Gas chromatography-mass spectrometry determination of polybrominated diphenyl ethers in complex fatty matrices from aquaculture activities.

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Abstract

Gas chromatography coupled to mass spectrometry in negative chemical ionization (NCI) mode has been applied to the quantification and reliable identification of polybrominated diphenyl ethers (PBDEs) in animal and vegetable samples from aquaculture activities. Matrices analyzed included fish fillet, fish feed, fish oil and linseed oil, which their fat content ranged from 5 to 100%. Solid phase extraction (using Florisil and silica cartridges) and normal-phase high performance liquid chromatography were tested for an efficient clean-up in order to obtain sample extracts free of interfering compounds. Combining sulphuric acid digestion and solid-phase extraction with Florisil led to the highest efficiency in the elimination of interferences from the extracts. The sample procedure developed, together with the application of GC-(NCI)MS for measurement, led to the satisfactory determination of PBDEs at $\mu\text{g Kg}^{-1}$ levels in complex aquaculture matrices with high lipid content. The use of a short and thin film-thickness fused-silica capillary column allowed to determine the problematic BDE 209 with satisfactory results. Three m/z ions were acquired for each analyte, which ensured a reliable identification of compounds detected in samples.

Keywords: polybrominated diphenyl ethers; fatty samples; gas chromatography, mass spectrometry, clean up, aquaculture matrices.

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31 **1. Introduction**

32 Polybrominated diphenyl ethers (PBDEs) are anthropogenic chemicals that are added to a
33 wide variety of consumer/commercial products in order to improve their fire resistance. For
34 this reason, PBDEs are commonly named as Brominated Flame Retardants (BFRs) due to
35 their capability in reducing fire in a widespread list of products. BFR's frequent employment
36 is to reduce successfully deaths related to the fire, injuries and property damage [1]. This fact,
37 together with their hydrophobic properties, contribute to the presence and bioaccumulation of
38 BFRs in environmental samples [2]. Analytical studies on BFRs have been focused on
39 environmental samples [3], as air, sewage sludge, sediments, soils and water, and on biota for
40 fish derivatives [4], and they are normally found in biological tissues at $\mu\text{g Kg}^{-1}$ levels [5,6].

41 Marine aquaculture has experienced strong development in the last decades as a consequence
42 of increased fish consumption by the world population and decreasing wild stocks. Fish used
43 as raw material for manufacture of fish feed ingredients (fish oils and meals) are a potential
44 source of PBDEs in fish feed, which can be bioaccumulated by aquatic species [4,7]. PBDEs
45 determination in samples and resources from aquaculture is important in order to establish the
46 contamination origin, and for a dietary exposure assessment and for protection of public
47 health [4,8,9].

48 The determination of PBDEs in marine matrices is difficult due to the low analyte levels
49 normally present and to the presence of interfering compounds, mainly fats, which are co-
50 extracted with the analytes. This makes necessary an efficiency clean-up before instrumental
51 measurement. Extraction of PBDEs in these samples is normally carried out using Soxhlet or
52 sonication [10,11]. The purification of fatty marine extracts is crucial to get satisfactory
53 analytical data and to reach the sensitivity required. An efficient clean-up can be performed

54 using gel permeation chromatography or solid phase extraction (SPE), as well as by acid
55 treatment or the use of acidified silica for lipid removal [12].

56 As regards analytical measurement, gas chromatography coupled to mass spectrometry is
57 normally the preferred technique for PBDEs, although liquid chromatography coupled to
58 mass spectrometry under different ionization modes has been also tested [1,13]. GC with
59 electron capture detector has been applied, but it has important limitations in selectivity
60 because the detection and identification is based only on retention time and matrix
61 interferences can appear [14]. Some authors have reported PBDEs measurements in
62 environmental analysis by GC-MS under single ion monitoring mode (SIM) [15-17]. In the
63 light of data reported, one of the most sensitive approaches seems to be high resolution GC
64 with negative chemical ionization (NCI) coupled to a single quadrupole MS in SIM mode,
65 while electron impact ionization may improve the selectivity thanks to the most abundant
66 fragmentation. NCI-MS may be less specific than EI-MS due to the Br⁻ ions formation, which
67 can be confused from interfering compounds in complex samples [14]. However, GC-
68 (NCI)MS increases sensitivity and accuracy in the determination of BDE 209, the most
69 difficult BDE from an analytical point of view, due to his particular behaviour on degradation
70 and sensitivity [18].

71 Analytical methodologies reported for the determination of PBDEs in marine fatty samples
72 are normally focused on tri, tetra, penta and hexa PBDEs congeners. Thus, BDE-47 (tetra-
73 BDE), 99 and 100 (penta-BDEs), have been usually detected in this kind of samples [19-21].
74 High brominated compounds with seven or more bromine atoms have proven to be more
75 difficult to be determined correctly. Thus, high PBDEs, such BDE-209, are not often included
76 in PBDE studies [22,23], possibly due to their tend to degradation in injectors and during gas
77 chromatographic separation process [21]. Nevertheless, most of commercial mixtures of
78 PBDEs reference standards also contain high-brominated congeners, as they are relevant as a

79 consequence of their higher lipophilic character and consequently their higher trend to be
80 bioaccumulated in fatty animal tissues.

81 As regards the determination of PBDEs in fish feed and their ingredients, Fajar et al. (2008)
82 [24] proposed a methodology for the determination of seven PBDEs in fish feed and
83 molluscs, at $\mu\text{g Kg}^{-1}$ levels. However, fish and vegetal oils used in the fish feed manufacture
84 and farmed fish are normally scarcely investigated in the bibliography.

85 In this work, we have developed a sensitive method for the reliable quantification and
86 identification of 12 PBDEs (including tri, tetra, penta, hexa, hepta PBDEs congeners and the
87 deca congener BDE-209), in aquaculture samples with fat contents ranging from 5 to 100 %.
88 The method is based on the use of gas chromatography coupled to mass spectrometry working
89 in negative chemical ionization mode and single ion recording (SIR). In order to obtain
90 extracts with very low content of fats and other interference compounds, the efficiency of SPE
91 and of normal-phase HPLC as clean-up techniques has been studied after applying a first
92 clean-up step by sulphuric acid digestion. The developed methodology has been applied to
93 real-world samples of fish feed, vegetable and fish oils, and fish fillets from feeding trials
94 with gilthead sea bream (*Sparus aurata L.*) as a part of the European Union project
95 “Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for consumers”
96 (AQUAMAX), Contract number: 016249-2. The acquisition of three selective m/z ions for
97 each analyte has allowed quantification and reliable identification of PBDEs, at low $\mu\text{g Kg}^{-1}$
98 level, in the different fatty samples analyzed.

99

100 **2. Experimental.**

101 **2.1. Materials and reagents.**

Standards of PBDEs, (50 mg L⁻¹ in isooctane) were obtained from Chiron As (Trondheim, Norway) with purity up to 99.5 %: triBDE (BDE28), tetraBDEs (BDE71, BDE47, BDE66), pentaBDEs (BDE100, BDE99, BDE85), hexaBDEs (BDE154, BDE153, BDE138), heptaBDE (BDE183) and decaBDE (BDE209). A standard mixture, which contained the 12 PBDEs (5 mg L⁻¹) was prepared by dilution of individual reference standard solutions with n-hexane, and it was stored in a freezer at -20 °C.

Working solutions of 12 PBDEs were prepared by diluting the standard mixture solution with n-hexane, and they were used for sample fortification and for preparation of calibration curves.

4,4-DDE D₈ from Dr Ehrenstorfer (Promochem, Wesel, Germany) was used as surrogate internal standard (IS). Working solutions (100µg L⁻¹ and 250µg L⁻¹) were prepared by dilution of the stock solution (100mg L⁻¹) with n-hexane and were stored at 4°C.

Ethyl acetate and n-hexane (ultratrace quality) were purchased from Scharlab (Barcelona, Spain). Sulphuric acid 95–98% was purchased from Scharlab. Anhydrous sodium sulfate of pesticide residue quality (Scharlab) was dried for 18 h at 300°C before use. Silica cartridges (1g) (Strata, Phenomenex, USA) and Supelclean LC-Florisil SPE tube (1g) (Sigma-Aldrich, Madrid, Spain) were used in SPE experiments.

119

2.2. Sample material.

Gilthead sea bream (*Sparus aurata* L.) specimens of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were cultured at the Instituto de Acuicultura de Torre la Sal, Spain (IATS, CSIC) and collected when fish accomplished commercial size (≈500g). The left-side fillets (denuded from skin and bone) were excised and stored at -20 °C until analysis.

125 Fish feed supplied to sea bream during feeding trials were stored at -20°C until analysis. Fish
126 oil and linseed oil used in fish feed manufacture, were also stored at -20°C until analysis.
127 Ingredients and approximate composition of fish feed are shown in **Table 1**.
128

129 **2.3. NPLC clean-up procedure.**

130 A normal-phase liquid chromatography (NPLC) clean up system previously applied by
131 Serrano et al. [25] was tested to remove the interferences in the sample extracts. The system
132 consisted on an LC Pump Master 305 (Gilson), two VICI Valco six-way high pressure valves
133 (Europe instruments, Schenk, Switzerland), a Rheodyne sampler injector valve (Colati,
134 CA) with 1mL loop, a Novapack silica column (150mm x 3.9 mm i.d., 4µm particle size;
135 Waters, Mildford, MA), and a Gilson FC 203B fraction collector. The mobile phase was
136 hexane at a flow rate of 1mL min⁻¹.

137 Firstly, 1-mL LC fractions after injecting PBDE standards were collected in order to know
138 their elution patterns in the LC clean-up. Results showed that all PBDEs eluted in the
139 fractions between 2-8 minutes. Therefore, the whole fraction eluting from 2 min. to 8 min.
140 was collected after injecting sample extracts and adjusted to 0.25 mL by evaporation under N₂
141 stream at 40°C. The overall procedure was controlled from the LC pump, programming the
142 times of valve activation automatically.

143

144 **2.4. SPE clean-up procedure.**

145 SPE was also tested as clean-up procedure for fatty samples using Florisil and silica
146 cartridges. 1-mL of PBDEs mix standard solution was loaded into the cartridge, previously
147 conditioned with 6mL of n-Hexane. Elution was carried with n-hexane and individual
148 fractions of 1mL were collected and analyzed by GC-MS. PBDEs eluted in the first 8mL

149 using Florisil cartridges. In the case of silica SPE cartridges, 10mL n-hexane were necessary
150 for elution of all 12 PBDEs. The whole eluate, containing all analytes, was evaporated under a
151 gentle nitrogen stream at 40°C, and the final residue was dissolved in 0.25 mL n-hexane in
152 both cases.

153

154 **2.5. GC instrumentation**

155 A GC system (Agilent 6890N; Agilent Palo Alto, USA) equipped with an autosampler
156 (Agilent 7683) was coupled to a triple quadrupole (QqQ) mass spectrometer (Quattro Micro
157 GC; Micromass, Boston, USA) operating in negative chemical ionization (NCI). The GC
158 separation was performed using a fused silica DB-1HT capillary column with a length of 15
159 m, an internal diameter of 0.25 mm and a film thickness of 0.1 μm (J&W Scientific, Folsom,
160 CA, USA). The injector temperature was set to 260°C. Splitless injections of 1 μL were
161 carried out. Helium (99.999%; Praxair, Spain) was used as carrier gas at a flow rate of 1 mL
162 min^{-1} . The source temperature was set to 200°C and a solvent delay of 3 min was selected.
163 QqQ system operated in Single Ion Recording (SIR) mode. Methane (99.9995%; Praxair,
164 Spain) was used as reagent gas with an optimal flow of 60%. Experimental conditions
165 selected for GC-(NCI)MS measurement are shown in **Table 2**. The oven temperature program
166 was as follows: 120°C (1 min); 10°C min^{-1} to 220°C; 20°C min^{-1} to 300°C (3min), 40°C min^{-1}
167 to 340°C (3min). Helium was used as carrier gas at 1 mL min^{-1} . A dwell time per channel
168 between 0.05 and 0.1 s was chosen. The application manager TargetLynx was used to process
169 the quantitative data obtained from calibration standards and samples.

170

171 **2.6. Recommended procedure.**

172 Before analysis, samples were thawed at room temperature and carefully ground using a mill
173 Super JS (Moulinex, France). Approximately 16 g of fish fillet or fish feed were homogenized
174 with the amount of anhydrous sodium sulphate necessary to remove water, and the blend was
175 spiked with 800 μ L of surrogate IS solution (250 μ g L⁻¹ 4,4-DDE-D₈). Extraction was
176 performed by refluxing in n-hexane for 4 hours at 80°C. After filtration (0.45 μ m), the extract
177 was pre-concentrated using a Kuderna-Danish apparatus until ca. 8 mL (2 g sample per mL n-
178 hexane).

179 A sulphuric acid digestion [25] was firstly applied to 1ml of the hexanic extract, in order to
180 remove most of the fats. In the case of oils, 0.5 g of fish oil or linseed oil were mixed with
181 1.5mL of n-hexane and were directly digested. Digestion was carried by adding 2mL of conc.
182 H₂SO₄ and shaking 1 min in a Vortex. The hexanic layer was collected, and the remaining
183 sulphuric phase was washed with 1mL hexane twice. Finally, the around 3mL cleaned-up
184 hexanic phase was adjusted to 1mL at 40°C under a gentle N₂ stream.

185 The 1-ml extract resulting from sulphuric acid digestion was passed through a 1 g Florisil
186 SPE cartridge, previously conditioned with 6 mL of n-hexane, and it was eluted with 8 mL n-
187 hexane. The eluate was evaporated under a gentle nitrogen stream at 40°C and the final
188 residue was reconstituted in 0.25 mL of n-hexane.

189 The final cleaned-up extracts obtained were injected into the Quattro Micro GC system
190 working in NCI (MS) mode under the experimental conditions shown in **Table 2**.
191 Quantification of samples was carried out using calibration with standards in solvent, using
192 4,4-DDE D₈ as surrogate internal standard for all congeners.

193

194 **2.7. Validation**

195 Validation of the method developed was performed by evaluating the following parameters:

196 – Linearity. The calibration curves were obtained by injecting reference standard solutions in
197 n-hexane by duplicate. The concentration range tested was 0.5–50 $\mu\text{g L}^{-1}$ (seven points) for all
198 congeners. Linearity was assumed when the regression coefficient was greater than 0.99 with
199 residuals randomly distributed and being lower than 30%.

200 – Accuracy. It was evaluated by means of recovery experiments, analyzing “blank” reference
201 samples (n=3) of each matrix investigated, which were spiked at two concentration levels:
202 0.25 $\mu\text{g Kg}^{-1}$ and 2.5 $\mu\text{g Kg}^{-1}$ (1.25 $\mu\text{g Kg}^{-1}$ and 12.5 $\mu\text{g Kg}^{-1}$ for BDE209) in fish fillet and
203 fish feed, and 1 $\mu\text{g Kg}^{-1}$ and 10 $\mu\text{g Kg}^{-1}$ (5 and 50 $\mu\text{g Kg}^{-1}$ for BDE209) in fish and linseed
204 oils. Previously, several samples were analyzed by triplicate to determine the concentration of
205 the analytes in the different sample matrices. Data showed that BDE 28 and BDE 47 were the
206 most frequently detected in fish fillet and fish feed, while in oils PBDEs were always below
207 the limits of quantification. From these analysis, we selected the “blank” samples with the
208 lowest levels of PBDEs, in such a way that PBDEs concentrations in the four blanks were
209 lower than the limits of quantification.

210 – Precision. The precision, expressed as the repeatability of the method, was determined in
211 terms of relative standard deviation (RSD, %) from recovery experiments at each fortification
212 level.

213 – Limit of quantification (LOQ) objective. It was established, from the quantification ion, as
214 the lowest concentration that was validated in spiked samples following the overall procedure
215 with satisfactory recovery (between 80-120%) and precision (RSD<20%) (0.25 $\mu\text{g Kg}^{-1}$ for
216 fish fillet and fish feed and 1 $\mu\text{g Kg}^{-1}$ for fish oil and linseed oil).

217 – Limit of detection (LOD). It was statistically estimated, from the quantification ion, as the
218 analyte giving a peak signal of three times the background noise from the sample
219 chromatograms at the lowest fortification level tested.

220 – Confirmation criteria: The Q/q ratio, defined as the intensity ratio between the
221 quantification ion (Q) and the confirmation ion (q), was used to confirm the identity of the
222 compounds detected in samples. Confirmation criteria was based on the European
223 Commission Decision (2002/657/CE) [26]. Briefly, to confirm a finding as an actual positive,
224 a maximum ratio tolerance $\pm 20\%$ was accepted when the relative intensity of the confirmative
225 transition was $>50\%$ as regards the quantitative one (Q/q ratio 1-2). For higher Q/q ratios, the
226 tolerances increased. Thus, maximum deviations of $\pm 25\%$ (relative intensity 20-50%, Q/q
227 ratio 2-5), $\pm 30\%$ (relative intensity 10-20%, Q/q ratio 5-10) and $\pm 50\%$ (relative intensity $\leq$
228 10%, Q/q ratio >10) were accepted. This criterion was originally defined on measures to
229 monitor certain substances and residues thereof in live animals and animal products, and it is
230 being increasingly used in other fields like environmental and biological samples analysis
231 [27-30]. Obviously, the agreement in the retention time in sample and reference standard was
232 also required to confirm a positive finding. The theoretical average Q/q ratios for each
233 compound were obtained as the mean value calculated from seven standard solutions injected
234 twice.

235

236 **3. Results and discussion.**

237 **3.1. Comparison between NPLC and SPE clean-up procedures**

238 As a consequence of the high lipid content and complexity of the matrices studied, it was
239 necessary a first clean-up of the extracts by means of acid digestion using conc. sulphuric
240 acid, which allowed us to remove around 90% of lipids. In spite of this, an amount of lipids

241 and other interfering compounds still remained in extracts making necessary a second clean-
242 up step to improve sensitivity and selectivity. The application of NPLC or SPE was efficient
243 to reduce interferences, and allowed us to achieve the low LODs pursued in this research.

244 NPLC and SPE (Florisil and silica) were tested in order to know the improvements of the
245 method in terms of LODs, LOQs, recoveries and matrix effects in the four sample matrices
246 fortified at 0.25 $\mu\text{g Kg}^{-1}$ (fish fillet and fish feed) or at 1 $\mu\text{g Kg}^{-1}$ (fish oil and linseed oil).
247 BDE 209 fortification level was 1.25 $\mu\text{g Kg}^{-1}$ and 5 $\mu\text{g Kg}^{-1}$, respectively.

248 Similar LODs were obtained with the three clean-up approaches, except for BDE 209 where
249 the lowest LODs were reached using Florisil SPE cartridges. **Figure 1** shows a comparison
250 between BDE 209 chromatograms obtained when applying different clean-up procedures to
251 fish fillet, fish feed, linseed and fish oil samples at the lowest fortified level (1.25 $\mu\text{g Kg}^{-1}$ for
252 fish fillet and fish feed, 5 $\mu\text{g Kg}^{-1}$ for oils). As expected, oil matrices presented more
253 interferences. The highest sensitivity and lower LODs were achieved when using Florisil in
254 all matrices. LODs after applying sulphuric acid treatment and Florisil SPE clean-up ranged
255 for the eleven selected PBDEs (in $\mu\text{g Kg}^{-1}$) from 0.03-0.10 (fish fillet), 0.03-0.19 (fish feed),
256 0.06-0.25 (fish oil) and 0.05-0.20 (linseed oil). LOD's for BDE 209 varied from 0.70 (fish
257 fillet and fish feed) to 0.90 $\mu\text{g Kg}^{-1}$ (fish feed and fish oil).

258 The analytical methodology developed presents high selectivity and sensitivity as
259 consequence of the efficient clean-up procedure applied, and the instrumental determination
260 by GC-(NCI)MS, reaching low LODs for all PBDEs selected in the four matrices studied.

261 In addition, recoveries of PBDEs using SPE clean-up with Florisil were mostly better than
262 those obtained after SPE-silica or NPLC clean-up (see **Table 3**). In some particular cases,
263 recoveries slightly higher than 120% were achieved suggesting a poor connection when using
264 DDE-D₈ as surrogate, specially after silica SPE and NPLC clean-up. However, after Florisil

265 SPE clean-up, recoveries at the four fortification levels tested in the four sample matrices
266 were typically between 80-120% with RSD lower than 15% with the exception of the lowest
267 level ($0.25\mu\text{g Kg}^{-1}$) in fish fillet, where RSD's around 25% were observed for several
268 compounds (**Table 3**).

269 **Figure 2 and 3** show illustrative GC-(NCI)MS chromatograms at the lowest level validated
270 for the sample matrices studied after applying the overall recommended analytical procedure.

271 **Figure 2** shows chromatograms for tri-BDE (28) and tetra-BDEs (47, 66, 71) and **figure 3**
272 shows chromatograms for penta-BDEs (85, 99, 100), hexa-BDEs (138, 153, 154), hepta-BDE
273 (183) and deca-BDE (209). As it can be seen, satisfactory sensitivity was obtained at the LOQ
274 level when using the quantification ion (Q) and the first confirmation ion (q_1), while the lower
275 abundance of the second confirmation ion (q_2) led to a notable decrease in sensitivity. In spite
276 of this, q_2 was also acquired to help in the confirmation of the identity of positive findings.

277

278 3.2. GC-(NCI)MS optimization

279 GC coupled to mass spectrometry is, in most cases, the preferred technique for the analysis of
280 organic pollutants in environmental samples [3,31]. In addition, the QqQ mass analyzer
281 working in tandem MS/MS mode provides high selectivity and sensitivity in the analysis of
282 complex environmental samples, which is an evident advantage in environmental analysis.
283 The use of two stages of mass analysis in MS/MS systems based on QqQ analyzers offers the
284 possibility of applying selected reaction monitoring (SRM), one of the most selective and
285 sensitive approaches. In spite of this, there are still some compounds that can not be
286 determined with enough sensitivity with this technique. Thus, the development of MS/MS
287 method in NCI mode for PBDEs was not feasible because of the poor MS spectra, which only
288 showed the clusters from the fragments $[\text{Br}]^-$ and $[\text{HBr}_2]^-$ [5]. The molecular cluster was not

289 observed or only constituted a minor peak. Therefore, the unique transition reasonable for the
290 majority of compounds would have been the fragmentation of $[\text{HBr}_2]^-$ to give a bromine
291 atom, with low sensitivity and poor selectivity. Consequently, a SIR method was optimized
292 by monitoring the three most intense peaks of the mass spectra, which corresponded to m/z 79
293 ($[\text{}^{79}\text{Br}]^-$), m/z 81 ($[\text{}^{81}\text{Br}]^-$), m/z 161 ($[\text{H}^{79}\text{Br}^{81}\text{Br}]^-$) and m/z 487 ($[\text{C}_6\text{O}^{79}\text{Br}_3^{81}\text{Br}_2]^-$) for BDE 209.

294 Chromatographic conditions for PBDEs in GC-MS were optimized by injecting standard
295 solutions in solvent. We studied the temperature program in order to achieve satisfactory
296 resolution and peak shape for the internal standard and 12 PBDEs, including the deca-
297 polybrominated diphenyl ether (BDE 209). A shorter and thin capillary column was used to
298 prevent the degradation of PBDEs due to the strong influence of this parameter on accuracy
299 and precision of the analysis. The overall procedure was tested for the analytical
300 characteristics shown in the validation section, obtaining satisfactory results.

301 The coelution of PBDE congeners under certain chromatographic conditions has been
302 reported. Thus, BDE 49 and BDE 71 are close-eluting on 100% Dimethylpolysiloxane GC
303 capillary column and might coelute in our DB-1HT column. Covaci et al. (2006) [12] have
304 performed a detailed study of PBDEs chromatographic behavior using different GC capillary
305 columns, but they reported no coelution of the two mentioned congeners. We did not test this
306 fact as BDE 49 was not studied in our work.

307 NCI ionization in SIR acquisition mode was applied to monitoring bromine ions: $[\text{}^{79}\text{Br}]^-$,
308 $[\text{}^{81}\text{Br}]^-$ and secondary fragments such $[\text{H}^{79}\text{Br}^{81}\text{Br}]^-$ and $[\text{C}_6\text{O}^{79}\text{Br}_3^{81}\text{Br}_2]^-$. GC-(NCI)MS was
309 proven to notably increase the selectivity, sensitivity and accuracy for PBDEs determination,
310 specially for BDE 209 [12,14]. The $[\text{Br}]^-$ mass fragment was the most intense ion, while the
311 molecular ion was normally missing. $[\text{H}^{79}\text{Br}^{81}\text{Br}]^-$ and $[\text{C}_6\text{O}^{79}\text{Br}_3^{81}\text{Br}_2]^-$ fragments were less
312 abundant than $[\text{Br}]^-$. Thus, $[\text{Br}]^-$ ($m/z=79$) was selected for quantification due to the higher

sensitivity reached, and $[\text{Br}]^-$ ($m/z=81$) was selected as confirmation ion. $[\text{H}^{79}\text{Br}^{81}\text{Br}]^-$ and $[\text{C}_6\text{O}^{79}\text{Br}_3^{81}\text{Br}_2]^-$ were selected for a more reliable confirmation of PBDEs in samples, but they presented some difficulties due to the lower abundance compared with $[\text{Br}]^-$ ions. Due to the notable differences in abundances, we obtained Q/q ratios around one for the relation with 79/81 (Q/q_1), but much higher Q/q ratios for 79/161 and 79/487 (Q/q_2). **Figure 2, 3 and 4** illustrate in fact the limitations for confirmation when using Q/q₂ ratio in samples at low concentration levels. In spite of this, we decided to acquire $m/z=161$ and 487 although in the most of cases the Q/q₂ ratios were higher than 20. Using the second confirmation ion was useful when the analyte was present at higher concentrations. Agreement in Q/q ratios between samples and reference standards is required to confirm positive findings [26]. The agreement in the retention time of a compound in the sample and in reference standard is also required to confirm a positive result.

325

3.3. Application to samples from bioaccumulation experiments

The overall optimized procedure was applied to the analysis (in triplicate) of 20 fish fillet samples, 11 fish feed, 1 fish oil, 1 linseed oil, 1 rapeseed oil and 1 palm oil used in aquaculture experiments. The results obtained are shown in **Table 4**. Most PBDE congeners were below the limit of detection and only a few samples presented positive findings in this study. The most frequently detected congeners were PBDE 28 and PBDE 47. **Figure 4** reveals positive findings of tetra, penta and hexa-PBDEs in fish fillets. Concentration levels found in fish fillets ranged from 0.25 to 4.7 $\mu\text{g Kg}^{-1}$. Some authors have also reported low PBDE levels in wild aquatic species (mud carp and crucian carp), with individual values from 1.3 $\mu\text{g Kg}^{-1}$ to 400 $\mu\text{g Kg}^{-1}$ [4]. Shawn et al. [16] reported mean concentrations of ΣPBDEs in farmed salmon samples from Maine (0.95 $\mu\text{g Kg}^{-1}$ ww, 7.3 $\mu\text{g Kg}^{-1}$ lw) and eastern

337 Canada ($0.85 \mu\text{g Kg}^{-1}$ ww, $6.3 \mu\text{g Kg}^{-1}$ lw) lower than data reported in our paper for fish
338 fillet.

339 PBDEs in fish feed ranged from 0.5 to $2.8 \mu\text{g Kg}^{-1}$, except for one sample that contained
340 BDE 209 at a $15.5 \mu\text{g Kg}^{-1}$ (**Figure 4**). Some authors reported low contamination by PBDEs
341 in aquatic species compared with the samples in our work [16,32]. Dietary intake studies of
342 lower PBDEs have shown that fish and animal products are important vectors of human
343 exposure, but data on higher brominated BDEs are scarce [33].

344 We did not find PBDEs in the oil samples analyzed at levels higher than the LOQ objective (1
345 $\mu\text{g Kg}^{-1}$; $5 \mu\text{g Kg}^{-1}$ BDE 209). This fact agrees with data reported by Domingo et al. (2008)
346 [8], where total load of PBDEs from marine species, oils and fats and other components from
347 the normal diet was low; e.g. the highest PBDE levels found in oils and fats was $0.59 \mu\text{g Kg}^{-1}$.
348 ¹.

349

350 4. Conclusions

351 An efficient and sensitive analytical methodology for the determination of PBDEs in fat
352 aquaculture matrices has been developed using an efficient clean-up step combining acid
353 digestion and SPE Florisil procedure previous to the injection of the extracts in GC-MS
354 system operating in NCI ionization and SIR mode acquisition. The procedure applied allowed
355 to reach LODs as low as 0.03 - $0.06 \mu\text{g Kg}^{-1}$ in samples. The most difficult task in this work
356 was to determine the BDE 209, as it required special chromatographic conditions to reach
357 good peak shape and satisfactory sensitivity. The acquisition of three m/z ions in SIR mode
358 offers a reliable confirmation of the identity of the PBDEs detected in samples. BDE 28 and
359 47 were the most commonly found in fish fillet and fish feed. Individual concentrations were

360 always below $5\mu\text{g Kg}^{-1}$, except for BDE 209 in one fish feed sample that reached up to 15.5
361 $\mu\text{g Kg}^{-1}$.

362

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380 **6. Figure Captions.**

381 **Figure 1.** GC-(NCI)MS chromatograms of BDE 209 for fish fillet, fish feed, linseed and fish
382 oils spiked at the lowest fortification level ($1.25 \mu\text{g Kg}^{-1}$ for fish fillet and fish feed, $5 \mu\text{g Kg}^{-1}$
383 for oils). Comparison of different clean-up procedures (Florisil and silica SPE, and NPLC)
384 applied after sulphuric acid treatment.

385 **Figure 2.** GC-(NCI)MS illustrative chromatograms at $0.25 \mu\text{g Kg}^{-1}$ level (fish fillet and fish
386 feed) following the recommended procedure. “Q” quantification ion, “q₁ and q₂” confirmation
387 ions. Q/q were in agreement with the criteria established.

388 **Figure 3.** GC-(NCI)MS illustrative chromatograms at $1 \mu\text{g Kg}^{-1}$ level (fish and linseed oils;
389 $5 \mu\text{g Kg}^{-1}$ for BDE-209) following the recommended procedure. “Q” quantification ion, “q₁
390 and q₂” confirmation ion. Q/q were in agreement with the criteria established.

391 **Figure 4.** Positive findings of PBDEs in samples of fish fillet, fish feed. Q/q were in
392 agreement with the criteria established.

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402 **7. References.**

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462 **Table 1.** Ingredients and chemical composition of fish feed analyzed.

Ingredient (%)	<i>FO</i>	<i>33VO</i>	<i>66VO</i>
Fish meal (CP 70%) ¹	15	15	15
CPSP 90 ²	5	5	5
Corn gluten	40	40	40
Soybean meal	14.3	14.3	14.3
Extruded wheat	4	4	4
Fish oil ³	15.15	10.15	5.15
Rapeseed oil	0	0.85	1.7
Linseed oil	0	2.9	5.8
Palm oil	0	1.25	2.5
Soya lecithin	1	1	1
Binder	1	1	1
Mineral premix ⁴	1	1	1
Vitamin premix ⁵	1	1	1
CaHPO ₄ ·2H ₂ O (18%P)	2	2	2
L-Lys	0.55	0.55	0.55
Proximate composition			
Dry matter (DM, %)	93.13	92.9	92.77
Protein (% DM)	53.2	52.81	52.62
Fat (% DM)	21.09	21	20.99
Ash (% DM)	6.52	6.69	6.57

463 ¹Fish meal (Scandinavian LT)

464 ²Fish soluble protein concentrate (Sopropêche, France)

465 ³Fish oil (Sopropêche, France)

466 ⁴Supplied the following (mg · kg diet⁻¹, except as noted): calcium carbonate (40% Ca)
467 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric
468 citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen
469 phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese
470 sulphate 30, sodium selenite 0.3.

471 ⁵Supplied the following (mg · kg diet⁻¹): retinyl acetate 2.58, DL-cholecalciferol
472 0.037, DL-α tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5,
473 riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate
474 2.5, vitamin B₁₂ 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline
475 chloride 500.

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480 **Table 2.** Experimental conditions of the optimized GC-(NCI)MS method.

t_R (min)	PBDE	Quantification Ion (Q) (m/z)	Confirmation Ion (q) (m/z)	q	Q/q ₁ ^b	Q/q ₂ ^b	Dwell time (sec)
7.4	4,4'-DDE-D ₈ ^a	289 [C ₁₄ D ₈ ³⁵ Cl ₃] ⁻					0.1
7.9	28	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	1.01(5)	>20	0.05
9.6	71	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	1.01(1)	>20	0.05
9.8	47	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	1.01(1)	>20	0.05
10.1	66	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	1.05(4)	>20	0.05
11.2	100	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	1.02(1)	>20	0.05
11.6	99	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	0.99(7)	>20	0.05
12.1	85	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	0.98(10)	>20	0.05
12.5	154	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	0.99(8)	>20	0.05
12.9	153	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	0.96(13)	>20	0.05
13.3	138	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	0.96(9)	>20	0.05
13.8	183	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 161 [H ⁷⁹ Br ⁸¹ Br] ⁻	q ₁ q ₂	1(7)	>20	0.05
16.5	209	79 [⁷⁹ Br] ⁻	81 [⁸¹ Br] ⁻ 487[C ₆ O ⁷⁹ Br ₃ ⁸¹ Br ₂] ⁻	q ₁ q ₂	0.98(6)	12.85(17)	0.05

481 ^aInternal Standard used as surrogate. ^bAverage value calculated from 2 injections of standard
482 solutions at seven concentration levels.

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Table 3. Recoveries (%) obtained in the analysis of PBDEs in fish fillet, fish feed, fish oil and linseed oil (n=3, at each fortification level) after Florisil SPE clean-up.

<i>Compound</i>	0.25µg kg ⁻¹ ^a		1µg kg ⁻¹ ^b		2.5µg kg ⁻¹ ^c		10µg kg ⁻¹ ^d	
	Fish Fillet	Fish Feed	Fish Oil	Linseed Oil	Fish Fillet	Fish Feed	Fish Oil	Linseed Oil
28	107(28)	92(6)	87(3)	79(11)	104(2)	99(11)	86(24)	108(9)
71	99(25)	95(16)	87(8)	86(13)	102(4)	103(15)	89(16)	87(23)
47	98(7)	80(4)	92(14)	89(19)	102(10)	98(9)	96(7)	108(3)
66	107(19)	103(3)	115(9)	105(7)	98(3)	103(2)	93(12)	112(5)
100	115(25)	104(6)	107(14)	102(18)	101(9)	116(8)	104(10)	119(3)
99	98(4)	119(5)	99(7)	119(14)	103(9)	114(10)	104(8)	117(5)
85	106(10)	115(4)	103(10)	98(16)	95(5)	119(8)	101(5)	116(12)
154	110(5)	117(9)	133(11)	101(10)	101(11)	126(6)	98(6)	111(5)
153	95(26)	108(13)	108(10)	108(3)	100(15)	107(8)	107(7)	118(15)
138	100(25)	102(6)	113(9)	111(3)	102(16)	132(6)	102(11)	119(1)
183	100(21)	110(5)	103(10)	109(2)	75(12)	100(8)	81(9)	99(7)
209	110(16)	84(15)	104(12)	106(9)	80(13)	116(8)	84(8)	109(8)

^a 1.25 µg kg⁻¹ for BDE 209. ^b 5 µg kg⁻¹ for BDE 209. ^c 12.5 µg kg⁻¹ for BDE 209. ^d 50 µg kg⁻¹ for BDE 209.

519 **Table 4.** PBDEs detected in aquaculture samples (n=3).
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BDE Congener	Fish Fillet		Fish Feed	
	Number of positives	conc. range ($\mu\text{g kg}^{-1}$)	Number of positives	conc. range ($\mu\text{g kg}^{-1}$)
28	7	0.25-0.5	4	0.25-0.3
71	3	0.25-0.5	4	0.25-0.8
47	14	0.25-4.7	4	0.25-2.8
66	1	0.6	3	0.25-0.3
100	2	0.4-0.7	4	0.25-0.7
99	2	0.6-0.9	4	0.25-0.9
85	2	0.4-0.7	4	0.25-0.6
154	2	0.4-0.6	4	0.25-0.6
153	2	0.4-0.6	4	0.4-0.9
138	1	0.7	2	0.25-0.3
183	1	0.6	2	0.25-0.3
209	-		1	15.5

521 Total number of samples analyzed: 20 fish fillet, 11 fish feed.
522 Although some PBDEs were detected in the oil samples, their levels
523 were below the limit of quantification.
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Figure 1.

FISH FILLET

FISH FILLET

FISH FEED

FISH FEED

Figure 2.

Figure 3.

Figure 4.