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Validation of Analytical Methods for Assessing Heavy Metal in Green Mussels (*Perna viridis*) from Major Mussel-Producing Regions in the Philippines

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Abstract **– Green mussels (*Perna viridis*) are an important marine resource in the Philippines, widely farmed for their economic and nutritional value. As filter feeders, they can accumulate heavy metals like cadmium (Cd), mercury (Hg), and lead (Pb), posing potential health risks. This study developed and validated gravimetric external calibration methods to quantify heavy metal levels in mussels from major producing regions using Graphite Furnace - Atomic Absorption Spectrophotometry (GF-AAS) for Cd and Pb, and Direct Mercury Analyzer (DMA) for Hg. Validation showed strong linearity (r > 0.995), with LOQs of 20.9 µg/kg (Hg), 175.9 µg/kg (Cd), and 208.7 µg/kg (Pb). Trueness was confirmed using NIST SRM for Oyster Tissue and spike recovery, with acceptable recovery ranges of 85 – 105 %. Measurement results revealed Hg and Pb levels in mussels were below regulatory limits, while Cd exceeded limits in one region. These methods support food safety monitoring and quality assurance in Philippine aquaculture.**

I. INTRODUCTION

Mussels are a significant aquaculture product in the Philippines, valued both economically and nutritionally. The primary species farmed in the Philippines is the Asian green mussel (*Perna viridis*), known locally as Tahong, which thrives in tropical and subtropical waters [1]. As a widely consumed seafood item, they provide a vital source of protein and livelihood for coastal communities. Mussel farming supports the livelihoods of coastal communities, with significant production in areas like Capiz, Cavite, Samar, and NCR. Their ease of cultivation and rapid growth have made them a staple in many regions, reinforcing their importance in the country's aquaculture industry.

However, due to their filter-feeding nature, green mussels are prone to accumulating contaminants from their surrounding environment, including heavy metals such as cadmium (Cd), mercury (Hg), and lead (Pb) [2]. These elements are persistent pollutants that can originate from natural sources as well as anthropogenic activities, including industrial discharges, mining, and agricultural runoff. Once introduced into marine ecosystems, these metals can be absorbed and concentrated in shellfish tissues, potentially leading to hazardous exposure levels in consumers.

A study was conducted in green mussels harvested around Manila Bay and reported the presence of Hg, Cd, and Pb in both dry and wet seasons [2]. Another study in 2003 reported the presence of Cd and Pb in green mussels harvested in Bacoor Bay, Cavite [3]. These findings on heavy metal contamination in seafood are a major public health concern. Chronic exposure to elevated levels of Cd, Hg, and Pb has been associated with serious health effects, including kidney damage, neurological disorders, and developmental issues [4]. The Philippine National Standard (PNS) for live and raw bivalve mollusks complies with the maximum levels of the Codex General Standard for Contamination and Toxins in Foods [5]. European Parliament published EC no. 1881/2006 which states the established maximum levels for certain contaminants in bivalve mollusks such as mussels, oysters, and scallops were regulated with the maximum allowable level for Hg, Cd, and Pb are 0.5 mg/kg, 1.0 mg/kg, and 1.5 mg/kg, respectively [6].

Accurate determination of heavy metal concentrations requires sensitive and validated analytical techniques. Graphite Furnace - Atomic Absorption Spectrophotometry (GF-AAS) is widely used for detecting trace levels of metals such as Cd and Pb, offering high sensitivity and specificity [7, 8]. Mercury, due to its volatile nature, is often quantified using Direct Mercury Analyzers (DMA), which allow for precise analysis without the need for extensive sample preparation [9]. Although these testing methods are available, they still need to be validated for specific matrices, such as green mussels, and for the contamination levels found in the Philippines..

This study addresses this need by optimizing and validating gravimetric external calibration methods using GF-AAS for Cd and Pb, and DMA for Hg in mussels. The validated methods were used to assess the heavy metal content in green mussels collected from major production regions in the Philippines. The results aim to support food safety monitoring programs and enhance quality assurance efforts in the local aquaculture industry.

II. MEASUREMENT METHOD

Reagents

All sample preparations were carried out using a gravimetric method, employing a *Mettler Toledo XSE205DU* semi-micro balance to ensure high precision and accuracy in weighing. The Type 1 ultrapure water utilized throughout the study was sourced from a *Millipore Milli-Q Integral 3* water purification system. Analytical-grade nitric acid (65 %) and hydrogen peroxide (30 %) were obtained from *Merck, Darmstadt, Germany*. Prior to use, the nitric acid underwent a double distillation process using the *Milestone DuoPur Acid Distillation System* to eliminate potential contaminants and ensure reagent purity. For calibration and quality control (QC) of analytical measurements, Standard Reference Materials (SRMs) certified by the National Institute of Standards and Technology (NIST), USA, were employed. Specifically, the calibration standards *NIST SRM 3133* for Hg analysis, *NIST SRM 3128* for Pb, and *NIST SRM 3108* for Cd determination and the matrix SRM *NIST SRM 1566b Oyster Tissue* for quality measures.

Sample Preparation

The preparation of mussel samples began by forcibly opening the shells to extract the soft tissues. The byssus, locally referred to as "balbas," was carefully removed before tissue collection to avoid contamination. Collected soft tissues were subsequently freeze-dried using a *Gamma 2-16 LSCplus Christ* freeze-dryer. The resulting freeze-dried material was milled and sieved. This series of steps yielded the final product: fine, freeze-dried mussel powder.

To quantify Cd and Pb concentrations in the mussels samples, a closed-vessel microwave digestion was performed using the *Milestone Ethos UP* system. The digestion process involved the addition of 9 mL concentrated double-distilled nitric acid to 0.50 g of sample. To ensure the effectiveness of digestion, samples were predigested for 2 hours at room temperature. After predigestion, 1 mL hydrogen peroxide was added and the microwave digestion was performed with the method parameters shown in Table 1. The method adhered strictly to the manufacturer's recommended procedure for mussel matrices, ensuring complete digestion of organic material and efficient release of trace metals for analysis. Digested samples were diluted to 25 g with type 1 water.

Table 1. Microwave Digestion System Program

Step	**Time (min)**	**Power (W)**	**Temperature (°C)**
1	15	1800	25 to 200
2	15	1800	200
cooldown	15		

Method Validation

The determination of heavy metal in lyophilized mussel tissue was done using *PerkinElmer PinAAcle 900T* GF-AAS for Cd and Pb analysis and *Milestone DMA-80* DMA for total Hg. The methods were validated following the single-laboratory validation approach outlined in the ISO/IEC 17025:2017 guidelines for method validation. These standards recommend assessing parameters such as linearity, precision, trueness, limit of detection (LOD), and limit of quantification (LOQ). Quantification of the analytes was carried out using external standard calibration, wherein a set of standards was analyzed to generate a calibration curve, which was then used to determine the concentrations of the samples.

Linearity was evaluated for each metal by analyzing a series of calibration standards across relevant concentration ranges. For Hg, nine (9) calibration standards were prepared with concentrations ranging from 10 to 50 µg/kg. An aliquot of 0.10 g from each standard was weighed into a nickel sample boat and analyzed in duplicate using DMA. For Cd, nine (9) calibration standards were prepared with concentrations from 0.50 to 3.50 µg/kg, while for Pb, six (6) calibration standards were prepared within the range of 1 to 25 µg/kg. Each Cd and Pb standard was weighed and analyzed in duplicate using GF-AAS. Calibration curves for Hg, Cd, and Pb were constructed by applying linear regression to the measured response data.

The precision of the method was evaluated through repeatability testing. Two mussel samples containing low and high concentration levels of Hg, Cd, and Pb were prepared and used for repeatability testing. Each metal in both samples was analyzed in seven sub-samples. From these, the mean, standard deviation, and percent relative standard deviation (% RSD) of the measurement results were calculated to assess consistency.

Trueness of the method for Cd and Hg determination was evaluated using *NIST SRM 1566b Oyster Tissue*, a certified reference material (CRM). For Cd analysis, ten replicates were analyzed following the same sample dissolution procedure applied to mussel tissue samples, while for Hg, the CRM was analyzed without sample preparation. For lead (Pb), method trueness was assessed through recovery by spiking, wherein blank mussel samples were spiked with Pb at 0.50 µg/kg and 6.60 µg/kg concentration levels. Spiked samples underwent the same preparation and analytical procedures as the test samples, and each level was analyzed in seven replicates. Recovery was calculated by comparing the measured concentrations to the CRM (for Cd and Hg) or known spiked values (for Pb), thereby providing an evaluation of method accuracy.

LOD refers to the lowest concentration at which the analyte can be detected by the instrument, though not accurately quantified, while LOQ is the lowest concentration at which the analyte can be both detected and reliably quantified. For the determination of the limits, a blank mussel sample was prepared and analyzed in ten replicates for Cd, Hg, and Pb. The limits were calculated based on the instrument responses obtained from these measurements. The method LOD and LOQ were calculated using the appropriate formula.

Sample collection and analysis of heavy metals in green mussels

Green mussels were collected from three of the Philippines' major mussel-producing regions, which include Samar (Region VIII), Capiz (Region VI), and Cavite (Region IV-A), to survey heavy metal content in mussel samples from these areas. In Samar and Capiz, three (3) distinct sampling stations with at least 2 sampling sites were visited as shown in Fig. 1 and 2. In Cavite, only 3 sampling sites (Fig. 3) were selected due to the availability of mussels ready for harvesting. At each site, mussel samples were collected in quantities ranging from 1 to 2 kg per station, with the help of local mussel farmers where applicable. Mussels purchased from Navotas and Taguig wet markets were also collected to assess public markets near coastal areas..

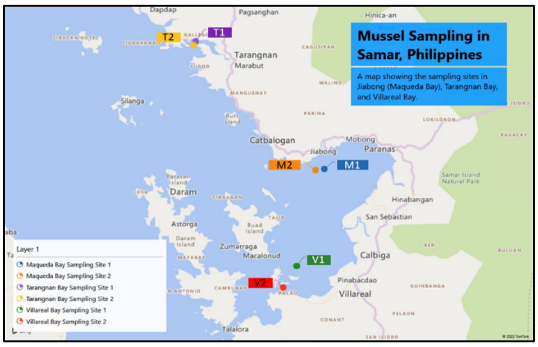

Fig. 1. Location of mussel sampling sites in Samar.

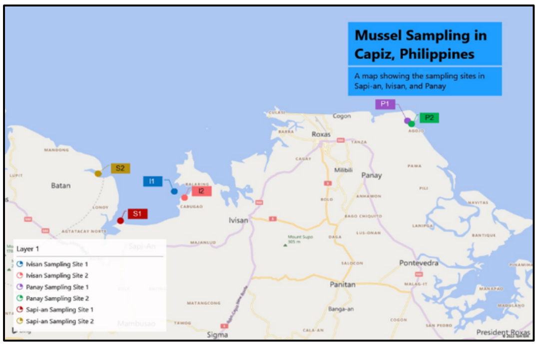

Fig. 2. Location of mussel sampling sites in Capiz.

Fig. 3. Location of mussel sampling sites in Cavite.

Following collection, the samples were processed following the procedure detailed in Section II.B of this study, which includes freeze-drying, homogenization, and sample preparation to ensure consistency and stability. The concentrations of Cd, Hg, and Pb in the prepared samples were then determined using the validated analytical methods.

III. RESULTS AND DISCUSSION

A. Method Validation for Hg in Mussels using DMA

The linearity assessment confirmed strong linear relationships for mercury detection with correlation coefficients (R) of 0.997, exceeding the acceptance criterion of R > 0.995. Residuals were randomly distributed, supporting the validity of the calibration curves. The method demonstrated high sensitivity with LOD and LOQ of 17.4 µg/kg and 20.9 µg/kg, respectively, using mussel samples with low mercury content. Trueness evaluation using NIST SRM 1566b yielded recoveries ranging from 89.2 % to 96.0 %, within the acceptable 80–110 % range, however, a slight negative bias was noted. Repeatability tests at 0.04 mg/kg and 1.81 mg/kg Hg levels showed % RSD values of 1.2 % and 1.1 %, respectively, indicating consistent performance across concentration ranges. The summary of the method validation results is presented in Table 2.

Table 2. Summary of Method Validation of Hg in Lyophilized Mussels using DMA

Parameters		Results	Acceptable Criteria
Linearity 10-50 µg/kg		R = 0.997	R ≥ 0.995
Repeatability (% RSD; n=7)	Low level	1. 2 %	< 17.3 %
	High level	1.1 %	< 9.8 %
Trueness (NIST SRM 1566b; n=10)		89.2-96.0 %	80-110 %
LOD (n=10)		17.4 µg/kg	-
LOQ (n=10)		20.9 µg/kg	-

B. Method Validation for Cd in Mussels using GF-AAS

The method for Cd determination in mussels demonstrated strong linearity, with a calibration curve built from five points yielding R of 0.998 over the range of 0.50 – 3.50 µg/kg, supported by a residual plot showing random distribution. Sensitivity parameters revealed a LOD of 2.2 µg/kg and LOQ of 175.9 µg/kg. Accuracy testing using SRM 1566b (2.48 mg/kg) produced recoveries between 85.86 % and 91.70 %, confirming acceptable trueness despite a slight negative bias likely due to sample weight loss. Precision was validated by repeatability tests at both 0.7 mg/kg and 4.0 mg/kg concentration levels, yielding % RSD of 3.3 % and 3.6 %, respectively, aligning with Horwitz criteria and demonstrating consistent method performance as shown in Table 3.

Table 3. Summary of Method Validation of Cd in Lyophilized Mussels using GF-AAS

Parameters		Results	Acceptable Criteria
Linearity 0.50 – 3.50 µg/kg		R = 0.998	R ≥ 0.995
Repeatability (% RSD; n=7)	Low level	3.3 %	< 11.4 %
	High level	3.6 %	< 8.7 %
Trueness (NIST SRM 1566b; n=10)		85.9-91.7 %	80-110 %
LOD (n=10)		2.2 µg/kg	-
LOQ (n=10)		175.9 µg/kg	-

C. Method Validation for Pb in Mussels using GF-AAS

The method for determining Pb in mussels showed strong linearity in the concentraion range of 1–25 µg/kg, with correlation coefficients of 1.000 exceeding the R > 0.995 criterion. Residual plots confirmed the appropriateness of the regression models with random distribution around zero. Sensitivity analysis using low-Pb samples indicated LOD and LOQ values of 142.2 and 208.7 µg/kg, respectively. Repeatability was confirmed with % RSD values of 10.1 % (0.3 mg/kg) and 2.9 % (5.0 mg/kg), which were within the Horwitz criteria. Recovery tests showed results within the AOAC-accepted range of 80–110%, with recoveries of 85.1–104.8 % for low and 87.9–100.1 % for high Pb concentrations, demonstrating the method's accuracy and reliability. The method validation results for Pb were summarized in Table 4.

Table 4. Summary of Method Validation of Pb in Lyophilized Mussels using GF-AAS

Parameters		Results	Acceptable Criteria
Linearity 1-25 µg/kg		R = 1.000	R ≥ 0.995
Repeatability (% RSD; n=7)	Low level	10.1 %	< 12.6 %
	High level	2.9 %	< 8.3 %
Recovery by Spiking (Low Level)		85.1-104.8 %	80-110 %
Recovery by Spiking (High Level)		87.9-100.1 %	80-110 %
LOD (n=10)		142.2 µg/kg	-
LOQ (n=10)		208.7 µg/kg	-

D. Assessment of Heavy Metals in Green Mussels from Samar, Capiz, and Cavite

A survey of mussel samples collected from various locations, including coastal sites in Samar (Region VIII), Capiz (Region VI), and Cavite (Region IV-A), was conducted to assess heavy metal contamination levels. Additional samples from Navotas and Taguig wet markets were included to assess public markets near coastal areas.

The measurement results were shown in Table 5, wherein Hg levels in all sampling sites were found to be below the maximum residue limit (MRL) of 0.50 mg/kg Hg, indicating compliance with safety standards. Pb was not detected in any of the samples. In contrast, Cd concentrations exceeded the maximum allowable limit of 1.0 mg/kg in samples obtained from Villareal and Jiabong, Samar. All other sites had cadmium levels below acceptable limits.

Table 5. Survey Results of Hg, Cd, and Pb Content in Mussels from Different Locations

Sampling station	Concentration (mg/kg)		
	Hg	Cd	Pb
Samar: Tarangnan	0.04	0.87	N.D.
Samar: Villareal	0.06	1.37	N.D.
Samar: Jiabong	0.05	1.17	N.D.
Capiz: Sapian	0.04	0.64	N.D.
Capiz: Ivisan	0.04	0.50	N.D.
Capiz: Panay	0.06	0.31	N.D.
Cavite 1	0.03	0.13	N.D.
Cavite 2	0.04	0.20	N.D.
Cavite 3	0.03	0.26	N.D.
Bicutan market	0.02	0.07	N.D.
Navotas market	0.01	0.09	N.D.

*N.D. = Not detected; below the detection limits

IV. CONCLUSIONS

The validated analytical methods for Hg, Cd, and Pb in mussel tissue demonstrated acceptable performance in terms of linearity, sensitivity, trueness, and precision, meeting international validation standards. The DMA showed high sensitivity and consistent recovery for Hg, while GF-AAS methods for Cd and Pb yielded robust calibration, acceptable detection limits, and repeatability in accordance with AOAC and Horwitz criteria.

Application of these validated methods to mussel samples from major production and market sites across the Philippines including Samar, Capiz, Cavite, Taguig, and Navotas provided critical insights into heavy metal contamination levels. Mercury concentrations across all sites were below the MRL, and lead was not detected in any sample, indicating minimal public health risk from these elements. However, cadmium levels in mussels from Villareal and Jiabong, Samar exceeded regulatory limits, highlighting localized contamination concerns that warrant further investigation and potential mitigation measures. Overall, the validated methods are suitable for routine monitoring of heavy metals in mussels, supporting national food safety and environmental surveillance efforts.

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