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Short communication

Improvements in the methylmercury extraction from human hair by headspace solid-phase microextraction followed by gas-chromatography cold-vapour atomic fluorescence spectrometry

P. Montuori, E. Jover, R. Alzaga, S. Diez, J.M. Bayona*

Environmental Chemistry Department, IIQAB-CSIC, Jordi Girona 18-26, E-08034 Barcelona, Spain

Abstract

Improvements in the methylmercury extraction from human hair by solid-phase microextraction followed by gas chromatography coupled to cold-vapour atomic fluorescence spectrometry (GC-CVAFS) have been carried out. They consisted in the optimisation of the digestion step prior to the aqueous-phase ethylation and in the GC-CVAFS interface set-up. The main digestion parameters such as acid type, concentration, temperature and time have been optimised for hair sample analysis, thereby avoiding methylmercury degradation. Moreover, the stability of the digested samples was evaluated to improve the sample throughput.

Keywords: Cold-vapour atomic fluorescence spectrometry; Atomic fluorescence spectrometry; Detection, GC; Headspace analysis; Methylmercury; Organomercury compounds

1. Introduction

Mercury is a naturally occurring element that is widespread in the environment. Human activity can also release mercury to the environment, increasing the amount that is currently cycled in the biosphere. In water or soil, microorganisms can convert inorganic mercury into a more toxic organic form, methylmercury. Methylmercury is primarily neurotoxic [1–4], uptake by humans being mainly through fish consumption [5]. Many industrialised countries have issued cautionary advisories about eating fish caught in waterways because of the presence of methylmercury [6]. In spite of persistent efforts by the industrialized countries over the last decades to reduce mercury emission, its decline is not yet reflected in the methylmercury levels [7]. To assess human exposure, body fluids such as blood [8,9] and urine [9] or tissues such as hair have been analysed [9,10]. Hair is a suitable indicator for biomonitoring of human exposure to mercury, which reflects organ mercury levels as well as dietary intake. A convincing relationship between the content of mercury in hair and its content in blood has been reported [9,11,12].

At present, methylmercury in hair is analysed through a three-step procedure: digestion, extraction and determination. Digestion is an essential step for the release of methylmercury bound to hair. In fact, the high affinity of methylmercury for hair is due mainly to the presence of cystine, which makes up approximately 14% of human hair weight. Methylmercury found in hair is bound either to cystine sulphur or to the sulphydryl (SH) group present in other amino acids [13]. Therefore, the digestion step has to be able to break the existing bonds between hair and methylmercury, avoiding methylmercury carbon–mercury bond cleavage. Two kinds of heated digestions have been used, either acidic [9,14] or basic [14,15], followed by extraction with hazardous solvents [9,15,16] or solid-phase extraction [17]. However, in order to avoid solvents, solid-phase microextraction (SPME) is the technique preferred [18]. When solvents are used, Hg compounds were determined either directly as chlorides [9] or bromides [15]. For headspace SPME, methylmercury in its ionic form is non-volatile, in which case a derivatisation step is needed prior to its extraction. Two different derivatising agents have already been used, such as sodium tetraethylborate [18–21] or sodium tetraphenylborate [21,22], permitting MeHg alkylation. Methylmercury is then analysed by GC using electron-capture detection (ECD) [9,23], instrumental neutron activation analysis (INAA), atomic absorption

* Corresponding author. Tel.: +34-93-400-6119; fax: +34-93-204-5904.
E-mail address: jbtqam@cid.csic.es (J.M. Bayona).

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spectrophotometry (AAS) [24], by GC using atomic emission detection (AED) [25], LC interfaced to inductively coupled plasma mass spectrometry (ICP-MS) [14], and the most sensitive GC with cold-vapour atomic fluorescence spectrometry (CVAFS) [18,21].

The aim of the present work consisted of improving an earlier methodology which used SPME and GC-CVAFS determination for the first time in the analysis of human hair [18], but has shown poor reproducibility for real samples. Therefore, it is expected that improving its reproducibility will make the methodology suitable for routine analysis. For this reason, special care has been taken for the hair digestion stability and its robustness. Important parameters, such as type of acid and concentration, temperature, time, volume and the digestion stability with storage time, have been optimised.

2. Experimental

2.1. Reagents and materials

Methylmercury chloride (CH_3HgCl, 99%) and mercury dichloride ($HgCl_2$, 99.9995%) were purchased from Strem (Newburgport, MA, USA), and phenylmercury chloride [$(C_6H_5)HgCl$, 97%] was from Fluka (Buchs, Switzerland). Sodium tetraethylborate ($NaBEt_4$, 98%) was purchased from Galab (Geesthacht, Germany). Analytical-grade sodium acetate (99%) and acetic acid (99.7%) were obtained from Sigma–Aldrich (Steinheim, Germany). Hydrochloric acid (25%), sulphuric acid (95%) and methanol for trace analysis were obtained from Merck (Darmstadt, Germany). Nitric acid (60%) was purchased from Quimivita (Barcelona, Spain).

Stock standards were prepared at 1000 mg/l (as Hg) in methanol and working solutions were prepared weekly by diluting the stock solutions with methanol in the range of 0.5–500 μg/l as Hg. All standards were stored at 4 °C. A fresh $NaBEt_4$ solution of 1% (w/v) was prepared daily in deionized water and stored at 4 °C. A buffer solution at pH 4.5 was prepared by mixing sodium acetate (0.2 M) and acetic acid (0.2 M). A certified human hair reference material from the National Institute of Environmental Studies of Japan (NIES, CRM No. 13) was purchased.

Dipentylmercury, which was used as the internal standard, was synthesised in the laboratory using mercury dichloride and the Grignard reagent pentylmagnesium bromide 2 M, which was obtained from Aldrich (Steinheim, Germany). Eighty microlitres of $HgCl_2$ (600 μg/g) and 10 μl of pentylmagnesiumbromide (2 M) were added to 2 ml of acetonitrile. After 20 min, the reaction was stopped by adding 2 ml of Milli-Q water and cooling down the solution with an ice bath. Finally, dipentyl Hg was extracted with 2 ml of hexane and percolated through a funnel containing sodium sulphate.

2.2. Apparatus

The GC analysis was carried out with a Carlo Erba 4130 (Milan, Italy) gas chromatograph interfaced to a CVAFS Tekran Model 2500 (Toronto, Canada). The injector was kept at 170 °C in the splitless mode (3 min). A 30 m × 0.25 mm i.d. fused-silica column coated with a 0.25 μm thick film of DB-5 (J&W Scientific, Folsom, CA, USA) was used. Initial column temperature was held at 50 °C for 1 min, programmed at 15 °C/min to 220 °C, which was maintained for 2 min. After the Hg forms were separated, the column effluent was fitted to a heated quartz tube (20 cm × 1 mm i.d.) by tubular oven at 800 °C (Raypa BAT 850, Barcelona, Spain), which converts the different mercury species to Hg^0 for detection by CVAFS. Argon gas 5.0 grade was used as the carrier gas (i.e. 3 ml/min) and also as a make-up gas (i.e. 70 ml/min) for the CVAFS detector connected to the system before the pyrolyzer. All the tubing fittings were of PTFE material. In parts 2 and 4, all the connections near the pyrolyzer have to be protected from too high temperatures using Kevlar fibre. Finally, data were acquired and processed by a Perkin-Elmer Nelson interfaced to a personal computer.

Injection of solvents in the apparatus has to be limited in order to avoid their pyrolysis in the quartz tube at high temperatures, which can cause some adsorptions and tube plugging. For this reason, when using SPME, no soot problems were observed after 100 injections.

2.3. Sample preparation and acid digestion

Hair samples were finely cut and placed in 100 ml beakers and ultrasonically washed with a commercial detergent solution. After vigorous rinsing with a large volume of deionized water, hair samples were rinsed with acetone and air-dried.

The digestion procedure was optimised and carried out as follows. Finely cut hair (20 mg) was placed in a 5 ml Pyrex tube and then acid solution was added (350 μl). The tube was capped non-tightly and then heated (25–100 °C) for 10–110 min. Digested samples were stored at 4 °C in the dark.

2.4. SPME sampling

After the digest was cooled down at room temperature, an aliquot (25 μl) was transferred to a 7 ml glass vial with 3 ml of acetate buffer solution (pH 4.5) containing a magnetic stirring bar. A 40 μl volume of dipentylmercury (14 ng/g), as internal standard, was added to the sample and also 50 μl (12 ng/g) of phenylmercury, which was used as ethylation quality control. Then, 500 μl of 1% aqueous $NaBEt_4$ solution was injected through the septum. A 100 μm polydimethylsiloxane fibre from Supelco (Bellefonte, PA, USA) was used for the SPME extraction which was carried out in the headspace during 20 min at 1200 rpm and immediately injected in the GC-CVAFS system. Quantification of hair samples was carried out by external calibration with a

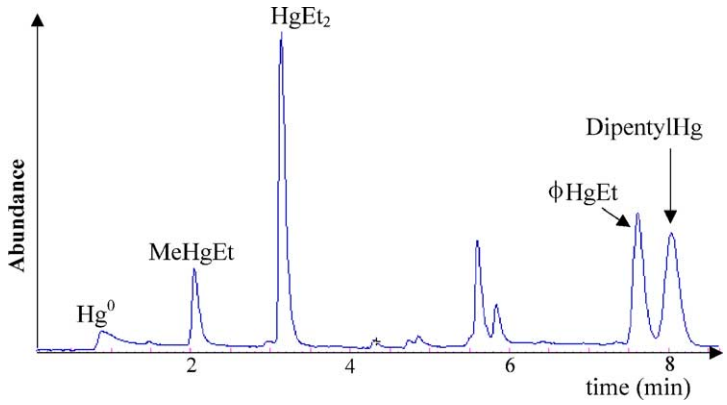

Fig. 1. GC-CVAFS chromatogram of a real hair sample.

$R^2 > 0.99$. No MeHg carryover was observed in the studied concentrations. In order to statistically evaluate the optimisation results, the SPSS 8.0 software (Chicago, IL, USA) has been used. Response surface has been plotted by using the Matlab 6.0 software (Natick, MA, USA).

3. Results

3.1. GC-CVAFS interfacing

The optimisation of the instrumental set-up has been carried out in order to obtain a robust and reliable methodology. Although several configurations were evaluated, the one exhibiting a better performance is described. The more critical parts of the system are the interface between the GC oven and the pyrolyzer and the used quartz tube. In fact a 20 cm × 1 mm i.d. quartz tube has been chosen to minimize dead volumes, avoiding band broadening by adding a make-up gas coaxially after the GC column effluent. This intermediate part of the apparatus working as the interface between the pyrolyzer and the GC system had to be thermally isolated in order to avoid the condensation of the less volatile analytes (i.e. phenylmercury and dipentylmercury). Finally, the chromatographic parameters such as carrier-gas flow rate and temperature program were optimised in order to perform baseline resolution of all the analytes in less than 10 min (Fig. 1).

3.2. Digestion optimisation

As a precedent methodology [18], it has been shown to be matrix dependent when applied to real samples; all the digestion parameters were optimised by using real non-spiked hair samples.

Several types of acids at different concentrations have been evaluated to carry out the digestion of human hair such as HCl (2, 5 and 10 M), HNO_3 (2, 5 and 10 M), H_2SO_4 (6 M), a mixture of HNO_3 and H_2SO_4 (5 M/3 M) and a mixture of HCl and H_2SO_4 (5 M/3 M). Also acetic acid has been tested as it has been recently reported for the determination of MeHg in biota [26]. Acid selection has to compromise between a complete hair matrix digestion and the methylmercury degradation. From the acids evaluated, a complete matrix digestion has been achieved for HNO_3 (1–10 M), H_2SO_4 (6 M) and the mixture of HCl with H_2SO_4 (5 M/3 M) and HNO_3 with H_2SO_4 (5 M/3 M). For all these acids, except for HNO_3, methylmercury was fully degraded to Hg^{2+}, which was detected as $HgEt_2$. For HNO_3, only in the 5 M solution, a satisfactory methylmercury recovery was observed (Fig. 2A). These data are consistent with the fact that an oxidant like HNO_3 will be efficient cleaving the S–H bonds in the hair structure but not strong enough to cleave the C–Hg bond, which was monitored by an increase in Hg^{2+} analysed as $HgEt_2$.

Another important parameter is the digestion temperature, which, due to its relevance, has been optimised independently. Therefore, several temperatures (25, 50, 75 and 100 °C) have been evaluated (Fig. 2B). The optimum

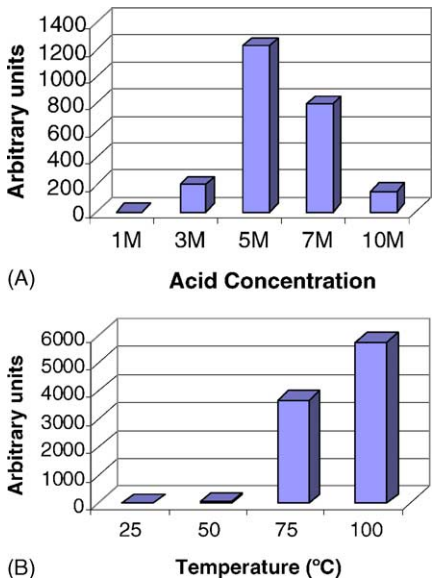

Fig. 2. (A) Dependence of MeHg extraction from human hair on HNO_3 concentration. (B) Dependence of extracted MeHg on the digestion temperature.

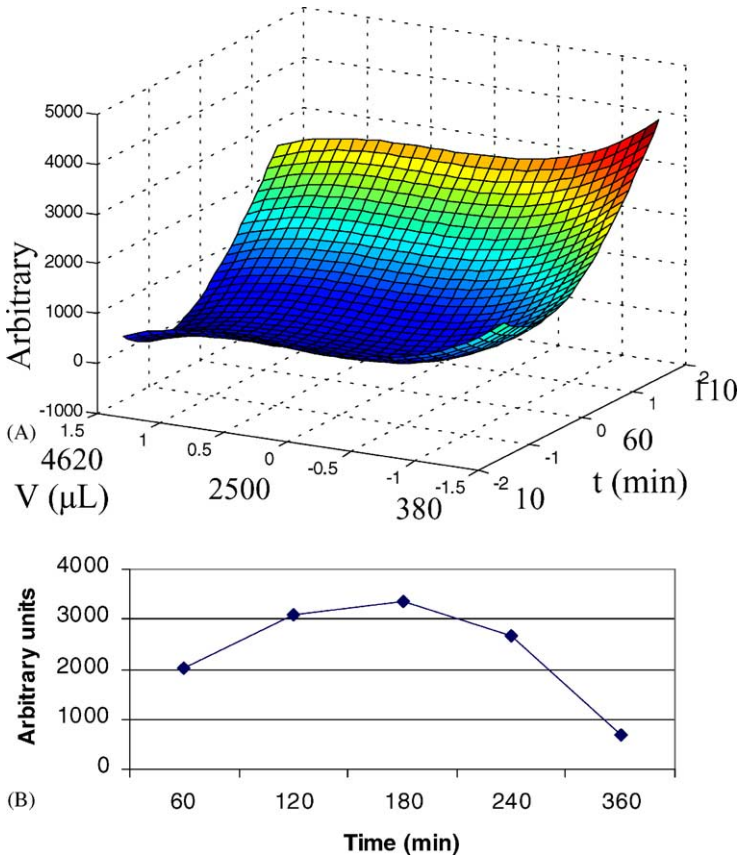

Fig. 3. (A) Three-dimensional representation of the modelling corresponding to the optimisation of the digestion time and acid volume used versus MeHg abundance for a hair sample. (B) Evaluation of the influence of longer digestion time on MeHg recovery.

condition was 100 °C, at which a complete matrix digestion was obtained. For safety reasons, no higher temperatures were evaluated.

In order to optimise the HNO_3 volume and digestion time conditions, a star experimental design with a central point has been carried out (three levels). Experiments were performed from 10 to 110 min (t) and HNO_3 volumes (V) from 380 to 4620 μl. A total of 11 experiences have been performed, including three that correspond to the central point. Modelling was carried out using a third-order polynomial equation. The simplified equation according to the statistical significance of the different terms fitted to the experimental results ($R^2 = 0.994$) and was as following:

$$\text{Area}_{\text{MeHg}} = 611 + 1001t + 16V - 29tV + 508t^2 + 186V^2 - 98t^3 - 237V^3 \quad (1)$$

From Eq. (1), a positive influence of the digestion time and a slight negative effect of acid volume on the extraction was apparent. The best results were obtained for extreme conditions, lowest acid volume (380 μl) and longest digestion time (110 min) (Fig. 3A). From these results, a higher methylmercury recovery was expected with longer digestion times. Therefore, this parameter alone has been optimised (60, 120, 180, 240 and 360 min) (Fig. 3B). The best results were obtained for 180 min, but only slight differences were observed from 120 to 180 min. Hence 120 min has been chosen as the optimum digestion time and 350 μl as the acid volume. For this acid volume, the optimum hair amount was 20 mg. In summary, optimum digestion conditions were an acid digestion using 20 mg of hair with 350 μl of 5 M HNO_3 at 100 °C for a duration of 120 min.

3.3. Sample analysis

In order to evaluate the developed methodology, 22 human hair samples with incurred MeHg and a certified reference material have been analysed. Digestion recoveries were calculated using a reference material analysis obtaining yields 75 ± 11% of the certified value. Ethylation reaction is very sensitive to pH and to the excess of ions in solution. Therefore, a relatively low concentrated buffer (0.2 M) was used. In order to maintain the optimum ethylation pH value, a 25 μl aliquot was transferred from the digestion to the SPME vial.

For these measurements, dipentylmercury has been used as internal standard. In order to control the ethylation, phenylmercury was used as quality control. In real samples, due to matrix effects, ethylation yield for phenylmercury is lower than methylmercury; thus a threshold value of

50% of ethylation yield for phenylmercury was established. Therefore, methylmercury values below that phenylmercury threshold were rejected. For the analysed samples ($n = 23$) monophenylmercury showed an average ethylation yield of $84 \pm 23\%$.

Repeatibility of a same digestion was better than the 5% ($n = 5$) showing the robustness of the analytical procedure. Also, the reproducibility of the methodology has been tested analysing several times the same sample in three different days obtaining a relative standard deviation (R.S.D.) below 15% ($n = 5$).

Finally, the stability of digested samples has been studied analysing three times the same digestion at different storage times at 4 °C, up to a month from its preparation. The obtained results show an R.S.D. lower than 10%.

Concentrations of the analysed samples ranged from 150 to 3499 ng/g, with a median value of 819 ng/g. Technique detection limit (blank level more than three times its standard deviation) was of 40 ng/g and the quantification limit evaluated as the lower concentration of the calibration plot was 110 ng/g.

4. Conclusion

In this paper, a methodology of analysing methylmercury in human hair in combination with HNO_3 digestion, SPME and GC-CVAFS determination is presented. The main goal is to obtain a robust non-invasive technique that will be suitable for routine analysis. Special attention has been focused on the optimisation of the main digestion parameters. Overall R.S.D., considering different digestions, analysed at different times was below 15%. Also, digestion stability up to a month will be helpful in order to automate the method of determination. The robustness of the developed methodology will be a helpful tool for implementation in epidemiological studies involving a large number of samples.

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