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PRACTICAL AND THEORETICAL CONSIDERATIONS FOR THE DETERMINATION OF $\delta^{13}C_{VPDB}$ VALUES OF METHYLMERCURY IN THE ENVIRONMENT

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ABSTRACT

RATIONALE: Analytical methods that can identify the source and fate of mercury and organomecury compounds are likely to be useful tools to investigate mercury in the environment. Carbon isotope ratio analysis of methylmercury (MeHg) together with mercury isotope ratios may offer a robust tool to study environmental cycling of organomercury compounds within fish tissues and other matrices.

METHODS: MeHg carbon isotope ratios were determined by gas chromatography/combustion-isotope ratio mass spectrometry (GC/C-IRMS) either directly or following derivatization using sodium tetraethylborate. The effects of normalisation protocol and of derivatization on the measurement uncertainty of the methylmercury $\delta^{13}C_{VPDB}$ values were investigated.

RESULTS: GC/C-IRMS analysis resulted in a $\delta^{13}C_{VPDB}$ value for an in-house MeHg reference material of $\delta^{13}C_{VPDB}$ = -68.3 ± 7.7 ‰ (combined standard uncertainty, k = 1). This agreed very well with the value obtained by offline flow-injection analysis-chemical oxidation-isotope ratio mass spectrometry of $\delta^{13}C_{VPDB}$ = -68.85 ± 0.17 ‰ (combined standard uncertainty, k = 1) although the uncertainty was substantially larger. The minimum amount of MeHg required for analysis was determined to be 20 μg.

CONCLUSIONS: While the $\delta^{13}C_{VPDB}$ values of MeHg can be obtained by GC/C-IRMS methods with or without derivatization, the low abundance of MeHg precludes such analyses in fish tissues unless there is substantial MeHg contamination. Environmental samples with sufficient MeHg pollution can be studied using these methods provided that the MeHg can be quantitatively extracted. The more general findings from this study regarding derivatization protocol implementation within an autosampler vial as well as measurement uncertainty associated with derivatization, normalisation to reporting scales and integration are applicable to other GC/C-IRMS-based measurements.

INTRODUCTION

Mercury (Hg) is very toxic to humans as well as animals; it can bio-accumulate in terrestrial and aquatic ecosystems and is therefore a pollutant of particular concern in the environment in general. There is little doubt that human activities have contributed to the levels of Hg in the natural environment, particularly during the last century.[1] While there is legislation in place to limit anthropogenic release of Hg and its use is being phased out where less toxic alternatives exist, there is still a requirement for measurement of mercury in the environment as well as the development of methods to trace Hg through ecosystems. These measurements underpin measures aimed at monitoring mercury pollution, such as the European Water Framework Directive and the Minamata Convention.[2,3]

While Hg can exist in many chemical forms within the environment such as elemental mercury (Hg^0) and oxidised mercury (Hg^{2+} and Hg_2^{2+}), it is organic forms of mercury such as methylmercury (MeHg) that are of particular interest when investigating Hg transport within the environment. Biological activity transforms inorganic Hg (iHg) into organic MeHg and it is the latter that bio-accumulates and biomagnifies in the food webs. Human exposure to MeHg largely comes from the consumption of fish and other seafood;[4] however, a better understanding of the source(s) and fate(s) of MeHg within the environment is required, particularly within materials such as fish tissues that may enter the human food chain.

For elements with more than one stable isotope, isotope ratio analysis can be used to follow elements or indeed compounds though biosynthetic pathways. While the environmental pathways and cycling of bio-element isotope ratios (H, C, N, O and S) have been more extensively studied,[e.g.5,6] isotope ratio analyses of elements such as Pb, Sr, Mo, Hg and U are also becoming more common.[e.g. 7,8] Such isotopic studies can measure isotope ratios at natural abundance or introduce isotopically labelled compounds into the element cycle as tracers that can then be followed through environmental compartments.

While mercury isotope ratios have the potential to help uncover how mercury passes through the environment,[e.g. 9-12] the isotope ratios of the organic moieties within MeHg and other organic forms of Hg also have the potential to aid understanding. For example, the study of the carbon isotope ratios of MeHg within fish tissues or other aquatic organisms, sediments or soils may reveal the source(s) of carbon within the methyl groups. While there have been some studies into the carbon isotope analysis of organometallic compounds using hydride generation,[e.g. 13, 14] the carbon isotope ratio analysis of MeHg or other organomercury compounds has not been extensively studied in the past, although Masbou and colleagues have published a comparison of three different approaches for the analysis of MeHg by gas chromatography/combustion-isotope ratio mass spectrometry (GC/C-IRMS),.[15] Thus far, however, carbon isotope ratios of MeHg that are fully traceable to the international Vienna Peedee Belemnite (VPDB) scale have not been reported. Furthermore, only standard deviations of replicate analyses have been provided in previous studies of MeHg carbon isotope ratios.[15] In addition, there is as yet no complete uncertainty budget reported for the determination of MeHg carbon isotope ratios and therefore it is unclear what the analytical limitations of such methods are.

In this work we have developed methods and derived uncertainty budgets for the fully VPDB-traceable carbon isotope ratio determination of MeHg with the aim of studying MeHg within fish tissues. Reference compounds/materials for method development were identified, then characterised for their bulk $\delta^{13}C_{VPDB}$ values by flow injection-chemical oxidation-isotope ratio mass spectrometry (FIA/CO-IRMS). Two methods for the determination of compound-specific MeHg $\delta^{13}C_{VPDB}$ values by GC/C-IRMS were developed, with and without derivatization. For the first time, the performance of the developed methods in terms of measurement uncertainty as well as normalisation and traceability considerations has been assessed. While the focus of this work has been on methods that can be applied to the study of MeHg extracted from fish

tissues, the implications for carbon isotope ratio analysis of MeHg within other environmental samples are also discussed.

MATERIALS and METHODS

Safety considerations

Methylmercury species are toxic and particularly hazardous at the elevated concentrations described below. Furthermore, derivatives of methylmercury such as methylethylmercury (MeEtHg) and other doubly substituted organomercury compounds such as dimethylmercury (Me_2Hg) and diethylmercury (Et_2Hg) are substantially more hazardous due to their volatility, and exposure to such compounds should be avoided wherever possible.

Specific procedures to minimise risk arising from mercury species and from other hazards were applied during this work and included (i) derivatization of Hg species using $NaB(Et)_4$ in tetrahydrofuran (THF) to avoid the need to prepare aqueous solutions of the derivatization agent which can result in the release of flammable gases; (ii) carrying out the derivatization within sealed autosampler vials to remove the need to transfer concentrated solutions of doubly substituted organomercury compounds between containers prior to instrumental analysis; (iii) modification of the GC/C-IRMS instrumentation to include a gold trap after the combustion reactor, with all vent lines being connected to exhaust systems; (iv) sealing of the autosampler waste vials to avoid release of organomercury compounds into the laboratory atmosphere; and (v) use of an on-column injector to remove the risk of derivatized MeHg species being vented during injection (which has the potential to occur with split/splitless injections).

Materials

Methylmercury chloride solids and aqueous solutions were obtained from Alfar Aesar (1000 $\mu g\ mL^{-1}$ solution in H_2O, part number 33553, Heysham, UK), VHG (1000 $\mu g\ mL^{-1}$ solution in

H_2O, part number VHG-MMC-25, Manchester, NH, USA) and Dr. Ehrenstorfer (solid, part number DRE-C15100000, Augsburg, Germany). The solid material from Dr. Ehrenstorfer was dissolved in 18 MΩ cm^{-1} water (Elga system, Veolia, Marlow, UK) to a concentration of 1000 µg mL^{-1} thereby resulting in solutions of three different MeHg materials, each at 1000 µg mL^{-1} in water being available for analysis. The 1000 µg mL^{-1} mono-elemental solution of Hg in dilute nitric acid was obtained from Romil (Waterbeach, UK).

FIA/CO-IRMS analysis

Flow injection analysis/chemical oxidation-Isotope ratio mass spectrometry (FIA/CO-IRMS) analysis was performed using a Dionex Ultimate 3000 HPLC system (pump and autosampler) coupled *via* an LC IsoLink chemical oxidation interface to a Delta V Advantage mass spectrometer (all from Thermo Scientific, Bremen, Germany). The mobile phase was 18 MΩ cm^{-1} water pumped at a flow rate of 500 µL min^{-1} while the oxidation reagents were phosphoric acid (1.5 M, prepared from 99 % orthophosphoric acid, Sigma Aldrich, Poole, UK) and sodium persulphate (1 g mL^{-1}, 99 %, Sigma Aldrich) were each pumped at 30 µl min^{-1}.

Raw isotope delta values for sample gas peaks were obtained using CO_2 working gas pulses introduced to the mass spectrometer *via* a separate open split within the LC IsoLink at the beginning (n = 3) and end (n = 3) of each run. These raw delta values were determined outside the instrumental software to allow use of the International Union of Pure and Applied Chemistry's (IUPAC) Commission on Isotopic Abundances and Atomic Weights (CIAAW)-recommended ^{17}O correction.[16] The raw delta values were corrected for the blank and then scale calibrated to the VPDB delta scale using reference materials (RMs) analysed at the beginning and end of each sequence. These RMs were ERM AE672a glycine (^{13}R = 0.010648 ± 0.000031; indicative $\delta^{13}C_{VPDB}$ = -42.12 ± 0.42 ‰, expanded uncertainties, k = 2, LGC Standards, Teddington, UK), USGS40 l-glutamic acid ($\delta^{13}C_{VPDB}$ = -26.39 ± 0.08 ‰, expanded

uncertainty, k = 2, USGS, Reston, VA, USA), IAEA-CH-6 ($\delta^{13}C_{VPDB}$ = -10.45 ± 0.07 ‰, expanded uncertainty, k = 2, IAEA, Vienna, Austria) and USGS41 l-glutamic acid ($\delta^{13}C_{VPDB}$ = +37.63 ± 0.10 ‰, expanded uncertainty, k = 2, USGS) that had been prepared as solutions in 18 MΩ cm^{-1} water at a concentration of approximately 4 mg mL^{-1} in terms of carbon. Measurement uncertainties were estimated following the Kragten numerical approach for all calculations.[17-19] For instrumentally measured terms such as integrated ion current ratios, the standard uncertainty was taken to be the standard deviation of repeat analyses from the same vial.

GC/C-IRMS analysis using derivatization

The derivatization protocol and chromatographic separation were based upon the work of Epov *et al*.[20,21] MeHg was ethylated using 1 mL of a 0.1 M acetic acid/sodium acetate buffer with pH 4 prepared from sodium acetate and acetic acid from Sigma Aldrich, added to a 2-mL GC autosampler vial and cooled in an ice bath. 0.2 mL of aqueous MeHg solution was added, followed by 20 µL of 10 % $NaB(Et)_4$ in THF (LGC Standards) and 0.5 mL of hexane (LGC Promochem, Teddington, UK) all of which had been previously cooled in an ice bath. The vial was sealed and gently shaken for five minutes and then stored in a sealed container at –5 °C until required for analysis.

GC/C-IRMS analysis was performed using a Trace GC Ultra gas chromatograph with on column injector and Triplus autosampler coupled *via* a GC IsoLink combustion interface and Conflo IV continuous flow interface to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Scientific). The backflush port of the GC IsoLink and the extraction port of the Conflo IV were connected to an extraction line. A gold trap (Alfa Aesar gold wires, 0.1 mm diameter, 99.95% purity, 1 m length, folded in half three times to give 8 strands of 12.5 cm length inside the gold trap) inside a narrow piece of stainless steel tubing was connected inline,

directly after the combustion reactor of the GC IsoLink, to trap any mercury that did not remain inside the reactor. GC separation of mercury species was performed using a MXT-1 capillary column (cross-bonded 100 % dimethyl polysiloxane, 15 m, 0.25 mm I.D., 0.25 µm film thickness, Restek, Bellefonte, PA, USA) using the following oven programme: initial temperature 40 °C, held for 7 min, ramp to 80 °C at 60 °C min^{-1}, hold for 6 min, ramp to 250 °C at 60 °C min^{-1} and hold for 1 min (total analysis time 17 min 50 s). The helium carrier gas flow rate was 2 mL min^{-1}. The injection volume was 1 µL taken directly from the upper hexane phase within the vial in which derivatization was performed, removing the need to transfer the volatile and highly toxic mercury species. No sample wash step was implemented within the autosampler method and the waste vials for the autosampler were sealed using crimp-caps with septa to avoid the volatile mercury species being released into the laboratory atmosphere. The backflush valve of the GC IsoLink was turned on at the start of each run, turned off at 150 s and then turned back on at 800 s to the end of each run.

The instrumental software (Isodat v 3.0, Thermo Scientific) was used for data acquisition and for integration of ion current chromatograms. Raw delta values for sample gas peaks were obtained using CO_2 working gas pulses introduced to the mass spectrometer *via* a separate open split within the Conflo IV at the beginning (n = 3) and end (n = 3) of each run. These raw delta values were determined outside of the instrumental software as before, using the CIAAW-recommended ^{17}O correction.[16] The raw delta values were then corrected for the presence of derivative carbon using a simple mass balance approach (as described elsewhere[19, 22-23]) either using an external standard iHg solution derivatized within the same sequence of analyses, or using an internal standard iHg solution added to the MeHg solution prior to derivatization, to determine the carbon isotopic composition of the ethyl derivatization group (following[15]). The corrected delta values for the MeHg were then scale calibrated using two MeHg external standard solutions analysed before and after the samples that had been previously calibrated

for carbon isotopic composition by FIA/CO-IRMS against secondary reference materials. All calculations were performed within a Kragten-type spreadsheet (KS) to allow the estimation of measurement uncertainty to be carried out.[17-19] For instrumentally measured terms such as integrated ion current ratios, the uncertainty was taken to be the standard deviation of repeat analyses from the same vial.

Before each analytical sequence, the backgrounds for *m/z* 18, 28, 32, 40 and 44 were checked to ensure that they were below the established thresholds, and pulses of working gas were used to assess the stability and linearity of the mass spectrometer response to carbon dioxide.

GC/C-IRMS without derivatization

GC/C-IRMS analysis of MeHg without derivatization was carried out using a Trace GC Ultra gas chromatograph linked to a MAT 253 isotope ratio mass spectrometer using a GC combustion III interface (all Thermo Scientific, Bremen, Germany). A ZB-1MS column (60 m x 0.25 mm I.D., 0.25 µm film thickness, Phenomenex) was pre-treated by injecting 1 µL 0.3 mM methanolic HBr ten times at 1 min. intervals. After the column pre-treatment, 1 µL of MeHg in benzene solution was injected into the instrument using a split/splitless injector in split mode (split ratio 1:10) held at 220 °C. The carrier gas (helium) flow rate was 10 mL min^{-1}. An isothermal GC oven programme was employed at 50 °C for 6 min to ensure elution of the Hg species. Backflush was used to divert the solvent peak. Backflush was on until 600 s and then off until the end of the analysis.

MeHg was injected in the form of a 1000 µg mL^{-1} solution in benzene. While the solid MeHgCl in-house reference materials (e.g. Dr. Ehrenstorfer) could be directly prepared as 1000 µg mL^{-1} solutions in benzene, the MeHg in-house reference materials obtained as aqueous solutions required an extraction step into benzene. This was achieved by the addition of an equal volume

of benzene to the MeHg aqueous solution, vigorous shaking and allowing the two phases to separate.

Raw data ($\delta^{13}C$ values on a scale defined by the working gas) were obtained using Isodat v3.0 and the Santrock Studley and Hayes (SSH) ^{17}O correction algorithm[24]. The working gas was introduced in pulses directly into the mass spectrometer at the beginning of each run.

RESULTS AND DISCUSSION

Characterization of in-house MeHg reference materials

GC/C-IRMS instrumentation does not provide structural information for the sample compounds and therefore a separate means of compound identification is required. This can consist of e.g. an organic mass spectrometer that is interfaced to same gas chromatograph with the column eluent split to allow simultaneous compound identification of and isotope ratio measurement.[25] Where such a system is not available, identification of compounds eluting from the gas chromatograph should be carried out on a separate GC/MS system that uses the same chromatographic column and oven programme. For simple analyses involving only few compounds such as the isotopic composition of MeHg from high-purity standard solutions, it is also possible simply to compare the chromatogram obtained for the material with that for a procedural blank. Provided that the sample preparation and instrumental analysis approaches have been well-studied in the past, it is likely that any peaks present in procedural blank are not the compound(s) of interest. If only one peak remains within the sample chromatogram that is not in the procedural blank and which is of the expected size based upon the amount of the high-purity standard compound injected, identification of peaks is straightforward.

In this work, the MeHg materials were of high purity (>95 % according to the manufacturers), while the derivatization and chromatographic separations had been previously studied and not found to produce significant amounts of byproducts.[20,21] Comparison of the chromatograms

for a procedural blank, derivatized MeHg solution and derivatized iHg solution (Figure 1) showed that only one peak was present in each of the derivatized solutions, thereby allowing identification of the peaks associated with the derivatized MeHg species. For the method using direct injection of MeHg without a derivatisation step, again only one peak was obtained for the analysis of MeHg (Figure 2) making identification of the MeHg peak straightforward.

To determine whether the carbon isotope ratios of MeHg in-house reference material solutions could be measured using a bulk isotope ratio analysis approach such as FIA/CO-IRMS, it was necessary to demonstrate that each of the three solutions predominately contained carbon in the form of MeHg. Five subsamples of each of the three MeHg in-house reference material solutions (VHG, Alfa Aesar and Dr. Ehrenstorfer) were derivatized within the same batch and then each analysed in triplicate by GC/C-IRMS. Procedural blanks (n=3) and an external standard iHg solution were also derivatized and analysed in triplicate. The chromatograms obtained for the derivatized MeHg solutions were examined for peaks not present within the procedural blanks or attributed to MeEtHg. The only peaks thus identified were attributed to the presence of Et_2Hg by comparison of retention times with those in iHg solutions also derivatized within the same batch (Figure 1).

These Et_2Hg peaks were very small, being < 15 % of the peak area of the MeEtHg peaks (often much smaller) and therefore < 12 % of the mass of MeEtHg represented by those peaks, and they could arise from the presence either of EtHg or of iHg in the MeHg in-house reference material solutions as both of these compounds would produce Et_2Hg upon derivatization. The Et_2Hg peaks arising from the analysis of MeHg in-house reference material solutions displayed raw $\delta^{13}C$ values = -30.6 ‰ with standard deviation of 6.7 ‰. This poor precision resulted from the very small nature of the Et_2Hg peaks, making run-to-run variability very high. The delta values were identical within measurement uncertainty to those of the Et_2Hg obtained from derivatization of the iHg solutions prepared and analysed within the same batch (raw $\delta^{13}C$ = -

27.34 ± 0.26 ‰). Only if the ethyl carbon isotope ratio of any purported EtHg present in the MeHg in-house reference material solutions were a close match to that of the derivatization agent would this be the case. There are therefore two possible causes of the small Et_2Hg peaks observed following derivatization of the MeHg in-house reference material solutions. First, there could be iHg within the MeHg standard solutions – indeed this has been observed during Hg isotope analysis of mercury species within the same MeHg standard solutions (Dmitriy Malinovskiy, personal communication). Secondly, it is possible for derivatization of MeHg in the presence of solvent to induce degradation artefacts in small amounts that could also be the cause of the small Et_2Hg peaks.

There was therefore no evidence to suggest the presence of other carbon-containing mercury species within the MeHg in-house reference material solutions. Hence characterisation of these solutions by a bulk isotope ratio technique such as elemental analyser-IRMS (EA-IRMS) or FIA/CO-IRMS to determine the carbon isotope ratio of the MeHg would be possible.

Quantitative conversion of MeHg to CO_2 during FIA/CO-IRMS

Quantitative conversion of a target compound or material into the appropriate analyte gas for analysis by IRMS is a prerequisite for determination of accurate isotope ratios. Indeed it is the study of analyte gas yield that has recently highlighted the potential for incomplete conversion of hydrogen within nitrogen- and other heteroatom-containing molecules to hydrogen gas during high temperature conversion using glassy carbon.[26-28] The simplest means to determine whether the yield of CO_2 is quantitative for a particular material is to compare the peak size obtained in the IRMS chromatogram for a given mass of the element within the target compound with the peak size for the same mass of the element within a material known to exhibit quantitative conversion (e.g. a reference material for isotope ratio). During FIA/CO-IRMS analysis, the peak area obtained for MeHg was compared with that of the RMs used for

normalisation and as quality control materials (two glycines, two L-glutamic acids and a sucrose) as all materials were analysed in known amounts in terms of carbon. All peak areas for the MeHg solutions were of similar peak area to those expected based upon the analysis of the RMs, suggesting that complete conversion of the MeHg to CO_2 had been achieved. The peak area data can be found in Table 1.

Determination of $\delta^{13}C_{VPDB}$ values for in-house MeHg reference materials by FIA/CO-IRMS

The three MeHg in-house reference material solutions (VHG, Alfa Aesar and Dr Ehrenstorfer) were analysed by FIA/CO-IRMS in the same sequence as four different normalisation reference materials to determine their $\delta^{13}C_{VPDB}$ values. Each material was analysed using five repeat injections from the same subsample of solution. The results can be found in Table 2, which also includes the obtained $\delta^{13}C_{VPDB}$ values for the normalization reference materials - these all agreed with the expected values listed above within uncertainty.

The calibration range afforded by the RMs was from $\delta^{13}C_{VPDB}$ = -42.12 ‰ to $\delta^{13}C_{VPDB}$ = +37.63 ‰ and therefore, for two of the MeHg in-house reference materials (VHG and Alfar Aesar), calibration *via* extrapolation was required. Given that the RMs covered just under 80 ‰ while the extrapolation was by up to 30 ‰, there was only a slight increase in measurement uncertainty compared with the MeHg in-house reference material within the calibration range. For each of the three MeHg solutions, over 85 % of the uncertainty obtained was due to the uncertainty in the reference material expected values. The remainder was from the measured parameters for the normalization RMs as well as for the MeHg solutions.

Consistency of derivatization for GC/C-IRMS analysis

Where derivatization is used prior to GC/C-IRMS analysis, the derivatization reaction must involve either no kinetic isotope effect (KIE) or a reproducible KIE for the correction for derivative carbon to be successful.[22,23] If the KIE is not reproducible, the reaction approach is unsuitable for GC/C-IRMS analysis. It therefore follows that repeated derivatization of subsamples of the same material using the same batch of derivatization agent should result in consistent raw delta values.

Five subsamples of same MeHgCl in-house reference material solution (VHG) were derivatized within a single batch using the same bottle of $NaB(Et)_4$ in THF. Each of the derivatized subsamples was then analysed in triplicate by GC/C-IRMS and raw delta values for MeEtHg obtained (Figure 3). One way analysis of variance (ANOVA) demonstrated no significant difference in the raw delta values between the subsamples (F_{stat} = 0.668, F_{crit} = 3.478, p = 0.63), demonstrating that the reaction was suitable.

The peak areas of these analyses were also very consistent despite their small size (average = 1.48 mV.s, standard deviation = 0.19 mV.s, n=15), suggesting that conversion of the MeHg species to CO_2 was consistent. To determine if this conversion and the derivatization and extraction into the hexane phase were also complete, the signal from the CO_2 produced from known amounts of derivatized MeHg species was compared with that obtained from analysis of known amounts of fatty acid methyl esters (FAMEs) - compounds that exhibit favourable combustion characteristics within the instrumentation (which was in the same tune state). The mass of carbon in the form of MeEtHg within each injection for the 15 analyses described above was approximately 58 ng (assuming that derivatization and extraction into the hexane were quantitative) with amplitudes of the associated carbon dioxide peaks of approximately 2.2 V (*m/z* 44, $3x10^8$ Ω amplification). For a range of even-numbered FAMEs from methyl myristate to methyl behenate, a signal amplitude of approximately 3.2 V was obtained for the

analysis of approximately 88 ng of carbon of each. The MeEtHg signal therefore represents approximately 104 % of the signal size expected based upon the FAME results, suggesting that complete conversion of MeEtHg to carbon dioxide has been achieved and that both derivatization and extraction into the hexane were quantitative.

Correction for derivative carbon and contribution to uncertainty

The addition of carbon from the $NaB(Et)_4$ to the MeHg must be accounted for in order to determine the $\delta^{13}C$ value of MeHg. This can be done by a simple mass balance approach as described elsewhere.[19, 22,23] This necessitates determination of the $\delta^{13}C$ value of the derivative carbon ($\delta^{13}C_d$). Where the derivatization agent has been prepared from solid $NaB(Et)_4$, the solid could be analysed by an offline approach such as EA-IRMS to determine the $\delta^{13}C_d$ value. As $NaB(Et)_4$ in THF was used, this cannot be done due to the carbon present in the THF and therefore an indirect approach to determination of the $\delta^{13}C_d$ value is required. As the derivatization procedure also converts any iHg present to diethylmercury (Et_2Hg), an external or internal iHg standard can be used to determine the $\delta^{13}C_d$ value.[15] The ethyl groups of both MeEtHg and Et_2Hg can be assumed to be identical provided that (i) the same lot of derivatization agent is used; (ii) derivatization is carried out within the same batch; and (iii) the derivatization agent is present in great excess.

While the correction for derivative carbon is straightforward, its application will contribute to the overall measurement uncertainty. The uncertainty associated with the correction can be estimated by using a KS or by using the traditional means to combine uncertainty components within the mass balance equation together as described elsewhere.[23] If one assumes that the isotopic compositions of the derivatized methylmercury compound and the derivative carbon (from derivatized iHg) can be measured to the same degrees of uncertainty regardless of isotopic composition (0.2 ‰); and that the isotopic composition of the methyl mercury is

identical for each different derivative option, it is possible to isolate how the number of carbon atoms within a derivative affects the uncertainty in the corrected delta value for MeHg (Figure 4).

The KS approach displays a linear increase in measurement uncertainty with increasing numbers of derivative carbon atoms, while the Docherty equation produces a polynomial increase. Regardless of the uncertainty estimation approach it is clear that increasing the number of derivative carbon atoms, increases the associated measurement uncertainty. The selection of derivatization approach (e.g. between the use of ethylation and propylation) is therefore a balance between increasing the measurement uncertainty from the correction for derivative carbon and increasing the signal amplitude which results in better precision of replicate injections. More generally there is also chromatographic performance to consider as this can be important in some applications of GC/C-IRMS;[e.g. 29] however. this aspect is of less importance during the study of MeHg.

One further consideration for the correction of derivative carbon is whether to use iHg as an internal standard or as an external standard for the determination of the isotopic composition of the derivative carbon. With the internal standard approach, iHg is added to each sample of MeHg prior to derivatization and the resulting Et_2Hg used during the derivative correction. The external standard approach uses a separate solution of iHg that is derivatized within the same batch as any MeHg samples. The internal standard approach does have the advantage that the two compounds, MeEtHg and Et_2Hg, pass through the combustion reactor and other parts of the instrumentation much closer in time, ensuring that instrumental conditions are as similar as possible. The advantage of the external standard approach is that should the iHg solution contain any MeHg, this would not contribute to the isotope ratios determined for any sample MeHg. There was no difference within measurement uncertainty between the values obtained for the VHG MeHg normalised using the other two MeHg in-house reference material

compounds when using a derivative carbon correction *via* an internal or external standard approach. This was because the raw delta value for the Et_2Hg derived from the internal standard (raw $\delta^{13}C$ = -27.35 ± 0.45 ‰) and external standard approaches (raw $\delta^{13}C$ = -27.34 ± 0.26 ‰) were also identical within measurement uncertainty for analyses within the same sequence.

Normalisation of GC/C-IRMS $\delta^{13}C$ values using in-house MeHg reference material solutions

Compound-specific carbon isotope ratios should be reported on the VPDB scale. Delta values obtained by GC/C-IRMS analyses against a working gas, whether or not this gas has been calibrated against reference materials, are not traceable to VPDB as the sample and working gases are not treated identically during analysis. Ensuring traceability to VPDB requires normalisation of raw delta values using at least two materials of known isotopic composition that are traceable to the reporting scale analysed within the same sequence.[19, 30] These should be isotopologues of the sample compound and should have isotopic compositions that span the expected range of the sample compound to allow calibration *via* interpolation rather than extrapolation. For the carbon isotope ratio analysis of individual compounds separated within a complex mixture this is potentially challenging due to the large number of reference materials that are necessary and therefore more pragmatic approaches have been recommended;[19] however, in the current case where only one compound (MeHg) is of interest, traceability to the reporting scale can be easily achieved while adhering to the ideal approach.

Provided that two methylmercury reference compounds can be sourced with differing isotopic composition, these can be used for normalisation if they are treated identically to the samples and analysed within the same sequence using the same batch of derivatization agent. Determination of the carbon isotopic composition of these MeHg reference compounds can be

carried out by some form of bulk carbon isotope ratio analysis such as EA-IRMS or FIA/CO-IRMS provided that there is very little carbon present in the material which is not in the form of MeHg. As discussed above, three different MeHg in-house reference materials (VHG, Alfa Aesar and Dr. Ehrenstorfer) were sourced and characterised for non-MeHg carbon and also for bulk carbon isotope ratios by FIA/CO-IRMS. Two were suitable to use for normalisation as they differed in $\delta^{13}C_{VPDB}$ values by just over 30 ‰ (Table 3) while the third (VHG) could be used as a quality control (QC) material as it was within this calibration range in terms of its $\delta^{13}C_{VPDB}$ value.

Normalisation of sample MeHg $\delta^{13}C$ values to the VPDB scale can be achieved using the offline FIA/CO-IRMS and online GC/C-IRMS measured values for the Alfa Aesar and Dr. Ehrenstorfer MeHg in-house reference material to create a normalisation plot. The maximum uncertainty introduced by normalisation is < 0.35 ‰ assuming that the GC/C-IRMS measurements of the normalisation reference materials and of the sample each have an associated uncertainty of 0.2 ‰ and that the sample can be calibrated *via* interpolation. Should sample MeHg $\delta^{13}C$ values lie outside the calibration range, there are two options: first. the calibration range could be extended (by sourcing a suitable MeHg commercially or by synthesis of ^{12}C- or ^{13}C-enriched MeHg and gravimetric mixing with the current MeHg in-house reference material to obtain the desired $\delta^{13}C$ value) or calibration *via* extrapolation could be employed. The latter is far easier and more cost effective; however, the additional uncertainty introduced by extrapolation must be accounted for.

This additional uncertainty can be estimated using a KS, where the isotopic compositions of the normalisation RMs as well as all input parameter standard uncertainties are held constant but the isotopic composition of the sample is varied. Using such an approach, the effect of each permil increase in distance from the 30 ‰ calibration range can be shown to result in an increase in the combined uncertainty of the scale-calibrated $\delta^{13}C$ value of 0.01 ‰ (Figure 5).

The effect of calibration range can also be investigated using a similar approach whereby a KS is used to determine the relationship between the $\delta^{13}C$ value range afforded by the normalisation RMs and the additional measurement uncertainty introduced by normalisation *via* extrapolation (Figure 5). It is clear that the smaller the difference in isotopic composition between the normalisation reference materials, the more rapidly the measurement uncertainty increases when extrapolation is applied. Extrapolation by 100 % of the calibration range doubles the uncertainty in the resulting delta value and, therefore, unless the increase in measurement uncertainty is fit-for-purpose, extrapolation should be avoided.

Use of matrix matched normalisation RMs and the need for other corrections

Application of the principle of identical treatment (PIT) ensures that samples and RMs are treated in the same way during analyses and are of the same matrix, thereby negating some biases and cancelling some contributions to measurement uncertainty.[31] Often in isotope analysis it is possible to use organic RMs for normalisation of organic samples, rather than exactly matching the sample and RM compound. In the situation where two exactly-matched compounds are available for normalisation with a further QC material also of the same compound as the sample, all of which are analysed at the same amount level, the PIT can be applied in the strictest sense and thereby separate calculation stages for blank correction, normalisation and correction for derivative carbon need not be applied. Simply creating a calibration plot of the measured (raw) and expected $\delta^{13}C$ values for the normalisation reference materials and applying this to the samples and QC materials of the same compound run within the same sequence is sufficient. In situations where the analyte compound is not an exact match for the compounds used for normalisation, separate calculation stages must be employed. Applying a normalisation directly to the measured data for the sample MeHg does not result in a value or measurement uncertainty that is different from that from the stepwise approach

involving separate derivative correction and normalisation stages. It is also the case that the measurement uncertainty budget remains the same, with the majority of the uncertainty coming from the raw ion current ratios for the MeEtHg peaks within the chromatograms.

Other measurement uncertainty considerations

When peaks are small, automated integration of ion current signals is typically less reliable than for larger amplitude signals and this can result in larger variation in peak areas between replicates even if these are repeated injections of the same solution. It is therefore important to capture this measurement uncertainty in a realistic way. While it has been previously suggested that the input parameters to a KS for the calculation of raw delta values from integrated ion current ratios should use the standard deviation of the mean of independent replicates,[18] this can lead to overestimation of measurement uncertainty in cases where there is significant variation in peak areas between replicates of the same sample. In such cases (e.g. small sample sizes with consequent difficulties with integration, or indeed for bulk measurements on materials for which it is difficult to weigh out the same amount for replicate analyses), it is better to calculate the raw delta values for each replicate analysis independently and then determine the mean later in the calculation process. This is because the raw *m/z* 45/44 and *m/z* 46/44 ratios will vary in the same way (they are correlated) and therefore there can be significant variation in these two ratios but far less variation in the raw delta value that they are used to calculate. Alternatively, the input data into the KS should not be raw ion current ratios, but rather the raw integrated ion currents individually, as this again would avoid the manifestation of correlation between the *m/z* 45/44 and *m/z* 46/44 ratios.

To illustrate the effect on measurement uncertainty of this correlation, the same raw instrumental data can be evaluated in two different ways: first, as described elsewhere taking the mean of the input ion current ratios,[18] and secondly, treating each replicate separately for

raw delta value calculation, then taking the mean and applying the remaining calculation stages such as normalisation. Doing this for three replicate injections of the Dr. Ehrenstorfer MeHg resulted in individual raw $\delta^{13}C$ values of -28.40 ± 0.20 ‰, -26.42 ± 0.20 ‰ and -27.67 ± 0.20 ‰ (combined uncertainty, k = 1). The mean of these three values with combined uncertainty is -27.50 ± 0.61 ‰ (combined uncertainty, k = 1), while using the mean ion current ratios as input data resulted in a raw delta value of -27.50 ± 0.82 ‰. This difference in obtained measurement uncertainty highlights the need to account for or avoid correlation between input parameters – particularly when the correlation would result in a reduced measurement uncertainty.

A further instance of correlation between input parameters arises from the use of reference materials of known delta values within the traceability chain for the MeHg in-house reference materials. For example, the indicative $\delta^{13}C$ value for ERM AE672a applied in this work was obtained by normalisation using reference materials including USGS40, IAEA-CH-6 and USGS41 – the other RMs used for FIA/CO-IRMS analyses in this work; the delta values of these reference materials are therefore correlated. Furthermore, the currently accepted delta values for USGS40, IAEA-CH-6 and USGS41 were all obtained during a single inter-laboratory exercise and therefore are also probably correlated.[32,33] The relationships between the $\delta^{13}C_{VPDB}$ values of commercially available RMs are complex and accounting for correlations between them is challenging. Neglecting to account for correlations in this instance will lead to a larger uncertainty which is a conservative rather than overly-optimistic result and therefore acceptable.

A final potential source of uncertainty is the so-called linearity effect. This occurs when a single material analysed over a range of different masses, produces different raw delta values and can be seen even for homogenous materials.[19] Where sample and RMs for normalisation and QC are not analysed in exactly the same mass, there will be an additional dispersion of the raw data obtained, leading to increased standard deviations of replicate analyses and increased

measurement uncertainty. The extent of the linearity effect can be determined via the analysis of increasing amplitude pulses of working gas – in this work, the linearity was typically less than 0.02 ‰ V^{-1} as measured on the middle Faraday collector monitoring *m/z* 45.

It is also important to check for linearity of the observed delta values for real samples. For CSIA of MeHg, the mass of sample injected depends on the concentration of solution as well as the injection volume. The concentration of solution (i.e. of derivatized MeHg present in the hexane phase) could be controlled by derivatization of different amounts of the stock 1000 µg mL^{-1} MeHg solutions. Figure 6 shows the relationships between the amount of derivatized MeHg injected on column and the signal size obtained, as well as against the raw delta value. There is a clear linearity effect present; however, the slopes are approximately 0.1 ‰ ng^{-1}. Provided that the mass of sample injected is controlled to the same extent as the x-axis error bars shown in Figure 6, the additional dispersion of raw delta values due to the linearity effect will be approximately 0.3 ‰.

Overall measurement uncertainty for the derivatization approach

The analysis of the VHG MeHg solution by GC/C-IRMS using the other two in-house MeHg solutions for calibration resulted in a $\delta^{13}C_{VPDB}$ value of -68.3 ± 7.7 ‰ (Table 3). This is a combined standard uncertainty from 15 measurements of each of the three MeHg solutions (two used for normalisation, one as a "sample") consisting of three repeat analyses of each of 5 independently prepared replicates. All MeHg signals were between 1 and 3 V in amplitude for *m/z* 44 with $3x10^8$ Ω amplification to determine the performance of the method at the lower amounts of MeHg. The mass of MeHg injected was controlled to within 3 ng (equivalent to the x-axis error bars of Figure 6). Data analysis was by simple normalisation using the raw $\delta^{13}C$ values for the MeEtHg of the two in-house normalisation reference materials together with the expected values determined by offline FIA/CO-IRMS (Table 3). The GC/C-IRMS result agrees

very well with the bulk value obtained by FIA/CO-IRMS analysis of $\delta^{13}C_{VPDB}$ = -68.85 ± 0.17 ‰ although the uncertainty is substantially larger. Examination of the uncertainty related to each stage of the calculations showed that the $\delta^{13}C_{VPDB}$ values for each of the five replicates of VHG MeHg solution could be obtained with a measurement uncertainty of less than 3 ‰ from repeated injections of the same solution (which included the uncertainty arising from normalisation and the assignment of the in-house MeHg reference material $\delta^{13}C_{VPDB}$ values). This measurement uncertainty of less than 3 ‰ for replicate injections of the same solution was also the case for the two MeHg standard solutions used for normalisation. The variability in results for the normalisation MeHg standard and MeHg sample solutions between the independently prepared solutions using different lots of derivatization agent and different derivatization batches was considerably higher and contributed over 85 % to the combined uncertainty. Substantial variability is to be expected when small signals of the order of 1 V for *m/z* 44 are integrated over a baseline that is not completely stable.

GC/C-IRMS analyses of MeHg without derivatization

Chromatographic separation of organomercury compounds can be achieved without derivatization.[29] While such methods require larger amounts of MeHg to produce the same signal size during GC/C-IRMS analysis (assuming that the same instrument, tune state and combustion reactor efficiency are used), correction would not be required for derivative carbon and therefore lower measurement uncertainty would result. As is shown in Figure 4, correcting raw delta values obtained with 0.2 ‰ standard uncertainty for derivative carbon results in a combined uncertainty of at least 0.4 ‰ – i.e. a doubling of the measurement uncertainty as a minimum. As derivatisation is therefore a significant component of uncertainty, investigation of instrumental methods for carbon isotope ratio analysis of MeHg that do not require derivatization is valuable. Furthermore, the use of such a method on the same materials as used

in the derivatization approach would act as independent confirmation of the results – in particular of the correction for derivative carbon – although both techniques rely on the FIA/CO-IRMS-derived known values for MeHg materials used for normalisation.

The data handling for this approach used a different ^{17}O correction (SSH as opposed to IUPAC); the effect of this on the raw delta values obtained is expected to be less than 0.06 ‰.[16,19] The SSH approach could be implemented within the instrumental software automatically, rather than requiring external calculations, which was an advantage. For normalised delta values traceable to VPDB obtained by GC/C-IRMS, the difference in values obtained between the ^{17}O algorithms is significantly smaller (<0.001‰) provided that the same algorithm is applied to samples and normalisation RMs.[19] The contribution of the ^{17}O correction to the measurement uncertainty for obtaining delta values for MeHg with or without derivatization was therefore deemed negligible.

Analysis of five separate subsamples of two of the MeHg in-house reference material solutions directly by GC/C-IRMS resulted in a mean raw $\delta^{13}C$ value of -42.92 ‰ with a standard deviation of 0.06 ‰ (Dr. Ehrenstorfer) and a mean raw $\delta^{13}C$ value of -73.47 ‰ with a standard deviation of 0.34 ‰ (VHG). The normalisation plot obtained using these two results, together with the expected values obtained by FIA/CO-IRMS (Table 3), has a slope of 1.12 and an intercept of 3.60. The slope is a function of the mass spectrometer tune state, while the intercept represents the difference between the actual and assigned $\delta^{13}C$ value for the working gas. The standard deviations are comparable with those obtained by analysis of MeHg derivatives within a single derivatization batch, but required much larger amounts of MeHg – Figure 2 shows the peak size obtained from the injection of 1000 ng of MeHg (note that the amplitudes of the signals in Figures 1 and 2 cannot be directly compared as they were obtained using different instrumentation as noted in the methods section). As discussed above, the uncertainty introduced by correcting for derivative carbon is approximately double that for a raw,

uncorrected $\delta^{13}C$ value and therefore in instances where sufficient MeHg is present and low uncertainty is required, the direct analysis approach would be more appropriate.

Amounts of MeHg required for GC/C-IRMS analysis

In GC/C-IRMS analysis, the lower limit for sample mass is typically around 10 ng of carbon on column.[19] This mass of carbon results in sufficient CO_2 ions being produced in the ion source (following combustion of the carbon within the sample compound to CO_2) to give a signal of 3 nA maximum intensity (equivalent to 1 V with $3x10^8$ Ω amplification) on *m/z* 44. The exact mass of carbon required on column will of course vary with mass spectrometer model, tune state of the instrument, etc.; however, this is a suitable estimate. Signals smaller than 1 V are typically the subject of poorly reproducible integration (fluctuations in the background or baseline are more significant) which results in larger variation in isotope ratio between repeat analyses of the same material.

To determine the minimum amount of MeHg required on column, varying amounts of each of the three MeHg in-house reference material solutions were derivatized and analysed. A plot of mass of carbon injected (in the form of MeEtHg) against signal size could then be created (Figure 6A). Given that each MeHg stock solution was 1000 µg mL^{-1}, the three plots should all overlap; however, there is a slight offset for the Dr. Ehrenstorfer MeHg. As this material was dissolved in-house to the nominal concentration of the other two solutions, this small offset is not unexpected. The minimum mass of carbon within the injection volume (1 µL) required to produce a 1 V signal was approximately 30 ng on column. Given that the hexane phase was 500 µL, this implies that 15 µg of carbon was within the hexane phase. Of this carbon, only one third is from MeHg while the rest is derivative carbon and therefore 5 µg of carbon from MeHg (equivalent to approximately 100 µg of MeHg) would be required.

The volume of the hexane phase could be reduced to 100 μL which would also reduce the amount of MeHg required proportionately to 20 μg; however. a further reduction in the volume of hexane would make direct sampling from this phase using the autosampler syringe more difficult. Use of vial inserts could further reduce the volumes of solution required without impacting the ease of direct sampling of the hexane phase; however, it would then be more difficult to ensure thorough mixing during the derivatization reaction, which would increase the risk of incomplete derivatization and associated fractionation. Introduction of a pre-concentration step between derivatization and analysis would be possible; however, the risk of losing some of the volatile and highly toxic mercury species would increase. Other options for increasing sensitivity and thereby reducing the mass of MeHg required include the use of propylation rather than ethylation. Propylation should increase the signal amplitude by factor of 33 % over ethylation but it requires larger correction for derivative carbon which has consequences in terms of measurement uncertainty as discussed above.

Given the need for a combination of multiple analyses from a single derivatization reaction, as well as independent derivatization reactions for a number of subsamples of the same MeHg, 20 μg is a pragmatic minimum amount of MeHg required. The combined standard uncertainty of ± 7.7 ‰ obtained for the VHG MeHg solution reported above represents a practical minimum uncertainty possible for the analysis of the carbon isotope ratio of MeHg at this amount level used. A reasonable assumption is that this amount of MeHg will need to be trebled to 60 μg should the GC/C-IRMS approach without derivatization described above be employed given the corresponding decrease in the number of carbon atoms. Where more MeHg is available for analysis, the uncertainty associated to the measurement results using the methods described will decrease as the signal amplitude can be increased.

Application of developed GC/C-IRMS methods to MeHg from fish tissues and other environmental samples

Analysis of MeHg from environmental samples such as fish tissues requires extraction of the MeHg from the matrix. Given the relatively large amount of MeHg required for the methods developed here it was important to determine whether any MeHg signal could be obtained from a reasonable amount of sample. Assuming that 20 μg of MeHg is the minimum amount required for GC/C-IRMS analysis using the ethylation approach, the applicability of the method described above to environmental samples such as fish tissues can be judged by comparison with the total amount of MeHg within commercially available reference materials certified for MeHg content. Table 4 provides a list of such materials together with the number of units requited to provide the 20 μg of MeHg. It is clear that very few of the fish tissue reference materials contain sufficient MeHg to allow carbon isotope analysis reliably by GC/C-IRMS with the methods described herein. The extraction and derivatization of MeHg from BCR-463 were performed using 1 g of material but no MeEtHg peak was seen above the baseline although the expected peak size was only a few hundred mV.

A number of freshly collected fish samples together with some archived fish tissues obtained from environmental specimen banks within Germany were also available through the European Metrology Programme for Innovation and Research (EMPIR) Joint Research Project: ENV51 "Traceability for Mercury Measurements" (MeTra).[34] These included roach and pike from Lake Stechlin, Germany collected in March 2015 and archived bream collected in 2013. The total Hg contents for these fish samples were between 60 and 1300 μg kg^{-1}.[34] Even at the upper end of this range, approximately 15 g of fish tissue would be required for each independent replicate analysis, substantially more than was available. It is important to note that fish samples collected from other riverine or marine ecosystems could be significantly more contaminated by MeHg than these reference materials and fish tissue samples. The methods

discussed herein do therefore have the potential to be useful for the analysis of severely polluted specimens.

MeHg can also be found in other environmental settings including sediments, soils and river water, for example in locations close to sites where gold is processed using Hg.[35] Table 4 also lists a number of non-fish reference materials certified for MeHg content and again the amount of MeHg present is generally too low for carbon isotope analysis using the methods described above. As with fish tissues, where levels of MeHg contamination are higher than for the reference materials listed, the methods described herein can provide useful means to study MeHg in these types of environmental samples.

Whether samples are fish tissues, sediments, or other environmental materials, important considerations for the application of the GC/C-IRMS methods described herein are quantitative extraction of the MeHg present to avoid fractionation and the presence of sufficient MeHg. Concentration levels of MeHg within environmental samples of 4 μg g^{-1}or greater would require 5 g (or less) of material for carbon isotope ratio analysis. Any extraction method will require validation or verification for use with new matrices to ensure that it does not introduce fractionation.

CONCLUSIONS

Methods for GC/C-IRMS carbon isotope analysis of MeHg have been developed including a novel approach that applied the derivatization protocol directly within an autosampler vial, rather than in a separate vessel with subsequent transfer. This provided a simple means to minimise exposure to highly toxic organomercury species and precluded the loss of volatile MeHg derivatives and it may be of use more widely for GC/C-IRMS analyses of other compounds where there are similar considerations. Similarly, while the measurement uncertainty considerations regarding normalisation and derivatization may be rarely applied

for MeHg analysis within environmental samples that are not heavily contaminated with MeHg at the μg g^{-1} level, the general approaches will be useful for all applications of GC/C-IRMS where normalisation or derivatization are applied. Assuming that 20 μg of MeHg can be extracted from an environmental sample, it is possible to determine the $\delta^{13}C_{VPDB}$ value with a combined standard uncertainty of around 7 ‰. This uncertainty will decrease the more MeHg is present for analysis, as run-to-run variability in integrated peak areas will be lower for larger peaks. Where sufficient MeHg can be extracted to apply the method which does not include derivatization, the achievable measurement uncertainty will also be lower.

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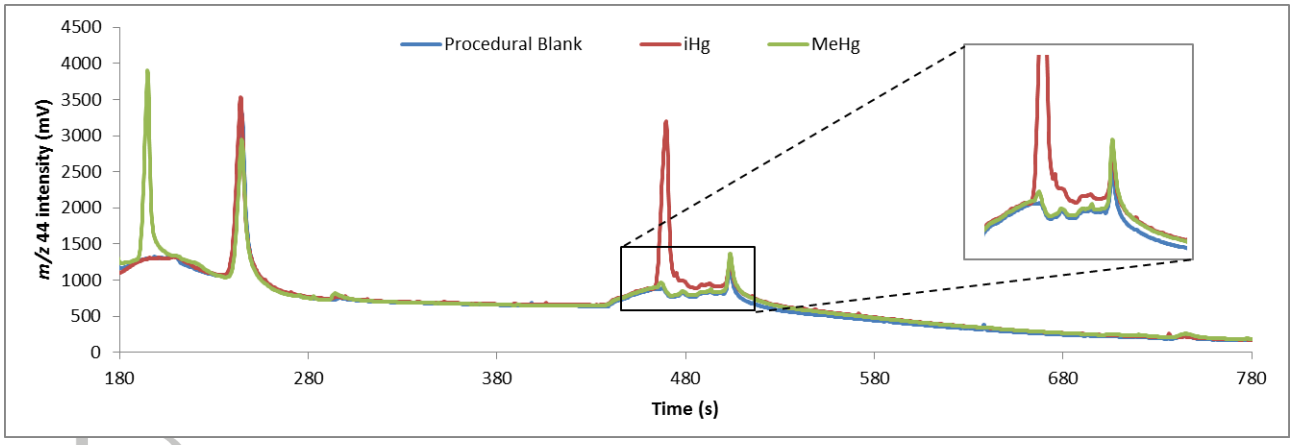

Figure 1 – Partial chromatograms of the *m/z* 44 intensity for a procedural blank (blue), MeHg (green) and iHg (red) in-house reference materials for the GC/C-IRMS method using derivatization and injection of solution. MeEtHg and Et_2Hg peaks are identified. The MeHg solution clearly contains a small amount of Et_2Hg (originally present as iHg or EtHg that have been derivatized) as highlighted in the insert.

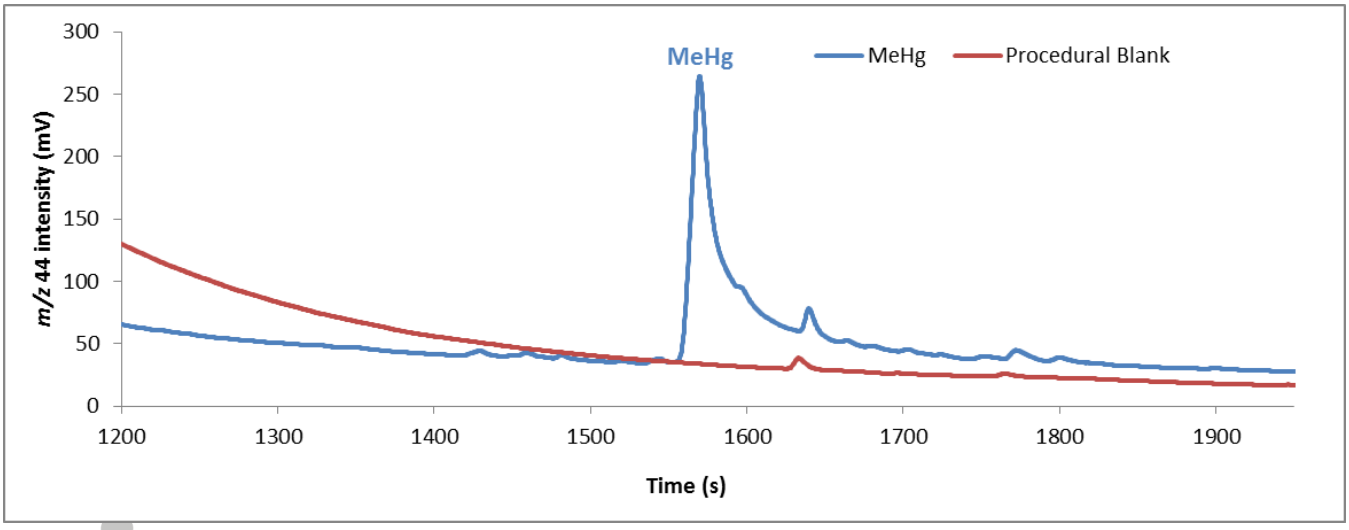

Figure 2 – Partial chromatograms of the *m/z* 44 intensity for a procedural blank and MeHg in-house reference material solution for the GC/C-IRMS method without derivatization. The MeHg peak is identified.

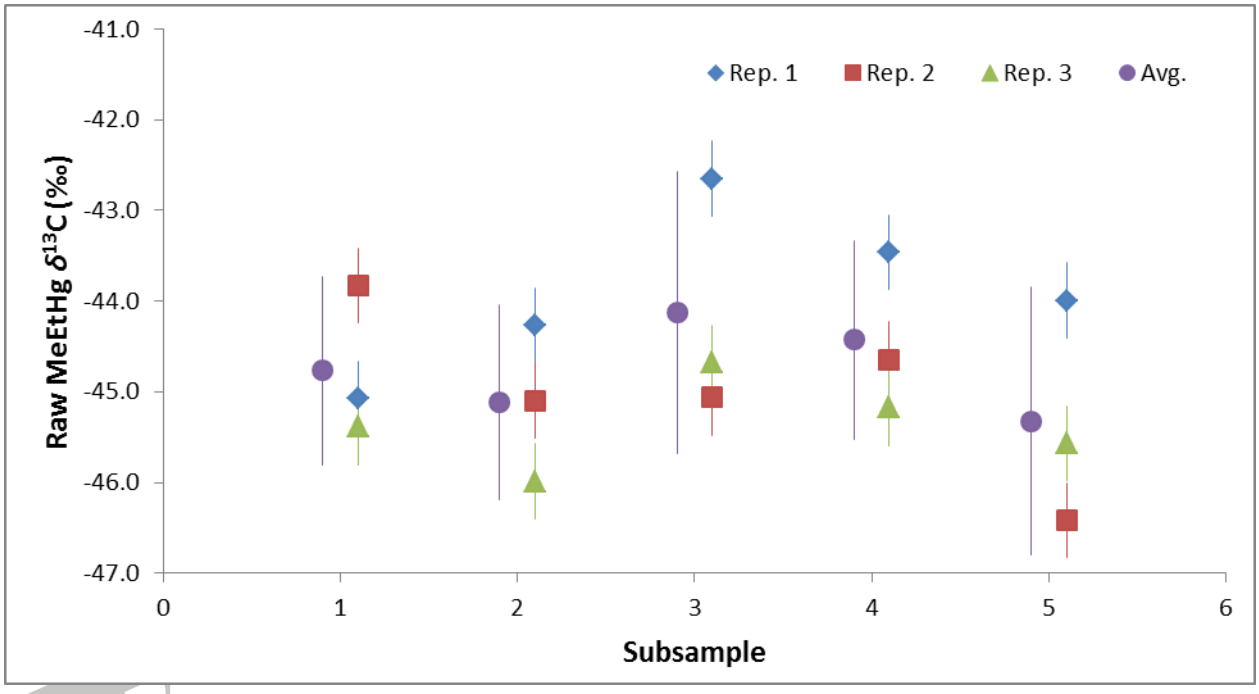

Figure 3 – Raw $\delta^{13}C$ values of MeEtHg (VHG) obtained from five subsamples of MeHg in-house reference material solution derivatized within the same batch of $NaB(Et)_4$ and each analysed in triplicate. Error bars show the expanded uncertainty (k = 2).

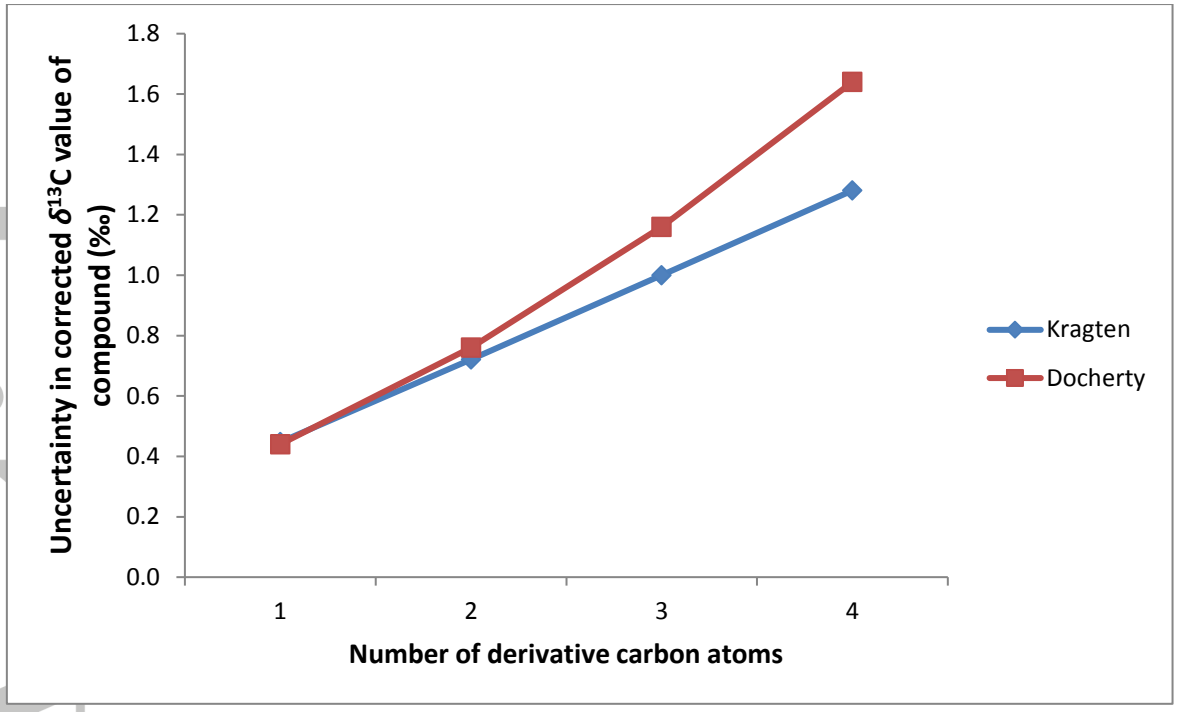

Figure 4 – The effect of increasing the number of derivative carbon atoms on the combined standard measurement uncertainty (k = 1) introduced in the corrected δ^{13}C values for MeHg. Two approaches for estimation of the measurement uncertainty are compared: Kragten and Docherty et al.[17,23]

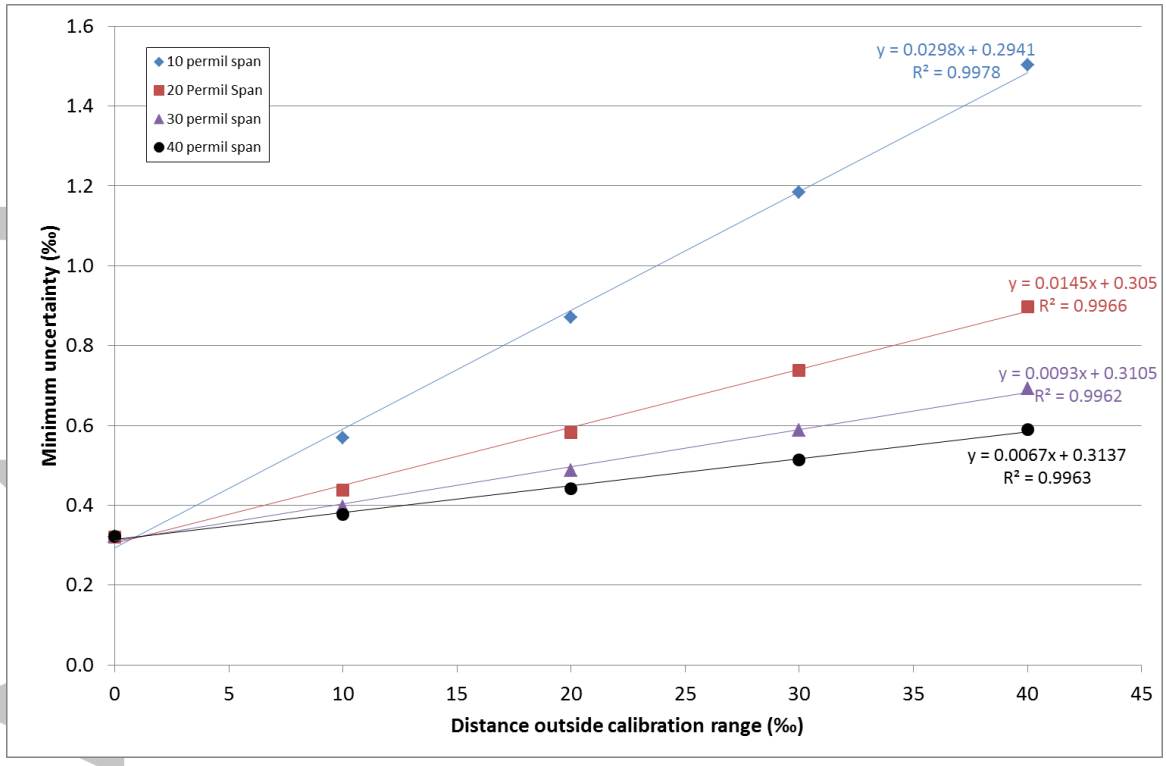

Figure 5 – The effect on measurement uncertainty introduced by normalisation arising from extrapolation (i.e. when raw $\delta^{13}C$ values for MeHg lie outside the calibration range afforded by the RMs) for pairs of RMs of increasing span in isotope ratio. The uncertainties shown are the minimum combined standard uncertainties with $k = 1$ resulting from normalisation.

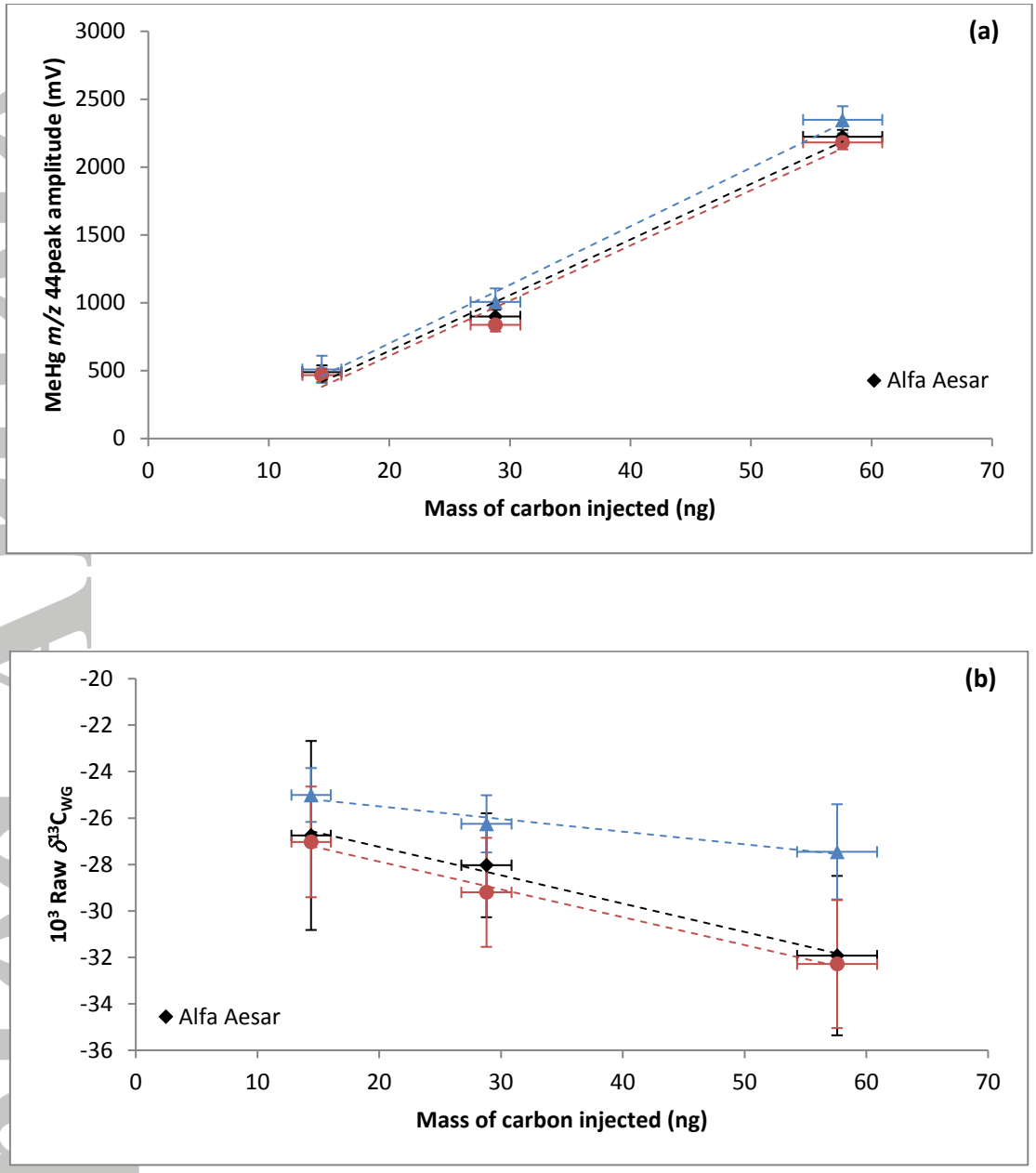

Figure 6 – The relationships between **(A)** mass of carbon in the form of MeEtHg injected on column and obtained peak amplitude as measured on the *m/z* 44 signal and **(B)** between mass of carbon in the form of MeEtHg injected on column and raw isotope delta value obtained. Each of the three MeHg standards is shown separately and the error bars show the expanded uncertainties (k = 2).

Table 1 – Peak areas (for all three Faraday collector signals combined) obtained for RMs and MeHg samples during FIA/CO-IRMS analyses together with amounts of carbon analysed. Peak areas for the MeHg were slightly higher than expected.

Material	Mass of C analysed (μg)	Peak Area (mV.s)
VHG MeHg	0.56	197
Alfa Aesar MeHg	0.56	174
Dr Ehrenstorfer MeHg	0.56	192
ERM-AE672a	0.40	110
USGS40	0.40	105
IAEA-CH-6	0.40	121
USGS41	0.40	114
in-house Glycine	0.16	50
	0.64	191

Table 2 – Calibrated $\delta^{13}C_{VPDB}$ values for the MeHg in-house reference materials obtained by FIA/CO-IRMS and GC/C-IRMS both with and without derivatization, as well as for the materials used for normalization during FIA/CO-IRMS. Uncertainties are the combined standard uncertainties with $k = 1$.

Material	$\delta^{13}C_{VPDB}$ (‰)			
	Expected	**FIA/CO-IRMS (i.e. bulk)**	**GC/C-IRMS with derivatization**	**GC/C-IRMS without derivatization**
VHG MeHg	n/a	-68.85 ± 0.17	-68.3 ± 7.7*	-68.85 ± 0.17*
Alfa Aesar MeHg	n/a	-72.52 ± 0.18	-72.5 ± 10.9	n/a
Dr Ehrenstorfer MeHg	n/a	-41.56 ± 0.12	-41.6 ± 5.3*	-41.56 ± 0.12*
ERM-AE672a	-42.12 ± 0.21	-42.07 ± 0.12	n/a	n/a
USGS40	-26.39 ± 0.04	-26.40 ± 0.09	n/a	n/a
IAEA-CH-6	-10.45 ± 0.04	-10.51 ± 0.08	n/a	n/a
USGS41	+37.63 ± 0.05	+37.65 ± 0.15	n/a	n/a

*These values are identical to those for FIA/CO-IRMS as they have been normalised using the FIA/CO-IRMS results (but the associated uncertainty may be different).

Table 3 – MeHg content of selected RMs (not necessarily certified for MeHg isotope ratio) together with indicative amounts required for analysis using the derivatization approach. Values were taken from the latest available certificates from the supplier.

Supplier	Reference Material	Nature	MeHg Concentration		Unit Size	Mass of MeHg per unit	Units required per analysis (20 µg MeHg required)
			value	unit		µg	
NIST	SRM 1947	Fish tissue	0.233 ± 0.010	mg kg^{-1}	8 g	1.86	11
	SRM 1946	Fish tissue	0.394 ± 0.015	mg kg^{-1}	8 g	3.15	6
	SRM 2974a	Mussel tissue	69.06 ± 0.81	µg kg^{-1}	5 g	0.35	58
	SRM 2976	Mussel tissue	28.09 ± 0.31	µg kg^{-1}	25 g	0.70	28
	SRM 955c	Caprine blood	4.5 ± 1	µg L^{-1}	2 mL	0.01	2222
NIES	CRM 13	Human Hair	3.8 ± 0.4	µg g^{-1}	3 g	11.40	2
IAEA	IAEA-085	Human Hair	21.9-23.9 (95% CI)	mg kg^{-1}	5 g	114.50	0.2
	IAEA-086	Human Hair	0.236-0.279 (95% CI)	mg kg^{-1}	5 g	1.29	16
	IAEA-407	Fish tissue	0.188-0.212 (95% CI)	mg kg^{-1}	25 g	5.00	4
	IAEA-452	Scallop	0.022 ± 0.004	mg kg^{-1}	8 g	0.18	114
IRMM	ERM-CE464	Tuna Fish	5.50 ± 0.17	mg kg^{-1}	15 g	82.50	0.2
	ERM - CC580	Sediment	75 ± 4	µg kg^{-1}	40 g	3.00	7
	BCR-463	Tuna Fish	3.04 ± 0.16	mg kg^{-1}	15 g	45.60	0.4