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Cooking and co-ingested polyphenols reduce *in vitro* methylmercury bioaccessibility from fish and may alter exposure in humans

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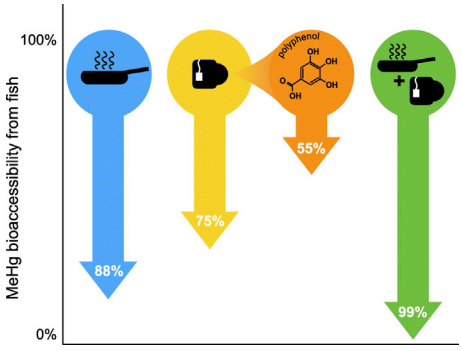
HIGHLIGHTS

- Cooking fish decreases *in vitro* MeHg bioaccessibility (BA) to 12%.
- Polyphenol-rich foods (tea, coffee) reduce MeHg BA to 25%.
- Purified polyphenols also reduce MeHg BA.
- Together, cooking and polyphenol-rich foods decrease MeHg BA by up to 99%.
- Once validated, BA assays could inform estimated intakes and consumer guidelines.

GRAPHICAL ABSTRACT



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ABSTRACT

Fish consumption is a major pathway for mercury exposure in humans. Current guidelines and risk assessments assume that 100% of methylmercury (MeHg) in fish is absorbed by the human body after ingestion. However, a growing body of literature suggests that this absorption rate may be overestimated. We used an *in vitro* digestion method to measure MeHg bioaccessibility in commercially-purchased fish, and investigated the effects of dietary practices on MeHg bioaccessibility. Cooking had the greatest effect, decreasing bioaccessibility on average to 12.5 ± 5.6%. Polyphenol-rich beverages also significantly reduced bioaccessibility to 22.7 ± 3.8% and 28.6 ± 13.9%, for green and black tea respectively. We confirmed the suspected role of polyphenols in tea as being a driver of MeHg's reduced bioaccessibility, and found that epicatechin, epigallocatechin gallate, rutin and cafeic acid could individually decrease MeHg bioaccessibility by up to 55%. When both cooking and polyphenol-rich beverage treatments were combined, only 1% of MeHg remained bioaccessible. These results call for *in vivo* validation, and suggest that dietary practices should be considered when setting consumer guidelines for MeHg. More realistic risk assessments could promote consumption of fish as a source of fatty acids, which can play a protective role against cardiovascular disease.

1. Introduction

A large proportion of the world's population depends on fish. Indeed, fish are estimated to provide 17% of animal proteins consumed by humans (and 6.7% of all proteins consumed worldwide) (Food and

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Agriculture Organization, 2016), and are an important source of vitamins, minerals and fatty acids, which can protect from cardiovascular disease (Mahaffey et al., 2011). However, fish consumption is one of the major pathways of human exposure to mercury (Hg) (Committee on Toxicological Effects of Methylmercury, National Research Council of the United States, 2010), which in its organic form of methylmercury (MeHg) is a potent neurotoxin (Clarkson and Magos, 2008). To protect at-risk populations, Hg blood guidelines have been established, derived from large-scale studies defining lowest adverse effect doses (Chapman and Chan, 2000; Legrand et al., 2010).

However, there is also a growing body of evidence suggesting that our understanding of Hg absorption in the body is incomplete. Current recommendations on fish consumption consider that the ingested dose of Hg from fish is equal to MeHg's – this assumes that 100% of Hg in fish is in the form of MeHg, and that MeHg's absorption rate is of 100% (Committee on Toxicological Effects of Methylmercury, National Research Council of the United States, 2010; Ha et al., 2016). This stems from older studies performed on human volunteers (Aberg et al., 1969) and on rats (Miettinen et al., 1971) with methylmercuric nitrate ($MeHgNO_3$). However, this may not be representative of MeHg speciation in fish, which is more likely bound to thiol groups included in proteins (Clarkson and Magos, 2008; Harris, 2003). Indeed, assuming that nearly all of Hg in fish is bioavailable may overestimate intake by 50% (Ha et al., 2016): while the absorption rate of solubilized MeHg may be high, not all MeHg is necessarily freed from the fish matrix into digestive fluids (*i.e.* made bioaccessible) and made available for absorption by the body following metabolism in the intestine by the gut microbiome or in the liver (bioavailable) (Afonso et al., 2015a). Thus, to postulate near total MeHg bioavailability overlooks processes that may occur before absorption and into systemic circulation. This is supported by studies reporting that Hg bioaccessibility is not positively correlated to concentration in the consumed food (Laird and Chan, 2013; Laird et al., 2009a). While biomarkers like blood or hair Hg show robust relationships to Hg intake (Abdelouahab et al., 2008; Cole et al., 2004; Kosatsky et al., 2000; Legrand et al., 2005; Mahaffey and Mergler, 1998), in most of these studies, Hg intake is estimated from food frequency questionnaires and the literature on the consumed fish species, rather than direct Hg measurements (Abdelouahab et al., 2008; Sunderland, 2007), meaning that exact Hg intake is frequently unknown. Furthermore, there is evidence that populations exhibit toxicological responses to Hg in different ways (Canuel et al., 2006a; Chapman and Chan, 2000). As Hg remains a contaminant of major concern (Mergler et al., 2007), it is critical we better understand its fate in the body. A cost-effective and non-invasive way of doing so is through *in vitro* bioaccessibility studies, to first investigate the fate of Hg in the gastrointestinal tract.

Many factors could be responsible for altering MeHg bioaccessibility from ingested food. Food matrix composition may affect the fate of MeHg in the body, with one study reporting that Hg from the flesh of a salmonid may be 6-fold more bioaccessible than that from marine mammalian organs (Laird et al., 2009a). Different levels of Hg bioaccessibility have also been reported for various fish species (H.-S. Wang et al., 2013). Fish handling by industries and by consumers could also alter bioaccessibility. While freezing can induce physicochemical changes to meat (Farouk et al., 2004; Sanza et al., 1999), it is widely used in fish processing to prevent spoilage (George, 1993), which could change MeHg bioaccessibility before fish become available on the market for purchase. Consumer-based food preparation can significantly transform meat, with cooking and drying reducing moisture, crude protein content and total lipids (Toyes-Vargas et al., 2016). Indeed, cooking has been found to reduce Hg and MeHg bioaccessibility (Afonso et al., 2015b; He and Wang, 2011; Jadán Piedra et al., 2016; Ouédraogo and Amyot, 2011; Torres-Escribano et al., 2010, 2011). *In vitro* studies have also suggested that foods rich in plant polyphenols (such as tea) may reduce MeHg bioaccessibility (He and Wang, 2011; Ouédraogo and Amyot, 2011; Shim et al., 2009). Dietary practices may thus alter the way MeHg is solubilized from food (bioaccessibility), and ultimately change its bioavailability. A better understanding of these processes could lead to easily implementable guidelines and recommendations to reduce Hg loading in fish-consuming populations.

The goal of this study was to explore how dietary practices can alter MeHg bioaccessibility, using an *in vitro* digestion model. We explored how various cooking techniques and the co-ingestion of polyphenol-rich foods could alter MeHg solubilization from food. We also investigated the role of specific polyphenols in driving this effect which had been hypothesized in the literature, but never confirmed. We also assessed the potential effect of combined dietary practices on MeHg bioaccessibility. Finally, we report how these dietary practices can affect MeHg intake and loading in the body, and propose ways to use this information to inform future research and guidelines.

2. Methods

2.1. Food items, co-ingested foods and polyphenols

Experiments were performed on swordfish, grouper, tuna and salmon filets obtained from fish markets in Montreal. These species were selected to reflect fish readily available to Canadian consumers year-round. Blueberries, coffee (Nescafé, Maxwell) and green and black teas of various brands (Twinnings, Stash, Green Sail, Salada) were purchased in Montreal supermarkets as were corn oil (Mazola) used for cooking treatments, and cornstarch (Ideal), used as a non-polyphenol control. Pure polyphenols (gallic acid (>97.5%), catechin (>98%), epigallocatechin gallate (>80%), theaflavin (>80%), rutin (>94%)) were obtained from Sigma-Aldrich.

2.2. Food preparation methods

Three cooking methods were tested: grilling, frying and boiling. Grilling was performed on a Teflon-coated pan, at 100 and 150 °C for 1 min. Frying treatments were conducted in 1 mL of corn oil, in glass vials heated on a burner for 1 min. Samples were boiled in 2 mL of ultrapure MilliQ water (>18.2 MΩ cm^{-1}) (EMD Millipore) in glass vials for 5 or 10 min. Temperature was monitored throughout cooking. For freezing, fish samples were subsampled immediately following their purchase and placed in glass vials, and kept at −20, −80 °C or flash frozen in liquid nitrogen (then kept at −80 °C). Glassware was rinsed with distilled water, soaked in a 45% HNO_3, 5% HCl (Fisher Scientific, ACS-pure) bath overnight and rinsed 3 times with MilliQ water before use.

For co-ingestion experiments, fish samples were digested simultaneously with either beverages or pure polyphenols. Beverages (tea, coffee, instant coffee) were prepared as per the manufacturer's instructions, and lyophilized overnight into a powder (Freezone6, Labconco). Powdered beverages were solubilized in 2 mL of MilliQ water, in two different doses: 40 mg or 120 mg, and were added to fish at the start of *in vitro* digestion experiments. In these experiments, controls were amended with 2 mL of MilliQ water to adjust the volume. Pure polyphenols were solubilized in 2 mL of dimethyl sulfoxide (DMSO), in amounts of 5 or 10 mg, and used in *in vitro* digestions. Controls with no polyphenols were also amended with 2 mL of DMSO, to account for volume increase.

2.3. Physiologically-based extraction test

Many *in vitro* digestion protocols exist to assess bioaccessibility of nutrients and dietary compounds (Dong et al., 2016; Minekus et al., 2014; Van de Wiele et al., 2007). We selected the Physiologically-based extraction test (PBET), adapted from Ruby et al. (1996) and Ouédraogo and Amyot (2011), to perform digestive simulations, as it has been used frequently for metals and Hg (Calatayud et al., 2012; Ouédraogo and Amyot, 2011; Siedlikowski et al., 2016). All digestive simulations were performed on 1.0 ± 0.1 g of fresh fish sample, in triplicate. Experimental solutions were prepared in acid-washed Teflon bottles prior to each PBET

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