COMPREHENSIVE STRATEGY FOR PESTICIDE RESIDUE ANALYSIS THROUGH THE PRODUCTION CYCLE OF GILTHEAD SEA BREAM AND ATLANTIC SALMON

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ABSTRACT

Plant ingredients and processed animal proteins are alternative feedstuffs for fish feeds in aquaculture. However, their use can introduce contaminants like pesticides that are not previously associated with marine Atlantic salmon and gilthead sea bream farming. This study covers the screening of around 800 pesticides by gas chromatography (GC) and liquid chromatography (LC) coupled to high resolution time-of-flight mass spectrometry in matrices throughout the entire marine food production chain. Prior to analysis of real-world samples, the screening methodology was validated for 252 pesticides to establish the screening detection limit. This was 0.01 mg Kg^{-1} for 113 pesticides (45%), 0.05 mg Kg^{-1} for 73 pesticides (29%) and >0.05 mg Kg^{-1} for 66 pesticides (26%). After that, a quantitative methodology based on GC coupled to tandem mass spectrometry with atmospheric pressure chemical ionization source (GC-APCI-MS/MS) was optimized for the pesticides found in the screening. Although several polar pesticides, of which pirimiphos methyl and chlorpyriphos-methyl were most dominant, were found in plant material and feeds based on these ingredients, none of them were observed in fillets of Atlantic salmon and gilthead sea bream fed on these feeds.

KEYWORDS

Fish, feed, processed animal products, screening, pesticides, quadrupole time-of-flight.

1. INTRODUCTION

The availability of wild fishery-derived raw materials is finite and the rapid and sustained growth rate of global aquaculture have forced the industry to explore alternative and more sustainable feed ingredients (Tacon and Metian, 2013). Much attention has been paid to plant ingredients and experimental evidence supports a successful replacement of marine feedstuffs at relatively high levels in most carnivorous farmed fish, such as Atlantic salmon (*Salmo salar*) and gilthead seabream *(Sparus aurata)* (Benedito-Palos et al., 2016; Ytrestøyl et al., 2015). Processed animal proteins (PAPs) from the rendering industry, re-authorised for use in aquafeeds in the European Union (EU) in 2013 (EC, 2013a), are another valuable source of feed ingredients for farmed marine fish (Hatlen et al., 2015).

The use of these alternative feed ingredients can introduce contaminants that were previously not associated with marine salmon and gilthead sea bream farming. One example are pesticides that are world-wide pre and post harvest used on crops or as anti-parasite agent in farming of terrestrial animals. Well known organochlorine pesticides (OCP) such as DDT and HCB have been mostly banned for agricultural use and are associated with fish oil (Berntssen et al., 2010; Friesen et al., 2008; Nácher-Mestre et al., 2009). These OCP pesticides have been replaced by less persistent and more water soluble pesticides (Seiber, 2002)**.** EU Maximum Residue Level (MRL) legislation for non-OCP pesticides comprises most food commodities (EC, 2005), but for feed ingredients and fish, specific harmonized EU MRLs are not yet established (EC, 2013b)**.** This emphasizes the need for data on the occurrence of pesticides in feed ingredients and the edible part of fish farmed on plant-based feeds. Extensive EU surveillance programmes exist on pesticide residues in food (EFSA, 2013). Several surveillance studies report on pesticides in terrestrial animals feed (i.e. (Gómez-Pérez et

al., 2015) as well as potential transfer of the pesticides to edible part of animals such as meat milk and eggs (Kan and Meijer, 2007; Leeman et al., 2007). For farmed fish, occurrence and feed-to-fillet transfer data on most (non POPs) pesticides, is limited (Lovell et al., 1996; Nácher-Mestre et al., 2014).

In addition to the above findings, the different physico-chemical characteristics of pesticides, together with the low concentration levels established by current legislation and the complexity of the matrices make necessary the use of last generation analytical techniques. Multi-residue methods (MRM) are applied with a clear tendency to liquid chromatography-mass spectrometry (LC-MS), although gas chromatography-MS (GC-MS) is still required to widen the number of compounds investigated. Thus, the combined use of GC and LC with tandem MS with last generation triple quadrupole (QqQ) instruments is one of the best options to get the sensitivity and selectivity required (Golge and Kabak, 2015; Hernández et al., 2013, Hernández et al., 2012). Additionally, accurate-mass full-spectrum data obtained by high resolution MS opens the possibility to provide a complete overview of pesticide pollution, and not only those compounds initially targeted can be investigated. LC coupled to quadrupole-time of flight (QTOF) MS with electrospray (ESI) source has been widely explored for the screening of polar contaminants, their metabolites and transformation products (TPs) (Hernández et al., 2015a; Hernández et al., 2014; Nácher-Mestre et al., 2013). In relation to GC-MS, the soft ionization obtained from atmospheric pressure chemical ionization (APCI) source in GC-MS instruments has offered attractive features for screening purposes (Portolés et al., 2014; Portolés et al., 2010). All this, thereby, opens fascinating perspectives in the analytical field (Hernández et al., 2015b; Pitarch et al., 2016) towards the screening of thousands of contaminants without standards (Castillo et al., 2016; Hernández et al., 2015b; Krauss et al., 2010).

The present work is based on our previous research on screening of pesticide residues (Nácher-Mestre et al., 2014, Nácher-Mestre et al., 2013) in farmed fish. A comprehensive strategy is presented for screening, identification and quantification of around 800 pesticides in commercially available plant and novel PAP feed ingredients and their transfer to the edible part of farmed Atlantic salmon and gilthead sea bream (two main species of the European aquaculture). The screening considers an initial qualitative validation of 252 pesticides using GC-(APCI)QTOF MS and UHPLC-(ESI)QTOF MS, followed by a target quantitative assessment by GC-(APCI)MS/MS QqQ for those pesticides identified in the qualitative validation.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All pesticides and isotopically labelled reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and Sigma Aldrich (St Louis, MO, USA). Isotopically labelled internal standards (ILIS) Hexachlorobenzene-$^{13}C_6$, Tebuconazole-D_6 and 4,4'-DDE-D_8 were also purchased from Dr. Ehrenstorfer. All standards had purities higher than 95%. Stock standard solutions (around 500 mg L^{-1}) were prepared in acetone and were stored at -20 °C. Twenty-two mixtures of pesticide standards (individual concentration of each pesticide around 50 mg L^{-1}) were prepared by dilution of stock individual solutions in acetone. Working standard solutions containing all pesticides were prepared by dilution of mixtures with acetone (for sample fortification in GC), hexane (GC injection), methanol (for sample fortification in LC) and water (instrument injection in LC). Stock standard solutions were stored at -20 ºC, whereas working solutions were stored at 4 ºC.

HPLC-grade water was obtained from a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). HPLC-grade methanol, HPLC-supragradient acetonitrile, acetone (pesticide residue analysis quality) and n-hexane (all ultra-trace quality) were purchased from Scharlab (Barcelona, Spain). Formic acid (HCOOH, content > 98%), sodium hydroxide (NaOH, reagent grade) and ammonium acetate (NH_4Ac, reagent grade) were supplied by Scharlab. Anhydrous magnesium sulfate (extra pure) and anhydrous sodium acetate (reagent grade) were purchased from Scharlab. Leucine enkephalin (used as lock mass in LC) and heptacosa (for GC calibration) were purchased from Sigma Aldrich.

QuEChERS commercial clean-up kits were purchased from Teknokroma (Barcelona, Spain). Each kit contains 50 mg of primary-secondary amine (PSA), 150 mg of anhydrous magnesium sulfate, and 50 mg of C_{18}, in 2 mL microcentrifuge tubes for d-SPE.

2.2 Samples

A total of 76 samples were studied in this work as detailed in **Table S1**. The list contains ingredients from different origin (plant, terrestrial animals and marine), and also feeds based on these feed ingredients, as well as fillets of Atlantic salmon and gilthead seabream reared on these feeds. Atlantic salmon and gilthead seabream were fed by the produced feeds for 7 and 18 months, respectively, and fillet samples were taken for analysis at the end of the exposure trial. The same feeds were provided throughout the feeding trial. The screening and quantification was performed on feed ingredients, feeds produced from the same feed ingredients, and fish fillets of fish fed on these feeds. The feed samples were analysed at the beginning of the trial and no stability assessment was made by analyzing the feed during storage.

142 Commercially available plant and marine feed ingredients were provided by BioMar
143 (Tech Center, Brande, Denmark) feed producer and PAPs from non-ruminants were
144 provided by the European Fat Processors and Renderers Association (EFPRA). All
145 PAPs were produced according the EU regulation for PAP intended for use as feed-
146 ingredients in animal feed (EC, 2001, EC, 2009). The ingredients selected represent the
147 novelties in fish feed compositions to reduce the inclusion of fish derivatives. Fish feeds
148 for feeding trials were based on plant feed ingredients, and not PAPs, as higher levels of
149 pesticide residues were found in plant feed ingredients (see section 3). The feeds were
150 produced by BioMar under commercial aquafeed production techniques based on high-
151 temperature extrusion processes, which potentially could affect pesticide residue levels.
152

153 **2.3 Wide scope screening work-flow**

154 The sample procedure applied for pesticides screening and quantification is illustrated
155 in **Fig. 1.** Briefly, samples were thawed at room temperature and 5 g were accurately
156 weighed and transferred to centrifuge tubes (50 mL). For GC-analysis, samples were
157 extracted with acetonitrile (10 mL) and the extract was subsequently left in a freezer (at
158 least for two hours to precipitate proteins and fix lipids to the tube walls). Then, a
159 QuEChERS clean-up step was carried out prior injection in the GC-system (Nácher-
160 Mestre et al., 2014). In the LC-screening, similar procedure was followed without any
161 purification or preconcentration step (**Fig. 1**). In this case, extraction of the samples was
162 carried out wih acetonitrile/water 80:20 (0.1% formic acid (Nácher-Mestre et al., 2013).

163 Pesticides found by the GC&LC-QTOF MS screening were subsequently confirmed
164 and quantified by GC-(APCI)MS/MS QqQ. Sample treatment was similar to that
165 applied for GC-screening with two slight variations: i) 1 g (instead of 5 g) of sample
166 was spiked with isotopically labelled internal standards and extracted with 2 ml of

167 acetonitrile (instead of 10 mL); ii) just before injection, 50 µL of the final acetonitrile
168 extract was diluted with 300 µL of acetone and 650 µL of hexane in order to make the
169 solution miscible.

170

171 **2.4 Screening validation.**

172 For qualitative analysis of GC-amenable compounds, an Agilent 7890A GC system
173 (Palo Alto, CA, USA) equipped with an Agilent 7693 autosampler was coupled to the
174 Xevo G2 QTOF (Waters, Manchester, UK), operating in APCI mode. (See more details
175 in supplementary information). For qualitative analysis of LC-amenable compounds, a
176 Waters Acquity UHPLC system (Waters, Milford, MA, USA) was coupled to a XEVO
177 G2 QTOF (Waters, Manchester, UK), with an orthogonal Z-spray-ESI interface
178 operating in both positive and negative ionization modes. (See more details in
179 supplementary information).
180 Validation of the two screening methods applied (GC-QTOF MS for 170 pesticides and
181 UHPLC-QTOF MS for 162 pesticides) was performed for qualitative purposes on the
182 basis of European analytical guidelines (Sanco, 2013). To this aim, at least twenty
183 sample matrices (including different feed ingredients, feed and fish) were spiked with
184 pesticides at two concentrations, 0.01 and 0.05 mg Kg^{-1} and, analyzed together with
185 their respective non-spiked samples ("blanks") and method blanks to assure absence of
186 contamination along the procedure. The main parameter evaluated was screening
187 detection limit (SDL), which was the lowest concentration for which each pesticide was
188 detected in 95% of the spiked samples tested (e.g. 19 out of 20 samples) independently
189 of its recovery and precision. The detection of the compound was made by using the
190 most abundant ion measured at its accurate mass (typically the molecular ion or
191 (de)protonated molecule) in the LE function. This implied that at least one *m/z* ion was

192 observed at the expected retention time (Rt) (deviation accepted ± 0.2 min, in
193 comparison with the reference standard) with mass error below 5 ppm.
194 The GC-QTOF MS qualitative screening had been previously validated for 131
195 pesticides in twenty aquaculture samples in a previous work (Nácher-Mestre et al.,
196 2014). In this work, validation was widened with 39 pesticides in relation to our
197 previous work and was tested for new sample matrices from the fish growing trials. To
198 this aim, 4 additional matrices (gilthead sea bream fillet, salmon fillet and two
199 additional fish feed) were spiked with the already validated 131 pesticides together with
200 the new 39 pesticides. For those 4 new matrices, a criteria of 4 positives out of 4
201 analyzed was required to accept the SDL. This allowed us to check and confirm the
202 SDLs previously established for the 131 pesticides, as well as establishing provisional
203 SDLs for the additional 39 pesticides studied. Furthermore, 6 PAP matrices were also
204 subjected to the same methodology, and spiked at the concentration levels indicated
205 above. With a requirement of 6 positives out of the 6 samples analyzed, provisional
206 SDLs were also established.

207 Regarding LC-QTOF MS screening, the validation has been performed in two steps as a
208 function of the availability of the samples. Similarly to GC-QTOF MS, in a first step,
209 the LC screening was qualitatively validated in twenty different sample matrices (feed
210 ingredients, feed and fish) spiked with 125 pesticides at two concentrations, 0.01 and
211 0.05 mg Kg^{-1}, and the SDL was established. The detection was made by using the
212 (de)protonated molecule, so at least one *m/z* ion was found at the expected Rt (deviation
213 accepted ± 0.2 min) with mass error below 5 ppm. The LC qualitative screening was
214 widened, in a second step, with 36 pesticides. Also, 4 additional samples (the same as in
215 GC-QTOF MS) were spiked with the already validated 125 compounds and 36 more
216 pesticides. This allowed us to confirm the SDLs already established in the first step and

217 also establish provisional SDLs for the 36 new pesticides. Additionally, 6 PAPs were
218 also spiked at the concentration levels cited above and for those samples a criteria of 6
219 out of 6 was required to establish a provisional SDL.

220

221 **2.5 Qualitative screening of aquaculture samples**

222 The overall strategy proposed was applied to the screening of aquaculture samples from
223 a multidisciplinary European funded project (EU Seventh Framework Programme
224 ARRAINA Project 288925). Samples analyzed covered the whole production chain of
225 Atlantic salmon and gilthead sea bream. For these purposes, plant and marine
226 ingredients as raw materials for aquafeed compositions from feed producers were all
227 studied (**Table S1**). Fish tissues from gilthead sea bream and salmon feeding trials were
228 analyzed in parallel. After injection of the sample extracts, full-spectrum acquisition
229 data generated at low and high collision energy (MS^E) were processed, using the
230 specialized application manager ChromaLynxXS (within MassLynx) in combination
231 with a home-made database containing 465 pesticides for GC and 527 for LC. Around
232 200 compounds were included in both databases; therefore, the total number of
233 pesticides searched in the comprehensive screening was near 800. The screening was
234 applied for those compounds that were qualitatively validated (thus, reference standards
235 were available) and also for those other pesticides included in the database, for which
236 reference standards were not available (suspect screening). The detection of a potential
237 positive was based on the presence of the (de)protonated molecule/molecular ion
238 (occasionally adducts), measured at its accurate mass, in the LE function (for both GC
239 and LC-QTOF). For this purpose, nw-XICs at the *m/z* of all compounds included in the
240 database were automatically performed in the LE function (150 ppm mass window)

241 (Hernández et al., 2015b). Data from HE function was used to confirm the identity
242 based to the presence of fragment ions.

243 When a sample was analyzed, the presence of chromatographic peak at the expected Rt,
244 together with the evaluation of the accurate-mass fragment ions and characteristic
245 isotopic ions, allowed the unequivocal confirmation of the identity of the compound
246 detected when the reference standard was available. In the case of suspect analysis, the
247 tentative identification was supported by MS/MS product ions reported in the literature
248 for the suspect compound (either in exact or nominal mass) and by the compatibility of
249 the fragment ion with the chemical structure of the candidate. Tentative identification
250 was finally confirmed by subsequent acquisition of the reference standard, which was
251 made at a later step. MassFragment software (Waters) was used to propose compatible
252 structures from accurate mass measurements of the observed fragment ions.

253 All compounds detected (only one ion with accurate mass and Rt agreement) and/or
254 identified (minimum two accurate-mass ions, with Rt and ion ratio agreement) were
255 included in the GC-(APCI)MS/MS quantitative method developed. Those compounds
256 from the suspect list that were just tentatively identified were also included in the target
257 quantitative method.

258

259 **2.6 Quantitative analyses of aquacultural samples**

260 Quantitative analysis of selected pesticides was performed in a GC system (Agilent
261 7890B, Palo Alto, CA, USA) equipped with an autosampler (Agilent 7693) and coupled
262 to QqQ mass spectrometer (Xevo TQ-S, Waters Corporation, Manchester, UK),
263 operating in APCI mode. (More details in supplementary information).

A quantitative method was optimized for those pesticides found in the screening of samples. Validation of the GC-(APCI)MS/MS method was performed for 12 out of 16 pesticides detected and/or identified in the samples. The remaining four compounds, flufenoxuron, tebufenozide, teflubenzuron and carbofuran-3OH, were not included in the quantification step as they are not GC-amenable compounds. Accuracy (estimated by means of recovery experiments) was evaluated by analyzing quality control (QC) samples spiked at 0.005 and 0.05 mg Kg^{-1} in 26 "blank" samples (corresponding to 19 different matrices). The limit of quantification (LOQ) was established as the lowest concentration for which the method showed satisfactory recovery (between 60 and 140%). Isotopically labeled internal standards were used to correct matrix effects and potential errors associated to sample manipulation (Portolés et al., 2017).

3. RESULTS AND DISCUSSION

3.1. Target and suspect screening of feed ingredients, feed and transfer to farmed fish

Regarding GC-QTOF screening validation, among the 131 pesticides already studied, 121 maintained the already established SDL: 0.01 mg Kg^{-1} (69 pesticides), 0.05 mg Kg^{-1} (34 pesticides) and > 0.05 mg Kg^{-1} (18 pesticides), and 4 improved/lowered this value (carbophenothion, chlorfenson, pendimethalin and tau-fluvalinate) (see **Table S2**). Only six pesticides did not pass the new criteria of 4 out of 4 in the new samples and sacrificed the SDL from 0.01 to >0.05 mg Kg^{-1} (diphenylamine and leptophos), or from 0.05 to >0.05 mg Kg^{-1} (chlorothalonil, heptachlor epoxide A, heptachlor epoxide B and propoxur). For the 39 additional pesticides studied in four new samples (including two fish feed and two fish fillets), a provisional SDL was established as 0.01 mg Kg^{-1} for 13 pesticides, 0.05 mg Kg^{-1} for 17 pesticides and >0.05 mg Kg^{-1} for 9 pesticides based on

289 the 4 out of 4 criteria. SDLs obtained for PAPs coincided with those of feed ingredients,
290 feed and fish for the great majority of analytes (82% of cases, corresponding to 141
291 compounds).

292 Regarding LC-QTOF screening validation, for the first 125 pesticides studied, SDLs
293 were established as 0.01 mg Kg^{-1} (49 pesticides), 0.05 mg Kg^{-1} (31 pesticides) and >
294 0.05 mg Kg^{-1} (25 pesticides) and 18 improved/lowered this value (see **Table S3**). Only
295 two pesticides (chlorpropham and parathion-ethyl) did not pass the new criteria of 4 out
296 of 4 in the new samples and sacrificed the SDL from 0.05 to >0.05 mg Kg^{-1}. For the
297 new 36 pesticides studied in four matrices, a tentative/provisional SDL was established
298 as 0.01 mg Kg^{-1} for 6 pesticides, 0.05 mg Kg^{-1} for 24 pesticides and >0.05 mg Kg^{-1} for 6
299 pesticides. SDL obtained for PAPs coincided with those for feed ingredients, feed and
300 fish in 136 cases (84%) and for the rest showed worst results except for hexaflumuron,
301 butachlor and omethoate.

302 In general, the evaluation of the SDL for ethoxyquin (ETQ) was troublesome due to the
303 presence of the analyte at high concentrations in the samples used for validation.

304 It is worth to mention that in some cases the same pesticide was included in both
305 screening methodologies, LC and GC. In those cases, the most favorable SDL was
306 selected. In this way, **Table 1** summarizes the final SDLs established for feed
307 ingredient, feed and fish for the 252 pesticides studied (removing duplicities resulting
308 from LC and GC analysis of the same compound). Overall, SDL values were 0.01 mg
309 Kg^{-1} for 113 pesticides (45%), 0.05 mg Kg^{-1} for 73 pesticides (29%) and a total of 66
310 pesticides could not be qualitatively validated (26%) at these levels. For most of them,
311 surely the method was not sensitive enough for the analyte/matrix tested, and higher
312 analyte concentrations (>0.05 mg Kg^{-1}) should be tested. In addition, some pesticides

313 and sample matrices might require specific sample treatments and/or measurement
314 conditions in order to reach the low concentration levels tested in this work.

315 All the samples described in the experimental section, which contain ingredients from
316 different origin (plant, terrestrial animals and marine), and also different feed
317 compositions and fish tissues, were analyzed following the recommended procedure by
318 both GC-(APCI)QTOF MS and UHPLC-(ESI)QTOF MS. **Fig. 2** illustrates the
319 pesticides detected, identified (confirmed with reference standard) and tentatively
320 identified in the screening of these aquaculture samples.

321 Pirimiphos methyl, was the compound more frequently identified by both techniques in
322 most ingredients (68% of plant protein, 75% plant oil ingredients and 17% of marine
323 ingredients) and in all the feed samples (salmon and sea bream). However, no residues
324 were found in the fish samples suggesting none feed to fish fillet transfer. Similarly,
325 chlorpyriphos methyl was detected, mainly by GC-(APCI)QTOF MS, in plant protein
326 (11%), plant oil ingredients (25%), gilthead sea bream feed (50%) and salmon feed
327 (25%) but not fish fillets. Foodborne chlorpyriphos-methyl is readily metabolized and
328 eliminated by fish, and its relative low biomagnification potential compared to POPs
329 (Varó et al., 2002) could explain the non-detectable fillet levels in fish that were fed on
330 low background levels in the present study. In addition, long term storage at high
331 temperatures could potentially affect the level of chlorpyrifos-methyl, but not
332 pirimiphos-methyl residues in corn products (White et al., 1997). In the present trial, no
333 assessment of the pesticide level during storage was made, which could have
334 contributed to possible absence of detectable pesticides in the fish fillets of fish fed on
335 the feeds. Earlier surveillance studies also identified chlorpyriphos methyl, and to a
336 lesser extend pirimiphos methyl, as some of the most frequent pesticide residues in
337 terrestrial animal feeds (Gómez-Pérez et al., 2015; Lovell et al., 1996). In contrast to the

present study, chlorpyriphos-methyl was also detected in fish from Taiwan markets and fish feeds were suggested to be the main source of this compound in farmed fish (Sun and Chen, 2008).

Other pesticides like the organochlorine pesticide HCB was found by GC-(APCI)QTOF MS in one marine origin ingredient, which is a well-known OCP pesticide that behaves as a POP with elevated levels in fish oil obtained from pelagic fish species (Berntssen et al., 2010). The none-OCP pesticides, tebuconazole, azoxystrobin, malathion and boscalid were found by UHPLC-(ESI)QTOF MS in plant-based ingredients (specially in plant oil ingredients). In contrast to chlorpyriphos-methyl and pirimiphos-methyl, these pesticides were not found in feed samples. The absence of these pesticides in feed while present in the plant-based feed ingredients is likely due to the dilution effect occurred when plant ingredients are mixed with other ingredients such as fish oil and meal to produce fish feeds, causing levels below SDL. Then, flufenoxuron, tebufenozide and teflubenzuron were identified (tebufenozide only detected) by UHPLC-(ESI)QTOF MS in gilthead sea bream feed samples (among 13 and 38% of the analyzed samples), but not in the feed ingredients used in these feeds or fillets of seabream fed on these feeds. Ethoxyquin, which use is currently authorized as a feed ingredient antioxidant supplement, was identified in all samples by both techniques except plant oil ingredients (75%) and animal origin ingredients (only in 5%).

All cited compounds had been included in the target screening list, as reference standards were available for them and had been previously included in the qualitative screening validation protocol. Oppositely, the fungicide fluazinam, included in the suspect list, was tentatively identified by UHPLC-(ESI)QTOF MS in two gilthead sea bream feed samples. **Fig. 3** illustrates the detection and tentative identification of this compound in a gilthead sea bream feed sample by UHPLC-QTOF MS. The deprotonated molecule of fluazinam was detected in the LE function in ESI negative

mode, with a mass error of -1.9 ppm. As the reference standard was not available, chemical structures for the most abundant fragment ions were suggested based on their accurate masses, using the MassFragment software (Waters). In the HE function, 2 fragments (*m/z* 415.9433 and 397.9768) were observed with chromatographic peaks at the same Rt, and mass errors lower than 1.2 ppm in relation to the theoretical predicted exact masses. All structures proposed for the fragments were compatible with the chemical structure of fluazinam and were in accordance with the isotopic pattern observed for the chlorine atoms present in the structure, making the identification even more reliable. Moreover, the tentative identification was supported by the MS/MS product ions reported in the literature (Pizzutti et al., 2009)**.** After this careful evaluation process, the reference standard was finally acquired and injected, allowing the ultimate confirmation of this compound in the sample.

3.2 Quantitative analysis of identified pesticides and feed-to fillet transfer

QC recoveries were obtained at 0.005 and 0.05 mg Kg^{-1} in 19 different matrices, ranging between 60% and 130% for most matrix/analyte combinations. A LOQ of 0.005 mg Kg^{-1} was obtained for azoxystrobin, boscalid, malathion, pirimiphos-methyl, chlorpyriphos-methyl and ethoxyquin-dimer (ETQ-D) while it was 0.05 mg Kg^{-1} for diphenylamine, tebuconazole and imazalil, this being the lower MRL established in the current legislation for crops that can be used as feed ingredients. Regarding ethoxyquin, the evaluation of its recovery was not feasible due to the presence of the analyte at high concentrations in the samples used for validation. For the remaining two compounds, fluazinam and hexachlorobenzene, the method was not suitable as they did not present satisfactory results in most of the matrices).

388 All compounds reported as identified by QTOF screening were confirmed and
389 quantified by GC-(APCI)MS/MS. The only exceptions were fluazinam (2 samples),
390 flufenoxuron (1 sample), teflubenzuron (1 sample) and carbofuran-3OH (1 sample)
391 which were identified by UHPLC-QTOF MS but could not be included in GC-
392 (APCI)MS/MS quantitative method due to their physico-chemical characteristics.
393 Additionally, there were another 37 detections in the screening, for which only one ion
394 measured at accurate mass at expected Rt was found and therefore their identity could
395 not be confirmed. 22 out of these 37 suspect positives could be confirmed and
396 quantified by QqQ while for rest seemed to be false detections. The greater sensitivity
397 of GC-MS/MS with QqQ in comparison to QTOF made it possible to report 47 new
398 positive findings that had not been detected previously or identified by QTOF
399 (ethoxyquin, ethoxyquin-dimer, boscalid, azoxystrobin, tebuconazole and imazalil). All
400 of them were quantified by QqQ at levels below 0.05 mg Kg^{-1} except for ethoxyquin
401 and ethoxyquin-dimer whose concentrations exceeded 0.05 mg Kg^{-1} in most of the
402 salmon fillets analyzed. As regards identification, all quantified pesticides were
403 identified by the use of three transitions and the compliance of at least one q/Q ratio.

404 **Table 2** summarizes the pesticide concentrations determined in the analyzed samples by
405 GC-(APCI)MS/MS. Ethoxyquin and ethoxyquin-dimer were found in all feed and fish
406 samples. Concentrations were above 0.5 mg Kg^{-1} in all feeds, in the range of 0.005 to
407 0.5 mg Kg^{-1} in salmon fillet and above 0.05 mg Kg^{-1} in gilthead sea bream. ETQ was
408 found at concentration levels above 0.05 mg Kg^{-1} in all ingredients with the exception of
409 one plant ingredient in the range of 0.005-0.05 mg Kg^{-1}. ETQ-D was found below
410 0.005 mg Kg^{-1} except four plant ingredients in the range of 0.005 to 0.05 mg Kg^{-1} and,
411 above 0.005 mg Kg^{-1} in all marine origin ingredients. Earlier studies also reported the
412 overall presence of synthetic antioxidants such as ETQ in commercial feed and ETQ

413 and ETQ-D in farmed fish including Atlantic salmon, halibut, cod, and rainbow trout,
414 with mean (min.-max.) ETQ feed levels of 10 (1.4-32) mg Kg^{-1} and mean (min.-max.)
415 ETQ and ETQ-D levels of 0.06 (0.013-0.17) and 0.7 (0.29-1.5) mg Kg^{-1}, respectively,
416 analyzed by means of HPLC coupled to fluorescence detection (Lundebye et al., 2010).

417 A concentration level around 0.01 mg Kg^{-1} of fungicides boscalid and azoxystrobin
418 were found in one feed sample (0.009 mg Kg^{-1} for both analytes), one PAP (0.007 and
419 0.008 mg Kg^{-1} respectively) and one plant oil (only boscalid at 0.007 mg Kg^{-1}) although
420 not exceeding its MRL. The organophosphorous insecticides pirimiphos-methyl and
421 chlorpyriphos-methyl were found in 66% and 25% of the feed samples in a range of
422 0.006-0.030 mg Kg^{-1} and 0.005-0.009 mg Kg^{-1}, respectively. The highest concentration
423 level of these two OP insecticides was found in a wheat gluten sample at 0.037 mg Kg^{-1}
424 for chlorpyriphos-methyl and 0.191 mg Kg^{-1} for pirimiphos-methyl. Additionally,
425 pirimiphos-methyl was also found in five plant oil and three plant ingredient samples at
426 concentration levels among 0.005-0.5 mg Kg^{-1}. No MRL exists for crop partly or
427 exclusively used for feed ingredients (EC, 2013a). Until specific feed ingredient MRLs
428 have been established, existing EU MRLs for food crop would apply, taking into
429 account an appropriate processing (EFSA, 2015). As no standard factors are known for
430 the processing of whole wheat into animal feed graded wheat gluten, no clear MRL can
431 be set. However the wheat MRLs for chlorpyriphos-methyl and pirimiphos-methyl are
432 respectively 3.0 and 5.0 mg Kg^{-1} which is respectively 25 and 135-fold higher than
433 levels found in present study. Regarding tebuconazole, it was found in two rapeseed oil
434 samples at concentration around 0.01 mg Kg^{-1}. As an illustrative example, **Fig. 4** shows
435 the GC-(APCI)MS/MS chromatograms obtained for the quantification and confirmation
436 of boscalid in one salmon feed (0.009 mg Kg^{-1}), azoxystrobin in a poultry blood meal
437 (0.008 mg Kg^{-1}) and chlorpyriphos-methyl in wheat gluten (0.037 mg Kg^{-1}). Also, **Fig.**

438 **S1 (a)** shows the GC-(APCI)MS/MS chromatograms obtained for the quantification and
439 confirmation of pirimiphos-methyl in wheat gluten at concentration level of 0.191 mg
440 Kg^{-1}. This ingredient is used to prepare a gilthead sea bream feed shown at **Fig. S1 (b)**
441 that contains the pirimiphos-methyl at concentration level of 0.007 mg Kg^{-1}. This feed
442 represents a total replacement of 80% of fish meal by plant meal and 84% of the fish oil
443 by alternative plant oils. The gilthead sea bream fish fillet reared on this feed does not
444 shown any trace of pirimiphos methyl (**Fig. S1(c)**).

445

446 **4. CONCLUSIONS**

447 The developed strategy faces the "universal" pesticide analysis in aquaculture field by
448 means of combined use of LC-QTOF MS and GC-QTOF MS for screening, followed
449 by confirmation and quantification by GC-(APCI)MS/MS with QqQ, as most pesticide
450 detected in the screening were GC-amenable. The strategy proposed is among the most
451 comprehensive and informative in the pesticide analysis context as it covers a large list
452 of pesticides from different families. The overall strategy is presented as a risk
453 assessment tool available for the feed industry in order to widen the knowledge of novel
454 and traditional ingredients, feed and edible parts of consumed animals. The most
455 dominant polar pesticides found in plant feed ingredients and feed based on these
456 ingredients were pirimiphos-methyl and chlorpyriphos metyl. These pesticides were not
457 found in the fillets of fish fed on these feeds.

458

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468

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627
628

629 **FIGURE CAPTIONS**

630 **Fig. 1**. Scheme of the sample procedure for screening and quantification of pesticides in
631 aquaculture field.

632 **Fig. 2**. Accumulated % of samples positives to pesticides detected or identified in the
633 screening of aquaculture samples by GC&LC QTOF MS

Fig. 3. Detection and identification of fungicide fluazinam by UHPLC-QTOF MS in a gilthead sea bream feed sample (the reference standard was not available at our laboratory in the time of the detection): (a) LE (bottom) and HE (top) spectra of the compound eluting at 13.96 min. Proposed elemental compositions for fragment ions; (b) extracted-ion chromatograms (150 ppm mass width) for protonated molecule in LE function and different fragment ions in HE function.

Fig. 4. GC-(APCI)MS/MS chromatograms obtained for the quantification and confirmation of boscalid in a) feed (0.009 mg Kg^{-1}); b) azoxystrobin in animal origin ingredient (0.008 mg Kg^{-1}) and; c) chlorpyriphos-methyl in plant oil (0.037 mg Kg^{-1}). Q: Quantification transition; q_i: qualification transitions. ✓ q/Q within accepted tolerances.

Table 1. SDLs obtained for each pesticide studied by GC-APCI-QTOF MS and UHPLC-ESI-QTOF MS

0.01 mg Kg^{-1}		**0.05 mg Kg^{-1}**	**> 0.05 mg Kg^{-1}**
2-Phenylphenol	Folpet	alpha-endosulphan	**Acetamiprid**
4-4'-Dichlorobenzophenone	**Haloxyfop-2-ethoxyethyl**	*alpha-HCH**	**Aldicarb sulfone**
Alachlor*	**Haloxyfop-methyl**	**Bensulide**	**Aldicarb sulfoxide**
Atrazine desethyl	**Imazalil**	beta-endosulfan	Aldrin
Atrazine desisopropyl	Iprodione	*beta-HCH**	**Azinphos-ethyl**
Atrazine/**Atrazine**	**Isoproturon**	**Boscalid**	**Benomyl**
Azinphos-methyl	**Linuron**	**Chlorsulfuron**	**Bifenazate**
Azoxystrobin	**Malaoxon**	Cyanazine	**Butachlor**
Bentazone (-)	Malathion/**Malathion**	*delta-HCH**	**Butocarboxym**
Bifenthrin*	Metalaxyl/**Metalaxyl**	Dieldrin	Captafol
Bromophos	Methiocarb*	**Diflubenzuron**	Captan*
Bromophos ethyl	Methoxychlor*	Dimethoate	**Carbendazim**
Bromopropylate	Metolachlor/**Metolachlor**	Dioxathion*	**Carbofuran-3-OH**
Buprofezin/**Buprofezin**	Metribuzin	**Diuron**	Carbophenothion
Cadusafos	Molinate	Endrin	Chlorfenson
Carbaryl	Oxyfluorfen	**Ethiofencarb**	**Chloridazon**
Carbofuran	**Paclobutrazol**	Ethion/**Ethion**	Chlorothalonil
Carfentrazone ethyl	Parathion ethyl	**Ethofumesate**	**Clothianidin**
Chinomethionat	Parathion methyl	**Ethoxyquin dimer**	Cyfluthrin
Chlorfenapyr	Pirimicarb/**Pirimicarb**	**Fenhexamid**	Cypermethrin
Chlorfenvinphos	Pirimiphos methyl/**Pirimiphos methyl**	**Fenoxaprop**	Deltamethrin
Chlorpropham*	Procymidone	Flucythrinate*	Diphenylamine
Chlorpyrifos ethyl	**Promecarb**	**Flufenoxuron**	Endosulfan sulfate
Chlorpyrifos methyl	**Propanil (-)**	*gamma-HCH **	**Ethiofencarb sulfone**
Clomazone	Propham*	*HCB*	**Ethiofencarb sulfoxide**
Coumaphos/**Coumaphos**	Propiconazole/**Propiconazole**	Heptachlor	Ethoxyquin/**Ethoxyquin**
Cyanophos	Propyzamide	**Hexaflumuron (-)**	Fenvalerate
Cyprodinil/**Cyprodinil**	**Pyridaphenthion**	lambda-Cyhalothrin	**Fluroxypyr**
Diazinon/**Diazinon**	**Pyrifenox**	Methamidophos	Heptachlor epoxide A
Dichlofenthion	Pyriproxyfen/**Pyriproxyfen**	**Methidathion**	Heptachlor epoxide B
Dichloran	Quinalphos/**Quinalphos**	**Mevinphos**	*Hexachlorobutadiene*
Dichlorvos/**Dichlorvos**	Resmethrin	**Monocrotophos**	**Hexythiazox**
Dicrotophos	Simazine/**Simazine**	Oxadixyl/**Oxadixyl**	**Imidacloprid**
Diflufenican	**Tebuconazole**	*p,p'-DDD**	Isodrin
Dimethomorph	**Teflubenzuron (-)**	*p,p'-DDE*	Leptophos
Endosulfan ether	Terbacil*	*p,p'-DDT**	**Lufenuron**
EPN	Terbumeton/**Terbumeton**	**Pendimethalin**	**MCPA (-)**
Ethalfluralin	Terbumeton desethyl	*Pentachlorobenzene*	**Methiocarb sulfone**
Etofenprox*	**Terbuthylazine**	Permethrin*	**Methiocarb sulfoxide**
Famphur	Terbuthylazine desethyl /**Terbuthylazine desethyl**	Phorate	**Methomyl**
Fenamiphos	Terbutryn/**Terbutryn**	Phosmet	*Mirex**
Fenarimol	**Tetraconazole**	**Quizalofop-ethyl**	**Omethoate**

Fenitrothion	Tetradifon	**Spiroxamine**	**Oxamyl**
Fenoxycarb	**Thiobencarb**	**Tebufenpyrad**	**Propamocarb**
Fenthion	Tolclofos methyl	Tefluthrin	Propetamphos
Fipronil/**Fipronil (-)**	Triadimefon	**Terbacil (-)**	Propoxur
Fluazifop-P-butyl	**Triflumizole**	Thiabendazole/**Thiabendazole**	**Simazine 2-hydroxy**
Fluazinam (-)	Trifluralin	**Thiacloprid**	tau-Fluvalinate
Fludioxonil* (-)	Vinclozolin	**Thiodicarb**	**Terbufos**
Flutriafol		**Thiophanate-methyl**	**Terbuthylazine 2-hydroxy**
		Triadimenol	**Thiamethoxam**
		Tridemorph	**Thiram**
			Tolyfluanid*/**Tolyfluanid**
			trans-Chlordane
			Trichlorfon
			Triforine

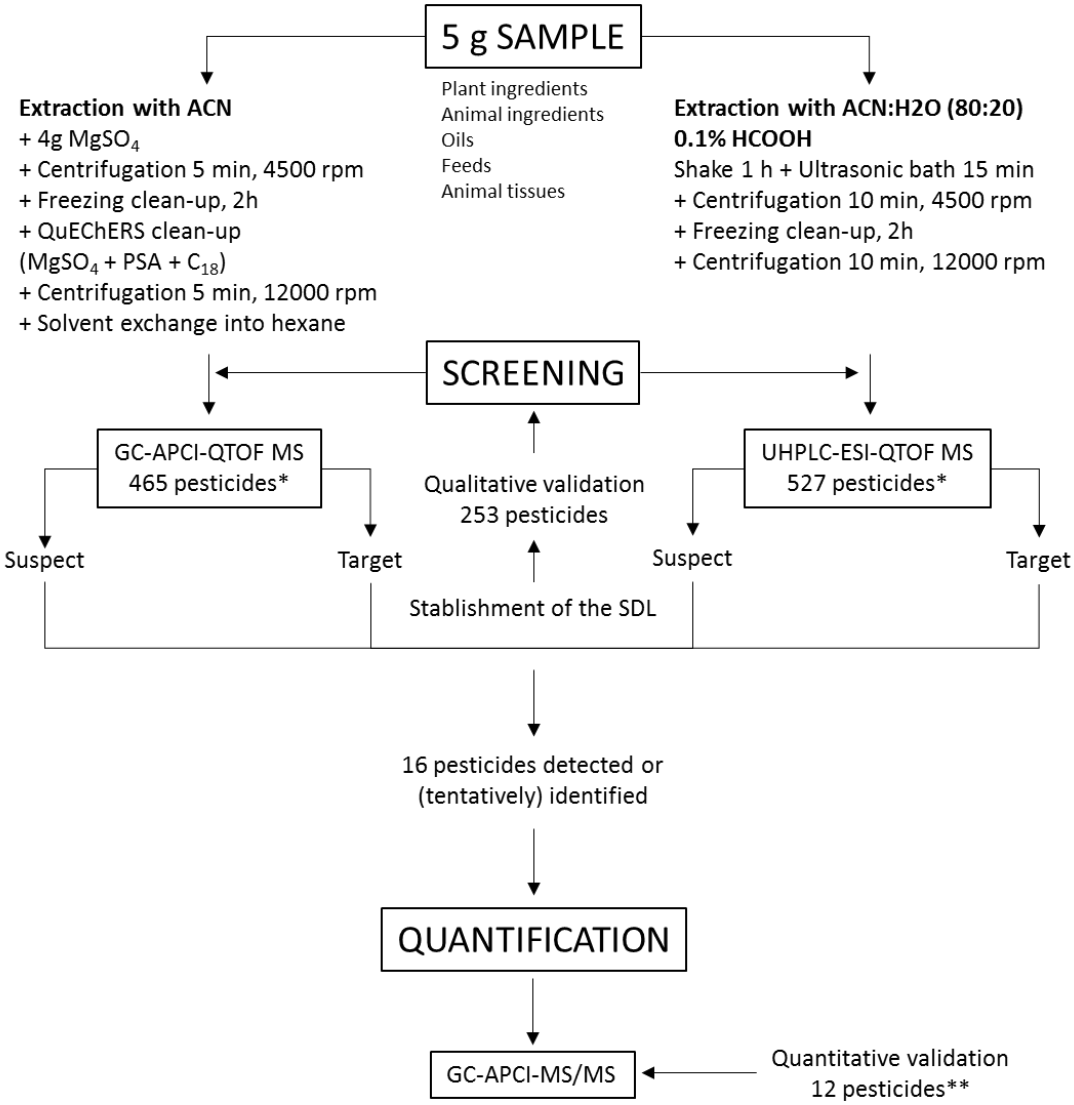
0.01 mg Kg^{-1}	**0.05 mg Kg^{-1}**	**> 0.05 mg Kg^{-1}**
Azaconazole/**Azaconazole**	**Aldicarb**	Acequinocyl
Bromuconazole	Bixafen/**Bixafen**	**Benoxacor**
Clodinafop-propargyl	Carbetamide/**Carbetamide**	Bromoxynyl/**Bromoxynil (-)**
Cyproconazole/**Cyproconazole**	Difenoconazole	Carbosulfan/**Carbosulfan**
Dimethachlor	Indoxacarb/**Indoxacarb**	Chlordecone
Epoxyconazol	**Ioxynil (-)**	**Dalapon (-)**
Fenpropimorph	Ioxynil-Octanoate	Flumetrine
Fluquinconazole	Iprovalicarb/**Iprovalicarb**	Oxydemeton-methyl
Isopyrazam	Isoxaben/**Isoxaben**	Spiromesifen
Mepanipyrim	Methabenzthiazuron/**Methabenzthiazuron**	**Trinexapac acid (-)**
Mephosfolan/**Mephosfolan**	Metrafenon/**Metrafenone**	
Metconazole	**Oxydemeton-methyl**	
Propazine/**Propazine**	Procloraz/**Procloraz**	
Prosulfocarb	Profenofos/**Profenofos**	
Tebuconazole	**Prothioconazole**	
	Pymetrozine	
	Pyraclostrobin/**Pyraclostrobin**	
	Quintocene	
	Tebufenozide	
	Tepraloxydim	
	Tepraloxydim	

italic: GC under charge transfer conditions
bold: LC
*: ion frangment
(-): ESI neg

Table 2. Number of samples with quantified values in the mentioned range

	Feed: sea bream (n=8)				Feed: salmon (n=4)				Fish: salmon (n=4)				Fish: sea bream (n=8)			
	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg
Diphenylamine	8				4				4				8			
HCB	8				4				4				8			
Ethoxyquin				8				4			4				7	1
Chlorpyriphos-methyl	6	2			3	1			4				8			
Pirimiphos-methyl	4	4				4			4				8			
Malathion	8				4				4				8			
Imazalil	8				4				4				8			
Tebuconazole	8				4				4				8			
Ethoxyquin dimer				8				4		2	2				4	4
Boscalid	8				3	1			4				8			
Azoxystrobin	8				3	1			4				8			

	Ingredients: animal origin (n=19)				Ingredients: marine origin (n=6)				Ingredients: plant oil (n=8)				Ingredients: plant (n=19)			
	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg	< 0.005 mg/kg	0.005-0.05 mg/kg	0.05-0.5 mg/kg	>0.5 mg/kg
Diphenylamine	19				6				7	1			17	2		
HCB	19				5	1			8				19			
Ethoxyquin			18	1			2	4			5	3		1	16	2
Chlorpyriphos-methyl	19				6				8				18	1		
Pirimiphos-methyl	19				6				3	5			15	1	3	
Malathion	19				6				8				19			
Imazalil	19				6				8				19			
Tebuconazole	19				6				6	2			19			
Ethoxyquin dimer	19					2	2	2	6	2			17	2		
Boscalid	18	1			6				7	1			19			
Azoxystrobin	18	1			6				8				19			



* Around 200 compounds were included in both databases; therefore, the total number of pesticides searched in the comprehensive screening was near 800.
** 4 out of 16 pesticides were not GC-amenable and should be determined by LC-MS/MS

Figure 1

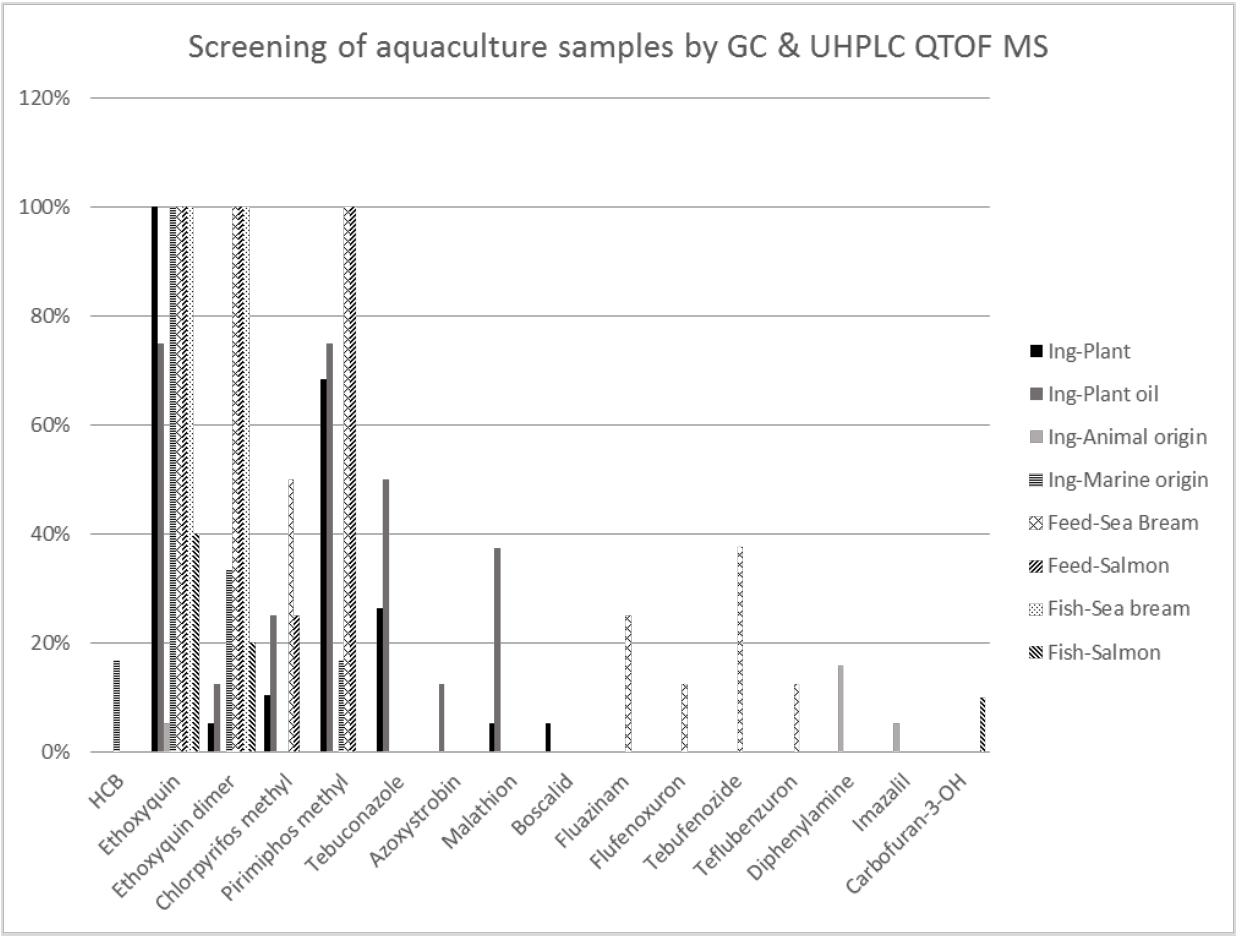
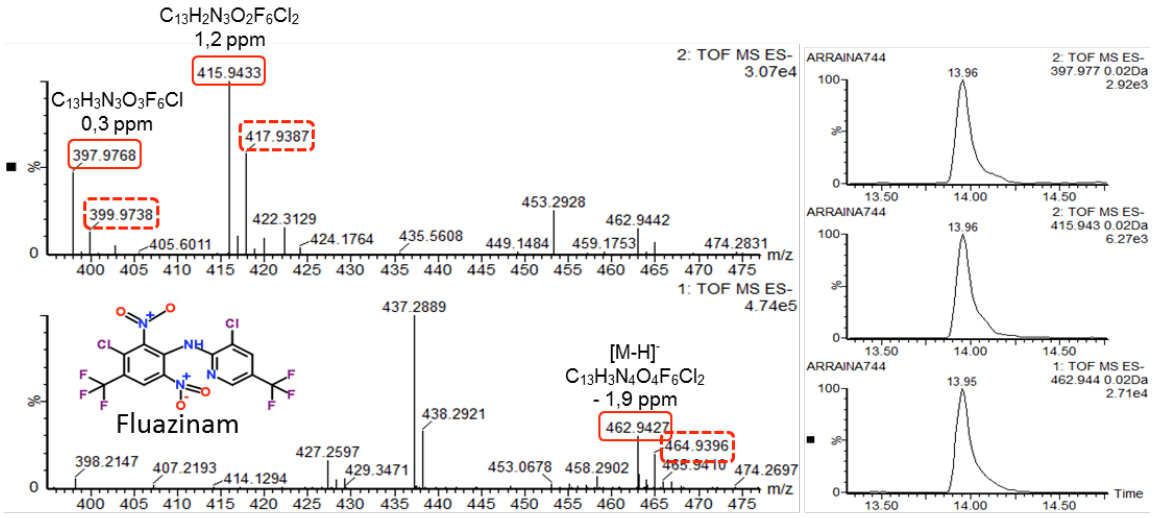

Screening of aquaculture samples by GC & UHPLC QTOF MS
120%
100%
80%
60%
40%
20%
0%
HCB
Ethoxyquin
Ethoxyquin dimer
Chlorpyrifos methyl
Pirimiphos methyl
Tebuconazole
Azoxystrobin
Malathion
Boscalid
Fluazinam
Flufenoxuron
Tebufenozide
Teflubenzuron
Diphenylamine
Imazalil
Carbofuran-3-OH
Ing-Plant
Ing-Plant oil
Ing-Animal origin
Ing-Marine origin
Feed-Sea Bream
Feed-Salmon
Fish-Sea bream
Fish-Salmon

Figure 2


C13H2N3O2F6Cl2
1,2 ppm
415.9433
C13H3N3O3F6Cl
0,3 ppm
397.9768
417.9387
399.9738
422.3129
405.6011
424.1764
435.5608
449.1484
453.2928
459.1753
462.9442
474.2831
2: TOF MS ES-
3.07e4
m/z
1: TOF MS ES-
4.74e5
437.2889
438.2921
Fluazinam
[M-H]-
C13H3N4O4F6Cl2
- 1,9 ppm
462.9427
464.9396
465.9410
398.2147
407.2193
414.1294
427.2597
429.3471
453.0678
458.2902
474.2697
ARRAINA744
2: TOF MS ES-
397.977 0.02Da
2.92e3
13.96
ARRAINA744
2: TOF MS ES-
415.943 0.02Da
6.27e3
13.96
ARRAINA744
1: TOF MS ES-
462.944 0.02Da
2.71e4
13.95
Time

(A) (B)

Figure 3

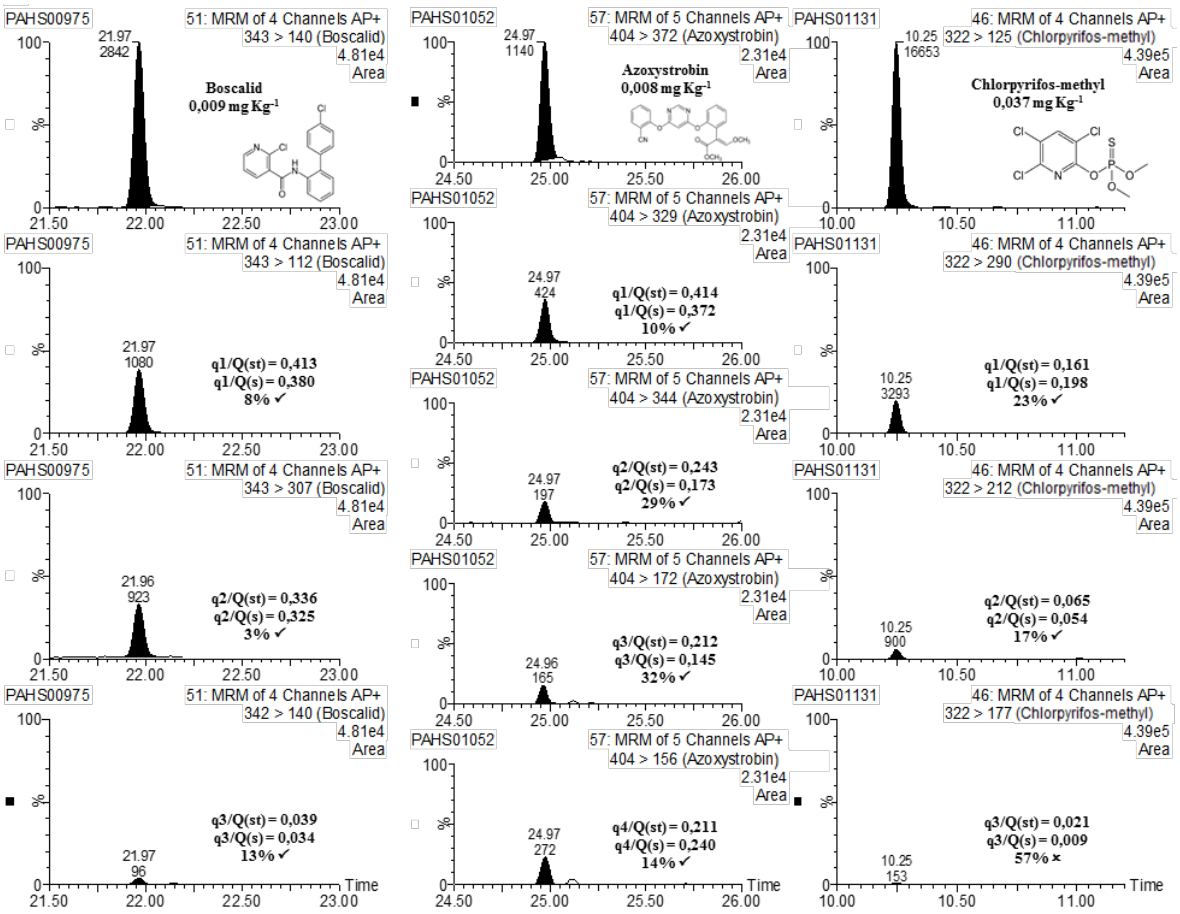

PAHS00975
51: MRM of 4 Channels AP+
343 > 140 (Boscalid)
4.81e4
Area
21.97
2842
Boscalid
0,009 mg Kg-1
343 > 112 (Boscalid)
21.97
1080
q1/Q(st) = 0,413
q1/Q(s) = 0,380
8% ✓
343 > 307 (Boscalid)
21.96
923
q2/Q(st) = 0,336
q2/Q(s) = 0,325
3% ✓
342 > 140 (Boscalid)
21.97
96
q3/Q(st) = 0,039
q3/Q(s) = 0,034
13% ✓
PAHS01052
57: MRM of 5 Channels AP+
404 > 372 (Azoxystrobin)
2.31e4
Area
24.97
1140
Azoxystrobin
0,008 mg Kg-1
404 > 329 (Azoxystrobin)
24.97
424
q1/Q(st) = 0,414
q1/Q(s) = 0,372
10% ✓
404 > 344 (Azoxystrobin)
24.97
197
q2/Q(st) = 0,243
q2/Q(s) = 0,173
29% ✓
404 > 172 (Azoxystrobin)
24.96
165
q3/Q(st) = 0,212
q3/Q(s) = 0,145
32% ✓
404 > 156 (Azoxystrobin)
24.97
272
q4/Q(st) = 0,211
q4/Q(s) = 0,240
14% ✓
PAHS01131
46: MRM of 4 Channels AP+
322 > 125 (Chlorpyrifos-methyl)
4.39e5
Area
10.25
16653
Chlorpyrifos-methyl
0,037 mg Kg-1
322 > 290 (Chlorpyrifos-methyl)
10.25
3293
q1/Q(st) = 0,161
q1/Q(s) = 0,198
23% ✓
322 > 212 (Chlorpyrifos-methyl)
10.25
900
q2/Q(st) = 0,065
q2/Q(s) = 0,054
17% ✓
322 > 177 (Chlorpyrifos-methyl)
10.25
153
q3/Q(st) = 0,021
q3/Q(s) = 0,009
57% x
Time
(A)
(B)
(C)

Figure 4