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Analysis of Mercury in Sequential Micrometer Segments of Single Hair Strands of Fish-Eaters

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Although it has been established that mercury (Hg) can be detected in single hair strands using laser ablation−inductively coupled plasma−mass spectrometry (LA−ICP−MS), calibration remains a challenge due to the lack of well-characterized matrix-matched standards. We concurrently evaluated two strategies for quantifying Hg signals in single hair strands using LA−ICP−MS. The main objective was to obtain time-resolved Hg concentrations in single hair strands of fish-eaters that would correspond to the changes of their body burden over time. Experiments were conducted using hair samples collected from 10 individuals. The first experiment involved the construction of a calibration curve with four powdered hair standard reference materials (SRMs) with a range of Hg concentrations (0.573−23.2 mg/kg). An internal standard, sulfur, as ^{34}S, was applied to correct for ablation efficiency for both the hair strands and the SRMs. Results showed a linear relationship ($R^2 = 0.899$) between the ratio of ^{202}Hg to ^{34}S obtained by LA−ICP−MS and the certified total Hg concentration in the SRMs. Using this calibration curve, average Hg concentrations of 10 shots within a 1-cm segment of a hair strand were calculated and then compared to the total Hg concentrations in the matched 1-cm segment as measured by cold vapor atomic absorption spectrometry (CV-AAS). A significant difference ($p < 0.05$) was observed. The difference could be attributed to the highly variable ablation/sampling process caused by the use of the laser on the hair powder SRM pellets and the difference in the physical properties of the SRMs. An alternative approach was adopted to quantify consecutive ^{202}Hg to ^{34}S ratios by calibrating the signals against the average Hg concentration of the matched hair segment as measured by CV-AAS. Consecutive daily Hg deposition in single hairs of fish eaters was determined. Results showed that apparent daily changes in Hg concentrations within a hair segment that corresponds to 1 month of hair growth. In addition, a significant decreasing or increasing time-trend was observed. The difference between the minimum and maximum Hg concentration within each individual corresponded to a change of 26−40%. Our results showed that LA−ICP−MS can be used to reconstruct time-resolved Hg exposure in micrometer segments of a single hair strand.

Introduction

Through natural events and human activities, mercury (Hg), a heavy metal constituent of the rocks within the earth's crust, is a ubiquitous pollutant of global importance. Various forms of Hg exist, each with their distinct toxicological profile and for which potential impacts should be considered carefully (*1*, *2*). Methylmercury (MeHg), the organic form of Hg, is biomagnified in aquatic food chains. It can be detected in people, particularly those who consume predatory fish and marine mammals. MeHg has been shown to be associated with neurological dysfunction in exposed populations, and continues to be a concern among fish-eating populations (*3*). Human fetuses are considered an especially high risk group because MeHg crosses the placental barrier to reach the highly susceptible developing brain (*1*, *3*).

A key step in the risk management of Hg is the assessment of exposure. Reliable data minimizes misclassification of exposure which leads to proper development of dose−response relationships (*4*), and public health decisions and recommendations (*5*). Blood and hair are the most commonly used body burden indices for biomonitoring. Hair to blood ratio have been modeled with hair concentrating on average approximately 250−300 times more Hg (*3*). However, the reported ratios obtained within and between populations are variable (*3*), as both indices are affected by measurement imprecision (*6*) and the time period of exposure represented by blood and hair are often different (*7*).

Scalp hair is a well-established matrix for assessing Hg exposure of individuals and has been extensively used as a biomarker of exposure in large epidemiological studies (*7*−*9*). It is easily obtained and is less invasive compared to blood collection. Assuming an average hair growth rate of approximately 1 cm a month (*10*), retrospective temporal analysis can retrace weeks and months of exposure (*11*, *12*), a feature not achievable with blood analysis. It is particularly useful for assessing fetal exposure as maternal hair samples of various lengths correspond to various periods of gestation (*13*). From an analytical perspective, detection of Hg is less problematic when using hair compared to blood as the Hg concentrates in hair (*14*).

Cold vapor atomic absorption spectrometry (CV-AAS), cold vapor atomic fluorescence spectrometry (CV-AFS) and inductively coupled plasma mass spectrometry (ICP−MS) are the most common methods to measure total Hg in hair (*14*). Five to 10 mg of hair is required and detection limits range from about 0.04 mg/kg with CV-AFS up to 0.4 mg/kg with CV-AAS (*15*, *16*). To obtain spatial resolution of centimeters (for biomonitoring purposes), 100−150 hair strands are required. The large requirement of strands can be intrusive to participants which can lead to low response rates, particularly in children.

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TABLE 1. Certified Mean and Range of Total Hg (mg/kg) in Hair Powder SRM Used in Experiment 1

identification	origin	mean	range
IAEA086	International Atomic Energy Agency, Vienna, Austria	0.573	0.534−0.612
NIES13	National Institute of Environmental Studies, Ibaraki, Japan	4.42	4.22−4.62
BCR397	Community Bureau of Reference, Commission of the European Communities, Brussels, Belgium	12.3	11.8−12.8
IAEA085	International Atomic Energy Agency, Vienna, Austria	23.2	22.4−24.0

Direct solid introduction techniques minimize the weaknesses imposed by methods which require chemical digestion prior to detection (*17*). In addition, the number of hair strands required for analysis can be reduced to a single hair strand. Single hair strand analysis offers certain advantages over hair-bundle analysis. The collection is less intrusive to participants (especially for children) and hair segmentation is more efficient. Furthermore, single hair strands of maternal scalp hair have been suggested to provide a better index of fetal exposure than analysis of a bundle of hair because the period and degree of exposure are more accurately defined (*18*).

Methods for measuring Hg in solid samples such as single hair strands include combustion gold amalgamation atomic absorption spectrometry (C-GA-AAS) (*17*), induction heating-electrothermal vaporizer with ICP−MS detection (IH-ETV-ICP−MS) (*19*), proton induced X-ray emission (PIXE) (*20*), and X-ray fluorescence (XRF) (*21*, *22*). C-GA-AAS and IH-ETV-ICP−MS are capable of measuring total Hg in 1- and 12-cm segments, respectively, of a single hair strand (*17*, *19*). However, the spatial resolution within a hair strand is not improved over the traditional methods of 1-cm measurements. PIXE and XRF are capable of spatial resolutions of millimeters of hair but are respectively limited by expensive instrumentation and relatively elevated detection limits (*13*, *19*, *22*).

Laser ablation−ICP−MS provides the advantages of the spatial solid micro LA sampling and high sensitivity multi-element ICP−MS capability for the analysis of Hg in single hair strands (*23*). The detection of Hg by LA−ICP−MS in micrometer segments of single hair strands has recently been established (*24*) with demonstrated application to reconstruct the hair-Hg profile of an individual affected by acute Hg intoxication (*25*). However, these studies provided quantitative measurements that have some limitations as the LA−ICP−MS signals were quantified with only one SRM with the assumption that the concentration of the internal standard is equivalent in the samples and the SRM. In addition, the feasibility of using this method to study continuous time-resolved changes in Hg body burden of individuals exposed to environmentally relevant concentrations has not been explored.

We evaluated two quantification approaches for single hair strand analysis using four standard reference materials as external standards and co-analysis of matched hair samples using CV-AAS. The main objective of this study was to obtain quantified continuous Hg concentrations in single hair strands of fish-eaters.

Materials and Methods

Experiment 1: Quantification with Pressed Pellet Standards. *Hair Samples and SRMs.* Four powdered hair SRMs with a wide range of certified Hg concentrations were purchased (Table 1). Fifty milligrams of powder was compressed into solid flat pellets with a manual hydraulic press with Specac evacuable pellet die assembly (Specac, Orpington, Kent, United Kingdom) at 8 t pressure.

TABLE 2. ICP−MS Operating Parameters

ICP−MS	
RF power	1000 W
sampling and skimmer cones	nickel
plasma gas flow rate	15 l min^{-1} Ar
auxiliary gas flow rate	1.2 l min^{-1} Ar
carrier gas flow rate	0.8 l min^{-1} Ar
signal measuring parameters	
detector mode	pulse
acquisition mode	peak hopping
sweeps per reading	1
readings per replicate	1000
replicates	1
MCA channels	1
dwell time	30 ms
total acquisition time	100 s

Scalp hair from five individuals was obtained from a larger pool of samples that were previously collected as part of two interdisciplinary studies (*26*, *27*). The Hg concentrations of the first cm next to the scalp were previously determined by conventional CV-AAS using the Farant et al. methodology (*15*) and ranged from less than 0.4 to 14.2 mg/kg. For confidentiality, they are labeled sample 1 to 5.

Instrumentation. A CETAC LSX-100 laser ablation system (CETAC Technologies, Omaha, NE) coupled with a Perkin-Elmer ELAN 6000 ICP−MS (SCIEX, Concord, ON, Canada) was used. An in-house adapter made of Teflon was used to connect the LSX-100 directly to the injector tube of the ELAN 6000 torch assembly. A sample holder was constructed in-house with four parallel trenches of 120 μm width, spaced 5 mm apart for aligning the single hair strands straight in the sample cell. Ends of the hair samples were secured to the sample holder with tape. The LSX-100 is a Q-switched frequency quadrupled Nd:YAG laser system, operating at 266 nm. The beam profile of the laser was 1 mm Gaussian with an 8−12 ns pulse width. A translation stage varied the position of the sample cell in the *X*, *Y*, and *Z* directions to adjust the focus and position of the laser with respect to the sample. The ablated sample was carried into the ICP−MS by an argon gas stream. The ablated crater was approximately 50 μm in diameter. To avoid breaking the hair, the energy of the laser shot was limited to one shot at 2 mJ per site.

The operating conditions for the ICP−MS are found in Table 2. Due to the lack of appropriate solid standards, the performance of the ICP−MS was not optimized for laser ablation but was instead optimized with conventional nebulization techniques.

Experimentation and Statistical Analysis. A single hair strand from each of the five individuals was ablated at 10 separate sites within the first cm next to the scalp. To verify the hair to hair variability, additional hair strands from three of the five individuals were also ablated in the same fashion (i.e., at the same sites) as the first hair strand. Each of the four pelletized hair powder SRM was ablated at 10 separate sites.

Sulfur was used as the internal standard. The relative abundances of the four S isotopes ^{32}S, ^{33}S, ^{34}S, and ^{36}S are 95.02%, 0.75%, 4.21%, and 0.02%, respectively. The most abundant S isotope, ^{32}S, was ruled out due to mass spectral interference of $^{32}O_2$. We also excluded ^{36}S because of spectral interference and low relative abundance. Both ^{33}S and ^{34}S were tested. The ^{34}S transient signals were relatively large whereas the ^{33}S signals were not distinguishable from baseline noise. Therefore, the ^{34}S isotope was retained as the internal standard. The concentration of S in three of the four reference material (IAEA086, NIES13, and BCR397) was also measured

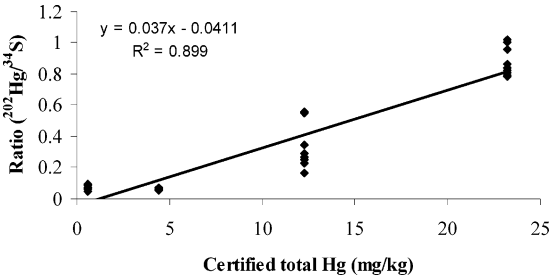

FIGURE 1. Calibration curve and linear regression equation using four SRMs. See Table 1 for the sources of the SRMs. The certified values are 0.573, 4.42, 12.3, and 23.2 mg/kg.

using a conventional sulfur/carbon analyzer (ELTRA CS-800, LECO Instruments). The most abundant Hg isotope, ^{202}Hg with 29.86% relative abundance, had a visible transient peak, and little spectral interference, and hence was retained for biomonitoring. For each ablation, peak areas of the transient signals for ^{202}Hg and ^{34}S were determined.

The calibration curve was obtained by plotting the observed ratio of ^{202}Hg to ^{34}S versus the certified concentrations of the four SRMs (Figure 1). The ^{34}S normalized ^{202}Hg of the single hair strands was quantified using the linear regression equation of the calibration curve. A *t*-test was then used to compare the Hg concentrations obtained by LA−ICP−MS (i.e., through the use of the calibration curve) and CV-AAS for split samples of each individual. Analysis of variance was used to test for differences in the ^{34}S normalized ^{202}Hg of replicate intra-individual hair strands. *p* values below the 0.05 α level were considered significant.

Experiment 2: Quantification of Sequential Daily Hg Deposition. *Hair Samples and Experimentation.* Hair samples were obtained from a large interdisciplinary project conducted in Brazil (*27*). One hair strand each from five individuals (labeled A to E) were used in this experiment. For each individual hair strand, the first centimeter proximal to the scalp was ablated at 30 consecutive spots such that each spot would reflect daily hair growth. Assuming that hair grows at 1 cm per month (*10*), each spot was continuously ablated (i.e., using the Line Scan Mode) to cover a spatial distance of 333 μm per spot. ^{202}Hg was monitored during each ablation. ^{34}S was also monitored as the internal standard and was used to normalize ^{202}Hg for each ablation.

An alternate approach to quantifying LA−ICP−MS signals was used whereby each ^{202}Hg to ^{34}S ratio is approximated into concentration using the following equation:

$$\frac{\text{Hg concentration of the entire 1-cm segment}}{\text{average of all } ^{202}\text{Hg}/^{34}\text{S}} \times {}^{202}\text{Hg}/^{34}\text{S}_x$$

x ranges from 1 to n where n samples (spots) were taken along the hair segment. For each of the five individuals, total Hg concentration in the matched first cm segment proximal to the scalp was measured by CV-AAS (*27*). Basic statistics were used to describe the data, and linear regression was applied to determine the overall trend.

Instrumentation. This experiment was performed using a CETAC LSX 200 laser ablation system (CETAC Technologies, Omaha, NE). The LXS-200 is similar to the 100 in most regards; however, the LSX 200 offers better control of spot size of the ablated crater (10−200 μm) and additional movement of the translation stage. The beam profile of the 266 nm laser was flat with a 6 ns pulse width. The ICP conditions are outlined in Table 2. Sensitivity of the LA−ICP−MS was previously explored using a single laser shot to ablate a single strand that had an average Hg concentration of 3.63 mg/kg. A detection limit of 0.2 mg Hg per kg of hair was obtained.

Quality Control. In both experiments 1 and 2, shot-to-shot reproducibility of the laser was tested by ablating single shots on a homogeneous piece of metal, a copper disc, and by monitoring the ^{63}Cu isotope. The relative standard deviation (RSD) for each run consistently ranged between 5 and 8%. A mass adjacent to ^{202}Hg, 205 m/z, was monitored during all analyses as a reference. The only isotope present at that mass is thallium (Tl) with concentrations in hair reported to be less than 5 ng g^{-1} (*28*), and thus would not be expected to contribute a detectable signal. For all analyses, no observable signal (change in baseline) was seen for ^{205}Tl. This indicates that the signal for Hg is not an artifact resulting from pressure pulse. Argon gas (i.e., blank) analyses were done every 10−12 ablations to ensure neither carry-over effect nor contamination. Blank runs did not produce an observable peak for ^{202}Hg, ^{34}S, or ^{205}Tl.

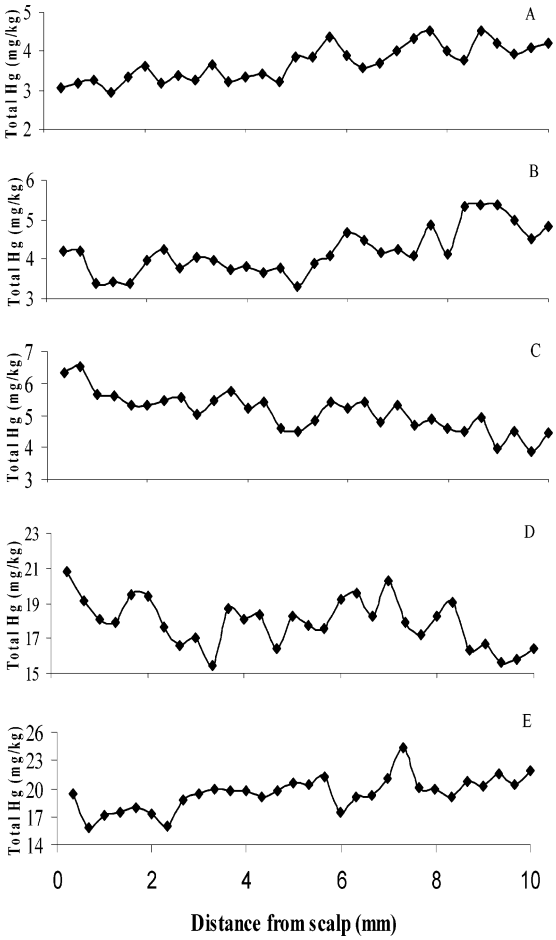

FIGURE 2. Consecutive daily Hg concentrations over 1 month (i.e., 1 cm) within a single hair strand of five individuals. The mean Hg concentrations in the 1-cm segments are: A = 3.7 mg/kg, B = 4.2 mg/kg, C = 5.1 mg/kg, D = 17.9 mg/kg, E = 19.5 mg/kg.

Results and Discussion

The main objective of this paper was to obtain quantified continuous daily Hg concentrations in single hair strands of fish-eaters for whom seasonal variation in Hg exposure as a result of differential seasonal fish intake (type and amount consumed) had previously been shown (*27*). Figure 2 shows the daily changes in Hg concentrations within a 1-month period. There was a significant decreasing or increasing overall trend observed for each individual. The range of daily Hg concentrations recorded within the monthly profile was as follows: individual A, 3.0−4.5 mg/kg; individual B, 3.3−5.4; individual C, 3.9−6.5 mg/kg; individual D, 15.4−20.8 mg/kg; individual E, 15.7−24.4 mg/kg). The difference between the minimum and the maximum concentration corresponded to a change of 26−40%. Since the RSD of the internal

TABLE 3. Comparison of Total Hg Concentrations (mg/kg) Obtained by CV-AAS and LA−ICP−MS for Five Individuals

	CV AAS[a]	LA-ICP−MS				
individual identification	total Hg (mg/kg)	mean[b]	SD[b]	min[b]	max[b]	*n* shots[c]
1	<0.4	4.7	0.4	4.0	5.2	10
2	0.7	4.6	1.1	2.7	6.8	10
3	1	5.8	2.3	2.3	10.0	10
4	3.6	5.3	0.5	4.4	6.0	10
5	14.2	10.9	0.8	10.0	12.1	10

[a] Total Hg concentration measured in the first centimeter closer to the scalp. [b] The mean, standard deviation (SD), minimum (min), and maximum (max) refer to total Hg (mg/kg) [c] This refers to 10 independent ablation spots in the first centimeter closest to the scalp.

standard, ^{34}S, within each individual only ranged from 2.2% to 5.8%, the larger variation in relative Hg concentration observed along the hair strand is attributed to changes in endogenous Hg deposition within the hair strand. Further, the trend was neither consistently decreasing nor consistently increasing, thereby confirming that the data could not be attributed to an artifact induced by a systematic effect of laser sampling. Dietary intake records corresponding to the estimated dates of hair Hg measurements were not obtained thus limiting our ability to correlate intake with hair Hg concentrations.

Quantification of LA−ICP−MS signals has always been a challenge due to lack of well-characterized matrix-matched standards. The use of an internal standard, an element with uniform concentration within the matrix of interest is necessary to correct for variations in the ablation process between and within samples. For hair analysis, S was deemed a suitable candidate as several S-containing amino acids (cysteine, methionine, and cysteic acid) are found in hair (*10*) and studies have shown S to be constant within a given hair strand (*24*) and across a Swedish population (*23*). In this experiment, the observed low RSD for ^{34}S within a given hair strand confirms the suitability of S as an internal standard.

To obtain matrix-matched standards for quantification, Rodushkin et al. (*23*) quantified LA−ICP−MS signals from a wisp of hair strands (approximately 0.5 cm length) using a Chinese human hair powder SRM (GBW07601) which was compressed into a hard pellet. Stadlbauer et al. (*25*), using CRM397 hair reference material, also used this approach to quantify LA−ICP−MS signals from single hair strands. In both studies, S concentrations in the sample and the standard were assumed to be equivalent. However, multi-point calibration is considered more reliable than the one-point calibration used in refs *23* and *25*. Thus, we used four powdered hair SRMs with a wide range of reported Hg concentrations to construct a calibration curve. A significant correlation ($R^2 = 0.899$, $p < 0.05$) between the ^{202}Hg to ^{34}S ratio obtained by LA−ICP−MS and the certified Hg concentrations in the four SRMs was observed (Figure 1). Using this calibration curve, LA−ICP−MS signals obtained from a hair strand were quantified and then compared to the total Hg concentrations in matched 1-cm hair segments that were previously measured by CV-AAS (*26*, *27*) (Table 3). Significant differences ($p < 0.05$) were noted between total Hg obtained by LA−ICP−MS and CV-AAS for the split samples.

The calibration curve did not display linearity, particularly at the low end of the spectrum (Figure 1). Since there were only four SRMs, it was not possible to fit a nonlinear calibration curve. Several factors may have influenced the nonlinearity. First, precision (i.e., the RSD) of the Hg content within each SRM, expressed as the ratio of ^{202}Hg to ^{34}S ranged from 10 to 40% (Table 4). Second, the ^{202}Hg to ^{34}S ratio of the SRMs NIES13 and IAEA086 were on average 0.06 and 0.07,

TABLE 4. Total Counts and Relative Standard Deviation (RSD) (%) of ^{202}Hg, ^{34}S, and the ^{202}Hg to ^{34}S Ratio for Each Pelletized SRM

		202Hg		34S		202Hg/34S		
identification	total Hg (mg/kg)[a]	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	*n*
IAEA086	0.573	6290	15.2	89440	26.1	0.07	20.3	10
NIES13	4.42	8856	12.1	145996	16.3	0.06	10.8	10
BCR397	12.3	11519	34.7	39106	48.6	0.32	40.3	10
IAEA085	23.2	47399	44.9	54351	46.2	0.87	10.0	10

[a] Certified value reported by manufacturer.

respectively even though the certified Hg concentration of NIES13 is 7.7 times higher than that of IAEA086 (Table 4). At first, this appeared to be due to significant differences in ^{34}S content between the four SRMs ($F = 28.8$; $p < 0.0001$). However, follow-up analysis of S concentration showed that levels of S in the SRMs were similar and averaged 4.52% (IAEA 086, 4.54%, 4.55%, and 4.65%; NIES 13, 4.54%; BCR 397, 4.33%). These S concentrations are also similar to the average reported in ref *23*. The poor precision of the calibration may be a byproduct of the highly variable ablation/sampling process caused by the use of the laser on the hair powder pellet. The variability could be observed visually under the microscope as craters differed significantly in appearance from shot-to-shot. The same variability was not observed when sampling hair strands.

We explored an alternate quantification protocol whereby consecutive ^{202}Hg to ^{34}S ratios obtained with LA−ICP−MS were calibrated against the average Hg concentration in matched hair samples measured by CV-AAS. The rationale was based on our previous results (*17*) showing that the Hg concentration of a 12-cm hair strand significantly correlated ($r = 0.98$ with a slope of 1.03) with the average of the 12 matched 1-cm segments despite variations in Hg concentrations between each 1-cm segment. Assuming that the same is true within shorter hair segments, the average of consecutive ^{202}Hg to ^{34}S ratios within a defined hair segment should correspond to the measured Hg concentrations in the same spatial segment. Using this calibration approach, consecutive Hg concentrations within a single hair strand that corresponded to sequential daily measurements over a 1-month period were obtained (Figure 2).

An important prerequisite for the use of this quantification approach is the affirmation that the variation in Hg content between hair strands within the same individual is low. In this study, we showed that there was no significant intra-individual difference of replicate sample analysis (10 shots) when matched 1-cm segments of three individuals were compared (Table 5). Our results are supported by ref *25*, which reported reproducible LA−ICP−MS Hg measurements in 10 intra-individual hair strands. Further, the RSD of Hg concentration (as determined by C-GA-AAS) between five hair strands within an individual was determined to be low, averaging $6.5 \pm 2.8\%$ (*17*). Taken together, these results justify the use of single hair strands for Hg analysis and lend support to the proposed quantification approach.

Overall, our results confirm that LA−ICP−MS can be used to obtain longitudinal elemental profiles along a single hair strand of fish-eaters. However, without an external method such as CV-AAS to calibrate LA−ICP−MS signals, the raw or ^{34}S normalized LA−ICP−MS signals cannot be used to compare different individuals. Although current laser technology generally provides relatively stable signals, variability does occur during the ablation process (*29*). The variability has been partially attributed to the physical properties (such as reflectivity and density) of the material being ablated (*23*, *29*−*31*). In this study, the color, the weight, and the width

TABLE 5. Intra-Individual Variations in ^{34}S Normalized ^{202}Hg Content

individual identification	strand identification	*n* shots[a]	mean $^{202}Hg/^{34}S$	F	*p*-value
2	a	10	0.13	1.19	0.319
	b	10	0.14		
	c	10	0.16		
3	a	10	0.17	1.91	0.167
	b	10	0.22		
	c	10	0.16		
4	a	10	0.16	1.41	0.251
	b	10	0.14		

[a] Intra-individual replicates were ablated at the same sites.

of the hair differed between individuals. Adjustment for such factors on LA−ICP−MS signals has yet to be developed but should be the focus of future studies. Moreover, accumulation of Hg in human scalp hair is a complex process that is influenced by Hg metabolism and scalp hair production, which are in turn affected by individual criteria such as age, gender, health status, and type of exposure (*10*, *25*, and references therein). Since there are still uncertainties regarding the relationship between oral exposure and measured Hg concentrations in biomarkers such as hair (*32*), improving calibration of the LA−ICP−MS technique could help address this key issue of individual variability, particularly over sensitive periods such as gestation.

Despite potential problems during the calibration process, the challenges are offset by rapid analysis, little or no sample preparation, and spatially resolved multi-element analysis on micrometer segments of single hair strands. The combination of C-GA-AAS and LA−ICP−MS offers the potential to use few hair strands to reconstruct sequential short term (such as daily) exposure. An important application of this method involves assessment of prenatal fetal exposure to Hg. For the developing fetus, a sequence of meals with relatively high Hg content may be the toxicological relevant time scale. Thus, measurements in 1-cm or longer segments of maternal hair (equivalent to months of exposure) may mask peak exposure. In this context, improved measurements of the time course of exposure on the scale of a few days may be especially relevant to the developing fetus and may be achieved with hair analysis by LA−ICP−MS.

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