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Editorial office
Food Control

Dear Editor,

Enclosed herewith you find the files of the paper entitled "Determination of Methylmercury and Inorganic Mercury in Brazilian and Spanish Seafood Samples by LC-UV-HG-AFS", to be considered for publication in Food Control as an original research paper.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) proposed a provisional tolerable weekly intake for $MeHg^{+}$ of 1.6 µg kg^{-1} in body weight. However, the European Commission asked the European Safety Authority (EFSA) to review the tolerable value of $MeHg^{+}$. Thus, the EFSA published in 2012 a scientific opinion on the risk of human exposure to mercury and methylmercury. According to new epidemiological studies in children, the EFSA Panel on Contaminants in the Food Chain (CONTAM) established a tolerable weekly intake (TWI) for $MeHg^{+}$ of 1.3 µg kg^{-1} in body weight, expressed as mercury.

Seafood is responsible for the highest source of Hg, especially $MeHg^{+}$, and thus the monitoring of Hg species prior consumption is important for future risk assessment analysis. Therefore, the goal of the study was to determine total Hg and Hg species in seafood samples comprising fish, crustaceans and bivalves. The study focused on the extraction, identification and accurate quantification of $MeHg^{+}$, the most toxic form, which was selectively separated and determined by LC-UV-HG-AFS. Sample preparation was optimized to be as simple as possible, but still provide adequate sensitivity and specificity for the routine analyses of seafood.

Sincerely yours,
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Highlights

- Total mercury and speciation in fish, crustaceans and bivalves.
- Methylmercury was the predominant species in fish samples.
- Four fish samples exceeded the maximum limit set by European regulation.

Method Development for the Simultaneous Determination of Methylmercury and Inorganic Mercury in Seafood

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Abstract

This paper reports the method development for the simultaneous determination of methylmercury ($MeHg^+$) and inorganic mercury (iHg) species in seafood samples. The study focused on the extraction and quantification of $MeHg^+$ (the most toxic species) by liquid chromatography coupled to on-line UV irradiation and cold vapour atomic fluorescence spectroscopy (LC-UV-HG-AFS), using HCl 4 mol L^{-1} as the extractant agent. Accuracy of the method has been verified by analysing three certified reference materials and different spiked samples. The values found for total Hg and $MeHg^+$ for the CRMs did not differ significantly from certified values at a 95% confidence level, and recoveries between 85% and 97% for $MeHg^+$, based on spikes,

were achieved. The detection limits (LODs) obtained were 0.001 mg Hg kg^{-1} for total mercury, 0.0003 mg Hg kg^{-1} for $MeHg^{+}$ and 0.0004 mg Hg kg^{-1} for iHg. The quantification limits (LOQs) established were 0.003 mg Hg kg^{-1} for total mercury, 0.0010 mg Hg kg^{-1} for $MeHg^{+}$ and 0.0012 mg Hg kg^{-1} for iHg. Precision for each mercury species was established, being ≤ 12 % in terms of RSD in all cases.

Finally, the developed method was applied to 24 seafood samples from different origins and total mercury contents. The concentrations for Total Hg, $MeHg^{+}$ and iHg ranged from 0.07–2.33, 0.003–2.23 and 0.006–0.085 mg Hg kg^{-1}, respectively. The established analytical method allows to obtain results for mercury speciation in less than one hour including both, sample pretreatment and measuring step.

Keywords: mercury speciation; methylmercury; inorganic mercury; seafood; certified reference materials (CRMs); LC-UV-HG-AFS.

1. INTRODUCTION

Within the elements that are toxic for humans and the environment, mercury is a well-known pollutant due to the high toxicity of its species (C. Ibáñez-Palomino, J. F. López-Sánchez, & A. Sahuquillo, 2012a; Leopold, Foulkes, & Worsfold, 2010). All Hg forms are toxic, with the organic species being in most cases more dangerous than the inorganic ones (Gochfeld, 2003; Leopold et al., 2010). However, it is very important to identify which chemical form is more or less toxic, so as to evaluate its impact on environment and human health (Ibáñez-Palomino et al., 2012a). The akyl compounds of Hg are more toxic than the inorganic ones, particularly methylmercury ($MeHg^{+}$), the most toxic species (Ibáñez-Palomino et al., 2012a; Leopold et al., 2010). $MeHg^{+}$

93 bioaccumulates in the food chain, with its concentration higher in some fish species
94 than in the water environment (Leopold et al., 2010).

95 Bioaccumulation occurs in most cases of human exposure (Gochfeld, 2003).
96 Seafood is responsible for the highest source of Hg, especially $MeHg^{+}$ (Baer et al.,
97 2011; Capar, Mindak, & Cheng, 2007). Concentrations higher than 10 mg kg^{-1} of
98 $MeHg^{+}$ are found in the muscle of some fish species (Von Burg & Greenwood, 1991).
99 The consumption of fish located at the top of the food chain is not recommended for
100 pregnant women, due to the potential risk of $MeHg^{+}$ contamination (Baer et al., 2011;
101 EFSA, 2004). As a consequence of $MeHg^{+}$ exposure, neurological problems in adults
102 and blindness and mental retardation in infants were reported in the victims of
103 Minamata disease (Gochfeld, 2003). Other countries, such as Iraq, Guatemala and
104 Brazil, also had serious problems with exposure to organic mercury (Amin-Zaki et al.,
105 1974; Dolbec, Mergler, Sousa Passos, Sousa de Morais, & Lebel, 2000; Gochfeld,
106 2003; Storelli, Busco, & Marcotrigiano, 2005).

107 The Codex Stan 193-1995, organized by the FAO (Food and Agriculture
108 Organization of the United Nations) and WHO (World Health Organization), stipulates
109 the maximum levels of methylmercury in fish and predatory fish (0.5 and 1 mg kg^{-1},
110 respectively) (CODEX STAN 193-1995, 2009). The Codex indicates the maximum
111 level for toxicants permitted in food trade internationally (CODEX STAN 193-1995,
112 2009). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) proposed a
113 provisional tolerable weekly intake for $MeHg^{+}$ of 1.6 µg kg^{-1} in body weight. However,
114 the European Commission asked the European Safety Authority (EFSA) to review the
115 tolerable value of $MeHg^{+}$ (EFSA, 2012). Thus, the EFSA published in 2012 a scientific
116 opinion on the risk of human exposure to mercury and methylmercury (EFSA, 2012).
117 According to new epidemiological studies in children, the EFSA Panel on Contaminants

in the Food Chain (CONTAM) established a tolerable weekly intake (TWI) for $MeHg^{+}$ of 1.3 μg kg^{-1} in body weight, expressed as mercury (EFSA, 2012).

Although the Commission Regulation (EC) Nº 1881/2006 does not provide limits for $MeHg^{+}$ concentration, total Hg limits of 0.5 mg kg^{-1} and 1 mg kg^{-1} are given for distinct seafood (according to seafood type) (Commission Regulation (EC) No 1881/2006). The Brazilian Normative Instruction Nº 14 (May 2009) regulates the maximum level of total Hg in fish farming and predator fish. The established limits are 1 mg kg^{-1} for predator fish and 0.5 mg kg^{-1} for fish farming (Damin, Santo, Hennigen, & Vargas, 2013; PNCRC, 2009).

The toxicity of metals and their bioavailability depend on the chemical form of the metals. Thus, an accurate analytical method for Hg speciation is required to assess the real toxicity of samples (Harrington, 2000). Mercury speciation is generally performed by chromatographic separation techniques coupled with different detectors (Zhang, Yang, Dong, & Xue, 2012). The chromatographic separation techniques include: gas chromatography (GC) (Barst et al., 2013; Kenšová, Kružíková, & Svobodová, 2012; Nevado et al., 2011), liquid chromatography (HPLC) (Batista, Rodrigues, De Souza, Oliveira Souza, & Barbosa Jr, 2011; Chen, Han, Cheng, Liu et al., 2013; Chen, Han, Cheng, Wang et al., 2013) and ionic chromatography (IC) (Shade & Hudson, 2005). The most commonly used detectors are: inductively coupled plasma mass spectrometry (ICP-MS) (Batista et al., 2011; Clémens, Monperrus, Donard, Amouroux, & Guérin, 2011), atomic absorption spectroscopy (AAS) (Naozuka & Nomura, 2011; Sarıca & Türker, 2012), atomic fluorescence spectrometry (AFS) (Nevado et al., 2011; Zhang, et al., 2012), electron capture detector (ECD) (Kehrig et al., 2009; Kenšová et al., 2012), microwave induced plasma-atomic emission spectrometry (MIP-AES) (Sanz, De Diego, Raposo, & Madariaga, 2003), atomic

emision detection (AED) (Kuballa, Leonhardt, Schoeberl, & Lachenmeier, 2011) and isotope dilution mass spectrometry (IDMS) (Demuth & Heumann, 2001).

The goal of the study was to determine total Hg and Hg species in seafood samples comprising fish, crustaceans and bivalves. The study focused on the extraction, identification and accurate quantification of $MeHg^+$, the most toxic form, which was selectively separated and determined by liquid chromatography coupled to on-line UV irradiation and cold vapour atomic fluorescence spectroscopy (LC-UV-HG-AFS). Sample preparation was optimized to be as simple as possible, but still provide adequate sensitivity and specificity for the routine analyses of seafood.

2. MATERIALS AND METHODS

2.1 Instruments

Total Hg was measured by an Agilent 7500ce ICP-MS (Agilent, Germany) with a BURGENER Ari Mist HP type nebulizer. For Hg speciation, a HPLC system with a quaternary pump and degasser (Agilent Technologies 1100, Waldbronn, Germany) equipped with a manual stainless steel sampler injector (Rheodyne 7725i) and a 100 µL sample loop was used. Mercury species (iHg and $MeHg^+$) were separated in an analytical RP-C_{18} column (ODS Hypersyl 250 mm × 4.6 mm id, 5 µm, Thermo Hypersil-Keystone). After separation, a photo-oxidation step was performed in a 12 meter-long × 0.5 mm id PTFE tube coiled around a UV lamp with 150 W of power irradiation (Heraeus TQ 150). The reduction step was achieved in a cold vapour generator (CV) 10004 (P.S. Analytical, Orpington, UK), in which the effluent is mixed with the reducing agent. The metallic mercury vapour obtained reaches the gas-liquid separator, from which it is dragged into the detector by an argon stream and dried in a

PermaPure membrane with nitrogen. A Merlin Mercury Atomic Fluorescence Detector, model 10023 (P.S. Analytical), was used for measurements. A microwave (Milestone Ethos Touch Control) was used for digesting and extracting the samples. The fish samples supplied by MAPA (Brazil) were lyophilized in a ModulyonD Freeze Dryer lyophilizer (Thermo Electron Corporation, USA) and milled in an A 11 Basic micro-mill (IKA – Werke, Germany).

2.2. Reagents and standards

Only analytical grade reagents were used in this study. The standards and reagents were prepared with doubly deionized water (Elix&Rios 5–15MΩ cm^{-1}, Total Organic Carbon <30 μg L^{-1}) obtained from the Milli-Q water purification system (Millipore, Bedford, MA, USA). An inorganic mercury stock standard solution of 1000 mg L^{-1} was prepared by dissolving appropriate amounts of mercury chloride, $HgCl_2$ (Merck, Darmstadt, Germany), in 1% (v/v) HNO_3, from 69% nitric acid (Panreac, Hiperpur). A methylmercury stock standard solution of 1000 mg L^{-1} was prepared by dissolving appropriate amounts of CH_3HgCl (Carlo Erba, Milan, Italy) in 3% Methanol (Panreac, p.a.). All stock standard solutions were stored at 4°C. The working standard solutions were prepared daily from the stock standard solutions by appropriate dilution. For cold vapour generation, $SnCl_2$ solution was prepared daily from tin chloride 2-hydrate (Panreac, p.a.) to 1.5% concentration, in 4% of HCl, from 35% hydrochloric acid (Panreac, Hiperpur). Mobile phase was prepared daily by dissolving appropriate amounts of pyrrolidinedithiocarbamate, APDC, (Fluka, p.a.) and ammonium acetate, NH_4CH_3COO, (Merck, p.a.) in water. pH was adjusted with diluted acetic acid (Panreac, p.a.) and then filtered in a 0.45 μm filter (HA-type Millipore). The final mobile phase composition was 20% of the APDC and NH_4CH_3COO solution and 80%

of methanol HPLC-gradient grade (Panreac, p.a.). For microwave digestion samples, 31% H_2O_2 (Merck, Selectipur) and 69% HNO_3 (Panreac, Hiperpur) were used. For microwave extraction, 4 M HCl was prepared from 35% hydrochloric acid (Panreac, Hiperpur).

2.3. Reference materials and samples

The following certified reference materials (CRM) were used for quality control: DOLT-4 (Dogfish), TORT-2 (Lobster Hepatopancreas) (both from the National Research Council, Canada) and BCR-463 (Tuna fish) (Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre, Geel, Belgium). DOLT-4 was also used to assess the selection of extractant agent.

Five fresh fish muscle samples were provided by the Laboratory of Trace Metals and Contaminants (LANAGRO/RS) of the Ministry of Agriculture, Livestock and Supply (MAPA/Brazil). These samples were initially washed with Milli-Q water, cut and then lyophilized for a period of 5 hours. They were then ground in a vibratory mill and sieved through 85 µm polyester mesh to improve the particle size distribution.

Eleven fish samples and a clam sample were supplied by the Public Health Agency Laboratory, Barcelona (ASPB, Barcelona, Spain). Three crustacean samples and four bivalve samples were purchased from local supermarkets in Barcelona, Spain, during 2013. All samples were washed with Milli-Q water, cut and homogenized using a blender (non-contaminating kitchen mixer; Multiquick 5 Hand Processor, Braun, Barcelona, Spain). After homogenization, samples were stored in the freezer at -18°C until analysis.

2.4. Procedures

2.4.1. Moisture determination

The moisture of samples was determined in triplicate by drying 0.5 g aliquots in an oven at 102 ± 3°C until constant weight. Moisture ranged from 5% (lyophilized samples) to 88% (fresh samples); all results were expressed as dry mass.

2.4.2. Total mercury analysis

The total mercury content in seafood and CRM samples was determined by ICP-MS following microwave digestion. Initially, 0.1 – 1 g of samples were weighed in digestion vessels, after which 8 mL of concentrated nitric acid and 2 mL of hydrogen peroxide were added. The microwave digestion procedure was as follows: 10 min from room temperature to 90°C, maintained for 5 min at 90°C, 10 min from 90°C to 120°C, 10 min from 120°C to 190°C and 10 min maintained at 190°C. After cooling to room temperature, the digested samples were diluted in water up to 20 mL.

Total Hg was measured in the digested samples by ICP-MS. Helium gas was used in the collision cell to avoid interference in the ICP-MS measurements. A solution of ^{9}Be, ^{103}Rh and ^{205}Tl was used as the internal standard. The samples were quantified by means of an external calibration curve from inorganic mercury standards. Analyses in triplicate were performed for each sample. For quality control purposes, the standards of the calibration curve were run before and after each sample series. The corresponding digestion blanks (one for each sample digestion series) were also measured. Quality control standard solutions at two concentrations were measured at the end of the sequence to ensure stable instrument sensitivity. To assess the accuracy of the ICP-MS method, three CRMs (DOLT-4, TORT-2 and BCR-463) were analysed.

2.4.3 Mercury speciation analysis

The mercury speciation content in seafood and CRM samples was determined by LC-UV-CV-AFS following microwave extraction. The samples and CRMs were weighed in digestion vessels (0.1 – 1 g) and 10 mL of hydrochloric acid 4 mol L^{-1} were added to perform a microwave-assisted extraction (MAE). The microwave extraction procedure was as follows: 2 min from room temperature to 100°C and 10 min maintained at 100°C. After cooling to room temperature, the extraction samples were filtered through paper filters (Whatman 40). Mercury species were measured in the extracts by LC-UV-CV-AFS. The performance characteristics of the hyphenated system are those described by Ibañez-Palomino et al. (2012). Mercury species in extracts were identified by comparison of retention times with standards. External calibration curves quantified $MeHg^{+}$ and iHg, according to the corresponding standards. All samples were analysed in triplicate. Extraction blanks were also analysed by LC-UV-CV-AFS in each work session. In each speciation run, two quality control standard solutions were measured at the end of the sequence to ensure stable instrument sensitivity. To assess the accuracy of the LC-UV-CV-AFS method, three CRMs (DOLT-4, TORT-2 and BCR-463) were analysed.

3. RESULTS AND DISCUSSION

3.1. Selection of extractant agent

This assay focused on the study of a quantitative species extraction method system for seafood matrices, suitable for the subsequent determination technique. Extraction methods performed by several authors during the last five years are summarised in Table 1. Both acidic and basic extraction methods are described. However, there is no knowledge about standardised extraction methods in seafood

matrices. Therefore, to perform the extraction of Hg species in seafood, a preliminary test selecting two different extractant agents was run, to assess the main Hg species extracted. However, there is knowledge about a standardised extraction method for sediments: EPA 3200 (EPA, 2005). This method uses HNO_3 4 mol L^{-1} as extractant agent. It has also been taken into account that, in almost half the studies summarised in Table 1, hydrochloric acid was used as the extractant agent. Therefore, the extractants tested were HNO_3 4 mol L^{-1}, such as EPA 3200 employs, and HCl 4 mol L^{-1}, as an adaptation of this method. The present method is based on a microwave-assisted extraction, whose procedure is described in section 2.4.3. For this study, the CRM DOLT-4 (Dogfish) was used. In DOLT-4, $MeHg^+$ and iHg were the species present in the extracts. Figure 1 shows chromatograms obtained from hydrochloric and nitric acid DOLT-4 extracts, in which Hg species are highlighted. Recoveries obtained for $MeHg^+$ were 95% and 86%, using HCl and HNO_3, respectively. On measuring iHg, the concentration obtained when using HCl was 46% of the total certified content; whereas, when using HNO_3, the figure was 85%. This increase could be attributed to the oxidising action of HNO_3. As $MeHg^+$ recovery with the HNO_3 extraction method is 9% less than recovery with HCl extraction, there is evidence that this difference could be caused by $MeHg^+$ conversion to iHg. Therefore, HCl 4 mol L^{-1} was selected as the extractant agent.

3.2 Quality parameters

3.2.1. Analysis of the total Hg

Three CRMs (TORT-2, DOLT-4 and BCR-463) were analysed to verify the accuracy of the proposed method. Concomitant analyses of TORT-2, DOLT-4 and BCR-463 verified the accuracy of the determination of total Hg (Table 2). The analysis

293 of one CRM for each sample group was used in total Hg measurements. The use of
294 CRMs guaranteed the quality control of acid digestion (sample pre-treatment). The
295 values for total Hg concentration, together with the corresponding certified value, are
296 given in Table 2. According to the Student's *t*-test, no significant difference at a 95%
297 confidence level was found in the data shown in Table 2. Repeatability was checked by
298 analysis of CRMs (different replicates) 6 times throughout the day (Table 2). The RSD
299 (%) values were: 7% for TORT-2 and 4% for DOLT-4. The instrument detection (LOD)
300 and quantification limits (LOQ) were calculated as three times the standard deviation
301 (3σ) and ten times the standard deviation signal (10σ) of ten digestion blanks,
302 respectively (Llorente-Mirandes, Calderón, Centrich, Rubio, & López-Sánchez, 2014).
303 The results obtained were 0.001 mg Hg kg^{-1} for LOD and 0.003 mg Hg kg^{-1} for LOQ.
304
305 **3.2.2 Analysis of Hg species**
306 The accuracy of the method proposed for $MeHg^{+}$ speciation was verified by
307 analysis of BCR-463 (Tuna fish), DOLT-4 (Dogfish liver) and TORT-2 (Lobster
308 hepatopancreas) CRMs. The values for each CRM are given in Table 2 and did not
309 differ significantly at a 95% confidence level from certified values. The amount of iHg
310 was also analysed in the BCR-463, DOLT-4 and TORT-2 CRMs. The recovery for each
311 CRM was calculated by comparing the sum of $MeHg^{+}$ and iHg concentration, obtained
312 by the proposed speciation method (LC-UV-HG-AFS) and total Hg concentration (ICP-
313 MS). The total Hg concentration was taken as 100% in the calculation of recovery
314 values. The recoveries analysed for CRMs showed a range between 80-102% (Table 2).
315 Additionally, standards of $MeHg^{+}$ were spiked in solid samples of tuna-2,
316 forkbeard, prawn-1, cockle and BCR-463. After addition of standards, the solid samples
317 were homogenized. The extraction procedure was carried out only 30 minutes after the

spiking procedure. The recoveries found for tuna-2, forkbeard, prawn-1, cockle and BCR-463 were 93 ± 3, 85 ± 5, 93 ± 2, 87 ± 4 and 97 ± 2 (mean % ± standard deviation, n=3), respectively. These recovery values were calculated according to the literature (Santoyo, Figueroa, Wrobel, & Wrobel, 2009) and show good recovery of $MeHg^{+}$. As an example, Figure 2 shows the chromatograms of tuna-2, forkbeard, prawn-1 and cockle. The tuna-2 was fortified with 0.20 mg Hg kg^{-1} of $MeHg^{+}$; the forkbeard, with 0.35 mg Hg kg^{-1} of $MeHg^{+}$; and the prawn-1 and cockle, with 0.10 mg Hg kg^{-1} of $MeHg^{+}$. As can be seen, $MeHg^{+}$ was recovered successfully from the four samples.

Limits of detection (LOD) and limits of quantification (LOQ) for mercury species were estimated. To calculate LOD and LOQ, the standard deviation of the base line and the chromatographic peak base of each analyte (SD_{BLANK}), multiplied by 3 or 10 (LOD and LOQ, respectively), were interpolated in the slope of the height calibration curve (C. Ibáñez-Palomino, J. F. López-Sánchez, & À. Sahuquillo, 2012b), which is expressed as: LOD = 3 SD_{BLANK}/slope; LOQ = 10 SD_{BLANK}/slope. The instrument limits were converted to sample limits by multiplying by the extraction dilution factor. The LODs were 0.0003 and 0.0004 mg Hg kg^{-1} for $MeHg^{+}$ and iHg, respectively. The LOQs were 0.0010 and 0.0012 mg Hg kg^{-1} for $MeHg^{+}$ and iHg, respectively.

3.3 Total Hg in samples

Total Hg concentration was determined in 24 seafood samples: 5 Brazilian fish samples and 19 Spanish seafood samples. The samples were classified as fish (n=16), crustaceans (n=3) and bivalves (n=5); the values found for total Hg in seafood samples are given in Table 3. Total Hg concentration ranged from 0.07–2.33 mg kg^{-1}, with the crustaceans and bivalves showing less total Hg than fish samples. Comparison of total

Hg concentration means showed that crustaceans and bivalves had 0.07 mg kg^{-1} dry mass (dm) and 0.12 mg kg^{-1} wet mass (wm), while fish had a mean of 0.71 mg kg^{-1} dm and 0.59 mg kg^{-1} wm. These results are consistent with the literature (Batista et al., 2011; Clémens et al., 2011; Krystek & Ritsema, 2006). According to Krystek and Ritsema (2006), significant differences in Hg levels are found in different seafood species analysed. Fish at high trophic levels in the food chain, like large predatory fish, accumulate more Hg and contain significantly higher concentration levels. Two predatory Brazilian fish samples (red porgy-1 and red porgy-2) and two predatory Spanish fish samples (tuna-3 and swordfish-1) showed the highest levels of total Hg: 1.63 ± 0.04 mg kg^{-1} (red porgy-1), 1.15 ± 0.01 mg kg^{-1} (red porgy-2), 2.33 ± 0.03 mg kg^{-1}(tuna-3) and 1.04 ± 0.03 mg kg^{-1} (swordfish-1).

The Brazilian government, through its Ministry of Agriculture, Livestock and Food Supply (MAPA), instituted a reference value of 0.5 mg kg^{-1} for total Hg in fish farming and 1 mg kg^{-1} for predator fish (Damin et al., 2013; PNCRC, 2009). Two of the five Brazilian samples (red porgy-1 and red porgy-2) were above the values recommended by the Brazilian government (Table 3). All Spanish samples had concentrations of total Hg below the maximum levels set by EC Regulation No 1881/2006 (Commission Regulation (EC) No 1881/2006), except for tuna-3 and swordfish-1 samples (2.33 ± 0.03 mg kg-1 and 1.04 ± 0.03 mg kg-1, respectively). These data demonstrate the need to carry out speciation in seafood samples to discern the more toxic species.

3.4 Hg species in seafood samples

The concentrations of MeHg+ found in the literature since 2009 are given in Table 1. These concentrations vary widely, depending on the extraction and detection

368 method. According to Table 1, the concentrations of $MeHg^+$ ranged from 0.001 to 3.2
369 mg kg^{-1} for seafood samples. However, bivalves, mollusks and crustaceans have lower
370 $MeHg^+$ concentration than fish. Zhang et al. (2012) found concentrations between 0.022
371 and 0.034 mg Hg kg^{-1} (in the form of $MeHg^+$) for mussel and clam samples. Clémens et
372 al. (2011) found concentrations of 0.001 and 0.033 mg Hg kg^{-1} (in the form of $MeHg^+$)
373 for mussel, oyster, scallop and shrimp; and Batista et al. (2011), of 0.003 and 0.037 mg
374 Hg kg^{-1} (in the form of $MeHg^+$) for mussel, octopus, shrimp and squid samples.

375 In this study, the Hg species were analysed from a selection of 24 seafood
376 samples, including crustaceans, bivalves and fish. The results are given in Table 4. For
377 all samples, the sum of $MeHg^+$ and iHg concentration (obtained by the proposed
378 speciation method, using LC-UV-HG-AFS) was compared with total Hg concentration
379 (obtained by ICP-MS). The total Hg concentration was taken as 100% in the calculation
380 of recovery values. All samples analysed showed recovery values between 88 and 120%
381 (Table 4), which are corroborated by the literature (Chen, Han, Cheng, Liu et al., 2013;
382 Clémens et al., 2011; Kenšová et al., 2012). Clémens et al. (2011) observed recoveries
383 between 90 and 110% for matrices with low-fat content. High recovery values were
384 observed for salmon, hake and whitefish samples (fatty samples), with values of 120%,
385 117% and 114%, respectively (Clémens et al., 2011). Thus, close correlation between
386 total and sum of species is achieved, regardless of sample matrix composition.

387 The presence of $MeHg^+$ was detected in 19 analysed samples. $MeHg^+$ was the
388 predominant form of mercury in all fish samples and one shellfish sample (prawn-1).
389 The clam-2 and cockle samples had only 13% and 36% of $MeHg^+$, respectively. The
390 mean values of percentage and concentration of $MeHg^+$ in fish and shellfish samples
391 were calculated. For fish, a mean percentage of 98%, a mean concentration of 0.71 mg
392 $MeHg^+$ kg^{-1} in dry mass (dm) and a mean concentration of 0.60 mg $MeHg^+$ kg^{-1} in wet

393 mass (wm) were found; and for shellfish, a mean percentage of 49%, a mean
394 concentration of 0.027 mg $MeHg^{+}$ kg^{-1} in dm and a mean concentration of 0.009 mg
395 $MeHg^{+}$ kg^{-1} in wm. Inside the fish sample group, the highest concentrations of $MeHg^{+}$
396 in wm were found for red porgy-1 and red porgy-2 (mean value 1.4 mg kg^{-1}), tuna-3
397 (2.23 mg kg^{-1}) and swordfish-1 (1.04 mg kg^{-1}). In shellfish, the highest levels of
398 $MeHg^{+}$ were found for prawn-1 (0.011 mg kg^{-1}). The concentrations of all samples were
399 within the maximum levels set by (EC) No. 1881/2006 for $MeHg^{+}$ (Commission
400 Regulation (EC) No 1881/2006), except for red porgy-1 and -2 (Brazilian fish samples),
401 tuna-3 and swordfish-1 (Spanish fish samples), which showed concentrations higher
402 than 1 mg kg^{-1}. In some samples, iHg was also identified. Table 4 shows that values of
403 iHg concentration ranged from 0.010 to 0.085 mg iHg kg^{-1} in wm in fish samples; and
404 from 0.006 to 0.016 mg iHg kg^{-1} in wm in shellfish samples. However, iHg was
405 quantified only in four of the eight shellfish samples. These data underline the
406 importance of speciation in seafood samples. Speciation makes it possible to establish
407 which the most harmful form to humans is and, therefore, whether the seafood is
408 suitable or not for consumption. Likewise, the need to introduce maximum levels of
409 $MeHg^{+}$ in seafood in Brazilian and European legislation should be considered in further
410 Directives.

411 According to data obtained in this work, and as described in the literature, the
412 concentrations of $MeHg^{+}$ are higher in fish than shellfish being predatory fish those
413 samples showing the highest values.

414 Higher $MeHg^{+}$ content in fish samples could be related to the fat content.
415 Methylmercury is a fat-soluble substance and therefore can be accumulated in the fatty
416 tissues more easily than inorganic mercury. Bluefish samples, such as salmon and tuna
417 with high fat content, present high levels of MeHg+. Whitefish and shellfish, with lower

418 fat content, present lower MeHg+ concentration and in the case of some shellfish
419 samples the predominant mercury species is iHg.

420 When comparing the concentrations found in this study for $MeHg^+$ in fish (Table
421 4) with the literature (Table 1), the values were similar (Chen, Han, Cheng, Liu et al.,
422 2013; Chen, Han, Cheng, Wang et al., 2013; Clémens et al., 2011; Montero-Alvarez,
423 Fernández de la Campa, & Sanz-Medel, 2014) or higher (Carrasco et al., 2011; Fu,
424 Wang, Zhou, & Jiang, 2010; Hajeb, Jinap, & Ahmad, 2010; Kenšová et al., 2012;
425 Kuballa, Moellers, Schoeberl, & Lachenmeier, 2011; Liang et al., 2011; Miklavčič et
426 al., 2011; Nevado et al., 2011; Qiu, Feng, Wang, Fu, & Shang, 2009; Santoyo et al.,
427 2009; Wang et al., 2010). The shellfish analysed had similar levels of $MeHg^+$ to those
428 found by Clémens et al. (2011). According to Fitzgerald et al. (2007), the behaviour of
429 Hg chemistry in the marine environment and the number of predatory fish analysed
430 explain the differences between the mean values of $MeHg^+$ found in several studies. The
431 conditions of the water environment, the age of each species and the time of exposure to
432 Hg contaminants are also factors that affect the results (Fitzgerald, Lamborg, &
433 Hammerschmidt, 2007). The results obtained are in agreement with those reported by
434 Kuballa et al. (2011), showing a great variability in $MeHg^+$ concentration in different
435 fish species. These differences reaffirm the need to monitor $MeHg^+$ concentrations in
436 seafood species more frequently and in different areas, in order to avoid human
437 contamination.

438

439 **4. CONCLUSION**

440

441 This study determined total Hg, $MeHg^+$ and iHg species in different seafood
442 samples, including fish, crustaceans and bivalves. Figures of merit (LOD, LOQ,

reproducibility and trueness) of the proposed LC-UV-HG-AFS procedure were satisfactory for the determination of $MeHg^+$ and iHg in fish and shellfish. $MeHg^+$ was the predominant species in all fish samples. The highest levels of $MeHg^+$ in fish were found in two Brazilian fish samples and two Spanish fish samples. All concentrations are below the maximum levels set by Regulation (EC) No. 1881/2006 for $MeHg^+$ except for these four fish samples, which showed concentrations higher than 1 mg kg^{-1}. Despite the lack of Brazilian legislation regulating the maximum levels of $MeHg^+$ in seafood, the present results have increased the availability of reliable results on $MeHg^+$ in seafood and could be used in further Directives on $MeHg^+$ in food commodities. Thus, the present method could be a valuable tool for food control laboratories that assess $MeHg^+$ in seafood samples.

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642 **Figure captions**

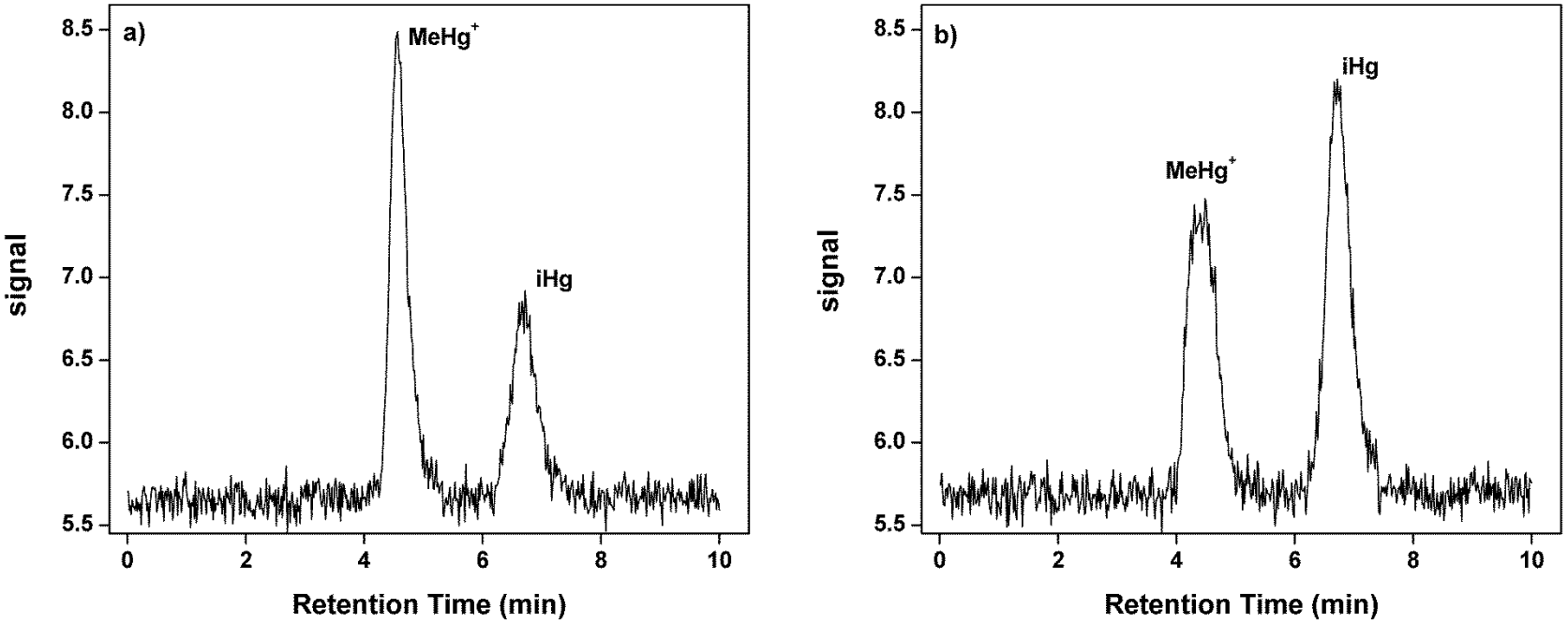
643

644 **Figure 1.** Chromatograms obtained for DOLT-4 using microwave assisted extraction
645 with (a) HCl 4 mol L^{-1} and (b) HNO_3 4 mol L^{-1}.

646

647 **Figure 2.** Chromatograms of **a)** cokle extract **b)** prawn-1 extract **c)** forkbeard extract **d)**
648 tuna- 2 extract (continuous line: non-spiked sample and dotted line: sample spiked with
649 $MeHg^{+}$) by LC-UV-HG-AFS.

650 **Figure 1**



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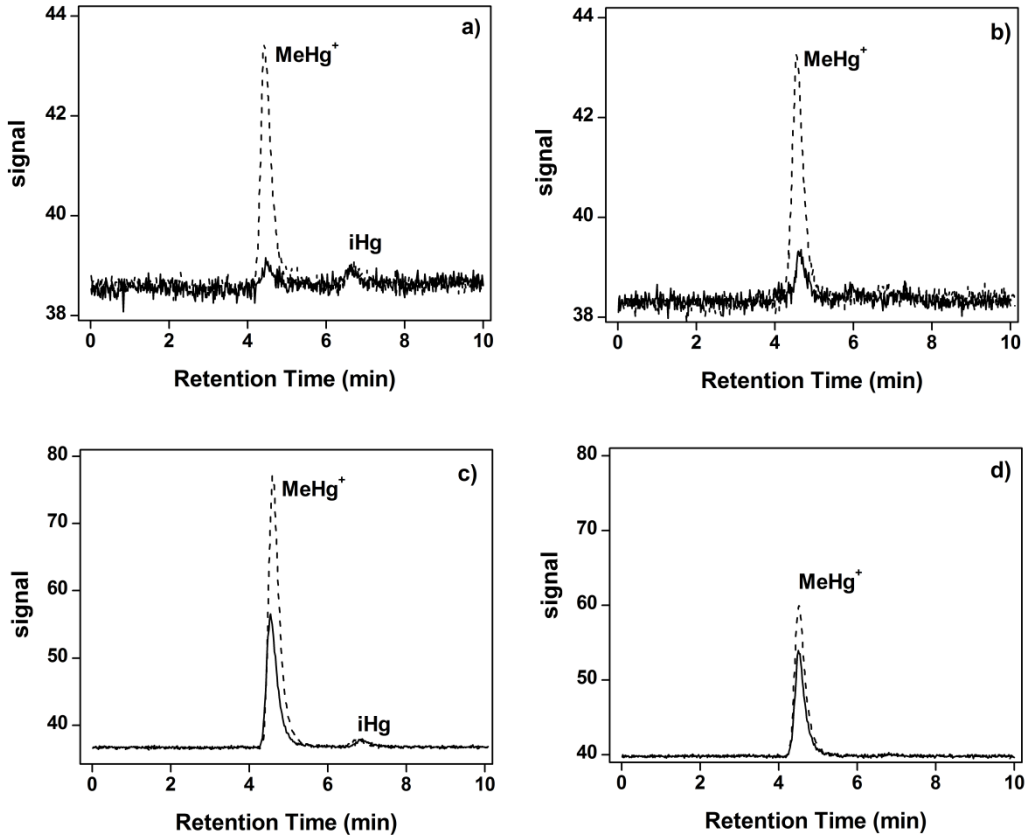
653 **Figure 2**



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656 **Table 1:** $MeHg^+$ concentrations in seafood samples found in literature since 2009.

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Type of seafood	Samples	Extraction procedure	Extracting agent	Technique	$MeHg^+$ (mg kg^{-1})	$MeHg^+$ %	Reference
Fish	Chub muscle	Stirring manually with a glass rod	Toluene	GC-EDC	<0.5	-	Sedláčková et al., 2014
Fish	Tuna Emperor fish Red grouper Bass Aquarium fish Snook black Grunt Bream Dogtooth herring Mackerel Nurse shark	Ultrasonication	2-mercaptoethanol, L-cysteine and HCl	ID-LC–ICP-MS	0.04- 1.92	83-98	Montero-Alvarez et al., 2014
Fish	Bearded brotula Tuna Pirarucu Salmon Whitemouth croaker Mullet	Microwave	L-cysteine	LC-CV-ICP-MS	0.01- 1.00	-	Schmidt et al., 2013

Fish	Arctic char Spotted gar Largemouth bass Bowfin Catfish	Water bath	HNO_3	GC-CVA-FS	0.5-1.5	-	Barst et al., 2013
Fish	Pomfret Hairtail Croaker Japanese seabass	Ultrasonication	HCl + l-cysteine	LC-ICP-MS	0.17-0.75	more than 86%	Chen et al., 2013b
Fish	Pomfret Hairtail Croaker Japanese seabass Silver carp Black carp Goldfish Northern snakehead	Ultrasonication	HCl + Sodium 3-mercapto-1-propanesulfonate	LC-ICP-MS	0.0032-0.75	more than 86%	Chen et al., 2013a
Fish	Tapertail Anchovy	Microwave	HCl	CE-ICP-MS	1.2-3.2	-	Zhao et al., 2012
Shellfish	Mussel Razor clam Baby clam	Ultrasonication	HCl	EVG-AFS	0.022-0.034	-	Zhang et al., 2012

Fish	Chub Pike Bream Roach Asp Carp Eel Perch Tench Trout Grayling	Stirring	Toluene	GC-ECD	0.05-0.8	46-100	Kenšová et al., 2012
Fish	Nase Carp Catfish	Microwave	TMAH	GC-AFS	0.001-1.16	60-88	Nevado et al., 2011
Fish	Red snapper Orange-spotted grouper Snubnose pompano	Wet Digestion	KOH–methanol	GC-CV-AFS	0.007-0.12	37-81	Liang et al., 2011
Fish	Sea fish from local markets (Wuhan, China)	Ultrasonication	HCl	LLME-CE-UV	0.004-0.027	-	Li et al., 2011
Fish	Saithe Salmon Smoked salmon Tuna Canned tuna	a) Solid–liquid extraction b) Microwave c) Extraction at room temperature	TMAH	ID-GC-ICP-MS	0.002-0.58	84-97	Clémens et al., 2011

Shellfish	Mussel Oyster Scallop Shrimp				0.001-0.033	28-98	
Fish	Catfish Carp	Water bath	KOH	HS-SPME-GC-AFS	0.76	74	Carrasco et al., 2011
Shellfish	Mussels Octopus Shrimps Squids	Ultrasonication	HCl + L-cysteine + 2-mercaptoethanol	LC-ICP-MS	0.003-0.037	-	Batista et al., 2011
Fish	Tuna				0.03-0.16		
Fish	German market	alkaline digestion	methanolic potassium hydroxide solution	GC-AED	0.006-0.5	14-100	Kuballa et al., 2011
Fish	Canned fish	According to reference	According to reference	GC-ECD	0.002-0.1	40-110	Miklavčič et al. 2011
Fish	Fish from Qinghai and Tibet plateau	Shaking	Alkaline extraction	LC-CV-AFS	0.1-0.6	84-89	Wang et al., 2010

Fish	Common carp Crucian carp Catfish Java tilapia Chinese soft shell turtle	Shaking	Alkaline extraction	LC-UV-AFS	0.1-0.4	35-76	Fu et al., 2010
Fish	Tuna and mackerel	Shaking and centrifugation	H_2SO_4 + KBr + toluene + cysteine	GC-ECD	0.29-0.69	70-82	Hajeb et al., 2010
Fish	Grass carp	Over digestion	KOH–methanol	CV-AFS	0.02-0.09	7.4-93	Qiu et al., 2009
Fish	King mackerel Red snapper	Ultrasonication	Perchloric acid + l-cysteine + toluene+methanol	LC-ICP-MS	0.05-0.3	80	Santoyo et al., 2009

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660 **Table 2.** Total mercury and mercury species in certified reference materials; concentrations are expressed as mg Hg kg^{-1} dry mass (mean ±
661 SD, n = 3).

Sample		Total Hg	$MeHg^{+}$	iHg	Sum of Hg species	Recovery %
TORT-2	**measured value**	0.30 ± 0.02	0.161 ± 0.010	0.081 ± 0.002	0.242 ± 0.012	**80**
	certified value	**0.27 ± 0.06**	**0.152 ± 0.013**			
DOLT-4	**measured value**	2.68 ± 0.11	1.27 ± 0.04	1.19 ± 0.02	2.46 ± 0.06	**92**
	certified value	**2.58 ± 0.22**	**1.33 ± 0.12**			
BCR-463	**measured value**	2.86 ± 0.15	2.78 ± 0.16	0.16 ± 0.20	2.94 ± 0.36	**102**
	certified value	**2.85 ± 0.16**	**3.04 ± 0.16**			

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Table 3. Total mercury in seafood samples, concentrations are expressed as mg Hg kg^{-1} wet mass (mean ± SD, n = 3).

Samples	Species	Trade name	Origin	Total Hg
Fish				
	Urophycis cirrata	White fish	Brazil	0.27 ± 0.01
	Pagrus pagrus	Red porgy-1	Brazil	1.63 ± 0.04
	Pagrus pagrus	Red porgy-2	Brazil	1.15 ± 0.01
	Merluccius hubbsi	Hake-1	Brazil	0.53 ± 0.01
	Merluccius gayi	Hake-2	Brazil	0.27 ± 0.01
	Phycis blennoides	Forkbeard	Spain	0.30 ± 0.02
	Sardina pilchardus	Sardine	Spain	0.040 ± 0.001
	Salmo sp.	Salmon-1	Spain	0.021 ± 0.001
	Salmo sp.	Salmon-2	Spain	0.023 ± 0.002
	Thunnus sp.	Tuna-1	Spain	0.32 ± 0.04
	Thunnus sp.	Tuna-2	Spain	0.14 ± 0.01
	Thunnus sp.	Tuna-3	Spain	2.33 ± 0.03
	Luvarus imperialis	Louvar	Spain	0.60 ± 0.04
	Xiphias gladius	Swordfish-1	Spain	1.04 ± 0.03
	Xiphias gladius	Swordfish-2	Spain	0.25 ± 0.03
	Xiphias gladius	Swordfish-3	Spain	0.56 ± 0.01
Crustaceans				
	Aristeus antennatus	Prawn-1	Spain	0.013 ± 0.002
	Aristaeopsis edwardsiana	Prawn-2	Spain	< LOQ
	Crangon crangon	Shrimp	Spain	< LOQ
Bivalves				
	Tapes pullastra	Clams-1	Spain	0.015 ± 0.001
	Tapes Decussatus	Clams-2	Spain	0.018 ± 0.001
	Mytilus edulis	Mussel	Spain	< LOQ
	Cerastoderma edule	Cockle	Spain	0.009 ± 0.002
	Ostrea sp.	Oyster	Spain	0.007 ± 0.001

Table 4. Mercury speciation analysis of selected seafood samples; concentrations are expressed as mg Hg kg^{-1} wet mass (mean ± SD, n = 3).

Sample	$MeHg^+$	%$MeHg^+$	iHg	Sum of Hg species	Recovery (%)
White fish	0.30 ± 0.02	100	<LOQ	0.30 ± 0.02	114
Red porgy-1	1.67 ± 0.04	96	0.061 ± 0.009	1.73 ± 0.05	105
Red porgy-2	1.13 ± 0.06	97	0.035 ± 0.001	1.17 ± 0.06	101
Hake -1	0.62 ± 0.02	97	0.019 ± 0.002	0.64 ± 0.02	117
Hake -2	0.31 ± 0.04	100	<LOQ	0.31 ± 0.04	114
Forkbeard	0.32 ± 0.01	98	0.010 ± 0.003	0.33 ± 0.01	109
Sardine	0.040 ± 0.002	100	<LOQ	0.040 ± 0.002	100
Salmon-1	0.022 ± 0.001	100	<LOD	0.022± 0.001	103
Salmon-2	0.025 ± 0.003	100	<LOD	0.025 ± 0.003	120
Tuna-1	0.30 ± 0.05	98	0.011 ± 0.003	0.31 ± 0.05	95
Tuna-2	0.136 ± 0.008	100	<LOD	0.136 ± 0.008	97

Tuna-3	2.23 ± 0.04	96	0.085 ± 0.004	2.31 ± 0.04	99
Louvar	0.64 ± 0.03	99	0.011 ± 0.001	0.65 ± 0.03	108
Swordfish-1	1.04 ± 0.04	98	0.02 ± 0.002	1.06 ± 0.05	102
Swordfish-2	0.26 ± 0.03	100	<LOD	0.26 ± 0.03	102
Swordfish-3	0.58 ± 0.04	100	<LOQ	0.58 ± 0.04	103
Prawn-1	0.011 ± 0.003	100	<LOD	0.011 ± 0.003	88
Prawn-2	<LOD	-	<LOD	-	-
Shrimp	<LOD	-	<LOD	-	-
Clams-1	<LOD	-	0.016 ± 0.004	0.016 ± 0.004	108
Clams-2	0.013 ± 0.001	13	0.008 ± 0.001	0.021 ± 0.002	110
Mussel	<LOD	-	<LOD	-	-
Cockle	0.003 ± 0.001	36	0.006 ± 0.002	0.009 ± 0.003	110
Oyster	<LOD	-	0.007 ± 0.001	0.007 ± 0.001	100