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Mercury and selenium in fish and shellfish: Occurrence, bioaccessibility and uptake by Caco-2 cells

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ABSTRACT

This study evaluates Hg and Se concentrations and bioaccessibility (element solubilised after simulated gastrointestinal digestion) in 16 raw seafood species consumed in Spain. The concentrations varied greatly (Hg, 3.8–1621 ng/g wet weight, ww; Se, 84–1817 ng/g ww). Only one sample of swordfish exceeded the Hg limit permitted in Spain (1 mg/kg), and for this sample the Hg/Se molar ratio and Se Health Benefit Value food safety criteria also indicated the presence of a risk. Bioaccessibility of Hg (35–106%) and Se (17–125%) was very variable and the Hg/Se molar ratio in the bioaccessible fraction was less than one for all samples. Transport by Caco-2 cells, an intestinal epithelium model, was also evaluated from the swordfish bioaccessible fraction. Hg and Se transport from the food was less than 14%, and cell retention was much greater for Hg (49–69%) than Se (8–12%).

1. Introduction

Seafood and shellfish are important sources of proteins, polyunsaturated lipids and phospholipids, and also of numerous micronutrients, group B vitamins, vitamins A and D and minerals (phosphorus, potassium, sodium, calcium, magnesium, iron and iodine). However, they may contain toxic trace elements, whose concentration varies according to the element and the kind of seafood product considered. One of the contaminants of greatest interest is mercury (Hg) and its most toxic chemical species, methylmercury (MeHg). Studies conducted in recent decades associate chronic exposure with methylmercury through consumption of seafood products with health risks. In view of its neurotoxic nature (Grandjean et al., 2010), women of childbearing age, pregnant women, breastfeeding women and children are considered risk populations. Large marine predators such as swordfish, tuna and shark have the highest methylmercury concentrations, with up to 4 mg/g wet weight (ww) reported in swordfish (Forsyth et al., 2004).

Seafood products are also an important source of another trace element, selenium (Se), which, unlike Hg, may be considered an essential element, although cases of poisoning after intake of Se have been reported (Yang et al., 1983). Selenium forms part of selenoenzymes and selenoproteins related with the synthesis of thyroid hormones (Arthur, 1991) and with processes that protect against oxidative stress (Zhou et al., 2009). Another role attributed to Se is that of reducing the toxicity of certain xenobiotics, particularly Hg. This protective effect seems to be due to the formation of Se–Hg complexes (Raymond and Ralston, 2004).

Most studies about Hg and Se in seafood products only determined their concentrations. However, in order to make a more accurate estimate the exposure to these elements it is necessary to consider bioavailability, the quantity of the element that reaches the systemic circulation after ingestion and that is available to carry out its biological activity. A first *in vitro* approach to the study of bioavailability can be made by determining bioaccessibility, the maximum concentration soluble in simulated gastrointestinal media that is available for subsequent processes of absorption into the intestinal mucosa. The system can be improved with the Caco-2 cell, which can be used to evaluate intestinal cell retention and transport processes and offers a more reliable approximation to the *in vivo* situation in estimating bioavailability at intestinal level. This model has been widely used to evaluate the mineral contribution (Ca, Fe, and Mg) of some food products and to study drug absorption. However, there are not many cases of application of this model to evaluate bioavailability of contaminants and trace elements from food. Studies on the bioaccessibility of Hg from seafood products have been conducted recently (Laird et al., 2009; Shim et al., 2009; Torres-Escribano et al., 2010; Torres-Escribano et al., 2011; He and Wang, 2011), but there are few studies that

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determine bioavailability of Hg from these foods either *in vitro* with the Caco-2 cell line (Hwang and Shim, 2008), or *in vivo*, using experimental animals (Endo et al., 2003; Berntssen et al., 2004). In the case of Se, there are few studies on its bioaccessibility from seafood products (Cabañero et al., 2007) or its oral bioavailability (Bügel et al., 2001; Fox et al., 2004).

The aim of the present study was to evaluate the Hg and Se concentrations and bioaccessibility in a wide variety of seafood products consumed in Spain. The uptake (cell retention and transport) by Caco-2 cell line of these two elements in swordfish samples was also studied.

2. Material and methods

2.1. Equipment

For Hg analysis, a microwave accelerated reaction system (MARS) from CEM (Vertex, Spain) was used for wet mineralization of samples and a continuous flow cold vapour generation atomic fluorescence spectrometer (CV-AFS) (Millennium Merlin model PSA 10.025, PS Analytical, UK) was used for Hg quantification.

For Se analysis, a muffle furnace (K1253, Heraeus, Spain) was used for dry ashing of samples and a atomic absorption spectrometer (model 3300, Perkin-Elmer, Spain) equipped with an autosampler (AS-90, Perkin-Elmer), a flow injection-hydride generation (FI-HG) system (FIAS-400, Perkin-Elmer), and an electrothermally heated quartz cell was used for quantification.

Other equipment used included a lyophiliser (Genesis SQ 35 EL, Virtis, USA), a sand bath (PL 5125, Raypa, Scharlau, Spain), a mechanical shaker (KS 125 Basic, IKA Labortechnik, Merck, Spain), a pH meter (pH 526, Multical, Spain), an orbital shaking water bath (Unitronic Orbital C, J.P. Selecta, Spain) and a centrifuge (RC-5B Superspeed Refrigerated Centrifuge, Sorvall, Du Pont).

2.2. Reagents

Deionised water (18.2 MΩ cm), obtained with a Milli-Q water system (Millipore Inc., Millipore Ibérica, Madrid, Spain) was used for the preparation of standards and reagents. Standard solutions of 1000 mg/L Hg(II) [$Hg(NO_3)_2$, VWR, Barcelona, Spain] and 1000 mg/L Se(IV) [SeO_4, Merck] were employed. A standard solution of 1000 mg/L MeHg was prepared by dissolving a commercially available salt of MeHgCl (Sigma–Aldrich, Madrid, Spain) in 50% (v/v) $MeOH/H_2O$.

Other reagents used, analytical or reagent grade, were: hydrochloric acid (Merck), nitric acid (Merck), sodium hydroxide (Merck), magnesium oxide (Prolabo, Barcelona, Spain), magnesium nitrate (Prolabo), ascorbic acid (Prolabo), potassium iodide (Prolabo), sodium tetrahydroborate (Panreac, Barcelona, Spain), hydrogen peroxide (Prolabo) and tin (II) chloride dihydrate (Scharlab, Scharlau Chemie, Barcelona, Spain). All material was treated with 10% (v/v) HNO_3 for 24 h, and then rinsed three times with deionised water before use.

Water of cellular grade (B. Braun Medical, S.A., Barcelona, Spain) was used throughout the *in vitro* digestion assay. Enzymes and bile salts for *in vitro* gastrointestinal digestion were purchased from Sigma: porcine pepsin (enzymatic activity 944 U/mg protein), porcine pancreatin (activity equivalent to 4× US Pharmacopoeia specifications/mg pancreatin) and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts).

2.3. Sample collection and preparation

Forty samples were analysed, corresponding to 16 species of fish and shellfish selected on the basis of their high domestic consumption in the Valencian Community (Spain) (Table 1). Samples fresh or frozen were purchased from Mercavalencia (Valencia, Spain) between May 2006 and April 2007. The edible portions of the raw samples were lyophilised, crushed, homogenized to a fine powder and stored at 4 °C until analysis.

2.4. Simulated gastrointestinal digestion

The simulated gastrointestinal digestion employed was a modification of the method described by Laparra et al. (2003). The equivalent of 10 g of fresh sample was weighed, 80 g of cell culture grade water was added and the pH was adjusted to 2.0 with 6 mol/L HCl. Freshly prepared pepsin solution (10% m/v pepsin in 0.1 mol/L HCl) was added to provide 0.001 g of pepsin/g fresh seafood. The sample was made up to 100 g with cell culture grade water, and incubated in a shaking water bath (120 strokes/min) at 37 °C for 2 h.

The pH of the gastric digests was then raised to pH 5 by drop-wise addition of 1 mol/L $NaHCO_3$. The pancreatin-bile extract mixture (0.4% m/v pancreatin and 2.5% m/v bile extract in 0.1 mol/L $NaHCO_3$) was added to provide 0.00025 g of pancreatin/g seafood and 0.0015 g of bile extract/g seafood, and the incubation at 37 °C continued for an additional 2 h. After the intestinal digestion step, the pH was adjusted to 7.2 by drop-wise addition of 0.5 mol/L NaOH. The digests were transferred to polypropylene centrifuge tubes and centrifuged at 10000 rpm for 30 min at 4 °C to separate soluble and precipitate. The total concentrations of Hg and Se in the soluble (bioaccessible) fraction were quantified, and bioaccessibility was determined by the following equation:

$$\text{Bioaccesibility} = \frac{(\text{Element in bioaccessible fraction})}{(\text{Element in seafood})} \times 100$$

The contribution of the enzymes and reagents to Hg and Se concentration in the bioaccessible fractions was also evaluated by digesting blanks.

For the uptake and transport assay with cells, the bioaccessible fraction was inactivated by heating for 4 min at 100 °C to inhibit sample proteases and then cooled by immersion in an ice bath. Glucose (Sigma, 5 mM final concentration) and HEPES (BioWhittaker; 50 mM final concentration) were then added to facilitate cell viability, and water or NaCl (Panreac) was added to adjust the osmolarity to 310 ± 10 mOsm/kg using a Freezing point osmometer (Osmomat 030, Berlin, Germany).

Table 1
Samples of fish and shellfish analysed in this study.

Category	Commercial name	Scientific name
Fish	Anchovy	*Engraulis encrasicolus*
	Anglerfish	*Lophius piscatorius*
	Blue whiting	*Micromesistius poutassou*
	Hake	*Merluccius merluccius*
	Salmon	*Salmo salar*
	Sardine	*Sardina pilchardus*
	Small hake	*Merluccius* spp.
	Sole	*Solea solea*
	Swordfish	*Xiphias gladius*
Shellfish	Clam	*Chamelea gallina*
	Cuttlefish	*Sepia* spp.
	Norway lobster	*Nephrops norvegicus*
	Mussel	*Mytilus edulis*
	Prawn	*Penaeus kerathurus*
	Shrimp	*Parapenaeopsis* spp.
	Squid	*Loligo* spp.

2.5. Cell culture

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, UK). The cells were mantained in 75 cm^2 flasks to which 10 mL of Minimum Essential Medium (MEM, Gibco BRL Life Technologies, Paisley, Scotland) at pH 7.4 were added. The MEM was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco), 1% (v/v) non-essential amino acids (Gibco), 0.6 g/L L-glutamine (BioWhittaker Europe, Verviers, Belgium), 1 mM sodium pyruvate (BioWhittaker), 0.22% (w/v) $NaHCO_3$ (Merck), 10 mM HEPES, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin (BioWhittaker), and 0.0025 mg/mL of fungizone (Gibco) (MEMc). Incubation conditions were 37 °C, 5% CO_2 and 95% relative humidity atmosphere. The culture medium was changed every two days. At 70% confluency the cells were harvested using trypsin–EDTA solution (2.5 g/L trypsin, Sigma; 0.2 g/L EDTA, Sigma) and reseeded.

2.6. Hg and Se uptake (retention and transport) by Caco-2 cell

Experiments were conducted for MeHg standard solution and bioaccessible fractions of seafood samples. For these studies, Caco-2 cells were seeded onto polycarbonate membrane filters, Transwell® inserts with 24 mm diameter and 0.4 μm pore size (Costar Corp., United States), at a density of 5×10^4 cells/cm^2. The Transwell® inserts were placed in six-well plates, creating a two-chamber system with an apical (upper) compartment and a basal (lower) compartment. Then 1.5 mL of MEMc was added to the apical chamber and 2 mL of MEM was added to the basal chamber and the cells were incubated at 37 °C, 5% CO_2 and 95% relative humidity atmosphere. Medium was changed every 48 h until cell differentiation (15–18 days after seeding).

Once differentiation had taken place, the culture medium was aspirated from the apical and basal chambers, and the cell monolayers were washed three times with phosphate-buffered solution (PBS) [NaCl, 140 mM; KCl, 2.7 mM; Na_2HPO_4, 6.4 mM; H_2KPO_4, 1.5 mM; all reagents from Merck]. Then 1.5 mL of MeHg standard prepared in MEMc [0.1 μg/L] or 1.5 mL of inactivated bioaccessible fraction of seafood was added to the apical chamber. Fresh MEMc (2 mL) was added to the basal side. The cell culture was incubated for 2 h or 4 h at 37 °C, 5% CO_2 and 95% relative humidity atmosphere. Afterwards, both the apical and the basal media were recovered from the inserts by aspiration, and Hg and Se were analysed in order to evaluate transepithelial transport. The cell surfaces of the monolayers were washed three times with PBS, detached with a trypsin–EDTA solution, and recovered with 0.5 mL of PBS. Hg and Se were analysed in the cell lysate in order to evaluate

retention. Hg and Se retention and transport percentages were calculated with respect to the initial quantity of Hg and Se added to the Caco-2 cell cultures. Control cells were used throughout each assay.

The integrity of the monolayer was monitored by measuring the transepithelial electrical resistance value (TEER) (Millicell electrical resistance system, Millicell-ERS; Millipore Iberia, Madrid, Spain), TEER was measured at different time points, including the start and end of the experiments and only cultures with TEER values >250 Ω/cm were used.

2.7. Determination of mercury

Mercury concentrations were determined by CV-AFS after a microwave acid digestion. Seafood samples (0.2 g), bioaccessible fraction (1.5 mL) and transport study samples (apical media, basal media and cells) were placed in a Teflon PFA vessel, treated with 4 mL of HNO_3 concentrate (14 mol/L) and 1 mL of H_2O_2 (30% v/v) and digested in the microwave system (800 W, 15 min, 180 °C). After that, the digest was placed in a beaker and allowed to rest all night to eliminate nitrous vapour. It was then filtered through Whatman No. 1 paper and made up to volume with 5% HCl (v/v). The analytical conditions used for mercury determination by CV-AFS were the following: reducing agent, 2% (m/v) $SnCl_2$ in 1.8 M HCl, 4.5 mL/min flow rate; carrier solution, 0.6 M HCl (v/v), 9 mL/min flow rate; carrier gas, argon 0.3 L/min flow rate, dryer gas, 2.5 L/min flow rate; range 100; filter 32.

The analytical characteristics of the method were evaluated in accordance with the following criteria. The limit of quantification was calculated by dividing ten times the standard deviation of the absorbance of nineteen reagent blanks by the slope of the standard calibration curve. Precision was assessed as within-day repeatability, with the performance of three independent analyses of three seafood products with different Hg concentrations (<0.1 μg/g, 0.1–1 μg/g and >1 μg/g wet weight). Trueness, expressed as recovery, was evaluated in triplicate in the same samples that were used to evaluate precision. The concentration level added was the same as the level found in the unspiked sample. Accuracy was evaluated by analysis of the DORM-2 certified reference material with each batch of samples. The food samples were only analysed if the concentrations found in the reference material were within the certified range.

The analytical characteristics of the method for the analysis of Hg in seafood were: quantification limit = 0.001 mg/kg wet weight; precision = 6%; trueness = 98%. The results obtained in the reference material were in good agreement with the certified values.

2.8. Determination of selenium

Selenium was determined by FI-HG-AAS after a dry ashing step. Samples [1 g of seafood sample, 5 mL of bioaccessible fraction and transport study samples (apical media, basal media and cells)] were treated with 1 mL of ashing aid suspension [20% m/v $MgNO_3$ and 2% m/v MgO] and 5 mL of 7 M HNO_3. The mixture was evaporated on a sand bath until total dryness, and placed in a muffle furnace at an initial temperature not exceeding 150 °C. The temperature was increased progressively to (425 ± 25) °C at the rate of 50 °C/h, and the maximum temperature was maintained for 12 h. The white ash was dissolved in 50% (v/v) HCl and then heated at 90 °C for 20 min.

The analytical conditions used for quantification were the following: FI-HG [reducing agent, 0.2% (m/v) $NaBH_4$ in 0.05% (m/v) NaOH, 5 mL/min flow rate; carrier solution, 1.2 M HCl, 10 mL/min flow rate; carrier gas, argon 0.13 L/min flow rate, dryer gas, 2.5 L/min flow rate]; AAS [wavelength, 196.0 nm; spectral bandpass, 0.2 nm; electrodeless discharge lamp system 2, lamp current setting 280 mA; cell temperature, 900 °C].

The criteria used in the evaluation of the analytical characteristics of the method for the analysis of Se in seafood were the same as those described above for Hg. The analytical characteristics of the method were: quantification limit = 0.003 mg/kg wet weight; precision = 8%; trueness = 97%. The results obtained in the reference material were in good agreement with the certified values.

3. Results and discussion

3.1. Hg and Se in seafood

The Hg and Se concentrations found in the 16 species of seafood analysed are shown in Table 2. The Hg concentrations vary between 3.8 (shrimp) and 1621 ng/g ww (swordfish). European legislation limits the maximum concentration of total Hg in seafood products to 0.5 mg/kg ww or 1 mg/kg ww, depending on the fish species considered (EC, 2008). The samples analysed comply with this legislation with the exception of one sample of fresh swordfish, which exceeds the maximum allowed for this kind of fish product (1 mg/kg ww). The Se concentrations vary between 84 ng/g ww (frozen cuttlefish) and 1817 ng/g ww (Norway lobster). It has been reported that Se, like Hg, is biomagnified throughout the food web (Kehrig et al., 2009). In our study, the median Hg levels are higher in the fish (48 ng/g ww) than in the shellfish (11 ng/g ww), a trend that has been reported in one previous paper (Chen and Chen, 2006). The median selenium concentration, however, is higher in the shellfish (306 ng/g ww) than in the fish (228 ng/g ww), which differs from the trend found by Chien et al. (2003) (shellfish < fish).

Hg is a contaminant that has been much studied in seafood marketed in Spain, and reported concentrations are similar to those found in the present study (Sahuquillo et al., 2007; Blanco et al., 2008; Yusà et al., 2008; Rubio et al., 2008; Torres-Escribano et al., 2010, 2011; Rodellar et al., 2010; Martorell et al., 2011). The studies cited report concentrations exceeding the limit permitted by the EC in samples of louvar, tuna, swordfish, gilthead sea bream, tope shark and hake. Research on Se in seafood marketed in Spain is sparse (Cabañero et al., 2007; Lavilla et al., 2008).

Recent reviews of epidemiological studies that evaluate the effect of exposure to MeHg on neurobehavioural development consider that the conclusions that have been drawn concerning a direct relationship between MeHg in ingested fish and the effects observed are not very firmly based (Choi et al., 2008; Ralston, 2008). The authors suggest that the lack of consistency comes from the failure to evaluate the effect of co-exposure to MeHg and beneficial compounds that seafood provides and that may counteract the effects of MeHg, such as selenium, vitamin E and polyunsaturated fatty acids (Choi et al., 2008; Ralston, 2008). Of all these, the effect of Se on the toxicity of MeHg is the one that has been most studied. An *in vivo* study with joint administration of the major species of Hg and Se in seafood, methylmercury–cysteine and selenomethionine, respectively, shows a possible amelioration of neurobehavioural impacts of Hg as a result of the presence of Se (Folven et al., 2009). Therefore, the molar ratio of Hg and Se in the diet appears to be an essential criterion of risk from Hg exposure rather than the Hg content alone (Kaneko and Ralston (2007)). It has been shown in experimental animals that Hg toxicity is more closely related with the Hg/Se molar ratio in brain and blood than with Hg concentrations in blood and hair, which provide a good reflection of MeHg exposure (Ralston et al., 2007). In a study conducted by Newland et al., (2006) on rats, the authors observed that conditions that produce a molar excess of Hg over Se in the brain result in the appearance of neurological signs, while conditions that produce molar excesses of Se do not produce these signs. In general, experiments performed on animals seem to indicate that dietary Hg/Se ratios significantly lower than 1 ensure that maternal export of Se to the foetus to prevent adverse neurodevelopmental outcomes in the offspring is unimpaired (Ralston, 2008).

Another parameter proposed to make it easier to interpret the risk/benefit of consuming a seafood product that contains Se and Hg is the Se Health Benefit Value (Se-HBV) (Ralston, 2008), calculated as:

$$\text{Se} - \text{HBV} = (\text{Se/Hg molar ratio x total Se}) - (\text{Hg/Se molar ratio x total Hg})$$

The sign of the Se-HBV indicates the health benefits (if positive values are obtained) or health risks (if the values are negative) related to the exposure, and the magnitude of this parameter is proportional to the expected benefits or risks. Application of these two parameters (Hg/Se molar ratio and Se-HBV) to the samples analysed in the present study (Table 2) gives molar ratios ranging between 0.005 and 0.632 and positive Se-HBVs for most of the samples (4–3820), indicative of health benefits according to Kaneko and Ralston (2007). There was only one sample, swordfish, in which the Hg/Se molar ratio was greater than one (1.21) and the Se-HBV was negative (−4.23), and this was also the sample that exceeded the maximum Hg limit permitted in the EU. In the literature negative Se-HBVs have only been reported in mako shark sample (Kaneko and Ralston, 2007).

Table 2
Mercury and selenium concentrations in seafood products (ng/g wet weight), Hg/Se molar ratio and Se Health Benefit Value (Se-HBV). Results expressed as mean ± standard deviation (n = 3). (n.a.: not analysed.).

Category	Product name (state)	Hg	Se	Molar ratio Hg/Se	Se Health Benefit Value (Se-HBV)
Fish	Anchovy (fresh)	42.5 ± 0.9	175 ± 4	0.096	23
		23.5 ± 0.1	162 ± 1	0.057	36
	Anglerfish (fresh)	133.0 ± 0.1	199 ± 4	0.263	9
		132.9 ± 0.1	242 ± 3	0.216	14
	Blue whiting (fresh)	85.2 ± 2.3	518 ± 18	0.065	101
		85.4 ± 2.2	1356 ± 15	0.025	693
	Hake (fresh)	44.0 ± 0.3	240 ± 4	0.072	42
		152.7 ± 0.6	128 ± 15	0.470	3
	Hake (frozen)	50.7 ± 0.6	143 ± 3	0.140	13
		274 ± 8	215 ± 12	0.502	5
	Salmon (fresh)	24.5 ± 1.3	103 ± 2	0.094	114
		28.1 ± 0.1	98 ± 4	0.113	11
	Sardine (fresh)	10.2 ± 0.6	268 ± 6	0.015	227
		43.4 ± 1.2	507 ± 24	0.034	191
	Small hake (fresh)	36.5 ± 0.7	n.a.	–	–
		73.1 ± 2.7	176 ± 12	0.163	14
	Small hake (frozen)	45.1 ± 3.4	406 ± 24	0.044	118
		21.5 ± 0.3	166 ± 2	0.051	41
	Sole (fresh)	17.0 ± 0.6	178 ± 10	0.038	60
	Sole (frozen)	14.6 ± 0.02	130 ± 4	0.044	37
	Swordfish (fresh)	518.6 ± 4.7	308 ± 5	0.663	4
		1621 ± 101	528 ± 20	1.21	−4
	Swordfish (frozen)	1004 ± 8	650 ± 14	0.608	11
		633 ± 57	394 ± 7	0.632	6
Shellfish	Clam (fresh)	5.5 ± 0.2	464 ± 39	0.005	1259
		10.8 ± 0.3	396 ± 16	0.011	467
	Cuttlefish (fresh)	37 ± 1	136 ± 7	0.107	16
		25 ± 0.7	106 ± 8	0.093	14
	Cuttlefish (frozen)	4.5 ± 0.4	84 ± 4	0.021	50
		8.9 ± 0.4	157 ± 8	0.022	89
	Mussel (fresh)	9.6 ± 0.8	626 ± 2	0.006	1313
		10.5 ± 0.2	137 ± 4	0.030	58
	Norway lobster (frozen)	27.8 ± 0.9	1817 ± 10	0.006	3821
		14.0 ± 1.4	309 ± 14	0.018	219
	Prawn (frozen)	19.7 ± 0.7	400 ± 10	0.019	261
		21.4 ± 1.0	391 ± 1	0.022	230
	Shrimp (frozen)	3.8 ± 0.1	303 ± 6	0.005	777
		8.6 ± 0.4	259 ± 14	0.013	251
	Squid (frozen)	11.7 ± 0.1	186 ± 7	0.025	95
		11.5 ± 0.4	333 ± 31	0.014	310

Other studies have previously considered the Hg/Se molar ratio in marine species (Dietz et al., 2000; Plessi et al., 2001; Endo et al., 2005; Cabañero et al., 2007; Burger and Gochfeld, 2011; Dang and Wang, 2011) and in estuary species (Kehrig et al., 2009) or freshwater fish (Peterson et al., 2009). In these studies the relationship between the Hg and Se levels varies among the species analysed, and both positive and inverse relationships have been reported (Kehrig et al., 2009; Burger and Gochfeld, 2011). However, the Hg/Se molar ratio is less than 1 for most of the species. Exceptions are samples of mako (Burger and Gochfeld, 2011), polar cod and cod (Dietz et al., 2000), red meat products from small cetacean species (Endo et al., 2005) and pikeminnows (Peterson et al., 2009), with Hg/Se molar ratios greater than 1. Since the evaluation of the Hg/Se ratio may improve knowledge of the risks associated with the consumption of fish, the mechanisms that condition the interaction between these two elements in aquatic organisms should be studied in detail. Recently, Dang and Wang (2011) studied antagonistic effects between Hg and Se in a marine fish in the framework of biokinetic processes. The study shows that assimilation of Hg(II) decreases in the presence of dietary Se, whereas assimilation of MeHg is relatively constant and independent of Se.

3.2. Bioaccessible concentrations and bioaccessibility of Hg and Se

Table 3 shows the bioaccessible concentrations (ng/g ww) of Hg and Se, the bioaccessibility (solubilised percentage with the respect to the concentration in the sample), and the Hg/Se molar ratios of the bioaccessible fractions obtained after applying an *in vitro* gastrointestinal digestion.

For Hg, the bioaccessible concentrations range between 3.6 (fresh mussel) and 752 ng/g ww (swordfish). The bioaccessibility ranges between 35% (sardine) and 106% (salmon); the median bioaccessibility in crustaceans (75%) is higher than the median bioaccessibility values found in bivalves (69%), fish (68%) and cephalopods (54%). For Se, the bioaccessible concentrations vary between 89 ng/g ww (fresh anchovy) and 1314 ng/g ww (Norway lobster). The Se bioaccessibility ranges between 17% (blue whiting) and 125% (angler fish); high median bioaccessibility values were found for the various types of products analysed [cephalopods 100%, fish 84%, crustaceans 82%, bivalves 73%].

Previous data about the bioaccessibility of Hg and Se from seafood are sparse. Research on Hg has concentrated mainly on predatory fish and there are few data for other fish (Cabañero et al., 2007; Laird et al., 2009; Torres-Escribano et al., 2010, 2011; He and Wang, 2011). These studies report very variable Hg bioaccessibilities (1–100%), which might be partly attributable to the conditions used by the various authors for the *in vitro* digestion method (pH, shaking time and conditions, quantity of enzymes, etc.). However, even for the same method and fish species the bioaccessibilities can be very different (38–83%) (Torres-Escribano et al., 2010), so that other factors, such as the composition of the food matrix, must be considered. With regard to Se, research on its bioaccessi-

Table 3
Bioaccessible concentrations of mercury and selenium (ng/g wet weight; mean ± standard deviation of two replicates), bioaccessibility (solubilised percentage after gastrointestinal digestion) and Hg/Se molar ratio in bioaccessible fractions. (n.a.: not analysed).

Category	Product name (state)	Hg bioaccessible concentrations	Hg bioaccessibility	Se bioaccessible concentrations	Se bioaccessibility	Molar ratio bioaccessible Hg/Se
Fish	Anchovy (fresh)	32.7 ± 2.2	77	89 ± 7	51	0.029
		20.2 ± 1.2	86	109 ± 6	67	0.015
	Anglerfish (fresh)	75.2 ± 4.1	57	248 ± 6	125	0.024
		77.0 ± 5.6	57	158 ± 13	65	0.038
	Blue whiting (fresh)	53.4 ± 0.3	62	469 ± 2	91	0.009
		58.3 ± 4.7	68	233 ± 15	17	0.020
	Hake (fresh)	35.4 ± 3.3	81	237 ± 10	99	0.012
		101 ± 8	66	113 ± 3	88	0.071
	Hake (frozen)	46.5 ± 1.1	92	151 ± 1	105	0.024
		163 ± 2	59	175 ± 4	82	0.074
	Salmon (fresh)	25.1 ± 1.6	102	119 ± 4	116	0.017
		29.9 ± 3.5	106	91 ± 1	93	0.026
	Sardine (fresh)	5.1 ± 0.4	50	143 ± 11	53	0.003
		15.1 ± 1.0	35	197 ± 6	39	0.006
	Small hake (fresh)	32.7 ± 1.3	89	n.a.	n.a.	–
		42.5 ± 6.8	58	139 ± 2	79	0.024
	Small hake (frozen)	47.4 ± 2.6	105	349 ± 12	86	0.011
		21.0 ± 1.2	98	159 ± 4	96	0.010
	Sole (fresh)	11.5 ± 0.2	67	148 ± 9	84	0.006
	Sole (frozen)	15.3 ± 1.0	105	124 ± 4	95	0.010
	Swordfish (fresh)	340 ± 5	66	296 ± 1	96	0.091
		672 ± 18	42	369 ± 11	70	0.144
	Swordfish (frozen)	752 ± 11	75	431 ± 9	66	0.138
		347 ± 0.6	55	283 ± 10	72	0.097
Shellfish	Clam (fresh)	n.a.	n.a.	338 ± 4	73	–
		8.7 ± 0.6	82	265 ± 18	67	0.003
	Cuttlefish (fresh)	23.1 ± 0.3	63	132 ± 7	98	0.014
		15.9 ± 1.4	65	114 ± 1	107	0.011
	Cuttlefish (frozen)	n.a.	n.a.	94 ± 3	112	–
		4.8 ± 0.6	54	109 ± 2	70	0.003
	Norway lobster (frozen)	11.2 ± 0.1	40	1314 ± 9	72	0.001
		11.3 ± 2.0	81	278 ± 12	90	0.003
	Mussel (fresh)	3.6 ± 0.2	38	n.a.	n.a.	–
		7.2 ± 0.5	69	141 ± 5	103	0.004
	Prawn (frozen)	17.0 ± 0.5	86	288 ± 13	72	0.005
		16.0 ± 0.8	75	290 ± 10	74	0.004
	Shrimp (frozen)	n.a.	n.a.	274 ± 9	90	–
		7.9 ± 0.9	92	229 ± 6	89	0.003
	Squid (frozen)	6.0 ± 0.4	51	170 ± 3	92	0.003
		6.2 ± 0.6	54	340 ± 23	102	0.001

bility from seafood is more sparse, with reported values ranging between 3% and 85% (Crews et al., 1996; Cabañero et al., 2007; Metian et al., 2009).

Most of the inorganic Hg and MeHg present in seafood are bound to sulphydryl groups of proteins. As for Se, in foods of animal origin there are specific selenium proteins where selenium is incorporated via selenide as selenocysteine, while selenomethionine is non-specifically incorporated as an analogue to methionine (EC, 2000). Cabañero et al. (2007) observed that after the gastrointestinal digestion processes the proteins were not completely hydrolysed, leaving peptide fragments that might not be soluble in the bioaccessible fraction, so that the Hg and Se bound to them would not be bioaccessible. The pepsin used in the simulated gastrointestinal digestion preferentially cleaves C-terminal linkages of Phe, Leu and Glu. It does not cleave at Val, Ala or Gly. Pancreatin contains many enzymes, including amylase, lipase, ribonuclease and trypsin. The last of these specifically hydrolyses peptide bonds at the carboxyl side of Arg and Lys residues. Therefore the amino acid composition of each of the seafood samples analysed may influence its bioaccessibility and explain the variations found for the same seafood with the same *in vitro* digestion method.

From the Hg and Se bioaccessible concentrations it is possible to make an estimate of the maximum quantity of the element that is available for subsequent absorption by the epithelial cells of the intestine. For some samples, the Hg bioaccessibility of less than 50% introduces a clear change in the risk estimate in comparison with estimates made on the basis of the concentration of Hg in the seafood. Se bioaccessibility is also low in some samples, which means a decrease in the contribution of this nutrient. In the bioaccessible fraction, the Hg/Se molar ratio (Table 2) is less than one (0.001–0.144) and the Se-HBV is positive (4–4960), and therefore, according to this parameter, none of the samples should be considered a health risk. The introduction of bioaccessibility alters the evaluation of the risk of the swordfish sample, for which the Se-HBV in the raw product was negative (Table 2; Section 3.1).

The estimation of the risk in raw products might vary after research that comes closer to the consumer's reality and considers the effect of cooking and the part that might be played by nutrients contributed by other foods that may accompany fish in a meal. In this respect, recent studies show that the cooking of seafood causes significant reductions in the bioaccessibility of Hg (Torres-Escribano et al., 2011; He and Wang 2011) and Se (Cabañero et al., 2007; Metian et al., 2009), although the molar ratios and Se-HBVs in the soluble fractions of the cooked seafood were not evaluated. Moreover, some research has shown that dietary factors such as wheat, green tea extracts and food supplements such as sodium copper chlorophyllin reduce the bioavailability of Hg (Hwang and Shim, 2008; Shim et al., 2009; He and Wang, 2011). It would be interesting to continue research in this area, which would contribute to a more realistic risk estimation.

Table 4
Mercury and selenium cell retention and transport from swordfish bioaccessible fractions and methylmercury standard. Results expressed as mean ± standard deviation of a minimum of three independent replicates.

Samples	Type	Element	Exposure time	Addition [a] (ng)	Cell retention (ng)	Basal medium (ng)	Uptake (%) [b]	
							Retention	Transport
Swordfish	Fresh (sample A)	Hg	2	249 ± 7	172 ± 21	7 ± 1	69 ± 8	3 ± 0.3
	Frozen (sample B)		2	201 ± 4	128 ± 8	8.3 ± 0.7	64 ± 4	4 ± 0.3
	Frozen (sample C)		2	30 ± 2	16 ± 1	3.9 ± 0.5	54 ± 4	14 ± 1.8
	Frozen (sample C)		4	30 ± 2	15 ± 1	3.1 ± 0.04	49 ± 1	10 ± 0.2
Swordfish	Fresh (sample A)	Se	2	72 ± 1	2.3 ± 0.2	5.5 ± 0.3	3 ± 1	8 ± 1
	Frozen (sample C)		2	36 ± 2	1.7 ± 0.1	4.1 ± 0.3	5 ± 1	12 ± 1
	Frozen (sample C)		4	36 ± 2	3.6 ± 0.1	4.2 ± 0.2	10 ± 1	12 ± 1
Methylmercury standard	–	Hg	2	248 ± 1	191 ± 20	3.1 ± 0.7	77 ± 8	1 ± 0.3

[a] Mercury or selenium content in the aliquot (1.5 mL) of bioaccessible fraction added to cell cultures.
[b] Percentages of retention or transport calculated with respect to the amount added.

3.3. Hg and Se uptake (retention and transport) by Caco-2 cells

The values of Hg and Se retention and transport in Caco-2 cells obtained for standards and the bioaccessible fraction of seafood samples are shown in Table 4.

The Hg present in the swordfish bioaccessible fraction has a high cell retention (49–69%) and a much lower transport to the basal compartment (<14%). There are differences in the transport, depending on the Hg concentration added to the culture. With high exposures (249 ng) the apical-basal transport is low (3%), whereas with lower levels of exposure (30 ng) the transport increases (14%). Moreover, accumulation and transport are not affected by longer exposure time, as the results obtained for sample C at 2 and 4 h show. For Se, low percentages were observed for cell retention (3–10%) and transport through the monolayer (8–12%). The increase in exposure time doubles the cell retention of Se but does not affect its transport to the basal compartment. It is notable that the cell retention of Se (3–10%) is much lower than the retention of Hg (49–69%), whereas transport is similar for the two elements.

The study of the MeHg standard shows percentages of retention (69%) and transport (3%) similar to those found in the swordfish bioaccessible fractions. This might indicate that MeHg is the major chemical species in the swordfish bioaccessible fraction added to the cell culture. In this respect, Cabañero et al. (2007) reported that MeHg does not degrade during gastrointestinal digestion, and Torres-Escribano et al. (2010) showed that after *in vitro* digestion of swordfish samples, bioaccessible MeHg concentrations represented 94% of the bioaccessible total Hg concentrations. Moreover, the similarity of the standard and swordfish uptake and transport values seems to indicate that other dietary constituents present in the bioaccessible fraction do not have a significant influence on the bioavailability of mercury.

With regard to the Hg and Se contents transported to the basal medium for the swordfish samples, it can be seen that they are similar for the two elements (sample A: 7 ng Hg and 6 ng Se; sample C: 3.9 ng Hg and 4.2 ng Se). In the case of Hg, the high cell retention might indicate greater bioavailability if the intracellular content is finally transported to the basal compartment. Also, the high retention of Hg could indicate that the intestinal epithelium acts as a barrier for absorption of this contaminant, although the toxicological effects on the epithelium should be evaluated.

4. Conclusions

This study shows that estimation of the risk associated with intake of Hg varies depending on the parameter employed to evaluate exposure to the contaminant: concentration in the raw food, bioaccessibility or transport by Caco-2 cells. This suggests that greater interest should be given to the bioavailability of Hg from foodstuffs. An in-depth evaluation should be made of the influence on bioavailability of the type of seafood considered, the cooking treatments applied and the other nutrients ingested with it. In order to do this, a priority would be to validate *in vitro* methods which could then be applied to a large number of samples to provide reliable data that would serve as a basis for risk evaluators to elucidate the effect of bioavailability on the estimation of risk from chemical contaminants. Further research is also needed to attain a better understanding of Hg absorption and the protective role of Se. This research would benefit frequent consumers of large marine predators and also the countries that market these species, which are currently facing serious problems of national and international distribution of catches because they exceed the legally permitted Hg limits as a result of contamination of the seas.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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