Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2016, **8**, 4263

Sequential factorial designs for method development of the determination of Cd and Pb in fish and shrimp by GF AAS after sample freeze-drying and tetramethylammonium hydroxide solubilization

Daiane Placido Torres,*[ab] Maristela Braga Martins-Teixeira,[c] Solange Cadore[b] and Helena Müller Queiroz[c]

The development and validation of a simple, reliable and fast method for the determination of cadmium and lead in fish and shrimp by GF AAS, following sample freeze-drying and tetramethylammonium hydroxide (TMAH) solubilization, is presented. The method development was achieved by sequentially applying factorial designs until optimization within the bilinear approximation of the method was accomplished. As such, seven experimental parameters were initially studied, which were considered important for the determination of cadmium and lead in seafood samples using TMAH solubilization. The validation of the method was completed in order to comply with international food regulations and method accreditation under ISO 17025. In this sense, the following parameters were evaluated: linearity, limits of detection and quantification, precision, recovery (trueness), specificity and robustness. Linearity of response was satisfactory for the concentration ranges of both analytes. The residuals for both elements were homoscedastic and independent, with normal distributions. Limits of quantification (LOQ), based on signal standard deviation for low-in-cadmium and lead samples, were 6.25 μg kg^{-1} and 31.25 μg kg^{-1}, respectively. These figures are in accordance with performance criteria required by the Commission Regulation (EU) no 836/2011. The repeatability of the method, calculated from the analysis of seven sample replicates at two concentration levels of cadmium and lead, by the same analyst, was usually better than 10%. Recovery was estimated from the repeatability evaluation, lying in the range of 84% to 99% for cadmium, and 94% to 107% for lead. Furthermore, a wide evaluation of the method robustness was performed during the method optimization. Thus, the results showed the suitability of the developed method for the determination of Cd and Pb in fish and shrimp by GF AAS, following TMAH solubilization for food control purposes. Additionally, the use of sequential factorial designs in order to achieve bilinear optimization of the method was proven to be a very valuable tool, by sharply reducing optimization time and the number of experiments.

Received 18th April 2016
Accepted 30th April 2016

DOI: 10.1039/c6ay01145d

www.rsc.org/methods

1. Introduction

"Toxic elements" are one of the oldest environmental problems. Today, there are new dimensions to the problem, such as the production of metals in developing countries leading to occupational exposure and exposure to the general public through the ambient air, drinking water, food and consumer products. In this sense, heavy metals in the marine environment, resulting from urban discharge, can remain in solution or suspension and precipitate to the bottom, or be taken up by organisms, thus creating a potential source of heavy metal pollution in the aquatic environment. Accordingly, metals can accumulate to toxic concentration levels and cause ecological damage and subsequently, be transferred to humans through the food chain. In this context, cadmium and lead are among the most dangerous metals and have been associated with serious adverse health effects.[1,2] Because of the toxicity of some trace elements, their presence in foodstuffs should be carefully evaluated to prevent toxicological risks.[3,4] It is therefore necessary to ensure that the quality and comparability of the analytical results generated by laboratories are approved for official residue and contaminant control. This could be

[a]*Brazilian Agricultural Research Corporation, Embrapa Clima Temperado, BR 392 km 78, Pelotas, Rio Grande do Sul, Brazil, 96010-970. E-mail: daiplacido@yahoo.com.br; Fax: +55 53 3275 8221; Tel: +55 53 3275 8284*

[b]*Chemistry Institute, University of Campinas, Unicamp. Campinas, SP, Brazil, 13083-970*

[c]*Laboratório Nacional Agropecuário – Lanagro-SP, Ministry of Agriculture, Livestock and Food Supply. Campinas, São Paulo, Brazil, 13100-105*

achieved by using quality assurance systems and specifically, by application of methods that are validated according to common procedures and performance criteria and by ensuring traceability to common standards or standards of common accord.[5] In this context, validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled.[6] Such requirements include establishing traceability of the measurements, use of validated methods for analyses, use of defined internal quality control procedures, participation in proficiency testing schemes and accreditation by an international standard, normally ISO/IEC 17025 norm.

From the perspective of method development and validation, it is imperative to have information on the independence and interdependence of the variables. Multivariate methods or factorial designs involve simultaneous combinations of a number of parameters, according to a predefined plan, and the number of experiments could be 2^n for a system with "n" factors, evaluated on two levels, either qualitative or quantitative. Thus, factorial designs are ideal when few variables are studied. In this sense, different approaches to experimental factorial designs can be applied, aiming to achieve an optimum method development.[7]

The aim of the present work is to develop a simple, fast and efficient method by employing factorial design tools for the determination of cadmium and lead in fish and shrimp samples by GF AAS, following TMAH treatment, in order to promote the official control of these contaminants in seafood. Most methods developed for the determination contaminants do not actually take into consideration the maximum levels established in food regulations. Additionally, when dealing with a method for official control purposes, it is very important to have simple and reliable methods available, since a considerable number of samples are analyzed continuously. In a second step, the method validation was completed in compliance with national (Brazilian) and international food legislations. In this sense, the procedures were based on Commission Decision (2002/657/EC),[5] Commission Regulation (EU) no 836/2011,[8] (EC) no 882/2004,[9] (EC) no 1881/2006,[10] and EC 629/2008 [11] and DOQ-CGCRE-008 (Revision 04/2011)[12] from INMETRO (Brazilian Institute of Metrology, Quality and Technology). Furthermore, the present work is aimed toward accreditation according to the ISO 17025 norm.[6]

2. Experimental

2.1. Instrumental

All measurements were carried out using an atomic absorption spectrometer with a transversely-heated graphite atomizer (AAnalyst 600 and AAnalyst 800 from Perkin Elmer, USA) with Zeeman background correction, and equipped with an autosampler (AS-800, also from Perkin Elmer) and electrodeless discharge lamps.

The following operating conditions were adopted: wavelength, 228.8 nm for Cd and 283.3 nm for Pb; slit-width, 0.7 nm for both elements; current, 230 mA (Cd) and 440 mA (Pb). Argon with a purity of 99.996% (White Martins, São Paulo, Brazil) was used as a protective gas, at a pressure of 400 kPa. Peak area was used for signal evaluation. Sample aliquots were weighed using an analytical scale AY220 (Shimadzu, Japan).

2.2. Reagents

All chemicals used were of at least analytical grade, and the solutions were prepared using high-purity water with a resistivity of 18.2 MΩ cm, obtained from a Milli-Q Plus water purification system (Integral 5, Millipore, USA). 65% nitric acid (Merck, Germany) was used, which was distillated in a quartz sub-boiling apparatus DuoPUR (Milestone, Italy). A 25% m/v tetramethylammonium hydroxide (TMAH) solution in methanol (Sigma, USA) was used to treat the samples before measurement. Reference solutions in the range of 2.0 to 10.0 μg L^{-1} for cadmium and 5.0 to 40.0 μg L^{-1} for lead were prepared daily by the appropriate sequential dilution of 1000 mg L^{-1} of inorganic analyte stock solutions (Fluka, Sigma-Aldrich). Daily verification of sensitivity was performed by using a reference solution of 5.0 μg L^{-1} for cadmium and 25.0 μg L^{-1} for lead, prepared by the appropriate sequential dilution of 1000 mg L^{-1} of inorganic analyte stock solution. Magnesium nitrate 1.0% m/v and ammonium dihydrogen phosphate 10% m/v (both from Perkin Elmer) were used as chemical modifiers after applying a dilution factor of ten.

Polypropylene (PP) flasks were used for sample preparation and storage (Sarstedt, Germany). Plastic and glass containers were washed with tap water and a diluted Extran solution (Merck). Afterwards, they were immersed in a 5% v/v HNO_3 solution for at least 48 h, and rinsed thoroughly with deionized water prior to their use.

2.3. Sample preparation

Fish (*Cynoscion microlepidotus*) and shrimp (*Litopenaeus vannamei*) samples were prepared by simple muscle homogenization using a stainless steel knife (30 cm, Mundial, Brazil), after removing skin, bone and viscera. For some specific investigations on sample homogenization during the robustness evaluation, samples were homogenized by using a mixer (model HC31, Black & Decker, Brazil). These samples, low in cadmium and lead, were purchased from farm Primar (Goianinha city, Rio Grande do Norte, Brazil). In order to maximize the contact surface of the sample with the alkaline reagent, samples were freeze-dried (freeze dryer LS3000, Terroni, Brazil) after the muscle homogenization and sample weighing steps. Tests were also performed without sample freeze-drying; however, this step was mandatory since neither sample solubilization nor quantitative extraction of the analytes occurred under these conditions, precluding the measurement step. In this context, about 4.0000 grams of the fresh sample were weighed directly into the PP tubes. After the freeze-drying step (usually over 16 h, according to further discussion) in the same PP tubes, 4.0 mL of deionized water were added, and after a few minutes, a volume of 4.0 mL of TMAH was added as well. Samples were left to stand for at least 2 h at room temperature and then the volume was made up to 25.0 mL with deionized water. The final TMAH concentration of the samples was 4.0% m/v.

2.4. Measurement conditions

The employed conditions for Cd and Pb determination were as follows: 20 μL of the sample or standard solution, 3 μg of $Mg(NO_3)_2$, plus 50 μg of $NH_4H_2PO_4$ as chemical modifiers in solution. The pyrolysis and atomization temperatures were optimized and the adopted conditions were 550/1300 °C for cadmium and 600/1400 °C for lead. The calibration standard solutions were prepared in 0.2% v/v nitric acid medium and the calibration ranges were from 1.0 to 10.0 μg L^{-1} for cadmium and 5.0 and 40.0 μg L^{-1} for lead. The fortification levels were 50.0 μg kg^{-1} for cadmium and 200 μg kg^{-1} for lead, which under the employed measurement conditions, correspond to 8.0 μg L^{-1} and 32.0 μg L^{-1}, respectively. These levels are in accordance with Codex Alimentarius[4] and European regulations.[10,11]

3. Results and discussion

3.1. Method development (robustness evaluation)

The employed method using TMAH for sample preparation (fish and shrimp), was based on the work of Torres *et al.*[13] For the optimization of the conditions for the determination of cadmium and lead in the samples, a sequential factorial design plan was employed; therefore, the most important factors that may influence the measurement results were selected and subsequently, the concentration range was chosen in a purpose-adapted way, according to the level of interest.[5] It is important to point out that throughout this work, the spiking of the samples was made simultaneously with cadmium and lead, since our purpose was to have the same sample preparation procedure for the reliable determination of both analytes by GF AAS. As such, all the investigations performed in the present work were executed for both Cd and Pb, considering method development and validation.

In addition to the factorial design investigations, the heating program of the graphite furnace was optimized according to Fig. 1. For this study, 3 μg of $Mg(NO_3)_2$ and 50 μg of $NH_4H_2PO_4$ were used as chemical modifiers. As expected, for both analytes, the standard solution presented a more effective interaction with the modifiers, which resulted in larger thermal stability in both the pyrolysis and atomization curves. Despite TMAH solubilization, this can be classified as a slurry technique, which represents one of the possibilities for solid sampling; similar thermal behavior was achieved for the standard solutions and fish samples. The adopted pyrolysis temperatures were 550 °C for cadmium and 600 °C for lead, and selected atomization temperatures were 1300 °C and 1400 °C for cadmium and lead, respectively.

Initially, for the method optimization, seven parameters (or factors) were investigated by using a fractional factorial design 2^{7-4}, according to recommendations by the Commission Decision 2002/657/EC,[5] DOQ-CGCRE-008 (ref. 12) and Torres *et al.*[14] This factorial design actually features the robustness evaluation (Youden test). Over the past few years, the authors of this paper have realized that evaluating robustness as the first step of a method validation is a valuable tool, which provides the chance to change the experimental factors in order to simplify the procedure, reduce the time of a specific step or save reagents before the validation is completed. With that in mind, the first investigation of the present validation method was robustness.

The following factors were chosen to be investigated: (1) sample mass (3.0 or 4.0 g); (2) proportion of sample mass in grams, to TMAH volume in mL (1 : 1 or 1 : 1.25); (3) freeze-drying time of the samples (36 or 24 h); (4) standing time after the addition of TMAH to the freeze-dried samples (18 h or 2 h); (5) sample homogenization (with knife or mixer); (6) centrifugation time of the sample (0 min or 20 min, after the TMAH step and dilution to the final volume with deionized water); (7) sample species (muscle of fish or shrimp). The design was performed with duplicate samples to evaluate experimental error. This choice provides enough degrees of freedom to make the evaluation of the design results possible *via* Pareto chart, instead of probability plot (normal graph for the effects values). The recovery of the analytes was calculated, considering the added concentrations of 50 μg kg^{-1} of Cd and 200 μg kg^{-1} of Pb as 100%, agreeing with the maximum levels established in the

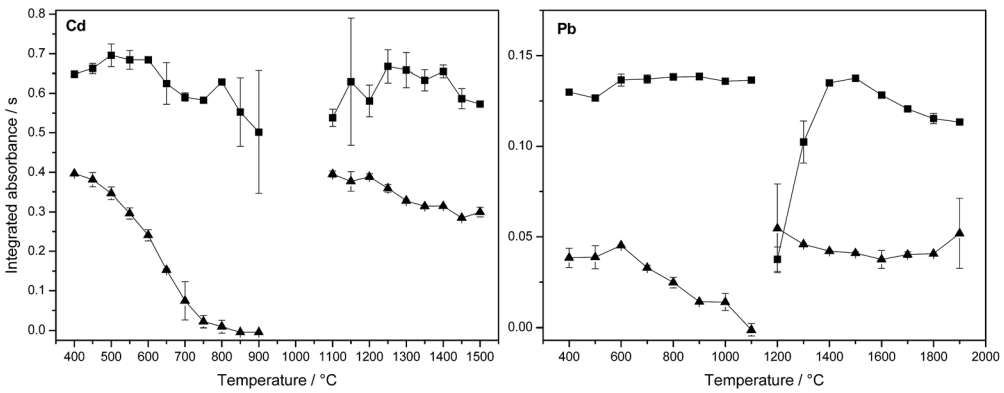

Fig. 1 Pyrolysis and atomization curves for Cd and Pb obtained from 0.2 g of freeze-dried fish, following solubilization with TMAH. (– ▲ –) Spiked samples: Cd, 5.0 μg L^{-1} and Pb, 20.0 μg L^{-1}; (– ■ –) standard solutions: Cd, 10.0 μg L^{-1} and Pb, 50.0 μg L^{-1}. Atomization temperatures employed for pyrolysis curves for cadmium and lead: 1250 °C and 1600 °C, respectively; pyrolysis temperatures employed for atomization curves: 500 °C (Cd) and 900 °C (Pb).

food regulations followed in the present work, according to the above discussion.

The evaluated factors that had significant influence on the recovery of the analytes are as follows. For cadmium (mean effect of 53.76 and mean standard error of 0.90), species > sample mass > freeze-drying time. For lead (mean effect of 123.00 and mean standard of error 2.91), freeze-drying time > sample mass > sample homogenization. Fig. 2 shows the Pareto charts obtained from this study. From these factors, the freeze-drying time can be easily changed with benefit to the method; however, this is not the case for the sample mass (mass reduction would negatively affect the sensitivity, since lower sample mass provides lower amounts of the analyte and therefore lower sensitivity), or the other factors that directly impact the sensitivity of the method, such as the spending of reagent (if the sample mass were increased), or species to be analyzed (as anticipated, fish and shrimp), or sample homogenization, which is much simpler when achieved using a knife.

In this context, the next step for the optimization of the method was the application of a new factorial design, aimed at assessing the parameters that previously presented statistical significance, which was named a refinement study and chosen to be a 2^{4-1} fractional factorial design.[15] Since the other factors did not present statistically significant effects for the evaluated levels, the following conditions were adopted for sample preparation: the ratio of sample mass to TMAH of 1 : 1 (*i.e.* 4.0 mL), elimination of the centrifugation step and standing time of either 2 h or 18 h, which is a very valuable tool, considering the availability of time for sample preparation before analysis. The 2^{4-1} factorial design was also performed in duplicate and the evaluation was simultaneous for fish and shrimp, with freeze-drying time of 16 h or 8 h, sample mass of 4.0 g or 3.0 g and sample preparation with knife or mixer, respectively. Fig. 3 presents the obtained Pareto charts for the refinement study. For this design, the decrease of sample mass influenced the Cd response. Therefore, the sample mass of 4.0 grams should be kept, under the evaluated conditions. For lead, the sample species was on the threshold of significance for an effect. As a result, a huge improvement on the method could be achieved for both elements and the significance of the effects was overcome by evaluating new levels for freeze-drying, for instance. This step seemed really important for the method since the larger this step is, the more rigid the samples are, thus making the sample solubilization step more difficult. Additionally, sample homogenization was no longer a significant factor (by reducing the freeze-drying time), and the knife homogenization was the adopted condition from this point on.

For a final evaluation of the method, it was investigated with a full factorial design, 2^3, with broader levels for the significant parameters of the 2^{4-1} design, which were (1) time of freeze-drying of 4 h or 8 h; (2) sample mass of 2.0 g or 5.0 g, and (3) species, fish or shrimp. This time, for the sake of simplicity, three central points were evaluated for each matrix instead of duplicating the experiments, as a measurement of the data dispersion. The obtained Pareto charts for this investigation are shown in Fig. 4. Only the species factor for Pb determination was significant. This design showed a very large variability of results, taking into account the levels tested for each investigated factor. However, for the evaluated levels, the species was once more significant for lead, which led to the decision to fix the sample mass at 4.0 g. As the freeze-drying factor had no significant effects, a drying time of 16 hours was adopted since this period refers to an overnight procedure, which is very convenient for the laboratory, considering the analytical frequency and the high level of noise produced when the equipment is running in the vacuum mode. At this point, the method for the determination of both analytes in fish and shrimp was considered optimized and the next step was the validation of the method according to Torres *et al.*,[16] as well as international food legislations EU 836/2011,[8] 2002/657/EC,[5] DOQ-CGCRE-008 from INMETRO[12] and ISO/IEC 17025.[6]

3.2. Method validation

In the present validation procedure, the parameters of linearity, specificity, limits of detection and quantification, precision, trueness (recovery) and robustness of the method have been evaluated in order to comply with the conventions established by national (Brazilian) and international food legislation. All

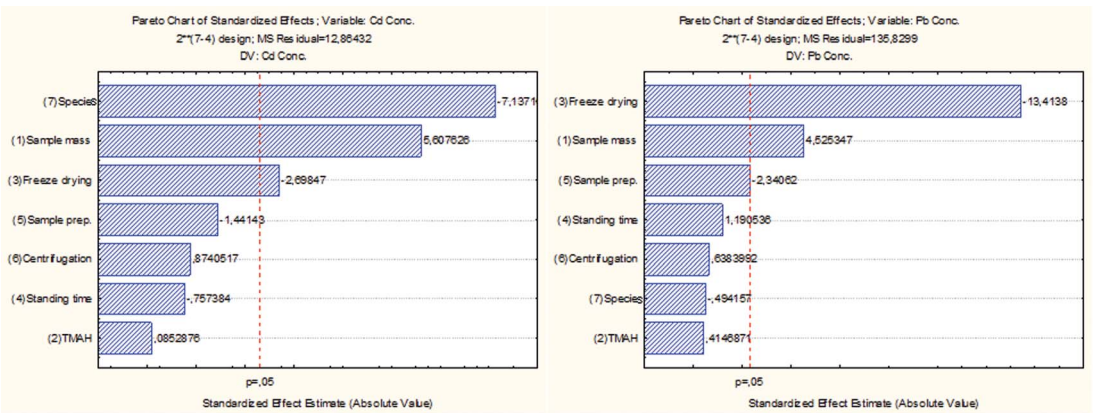

Fig. 2 Investigation of the factors evaluated for cadmium and lead determination in samples solubilized using TMAH using the 2^{7-4} factorial design.

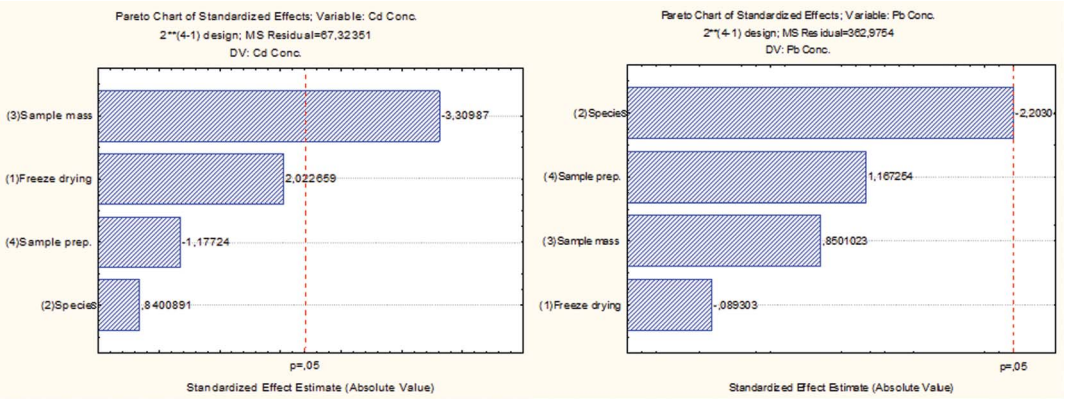

Fig. 3 Investigation of the factors involved on the refinement study for Cd and Pb determination in fish and shrimp by GF AAS using the 2^{4-1} factorial design.

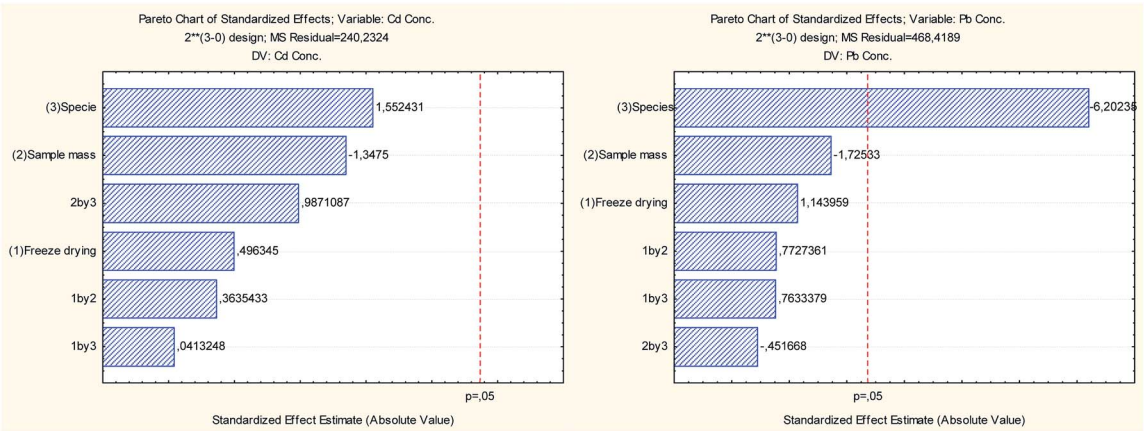

Fig. 4 Final refinement study of the statistically significant factors for the 2^{4-1} design using a 2^3 factorial design with central point for cadmium and lead determination by GF AAS.

spikings were performed according to the maximum levels (MLs) established by Codex Alimentarius 2010 (ref. 4) and/or European Community Regulations (EC) no 1881/2006 (ref. 10) and (EC) no 629/2008,[11] always adopting the lowest ML, which is a conservative stance, in the event of multiple values for the different species. In this context, the adopted values were 200 μg kg^{-1} for lead in fish, and 50 μg kg^{-1} or 100 μg kg^{-1} for cadmium (according to fish species) and 500 μg kg^{-1} for both cadmium and lead in shrimp.

3.2.1. Linearity. Most equipment establish their linear dynamic range. However, it is necessary to verify how far the range of the analyte concentration coincides with the linear dynamic range, and ensure that no other phenomenon has an adverse impact on response.[12] Subsequently, the calibration curve can be established from the concentrations in which the linearity is observed. Moreover, working under isothermal atomization conditions (*e.g.* transverse heated graphite tube with integrated L'vov platform), in combination with the usage of chemical modifiers and Zeeman-background correction, will allow quantification by means of a calibration curve, based upon the measuring of aqueous standard solutions.[5] In this context, for the construction of the calibration curve, five and six concentration levels were used for lead and cadmium, respectively, each one prepared in seven replicates from seven independent intermediate solutions. An important recommendation when measuring elements is that the first point of the calibration curve must correspond to the limit of quantification. The concentration levels investigated were 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 μg L^{-1} for cadmium, and 5.0, 10.0, 20.0, 30.0 and 40.0 μg L^{-1} for lead.

The presence of outliers for each concentration level was checked by the use of the Dixon test. After that, the evaluation of the residuals for homoscedasticity, independence and normality was performed by applying the Levene, Durbin–Watson and Anderson–Darling tests, respectively. Additionally, a minimum correlation coefficient of 0.995 must be achieved for the regression of the evaluated concentration range. Table 1 presents the results obtained for Cd and Pb in this study.

Initially, the calibration range investigated for lead was between 5.0 and 50.0 μg L^{-1}; however, for this concentration range it was not possible to satisfy the requirements of normality and homoscedasticity of the data, even after three

Table 1 Statistical tests for the verification of the linear model assumption covering the calibration ranges for cadmium and lead by GF AAS. Conditions: $n = 7$ for each concentration level, with three readings for each reference solution

Statistical test	Levene (homoscedasticity)		Durbin–Watson (random residues)	Anderson–Darling (data normality)	Correlation coefficient
Evaluated parameter	F_{found}	F_{table}	D^a	p-Value^b	R^2
Cadmium	1.1810	2.5336	1.7346	0.4582	0.9974
Lead	1.5788	2.6896	2.1089	0.1727	0.9955

Criteria: ^{a} $D \geq 1.5$ – independent residues; ^{b} p-value ≥ 0.05 – normal data.

attempts. This problem was then circumvented by eliminating the highest calibration point, which presented the highest variability, indicating the heteroscedasticity of this concentration level, compared to the other studied levels. According to the data presented in Table 1 for the linearity evaluation for cadmium and lead, it is possible to assume that the model correctly represents the relationship between the analyte concentrations and the absorbance at the tested levels. The first point of the calibration curve must correspond to the limit of quantification, which represents 6.25 μg kg^{-1} and 31.25 μg kg^{-1} in the cadmium and lead samples, respectively, according to further discussion. The ML for each analyte represents, under the measurement conditions, concentrations of 8.0 and 32.0 μg L^{-1} for cadmium and lead, respectively. Therefore, the proposed calibration ranges for both elements were fitted for purpose since they covered the range from LOQ up to 1.25 ML.

Daily calibration was achieved by using two standard solutions in 0.2% v/v HNO_3 of 2.0 μg L^{-1} and 10.0 μg L^{-1} for cadmium and 20.0 μg L^{-1} and 50.0 μg L^{-1} for lead. Furthermore, the sensitivity of the equipment was checked daily based on the characteristic mass before any further procedure. In this sense, according to the manufacturer's specifications, a deviation of ±20% of the target value was acceptable. If this criterion could not be achieved, the measurement procedure was aborted for the day. Additionally, every day a third standard solution of each analyte from a different standard lot had to be prepared in order to check the obtained calibration curve. The acceptability criterion is ±10% deviation of the nominal concentration for the new solution against the concentration calculated from the calibration curve, with a precision equal to or better than 10%. If this criterion could not be achieved, an additional standard solution from a different lot would be prepared and analyzed, and if the problem persisted, no official measurement was allowed to be executed for the day.

3.2.2. Limit of quantification. According to the Commission Regulation (EC) no 836/2011,[8] the limit of quantification (LOQ) is the lowest content of the analyte that can be measured with reasonable statistical certainty, *i.e.*, 95%. If both trueness and precision are constant over a concentration range around the limit of detection (LOD), then the limit of quantification is numerically equal to six or ten times the standard deviation of the mean of blank determinations ($n > 20$). Furthermore, the LOQ must be less than one fifth of the ML in regulations; *i.e.* less than 10.0 μg kg^{-1} for Cd and 40.0 μg kg^{-1} for Pb. For the present sample preparation procedure (4.0000 g of sample and a final volume of 25.0 mL), these concentration values mean 1.6 μg L^{-1} for Cd and 6.4 μg L^{-1} for Pb. Since these values are not easily achieved by dilution on an autosampler and the LOQ value can be lower than these figures, the option was to decrease these values to 1.0 μg L^{-1} for Cd and 5.0 μg L^{-1} for Pb (meaning 6.25 μg kg^{-1} for Cd and 31.25 μg kg^{-1} for Pb, in the samples). Hence, all spikings for the LOQ confirmation were performed in accordance with these concentration levels.

In order to establish the limit of quantification, 25 blank samples of fish muscle were prepared, as previously described in the method optimization section, without addition of the analytes. Sample preparation and analysis were conducted on two different days. Following this experiment, the calculated LOD and LOQ for lead was 8.77 μg kg^{-1} and 29.23 μg kg^{-1}, respectively. For cadmium, a negative value for the SD was obtained, leading to a negative value for the LOD and LOQ, which obviously does not present any chemical meaning. This behavior can be attributed to the difference in the signal obtained from the first day of analysis to the second. However, since for both elements all measurements resulted in very low absorbance values (basically much lower than 0.01 s), the confirmation of the LOQ values for both elements by using spiked samples assumes a very important role, and therefore, this experiment was successful.

Accordingly, the confirmation of the LOQ must be performed, which was achieved in this work with the analysis of seven spiked blank samples and seven standard solutions at the level proposed for the LOQ.[16] According to the regulation 2002/657/EC,[5] for elements, the recovery data are only acceptable when they are within ±10% of the target value. Table 2 shows the recovery values for both standard solutions and fish samples spiked in the LOQ level for Cd and Pb, as well as the

Table 2 Results obtained for the confirmation of the LOQ values for Cd and Pb, following measurements on fish and standard solutions. Conditions: $n = 7$ for each concentration level, with three readings for each sample/reference solution

Analyte	Medium	Spiked level	Recovery/%	DF^a	F_{found}	F_{table}
Cadmium	Standard	1.0 μg L^{-1}	107.7	5	3.52	4.95
	Fish	6.25 μg kg^{-1}	97.8	6		
Lead	Standard	5.0 μg L^{-1}	96.0	5	1.78	4.95
	Fish	31.25 μg kg^{-1}	93.9	5		

^{a} Degrees of freedom. F-Test 0.05% significance level.

values obtained from the *F*-test of equality of variances. Since the values found for the *F*-test for both elements were lower than the tabulated values ($F_{0.05;\nu_1;\nu_2}$), it is possible to state that the standard deviations are equal when comparing fish samples and standard solutions for the mentioned concentration range. Considering the results from the *F*-test and *t*-test for a 95% confidence level, as well as the adequate recovery range achieved in all analyses for both analytes, it is safe to assume that the average for both standard solutions and fish samples are statistically identical, and this is implied simultaneously in the absence of interferences, adequate precision and trueness, as well as sensitivity at the LOQ level.

3.2.3. Specificity, precision and trueness. A method should be able to distinguish between the analyte and other substances under the experimental conditions. It also needs to present adequate precision, which is the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions, in addition to trueness, which is the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Trueness is usually expressed as bias. The measure of precision is usually expressed in terms of imprecision and computed as standard deviation of the test result. Accuracy is determined from the trueness and precision.[5] In the present work, the evaluation of trueness was made through the recovery values obtained for the investigated concentration levels from the repeatability study.

As mentioned before, a strong control of interference when dealing with inorganic contaminants is achieved under STPF conditions (isothermal atomization, optimization of the atomization conditions, use of chemical modifiers, among others).[5,17] In order to prove the absence of interference, seven spiked blank fish samples and seven standard solutions were analyzed in two concentration levels, 0.2 ML and 1.0 ML. This experiment was performed one more time for fish samples only, by the same analyst and within a short period of time, in order to evaluate the method's repeatability. According to the availability of the equipment and the total amount of spiked samples to be analyzed for each analyte, the specificity experiment was performed with an interval of one week for cadmium and four days for lead. Similarly, repeatability evaluation was performed on two consecutive days for Cd and with an interval of three days for Pb. Considering that only two concentration levels have to be evaluated for lead in fish, it was possible to measure the respective standard solutions for this element on the first day of the repeatability experiment. The same was not possible for cadmium, considering the two MLs for fish in the regulations.[10,11] Consequently, a third day was needed to read the spiked fish samples for comparison with the respective standard solutions.

The results for this extensive study are presented on Table 3. The acceptability criteria are as follows: for concentrations between 10 and 100 μg kg^{-1}, the recovery values should be between 70 and 110%, with RSD ≤20.0%; for concentrations higher than 100 μg kg^{-1}, the recovery values should be between 80 and 110%, with RSD ≤15.0%.[5] In view of the fact that these criteria have been fulfilled for all evaluated levels for Cd and Pb, it is possible to state that the method for both elements presents adequate precision and trueness, being also free of interference. Additionally, the Horwitz ratio (HorRat value) is another parameter that also has to be calculated according to (EU) no 836/2011.[8] The HorRat value is obtained from the ratio between the observed RSD under repeatability conditions and the RSD value estimated from the Horwitz equation. The equation is as follows:

$$\mathrm{RSD} = 2^{(1-0.5\log C)}$$

where *C* is the mass fraction expressed as a power of 10.[5]

Moreover, from the same experiment, it was possible to complete the *F*-test as an additional tool for specificity evaluation. The values found for the test showed that fish samples prepared as slurries with TMAH have no statistical difference from the standard solutions for both analytes under the optimized measurement conditions. Only one exception was found for the level 1.0 ML(2) for Cd (capture fish), which presented

Table 3 Results obtained for cadmium and lead from repeatability and specificity evaluation, stated according to MLs and HorRat values

Analyte	Cadmium						Lead					
Sample	Mean (±SD)[a]/ μg kg^{-1}	Rec.[a]/%	$HorRat_r$	Degrees of freedom[b]/ $\nu_1;\nu_2$	F_{found}[c]	F_{table}	Mean (±SD)[a]/ μg kg^{-1}	Rec.[a]/%	$HorRat_r$	Degrees of freedom[b]/ $\nu_1;\nu_2$	F_{found}[c]	F_{table}
Fish 0.2 ML(1)	9.40 (±0.86)	94.0	0.29	6;6	2.35	4.28	37.63 (±3.34)	94.1	0.34	6;6	3.18	4.95
Standard 0.2 ML(1)	9.87 (±0.41)	98.7	—	6;6			40.10 (±2.00)	100.25	—	6;5		
Fish 1 ML(1)	44.59 (±4.37)	89.2	0.39	6;6	1.42	4.28	207.82 (±7.17)	103.7	0.17	6;5	1.56	5.05
Standard 1 ML(1)	44.94 (±1.56)	89.9	—	6;6			213.64 (±6.09)	106.8	—	5;5		
Fish 0.2 ML(2)	17.35 (±1.22)	86.8	0.24	6;6	1.32	4.39	—	—	—	—	—	—
Standard 0.2 ML(2)	16.83 (±0.49)	84.1	—	5;6			—	—	—	—	—	—
Fish 1 ML(2)	87.18 (±7.09)	87.2	0.36	6;6	6.92	4.28	—	—	—	—	—	—
Standard 1 ML(2)	93.90 (±5.31)	93.9	—	6;6			—	—	—	—	—	—

[a] Values obtained from analyses on days 1 and 2. [b] For fish: ν_1 and ν_2 correspond to the first and second day of measurement for the repeatability study, respectively. For standards: ν_1 and ν_2 represent the comparison between the spiked fish samples and the respective standard solutions. [c] *F*-Test calculated from the analysis of spiked fish samples against the respective levels for the standard solutions; 0.05% significance level for *F*-test.

Table 4 Results for the extension of experiment scope. The spiking of shrimp and fish samples according to MLs for Cd and Pb in shrimp (500 μg kg^{-1})

Element	Cadmium					Lead				
Parameter	Mean (±SD)/μg kg^{-1}	Recovery[a]/%	F_{found}	F_{table}	DF[b]	Mean (±SD)/μg kg^{-1}	Recovery[a]/%	F_{found}	F_{table}	DF[b]
Fish 0.2 ML	94.82 (±2.12)	94.8	2.47	4.28	6	91.66 (±6.32)	91.7	1.77	5.05	5
Shrimp 0.2 ML	93.81 (±3.33)	93.8			6	91.95 (±8.41)	92.0			5
Fish 1.0 ML	449.11 (±7.12)	89.8	1.81	4.95	6	457.01 (±60.39)	91.4	2.48	5.05	5
Shrimp 1.0 ML	451.47 (±5.29)	90.3			5	497.34 (±38.33)	99.5			5

[a] Recovery calculated against the calibration curve. [b] Degrees of freedom. *F*-Test 0.05% significance level.

a value for the *F*-test that was higher than the corresponding tabulated value, at a 0.05% significance level. This higher variance is probably associated with the autosampler dilution, which had to be employed at this level in order to achieve the linear range of the calibration curve, and is certainly more critical for samples than standard solutions. To overcome this drawback, an alternative would be the manual dilution of the samples spiked at this high concentration level of 1.0 ML(2) for Cd, which was not executed in the present work. Additionally the RSD values were lower than 10.0% for all evaluated levels of both analytes, and the recovery ranges were in agreement with the requirements in all cases. As a consequence, it is possible to affirm the suitability of the method for the proposed use, as well as its accuracy, which is supported by the precision and trueness of the present analytical method.

3.2.4. Extension of the scope. The proposal of this experiment, as part of the validation procedure, is to extend the suitability of a well-established and validated method to a similar matrix. For the present work, the aim was to investigate whether the method, initially developed and validated for the determination of Cd and Pb in fish samples by GF AAS, could work with the same performance for shrimp samples. For this assessment, fourteen spiked blank samples of each matrix, namely fish and shrimp, were arranged into two concentration levels (0.2 ML and 1.0 ML for shrimp, which is 500 μg kg^{-1} for both analytes) and analyzed, which resulted in total, in an experiment with twenty eight spiked samples of fish and shrimp. This assay is an extension of the recommendations for specificity evaluation,[16] found in Commission Decision (2002/657/EC)[5] and Commission Regulation (EC) no 836/2011,[8] in order to extend the scope of the validated method to a similar matrix of the same species (*i.e.*, muscle). The results for this study are presented in Table 4. The acceptability criteria for the concentration range of this study are that recovery values should be between 80 and 110% with RSD ≤15.0%.[5]

For lead, the recovery range for both evaluated levels and matrices was from 91.4% to 99.5%, with a precision better than 8.5% in all cases, except for fish with 1.0 ML, which presented RSD equal to 13.2%. For cadmium, the recovery range was between 89.8% and 94.8%, with a precision better than 3.6%. Moreover, in all cases the *F*-test resulted in calculated values lower than the corresponding tabulated values, at a 0.05% significance level, statistically proving the equality between these matrices, as well as the suitability of the employed conditions for analysis of both fish and shrimp samples. Such behavior was expected, since the fish muscle was extensively studied during the method development, and in all instances, satisfactory results were achieved. From these results, it is safe to state that the method is appropriate for the determination of both analytes in fish, as well as shrimp, by GF AAS following TMAH solubilization of the freeze-dried muscles.

4. Conclusions

The employment of sequential factorial designs for the refinement of the evaluated significant factor levels aimed at method development for the determination of cadmium and lead in the studied seafood samples, solubilized with TMAH by GF AAS for food control purposes, has proven to be a highly valuable tool, by meaningfully reducing the number of experiments, as well as the time elapsed to accomplish the method optimization. However, when considering the validation procedure of an analytical method in order to comply with internationally recognized food regulations, according to the exposed data, it is noteworthy how laborious, time-consuming and expensive the process is.

The fitness for purpose of the developed and validated method for the determination of Cd and Pb in fish and shrimp samples by GF AAS is supported by the investigations on linearity, limits of detection and quantification, precision (repeatability), recovery (trueness), accuracy (precision plus trueness) specificity and robustness, which all fulfilled the required criteria established on the thoroughly mentioned foodstuff protocols. Therefore, the present method is apposite for the controlled analysis of the mentioned foodstuffs, even as it provides values for the establishment of internal quality control parameters for the supervision of the performance of routine analyses.

Acknowledgements

Authors are thankful to Brazilian Ministry of Agriculture, Livestock and Food Supply for financial support and to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) for a scholarship (D. P. Torres). Invaluable contribution is acknowledged to R. E. Bruns for the help with the factorial designs and their discussions.

References

1 G. F. Nordberg, B. A. Fowler, M. Nordberg and L. Friberg, *Handbook on the Toxicology of Metals*, Academic Press, San Diego, 3th edn, 2007.
2 N. Bilandzic, M. Dokic and M. Sedak, Metal content determination in four fish species from Adriatic Sea, *Food Chem.*, 2011, **124**, 1005–1010.
3 L. Noel, V. Dufailly, N. Lemahieu, C. Vastel and T. Guerin, Simultaneous analysis of cadmium, lead, mercury, and arsenic content in foodstuffs of animal origin by inductively coupled plasma/mass spectrometry after closed vessel microwave digestion: method validation, *J. AOAC Int.*, 2005, **88**, 1811–1821.
4 CODEX STANDARD, CODEX general standard for contaminants and toxins in food and feed, CODEX STAN 193–1995, 2010.
5 European Commission. 2002. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results – 2002/657/EC.
6 International Organization for Standardization (ISO). ISO/IEC 17025:2005 – General requirements for the competence of testing and calibration laboratories.
7 J. Moreda-Pineiro, C. Moscoso-Perez, P. Lopez-Mahia, S. Muniategui-Lorenzo, E. Fernandez-Fernandez and D. Prada-Rodriguez, Multivariate optimization of hydride generation procedures for single element determinations of As, Cd, Sb and Se in natural waters by electrothermal atomic absorption spectrometry, *Talanta*, 2001, **53**, 871–883.
8 Commission Regulation (EU) No 836/2011, Laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(*a*)pyrene in foodstuffs.
9 Regulation (EC) No. 882/2004 of the European Parliament and of the Council, 2004, Official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.
10 Commission Regulation (EC) No. 1881/2006, Setting maximum levels for certain contaminants in foodstuffs.
11 Commission Regulation (EC) No 629/2008, amending Regulation (EC) No 1881/2006, Setting maximum levels for certain contaminants in foodstuffs.
12 INMETRO. Orientação sobre validação de métodos de ensaios químicos – DOQ-CGCRE-008. Revisão 04, Julho, 2011.
13 D. P. Torres, M. A. Vieira, A. S. Ribeiro and A. J. Curtius, Determination of inorganic and total mercury in biological samples treated with tetramethylammonium hydroxide by cold vapor atomic absorption spectrometry using different temperatures in the quartz cell, *J. Anal. At. Spectrom.*, 2005, **20**, 289–294.
14 D. P. Torres, M. B. Martins-Teixeira, E. F. Silva and H. M. Queiroz, Method development for the control determination of mercury in seafood by solid-sampling thermal decomposition amalgamation atomic absorption spectrometry (TDA AAS), *Food Addit. Contam., Part A*, 2012, **29**(4), 625–632.
15 R. E. Bruns, I. S. Scarminio and B. de Barros Neto, *Statistical Design – Chemometrics*. Elsevier, Amsterdam, The Netherlands, 2006.
16 D. P. Torres, M. B. Martins-Teixeira, S. Cadore and H. M. Queiroz, Method validation for control determination of mercury in fresh fish and shrimp samples by solid sampling thermal decomposition/amalgamation atomic absorption spectrometry, *J. Environ. Sci. Health, Part B*, 2015, **50**(7), 514–522.
17 B. Welz and M. Sperling, *Atomic Absorption Spectrometry*. Wiley-VCH, Weinheim, Germany, 1999.