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Method Validation of Total Reflection X-ray Fluorescence (TXRF) Analysis as a Screening Method for Mercury (Hg) in Philippine Milkfish

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***Abstract* – Mercury (Hg) is a naturally occurring element that is released to bodies of water through anthropogenic activities, industrial discharges, or from the existing Hg in soils. Its presence can cause bioaccumulation in aquatic species, which poses potential health risks when taken for human consumption. The most common method for elemental analysis in fish samples is inductively coupled plasma (ICP) spectroscopy, which is highly accurate but has high cost and analysis time. Recently, X-ray fluorescence (XRF) has gained attention for its heavy metal detection capability, which is cheaper and requires less sample preparation compared to ICP. For this study, analysis of Hg content in milkfish using Total Reflection X-ray Fluorescence (TXRF) spectrometry was validated using Philippine Reference Material (PRM) 2002-As, Cd, Hg & Pb in Milkfish produced by the DOST-ITDI National Metrology Division with a recovery range of 93-104%. The relative standard deviation (RSD) of the collected dataset is less than 10%, and the calculated expanded uncertainty was 0.08 ppm. For confirmation, DORM-5 was analyzed, resulting in 98.885% Hg recovery. Overall, the study showed that the TXRF analysis is a cheaper and reliable alternative method in detecting trace Hg content in milkfish samples; therefore, making it suitable for screening and evaluation of mercury in milkfish samples.**

I. Introduction

According to the Bureau of Fisheries and Aquatic Resources (BFAR), milkfish (*Chanos chanos*) is considered one of the most significant fish species farmed in the Philippines [1]. It can be cultivated in freshwater, brackish water, and certain marine environments. However, milkfish raised in freshwater systems are more susceptible to heavy metal accumulation than those in marine environments, as industrial waste pollutants are initially introduced into freshwater ecosystems.

Mercury (Hg) is a naturally occurring element with a high atomic weight and a density at least five times greater than that of water [2, 3]. It is recognized as one of the major contributors to water pollution due to its toxicity, which can harm the lungs, kidneys, skin, and eyes, as well as impact the nervous, digestive, and immune systems [4]. The mercury in aquatic ecosystems is influenced by the existing Hg in soils and Hg released due to anthropogenic activities, such as fossil fuel and coal combustion, gold mining, metal smelting, cement production, and industrial discharges. Organisms exposed to environments with Hg result in bioaccumulation influenced by the organisms' growth rate; rapid growth may lead to the dilution of Hg in tissues, while a short lifespan limits its bioaccumulation over time. Since Hg cannot be broken down, it also accumulates and magnifies through the food chain, and its increasing concentration may pose health risks to consumers [5, 6].

The most common techniques used for elemental analysis of fish samples include the use of inductively coupled plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS), which provides high accuracy [7, 8, 9]. However, testing using ICP analysis would incur high costs and longer analysis time due to sample preparation, which includes digestion of the sample [10].

Recently, it has been reported that X-ray fluorescence (XRF) spectrometry is one of the most effective and widely used techniques in determining heavy metals due to its non-destructive, fast, and continuous measurements [11, 12, 13]. In particular, the total reflection XRF spectroscopy (TXRF) is a specialized and well-established analytical technique used for multi-element determination in various samples, particularly in liquids and small powder samples [14]. It offers a wider range of elemental detection and higher resolution in terms of its limit of detection compared to a standard Energy Dispersive XRF (EDXRF) machine, which can reach ppm to sub-ppb level [15, 16]. The x-ray produced in the TXRF provides characteristics of each element, and its intensities are proportional to its concentrations in the sample [17]. It is

highly advantageous in that its determination of concentration is unaffected by matrix effects, has lower sensitivity reaching up to ppb level, and requires a small amount of sample (ug or uL) for analysis [18, 19]. In this study, method validation of TXRF for determining the Hg content in milkfish samples was conducted using Philippine Reference Material (PRM) 2002-As, Cd, Hg & Pb in Milkfish.

II. METHODOLOGY

A. Reagents and materials

The TXRF used for the analysis is the Bruker S2 Picofox, a compact and transportable TXRF spectrometer used for ultra-trace element analysis. It also uses 30-mm diameter quartz discs as sample carriers. All glasswares, including the quartz discs, were cleaned by submerging in a 5% RBS 50 solution, a special detergent for glass, and a 20% nitric acid bath.

For sample preparation, octylphenol ethoxylate (Triton X100) solution was used for suspending solid particles, and the internal standard is 1000 mg/L yttrium (Y), which is an element not typically found in milkfish and is even used as an inert marker for digestability studies with fish because it is indigestible [20]. The milkfish samples were PRM-2002 and DORM-5. The PRM-2002 is made from dried milkfish meat spiked with heavy metals, including arsenic, lead, mercury, and cadmium, while DORM-5 is fish meat protein with different elements, including a small amount of mercury.

B. Sample Preparation

To prepare the samples, 0.1g of sample (either PRM-2002 or DORM-5) was weighed with an analytical balance. It is mixed with 10 µL of 1000 mg/L yttrium standard and 5 mL of 1% by volume Triton X-100 solution. The sample is placed in a sonicator at 5-minute intervals for at least 3 times, agitating the mixtures using a vortex shaker in between to break up clumps of the solid sample and ensure that fine particles are suspended in the sample mixture.

The samples were also agitated with the vortex shaker to ensure that solid particles were suspended in the liquid right before transferring 10 µL into a siliconized quartz disc. The samples were allowed to air-dry until no liquid was visible, and about a 1cm diameter of solid sample particles was thinly laid on the center of the disc.

C. TXRF Parameters

The analysis was set to 2000 seconds to increase the count rate per element and improve the quantification of elements even in low amounts. The calibration constant, also referred to as sensitivity, used for the target elements (Hg) and internal standard (Y) is listed in Table 1 together with other analysis parameters.

Table 1. Parameters set for the analysis of PRM-2002 using TXRF.

Parameter	Unit	Value
X-ray voltage	kV	50
X-ray current	µA	1000
Duration of scan	s	2000
Amount of IS (Y)	µL	10
Hg sensitivity factor	-	0.52
Y sensitivity factor	-	1.780218

III. RESULTS AND DISCUSSION

A. Calculation of concentration

The Bruker S2 Picofox TXRF spectrometer is accompanied by the S2 Picofox software for operating the TXRF spectrometer, adjusting analysis parameters, and exporting the results. The software produces a spectrum graph as well as numerical quantifications of the counts under the curve for detected elements in terms of net area and concentration. To take account of possible concentrations in a blank solution, the net area under the characteristic emission peak of each target element was used to calculate the elements' concentration. For every sample, a blank sample (containing only 10 µL yttrium standard and 5 mL of 1% Triton X-100 solution) was analyzed with the same analysis parameters. A representative spectrum of a blank sample and PRM-2002 sample scans are shown in *Fig. 1*.

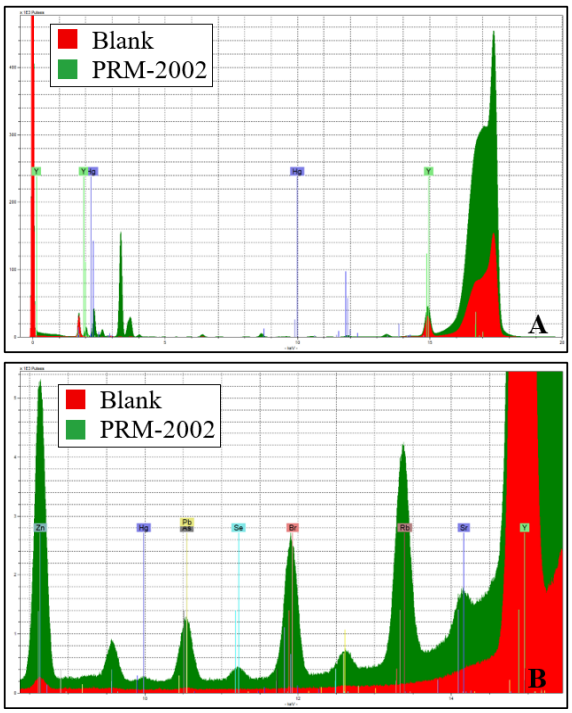

Fig. 1. TXRF spectra containing a blank sample and a PRM-2002 sample scan: (A) full spectra, (B) zoomed in to observe the target element, Hg.

In the zoomed-in image, a slight peak near 10 keV can be seen from the PRM-2002 spectrum. This peak is the characteristic emission line of Hg, which is exactly at 9.989 keV [21]. The S2 Picofox software readily produces the net area of each detected element after the analysis.

Since the PRM-2002 sample is a multi-element sample, some fluorescence peaks overlap with others, like the arsenic (As) and lead (Pb) peaks in *Fig. 1*. To correct the intensities, the spectrum is separated into the individual element lines through deconvolution. The Bruker S2 Picofox software uses the SuperBayes deconvolution routine, which relies on measured mono-element profiles to determine peak intensities. The net area values of the blank samples and the PRM-2002 samples were all recorded for calculating Hg concentrations. The Hg net area in the blank samples was then subtracted from the Hg net area of their respective PRM-2002 samples before calculation.

Eq. 1 is used to calculate the concentration of the target heavy metal (C_{Hg}) using the following values reported by the TXRF:

A_{Hg} = net area of the heavy metal
S_{Hg} = sensitivity factor of Hg
C_Y = concentration of y std
A_Y = net area of y std
S_Y = sensitivity factor of y std

$$C_{Hg} = (C_Y \bullet A_{Hg} \bullet S_Y) / (A_Y \bullet S_{Hg}) \quad (1)$$

Table 2 shows the calculated average concentrations of As and Hg expressed in ppm, together with total recovery percentage as calculated using Eq. 2, where C_{ref} is the known concentration of the reference material.

$$\text{Total \% Recovery} = 100\ (C_{Hg} / C_{ref}) \quad (2)$$

The recovery percentages of the samples are within the 80-110% range, which is the acceptable mean recovery for amounts between 100 ppm and 100 ppb [22].

Table 2. Calculated concentrations of Hg in multiple runs of PRM-2002 samples.

PRM Sample	Hg (ppm)	Hg Recovery (%)
1	0.503	102.653
2	0.502	102.449
3	0.468	95.510
4	0.500	102.041
5	0.507	103.469
6	0.506	103.265
7	0.483	98.571
8	0.489	99.796
9	0.473	96.531
10	0.460	93.878
Average	0.489	99.820

Table 2 shows the Hg concentrations calculated using equation 1 for the net area obtained from the TXRF analysis listed in Table 3. The calculated overall average was 0.489 ppm. It also shows that there are different variations in Hg recovery, which could be from the noise signals during the TXRF analysis, sample particle size, homogeneity of sample suspension, thickness of the deposited sample on the quartz discs, and matrix effects.

To account for errors during the analysis, the Westgard rules are implemented for quality control. This method can monitor possible significant deviations of data points or runs and quickly evaluate the accuracy and precision of the collected data. *Fig. 2* shows the ten (10) data points plotted in the Levey-Jennings chart. There are several Westgard rules e.g. a run is rejected if the measurement exceeds +/- 3s (s = standard deviation), runs are ejected if two (2) consecutive runs exceed mean +/- 2s, reject when four (4) consecutive runs exceed either mean +1s or -1s, reject if at least six (6) consecutive runs fall on one side of the mean, etc. Violation of the rules increases suspicion of the measurement method's accuracy and precision. Ultimately, the violations could lead to the analytical runs being deemed unacceptable [23]. As shown in *Fig. 2,* the data points did not exceed the ±1s range, and no six consecutive runs fell on one side of the mean, which indicates that the runs are acceptable and the method has good precision in detecting Hg in milkfish samples, even in low concentration amounts.

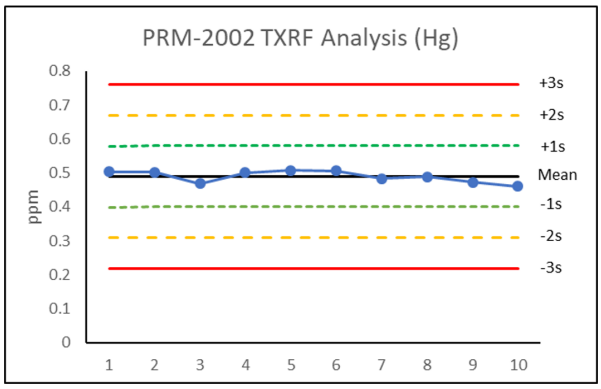

Fig. 2. Levey-Jennings chart for TXRF analysis results of Hg in PRM-2002 samples.

B. Relative Standard Deviation

The relative standard deviation (RSD) or coefficient of variation (CV) of the TXRF analysis for every element is also calculated (Eq. 3) to evaluate the precision of the analysis.

$$RSD = (sd / \bar{x}) \bullet 100\% \quad (3)$$

sd = Standard deviation
$\bar{x}$ = Mean

The RSD is 3.52%, which indicates that the collected dataset is relatively precise and consistent. This also suggests that TXRF is capable of analyzing Hg in milkfish samples. Therefore, it is suitable for the detection and initial evaluation of Hg contamination in milkfish samples.

C. Measurement Uncertainty

The Nordtest method was used for estimating the measurement uncertainty, more specifically, the method that uses a CRM. This method combines uncertainty due to random effects, u(Rw), and uncertainty from bias, u(bias), to calculate the standard uncertainty. The Nordtest TR 569 visualizes the errors in a ladder where each step adds to the measurement uncertainty. Steps 1 (method bias) and step 2 (laboratory bias) are estimated using u(bias), while step 3 (day-to-day variation) and step 4 (repeatability) contribute to u(Rw) [24].

The uncertainty from random bias comes from the standard deviation of repeated analyses over time. Since the analyses of PRM-2002 samples were done over a couple of months, the data collected can be used for determining the u(Rw). On the other hand, the u(bias) requires the root mean square of bias, RMS_{bias}, and uncertainty of the reference material, u(Cref).

First, the biases of all TXRF analyses were calculated using Eq. (4) wherein C_{lab} is the data values obtained from the analysis and Cref is the reference value. Then the biases and number of analyses, n, are used to get the RMS_{bias} using Eq. 5.

$$\text{bias} = C_{lab} - C_{ref} \tag{4}$$

$$RMS_{bias} = \sqrt{\frac{\Sigma(bias)^2}{n}} \tag{5}$$

Second, the uncertainty of individual reference values, $u(Cref_i)$, were calculated from individual reference standard deviation, s_i, and number of analyses, n, as shown in Eq. (6). Then the $u(Cref_i)$ is used to calculate u(Cref) using Eq. (7). Since only one CRM was used in the analyses, the u(Cref) is also equal to the reference standard deviation.

$$\Sigma u(Cref_i)^2 = \frac{s_i}{\sqrt{n_i}} \tag{6}$$

$$u(Cref) = \sqrt{\frac{\Sigma u(Cref_i)^2}{n}} \tag{7}$$

Once the RMS_{bias} and u(Cref) were calculated, they were combined for the u(bias) as shown in Eq. (8).

$$u(bias) = \sqrt{RMS_{bias}{}^2 + \frac{s_{bias}^2}{n} + u(Cref)^2} \tag{8}$$

Eq (8) is a variation of the u(bias) calculation formulated for analysis using only one CRM. In this equation, s_{bias} is the standard deviation of the biases.

Combining u(Rw) and u(bias) using Eq (9) gives the combined standard uncertainty, u_c; and, finally, the expanded uncertainty (U) at 95% confidence level with a coverage factor (k =2) using Eq (10).

$$u_c = \sqrt{u(Rw)^2 + u(bias)^2} \tag{9}$$

$$U = u_c \bullet k \tag{10}$$

Table 3 summarizes the two main uncertainty components, which are uncertainty due to random effects and uncertainty from bias. It also includes the standard uncertainty and the expanded uncertainty.

Table 3. Nordtest method calculated uncertainty components ($u(Rw)$ and $u(bias)$), standard uncertainty (u_c), and expanded uncertainty (U) in ppm.

$u(Rw)$	$u(bias)$	u_c	U
0.017	0.039	0.042	0.083

Using the Nordtest method, the calculated expanded uncertainty was 0.49 ± 0.08 ppm.

D. Analysis of DORM-5

On the certificate, the DORM-5 obtained from the National Research Council Canada contains 0.316 ± 0.017 ppm Hg. A summary of the calculated Hg concentration and recovery for each replicate, as well as the average value for the sample, is shown in Table 4. The average concentration calculated was 0.312 ppm, with a 98.885% recovery.

Table 4. DORM-5 sample TXRF spectroscopy calculated concentration and % recovery.

Sample	Hg (ppm)	Hg Recovery (%)
DORM-5 (1)	0.319	100.991
DORM-5 (2)	0.311	98.316
DORM-5 (3)	0.308	97.349
Average	0.312	98.885

IV. CONCLUSION

In conclusion, the study demonstrated that TXRF analysis is a reliable method for detecting Hg content in milkfish samples, even at low concentrations. The TXRF analysis of PRM-2002 samples yielded an average concentration of 0.49 ppm. The measurement uncertainty calculated using the Nordtest Method was determined to be 0.49 ± 0.08 ppm. To validate the accuracy of the method, a DORM-5 sample was analyzed, which achieved a recovery rate of 98.885%. These findings confirm that TXRF is not only a rapid and accurate technique but also a suitable screening method for Hg detection in milkfish samples. This will be very useful to assess possible Hg contamination in milkfish (*Chanos chanos*) and ensure safe consumption.

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