



Published in final edited form as:

*Anal Bioanal Chem.* 2014 August ; 406(20): 5039–5047. doi:10.1007/s00216-014-7907-4.

## Measurement of Mercury Species in Human Blood using Triple Spike Isotope Dilution with SPME-GC-ICP-DRC-MS

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### Abstract

The measurement of different mercury compounds in human blood can provide valuable information about the type of mercury exposure. To this end, our laboratory developed a biomonitoring method for the quantification of inorganic (iHg), methyl (MeHg) and ethyl (EtHg) mercury in whole blood using a triple spike isotope dilution (TSID) quantification method employing capillary gas chromatography (GC) and inductively coupled dynamic reaction cell mass spectrometry (ICP-DRC-MS). We used a robotic CombiPAL<sup>®</sup> sample handling station featuring twin fiber-based solid phase microextraction (SPME) injector heads. The use of two SPME fibers significantly reduces sample analysis cycle times making this method very suitable for high sample throughput, which is a requirement for large public health biomonitoring studies. Our sample preparation procedure involved solubilization of blood samples with tetramethylammonium hydroxide (TMAH) followed by the derivatization with sodium tetra(n-propyl)borate (NaBPr<sub>4</sub>) to promote volatility of mercury species. We thoroughly investigated mercury species stability in the blood matrix during the course of sample treatment and analysis. The method accuracy for quantifying iHg, MeHg and EtHg was validated using NIST standard reference materials (SRM 955c Level 3) and the Centre de Toxicologie du Québec (CTQ) proficiency testing (PT) samples. The limit of detection (LOD) for iHg, MeHg and EtHg in human blood was determined to be 0.27, 0.12, and 0.16 µg/L, respectively.

### Keywords

speciation; mercury; blood; biomonitoring; isotope dilution; SPME; GC; ICP-MS

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### Disclosure statement

Mention of company or product names does not constitute endorsement by the Centers for Disease Control and Prevention (CDC) or the U.S. Department of Health and Human Services.

## Introduction

Mercury is a non-essential toxic element occurring in the environment in different chemical forms including elemental (Hg), inorganic (iHg) and various organic forms such as methyl mercury (MeHg), ethyl mercury (EtHg), and others. Mercury adversely affects human well-being; the manner in which it does depends on the chemical form of mercury (“species”), the route of entry into the body, length of exposure, and the age and susceptibility of the exposed person [1]. The most accepted biomarker of mercury exposure in humans is the direct measurement of mercury species in blood, which can aid in the identification of the exposure source and help guide treatment options [2,3]. From the perspective of improving the value of speciated mercury data from human exposure studies, it is essential that analytical laboratory performance benchmarks continue to advance.

Our laboratory supports a number of public health biomonitoring studies, such as the National Health and Nutrition Examination Survey (NHANES) [4] which includes the measurement of mercury species in human blood. National biomonitoring studies should measure any national or subpopulation initial exposures and monitor ongoing trends of analytes or their species that are of medical or public health concern. Additional analytical requirements include the ability to have the limit of detection (LOD) be approximately less than one third of the expected population mean; this ensures that the measurements are “fit for purpose”. Thus, it is clear that fast and robust methods with high accuracy, high precision, and low detection limits are important requirements for meeting the objectives of national surveys.

Solid-phase microextraction (SPME) paired with gas chromatography (GC) and inductively-coupled plasma mass spectrometry (ICP-MS) has grown to become one of the more reliable technologies for the mercury speciation of biologic samples [2,5]. Despite the advances in instrumentation and sample introduction techniques, the mercury speciation methodology continued to be plagued by spontaneous *in vitro* transformation reactions resulting in unwanted changes, especially the dealkylation of organomercury compounds in biologic samples during processing or storage. Triple spike isotope dilution (TSID) is a particular useful technique that puts a number on how far species transformations have proceeded, so the analyst can apply the proper corrections [6]. Central to TSID design is that “spike” solution containing mixture of three mercury species standards, each synthesized to be enriched with different isotopes of mercury ( $^{199}\text{HgCl}_2$ ,  $\text{CH}_3^{200}\text{HgCl}$  and  $\text{C}_2\text{H}_5^{201}\text{HgCl}$ ) must be added to each patient sample and equilibrated before mercury transformation reactions occur. Since the spike is chemically identical to the native form of mercury species contained in the sample, chemical transformations can be measured and mathematically corrected [7,8].

In this paper, we describe the development and characterization of a method for measuring iHg, MeHg and EtHg species in whole blood, which is intended for high throughput use in support of large human biomonitoring surveys. The TSID methodology offers us major improvements in accuracy, precision and LOD over our previous methods. We set goals for speed and efficiency, and we invested in a uniquely configured robotic CombiPAL<sup>®</sup> sample handling station featuring two SPME heads on the same workstation, which work together

in tandem. This arrangement allows the robot to perform a greater number of GC injections per hour without sacrificing optimum fiber extraction time. We validated our method for accuracy and precision using NIST standard reference material (SRM 955c Level 3) and Centre de Toxicologie du Québec (CTQ) proficiency testing (PT) samples. Further, we thoroughly investigated the stability of mercury species in whole blood during sample storage, preparation and analysis.

## Experimental section

Detailed description of the experimental section can be found in Supplemental Information (SI).

### Sample preparation

All reagents used were of analytical or reagent grade purity. Mercury speciation analysis of each 100  $\mu\text{L}$  aliquot of blood sample began with the addition of 100  $\mu\text{L}$  of spike solution containing known amounts of enriched  $^{199}\text{HgCl}_2$ ,  $\text{CH}_3^{200}\text{HgCl}$  and  $\text{C}_2\text{H}_5^{201}\text{HgCl}$  isotopic standards (Applied Isotope Technologies (AIT), Sunnyvale, CA, USA), and immediately mixed by vortexing. After addition of 500  $\mu\text{L}$  of tetramethylammonium hydroxide (TMAH), 25% w/w in methanol (Alfa Aesar, Ward Hill, MA, USA), the samples were put in an 80  $^\circ\text{C}$  convection oven (FREAS Model 605, Thermo Fisher Scientific, USA) for 24–26 hours. Next, 200  $\mu\text{L}$  of the solubilized sample was transferred to a 20 mL glass SPME analysis vial (Wheaton, Millville, NJ, USA) followed by the additions of 7.7 mL of 0.1 M sodium acetate buffer, pH 4.75, (bringing the final pH to 5–6) and 250  $\mu\text{L}$  of 0.2% w/v of sodium tetra(n-propyl)borate ( $\text{NaBPr}_4$ ) (ABCR, Germany). During the sample preparation procedure, the relative standard deviation (RSD) value associated with using the analytical balance was 0.002% (N=10, 1g NIST certified weight), pipetting whole blood was 0.77% (N=10, 100  $\mu\text{L}$ ), and pipetting the aqueous spike solution was 0.72% (N=10, 100  $\mu\text{L}$ ). (See Figure S1 in SI for a diagrammatic representation of sample preparation and analysis.)

We prepared quality control (QC) samples using donated human whole blood purchased from Tennessee Blood Services (Memphis, TN, USA). “Low” (LB QC) and “high” (HB QC) pool contained concentrations of 0.8, 0.7, 0.6 and 2.2, 3.7, 1.2  $\mu\text{g/L}$  for iHg, MeHg and EtHg, respectively (see SI, **Table S1** for exact concentrations of each mercury species and detailed description of QC preparation). The empirical expanded uncertainties,  $U$  ( $k=2$ ), for reported concentrations ( $\mu\text{g/L}$ ) of iHg, MeHg and EtHg determined by our analytical method are  $\pm 0.16$ ,  $\pm 0.11$ , and  $\pm 0.13$  for LB QC;  $\pm 0.34$ ,  $\pm 0.34$ , and  $\pm 0.21$  for HB QC (**Table S1** in SI).

### Instrumentation and data processing

Our laboratory measured the mercury analytes with an ELAN<sup>®</sup> DRC<sup>™</sup> II ICP-MS (PerkinElmer Life Sciences, Shelton, CT, USA) operated in DRC mode (Argon gas, 0.3 mL/min) which enhanced the mercury signal intensity (presumably by collisional focusing) [9], (see **Table 1** for instrument settings and parameters). We used a PerkinElmer<sup>®</sup> Clarus 500<sup>™</sup> gas chromatograph (PerkinElmer Life Sciences, Shelton, CT, USA) equipped with a GC capillary column (Perkin Elmer<sup>®</sup> Elite-5 30m, 0.25mm ID, 0.25 $\mu\text{m}$  of 5% diphenyl, 95%

polydimethylsiloxane, Shelton, CT, USA). The GC was coupled to the quadrupole ICP-MS by a heated GC transfer line (Redshift®, Italy, purchased through PerkinElmer Life Sciences). The transfer line's capillary tubing was threaded into the ICP-MS injector, stopping approximately 0.5 - 1 cm from the tip of the injector. This arrangement took advantage of the simplicity of direct injection, thereby avoiding the aerosol-gas mixing dynamics of a spray chamber that could negatively affect peak resolution and signal strength. Alternatively, the lack of a spray chamber meant we could not analyze liquid standards without removing the transfer line. We optimized the GC parameters for optimum peak retention and resolution (**Table 1** and **Figures S2** in SI). A CombiPAL® robotic sample processing workstation (CTC Analytics, Zwingen, Switzerland) performed SPME extractions and injections into the GC [10] (**Table 1**).

ICP-MS intensity signals of the three mercury species (iHg, MeHg and EtHg) were measured at m/z 198, 199, 200, 201 and 202. The  $^{198}\text{Hg}/^{202}\text{Hg}$ ,  $^{199}\text{Hg}/^{202}\text{Hg}$ ,  $^{200}\text{Hg}/^{202}\text{Hg}$ , and  $^{201}\text{Hg}/^{202}\text{Hg}$  peak area ratios were calculated and exported into a Microsoft Excel spreadsheet originally developed by Ouerdane and colleagues [11-13] where a deconvolution algorithm mathematically corrects for mercury species interconversions. Final mercury species concentrations were calculated using these isotope dilution equations. (Isotopic impurities associated with isotopically enriched mercury standards, provided in AIT certificate of analysis, were taken into the account.) Internal mass bias correction was applied according to the Meija and coworkers technique [14].

Additionally for method validation experiments, we used one-way ANOVA statistical data analysis to compare different experimental conditions. Scenarios with significant p-values (<0.05) were tested further for pairwise comparisons [15].

## Results and discussion

The biomonitoring method described in this work quantifies iHg, MeHg and EtHg in whole blood using TSID-SPME-GC-ICP-DRC-MS. The sample preparation procedure involves addition of spike solution (isotopically labeled mercury species) to blood samples, followed by solubilization of blood with TMAH. Next, mercury species are derivatized with NaBPr<sub>4</sub> and the resulting volatile reaction products are sampled with a SPME fiber, followed by analysis using GC-ICP-MS (**Figure S1** in SI presents the scheme of analytical procedures).

### Sample preparation conditions

**TSID spike solution**—To calculate the ideal amount of spike to add to a sample, it is beneficial to know in advance the concentration of the analyte in question [6]. However, because of our need to develop a high throughput method and seeing the impracticability of prescreening a large number of samples for the purpose of calculating individual spike amounts for each sample, we pursued a different route. Specifically, we used one spike concentration that would work for most samples with mercury species present within a certain concentration range. Therefore, for each batch analysis we gravimetrically prepared a single spike solution having a precisely determined concentration of each isotope. We kept these concentrations as close as possible to 1 µg/L. This gave isotope ratios ( $^{199}\text{Hg}/^{202}\text{Hg}$ ,  $^{200}\text{Hg}/^{202}\text{Hg}$ , and  $^{201}\text{Hg}/^{202}\text{Hg}$ ) between one and three for HB QC (**Table S2**

in SI). Previous researchers have used spike:analyte ratios between one and three while keeping the uncertainty in their TSID measurements to a minimum [8,16]. We chose the spike:analyte ratio optimization for the HB QC sample because this sample's total mercury concentration is not far from 5.13  $\mu\text{g/L}$ , the 95<sup>th</sup> percentile of total blood mercury in the U.S. population [17].

To evaluate the effect of altering spike concentration, we analyzed LB QC and HB QC using spike solutions containing 0.5, 1.0, 2.5, 5.0, and 10.0  $\mu\text{g/L}$  of each isotopically enriched mercury species (**Table S2** in SI). **Figures 1a** and **b** show that for each spike level the measured concentrations fell inside the statistical control limits for each QC pool (**Table S1** in SI). For HB QC (**Figure 1a**), the measured mercury species concentrations remain constant throughout the entire 0.5–10.0  $\mu\text{g/L}$  spike range; there is more absolute variability for LB QC but the relative variability is not much different from HB QC. Additionally, as will be discussed further in the *dynamic analytical range* section, using this method, we can analyze higher mercury concentrations using the 1  $\mu\text{g/L}$  spike. Furthermore, in this experiment we calculated p-value to determine whether a statistically significant difference existed between the various spike concentrations that were tested. MeHg in the LB QC showed significance ( $p=0.04$ ), **Table S3** in SI. In the step-down tests, our new significance level was  $0.05/10=0.005$ . We found that for MeHg in the LB QC, the mean at the higher spike level (10  $\mu\text{g/L}$ ) was significantly lower than the mean at the lower spike level of 1  $\mu\text{g/L}$  (mean of 0.640  $\mu\text{g/L}$  vs. 0.733  $\mu\text{g/L}$ ,  $p=0.0042$ ). However, it is important to note that statistical significance does not necessarily signify scientific importance. Since both 0.640  $\mu\text{g/L}$  and 0.733  $\mu\text{g/L}$  are values that are well within our statistical control limits established for MeHg in our LB QC pool, we affirm our conclusion that there is no definitive evidence that one spike ratio is better than the other in the range of 0.5 to 10.0  $\mu\text{g/L}$  for our method.

**Solubilization**—Due to the amount of protein and fat in blood, it is often necessary to use a strong acid or base to release metallic species [16]. We used TMAH to solubilize protein and release bound mercury species. At room temperature, it took approximately 72 hours to solubilize blood before iHg, MeHg and EtHg could be quantified (EtHg to iHg conversion of  $63 \pm 9\%$ ,  $N=12$ ). To decrease the solubilization time, the blood samples were subjected to thermal treatment (“convection oven”) at 40°, 60°, 80°, 100° C for 24 hours. We found 80° C to be the optimum temperature providing highest mercury peak intensities (a detailed description can be found in the *Sample solubilization* section in SI). We evaluated the effect of oven time on the effectiveness of blood sample solubilization. LB and HB QC material were kept at 80° C for 4, 8, 16, 20, 24 and 26 hours. **Figures 1c** and **d** present the MeHg results for this experiment. At 4 hours, we were not able to measure mercury species in LB QC and HB QC. At 8 hours, some measurements were possible but peak intensities were low, thus adversely affecting the calculated concentrations of the mercury species, especially for LB QC. After 16 hours, mercury species in LB and HB QC samples could be reliably measured as evidenced by results that fell inside their statistical control limits (**Figures 1c**). After 20, 24 and 26 hours, the results were again inside the QC statistical control limits. As expected, the MeHg chromatographic peak area increased as a function of the solubilization time (**Figure 1d**). iHg and EtHg showed the same trend (data not shown). We selected a 24 to 26 hours oven time to obtain maximum peak intensities. We observed no clear increase in

standard deviation values for different solubilization times. There was no influence of solubilization time on the sample degradation with EtHg to iHg conversion of  $61 \pm 6$ ,  $60 \pm 5$ ,  $59 \pm 4$ , and  $58 \pm 2$  % at 16, 20, 24, and 26 hours, respectively. Please note these are “final” conversion percentages after all sample preparation steps and analysis (solubilization, derivatization, and injection of SPME into heated GC injector). During the solubilization step, EtHg to iHg conversion percent is approximately half of the final value. A detailed experimental description can be found in *Sample solubilization* section in SI. Furthermore, in this experiment, we used a p-value to evaluate the statistical significance between different solubilization times. EtHg in the LB QC showed significance ( $p=0.02$ ) (**Table S3** in SI). In the step-down tests, our new significance level was  $0.05/6=0.008$ . The mean at a solubilization time of 24 hours was significantly lower than the mean at 20 hours ( $0.522 \mu\text{g/L}$  vs.  $0.635 \mu\text{g/L}$ ,  $p=0.02$ ). Even in the presence of a statistically significant difference, it is important to note that both  $0.522 \mu\text{g/L}$  and  $0.635 \mu\text{g/L}$  are both within the EtHg statistical control limits established for LB QC (**Table S1** in SI). Therefore, we state that the difference in the EtHg results between 20 and 24 hours is unimportant.

**SPME sampling**—In our method, we selected headspace sampling to prolong the lifetime of the SPME fiber by minimizing its exposure to the sample matrix [18]. Parameters such as volatility of analytes, the concentration of derivatization reagent, pH of the solution, extraction time and temperature, and desorption conditions that govern efficiency of SPME extraction were optimized (**Figures S3** and **S4** in SI).

### Analytical characteristics of the method and stability studies

**Freeze-thaw stability**—In practice, it is occasionally necessary to repeat the analysis of some blood samples, which requires a second thawing and refreezing of the remaining sample. Some samples may undergo three or four freeze-thaw cycles. Therefore, we studied the stability of mercury species in blood after five freeze-thaw cycles. Groups of frozen LB QC and HB QC samples were thawed and refrozen up to five times (replicates of six) then analyzed for the mercury species concentration using our method. All samples after the 1st, 2nd, 3rd, 4th, and 5th freeze-thaw cycles provided mercury species concentrations that were within the statistical control limits established for each QC pool (**Figure S5** and **Table S1** in SI). Thus, we concluded that mercury species are stable in blood after the repeated freeze-thaw actions.

**Room temperature stability**—In the field where sample collections occur, it can be difficult to control the time lapse between the blood draw and the freezing of the sample. Because of this variability in time before samples get frozen, we investigated the stability of mercury species stored at room temperature. At various times equal to 0, 4, 8, 16, 20 and 24 hours prior to analysis, we thawed out LB QC and HB QC samples in groups of three which were kept at  $20\text{--}23 \text{ }^\circ\text{C}$  until all were analyzed for mercury species. Our measurements demonstrated that the aged samples produced mercury concentrations that were within our statistical control limits established for each QC pool at all time periods (**Figure S6** and **Table S1** in SI). The obtained result has confirmed that IHg, MeHg and EtHg species are stable in the blood matrix for up to 24 hours at room temperature.

**Stability following TMAH addition**—If an analytical run needs to be repeated due to a problem not associated with sample preparation, it is appropriate to re-prepare the samples beginning with saved TMAH solubilized extract. In addition to saving time, it avoids the problem of re-thawing the same samples. To answer the question of whether mercury species are stable in TMAH, we repeatedly tested batches of LB QC and HB QC solubilized in TMAH addition for four months. The most important factor affecting stability for these samples is mercury species transformations. During routine sample pretreatment and analysis, approximately 35-55% of EtHg transforms to iHg (calculated using the deconvolution spreadsheet). We stored TMAH solubilized LB QC and HB QC (for QC pool concentrations refer to **Table S4** in SI) at 20–23 °C and retested them periodically (in triplicate) for 134 days. **Figure 2a** displays the percentages of EtHg to iHg transformation as a function of time. The extent of EtHg to iHg transformation increased from 55% to 90% after four months. Interestingly, even at transformation up to 80%, LB QC and HB QC yielded concentrations within our statistical control limits for all three mercury species (**Figures 2b, c, and d**). When EtHg to iHg transformation exceeded 80%, results for EtHg and iHg started to fall outside their statistical control limits. At transformations exceeding 90%, it became difficult to detect EtHg peaks in the chromatograms, making the calculation of the other mercury species in the same chromatogram impossible. **Figure 2d** shows that there is little change in the concentration distribution of MeHg as EtHg transforms into iHg. To conclude, the blood solubilized in TMAH is sufficiently stable to be reanalyzed for up to two months when stored at 20–23 °C. However, we have to take into the account that as the conversion rate reaches 80%, more error is seen with the analytical results obtained [19,7].

**Final extract stability**—As a part of our robustness testing, we wanted to determine if repeated SPME fiber extractions made on the same SPME vial would give consistent results. The CombiPal® autosampler was programmed to perform five consecutive fiber extraction/GC injection cycles on the same SPME vial at ~20 min intervals. This was done using four samples: LB QC, HB QC, LB QC diluted ten times with base blood (10X, concentration LOD for the method) and NIST SRM 955c Level 3. As expected, we observed a large incremental decrease in all peak areas following each re-extraction cycle (see **Figure S7** and *sample recovery section* in SI). Nonetheless, the concentrations measured for the first extraction/GC injection cycle could be reproduced by each subsequent cycle for each sample. Further, it is important to know how stable are the mercury species in 20 mL SPME vials once they are propylated, in case of a prolonged delay before the instrument is started. To address this, we prepared thirty samples of LB QC and HB QC for analysis through all steps including derivatization. The first ten samples were immediately analyzed while analysis of the remaining samples was delayed for 24 and 48 hours. The concentrations resulting from 24 and 48 hour delayed analysis fell outside the established QC limits (a finding confirmed by additional experimentation). Thus, we concluded that a steady loss of derivatized mercury species occurred. Therefore, it is important to avoid a prolonged delay of sample analysis once they have been derivatized.

**Spike solution stability**—Avoiding daily preparations of the spike solution can improve our method's efficiency. Therefore, we tested the stability of the spike solution over the period of 29 days by observing changes in LB and HB QC concentrations prepared using the

same spike solution (**Figure S8** in SI, four spike solutions were tested). LB and HB QC samples (four of each) were prepared with the same spike solution on days 1, 8, 15, 22, and 29. All four spike solutions produced QC sample results for each species that were within our statistical quality control limits (**Figure S8, Table S1** in SI). Thus, we concluded that it is safe to use a single preparation of spike solution for up to one month.

Additionally for freeze-thaw cycles, room temperature stability, and spike solution stability studies certain differences between the experimental conditions were statistically significant (**Table S3** in SI). However, they were small and did not result in concentrations that exceeded their respective statistical control limits (detailed description can be found in SI).

**Dynamic analytical range**—Fundamental to the method performance is the linearity of signal response (i.e., dynamic range). In this method's typical measurement protocol, we use pooled whole human blood (base blood) to dilute and reanalyze samples with individual mercury species concentrations > 10 µg/L. (This originates from the protocol of the CDC method used to measure total mercury.) Base blood has been predetermined to have no or minimal amounts of mercury species present. We wanted to establish whether or not there is a statistically significant difference between diluted and non-diluted elevated samples if the spike concentration is kept the same for all samples at 1 µg/L. Thus, we performed experiments analyzing CTQ PT samples with concentrations ranging from 10 – 90 µg/L in non-diluted and diluted (bringing concentration below 10 µg/L) forms (**Figure 3a** and **Table S5** in SI). The results for diluted samples compared well to the non-diluted ones with a slope of 0.97 and  $r^2=0.985$  (**Figure 3a**). A Bland–Altman (B-A) difference plot comparing these two conditions displayed no clear bias (**Figure S9a** in SI). Furthermore, in this experiment we used a p-value to evaluate if there is a statistically significant difference between the diluted and non-diluted samples. A p-value of 0.4, (> 0.05) indicated that there is no statistically significant difference between the diluted and non-diluted samples. We compared the within-condition variability by calculating the coefficient of variation (CV %) for each condition (diluted vs. non-diluted) to be 8.27 vs. 4.04, respectively. Variability of results were higher (approximately double the value) under the dilute conditions. As of now, our laboratory is working on collecting enough patient data to show that in a selected concentration range, there is no need for diluting elevated samples with base blood prior to analysis.

**Analytical figures of merit**—The LODs for iHg, MeHg and EtHg were calculated to be 0.27, 0.12, 0.16 µg/L, respectively. These limits are at least three times lower than the U.S. population average for total mercury blood concentrations [17] and are comparable to other published mercury speciation methods [20,21]. We evaluated short-term within-run analytical precision for this method by replicate analyses (n=8) of LB and HB QC samples for iHg, MeHg and EtHg species. The average RSD was approximately 3% (**Figure S10** in SI). Uncertainty associated with the sample preparation procedure (weighing and pipetting) sum up to approximately 1 % RSD. More significant uncertainty contributions include instrumental drift, SPME sample introduction, and error associated with peak integration.

We verified the accuracy of the proposed methodology by analyzing whole blood NIST SRM 955c Level 3 and CTQ PT samples (**Table 2** and **Figure 3c**, respectively). The

obtained NIST concentration values are the average of N=60 independent replicates collected over ten months on two separate instruments by two different analysts. The empirical expanded uncertainties, U (k=2), for reported concentrations ( $\mu\text{g/L}$ ) of iHg, MeHg and EtHg for NIST determined by our analytical method are  $\pm 1.6$ ,  $\pm 0.6$ , and  $\pm 0.6$  respectively. NIST results for mercury species produced by this method fall within the allowable certified limits. We obtained RSD percentages of 8.3, 6.2, and 6.3 for iHg, EtHg and MeHg respectively (**Table 2**). It is important to note that these RSD values are without application of mass bias correction, **Table 2**. Additionally, we analyzed twenty-eight CTQ PT samples (refer to **Table S5** in SI for the exact concentrations). These samples only contained iHg and MeHg. The results analyzed by our method compared very well to certified target values with a slope of 1.05 and  $r^2=0.986$  (all falling within certified ranges). B–A difference plot comparing certified CTQ values to the results from this method displayed no clear bias, **Figure S9b** in SI. The calculated p-value of 1 indicated that there was no statistically significant difference between certified values and values produced by this method.

Our choices of mass bias correction techniques were limited by the design of our GC-ICP-MS interface. Instruments have been described that use a specially designed transfer line–torch interface that incorporate an off-axis liquid spray chamber allowing liquid standards to be freely analyzed without removing the transfer line. Because we did not have this unique GC-ICP-MS interface, we could not analyze liquid external standards for mass bias correction unless we removed the transfer line and installed a spray chamber in its place. If performed in this manner, mass bias measurements using wet plasma can only be cautiously extrapolated to make estimates of the mass bias affecting chromatographically separated isotopes measured under dry conditions. Therefore, we alternatively employed an internal mass bias correction calculation (a form of the Russell exponential function) which depends merely on measuring the departure of the sample's own isotope intensity pattern (not ratios) from the expected (pure component) pattern for each chromatographic peak [22]. Excel Solver® was used to iteratively find the mass bias correction coefficient K that gives the aggregate minimum of the sum squared error terms calculated from a multiple regression fit of the observed peak isotope patterns. Internal mass bias determinations are, in principle, free from errors coming from instrument instability and other temporally related causes of imprecision.

After finalizing the instrument parameters and optimization procedure, we monitored K while analyzing numerous blood samples including internal QC, NIST 955c Level 3, and other samples over the course of several months. What we found is, with only a few exceptions, K did not significantly differ from zero. Using NIST 955c Level 3 as an example, after 55 independent single sample measurements (i.e., each done on a separate day), the one-sample T-Test mean (confidence intervals,  $\alpha=0.05$ ) for K was  $-0.0009$  ( $-0.0027$ ,  $0.0009$ ) indicating that K was not insignificantly different from zero. Even when the differences in final resulting concentrations between mass bias corrected and uncorrected results were statistically compared using paired T-Test ( $\alpha=0.05$ ,  $n=55$ ), no significant differences were found ( $p=0.21$ ,  $0.12$  and  $0.23$  for iHg, MeHg and EtHg, respectively). On the other hand, correcting for mass bias gave slightly larger run-to-run variation (corrected

v. uncorrected RSD): iHg, 9.6% v. 8.2%; MeHg, 7.2% v. 6.3%; EtHg, 8.2% v. 6.8%. We realized that there would be no practical benefit by routinely correcting for mass bias for the following reasons: 1) the change in concentration resulting from mass bias correction was <1% compared to the overall run-to-run variability and 2) applying mass bias correction caused run-to-run variability to noticeably increase. Therefore, we decided to stop making corrections for mass bias and instead monitor K as a quality assurance indicator.

## Conclusions

Our laboratory developed a rapid, accurate and precise biomonitoring method for the quantification of iHg, MeHg and EtHg in whole blood using TSID-SPME-GC-ICP-DR-MS. We proved that optimized headspace extraction by a dual arm SPME setup is a fast and sensitive approach in providing capability for high sample throughput. Additionally, the TSID technique clearly demonstrated its superior capability in overcoming precision shortcomings historically associated with the SPME sampling technique. The method's within-run average analytical precision (RSD %) for all three mercury species in LB and HB QC samples analyzed after eight replicates was approximately 3%. We achieved interference free GC separation of the three mercury analytes with excellent selectivity. With the optimized sample preparation, SPME sampling, GC-separation, and ICP-MS detection we determined the limits of detection for iHg, MeHg and EtHg species in human blood matrix to be 0.27, 0.12, 0.16 µg/L, respectively. We validated the accuracy of the method through the analysis of certified reference materials and proficiency testing samples. An accurate and high throughput mercury speciation method is a necessary tool for providing valuable information about the source of mercury exposure in humans. Future work with this method will include reducing the solubilization time to make the method better suited for high sample throughput and faster response times.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

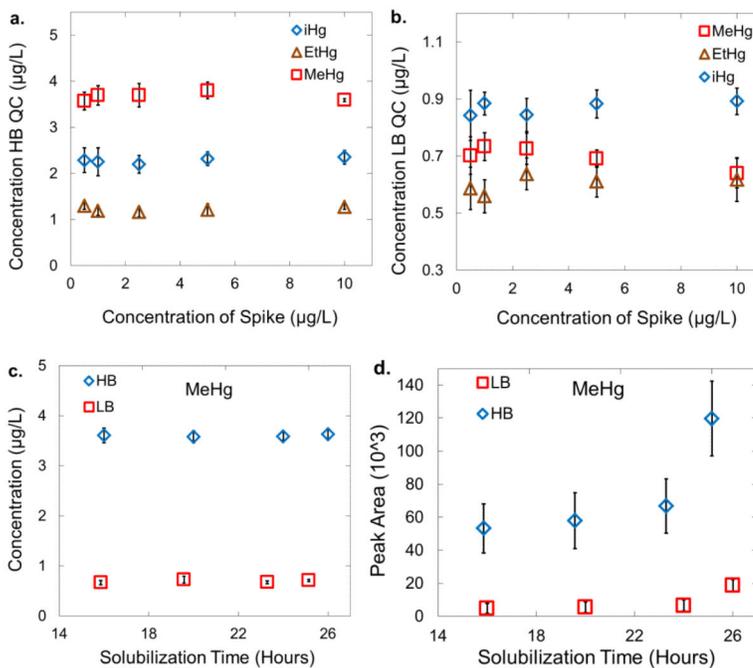
## Acknowledgements

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention and the U.S. Department of Health and Human Services. The authors are thankful to Joshua Godshaw (ORISE Fellow) for helping with sample analysis and data reprocessing.

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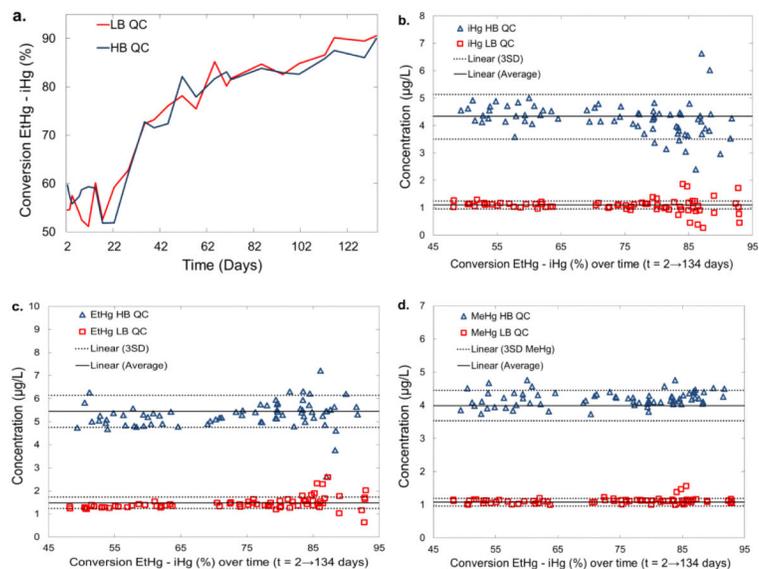
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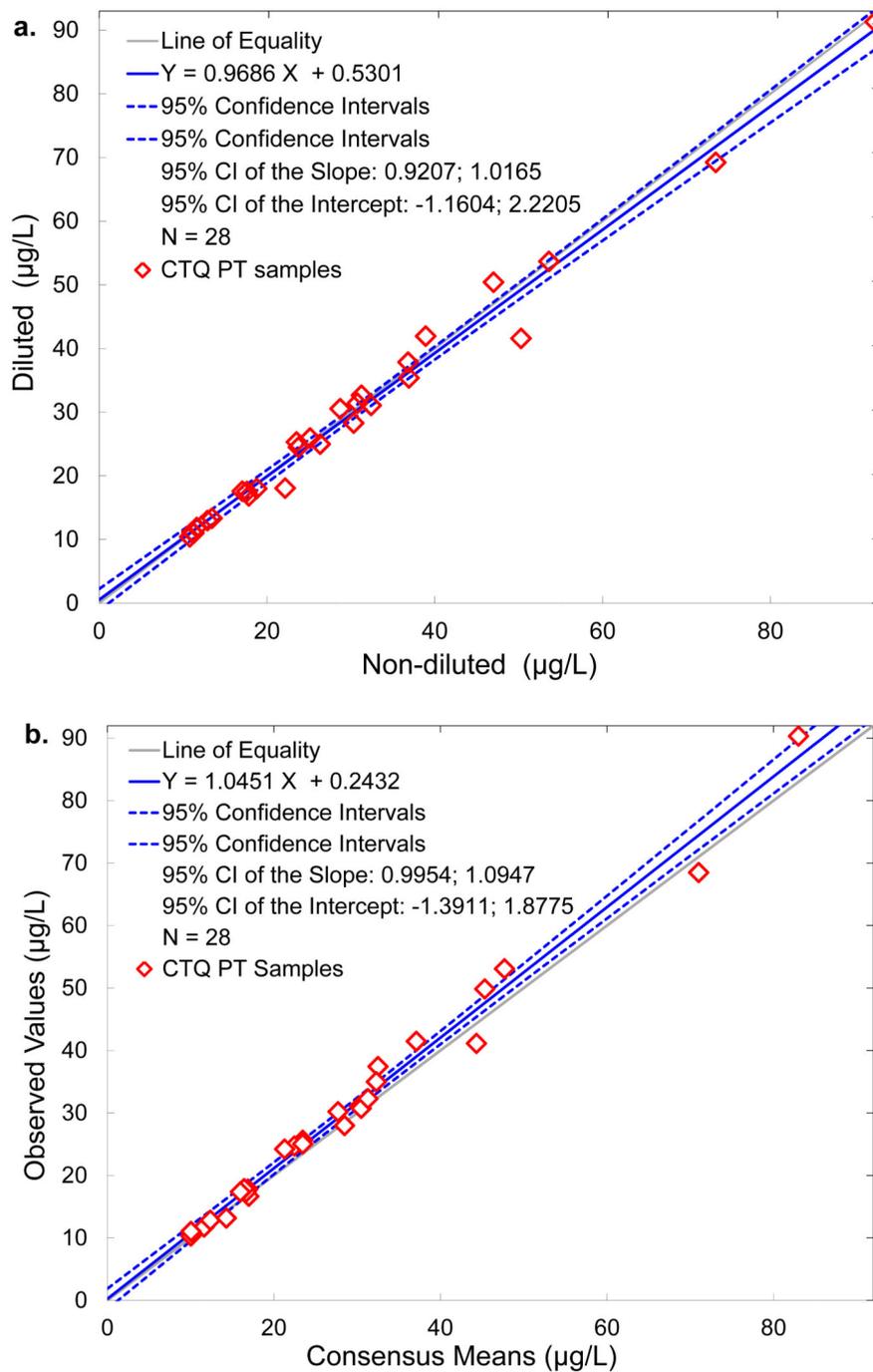


**Figure 1.**

Concentration changes of iHg, MeHg and EtHg in (a) HB QC and (b) LB QC samples prepared with spike solutions containing different concentrations of isotopically enriched mercury standards (0.5, 1.0, 2.5, 5.0, and 10.0  $\mu\text{g/L}$ ). The results shown in the graphs are the averages of all replicates (N=6) at each of the different spike concentrations. (c) Concentration of MeHg in HB and LB QC material as a function of sample solubilization times (4, 8, 16, 20, 24 and 26 hours). (d) Changes in MeHg peak area as a function of the sample solubilization times. The results shown above are the averages of the replicates (N=6) from the different solubilization time intervals. The 95<sup>th</sup> percentile confidence intervals are shown as error bars.



**Figure 2.** Blood solubilization stability study. (a) Monitoring EtHg to iHg species interconversions percentages as a function of time. (b), (c), and (d) Present iHg, MeHg and EtHg concentrations, respectively, in HB and LB QC material as species conversion percentages (EtHg to iHg) increase over time ( $t = 2 \rightarrow 134$  days). The solid black line represents established QC concentrations while the dotted line represents the QC limits. (A different QC pool is used for this experiment; see **Table S4** in SI for exact concentrations. This pool was used prior to the characterization of a new QC pool, **Table S1** in SI.)



**Figure 3.** (a) Comparison of dilute and non-diluted CTQ PT samples (slope=0.97 and  $r^2=0.985$ , p-value of 0.4), (b) comparison of observed values vs. consensus means for CTQ PT samples (slope=1.05 and  $r^2=0.986$ , p-value of 1).

**Table 1**

Optimal operating conditions for mercury speciation method.

ICP-DRC-MS	
Carrier Ar gas flow	1.5 L/min
Auxiliary Ar gas flow	1.2 L/min*
Plasma Ar gas flow	15 L/min*
Lens voltage	4.75 V*
RF Power	1450 W*
DRC Mode QRO	- 10 V*
DRC Mode CRO	- 1V*
DRC Mode CPV	- 55 V*
Cell Gas A	Ar at 0.3 mL/min*
DRC rejection parameter q (RPq)	0.3*
Scanning mode	Peak hopping
Dwell time	25 ms
Sweeps per reading	1
Readings per replicate	2725
Number of replicates	1
Dead time	60 ns
Isotopes monitored	198, 199, 200, 201, 202
Measurement time per sample	20 min
GC	
GC injector temperature	220°C (1 min), max ramp to 280°C, hold
Carrier gas / flow rate	He / 2 mL/min
Oven program	75°C (1 min) to 250°C (2 min) (at rate = 45 °C/min)
Transfer line temperature	250°C
Injection mode	splitless
Autosampler	
Needle penetration	35.0 mm
Extraction temperature	Room temperature (approximately 25.7°C)
SPME	
Fiber coating	100 µm PDMS
Sampling mode	Head space
Enrichment Time	1200 s
Desorption Time	420 s
GC Cooling Down	180 s

\* Suggested starting values only. Optimum parameters will depend on outcome of the optimization procedure and the instrument being used.

**Table 2**

Certified mean mercury concentration values ( $\mu\text{g/L}$ ) provided by the National Institute of Standards and Technology (NIST) and mean mercury concentration values ( $\mu\text{g/L}$ ) produced by our method at CDC for reference material NIST SRM 955c Level 3 (Caprine blood). The obtained concentration values are the average of  $N=60$  independent replicates collected over ten months on two separate instruments by two different analysts.

NIST SRM 955c Level 3	EtHg	iHg	MeHg	Total Hg
	$\mu\text{g/L}$			
CDC Mean	5.0	9.1	5.0	19.1
Standard Deviation	0.3	0.8	0.3	0.9
Relative Standard Deviation (%)	6.2	8.3	6.3	4.6
NIST Certified Mean	5.1	9.0	4.5	17.8
Expanded Error (k=2)	0.5	1.3	1.0	1.6

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