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1 **Sensitive determination of methylmercury $\delta^{13}\text{C}$ compound specific stable**
2 **isotopic analysis by purge and trap gas chromatography combustion**
3 **isotope ratio mass spectrometry**

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10

11 **Abstract**

12 Despite several decades of mercury research, answering fundamental questions on
13 where and how methylmercury (CH_3Hg) toxin is naturally produced in aquatic ecosystems, is
14 still highly challenging. Investigating complex and/or coupled processes in the context of
15 global changes requires new high-resolution analytical tools. The purpose of the compound
16 specific carbon stable isotopic analysis ($\delta^{13}\text{C}$ -CSIA) of the methyl group of methylmercury
17 (CH_3Hg), is to explore how the carbon cycle contributes to CH_3Hg sources and formation
18 pathways. The main problem associated with recent CH_3Hg $\delta^{13}\text{C}$ -CSIA methods is the limited
19 sensitivity when using Liquid Injection (LI)-GC-C-IRMS techniques, requiring several
20 micrograms of CH_3Hg (as Hg). In this work, we present the development and application of
21 an original Purge-&-Trap system (PT) coupled to a GC-C-IRMS with the purpose of
22 transferring and analyzing the total amount of CH_3Hg available in a sample vial in the low
23 nanogram range. The new PT-GC-C-IRMS system enhance the sensitivity by a factor better
24 than 200, relative to LI-GC-C-IRMS, by minimizing the sample mass requirements. The
25 $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ values obtained, following the same sample derivatization approach coupled to PT-
26 GC-C-IRMS ($-53.5 \pm 1.9 \text{ ‰}$), were in good agreement with the ones obtained in a previous
27 study ($-53.8 \pm 1.1 \text{ ‰}$). The standard solution was prepared from the same salt, requesting only
28 25 to 200 ng of CH_3Hg (as Hg). This new methodology represents a milestone towards the
29 analysis of large array of biological samples displaying CH_3Hg concentrations in the low-mid

30 ng/g range, in order to explore the meaning of the carbon stable isotopic signature of CH₃Hg
31 in the environment.

32 Keywords: methylmercury, carbon isotopes, purge and trap, isotope ratio mass spectrometry

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34

35 **1. Introduction**

36 Methylmercury (CH₃Hg) is considered the highest neurotoxic form of mercury due to
37 its effects in the development of the nervous system [1]. Fish consumption is, currently, the
38 main known source of CH₃Hg exposure in humans [2]. Mercury levels in fish are of public
39 concern and some guidelines were adopted. In 2001, the European Union (Decision
40 466/2001/EC) set a maximum level of 0.5 µg Hg g⁻¹ (wet weight) [3]. The U.S. Food and
41 Drug Agency adopted the concentration of 1 µg g⁻¹ for MeHg, as the limit from which fish
42 should not be consumed, due to its associated health risk. [4] The U.S. Environmental
43 Protection Agency established a standard value for the daily MeHg dose of 0.1 µg kg⁻¹ of
44 body weight [5]. Once inorganic Hg is methylated, it is rapidly accumulated in the foodweb
45 and biomagnified in the trophic chain [6]. Even though the concentrations of this compound
46 have been widely studied in both marine and fresh aquatic ecosystems, processes governing
47 how, where, and when it is formed prior to its incorporation in the food web remain still
48 unclear [7]. Methylation can occur by both biotic and abiotic mechanisms in the water column
49 [7–10]. In addition, the concentration increases with depth being more pronounced in the area
50 close to the sediment compartment, with greater particulate and bacterial activity [11]. The
51 recent discovery of methylation genes [12] has confirmed the consensual idea that the
52 bacterial origin of CH₃Hg was likely one of the main production pathways. However, the
53 presence of these genes may not be the most relevant factor in methylation. Invertebrate
54 digestive tracts, permafrost soils or extreme environments, has been also revealed as potential

55 methylation environments, suggesting multiple routes for MeHg entry into food webs [13].
56 The presence of organic matter seems to increase MeHg bioaccumulation in the trophic chain
57 [14]. A recent work has found that the methylation mechanism is controlled by the presence
58 of Hg-ligand binding [15]. So far, it is still difficult to precisely deconvolute the individual
59 pathways governing CH₃Hg origin and production in complex ecosystems. New and more
60 resolute analytical tools capable of providing new insights on CH₃Hg origin and fate are
61 much needed.

62 The recent development of compound-specific stable isotope analysis of
63 organometallic compounds has shed some light on the formation of this class of compounds
64 and opens a new source of knowledge about tracing their environmental behavior [16]. The
65 interest of carbon isotope analysis to elucidate the fate of organometallic compounds, is
66 shown in the large isotope fractionation observed due to the abiotic transformation of the
67 transferred methyl group in the case of arsenic compounds [17]. The study of the carbon
68 source and isotope fractionation of the C atom embedded in methyl group of CH₃Hg could
69 provide new information to understand the fundamentals of Hg methylation processes, and
70 CH₃Hg origin [18].

71
72 Gas chromatography coupled to isotope ratio mass spectrometry (GC-C-IRMS) has
73 been revealed as a powerful technique to identify biogeochemical pathways for multiple
74 organic analytes [19]. Recently, Masbou and coworkers [18] presented the first approach to
75 determine carbon isotope ratios for CH₃Hg compound. The limiting factor for the application
76 of these methodologies is the amount of CH₃Hg needed, and by extent the sample mass
77 requested for analysis. The recommendations for the precise measurement of carbon isotope
78 ratios ($\delta^{13}\text{C}$) on individual organometallic molecules require a signal higher than 0.5 V [20].
79 These sensitivity conditions are highly restrictive in the case of CH₃Hg which holds a single

80 carbon atom and require a minimum injection of 100-300 ng of CH₃Hg (as Hg). In the case of
81 liquid injection techniques, where only a μL fraction of solvent is injected per sample, and
82 even after manual preconcentration under a nitrogen stream, this means extracting initially
83 about 20 μg of CH₃Hg for each individual sample [18]. One of the instrumental possibilities
84 to enhance the sensitivity is to maximize the preconcentration step of the analytes prior to its
85 introduction in the GC column [21,22]. A programmable temperature vaporizing (PTV)
86 injector is designed to concentrate volatile analytes contained in a high volume of sample and
87 corresponding solvent. However, the preconcentration is not only limited to the volatile
88 analytes but also to the impurities with carbon content, thus increasing as well as the signal
89 background in the case of GC-C-IRMS. Alternatively, a purge and trap (PT) approach which
90 consisting of quantitatively purging and accumulating a volatile compound of interest present
91 into a sample vial onto an analytical trap prior to its desorption and analysis could help to
92 decrease the limits of detection [22]. This methodology allows to separate the volatile
93 compounds of interested from its matrix, and to eliminate the need for a solvent, therefore
94 minimizing the contribution of carbon blank. Zwank and coworkers demonstrated the power
95 of the PT in reducing the initial amount of analyte required for the analysis [23]. The
96 capability of PT method including a secondary cryofocussing step coupled to GC-C-IRMS
97 analysis, was previously demonstrated by Wuerfel and coworkers for carbon isotope
98 fractionation studies in the case of arsenical compounds [17,24]. The efficiency of this purge
99 and trap methodology was previously evaluated by Diaz-Bone and coworkers by GC-MS
100 [25]. The other limiting factor associated with this type of analysis is the lack of existing
101 reference standard solution, and beyond, true natural reference materials for which certified
102 and/or published *in house* reference δ¹³C values for organometallic standards, and in
103 particular CH₃Hg are available.

104

105 The aim of this work was to (i) test and optimize a unique PT device equipped with a
106 LN₂ cooled microtrap technology coupled with a GC-C-IRMS for the precise, quantitative
107 and accurate determination of $\delta^{13}\text{C}$ for both volatile organic compounds (VOC), and
108 derivatized (ethylated) CH₃Hg present at low concentration levels *in house* CH₃Hg reference
109 solutions, (ii) document the sensitivity improvement of the PT-GC-C-IRMS method relative
110 to LI-GC-C-IRMS analysis for $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ determination [18], and (iii) to discuss the
111 agreement and precision obtained by both methods when analyzing a standard solution
112 prepared from the same CH₃Hg salt source.

113

114 **2. Experimental**

115 2.1 Material and methods

116 The primary VOC standard solution used in this work consisted of a 200 $\mu\text{g g}^{-1}$
117 benzene, ethylbenzene, toluene, and (m, o, p) xylenes (BTEX) compound mixture in
118 Methanol (MeOH), purchased from Restek (Bellefonte, PA, USA). This standard solution
119 was primarily diluted in MeOH, and a secondary standard of 200 ng g^{-1} in water, prepared on
120 a daily basis was used. About 50 to 200 μL of this secondary BTEX standard solution was
121 diluted into a final volume of 10 mL Milli-Q water in a 15 mL PT glass vials equipped with a
122 silicon Teflon septum.

123 The primary CH₃Hg reference standard solution used in this study was prepared from
124 a methylmercury chloride salt (Sigma-Aldrich; Milwaukee, WI, USA) dissolved in a 10%
125 (v/v) methanol/water solution. This CH₃Hg salt source is identical to the employed in our
126 previous study [18] with an estimated $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ value of -53.8 ± 1.1 ‰. An inorganic Hg
127 standard solution (Hg(II)) NIST SRM 3133 (National Institute of Standards and Technology,
128 Gaithersburg, MD, USA) was also employed in this work. Secondary CH₃Hg and Hg(II)
129 standard solutions of approximately 1000 ng Hg/g each were prepared in 1% w/w bi-distilled

130 HCl. About 25 to 200 μL of these standard solutions were added to 9 mL of MQ water into a
131 15mL PT glass vials equipped with a with silicone Teflon septa, and followed by the addition
132 of 1 mL of pH4; 0.1M Citric acid- Sodium citrate buffer (salts purchased from Sigma-
133 Aldrich; Milwaukee, WI, USA) [26]. These solutions were ethylated by adding 50 to 250 μL
134 of NaBEt_4 0.1% (Merseburger Spezialchemikalien, Germany) to form CH_3HgEt and HgEt_2
135 compounds, respectively. The sodium tetraethylborate salt (NaBEt_4) was diluted to 20% in
136 Milli-Q, aliquoted in vials of 1 mL and stored in the freezer at -18°C until use. The solution
137 was cleaned, before its dilution to 0.1%, by means of a liquid-liquid extraction with hexane
138 and two subsequent dilutions in order to eliminate the traces of hexane in the reagent. This
139 clean-up step allows to eliminate some organic interferents (See ESI Section and Figure S.3).
140 This procedure is performed just before the preparation of the solutions for analysis, with
141 frozen stored aliquots of the reagent that should melt prior to the cleaning with hexane.

142 All the glass labware, new and reused, employed in this work was cleaned by
143 pyrolysis before use. Millipore $18.6 \text{ M}\Omega\cdot\text{cm}^{-1}$ ultrapure water and bi-distilled hydrochloric
144 acid were used for the preparation of all solutions.

145

146 2.2 Instrumentation

147 The measurement of the $\delta^{13}\text{C}$ values for ethylated $\text{Hg}(\text{II})$ and CH_3Hg , and BTEX compounds
148 was performed using a gas chromatograph (GC, Trace 1310, Thermo Scientific) equipped
149 with a DB-5MS capillary column (Agilent J&W Scientific, 5% diphenyl, 95%
150 dimethylsiloxane, $30 \text{ m} \times 0.25 \text{ mm i.d.} \times 0.25 \mu\text{m}$), coupled to an isotope ratio mass
151 spectrometer (MAT 253 IRMS, Thermo Scientific), using a GC Isolink combustion reactor
152 and a Conflow IV (Thermo Scientific) interface. The GC-C-IRMS was interfaced with an
153 automated Purge and Trap unit (VSP4000, IMT Innovative Messtechnik, Vohenstrauß,
154 Germany) directly connected to the GC column by the mean of a heated transfer line. The PT

155 unit was equipped with a Peltier water trap (PWT) to remove water vapors and a micro Tenax
156 trap (cooled at -50°C by LN₂) was used as a sorbent to trap volatile organic compounds. The
157 GC conditions and the set-up of the PT unit are detailed in Table S.1 of the ESI.

158

159 2.3 Measurement of carbon isotope ratios

160 The $\delta^{13}\text{C}$ values measured in this work are expressed relative to a high purity CO₂
161 reference gas which was previously calibrated by liquid injections of a $\delta^{13}\text{C}$ certified C15-
162 C13-C20 alkane mix standard solution (Chiron, Norway). The $^{13}\text{C}/^{12}\text{C}$ ratios measured for
163 each BTEX and ethylated Hg(II) and CH₃Hg compounds are normalized to the Vienna Pee
164 Dee Belemnite (VPDB) reference standard and expressed in δ notation as shown in Equation
165 1.

$$166 \quad \delta^{13}\text{C} = \left(\frac{\left(\frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{sample}}}{\left(\frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{VPDB}}} - 1 \right) \times 1000 \text{ (‰)} \quad \text{Eq. 1}$$

167 Before each analytical session, IRMS linearity and stability tests were conducted as
168 part of the daily maintenance checks.

169 Since $\delta^{13}\text{C}$ values can be determined for ethylated Hg(II) and CH₃Hg compounds, the
170 estimation of the endogenous $\delta^{13}\text{C}$ values of the methyl group of CH₃Hg is estimated
171 according to Equation 2 (see ref. [18] for details),

172

$$173 \quad \delta^{13}\text{C}(\text{CH}_3\text{Hg}) = \delta^{13}\text{C}(\text{CH}_3\text{HgEt}) \times 3 - \delta^{13}\text{C}(\text{HgEt}_2) \times 2 \quad \text{Eq. 2}$$

174

175 2.4 Experimental strategy for assigning reference $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ values for trace amounts of
176 CH₃Hg by PT-GC-C-IRMS.

177

178 In order to ensure accurate and traceable $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ measurements by PT-GC-C-IRMS
179 analysis, a two steps approach was considered. Firstly, a diluted BTEX standard solution, was
180 used to optimize the purging conditions to ensure a quantitative purging and trapping of each
181 compound by the optimized PT method. BTEX act as a secondary standard for $\delta^{13}\text{C}$ analysis
182 of volatile compounds, that can be analyzed directly by PT-GC-C-IRMS, displaying boiling
183 points in the range of ethylated Hg compounds. Secondly, we applied the validated purging
184 conditions to Hg compounds with checking that peaks intensities for both CH_3HgEt and
185 Et_2Hg normalized to their carbon contents was similar to that of BTEX compounds to ensure
186 a quantitative derivatization and purging of ethylated Hg compounds in solution. Every five
187 measurements of a solution of ethylated CH_3Hg and Hg (II), a solution of BTEX was
188 introduced into the system with the aim of controlling the traceability and accuracy of the
189 measurements. The optimized conditions were then used to assign reference $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ values
190 to the CH_3Hg reference solution prepared in this study and traceable to the of our previous
191 study [18]. All results were obtained by performing a partial oxidation of the combustion
192 reactor (Seed Oxidation), between each run. The oxidation of the combustion reactor was
193 performed on daily basis at the end of each analytical session.

194

195 **3. Results and discussion**

196

197 **3.1 Evaluation of the purging and trapping efficiencies of BTEX for $\delta^{13}\text{C}$ analysis by PT-** 198 **GC-C-IRMS**

199 The purging conditions were optimized to obtain the maximum purging and transfer
200 yield of the VOC compounds from the solution to the analytical trap, and from the trap to the
201 GC-C-IRMS. BTEX (benzene, toluene, ethylbenzene and o,p,m-xylene), were the organic
202 compounds selected for this evaluation, due to the possibility of employing similar operating

203 conditions as for Hg compounds (See Table S.1. of the ESI). Benzene was not analyzed due
204 to its proximity in the elution to the peak of MeOH, which is the dilution media of the BTEX
205 stock solution. These two early peaks were left out from the chromatograms by switching on
206 the backflush valve position into straight mode after 200 sec, in order to avoid saturation of
207 the detector with carbon inputs. The consideration of the other four volatile organic
208 compounds is sufficient for method validation.

209 An assay was performed to determine the purging time that gives the maximum intensity
210 (peak areas per ng of C added to the solution) for each compound. The results are illustrated
211 in Figure 1. The carbon content of each compound in the purging solution was set to about 15
212 ng of C (about 16 ng of each BTEX), which provide enough sensitivity for precise $\delta^{13}\text{C}$ CSIA
213 measurements. The purging time varied from 5 to 20 min using a He purge flow of 20
214 ml/min. The desorption time of the tenax trap was set to 235°C during 30 s to achieve a
215 complete transfer of BTEX compounds from the analytical trap to the GC-C-IRMS by the
216 mean of the heated transfer line. Five replicates of each compound, with the corresponding
217 purging time, were analyzed to obtain the uncertainty, expressed as 2SD. As the graphs show,
218 the normalized peak area (V.s-1/ng C), for each compound in the purging vial, increases
219 proportionally up to 15 min of purge with no further improvements between 15 and 20 min.
220 This suggests that the quantitative trapping of BTEX compounds is achieved after 15 minutes
221 of purge. Thus, the purging time was set to 20 min using a He purge flow of 20 mL/min to
222 achieve a quantitative transfer and trapping of the BTEX compounds prior to GC-C-IRMS
223 analysis. The $\delta^{13}\text{C}$ values for BTEX were found unchanged during the different purging
224 times, indicating the absence of fractionation artefact resulting from the incomplete purging,
225 and thus transfer of BTEX from the liquid phase to the analytical trap prior to GC-C-IRMS
226 analysis. The chromatograms illustrated in Figure 2.a. showed that three of the BTEX
227 compounds were perfectly resolved, except for the m-xylene and p-xylene compounds,

228 displaying nearly identical boiling points and coeluting at 403 s. This coelution is confirmed
229 by the higher peak height relative to the three other compounds.

230

231 The linearity of the measurements was evaluated by analyzing four different
232 concentration levels (10, 20, 30 and 40 ng of C) of the BTEX standard solution in Milli-Q
233 water. The results are shown in Figure 3, showing that the peak areas of each individual
234 compound increase linearly as a function of the amount of BTEX (ng of C) compounds in
235 solution ($r^2=0.99$). These results indicate a quantitative purging, transfer and analysis of the
236 different analytes, independent of its respective concentration in the solution. The numerical
237 data for $\delta^{13}\text{C}$ values of BTEX are presented in Table S.2 of the ESI. The relative similar
238 intensity for the four compounds, present at the same concentration in solution, suggests that
239 there is no compound specific preferential purging, and that the purging and trapping
240 conditions are efficient for a wide range of volatile compounds. The quantitative purging
241 conditions lead to consistent $\delta^{13}\text{C}$ values for each BTEX compounds over the concentration
242 range investigated, evidencing the absence of fractionation bias by the PT device, and its
243 coupling to the GC-C-IR-MS. As expected, $\delta^{13}\text{C}$ measurements precision increased with the
244 concentration of BTEX in solution. Overall and across the BTEX concentration range
245 analyzed (10-40 ng of C), the standard deviation from the average ranged from 0.08-0.47 ‰
246 (as $\pm 2\text{SD}$, $n=20$ replicates). The instrument specifications suggest a precision in the range of
247 the instrument (0.25‰ as SD ($n=5$)). This indicates that the coupling of the PT module to the
248 GC-C-IRMS does not induce any significant additional analytical bias in the case of volatile
249 compounds relative to classical liquid injection techniques. Our in house $\delta^{13}\text{C}$ values for the
250 four BTEX compounds are summarized in Table 1. The reference values obtained for BTEX
251 standards were -27.5 ± 0.4 ‰ for toluene, -28.9 ± 0.5 ‰ for ethylbenzene, -26.4 ± 0.3 ‰ for
252 m,p-xylene and -27.4 ± 0.4 ‰ for o-xylene (uncertainties expressed as 2SD for $n=45$

253 measurements). The $\delta^{13}\text{C}$ reference values and sensitivity values estimated for the different
254 BTEX compounds, are employed as quality control over time to ensure that the system is
255 working properly among different analytical sessions, when analyzing Hg compounds.

256

257 **3.2. Evaluation of the quantitative purging and transfer of ethylated CH_3Hg and Hg(II)** 258 **to the GC-C-IRMS**

259 We have compared the sensitivities obtained for ethylated Hg compounds and BTEX
260 when expressed as ng of C added to the solution. The comparison was done for the optimized
261 purge and trap conditions obtained for BTEX compounds. Figure S.2. in the ESI, showed that
262 the purge and trap of ethylated mercury compounds provides nearly identical sensitivity
263 estimates ($0.19 \pm 0.02 \text{ V}\cdot\text{s}^{-1}/\text{ng}$ of C for n=60 replicates of CH_3HgEt) relative to those
264 measured for BTEX ($0.18 \pm 0.02 \text{ V}\cdot\text{s}^{-1}/\text{ng}$ of n=45 replicates of C signal for toluene). This
265 indicates that there are no specific differences in the purge and transfer between derivatized
266 Hg compounds and volatile compounds. Therefore, Hg compounds are quantitatively
267 derivatized in solution, purged, and analyzed by PT GC-C-IRMS in the same way as BTEX.

268 The linearity for these measurements was evaluated by analyzing standards prepared
269 at five concentration levels (25, 50, 100,150 and 200 ng of Hg, which corresponds to 5 (6),10
270 (12), 20 (24), 30 (36), and 40 (48) ng C for MeHg (Hg(II)) in Milli-Q, as described in the
271 material and method section. The results showed in Figure 4, correspond to 11 independent
272 replicates for each level of concentration, except in the case of 25 ng with only 8 replicates.
273 The linearity curves expressed as peak areas vs. ng of ethylated Hg compound present a
274 regression coefficient (r^2) better than 0.99. These results suggest a quantitative transfer of the
275 analytes and the absence of isotopic fractionation, independent of its amount in the solution.

276 The numerical data for $\delta^{13}\text{C}$ measurements is presented in the Table S.3 of the ESI.
277 The average values range from -29.6 to -30.6 and -38.0 to -38.4 ‰ for $\delta^{13}\text{C}_{\text{HgEt}_2}$ and

278 $\delta^{13}\text{C}_{\text{CH}_3\text{HgEt}}$ respectively. Overall, the estimated values for CH_3Hg $\delta^{13}\text{C}$ range from -53.6 to -
279 54.8 for this assay, which is within the uncertainty range previously measured for the same
280 MeHg salt source [18]. The chromatogram for CH_3Hg and Hg(II) is represented in Figure 2.b.
281 The stability of the mercury compounds ethylated in aqueous solution has been demonstrated
282 along the measurements performed in this work (Figure S.1. of the ESI).

283

284 The uncertainty ($\pm 2\text{SD}$) associated with $\delta^{13}\text{C}$ measurements range from 3.5 to 5.5 and
285 2.6 to 3.3 ‰ for HgEt_2 and CH_3HgEt respectively. This uncertainty is significantly higher
286 than the uncertainty observed for BTEX compounds, despite covering a similar concentration
287 range (0-50 ng of C). The higher uncertainties for ethylated Hg compounds may be associated
288 to the fact that the analysis of these compounds involves an additional derivatization step
289 prior to purging, relative to BTEX. Thus, this variability could be associated with the
290 derivatization conditions. However, we found that despite Hg(II) acts as an internal standard
291 capable of correcting the additional C atom, originating from NaBEt_4 , it also minimizes the
292 uncertainties associated with the derivatization step to estimate $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$. It is observed that
293 the measurement precision ($\pm 2\text{SD}$) associated with the estimated $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ value, range from
294 1.0 to 2.7, which is much lower than the determined for ethylated CH_3Hg and Hg(II)
295 compounds (Figure 4.a. for ethylated CH_3Hg and 4.b. for ethylated Hg(II)).

296

297 **3.3 Evaluation of different derivatization conditions to minimize $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ uncertainty.**

298 The most common way of derivatizing Hg compounds is the alkylation through
299 alkylborate reagents. In this type of analyses, it is preferable to use of the minimum amount of
300 reagent to limit the carbon blank contribution, associated with the reagents. Because of that,
301 we have first estimated that the concentration of 0.1% of NaBEt_4 in Milli-Q was enough to
302 accomplish a quantitative derivatization of the analytes. The assay was performed for a

303 solution of Hg compounds of 100 ng of Hg (20 and 24 ng of C for CH₃Hg and Hg(II)
304 respectively when ethylated). The drawback of this reaction is the addition of carbon atoms to
305 the molecule with the consequent impossibility of the direct measurement of the $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$
306 value. For this reason, and as specified before (18), we also used Hg(II) as an internal
307 standard to correct for the $\delta^{13}\text{C}$ value of the exogenous alkyl group contribution coming from
308 the reagent (see Eq. 2). Previous work showed that the amount of the derivatization reagent is
309 known to be responsible for the reduction and transformation of Hg compounds when
310 derivatized Hg species are extracted into an organic solvent prior to GC-ICP-MS analysis
311 [27]. Our results suggest that this is not the case when considering PT approaches, in the
312 absence of solvent during the derivatization reaction since both the peaks intensities for both
313 CH₃HgEt and HgEt₂ and their respective $\delta^{13}\text{C}$ values were nearly identical across a wide
314 range of derivatizing agent amounts covering between 50 and 250 μL (Figure 5). This also
315 suggests that the derivatization reaction does not induce significant fractionation artefacts in
316 the range of the amount of NaBEt₄ added. However, in this assay we observed that for higher
317 amounts than 100 μL of NaBEt₄ (0.1%), the precision of the $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ was much lower than
318 the precision reached when using the lowest amount of NaBEt₄ (50 μL).

319 The $\delta^{13}\text{C}$ values obtained for 60 independent replicates of Hg compounds are detailed
320 in Table 1, with an estimated $\delta^{13}\text{C}_{\text{CH}_3\text{Hg}}$ value of -53.5 ± 1.9 ‰. The results were compared
321 with the values obtained in a previous approach by liquid injection of the same Hg
322 compounds analytes in hexane [18]. The summary of all the results, obtained for different
323 concentrations and different volumes of NaBEt₄, shows that they are in agreement ($-53.8 \pm$
324 1.1 ‰) with the value obtained previously for the same CH₃Hg source material. The
325 comparison of these results allows us to conclude that the methodology employed here does
326 not fractionate Hg compounds, giving rise to accurate and precise $\delta^{13}\text{C}$ values of CH₃Hg.

327

328 **3.4 Sensitivity improvement of the PT-GC-C-IRMS method relative to LI-GC-C-IRMS**

329 The method performance of the new PT-GC-C-IRMS approach developed in this
330 study is compared in Table 2 to the results obtained previously by LI-GC-C-IRMS, with
331 analyzing a reference solution prepared from the same CH₃Hg salt source as in our previous
332 study [18]. Both methods provide $\delta^{13}\text{C}$ values of the methyl group of CH₃Hg in good
333 agreement and exhibit similar precision (2SD of 1.9 and 1.3 ‰, respectively). This is
334 consistent with the fact that for both approaches, the same final amount of CH₃Hg is injected
335 in the GC-C-IRMS. However, these results clearly show the higher efficiency and sensitivity
336 of the PT approach over the LI method, requiring about 200 times lower initial amounts of
337 CH₃Hg to finally inject the same quantity (25-200 ng) in the GC-C-IRMS. This achievement
338 represents a milestone, indicating that PT-GC-C-IRMS is likely the method of choice towards
339 analyzing different types of natural biological materials exhibiting CH₃Hg concentrations in
340 the low-mid ng/g range such as invertebrates, mollusks, fish, and birds. These taxa represent
341 the majority of the fauna in continental and marine ecosystems, and thus the resource of
342 samples that can be used to document CH₃Hg carbon isotopic fractionation in the
343 environment.

344

345 **4. CONCLUSION**

346 This work demonstrates the application of a Purge & Trap system coupled to a GC-C-
347 IRMS to measure carbon isotope ratios of CH₃Hg. The simultaneous measurement of Hg(II),
348 as internal standard, also allows to the correction of the carbon contribution of the
349 derivatization reagent, as well as improving measurement precision. The comparison of the
350 $\delta^{13}\text{C}$ values obtained for the same “in house” standard solution in this study with a previous
351 work, demonstrates also that the PT system does not fractionate the $\delta^{13}\text{C}$ values of the
352 ethylated mercury compounds. A 200-fold improvement in sensitivity was achieved by PT-

353 GC-C-IRMS compared to LI-GC-C-IRMS. The employment of BTEX as a secondary quality
354 control standard along the measurements allow us to verify the efficiency of the transfer of
355 the Hg analytes. Therefore, the methodology developed here could provide precise and
356 accurate information to explore and understand the meaning of the carbon signature of
357 CH₃Hg. Future studies should focus on measuring carbon isotope ratios of CH₃Hg in real
358 samples and in natural certified reference materials. These studies must include the
359 development of clean, quantitative and selective extraction procedure for methylmercury
360 which are compatible with the ethylation procedure for PT-GC-C-IRMS analysis.

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Figure Caption

484 **Figure 1.** Variation of peak areas per ng of C ($\pm 2SD$) added and $\delta^{13}C$ values ($\text{‰} \pm 2SD$) for
485 $n=5$ replicates for each different purging time of the solution from 5 to 20 min, for: a) toluene;
486 b) ethylbenzene; c) m,p-xylene and d) o-xylene.

487 **Figure 2.** Chromatogram obtained for: (a) purged BTEX solution of 15 ng of C (about 16 ng
488 of each compound in Milli-Q) added to the solution for the best conditions assayed; (b)
489 purged solution of 100 ng of CH_3Hg and $Hg(II)$ (20 and 24 ng of C respectively).

490 **Figure 3.** Linearity curves for BTEX (Peak area vs ng of compound (ng of C)) and $\delta^{13}C$
491 values for different concentrations in solution: a) toluene; b) ethylbenzene; c) m,p-xylene and
492 d) o-xylene. The uncertainties are expressed as $\pm 2SD$ from five independent replicates.

493 **Figure 4.** Linearity curves for ethylated CH_3Hg and $Hg(II)$ (Peak area vs. ng of compound
494 (ng of C)) and $\delta^{13}C$ values for different concentrations in solution: a) Ethylated CH_3Hg
495 (CH_3HgEt) and CH_3Hg estimated $\delta^{13}C$ values; b) Ethylated $Hg(II)$. The uncertainties are
496 expressed as $\pm 2SD$ from 11 independent replicates in each level of concentration, except for
497 25 ng of C level with 8 replicates.

498 **Figure 5.** Evaluation of the volume of derivatization reagent ($NaBEt_4$ 0.1%) for the reaction
499 with 100 ng of CH_3Hg and $Hg(II)$, in order to obtain the best peak areas per ng of C added
500 and the more reproducible $\delta^{13}C$ values. The uncertainties are expressed as $\pm 2SD$ from five
501 independent replicates in each level of concentration.

Figure 1

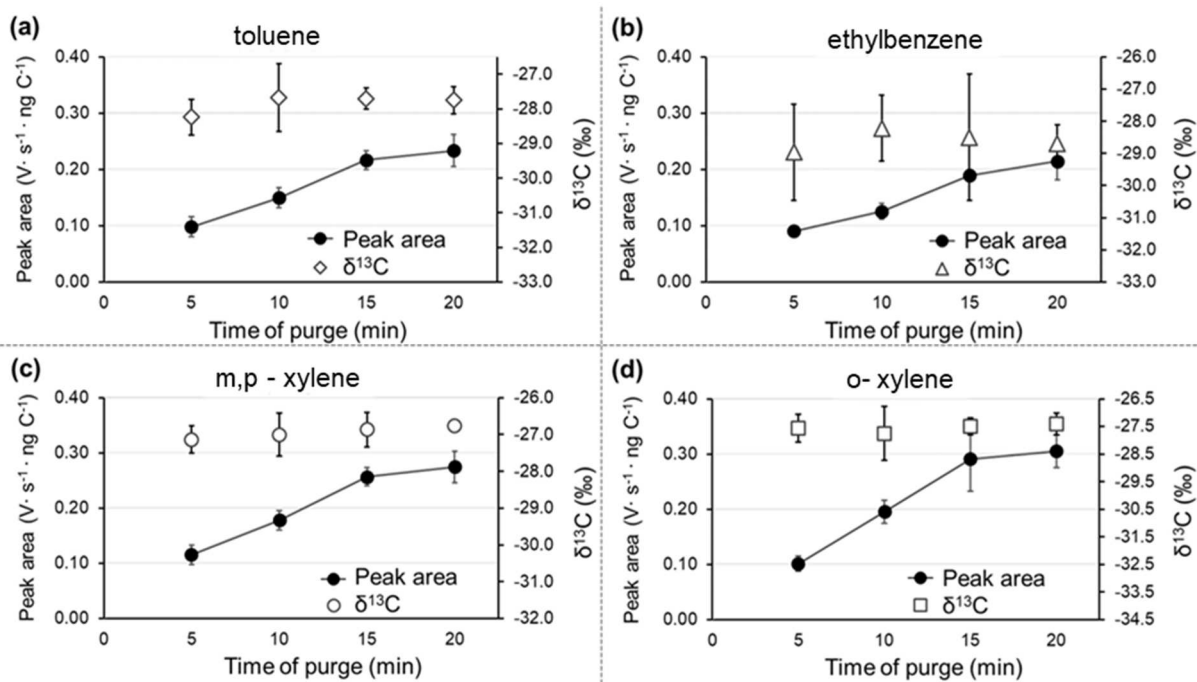


Figure 2

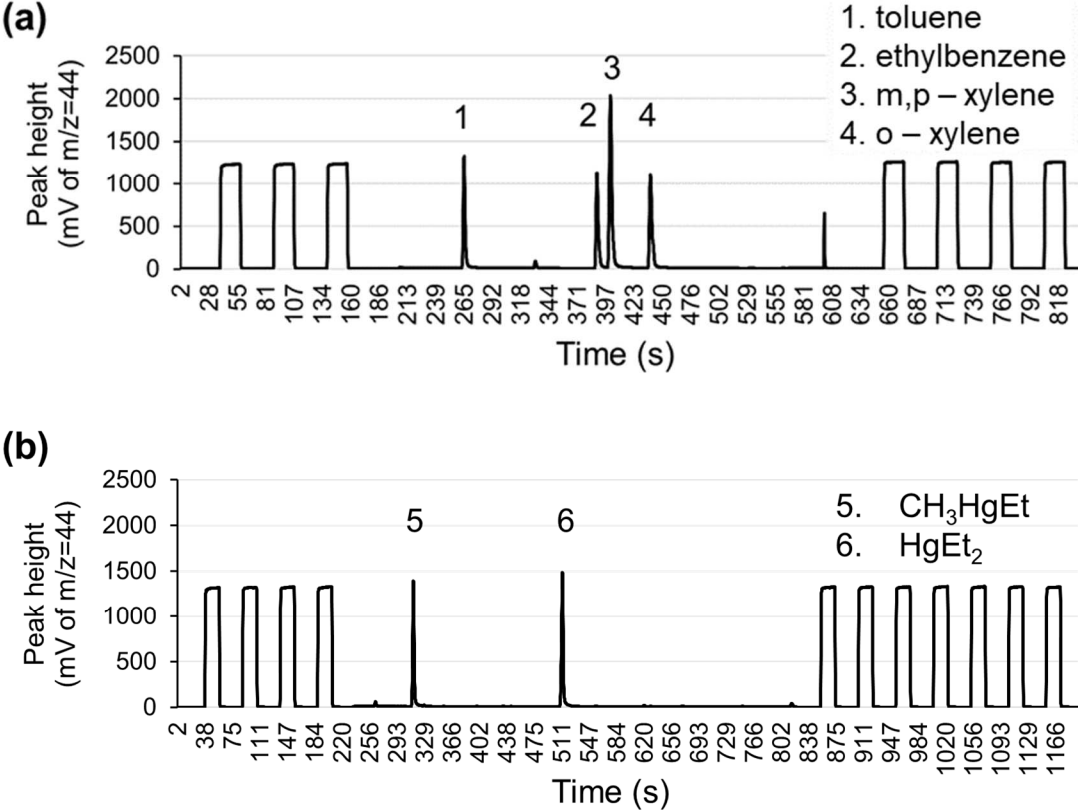


Figure 3

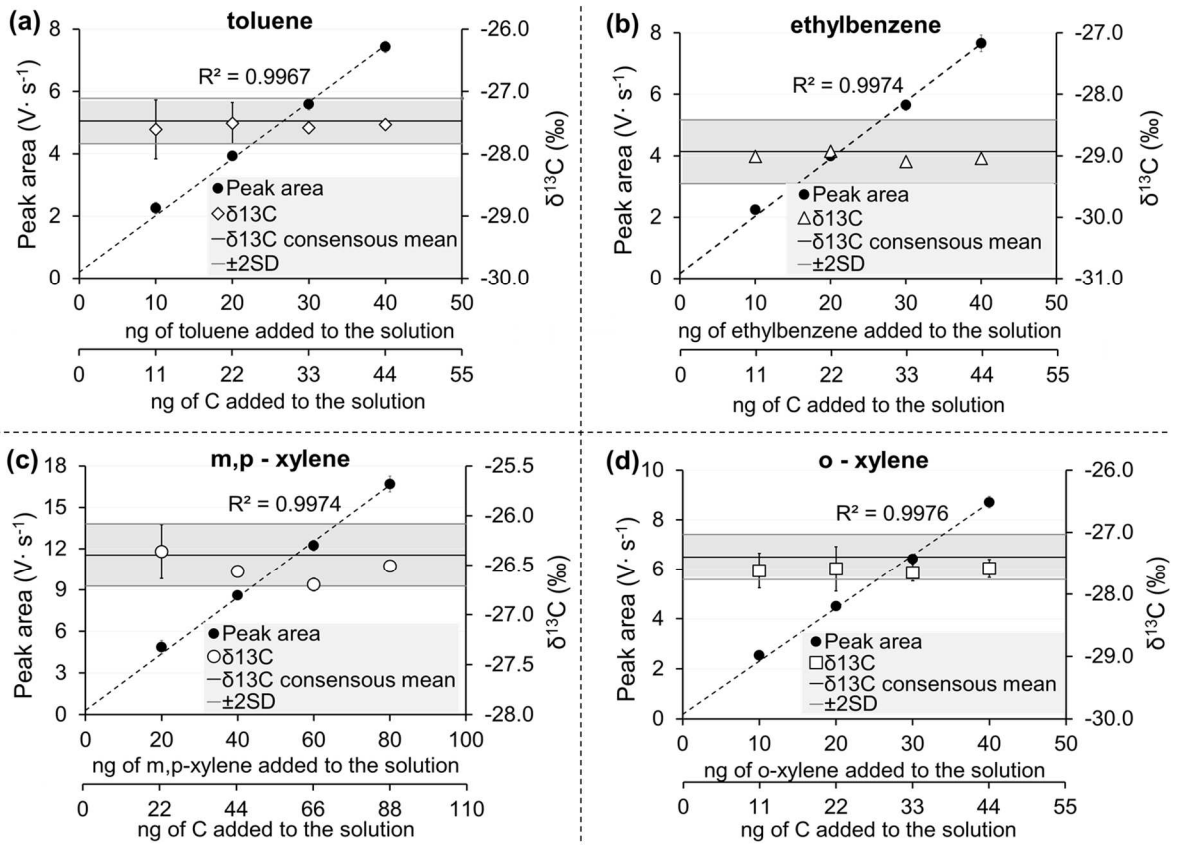


Figure 4

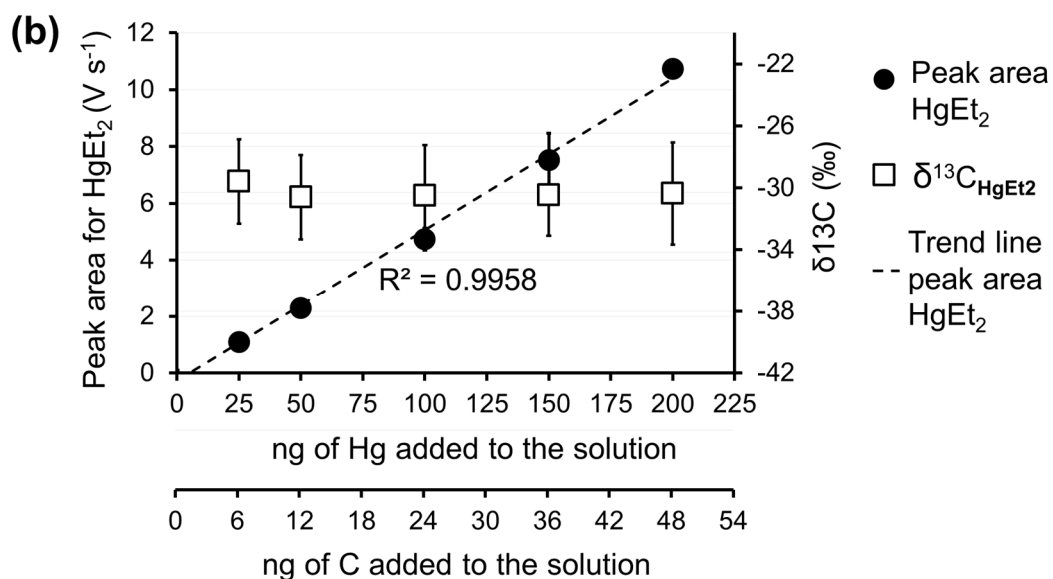
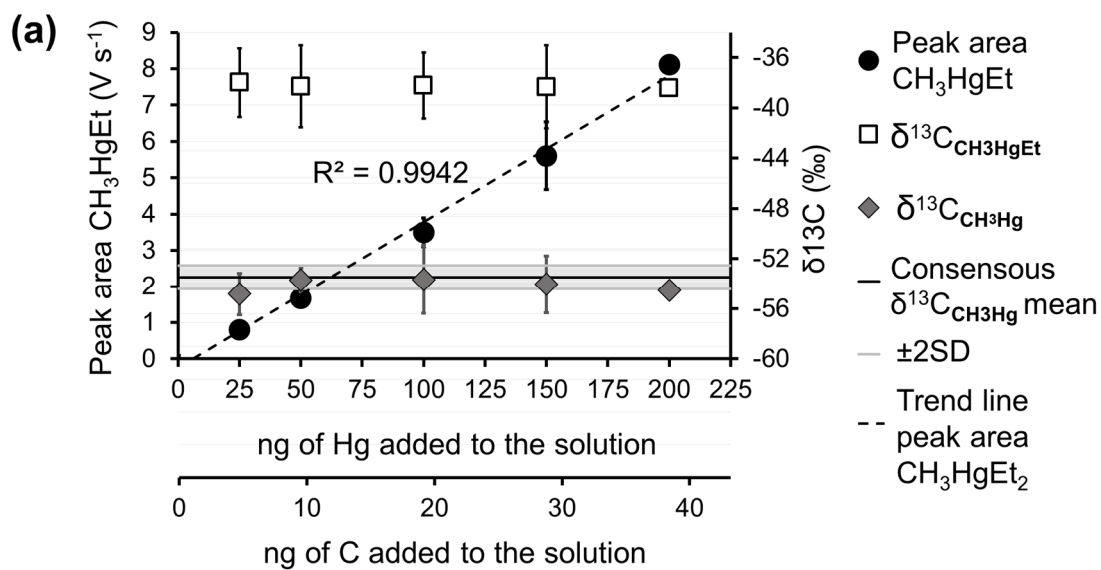


Figure 5

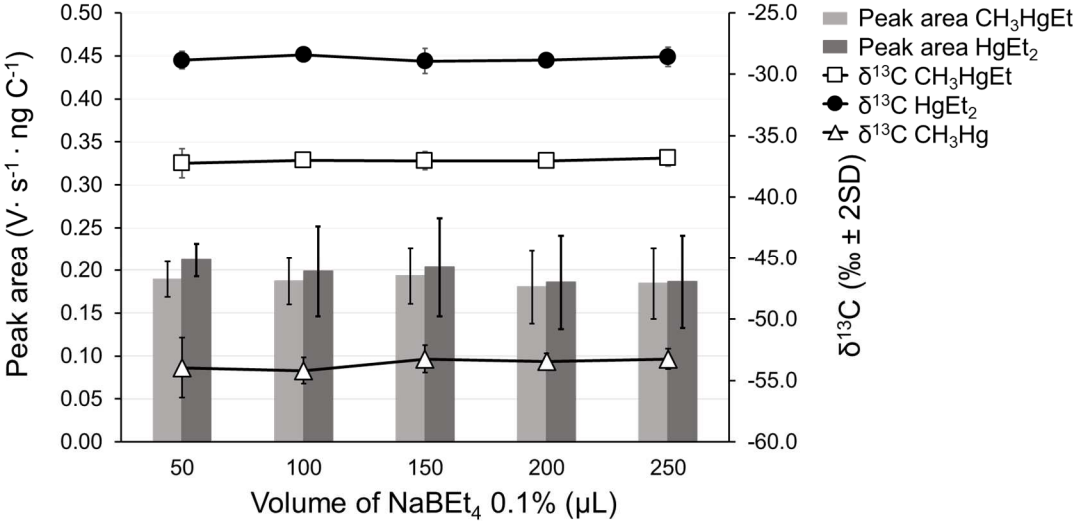


Table 1. “In-house” reference $\delta^{13}\text{C}$ values for BTEX and ethylated mercury compounds (CH_3HgEt and HgEt_2) and corrected $\delta^{13}\text{C}$ for CH_3Hg (isotope ratios in ‰ with uncertainties expressed as 2SD from the average of the N corresponding replicates).

$\delta^{13}\text{C}$ (‰)	Toluene	Ethylbenzene	p,m-Xylene	o-Xylene	CH_3HgEt	HgEt_2	CH_3Hg	CH_3Hg [18]
N	45	45	45	45	71	71	71	32
Average	-27.5	-28.9	-26.4	-27.4	-38.6	-31.1	-53.5	-53.8
2SD	0.4	0.5	0.3	0.4	2.6	4.2	1.9	1.1

Table 2. Method performance of the PT-GC-C-IRMS method relative to classical LI-GC--IRMS for the determination of the $\delta^{13}\text{C}$ value of the methyl group of CH_3Hg on the same reference standard solution.

Reference	Preparation and injection Methods	Derivatization Technique	Initial amount of CH_3Hg (ng, as Hg)	Solvent Vol., (Inj. Vol.)	Amount of CH_3Hg injected (ng, as Hg)	$\delta^{13}\text{CCH}_3\text{Hg}$ ($\text{‰} \pm 2\text{SD}$)
Masbou et al., 2015[18]	Liq.-Liq. Extraction, Liq. Inj. GC-C-IRMS	Ethylation	5000-80000	0.5mL (1 μL)	10-110	-55.1 \pm 0.9 (-53.8 \pm 1.1*)
This Study	In vial purging, PT-GC-C-IRMS	Ethylation	25-200	-	25-200	-53.5 \pm 1.9

*Consensus mean obtained by 3 independent methods (See Masbou et al., 2015 for details)