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Qualitative Screening of Undesirable Compounds from Feeds to Fish by Liquid Chromatography Coupled to Mass Spectrometry

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ABSTRACT: This paper 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 extraction of 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 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 (on the basis of 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 either reprocessing or injecting the samples. This allowed the detection and tentative identification of other organic undesirables different from those included in the validated list.

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

INTRODUCTION

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

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

The results obtained in a previous project (www.aquamaxip.eu), based on target analysis focused on persistent organic pollutants (POPs), demonstrated that organochlorine compounds, polycyclic aromatic hydrocarbons, and polybrominated diphenyl ethers were present in feed and raw materials for sea bream and also in sea bream fillets at trace levels.[7,8,11,13] In the present research, the analytical strategy was directed toward a multiclass screening able to easily and rapidly detect and identify a large number of suspected compounds in the samples studied. To this aim, a generic and rapid nondestructive extraction was applied trying to avoid possible losses of the compounds of interest during the sample treatment. The method developed has 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, facilitates performing wide-scope screening using target and nontarget approaches.[17] Moreover, it is possible to make a retrospective data evaluation at any time

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to search for additional compounds without the need to perform additional analyses. QTOF MS allows working under MS^E mode, that is, 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 the 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 feed.[23,24] In fact, LC-MS techniques have not been used much for analysis of this type of fatty sample. 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 such as specific flame retardants and perfluorinated compounds.[25,26] With regard to LC-TOF MS, very little has been published in the marine field.[27,28] Villar-Pulido et al.[27] reported a multiclass detection methodology to detect antibiotics and veterinary drugs in shrimp, and Peters et al.[28] reported a multiresidue screening of veterinary drugs in several fish samples showing that TOF is one of the most powerful tools for multicompound analysis.

The aim of the present work is to develop a 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 by 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.

■ MATERIALS AND METHODS

Reagents and Chemicals. In this work, up to 35 antibiotics, 36 pesticides, and 11 mycotoxins were selected as representative compounds to validate the methodology. Reference standards of sulfamethoxazole, sulfamethazine, sulfadiazine, and sulfathiazole were from Acros 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 the antibiotics were supplied by Sigma-Aldrich (St. Louis, MO, USA) or Fluka (Buchs, Switzerland). All antibiotic standards presented purity >93%. Pesticide reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Riedel-de Haën (Seelze, Germany). or Sigma-Aldrich (St. Louis, MO, USA). All mycotoxin 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 of antibiotic quinolones, which were dissolved in methanol and required the addition of 100 μL of 1 M sodium hydroxide for their proper dissolution. With regard to pesticides, individual stock standard solutions were prepared by dissolving solid standard in acetone. Working solutions of antibiotics, pesticides, and mycotoxins, respectively, were obtained after individual stock solutions of each family were mixed and diluted 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 refrigerator.

HPLC grade water was obtained from a Milli-Q 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 (NH_4Ac, 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.

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. With regard to 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. The mobile phase consisted of a water/methanol gradient both with 0.01% HCOOH and 0.1 mM NH_4Ac. 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 10000 at full width at half-maximum (fwhm), at m/z 556.2771. MS data were acquired over 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 a 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 a second one, the high-energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV to promote in-source fragmentation. The LE and HE function settings were for a scan time of 0.2 s and an interscan delay of 0.05 s.

Calibrations were conducted from m/z 50 to 1000 with a 1:1 mixture of 0.05 M 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 (2 mg/L) in acetonitrile/water (50:50) at 0.1% HCOOH pumped at 30 μL/min through the lock-spray needle. A cone voltage of 95 V 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 of 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.

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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	

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Table 1. continued

	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]
Pesticides														
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 A	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).

However, as this deviation is also applied during mass axis calibration, there is no negative impact on the mass errors presented in this paper. MS data were acquired in centroid mode and were processed by the ChromaLynx XS application manager (within MassLynx v 4.1; Waters Corp.).

A triple-quadrupole analyzer (Waters Corp.) operating in MS/MS was used for the analysis of positive samples from the screening. Drying gas as well as nebulizing gas was nitrogen generated from pressurized air in a N_2 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. Column temperature 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 (Bagnolet 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 of acetonitrile/water (80:20) 0.1% HCOOH. After the samples had been shaken (S.B.S. Instruments S.A, Barcelona, Spain) for 1 h, tubes were placed in an ultrasonic bath during 15 min 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 h) 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 and 100 μg/kg, and analyzed together with their nonspiked samples ("blanks"). Additionally, two method blanks were analyzed to ensure that no laboratory contamination was introduced in the procedure. It is noteworthy that mycotoxins were evaluated only 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) independent of its recovery and precision (details in Table 1). The

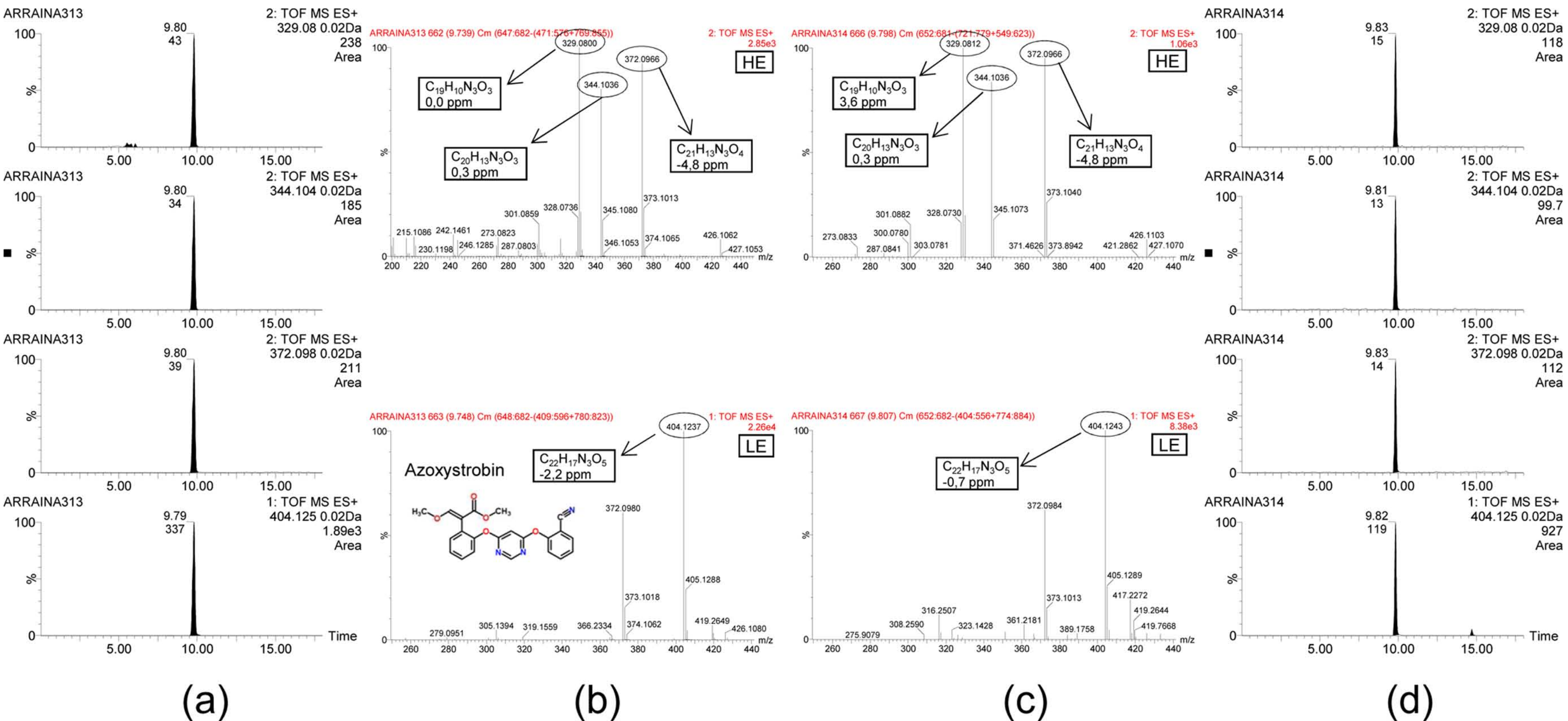

Figure 1. Azoxystrobin standard at 50 ng/mL in solvent: (a) nw-XIC for protonated molecule in LE and main fragment ions in HE; (b) ESI+ accurate LE and HE spectra; elemental composition and mass errors of main ions. Fish spiked at 20 μg/kg: (c) ESI+ accurate LE and HE spectra; elemental composition and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

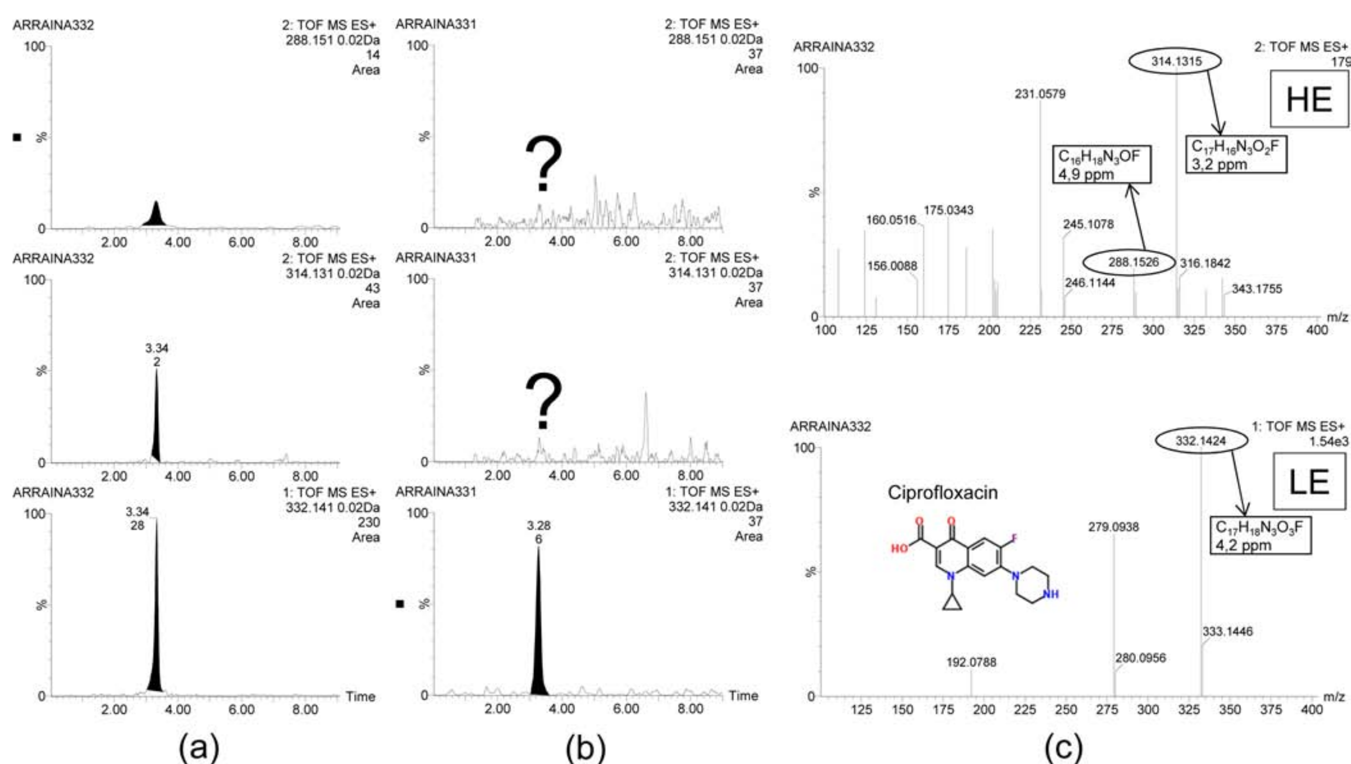

Figure 2. (a) nw-XICs for the protonated molecule and two main fragment ions for ciprofloxacin standard (50 ng/mL in solvent); (b) nw-XICs for ciprofloxacin in a feed spiked at 100 μg/kg (final extract concentration = 50 ng/mL); (c) experimental ESI+ accurate mass spectra (LE and HE) for ciprofloxacin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

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% with respect to standard), measured at accurate mass (mass error < 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, to investigate the presence of any organic compound in complex matrices, cleanup steps are usually incorporated into the analytical process, 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 physicochemical characteristics. In addition, because cleanup is avoided, potential analyte losses are minimized. The screening was focused on the 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 during 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 for which reference standards were available at our laboratory. Formerly, LC-MS/MS methodology was developed for their quantification at low levels, for example, antibiotics and pesticides in water and mycotoxins in food.[31−33]

Chromatography Optimization. Methanol and acetonitrile with different formic acid and ammonium acetate contents were tested as organic solvents for chromatographic optimization, to achieve 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, in both water and methanol mobile phase solvents. The use of NH_4Ac (0.1 mM) as a modifier improved the chromatographic behavior and sensitivity for the great majority of the compounds studied in line with previously reported data.[31−33]

With regard to the organic content of the sample extract injected into the LC-MS system, different dilutions with water were tested 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.

Validation. Table 1 shows the number of positive/negative findings for all analytes at each spiked level in feed and fish samples. The SDL and LOI for a given compound were

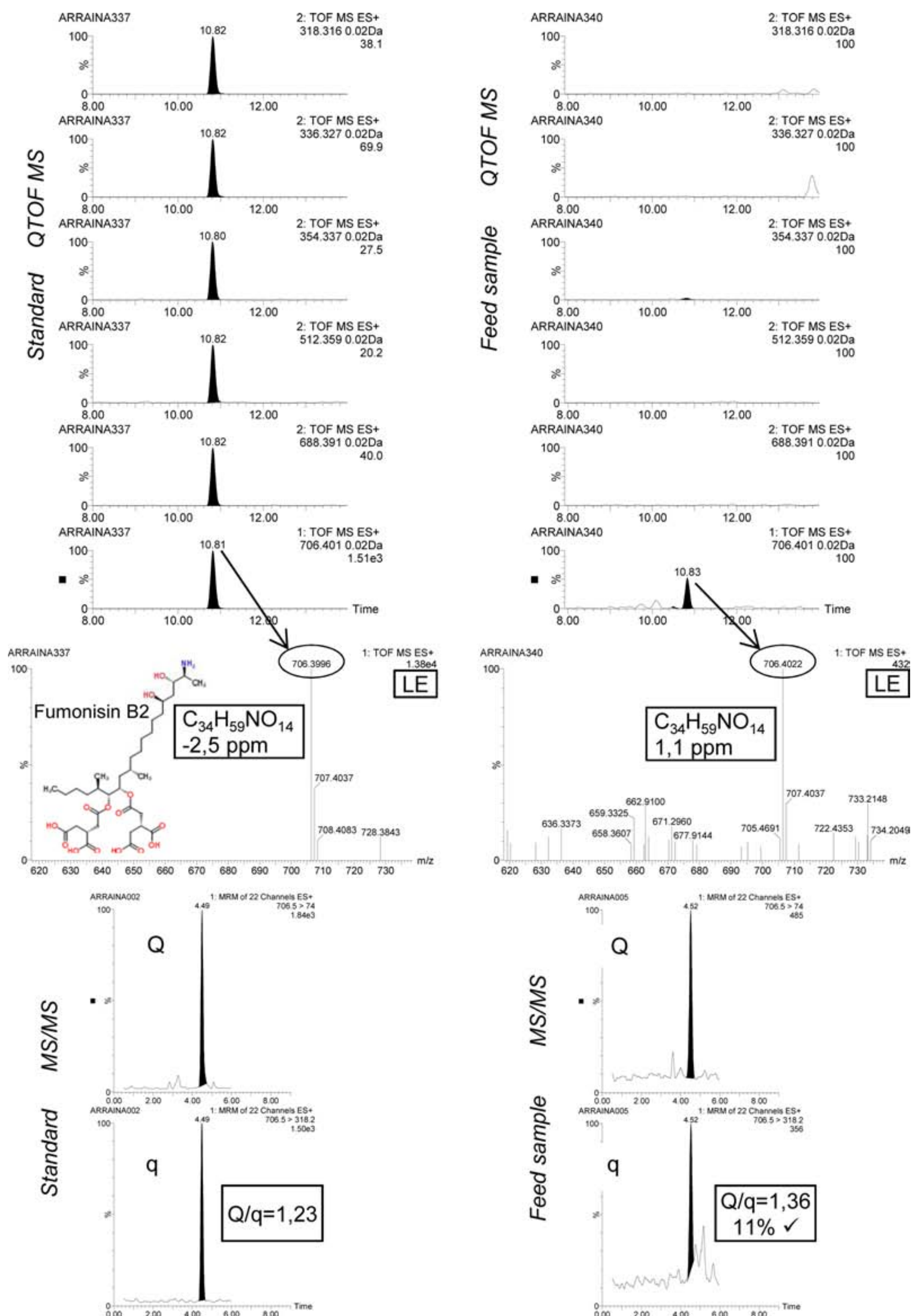

Figure 3. Confirmation of fumonisin B2 in a feed sample. (Top) nw-XICs for protonated molecule and fragment ions of fumonisin B2 for the standard (50 ng/mL) and feed extract, respectively. (Middle) Accurate mass LE spectrum of fumonisin B2 corresponding to $[C_{34}H_{59}NO_{14}]^+$ for both standard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

achieved, for a given spiked level, when a score of 10/0 was obtained according to the criteria established. As expected, fish matrix (fillet) presented better SDL and LOI in comparison to the more complex matrix of feed. Several quinolone antibiotics could not be identified in most of the feed samples, as well as tetracyclines and sulfonamides, in such a way that no LOI values were proposed. However, the detection of these compounds was feasible with SDL of 20 or 100 μg/kg. A more selective sample treatment seems necessary and/or the use of newer and more sensitive QTOF analyzer (e.g., Xevo G2 QTOF by Waters Corp.) to reach unequivocal identification at low parts per billion levels for these compounds in fish feed.

In contrast to feed, a LOI of 20 μg/kg could be achieved for the great majority of targeted compounds in fish. As an

example, Figure 1 shows the LE and HE TOF MS spectra for a fish sample spiked with azoxystrobin at 20 μg/kg. The chromatograms for the predominant m/z ions are also depicted at the lowest level studied. The presence of at least two chromatographic peaks at expected retention times allowed the unequivocal identification in the samples. Moreover, the low mass errors (<4.8 ppm) for the protonated molecule and the most abundant fragments supported the identification.

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

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

In relation to the maximum residue limits (MRLs), only a few compounds have MRLs established in feed 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 below 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, whereas 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 apply only 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 MRLs are set at 100 and 5000 μg/kg, respectively. MRLs for aflatoxin B1 and the sum of fumomisins B1 andB2 are set at 10 μg/kg, whereas the lowest concentration tested in validation was 20 μg/kg. Our results showed that detection at 10 μg/kg should not be much of a 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) showed only the ion corresponding to $[M + H]^+$. An 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. To evaluate the applicability of the method for routine analysis, 10 feed samples and 11 fish fillets were analyzed apart from the nonspiked 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 of 11 fish fillets; fumonisin B2 was found in 2 and zearalenone in 1 of 10 feeds; pirimiphos-methyl was detected in 8 of 10 feeds and in 2 of 11 fish fillets. In all of 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 μg/kg (SDL) and 100 μg/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 μg/kg (LOI), as it could not be identified with two ions. Although the SDL was also set at 20 μg/kg, surely this empirical value could have been decreased if lower concentrations had been tested.

With regard to fish feed, two mycotoxins were detected, fumonisin B2 and zearalenone, at predictable concentrations between 20 μg/kg (SDL) and 100 μg/kg (LOI). Pirimiphos-methyl was found in several feeds, at a predictable concentration below 20 μg/kg (LOI).

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

To confirm the presence of the compounds detected, the sample extracts were reanalyzed using a highly sensitive technique, that is, 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 compound 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 $[C_{34}H_{59}NO_{14}]^+$. However, no fragment ions were found in the feed sample, whereas up to four were observed in the standard (50 ng/mL). The high differences in sensitivity between the protonated molecule and the fragment ions for fumonisin B2 are remarkable. Accurate-mass LE spectra for $[C_{34}H_{59}NO_{14}]^+$ for both standard and feed samples 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, that is, 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 homemade database containing around 1000 compounds. Different strategies were followed depending on the availability or not of the reference standard.[34]

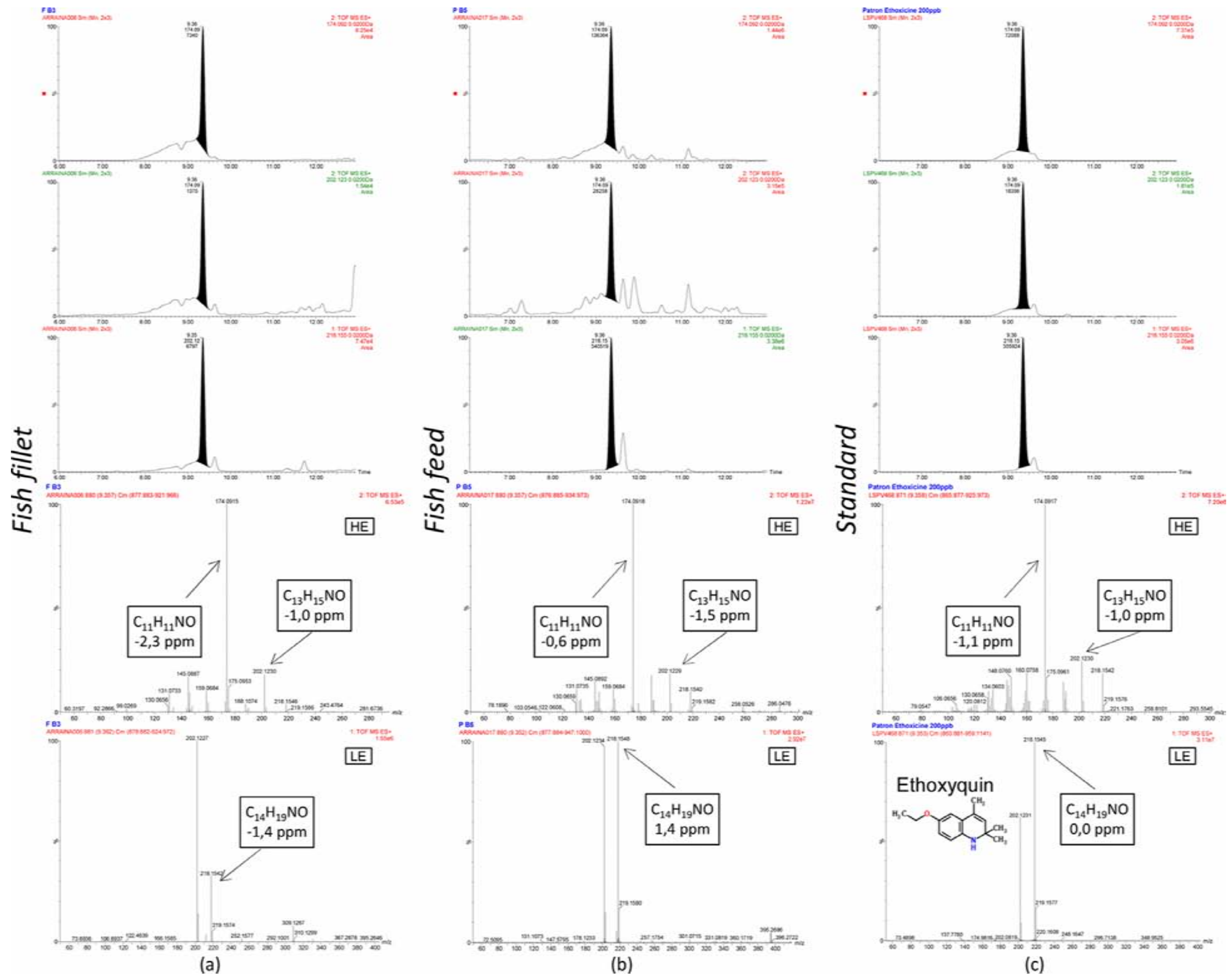

Figure 4. nw-XICs for protonated molecule and fragment ions and accurate mass spectra (both LE and HE) for ethoxyquin in (a) fish fillet, (b) fish feed, and (c) standard (200 ng/mL), respectively.

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 of 21 fish samples and in 12 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 the contrary, when the reference standard was unavailable at our laboratory, 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). In this way, several mycotoxins such as 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 MRLs 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 μg/kg). With regard to 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 $[M + H]^+$ 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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Notes

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