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Assessment of mercury speciation in feathers using species-specific isotope dilution analysis

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Abstract:

Seabirds are considered as effective sentinels of environmental marine contamination and their feathers are extensively used as non-lethal samples for contaminant biomonitoring. This tissue represents the main route for mercury (Hg) elimination in seabirds and contains predominantly methylmercury (MeHg). In this work, we developed a robust analytical technique for precise and accurate simultaneous quantification of MeHg, inorganic Hg (iHg) and consequently total Hg (THg), in feathers by gas-chromatography (GC)-ICPMS analyses using species-specific isotope dilution technique. An optimisation of the extraction method was carried out by testing different extraction systems, reagents and spiking procedures using an internal reference feather sample. The procedure was validated for MeHg and THg concentrations with a human hair certified reference material. Microwave nitric acid extraction with spike addition before the extraction provided the best recovery and was chosen as the most appropriate species simultaneous extraction method (SSE). An additional assessment was performed by comparison of our developed extraction method and a MeHg specific extraction technique (MSE) classically used for Hg speciation studies on feathers. The developed method was applied to feather samples from a large number of seabirds from the Southern Ocean (penguins, albatrosses, petrels and skuas) to investigate the variability of Hg speciation across a large range of Hg exposure conditions and concentrations. In all cases, MeHg accounted for > 90% of THg, thus verifying the predominance of organic Hg over iHg in feathers.

Keywords: seabirds, Southern Ocean, GC-ICPMS, methylmercury, inorganic mercury, inter-species transformations, keratin

1. Introduction

Mercury (Hg) is a globally distributed pollutant of major concern for humans and wildlife, whose toxicity is known to be dependent on its molecular speciation. Methylmercury (MeHg) is considered the most toxic Hg species and, once acquired by dietary uptake, it accumulates in organisms and biomagnifies within the food webs [1,2]. In aquatic systems, anaerobic microorganisms such as sulphate and iron reducing bacteria (SRB and IRB) transform inorganic Hg (iHg) in MeHg [3–5], resulting in its incorporation into the food chain. Hence top predators, particularly those linked to aquatic ecosystems, are at highest risk for increased dietary Hg exposure, especially MeHg, leading to potential Hg-related health effects [6] and to consequences at the population level [7].

Birds have been extensively used as effective bioindicators of Hg contamination in the environment, particularly of marine ecosystems [8]. Due to their high position on the aquatic food webs and their long life span, seabirds accumulate significant levels of Hg in their tissues. Feathers are interesting samples to analyse because they represent the main route of Hg elimination in seabirds, so contain most of their Hg body burden [9–12]. Moreover, feathers can be easily and non-destructively sampled on live individuals. During moult, most of the Hg stored within internal tissues (70-90%) is remobilised into growing feathers [11], where it is sequestered in the sulphhydryl groups of the keratin molecules and cannot be reincorporated into internal tissues. Once bound to keratin, Hg is physically and chemically stable [13] and resistant to a variety of rigorous treatments [14]. Although other metals such as lead or cadmium are supposed to be incorporated in feathers by atmospheric input, the homogenous distribution pattern of Hg previously observed in feathers has led to hypothesize that Hg contamination is more likely due to endogenous causes (food and physiology) and is not affected by atmospheric exposure [15]. Nevertheless, gaseous Hg adsorption has been recently demonstrated in human hair under Hg vapour exposure [16,17], suggesting the possibility of direct Hg deposition also in feathers under high gaseous Hg ambient concentrations which could potentially have a repercussion on the use of bird feathers from museum collections for retrospective investigation

on Hg temporal trends. However, this process is unlikely to occur in feathers of seabirds inhabiting non-contaminated areas. The first research study of Hg speciation in feathers was carried out by Thompson and Furness [18] in various seabird species, in which they found that Hg incorporated into this tissue was mainly composed of MeHg (77-118%). A dominance of MeHg in feathers was also observed in following studies [19–21]. Hg has been therefore assumed to be present almost exclusively under its organic form in feathers while no accurate determination of iHg has been achieved so far. Total Hg concentrations (THg) are often measured for MeHg quantification in feathers as an economical alternative to speciation analyses. Actually, direct analyses of MeHg in feathers are usually considered on studies focused on temporal variations on Hg concentrations using historical feather collections from museums, e.g [22–25]; where a potential contamination of iHg has been found to be produced by the successive application of preservatives containing HgCl_2 and methyl-bromide [26]. Analyses of Hg speciation in feathers are therefore essential to better evaluate Hg exposure and metabolic processes in birds.

Different analytical methods for Hg speciation in feather samples have been reported in previous studies. Thompson and Furness [27] proposed the first method to determine concentrations of THg and MeHg in feathers by MeHg selective extraction (adapted from Uthe et al. [28]) for subsequent analyses by cold-vapour atomic fluorescence spectroscopy (CV-AFS). This procedure has been widely used by studies of Hg speciation in feathers [19,20,22,29–31]. More developed analytical techniques were later performed by using gas chromatography in order to separate Hg species: gas chromatography coupled to atomic fluorescence spectroscopy (GC-AFS) [32,33]; gas chromatography coupled to electron-capture detector (GC-ECD) [34], and single isotope dilution analysis by gas chromatography coupled to ICP-MS (S-IDA-GC-ICPMS) with single isotope spike (only isotopically labelled MeHg was added) [26]. However, these analytical techniques do not allow correcting possible losses or transformations between Hg species and provide uniquely the quantification of MeHg and THg concentrations, so the determination of iHg concentrations needs to be calculated as the

difference between both compounds' concentrations. A synthesis of the results published in previous studies have been compiled in Table 1. It can be observed that in some cases MeHg proportion values of feathers exceed 100% of THg, indicating a lack of accuracy or precision in MeHg quantification by classical techniques. This observation enhances the interest of our developed method for accurate and precise Hg species analyses.

The quantification of both Hg species involves an analytical challenge due to potential losses or species transformation reactions (i.e., MeHg demethylation or iHg methylation processes) which could occur during the whole analytical procedure, leading to erroneous results in the quantification of Hg species concentrations [35]. GC-ICPMS using a double isotopic dilution method provides the simultaneous measurement of both MeHg and iHg, and subsequently THg as $THg = MeHg + iHg$, with high precision [36,37]. Obtained data can also be processed by Isotope Pattern Deconvolution (IPD), a general model for isotope dilution that takes into consideration both spikes (isotopically enriched solutions) and natural species, and enables the determination of potential interconversion reactions and the consequent correction of Hg species concentrations [35]. Accordingly, isotope dilution methods guarantee a better precision and accuracy than conventional quantification by external calibration [37]. Previous studies published method developments (extraction and derivatisation) for Hg speciation by ID-GC-ICPMS in biological samples, such as seafood [37] or human hair [38]. Preliminary work was carried out on human hair thus allowing an initial approach of Hg species analyses on keratin samples by evaluating different extraction methods [38].

Our research work includes the assessment of two keratin-based materials (feathers and hair) and considers further analytical strategies, such as the isotopically enriched spiking technique in all the extraction methods tested or the additional evaluation of classic MeHg selective extraction. This study evaluates in depth analytical performances on keratin samples and provides information about non-desirable reactions occurring during both extraction and derivatisation of each analytical procedure tested. Hg speciation in feathers of a great number of

seabirds from the Southern Ocean have been successfully determined by applying the developed method.

2. Experimental

2.1 Feather samples and reference materials

Due to the non-existence of commercialised feather reference material, we prepared a pool sample of feathers collected from different individuals of king penguin (*Aptenodytes patagonicus*) from Crozet Islands which was used as internal reference standard (IRM) for our laboratory feather analyses and named P-KP. For the validation of the results, all the analyses were performed on a human hair certified reference material (NIES-13). Human hair has been chosen as the most appropriate matrix for validation of feather analysis since they have a similar composition, almost completely composed of keratin. NIES-13 presents high Hg concentrations and contains ~90% of MeHg ($[THg] = 4420 \pm 200 \text{ ng}\cdot\text{g}^{-1}$ and $[MeHg] = 3800 \pm 400 \text{ ng}\cdot\text{g}^{-1}$, certified values).

The evaluation of MSE and SSE methods was accomplished with feather samples of two marine bird species: the white-chinned petrel (*Procellaria aequinoctialis*) and Antarctic prion (*Pachyptila desolata*). A pool of feathers from a raised pheasant (*Phasianus colchicus*) was used as a control since terrestrial birds are known to accumulate lower amounts of Hg in their tissues than aquatic birds [8].

The developed SSE method was applied to feathers from several seabird species exhibiting a large range of Hg concentrations. The selection of marine birds comprises seven species of penguins: emperor (*Aptenodytes forsteri*), king (*A. patagonicus*), Adélie (*Pygoscelis adeliae*), gentoo (*P. papua*), macaroni (*Eudyptes chrysolophus*), southern rockhopper (*E. chrysocome filholi*) and northern rockhopper (*E. chrysocome moseleyi*) penguins, and the wandering albatross (*Diomedea exulans*), northern (*Macronectes halli*) and southern (*M. giganteus*) giant petrels, Antarctic prion (*Pachyptila desolata*) and Antarctic (*Catharacta maccormicki*) and subantarctic (*C. lönnbergi*) skuas. Feather sampling was conducted in four sites of the French

Southern and Antarctic Territories: Adélie Land (66°40'S, 140°10'E), Crozet Islands (46°26'S, 51°45'E), Kerguelen Islands (49°21'S, 70°18'E) and Amsterdam Island (37°50'S, 77°31'E). Feather sampling dates of each seabird species are indicated in Table 4.

2.2 Sample preparation and extraction procedures

Feathers were cleaned in a 2:1 chloroform:methanol solution for 5 min in an ultrasonic bath, followed by two methanol rinses to remove surface impurities, and then oven dried at 50°C during 48h [39]. They were afterwards well homogenised in order to acquire accurate analytical results avoiding within-feather variation in Hg sequestration, which could produce fluctuations in observed Hg measurement [15]. Feathers were finely cut with scissors to obtain a homogenous sample. In the particular case of king penguin (P-KP), white-chinned petrel and Antarctic prion (used for the extraction method assessment since more quantity of sample was available), feathers were cut with scissors and additionally grinded in a planetary ball mill (Retsch PM400) at 400 rpm. We noted that during homogenization with planetary ball mill, a potential contamination of iHg could occur and therefore, this homogenisation method was later discarded.

Different reagents were tested for the optimisation of the extraction method: acid digestion using nitric acid (HNO_3 ·6N, INSTRA quality) and alkaline digestion by tetramethylammonium hydroxide (25% TMAH in H_2O , Sigma Aldrich). Sample amounts between 0.20-0.25 g were digested in 5 mL of reagent. Two different extraction systems were also tested: microwave (MW) and Hotblock (HB) (Figure 1). Microwave-assisted extraction was performed using a CEM microwave system (Discover SP-D, CEM Corporation) coupled to an autosampler Explorer 4872 96 (USA). The extraction was carried out in CEM Pyrex vessels by 1 min of warming up to 75°C and 3 min at 75°C with magnetic agitation to homogenise the samples. HB extractions were performed in closed PFA vessels (Savillex) of 50 mL at 85°C during 2 hours in a SC100-36 Hotblock (Environmental Express, South Carolina, USA). The addition of isotopic enriched standard solutions was tested before and after the extraction process. In the case of spike addition before extraction, standard solutions were added directly

to the solid sample whereas for spike addition after extraction solutions were added to the extract. All samples were extracted in triplicate.

Prior to Hg species analyses, samples were derivatized at pH 4 by ethylation using sodium tetraethylborate (NaBEt_4 , 5%), in order to produce volatile ethylated forms of Hg that could be separated by gas chromatography, and then extracted in isooctane by mechanical shaking using an orbital shaker during 20 min. Hg species analyses were carried out by GC-ICPMS Trace Ultra GC equipped with a Triplus RSH autosampler coupled to an ICP-MS XSeries II (Thermo Scientific, USA) as detailed in previous works [35].

2.3 Total Hg analyses

Total Hg concentrations were quantified by using an advanced Hg analyser spectrophotometer (AMA-254, Altec). Homogenised samples (aliquots between 10 and 15 mg) were analysed after thermal destruction and gold amalgamation (drying time 60 s, decomposition time 180 s, waiting time for quantitative trapping of released mercury on the gold amalgamator 45 s). A matrix dependent calibration was performed with human hair reference material (NIES-13) by addition of different masses of sample, following EPA method 7473 [40]. This calibration was validated by quantification of a second human hair reference material (IAEA-086), providing an accuracy of $92 \pm 5\%$ ($n=5$) relative to recommended reference value. Feather THg concentrations were calculated by this calibration in order to correct matrix effects associated to keratinised samples. Several blanks were analysed at the beginning of each analytical session. Limit of detection (LOD), calculated for blank average values (15 blanks) plus three times the standard deviation (SD) of these blanks (IUPAC), was 0.15 ng g^{-1} .

2.4 Quantification methods for isotopic dilution calibration

Hg species concentrations were determined by different quantification approaches in order to deeply assess analytical performances on keratin samples. Two quantification methods for isotope dilution technique were used: single-IDA and IPD. The concentrations were calculated by both methods and the transformation factors were calculated using IPD, allowing to evaluate interconversion reactions (M% and D%) that occur during both extraction and derivatisation of

each analytical procedure tested. For double isotope dilution technique, the sample is spiked with known amounts of two isotope tracers (in this case ^{199}iHg and $^{201}\text{MeHg}$) to alter the natural isotopic abundance of the studied endogenous species (^{202}iHg and $^{202}\text{MeHg}$). Quantification is then based on the measurement of the mixed isotope ratios, as explained elsewhere [36,37]. Single-IDA model consists on the specific measurement of Hg species separately. Only two isotopes are considered for the quantification of each Hg species ($R^{202/201}$ for MeHg and $R^{202/199}$ for iHg). IPD takes into account all the different isotopic patterns of both spikes and endogenous species, providing the determination of possible inter-species transformations (M% and D%) and the consequent correction of concentrations [37]. The reported results of [THg] were calculated as the sum of [MeHg] and [iHg] determined by ID-GC-ICPMS, and were compared to [THg] determined by AMA-254 in order to evaluate their similarity and verify the recovery of the extraction.

2.5 Adaptation of MeHg selective extraction method (MSE)

A specific extraction technique of MeHg was applied in feather samples for the analysis of Hg speciation by ID-GC-ICPMS. The method, adapted from Uthe et al. [28], consists in a first extraction of MeHg in an organic phase (toluene) followed by a reverse extraction in aqueous phase. P-KP and feather samples from different marine bird species (Antarctic prion and white-chinned petrel) and one terrestrial species (pheasant) were used to assess the selectivity of this method, as they exhibit a wide range of Hg concentrations and different Hg species distribution. NIES-13 was also extracted in triplicate and analysed.

Firstly, 0.15-0.20 g of feather samples were extracted by alkaline extraction in a HB system in Savillex vessels with sodium hydroxyde (NaOH [reagent grade], 10M, 4 mL) during 2 h at 60°C, following Thompson et al. [27]. Then, 0.5 mL of extract was diluted with 4.5 mL of milliQ water in 50 mL tubes and neutralized afterwards with 0.1 mL of sulfuric acid (H_2SO_4 , ACS Grade 95-98%). 5 mL acidic NaBr (30% w/w NaBr [Ultragrade, 99.5%] in H_2SO_4 4 M), 10 mL of aqueous CuSO_4 (2.5% w/w [reagent grade, 99%]) and 10 mL of toluene (anhydrous, 99.8%) were added to the extracts. Samples were agitated at 420 rpm for 1 hour in an orbital

shaking table. Secondly, an amount of 4 mL of toluene (MeHg passes into the organic phase) was transferred in a Falcon tube with 4 mL of sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$, 0.005 M [ACS grade, 98%]). Samples were vortexed during 1 min. An aliquot of sodium thiosulphate (MeHg-thiosulphate) was collected in 5 mL tubes and kept at 4°C until analyses. The addition of isotopically enriched solutions ($^{201}\text{MeHg}$ and ^{199}iHg) was tested in two different steps: 1) before NaOH extraction and 2) before specific MeHg extraction (after NaOH extraction) (Figure 2). Prior to derivatisation, the addition of a solution of HCl to the MeHg-thiosulphate extract is required to reduce competition with sulphur groups during derivatisation, then different concentrations of HCl solution were tested. The concentrations of the NaBEt_4 solution for ethylation were as well optimised. These tests were performed and validated for NIES-13. The best results were achieved when adding 2 mL of HCl solution (5% v/v) and 200 μL of NaBEt_4 (5% v/v) to 200 μL of thiosulphate extract. The limit volume of thiosulphate extract for derivatisation is 500 μL , since a higher volume involved matrix effects.

2.6 Statistical tests for environmental feather samples

Statistical analyses were performed using XLSTAT 2016. Normality and homoscedasticity were firstly checked for the whole dataset by using Shapiro-Wilk and Breusch-Pagan tests, respectively. Since not all the results all the samples presented a normal distribution and homoscedasticity, non-parametric test was used (Kruskal-Wallis coupled to Conover-Iman procedure with Bonferroni correction). The significance level was fixed of $\alpha=0.05$ for all tests. Values are means \pm SD.

3. Results and discussion

3.1 Comparison of speciation extraction procedures: optimisation

Results of $[\text{MeHg}]$, $[\text{iHg}]$ and $[\text{THg}]$ (as $[\text{MeHg}]+[\text{iHg}]$) determined by - single-IDA and IPD and transformation factors (M% and D%) calculated by IPD for each selected extraction procedure are presented for both reference materials NIES-13 (Table 2) and P-KP (Table 3).

All the extraction methods tested provided satisfactory results for Hg concentrations for the two reference materials, except acid extractions with spike addition after extraction, which resulted in an insufficient recovery for both Hg species. In the case of NIES-13, recoveries for MW acid extractions with spike addition after extraction were $80.6\pm1\%$ and $76.5\pm4.6\%$ for MeHg and THg, respectively, while recoveries of $96.0\pm1.2\%$ and $95.9\pm0.2\%$ were achieved for spike addition before extraction. For P-KP, differences between both spiking procedures were not as remarkable as for NIES-13, but better results of THg were obtained when spike addition was added before ($[\text{THg}]=3899\pm62 \text{ ng}\cdot\text{g}^{-1}$) than after MW acid extraction ($[\text{THg}]=3249\pm118 \text{ ng}\cdot\text{g}^{-1}$). This result indicates that spike addition before extraction is highly recommended for hair and feather samples for correcting possible losses and/or species interconversion processes occurring also during the acid extraction step.

TMAH extracts exhibited lower sensitivity for both keratinised matrixes, which consequently induced a higher standard deviation (SD) in determination and a source of error in quantification, even if no substantial alteration of the obtained isotopic ratio was observed. This response demonstrates the occurrence of undesirable competing reactions during derivatisation when using TMAH as extraction reagent. In an acid medium, MeHg from the spike solution is less complexed with the thiol ligands of keratin. Thus, MeHg is more available for ethylation so the derivatization step will be more effective. In the opposite case, in an alkaline medium there is a stabilization of the MeHg by the complexes formed with the thiols, a minor degradation exists but, at the same time, MeHg will be less reactive and there could be interferences by other derivatized molecules (competition) [41]. Thus, when extracting keratinised samples in alkaline medium the risk of error by integration of attenuated peaks must be taken into account.

Generally, no significant differences in concentration values were observed between Dsingle-IDA and IPD quantification methods for our two reference materials. The only exception was found in the case of NIES-13 MW TMAH with spike addition before extraction. The correction of a substantial D% factor by IPD ($26.6\pm1.0\%$) involved a significant difference on iHg concentrations calculated by single-IDA ($1463\pm398 \text{ ng}\cdot\text{g}^{-1}$) and IPD ($574\pm68 \text{ ng}\cdot\text{g}^{-1}$).

This phenomenon also occurred under the same extraction conditions for P-KP, which exhibited a D% of $11.3 \pm 10.4\%$. According to the results obtained for the rest of extraction methods when spiked solutions were added before extraction, lower but also important D% factors were obtained: MW acid ($5.5 \pm 1.0\%$ and $4.5 \pm 0.2\%$), HB acid ($8.4 \pm 0.1\%$ and $3.3 \pm 0.8\%$) and HB alkaline ($7.7 \pm 0.3\%$ and $6.5 \pm 1.5\%$) for NIES-13 and P-KP samples, respectively. Indeed, for the same extraction conditions but spike addition after extraction, no significant D% were observed on hair and feathers. This result means that significant demethylation reactions mainly occurred during extraction, particularly in the case of MW extraction with TMAH. This could be explained by an influence of different behaviour or transformation rates between endogenous Hg and isotopically enriched Hg from spike solutions [42]. Differences of complexation and lability patterns between Hg from matrix and enriched Hg potentially occur during the extraction procedure, affecting the accuracy of Hg analysis by species-specific isotope dilution. In the previous assessment on human hair samples [38], demethylation during extraction was uniquely observed for HNO₃ oven extraction at 80 °C ($4.6 \pm 2.5\%$). Contrary to our results, no demethylation reactions occurred during TMAH extraction.

Concerning the results obtained for procedures with spike addition after extraction, a significant M% was observed for alkaline extractions of P-KP by both systems: MW ($8.8 \pm 1.9\%$) and HB ($11.9 \pm 8.6\%$). Much lower M% factors were obtained in the case of hair TMAH extracts by MW ($3.0 \pm 1.5\%$) and HB ($1.3 \pm 1.0\%$). Therefore, important methylation reactions occurred in feather TMAH extracts when spike was added after but not when it was added before. It should also be considered that an additional source of error could exist in quantification of TMAH extraction due to low-sensitivity. No significant D% was obtained for extractions with spike addition after extraction, which means that demethylation artefacts barely occurred during derivatisation step whatever the reagent used. Laffont et al. [38] did not observe methylation but considerable demethylation ($4.2 \pm 0.8\%$) during derivatisation of hair TMAH extracts. Notable demethylation reactions occurred during extraction whatever the reagent and extraction system used, and particularly when using TMAH. Due to the existence of important

inter-species conversion reactions during extraction and derivatization of keratin-based material samples, the addition of the enriched solutions before acid or alkaline extraction is highly recommended independently of the method used.

3.2 Comparison of analytical performances

Since no significant differences were observed between MW and HB extraction systems, MW was chosen as the most suitable extraction system since it provides a better-quality control of the extraction, permitting a homogenisation of the sample by electromagnetic stirring and automatic and individually controlled temperature and pressure conditions. Results of MW extraction with spike addition before extraction were evaluated in order to choose the most appropriate reagent (Table S1). The sensitivity was assessed by the measurement of calibration slopes calculated as the relation of the peak area obtained for $^{202}\text{MeHg}$ (counts per second, cps) divided by the concentration of MeHg injected ($\text{ng}\cdot\text{L}^{-1}$) for the injection in 2 μL of isooctane. Much higher sensitivity was obtained for HNO_3 than for TMAH extracts obtained for both hair and feathers. Better precision (RSD) was achieved by HNO_3 extraction than by TMAH for the two reference samples. Such important difference is also a consequence of lower sensitivity achieved after alkaline extraction. Accuracy was calculated by recoveries of MeHg and THg relative to NIES-13 certified values. Although the most satisfactory recoveries (for both MeHg and THg of NIES-13) were obtained for TMAH MW extraction with spike addition before the extraction by IPD quantification, this kind of extraction was refused as a pertinent method due to the low sensitivity obtained when using TMAH reagent. Nitric acid MW extraction with spike addition before extraction provided much higher sensitivity and precision, consequently it was chosen for simultaneous species extraction (SSE) on hair and feather samples. For this selected SSE method, mean recoveries for MeHg and THg in NIES-13 quantified by single-IDA ($96.0\pm1.2\%$ and 95.9 ± 0.2) and IPD ($94.2\pm1.4\%$ and $93.7\pm2.3\%$) were satisfactory and very similar, suggesting the possibility of using both quantification approaches as valid analytical solutions. Due to practical reasons, single-IDA was chosen in this study to avoid the long data treatment required by IPD. Nevertheless, IPD permits the calculation of conversion reactions

among Hg species and the consequent correction of concentrations related to these transformations and therefore, it is considered a more powerful approach. Indeed, IPD generally provides more accurate results than a single-IDA except under particular circumstances in which Hg species concentrations are substantially different within the same sample [43]. In the rest of cases, the level of accuracy of IPD is higher, although it is less precise because the correction of species interconversion is carried out at the expense of the precision of the obtained amount of interconverting analytes [44]. In conclusion, since satisfactory results were obtained either using single-IDA or IPD in hair and feather samples, IPD remains a reference accurate method for metrology and analytical development while single-IDA can be easily used for routine monitoring analyses.

3.3 Long-term internal reproducibility of SSE method by ID-GC-ICPMS

An evaluation of long-term internal reproducibility and repeatability of the optimised method was performed on human hair certified reference material (NIES-13) and on our internal reference material for feather samples (P-KP). Extractions by SSE method were performed in triplicate. Internal reproducibility was assessed for triplicate injection of the three extracts of each reference material (n=9), prepared following identical protocols and by the same operator and equipment in each analytical session, with 1-6 months of interval (Figure S1). Since the term reproducibility implies the involvement of measurement by different operators and laboratories, we use the term internal reproducibility. The precision, calculated as the mean RSD, was 1.38% and 1.49% for MeHg and 5.33% and 1.71% for iHg for NIES-13 and P-KP, respectively. Repeatability was estimated by analysing in triplicate the same extract the same day, under identical conditions (n=3). The mean RSD was 1.24% and 1.64% for MeHg and 7.28% and 3.04% for iHg in the case of NIES-13 and P-KP, respectively. A previous analytical publication which developed this method for application to human hair samples [38] obtained a repeatability for triplicate injection of IAEA-86 (n=6) between 1.0–2.5% for MeHg and 0.5–1.8% for iHg. Accuracy was evaluated using NIES-13 and calculated as the recovery of measured MeHg and THg concentrations relative to certified values. MeHg and THg mean

recovery for all the analytical sessions (i.e. average of all the injections), was $94\pm 2\%$ and $98\pm 3\%$, respectively. Laffont et al. [38] obtained mean recoveries of 98 and 88% for the reference human hair sample (IAEA-086). Limits of detection (LOD) and quantification (LOQ) were calculated as the sum of spiked $\text{HNO}_3\cdot 6\text{N}$ blank average values analysed in triplicate (15 blanks of extraction) plus three times the standard deviation (SD) of these blanks for LOD or ten times for LOQ (IUPAC). For extraction of 0.25 g of hair or feathers in 5 mL of $\text{HNO}_3\cdot 6\text{N}$, LOD obtained is 3.24 ng g^{-1} and 11.62 ng g^{-1} and LOQ is 9.04 ng g^{-1} and 30.37 ng g^{-1} , for MeHg and iHg, respectively.

3.4 Intercomparison methods for THg concentrations quantification

Results of THg concentrations were compared for direct quantification by AMA-254 and for the sum of Hg compounds by speciation analyses (SSE) (Table S2). P-KP feather sample was analysed several times in order to obtain a representative THg concentration value ($[\text{THg}]=3816\pm 275\text{ ng}\cdot\text{g}^{-1}$, $n=12$). Feather THg concentrations obtained by both methods were satisfactory, with recoveries higher than 97% when comparing AMA-254 to SSE values. Precision (RSD) was higher by SSE method (0.3-1.6%) than by AMA-254 (4.3-7.2%) even if for SSE method analyses are performed for triplicate extraction.

3.5 Intercomparison between MeHg selective extraction (MSE) and simultaneous species extraction (SSE)

Feather samples were extracted by our previously optimised method for Hg speciation in feathers (SSE) and by MeHg selective extraction (MSE). Results of MeHg concentrations for both methods are given in Table S3. Recoveries of MSE for NIES-13 were calculated relative to MeHg certified values. For the rest of the samples, recoveries were calculated in function to MeHg concentrations obtained by SSE method. Non-significant differences between the mean concentrations obtained for blank extractions and iHg fractions quantified by isotope dilution were found for the MSE extracts, meaning that exclusively MeHg was extracted. We observed notable differences between concentrations values for both types of spiking procedures for MSE. In all cases, spike addition before NaOH extraction provided better results. For NIES-13

recoveries of [MeHg] were satisfactory for SSE ($96\pm3\%$) and MSE with spike addition before ($97\pm6\%$), but not sufficient for MSE with spike addition after extraction ($80\pm4\%$). P-KP presented recoveries of [MeHg] of $76\pm7\%$ and $82\pm10\%$ for MSE with spike addition after and before extraction, respectively. Precision (as RSD) was similar for MSE with spike addition after extraction than before extraction. For NIES-13 and P-KP reference samples, extracted in triplicate, precision of MSE with spike after extraction was 4.7% and 9.0%; whereas precision obtained for MSE with spike addition before was 6.3% and 12.3%, respectively. For feather samples, extracted only once, mean RSD was 3.2% (2.6-4.0%) and 2.2% (1.8-4.8%) for spike addition after and before, respectively. Better results were achieved for MSE with spike addition before NaOH extraction as it enables the correction of methylation or demethylation reactions occurring during the whole procedure, which in the case of MSE involves several steps that could induce undesirable interconversion reactions.

A logarithmic representation of obtained [MeHg] values for all the samples tested is shown in Figure 3. Since the addition of spike before NaOH extraction resulted in better recoveries, only these results of MSE are plotted to compare to SSE. The precision in MeHg quantification appeared to be much higher for SSE method for reference materials (1.26% for NIES-13 and 1.53% for P-KP) and feather samples (mean value 1.17% (0.27-2.32%)). This could mean that MSE is less precise, maybe due to its higher complexity and elevated number of analytical steps. As a general trend, results for both extraction methods agree with the expected values for reference materials and matched for the rest of feathers samples ($R^2=0.997$).

MSE can be thus considered as an efficient and valid method to quantitatively extract MeHg in hair and feather samples covering a great range of Hg concentrations. However, it is important to highlight that, in this study, MeHg extracted by MSE has been measured by isotope dilution and not by external calibration, allowing to obtain more precise measurements and to correct possible losses or transformations during the whole analytical procedure and providing a more precise quantification of MeHg concentrations. Determination of Hg concentrations by external calibration, as it was performed by previously published studies on Hg speciation using

MSE [18–20,45], could induce a lack of accuracy and precision and therefore errors in MeHg quantification.

4. Hg speciation in feathers from Southern Ocean seabirds

The optimised SSE method was applied on feather samples from a large number of marine birds from the Southern Ocean (details in section 2.1). This selection of seabird species exploiting different ecological characteristics (such as feeding habits, trophic positions and thus different Hg exposure conditions) permits to use our developed speciation method in feathers covering a large range of Hg concentrations (from 375 ± 87 to 39924 ± 29412 ng THg·g⁻¹, corresponding to Adélie penguins and wandering albatrosses, respectively). Values of MeHg, iHg and THg concentrations (as MeHg+iHg) are presented in Table 4. THg concentrations values obtained by AMA-254 and those calculated as the sum of MeHg and iHg concentrations by SSE method for all the feather samples were highly correlated (Pearson's correlation, $r=0.985$, $p<0.001$, $n=175$) (Figure S2).

Generally, penguins displayed the lowest feather MeHg concentrations whereas southern and northern giant petrels, subantarctic skuas and wandering albatrosses presented significantly higher MeHg concentrations (Kruskal-Wallis, $H=156.27$, $p<0.0001$, $n=175$). Considering the proportion of MeHg in the feathers, no substantial differences were found among the different analysed species (Kruskal-Wallis, $H=73.06$, $p<0.0001$, $n=175$), with MeHg being the major Hg compound for all the individuals. As it was expected, all the feathers displayed more than 80% of Hg as MeHg. This result is coherent with a dominant presence of MeHg in feathers obtained in previous studies on seabirds from different localities [19–21]. It indicates that Hg speciation in feathers is not influenced by the levels of Hg concentrations, with MeHg being the most abundant compound since it is preferentially excreted via feathers for detoxification purposes (e.g, [46,47]). Despite the predominance of MeHg in seabird feathers, the amounts of iHg appeared to be non-negligible (reaching almost 20% of total Hg in some individuals). This result highlights the necessity of measuring both Hg compounds in feathers to better investigate Hg exposure and metabolic response of birds.

5. Conclusions

A method for the simultaneous determination of Hg speciation in feathers was optimised and validated. The evaluation of different extraction procedures, spiking strategies and quantification methods was performed, concluding that nitric acid microwave assisted extraction with spike addition before the extraction was found the most adequate for feathers (and hair) samples. Both single-IDA and IPD quantification methods are proposed as valid analytical approaches for either routine analysis or monitoring issues (single-IDA) or metrology and analytical development purposes (IPD). In our case, due to the high number of feather samples, single-IDA was favoured because it is a more practical option. The developed method demonstrates the capability of the GC-ICPMS by using species-specific isotope dilution for the precise and accurate measurement of MeHg, iHg and thus THg as MeHg+iHg concentrations and the correction of potential transformations between MeHg and iHg compounds during the different analytical steps. It was successfully applied in environmental feather samples where MeHg appeared to be the major species for all the feathers analysed independently of THg concentrations. This finding fits well with the evidence that seabirds excrete MeHg in moulting feathers as a Hg detoxification strategy. However, non-negligible amounts of iHg were present in feathers from some individuals. This finding, together with the existence of accidental iHg contamination in feather museum collections, supports the recommended application of methods measuring both Hg compounds' concentrations in feathers.

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Table 1. Synthesis of main previous studies on Hg speciation in feathers in order of publication. Result values are represented as mean±SD.

Hg speciation approach	Analytical method	Species	Region	n	THg ($\mu\text{g}\cdot\text{g}^{-1}$)	iHg ($\mu\text{g}\cdot\text{g}^{-1}$)	MeHg ($\mu\text{g}\cdot\text{g}^{-1}$)	MeHg (%)	Ref
Hg species measured: MeHg, THg MeHg specific extraction	CV-AFS	Wandering albatross complex	Gough Island (South Atlantic Ocean) Marion Island (Indian Ocean)	26	30.7±11.7	n/a	29.2±11.6	95	[18]
		Sooty albatross	Gough Island (South Atlantic Ocean)	7	9.4±3.9	n/a	9.1±4.0	97	
		Northern fulmar	Foula (Shetland)	15	1.8±0.8	n/a	2.0±0.7	111	
		European shag	Foula (Shetland)	14	1.7±0.7	n/a	2.0±0.8	118	
		Great skua	Foula (Shetland)	14	6.8±4.4	n/a	7.3±5.5	107	
		Arctic skua	Foula (Shetland)	9	2.2±1.7	n/a	1.7±1.8	77	
		Kittiwake	Foula (Shetland)	14	2.4±0.6	n/a	2.2±0.7	92	
		Razorbill	Foula (Shetland)	16	2.1±0.3	n/a	2.1±0.6	100	
		Common guillemot	Foula (Shetland)	17	1.5±0.4	n/a	1.7±0.5	113	
		Puffin	Foula (Shetland)	10	5.2±2.7	n/a	5.1±2.1	98	
Hg species measured: MeHg, THg MeHg specific extraction	CV-AFS	Oldsquaw	Chaun (Northeast Siberia)	5	0.7±0.2	n/a	0.9±0.2	128	[19]
		Herring gull	Chaun (Northeast Siberia)	5	6.1±4.6	n/a	6.5±4.5	106	
		Arctic tern	Chaun (Northeast Siberia)	5	0.9±0.1	n/a	1.1±0.1	122	
Hg species measured: MeHg, THg MeHg specific extraction	CV-AFS	Great egret	Everglades (Florida)	6	2.0±0.2	n/a	n/a	120	[31]
Hg species measured: MeHg, THg MeHg specific extraction	GC-AFS	Arctic tern	New Brunswick (Canada)	5	0.9±0.5	n/a	0.8±0.3	95±15	[21]
		Common murre	New Brunswick (Canada)	5	1.0±0.4	n/a	1.2±0.3	133±33	
		Common tern	New Brunswick (Canada)	5	1.4±0.6	n/a	1.6±1.8	114±65	
		Razorbill	New Brunswick (Canada)	5	1.8±0.7	n/a	1.1±0.4	82±35	
		Atlantic puffin	New Brunswick (Canada)	5	4.9±2.8	n/a	1.6±0.7	100±50	
		Leach's storm petrel	New Brunswick (Canada)	5	4.9±2.8	n/a	5.3±3.4	99±40	
Hg species measured: MeHg, iHg, THg Chromatographic separation	ID-GC-ICPMS	Black-footed albatross (post-1990)	Pacific Ocean	10	47.9±35.7	0.5±1.2	42.1±30.9	89±14	[26]
Hg species measured: MeHg, THg MeHg specific extraction	CV-AFS	Ivory gulls (post-1975)	Canada	5	3.5±2.1	n/a	2.7±2.2	68±37	[24]
Hg species measured: MeHg, THg Chromatographic separation	GC-ECD	Magellanic penguins	Southern Brazilian coast	22	0.8±0.4	n/a	0.6±0.3	n/a	[34]
Hg species measured: MeHg, iHg, THg Chromatographic separation	GC-AFS	Ivory gulls	Canada	4	11.6±6.5	n/a	9.4±5.3	81±4	[33]
		Glaucous gull	Canada	4	2.3±1.7	n/a	2.1±1.7	91±4	
		Black-legged kittiwake	Canada	2	3.6±0.9	n/a	3.3±0.9	91±n/a	
		Common eider	Canada	10	0.6±0.2	n/a	0.5±0.2	87±2	
		Thick-billed murre	Canada	10	1.9±0.6	n/a	1.7±0.6	90±1	
		Northern fulmar	Canada	10	2.7±0.7	n/a	2.4±0.6	88±1	

Table 2. Results of Hg species concentrations and recoveries obtained for the different extraction methods tested in CRM (NIES-13) and calculated by the two different calculation methods (D-IDA and IPD). Species interconversion factors were calculated by IPD. N is referred to number of extractions.

NIES-13	Human hair CRM				Concentrations (ng·g ⁻¹)				Recoveries (%)		Interconversion factors (%)							
Calculation method	System	Reagent	Spike addition	n	MeHg		iHg		THg		MeHg (%)		THg(%)		M (%)		D (%)	
			Certified values		3800 ± 400				4420 ± 200									
Single-IDA	Microwave	HNO ₃ ·6N	after extraction	3	3064 ± 55		319 ± 148		3383 ± 158		81 ± 1		77 ± 5					
			before extraction	3	3647 ± 46		591 ± 53		4238 ± 52		96.0 ± 1.2		95.9 ± 0.2					
	Microwave	TMAH	after extraction	3	3467 ± 236		594 ± 8		4202 ± 104		96 ± 3		95 ± 2					
			before extraction	3	3651 ± 417		1463 ± 398		5114 ± 589		96 ± 11		116 ± 13					
	HotBlock	HNO ₃ ·6N	after extraction	3	3022 ± 157		661 ± 16		3683 ± 149		80 ± 4		83 ± 3					
			before extraction	3	3403 ± 28		621 ± 64		4025 ± 48		90 ± 1		91 ± 1					
	HotBlock	TMAH	after extraction	3	3364 ± 150		845 ± 99		4210 ± 192		89 ± 4		95 ± 4					
			before extraction	3	3549 ± 263		964 ± 257		4514 ± 499		93 ± 7		102 ± 11					
IPD	Microwave	HNO ₃ ·6N	after extraction	3	3022 ± 18		218 ± 53		3240 ± 62		79.5 ± 0.5		73 ± 1		2.8 ± 1.2		0.3 ± 0.1	
			before extraction	3	3578 ± 53		565 ± 82		4143 ± 103		94 ± 1		94 ± 2		1.5 ± 1.2		5.5 ± 1.0	
	Microwave	TMAH	after extraction	3	3574 ± 104		616 ± 56		4191 ± 92		96 ± 1		96 ± 2		3.0 ± 1.5		1.3 ± 0.6	
			before extraction	3	3803 ± 112		574 ± 68		4377 ± 44		100 ± 3		99 ± 1		1.0 ± 0.3		26.6 ± 1.0	
	HotBlock	HNO ₃ ·6N	after extraction	3	2826 ± 23		704 ± 9		3529 ± 31		74 ± 1		80 ± 1		0.7 ± 0.2		-1.1 ± 0.1	
			before extraction	3	3421 ± 45		479 ± 22		3900 ± 23		90 ± 1		88 ± 1		1.0 ± 0.3		8.4 ± 0.1	
	HotBlock	TMAH	after extraction	3	3146 ± 186		654 ± 37		3800 ± 148		83 ± 5		86 ± 3		1.3 ± 1.0		2.9 ± 0.4	
			before extraction	3	3346 ± 32		494 ± 55		3840 ± 87		88 ± 1		87 ± 2		2.7 ± 1.4		7.7 ± 0.3	

Table 3. Results of Hg species concentrations and recoveries obtained for the different extraction methods tested in feather internal reference sample (P-KP) and calculated by the two different calculation methods (D-IDA and IPD). Species interconversion factors were calculated by IPD. N is referred to number of extractions.

P-KP	King penguin feathers (IRM)			<i>n</i>	Concentrations (ng·g ⁻¹)			Interconversion factors (%)			
					MeHg	iHg	THg	M (%)		D(%)	
Calculation method	System	Reagent	Spike addition								
THg AMA-254 (n=12)							3816 ± 275				
Single-IDA	Microwave	HNO ₃ ·6N	after extraction	3	2219 ± 50	1029 ± 106	3249 ± 118				
			before extraction	3	2539 ± 39	1360 ± 49	3899 ± 62				
	Microwave	TMAH	after extraction	3	2461 ± 159	928 ± 83	3389 ± 179				
			before extraction	3	2581 ± 256	1592 ± 311	4173 ± 237				
	HotBlock	HNO ₃ ·6N	after extraction	3	2238 ± 40	1267 ± 77	3506 ± 87				
			before extraction	3	2376 ± 22	1315 ± 13	3691 ± 28				
	HotBlock	TMAH	after extraction	3	2733 ± 70	1003 ± 50	3737 ± 86				
			before extraction	3	2384 ± 192	1438 ± 137	3822 ± 225				
IPD	Microwave	HNO ₃ ·6N	after extraction	3	2175 ± 43	1162 ± 91	3337 ± 101	1.3 ± 1.1	0.5 ± 0.7		
			before extraction	3	2585 ± 22	1405 ± 46	3990 ± 51	0.3 ± 0.3	4.5 ± 0.2		
	Microwave	TMAH	after extraction	3	2161 ± 23	964 ± 67	3125 ± 71	8.8 ± 1.9	0.2 ± 0.3		
			before extraction	3	2505 ± 284	1217 ± 114	3722 ± 323	0.1 ± 0.8	11.3 ± 10.4		
	HotBlock	HNO ₃ ·6N	after extraction	3	2165 ± 106	1240 ± 77	3404 ± 131	2.8 ± 1.5	0.2 ± 0.2		
			before extraction	3	2374 ± 24	1194 ± 24.4	3568 ± 40	0.5 ± 0.2	3.3 ± 0.8		
	HotBlock	TMAH	after extraction	3	2118 ± 138	1110 ± 47	3228 ± 145	11.9 ± 8.6	1.0 ± 0.9		
			before extraction	3	2452 ± 156	1266 ± 90.9	3718 ± 184	1.4 ± 1.3	6.5 ± 1.5		

Table 4. Results of MeHg, iHg and THg concentrations obtained for feathers of 13 seabird species of the Southern Ocean. Values are expressed as mean±SD. N means number of individuals analysed. Groups with different letter presented statistically different values (Kruskal-Wallis).

Species	Locality	Sampling dates	Status	Chick diet	n	THg		iHg	MeHg					
						(ng g ⁻¹)		(ng g ⁻¹)	(ng g ⁻¹)	Statistic	%	Statistic		
Spheniscidae														
Emperor penguin	Terre Adélie	Nov 2011	Chicks	pelagic fish	10	812±100	(662-1047)	48±19	764±96	A,B	94±2	(88-97%)	B,C,D,E	
King penguin	Crozet	Oct 2011	Adults	pelagic fish	11	2291±704	(1658-4141)	178±82	2113±633	D,E,F	92±2	(89-95%)	A,B,C,D	
Adélie penguin	Terre Adélie	Feb 2012	Adults	pelagic crustaceans & fish	10	375±87	(289-532)	28±16	347±76	A	93±3	(88-98%)	A,B,C,D,E	
Gentoo penguin	Crozet	Oct 2011	Adults	crustaceans & fish	11	4330±1853	(1548-7590)	430±181	3899±1686	E,F	90±2	(87-92%)	A	
Macaroni penguin	Crozet	Jan 2012	Adults	pelagic crustaceans & fish	10	2274±244	(1852-2575)	222±49	2053±231	F,G	90±2	(87-94%)	A	
Southern rockhopper penguin	Crozet	Feb 2012	Adults	pelagic crustaceans & fish	10	1388±243	(1055-1685)	110±36	1279±214	A,B,C	92±2	(90-95%)	A,B,C	
Northern rockhopper penguin	Amsterdam	Nov 2011	Adults	pelagic crustaceans, fish & squid	10	1692±241	(1250-2033)	122±43	1571±243	B,C,D	93±3	(88-95%)	A,B,C,D,E	
Diomedeiidae														
Wandering albatross	Crozet	Dec 2007-Mar 2008	Adults	cephalopods	10	39924±29412	(6334-91651)	3044±2846	35880±28229	J	90±6	(81-99%)	A,B,C	
Procellariidae														
Northern giant petrel	Crozet	Jan 2009	Chicks	seabirds	10	5760±1675	(3935-8867)	447±145	5317±1543	G,H	92±1	(90-94%)	A,B,C	
Northern giant petrel	Crozet	Nov 2008	Adults	seabirds	10	12714±6904	(4730-28823)	541±364	12173±6563	I,J	96±1	(90-94%)	E	
Southern giant petrel	Crozet	Feb-Mar 2009	Chicks	seabirds	11	5795±821	(4899-7733)	532±146	5263±829	G,H	91±3	(84-93%)	A,B	
Southern giant petrel	Crozet	Oct 2009	Adults	seabirds	10	11082±4623	(5066-19966)	587±301	10495±4438	I,J	95±2	(89-96%)	C,D,E	
Antarctic prion	Kerguelen	Jan 2012	Adults	crustaceans	10	2568±918	(1219-3985)	152±68	2416±888	D,E,F	92±1	(89-94%)	A,B,C	
Stercorariidae														
Antarctic skua	Terre Adélie	Dec 2011-Jan 2012	Chicks	penguins	11	1933±284	(1338-2393)	165±80	1817±266	C,D,E	92±3	(84-95%)	A,B,C	
Subantarctic skua	Kerguelen	Dec 2011	Chicks	petrels	10	6978±116	(4134-8210)	500±189	6493±1082	H,I	93±2	(89-96%)	A,B,C,D,E	
Subantarctic skua	Crozet	Jan-Feb 2012	Chicks	penguins & rats	11	4911±162	(2293-6328)	273±173	4642±1507	G,H	95±2	(89-97%)	C,D,E	
Subantarctic skua	Amsterdam	Dec 2011	Chicks	unknown (seabirds?)	10	12366±2443	(9271-17667)	584±130	11782±2376	I,J	95±1	(94-96%)	D,E	

Figure 1: Optimisation procedure for Hg speciation analyses in feathers: scheme of the extraction methods tested

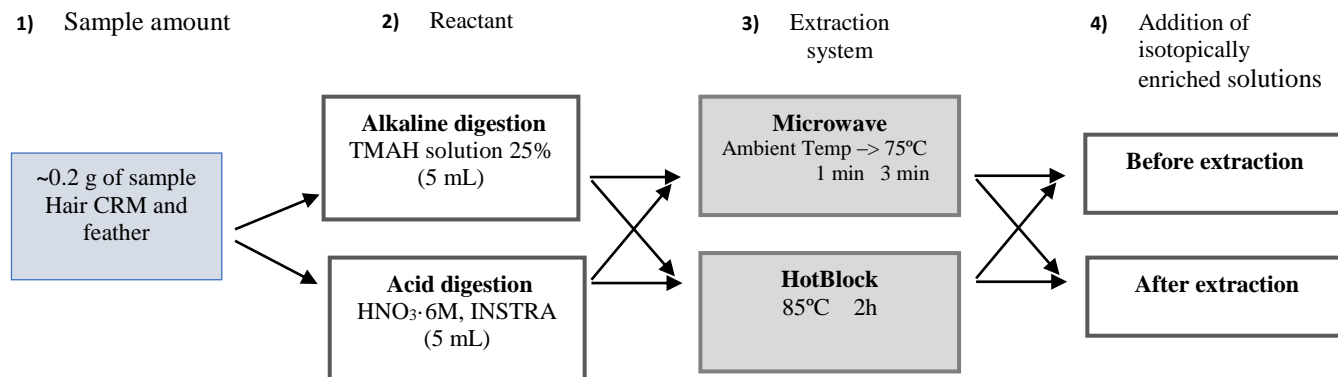


Figure 2: Sample preparation flow chart of the two methods compared: speciation extraction and MeHg specific extraction in human hair and feather samples.

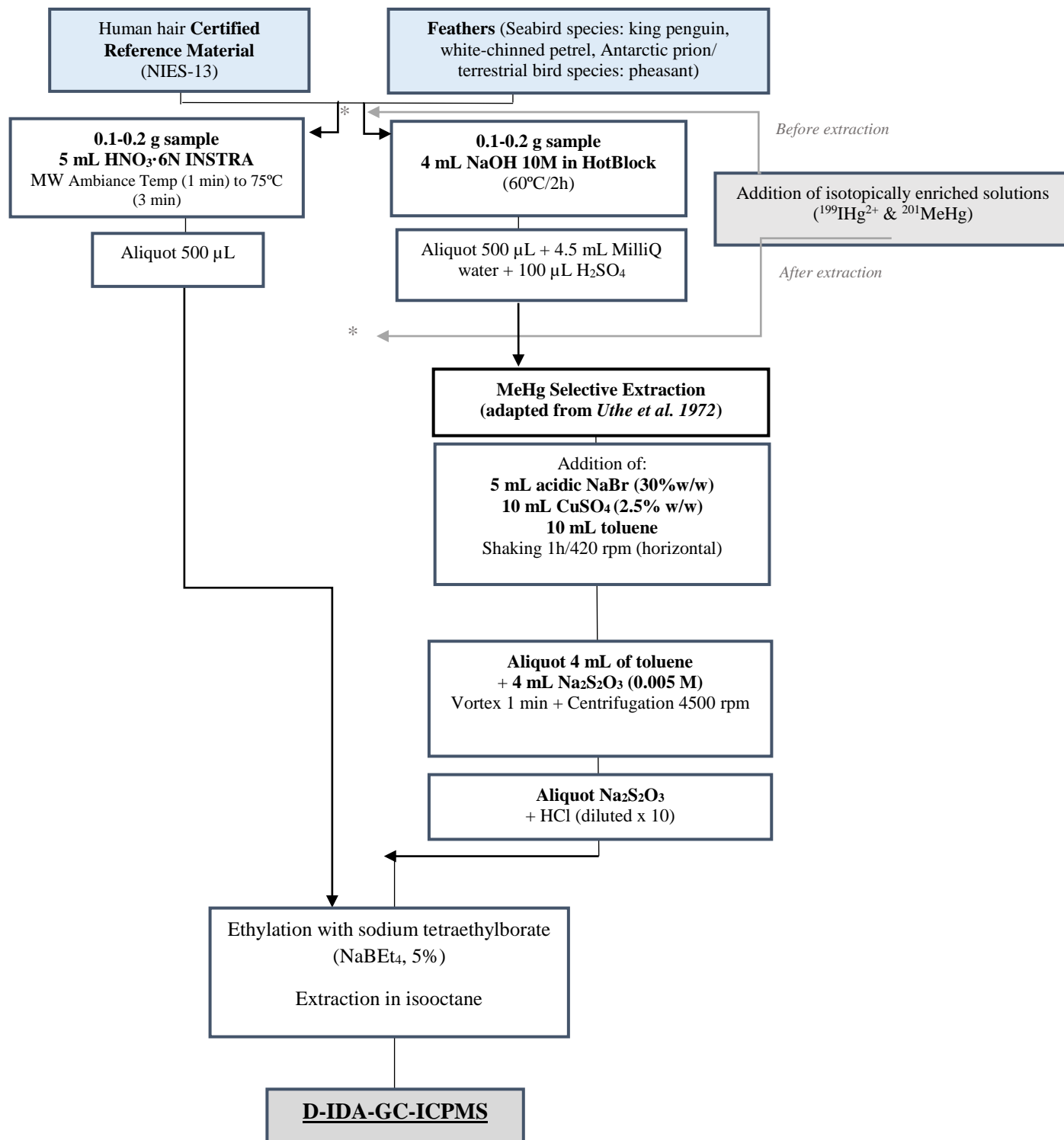
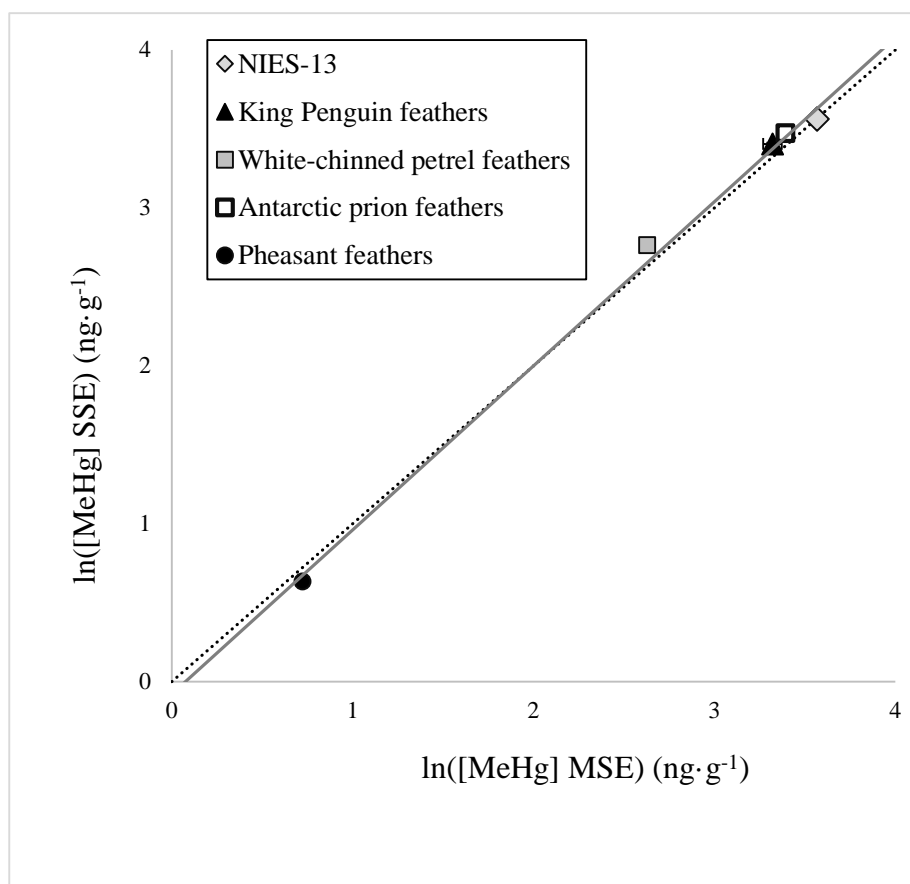


Figure 3: Comparison of MeHg concentrations ($\text{ng}\cdot\text{g}^{-1}$, logarithmic representation) obtained by simultaneous species extraction (SSE) and MeHg selective extraction (MSE) methods by isotope dilution analyses with spike addition before extraction in certified reference material (NIES-13) and feather samples for the key species studied. Trend line $y = 1.0399x - 0.0798$ (Pearson's correlation, $r=0.998$, $p<0.001$, $n=5$)



Supplementary material

Assessment of mercury speciation in feathers using species-specific isotope dilution analysis

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Table S1. Comparison of analytical performances for acid and alkaline reagents by using microwave assisted extraction (3 extractions) of both studied matrixes human hair CRM (NIES-13) and feathers IRM (P-KP).

Matrix	Method	Sensitivity (cps/ng·L ⁻¹)	Precision (%)		Accuracy (%)	
			MeHg	iHg	MeHg	THg
Hair (NIES-13)	MW TMAH	1.39E+04	10.70 ± 1.45	7.75 ± 1.34	96.0 ± 10.9	115.7 ± 13.3
	MW HNO ₃ 6N	8.14E+05	0.38 ± 0.05	1.59 ± 0.31	95.9 ± 1.2	95.9 ± 0.2
Feathers (P-KP)	MW TMAH	6.53E+04	5.83 ± 2.27	3.76 ± 1.28		
	MW HNO ₃ 6N	1.05E+06	1.30 ± 0.34	1.58 ± 0.23		

Table S2. Comparison of THg concentrations obtained by the developed speciation method (SSE) and AMA-254 analyses in feather samples. N for SSE method is referred to number of extractions (analysed in triplicate). N for AMA-254 is referred to number of analyses.

Sample	THg SSE (ng g ⁻¹)				THg AMA-254 (ng g ⁻¹)				Rec (%) as (AMA-254/SSE)*100
	Mean	SD	RSD (%)		Mean	SD	RSD (%)		
P-KP	3899	62	1.6	(<i>n=3 ext</i>)	3816	275	7.2	(<i>n=12</i>)	98
P-WCP02	724	6	0.8	(<i>n=3 ext</i>)	706	33	4.7	(<i>n=3</i>)	98
P-FA09	3711	12	0.3	(<i>n=3 ext</i>)	3589	155	4.3	(<i>n=3</i>)	97

Table S3: Comparison of MeHg concentrations obtained for simultaneous species extraction (SSE) and MeHg selective extraction (MSE) methods by isotope dilution analyses with spike addition before extraction in Certified Reference Materials (NIES-13) and feather samples for the key species studied. N is referred to number of extractions

Sample			<i>n</i>	MeHg (ng·g ⁻¹)			Recovery (%) MSE/SSE*100		
NIES-13	Human hair CRM	<i>Certified values</i>		3800	±	400			
		SSE	3	3647	±	46	96	±	3
		MSE spike after	3	3045	±	142	80	±	4
		MSE spike before	3	3687	±	233	97	±	6
P-KP	King penguin feathers	SSE	3	2539	±	39			
		MSE spike after	3	1971	±	9	76	±	7
		MSE spike before	3	2108	±	260	82	±	10
P-WCP02	White-chinned petrel feathers	SSE	1	579	±	7			
		MSE spike after		NA					
		MSE spike before	1	425	±	17	73	±	3
P-FA09	Antarctic prion feathers	SSE	1	2966	±	8			
		MSE spike after	1	1883		35	63	±	1
		MSE spike before	1	2472	±	64	83	±	2
P-FSN	Pheasant feathers	SSE	1	4.3	±	0.1			
		MSE spike after	1	4.3	±	0.2	100	±	4
		MSE spike before	1	5.3	±	0.1	123	±	3

Figure S1: Control charts of measured MeHg and THg concentrations for reference materials NIES-13 and P-KP for each analytical session.

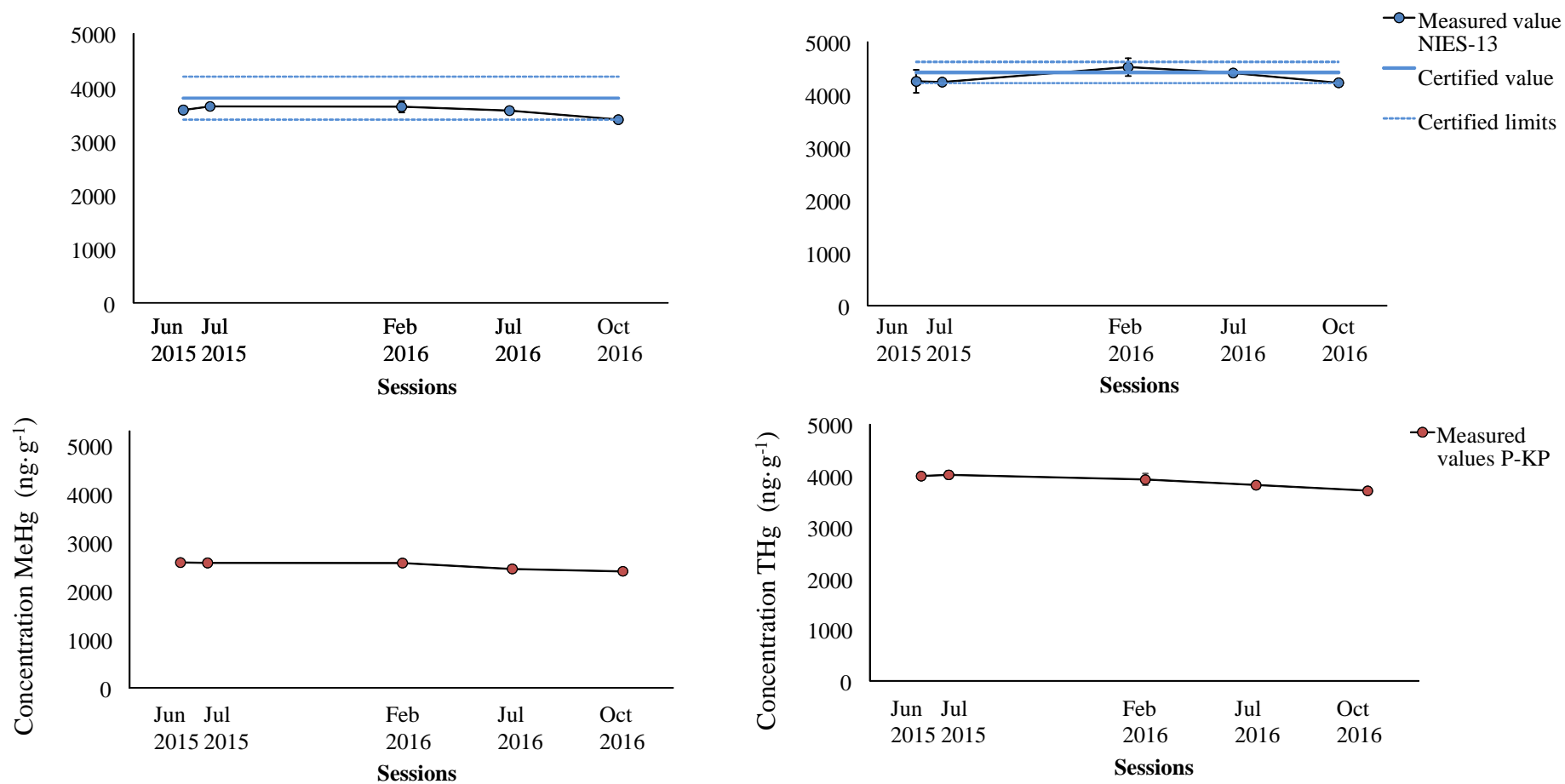


Figure S2. Comparison of THg concentrations ($\text{ng}\cdot\text{g}^{-1}$, logarithmic representation) obtained by simultaneous species extraction SSE (MeHg+iHg) and by AMA-254 for all feather samples of the 13 seabird species studied ($n=175$ individuals).

