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1 **Qualitative screening of undesirable compounds from feeds to fish by**

2 **liquid chromatography coupled to mass spectrometry.**

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Abstract

This article describes the development, validation and application of a rapid screening method for the detection and identification of undesirable organic compounds in aquaculture products. A generic sample treatment was applied without any purification or preconcentration step. After extracting the samples with acetonitrile/water 80:20 (0.1% formic acid), the extracts were centrifuged and directly injected in the LC-HRMS system, consisting of ultra-high performance liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (UHPLC-QTOF MS). A qualitative validation was carried out for over 70 representative compounds, including antibiotics, pesticides and mycotoxins, in fish feed and fish fillets spiked at 20 μg/Kg and 100 μg/Kg. At the highest level, the great majority of compounds were detected (using the most abundant ion, typically the protonated molecule) and unequivocally identified (based on the presence of two accurate-mass measured ions). At the 20 μg/Kg level, many contaminants could already be detected although identification using two ions was not fully reached for some of them, mainly in fish feed due to the complexity of this matrix. Subsequent application of this screening methodology to aquaculture samples made it possible to find several compounds from the target list, such as the antibiotic ciprofloxacin, the insecticide pirimiphos-methyl and the mycotoxins fumonisin B2 and zearalenone. A retrospective analysis of accurate-mass full-spectrum acquisition data provided by QTOF MS was also made, without neither reprocessing nor injecting the samples. This allowed the detection and tentative identification of other organic undesirables different than those included in the validated list.

Keywords: aquaculture, fish feed, liquid chromatography, mass spectrometry, screening, organic contaminants, QTOF MS, qualitative validation

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53 **INTRODUCTION**

54 Numerous undesirable organic contaminants have been regulated by European guidelines in the
55 food safety field. [1-3] Updated guides have included mycotoxins and antibiotics which should be
56 monitored as regards the risk management in animal feed. [4, 5] Moreover, the great majority of
57 feeds for animal farming contain plant raw materials which may contain residues of pesticides,
58 frequently used in agriculture practices. This fact raises the need to develop analytical strategies
59 based on a multiclass screening able to monitor many undesirables from different chemical
60 families in a single method.

61 Aquaculture represents only one example of animal farming. It has undergone a notable growth
62 rate, mainly due to the decrease in marine wild fish stocks and the increase in consumption of
63 seafood.[6] The huge demand of fish raw materials to produce fish feed in aquaculture, makes it
64 necessary to find alternatives for new fish feed production. This implies new raw materials, new
65 feed formulations and, as a consequence, wide research on their application in aquaculture.[7,8] It
66 is necessary to ensure that new generations of feed and seafood are safe and healthy for fish
67 growing, and also that farmed fish for human consumption is free from banned undesirables or
68 that contains concentrations lower than maximum limits established.[4, 5] New undesirable
69 substances could be in the new final product in addition to others commonly found in marine
70 samples.[9-13]

71 The results obtained in a previous project (www.aquamaxip.eu) on the basis of target analysis,
72 focused on persistent organic pollutants (POPs), demonstrated that organochlorine compounds,
73 polycyclic aromatic hydrocarbons and polybrominated diphenyl ethers were present in feed and
74 raw materials for sea bream and also in sea bream fillets at trace levels.[7, 8, 11, 13] In the present
75 research, the analytical strategy was directed toward a multiclass screening able to easily and
76 rapidly detect and identify a large number of suspected compounds in the samples studied. To
77 this aim, a generic and rapid non-destructive extraction was applied trying to avoid possible
78 losses of the compounds of interest during the sample treatment. The method developed has
79 been tested in some of the most common fish species in Europe: salmon (*Salmo salar*), sea bass

80 (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), sole (*Solea solea*) and turbot (*Scophthalmus*
81 *maximus*), together with commercially available feeds for these species. The methodology was
82 qualitatively validated on the basis of European analytical guidelines.[14-16]

83 LC-QTOF MS has shown strong potential for screening and confirmation of organic
84 contaminants in the environment.[17-22] Full spectrum acquisition sensitivity, together with its
85 excellent mass accuracy, facilitate performing wide-scope screening using target and non-target
86 approaches.[17] Moreover, it is possible to make a retrospective data evaluation at any time
87 searching for additional compounds without the need for performing additional analysis. QTOF
88 MS allows working under MS^E mode, i.e. simultaneous acquisition at low (LE) and high
89 collision energy (HE), which provides useful information on the (de)protonated molecule
90 (commonly at LE) and on the main fragment ions (commonly at HE). On the basis of this
91 information, and on isotopic distribution observed in the spectra, the reliable identification of
92 the compounds detected in the samples is feasible.

93 Until now, LC-QTOF MS has been scarcely employed for monitoring the presence of organic
94 contaminants in fish origin raw materials, fish and feed [23-24]. In fact, LC-MS techniques have not
95 been used much for analysis of this type of fatty samples. The vast majority of papers reported
96 in the marine field are focused on the determination of POPs using GC-MS. In a few cases, LC-
97 MS has been applied for compounds like specific flame retardants and perfluorinated
98 compounds.[25, 26] As regards LC-TOF MS, very little has been published in the marine field [27, 28].
99 Villar-Pulido et al.[27] reported a multiclass detection methodology in order to detect antibiotics
100 and veterinary drugs in shrimps and Peters et al. [28] reported a multi-residue screening of
101 veterinary drugs in several fish samples showing that TOF is one of the most powerful tools for
102 multicompound analysis.

103 The aim of the present work is to develop modern screening methodology that allows the rapid
104 detection and identification of a large number of LC-(ESI)-amenable undesirable compounds in
105 animal feed and fish. To achieve this outcome, a generic sample extraction followed by
106 UHPLC-QTOF MS has been used, and the procedure has been validated selecting

107 representative undesirables from antibiotics, pesticides and mycotoxins. Moreover, the use of
108 LC-MS/MS was assayed for confirmation of positive samples that were detected by QTOF
109 screening but were present at very low concentration levels. The application of QTOF MS for
110 post-target screening of many other contaminants not included in the validated list was
111 evaluated.

112

113 **MATERIAL AND METHODS**

114 **Reagents and chemicals.** In this work, up to 35 antibiotics, 36 pesticides and 11 mycotoxins
115 were selected as representative compounds in order to validate the methodology. Reference
116 standards of sulfamethoxazole, sulfamethazine, sulfadiazine and sulfathiazole were from Across
117 Organics (Geel, Belgium). Enrofloxacin, moxifloxacin and ciprofloxacin were from Bayer
118 Hispania (Barcelona, Spain). Sarafloxacin, marbofloxacin and pefloxacin were provided by Fort
119 Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid Spain) and Aventis Pharma
120 (Madrid, Spain), respectively. The rest of antibiotics were supplied by Sigma-Aldrich (St Louis,
121 MO, USA) or Fluka (Buchs, Switzerland). All antibiotic standards presented purity higher than
122 93%. Pesticide reference standards were purchased from Dr. Ehrenstorfer (Augsburg,
123 Germany), Riedel-de Haën (Seelze, Germany) or Sigma-Aldrich (St. Louis, MO, USA). All
124 mycotoxins standards (>99% purity) were supplied by Sigma Aldrich (Madrid, Spain).

125 For antibiotics and mycotoxins, individual stock standard solutions were prepared by dissolving
126 solid standard in acetonitrile with the exception for antibiotic quinolones, which were dissolved
127 in methanol and required the addition of 100 μL of 1M sodium hydroxide for their proper
128 dissolution. Regarding pesticides, individual stock standard solutions were prepared by
129 dissolving solid standard in acetone. Working solutions of antibiotics, pesticides and
130 mycotoxins, respectively, were obtained after mixing individual stock solutions of each family
131 and diluting with water to give a final concentration of around 500 ng/mL for sample
132 fortification and injection in the chromatographic system. Stock solutions were stored in a
133 freezer at -20 ºC and working solutions were stored in a fridge.

134 HPLC-grade water was obtained from a MilliQ water purification system (Millipore Ltd.,
135 Bedford, MA, USA). HPLC-grade methanol, HPLC-grade acetonitrile and acetone for residue
136 analysis were purchased from Scharlau (Barcelona, Spain). Formic acid (HCOOH, content >
137 98%) and ammonium acetate (NH_4Ac, reagent grade) were supplied by Scharlau.

138

139 **Samples.** Commercially available fish feeds for sea bream, salmon, sole, sea bass and turbot
140 were used for validation purposes. These feeds represent the new trends of alternative feed
141 production in European aquaculture. For a given species, two pellet sizes representative of those
142 used over the course of the production cycle were selected, giving a total number of 10 samples
143 subjected to validation. Samples were stored at -20°C until analysis.

144 Then cultured fish were selected for validation consisting of six sea breams with different
145 weights, collected from the Instituto de Torre la Sal, Castellón, Spain (IATS, CSIC), and four
146 commercially available cultured fishes of salmon, sole, sea bass and turbot that were purchased
147 directly from city supermarkets. The fillets (denuded from skin and bone) were excised and
148 stored at -20 ºC until analysis.

149 In addition to the samples used for validation, the developed methodology was applied to other
150 feeds and fishes. Five experimental sea bream feeds with different plant compositions were
151 collected from IATS. Additionally, three feeds for floating turbot, sole and sea bass were
152 collected from IATS experiments and two salmon feeds were also obtained from salmon
153 growing experiments. As regards fish, eight fish samples (panga, pollack, salmon, sole, sea
154 bass, sea bream and turbot fillets and fish fingers) were directly purchased from supermarkets
155 and three sea bream fillets from other growing experiments were also collected from IATS
156 facilities.

157

158 **Liquid Chromatography.** A Waters Acquity UHPLC system (Waters, Milford, MA, USA)
159 was employed for chromatographic separation using an Acquity UHPLC BEH C18 1.7 µm
160 particle size analytical column 2.1×100 mm (Waters) at a flow rate of 300 µL/min. Mobile

161 phase consisted of water/methanol gradient both with 0.01% HCOOH and 0.1mM NH_4Ac. The
162 percentage of organic modifier (B) was changed linearly as follows: 0 min, 10 %; 14 min, 90 %;
163 16 min, 90 %; 16.01 min, 10 %; 18 min, 10 %. The column temperature was set to 60 ºC.

164

165 **Mass spectrometry.** A hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (Q-
166 oaTOF Premier, Waters Micromass, Manchester, UK), with an orthogonal Z-spray-ESI
167 interface operating in positive ion mode was used. TOF MS resolution was approximately
168 10,000 at full width half maximum (FWHM), at *m/z* 556.2771. MS data were acquired on the
169 *m/z* range of 50-1000. The microchannel plate (MCP) detector potential was set to 2050 V. A
170 capillary voltage of 3.5 kV and cone voltage of 25 V were used. Collision gas was argon
171 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 350 ºC and the source
172 temperature to 120 ºC. For MS^E experiments, two acquisition functions with different collision
173 energies were created: the low energy function (LE), selecting a collision energy of 4 eV, and
174 the second one, the high energy (HE) function, with a collision energy ramp ranging from 15 eV
175 to 40 eV in order to promote in-source fragmentation. The LE and HE functions settings were
176 for both a scan time of 0.2 s and an inter-scan delay of 0.05 s.

177 Calibrations were conducted from *m/z* 50 to 1000 with a 1:1 mixture of 0.05M NaOH:5%
178 HCOOH diluted (1:25) with acetonitrile:water (80:20), at a flow rate of 10 mL/min. For
179 automated accurate mass measurement, the lock-spray probe was used, using as lockmass a
180 solution of leucine enkephalin (2mg/L) in acetonitrile:water (50:50) at 0.1% HCOOH pumped
181 at 30 μL/min through the lock-spray needle. A cone voltage of 95V was selected to obtain
182 adequate signal intensity for this compound (~500 counts). The protonated molecule of leucine
183 enkephalin at *m/z* 556.2771 was used for recalibrating the mass axis and ensuring a robust
184 accurate mass measurement along time. It should be noted that all the accurate masses shown in
185 this work have a deviation of 0.55 mDa from the "true" value because MassLynx software uses
186 the mass of hydrogen instead of a proton when calculating $[M+H]^+$ accurate mass. However, as
187 this deviation is also applied during mass axis calibration, there is no negative impact on the
188 mass errors presented in this article. MS data were acquired in centroid mode and were

189 processed by the ChromaLynx XS application manager (within MassLynx v 4.1; Waters

190 Corporation).

191 A triple quadrupole analyser (Waters Corp., Milford, MA, USA) operating in MS/MS was used

192 for the analysis of positive samples from the screening. Drying gas as well as nebulising gas

193 was nitrogen generated from pressurized air in a N_2 LC-MS (Claind, Teknokroma, Barcelona,

194 Spain) and the collision gas was argon (99.995%; Praxair, Madrid, Spain) with a pressure of

195 approximately 4.10^{-3} mbar in the collision cell. A capillary voltage of 3.5 kV in positive

196 ionization mode was applied. The desolvation gas temperature was set to 500ºC and the source

197 temperature to 120ºC. Temperature column was set to 40ºC. Dwell times of 0.030 s/scan were

198 chosen. TargetLynx application manager (MassLynx v 4.1) software was used to process the

199 data obtained from standards and samples.

200

201 **Recommended analytical procedure.** Before analysis, feed samples were thawed at room

202 temperature and ground using a Super JS mill from Moulinex (Bagnolet Cedex, France). Fish

203 fillets were also thawed at room temperature and processed in a crushing machine (Thermomix,

204 Vorwerk España M.S.L., S.C., Madrid). As a result, homogenized samples were obtained in

205 both cases. The recommended procedure was the following: 5 g of sample was accurately

206 weighed (precision 0.1 mg), transferred to centrifuge tubes (50 mL) and homogenized in a

207 Vortex with 10 mL acetonitrile:water (80:20) 0.1% HCOOH. After shaking the samples (S.B.S.

208 Instruments S.A, Barcelona, Spain) for one hour, tubes were placed in an ultrasonic bath during

209 15 minutes followed by centrifugation at 4500 rpm for 10 min (Consul centrifuge, Orto-Alresa,

210 Madrid, Spain). Approximately 2 mL of supernatant extract was transferred to an eppendorf vial

211 and stored in a freezer (minimum 2 hours) in order to precipitate proteins. Expired this time, the

212 extract was centrifuged again at 12000 rpm for 10 min. Finally, the supernatant extract was

213 injected into the UHPLC-QTOF MS system.

214

215 **Method validation.** Validation of the screening method was performed for qualitative purposes
216 on the basis of European analytical guidelines.[14-16] Ten different samples of each feed and fish
217 were spiked at two levels, 20 µg/Kg and 100 µg/Kg, and analyzed together with their non-
218 spiked samples ("blanks"). Additionally, two method blanks were analyzed to assure that no
219 laboratory contamination was introduced in the procedure. It is noteworthy that mycotoxins
220 were only evaluated in feed as their presence was not expected in fish.

221 The screening detection limit (SDL) and limit of identification (LOI) were investigated as the
222 main validation parameters to estimate the threshold concentration at which detection and
223 identification become reliable, respectively. These parameters were established as the lowest
224 concentration tested at which a compound was detected/identified in all spiked samples under
225 study (n=10, at each level) independently of its recovery and precision (details in **Table 1**). The
226 detection was made by using the most abundant ion measured at its accurate mass (typically the
227 protonated molecule). For the reliable identification, the presence of two *m/z* ions was required.
228 This means that, at least, one peak (SDL) and two peaks (LOI) had to be observed in the
229 respective narrow-window eXtracted Ion Chromatogram (nw-XIC), at the same retention time
230 (tolerance of ±2.5% respect to standard), measured at accurate mass (mass error lower than 5
231 ppm), respectively. **Table 1** shows the results obtained for all target compounds at each spiked
232 level in both fish and feed. The values resulting for SDL and LOI are also shown.

233

234 **RESULTS AND DISCUSSION**

235 Fish feed and fish are complex samples that contain a large number of matrix components such
236 as lipids and proteins besides other organic compounds which are likely to hamper our
237 identification of analytes. Consequently, in order to investigate the presence of any organic
238 compound in complex matrices, clean-up steps are usually incorporated into the analytical
239 process, in order to improve sensitivity and selectivity. [29, 30] Therefore, it is a challenge to
240 perform reliable analysis directly on sample extracts without any purification step. In this work,
241 the objective was exactly this: to perform the screening of emerging compounds from different
242 families such as antibiotics, pesticides and mycotoxins, among others, in sample extracts

243 obtained after a generic extraction with acetonitrile-water. In this way, we pursued the
244 extraction of as many compounds as possible, from different chemical families and with
245 different physico-chemical characteristics. In addition, avoiding clean-up, potential analyte
246 losses are minimized. The screening was focused on detection and identification of analytes in a
247 single analysis; as a consequence, no recoveries and precisions were calculated in this work.
248 Obviously, compounds subjected to investigation had to satisfy the requirements for LC-MS
249 analysis: to be LC-amenable and satisfactorily ionized in the atmospheric pressure ionization
250 (API) source employed (in our case, ESI+), and not be lost along the overall analytical
251 procedure applied.

252 In this work, the study was made on 35 antibiotics, 36 pesticides and 11 mycotoxins selected
253 among the most widely investigated in the environmental and food safety fields, and whose
254 reference standards were available at our laboratory. Formerly, LC-MS/MS methodology was
255 developed for their quantification at low levels, e.g. antibiotics and pesticides in waters and
256 mycotoxins in food.[31-33]

257

258 **Chromatography optimization.** Methanol and acetonitrile with different formic acid and
259 ammonium acetate content were tested as organic solvents for chromatographic optimization,
260 looking for a compromise between chromatographic behavior (peak shape) and sensitivity.
261 Most of the compounds presented better peak shape and ionization yield when methanol was
262 used instead of acetonitrile. An increased peak area was observed for many analytes when a
263 small amount of HCOOH was added, both in water and methanol mobile phase solvents. The
264 use of NH_4Ac (0.1mM) as a modifier improved the chromatographic behavior and sensitivity
265 for the great majority of the compounds studied in the line of previous data reported.[31-33]

266 Regarding the organic content of the sample extract injected into the LC-MS system, different
267 dilutions with water were tested in order to achieve 20%, 40% and 80% acetonitrile. Finally, the
268 injection of 20 µL of the extract with 80% organic content (no dilution) was selected as a
269 compromise between peak shape and sensitivity.

270

271 **Validation. Table 1** shows the number of positive/negative findings for all analytes at each
272 spiked level in feed and fish samples. The SDL and LOI for a given compound were achieved,
273 for a given spiked level, when a score of 10/0 was obtained according to the criteria established.
274 As expected, fish matrix (fillet) presented better SDL and LOI in comparison to the more
275 complex matrix of feed. Several quinolone antibiotics could not be identified in most of the feed
276 samples, as well as tetracyclines and sulfonamides, in such a way that no LOI were proposed.
277 However, the detection of these compounds was feasible with SDL of 20 or 100 µg/Kg. A more
278 selective sample treatment seems necessary and/or the use of newer and more sensitive QTOF
279 analyzer (e.g. Xevo G2 QTOF by Waters Corp.) in order to reach unequivocal identification at
280 low ppb levels for these compounds in fish feed.

281 Opposite to feed, a LOI of 20 µg/Kg could be achieved for the great majority of targeted
282 compounds in fish. As an example, **Figure 1** shows the LE and HE TOF MS spectra for a fish
283 sample spiked with azoxystrobin at 20 µg/Kg. The chromatograms for the predominant *m/z* ions
284 are also depicted at the lowest level studied. The presence of at least two chromatographic peaks
285 at expected retention time allowed the unequivocal identification in the samples. Moreover, the
286 low mass errors (below 4.8 ppm) for the protonated molecule and the most abundant fragments
287 supported the identification.

288 Four compounds (chlortetracycline, sulfamethoxazole, methomyl and molinate) could neither be
289 detected nor identified in fish at the levels tested. For these compounds, another sample
290 treatment and/or a more sensitive instrument might be required.

291 Several undesirable compounds could not be identified in feeds. In these cases, only typically
292 the $[M+H]^+$ ion was observed, so the compound was detected although not fully identified
293 according to the criteria established in the work. Higher collision energy values were tested but
294 no fragment ions were finally obtained, suggesting that the sample matrix might affect
295 fragmentation of trace analytes.

296 In relation to the Maximum Residue Limits (MRLs), only a few compounds have MRLs
297 established in feed and/or in fish (see **Table1**). In general, the method can be considered as
298 satisfactory for screening of antibiotics in fish, as both the SDL and LOI were bellow or the
299 same as the MRL in most of cases. Oxacillin and oxytetracycline could be detected at regulatory
300 levels using one accurate-mass ion ($M+H^+$), and penicillin G was detected at 100 µg/kg while
301 the MRL was 50 µg/kg. Only two regulated antibiotics, chlortetracycline and sulfamethoxazole,
302 could not be detected in fish as stated above. The wide majority of compounds included in the
303 screening are unregulated in fish feed, as MRLs only apply to four mycotoxins (see **Table 1**),
304 which were detected at 20 µg/kg (deoxynivalenol at 100 µg/kg). This is satisfactory for
305 zearalenone and deoxynivalenol, as their MRL are set up at 100 and 5000 µg/kg respectively.
306 MRLs for aflatoxin B1, and the sum of fumomisin B1+B2, are set up at 10 µg/kg, while the
307 lowest concentration tested in validation was 20 µg/kg. Our results showed that detection at 10
308 µg/kg should not be much problem taking into account the signal observed for these compounds
309 at the lowest level assayed.

310 **Figure 2** shows illustrative chromatograms for ciprofloxacin: apart from the protonated
311 molecule, the standard in solvent (50 ng/mL) hardly showed two fragment ions at the expected
312 retention time. However, the feed spiked at 100 µg/Kg (extract concentration 50 ng/mL) only
313 showed the ion corresponding to $[M+H]^+$. Experimental ESI+ accurate mass spectrum is also
314 presented for the standard, with mass errors for the fragment ions below 4.9 ppm. In this way,
315 ciprofloxacin could be satisfactorily detected in feed (SDL established at 100 µg/Kg) but no
316 LOI could be proposed demonstrating the difficulties to identify this compound in feed due to
317 the absence of fragment ions.

318

319 **Screening results in fish feed and fish fillet samples.** In order to evaluate the applicability of
320 the method for routine analysis, 10 feed samples and 11 fish fillets were analyzed apart from the
321 non-spiked samples used for validation. In a first step, only the target list of validated
322 compounds was searched for. Several compounds were detected in the samples: ciprofloxacin
323 was detected in 1 out of 11 fish fillets; fumonisin B2 was found in 2 and zearalenone in 1 out of

324 10 feeds; pirimiphos-methyl was detected in 8 out of 10 feeds and 2 out of 11 fish fillets. In all
325 these cases, the $[M+H]^+$ ion at the expected retention time was observed in the LE function. The
326 concentration levels found in the samples seemed to be very low as only the most abundant ion,
327 protonated molecule, was observed. The antibiotic ciprofloxacin was detected only in one
328 sample of fish fillet. Its concentration in the sample must have been between 20 µg/Kg (SDL)
329 and 100 µg/Kg (LOI), as it could be detected although not fully identified with additional
330 fragment ions. In two fish samples, the insecticide pirimiphos-methyl was detected, at a
331 predictable concentration below 20 µg/Kg (LOI), as it could not be identified with two ions.
332 Although the SDL was also set-up at 20 µg/Kg, surely this empirical value could have been
333 decreased if lower concentrations had been tested.

334 As regards fish feed, two mycotoxins were detected, fumonisin B2 and zearalenone, at
335 predictable concentrations between 20 µg/Kg (SDL) and 100 µg/Kg (LOI). Pirimiphos-methyl
336 was found in several feeds, at a predictable concentration below 20 µg/Kg (LOI).

337 Quality Controls (QCs) were analyzed in every batch of real sample analysis consisting of
338 selected samples spiked at 20 µg/Kg and 100 µg/Kg with all the target analytes. QCs were used
339 for quality control purposes to support the performance of the screening method.

340 In order to confirm the presence of the compounds detected, the sample extracts were
341 reanalyzed using a highly sensitive technique, i.e. LC-MS/MS with triple quadrupole, searching
342 only for the analytes found by QTOF MS. The analytical methodology was based on that
343 previously reported for this type of compounds in environmental and/or food matrices. [31-33] It is
344 noteworthy that all positives reported by QTOF MS were confirmed by LC-MS/MS acquiring
345 two transitions per compound and by the agreement in Q/q ratios in comparison with standards.
346 This fact reveals that detection with one accurate-mass ion and retention time allows a tentative,
347 rather reliable, identification minimizing the number of positives that need to be
348 confirmed/quantified in a subsequent analysis.

349 **Figure 3** shows an illustrative example of fumonisin B2, which was detected in feed by QTOF
350 MS and later confirmed by MS/MS. A chromatographic peak was observed at the expected
351 retention time (10.8 min) for the protonated molecule $[C_{34}H_{59}NO_{14}]^+$. However, no fragment

352 ions were found in the feed sample, while up to four were observed in the standard (50 ng/mL).
353 It is remarkable the high differences in sensitivity between the protonated molecule and the
354 fragment ions for fumonisin B2. Accurate mass LE spectra for $[C_{34}H_{59}NO_{14}]^+$ for both standard
355 and feed sample showed low mass errors in standard (2.5 ppm) and in feed sample (1.1 ppm).
356 **Figure 3** (bottom) also shows the LC-MS/MS chromatograms for this feed sample for the two
357 transitions acquired (Q quantification; q confirmation). Ultimate analyte confirmation was
358 carried out by comparison of the Q/q intensity ratios in standards and in samples, which were
359 within the maximum tolerances established.[15]

360 Thanks to the accurate-mass full-spectrum acquisition capabilities of the TOF analyzer, it was
361 feasible to investigate the presence of a wider list of pesticides, antibiotics and mycotoxins.
362 Moreover, other compound families not included in the preliminary target screening were also
363 investigated in the samples using a post-target approach, i.e searching for the presence of a
364 given compound after MS data acquisition. The presence of the protonated molecule was
365 evaluated in the samples, making use of a home-made data base containing around 1,000
366 compounds. Different strategies were followed depending on the availability or not of the
367 reference standard.[34] When standards were available at our laboratory, information about
368 retention time, fragmentation, and adduct formation was also included in the target list for those
369 compounds to facilitate and enhance reliability in the identification/elucidation process. As an
370 example, the preservative ethoxyquin was identified in 5 out of 21 fish samples, and 12 out of
371 20 feed samples. This compound is used as a pesticide in agriculture and as a preservative in
372 animal feed. **Figure 4** shows the identification of ethoxyquin in a post-target way. As can be
373 seen, three peaks were observed in the chromatograms at the exact masses of the protonated
374 molecule and of two fragment ions, at the same retention time. Mass errors lower than 2.3 ppm
375 were obtained in all cases, giving high reliability to the identification. On contrary, when the
376 reference standard was unavailable at our lab, a tentative identification was made based on the
377 interpretation of MS data (typically the presence of fragment ions in the HE spectra, their
378 compatibility with the chemical structure of the candidate, isotopic pattern and available
379 literature). By this way, several mycotoxins like agroclavine, altenuene, beauvericin,

380 chanoclavine, citrinin, dihydrosergol, emodin, enniatin B and lysergol were found in some feed
381 samples. These mycotoxins are typically found in cereals and moldy samples, but they are not
382 regulated; so maximum residue levels have not been established yet. No reference standards
383 were available at our laboratory for these mycotoxins; therefore, the unequivocal confirmation
384 was not feasible, although their tentative identification was made after exhaustive mass
385 interpretation of data. In the light of these findings, a more detailed study seems necessary to
386 confirm the presence of mycotoxins in fish feed.

387 In summary, the multiclass screening methodology has been validated for around 70 compounds
388 from these families. Selectivity of the screening was supported by accurate mass measurements
389 provided by QTOF MS, which allowed using nw-XICs (± 0.02 Da) at selected m/z ions. The
390 vast majority of the compounds investigated were properly detected and identified in fish at the
391 two spiked levels (20 and 100 μg/Kg). Regarding feed, more difficulties were found, although a
392 great representation of the different families was satisfactorily validated. Despite the large
393 number of targeted analytes that were detected at the two concentrations tested, in some cases
394 (especially in the more complex feed matrices), the LOI could not be proposed, as only the
395 [M+H]+ ion was observed. In those cases, additional analysis would be required (e.g. by LC-
396 MS/MS with QqQ) for confirmation and quantification of the compound detected in the sample.

397

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Table 1. Validation results. Detection and identification limits in spiked feed and fish at two concentration levels. SDL and LOI obtained according to the established criterion.

		Positive/negative results													
		Feed (n=10)							Fish (n=10)						
		Detection			Identification				Detection			Identification			
	Compound	20 µg/Kg	100 µg/Kg	SDL (µg/Kg)	20 µg/Kg	100 µg/Kg	LOI (µg/Kg)	LMR[a]	20 µg/Kg	100 µg/Kg	SDL (µg/Kg)	20 µg/Kg	100 µg/Kg	LOI (µg/Kg)	LMR[b]
ANTIBIOTICS	Azithromycin	10/0	10/0	20	0/10	0/10	-		10/0	10/0	20	10/0	10/0	20	
	Chlortetracycline	0/10	0/10	-	0/10	0/10	-		0/10	0/10	-	0/10	0/10	-	100
	Ciprofloxacin	2/8	10/0	100	2/8	3/7	-		10/0	10/0	20	8/2	10/0	100	100[c]
	Clarythromycin	10/0	10/0	20	1/9	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Clindamycin	10/0	10/0	20	8/2	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Cloxacillin	1/9	10/0	100	0/10	0/10	-		0/10	10/10	100	0/10	0/10	-	300
	Dicloxacillin	0/10	0/10	-	0/10	0/10	-		0/10	10/0	100	0/10	10/0	100	300
	Doxycycline	0/10	0/10	-	0/10	0/10	-		0/10	10/0	100	0/10	10/0	100	
	Enrofloxacin	10/0	10/0	20	0/10	1/9	-		10/0	10/0	20	10/0	10/0	20	100[c]
	Erythromycin A	10/0	10/0	20	1/9	10/0	100		10/0	10/0	20	10/0	10/0	20	200
	Flumequine	10/0	10/0	20	2/8	10/0	100		10/0	10/0	20	10/0	10/0	20	600
	Furaltadone	2/8	10/0	100	2/8	3/7	-		10/0	10/0	20	7/3	10/0	100	
	Furazolidone	10/0	10/0	20	3/7	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Lincomycin	3/7	10/0	100	0/10	2/8	-		10/0	10/0	20	10/0	10/0	20	100
	Marbofloxacin	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	10/0	10/0	20	
	Moxifloxacin	10/0	10/0	20	1/9	2/8	-		10/0	10/0	20	10/0	10/0	20	
	Nalidixic acid	10/0	10/0	20	1/9	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Norfloxacin	3/7	10/0	100	2/8	4/6	-		10/0	10/0	20	6/4	10/0	100	
	Ofloxacin	10/0	10/0	20	4/6	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Oxacillin	1/9	3/7	-	0/10	0/10	-		0/10	10/0	100	0/10	6/4	-	300
	Oxolinic acid	3/7	10/0	100	0/10	0/10	-		10/0	10/0	20	0/10	10/0	100	100
	Oxytetracycline	0/10	0/10	-	0/10	0/10	-		0/10	10/0	100	0/10	0/10	-	100
	Pefloxacin	2/8	10/0	100	1/9	1/9	-		10/0	10/0	20	3/7	10/0	100	
	Penicillin G	0/10	3/7	-	0/10	0/10	-		0/10	10/0	100	0/10	4/6	-	50
	Pipedimic acid	3/7	10/0	100	3/7	10/0	100		10/0	10/0	20	7/3	10/0	100	
	Piperacillin	7/3	10/0	100	7/3	10/0	100		10/0	10/0	20	0/10	10/0	100	
	Roxythromycin	10/0	10/0	20	0/10	0/10	-		10/0	10/0	20	10/0	10/0	20	
	Sarafloxacin	3/7	10/0	100	0/10	0/10	-		10/0	10/0	20	10/0	10/0	20	
	Sulfadiazine	4/6	6/4	-	2/8	3/7	-		10/0	10/0	20	10/0	10/0	20	100[d]
	Sulfamethazine	10/0	10/0	20	1/9	3/7	-		10/0	10/0	20	10/0	10/0	20	100[d]
	Sulfamethoxazole	3/10	10/0	100	1/9	1/9	-		0/10	0/10	-	0/10	0/10	-	100[d]
	Sulfathiazole	10/0	10/0	20	0/10	0/10	-		0/10	10/0	100	0/10	10/0	100	100[d]
	Tetracycline	0/10	0/10	-	0/10	0/10	-		1/9	10/0	100	1/9	10/0	100	100
	Trimethoprim	10/0	10/0	20	3/7	4/6	-		10/0	10/0	20	10/0	10/0	20	100
	Tylosin A	10/0	10/0	20	1/9	3/7	-		10/0	10/0	20	7/3	10/0	100	100
PESTICIDES	Acetamiprid	2/8	7/3	-	0/10	4/6	-		10/0	10/0	20	10/0	10/0	20	
	Alachlor	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	10/0	10/0	20	
	Atrazine	10/0	10/0	20	3/7	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Azinphos-methyl	4/6	10/0	100	4/6	10/0	100		10/0	10/0	20	0/10	0/10	-	
	Azoxystrobin	10/0	10/0	20	4/6	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Bromacil	4/6	10/0	100	4/6	10/0	100		10/0	10/0	20	4/6	10/0	100	
	Buprofezin	1/9	10/0	100	1/9	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Carbaryl	2/8	10/0	100	2/8	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Carbendazim	3/7	10/0	100	1/9	1/9	-		10/0	10/0	20	10/0	10/0	20	
	Carbofuran	8/2	10/0	100	0/10	4/6	-		10/0	10/0	20	10/0	10/0	20	
	Cyprodinil	2/8	10/0	100	2/8	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Dimethoate	0/10	0/10	-	0/10	0/10	-		0/10	10/0	100	0/10	0/10	-	
	Diuron	2/8	10/0	100	0/10	4/6	-		10/0	10/0	20	10/0	10/0	20	
	Fenarimol	0/10	10/0	100	0/10	0/10	-		10/0	10/0	20	10/0	10/0	20	
	Hexythiazox	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	2/8	10/0	100	
	Imazalil	0/10	10/0	100	0/10	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Imidacloprid	7/3	10/0	100	7/3	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Isoproturon	0/10	7/3	-	0/10	6/4	-		10/0	10/0	20	10/0	10/0	20	
	Malathion	0/10	4/6	-	0/10	4/6	-		10/0	10/0	20	10/0	10/0	20	
	Metalaxyl	1/9	1/9	-	0/10	0/10	-		10/0	10/0	20	10/0	10/0	20	
	Methidathion	0/10	1/9	-	0/10	0/10	-		10/0	10/0	20	6/4	10/0	100	
	Methiocarb	3/7	10/0	100	3/7	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Methomyl	0/10	2/8	-	0/10	0/10	-		0/10	3/7	-	0/10	0/10	-	
	Metolachlor	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	10/0	10/0	20	
	Molinate	6/4	10/0	100	2/8	7/3	-		0/10	0/10	-	0/10	0/10	-	
	Pirimicarb	4/6	10/0	100	3/7	3/7	-		10/0	10/0	20	10/0	10/0	20	
	Pirimiphos-methyl	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	10/0	10/0	20	
	Propanil	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	10/0	10/0	20	
	Pyridaphenthion	2/8	10/0	100	2/8	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Simazine	0/10	10/0	100	0/10	2/8	-		10/0	10/0	20	10/0	10/0	20	
	Terbumeton	1/9	10/0	100	1/9	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Terbuthylazine	0/10	7/3	-	0/10	6/4	-		10/0	10/0	20	10/0	10/0	20	
	Terbutryn	3/7	10/0	100	3/7	10/0	100		10/0	10/0	20	10/0	10/0	20	
	Thiabendazole	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	10/0	10/0	20	
	Thiobencarb	10/0	10/0	20	10/0	10/0	20		10/0	10/0	20	10/0	10/0	20	
	Triadimenol	1/9	7/3	-	1/9	6/4	-		10/0	10/0	20	3/7	10/0	100	
MYCOTOXINS	Aflatoxin B1	10/0	10/0	20	10/0	10/0	20	10							
	Aflatoxin B2	10/0	10/0	20	1/9	10/0	100								
	Aflatoxin G1	10/0	10/0	20	3/7	10/0	100								
	Aflatoxin G2	10/0	10/0	20	1/9	2/8	-								
	Deoxynivalenol	2/8	10/0	100	0/10	0/10	-	5000							
	Fumonisin B1	10/0	10/0	20	0/10	1/9	-	10[e]							
	Fumonisin B2	10/0	10/0	20	2/8	10/0	100	10[e]							
	HT-2 Toxin	0/10	0/10	-	0/10	0/10	-								
	Ochratoxin Alpha	10/0	10/0	20	2/8	4/6	-								
	T-2 Toxin	0/10	0/10	-	0/10	0/10	-								
	Zearalenone	10/0	10/0	20	7/3	10/0	100	100							

a= LMR for feed (µg/Kg) [4], b= LMR for fish (µg/Kg) [5], c= Sum Ciprofloxacin+Enrofloxacin, d= Sum Sulfonamides, e= Sum (Fum B1+Fum B2)

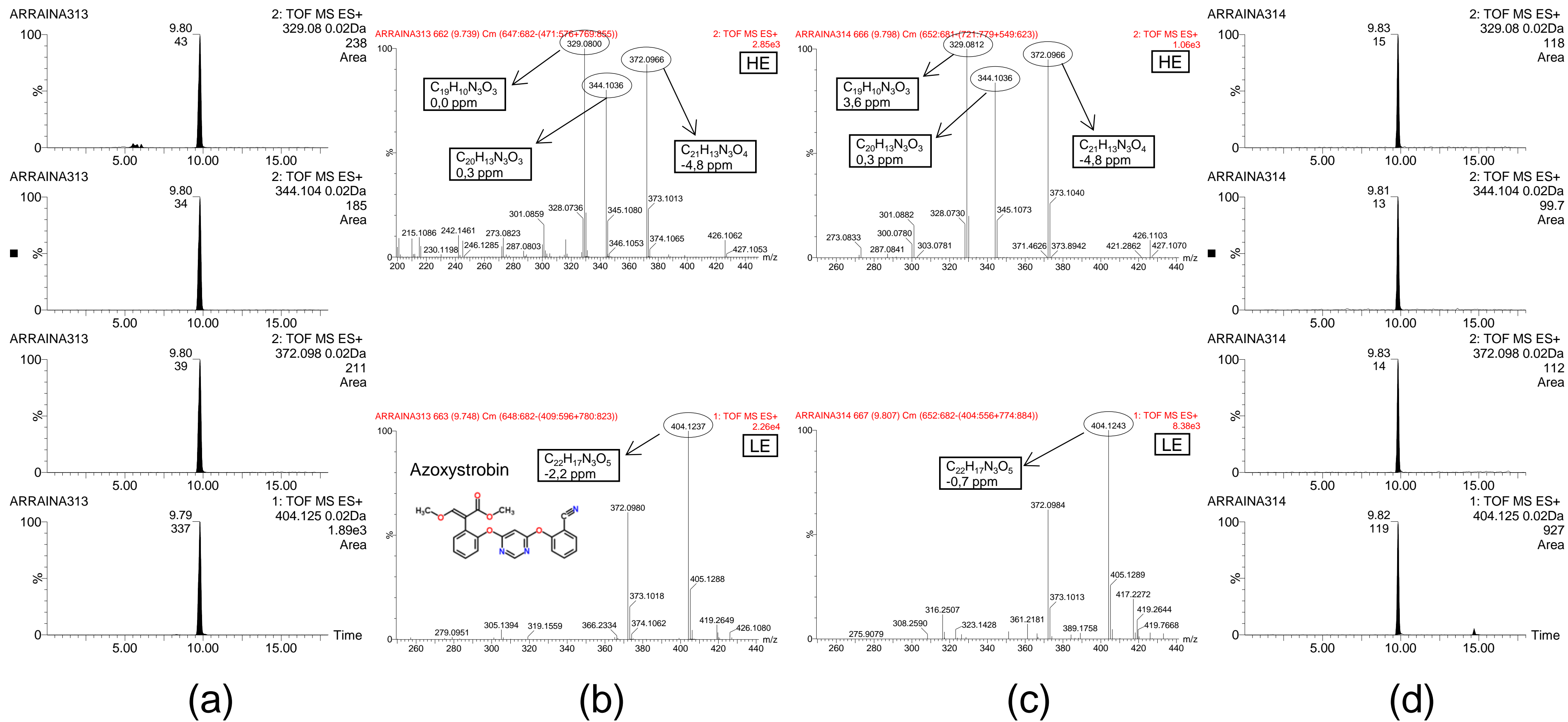
Figure captions.

Figure 1. Azoxystrobin standard at 50 ng/mL in solvent: (a) nw-XIC for protonated molecule in LE and main fragment ions in HE, (b) ESI+ accurate LE and HE spectra; elemental composition and mass errors of main ions. Fish spiked at 20 µg/Kg: (c) ESI+ accurate LE and HE spectra; elemental composition and mass errors of main ions, (d) nw-XIC for protonated molecule in LE and main fragment ions in HE.

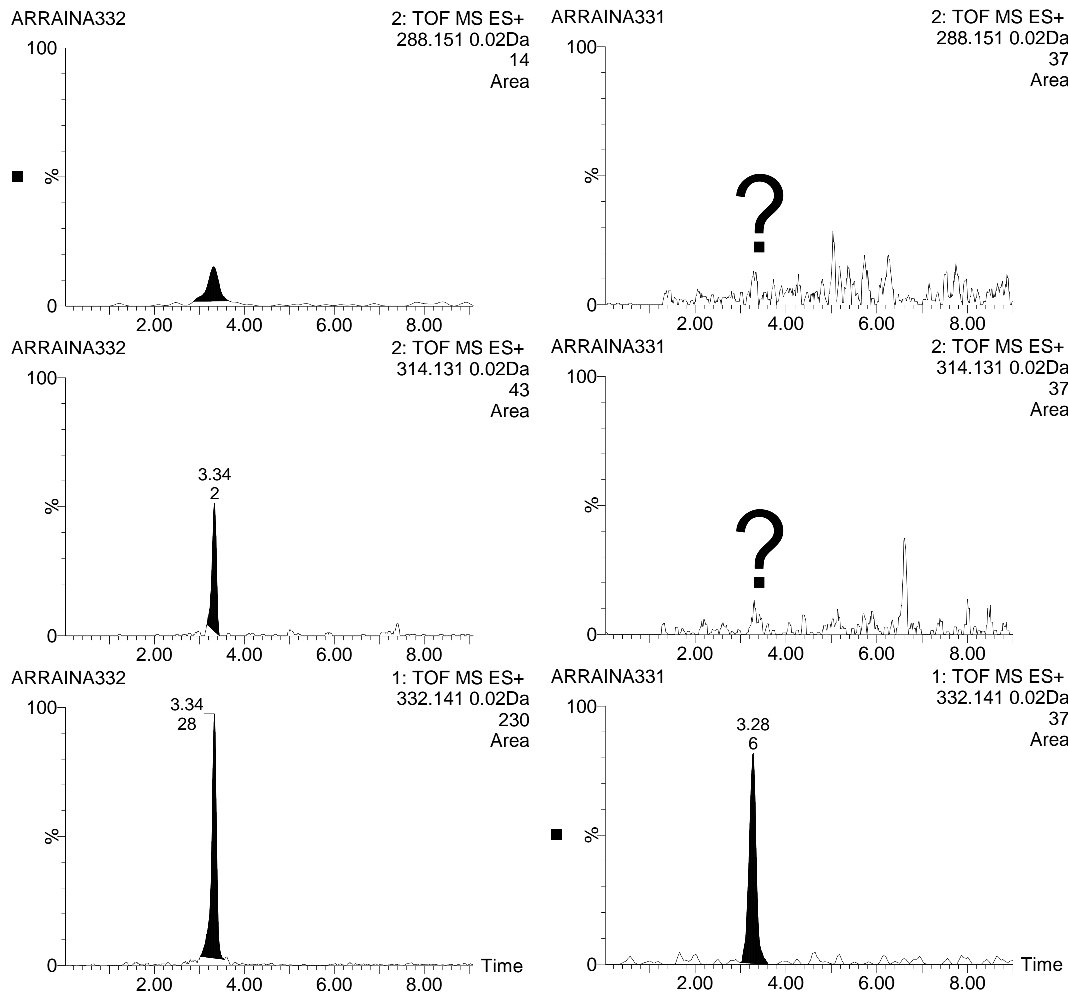
Figure 2. (a) nw-XICs for the protonated molecule and two main fragment ions for ciprofloxacin standard (50 ng/mL in solvent), (b) nw-XICs for ciprofloxacin in a feed spiked at 100 µg/Kg (final extract concentration 50 ng/mL) and, (c) experimental ESI+ accurate mass spectra (LE and HE) for ciprofloxacin standard.

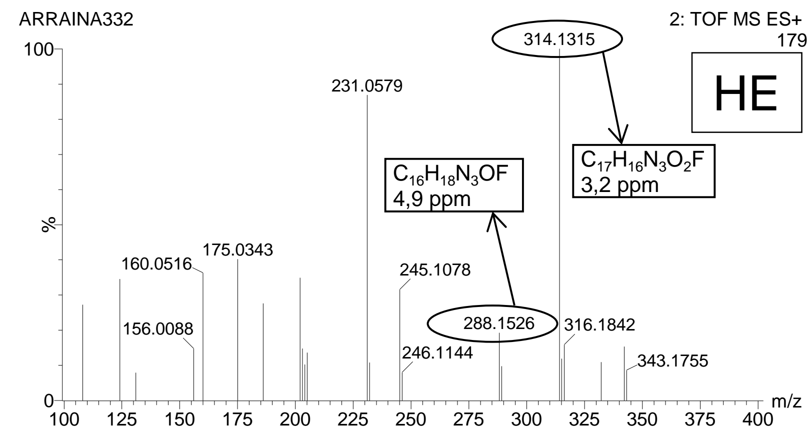
Figure 3. Confirmation of fumonisin B2 in a feed sample. Top: nw-XICs for protonated molecule and fragment ions of fumonisin B2 for the standard (50 ng/mL) and feed extract, respectively. In the middle: Accurate mass LE spectrum of fumonisin B2 corresponding to $[C_{34}H_{59}NO_{14}]^+$ for both standard and feed. Bottom: LC-MS/MS chromatograms for the standard (50 ng/mL) and feed extract, respectively. ✓: Q/q ratio within tolerance limits.

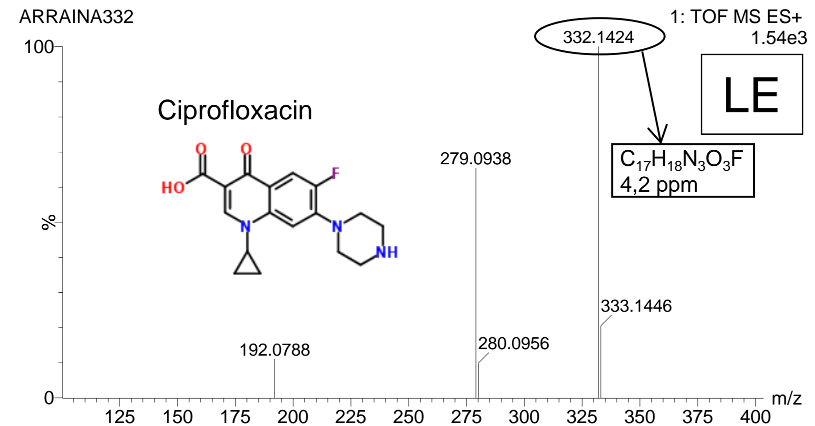
Figure 4. nw-XICs for protonated molecule and fragment ions and accurate mass spectra (both LE and HE) for ethoxyquin in (a) fish fillet, (b) fish feed and (c) standard (200 ng/mL), respectively.



589
590 Figure 1.







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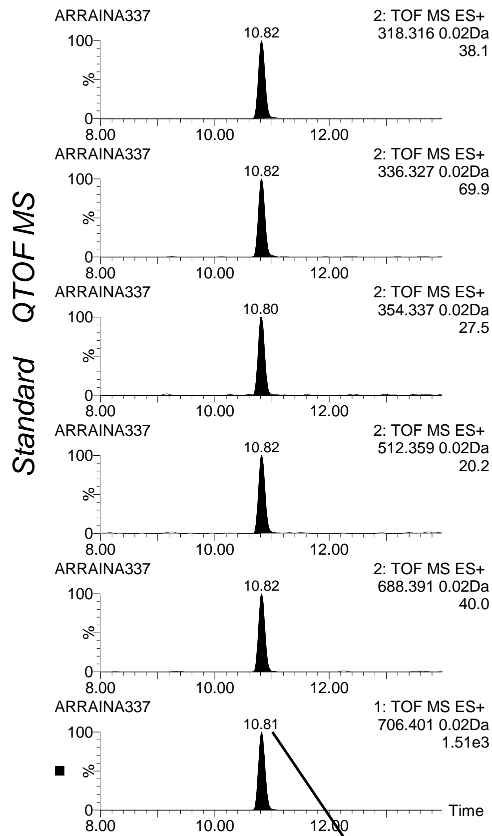
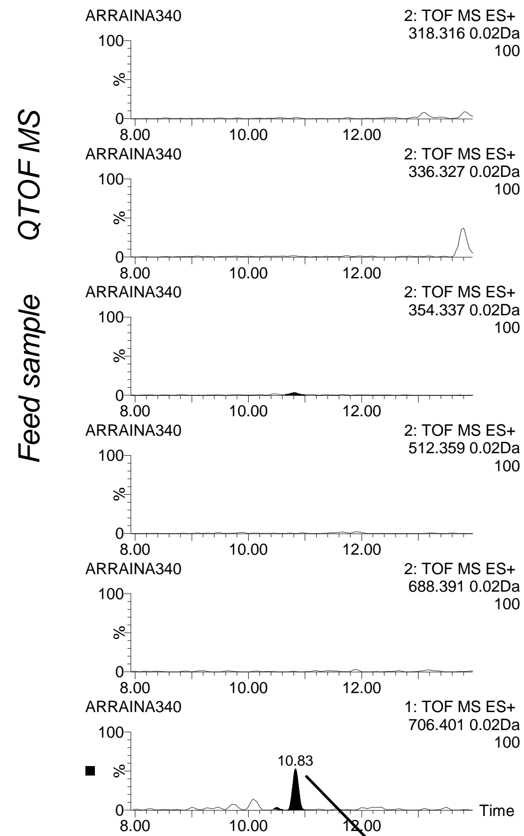
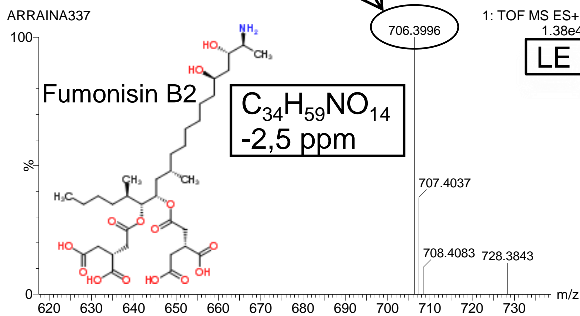
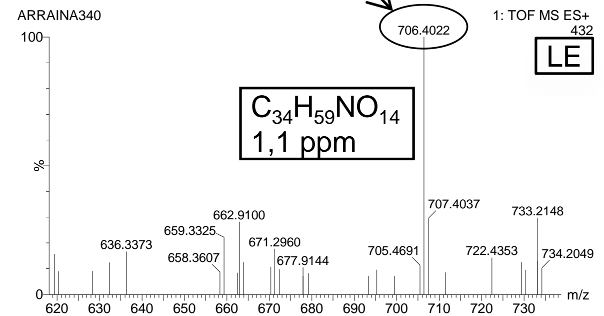
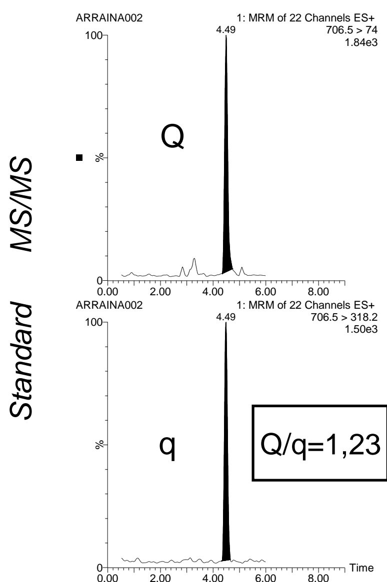
592 Figure 2.

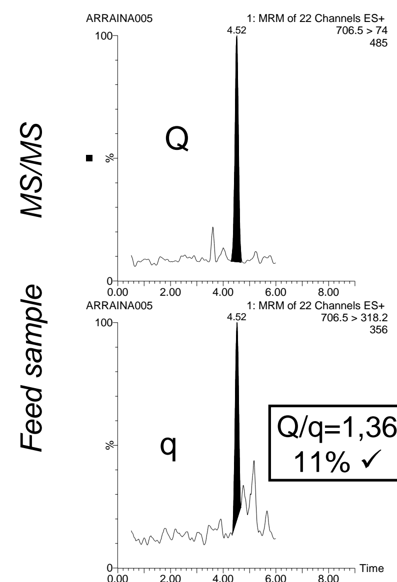












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595 Figure 3.
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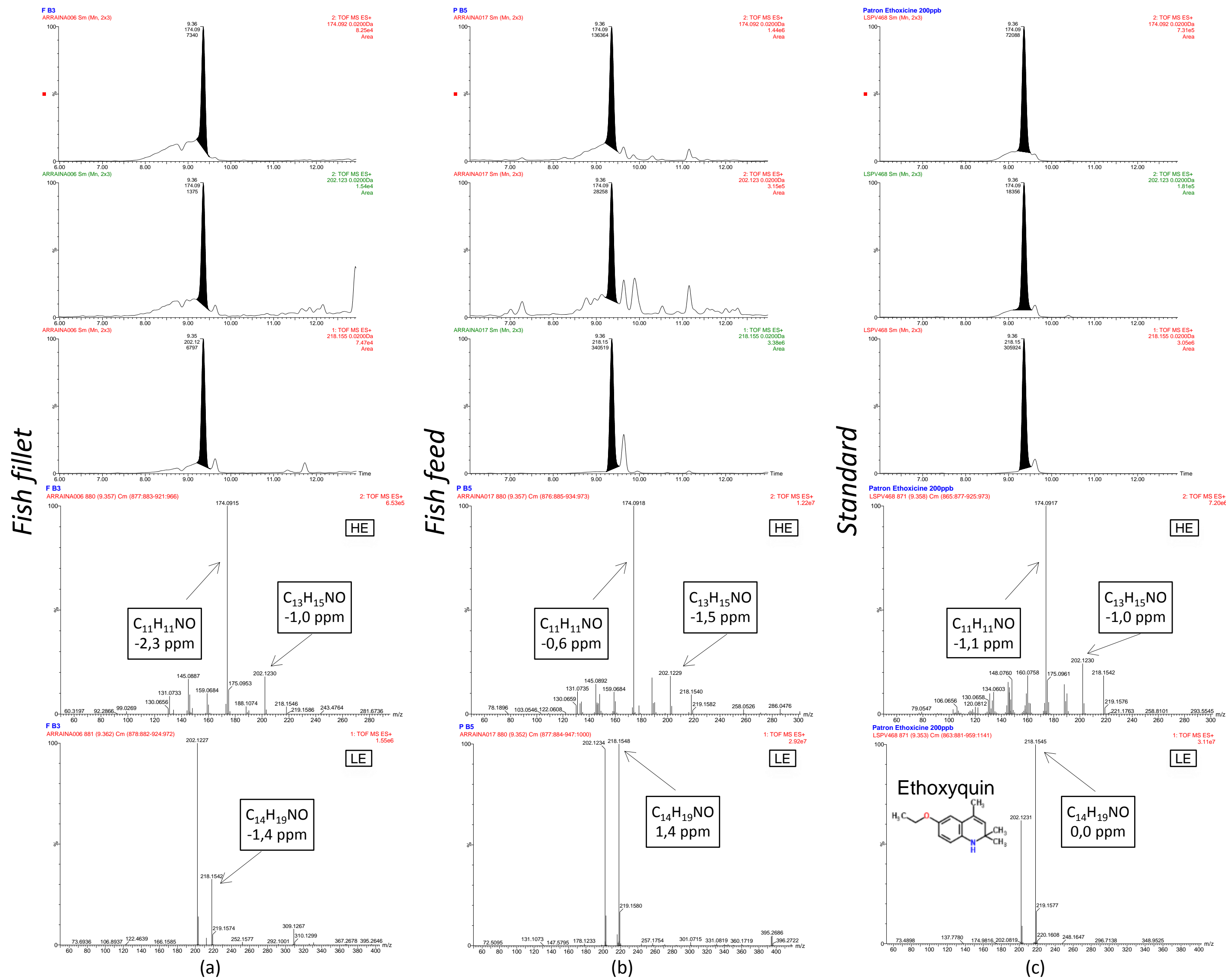

597 Figure 4.