**Qualitative screening of undesirable compounds from feeds to fish by
liquid chromatography coupled to mass spectrometry.**

Jaime Nácher-Mestre^a, María Ibáñez^a, Roque Serrano^a, Jaume Pérez-
Sánchez^b, Félix Hernández^{a*}

*^aResearch Institute for Pesticides and Water (IUPA). Avda. Sos Baynat, s/n. University Jaume I,
12071 Castellón, Spain*

*^bInstitute of Aquaculture of Torre la Sal (IATS), CSIC, 12595 Ribera de Cabanes, Castellón,
Spain*

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

* Corresponding author. Tel. +34-964-387366; e-mail address: felix.hernandez@qfa.uji.es

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

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

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

Until now, LC-QTOF MS has been scarcely employed for monitoring the presence of organic contaminants in fish origin raw materials, fish and feed²³⁻²⁴. In fact, LC-MS techniques have not been used much for analysis of this type of fatty samples. The vast majority of papers reported in the marine field are focused on the determination of POPs using GC-MS. In a few cases, LC-MS has been applied for compounds like specific flame retardants and perfluorinated compounds.^{25, 26} As regards LC-TOF MS, very little has been published in the marine field^{27, 28}. Villar-Pulido et al.²⁷ reported a multiclass detection methodology in order to detect antibiotics and veterinary drugs in shrimps and Peters et al.²⁸ reported a multi-residue screening of veterinary drugs in several fish samples showing that TOF is one of the most powerful tools for multicomponent analysis.

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

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

MATERIAL AND METHODS

Reagents and chemicals. In this work, up to 35 antibiotics, 36 pesticides and 11 mycotoxins were selected as representative compounds in order to validate the methodology. Reference standards of sulfamethoxazole, sulfamethazine, sulfadiazine and sulfathiazole were from Across Organics (Geel, Belgium). Enrofloxacin, moxifloxacin and ciprofloxacin were from Bayer Hispania (Barcelona, Spain). Sarafloxacin, marbofloxacin and pefloxacin were provided by Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid Spain) and Aventis Pharma (Madrid, Spain), respectively. The rest of antibiotics were supplied by Sigma-Aldrich (St Louis, MO, USA) or Fluka (Buchs, Switzerland). All antibiotic standards presented purity higher than 93%. Pesticide reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Riedel-de Haën (Seelze, Germany) or Sigma-Aldrich (St. Louis, MO, USA). All mycotoxins standards (>99% purity) were supplied by Sigma Aldrich (Madrid, Spain). For antibiotics and mycotoxins, individual stock standard solutions were prepared by dissolving solid standard in acetonitrile with the exception for antibiotic quinolones, which were dissolved in methanol and required the addition of 100 µL of 1M sodium hydroxide for their proper dissolution. Regarding pesticides, individual stock standard solutions were prepared by dissolving solid standard in acetone. Working solutions of antibiotics, pesticides and mycotoxins, respectively, were obtained after mixing individual stock solutions of each family and diluting with water to give a final concentration of around 500 ng/mL for sample fortification and injection in the chromatographic system. Stock solutions were stored in a freezer at -20 °C and working solutions were stored in a fridge.

HPLC-grade water was obtained from a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). HPLC-grade methanol, HPLC-grade acetonitrile and acetone for residue analysis were purchased from Scharlau (Barcelona, Spain). Formic acid (HCOOH, content > 98%) and ammonium acetate (NH₄Ac, reagent grade) were supplied by Scharlau.

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

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

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

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

phase consisted of water/methanol gradient both with 0.01% HCOOH and 0.1mM NH₄Ac. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10 %; 14 min, 90 %; 16 min, 90 %; 16.01 min, 10 %; 18 min, 10 %. The column temperature was set to 60 °C.

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

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

processed by the ChromaLynx XS application manager (within MassLynx v 4.1; Waters Corporation).

A triple quadrupole analyser (Waters Corp., Milford, MA, USA) operating in MS/MS was used for the analysis of positive samples from the screening. Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N₂ LC-MS (Claind, Teknokroma, Barcelona, Spain) and the collision gas was argon (99.995%; Praxair, Madrid, Spain) with a pressure of approximately 4.10⁻³ mbar in the collision cell. A capillary voltage of 3.5 kV in positive ionization mode was applied. The desolvation gas temperature was set to 500°C and the source temperature to 120°C. Temperature column was set to 40°C. Dwell times of 0.030 s/scan were chosen. TargetLynx application manager (MassLynx v 4.1) software was used to process the data obtained from standards and samples.

Recommended analytical procedure. Before analysis, feed samples were thawed at room temperature and ground using a Super JS mill from Moulinex (Bagnole Cedex, France). Fish fillets were also thawed at room temperature and processed in a crushing machine (Thermomix, Vorwerk España M.S.L., S.C., Madrid). As a result, homogenized samples were obtained in both cases. The recommended procedure was the following: 5 g of sample was accurately weighed (precision 0.1 mg), transferred to centrifuge tubes (50 mL) and homogenized in a Vortex with 10 mL acetonitrile:water (80:20) 0.1% HCOOH. After shaking the samples (S.B.S. Instruments S.A, Barcelona, Spain) for one hour, tubes were placed in an ultrasonic bath during 15 minutes followed by centrifugation at 4500 rpm for 10 min (Consul centrifuge, Orto-Alresa, Madrid, Spain). Approximately 2 mL of supernatant extract was transferred to an eppendorf vial and stored in a freezer (minimum 2 hours) in order to precipitate proteins. Expired this time, the extract was centrifuged again at 12000 rpm for 10 min. Finally, the supernatant extract was injected into the UHPLC-QTOF MS system.

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

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

RESULTS AND DISCUSSION

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

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

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

Chromatography optimization. Methanol and acetonitrile with different formic acid and ammonium acetate content were tested as organic solvents for chromatographic optimization, looking for a compromise between chromatographic behavior (peak shape) and sensitivity. Most of the compounds presented better peak shape and ionization yield when methanol was used instead of acetonitrile. An increased peak area was observed for many analytes when a small amount of HCOOH was added, both in water and methanol mobile phase solvents. The use of NH₄Ac (0.1mM) as a modifier improved the chromatographic behavior and sensitivity for the great majority of the compounds studied in the line of previous data reported.³¹⁻³³

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

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

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

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

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

As regards fish feed, two mycotoxins were detected, fumonisin B2 and zearalenone, at predictable concentrations between 20 $\mu\text{g/Kg}$ (SDL) and 100 $\mu\text{g/Kg}$ (LOI). Pirimiphos-methyl was found in several feeds, at a predictable concentration below 20 $\mu\text{g/Kg}$ (LOI).

Quality Controls (QCs) were analyzed in every batch of real sample analysis consisting of selected samples spiked at 20 $\mu\text{g/Kg}$ and 100 $\mu\text{g/Kg}$ with all the target analytes. QCs were used for quality control purposes to support the performance of the screening method.

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

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

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

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

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

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

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

Positive/negative results															
		Feed (n=10)						Fish (n=10)							
Compound	Detection			Identification			LMR ^a	Detection			Identification			LMR ^b	
	20	100	SDL	20	100	LOI		20	100	SDL	20	100	LOI		
	µg/Kg	µg/Kg	(µg/Kg)	µg/Kg	µg/Kg	(µg/Kg)		µg/Kg	µg/Kg	(µg/Kg)	µg/Kg	µg/Kg	(µg/Kg)		
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	
	Metaxyl	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	-	
Pyrimicarb	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		
Terbutylazine	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.

(a)

(b)

(c)

Figure 2.

Figure 3.