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**RESEARCH ARTICLE**

# Optimization of selected heavy metals removal from anchovies using trisodium citrate and peracetic acid as chelating agent

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**Abstract**

Anchovy is widely used in many dishes and are among the most consumed marine fish among Malaysians. Anchovies are one of the marine organisms that can accumulate pollutants including heavy metals. Numerous studies were conducted to minimize the heavy metals concentration in seafood such as various cooking and processing methods, and advanced treatments with the aid of chelating agents. Among the effective methods to remove heavy metals from seafood is by chelating techniques. Previous research conducted showed that the removal of heavy metals from seafood with nanomaterials such as chelating agents enhances the quality of the seafood with reduced heavy metals concentration as well as minimum purification costs. In this research, combination of trisodium citrate and peracetic acid were used as the chelating agent to perform a series of optimization treatments to remove toxic heavy metals including arsenic (As), lead (Pb), cadmium (Cd), and chromium (Cr). The optimization treatment was conducted by using Box-Wilson central composite design (CCD) which is a model in response surface methodology (RSM). Among the parameters that were optimized in this study are temperature between 29.0 to 37.0°C, pH between 3 to 7, treatment time between 5 to 60 minutes, and dosage of chelating agent between 1000 to 2000 mL/Kg. The anchovy samples were prepared according to a method adopted from AOAC 999.10 standard method for elements cadmium, lead, arsenic, and chromium. Inductively coupled plasma optical emission spectrophotometer (ICP- OES) was used to determine the heavy metals concentration in untreated and treated anchovy samples. Analysis of variance (ANOVA) showed all the four models designed are significant and influence the percentage removal of heavy metals. The Design-Expert software based on CCD RSM suggested a total of 97 solutions according to the four independent variables. Based on the solutions suggested, an optimized condition selected with highest desirability at 0.644 with percentage removal of arsenic at 77%, lead 39%, cadmium 54%, and chromium 18%.

**Keywords:** Anchovy, Chelating Agent, Response Surface Methodology, Analysis of Variance.

## INTRODUCTION

The marine organisms such as fishes, prawns, and cockles that are from coastal areas where high possibilities of industrial discharge could be found usually contain high amounts of heavy metal pollutants. Bioaccumulation of heavy metal contaminants in bivalve organisms would induce the initiation of toxic heavy metals elements entering the human food chain and frequent consumption of foods containing heavy metals could negatively affect human health including kidney and brain damage as well as carcinogenesis (Otchere, 2019).

The usage of chelating agents to remove heavy metal pollutants could overcome this issue. However, a suitable chelating agent is required that is safe to be used for seafood that will be consumed after the cleaning process. The chelating agent should be able to remove various heavy metals pollutants with reliable and sufficient concentration that the seafood is safe to be consumed with heavy metals concentrations according to the Codex Alimentarius collection of food standards (CODEX), Malaysian Food Act Regulation 1985 (Regulations, 1985), and Commission Regulation of EU (2006) (Van Der Meulen, 2018) permissible limit.

This research work is aimed to prepare a functional seafood wash that can remove heavy metal contamination from the selected aquatic organisms. Firstly, the content of heavy metals in anchovy was analyzed. The next objective of this research is to optimize the treatment for heavy metals removal from anchovy using response surface methodology (RSM), and analyze the percentage removal of the heavy metals from anchovy.

The project was first begun with the purchase of anchovy. The anchovy samples were washed with ultra-pure water and dried until constant weight obtained. Microwave digestion of the anchovy samples were conducted with concentrated nitric acid and the heavy metals concentration of lead, cadmium, arsenic, and chromium were detected using Inductively coupled plasma optical emission spectrophotometer (ICP-OES) to identify the concentration of heavy metals present in the anchovy samples before treatment. The optimization of the treatment process involved the time used for the treatment, temperature, pH, and the chelating agent dosage. The anchovy samples with various factors stated were analyzed to determine the optimum condition for the treatment to be conducted for maximum reduction of heavy metals.

This study effectively determined the optimization of the treatment needed for anchovy samples to remove the heavy metals pollutants by achieving the permissible limit set by the Codex Alimentarius collection of food standards (CODEX), Malaysian Food Act, and Regulation 1985 as shown in Table 1 (Van Der Meulen, 2018; Regulations, 1985; Committee, 2015). The successful formulation of the chelating agent could synthesize a solvent that can be even used in households or commercially as a seafood wash to remove heavy metal pollutants and bacteria quickly before cooking.

**Table 1.** Food standards of heavy metals permissible limit in seafood

| Heavy Metals | Standards | | |
|---|---|---|---|
| | Commission Regulation of EU | Malaysian Food Act Regulation 1985 | Codex Alimentarius collection of food standards (CODEX) |
| Pb (μg/g) | 1.50 | 2.00 | 2.00 |
| As (μg/g) | 1.00 | 1.00 | 1.00 |
| Cd (μg/g) | 1.00 | 1.00 | 2.00 |
| Cr (μg/g) | 0.50 | 1.00 | 0.50 |

## MATERIALS AND METHODS

### Sampling of Anchovy

Anchovy was purchased freshly from a wet market available in Taman Universiti, Skudai. The anchovy sample was kept clean in an airtight plastic bag and brought to the analytical laboratory at the Faculty of Science, Universiti Teknologi Malaysia (UTM). The sample was stored in a dry and clean place until further analysis was conducted.

### Chemicals and Reagents

The reagent tested in this study was Molluscure® Seafood Wash which contains a combination of chelating agents including trisodium sulphate dihydrate and peracetic acid. 65% of nitric acid, HNO3 was used in the digestion process of the anchovy samples. The calibration was done by using standard solutions of lead, arsenic, cadmium, antimony, and chromium. Sodium hydroxide NaOH (1 M) was used to adjust the pH value of the chelating agent.

### Instrument and Apparatus

Inductively coupled plasma optical emission spectrophotometer (Avio® 200 ICP Optical Emission Spectrometer) was used for heavy metal detection. A pH meter was used to examine the pH of the chelating agent solution and identify the effect of pH on the heavy metal removal from the anchovy sample. Additionally, a thermometer was used to adjust the temperature needed for the chelating agent. Besides, glassware apparatus such as beakers, test tubes, volumetric flasks, measuring cylinders, droppers, pipettes were used during the experiment. Microwave digester (Titan MPS Microwave Sample Preparation System) was used for complete digestion of the sample. An analytical balance was used for accurate measurement of weight of samples and chemicals.

### Sample Preparation

The anchovy samples were washed with ultra-pure water. The sample was dried in an oven at temperature 50°C until a constant mass was obtained. Before analysis, the composite sample of each species was prepared by mixing and grinding homogeneously the prepared samples. Microwave digestion method adopted from AOAC 999.10 standard method was selected to perform for elements cadmium and lead and in house method based on AOAC 999.10 for elements arsenic and chromium. 0.5 g of the total weight of homogeneously grinded anchovy sample was undergone microwave digestion with 5 mL of analytical grade 65% nitric acid (HNO3) and 2 mL hydrogen peroxide (H2O2). After complete digestion of the sample, a clear solution was formed, and the sample was cooled. The sample solution was then diluted to 25 mL volumetric flask with deionized water. The sample is prepared for heavy metal analysis to detect lead, arsenic, cadmium, and chromium using ICP-OES.

### Preparation of Chelating Agent Solution

The chelating agents that were used for the treatment of the anchovy sample in this study are the combination of trisodium citrate dihydrate, C6H5Na3O7.2H2O and peracetic acid, CH3CO3H. These edible chelating agents were prepared at different dosage to determine an optimized concentration for maximum heavy metal removal from the sample. Three different dosages of the chelating agents were prepared at 1000 mL/Kg, 1500 mL/Kg, and 2000 mL/Kg. Each different dosage of the chelating agent was prepared based on the constant mass of sample used which was 30 g and the volume of the chelating agent changed from 30 mL to 60 mL to obtain the dosage from 1000 mL/Kg to 2000 mL/Kg.

### Heavy Metals Analysis Using ICP-OES

Following the microwave digestion, heavy metals analysis of the anchovy samples was conducted by using Inductively coupled plasma optical emission spectrophotometer (ICP-OES). ICP- OES was used in this experiment due to its low detection limit up to part per million (ppm) and could detect multiple elements in a solution. ICP multi element standard solution was used as the standard solution for calibration purposes.

### Optimization of Treatment to Remove Heavy Metals from Anchovy

The heavy metals in anchovy samples were removed by chelation method using the combination of trisodium citrate dihydrate, C6H5Na3O7.2H2O and peracetic acid, CH3CO3H. An optimum treatment condition to determine suitable pH, chelating agent concentration, temperature, and treatment time to remove heavy metal pollutants and bacteria to meet the permissible limit of the food standards. Hence, a series of experiments was done by alternating the four factors as shown in Table 2.

**Table 2.** Experimental parameters for heavy metals removal

| Experimental Parameter | Experimental Variable |
|---|---|
| Dosage of Chelating Agent (mL/Kg) | 1000 to 2000 |
| Treatment Time (Minute) | 5 to 60 |
| Temperature (°C) | 29.0 to 37.0 |
| pH of Solution | 3 (Acidic) to 7 (Neutral) |

### Method Validation

Limit of detection (LOD) can be defined as the lowest amount or concentration of a component that can be effectively differentiated from the limit of blank (LOB) that represents the concentration below than 95% of measurement signals detected when continuously measuring a blank sample. Limit of quantitation (LOQ) defined as the least amount or the lowest concentration of a compound which able to be determined with given analytical procedure and established accuracy, precision, and uncertainty [27]. Both LOD and LOQ was calculated based on the standard deviation, of the standard solution used to obtain the calibration equation.

## RESULTS AND DISCUSSION

### Determination of Limit of Detection and Quantitation

Table 3 below shows the LOD and LOQ obtained for arsenic (As), lead (Pb), cadmium (Cd), and chromium (Cr) based on the calibration curve plotted from the standard solutions used in ICP-OES.

**Table 3.** LOD and LOQ of heavy metals

| Elements | Standard Deviation | LOD (ppm) | LOQ (ppm) | RSD (%) |
|---|---|---|---|---|
| As | 0.0031 | 0.010 | 0.031 | 1.05 |
| Pb | 0.0052 | 0.017 | 0.052 | 1.73 |
| Cd | 0.0012 | 0.004 | 0.012 | 0.41 |
| Cr | 0.0022 | 0.007 | 0.022 | 0.75 |

### Concentration of heavy metals in untreated and blank solution anchovy

The table 4 below shows the concentration (mean ± standard deviation) of heavy metals in untreated anchovy replicate samples obtained using ICP-OES.

**Table 4.** Concentration of heavy metals in untreated anchovy samples

| Heavy Metals (ppm) | As | Pb | Cd | Cr |
|---|---|---|---|---|
| Sample 1 | 2.578 | 1.146 | 0.621 | 0.837 |
| Sample 2 | 2.366 | 1.132 | 0.636 | 0.84 |
| Sample 3 | 2.535 | 1.145 | 0.627 | 0.844 |
| mean ± standard deviation | 2.493 ± 0.092 | 1.141 ± 0.006 | 0.628 ± 0.006 | 0.84 ± 0.003 |

Table 5 shows the concentration (mean ± standard deviation) of heavy metals treated with blank solution anchovy replicate samples obtained using ICP-OES.

**Table 5.** Concentration of heavy metals in anchovy in ultra-pure water treatment

| Heavy Metals (ppm) | As | Pb | Cd | Cr |
|---|---|---|---|---|
| Replicate 1 | 1.188 | 0.961 | 0.467 | 0.772 |
| Replicate 2 | 1.196 | 0.975 | 0.457 | 0.773 |
| Replicate 3 | 1.195 | 0.989 | 0.48 | 0.786 |
| mean ± standard deviation | 1.193 ± 0.004 | 0.975 ± 0.011 | 0.468 ± 0.009 | 0.777 ± 0.006 |
| Percentage Removal (%) | 52.15 | 14.55 | 25.47 | 7.5 |

## Treatment of Anchovy by Using Chelating Agent

Combination of trisodium citrate and peracetic acid solution were used to treat the anchovy samples. The experiments in this research were designed using Box-Wilson central composite design (CCD) by response surface methodology with the aid of Design Expert software version 11. Four independent variables or parameters were inserted in this software to design the experiments including temperature (A), pH (B), treatment time (C), and dosage of chelating agent (D). There were four responses set in the software which are the percentage removal of arsenic, lead, cadmium, and chromium. Based on the designed experiment from CCD-RSM, a total of 30 experiments were proposed to obtain the optimized condition based on the four independent variables stated.

## Analysis of Variance (ANOVA) for the Removal of Arsenic

The percentage removal of As from anchovy samples has been analyzed by analysis of variance (ANOVA) with a default 5% level of significance. The model based on ANOVA was significant with p- value at <0.0001 which is less than 0.05 significant level that supports the quadratic model proposed which describes the correlation between response and independent variables. The F-value is 27.33 which is greater than tabulated F0.05 (13,16) at 2.60 shows that the null hypothesis is denied. Based on the fit statistics from the RSM, the R2 value obtained from the generated quadratic model is 0.9569 that shows there is a good relationship between predicted and actual values.

## Analysis of Variance (ANOVA) for the Removal of Lead

The percentage removal of Pb from anchovy samples has been analyzed by analysis of variance (ANOVA) with a default 5% level of significance. The model based on ANOVA was significant with p- value at <0.0001 which is less than 0.05 significant level that supports the quadratic model proposed which describes the correlation between response and independent variables. The F-value is 13.13 which is greater than tabulated F0.05 (14,15) at 2.40 shows that the null hypothesis is denied. Based on the fit statistics from the RSM, the R2 value obtained from the generated quadratic model is 0.9246 that shows there is a good relationship between predicted and actual values.

## Analysis of Variance (ANOVA) for the Removal of Cadmium

The percentage removal of Cd from anchovy samples has been analyzed by analysis of variance (ANOVA) with a default 5% level of significance. The model based on ANOVA was significant with p- value at <0.0001 which is less than 0.05 significant level that supports the quadratic model proposed which describes the correlation between response and independent variables. The F-value is 57.21 which is greater than tabulated F0.05 (14,15) at 2.40 shows that the null hypothesis is denied. Based on the fit statistics from the RSM, the R2 value obtained from the generated quadratic model is 0.9816 that shows there is a good relationship between predicted and actual values.

### Analysis of Variance (ANOVA) for the Removal of Chromium

The percentage removal of Cr from anchovy samples has been analyzed by analysis of variance (ANOVA) with a default 5% level of significance. The model based on ANOVA was significant with p-value at <0.0001 which is less than 0.05 significant level that supports the quadratic model proposed which describes the correlation between response and independent variables. The F-value is 10.61 which is greater than tabulated F0.05 (14,15) at 2.40 shows that the null hypothesis is denied. Based on the fit statistics from the RSM, the R2 value obtained from the generated quadratic model is 0.9083 that shows there is a good relationship between predicted and actual values.

### Optimization of Removal of Heavy Metals from Anchovy

The optimization of removal of heavy metals from anchovy was demonstrated according to the numerical values of variables suggested by the Design-Expert software based on CCD RSM for all the responses combined. According to Table 6, the temperature was set to minimum in order to obtain the maximum removal rate of heavy metals at room temperature 29.0°C. Besides, pH was set to maximum at 7 in order to conduct the cleaning process at neutral pH that is safer for contacting. The treatment time was set to minimum at 5 minutes in order to reduce the waiting time for the cleaning process. The dosage of chelating agent was set to minimum level in order to reduce the cost and at the same time achieve a better removal rate of heavy metals. The level of importance set for removal of heavy metals is according to the highest content of heavy metals in the untreated sample. The importance level 5 shows highest priority to remove the heavy metal meanwhile importance level 2 shows lowest priority.

**Table 6.** Optimized parameter and range proposed by CCD RSM

| Constraints | Goal | Lower Limit | Upper Limit | Importance |
|---|---|---|---|---|
| Temperature (°C) | minimize | 29 | 37 | 3 |
| pH | maximize | 3 | 7 | 3 |
| Time (minute) | minimize | 5 | 60 | 3 |
| Dosage (mL/Kg) (%) | minimize | 1000 | 2000 | 3 |
| Removal of As (%) (%) | maximize | 49.9 | 100 | 5 |
| Removal of Pb (%) (%) | maximize | 17.8 | 53.6 | 4 |
| Removal of Cd (%) (%) | maximize | 46.5 | 65.8 | 3 |
| Removal of Cr (%) (%) | maximize | 5.6 | 28.6 | 2 |

The Design-Expert software based on CCD RSM suggested a total of 97 solutions according to the parameters in Table 6. Based on the solution suggested, an optimization condition selected with highest desirability at 0.644 is shown in Table 7 and Figure 1 with percentage removal of arsenic at 77%, lead at 39%, cadmium at 54%, and chromium at 18%.

**Table 7.** Suggested solution parameters and desirability

| Constraints | Solution suggested |
|---|---|
| Temperature (°C) | 29.718 |
| pH | 7.0 |
| Time (minute) | 34.192 |
| Dosage (mL/Kg) | 1000.004 |
| Removal of As | 77.176 |
| Removal of Pb | 39.030 |
| Removal of Cd | 54.460 |
| Removal of Cr | 18.726 |
| Desirability | 0.644 |

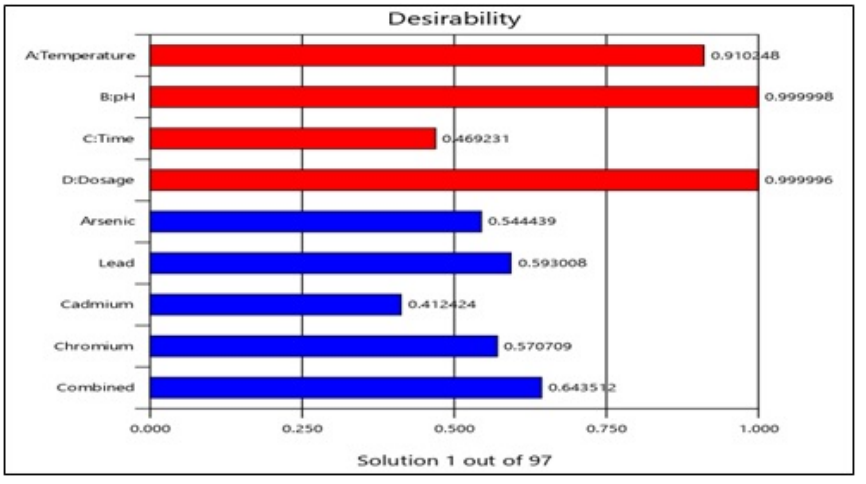


**Figure 1.** Suggested parameters and desirability of heavy metals removal.

## CONCLUSIONS

The responses inserted in the Design Expert software were the percentage removal of the heavy metal arsenic, lead, cadmium, and chromium. The analysis of variance (ANOVA) of each heavy metal shows that the models stimulated are significant and the four variables influence the heavy metals removal independently. The maximum percentage removal of arsenic was 100%, lead 53.6%, cadmium 65.8%, and chromium 28.6% at different combinations of parameters. Based on the suggested optimized conditions with desirability of 0.644 at temperature 29.7 °C, pH 7.0, treatment time 34.2 minutes, and chelating agent dosage 1000.0 mL/Kg, the percentage removal of arsenic was 77.18%, lead 39.03%, cadmium 54.46%, and chromium 18.73%. Similar experiments should be considered for other marine organisms that are affected by heavy metal pollutants such as prawns, oysters, and crabs to investigate the effectiveness of the chelating agents used in this research. Thus, a universal chelating agent solution or seafood wash could be created to remove the heavy metal pollutants from most of the marine organisms.

### Author Contribution

Pavitran Jaganazan: Conceptualization, writing draft, writing review dan editing; Faizuan B Abdullah: Data curation, formal analysis.

### Conflict of Interest

There is no conflict of interest in this study.

### Acknowledgment

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### Data Availability

We thank all respondents involved in this research project.

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