



# Determination of mercury in fish otoliths by cold vapor generation inductively coupled plasma mass spectrometry (CVG-ICP-MS)<sup>☆</sup>

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## ABSTRACT

A method based on cold vapor generation inductively coupled plasma mass spectrometry (CVG-ICP-MS) has been developed for determination of inorganic mercury, Hg(II), and total mercury in fish otoliths. Sodium borohydride (NaBH<sub>4</sub>) was used as the only reducing agent and its concentration was optimized across an acidity gradient to selectively reduce Hg(II) without affecting methylmercury, CH<sub>3</sub>Hg(I). Inorganic Hg was quantitatively reduced to elemental mercury (Hg<sup>0</sup>) with  $1 \times 10^{-4}\%$  (m/v) NaBH<sub>4</sub>. CH<sub>3</sub>Hg(I) required a minimum of 0.5% (m/v) NaBH<sub>4</sub> for complete reduction. Increasing the HCl concentration of solution to 5% (v/v) improved the selectivity toward Hg(II) as it decreased the signals from CH<sub>3</sub>Hg(I) to baseline levels. Potassium ferricyanide solution was the most effective in eliminating the memory effects of Hg compared with a number of chelating and oxidizing agents, including EDTA, gold chloride, thiourea, cerium ammonium nitrate and 2-mercaptoethylamine chloride. The relative standard deviation (RSD) was less than 5% for 1.0  $\mu\text{g L}^{-1}$  Hg(II) solution. The detection limits were 4.2 and 6.4 ng L<sup>-1</sup> (ppt) for Hg(II) and total Hg, respectively. Sample dissolution conditions and recoveries were examined with ultra-pure CaCO<sub>3</sub> (99.99%) spiked with Hg(II) and CH<sub>3</sub>HgCl. Methylmercury was stable when dissolution was performed with up to 20% (v/v) HCl at 100 °C. Recoveries from spiked solutions were higher than 95% for both Hg(II) and CH<sub>3</sub>Hg(I). The method was applied to the determination of Hg(II) and total Hg concentrations in the otoliths of red emperor (CRM 22) and Pacific halibut. Total Hg concentration in the otoliths was  $0.038 \pm 0.004 \mu\text{g g}^{-1}$  for the red emperor and  $0.021 \pm 0.003 \mu\text{g g}^{-1}$  for the Pacific halibut. Inorganic Hg accounted for about 25% of total Hg indicating that Hg in the otoliths was predominantly organic mercury (e.g., methylmercury). However, as opposed to the bioaccumulation in tissues, methylmercury levels in otoliths was very low suggesting a different route of uptake, most likely through the deposition of methylmercury available in the water.

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## 1. Introduction

Fish otoliths are calcium carbonate minerals (as aragonite) in the head of fish that aid in balance and hearing to the fish [1,2]. These aragonite minerals grow throughout the life of fish by deposition of calcium carbonate in concentric layers on a proteinaceous matrix. In the meantime, trace elements from the surrounding water successively incorporate into the newly forming aragonite layer. The aragonite polymorph is not susceptible to resorption. Therefore, the temporal concentrations of the trace elements (so-called fingerprints) remain unchanged throughout the fish's lifetime, and

consequently integrate over the fish's life history when a whole is dissolved [1–7].

Trace elements and heavy metals make up less 1% (by mass) of an otolith. With the exception of Sr, their concentrations range from low ng g<sup>-1</sup> to a few  $\mu\text{g g}^{-1}$  [3,8–14]. The utilization of trace elements as robust biological tags in otolith micro-chemical analysis has been largely due to the use of inductively coupled plasma mass spectrometry (ICP-MS) as a highly sensitive tool. Nevertheless, accurate determination of most trace elements and heavy metals, with the exception of relatively abundant Mg, Cu, Mn and Zn, from the otoliths is still a challenging task by direct analysis [3,4,6,8–10]. Various analytical methods, including isotope dilution [4,15], solvent extraction [16], solid phase extraction [6,17–20], co-precipitation [21] and hydride generation [22] have been developed to overcome the difficulties associated with low elemental concentrations in a highly saline calcium matrix.

Mercury (Hg) in the aquatic ecosystems mainly originates from the deposition of atmospheric Hg released from the anthropogenic

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activities [23–25]. Inorganic Hg in water is converted by bacteria to highly toxic methylmercury that accumulates in the sediments [23,25–27]. While microorganisms, such as phytoplankton and zooplankton ingest methylmercury ( $\text{CH}_3\text{Hg}$ ) from water, dietary uptake is the major route of exposure of fish to methylmercury [25,28–30]. It is now well-documented that most Hg in fish tissue is in the form of methylmercury, although total body burden could vary with geological, biological and physiological differences among species [31–33].

The concentrations for a number of minor and major elements (Al, Na, Cl, Sr, Ca, Si) in bluefin tuna otoliths were reported to vary with total Hg body burden [34]. Further, laboratory exposures conducted with different fish indicate that the uptake of Hg into otoliths is related with its concentration in the water [35]. To date, however, Hg has not been considered as a biological tracer in otolith microchemistry, which is due in part to measurement difficulties and relatively low sensitivity of solution-based ICP-MS to this element. Thus, there is no information about the chemical forms of Hg in fish otoliths and whether otolith Hg could aid in population studies or not.

In this study, we have developed a cold vapor generation method for determination of Hg(II) and total Hg in otoliths by ICP-MS in an attempt to elucidate the chemical forms of Hg in fish otoliths, and to provide an insight about its source and utility in otolith microchemistry. Sodium borohydride ( $\text{NaBH}_4$ ) was used as reducing agent to discriminate between the Hg(II) and total Hg levels. Studies were performed with a number of chelating and oxidizing reagents to eliminate the memory effects. Effects of acid dissolution on the stability and recoveries of Hg species were examined by spiking Hg(II) and  $\text{CH}_3\text{HgCl}$  to ultra-pure calcium carbonate. The method was applied to the determination of Hg(II) and total Hg in otolith samples from two different oceanic fish species, red emperor and Pacific halibut.

## 2. Experimental

### 2.1. Reagents and solutions

Deionized water produced by Barnstead™ E-Pure system with minimum resistivity of  $17.8 \text{ M}\Omega \text{ cm}$  was used throughout. A  $1.0 \mu\text{g mL}^{-1}$  Hg(II) solution was prepared from a  $1000 \mu\text{g mL}^{-1}$  standard solution (Sigma Aldrich) and stored in 5% (v/v)  $\text{HNO}_3$  (Trace metal grade, Fisher Scientific). Methylmercury chloride ( $\text{CH}_3\text{HgCl}$ ) solution ( $1000 \mu\text{g mL}^{-1}$  in water) was purchased from Alfa Aesar (99.99%). A  $1.0 \mu\text{g mL}^{-1}$   $\text{CH}_3\text{HgCl}$  stock solution was prepared and stored in water. All experimental solutions and calibration standards were prepared from these standard solutions. Trace metal grade hydrochloric acid (HCl, BDH Chemicals) was used for dissolution of samples and preparation of experimental solutions. Sodium borohydride ( $\text{NaBH}_4$ , 99.9%, Sigma Aldrich) was used as reducing agent. Aqueous solutions of  $\text{NaBH}_4$  were stabilized in 0.1% (m/v) NaOH (99.9%, Sigma Aldrich). All other reagents, including ethylenediaminetetraacetic acid (EDTA), potassium ferri-cyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ], thiourea, L-cysteine, 2-mercaptoethyamine chloride, cerium(IV) ammonium nitrate [ $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$ ], and gold chloride ( $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ ) were reagent grade.

### 2.2. Instrumentation

A Varian 820MS ICP-MS instrument (Varian, Australia) was used. The instrument was equipped with a peltier-cooled double-pass glass spray chamber, micromist nebulizer, standard one-piece, low flow, ball-and-socket connection quartz torch, standard Ni sampler and skimmer cones, patented Collision Reaction Interface (CRI), a unique  $90^\circ$  ion mirror delivering exceptional sensitivity,

**Table 1**

Operating parameters for Varian 820-MS ICP-MS instrument for cold vapor generation.

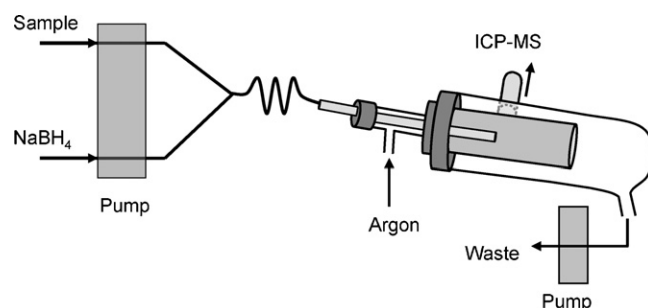
ICP-MS	
RF power (kW)	1.4
Plasma Ar flow ( $\text{L min}^{-1}$ )	18
Auxiliary Ar flow ( $\text{L min}^{-1}$ )	1.8
Nebulizer Ar flow ( $\text{L min}^{-1}$ )	1.2
Sheath Ar flow ( $\text{L min}^{-1}$ )	0.1
Sampling depth (mm)	6
Pump rate (rpm)	25
Stabilization time (s)	50
Spray chamber temperature ( $^\circ\text{C}$ )	3
Scan mode	Peak hopping
Dwell time (ms)	20
Points/peak	3
Scans/peak	5
Scans/replicate	10
CRI gas flow	0
Isotopes measured	$^{200}\text{Hg}$ and $^{202}\text{Hg}$
Vapor generation	
Sample acidity (% (v/v) HCl)	5.0
Sample flow rate ( $\text{mL min}^{-1}$ )	2.0
$\text{NaBH}_4$ flow rate ( $\text{mL min}^{-1}$ )	1.0

all-digital detector; Discrete Dynode Electron Multiplier (DDEM, Model AF250, ETP Australia) providing nine decades of linear dynamic range. Samples were introduced manually. The instrument was optimized daily for sensitivity, doubly charged ions (<2%) and oxides (<3%) with  $5 \mu\text{g L}^{-1}$   $^{138}\text{Ba}$ ,  $^{25}\text{Mg}$ ,  $^{115}\text{In}$ ,  $^{140}\text{Ce}$ ,  $^{208}\text{Pb}$  solution. Data collection was achieved by ICP-MS Expert software package (version 2.2 b126). The operating parameters of the instrument are summarized in Table 1.

Schematic diagram of the cold vapor generation manifold is illustrated in Fig. 1. The stand-alone spray chamber with 100 mL inner volume was used as gas–liquid separator. The nebulizer housing was fitted with a T-adaptor through which the sample line was extended into the spray chamber (Fig. 1). Nebulizer argon was introduced as carrier gas through the T-inlet. Tygon pump tubings were used for sample (1.14 mm i.d., red/red) and  $\text{NaBH}_4$  (0.76 mm i.d., black/black). The waste line (2.79 mm i.d., purple/white) was on a separate peristaltic pump (Ismatec). The reaction line (e.g., transfer line) was 30 cm long PTFE tubing (0.8 mm i.d.) extending from the mixing point of sample solution and  $\text{NaBH}_4$  lines into the spray chamber.

### 2.3. Otolith samples

Fish otolith certified reference material (CRM 22) was purchased from the National Institute of Environmental Studies (NIES), Japan. The material was prepared from sagittal otoliths



**Fig. 1.** Schematic diagram of cold vapor generation manifold and gas–liquid separator (GLS). The GLS is the stand-alone double pass spray chamber. Optimum CVG conditions: sample acidity = 5% (v/v) HCl;  $\text{NaBH}_4 = 1 \times 10^{-4}\%$  (m/v) for Hg(II) and 0.5% (m/v) for total Hg.

of emperor snapper (*Lutjanus sebae*) collected from the northwest coast of Western Australia. It is certified for Ba, Ca, K, Mg, Na and Sr along with indicative values for Cd, Cu, Pb and Zn. Otolith samples of adult Pacific halibut (*Hippoglossus stenolepis*) collected from the eastern Pacific Ocean were kindly provided by NOAA James Howard Marine Laboratory, Sandy Hook, NJ.

#### 2.4. Otolith dissolution procedure

The otolith sample of emperor snapper was received as fine powder. Those of Pacific halibut were ground using agate mortar and mill, passed through 120 mesh (125  $\mu\text{m}$ ) stainless steel sieves, and stored in an acid-cleaned plastic container. Approximately 50 mg powdered otolith sample was placed in teflon tubes (60 mL inner volume). Each sample was first wetted with water and slowly dissolved with 0.5 mL of 5% (v/v) HCl. Then, 3 mL of 20% (v/v) HCl was added to the pre-dissolved samples. The tubes were screw-capped tightly and heated 100 °C for 1 h on a 48-well digestion block (Digiprep MS, SCP Science, Champlain, NY). At the conclusion of heating, the tubes were cooled to room temperature, and the contents were diluted to 12 mL yielding a solution in about 5% (v/v) HCl.

#### 2.5. Optimization of vapor generation conditions

The experimental conditions for generation of Hg vapor were carried out by a univariate procedure. Initially, the acidity of the solutions was examined from 0% to 5% (v/v) HCl for 10  $\mu\text{g L}^{-1}$  Hg(II) and  $\text{CH}_3\text{HgCl}$  solutions by using 1% (m/v)  $\text{NaBH}_4$  as reductant. Then, the concentration of  $\text{NaBH}_4$  solution was optimized at the optimum HCl concentration to affect Hg vapor generation from 10  $\mu\text{g L}^{-1}$  of Hg(II) and  $\text{CH}_3\text{HgCl}$  solutions. Flow rates of carrier gas and sample solution were examined for the optimum conditions for HCl and  $\text{NaBH}_4$ . Carrier argon flow rate was adjusted for the highest Hg signals by tuning the nebulizer argon flow rate from 0.8 to 1.5  $\text{L min}^{-1}$ . The flow rate of sample solution was varied from 0.7 to 2.6  $\text{mL min}^{-1}$  for 10  $\mu\text{g L}^{-1}$  Hg(II) solution to find the optimum settings.

#### 2.6. Examining memory effects and washout conditions

Signal stability and memory effects were investigated for 10  $\mu\text{g L}^{-1}$  Hg(II) solution which was reacted on-line with 1% (m/v)  $\text{NaBH}_4$  for about 150 s followed by a series of washout solutions to remove the residual Hg. These solutions included gold chloride solution (10  $\mu\text{g mL}^{-1}$  Au), 0.2% (m/v) EDTA, 0.5% (m/v) thiourea, 0.5% (m/v) L-cysteine, 0.5% (m/v) 2-mercaptoethylamine chloride, 0.5% (m/v) cerium(IV) ammonium nitrate, and 0.5% (m/v) potassium ferricyanide. They were all run through the sample line and mixed on-line with the  $\text{NaBH}_4$  solution just as the sample solutions.

#### 2.7. Examining methylmercury stability and matrix effects

The effect of acid dissolution on the stability of  $\text{CH}_3\text{Hg(I)}$  was investigated for 10  $\mu\text{g L}^{-1}$   $\text{CH}_3\text{HgCl}$  solutions prepared in 10 mL of 5, 10, 20, and 50% (v/v) HCl in 50-mL polypropylene tubes. The tubes were screw-capped and heated on the digestion block at 100 °C for 1 h. All solutions were first cooled to room temperature and then analyzed for Hg(II) and total Hg by CVG-ICP-MS. To determine Hg(II) concentration, 1% stannous chloride ( $\text{SnCl}_2$ , 98%) in 5% (v/v) HCl solution was used as reducing agent.

Acid dissolution of fish otoliths yields high levels of calcium (as chloride). Thus, the effect of calcium matrix was investigated on the recoveries of Hg(II) and  $\text{CH}_3\text{Hg(I)}$  in the last stage of the optimization studies. In this case, appropriate masses of pure  $\text{CaCO}_3$  (99.99%) were weighed into teflon tubes and were spiked with

either Hg(II) or  $\text{CH}_3\text{HgCl}$  to yield 10  $\mu\text{g L}^{-1}$  concentrations of each in the analysis solution. The contents were dissolved similarly as described by adding 20% (v/v) HCl slowly. The solutions were then heated in closed-tubes at 100 °C for 1 h, cooled to room temperature and then completed to 12 mL with water. Three replicate measurements were made for both Hg species by CVG-ICP-MS.

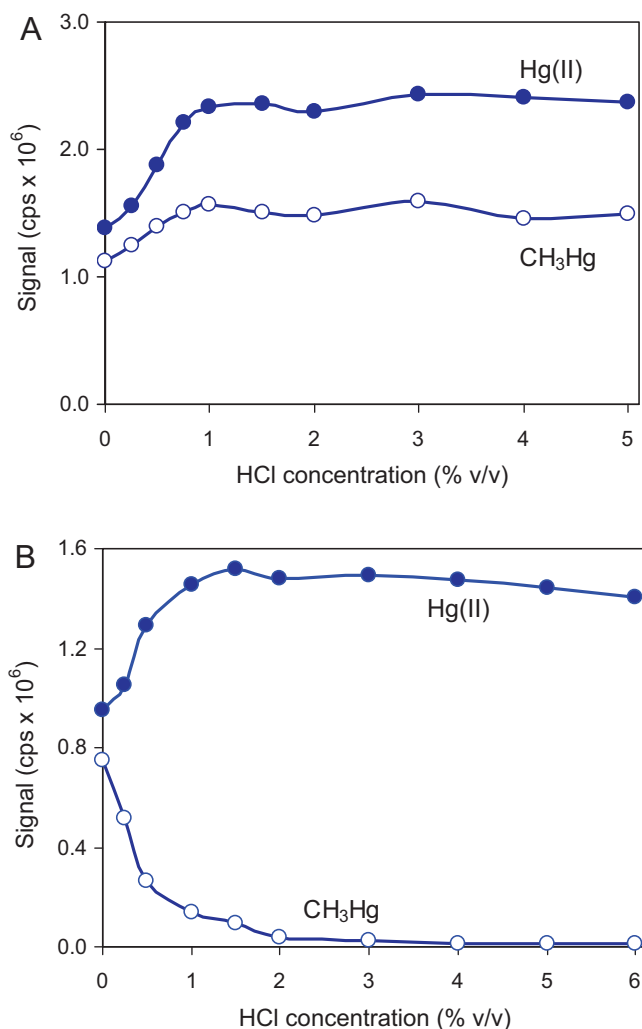
### 3. Results and discussion

#### 3.1. Optimum conditions for vapor generation

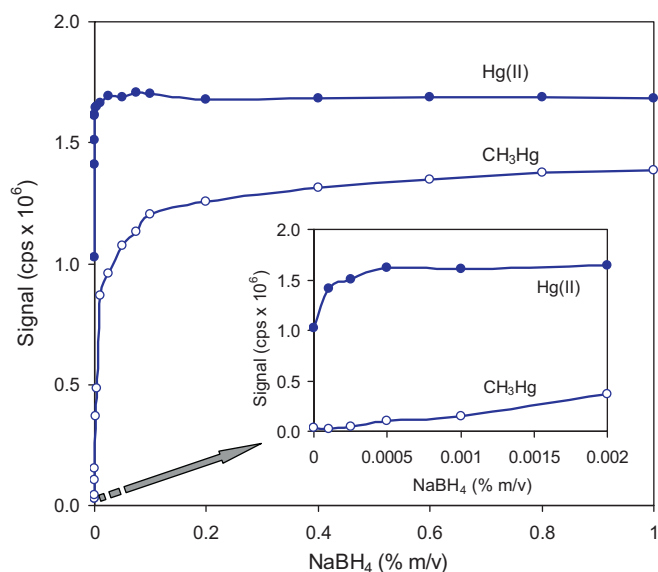
Cold vapor generation (CVG) has been the preferred method for determination of Hg by atomic absorption (AAS) and atomic fluorescence spectrometry (AFS) as it offers improved sensitivity and lower detection limits [36–41]. Unlike many other heavy metals, direct determination of Hg by ICP-MS suffers from memory effects due to the adsorption of Hg throughout the sampling system. In addition, ICP-MS exhibits relatively low sensitivity to Hg due to the low abundances of natural Hg isotopes and its high first ionization potential (10.4 eV). Thus, CVG has also been interfaced to ICP-MS as an attractive sample introduction method to enhance the sensitivity in determination of this ubiquitous and toxic element [42–45]. Acidic stannous chloride ( $\text{SnCl}_2$ ) solution is frequently used as reductant provided that all Hg species in the sample are converted to Hg(II) by means of vigorous acid digestion or reacting with strong oxidizing agents (e.g.,  $\text{KMnO}_4$ ) [38,41–43]. In this study, we opted for  $\text{NaBH}_4$  as it can reduce both inorganic and organic forms of Hg [36,39,44]. Further, the reaction of  $\text{NaBH}_4$  with acids produces water vapor and hydrogen gas as by-products, which is also advantageous to avoid possible contamination to the sampling interface of ICP-MS instrument from using  $\text{SnCl}_2$  at percent levels.

Initial experiments showed that the generation of Hg vapor from Hg(II) and  $\text{CH}_3\text{Hg(I)}$  took place robustly in a wide acidity interval (Fig. 2A). Signals increased up to 1% (v/v) HCl and remained almost flat with further increase to 5% (v/v) HCl. The signal intensity from 10  $\mu\text{g L}^{-1}$   $\text{CH}_3\text{HgCl}$  was always about 80% of that from 10  $\mu\text{g L}^{-1}$  Hg(II), which was proportional to the stoichiometric composition of Hg in  $\text{CH}_3\text{HgCl}$ . However, Fig. 2A clearly indicated that  $\text{CH}_3\text{Hg(I)}$  was quantitatively converted Hg vapor as was Hg(II) when solution acidity was above 1% (v/v) HCl.

For optimization of  $\text{NaBH}_4$  concentration, Hg(II) and  $\text{CH}_3\text{HgCl}$  solutions were acidified to 2% (v/v) HCl. The signal profiles from Hg(II) and  $\text{CH}_3\text{Hg(I)}$  are illustrated in Fig. 3. The reduction of Hg(II) occurred even in the presence of trace levels of  $\text{NaBH}_4$ , while signals for  $\text{CH}_3\text{Hg(I)}$  increased steeply with increasing  $\text{NaBH}_4$  up to 0.1% (m/v) and then leveled off above 0.2% (m/v)  $\text{NaBH}_4$ . For quantitative reduction of  $\text{CH}_3\text{Hg(I)}$ , 0.5% (m/v)  $\text{NaBH}_4$  was sufficient. The inset figure is the blow-up illustration of signal profiles up to 0.002% (m/v)  $\text{NaBH}_4$ . This pattern indicated that  $\text{NaBH}_4$  concentration of about  $1 \times 10^{-4}\%$  (m/v) was adequate to achieve quantitative reduction of Hg(II) to Hg vapor. These results are consistent with those reported by Segade and Tyson [39] who first utilized this approach for determination of Hg(II) and total Hg content in various samples. Under same conditions, the reduction of  $\text{CH}_3\text{Hg(I)}$  was negligible (ca.  $3\text{--}4 \times 10^4$  cps) which was approximately 2% of the signal from 10  $\mu\text{g L}^{-1}$  Hg(II). Considering  $\text{ng g}^{-1}$  levels of total Hg in otoliths, the contribution from  $\text{CH}_3\text{Hg(I)}$  could be significant to confound accurate determination of inorganic Hg levels as well as deteriorate the detection limits. Thus, the solution acidity was reexamined for the Hg(II) and  $\text{CH}_3\text{HgCl}$  solutions using  $1 \times 10^{-4}\%$  (m/v)  $\text{NaBH}_4$  as reductant. The results are illustrated in Fig. 2B. As occurred with 1% (m/v)  $\text{NaBH}_4$  (see Fig. 2A), signals for Hg(II) increased with increasing acidity up to 2% (v/v) HCl and then remained relatively constant. In contrast, signals from  $\text{CH}_3\text{Hg(I)}$  were at the maximum when  $\text{CH}_3\text{HgCl}$  was in water (e.g., 0%, v/v) and rapidly decreased with



**Fig. 2.** The effect of HCl concentration on Hg signals from  $10 \mu\text{g L}^{-1}$  Hg(II) and CH<sub>3</sub>HgCl solutions. (A) Initial optimization of HCl concentration using 1% (m/v) NaBH<sub>4</sub>. (B) Re-examination of HCl concentration on signal profiles with  $1 \times 10^{-4}$ % (m/v) NaBH<sub>4</sub>.



**Fig. 3.** The effect of NaBH<sub>4</sub> concentration on Hg signal from  $10 \mu\text{g L}^{-1}$  Hg(II) and CH<sub>3</sub>HgCl solutions in 2% (v/v) HCl. The inset figure is the blow-up from 0% to 0.002% (m/v) NaBH<sub>4</sub>.

increasing HCl concentration. For 5% and 6% (v/v) HCl solutions, signals were around  $5 \times 10^3$  to  $1 \times 10^4$  cps that were within the vicinity of blanks signals from 5% (v/v) HCl solutions. The concentration of HCl was increased up to 12% (v/v), but did not show any improvement in the background Hg signals, and thus the solutions were maintained at 5% (v/v) throughout.

The flow rates of the carrier argon and sample solution were optimized for Hg(II) solutions in 5% (v/v) HCl. Optimum flow rate for sample solution was around  $2 \text{ mL min}^{-1}$  when reacted on-line with 1% (m/v) NaBH<sub>4</sub> solution running at  $1 \text{ mL min}^{-1}$ . Signals did increase to some extent with higher flow rates but at the cost of more sample solution, which was not desirable as it would require dilution of otolith solutions containing very levels of Hg. The flow rate of carrier argon was more influential on signal intensity and stability. Optimum value was  $1.2 \text{ L min}^{-1}$  for both  $1.0 \times 10^{-4}$ % and 1% (m/v) NaBH<sub>4</sub>. Plasma stability was not affected from the vigorous reaction of 1% (m/v) NaBH<sub>4</sub> with 5% (v/v) HCl since water vapor was removed efficiently by the peltier-cooled spray chamber ( $3^\circ\text{C}$ ).

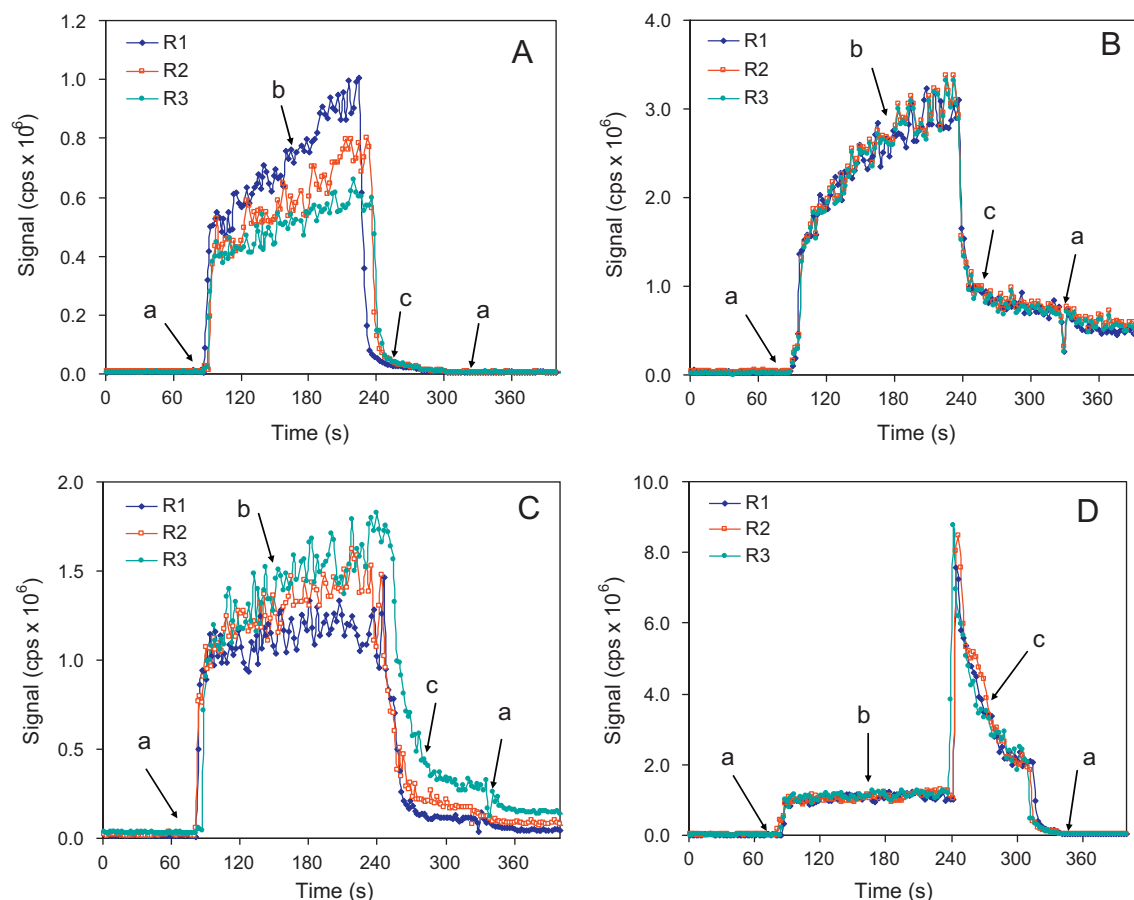
### 3.2. Memory effects

Mercury determination by ICP-MS is known to suffer from memory effects that are attributed to mainly adsorption of Hg(II) through the sampling interface and sample introduction system [43,46,47]. In conventional solution-based measurements, the memory effects were eliminated by adding gold chloride (HAuCl<sub>4</sub>) to sample and washout solutions [48]. L-Cysteine was also reported to be effective among a number reagents used to facilitate the washout of Hg from the sampling interface of ICP-MS instrument [47].

The memory effects in CVG-ICP-MS were said to be lower because of the gaseous nature of Hg introduced to the spectrometer and the reduced surface area of sample introduction components interacting with ionic Hg [42,43]. Our results, however, showed that memory effects in CVG were severe just as in solution mode to deteriorate the precision and detection limits. The residual signals from the introduction  $10 \mu\text{g L}^{-1}$  Hg(II) solution varied between 300,000 and 400,000 cps. This background gradually decreased to about 50,000 cps within 10 wash cycles with 5% (v/v) HCl or 5% (v/v) HNO<sub>3</sub>, but was still far above the blank signals observed from 5% (v/v) HCl (~5000 cps). It was interesting to note that such background was not observed from CH<sub>3</sub>HgCl that was washed out from the sampling and CVG components readily.

The signal profiles from three replicate runs for  $10 \mu\text{g L}^{-1}$  Hg(II) and the performances of washout solutions are illustrated in Fig. 4. Despite the fact that gold chloride (HAuCl<sub>4</sub>) is the most referred reagent for Hg washout, signal stability was highly variable when gold chloride solution ( $10 \mu\text{g mL}^{-1}$  Au) was run through the system (Fig. 4A). Signals repeatedly decreased in each washout, which could be due to the fact that Hg(II) amalgamated with residual gold throughout the sampling tubing and fittings. Signals tended to increase in subsequent runs, but the variation in signal intensity was substantial. Moreover, the depression in Hg signals was so prominent after repeated washout such that it was necessary to replace the pump tubing and teflon lines to recover the sensitivity. Washout with 0.5% (m/v) L-cysteine did not cause such depression, but the cleaning of residual Hg was inefficient as can be seen from the high background signals (~500,000 cps) following the washout with 0.5% (m/v) L-cysteine solution (Fig. 4B). Cerium(IV) ammonium nitrate (0.5% m/v), as a strong oxidizing agent, performed better than L-cysteine. However, extended wash was necessary to decrease the signals to baseline levels (Fig. 4C). The other washout solutions, including 0.2% (m/v) EDTA, 0.5% (m/v) 2-mercaptoethylamine chloride, and 0.5% (m/v) thiourea performed similarly to L-cysteine, and thus were not effective in removing the memory effects. Potassium ferricyanide solution, 0.5% (m/v) K<sub>3</sub>Fe(CN)<sub>6</sub> in 5% (v/v) HCl, was the most effective among





**Fig. 4.** Removal of residual Hg with various washout solutions. Signals during continuous CVG runs: a: 5% (v/v) HCl; b: 10 µg L<sup>-1</sup> Hg(II); c: washout solution. Washout solutions are: (A) gold chloride (10 µg mL<sup>-1</sup> Au) in 5% (v/v) HCl; (B) 0.5% (m/v) L-cysteine in 2% (v/v) HCl; (C) 0.5% (m/v) cerium(IV) ammonium nitrate in 2% (v/v) HCl; (D) 0.5% (m/v) potassium ferricyanide in 5% (v/v) HCl.

the solutions examined (Fig. 4D). The signal intensity abruptly increased when the ferricyanide solution was run and reacted with 1% (m/v) NaBH<sub>4</sub> solution, suggesting that the Hg adsorbed along the sampling system was brought to a form, most likely Hg(II), to react with the reductant. The signals decayed during the washout for about 90 s then immediately dropped to the background levels during 5% (v/v) HCl run (see Fig. 4D), indicating that K<sub>3</sub>Fe(CN)<sub>6</sub> solution effectively removed the residual Hg from the sampling components. Washout with potassium ferricyanide solution was also advantageous as it afforded better sensitivity and stability in signals. Signals for Hg were stable during the replicate runs (RSD < 5%), unlike the steady increase occurred after the washout with gold chloride solution. Additional experiments to optimize the concentration of K<sub>3</sub>Fe(CN)<sub>6</sub> solution showed that 0.2% (m/v) solution was sufficient for successful washout of the residual Hg within 45–60 s.

### 3.3. Effects of acid dissolution on methylmercury stability

Otoliths are primarily calcium carbonate with about 3–4% organic matrix that could contain elemental species, including inorganic Hg and methylmercury. Digestion in acid is thus essential for complete extraction of elemental species to the solution. In case of Hg, it is also critical to utilize acid digestion/dissolution conditions without altering the organic mercury (e.g., CH<sub>3</sub>Hg) levels in the otoliths. Table 2 summarizes the composition of Hg(II) and CH<sub>3</sub>Hg(I) in solutions when CH<sub>3</sub>HgCl (10 µg L<sup>-1</sup>) is digested in various HCl solutions at 100 °C. Inorganic Hg was measured by using 1% (m/v) SnCl<sub>2</sub> solution in 5% (v/v) HCl as reductant, whereas 0.5%

**Table 2**

Effect of HCl concentration on the stability of methylmercury (10 µg L<sup>-1</sup> CH<sub>3</sub>HgCl) during heating at 100 °C in screw-capped teflon tubes. Values are mean ± standard deviation (n = 3).

HCl concentration (% v/v)	Hg(II) (µg L <sup>-1</sup> )	CH <sub>3</sub> HgCl (µg L <sup>-1</sup> )
5	0.04 ± 0.01	9.6 ± 0.4
10	0.12 ± 0.05	9.4 ± 0.2
20	0.14 ± 0.05	9.6 ± 0.2
50	0.72 ± 0.10	9.1 ± 0.3

(m/v) NaBH<sub>4</sub> was adopted for CH<sub>3</sub>Hg(I). The concentration of Hg(II) was not significant when CH<sub>3</sub>HgCl was digested in up to 20% (v/v) HCl. Average Hg(II) concentration was about 0.14 µg L<sup>-1</sup>, indicating that dissolution of an otolith sample in 20% (v/v) HCl would not induce any significant changes to methylmercury levels. Treatment with 50% (v/v) HCl was not suitable as it yielded higher levels of Hg(II) up to 0.72 µg L<sup>-1</sup> due to the mineralization of CH<sub>3</sub>HgCl.

### 3.4. Interferences and analytical performance

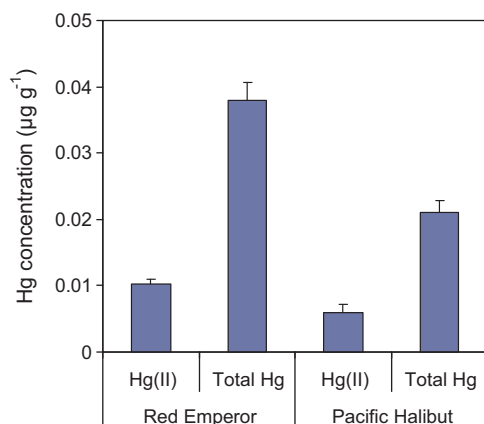
The effect of calcium matrix on the recoveries of Hg(II) and CH<sub>3</sub>Hg(I) is shown in Table 3. These results were obtained from the dissolution of ultra-pure CaCO<sub>3</sub> (99.99%) samples in 20% (v/v) HCl that were spiked with Hg(II) and CH<sub>3</sub>HgCl solutions. No significant interference was observed from up to 20,000 µg mL<sup>-1</sup> Ca(II) (e.g., 2%, m/v). The recoveries for Hg(II) and CH<sub>3</sub>Hg(I) were better than 95% by using the aqueous external calibration.

The memory effects were minimal at Hg concentrations less than 1.0 µg L<sup>-1</sup> when 0.2% (m/v) K<sub>3</sub>Fe(CN)<sub>6</sub> solution was run

**Table 3**

Effect of calcium matrix (as calcium chloride) on the recoveries of inorganic Hg and methylmercury. Ultra-pure  $\text{CaCO}_3$  (99.99%) was spiked with either Hg(II) or  $\text{CH}_3\text{HgCl}$  and then dissolved in 20% (v/v) HCl by heating at 100 °C. Analysis solution contained  $10 \mu\text{g L}^{-1}$  Hg(II) or  $\text{CH}_3\text{HgCl}$  in 5% (v/v). Values are mean  $\pm$  standard deviation ( $n = 3$ ).

Ca(II) ( $\mu\text{g mL}^{-1}$ )	Hg(II) (%)	$\text{CH}_3\text{Hg}$ (%)
500	$98.7 \pm 2.1$	$98.0 \pm 3.0$
2000	$96.2 \pm 3.0$	$96.8 \pm 1.8$
5000	$97.0 \pm 2.5$	$97.2 \pm 3.1$
10,000	$96.3 \pm 1.5$	$98.5 \pm 2.0$
20,000	$98.1 \pm 2.6$	$96.5 \pm 3.2$



**Fig. 5.** Inorganic Hg and total Hg content in the otoliths of red emperor (CRM 22) and Pacific halibut. Values are mean  $\pm$  standard deviation for five replicate measurements.

through the CVG manifold for 45–60 s. Under these conditions, the detection limits based on the 3 times the standard deviation of blank ( $n = 12$ ) were  $4.2 \text{ ng L}^{-1}$  and  $6.4 \text{ ng L}^{-1}$  for Hg(II) and total Hg. The relative standard deviation ( $n = 5$ ) was lower than 5% at  $1.0 \mu\text{g L}^{-1}$  level for Hg(II) and  $\text{CH}_3\text{Hg(I)}$ . The detection limits were mainly limited by the background signals of the blank solution (5% (v/v) HCl). Yet, they were comparable to those obtained by isotope dilution (ID) CVG-ICP-MS ( $\sim 1 \text{ ng L}^{-1}$ ) using  $\text{SnCl}_2$  as reductant [42,43], which offers better precision and reproducibility due to the nature of ID analysis.

### 3.5. Mercury composition of fish otoliths

The otolith certified reference material of red emperor (CRM 22) and the otolith samples extracted from Pacific halibut were digested according to the optimized dissolution conditions and were analyzed for Hg(II) and total Hg by using  $1 \times 10^{-4}\%$  (m/v)  $\text{NaBH}_4$  and 0.5% (m/v)  $\text{NaBH}_4$ , respectively. Calibration was performed with standards that contained Hg(II) and  $\text{CH}_3\text{HgCl}$  at equal concentrations from 0 to  $1.0 \mu\text{g L}^{-1}$ . The results for Hg(II) and total Hg are illustrated in Fig. 5. The concentrations were at sub- $\mu\text{g g}^{-1}$  levels for the otoliths of both species. For red emperor, Hg(II) and total Hg concentrations were  $0.010 \pm 0.001 \mu\text{g g}^{-1}$  and  $0.038 \pm 0.004 \mu\text{g g}^{-1}$ , respectively. Mercury levels in the Pacific halibut otoliths were even lower;  $0.006 \pm 0.001 \mu\text{g g}^{-1}$  for Hg(II) and  $0.021 \pm 0.003 \mu\text{g g}^{-1}$  for total Hg. Because of these significantly low concentrations, Hg(II) levels were verified with an alternate method using 1% (m/v)  $\text{SnCl}_2$  in 5% (v/v) HCl as a highly selective reductant that reduces only Hg(II) to elemental Hg. The results were  $0.008 \pm 0.001 \mu\text{g g}^{-1}$  for the red emperor and  $0.004 \pm 0.001 \mu\text{g g}^{-1}$  for the Pacific halibut. These values were not significantly different from those obtained with  $1 \times 10^{-4}\%$  (m/v)  $\text{NaBH}_4$  and thus confirmed that the contribution to Hg(II) from organic Hg was not significant.

Laboratory experiments indicate that uptake of heavy metals, including Cu, Pb and Hg into fish otoliths is proportional to their concentrations in the water [35,49,50]. On the other hand, mercury in fish tissue is mainly from the dietary uptake at the base of the food chain and is known to bioaccumulate (as methylmercury) two to three orders of magnitude higher levels than its concentration in the water. For instance, total Hg as methylmercury in tissues of Pacific halibut ranges from 0.15 to  $0.45 \mu\text{g g}^{-1}$  averaging to about  $0.241 \mu\text{g g}^{-1}$  [51,52]. These concentrations are about an order of magnitude higher than total Hg ( $0.021 \pm 0.003 \mu\text{g g}^{-1}$ ) found in the otoliths of adult halibut, although most Hg in the otoliths is also methylmercury. Such a large difference could point to the fact that Hg in otoliths may not be influenced from the dietary uptake, nor could it be related to Hg levels in tissues. As such, it is presumably related with the Hg available in the water.

Evidently, the information gathered from the otoliths of red emperor and Pacific halibut demonstrated that Hg in the otoliths was predominantly organic mercury (e.g., methylmercury). Inorganic Hg was present only at sub- $\text{ng g}^{-1}$  levels. Further, total Hg levels were distinctly different between the two otolith samples, which could be attributed to the geo-chemical differences between the habitats of the two fish species (e.g., eastern Pacific Ocean for halibut and northwest coast of Western Australia for the red emperor) [2,3,6,8,50]. This result is consistent with the laboratory exposure studies [35] and also supports the assumption above that Hg content in otoliths is most likely mediated by Hg available in the water.

## 4. Conclusion

A cold vapor generation procedure has been developed and applied to the determination of Hg species and their composition in fish otoliths by ICP-MS. It is demonstrated that careful optimization of  $\text{NaBH}_4$  concentration as a strong reducing agent affords selective determination of Hg(II) and total Hg levels. Potassium ferricyanide provides rapid and effective removal of residual Hg from the sample introduction system. It proved to be better washout solution for CVG determinations of Hg compared with gold chloride and thiol-containing chelating agents. The procedure is highly robust, free of memory effects and offers sufficiently low detection limits for accurate determination of small concentrations of Hg in otoliths by ICP-MS.

The results suggest that fish otoliths contain Hg at  $\text{ng g}^{-1}$  levels, which is largely methylmercury. In addition, despite its very low concentrations, Hg content in the otoliths could be significantly different provided that the habitats of the fish are different as in the case of red emperor (*L. sebae*) and Pacific halibut (*H. stenolepis*). It is therefore assumed that Hg concentration in fish otoliths could be a useful chemical tag in otolith microchemistry. It is important to note that this assumption is based on the fact that otolith Hg originates through deposition from the water, and is not biomagnified (e.g., methylated) in the otolith. Although the results ascertain the presence of methylmercury in the otoliths, more studies should be conducted through controlled laboratory exposures to elucidate if methylmercury concentration in otoliths is related to its concentration in the water or is derived from the methylation of inorganic Hg.

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