

***In vitro* evaluation of dietary compounds to reduce mercury bioavailability**

Carlos Jadán-Piedra, Dinoraz Vélez, Vicenta Devesa*

Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Av. Agustín Escardino 7, 46980 Paterna (Valencia), Spain.

* To whom correspondence should be addressed (telephone (+34) 963 900 022; fax (+34) 963 636 301; e-mail: vdevesa@iata.csic.es)

ABSTRACT

Mercury in foods, in inorganic form [Hg(II)] or as methylmercury (CH₃Hg), can have adverse effects. Its elimination from foods is not technologically viable. To reduce human exposure, possible alternatives might be based on reducing its intestinal absorption. This study evaluates the ability of 23 dietary components to reduce the amount of mercury that is absorbed and reaches the bloodstream (bioavailability). We determined their effect on uptake of mercury in Caco-2 cells, a model of intestinal epithelium, exposed to Hg(II) and CH₃Hg standards and to swordfish bioaccessible fractions. Cysteine, homocysteine, glutathione, quercetin, albumin and tannic reduce bioavailability of both mercury species. Fe(II), lipoic acid, pectin, epigallocatechin and thiamine are also effective for Hg(II). Some of these strategies also reduce Hg bioavailability in swordfish (glutathione, cysteine, homocysteine). Moreover, extracts and supplements rich in these compounds are also effective. This knowledge may help to define dietary strategies to reduce *in vivo* mercury bioavailability.

Keywords: mercury; food; swordfish; bioavailability; dietary strategies

1. INTRODUCTION

Diet is the main source of exposure to mercury (Hg) for the general population. This contaminant is present in foods as methylmercury (CH₃Hg) or inorganic mercury [Hg(II)]. It has been reported that tuna, swordfish, cod, whiting, and pike were the major contributors to CH₃Hg dietary exposure in the adult European population (EFSA, 2012). Some studies link high prenatal exposure to CH₃Hg, as a result of the mother consuming seafood products with elevated levels of this species of mercury, with deficits in neurobehavioral development (Grandjean et al., 2002). Therefore, some public health agencies recommend avoiding or reducing consumption of certain kinds of seafood in susceptible population groups (children, pregnant and breastfeeding women) (Health Canada, 2008). This recommendation should not be associated with a decrease in consumption of seafood for the general population, given the high nutritional value of these food products (EFSA, 2012).

The existing concern has led to investigation of alternatives to reduce Hg dietary exposure. Methods have been developed to remove Hg from seafood products before they are marketed by the addition of leaching and chelating agents (Schab et al., 1978; Aizpurúa et al., 1997; Hajeb and Jinap, 2012). The conditions applied (long times, acid pHs, sliced or minced fish) have not made their industrial scale-up advisable. As an alternative, there has been research on the possibility of reducing the amount of Hg ingested that reaches the systemic circulation. These approaches have concentrated on searching for strategies to reduce the Hg that is in soluble form after digestion and therefore available for absorption (bioaccessibility). Using a simulated human digestion approach, Jadán-Piedra et al. (2016a) showed that addition of tannic acid, cellulose, lignin, or pectin during digestion of swordfish and tuna significantly reduces Hg bioaccessibility (30–98% reduction). Later studies by the same research group showed that some strains of lactic acid bacteria and of *Saccharomyces cerevisiae* are also effective in reducing bioaccessibility from foods (Jadán-Piedra et al., 2017a,b).

Another way of pursuing this objective is by acting directly on intestinal absorption. This process is dependent on the physicochemical state of the substance, the lumen environment, the metabolic

activity and functions of the intestinal cell, and the structure of the absorbing surface (Levine, 1970). The chemical form of Hg is a determining factor in its absorption. The absorption of Hg(II) present in food has been reported to be less than 15% in experimental animals (Piotrowski et al., 1992), and in human volunteers who ingested an oral tracer dose of Hg(II) nitrate given either in aqueous solution or bound to protein (Rahola et al., 1973). In contrast, about 95% of the CH₃Hg in fish ingested by humans, and about 95% of methylmercuric nitrate given orally to volunteers is absorbed (NRC, 2000). Another decisive factor is the lumen environment; interactions of the element with components of the diet or with intestinal microbiota present in the lumen can play a crucial role in its absorption. In fact, it has been shown that the activity of intestinal bacteria facilitates fecal excretion of CH₃Hg owing to its demethylation to Hg(II) (Rowland et al., 1984).

The aim of this study is to seek dietary components capable of reducing the amount of Hg that is absorbed by the intestinal epithelium (bioavailability) using human colon Caco-2 cells as a model of the intestinal epithelium.

2. MATERIALS AND METHODS

2.1. Bioaccessibility of Food Products. Fillets of swordfish (*Xiphias gladius*) cooked in a frying pan without additional ingredients were submitted to *in vitro* gastrointestinal digestion, following the protocol described by Jadán-Piedra et al. (2016b). Five grams of sample were weighed and deionized water was added to make a final volume of 50 mL. The pH was adjusted to 2.0 with 6 M HCl, and a solution of pepsin (0.1 g of pepsin/mL prepared in 0.1 M HCl) was added to obtain 0.01 g of pepsin/50 mL of digestion solution. The mixture was incubated at 37 °C for 2 h with constant shaking (120 rpm).

The digest was then submitted to the intestinal digestion step. The pH was increased to 6.5 by means of NH₃ 25% (Panreac), and a solution of pancreatin and bile extract was added (0.004 g/mL of pancreatin and 0.025 g/mL of bile extract in 0.1 M NH₄HCO₃) to obtain a final concentration of

1.25 mg of pancreatin/50 mL of digestion solution and 7.5 mg of bile extract/50 mL of digestion solution. The mixture was incubated at 37 °C for 2 h with constant shaking (120 rpm). After the digestion, the samples were transferred to tubes and centrifuged (10000 rpm, 4 °C, 30 min), and the soluble fraction was collected to be used in the study.

The enzymes and bile salts used were purchased from Sigma (Spain): porcine pepsin (enzyme activity 944 U/mg protein), porcine pancreatin (activity equivalent to 4 × US Pharmacopeia specifications/mg pancreatin), and bile extract (glycine, taurine conjugates, and other bile salts).

2.2. Caco-2 cell culture. Caco-2 cells were acquired from the European Collection of Cell Cultures (ECACC; number 86010202, UK). Cell maintenance was performed in 75 cm² flasks, using Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/L) and glutamine at pH 7.2. The DMEM was supplemented with 10% (v/v) of fetal bovine serum, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.0025 mg/L of amphotericin B (DMEMc). All reagents used were acquired from Hyclone (Fisher, Spain).

The cells were incubated at 37 °C in an atmosphere with 95% relative humidity and 5% CO₂ flow. The medium was changed every 2–3 days. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 mg/mL) and EDTA (ethylenediaminetetraacetic acid, 0.2 mg/mL) (Hyclone), and reseeded at a density of 1 × 10⁴ cells/cm². The cells were used between passages 10 and 25.

Transport assays were performed in 6-well plates with inserts with a polyester membrane (diameter 24 mm, pore size 0.4 µm; Transwell®, Corning, Cultek, Spain). In this system the Caco-2 cells are seeded on the porous support of the insert which separates the well into two compartments: apical (upper) and basolateral (lower). The cells were seeded at a density of 7.5 × 10⁴ cells/cm² and they were supplemented with 1.5 mL of DMEMc in the apical chamber and 2 mL of DMEMc in the

basolateral chamber. The cells were incubated at 37 °C, 5% CO₂ and 95% relative humidity, and the medium was changed every 2–3 days until cell differentiation took place (9 days post seeding).

2.3. Determination of Apparent Permeability Coefficients (P_{app}) and Cellular Retention. The transport assays were performed using standard solutions of Hg(II) and CH₃Hg or the bioaccessible fraction of swordfish. The standard solutions were prepared from Hg(NO₃)₂ (1000 mg/L, Merck, Spain) and CH₃HgCl (1000 mg/L, Alfa Aesar, Spain) in Hanks' balanced salt solution medium with NaCO₃ (HBSS) (Hyclone) supplemented with 10 mM of HEPES (pH 7.2). The bioaccessible fractions were inactivated for the transport assay by heating for 5 min at 90 °C to inhibit sample proteases. Glucose (final concentration 1 g/L, Sigma) was added to facilitate cell viability. NaCl (5 mM, Panreac) was used to adjust the osmolarity to 300 ± 30 mOsm/kg, using a freezing point osmometer (Automatic Micro-Osmometer Type 15 Löser, Löser Messtechnik, Germany).

Bioaccessible fractions or Hg standards (1.5 mL) were added to the apical compartment, with or without the dietary components to be assayed (Table S1, supplementary data), and HBSS-HEPES (2 mL) was added to the basolateral side. At the established times (45, 60, 90, and 120 minutes), aliquots were removed from the basolateral compartment and were replaced by the same volume of HBSS-HEPES. Total Hg was determined in aliquots of the basolateral medium removed at each time and in the apical medium and cell recovered at the end of the experiment.

The apparent permeability coefficients (P_{app}) were calculated by using equation 1.

$$P_{app} = (dC/dt) (V_r/AC_o) \text{ (Eq.1)}$$

where:

dC/dt is the flow (µg/s) determined by the linear slope of the equation that governs the variation in the concentrations of Hg, corrected with dilution, against time.

V_r is the volume of the acceptor compartment (2 mL).

A is the surface occupied by the cell monolayer (4.71 cm²).

C_o is the initial concentration of Hg in the donor compartment (0.5 mg/L for standards).

2.4. Cell Monolayer Integrity. During the transport assays, cell monolayer integrity was evaluated by measuring (a) transepithelial electrical resistance (TEER) at various points in the study, including the start and end of the experiment, (b) the P_{app} of the paracellular transport marker Lucifer Yellow (LY, Sigma), (c) the cellular viability at the end of the assay.

To determine the LY P_{app} , the compound was added at a concentration of 100 μ M to the apical compartment in all the treatments. The fluorescence of the LY transported to the basolateral side was measured with a fluorescence microplate reader (PolarSTAR OPTIMA, BMG-Labtech, Germany) at excitation/emission wavelengths of 485/520 nm. Cell viability was determined by staining with 0.4% (v/v) trypan blue (Sigma).

The assays were considered valid if: a) TEER values were not modified by more than 25% with respect to the initial values; b) the LY P_{app} was below 2×10^{-7} cm/s; c) cellular viability \geq 80%.

2.5. Determination of Hg. Bioaccessible fractions, media, and cell lysates were placed in a Teflon perfluoroalkoxy (PFA) vessel and treated with 1 mL of HNO₃ (14 M, Merck) and 1 mL of H₂O₂ (30% v/v, Panreac). The Teflon PFA vessel was irradiated at 800 W (15 min at 180 °C) in a microwave accelerated reaction system (MARS) from CEM (Vertex, Spain). At the end of the digestion program, the digest was placed in a tube and allowed to rest all night to eliminate nitrous vapor. It was then made up to volume (9-15 mL) with 0.6 M HCl.

For mercury quantification, a cold vapor generation-atomic fluorescence spectrometer (CV-AFS) (PSA 10.025 Millennium Merlin, PS Analytical, UK) was employed. The analytical conditions were the following: reducing agent, 2% (w/v) SnCl₂ (Scharlau, Scharlab, Spain) in 1.8 M HCl, 4.5 mL/min flow rate; carrier solution, 0.6 M HCl, 9 mL/min flow rate; carrier gas, argon 0.3 L/min flow rate; dryer gas, air 2.5 L/min flow rate; specific Hg lamp; fixed 254 nm filter. Quantification

was carried out against an external calibration curve (0.1–2 ng/mL). The analytical performance of the equipment used for Hg quantification was evaluated by analysis of a water certified reference standard (RTC, QCI-049-1) supplied by LGC Standards (Spain) [Hg certified value = (40.8 ± 1.19) $\mu\text{g/L}$]. The results obtained in the reference material were in good agreement with the certified values: (40.2 ± 1.0) $\mu\text{g/L}$.

In all the assays conducted a mass balance was performed, comparing the Hg added to the cells initially and the Hg quantified at the end of the transport assays in the apical and basolateral media and in the cells. The results show recoveries ranging between 85 and 99%.

2.6. Statistical Analysis. All tests were performed at least in triplicate in independent cultures. The results were subjected to statistical analysis by one-factor analysis of variance (ANOVA) with the Tukey HSD *post hoc* multiple comparison test or using the Student t-test (SigmaPlot version 13.0). Differences were considered significant for $p < 0.05$.

3. RESULTS

All the results presented in this section correspond to treatments in which the TEER, LY permeability, and cell viability remained within the ranges considered optimal (section 2.4).

3.1. Effect of Dietary Components on Apparent Permeability of Hg in Aqueous Solution

3.1.1. Effect on apparent permeability of aqueous solutions of Hg(II). The compounds used to attempt to reduce cellular transport of Hg(II) were selected on the basis of three criteria: a) the possibility that they would compete for transporters of divalent cations [salts of Zn and Fe(II)]; b) the possibility that they would compete for transporters of amino acids, because it has been reported that Hg(II) bound to Cys (Cys-S-Hg-S-Cys), one of the major forms of Hg in biological samples, has a structure similar to that of cystine and therefore it can be transported by transporters of that

amino acid (Bridges et al., 2004) (methionine, cysteine, phenylalanine, homocysteine, arginine, lysine); c) the possible formation of complexes with Hg(II), which could lead to modification of its transport (glutathione, albumin, xylan, lipoic acid, DHLA, pectin, cysteine, homocysteine, quercetin, tannic acid).

Figure 1 shows the percentages of the Hg(II) P_{app} in cells co-exposed to Hg(II) and the various dietary components with respect to the permeability value obtained in cells treated only with Hg(II), which was considered the control treatment. For 70% of the components the P_{app} was lower than that of the cells treated only with Hg(II), showing that they reduce transport of Hg(II) through the monolayer of intestinal cells. The greatest reductions were produced in the presence of compounds with thiol groups, such as GSH ($97 \pm 3\%$), homocysteine ($94 \pm 2\%$), cysteine ($93 \pm 1\%$), DHLA ($89 \pm 2\%$), or lipoic acid ($83 \pm 2\%$). There were also considerable reductions with Fe(II) ($65 \pm 4\%$) and with more complex compounds such as thiamine ($83 \pm 9\%$), pectin ($78 \pm 2\%$), quercetin ($73 \pm 1\%$), epigallocatechin ($64 \pm 8\%$), and tannic acid ($57 \pm 16\%$).

Many of the reductions in transport were accompanied by decreases in the intracellular content of Hg(II) (figure 2), with particularly notable reductions produced by thiolated compounds [GSH ($98 \pm 1\%$), cysteine ($97 \pm 0.2\%$), and homocysteine ($94 \pm 1\%$)].

3.1.2. Effect on the apparent permeability of aqueous solutions of CH₃Hg. The criteria for the selection of the compounds used to reduce the permeability of the organic form were the same as those mentioned earlier for Hg(II), with the exception of those connected with competition for transporters of divalent cations.

Figure 3 shows the percentages of the CH₃Hg P_{app} in cells treated with CH₃Hg and the dietary components with respect to cells treated only with this form of mercury. The number of compounds capable of modifying this parameter in the cells treated with CH₃Hg was much lower than that of the compounds capable of altering this parameter in cells treated with Hg(II). Of the 21 components

analyzed, only co-exposures with Cys (20 mg/L), GSH (1 mM), homocysteine (5 mg/L), albumin (20 mg/L), tannic acid (20 mg/L), and quercetin (5 mg/L) produced significant reductions in permeability with respect to the cells treated only with CH₃Hg. The reductions in the percentages of P_{app} were: GSH (94 ± 10)%, Cys (79 ± 4)%, tannic acid (45 ± 6)%, homocysteine (29 ± 2)%, quercetin (24 ± 9)%, and albumin (21 ± 8)%.

Reductions in cellular accumulation (figure 4) were produced in those treatments in which permeability was also reduced, as in the case of Hg(II). The largest decreases were obtained with GSH (98 ± 0.1)%, Cys (91 ± 2)%, tannic acid (30 ± 6)%, albumin (28 ± 8)%, and homocysteine (27 ± 5)%.

3.2. Influence of Dietary Supplements and Plant Extracts on Hg(II) and CH₃Hg Apparent Permeability. Assays were performed with dietary supplements and plant extracts that contained one of the components that had been successful in reducing the P_{app} of the forms of mercury (section 3.1). Accordingly, we evaluated the reduction in absorption of standards of Hg(II) and CH₃Hg in the presence of extracts rich in tannic acid and/or epigallocatechin (green tea and grape seeds), and supplements of Cys, GSH, thiamine, and lipoic acid.

Supplements of lipoic acid and thiamine did not produce statistically significant reductions in Hg(II) and CH₃Hg permeability (data not shown). The other components were effective in reducing absorption of Hg(II) (figure 5) [green tea extract (median: 72%), grape seed extract (median: 66%), and Cys/GSH supplement (median: 32%)]. Reductions in CH₃Hg permeability were only achieved in the presence of Cys/GSH supplement (median: 57%) and grape extract (median: 32%) (Figure 5).

Of all the treatments performed with supplements and extracts, only the Cys/GSH supplement significantly reduced the intracellular contents of Hg(II) (43–65%, median: 60%) and CH₃Hg (87–87%, median: 86%).

3.3. Influence of Food Components and Supplements on the Apparent Permeability of Hg present in Foods. The data obtained in the assays with Hg standards were used to select the components and supplements or extracts to be assayed with the bioaccessible fraction of swordfish. According to the data in the literature (Cabañero et al., 2007), CH₃Hg is the major species in swordfish, which is why we used supplements that affect transport of this organic form: GSH, Cys, homocysteine, tannic acid, quercetin, GSH/Cys supplement, and grape seed extract.

Table 1 shows the effect of these components or supplements on the permeability of the Hg present in the bioaccessible fraction of swordfish. As occurred in the standard solutions, GSH was the most effective component in reducing P_{app}, followed by cysteine and homocysteine. However, tannic acid and quercetin had no effect. The substantial reduction in transport of Hg that was produced in the presence of the two dietary supplements assayed (range: 29–80%) is particularly noteworthy.

4. DISCUSSION

The mechanisms involved in intestinal transport of Hg(II) and CH₃Hg are different. The movement of HgCl₂ is mainly carrier-mediated. Vázquez et al. (2015a) showed that DMT1, a transporter of divalent cations and the main mechanism of intestinal absorption of non-heme Fe, is involved in its transport. As for CH₃HgCl, because of its lipophilic nature it has been postulated that part of the absorption may take place through passive diffusion across the cell (Vázquez et al., 2014). Furthermore, it has been shown that mercury species have a high affinity for thiol groups, and it has been indicated that these species may be the major forms of Hg in foods, tissues and body fluids (Ballatori and Clarkson, 1985; Harris et al., 2003). Some studies point out that these complexes are transported by transporters of amino acids or transporters of organic anions because they have a structural similarity to the substrates of those transporters (Ballatori, 2002).

The fact that transport of Hg is mediated by transporters of nutrients suggests the possibility that its absorption across the intestinal epithelium could be reduced by processes of competition with other components of the diet. Moreover, the use of dietary compounds that form complexes with Hg may also affect transport by modifying the chemical form in which the metal is present in the lumen. In this study, bearing both possibilities in mind, we conducted assays in cellular models of the intestinal epithelium, using divalent cations or amino acids as possible competitors with the transport of these two species of mercury, and also other dietary compounds that are capable of forming complexes with Hg or with cations with similar physicochemical characteristics. The results obtained show that inhibition of CH₃Hg transport is achieved mainly with compounds that have thiol groups in their structure (cysteine, homocysteine, GSH, and albumin), with reductions of as much as 89% for GSH, and 55% and 25% in the presence of tannic acid and quercetin, respectively. The spectrum of compounds that reduce absorption of Hg(II) is much greater; in addition to compounds with thiol groups, the substantial effect of Fe salts, tannic acid, quercetin, and thiamine is noteworthy. GSH is also the most effective compound (97% reduction).

The reductions in the permeability of the forms of mercury that were demonstrated with tannic acid and quercetin are probably due to the formation of complexes with low solubility. Quercetin is a food flavonoid that is abundant in onions, tea, tomatoes, and apples (Aherne and O'Brien, 2002), has low solubility in water, and therefore has low intestinal absorption (Cai et al., 2013). This flavonoid forms complexes with a large number of divalent cations through the 5-OH and 4-carbonyl groups, whose stability depends on the metal ion assayed (Liu and Guo, 2015). Although, it has been reported that interaction with metals increases the solubility of this flavonoid, the solubility is still very low. Using concentrations of quercetin similar to those ingested by the population, in this study there was a substantial reduction in transport and cellular retention of Hg in intestinal cells. Its effect on Hg has not been studied previously, but possibly the formation of complexes with low solubility may be the main mechanism by which quercetin reduces transport of Hg across the cell monolayer.

On the other hand, Lesjak et al. (2014) showed that quercetin reduces intestinal absorption of Fe(II) by chelation, but that it also acts by reducing expression of ferroportin, a transporter of non-heme Fe in the basolateral domain of enterocytes. Therefore, in addition to its binding ability, there is also a molecular mechanism that affects the Fe(II) transporter. It cannot be ruled out that a similar effect may have taken place in the present study, given that Hg(II) uses non-heme Fe transporters in its transport in intestinal cells (Vázquez et al., 2015a). With regard to the effect of tannic acid, a compound that occurs naturally in several beverages and many vegetables (Gülçin et al., 2010), it has a high number of carboxylic groups, potential binding sites for cations. In fact, it has been shown that it binds to Hg(II) and CH₃Hg in aqueous solution (Torres et al., 1999); it has even been demonstrated that the solubility of the forms of mercury is reduced in the presence of tannic acid at concentrations similar to those used in this study (Jadán-Piedra et al., 2016a).

Another mechanism for reducing Hg permeability may be connected with the formation of soluble complexes whose transport is less than that of the saline forms of the mercury species. This may be the case with compounds that have thiol groups, such as cysteine, homocysteine, albumin, and GSH. Several studies suggest that the forms of Hg conjugated with thiol groups are the forms mainly transported through various tissues (Zalups, 2000; Ballatori, 2002), possibly because they are considered to be the most abundant forms in the systemic circulation. However, the data on the magnitude of the transport of Cys/Hg complexes are contradictory. Vázquez et al. (2014, 2015b) showed that in the presence of derivatives of cysteine the transport of HgCl and CH₃HgCl is significantly reduced. Endo et al. (1991) demonstrated a substantial reduction of intestinal absorption of Hg in the presence of Cys in *in situ* assays. However, in oral exposure of rats to CH₃HgCl or CH₃Hg-Cys, Mori et al. (2012) did not observe differences in plasma concentrations of Hg. Similarly, in another *in vivo* study, Roos et al. (2010) observed greater accumulation of the metal in liver and brain of mice treated with CH₃Hg-Cys and greater renal accumulation in the groups treated with CH₃HgCl. For CH₃Hg, the formation of complexes with Cys involves a change

in the degree of lipophilicity and possibly a change in the transport pathway, from passive diffusion (Vázquez et al., 2014) to carrier-mediated transport (Ballatori, 2002), which might lead to less transport because of the existence of a transport mechanism subject to possible competition and saturation.

The reduction in Hg(II) permeability produced by ferric salts is possibly a result of competition for the same transport mechanisms. The main transporter of non-heme Fe present in the apical domain of human enterocytes is DMT1. This transporter has affinity for a series of metal cations, including Hg(II) (Vázquez et al., 2015a). Therefore the presence of Fe(II) salts in concentrations much higher than those of Hg(II) may lead to a reduction in transport of the toxic metal as a result of competition. This effect on Hg(II) permeability has also been shown in the presence of Mn(II) (Vázquez et al., 2015b), another substrate that has high affinity for DMT1 (Garrick et al., 2006). However, the effect does not appear when Zn(II) is added to the medium. It must be emphasized that Zn may also be a substrate of DMT1, although this transporter has less affinity for Zn(II) than for Fe(II) or Mn(II) (Garrick et al., 2006; Espinoza et al., 2012); in fact, Zn is transported in the intestine mainly by zrt- and irt-like proteins (ZIP) (Ford, 2004). This may be the cause of the lack of effect of Zn sulphate on intestinal transport of Hg(II) observed in the present study.

Irrespective of the mechanism involved in modifying the permeability of the forms of mercury, in the present study we have found compounds that are capable of reducing the bioavailability of Hg and that also have the advantage of forming part of foods, so their use as strategies to reduce exposure to this metal should not pose any risk. It should be noted that the supplements rich in some of these compounds that were assayed in this study (Cys/GSH supplement and grape seed extract) and that have high effectiveness (32–72%) are authorized for sale, which makes their use as a dietary strategy more viable. Moreover, it was seen that they are effective not only in aqueous solutions but also in the presence of foods, matrices with greater complexity, in which the nutrients present might cause the metal/dietary strategy interactions to vary.

Finally, it must be emphasized that most of the compounds that are effective in reducing Hg permeability in intestinal cells are compounds with a high antioxidant capacity, which may counteract the stress generated by Hg. *In vivo* studies have shown that administration of quercetin (5–50 mg/kg body weight/day) reduces the stress generated by CH₃Hg (30 µg/kg body weight/day, for 45 days), with recovery of levels of GSH and glutathione peroxidase activity (GPx) in exposed rats (Barcelos et al., 2011). The antioxidant effect of tannic acid has been shown in *in vivo* studies in which the animals were exposed to Cd and Pb (Mishra et al., 2015; Tüzmen et al., 2015), metals whose toxicity mechanisms are similar to those described for the forms of mercury.

The data obtained in the present study show that dietary consumption of compounds with thiol groups, flavonoids such as quercetin, polyphenols such as tannic acid, or plant extracts or supplements rich in them may be a suitable strategy to reduce oral bioavailability of Hg. It is necessary to evaluate whether this effect observed *in vitro* is confirmed *in vivo*, when the compounds present in the lumen, the activity of the intestinal microbiota, and the metabolism of intestinal cells may alter the interactions observed in the *in vitro* model.

The compounds assayed should not be harmful, because at the concentrations employed in the present study they form part of a normal diet; however, before proposing their use as dietary strategies for reducing absorption of Hg it is necessary to verify that their application *in vivo* does not produce adverse effects. It has been seen that some of these compounds may interfere with absorption of minerals. Reductions in absorption of Fe(II) in rodents have been reported in the presence of quercetin (Lesjak et al., 2014) and tannic acid (Afsana et al., 2004). A reduction in absorption of essential elements is not desirable if we bear in mind that this kind of strategy may be employed in future population interventions.

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FIGURE CAPTIONS

Figure 1. Effect of dietary components on transport of Hg(II). Bars represent apparent permeability coefficients (P_{app}) of Caco-2 cells co-exposed to Hg(II) (0.5 mg/L) and various food components for 2 h. Values expressed as percentages with respect to cells treated only with Hg(II) (mean \pm SD, n = 3). Asterisks indicate statistically significant reductions with respect to the treatments only with Hg(II) ($p < 0.05$).

Figure 2. Effect of dietary components on cellular retention of Hg(II). Bars represent intracellular contents in Caco-2 cells co-exposed to Hg(II) (0.5 mg/L) and various dietary components for 2 h. Values expressed as percentages with respect to cells treated only with Hg(II) (mean \pm SD, n = 3). Only those components that produced statistically significant reductions are shown ($p < 0.05$).

Figure 3. Effect of dietary components on transport of CH₃Hg. Bars represent apparent permeability coefficients (P_{app}) of Caco-2 cells co-exposed to CH₃Hg (0.5 mg/L) and various dietary components for 2 h. Values expressed as percentages with respect to cells treated only with CH₃Hg (mean \pm SD, n = 3). Asterisks indicate statistically significant differences with respect to the treatments only with CH₃Hg ($p < 0.05$).

Figure 4. Effect of dietary components on cellular retention of CH₃Hg. Bars represent intracellular contents in Caco-2 cells co-exposed to CH₃Hg (0.5 mg/L) and various dietary components for 2 h. Values expressed as percentages with respect to cells treated only with CH₃Hg (mean \pm SD, n = 3). Only those components that produced statistically significant reductions are shown ($p < 0.05$).

Figure 5. Effect of dietary supplements or plant extracts on transport of Hg(II) and CH₃Hg. Bars represent percentages of reduction in apparent permeability coefficients (P_{app}) in Caco-2 cells co-exposed for 2 h to Hg(II) or CH₃Hg (both 0.5 mg/L) and various plant extracts or dietary supplements. Values expressed as percentages with respect to cells treated only with Hg (mean \pm SD, n = 3). Asterisks indicate statistically significant differences with respect to the values obtained in cells treated only with Hg standards ($p < 0.05$).

Table S1. Dietary compounds used in the study.

Dietary compounds	Concentration (aqueous standards)	Concentration (food matrix)	Brand
Bovine serum albumin	20 mg/L	n.a.	Biowest
Catechin monohydrate	5 mg/L	n.a.	Fluka
Corn starch dextrin	10 mg/L	n.a.	Sigma
DL-Homocysteine (HCys)	5 mg/L	5 mg/L	Sigma
Epigallocatechin	5 mg/L	n.a.	Sigma
Glutathione (GSH)	1 mM	1 mM	Sigma
Iron(II) sulfate [Fe(II)]	50 mg/L	n.a.	Sigma
L-Arginine (Arg)	20 mg/L	n.a.	Sigma
Lecithin (granular)	10 mg/L	n.a.	Sigma
(±)- α -Lipoic acid	10 mg/L	n.a.	Sigma
Reduced lipoic acid (DHLA)	50 mg/L	n.a.	Sigma
L-Methionine (Met)	20 mg/L	n.a.	Merck
L-Phenylalanine (Phe)	20 mg/L	n.a.	Merck
Lysine monohydrate (Lys)	20 mg/L	n.a.	Merck
Lys + Phe	20 mg/L+ 20 mg/L	n.a.	Merck
Pectin (from apple)	50 mg/L	n.a.	Sigma
Quercetin dihydrate	5 mg/L	5 mg/L	Sigma
Selenium dioxide [Se(IV)]	0.5 mg/L	n.a.	Merck
Tannic acid	20 mg/L	20 mg/L	Merck
Thiamine hydrochloride	25 mg/L	n.a.	Sigma
Xylan (from oat flakes)	20 mg/L	n.a.	Sigma
Zinc sulfate [Zn(II)]	50 mg/L	n.a.	Panreac
Dietary supplements or extracts			
Benfotiamine supplement	100 mg/L	n.a.	Life Extension
Grape seed extract	100 mg/L	100 mg/L	Plantextrakt
Green tea extract	100 mg/L	n.a.	Plantextrakt
GSH/Cys/vitamin C supplement	100 mg/L	100 mg/L	Life Extension
Lipoic acid supplement	100 mg/L	n.a.	Life Extension

n.a.: not assayed.

Table 1. Effect of the dietary strategies (food compounds, dietary supplements, plant extracts) on the transport of Hg present in the bioaccessible fraction of swordfish. Apparent permeability coefficients (P_{app}) of the Caco-2 monolayers exposed for 120 min to the bioaccessible fractions in the presence or absence of the dietary strategies, and reduction in Hg P_{app} in the presence of those compounds (mean \pm SD; n = 4–6). Asterisks indicate statistically significant differences in the P_{app} values with respect to the assays performed without dietary strategy ($p < 0.05$).

	P_{app} ($\times 10^{-5}$ cm/s)	P_{app} reduction (%)
Food components		
Without component	1.39 \pm 0.40	
Cysteine	0.87 \pm 0.14	37.5 \pm 10.0*
GSH	0.65 \pm 0.37	53.7 \pm 21.7*
Homocysteine	0.91 \pm 0.13	34.6 \pm 9.6*
Tannic acid	1.92 \pm 0.81	—
Quercetin	1.50 \pm 0.40	—
Supplements or extracts		
Without component	3.69 \pm 0.41	
Cys/GSH supplement	2.50 \pm 0.34	37.0 \pm 10.6*
Grape seed extract	1.64 \pm 0.82	63.8 \pm 22.6*

Figure 1

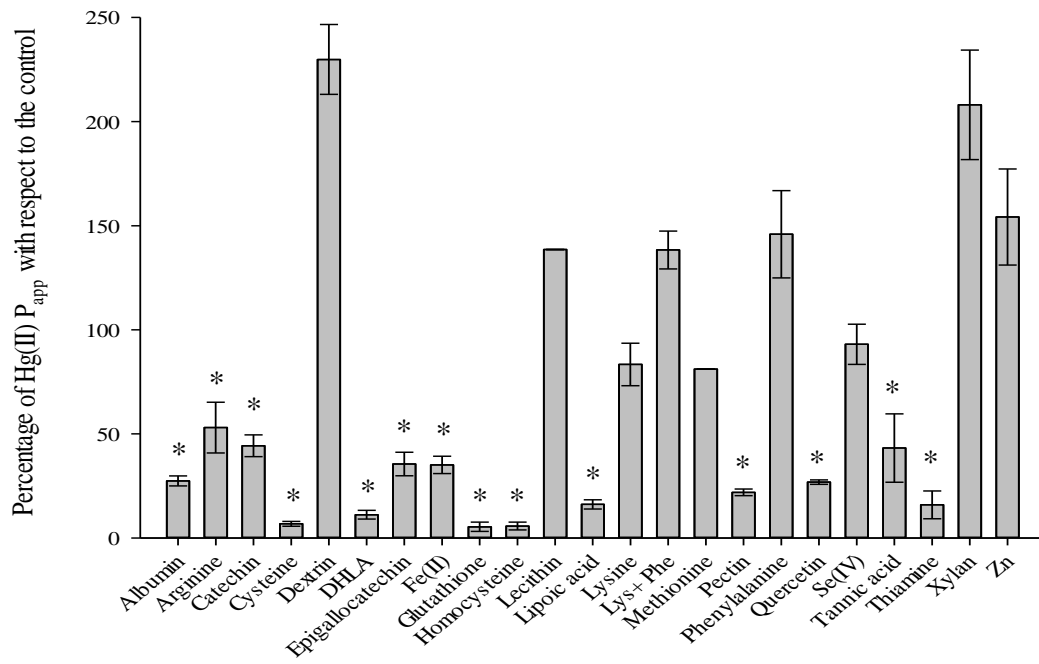


Figure 2.

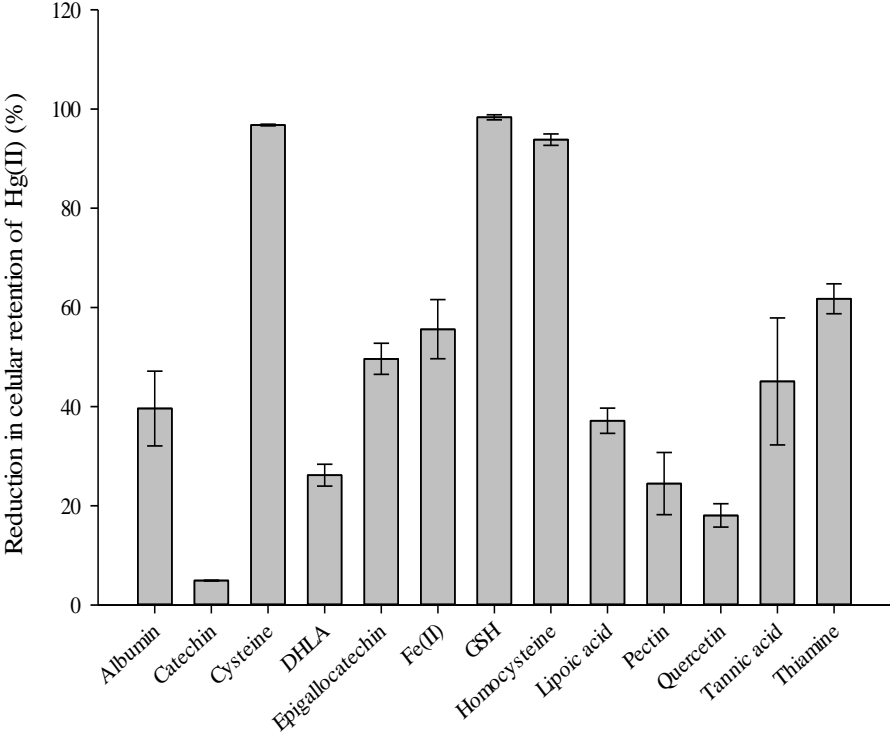


Figure 3.

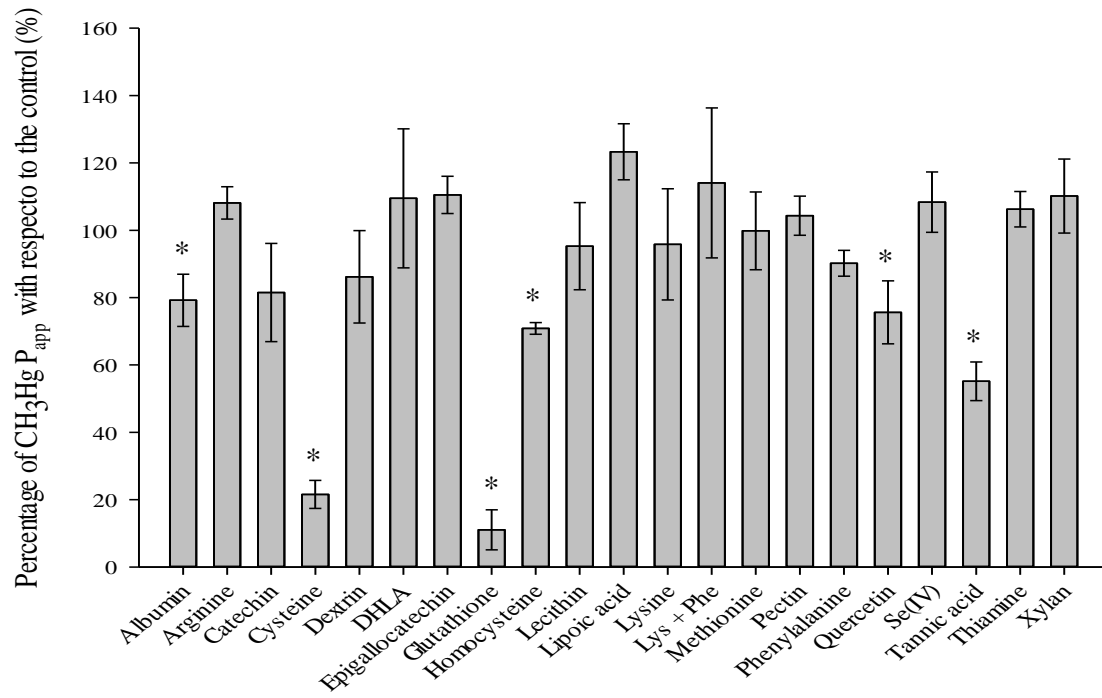


Figure 4.

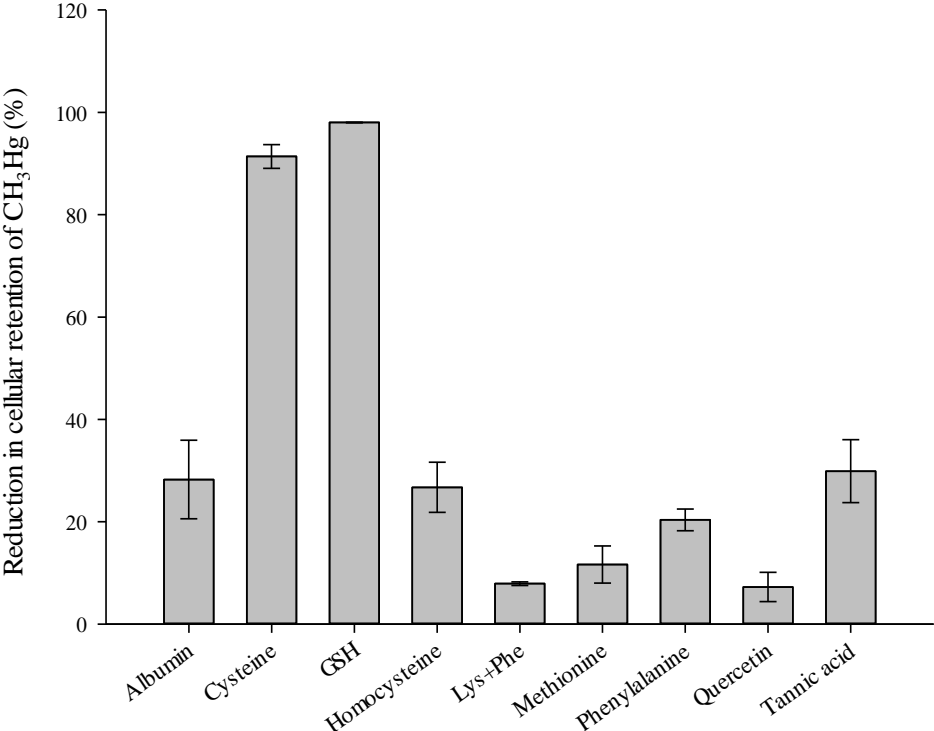


Figure 5.

