No transfer of the non-regulated mycotoxins, beauvericin and enniatins, from feeds to farmed fish reared on plant-based diets

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Abstract

Concern about the risk of exposure to emerging plant-derived mycotoxins such as beauvericin and enniatins has been addressed by the European Commission who requested the European Food Safety Authority for a scientific opinion on their risk to human and animal health. The studied mycotoxins were found in feeds with enniatin B and beauvericin at average concentrations of 19.9 μg/kg and 30 μg/kg, respectively. In all cases, concentrations of all the mycotoxins analyzed were below quantification limits (< 0.1 μg/kg) in fish samples (n = 82). The present work provides comprehensive and traceable data of emerging mycotoxins in plant-based aquafeeds and fish reared on the feeds, responding to increasing concerns about safety of farmed fish fed on sustainable feeds. On the basis of data reported, there was no transfer of the emerging mycotoxins, beauvericin and enniatins, from feeds to fish and so, no risk for human consumption.

Keywords: mycotoxins, fish, feed, liquid chromatography, mass spectrometry, transfer, Atlantic salmon, sea bream.

1. Introduction

Fish consumption has shown continuous growth due to increasing demand, world market availability, and consumer trends related to concerns for healthy eating (FAO, 2014). This expansion, combined with the limited availability of marine raw materials, fishmeal and fish oil, has forced the European aquafeed industry to explore alternative, sustainable sources of protein and lipid for feeds. Consequently, the most important European farmed fish species, including Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*), are currently being farmed successfully using feeds with a very high inclusion of plant-based ingredients, plant meals and vegetable oils, with only a low inclusion level of marine ingredients (Liland et al., 2013; Shepherd et al., 2017; Simó-Mirabet et al., 2018). However, these achievements have also brought concerns about the safety of market fish, of relevance not only for European food authorities and farmers, but also consumers. These concerns about potential risks to human health and animal welfare led to considerable research to determine what contaminants are of relevance in aquaculture based on changing feed formulations over the last decade or more (Nácher-Mestre et al., 2018; Simó-Mirabet et al., 2018).

The use of plant-based feeds can introduce contaminants that were not previously associated with fish farming utilizing traditional marine feed ingredients. Mycotoxins represent an important group of naturally-derived plant contaminants found world-wide in cereals, and consequently introduced into the new, alternative and sustainable aquafeeds. Concern about the risk of exposure to these mycotoxins has been partially addressed (EC, 2002; EFSA, 2011; EU, 2006) in research conducted primarily to protect consumers, but also animal health, in the context of improvement of food safety and farming industry efficiency. Anater et al. collected the presence of residues of mycotoxins in fish and estimated risks for public health (Anater et al., 2016). These analysis in wild European sea bass (*Dicentrarchus labrax L.)*, sea bream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis niloticus*), under natural occurrence, resulted in non-detectable levels of mycotoxins in muscle (Anater et al., 2016). Moreover, recent studies carried out on farmed Atlantic salmon and gilthead sea bream revealed that current regulated mycotoxins were not transferred from feeds to the edible fillets of the fish after representative feeding trials (Nácher-Mestre et al., 2015; Johny et al., 2019). However, other studies reported the presence of the mycotoxins, enniatins A1, B and B1, in market sea bass and sea bream fillets and also in liver, head and viscera (Tolosa et al., 2013, 2014). The above cited studies could be considered as the current state-of-the-art of knowledge regarding mycotoxins in farmed fish, but this requires to be extended to other European farmed fish species, including an assessment of the potential transfer of mycotoxins from feed to the edible part of the fish in controlled feeding trials. This accounts in particularly to new and emerging mycotoxins that have not yet been assessed.

In fact, a European Food Safety Authority (EFSA) Scientific Opinion has already indicated the potential risks to human and terrestrial animal health due to the presence of emerging mycotoxins, beauvericin and enniatins, in food and feed (EFSA, 2014). However, exposure estimated to beauvericin and enniatins for farmed fish or their potential feed-to-fillet transfer have not been made in the EFSA opinion (EFSA, 2014). These mycotoxins are more lipophilic than currently regulated mycotoxins and so they could be potentially deposited and accumulated in edible fillets and other essential fish organs.

Taking into account the above justification, the development of analytical strategies to monitor and quantify these natural toxins in feeds and fish fed plant-based diets should be carried out and, indeed, is expected by Authorities (EFSA, 2014). In addition, data on emerging mycotoxins in sustainable feeds and fish fed these feeds is required by the European aquaculture industry and regulatory authorities to increase relevant knowledge for future actions and regulations. For this purpose, the use of liquid chromatography coupled to mass spectrometry working in tandem mode (LC-MS/MS) are consistent with EFSA recommendations as matrix effects can be better addressed with this instrumentation, and these methods are able to perform reliable quantification at concentrations below 1 μg/kg (EFSA, 2014). Thus, LC-MS/MS makes feasible the transfer of acquired knowledge of relevance to emerging mycotoxins in farmed fish fed plant-based diets to the European aquaculture industry. The present study utilized ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to quantify levels of the emerging mycotoxins, enniatins and beauvericin, in feeds formulated with high levels of plant-derived ingredients and in Atlantic salmon and gilthead sea bream reared to market size on these feeds.

2. Materials and methods

2.1. Reagents and chemicals

HPLC-grade methanol (MeOH), HPLC-supergradient acetonitrile, formic acid (>98%), ammonium acetate (NH_4Ac), reagent grade were obtained from Flucka (Buchs, Switzerland). HPLC-grade water was obtained from a Milli-Q water purification system (Millipore Ltd., Bedford, MA, USA). Analytical standards for the emerging mycotoxins (≥ 95 %, HPLC) beauvericin (BEA), enniatin A (EnA), enniatin A1 (EnA1), enniatin B (EnB) and enniatin B1 (EnB1) were purchased from Sigma Aldrich (Gillingham, UK).

2.2. Samples

A total of 21 non-spiked feeds (17 for Atlantic salmon and 4 for sea bream) formulated with high percentages of plant proteins and oils were analyzed from controlled feeding trials conducted at the University of Stirling (UoS, Scotland), Institute of Marine Research (IMR, Norway) and the Institute of Aquaculture Torre de la Sal (IATS, Spain) within the framework of the EU ARRAINA Project and UK TSB Beans4Feeds project. Briefly, in experimental trials conducted by UoS, Atlantic salmon were fed with 6 different diet formulations (corresponding to 14 feeds), with fish sampled at 3, 6 and 11 months' post application of experimental feeds. A total of 50 samples (each sample was pooled from 5 fillets) were analyzed from dietary treatments, from intermediate and final sampling points, covering juveniles (250 g) to market fish size (2.6 kg). In parallel in this trial, 18 samples of whole salmon (pools of two fish) were also analyzed. In addition, two trials with freshwater salmon (parr) and seawater-adapted salmon (post-smolt) were performed by IMR with 3 different dietary formulations (3 feeds analyzed), in triplicate tanks/cages over a 11 month period, covering freshwater start feeding salmon (18 g) to seawater transferred post smolt (480 g). At the end of the trial, 10 fish from each cage were sampled and muscle samples were pooled. From those stored, a total of 10 available samples were analyzed. In the case of sea bream trial, juveniles of Atlantic origin (Ferme Marine de Douhet, France) were acclimated for four weeks to the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS, Spain). During this initial period, fish were fed with a standard diet (Efico YM 568 1.9 mm, BioMar). Then, fish of 13–16 g initial mean body weight were distributed in 2500 L tanks in triplicate groups of 180 fish each. Oxygen content of outlet water remained higher than 75% saturation, and day-length and water temperature followed the natural changes at IATS latitude (40º 5'N; 0º 10'E). Sea bream was fed with 4 different diet formulations (4 feeds analyzed) offered to visual satiety 1–2 times per day and 3–6 days per week from May 2013 to October 2014 (18-month feeding trial), according to the changes in fish size and season. At the end of trial, 20 fish of market size (0.8 – 1 kg) were randomly sampled and pools of 5 fish per each dietary condition were analyzed (4 fish samples in total). More specific details of the salmon (De Santis et al., 2016; Hemre et al., 2016; Taylor et al., 2019; Vera et al., 2019) and sea bream (Simó-Mirabet et al., 2018) trials can be found in the previously published studies.

2.3. Processing of samples and mycotoxin extraction

The samples of feeds and fish analyzed were collected at appropriate times during and at the end of the feeding trials carried out between 2014 and 2016 (De Santis et al., 2016; Hemre et al., 2016; Simó-Mirabet et al., 2018; Taylor et al., 2019; Vera et al., 2019). Samples of feeds were ground in a blender (Waring Laboratory Science, Winsted, CT, USA) and stored at -40 ºC prior to analysis. Whole salmon samples (pools of 2 fish) and salmon and sea bream fillet samples (pools of 5 fish) were homogenized in a blender (Waring Laboratory Science) to produce homogenous pates that were stored at -70 ºC prior to analysis. Samples of 2.5 g of homogenized feed, fish or fillet were accurately weighed and subsequently extracted with 10 ml acetonitrile using an automatic mechanical shaker at room temperature for 90 min. The extract was then centrifuged (5 min, 4500 rpm) followed by 1:4 dilution with water (1:10 dilution in feed matrix) prior analysis by LC-MS/MS.

2.4. UHPLC-MS/MS

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was coupled to a triple quadrupole mass spectrometer (Xevo TQS, Waters, Manchester, UK), using a Z-spray-ESI interface operating in positive ionization mode. The UHPLC separation was performed using an Acquity UPLC® BEH C18 analytical column 50 x 2.1 mm, 1.7 µm particle size (Waters) at a flow rate of 400 µL/min. The mobile phase consisted of water and methanol containing both solvents 5 mM ammonium acetate and 0.1 % formic acid as modifiers. Chromatographic separation was carried out in gradient mode, changing the percentage of organic solvent as follows: 0 min, 60 %; 4 min, 90 %; 4.1 min, 60 %; 6.0 min, 60 %. The column temperature was set at 40 ºC and sample injection volume was 10 µL.

Cone voltage and collision energy were optimized by acquisition of full-scan MS and MS/MS spectra of 1 ng/mL targeted mycotoxin reference standard. Three Selected Reaction Monitoring (SRM) transitions using as a precursor ion the $[M+H]^+$ were proposed for quantification (Q_i) and confirmation purposes (q_i). The UHPLC-MS/MS conditions are detailed in Table 1.

2.5. Analytical method validation

Method validation was carried out in 3 different matrices (feed, fish fillet and whole fish). Investigated compounds were beauvericin and enniatins A, A1, B and B1 at 1 µg/kg (25 ng/L in extract) and 10 µg/kg (250 ng/L in extract) in feed. For fish fillet and whole fish the methodology was validated at 0.1 µg/kg (6.25 ng/L in extract) and 1 µg/kg (62.5 ng/L in extract).

Linearity of the method was evaluated taking six matrix-matched standard solutions (from 2.5 ng/L to 10000 ng/L) for the 5 selected mycotoxins injected in triplicate. Correlation coefficients higher than 0.99 with residuals lower than 20 % were required for satisfactory linearity. Recoveries and precision (repeatability, expressed as relative standard deviation (RSD) in %), were determined within-day by analyzing spiked blank samples (n = 5) at the spiking levels. Acceptable recoveries and RSDs were established according to SANTE/11945/2015 guidelines for analytical quality control and method validation procedures (expected and desirable recoveries between 70 and 120 % with RSD lower than 20 %) (SANTE, 2015).

Limits of quantification (LOQ) were established at the lowest concentration validated, at 1 µg/kg for feed and 0.1 µg/kg in the case of fillets and whole fish. The specificity of the method was evaluated by analyzing a blank procedure, a processed blank sample and the same blank spiked at the lowest concentration validated for each studied matrix. Satisfactory results were considered when the signal obtained from blank samples was lower than 30 % of the signal at LOQ.

Quantification was carried out by means of matrix matched calibration to ensure an accurate determination compensating for matrix effect. Matrix effects were evaluated by comparison of calibration curves slopes when slopes were prepared in neat solvent and in matrix.

$$Matrix\ effect\ (\%) = \left(\frac{Matrix\ calibration\ slope}{Solvent\ calibration\ slope} - 1\right) x\ 100$$

Matrix effects were calculated in percentage. Positive values indicated signal enhancement, whereas negative values were associated to signal suppression. Matrix matched calibration consisted of blank extracts (900 µL) spiked with 100 µL of the corresponding calibration mix standard. Data obtained from analyzed samples was processed both automatically and manually by the TargetLynx application manager (within MassLynx v 4.1; Waters Corporation).

The q_i/Q ratios considered as the ratio between the intensity of the identification transition (q_i) and the quantification transition (Q), were used to additionally confirm peak identity in all samples analyzed (Table 1). According to SANTE/11945/2015 guidelines, q_i/Q transitions were accepted for identification purposes when the q_i/Q ratios were below 30%, in comparison with the experimental q_i/Q value calculated from solvent standards (SANTE, 2015).

3. Results and Discussion

3.1. UHPLC-MS/MS

The use of UHPLC-MS/MS has experimented an increasing use in the last years thanks to their extraordinary sensitivity and the short run time analysis achieved for a limited number of target mycotoxins (Beltrán et al., 2009, 2013). Despite of this fact, limitations for multi-mycotoxins have been found to get enough points per peak in MS/MS data acquisitions (Malachova et al., 2014). In our case, UHPLC with the use of a C18-50mm column working with a flow rate of 400 µL/min allowed the analysis of the five selected mycotoxins in only 6 min run time. Acceptable peak shapes with at least 12 points per peak were obtained for the five mycotoxins studied. This was also feasible with the gradient mode selected, starting with 60% of organic solvent.

The addition of modifiers in the mobile phase is crucial to improve the ionization of selected compounds in source and to obtain the maximum sensitivity in target analysis. In the present study, the addition of formic acid, acetic acid and ammonium acetate was studied to test the MS/MS performance and to enhance SRM transitions selecting $[M+H]^+$ and/or $[M+NH_4]^+$ as precursor ions, instead of sodium adducts. These additives are commonly included, in mobile phase and/or extraction solvent, in multi-mycotoxin methods (Beltrán et al., 2009, 2013; de Souza et al., 2013; Malachova et al., 2014; Sulyok et al., 2007). It has also been demonstrated that the use of ammonium acetate in the mobile phase is crucial to promote $[M+H]^+$ instead of sodium adducts in the case of aflatoxins (Beltrán et al., 2009, 2013). Acidic conditions are common in multi-mycotoxin methods and are necessary for the extraction and determination of fumonisins by LC-ESI-MS/MS (Beltrán et al., 2009, 2013; de Souza et al., 2013; Malachova et al., 2014; Sulyok et al., 2007). In the analysis of beauvericin and enniatins, some authors have also applied these modifiers suggesting their use to improve peak shape and analyte response (Decleer et al., 2016; Renaud et al., 2017; Tolosa et al., 2013, 2014, 2019).

In the present work, study of the ionization of the individual selected mycotoxins with different combinations and concentrations of modifiers, showed the presence of $[M+H]^+$ as well as their corresponding ammonium $[M+NH_4]^+$ and sodium $[M+Na]^+$ adducts. The addition of formic acid (0.1 %) and 5 mM ammonium acetate increased the intensity of $[M+H]^+$ in the source instead of $[M+Na]^+$ and $[M+NH_4]^+$ adducts. The study of SRM transitions confirmed that the sensitivity of transitions with $[M+H]^+$ as a precursor ion was significantly higher in comparison with the peak responses obtained for $[M+Na]^+$ and $[M+NH_4]^+$. Figure 1 illustrates the peak responses for the two most sensitive SRM transitions using each precursor ion for beauvericin in the three studied matrices, spiked at 1µg/kg. In all cases, transitions from the $[M+H]^+$ showed higher sensitivity than those coming from the adducts, specially the $[M+NH_4]^+$.

For both enniatins and beauvericin, at least three MS/MS transitions were proposed. For beauvericin, as only two transitions from the protonated ion were feasible, a transition from the sodium adduct was proposed for qualifying purposes. EnB1 and EnB showed the most sensitive SRM transition from their corresponding protonated molecule to the product *m/z* ion 86.1. However these transitions were not selected for quantification purposes due to the presence of higher noise (Table 1).

3.2. Sample treatment.

Solid-liquid extraction with mixtures of acetonitrile/water (80:20, v/v) was initially tested, following a similar protocol carried out in previous experiments with marine samples (Nácher-Mestre et al., 2015). Extraction efficiency was studied comparing the signal obtained with spiked samples and blank extract spiked prior injection at 1 µg/kg (62.5 ng/L in the extract for fish and fillet, and 25 ng/L in the extract for feed). The addition of modifiers such as formic acid and/or ammonium acetate in the extraction solvent was also tested. As illustrated in Figure 2, the use of acidic extraction solvent decreased the signal obtained whilst ammonium acetate did not produce significant changes in the extraction efficiency.

Non-polar enniatins and beauvericin presented lower recoveries (< 70 %) when water was introduced in the extraction solvent, in comparison with the single use of acetonitrile. However, the use of acetonitrile could promote the extraction of higher amounts of fatty acids, increasing matrix effects. To minimize matrix effects (usually observed as signal suppression, Figure 3), the extracts were diluted after sample extraction to minimize the interferences from the matrix and also, reduce the percentage of organic content in the injected extract. Thus, chromatographic peak shape was improved, avoiding the observed peak fronting in the most polar mycotoxins.

3.3. Method validation

Validation of analytical methods for fish feed, flesh fillets and whole fish samples was carried out in accordance with the recommendations of the EU Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed (EFSA, 2014). Each matrix was validated at two concentration levels. Feed samples were validated at 1 and 10 µg/kg, while flesh and whole fish were at 0.1 and 1 µg/kg. The validation of feed was not feasible below 1µg/kg due to the absence of blank feeds (i.e. none of the feeds were mycotoxin free). In addition, high interferences were found at low concentration levels in this complex matrix, probably derived from the fatty content and pigments. Although the proposed method was not able to selectively extract the mycotoxins, extracting also undesired matrix interferences, the dilution step greatly helped to reduce signal suppression from the matrix.

Five replicates were prepared at each level. In addition, a sample blank and a procedure blank (only solvents and/or reactants) were injected during the sequence of analysis.

Matrix-matched calibration showed good linearity in the range from 2.5 ng/L up to 10 ng/mL with regression coefficients above 0.995 for both enniatins and beauvericin with residuals lower than 20 % and without any clear trend in their distribution. Table 2 shows the recoveries obtained for the three studied matrices at the different spiked concentrations. Recoveries from the five replicates were above 70 % and below 109 % with maximum RSDs below 19 %. These results obtained are in agreement with recent articles on this discipline that also obtained satisfactory recoveries with acceptable deviations (Johny et al., 2019; Tolosa et al., 2019).

To deal with the specificity of the method, a blank procedure, a processed blank sample and a blank sample spiked at the lowest concentration (five replicates analyzed) were injected and compared. In this case, no peaks were found in the blanks selected for fish and whole fish samples and, therefore, the rules proposed in the validation section for specificity were satisfactory. This was not the case for feed where it was not feasible for EnA1, EnB, EnB1 and Bea, as no real blank was found from the large list of collected feed.

The limits of detection (LOD) were estimated by using the *S/N* ratios of 3, from the SRM chromatograms of samples spiked at the lowest concentration. LOD values ranged from 0.01 to 0.04 µg/kg while LOQ proposed were the lowest spiked concentrations with acceptable accuracy by applying the complete analytical method, 1 µg/kg for feed and 0.1 µg/kg for flesh and whole fillet (Table 2). Although LOD are not included in SANTE guidelines it gives an overview about the analytical capabilities of LC-MS/MS for detection at trace levels in complex matrices.

The wide majority of matrix-analyte combination suffered from matrix suppression (Figure 3). Higher matrix effects were observed for feed and whole fish, showing signal suppression higher than 50 % for beauvericin in feed and EnA in whole fish. Regarding fish fillet, matrix effects were lower, probably due to the lower presence of matrix co-extracted components such as lipids and proteins.

For accurate quantification, co-eluted matrix interferences must be minimized or compensated. Different approaches have been used to deal with matrix effects such as the dilution of samples and subsequently, minimizing the amount of co-eluted matrix introduced into the MS system (Decleer et al., 2016; Panuwet et al., 2016; Renaud et al., 2017). However, dilution is limited as it reduces sensitivity and increases limits of detection. In the case of feed, 10-fold dilution was applied to reduce co-eluted matrix and, 4-fold dilution was applied to fish samples. The use of isotopically labelled analogue of the target analyte as internal standard is the most effective way to compensate matrix effects (Panuwet et al., 2016). However, for the selected compounds, internal labelled standards were not commercially available. Thus, quantification was carried out using matrix-matched calibration, after applying the corresponding dilution.

3.4. Application to sample analysis

The proposed method was applied to the analysis of samples derived from feeding trials carried out on sea bream and Atlantic salmon (further details of samples in Material and methods) (De Santis et al., 2016; Hemre et al., 2016; Simó-Mirabet et al., 2018; Taylor et al., 2019; Vera et al., 2019). Mycotoxins were detected in all the feeds. The highest values were found for Bea (80.4 μg/kg) while EnA was in all cases below LOQ. Bea, EnB and EnB1 were found in all feeds while EnA1 was quantified in 20 feed samples with concentrations up to 3.8 μg/kg. EnB1 was found in the concentration range between 2.4 - 10.9 μg/kg while EnB was between 8.0 - 32.8 μg/kg.

Neither whole fish nor fillet samples contained the studied mycotoxins at any detectable concentration. Therefore, no feed to fillet transference of the parent compound was detected. This evidence suggests no risk for human consumption but there could still be some concern that the molecules studied might be metabolized and/or deposited in organs at concentrations below those established in the current research (Anater et al., 2016; Glencross et al., 2019).

Figure 4 shows the SRM chromatograms from different sea bream and salmon feeds. Reliable quantification and identification was carried out due to the acquisition of three SRM transitions with q_i/Q ratios in agreement with values obtained from standards (SANTE, 2015).

4. Conclusions

Advanced analytical methodology based on the use of liquid chromatography coupled to tandem mass spectrometry was developed to analyze the occurrence of emerging mycotoxins, enniatins A, A1, B and B1 and beauvericin, in samples derived from feeding trials in farmed Atlantic salmon and gilthead sea bream. The methodology proposed allowed the determination of these emerging mycotoxins at low limits in complex fatty samples by acquiring three SRM transitions per compound in a run time of only 6 min. The use of UHPLC-MS/MS resulted as an extraordinary option to deal within medium-short list of target mycotoxins with short run time analysis. Mycotoxins were found in feed at concentrations lower than 112 µg/kg (expressed as the sum of the five mycotoxins). The results based on the use of new advanced analytical methodology showed that samples of Atlantic salmon and sea bream reared on these feeds did not present any detectable parent-mycotoxin, neither in the fillet (salmon and bream), nor in whole fish (salmon). Therefore, no feed to fillet transfer of the parent mycotoxin compounds was detected.

Next challenges for the analysis of emerging mycotoxins will probably address the identification of metabolites in target animal tissues where it is interesting to realize how can interact in biological routes.

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Conflict of interest statement

The authors of this manuscript have declared no conflict of interest.

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Table 1. Experimental MS/MS conditions for the studied mycotoxins.

Mycotoxin	**Retention time (min)**	**Precursor ion (*m/z*)**	**Product ion (*m/z*)**	**Collision energy (eV)**	**Cone voltage (V)**	**q_1/Q**	**q_2/Q**
Enniatin B (EnB)	3.0	$[M+H]^+$ 640.2	196.0 (Q)	20	40	0.63	1.62
			214.3 (q_1)	20			
			86.0 (q_2)	60			
Enniatin B1 (EnB1)	3.3	$[M+H]^+$ 654.4	196.3 (Q)	60	40	0.57	1.41
			100.1 (q_1)	30			
			86.1 (q_2)	60			
Beauvericin (Bea)	3.4	$[M+H]^+$ 784.4	134.1 (Q)	50	40	0.40	0.39
			244.0 (q_1)	30			
		$[M+Na]^+$ 806.5	384.2 (q_2)	50			
Enniatin A1 (EnA1)	3.5	$[M+H]^+$ 668.2	100.1 (Q)	60	40	0.75	0.46
			210.0 (q_1)	60			
			86.2 (q_2)	20			
Enniatin A (EnA)	3.7	$[M+H]^+$ 682.4	100.0 (Q)	60	40	0.85	0.27
			210.0 (q_1)	30			
			228.0 (q_2)	30			

Q: quantification transition, q_i: qualitative transitions

Table 2. Main recoveries (n=5), LOD and LOQ proposed for the studied mycotoxins in matrices from the aquaculture.

	Feed						**Fish fillet**						**Whole fish**					
	1 µg/kg		10 µg/kg		LOD (µg/kg)	LOQ (µg/kg)	0.1 µg/kg		1 µg/kg		LOD (µg/kg)	LOQ (µg/kg)	0.1 µg/kg		1 µg/kg		LOD (µg/kg)	LOQ (µg/kg)
	Rec (%)	RSD	Rec (%)	RSD			Rec (%)	RSD	Rec (%)	RSD			Rec (%)	RSD	Rec (%)	RSD		
EnA	70	8	70	19	0.04	1	100	13	85	5	0.01	0.1	98	8	107	14	0.01	0.1
EnA1	85	16	72	13	0.04	1	81	13	82	7	0.02	0.1	78	19	100	8	0.01	0.1
EnB	76	9	78	7	0.03	1	76	9	78	7	0.01	0.1	76	8	96	7	0.02	0.1
EnB1	95	11	77	3	0.03	1	87	11	91	8	0.02	0.1	99	19	93	14	0.03	0.1
Bea	93	19	72	14	0.03	1	86	11	92	10	0.03	0.1	104	7	109	11	0.03	0.1

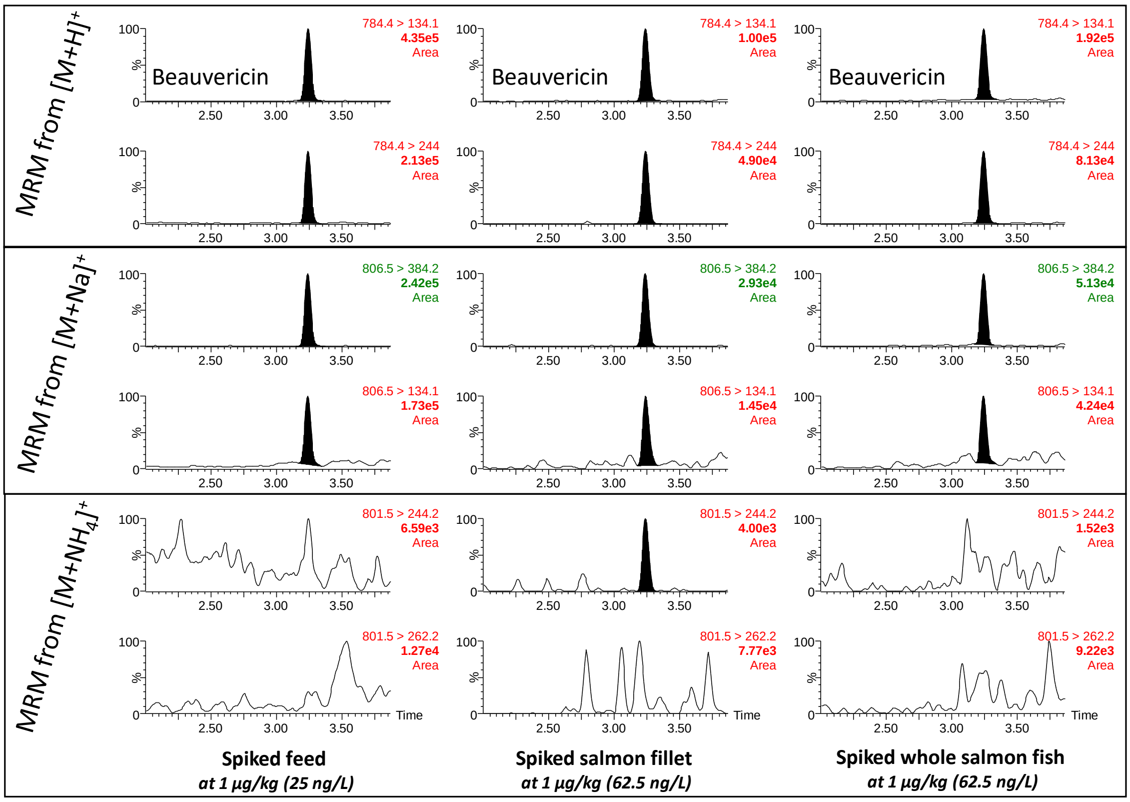


Figure 1. SRM chromatograms for beauvericin spiked at 1µg/kg in feed, salmon fillet and whole salmon fish. Chromatograms for two selected SRM transitions are shown for each precursor ion ($[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$) were illustrated for the three studied matrices.

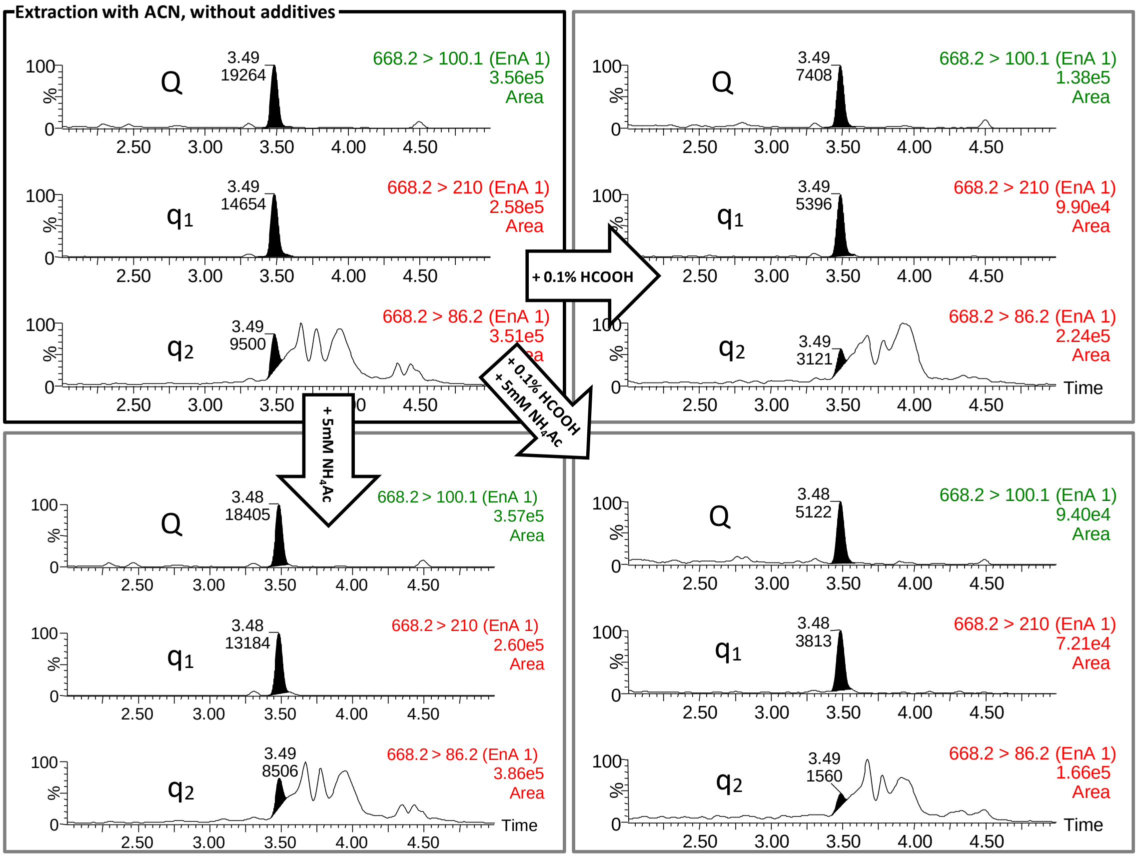


Figure 2. SRM chromatograms for EnA1 in feed spiked at 1µg/kg. Effect on sensitivity produced by the addition of modifiers in the extraction solvent.

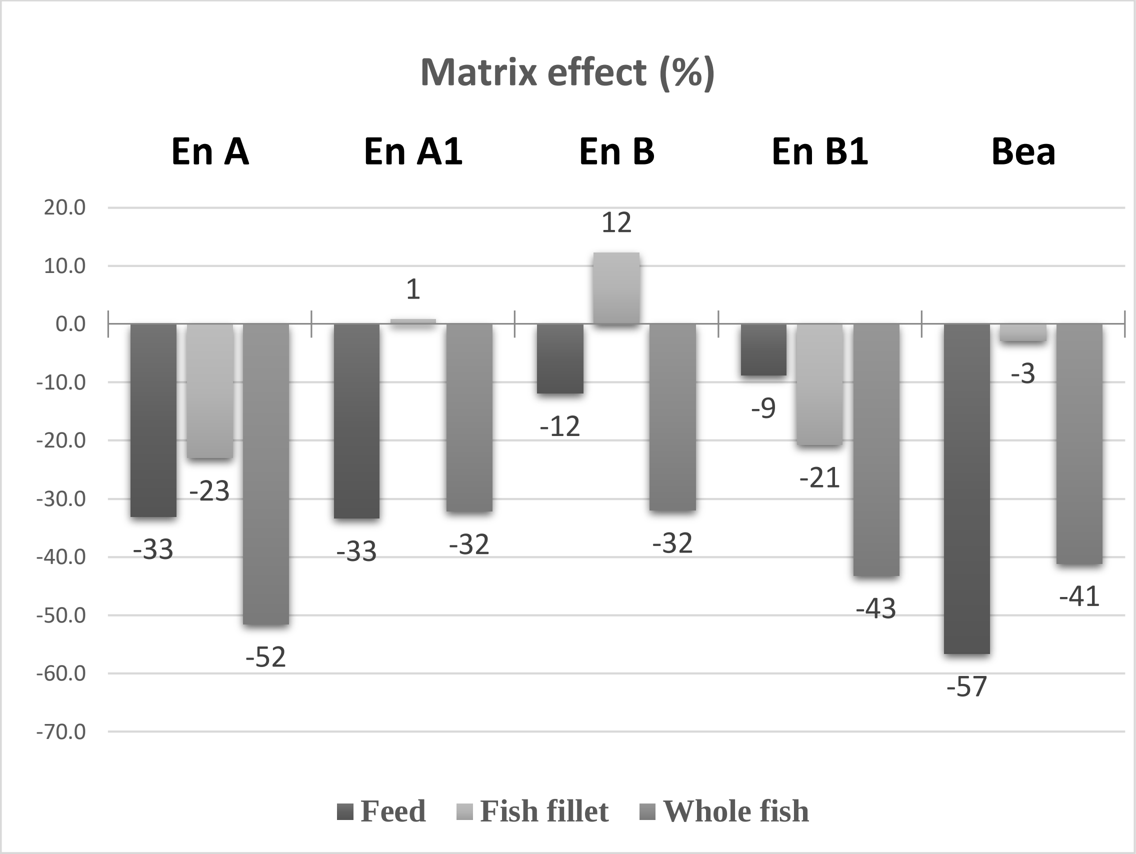
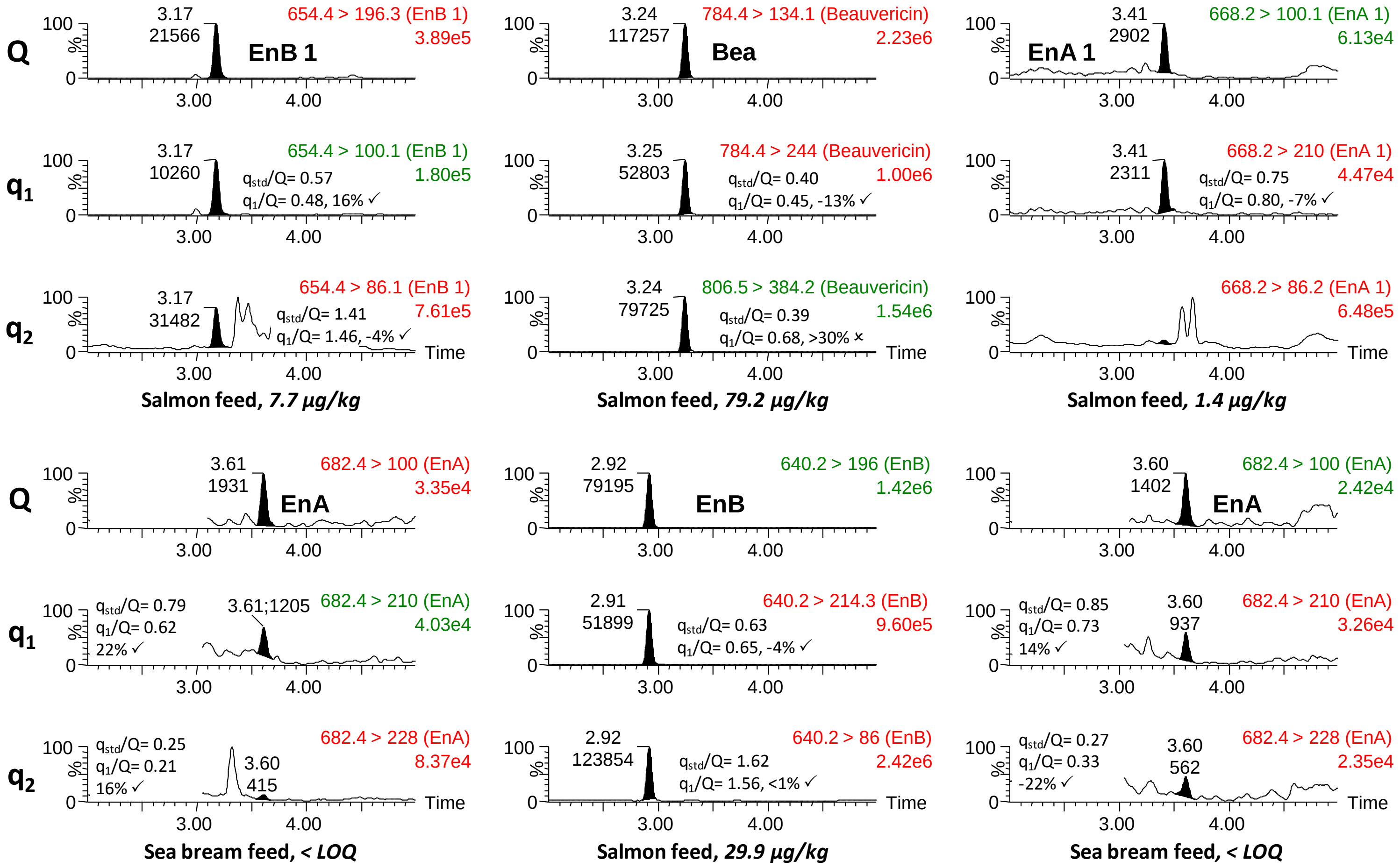


Figure 3. Matrix effects calculated in percentage for mycotoxins in the three studied matrices. Positive values indicated signal enhancement, whereas negative values were associated to signal suppression.



Salmon feed, *7.7 µg/kg*

Salmon feed, *79.2 µg/kg*

Salmon feed, *1.4 µg/kg*

Sea bream feed, *< LOQ*

Salmon feed, *29.9 µg/kg*

Sea bream feed, *< LOQ*

Figure 4. SRM chromatograms for enniatins and beauvericin in feed samples. Experimental q_i/Q calculated in real samples were compared with the q_i/Q obtained injecting the standards in the same sequence.