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POLLUTANT ANALYTIC

Verification of the atomic absorption spectroscopy with graphite furnace analytical method for the quantification of cadmium in cocoa almonds (Theobroma cacao)

Verificación del método analítico de espectroscopía de absorción atómica con horno de grafito para la cuantificación de cadmio en almendra de cacao (Theobroma cacao)

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Resumen

El método de espectroscopía de absorción atómica (AA) de llama para la determinación de cadmio (Cd) en almendra de cacao (*Theobroma cacao*) utilizado por Agrocalidad es tóxico para el ser humano y el ambiente; por ello, se pretende utilizar el método de espectroscopia de absorción atómica con horno de grafito (GFAAS) por ser más confiable y seguro. Así, se realizó la verificación de cuatro parámetros de desempeño del método GFAAS para cuantificar Cd en almendra de cacao utilizando material de referencia certificado (MRC) y muestras provenientes de cuatro fincas (A, B, C, D) ubicadas en la zona cacaotera de Ecuador, cantón Flavio Alfaro, provincia de Manabí. Se realizó una prueba inter-laboratorios y finalmente se elaboró el protocolo (PEE/B/14). Sobre el MRC (Cód. 07206B y 07167A) se verificó: linealidad, precisión, veracidad e incertidumbre de acuerdo con la *Guía Eurachem* de Eurolab España et al. (2016), y con el estándar IRAM 35050 (2001) se encontró linealidad entre 0 y 8 ppb con R^2=0.9988; desviación estándar de 0.0005 y 0.0022 respectivamente; sesgo en 0.007 y porcentaje de recuperación de 109.75; la incertidumbre estándar de 0.00013 y 0.00082. El contenido de Cd en las muestras de la finca A con 0.54 ppm, las Fincas B-D con 0.26 ppm y 0.15 ppm en la finca C. En la prueba inter-laboratorios se estableció la misma concentración de cadmio para la muestra C3 y, de acuerdo con lo estipulado por la Unión Europea, el cacao de las cuatro fincas podría ser exportado sin restricciones.
Palabras clave: Cadmio, cacao, verificación, análisis, método.

Abstract

The flame atomic absorption (AA) spectroscopy method for the determination of cadmium (Cd) in cocoa almond (*Theobroma cacao*) used by Agrocalidad is toxic to humans and the environment, reason for which the atomic absorption spectroscopy method with graphite furnace (GFAAS) was used, because it is more reliable and safer. Thus, four performance parameters of GFAAS method were used to quantify Cd in cocoa almond, by using certified reference material (MRC) and samples from four farms (A,B,C,D) located in the most important cocoa area of Ecuador, Flavio Alfaro city, province of Manabi. An interlaboratory test was performed and finally a protocol (PEE/B/14) was developed. Using the MRC (Code 07806B and 07167) was verified: linearity, precision, veracity and uncertainty in accordance with international standards, *Eurachem Guide* of Eurolab España et al. (2016), and with the standard IRAM 35050 (2001) were found linearity between 0 and 8 ppb with R^2 = 0.9988; standard deviation 0.0005 and 0.0022, respectively; slant was 0.007 and the recovery percentage was 109.75; standard uncertainty was 0.00013 and 0.00082. The content of Cd in samples from farm A was 0.54 ppm, B and D farms 0.26 ppm and 0.15 ppm in farm C. In the interlaboratory test, the same concentration of Cd was established for simple C3 and, in accordance with the stipulated by the European Union, cocoa from the four farms could be exported without restrictions.
Keywords: Cadmium, cocoa, verification, analysis, method.

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1 Introduction

Cocoa beans are the seeds of the *Theobroma cacao* tree (native to the Amazon Region of South America), which are widely consumed worldwide (Almeida and Valle, 2007). It is an important neotropical, perennial crop growing at 20° north and 20° south of the equatorial line (Shavez Beg et al., 2017). It is grown at a height of less than 400 m.a.s.l; the optimal temperature ranges from 22 $°C$ to 30 $°C$, depending on the variety (Damatta et al., 2018), and rain should be at least 1500 to 2500 mm of water per year. The almonds are brown and are covered on the outside by a sweet white mucilage that is edible (Waizel-Haiat et al., 2012); grains are processed to obtain chocolate liqueur, cocoa powder and cocoa butter, which are the main ingredients of chocolate and a wide range of products such as cocoa drinks, ice cream, bakery products that distribute a distinctive flavor to derivatives (Dasgupta and Klein, 2014). Cocoa beans are the raw material for the multibillion-dollar industry that produces chocolate and confectionery products, the economic importance of the chocolate industry in the world market has recently been revised (Squicciarini and Swinnen, 2016) rising 13% from 2010 to US$101 billion in 2015, with Switzerland as the highest consumer rate (Wickramasuriya and Dunwell, 2018).

Ecuador, because of its geographical conditions and its richness in biological resources, is the quintessential producer of fine-scented cocoa (63% of world production) from the national variety, whose flavor has been recognized for centuries on the international market. This type of grain is used in all refined chocolates. Of the total Ecuadorian export, 75% is estimated to be fine-scented cocoa while the remaining 25% belong to other varieties such as CCN51. Ecuador ranks as the most competitive country in Latin America in this field, followed by Venezuela, Panama and Mexico, which are countries that have gradually increased their share of the global market (Anecacao, 2019).

Various attributes of grain quality, both physical and chemical, are required by manufacturers, cocoa buyers and control bodies to encourage the cocoa community towards better quality production (CAOBISCO/ECA/FCC, 2015). These quality characteristics include taste, purity and health (e.g. bacteria-free, infestation, allergens, mycotoxins, heavy metals and pesticide residues), physical characteristics (e.g. consistency, yield of grain edible material, size and uniformity, shell content, fat content and moisture content) and the characteristics of cocoa butter (e.g. free of fatty acid) (Dasgupta and Klein, 2014). Some of the attributes of grain quality, such as the total fat content, acidity, total phenols, organic acids, heavy metals, amino acids, caffeine, theobromine, pH, sugars, macro content and micronutrients, have been considered in the proposal Cocoa Quality Index (CQI) for some types of grain (Araujo et al., 2014).

Heavy metals are defined as elements with a density of more than 5 g/cm^3 (Navarro-Aviñó et al., 2007). Copper, iron, magnesium and zinc in low concentrations are essential for biochemical and physiological processes in plants, while arsenic, cadmium and lead do not have a known role in them (Ali et al., 2013). The accumulation of heavy metals in plants is affected by several factors such as pH, organic matter content and soil texture, plant genotype and heavy metal content in the growth medium. Latin America has the highest levels of heavy metals in cocoa beans, especially cadmium and lead (Bertoldi et al., 2016), compared to other producers in the world. The presence of heavy metals in cocoa beans represents a threat to cocoa producers, as a high heavy metal content could affect the export of the grains.

Chocolate has been attributed to the achievement of optimal human health and development, due to its high content of flavonoids that are crucial to reducing the risk or in turn delaying the development of cardiovascular disease, cancer and others related to age (Cooper et al., 2008).

In 2014, the European Union announced, through the Regulation (EU) No. 488/2014, plans to implement regulations on chocolate and cocoa products containing excessive levels of cadmium (Cd), which entered into force on January 1, 2019 (Commission Regulation EU, 2014). Non-compliance with regulations would have significant economic and social consequences for cocoa-producing countries, such as Ecuador, from the date of entry into force of that regulation. The Cd has received attention in the last decade due to its importance in food quality/safety, and in human health, since the consumption of foods high in this heavy metal could

lead to renal tubular dysfunction, the formation of kidney stones, the alteration of calcium metabolism and skeletal, endocrine, reproductive and respiratory defects (Järup and Akesson, 2009).

Analytical techniques for the determination of Cd are based on the use of reagents that act on the grain digestion and that are highly hazardous. One of the reagents of frequent use is *Agua Regia* $(HCl : HNO_3)$, which is subjected to heat resulting in irritating and corrosive vapors; the protocol used corresponds to an adaptation of the official AOAC method: 999.11 (AOAC, 2005).

As a more effective analytical method and less toxic to humans and the environment, this paper verified the performance of the graphite furnace atomic absorption spectroscopy (GFAAS) method in the determination of Cd in cocoa almond (*Theobroma cacao*), by determining linearity, accuracy and veracity (Eurolab España et al., 2016) and uncertainty (IRAM 35050, 2001) on certified reference material, and the analytical protocol for the determination of the concentration of Cd in cocoa almond samples was validated; in addition, an inter-lab comparison test was performed.

2 Materials and methods

First Phase: the verification of the analytical method was performed by evaluating the linearity, accuracy, veracity and uncertainty parameters, based on certified material MRC 07206B and 07167A.

Second phase: cocoa samples (ears) were collected by designated AGROCALIDAD personnel, on four farms in the country's cocoa area: Flavio Alfaro-Manabí, Ecuador, referred to as A, B, C, D. Subsequently, the corresponding samples were analyzed in the Nutritional Science and Microbiology Laboratory to determine their Cd content, after having verified the calibration status of the instruments and volumetric materials to be used.

Third phase: the PEE/B/14 protocol for the determination of Cd in cocoa almond by GFAAS was developed, containing the sample preparation process (cocoa ear) from their reception, drying, homogenization, microwave digestion and subsequent reading in the graphite oven spectrometer.

2.1 Verification of the analytical method

The verification of the analytical method was done as reference to the Eurolab Spain Eurachem Guide Eurolab España et al. (2016), and to the IRAM 35050 (2001), following the PEE/B/14 protocol.

2.1.1 Linearity

A curve with 10 concentrations was developed from the Cd standard of 20 ppb, the concentrations used were 0 to 18 ppb. In the curve, the theoretical concentration was expressed in $\mu g/L$ on the axis of the "x", and on the axis of the "y" the calculated concentration was expressed in $\mu g/L$; from the resulting curve, the linear range was chosen and a new curve was drawn. In this linear range, a white, six different dilutions of the standard of 20 ppb and finally the MRC 07206B and 07167A were evaluated by triplicate.

2.1.2 Accuracy

Fifteen readings were made for each MRC 07206B and 07167A and the standard deviation (s) of each material was calculated, using the following formula:

$$s = \sqrt{\frac{1}{n-1}\sum_{k=1}^{n}(x-\bar{x})^2} \quad (1)$$

Standard deviation (s)
Where:
$n =$ number of total measurements of the reference material.
$\bar{x} =$ average of the readings.
$x =$ each measurement performed.

2.1.3 Veracity

Ten readings for MRC 07206B, ten readings for three digested targets and ten readings for three whites added with 2, 3 and 4 ppb of Cd were done; in addition; ten readings were also performed on three samples analyzed. In the first instance, by the method normally used by the laboratory, using acid digestion with *Agua Regia* $(HCl : HNO_3)$, followed by a reading using flame atomic absorption spectroscopy (AA) and, secondly, by the verified GFAAS method and the analysis protocol proposed in this research.

The data obtained for the MRC 07206B proceeded to perform the calculations with the following formulas provided by Eurolab España et al. (2016):

$$b = \bar{x} - x_{ref} \tag{2}$$

Bias

$$b(\%) = \frac{\bar{x} - x_{ref}}{x_{ref}} \times 100 \tag{3}$$

Bias in Percentage

$$R(\%) = \frac{\bar{x}}{x_{ref}} \times 100 \tag{4}$$

Relative Recovery
Where:
$\bar{x}$ = mean of the readings of the MRC 07206B.
x_{ref} = theoretical value that the certificate grants to the reference material.
The calculated values obtained a measure of bias taking into account the effects of the laboratory compared to the bias method data.
For the analysis of the data obtained from the measurements of digested and added whites, the following formula Eurolab España et al. (2016) was used:

$$R'(\%) = \frac{\bar{x}' - \bar{x}}{x_{addition}} \times 100 \tag{5}$$

Relative retrieval of additions
Where:
$\bar{x}'$ = average value of the added whites.
$\bar{x}$ = is the average value of the digested whites.
$x_{addition}$ = concentration value added.
Finally, with the data obtained from the reading of samples by the method normally used in the laboratory, the verified GFAAS method and the proposed analysis protocol, the calculations were performed based on the following equations Eurolab España et al. (2016):

$$b = \bar{x} - \bar{x}_{ref} \tag{6}$$

Bias

$$b(\%) = \frac{\bar{x} - \bar{x}_{ref}}{\bar{x}_{ref}} \times 100 \tag{7}$$

Bias in Percentage

$$R(\%) = \frac{\bar{x}}{\bar{x}_{ref}} \times 100 \tag{8}$$

Relative Recovery
Where:
$\bar{x}$ = means of the sample readings by the old method.
$\bar{x}_{ref}$ = average sample readings by the proposed new method.
The measurement of bias was obtained according to the verified GFAAS method and the proposed analysis protocol, in order to demonstrate its effectiveness and replace the method normally used by AGROCALIDAD.

2.1.4 Uncertainty

10 readings of the concentration of the reference material were performed and the uncertainty values were calculated with the following formulas (IRAM 35050, 2001):

$$\bar{I} = \frac{1}{n} \sum_{k=1}^{n} I_k \tag{9}$$

Average (I) of readings
Where:
n = number of measurements of the reference material.
I_k = each measurement of the reference material

$$S(I_k) = \sqrt{\frac{1}{n-1} \sum_{k=1}^{n} \left(I_k - \bar{I}\right)^2} \tag{10}$$

Standard deviation (I_k)
Where:
n = number of total measurements of the reference material.
$\bar{I}$ = the average of the readings.
I_k = each measurement performed.

$$S\left(\bar{I}\right) = \frac{S(I_k)}{\sqrt{n}} \tag{11}$$

Standard uncertainty $S(I)$
Where:
$S(I_k)$ = the standard deviation
n = number of total measurements of the reference material.

2.2 Determination of cadmium in cocoa almond

With samples obtained from Farms A, B, C and D, the elaborate protocol (Procedure PEE/B/14) was done for the determination of Cd in cocoa almonds, detailing:

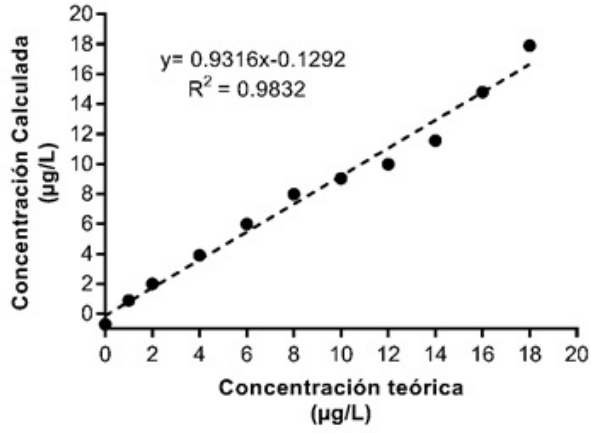

Figure 1. Linear range evaluated with different Cd standard concentrations

1. *Sample processing:* The cocoa almonds were dried on the stove at a temperature of 150 $^{\circ}C$, for a period of 24 hours and it was verified that the shell is easily peeled off, otherwise drying was continued for an additional 12 or 24 hours and a new verification was performed before removing cocoa from the stove. The peeling was done manually with the hot samples to make it easier to husk. To peel the grain, it was pressed with the fingertips or gently striking with a mortar. Approximately 20 g of sample was processed in the mill, until a homogeneous product was obtained. The processed sample was stored in polypropylene bottles with the sample code.
2. *Digestion:* Accurately 0.5 g of dry sample was weighted in the digestion container and microwave digestion was subjected to the parameters indicated by the equipment.
3. *Preparation of standards:* 20 ml of 1000 ppb Cd mother solution was prepared. 2 ml of 1000 $\mu g/ml$ Cd standard was taken with volumetric pipette and graduated to 20 ml with 1 % of nitric acid. From this solution, the graphite furnace atomic absorption equipment automatically performed the corresponding dilutions (1-5 ppb).
4. *Reading of the samples:* The concentration of each encoded sample on the computer was measured. The equipment was programmed to perform triplicate readings on the same sample and the result was expressed in parts per billion (ppb).

2.3 Statistical analysis

The statistical analysis was carried out taking into account each method verification parameter: linearity, accuracy, veracity and uncertainty, each of which provides formulas for the calculation of means, bias, relative bias and recovery. In addition, a variance analysis was performed in the determination of Cd in cocoa almonds to determine whether there are significant differences between the samples obtained on each farm. The statistical program Graphpad Prism 7 was used.

3 Results

3.1 Verification of the analytical method

3.1.1 Linearity

In Figure 1, the theoretical concentration (0-18 ppb) was plotted versus that provided by the equipment

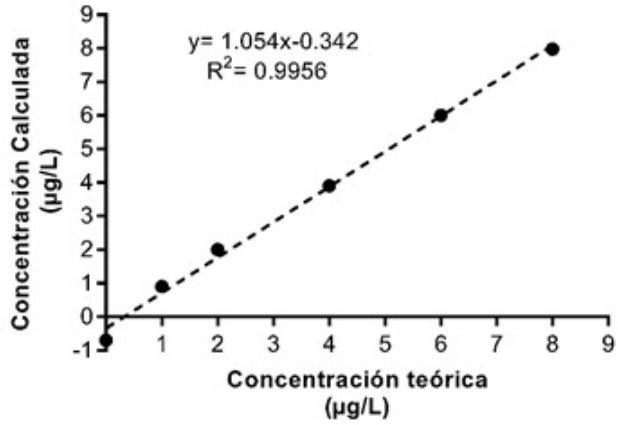

Figure 2. Linear range of the method 0-8 ppb.

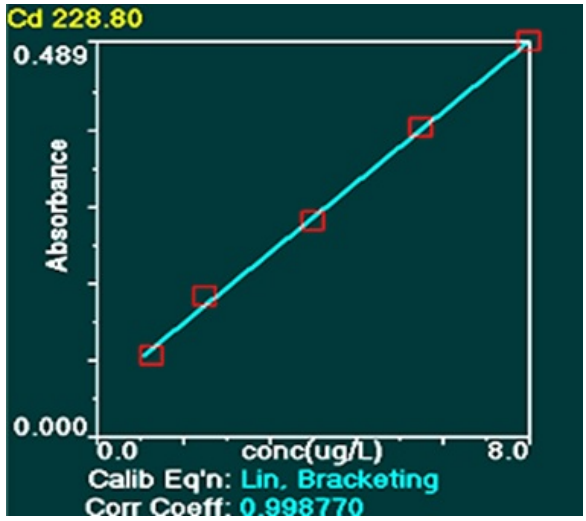

Figure 3. Linear range obtained by the equipment

(calculated), and a visual analysis was performed in which the linear range was established to be between 0 and 8 ppb (Figure 2 and 3).

The linear range is between 0 and 8 ppb (Figure 2), with a residuality coefficient of 0.9956, acceptable for analytical procedures.

The GFAAS method yielded a calibration curve (Figure 3), which presented a residuality coefficient of 0.998770, much more accurate than the one calculated, thus it only uses the points of interest, eliminating background errors. To confirm the linearity of the method and following the procedure of the Eurachem Guide (2016), a target and six known concentration solutions were measured in the linear range given by the equipment, which is represented in Figure 4. Figure 4.A. shows that the residuality coefficient was 0.9964; Figure 4.B. 0.9976 and Figure 4.C. 0.9952, confirming the linearity of the method.

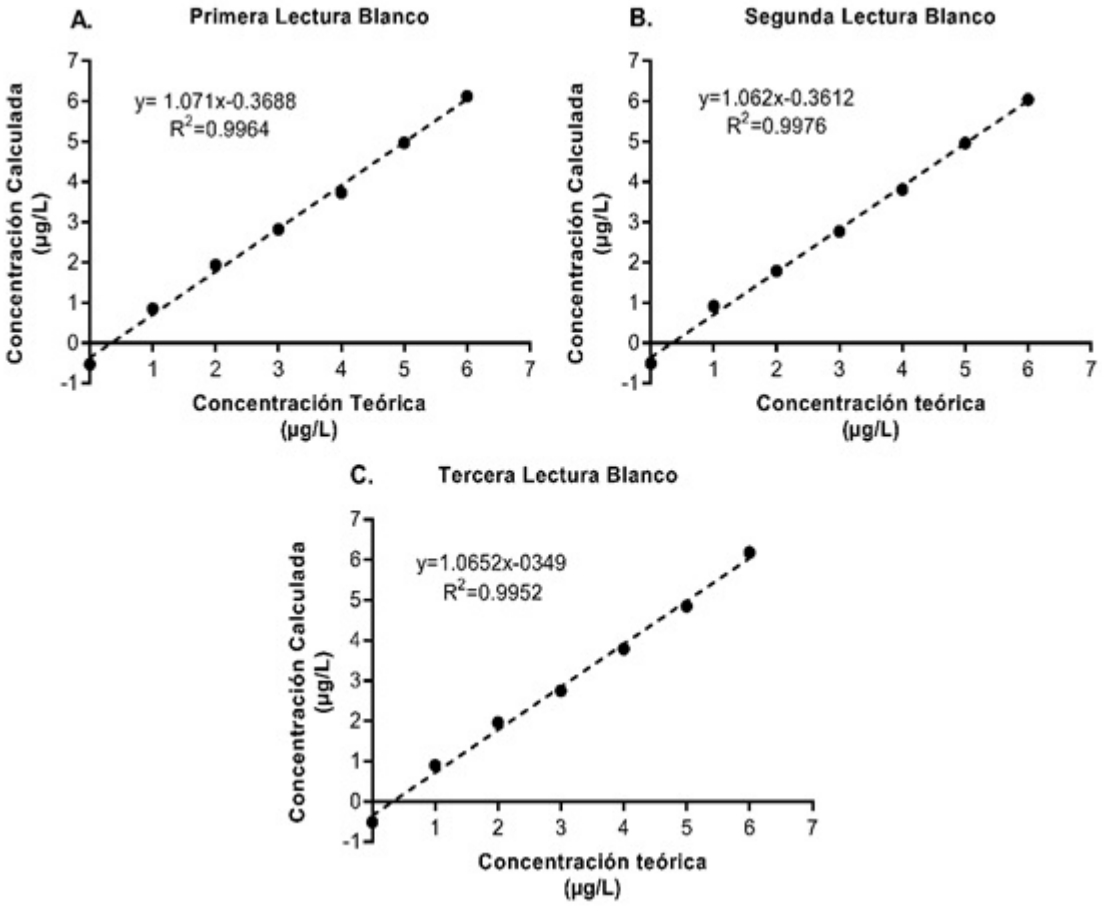

Figure 4. A. First reading of a white and six Cd standards; B. Second reading of a white and six Cd standards; C. Third reading of a white and six Cd.

3.1.2 Accuracy

The accuracy of the analyst, equipment and analytical method were measured by 15 readings of two reference certified materials 07206B and 07167A, obtaining a minimum and less standard deviation compared to the obtained by the manufacturer of the equipment, which was 0.02 as seen in Table 1.

3.1.3 Veracity

The readings of the certified reference material 07167A were made in the measurement of veracity; on the basis of the data obtained the mean, bias, relative bias in percentage and relative recovery were calculated, using as a reference value the data provided by the reference material certificate, as shown in Table 2.

In addition, ten readings of three whites and ten readings of the same white added with 2, 3 and 4 ppb of Cd were made, thus obtaining the mean and percentage of recovery, as shown in Table 3.

Three sample readings were finally made by the method normally used by the laboratory (AA), by the verified GFAAS method and the proposed protocol, as shown in Table 4.

Table 4 shows that the bias in the reading of the three samples by the two methods is minimal and the recovery percentage is high, which gives reliability to the GFAAS method.

Table 1. Concentration of cadmium obtained from 15 readings of two certified cocoa powder reference materials.

Reading number	CERTIFIED REFERENCE MATERIAL			
	Reading obtained by the equipment		Calculated Final Concentration	
	07206B ($\mu g/L$)	07167A ($\mu g/L$)	07206B (mg/kg)	07167A (mg/kg)
1	1.608	1.656	0.0803	0.0820
2	1.605	1.794	0.0801	0.0888
3	1.612	1.604	0.0805	0.0794
4	1.610	1.626	0.0804	0.0805
5	1.609	1.656	0.0803	0.0820
6	1.632	1.614	0.0815	0.0799
7	1.605	1.637	0.0801	0.0810
8	1.615	1.644	0.0806	0.0814
9	1.610	1.655	0.0804	0.0819
10	1.603	1.645	0.0800	0.0814
11	1.608	1.656	0.0803	0.0820
12	1.603	1.702	0.0800	0.0842
13	1.611	1.668	0.0804	0.0826
14	1.622	1.644	0.0810	0.0814
15	1.631	1.656	0.0814	0.0820
Standard deviation (*s*)			0,0005	0,0022

Note: Values are reported in milligrams per kilogram. The final calculated concentration was based on the reading of the equipment by the capacity volume and dilution factor, divided for the weight of the sample per thousand.

3.1.4 Uncertainty

Cd concentration of two reference materials 07206B and 07167A was measured ten times, and the mean, standard deviation and standard uncertainty (Table 5) was calculated.

The uncertainty calculated by measuring the Cd concentration of MRC (07206B and 07167A) is 0.00013 and 0.00082, respectively (Table 5), indicating that measurements made were not affected by systematic errors in the process.

3.2 Determination of cadmium in cocoa almonds

Samples from four farms located in Alfaro-Manabí estates named A, B, C and D (Table 6) were analyzed, collecting 15 samples from farm A, 9 samples from farm B, 4 from farm C and 5 of farm D, according to internal agroquality protocols, establishing the following hypothesis:

Null hypothesis: the concentration of Cd in cocoa almonds from the four farms is not significantly different.

Alternative hypothesis: the concentration of Cd in cocoa almonds of at least one farm differs significantly from the others.

After performing the analysis of variance (Table 7) being $p < 0,05$, the alternative hypothesis is accepted, i.e., the concentration of Cd in cocoa almond of at least one farm differs from the others.

Farm A that has on average 0.546 mg/Kg, being the highest value compared to the other farms. It should be mentioned that between Farm B and D there are no significant differences since in the two farms the concentration of Cd in cocoa almond was 0.260 mg/Kg. Farm C has the lowest concentration of Cd in cocoa almond, being 0.146 mg/Kg.

Table 2. Cadmium concentration obtained from 10 readings of the certified cocoa powder reference material

Reading	Reference Material	
	07167A $(\mu g/L)$	07167A (mg/Kg)
1	1.614	0.080
2	1.637	0.081
3	1.655	0.082
4	1.655	0.082
5	1.645	0.081
6	1.656	0.082
7	1.612	0.080
8	1.668	0.083
9	1.623	0.080
10	1.644	0.081
Media		0.081
Reference Value		0.074
Bias		0.007
Relative bias in percentage		9.75
Relative recovery		109.75

Note: The values reported by the equipment are in $\mu g/L$. The calculated final concentration (mg/kg), is based on the reading of the equipment by the volume of capacity and dilution factor, divided for the weight of the sample per thousand.

Table 3. Reading of three whites and whites added with 2, 3 and 4 ppb of cadmium

No. Reading	WHITES $(\mu g/L)$			ADDED WHITES $(\mu g/L)$		
	B2V	**B3V**	**B4V**	**BA2V**	**BA3V**	**BA4V**
1	−0.5278	−0.7799	−0.4077	2.098	3.115	3.780
2	−0.5017	−0.7791	−0.3903	2.084	3.185	3.898
3	−0.5106	−0.7442	−0.4038	1.962	3.247	4.165
4	−0.5613	−0.7247	−0.4415	1.939	3.025	4.036
5	−0.4176	−0.7153	−0.4453	1.962	3.004	4.054
6	−0.5200	−0.7298	−0.4692	1.963	3.027	4.076
7	−0.6586	−0.7062	−0.4406	1.876	3.057	4.185
8	−0.5363	−0.7423	−0.4894	1.878	2.947	4.196
9	−0.5810	−0.7847	−0.4962	1.890	3.030	4.223
10	−0.5134	−0.7700	−0.4194	1.800	3.137	4.263
Media	−0.53283	−0.74762	−0.44034	1.945	3.077	4.088
Recovery $(\%)$				123.902	127.501	113.199

Note: The units are reported in $\mu g/L$. in the B2V code means: White number two veracity. BA2V stands for, BA2: White added with 2 ppb of Cd; V: veracity.

3.3 Inter-laboratory test

The sample with code C3 was sent to UBA Laboratories located in the city of Guayaquil, Ecuador. The reported result was 0.11 ppm of Cd, which coincides with the result obtained in Laboratory of Nuritional Science and Microbiology of Agroquality.

Table 4. Cadmium concentration in three samples evaluated by GFAAS and AA

Reading	GFAAS Sample Reading			AA Sample Reading		
	A8A	**B23A**	**D6A**	**A8A**	**B23A**	**D6A**
1	1.356	0.174	0.991	1.348	0.178	0.985
2	1.348	0.177	0.996	1.387	0.174	0.981
3	1.358	0.178	0.989	1.356	0.180	0.964
4	1.363	0.175	0.983	1.364	0.173	0.994
5	1.361	0.176	0.987	1.377	0.178	0.997
6	1.371	0.174	0.981	1.374	0.180	0.999
7	1.376	0.180	0.998	1.371	0.171	0.997
8	1.368	0.182	0.993	1.350	0.178	0.986
9	1.373	0.182	1.001	1.336	0.173	0.992
10	1.366	0.180	0.989	1.342	0.173	0.988
Media	**1.364**	**0.178**	**0.991**	**1.361**	**0.176**	**0.988**
Bias	0.004	0.002	0.003			
Bias %	0.26	1.16	0.29			
R %	100.26	101.16	100.29			

Note: Units are expressed in mg/Kg. The code A8A means: A: The name given to the Farm; 8A: sample numbering.

Table 5. Cadmium concentration of two reference materials

Reading number	CERTIFIED REFERENCE MATERIAL			
	Reading obtained by the equipment		Calculated Final Concentration	
	07206B $(\mu g/L)$	07167A $(\mu g/L)$	07206B (mg/Kg)	07167A (mg/Kg)
1	1.608	1.656	0.0803	0.0820
2	1.605	1.794	0.0801	0.0888
3	1.612	1.604	0.0805	0.0794
4	1.610	1.626	0.0804	0.0805
5	1.609	1.656	0.0803	0.0820
6	1.632	1.614	0.0815	0.0799
7	1.605	1.637	0.0801	0.0810
8	1.615	1.644	0.0806	0.0814
9	1.610	1.655	0.0804	0.0819
10	1.603	1.645	0.0800	0.0814
Media			0.0804	0.0818
Standard deviation			0.0004	0.0026
Standard uncertainty			0.00013	0.00082

Note: Values are expressed in milligrams per kilogram. The calculated final concentration is based on the reading of the equipment by the capacity volume and dilution factor, divided for the weight of the sample per thousand.

4 Discussion

The verification of the analytical method of atomic absorption spectroscopy with graphite furnace in the Laboratory of Nutritional Science and Microbiology of Agroquality, complied with the performance parameters: linearity, precision, veracity according to the Eurachem Guide (Eurolab España et al., 2016) and uncertainty according to the Guide of the Uncertainty Calculation of the Argentine Institute of Standardization and Certification IRAM 35050 (2001). Linearity was worked with a curve in

Table 6. Concentration of cadmium in cocoa almonds of Farms A, B, C and D

Sample	Cad concentration (mg/Kg)			
	Farm A	Farm B	Farm C	Farm D
1	0.60	0.60	0.12	0.12
2	0.79	0.10	0.18	0.11
3	0.60	0.01	0.11	0.24
4	0.88	0.34	0.17	0.30
5	0.74	0.30		0.54
6	0.89	0.40		
7	0.50	0.40		
8	0.54	0.09		
9	0.55	0.11		
10	0.49			
11	0.54			
12	0.15			
13	0.13			
14	0.40			
15	0.39			

Note: Values are expressed in milligrams per kilogram. The sample numbers do not correspond to the numbering expressed to the samples on the labeling

a range of 0 to 8 ppb, whose $R^2 = 0.998770$, which by its proximity to $+1$ indicates that there is a "perfect" correlation between the variables being acceptable for analytical measurements (Gaddis and Gaddis, 1990).

Accuracy for two certified reference materials (07206B and 07167A) yielded a standard deviation of 0.0005 and 0.0022, respectively, implying that the measurements made by the analyst were accurate and repeatable. As for the veracity, it was found that the proposed methodology yields result similar to the non-standardized and toxic methodology that the Laboratory of Nutritional Science and Microbiology Agroquality used for the analysis of Cd, since the bias between the two methodologies was minimal and the recovery rate was greater than 100%. This is in agreement with what Ospina and Zapata (2012) mentioned, who point out that the recovery percentage in an analytical method should be in a range of 80% to 120%. In addition, it is comparable with the validation of (Lo Dico et al., 2018) in which they obtain a recovery percentage of 110%. The standard uncertainty was 0.00013 and 0.00082, which gives reliability to the proposed method and leads to the assumption that working in a closed environment and with the other laboratory equipment turned off leads to minimizing errors as mentioned (Gonzaga, 2016).

Table 7. Summary of the analysis of variance

Groups	Count	Sum	Average	Variance
Farm A	15	8.190	0.546	0.051
Farm B	9	2.350	0.261	0.038
Farm C	4	0.585	0.146	0.001
Farm D	5	1.310	0.262	0.031

A related investigation is that of Acosta and Pozo (2013) in which the official Method AOAC 999.11 was applied, where the verification of performance parameters in the Agroquality laboratory is not evident, thus the results are unreliable. However, the same author recommends using for the digestion of the sample, a type of microwave digestion which is faster and more effective; therefore, in this research this methodology of digestion based on the standard was used (AOAC, 2010), obtaining good results. In related research, the technique of flame-coupled spectroscopy with perchloric digestion such as Mite et al. (2010), and a pre-coupled nitric digestion followed by atomic emission spectroscopy reading is used (ICP-MS) from Chavez et al. (2015). However, ICP-MS can be used after microwave digestion, which gives greater specificity of the analysis (Lo Dico et al., 2018).

As stipulated by the European Union (0.8 ppm of Cd in cocoa powder), which entered into force in January 2019, the cocoa from the four farms presented in this study could be exported without restriction, having been at its levels (0.546 A; 0.261 B; 0.146 C; 0.262 D) within the allowed range. They also comply with the codex Committee on Food Pollutants (CCCF), which, at the 9th session in the Netherlands, stipulates a limit of 1.5 ppm (CCCF, 2015).

According to Chavez et al. (2015) the difference in the content of Cd in the four farms can be attributed to the existence of heavy metal contamination in the irrigation water or soil contamination (Rankin et al., 2005). On the other hand, Acosta and Pozo (2013) refer to the location of the crops, i.e., if they are near the paved road, heavy metals are more likely to accumulate in both soil and aerial parts of the plants, because of emissions from vehicles circulating into the atmosphere, which would not happen with farms far from the road. In addition to the above, Mite et al. (2010) note that Cd contamination of cocoa almonds can also be caused by burning urban waste, use of urban sludge in agriculture, agrochemicals and, pollution by petroleum derivatives when drying cocoa on the roads. Argüello et al. (2019) in a recent investigation mention that the concentration of Cd in cocoa almonds may vary from farm to farm with respect to the genotype cultivated (CCN-51 vs. National).

Although this study did not focus on the determination of Cd in cocoa almonds but on a validation of the analytical method for quantifying this metal, it has been possible to contribute significantly to the development of subsequent studies where GFAAS is used and, investigate the traceability of the Cd in the almonds, it being clear that the soil is the largest supply of this metal, being able to implement mitigation strategies, such as bioremediation.

5 Conclusions

The performance parameters of the analytical method of atomic absorption spectroscopy with graphite furnace were successfully verified, thus ensuring that the method is reliable and that it can be used in the Laboratory of Nutritional Science and Microbiology of Agroquality. The developed protocol (PEE/B/14) allows the correct application of the method in the analysis of Cd in cocoa Almond, since it defines a standard operating procedure that ensures faithful compliance with the test requirements for reliable results. Furthermore, differences were evident in the concentration of Cd in cocoa almonds of the farms under study, which are related to the source of the samples. However, all the samples analyzed revealed lower concentrations of Cd than the stipulated by the European Union (0.8 ppm of Cd in cocoa powder); in addition, they comply with the CCCF's standard, a limit of 1.5 ppm, so this cocoa could be exported unrestricted.
The inter-laboratory test carried out confirms the validity of the method verified in this investigation. In view of the difference in the concentration of Cd according to the source of the samples, it is advisable to carry out other investigations concerning the factors involved in the contamination of this element in cocoa.

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