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Mercury–Selenium Species Ratio in Representative Fish Samples and Their Bioaccessibility by an In Vitro Digestion Method

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Abstract The potential toxicity of mercury (Hg) content in fish has been widely evaluated by the scientific community, with Methylmercury (MeHg) being the only legislated species (1 mg kg^{-1}, maximum concentration allowed in predatory fish). On the other hand, selenium (Se) is recognized to decrease its toxicity when both elements are simultaneously administrated. In the present paper, the total content of Se and Hg and their species in fish of high consumption, such as tuna, swordfish, and sardine, have been evaluated. The percentage of MeHg is higher than 90% of total Hg content. The results show that, for all of them, the Se/Hg ratio is significantly higher than one, being the maximum ratio for sardine. As only studying the bioaccessible fraction the extent of a toxic effect caused by an element can be predicted, the bioaccessibility of both analytes through an in vitro digestion method has been carried out. The results show that MeHg in all fishes is very low bioaccessible in both gastric and intestinal digestion. Because the MeHg bioaccessible fraction might be correlated to the Se content, the potential toxicity cannot be only related to the total Hg content but also to Se/Hg ratio.

Keywords Mercury · Selenium · MeHg · Speciation · Bioaccessibility

Introduction

Mercury (Hg) is a widely distributed and persistent pollutant in the environment and is one of the most highly bioconcentrated trace metals in the human food chain. Among all the species, methylmercury (MeHg) is the organomercury compound most commonly found in fish and is recognized as a major environmental pollutant and health hazard for humans because of its easy penetration through biological membranes, efficient bioaccumulation, high stability, and long-term elimination from tissues [1]. Therefore, the exposure to MeHg supposes a risk for human health because of its teratogenic, immunotoxic, and, most

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importantly, neurotoxic effects [2–4]. As a result, risk assessment and risk prevention of human exposure to Hg is important in food safety.

One of the natural components of fish that may protect against Hg toxicity is selenium (Se) [3, 4]. Interaction of Se with Hg has long been investigated, yet, it is still incompletely understood [5]. As reported by Parizek and Ostadalova [6], and subsequently confirmed by many other researchers, simultaneous administration of selenite counteracts the negative impacts of exposure to inorganic Hg, particularly in regard to neurotoxicity, fetotoxicity, and developmental toxicity [7]. In addition to the antagonism by Se of the toxicity of inorganic Hg, its detoxifying effect on methylmercury has attracted the attention of many scientists in heavy metal toxicology [8].

Se is an essential micronutrient for animals and humans. To date, the major biological functions of Se are attributed to its antioxidative properties and its roles in the regulation of thyroid hormone metabolism and cell growth [9].

It is recognized that the toxic or beneficial effects from Hg and Se are not governed by their total concentration, instead, it comes from the species that can interact efficiently with sites on biological ligands. The identification and quantification of Hg and Se species provides information about bioaccumulation, detoxification, and toxicological implications [10]. Therefore, the interest in Hg and Se speciation in food and biological samples is currently increasing. Consequently, food safety and nutritional quality depend not only on the determination of total levels but also on the speciation of the trace elements that exist in foodstuffs. As a result, it is necessary to develop rapid, sensitive, and accurate methods for the extraction, separation, identification, and quantification of Hg and Se species in food samples.

Solid sample pretreatment has been the Achilles' heel of the analytical process, almost from the era of alchemy to the present time, mainly because of high time [11–15] and solvent consumption, high cost of the equipment and risks of interferences [16], losses [17], and species transformation. In fact, very recently surprising discoveries have reported that inorganic Hg or MeHg present in the samples may be accidentally converted into MeHg or dimethylmercury during sample preparation, extraction, derivatization, and separation [18–22]. This leads to the conclusion that organic Hg species levels determined in samples may have been overestimated in the past, and therefore, it constitutes a source of error that should be studied and minimized.

On the other hand, considerable interest has been expressed for shortened and simplified sample preparation procedures for Hg and Se analysis. In this respect, sonochemistry (chemistry enhance by ultrasound) has emerged as an interesting alternative to traditional sample pretreatment methods (hot plate and microwave digestion) for total metal and species determination in different matrices. Ultrasound could facilitate the analytical determination of analytes as it diminishes matrix effects [23] and accelerates various steps, such as dissolution, fusion, and leaching in the analytical process [24].

Because the extent of the toxic effects caused by heavy metals is regulated by the forms in which elements reach the absorption site, instead of their total concentration, bioavailability studies result of high interest when a toxicological study is carried out.

Whereas information on the Hg and Se content of foods is reasonably adequate, knowledge of Hg and Se bioavailability is incomplete. This knowledge gap continues because accurate measurement of Hg and Se bioavailability can be difficult, expensive, and time consuming [25].

In vitro experiments offer an appealing alternative to human and animal studies [26]. Nowadays, there are well established in vitro methods for estimating element solubility in animals [27]. The results from most in vitro methods are based on the formation of

digestion products that are soluble and not precipitated by precipitating agents or dialyzable [28]. In this way, the bioaccessible fraction is determined, which is the maximum concentration soluble in simulated gastrointestinal media that is available for subsequent processes of absorption into the intestinal mucosa [29].

This work summarizes the Se and Hg quantification and speciation in fish samples commonly consumed in Spain and Portugal and the subsequent in vitro enzymolysis of the samples to broadly simulate human gastrointestinal digestion carried out in our investigation group during the last years.

Experimental Procedure

Instrumentation

An atomic fluorescence spectrometer (AFS, Merlin 10.023, P.S. Analytical Ltd., Orpington, Kent, UK) was used to determine the total Hg content. Hg vapor was generated in a flow injection system. A gas chromatograph (GC, Perkin Elmer, model 8410, Ltd. England) was hyphenated to the AFS detector for the speciation of organomercury compounds. Separation of organomercury compounds was carried out in a gas chromatograph with an on-column injector. The chromatograph was fitted with a nonpolar capillary fused silica column SGL-1 (15 m×0.53 mm i.d.) coated with 1.5 μm dimethylpolysiloxane (Sugelabor S.A., Spain).

A pyrolyzer unit 10.558 (P.S. Analytical Ltd., Orpington, Kent, UK) with a temperature control module was used as the interface between GC and AFS to convert the organomercurial compounds to atomic Hg vapor.

An AFS (Excalibur, P.S. Analytical Ltd., Orpington, Kent, UK) was used to determine the total Se content. Se hydride was generated in a flow injection system. An inductively coupled plasma mass spectrometer (ICP-MS, HP-4500 Plus, Tokyo, Japan) fitted with a Babington nebulizer and a Scott double-pass spray chamber was used for Se species quantification after chromatographic separation.

A PU-2089 high performance liquid chromatography (HPLC) pump (Jasco Corporation, Tokyo, Japan) fitted with a six-port sample injection valve (model 7725i, Rheodyne) with a 100-μl injection loop was used for chromatographic experiments. Separations were carried out in a Hamilton PRP-X200 (10 × 250 mm; Reno, NV, USA) for cationic exchange chromatography.

A Sonoplus ultrasonic homogenizer (Bandelin, Germany) fitted with a high frequency generator 2200 was used for Se species extraction. The homogenizer was equipped with a titanium micro tip of 3 mm in diameter, and the power was set to 20 W. The frequency was fixed at 20 kHz.

For molecular weight fractionation, 10-kDa cut-off filters (Millipore, Bedford, MA, USA) and an Eppendorf (Hamburg, Germany) Centrifuge 5804, F34-6-38, were used.

Reagents

All reagents were of analytical grade and were used without further purification. Inorganic Hg standard solutions were prepared by dilution of a stock Hg(II) solution (1,000 mg l^{-1}; Merck, Darmstadt, Germany) in deionized Milli-Q water (Millipore, Ohio, USA). Standard stock solution of 1,000 mg l^{-1} of methylmercury chloride (Alfa Aesar, Karlsruhe, Germany) was prepared in methanol (HPLC grade, Scharlau, Barcelona, Spain). These

solutions were stored in amber vials at −18°C and diluted with methylene chloride (HPLC grade, Scharlau) to obtain working standard solutions.

Stannous chloride (3% w v^{-1}), used as a reducing agent for Hg(II) in CV-AFS, was prepared daily by dissolving the appropriate mass of anhydrous stannous chloride (Merck) in 3 M of hydrochloric acid.

Potassium bromide, copper(II) sulphate and sodium thiosulphate (Merck) were used in sample preparation for Hg speciation.

Inorganic Se solutions were obtained by dissolving sodium selenite and sodium selenate (Merk) in deionized Milli-Q water (Millipore, Ohio, USA). Selenocystine and selenomethionine (Sigma Chemicals, St. Louis, MO, USA) were dissolved in 3% hydrochloric acid and deionized Milli-Q water, respectively. Trimethylselenonium chloride was synthesized in our laboratory. Stock solutions of 10 mg l^{-1} were stored in the dark at 4°C. Working standard solutions were prepared daily by dilution.

For hydride generation atomic fluorescence spectrometry studies, sodium borohydride (Sigma-Aldrich, Steinheim, Germany) was prepared in 0.3% sodium hydroxide (Merck). Three molar hydrochloric acid solution was prepared by diluting the appropriate volume of concentrated HCl (Merck).

For the HPLC and inductively coupled plasma mass spectrometry (HPLC-ICP-MS) studies, the mobile phase was 4 mM pyridine (Merck) in 3% methanol (SDS, Barcelona, Spain).

For the enzymatic hydrolysis procedure, Tris-HCl buffer (pH=7.5) and the nonspecific *Streptomyces griseus* (Pronase E; Merck) were used to prepare the fish tissue samples.

Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA): pepsin (porcine), pancreatin (porcine), and bile salts. α-amylase was purchased from Merck.

H_2O_2 (35%) from Panreac and HNO_3 (65%) were used for acid digestion of samples. Helium c-50 was used as a carrier gas, and argon (purity=99.9999%) was used as a make up gas, sheath gas and carrier gas at the transfer line and AFS, respectively (Carburos Metálicos, Spain).

Samples

Fish samples: swordfish (*Aphanopus carbo*), sardine (*Sardina pilchardus*), and tuna (*Thunnus* spp.) were purchased at a Spanish market or collected in docks (Sesimbra, Portugal) just before delivery to consumers following the recommendations of European Commission 2001/22/CE (JO CE, 2001). The skin and bones were removed, then the edible (muscle) portions of fishes were immediately blended and frozen at −18°C. These tissues were then oven dried at 40°C for 2 days and stored at −18°C until analysis. Analyses were performed avoiding UV radiation because of its detrimental effects on organomercurials.

Procedures

Figure 1 briefly describes an overview of the procedure for total Hg and Se determination and further speciation.

Total Hg Quantification

To determine the total Hg content, the dry samples (0.2 g) were digested with 2 ml of nitric acid and 0.5 ml of hydrogen peroxide (35%) in an analytical microwave oven at 43% power

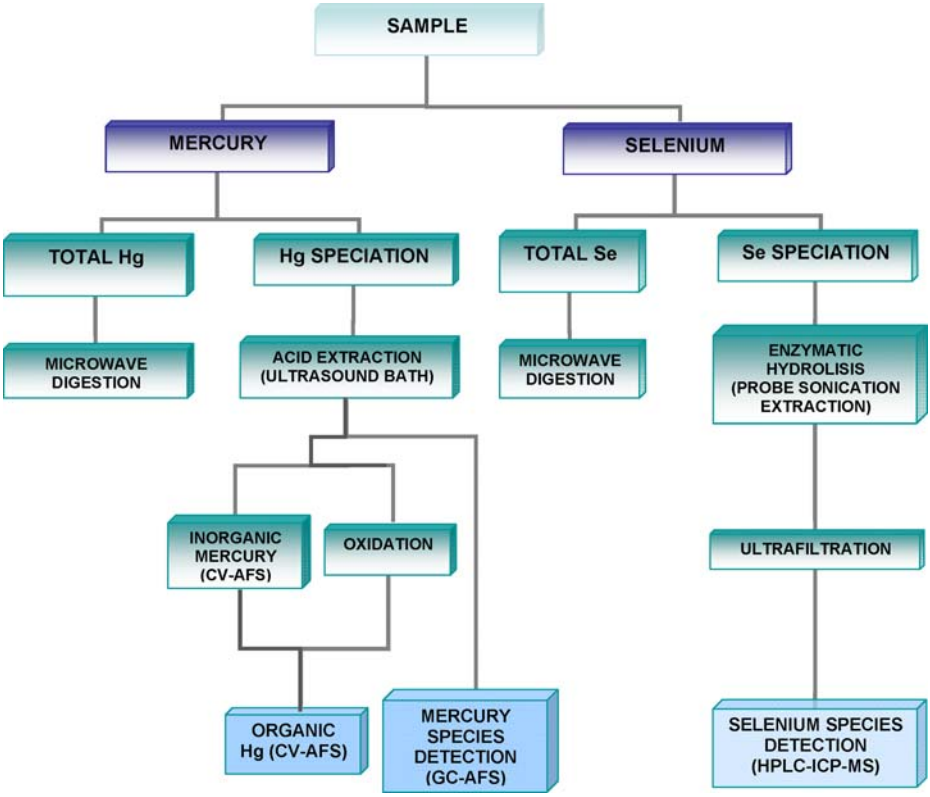

Fig. 1 Flow chart of the sample treatments used for mercury and selenium speciation in fish samples

output. The pressure was held at 20 psi for 15 min, at 40 psi for 30 min, and finally, at 85 psi for 1 h.

Total Hg concentration was determined by both external and standard addition calibrations of the signal obtained by the continuous Hg cold vapor system connected to AFS equipment (CV-AFS). A flow rate of 2.5 ml min^{-1} (3 M HCl) and a similar flow rate of the reductant solution (3% $SnCl_2$ in 15% HCl) were used to generate the Hg cold vapor.

Hg Speciation

Hg species leaching was performed following an acid leaching procedure developed previously [22].

Acid leaching A 300-mg portion of samples and 5 ml of 5 M hydrochloric acid were placed in a borosilicate glass tube and sonicated for 5 min.

Hg speciation After neutralization of the HCl extract, Hg species were converted into their bromide derivates (RHgBr).

Organomercury species were extracted into an organic phase by adding 5 ml of methylene chloride and shaking the mixture for 5 h. The samples were then centrifuged for 10 min. A 4-ml aliquot of the organic solvent layer containing the extracted organomercury

was transferred to a glass vial, and 1 ml of 0.01 M sodium thiosulfate solution was added. The solution was mixed for 20 min and subsequently centrifuged at 1,575×*g*.

Aqueous layer (800 μl) was placed in a 3-ml polyethylene vial, and 300 μl of KBr/$CuSO_4$ and 300 μl of CH_2Cl_2 were added. Each vial was manually shaken for 1 min, centrifuged, and 0.1–0.2 ml of the organic extract was placed in a 2-ml borosilicate glass vial.

Detection Organic Hg speciation was performed with the coupling GC-pyrolyzer-AFS.

Helium with a flow rate of 10 ml min^{-1} was used as the carrier gas. The temperatures were 250°C for the injector and 40°C with a ramp of 15°C min^{-1} up to 200°C for the oven.

For AFS detection, argon was used as make-up gas and sheath gas at flows of 60 and 300 ml min^{-1}, respectively.

Total organomercury determination After acid leaching, the total organomercury content in the supernatants was determined by difference between total Hg content (after digestion with HNO_3 and H_2O_2) and inorganic Hg content, by using stannous chloride as a selective reductant.

Total Se Quantification

The acid digestion procedure used for Se quantification was the same as that described for total Hg quantification. Se(VI) was reduced to Se(IV) by adding concentrated hydrochloric acid (6 M final concentration) to the digest and heating at 95°C for 1 h. The solutions were then diluted to 25 ml with Milli-Q-water.

Total Se concentration was determined by the continuous Se hydride system connected to AFS equipment. A flow rate of 1.5 ml min^{-1} (3 M hydrochloric acid) and a similar flow rate of the reductant solution (1% sodium tetrahydroborate w v^{-1}) were used to generate the Se hydride.

Se Speciation

Se species leaching was performed following an enzymatic leaching procedure developed previously [30].

Enzymatic hydrolysis Fish samples were enzymatically hydrolyzed following a probe sonication extraction. Fish samples (0.1 g) were placed in a 10-ml Teflon vial. Then, it was added 20% of the sample weight of the nonspecific protease *S. Griseus* (Pronase E) in 3 ml of the extraction media (Tris-HCl buffer, pH=7.5). The mixture was sonicated during 120 s at 20 W of power intensity. The resulting suspension was centrifuged at 4,000 rpm during 20 min, and aliquots of supernatant were taken for Se speciation.

Ultrafiltration To enhance the clean-up procedure, the supernatants were processed through a 10-kDa cut-off filter and diluted to 10 ml.

Quantification Se species were quantified by HPLC-ICP-MS under the following operating conditions (coolant Ar flow rate, 15.0 l min^{-1}; auxiliary Ar flow rate, 1.1 l min^{-1}; nebulization Ar flow rate, 1.3 l min^{-1}; sample flow rate, 1.0 ml min^{-1}; nebulizer type, Babington). For this purpose, a cationic exchange column using 4 mM pyridine at pH=2.8

and 4.7 as a mobile phase was used. The analytical peaks were evaluated in terms of peak area by the standard addition method at m/z 82 and 78.

In Vitro Gastrointestinal Digestion Method

The in vitro digestion method used (Fig. 2) was based on a previously developed method [31]. About 25 g of sample were placed in a 250-ml Erlenmeyer flask with 75 ml of gastric juice (6% w v^{-1} pepsin in 0.15 M NaCl, acidified with HCl to pH 1.8) and shaken for 1 min for initial degassing. The mixtures were then held in a thermostatic water bath for 4 h at 37°C, shaking periodically. After 1 h, the pH was checked and adjusted to 3 with 6 M hydrochloric acid.

After gastric digestion, saturated sodium bicarbonate was added to raise the pH to 6.8. Then, 50 ml of intestinal juice (1.5% pancreatin, 0.5% amylase, and 0.15% bile salts w v^{-1}, in 0.15 M NaCl) were added, and the mixture was energetically shaken for 1 min and left in a thermostatic water bath for 4 h at 37°C, shaking periodically. Once gastric/gastrointestinal

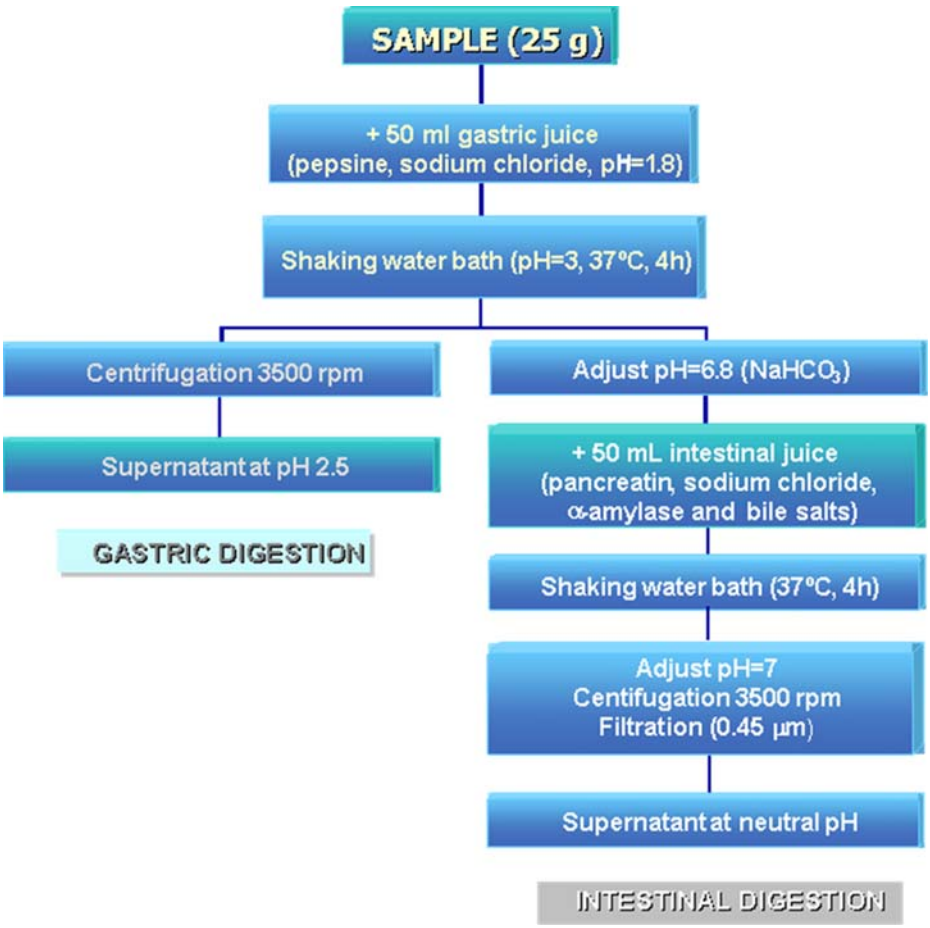

Fig. 2 Flow chart of the experimental protocol of the enzymolysis approach

digestion was completed, a 10-ml aliquot of the suspension was transferred to a polypropylene tube and centrifuged at 1,575×*g* for 1 h. The supernatant was filtered through a 0.45-μm Millipore filter to reduce any effect from microbial activity, and both supernatants and precipitates were stored in the dark at 4°C until analysis.

Gastric and intestinal digestion blanks were obtained by adding 75 ml of gastric juice to 25 ml of Milli-Q water and 50 ml of intestinal juice, respectively, and the above procedure was applied. In addition, some blanks were spiked before enzymolysis with SeMet and MeHg to determine the recovery of the added Se and Hg at the end of the gastric and gastrointestinal stages.

Consumption Limits

Monthly consumption limits for tuna, swordfish, and sardine were determined using methods from USEPA [32]. Limits were calculated using the equation:

$$\mathrm{CRm} = \frac{(\mathrm{Rf\,D})(\mathrm{BW})}{\mathrm{Cm}} \times \frac{30.44\mathrm{days/month}}{\mathrm{IR}}$$

Where RfD is the reference dose (1.10^{-4} mg kg^{-1} day^{-1}), BW the body weight (70 kg), Cm the concentration in fish (mg kg^{-1}), and IR is the ingestion rate (0.227 kg $meal^{-1}$).

Validation of the Results

In the present work, two certified reference materials were employed for validation of the methodologies used. Method validation for Hg was performed by using the reference material CRM-463, certified for methylmercury (2.85+0.16 μg g^{-1}), from the Community Bureau of Reference of European Commission (BCR); whereas for total Se, a marine tissue reference material (Murst-ISS A2) certified for total Se (7.37+0.91 μg g^{-1}) was used.

Statistical Analysis

A one-factor analysis of variance was applied to detect possible differences in total, organic, bioaccessible Se and Hg content, and in bioaccessibility between the three studied fish. A significance level of $p<0.05$ was adopted for all comparisons. Statgraphics Plus version 6.0 (Statistical Graphics) was used for the statistical analysis.

Results and Discussion

Sample Pretreatment

The sample pretreatment step is usually the most labor intensive part of the analytical method. Indeed, the physical treatment of the sample (e.g., grinding to decrease particle size, drying to eliminate the water, exposure to changes in ambient temperature or daylight) may give rise to losses of volatile analyte components [17]. To achieve the best conditions for trace Hg analysis, an evaluation of different drying sample treatment was carried out previously [22] (oven dried, microwave-oven dried, and freeze dried). The results indicated that the only pretreatment that did not lead to Hg volatilization was oven dried, and therefore, fish tissues were oven dried at 40°C for 2 days to eliminate its water content.

Total and Hg Speciation in Fish Samples

Total Hg content of the samples was determined by FI-CV-AFS to evaluate the Hg exposure through fish consumption. Table 1 shows total Hg contents in muscle of various fish species. The Hg content varied between 0.060 and 0.45 mg g^{-1}, with significant differences ($p<0.05$) in concentration depending on the type of fish analyzed (swordfish> tuna≫sardine). Such variability might be explained by the interference of biotic parameters such as age, size, sex, metabolism, and feeding habits [33–35] that affect the bioaccumulative process of Hg in fish.

All these values are within the European Commission Regulations 466/2001 and 221/2002 [36, 37], which forms part of EC food hygiene legislation and sets the maximum limit for Hg in whole fresh fish at 0.5 mg kg^{-1}, except mainly for predatory species, which may have higher Hg concentration (1.0 mg kg^{-1}). The value for Hg for these species was comparable with other Hg data found in fish [38–40].

With the exception of occupational exposure, fish are acknowledged to be the single largest source of Hg for man [41]. Fish is therefore a product for which suitable measures should be taken to provide chemical monitoring of the risks deriving from its consumption.

Since the toxic effect of Hg depends not only on its total concentration but also on its species, Hg speciation was performed. Hg species extraction was conducted after acid leaching (hydrochloric acid) combined with ultrasound bath extraction.

Several procedures based on alkaline digestion have been used for the analysis of Hg speciation but occasionally have shown overestimation of methylmercury and dimethylmercury concentration [18, 22, 42]. Therefore, to perform Hg species extraction, acid extraction (hydrochloric acid) combined with an ultrasound bath extraction was selected. Several variables, such as acid concentration (0–7 M), volume of extractant (0–7 ml), reagents, and sonication exposure time (0–15 min), were previously optimized [22]. Once total Hg extraction was carried out, determination of inorganic and total Hg was achieved in the acid extract by CV-AFS. The results obtained showed that organomercury compounds comprised more than 93% from the total Hg occurring in fish samples.

To evaluate the organomercury compounds, after applying the HCl extraction procedure, Hg speciation of the extracts with GC coupled to pyrolysis with atomic fluorescence detection was carried out. A chromatographic analysis was performed on different fish samples and only MeHg was detected. This fact is in good agreement with the literature, as MeHg is the only organomercury compound found in fish [43].

The amount of Hg found depends on the fish, but the percentage of MeHg (Table 1) in all samples is higher than 92%. No significant differences were found between this value and the one given above for total organomercury compounds.

Table 1 Total Mercury Concentrations Found in Fish Samples by CV-AFS and MeHg Concentration Found After Acid Hydrolysis of Fish Samples by GC-AFS

Sample	Moisture (%)	Total Hg fresh weight (μg g^{-1})	Total Hg dry weight (μg g^{-1})	MeHg dry weight (μg g^{-1})	Percentage of MeHg (%)
Tuna	64	0.37±0.02	1.03±0.03	0.95+0.06	92
Swordfish	78	0.45±0.02	2.04±0.03	1.89+0.07	93
Sardine	76	0.060±0.002	0.25±0.02	0.23+0.05	92
CRM-463[a]	3	–	2.87±0.07	2.87±0.07	

Results expressed as mean values ± standard deviation, $n=5$

[a] Certified value, 2.85±0.16 μg g^{-1}

The method employed was validated by the analysis of the standard reference material, CRM 463 tuna fish. No significant differences were found between the certified value and the one provided by the acid leaching method (GC-AFS) at 95% confidence level.

According to this data, consumption limits for MeHg for adults were calculated. Following the USEPA [32] recommendations, the consumption of these fishes should be restricted. Two meals of tuna or swordfish (0.227 kg) or 13 meals of sardine may be eaten per month. Sardines are, therefore, preferable as source of protein for humans because of its low Hg content.

It is important to notice that these data have not taken into account the results of bioaccessible studies, which clearly demonstrate that the bioaccessibility of MeHg is lower than expected (see "Hg and Se Bioaccessibility") and so is the potential risk associated to these fishes.

Total and Se Speciation in Fish Samples

Se status relies on the dietary Se intake and the element bioavailability. Therefore, total Se and species determination in fish samples is of special concern.

Total Se content of the fish samples was determined to evaluate the Se exposure of fish consumers. The results obtained are shown in Table 2.

Total Se concentration in fish tissue varied between 0.36 (swordfish) and 0.72 μg g^{-1} (tuna). The value for Se in these fish species was comparable with other Se data for fish [44–46] and agrees with the finding that Se content in the edible part of fish products varies within a rather small range from 0.2 to 0.9 mg kg^{-1} (Food Standards Agency).

The quality assurance procedures for these analyses included the measurement of a marine tissue reference material (Murst-ISS A2). Because, at the 95% confidence level, no significant difference was detected between the certified value and the experimental one, the method used was considered accurate for total Se determination.

According to the data reported here, fish is a good source of Se and could substantially contribute to meeting the Se requirements for adult humans presented by the World Health Organization (WHO [47]; 0.39 and 0.42 μg g^{-1} body weight for adult men and women, respectively).

With regard to these results, consumption limits for Se calculated for adults according to the USEPA [32] recommendations indicate that unrestricted fish meals (>50) may be eaten per month for the three fishes, unlike what happened with Hg.

Because Se toxicity and bioaccessibility depends on the chemical form of Se, the chemical species of Se present must be known before any conclusions can be drawn about the toxic or beneficial effects of Se from fish.

Table 2 Total Selenium Concentrations Found in Fish Samples by HG-AFS and SeMet Concentration Found After Enzymatic Hydrolysis of Fish Samples by HPLC-ICP-MS

Sample	Moisture (%)	Total Se fresh weight (μg g^{-1})	Total Se dry weight (μg g^{-1})	SeMet dry weight (μg g^{-1})	Percentage of SeMet (%)
Tuna	64	0.72±0.01	2.00±0.03	0.90±0.03	45
Swordfish	78	0.36±0.02	1.63±0.04	1.47±0.04	90
Sardine	76	0.41±0.02	1.71±0.02	0.46±0.04	27
Murst-ISS A2[a]	2	–	7.42±0.52	–	

Results expressed as mean values ± standard deviation, $n=5$

[a] Certified value, 7.37±0.91 μg g^{-1}

The use of appropriate methods of protein hydrolysis is of key importance in speciation analysis irrespective of the method of Se species determination used. Conditions must be carefully chosen to achieve complete protein hydrolysis with minimal concomitant destruction of the Se species. Generally, the use of enzymatic hydrolysis processes has shown better results in the release of Se species from biological solid samples [48] than basic (tetramethylammonium hydroxide) or acid (hydrochloric acid) hydrolysis that led to Se species degradation [49, 50]. In addition, several techniques like ultrasound can enhance the activity of the enzyme, shortening substantially enzymatic incubation time, and thus lead to a fast species extraction method keeping its integrity.

Because of this, Se species extraction was performed after an enzymatic hydrolysis enhance by probe sonication developed previously [30] and adopted for the fish being studied. Several variables such as: sonication time, extraction media, temperature, sample/enzyme mass ratio, ultrasound amplitude, and sample mass were previously optimized.

The enzymatic digestion specified in "Experimental Procedure" followed by ultrafiltration with 10-kDa cut-off filters was applied to the fish samples to identify and quantify the selenoamino acids and inorganic Se species. For swordfish, no Se loss was detected in this step (Se recoveries, 90–96%), which indicates that the molecular weight of most of the Se species extracted during the hydrolysis was lower than 10 kDa. On the other hand, the Se recoveries for tuna and sardine were not quantitative, which means that some Se may remain in peptide form. This explanation stems from the knowledge that, during the enzymatic hydrolysis of proteins, some peptide bonds can remain intact, depending on the cleavage specificity of the enzyme [51]. This could be the reason a Se fraction with a molecular weight higher than 10 kDa remained after the enzymatic hydrolysis step.

To ensure that no Se compounds lower than 10 kDa were retained in the filters, the total Se content in the extracts (spiked with the standards) after filtration was determined. The results, with an average recovery of 97±3% of total Se, showed that significant Se losses did not occur with this sample treatment.

In this study, five standard Se compounds and two mobile phases were tested for Se separation using a cationic exchange column. If a species is identified under two different chromatographic conditions, its identity can be stated with more certainty. Because of this, different experiments were carried out to provide good resolution for standard solutions

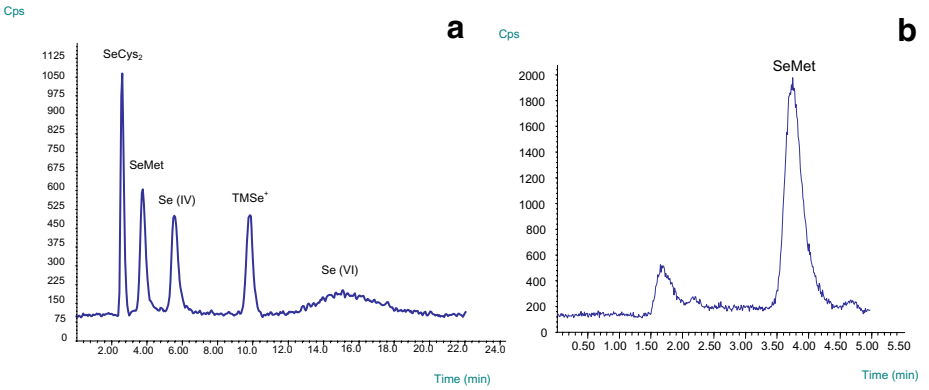

Fig. 3 Chromatograms of **a** 10 μg l^{-1} of Se species obtained for cationic exchange chromatography at pH value 4.7 and **b** Se species found after enzymatic hydrolysis of swordfish at pH value 4.7

using a mobile phase at two pH values (2.8 and 4.7). Chromatographic analyses were performed on the fish samples. Figure 3 shows the chromatograms obtained for the standards and a representative sample. Two peaks could be differentiated in each of the evaluated samples. The first peak was unidentified, and it could not be attributed to any of the Se species tested ($SeCys_2$, Se(IV), Se(VI), and $TMSe^+$); so, it could correspond to any of the Se species that elutes in the dead volume. The second peak was identified as SeMet, the only selenoamino acid found in all samples. The identification of the peaks was carried out by the spiking procedure. The same chromatographic profiles were obtained by the two chromatographic methods used (at pH 2.8 and 4.7) in all the samples. In addition to that, an evaluation of SeMet stability in enzymatic extracts was carried out. Results showed that this species was stable for at least 48 h after sonication at room temperature.

The total amount of SeMet varied depending on the type of fish. In swordfish, 90% of the total Se was found to be SeMet, whereas tuna and sardine had lower percentages of SeMet (45 and 27%, respectively). The value for SeMet for these species was comparable with other SeMet contents found in previous works [48].

In spite of the highest SeMet content found is swordfish, it can also be seen (Table 3) that the highest value of SeMet/MeHg ratio, as well as Se/Hg ratio, is for sardine and the lowest one for swordfish.

Hg and Se Bioaccessibility

The extent of the toxic effects caused by Hg and the toxic/beneficial effects caused by Se are not governed by their total concentration but rather regulated by the forms of the metal that interact with sites on the biological ligands. Consequently, total determination and speciation of Hg and Se in the gastrointestinal tract is essential to understand and predict its availability for absorption.

To study Hg and Se bioaccessibility from human diet, an in vitro enzymolysis simulating the human gastrointestinal digestion of fish was carried out.

The recoveries of endogenous Hg and Se from fish in the gastric extract (pH 2.0) differed significantly ($p<0.05$) from the gastrointestinal (pH 6.8) supernatants and varied depending on the type of fish. This might be due to differences in the composition of the fish, as proteins and fat content may affect the solubility of Hg and Se or to a different capability of the enzymes in the in vitro method for releasing the trace elements existing in each sample.

A low percentage of the Hg in the fish was found bioaccessible (<20%) in both the simulated stomach and intestinal digestion. On the other hand, between 50 and 83% of the Se in fish was bioaccessible in the simulated gastrointestinal digestion. The very low

Table 3 Molar Se/Hg, SeMet/MeHg, $[Se/Hg]_{bioaccessible}$, and $[SeMet/Hg]_{bioaccessible}$ Ratios Found in Fish Samples After Mineralization, Acid/Enzymatic Hydrolysis, and In Vitro Gastrointestinal Digestion

Sample	Se/Hg molar ratio	SeMet/MeHg molar ratio	$[Se/Hg]_{bioaccessible}$ molar ratio	$[SeMet/Hg]_{bioaccessible}$ molar ratio
Tuna	4.9	2.4	27.5	10.4
Swordfish	2.0	1.9	9.3	1.7
Sardine	17.3	4.7	126.3	25.3

Results expressed as mean values ± standard deviation, $n=5$

bioaccessible Hg content (10%) and the high bioaccessible Se content (83%) in sardine is a further reminder of the benefit from this product compared with the two other fish.

These results are in conflict with earlier experiments on animals under MeHg administration, in which the high absorption and therefore bioavailability of MeHg is addressed. The low Hg recovery obtained in this study could be attributed to the low ability of enzymes in the in vitro method to release the Hg existing in each of the samples, perhaps because of the fact that Hg is complexed by Se rather than to a lack of bioaccessibility of the MeHg itself. In fact, a recovery of 89% was obtained from in vitro enzymolysis of a sample spiked with MeHg.

It has been previously suggested that Se is involved in the protection of Hg by forming complexes containing the two elements such as tiemannite [52], Se–Hg–S species [53], or Se–Hg precipitates.

In this study, Se has been found in excess together with a low MeHg bioaccessibility in all samples; as a result, this fact supports a significant antagonistic effect of Se on Hg toxicity.

In the three samples, a mass balance was performed after application of the in vitro digestion method. Both the soluble fraction and nonsoluble fraction resulting from application of the in vitro digestion method were analyzed. The mass balance results for Hg and Se were higher than 91%.

Hg and Se Speciation of the Gastrointestinal Extracts

To evaluate whether the in vitro digestion method employed keeps the integrity of the Hg and Se species present in the initial product or brings some transformation, Hg and Se speciation of the gastrointestinal extracts was carried out.

To evaluate possible transformation of Hg species, mainly MeHg, following the gastrointestinal procedure, the extracts were directly analyzed by selective reduction with 3% (w/v) stannous chloride and measurement by CV-AFS. Total Hg was determined by the same procedure but with previous HNO_3–H_2O_2 digestion of the extract. Organic Hg was calculated as the difference between the values obtained in the two steps. The results obtained showed that the bioaccessible Hg was not transformed into inorganic forms; so, it remained as organomercury after in vitro gastrointestinal digestion.

To evaluate possible transformation of Se species following the gastrointestinal procedure, the extracts were directly analyzed by HPLC-ICP-MS.

The identification of the peaks was carried out by the spiking procedure. The first peak was unidentified and could not be attributed to any Se species tested. SeMet was found (second peak) to be the dominant Se species, being the only selenoamino acid found in both extracts (gastric and gastrointestinal). The chromatographic profiles obtained by either chromatographic method were identical.

Speciation analysis of the gastrointestinal digestion extracts show that the amount of SeMet varied depending on the type of fish. Tuna had a higher bioaccessible SeMet concentration (0.320 ± 0.005 μg g^{-1}) than swordfish and sardine (0.245 ± 0.005 μg g^{-1} and 0.239 ± 0.006 μg g^{-1}, respectively). However, tuna, swordfish, and sardine had similar percentages of bioaccessible SeMet (≈15%) relative to the total Se content. Unlike what happened when Se speciation was performed on fish samples using Pronase E (SeMet content varied within the fish species), in vitro digestion resulted in the same percentage of bioaccessible SeMet regardless of the sample analyzed, although Se bioaccessibility varied depending on the fish species. Therefore, bioaccessible SeMet may be located in proteins of similar nature and accessibility.

Se/Hg Ratios

Although swordfish and sardine had similar Se contents, the Se/Hg molar ratio varies (Table 3) from 2 (swordfish) to 17 (sardine). As a consequence, taking into account its total Se and Hg content, sardine is the preferred fish species because of its low Hg content and considerable Se content. The same behavior was observed when simulating in vitro gastrointestinal digestion. The $[Se/Hg]_{bioaccessible}$ ratios varied to the same extent as they did above, showing the following order: sardine>tuna>swordfish (126.3, 27.5, and 9.3, respectively). Furthermore, although the highest $SeMet_{bioaccessible}$ content was found in tuna, the highest values of $[SeMet/Hg]_{bioaccessible}$ ratio (25.3) and $[Se/Hg]_{bioaccessible}$ ratio were in sardine, and the lowest value in swordfish (1.7). Therefore, sardine consumption would be preferable to tuna and swordfish.

Future Studies

In preliminary studies carried out in our laboratory, it has been found that functional feed enriched in Se increases the concentration level of both total Se and SeMet in fish (African catfish, *Clarias gariepinus)*, but its effect on Hg bioaccumulation has not been yet studied. More research in this field is necessary.

Therefore, further studies targeted on in vivo experiments conducted to evaluate the effect of Se on MeHg bioaccumulation in highly polluted fishes are proposed to clarify their interaction.

Conclusions

This paper provides information about the concentration of total Hg and Se and its species found in highly consumed fish.

The levels of total Hg found are compatible with the maximum level allowed by the European legislation, being more that 92% of the total Hg as MeHg. In addition, the average concentrations of Se are important to ensure the recommended daily amount.

SeMet was the only selenoamino acid identified in the three species after an enzymatic hydrolysis process and in vitro digestion.

Se and Hg bioaccessibility was found to be dependent on the type of fish analyzed because of different degradation effectiveness of the food matrix and consequent release of these elements. Simulated human gastric and intestinal digestion led to a high Se bioaccessibility and low Hg bioaccessibility, and no modification during digestion of the Hg and Se species was found for all samples. Thus, the potential toxicity of MeHg in fish, such as tune, could significantly be decreased by an antagonistic effect of Se.

Regardless of the sample, it was found that bioaccessible SeMet accounted for the same percentage of the total Se. Therefore, bioaccessible SeMet may be located in proteins of similar accessibility in all the samples. The favorable ratio between SeMet and MeHg found in sardine increases the nutritional importance of this fish. Thus, its consumption would be preferable within the other two fishes studied.

The molar ratios Se/Hg, [Se/Hg]bioaccessible, and [SeMet/Hg]bioaccessible showed the same order: sardine>tuna>swordfish. In conclusion, the most favorable fish for human consumption can be predicted by calculating the molar ratio Se/Hg.

In conclusion, the potential toxicity of fish cannot be evaluated independently by analyzing the total Hg or MeHg content but also the Se content that could significantly influence the Hg bioaccessible fraction.

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