

1 **Investigation of pharmaceuticals in processed animal by-products by**  
2 **liquid chromatography coupled to high-resolution mass spectrometry.**

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22 **Abstract**

23 There is an on-going trend for developing more sustainable salmon feed in which  
24 traditionally applied marine feed ingredients are replaced with alternatives. Processed  
25 animal products (PAPs) have been re-authorized as novel high quality protein  
26 ingredients in 2013. These PAPs may harbor undesirable substances such as  
27 pharmaceuticals and metabolites which are not previously associated with salmon  
28 farming, but might cause a potential risk for feed and food safety. To control these  
29 contaminants, an analytical strategy based on a generic extraction followed by ultra-  
30 high performance liquid chromatography coupled to high resolution mass spectrometry  
31 (UHPLC-HRMS) using quadrupole time-of-flight mass analyzer (QTOF MS) was  
32 applied for wide scope screening. Quality control samples, consisting of PAP  
33 commodities spiked at 0.02, 0.1 and 0.2 mg/kg with 150 analytes, were injected in every  
34 sample batch to verify the overall method performance. The methodology was applied  
35 to 19 commercially available PAP samples from six different types of matrices from the  
36 EU animal rendering industry. This strategy allows assessing possible emergent risk  
37 exposition of the salmon farming industry to 1005 undesirables, including  
38 pharmaceuticals, several dyes and relevant metabolites.

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40 **KEYWORDS:** aquaculture, processed animal products, salmon, liquid  
41 chromatography, screening, undesirables, pharmaceuticals, dyes, quadrupole time-of-  
42 flight.

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## 46 INTRODUCTION

47 Estimated global production of farmed salmon (including *Salmo salar*, *Oncorhynchus*  
48 *kisutch*, *O. tshawytscha*) in 2010 was approximately 1.8 million metric tonnes with  
49 expected production of around 2.9 million metric tonnes in 2020 (Tacon et al., 2008).  
50 Furthermore, consumption of all species of farmed fish is expected to exceed that of  
51 feral fish (FAO, 2014). Traditional commercial feed for farmed salmon and rainbow  
52 trout are based on marine feed ingredients extracted from pelagic fish stocks. Concern  
53 of pressure on feral fish stocks and limited fish meal and fish oil availability to supply a  
54 rapidly growing aquaculture industry has led to the development of aquafeeds in which  
55 marine resources are replaced with alternative feed ingredients to develop more  
56 sustainable marine aquafeeds (Tacon et al., 2008; Torrissen et al., 2011; Waago et al.,  
57 2013). On a global basis, processed animal products (PAP) from the rendering industry  
58 constitute one of the largest sources of high quality animal protein available for animal  
59 feed production (Toldra et al., 2012). The use of PAPs such as feather meal, poultry by-  
60 product meal, pork meat and bone meal, and poultry and pork blood meal have been  
61 shown to be relevant nutritional replacements of fish meal for many cultured fish  
62 species including salmonids (Brandsen et al., 2001; Rosenlund et al., 2001; Yanik et al.,  
63 2003; Rahnema et al., 2007; Wilson et al., 2007; Friesen et al., 2008; Poppi et al., 2011;  
64 Burr et al., 2012). However, following the peak outbreaks of transmissible spongiform  
65 encephalopathies (TSE) in the UK in the early 1990's, the use of PAPs in all animal  
66 feed was banned in the European Union (EU) in 2001 (EC, 2001; EC, 2003a).  
67 Following a bovine spongiform encephalopathies (BSE) risk assessment by the  
68 European Food Safety Authorities (EFSA) (EFSA, 2011), the EU set out a working plan  
69 for the re-authorization of the use of non-ruminant PAPs in animal feed, initially for  
70 aquafeeds in 2013 (EC, 2013a).

71 In EU, the use of veterinary drugs is regulated according to EU legislation. Permitted  
72 residue levels of pharmaceutical substances (EC, 2009a) and mandatory monitoring  
73 activity (EC, 1996) have been established for all food producing animals. These  
74 regulations affect the legal addition of pharmaceuticals to animal feed, including the  
75 prohibition of substances in feeds that have hormonal or thyreostatic action as well as  $\beta$ -  
76 agonist (EC, 2003b). In addition, the supplementation of coccidiostats or histomonostats  
77 as feed additive causing unavoidable carry-over in non-target feed is regulated in the  
78 EU (EC, 2009b). In Norway, the use of pharmaceuticals in Atlantic salmon farming is  
79 under strict control and all sales of pharmaceuticals in animal farming have to be  
80 reported to the Norwegian Food Safety Authority. Open and reliable statistics on the  
81 consumption of therapeutic agents in aquaculture is only available from some nations.  
82 As an example, the registered use of pharmaceuticals in Norwegian aquaculture in 2014  
83 included the antimicrobials florfenicol and oxolinic acid, the anti-parasitic agents  
84 azametifos, cypermetrin, deltametrin, diflubenzuron, teflubenzuron, emamectin,  
85 praziquantel and hydrogen peroxide, as well as the anaesthetic agents benzocaine,  
86 metacaine and isoeugenol (available at <http://www.fhi.no/artikler/?id=114175>) (Grave  
87 et al., 2008).

88 The use of PAPs in salmon feed can potentially introduce new chemical undesirables  
89 that have not been previously associated with farmed Atlantic salmon. Of special  
90 interest are pharmaceuticals such as antibacterials, which are used in all sectors of  
91 farming, including poultry and swine production (Toldra et al., 2012). Other  
92 pharmaceutical agents used in terrestrial farmed animals include antiparasitic agents  
93 such as coccidiostats, which are added to poultry feed to cope with protozoa as well as  
94 enhancing animal growth (Ruff, 1999; Chapman, 2014). It should be kept in mind that  
95 the number of pharmaceuticals used in terrestrial animals is more diverse than those  
96 used for fish, and that there are substantial differences in the prescribing patterns of

97 veterinary agents between countries and regions (EMA, 2011). Earlier screening studies  
98 on feather meal from the USA (10 samples) and China (2 samples) for 59  
99 pharmaceuticals, showed the presence of six classes of antimicrobials including  
100 fluoroquinolones, tetracyclines, folic acid antagonists, and streptogramins (Love et al.,  
101 2012). Two of the main antibacterials were enrofloxacin and ciprofloxacin, and studies  
102 in EU also showed the occurrence of these substances in poultry and pork PAPs  
103 (Berntssen et al., 2014). In addition to potentially direct adverse health effects of  
104 pharmaceutical residues in food and non-compliance with food legislation, concern has  
105 been raised for the development of resistant pathogenic microorganisms when exposed  
106 to low non-clinical residue levels (Reig et al., 2009; Blazquez et al., 2012; Gillings et  
107 al., 2013).

108 The list of prohibited and allowed antibiotics in the EU includes many substances which  
109 may co-occur in samples of animal origin. This requires the use of comprehensive  
110 screening methods for detection of these substances (Bohn et al., 2013; Nacher-Mestre,  
111 et al., 2013; Masiá et al., 2014; Turnipseed et al., 2014; Boix et al., 2014). Therefore,  
112 generic sample treatment is advisable to cover as many compounds as possible during  
113 the experimental process. High resolution mass spectrometry (HRMS) allows the  
114 acquisition of full-spectrum accurate-mass data using analyzers such as quadrupole  
115 time-of-flight (QTOF). The coupling of liquid chromatography (LC) with QTOF MS is  
116 nowadays one of the most efficient analytical tools to face the investigation of large  
117 number of medium-high polar organic contaminants and residues in food-safety and  
118 related fields (Ibañez et al., 2012). The present study reports a wide-scope qualitative  
119 screening approach for 1005 permitted and prohibited pharmaceutical residues (also  
120 including metabolites) and dyes in commercially available EU produced PAPs with  
121 potential use in aquafeed.

122 **MATERIAL AND METHODS**

123 **Reagents and chemicals.** 150 reference standards were purchased from Acros Organics  
124 (Geel, Belgium), Aventis Pharma (Madrid, Spain), Bayer Hispania (Barcelona, Spain),  
125 Cerilliant (Round Rock, TX, USA), Fluka (Buchs, Switzerland), Dr. Ehrenstorfer  
126 (Augsburg, Germany), Fort Dodge Veterinaria (Gerona, Spain), National Measurement  
127 Institute (Pymble, Australia), Riedel-de Haën (Seelze, Germany), Sigma Aldrich (St  
128 Louis, MO, USA), Vetoquinol Industrial (Madrid, Spain), and Witega (Berlin,  
129 Germany). All reference materials had purities higher than 98% (w/w), except for  
130 marbofloxacin and pefloxacin, which had purities higher than 93%.

131 HPLC-grade water was obtained from a MilliQ water purification system (Millipore  
132 Ltd., Bedford, MA, USA). HPLC-grade methanol, HPLC-supragradient acetonitrile and  
133 acetone for residue analysis were purchased from Scharlab (Barcelona, Spain). Formic  
134 acid (HCOOH, content > 98%), ammonium acetate (NH<sub>4</sub>Ac, reagent grade) and sodium  
135 hydroxide (NaOH, reagent grade) were supplied by Scharlab. Leucine enkephalin was  
136 purchased from Sigma Aldrich.

137

138 **Samples.** Commercially available PAPs were studied in this work. A total of 19  
139 available non-ruminant PAPs from 6 different types of matrices were provided by the  
140 European Fat Processors and Renderers Association (EFPPRA). The samples had been  
141 produced in different rendering factories. All PAPs were produced according to EU  
142 regulation for PAPs intended for use as feed ingredients (EC, 2001; EC, 2009c). All  
143 PAPs were “category 3 products” which are fit for human consumption at the point of  
144 slaughter as defined by EU-legislation (EC, 2009c). The PAPs obtained included six  
145 different matrices: poultry blood meal (n=4), poultry meal (n=4), feather meal (n=3),  
146 pork blood meal (n=3), pork meal (n=3) and pork greaves (n=2). Samples were stored at

147 -20°C until analysis (within one week). The PAPs included are all produced in central  
148 Europe and are firmly considered to be used for future aquafeeds after the lift of the ban  
149 on these products in the EU food supply chain.

150

151 **Instrumentation.** A Waters Acquity UHPLC system (Waters, Milford, MA, USA) was  
152 coupled to a quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2  
153 QTOF, Waters Micromass, Manchester, UK), with a Z-spray-ESI interface, operating in  
154 both positive and negative ionization modes. An Acquity UHPLC BEH C18 1.7  $\mu\text{m}$   
155 particle size analytical column 2.1 $\times$ 100 mm (Waters) at a flow rate of 300  $\mu\text{L}/\text{min}$  was  
156 employed for chromatographic separation. Mobile phase consisted of water/methanol  
157 gradient both with 0.01% HCOOH and 0.1mM NH<sub>4</sub>Ac. The percentage of organic  
158 modifier (B) was changed linearly as follows: 0 min, 10 %; 14 min, 90 %; 16 min, 90  
159 %; 16.01 min, 10 %; 18 min, 10 %. The column temperature was set at 40 °C. Hybrid  
160 TOF MS resolution was approximately 20,000 at full width half maximum (FWHM), at  
161  $m/z$  556. MS data were acquired in the  $m/z$  range of 50-1000. A capillary voltage of 0.7  
162 kV in positive mode and 2.5 kV in negative mode were used with a cone voltage of 20  
163 V. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface  
164 temperature was set to 650 °C and the source temperature at 130 °C.

165 For MS<sup>E</sup> experiments (also known as all-ion fragmentation or broadband collision-  
166 induced dissociation by other manufacturers), two sequential acquisition functions were  
167 created: in the low energy function (LE), a collision energy of 4 eV is selected,  
168 obtaining a conventional full spectrum where intact (de)protonated molecules/adducts  
169 are commonly observed ; in the high energy function (HE), a linear collision energy  
170 ramp from 15 to 40 eV is applied in order to induce ion fragmentation. In this way,  
171 fragmentation information is obtained in advance for all compounds in a single run

172 without the need for re-injecting the sample in MS/MS mode. Scan time of 0.4 s was  
173 selected.

174 Calibrations were conducted daily from  $m/z$  50 to 1000 with a 1:1 mixture of 0.05M  
175 NaOH:5% HCOOH diluted (1:25) with acetonitrile:water (80:20), at a flow rate of 20  
176  $\mu\text{L}/\text{min}$ . For automated accurate mass measurement (i.e. real-time calibration), a  
177 solution of leucine enkephalin (10 mg/L) in acetonitrile:water (50:50) at 0.1% HCOOH  
178 was used as lock mass and pumped at 30  $\mu\text{L}/\text{min}$  through the lock-spray needle. The  
179 (de)protonated molecule of leucine enkephalin at  $m/z$  556.2771 in positive mode and  
180  $m/z$  554.2615 in negative mode was used for recalibrating the mass axis and ensuring a  
181 robust accurate mass measurement along time. MS data were acquired in centroid mode  
182 and processed by the ChromaLynx XS application manager (within MassLynx v 4.1;  
183 Waters Corporation).

184

185 **Recommended analytical procedure.** A generic sample procedure has been expanded  
186 to PAPs following a screening strategy previously validated for aquaculture matrices  
187 (Nácher-Mestre et al., 2013) and feed samples (Boix et al., 2014). Briefly, PAPs were  
188 thawed at room temperature and 2.5 g of sample (dry weight) was accurately weighed  
189 (precision 0.01g) in centrifuge tubes (50 mL) and homogenized in a Vortex with 10 mL  
190 acetonitrile:water (80:20, v/v) 0.1% HCOOH. After shaking the samples for one hour,  
191 the tubes were placed in an ultrasonic bath during 15 minutes followed by  
192 centrifugation at 4500 rpm for 10 min. Then, an aliquot of 1 mL of supernatant extract  
193 was transferred to an eppendorf vial, diluted with 1 mL of HPLC-grade water and stored  
194 in a freezer (minimum 2 hours) to precipitate proteins. After that, the extract was again  
195 centrifuged at 12000 rpm for 10 min and supernatant was transferred to another vial.

196 Finally, 20  $\mu$ L of the extract (final composition acetonitrile:water (40:60, v/v) 0.05%  
197 HCOOH) was injected into the UHPLC-QTOF MS.

198

199 **Quality assurance.** Sample batches consisted of 20-30 vials, including 4 standards  
200 (only for qualitative purposes) and quality control samples (QCs). The reliability of the  
201 qualitative screening was assessed by the analysis of three QCs per matrix at different  
202 concentration levels. To this aim, six different PAPs (each representing the matrices  
203 studied, see samples section) were spiked with 150 pharmaceutical agents (including 4  
204 dyes, see Sup. info for more details) at three different concentration levels (0.02, 0.1 and  
205 0.2 mg/kg), and analyzed together with their respective “blanks”. Additionally, two  
206 method blanks were analyzed to assure that no laboratory contamination was introduced  
207 in the procedure. This approach was carried out following the spirit of European  
208 guidelines (EC, 2002; EC, 2013b).

209

## 210 **RESULTS AND DISCUSSION**

211 **UHPLC-QTOF MS screening.** Satisfactory chromatographic peaks, i.e. at least 10  
212 data points per peak, for all 150 compounds were obtained. Formic acid (0.01 %) was  
213 added to both water and methanol mobile phase solvents, favoring the formation of the  
214 protonated molecule in positive ionization mode. The addition of ammonium acetate in  
215 the mobile phase allowed improving peak shapes (Gracia-Lor et al., 2010). In general,  
216 the best results in terms of sensitivity were obtained under positive ionization mode  
217 selecting the protonated molecule as the main diagnostic ion.

218 With the previous chromatographic conditions, the screening was applied on the basis  
219 of a compound database that is being continuously updated (Nácher-Mestre et al., 2013;  
220 Boix et al., 2014). At this stage, new pharmaceuticals were included into our previous

221 lists in order to investigate their possible presence in PAP matrices of interest for  
222 salmon aquafeed. Individual standard solutions were injected to study MS  
223 fragmentation of each target analyte, achieving a final list of 150 analytes, which were  
224 also used for quality control of the qualitative screening. The selected compounds were  
225 representative from different families with quite different chemical-physical  
226 characteristics and were used to test the applicability of the screening. Briefly, 75% of  
227 the 150 compounds used in QC analysis were detected in all matrices at 0.2 mg/kg and  
228 65% at 0.1 mg/kg, decreasing down to 30% at the lowest spiking level of 0.02 mg/kg.  
229 Many of the compounds that could not be detected in all spiked samples were however  
230 detected in some of them, sometimes even in 5 out of 6 samples. In those cases, we did  
231 not consider the screening as satisfactory at the concentration level tested, as the  
232 requirement was to have 100% of positive samples detected. This was mostly due to the  
233 high variability in matrix composition between the samples used for preparing QCs.

234 In addition to these 150 compounds, up to 855 compounds were searched in the samples  
235 making a total target list of 1005 compounds. The compound database used can be  
236 found in supporting information, including Retention time (Rt) (when the reference  
237 standard was available) and the elemental composition used for characterization. With  
238 so high number of compounds, it might be possible that some of them co-elute (for  
239 example, sulfaquinoxaline and sulfathiazole at 6.51 min). Under this situation, UHPLC  
240 was valuable for choosing almost co-eluting fragment ions that would correspond to the  
241 same precursor avoiding spectrum interferences that would complicate the identification  
242 process. Not only the retention time but also the chromatographic peak shape were  
243 taken into account to match each fragment ion to the correct “precursor ion”. Anyway,  
244 as a hybrid QTOF was used, additional MS/MS experiments could be performed, if  
245 necessary, in analysis of real-world samples for confirmation of positives in case of

246 doubt. For adequate retention time comparison, LC conditions used (LC column, mobile  
247 phase and gradient) should be the same than those used when creating the database.

248 **Detection and identification criteria.** Figure 1 illustrates the criteria used in this work.

249 The parameters considered for a satisfactory detection and identification were  $R_t$ , mass

250 accuracy (i.e. mass errors),  $q_i/Q$  ratios ( $Q$ : the most abundant ion –commonly the

251 (de)protonated molecule;  $q_i$ : fragment ion –or occasionally an adduct) and the isotopic

252 distribution pattern (especially for Cl and Br) (Hernández et al., 2015). Requirements

253 and interpretation of results were in agreement with Commission Decision

254 2002/657/EC, which applies to the monitoring of certain substances and residues

255 thereof in live animals and animal products (EC, 2002). In addition, SANCO guideline

256 for pesticide residue analysis in food was of help in terms of mass accuracy

257 requirements (EC, 2013b). The key parameters when testing the screening were

258 detection and identification of the compound in the sample at the concentration levels

259 tested. Neither recovery nor precision were calculated (contrarily to quantitative

260 methods), which is in the line of the guidelines used nowadays in food safety or doping

261 control analysis (EC, 2002; EC, 2013b; FDA, 2015; WADA 2010). Several situations

262 were considered in the screening depending on the reference standard availability

263 (Figure 1):

264 1) Reference standard available:

- 265 - Detection was considered satisfactory when the most abundant ion ( $Q$ ),  
266 commonly the (de)protonated molecule, was found at the expected  $R_t$  (deviation  
267 accepted  $\pm 2.5\%$  in comparison with the reference standard) (EC, 2002) and  
268 mass error was below 5 ppm (named as “d1”) (EC, 2013b). Another likely  
269 situation for detection was to find two representative  $m/z$  ions (i.e. the most  
270 abundant ion ( $Q$ ) and/or minor fragment/adduct ions ( $q$ )) for the target

271 compound at the expected  $R_t$ , but with mass errors between 5-20 ppm (named as  
272 “d2”). The latter situation seemed to occur when the signal intensity was low  
273 (favored at low analyte concentrations). In that case, an additional effort is  
274 recommended to investigate more accurate-mass ions and/or repeat sample  
275 injection, if possible.

276 - Identification was based on the presence of at least two representative  $m/z$  ions  
277 (Q, q) at the expected  $R_t$  ( $\pm 2.5\%$ , in comparison with the reference standard)  
278 with mass errors below 5 ppm. Additionally, q/Q ratios should fit with those for  
279 reference standards within tolerance limits admitted which range from 20% to  
280 50 % depending on the q/Q ratio (<0.1:50%, 0.1-0.2:30%, 0.2-0.5:25% and,  
281 >0.5%:20%) (EC, 2002). Identification under these conditions was highly  
282 reliable and it was considered as the ideal situation.

283 In both cases, the characteristic isotopic pattern (e.g. when ions as Cl or Br are  
284 present) should be observed.

285 2) Reference standard not available:

286 - Tentative identification could be made when an expected ion with mass error below  
287 5 ppm was observed, together with its characteristic isotopic pattern. Subsequently,  
288 the fragment ions ( $q_i$ ) (or characteristic isotopic ions) were evaluated. For this  
289 purpose, different possibilities are available, such as comparing experimental  
290 MS(/MS) spectra or the main fragment ions with those reported in the literature  
291 (massbank, METLIN public library), or justifying the accurate-mass fragments  
292 taking into account the structure of the molecule. So, tentative identification  
293 required the presence of one or more fragment ions (q) compatible with the  
294 chemical structure of the candidate (mass error < 5 ppm) and/or in agreement with  
295 previous data reported in literature. Retention time prediction may also help in this

296 process (focusing research only on “predicted” peaks) (Bade et al., 2015). Although  
297 tentative identification was strongly supported by accurate-mass data obtained, the  
298 confirmation of the identity requires the injection of the reference standard.

299

300 **Screening of PAP samples.** The HRMS screening developed allows research  
301 laboratories to apply a cost-effective strategy to extend their analytical scope to analytes  
302 which might potentially be present in the samples even without the need of having all  
303 reference standards (EC, 2013b). In this way, laboratories do not need to acquire all  
304 reference standards before analysis, with the subsequent problems of availability (e.g.  
305 metabolites and transformation products), costs and expiry dates. Accurate-mass full-  
306 spectrum acquisition capabilities of the TOF analyzer made feasible the investigation of  
307 many undesirables in a single run injection. In absence of standards, the only  
308 information available for data processing was the elemental composition (i.e. accurate-  
309 mass), but a tentative identification could be made if sufficient information was  
310 obtained from analyses (see Figure 1).

311 With the objective to support the reliability of the screening procedure, figure 2 shows a  
312 sample matrix (poultry meal) fortified with the antibiotic enrofloxacin. The standard of  
313 this fluoroquinolone was previously injected, selecting two representative  $m/z$  ions, the  
314 protonated molecule (Q) and one fragment ion (q) for the reliable identification (Figure  
315 2-A). Figure 2-B shows the same  $m/z$  ions of a quality control sample at 0.1 mg/kg  
316 supporting the suitability of the method at this concentration level (mass deviations and  
317 q/Q were satisfactory). Finally, figure 2-C shows the identification of enrofloxacin in a  
318 PAP sample. The presence of two  $m/z$  ions, measured at the expected retention time,  
319 with acceptable mass deviations and appropriate q/Q ratios, allowed to identify this  
320 compound in that sample.

321 After applying the described screening strategy to 19 PAP samples, pharmaceutic  
322 agents like monensin, flumequine, enrofloxacin, trimethoprim, tylosin A,  
323 acetaminophen, salicylic acid, oxyphenylbutazone, and the dye leucocrystal violet  
324 (metabolite of the crystal violet dye) were found (Table 1).

325 Monensin is an antibiotic also allowed as a coccidiostat feed additive with an  
326 established maximum residue level (MRL is 1.25 mg/kg for feed materials) for  
327 unintended cross-contamination to non-target animal feed (EC, 2009b). The antibiotics  
328 flumequine, enrofloxacin, trimethoprim and tylosin A, are authorized pharmaceutical  
329 products in the EU with set MRLs in fish products (EC, 2009a); however in Norway,  
330 these pharmaceuticals have not been registered for aquaculture use for the last decade  
331 (Grave et al., 2008). The analgesics acetaminophen (paracetamol) is allowed to use for  
332 porcine, and salicylic acid is allowed to use for all food producing species except fin  
333 fish. Oxyphenylbutazone is a non-steroidal anti-inflammatory drug (NSAID) which is  
334 not listed as an authorized drug in the EC directive (EC, 2009b), and crystal violet is a  
335 potential carcinogenic pharmaceutical triphenylmethane dye, illegal for use in food-  
336 producing animals in the EU (Serratos et al., 2006). As opposed to the other  
337 pharmaceuticals identified in the study, crystal violet is not allowed to be used in the  
338 food production chain in the EU, and its potential source in PAPs is hence unclear.  
339 However, crystal violet can be unintentionally introduced as marker dye to identify treated  
340 farm animals. Outside the EU it is used as an antimicrobial agent to prevent the fungal  
341 growth in animal feeds (Mani and Bharagava, 2016).

342 Figure 3 illustrates the identification of trimethoprim, flumequine and salicylic acid in  
343 different PAP samples analyzed. Figure 3-A corresponds to trimethoprim found in  
344 feather meal. Three representative  $m/z$  ions with mass deviations below 5 ppm were  
345 observed together with acceptable  $q/Q$  ratios. Figure 3-B corresponds to flumequine,  
346 also in feather meal, where two representative  $m/z$  ions were present with acceptable

347 mass errors and  $q/Q$  deviations (<25%). However, in this sample the fragment with  $m/z$   
348 174.036 was not clearly observed. Finally, figure 3-C shows the identification of  
349 salicylic acid in pork blood meal with again two representative  $m/z$  ions. The fact that  
350 salicylic acid is a small molecule made that few fragments were available under the  
351 experimental conditions applied. Both salicylic acid and acetylsalicylic acid are allowed  
352 to be used in all food producing species except fin fish or use in animals from which  
353 milk or eggs are produced for human consumption (EC, 2009a). The presence of this  
354 analgesic might be related to the previous administration of acetylsalicylic acid in  
355 animals, a known pain reliever, anti-inflammatory and antipyretic drug, producing  
356 salicylic acid as a metabolite. Besides this, salicylic acid is also used as an agent against  
357 fungal diseases in the skin of animals. However, since salicylic acid is known to be  
358 found in several plants (Venema et al., 1996; Scotter et al., 2007), it could also be  
359 present in feed ingredients for livestock and subsequently in by-products from livestock  
360 animals. Furthermore, salicylic acid has been previously detected in man and animals  
361 not medicated by salicylic acid derived therapeutics (Paterson et al., 2008).

362 Figure 4 illustrates two situations where the compounds detected in poultry blood meal  
363 could not be fully identified. Figure 4-A shows two  $m/z$  ions for leucocrystal violet but  
364 the  $m/z$  239.1548 ( $q_1$ ), corresponding to the fragment  $C_{16}H_{18}N_2$ , presented mass error  
365 higher than 5 ppm and further study of other fragment ions failed. In addition,  $q_1/Q$  ratio  
366 was higher than 20% (tolerance permitted for  $q/Q > 0.5$ ). Therefore, the identification  
367 criteria were not met, and we considered this compound as detected but not identified  
368 (d2 following criteria from Figure 1). A similar situation occurred with tylosin A  
369 (Figure 4-B) for which three representative ions were found at the same  $R_t$ , in  
370 agreement with the standard. However, it was reported only as detected (d2) because all  
371 the ions presented high mass errors. This might be due to the high mass of this  
372 compound, very close to the higher end of the mass axis calibration range. Although it

373 seemed that both compounds were present in the sample, they could not be identified in  
374 a reliable way; therefore, further analysis should be carried out for confirmation of their  
375 identity.

376 The data processed also revealed the suspicious presence of ciprofloxacin and dyes such  
377 as crystal violet, malachite green and leucomalachite green in PAPs, but with such a  
378 low intensity that a second target analysis with a higher sensitivity technique (e.g. LC-  
379 MS/MS with triple quadrupole) would be necessary. Thus, further work is required for  
380 those compounds identified (or tentatively identified) by this qualitative screening in  
381 order to confirm their presence and determine the concentration levels in the samples.  
382 This could be ideally made by LC-MS/MS using target quantitative methods developed  
383 for the compounds revealed by QTOF MS screening.

384 In conclusion, the LC-HRMS screening methodology developed in this work has  
385 revealed the presence of some pharmaceutical agents and marker dyes in re-authorized  
386 PAPs that might be introduced in novel salmon feed. The qualitative validation was  
387 performed for 150 compounds, and it was satisfactory for a notable number of analytes.  
388 Most of compounds that could not be detected in 100% of the spiked samples were  
389 however detected in several of them (in some cases up to 5 out of 6 samples),  
390 illustrating that the screening was satisfactory in nearly all matrices tested. The lack of  
391 sensitivity seemed to be the main reason for the non-detections, a drawback that can be  
392 solved with the last-generation HRMS instruments that offer much better sensitivity than  
393 the QTOF MS used in the present work.

394 Although the screening was tested for a limited number of compounds, it might be  
395 assumed to be applicable to the wide majority of the remaining compounds included in  
396 the database, as the contaminants used for validation covered a broad range of  
397 physicochemical properties. Obviously, the only way to ensure a completely reliable

398 screening would be performing a full validation for all compounds, a task that seems  
399 extremely complicated when dealing with hundreds of analytes. Yet, in the present  
400 work, one can ensure the positive findings owing to the power of accurate-mass full  
401 spectrum acquisitions in HRMS, reaching reliable tentative identifications even when  
402 the standard is not available. Nevertheless, these tentative identified compounds should  
403 be confirmed by acquiring the reference standard in a subsequent step. A major  
404 advantage of this approach is that laboratories do not need to purchase hundreds of  
405 standards for compounds that in many cases will never be found in the samples. In fact,  
406 standards only need to be purchased in a subsequent step on the basis of the tentative  
407 identifications in the samples. However, this type of screening has the drawback that  
408 false negative results may occur for the compounds that have not been subjected to  
409 validation. The proposed strategy is of interest for food safety and public health control  
410 authorities as the presence of unintended background levels of illegal pharmaceutical  
411 dyes, unreported antibiotics, or unauthorized anti-inflammatory agents in salmon feed  
412 ingredients might give cause to legal actions. The approach presented in this paper can  
413 be considered as a useful risk assessment tool for feed industry in order to widen the  
414 knowledge on novel ingredients and also traditional ingredients under use. Also of  
415 interest is the fact that accurate-mass full-spectrum acquisition allows widening the  
416 search to other compounds not included in this work. This can be done at any time in  
417 the future by retrospective evaluation of data obtained without additional analysis. In  
418 the near future, routine monitoring and quantification of the compounds detected will be  
419 carried out due to the potential implications in food safety.

420

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430 assistance regarding retention time prediction.

431

#### 432 **SUPPORTING INFORMATION**

433 The compound database used in this work can be found in supporting information,  
434 including Retention time (Rt) (when the reference standard was available) and the  
435 molecular composition used for characterization.

436

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617 **Figure captions.**

618

619 **Figure 1.** Scheme of detection and identification criteria for HRMS screening used in  
620 this work.

621 **Figure 2.** nw-XICs for enrofloxacin in: (A) standard in solvent at 250 ng/mL; (B)  
622 quality control of poultry meal A at 0.1 mg/kg; (C) poultry meal C sample. In each case,  
623 chromatograms for the LE function (bottom) and HE (top) are shown to illustrate the  
624 presence of the protonated molecule (LE) and fragment ions (HE). ✓ Accurate mass  
625 deviations within tolerance limits (below 5 ppm). ✓  $q_i/Q$  ratio tolerance accepted by  
626 Com. Decision 2002/657/EC.

627 **Figure 3.** nw-XICs illustrating the identification of: (A) trimethoprim in feather meal;  
628 (B) flumequine in feather meal; (C) salicylic acid in pork blood meal. ✓ Accurate mass  
629 deviations within tolerance limits (below 5 ppm). ✓  $q_i/Q$  ratio tolerance accepted by  
630 Com. Decision 2002/657/EC.

631 **Figure 4.** nw-XICs illustrating the detection of: (A) leucocrystal violet in poultry blood  
632 meal A; (B) tylosin A in poultry blood meal. ✓/✗ Accurate mass deviations  
633 within/without tolerance limits. ✓/✗  $q_i/Q$  ratio tolerance accepted/not permitted by  
634 Com. Decision 2002/657/EC.

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637 **Table 1. Compounds detected and identified from the LC-HRMS screening.**

<b>Pharmaceuticals &amp; Dyes</b>	Poultry blood meal A	Poultry blood meal B	Poultry blood meal C	Poultry blood meal D	Poultry meal A	Poultry meal B	Poultry meal C	Poultry meal D	Feather meal A	Feather meal B	Feather meal C	Pork blood meal A	Pork blood meal B	Pork blood meal C	Pork meal A	Pork meal B	Pork meal C	Pork greaves A	Pork greaves B
Acetaminophen	-	-	-	-	-	d1	-	d1	-	-	-	-	-	-	-	-	-	d1	-
Enrofloxacin	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	-	-	-	-
Flumequine	-	-	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	-	-
Leucocrystal violet	d2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Monensin	-	-	-	-	-	-	d1	-	-	-	-	-	-	-	-	-	-	-	-
Oxyphenylbutazone	-	d1	-	-	-	-	-	-	-	-	-	-	-	d1	-	-	-	-	-
Salicylic acid	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	d1	d1	d1	d1	d1
Trimethoprim	-	-	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	-	-
Tylosin A	-	-	-	d2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

638 d: detected (d1 and d2 are defined in Figure 1); ✓: identified.

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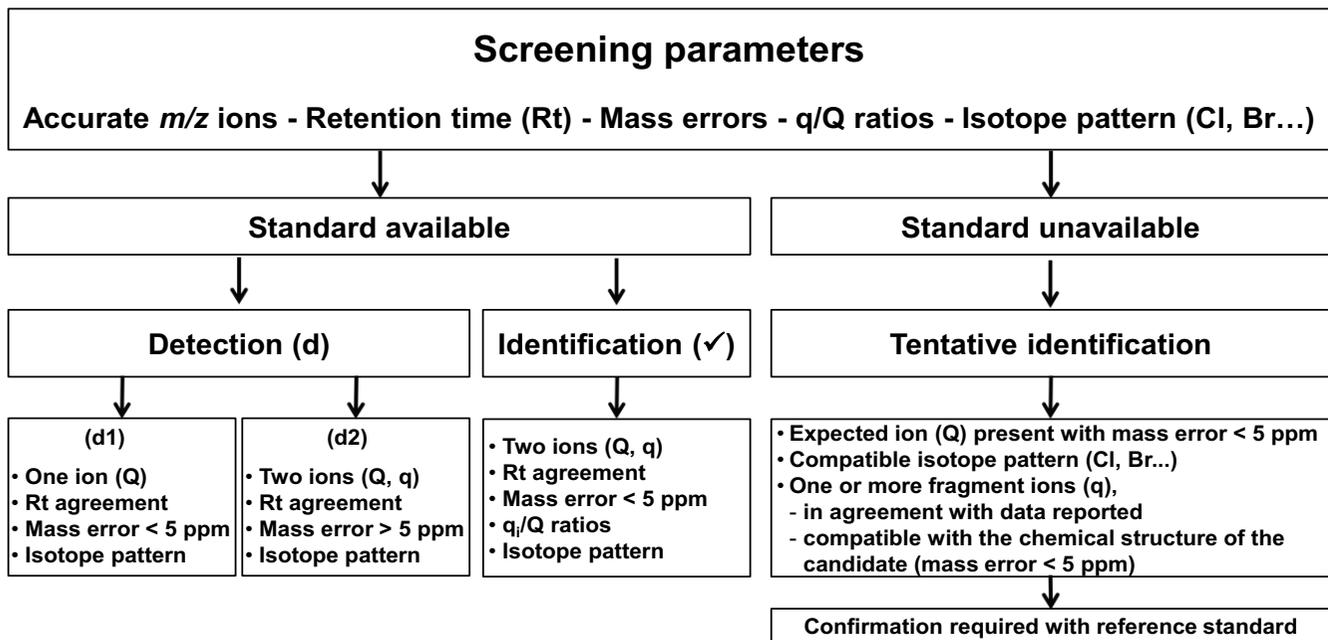
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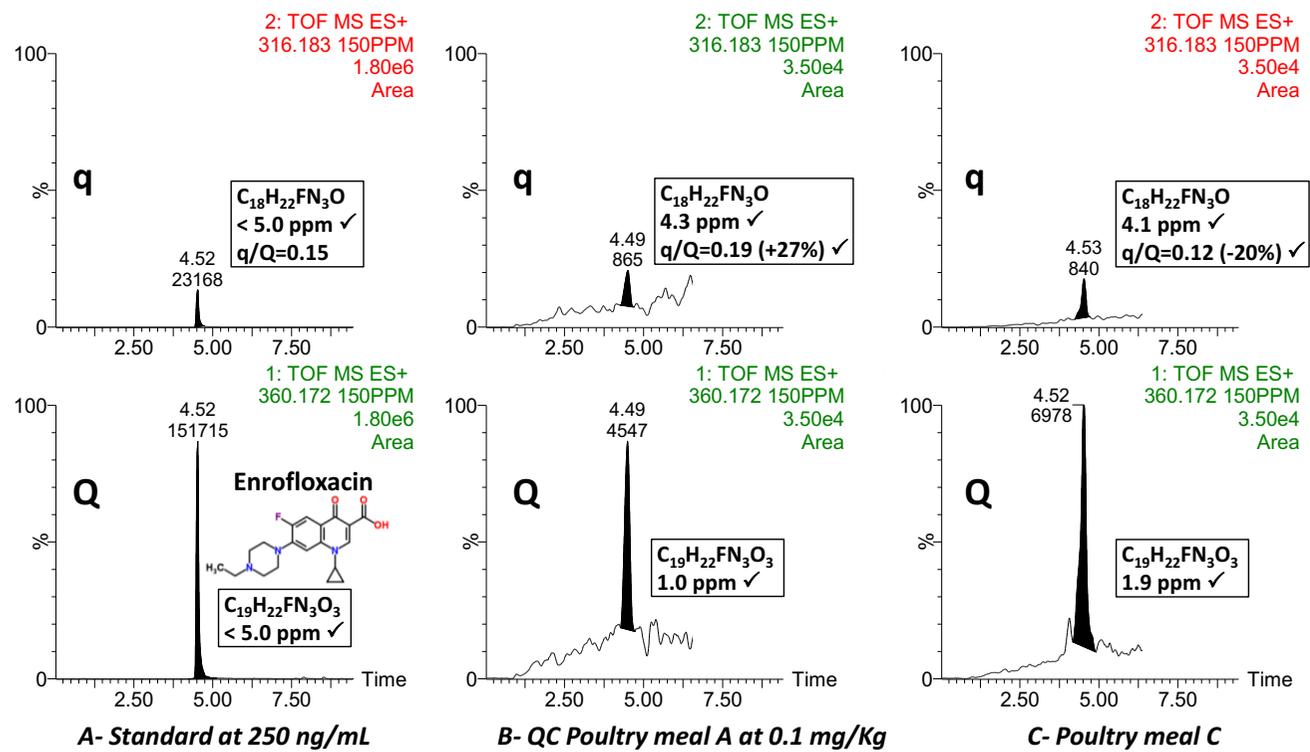
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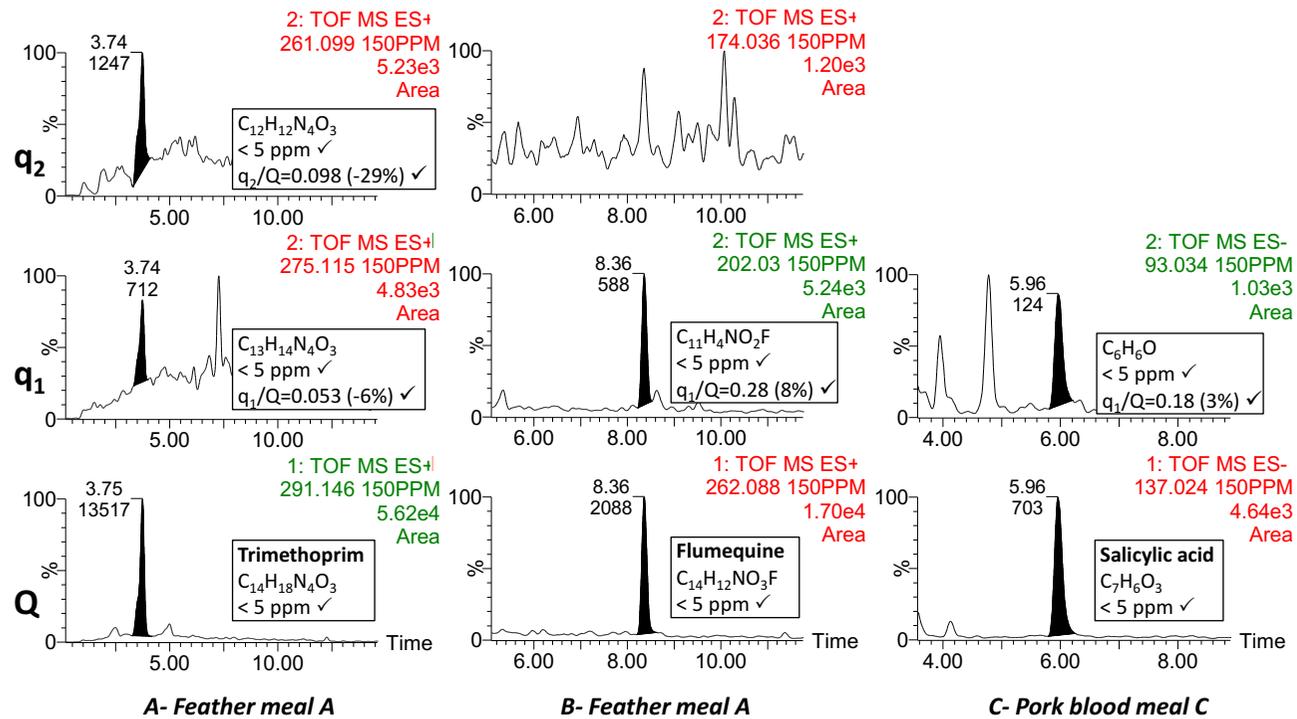
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646 **Figure 1.**



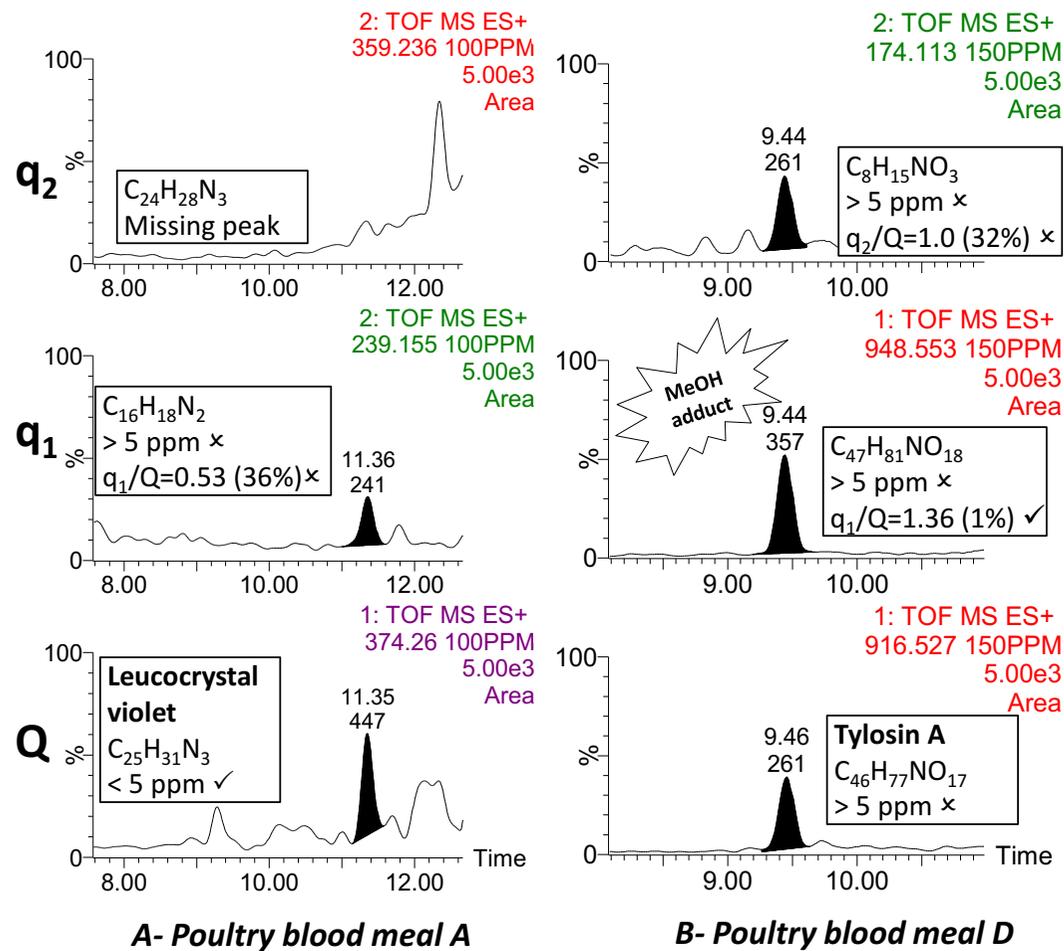
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648 **Figure 2.**



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650 **Figure 3.**



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652 Figure 4.