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DETERMINATION OF MERCURY SPECIATION IN FISH TISSUE WITH A DIRECT MERCURY ANALYZER

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Abstract: Knowledge of Hg speciation in tissue is valuable for assessing potential toxicological effects in fish. Direct Hg analyzers, which use thermal decomposition and atomic absorption spectrometry, have recently gained popularity for determining organic Hg after procedural solvent extraction from some environmental media, although quantitative recovery from lipid-rich materials, such as fish liver, has been problematic. The authors developed a new method by which organic Hg in fish liver and muscle is estimated by the difference between direct measurements of inorganic Hg in an acid extract and total Hg in whole tissue. The method was validated by analysis of a certified reference material (DOLT-4 dogfish liver) and naturally contaminated fish tissues with comparison to an established Hg speciation method (gas chromatography cold vapor atomic fluorescence spectrometry). Recovery of organic Hg from DOLT-4, estimated by difference, averaged 99 ± 5% of the mean certified value for methylmercury. In most liver samples and all muscle samples, estimates of organic Hg from the proposed method were indiscernible from direct speciation measurements of methylmercury (99% ± 6%). Estimation of organic Hg by the difference between total Hg and inorganic Hg was less accurate in liver samples with a high percentage of inorganic Hg (90%). This was because of the increased uncertainty that results from estimating a third value (i.e., organic Hg) by using the difference between two large concentrations (inorganic and total Hg). The proposed method is a useful tool for examining the speciation of Hg in fish muscle and liver, and by extension, potentially other tissues and environmental media. *Environ Toxicol Chem* 2013;32:1237–1241. © 2013 SETAC

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INTRODUCTION

Mercury is an environmental contaminant that poses health risks to humans and wildlife [1,2]. In aquatic ecosystems, the conversion of inorganic Hg (Hg[II]) to methylmercury (MeHg) and the subsequent biomagnification of MeHg in food webs results in high concentrations in fish [3]. Humans are primarily exposed to MeHg through the consumption of fish and shellfish; accordingly, risk assessments and monitoring programs are based on the concentration of MeHg in fish muscle or whole body [4]. Methylmercury contamination of aquatic environments is extensive and negatively affects fish health; a recent study, for example, estimated that more than 40% of walleye (*Sander vitreus*) populations in the Great Lakes region of North America are at risk of MeHg toxicity [5]. Methylmecury has been shown to negatively impact survival, growth, behavior, and reproduction of fish [6–10]. Additional effects have been observed in the liver of fish, where Hg causes damage through oxidative stress [11]. Ingested MeHg passes through the intestinal wall to blood, in which it is transported by the portal vein to the liver. The liver therefore has a first pass at either accumulating or detoxifying MeHg before it is circulated to other tissues. Primary detoxification of MeHg involves complexation with glutathione before excretion into bile and transfer to the intestine [12,13]. Hepatic demethylation of MeHg, which has been shown to occur in marine mammals, also may be carried out in fish [14,15]. Although Hg(II) is less likely than MeHg to bioaccumulate and biomagnify, several studies have observed greater occurrence of liver damage with increasing Hg(II) in fish [11,16,17]. Thus, knowledge of Hg speciation is valuable for assessing potential toxicological effects in fish.

Multiple analytical techniques may be used for Hg speciation analysis in tissues, including, for example, high-performance liquid chromatography–inductively coupled plasma mass spectrometry [18], Mercury-thiourea complex liquid chromatography cold vapor atomic fluorescence spectrometry [19], and gas chromatography cold vapor atomic fluorescence spectrometry (GC-CVAFS) [20]. Recently, direct Hg analyzers, such as the Milestone DMA-80, have gained popularity for analysis of total Hg because of their ability to analyze both liquid and solid matrices effectively, high sample throughput, and relatively low detection limits and cost [21,22]. The DMA-80 analysis involves thermal decomposition followed by gold amalgamation and detection with atomic absorption spectrometry. Several studies also have used direct Hg analysis to quantify MeHg (or organic Hg) in various sample matrices [23–26]. Often this is done by chemically digesting the material, procedurally isolating MeHg from forms of Hg(II) by extraction with an organic solvent, back-extracting MeHg into aqueous solution, and measuring total Hg in the extract with a direct analyzer [23–26]. Measured total Hg in the final extract is interpreted as either MeHg or organic Hg.

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Prior studies have had varying degrees of success with quantitative extraction of organic Hg from various matrices for direct Hg analysis. Nam and Basu [25] digested biological reference materials with a tris-buffered protease solution that was then treated with NaOH, cysteine, $CuSO_4$, and acidic NaBr, followed by organic Hg extraction with toluene and back extraction into an aqueous solution for analysis with a DMA-80. Recoveries of organic Hg from the reference materials were excellent and ranged between 86% and 107% relative to certified values for MeHg [25]. Maggi et al. [24] used a different digestion method with hydrobromic acid, followed by toluene extraction, back extraction with an L-cysteine solution, and analysis by DMA-80 to analyze various certified reference materials as well as environmental samples. Using this approach, they recovered more than 80% of MeHg from multiple reference materials, but recovery averaged only 74% from dogfish liver reference material (DOLT-3). Similarly, Scerbo and Barghigiani [23] noted an increased degree of variation in the recovery of organic Hg from liver reference material DOLT-1, which they attributed to the relatively high lipid content of fish liver that affected quantitative back extraction with L-cysteine [23].

We propose and validate a new method for the quantification of Hg(II) in fish muscle and liver with a direct Hg analyzer. Tissue is digested in acid with microwave heating, and organic Hg is extracted with toluene. The novel aspect of this approach is that the remaining acidic fraction, containing Hg(II), is analyzed instead of organic Hg in the nonpolar fraction. This approach, in contrast to prior methodologies, does not involve an additional step of analyte back extraction into an aqueous phase, and it thereby minimizes both random errors and the potential for procedural biases associated with lipid-rich matrices from which back-extraction may not be quantitative, as observed by others [23,24]. Measured Hg(II) concentrations are subtracted from those of total Hg, determined with the same instrument, to estimate MeHg by difference. Also in contrast to prior studies, this method was validated with naturally contaminated fish muscle and liver tissue that were analyzed with the proposed technique and an established speciation method (GC-CVAFS), in addition to analysis of a certified reference material.

MATERIALS AND METHODS

Reagents and standards

Reagents used for digestion and extraction included high-purity HCl (J.T. Baker, A.C.S. grade) and toluene (Fisher, A.C. S. grade). Calibration standards and known additions of Hg were made with a solution traceable to the US National Institute of Standards and Technology (US NIST). All dilutions were made with reagent-grade water (resistivity $\geq$ 18 MΩ-cm).

Sampling and sample preparation

Arctic char (*Salvelinus alpinus)* livers were collected from 5 lakes on Cornwallis Island (Nunavut, Canada) in July 2009 (Amituk Lake) and 2010 (Small, 9 Mile, North, and Char lakes). Spotted gar (*Lepisosteus oculatus*), largemouth bass (*Micropterus salmoides)*, bowfin (*Amia calva*), and channel catfish (*Ictalurus punctatus*) were collected from Caddo Lake (Texas/Louisiana, USA) in June and July of 2007 and 2011. Muscle and liver samples were dried and homogenized before Hg analysis. Extractions and analyses of certified reference material DOLT-4 (dogfish liver), which has a lipid content of 6.3% by mass, also were used to validate the proposed method.

Mercury analyses

DMA-80 total Hg analyses. All tissue samples were analyzed for total Hg at Institut National de la Recherche Scientifique -Centre Eau Terre Environnement with a DMA-80 (Milestone). Our quality assurance protocol included calibration with US NIST-traceable standards, analyses of certified reference materials from the National Research Council of Canada (MESS-3 marine sediment, TORT-2 lobster hepatopancreas, and DOLT-4 dogfish liver), and duplicate samples. All analyses of total Hg in reference materials were within certified ranges, and the relative difference between duplicate samples averaged 1.2% ($n = 15$).

DMA-80 Hg(II) analyses of fish muscle and liver. Our sample digestion method for Hg speciation analysis was modified from that of Ashoka and colleagues [27]. Briefly, between 50 and 100 mg dried sample was weighed accurately into a 50-mL centrifuge tube to which was added 1 mL 12 M HCl. Samples in capped tubes were irradiated in a conventional 1100 W microwave oven at 30% power for 30 sec and then allowed to cool for 5 min and irradiated again at 30% power for 30 sec. After cooling, 5 mL toluene was added to each sample, the tubes capped and shaken with a wrist-action shaker for 20 min, and then centrifuged at 966 *g* for 15 min. Supernatant toluene was transferred to a separate 50-mL tube. The acidic fraction remaining after toluene extraction of the original digestate was diluted 5-fold with reagent-grade water, and an aliquot was added to a quartz analytical vessel for quantification of ionic Hg with a DMA-80. Continual analysis of samples containing the maximum allowable HCl concentration (10%) can increase corrosion of the instrument. We chose to reduce the HCl concentration of our samples, by dilution with reagent-grade water, to approximately 7% to reduce the risk of corrosion to instrument components. Quality assurance analyses included reagent blanks, known additions to reagent blanks (10, 20, 100, and 1 000 ng), and duplicate samples. Recovery of known additions ($n = 10$) averaged 92% relative to calibration standards, reagent blanks had no detectable amount of Hg, and the relative difference between duplicate samples averaged 2.1% ($n = 10$). The method detection limit was 0.41 ng Hg and was estimated by analyzing 7 replicates of known Hg additions to reagent solutions (20 ng Hg) and multiplying the standard deviation among replicates by 3.14, the *t* value for a 99% confidence interval.

MeHg and total Hg analyses of fish muscle and liver by CVAFS. Subsamples of Arctic char liver ($n = 20$), spotted gar liver ($n = 4$), bowfin liver ($n = 3$), catfish liver ($n = 3$), largemouth bass liver ($n = 3$), and muscle ($n = 3$) were digested with 7 mL 4.57 N HNO_3 for 12 h in a 60 °C water bath [28]. Digestates were analyzed for MeHg after derivatization with sodium tetraethylborate by flow-injection GC-CVAFS [20,29]. Analyses were calibrated with MeHg standards and blanks taken through the digestion procedure. Methylmercury standards were calibrated against elemental Hg [30] and an US NIST-traceable Hg(II) solution. Relative difference between duplicate digestates of the same sample ($n = 13$) averaged 3.2%. All analyses of 2 standard reference materials (TORT-2 and DORM-3 fish protein; $n = 9$ each) were within the certified ranges.

Total Hg also was measured in digestates of all fish tissues by dual-Au amalgamation CVAFS [31] after HNO_3 digestion and BrCl oxidation [28]. Analyses were calibrated with an Hg(II) solution traceable to US NIST. All analyses of total Hg in TORT-2 and DORM-3 ($n = 9$ each) were within certified ranges, and

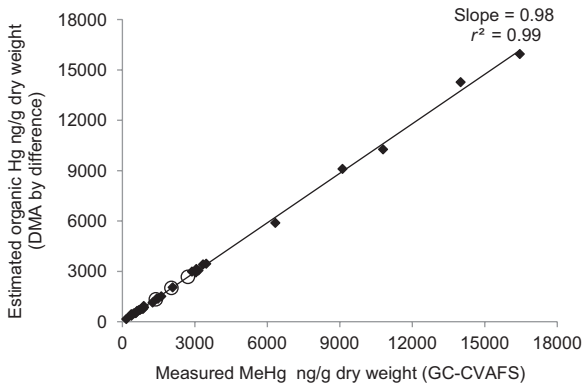

Figure 1. Comparison of MeHg measured by gas chromatographic cold-vapor atomic fluorescence spectrometry (GC-CVAFS) and estimated organic Hg (by DMA-80 with proposed method) in fish liver (diamonds) and muscle tissue (circles).

the relative difference between duplicate digestates of the same sample ($n = 18$) averaged 2.5%.

RESULTS AND DISCUSSION

Method validation with certified reference material

DOLT-4 has lipid (6.3% by mass), MeHg (1 330 ng/g), and total Hg (2 580 ng/g) contents that are similar to those in livers of many other fish species and should therefore pose a representative analytical challenge. Measured concentrations of Hg(II) in the acidic fraction of DOLT-4 digestates (1 243 ± 66 ng/g) were not significantly different from those estimated by difference between mean certified values for total Hg (2 580 ± 220 ng/g) and MeHg (1 330 ± 120 ng/g; paired *t* test, $p = 0.69$). Recovery of Hg(II) from DOLT-4 averaged 99 ± 5% of the difference between mean certified values ($n = 8$). Organic Hg can be estimated as the difference between measured concentrations of total Hg and Hg(II). With the proposed method, the mean estimated recovery of organic Hg from DOLT-4, by difference, was 100 ± 5% of the certified mean value for MeHg. This is a substantial improvement over our own attempts (70 ± 2%), and those of others [23,24], to determine organic Hg directly in organic solvent extracts of liver.

Hg in naturally contaminated fish tissues

Organic Hg in char, catfish, bowfin, and largemouth bass livers and muscle was estimated by the difference between measured concentrations of total Hg and Hg(II) and compared with direct speciation analysis of MeHg by GC-CVAFS. In these samples, concentrations of total Hg ranged from 460 to 40 700 ng/g dry weight and Hg(II) from 49 to 36 700 ng/g. Mercury (II) comprised a relatively minor fraction of total Hg in Arctic char livers (mean, 14%), largemouth bass livers (mean, 42%), and largemouth bass muscle (mean, 10%). In contrast, Hg (II) was the major Hg species in livers of bowfin (mean, 81%), channel catfish (mean, 83%), and spotted gar (mean, 96%). Variation in Hg speciation (inorganic and organic) has been observed in fish livers both across and within species [32–35], and the reason for this variation is the subject of current research. Estimates of MeHg, calculated by the difference between measured concentrations of total Hg and Hg(II) with the proposed method, were in excellent agreement with direct speciation measurements of MeHg by established GC-CVAFS techniques (mean recovery, 99% ± 6%; Figure 1) for all liver and muscle samples except spotted gar liver.

Hg in spotted gar livers

In contrast to the other fish livers analyzed, spotted gar had much greater concentrations of total Hg (mean, 28 200 ± 12 100 ng/g dry wt), of which only a small fraction was as organic Hg (mean, 4.1%; Table 1). In spotted gar livers, concentrations of total Hg measured by DMA-80 and CVAFS differed by less than 3%, which, as noted, was comparable to the procedural variability between duplicate samples for both methods. However, concentrations of organic Hg in gar livers, estimated by the difference between measured Hg(II) and total Hg, averaged 183% greater than those determined by GC-CVAFS. These differences in estimated organic Hg versus measured MeHg are likely attributable to the increased uncertainty that results from estimating a third value (i.e., organic Hg) by difference between two large values (Hg[II] and total Hg). The proposed method was developed to measure Hg(II), and despite the difficulties in estimating organic Hg in gar livers, the method accurately quantifies inorganic Hg. Interestingly, calculated Hg (II) (calculated by subtracting MeHg from total Hg measured by GC-CVAFS) and measured Hg(II) were within 6% of one another, suggesting that Hg(II) was accurately measured in these samples.

To avoid analysis of a complete fish liver or muscle, samples are routinely homogenized and subsampled for Hg analysis. Analysis of homogenized subsamples provides a way to estimate the true Hg concentration of a complete tissue. We analyzed duplicate samples of fish liver and muscle tissue for both total Hg and Hg(II). The percentage of difference between duplicate samples was 1.2% for total Hg and 2.1% for Hg(II) analyses. These small percent differences between duplicate samples demonstrate that our tissues were well homogenized and all subsamples taken were representative of the complete tissue. Some variation in Hg concentration is expected, but it prevents the accurate estimation of MeHg in high percent Hg(II) samples

Table 1. Comparison of mercury speciation determined with the proposed DMA-80 method and gas chromatographic cold-vapor atomic fluorescence spectrometry (GC-CVAFS) technique in livers of 4 spotted gar from Caddo Lake (TX/LA)

	DMA-80			GC-CVAFS	
Fish	Total Hg (ng/g dry wt)	Hg(II) (ng/g dry wt)	Estimated MeHg[a] (ng/g dry wt)	Measured MeHg (ng/g dry wt)	MeHg percentage of recovery DMA vs GC-CVAFS
Spotted gar 1	16 229	15 287	942	581	162.1
Spotted gar 2	19 661	18 259	1 402	1 226	114.4
Spotted gar 3	36 374	33 851	2 524	1 310	192.6
Spotted gar 4	40 716	36 735	3 982	1 510	263.7

[a]Methylmercury estimated by subtracting inorganic mercury from total mercury.

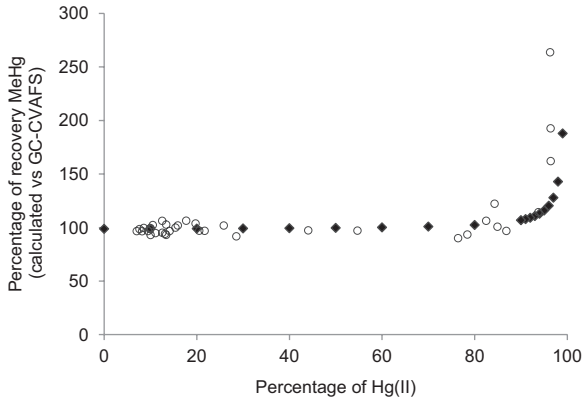

Figure 2. Comparison of percentage of recovery of MeHg (calculated vs measured by gas chromatographic coldvapor atomic fluorescence spectrometry [GC-CVAFS]) and percentage of inorganic Hg. Diamonds represent hypothetical samples, and circles represent samples from the present study.

(>90%), such as spotted gar. By using the percent differences between duplicate samples as an estimate for error, we were able to model percentage of recovery of MeHg versus percentage of Hg(II) for a group of hypothetical samples and determine the point at which our method no longer provides accurate estimates of MeHg (Figure 2). These hypothetical samples were assigned differing total Hg concentrations (500, 5 000, or 50 000 ng/g) and percentage of Hg(II) concentrations (0–100%) before their values were adjusted using our calculated percentage differences. Both MeHg and adjusted MeHg values were calculated (by difference between total Hg and Hg[II]), and their percentages of recovery are presented in Figure 2. Based on the hypothetical data and the data from the present study, poor estimation of MeHg appears to be independent of total Hg concentration.

Thus, the proposed method is an excellent approach to directly quantify Hg(II) in fish liver and muscle and, from that, to estimate organic Hg in samples with less than 90% Hg(II). The robustness of this method is supported by the accurate estimation of organic Hg across a range of organic and total Hg concentrations and widely varying percentage of organic Hg values. Greater uncertainty of organic Hg estimates is likely to occur when Hg(II) is the major fraction of a large total Hg concentration, as in the case of spotted gar liver. A recent study by Chumchal and colleagues [35] also reported elevated total Hg concentrations and high percentage of Hg(II) in spotted gar livers from Caddo Lake. Aside from these two studies, no reported cases exist of such elevated total Hg concentrations coupled with high percentage of Hg(II) in fish liver. In addition to the present study, other studies have noted that MeHg is the predominant form of Hg in fish muscle tissue [11,36]. Thus, the total Hg concentrations and high relative Hg(II) concentrations found in spotted gar livers are not common to most fish, meaning the proposed method is appropriate for estimating organic Hg in most fish liver and muscle samples. The lack of certainty in estimated organic Hg concentrations in spotted gar livers is not overwhelmingly significant from an ecotoxicological perspective because the toxicological threat of organic Hg in gar liver is likely outweighed by the much more abundant inorganic Hg species. Nevertheless, estimates of either organic Hg or MeHg in samples with relatively high fractions of Hg(II) and elevated total Hg concentrations should be interpreted with caution, and a more traditional direct measure of MeHg may be more appropriate.

CONCLUSION

We have described a new low-cost and rapid (~60 min) procedure for the determination of Hg(II) and organic Hg in fish liver and muscle tissue by direct Hg analysis. Cost estimates of the proposed method should include the requirement of two analyses (total Hg and Hg[II]) to obtain organic Hg by difference. In addition, the method allows for the generation of percentage of MeHg data that can be of assistance in biomagnification and demethylation studies. Other studies have used the DMA-80 to measure MeHg in fish tissues [23–26], but these techniques use many hazardous or greater quantities of chemicals that contribute to the cost of analysis and waste disposal. Moreover, some prior techniques appear to have shortcomings related to quantitative extraction of organic Hg from lipid-rich biological materials such as fish liver. The proposed method was validated with analyses of reference material DOLT-4 and naturally contaminated fish liver and muscle that were compared with direct speciation measurements by GC-CVAFS. Greater uncertainty of organic Hg, and perhaps a positive bias, was observed for livers in which Hg(II) constituted most elevated total Hg concentrations. Therefore, estimates of either organic Hg or MeHg in samples with greater than 90% Hg(II) should be interpreted with caution and evaluated for methodological bias.

With further development, this method should be useful to determine Hg(II) and estimate organic Hg in other tissues, organisms, and environmental media. Application of the method should be limited to materials with Hg concentrations that, for a given mass or volume, will exceed the method detection limit of approximately 0.4 ng Hg.

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