Validation of the Analytical Procedure for Quantitative Determination of Four Trace Metals (As, Cd, Pb and Hg) in Fish Lipids Using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

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Received (in XXX, XXX) Xth XXXXXXXXX 200X, Revised Xth XXXXXXXXX 200X , Accepted Xth XXXXXXXXX 200X
First published on the web Xth XXXXXXXXX 200X, DOI: xx.xxxx/xxxxxx

Abstract : The objective of the present study was to validate the analytical procedure for the quantitative determination of four trace metals (As, Cd, Pb, and Hg) in extracted fish lipids using Inductively Coupled Plasma-Mass Spectrometry, ICP-MS. The extracted lipids using Bligh and Dyer method were digested by means of microwave-assisted acid digestion and introduced into an optimized ICP-MS instrument. The validation of the analytical method was carried out in accordance with the international standards and guidelines outlined in the European Pharmacopeia (2022), which included specificity, selectivity, linearity, limit of detection, limit of quantification, precision, and accuracy. The linearity ranges of the calibration curves were $R^2 > 0.999$, while the relative standard deviation (%RSD) for precision was within 5%. All targeted trace metals have shown mean recoveries between 88.0%–114.9%. The obtained LOD and LOQ values for this analytical protocol indicated the ability to detect and quantify of As, Cd, Pb, and Hg at trace levels. The overall validation confirms the described analytical method was appropriate for routine analyses of As, Cd, Pb, and Hg in fish lipids.

Keywords : Method validation, Fish lipids, Trace metals, ICP-MS

Introduction

The lipidaceous fraction derived from fatty fish is generally referred to as fish oil and is identified as one of the major natural sources of omega-3 polyunsaturated fatty acids.[1] The numerous speculated health benefits associated with consuming fish oil have been proven by many researchers in the past few decades due to the presence of long-chain omega-3 polyunsaturated fatty acids (PUFA), including EPA and DHA. Omega-3 can be used to prevent and treat several health problems. viz. Coronary artery disease, dyslipidemia, high blood pressure, platelet aggregation, mental disorder, arthritis, autoimmune disorders, obesity, and diabetes mellitus type-2.[2-4] In addition to that omega-3 ensures the proper neural development in fetal and infants.[5,6]

Due to pollution of the aquatic environment, some of marine fishes can accumulate a significant amount of trace metals in their body. Consequently, the concentration of trace metals in fish lipids (fish oil) may be elevated, making it a significant contributor to human exposure to trace metals.[7,8] As a result, the trace metal contamination in fish oil may negate the health benefits of omega-3 fatty acids in fish oil. Thus, constant monitoring of trace metal levels in fish oil with reliable analytical techniques generally assures the safety of a consumer. Therefore, it is important to find a rapid, simultaneous, precise, and accurate analytical method in order to quantitative determination of toxic trace metals in fish oil.

Inductively coupled plasma-mass-spectrometry (ICP-MS) has been gaining popularity as the pre-eminent technique capable of determining element concentrations with low detection limits ranging from µg/L to ng/L levels. ICP-MS is a multi-element tool that offers great advantages. viz. simple sample preparation, high throughput, short time of analysis of the elements, relatively free from interferences, high precision, and high accuracy. Due to the above advantages, ICP-MS has emerged as one of the most well-liked detection systems and is frequently employed in a wide range of research domains. such as, scientific research, clinical, pharmaceutical, forensic sciences, food, material, environmental, fertilizer, chemical, and nuclear industries.[9-12]

The objective of this work was to validate the analytical procedure for the quantitative determination of four trace metals (As, Cd, Pb, and Hg) in extracted fish lipids by using, ICP-MS. In this experiment, the analytical method was validated based on the European Pharmacopeia (2022) international guidelines.[13-16] The validation included the performance parameters namely, selectivity and specificity, correlation coefficient, linearity, the limit of detection (LOD) and limit of quantification (LOQ), precision, and accuracy.

Experimental

Reagents and Chemicals

All solutions for the validation study such as, non-spiked samples, spiked samples and calibration standards were prepared using de-ionized water which was obtained by running distilled water through a Millipore Milli-Q water purification system. The standard solutions which are used for the generation of calibration curves were made by volumetrically diluting (2% volume fraction of ultrapure nitric acid as diluent) the single standard solution 100 mg/L of As, Cd, Pb, and Hg procured from Perkin Elmer, Inc. Shelton, USA. Concentrated nitric acid (65% volume fraction of HNO_3 TraceSELECT, Honeywell, France) and hydrogen peroxide (30% volume fraction of H_2O_2 Suprapur, Supelco, Germany) were used to lipid digestion purpose. Spike solutions were prepared by spiking the sample before digestion with the 100 mg/L single standard solutions.

Extraction of fish lipid samples

The procedure was validated on fish lipid samples which extracted from the three fish species were obtained from Trincomalee fish market in Sri Lanka. Fish species: 1- *Nemapteryx caelata* (Engraved catfish), 2- *Sardinella gibbose* (Goldstripe sardinella) and 3- *Amblygaster sirm* (Trenched sardinella) were subjected to extract fish lipids. Total lipids were extracted from the fish muscle according to Bligh and dyer (1959).[17,18] About 25 g of fish sample was homogenized with 50 mL of Methanol and then 25 mL of Chloroform about for 2 minutes. Another 25 mL of Chloroform was then added to it and homogenized for another 1 minute. Then 25 mL of de-ionized water was added and it was homogenized for another 1 minute. The homogenate was filtered through filter paper (Whatmann, Pore size-11 µm) using a Buchner funnel under suction. The filtrate was collected and the residue was subjected to another round of homogenization with Chloroform, Methanol, and water with a volume of 25: 25: 12.5 mL. The filtrates from both rounds were pooled in a 100 mL measuring cylinder and allowed for a few minutes for complete separation and clarification. After allowing the filtrate to separate into two layers, the upper alcoholic layer was removed using a dropper. Then lower Chloroform layer was transferred in to sampling tubes and Chloroform layer was then evaporated in an oven for 1 hour at 70 ^{0}C. The extracted fish lipid samples were collected in plastic sampling tubes and stored at 4-5 ^{0}C in a refrigerator until microwave digestion.

Digestion of fish lipid samples

Microwave digestion of the extracted fish lipid samples for ICP-MS analysis was carried out using the closed vessel microwave digestion system (Model-ETHOS EASY-49030, Milestone, Italy) according to the following procedure. A 0.05 to 0.1 g fish lipid samples were weighed out in the pre-cleaned digestion reaction vessel. 5 mL of HNO_3 and 1 mL of H_2O_2 were added to each vessel. Prior to digestion, all samples were spiked with 250 µL of a 1000 µg/L gold solution to stabilize mercury and arsenic during the digestion process. All the vessels were tightly sealed and placed in the rotor. Finally, the rotor was then placed inside the microwave chamber, and the digestion program was executed in accordance with the method depicted in **Table 1**. After digestion, reaction vessels were allowed to cool (door opening temperature < 50 ^{0}C), and then digestate was transferred into acid-clean 25 mL polypropylene tubes. All the vessels were washed using 2% volume fraction of HNO_3 acid and pooled with digestate. The digestate was made up to 25 mL with 2% volume fraction of HNO_3 acid and filtered through a 0.45 µm syringe filter. Finally, filtered digestates were stored in the refrigerator until ICP-MS analysis. The same digestion procedure was followed while preparing spiked samples and method blanks.

Table 1. Operating conditions of microwave digestion system

Step	Time (min)	Temperature (^{0}C)	Power (W)
01	20	200	1800
02	15	200	1800
03		Cooling	

Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

PerkinElmer®, USA, Nexion 2000 quadrupole-based ICP-MS instrument was used for the detection and quantification of As, Cd, Pb, and Hg. Detailed operating conditions for measuring the isotopes are given in **Table 2**.

Table 2. ICP-MS operating conditions

Parameter	Conditions and values
Spray chamber	Cyclonic
Nebulizer	Meinhard
Interface	Pt cones
Mass analizator	Quadrupole
Detector	Dual
Scanning mode	Peak hopping
RF power (W)	1,200
Ar gas flow rates (L/min)	
Plasma	15
Auxiliary	1.2
Nebulizer	0.94
Lens voltage (V)	7.75
Resolution (amu)	0.7
Replicate time (s)	1
Dwell time (ms)	50
Sweeps	20
Number of Replicates	3
Reading	1
Isotopes	^{91}AsO, ^{111}Cd, ^{208}Pb, ^{202}Hg

The Syngistix software (version 3.1) equipped with ICP-MS was used to data acquisition and process. DRC (Dynamic Reaction Cell) mode with 0.6 mL/min Oxygen gas flow was

used to As determination and KED (Kinetic Energy Discrimination) mode with helium (He) cell gas line was used to Cd and Hg determination (He gas flow is approximately 3.5 mL/min). Pb measurements were performed in a standard mode. ICP-MS tuning solution was used to instrument optimization before every analysis.

Results Discussion

Selectivity and specificity

Selectivity and Specificity refer to the capability to unambiguously discriminate and measure the target analyte in the presence of other expected component entities within the sample matrix.[19,20] The Specificity of ICP-MS is reliant on the resolving power of the mass filter (quadrupole), undesirable spectral and non-spectral interferences (impurities, degradants, or matrix) because, potential occurrence of adulteration of the assessed elemental composition of the samples. Selectivity of the present ICP-MS method was established by excellent separation of the targeted element (responses) with minimal possible interferences. Daily performance check was carried out analysis basis according to the recommendations provided by the ICP instrument's manufacturer to make certain adequate instrumental resolution viz. stability, doubly charged ions (typically by monitoring cerium 2+/cerium ratio [i.e., Ce 2+ /Ce]), oxide levels (typically by monitoring cerium oxide/cerium ratio [i.e., CeO /Ce]), mass calibration, detection limits, and resolution. In addition, appropriate isotopes were chosen in our work to reduce matrix-induced isobaric interferences. Determination of As was done by as AsO using Dynamic Reaction Cell (DRC) mode with Oxygen gas to eliminate the polyatomic ion ArCl originating from Ar and Cl causes interference. The Kinetic Energy Discrimination (KED) mode with a helium (He) gas cell was used to determine Cd and Hg by removing polyatomic interferences.

Range of linearity and calibration curve

The term 'linearity' of an analytical method refers to the ability to generate signals that exhibit a direct, proportional relationship with the concentration of the analyte under investigation, within a specified concentration range.[19,20] It is important to establish the linearity of the analytical method across a specified concentration range in order to obtain test results with suitable accuracy. The calibration curves were generated based on measurement data from 6 to 8 standards and linearity were assessed by inspecting the linear correlation coefficients of each generated calibration curves. The calibration curves were processed by using the Perkin elmer's syngistix software (version 3.1) of ICP-MS. Linearity was deemed acceptable if the correlation coefficient (R^2) was equal to or greater than 0.999.

Limits of detection (LOD) and limits of quantification (LOQ)

The Limit of Detection (LOD) of a specified analytical approach is defined as the minimum concentration of constituent in the sample that can be detected by the detector, but it may not be feasible to quantify as an exact value under the established experimental conditions whereas, the Limit of Quantification (LOQ) of a particular analytical procedure refers to the minimum concentration of the constituent present in the sample that can be detected and measured with suitable precision and accuracy.[19,20] The LODs for the procedure were determined by calculating three times the standard deviation (SD) from seven measurements of independently prepared method blank solutions, and the LOQs were established as 10 SD. The results determined for LOD and LOQ are summarised in **Table 3.**

Table 3. Results of determination of LOD and LOQ

Reagent blanks	Trace metal concentration / (µg/l)			
	As	Cd	Pb	Hg
RB 1	0.046	0.020	0.710	0.013
RB 2	0.040	0.019	0.704	0.013
RB 3	0.040	0.019	0.726	0.011
RB 4	0.039	0.018	0.723	0.011
RB 5	0.039	0.018	0.734	0.012
RB 6	0.036	0.017	0.716	0.013
RB 7	0.036	0.017	0.722	0.012
SD	0.003	0.001	0.010	0.001
LOD	0.010	0.003	0.031	0.002
LOQ	0.033	0.009	0.103	0.007

According to the **Table 3**, The LOD and LOQ values for the four metals have been acquired, which enables the detection and quantification of these metals in fish lipids at low concentrations. It was verified that the concentrations of all prepared samples are above the LOQs of As, Cd, Pb, and Hg.

Table 4 shows data obtained for the calibration curves and correlation coefficients of generated calibration curves. According to **Table 4**, the correlation coefficient (R^2) is 0.999 for As, 0.999 for Cd, 0.999 for Pb, and 0.999 for Hg. These correlation coefficients meet the requirements for admissibility, $R^2 \geq 0.999$. It can be concluded that the calibration curves for As, Cd, Pb, and Hg were linear in the respective calibration ranges. **Figures I, II, III, and IV** represent the calibration graphs for As, Cd, Pb, and Hg, respectively.

Table 4. Results of determination of linearity of calibration curves

Standard No	As		Cd		Pb		Hg	
	Standard level (µg/L)	ICP reading (µg/L)	Standard level (µg/L)	ICP reading (µg/L)	Standard level (µg/L)	ICP reading (µg/L)	Standard level (µg/L)	ICP reading (µg/L)
Standard 1	0.5	0.5	0.01	0.01	0.1	0.1	0.01	0.01
Standard 2	1.0	1.0	0.05	0.05	0.5	0.5	0.02	0.02
Standard 3	5.0	5.0	0.10	0.10	1.0	0.9	0.04	0.04
Standard 4	10.0	10.2	0.50	0.51	5.0	5.0	0.06	0.06
Standard 5	25.0	24.9	1.00	0.80	10.0	10.0	0.08	0.07
Standard 6	50.0	49.7	5.00	4.97	25.0	25.3	0.10	0.10
Standard 7	75.0	76.3	-	-	-	-	0.50	0.49
Standard 8	100.0	100.5	-	-	-	-	-	-
Correlation (R^2)	0.999		0.999		0.999		0.999	

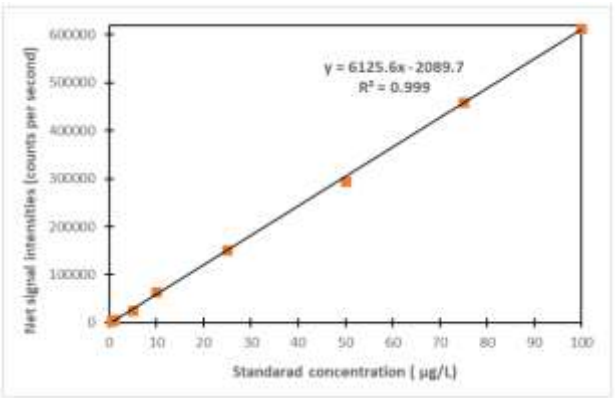

Figure I. Calibration curve for As

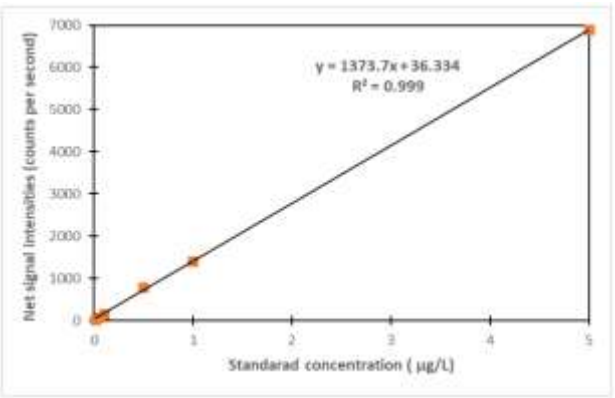

Figure II. Calibration curve for Cd

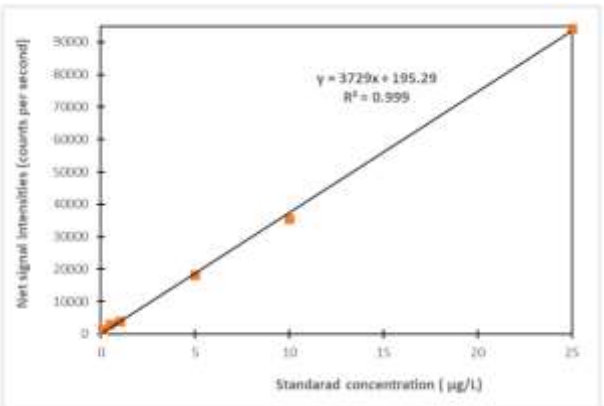

Figure III. Calibration curve for Pb

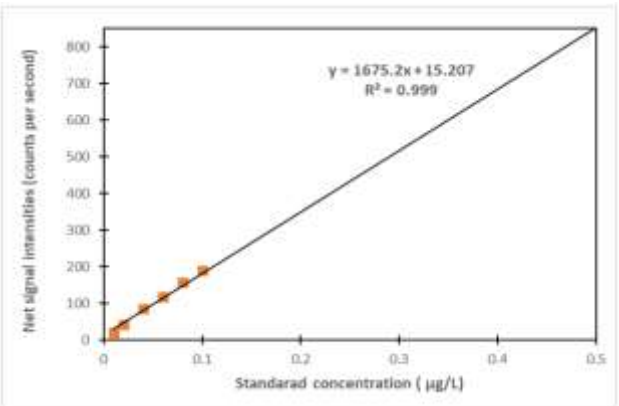

Figure IV. Calibration curve for Hg

Repeatability (single laboratory precision)

Repeatability represents a quantification of the level of concurrence between replicate test outcomes obtained through the application of the same operating conditions, by the same analyst, in the same laboratory, on the same sample material, and within a short time intervals.[19,20] The repeatability (single laboratory precision) of each metal was assessed using the relative standard deviation based on ten measurements of a homogeneous samples, covering three concentration levels (Low, Mid, and High) within the established range for the procedure. The equations used to calculate repeatability are shown as follows: Equation (1) for the mean ($\bar{X}$) , Equation (2) for the standard deviation (SD), and Equation (3) for the relative standard deviation (RSD).The repeatability values of the metals studied under this work are shown in **Table 5.**

$$\bar{X} = \frac{\sum_{i=1}^{n} x_i}{n} \quad (1)$$

$$SD = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N-1}} \quad (2)$$

$$RSD = \frac{SD}{\bar{X}} \quad (3)$$

Table 5. Results of determination of repeatability

No of replicates	As (mg/kg)			Cd (mg/kg)			Pb (mg/kg)			Hg (mg/kg)		
	Low	**Mid**	**High**	**Low**	**Mid**	**High**	**Low**	**Mid**	**High**	**Low**	**Mid**	**High**
1	9.173	19.917	32.784	0.012	0.057	0.430	0.254	0.684	1.224	0.005	0.007	0.017
2	9.450	20.253	33.178	0.012	0.057	0.422	0.254	0.684	1.241	0.005	0.007	0.017
3	9.289	19.703	34.240	0.012	0.057	0.424	0.246	0.690	1.228	0.005	0.007	0.018
4	9.223	19.983	33.230	0.012	0.058	0.432	0.242	0.689	1.234	0.004	0.007	0.018
5	9.476	20.228	33.572	0.012	0.057	0.423	0.254	0.688	1.229	0.005	0.006	0.018
6	9.359	19.804	34.592	0.011	0.058	0.426	0.233	0.681	1.247	0.005	0.007	0.019
7	9.232	20.130	33.015	0.013	0.057	0.430	0.254	0.686	1.200	0.004	0.007	0.018
8	9.446	20.279	33.586	0.012	0.058	0.421	0.249	0.688	1.240	0.005	0.007	0.017
9	9.304	19.895	34.943	0.011	0.059	0.423	0.242	0.694	1.238	0.005	0.007	0.018
10	9.450	19.970	33.264	0.013	0.058	0.424	0.251	0.685	1.279	0.005	0.007	0.018
Mean	9.340	20.016	33.640	0.012	0.058	0.426	0.248	0.687	1.236	0.005	0.007	0.018
SD	0.111	0.198	0.716	0.001	0.001	0.004	0.007	0.004	0.020	0.000	0.000	0.001
RSD (%)	1.19	0.99	2.13	4.37	1.38	0.94	2.89	0.53	1.62	4.16	2.93	4.25

According to **Table 5**, one may conclude that the results obtained for the RSDs are as follows: 1.19%, 0.99%, and 2.13% for arsenic; 4.37%, 1.38%, and 0.94% for cadmium; 2.89%, 0.53%, and 1.62% for lead; and 4.16%, 2.93%, and 4.25% for mercury, respectively. The admissibility condition for repeatability should be less than 5% (RSD ≤ 5). Therefore, the above values fulfil admissibility criteria and method can consider as precise.

Accuracy (Spike Recovery)

Accuracy serves as a benchmark for evaluating the true nature of an analytical method, by gauging how closely the measured value aligns with either a widely-accepted reference value or a conventionally-defined 'true' value.[19,20] Recovery study was carried out to evaluate the accuracy of the method or effectiveness of this procedure by means of fortified analytical portion (FAP) method. It was done by spiking the target elements (As, Cd, Pb, and Hg) into test samples with the appropriate quantities. Samples were spiked with three concentration levels (low, mid, and high) covering the established range of the corresponding calibration curves, and analyzed in triplicate at each level. The spike recovery in mathematical terms can be expressed using Equation (4), wherein C_{spike} denotes the level of the analyte in the spiked sample, C_{sample} represents the level of the same analyte in an unfortified sample, and C_{add} denotes the added level of the analyte in the spiked sample. The spiking samples were prepared in triplicate and the recovery data obtained are shown in the **Table 6.**, **Table 7.**, **Table 8.**, and **Table 9.**

$$\%Recovery = \frac{Cspike - Csample}{Cadd} \times 100 \quad (4)$$

Table 6. Spike recovery results for Arsenic

Lipid sample	Replicate	Spiked level (mg/kg)	Measured level (mg/kg)		Spike recovery (mg/kg)	Recovery %	Mean recovery %
			Non spiked sample	Spiked sample			
1	Low spiked-1	0.473	8.942	9.350	0.408	86.3	97.0 ± 9.8
	Low spiked-2	0.483		9.450	0.509	105.4	
	Low spiked-3	0.476		9.415	0.473	99.4	
	Mid spiked-1	12.136	8.804	20.244	11.440	94.3	95.8 ± 1.5
	Mid spiked-2	12.336		20.253	11.448	92.8	
	Mid spiked-3	11.981		20.286	11.482	95.8	
	High spiked-1	26.549	8.893	33.136	24.243	91.3	89.9 ± 1.8
	High spiked-2	26.834		33.178	24.285	90.5	
	High spiked-3	27.675		33.199	24.305	87.8	
2	Low spiked-1	0.484	9.207	9.682	0.475	98.3	103.6 ± 5.7
	Low spiked-2	0.473		9.693	0.487	102.8	
	Low spiked-3	0.449		9.699	0.492	109.6	
	Mid spiked-1	15.400	9.268	24.003	14.736	95.7	94.9 ± 2.2
	Mid spiked-2	15.723		23.802	14.535	92.4	
	Mid spiked-3	15.060		23.811	14.543	96.6	
	High spiked-1	27.675	9.243	34.856	25.614	92.6	92.9 ± 1.9
	High spiked-2	28.195		34.950	25.707	91.2	
	High spiked-3	27.027		34.912	25.669	95.0	
3	Low spiked-1	0.568	8.973	9.554	0.580	102.1	96.6 ± 8.8
	Low spiked-2	0.572		9.552	0.578	101.1	
	Low spiked-3	0.580		9.475	0.501	86.4	
	Mid spiked-1	15.306	8.998	22.385	13.387	87.5	88.0 ± 0.5
	Mid spiked-2	15.432		22.637	13.639	88.4	
	Mid spiked-3	15.593		22.746	13.748	88.2	
	High spiked-1	30.303	8.998	37.056	28.058	92.6	91.4 ± 1.0
	High spiked-2	30.612		36.781	27.783	90.8	
	High spiked-3	30.928		37.090	28.092	90.8	

Table 7. Spike recovery results for Cadmium

Lipid sample	Replicate	Spiked level (mg/kg)	Measured level (mg/kg)		Spike recovery (mg/kg)	Recovery %	Mean recovery %
			Non spiked sample	Spiked sample			
1	Low spiked-1	0.002	0.010	0.012	0.002	102.9	106.6 ± 3.7
	Low spiked-2	0.002		0.012	0.002	106.6	
	Low spiked-3	0.002		0.012	0.002	110.3	
	Mid spiked-1	0.043	0.009	0.057	0.048	111.9	111.6 ± 0.7
	Mid spiked-2	0.043		0.057	0.048	110.8	
	Mid spiked-3	0.043		0.057	0.048	112.1	

	High spiked-1	0.406	0.010	0.430	0.420	103.5	
	High spiked-2	0.406		0.422	0.412	101.5	102.4 ± 1.0
	High spiked-3	0.405		0.424	0.414	102.2	
	Low spiked-1	0.002	0.014	0.016	0.002	94.5	
	Low spiked-2	0.002		0.016	0.002	88.6	93.6 ± 4.6
	Low spiked-3	0.002		0.016	0.002	97.6	
	Mid spiked-1	0.046	0.016	0.059	0.043	92.9	
2	Mid spiked-2	0.046		0.060	0.044	95.3	92.3 ± 3.3
	Mid spiked-3	0.046		0.057	0.041	88.8	
	High spiked-1	0.439	0.014	0.459	0.445	101.3	
	High spiked-2	0.439		0.476	0.462	105.0	104.0 ± 2.3
	High spiked-3	0.440		0.479	0.464	105.5	
	Low spiked-1	0.003	0.017	0.021	0.004	113.4	
	Low spiked-2	0.003		0.021	0.003	112.2	112.4 ± 1.0
	Low spiked-3	0.003		0.020	0.003	111.5	
	Mid spiked-1	0.050	0.017	0.067	0.050	101.1	
3	Mid spiked-2	0.049		0.065	0.048	98.8	100.2 ± 1.2
	Mid spiked-3	0.048		0.065	0.048	100.7	
	High spiked-1	0.606	0.015	0.649	0.634	104.7	
	High spiked-2	0.595		0.649	0.634	106.6	105.9 ± 1.1
	High spiked-3	0.585		0.638	0.623	106.5	

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Table 8. Spike recovery results for Lead

Lipid sample	Replicate	Spiked level (mg/kg)	Measured level (mg/kg)		Spike recovery (mg/kg)	Recovery %	Mean recovery %
			Non spiked sample	Spiked sample			
	Low spiked-1	0.045	0.205	0.254	0.049	110.2	
	Low spiked-2	0.045		0.254	0.049	110.5	109.1 ± 2.1
	Low spiked-3	0.045		0.253	0.048	106.7	
	Mid spiked-1	0.431	0.206	0.684	0.478	110.8	
1	Mid spiked-2	0.430		0.684	0.477	110.9	111.4 ± 0.9
	Mid spiked-3	0.430		0.690	0.483	112.5	
	High spiked-1	1.015	0.209	1.224	1.015	100.0	
	High spiked-2	1.014		1.241	1.033	101.9	100.8 ± 1.0
	High spiked-3	1.013		1.228	1.019	100.6	
	Low spiked-1	0.045	0.221	0.270	0.049	108.5	
	Low spiked-2	0.045		0.270	0.048	107.2	106.8 ± 2.0
	Low spiked-3	0.045		0.269	0.047	104.6	
	Mid spiked-1	0.462	0.222	0.755	0.533	115.3	
2	Mid spiked-2	0.462		0.753	0.531	114.9	114.9 ± 0.5
	Mid spiked-3	0.461		0.750	0.528	114.4	
	High spiked-1	1.097	0.223	1.329	1.107	100.8	
	High spiked-2	1.098		1.338	1.116	101.6	98.5 ± 4.6
	High spiked-3	1.100		1.249	1.026	93.2	

3	Low spiked-1	0.062	0.268	0.328	0.060	96.6	101.8 ± 2.7
	Low spiked-2	0.062		0.329	0.061	98.0	
	Low spiked-3	0.062		0.331	0.063	101.8	
	Mid spiked-1	0.498	0.275	0.812	0.538	108.0	106.4 ± 1.6
	Mid spiked-2	0.490		0.797	0.522	106.5	
	Mid spiked-3	0.481		0.779	0.504	104.8	
	High spiked-1	1.515	0.271	1.762	1.491	98.4	96.8 ± 1.5
	High spiked-2	1.488		1.707	1.436	96.5	
	High spiked-3	1.462		1.666	1.395	95.4	

Table 9. Spike recovery results for Mercury

Lipid sample	Replicate	Spiked level (mg/kg)	Measured level (mg/kg)		Spike recovery (mg/kg)	Recovery %	Mean recovery %
			Non spiked sample	Spiked sample			
1	Low spiked-1	0.002	0.003	0.005	0.002	91.4	95.8 ± 4.0
	Low spiked-2	0.002		0.005	0.002	96.6	
	Low spiked-3	0.002		0.005	0.002	99.3	
	Mid spiked-1	0.004	0.003	0.007	0.004	113.2	110.7 ± 3.5
	Mid spiked-2	0.004		0.007	0.004	106.7	
	Mid spiked-3	0.004		0.007	0.004	112.2	
	High spiked-1	0.017	0.002	0.017	0.015	86.8	90.5 ± 3.8
	High spiked-2	0.017		0.017	0.015	90.4	
	High spiked-3	0.017		0.018	0.016	94.3	
2	Low spiked-1	0.001	0.024	0.025	0.001	95.3	95.2 ± 0.2
	Low spiked-2	0.001		0.025	0.001	95.3	
	Low spiked-3	0.001		0.025	0.001	95.0	
	Mid spiked-1	0.002	0.024	0.026	0.003	104.7	100.6 ± 3.7
	Mid spiked-2	0.002		0.026	0.002	97.4	
	Mid spiked-3	0.002		0.026	0.002	99.9	
	High spiked-1	0.012	0.022	0.034	0.011	95.6	95.2 ± 1.5
	High spiked-2	0.012		0.034	0.011	96.5	
	High spiked-3	0.012		0.033	0.011	93.6	
3	Low spiked-1	0.002	0.011	0.013	0.002	105.8	98.4 ± 6.6
	Low spiked-2	0.002		0.013	0.002	96.0	
	Low spiked-3	0.002		0.013	0.002	93.4	
	Mid spiked-1	0.004	0.012	0.015	0.003	91.6	93.6 ± 2.0
	Mid spiked-2	0.004		0.016	0.003	95.5	
	Mid spiked-3	0.004		0.016	0.003	93.6	
	High spiked-1	0.019	0.011	0.032	0.021	109.3	111.9 ± 2.7
	High spiked-2	0.019		0.033	0.022	114.6	
	High spiked-3	0.019		0.033	0.021	111.8	

The recovery percentages (Table 6, 7, 8, and 9) of the targeted metals in extracted fish lipids were obtained by comparing the analyte's level in the spiked and non-spiked samples which is acquired from the calibration curve, to the metal's spike level. The mean percentage (%) recoveries were found between 88.0 ± 0.5 to 114.9 ± 0.5 % for all 4 elements. The recoveries were found within the acceptance range (80- 120%) and the method was found to be accurate. The trace metal levels in extracted fish lipids are shown in **Table 10**.

Table 10. Trace metals levels in extracted fish lipids

Lipid sample	Trace metal level (mg/kg)			
	As	Cd	Pb	Hg
1	8.893 ± 0.070	0.010 ± 0.000	0.207 ± 0.002	0.003 ± 0.001
2	9.239 ± 0.031	0.015 ± 0.001	0.222 ± 0.001	0.023 ± 0.001
3	8.990 ± 0.014	0.016 ± 0.001	0.271 ± 0.003	0.012 ± 0.001

The maximum accepted levels (MAL) for Cd, Pb, and Hg in omega-3 fish oil supplements, as established by the European Pharmacopeia (EP), were at 1.0, 3.0, and 0.1 mg/kg, respectively.[21] The Cd, Pb, and Hg levels in the three analyzed lipid samples were below the MAL values recommended by the EP. In general, marine fish naturally contain high levels of total arsenic, and it can biomagnify as trophic levels increase in the aquatic food chain.[22] A previous study found that the total arsenic content in Japanese sardine oil, krill oil, Japanese common squid oil, and anchovy oil was 9.68, 5.57, 19.6, and 15.5 mg/kg, respectively.[23] The total arsenic levels in fish lipids are consistent with those previously reported in the literature.

Conclusions

An ICP-MS method has been validated according to the European Pharmacopeia (2022) international guidelines to measure the levels of Arsenic, Cadmium, Lead, and Mercury in fish lipids. This method demonstrates excellent selectivity and linearity for the determination of target metals in the respective ranges. The Low LOD and LOQ values obtained in this study verified that the method is capable of detecting and measuring the target metals in fish lipids at trace levels. The results of recovery and repeatability confirmed that the method is accurate and precise. In summary, the validated ICP-MS technique is suitable for the simultaneous quantification of Arsenic, Cadmium, Lead, and Mercury in fish lipids and can be employed for the quantification of these metals in commercial fish oil.

Acknowledgment

Financial Assistance of World Bank AHEAD RIC Project no 28 “Encapsulation of Omega -3 fish oil from Sri Lankan Fishes and Development of Omega -3 Fortified Foods” of Faculty of Applied Sciences, Rajarata University of Sri Lanka, Mihintale is acknowledged.

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