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A New Method for Total Mercury and Methyl Mercury Analysis in Muscle of Seawater Fish

G. Carbonell · J. C. Bravo · C. Fernández · J. V. Tarazona

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Abstract In this work we have developed a cost-effective method for the analysis of methyl mercury (MeHg) in seawater fish muscle. The novelty of this method lies in the use of microwave-assisted extraction with acidic solution (HCl), addition of toluene, and subsequent extraction with cysteine acetate solution where only MeHg is present because of its affinity for cysteine groups. MeHg in cysteine phase and total mercury in the homogenate muscle tissue were determined using a direct Hg analyzer (DMA-80). Validation, precision, and accuracy of the method were evaluated and monitored with a tuna fish certified reference material (CRM 463) containing MeHg.

Keywords Methyl mercury · Microwave extraction · Mercury · Direct mercury analyzer

There is a growing interest in determining mercury (Hg) levels in the marine environment as well as in fish for human consumption (Ashraf 2004; Mishra et al. 2007). Methyl mercury (MeHg) in particular is the most toxic and bioaccumulative form of mercury in food webs (Krabbenhoft et al. 2006) and it is the predominant chemical form making up 80%–90% of the total mercury present in fish muscle tissue (Houserová et al. 2007). Hence, fish and other organisms at the end of the food chain constitute the major source of MeHg in the human diet (Plessi et al. 2001). As outcome of this risk, MeHg has been classically monitored in fish, and methodologies for mercury speciation have increased significantly since the early 1990s. Currently, microwave-assisted extraction (MAE) has provided an efficient alternative strategy to conventional techniques for solvent extraction of a large amount of organometals compounds (Vazquez et al. 1997; Alonso-Rodríguez et al. 2006). The analytical techniques most frequently applied for Hg speciation analysis involve GC (Gas Chromatography), GC–ICP-MS (Gas Chromatography–Inductively Coupled Plasma Mass Spectrometry), supercritical fluids chromatography (SFC), ion chromatography (IC), HPLC–CVAAS (High Performance Liquid Chromatography–Cold Vapor Atomic Absorption Spectrometry) or ICP-MS systems. Several authors recommended a back-extraction of mercury species from organic solvents to cysteine or sodium thiosulphate aqueous solutions (Westöö 1968; Padberg et al. 1993; Aceto el al. 1995; Morita et al. 1998; Xia et al. 2007). Because MeHg is the most common organomercury compound in biological materials (Houserová et al. 2007), here we offer a new simple and cost-effective method to determine MeHg. Basically, this method combines the microwave assisted extraction followed by clean-up with cysteine acetate solution and MeHg quantification by a direct mercury analyzer (DMA).

Materials and Methods

We used a laboratory microwave system (Ethos SEL, Milestone, Monroe, CT) equipped with a temperature and pressure feedback control, and magnetic stirring capability with the possibility of a simultaneous multi-extraction. Medium-pressure closed digestion vessels (high purity TFM, a thermally resistant form of Teflon) were used for samples extraction.

G. Carbonell (✉) · J. C. Bravo · C. Fernández · J. V. Tarazona
Laboratory for Ecotoxicology, Department of the Environment, INIA, Crta. La Coruña km 7.5, 28040 Madrid, Spain
e-mail: carbonel@inia.es

Hg analyses – total Hg and MeHg – were performed using a direct Hg analyzer (DMA80, atomic absorption spectrophotometer, Milestone, Wesleyan University, Middletown, CT, USA). The results of this detection system were previously validated for solid and liquid matrices (EPA 7473). Sodium acetate (99.5%), L-cysteine chlorhidrate (99%), toluene (99.5%), and hydrochloric acid (30%) from Sigma Chemical Co. (St. Louis, MO), were used. Acetate cysteine (1%) was obtained by mixing L-cysteine chlorhidrate (2%) and sodium acetate (2%) v/v.

A certified reference material, a tuna fish CRM 463 obtained from the European Commission Community Bureau of Reference, was used to check the accuracy of the method. Three pelagic marine fish species were selected: sardine (*Sardina pilchardus*), anchovy (*Engraulis encrasicolus*), and tuna fish (*Thunnus thynnus*). The samples were purchased in a municipal fish market.

All materials used for Hg analysis in this study were acid-washed with 10% HNO_3 and carefully rinsed with ultra pure water (Milli-Q system, Bedford, MA). For the sardine and anchovy, analyses were carried out individually considering a sample size of six animals. For the tuna fish, six samples (500 g of the edible muscle) were purchased in six different fish markets.

For total (THg) and MeHg analyses, 0–20 g wet weight (w/w) was placed in a Teflon potter in order to obtain a homogenate. A total of 50–100 mg of homogenate muscle was loaded in quartz boats and introduced into the DMA-80 system in order to analyze THg. MeHg extraction from muscle tissue is based on a microwave assisted extraction in acidic conditions with toluene previously described by Vazquez et al. (1997) with some modifications. Briefly, a fraction of homogenate muscle (4–5 g w/w) was directly weighed into the TMF vessel, 750 µl of hydrochloric acid (30%), 1,000 µl of Milli-Q water and 10 ml of toluene were added. The addition of water, Wefflon TM heating bottom and Wefflon TM stir bars facilitate the establishment of constant temperature in the vessel. To control the temperature, a sensor was placed inside one of the vessels containing a fish muscle sample. The temperature program was as follows: first, the temperature reached 110°C in 10 min, and then the temperature was held constant for 10 min. After cooling, 4 ml of toluene phase was transferred to a 15 ml caped glass tube with 2 ml of 1% cysteine acetate solution. After 2 min of stirring, two-phase separation was obtained by centrifugation. The upper toluene layer was removed with a syringe, and the cysteine acetate phase containing MeHg was analyzed. Volumes (50–100 µl) of the cysteine acetate phase were loaded in quartz boats and introduced into the DMA-80 system with thermal combustion (drying 60 s at 300°C, thermal decomposition 180 s at 850°C. The amalgamator selectively traps Hg, after the system is flushed with oxygen to remove any remaining gases or decomposition products (60 s); the amalgamator is rapidly heated (12 s), releasing Hg vapor. Absorbance is measured at 253.7 nm as a function of mercury concentration (EPA 1998). To correct for possible mercury contamination during the analysis the mercury concentration of a blank (cysteine acetate) was subtracted from sample Hg concentrations.

Results and Discussion

The instrument, a DMA-80, provides two working ranges for Hg detection: 0–40 and 40–600 ng. Each range is calibrated independently to optimize response over the entire dynamic range. Calibration samples containing 0, 10, 20, 30, and 40 µl of 1 or 10 ppm Hg were processed to calibrate the instrument for 0–40 and 40–400 ng, respectively. The limits of quantification (LOQ) and detection (LOD) were 0.6 and 0.24 µg kg^{-1}, respectively. The LOQ was established by the lowest calibration point; the LOD was 2.5 times lower than the LOQ when the signal/noise ratio was higher than 10. THg and MeHg concentrations for a certified reference material CRM 463, a tuna fish, are shown in Table 1.

Analyses of the certified reference material CRM 463 showed recoveries of 96% (3.17% VC) and 92% (2.53% VC) for THg and MeHg, respectively. Results are in consonance with those of the certified values, showing the proposed method for MeHg extraction as an adequate procedure for measuring this form of Hg in the muscle of the seawater fish. The whole process is quite straight forward, with excellent recoveries while the analyses for THg and MeHg can be made in the same analytical system.

THg and MeHg concentrations in the muscle tissue of sardine, anchovy and tuna are shown in Table 2. The highest THg concentrations were observed in the following order: tuna fish, anchovy and sardine. Our data reported herein are comparable to THg concentrations in species purchased in Catalonia, Spain (Martí-Cid et al. 2007), and in European pilchard (*S. pilchardus)* from Tunisia and other Mediterranean regions with values ranging from 0.09 to 0.75 µg g^{-1} w/w (Joiris et al. 1999). The mean of THg in anchovy samples analyzed in this study (0.038 ± 0.004 µg g^{-1}) was is in the range of data reported by the US FDA Monitoting Program (1990–2004) with an average of 0.043 µg g^{-1} w/w. The maximum Hg concentration was found in the muscle tuna fish muscle (0.244 ± 0.011 µg g^{-1} wet wt) probably resulting from biomagnifications within predatory species. Hg and MeHg values in tuna fish observed in this research are in the middle of the range reported by an FDA evaluation (December 2003). In that study, Albacore 'white' canned

Table 1 Analysis for mercury and methyl mercury in CRM 463

Parameter	Analyzed		Certified	
	Total mercury (mg kg^{-1} dry wt)	Methylmercury (mg kg^{-1} dry wt)	Total mercury (mg kg^{-1} dry wt)	Methylmercury (mg kg^{-1} dry wt)
Average value	2.72	2.80	2.85	3.03
SD	0.09	0.07	0.15	0.16
Variability coefficient (%)	3.17	2.53		
Recovery (%)	95.61	91.99		
Sample/replicates	6	6		

Table 2 Total and methyl mercury (mg kg^{-1} wet wt) in muscle of seawater fish species

Number	Sardine		Anchovy		Tuna fish	
	Total mercury (mg kg^{-1} wet wt)	Methylmercury (mg kg^{-1} wet wt)	Total mercury (mg kg^{-1} wet wt)	Methylmercury (mg kg^{-1} wet wt)	Total mercury (mg kg^{-1} wet wt)	Methylmercury (mg kg^{-1} wet wt)
1	0.027	0.020	0.045	0.034	0.235	0.265
2	0.030	0.019	0.037	0.032	0.257	0.267
3	0.024	0.017	0.033	0.030	0.256	0.268
4	0.021	0.017	0.039	0.029	0.235	0.259
5	0.023	0.019	0.036	0.031	0.236	0.257
6	0.030	0.018	0.040	0.030	0.246	0.257
Average value	0.026	0.018	0.038	0.031	0.244	0.262
SD	0.004	0.001	0.004	0.002	0.011	0.005
Variability coefficient (%)	14.79	6.49	10.21	5.79	4.33	1.87
MeHg (%)		72.66 ± 1.04		80.39 ± 2.03		106.28 ± 0.18
Sample/replicates	6	6	6	6	6	6

tuna were 0.358 μg g^{-1} wet wt versus 0.123 μg g^{-1} wet wt in the smaller 'light' tuna. The levels of mercury in the tuna fish we analyzed were also in the range of data reported by the US Food and Drug Administration, Monitoring Program 1990–2004 (Tables 3 and 4).

Yamshita et al. (2005) have reported high Hg concentrations for Atlantic Bluefin Tuna (*T. thynnus*; 0.42 ± 0.06 μg g^{-1} wet wt), Pacific Bluefin Tuna (0.59 ± 0.34 μg g^{-1} wet wt), and bigeye tuna (*Thunnus obesus*; 0.98 ± 0.34 μg g^{-1} wet wt). The percentage of THg expressed as MeHg represented 106% ± 0.18%; 80.39% ± 2.3%, and 72.66% ± 1.04% in tuna fish, anchovy, and sardine, respectively. These data are in agreement with data from previous studies (Rai et al. 2002; Houserová et al. 2007) suggesting that most of the Hg in fish muscle is present as MeHg.

Although all samples analyzed in this study showed Hg concentrations below the typical international marketing limit (i.e., 0.5 μg Hg g^{-1} w/w fish muscle), the values for Tuna fish are higher than that reccommended by the World Health Organizations (i.e., 0.2 μg Hg g^{-1} w/w) for vulnerable human consumers (WHO 1990).

Table 3 Mercury concentrations in fish and shellfish

Hg (mg kg^{-1} wet wt)						
Species	Sample (n)	Average	SD	Min	Max	cv (%)
Anchovy	40	0.043	NA	NA	0.340	NA
Mullet	191	0.046	NA	NA	0.130	NA
Herring	38	0.044	NA	NA	0.135	NA
Herring[a]	71	0.026	0.005	0.005	0.170	19.2
Herring[b]	8	0.026	0.017	0.012	0.062	63.9
Sardine	29	0.017	0.007	0.004	0.035	43.80
Tuna canned albacore	219	0.354	0.114	0.029	0.853	219
Tuna canned light	209	0.115	0.113	0.000	0.723	98.2
Tuna canned light chunk	100	0.110	0.030	0.000	0.498	26.9
Tuna (fresh/frozen)	32	0.678	0.295	0.137	1.300	43.5
Shark	27	1.122	0.511	0.380	2.100	45.5
Swordfish	162	1.035	0.509	0.000	3.005	49.1

Data obtained form the US FDA, Monitoring Program (1990–2004)

[a] Baltic species (1996–1998)

[b] Barska and Skrzynski (2003)

Table 4 Methyl mercury concentrations in fish and shellfish

MeHg (mg kg^{-1} wet wt)						
Species	Sample (n)	Average	SD	Min	Max	cv (%)
Tuna canned light chunk	8	0.055	0.048	0.000	0.100	87.5
Tuna (fresh)	54	0.195	0.205	0.000	0.900	105.2
Tuna (canned)	214	0.172	0.154	0.000	0.752	89.5
Tuna (fresh/frozen)	64	0.284	0.245	0.000	0.960	86.5
Shark	86	3.083	0.440	0.000	2.390	14.3
Swordfish	448	0.957	0.505	0.000	3.220	52.8
Oyster	34	0.012	0.044	0.000	0.250	359.9
Crab	43	0.030	0.046	0.000	0.150	153.5

Data obtained form the US FDA, Monitoring Program (1990–2004)

The most relevant advantage of this method is the short processing time (<120 min) to extract and analyze a considerable number of samples (10). In addition precise and rapid determination of both chemical forms (THg and MeHg) in fish tissues can be performed using the same analytical system thus reducing the variability in reported results.

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