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RESEARCH

Human hair certified reference material for total mercury, methylmercury, and trace element analyses

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Abstract

The National Institute for Environmental Studies (NIES) developed the NIES CRM No. 13-a, a new certified reference material for human hair, using scalp hair from Asian females. This CRM represents a significant advancement in support of global mercury exposure assessments and offers unparalleled reliability and scope compared with existing materials. We aimed to provide a comprehensive overview of the preparation, certification, and application of NIES CRM No. 13-a. In total, 806 bottles (3 g each) were produced, with thorough homogenization ensured through sieving and blending. Certified values for total mercury (1.06 ± 0.07 mg/kg), methylmercury (0.858 ± 0.075 mg/kg), and key trace elements (arsenic, cadmium, lead, selenium, and zinc) were determined through extensive collaborative analyses involving 20 laboratories. Additional reference values were provided for calcium, magnesium, sodium, sulfur, antimony, barium, copper, iron, and manganese. Rigorous stability and homogeneity assessments demonstrated the stability of the CRM for over 10 years and consistency across sample units, even for challenging elements such as selenium. The CRM also includes information values of stable mercury isotope ratios, reflecting their growing importance as exposure tracers. This enhancement in accuracy and traceability facilitates accurate mercury and trace element assessments in human hair, enabling improved biomonitoring of mercury exposure, dietary studies, toxicological evaluations, human health risk evaluations, and regulatory compliance.

Keywords Human hair · Certified reference material · Methylmercury · Total mercury · Quality assurance/quality control · Isotope

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Introduction

The release of mercury (Hg) into the environment has become a pressing issue in the public health and environmental safety field because of its extensive presence in air, land, and water systems. In aquatic ecosystems, inorganic Hg is converted to the highly toxic methyl Hg (MeHg) through microbiological processes, leading to bioaccumulation within fish, particularly in the relatively large predatory species commonly consumed by humans. Consequently, human exposure to Hg, primarily through the consumption of contaminated fish and seafood, poses significant health risks. Monitoring Hg levels in human hair is a valuable and noninvasive method for assessing individual exposure. Hair provides a stable, long-term record of organomercury concentrations and dietary intake, making it particularly useful for large-scale population studies [1–3]. Unlike other biomarkers, once MeHg is incorporated into the hair, it remains fixed and does not return to the bloodstream, ensuring its reliability as a noninvasive biomarker for the long-term monitoring of Hg exposure. Because fish is the primary dietary source of MeHg in human hair, accurate determination of MeHg levels is critical for assessing dietary exposure. However, total Hg (THg) measurements often include atmospheric Hg surface contamination. The increasing global demand for reliable data on Hg exposure highlights the critical need for readily available and well-characterized certified reference materials (CRMs) for THg and MeHg analyses.

These reference materials are essential for maintaining the precision and veracity of Hg speciation analyses, particularly considering the various sources and exposure routes linked to each form of Hg. Over the years, several institutions, including the International Atomic Energy Agency (IAEA), the National Institute for Minamata Disease (NIMD), and the European Reference Materials (ERM), have produced CRMs for human hair that are certified for Hg species. In addition, the United States Geological Survey (USGS) distributes reference materials to which stable isotope ratios are assigned. Precise and reliable Hg speciation analyses necessitate high-quality CRMs. Various institutions provide CRMs for human hair; however, the newly developed NIES CRM No. 13-a offers significant advantages. It features a substantially broader range of certified and reference values, including key trace elements essential for comprehensive toxicological assessment (e.g., antimony, barium, and manganese), and uniquely incorporates information values of stable Hg isotope ratios. This enhanced scope and accuracy improve the reliability of Hg exposure assessments. Table 1 presents the composition of NIES CRM No. 13-a and other RMs. The National Institute for Environmental Studies (NIES) has played a leading role in the development of CRM by establishing key milestones in the field. Since the development of the first Japanese environmental CRM (Pepperbush, NIES CRM No.1) in 1979, NIES has consistently advanced CRM technology, notably through the development of human hair CRMs since 1985 (NIES CRM No. 5) [4, 5]. A previous human hair CRM (NIES CRM No. 13) represents significant advancement, offering certified values for MeHg and THg, reference values

Table 1 Standard human hair materials and their assigned items

Reference material	NIES CRM No. 13-a	NIMD-01	IAEA-085	IAEA-086	ERM-DB001	USGS 42	USGS 43
Year of manufacture	2024	2022	2000	2000	2013	2019	2019
Raw material of scalp hair	East-Asian females	Vietnamese males	European (addition of MeHg)	European	Belgium	Tibetan	Indian
Certified value	THg, MeHg, As, Cd, Pb, Se, Zn	THg, MeHg, Cu, Zn, Se			As, Cd, Cu, Hg, Pb, Se, Zn		
Reference value	Ca, Mg, Na, S, Sb, Ba, Cu, Fe, Mn	As	THg, Fe, Zn, MeHg	THg, Fe, Zn, MeHg			
Informative value	Hg isotope		Ca, Cu, Mg, Mn, Sc, Se	Ca, Cu, Mg, Mn, Sc, Se	34 trace elements, and MeHg	δ^2H, $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$	δ^2H, $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$
THg (mg/kg)	1.06 ± 0.07	0.794 ± 0.050	23.2 (21.9–23.9)	0.573 (0.534–0.612)	0.365 ± 0.028		
MeHg (mg/kg)	0.858 ± 0.075	0.634 ± 0.071	22.9 (22.4–24.0)	0.258 (0.236–0.279)	0.240		
Reference	This study	[14]	[15]	[15]	[16]	[17]	[17]

for important trace elements, and information values for stable Hg isotope ratios [6], thereby significantly improving the accuracy of Hg exposure assessments.

The primary objective of this study is to provide a comprehensive overview of the preparation, certification, and application of NIES CRM No. 13-a, a new certified reference material for human hair that significantly expands the scope of Hg exposure assessments. This CRM uniquely incorporates certified stable Hg isotope ratios and certified values for an expanded suite of trace elements, significantly enhancing its versatility. This essential quality control tool for analyzing Hg speciation in human hair further extends its utility by including reference values for various toxicologically significant elements, as well as informational values for the stable Hg isotope ratio, which are valuable for investigating exposure sources. No other currently available human hair CRM offers a comprehensive set of certified values. By providing this extensive dataset, NIES CRM No. 13-a enhances its applicability to a wide range of studies, from Hg speciation and trace element analysis to broader inquiries in the environmental and health sciences.

Materials and methods

Sample preparation

A new CRM using Asian female scalp hair was developed for large-scale processing to ensure optimal homogenization. To secure sufficient raw material while minimizing variability, long hair from female donors was selected because collecting the necessary amount from males was difficult, as they generally have shorter hair. Hair from individuals with similar racial and environmental backgrounds was used; however, no specific selection criteria were applied based on Hg exposure risks or dietary habits. Additionally, only the hair that had not been dyed or subjected to chemical treatment was selected to reduce potential variability. Although the commutability of this CRM to male hair was not specifically assessed, no significant matrix differences are expected between male and female hair. Given that mercury exposure-related studies commonly include both sexes and various ethnic groups, users are encouraged to select the most suitable CRM based on their analytical purpose and target population. In this phase, 5 kg of hair was first washed by hand in a 0.3% neutral detergent (Contaminon N, Wako Pure Chemical Industries, Osaka, Japan). The detergent was completely removed by rinsing with distilled water. The clean hair was dried in an oven at 50 °C overnight. Then, 4.6 kg of pre-ground hair was obtained using a roll crusher (RP-150, Seishin Co. Ltd., Fukuoka, Japan) and pin mill (PIN MILL-4, Seishin Co. Ltd., Fukuoka, Japan). This initial step was crucial for breaking down hair fibers, allowing for more effective subsequent grinding and ensuring a uniform sample.

After the pre-grinding process, 4 kg of the resulting hair powder was sieved through a 500-µm mesh screen to eliminate larger particles, facilitating more even refinement. The screened material was then finely milled using a cryogenic air jet mill (TURBO Mill, Toho Reinetsu Co. Ltd., Aichi, Japan) that employed a classifying rotor to achieve the desired particle size. This final milling step resulted in 2.5 kg of hair powder with a particle size of less than 74 µm, a critical specification for maintaining the integrity and homogeneity required for the CRM.

Once the hair powder was finely milled, it was collected in a stainless-steel barrel and subjected to thorough continuous homogenization using a Rocking Mixer (TMHS-100S, Seiwa Giken Co. Ltd., Osaka, Japan) for 24-h. This extended blending ensured that the particle size distribution was consistent throughout the sample.

Following homogenization, the prepared hair powder was portioned into 806 pre-cleaned borosilicate bottles, each containing 3 g of the sample. To ensure the stability and integrity of the CRM, the bottles were sterilized via ^{60}Co-irradiation (20 kGy). Finally, the samples were stored at 5 °C in a dark environment to preserve their quality until they were needed for further analysis.

Because the certified and reference values were expressed on a dry weight basis, the moisture content was measured to correct the analytical values. For this, 0.2 g aliquots of the powdered material were placed in capped weighing bottles and dried for 4 h at 85 °C [7, 8]. After drying, the bottles were transferred to a silica-gel desiccator and cooled for 30 min. The percentage of weight loss was calculated as the moisture content, which was determined to be approximately 8%.

Instrumental analyses

This section outlines the analytical methods employed at the NIES to measure THg, trace elements, and Hg isotope ratios. The MeHg analyses were conducted by IDEA Consultants, Inc.

Of the 806 bottles, one was randomly selected for the minimum sample test to determine the smallest sample amount required for sufficient analytical precision, ensuring that even small sample sizes provided reliable and accurate measurements. The homogeneity and short- and long-term stability tests were conducted based on the minimum analytical use of the samples determined for this analytical method. The accuracy and precision of the analytical methods were verified by analyzing NIMD-01, IAEA-086, and NIES CRM No. 13 reference materials with known values, using the same procedures as those used for the samples. A comparison of our results with certified and reference values

demonstrated good agreement, confirming the reliability of the measurements.

THg analysis

THg was determined via cold vapor atomic absorption spectrometry (CVAAS) using an MA-3000 analyzer (Nippon Instruments Corp., Osaka, Japan), a widely established technique for Hg analysis in environmental samples, including biological matrices. This method employs a direct thermal decomposition-gold amalgamation technique to efficiently trap and quantify Hg vapor released from the sample. The collected Hg is released by heating the Hg collection tube, thus converting it back into Hg gas. The absorbance of Hg gas was measured using CVAAS at a wavelength of 253.7 nm, allowing for the precise quantification of THg content.

Nine aliquots were analyzed per bottle, with three replicates each for sample weights of 10, 20, and 50 mg. This range of sample weights was tested to determine the minimum sample amount required to achieve sufficient analytical precision and ensure reliable and accurate THg measurements even with smaller sample sizes. The Hg analyzer was calibrated using dilute solutions ranging between 0 and 50 ng that were prepared with L-cysteine (guaranteed reagent from NACALAI TESQUE, INC., Kyoto, Japan), a mercury standard solution (1000 mg/L, Kanto Chemical Co., INC., Tokyo, Japan), and pure water obtained from the Milli-Q water system (Japan Millipore Ltd., Tokyo, Japan). The powdered samples were combusted to 850 °C. During combustion, oxygen gas was flowed at a rate of 0.4 L/min to carry the released gases through a catalyst filter to the amalgamator, where Hg was collected. After the combustion was complete, the amalgamator was heated to over 600 °C within 30 s to release the Hg, which was then carried to the detection cells with an oxygen flow of 0.2 L/min. Both the standard and samples were prepared in the same manner, with no differences in sample preparation procedures. Moreover, this study did not use additives, such as oxidation promoters or reducing agents. The validity of the method was confirmed by measuring a certified reference material with a known total Hg concentration and ensuring that the obtained results were consistent with the certified or reference values.

A comprehensive approach was used to minimize contamination risks throughout the analysis. This included pre- and post-analysis heating of the instrument, along with the routine inclusion of method blanks and control samples in each analytical run to monitor and mitigate contamination.

MeHg analysis

MeHg was quantified by gas chromatography using an electron capture detection system (GC-ECD) (G2700, Yanaco Analytical Systems Inc., Kyoto, Japan). As described by Akagi and Nishimura [9], this method is a well-established and widely accepted technique for MeHg analysis aligned with the analytical manual of Ministry of the Environment, Japan (https://www.env.go.jp/chemi/report/h15-04/). Information about the reagents is as follows: toluene and ethanol (pesticide and PCB analysis grade, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), hydrochloric acid (toxic metal analysis grade, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), sodium hydroxide (special grade reagent, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), L-cysteine hydrochloride (toxic metal analysis grade, Kanto Chemical Co., Inc., Tokyo, Japan), and methylmercury chloride (special grade reagent, Kanto Chemical Co., Inc., Tokyo, Japan). GC-ECD offers high sensitivity and selectivity for MeHg, thereby minimizing interference from other compounds often present in complex biological samples. A hair sample (approximately 10 mg) was carefully weighed and transferred to a 10-mL screw-lid round-bottom centrifuge tube. To facilitate infiltration, two drops of ethanol were added to the sample, followed by the insertion of a small amount of glass or cotton wool using a glass rod that was lightly pressed to secure the sample. Subsequently, 3 mL of 2 M HCl was added gently onto the cotton to avoid disturbing the sample and heated in a thermostatic bath at 100 °C for 5 min to elute MeHg from the hair. After heating, the mixture was allowed to cool before being inverted and mixed thoroughly. The sample was then centrifuged at $260 \times g$ for 3 min using Hitachi Himac CT6D (Eppendorf Himac Technologies Co., Ltd., Ibaraki, Japan). Following centrifugation, 1 mL of the supernatant was transferred to a 10 mL conical centrifuge tube equipped with a co-stopper. Subsequently, 2 mL of toluene was added and the mixture was shaken for 3 min to extract MeHg from the HCl phase into the toluene phase. After shaking, the sample was centrifuged at $260 \times g$ for an additional 3 min, and the lower phase was carefully aspirated to obtain the test solution. For the preparation of calibration standards, 0 and 0.10 mL of a MeHg/cysteine solution (equivalent to 100 ng of Hg) were separately added to two 10 mL screw-lid centrifuge tubes. To each tube, 2 M HCl was added to achieve a final volume of 3 mL. The prepared solutions were then processed using the same method as the test solution to create both an empty test solution and a MeHg standard test solution.

Nine aliquots were analyzed from each bottle, with three aliquots for each weight category (weighing approximately 10, 20, and 50 mg). This range of sample weights was tested to determine the minimum sample amount required to achieve sufficient analytical precision and ensure reliable and accurate MeHg measurements, even with smaller sample sizes.

Trace element analysis

The analysis of toxic and essential trace elements, including arsenic (As), cadmium (Cd), lead (Pb), selenium (Se), zinc (Zn), and others, in NIES CRM No. 13-a was conducted using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 8800, Agilent Technologies Inc., Santa Clara, CA, USA) or inductively coupled plasma optical emission spectrometry (ICP-OES; ICPE-9820, Shimadzu Corp., Kyoto, Japan), depending on the element of interest. To prepare the samples for analysis, an acid digestion procedure was performed to break down the hair matrix and release the elements into solution.

For acid digestion, 3 mL of nitric acid (HNO_3) was added to the sample powder and allowed to sit overnight. Subsequently, 1 mL of perchloric acid ($HClO_4$) was added and the mixture was heated at 180 °C for 5 h on a hotplate. Following this step, an additional 3 mL of HNO_3 and 1 mL of $HClO_4$ were introduced, and the solution was further heated at 180 °C for 1 h, followed by 4 h at 200 °C. Finally, 0.3 mL of hydrofluoric acid (HF) was added, and the mixture was heated at 220 °C for 1 h, after which it was dried at the same temperature. All acids used in this study were Ultrapure-100 and purchased from Kanto Chemicals, Ltd. (Tokyo, Japan). The pure water used was prepared with a Milli-Q system (Japan Millipore Ltd. Tokyo, Japan). This process ensured the complete dissolution of the hair samples, allowing for accurate quantification of the target elements. The digested solutions were then analyzed via ICP-MS or ICP-OES to measure the trace element concentrations.

Nine aliquots were analyzed, with three aliquots taken from each weight category (weighing approximately 20, 50, and 100 mg). This range of sample weights was tested to determine the minimum amount required to achieve sufficient analytical precision and ensure that accurate and reliable measurements could be obtained, even with smaller sample sizes.

Stable isotope information values: Hg isotope analysis

Standard reference materials NIST SRM 3133 (mercury standard solution) and NIST SRM 997 (thallium isotopic standard) were purchased from the National Institute of Standards (NIST, Gaithersburg, MD, USA). Tin chloride dihydrate ($SnCl_2 \bullet 2H_2O$) used for Hg isotopic measurements was purchased from Kanto Chemicals (Tokyo, Japan). A multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS; Nu Plasma II, Nu Instruments Ltd, Wrexham, UK) was interfaced with an Aridus II desolvating nebulizer for Tl introduction and an HGX-200 cold vapor (CV) system (both from Teledyne CETAC Technologies, Omaha, NE, USA) for Hg^0 generation. Hg^0 and Tl dry aerosols (introduced Tl concentration = 50 µg/L) were mixed at the outlet of the CV generation system before being introduced into the plasma. Further measurement protocols are described by Yamakawa et al. [6]. Hg isotopic composition is determined by the measurement of isotope ratios that are generally reported as δ values. δ values represent deviations in an isotope ratio in parts per thousand (denoted as ‰) from that of a standard, NIST SRM 3133. All sample analyses were bracketed by the analysis of an Hg isotopic standard solution (NIST SRM 3133). Calibration against the standard ensured the accuracy of the isotope ratio measurements, with deviations consistently below 0.2‰. The Hg isotopic ratios were calculated relative to the mean of the bracketing standards using the following equation [10]:

$$\delta^{***}\mathrm{Hg}\,() = \left[\left(^{***}\mathrm{Hg}/^{198}\mathrm{Hg}\right)_{\mathrm{sample}}/\left(^{***}\mathrm{Hg}/^{198}\mathrm{Hg}\right)_{\mathrm{NIST\ SRM\ 3133}} - 1\right] \times 1000$$

where *** represents one of five other possible isotopic mass numbers for Hg (199, 200, 201, 202, and 204). In this study, the mass independent fractionation (MIF) factor is reported using the capital delta notation (Δ) as the difference between the measured δ^{***}Hg and the same parameter's theoretically predicted value using the following relationship:

$$\Delta^{***}\mathrm{Hg}\ (‰) = \delta^{***}\mathrm{Hg} - \left(\beta \times \delta^{202}\mathrm{Hg}\right)$$

where β represents the equilibrium mass dependent fractionation (MDF) factor, which is equal to 0.252, 0.502, 0.752, and 1.493 for ^{199}Hg, ^{200}Hg, ^{201}Hg, and ^{204}Hg, respectively [10].

Homogeneity testing

A homogeneity test was performed for the candidate CRM following ISO Guide 35. Stratified random sampling was employed as the sampling method for the homogeneity test. According to ISO Guide 35, 806 bottles were divided into 12 groups (67 bottles/group), and one bottle from each group was randomly selected. Twenty-four aliquots (two aliquots from each bottle) were analyzed for MeHg, THg, and trace elements. Analysis of variance (ANOVA) was applied to the measurement results to statistically evaluate the between-bottle and within-bottle variations. The obtained mean squares for between-bottle (MS_{among}) and within-bottle (MS_{within}) variances were used to calculate the between-bottle standard deviation (s_{bb}) and analytical repeatability-based estimate of between-bottle variation (u_{bb}) using the following equations:

$$s_{\mathrm{bb}} = \left(\left(MS_{\mathrm{among}} - MS_{\mathrm{within}}\right)/n\right)^{0.5}, \quad (1)$$

$$u_{bb} = \left(MS_{\mathrm{within}}/n\right)^{0.5} x \left(2/v_{MS\mathrm{within}}\right)^{0.25}, \quad (2)$$

where n is the number of aliquots and $v_{MS\text{within}}$ is the degrees of freedom of MS_{within}. Following the recommendation of ISO Guide 35, the larger of s_{bb} and u_{bb} was used as the standard uncertainty contribution from potential inhomogeneity (u_{hom}). This approach ensures that the homogeneity contribution to the overall uncertainty is conservatively estimated and considers both actual between-bottle variability and potential analytical uncertainty.

Stability testing

To evaluate the stability of hair material, we analyzed the concentrations of MeHg, THg, and other trace elements. Random sampling was used for stability testing. Long-term stability was examined to determine whether the material could be effectively used as a CRM. For this evaluation, four bottles were stored at 5 °C for a full year, and samples were taken for analysis at intervals of 0, 3, 6, and 12 months to observe any potential changes in the concentrations of the elements over the duration. The criteria for determining stability were based on acceptable variability limits for the element concentrations. Additionally, a t test was conducted, resulting in a p value > 0.05, indicating that no significant differences were observed.

Short-term stability tests were conducted to ascertain the durability of the materials under typical shipping conditions. The individual bottles were stored at different temperatures (–30, + 5, and + 50 °C) for a two-week period. Samples from these bottles were analyzed to detect variations in elemental concentrations under these conditions.

For each stability test, three individual subsamples from each bottle were digested, and three replicate analyses were conducted for each subsample to ensure the accuracy and consistency of the results obtained.

Collaborative analysis

Fourteen organizations (consisting of twenty laboratories) analyzed candidate human hair CRM for MeHg, THg, and trace elements using various techniques. Following the ISO Guide 35, multiple laboratories and methods were employed to minimize bias, and statistical analysis, including outlier handling, ensured data reliability. The certified values and uncertainties were determined according to ISO Guide 35. Two randomly selected bottles were sent to each participating laboratories, and three replicate analyses were performed for each bottle. To assess the instrument accuracy, the analyses were repeated on different days. Laboratory validation was performed using one of the three replicates (NIES CRM No. 13, IAEA-086, and NIMD-01). The moisture content was also measured in each laboratory. Although variations in instrumentation and environmental conditions among laboratories present inherent challenges in collaborative studies, the standardized analytical protocol and inclusion of CRM analyses helped mitigate these potential sources of error and improve the comparability of results.

Uncertainty

The calculation of CRM uncertainty follows ISO Guide 35, which provides a standardized framework for estimating and combining the uncertainties associated with CRM certification. The combined standard uncertainty (u_{CRM}) of the certified value of the candidate CRM (NIES CRM No. 13-a) was calculated as follows:

$$u_{\text{CRM}} = \left(u_{\text{hom}}^2 + u_{\text{lts}}^2 + u_{\text{char}}^2\right)^{0.5}, \tag{3}$$

where u_{hom} is the uncertainty associated with the component from batch homogeneity, u_{lts} is the long-term stability uncertainty, and u_{char} is the uncertainty from the characterization. To evaluate the characterization uncertainty from different techniques, we first performed measurements using multiple independent analytical methods, such as CVAAS and ICP-MS. For each technique, the mean value (y_i) was calculated. We then calculated the characterization uncertainty (u_{char}) based on the standard deviation of these mean values (following ISO Guide 35) using the following formula:

$$u_{\text{char}} = \frac{s(y)}{\sqrt{p}} = \frac{1}{\sqrt{p}}\sqrt{\frac{\Sigma\left(\mathrm{y}_i - y_{\text{char}}\right)^2}{p-1}}, \tag{4}$$

where $s(y)$ is the standard deviation of the p mean values y_i, y_{char} is the unweighted mean of all y_i, and p is the number of datasets (number of techniques or laboratories). This equation represents the standard error of the mean when combining p independent mean values obtained from different characterization techniques and reflects the uncertainty due to the variation among the techniques.

These components are critical for establishing the overall uncertainty of the certified CRM values and ensuring the reliability and traceability of the assigned values.

The expanded uncertainty (U_{CRM}) was then obtained by multiplying the combined standard uncertainty by coverage factor k, as follows:

$$U_{\text{CRM}} = k \cdot u_{\text{CRM}}, \tag{5}$$

Coverage factor k was determined based on the assumed distribution function and required coverage probability. In most cases, a normal distribution is assumed with a 95% coverage probability, leading to $k = 2$.

In this study, short-term stability uncertainty (u_{sts}) was not included in the uncertainty budget. This decision was based on experimental evaluation under potential transportation conditions, which demonstrated that short-term stability did not significantly affect the overall uncertainty of the CRM.

Results and discussion

Stability

The stability of the hair powder was evaluated by analyzing the concentrations of THg, MeHg, and other trace elements, which showed no significant trends ($p \geq 0.05$ by t test). The results of long- and short-term stability of THg and MeHg are shown in Fig. 1a and b, respectively. To evaluate the statistical significance of the slope in the long-term stability study, a t test was performed to determine whether the slope significantly deviates from zero. The test statistic (t_{b1}) was calculated using the following equation, as referenced in ISO Guide 35:

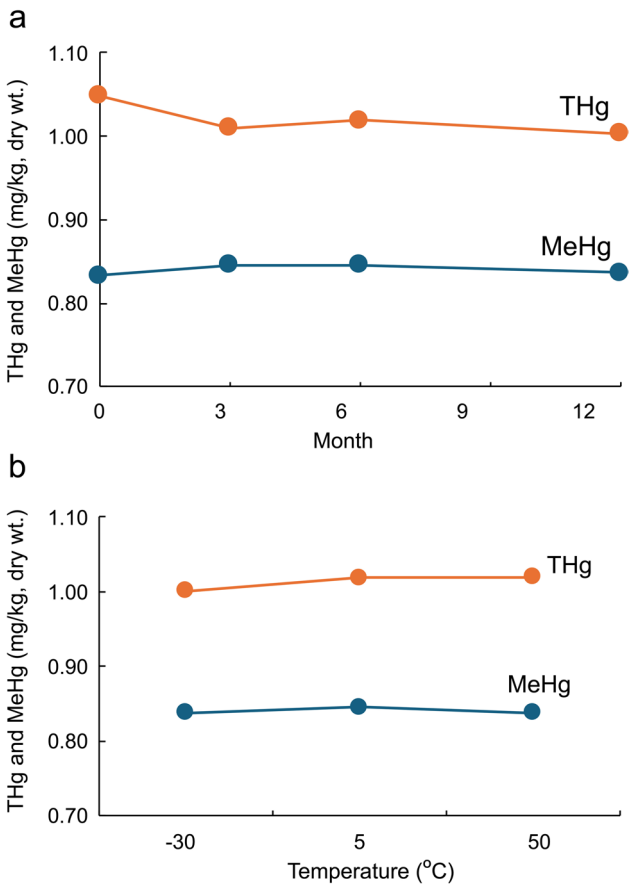

Fig. 1 **a** Long-term and (**b**) short-term stability examinations of THg and MeHg

$$t_{b1} = |b_1| / s(b_1), \tag{6}$$

where b_1 is the estimated slope and $s(b_1)$ is its standard error. The calculated t_{b1} value was then compared with the two-tailed critical value of Student's t-distribution at a 95% confidence level with $n-2$ degrees of freedom. If t_{b1} exceeds the critical value, then the slope is considered significantly different from zero.

Regarding the estimation of long-term stability uncertainty (u_{lts}), since the t test indicated no significant trend in the long-term stability study, it was assumed that the material remained stable over time. Furthermore, referring to NIES CRM No. 13, the previous human hair CRM that has remained stable for over 26 years, we estimated that the chemical composition of human hair reference materials remains stable for at least 10 years. Thus, the validity period of NIES CRM No. 13-a was set to 10 years.

Long-term stability is critical to obtaining reliable Hg biomonitoring data. End users should store the CRM at 5 °C in a clean, dark environment and in its original sealed container. Refrigeration or freezing is recommended; careful handling is essential to prevent contamination. Detailed handling and storage instructions accompany each batch of CRM, provided in the Certificate, and were also shared with the laboratories receiving the samples. Short-term stability testing simulated the conditions experienced during the CRM transport, accounting for potential temperature fluctuations. While CRMs are typically stored at 5 °C in the dark environment, transit conditions—particularly for international shipments—can expose them to ambient temperatures. The consistent THg, MeHg, and trace element concentrations observed across a wide temperature range (from −30 to + 50 °C) demonstrate the material's ability to withstand temperature fluctuations during short-term transport.

Minimum requirement for measurements and homogeneity examinations

The sample weights between bottles ranged from 10 to 50 mg, with relative standard deviations of less than 1% for THg and 2% for MeHg, as shown in Table 2. While no signs of heterogeneity were observed at the 10 mg level, a minimum sample size of 20 mg was recommended to account for the variability introduced by analytical procedures and equipment (Table 2). Consequently, a sample weight of at least 20 mg was employed for all subsequent analyses of the NIES CRM to ensure the reliable homogeneity of THg and MeHg.

For trace element analysis, a sample weight range of 20–100 mg was confirmed to be appropriate (Table 2). Specifically, As, Cd, Pb, and Zn showed good analytical precision at sample weights of 50 mg or more. In contrast, the relative standard deviation (RSD) for Se from sample weights

Table 2 Minimum requirements for measurements

	Sample amount (mg)	Mean (mg/kg)	SD	RSD (%)
THg	10	1.064	0.004	0.4
	20	1.068	0.004	0.3
	50	1.069	0.005	0.4
MeHg	10	0.826	0.010	1.2
	20	0.842	0.015	1.7
	50	0.804	0.018	2.2
As	20	0.236	0.005	2.3
	50	0.231	0.003	1.5
	100	0.233	0.006	2.7
Cd	20	0.156	0.011	6.9
	50	0.157	0.004	2.7
	100	0.153	0.006	4.0
Pb	20	7.226	0.049	0.7
	50	7.185	0.054	0.8
	100	7.291	0.087	1.2
Se	20	0.404	0.021	5.1
	50	0.397	0.024	6.2
	100	0.378	0.016	4.2
Zn	20	337.483	1.125	0.3
	50	337.544	0.445	0.1
	100	339.283	0.203	0.1

SD, standard deviation; *RSD*, relative standard deviation

between 20 and 50 mg was greater than those for the other trace elements. The determination of Se using ICP-MS poses challenges owing to interference from molecular ions and a relatively high ionization potential, leading to low ionization efficiency (approximately 30%) in the plasma, as reported by Lam et al. [11]. Therefore, to assess Se levels in hair, it is advisable to increase the sample size used or consider alternative measurement methods, such as hydride generation-atomic absorption spectrometry (HG-AAS), to enhance the accuracy of the results. The standard deviation (s_r) of repeatability exceeds 5%. Its uncertainty is also included in the uncertainty of the certified value and is homogeneous within its uncertainty. The minimum usage in the analysis presented here strikes a balance between the practical ease of use and the reliability of the analysis. A summary of the homogeneity test results is provided in Table 3. The uncertainty component of the batch homogeneity was less than 1.0%. NIES CRM No. 13-a exhibits excellent homogeneity at the recommended minimum sample size of 20 mg for both Hg and MeHg, which is comparable to that of NIMD-01, and 50 mg for trace elements. This significant reduction in sample size compared to our previous CRM (NIES CRM No. 13: 120 mg) improves analytical efficiency and minimizes donor burden without compromising data quality across various Hg analysis methods.

Certifications

The certified values for THg, MeHg, and trace elements in the human hair reference materials represent a significant advancement in human hair analysis. These values were established using extensive analytical data obtained from at least eight independent methods provided by various collaborating laboratories. Table 4 presents a summary of the analytical techniques used to certify each element. All values were determined based on dry mass. Outliers were identified and excluded using the *z*-score method, which was chosen because of its stricter rejection criteria for datasets with eight or more observations compared to the single Grubbs test. The *z*-score was adopted because it applies a fixed threshold of 2, while the Grubbs test threshold varies with sample size. Although ISO Guide 35 recommends the Grubbs test, the *z*-score method was deemed appropriate for this dataset to ensure a stricter and more consistent outlier rejection approach.

Table 3 Homogeneity tests

Element	Sample amount (mg)	Concentration (mg/kg)	MS_{among} (mg^2/kg^2)	MS_{within} (mg^2/kg^2)	s_r (%)	s_{bb} (%)	u_{bb} (%)
THg	20	1.04	0.00014	0.00048	2.1	–	0.34
MeHg	20	0.848	0.00154	0.00061	2.9	2.6	0.48
As	50	0.238	0.00002	0.00002	2.1	–	0.34
Cd	50	0.163	0.00003	0.00002	2.7	1.3	0.44
Pb	50	7.60	0.05842	0.03698	2.5	1.4	0.41
Se	50	0.437	0.00050	0.00067	5.9	–	0.97
Zn	50	352	18.6	10.7	0.93	0.56	0.15

MS_{among}, among-bottle mean square; MS_{within}, within-bottle mean square; s_r, relative repeatability standard deviation in analysis of the variance; s_{bb}, relative between-bottle standard uncertainty; and u_{bb}, relative between-bottle variance incorporating the effect of analytical variation

Table 4 Analytical methods of THg, MeHg, and other trace elements in NIES CRM No. 13-a Human Hair by collaborating laboratories

Element	Instrument	Sample preparation	Delivered data	Accepted data	Mean from accepted results
THg	CVAAS	HNO_3 microwave	1	1	1.063
	ICP-MS	HNO_3 hot plate	3	2	1.067 ± 0.114
	ID-GC-ICP-MS	HNO_3 hot plate	1	1	1.151
	TD-CVAAS	N/A (no pretreatment)	6	5	1.033 ± 0.023
MeHg	GC-CV-AFS	HNO_3 hot plate/ethylation; aqueous ethylation	4	4	0.819 ± 0.059
	GC-ECD	HCl leaching/toluene extraction	1	1	0.800
	ID-GC-ICP-MS	HNO_3 oven	2	2	0.965 ± 0.047
	LC-ICP-MS	TMAH block heater	2	1	0.857
As	ICP-MS	HNO_3, $HNO_3 + H_2O_2$, $HNO_3 + HF$, $HNO_3 + HCl + H_2O_2$, $HNO_3 + H_2O_2 + HF$, $HNO_3 + HClO_4 + HF$, hot plate, hot dry bath, microwave	10	9	0.255 ± 0.012
	HG-AAS	$NaBH_4$	1	1	0.251
Cd	ICP-MS	HNO_3, $HNO_3 + H_2O_2$, $HNO_3 + HF$, $HNO_3 + HCl + H_2O_2$, $HNO_3 + H_2O_2 + HF$, $HNO_3 + HClO_4 + HF$, hot plate, hot dry bath, microwave	10	9	0.165 ± 0.006
Pb	ICP-MS	HNO_3, $HNO_3 + H_2O_2$, $HNO_3 + HF$, $HNO_3 + HCl + H_2O_2$, $HNO_3 + H_2O_2 + HF$, $HNO_3 + HClO_4 + HF$, hot plate, hot dry bath, microwave	10	9	7.42 ± 0.31
Se	ICP-MS	HNO_3, $HNO_3 + H_2O_2$, $HNO_3 + HF$, $HNO_3 + HCl + H_2O_2$, $HNO_3 + H_2O_2 + HF$, $HNO_3 + HClO_4 + HF$, hot plate, hot dry bath, microwave	10	9	0.475 ± 0.037
	HG-AAS	$NaBH_4$	1	1	0.373
Zn	ICP-OES	$HNO_3 + H_2O_2$, $HNO_3 + HCl + H_2O_2$, $HNO_3 + H_2O_2 + HF$, $HNO_3 + HClO_4 + HF$, hot plate, hot dry bath, microwave	4	4	347 ± 12
	ICP-MS	HNO_3, $HNO_3 + H_2O_2$, $HNO_3 + HF$, $HNO_3 + HCl + H_2O_2$, $HNO_3 + H_2O_2 + HF$, $HNO_3 + HClO_4 + HF$, hot plate, hot dry bath, microwave	6	5	329 ± 2

THg

For the collaborative analysis of THg, the results from 11 laboratories were employed. Seven of the laboratories provided analytical values using CV atomic absorption spectrometry (CVAAS), two laboratories provided analytical values using ICP-MS, and one laboratory provided analytical values using isotope dilution ICP-MS (ID-ICP-MS). The mean value and 95% confidence interval (CI) were calculated to be 1.06 ± 0.07 mg/kg. The certified value for the total Hg in the reference material (RM) was determined to be 1.06 ± 0.07 mg/kg. Of the 11 institutions, Lab (J) and Lab (K) were rejected because the analytical results of similar RMs measured as part of the analytical precision control deviated from the certified values (Fig. 2a). Labs (H) and (I) were not rejected because the standard measured at the same time was within the range of certified values ($\pm 10\%$ of the certified value), did not qualify as an outlier in the rejection test of the joint study, and was within the range in the 2SD of the testing institution.

MeHg

MeHg data were obtained from nine laboratories: four by GC-cold vapor atomic fluorescence spectroscopy (GC-CV-AFS), two by ID-GC-ICP-MS (isotope dilution-gas chromatography-inductively coupled plasma mass spectrometry), one by LC-ICP-MS (liquid chromatography-inductively coupled plasma mass spectrometry), and one by GC-ECD. The results from the various methods were in agreement, and the certified value for MeHg was determined to be 0.858 ± 0.075 mg/kg dry weight as Hg. Of these nine institutions, we rejected Lab (I) because it significantly exceeded the lower limit of uncertainty. For Labs (A) and (H), the results were adopted because the results for similar RMs were within the certified values (Fig. 2b).

Other trace elements

Extensive analyses have been conducted on the nutritional and toxicological significance of several heavy metals, including As, Cd, Pb, Se, and Zn, in human hair.

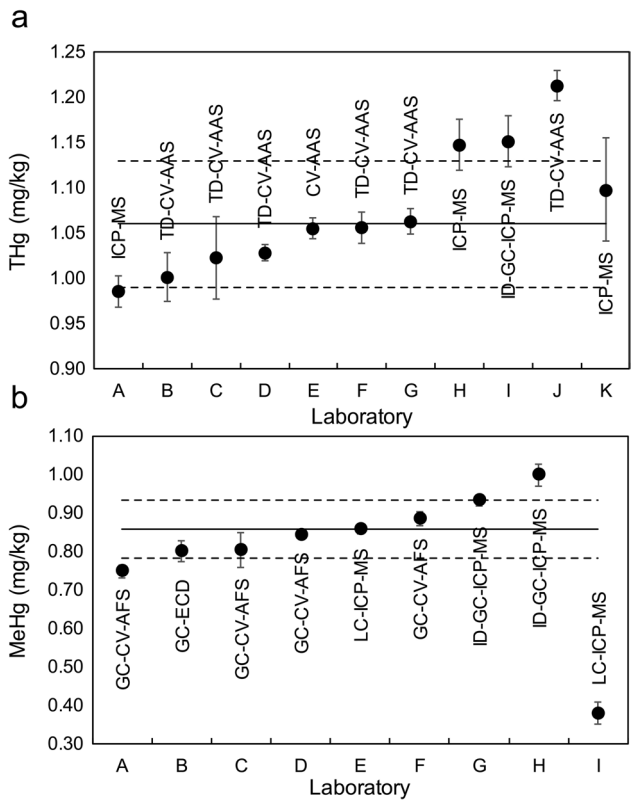

Fig. 2 Results for collaborative measurements of (**a**) THg and (**b**) MeHg

Table 5 presents the certified values obtained from ICP-MS, ICP-OES, and HG-AAS: As = 0.255 ± 0.016 mg/kg, Cd = 0.165 ± 0.012 mg/kg, Pb = 7.42 ± 0.62 mg/kg, Se = 0.463 ± 0.052 mg/kg, and Zn = 337 ± 22 mg/kg. For one laboratory that performed the ICP-MS analysis, we rejected the data because the results for similar RMs did not fall within the range of certified values.

Table 6 lists the budgets of the combined uncertainties. The uncertainty associated with the certified values is expressed as the expanded uncertainty (U_{CRM}), which was calculated by multiplying the combined standard uncertainty (u_{CRM}) by a coverage factor ($k = 2$). This coverage factor corresponds to a confidence level of approximately 95%, assuming a normal distribution.

Reference values for trace elements

In addition to the certified values, reference values were assigned to several elements including calcium (Ca), magnesium (Mg), sodium (Na), sulfur (S), antimony (Sb), barium (Ba), copper (Cu), iron (Fe), and manganese (Mn). These values were determined based on one or two independent analytical methods, ensuring satisfactory agreement across laboratories (Table 6): Ca = 0.14 ± 0.01%, Mg = 0.031 ± 0.002%, Na = 0.058 ± 0.005%, S = 4.7 ± 0.3%, Sb = 0.062 ± 0.005 mg/kg, Ba = 11 ± 1 mg/kg, Cu = 19 ± 2 mg/kg, Fe = 71 ± 8 mg/kg, and Mn = 37 ± 2 mg/kg.

Table 5 Certified and reference values of NIES CRM No. 13-a

Element	Unit	Certified value	Uncertainty
THg	mg/kg	1.06	0.07
MeHg (as Hg)	mg/kg	0.858	0.075
As	mg/kg	0.255	0.016
Cd	mg/kg	0.165	0.012
Pb	mg/kg	7.42	0.62
Se	mg/kg	0.463	0.052
Zn	mg/kg	337	22
Element	Unit	Reference value	Uncertainty
Ca	%	0.14	0.01
Mg	%	0.031	0.002
Na	%	0.058	0.005
S	%	4.7	0.3
Sb	mg/kg	0.062	0.005
Ba	mg/kg	11	1
Cu	mg/kg	19	2
Fe	mg/kg	71	8
Mn	mg/kg	37	2

The uncertainties associated with the certified values represent expanded uncertainties with a coverage factor of $k = 2$, corresponding to an approximately 95% confidence interval (i.e., half-width of the interval). The reference values were not treated as certified values because they did not meet the certification criteria; however, they represent the characteristics of this CRM in the same manner as the certified values

Table 6 Budgets of the combined relative uncertainties, U_{CRM} (%)

Element	u_{hom}	u_{lts}	u_{char}	u_{CRM}	Laboratories	U_{CRM}
THg	0.34	2.48	1.84	3.11	9	6.22
MeHg	2.55	1.15	3.29	4.32	8	8.64
As	0.34	2.56	1.42	2.94	10	5.89
Cd	1.27	2.99	1.12	3.44	9	6.87
Pb	1.36	3.65	1.38	4.13	9	8.26
Se	0.97	4.23	3.50	5.57	9	11.14
Zn	0.56	2.25	2.15	3.16	9	6.32

The inclusion of reference values for noncertified elements is crucial for providing a more comprehensive framework for research and regulatory applications. These reference values can facilitate the assessment of elemental concentrations in human hair and provide baseline data that are critical for health assessments and environmental monitoring. However, importantly, the number of laboratories contributing to these reference values is limited to fewer than five in certain cases. Although this raises concerns regarding the robustness of the reference values, it was decided that no additional analysis would be conducted to expand this dataset.

Cu was a notable exception in our analysis, as it was measured in ten institutions. However, the *t* test results from the long-term stability testing indicated a significant slope, suggesting potential variability in the measurements. This finding emphasizes the need for ongoing monitoring and verification of the stability of this element to ensure that the reference value remains accurate over time. Assigning reference values to additional elements enhances the versatility and adoption of CRM by providing richer datasets that meet the requirements of diverse research fields. Expanding the scope of reference values will not only improve CRM utility but also foster increased confidence among users regarding the integrity and applicability of the data in both regulatory and research settings.

Information values for stable Hg isotope

In this study, the δ^{202}Hg value for NIES CRM No. 13 was determined to be 2.17 ‰ (2SD = 0.13, $n = 22$) (Table 7). Yamakawa et al. [6] reported a δ^{202}Hg value of 1.89 ‰ (2SD = 0.10, $n = 10$), which is lower than the values obtained in our study. According to Yamakawa et al. [6], the reproducibility of NIST SRM 3133 was approximately 0.2 ‰ for a 5 ppb Hg solution, indicating that the actual variation in measurements could be greater. Furthermore, other laboratories have reported δ^{202}Hg values of 1.95 ‰ (taken at University of Pau) [6], 2.08 ‰ [12], and 1.84 ‰ [13]. These discrepancies may have been influenced by variations in sample preparation, instrument calibration, and analytical methodologies. In this study, we implemented stringent quality control procedures, including replicate measurements and calibration with certified reference materials to mitigate potential sources of variability. The measurements for IAEA-085 obtained in this study also aligned well with these findings, reinforcing the reliability of the analytical methods employed and the validity of our results. Hg isotopes are valuable tracers for assessing Hg exposure pathways, significantly elucidating the sources and pathways of Hg exposure in human populations. For instance, studies have demonstrated how variations in Hg isotopes can be used to distinguish between Hg originating from atmospheric deposition and that derived from local industrial sources. Such information is essential for evaluating health risks and developing targeted strategies to mitigate Hg exposure.

Conclusions

The development of the NIES CRM No. 13-a Human Hair represents significant advancement in the accurate analysis of THg, MeHg, and trace elements in human hair. This CRM serves as a vital tool for researchers and laboratories, ensuring precise measurements essential for assessing human exposure to these elements. NIES CRM No. 13-a provides certified values for THg and MeHg, reference values for a range of trace elements, and informational values for stable Hg isotopes, making it superior to other available CRMs. Due to the limited number of joint analyses, certain elements were designated as reference values. In addition, interference issues during Se analysis required a minimum sample mass of 50 mg.

To ensure metrological traceability, all measurements related to CRM production were linked to SI units through calibration with CRMs provided by institutions such as NIST and IAEA. The measurement instruments, including ICP-MS and CVAAS, were regularly calibrated using traceable standards to maintain accuracy. The certified values of our CRM were established through a rigorous process involving multiple analytical techniques and independent measurements. Measurement uncertainty was evaluated in

Table 7 Hg isotopic compositions of NIES CRM No. 13, 13-a, IAEA-085, and NIST SRM 8610

Sample	Sample preparation		*n*	$\delta^{199}Hg$	$\delta^{200}Hg$	$\delta^{201}Hg$	$\delta^{202}Hg$	$\delta^{204}Hg$	$\Delta^{199}Hg$	$\Delta^{200}Hg$	$\Delta^{201}Hg$	$\Delta^{204}Hg$	Recovery (%)
NIES CRM No. 13	HNO_3 + HCl (HotBlock)	Mean	9	2.46	1.20	3.20	2.20	3.10	1.91	0.09	1.54	−0.18	83.4%
		2SD		0.07	0.06	0.11	0.14	0.20	0.06	0.06	0.06	0.11	8.9%
	HNO_3 + HCl + H_2O_2 + BrCl (HotBlock)	Mean	11	2.44	1.17	3.19	2.17	3.09	1.89	0.08	1.55	−0.15	82.1%
		2SD		0.10	0.08	0.10	0.13	0.17	0.08	0.05	0.06	0.11	7.2%
	HNO_3 + HCl + H_2O_2 + BrCl (Microwave)		2	2.42	1.14	3.19	2.18	3.10	1.87	0.04	1.55	−0.16	83.9%
				2.44	1.14	3.15	2.13	3.04	1.90	0.07	1.55	−0.13	85.1%
	Mean		22	2.44	1.17	3.19	2.17	3.09	1.90	0.08	1.56	−0.15	81.9%
	2SD			0.10	0.08	0.11	0.13	0.16	0.08	0.05	0.06	0.11	7.1%
	Measured at NIES [6]			*2.13*	*0.98*	*2.77*	*1.89*	*2.76*	*1.65*	*0.04*	*1.36*	*−0.04*	
	Measured at IPREM [6]			*2.30*	*1.06*	*2.96*	*1.95*		*1.81*	*0.08*	*1.50*		
	Renedo et al. [12]			*2.30*	*1.13*	*3.01*	*2.08*	*2.87*	*1.75*	*0.04*	*1.42*	*−0.08*	
	Wang et al. [13]						*1.84*		*1.75*	*0.06*	*1.44*		
Sample	Sample preparation		*n*	$\delta^{199}Hg$	$\delta^{200}Hg$	$\delta^{201}Hg$	$\delta^{202}Hg$	$\delta^{204}Hg$	$\Delta^{199}Hg$	$\Delta^{200}Hg$	$\Delta^{201}Hg$	$\Delta^{204}Hg$	Recovery (%)
NIES CRM No. 13-a	HNO_3 + HCl (HotBlock)	Mean	9	0.63	0.55	1.00	0.99	1.42	0.38	0.05	0.26	−0.06	85.3%
		2SD		0.09	0.09	0.15	0.10	0.12	0.07	0.07	0.09	0.07	6.6%
	HNO_3 + HCl + H_2O_2 + BrCl (HotBlock)	Mean	15	0.64	0.54	0.99	0.97	1.37	0.38	0.05	0.26	−0.08	83.2%
		2SD		0.08	0.08	0.14	0.10	0.15	0.06	0.05	0.09	0.10	7.6%
	HNO_3 + HCl + H_2O_2 + BrCl (Microwave)	Mean	6	0.65	0.56	1.00	0.99	1.38	0.40	0.06	0.25	−0.10	81.3%
		2SD		0.05	0.04	0.06	0.03	0.04	0.05	0.04	0.05	0.05	6.1%
	Mean		30	0.64	0.55	1.00	0.98	1.38	0.39	0.05	0.26	−0.09	82.0%
	2SD			0.08	0.07	0.12	0.10	0.14	0.06	0.05	0.07	0.09	7.2%
Sample	Sample preparation		*n*	$\delta^{199}Hg$	$\delta^{200}Hg$	$\delta^{201}Hg$	$\delta^{202}Hg$	$\delta^{204}Hg$	$\Delta^{199}Hg$	$\Delta^{200}Hg$	$\Delta^{201}Hg$	$\Delta^{204}Hg$	Recovery (%)
IAEA-085	HNO_3 + HCl + H_2O_2 + BrCl (HotBlock)		2	−0.10	−0.16	−0.30	−0.41	−0.63	0.00	0.05	0.01	−0.01	86.2%
				−0.16	−0.20	−0.40	−0.44	−0.67	−0.05	0.02	−0.07	−0.01	88.5%
	Yamakawa +, 2016			*−0.13*	*−0.19*	*−0.32*	*−0.38*	*−0.56*	*−0.03*	*0.00*	*−0.03*	*0.00*	
	Evop +, 2008			*−0.12*	*−0.18*	*−0.31*	*−0.37*		*−0.02*	*0.01*	*−0.03*		
	Laffont +,2009			*−0.12*	*−0.18*	*−0.31*	*−0.37*		*−0.03*	*−0.01*	*−0.03*		
Sample			*n*	$\delta^{199}Hg$	$\delta^{200}Hg$	$\delta^{201}Hg$	$\delta^{202}Hg$	$\delta^{204}Hg$	$\Delta^{199}Hg$	$\Delta^{200}Hg$	$\Delta^{201}Hg$	$\Delta^{204}Hg$	
NIST SRM 8610	Mean		10	−0.14	−0.22	−0.41	−0.50	−0.77	−0.01	0.03	−0.04	−0.03	
	2SD			0.07	0.06	0.09	0.07	0.15	0.08	0.05	0.07	0.08	

The numbers were italicized to distinguish them from the results obtained in our study

n refers to the number of measurements performed for each sample

accordance with ISO Guide 35 to ensure reliability. Furthermore, interlaboratory comparisons were conducted to validate the measurement results and confirm their consistency with internationally recognized reference materials. To maintain high-quality standards, our CRM production adhered to stringent guidelines to ensure homogeneity, stability, and suitability for environmental and analytical applications.

This reference material has diverse applications, including environmental health, biomonitoring, and human exposure assessment. To maximize its utility, users are encouraged to adhere to strict analytical protocols and quality control measures. The use of NIES CRM No. 13-a is expected to enhance the reliability and accuracy of analytical results, thereby improving assessments of human exposure to hazardous substances. Additionally, it is anticipated to facilitate global mercury monitoring, contribute to more stringent regulatory standards, and promote international collaboration to combat mercury exposure risks.

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Author contributions AY contributed to conceptualization, project administration, supervision, and visualization; AY, KN, KO, MU, TI, and TS were involved in methodology and data curation; AY, KN, KO, MU, TI, MK, MI, KI, AD, ET, MH, AA, PK, EB, MH, RM, KH, PL, LS, PR, LH, NT, YL, and KU contributed to formal analysis; AY and TI were involved in investigation and validation; KI and AY contributed to accounting procedure; AY and KN were involved in writing—original draft; and all authors contributed to writing—review and editing.

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Data availability All data analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare no competing interests.

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