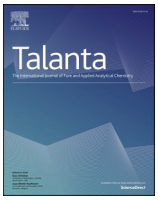
Talanta 270 (2024) 125612

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



A high-throughput, straightforward procedure for biomonitoring organomercury species in human hair

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ARTICLE INFO

Handling editor: J. Wang

Keywords:
Methylmercury
Mercury speciation
Chromatography
ICP-MS
Biomonitoring
Human hair

ABSTRACT

Mercury is a pervasive and concerning pollutant due to its toxicity, mobility, and tendency to biomagnify in aquatic and terrestrial ecosystems. Speciation analysis is crucial to assess exposure and risks associated with mercury, as different mercury species exhibit varying properties and toxicities. This study aimed at developing a selective detection method for organic mercury species in a non-invasive biomonitoring matrix like human hair. The method is based on frontal chromatography (FC) in combination with inductively coupled plasma mass spectrometry (ICP-MS), using a low pressure, homemade, anion exchange column inserted in a standard ICP-MS introduction system, without requiring high-performance liquid chromatography (HPLC) hyphenation. In addition to the extreme simplification and cost reduction of the chromatographic equipment, the proposed protocol involves a fast, streamlined and fully integrated sample preparation process (in contrast to existing methods): the optimized procedure features a 15-min ultrasonic assisted extraction procedure and 5 min analysis time. Consequently, up to 100 samples could be analyzed daily, making the method highly productive and suitable for large-scale screening programs in public and environmental health. Moreover, the optimized procedure enables a limit of detection (LOD) of 5.5 μg/kg for a 10 mg hair microsample. All these features undeniably demonstrate a significant advancement in routine biomonitoring practices. To provide additional evidence, the method was applied to forty-nine human hair samples from individuals with varying dietary habits successfully finding a clear correlation between methylmercury levels (ranging from 0.02 to 3.2 mg/kg) in hair and fish consumption, in line with previous literature data.

1. Introduction

Mercury has since long been recognized as a widespread and high concern pollutant [1,2], due to its toxicity [3], mobility [4] and tendency to biomagnify along the food chain [5]. Both aquatic and land ecosystems have been contaminated by this element, resulting in measurable effects on both marine [6]and terrestrial [7] organisms. Effects in humans are also well documented [8–10], but we are far from establishing clear exposure/outcomes relationships for all the exposure scenarios, calling for a continuous effort in advancing our knowledge and reducing Hg emissions [11,12].

The rich speciation of mercury adds complexity to the picture: terrestrial [13,14] and aquatic [15,16] processes contribute to the production and interconversion of Hg species that show differing chemicophysical properties, toxicity and mobility in the environment (see e.g. Ref. [17], and previously cited references). Accordingly, mercury speciation analysis is needed for reliable health risk assessment and to evaluate exposure sources, with methylmercury (MeHg) being mainly associated to Hg-contaminated food [18] and inorganic mercury to industrial or artisanal activities where significant amounts of mercury are manipulated (see e.g. Ref. [19]).

In this context, biomonitoring is clearly pivotal to understand exposure, evaluate risk and informing individuals and decision makers for action. Despite blood may be perceived as the most adequate matrix, sample collection is intrinsically invasive and requires specialized personnel, also involving extensive pretreatment [20]. Accordingly, non-invasive biomonitoring, namely the analysis of biomatrices like saliva, urine, feces, and hair, has emerged as an alternative, uncomplicated approach [21]. The detection of mercury species in human hair proved a reliable record of chronic exposure to mercury species from diet, which is the major exposure route in the non-occupationally exposed population [20]. Mercury detection in scalp hair is also

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https://doi.org/10.1016/j.talanta.2023.125612
Received 6 October 2023; Received in revised form 21 December 2023; Accepted 27 December 2023
Available online 29 December 2023

facilitated as Hg naturally preconcentrates in this tissue, where it is approx. 250-times higher than in blood [22].

Accordingly, the development of analytical procedures for the selective detection of mercury species attracted much attention (see the recent reviews [23–25]). Focusing on the analysis of human hair, hyphenated techniques combining HPLC or gas chromatography (GC) separation with sensitive detection system (e.g., atomic fluorescence spectroscopy [26,27], electron capture detector [28,29] and ICP-MS [30–32]) are widely preferred over non-chromatographic strategies being faster, automated and generally enabling the detection of organomercury species down to μg/kg levels [26–33]. Despite their high performance, these approaches come with numerous drawbacks, especially when seeking routine and high-throughput analysis. The main challenge lies in the extensive sample preparation process, particularly for GC-based strategies. This involves multiple time-consuming steps such as solid-liquid extraction, transfer into organic phases, derivatization, and head-space sampling. These steps often require operator supervision and caution due to the use of potentially hazardous chemicals (see organic solvents and alkylating agents). In contrast, HPLC strategies typically involve simpler, quicker, and safer sample manipulation. However, this comes at the cost of detection capability, as most HPLC methods do not require preconcentration steps, unlike many GC methods: this aspect may hinder the quantification of baseline levels for human hair. Additionally, although widely ignored, the high resolution provided by HPLC may be superfluous in the specific case of organomercury species determination. Based on this consideration, we recently demonstrated the suitability of the Frontal Chromatography ICP-MS (FC-ICP-MS) [34–37], which avoids the use of HPLC by simply introducing a low-pressure column. Such a simplification not only reduce the instrumental equipment complexity and costs but, more importantly, enables the rapid and selective determination of MeHg in biological samples [35]. Nevertheless, limited detection capabilities were achieved restricting the application of the published procedure to samples with relatively high methylmercury levels (e.g., tissues from apex predators in the marine food chain, LOD = 40 μg/kg based on 10 mg solid sample [35]), i.e., not being suitable for the analysis of hair samples.

The present paper aims at developing, validating and applying a procedure for the selective detection of organic mercury species in human hair, using the minimum sample mass, simplifying the sample treatment, minimizing the analysis time, and affording easy operations. The procedure was finally applied to the determination of methylmercury in scalp hair from individuals with different dietary habits, confirming fish consumption as the main source of organomercury in scalp hair.

2. Experimental

2.1. Standards, reagents, and certified reference materials

Ultrapure hydrochloric acid (HCl) and nitric acid (HNO_3) were obtained by a Milestone sub-boiling distillation apparatus (see Ref. [38] for details). Ultrapure water produced by a Sartorius Arium mini–UV Lab Water System was used throughout for standard and solution preparation. MeHg standard solutions were prepared by diluting a 1000 ± 20 mg L^{-1} MeHg standard solution (Alfa Aesar, concentration referred to MeHgCl), whereas a 10 mg L^{-1} Hg standard solution (TraceCERT®, Sigma-Aldrich) was used for inorganic mercury standards. Lutetium (Lu) obtained from a 1000 mg L^{-1} standard solution (TraceCERT®, Sigma-Aldrich) was used as the internal standard after adequate dilution. Solid thiourea (TU, 99 % pure) and hydrobromic acid (48 %) were obtained from Carlo Erba Reagents. All dilutions were performed by weighing on a 2-digit precision balance.

Method validation was achieved by analyzing the certified reference material NIMD-01 Mercury in human hair from the National Institute for Minamata Disease, which is certified for both total Hg (0.794 ± 0.050 mg/kg) and methylmercury (0.634 ± 0.071 mg/kg as Hg) content [39].

The method was also applied to four certified reference materials (CRMs) from the marine food web to show that the method may be also applied to matrices other than human hair. The CRMs are tuna fish muscles (BCR-463 and ERM-CE464 from the Institute for Reference Materials and Measurements, IRMM, Belgium), plankton (BCR 414 from IRMM, Belgium), and one dogfish liver (DOLT-5 from the Canadian National Research Council).

2.2. Total mercury and MeHg extraction procedure

Total mercury concentration in the samples was determined by microwave-assisted digestion using a Milestone ETHOS One, followed by traditional, i.e., without the chromatographic column, ICP-MS analysis. A mixture of 0.5 mL pure HNO_3 and 0.5 mL pure HCl produced by sub-boiling distillation [38] was used for sample digestion in a multi-batch system (see our previous work [40] for a detailed description). A simple digestion program involving a 20-min ramp from room temperature to 110 °C followed by 30 min fixed at 110 °C was used.

MeHg extraction was performed by using a solution including HCl 0.5 M, thiourea 3.3 mM and HBr 0.037 M, whose composition was optimized as reported in the Results and discussion section. The extraction was performed on approx. 20 mg of each hair sample in a 10 mL polypropylene test tube. The analyzed masses of CRMs were as follows: 20 mg NIMD-01, 250 mg of DOLT-5, 100 mg of BCR-414 (as specified by the certificates of the CRMs), 20 mg of BCR-463, 10 mg of ERM-CE464 (that is approximately one-tenth of the amounts indicated by the certificates of the CRMs). Five mL of the extraction solution were added to each test tube and the dispersion was sonicated at room temperature for 15 min (Branson 5800 ultrasonic bath). The resulting suspension was either filtered through a 0.45 μm syringe filter (Millex, mixed cellulose esters) when human hair was involved or centrifuged (4000 rpm for 10 min, ALC 4206 centrifuge) if samples from the marine food web were analyzed. Two blank samples and one aliquot of the certified reference material NIMD-01 Mercury in human hair were analyzed per batch. Sample solutions were spiked with the lutetium solution (internal standard, 1 μg/kg) to correct potential sensitivity drifts and matrix effects. Low-Density Polyethylene (LDPE) bottles and test tubes were decontaminated prior and after any extraction batch through a 3-stage process: i) soaking in a 0.4 % w/w detergent solution (Nalgene L900) for one week; ii) soaking in a HNO_3 solution (2 %, w/w) for one week; iii) soaking in a HCl solution (pH = 2) for one week. Bottles and test tubes were thoroughly rinsed with ultrapure water between each step and before use.

2.3. Sample collection and pretreatment

Hair samples were collected on a voluntary base from the students of the Chemistry program at the University of Insubria and their relatives. The call for donation was spread by the Instagram page of our research group (https://www.instagram.com/analiticacomo) and directly at the faculty premises. Hair were either directly provided by the donors or cut in our laboratory: in any case, donors had to fill in a questionnaire (see Table S1) investigating the sources of mercury intake, possible hair treatments, which are known to interfere with mercury concentration in hair [41], and personal data (gender, weight, height, smoking habits and presence of mercury based dental fillers) to assess the Hg intake per kg body weight.

A total of 49 hair samples and questionnaires were collected. Hair samples were firstly suspended in acetone to remove lipids, dried at room temperature in a laminar flow hood and subsequently cut into short pieces by a ceramic knife.

2.4. Instrumental setup and analysis protocol

A Thermo Scientific ICAP Q ICP-MS was used for element detection. The selectivity for methylmercury is obtained by inserting a short

column between the peristaltic pump used for sample uptake and the nebulizer of the ICP-MS (internal diameter 2.5 mm, length 20 mm), which contains a strong anionic exchange resin (AmberChrom® 1X2 chloride form 200−400 mesh, Sigma-Aldrich). A 0.19 M thiourea solution was added post-column to reduce the well-known Hg memory effect by using a Y-shaped connector. ^{202}Hg and ^{175}Lu signals were acquired and all the ICP-MS measurements were performed using a He-collision cell in kinetic energy discrimination (KED) mode: operating conditions are summarized in Table S2.

The analysis protocol was as follows. The Hg and Lu signals are acquired for 20 s, then a 0.2 % m/v thiourea solution is passed through the column for 95 s to wash the column, the spray chamber and the lines, and finally the blank solution (0.5 M HCl, 37 mM HBr, 3.3 mM TU) is passed for 95 s to recondition the column and establish the baseline signal. The autosampler tip undergoes a brief 5-s rinsing process employing a blank solution (comprising 0.5 M HCl, 37 mM HBr, and 3.3 mM TU) through the autosampler's washout function. The analysis sequence is reported in Scheme 1 and takes 305 s (approx. 5 min).

MeHg concentrations are expressed as mass concentration of MeHg and all the uncertainties reported were estimated as one standard deviation calculated over three replicated measurements.

2.5. Estimation of the mercury weekly intake

Questionnaire data were used to provide a rough estimate of the mercury weekly intake for each donor. The weekly mercury intake per kg body weight was calculated as follows, assuming a portion of rice or fish is equal to 0.1 kg:

$$\text{Weekly intake} = \frac{\text{Ingested Hg } (\mu g)}{\text{Body weight (kg)}} = \frac{\text{weekly consumption frequency} \bullet 0.1 \text{ Kg} \bullet \text{Hg concentration in food } \left(\frac{\mu g}{kg}\right)}{\text{Body weight (kg)}}$$

where the weekly consumption frequency is the number of food portions consumed per week as obtained from the questionnaire, 0.1 kg is the assumed weight of each portion (according to the U.S. Food and Drug Administration reference amounts customarily consumed per eating occasion [42]) and the concentrations in food (rice and different fish and seafood) were obtained from the literature [43,44] and are reported in Table S3 (along with additional information from the questionnaire) and Fig. S1. The estimation is clearly an approximation of the real Hg intake.

3. Results and discussion

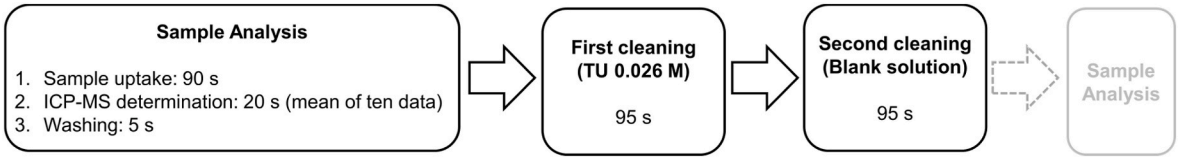
3.1. Method outline

The definition of an extraction solution that could be directly analyzed by Frontal Chromatography – ICP-MS to selectively determine methylmercury by blocking inorganic Hg (iHg) on an anion exchange resin was the main goal of the research: it would afford a fast and sensitive (i.e., without dilution) method with minimal requirements from the operator side. The literature reports several solution compositions, but some of them are not compatible with ICP-MS analysis (5 M HCl [45] that we also employed in our previous work after 1:10 dilution [35]), or with the chromatographic separation on a short, low efficiency column (e.g., Refs. [46–50]). HCl concentration should be accordingly reduced and supplementary, sulfur-based complexant(s) added to enable the extraction of Hg species: mercaptoethanol, cysteine and thiourea [51–55] were employed. We decided to employ 0.5 M HCl (perfectly compatible with ICP-MS analysis [35]) and thiourea as the additional complexant as it strongly binds mercury species even at acidic pH [56], which are required for an efficient extraction and ICP-MS analysis. Unfortunately, no separation is possible under these conditions as thiourea binds both Hg(II) and methylmercury more strongly than chloride ions, leading to the formation of $Hg(TU)_n^{2+}$ (with n ranging from 1 to 4), and $MeHgTU^+$ (see Table S4 [57–59]). The latter species are positively charged and cannot clearly be separated on an anion exchange column. We decided to add a further complexant to induce the formation of anionic species of iHg, which should be blocked onto the stationary phase, while keeping methylmercury bound to thiourea and thus unretained by the resin. Bromide was selected as it is already used for Hg extraction [60,61] and it binds Hg(II) more strongly than chloride (see Table S4 [58,59]).

3.2. Optimization of the extractant composition

As a starting point to select the useful bromide and thiourea concentrations, the distribution diagrams of both Hg(II) and MeHg species were calculated as a function of a wide range of thiourea (10^{-1}-10^{-5} M range) and bromide (1-10^{-4} M range) concentrations at a 0.5 M HCl (Fig. 1, calculated by Hydra-Medusa chemical equilibrium software developed by Ignasi Puigdomènech [62]).

Hg(II) shows a rich speciation under the investigated conditions: as expected, bromide species prevail for high Br^- and low TU concentrations, whereas $HgTU_n^{2+}$ predominate when TU concentration is raised. Differently, methylmercury is strongly bound by thiourea under all the investigated experimental conditions. Based on data reported in Fig. 1, a design of experiment (DoE) was set up to define the combination of bromide and TU concentrations that keep iHg in anionic form: MeHg is in cationic form according to the data in Fig. 1 (but see below) and thus should not be retained by the anion exchange resin under the explored TU and bromide concentrations. Accordingly, nine possible extractant solutions were prepared by systematically varying the concentrations of bromide and thiourea in a wide concentration range (see stars in Fig. 2 a & b) and measuring the frontal chromatograms for both Hg(II) and MeHg separately (see examples in Fig. 2 c & d). The results are reported in Fig. 2: for MeHg, we report the time required for the signal to reach 95 % of the plateau signal, aiming at the lowest possible time, whereas the Hg(II) eluted fraction is reported for Hg(II), ideally aiming at zero elution.



Scheme 1. Schematic representation of an entire analysis sequence (overall time 305 s).

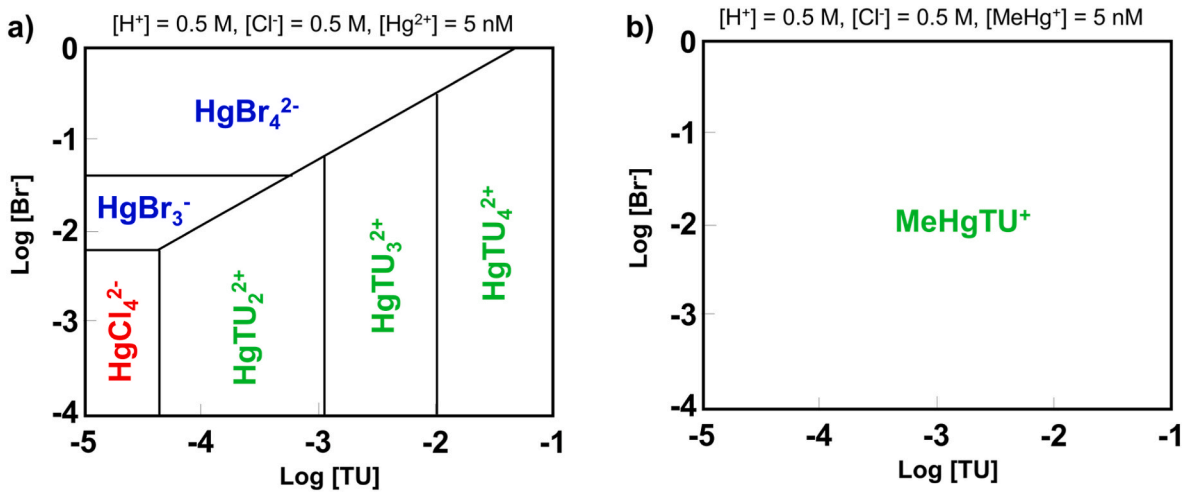


Fig. 1. Predominance area diagram obtained by Hydra-Medusa software (Hydra version: August 18, 2009; Medusa version: December 16, 2010) for (a) Hg and (b) MeHg species using the stability constants summarized in Table S4 (temperature = 25 °C, ionic strength = 0).

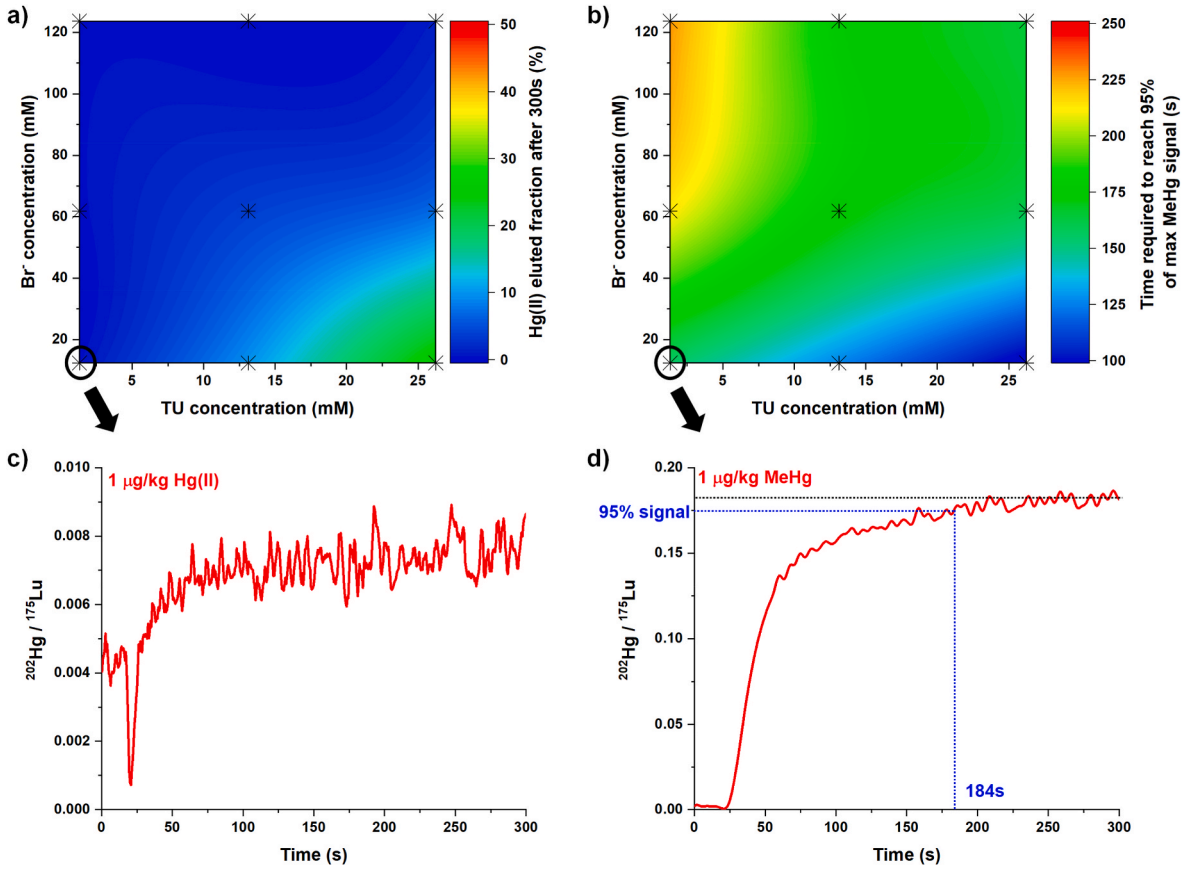


Fig. 2. (a–b) Contouring maps (obtained by using Origin 2018 software) showing (a) the eluted fraction of Hg(II) after a 300 s analysis (expressed as relative percentage respect to Hg(II) total content) and (b) the time required by eluted MeHg species to reach a 95 % signal (compared to the maximum stable signal). Both plots are visualized as a function of bromide and thiourea concentration (HCl concentration kept constant at 0.5 M). Stars represent the performed experiments. (c–d) Frontal chromatograms observed for the most promising condition (12 mM HBr and 1.3 mM TU, see black circles) for solutions containing (c) 1 µg/kg of Hg(II) and (d) 1 µg/kg of MeHg.

Fig. 2 a & b clearly show that thiourea promotes the fast elution of both species, whereas bromide increases the elution time. The latter results are in full agreement with Hg(II) forming cation species in the presence of TU and negatively charged species with bromide (see Fig. 1). Data for MeHg are instead not coherent with calculations, as its elution profile should be independent from bromide and thiourea concentrations according to Fig. 1 ($MeHgTU^+$ always prevailing in the investigated experimental space). Understanding the reasons for this behavior is outside the aim of this work, but MeHg may also form anionic species as reported in the literature [63]. The most promising part of the experimental space is the lower left quarter: the upper half, where C_{HBr}>0.5 %, shows complete blockage of Hg(II) at the expenses of

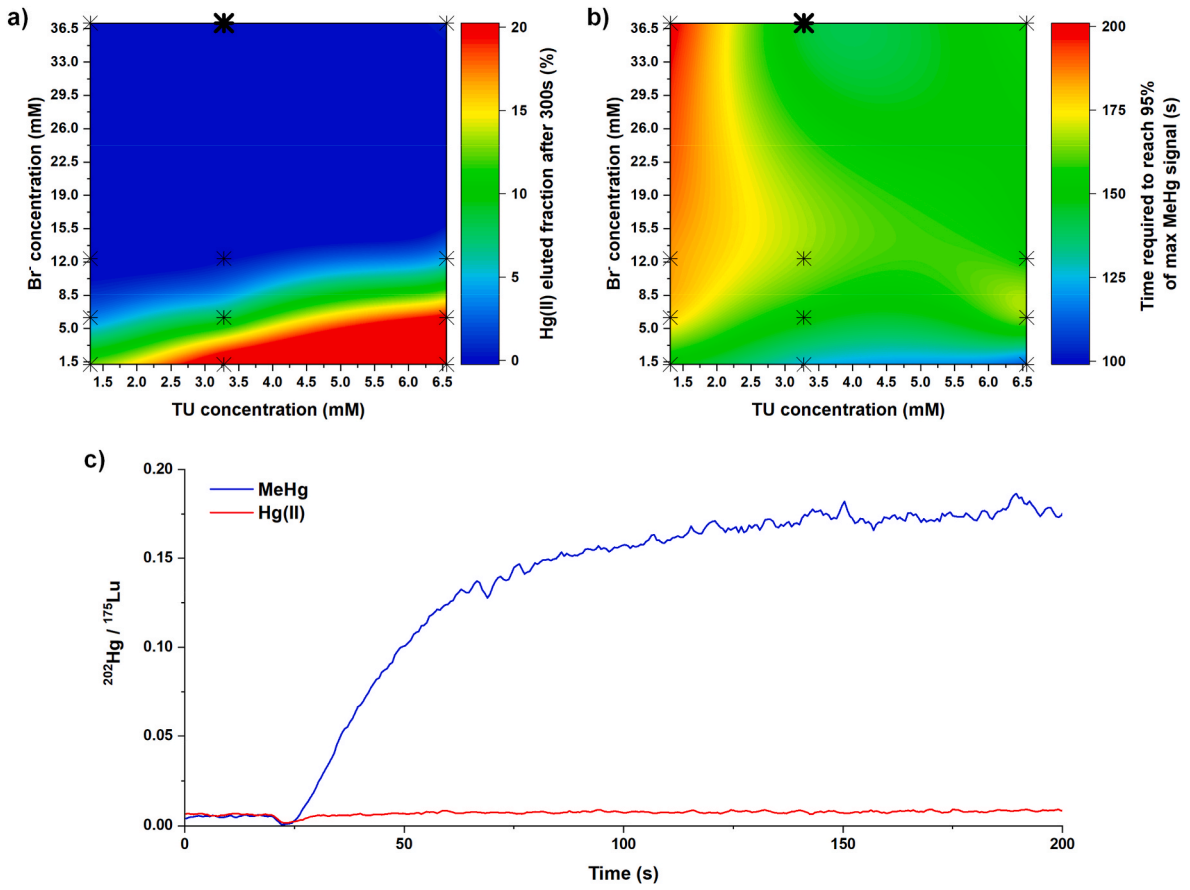


Fig. 3. (a–b) Contouring maps obtained by a second DoE showing (a) the eluted fraction of Hg(II) after a 300 s analysis (expressed as relative percentage respect to Hg(II) total content) and (b) the time required by eluted MeHg species to reach a 95 % signal (compared to the maximum stable signal). Both plots are visualized as a function of bromide and thiourea concentration (HCl concentration kept constant at 0.5 M). Stars represent the performed experiments. (c) Frontal chromatograms observed for the optimized condition (37 mM HBr and 3.3 mM TU, see bold star) for solutions containing 1 μg/kg of Hg(II) and 1 μg/kg of MeHg.

a high elution time for MeHg, whereas in the lower right quarter HBr concentration is too low and TU concentration too high for Hg(II) to be completely retained. Accordingly, a second DoE was setup to better explore this part of the experimental space and to find the best eluent composition: results are reported in Fig. 3 a & b.

The experimental conditions ensuring the best performances are accordingly 37 mM HBr and 3.3 mM TU, which features the complete blockage of Hg(II) and a fast elution of MeHg (130 s to reach 90 % of the plateau value, see Fig. 3c for typical chromatograms in these conditions).

3.3. Memory effect attenuation

Memory effects are often a problem in mercury analysis by ICP-MS, due to the reduced washout time of this element [64]. We demonstrated in a previous work that the on-line addition of a 0.19 M thiourea solution after the column strongly reduces the washout time of mercury in the spray chamber (see our previous work [35]). An efficient wash of the system between samples is also needed to avoid cross contamination. Nevertheless, simply fluxing a blank solution (0.5 M HCl, 37 mM HBr, 3.3 mM TU) for 5 min could not restore the baseline Hg signal after a 5 μg/L MeHg was analyzed. Accordingly, a TU solution was then fluxed after the standard and before the blank to accelerate Hg washout: a much faster decrease to the baseline value was observed. Moreover, the TU solution removes Hg(II) blocked in the column, if present, thus avoiding its overload and consequent slow mercury release. The optimal TU concentration, 26 mM, was established by selecting the concentration above which no further improvement was achieved. A 90 s flushing with the TU solution followed by an equal flushing with the blank enabled an efficient restoring of the baseline even after solutions with high concentrations of MeHg. The entire analysis sequence for each standard/sample accordingly requires 5 min and it is reported in Scheme 1 in the Experimental section.

3.4. Method performances

The detection capabilities, limit of linearity and mostly the selectivity of the method were investigated: defining the MeHg and iHg ranges where the method provides consistent results is clearly of the utmost in the contest of speciation analysis.

The lowest MeHg concentration that may be detected, was determined by the common IUPAC methodology [65,66] by ten replicate measurements of a 100 ng/kg MeHg standard solution: the limit of detection is defined as three time the standard deviation of the low concentration standard (no signal was visible in the blank). The calculated value is 11 ng/kg in the solution: if a sample mass of 250 mg (i.e., as suggested for CRM analysis) is extracted in 5 mL, the LOD in the solid sample is 0.22 μg/kg; if only a microsample of e.g. 10 mg only is available, the LOD raises to 5.5 μg/kg. Accordingly, the LOD is about ten times lower with respect to our previous work (Spanu et al., 2022) thanks to the direct, i.e. without dilution, analysis of the extraction solution. As the consequence, the detection capabilities of this approach are so far the best ones if methods without a preconcentration step are considered (see the review papers [23,24,67], plus [35] that summarizes the best performing speciation methods; see section 3.6).

The limit of linearity was verified up to 10 μg/kg with standard

solutions ($r = 0.99985$, $p < 0.001$, $n = 5$): higher concentrations are not expected when microsamples are analyzed. Reproducibility was also in line with expectances from an ICP-MS analysis: percentage relative standard deviations (RSD%) for 0.1 and 1 μg/kg were 2.24 % and 0.97 %, respectively when ten replicates of standard solutions were analyzed (see below the Validation section for the reproducibility when samples were analyzed).

Finally, we systematically investigated the Hg(II)/MeHg space to define the conditions in which MeHg may be selectively detected in the presence of inorganic mercury. Environmental (biological tissues, soils, sediments, etc.) and human-derived samples shows a wide range of mercury concentrations and of iHg to MeHg ratios: the latter ranges from almost 100 % MeHg (shark muscles [68,69]) to almost 100 % inorganic mercury in soils [70,71], with human hair in between [72]. Accordingly, we analyzed 20 standard solutions containing iHg in the range 0.1–10 μg/kg and MeHg in the range 0.1–10 μg/kg: the recovery of MeHg is reported in the contouring plot in Fig. 4.

Fig. 4 clearly shows that Hg(II) never interfere with MeHg determination when Hg(II) concentration is below around 5 μg/kg (see left half of Fig. 4 where the recovery falls in the range 85%–120 %). Unacceptably high recoveries are instead registered when Hg(II) concentration is higher than 5 μg/kg for very low levels of MeHg. A deeper analysis of the data indicates that the presence of Hg(II) induces an increase in the MeHg concentration proportional to the Hg(II) concentration: 10 μg/kg induces an overestimation of 0.2 μg/kg on average, whereas no effect was detected for the 0.1 and 1 μg/kg concentration levels. The latter feature is well explained by the chromatographic stationary phase failing to fully block Hg(II), leaving a small portion of Hg(II) unretained: if Hg(II) concentration is low, this small concentration is undetected, whereas a significant signal is registered when Hg(II) concentration approaches 10 μg/kg.

Concluding, MeHg may be reliably detected in the presence of Hg(II) if Hg(II) concentration is below 5 μg/kg or MeHg is the prevailing species (e.g., the ratio MeHg to Hg(II) is above 10).

3.5. Validation

The extractant composition was so far optimized to achieve a fast and reliable separation, with the components HCl, HBr, and TU closely resembling those in the currently used mercury extractants [47,61,73]. Nonetheless, the extractant capacity to quantitatively extract mercury species from relevant matrices requires demonstration. Accordingly, eleven 20 mg aliquots of the NIMD-01 reference material certified for methylmercury content [39] were extracted with the 0.5 M HCl, 37 mM HBr, 3.3 mM TU solution and analyzed by the described procedure. Four more reference materials were also analyzed to check the possible extension of the procedure to environmental matrices (the BCR-414 CRM is not certified for MeHg content, but literature values are available [74,75]). The extraction kinetics was assessed: Fig. S2 shows the effect of the extraction time (15, 30 and 60 min) on the recovery of methylmercury for NIMD-01. No clear trend is visible and quantitative extraction is achieved in any case: the lowest extraction time (15 min) was accordingly adopted. Table 1 reports the results of the analyses of the reference materials, with special emphasis on the NIMD-01 CRM.

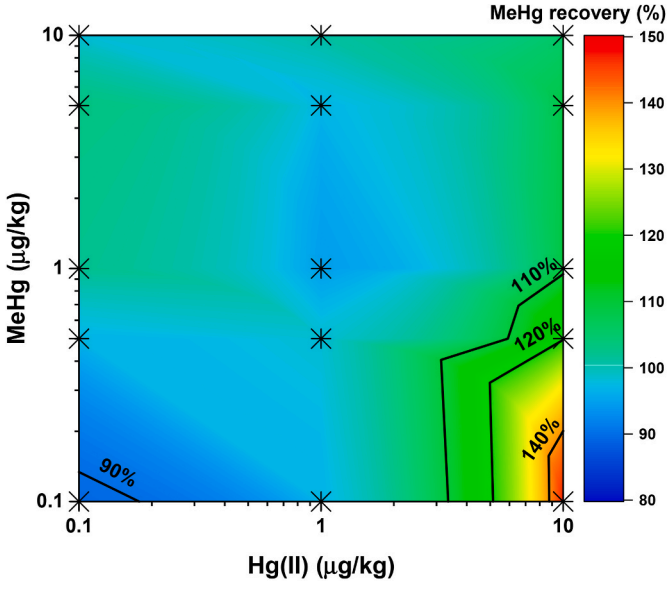


Fig. 4. Contouring map of MeHg recovery as a function of MeHg and Hg concentration. Stars represent the performed experiments.

The absence of any statistically significant difference was verified in accordance with the European References Materials (ERM) Application Note 1 [76]. The results obtained by the proposed procedure were not significantly different from the certified value for the human hair CRM, validating the unbiased detection of MeHg in human hair: analogously, also total Hg determined by microwave-assisted acid digestion followed by ICP-MS was not statistically different from the certified value (0.740 ± 0.050 mg/kg, as determined by 12 replicates, and 0.794 ± 0.050 mg/kg, respectively). The precision expressed as percentage relative standard deviation (RSD%) is around 3.3 % for the 5 analyzed CRM aliquots. The results for the analysis of CRMs of different matrices from the marine food web are also reported in Table 1: the absence of bias strongly support the extension of this procedure to the selective determination of MeHg in biological samples such as plankton, fish liver and muscle, fostering the understanding of the trophic transfer of this bioaccumulated and biomagnified toxicant.

3.6. Comparison with existing analytical methods

Table 2 compares the detection capabilities (LOD) and the sample throughput (pretreatment and analysis time) of the present method with literature ones.

Generally, hyphenated techniques incorporating HPLC and GC separation, followed by detection using an atomic or mass spectrometer outperform non-chromatographic techniques in terms of analysis time, degree of automation, and sensitivity. Among them, GC-based techniques employing preconcentration strategies such as headspace analysis and microextraction, are mostly employed owing to their lower limits of detection compared to HPLC-based strategies. However, the steps preceding GC determinations are complex, time-consuming and in contrast with the principles of green analytical chemistry. A part from the acid-assisted (or alkaline) extraction of Hg species from the solid matrix which is a common step in all the strategies reported in Table 2, GC-based procedures require a back-extraction in organic solvent followed by derivatization by alkylation: the latter procedures require hazardous chemicals (CH_2Cl_2, iso-octane and toluene are all toxic solvents and the alkylating agent $NaBEt_4$ is toxic, highly reactive and inflammable). Overall, sample treatment involves extensive sample and chemical manipulations which may take several hours (ranging from 40 min to more than 12 h, see Table 2), questioning the adoption of GC-based techniques for routine analysis. Moreover, biomonitoring studies [77–79] showed that mercury baseline concentrations in human hair are higher than a few μg/kg: LODs down to tens of ng/kg at the expenses of such a complex and time consuming sample preparation seems unnecessary in most cases. In this work, this baseline level can be accurately determined by an extremely simple, straightforward, and rapid strategy (15 min extraction + 5 min of analysis time), which has the additional advantage of processing microsamples down to 10 mg, a relevant feature when treating biological tissues.

On the other hand, HPLC-ICP-MS strategies require analogous analysis times (e.g., 10 min extraction + 8 min analysis time [31]), but attain a significantly higher limit of detection (50 μg/kg vs. 5.5 μg/kg on a 10 mg sample mass basis): this eminent LOD value is achieved by the

Table 1
MeHg concentrations determined by replicated analyses of CRMs. Concentrations are expressed as Hg content.

CRM	N° of replicates	Sample description	Certified total Hg concentration (mg/kg)	Certified MeHg concentration (mg/kg)	Measured MeHg concentration (mg/kg)
NIMD-01	5	Human hair	0.794 ± 0.050	0.634 ± 0.071	0.670 ± 0.022
BCR-414	4	Plankton	0.276 ± 0.018	0.235 ± 0.007 [74] 0.210 ± 0.019 [75] 0.190 ± 0.003 [75]	0.189 ± 0.009
DOLT-5	5	Dogfish liver	0.44 ± 0.18	0.119 ± 0.058	0.113 ± 0.003
BCR-463	3	Fish muscle	2.85 ± 0.15	2.83 ± 0.15	2.99 ± 0.10
ERM-CE464	3	Fish muscle	5.24 ± 0.10	5.12 ± 0.16	5.5 ± 0.16

integration of sample preparation and analytical determination, that is, by directly analyzing the extraction solution without any dilution.

Finally, the Frontal Chromatography is unquestionably more straightforward and economical in comparison to both HPLC and GC equipment.

3.7. Methylmercury in human hair

Forty-nine hair samples were analyzed for methylmercury concentration according to the defined procedure: is the results are reported in Table 3. Whenever enough sample mass was available, three replicate extractions were performed to evaluate the reproducibility of the data. As expected, the RSD% decreased with increasing MeHg concentration: 10 % for MeHg concentrations around 0.3 mg/kg, 5 % for samples in the range 0.5–0.9 mg/kg and 2 % for the high concentration ones (above 1 mg/kg). Total mercury concentration was also determined following conventional microwave assisted digestion (see the Experimental section for details and Table 3 for concentrations).

As regards the analysis of the data, the role of possible confounding factors (sex, age, weight, height, and smoking habits) was firstly evaluated before any correlation with dietary habits was assessed. This assessment is fundamental to exclude that any of the mentioned variables affects measured Hg concentrations (a further factor, i.e., hair treatment, was also considered: see in the following). As a results, none of these factors showed significant correlation with total mercury concentration (Table S5). The lack of any effect of these variables on Hg hair concentrations is clearly restricted to this set of data and cannot be generalized. The correlation of the data with dietary habits was then evaluated. The first striking point is the difference between fish consuming and non-fish consuming individuals: the average mercury concentrations is 0.85 ± 0.44 mg/kg in the first group (average ± one standard deviation), whereas a twenty times lower value was determined for non-fish-eating persons (0.045 ± 0.044 mg/kg, average ± one standard deviation). Standing the previous results on possible confounding variables, these results may be reliably associated with fish consumption, as also demonstrated by several previous studies [80–83]. In addition, twenty-one and six individuals showed values higher than the international limits for Hg in hair recommended by the United States Environmental Protection Agency (USEPA) (1.0 mg/kg) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2.3 mg/kg), respectively [84], pointing to a possible health risk. However, all the samples are far below the estimated no-observable-adverse-effect levels (NOAEL) which are higher than 10 mg/kg [3].

The questionnaire (see sections 2.3 and 2.5) also enabled an estimation of the weekly mercury intake (see Table S3): its correlation with mercury concentration in hair is highly significant, especially when treated hair samples are excluded ($r = 0.684$, $n = 32$, $p < 0.001$; not excluding treated hair: $r = 0.382$, $n = 49$, $p < 0.01$). Hair treatments (decolorization, coloring and permanent) are indeed well known to alter Hg levels in hair and will be accordingly excluded from further evaluations [41]. Fig. 5 shows the correlation between methylmercury concentration in hair and the estimated weekly intake per kilogram body weight: here again, the correlation is highly significant ($r = 0.716$, $n = 32$, $p < 0.001$). The exclusion of high leverage data (five data with methylmercury higher than 1.5 mg/kg) lowers the correlation significance, which nonetheless remains very high ($r = 0.582$, $n = 27$, $p < 0.001$).

The percentage of methylmercury over total Hg is highly variable, from 5 % to 100 %, with values below 20 % associated to three vegan or vegetarian individuals and one omnivorous: fish consumption is known to be the main source of mercury in the form of methylmercury. Moreover, the concentrations of total mercury and methylmercury are strongly correlated ($r = 0.857$, $n = 34$, $p < 0.001$), with an average ratio of 62 % MeHg. The latter data is consistent with the prevalence of methylmercury in fish tissues, but usually higher MeHg percentages are registered in fish muscle, with only limited variations as a function of fish species: 67%–100 % [85], 95 % [86], 67%–93 % in a recent study [87]. This discrepancy may be due to the partial demethylation of MeHg in the digestive tract of humans, as revealed by using Hg isotopes for source apportionment of mercury species [88]. In general, the assumption that MeHg coincide with total Hg in hair may be misleading in exposure and risk assessment, leading to an overestimation of the health risk, whatever the reason for the significant difference between MeHg and total Hg concentrations may be (demethylation, low MeHg in the consumed fish species, environmental exposure other than diet [19,88]).

4. Conclusions

A novel and advantageous ICP-MS-based method was developed, optimized, and applied for the selective detection of organomercury species in human hair. It features a fast and simple extraction (15 min sonication at room temperature), syringe filtration and direct analysis by Frontal Chromatography – ICP-MS with an analysis time of 5 min. The proposed procedure successfully cope with organomercury ultratraces determination by providing 1) adequate detection capabilities for microsamples (5–10 mg) allowing the quantification of extremely low MeHg concentrations (limit of detection 5.5 μg/kg for a 10 mg sample); 2) selectivity for MeHg notwithstanding the high variability of MeHg/total Hg ratios (12%–100 %); 3) high throughput for large scale investigations (approximate productivity of 100 samples/day).

These characteristics collectively mark the presented analytical method as a significant advancement in this field, particularly when considering the labor-intensive and chemical-intensive sample preparation demanded by most existing techniques, which cannot reach the combined high level of productivity and sensitivity demonstrated in this work. These features were clearly demonstrated in a local case study.

Detection capabilities should be further increased if the procedure is to be extended to very low concentration samples like unpolluted waters, soils and sediments. The latter show methylmercury concentrations in the low ng/kg (waters) or μg/kg (solid matrices) range and cannot be analyzed notwithstanding the very low limit of detection offered by the proposed procedure: work is ongoing to preconcentrate MeHg and iHg or methylmercury selectively on an anion exchange resin prior to actual determination.

Table 2
Comparison of the analytical features of the proposed method with those of existing chromatographic procedures for the determination of organomercury species in human hair samples.

Analytical Technique	Sample preparation	Extraction time	Chromatographic run time	LOD(*)	Ref.
HPLC-ICP-MS	i) Microwave-assisted digestion: Hair sample amount: 100 mg Solution: 2 mL HNO_3 + 1 mL H_2O_2 Microwave power max: 600 W Treatment time: 29 min + overnight cooling down	>12 h	>10 min	5000 μg/kg	[32]
GC-CV-AFS	i) Digestion: Hair sample amount: 100 mg Solution: 2 mL HCl (2 M) Temperature: 100 °C Treatment time: 15min (+ cooling time) ii) Derivatization 3 mL acetate buffer (pH 4.5) and 300 μL 1 % $NaBEt_4$ added to the digestion solution for etyhlation iii) Headspace SPME 10 min sampling time (fiber exposure)	55 min (**)	20 min	500 μg/kg	[26]
HPLC-ICP-MS	i) Ultrasound-assisted extraction: Hair sample amount: 50 mg Solution: 10 mL 0.10 % v/v HCl + 0.05 % m/v L-cysteine + 0.10 % v/v 2-mercaptoethanol Treatment time: 10 min	10 min	8 min	50 μg/kg	[31]
HI-GC-AFS	Method 1: i) Alkaline digestion: Hair sample amount: 100 mg Solution: 2 mL TMAH Temperature: 85 °C Treatment time: 3h ii) Back-extraction: Solution: 10 mL CH_2Cl_2 + 1.5 mL HCl Treatment time: 30 min followed by back-extraction at 50 °C under N_2 (water removal and solvent evaporation) iii) Derivatization: Solution: Acetate buffer + $NaBEt_4$ Treatment time: 60–300 min Method 2: i) Acid digestion: Hair sample amount: 100 mg Solution: 5 mL H_2SO_4 (5 %) + 18%KBr + 1 mL $CuSO_4$ (1 M) Treatment time: 20 min ii) Back-extraction: Solution: 10 mL CH_2Cl_2 + 1.5 mL HCl Treatment time: 30 min followed by back-extraction at 50 °C under N_2 (water removal and solvent evaporation) iii) Derivatization: Solution: Acetate buffer + $NaBEt_4$ Treatment time: 60–300 min	Method 1: 270–510 min Method 2: 110–350 min	8 min	0.08 μg/kg	[27]
GC-ECD	i) Extraction: Hair sample amount: 15–30 mg Solution: 10 mL HCl 6 M Treatment time: overnight ii) Back-extraction: Solution: 0.5 mL toluene Treatment time: n.a.	>12h	4 min	0.6 μg/kg	[29]
ID-GC-ICP-MS	i) Microwave-assisted extraction: Hair sample amount: 20 mg Solution: TMAH (25 %) Treatment time: 6 min (+ cooling time) Temperature: 75 °C ii) Derivatization: Solution: 4 mL acetate buffer (pH 4) + 100 μL $NaBPr_4$ (20 %) +2–6 mL Isooctane Treatment time: 5 min	41 min (**)	>5 min	54 μg/kg	[30]
GC-ECD	i) Extraction: Hair sample amount: 20 mg Solution: 3 mL HCl 2 N Treatment time: 5 min (+ cooling time) Temperature: 100 °C Pre-treatment: 2 drops of ethanol ii) Back-extraction: Solution: 2 mL toluene Treatment time: 3 min	38 min (**)	n.a.	n.a.	[28]
GC-PD-OES	i) Extraction: Sample hair amount: 100–150 mg	>12 h	7 min	10 μg/kg	[33]

(*continued on next page*)

Table 2 (*continued*)

Analytical Technique	Sample preparation	Extraction time	Chromatographic run time	LOD(*)	Ref.
	Solution: 1.5 mL HNO_3 (20 %) Treatment time: 12 h Temperature: 60 °C ii) Derivatization Solution: acetate buffer (pH = 4.5) + 0.2 mL of 1 %(w/v) $NaBEt_4$ Treatment time: 1min iii) Headspace SPME 10 min sampling time (fiber exposure) at 25 °C				
FC-ICP-MS	Ultrasound-assisted extraction: Hair sample amount: 10–20 mg Solution: 5 mL HCl 500 mM + HBr 37 mM + TU 3.3 mM Treatment time: 15 min	15 min	5 min	5.5 μg/kg	This work

(*) LOD re-adapted on a 10 mg hair sample mass basis.
(**) Cooling time after high temperare extraction/digestion is considered equal to 30 min.
TMAH: Tetramethylammonium hydroxide, ID: Isotope Dilution, HI: Headspace Injection, PD-OES: Point Discharge-Optical Emission Spectroscopy, CV: Cold Vapour, AFS: Atomic Fluorescence Spectroscopy, ECD: Electron Capture Detector.

Table 3
General statistics on total Hg and MeHg concentration found in human hair samples (n = 49).

Statistics	Total Hg (mg/kg)	MeHg (mg/kg)	MeHg/Total Hg (%)
Range	0.14–5.8	0.02–3.2	5.2–123.5
Median value	0.84	0.65	63.8
Mean value	1.2	0.77	64.6
Standard deviation	1.1	0.69	32.1

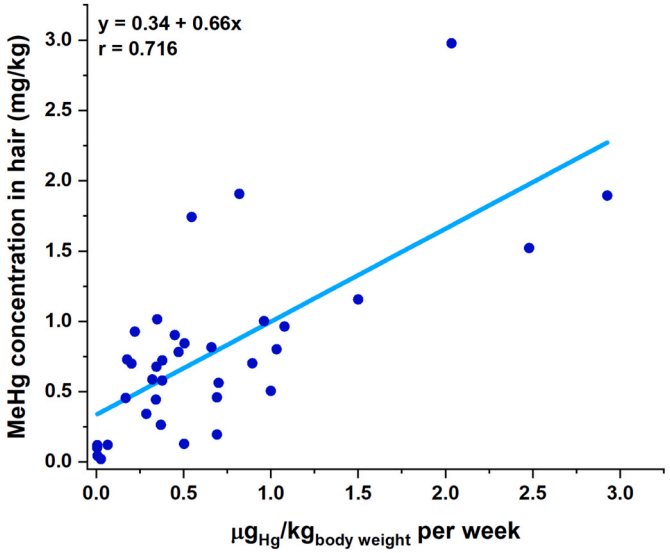


Fig. 5. Correlation between MeHg concentration in hair and the estimated weekly intake per kilogram body weight.

CRediT authorship contribution statement

Davide Spanu: Data curation, Investigation, Methodology, Visualization, Writing - review & editing. **Laura Butti:** Investigation, Methodology. **Sandro Recchia:** Conceptualization, Supervision. **Carlo Dossi:** Methodology, Validation. **Damiano Monticelli:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank the hair donors who made the of the case study possible. We would also like to thank Ms. S. Ianniello, Ms. M. Russo and Ms. A. P. Ciavarella for assisting in mercury analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2023.125612.

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