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Research Article

Total Reflection X-Ray Fluorescence Spectroscopy (TXRF) Method Validation: Determination of Heavy Metals in Dietary Supplements

Blanca G. Beltrán,[1] **Victor Ramos-Sanchez**,[2] **David Chávez-Flores**,[2] **Rogelio Rodríguez-Maese**,[3] **and Edwin Palacio**[4]

[1]*Faculty of Nursing and Nutriology, Chihuahua Autonomous University, Chihuahua 31125, Mexico*
[2]*Faculty of Chemical Sciences, Chihuahua Autonomous University, Chihuahua 31125, Mexico*
[3]*Environment and Energy Department, Advances Materials Research Center (CIMAV) S.C, Miguel de Cervantes 120, Chihuahua 31109, Mexico*
[4]*Laboratory of Environmental Analytical Chemistry, University of the Balearic Islands, Cra. Valldemossa km 7'5 Palma de Mallorca, Palma, Spain*

Correspondence should be addressed to Blanca G. Beltrán; bbeltran@uach.mx

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Dietary supplements may contain heavy metals with the property of bioaccumulation in humans. The aim of this research was to validate and apply two analytical methods to determine Pb, As, Cr, and Hg in dietary supplements by Total Reflection X-ray Fluorescence Spectroscopy (TXRF). Methods validation was conducted through a multivariate analysis using a central composite design (CCD) and a desirability function. Critical values for each study variable were established. The TXRF_DS_1 method was proposed for Pb, As, and Cr determinations, while the TXRF_DS_2 was established for Hg analysis. The digestion method with an acid mixture ($HNO_3 + HCl + H_2O_2$) was used to break down the organic material of dietary supplements. A solution of 10 $\mu g\,L^{-1}$ Ga was used as an internal standard. Excellent analytical performance was obtained as LODs of 0.59, 0.41, 0.57, and 0.75 $\mu g\,L^{-1}$ and LOQs of 1.95, 1.35, 1.90, and 2.50 μgL^{-1} for Pb, As, Cr, and Hg, respectively. Calibration curves showed a good linearity for all elements (R^2>0.999). Excellent accuracy and precision in measurements (% RSD) was achieved. The real and spiked samples analysis demonstrated the applicability of the TXRF technique (percentage recovery 91–108%). Besides, two samples were analyzed in a comparison study between the TXRF_DS_1 method and the ICP-OES method. The results obtained showed good agreement between both techniques. The TXRF technique allows the analysis of toxic heavy metals in dietary supplements, which are marketed in a wide variety of presentations.

1. Introduction

Dietary supplements are products that contain a "dietary ingredient" which includes vitamins, minerals, and amino acids, as well as other substances that can be used to supplement the diet [1]. They can be prepared from decoctions from fresh or dried crude herbs from algae or plants, but are usually made into different presentations, including powders, tablets, capsules, energy bars, and liquids [2]. The consumers believe that since these products contain ingredients that come from plants, they are safe and without any adverse effect [3]. However, many ingredients are derived from natural products, which can be toxic due to the presence of heavy metals.

Dietary supplements could contain heavy metals due two fundamental reasons. (a) Their distribution is not uniform, such that some soils may contain higher concentrations derived from natural components or geological sources as well as from human activities or anthropogenic causes. Taken up by plants, heavy metals may enter the food chain in

significant amounts. Hence, people could be at risk of adverse health effects from consuming dietary supplements made with plants grown in soils containing elevated metal concentrations [4]. (b) Contamination may occur during the production processes (manufacturing, handling, storage, processing, or distribution). Heavy metals such as Pb, As, Hg, and Cr are most commonly the subject of attention in manufacturing dietary supplements [4, 5].

The botanical ingredients in dietary supplements may contain elevated concentrations of As, contributing to the overall exposure to this element [5]. Arsenic exposure affects all organ systems, including the cardiovascular, dermatologic, nervous, renal, gastrointestinal, and respiratory systems. Also, it is now well accepted that exposure to high levels is associated with an increased risk of various types of cancers, most notably, skin, urinary bladder, and lung [6].

Lead is poisonous to humans and can affect people of any age or health status. Lead is especially harmful to vulnerable populations, including infants, young children, pregnant women, and their fetuses, and others with chronic health conditions [7]. On the other hand, Pb poisoning mainly affects the nervous system (headache, decreased attention span, irritability, loss of memory, and dullness are the early symptoms) [8].

Chromium is a mineral nutrient that is essential for the proper functioning of many systems in the human body. Some studies have observed that Cr (III) could improve insulin action, but the relation between Cr and insulin is not well known. This element also could participate in lipid metabolism and could have some effect on body composition [9]. The major problem with some dietary supplements is that their labels do not show the concentration of either the oxidation estate of Cr. The oxidation state should consider when evaluating the toxicity of Cr compounds. Due to this, metal may form complexes with peptides, proteins, and DNA, resulting in DNA-protein cross-links, DNA strand breaks, and alterations in cellular signaling pathways, which may contribute to the toxicity and carcinogenicity of chromium compounds. Meanwhile, the products of metabolic reduction of Cr (VI) (free radicals and Cr IV and V), and the newly generated Cr (III), are thought to be in part responsible for the carcinogenic effects seen in human and animal studies [10].

Abdulla et al. presented reports of mercury contamination of dietary supplements [11]. Mercury interferes with many enzymatic reactions, causes problems in migration and division of cells, and is responsible for cell damage, or even death [12].

Heavy metals, even at trace levels, are very harmful to human beings. Thus, their analytical determination is a very critical and essential topic, which has attracted considerable attention [7], particularly due to the actual amount of Pb, As, Cr, and Hg contained in these dietary supplements, which could exceed the acceptable standard. The standards defined by different international organizations presented discrepancies in what concerns the listing of elements and compliance limits for each element. The World Health Organization (WHO) guidelines proposed Pb permissible levels ($10\,\mu g{\cdot}g^{-1}$) in medicinal raw plant materials and dietary supplements [13], whereas, for plant-based food supplements, the European Commission set limits of $3\,\mu g{\cdot}g^{-1}$ and $0.1\,\mu g{\cdot}g^{-1}$for Pb and Hg, respectively [14]. The United States Pharmacopeia (USP) recommended permissible limits (in $\mu g{\cdot}g^{-1}$) for Pb (0.5), As (0.15), and Hg (1.5) in dietary supplements [15, 16]. Besides, the growing interest, popularity, and the lack of quantitative and qualitative research on the composition of supplements before being place into the market are important topics to attend [17].

The most common techniques used for the elemental analysis in dietary supplements include inductively coupled plasma optical emission spectrometry (ICP-OES) [18], inductively coupled plasma mass spectrometry (ICP-MS) [3, 19, 20], wavelength dispersive-x-ray fluorescence spectrometry (WD-XRF) [16], scanning electron microscopy-energy dispersive x-ray spectrometry (SEM-EDX), and ion chromatography (IC) [21]. However, to our knowledge, there is not any publication about the validation of an analytical method for heavy metals determination in dietary supplements by Total Reflection X-ray Fluorescence Spectroscopy (TXRF).

TXRF is a well-established analytical technique for multielement determination in various sample types, especially liquids and powdered microsamples. The x-ray emitted in the TXRF is characteristic of each individual element and its intensities are proportional to their concentrations in the sample [22]. The incoming radiation is incident on the sample at less than the critical angle and is totally reflected; this is one difference with the XRF technique. Advantages include the element and its concentration being unaffected by matrix effects, its low sensitivity (ppb), requiring small amounts of sample (μg or μL), the use of small quantities of reagents, and not utilizing argon or nitrogen gases. The low background levels result in improved LODs and even 2 pg may be detected for a variety of elements with a counting time of 1,000 s [23–25]. Actually, the development and commercialization of TXRF instrumentation, which offers simple operations with a low-cost compact design, have promoted its application in many different fields [24].

In view of these premises, the aim of this work was the validation and application of a simple and accurate methodology to determine the content of Pb, As, Cr, and Hg in dietary supplements by TXRF. Also, there were two research purposes, the first being to contribute to the risk assessment analysis regarding the consumption of dietary supplements and the second being to propose the TXRF technique as a powerful tool for quality standardization and control of both raw material and finished product.

2. Materials and Methods

2.1. Reagents and Standard Solutions. The concentrated nitric acid (HNO_3, 69% w/w), ethylenediaminetetraacetic acid (EDTA, 99.4–100.6% w/w), hydrochloric acid (HCl, 36.5–38 % w/w), and hydrogen peroxide (H_2O_2, 30% w/w) were obtained from J.T. Baker (Mexico). ICP standard stock solutions contained $1000\,mg{\cdot}L^{-1}$ of Cr, As, and Hg which were purchased from Cresent Chemical Co., Inc. (Mexico).

The Pb and Ga standard solutions were acquired from Sigma Aldrich (Mexico). Millipore-Q water (Milli-Q plus, 18.2 MΩ·cm^{-1}) was used for standard and reagent preparations.

Individual single-element stock solutions containing 1000 mg·L^{-1} Pb, As, Cr, Hg, and Ga were used as calibrations standards. Gallium was employed as an internal standard (IS) to improve the precision of quantitative analysis. For each element, six different known concentrations were prepared into 10 mL volumetric flasks and filled to the mark with 0.31 mol·L^{-1} of HNO_3 y 10 μg·L^{-1} of Ga (IS) solution. For Hg analysis, was utilized a dilute solution which contains 0.01 mol·L^{-1} of HNO_3, 0.01 mol·L^{-1} of EDTA, and 10 μg·L^{-1} of Ga. All glassware was kept overnight in HNO_3 (10 % v/v) and rinsed several times with Millipore-Q water before its use to avoid cross-contamination.

2.2. Dietary Supplement Samples' Pretreatment. Different mixtures have been reported for the digestion procedure of dietary supplements. The most common compounds were the concentrated HNO_3 [19], mixtures of $HNO_3 + HCl$, and concentrated $HNO_3 + H_2O_2$ [3, 20]. The present work utilized a digestion procedure using concentrated $HNO_3 + HCl + H_2O_2$ for the determination of Pb, As, Cr, and Hg in six dietary supplements [2, 24].

Four dietary supplements, fat burner (A-01), laxative (A-02), *tejocote* root (A-03), and Omega 6, 12 oil (A-04), were randomly collected from local Mexican suppliers. The selection criterion was based on high consumption and popularity in the treatment of chronic diseases (diabetes, high blood pressure, cancer, and obesity). The composition of all dietary supplements includes organic (carbohydrates, proteins, lipids, and vitamins) and inorganic compounds (Zn, Cu, Fe, Ca, among others). For confidentiality reasons, the studied products shall not be identified.

The digestion procedure consisted of weighing approximately 0.020–0.050 g of each sample in polypropylene tubes. Then, 5 mL of H_2O and 1 mL nitric acid concentrated solutions were added into glass testing tubes. Reagents and sample mixtures were allowed to stand for 15 minutes. Subsequently, 100 μL of H_2O_2, 485 μL of concentrated HNO_3, and 100 μL of concentrated HCl were incorporated into tubes, which were covered with their respective stopper. A vortex was used to shake the samples in the testing tubes, which were then placed into a bathwater at 80°C for eight hours. After acid digestion, samples were filtered. Finally, samples were prepared into 25 mL volumetric flasks and filled to the mark with 0.31 mol·L^{-1} of HNO_3 and 10 μg·L^{-1} of Ga (IS) solution. For Hg analysis, a dilute solution was utilized which contains 0.01 mol·L^{-1} of HNO_3, 0.01 mol·L^{-1} of EDTA, and 10 μg·L^{-1} of Ga. Blank samples were made to identify any possible source of contamination. Analyses were carried out in triplicate, and analytical blanks were prepared by following the same procedure used for the samples.

2.2.1. Analysis of Certified Reference Material. The USP mentioned some names of plants to develop standard mixtures that can be used in validation studies [15]. However, dietary supplements are usually made into different presentations, including powders, tablets, capsules, energy bars, liquids, and oils, among others [2], which implies a wide variety of matrices. This is because it is difficult to find certified reference materials, with the validation purpose. However, a BCR-610 (groundwater, CRM) was analyzed, adjusting the HNO_3 concentration to 0.30 mol L^{-1} and added Ga as IS, without any prior digestion procedure. Samples were examined in triplicate by the TXRF_1 method.

2.2.2. Intercomparison Studies between TXRF and ICP-OES. All chemicals utilized were of analytical reagent grade. Hydrogen peroxide (H_2O_2, 30% w/w) and nitric acid (65% w/w) were obtained from Scharlau (Barcelona, Spain) as well as standard solutions of As, Cr, and Pb (1000 mg·L^{-1} in 2% of HNO_3). Two samples (A-05 and A-06) were acquired from local Spain suppliers and employed for the intercomparison studies between TXRF and ICP-OES methods. The purpose was an estimate of the accuracy in the measurements of both methods. Student's *t*-test was utilized to find significant differences in the metal determinations.

According to its label, the A-05 sample is a dietary supplement utilized for the organism detoxification, while the A-06 (Fat Burner) is used to increase acutely fat metabolism and for the loss of weight. Both samples were weighed (0.5 g approximately) into a microwave Teflon vessel, to which 1 mL of H_2O_2, 1 mL of HCl, and 3 mL of HNO_3 were added. After digestion, they diluted with 25 mL of water and analyzed by ICP-OES (Optimal 5300 DV PerkinElmer Inc.). In the TXRF method, A-05 and A-06 samples were pretreated by following the procedure indicated in Section 2.2.

2.3. Instrumentation and Experimental Procedure. The TXRF measurements were performed with a commercial S2 PICOFOX TXRF spectrometer (Bruker AXS Microanalysis GmbH, Berlin, Germany), equipped with either a molybdenum (Mo) x-ray tube operated at 600 μA and 50 kV. The automatic sample changer (holder) allows up to 25 sample carriers to be loaded and analyzed. The software used in the instrument control, data collection, and data analysis was *Spectra PICOFOX®7*. The spectral lines utilized: $L_{-\alpha}$ 10.5 KeV, $K_{-\alpha}$ 5.4 KeV, $L_{-\alpha}$ 9.2 KeV, $K_{-\alpha}$ 10.5 KeV *y*, and $L_{-\alpha}$ 10 KeV, for Pb, Cr, Ga, As, and Hg, respectively. Sample carriers and glassware were subjected to a strict cleaning protocol. The experimental procedure applied was that reported by Beltrán and Cols. [24].

2.4. Design of the Experiments. A response surface methodology involves several experimental techniques for the evaluation of the relationship between a group of controlled experimental factors and measured responses based on one or more criteria. Besides, it can be used to estimate the effect of individual parameters, the interaction of variables, and the optimum conditions for responses [17]. A central composite

design (CCD) was used to investigate the impact of the selected parameters on the TXRF measurements.

The HNO_3 concentration ($mol{\cdot}L^{-1}$), EDTA concentration ($mol{\cdot}L^{-1}$), and sample-reading time (s) were selected as the independent variables, while analytical signals (Pb, As, Cr, and Hg) were considered as the response (dependent variables). The independent variables vary between the lowest level of–*l*, center level 0, and the highest level of +*l*. Then, their studied ranges were as follows; HNO_3 concentration ($0.01–1\ mol{\cdot}L^{-1}$), EDTA concentration ($0 – 0.1\ mol\,L^{-1}$), and sample-reading time (100 – 500 s). The range of independent variables was based on preliminary experiments and determined literature [24]. The experimental design points consist of the 2^3 factorial points, six axial points, and six central points. Center points were used to determine the reproducibility of the data and the experimental error [17]. Results were examined by the software Minitab®17. Twenty experiments were performed to establish the optimal conditions utilizing the minimum reagents concentration and lower reading time.

2.5. Figures of Merit. Figures of merit considered in this work were (the linearity and range, limit of detection, limit of quantification, accuracy, and precision) according to the recommendations of IUPAC [26].

Calibration curves were prepared for each of the metals and running a range of concentration between 2 and $100\ \mu g{\cdot}L^{-1}$ (Pb, As, and Cr), and for Hg analysis in a range $5–100\ \mu g{\cdot}L^{-1}$. It plotted on the *y*-axis (analyte peak area/Ga peak area) against the corresponding ones (*x*-axis). The concentration of the analyzed samples was estimated by the assessment of the calibration curve data corresponding to each element: *m* (the slope of the calibration curve) and *b* (interception with the *y*-axis).

The LODs and LOQs were calculated as 3 s/m and 10 s/m, respectively, where *s* is the standard deviation of the analytical signal of ten blanks [26]. In addition, regression equations, determination coefficient (R^2), analysis frequency (h^{-1}) were estimated. The precision was considered in terms of the intra-day repeatability and intra-day reproducibility by the use of $20\ \mu g{\cdot}L^{-1}$ concentration level for all analytes and evaluated as the percentage of relative standard deviation (% RSD).

2.6. Analysis of Real and Spiked Samples. The accuracy of the TXRF_DS_1 and TXRF_DS_2 methods were evaluated by the recovery percentage of heavy metals by known amount added to selected dietary supplements. Samples (A-01, A-02, A-03, and A-04) were spiked with a standard solution of $20\ \mu g{\cdot}L^{-1}$ Pb, As, Cr and Hg during the digested process. Three replicates of each spiked samples were analyzed by TXRF under optimum conditions.

3. Results and Discussion

3.1. Optimization of the TXRF Variables. Table 1 shows the experimental design with 20 experiments with their analytical responses. The experiments were run randomly. The ANOVA table showed a low fit for the tested models (Lineal, lineal + interactions, lineal + squares, and full quadratic). Thus, such as alternative, the optimal values of the variables that affected the proposed TXRF methods were obtained utilizing the desirability function. The function can be maximized by using a random starting point and then proceeding along the steepest slope up to a maximum. Starting from several points in the design space increases the chance of achieving the "best" maximum among all possible maxima of the function [27]. This mathematical model transforms each response into an individual desirability (d_i) value, coded from 0 (undesired response) to 1 (desired response). In this analysis, ($d_i = 1$) corresponds to a desired response (maximum analytical signal for all elements), while ($d_i = 0$) represents a minimum analytical signal. Finally, the individual desirability of each analyte was combined into a single response, which means the overall desirability (CD) through the geometric mean [16]. The best experimental conditions were chosen according to the higher composite desirability (CD).

The analysis of data obtained in the experiment design included the four dependent variables and their response. In the first analysis, the CD value was low (0.73). Probably, due to the Hg response had a different behavior than the other elements, that is the "EDTA concentration" variable was not significant for Pb, As and Cr responses, but it was for Hg. Therefore, a second analysis was carried out, excluding Hg data. In this case, the best local maximum was found to be at the sample-reading time of 500 s, $0.31\ mol\,L^{-1}$ HNO_3, $0\ mol\,L^{-1}$ EDTA, while the CD value was 0.9802. However, looking for a compromise between the sensitivity of the technique and the lamp lifetime, the variable "sample-reading time" was finally set to 401 s, without the desirability value (0.9170) being affected (Figure 1). The desirability of 0.9170 indicates that the estimated function may express the desired conditions and the experimental model. Under these conditions, the method was called TXRF_DS_1.

The individual analysis for Hg analytical response showed that the best local maximum was found to be at the sample-reading time of 431 s, HNO_3 concentration of $0.01\ mol{\cdot}L^{-1}$, EDTA concentration of $0.01\ mol{\cdot}L^{-1}$, and CD value = 1. In the same way, the sample-reading time variable was established in 401 s and the CD value was not affected (Figure 2). The Hg analysis method was called TXRF_DS_2.

3.2. Figures of Merit. Under the optimal conditions, the analytical performance of the proposed procedure was evaluating. Four calibration curves were built. Table 2 shows the linear calibration ranges and their respective determination coefficient (R^2), LODs, LOQs, repeatability, and reproducibility for all analyses using TXRF_DS_1 and TXRF_DS_2 methods. The calibration plots are depicted in Figure 3.

The limit of detection (LOD) is the lowest concentration that can be detected, but not necessarily quantified. Detection limits for Pb, As, Cr, and Hg were lower than LODs obtained with an ICP-OES method for the quantitative analysis of toxic elements in some dietary supplements and

Table 1: Experimental conditions and values obtained through the CCD.

	Independent variables			Analytical signals (CPS)[a]			
Runs no.	EDTA mol·L^{-1}	Time (s)	HNO_3 mol·L^{-1}	Cr	As	*P*b	Hg
1	0.005	300	0.505	680.3	2260.3	884	830
2	0.005	300	0.505	756.3	1870.7	884	830
3	0.005	300	0.010	335.5	2230.3	1104	883.5
4	0.010	100	1	458.5	709	247.3	198
5	0.010	500	1	937	3377	1194.7	883.3
6	0.010	300	0.505	954	3741.7	1450.7	1343
7	0	300	0.505	978.3	3371.7	1905.7	204
8	0.005	100	0.505	199.3	768.7	395	126.3
9	0	500	0.010	1093	4033.7	2539	238.3
10	0.005	300	1	901	3036.5	1168	589
11	0	100	1	291.7	751.7	464	189
12	0.010	100	0.010	222.7	872.7	210	212
13	0.005	300	0.505	680.3	2260.3	884	830
14	0.005	300	0.505	756.3	1870.7	847	830
15	0	100	0.010	402.7	1210.3	623.3	97
16	0.005	500	0.505	878	3196.5	1256.5	597
17	0.010	500	0.010	954	3741.7	1450.7	1343
18	0	500	1	1203	3782	2303.5	232
19	0.005	300	0.505	761.3	2239	884	830
20	0.005	300	0.505	884	2313	884	830

[a]Counts per seconds, CPS.

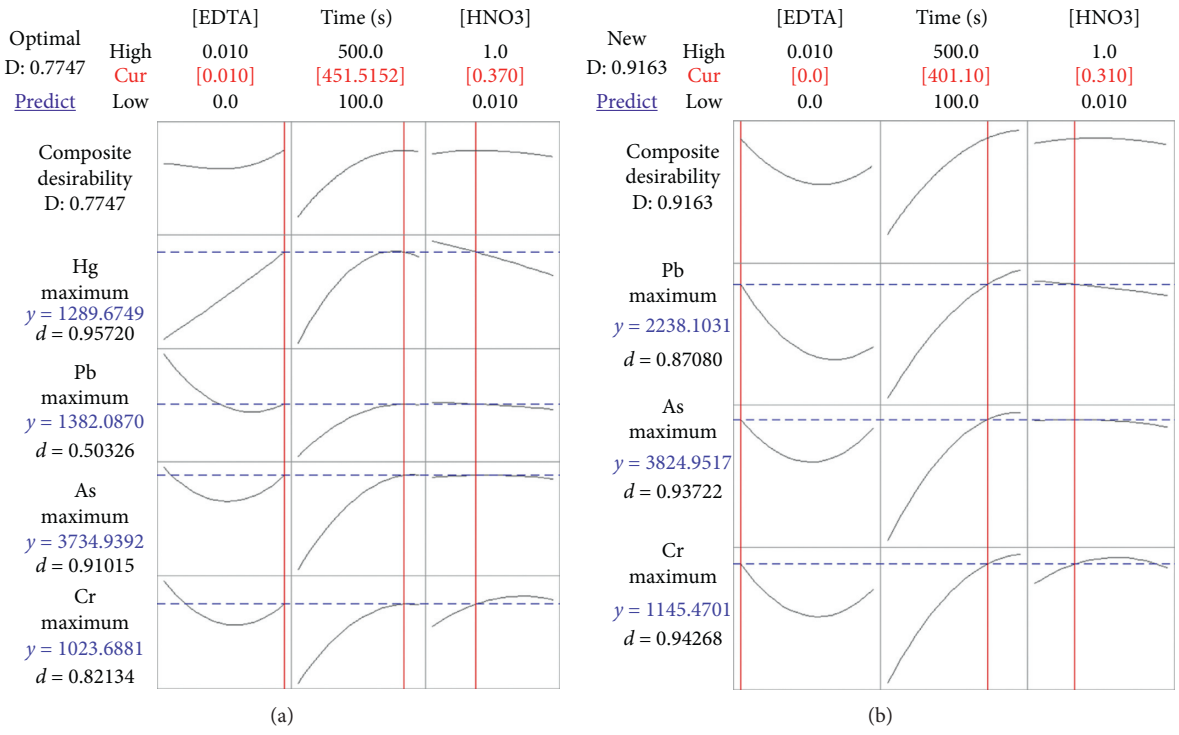

Figure 1: Response optimization plots. (a) First analysis with analytical responses Pb, Cr, As, and Hg (CD = 0.7774). (b) Second analysis without Hg analytical response (CD = 0.9163).

diet products [18]. However, the excellent analytical sensitivity that ICP-MS allows is indisputable. Table 3 shows other methods that had better LODs than those achieved in proposed TXRF methods [3, 19]. Nevertheless, these methods utilized a larger amount of samples and reagents for digestion procedures in comparison with TXRF_DS_1 and TXRF_DS_2 methods. Besides, our methods use a final dilution of samples between 10 and 25 mL (4-fold lower than other proposed methods). In addition, a sample volume of 10 μL was required, which contributes to the minimization of residues. The TXRF neither requires an argon gas nor hydride generation system. Moreover, all

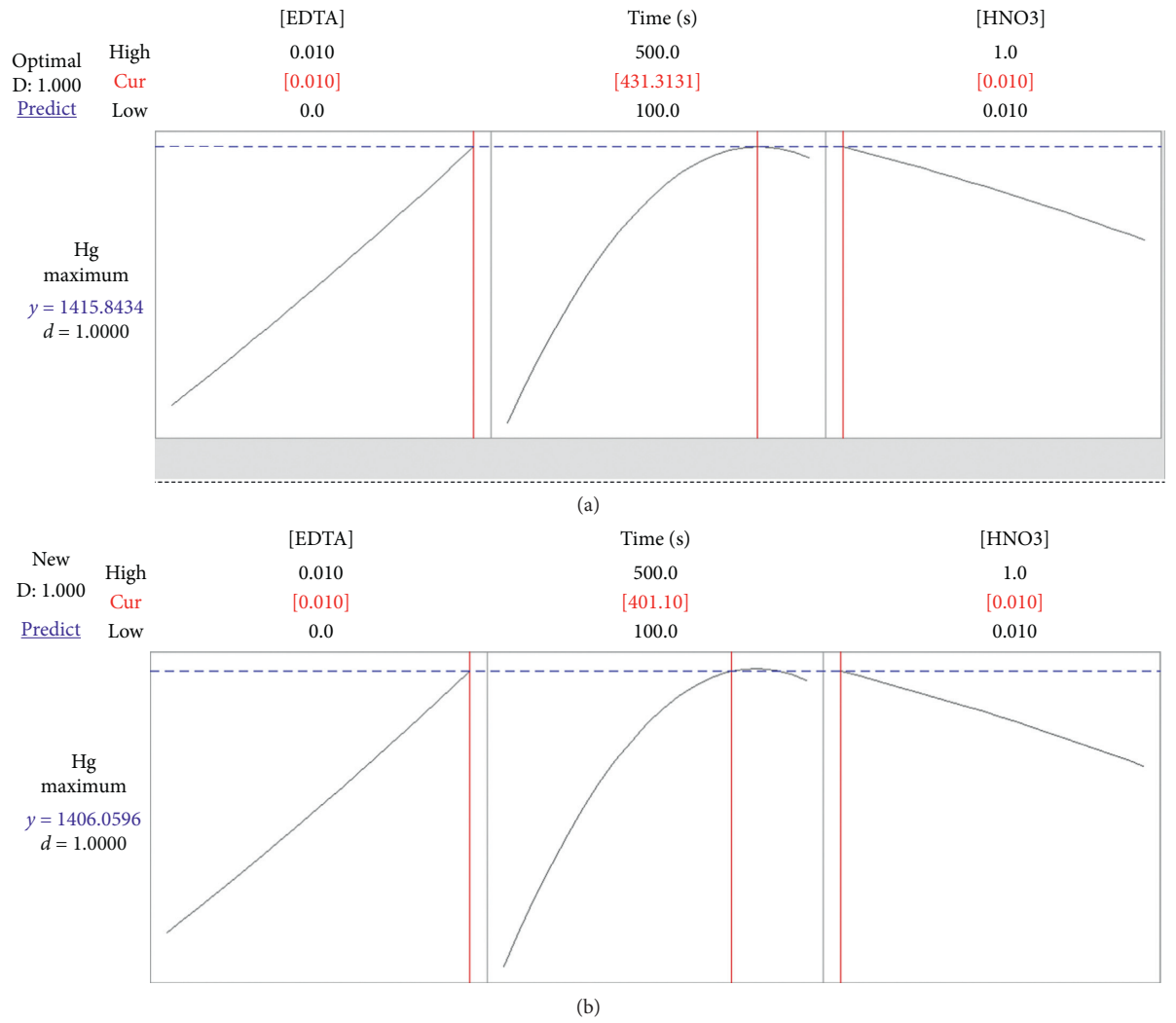

Figure 2: Response optimization plots. (a) First analysis with Hg analytical response Hg (CD = 1). (b) Second analysis modifying the reading time (CD = 1).

Table 2: Figures of merit of TXRF methods.

Element	LOD $\mu g \cdot L^{-1}$	LOQ $\mu g \cdot L^{-1}$	Repeatability % RSD	Reproducibility %RSD	Lineal range $\mu g \cdot L^{-1}$	R^2
Pb[a]	0.59	1.95	3.31	3.26	1.95–100	0.9999
As[a]	0.41	1.35	1.59	1.96	1.35–100	0.9999
Cr[a]	0.57	1.90	5.11	5.27	1.90–100	0.9999
Hg[b]	0.75	2.50	1.71	4.77	2.50–100	0.9996

Analytical parameters for [a]TXRF_DS_1 method and [b]TXRF_DS_2 method.

LODs were lower than levels suggested by international organizations (WHO and US Pharmacopeia) [13, 15].

The LOQs were calculated and produced a peak with ten times the signal-noise ratio for Pb, As, Cr, and Hg, respectively, and were in the range of 1.35–2.50 $\mu g \cdot L^{-1}$.

The precision under intraday repeatability and intraday reproducibility was assessed as the relative standard deviation (% RSD). The intraday repeatability was calculated based on five consecutive measurements of a standard solution (20 $\mu g \cdot L^{-1}$) using the same measurement procedure and the same operating conditions. The intraday reproducibility can be obtained with stated precision by five consecutive measurements of (20 $\mu g \cdot L^{-1}$) standard solution in a different analysis day. For all elements retained in the analysis, % RSD values are lower than 20%, as recommended by the US Pharmacopeia [28]. Table 2 shows figures of merit for the proposed methods. The calibration curves achieved statistically satisfactory results ($R^2 > 0.999$). The analysis frequency was nine samples per hour.

3.3. Analysis of Certified Reference Material. To complete the validation of the TXRF_DS_1 method, a BCR-610 certified reference material was analyzed for Pb and As. The results

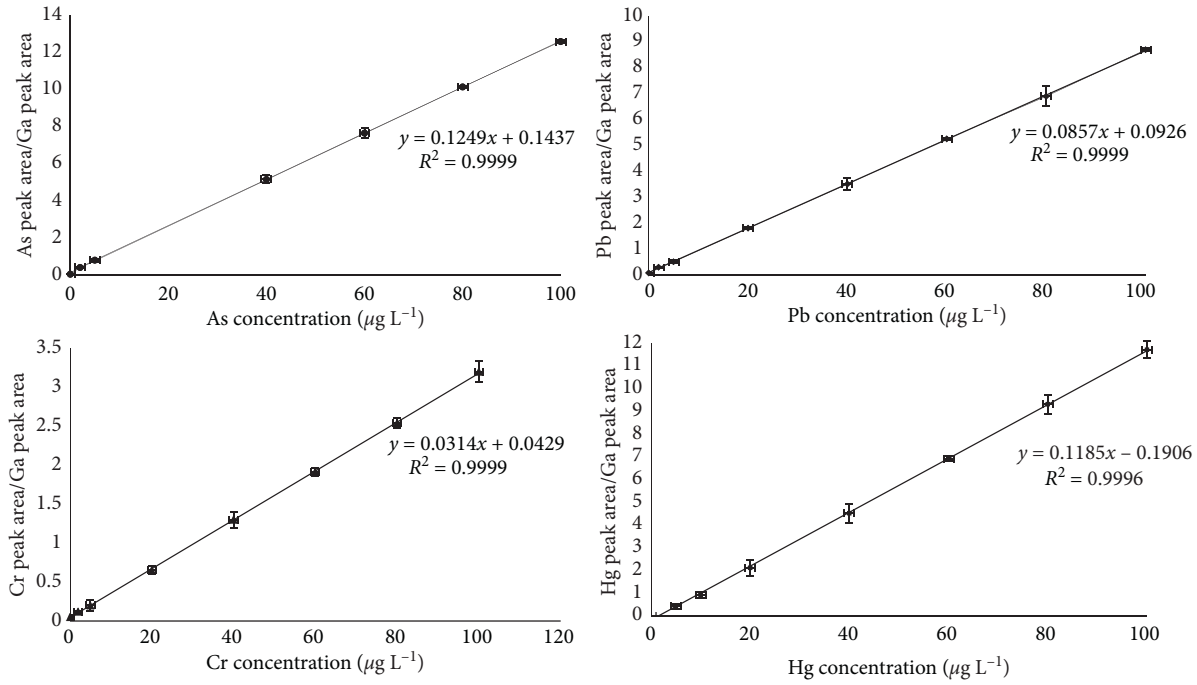

Figure 3: Calibration curves for As, Pb, and Cr. TXRF_DS_1 method optimal conditions: 0.31 mol·L^{-1} of HNO_3 and 10 μg·L^{-1} of Ga (IS) solution. Calibration curve for Hg under TXRF_DS_2 method optimal conditions: 0.01 mol·L^{-1} of HNO_3, 0.01 mol·L^{-1} of EDTA, and 10 μg·L^{-1} of Ga. The error bars represent the standard deviation ($n = 3$) for each point.

Table 3: Comparison of methods for Pb, As, Cr, and Hg determinations in dietary supplements.

Analytical technique	Digestion procedure[a]	LODs (μg·L^{-1})				Observations	Ref.
		Pb	As	Cr	Hg		
TXRF	0.020–0.050 g 485 μL HNO_3 100 μL HCl 500 μL H_2O_2	0.59	0.41	0.57	0.75	No requirement of argon gas; 10–25 mL[b]	Present work 2020
ICP-OES	0.5 g[a] 9 mL HNO_3 3 mL HCl	5	5	2.5	1	As and Hg analysis by HG	[18]
ICP-MS	0.3^{-1} g[a] 3–5 mL HNO_3	0.20	0.004	0.011	—	100 mL[b]	[19]
ICP-MS	15 g[a] 7.5 mL HNO_3 2.5 mL HCl	0.002	0.010	—	—	100 mL[b]	[3]

[a]Grams of sample; [b]final dilution volume of samples after digestion procedure.

were obtained by three replicates ($n = 3$) and expressed as the means and their standard deviation. The t-test for the comparison of means revealed that there were no significant differences at a 95% confidence level between the values obtained (7.85 ± 0.04 μg·L^{-1} and 10.79 ± 0.40 μg·L^{-1} for Pb and As, respectively) and the certified values (7.78 ± 0.13 μg·L^{-1} for Pb and 10.40 ± 0.25 μg·L^{-1} for As).

3.4. Intercomparison Studies between TXRF and ICP-OES. Two dietary supplements were analyzed in the intercomparison study between TXRF and ICP-OES methods (Table 4). Student's t-tests were performed to compare the experimental data obtained between the TXRF-DS_1 method and ICP-OES method. Sample pretreatment was carried out according to Section 2.2.2. First, in the A-05 sample, the Pb concentrations were in agreement in both methods; in the case of Cr and As determinations, it was not possible to establish a comparison, because some values were below ICP-OES LOQ. The results found for Pb and Cr determinations in the A-06 sample showed that there were no significant differences at a confidence level of 95% for $n = 3$. However, As was not detected by ICP-OES. The results demonstrated that the accuracy of the TXRF_DS_1 method is acceptable.

3.5. Analysis of Real and Spiked Samples. The developed procedures were applied for the determination of Pb, As, Cr, and Hg in real and spiked dietary supplements.

Table 4: Intercomparison studies between TXRF and ICP-OES methods.

Sample	Methods comparison[a]		
	Element	ICP-OES ($\mu g \cdot L^{-1}$)	TXRF-DS-1 ($\mu g \cdot L^{-1}$)
A-05	Pb	0.57 ± 0.32	0.54 ± 0.16
	Cr	≤LOQ	≤LOQ
	As	≤LOQ	0.16 ± 0.030
A-06	Pb	0.70 ± 0.36	0.25 ± 0.023
	Cr	57.56 ± 1.50	58.61 ± 1.54
	As	≤LOQ	0.38 ± 0.070

[a]Results are expressed as the mean value ± s (n = 3). s, standard deviation.

Table 5: Results of the recovery study in the dietary supplements.

Sample[a]	Metal	[b]Analyzed sample $\mu g \cdot L^{-1}$	[b]Spiked sample $\mu g \cdot L^{-1}$	% recovery
A-01	Pb	2.64 ± 0.17	22.92 ± 0.59	101
	As	1.51 ± 0.059	22.47 ± 1.13	105
	Cr	31.61 ± 0.46	52.15 ± 0.45	103
A-02	Pb	1.77 ± 0.11	19.88 ± 0.078	91
	As	0.45 ± 0.015	22.17 ± 0.45	108
	Cr	18.63 ± 0.31	39.65 ± 0.32	105
A-03[c]	Hg	0.37 ± 0.30	20.15 ± 0.10	99
A-04[c]	Hg	0.10 ± 0.0080	20.40 ± 0.30	101

[a]The results are reported as the Pb, As, Cr, and Hg concentration in the analyzed solutions. [b]Results are expressed as the mean value ± s ($n = 3$). s, standard deviation. [c]Samples analyzed with TXRF_DS_2.

Sample pretreatment was carried out according to Section 2.2. Concentrations of the target analytes in samples were obtained by using calibration curves and are shown in Table 5. The obtained recoveries of Pb, As, and Cr in A-01 and A-02 were ranging from 91% to 105%. The Hg concentrations in these samples were less than the proposed LOD. These samples were analyzed by the TXRF_DS_1 method. The analysis recoveries of Hg were probed once more in the two samples A-03 and A-04 by the method TXRF_DS_2. The high recovery values indicated the absence of analyte loss during the sample preparation step and that sensitivity was not influenced by the dietary supplements matrix.

Sample concentrations were expressed as μg of heavy metals per gram of sample. For sample A_01, we obtained $1.12\ \mu g \cdot g^{-1}$ Pb, $0.65\ \mu g \cdot g^{-1}$ As, and $13.62\ \mu g \cdot g^{-1}$ Cr. On the other hand, sample A_02 contained 1.32, 0.37, and $13.69\ \mu g \cdot g^{-1}$ of Pb, As, and Cr, respectively. Mercury was not detected in these two samples. For samples A_03 and A_04, the Hg contents were $0.42\ \mu g \cdot g^{-1}$ and $0.12\ \mu g \cdot g^{-1}$. These samples were analyzed with the TXRF_DS_2 method, exclusively for Hg.

The results show that methods TXRF_DS_1 and TXRF_DS_2 are suitable options to quantify without any problem to heavy metals at trace levels, even below the maximum permissible limits imposed by international standards [13–16]. The TXRF technique is presented as a useful tool for monitoring heavy metals in food supplements, through which the quality of these products can be guaranteed before going to market and consumers ingest them.

4. Conclusions

The scientific and regulatory challenges in terms of the quality, safety, and efficacy of dietary supplements are common to all countries as the marketplace for them becomes increasingly global. In this study, two methods for quantitative analysis for the determination of toxic metals (Pb, As, Cr, and Hg) were developed, validated, and applied for the analysis of dietary supplements samples. It utilized a central composite design and desirability function for the method validation. Results obtained as linearity, accuracy, precision, LODs, and LOQs were within satisfactory borders, because we recommend TXRF_DS_1 and TXRF_DS_2 methods for heavy metal determinations in dietary supplements at trace level. No significant differences were found in the inter-comparison studies between TXRF and ICP-OES methods. TXRF methods improve the economy of laboratories in the research centers, universities, and industry. As they are environmentally friendly since the consumption of samples and reagents is minimum, they do not use toxic reagents, and it is not necessary to use carrier gases. These characteristics allow their use in toxicological, environmental, biological, and food studies. Finally, this research proposes two methods for the standardization and control of the quality of both the raw material and the dietary supplement finished product before human consumption.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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