Influence of Physiological Gastrointestinal Parameters on the Bioaccessibility of Mercury and Selenium from Swordfish

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ABSTRACT

Swordfish tend to accumulate mercury (Hg), but they are rich in selenium (Se), an element that can counteract toxic effects of Hg. The aim of the present study was to evaluate the concentrations and bioaccessibility of Hg and Se in cooked swordfish and to identify the digestion parameters (pH, residence time, enzyme concentrations) that may influence their bioaccessibility. The Hg concentrations ranged between 0.17 and 2.82 mg/kg wet weight (ww) and the bioaccessibility between 14 and 92%. The range for Se concentrations was narrower (0.29–1.17 mg/kg ww), with a bioaccessibility that was generally greater than that of Hg (59–103%). Most of the solubilization took place in the gastric step, where an acid pH and the increase in the pepsin concentration led to greater bioaccessibility, especially for Hg. In the intestinal stage, the most notable effect was the decrease in the bioaccessibility of Hg in the presence of bile salts.

Keywords: bioaccessibility; mercury; selenium; swordfish; molar ratio Hg/Se.

INTRODUCTION

Food is the main source of exposure to mercury (Hg) for most of the population. Large predatory fish, such as marlin, shark, swordfish and tuna, are the foods that contribute most to intake of this contaminant.^{1,2} Methylmercury (CH₃Hg), the major mercury species in seafood products, is considered a probable carcinogen for humans by the International Agency for Research on Cancer.³ To respond to the risk associated with the presence of Hg in food the agencies responsible for health and food safety use various strategies: establishing Hg concentration limits for foods, almost exclusively established for fish; proposing a tolerable weekly intake of CH₃Hg and recommending limited consumption of certain fish for population groups at risk (children, pregnant women and breastfeeding women).^{1,4} Various epidemiological studies have linked high intake of CH₃Hg through seafood products with the development of certain pathologies,⁵ emphasizing its neurotoxicity.⁶ A recent article by Grandjean and Herz⁷ discusses these studies and indicates that they present too many uncertainties for one to be able to confirm a direct cause-effect relation. For example, seafood products with high levels of CH₃Hg are also important sources of beneficial nutrients such as selenium (Se) and long-chain polyunsaturated fatty acids. *In vitro* and *in vivo* studies show that a molar excess of Se over Hg reduces the toxic effect of the contaminant.⁸

The Hg and Se present in seafood products are mainly bonded to proteins and therefore absorption of them is conditioned by their solubilization as food passes through the digestive tract. Gastrointestinal digestion consists of a series of enzymatic steps at various pH levels, which release the nutrients and contaminants from foods, thus favouring their subsequent intestinal absorption. Studies on the bioaccessibility (solubilization of a compound through the gastrointestinal digestion process) of Hg from food have concentrated mainly on seafood products.⁹⁻¹⁴ Research on raw products shows great variability in the bioaccessibility of mercury (9–106%), even for a given species of fish and the same *in vitro* gastrointestinal

digestion method.¹² Moreover, other factors that reduce the solubility of Hg have been reported and should be taken into account for a more accurate bioaccessibility evaluation. Examples are the cooking of fish^{12,13,15} and co-consumption of foods containing phytochemicals (tea extract, soy, bran) at the same time as fish.¹⁵⁻¹⁶ For Se, bioaccessibility studies have been conducted on vegetables, cereals and seafood products.^{9,13,17-19} As with Hg, the bioaccessibility of Se depends on factors such as cooking, the type of food matrix and other factors such as fat content and heavy metals such as Cd.^{17,19}

Bioaccessibility studies are conducted by means of static or dynamic *in vitro* assays that attempt to emulate this complex process. Existing methods do not try to reproduce the conditions of each of the compartments of the digestive tract faithfully, but reproduce the key processes and/or factors in gastrointestinal digestion.²⁰ Most of the research on the bioaccessibility of toxic trace elements uses static models, which are more versatile and easy to apply in the laboratory, but they simplify the gastrointestinal process. This hampers evaluation of the effect that changes in particular parameters of the process (pH, food residence time in the tract, concentrations of digestive enzymes) have on the bioaccessibility of the elements of interest. There is earlier research on how changes that take place in these factors condition the solubilization of trace elements such as As and F,²¹⁻²² but their effect on Hg and Se is not known.

The aim of this study was to evaluate the concentrations of Hg and Se in samples of cooked swordfish and their bioaccessibility, and to study the effect that the digestion steps (gastric and intestinal) and certain physiological parameters of the gastrointestinal digestion (pH, residence time and enzyme concentrations) have on the solubilization of these elements.

MATERIALS AND METHODS

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

For Se analysis, a muffle furnace (K1253, Heraeus, Spain) was used for dry ashing of samples and an 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 for quantification.

Other equipment used included 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).

Reagents. Deionized water (18.2 M Ω cm), obtained with a Milli-Q water system (Millipore, Spain), was used for preparation of standards and reagents. Standard solutions of 1000 mg/L Hg(II) [Hg(NO₃)₂, Merck, Spain] and 1000 mg/L Se(VI) [SeO₄, Merck] were employed.

Other reagents used, analytical or reagent grade, were: hydrochloric acid (Merck), nitric acid (Merck), sodium hydroxide (Merck), magnesium oxide (Prolabo, Spain), magnesium nitrate (Prolabo), ascorbic acid (Prolabo), potassium iodide (Prolabo), sodium tetrahydroborate (Panreac, Spain), hydrogen peroxide (Prolabo) and tin(II) chloride dihydrate (Scharlab, Scharlau Chemie, Spain). All material was treated with 10% (v/v) HNO₃ for 24 h, and then rinsed three times with deionized water before use.

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 x US Pharmacopoeia specifications/mg pancreatin), bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts) and lipase from porcine pancreas (Type II, 100-500 units/mg protein).

Samples. Thirty-five fillets (120 to 150 g) of frozen swordfish (*Xiphias gladius*) were purchased in supermarkets in the city of Valencia, each with a different batch number. Each fillet was defrosted and cooked in a frying pan without additional ingredients. The inedible parts were then removed and the resulting sample was ground, homogenized and stored at 4 °C until the analysis.

Quality control was performed by analysing three certified reference materials: water RTC QC1014 Trace Metals AA Sample 1 (LGC Standards, Spain), fish protein (DORM-3, National Research Council of Canada, NRCC) and lobster hepatopancreas (TORT-2, NRCC).

***In vitro* Gastrointestinal Digestion.** All cooked samples were subjected to an *in vitro* gastrointestinal digestion process. The method described by Laparra et al.²³ was used, with slight modifications. To simulate the gastric step, 10 g of cooked sample was weighed and 85 g of deionized water was added. The pH was adjusted to 2.0 with 6 mol/L HCl. A solution of pepsin (0.1 g of pepsin/mL prepared in 0.1 mol/L HCl) was added in order to obtain 0.002 g of pepsin/g of sample (equivalent to 0.02 g of pepsin/100 g of solution to be digested). The mixture was made up to a weight of 100 g with deionized water and incubated at 37 °C for 2 h with constant shaking (120 rpm/min).

The digest was then submitted to the intestinal step by increasing pH to 6.5 by means of 1 mol/L NaHCO₃ and adding a solution of pancreatin and bile extract (0.004 g/mL of

pancreatin and 0.025 g/mL of bile extract in 0.1 mol/L NaHCO₃) to obtain a final concentration of 0.00025 g of pancreatin/g of sample and 0.0015 g of bile extract/g of sample (equivalent to 0.0025 g of pancreatin/100 g of solution to be digested and 0.015 g of bile extract/100 g of solution to be digested). The mixture was incubated at 37 °C for 2 h with constant shaking (120 rpm/min).

After the digestion, the samples were transferred to tubes and centrifuged (10000 rpm, 4 °C, 30 min). The total concentrations of Hg and Se were quantified in the soluble fraction obtained (bioaccessible fraction) and the bioaccessibility was determined by means of the following equation:

$$\text{Bioaccessibility} = [A/B] \times 100$$

where A is the concentration of Hg or Se in the bioaccessible fraction and B is the concentration of Hg or Se in the cooked product. *In vitro* gastrointestinal digestion was assayed in duplicate for each cooked swordfish sample. The contribution of the enzymes and reagents to Hg and Se concentration in the bioaccessible fractions was also evaluated by the analysis of blanks of digestion.

Influence of Digestion Step on Solubilization of Hg and Se. The influence of the digestion step on the bioaccessibility (gastric or intestinal) was evaluated in 5 different samples of cooked swordfish.

The gastric digestion step was emulated in one portion of the sample following the protocol described previously. Another portion of each sample was subjected to the complete digestion process (gastric and intestinal steps). The concentrations of Hg and Se were quantified in the soluble fraction obtained from each step.

Influence of Gastric Parameters on Solubilization of Hg and Se. The effect of changes in pH, pepsin concentration and digestion time on the solubilization of Hg and Se were evaluated independently. Each parameter was tested in 6 swordfish cooked samples. Gastric digestion step was applied under the conditions described above, changing in each case the values of the parameter to be studied. A total of 4 pHs (2, 3, 4, 5 and 6), 3 digestion times (2, 4 and 6 h) and 6 concentrations of pepsin (0.001, 0.002, 0.004, 0.006, 0.008 and 0.013 g pepsin/g sample tested, equivalent to 0.01, 0.02, 0.04, 0.06, 0.08 and 0.13 g pepsin/100 g of solution to be digested) were evaluated. After the gastric phase, the intestinal step was applied in the conditions described previously. The digest obtained was centrifuged (10000 rpm, 30 min, 4 °C) and Hg and Se concentrations in the soluble fraction were analysed.

Influence of Intestinal Parameters on Solubilization of Hg and Se. The gastric step was applied to cooked swordfish samples as described previously. Subsequently the intestinal step was applied under the standard conditions varying in each case the parameter under study (bile salt concentration, pancreatic lipase concentration and intestinal digestion time).

The effect of bile salts was studied in 6 samples to which intestinal step was applied without bile salts and with various concentrations of salts (0.003, 0.1 and 0.3 g of bile extract/g of sample, equivalent to 0.03, 1 and 3 g of bile extract/100 g of solution to be digested). The effect of lipase was tested in 3 samples in which the intestinal digestion was carried out without lipase and with increasing concentrations of lipase (0.001, 0.005 and 0.01 g lipase/g of sample, equivalent to 0.01, 0.05 and 0.1 g lipase/100 g of solution to be digested). Finally, in 4 samples of cooked swordfish several intestinal digestion times were assayed (2, 4 and 6 h). The digests obtained were centrifuged (10000 rpm, 30 min, 4 °C) and Hg and Se concentrations in the soluble fraction were analysed.

Determination of Hg. Samples of cooked swordfish (0.5 g) and the bioaccessible fractions (4 mL) were placed in Teflon reactors and 4 mL of 14 mol/L HNO₃ and 1 mL of H₂O₂ (30% v/v) were added and irradiated (180 °C, 15 min) in a microwave system.¹³ The digest obtained was allowed to rest for 12 h to eliminate nitrous vapour. Then it was made up to volume with 0.6 mol/L HCl and Hg was quantified by CV-AFS. The analytical conditions employed were: reducing agent, 2% (w/v) SnCl₂ in 1.8 mol/L HCl, 4.5 mL/min; reagent blank, 0.6 mol/L HCl, 9 mL/min; carrier gas, argon, 0.3 L/min; dryer gas, air, 2.5 L/min; delay time, 15 s; analysis time, 40 s; memory wash time, 60 s.

The concentration was quantified against a Hg calibration curve for 0–2 µg/L. The analytical characteristics of the method were: quantification limit=0.001 mg/kg wet weight; precision=6%; trueness=98%. Quality control for quantification by CV-AFS was performed by analysing RTC QC1014 reference material with a certified Hg concentration of 40.8 ± 1.19 µg/L. Quality control for the complete analytical method (sample decomposition and quantification) in each batch of samples was performed by analysing DORM-3 (certified Hg 0.382 ± 0.060 mg/kg; obtained Hg: 0.368 ± 0.035 mg/kg; n=20).

Determination of Se. The cooked samples (1 g) and the bioaccessible fractions (10 mL) were treated with 1 mL of ashing aid [20% w/v MgNO₃ + 2% w/v MgO] and 5 mL of 7 mol/L HNO₃ (v/v).¹³ Subsequently, samples were evaporated to dryness in a sand bath and mineralized (425 °C, 12 h) using a muffle furnace. The mineralizing process was repeated until white ash was obtained, and this ash was dissolved in 6 mol/L HCl and then heated to 90 °C for 20 min to proceed with the reduction of Se(VI) to Se(IV).

Se was quantified by HG-AFS employing the following conditions: reducing agent, 1.2% (w/v) NaBH₄ in 0.4% (w/v) NaOH, 4.5 mL/min; reagent blank, 4.5 mol/L HCl, 9 mL/min;

carrier gas, argon, 0.3 L/min; dryer gas, air, 3.0 L/min; delay time, 10 s; analysis time, 20 s; memory wash time, 40 s.

The concentration was quantified against a Se calibration curve for 0–10 µg/L. The analytical characteristics of the method were: quantification limit=0.003 mg/kg wet weight; precision=8%; trueness=97%. Quality control for quantification by HG-AFS was performed by analysing RTC QC1014, which had a certified Se concentration of 27.5 ± 0.843 µg/L. Quality control for the complete analytical method (dry ashing and quantification) in each batch of samples was performed by analysing TORT-2 (certified Se: 5.63 ± 0.67 mg/kg; obtained Se: 5.47 ± 0.28 mg/kg; n=20).

Statistical analysis. The statistical analysis was performed by means of a one-factor analysis of variance (ANOVA) with multiple post hoc comparisons using the Tukey HSD test (SigmaPlot, version 12). Differences were considered significant for $p < 0.05$.

RESULTS

Hg and Se Concentrations in Swordfish and their Bioaccessibility. Total concentration of Hg in the cooked swordfish samples (Table 1) ranged between 0.16 and 2.82 mg/kg wet weight (ww), with a median of 0.65 mg/kg ww. The range for total Se concentrations was narrower (0.29–1.17 mg/kg ww, median 0.54 mg/kg ww; Table 1).

The solubilization of the two elements after the gastrointestinal digestion is shown in Table 1. The Hg concentrations in the bioaccessible fraction ranged between 0.07 and 1.54 µg/g ww, corresponding to very variable bioaccessibilities (14–92%), with a low median (40%). In the literature there are reports of similar Hg bioaccessibilities in cooked swordfish: 35–49%.^{12,14} In contrast, the Se concentrations in the soluble fraction ranged between 0.20 and

1.02 $\mu\text{g/g ww}$, with bioaccessibilities that were greater and less variable than those observed for Hg (59–103%; median 81%).

A parameter proposed to make it easier to interpret the risk/benefit of consuming a seafood containing Hg is the molar ratio of Hg/Se in the product.²⁴ The Hg/Se molar ratios in the cooked swordfish (Table 1) varied between 0.10 and 2.25, with a median of 0.47. Only 12% of the cooked samples had a Hg/Se molar ratio greater than one. After the gastrointestinal digestion there were changes in this molar ratio. In the soluble fraction all the samples had Hg/Se molar ratios that were less than one (0.07–0.96; median 0.24).

Influence of the Digestion Step on Bioaccessibility of Hg and Se. Figure 1 shows the concentrations of Hg (1A) and Se (1B) in the cooked samples, and the concentrations in the soluble fractions resulting from applying a gastric digestion or a complete gastrointestinal digestion.

At the end of the gastric step the Hg concentrations in the soluble fraction ranged between 0.38 and 1.29 mg/kg ww (16–100% of the initial concentration in the sample). At the end of the intestinal step (bioaccessible concentrations between 0.21 and 1.07 mg/kg ww; bioaccessibility 15–85%) there was a statistically significant decrease in the solubilized content in 4 of the 5 samples analysed (reduction of 17–58% with respect to the gastric step). For Se, the solubilized concentrations after the gastric digestion ranged between 0.46 and 0.53 mg/kg ww, corresponding to 75–100% of the Se present in the cooked swordfish. The subsequent intestinal step (bioaccessible concentrations 0.42–0.51 mg/kg ww; bioaccessibility 63–100%) significantly reduced the solubilized concentration in only two of the samples, although the reductions were less than those observed for Hg.

Influence of Gastric Parameters on Solubilization of Hg and Se. Figure 2 shows the percentage of bioaccessibility of Hg (2A) and Se (2B) after applying the gastric digestion step at various pHs. The results show that pH was a decisive factor in the solubilization. The application of a $\text{pH} \geq 3$ in the gastric step reduced the solubilization of Hg. The effect was remarkable, with the change from pH 2 to pH 3 producing a reduction of 82% in solubilized Hg. For Se, the decrease was less marked, with reductions of 33–51% in the solubilized concentration at pH 4 with respect to pH 2. At pH 5 or 6, the bioaccessibilities of Hg and Se did not vary with respect to pH 4 (data not shown).

The effect of pepsin on the bioaccessibility of both elements is shown in Figure 3. When the gastric step was applied without pepsin, the solubilized Hg percentage was very low (<5%) (Figure 3A). Increasing the concentration of this enzyme to 0.06 g/100 g of solution brought about a significant increase in the bioaccessibility of the element. For Se, however, the effect of pepsin was more moderate. Although without this enzyme the bioaccessibility was low (19–57%), once the enzyme was present in the digestion the changes in pepsin concentration did not significantly affect the bioaccessibility of most of the samples analysed.

The study on the effect of the duration of the gastric step was conducted using the customary times for emptying the stomach.²⁵ Once again this factor affected the bioaccessibility of the two elements differently. For Se, solubilization was almost complete after 2 h of digestion (81–99%) and the gastric digestion time did not alter the bioaccessibility. In contrast, the bioaccessibility of Hg in some of the samples increased with gastric residence time (Figure 4), an effect that might be conditioned by the solubilization that existed after 2 h. In fact, there were only notable increases with residence time for samples whose bioaccessibility after 2 h was less than 65% (1.3–1.9 times greater after 6 h than after 2 h).

Influence of Intestinal Parameters on Solubilization of Hg and Se. The concentration of bile salts varies during intestinal digestion.²⁵ In the present study the digestion of samples of cooked swordfish was performed by applying the intestinal step without bile salts and with three increasing concentrations (0.3, 10 and 30 g/L, equivalent to 0.03, 1 and 3 g salts/100 g solution to be digested). Figure 5 shows the bioaccessibilities of Hg (5A) and Se (5B) obtained in these conditions. The presence of bile salts significantly reduced the bioaccessibility of Hg in all the samples analysed and the decrease was concentration dependent. At the highest concentration assayed (3 g of bile extract/100 g), the reduction in bioaccessibility with respect to the bioaccessibility without bile salts ranged between 25 and 94%. For Se, the presence of bile salts and the various concentrations employed did not have a marked effect on bioaccessibility, although there were significant increases in some samples.

The study on the influence of pancreatic lipase on the bioaccessibility of Hg and Se was conducted without this enzyme and with the reported concentrations in the duodenum during digestion of foods with various concentrations of fat.²⁵ The results (Figure 6) show that at the highest pancreatic lipase concentrations there was an increase in the bioaccessibility of both elements, with the effect for Hg (increases of 12 to 94%, Figure 6A) being greater than the effect for Se (increases of 2 to 21%, Figure 6B).

Finally, the effect of the duration of the intestinal digestion step (2, 4 and 6 h) on the bioaccessibility of Hg and Se was analysed. For Hg (Figure 7), after 4 hours of intestinal digestion there was a significant decrease in bioaccessibility with respect to the values found after 2 h of digestion (reduction of 28–49%). When the digestion was prolonged to 6 h there was an increase in the bioaccessibility of Hg in most of the samples. For Se, there were no notable variations in bioaccessibility related to intestinal digestion time (data not shown).

DISCUSSION

Food safety risks associated with the presence of Hg in fish and especially in predatory fish are object of continuing interest for health authorities. The latest safety evaluations made by EFSA and FAO/WHO recommend increasing the number of methylmercury and inorganic mercury data in all food groups that contribute significantly to overall dietary exposure.¹⁻² The information available so far indicates that, for example, in Europe high consumers of fish meat may exceed the methylmercury tolerable weekly intake by up to approximately six-fold.² The concentrations of Hg in predatory fish marketed in Europe frequently exceed the legal limits (1 mg/kg ww), especially in swordfish.²⁶ Although these limits are established for the fresh product, knowledge of the concentrations in the product as consumed provides information of interest. In our study, 23% of the cooked samples analysed exceeded the value of 1 mg/kg ww. Cooking may have had the effect of concentrating the element, as was found in previous studies, which showed that after various cooking treatments Hg concentrations increased by a factor ranging between 1.1 and 1.8.^{12, 27-29}

Consumption of one portion (150 g) of the swordfish samples analysed in this study would give a mercury intake ranging between 24 and 423 µg, most of which could be assumed to be in the form of CH₃Hg. Even if this consumption were sporadic, for the cooked samples exceeding 0.7 mg/kg ww it would give an adult population intake of CH₃Hg exceeding the tolerable weekly intake (1.6 µg/kg of body weight).¹ This problem would be aggravated in the child population. These results indicate that, even though swordfish complies with the legislative regulations, it represents a food risk. However, the health benefits of consuming fish are well known,³⁰ and the debate on mercury/fish and risk/benefit is open. EFSA's recent scientific opinion on mercury says specifically that "in order to decrease the uncertainty in the point of departure derived from the epidemiological studies, more reliable definition of the dose response taking confounding factors into account is needed".² Selenium may be one of

the factors to be evaluated. Earlier studies have shown that Se is capable of protecting against the toxic effect of Hg,³¹ although the specific mechanisms of this effect, in which the chemical species of both elements must be considered, have not been completely clarified.³² Some studies indicate that the Hg/Se molar ratio in the diet is a more suitable parameter than the individual concentrations of Hg when it comes to making estimates about the risks associated with exposure.²⁴ Specifically, it has been shown that a Hg/Se molar ratio greater than one is associated with a lower toxic effect of Hg.²⁴ Studies that provide information about the Hg/Se molar ratio are scarce at present^{13,33-36} in comparison with the extensive literature about Hg concentrations.

The results obtained in the present study show that swordfish is an important source of Se. Consumption of the samples analysed would provide between 0.36 and 1.17 mg/kg of Se, which in most cases would supply the recommended daily intake for this element (54-65 µg/day).³⁷ With regard to the Hg/Se molar ratio, some of the samples whose Hg concentration exceeded the legislated limit had a Hg/Se molar ratio less than one, and therefore the introduction of this parameter would indicate a smaller risk.

On the other hand, evaluation of the risk associated with the presence of contaminants generally does not consider the steps after ingestion, which alters the quantity of contaminant that finally reaches the systemic circulation. One of these processes is gastrointestinal digestion, which released the contaminant from the food matrix and solubilizes for subsequent absorption. The present study shows that, for most of the samples, after application of an *in vitro* gastrointestinal digestion the solubilized Hg did not exceed 50% of the initial concentration in the swordfish. If the solubilized concentrations after gastrointestinal digestion (0.07–1.54 µg/g) were used to evaluate the risk, only one of the samples would exceed the recommended intake.

Furthermore, the results obtained show that the solubilization of Se was greater than that of Hg in a given seafood product. This means that in most cases the bioaccessible concentrations of Se exceeded the bioaccessible concentrations of Hg, giving Hg/Se molar ratios less than one in the bioaccessible fraction. On the basis of these results, the quantity of Se that could reach the systemic circulation might be greater than the quantity of Hg, and this might counteract the toxic effect of Hg, as indicated by earlier studies.³¹ However, in view of the complexity of the process of absorption by the gastrointestinal epithelium, further studies are needed to make an *in vivo* evaluation of the quantity of both elements that reaches the bloodstream after ingestion of seafood in order to confirm this point.

At present there is no method for estimating bioaccessibility that has been validated by interlaboratory studies. The protocols that are applied have been shown to be valid for major nutrients,³⁸ but for minor contaminants it is not known whether they generate data that can be extrapolated to *in vivo* conditions. Static bioaccessibility models, which are used most because of their simplicity, do not take into account the alterations in the parameters of the digestive process that occur *in vivo*. For example, most of them maintain pH≈2 during the gastric step, whereas *in vivo* there is an increase in pH in the stomach after consumption of food. Depending on the food and the individual, the pH may reach values close to neutral in the initial stage of digestion, subsequently decreasing to an acid pH about 2 hours after the food is ingested.³⁹⁻⁴⁰ The results obtained in our study show that the greatest solubilization of Hg and Se took place at pH 2, and that there were substantial decreases in bioaccessibility at higher pHs. The use of pH 2 throughout the *in vitro* gastric step favours solubilization and might lead to overestimates of the bioaccessibility of both elements, with implications for the risk/benefit estimation.

Various studies indicate that in fish Hg and Se are present in the protein fraction of the food,⁴¹⁻⁴³ and therefore their solubilization during digestion might require protein hydrolysis.

The optimum pH for pepsin ranges between 1.5 and 2.5,⁴⁴ and at pH 4 there is a considerable decrease in the rate of protein digestion.⁴⁵ The reduction in the solubilization of Se and Hg shown at pH>2 might be due to the low enzyme activity of gastric pepsin in these pH conditions. In this study we have seen that in optimum pH conditions the absence of pepsin produces low bioaccessibility. Thus pepsin is a decisive factor in the solubilization of these two elements. The result obtained for Hg is noteworthy, with <5% solubilization in the absence of this protease.

Most of the static methods use the fasting concentration of pepsin in the stomach (0.11–0.22 mg/mL).^{23,40} This does not emulate the *in vivo* digestion process, in which there is an increase in pepsin in the presence of food (fasted: 0.11 –0.22 mg/mL; fed: 0.26–0.58 mg/mL).⁴⁶ Our work shows that the increase in the pepsin concentration during the gastric step brings about an increase in bioaccessibility of Hg and, to a lesser extent, Se. Therefore the use of static methods in fasting conditions may lead to an underestimate of solubilization.

It was the gastric step that determined the solubilization of Hg and Se from the swordfish. The subsequent intestinal step did not increase the solubilization of Se, but it did produce a significant decrease in solubilization of Hg in some samples. It was considered that it would be of interest to evaluate whether changes in some factors of the intestinal step alter this solubilization. One of the particularly important factors in the digestion of foods with a high lipid content is bile salts. The small intestine is the part of the gastrointestinal tract where the majority of lipolysis and proteolysis takes place, and bile salts delivered with the bile into the duodenum can play a significant role in the colloidal aspects of both types of the digestive processes.⁴⁷ The concentration of bile salts varies during intestinal digestion. In fasting conditions concentrations of 5 g/L of chyme have been reported, and these values increase to 30 g/L for a standard meal and 60 g/L for a fatty meal.²⁵ Swordfish is a semi-fatty seafood (fat content 4–7 g/100 g) and therefore one might expect that the presence of bile salts would

increase the bioaccessibility of its components. This happened for Se, which underwent an increase of as much as 152% in the presence of bile salt concentrations similar to those present in the intestine during the digestion of food with a high fat content.²⁵ However, there was an opposite effect for Hg, because the increase in the concentration of bile salts reduced the solubilized Hg by as much as 94%.

CH₃Hg, the major species in seafood products,² has an affinity for taurine,⁴⁸ an amino acid that forms part of taurine conjugated bile acids such as chenodeoxycholytaurine, deoxycholytaurine and cholytaurine. Perhaps a complex may be formed between CH₃Hg and these bile acids. It is known that the solubility of bile acids depends on the pH and the presence of ions such as calcium.⁴⁹ If these acids precipitated, they would insolubilize Hg, explaining the decrease in bioaccessibility of Hg in the presence of increasing concentrations of bile salts.

The presence of pancreatic lipase also favours the solubilization of Hg and Se. This enzyme is responsible for most of the lipolysis that takes place in the intestinal tract.⁵⁰ Its concentration also increases during digestion of fatty products.²⁵ In many *in vitro* studies this enzyme is not added to the model; instead, pancreatin is used, which has a much lower lipase activity than the activity present in the digestive tract in fasting conditions.²⁵ In our study, the addition of lipase led to a greater release of Hg and Se from the matrix, which might be partly due to better digestion of the fatty component of swordfish. In order to function properly, this type of lipase needs a protein, colipase, which is believed to be involved in favouring the contact of soluble lipase with the water-oil interface.⁵⁰ Colipase was not used in our study, so it is possible that lipase activity may be more decisive than is shown here.

The last factor to be evaluated was the gastric and intestinal transit time, which can vary between 1 and 6 h in normal conditions.²⁵ Our study indicates that the effect of residence time was only significant for Hg, although it did not have the same magnitude for all the samples.

In general, solubilization of the element increased in the gastric step and decreased in the intestinal stage.

In view of the results obtained, it might be concluded that the estimation of the risk associated with ingestion of Hg from swordfish might be altered substantially if account were taken of the contribution of Se in this fish and the bioaccessibility of the two elements. Furthermore, the bioaccessibility of Hg and Se is affected by the conditions used in the gastrointestinal model. Both elements respond differently to these variations, and their solubilization is not affected by the same factors. Consequently the effect of the gastrointestinal physiological parameters on the bioaccessibility of Hg should be taken into account.

FUNDING SOURCES

This work was supported by the Spanish Ministry of Economy and Competitiveness (AGL2012-33461), for which the authors are deeply indebted. Carlos Jadán Piedra received a Personnel Training Grant from SENESCYT (Ecuadorian Ministry of Higher Education, Science, Technology and Innovation) to carry out this study.

ACKNOWLEDGMENT.

The authors are grateful to Beatriz Estévez Cuesta for help provided in performing the experimental part of this study.

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Table 1. Hg and Se (mg/kg wet weight, ww) and Hg/Se Molar Ratio in Samples of Cooked Swordfish (Mean $\pm$ Standard Deviation), Hg and Se Concentrations in the Bioaccessible Fraction (mg/kg ww), Bioaccessibility (Percentage; Value in Parentheses) and Hg/Se Molar Ratio in the Bioaccessible Fraction of Samples of Cooked Swordfish (Minimum and Maximum Values, Mean Value and Standard Deviation, Median Value; n=35).

	Cooked swordfish			Bioaccessible fraction of cooked swordfish				
Sample (n=35)	Hg (mg/kg, ww)	Se (mg/kg, ww)	Molar ratio Hg/Se	Hg (mg/kg, ww)	Hg Bioaccessibility (%)	Se (mg/kg, ww)	Se Bioaccessibility (%)	Molar ratio Hg/Se
min	0.17	0.29	0.1	0.07	14	0.2	59	0.07
max	2.82	1.17	2.25	1.54	92	1.02	103	0.96
mean	0.80 $\pm$ 0.52	0.58 $\pm$ 0.17	0.57 $\pm$ 0.40	0.38 $\pm$ 0.34	45 $\pm$ 24	0.48 $\pm$ 0.18	81 $\pm$ 19	0.30 $\pm$ 0.23
median	0.65	0.55	0.47	0.28	40	0.46	81	0.24

Figure 1. Concentrations of Hg (1A) and Se (1B) in five samples of cooked swordfish (striped bars) and in the solubilized fraction obtained after application of gastric digestion (white bars) and gastrointestinal digestion (grey bars). Values expressed in mg/Kg wet weight (mean $\pm$ standard deviation). For each sample, different letters above the bars indicate significant differences between the solubilized concentrations in the gastric and gastrointestinal digestions ($p < 0.05$).

Figure 2. Variations in bioaccessibility (%) of Hg (2A) and Se (2B) in relation to pH applied during the gastric step of digestion of six samples of cooked swordfish [pH 2 (striped bars); pH 3 (white bars); pH 4 (grey bars)] (mean $\pm$ standard deviation). For each sample, different letters above the bars indicate significant differences between the bioaccessibilities at each pH ($p < 0.05$).

Figure 3. Effect of pepsin concentration used in the gastric step (g pepsin/100 g solution to be digested) on bioaccessibility (%) of Hg (3A) and Se (3B) in six samples of cooked swordfish (mean $\pm$ standard deviation).

Figure 4. Effect of time of application of the gastric digestion step on bioaccessibility (%) of Hg in six samples of cooked swordfish [2 h (striped bars); 4 h (white bars); 6 h (grey bars)]. Values expressed as mean $\pm$ standard deviation. For each sample, different letters above the bars indicate significant differences between the bioaccessibilities at each digestion time ($p < 0.05$).

Figure 5. Effect of bile salt concentration used in the intestinal step on bioaccessibility (%) of Hg (5A) and Se (5B) in six samples of cooked swordfish [without salts (striped bars); 0.03 g salts/100 g solution to be digested (white bars); 1 g salts/100 g solution to be digested (dotted bars); 3 g salts/100 g solution to be digested (grey bars)]. Values expressed as mean $\pm$ standard deviation. For each sample, different letters above the bars indicate significant differences between the bioaccessibilities at each bile salt concentration ($p < 0.05$).

Figure 6. Effect of pancreatic lipase concentration used in the intestinal step on bioaccessibility (%) of Hg (6A) and Se (6B) in three samples of cooked swordfish [without lipase (striped bars); 0.01 g lipase/100 g solution to be digested (white bars); 0.05 g lipase/100 g solution to be digested (dotted bars); 0.1 g lipase/100 g solution to be digested (grey bars)]. Values expressed as mean $\pm$ standard deviation. For each sample, different letters above the bars indicate significant differences between the bioaccessibilities at each lipase concentration ($p < 0.05$).

Figure 7. Effect of intestinal digestion time on bioaccessibility (%) of Hg in four samples of cooked swordfish [2 h (striped bars), 4 h (white bars), 6 h (grey bars)]. Values expressed as mean $\pm$ standard deviation. For each sample, different letters above the bars indicate above the bars significant differences between the bioaccessibilities at each time ($p < 0.05$).