Applications de la méthode à des études sur la spéciation du mercure dans des matrices biologiques

I. Introduction

Cette troisième partie du manuscrit a pour but de montrer la viabilité et la justesse de la méthode ainsi que son applicabilité en routine sur des matrices réelles diverses de produits de la pêche. Par conséquent, après validation de la méthode d'analyse, des contrôles qualités internes (CQI) ont été mis en place et compléter par des contrôles qualités externes (CQE) conformément à la norme ISO 17025:2005 (ISO 2005) et au système d'assurance qualité (AQ) mis en place au sein du LNR « métaux » de l'ANSES pour toutes nos activités, qu'elles soient de référence ou de recherche (Cf. également chapitre IV).

Par la suite, une première application a été menée au sein du laboratoire LCABIE sur des échantillons réels lyophilisés mais non dégraissés (contrairement aux MRC étudiés), d'anguilles et civelles prélevés dans l'estuaire de l'Adour situé au sud-ouest de la France afin de s'assurer que la méthode de spéciation est applicable à l'analyse d'échantillons réels (Cf. Article I). Nous nous sommes ensuite questionné sur l'influence de la lyophilisation sur la distribution des espèces mercurielles car ce procédé, utilisé dans l'étude précédente, est couramment utilisé pour l'échantillonnage. Cette application a été menée sur différentes matrices de produits de la pêche et en collaboration avec le LCABIE. Pour finir et d'après les observations faites après étude du

procédé de lyophilisation, nous avons décidé d'analyser les échantillons lyophilisés de l'étude EAT 2, plutôt que les matières fraiches (Millour et al., 2011) (Cf. Article II). Les données d'occurrence ainsi obtenues pourraient par la suite être utilisées pour une évaluation plus juste de l'exposition humaine au MeHg via la consommation de produits de la pêche

II. Contrôles qualités internes (CQI) mis en place

Lors de chaque série d'analyses d'échantillons réels, un blanc d'extraction pour surveiller une éventuelle contamination ou détecter des effets de mémoire, un MRC pour vérifier la justesse et la mesure du rapport isotopique $^{203}Tl/^{205}Tl$ pour calculer le biais en masse et vérifier la stabilité du signal dans le temps, sont systématiquement utilisés comme CQI. Enfin, les concentrations et les abondances isotopiques des spikes ^{199}iHg et $^{201}MeHg$ sont vérifiées par dilution isotopique inversée et par analyse des abondances isotopiques (Rodriguez-Gonzalez et al., 2005).

Concernant le suivi de la justesse, une carte de contrôle a été mise en place sur le DOLT-4 selon la procédure CIME VII.03 du 05 décembre 2008 « Cartes de contrôles » et la norme NF X 06-031-0 : décembre 1995 (AFNOR 1995) (Figure 38). Les limites de surveillance sont calculées comme M ± 2s, les limites d'action comme M ± 3s, l'intervalle de confiance comme M ± (k x CVR x M) avec M la valeur certifiée, s l'écart-type, k = 3 (p = 99%) et CVR le coefficient de fidélité intermédiare de reproductibilité estimé lors de la validation de la méthode à 1,5% pour le MeHg et 2,2% pour le THg.

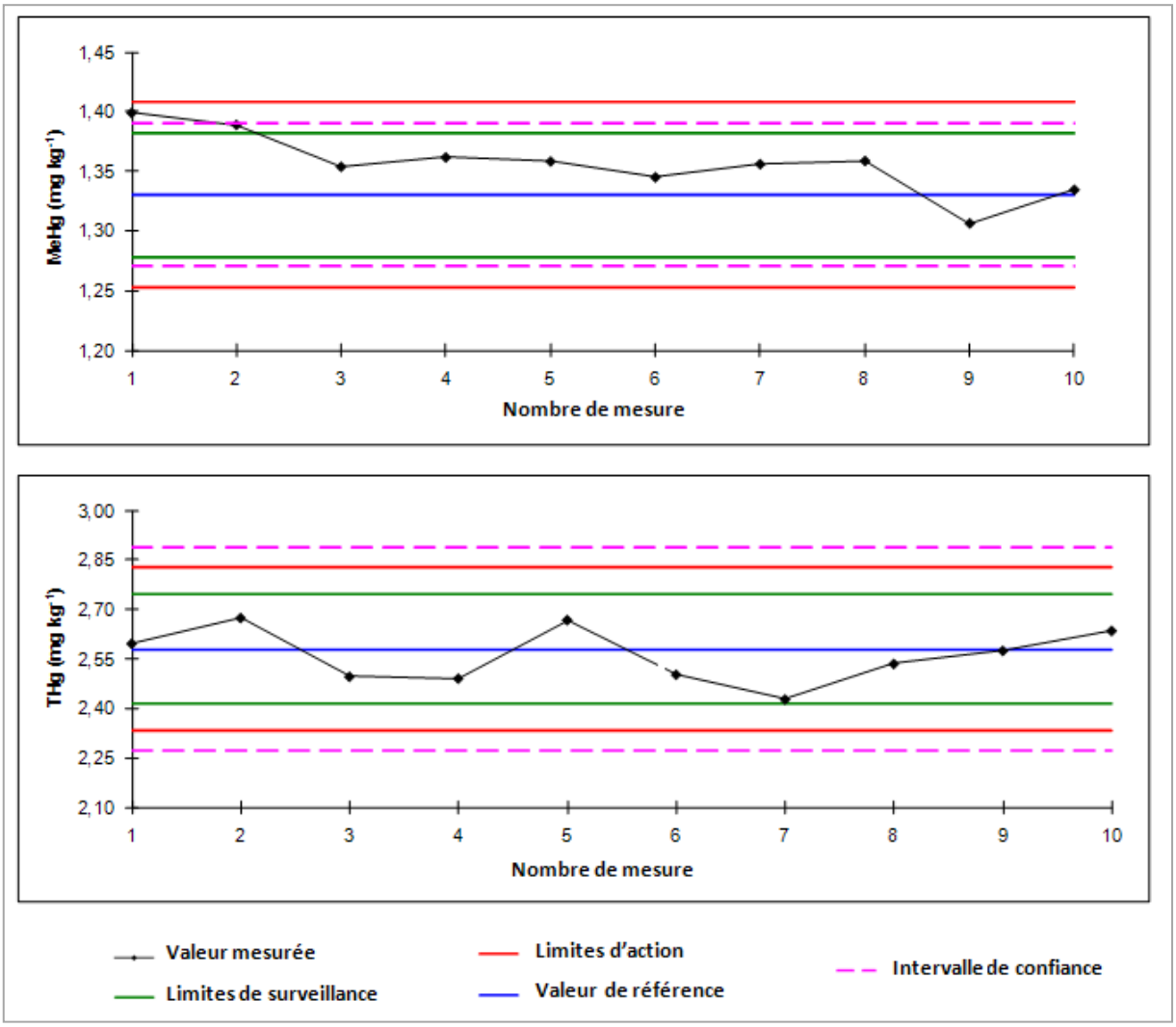

Figure 38 : Cartes de contrôle du DOLT-4 pour le MeHg et le HgT (n = 10).

Les résultats indiquent que les concentrations trouvées sont généralement dans l'intervalle de confiance du fabriquant. Les valeurs moyennes obtenues sur le DOLT-4 pour le MeHg et HgT sont respectivement de 1,36 et de 2,56 mg kg^{-1} avec un écart-type relatif de 1,9% et de 3,2%, respectivement (n = 10). Cependant, il faut noter que les deux premières valeurs de MeHg se situent entre la limite supérieure de surveillance et la limite supérieure d'action, mais comme les limites d'action restent inférieures à l'intervalle de tolérance de la valeur certifiée du DOLT-4 (1,33 ± 0,12 $mg.kg^{-1}$), aucune action corrective n'a été nécessaire. Ces données démontrent une justesse satisfaisante de la méthode lors de l'analyse d'échantillons réels.

Aucune dérive de la stabilité du signal des isotopes du thallium n'a été mise en évidence lors de ces analyses et le contrôle des blancs n'a montré aucune contamination significative en mercure lors de ces essais.

III. Contrôles qualités externes (CQE) effectués

Les CQE sont complémentaires aux CQI mis en place, afin d'évaluer régulièrement la méthode sur des échantillons réels à teneurs inconnues de l'élément d'intérêt. Ce type d'exercice est communément appelé « Essais interlaboratoires d'aptitude (Eilas) ou essais d'aptitude » ou « Comparaison interlaboratoires (CIL) », à ne pas confondre avec « Essais interlaboratoires de validation ». Selon les organismes organisateurs d'Eilas, la matrice et le contaminant d'intérêt, plusieurs dizaines à plusieurs centaines de laboratoires y participent. Après traitement statistique et éventuellement, élimination des valeurs aberrantes, une valeur consensuelle correspondant à la moyenne des valeurs observées, sert de valeur de « référence ». Les Eilas permettent à chaque participant de vérifier sa justesse et de comparer ses données au moyen d'une évaluation externe et d'entreprendre d'éventuelles actions correctives, en cas de résultat non-conforme. Les résultats de chaque participant sont généralement évalués en calculant des Z-scores à partir de l'équation 11 :

$$Z = \frac{\left(\overline{Xf} - \overline{Xc}\right)}{\left(s_R \middle| \sqrt{n}\right)} \qquad (11)$$

avec $\overline{Xf}$ = résultat expérimental, $\overline{Xc}$ = valeur certifiée, s_R = écart-type de reproductibilité, n = nombre de résultats expérimentaux

Les Z-scores sont interprétés comme :

- $|Z| \leq 2$, résultats conformes
- $2 < |Z| \leq 3$, résultats questionnables
- $|Z| > 3$, résultats non conformes.

Au cours de ces travaux, nous avons participé à 2 Eilas organisés par le CSL-FAPAS (FAPAS, 2010) sur un échantillon de thon en conserve, en mai 2010 et par l'IRMM (Geel, Belgique), sur le matériau IMEP-109 de produit de la pêche pour déterminer, entre autres, le MeHg et HgT.

L'Eila organisé par le FAPAS a compté 56 participants issus de 27 pays dont 16 laboratoires ont participé à la détermination de la teneur en MeHg. Cinq techniques analytiques différentes ont été utilisées pour la détermination du MeHg (analyseur automatique de Hg, CV/HG-AAS, GC-ECD, GC-ICP-MS et HPLC-ICP-MS). Le tableau 5 de l'article II présente les valeurs cibles et les

résultats obtenus. En complément, la figure 39 présente les Z-scores obtenus pour le MeHg par les 16 laboratoires participants. Le code laboratoire CIME est le n°15.

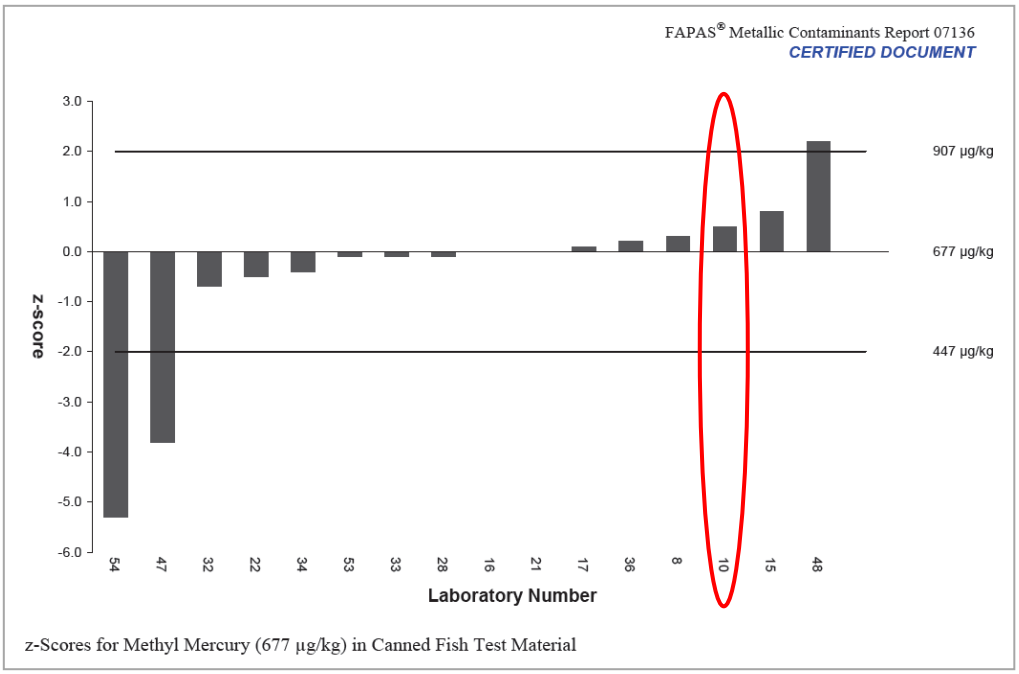

Figure 39 : Z-scores pour le MeHg sur l'échantillon de thon en conserve (FAPAS, 2010)

Sur 16 laboratoires participants, 13 laboratoires, dont le notre (Z-score = 0,8), ont obtenu des résultats conformes. Le laboratoire n°48 a eu un résultat questionnable et les laboratoires n°47 et 54 ont eu des résultats non conformes. Parmi les laboratoires jugés conformes, 5 d'entres eux (n° 10, 16, 17, 32 et 33) ont utilisé une méthode accréditée.

Concernant l'Eila IMEP 109 (IRMM, 2010), 38 participants issus de 27 pays ont participé mais seuls 5 laboratoires ont déterminé la teneur en MeHg dans l'échantillon. Un couplage HPLC-ICP-MS a été utilisé par les laboratoires n°13 et 38. Le laboratoire n°7 a utilisé un analyseur de mercure et détection SAA. Enfin, notre laboratoire (n° 1) et le laboratoire n° 10 ont utilisé un couplage GC-ICP-MS. La figure 40 présente les teneurs en MeHg observées (mg kg^{-1}). La ligne noire présente la teneur certifiée du MRC DOLT-4 ($X_{réf}$) qui a été utilisé lors de cet Eila. L'intervalle de tolérance du MRC ($X_{réf} \pm U_{réf}$) est spécifié par les lignes vertes. L'intervalle cible défini par les organisateurs de l'Eila est caractérisé par les lignes rouges.

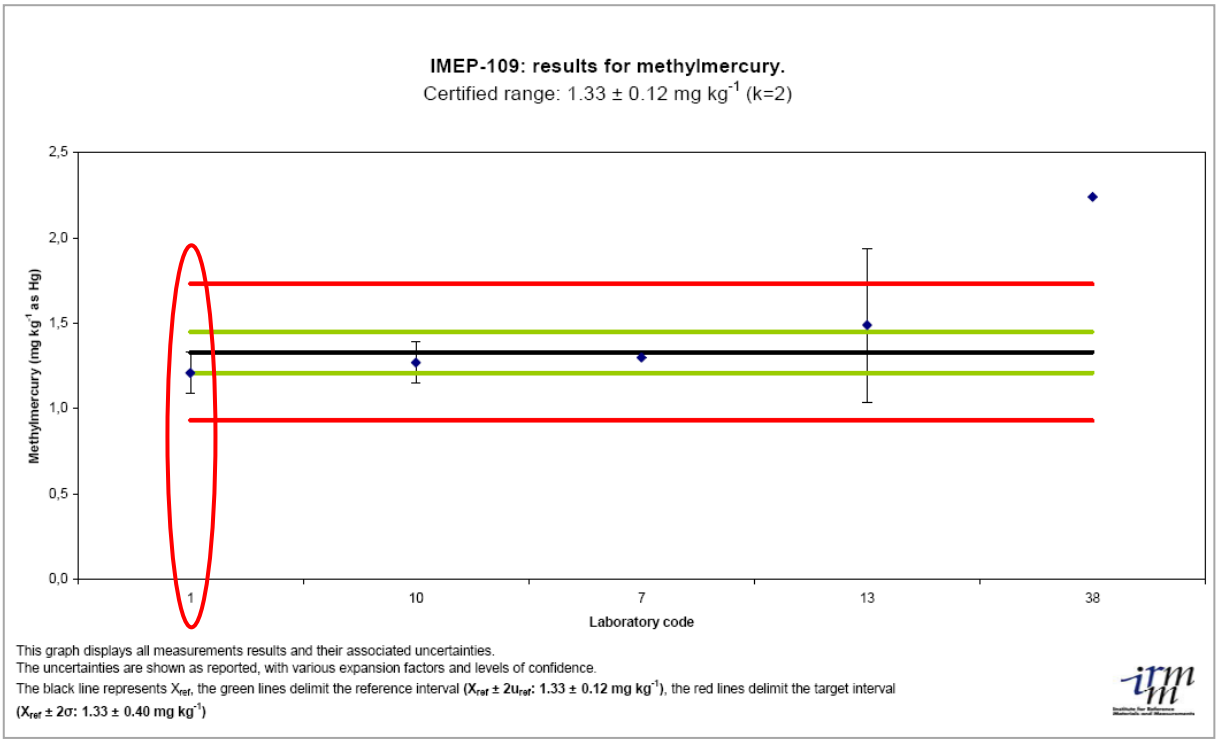

Figure 40 : Teneurs en MeHg obtenus de l'IMEP-109 (IRMM, 2010)

Les teneurs en MeHg observées sont conformes pour 4 laboratoires dont le notre (Z-score = 0,6), et le laboratoire n° 38 a rendu un résultat non-conforme avec un Z-score > 3.

Les résultats obtenus pour le MeHg et le HgT lors de ces 2 Eilas sur des échantillons de poissons sont satisfaisants, démontrant la justesse de notre méthode validée. A noter qu'aucun Eila n'a porté sur les autres produits de la mer (mollusques, coquillages…) au cours de cette période. Nous avons également participé à un troisième Eila organisé par le FAPAS sur une matrice de poisson, dont le traitement des données statistiques est actuellement en cours.

IV. Applications

IV.1 Etude des échantillons lyophilisés d'anguilles et de civelles

Cette étude a été menée au sein du laboratoire de Pau lors de mon stage de formation de 3 mois sur la technique GC-ICP-MS. Cette étude a fait l'objet d'un article (Article I) sous presse dans le journal « International Journal of Environmental Analytical Chemistry ».

IV.1.1 Contexte de l'étude

L'anguille est un poisson migrateur de grand intérêt écologique. Selon son âge, son alimentation varie (d'abord du plancton puis des crustacés, des mollusques et des poissons) ainsi que sa physionomie (poids : jusqu'à 3 kg, longueur : jusqu'à 1,5 m, longévité : 10 ans, graisse : 30% poids corporel en moyenne pour les anguilles argentées de plus de 500 g) font qu'elle tend à bioaccumuler et bioamplifier les polluants dans son organisme. Elle devient donc un parfait indicateur de pollution de l'environnement aquatique de part ses évolutions marines et estuariennes (lieux et durées) qui peuvent être évaluées par l'étude de ses otolithes (os de l'oreille interne) (Tabouret et al., 2010, Daverat et al., 2011).

Depuis les années 1980, la population des anguilles diminuent drastiquement en raison de surpêches et de changements climatiques (Dekker, 2000, Feunteun 2002). Elles sont classées comme espèces menacées d'extinction depuis juin 2007. Le cycle biologique de l'anguille européenne (*Anguilla anguilla*) est détaillé dans l'article I et résumé dans la figure 41.

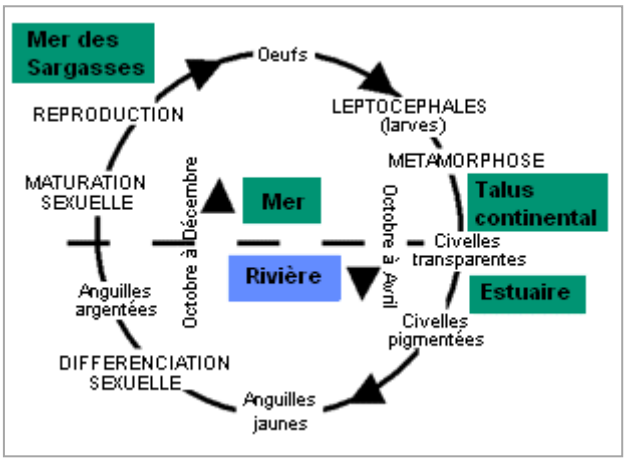

Figure 41 : Cycle biologique de l'anguille (source : http://www.rhone-mediterranee.eaufrance.fr/milieux-continentaux/poissons/anguille-migrateur.php)

Dans cette étude, les teneurs en espèces Hg et butylétains (BuSn) d'échantillons réels d'anguilles et de civelles ont été déterminées par analyse GC-ICP-MS et quantifiées par D-IDMS et IPD. Les échantillons étudiés concernaient 2 stades de l'espèce *Anguilla anguilla* (civelles et anguilles jaunes) prélevés le long de l'estuaire de l'Adour dans le Sud-ouest de la France. Les civelles ont été prélevées la nuit en milieu marin, à Moliets et dans des rivières, à Urt en décembre 2004 et avril 2005. Les anguilles jaunes ont été prélevées le long de la rivière de l'Adour, à Redon, Saint Laurent de Gosse, Termi et Cauneille de juillet 2005 à juillet 2007. Le muscle des anguilles jaunes et les civelles entières ont été séparément lyophilisés et homogénéisés avant analyse. Une description plus détaillée des matériels et méthodes utilisés pour cette étude, ainsi qu'une présentation des sites de prélèvement, de la méthode analytique et des résultats obtenus sont faites dans l'article I. Seuls les résultats en mercure sont présentés et discutés dans ce paragraphe. Il est à noter qu'à notre connaissance, aucun article dans la littérature n'a concerné des études de spéciation du Hg dans les civelles (stade juvénile de l'anguille). Les résultats présentés ici sont les premières données d'occurrence pour cette matrice.

IV.1.2 Résultats expérimentaux

Les résultats de cette étude sont présentés dans l'article I.

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Simultaneous determination of mercury and butyltin species using a multiple species-specific isotope dilution methodology on the European, *Anguilla anguilla* glass eel and yellow eel

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A methodology to simultaneously determine mercury (MeHg, IHg) and butyltin (TBT, DBT, MBT) compounds in eel samples was assessed and validated using multiple isotopically enriched species. The developed methodology was able to analyse simultaneously the organometal species accurately and precisely and to correct for the potential transformations/degradations of the different species during the various steps of the analytical procedure. Low detection limits were achieved (0.007–0.17 µg Hg kg^{-1} for mercury (Hg) species and 0.42–0.71 µg Sn kg^{-1} for tin (Sn) species) allowing analysis of low-mass samples and thus the analysis at the individual organism scale, including glass eels for which samples dry weight ranged from 60 to 100 mg. The methodology was validated with certified reference materials (BCR-464, BCR-477, BCR-710, DOLT-4 and NIST-2977) and applied to the analysis of these pollutants in two developmental stages of the European eel, *Anguilla anguilla*: individual whole glass eels and muscle tissue from yellow eels. The Adour estuary (South-west France) was selected to monitor the bioaccumulation of organometal species in these organisms, according their developmental stage, their morphological parameters and the sampling site. The results suggest that the accumulation of methylmercury in glass eel tissue is related to weight, with higher concentrations in smaller individuals. Butyltin concentrations were very close to the limit of detection, and no significant differences were detected between glass and yellow eels.

Keywords: speciation; mercury; butyltin; eel; isotopicdilution

1. Introduction

The European eel (*Anguilla anguilla*) is a catadromous species of major ecological and economic significance [1]. Over the last three decades, it has suffered a dramatic population decline [2], being outside safe biological limits throughout all of Europe [3].

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Different factors have been involved in this decline, including overfishing of glass eels, climate/current changes, habitat destruction, migration barriers and pollution [4,5].

A. anguilla has a life cycle that goes through different stages and habitats, i.e. different environmental conditions (oxygen availability, salinity, exposure to anthropogenic compounds) [6]. This species spawns in the Sargasso Sea and the resulting larvae (leptocephali) use ocean currents (mainly the Gulf Stream) to migrate to European coastal areas. The leptocephalus stage is a key developmental stage, characterised by the accumulation of energy stores for metamorphosis [7] and estuarine migration. When the leptocephali reach the continental shelf, they metamorphose into glass eels which enter estuaries mostly from October to April/May on the French Atlantic coast and then migrate up estuaries without feeding [8]. They progressively acquire green and brown pigments and become yellow eels that colonise the watershed for a long freshwater growth phase. Eels then undergo a second metamorphosis and become silver eels, and return to the Sargasso Sea where they reproduce and die [9]. Because glass eels and silver eels do not feed during estuarine and reproductive migrations, both leptocephali and yellow eels must accumulate energy reserves during their growth phase [10]. In addition, yellow eels feed on benthic macro-invertebrate predators during their early years, and are thus exposed to contaminants accumulated and adsorbed in sediments [10]. When they reach more than 30–40 cm, eels become top piscivorous predators[11] and can swim throughout the entire water column. Therefore, due to its high fat content and local benthic feeding behaviour, the sub-adult stage is considered extremely prone to the bioaccumulation of pollutants [12], and has been used as an indicator of pollution in aquatic environments [13–17].

Industrial contamination of river systems by toxic metals can have long-term effects on the metal body burdens of resident fish. This is particularly true in the case of mercury (Hg), which is not only converted from a relatively toxic inorganic species to a very toxic organometal species (methylmercury, MeHg) at low concentration levels, but also bioaccumulates and biomagnifies in aquatic biota [18,19]. The behaviour of organotin compounds (OTs), i.e. tributyltin (TBT), is similar. OTs have been considered endocrine-disrupting chemicals and they have been extensively used as biocides in wood preservatives, antifouling paints and fungicides [20], all released directly to the aquatic environment. Fish and seafood products can be important sources of energy, protein, ω-3 fatty acids, trace elements and vitamins, but also of environmental contaminants [21–23] such as Hg and Sn species that are bound to proteins in biological tissues. Therefore, the accumulation of organomercury and tin (Sn) compounds through food chain has been found in organisms at higher trophic levels, i.e. fish, marine mammals, birds and humans. Moreover, their uptake within a given population can vary with size and age and it is therefore valuable in ecosystem studies to analyse individual organisms.

The determination of organometal species in complex environmental matrices requires extensive sample preparation prior to instrumental analysis, and is one of the main concerns in speciation analysis. The sample preparation step, including extraction of the species from the matrix and derivatisation, is prone to non-quantitative reactions, unpredictable analyte losses, contamination and species interconversions [24,25]. Optimised sample preparation is necessary, not only to reduce the time involved, but also because each step adds a potential source of error. Sample preparation becomes even more critical at the low concentrations required for ultra-trace level analysis, as it can account for a significant proportion of the variability of a particular methodology [26]. The use of speciated isotope dilution for the determination of a wide range of elemental species, even in complicated matrices [27], can overcome these problems in

sample preparation. This quantification approach is considered a definitive method that provides highly accurate and precise data and can also correct for possible losses and species transformations. Recently, several methodologies based on the use of multiple-spiking species-specific isotope dilution analysis have been developed [28–30]. Depending on the number of species to be analysed simultaneously and the transformation rates to be estimated, different numerical approaches can be used. A recent numerical approach based on isotopic pattern deconvolution developed by Rodríguez-González *et al.* [30,31] has been demonstrated to be a powerful and versatile approach, not only for monitoring the species degradation processes, but also for accurate quantification.

In this study, we optimised several steps of the sample preparation procedure and compared two species determination approaches.Owing to small quantities of sample and low concentration levels, samples were analysed using gas chromatography - inductively coupled plasma-mass spectrometry (GC-ICP-MS) because it offers high sensitivity and low detection limits. In combination with isotope dilution, the methodology validated was used to assess the impact of these contaminants in two developmental stages of European eels from the Adour estuary, i.e. glass and yellow eels. Both stages were analysed to determine the concentrations of organomercury and organotin compounds and establish differences in the accumulation patterns of MeHg and butyltin compounds. The glass and yellow eels were collected in different locations along the Adour estuary (south-western France), which is influenced by urban development and industrial activities downstream, and agriculture and fisheries activities upstream (the river has several floodplains which are natural habitats for *A. anguilla*) [32].

2. Experimental

2.1 *Environmental samples*

The samples studied in this work were two stages of the species *Anguilla anguilla* (glass eels and yellow eels) collected along the Adour River estuary in south-western France (see Figure 1). The estuary is affected by a dynamic macrotidal regime, reaching up to

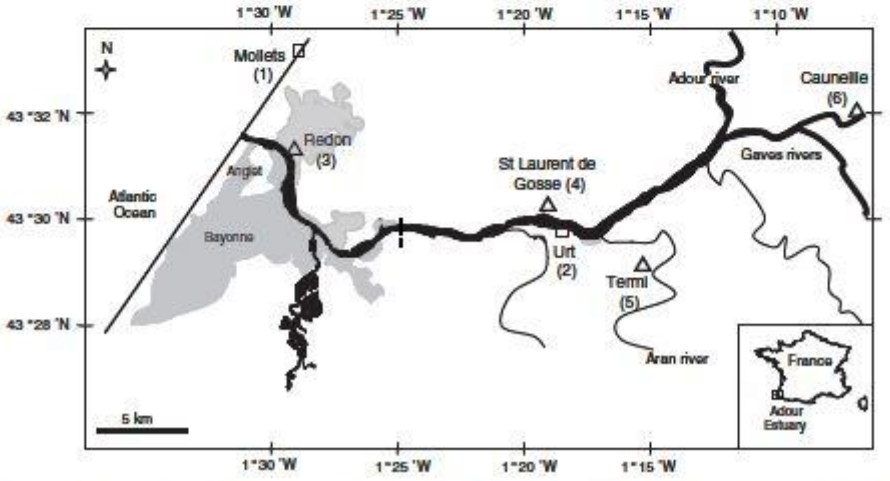

Figure 1. Map of the sampling sites. Glass eel sampling sites are indicated by open squares (□) and yellow eel sampling sites by open triangles (Δ).

70 km upstream. The upstream part of the estuary flows through agricultural areas, while the downstream part is within the urban development of the city of Bayonne and is subject to urban and industrial inputs. In a previous study, Stoichev *et al.* [33] reported chronic Hg contamination in sediments with some specific sedimentation areas where inorganic mercury (IHg) can be converted into MeHg, especially during low discharge periods and warm weather conditions. The determination of Hg and butyltin (BuSn) species concentrations in sediments in the Adour estuary has shown concentrations ranging 0.01–0.23 ng Hg g^{-1} for MeHg, 25–477 ng Hg g^{-1} for IHg, 0.09–8.95 μg Sn g^{-1} for TBT and 0.43–2.23 μg Sn g^{-1} for DBT [34]. For benthic macrofauna in the Adour estuary concentrations were less than 0.12 μg Hg kg^{-1} for MeHg, ranging from 25 to 200 μg Hg kg^{-1} for IHg, 0.25 to 0.40 μg Sn kg^{-1} for TBT and 20 to 30 μg Sn kg^{-1} for dibutyltin (DBT) [34]. Other study carried out by Lespes *et al.* [35] reported BuSn concentration values along the Adour estuary in sediments ranging <0.1–3.6 μg Sn kg^{-1} for TBT, <0.1–7 μg Sn kg^{-1} for DBT and 13–39 μg Sn kg^{-1} for MBT and in dissolved phase 0.2–30 ng Sn L^{-1} for TBT, <0.2–33 ng Sn L^{-1} for DBT and 0.7–35 ng Sn L^{-1} for MBT. In addition, Arleny *et al.* [36] found values ranging from 80 to 450 μg Hg kg^{-1} for MeHg and from 3 to 160 μg Hg kg^{-1} for IHg in European yellow eels caught in the same estuary. Therefore, *A. anguilla* appears to be an effective bioaccumulator of MeHg, even in moderately contaminated aquatic environments.

Glass eels were collected at night in two sites: station 1 (Moliets), a marine site, which is a sandy beach on the Atlantic coast, 30 km away from the mouth of the Adour River, in December 2004 ($N = 20$); station 2 (Urt), located 21 km from the mouth of the Adour River, i.e. half way between the mouth of the river and the upstream limit of tidal influence (salinity <0.5 ‰), in December 2004 ($N = 20$) and in April 2005 ($N = 20$).

Yellow eels were sampled from four sites along the river. At station 3 (Redon), located at the mouth of the river, under tidal influence and affected by urban and industrial activities, yellow eels were caught in July 2005 ($N = 12$) and in July 2007 ($N = 2$). At station 4 (Saint Laurent de Gosse), a human-modified floodplain subject to agricultural activities that is connected to saline water and flooded twice a year, the individuals were collected in July 2006 ($N = 3$). At station 5 (Termi), another floodplain connected to the Adour River by a tributary and not affected by saline water, yellow eels were caught in April 2006 ($N = 3$), in July 2006 ($N = 3$), in October 2006 ($N = 3$) and in July 2007 ($N = 3$). At Station 6 (Cauneille), one of the three main tributaries of the Adour River, outside any tidal influence, the individuals were sampled in October 2006 ($N = 2$).

On the morning following their capture, glass eels were transported to the laboratory, anaesthetised by immersion in a solution of 0.036 mL L^{-1} clove oil, weighed (Sartorius CP 153 balance, ±1 mg), and their length was measured. Then, they were killed by lethal immersion and immediately frozen at −20°C until lyophilisation. Glass eels weighed around 70 mg (dry weight) and each individual was ground by hand. The yellow eels were transported to the laboratory in cool boxes and then dissected. Eel muscle tissues were lyophilised and homogenised before analysis.

2.2 *Chemicals*

TBT chloride (96%), DBT dichloride (97%) and monobutyltin trichloride (MBT) (95%) were obtained from Sigma-Aldrich, whereas the MeHg standard was obtained from Strem Chemicals (Newburyport, MA, USA). The stable isotopes and isotopically enriched

species used were purchased from ISC Science (Oviedo, Spain): IHg enriched in ^{199}Hg (91%), MeHg enriched in ^{201}Hg (96.5%) and a mix of MBT, DBT and TBT enriched in ^{119}Sn (82.4%). DBT enriched in ^{118}Sn and TBT enriched in ^{117}Sn were obtained from LGC Limited (Teddington, UK). All stock solutions were kept in the dark at −20°C until use and were prepared by dissolving the corresponding salt either in acetic acid for butyltin compounds or in methanol for MeHg and in ultrapure water (1% HCl) for IHg. Working solutions of the butyltin compounds were prepared daily before analysis by dilution of the stock solutions with a 3:1 mixture of acetic acid:methanol. For Hg, the working solutions were prepared daily by dissolving the corresponding stock solution with 1% HCl in ultrapure water.

Hydrochloric acid (HCl, 33–36%, ultrex® II ultrapure reagent) and glacial acetic acid (HAc, Instra-analyzed®) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Methanol (MeOH, chromasolv®) was obtained from Sigma-Aldrich (Seelze, Germany). Ammonium hydroxide (NH_4OH, puriss p.a.) and tetramethylammonium hydroxide (TMAH, 25% in water, traceselect) were purchased from Fluka (Steinheim, Germany) and sodium acetate trihydrate (NaAc, puriss p.a.) from Riedel-de-Haën (Seelze, Germany). Sodium tetraethylborate and sodium tetrapropylborate (98%) were purchased from Galab (Geesthacht, Germany).

The certified reference materials used for validating the methodology were BCR-464 (tuna fish, Hg species), BCR-477 (mussel tissue, BuSn species), BCR-710 (oyster tissue, BuSn species) purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), DOLT-4 (dogfish liver, Hg species) obtained from the National Research Council Canada (NRCC, Ottawa, Canada) and SRM-2977 (mussel tissue, Hg species) from the National Institute of Standards and Technology (NIST, Gaithersburg, USA).

2.3 *Intrumentation*

The extraction of the organometallic species from solid matrices was carried out using an Explorer focused microwave system from CEM Corporation (Mathews, NC, USA); this system ensures accurate control of temperature and pressure inside the glass vial. The simultaneous determination of Hg and BuSn species was performed in a Thermo XSeries 2 inductively coupled plasma-mass spectrometer (ICP-MS) coupled to a gas chromatograph (GC) (Thermo Fisher, Waltham, MA, USA) by a commercial GC-ICP-MS interface (Silcosteel®,0.5 m length, inner i.d. 0.28 mm and o.d. 0.53 mm, outer i.d. 1.0 mm and o.d. 1,6 mm, Thermo Fisher). The sample (2 µL) was injected in the splitless mode at 250°C into a (30 m x 0.53 mm × 1 µm) MXT® Silcosteel® column (Restek, Bellefonte, PA, USA). The carrier gas used was helium at 25 mL min^{-1}. The temperature program used for the chromatographic separation was as follows: temperature increase at 60°C min^{-1} from 60°C to 280°C. The ICP-MS parameters used for the analysis were as follows: nebulizer, plasma and auxiliary flows 0.6, 15 and 0.9 L min^{-1}, respectively; radiofrequency power 1250 W; isotopes for Hg analyses 198, 199, 200, 201 and 202 (Tl, 203 and 205); isotopes for Sn analyses 116, 117, 118, 119 and 120 (Sb, 121 and 123); a dwell time of 25 ms for Hg and Sn isotopes and 5 ms for Tl and Sb. The performance of the instrument was optimised with liquid standards, and the isotopes of Tl and Sb were measured to check the mass bias in each chromatographic run [37].

2.4 *Sample preparation and analysis*

A 250 mg subsample for analyses of yellow eels or whole glass eels was accurately weighed in an extraction tube and 5 mL of TMAH were added to the tube. The appropriate amount of isotopically enriched Hg (^{199}IHg and ^{201}MeHg) and BuSn species (^{118}DBT and ^{117}TBT) was added. The microwave extraction conditions were 70°C for 4 minutes with stirring [38]. After cooling, the extracts were centrifuged at 3500 rpm for 5 min and an aliquot of the extracted solution was added to 22 mL glass vials containing 5 mL of HAc/NaAc buffer (pH 5, 0.1 mol L^{-1}). The pH was then re-adjusted with ultrapure HCl to pH 5 for simultaneous analysis. Propylation of the species was carried out by adding 1 mL of iso-octane and 0.25 mL of $NaBPr_4$ 5% (w/v) freshly prepared daily, and the vial was manually shaken for 5 min. The organic phase was recovered, transferred to a chromatographic vial and kept at −20°C until analysis. The GC-ICP-MS analysis was then performed within 24 h [39]. At least one blank was also run in each batch of analyses.

The concentrations and the transformation factors were calculated using the isotope pattern deconvolution (IPD) approach previously applied for quantification of BuSn species [31] and Hg species [30]. The concentrations were compared to those calculated using the classical isotope dilution analysis (IDA) approach [34].

3. Results and discussion

3.1 *Evaluation and validation of the methodology using multiple isotopically enriched species*

Various steps of the sample preparation procedure were evaluated: the extraction and derivatisation steps were studied because they are the main processes during which species transformations can occur. Species transformations were assessed by adding the multiple spike solution before and after extraction. After comparison of the two spike addition conditions, the analytical step during which the transformation takes place (extraction or derivatisation) was determined as well as the type of conversion (methylation of IHg, demethylation of MeHg and debutylation of BuSn species) that may have occurred.

The type of derivatisation reagent (sodium tetraethyl borate or sodium tetrapropyl borate) was also studied because the use of different reagents can affect derivatisation performance and induce conversion between species [40]. The spiking procedure and the derivatisation reagent effect were evaluated by comparing concentrations and transformation factors obtained with the two different quantification approaches described above.

Hg species - Table 1 gives the results obtained for the certified reference materials for the different test conditions. The experimental concentrations obtained by classical isotope dilution (IDA) approaches are not significantly different ($p > 0.05$) from the certified values for total Hg and MeHg in DOLT-4, except for MeHg when spike was added after extraction with any derivatisation reagent. Concentrations obtained from IPD approach were statistically different ($p < 0.05$) from certified values in most of the conditions tested. For tuna fish (BCR-464) only the propylated extract spiked before the extraction matched the certified value, probably owing to the complexity of this matrix. This can be explained by a non-quantitative extraction for this complex matrix under the extraction conditions used in this study. Even if the isotopically enriched analogues are added at the beginning of the procedure, the IDA and IPD approaches are not able to properly correct the concentration values when the analytes are not extracted completely.

Table 1. Concentrations (μg Hg kg^{-1} dry weight ± standard deviation ($N = 3$)) obtained for Hg speciation in several certified reference materials under different conditions (spiking before/after the extraction and ethylation/propylation) using either the isotope dilution (IDA) or isotope pattern deconvolution (IPD) approach. % met, percentage of methylation (IHg transformation to MeHg); % demet, percentage of demethylation (MeHg transformation to IHg).

	Derivatisation reagent	Spiking procedure	IDA		IPD			
			Total Hg	MeHg	Total Hg	MeHg	% met	% demet
BCR 464	Certified value		5240 ± 100	5117 ± 158				
	ethylation	before	4825 ± 192	4625 ± 158	4635 ± 734	4497 ± 661	423 ± 243	4 ± 3
		after	4503 ± 102	4314 ± 88	4404 ± 147	4232 ± 143	117 ± 21	0.2 ± 0.2
	propylation	before	5101 ± 157	4927 ± 155	4983 ± 370	4859 ± 364	205 ± 120	4 ± 0.9
		after	4685 ± 319	4569 ± 233	4483 ± 685	4375 ± 683	108 ± 37	0.3 ± 0.1
DOLT-4	Certified value		2580 ± 220	1330 ± 120			108 ± 37	
	ethylation	before	2581 ± 156	1228 ± 44	2601 ± 174	1228 ± 119	8 ± 6	3 ± 2
		after	2513 ± 67	1177 ± 27	2547 ± 58	1177 ± 31	0.9 ± 0.7	3.5 ± 0.8
	propylation	before	2615 ± 210	1280 ± 61	2628 ± 202	1265 ± 112	8 ± 4	4 ± 2
		after	2756 ± 256	1586 ± 44	2777 ± 188	1634 ± 61	2 ± 2	3.0 ± 0.8
BCR-710	Certified value		260 ± 50*	107 ± 8				
	ethylation	after	235 ± 16	95 ± 8	228 ± 13	92 ± 11	5 ± 1	8 ± 5
	propylation	after	255 ± 22	96 ± 14	267 ± 45	105 ± 14	4 ± 2	4 ± 2
NIST-2977	Certified value		101 ± 4	36.6 ± 1.0				
	propylation	before	99 ± 7	37 ± 2	101 ± 3	37 ± 1	1 ± 1	4 ± 1

*indicative value.

Regarding the spiking procedure, experimental values for total Hg and MeHg for both calculation approaches were not significantly different from the certified materials values when the enriched species were added before extraction in the case of DOLT-4. The methylation and demethylation factors were generally higher when the spike was added to the sample before the extraction step. IHg was partially methylated during the extraction process with yields reaching 8% for DOLT-4. Similar methylation yields during extraction have been described by Hammerschmidt and Fitzgerald [41]. In contrast, demethylation factors were similar for both spiking procedures for DOLT-4, suggesting that demethylation is not related to the extraction procedure, but to the derivatisation step. In the case of BCR-464, demethylation generally occurred during the extraction step under both ethylation and propylation conditions. Aberrant methylation factors for tuna fish can be attributed to very low concentration of IHg (123 ng g^{-1}) compared to high concentration of MeHg (5117 ng g^{-1}). As reported before by Monperrus *et al*. for IDA calculation [29], these results demonstrate that IPD methodology does not provide accurate results for the quantification of methylation when there is a huge difference between MeHg and IHg concentrations. Species transformations, especially in the case of an extremely high excess of one of the elemental species, must be carefully monitored in order to obtain accurate results [42]. In this case, the high methylation factors are due to an overestimation of the 199MeHg from the spiked 199IHg in the presence of high natural levels of MeHg. The results from DOLT-4 BCR-464, BCR-710 and NIST-2977 confirm that methylation or demethylation reactions strongly depend on the matrix, as Hinojosa Reyes *et al*. [43] demonstrate for various fish tissues.

Regarding the derivatisation reagent, for DOLT-4, no significant differences ($p > 0.05$) were found between ethylation and propylation when the spike was added before the extraction but significant differences were observed when the spike was added after the extraction step for both THg and MeHg, and only for THg in the case of BCR-710. In the case of BCR-464, ethylation showed significant differences ($p < 0.05$) from propylation in both spiking before and after the extraction and for both THg and MeHg. As reported in other studies [30,44], the ethylation reagent can induce demethylation or methylation during the derivatisation step. For the studied CRMs, transformation factors were not significantly different between both derivatisation reagents for BCR-464 but they were differences for DOLT-4 in demethylation factor when the spike was added after extraction and for the oyster tissue in methylation factor.

No significant differences between the concentrations calculated using the two quantification approaches were observed. However, although the IDP approach properly corrects for transformations among species and gives the corrected concentrations given these transformations, results were more repeatable using the IDA calculation, with standard deviations ranging between 2.0–4.9% and 2.9–15.8% for MeHg calculated by IDA and IPD, respectively, and between 2.1–13.6% and 2.9–16.1% for IHg, respectively. This can be explained by the poor repeatability of the transformation factors which affect the repeatability of the degradation-corrected concentrations of total Hg and MeHg. The repeatability of the transformation factors may be affected by the complexity of the mathemathical IPD model, which takes the contribution of all the monitored isotopes into account.

BuSn species – Table 2 gives the results for oyster tissue (BCR 710) and mussel tissue (BCR 477). For oyster tissue, ethylation and propylation reagents were tested adding the spike after the extraction step. IDA concentrations were not statistically different ($p > 0.05$) from the certified values for both derivatisation reagents, except for DBT

Table 2. Concentrations ($\mu g\,Sn\,kg^{-1}$ dry weight ± standard deviation ($N=3$)) obtained for butyltin species in certified reference materials using the isotope dilution analysis (IDA) or isotope pattern deconvolution (IPD) approach. % debutyl, percentage of debutylation (TBT transformation to DBT); % butyl, percentage of butylation (DBT transformation to TBT).

			IDA		IPD			
	Derivatisation reagent	Spiking procedure	TBT	DBT	TBT	DBT	% debutyl	% butyl
BCR-710	Certified value		54 ± 8	42 ± 8				
	ethylation	after	52 ± 7	33 ± 7	47 ± 8	30 ± 6	−3.8 ± 2.0	−3.3 ± 8.3
	propylation	after	51 ± 4	62 ± 10	47 ± 8	58 ± 10	0.2 ± 4.9	3.8 ± 9.3
BCR-477	Certified value		901 ± 78	785 ± 61				
	ethylation	before	851 ± 25	781 ± 17	778 ± 26	736 ± 14	0.7 ± 0.4	−0.1 ± 1.0
		after	784 ± 94	592 ± 67	736 ± 90	553 ± 61	1.7 ± 0.2	−0.05 ± 1.4

using propylation. For mussel tissue, spike addition was studied with the ethylation reagent only. The addition of the spike before the extraction gave IDA concentrations in agreement with the certified values for both TBT and DBT. This can be attributed to a better equilibrium between endogenous and spiked species when the spike was added before the extraction. The addition before showed significant differences from the addition after the extraction for both TBT and DBT species.

Regarding butylation/debutylation factors, no significant yields were observed except for mussel tissue. Debutylation factors were different ($p < 0.05$) [Q] ital between ethylation and propylation and, between spike before and after the extraction. Very low debutylation factors were detected (0.7–1.7%) in the case of mussel tissue. The ethylation reaction showed a more coherent transformation yield, because butylation reactions are very improbable. The results determined by IPD and IDA were comparable with similar relative standard deviations ranging from 2.2 to 12.0% and 1.9 to 12.2%, respectively.

The overall results for CRMs confirm that the sooner the enriched species are added to the matrix, the more their behaviour is similar to the natural species against transformations or non-quantitative reactions. Thus, to correct for possible losses/transformations of species, the spike should be added before the extraction step. These results also highlight that transformation yields strongly depend on the matrix. Methodologies using isotopically enriched species are thus necessary to evaluate the analytical procedure for any real sample. Therefore, the simultaneous analysis of speciation of Hg and BuSn compounds in real fish samples was performed by adding the spike before the extraction step and using tetrapropyl borate as the derivatisation reagent. It should be noted that when the analysis of speciation for only BuSn species wants to be performed, tetraethyl borate should be used as derivatisation reagent.

3.2 *Analytical performance of the simultaneous analysis*

The limits of detection (LODs) of the methodology were determined by isotope dilution in spiked extraction blanks. The LODs were calculated based on blank runs ($N=9$) as three times the standard deviation of the blank, following IUPAC recommendations. Contributions to the blank thus come from reagents and vessels and were significantly

Table 3. Analytical performance of the Hg and Sn speciation analysis. Recoveries are expressed as percentages, and LODs as $\mu g\,Hg\,kg^{-1}$ or $\mu g\,Sn\,kg^{-1}$ ($N=9$). The repeatability values shown are relative standard deviations (%).

Recovery	THg	MeHg	TBT	DBT
BCR 464	89	89	–	–
DOLT 4	101	93	–	–
BCR-710	95	94	94	89
NIST 2977	98	101	–	–
BCR 477	–	–	95	100
Repeatability	2.1–8.0	2.7–8.3	2.9–7.0	2.2–6.3
LODs	0.24	0.07	0.58	0.42

higher for IHg and butyltin species than for MeHg (Table 3). LODs for the transformation factors were determined using isotope deconvolution patterns in standard solutions containing 1 $\mu g\,L^{-1}$ of each Hg and BuSn species. They were calculated as three times the standard deviation of the transformation yields for the standard solutions. Low LODs for the transformation factors were obtained (0.7% for demethylation, 0.3% for methylation, 0.5% for debutylation and 0.4% for butylation).

Accuracy and precision of the multiple-spike methodology proposed in this study were estimated by analysing the different CRMs following the best assay conditions as determined above (isotopically enriched species added before extraction and propylborate used as derivatisation reagent for the simultaneous analysis of Hg and BuSn species). At least three independent replicates of each CRM were carried out to evaluate recovery and repeatability. Recoveries were estimated at 79% for DBT in BCR-710 and up to 101% for THg in DOLT-4 by IDA approach. Repeatability of the assay ranged from 2.1 to 8.3% for Hg species and from 2.2 to 7.0% for BuSn species. Thus, the assay conditions determined here were adequate for analysing organomercury and butyltin species at very low concentration levels in various animal tissues (mussel, oyster, tuna or fish liver). The very low LODs achieved by this methodology allow the analysis of Hg and butyltin species in fish samples even for samples of very low mass.

3.3 *Assessment of the multiple-spike methodology in real eel samples*

The eel samples were analysed by the analytical multiple-spike methodology described herein and the two quantification approaches (IDA and IPD) were applied. The results were compared in terms of accuracy and precision of the concentration values. The results of the analysis on 10 yellow eel individuals and 10 glass eel individuals injected in triplicate are given in Table 4. The eels were randomly selected from the different sampling sites. The IHg concentration values obtained using IPD were significantly different (Student's t test, $p < 0.05$) from those obtained by IDA, suggesting that IPD provides a better correction of the degradation processes. In contrast, the MeHg concentrations were not different (Student's t test, $p > 0.05$) whether they were calculated using IDA or IPD. Regarding the interconversion processes, demethylation reactions generally took place during the analysis procedure, ranging from 0.5 to 3.9%. Higher and not-so-homogeneous percentages of methylation were calculated, typically from 1.0 to 9.5%, but also up to

Table 4. Concentrations (μg Hg kg^{-1} dry weight ± standard deviation (N = 3)) obtained for Hg speciation in real eel samples using the isotope dilution (IDA) or the deconvolution pattern (IPD) approach. % met, percentage of methylation; % demet, the percentage of demethylation.

		IDA		IPD			
		IHg	MeHg	IHg	MeHg	% met	% demet
Yellow eel	Stream water	23.5 ± 0.9	601 ± 18	10.5 ± 0.1	597 ± 10	7.7 ± 3.6	2.0 ± 0.2
	(Termi, station 5)	27.6 ± 0.4	673 ± 17	15.3 ± 2.0	829 ± 13	<LOD	2.4 ± 0.2
		51.1 ± 0.7	1444 ± 40	15.8 ± 5.7	1244 ± 15	9.3 ± 2.4	2.3 ± 0.2
		24.9 ± 1.5	385 ± 4	8.1 ± 0.5	391 ± 2	9.5 ± 1.0	3.6 ± 0.2
		23.4 ± 0.7	431 ± 6	17.8 ± 2.3	534 ± 9	<LOD	2.2 ± 0.2
	Estuary	14.1 ± 0.1	329 ± 9	7.7 ± 0.4	308 ± 2	4.5 ± 1.6	1.9 ± 0.2
	(Redon, station 3)	28.7 ± 1.9	610 ± 14	9.3 ± 1.9	635 ± 16	<LOD	3.4 ± 0.4
		29.1 ± 2.2	574 ± 8	14.6 ± 1.9	591 ± 10	2.9 ± 2.8	2.6 ± 0.3
		41.9 ± 0.2	1125 ± 32	15.1 ± 3.2	1082 ± 27	<LOD	2.7 ± 0.3
		62.8 ± 3.4	960 ± 25	22.2 ± 5.4	1004 ± 31	6.0 ± 4.1	3.9 ± 0.4
Glass eel	Estuary	15.6 ± 0.3	137 ± 1	10.9 ± 0.4	138 ± 2	1.0 ± 0.9	3.5 ± 0.1
	(Urt, station 2)	21.3 ± 0.4	150 ± 2	18.0 ± 0.4	150 ± 1	1.3 ± 0.6	1.6 ± 0.1
		10.0 ± 1.8	126 ± 5	7.8 ± 2.2	121 ± 3	47 ± 11	0.7 ± 0.5
		14.5 ± 1.5	154 ± 12	13.9 ± 1.0	156 ± 8	22 ± 1	0.5 ± 0.1
		18.2 ± 1.8	147 ± 25	16.5 ± 1.8	143 ± 20	15 ± 15	0.9 ± 0.1
	Sea	11.9 ± 1.2	111 ± 14	10.4 ± 1.6	111 ± 7	<LOD	2.0 ± 1.4
	(Moliets, station 1)	7.5 ± 0.7	81 ± 6	7.5 ± 0.5	83 ± 9	<LOD	0.8 ± 0.4
		22.8 ± 0.5	149 ± 1	18.6 ± 0.5	150 ± 1	<LOD	3.1 ± 0.2
		17.6 ± 0.7	118 ± 2	14.5 ± 0.4	119 ± 2	<LOD	3.0 ± 0.1
		15.6 ± 0.3	137 ± 1	10.9 ± 0.4	138 ± 2	1.0 ± 0.9	3.5 ± 0.1

see Figure 1 for sampling site locations.

47% in the case of glass eels caught in Urt. As in section 3.1. the best option selected regarding accuracy and repeatability was IDA approach, we only took into account the concentrations calculated by this approach from now on.

3.4 *Concentration levels in glass and yellow eel samples from the adour river estuary*

3.4.1 *Concentration levels in two eel life cycle stages*

The mean values of Hg and butyltin compounds found in glass eels and yellow eels at the different sampling sites are summarised in Table 5. First, concentrations of each pollutant were very similar in both stages of *A. anguilla* and for all sampling sites. No significant differences were observed among the four sampling sites for yellow eels or among the two glass eel sampling sites (Student's t test, $p > 0.05$). Hg species concentrations in yellow eels were roughly six times higher than in glass eels, independent of the sampling site. Thus, eels accumulate not only MeHg but also IHg in their tissues. This relationship between Hg accumulation (both MeHg and IHg) and age is in agreement with other studies [45,46] where accumulation was studied.

In contrast, TBT concentrations were higher in glass eels than in yellow eels, suggesting that TBT does not accumulate in eels and dissipates from the tissues. TBT concentrations in eels caught in the downstream end of the estuary near Redon were slightly higher than those collected in other upstream river sites. The downstream estuary is located in an

Table 5. Mean values ± standard deviation (µg kg^{-1} dry weight) obtained for glass and yellow eels for each sampling site.

		N	MeHg	IHg	% MeHg	TBT	DBT	MBT	Length range (mm)	Weight range (g)
Yellow eel	Redon (station 3)	14	652 ± 265	33 ± 13	95 ± 1	1.3 ± 1.3	7 ± 5	2.3 ± 0.9	240–550	26–287
	St Laurent de Gosse (station 4)	3	488 ± 160	37 ± 8	93 ± 1	0.8 ± 0.3	<LOD	0.9 ± 0.4	280–550	31–231
	Termi (station 5)	12	705 ± 312	34 ± 14	95 ± 1	0.9 ± 0.2	13 ± 10	1.0 ± 0.3	220–392	15–145
	Cauneille (station 6)	2	630 ± 60	26 ± 7	96 ± 1	0.5 ± 0.1	40 ± 16	0.7 ± 0.3	336–374	62–85
Glass eel	Urt (station 2)	40	116 ± 32	4.5 ± 2.5	96 ± 2	5.6 ± 2.0	5.6 ± 3.2	2.4 ± 1.9	62–80	0.04–0.1
	Moliets (station 1)	20	117 ± 21	6.6 ± 5.1	93 ± 5	8.2 ± 4.3	2.7 ± 2.3	2.2 ± 2.0	63–81	0.05–0.1

see Figure 1 for sampling site locations.

industrial and urban area and is subject to direct anthropogenic inputs [47]. For glass eels, the concentration of TBT was higher in individuals caught seaward when compared to the more upstream samples. Ohji *et al.* [48] described a similar pattern for liver tissues in *A. japonica*.

Table 6 shows that only a few studies have determined Hg and BuSn species in eels. MeHg and total Hg concentration values found in the present study were similar to those found by Arleny *et al.* [36] in the same area of the Adour estuary and those found by Redmayne *et al.* [49] for the same eel life cycle stage. Concentrations found in muscle tissue by Le *et al.* [46] in Vietnam are higher but the eels analysed in their study were in a more advanced life cycle stage (silver eel). The elevated levels of Hg found in their study can be related to the high accumulation rate in prey consumed by eels and to the energy-storing phase of young eels. Moreover, the liver of silver eels accumulates mainly IHg, which may be related to the detoxification and excretion function of the liver, while muscle has a high affinity for organic Hg, i.e. MeHg [18,45].

Only a few studies on butyltin species have been conducted in eels. The values found in this work are of the same order as those found by Guérin *et al.* [50] in eels bought at four markets in four French cities. The values from Ohji *et al.* [48] are higher than those that we report here because they were analysed in the eel liver, which is one of the organs in which organometals accumulate. Moreover, the values of BuSn concentrations found in the present study were much lower than those found in the literature for animal tissues (snails, mussels, oyster, fish and macro-invertebrates) from European estuaries [34,51–53].

3.4.2 *Bioaccumulation versus biometric factors*

In Figure 2a, MeHg concentrations in yellow eel samples are plotted against individual eel length. The concentrations increased with length (r^2 0.7741, correlation coefficient 0.8798) showing a statistically significant relationship between MeHg concentration and eels' length at the 99% confidence level ($p < 0.01$). This corroborates a bioamplification of MeHg according to length, as previously observed by Arleny *et al.* [36].

In the case of glass eels, even if an inverse statistically significant relationship between MeHg concentrations and their length (Figure 2b) was observed at the 99% confidence level ($p < 0.01$), the correlation between the variables was relatively weak (correlation coefficient 0.4397). This relationship occurred both for marine and estuarine glass eels, suggesting that its origin is not related to estuarine migration but to sea-stage. MeHg may have been passively assimilated by leptocephali through their gills or through feeding during oceanic migration. However, if assimilated through feeding, accumulation should have increased with weight: the more leptocephali consume, the more they accumulate. On the other hand, this result may also come from maternal transfer of Hg. Eels may have accumulated pollutants during their entire life cycle and store them in adipose tissue. During reproductive migration, the silver eel does not feed and relies on its energy stores. When fat is being mobilised, accumulated pollutants are concentrated, as demonstrated for PCBs in the European silver eel [54]. Thus, when arriving at the spawning site, a high level of organic pollutants may be maternally transferred to eggs. During leptocephalus oceanic migration, a dilution effect may occur with weight gain. The more they consume, the more their MeHg concentration is diluted. However, MeHg contamination may also restrict growth (development, haematology, appetite and behaviour) depending on individual variation as noted above [55,56]. Further investigations are now required to address this issue.

Table 6. Concentration values found in the present study and in the literature for eel muscle or liver[a] tissue (µg Hg/Sn kg^{-1} wet weight).

Species	Location	MeHg	Total Hg	TBT	DBT	MBT	Reference
A. anguilla (glass eel)	Adour estuary (France)	20–59	21–62	0.8–4.3	0.2–2.5	0.2–2.3	this work
A. anguilla (yellow eel)	Adour estuary (France)	77–361	83–374	0.2–1.0	0.3–8.1	0.2–0.7	
A. anguilla	River (UK)	–	104–255	–	–	–	Edwards *et al.* (1999) [18]
A. dieffenbachii	River (New Zealand)	80–500	117–651	–	–	–	Redmayne *et al.* (2000) [49]
A. anguilla	River (Belgium)	–	60–245	–	–	–	Maes *et al.* (2005) [12]
A. anguilla (yellow eel)	Adour estuary (France)	80–450	120–480	–	–	–	Arleny *et al.* (2207) [36]
A. anguilla	Lesina lagoon (Italy)	–	130–240	–	–	–	Storelli *et al.* (2007) [14]
A. marmorata (silver eel)	River (Vietnam)	510	389–623	–	–	–	Le *et al.* (2010) [46]
		160[a]	225–1004[a]				
A. anguilla	Markets? (France)	–	–	1.6	1.1	0.1	Guérin *et al.* (2007) [50]
A. japonica (yellow eel)	Estuary (Japan)	–	–	12–27[a]	18–85[a]	13–75[a]	Ohji *et al.* (2006) [57]

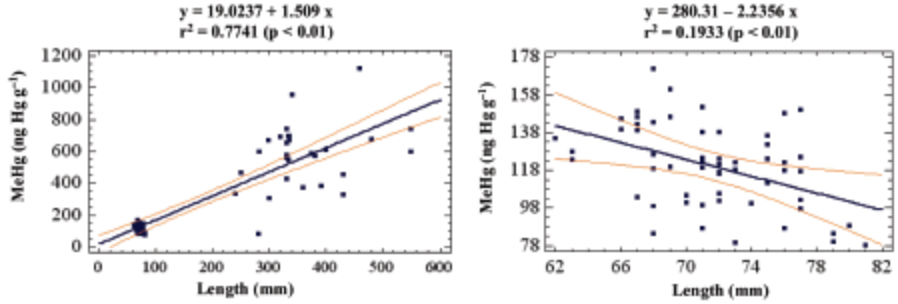

Figure 2. MeHg concentrations (ng Hg g^{-1} dry weight) according to individual length (a) in yellow eels and (b) in glass eels.

4. Conclusions

A methodology for the simultaneous determination of organomercury and butyltin compounds in eels was optimised. The developed methodology was able to accurately and precisely analyse organometal species using multiple species-specific isotope dilution and apply appropriate corrections for potential transformations between species during the analytical procedure. Low detection limits were achieved (0.007–0.17 µg Hg kg^{-1} for Hg species and 0.42–0.71 µg Sn kg^{-1} for Sn species) allowing the analysis of individual samples with low mass, even in the case of early young life cycle stages, where samples may only weigh 60–100 mg. This type of methodology is necessary to directly correlate the contaminant load with individual biological and behavioural parameters that can fluctuate greatly among individuals.

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IV.1.3 Discussion

Malgrè des taux de méthylation élevés (1,0 à 9,5%, résultats présentés dans le tableau 4 de l'article I), les concentrations en MeHg calculées par IPD et par D-IDMS ne sont pas significativement différentes. Par contre, une différence de quantification entre la D-IDMS et l'IPD a été constatée pour le iHg (taux de déméthylation = 0,5 à 3,9%). Les résultats IPD ont donc été conservés. Les principaux résultats de l'étude sont présentés dans le tableau 5 de l'article I et ont été reportés ci-dessous (tableau 22).

Tableau 22: Valeurs moyennes obtenus pour les anguilles et civelles (en µg Hg kg^{-1})

	Localisation	n	MeHg	iHg	%MeHg	Longueur (mm)	Poids (g)
Anguilles jaunes	Estuaire	14	652 ± 265	33 ± 13	95 ± 1	240 - 550	26 - 287
	St Laurent de Gosse	3	488±160	37 ± 8	93 ± 1	280 - 550	31 - 231
	Cours d'eau	12	705 ± 312	34 ± 14	95 ± 1	220 - 392	15 - 145
	Cauneille	2	630 ± 60	26 ± 7	96 ± 1	336 - 374	62 - 85
Civelles	Estuaire	40	116 ± 32	4.5 ± 2.5	96 ± 2	62 - 80	0.04 - 0.1
	Mer	20	117 ± 21	6.6 ± 5.1	93 ± 5	63 - 81	0.05 - 0.1

Pour chaque stade de l'anguille, les concentrations en iHg et MeHg sont similaires, quelque soit les sites de prélèvement. Les concentrations en MeHg et iHg des anguilles jaunes sont en moyenne 6 fois supérieures aux concentrations mesurées dans les civelles, confirmant un phénomène de bioaccumulation du Hg relatif à l'alimentation, au poids, à l'âge et au taux de graisse de l'organisme. Ces observations sont renforcées par une étude de la concentration en Hg en fonction du poids de l'anguille (Cf. article I). Il est en effet constaté que les teneurs en MeHg augmentent avec le poids corporel.

Les données individuelles de chaque anguille et civelle montrent une nette différence de pourcentage de MeHg entre les deux stades (tableau 23).

Tableau 23 : Pourcentages de MeHg d'après les données par IPD du tableau 4 de l'article I

		iHg	MeHg	∑Hg	% MeHg
Anguilles	Cours d'eau	10,5	597	608	98
		15,3	829	844	98
		15,8	1244	1260	99
		8,1	391	399	98
		17,8	534	552	97
	moyenne	**13,5**	**719**	**733**	**98**
	sd	4,0	333	335	1
	Estuaire	7,7	308	316	98
		9,3	635	644	99
		14,6	591	606	98
		15,1	1082	1097	99
		22,2	1004	1026	98
	moyenne	**13,8**	**724**	**738**	**98**
	sd	5,7	318	323	1
Civelles	Mer	10,9	138	149	93
		18,0	150	168	89
		7,8	121	129	94
		13,9	156	170	92
		16,5	143	160	90
	moyenne	**13,4**	**142**	**155**	**91**
	sd	4,1	13	17	2
	Estuaire	10,4	111	121	91
		7,5	83	90,5	92
		18,6	150	169	89
		14,5	119	134	89
		10,9	138	149	93
	moyenne	**12,4**	**120**	**133**	**91**
	sd	4,3	26	29	2

En moyenne, 98% du HgT est présent sous forme méthylé chez les anguilles prélevées dans les cours d'eau et l'estuaire, contre 91% chez les civelles. Ces résultats laissent supposer un phénomène de bioamplification.

Une discussion de comparaison entre les résultats obtenus pour les anguilles lors de cette étude et les résultats retrouvés dans la littérature a été faite dans l'article I. Les teneurs et pourcentages en MeHg déterminés dans cette étude sont généralement en accord avec les données présentées dans d'autres travaux.

IV.1.4 Conclusion

La méthode analytique développée dans cette étude a été appliquée avec satisfaction à des échantillons réels et s'est montrée suffisamment sensible pour permettre l'analyse des faibles masses de civelles (60 à 100 mg). De plus, la comparaison faite entre une quantification par IPD et une quantification par D-IDMS a mis en évidence des phénomènes de méthylation du iHg et donc une quantification plus juste et précise par IPD. Enfin, en plus de l'intérêt de cette étude a montrer l'applicabilité de la méthode d'analyse de spéciation du Hg dans des matrices réelles, l'analyse de deux stades d'évolution de l'anguille a permis d'observer le caractère de bioaccumulation et de bioamplification du MeHg dans cette espèce.

IV.2 Impact de la lyophilisation sur la spéciation du Hg

IV.2.1 Contexte de l'étude

Comme cela fût le cas pour l'application précédemment présentée et pour la majeure partie des applications en HgT et en spéciation du Hg dans les produits de la pêche, les analyses ont généralement lieu sur des échantillons lyophilisés et homogénéisés (Arleny et al., 2007, Mishra et al., 2007, Bustamante et al., 2010, Batista et al., 2011). Cette étape de prétraitement a pour but d'homogénéiser et de faciliter le conditionnement et l'éventuel transport des échantillons. Pourtant et malgré une utilisation intensive de ce procédé d'échantillonnage, seulement deux études semblent s'être intéressées au comportement des échantillons lyophilisés par rapport aux échantillons frais (Yu et Yan, 2003, Point et al., 2007). Yu et Yan (2003) ont évalué, parmi d'autres paramètres, l'influence de la texture de la matrice (fraîche ou lyophilisée) sur la stabilité du iHg et du MeHg lors du stockage des échantillons. Les résultats ont indiqué que les échantillons frais et lyophilisés étaient équivalents en terme de stabilité des espèces Hg mais qu'une congélation – décongélation répétée des matrices fraiches favorisait la perte de MeHg. Enfin, l'étude de Point et al. (2007) sur la quantification par S-IDMS et D-SS-IDMS, des teneurs en iHg, MeHg et HgT sur 3 MRCs lyophilisés et 2 MRCs frais a montré une surestimation du MeHg dans les échantillons lyophilisés en raison de l'acide utilisé pour l'extraction, montrant un comportement différent de ce type de matrice par rapport aux matrices fraîches. En raison du peu de données publiées dans la littérature, une évaluation des pertes potentielles ou transformations inter-espèces ayant pu survenir durant la lyophilisation a été réalisée.

Dans un souci de clarté et afin d'alléger ce paragraphe, la description des conditions expérimentales appliquées a été faite en annexe (Annexe I).

IV.2.2 Résultats expérimentaux et discussion

Les résultats de l'étude, obtenus par les deux partenaires, sont présentés dans le tableau 24. Les plages bleues correspondent aux résultats d'analyses menées sur les échantillons frais (étape 1), les plages blanches correspondent aux échantillons marqués isotopiquement puis lyophilisés (étape 2) et les plages orange représentent les échantillons lyophilisés puis marqués isotopiquement (étape 3).

Tableau 24 : Concentrations et écart-types en MeHg et HgT (mg kg^{-1}) des 2 laboratoires

	Analyses		MeHg	∑ Hg	% MeHg	M (%)	D (%)
Julienne	D-IDMS	CIME	0,462 ± 0,014	0,481 ± 0,017	96	-	-
	IPD		0,457 ± 0,005	0,464 ± 0,007	98	3 ± 6	1 ± 0,3
	D-IDMS	LCABIE	0,430 ± 0,009	0,531 ± 0,035	81	-	-
	IPD		0,435 ± 0,009	0,443 ± 0,019	98	-13 ± 5	23 ± 4
	D-IDMS	CIME	0,379 ± 0,036	0,410 ± 0,033	92	-	-
	IPD		0,368 ± 0,031	0,389 ± 0,023	95	-0,1 ± 4	3 ± 0,3
	D-IDMS	LCABIE	0,395 ± 0,020	0,472 ± 0,055	84	-	-
	IPD		0,393 ± 0,019	0,401 ± 0,022	98	2 ± 3	15 ± 7
	D-IDMS	CIME	0,510 ± 0,014	0,536 ± 0,017	95	-	-
	IPD		0,469 ± 0,005	0,481 ± 0,005	98	349 ± 127	-2 ± 1
	D-IDMS	LCABIE	0,387 ± 0,049	0,483 ± 0,028	80	-	-
	IPD		0,384 ± 0,047	0,390 ± 0,044	98	-1 ± 1	24 ± 9
Thon	D-IDMS	CIME	0,880 ± 0,028	0,912 ± 0,029	96	-	-
	IPD		0,899 ± 0,007	0,930 ± 0,012	97	-127 ± 6	5 ± 0,2
	D-IDMS	LCABIE	0,811 ± 0,010	0,939 ± 0,020	86	-	-
	IPD		0,809 ± 0,007	0,838 ± 0,012	100	-1 ± 1	13 ± 6
	D-IDMS	CIME	0,768 ± 0,023	0,817 ± 0,022	94	-	-
	IPD		0,762 ± 0,008	0,794 ± 0,004	96	59 ± 70	-0,002 ± 0,12
	D-IDMS	LCABIE	0,723 ± 0,048	0,820 ± 0,043	88	-	-
	IPD		0,719 ± 0,038	0,738 ± 0,038	97	-0,4 ± 2	10 ± 4
	D-IDMS	CIME	0,904 ± 0,040	0,949 ± 0,045	95	-	-
	IPD		0,914 ± 0,081	0,953 ± 0,092	96	90 ± 243	-2 ± 1
	D-IDMS	LCABIE	0,708 ± 0,026	0,772 ± 0,033	92	-	-
	IPD		0,715 ± 0,034	0,738 ± 0,038	97	-4 ± 3	-4 ± 3
Bar loup	D-IDMS	CIME	0,404 ± 0,022	0,424 ± 0,020	95	-	-
	IPD		0,405 ± 0,009	0,417 ± 0,011	97	-54 ± 73	2 ± 0,1
	D-IDMS	LCABIE	0,336 ± 0,020	0,426 ± 0,038	79	-	-
	IPD		0,330 ± 0,017	0,335 ± 0,011	99	-1 ± 3	26 ± 6
	D-IDMS	CIME	0,360 ± 0,001	0,377 ± 0,003	95	-	-
	IPD		0,354 ± 0,010	0,364 ± 0,010	97	8 ± 17	-0,3 ± 1
	D-IDMS	LCABIE	0,325 ± 0,015	0,364 ± 0,024	89	-	-
	IPD		0,324 ± 0,013	0,328 ± 0,013	99	-3 ± 5	10 ± 2
	D-IDMS	CIME	0,409 ± 0,014	0,428 ± 0,017	96	-	-
	IPD		0,378 ± 0,015	0,385 ± 0,014	98	660 ± 148	2 ± 0,3
	D-IDMS	LCABIE	0,316 ± 0,009	0,367 ± 0,029	86	-	-
	IPD		0,317 ± 0,01	0,320 ± 0,011	99	-2 ± 2	15 ± 5

Les taux de M calculés sont peu exploitables, principalement en raison d'écart-types importants, probablement dus aux grandes différences de concentrations entre l'iHg et le MeHg comme expliqué par Monperrus et al (2008) et précédemment dans la partie II paragraphe VI.4.

Les résultats obtenus par le laboratoire CIME n'indique aucune réaction de déméthylation, quelque soit la matrice étudiée ou la procédure analytique utilisée, à l'exception d'un taux de D de 5% noté pour le thon lors de l'analyse du produit frais. Par contre, les résultats du laboratoire LCABIE indiquent d'important taux de D (D moyen = 16%) sur l'ensemble des échantillons, à l'exception du thon lyophilisé puis spiké (D = -4 ± 3%). Des taux de D minimum de 10% pour le thon et le bar-loup spikés puis lyophilisés et un taux maximum de 26% pour le bar-loup frais sont observés. Le phénomène de D est constaté quelque soit la procédure analytique utilisée et la matrice étudiée, il semble donc que ces réactions soient liées à la méthode d'extraction MAE comme cela a été précédemment constaté lors de l'étude présenté dans l'article I.

La figure 42 présente les résultats de chaque matrice analysée en spéciation par les 2 laboratoires. Les résultats en rouge et bleu foncés correspondent aux concentrations en iHg et MeHg calculés par D-IDMS du laboratoire CIME et ceux en clairs correspondent aux résultats du laboratoire LCABIE calculés par IPD. L'appellation « Frais » correspond à l'étape 1, « Spikes + lyoph » à l'étape 2 et « Lyoph + spikes » à l'étape 3. Les barres vertes représentent les concentrations en mercure total des échantillons frais par une méthode ICP-MS accréditée (Noël et al., 2005).

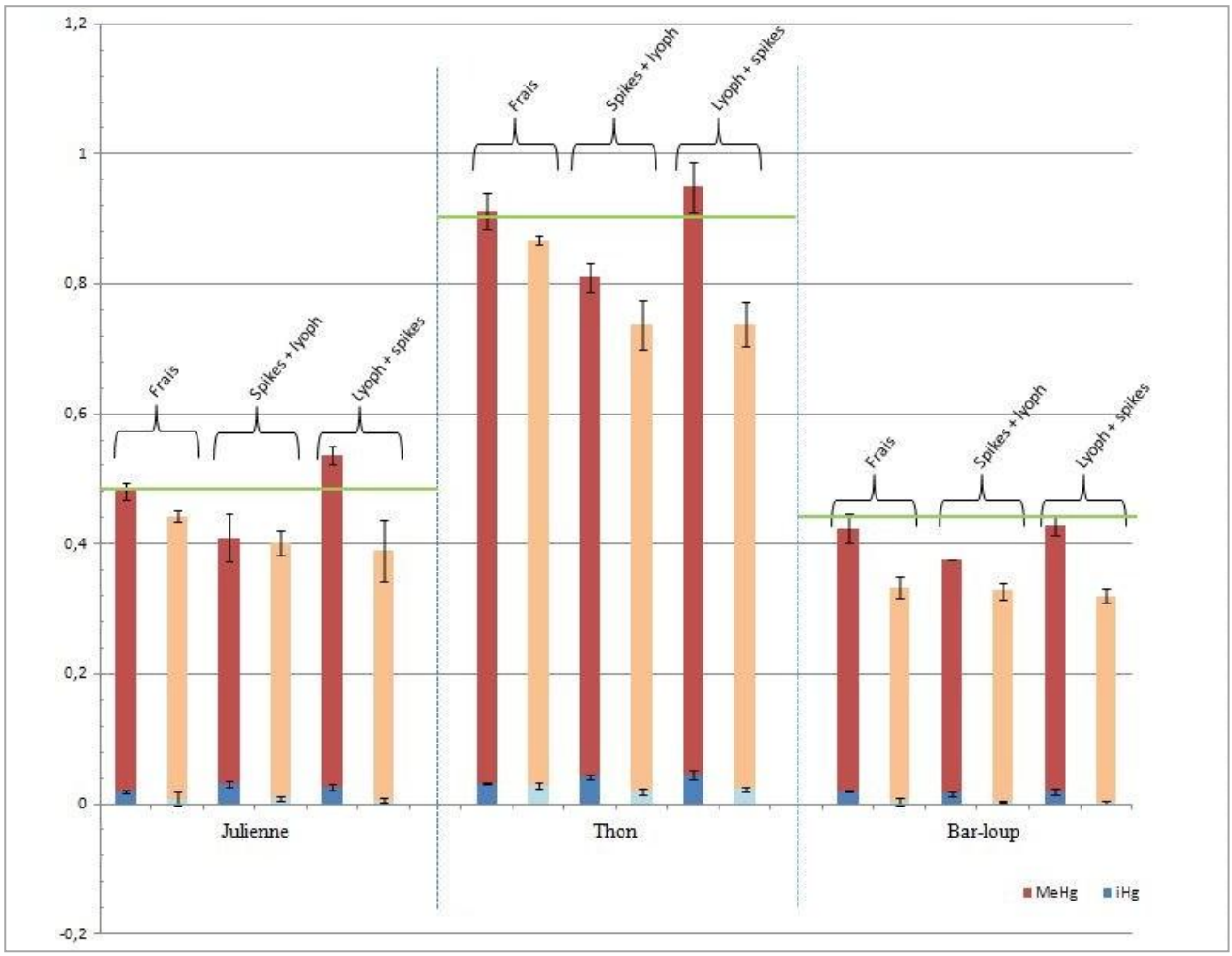

Figure 42 : Concentrations en iHg et MeHg (mg.kg^{-1}) dans les trois poissons analysés.

Concernant les résultats du laboratoire CIME, une diminution des teneurs en MeHg est observée sur les échantillons marqués isotopiquement puis lyophilisés (étape 2). Par contre, les teneurs en MeHg des échantillons frais (étape 1) et lyophilisés puis spikés (étape 3) sont similaires et la somme des espèces iHg et MeHg est proche de la valeur quantifiée en total. Si des pertes ou transformations inter-espèces étaient survenues au cours de l'étape de lyophilisation, une diminution des teneurs en espèces Hg aurait été observée au niveau de l'étape 3 où les spikes ont été ajoutés après lyophilisation et donc où les résultats finaux ne sont pas corrigés des éventuelles modifications ayant pu survenir au cours de ce traitement. Il ne semble donc pas y avoir d'influence de la lyophilisation sur les espèces Hg. Par contre, des diminutions des teneurs en espèces Hg sont constatées dans l'étape 2, où la lyophilisation est contrôlée par IDMS. Il est donc probable que la perte apparente observée en MeHg lors de l'étape 2 soit en fait due à une quantification faussée liée à un équilibre isotopique incomplet entre les spikes et les espèces endogènes. Il faut toutefois noter que cela ne remet pas en question la méthode analytique développée et validée lors de ces travaux

de thèse car dans le cas présent, l'équilibre isotopique doit se faire entre une matrice fraîche et des spikes en solution, dans un milieu où le solvant d'extraction est absent.

Pour le laboratoire LCABIE, une lègére décroissance des teneurs en MeHg est observée entre l'étape 1 et les étapes 2 et 3, uniquement significative sur le thon avec des valeurs similaires entres les étapes 2 et 3. Ces pertes en espèces Hg étant constatées pour l'étape 2 mais également pour l'étape 3, elles ne peuvent pas être imputables à la lyophilisation, qui ne semble pas modifier pas la distribution des espèces Hg. Ces pertes semblent plutôt liées à la nature de la matrice, les matrices lyophilisées réagissant différemment des matrices fraîches face à la méthode analytique utilisée par le laboratoire..

Pour les deux laboratoires, les teneurs en HgT des échantillons lyophilisés sont en accord avec les teneurs en HgT des échantillons frais. Cela renforce l'hypothèse stipulant qu'il ne semble pas y avoir de pertes d'espèces durant le processus de lyophilisation.

Les différences observées entre les 2 laboratoires s'expliquent probablement par la différence des méthodes de lyophilisation utilisées (masses et support de lyophilisation différents) (Cf. Annexe I). Ces résultats semblent confirmer ceux de Yu et Yan, (2003) qui concluent que les résultats observés sur des échantillons frais ou lyophilisés étaient équivalents sauf si les échantillons frais subissaient une congélation – décongélation trop répétée.

IV.2.3 Conclusion

Cette étude a été menée conjointement par les laboratoires LCABIE et CIME et les résultats obtenus laissent supposer qu'il ne semble donc pas y avoir de pertes ni de modification d'espèces durant le processus de lyophilisation. Néanmoins, afin de pouvoir confirmer cette hypothèse, cette étude nécessite la participation d'un plus grand nombre de laboratoires et la mise en place d'une procédure analytique unique. Idéalement, l'étude d'un plus grand nombre de matrices permettrait d'avoir une vision plus globale de l'éventuelle influence de la lyophylisation sur les produits de la pêche. A ce jour, nous avons décidé de poursuivre nos travaux en analysant les échantillons lyophilisés des produits de la pêche de l'EAT 2 plutôt que les échantillons frais (EAT 2, 2011).

IV.3 Etude des échantillons lyophilisés de l'EAT 2

IV.3.1 Contexte de l'étude

En 2000, Le ministère de l'agriculture et de la pêche ainsi que l'institut national de recherche agronomique (INRA) ont décidé d'implanter en France la méthode dite « d'étude de l'alimentation totale (EAT) », méthode standardisée et recommandée par l'Organisation Mondiale de la Santé (OMS) et qui vise à rechercher diverses substances chimiques susceptibles d'être présentes dans les aliments « tels que consommés ». Les EAT sont des études nationales de surveillance des expositions alimentaires qui évaluent les risques liés à des substances chimiques d'intérêt en termes de santé publique. Elles sont construites, d'après des enquêtes de consommation, à partir de paniers représentatifs des consommations alimentaires des français.

Les EAT apportent des informations sur la composition et/ou la contamination des aliments, ainsi que sur leurs apports nutritionnels et permettent une évaluation des risques sanitaires des populations liée aux aliments étudiés. Une prise de décisions en matière de contrôle, de réglementation des produits chimiques et de sécurité des produits alimentaires, aussi bien au niveau national, qu'européen ou international est alors faite. Pour le gestionnaire du risque, ces études apportent les éléments scientifiques permettant de réviser ou d'établir une réglementation afin de réduire l'exposition du consommateur. Dans le cas d'une substance bénéfique, elles orientent les recommandations de consommation permettant d'optimiser les apports.

Une première EAT française (EAT 1) a été réalisée entre 2000 et 2004 par l'INRA, en collaboration avec l'AFSSA (Leblanc et al., 2005) et une seconde EAT (EAT 2) a été entreprise par l'AFSSA en 2006, incluant un nombre plus large de substances à rechercher et l'ensemble des régions administratives du territoire métropolitain (contre 3 grandes villes dans l'EAT 1). L'élaboration des paniers représentatifs des consommations alimentaires des français de l'EAT 2 s'appuie sur des données de l'enquête INCA 2 (seconde enquête individuelle et nationale sur les consommations alimentaires) réalisée en 2006-2007.

Dans ces travaux, les échantillons lyophilisés et non dégraissés de produits de la pêche de l'EAT 2 ont été étudiés. 34 échantillons de mollusques et crustacés (10 moules, 4 huîtres, 16 crevettes et 4 coquilles Saint-Jacques) et 28 échantillons de poissons (6 thons, 16 saumons, 6 lieu-colins) ont été analysés par D-IDMS et D-SS-IDMS et par GC-ICP-MS. Les matériels et méthodes utilisés ainsi que les résultats obtenus sont discutés dans l'article II. Les matériels et méthodes utilisées sont discutés dans l'article II et des informations complémentaires relatives à la procédure de traitement d'échantillon ont été ajoutées en annexe (Annexe II). Il est à noter que les résultats de 3 échantillons de poissons panés non présentés dans cet article indiquent des concentrations très faibles de MeHg ($[MeHg_{moy}] = 24\ \mu g.kg^{-1}$ avec % MeHg ≈ 100%).

IV.3.2 Résultats expérimentaux

Les résultats de cette étude sont présentés dans l'article II.

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ORIGINAL PAPER

Mercury speciation analysis in seafood by species-specific isotope dilution: method validation and occurrence data

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Abstract Methylmercury (MeHg) and total mercury (THg) in seafood were determined using species-specific isotope dilution analysis and gas chromatography combined with inductively coupled plasma mass spectrometry. Sample preparation methods (extraction and derivation step) were evaluated on certified reference materials using isotopically enriched Hg species. Solid–liquid extraction, derivation by propylation and automated agitation gave excellent accuracy and precision results. Satisfactory figures of merit for the selected method were obtained in terms of limit of quantification (1.2 μg Hg kg^{-1} for MeHg and 1.4 μg Hg kg^{-1} for THg), repeatability (1.3–1.7%), intermediate precision reproducibility (1.5% for MeHg and 2.2% for THg) and trueness (bias error less than 7%). By means of a recent strategy based on accuracy profiles (β-expectation tolerance intervals), the selected method was successfully validated in the range of approximately 0.15–5.1 mg kg^{-1} for MeHg and 0.27–5.2 mg kg^{-1} for THg. Probability β was set to 95% and the acceptability limits to ±15%. The method was then applied to 62 seafood samples representative of consumption in the French population. The MeHg concentrations were generally low (1.9–588 μg kg^{-1}), and the percentage of MeHg varied from 28% to 98% in shellfish and from 84% to 97% in fish. For all real samples tested, methylation and demethylation reactions were not significant, except in one oyster sample. The method presented here could be used for monitoring food contamination by MeHg and inorganic Hg in the future to more accurately assess human exposure.

Keywords Speciation · Methylmercury · Seafood · GC/ICP-MS · Species-specific isotope dilution analysis

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Introduction

Mercury (Hg) is a toxic compound that can contaminate humans through food, particularly via fish consumption. Seafood is the first source of human contamination by methylmercury (MeHg), the most toxic form of Hg. Over 90% of MeHg is absorbed through the gastrointestinal tract and rapidly transferred into the bloodstream due to its high lipophilicity [1]. MeHg is able to cross the blood–brain and placenta barriers, which make it neurotoxic and teratogenic [1]. The neurotoxicity of MeHg has been known since the poisoning of the population in Minamata, Japan, in the 1960s and since two other massive contamination episodes in Niigata, Japan, and Iraq [2].

As a safeguard for human health, guidelines and regulations stipulating maximum permissible levels of mercury in fish (0.50 or 1 mg kg^{-1} essentially for predatory fish) and seafood (0.50 mg kg^{-1}) have been set by Regulation (EC) No. 629/2008 [3] to limit dietary exposure of consumers. In 2003, the Joint FAO/WHO Expert

Committee on Food Additives (JECFA) established a provisional tolerable weekly intake (PTWI) of 1.6 μg MeHg/kg bw and of 5 μg total mercury (THg)/kg bw [4]. The PTWI for THg was withdrawn in 2010 by the JECFA and replaced by a PTWI for inorganic mercury (Hg(II)) of 4 μg (Hg)/kg bw/week [5]. The new PTWI for inorganic mercury is considered to be applicable for dietary exposure to THg from foods other than fish and shellfish. For dietary exposure to mercury from fish and shellfish, the previously established PTWI for MeHg should be applied.

The methods developed to perform Hg speciation analysis involve coupling a powerful separation technique (liquid or gas chromatography, capillary electrophoresis) with a selective and sensitive (elemental) detection method (fluorescence spectrometry, atomic emission spectrometry or mass spectrometry) [6, 7]. Gas chromatography combined with inductively coupled plasma-mass spectrometry (GC/ICP-MS) has rapidly dominated the field of trace and ultra-trace elemental analysis [8–10], despite the availability of cheaper, sufficiently powerful, but less versatile competing technologies (e.g. pyrolyzer-atomic fluorescence spectrometry, microwave-induced plasma-atomic emission spectrometry and electron ionisation mass spectrometry) [6, 7, 11]. The GC/ICP-MS technique has the high resolving power of capillary GC technology, which offers excellent separation of Hg species, and the sensitive multi-elemental detection capabilities of ICP-MS. Furthermore, this system of detection offers the possibility to perform speciated isotope dilution mass spectrometry [7, 12].

Most of developed sample preparation method involves a lyophilisation/homogenisation step, extraction and/or enrichment of the matrix, derivatisation and occasionally sample clean-up. Each step is critical to the viability and comparability of final results due to the possible occurrence of methylation (M) and demethylation (D) reactions during sample preparation [7, 10, 12]. The main extraction methods used are solid/liquid extraction (SLE), microwave-assisted extraction (MAE), extraction using sonication (US) and alternative methods such as solid-phase micro-extraction (SPME) [8, 9, 13]. However, few analytical methods have been optimised with supercritical fluid extraction (SFE), pressurised liquid extraction (PLE) or stir-bar sorptive extraction (SBSE) [9, 13]. In the last decade, MAE and US extraction techniques have been increasingly used because they are rapid, efficient and have an excellent recovery rate; furthermore, they require only small amounts of solvents.

Recently, the development of multiple isotope dilution procedures (M-IDA) has drastically improved the accuracy and quality of Hg speciation analysis [7, 8, 12]. The sample is spiked with two isotope tracers (^{199}Hg(II) and ^{201}MeHg) that react identically to the studied endogenous species, thereby controlling all losses or transformation reactions (i.e. M and D) that may occur during the analytical procedure. The quantification is based on measuring the altered isotopic ratio; quantitative extraction is therefore not necessary. Provided that the isotopically enriched exogenous species and the isotopically natural endogenous species are present in an equilibrated and equivalent state, spikes play the role of 'ideal' internal standards, reacting and transforming as species under investigation [12]. Consequently, because spikes are added in a known amount, artefacts can be observed, quantified and corrected. M-IDA is the only technique of quantification by which the analytical procedure can be monitored with high precision, thereby providing excellent accuracy.

The objectives of this work were first to optimise the analytical procedure among different sample preparation techniques (comparison of three techniques of extraction, two techniques of derivation and three techniques of agitation) and three methods for calculating isotope dilution for the determination of MeHg and Hg(II) in seafood samples. Second, the figures of merit of this optimised method were evaluated in terms of its limits of quantification (LOQ), accuracy, repeatability and intermediate precision reproducibility. Third, samples of the second French Total Diet Study (TDS) were analysed to assess MeHg levels and transformation rates according to tested matrices and to provide data on the occurrence of Hg in fish and shellfish.

Experimental

Instrumentation

GC/ICP-MS analysis was performed with an 6890N gas chromatographic system equipped with an on-column injector (Agilent, Massy, France), a MXT-1 guard column and a MXT-1 column (30 m, i.d. 0.53 mm; *df*, 1 μm; Restek, Lisses, France). The chromatographic system was coupled to an X-Series^{II} ICP-MS instrument via a commercial GC/ICP-MS interface (all Thermo Scientific, Courtaboeuf, France). This instrument configuration accepts dual sample introduction (gaseous samples and liquid standards). Torch position and ion lenses of the ICP-MS system were optimised daily by performing short-term stability tests with a 1-μg L^{-1} tuning solution (containing especially mercury (Hg) and indium (In)) to maximise Hg signal and stability. Mass bias was evaluated by measuring the ^{203}Tl/^{205}Tl ratio during each chromatographic run. Signals were monitored in the Time Resolved Analysis (TRA) mode of the ICP software. Further details of instrument settings are given in Table 1. Other equipment include the following: a closed-vessel microwave digestion system (Multiwave, Anton-Paar, Courtaboeuf, France) equipped with 80 mL quartz vessels (80 bar

Table 1 Instrument operating conditions for the GC/ICP-MS system

ICP-MS parameters	
Gas flow rate	
Plasma	15 L min^{-1}
Auxiliary	0.9 L min^{-1}
Nebulizer	0.5 L min^{-1}
Plasma power	1,450 W
Isotopes; dwell times	Hg (202, 201, 200, 199 and 198); 30 ms Tl (205 and 203); 5 ms
GC parameters	
Column	MXT Silcosteel 30 m: i.d. 0.53 mm; *df*, 1 µm
Injection port	On-column
Injection temperature	
Initial temperature	75 °C
Initial time	0 min
Ramp rate	120 °C min^{-1}
Final temperature	250 °C
Injection volume	1 µL
Carrier gas flow	He 25 ml min^{-1}
Make-up gas flow	Ar 300 ml min^{-1}
Oven temperature	
Initial temperature	60 °C
Initial time	1 min
Ramp rate	60 °C min^{-1}
Final temperature	280 °C
Transfer line temperature	280 °C

operating pressure), a *Digi*PREP digestion system (SCP Science, Courtaboeuf, France) equipped with 50 mL *Digi*tubes, a REAX 2 rotary shaker (Heidolph, Saint Herblain, France), a Seven easy pH meter, an AT460 Delta range balance (all Mettler Toledo, Viroflay, France) and a Universal 32R centrifuge (Hettich, Tuttlingen, Germany).

Chemicals

All solutions were prepared with analytical reagent-grade chemicals and ultra-pure water (18 MΩ cm) generated by purifying distilled water with the Milli-Q™ PLUS system combined with an Elix 5 pre-system (Millipore S.A., St. Quentin-en-Yvelines, France). Methanol (HPLC gradient grade, Sigma-Aldrich, St. Quentin Fallavier, France), nitric acid (Suprapur, 67%, Merck), hydrochloric acid (Trace analysis grade, 37%, Fischer Scientific) and ammonia solution (Suprapur, 25%, Merck, Darmstadt, Germany) were used to stabilise species or to adjust pH. Standard solutions of the individual Hg species with an Hg concentration of 1,000 mg (Hg) L^{-1} were prepared by dissolving mercury (II) chloride (≥99.999%, Sigma-Aldrich) in 1% hydrochloric acid and MeHg chloride (Pestanal grade, Sigma-Aldrich) in methanol. Each stock solution was further diluted to 100 or 10 mg L^{-1}. Enriched standard solutions of the individual Hg species with an Hg concentration of 100 or 10 mg (Hg) L^{-1} were prepared by diluting Hg standard solutions enriched in ^{199}Hg (91.71%, ISC Science, Oviedo, Spain) or $^{201}MeHg$ enriched mercury (96.5%, ISC Science) in 1% hydrochloric acid. Stock solutions were stored in the dark at 4 °C to prevent decomposition or oxidation. Tetramethylammonium hydroxide (TMAH, Purum, 25%, Sigma-Aldrich) was used as solvent extraction. Buffer solution of pH 4 was prepared by dissolving sodium acetate in Milli-Q water and adjusting to pH 4 with glacial acetic acid. One percent (*w*/*v*) solution of sodium tetrapropylborate ($NaBPr_4$) used for derivatisation was prepared every 6 h and kept in the dark. Isooctane (analytical reagent grade, Merck) was used for the analyte transfer phase. An internal standard solution was prepared with 1,000 mg L^{-1} standard stock solutions of indium (In), purchased from Analytika (Prague, Czech Republic) to check the sensitivity of the couple and the stability of the signal. Inorganic thallium was obtained from Spex Certiprep (Metuchen, NJ, USA) and used to correct for mass bias during the mercury isotope ratio measurements.

Reference materials

Certified reference materials (CRMs) DOLT-4 (Dogfish liver) and TORT-2 (Lobster hepatopancreas) from the National Research Council of Canada (CNRC) and BCR-464 (Tuna fish) from the Institute for Reference Materials and Measurements (IRMM) were purchased from Promochem (Molsheim, France). All samples were used as provided without further grinding.

Seafood samples

All the seafood composite samples from the second French TDS were analysed: fish (saithe, salmon, smoked salmon, tuna and canned tuna), mollusc (mussel, oyster and scallop) and crustacean (shrimp) products. Each of the samples was composed of up to 15 sub-samples of equal weight of the same food item and was prepared 'as normally consumed'. Only the edible part was used to prepare the sample (i.e. inedible parts fish, bones, fish skin, shells etc. were discarded). The core foods were then prepared as consumed (i.e. smoked salmon or steamed fresh salmon, oven-cooked fresh tuna or canned tuna in oil or brine, cooked saithe, raw oysters, boiled shrimp and mussels and steamed scallops). Therefore, in the TDS study, the impact of home-cooking was taken into account with regard to possible decomposition of less stable chemicals and the formation of new ones [14].

Total mercury determination

Aliquots of ~0.150–0.300 g sample were separately weighed in quartz vessels in duplicate, and 3 mL nitric acid and 3 mL ultra-pure water were added. The digestion program was performed as described previously [15]. After cooling, sample solutions were quantitatively transferred into calibrated 50-mL polyethylene flasks. Before final dilution with water to 50 mL, 1 mg L^{-1} internal standard solution (Re, Bi) was added to a final concentration of 2 μg L^{-1} to allow drift correction and to compensate for possible matrix effects. Total Hg concentration in extracted samples was determined by ICP-MS according to a validated and accredited "in-house" method [16]. Quantification was performed by external calibration using five aqueous Hg standard solutions from 0 to 20 μg L^{-1}.

Mercury speciation

In a recent study, the spiking procedure (before or after extraction) and the derivatisation reagent (ethylation or propylation) effect were evaluated. The results on CRMs indicated that concentration values were slightly more accurate to certified values when using tetrapropylborate and that the spike should be added before the extraction in order to correct for possible losses/transformations of the species during the extraction (Navarro et al., submitted). Therefore, propylation and spiking prior to extraction were used in this study to evaluate three different extractions and three different agitation methods.

For each extraction method tested, aliquots of approximately 0.250 g freeze-dried samples were separately weighed in appropriate vessels (*Digi*tubes, microwave digestion vessels or polyethylene flasks for the three extraction methods, see below), and appropriate weighed amounts of ^{199}Hg(II) and ^{201}MeHg were added so that the resulting isotope ratios of $^{199}Hg/^{202}Hg$ for Hg(II) and $^{201}Hg/^{202}Hg$ for MeHg were close to unity. Then, 5 mL of TMAH 25% (*w*/*v*) was added, and one of the three following extraction methods was used:

1. Solid–liquid extraction (SLE): *Digi*tubes were closed and placed into the digestion system. The samples were heated and maintained at 85 °C for 2 h.
2. Microwave-assisted extraction (MAE): The vessels were closed and placed into the microwave system. The samples were irradiated at 60 W for 3 min.
3. Extraction at room temperature (ERT): The tubes were closed and placed under the hood. The samples were left 24 h without agitation or heating.

After cooling, an aliquot of extracts produced from each of the three extraction methods was transferred into 22-mL glass tubes containing 5 mL of acetate buffer solution of pH 4, and the pH was adjusted to 4 by adding HCl. Species were simultaneously derivatised and extracted by adding 0.5 mL of 1% $NaBPr_4$ (*w*/*v*) and 1 mL of isooctane. The mixture was then manually shaken for 5 min, vortexed for 5 min or placed in a rotary shaker to be agitated for 7 min. The samples were centrifuged (2,500 rpm for 5 min), and the organic phase was transferred to an injection vial and, if necessary, stored at −18 °C until GC/ICPMS analysis. Each sample was extracted three times, and each extract was injected in triplicate.

The GC separation was performed at a He flow rate of 25 mL min^{-1}, using a gradient temperature of between 60 °C and 280 °C (Table 1). For quantification using peak area, the chromatographic software (PlasmaLab) of the ICP-MS instrument was used. Isotope ratios $^{199}Hg/^{202}Hg$ and $^{201}Hg/^{202}Hg$ were measured for both Hg species. Concentrations of unknown species and transformation factors (i.e. methylation and demethylation) were estimated using species-specific isotope dilution quantification as previously described [12].

Calculations and statistical methods

The concentrations of mercury species are expressed in milligrammes of Hg per kilogramme of dry mass for certified reference materials or in microgrammes of Hg per kilogramme of fresh mass for real samples. The calculated average moisture of the 62 seafood samples was 71%. All TDS samples were analysed in triplicate. When concentrations were below the limit of detection (LOD) or LOQ, the values were taken as being equal to the LOD or LOQ, respectively.

Results and discussion

Comparison of extraction procedures

As a compromise between extraction efficiency, duration and Hg species integrity, three common extraction procedures (MAE, SLE using a digestion system and ERT) were compared in terms of accuracy, precision and interspecies transformation. These procedures were selected because they greatly differ in their duration and their power. Indeed, MAE is very fast but aggressive, SLE requires more time, but is gentler and ERT is not aggressive but is time-consuming. Table 2 shows the concentrations of extracted MeHg and THg (∑MeHg+Hg(II)) in three CRMs (TORT-2, DOLT-4 and BCR-464). MeHg and THg concentrations obtained by the three extraction procedures were generally close to the certified values, with slightly lower standard deviations obtained by SLE, indicating better reproducibility

Table 2 MeHg and THg concentrations (milligrammes per kilogramme; ±SD) calculated by double species-specific isotope dilution analysis for each extraction condition in three certified reference materials

		MeHg	THg	*M* (%)	*D* (%)
TORT-2	Certified values	0.152±0.013	0.270±0.060	–	–
	SLE	0.160±0.017	0.285±0.032	3	3
	ERT	0.189±0.029	0.324±0.050	7	4
	MAE	0.152±0.036	0.328±0.052	4	9
DOLT-4	Certified values	1.33±0.12	2.58±0.22	–	–
	SLE	1.35±0.13	2.44±0.19	−1	2
	ERT	1.45±0.27	2.56±0.23	4	2
	MAE	1.53±0.25	2.60±0.30	−6	2
BCR-464	Certified values	5.12±0.17	5.24±0.10	–	–
	SLE	5.24±0.39	5.30±0.39	−191	0
	ERT	5.27±0.98	5.45±0.89	−248	4
	MAE	5.10±0.45	5.16±0.46	−27	1

M (percent) is the percentage of methylation (transformation of Hg(II) to MeHg), and *D* (percent) is the percentage of demethylation (transformation of MeHg to Hg(II)) ($n=5$)

and repeatability. More transformation reactions were observed when ERT was used, with a maximum of 7% of methylation in TORT-2. For the same CRM, 9% of demethylation after MAE was observed. Methylation rates for BCR-464 suggest that a high quantity of Hg(II) was methylated under the three extraction methods used, but these aberrant rate factors may be due to the low Hg(II) concentration (0.12 mg kg^{-1}) compared to MeHg (5.12 mg kg^{-1}), as previously explained [17]. After SLE, transformation reactions were generally negligible (<2%) except for TORT-2 where 3% of methylation and demethylation were observed, although their rates were very low. Therefore, SLE was chosen as the best compromise between duration, precision and induced transformation for the remainder of this work.

Comparison of the modes of agitation

To make the derivatisation step easier and more repeatable, the conventional technique of agitation (i.e. rigorous manual agitation (RMA) for 5 min) was compared with two other techniques: vortex agitation (VA) for 5 min and rotary agitation (RA) for 7 min. The time used for rotary agitation was first optimised by analysing DOLT-4 (between 7 and 15 min, data not shown). Since the results obtained using the three modes of agitation on the three CRMs ($n=6$ on different days) indicated no significant differences (Table 3) and no significant transformation reactions, rotary agitation was chosen for the remainder of this work.

Comparison of quantification methods

Three types of quantification methods, i.e. double isotope dilution analysis (D-IDA) [18], double species-specific isotope dilution analysis (D-SS-IDA) [17] and isotope pattern deconvolution (IPD) [11], were assessed on TORT-2, DOLT-4 and BCR-464. D-IDA determines Hg (II) and MeHg concentrations separately and therefore corrects for lack of recovery and analyte losses, but not for any potential transformation reactions [12]. D-SS-IDA and IPD methodologies provide both Hg(II) and MeHg concentrations simultaneously, and are generally used to control M and D reactions during the analytical procedure [11, 17]. Table 4 shows the concentrations of extracted MeHg and THg (∑MeHg+Hg(II)) in the three CRMs. MeHg and THg concentrations obtained by the three quantification methods matched certified values. Potential transformation reactions quantified by D-SS-IDA and IPD were not significantly different and, for all CRMs, M and D reactions were negligible or very low (<4%). Consequently, D-SS-IDA or IPD mathematical models can be used when information on M and D reactions is needed.

Table 3 MeHg and THg concentrations (milligrammes per kilogramme; ±SD) for each agitation conditions in three certified reference materials ($n=6$)

		MeHg	THg
TORT-2	Certified value	0.152±0.013	0.270±0.060
	RMA	0.165±0.007	0.279±0.010
	VA	0.166±0.009	0.284±0.009
	RA	0.163±0.005	0.275±0.008
DOLT-4	Certified value	1.33±0.12	2.58±0.22
	RMA	1.41±0.04	2.60±0.04
	VA	1.37±0.05	2.61±0.07
	RA	1.35±0.04	2.53±016
BCR-464	Certified value	5.12±0.17	5.24±0.10
	RMV	5.24±0.09	5.35±0.09
	VA	5.12±0.08	5.24±0.08
	RA	5.17±0.08	5.27±0.08

Table 4 MeHg and THg concentrations (milligrammes per kilogramme; ±SD) obtained on certified reference materials

		MeHg	THg	*M* (%)	*D* (%)
TORT-2	Certified values	0.152±0.13	0.270±0.060	–	–
	D-IDA	0.165±0.007	0.292±0.013	–	–
	IPD	0.160±0.002	0.272±0.004	4	3
	D-SS-IDA	0.164±0.017	0.288±0.023	0	2
DOLT-4	Certified value	1.33±0.12	2.58±0.22	–	–
	D-IDA	1.29±0.09	2.51±0.05	–	–
	IPD	1.28±0.12	2.39±0.10	3	4
	D-SS-IDA	1.29±0.15	2.47±0.29	−1	3
BCR-464	Certified values	5.12±0.17	5.24±0.10	–	–
	D-IDA	5.10±0.30	5.24±0.35	–	–
	IPD	5.18±0.35	5.28±0.35	−196	3
	D-SS-IDA	5.27±0.33	5.33±0.33	−65	1

M (percent) is the percentage of methylation (transformation of Hg(II) to MeHg), and *D* (percent) is the percentage of demethylation (transformation of MeHg to Hg(II)) (n=3)

Validation parameters

The optimised conditions for the analysis of mercury speciation in seafood samples by GC/ICP-MS were used to validate the quantification method by D-IDA. The criteria performance of the speciation method, including LOQs, repeatability and intermediate precision reproducibility were defined. Accuracy profiles estimated according to two standards are presented in Fig. 1 [19].

Limits of quantification

The LODs and LOQs (in microgrammes (Hg) per kilogramme) were defined, respectively, as three and six times the standard deviation of the average from the spiked blank samples (n=21) quantified on different days over several months, following quantification of isotope dilution. So, the LOQs estimated in this study (MeHg, 1.2 μg Hg kg^{-1}; THg, 1.4 μg Hg kg^{-1}) were obtained under robust conditions. These LOQs were sometimes in good agreement or better than those previously reported for marine samples [11, 20, 21] and sometimes higher than those previously obtained under short-term conditions [17, 22, 23]. The large variability among reported LOQs can be attributed to differences in methods of evaluation, such as the number of blanks analysed and LOQs estimated under short- or long-term conditions.

Precision under repeatability and intermediate precision reproducibility conditions

Repeatability ($_r$) and intermediate precision reproducibility ($_R$) were evaluated on TORT-2, DOLT-4 and BCR-464 spiked with ^{199}Hg(II) and ^{201}MeHg. Repeatability was estimated by using the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. Repeatability variance (s_r^2) represents intra-series variation and is calculated following Eq. 1:

$$s_r^2 = \frac{\mathrm{SCE_r}}{I(J-1)} \quad (1)$$

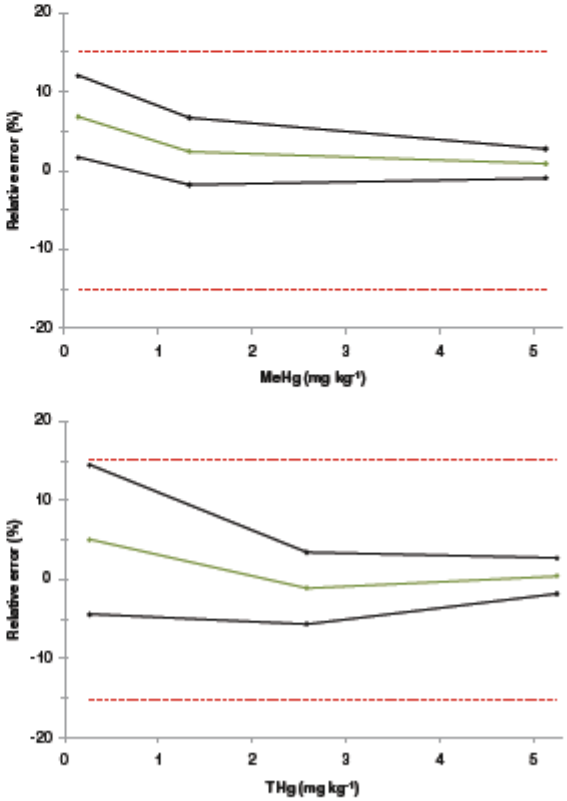

Fig. 1 Accuracy profile for MeHg and THg; β=95%, λ (acceptance limits)=±15%

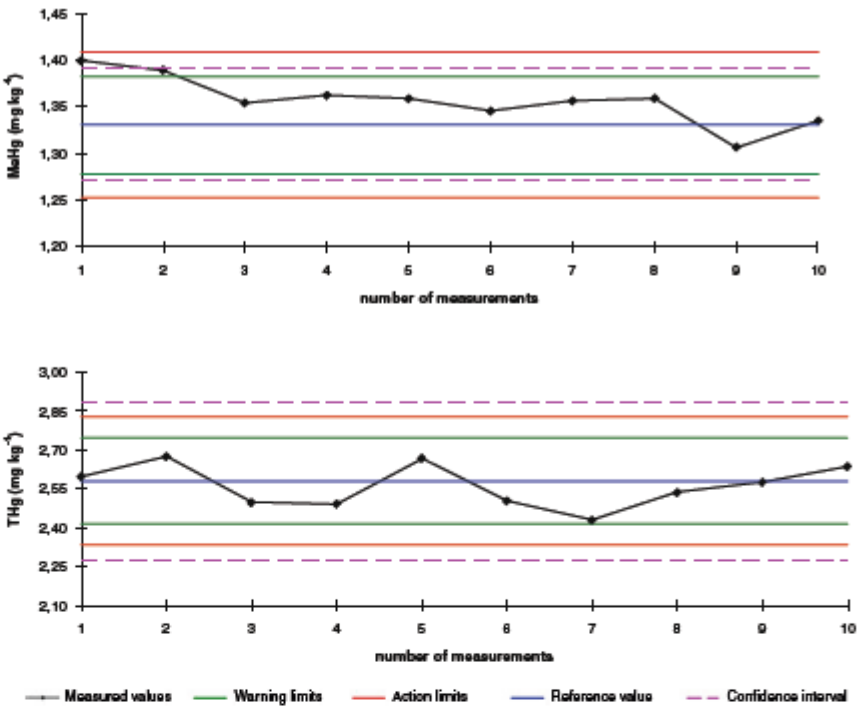

Fig. 2 Control charts for MeHg and THg in DOLT-4 (n=10). Warning limits were calculated as M±2 s, action limits as M± 3 s, confidence interval as M± (k×CV_R×M) where M is the certified value, s the standard deviation, k=3 (P=99%) and CV_R the intermediate precision coefficient of variation set to 1.5% for MeHg and 2.2% for THg

with SCE_r=sum of intra-series squared deviations from the mean, I=number of series analyzed and J=number of repetition.

Intermediate reproducibility variance (s_R^2) was estimated from results obtained with the same method in the same laboratory within long intervals of time. Intermediate reproducibility variance was calculated as $s_R^2=s_L^2+s_r^2$. s_L^2 is the variance that measures sample variations and is calculated following Eq. 2:

$$s_L^2 = \frac{\frac{SCE_L}{I-1} - s_r^2}{J} \quad (2)$$

with SCE_L=sum of inter-series squared deviations from the mean.

Repeatability was estimated by analysing the CRMs in duplicate on the same day by the same operator (n=6). The mean estimated CV_r were 1.3% and 1.7% for MeHg and THg, respectively. The intermediate precision reproducibility was investigated on the same CRMs analysed six times in duplicate on different weeks, over a period of 6 months. The average CV_R values were 1.5% and 2.2% for MeHg and THg, respectively. Compared to the literature, CV_r and CV_R were in good agreement or better than those previously reported in marine samples [20, 21, 23].

Accuracy profile

A recent validation strategy based on the accuracy profiles was applied to demonstrate the ability of the tested method to quantify MeHg, Hg(II) and THg [19, 24]. The probability β was set to 95%, which means that, on average, 95% of the future results will fall in the computed tolerance intervals. The acceptance limits were set to ±15%,

Table 5 Results from proficiency test schemes

		MeHg	Z-score	THg	Z-score
FAPAS 07136	Assigned value	0.677±0.115		0.774±0.129	
Canned Fish	Result	0.767	0.8	0.841	0.6
IMEP-109	Assigned value	1.33±0.20		2.58±0.39	
Fish	Result	1.21	−0.6	2.74	0.3

Table 6 Concentrations of Hg in shellfish (micrograms per kilogramme wet mass) as determined by GC/ICP-MS and ICP-MS

	GC/ICP-MS			ICP-MS		
	Hg(II)	MeHg	∑Hg species	%MeHg	THg	Recovery (%)
Mussels	8.3	5.0	13	37	15	87
	3.3	8.6	12	73	12	99
	8.0	9.8	18	55	15	116
	13	5.8	19	30	23	84
	6.0	8.3	14	58	15	96
	15	7.5	22	34	24	93
	7.9	7.7	16	49	16	98
	3.5	3.7	7.2	51	<8[a]	–
	5.2	26	32	84	32	97
	18	6.9	25	28	31	81
Mean (n=10)	8.8±5.0	8.9±6.3	18±7	50±19	19±8	95±10
Oysters	<0.70[a]	8.9	10	86	9.8	105
	3.9	7.2	11	65	15	73
	2.1	11	13	85	14	92
	<1.4[a]	9.0	10	90	13	78
Mean (n=4)	2.0±1.4	9.0±1.6	11±1	82±11	13±2	87±14
Great scallops	2.5	5.0	7.5	67	10	75
	4.2	9.5	14	69	14	98
	<1.4[a]	2.9	4.3	67	<8[a]	–
	2.0	1.9	3.9	49	<8[a]	–
Mean (n=4)	2.5±1.2	4.8±3.4	7.4±4.7	63±9	10±3	87±16
Shrimp	2.2	20	22	90	26	85
	1.8	30	32	94	34	95
	<1.4[a]	23	24	96	28	85
	2.2	20	22	90	27	81
	1.6	28	29	94	34	85
	<0.70[a]	27	28	98	28	99
	<1.4[a]	29	30	97	27	111
	3.5	27	30	89	32	95
	2.3	21	24	90	23	103
	<0.70[a]	33	34	98	38	89
	1.6	28	30	95	37	81
	<1.4[a]	33	34	97	29	116
	1.8	4.2	5.9	70	<8[a]	–
	1.5	31	32	95	27	119
	1.4	26	28	95	28	98
	<1.4[a]	25	26	96	25	103
Mean (n=16)	1.7±0.7	25±7	28±4	93±7	28±7	96±12
Total mean (n=34)	3.9	16	20	75	22	94
Median	2.2	10	22	86	24	95
P90	8.2	30	32	97	33	112
Minimum	<0.70	1.9	3.9	28	<8[a]	73
Maximum	18	33	34	98	38	119
n<LOQ	9	0	0	–	0	–

[a] Values below the LOD or LOQ

Table 7 Concentrations of Hg in fish (microgrammes per kilogramme wet mass) as determined by GC/ICP-MS and ICP-MS

	GC/ICP-MS			ICP-MS		
	Hg(II)	MeHg	∑Hg species	%MeHg	THg	Recovery (%)
Saithe	3.0	35	38	92	35	108
	4.4	74	78	94	72	109
	2.0	57	59	97	66	89
	2.0	47	49	96	58	85
	2.7	83	86	97	74	116
	7.7	73	81	91	82	98
Mean (n=6)	3.6±2.2	62±18	65±19	95±3	65±17	101±12
Tuna	54	588	642	92	727	88
	30	230	260	88	263	99
	33[a]	170	203	84	226	90
	14[a]	145	159	91	191	83
	16[a,b]	219	235	93	207	114
	10[a]	154	164	94	164	100
Mean (n=6)	26±16	251±169	277±183	90±4	296±214	96±11
Salmon	3.6	33	36	90	33	111
	2.0	29	31	93	27	117
	2.2	46	48	95	42	114
	2.1	28	30	93	25	120
	2.8	32	35	92	31	113
	3.9	39	43	91	42	102
	4.0	41	45	91	41	111
	2.0	48	50	96	42	119
	3.0	34	37	92	26	140
	1.8	39	41	96	39	106
	1.9	42	44	96	45	97
	3.2	37	40	92	42	95
	2.7	47	49	95	37	132
	1.4[b]	45	46	97	38	121
	3.2	48	51	94	42	121
	2.3	46	48	95	42	114
Mean (n=16)	2.6±0.8	40±7	42±7	94±2	37±7	115±12
Total mean (n=28)	7.9	90	97	93	99	108
Median	3.0	47	49	93	42	110
P90	20	185	213	96	213	121
Minimum	1.4	28	30	84	25	83
Maximum	54	588	642	97	727	140
n<LOQ	0	0	0	–	0	–

[a] Canned
[b] Smoked

which is lower than the regulatory requirements for food analysis. Figure 1 shows that tolerance intervals and trueness bias increased with decreasing concentrations for the three CRMs studied (TORT-2, DOLT-4 and BCR-464). The trueness bias ranged from 0.5% (BCR 464) to 5% (TORT-2) for THg and from 1% (BCR 464) to 7% (TORT-2) for MeHg. All the β-expectation tolerance intervals were comprised within the acceptability limits. Therefore, the analytical method can be declared as valid in the range of approximately 0.15–5.1 mg kg^{-1} for MeHg and 0.27–5.2 mg kg^{-1} for THg.

Uncertainty of measurement

Based on these CV_R values, uncertainties can be defined as twice the CV_R (percent; k=2, P=0.95). Therefore, uncertainties of the method were estimated on average at 3% and 4.4% for MeHg and THg, respectively.

Table 8 Comparison of the range (mean) and percentage levels of MeHg quantified in fish and shellfish (microgrammes Hg per kilogramme wet mass)

Group	Origin	Number species	Number samples	MeHg	∑Hg species	%MeHg	References
Fish	France	3	28	28–588 (90)	30–642 (97)	84–97 (93)	This work
	France	41	108	10–944 (169)	–	70–100	[29]
	Germany	32	536	6–567 (38)	–	14–100 (70)	[30]
	Slovenia	27	52	2–1,120 (127)	3–1,110 (150)	40–110 (80)	[31]
	Spain	14	25	54–596	–	–	[32]
	Italy	15	2,880	0–1,740 (314)	0–1,870 (356)	52–100 (88)	[33]
	Ghana	24	–	9–107	–	–	[34]
	Malaysia	2	69	(378)	(459)	70–82 (77)	[35]
	Hong Kong	89	280	3–1,010 (72)	3–1,370 (91)	–	[36]
	China	13	148	40–590 (260)	10–660 (180)	59–84 (74)	[37]
	India	7	–	8.0–16 (13)	8.7–17 (15)	71–95	[38]
	USA	9	–	(13–278)	(16–292)	93–98 (96)	[39]
	Canada	9	112	9–2,346 (342)	20–2,729 (542)	30–94 (64)	[40]
Shellfish	France	4	34	1.9–33 (16)	3.9–34 (20)	28–98 (75)	This work
	France	18	47	3–219 (54)	–	–	[29]
	Italy	1	–	15–51	35–115	33–91	[41]
	Brazil	4	14	3.8–37 (15)	3.8–40 (16)	–	[42]
	India	3	–	(34)	(48)	–	[38]
	China	3	–	17–24	–	–	[43]
	China	7	7	(56–84)	–	–	[44]

Application

Quality assurance

Following the proposed GC/ICP-MS procedure, sample solutions were analysed in batches including internal quality controls (IQC), e.g. a reagent blank, to monitor possible cross-contamination or memory effects [25], CRMs to check trueness, $^{203}Tl/^{205}Tl$ measurements to check mass bias or to evaluate detector dead time [12]. Concentrations and isotopic abundances of the spike solutions of $^{199}Hg(II)$ and $^{201}MeHg$ were checked for each set of analyses by reverse isotope dilution analysis [17]. The mean values obtained on DOLT-4 for MeHg and THg were 1.36 and 2.56 mg kg^{-1} with a relative standard deviation of 1.9% and 3.2%, respectively (n=10). Control charts indicate that the concentrations found were usually well within the confidence interval (CI; Fig. 2). Although the two first values of MeHg were in the warning limits (M±2 s), the action limits (M±3 s) were lower than the tolerance interval of the DOLT-4 certified value (1.33±0.12), demonstrating the accuracy and precision of this method.

External quality controls

To ensure and confirm the analytical trueness of the THg and Hg speciation methods, the laboratory regularly participated in proficiency test schemes (PTs) such as the Central Science Laboratory-Food Analysis Performance Assessment Scheme (CSL-FAPAS), the Community Reference Laboratory for Heavy Metals (Instituto Superiore di Sanità) or the Institute for Reference Materials and Measurements. The results for THg in fish and shellfish obtained by ICPMS were considered satisfactory with a Z-score in the range −2 and +2 [26]. The results for speciation analysis in two fish samples were also considered satisfactory (Table 5). No PTs on shellfish were available during this study.

Occurrence data

A selection of seven different seafood samples from the second French TDS (n=62) was analysed for their MeHg and THg content. The samples selected correspond to the most consumed species in the general French population and were prepared as consumed [14]. The average fat content for fish was 12.4% for smoked salmon, 11.8% for steamed salmon, 8% for canned tuna, 5% for oven-cooked tuna, 1.0% for saithe and for shellfish, 3.0% for boiled mussels, 1.4% for steamed scallops, 1.1% for boiled shrimp and 0.53% for raw oysters [27]. Each set of data were treated using D-IDA and D-SS-IDA because transformation reactions are matrix dependent [11, 12]. The individual results are presented in Table 6 for shellfish and Table 7 for fish (THg levels by ICP-MS). No significant M and D

factors were determined, except for one out of four oyster samples that presented a demethylation rate of 18% (data not shown). Furthermore, MeHg and THg concentrations obtained using the two modes of calculations were similar.

MeHg was quantified in all samples, but mainly at low concentrations. The average percentage of MeHg varied between 50% in mussel and 95% in saithe samples. Except in shrimp, the percentage of MeHg was generally more variable in the four shellfish species (range, 28–98%), notably in mussel samples (range, 28–84%), than in the three fish species analysed (range, 84–97%). Shellfish contained less MeHg than fish. A mean level of 16 μg MeHg kg^{-1} wet mass (wm) was found in shellfish, compared to 90 μg kg^{-1} in fish. The highest levels of MeHg in fish and shellfish were found in tuna (581 μg kg^{-1}) and in shrimp (33 μg kg^{-1}) samples, respectively. All concentrations fell much below the maximum levels set by Regulation (EC) No. 629/2008 [3], which is reassuring considering that these results were obtained from composite samples. In shellfish, shrimp was on average the most contaminated by MeHg (25 μg kg^{-1}), followed by oysters, mussels (9 μg kg^{-1}) and great scallops (5 μg kg^{-1}), while for the fish, tuna samples were on average the most contaminated by MeHg (249 μg kg^{-1}), followed by saithe (62 μg kg^{-1}) and salmon (40 μg kg^{-1}) samples. The recovery rates observed among THg levels quantified by a previously validated and accredited ICP-MS method for THg [16] and the sum of both MeHg and Hg(II) obtained by this speciation method indicated, on average, a good correlation between the two methods. Nevertheless, seven of 62 recovery rates were not found in the 80–120% range (corresponding to the confidence interval of the least precise ICP-MS method calculated with the CV_R of 10% at P=0.95). Ratios below 80% were observed for low-fat matrices and low concentrations in two oyster samples and one great scallop sample. Ratios above 120% were observed for fatty matrices on four salmon samples, with two values at 121%. However, only two ratio values on salmon (140% and 132%) exceeded the confidence interval of 70–130% calculated at P=0.99 (k=3). These concentrations were generally in good agreement with those found previously (on THg) in the first and the second French TDS [28].

Compared with recent previous studies dealing with occurrence data in food, the percentages of MeHg were generally greater in fish than in shellfish, and the highest levels of MeHg were found in predatory fish (Table 8) [29–44]. The levels observed in this study were similar [30, 36, 37, 39, 41–43] or lower [29, 31, 33, 35, 40, 44] than those found previously. The differences between studies are generally due to the number of predatory fish analysed and are also linked to the complex Hg biogeochemistry in the marine environment, e.g. due to the marine trophic chain in which the organism has been collected, the age and exposure time of each individual organism and also the amount of direct anthropogenic pollution prevailing in the seafood organism's ecosystem (mainly in the form of inorganic Hg) [45]. In fish, the mean MeHg percentages ranged from 64% to 96% (Table 8), with minimum percentages higher than 55%, except in some studies [30, 31, 33, 40]. According to Kuballa et al. [30] and Forsyth et al. [40], MeHg proportion varies widely and also could be dependent on the fish species. This is in contradiction with previous opinions who stated that virtually all (>95%) of the mercury present is in the form of MeHg, and this emphasizes that the use of a fixed conversion factor to estimate MeHg levels from total Hg determinations may not provide accurate exposure estimates. This reinforces the need to quantify directly Hg species by accurate and precise speciation methods to improve health risk assessments.

Conclusions

In this work, a recently developed strategy based on accuracy profiles was applied to demonstrate the ability of the GC/ICP-MS method to quantify MeHg and THg concentrations in the range of 0.15–5.12 mg kg^{-1} for MeHg and 0.27–5.24 mg kg^{-1} for THg (∑MeHg+Hg(II)). Figures of merit (LOQ, repeatability, intermediate precision reproducibility and trueness) of the proposed GC/ICP-MS procedure were satisfactory for the determination of MeHg, Hg(II) and THg in fish and shellfish. Data treatment using D-SS-IDA provided accurate results and effectively corrected for transformation reactions. The procedure was successfully applied to various real samples, e.g. mussels, oysters, shrimps and different types of more or less fatty fish.

In the future, this analytical method should be extended and used to estimate inorganic mercury in food matrices other than fish products to estimate dietary exposure to this Hg species with the new, recently established PTWI for inorganic mercury (Hg(II)) of 4 μg (Hg)/kg bw/week [5].

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IV.3.3 Résultats complémentaires et discussion

Les données obtenues par analyse des échantillons de produits de la pêche de l'EAT 2 ont été traitées par D-IDMS et D-SS-IDMS afin d'évaluer l'impact de ces nouvelles matrices sur les réactions de méthylation (M) et déméthylation (D). La D-SS-IDMS a été choisie plutôt que l'IPD pour alléger le traitement des données en raison du nombre conséquent d'échantillons analysés. Les résultats sont présentés dans le tableau 25 (les données individuelles sont présentées dans les tableaux 6 et 7 de l'article II).

Tableau 25 : Concentrations en Hg (µg kg^{-1}) des échantillons de produits de la pêche EAT 2

Espèces	n	Mode de calcul	MeHg	∑espèces Hg	% MeHg	M (%)	D (%)
Moule	10	D-IDMS	11 ± 5,4	18 ± 7,4	59 ± 15	-	-
		D-SS-IDMS	8,9 ± 6,3	18 ± 7,0	50 ± 19	4 ± 26	-18 ± 41
Huître	4	D-IDMS	6,5 ± 2,4	11 ± 2,6	84 ± 13	-	-
		D-SS-IDMS	9,0 ± 1,6	11 ± 1	82 ± 11	-151 ± 85	18 ± 59
Coquille Saint-Jacques	4	D-IDMS	5,1 ± 3,2	7,5 ± 4,6	68 ± 5	-	-
		D-SS-IDMS	4,8 ± 3,4	7,4 ± 4,7	63 ± 9	18 ± 32	-1 ± 27
Crevette	16	D-IDMS	25 ± 7,2	28 ± 7,4	90 ± 6	-	-
		D-SS-IDMS	25 ± 6,9	28 ± 4	93 ± 7	4 ± 93	4 ± 3
Lieu-colin	6	D-IDMS	66 ± 17	72 ± 17	91 ± 3	-	-
		D-SS-IDMS	62 ± 18	65 ± 19	95 ± 3	-27 ± 110	-0,3 ± 1,1
Thon	6	D-IDMS	254 ± 163	281 ± 179	90 ± 4	-	-
		D-SS-IDMS	251 ± 169	277 ± 183	90 ± 4	17 ± 38	0,5 ± 1,9
Saumon	16	D-IDMS	40 ± 7,7	43 ± 7,6	93 ± 2	-	-
		D-SS-IDMS	40 ± 6,8	42 ± 6,7	94 ± 2	-45 ± 88	0,4 ± 1,8

D'importantes fluctuations des taux moyens de M et de D sont observées. Ces fluctuations sont également significatives au niveau du triplicata d'injection d'un même échantillon, comme constaté précédemment sur les MRCs (partie II, paragraphe VI.4.). Néanmoins, à l'exception d'un échantillon d'huître qui présente un taux de D de 18% (résultats par échantillon non montrés), ces taux restent négligeables pour l'ensemble des échantillons. Les teneurs en iHg et MeHg déterminées par D-IDMS sont généralement similaires aux concentrations calculées par D-SS-IDMS, ce qui confirme une faible influence des réactions de transformation sur les résultats finaux.

Les pourcentages moyens de MeHg déterminés par D-IDMS sont similaires à ceux déterminés à partir des données D-SS-IDMS, à l'exception des moules où un pourcentage moyen de MeHg plus élevé (59% contre 50%) est calculé. Les taux de M et D pour cette matrice sont négligeables, ils n'expliquent donc pas cette différence. Par contre, les variations de pourcentage

entre les deux méthodes de calculs sont observées sur les échantillons les moins concentrés. Il est donc probable que ces différences soient liées aux incertitudes de mesure.

Le pourcentage moyen de MeHg dans les mollusques et crustacés est de 75% avec d'importantes variations inter-espèces mais également intra-espèces. Le pourcentage minimum de MeHg a été mesuré dans un échantillon de moule (28%) et le maximum dans un échantillon de crevette (98%). Les teneurs moyennes de MeHg sont comprises entre 1,9 µg kg^{-1} et 33 µg kg^{-1}, avec une teneur moyenne de 16 µg kg^{-1}. Les concentrations les plus elevées sont mesurées dans les crevettes.

Le pourcentage moyen de MeHg dans les poissons (93%) est plus élevé que dans les mollusques et crustacés. Les variations inter-espèces et intra-espèces sont plus faibles avec un pourcentage en MeHg minimal de 84% dans un échantillon de thon et un pourcentage maximal de 97% dans un échantillon de saumon et un de lieu-colin. Les teneurs moyennes de MeHg sont comprises entre 28 µg kg^{-1} et 588 µg kg^{-1}, avec une teneur moyenne de 90 µg kg^{-1}. Les concentrations les plus fortes sont mesurées dans le thon.

La fiabilité des résultats a été évaluée en comparant les teneurs en HgT obtenues en sommant les teneurs des espèces Hg déterminées par spéciation, avec les teneurs en HgT déterminées par la méthode ICP-MS accréditée (Noël et al., 2005). Les résultats de cette comparaison sont présentés dans les tableaux 6 et7 de l'article II. En moyenne, les teneurs en HgT déterminées par spéciation sont en accord avec les teneurs déterminées en total.

Les teneurs et pourcentages en MeHg des échantillons ont également été comparées aux données de la littérature (cf. tableau 8 de l'article II). Cette comparaison comprend peu d'études en raison de deux difficultés rencontrées. La première concerne la nature des données de MeHg. En effet, plusieurs études déterminent la teneur en HgT dans l'échantillon et utilise une hypothèse de pourcentage de MeHg présent dans le HgT (Cf. partie I, paragraphe III.8.) (AFSSA 2002, Usydus et al., 2009, Ström et al., 2010). La seconde réside dans l'origine et le domaine d'application de l'étude. Plus particulièrement pour les mollusques et crustacés que pour les poissons, ces produits de la pêche sont régulièrement utilisés comme indicateur de pollutions environnementales. Par conséquent, ils sont le plus souvent prélevés dans des milieux contaminés et sont peu susceptibles d'être consommés. Ils n'ont donc pas d'intérêt alimentaire. Les articles entrant dans ces deux catégories n'ont pas été pris en considération.

En général, les pourcentages en MeHg déterminés dans cette étude sont similaires ou supérieures aux valeurs de la littérature alors que les teneurs en MeHg sont soit similaires, soit inférieures (cf. tableau 8 de l'article II). Comme expliqué dans l'article II, les différences entre les études sont principalement liées au nombre de poissons prédateurs analysés et à la biogéochimie

complexe du Hg dans le milieu marin (par exemple, chaîne trophique marine dans lequel l'organisme a été recueilli, son âge, le temps de l'exposition et le niveau de contamination anthropique).

IV.3.4 Conclusion

Les résultats obtenus par le couplage ID-GC-ICP-MS sur des échantillons de produits de la pêche lyophilisés et non dégraissés de l'EAT 2 sont à la fois en bonne corrélation avec les valeurs en HgT quantifié par une méthode ICP-MS accréditée et en bon accord avec les données de la littérature.

V. Conclusions et perspectives de la partie III

Pour une utilisation en « routine » de la méthode validée sur des échantillons réels, des contrôles qualités internes ont été mis en place et la justesse de la méthode a été évaluée par le biais de 2 contrôles qualités externes avec des résultats satisfaisants sur des matrices de poisson.

Par la suite, l'applicabilité de la méthode a été évaluée sur des échantillons réels. Dans un premier temps, des essais ont été réalisés avec satisfaction sur des échantillons lyophilisés non dégraissés, d'anguilles et de civelles, au sein du laboratoire LCABIE. Les résultats sont présentés dans l'article I. Parmis les observations faites, cette étude a clairement montré l'avantage d'utiliser la déconvolution isotopique par rapport à la dilution isotopique classique car elle a permis d'obtenir des résultats finaux plus justes et précis, car corrigés des transformations inter-espèces engendrées par l'étape d'extraction.

Dans un second temps, une étude sur l'influence de la lyophilisation sur les espèces Hg dans les produits de la pêche a été menée aux laboratoires CIME et LCABIE dans le but de déterminer l'impact de la lyophilisation sur la distribution naturelle des espèces Hg. Des résultats différents ont été obtenus par les deux laboratoires, probablement en raison des méthodes de lyophilisation utilisées (masses et support de lyophilisation différents). Néanmois, cette évaluation a permis de constater qu'il ne semble pas y avoir de pertes ni de modification d'espèces durant le processus de lyophilisation. Toutefois, de nouvelles expérimentations pourraient être envisagées afin de renforcer les premières constatations faites, en augmentant le nombre de laboratoires participants, en définissant une procédure unique de lyophilisation et sur un plus grand nombre de matrices.

Enfin, la méthode validée a été utilisée avec succès sur les échantillons lyophilisés et non dégraissés des produits de la pêche de l'EAT 2, au sein du laboratoire CIME. Les conclusions de cette étude sont présentées dans l'article II. Les résultats obtenus ont montré que, pour les sept

matrices différentes analysées, la dilution isotopiques classique a permis une quantification juste, au regard des résultats retrouvés dans la littérature et des résultats déterminés par analyse de l'élement mercure sans spéciation et donc nous conforte dans la constatation que la méthode analytique développée et présentée dans ces travaux ne semble pas engendrer de transformations inter-espèces. En perspective, ces données d'occurrence pourraient être utilisées pour une évaluation plus juste de l'exposition humaine au MeHg via la consommation de produits de la pêche, lors du traitement de l'ensemble des données de l'étude EAT 2 dont le rapport final est en cours de rédaction. Enfin, il serait intéressant d'analyser également les échantillons frais des produits de la pêche de l'EAT 2 afin de comparer les résultats avec les échantillons lyophilisés et de conforter ou non les résultats sur l'influence de la lyophilisation sur les espèces Hg.