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Double Spike Isotope Dilution GC-ICP-MS for Evaluation of Mercury Species Transformation in Real Fish Samples Using Ultrasound-Assisted Extraction

Diego Esteban-Fernández,*[,†] Manuela Mirat,[‡] M. Ignacia Martín de la Hinojosa,[‡] and J. Ignacio García Alonso[§]

[†]Department of Chemistry, Humboldt Universitaet zu Berlin, Brook-Taylor Strasse 2, 12489 Berlin, Germany

[‡]Laboratorio Arbitral Agroalimentario del Ministerio de Agricultura Alimentación y Medio Ambiente, Ctra. Coruña Km 10.700, 28023 Madrid, Spain

[§]Faculty of Chemistry, Department of Physical and Analytical Chemistry, University of Oviedo, Julián Clavería 8, 33006 Oviedo, Spain

ABSTRACT: Sample preparation continues being a key factor to obtain fast and reliable quantification of Hg species. Assisted procedures enhance the efficiency and reduce the extraction time; however, collateral species transformations have been observed. Moreover, differential interconversions have been observed even between similar matrixes, which introduce an important uncertainty for real sample analysis. Trying to minimize Hg species transformations, we have tested a soft ultrasound-assisted extraction procedure. Species quantification and transformations have been evaluated using double spike isotope dilution analysis (IDA) together with gas chromatography inductively coupled plasma mass spectrometry (GC-ICP-MS) for a CRM material (Tort-2) and shark and swordfish muscle samples. Optimum extraction solution and sonication time led to quantitative extraction and accurate determination of MeHg and IHg in a short time, although different behaviors regarding species preservation were observed depending on the sample. Negligible species transformations were observed in the analysis of the CRM, while a small but significant demethylation factor was observed in the case of real samples. In comparison with other extraction procedures, species transformations became smaller, and fewer differences between fish species were found. Similar results were obtained for fresh and lyophilized samples of both fish samples, which permit one to analyze the fresh sample directly and save time in the sample preparation step. The high grade of species preservation and the affordability of the extraction procedure allow one to obtain accurate determinations even for routine laboratories using quantification techniques, which do not estimate species transformations.

KEYWORDS: *methylmercury, double isotope dilution analysis, GC-ICP-MS, species transformation, real fish samples, ultrasound bath extraction*

■ INTRODUCTION

Mercury is found in the environment in different chemical forms and concentrations. The most common Hg species found in environmental and biological samples are inorganic mercury (IHg) and methylmercury (MeHg).[1] Alkylated Hg-species are much more toxic than inorganic or elemental Hg due to their capability to cross cellular membranes and ease of accumulating in tissues.[2] Among all of these species, MeHg stands out because of its abundance in nature, toxicity, efficient bioaccumulation, high stability, and long lifetime in tissues.[3] MeHg is bioaccumulated through aquatic food chain, and relatively high concentration could be found in large predatory fish. IHg bioaccumulation is lower than MeHg, resulting in a higher proportion of MeHg in fish (ca. 95%) than in water (ca. 10%) or in the previous organisms in the food chain as phytoplankton (ca. 15%) or zooplankton (ca. 30%).[4] The fact that MeHg is mainly found in muscle tissue also points out that not only liposolubility works as a bioaccumulation mechanism.

Fish and its products make up a large part of the diet for many populations around the world and constitute an important supply of nutrients. For this reason, consumption of contaminated fishery products and fish muscle represents the first source of exposure to MeHg. Neurotoxicity and teratogenic effects are some of the side effects related to Hg consumption. Moreover, the concentration cannot be reduced by skinning, trimming, or cooking the mentioned foodstuffs, so the only way to prevent the exposure is to perform control analyses to limit Hg intake.

For several years now, governments of different continents have been promoting the routine measurement of MeHg in fish for human consumption.[5,6] To this date, no legislation establishing maximum levels for MeHg in fish has been published in the European Union, although some specific workshops and national reference laboratories (NRL) meetings have been held to check the capacity of the European members to tackle this speciation analysis.[7] Only maximum levels for total Hg are considered (between 0.5 and 1.0 mg/kg depending on the fishery products) in the Commission Regulation (EC) no. 1881/2006 and (EC) no. 629/2008.[8,9] Considering the

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high toxicity of MeHg and that more restrictive future regulations are expected, the development of reliable and fast analytical methodologies for routine determinations of MeHg in fish samples is a requirement.

A wide variety of analytical methodologies have been developed to analyze total contents of Hg and Hg-species in biological samples. Mercury speciation analyses are usually tackled employing hyphenated techniques that combine some separation techniques, such as liquid (LC) or gas chromatography (GC), with Hg-selective detectors. LC methodologies present easy sample preparation and simple interfaces for selective detectors as ICP-MS. GC-ICP-MS offers higher sensitive determinations and allows one to employ SPME as preconcentration and sampler device.[10] Atomic absorption spectrometry (AAS),[11] atomic fluorescence spectrometry (AFS),[12,13] atomic emission spectrometry (AES),[14] microwave-induced plasma optical emission (MIP-OES),[15] inductively coupled plasma optical emission spectrometry (ICP-OES),[16] ICP-MS,[17] and GC–MS[18] have been used as selective elemental detectors for Hg speciation. Nowadays, the most interesting detector for Hg determinations is ICP-MS because of special properties such as high sensitivity, high selectivity, versatility in terms of coupling to separation techniques, and capacity to detect simultaneously different isotopes. This latter feature allows one to perform isotope dilution analysis (IDA) obtaining very accurate measurements as well as correcting possible species transformations.[17,19]

Sample preparation is actually the key step for Hg speciation. The extraction procedure must be fast, robust, efficient, and preserve original species.[18,20] Usually alkaline[21] or acid leaching[22] is used to extract Hg-species from biological tissues. Even enzymatic digestion procedures have been used to improve the extraction yield.[23] Elevated temperatures, microwave-assisted,[24] or ultrasound-assisted extractions[25] provide better extraction efficiency and reduce extraction time and solvent consumption. However, the species transformations produced in each of these extraction procedures can lead to inaccurate results when Hg species are determined[20] and traditionally have been not evaluated. Without the consideration of species interconversions, the data resulting from the sum of species are not enough to validate the quantification of the Hg-species. In past years, some authors have studied in parallel the extraction efficiency and the species preservation. For instance, variable extraction yields and grades of interconversion for ERM-CE464 reference material comparing extraction solutions and procedures have been reported.[20] On the other hand, accelerated-extraction methodologies based on focused microwave (MW) systems have shown different demethylation of the MeHg depending on the Certified Reference Material (CRM) analyzed.[18] These results demonstrated that the applied procedures do not allow optimum extraction conditions for all types of matrixes. Therefore, validation of the extraction method with not completely representative CRMs can lead to inaccurate determinations in other matrixes as those coming from real fishery products.[26] Unfortunately, only a few studies dealing with real fish samples consider the stability of the species during the extraction procedure,[17,27] and more works are necessary for deepening understanding of the effect of extraction procedures in species transformations.

The ultimate objective of this work is the accurate measurement of Hg-species in real samples trying to minimize the uncertainties coming from the species transformations produced during the extraction procedure. On the basis of soft ultrasound-assisted extractions, this work looks for extraction solutions and experimental conditions that can preserve species in different sample matrixes without sacrifice the extraction speed and efficiency. A recently developed IDA mathematical treatment called isotope pattern deconvolution (IPD)[28] has been employed to quantify Hg-species and their transformations using double spike isotope dilution data from GC-ICP-MS analysis. Once the quantitative extraction and the absence of significant transformations of Hg-species have been demonstrated using IPD, an external calibration or an internal addition calibration may be used on a routine basis. Fresh and dried fish muscle was analyzed, and species transformations differences with CRMs were assessed.

MATERIALS AND METHODS

Instrumentation. Two different elemental detectors were employed in the present work for speciation and total amounts measurements. An Agilent 6890N gas chromatograph equipped with a 7683B Series injector (Agilent Technologies, Waldbronn, Germany) was coupled to an Agilent 7500 ICP-MS (1250 W RF power, 0.14 L min^{-1} Ar carrier gas, 0.41 L min^{-1} Ar/O_2 makeup gas, 0.066 s integration time) for speciation analysis. Isotopes ^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, and ^{202}Hg were measured. The Hg-species were separated in a HP-5MS SV capillary column (30 m × 0.25 mm i.d. × 0.50 μm, Agilent), and the oven temperature was programmed from 50 °C (held for 1.5 min) to 150 at 45 °C/min and to 230 at 30 °C/min. Sample volumes of 2 μL were injected using pulsed splitless mode.

Total Hg content was performed via a DMA-80 Direct Mercury Analyzer (Milestone Inc., Shelton, U.S.) working in the lower range mode at 253.65 nm. An Allegra 21 centrifuge (Beckman Coulter, U.S.) was employed to separate the leachate from the solid during the initial extraction step. This centrifuge was also used to help the separation of the organic and aqueous phases during the liquid–liquid derivatization/extraction step. For fresh fish sample, homogenization and mixing was performed using a Büchi inert mixer B-400 (Büchi Labortechnik AG, Flawil, Switzerland).

Reagents and Materials. Milli-Q water (Millipore, Billerica, U.S.) was used for the preparation of all solutions. Spike solutions MeHg (^{201}Hg-enriched) and IHg (^{199}Hg-enriched) were obtained from Innovative Solutions in Chemistry (ISC-Science, Oviedo, Spain). Natural abundance Hg^{2+} (Romil Pure Chemistry, PRIM-AG-XTRA) was used for total quantification calibration. The isotopic abundances of natural Hg as well as the ^{201}Hg and ^{199}Hg-enriched spikes are included in Table 1.

For the extraction of Hg-species from fish samples, two acid and two alkaline solutions were tested as extraction reagents: i) HCl (Merck, Darmstadt, Germany) 5 mol L^{-1}, (ii) saturated NaCl (Merck) solution with 60 mmol L^{-1} HCl, (iii) 3.0 mol L^{-1} KOH (Panreac, Barcelona, Spain) in MeOH (Merck), and (iv) 25% (w/v) TMAH in H_2O (Fluka, St. Louis, MO). The lobster hepatopancreas reference

Table 1. Isotope Abundance of Natural Hg, ^{199}Hg-Enriched IHg Spike, and ^{201}Hg-Enriched MeHg Spike

	isotope abundance (%)		
isotope	natural Hg	^{201}Hg-enriched MeHg spike	^{199}Hg-enriched IHg spike
^{196}Hg	0.15	<0.01	0.11
^{198}Hg	9.97	0.04	1.58
^{199}Hg	16.87	0.11	91.71
^{200}Hg	23.10	0.89	4.87
^{201}Hg	13.18	96.49	0.73
^{202}Hg	29.86	2.37	0.87
^{204}Hg	6.87	0.09	0.13

material Tort-2 (National Measurement Standards, Ontario, Canada), certified for MeHg (0.152 ± 0.013 mg/kg) and total Hg (0.270 ± 0.06 mg/kg) was employed to validate the quantification methodology. Shark and swordfish muscle samples from the Bay of Biscay were analyzed before and after drying. The employed CRM is a fishery product with a IHg:MeHg ratio similar to the studied predatory fish, although it is not a fish tissue.

Sodium tetrapropyl borate (ABCR GmbH, Karlsruhe, Germany), 2% (w/v), was used for the derivatization step. It was prepared daily in 0.2 mol L^{-1} sodium hydroxide (Merck) and kept refrigerated until use. A buffer solution of 1.1 mol L^{-1} sodium acetate (Merck) adjusted to pH 5 with acetic acid (Merck) was used to fix the pH during the derivatization step.

Extraction and Derivatization Procedures. About 100 mg of fish sample was extracted with 4 mL of the extraction solutions in the ultrasonic bath. Every extraction was performed with the same number of vials inside the bath to ensure constant experimental conditions. Furthermore, a constant extraction temperature of 50 °C was maintained during the whole working day. This experimental setup was designed to improve the reproducibility of the method. Extraction times of 5, 15, 30, and 60 min were tested with the four different extraction solutions. To correct for species transformation and losses, enriched solutions of MeHg and IHg were spiked at the beginning of the procedure, keeping sample/spike ratios between 1:1 and 1:3. Sample extractions were performed 15 min after spiking and shaking of the sample.

After the sonication step, the sample was centrifuged, and 1 mL of the supernant was derivatized. For that purpose, 4 mL of sodium acetate buffer solution, 0.4 mL of sodium tetrapropyl borate solution, and 1 mL of hexane were mixed with the aqueous extract. After gentle shaking during 10 min, the organic phase was collected (sometimes after centrifugation) and analyzed directly by GC-ICP-MS. Alternatively, a preconcentration step of the organic extract can be performed by evaporation under N_2 stream. Each sample was injected three times in the GC-ICP-MS instrument, and for each experimental condition three independent samples were extracted.

Procedure for Total Hg Using the DMA. Total content measurements by direct mercury analyzer (DMA) do not require sample preparation, and liquid or solid samples can be measured directly after calibration with solid and liquid standards in different concentration ranges. The liquid or solid sample is introduced into the sample boat and weighed. Intensive heating of the sample produces Hg vapors that are trapped on a gold amalgamator and later desorbed for quantification. The instrument calculates the concentration of total Hg taking into account the sample weight and the atomic absorption spectrophotometric response at 253.65 nm. The total Hg concentrations of each extract and fresh and dried fish were measured employing this technique.

Fish Drying. Both fresh and dried fish samples were analyzed. A significant piece of fresh fish muscle was homogenized with a metal-free mixer. A part of this sample was oven-dried at 40 °C for 24 h up to constant weight[30] and finally converted into powder by agate mortar grinding. The other part of the fresh sample was extracted directly after homogenization.

Mathematical Procedure: Isotope Pattern Deconvolution. The calculation procedure used has been described in detail elsewhere.[29] For ICP-MS detection, in contrast to GC–MS, an additional internal mass bias correction step was required. For this purpose, the peak areas for MeHg and IHg in the spiked sample were measured at five Hg-isotopes of mass 198, 199, 200, 201, and 202. The peak areas were then corrected for mass bias using the equation:

$$\text{area}^{i}_{corr} = \text{area}^{i}_{exp} \times e^{-k\Delta M_i} \quad (1)$$

where the measured peak areas (area_{exp}) were corrected using a given tentative mass bias factor k and the nominal mass difference, ΔM_i, between the i isotope and a reference isotope (in this case mass 200) using the exponential mass bias correction equation. The isotope abundances, A^{i}_{m}, for each Hg-species in the spiked sample were calculated by dividing each corrected peak area by the sum of all peak areas. Finally, the measured abundances for each Hg peak were deconvoluted into three molar fractions (x_{nat}, x_{MeHg}, and x_{IHg}) by multiple linear regression using the equation:

$$\begin{bmatrix} A^{198}_{m} \\ A^{199}_{m} \\ A^{200}_{m} \\ A^{201}_{m} \\ A^{202}_{m} \end{bmatrix} = \begin{bmatrix} A^{198}_{nat} & A^{198}_{MeHg} & A^{198}_{IHg} \\ A^{199}_{nat} & A^{199}_{MeHg} & A^{199}_{IHg} \\ A^{200}_{nat} & A^{200}_{MeHg} & A^{200}_{IHg} \\ A^{201}_{nat} & A^{201}_{MeHg} & A^{201}_{IHg} \\ A^{202}_{nat} & A^{202}_{MeHg} & A^{202}_{IHg} \end{bmatrix} \cdot \begin{bmatrix} x_{nat} \\ x_{MeHg} \\ x_{IHg} \end{bmatrix} + \begin{bmatrix} e_{198} \\ e_{199} \\ e_{200} \\ e_{201} \\ e_{202} \end{bmatrix} \quad (2)$$

where the reference isotope abundances A^{i}_{nat}, A^{i}_{MeHg}, and A^{i}_{IHg} are given in Table 1. Finally, the "best" value of the mass bias factor k was obtained by minimizing the square sum of the residuals of the multiple linear regression using the SOLVER application in Excel as described previously.[28,29] Finally, degradation corrected concentrations for MeHg and IHg and the degradation factors F1 (IHg to MeHg) and F2 (MeHg to IHg) in the sample were calculated as described previously.[29]

RESULTS AND DISCUSSION

Evaluation of Extraction Conditions. For the evaluation of the different acidic and alkaline extraction media using a fast ultrasonic extraction, the reference material Tort-2 was extracted during 30 min at 50 °C. Both, DMA and extraction/derivatization procedure using GC-ICP-MS were employed. The results obtained are shown in Table 2. The DMA is an easy, quick, and matrix-independent technique to measure total Hg contents in liquid and solid samples, which is being employed in our laboratory for accredited methodologies and allowed us to calculate the total Hg extraction yield in comparison to the certified value. The results shown are expressed as mg of Hg per kg of Tort-2 material. Up to 12 samples could be processed simultaneously without differences in the extraction efficiency depending on the position in the US bath (data not shown). As can be observed in Table 2, the total extraction yield can be considered quantitative employing both TMAH 25% and HCl 5 M. On the other hand, the extraction efficiencies obtained for KOH/MeOH (ca. 83%) and for NaCl/HCl (ca. 24%) can be considered insufficient. Although these two extraction solutions are not suitable for quick and efficient extractions using sonication, it has been previously used successfully for the quantification of Hg-species employing more drastic experimental conditions.[9,30]

Regarding the quantification of the different Hg species, double spike IDA has been used as tool to evaluate the species transformations produced with each extraction procedure. All concentration values obtained using the double spike IDA GC-ICP-MS procedure were in good agreement with the MeHg and total Hg certified reference values. The IDA technique provides accurate results correcting incomplete processes or instrumental errors and drifts. Furthermore, the double spike strategy allows taking into account species interconversion. Even for nonquantitative extractions, as in the case of NaCl/HCl and KOH/MeOH solutions, good results were obtained using double spike IDA GC-ICP-MS. This interesting behavior points out the isotopic equilibration between the endogenous Hg and the spiked Hg even with not complete extractions. The low overall extraction yields are corrected by adding the enriched species at the beginning of the procedure and achieving species isotopic equilibrium. Although a complete explanation of this behavior was not found, solution

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Table 2. Total Hg and Species Concentrations (mg of Hg per kg of CRM) at Different Extraction Times and with Different Extraction Solutions in Lobster Hepatopancreas Reference Material (Tort-2) Measured by DMA and GC-ICP-MS, Respectively[a]

		DMA	GC-ICP-MS				
procedure	extraction time (min)	total Hg in extract (mg/kg)	IHg (mg/kg)	MeHg (as Hg) (mg/kg)	sum of species (mg/kg)	F1	F2
NaCl/HCl	30	0.064 ± 0.003 (23.8 ± 1.0)	0.120 ± 0.003	0.149 ± 0.004 (98.0 ± 2.6)	0.269 ± 0.001 (99.6 ± 0.4)	<0.3	5.0 ± 1.2
KOH/MeOH	30	0.224 ± 0.011 (82.9 ± 4.2)	0.127 ± 0.003	0.146 ± 0.003 (96.1 ± 2.0)	0.272 ± 0.001 (100.7 ± 0.4)	<0.3	1.2 ± 0.5
TMAH 25%	30	0.273 ± 0.002 (101.2 ± 0.6)	0.118 ± 0.001	0.150 ± 0.001 (98.7 ± 0.7)	0.268 ± 0.002 (99.3 ± 0.7)	<0.3	12.3 ± 2.5
HCl 5 M	30	0.266 ± 0.001 (98.4 ± 0.2)	0.118 ± 0.001	0.150 ± 0.001 (98.7 ± 0.7)	0.268 ± 0.002 (99.3 ± 0.7)	<0.3	0.69 ± 0.04
HCl 5 M	5	0.269 ± 0.003 (99.6 ± 1.2)	0.116 ± 0.001	0.151 ± 0.001 (99.3 ± 0.7)	0.267 ± 0.001 (98.9 ± 0.4)	<0.3	<0.3
HCl 5 M	15	0.271 ± 0.003 (100.5 ± 1.0)	0.118 ± 0.001	0.149 ± 0.001 (98.0 ± 0.7)	0.267 ± 0.002 (98.9 ± 0.7)	<0.3	<0.3
HCl 5 M	60	0.271 ± 0.007 (100.5 ± 2.5)	0.117 ± 0.001	0.148 ± 0.001 (97.4 ± 0.7)	0.265 ± 0.002 (98.1 ± 0.7)	<0.3	0.87 ± 0.09

[a]Recoveries (in %) with respect to the certified values are indicated in parentheses. Methylation of the IHg and demethylation of the MeHg are represented by F1 and F2, respectively (in %). The uncertainties given correspond to the standard deviation of $n = 3$ independent extractions. The reference material Tort-2 is certified for MeHg (0.152 ± 0.013 mg/kg) and total Hg (0.270 ± 0.06 mg/kg).

equilibration followed by readsorption processes could take place.

The interconversion factors F1 and F2 were used to evaluate the species transformations during the extraction, with F1 being the factor related to the methylation of the IHg and F2 the factor that informs about the conversion of MeHg to IHg. The results obtained from the isotope pattern deconvolution approach are also given in Table 2. The accuracy of the results calculated by IPD is excellent because not only a pair of isotopes but all of the Hg isotopes are considered to determine the unknown concentrations. As can be observed, there was not significant methylation of IHg in the evaluated extracts. However, MeHg demethylation was clearly detected for some of the extraction solutions. Especially high was the F2 value for the TMAH 25% solution, exceeding 10% of demethylation of the original MeHg. Also, NaCl/HCl showed an appreciable transformation in the same direction. These results point out that demethylation can take place relatively easily under different experimental conditions, while methylation processes are less common and require more special conditions to occur, as happens in the nature where it is mainly produced by the action of sulfate reducing bacteria. The double spike procedure was able to correct for this high demethylation factor, and the measured MeHg concentrations were within the uncertainty of the certified value. Similar results using TMAH 25% and focused microwave extraction was obtained by Castillo et al.[18] via GC−MS.

Therefore, extraction solutions play a fundamental role in the preservation of the original species as well as in the efficiency of the ultrasound-assisted extractions. From the point of view of stability of the species and quantitative extraction, 5 mol L^{-1} HCl seems to be the extractant of choice in combination with ultrasonic extraction. Thus, we evaluated also different extraction times from 5 to 60 min using this extractant, and the results are given in Table 2. The results indicate that all of the assayed extraction times lead to similar results in terms of total recovery, species concentrations, and negligible transformation factors. For this reason, 5 min extraction was selected as the optimum extraction time for the lyophilized Tort-2 material. Moreover, the quantitative recovery and species preservation of this acid leaching procedure would enable quantification employing conventional single isotope IDA without species correction or even using standard additions, or external calibration.

Real Fish Samples. Many times, the optimized parameters for standards or even certified reference materials are far from the ideal conditions for biological samples.[26] Therefore, every analytical methodology should be finally applied to real samples. To check the feasibility of the procedure, the method was applied to real samples of fresh and dried fish. Samples of swordfish and shark were analyzed using the detailed procedure and 5 mol L^{-1} HCl as extraction solution. Complete results including fresh and dried sample and two different extraction times (5 and 15 min) are given in Table 3. The moisture was determined by oven drying of the samples to constant weight with 79.2 ± 0.3% and 74.1 ± 0.7% being the values for swordfish and shark, respectively. The results shown for dried samples were corrected for water content with the values of concentration expressed as mg of Hg per kg of the original tissue.

The results obtained by DMA showed good agreement between total Hg measured directly in the solid sample and total Hg measured in the extracts for both the fresh and the dried fish samples (Table 3). This means that a complete extraction of the Hg species occurred as it happened with the CRM. For the GC-ICP-MS method, the results for the sum of species were in good agreement with the DMA results. Therefore, both swordfish and shark fresh and dried samples can be quantitatively extracted with the selected acid leaching.

GC-ICP-MS analyses showed that, as can be expected, the concentration of IHg in all samples was very low, below 2% of the total Hg concentration (Table 3). Other authors have also described high MeHg percents of ca. 95%[4] in fishery products. The combination of speciation analysis with double spike isotope dilution allows us to increase the accuracy of the quantification due to the introduction of the species trans-

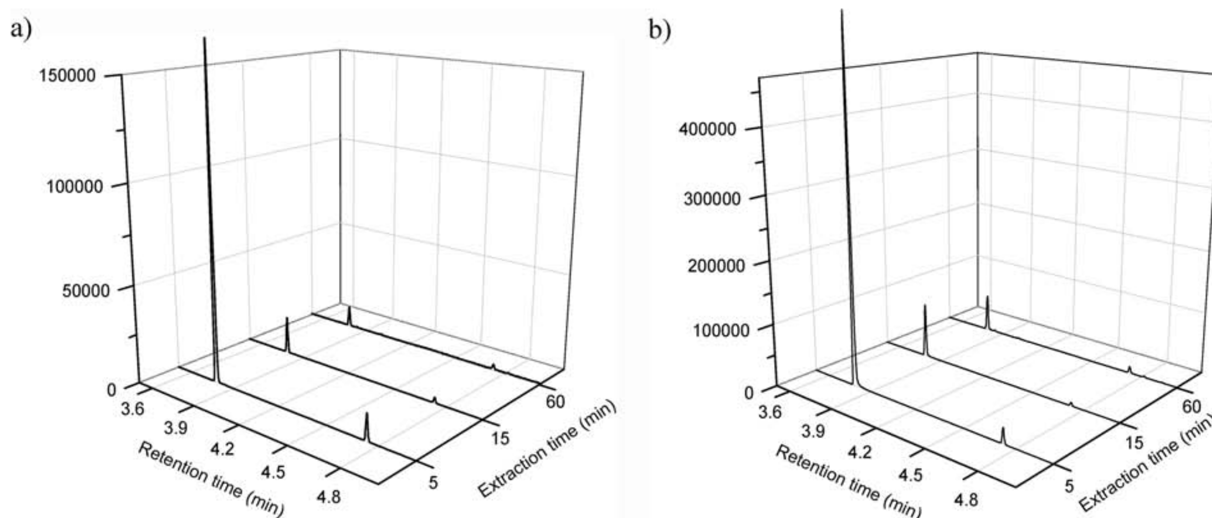

Figure 1. GC-ICP-MS chromatogram from (a) dried and (b) fresh shark extracted with HCl 5 M in ultrasonic bath for 5, 15, and 60 min. Signal at 60 min multiplied by a factor of 10.

Table 3. Total Hg and Species Concentrations (mg of Hg per kg of fresh sample) in Dry and Fresh Swordfish and Shark Measured by DMA and GC-ICP-MS, Respectively[a]

		DMA		GC-ICP-MS				
sample	extraction time (min)	total Hg in solid sample (mg/kg)	total Hg in extract (mg/kg)	IHg (mg/kg)	MeHg (as Hg) (mg/kg)	sum of species (mg/kg)	F1	F2
dry swordfish	5	0.699 ± 0.012	0.696 ± 0.004 (99.6 ± 0.6)	0.009 ± 0.001	0.686 ± 0.001 (98.1 ± 0.1)	0.695 ± 0.001	<0.3	3.30 ± 0.20
	15		0.699 ± 0.020 (100.0 ± 2.9)	0.010 ± 0.001	0.686 ± 0.005 (98.1 ± 0.7)	0.697 ± 0.005	<0.3	4.55 ± 0.18
fresh swordfish	5	0.704 ± 0.015	0.683 ± 0.037 (97.0 ± 5.3)	0.010 ± 0.001	0.684 ± 0.007 (97.9 ± 1.0)	0.695 ± 0.006	<0.3	5.24 ± 0.33
	15		0.702 ± 0.021 (99.7 ± 3.0)	0.010 ± 0.001	0.687 ± 0.007 (98.3 ± 1.0)	0.697 ± 0.006	<0.3	5.84 ± 0.66
dry shark	5	1.694 ± 0.011	1.691 ± 0.026 (99.8 ± 1.5)	0.015 ± 0.001	1.677 ± 0.001 (99.0 ± 0.1)	1.692 ± 0.001	<0.3	3.07 ± 0.18
	15		1.686 ± 0.006 (99.5 ± 0.4)	0.014 ± 0.001	1.678 ± 0.006 (99.1 ± 0.3)	1.692 ± 0.006	<0.3	4.61 ± 0.08
fresh shark	5	1.685 ± 0.008	1.682 ± 0.011 (99.8 ± 0.7)	0.014 ± 0.001	1.673 ± 0.006 (99.3 ± 0.4)	1.687 ± 0.006	<0.3	3.91 ± 0.02
	15		1.691 ± 0.006 (100.4 ± 0.4)	0.014 ± 0.001	1.674 ± 0.008 (99.3 ± 0.5)	1.688 ± 0.008	<0.3	5.34 ± 0.11

[a]Ultrasonic bath extractions during 5 or 15 min using HCl 5 M as extraction solution. Recoveries (in %) with respect to the concentrations measured directly in the solid sample are indicated in parentheses. Methylation of the IHg and demethylation of the MeHg are represented by F1 and F2, respectively (in %). The uncertainties given correspond to the standard deviation of $n = 3$ independent extractions.

formation correction factors. Thus, MeHg abundances around 98% and 99% have been determined for swordfish and shark, respectively, which are slightly higher values than those reported in previous publications. Furthermore, both dry and fresh samples showed similar IHg and MeHg concentrations and absolute recoveries, indicating that the extraction procedure could be applied to both types of samples with satisfactory results and that Hg species are not lost during the drying procedure. This supposes a significant advantage for routine laboratories that can avoid the drying step and save time.

Concerning species transformations, real samples showed slightly higher demethylation than the CRM material Tort-2 under the same extraction conditions. For Tort-2, the F2 values were always lower than 1%, while for the real samples mean F2 values lower than 4% were calculated for the optimum extraction conditions. An increase of the demethylation was observed using higher extraction time, which was not observed with the reference material and remark the need of reoptimization of the method using real samples. Once again, different transformations of the species were observed depending on the analyzed matrix. However, the tested ultrasound-assisted extraction reduces considerably the demethylation

differences between real samples and CRM. These results improve previous works dealing with reference materials extracted by focused MW, where higher absolute demethylation factors and discrepancy between matrixes were reported.[18] Consequently, acceptable deviations from the real values can be obtained using the optimized extraction method and quantifying with techniques that do not consider species transformations. It can be concluded that, although it is still necessary to have the evaluation of more fish species, the soft extraction conditions employed in the ultrasound bath bring closer the interconversion behaviors of the different matrixes.

On the other hand, the results for fresh and dried samples for both fish were similar, indicating that the same factors affect all of the analyzed real samples. The CRM manufacturer sample treatment consisted of a sterilization process using radiation and an extraction with acetone to remove the oil. These preparation treatments could be related to the lower demethylation factors of the Tort-2 samples, although more experiments are necessary to study this hypothesis. As in the case of Tort-2, F1 factor is negligible corroborating that no significant methylation of the IHg takes place in any studied sample.

It is worth mentioning that the sensitivity of the whole procedure is strongly affected by the extraction time. Longer extraction times lead to lower absolute ICP-MS signals for the Hg species. Fortunately, after isotope equilibrium is attained, the response of the ICP-MS does not affect the final quantitative results. Both fresh and dried samples are affected by the same effect, although this behavior was not observed in the Tort-2 material and is another reason to doubt methodologies optimized only for CRMs. This differential behavior could also be related to the oil extraction carried out by the manufacturer in the preparation of the CRM material. Sample degradation or higher coextraction of fats and high-boiling hydrocarbons could produce matrix effects as well as reduce derivatization and organic extraction efficiency, explaining the signal evolution with the extraction time. Figure 1 shows the chromatograms obtained for both the fresh (Figure 1a) and the dried shark (Figure 1b), increasing the extraction time from 5 to 60 min. At 15 min, the signal is only ca. 10% of the response obtained with 5 min extractions. Cleanup of the organic phase by, for instance, Florisil solid phase extraction cartridges, improved the results for long extraction times. This alumina-based material allows increasing the signal of the Hg species until reaching one-half of the expected signal, which cannot solve completely the problem related to high extraction times. However, the excellent recoveries, sensitivity, and the small F2 factors achieved after 5 min sonication can be considered satisfactory without requiring additional cleaning steps. The drastic changes in the sensitivity of the method at different extraction times force one to use quantification techniques that correct these strong matrix effects. As it has been demonstrated in this work, double spike IDA overcomes this problem. Even single IDA could provide accurate results with small F1 and F2 factors as those found in this work. Moreover, more affordable quantification strategies can be proposed as alternatives for routine laboratories. Quantification techniques based on internal standards or standard additions could also be employed as long as the additions take place just after the quantitative extraction of the Hg species.

To conclude, it must be highlighted that the soft sonication conditions have allowed the better control of the species transformations and have provided simplicity, speed, efficiency, and low cost to the procedure. Thus, routine analysis of fresh fish samples could be tackled with extraction procedures as short as 5 min with much reduced demethylation levels. The general availability of ultrasonic baths together with the possibility of using conventional quantification techniques increases the versatility of the analytical method.

AUTHOR INFORMATION

Corresponding Author

*Tel.: 0049 30 20937164. Fax: 0049 30 20936985. E-mail: diesteban77@yahoo.es.

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Notes

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REFERENCES

(1) Gochfeld, M. Cases of mercury exposure, bioavailability, and absorption. *Ecotoxicol. Environ. Saf.* **2003**, *56*, 174−179.

(2) Kim, D.; Wang, Q.; Sorial, G. A.; Dionysiou, D. D.; Timberlake, D. A model approach for evaluating effects of remedial actions on mercury speciation and transport in a lake system. *Sci. Total Environ.* **2004**, *327*, 1−15.

(3) Lindqvist, O.; Johansson, K.; Aastrup, M.; Andersson, A.; Bringmark, L.; Hovsenius, G.; Hakanson, L.; Iverfeldt, A.; Meili, M.; Timm, B. Mercury in the Swedish environment - Recent research on causes, consequences and corrective methods. *Water, Air, Soil Pollut.* **1991**, *55*, 23−30.

(4) Watras, C. J.; Bloom, N. S. Mercury and methylmercury, in individual zooplankton: Implications for bioaccumulation. *Limnol. Oceanogr.* **1992**, *37*, 1313−1318.

(5) European Food Safety Authority (EFSA) journal 34, 2004; pp 1−14; http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620763245.htm.

(6) Health and consumer protection directorate-general (2004) of the European Commission, Brussels; http://ec.europa.eu/food/food/chemicalsafety/contaminants/information_note_mercury-fish_12-05-04.pdf.

(7) Publications Office of the European Union, 2010, Intitute for Reference Materials and Measurements (irmm), DOI 10.2787/33057; http://publications.jrc.ec.europa.eu/repository/handle/111111111/15484.

(8) Commission regulation (EC) no. 1881/2006 (2006), Official Journal of the European Union; http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_364/l_36420061220en00050024.pdf.

(9) Commission regulation (EC) no. 629/2008 (2008), Official Journal of the European Union; http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:173:0006:0009:EN:PDF.

(10) Centineo, G.; Blanco González, E.; García Alonso, J. I.; Sanz-Medel, A. Isotope dilution SPME GC/MS for the determination of methylmercury in tuna fish samples. *J. Mass Spectrom.* **2006**, *41*, 77−83.

(11) Qvarnström, J.; Tu, Q.; Frech, W.; Lüdke, C. Flow injection-liquid chromatography-cold vapour atomic absorption spectrometry for rapid determination of methyl and inorganic mercury. *Analyst* **2000**, *125*, 1193−1197.

(12) Li, Y.; Yan, X. P.; Dong, L. M.; Wang, S. W.; Jiang, Y.; Jiang, D. Q. Development of an ambient temperature post-column oxidation system for high-performance liquid chromatography on-line coupled

with cold vapor atomic fluorescence spectrometry for mercury speciation in seafood. *J. Anal. At. Spectrom.* **2005**, *20*, 467−472.

(13) Gómez-Ariza, J. L.; Lorenzo, F.; García-Barrera, T. Sample treatment selection for routine mercury speciation in seefood by gas chromatography-atomic fluorescence spectroscopy. *Appl. Organomet. Chem.* **2005**, *19*, 600−604.

(14) Percy, A. J.; Korbas, M.; George, G. N.; Gailer, J. Reversed-phase high-performance liquid chromatographic separation of inorganic mercury and methylmercury driven by their different coordination chemistry towards thiols. *J. Chromatogr., A* **2007**, *1156*, 331−339.

(15) Gebersmann, C.; Heisterkamp, M.; Adams, F. C.; Broekaert, J. A. C. Two methods for the speciation analysis of mercury in fish involving microwave-assisted digestion and gas chromatography-atomic emission spectrometry. *Anal. Chim. Acta* **1997**, *350*, 273−285.

(16) Li, Y.; Hu, B. Sequential cloud point extraction for the speciation of mercury in seafood by inductively coupled plasma optical emission spectrometry. *Spectrochim. Acta, Part B* **2007**, *62*, 1153−1160.

(17) Monperrus, M; Rodríguez González, P.; Amouroux, D.; García Alonso, J. I.; Donard, O. F. X. Evaluating the potential and limitations of double-spiking species-specific isotope dilution analysis for the accurate quantification of mercury species in different environmental matrices. *Anal. Bioanal. Chem.* **2008**, *390*, 655−666.

(18) Castillo, A.; Rodríguez-González, P.; Centineo, G.; Roig-Navarro, A. F.; García Alonso, J. I. Multiple spiking species-specific isotope dilution analysis by molecular mass spectrometry: simultaneous determination of inorganic mercury and methylmercury in fish tissues. *Anal. Chem.* **2010**, *82*, 2773−2783.

(19) Clémens, S.; Monperrus, M.; Donard, O. F. X.; Amouroux, D.; Guérin, T. Mercury speciation in seafood using isotope dilution analysis: A review. *Talanta* **2012**, *89*, 12−20.

(20) Reyes, L. H.; Mizanur Rahman, G. M.; Fahrenholz, T.; Skip Kingston, H. M. Comparison of methods with respect to efficiencies, recoveries, and quantitation of mercury species interconversions in food demonstrated using tuna fish. *Anal. Bioanal. Chem.* **2008**, *390*, 2123−2132.

(21) Nevado, J. J. B.; Rodríguez Martín-Doimeadios, R. C.; Guzmán Bernardo, F. J.; Jiménez Moreno, M. Determination of mercury species in fish reference materials by gas chromatography-atomic fluorescence detection after closed-vessel microwave-assisted extraction. *J. Chromatogr., A* **2005**, *1093*, 21−28.

(22) Hintelmann, H.; Nguyen, H. T. Extraction of methylmercury from tissue and plant samples by acid leaching. *Anal. Bioanal. Chem.* **2005**, *381*, 360−365.

(23) Ebdon, L.; Foulkes, M. E.; Le Roux, S.; Muñoz-Olivas, R. Cold vapour atomic fluorescence spectrometry and gas chromatography-pyrolysis-atomic fluorescence spectrometry for routine determination of total and organometallic mercury in food samples. *Analyst* **2002**, *127*, 1108−1114.

(24) Clay Davis, W.; Vander Pol, S. S.; Schantz, M. M.; Long, S. E.; Day, R. D.; Christopher, S. J. An accurate and sensitive method for the determination of methylmercury in biological specimens using GC-ICP-MS with solid phase microextraction. *J. Anal. At. Spectrom.* **2004**, *19*, 1546−1551.

(25) Lemos Batista, B.; Rodrigues, J. L.; de Souza, S. S.; Oliveira Souza, V. C.; Barbosa, F., Jr. Mercury speciation in seafood samples by LC-ICP-MS with a rapid ultrasound-assisted extraction procedure: Application to the determination of mercury in Brazilian seafood samples. *Food Chem.* **2011**, *126*, 2000−2004.

(26) Ibánez-Palomino, C.; López-Sánchez, J. F.; Sahuquillo, A. Certified reference materials for analytical mercury speciation in biological and environmental matrices: Do they meet user needs?; a review. *Anal. Chim. Acta* **2012**, *720*, 9−15.

(27) Pedrero, Z.; Mounicou, S.; Monperrus, M.; Amouroux, D. Investigation of Hg species binding biomolecules in dolphin liver combining GC and LC-ICP-MS with isotopic tracers. *J. Anal. At. Spectrom.* **2011**, *26*, 187−194.

(28) Rodríguez-Castrillón, J. A.; Moldován, M.; García Alonso, J. I.; Lucena, J. J.; García Tomé, M. L.; Hernández-Apaolaza, L. Isotope pattern deconvolution as a tool to study iron metabolism in plants. *Anal. Bioanal. Chem.* **2008**, *390*, 579−590.

(29) Rodríguez-González, P.; García Alonso, J. I. Recent advances in isotope dilution analysis for elemental speciation. *J. Anal. At. Spectrom.* **2010**, *25*, 239−259.

(30) Cabañero Ortiz, A. I.; Madrid Albarrán, Y.; Cámara Rica, C. Evaluation of different sample pre-treatment and extraction procedures for mercury speciation in fish samples. *J. Anal. At. Spectrom.* **2002**, *17*, 1595−1601.