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1 **Quantification of light Polycyclic Aromatic Hydrocarbons in** 2 **seafood samples using on-line Dynamic Headspace extraction,** 3 **ThermoDesorption, Gas Chromatography tandem Mass** 4 **Spectrometry, based on an isotope dilution approach**

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18 **Abstract**

19 The aim of our work was to develop an analytical strategy to quantify naphthalene,
20 acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene in fish products by on-line
21 dynamic headspace extraction, followed by thermodesorption injection and gas chromatography
22 analysis coupled with tandem mass spectrometry using electron ionization mode (DHS-TD-GC-
23 EI-MS/MS). The developed protocol used 1 g of freeze-dried or oil sample supplemented with
24 perdeuterated light PAHs. The sample was heated at [90 - 100°C], the headspace of the sample
25 was swept by nitrogen and the trapping of the PAHs was carried out on a Tenax-type adsorbent
26 placed at 25°C. Analytes were thermodesorbed at 300°C from the dried adsorbant and then
27 cryofocused on a cooled injection system (CIS) at -25°C before injection (12°C s⁻¹ up to 300°C).
28 The chromatographic separation of PAHs was carried out on a 5-MS type column (30 m ×
29 0.25 mm, 0.25 µm) and the acquisition of the signals was performed in SRM following the
30 transitions, involving the loss of one or two hydrogen atoms from the molecular ion. In view of
31 the principle of extraction, the calibration curve was performed on a representative matrix or
32 using the standard addition method. Quantification limits were determined between 0.01 and
33 0.6 ng g⁻¹ of matrix from the method blank results. The method was validated by a series of
34 multi-level supplemented matrix assays and by the analysis of a reference material from an inter-
35 laboratory test (mussels, IAEA-432). The average of the expanded measurement uncertainty was
36 from 9 to 44% for the four lightest PAHs, except for fluorene when the sample incubation was
37 set at 90°C. Occurrence measurements were performed on almost two hundred samples of

38 molluscs, echinoderms and fish. The results have shown a quantification frequency greater than
39 66% for naphthalene and fluorene, at concentrations below a few ng g^{-1} of dry matter of fishery
40 products. With this methodology, the light PAHs occurrence can now be measured in a wider
41 range of foodstuffs in order to better characterize their contamination trends and the associated
42 risk simultaneously.

43
44

45 Keywords :

46 Light Polycyclic Aromatic Hydrocarbons (PAHs); Dynamic HeadSpace (DHS); Thermo
47 Desorption (TDU); GC-MS/MS; isotope dilution; seafood

48
49 Highlights :

- 50 • A DHS-TDU-GC-MS/MS method was developed to quantify light PAHs in seafood
- 51 • Two methods are now available, one is more accurate and the second more robust
- 52 • Validation was performed to determine measurement uncertainties at $[1-20] \text{ ng g}^{-1} \text{ dw}$
- 53 • A first occurrence level was measured in around 200 seafood samples

54

55 **1. Introduction**

56 Polycyclic Aromatic Hydrocarbons (PAHs) are organic compounds constituted of at least two
57 condensed aromatic hydrocarbonated rings. Their natural and anthropic sources, resulting from
58 pyrogenic and petrogenic inputs, lead to their global release into the environment [1-3]. Food
59 processing can also induce PAHs contamination, as in grilled food or in smoked fish [4].
60 Moreover, food from animal origin can be contaminated by bioaccumulation of PAHs all along
61 the food chain, because of their lipophilic properties [5]. PAHs human exposure is of major
62 concern since their toxicity was demonstrated in regard to their carcinogenicity, teratogenicity
63 and mutagenicity [6].

64 The US Environmental Protection Agency first, and then the European Food Safety Agency,
65 have determined priority lists which are more focused on the heaviest PAHs [7], because they
66 represent greater harmful effects [8]. Therefore, the PAHs exposure assessment studies found in
67 literature more often reported occurrence of « heavy PAHs » with at least four benzene rings [9].
68 However, light PAHs, made up of 2 or 3 condensed rings (cf. Fig. 1), are also of scientific
69 concern. Actually, naphthalene (NAP) is classified 2B whereas acenaphthene (ACE), fluorene
70 (FLU), phenanthrene (PHE) and anthracene (ANT) are classified 3 in the International Agency
71 for Research on Cancer (IARC) classification [10]. Their occurrence in the total environment
72 was already reported thanks to the US EPA list which includes these compounds. Obviously,

73 their human exposure is more correlated to air contamination because of their volatility,
74 particularly naphthalene, fluorene and phenanthrene [11]. Nevertheless, food remains a
75 significant way of Human exposure. A Spain dietary exposure study conducted in 2006 has
76 reported the prevalence of light PAHs compared to heavy PAHs in food [12] . Human
77 biomonitoring studies have also confirmed the relevance to monitor these light PAHs. Actually,
78 Thai *et al.* [13] found significant amount above the tens of $\mu\text{g L}^{-1}$ of OH-naphthalene in urine in
79 the Australian population. This biomarker was present at concentrations ten times higher than
80 those observed for the other metabolites of light PAHs.

81 Multiresidue methods have been developed in the last two decades to determine and monitor
82 PAHs exposure according to the regulation, mainly using GC-MS/MS analysis [14, 15] to
83 discriminate isomers. In most cases, the sample preparation included Pressurized Liquid
84 Extraction (PLE) [16] or Soxhlet extraction [17] to maximize recovery yields before purification.
85 However, these extraction steps are critical for light PAHs. On the one hand, losses of light
86 PAHs were demonstrated during solvent evaporation. On the other hand, naphthalene
87 contamination remains an important issue in the laboratory because of its ubiquitous behaviour.
88 PLE instruments but also evaporators and indoor air could contribute to increase the
89 environmental contamination of naphthalene. Many different sample preparation methods have
90 been proposed in the literature [18], among which SPME has been suggested as an interesting
91 alternative to monitor light PAHs . They described mixtures of DVB, Carbon and PDMS
92 adsorbents to achieve PAHs extraction from vegetable oil [19, 22], milk [20], fish [21] or
93 smoked food [23]. The sample preparation was carried out in a closed headspace vial where the
94 external contamination was kept under control. Moreover, no additional evaporation step was
95 required and the light PAHs were preserved on the SPME fiber before analysis. However, the
96 enrichment capability of SPME is limited to its stationary phase quantity and to the partition
97 coefficient of PAHs between the gaseous and the stationary phases during the extraction step.
98 SPME fibers are also known as brittle and could present carry-over issues. More recently,
99 headspace mode of liquid phase microextraction (HS-SDME) was described as easy to
100 implement with automation ability for PAHs extraction from water and environmental samples
101 [24, 25]. However, the partition coefficient between liquid and gaseous phase could also limit the
102 enrichment capability and, thus, the sensitivity of the method.

103 Therefore, to shift the balance of PAH partitioning from the sample to the gaseous phase,
104 Dynamic HeadSpace (DHS) extraction can be performed by continuously sweeping the
105 headspace of the sample with a significant quantity of gas. Then, the extracting gas is loaded on
106 a selective adsorbent where analytes are trapped. Thermodesorption of analytes from the trap is
107 then required before cryofocalisation and GC-MS analysis. This approach has already been used

108 for volatile compounds determination in ham [26] based on the work of Barcarolo and Casson
109 [27], and also for wine studies [28, 29], for olive oil characterisation [30] or for biomonitoring
110 studies such as chlorinated contaminants determination in human urine [31]. As a result,
111 sensitivity was significantly improved in comparison to static headspace techniques. Moreover,
112 the authors have validated their DHS-TDU-GC/MS methods and have demonstrated a promising
113 intermediate precision for this kind of non-targeted approach [29, 30].

114 The aim of our study was to assess the DHS-TD-GC/MS/MS approach for light PAHs
115 quantification in seafood which is quite challenging because of the complexity and diversity of
116 such matrix. The final goal of our work is to cover the whole PAHs list, monitoring the four
117 lightest PAHs with this new approach and the other ones with the conventional method
118 published by Veyrand et al. in 2007 [14] in parallel.

119

120 **2. Experimental**

121

122 *2.1 Reagents and material*

123 Toluene of picograde quality was obtained from LGC (Wesel, Germany). The native compounds
124 (Naphthalene ; Acenaphthylene (ACY) ; Acenaphthene ; Fluorene ; Phenanthrene ; Anthracene ;
125 Fluoranthene ; Pyrene ; Benz[a]anthracene ; Chrysene ; Benzo[b]fluoranthene ;
126 Benzo[k]fluoranthene ; Benzo[a]pyrene ; Indeno[1,2,3-cd]pyrene ; Benzo[ghi]perylene ;
127 Dibenz[a,h]anthracene) and the perdeuterated internal standard compounds (Naphthalene-d₈ ;
128 Acenaphthylene-d₈ ; Acenaphthene-d₁₀ ; Fluorene-d₁₀ ; Phenanthrene-d₁₀ ; Anthracene-d₁₀ ;
129 Fluoranthene-d₁₀ ; Pyrene-d₁₀ ; Benz[a]anthracene-d₁₂ ; Chrysene-d₁₂ ; Benzo[b]fluoranthene-
130 d₁₂ ; Benzo[k]fluoranthene-d₁₂ ; Benzo[a]pyrene-d₁₂ ; Indeno[1,2,3-cd]pyrene-d₁₂ ;
131 Benzo[ghi]perylene-d₁₂ ; Dibenz[a,h]anthracene-d₁₄) were purchased from Dr Ehrenstorfer
132 (Augsburg, Germany). The concentration of these commercial mixtures was 100 ng μL⁻¹ in
133 toluene. Successive dilutions by ten or twenty were prepared in toluene at 10, 1 and 0.1 ng μL⁻¹
134 for the native compounds and at 10, 1 and 0.05 ng μL⁻¹ for the labelled ones. Mixtures of native
135 and labelled compounds were prepared in toluene at a constant concentration of 50 pg μL⁻¹ for
136 labelled compounds and 5, 10, 25, 50, 100, 250, 500, 1000 and 2500 pg μL⁻¹ for native ones.

137 For Dynamic HeadSpace extraction, 20 mL headspace vials with screw caps were purchased
138 from Gerstel (Mülheim, Germany).

139 Samples of mollusc, echinoderm, algae oil and fish were collected in 2017 and 2018 by the
140 French chemical monitoring network (ROCCH) managed by the French Marine Science
141 Research Institute along the French coasts, and by the departmental civilian population
142 protection services as part of the French control plan.

143

144 2.2. *Sample pretreatment*

145 To avoid the risk of contamination, samples were prepared in an air-conditioned room away
146 from solvent vapor. After freeze-drying, each sample was weighed in order to determine its
147 water content. One gram of the ground dried sample was introduced in a 20 mL headspace vial
148 and spiked with 20 μL of a labelled internal standard solution at 50 $\text{pg } \mu\text{L}^{-1}$. A few samples of
149 algae oil (expanded scope of seafood) were analyzed using the same principle associated to
150 standard addition method. To this end, four vials were prepared with 1 g of the same oil sample.
151 Twenty microliters of the labelled internal standard solution at 50 $\text{pg } \mu\text{L}^{-1}$ were added into the
152 first vial, whereas the three other ones received 20 μL of solutions at 50, 250 or 1000 $\text{pg } \mu\text{L}^{-1}$ of
153 native PAHs and systematically 50 $\text{pg } \mu\text{L}^{-1}$ of labelled ones. Simultaneously, a method blank
154 was prepared introducing only 20 μL of a labelled internal standard solution at 50 $\text{pg } \mu\text{L}^{-1}$, at the
155 bottom of the headspace vial. For all the dried matrices, 20 μL of a labelled internal standard
156 solution at 50 $\text{pg } \mu\text{L}^{-1}$ were loaded on the matrix. Each vial was then screwed thoroughly to
157 avoid any losses of the headspace phase during the extraction step. Before placing the vials into
158 the autosampler, they were gently shaken manually without reversing the vial, in order to
159 enhance the spiking solution impregnation and the homogeneity of the sample.

160

161 2.3. *On-line Dynamic Headspace extraction*

162 Firstly, to prevent any issue on the automatic dynamic headspace instrument, several precautions
163 were implemented in our lab. Each headspace septum was pierced only once. The dry purge
164 septum cap was changed each sequence or everyday if the sequence was longer, to keep a good
165 tightness and to keep the required flow rate stable.

166 Then, two DHS methods were developed in this work, called Method 1 and Method 2.

167 Method 1: The vial was incubated during 10 minutes at 100°C under stirring (500 rpm) before
168 extraction. The sample was then maintained at 100°C during extraction without stirring. To
169 achieve analyte extraction, 500 mL of nitrogen were used as sweep gas at 75 mL min^{-1} . The
170 Tenax TA trap and the DHS transfer line were set at 25°C and 150°C during the extraction step
171 respectively. The Tenax TA trap was then slightly heated at 50°C and dried with 1 L of nitrogen
172 at 50 mL min^{-1} before thermodesorption.

173 Method 2: The vial was incubated during 10 minutes at 90°C under stirring (500 rpm) before
174 extraction. The sample was then maintained at 90°C during extraction without stirring. To
175 achieve analyte extraction, 2 L of nitrogen were used as sweep gas at 100 mL min^{-1} . The Tenax
176 TA trap and the DHS transfer line were set at 25°C and 150°C during the extraction step

177 respectively. The Tenax TA trap was then slightly heated at 50°C and dried with 2 L of nitrogen
178 at 100 mL min⁻¹ before thermodesorption.

179

180 *2.4. Thermodesorption injection*

181 The trap was thermodesorbed in the TDU programmed from 30°C (0.1 min) up to 300°C (5 min)
182 at 60°C min⁻¹. The TDU transfer line was set at 300°C and analytes were refocused in the CIS
183 equipped with a baffle liner and cooled at -25°C by a chiller. GC injection was performed
184 thanks to a fast heating of the CIS up to 300°C (5 min) at 12°C s⁻¹.

185

186 *2.5. DHS-TDU-GC-MS/MS*

187 A robotic arm MultiPurpose Sampler (MPS) was equipped with a DHS system [32] and a
188 ThermoDesorption Unit (TDU) coupled to a Cooled Injection System (CIS), all from Gerstel
189 company (Mülheim, Germany) [33]. The GC-MS/MS instrument consisted in a gas
190 chromatograph Agilent 7890 Series coupled to a triple quadrupole analyzer Agilent 7000
191 operating in electron ionization (70 eV). Gas chromatography was performed on a non-polar
192 column DB-5MS (30 m × 0.25 mm, 0.25 µm) purchased from Agilent (Santa Clara, CA USA).
193 Helium was used as carrier gas at a constant flow rate of 1 ml min⁻¹. The gradient of temperature
194 was 90°C (2 min), 10°C min⁻¹ to 240°C (0 min) and 40°C min⁻¹ to 320°C (10 min). The GC
195 transfer line was maintained at 300°C. Temperature of the source was kept at 230°C. A mix of
196 Nitrogen and Helium was used as collision gas. Two transitions per PAH were selected (cf.
197 Table 1 and Fig.S1).

198

199 *2.6 Identification*

200 The following requirements have to be fulfilled before light PAHs quantification [34]: presence
201 of internal standard (S/N > 3), acceptable relative retention time of the analyte (tolerance of
202 ±0.5%), detection of the two diagnostic transitions (S/N > 3), acceptable intensity ratio of the
203 two diagnostic transitions (tolerance from 20 to 50% depending on the ratio).

204

205 *2.7 Quantification*

206 The quantification was based on the isotope dilution approach. Each native compound amount
207 was determined taking into account each corresponding labelled compound as internal standard.
208 The calibration was performed using a similar matrix spiked at 10 different levels for native
209 compounds including 0 and constant level for labelled ones (cf. Fig. S2a), as described in the
210 “reagents and material” part. The calibration used an unweighted method and each calibration
211 point was injected once, at the beginning of each sequence. As calibration curves were built with

212 a pool of similar matrix, only slopes were used to determine concentrations. Using the standard
213 addition method, the calibration curves being built with the sample matrix, amounts were
214 calculated dividing intercepts by slopes (cf. Fig. S2b). Method blanks were quantified with a
215 calibration without matrix.

216

217 *2.8 Validation procedure*

218 Each validation was based on three series of four-level supplemented matrix assays (pool of
219 dried mussels) at 0, 1, 5 and 20 ng for native compounds, resulting in n=6, 10, 6 and 6 samples
220 respectively. A reference material coming from an inter-laboratory test (mussels, IAEA-432) was
221 included once in each series. Each performance criterion was assessed at each level and
222 averaged. We classically used the following definitions: the limit of detection (LOD)
223 corresponds to amounts for which the first transition presents a signal-to-noise ratio (S/N) of 3,
224 and limit of quantification (LOQ) corresponds to amounts for which the second transition
225 presents a S/N of 3 [35]. The linearity was assessed on 10 calibration levels at 0 ng and from 0.1
226 to 50 ng. Absolute recovery rates were determined on labelled compounds (at constant amount of
227 spiking) using matrix and matrixless tests.

228

229 **3. Results and discussion**

230 *3.1 Sample preparation*

231 **3.1.1. Solvent addition and sensitivity**

232 PAH quantification requires the addition of native standard solutions in calibration samples and
233 internal standard solution in all the samples. Thus, toluene (solvent used for standard dilutions)
234 was added in all samples. This solvent can become a competitor against the target analytes on the
235 adsorbent during the extraction. Then, trapping recoveries of analytes can be affected by the
236 solvent volume added in the sample. In our study, toluene could saturate the adsorbent because
237 of its volatility at 90 or 100°C (sample temperatures during extraction). In order to determine the
238 more adapted volume for standard spiking, the first tests were performed without any matrix.
239 Standard solution volumes of 20, 50 and 100 µL containing the same quantity of PAHs were
240 introduced into headspace vials. We found that the less solvent volume there was, the more
241 intense the signal to noise ratio was for the light PAHs (factor 200 to 7 from naphthalene to
242 pyrene, data not shown). The solvent of the spiking solution, toluene, is also volatile. Thus, there
243 is a competition between toluene and light PAHs, about volatility but above all adsorption on the
244 trap. This explain why, for an equal amount of light PAHs, responses (areas and S/N ratios)
245 increase when the solvent volume decreases. The lowest volume was chosen but we did not try
246 under a 20 µL volume to ensure a convenient use and a satisfactory pipetting uncertainty.

247 Therefore, each spiking level, of native and labelled compounds, requires using one spiking
248 solution, in order to ensure that the same volume of 20 μL is added. Thus, we prepared ten
249 different solutions: one containing only labelled compounds to spike samples and blanks, and
250 nine solutions containing labelled compounds at the same concentration and native ones at
251 various concentrations.

252 **3.1.2. Elimination of water traces**

253 After freeze-drying, the homogenized dried sample was kept at room temperature until the
254 analysis. Dried samples were kept in the freezer for several weeks and samples were slightly
255 rehydrated because of the relative humidity in the closed chamber. Then, traces of moisture led
256 to a plugging, located in the CIS at -25°C , by ice formation. To avoid this critical point, we now
257 recommend to perform an additional frozen cycle when dried samples are not analyzed directly
258 after drying. Moreover, we advise to limit the dried samples preservation time after freeze-drying
259 to one week at room temperature to avoid any moisture re-capture. Furthermore, we did not
260 observe any light PAH amounts loss during storage at room temperature. Freeze-drying yields
261 were studied (cf. Fig. S3) by comparing responses of the four lightest labelled PAHs with or
262 without freeze-drying. Results showed that there is no impact on PAHs from 3 cycles, whereas a
263 2 cycles PAH (NAP) freeze-drying induced some losses. We have to keep this weakness in mind
264 for NAP.

265 **3.1.3. Sample size optimization**

266 Several tests with different sample weights (0.25, 0.5, 1 and 2 g) were assessed. Areas of
267 chromatographic peaks were not importantly different between sample sizes (cf. Fig. 2a).
268 However, a higher variability occurred for a sample size of 2 g, probably correlated to the first
269 observable matrix effects. Considering the relatively low impact of the sample weight, 1 g of
270 dried matrix was finally set in the method, to ensure a better representative sample weight and to
271 facilitate the conversion between amount and concentration simultaneously.

272

273 *3.2 On-line Dynamic Headspace optimization*

274 **3.2.1. Nature of the adsorbent**

275 The nature of the trap adsorbent was assessed on Tenax TA, Tenax GR and Carbopack B/X,
276 after Method 1 extraction on a mussel sample spiked at 20 ng g^{-1} . Tenax TA proved to be an
277 efficient option compared to Tenax GR and Carbopack B/X, particularly for the four lightest
278 PAHs (cf Fig. 2b). Despite a better repeatability of PAH recoveries with the Tenax GR, Tenax
279 TA gave the highest responses, with a gain of 30% for acenaphthene.

280 **3.2.2. Incubation parameters**

281 The incubation allows reaching the equilibrium point of PAH partition between the solid phase
282 (the sample) and the gas phase (headspace). This step remains crucial to lead an efficient

283 headspace extraction afterwards. To reach this equilibrium, we have played on incubation
284 temperature, from 60 to 120°C with an incubation time of 10 min (cf Fig. 2c and 2d), and
285 incubation time, i.e. 10 and 20 min with an incubation temperature of 100°C (cf. Fig. 2e). First
286 of all, Method 1 was applied on a spiked mussel (20 ng g⁻¹) at four incubation temperatures from
287 90 to 120°C by step of 10°C (cf. Fig. 2c). The best responses were obtained at 100°C for the four
288 lightest PAHs, 90°C also being acceptable regarding intensities and standard deviation
289 overlapped with results obtained at 100°C. The experiment was then reproduced on incurred
290 mussel sample, in order to observe the PAH behaviour in a naturally contaminated matrix (cf.
291 Fig 2d). In this experiment, Method 2 was used. The results confirmed the efficient incubation
292 temperature of 90°C for NAP, ACY and ACE, while 100°C showing that preferable for the
293 heaviest PAHs. Incubation temperatures below 90°C were also assessed as they could prevent
294 potential water traces into the headspace, but PAH extraction efficiencies became drastically low
295 from 80°C, with a loss of 50% of FLU compared to 100°C. Therefore, Method 1 was set with an
296 incubation temperature of 100°C, whereas Method 2, elaborated to minimize water residue, used
297 an incubation temperature of 90°C. Finally, incubation time above 10 min was not required as
298 shown in Fig. 2e. This shortest time was chosen to minimize the time of analysis.

299 **3.2.3. Trapping parameters**

300 To improve PAH recoveries, we have investigated the parameters to reach an efficient purge of
301 the headspace to move the balance between solid and gas phases, without exceeding the
302 breakthrough volume of the trap. A preliminary experiment was led according to Method 1
303 without any repetition on a spiked mussel matrix (20 ng g⁻¹) to assess trapping flows between 25
304 and 75 mL min⁻¹ and trapping volumes between 0.25 and 0.75 L (cf. Fig. 2f). The parameters
305 kept in Method 1 were the last tested, i.e. swept sample headspace by a 0.5 L of nitrogen at a
306 75 mL min⁻¹. As the incubation temperature of Method 1 was set at 100 °C, additional nitrogen
307 was not allowed without capturing water traces from sensitive samples (cf. 3.1.2). However, we
308 investigated using more sweeping gas with Method 2 where the incubation temperature was set
309 at only 90°C. This time, the experiment was led on an incurred sample (n=3) with trapping flows
310 between 50 and 100 mL min⁻¹ and trapping volumes between 0.5 and 2 L (cf. Fig. 2g). PAH
311 enrichment was proportional to the volume of nitrogen used. We choose to use 2 L of nitrogen at
312 100 mL min⁻¹ flow to prevent any water traces, without exceeding the breakthrough volume of
313 the trap.

314 **3.2.4. Dry purge parameters**

315 Nitrogen volume and flow rate for the dry purge were set according to the Gerstel
316 recommendations in Method 1 (1 L at 50 mL min⁻¹) [32]. To optimize the drying of the trap, we
317 increased the drying gas volume up to 4 L at flow rates up to 100 mL min⁻¹ (cf. Fig. 2h), without

318 reaching the breakthrough volume. Nevertheless, a 2 L dry purge volume at a 100 mL min⁻¹ dry
319 purge flow rate was chosen to gain time.

320 **3.2.5. General consideration on DHS optimization**

321 The DHS optimization was finally a compromise between the highest possible responses for at
322 least the four lightest PAHs without reaching the breakthrough volume, including the lowest
323 moisture content and in the shortest analysis time. Two slightly different combinations of
324 parameters were finally chosen, i.e. Method 1 and Method 2, with complete validation for each
325 one. In summary, Method 2 was chosen to provide a better robustness without the risk of
326 plugging the GC injector with ice, whereas a few samples followed an additional freeze-drying
327 for correct use of the Method 1.

328

329 *3.3 Thermo Desorption injection*

330 TDU and CIS initial temperatures were optimized to improve the chromatographic peak shape,
331 mainly for Naphthalene which is the lightest PAH. Several tests with different initial
332 temperatures of TDU (from 20 to 40°C) and CIS (from -32°C to -20°C) were assessed (cf. Fig.
333 S4). The lower these temperatures are, the better the shape of the peak is. The optimization gave
334 better results for an initial temperature of the CIS at -32°C. However, this temperature was too
335 long to reach for routine purposes. A refocusing temperature of -25°C in the CIS was then
336 chosen (results shown in Fig S1). In parallel, the initial TDU temperature less impacts the
337 chromatographic peak shape than those of the CIS. Then, initial TDU temperature of 30°C was
338 set to keep a reasonable cooling delay. Under these conditions, the peak shape of NAP is still
339 improvable. Additional focusing could be achieved by applying a starting oven temperature
340 below 90 °C. However, these lower temperatures were not evaluated in this study. Indeed, this
341 peak shape was considered acceptable. In addition, we did not want to lose more time during GC
342 cooling.

343 Finally, we have optimized the throughput of the analysis. As the bottleneck resulted in the
344 number of traps used, we assessed the intermediate precision using two different traps containing
345 the same adsorbant, i.e. Tenax TA. No significant variability was observed when an incurred
346 mussel sample was analysed with these two different traps (cf. Fig. S5). Therefore, during a
347 sequence, the alternative use of two traps saved time and kept the analysis duration to a
348 minimum, with a total overlap of the next DHS cycle (incubation, trapping and dry purge) with
349 the last GC-MS/MS analysis.

350

351 *3.4 MS/MS optimisation*

352 The mass spectrometry method was developed for light PAHs according to the same principle as
353 described by Veyrand et al. [14] for heaviest ones. Briefly, the acquisition of the signals was
354 ensured in selected reaction monitoring (SRM) following the transitions involving the loss of one
355 $[M-H]^+$ or two hydrogen atoms $[M-H_2]^+$ from the molecular ion $[M]^+$, or one or two deuterium
356 atoms for the labeled compounds. Under high energy voltage applied in the collision cell,
357 another specific daughter ion was $[M-C_2H_2]^+$.

358

359 *3.5 Performances*

360 Results of validations are summarized in Tables 3 and 4. In the light PAHs specific case, the
361 limiting factor was contamination measured by the method blank which represents the
362 environmental contamination level during the batch analysis and which is above S/N of 3.
363 Therefore, LOD were not of interest. LOQ were set at 1 ng in the vial to keep a degree of
364 confidence according to the maximum content of PAH measured in method blanks at 0.67 ng.
365 This amount of 1 ng is also the low level of supplemented matrix assays. These limits are fully
366 compatible with the amount range potentially met in seafood [12].

367 Regarding linearity performances, determination coefficients (R^2) were better than 0.98 for all
368 analytes, except for phenanthrene in two calibration curves of the second validation.

369 Absolute recovery rates were determined on labelled compounds (at constant amount of spiking)
370 using matrix and matrixless tests. Indeed, absolute recovery rates in matrix samples on the basis
371 of Dynamic headspace extraction is the ratio of the response of a compound in a matrix test on
372 the response of the same compound in a matrixless test. Results are shown in Table 2. Absolute
373 recovery rates in mussel ground dried samples were between [9-45] % and [13-62] % for method
374 Method 1 and method Method 2 respectively. These results were expected since the principle of
375 extraction is based on the balance between the volatility and the adsorption of the compounds in
376 the matrix. Absolute recoveries are classically below 10% with headspace method, and increase
377 with Dynamic headspace method due to enrichment. In spite of weak values for a few
378 compounds, accurate calculated amounts were guaranteed for native compounds by the use of
379 isotope dilution method with labelled compounds corresponding to each native compound. The
380 isotope dilution method allows to automatically correct the amounts in each sample whatever the
381 matrix. Moreover, the GC-MS/MS sensitivity has compensated some low recovery rates to keep
382 satisfactory response levels. The intermediate precision was below 15% for most of the light
383 PAHs, except for naphthalene, fluorene and phenanthrene in the second validation. The bias
384 values on the supplemented matrix were roughly sufficient, with better results at higher levels of

385 spiking and for Method 1. Quantification in the reference material was within the target range
386 (from 1.5 to 27 ng g⁻¹) when given for most light PAHs, except for fluorene in the second
387 validation (cf. Table 4). Indeed, we assume that an unexpected contamination could occur in the
388 sample without any detection in the blank sample. The result is then an extreme bias value.
389 Taking into account all uncertainties contributions, including the uncertainty component
390 associated to the concentrations of the standard solutions (3.2/3.2/3.3% at low/middle/high
391 level), the expanded measurement uncertainties, with a coverage factor of 2 (confidence level of
392 95%), were ranging from 8 to 73% for Method 1, depending on the quantified analytes and the
393 level. Method 2 gave higher uncertainties, particularly for fluorene. We assume that a kind of
394 unexpected contamination occurs frequently for PHE and FLU. Indeed, their intermediate
395 precision error and associated bias were relatively high, leading to extremely high-expanded
396 uncertainties, particularly when method 2 is used. The next stage is now to monitor this
397 suspected contamination to treat its sources. For the next batches of analysis, a recommendation
398 could be to use Method 2 in routine analysis to prevent any ice formation in the CIS and to use
399 Method 1 for confirmatory purposes, particularly for fluorene quantification, in order to increase
400 the intermediate precision of measurement.

401

402 *3.6 Light PAHs Occurrence*

403 Analyses were performed with Method 1 on almost two hundred samples of molluscs,
404 echinoderms and fishes. The aim of this work was to assess the methodology and to estimate the
405 order of magnitude of light PAHs amounts in seafood simultaneously. The four lightest PAHs
406 amounts were reported in Fig. 3, taking a value equal to zero when the compound was not
407 quantified (lowerbound). These results have highlighted a quantification frequency greater than
408 66% for naphthalene and fluorene at amounts of up to 8 ng g⁻¹ of dry matter.

409

410 **Conclusions**

411 The work carried out has led to develop two methods to determine light PAHs both in the
412 mollusc matrix and in other matrices such as oils, then proceeding by standard addition method.
413 The developed methods were validated for the lightest PAHs: NAP, ACY, ACE, FLU but also
414 PHE and ANT. Analytical performances matched with the levels of contamination observed in
415 seafood. In addition, the first analyses produced with this method show the interest of continuing
416 to monitor these parameters in seafood and more generally on a wide range of foodstuffs in order
417 to better characterize the associated risk to light PAHs dietary exposure.

418

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422

423

424

425 **References**

426

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531

532 **Figure Captions**

533 Fig. 1. Chemical structure of the light PAHs

534 Fig. 2. (a) PAHs response in matrix spiked at 1 ng regardless of the sample weight (n = 2 ; SD in
535 brackets); (b) PAHs response in matrix spiked at 20 ng in relation to adsorbent types (n = 3 ; SD
536 in brackets); PAHs response (c) in matrix spiked at 20 ng and (d) in incurred mussel in relation
537 to incubation temperature (n = 2(c) and n=3(d) ; SD in brackets); (e) PAHs response in matrix
538 spiked at 20 ng in relation to incubation times (n = 2 ; SD in brackets); (f) PAHs response in
539 matrix spiked at 20 ng in relation to trapping volume and flow rates; (g) PAHs response in
540 incurred mussel sample in relation to trapping volume and trapping flow(n = 3 ; SD in brackets);
541 (h) PAHs response in matrix spiked at 2 ng in relation to drying purge volume and flow rate (n =
542 3 ; SD in brackets)

543 Fig. 3. Lowerbound occurrences in ng g⁻¹ of dry matter by matrix type

544 Fig. S1. Extracted ion chromatograms from (a) standard solution at 50/1 ng native/labelled, (b)
545 pool of matrix spiked with 0/1 ng native/labelled, (c) pool of matrix spiked with 1/1 ng
546 native/labelled and (d) pool of matrix spiked with 50/1 ng native/labelled

547 Fig. S2a. Calibration curves with direct calibration method on a pool of seafood matrices

548 Fig. S2b. Calibration curves with standard addition method in an algae oil sample

549 Fig. S3. Response of the lightest labelled PAHs with or without freeze-drying step (SD in
550 brackets)

551 Fig. S4. Naphthalene peak shapes in relation to initial temperatures of TDU and CIS, from 20 to
552 40 °C and from -32 to -20 °C respectively

553 Fig. S5. Response of incurred matrix in relation of the trap used (n = 3 ; SD in brackets)

554

555 **Table list**

556 Table 1: Monitored transitions, indicative ratios and retention times of light PAHs. (IS : Internal
557 Standard ; A : Analyte ; RT : retention time.)

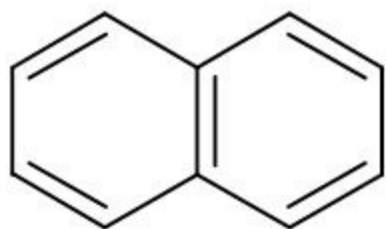
558 Table 2 : Absolute recovery rates of labelled light PAHs (determined at constant amount using
559 matrix and matrixless tests).

560 Table 3: Performances of light PAHs for methods 1 and 2. Uncertainties are given for each level
561 of concentration and average uncertainty is indicated in bold (Low level (1 ng)/Middle level (5
562 ng)/High level (20 ng)/Average)

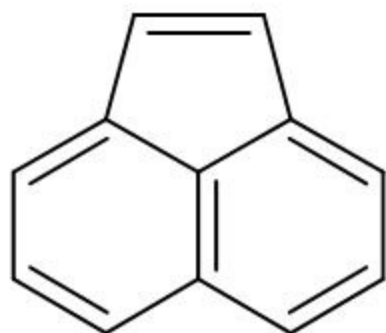
563 Table 4: quantified values vs. reference values in RM IAEA-432. (✓ : in the target range ; ✗ :
564 out the target range.)

565

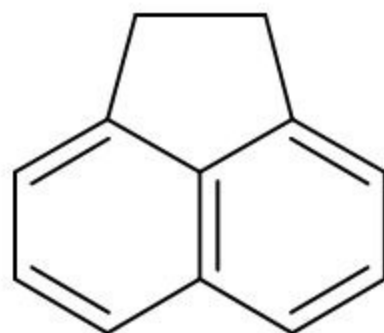
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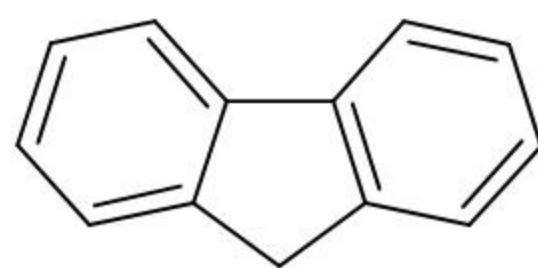
Naphthalene
(NAP)



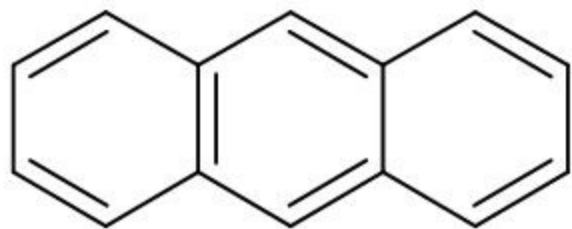
Acenaphthylene
(ACY)



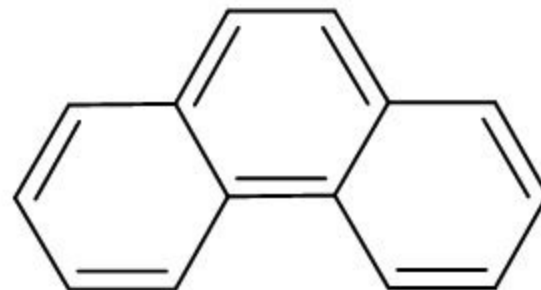
Acenaphthene
(ACE)



Fluorene
(FLU)



Phenanthrene
(PHE)



Anthracene
(ANT)

Fig. 2a.

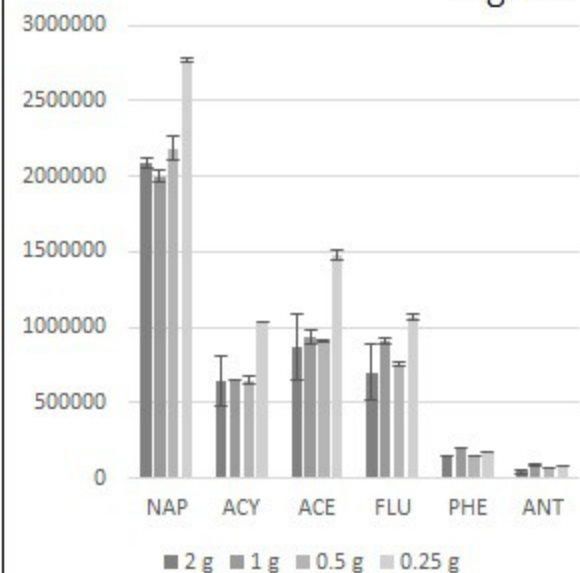


Fig. 2b.

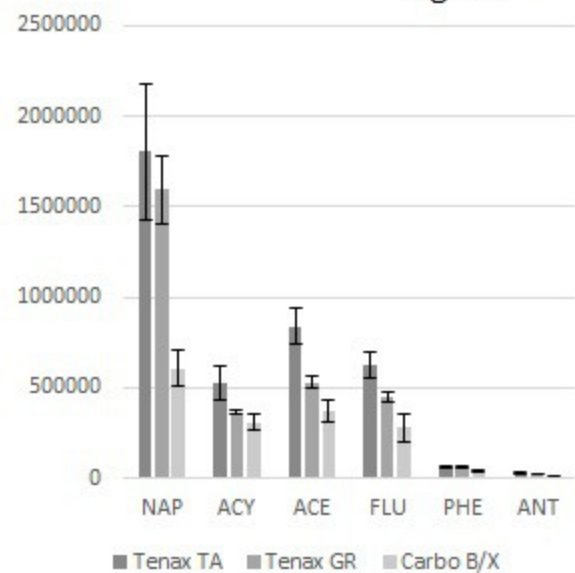


Fig. 2c.

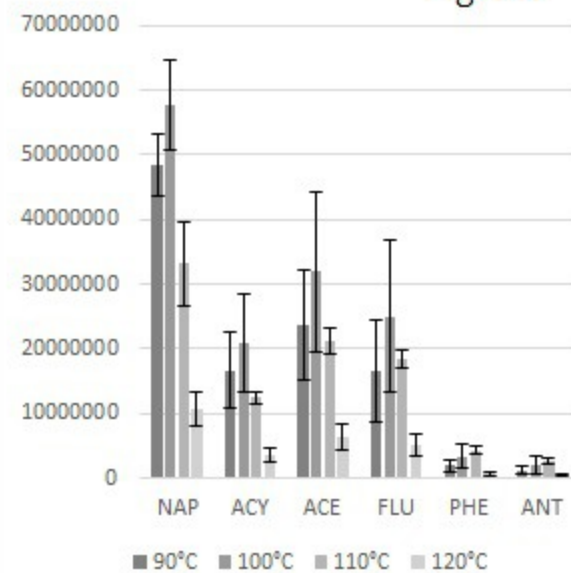


Fig. 2g.

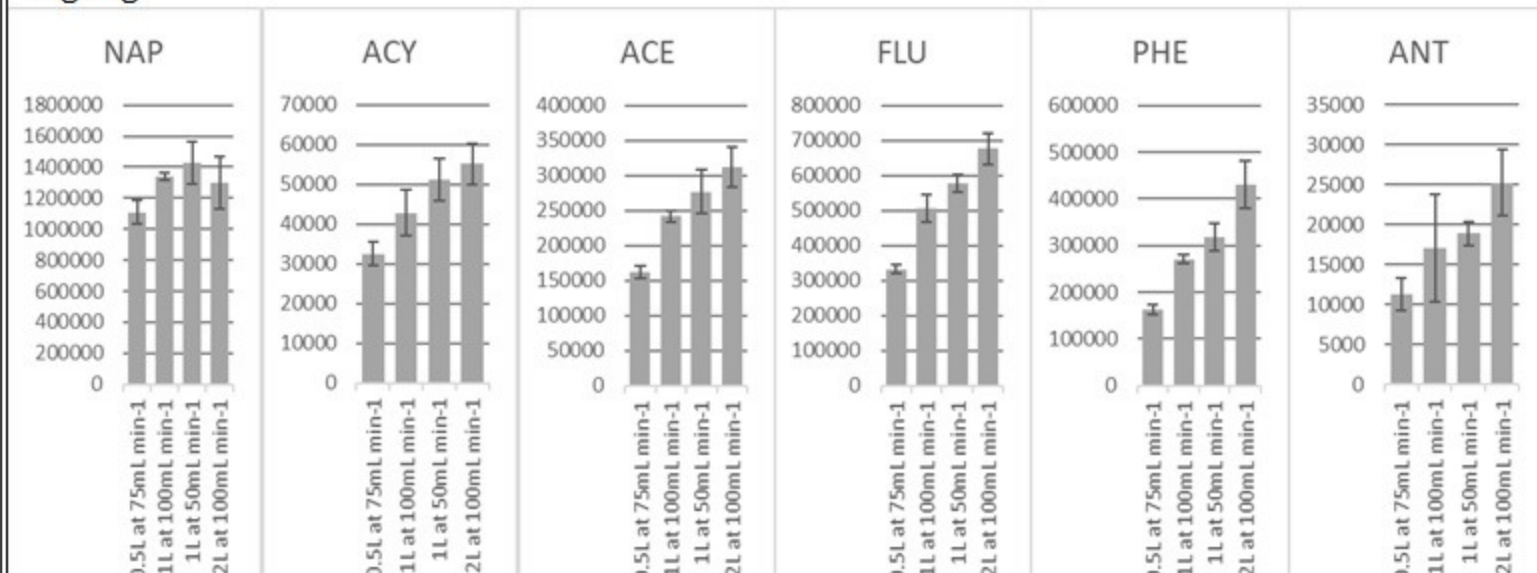


Fig. 2d.

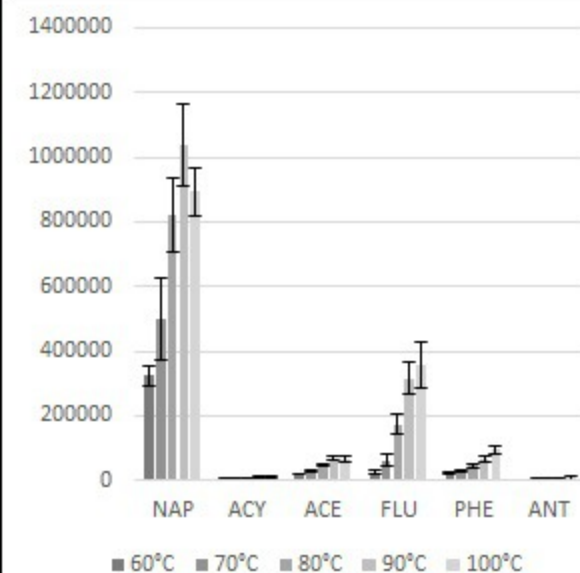


Fig. 2e.

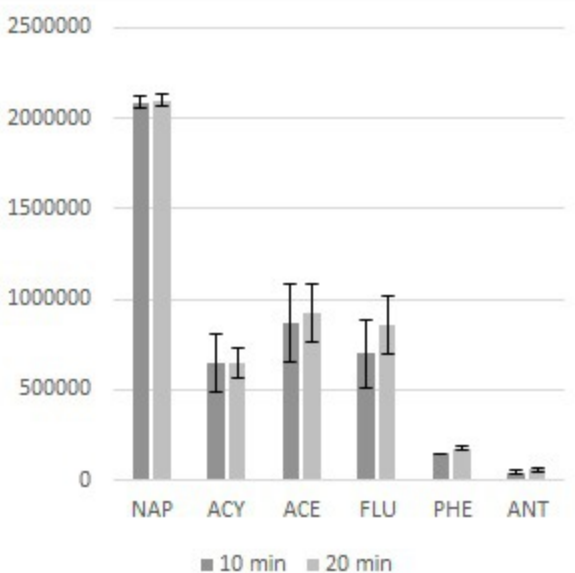


Fig. 2f.

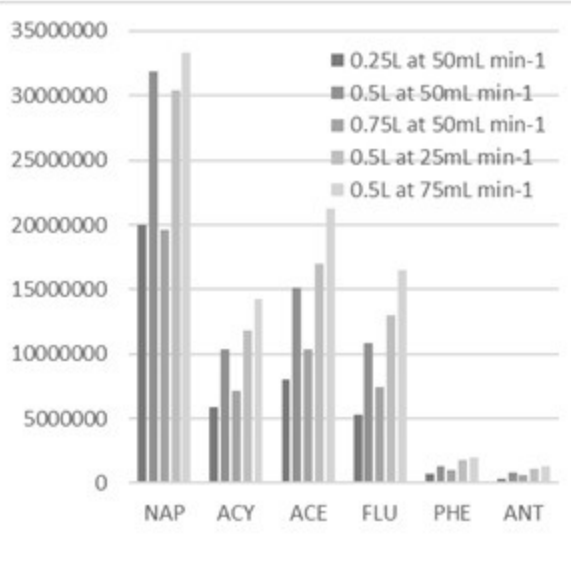
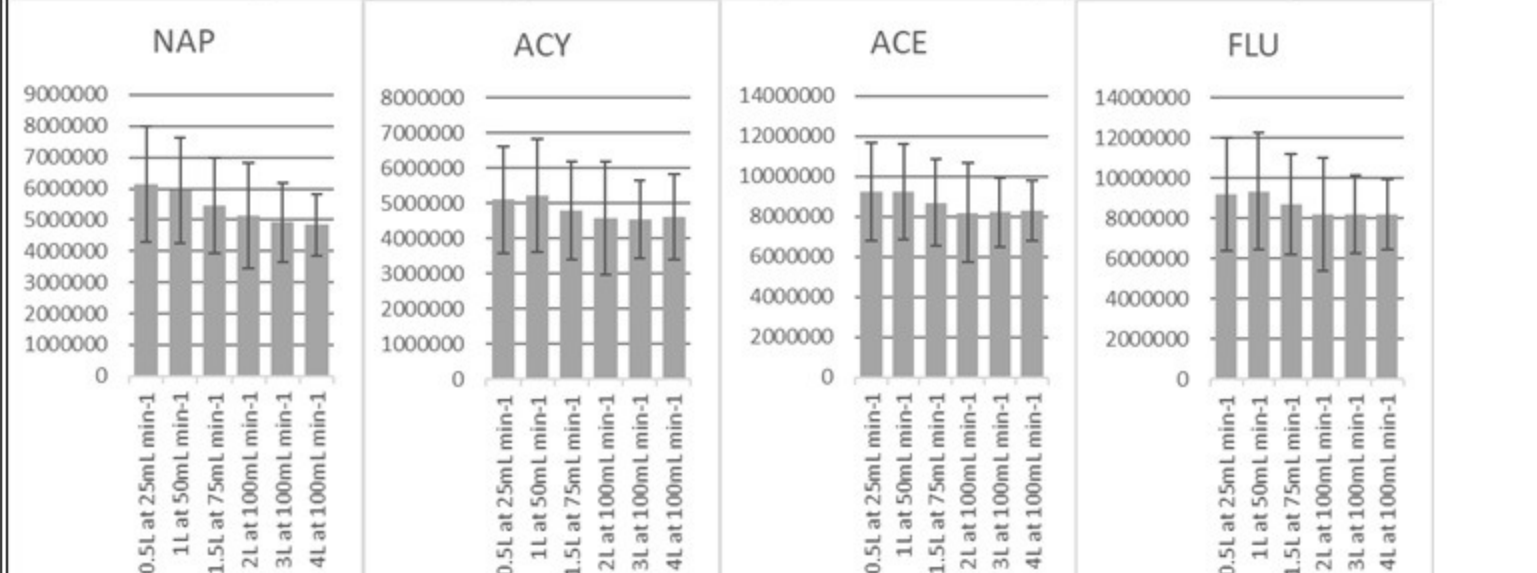


Fig. 2h.



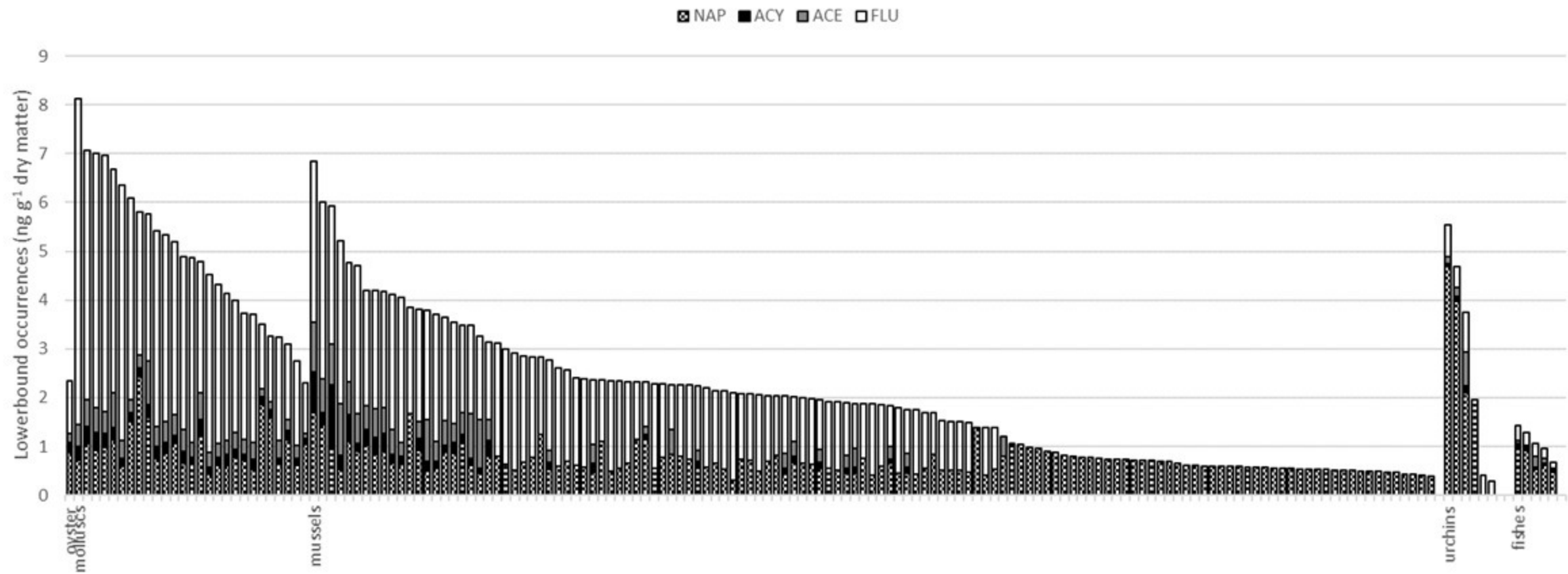


Table 1: Monitored transitions, indicative ratios and retention times of light PAHs. (IS : Internal Standard ; A : Analyte ; RT : retention time.)

Compounds	Type	Transition 1	Collision T1 (eV)	Transition 2	Collision T2 (eV)	Indicative Ratio (T2/T1)	Indicative RT (min)	Window
Naphthalene-d ₈	IS	136..1>108..1	25	136..1>134..1	20		6..40	1
Naphthalene	A	128..1>102..0	25	128..1>127..1	20	0.473	6..43	
Acenaphthylene-d ₈	IS	160..1>158..1	25	160..1>132..1	30		9..83	2
Acenaphthylene	A	152..1>151..1	25	152..1>126..0	30	0.439	9..86	
Acenaphthene-d ₁₀	IS	164..1>162..1	20	164..1>160..1	40		10..17	3
Acenaphthene	A	154..1>153..1	20	154..1>152..1	40	0.553	10..24	
Fluorene-d ₁₀	IS	176..1>174..1	25	176..1>172..1	40		11..42	4
Fluorene	A	166..1>165..1	25	166..1>164..1	40	0.189	11..48	
Phenanthrene-d ₁₀	IS	188..1>160..1	30	188..1>184..1	35		13..72	5
Phenanthrene	A	178..1>152..1	30	178..1>176..1	35	1.106	13..76	
Anthracene-d ₁₀	IS	188..1>160..1	30	188..1>184..1	35		13..86	
Anthracene	A	178..1>152..1	30	178..1>176..1	35	1.305	13..91	

Table 2 : Absolute recovery rates of labelled light PAHs (determined at constant amount using matrix and matrixless tests).

Compounds	Absolute recovery rate	Absolute recovery rate
	with Method 1 parameters validation	with Method 2 parameters validation
Naphthalene-d ₈	45%	13%
Acenaphthylene-d ₈	30%	62%
Acenaphthene-d ₁₀	37%	53%
Fluorene-d ₁₀	25%	41%
Phenanthrene-d ₁₀	9%	21%
Anthracene-d ₁₀	11%	36%

Table 3: Performances of light PAHs for Methods 1 and 2. Uncertainties are given for each level of concentration and average uncertainty is indicated in bold (Low level (1 ng)/Middle level (5 ng)/High level (20 ng)/Average)

Compounds	LOQ	Linearity R ²	Intermediate precision RSD (%)	Bias (%)	Expanded measurement uncertainty U k=2 (%)
	method blank (ng g ⁻¹ dw)				
Method 1					
Naphthalene	0.17	1.000	2.9/2.0/2.9/ 2.6	4.0/0.3/-2.2/ 0.7	9.9/7.5/9.1/ 8.8
Acenaphthylene	0.38	1.000	16.0/6.5/5.1/ 9.2	12.9/5.9/-1.3/ 5.8	36.0/15.9/12.3/ 21.4
Acenaphthene	0.43	0.999	2.0/1.9/3.9/ 2.6	10.5/2.8/-2.2/ 3.7	14.3/8.0/10.6/ 11.0
Fluorene	0.64	0.999	8.5/2.9/2.5/ 4.6	6.1/2.4/-2.6/ 1.9	19.5/9.0/8.8/ 12.4
Phenanthrene	0.60	0.990	21.7/16.5/5.9/ 14.7	-50.6/-3.3/-5.9/ -19.9	73.1/33.8/15.2/ 40.7
Anthracene	0.20	0.998	10.9/6.2/4.9/ 7.3	7.5/-2.0/-8.2/ -0.9	24.3/14.1/15.2/ 17.9
Method 2					
Naphthalene	0.40	0.997	26.9/20.4/7.3/ 18.2	-30.9/-6.5/-16.3/ -17.9	65.0/41.9/24.6/ 43.8
Acenaphthylene	0.01	1.000	3.6/2.8/3.3/ 3.2	-2.8/1.5/1.4/ 0.0	10.1/8.6/9.4/ 9.4
Acenaphthene	0.01	0.999	10.5/9.6/5.0/ 8.3	-5.9/6.3/5.7/ 2.1	22.9/21.4/13.7/ 19.3
Fluorene	0.02	0.995	27.6/35.6/43.7/ 35.6	59.1/38.6/67.6/ 55.1	88.0/84.2/117.4/ 96.5
Phenanthrene	0.05	0.896	99.2/71.1/79.5/ 83.3	-38.8/16.2/17.0/ -1.9	203.6/143.5/160.4/ 169.2
Anthracene	0.04	0.996	13.5/12.4/4.2/ 10.0	-22.1/6.2/6.1/ -3.3	37.7/26.6/12.8/ 25.7

Table 4: quantified values vs. reference values in RM IAEA-432. (✓ : in the target range ; ✖ : out the target range.)

Compounds	Reference values (ng g ⁻¹)	Amounts	Amounts	Bias	Bias
		with Method 1 parameters validation (ng g ⁻¹)	with Method 2 parameters validation (ng g ⁻¹)	with Method 1 parameters validation (%)	with Method 2 parameters validation (%)
Naphthalene	15 ± 18	13 ✓	7 ✓	-11	-53
Acenaphthylene	-	1.8	0.3	-	-
Acenaphthene	-	2.5	0.7	-	-
Fluorene	4.1 ± 2.2	3.9 ✓	26.2 ✖	-5	539
Phenanthrene	27 ± 21	16 ✓	8 ✓	-39	-69
Anthracene	1.5 ± 1.1	1.1 ✓	1.0 ✓	-25	-36