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Mercury speciation in hair by headspace injection–gas chromatography–atomic fluorescence spectrometry (methylmercury) and combustion-atomic absorption spectrometry (total Hg)

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ABSTRACT

The speciation of Hg in human hair was carried out with combustion-atomic absorption spectrometry for total Hg (THg) and headspace–gas chromatography–atomic fluorescence spectrometry (HS–GC–AFS) for methylmercury (MMHg).

The determination of total Hg in hair was carried out with the AMA analyzer (Advanced Mercury Analyser 254). Accuracy and reproducibility were assessed on a Certified Reference hair sample (IAEA-086 CRM), yielding, respectively, a recovery of 97.5% and a RSD of 3.2%. Analyses of 10 blank measurements resulted in a detection limit of 1.5 $ng\,g^{-1}$ of THg for a 20 mg sample of human hair.

MMHg concentrations in hair were assessed with HS–GC–AFS in a single analysis step. Either acid or alkaline extraction can be applied because they yielded very similar results on a IAEA-086 CRM: we observed a recovery of 103% and a RSD of 7% with acid extraction and a recovery of 110% and a RSD of 9% with alkaline extraction. Optimization of the headspace vial, injection and GC parameters is described. The detection limit of the MMHg determination in human hair, which amounts to 0.04 $ng\,g^{-1}$ for a 20 mg sample, is far below the concentrations observed in natural samples.

The number of samples that can be analyzed per hour, respectively, amounts to 8 for THg and 4 for MMHg. Finally, Hg speciation in natural human hair samples was carried out by combining both AMA and HS–GC–AFS analysis methods. THg levels were at the $\mu g\,g^{-1}$, level, with an average MMHg fraction of about 70%.

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1. Introduction

Mercury (Hg) is one of the most hazardous environmental pollutants, but its toxicity is dependent on its chemical form. MMHg compounds, the main mercury species in seafood, are considerably more toxic than elemental Hg and inorganic Hg salts. During the last decade improvements in analytical techniques as well as reaction oriented environmental research have considerably improved the knowledge on the Hg biogeochemical cycling and the impact of Hg exposure on human health. To reduce the risks of mercury intoxication, limits of Hg levels in fish as well as consumption advisory limits have been compelled by EU, USA and Canada amongst others. Hair is a suitable indicator for the monitoring of human exposure by mercury, especially resulting from dietary intake [1]. Collection, storage and transportation of hair samples are easy and simple. A convincing relationship between the content of mercury in hair versus its content in blood has been reported in several studies [2–4] despite Hg levels in blood are an indicator of ongoing or recent exposure, while Hg levels in hair represent a much longer time frame, potentially several years (the average scalp hair growth has been reported as 1 cm per month). In addition, compared to blood or urine, that are other indicators used to monitor human exposure by Hg, hair samples contain generally higher mercury concentrations, allowing more accurate results [5,6]. Based on impact studies of Hg on human health, WHO proposed a minimum threshold value for methylmercury in hair (50 $\mu g\,g^{-1}$) which is lowered by US-EPA to 10 $\mu g\,g^{-1}$ [7].

Speciation of Hg in hair can be achieved via a total Hg analysis on the one hand and a quantification of the organomercury compounds on the other hand. For total Hg analysis several methods exist including non-destructive ones such as Neutron Activation Analysis (NAA) [8], total destructive ones such as an aqueous mineralization step followed by AFS [9] or ICP-MS [10] or a total thermal decomposition such as performed with the AMA analyzer [11].

The analysis of organomercury compounds in biological samples such as hair requires the extraction, separation and detection of

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those compounds. For releasing organomercury species from a biological matrix, either a strong basic solution such as KOH/methanol [12] or a strong acid can be applied [13]. Since most mercury compounds are very volatile or can easily be derivatized to volatile compounds, GC is still the preferential separation method to assess organomercury species in biological or sediment samples. It also allows much lower detection limits than LC.

After extraction of the organomercury compounds from the sample matrix, clean-up is performed before injection into the GC, except in case of the HS–GC–AFS method. Removal of those unwanted compounds is achieved by various clean-up methods such as for example distillation [14], extraction and back-extraction with organic and aqueous solutions [15], static or dynamic headspace extraction on SPME or Tenax [16–18]. Since more than 25 years we avoid these additional, laborious clean-up steps for determination of organomercury compounds in fish, plants, suspended matter or sediments via a derivatization of the organomercury compounds in the headspace vial (either iodation or ethylation) and a direct injection of those derivatized compounds in the GC [13,19–21]. However, for each specific matrix such as fish, plant material, sediment, hair, etc., optimization of the sample extraction as well as the HS–GC–AFS parameters has to be performed. In this paper we describe the application of the new full automated headspace system as well as the optimization of all analytical parameters involved in the speciation of Hg in hair.

2. Methods and materials

2.1. Sampling

Hair of adolescents and mothers of newborns living in Flanders (Belgium) was collected. Hair from the neck is preferred because it is generally less contaminated than front face hair. A stainless steel scissor was used to cut the hair samples (about 0.5–1 g), they were stored in plastic bags with identification tags and shipped to the laboratory for further treatment and analysis. In the laboratory, the hair strands were further cut into smaller pieces (2–3 mm) and cleaned as follows: samples were washed five times with diluted laboratory detergent (1:200, v/v) rinsed three times with Milli-Q water (Millipore, >18 MΩ cm), and at the end rinsed with acetone (Analytical grade, Merck). Finally, the hair samples were dried in an oven at 60 °C overnight. The pre-washed hair samples were stored in clean plastic bags.

For optimization of the analytical methods and quality control (QC) of the analyses, a Certified Human Hair Reference Material, IAEA-086 was purchased from the International Atomic Energy Agency (IAEA, Austria).

2.2. Total Hg determination

Total mercury was measured with an Advanced Mercury Analyser 254 (AMA 254) from Altex Ltd., Czech Republic. This fully automated instrument combines the preconcentration of Hg on a gold amalgamator after catalytic combustion of the sample, with atomic absorption spectroscopy and does not require a pre-treatment step. The instrument uses two calibration ranges: 0.05–40 and 40–600 ng. It automatically changes to the higher calibration range if an absorption higher than 0.8 is measured. The procedure we applied is similar to that described by Dıez et al. [11].

2.3. MMHg determination

2.3.1. Extraction

2.3.1.1. Reagents. CH_2Cl_2 (Merck, SupraSolv); tetramethylammoniumhydroxide (TMAH, 25%, ACROS), $CuSO_4 \cdot 5H_2O$ (Merck, pa), KBr (Merck, pa), H_2SO_4 (96%, Merck, pa) and HCl (Merck, suprapur) are used.

A $CuSO_4$ solution (1 M), an 18% (w/v) KBr solution and a 5% (v/v) H_2SO_4 solution were prepared from the purchased reagents and dilution with Milli-Q water.

2.3.1.2. Extraction procedures. Alkaline extraction procedure: a 20 mL FEP bottle containing 2 mL TMAH and 0.1 g hair sample was placed in the oven at 85 °C for 3 h. Once cooled down, 10 mL CH_2Cl_2 and 1.5 mL HCl (Suprapur) were added; the mixture was shaken for 15 min, and centrifuged for another 15 min at 3000 rpm. The water layer was removed and MMHg species in the CH_2Cl_2 layer were back-extracted in Milli-Q water by solvent evaporation at 50 °C under a constant N_2 flow.

Acid extraction procedure: 5 mL of a 5% H_2SO_4 and 18% KBr mixture and 1 mL $CuSO_4$ (1 M) were added to 0.1 g hair sample in a 20 mL FEP bottle and shaken for 20 min. After adding 10 mL of CH_2Cl_2, the mixture was shaken again for 1 h, followed by centrifugation for 15 min at 3000 rpm. The water layer was removed and MMHg species in the CH_2Cl_2 layer were back-extracted in Milli-Q water by solvent evaporation at 50 °C under a constant N_2 flow.

2.3.2. Headspace vial reactions

2.3.2.1. Reagents and standards. A 100 ppm MMHg stock solution is prepared from a 1000 ppm Hg stock solution (1000 ppm, Alfa) in Milli-Q water and stored in a brown glass bottle at 4 °C. Working standard solutions of 5, 10, 20, 40 ng L^{-1} are prepared daily. One gram of sodium tetraethylborate ($NaBEt_4$, Strem Chemicals) is dissolved in a 100 mL, 2% KOH solution, which was cooled for 2 h in the deep freezer. This 1% $NaBEt_4$ solution is further diluted ten times and these solutions are stored in 20 mL FEP bottles in the deep freezer. The 0.1% $NaBEt_4$ deep frozen reagent is stable for several weeks, but once in use its lifetime is limited to one day. Acetate buffer solution is prepared in a FEP bottle by dissolving 272 g of sodium acetate and 118 mL of glacial acetic acid in 1 L Milli-Q water.

2.3.3. HS–GC–AFS analysis

The analytical system for the determination of Hg species consists of a Perkin Elmer Turbo Matrix 40 Trap headspace sampler coupled to a Perkin Elmer Clarus 500 gas chromatograph through a heated fused silica transfer line. Ar (Oxhydrique 5.0) is used as the carrier gas. The outlet of the GC is coupled to an atomic fluorescence detector (TEKRAN 2500) via a pyrolytic column. The Perkin Elmer Turbo Matrix 40 headspace sampler consists of a sample carousel with a capacity of 40 samples and an oven to regulate the temperature of up to 12 samples simultaneously, maximizing sample throughput. The Perkin Elmer Turbo Matrix 40 headspace sampler, with built-in analyte trapping capability, maximizes the extraction and transfer of headspace vapor into the GC column, thereby lowering the detection limit, compared to the semi-automated Perkin-Elmer HS-40XL. The heated fused silica transfer line is directly connected to the packed GC column (on-column injection). The GC column consists of an 80-cm long Teflon tube (OD 1/4″, ID 3 mm) filled with 10% OV3 on Chromosorb W-AW DMSC (60/80 mesh) (Altech). The 22-mL Pyrex glass headspace vials are closed with Teflon-coated butyl rubber septa and Al caps. Headspace, chromatographic and detection parameters were optimized, without the trap since in that configuration the method appeared sensitive enough for natural hair samples, by using working standard solutions in the range of 5–40 ng L^{-1}. Higher concentrations were not tested.

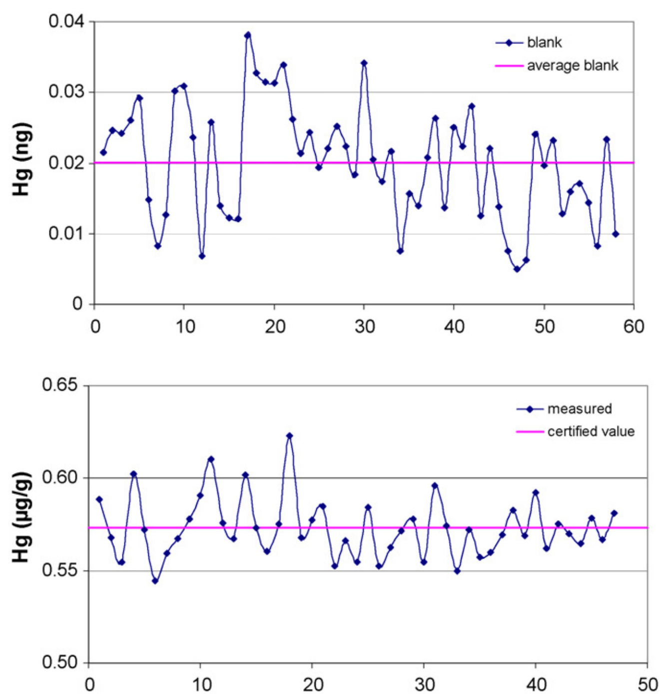

Fig. 1. Control charts of total Hg for the blank and the certified reference material IAEA-086 (certified value: 0.573 μg g^{-1}).

3. Results

3.1. Total Hg

The AMA has an internal calibration system that automatically selects the most appropriate concentration range. The hair samples we analyzed with the AMA felt all in the sensitive range (0.05–40 ng).

The theoretical detection limit of the AMA analyzer, reported by the instrument producer, equals 0.01 ng Hg, but it is unclear how this value was obtained. In our laboratory, the detection limit (DL), defined as three times the standard deviation on ten blank measurements equals 0.03 ng Hg, thus three times higher than the theoretical one. This limit of detection corresponds to a concentration of 1.5 ng g^{-1} in a sample of 20 mg, which is about half that of Dıez et al. [11]. Blanks were regularly inserted between samples to ensure that there was no carry-over of Hg between them.

A quality control of the analytical procedure was performed by analyzing a human hair certified reference material (CRM IAEA-086), with a total Hg concentration ranging between 0.534 and 0.612 μg g^{-1}, at the beginning and at the end of each batch of samples ($n = \pm 10$). The accuracy and reproducibility of the method, inferred from the CRM measurements (Fig. 1), were respectively 97.5 and 3.2% ($n = 48$).

3.2. MMHg

The headspace system can be used with or without the trap mode but in our case the MMHg concentrations in the hair samples were high enough so that no trap was needed. After the optimization of the system, calibration curves were established and CRM and real hair samples analyzed.

3.2.1. Optimization of the system

Ethylation parameters (concentration of ethylating agent, reaction time), headspace parameters (thermostatic heating time, temperature, pressurization time, injection time, sample volume), and GC parameters (column temperature, gas flow rate) were optimized by varying one parameter while holding the others constant. Initial headspace parameter values were based on default values suggested by the headspace manufacturer as well as our previous experiences with semi-automated headspace analysis of ethylated organomercury compounds [21,22]. 10 mL of working standard solution of 5, 10, 20, 40 ng L^{-1} or 0.1– 5 mL aqueous sample extract were transferred to the headspace vials and diluted to 10 mL. Next, 60 μL acetate buffer and 100 μL $NaBEt_4$ were then added. Finally, the vials were sealed with Teflon-coated butyl rubber septa and Al crimp caps and allowed to react for 1 h before analysis.

3.2.2. Optimization of the ethylation parameters

The pH was held at 4.9, which is in the middle of the optimal pH range of 3–7. The concentration of ethylating agent within the range 0.5–100 μg L^{-1} in the headspace vial had little effect on the signal. We kept the concentration at 10 μg L^{-1} throughout analysis. A minimum reaction time of 60 min at 20 °C was found to be necessary to obtain a stable signal. A further increase of the reaction time to 300 min slightly raised the signal, but reduced too much the throughput of samples. Every four samples, a 20 ng L^{-1} standard solution was inserted as a control sample.

3.2.3. Optimization of the headspace parameters

The distribution of the ethylated compounds between the gas and liquid phases in the headspace vials is determined by the gas–liquid partitioning coefficient, the equilibration temperature, and the gas to vapor phase ratio. Peak heights increased with increasing temperature, and the optimal temperature was set at 70 °C. Further increase in the temperature leads to increased water vapor injection and peak tailing of the Hg peak. The optimal thermostatic heating time was 5 min. With the pressure-balanced injection technique, the amount of headspace transferred to the GC column is determined by the injection time and carrier gas flow rate. The signal increased with an increase of the injection time (and volume) and was optimal at 15 s. The optimal sample volume for the 22-ml headspace vials is 6–12 ml and was held at 10 ml throughout analysis (a gas to vapor phase ratio of one). In order to avoid condensation the temperature of the injection needle and transfer line were held at 100 and 105 °C, respectively.

3.2.4. Optimization of the GC parameters

A temperature programming method was chosen rather than an isothermal procedure in order to improve the separation between the void peak including Hg, ethylmethylmercury (derivatized MMHg) and diethylmercury (derivatized Hg^{2+}) without increasing analysis time (Fig. 2). The temperature program is the following: an initial temperature of 50 °C hold for 1 min; an increase to 100 °C in 3 min; a further increase to 120 °C in 4 min; and finally a temperature decrease to 50 °C within half a minute. An optimal gas flow rate of 30 mL min^{-1} was maintained throughout analysis.

3.3. Application of the system

3.3.1. Calibration curves

Calibration curves are linear in the range 5–40 ng L^{-1}. The reproducibility of the method was tested on 10 replicates of 20 ng L^{-1} and yielded a RSD of 5%. The limit of detection, defined as for the total Hg analysis (see here and above), was also assessed on 10 replicates of a procedural blank and equals 0.08 ng L^{-1} as Hg. This limit of detection corresponds to a concentration of 0.04 ng g^{-1} in a hair sample of 20 mg. While this LOD is still comparable to the LOD of 0.3 ng g^{-1} for biological materials, including hair, reported by Liang et al. [23] it is much lower than the LOD in hair samples reported by Gibičar et al. [18] and Montuori et al. [16], amounting respectively

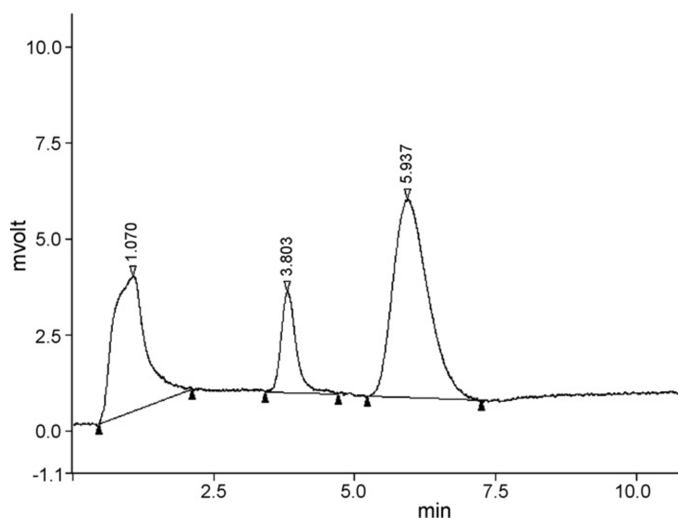

Fig. 2. Chromatogram of the derivatized mercury species: first peak is metallic mercury, second peak is methylethylmercury, third peak is diethylmercury.

to 5 and 40 ng g^{-1}. The direct headspace injection into the GC column allows working at low temperatures which may explain the lower and more stable blank signals and hence lower LODs.

3.3.2. Analysis of the CRM (IAEA-086)

The extraction methods were validated by the analysis of the IAEA-086 CRM. The results obtained with the acid extraction amounted to 0.265 ± 0.018 μg g^{-1}, with the alkaline extraction method to 0.283 ± 0.025 μg g^{-1}. These results also agree, within the observed uncertainties, with the value reported for the CRM (0.258 ± 0.022 μg g^{-1}): a recovery of 103% and a RSD of 7% for the acid extraction method and a recovery of 110% and a RSD of 9% for the alkaline one. Since the alkaline extraction procedure has somewhat less good recovery and RSD values on the CRM sample, it necessitates a longer reaction time and it could damage the PFA and FEP vessels due to the strong alkaline reagents at 85 °C, the acid procedure was chosen to extract MMHg from the hair samples. Similar studies on CRMs yielded a recovery of 75 ± 11% and a RSD below 15% for Montuori et al. [16] and recoveries of 131 and 116% and a RSD from 5 to 15% for Gibičar et al. [18].

3.3.3. Analysis of a limited number of participants in a human biomonitoring study

The Flemish Center of Environment and Health organized a biomonitoring study in 2009–2010 of 200 adolescents (14–16 years old) and of 250 newborns and their mothers (18–42 years old). A paper describing the Hg and MMHg results in hair of all participants in the biomonitoring study is in preparation. We present here as an example the THg and MMHg concentration found in hair of 5 adolescents and 5 mothers taking part in the study and randomly selected (Table 1). Total Hg concentrations vary between 0.29 and 1.51 μg g^{-1} for the mothers and from 0.13 to 0.36 μg g^{-1} for the adolescents. MMHg concentrations vary between 0.17 and 1.31 μg g^{-1} for the mothers and from 0.11 to 0.21 μg g^{-1} for the adolescents. Total Hg and MMHg concentrations in hair of mothers are much higher than in hair of adolescents. Also the MMHg fraction is quite higher in hair of mothers (79%) than of adolescents (63%).

The values we observed in our adolescents are at the lower end of the results (total Hg and MMHg in hair) for children in different areas in Spain, reported by Dıez et al. [24] and are much lower than the total Hg concentrations in children and adults living in the Amazon Basin, Negro River, reported by Barbosa et al. [25]. Our average MMHg/total Hg ratio of around 65% is in agreement with the value observed by Dıez et al. [24].

Table 1
Concentrations of total Hg and MMHg in human hair of five random participants from the Flemish Biomonitoring Study (2009–2010). Mothers (total $n = 250$; 18–42 years old) and adolescents (total $n = 200$; 14–16 years old).

Sample ID	HgT (μg g^{-1})	MMHg (μg g^{-1})	MMHg/HgT
Mothers			
Participant 1	0.285	0.166	0.58
Participant 2	0.746	0.469	0.63
Participant 3	1.512	1.313	0.87
Participant 4	0.526	0.524	1.00
Participant 5	0.374	0.328	0.88
Adolescents			
Participant 1	0.178	0.117	0.65
Participant 2	0.280	0.141	0.50
Participant 3	0.130	0.112	0.86
Participant 4	0.272	0.206	0.76
Participant 5	0.361	0.140	0.39

4. Conclusion

The analysis procedure for Hg in hair – total Hg with AMA and MMHg with HS–GC–AFS – is sensitive, accurate and reproducible. For total Hg, the calibration curves both in the low and high range are linear ($r > 0.99$). The detection limit on the AMA defined as three times the standard deviation on ten blank measurements was equal to 0.03 ng Hg and corresponds to a concentration of 1.5 ng g^{-1} in a sample of 20 mg. The accuracy and reproducibility of the method were assessed on a CRM (IAEA-086) and yielded, respectively, 97.5 and 3.2%.

For MMHg, calibration curves are linear in the range 5–40 ng L^{-1}. The limit of detection, defined as for the total Hg analysis (see here above), was equal to 0.08 ng L^{-1} as Hg and corresponds to a concentration of 0.04 ng g^{-1} in a sample of 20 mg, which is (much) better than LODs reported in literature. The accuracy and reproducibility of the method were assessed on the CRM (IAEA-086) with an acid and an alkaline extraction method: the acid method yielded a slightly better result with respectively a recovery of 103% and a RSD of 7%.

The ethylation step is without any doubt the most problematic one in the whole HS–GC–AFS analysis procedure. The ethylating reagent ages relatively fast (every day a new solution has to be prepared and the original product also slowly deteriorates even at low temperature), it may contain several impurities including Hg species, the ethylating reaction should be performed in a relatively narrow pH range (use of a buffer is necessary and pH should be controlled before adding the ethylating reagent) and constituents other than mercury may compete for the available amount of ethylating reagent. We have tested the possible drawbacks related to the ethylating reagent mentioned before, by analyzing frequently the CRM and by analyzing several dilutions of the same hair sample extract. In a seldom case a new solution of ethylating reagent had to be prepared.

The most important advantage of our method is that conversely to all other methods reported in literature it does not require a clean-up procedure prior to injection into the GC. In a routine way, we are able to analyze eight hair samples per hour with the AMA (THg) and four hair sample extracts per hour with the HS–GC–AFS (MMHg). The method was finally applied in a biomonitoring study in Flanders (Belgium). For five participants selected at random we found following results: total Hg concentrations in hair from adolescents varied between 0.13 and 0.36 μg g^{-1} (MMHg levels varied between 0.11 and 0.21 μg g^{-1}) while in mothers of newborns they varied between 0.29 and 1.51 μg g^{-1} (MMHg levels varied between 0.17 and 1.31 μg g^{-1}).

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