Determination of methylmercury in cryptogams by means of GC-AFS using enzymatic hydrolysis

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ABSTRACT

Since methylmercury is a highly toxic compound, there is undoubtedly a need for the monitoring of methylmercury in the ecosystem. However, its isolation from the organic matrix using an appropriate analytical procedure and sensitive detection technique are necessary due to trace levels of methylmercury in biomonitors. This study focuses on the determination of methylmercury in plant matrices by means of GC-AFS. The developed extraction procedure is based on the enzymatic hydrolysis of the matrix by cellulase, followed by the extraction of methylmercury in hydrochloric acid and the extraction of derivatized methylmercury into the organic phase. The limit of detection of methylmercury in environmental samples was $4 \, \mu g \, kg^{-1}$. The method demonstrated sufficient precision, accuracy, and repeatability with respect to the determination of methylmercury in cryptogams. High contents of methylmercury (up to $60.9 \pm 4.4 \, \mu g \, kg^{-1}$) were determined in cyanobacterial mats from James Ross Island (Antarctic Peninsula). Thus, freshwater lakes and wetlands in Antarctica can be sources of methylmercury for the local ecosystem.

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1. Introduction

Mercury is a global pollutant whose concentration in ecosystems is growing. The contamination of polar regions caused by long-range transport of Hg and its deposition in cold regions of the Earth poses a significant environmental risk due to the high bioaccumulation of Hg and its forms. The biogeochemical cycle of Hg is already very well explored [1–8] as well as the atmospheric chemistry in Antarctica regarding Atmospheric Mercury Depletion Events (AMDEs) [1,2]. Generally, Hg concentrations in biomonitors are commonly measured to assess Hg deposition from the atmosphere. Antarctic regolith has been found to be unusable as an indicator because of its low Hg retention [4]. The deposition of bioavailable Hg is increased in deglaciated Antarctic regions; therefore, Hg contents in Antarctic cryptogams are the same or higher than in the Northern Hemisphere [2,9]. Lakes and wetlands with colonies of microorganisms (cyanobacterial mat) represent an important part of the biomass occurring on land. In addition to lichens and mosses, cyanobacterial mats are major accumulators of Hg from melt water during the summer season [1,3]. Cyanobacterial mats have a complex internal structure and are often formed by a large number of species of cyanobacteria, algae, and diatoms [3], which results in a variable ability to accumulate metals.

The biotic and abiotic formation of methylmercury (MeHg) are key processes with respect to Hg speciation changes in the aquatic environment [8]. Methylmercury is formed primarily in anoxic aquatic environments, especially in sediments [5,7]. Biogeochemical changes in the ecosystem are particularly noticeable in lakes without any outflow because they accumulate dissolved compounds and solid particles [1]. The rate of methylation depends on the bioavailability of Hg^{2+} and the activity of methylating microorganisms [5]. The intake of metals by organisms in water involves two steps: the reaction of metal ions with reactive sites on the biological membrane and transport into the organism. However, there is competition with dissolved ligands (e.g. sulphides) in the water column and colloids [10].

A large number of analytical procedures for the determination of MeHg in sediments have been published; for these, see the review published by Jagtap and Maher [11]. Substantially fewer studies are concerned with the determination of MeHg in cyanobacterial mats [3] or in lichens [9]. The quantitative extraction of MeHg from the matrix is necessary for its determination in environmental samples. During the extraction step, Hg speciation must not be changed. Acid and alkaline extraction, and also distillation were used to extract MeHg from the sample matrix [6,12–14]. HCl [9], HBr [15], HNO_3 [16,17], and H_2SO_4 [18] were used for the acid extraction of MeHg in plant material.

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Methanolic solutions of KOH are most commonly used for alkaline extraction [12,19,20].

A number of analytical methods for the determination of MeHg have been used [14], of which gas chromatography (GC) [21] or high performance liquid chromatography (HPLC) [13] are the most commonly used separation techniques. Atomic fluorescence spectrometry (AFS) [3,9,21], cold vapor atomic fluorescence spectrometry (CV-AFS) [12,13,17,19,20,22,23], microwave-induced plasma atomic emission spectrometry (MIP-AES) [21], inductively coupled plasma mass spectrometry (ICP-MS) [16,24], atomic absorption spectrometry (AAS) [18], and electron capture detection (ECD) [15] are used as the detection techniques. The derivatization of Hg species is necessary for the most frequent determination of MeHg by a tandem technique involving GC. The ethylation or phenylation of MeHg with sodium tetraethylborate or sodium tetraphenylborate is carried out at a pH of 4.5–5 adjusted with acetate buffer, acids, or bases [3,16,19,21,25]. Derivatization is followed by extraction into the organic phase (e.g. hexane, toluene), thereby also concentrating the analyte prior to GC separation [14,15,21]. Besides analytical procedures using extract purification [20], pre-concentration by solid phase extraction [13] and a non-chromatographic separation technique (liquid/liquid extraction) [14,18] were developed for the determination of MeHg in environmental samples.

The main objective of this research was to develop a new analytical method for the determination of trace levels of MeHg in plant material using gas chromatography coupled to atomic fluorescence spectrometry (GC-AFS). The second aim was to study MeHg bioaccumulation in the Antarctic cyanobacterial mat.

2. Material and methods

2.1. Reagents and standards

All chemicals were obtained from Sigma-Aldrich. Cellulase from *Aspergillus niger* was used for the enzymatic hydrolysis of samples. The following chemicals were used for MeHg extraction, the treatment of extracts, and derivatization: HCl (≥37%, Hg ≤ 0.0000005%), NaOH (≥98%, Hg ≤ 0.000005%), CH_3COOH (≥99.8%), CH_3COONa (ACS reagent), $(CH_3CH_2)_4BNa$ (97%). Hexane (≥99%) was used for the pre-concentration of the analyte.

CH_3HgCl and Mercury Standard for AAS (1000 $\pm$ 4 mg L^{-1} Hg in 2 mol L^{-1} HNO_3) were used for the preparation of calibration solutions. The standard of MeHg (1 g L^{-1}) was prepared by the dissolution of CH_3HgCl in methanol (99.9%). Calibration solutions of MeHg were prepared by dilution of the standard in deionized water. An aqueous solution containing Hg^0 was prepared by saturating deionized water with metallic mercury.

Certified reference material (CRM) of BCR-482 Lichen (IRMM, Belgium) with a total Hg content of 0.48 $\pm$ 0.02 mg kg^{-1} was used during the development of the analytical method.

2.2. Samples of cyanobacterial mat

Samples of the Antarctic cyanobacterial mat from James Ross Island (Antarctic Peninsula) were studied in this work. The characteristics of the studied area are described in the literature [4,9,26]. Samples were collected in the deglaciated area of the Ulu Peninsula from Johnson Mesa Lake, Monolith Lake, the Interlagos ponds, and seepage near J.G. Mendel Czech Antarctic Station. Detailed description of the localities, and of the sampling and sample characteristics (including species of microorganisms) are reported in Coufalík et al. [26]. Lyophilized and homogenized samples were used for the analysis.

2.3. Extraction procedure

The following aspects of the extraction procedure were considered: the quantitative extraction of MeHg; the avoidance of losses,

contamination, and speciation changes; and the pre-concentration of trace contents. Accordingly, a suitable extraction method should include the use of sufficient sample weights, the use of the maximum volume of sample extract, and an advantageous ratio between aqueous and organic phases.

The developed extraction procedure is presented in Fig. 1. One hundred milligram of homogenized sample or CRM was weighed into a centrifuge tube and suspended in an acetate buffer (pH = 5) prepared from acetic acid and sodium acetate. An ultrasonic bath was used to thoroughly wet the sample particles. One milligram of cellulase (freshly dissolved in deionized water) was added to the sample and the sample was incubated in a water bath placed in an oven at 40 °C for 24 h. Subsequently, acid extraction with hydrochloric acid was performed in an ultrasonic bath for 4 h. The temperature of the ultrasonic bath was maintained at about 40 °C. After sample centrifugation, 3 mL of the extract was used for analysis. Follow-up treatment of the aqueous extract was carried out in a Kimble Mini Reaction Vial with a Screw Cap Mininert Valve (10 mL, Chromatography Research Supplies, USA). (This vial is suitable for the work with volatile analytes.) The extract was diluted with deionized water and a portion of the hydrochloric acid in the extract was neutralized by the addition of sodium hydroxide solution. Solid sodium acetate was added to the solution and the vial was sealed. The solution had a pH = 5. The pH of the solutions was monitored using a S220 SevenCompact pH Meter (Mettler Toledo). Hexane and derivatizing agent were added via the septum. (An aqueous solution of sodium tetraethylborate was prepared under an argon atmosphere every day and stored in a CERTAN capillary bottle.) MeHg was extracted into the organic phase. The determination of MeHg in the prepared extracts was performed on the same day. The removal of 4 μL of extract for analysis was performed using a Hamilton syringe with the vial turned upside down. Thus, the hexane phase created a thicker layer for convenient offtake in the conical part of the vial. In addition, the risk of analyte losses through the headspace was minimized.

For maximum pre-concentration of the analyte in the organic phase, the following conditions were changed during optimization of the procedure: the amount of sample (10–100 mg), the pH of the buffer during enzymatic hydrolysis (pH = 4 or pH = 5), the amount of enzyme (1–4 mg), the amount of hexane (0.2–1 mL), and the amount of derivatizing agent (0.1–0.5 mL) and its concentration (1–2%, w/v). Filtration using Whatman quartz filters (Grade GF/B) was performed in the case of insufficient separation of the solid sample during centrifugation.

2.4. Determination of mercury

An Agilent Technologies 6890 N Network gas chromatograph (USA) with a PSA 10.750 atomic fluorescence spectrometer (UK) was used for the determination of MeHg in extracts. In this commercial system, the detector was coupled to gas chromatograph via a pyrolysis oven held at 800 °C. The source of radiation in the atomic fluorescence detector was a mercury lamp with a wavelength of 253.7 nm. The HP-5 capillary column had an inner diameter of 0.25 mm and a length of 30 m. For MeHg determination, 4 μL of sample was injected in Splitless mode at an injector temperature of 220 °C. The column was heated according to the following temperature program: from 50 °C to 130 °C at 13 °C min^{-1}, from 130 °C to 230 °C at 100 °C min^{-1}. Argon at a flow rate of 0.9 mL min^{-1} was used as the carrier gas. Data were evaluated in the Agilent ChemStation.

The total Hg content in samples was determined by an AMA-254 mercury analyzer (Altec, Czech Republic). This atomic absorption spectrometer allows the dosing of solid and liquid samples. The Hg content in solid samples was determined for weights of about 100 mg. The total Hg content in liquid solutions was determined from volumes of 100 μL.
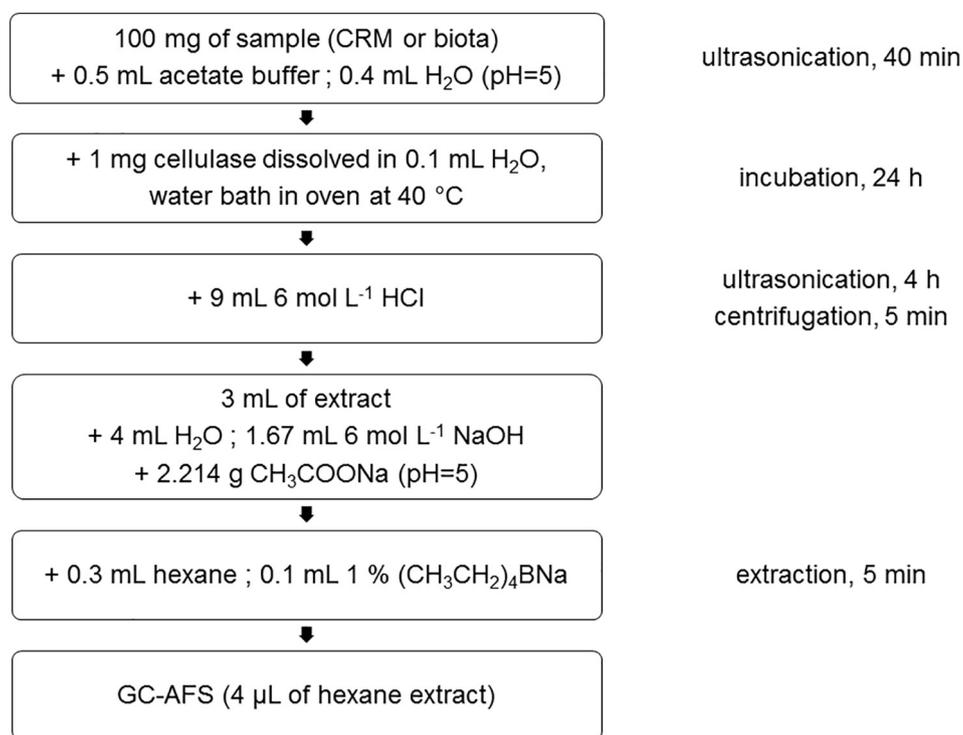
Fig. 1. The scheme of the extraction procedure.

2.5. QA/QC control

Emphasis was placed on the efficiency of MeHg extraction and the monitoring of potential contamination or losses during the individual extraction steps. The stabilities of the calibration solutions and derivatizing agent were also monitored.

Laboratory glass was cleaned in diluted HNO_3 for 24 h, rinsed with deionized water, and heated in the oven to 250 °C for 2 h. The work was carried out in a clean laboratory equipped with HEPA filters. Blank measurements were performed among the measurements of samples and calibration solutions; the preparation of blanks included all extraction steps as samples and standards. The limit of detection (LOD) and the limit of quantification (LOQ) of the method were determined as three times, and ten times the standard deviation (SD) of the experimental blanks, respectively. Potential Hg losses or contamination of the blank from the glassware were continuously monitored using rinses with a clean solution of HCl in which the total Hg content was determined. Control rinses were performed randomly.

The linearity of calibration curve was observed across the range of 0.1–10 ng MeHg (in absolute amounts); the first calibration point was measured near the LOD of the method. Fresh calibration solution (0.1 mg L^{-1}) was prepared daily by dilution of the standard. The retention times of Hg^0, MeHg, and Hg^{2+} were determined using deionized water with Hg^0 and standards of MeHg and Hg^{2+}. For the assessment of possible matrix interference, calibration using standard solutions was compared with that using a standard addition method. As no significant difference in the slopes of the two calibration methods was observed, the quantification of MeHg in real samples was performed against the calibration curve.

The method was developed using a CRM BCR-482. The extraction yield was assessed according to MeHg spikes to 100 mg of BCR-482 and the selected sample of cyanobacterial mat (Interlagos ponds). The contents of MeHg in samples of cyanobacterial mat were determined from three analyses.

3. Results and discussion

3.1. Determination of MeHg by GC-AFS

The determination of MeHg using the developed procedure demonstrated sufficient accuracy, trueness, repeatability, and linearity, as well as a sufficient LOQ with respect to the determination of trace concentrations of MeHg in the organic matrix of cryptogams. Fig. 2a presents the chromatograms of Hg species corresponding to Hg^0 (t_R = 2.00 min), MeHgEt (t_R = 3.45 min, i.e. ethylated MeHg), and HgEt$_2$ (t_R = 4.37 min, i.e. ethylated Hg^{2+}). The peaks of Hg^0 and Hg^{2+} in Fig. 2a correspond to the additions of Hg^0 in deionized water and the standard of Hg^{2+}. The peaks of MeHg correspond to the calibration points for 0.3, 3, 5, and 10 ng MeHg (in absolute amounts). Linear regression equation, LOD and LOQ of MeHg are given in Table 1. The range of the calibration curve (0.1–10 ng MeHg) corresponded to 3–333 ng g^{-1} MeHg in the solid sample. No MeHg was detected during blank measurements; thus, any contamination by MeHg or Hg methylation during the extraction procedure were avoided.

The extraction recovery of MeHg and the accuracy of determination in the presence of the organic matrix were determined by spikes (10 ng MeHg) to three parallel weights of 100 mg BCR-482 and 100 mg of cyanobacterial mat (Interlagos ponds); the recoveries were 99.5–103% and 99.0–101.8%, respectively. Thus, there were no losses or speciation changes of MeHg during the extraction. The repeatability was also determined by spikes to BCR-482; the relative standard deviation (RSD) of the determination was 3% for six parallel analyses.

The MeHg content in the CRM BCR-482 was determined according to the calibration curve in unspiked and spiked (10 ng MeHg) samples (Fig. 2b). The content of MeHg in unspiked CRM BCR-482 was 30.9 ± 0.9 µg kg^{-1}. The MeHg content in BCR-482 after spike subtraction was within the quoted uncertainty. The MeHg content in mat from the Interlagos ponds was 17.0 ± 1.4 µg kg^{-1} (Table 2). The MeHg content in the spiked sample also corresponded to the content determined after the subtraction of the spike.
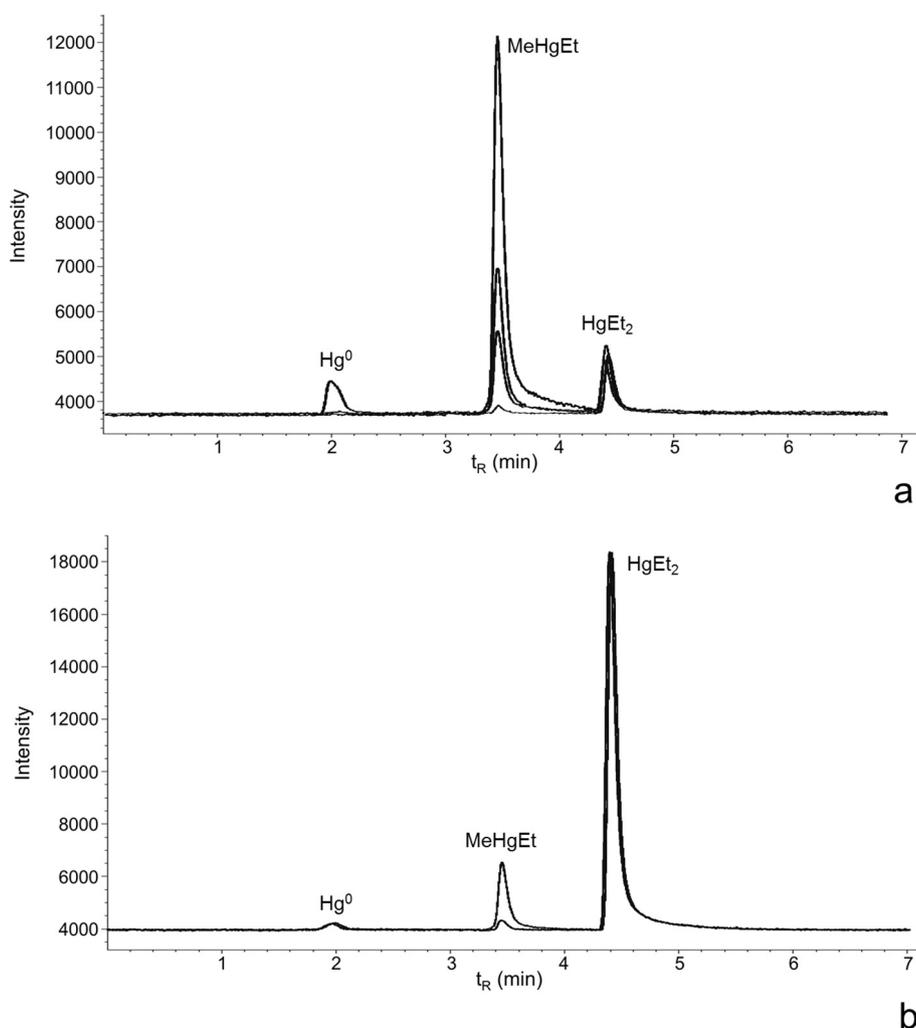
Fig. 2. GC chromatograms: a – calibration solutions of Hg^0 ($t_R = 2.00$), MeHg ($t_R = 3.45$), and Hg^{2+} ($t_R = 4.37$); b – spiked and unspiked CRM BCR-482.

The optimization of extraction parameters was performed regard to the extraction recovery of MeHg in spiked samples. One milligram of cellulase was sufficient for the enzymatic hydrolysis of 100 mg of sample. Enzymolysis at pH = 4 and pH = 5 did not differ; an acetate buffer with a pH of 5 was selected. Derivatization of spiked samples was performed quantitatively with 100 μL of 1% sodium tetraethylborate, higher amounts of derivatizing agent were unnecessary. Two hundred microliter of hexane can be used for the maximum concentration of the analyte; however, the use of 300 μL of the organic phase allows easier sampling. Larger volume of organic phase deteriorates LOD.

The acid extraction of samples in 6 mol L^{-1} HCl followed by ethylation and extraction in hexane was used to determine MeHg levels in microorganisms and in lichens [9,25]. However, this procedure only works well with higher dilutions of the sample in acetate buffer and a larger volume of the final organic phase. The MeHg content in the sample must be sufficient for such dilution. Otherwise, a higher volume of the extract should be used and the analyte should be concentrated

into a small volume of the organic phase. However, this approach increases matrix effects. Because of the low Hg contents in Antarctic cyanobacterial mat, extraction with the highest pre-concentration factor was necessary. Thus, three milliliter of HCl extract was used for MeHg determination when using a small volume of the organic phase. (3 mL of extract had to be diluted with 4 mL of water because of the high content of inorganic salts in the treated sample for MeHg derivatisation.)

In the first experiments, a very low recovery (about 5%) was found for spiked BCR-482, but the recovery increased (22%) in the case of lower sample weights (10 mg). A dense foam formed during the extraction of MeHg from the aqueous phase into hexane and the hexane was turbid. A distinct precipitate formed in 1 mL of hexane in the case of a higher dilution of aqueous extract. Precipitate formation was also observed for samples of cyanobacterial mat. The emerging precipitate from the organic matrix made the analysis of environmental samples impossible.

90–95% of the thallus of lichens is comprised of hyphae. The photobionts in lichens are often composed of cyanobacteria (e.g., Nostoc, Calothrix) [27], whose presence was confirmed in mat samples [26]. Lichens have high contents of polysaccharides, chitin, lichenin, isolichenin, hemicellulose and other substances [27]. In general, carbohydrates protect the organisms from environmental stress and increase their tolerance to temperature changes [28]. Lichenin ("moss starch") undergoes enzymolysis with cellulase [29]. Therefore, a modified form of enzymatic hydrolysis [30] was employed prior to the extraction of MeHg to prevent the formation of precipitate in the hexane phase.

Table 1
Linear regression equations, LOD and LOQ of methylmercury and total mercury.

Mercury species	Linear regression equation	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
MeHg	$y^a = 4106x$	0.998	4	12.6
Total Hg	$y^b = 0.0995x$	0.999	0.1	0.3

[a] y – peak area, x – Hg (ng); GC-AFS.
[b] y – peak height, x – Hg (ng); mercury analyzer.

Table 2
Contents[a] of total mercury and methylmercury in samples of cyanobacterial mat.

Sample	Total Hg ($\mu g\ kg^{-1}$)	MeHg$^+$ ($\mu g\ kg^{-1}$)	MeHg$^+$ (%)
Johnson Mesa L. – site A	88.5 ± 0.1	–	–
Johnson Mesa L. – site B	309 ± 5.6	13.7 ± 0.7	4.4 ± 0.2
Monolith L. inlet – site A	37.2 ± 1.0	15.0 ± 0.3	40.2 ± 0.7
Monolith L. inlet – site B	26.0 ± 1.0	11.6 ± 3.4	45 ± 13
Interlagos ponds	46.4 ± 1.0	17.0 ± 1.4	36.7 ± 3.1
Seepage – site A	80.9 ± 0.4	60.9 ± 4.4	75.3 ± 5.4
Seepage – site B	25.6 ± 1.1	–	–
Seepage – site C	27.2 ± 1.2	9.8 ± 1.8	36.1 ± 6.8

– the content under LOD.

[a] $\bar{x} \pm SD$; $N = 3$.

Precipitation or turbidity in extracts did not occur thanks to the activity of cellulase from *Aspergillus niger*. The proposed procedure of the enzymatic hydrolysis of the organic matrix appears to be universally applicable to samples with similar chemical compositions to lichens (algae, cyanobacteria and bryophytes), these also having a high carbohydrate content [28].

3.2. Bioaccumulation of MeHg in cyanobacterial mat

After verification of the analytical method, total Hg and MeHg were determined in samples of Antarctic cyanobacterial mat. The results are summarized in Table 2. The determination of total Hg by the AMA-254 analyzer was verified using CRM; the content in BCR-482 was 0.484 ± 0.004 mg kg^{-1}, which corresponded to the certified value. (LOD and LOQ of total Hg are in Table 1.) Total Hg contents in mat samples ranged from 25.6 µg kg^{-1} to 308.7 µg kg^{-1}; the RSD of the determination reached a maximum value of 4.5%.

The MeHg contents in mats ranged from 9.8 to 60.9 µg kg^{-1}; two samples had contents below the LOD. The RSD of the determination reached a maximum of 8.2% at values above the LOQ (12.6 µg kg^{-1}). MeHg formed a significant proportion of total Hg in most samples.

The contents of total Hg in regolith and sediments on James Ross Island range between 2.7 and 11.3 µg kg^{-1}, which can be considered as a background value in this part of Antarctica [4]. Sea spray was confirmed to have a small influence in the case of cyanobacterial mats in the studied localities [26]. Sea spray is not a significant source of Hg in this area according to the analysis of *Usnea antarctica* lichens. A large proportion of Hg in the local ecosystem likely originates from atmospheric deposition [9].

Generally, total Hg contents in Antarctic cyanobacterial mat can be up to hundreds of µg kg^{-1}. *Usnea* lichens accumulate higher Hg concentrations than the mat in the same area [2]. Concentrations of total Hg in mat samples (Table 2) were comparable to other areas in Antarctica [2] and significantly lower than concentrations in *Usnea antarctica* lichens in the studied area [9]. Hg concentrations in cyanobacterial mat may not be associated with their biological activity but with the growth rate of microbial colonies [3]. The bioaccumulation of Hg in mat can be increased by organic sulfur and sulphides [1]. Nevertheless, the determined Hg contents in samples did not demonstrate any correlation with sulfur [26] in this case.

The determined MeHg concentrations in cyanobacterial mat were comparable to MeHg concentrations in *Usnea antarctica* lichens [9]. Thus, the ratio of MeHg to total Hg in mats was very high, even in comparison with cyanobacterial mats in other Antarctic regions [3]. Increased methylation of Hg often occurs at elevated temperatures and with the prolongation of thermally favourable conditions [5]. It is probable that the biological activity of microorganisms methylating inorganic Hg is high because of the temperatures of the surface water in the studied area during the summer season [26].

4. Conclusion

The developed analytical procedure enables the determination of trace levels of MeHg in plant material by means of GC-AFS. The activity of *cellulase* enzyme caused the hydrolysis of the organic matrix, which avoided precipitate formation in the organic phase during the extraction of MeHg. The procedure was developed on lichen, algae and cyanobacteria, and, thus, can be used for organic matrices with a similar chemical composition. The method offers sufficient precision, accuracy, and repeatability with respect to measurements of environmental samples. MeHg concentrations in Antarctic cyanobacterial mat were determined. Cyanobacterial mats on James Ross Island exhibited very high contents of MeHg, which indicated ongoing methylation processes.

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