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Elaboration of reference material for cadmium and arsenic in hydrobiological products, for the purpose of being used in a laboratory intercomparation program

Elaboración de un material de referencia para cadmio y arsénico en productos hidrobiológicos, para propósito de ser utilizado en un programa de intercomparación de laboratorios

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ABSTRACT

Reference materials (RM) are tools used in the comparability and traceability of measurements. They are widely used by laboratories for method validation and quality control of assay. Chile must evaluate the performance of laboratories that analyzing metals in fishery products, despite RM have high prices and are scarce. For that reason, a RM in a hydrobiological product was developed. Reference values for arsenic and cadmium elements for a fishmeal were assigned. The measurement methods for characterization of the material were Inductively Coupled Plasma Mass Spectrometry, Atomic Absorption Spectrometry and Neutron Activation Analysis. Reference values with their expanded uncertainty (U) were established for arsenic 2.64 ± 0.42 mg/kg (U; k = 2) and for cadmium 0.86 ± 0.16 mg/kg (U; k = 2). Homogeneity and stability of the RM allowed its use in a proficiency test for eleven food control laboratories. Results for median were 2.114 mg/kg for arsenic, and 0.863 mg/kg for cadmium. The performance values of the participants were evaluated with a z score obtaining 60% satisfaction for arsenic and 73% for cadmium. The material demonstrated to be suitable for use in interlaboratory proficiency assay.

Keywords. Laboratory Proficiency Test, Reference Material, Arsenic, Cadmium, Fish Flour.

RESUMEN

Materiales de referencia (MR) son herramientas utilizadas en la comparabilidad y trazabilidad entre mediciones. Laboratorios los utilizan ampliamente en validación de métodos y control de calidad. Chile debe evaluar el desempeño de los laboratorios que analizan metales en productos pesqueros, a pesar de los altos precios y escasez del MR. Por esa razón, se desarrolló un MR en producto hidrobiológico. Se asignaron valores de referencia para arsénico y cadmio en harina de pescado. Los métodos de medición para la caracterización del material fueron Espectrometría de Masas de Plasma Acoplado Inductivamente, Espectrometría de Absorción Atómica y Análisis de Activación de Neutrones. Se establecieron valores de referencia con su incertidumbre (U) para arsénico 2.64 ± 0.42 mg/kg (U; k = 2) y para cadmio 0.86 ± 0.16 mg/kg (U; k = 2). La homogeneidad y estabilidad del MR permitieron su uso en una prueba de aptitud para once laboratorios de control de alimentos. Las medianas fueron 2,114 mg/kg para arsénico y 0,863 mg/kg para cadmio. Se evaluaron los rendimientos de los participantes con un estadístico de puntaje z satisfactorio del 60% para el arsénico y 73% para el cadmio. El material demostró ser adecuado para uso en ensayo de aptitud de intercomparación.

Palabras clave. Ensayos de Aptitud de Laboratorios, Material de Referencia, Arsénico, Cadmio, Harina de Pescado.

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INTRODUCTION

The *Instituto de Salud Pública de Chile* ("ISP", Public Health Institute of Chile) has the mission of developing certified reference materials and reference materials in food chemistry, to provide metrological traceability to measurement laboratories.

Reference materials (RM) are crucial for laboratory quality assurance. They are widely used for the internal quality control of analytical tests, in the validation of methodologies and as test items in proficiency tests[1]. Proficiency testing is a type of systems audit or external assessment, a check on the entire laboratory testing system. The term "check testing" is sometimes used for this activity. Proficiency test may also be used to assign values to reference materials or to determine their suitability for use. Proficiency test samples may, in many cases, also be considered reference materials[2]. Certified reference materials (CRM) and reference materials, in addition to being homogeneous and stable, have the advantage of having more exact values, lower uncertainties and metrological traceability, providing greater reliability and comparability of measurements. CRM and RM production is in accordance with ISO 17034[3], and requires very exact and precise methods.

The ISP conducts proficiency test as part of an external evaluation program of quality (PEEC, by its Spanish acronym) mainly aimed to public or private food audit and control laboratories authorized by the Chilean State to this goal. The objective of this program is to assess the performance of the national laboratories in relation to the analysis made, according to the quality assurance system ISO/IEC 17025[4]. Proficiency tests program focus on the control of food components, additives, and contaminants that are consumed by the population and are of national interest due to its impact in both economic and public health system.

According to figures put out by the Food and Agriculture Organization of the United Nations (FAO) in 2004, Chile was the sixth producer country in the world, with a production in fishery and aquaculture industry of 4% of the worldwide volume[5] that year. Nevertheless, National Food Consumption Surveys (ENCA, by its Spanish acronym) in 2016 revealed that the fish consumption is scarce as no more than the 15% of the Chilean population reports its consumption, with a clear socioeconomic gradient[6].

The Health Sector has emphasized in the promotion of fish consumption, especially in children, youngsters, and older adults to bear obesity problems in the population. This way, it is expected that fish integrated in the daily diet would be a healthy contribution for the consumer, and not a source of risk to develop diseases. For that reason, monitoring, control, and audit processes are used to assure the safety of these nourishments from sea to fork.

Sanitary Food Regulation (RSA) of the Ministry of Health of Chile ("MINSAL", *Ministerio de Salud*) establishes in its Title IV, section I that the presence of arsenic in fresh, refrigerated, frozen and preserved fish[7] must not exceed the maximum limit of 1 mg/kg, cadmium is not mentioned for these products. At an international level, the *Codex Alimentarius* have recommended for cadmium a maximum limit (ML) in food in a range that goes from 0.05 mg/kg (vegetables) to 2 mg/kg (marine bivalve molluscs). In the same way, for arsenic the recommended MLs range from 0.1 to 0.5 mg/kg in food, with no ML values for matrixes of marine origin[8]. In this context, both public and private laboratories must conduct their laboratory tests in a way to guarantee quality and comparability of their results, so decisions based on these outcomes are appropriate.

The *Servicio Nacional de Pesca* ("SERNAPESCA", National Fisheries Service) and the MINSAL raise the necessity of incorporate in the PEEC a proficiency test round in order to determine the presence of metals in foodstuff coming from fishery industry. Facing this need, the ISP, through the Designated Metrology Laboratory, together with the Chemical Metrology Section of the *Comisión Chilena de Energía Nuclear* ("CCHEN", Chilean Nuclear Energy Commission) developed a traceable reference material for elements in hydrobiological product (fish meal). There are several methods for the determination of elements in food, so to characterize the reference material, three techniques were selected with the capacity to measure traces of these substances (Cd and As): Neutron Activation Analysis (NAA), Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Graphite Furnace Atomic Absorption Spectroscopy (GFAAS). These methods are sensitive and accurate enough to characterize a reference material.

As a result of the increase in both national and international quality standards compared to exports and imports at the food industry level, new needs have been generated to comply with adequate quality and to comply with these, a demand has also been generated in the quality assurance of the laboratories. The demand for new CRM and RM traceable to the International Systems of Units (SI) has been growing in all areas, especially in the food industry, due to the variability of measurands and food matrices, access to these materials is hampered by the lack of supply and/or their high cost. The laboratories in charge of food control must demonstrate that the methods used are duly validated for the analytes and food matrices required. Food matrices are generally complex, so it is essential to use RM/CRM as a support to be able to demonstrate with accuracy and reliability the results obtained by these methods. The major limiting factor for the general use of RMs in food analysis laboratories is their restricted availability considering the huge range of possible analyte/concentration level/matrix combinations of the real samples to be analyzed[9].

RMs can be used to a great extent for internal quality control purposes in a food testing laboratory in control charts, in method validation and in ring tests, among others. However, in many cases they are not available in the market or are expensive due to production costs, and also because in most cases they must be imported. For these reasons, not all the needs for quality assurance tools for food control laboratories are covered in accordance with the requirements of ISO/IEC 17025. Metrological traceability and quality assurance in food production play an important role for food trade. Hence the importance that national metrology institutes (NMIs) or designated metrology institutes (DIs) can produce RMs and CRMs, according to the main analytical needs of their countries, ensuring the accessibility of national laboratories to these, and in this way to respond to the requirements of public and private food control laboratories, thus supporting a more reliable food trade.

The production of reference materials is a complex process, where producers must comply with the requirements of ISO/IEC 17025, ISO 17034 and ISO Guide 35, so that the RM/CRM produced satisfy the requirements of metrological traceability and uncertainty for the intended purpose. Scientific work aimed at the production of new RMs will contribute to reduce existing gaps in the market, allowing better access to RMs for national public and private laboratories.

Expanding the production of new RMs in food matrices, focused to certain analytical needs derived from "Food Surveillance Programs", such as nutrients, pollutants, residues and toxins, allows directing efforts towards developing RMs for assay processes critical associated with food control, generating tools helping to improve the quality and safety of food, for the benefit of consumer protection and the confidence of the food trade.

The aim of this scientific work is to present the elaboration of a traceable reference material to SI for determining As and Cd in fishmeal, destined to be used in a proficiency test to support the quality assurance of food analysis laboratories, in charge of the control and surveillance of the production of the fishing industry in Chile.

MATERIAL AND METHODS

Candidate Reference Material Preparation

The ISP used fish meal as a matrix for the elaboration of the reference material, because its composition characteristics (66% protein, 12% total fat, and moisture less than 10%) and its dry powder state facilitate its handling and preservation. Fish meal belonging to a sample of 4 kg from the same set was processed through drying, sieving and homogenizing. Subsequently, it was divided, packed and labeled in amber glass bottles of 35 g each, which were exposed to a minimum dose of gamma irradiation of 25 kGy for their sterilization in the CCHEN. 93 units of candidate reference material bottles were obtained.

The produced units were stored in a desiccator to room temperature between 20 and 27°C.

The elaboration and assessment processes of the reference material were in accordance with the quality standards ISO 17034[3], ISO Guide 35[10] and ISO/IEC 17025[4].

Reference Materials and testing methods

Testing methods used for the assessment of the reference material were validated by relevant authorities in order to be used for this purpose. The ISP was in the charge of the Analysis of food elements by ICP-MS for microwave digestion[11]. The ISP is a National Designated Metrology Laboratory of the *National Metrology Network* of Chile (RNM, its acronym in Spanish: *Red Nacional de Metrología*), complying with the normative requirements of ISO/IEC 17025 and ISO 17034.

The CCHEN was responsible for the execution of the tests following the NAA[12,13], ICP-MS[10], and GFAAS[14]. The CCHEN is a reference laboratory for nuclear techniques in Chile, under an ISO/IEC 17025 quality management system and is recognized by the International Atomic Energy Agency (IAEA) as a laboratory of high performance level for its good participation in the proficiency testing that this agency organizes annually.

The processing of the samples of the candidate reference material consisted of taking a portion of 0.2 to 0.5 g of dry sample in a microwave glass, adding 1 mL of water, 8 mL of high purity nitric acid (HNO_3 65% v/v) and 2 mL of high purity hydrogen peroxide (H_2O_2 30% v/v), letting it stand closed at room temperature under a hood for at least 12 hours and then placed in the microwave digester in a gradient program from 250 to 500 watts for 35 minutes. The digestion solution was transferred to a 25 mL volumetric flask with reagent grade water, for analysis by the defined method.

The SI traceable NIST (National Institute of Standards and Technology) reference materials that were used as calibration standards for the above mentioned methods were:

- *Cadmium (Cd) Standard Solution* SRM® 3108 Lot No. 130116.
- *Arsenic (As) Standard Solution* SRM® 3103a Lot No. 100818.

For tests quality control purposes, the *Fish protein certified reference material for trace metals* DORM-4 of NCR-CNRC (National Research Council Canada) traceable to SI, containing arsenic in 6.80 ± 0.64 mg/kg (U; $k = 2$) and cadmium in 0.306 ± 0.015 mg/kg (U; $k = 2$) was used in parallel to samples. The analytical results reported by the ISP and CCHEN were accepted, when the recovery rate of the material was between 80-110%.

Humidity was determined by the coulometric Karl Fischer method[15-17] using a certified standard oven 1%, Apure®, Merck®. Traceability to the SI of mass and volume measurements is to the National Mass Metrology Laboratory of Chile (CESMEC S.A.).

Homogeneity and stability study

A stratified random sample was taken from the set produced from the candidate reference material, obtaining a total of 25 bottles for the material characterization and assessment. Homogeneity and stability studies were statistically conducted in accordance with the ISO Guide 35[10] guidelines.

The ISP was responsible for conducting the homogeneity and stability studies. The number of samples required for the purposes of the homogeneity study was established based on 10% of the batch size, that is, the total number of units (bottles) produced by the batch ($N_{prod} = 93$ units), this due to the fact that the batch size was less than 100 units[10]. Finally, the material homogeneity study involved the analysis of 9 duplicate samples (bottles) of arsenic and cadmium elements with the ICP-MS method under repeatability conditions, which was previously validated for this purpose. For analysis of variance (ANOVA) of the duplicated data, the acceptability criteria were established as *F-value* must be less than *F-table*, with a 95% percent of confidence interval[10]. The study of the homogeneity of the material was carried out between and within the bottles, with their respective uncertainties.

The material stability study was established with the same method used in homogeneity study. The temperature for homogeneity and stability study was established between 20 to 25°C. For the evaluation of stability, a classical stability study was carried out in a short-term period of 10 days and 70 days, a long-term

period of 150 days, 200 days, and 250 days. For this, 6 bottles were analyzed in duplicate, 3 in the short-term study and 3 in the long-term stability study in intermediate conditions of measurement by ICP-MS Method.

Reference material characterization

Three analytical methodologies were used for the material characterization, since there is no primary method such as isotopic plasma mass dilution (IDMS). The ISP conducted the analysis of 9 bottles in duplicate and repeatability conditions with ICP-MS Method.

The CCHEN analyzed in duplicate and repeatability conditions 5 bottles by GFAAS for cadmium, 9 bottles NAA for arsenic and 10 bottles by ICP-MS for both elements, the treatment of the samples was carried out by microwave digestion. The methods were previously validated by ISP and CCHEN for their intended purpose.

The allocation of the value of the reference material was made in accordance with the ISO *Guide 35* and the associated uncertainty was determined according to the GUM[18].

The reference assigned value was obtained from the average of results found for the three methods in each case, which was calculated as the Equation 1:

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

where: $\bar{X}$ = average of the results reported by the ISP and CCHEN methods; x_i = average of the result reported by each method; n = number of methods.

Expanded uncertainty of the assigned value was determined as the combined uncertainty of characterization, homogeneity, and stability applying an coverage factor for a 95% of confidence interval[10,19,20], k = 2, that is, it was calculated as the Equations 2-6:

$$U = k \times u_c = 2 \times \sqrt{u_{char}{}^2 + u_{hom}{}^2 + u_{stab}{}^2} \quad (2)$$

where: u_{char} = standard uncertainty associated with material characterization; u_{hom} = standard uncertainty associated with homogeneity; u_{stab} = standard uncertainty associated with stability; k = coverage factor; k = 2: corresponds to a level of confidence of approximately 95%.

$$u_{char} = \sqrt{u^2_{c\,(I)} + u^2_{c\,(II)} + u^2_{c\,(III)}} \quad (3)$$

where: $u_{c\,(I)}$ = standard uncertainty of the measurement of bottles (n = 10) by the ISP; $u_{c\,(II)}$ = standard uncertainty of the measurement of bottles (n = 10) by the CCHEN method 1; $u_{c\,(III)}$ = standard uncertainty of the measurement of bottles (n = 10) by the CCHEN method 2;

$$u_{hom} = \sqrt{u^2_{bb}} \quad (4)$$

where: u_{bb} = standard uncertainty between-bottles, obtained from ANOVA. When ANOVA mean square between-bottles is less to Mean Square within-bottles, that is, MSB < MSW and the Equation 5 is used:

$$u_{bb} = \sqrt{\frac{MSW}{n_0}} \times \sqrt[4]{\frac{2}{v_{MSW}}} \quad (5)$$

where: n_0 = duplicates (n = 2); υ = degrees of freedom of MSW, number bottles in homogeneity test minus 1.

$$u_{stab} = s_{(b1)} \times t \quad (6)$$

where: $s_{(b1)}$ = standard deviation of slope of lineal regression of stability study; t = time interval the RM is considered stable, i.e., 250 days.

Proficiency test

Once its homogeneity and stability had been verified, it was determined that the material was suitable for the purposes of the proficiency test.

Reference material was sent to eleven national laboratories for its analysis in a proficiency test round as per the ISO/IEC 17043[21]. Reported data by the participants were requested on a dry basis for both analytes and were assessed according to ISO 13528, using the z-score statistics[22] using Equation 7:

$$z = \frac{(x - X)}{\widehat{\sigma}} \quad (7)$$

where: z = z score; X = assigned value for proficiency test (mg/kg); x = result of the participant (mg/kg); $\widehat{\sigma}$ = standard deviation for proficiency assessment (mg/kg).

Standard deviation for proficiency test round ($\widehat{\sigma}$) was established as the Horwitz standard deviation, determined as the Equation 8[23]:

$$CV_R, \% = RSD_R, \% = 2^{(1-0,5 log C)} \quad (8)$$

where: C is the concentration of analyte expressed as dimension less mass fraction (g/g); CV_R or RSD_R is a coefficient of variation of reproducibility, it is the average ratio of the standard deviation to the mean of assigned value for proficiency test mean (% average) (Equation 9).

$$CV_R = RSD_R = \frac{RSD_R}{100} \quad (9)$$

The standard deviation estimated from the Horwitz Equation for proficiency test is calculated with the Equation 10:

$$\widehat{\sigma} = \sigma_h = \bar{X} \times CV_R \quad (10)$$

where: $\widehat{\sigma}$ = standard deviation for proficiency assessment (mg/kg).

For the purposes of this procedure, the z-score values, considering the assigned value obtained from the reference value of the material produced.

For purposes of the suitability of the reference material, it was established that the standard uncertainty of the RM (assigned value) developed for proficiency testing purposes will be less than the target standard deviation (Horwitz) of the proficiency testing round, i.e (Equation 11):

$$u_{RM} < \hat{\sigma} \quad (11)$$

where: u_{RM} = standard uncertainty of the reference material (k = 1); $\widehat{\sigma}$ = standard deviation for proficiency assessment (mg/kg).

The mean values of cadmium and arsenic were calculated from the results of the participating laboratories with the Equation 12:

$$\bar{X} = \frac{\sum_{i=1}^{n} x_i}{n} \quad (12)$$

where: $\bar{X}$ = average of the reported results by laboratories (mg/kg); x_i = reported result by the laboratory (mg/kg); n = number of laboratories.

Regarding the number of participants, the median was calculated as follows:

- if n is odd, the median (Me) is determined as the Equation 13:

$$Me = X_{\frac{n+1}{2}} \quad (13)$$

- if n is even, the median (Me) is determined as the Equation 14:

$$Me = \left(x_{\frac{n}{2}} + x_{\frac{n}{2}+1}\right) \quad (14)$$

where: Me = median of the results reported by laboratories; x = value of the results in the position $\frac{n}{2}$ or $\frac{(n+1)}{2}$; n = number of laboratories.

The standard deviation of the median (MADe) was established as the Equation 15:

$$MADe = 1{,}483 \times [Me(xi - Me)] \quad (15)$$

where: Me = median of the results reported by laboratories; xi = results of laboratories.

The Grubbs[24] statistical test was used to identify outliers. Data were not excluded for the assessment, because they do not affect the assignment of the value for the performance evaluation, the assigned value was the reference value of the material.

RESULTS

Humidity

The results obtained by the humidity measuring method delivered a 5.4% of water content in fish meal in the three samples analyzed in duplicated (n = 6) (CV = 2.87%). Results of elements measurement were reported in a dry basis.

Homogeneity of the candidate reference material

The homogeneity of the reference material was assessed in relation to between and within-bottles homogeneity. The results obtained from the nine samples in duplicate they are presented in **Table 1**.

Table 1. Results of the homogeneity study of arsenic and cadmium in fishmeal

# Bottle	Arsenic		Cadmium	
	I (a)	II (b)	I (a)	II (b)
3	2.69	2.64	0.868	0.841
5	2.53	2.49	0.823	0.864
21	2.54	2.64	0.796	0.923
37	2.72	2.83	0.864	0.853
49	2.66	2.98	0.864	0.930
57	2.59	2.68	0.872	0.850
63	2.88	2.57	0.828	0.839
78	2.61	2.63	0.912	0.871
84	2.74	2.53	0.854	0.824
Average Homogeneity, $X_{Homogeneity}$, mg/kg	2.664		0.860	
Homogeneity Standard Uncertainty, u_{hom}, mg/kg	0.035		0.019	
Minimum Value, Min, mg/kg	2.494		0.796	
Maximum Value, Max, mg/kg	2.985		0.930	
Variance within-bottle, S^2_{an}	0.015 71		0.001 46	
Variance between-bottle, S^2_{am}	0.00125		0.018	
Paired Samples, m	9		9	
Calculated F value, F_{obs}	1.16		0.65	
F critical value, F_{table}	3.23		3.23	
$F_{obs} < F_{table}$	Yes		Yes	
p-value ($\alpha = 0.005$)	0.412		0.724	
Is homogeneous?	Yes		Yes	
Cochran's test — Value C	0.379		0.611	
Cochran's test — Value C critical, C_{critic}	0.638		0.638	
Cochran's test — $C < C_{critic}$	No outliers		No outliers	

The outliers' assessment was conducted with Cochran test, concluding that there was not presence of outliers, because the *C* values obtained of 0.379 for arsenic and 0.611 for cadmium were lower than the critical *C* of 0.638 for a 95% of confidence for the nine pair data (*m*). RM homogeneity value and the standard uncertainty (u_{hom}; $k = 1$) of each metal were: arsenic 2.664 ± 0.035 mg/kg and for cadmium 0.860 ± 0.019 mg/kg.

Stability of the candidate reference material

For the stability study under controlled temperature conditions and in intervals of established time, the material demonstrated to be stable in a period of 250 days for both measurands. The stability standard uncertainty (u_{stab}) obtained from the measuring was ± 0.14 mg/kg for arsenic and ± 0.056 mg/kg for cadmium.

In a linear regression for a 95% of confidence, a *p-value* higher than $\alpha = 0.05$ indicates there is no significant difference in the regression, meaning that, the material was stable, obtaining a *p-value* 0.384 for arsenic and a *p-value* 0.732 for cadmium. The absolute value of the slope $|b_1|$ for arsenic is 0.000 5 and for cadmium 0.000 08. The values of the Student's t value for 95% confidence interval for 4 degrees of freedom was 2.78, the t value multiplied by the standard deviation of the slope of regression were 0.001 5 for arsenic and 0.000 62 for cadmium (**Table 2**). The stability values were established for arsenic 2.58 ± 0.14 mg/kg (u_{stab}; $k = 1$) and for cadmium 0.832 ± 0.056 mg/kg (u_{stab}; $k = 1$).

Table 2. Results of stability study for arsenic and cadmium in fishmeal

Test	Arsenic	Cadmium
Average, $\bar{X}$, mg/kg	2.58	0.832
Slope, b_1	-0.000 5	-0.000 08
Intercept, b_o	2.641	0.842
Sample size (i.e., number of observations), n	6	6
Degrees of freedom for this test, df = n-2	4	4
Stability uncertainty, $u_{stability}$	0.14	0.056
p-value	0.384	0.732
$\|b_1\|$	0.000 5	0.000 08
t-Student critical, $t_{0.95\,,\,n-2}$	2.78	2.78
Standard deviation of slope, s (b_1)	0.000 545	0.000 224
$t_{0.95\,,\,n=2}$. s (b_1)	0.001 5	0.000 62
$\|b_1\| < t_{0.95\,,\,n=2}$. s (b_1)	Yes	Yes
Is stable?	Yes	Yes

Characterization of the candidate reference material

The characterization of the candidate reference material from the laboratories allowed the value assignment of the reference material. It was established based on the mean of the results obtained in the three reference methods used for ISP and CCHEN (**Figure 1**). The uncertainty of characterization was obtained according to GUM[17], combining the standard uncertainty obtained from the measurements made with the 3 methods with which the elements were analyzed. From this data, the obtained values of 2.64 ± 0.16 mg/kg (u_{char}; k = 1) for arsenic and 0.862 ± 0.052 mg/kg (u_{char}; k = 1) for cadmium.

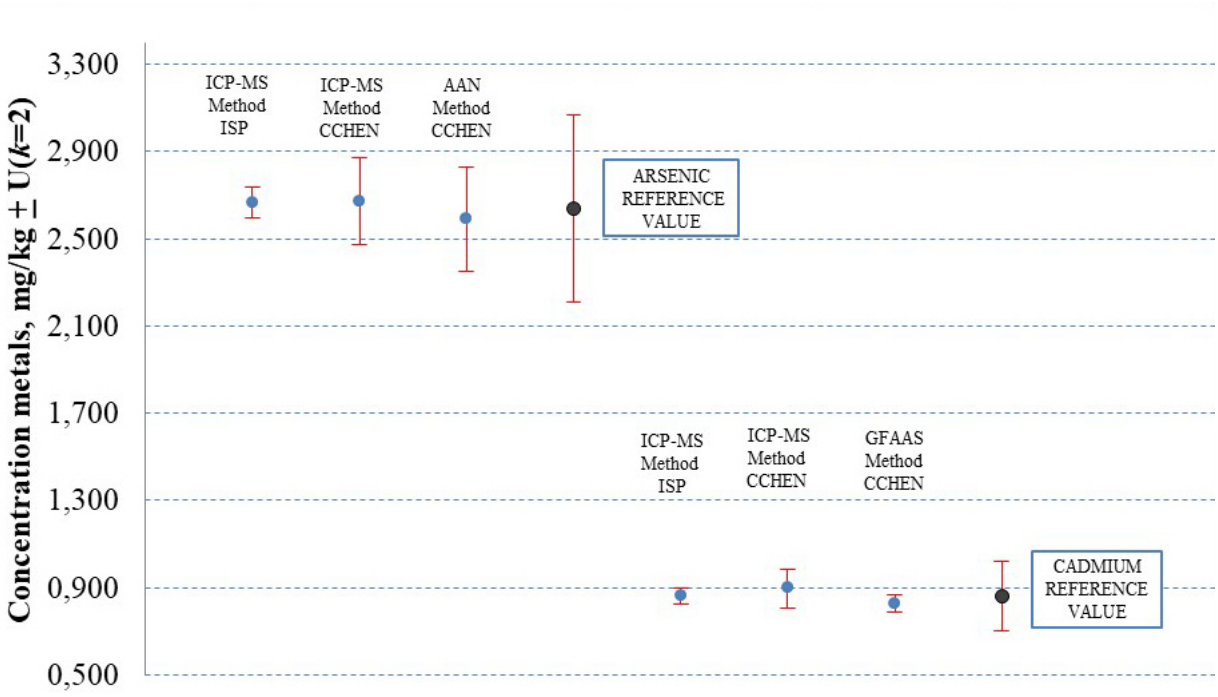

Figure 1. Comparison of results for different methods in the study of characterization of arsenic and cadmium – *RM fishmeal*

Material reference value

The combined uncertainty associated to the reference value was obtained through the combined uncertainty of the characterization, homogeneity and stability (**Table 3**). Reference values were established for arsenic 2.64 ± 0.42 mg/kg (U; k = 2) and for cadmium 0.86 ± 0.16 mg/kg (U; k = 2). The standard uncertainty (u_{RM}, k = 1) of the RM reference material was ± 0.21 mg/kg for arsenic and ± 0.079 mg/kg for cadmium.

Table 3. Value assignment for the reference material – *RM Fishmeal*

Test	Arsenic (As) mg/kg		Cadmium (Cd) mg/kg	
	C	$u_{(k=1)}$	C	$u_{(k=1)}$
Characterization	2.64	0.16	0.862	0.052
Homogeneity	2.664	0.035	0.860	0.019
Stability	2.58	0.14	0.832	0.056
Certified value	C	$U_{(k=2)}$	C	$U_{(k=2)}$
	2.64	**0.42**	**0.86**	**0.16**

C: concentration (mg/kg), u: standard uncertainty (mg/kg), U: expanded uncertainty (mg/kg).

Proficiency test

In the proficiency test, the results reported by the laboratories (n = 10) for arsenic were: Lab#1 2.771 mg/kg, Lab#2 1.742 mg/kg, Lab#3 2.087 mg/kg, Lab#4 1.610 mg/kg, Lab#5 2.140 mg/kg, Lab#6 3.350 mg/kg, Lb#7 2.730 mg/kg, Lab#8 2.400 mg/kg, Lab#9 1.023 mg/kg and Lab#10 1.742 mg/kg. For cadmium the results (n = 11) were: Lab#1 0.866 mg/kg, Lab#2 0.911 mg/kg, Lab#3 0.827 mg/kg, Lab#4 0.800 mg/kg, Lab#5 0.870 mg/kg, Lab#6 0.453 mg/kg, Lab#7 0.560 mg/kg, Lab#8 0.885 mg/kg, Lab#9 0.860 mg/kg, Lab#10 1.406 mg/kg and Lab#11 0.863 mg/kg. The laboratories reported the use of the hydride generation method of atomic absorption spectrometry for the determination of arsenic and the flame atomic absorption spectrophotometric method for the analysis of cadmium.

In the case of arsenic, the average from the participants was 2.160 mg/kg with a median of 2.114 mg/kg with a minimum reported value of 1.023 mg/kg and a maximum of 3.350 mg/kg. On the other hand, for cadmium the average was 0.846 mg/kg and the median 0.863 mg/kg, with a minimum reported value of 0.453 mg/kg and a maximum of 1.406 mg/kg. The robust standard deviations (MADe) were 0.649 mg/kg for arsenic and 0.053 mg/kg for cadmium. After applying the Grubbs test to both cases, it was identified that there were three outliers for cadmium (Lab#6, Lab#7 and Lab#10) and that there were no outliers for arsenic. The outliers were not excluded from the performance evaluation, since they did not affect the objective standard deviation that was established through the Horwitz statistical model.

The standard deviation of the Horwitz for proficiency test was 0.365 mg/kg for arsenic and 0.141 mg/kg for cadmium based on the reference value. The performance obtained by the laboratory by z-score, it is observed that most laboratories have a satisfactory evaluation for both elements (6 laboratories satisfactory for arsenic and 8 for cadmium), only an unsatisfactory result was obtained for cadmium (Lab#10) and one for arsenic (Lab#9) (**Figure 2**).

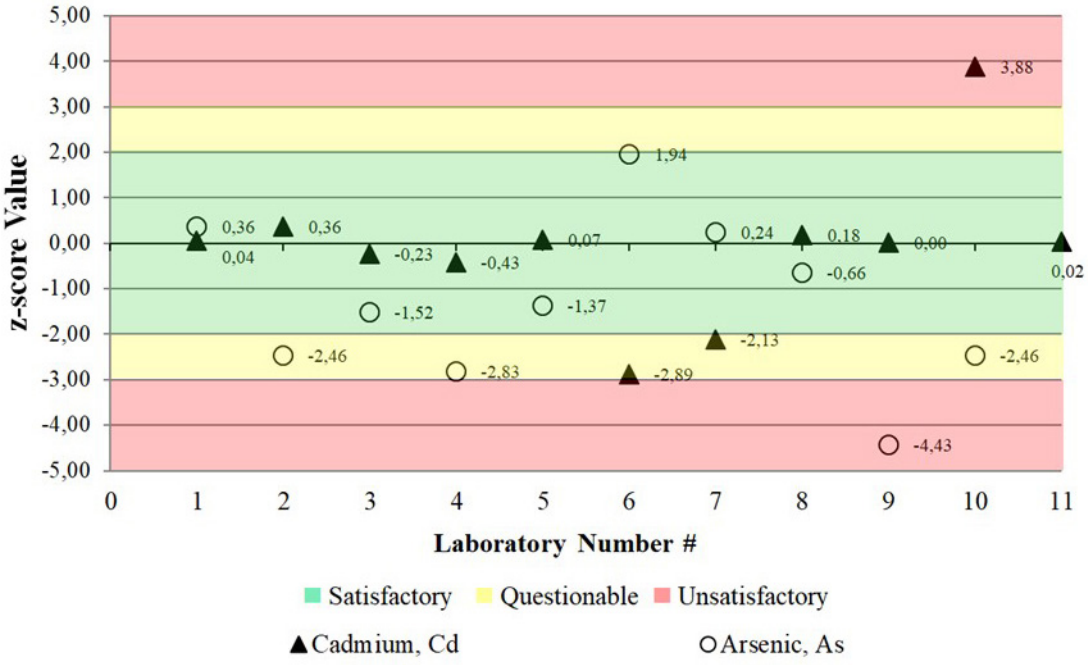

Figure 2. Performance z-score for cadmium and arsenic

DISCUSSION

The results obtained in the evaluation study of the reference material according to the ISO Guide 35 demonstrated its homogeneity, due to the ANOVA analysis, which revealed that the observed F was less than the critical F of the table for a 95% confidence interval[10], that is, $\alpha = 0.05$, with a *p-value* of 0.412 for arsenic and 0.723 for cadmium (**Table 1**).The results when applying Cochran's test in the evaluation of both elements, it is observed that the calculated value is smaller than the critical value *C* ($C_{crit} = 0.638$): arsenic $C = 0.379$ and cadmium $C = 0.611$. Therefore, we maintain the null hypothesis of homogeneity of variance[25].

No outliers were detected in the study, based on this and the results of the variances and mean values, the RM proved to be sufficiently homogeneous for the intended purpose, to be used in proficiency testing. The RM homogeneity value for arsenic was 2.664 ± 0.035 mg/kg (u_{hom}; $k = 1$) and for cadmium it was 0.860 ± 0.019 mg/kg (u_{hom}; $k = 1$).

The results of the stability study showed that in the regression obtained (**Table 2**), the absolute value of the slope $|b_1|$ for both measurands is less than a standard deviation value of the linear regression slope for the value of the factor t with 95% of confidence, for degrees of freedom n-2, i.e. $|b_1| < [t_{0.95,\,n=2} \cdot s\,(b_1)]$, indicating that no instability was observed in the samples. The RM stability study demonstrated a shelf life of 250 days, 8 months.

In the characterization study, as seen, the greatest contribution to the overall pooled uncertainty came from the method characterization uncertainty for arsenic, while the contribution from the stability uncertainty was more significant for cadmium. For both arsenic and cadmium, the characterization results obtained by both laboratories (CCHEN and ISP) and their uncertainty are comparable to each other, as can be seen in **Figure 1**.

The obtained statistical results and evaluations prove that the reference material for elements in fish meal, resultant of the ISP and the CCHEN collaboration, is homogeneous, stable and traceable

to the SI, allowing the definition of reference values for arsenic 2.64 ± 0.42 mg/kg (U; k = 2), and for cadmium 0.86 ± 0.16 mg/kg (U; k = 2). The standard uncertainty of the reference material (k = 1) met the defined criteria to be used for the proficiency test ($u_{RM} < \widehat{\sigma}$). The reference value for cadmium is below the regulated ML in marine bivalve molluscs (2 mg/kg), in the case of arsenic the reference value is above the range of MLs in food in Chile (0.1 to 0.5 mg/kg). Therefore, the reference material would satisfy the analytical requirements of the laboratories in terms of its concentration level, food matrix and metrology traceability. The results obtained demonstrated the suitability of the material to be used in a proficiency test.

The ISP organized a proficiency testing round in which 11 food testing laboratories participated, but only 10 reported values for arsenic. The median of the participating laboratories of 2.144 mg/kg and mean of 2.160 mg/kg for arsenic, are below the reference value of arsenic of 2.64 ± 0.42 mg/kg (U; k = 2), in the case of cadmium the value of the median of the participants 0.843 mg/kg and the mean of 0.846 mg/kg that are within the reference value for cadmium [0.86 ± 0.16 mg/kg (U; k = 2)]. The Horwitz standard deviation obtained for the proficiency test ($\widehat{\sigma}$) was 0.141 mg/kg for cadmium and 0.365 mg/kg for arsenic. No outliers were detected in the participants' results when applying the Grubbs test in arsenic, however; in cadmium 3 outliers were detected.

The performance evaluation z-score is based on statistics from a normal distribution meaning that about 95% of the data points will be within plus or minus two target standard deviations (± $2\widehat{\sigma}$). If a participant's z-score is outside | z-score | ≥ 2 there is a 1 in 20 chance that the result is in fact that your result is in fact an acceptable result from the end of the distribution. If a participant's z-score is outside | z-score | ≥ 3, the probability that your result is actually acceptable is only 1 in 300[26].

The performance of the laboratories in the analysis of arsenic was 60% satisfactory (|z-score|≤ 2), 30% questionable and 10% unsatisfactory (|z-score| ≥ 3). In the case of cadmium, 73% of the laboratories had a satisfactory evaluation however, 18% obtained a questionable score and 9% unsatisfactory. It is observed that for cadmium and for arsenic, only one laboratory has an unsatisfactory result. The proficiency test allowed evaluating the analytical performance of the fisher product control laboratories, and in this way, the laboratories were able to detect needs for improvement in their internal laboratory quality assurance system. The proficiency test organized with this RM provided the authorities with an independent and reliable tool to assess the competence of the national laboratories in charge of the control of fishery products.

CONCLUSION

The produced fish meal reference material met the requirements established in the ISO 17034 standard, allowing its subsequent certification. Also, the material demonstrated its adequacy by meeting the requirements established in the ISO/IEC 17043 standard, to be used in proficiency tests.

The results of the performance assessment revealed that only 60% of the laboratories obtained satisfactory results for arsenic and 73% for cadmium, which reveals that control processes in laboratories that conduct these determinations must be improved, as well as the need of the use of reference material and certified reference material in their validation processes. Intercomparison test allowed these laboratories to identify failings in their performance. According to ISO/IEC 17025 requirements standard, they should set corrective actions to improve their process.

Intercomparison tests are tools that allow to state and accreditation organizations to evaluate the performance of the authorized or accredited laboratories and this way request the necessary improvements so that their results are reliable and comparable for decision making.

The reference material *RM* Fishmeal Lot #12013 with reference values for arsenic 2.64 ± 0.42 mg/kg (U; k = 2), and for cadmium 0.86 ± 0.16 mg/kg (U; k = 2) allows to deliver a homogeneous, stable, and

traceable to SI reference material to laboratories, in order to be nationally used for proficiency testing and the validation of testing methods.

CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

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Not declared.

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