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Dedicated to Professor Emil Cordoș
on the occasion of his 80th anniversary

EVALUATION OF THE ANALYTICAL CAPABILITY OF THERMAL DESORPTION ATOMIC ABSORPTION SPECTROMETRY METHOD USED FOR MERCURY DETERMINATION IN SEAFOOD

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ABSTRACT. Mercury is recognized as a highly toxic and widespread element in environment that can be transferred in the whole food chain. Thus, the content of mercury in foodstuff become of great interest. The aim of this paper is to assess the analytical capability and validation of the method for quantitative determination of total mercury (Hg) in seafood using thermal desorption atomic absorption spectrometry (TD-AAS). TD-AAS is a simple technique which does not require sample digestion prior to analysis. The main figures of merit such as selectivity, linearity, limit of detection (LoD), limit of quantification (LoQ), working range, accuracy and precision were studied and discussed in relation with the requirements in the Commission Decision 2002/657/EC and Commission Regulations 2011/836/EU and 2007/333/EC. Measurement uncertainty was estimated using top-down approach and was compared with the maximum uncertainty value calculated as specified in the Commission Decision 2002/657/EC. LoD estimated using 3s criterion was found to be 3.0 μg kg^{-1}, while LOQ 9.0 μg kg^{-1}. The recovery (%), estimated by using the certified reference material BCR-463 Tuna Fish, was 95 ± 5.0 %, whereas recovery (%) estimated using spiked samples was 92 ± 5.6 %. Standard deviation of repeatability (sr) was 5.6% (n=10 parallel samples), while standard deviation of within-laboratory reproducibility (sR) was 9.8 % (n=10 parallel samples), which correspond to HorRat's index for repeatability and reproducibility of 0.28 and 0.50, respectively. The estimated expanded relative uncertainty (k=2) was 15.6 %. The obtained figures of merit fulfil the requirements of the European legislation, and demonstrate

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that the laboratory can properly apply the method in order to achieve accurate results. The paper represents a model for the method validation in analytical laboratories in order to check the fit for purpose of analytical methods.

Keywords: *mercury, uncertainty estimation, validation, seafood, TD-AAS*

INTRODUCTION

Elemental mercury (Hg) and its organic and inorganic species are well-known as being highly toxic to the living organisms even in low concentrations, and have no known physiological function. In addition, Hg is recognised to have a high bioaccumulation factor [1-3]. Hg is released into environment through atmospheric paths, from both natural (volcanic emissions, oceans, vegetation, wetlands), and anthropogenic (mining, use of pesticides, burning of fossil fuels, chemical industry, etc.) sources [4-7]. Once released into environment, Hg persists for very long time, and circulates between atmosphere, water, sediments, soil and biota in different forms [8]. This behaviour has led to increased concentration of Hg in ocean water and ultimately, to its accumulation in seafood, which imply an increased interest for its determination at low concentrations.

Hg toxicity depends also on its chemical form. The principal chemical forms in the environment are: elemental Hg, divalent inorganic Hg, methyl Hg, and dimethyl Hg. Although both inorganic and organic species of Hg are toxic [9], the organic compounds were found to be the most toxic [1]. Studies in literature present Hg speciation in environmental and food sample both by chromatographic and non-chromatographic techniques [10-12]. However, Commission Regulation 1881/2006/EC [13] set the maximum level for total Hg in foodstuff, while Decisions 2007/333/EC and 2002/657/EC [14,15], impose strict requirements for the analytical performance and results interpretation for laboratories that analyse contaminants in foodstuff. Thus, the existence of highly precise and accurate analytical techniques with well-defined figures of merit for food analysis is a real need. The most commonly used analytical techniques for the determination of Hg in solid samples are based on wet digestion [16,17], but these techniques are time and reagents consuming. A good alternative to wet digestion is the use of reagent-free methods as they are based on thermal decomposition of solid samples or extraction and pre-concentration of liquid samples [5, 18-20]. Thermal desorption atomic absorption spectrometry (TD-AAS) is based on thermal decomposition of sample, mercury reduction using a catalyst, followed by Hg vapour trapping on a gold amalgamator. Subsequently, Hg is desorbed and

transported in a measuring cell where its concentration is measured by atomic absorption spectrometry [5]. Due to its advantages, this method has been employed for the determination of total Hg traces in food samples [21-25].

The aim of this study was to perform a detailed validation of total Hg determination in seafood by TD-AAS analysis applied according to EPA Method 7473 [26] in relation with the demands of the Decisions 2007/333/EC, 2011/836/EC and 2002/657/EC on the determination of toxic elements in food. When presenting the measurement results, it is necessary to evaluate their confidence intervals [27-29]. The estimation of measurement uncertainty was made, based on the evaluation of its uncertainty components, and their combining using the law of propagation of uncertainty. The paper is important for the routine analytical laboratories since it presents all the steps necessary to demonstrate the fit-for-purpose and to evaluate the measurement uncertainty for mercury determination in seafood.

RESULTS AND DISCUSSION

Method validation

The validation of the analytical procedure for Hg determination in seafood samples was performed by evaluating the main figures of merit: selectivity, linearity, limit of detection (LoD), limit of quantification (LoQ), working range, trueness/accuracy (including matrix effect), precision (repeatability) and measurement uncertainty.

Selectivity was verified by measuring blank samples (5 % HCl) in the absence of the analyte. Results revealed that there was no significant growth of absorbance signal at the wavelength of the Hg absorption (253.65 nm) when the blank samples were introduced into the instrument. The selectivity in this technique is assured both by the amalgamation step, which is a selective reaction for mercury, and by using of a characteristic wavelength for mercury [30]. The absorbance signal for released Hg in function of time, registered after amalgamator heating, is presented in Figure 1. The first peak corresponds to Hg measured in high sensitivity cell, while the second peak corresponds to low sensitivity cell (see Experimental section).

Linearity of the calibration curve constructed using 6 levels of concentration (0 - 0.050 µg Hg) was tested. The calibration curve was produced by injecting different weights of 100 µg L^{-1} or 1,000 µg L^{-1} Hg aqueous standards in 5 % HCl into the nickel-sampling boat. The calibration curve represents the absorbance in function of mass of injected Hg (ng). The determination coefficient, r^2 was 0.9991 and the residual error was smaller than 10%, indicating a good linearity of the method.

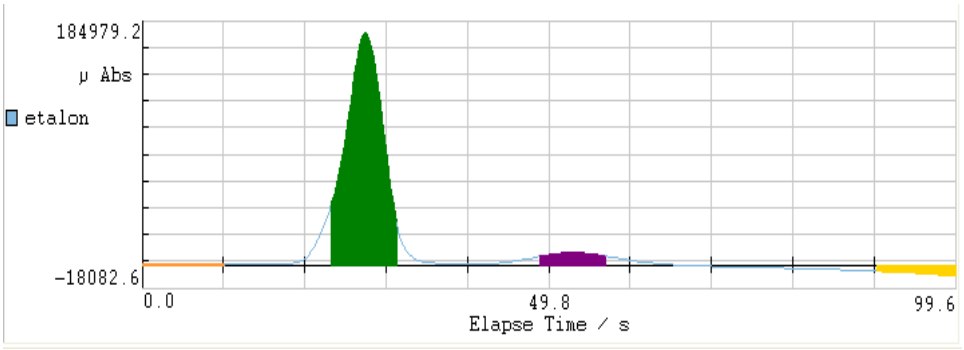

Figure 1. Typical absorbance signal for Hg determination

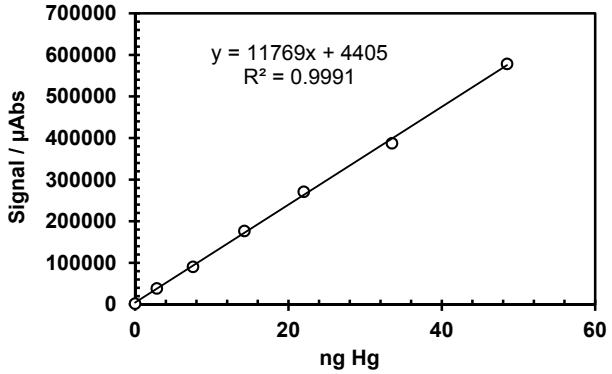

Figure 2. Calibration curve for Hg determination by TD-AAS

Limit of Detection (LoD) and Limit of Quantification (LoQ). LoD was calculated on the basis of 3 s criterion ($LoD=3s_B/m$), where m was the slope of calibration curve and s_B the standard deviation of 10 successive measurements of blank (5 % HCl). The LoQ was calculated as being $9s_B/m$. LoD was found to be 0.30 ng Hg, which means, for 100 mg sample a LoD of 3.0 μg kg^{-1}. LoQ was calculated to be 0.90 ng Hg (9.0 μg kg^{-1} if 100 mg of sample is analysed). This value was verified by analysing spiked solutions at the Hg content level equal to the evaluated LoQ. Relative standard deviation for ten replicates at this level of concentration was 17.5 % and recovery in confirmation of lower working range concentration was 90 %, what is satisfactory performance (targeted repeatability expressed as relative standard deviation (RSD) below 20 %, and recovery between 85-115 %). The method fulfils the requirements for Hg determination in foodstuffs (Decision 2007/333/EC): LoD and LoQ are less than one tenth and one fifth respectively, from the maximum level of 500 μg kg^{-1} Hg in fishery products (Decision 2006/1881/EC).

For the working range, at the lower end of the range, the restrictive factor is LoQ, while, at the upper end, limitations are imposed by various effects depending on the instrument response. For high sensitivity cell, the calibration curve is linear up to 50 ng Hg. If 100 mg sample is weighted and introduced in the system, the upper limit of working range is 5000 μg kg^{-1}, thus maximum level of 500 μg kg^{-1} Hg in fishery products and crustaceans (Decision 2006/1881/EC), can be easily measured by TD-AAS. Moreover, the upper limit of the working range can be extended by analysing less amount of sample or by using the low sensitivity cell of the instrument.

The *accuracy* of a method is acceptable if the mean analyte concentration measured in a CRM falls within ±10 % of the target value according to Commission Decision 2002/657/EC. Accuracy was studied by evaluating the recovery of a fish CRM (BCR-463 Tuna Fish). Thus, 5 parallel samples of CRM were analysed in order to determine the methods accuracy. Average recovery for fish CRM was 94 % with relative standard deviation of 5.0 % (n = 5 parallel samples). In addition, trueness was evaluated using the recovery for real fish samples spiked with known content of Hg. To each fish sample, amounts of 10 ng Hg were added. The recovery rate was calculated by taking into account the found concentrations in the enriched samples and the added concentration. The average recovery for spiked fish samples was 92% with a relative standard deviation of 5.6 % (n = 5 parallel samples). The results of Hg recovery for food CRM and spiked samples determined against aqueous curves confirmed that the method has no matrix effect.

The *precision* of Hg determination was verified in terms of compliance with the HorRat's index, calculated as the ratio of the relative standard deviation (RSD) found within the repeatability assay of test samples, and the predicted standard deviation (PRSD), calculated using the Horvitz's equation [31]:

$$PRSD = 2^{(1-0,5logC)} \quad (1)$$

where C is the half of the maximum mass fraction of Hg in fish tissue (2.5×10^{-7}) [9].

The repeatability of a method complies with requirements in Commission Decision (2007/333/EC) and Commission Regulation (2011/836/EU) if the HorRat index calculated as the RSD/PRSD ratio is less than 2 for Hg concentrations higher than 100 μg kg^{-1}. Precision was assessed both in terms of repeatability and reproducibility. For the repeatability study, the results were obtained by analysing 10 parallel samples by a single operator using the same equipment, while for the reproducibility study, a sample was measured in 10 different days by different operators using the same equipment. RSD for repeatability (RSD_r) was 5.6%, while RSD for reproducibility (RSD_R) was 9.8%

that correspond to $HorRat_r$ index of 0.28 and $HorRat_R$ index of 0.50, which denotes satisfactory performance. Summary of the results is presented in Table 1.

Table 1. Results of method validation for the measurement of Hg in seafood by TD-AAS method

Validation parameter	Results
Selectivity	No interfering signal
Linearity	R^2 = 0.9991
Limit of detection	3.0 μg kg^{-1}
Limit of quantification	9.0 μg kg^{-1}
Working range	9.0 – 5000 μg kg^{-1} (can be extended)
Trueness (recovery)	94% for CRM; 92% for spiked samples
RSD_r	5.6% (n=10 parallel samples)
RSD_R	9.3% (n=10 parallel samples)
$HorRat_r$ *index*	0.28
$HorRat_R$ *index*	0.50

Measurement uncertainty evaluation

In brief, the steps of the method are as shown in Figure 3.

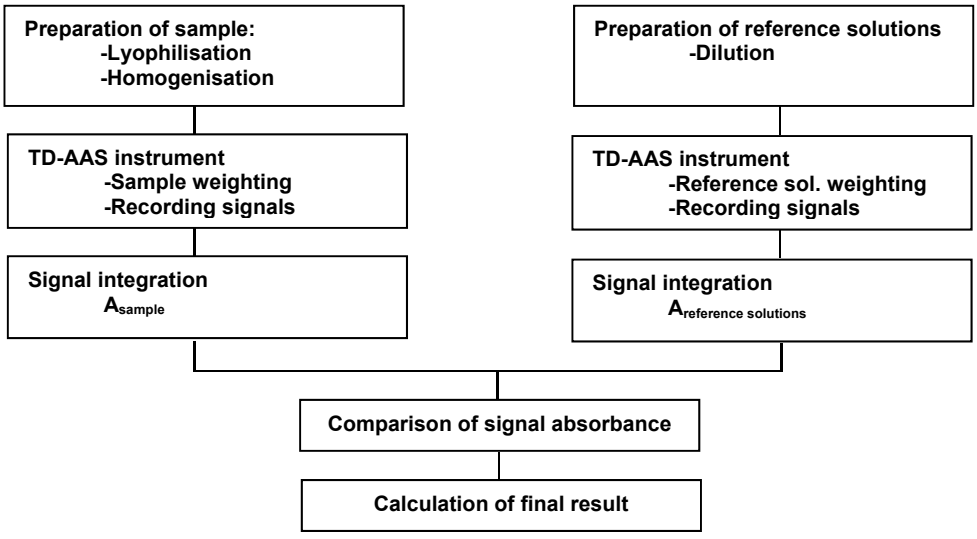

Figure 3. Experimental procedure for the measurement of Hg

Measurement uncertainty evaluation was based on method validation data (the "top down approach"), assuming that they comprise the total analytical procedure [26]. The identified main sources of measurement uncertainty were uncertainty of calibration reference materials (Ci), uncertainty of delivered volumes, uncertainty of weighted reference solutions and sample, uncertainty of the calibration curve, and accuracy and repeatability of the method, as presented in Figure 4 – cause and effects diagram (fishbone diagram).

Trueness of the method was determined by recovery study on CRM. The precision of the procedure represents a substantial source of measurement uncertainty and therefore requires detailed consideration in order to avoid over or underestimation of the combined uncertainty. Sources of uncertainty such as those arising from balances, volumetric measuring devices and influences of environmental conditions were covered by the within-laboratory repeatability. Following these assumptions, the total uncertainty of the method was composed of a contribution from the accuracy of the method (bias) and contribution from repeatability study in order to cover all the relevant uncertainty sources.

To estimate trueness of the method, recovery calculated from CRM analysis was used. Standard uncertainty associated to bias was calculated from Eq. 2:

$$u(B) = \sqrt{B^2 + u(C_R)} \tag{2}$$

where B is deviation from true value (140 μg kg^{-1}), u(C_R) is uncertainty of the certified reference material tested (80 μg kg^{-1}).

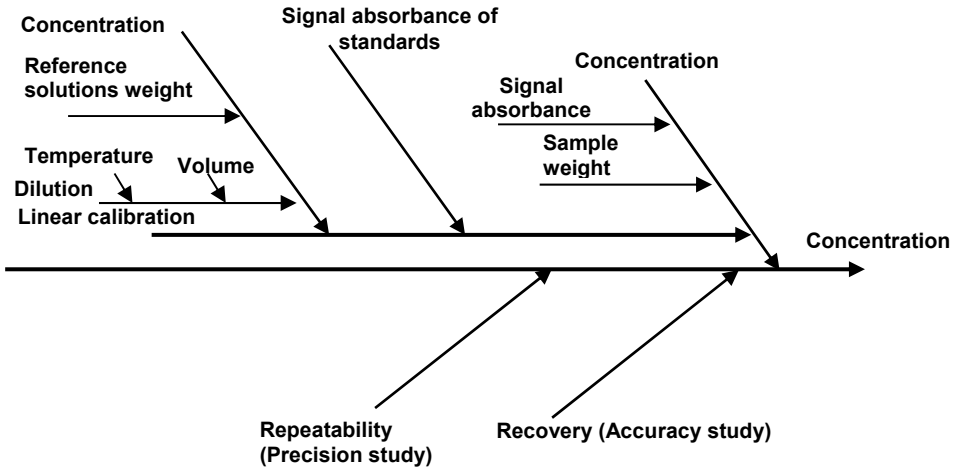

Figure 4. Cause and effects diagram (fishbone diagram) of uncertainties in measurement of Hg using TD-AAS

Uncertainty associated to method bias was calculated to be 160 μg kg^{-1} (5.6 %). Combined uncertainty was calculated following the Eq. 3:

$$u(Hg) = \sqrt{u(B)^2 + u(R_w)^2} \quad (3)$$

where $u(R_W)$ is the standard deviation resulted from within-laboratory repeatability study, for a real fish sample (average ± s = 329 ± 18 μg kg^{-1}). Before to be combined, the two components were transformed to relative standard uncertainties. Combined uncertainty u(Hg) was calculated to be 7.8%. The expanded uncertainty (U_E) resulted by multiplying u(Hg) by the coverage factor (k=2) which indicate the confidence interval expected to include 95% of results attributable to the measurand was 15.6%.

According to Commission Regulation (2011/836/ EU), the combined standard measurement uncertainty u(Hg) should be less than the maximum standard measurement uncertainty (Ut), calculated with the formula:

$$Ut = \sqrt{(\frac{LOD}{2})^2 + (\alpha\, c)^2} \quad (4)$$

where LoD is the limit of detection of the method (μg kg^{-1}); C is the concentration of interest (μg kg^{-1}); α is a numeric factor depending on the value of C (α = 0.18 for concentrations ranged between 51 - 500 μg kg^{-1} Hg). For the average concentration for the real sample of 329 μg kg^{-1} calculated Ut was 59 μg kg^{-1}, which represent 17.9%. Consequently, combined standard measurement uncertainty u(Hg) of 7.8% calculated for our method is well below Ut, which indicates satisfactory performance.

Real seafood samples analysis

Seafood samples were purchased from several supermarkets from Cluj-Napoca. In laboratory, the samples were lyophilised and analysed directly by TD-AAS. The average measured concentrations are presented in Table 2. The Hg concentrations ranged between 112 – 411 μg kg^{-1} wet weight (the higher Hg concentration was found in Hake), in the same order of magnitude with the results reported by Miclean et al. [32]. However, Hg concentrations in seafood samples were, in all cases, below the maximum level of 500 μg kg^{-1} wet weight set in Decision 2006/1881/EC, accordingly their consumption do not pose acute risks for consumers' health.

Table 2. Concentrations of total Hg measured in seafood samples (average ± U_E, k=2, n=5 parallel samples)

Sample type	**Hg** (μg kg^{-1} wet weight)
Hake *(Merluccius merlucius)*	411 ± 64
Shrimps (*Pandalus Borealis*)	310 ± 48
Pink shrimp *(Pandalus Borealis)*	112 ± 17
Squid *(calamarium)*	329 ± 51
Pangasius (*Pangasius buchanani*)	135 ± 21
Marbled rockcod (*Notothenia rossii*)	220 ± 34

CONCLUSIONS

The paper presents all the steps necessary to validate and to evaluate the measurement uncertainty for Hg determination in seafood using TD-AAS, a simple technique which require no sample digestion prior analysis. The studied figures of merit fulfil the requirements in terms of selectivity, linearity, LoD and LoQ, accuracy, and precision set out in the to the requirements in the Commission Decision 2002/657/EC and Commission Regulations 2011/836/EU and 2007/333/EC. The method was validated to be used for concentrations between 9.0 – 5000 μg kg^{-1}. TD-AAS techniques provide LoQ well below the maximum admitted concentration of Hg in seafood, which make it suitable to measure its concentrations at the imposed limits, and in addition for monitoring studies of Hg trace levels in seafood samples. Accuracy was studied by evaluating the recovery for a fish CRM and also by evaluating the recovery for spiked seafood samples. The recoveries for both CRM and spiked samples were in the target imposed by Commission Decision 2002/657/EC (90-110%). The precision of Hg determination was verified in terms of compliance with the HorRat's index calculated both for repeatability and reproducibility, and satisfactory results were obtained. Expanded uncertainty, estimated using top-down approach using the data from accuracy and precision studies, was 15.6% for a coverage factor k= 2. The combined standard uncertainty was less than the maximum standard measurement uncertainty calculated according to Commission Regulation (2011/836/ EU), indicating satisfactory performance of the method. It was demonstrated that the method can be applied in the laboratory for the designed purpose, determination of Hg in seafood by TD-AAS.

EXPERIMENTAL SECTION

Reagents, Standard Solutions and CRM

Stock standard solutions of mercury (1000 μg mL^{-1}) purchased from Merck (Darmstadt, Germany) was used for instruments calibration. Ultrapure water (18 MΩ cm^{-1}) obtained from a Millipore Direct Q3 (Millipore, France) and 30% (w/w) HCl ultrapur (Merck, Darmstadt, Germany) were used for all dilutions. A fish CRM BCR-463 (tuna fish) purchased from LGC Promochem (Wesel, Germany) was analysed to assess the accuracy of Hg determination. Oxygen (99.999%) for Hydra-C Analyzer supplied by Linde Gas SRL Cluj-Napoca, Romania was used.

Instrumentation and analytical method

The direct measurements of mercury from solid samples were carried out using an Automated Direct Hg Analyzer Hydra-C (Teledyne Instruments, Leeman Labs, USA). A block diagram of the instrument is presented in Figure 5 [33].

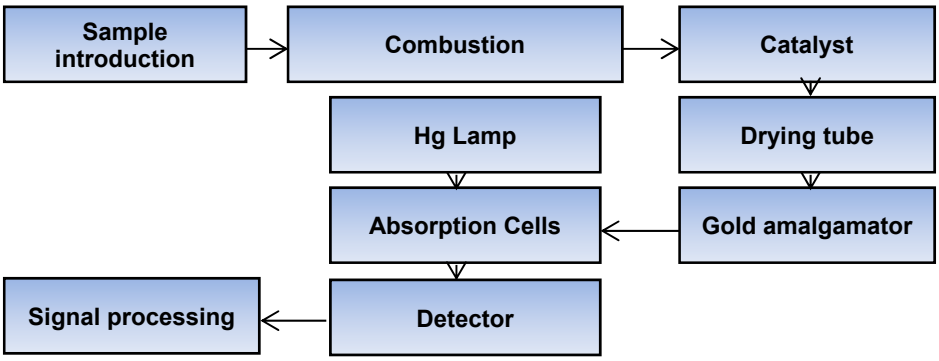

Figure 5. Block diagram of the TD-AAS instrument

The analyser includes a furnace module for the thermal decomposition of sample, an amalgamation trap, and a unit to measure the absorbance (AAS module). Determinations of Hg were performed using up to 100 mg dry sample weighted in nickel boats with a precision of ± 0.1 mg. The instrumental settings used for the Hg analyser for the all determinations are presented in Table 3.

Table 3. Instrumental setting for Hg determination in seafood using TD-AAS system

Parameter	Setting
Sample weight	100 ± 0.1 mg
Drying temperature/time	300°C / 45 sec.
Decomposition temperature/time	800°C / 150 sec.
Catalyst temperature	600°C
Catalyst Wait Period	60 sec.
Gold Trap temperature/time	700°C / 30 sec.
Measurement time	90 sec.
Oxygen Flow rate	300 min L^{-1}

Real seafood samples were purchased from supermarkets from Cluj-Napoca, Romania and were lyophilised prior to analysis using a FreeZone 2.5 Liter Benchtop Freeze Dry System (Labconco, USA).

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