APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2005; **19**: 600–604
Published online 24 February 2004 in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.868

Sample treatment selection for routine mercury speciation in seafood by gas chromatography–atomic fluorescence spectroscopy

J. L. Gómez-Ariza*, F. Lorenzo and T. García-Barrera

Departamento de Química y Ciencia de los Materiales, Facultad de Ciencias Experimentales, Universidad de Huelva, Campus de El Carmen, 21007-Huelva, Spain

Received 20 August 2004; Accepted 21 October 2004

A general analytical strategy for mercury speciation in seafood samples has been proposed to increase sample throughput. This consists of the initial determination of total mercury content, and then mercury speciation using gas chromatography coupled to atomic fluorescence spectroscopy. The appropriate sample treatment for mercury speciation is selected between a method based on aqueous ethylation with sodium tetraethylborate (Approach A: a rapid methodology for samples with methylmercury concentrations between 150 and 2000 ng g^{-1}) and another one based on the determination of organomercury chlorides (Approach B: a much more time-consuming methodology, applicable to samples with methylmercury at 1.2–200 ng g^{-1}). Both procedures have been used together for the analysis of bivalves and fish samples. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: methylmercury; seafood; gas chromatography; atomic fluorescence

INTRODUCTION

Mercury is an element of undoubted significance, since it is considered as one of the most toxic in the periodic table. The toxicity of mercury not only depends on its concentration, but also on the chemical form in which it is present.[1,2] It is well known that alkylated species of mercury are much more toxic than inorganic or elemental mercury, since they can easily cross the cellular membranes and accumulate with high efficiency in organisms.[3] In addition, alkylmercury is not well metabolized, and tends to be bioaccumulated and biomagnified,[4] increasing its concentration in top predators in the food chain.

The form of mercury studied most is methylmercury. This species is arises from both natural and anthropogenic origins. The main natural sources of methylmercury in the aquatic ecosystems are benthic microorganisms (bacteria and sulfate reducers), which transform inorganic mercury into its methylated form.[5–7] Molluscs and fish also contribute to the production of methylmercury from inorganic mercury.[8] Formerly, humans have used alkylmercury for agricultural purposes, drugs and in the chemical industry.[9,10] Nowadays, the use of organomercuric compounds has been reduced or eliminated, since the extreme toxicity of these species has caused several accidents during the 20th century. One of the most significant cases occurred in Minamata Bay (Japan), from the mid-1950s, the 1960s and 1970s, where thousands of people were affected. Latterly, in the 1970s, another important incident happened in Iraq, where more than 6000 people were injured, because they used seeds treated with methylmercury for their own consumption.[11] Mercury (and especially methylmercury) produces severe damage to health. Therefore, in extreme situations, mercury produces severe congenital effects, and infants born to mothers with high mercury content may suffer cerebral palsy, blindness or mental retardation.[12]

Owing to the toxicity of mercury and methylmercury, several international organizations, like the World Health Organization (WHO), have proposed maximum limits for mercury intake. In this way, the maximum value for mercury ingestion per week has been set at 0.3 mg per person, with no more than 0.2 mg as methylmercury.[13] The European Commission Regulation 466/2001/EC (amended by Regulation 221/2002/EC) came into effect on April 2002,

*Correspondence to: J. L. Gómez-Ariza, Departamento de Química y Ciencia de los Materiales, Facultad de Ciencias Experimentales, Universidad de Huelva, Campus de El Carmen, 21007-Huelva, Spain.
E-mail: ariza@uhu.es
Contract/grant sponsor: Ministerio de Ciencia y Tecnología (MCyT);
Contract/grant number: REN2002-04366-C02-02.

and sets maximum levels for mercury in bivalve molluscs at 0.5 mg kg^{-1} wet weight. However, no explicit limits were provided for methylmercury or other organometallic mercury species, possibly due to the scarce application of speciation for routine procedures.

Since recommended limits for mercury intake are being reduced progressively, monitoring of this element and its species (mainly methylmercury) is a requirement. The development of routine methodologies to detect mercury at trace and ultratrace levels is necessary. Inductively coupled plasma mass spectrometry has an excellent sensitivity, but it requires skilled personal for its handling and is strongly affected by the matrix of the samples.[14] Alternatively, detectors based on atomic fluorescence spectroscopy (AFS) are much more robust and are sensitive enough for the analysis of environmental samples. Moreover, atomic fluorescence detectors are inexpensive when compared with plasma-based detectors, which represent an additional disincentive.

Finally, sample treatment is also a critical feature in mercury speciation, and this dramatically affects the sensitivity, selectivity and accuracy of the entire method.

Two methodologies are usually proposed for mercury speciation with gas chromatography (GC). The first one is based on the alkylation of mercury species, in order to improve volatility of the inorganic species by adding an ethyl, propyl, phenyl or any other alkyl group to the molecule. The most traditional alkylating methods are based on Grignard reactions,[15] but these procedures are time consuming, and skilled personnel are required. More recently, aqueous alkylating reagents have become usual in the laboratories all around the world.[16] Tetraalkylborates, such as tetraethyl- or tetraphenyl-borates, are the most used.

Alternatively, other workers have introduced variations on the well-established methodology proposed by Westöö during the late 1960s[17,18] based on the determination of organomercury halides.

However, comparisons between both methods have been little studied (to our knowledge), and their complementary use for mercury speciation has not been considered yet. In this study, both approaches have been used and compared for the determination of methylmercury in seafood, using the coupling between a gas chromatograph and an atomic fluorescence detector (GC–AFS). The main aim of this work is to achieve the highest sample throughput, reducing as much as possible the time employed for sample treatment and analysis, to design a final analytical strategy to assist in routine speciation of mercury in seafood.

EXPERIMENTAL

Sampling and pretreatment of seafood

Samples of three bivalves (*Chamelea gallina, Donax trunculus* and *Scrobicularia plana*) and fish (*Pomadasys incisus, Merluccius merluccius* and *Engraulis encrasicolus*) from the Andalusian coast (south Spain), were obtained from the local market.

The bivalves were depurated in seawater, in order to eliminate residual sand and dust. Then, the shell was removed and the clean animals were lyophilized. Finally, the samples were milled and stored at −20 °C. In fish, muscles were separated from bones, and tissues were lyophilized and stored similarly to the bivalves.

Sample moisture was calculated, and the average median value (80%) was used throughout to express the results on a wet basis.

Instrumentation

An atomic fluorescence detector (Merlin Mercury Detector, model 10.023S, PS Analytical Ltd, Orpington, Kent, UK) was used for the determination of mercury and its species. This instrument was coupled as required for total mercury or mercury speciation. A home-made flow-injection cold-vapour (FI-CV) device was used for total mercury determination. The FI-CV-AFS consisted of a low-pressure injection valve (Omnifit, Cambridge, UK), provided with a 125 µl loop, a gas–liquid separator system (PS Analytical), a dryer hygroscopic membrane (Permapure MD110-12 FP, Perma Pure Inc., Toms River, NJ, USA) and the AFS system mentioned above. The aqueous solutions involved in the system were driven by a peristaltic pump (Gilson Minipuls 3, Villiers le Ber, France) equipped with Tygon tubes. The samples were incorporated in an acidified aqueous solution (2% HNO_3 v/v) at a flow rate of 6 ml min^{-1}. Then, the reducing reagent 2% ($SnCl_2$ in acidic media, 3 ml min^{-1}) was added online, to transform mercury into the elemental form. Latterly, an argon stream was introduced to assist the separation of the volatile elemental mercury from the liquid phase. The mixture was driven to the gas–liquid separator, and the gaseous stream directed into the hygroscopic membrane to remove any residual humidity, and finally introduced into the AFS detector.

For mercury speciation, a Varian CP-3800 gas chromatograph (Varian Iberica, Spain) equipped with a split–splitless injector (model 1079) and a non-polar capillary column (CP-SIL 5 CB, 15 m × 0.53 mm × 1.5 µm, Chrompack, Middelburg, Netherlands) were used. The gas chromatograph was interfaced with the AFS detector through an integral pyrolyser (PS Analytical, model 10.565), where mercury species, already separated in the chromatographic column, were atomized. Finally, the outlet of the pyrolysis unit was introduced in a stream of argon, which drove mercury to the AFS detector. High-purity helium (99.999%) was used as carrier in the gas chromatograph. The injection volume was preset at 1 µl. Operational conditions for the GC–pyro-AFS device are described in Table 1.

Reagents

All reagents were dissolved in ultra-pure water produced with a Milli-Q Gradient system (Millipore, Watford, UK). The reagents used throughout were at least of analytical grade.

Table 1. Operating conditions for mercury speciation by GC–pyro-AFS with CP-SIL 5 (15 m × 0.53 mm × 1.5 μm) GC column

Injector volume	1 μl, splitless
Injector temperature	300 °C
Carrier gas flow (He)	13 ml min^{-1}
Oven temperature	
Initial temperature	40 °C (2 min)
Ramp	20 °C min^{-1}
Final temperature	300 °C (2 min)
Pyrolysis temperature	850 °C
Make-up gas flow (Ar)	120 ml min^{-1}
Sheath gas flow (Ar)	145 ml min^{-1}

Nitric acid (2% v/v) was prepared by diluting appropriate amounts of 65% HNO_3 (Merck, Darmstadt, Germany). This solution was used as carrier for FI-CV-AFS. Tin chloride 98% (Sigma–Aldrich Chemie, Steinhelin, Germany) was dissolved with concentrated hydrochloric acid (Merck), and made up with Milli-Q water. The final concentration was 2% (w/v) $SnCl_2$ in 3% (v/v) HCl. Mercury standards for total mercury determination were prepared daily by appropriate dilution of a stock solution containing 1000 mg l^{-1} Hg^{2+} as $Hg(NO_3)_2$, from Merck.

Potassium hydroxide, sulfuric acid, copper sulfate and potassium bromide were obtained from Merck, as was hexane and dichloromethane (for gas chromatography). Methylmercury chloride (MeHgCl) and ethylmercury chloride (EtHgCl) were obtained from Alfa (Johnson Matthey GmbH, Karsruhe, Germany), diphenylmercury (Ph_2Hg) from Sigma–Aldrich and sodium tetraethylborate 98% ($NaBEt_4$) from Strem Chemicals (Bischheim, France). Mercury standard solutions of 1000 mg l^{-1} were prepared separately by dissolving the correct amounts of MeHgCl, EtHgCl and Ph_2Hg in dichloromethane. These stock solutions were stored at 4 °C in the dark. The working standard solutions were prepared daily by diluting with appropriate amounts of hexane or dichloromethane.

Sample treatment

Total mercury determination

1.0 g of each sample was accurately weighed and transferred to Teflon digestion bombs. Then, 5 ml of concentrated HNO_3 was added; the bombs were tightly closed and left in contact overnight for sample predigestion, which enhances the accuracy of the method. After that, the bombs were placed in a domestic microwave oven at 700 W for 3 min. Then, irradiation was stopped for 10 min to avoid overpressure, and the cycle was repeated twice more. Finally, the bombs were cooled at room temperature and then opened. The digests were transferred to volumetric flasks and made up with ultra-pure water to 25 ml.

Mercury speciation

Approach A: derivatization with $NaBEt_4$. Small aliquots, 50–100 mg, of lyophilized samples were accurately weighed in Teflon centrifuge tubes. 5 ml of aqueous KOH (25%, w/v) was added and then sonicated for 2 h. Later, the extract was neutralized by adding concentrated HCl. After that, 20 ml of 0.5 mol l^{-1} ammonia buffer solution (pH 8.0) was added. 1 ml hexane and 100 μl internal standard (Ph_2Hg, 1 μg ml^{-1} in hexane) were also added. Finally, 3 ml aqueous $NaBEt_4$ (0.6%, w/v) was used for the ethylation of mercury species. The final mixture was hand shaken for 10 min and then centrifuged for 20 min at 10 000 rpm. An aliquot of the upper organic phase was collected and injected into the GC–pyro-AFS system as soon as possible (the analysis was always performed before 24 h). The injection volume was 1 μl.

Approach B: mercury speciation without ethylation. Larger aliquots (0.5–1.0 g) of lyophilized samples were extracted with 5 ml KOH (25%, w/v) in the same manner as Approach A. Then, 2.5 ml concentrated HCl was added, together with 4 ml of an aqueous solution containing KBr (18%, w/v), 0.25 mol l^{-1} $CuSO_4$ and H_2SO_4 (5%, v/v). The mixture was homogenized by manual shaking for a few seconds, and then centrifuged for 10 min at 10 000 rpm. The aqueous supernatant was transferred to centrifuge tubes and 4 ml of dichloromethane was added. Then, the sample was shaken in a vortex for 4 h. The lower organic layer containing the mercury species was carefully collected with a Pasteur pipette and transferred to a glass vial, where 1 ml of 0.03 mol l^{-1} $Na_2S_2O_3$ was added to extract mercury compounds into the aqueous phase. The sample was shaken for 1 h in a vortex shaker and an exactly measured volume of thiosulfate (typically 0.6 ml from the upper layer) was transferred to a new glass vial, where 0.2 ml of dichloromethane and 0.3 ml of the aqueous solution containing KBr and $CuSO_4$ as mentioned above were added. The vial was closed and hand shaken for 10 min. Finally, an aliquot of the organic layer was recovered and 1 μl was injected into the GC–AFS device.

Validation

Validation of the analytical methods was performed with two certified reference materials (CRMs): DORM-2 (dogfish muscle) and TORT-2 (lobster hepatopancreas), both from the National Research Council, Ottawa, Ontario (Canada). These two CRMs were treated in the same manner as the samples. In addition, spiking experiments were performed to evaluate recoveries. Spikes were equivalent to the amount of mercury in the samples. Spikes with inorganic mercury were used for total determination of mercury, and methylmercury in methanol was employed for speciation. The results are shown in Table 2.

Figures of merit

Total mercury determination (FI-CV-AFS)

The detection limit for total mercury determinations (calculated as 3σ) was 0.23 ng g^{-1} (concentration referred

Table 2. Validation of total mercury and mercury speciation methods ($n = 3$)

		$\overline{X} \pm \sigma$ (mg kg^{-1})		
		Certified value	Experimental value	Recovery (%)
Total Hg	DORM-2	4.64 ± 0.26	4.62 ± 0.20	99
	TORT-2	0.27 ± 0.06	0.26 ± 0.02	92
$MeHg^+$	DORM-2[a]	4.47 ± 0.32	4.36 ± 0.26	94
	TORT-2[b]	0.152 ± 0.013	0.146 ± 0.006	96

[a] Approach A.
[b] Approach B.

to on a wet basis). The relative standard deviation of 10 consecutive injections of a standard solution containing 10 ng ml^{-1} inorganic mercury was 3%. Calibration curves in the range 50–1000 pg ml^{-1} were performed. Other calibration curves embracing the range 0.6–20 ng ml^{-1} were obtained when necessary, after changing the AFS gain.

Mercury speciation with Approach A (GC–AFS)

Methylmercury and inorganic mercury can be determined by this methodology, since both of them are ethylated. The species produced from $MeHg^+$ is EtMeHg, and from inorganic mercury it is Et_2Hg. The detection limit for methylmercury (using 0.1 g lyophilized sample) was 45 ng g^{-1}, and for inorganic mercury it was 20 ng g^{-1} (wet basis). The relative standard deviation (RSD) of 10 sequential injections of standard solutions containing 100 ng ml^{-1} for each of this species, derivatized as referred to before, was 4.9% and 6.1% respectively. Linear calibration curves were obtained between the detection limits and 500 ng ml^{-1} (for inorganic mercury) and 400 ng ml^{-1} (for methylmercury).

Mercury speciation with Approach B (GC–AFS)

At least two mercury species can be determined by this approach: methylmercury and ethylmercury. Both species are injected into the chromatograph as chlorides. Inorganic mercury could not be determined because of the high boiling point of $HgCl_2$ (302 °C). The detection limits for MeHgCl and EtHgCl, referred to 1.0 g of lyophilized sample, were 0.36 ng g^{-1} and 0.59 ng g^{-1} respectively (as metal and on a wet basis). The precision of the method was calculated by injecting 10 standard solutions at 50 ng ml^{-1} containing each species, and the calculated RSD was 3.4% for methylmercury and 1.7% for ethylmercury. The linear dynamic range was found to be between the detection limits and 500 ng ml^{-1} for both species, with correlation coefficients better than 0.99.

Blanks were performed in each batch of samples. No peaks were detected when they were injected.

RESULTS AND DISCUSSION

Applicability range of the methods: analytical strategy for mercury speciation

Total mercury can be determined in samples over a wide range of concentrations, since both large and small amounts of samples can be digested without interferences. At least 50 mg of lyophilized sample was considered for the digestion to avoid losses of reproducibility related to particle size. The biggest aliquots consisted of 1.0 g dry samples. The applicability concentration range was established to be between 0.77 and 4000 ng g^{-1} (wet weight).

For mercury speciation, Approach A should be used with small amounts of sample. Aliquots of 50–100 mg lyophilized samples were treated without detectable interferences. Larger amounts of sample were not assayed, to prevent chromatographic column degradation, since this procedure does not include any clean-up step. Considering the linear range of this approach and the sample weights used, the applicability range was established to be 150–2000 ng g^{-1} (wet basis) for methylmercury determination.

Since Approach B includes a clean-up stage, larger amounts of sample can be treated. With this method, 0.5–1.0 g of lyophilized samples could be extracted without any problem or any apparent change of sensitivity. The application range for methylmercury is between 1.2 and 200 ng g^{-1}, as metal on a wet basis. Ethylmercury remained undetected in all cases.

As mentioned before, the applicability range for each analytical approach is different, but complementary. Approach A is suitable for samples with higher methylmercury content, and Approach B is suitable for low organomercury content. However, samples with methylmercury concentrations in the range 150–200 ng g^{-1} can be analysed by both approaches.

A critical aspect is the time of analysis, since Approach A is much faster than Approach B. The entire procedure using the first methodology, including extraction, derivatization and analysis, takes approximately 3 h, whereas the second methodology needs about 7 h. However, Approach B allows the analysis of samples with much lower mercury content, because of the larger amounts of sample used. This is possible because of the clean-up step based on the preconcentration and extraction of mercury into thiosulfate and re-extraction into an organic solvent (dichloromethane).

Therefore, the strategy proposed to choose the most suitable methododology for the determination of methylmercury (the most common mercury species in shellfish and fish samples is as follows. (a) Total mercury content analysis has to be performed in order to provide a preliminary assessment about the most suitable methodology for speciation analysis. An approximation is made considering that most mercury (over 80%) is present in bivalves and fish as methylmercury.[19] (b) Under the assumption that methylmercury content is of the same order of magnitude as total mercury, the most suitable analytical approach can be selected, using the applicability range previously established. The final purpose is to perform the analysis for mercury speciation as fast as possible and to

improve sample throughput. (c) Consequently, Approach A is the preferred methodology, although Approach B has to be used when the mercury concentration is low.

Application of the proposed strategy to real samples

Real samples of bivalves and fish obtained from the local market were used for the application of the proposed strategy for mercury speciation. Results on total mercury concentrations, collected in Table 3, show that mercury content in fish was much higher than in bivalves. According to the suggestions from the analytical strategy, bivalves could only be analysed by Approach B, whereas fish samples could be determined by either Approach A or B. In order to evaluate the applicability of each methodology, fish samples were treated with both procedures.

As is shown in Table 3, methylmercury constitutes 42–44% of total mercury in bivalves and 83–89% in fish. The higher presence of methylmercury in fish is related to their higher position in the food chain with respect to bivalves. In addition, results obtained with both approaches in fish samples are in good agreement.

CONCLUSIONS

A general analytical strategy for the determination of organomercury in seafood by GC–AFS is proposed. This approach is based on the preliminary determination of total mercury in the samples. Speciation can be performed by a rapid (but less sensitive) methodology based on aqueous ethylation (Approach A), or by a highly sensitive (but time-consuming) approach based on the procedure proposed by Westöö (Approach B). The most suitable methodology for mercury speciation analysis is selected according to total mercury content.

The methodologies proposed in this study were applied to the analysis of bivalves and fish samples from the local market, with good agreement between the results from Approaches A and B. Therefore, the application of the proposed strategy is justified, with the main goal of reducing the time of analysis and assisting with routine speciation assessment of mercury in seafood being obtained.

Table 3. Total and methylmercury determination in seafood ($n = 3$)

Approach	Sample	Concentration ($\mu g\ kg^{-1}$)
Total mercury	*Chamelea gallina*	6.32 ± 0.24
	Donax trunculus	10.76 ± 0.18
	Scrobicularia plana	20.92 ± 0.36
	Pomadasis incisus	300 ± 18
	Merluccius merluccius	244 ± 18
	Engraulis encrasiculus	199 ± 20
Approach A	*Pomadasis incisus*	262 ± 11
	Merluccius merluccius	205 ± 10
	Engraulis encrasiculus	175 ± 18
Approach B	*Chamelea gallina*	2.8 ± 0.18
	Donax trunculus	4.56 ± 0.38
	Scrobicularia plana	9.26 ± 0.36
	Pomadasis incisus	256 ± 13
	Merluccius merluccius	202 ± 12
	Engraulis encrasiculus	180 ± 14

Acknowledgements

We thank the Ministerio de Ciencia y Tecnología (MCyT) for financial support (project REN2002-04366-C02-02). F. Lorenzo and T. Garcia-Barrera thank the Junta de Andalucia and the University of Huelva for predoctoral scholarships.

REFERENCES

1. Palenzuela B, Manganiello L, Ríos A, Valcárcel M. *Anal. Chim. Acta* 2004; **551**: 289.
2. Horvat M, Nolde N, Fajon V, Jereb V, Logar M, Lojen S, Jacimovic R, Falnoga I, Liya Q, Faganeli J, Drobne D. *Sci. Total Environ.* 2003; **304**: 231.
3. Kim D, Wang Q, Sorial GA, Dionysiou DD, Timberlakeb D. *Sci. Total Environ.* 2004; **327**: 1.
4. Río Segade S, Tyson JF. *Spectrochim. Acta Part B* 2003; **58**: 797.
5. Rapsomanikis S. Mercury. In *Environmental Analysis Using Chromatography Interfaced with Atomic Spectroscopy*, Harrison RM, Rapsomanikis S (eds). Ellis Howood: Chichester, 1989; 297–317.
6. Rapsomanikis S, Weber JH. In *Organometallic Compounds in the Environment*, Craig PJ (ed.). Longman: Harlow, 1986; 279.
7. Monson BA, Brezonik PL. *Biogeochemistry* 1998; **40**: 147.
8. Gerbersmann C, Heisterkamp M, Adams FC, Broekaert JAC. *Anal. Chim. Acta* 1997; **350**: 273.
9. Zhou HY, Wong MH. *Water Res.* 2000; **34**: 4234.
10. Marvin C, Painter S, Williams D, Richardson V, Rossmann R, Van Hoof P. *Environ. Pollut.* 2004; **129**: 131.
11. WHO. Effects on man. In *Environmental Health Criteria 101: Methylmercury*. World Health Organization: Geneva, 1990; 68–99.
12. Gochfeld M. *Ecotoxicol. Environ. Saf.* 2003; **56**: 174.
13. Dickman MD, Leung KCM, Koo LC. *Mar. Pollut. Bull.* 1999; **39**: 352.
14. Armstrong HEL, Corns WT, Stockwell PB, O'Connor G, Ebdon L, Evans EH. *Anal. Chim. Acta* 1999; **390**: 245.
15. Ipolyi I, Massanisso P, Sposato S, Fodor P, Morabito R. *Anal. Chim. Acta* 2004; **505**: 145.
16. Montuori P, Jover E, Alzaga R, Diez S, Bayona JM. *J. Chromatogr. A* 2004; **1025**: 71.
17. Westöö G. *Acta Chem. Scand.* 1967; **20**: 1790.
18. Westöö G. *Acta Chem. Scand.* **22**: 1968; 2131.
19. Gilmour CH, Henry EA, Mitchell R. *Environ. Sci. Technol.* 1992; **26**: 2281.