3] Mercury and methylmercury are neurological toxicants to humans. Human exposure to mercury is associated with slow development, blindness, cerebral palsy, and other birth defects.[4,5] The population believed to be the most vulnerable to hazards from mercury consumption are young children and women who are pregnant or nursing. [6] Human exposure to mercury is primarily through the consumption of fish,[5,7] where it is mainly present in the form of organic mercury.[8−11]

Seafood is a healthy dietary choice, and consumers should be encouraged to eat more fish for valid health reasons. Such dietary advice must be accompanied by clear and useful guidance on mercury in fish, to help consumers choose wisely, so they can both benefit from the nutritional advantages of fish consumption and minimize mercury exposure. It is widely known that the population of Malaysia has a fish-based diet.[12] Previous studies reported that mercury contamination in marine fish and shellfish in Malaysia exceeded provisional tolerable weekly intake of 5 μg/kg of total mercury and 1.6 μg/kg of methylmercury recommended by WHO.[13−17] Therefore due to the toxicity of mercury and methylmercury to human, this research tried to develop a protocol to remove mercury contamination in edible fish.

Methylmercury is found in fish muscle (fillets) bound to proteins. Therefore, common treatments (skinning, trimming, removing fat, cooking, frying, microwaving, or breading the fish) does not significantly reduce the mercury concentration.[18−22] Previous studies showed that cooking and canning can increase the mercury levels in fish.[19,23]

Some studies have reported methods to reduce mercury from edible fishes with variable results. Different agents and media have been used to reduce mercury contamination. They have used different solutions and media such as acid and alkaline solutions, cysteine and homocysteine, alcoholate solution under heating, organic sulfur complexing agent, dry crushed shell membrane, ascorbic acid, and pectin solution.[24−39] However, the combined effect of acidic and alkaline solution with mercury leaching agents, such as cysteine, EDTA, and salt (NaCl) has not been reported so far. The objective of this study was to develop a mixed solution formulation with industrial application to remove mercury from fish tissue. Response surface methodology (RSM) was used to optimize the mixed solution in order to get the highest mercury removal. Techniques such as response surface methodology (RSM) and mixture designs are useful in formula optimization.[40]

MATERIALS AND METHODS

Chemicals and Materials. All reagents were of analytical reagent grade. Total mercury standard was purchased from Fluka (Tokyo, Japan), BCR-463 (total and methylmercury in tuna fish) from Unit for Reference Materials (EC-JRC-IRMM, Geel, Belgium), L-cysteine from Fluka (Tokyo, Japan), and hydrochloric acid 37% (R&M: 1386-80), nitric acid 65% (R&M: 1401-80), L-cysteine hydrochloride monohydrate (food grade), sodium hydroxide (NaOH), sodium chloride (NaCl), disodium ethylene diaminetetraacetic acid (EDTA) ($C_{10}H_{14}N_2Na_2O_8$), tetrabutylammonium bromide (TBAB), ethyl ether, acetonitrile (HPLC grade), copper sulfate penthydrate ($CuSO_4$), and phosphoric acid (analytical reagent grade) from Merck (Darmstadt, Germany).

Total mercury standard solutions were prepared using deionized water (ELGA LabWater, Marlow, U.K.). Mercury stock standard solution (1000 mg/L) was prepared by dissolving 0.0677 g of $HgCl_2$ in the 3% HCl in a 100 mL digestion flask. The working solutions were freshly prepared by diluting an appropriate aliquot of the stock solution through intermediate solutions using 3% HCl. EDTA stock solution (500 μg/mL) was prepared by dissolving the appropriate

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amount of EDTA disodium salt in water and stored in the refrigerator (4 °C) in a plastic bottle. The working standard solutions were daily prepared by appropriate dilution with water.

Instrumentation. Total mercury was determined in all the digested samples using a cold vapor atomic absorption spectrophotometry flow injection mercury/hydride analyzer (FIAS 100, Perkin-Elmer), equipped with a hollow cathode mercury lamp operated at a wavelength of 253.7 nm and a quartz absorption cell. CV-AAS was calibrated using two sets of standard solutions of 0, 5, 10, 15 ng/L of mercury for the first and second calibration intervals, respectively. The correlation coefficient was higher than 0.996.

EDTA were determined by using high performance gas chromatography (Waters, Milford, MA, USA), which was connected to a photodiode-array detection detector (model 486, Milford, MA, Waters, USA) and C-18 column (4.6 × 250 mm).The mobile phase contained 0.02% phosphate buffer solution (pH 2.4, 0.175 mM), tetrabutylammonium bromide (TBAB), and 5% acetonitrile. The flow rate was 1 mL/min, and UV detection was 257 nm. A 10 μL volume of sample was injected to HPLC. HPLC was calibrated using five-point standard calibration solutions in the range of 0.05−300 μg/mL. The correlation coefficient was more than 0.994.

Experimental Design. The mercury reducing mixed solution contained hydrochloric acid, sodium hydroxide, cysteine, EDTA, and salt (NaCl). A five variable parametric study was employed for the central composite design (CCD) analysis with pH (1−6.5), cysteine (0−2.5 w/v %), EDTA (0−550 w/v %), and NaCl (0−1 w/v %); time (5−30 min). The experimental design included 32 experiments of five variables at five levels ($-\alpha$, −1, 0, +1, $+\alpha$). Table 1 shows the coded and actual levels of the variables employed in the design matrix. The experimental design, consisting of 32 different experiments, is shown in Table 2. Experiments were performed in random order.

Table 1. Uncoded and Coded Independent Variables Used in RSM Design for Mecury Reduction

		coded levels				
symbol	indep variable	$-\alpha$	−1	0	+1	$+\alpha$
X_1	pH (HCl and NaOH)	1.00	2.38	3.75	5.13	6.50
X_2	concn of cysteine (%)	0	0.63	1.25	1.88	2.50
X_3	concn of EDTA (mg/L)	0	137.5	275.0	412.5	550.0
X_4	NaCl (0−1%)	0	0.25	0.50	0.75	1.00
X_5	time (min)	5	11.25	17.50	23.75	30.00

Sample Preparation. Samples of short-bodied mackerel were purchased from a wet market in Selangor, Malaysia. Fresh fish samples (17−22.5 cm in length and 130−185 g in weight) were washed, skinned, filleted (manually) and stored in a freezer at −20 °C. Raw fish fillets of 50−70 g were dipped in mixed solutions, containing different concentrations of hydrochloric acid, sodium hydroxide, cysteine, EDTA, and salt, for different durations of time as dictated by experimental design. The quantity of slurry was sufficient to submerge the whole fish fillet (the ratio of solution to fish was 2:1). The solution was then decanted, and flesh was subsequently rinsed with water. pH was adjusted using different concentrations of hydrochloric acid and sodium hydroxide (Table 3). Different concentrations of hydrochloric acid and sodium hydroxide were prepared and mixed in equal volumes to obtain 100 mL of final solutions with the desirable pH.

Mercury and EDTA Extractions in Fish Samples. Total mercury was extracted in all the samples following the method described by Hajeb et al.[10] The fish samples were homogenized by repeated chopping and mixing of the frozen tissue followed by blending using a commercial blender that had been cleaned and rinsed with dilute nitric acid and deionized water prior to use. The homogenized samples (0.5 g wet) were weighed in digestion tubes, and 5 mL of HNO_3 (65%) was added before the mixture was digested at 40−90 °C for 3 h. Digested samples were then cooled and subsequently diluted to 40 mL volume with deionized water.

Table 2. Experimental Points of the Central Composite Design for Mercury Reduction

expt no.	pH	cysteine (%)	EDTA (mg/L)	NaCl (%)	time (min)
1	−1	+1	+1	−1	+1
2	0	$+\alpha$	0	0	0
3	0	0	$+\alpha$	0	0
4	+1	−1	+1	+1	−1
5	$+\alpha$	0	0	0	0
6	0	0	0	0	$-\alpha$
7	+1	+1	+1	+1	+1
8	0	0	0	0	0
9	+1	−1	+1	−1	+1
10	0	0	0	0	0
11	0	0	0	$+\alpha$	0
12	−1	−1	−1	−1	+1
13	0	0	$-\alpha$	0	0
14	0	0	0	0	$+\alpha$
15	+1	+1	−1	−1	+1
16	−1	−1	+1	+1	+1
17	$-\alpha$	0	0	0	0
18	+1	+1	+1	−1	−1
19	0	0	0	0	0
20	+1	−1	−1	+1	+1
21	−1	+1	−1	+1	+1
22	−1	−1	−1	+1	−1
23	0	0	0	0	0
24	0	0	0	0	0
25	+1	+1	−1	+1	−1
26	0	$-\alpha$	0	0	0
27	0	0	0	$-\alpha$	0
28	−1	+1	−1	−1	−1
29	0	0	0	0	0
30	−1	+1	+1	+1	−1
31	−1	−1	+1	0	−1
32	+1	−1	−1	0	−1

Table 3. pH Adjustment Using Different Concentration of HCl and NaOH

pH	1	2.36	3.75	5.15	6.5
HCl (%)	1.00	0.50	0.50	0.20	0.20
NaOH (g/100 mL)	0.05	0.12	0.20	0.15	0.25

In order to identify the maximum residue limit of EDTA, a preliminary study was done on EDTA absorption in fish tissue. The maximum addition limit in seafood is 250 mg/kg.[41] A 20 g sample of homogenized fish tissue was extracted with four 20 mL portions of water (by mixing with water for 10 min). After each extraction, samples were centrifuged for 15 min at 4000 rpm. The collected supernatants then were combined in a separating funnel and washed with 100 mL of ethyl ether. The aqueous layers were collected in a graduated cylinder, and the volume was recorded. A 20 mL aliquot of this solution was diluted with 1 mL of $CuSO_4$ solution and made to 25 mL with water. Samples were then centrifuged to precipitate (15 min at 4000 rpm) the proteins. Supernatants were finally filtered and injected to HPLC-PDA for EDTA analysis.

EDTA was analyzed using high performance gas chromatography (Waters, Milford, MA, USA), connected with a photodiode-array detection detector (model 486, Waters, USA). A C-18 column (4.6 × 250 mm) was used to detect EDTA. The mobile phase consisted of 0.02 phosphate buffer solution (pH 2.4, 0.175 mM), tetrabutylammonium bromide (TBAB), and 5% acetonitrile. The flow rate was 1 mL/

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Table 4. Design Matrix, Experimental Values, and Predicted Values in the Screening Design for Mercury Reduction

						mercury level (μg/g)		mercury removal (%)	
run order	pH	cys (%)	EDTA (mg/L)	NaCl (%)	time (min)	before expt	after expt	exptl	predicted
1	2.38	1.88	412.5	0.25	23.75	1.35	0.57	58.2	57.9
2	3.75	2.50	275.0	0.50	17.50	1.92	0.41	78.9	77.9
3	3.75	1.25	550.0	0.50	17.50	0.99	0.38	61.6	61.1
4	5.13	0.63	412.5	0.75	11.25	1.04	0.48	53.4	54.0
5	6.50	1.25	275.0	0.50	17.50	0.80	0.41	48.1	46.7
6	3.75	1.25	275.0	0.50	5.00	1.56	0.46	70.5	70.3
7	5.13	1.88	412.5	0.75	23.75	1.26	0.51	59.8	60.8
8 CP[a]	3.75	1.25	275.0	0.50	17.50	1.40	0.13	90.4	90.0
9	5.13	0.63	412.5	0.25	23.75	1.10	0.50	54.3	54.2
10 CP	3.75	1.25	275.0	0.50	17.50	0.93	0.10	89.4	90.0
11	3.75	1.25	275.0	1.00	17.50	0.57	0.15	73.1	72.1
12	2.37	0.63	137.5	0.25	23.75	0.69	0.15	78.3	77.1
13	3.75	1.25	0.0	0.50	17.50	0.73	0.22	70.1	70.8
14	3.75	1.25	275.0	0.50	30.00	1.85	0.48	73.9	74.3
15	5.13	1.88	137.5	0.25	23.75	1.02	0.44	56.7	56.8
16	2.38	0.63	412.5	0.75	23.75	1.11	0.37	66.5	66.2
17	1.00	1.25	275.0	0.50	17.50	1.51	0.43	71.3	72.9
18	5.13	1.88	412.5	0.25	11.25	1.10	0.40	63.3	63.9
19 CP	3.75	1.25	275.0	0.50	17.50	1.93	0.18	90.6	90.0
20	5.13	0.63	137.5	0.75	23.75	0.92	0.44	52.0	52.1
21	2.38	1.88	137.5	0.75	23.75	0.77	0.15	80.4	80.3
22	2.38	0.63	137.5	0.75	11.25	0.68	0.23	66.3	65.8
23 CP	3.75	1.25	275.0	0.50	17.50	1.69	0.15	91.3	90.0
24 CP	3.75	1.25	275.0	0.50	17.50	1.45	0.16	89.2	90.0
25	5.13	1.88	137.5	0.75	11.25	1.37	0.57	58.7	59.6
26	3.75	0.00	275.0	0.50	17.50	1.59	0.61	62.0	63.1
27	3.75	1.25	275.0	0.00	17.50	1.50	0.49	67.2	68.3
28	2.38	1.88	137.5	0.25	11.25	1.21	0.22	81.9	81.4
29 CP	3.75	1.25	275.0	0.50	17.50	0.67	0.07	89.4	90.0
30	2.38	1.88	412.5	0.75	11.25	0.79	0.27	65.9	66.2
31	2.38	0.63	412.5	0.25	11.25	0.98	0.43	55.6	54.9
32	5.13	0.63	137.5	0.25	11.25	0.73	0.41	43.9	43.6

[a]CP: Center point.

min, and UV detection was 257 nm. Ten microliters of sample was injected to HPLC.

Verification of the Model. Optimal conditions for maximum mercury removal depending on cysteine concentration, EDTA concentration, salt concentration, exposure time, and pH were obtained using RSM. The fish fillet was treated with the optimal conditions, and the mercury reduction was determined. The experimental and predicted values were compared in order to determine the validity of the model.

The single response optimization was carried out to visualize the significant ($p < 0.05$) interaction effects of independent variables on the response variables and to predict the levels of targeted variables resulting in the maximum removal of mercury content of fish flesh.

Recovery, Limit of Detection (LOD), and Limit of Quantification (LOQ). Recovery studies were made in order to detect mercury losses or contamination during sample treatment and matrix interferences during the measurement step. Recovery of total mercury at lower levels was determined by spiking 5, 10, and 15 ng of mercury to digested samples of fish fillet. The resulting solutions were analyzed for mercury concentration. The reliability of the analytical methods was tested by measuring mercury in reference material (CRM 463-total mercury and methylmercury in tuna fish) in seven replications. Recovery of EDTA was determined by spiking 100, 200, 400, and 600 mg/kg of EDTA to blended samples of fish fillet.

The limit of detection (LOD) and limit of quantification (LOQ) for total mercury and EDTA were determined by serial dilutions of the lowest calibrator concentration and established at a ratio of signal/noise (S/N) > 3 and signal/noise (S/N) > 10, respectively.

Statistical Analysis. A second-order polynomial equation was developed to study the effects of the variables on the mercury removal yields in terms of linear, quadratic, and cross product terms. The equation is of the general form (eq 1)

$$Y = A_0 + \sum_{i=1}^{N} A_i X_i + \sum_{i=1}^{N} A_{ii} X_i X_i + \sum_{i=1}^{N-1} \sum_{j=i+1}^{N} A_{ij} X_i X_j \quad (1)$$

where Y is the mercury reduction yield (%), X_i is the variable, A_0 is a constant term; A_i are the coefficients for the linear terms, A_{ii} are the coefficients for the quadratic terms, A_{ij} are the coefficients for the cross product terms, and N is the number of variables.

Analysis of variance (ANOVA) was performed to evaluate significant differences between independent variables, to determine regression coefficients and statistical significance of model terms and fitting the mathematical models to the experimental data. Multiple regression coefficients were determined by employing the least-squares technique to predict linear and quadratic polynomial models for the response variables studied. To visualize the relationships between the responses and the independent variables, surface response and counter plots of the fitted polynomial regression equations were generated. The experimental design matrix, data analysis, and optimization procedure were performed using the Minitab v. 13.2 statistical package (Minitab Inc., PA, USA).

The mathematical models were obtained by applying Minitab program to perform the multivariate regression analysis on the mercury removal data for each design point. These second-order equations quantitatively describe the relationship between the

responses and independent variables, so that mercury reduction can be predicted at any point within the factor domain, even though that point has not been included in the design.

The ANOVA results present the effect and regression coefficients of individual linear, quadratic, and interaction terms that were individually determined. The significance of the equation parameters for each response variable was also assessed by *F*-ratio at a probability (p) of 0.05. The adequacy of the models was determined using model analysis, coefficient of determination (R^2) analysis.

RESULTS AND DISCUSSION

The reliability of the analytical method tested by measuring total mercury in reference material was determined to be 96.54%. The concentration of total mercury in the reference material was reported to be 2.85 μg/g. The recovery for total mercury and EDTA by spiking was measured to be between 89 and 113% and between 91 and 105%. The detection limit (LOD) was 1.1 ng/g and 0.02 μg/g for total mercury and EDTA, respectively. The quantification limit (LOQ) was found to be 3 ng/g and 0.05 μg/g for total mercury and EDTA, respectively.

Fitting the Response Surface Models. The actual set of experiments undertaken as per CCD with uncoded values, the mercury reduction yields obtained, and the mercury reduction yields predicted by the design is given in Table 4. The experimental data fitted the second-order polynomial equation well as indicated by an R^2 value of 0.997 (Table 5). The final predictive equation is

$$\begin{aligned} Y = {} & 90.0267 - 6.5563X_1 + 3.6854X_2 - 2.4271X_3 \\ & + 0.9437X_4 + 0.9979X_5 - 7.5642X_1X_1 - 4.8767X_2X_2 \\ & - 6.0267X_3X_3 - 4.9517X_4X_4 - 4.4392X_5X_5 \\ & + 0.9531X_1X_2 + 5.0094X_1X_3 + 0.0406X_1X_4 \\ & - 0.6406X_1X_5 - 1.2406X_2X_3 - 0.0844X_2X_4 \\ & - 2.9156X_2X_5 + 1.0969X_3X_4 - 0.9969X_3X_5 \\ & - 0.7219X_4X_5 \end{aligned} \quad (2)$$

As shown in eq 2, the second-order polynomial regression equation (full quadratic) was fitted for predicting the mercury removal.

Analysis of variance (ANOVA) was used to evaluate the significance of the coefficients of the quadratic polynomial models (Table 5). For any of the terms in the models, a small *p*-value would indicate a more significant effect on the respective response variables. Thus, the variable with the largest effect on the mercury removal was the linear term pH, cysteine, EDTA, and salt ($P < 0.05$).

Interpretation of Response Surface Model. To visualize the effect of the independent variables on mercury removal, surface response of the quadratic polynomial models was generated by varying two of the independent variables within the experimental range while holding the other two constant at the central point.

Figure 1a shows the surface plot where the mercury removal increased as NaCl concentration increased up to 0.5% and exposure period up to 18 min, and it declined by increasing these two factors thereafter. Figure 1b shows the interaction effects of EDTA and time where mercury reduction increased with raising concentration of EDTA and time to 275 mg/L and 18 min.

The interaction between EDTA and salt (Figure 1c) produced the highest mercury reduction of 83% at EDTA and salt concentration of 340 mg/L and 0.5%, respectively. The interaction effects of cysteine and exposure time on mercury removal are shown in Figure 1d. As it is shown in Figure 1e,g, the interaction effects of cysteine with salt and EDTA on mercury removal were weak. The highest mercury removal can be achieved at cysteine concentration of 1.25% and 18 min of exposure time. Figure 1f shows that mercury elimination increased as pH was raised from 1 to 3.75. Figure 1h also shows the best level of pH and salt to reduce mercury in fish fillet.

The Main Effect of Independent Variables. The application of response surface methodology (RSM) allowed us to study the main and possible interaction effects between mercury reducing agents. The main effects of the five target variables as well as their quadratic effects were found to be significant ($p < 0.05$). Therefore, they all should be considered as important controlling factors for reducing mercury level. When the effects of variables were compared, cysteine and EDTA were found more effective on mercury reduction (Table 5). As Figure 1 shows, removal of mercury is increased by increasing the concentration of cysteine to 1.25% and it has a negative effect thereafter.

Various studies have used cysteine solution to eliminate mercury from fish and seafood.[26,31,33,34,37−39] Aizpurua et al. [26] claimed that there is no significant reduction of mercury from

Table 5. Analysis of Variance of the Regression Coefficients of the Fitted Quadratic Equations for Mercury Reduction

variable[a]	regression coeff	*P*-value
a_0	90.027	0.004[b]
	Linear	
a_1	−6.556	0.011[b]
a_2	3.685	0.001[b]
a_3	−2.427	0.000[b]
a_4	0.944	0.025[b]
a_5	0.998	0.025[b]
	Quadratic	
a_{11}	−7.564	0.001[b]
a_{22}	−4.877	0.000[b]
a_{33}	−6.027	0.001[b]
a_{44}	−4.952	0.000[b]
a_{55}	−4.439	0.000[b]
	Interaction	
a_{12}	0.953	0.014[b]
a_{13}	5.009	0.001[b]
a_{14}	0.041	0.903[c]
a_{15}	−0.641	0.075[c]
a_{23}	−1.241	0.003[b]
a_{24}	−0.084	0.801[c]
a_{25}	−2.916	0.000[b]
a_{34}	1.097	0.006[b]
a_{35}	−0.997	0.011[b]
a_{45}	0.722	0.049[c]
R^2	0.997	
R^2(adj)	0.991	
regression (*F*-value)		0.000[b]

[b]Significant ($p < 0.05$). [c]Not significant ($p > 0.05$). [a]a_0 is a constant; a_i, a_{ii}, and a_{ij} are the linear, quadratic, and interactive coefficients of the quadratic polynomial equations, respectively.

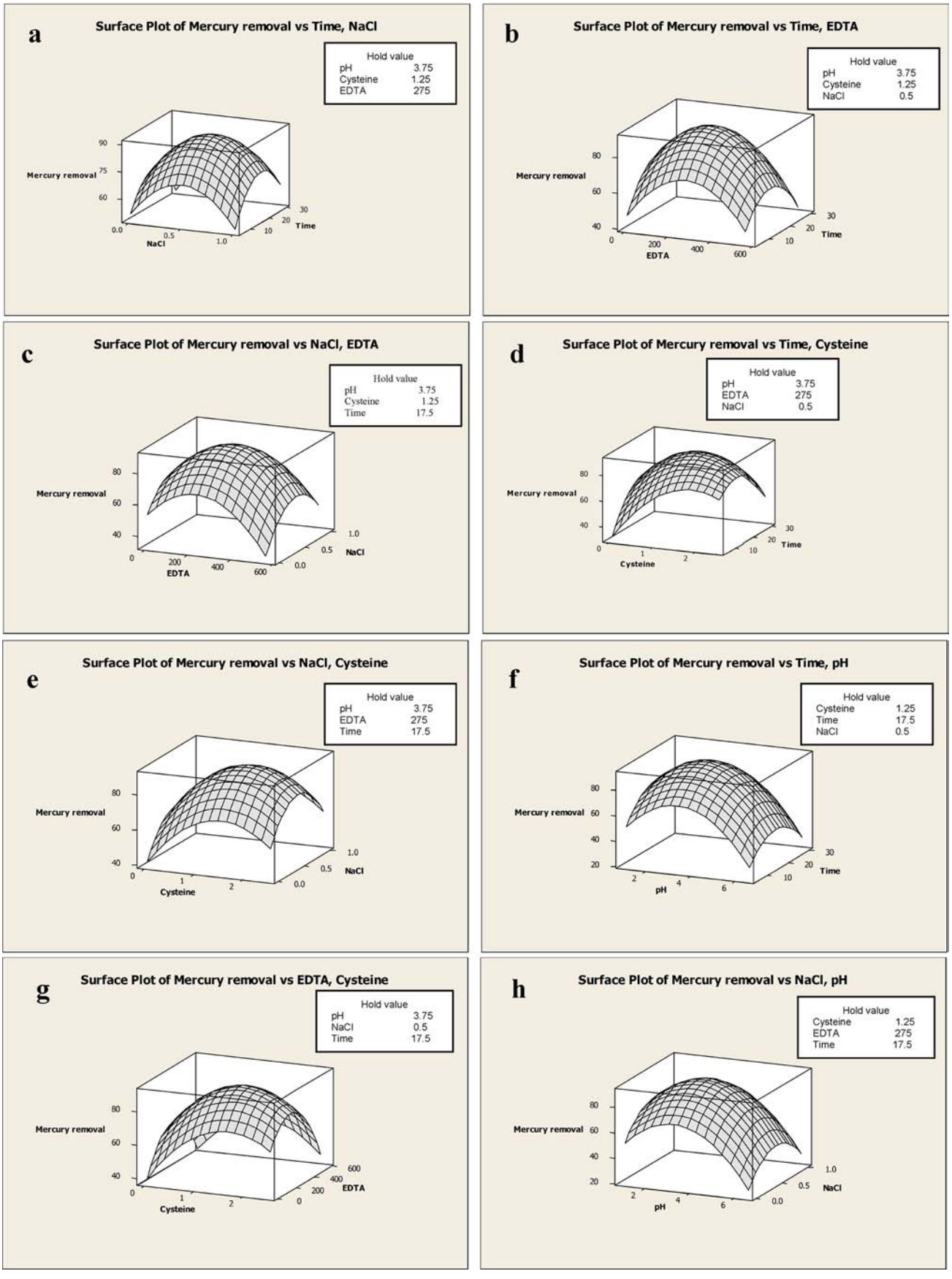

Figure 1. continued

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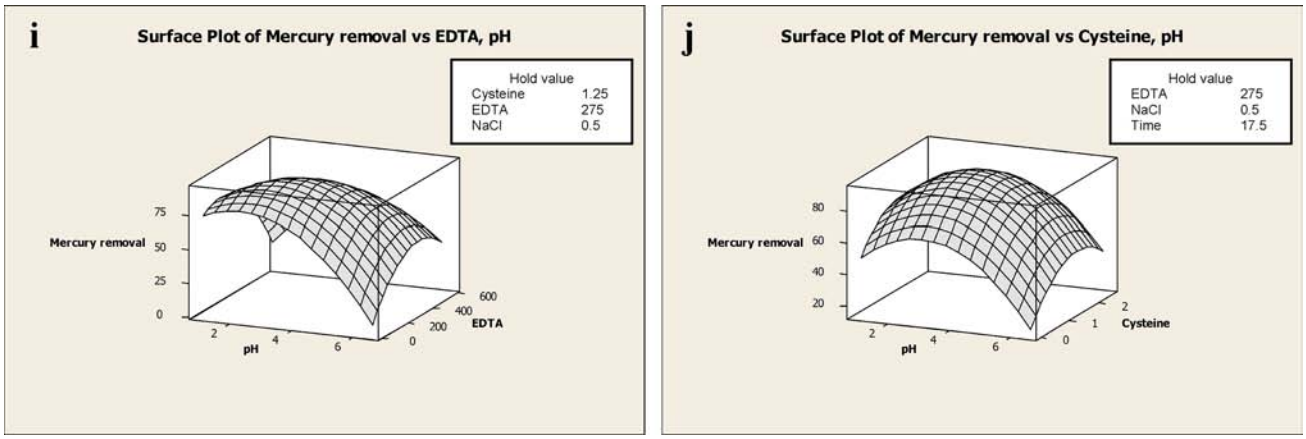

Figure 1. Three-dimensional response surface showing the effect of the pH, cysteine concentration, salt concentration, EDTA concentration, and exposure time on removal of mercury in fish fillet. Response surface plots for mercury removal as a function of (a) salt and time, (b) EDTA and time, (c) EDTA and salt, (d) cysteine and time, (e) cysteine and salt, (f) pH and time, (g) cysteine and EDTA, (h) pH and salt, (i) pH and EDTA, (j) pH and cysteine.

slices of blue shark using a 0.5% cysteine solution. The efficiency of 0.5% cysteine at pH 7.0 in removing mercury from minced shark was 40−45%. However other authors have shown a significant reduction in mercury in sliced fish following treatment with cysteine. Lipre[34] immersed cod fillets in 0.1% and 1.0% cysteine solutions (1:2) for 24 h, reducing the mercury by 40 and 44%, respectively. Yannai and Saltzman[39] treated precooked slices of tuna, 3 cm thick with 0.33% cysteine (1:7.5) at pH 2.0−2.2 for 24 h, removing 55% of the mercury. At a lower pH level (pH 1.5) 79% was removed. Schab et al.[33] removed 47% of the mercury of sliced precooked yellow tuna 2−3 cm thick, using 0.1% cysteine (1:3.3) at pH 2.2 for 1 h. Some of those reports did not involve sliced tissue (fillets or steaks) but only shredded, minced, or comminuted fish tissue where one would expect higher mercury removal than in fillets or steaks because of the cysteine solutions reaching more surface area. Mercury readily reacts with proteins and the sulfur atom of the sulfhydryl group in cysteine. The nitrogen (N) atom of the amino (NH_2) group and the oxygen (O) atom of the carboxyl (COOH) group also provide good ligands to mercury.[42]

Extracting solutions containing cysteine have been used above pH 4.5 by Spinelli et al.[37] and above pH 1.4 (i.e., below 0.040 N acidity) by Ohta et al.[31] while Schab et al.[33] claim that, for cysteine solutions, it appears that there is no advantage to lowering the pH level below 0.5. According to them it is presumably because strongly protonating the sulfur in the sulfhydryl groups blocks their ability to capture mercury ions. In this study the best pH to remove mercury was 3.75. Lower pH and higher pH have negative effects on mercury reduction. However lowering the pH results in softening and swelling of the fish flesh, which is not practicable. Lowering the pH dissociates the binding between CH_3−Hg and S− group in the protein.[42] The influence of pH on molecular binding site showed that of the three sites available for coordination only the thioether is bound at pH < 2 where the carboxylate and amino site are protonated. As the pH is raised the CH_3Hg^+ ion migrates to the COO− and NH_2 groups with the latter being strongly favored above pH = 8. The Hg−N bond length and the corresponding Hg−N bond distance in $[CH_3Hg(py)]NO_3$ prove unambiguously that methylmercury is capable of strong bonding to amine groups. It also influences protein chemistry via non-sulfhydryl interactions. In the pH range 3.5−8 competitive binding to COO− and $-NH_2$ is apparent while for 1 < pH < 3.5 the dominant species is $CH_3HgOOCCH_2{}^+NH_3$ and for 8 < pH < 12 most of the methymercury is in the form $CH_3Hg^+NH_2CH_2COO^-$.[43,44]

EDTA also showed significant impact on mercury elimination in fillets of short-bodied mackerel. Figure 1 shows that the highest mercury reduction can be achieved by EDTA concentration at 275 mg/L. Nevertheless Okazaki et al.[30] stated that EDTA yielded no significant effect on mercury removal from shark flesh. The degree of EDTA−mercury complex formation normally depends upon the hydrogen ion concentration or pH of the surrounding environment and the stability of the particular mercury−ligand complex. Effectively there is competition between the hydrogen ions and the metal ions in the system. A decrease in pH results in an increase in the deprotonation of EDTA and hence an increase in the concentration of the $(EDTA)^{4-}$ ion. Therefore more ligands are available for mercury to bond with.[45] The stability constant of EDTA with mercury (21.5) is higher than its stability with S− (21.2),[46] therefore more mercury ion can be chelated by EDTA.

Our research shows that salt can also significantly affect mercury removal in fish fillet. Aizpurua et al.[26] as well removed 40% mercury in minced shark treated by salt solution (0.1 M NaCl). This study managed to remove mercury up to 90% at 18 min exposure time with the proposed solution (Figure 1a−h, while other studies exposed the fish flesh for longer periods of time (24 h) and mercury reduction was 79%.[34,39]

The Interaction Effect of Independent Variables. In addition to the quadratic effects, the presence of significant ($p < 0.05$) interaction effects of independent variables in the amount of mercury reduced confirmed a potentially nonlinear relationship between the mercury chelating agents and the resulting mercury removal. Figure 1 exhibited how significant ($p < 0.05$) interaction effects of independent variables influenced the mercury reduction in fish fillet. The presence of curvature in 3D response surface plots could be interpreted by the quadratic effects of independent variables. Besides the quadratic effect, the presence of different curvature shapes presented in the release curves exhibited that mercury reduction was influenced not only by the main target variables but also by the type and individual characteristics of each reducing agent. Using more mercury chelating agents compared to the other studies helps

us to remove higher amounts of mercury contamination from fish flesh.

Optimization of Mercury Reduction in Fish Samples. The single response optimization was carried out to visualize the significant ($p < 0.05$) interaction effects of independent variables on the response variables and to predict the levels of targeted variables resulting in the maximum removal of mercury content in fish flesh. The overall optimal conditions leading to the maximum mercury removal (91.15 ± 0.73) were predicted to be obtained at combined level of 1.25% cysteine, 275 (mg/L) EDTA, 0.5% NaCl, 18.17 min, and pH of 3.75.

In conclusion, a method was proposed for reducing mercury in fish fillet using mixed solutions containing mercury absorbent agents. It can remove mercury from fish fillet up to 91%. Effects of pH, cysteine concentration, EDTA concentration, salt concentration, and time on mercury removal were found to be significant. Cysteine and EDTA were identified as the complexing agents with the greatest potential for use. Response surface methodology was successfully applied to optimize the mixed solution to eliminate mercury from fish fillet. The high coefficients of determination of the polynomial model showed that the model fitted the experimental data well. The mercury removing agents, including pH, cysteine concentration, EDTA concentration, salt concentration, and time, were optimized for better mercury removal from fish fillet. The optimal conditions were determined to be pH of 3.75, 1.25% cysteine, 275 (mg/L) EDTA, 0.5% NaCl, and 18.17 (min) exposure time. This proposed solution can be employed in fish industries to reduce mercury in highly contaminated fish, considering that the proposed mercury chelating agents, including hydrochloric acid, cysteine, and EDTA, are not home used food grade stuff. The protocol described here does not cause a considerable loss in protein due to protein denaturation. The solution does not have an adverse effect on nutrition and safety of the fish, as no toxic substances are used. The observations on the quality of treated fillet showed that this solution does not alter the aroma and color of fish. The applied mercury removing agents are all of food grade. Cysteine is a naturally occurring amino acid in the fish flesh. Ethylenediaminetetraacetic acid (EDTA) is permitted in a variety of foods including seafood.

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Notes

The authors declare no competing financial interest.

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