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Improvements and application of a modified gas chromatography atomic fluorescence spectroscopy method for routine determination of methylmercury in biota samples

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ABSTRACT

Improvements to the application of a combined solid-phase microextraction followed by gas chromatography coupled to pyrolysis and atomic fluorescence spectrometry method (SPME–GC–AFS) for methylmercury (MeHg) determination in biota samples are presented. Our new method includes improvements in the methodology of determination and the quantification technique. A shaker instead of a stirrer was used, in order to reduce the possibility of sample contamination and to simplify cleaning procedures. Then, optimal rotation frequency and shaking time were settled at 800 rpm and 10 min, respectively. Moreover, the GC–AFS system was equipped with a valve and an argon heater to eliminate the effect of the decrease in analytical signal caused by the moisture released from SPME fiber. For its determination, MeHg was first extracted from biota samples with a 25% KOH solution (3h) and then it was quantified by two methods, a conventional double standard addition method (AC) and a modified matrix-matched calibration (MQ) which is two times faster than the AC method. Both procedures were successfully tested with certified reference materials, and applied for the first time to the determination of MeHg in muscle samples of goosander (*Mergus merganser*) and liver samples of white-tailed eagle (*Haliaeetus albicilla*) with values ranging from 1.19 to 3.84 mg/kg dry weight (dw), and from 0.69 to 6.23 mg kg^{-1} dw, respectively.

1. Introduction

Organometallic compounds of mercury are some of the most toxic substances in aquatic systems. Methylmercury (MeHg) is the most common and toxic form of organomercury compounds, and has a dangerous tendency to bioaccumulate in aquatic food chains [1,2]. Determination of total mercury (THg) in birds' tissues [3–6] has been more often performed than the determination of MeHg [7–9]. Nevertheless, it is clear that the determination of MeHg is almost mandatory for risk assessment, since MeHg data facilitates a more complete understanding of toxic effects and risk to biota. The most frequently used separation technique for MeHg determination in environmental samples is gas chromatography (GC). In most cases the gas chromatograph is coupled to atomic emission spectrometry (AES) [10], atomic fluorescence spectrometry (AFS) [11] or mass spectrometry (MS) [12,13]. AFS enables the determination of mercury at the pg level in environmental samples. To determine MeHg using a GC–AFS method, the conversion of mercury to volatile and non-polar alkyl derivatives is required [14–16]. The most common derivatisation methods are ethylation of $MeHg^+$ and Hg^{2+} ions in the water phase by sodium tetraethylborate ($NaBEt_4$) [17,18], phenylation with $NaBPh_4$ [19] and propylation with $NaBPr_4$ [20]. Then, the MeHg derivative is extracted from the headspace with a SPME fiber, being the 100 μm polydimethylsiloxane (PDMS) the most common type of fiber employed for MeHg determination in environmental samples [21–23]. In this regard, the most common derivatisation procedures used for mercury have been reviewed [24,25]. Finally, volatile mercury derivatives are easily separable by gas chromatography [26–28], and after GC separation, MeHgEt is decomposed by means of a pyrolyzer at 800 °C and determined with a AFS detector.

The main aim of this paper was to improve the above-mentioned methodology of MeHg determination in biota samples, mainly focused on the quantification technique and the method of determination. Firstly, determination of MeHg in biological samples with the SPME–GC–AFS method requires releasing of MeHg from the sample into solution by means of alkaline digestion with

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KOH at 60 °C. Unfortunately, this method produces some matrix effects that have been reported and overcome by standard-addition calibration [18]. The standard-addition (AC) method enables one to minimize the influence of the matrix, but the method is time-consuming. On the other hand, owing to instability of the detector signal over a longer period of time, the typical and much faster external calibration (EC) method cannot be used with an AFS detector. The ageing of the detector lamp, and the electronic signal-level correction, change the signal-level in time, especially after turning off/on the detector. An internal standard method helps to correct the signal fluctuations, but complicates the analytical procedure, increases the total error and the cost of the determination.

To speed up the determination of MeHg while maintaining the accuracy and precision of standard-addition methods, and taking into account the limitations of the AFS detector, a modified matrix-matched method of quantification (MQ) was applied.

The correctness of determinations in the MQ method is controlled by calibration-line correlation R^2 and Certified Reference Material (CRM). This method permits to perform twice the amount of daily determinations of MeHg in biota samples.

To make the determination simpler and to reduce the possibility of sample contamination, the stirrer (used at the stage of derivatisation and SPME extraction) was replaced with a shaker. To apply the shaker in the SPME–GC–AFS method, a special holder for the fiber was constructed and successfully tested.

To eliminate the decrease of peak signal, caused by moisture condensing on the optics of AFS detector, the solutions proposed by Yang et al. [29] for a Tenax trap were successfully applied to the procedure with PDMS fiber. The main improvement was the argon heater-module that accelerates the detector-drying process and speeds up the determination process.

Finally, the improved methodology was successfully applied to the determination of MeHg in several unique biota samples.

2. Experimental

2.1. Instrumentation

Determination of MeHg was performed using the system shown in Fig. 1. The system consists of a gas chromatograph (Hewlett Packard 5890 I) – Fig. 1a, equipped with a 15 m, 0.53 mm ID column – Rtx-1 Restek. The outlet of the GC was coupled to an atomic fluorescence detector Tekran 2500 (Fig. 1b) via a home-made 20 cm-long pyrolysis unit (Fig. 1c).

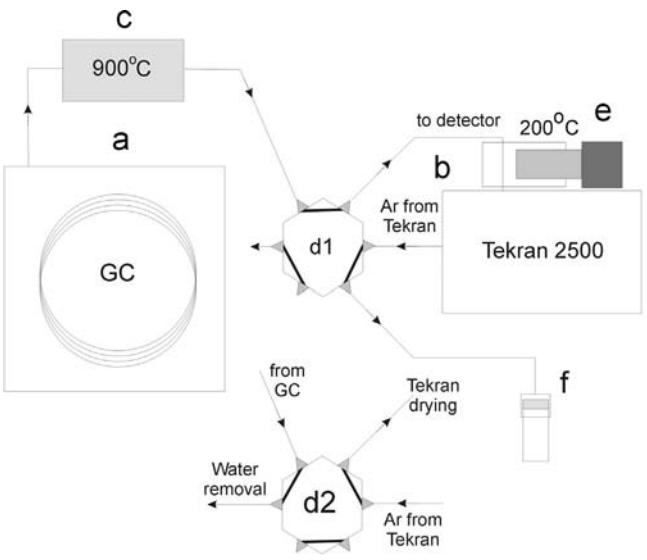

Fig. 1. System for determination of methylmercury: gas chromatograph (a), Tekran 2500 detector (b), pyrolyser unit (c), valve – measure mode (d1), valve – drying and moisture removing mode (d2), argon heater (e), and argon cap (f).

In order to avoid the decrease of peak signal, caused by moisture condensation into a Tekran detector, the modified system for Tenax-GC–AFS system [29] was enhanced and applied to the SPME–GC–AFS system. A 4-way valve (Fig. 1; d1,d2) was added to the typical GC–AFS determination system. The valve enables one to remove moisture at the beginning of the chromatographic separation, and to dry the detector between experimental runs. To shorten the time of a single determination, an argon-heating device (Fig. 1e) was additionally added. This modification increases the efficiency of the detector drying. According to our knowledge this was the first application of a moisture-removal procedure to the SPME–GC–AFS system.

To protect $NaBEt_4$ solution against oxygen during the MeHg measurements, and to extend its time of use, an argon hood (Fig. 1f) connected to the Tekran 2500 argon line was applied. During derivatisation and SPME extraction, a stirrer with a special home-made SPME setup was substituted by a shaker (OS 2 basic, Labart). An Automated Mercury Analyzer MA-2 (Nippon Corporation) was used for the total mercury determination.

2.2. Reagents and standards

All reagents and standards were of an analytical grade or higher. Deionised water was purified by using the HLP5 (Hydrolab, Poland) system. The derivatisation agent, sodium tetraethylborate ($NaBEt_4$, 97%, 1 g), was obtained from Acros Organics (Geel, Belgium). Air-tight microsyringes (100 μL, Hamilton) were used to introduce a 1% solution of derivatisation agent to the vial. Working MeHg solutions (in the range 1–40 ng/mL) were prepared by diluting a certified methylmercury standard (1000 mg kg^{-1}, Alfa Aesar, Karlsruhe, Germany).

Solid-phase microextraction fibers were purchased from Supelco (Bellefonte, PA, USA). Fibers with a 100 μm polydimethylosiloxane (PDMS) coating were used in all experiments. Argon 5.0 has been used as a carrier gas and for Tekran drying. A 25% (w/v) aqueous potassium hydroxide solution (Baker, Deventer, Holland) was used for MeHg extraction from goosander muscle samples.

Literature data [30] suggest that the concentration of MeHg standard solutions may change in time. The real concentration of MeHg in working standard solutions was controlled systematically according to the following procedure. In the MeHg standard, the THg concentration was determined with MA-2 mercury analyzer, the concentration of Hg^{2+} with a double standard-addition method, whereas the concentration of MeHg was the difference between both concentrations.

2.3. Procedure

2.3.1. Preparation and preservation of 1% $NaBEt_4$ solution

The fresh $NaBEt_4$ solution is oxygen-sensitive, and therefore should be prepared daily. To reduce the time spent on reagent preparation, a new procedure for its preparation and protection was developed. A few 20 mL glass vials (cleaned at 600 °C) were weighed and placed in a glove-box filled with argon. About 0.03 g of $NaBEt_4$ powder was added to each vial. Then vials were weighted again to calculate the amount of $NaBEt_4$ in each of the vials. Vials were sealed by septum and stored in a dark bottle at room temperature in an argon atmosphere until needed. In order to prepare a 1% $NaBEt_4$ solution, an appropriate volume of deionised and deoxygenated water was added into the vial through the septum by using a Hamilton microsyringe.

During the measurements, $NaBEt_4$ solution was sucked through the septum. The argon cup (Fig. 1f) was applied to protect the $NaBEt_4$ solution against oxygen. Solution prepared according to the above procedure can be used for two days.

2.3.2. Reduction of carry-over effect

To reduce the carry-over effect two procedures were used: elimination of stirring bar during derivatisation reaction and SPME extraction, and cleaning of vials at 600 °C. In SPME procedures, intensive stirring of solution in the derivatisation and extraction steps is required, for which the magnetic stirring bars are commonly used [31,32]. From an analytical point of view, any object that is in contact with the analyzed solution increases the risk of contamination of the sample. In fact, during our experiments using a stirring bar – which is covered by polytetrafluoroethylene (PTFE) – a memory effect was experimentally observed. To avoid possible contamination by means of the PTFE, the magnetic stirrer was replaced by a shaker. In such cases, the use of SPME fiber required a special holder to allow the immobilization of fiber during shaking is required. For this purpose a special setup (Fig. 2) was built and tested. The setup consists of two typical metallic vial caps with a punctured aluminum plate in between them and a polyethylene conical tube. The connection of all parts was made with double shrinkable tubing. Before extraction, a typical SPME fiber holder was inserted into the polyethylene tube (with simultaneous septum perforation).

The second method of carry-over effect reduction was temperature decomposition of MeHg traces. All vials, before use, were washed with HNO_3, rinsed with deionised water and heated for 3 h in 600 °C in the electric furnace to decompose and release any traces of Hg.

2.3.3. Sample collection and preparation

The detailed description of goosander (Mergus merganser) samples collection was described earlier [6]. Briefly, individuals of goosander were collected in 2005. The collected birds fed in the ice-free part of Lake Dąbie (Northwest Poland), and became accidentally trapped by stationary fishing nets deployed in the lake, and drowned. Muscle samples were used for Hg determination.

Samples of White-tailed Eagles (*Haliaeetus albicilla*) come from central and northern Poland and were collected between 2005 and 2012. The study was conducted on liver samples of birds that died of natural causes or by accident.

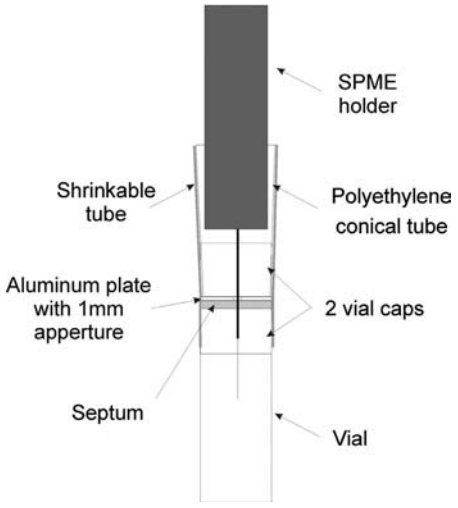

Fig. 2. Setup for SPME fiber used with a shaker.

Biological (muscle or liver) samples of about 10 g were dried to a constant weight and the samples were pulverized in a Pulverisette 6 planetary grinder.

The sample digestion procedure for MeHg determination was based on the method developed by Cai et al. [33], and modified by Carrasco et al. [18]. About 30 mg of biological sample or CRM was placed in a 20 mL vial, then 8 mL of 25% (w/w) aqueous KOH solution was added. The vial with mixture was placed in the ultrasonic bath for 1 min to homogenize the mixture. Then mixture was heated in a water bath at 60 °C for 180 min. All digested samples were analyzed within 7 days.

2.3.4. SPME sampling and detection

The 50 μL aliquot of a digested sample was transferred to the 20 mL glass vial with acetic-acetate buffer solution (pH 5.0). 100 μL of a 1% $NaBEt_4$ solution was added through the septum. The vial was placed in a shaker for 10 min (800 rpm), then the PDMS fiber was introduced into the headspace. Rotation frequency was changed to 500 rpm and the content of the vial was shaken for 5 min. Afterwards the fiber was withdrawn from the sample headspace and immediately placed in the injection port of the Gas Chromatograph. Organic mercury compounds were thermally desorbed from the fiber at 170 °C. Desorption time was 45 s. Immediately after introducing fiber into the injection port of the GC, the valve was switched to position d2 (Fig. 1). Then, with this valve position, water and Hg^0 from the fiber were re-directed to flow outside the system. After 1 min, the valve was switched to position d1 and GC column was connected to the Tekran detector. During the GC separation the column was kept at 30 °C for 2 min, then heated to 80 °C (25 °C/min) and kept at 80 °C for 5 min. The detector was dried between measurements for 4 min (valve position d1) with heated argon (flow of 750 mL/min.). After drying, the argon flow was reduced to 30 mL/min.

2.4. MeHg concentration determination in biota samples

To determine the concentration of MeHg in the biota samples, two methods were applied: a conventional double standard addition (AC) method and a modified matrix-matched method of quantification (MQ). Both methods were optimized to give as short as possible determination time while maintaining its quality control.

2.4.1. Optimized AC method

The MeHg concentration in the KOH extract of biota samples was measured twice using the method described before. If the difference between both analyses was more than 5%, then more analyses were performed. Then, the first standard solution was added to the samples and the measurement of MeHg was performed once. Subsequently a second standard solution was added to the sample and two measurements were performed. As above, more measurements were added if the difference between results was more than 5%. The correlation (R^2) of the AC line should be at least 0.9995 (in most cases R^2 was 0.9999). The MeHg concentration in sample was calculated based on the obtained AC line. The AC method enables to measure up to 5 samples per day.

2.4.2. Modified MQ method

The AC method is precise, but it is time-consuming. Therefore, a faster MQ method was developed and tested. In the first step of the method THg is determined in the series of 8–10 biota samples (in KOH extract) with an AM-2 Hg analyzer. The MeHg was then determined with the AC method in the sample with the lowest THg. The AC line (with a minimum R^2 0.9995) is used to develop an external calibration line (MQ line) for the rest of the biota samples. Each sample was measured twice. Again, if the difference

between results was more than 5%, then more measurements were added. The external calibration line was used only within one day. Using one of the samples for the preparation of an external calibration line eliminates the potential influence of the matrix on the quality of the determination of MeHg. To control the external calibration line, the CRM was added to the each series of 8–10 biota samples.

3. Results and discussion

3.1. Stirring and shaking effectiveness during SPME

From an analytical point of view, a less-complicated analytical procedure is safer, and for this reason the stirrer was replaced with a shaker, since it eliminates the possible contamination input by the use of a stirring bar, and simplifies the cleaning procedure.

To be sure that the replacement does not decrease the quality of results, then the effectiveness and reproducibility of shaking in comparison to stirring were tested in the following experiment.

An aliquot of 0.7 ng of MeHg in 50 μL of standard solution was transferred to a 20 mL glass vial with 2.9 mL of acetic-acetate buffer solution. A PTFE stirring bar (7 mm long) was placed in the vial. Then SPME setup (with septum) was tightly screwed on, and 50 μl of $NaBEt_4$ were injected into the vial via septum. Derivatisation and SPME steps were performed simultaneously with such a setup, PDMS fiber was inserted to the vial for 10 min in each experiment. In the first three experiments the solution was stirred with a stirring bar (1000 rpm) and in the next three experiments with a shaker (500 rpm). The average MeHg peak areas for stirrer and shaker were 30.32 ± 0.72 ($n=3$) and 30.21 ± 0.22 ($n=3$), respectively. The peaks signals were very similar, and the reproducibility is even better when using a shaker.

Several hundred measurements proved that this home-made setup was able to keep stable a typical Supelco SPME fiber holder during the shaking up to 500 rpm. The method with shaker can be applied in any HS-SPME application.

3.2. Selection of the optimal rotation frequency and shaking time

When selecting the rotation frequency, one should to take into account the technical limitations of the developed system (eg. safety of a SPME fiber placed in a SPME setup) and the need to maximize the analytical signal. In the following experiment four rotation frequencies (200, 400, 600, 800 rpm), during 10 min of derivatisation reaction, were tested. The analytical signal (peak area) using 800 rpm was higher (34.23) in comparison with frequencies from 200 to 600 rpm (20.06–32.48).

The comparison of peak signals obtained at different times of shaking (at 800 rpm) during the derivatisation reaction was also tested. Peak areas for times 7, 10, 15 min. were 31.46 ± 0.47 ($n=3$), 33.04 ± 0.30 ($n=3$) and 33.39 ± 0.48 ($n=3$), respectively, therefore there is no significant difference between 10 and 15 min. Additionally, 10 min of shaking is well-matched with other parts of the analytical procedure, especially with the time of chromatographic separation and the next sample preparation. As a result, 10 min of shaking was chosen as a reasonable compromise between time and the level of analytical signal.

3.3. The effectiveness of drying and cleaning procedures

The drying procedure eliminated very efficiently the analytical signal decrease, caused by the moisture, which could condense on the optical parts of the Tekran detector and increased the reproducibility of the measurements. Differences in chromatograms with (a) and without (b) the moisture-removal procedure are shown in Fig. 3.

3.4. Comparison of AC and MQ methods

The MQ method is based on two predictions: sample matrices are similar, and the AFS signal detector, which has been dried between measurements, is suitably stable throughout one experimental day, which is the time when the external calibration line is applied. To accurately compare the AC and MQ methods, six MQ lines were obtained from six AC lines. The AC lines come from MeHg determination in samples M1 to M3 (first day of experiment) and M4 to M6 (second day of experiment) (Table 1).

The parameters of AC lines are shown in columns 2–4 of Table 1, whereas estimated MeHg concentrations with an AC method (samples M1–M6), are shown in the fifth column. The slope (S) of AC lines decreases in subsequent experimental days. The average slope was 99.38 in the first experimental day and 89.58 the second day. The correlation (R^2) and %RSD of predicted concentration for the AC lines ranging from 0.9996 to 0.9999 and from 0.8 to 3.3%, respectively.

The six developed AC lines were used as external calibration lines (MQ method) to recalculate the amount of MeHg in two series of samples (M1, M2, M3 and M4, M5, M6). Then, data in columns 6–8 in Table 1 represent the amount of MeHg (and its relative error) calculated with MQ lines. The differences between results generated with AC and MQ methods are in a range from 0.2 to 6.7% (with an average value of 2.5%).

3.5. Method validation and application to real samples

The reproducibility of MeHg determination with our modified SPME–GC–AFS method is very high. The relative standard deviations

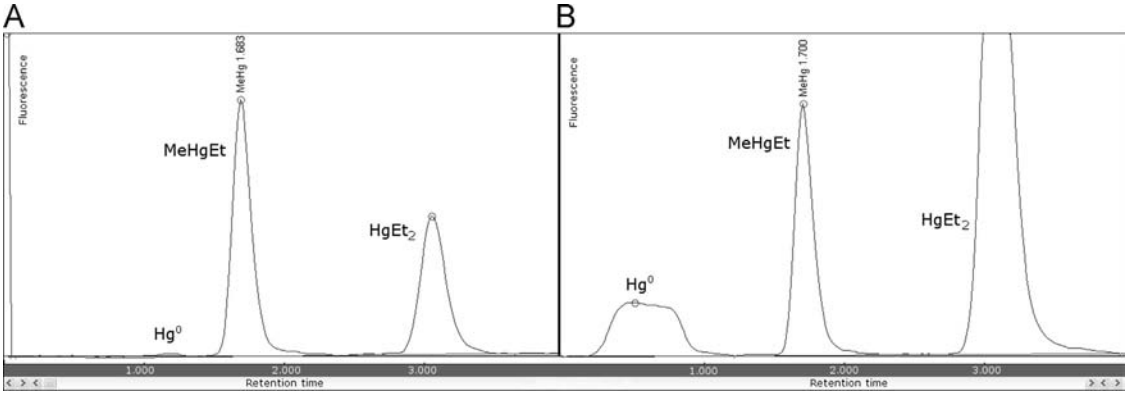

Fig. 3. Typical chromatograms obtained for the same sample of goosander muscle with (a) and without (b) use the procedure of moisture removal during measurements.

Table 1
Comparison between the concentration of MeHg calculated with double standard addition (AC) and a modified matrix-matched calibration (MQ) methods during two consecutive days. Values between parentheses are the differences between results generated by means of both methods.

	AC lines					MQ lines		
	Sample	Slope	R^2	%RSD	MeHg (ng)	MeHg (ng)		
						M1	M2	M3
Experimental day 1	M1	100.52	0.9999	1.4	0.298	0.301 (1.0)	0.299 (0.2)	0.314 (4.9)
	M2	100.52	0.9998	3.3	0.263	0.260 (1.0)	0.258 (2.0)	0.271 (2.8)
	M3	97.11	0.9999	1.6	0.234	0.223 (1.1)	0.221 (0.6)	0.232 (4.1)
						M4	M5	M6
Experimental day 2	M4	92.41	0.9999	0.8	0.630	0.633 (0.5)	0.652 (3.4)	0.667 (5.6)
	M5	89.52	0.9998	1.1	0.743	0.717 (3.7)	0.738 (0.6)	0.755 (1.6)
	M6	86.81	0.9996	2.0	0.571	0.535 (6.7)	0.551 (3.5)	0.564 (1.2)

(RSDs) for MeHg using the procedure with water removal in standard solution (130 pg/mL) was 1% ($n=7$) in comparison with 8% ($n=7$) for procedure without water removal. The %RSD of MeHg determination in goosander muscle sample was 3% ($n=3$). Moreover, during the routine determination ($n=45$) difference between two MeHg peaks area was in range from 96% to 103%.

The technique detection limit, calculated as three times the baseline noise in real sample chromatograms, was 12 ng/g, and the quantification limit, evaluated as ten times the S/N, was 39 ng/g. In the routine determination of MeHg in the goosander muscles samples the linear relationship between concentration and signal was up to 1.5 ng/mL.

The procedure was tested in different days with certified reference materials, DORM-2 (MeHg: 4.47 ± 0.32 mg/kg) and DOLT-4 (MeHg 1.33 ± 0.12 mg/kg). The MeHg concentration in both CRMs was 4.38 ± 0.12 mg/kg ($n=4$) and 1.37 ± 0.03 mg/kg ($n=4$), respectively, in good agreement with certified values.

The reproducibility of the procedure with detector drying and moisture removal in the case of environmental samples was very high, and the average RSD% of predicted concentration in the standard addition method of MeHg was 3%.

3.5.1. Application of AC method for goosander samples

The typical procedure of MeHg determination with the AC method was improved and successfully applied to 16 samples of goosander muscles. MeHg concentration ranged from 1.19 to 3.84 mg/kg and THg was in the range of 1.49 to 3.95 mg/kg. Percentages of MeHg in THg were in the range of 70% to 98%, which is consistent with the results of MeHg in the muscles of other piscivorous birds [9].

3.5.2. Application of MQ method for white-tailed eagle samples

The MQ method (described in Section 2.4.2) was successfully applied for MeHg determination in liver samples of white-tailed eagles (*Haliaeetus albicilla*). In the first step, THg was determined in 5 samples (with MA-2 analyzer). In the second step, the concentration of MeHg was determined in the sample with the lowest total Hg level (L0). For this measurement the AC method was applied. The concentration of MeHg in L0 was 0.71 mg/kg, the R^2 of the line was 0.9999 and the SD of predicted concentration was 0.005 mg/kg. The AC line was used to create an external calibration line (MQ line). The MQ line was checked with DOLT-4 CRM. The MeHg level was 1.38 mg/kg (certified value 1.33 ± 0.12 mg/kg). Subsequently, MeHg was determined in eagle liver samples (L1–L4) with the SPME–GC–AFS method. The concentration of MeHg in samples L1–L4 was calculated using the MQ line ($r^2=0.9999$) and the results ranged within 0.69–6.23 mg/kg, being 0005 the standard error of the predicted concentration value for each signal value in the regression. The reproducibility of the measurements, in the case of using the heating Ar to dry the detector before each measurement and at the beginning of the experimental day, usually allows one to limit the number of repetitions to two. The differences in the peak area of MeHg from two consecutive runs of a sample ranged from 1 to 1.5%. The MQ method enables to measure up to 10 samples per day, whereas the AC method enables to measure 5 samples per day.

4. Conclusions

A series of improved procedures were elaborated, tested and applied to the common SPME–GC–AFS methodology to determine MeHg in unique biota samples. Some of the modifications include improvements on the quantification technique and the method of determination. Modifications for the removal of moisture from the Tenax-GC–AFS system, have been successfully tested in our system and additionally enriched with an argon heating unit, which accelerates the detector-drying process. After detector modification and a selection of the optimal parameters of detector drying procedure, the repeatability of the MeHg determination procedure became high. The RSD for the procedure with water removal in standard solution (130 pg/mL) was 1% ($n=7$) in comparison with this without water removal (RSD=8%, $n=7$). On the other hand, the RSD of MeHg determination in goosander muscle sample was 3% ($n=3$).

The application of a shaker, with a special SPME fiber holder instead of stirrer during derivatisation and extraction steps, reduces the possibility of sample contamination, and simplifies the cleaning procedure.

For a faster determination of MeHg the MQ method of MeHg quantification was elaborated and tested. This method is especially useful in the case of AFS detectors, and the differences between results generated with AC and MQ methods are up to 7%.

Our methodology was successfully applied for the first time to the determination of MeHg in muscle samples of goosander and liver samples of white-tailed eagle with values ranging from 1.19 to 3.84 mg/kg dry weight (dw), and from 0.69 to 6.23 mg/kg dw, respectively. According to the authors' knowledge this is the first time that MeHg has been analyzed in such special samples of biota.

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