Development of a Common Procedure for the Determination of Methylmercury, Ethylmercury and Inorganic Mercury in Human Whole Blood, Hair and Urine by Triple Spike Species-Specific Isotope Dilution Mass Spectrometry

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Abstract

We report the first common methodology for the simultaneous determination of methylmercury (MeHg), ethylmercury (EtHg) and inorganic mercury (Hg(II)) in human blood hair and urine. With the exception of the initial sample mass (0.15 g for blood, 0.5 g for urine and 0.1 g for hair), the same sample preparation and GC-ICP-MS measurement conditions are employed for the three matrices providing experimental values in agreement with the certified values in the analysis of NIST SRM 955c (*Caprine blood*) Level 3 and the certified human hairs IAEA 085 and IAEA 086. Also, the method provides quantitative recoveries for the three Hg species in the analysis of fortified human urine samples at 1, 2 and 5 ng Hg g^{-1}. Mercury species concentrations for levels 2 and 4 of SRM 955c are reported here for the first time. A systematic interconversion of EtHg into Hg(II) was obtained for all matrices reaching values up to 95% in blood, 29% in hair and 11% in urine. MeHg dealkylation was also observed in a lesser extent in blood and hair analyses but it was not observed when analyzing urine samples. Hg methylation was not observed in any matrix. The amount of $NaBPr_4$ added for derivatization has been found to be the main factor responsible for Hg species interconversion. This work demonstrates for the first time, that experimental conditions optimized for SRM 955c (caprine blood) are not valid for human blood samples as the optimum initial sample amount for a real sample is more than three times lower than that for SRM 955c.

INTRODUCTION

Mercury presents a highly complex biogeochemical cycle as it exists in different chemical forms and oxidation states in many terrestrial and aquatic ecosystems[1].. Humans are exposed to elemental mercury (Hg^0) when it is released to the atmosphere by different natural processes such as volcanic eruptions and anthropogenic activities like fossil fuel combustion. Dental amalgams may release elemental mercury into the human body[2] which can be eventually oxidized to Hg(II) by catalase reaction in the erythrocytes[3]. Methylmercury (MeHg) is a highly neurotoxic compound formed from the biotic methylation of inorganic mercury (Hg(II)) at the water-sediment interface in aquatic ecosystems. It can be biomagnified through the food chain by a factor of 10^5-10^7 in predatory fish causing adverse effects in humans and wildlife via fish consumption[4]. Ethylmercury (EtHg) has similar physicochemical properties than MeHg[5] and is used in multidose vials of vaccines as a preservative due to its bactericidal and fungicidal properties..

Most of the current clinical studies of Hg exposure in humans are exclusively focus on the determination of total Hg concentrations. However, speciation analysis provides valuable information not only on the distribution of Hg species among different populations but also on possible contamination pathways. Blood, urine and hair are usually analyzed to evaluate mercury exposition in humans as each of these matrices may provide specific information about the source of mercury contamination. For example, organomercury compounds are preferentially found in blood and hair but they can also be excreted and detected in urine. Hg(II) is accumulated in kidneys and excreted in urine, while its levels in blood reflects short term exposure[6]. In addition, stable isotope analysis showed that inorganic mercury levels in urine are also consequence of demethylation of methylmercury[7]. Total mercury levels in hair correspond mainly to MeHg[8]

although the environmental or occupational exposure to gaseous Hg^0 could increase Hg(II) concentrations[9].

The development of efficient tools for the monitoring of environmental and occupational exposure of humans to Hg is important. However, such tools should be able to discriminate between the different compounds in which Hg is present in the environment.. The main problem arises from the possible occurrence of matrix-dependent alkylation and dealkylation reactions during the sample preparation and separation steps, leading to erroneous determinations. The application of species-specific isotope dilution procedures has drastically improved the quality of data on mercury speciation analysis in real samples. The use of multiple spiking enables not only highly accurate and precise determinations but also the quantification of the extent of interconversion reactions between Hg compounds. For this purpose, all measured compounds must be isotope diluted with a different mercury isotope in such a way that labelled and endogenous compounds must be completely mixed before the occurrence of any transformation reaction. In the case of Hg, double spiking approaches have been employed to determine MeHg and Hg (II) mainly in environmental samples. So far, the analysis of hair samples has been only reported by Rahman et al[10] and Laffont et al[11] using a double spike approach to determine MeHg and Hg(II). When EtHg must be also determined a triple spiking procedure is required. Three previous works have presented a triple spiking approach for Hg speciation analysis in human samples thus far. Davis and Long[12] applied a triple spike procedure for the certification of the standard reference material NIST SRM 955c (*Caprine blood*) whereas Sommer et al[13] and Rahman et al[14] applied a triple spike procedure combined with SPME-GC-ICP-MS to the determination of MeHg, EtHg and Hg(II) in human blood samples. However, those procedures were not tested in other human samples such as urine or hair thus far.

When developing the present work, we have considered two critical aspects. First, interconversion reactions occurring during Hg speciation analysis in environmental and biological samples are matrix dependent and hence the application of multiple spiking procedures is mandatory to achieve accurate results[15]. Secondly, the simplification and normalization of the sample preparation conditions facilitate the implementation of such sophisticated approaches for a routine clinical basis. Our first objective was therefore the development of the first common procedure for the simultaneous quantification of MeHg, EtHg and Hg(II)) in human blood, urine and hair. The methodology was applied to the analysis of NIST SRM 955c (caprine blood) and the human hair reference materials IAEA 085 and IAEA-086. Due to the lack of a certified urine, recovery studies in real samples for the three mercury compounds were carried out. The very low levels of Hg compounds in human samples and the complexity of the matrices compelled us to exhaustively optimize all sample preparation steps to achieve the highest sensitivity. Pursuing this objective, we report for the first time that optimized conditions for the analysis of SRM 955 are not valid for the analysis of real human blood samples.

EXPERIMENTAL

Instrumentation

The alkaline digestion of the samples was performed using a focused microwave Explorer Hybrid from CEM Corporation (Matthews, NC, USA). A gas chromatograph model Agilent 6890N (Agilent Technologies, Tokyo, Japan) fitted with a split/splitless injector and a DB-5MS capillary column from Agilent J&W Scientific (cross-linked 5% diphenyl, 95% dimethylsiloxane, 30 m × 0.53 mm i.d. × 1.0 μm) was coupled to an Inductively Coupled Plasma Mass Spectrometer Agilent 7500ce using a laboratory-made transfer line[16]. More details on the instrumentation and instrumental conditions of the GC-ICP-MS system are described Table S1 of the Supporting information.

Reagents and materials

Blood Standard Reference Material 955c (*Caprine Blood*) was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The Certified Reference Materials of human hair IAEA-085 and IAEA-086 were purchased from the International Atomic Energy Agency (IAEA, Vienna, Austria). MeHg enriched in ^{201}Hg, Hg(II) enriched in ^{199}Hg and EtHg enriched in ^{198}Hg were obtained from ISC-Science (Oviedo, Spain). Detailed information about all reagents employed in this work is given in the Supporting Information. A thorough characterization of the isotopically enriched Hg compounds was carried out, particularly for EtHg for which there is no commercially available natural abundance standard. Details on the characterization of the spike solutions are also given in the Supporting Information (Figures S1 to S4 and Table S2).

Procedures

Focused microwave assisted digestion

Samples were directly weighed in 10 mL disposable glass vials. The optimum amount of sample was 0.15 g for blood, 0.5 g for urine and 0.1 g for hair analysis. Immediately, appropriate amounts of ^{201}Hg-enriched MeHg, ^{198}Hg-enriched EtHg and ^{199}Hg-enriched Hg(II) were added to obtain an amount ratio of endogenous vs labelled compound between 0.1 and 10. Then 3 mL of 25% TMAH was added and a magnetic stir bar was introduced into the vial. The vial was closed with a Teflon cap and placed in the microwave cavity. The focused microwave assisted extraction of the sample was performed using a fixed power of 35 W for 4.5 min.

Derivatization, purification and preconcentration of the samples.

After digestion, the whole extract was transferred to a 22 ml clear glass vial containing 4 mL of an acetic acid/sodium acetate buffer (0.1 M, pH 4). Then, the pH was adjusted to pH 4 using sub-boiled HCl 10%. For derivatization, a 20% solution of the derivatization reagent in THF was

previously aliquoted and stored at -18ºC. Before each sample preparation session, the reagent was diluted ten times with ultrapure water to ensure that each aliquot was thawed only once. The derivatization and liquid-liquid extraction was carried out adding 0.4 mL of a 2% w/v sodium(tetra-n-propyl)borate in Milli-Q water and 1 ml of hexane. Simultaneous propylation and extraction into hexane was accomplished after five minutes of manual shaking. Then, the sample was centrifuged at 5000 rpm for 5 min, and most of the organic layer was transferred to another vial. The sample was then cleaned up using a homemade Florisil® column[17] and stored in a 2 mL clear glass vial at -18 ºC until analysis. Just before the GC-ICP-MS injection the sample was pre-concentrated under a gentle stream of nitrogen. A scheme of the whole sample preparation procedure is showed in Figure S5 of the Supporting Information.

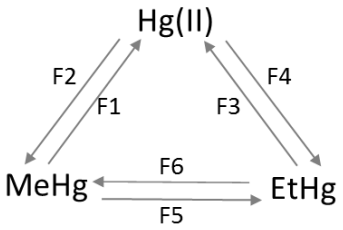
Measurement of the isotopic composition of the samples by GC-ICP-MS

Daily optimization of the ion lenses was performed after the connection of the GC to the ICP-MS by using the signal of $^{36}Ar^{40}Ar^{+}$ and $^{38}Ar^{40}Ar^{+}$. Integration of the chromatographic peaks was carried out using the commercial software supplied with the ICP-MS instrument. The integration time per isotope was 80 ms and the isotopes measured were 198, 199, 200, 201 and 202. Mass bias correction was performed using an internal correction based on the minimization of the square sum of the residuals of the multiple linear regression employed for quantification as explained elsewhere[18].

Triple Spike Isotope Dilution Equations

For the calculation of the concentration of the three mercury compounds it was assumed that the isotopic composition in a mixture of natural abundance and labeled compounds is a linear combination of the isotope patterns of the different constituents of the mixture. Thus, applying multiple linear regression the molar fraction of natural abundance and isotopically enriched

compounds can be calculated for each chromatographic peak. The general equations for triple spike isotope dilution are based on those previously developed for simultaneous determination of tin compounds[19]. If we consider a system of three interconverting mercury species as described in Equation 1 we can define six intercoversion factors from F1 to F6.



(1)

Due to its high volatility and the absence of an isotopically enriched analogue Hg^0 was not considered in this model. Nevertheless, transformations to Hg^0, although not quantified, are corrected by the proposed isotope dilution approach. Expressing the isotope abundances of the isotope diluted sample as a function of the Hg natural isotope abundances and the isotope abundances of the isotopically enriched tracers we can define for each chromatographic peak a matrix equation similar than Equation 2 that can be solved by multiple linear regression.

$$\begin{bmatrix} A_m^{198} \\ A_m^{199} \\ A_m^{200} \\ A_m^{201} \\ A_m^{202} \end{bmatrix} = \begin{bmatrix} A_{nat}^{198} & A_{MeHg}^{198} & A_{Hg(II)}^{198} & A_{EtHg}^{198} \\ A_{nat}^{199} & A_{MeHg}^{199} & A_{Hg(II)}^{199} & A_{EtHg}^{199} \\ A_{nat}^{200} & A_{MeHg}^{200} & A_{Hg(II)}^{200} & A_{EtHg}^{200} \\ A_{nat}^{201} & A_{MeHg}^{201} & A_{Hg(II)}^{201} & A_{EtHg}^{201} \\ A_{nat}^{202} & A_{MeHg}^{202} & A_{Hg(II)}^{202} & A_{EtHg}^{202} \end{bmatrix} \times \begin{bmatrix} X_{nat} \\ X_{MeHg} \\ X_{Hg(II)} \\ X_{EtHg} \end{bmatrix} + \begin{bmatrix} e^{198} \\ e^{199} \\ e^{200} \\ e^{201} \\ e^{202} \end{bmatrix} \quad (2)$$

The solutions to the three systems of equations are twelve molar fractions (four for each system as described in Equation 2) corresponding to the natural and labelled Hg species in each chromatographic peak that will allow the calculation of the extent of the interconversion reactions and the species concentrations. More details on the calculation procedure and on the development of the mathematical equations are given in the Supporting Information

RESULTS AND DISSCUSION

Selection of the focused microwave assisted digestion conditions

Optimum digestion conditions must ensure that the endogenous Hg species are completely released from the sample matrix and mixed with the added enriched species in the liquid phase. During this process the identity and concentration of the enriched Hg species must be prevailed to obtain accurate results. Based on previous works[19] we selected TMAH and focused microwaves to perform the digestion of the human samples in a short period of time. Two different microwave programs described previously for Hg speciation using the same system were employed: a constant temperature program at 70ºC[20] and a fixed power program at 35W with a maximum allowable temperature of 100ºC[12]. Both MW programs were applied to the analysis of levels 1 and 4 of SRM 955c (*caprine blood*), human hair samples, human urine samples and blanks. The interconversion factors obtained in all samples at different digestion conditions are summarized in Table S3 of the Supporting Information which shows a systematic degradation of EtHg and MeHg to Hg(II) under all digestion conditions tested. The dealkylation of EtHg was in all cases higher than that of MeHg. Dealkylation for EtHg ranged from 3 to 74% whereas that for MeHg ranged from 0.2 to 9.2 %. When comparing the different matrices, we observed that the lowest dealkylation factors were obtained when analyzing urine. Indeed, an almost negligible MeHg demethylation was obtained for urine under all digestion conditions tested. EtHg dealkylation was lower than 14% except when performing a room temperature digestion during 2 hours in which we obtained 41% conversion of EtHg into Hg(II). In this case the long digestion time might explain the high dealkylation compared to the values obtained by microwave extraction. EtHg dealkylation was significantly high in hair and blood samples reaching up to 74% when digesting hair at 70ºC for 4 minutes. These results confirm that the interconversion between Hg species during sample preparation depends not only on

the experimental conditions but also on the matrix type[21]. We finally decided to work with a fixed power o 35W during 4.5 minutes as the extraction cycle between samples was faster than when setting a constant temperature in the microwave digestor.

Optimization of the derivatization procedure

The pH, the sample matrix and the added amount of the propylation reagent ($NaBPr_4$) strongly affect the derivatization yield for the propylation of mercury compounds. For example, De Smaele and coworkers[22] reported that $NaBPr_4$ decomposed under pH 4 leading to an unreactive compound (BPr_3). Indeed, it has been demonstrated that the highest derivatization yield for organomercury species was obtained at pH 4[22, 23]. Providing an exhaustive control of the pH is achieved, it is important to take also into account that different constituents of the biological sample matrix may consume $NaBPr_4$ before it reacts with the target analytes[24]. According to this, different experiments were carried out to optimize the derivatization procedure.

Optimization of the extract volume in blood

Matrix effects during derivatization are more critical in blood samples due to its higher complexity in comparison with hair and urine. Therefore, we selected level 4 of NIST SRM 955c to optimize the extraction procedure. In all cases the digestion of the blood was performed with 4 mL of 25% TMAH; then increasing volumes of the extract, namely 0.5, 1, 2, 3 and the whole extract (ca. 4 mL) were derivatized with a fixed volume of 400 µL of 2% $NaBPr_4$ in water. Figure 1 shows the interconversion factors obtained for the dealkylation of MeHg and EtHg (%). The rest of interconversion factors were negligible. As can be observed, the highest dealkylation factors are obtained analyzing 1 ml of extract. Assuming an absence of signal drift of the GC-ICP-MS system during the measurement session, a comparison of the derivatization efficiency between samples can

be carried out calculating the ratio of the peak area for the isotopes 198, 199, and 201 in EtHg, Hg(II) and MeHg peaks, respectively per ng of added enriched compound. According to the results presented in Figure 1, the lowest dealkylation factors and the higher sensitivity was obtained when derivatizing the total extract volume. Note that error bars in Figure 1 correspond to n=2 independent replicates.

Optimization of the volume of $NaBPr_4$ in blood and urine

The volume of $NaBPr_4$ employed for derivatization was optimized for blood and urine. For this purpose, we added increasing volumes of the reagent (from 400 µL to 1 mL) to four replicates of the SRM NIST 955c Level 4 and to four replicates of the same human urine sample. In these cases the total extract volume was used for derivatization. It is worth noting that each set of data correspond to the same analytical session. The results from different sessions could not be compared due to the different characteristics of the derivatization reagent in terms of degradation. When $NaBPr_4$ is stored immediately after dilution it degrades slowly and the reactivity decreases over time affecting the derivatization efficiency and the transformation factors. Due to this fact the experiments were performed with a freshly prepared derivatization reagent. Similarly to the previous results the main interconversions observed where those of EtHg to Hg(II) and MeHg to Hg(II). Figure 2a shows that both interconversion factors increase with the amount of the derivatization reagent. When comparing the peak areas per ng of labelled compound the best sensitivity was obtained using 600 µL of the derivatization reagent but the lowest interconversion was obtained when using 400 µL. When using higher volumes of the derivatizing reagent, the signals decreased significantly. The increment in the volume of derivatization reagent not only caused analyte interconversions, but also other reactions such as analyte reductions to Hg^0. For example, Figure S6 of the Supporting Information shows the GC-ICP-MS chromatograms obtained when adding increasing volumes of $NaBPr_4$ to the same blood sample (0.5, 1, 2 and 3 mL). As can

be observed, the chromatograms showed the formation of a volatile Hg species, most likely Hg^0 due to the short retention time, with a mixed isotopic composition obtained from the reduction of mainly EtHg and Hg(II) by the derivatization reagent. When adding 3 mL of the derivatization reagent the EtHg and Hg(II) peaks disappeared completely from the chromatogram.

The volume of the derivatization reagent was also optimized for the analysis of urine samples. During previous experiments in urine samples we observed similar derivatization yields than those obtained in standards, indicating a very low matrix effect. Therefore this study was carried out adding volumes of $NaBPr_4$ from 100 to 400 µL. **Figure 2b** shows that the interconversion factors in urine were lower than 18% and that the sensitivity obtained was not significantly dependent on the derivatization reagent. The decrease of the sensitivity for Hg(II) when adding 200 µL can be considered as an artefact as similar peak areas per ng of labelled compound were obtained at 100, 300 and 400 µL. Due to the absence of any significant influence, a derivatization volume of 400 µL was selected for future experiments in order to fix the same experimental conditions for blood and urine samples.

Determination of the blank values in blood and urine analyses

Due to the absence of human samples free of mercury species, the blank values were calculated by Triple Spike IDMS adding proper amounts of ^{201}Hg-enriched MeHg, ^{199}Hg-enriched Hg(II) and ^{198}Hg-enriched EtHg to blanks. For this purpose, we considered a theoretical sample weight of 0.5 g employed in the previous analyses of SRM 955c and urine samples. We evaluated the contribution to the blank of two potential contamination sources: the disposable glass vials employed during the focused microwave assisted digestion and the use of TMAH. First we analyzed six blanks using precleaned vials with 10% HCl and six brand new non-precleaned vials in the presence of TMAH. Table S.4 shows that there was no significant difference between the blank values for the three Hg species when using the precleaned vials in comparison with the non-precleaned vials. Thus, we

analyzed six additional blanks using non-precleaned vials but removing the digestion reagent TMAH. As can be observed, the blank values for EtHg, MeHg and Hg(II) reduced to negligible levels. So, the extraction reagent was the main contributor to the blanks observed. Although we observed similar blank values with other batches we did not perform any study with different reagent purities. Multiplying by 3 the standard deviation of the blank values obtained using non-precleaned vials, detection limits of 0.11, 0.02 and 0.11 ng Hg g^{-1} were obtained for Hg(II), MeHg and EtHg, respectively. Figure S7 shows a representative GC-ICP-MS chromatogram of a blank spiked with 0.5 ng of each labeled species.

Analysis of the certified reference material NIST SRM 955c (Caprine Blood).

The proposed triple spike IDMS methodology was applied to the analysis of Levels 2, 3, and 4 of the SRM 955c (caprine blood). Level 1 was not analyzed because the concentration of the three Hg species was below the method detection limit. We analyzed n=15, 12 and 13 independent replicates for Levels 2, 3 and 4, respectively using at least four different vials of each concentration level. Table 1 shows the average concentration values with associated uncertainties calculated as 1s standard deviation obtained for MeHg, Hg(II), EtHg and the range of the interconversion factors obtained for each level. Tables S5, S6 and S7 in the Supporting Information show the concentrations and the interconversion factors obtained in all individual replicates of Levels 2, 3 and 4, respectively. The total mercury concentration (THg) corresponds to the sum of concentration of the three mercury species. The standard deviation of the THg concentration (SD_{THg}) was calculated using equation (3) where $SD_{Hg(II)}$, SD_{MeHg} and SD_{EtHg} correspond to the standard deviations of the individual concentrations of Hg(II), MeHg and EtHg, respectively.

$$SD_{THg} = \sqrt{[SD_{Hg(II)}]^2 + [SD_{MeHg}]^2 + [SD_{EtHg}]^2} \quad (3)$$

Table 1 shows also that THg values were in good agreement with the certified values reported by NIST in Levels 2, 3 and 4. In addition, the experimental values obtained for Hg(II), MeHg and EtHg in Level 3 were in agreement with the compound-specific certified values. It should be stressed that this work reports for the first time concentrations of MeHg, EtHg and Hg(II) for Levels 2 and 4 of SRM 955c. Typical GC-ICP-MS chromatograms of the three concentration levels are presented in Figure S8 of the Supporting Information. Concerning the interconversion factors, a systematic dealkylation of EtHg and MeHg to form Hg(II) was observed in all replicates. EtHg dealkylation was the most important reaction since the interconversion percentages ranged from 12 to 72% whereas dealkylation of MeHg ranged from 1 to 15%. As mentioned before, the state of the derivatization reagent might explain the high variability found for these factors between different replicates and analytical measurement sessions (see Tables S5, S6 and S7). Table 1 shows that the rest of interconversion factors were found to be negligible. Relative standard deviations from 3.2 to 16% were obtained reflecting the challenge of quantifying Hg species at the very low ng g^{-1} range in such a complex matrix. This is in agreement with previous works reporting that the correction of analytes interconversion by multiple-spiking isotope dilution is possible at the expense of the precision of the initial amount estimates[25].

Analysis of the certified reference materials IAEA-085 and IAEA-086 (human hair).

Certified human hair reference materials IAEA-085 and IAEA-086 were analyzed by the proposed methodology performing n=4 and n=8 independent replicates, respectively. Table 1 shows that MeHg and THg values were in good agreement with the certified values. This work also reports for the first time EtHg values for both materials. Preliminary experiments showed that EtHg concentration was lower than 10 ng g^{-1} in IAEA 085 whereas the certified concentration of MeHg was 22.9 µg g^{-1}. According to Monperrus et al[15] such difference between species concentrations limits the capabilities of multiple spiking approaches and might explain unusual interconversion

reactions such as the transalkylation of EtHg into MeHg obtained in IAEA 085 (see Table 1). To avoid anomalous conversion rates in the analysis of IAEA 086, we decided to add similar amounts of labelled EtHg and Hg(II) despite the risk of EtHg overspiking. As can be observed in Table 1, the EtHg dealkylation to form Hg(II), was the most important reaction since the interconversion percentages ranged from 9 to 32% whereas dealkylation of MeHg ranged from 1 to 4%. Table S8, shows the concentrations and the interconversion factors obtained in all individual replicates of hair reference materials IAEA-085 and IAEA-086 and Figure S9 shows typical GC-ICP-MS chromatograms for the analysis of both materials.

Recovery studies in fortified human urine samples.

Due to the lack of a Certified Reference Material for Hg species in urine we performed recovery experiments in fortified real samples to evaluate the accuracy and precision of the proposed methodology. After collection, urine samples were homogenized, aliquoted and immediately frozen. Thus, precipitation of different compounds in the urine was avoided before the sample preparation procedure and it was possible to analyze the same sample in different measurement sessions. Recovery experiments were performed at three different concentration levels from 1 to 5 ng Hg g^{-1} for each species. Due to the absence of a certified natural abundance standard for EtHg, we characterized the commercial EtHg standard using the same strategy followed to characterize the ^{198}Hg-enriched EtHg standard explained in the Supporting Information. The calculation of the recovery values required the previous determination of the background concentration of the three Hg species in the urine sample. It was observed that the concentration of Hg(II) was 1.03 ng g^{-1} while those of EtHg and MeHg of the same order of the detection limit of the procedure. Table 1 shows that recovery values from 96.0 to 99.1% were obtained with relative standard deviations from 1.5 to 8%. Table S9 shows the individual theoretical and experimental concentrations and associated recoveries for the fortified real urine samples at 1, 2, 5 ng g^{-1}. The highest relative

standard deviations were found for the lowest concentration level and for EtHg determination, similarly than for blood and hair analysis. The use of triple spike isotope dilution is justified also in urine matrices since significant conversions reaching up to 11% from EtHg to Hg(II) were observed. In contrast to hair and blood, dealkylation of MeHg was found to be negligible. Figure S10 shows typical GC-ICP-MS chromatograms of the urine sample fortified at 1, 2, 5 ng g^{-1}.

Application of the methodology to the analysis of whole human blood samples

The optimized sample preparation conditions were applied to the analysis of real human blood samples. In each measurement session we analyzed Level 2 of NIST SRM 955c for quality control purposes. During the measurement sessions it was found that the intensity of the isotopically labelled compounds was drastically decreased in human blood samples when compared with the reference material under the same extraction and derivatization conditions. Figures 3a and 3b show typical GC-ICP-MS chromatograms of a digested aliquot of SRM 955c caprine blood (Level 2) and a real sample, respectively, using in both cases 0.5 g of sample. As can be observed, although the same amount of labelled compounds was employed in both samples, the intensity of the chromatographic peaks for the isotopic tracers was decreased more than 15 times. According to these results, previous optimized sample preparation conditions for the analysis of SRM 955c might not be adequate for the analysis of every type of whole blood matrix. SRM 955c consists of frozen caprine blood containing spiked inorganic mercury, ethylmercury and methylmercury. Thus, Hg species may not be bonded or incorporated into the same sample constituents as the endogenous compounds in real samples. According to this, an in agreement with previous works[13], further optimizations of the analytical methodology were carried out with real human blood samples.

Particular effort was then paid to solve this analytical problem as the effect described in Figures 3a and 3b was observed in 24 out of 25 analyzed human blood samples. We modified first the focused microwave conditions to check for an incomplete digestion of the blood samples. Using the same sample of Figure 3 we increased the digestion time from 4.5 minutes to 10 minutes maintaining a fixed power of 35W. As can be observed in Figure S11 we did not observed any increase in the sensitivity for the three Hg species when increasing the digestion time. The next step was to reduce the initial sample amount to check for matrix effects during the derivatization step. Initial amounts of the same sample from 0.1 to 0.5 g were digested and derivatized using the recommended procedure. As can be observed in Figure 4 when using 0.5 g of sample the instrumental sensitivity for the three label species was very low and EtHg dealkylation reached almost 100% whereas that for MeHg into Hg(II) reached 76%. According to the results shown in Figure 4, 0.150 g of sample provided the best intensities for the labelled species and the lowest dealkylation reactions (26% and 3% for EtHg and MeHg dealkylation, respectively). Figure 3 shows the different behavior of NIST SRM 955c compared to real samples. When analyzing 0.150 g of sample the opposite effect is obtained (Figures 3c and 3d) than when using 0.5 g of sample. In any case, results in agreement with the reference values were also obtained for NIST SRM 955c Level 3 with 0.15 g of sample. Finally, using 0.15 g of the same real blood sample we optimized the derivatization reagent volume. Figure S12 shows that, similarly to the analysis of the SRM 955c, when adding a high amount of the derivatization reagent we observed a high suppression of the signal and high conversion rates between species. We finally selected 400 microliters of $NaBPr_4$ as the volume which provided the lowest interconversion factors (31% and 4.4% for EtHg and MeHg dealkylation into Hg(II), respectively) and an adequate sensitivity for the three Hg species.

CONCLUSIONS

This work demonstrates that the accurate determination of Hg species in human samples undoubtedly requires the application of multiple spiking species-specific IDMS. For all matrices and under all samples preparation conditions assayed, we have observed important conversions of MeHg and EtHg into Hg(II) which could not be corrected for using any other calibration technique including single spiking IDMS. We also report for the first time important procedural differences between the analysis of certified reference material SRM 955c and real blood samples. Hg species in real samples are endogenously associated to proteins or other matrix constituents whereas SRM 955c is a fortified caprine blood sample. Using the optimum initial sample amount for the analysis of SRM 955c a severe signal suppression effect is obtained in real samples. In any case, the amount of derivatization reagent $NaBPr_4$ is critical for species interconversion and has to be specifically optimized for each type of matrix. Indeed, a direct relationship between the quantity added to the sample and the transformation of EtHg and MeHg into Hg(II) that has been systematically observed in all matrices. In addition, when using an excess of $NaBPr_4$ we have observed the complete reduction of Hg species into Hg^0.

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Figures and Tables

Figure 1. Percentages of conversion of MeHg and EtHg into Hg(II) and peak area per added ng of ^{201}MeHg, ^{199}Hg(II) and ^{198}EtHg obtained in the analysis of level 4 of NIST SRM 955c when adding 400 µL of $NaBPr_4$ 2% to different volumes of the extract. Error bars correspond to n=2 independent replicates analyzed in the same measurement session.

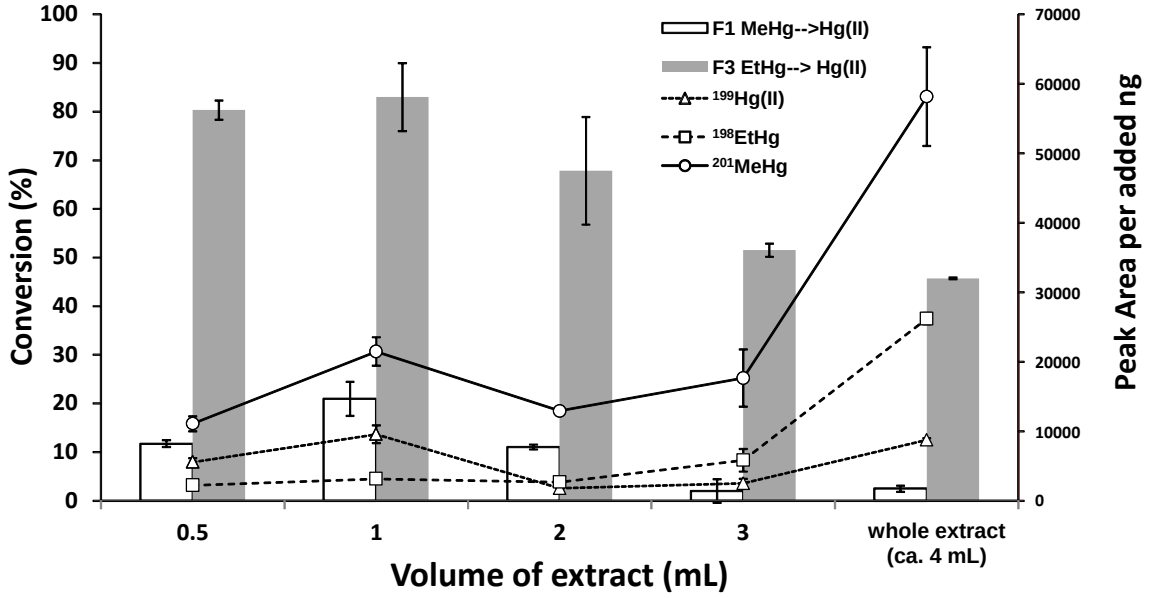
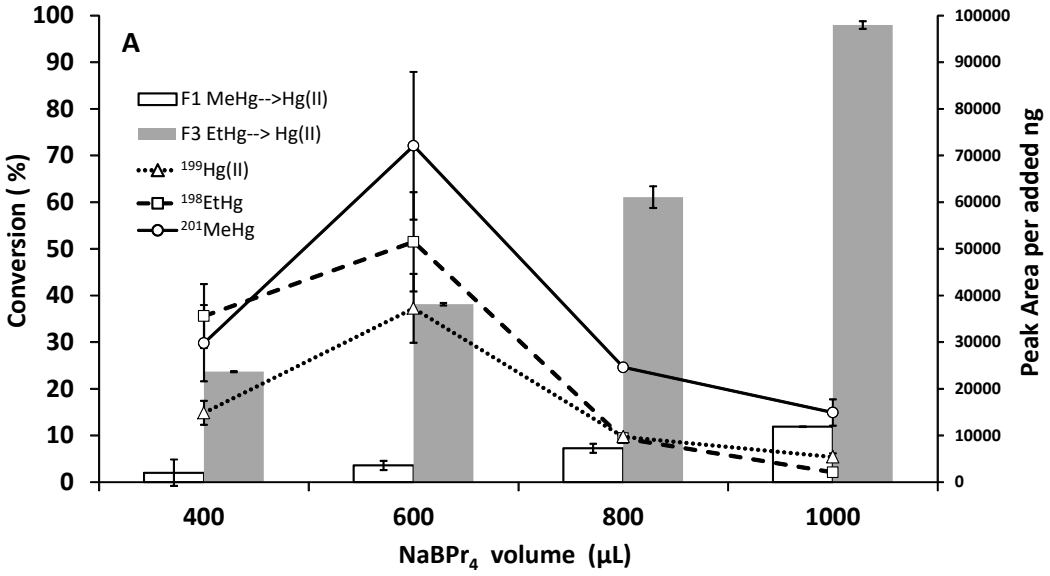


Figure 2. Percentages of conversion of MeHg and EtHg into Hg(II) and peak area per added ng of ^{201}MeHg, ^{199}Hg(II) and ^{198}EtHg obtained when adding different volumes of $NaBPr_4$ in the analysis of a) 4 mL of extract of level 4 of NIST SRM 955c and b) 4 mL of extract of the same human urine sample. Error bars correspond to n=2 independent replicates analyzed in the same measurement session.



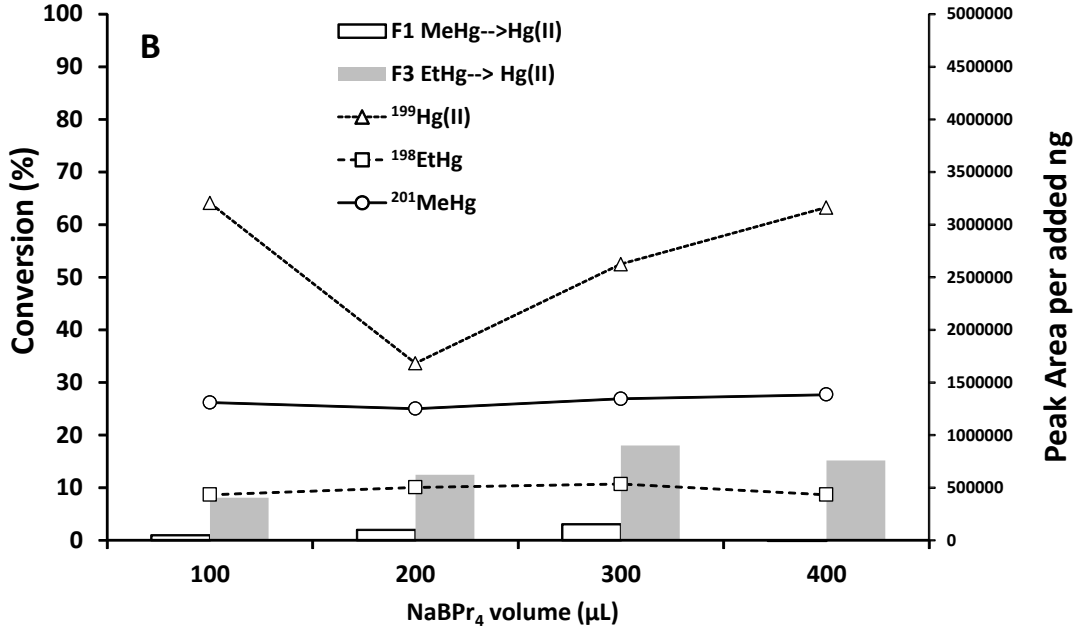


Figure 3. Comparison between the chromatograms obtained for the spiked NIST SRM 955c (Caprine Blood) and one spiked human blood sample for different weights taken. Figures A and B correspond to 0.500 g of sample for NIST SRM 955c and human blood, respectively. Figures C and D correspond to 0.150 g of sample for NIST SRM 955c and human blood, respectively.

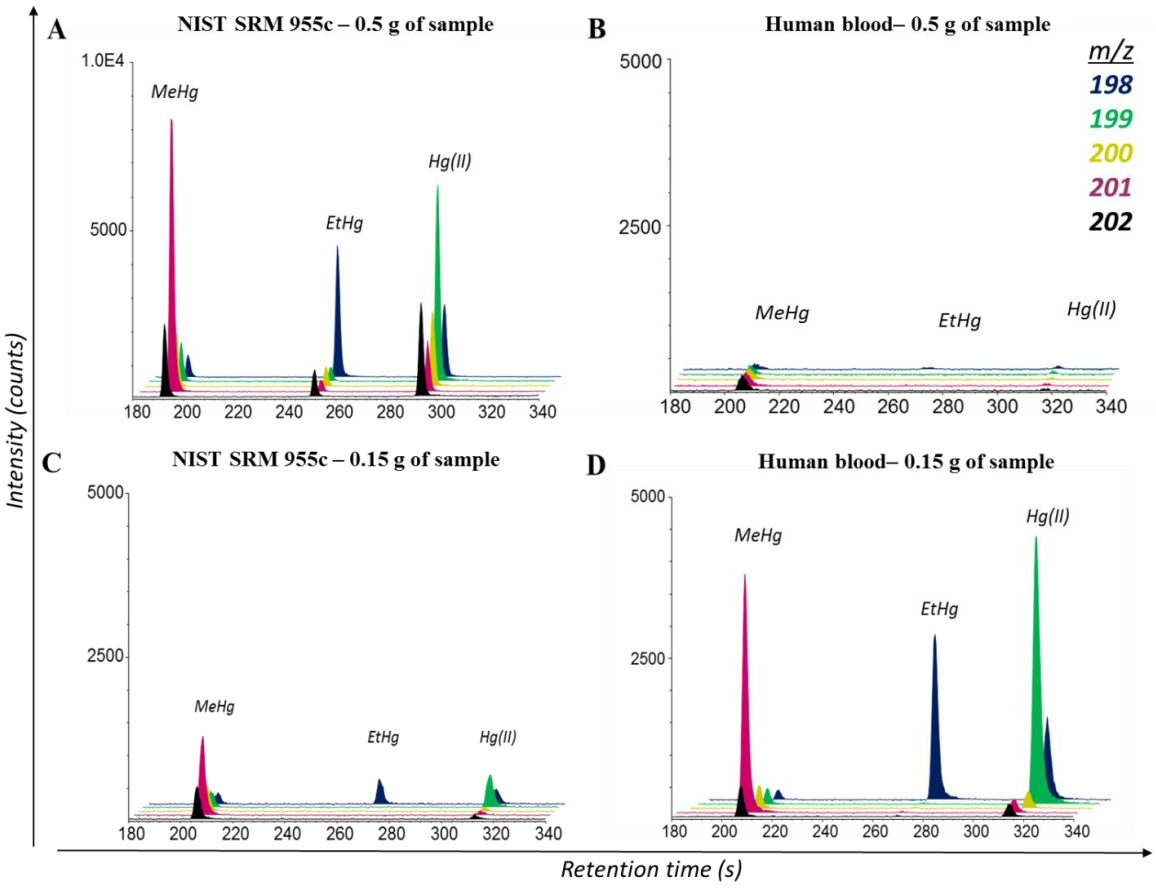


Figure 4. Percentages of conversion of MeHg and EtHg into Hg(II) and peak area per added ng of ^{201}MeHg, ^{199}Hg(II) and ^{198}EtHg obtained when adding different volumes of $NaBPr_4$ in the analysis of a human blood sample.

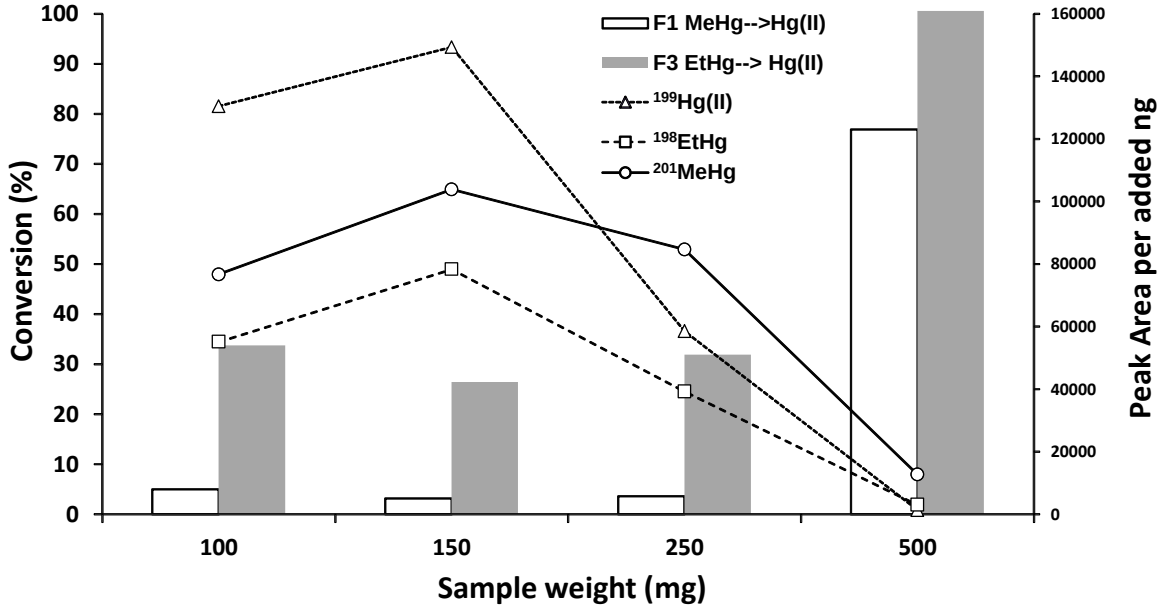


Table 1. Average concentration values of MeHg, EtHg and Hg(II) and range of interconversion factors obtained in the analysis of Levels 2,3 and 4 of certified caprine blood NIST SRM 955c, certified human hairs IAEA-085 and IAEA-086 and recovery values in the analysis of human urine fortified at 1, 2 and 5 ng g^{-1}. The range of interconversion factors (%) obtained for each matrix is also given. Uncertainty of the concentration values is expressed as 1s standard deviation of the analyzed replicates.

		Hg(II)	MeHg	EtHg	THg	% MeHg→Hg(II)	% Hg(II)→MeHg	% EtHg→Hg(II)	% Hg(II)→EtHg	% MeHg→EtHg	% EtHg→MeHg
SRM 955c Level 2 n=15	Experimental (ng Hg g^{-1})	2.09 ± 0.33	1.87 ± 0.30	1.40 ± 0.09	5.36 ± 0.46	2.0→15	-0.7→0.6	29→96	-0.6→0.2	-0.1→0.4	-0.5→0.3
	Certified	-	-	-	4.70 ± 0.72						
SRM 955c Level 3 n=12	Experimental (ng Hg g^{-1})	9.53 ± 0.57	4.77 ± 0.15	3.97 ± 0.40	18.27 ± 0.72	1.6→6.5	-0.5→0.3	22→69	-0.4→0.2	-0.3→0.2	-0.3→0.8
	Certified	8.55 ± 1.23	4.27 ± 0.95	4.81 ± 0.45	16.91 ± 1.52						
SRM 955c Level 4 n=13	Experimental (ng Hg g^{-1})	19.96 ± 0.80	7.02 ± 0.47	5.90 ± 0.73	33.03 ± 1.42	1.8→7.5	-0.9→0.2	24→72	-0.3→1.9	-0.3→0.4	-0.9→0.5
	Certified	-	-	-	32.20 ± 1.99						
IAEA-085 n=4	Experimental (µg Hg g^{-1})	0.67 ± 0.02	23.2 ± 0.3	0.01 ± 0.00	24.0 ± 0.3	1.1→3.9	-4.7→4.5	9.1→23	-0.7→-0.6	0.0→0.0	-5.9→4.5
	Certified	-	22.9 ± 1.0	-	23.2 ± 0.8						
IAEA-086 n=8	Experimental (µg Hg g^{-1})	0.278 ± 0.013	0.277 ± 0.006	0.004 ± 0.001	0.558 ± 0.014	1.5→3.1	-0.3→0.1	12→32	-7.0→0.1	-0.5→0.0	-1.1→11
	Certified	-	0.258 ± 0.022	-	0.573 ± 0.039						
Urine 1 ng g^{-1} n=4	Recovery (%)	97.4 ± 6.0	98.3 ± 1.9	93.8 ± 8.0	--	-1.0→0.5	-1.4→0.6	6.9→11.2	-1.2→-0.6	-0.6→0.3	-1.4→-0.2
Urine 2 ng g^{-1} n=4	Recovery (%)	97.3 ± 4.7	96.9 ± 1.5	99.1 ± 5.0	--	-0.2→0.1	-0.3→0.0	5.7→7.3	-1.2→1.0	-0.3→0.0	-0.1→0.0
Urine 5 ng g^{-1} n=4	Recovery (%)	98.1 ± 3.4	96.0 ± 1.5	98.1 ± 2.8	--	0.1→0.5	-0.5→0.0	5.4→7.4	-1.0→-0.2	-0.3→0.0	-0.6→-0.1