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Assessment of Modified Matrix Solid-Phase Dispersion as Sample Preparation for the Determination of CH_3Hg^+ and Hg^{2+} in Fish

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Supporting Information

ABSTRACT: This paper reports, for the first time, the development of an analytical method employing modified matrix solid-phase dispersion (MSPD) for the extraction of CH_3Hg^+ and Hg^{2+} species from fish samples. Separation and determination of mercury species were performed by gas chromatography coupled to mass spectrometry (GC/MS). Important MSPD parameters, such as sample mass, type and mass of solid support, concentration of extraction solution (HCl and NaCl), and stirring time, were investigated by the response surface methodology. The derivatization step and the separation of mercury species were also evaluated for the determination by GC/MS. Quantitative recoveries were obtained with 0.2 g of fish sample, 0.5 g of SiO_2 as the solid support, 0.5 mol L^{-1} NaCl and 4.2 mol L^{-1} HCl as the extraction solution, and 1 min stirring time. The MSPD method showed to be suitable for the extraction and determination of mercury species in certified reference materials of dogfish liver (DOLT-3) and dogfish muscle (DORM-2). It had good agreement (about 99%) with the certified values, and the relative standard deviation was lower than 9.5%. The limits of detection were 0.06 and 0.12 $\mu g\ g^{-1}$, for CH_3Hg^+ and Hg^{2+}, respectively. A matrix effect was observed, and the quantification was carried out by the matrix-matched calibration. The method was applied to tuna fish (*Thunnus thynnus*), angel shark (*Squatina squatina*), and guitarfish (*Rhinobatos percellens*) samples. The results of the mercury speciation by MPSD and GC/MS were compared to the total mercury concentration determined by flow injection cold vapor generation inductively coupled plasma mass spectrometry, after microwave-assisted digestion. Agreement ranged from 102% to 105%.

Mercury is considered a highly toxic element whose toxicity depends on its chemical forms. The main mercury species that can be found in the environment are elemental mercury (Hg^0), divalent inorganic mercury (Hg^{2+}), and methylmercury (CH_3Hg^+), which is the most toxic species.[1] After ingestion by humans, CH_3Hg^+ rapidly binds to erythrocytes and spreads to several body tissues.[2] Its lipophilic characteristic enables it to be easily absorbed by the gastrointestinal tract, to cross the blood−placenta barrier, and to concentrate in the central nervous system.[3,4] In fish, CH_3Hg^+ is the most common species, mainly due to its capacity for bioaccumulation, providing the major route of human exposure.[5]

For the speciation analysis in biological tissues, the most critical step can be attributed to the analyte extraction from the matrix, since the integrity of the different chemical forms must be guaranteed.[6] Extraction procedures based on acid leaching,[7−9] alkaline extraction,[10−12] complexing (L-cysteine) solutions,[8,9,12] or mixture of acid solutions and inorganic salts (Westöö method),[13] with the aid of sonication,[8,14,15] microwave radiation,[7,9,11] or conventional heating[10,16] have been evaluated to promote the extraction of mercury species from fish tissues. However, these methods demand a long extraction time or show low efficiency when they are not assisted by alternative energy, such as ultrasound or microwave. Both, but mainly the former, can increase the possibility of CH_3Hg^+ being converted to Hg^{2+}, depending on the conditions; thus, a careful study of species stability is required.[15] Additionally, studies have shown that the use of tetramethylammonium hydroxide (TMAH), as an extraction solution, can promote the methylation of Hg^{2+}, which can overestimate the results for CH_3Hg^+.[17,18]

Matrix solid-phase dispersion (MSPD), which was developed by Barker et al.,[19] in 1989, consists in blending a viscous, solid, or semisolid sample and a solid support (e.g., silica, SiO_2, or C18). The mixture is blended/homogenized in a mortar with a pestle, where the solid support acts as an abrasive agent that disrupts the gross architecture of the sample and breaks the material into smaller pieces. Blending and the presence of a solid support provide the dispersion of sample components on the surface of the particle. After blending, the material is transferred and packaged into a cartridge for further elution with a suitable solvent.[20] MSPD has been successfully applied to the extraction of organic compounds from food matrices.[21−23] However, few studies of element and/or species extraction have been published.[24−26] MSPD has shown

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advantages over the methods available for mercury speciation based on the use of sonication, microwave radiation, or conventional heating because it can be carried out at room temperature and atmospheric pressure, factors that guarantee the species integrity.[27] In addition, other factors such as quantitative extraction, low extraction time, use of reagents with low toxicity, and low waste generation have increased the interest in the development of new sample preparation methods. Therefore, MSPD appears as a novel alternative.

Mercury species have been determined by hyphenated techniques, such as liquid chromatography inductively coupled plasma mass spectrometry (LC/ICPMS),[14,15] gas chromatography inductively coupled plasma mass spectrometry (GC/ICPMS),[28,29] gas chromatography coupled to mass spectrometry (GC/MS),[30−32] gas chromatography coupled to atomic fluorescence spectroscopy (GC/AFS),[10,33] and gas chromatography microwave-induced plasma coupled to atomic emission spectroscopy (GC/MIP-AES).[18,34] The main advantage of GC by comparison with LC is the quantitative elution of analytes from the chromatographic column to the detector without any nebulization step, a fact that improves the limits of detection (LODs). However, a drawback of the GC technique is the requirement of derivatization of the ionic mercury species to obtain volatile and thermally stable forms,[35] mainly when the inorganic form is present. Sodium tetraethylborate ($Na[B(C_2H_5)_4]$),[30,36] sodium tetra *n*-propylborate ($Na[B(C_3H_7)_4]$),[5,31] and sodium tetraphenylborate ($Na[B(C_6H_5)_4]$)[32,34] have been described as derivatizant reagents for the alkylation of mercury species whereas ethylation and propylation are the most common derivatization reagents.[35] However, $Na[B(C_6H_5)_4]$ is more stable in water solution than $Na[B(C_2H_5)_4]$, and its derivatives are more thermostable for GC separation, due to the fact that the $RHg-C_6H_5$ bond is stronger than the $RHg-C_2H_5$ one.[33]

In this study, modified MSPD has been proposed, for the first time, as a novel extraction method of mercury species (CH_3Hg^+ and Hg^{2+}) from fish tissues. Separation and determination of mercury species were performed by GC/MS. The influence of important MSPD parameters, such as sample mass, type and mass of the solid support, concentration of the extraction solution, and stirring time were exhaustively investigated with the assistance of the surface response methodology. Results obtained from the sum of mercury species in angel shark (*Squatina squatina*), guitarfish (*Rhinobatos percellens*), and tuna fish (*Thunnus thynnus*) samples were compared to the results of total mercury obtained by flow injection cold vapor generation inductively coupled plasma mass spectrometry (FI-CVG-ICPMS) after microwave-assisted digestion (MAD). The accuracy was verified by applying the proposed method to the analysis of certified reference materials (CRMs) of dogfish muscle (DORM-2) and dogfish liver (DOLT-3).

EXPERIMENTAL SECTION

Instrumentation. The separation and determination of mercury species were carried out by a gas chromatograph coupled to a mass spectrometer (Shimadzu, model GC-MS-QP2010 Plus, Columbia, MD, USA) equipped with an AOC-20i auto sampler and a mass spectrometer detector with quadrupole mass filter. Mass spectrometry was performed in the electron impact mode with 70 eV. GC separation was carried out in an Rtx-5MS analytical column (30 m × 0.25 mm i.d) with 0.25 μm film thickness (Restek, Bellefonte, PA, USA). The mass spectrometer was operated in the selective ion monitoring (SIM) mode. Helium (99.999% purity) was used as the carrier gas (White Martins, São Paulo, Brazil). The total mercury concentration in digests was determined by CVG-ICPMS with a homemade flow injection system, whose construction was described in a previous study.[37] Mercury determination was carried out by an inductively coupled plasma mass spectrometer (Perkin−Elmer Sciex, model ELAN DRC II, Thornhill, Canada), equipped with a quartz torch (injector tube 2 mm i.d.) and platinum cones. ICPMS parameters were adjusted to obtain the highest signal to background ratio for ^{202}Hg. Samples were digested by a microwave oven Multiwave 3000 (Anton Paar, Graz, Austria). The laboratory equipment comprised an analytical balance (Bioprecisa, model FA210N, Curitiba, Brazil), a pH meter (Hanna, model pH21, São Paulo, Brazil), and a vortex stirrer (B. Braun Biotech Int., model Certomat MV, Melsungen, Germany).

Reagents. Ultrapure water with resistivity 18.2 MΩ cm^{-1} was obtained from a Milli-Q system Direct-Q UV3 (Millipore, Bedford, MA, USA). Nitric acid and hydrochloridric acid were purchased from Merck (Darmstadt, Germany) and purified by a sub-boiling system (Milestone, model Duopur, Bergamo, Italy). Sodium chloride and glacial acetic acid were purchased from Merck. A reference stock solution of Hg^{2+} with 1000 mg L^{-1} (as Hg) in 2% (v/v) HNO_3 was prepared by dissolving $HgCl_2$ (Merck). A reference stock solution of CH_3Hg^+ containing 1000 mg L^{-1} (as Hg) in methanol was prepared by dissolving CH_3HgCl purchased from Sigma−Aldrich (St. Louis, MO, USA) and stored in polypropylene vessels at 4 °C in the dark. For derivatization of mercury species, $Na[B(C_6H_5)_4]$ (Sigma−Aldrich) with purity above 99.5% was used. Sodium acetate, methanol, and *n*-hexane were purchased from J.T. Baker (Phillipsburg, NJ, USA). MSPD solid supports were octadecyl-functionalized silica gel (C18), purchased from Macherey−Nagel (Düran, Germany) with 45 μm particle size and 60 Å pore size, and silica gel (SiO_2), purchased from ACROS organics (Geel, Belgium) with particle size 35−70 μm and 60 Å pore size.

For total mercury determination by FI-CVG-ICPMS, analytical solutions ranging from 0.025 to 20 μg L^{-1} (as Hg), from Merck, were prepared in 5% (v/v) HNO_3 (Merck). For the cold vapor generation system, sodium tetrahydroborate ($NaBH_4$), purchased from Vetec (Duque de Caxias, Brazil), was prepared in 0.01% NaOH (Merck). Reference solutions for instrument calibration and optimization of the method were prepared fresh daily. All solutions were stored in polypropylene vessels, which were previously cleaned by immersion in 20% (v/v) HNO_3 solution for 24 h and rinsed with ultrapure water before use.

Fish Samples. Samples of angel shark (*Squatina squatina*), guitarfish (*Rhinobatos percellens*), and tuna fish (*Thunnus thynnus*) were purchased in a local supermarket in the forms of fresh steaks or muscles (~500 g of each fish). Samples were homogenized by mechanical blending using a stainless steel mixer (during 30 s with short cycles of 5 s), oven-dried at 75 °C for 24 h,[38] and then, ground in a mortar with a pestle. CRMs of dogfish muscle (DORM-2) and dogfish liver (DOLT-3) were purchased from the National Research Council of Canada (Ottawa, Canada) and were used to evaluate the accuracy and the precision of the method. For optimization of the MSPD procedure, a tuna fish sample was used. In this case, the original concentration of CH_3Hg^+ and Hg^{2+} was obtained according to

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Schmidt et al.,[38] using 10 mL of 0.6% (w/v) L-cysteine as extraction solution and a LC/ICPMS for species determination.

GC/MS Measurements. Separation and determination of the mercury species were performed by GC/MS in operating conditions, which are shown in Table 1. On the basis of a study developed by Mishra et al.,[32] the fragments m/z 51, 77, 91, 217, 279, and 294, and 51, 77, 154, 179, and 356 were used to monitor, in the SIM mode, both species $CH_3Hg(C_6H_5)$ and $Hg(C_6H_5)_2$, respectively. A split insert was used, and the split ratio was evaluated from 1:1 to 1:7. The initial temperature (30−130 °C) and the heating rate (20−50 °C min^{-1}) were also evaluated; the criterion was the lowest retention time. Derivatization variables, such as pH of acetate buffer (3.0−8.0), volume of 1% (w/v) $Na[B(C_6H_5)_4]$ solution (0.5−2.0 mL), and stirring time (0.5−15 min), were evaluated by comparing the peak areas. The acquisition and treatment of instrument data were performed by the software GCMS solution, version 2 (Shimadzu). The comparison between two averages was performed by the student t-test whereas the Tukey−Kramer test, with the aid of the software Statistic 8.0 (copyright 1984−2007, Statsoft Inc.), was used for comparing three or more averages. Data treatment obtained after MSPD multivariate optimization was also performed by the software Statistic 8.0.

Table 1. Operation Conditions of GC/MS and FI-CVG-ICPMS

GC/MS	
carrier gas flow rate (mL min^{-1})	14.5
linear velocity (cm s^{-1})	45.2
pressure (kPa)	121.8
injection temp (°C)	200
initial temp (°C)	130 for 5 min
heating rate (°C min^{-1})	50
final temp (°C)	270 for 2.5 min
ion source temp (°C)	230
interface temp (°C)	250
ionization (eV)	70
$CH_3HgC_6H_5$ (m/z)	51, 77, 91, 217, 279, 294
$Hg(C_6H_5)_2$ (m/z)	51, 77, 154, 279, 356
FI-CVG-ICPMS	
RF power (W)	1400
plasma gas flow rate (L min^{-1})	15
auxiliary gas flow rate (L min^{-1})	1.2
nebulizer gas flow rate (L min^{-1})	1.05
spray chamber	cyclonic
nebulizer	concentric
sampler and skimmer cones	Pt
ion lens	auto lens on
isotope (m/z)	^{202}Hg
dwell time (ms)	250
carrier solution (HCl) conc (mol L^{-1})	1 (8.0 mL min^{-1})
reductant solution ($NaBH_4$) conc (%, w/v)	0.1 (3.7 mL min^{-1})

Derivatization and MSPD Procedures. The sample was blended with a suitable amount of silica (SiO_2) in a ceramic pestle with a mortar for 5 min to obtain a homogeneous mixture that was transferred to a 15 mL polypropylene vessel. Afterward, 3 mL of the extraction solution was added to the vessel. Additionally, the mortar surface was washed with 2 mL of the same extraction solution. The suspension was vortex stirred for 1 min and centrifuged for 10 min at 3000 rpm. The supernatant was transferred to another polypropylene vessel with 2 mL of acetate buffer at pH 5.0; 1 mL of 1% (w/v) $Na[B(C_6H_5)_4]$ and 1 mL of n-hexane were added. The mixture was vortex stirred again for 0.5 min and then centrifuged for 10 min at 3000 rpm. The organic phase was transferred to glass vessels for further injection into the GC/MS instrument. It is important to point out that the original MSPD method[19] was modified: the extraction of mercury species was carried out directly in the polypropylene vessel; thus, not only the use of mixture packing in the cartridges but also the use of a manifold system for elution was eliminated.

Total Mercury Determination by FI-CVG-ICPMS. Total mercury determination was performed for the comparison between the sum of mercury species concentration, obtained by GC/MS after MSPD, and the concentration of total mercury, determined by FI-CVG-ICP-MS after MAD. Initially, 0.2 g of ground and dried fish samples were digested (n = 3) in a microwave oven with quartz closed vessels by adding 6 mL of concentrated HNO_3. The heating program was adapted from Pilz et al.,[15] and the samples were subject to the following conditions: (i) 0−1000 W (10 min of ramp); (ii) 1000 W for 10 min; and (iii) 0 W for 20 min (cooling step). During sample digestion, the temperature reached about 230 °C. Digests were completed up to 30 mL with ultrapure water, and the total mercury was determined by FI-CVG-ICPMS. Parameters of ICPMS were adjusted in order to obtain the highest signal to background ratio. Operation conditions for total mercury determination are described in Table 1.

RESULTS AND DISCUSSION

Optimization of CH_3Hg^+ and Hg^{2+} Separation and Determination by GC/MS. Fragments (m/z) used for mercury species (CH_3Hg^+ and Hg^{2+}) quantification in the SIM mode were m/z 294 and 356 for $CH_3HgC_6H_5$ and $Hg(C_6H_5)_2$, respectively. The initial temperature of the chromatograph heating program was evaluated from 30 to 130 °C. The temperature at 130 °C showed a decrease of up to 7 min in the retention time for both species; thus, the total time of analysis decreased in 5 min. The heating rate was evaluated from 20 to 50 °C min^{-1}, and the rate of 50 °C min^{-1} showed a decrease in the retention time of up to 2.3 min for the least volatile species ($Hg(C_6H_5)_2$), which eluted in 4.6 min; the $CH_3HgC_6H_5$ species eluted in 8.8 min. It is important to point out that values higher than 130 °C (initial temperature) and 50 °C min^{-1} (heating rate) were not studied, since these values showed optimum conditions.

Derivatization parameters were evaluated by comparing peak areas after a spike of 1 μg of CH_3Hg^+ and Hg^{2+}, as Hg, in an aqueous medium. The effect of the derivatizing volume was evaluated from 0.5 to 2.0 mL by employing 1% (w/v) $Na[B(C_6H_5)_4]$ solution in order to ensure an excess amount. In the volume range (0.5−2 mL) of the derivatizing solution under evaluation, no significant difference was observed (p > 0.05). The volume of 0.5 mL was chosen for further studies. Similarly, Mao et al.[29] used 1 mL of 1% (w/v) $Na[B(C_6H_5)_4]$ for the determination of CH_3Hg^+ and $C_2H_5Hg^+$ by GC/AFS and GC/ICPMS. Other authors have used 2 mL of 1% (w/v) $Na[B(C_6H_5)_4]$ for the determination of CH_3Hg^+ in seafood samples by GC coupled to atomic emission spectroscopy (AES).[34,39] The effect of the stirring/reaction time was also evaluated (ranging from 0.5 to 15 min), and no significant difference (p > 0.05) was found. Thus, in order to minimize the derivatization time, 0.5 min was chosen for further studies.

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Table 2. 2^{5-1} Fractional Factorial Design Matrix and CH_3Hg^+ and Hg^{2+} Recoveries

treatment	sample mass (g)	solid support mass (g)	HCl conc (mol L^{-1})	NaCl conc (mol L^{-1})	solid support (5=1234)[a]	CH_3Hg^+ recovery (%)	Hg^{2+} recovery (%)
1	−1 (0.25)	−1 (0.5)	−1 (1.0)	−1 (0.5)	+1 (SiO_2)	56	6
2	+1 (0.75)	−1 (0.5)	−1 (1.0)	−1 (0.5)	−1 (C18)	32	3
3	−1 (0.25)	+1 (1.5)	−1 (1.0)	−1 (0.5)	−1 (C18)	21	5
4	+1 (0.75)	+1 (1.5)	−1 (1.0)	−1 (0.5)	+1 (SiO_2)	21	2
5	−1 (0.25)	−1 (0.5)	+1 (5.0)	−1 (0.5)	−1 (C18)	10	30
6	+1 (0.75)	−1 (0.5)	+1 (5.0)	−1 (0.5)	+1 (SiO_2)	24	31
7	−1 (0.25)	+1 (1.5)	+1 (5.0)	−1 (0.5)	+1 (SiO_2)	28	34
8	+1 (0.75)	+1 (1.5)	+1 (5.0)	−1 (0.5)	−1 (C18)	13	12
9	−1 (0.25)	−1 (0.5)	−1 (1.0)	+1 (1.5)	−1 (C18)	43	10
10	+1 (0.75)	−1 (0.5)	−1 (1.0)	+1 (1.5)	+1 (SiO_2)	36	2
11	−1 (0.25)	+1 (1.5)	−1 (1.0)	+1 (1.5)	+1 (SiO_2)	28	8
12	+1 (0.75)	+1 (1.5)	−1 (1.0)	+1 (1.5)	−1 (C18)	17	2
13	−1 (0.25)	−1 (0.5)	+1 (5.0)	+1 (1.5)	+1 (SiO_2)	11	52
14	+1 (0.75)	−1 (0.5)	+1 (5.0)	+1 (1.5)	−1 (C18)	9	13
15	−1 (0.25)	+1 (1.5)	+1 (5.0)	+1 (1.5)	−1 (C18)	4	29
16	+1 (0.75)	+1 (1.5)	+1 (5.0)	+1 (1.5)	+1 (SiO_2)	25	26
17	0 (0.50)	0 (1.0)	0 (3.0)	0 (1.0)	+1 (SiO_2)	46	48
18	0 (0.50)	0 (1.0)	0 (3.0)	0 (1.0)	+1 (SiO_2)	44	32
19	0 (0.50)	0 (1.0)	0 (3.0)	0 (1.0)	+1 (SiO_2)	36	32
20	0 (0.50)	0 (1.0)	0 (3.0)	0 (1.0)	−1 (C18)	22	14
21	0 (0.50)	0 (1.0)	0 (3.0)	0 (1.0)	−1 (C18)	24	14
22	0 (0.50)	0 (1.0)	0 (3.0)	0 (1.0)	−1 (C18)	21	13

[a]Identity matrix: represents the multiplication of the first four columns in the fractional factorial design.

Table 3. Central Rotatable Composite Design Matrix, CH_3Hg^+ and Hg^{2+} Observed and Predicted Recoveries, and the Relative Deviations of Regression Models

treatment	sample mass (g)	HCl conc (mol L^{-1})	stirring time (min)	observed CH_3Hg^+ recovery (%)	observed Hg^{2+} recovery (%)	predicted CH_3Hg^+ recovery (%)	predicted Hg^{2+} recovery (%)	relative deviation CH_3Hg^+ (%)	relative deviation Hg^{2+} (%)
1	−1 (0.2)	−1 (1.8)	−1 (3.8)	129	35	118	49	9	40
2	1 (0.5)	−1 (1.8)	−1 (3.8)	89	10	101	19	14	79
3	−1 (0.2)	1 (4.2)	−1 (3.8)	79	98	95	97	19	2
4	1 (0.5)	1 (4.2)	−1 (3.8)	81	66	77	67	4	1
5	−1 (0.2)	−1 (1.8)	1 (12.2)	118	33	118	49	0	46
6	1 (0.5)	−1 (1.8)	1 (12.2)	105	11	101	19	4	68
7	−1 (0.2)	1 (4.2)	1 (12.2)	102	89	95	97	7	8
8	1 (0.5)	1 (4.2)	1 (12.2)	51	57	77	67	51	16
9	−1.68 (0.1)	0 (3.0)	0 (8.0)	112	99	112	92	0	7
10	1.68 (0.6)	0 (3.0)	0 (8.0)	104	43	83	42	20	3
11	0 (0.35)	−1.68 (1.0)	0 (8.0)	97	16	117	1	21	96
12	0 (0.35)	1.68 (5.0)	0 (8.0)	78	80	78	81	0	2
13	0 (0.35)	0 (3.0)	−1.68 (1.0)	103	68	98	67	5	2
14	0 (0.35)	0 (3.0)	1.68 (15.0)	109	69	98	67	10	3
15	0 (0.35)	0 (3.0)	0 (8.0)	85	67	98	67	15	0
16	0 (0.35)	0 (3.0)	0 (8.0)	107	69	98	67	9	4
17	0 (0.35)	0 (3.0)	0 (8.0)	100	62	98	67	2	7

Similarly, Cai et al.[33] studied the time of the phenylation reaction of CH_3Hg^+ from 5 to 40 min, and no influence was observed for this time interval. pH is another important parameter for the derivation reaction: pH values from 3 to 7 were evaluated, and no significant difference ($p > 0.05$) was found. Other authors[33,34] have also studied the pH effect from 4 to 10 in phenylation reactions of mercury species, and the same effect was found. However, most studies have used pH 4.5 or 5.0 in phenylation reactions.[29,33,36,39] Therefore, pH 5 was chosen for further studies.

MSPD Optimization. *Initial Studies.* In this study, MSPD was proposed for the first time for the extraction of CH_3Hg^+ and Hg^{2+} species from fish tissues. The preliminary experiments were based on the studies developed by Moreda-Piñeiro et al.[25] and Rodrigues et al.[21] Initially, the original MSPD was carried out: 0.5 g of sample; 1 g of C18 as the solid support; 5 min of dispersion time (time of blending); and 15 mL of elution solvent. The elution solvents evaluated were methanol 20, 50, 60, and 80% (v/v) and acetonitrile. However, in these conditions, CH_3Hg^+ and Hg^{2+} recoveries were below 1%. This difficulty regarding the extraction may be attributed to the ionic nature of CH_3Hg^+ and Hg^{2+} species, since these solvents have characteristics that enabled them to extract molecular compounds.

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Therefore, 5 mL of 1% (w/v) L-cysteine solution as extraction solution, in the same previously evaluated conditions, was tested. In these conditions, CH_3Hg^+ and Hg^{2+} recoveries were also below 1%, probably due to the low extraction time (about 1 min). In some cases, extraction times higher than 2 h (and even heating) are required for the extraction of CH_3Hg^+ and Hg^{2+} species from fish samples by an L-cysteine solution.[16,38]

On the basis of studies developed by Westöö[40] and Duarte et al.,[13] which used inorganic salts and acids for the extraction of CH_3Hg^+ and Hg^{2+} species, an experiment was carried out with 5 mL of extraction solution 3 mol L^{-1} HCl and 1 mol L^{-1} NaCl, and C18 and SiO_2 as the solid support, using the modified MSPD. This condition showed recoveries between 4% and 19%. Thus, a systematic study was carried out for the optimization of the main variables with the assistance of surface response methodology in order to improve the recoveries for CH_3Hg^+ and Hg^{2+}. Initially, a 2^{5-1} fractional factorial design was applied in order to perform a screening of significant variables on the mercury species extraction.

2^{5-1} Fractional Factorial Design. The influence of important MSPD variables for the mercury species recoveries, such as sample mass (0.25−0.75 g), mass (0.5−1.5 g) and type of solid support (SiO_2 and C18), HCl concentration (1.0−5.0 mol L^{-1}), and NaCl concentration (0.5−1.5 mol L^{-1}) were evaluated in 2 levels through of a fractional factorial design with 22 treatments (6 central point and triplicate for each solid support type, Table 2), in which the recoveries were up to 56% for both species. The influence of variables was evaluated through the analysis of effects (90% confidence level), as shown in Table 4. Among the variables under study, only sample mass, HCl concentration, and type of solid support showed significant effect ($p < 0.1$). The increase of the sample mass from 0.25 to 0.75 g showed a decrease in the Hg^{2+} recovery (about 10%). As a result, sample mass ranging from 0.1 to 0.6 g was studied in the next experimental design. The mass of the solid support was not significant in the range under investigation (0.5−1.5 g); 0.5 g was kept for the further study. HCl concentration (1 and 5 mol L^{-1}) was the most significant variable for both mercury species. For CH_3Hg^+, a negative effect (−16%) was observed; it showed the tendency to decrease the CH_3Hg^+ recovery as the HCl concentration increased. On the other hand, the Hg^{2+} species showed a positive effect (24%). Thus, the same HCl concentration range was evaluated in further studies. NaCl concentration varied from 0.5 to 1.5 mol L^{-1}. Since no significant effect for both mercury species was observed, 0.5 mol L^{-1} NaCl was kept for the next experimental design in order to decrease the reagent consumption. C18 and SiO_2 were chosen as solid supports in order to evaluate materials with different polarities and to study their behavior in the CH_3Hg^+ and Hg^{2+} species extraction in different extraction conditions. Mainly CH_3Hg^+, SiO_2, rather than C18, caused a significant increase in the recovery (about 10%). Therefore, SiO_2 was chosen as the solid support for further experiments. Therefore, a CRCD was applied only with the significant variables with the results obtained in the 2^{5-1} fractional factorial design.

Table 4. Analysis of Effects on the Variables Studied in the 2^{5-1} Fractional Factorial Design

	CH_3Hg^+			Hg^{2+}		
factor	effect	std error	p value	effect	std error	p value
mean	26	2	0	19	2	0
sample mass (g)	−3	5	0.5802	−10	5	0.0721
solid support mass (g)	−8	5	0.1693	−4	5	0.4862
HCl conc (mol L^{-1})	−16	5	0.0098	24	5	0.0004
NaCl conc (mol L^{-1})	−4	5	0.4568	2	5	0.6500
solid support type (C18 and SiO_2)	10	5	0.0891	7	5	0.1972

Central Rotatable Composite Design. On the basis of the results obtained in the 2^{5-1} fractional factorial design, the significant variables such as sample mass (0.2−0.5 g) and HCl concentration (1.0−5.0 mol L^{-1}) were again evaluated in new ranges with the addition of stirring time (1−15 min) as a variable, using a central rotatable composite design (CRCD). The CRCD was composed by 17 treatments with 6 experiments at axial points (−1.68 as the lowest level and 1.68 as the highest level) and 3 experiments at central points, as shown in Table 3. The variables solid support mass, NaCl concentration, and type of solid support were fixed at 0.5 g, 0.5 mol L^{-1}, and SiO_2 as solid support, respectively. When the CRCD was applied, the recoveries increased considerably, ranging from 51% to 129% and from 10% to 99% for CH_3Hg^+ and Hg^{2+}, respectively, by comparison with the first factorial design. The regression models were evaluated by ANOVA with 95% confidence level, employing the usual Fisher F-tests, according to Table 5. It is important to mention that the regression models that were simplified by removing terms that were not statistically significant ($p > 0.05$) are represented by the following equations:

$$R_{org} = 97.6 - 8.5m_s - 11.6[HCl]$$

$$R_{ino} = 66.8 - 15.1m_s + 24.1[HCl] - 9.1[HCl]^2$$

where R_{org} is the CH_3Hg^+ recovery, R_{ino} is the Hg^{2+} recovery, m_s is the sample mass, and [HCl] is the HCl concentration. The Fisher F-test (Table 5) revealed high significance for the regression models, since the computed F values were 2.1 and 16.1 times higher than the tabulated F values for CH_3Hg^+ and Hg^{2+}, respectively. The coefficients of determination (R^2) were 0.85 for CH_3Hg^+ and 0.91 for Hg^{2+}, indicating that only 15% and 9% of the overall variation was not explained by the regression models for CH_3Hg^+ and Hg^{2+} species, respectively.

Table 5. ANOVA Parameters[a]

	CH_3Hg^+					Hg^{2+}				
variation source	SS	DF	MS	F_{com}	F_{tab}	SS	DF	MS	F_{com}	F_{tab}
regression	2851.6	2	1425.8	7.9	3.7	11954.2	3	3984.7	54.9	3.4
residual	2514.2	14	179.6			943.0	13	72.5		
total	5365.7	16				12897.2	16			

[a]SS: sum of squares; DF: degrees of freedom; MS: mean squares; F_{cal}: computed F value; F_{tab}: tabulated F value.

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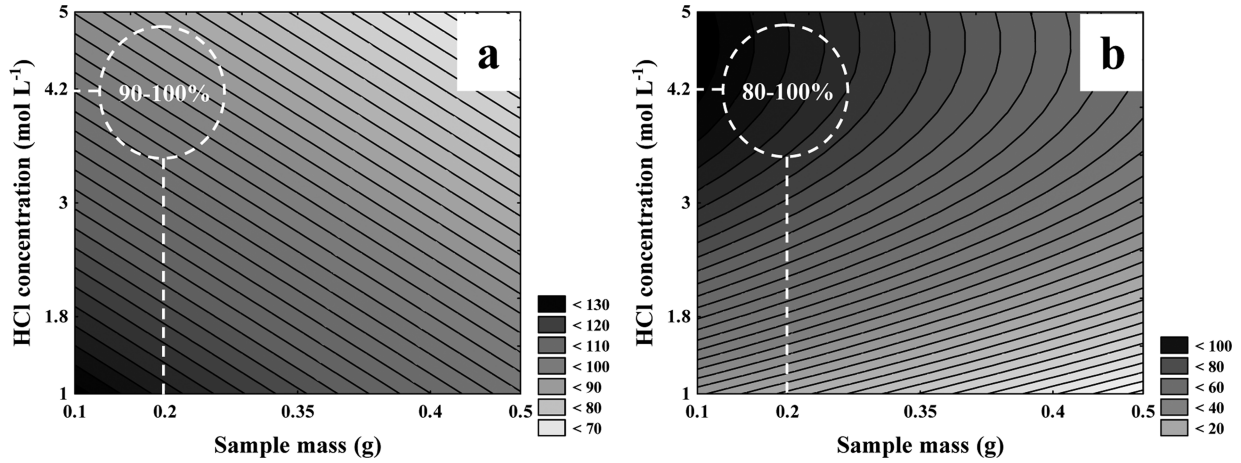

Figure 1. Response profiles representing the recoveries for (a) CH_3Hg^+ and (b) Hg^{2+} species as a function of variables HCl concentration and sample mass.

Table 6. Results for CH_3Hg^+ and Hg^{2+} Species Determination in Fish Tissues and CRMs by GC/MS after MSPD Extraction and Total Mercury Determination by FI-CVG-ICPMS after MAD[a]

sample	found value for CH_3Hg^+	found value for Hg^{2+}	sum of species	certified value CH_3Hg^+	certified value[b] Hg^{2+}	total[c]
DOLT-3	1.57 ± 0.15	1.76 ± 0.15	3.33 ± 0.21	1.59 ± 0.12	1.78 ± 0.18	3.37 ± 0.14
DORM-2	3.93 ± 0.09	0.39 ± 0.03	4.32 ± 0.09	4.47 ± 0.32	0.17 ± 0.41	4.64 ± 0.26
angel shark	0.37 ± 0.05	0.17 ± 0.04	0.54 ± 0.04	–	–	0.47 ± 0.05
guitarfish	0.52 ± 0.02	0.12 ± 0.01	0.64 ± 0.02	–	–	0.63 ± 0.03
tuna fish A	0.83 ± 0.05	0.33 ± 0.04	1.16 ± 0.06	–	–	1.09 ± 0.08
tuna fish B	0.25 ± 0.07	0.25 ± 0.05	0.50 ± 0.09	–	–	0.47 ± 0.05

[a]Values represent the mean in $\mu g\ g^{-1}$ and standard deviation (uncertainty for CRMs), $n = 3$. [b]Values calculated by the difference between total mercury and CH_3Hg^+ content. [c]Total mercury values determined by FI-CVG-ICPMS after the microwave-assisted digestion procedure.

The response profiles that represent the recoveries of CH_3Hg^+ and Hg^{2+} as functions of the significant variables (HCl concentration and sample mass) are shown in Figure 1. It is worth mentioning that, when 4.2 mol L^{-1} HCl and 0.2 g of sample mass are used, CH_3Hg^+ and Hg^{2+} show suitable recoveries, ranging from 79% to 102% for CH_3Hg^+ and from 89% to 98% for Hg^{2+}. Treatments 3 and 7 (Table 3) represent the optimum conditions; the relative deviations of the regression model were lower than 19% and showed good agreement with the experimental data. Treatment 9 also showed good recoveries (112% for CH_3Hg^+ and 99% for Hg^{2+}), but the relatively low sample mass (0.1 g), by comparison with 0.2 g (treatments 3 and 7), will result in the decrease in the LOD for both species, and thus, the conditions of treatments 3 and 7 (4.2 mol L^{-1} HCl and 0.5 mol L^{-1} NaCl) were chosen as the optimum ones. The stirring time was not added to the models because it was not significant ($p > 0.05$) for any mercury species. As a result, the stirring time was fixed at 1 min.

Serafimovski et al.[7] used a solution of 5.0 mol L^{-1} HCl for the extraction of CH_3Hg^+ and Hg^{2+}. However, the authors used the microwave-assisted extraction for 10 min at 50 °C for a quantitative extraction without any conversion of mercury species. Likewise, Reyes et al.[41] employed a mixture of 5.0 mol L^{-1} HCl and 0.25 mol L^{-1} NaCl with microwave irradiation for 10 min at 60 °C. An advantage of the present study, by comparison with the methods assisted by microwave or ultrasound is the dispersion step (blending), since the extraction does not require the use of additional instrumentation. Moreover, 5 min of dispersion time and 1 min of stirring time were enough to carry out quantitative extraction, which represents a lower extraction time by comparison with the studies that were previously cited. The modified MSPD also brought advantages to the method, since the elution step was eliminated, the variation among the extractions decreased, and the analysis got faster.

Analytical Performance. LODs and limits of quantification (LOQs) of the method were calculated according to the signal-to-noise (S/N) ratio, using 3 and 10 times the S/N ratio, respectively. The LODs were 0.06 $\mu g\ g^{-1}$ for CH_3Hg^+ and 0.12 $\mu g\ g^{-1}$ for Hg^{2+} whereas the LOQs were 0.20 $\mu g\ g^{-1}$ for CH_3Hg^+ and 0.40 $\mu g\ g^{-1}$ for Hg^{2+}. The method was linear between 0.05 and 1.25 mg L^{-1} for CH_3Hg^+ and between 0.08 and 0.4 mg L^{-1} for Hg^{2+} with coefficients of regression (R^2) higher than 0.991.

It is important to mention that a study of the matrix effect was performed by comparing the slopes of calibration curves prepared in aqueous solution and using the MSPD extract (both the calibration curves were prepared considering a further derivatization step). The results indicate significant signal suppression (43%) for CH_3Hg^+ and signal enrichment (16%) for Hg^{2+}. The matrix effect is poorly discussed in the literature for the determination of CH_3Hg^+ and Hg^{2+} by GC/MS. However, the matrix effect observed in this study was similar to the one observed by Zachariadis and Kapsimali,[42] who studied the matrix effect on sensitivity for the determination of the same species by GC/MS in human urine, saliva, and serum. The authors, who carried out the comparison of calibration curves, stated that higher sensitivity was observed when the calibration curves were prepared in urine due to less matrix interference by comparison with serum samples. The authors attributed this effect to the presence of

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blood proteins that form complexes with mercury. In addition, they mention that CH_3Hg^+ peaks were lower than Hg^{2+}, the same behavior that was observed in the present study. According to Lemes and Wang,[43] CH_3Hg^+ species are almost totally bound to cysteine in fish tissues. Thus, for matrix effect compensation, the determination of CH_3Hg^+ and Hg^{2+} must be carried out by the matrix-matched calibration.

Accuracy and precision were evaluated with CRMs DORM-2 (dogfish muscle) and DOLT-3 (dogfish liver). Results are shown in Table 6. After the application of the proposed (and optimized) method to CRMs, a good agreement (about 99% for both species) with the certified values was obtained, with a relative standard deviation (RSD) lower than 9.5% for both CRMs. The proposed method was also applied to the analysis of angel shark (*Squatina squatina*), guitarfish (*Rhinobatos percellens*), and tuna fish (*Thunnus thynnus*) samples (Table 6). The sum of mercury species was compared to the results for total mercury determination by FI-CVG-ICPMS after MAD. Table 6 shows that the sum of mercury species and the total mercury values were similar ($p > 0.05$). Mercury species concentration in fish tissues were between 0.25 and 0.83 $\mu g\ g^{-1}$ for CH_3Hg^+ and between 0.12 and 0.33 $\mu g\ g^{-1}$ for Hg^{2+}.

CONCLUSIONS

Modified MSPD and GC/MS have been successfully applied to mercury species (CH_3Hg^+ and Hg^{2+}) extraction and determination in fish tissues. The use of 5 mL of a mixture of 4.2 mol L^{-1} HCl and 0.5 mol L^{-1} NaCl with 1 min of stirring time in a vortex after 5 min of blending time (dispersion step) for CH_3Hg^+ and Hg^{2+} species extraction showed suitable recoveries. By comparison with the methods assisted by microwave radiation, ultrasound, and/or conventional heating, the modified MSPD has shown some advantages, such as easiness, quickness, and low cost, over the other methods. The modified MSPD has brought advantages by comparison with the original MSPD, since avoiding the use of toxic organic solvents for analyte elution, allowing the use of aqueous solution (diluted hydrochloric acid). The study of the matrix effect showed that the GC/MS calibration should be performed with the MSPD extract. Accuracy was evaluated by the analysis of CRMs (fish muscle and liver) and showed agreement with certified values (about 99%), with RSDs below 9.5%. Therefore, this study showed that MSPD can be considered an important and innovative sample preparation method for mercury speciation studies in fish tissues and should be studied for the speciation of other elements from different biological and environmental matrices.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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