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Development of an analytical method to detect metabolites of nitrofurans Application to the study of furazolidone elimination in Vietnamese black tiger shrimp (*Penaeus monodon*)

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ABSTRACT

Nitrofurans, banned antibiotics in the European Union (EU), have often been found in imported aquaculture products in the past and are still found nowadays according to the Rapid Alert System for Feed and Food (RASFF) of the European Commission. A quantitative method based on liquid chromatography coupled to isotopic dilution tandem mass spectrometry (LC–IDMS/MS) was developed for the determination of the residues of four nitrofuran antibiotic residues in shrimps. The experimental protocol consisted of an acid-catalysed release of protein-bound metabolites, followed by derivatisation with 2-nitrobenzaldehyde (NBA). Then, a double liquid–liquid extraction with ethyl acetate was performed before LC–IDMS/MS analysis by positive electrospray ionisation (ES+) with multiple reaction monitoring (MRM) of two transitions per compound. An "in-house" validation of the method for shrimp analysis was conducted according to the EU criteria for the analysis of veterinary drug residues in foods. The decision limits (CCalpha) were 0.08–0.36 μg kg^{-1} and the detection capabilities (CCbeta) were 0.12–0.61 μg kg^{-1}, which are both below the minimum required performance limit (MRPL) set at 1 μg kg^{-1} by the EU. The developed method was applied to evaluate the elimination of furazolidone residues in shrimp muscles after a contamination experiment. After 28 days of decontamination, a concentration of 115 μg kg^{-1} of furazolidone metabolite 3-amino-2-oxazolidinone (AOZ) was still measured in the shrimp muscle.

1. Introduction

There are several important concerns with regard to the use of antibiotics and chemotherapeutic agents in aquaculture, such as food safety, aquaculture product quality, and environmental impacts related to the dispersion of chemicals and drugs into water and sediments, as well as their effects on aquatic communities. The lack of toxicological and elimination kinetics data obtained for cultured organisms and within the aquaculture system itself, and the lack of alternative for chemical application are a reality. Very few studies of antibacterial drug depletion in farmed fish and shrimp are available. In the literature, the pharmacokinetics of furazolidone and its metabolite 3-amino-2-oxazolidinone (AOZ) are reported only for some fish species such as Nile tilapia (*Oreochromis niloticus*) (Xu et al., 2006), orange-spotted grouper (*Epinephelus coioides*) (Guo et al., 2007), or *Carassius Aurats* (Han et al., 2009).

The fishery and aquaculture sectors have become the leading export sectors in Vietnam, with marine shrimp farming having the most important activity in aquaculture. Tiger shrimps (e.g., *Litopenaeus vannamei* and *Penaeus monodon*) are the main contributors to shrimp production, accounting for 70–80% of produced shrimps. The expansion of shrimp culture led to the increased use of chemicals (pesticides) and drugs (antimicrobials) from which residue levels are affecting or threatening export acceptability of products. Within a research programme involving Belgian and Vietnamese universities, confirmatory methods for pesticide and antimicrobial residue measurements in shrimps are under development.

From early shrimp production practice investigations, it appeared that the nitrofurans are potentially among the most commonly used antimicrobials in extensive modes of production. In Vietnam, these antibiotics are used during the culture of shrimp, mainly to prevent (prophylactic use) and treat (therapeutic use) bacterial diseases (Tu et al., 2006, 2009).

Nitrofurans are synthetic broad spectrum antimicrobial agents that have been used in the past for breeding or in animal production (McCracken and Kennedy, 1997a; McCracken et al., 1995). They are

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effective against Gram-negative and Gram-positive bacteria. They also have anti-protozoal activity (Gräslund and Bengtsson, 2001). Nitrofurans have been extensively used in fish and shrimp farming (GESAMP, 1997).

In 1993, the European Union decided to ban the use of nitrofurans (furaltadone, nitrofurazone, nitrofurantoin and furazolidone) because of the potential mutagenic and carcinogenic effects of their metabolites (3-amino-5-morpholinomethyl-1,3-oxazolid-2-one (AMOZ), semicarbazide (SEM), 1-aminohydantoin (AHD) and 3-amino-2-oxazolidinone (AOZ), respectively) (Anonymous, 1993; van Koten-Vermeulen et al., 1993). Once administered, nitrofurans are quickly transformed into DNA and protein covalently-bound metabolites, complicating their quantitative analysis. However, the metabolite–protein bond can be hydrolysed to permit the extraction of the free residues (Horne et al., 1996). In 2003, the Commission Decision 2002/657/EC fixed a minimum required performance limit (MRPL) of 1 μg kg^{-1} for each free residue of nitrofurans in aquaculture products and in poultry meat (Anonymous, 2002).

There is serious concern about the consumption of shrimps containing forbidden substances such as nitrofuran metabolites as they are still found in imported aquaculture products nowadays according to the Rapid Alert System for Feed and Food (RASFF) of the European Commission. Indeed, for the last ten years, 74 alerts and 96 border rejections concerning nitrofuran metabolites in crustaceans and associated products have been reported in the RASFF database. For banned substances, these MRPL values are used as a limit of acceptability for imported products coming from various countries in the EU, according to regulation 2005/34/EC (Anonymous, 2005). For this reason, in this work, we shall present a quantitative method of analysis of the sum of free and bound nitrofuran residues using liquid chromatography combined with tandem in space mass spectrometry (LC–MS/MS) and its validation performed according to the Decision of the Commission 2002/657/EC (Anonymous, 2002). The developed method was applied to shrimp muscles after a contamination/decontamination experiment with furazolidone to evaluate the amount of nitrofuran metabolite that could be found in shrimps exported from Vietnam.

2. Experiment

2.1. Chemicals and solvents

1-Aminohydantoin·HCl (AHD·HCl, purity: >95%), 3-amino-5-morpholinomethyl-1,3-oxazolid-2-one (AMOZ, purity: >95%), d_2-aminohydantoin·HCl (AHD-d_2·HCl, purity: >95%), 5-(morpholinomethyl)-amino-2-oxazolidinone-d_5 (AMOZ-d_5, purity: >95%), and 3-amino-2-oxazolidinone-4,4,5,5-d_4 hydrochloride salt (AOZ-d_4·HCl, purity: 98%) were purchased from Chemical Synthesis Services (United Kingdom). Semicarbazide hydrochloride (SEM·HCl, purity: >95%) and 3-amino-2-oxazolidinone (AOZ, purity: >99%) were purchased from Aldrich (St-Louis, MO, USA). Semicarbazide hydrochloride [1,2-$^{15}N_2$, ^{13}C] (SEM-^{15}N–^{13}C·HCl, purity: >99%) was purchased from WITEGA (Berlin, Germany). The three deuterated compounds and ^{15}N–^{13}C labelled SEM were used as internal standards in the quantitative analysis. HiPersolV HPLC water was obtained from BDH Laboratory supplies (Poole, England). LC–MS-grade acetonitrile, LC–MS-grade methanol and HPLC-grade ethyl acetate were obtained from Biosolve (Valkenswaard, The Netherlands). 2-nitrobenzaldehyde (NBA) (98%) was purchased from Sigma (St-Louis, MO, USA). Acrodisc® 25 mm syringe filters (with 5 μm Versapor® membrane) were purchased from Pall Life Sciences, MI, USA.

2.2. Standard solutions

Eight individual stock solutions (1 mg/ml) were prepared by dissolving 5–15 mg of each nitrofuran standard in HPLC grade water. Eight intermediate solutions (10 μg/ml) were obtained by diluting 50 μl of each stock solution with 4.95 ml of HPLC grade water. A pool containing AHD·HCl, AMOZ, SEM·HCl and AOZ at a concentration of 0.01 μg/ml was prepared by diluting in a 100 ml volumetric flask 100 μl of each of the four 10 μg/ml solutions with 99.6 ml of HPLC grade water. A second pool containing AMOZ-d5, AOZ-d4·HCl and SEM-^{15}N–^{13}C·HCl at a concentration of 0.05 μg/ml and AHD-d2·HCl at a concentration of 0.1 μg/ml was prepared by diluting 50 μl of the AMOZ-d5, AOZ-d4·HCl and SEM-^{15}N–^{13}C·HCl 10 μg/ml solutions and 100·μl of the AHD-d2·HCl 10 μg/ml solution with 9.75 ml of HPLC grade water. All of the standard solutions were kept for maximum of 6 months at +4 °C.

2.3. Samples used for the validation of the method

For the validation of the method, four types of shrimps were bought in a Belgian supermarket in Spring 2004: tails of Vietnamese shrimps, two kinds of medium Indonesian shrimps and Bangladesh shrimps. A pool of the shrimps of each type, typically 100 g, was created by peeling and blending the shrimps until homogeneous in a mechanical blender (Moulinex, Germany). Then, each type of the samples was labelled and stored at −25 °C until analysis.

Those shrimp samples were checked, using the developed method, as "blank" regarding their nitrofuran metabolites content. They were then used as blank samples and spiked quality control (QC) samples for validating the developed method and for the calibration curves (see Section 2.6).

2.4. Sample preparation

One gram of shrimp was homogenised and spiked with a solution containing the four internal standards (as described in Section 2.1) at a concentration corresponding to 2 μg kg^{-1}. Hydrolysis of the metabolite–protein bond was performed using 5 ml of 0.2 M HCl and a derivatisation was performed by adding 50 μl of 0.1 M 2-nitrobenzaldehyde. Hydrolysis and derivatisation took place at the same time during an overnight incubation period of 16 h at 37 °C. After cooling the solution to room temperature, 500 μl of 2 M NaOH and 1 ml of 0.5 M phosphate buffer were added to adjust pH of the solution to a value included between 6.3 and 7. Nitrofuran derivatives were extracted with 2×4 ml of ethyl acetate followed by evaporation of the solvent to dryness under nitrogen and the addition of 300 μl of HPLC grade water. Finally, the solution was filtered through an Acrodisc® filter and transferred into an injection vial.

2.5. LC–MS/MS analysis

A 2690 Alliance Separation Modules (Waters, Milford, MA, USA) integrated autosampler, solvent delivery system and column heater coupled to a Quattro Ultima Platinum triple–quadrupole mass spectrometer (Micromass, Manchester, UK) were used for LC/MS–MS analysis. The LC column used was a Symmetry C_{18} (2.1×150 mm, 3.5 μm), with a Symmetry C_{18} guard column (2.1×10 mm, 3.5 μm), both from Waters Corporation, Milford, MA, USA. The mobile phase was acetonitrile (solvent A) and water containing 0.1% acetic acid (solvent B). The gradient elution conditions were: from 10% to 80% of solvent A between 0 and 17 min; then, conditions were held for one minute and the contribution of solvent A was decreased to 10% over 10 min. The oven temperature was set at 40 °C and the injection volume was 20 μl. The flow rate was 0.4 ml/min, with a split of 1:1 prior to the MS source.

The mass spectrometer was equipped with an electrospray ionisation (ESI) interface, used in positive ionisation mode. Derivatised native and deuterated standard solutions were synthesised in our laboratory and were infused in the mass spectrometer to optimise the MS tune parameters: capillary: 3.2 kV, source temperature: 125 °C, desolvation temperature: 250 °C, cone gas flow: 50 l/h, desolvation

gas flow: 620 l/h, collision cell pressure: 2×10^{-3} mbar, multiplier: 650 V. For the transition parameters, the Commission Decision 2002/657/EC specifies that a minimum of four identification points is required for the confirmation of substances classified in group A of Annex I of Directive 96/23/EC, including nitrofuran residues (Anonymous, 1996). As low resolution tandem mass spectrometry was used, this requirement was fulfilled by following a precursor and two daughter ions for each compound. The derivatised forms of the analytes were detected using the Multiple Reaction Monitoring (MRM) mode, with two transitions for each native compound. For the internal standards, only one transition per compound was followed.

Results were calculated using Quanlynx Software (Micromass).

2.6. Calibration curves

Six blank samples of shrimps (see Section 2.3) spiked with increasing concentrations (0, 0.5, 1, 2, 3 and 5 μg kg^{-1}) of each nitrofuran metabolite and with a pool of isotopically-labelled internal standards, each at a concentration of 2 μg kg^{-1}, were extracted simultaneously with the samples. The extracts of these six samples were used to construct the calibration curves: response (area of native compound and labelled compound ratio) versus concentration of native compound. Calibration points were injected before each series of samples and the extract spiked at a concentration corresponding to MRPL (1 μg kg^{-1}) was reinjected after the series of samples.

The choice of the best regression model was studied using the statistic F-test, which is also known as Mandel fitting test (Mandel, 1964).

2.7. Limits of detection (LOD) and of quantification (LOQ)

The limit of detection (LOD) and the limit of quantification (LOQ) were determined with the formulas: $LOD = 3*B/A$ and $LOQ = 10*B/A$, where B is the residual standard deviation (y-intercept) and A is the slope of the curve (Feinberg, 1996; Willetts and Wood, 2000).

2.8. Confirmation criteria

Nitrofuran metabolites were considered as positively identified in shrimp samples if the ratio between the chromatographic retention time of the analyte and that of the corresponding IS, i.e. the relative retention time (RRT)of the analyte, corresponded to that of the average retention time of the calibration solutions within a ±2.5% tolerance and the peak area ratio of the two transitions of the native analytes corresponded to that of the averaged transition ratio of the calibration solutions within the tolerances set by the Commission Decision 2002/657/EC (Anonymous, 2002).

2.9. "In-house" method validation

A quantitative validation method was performed according to the Commission Decision 2002/657/EC and following the most commonly used approaches (Feinberg, 1996; Willetts and Wood, 2000).

Extractions of shrimp samples and injections of sample extracts were performed as follows, according to the method validation purpose:

- Day 1: a calibration curve consisting of six blank shrimp samples spiked with a solution of nitrofuran metabolites and with a solution of isotopically-labelled internal standards (see Section 2.6), twenty blank samples and twenty quality control samples (QCs), i.e. blank shrimps spiked with a solution containing the four nitrofuran residues at a concentration corresponding to their MRPL, were analysed (determination of selectivity, specificity, repeatability and CCα).
- Day 2: a calibration curve consisting of six blank shrimp samples spiked with a solution of nitrofuran metabolites and with a solution of isotopically-labelled internal standards (see Section 2.6) and twenty QCs spiked at a concentration corresponding to the decision limit (CCα) were analysed (determination of CCβ).

The twenty blank samples, as well as the QCs, were composed of four batches of five samples, with each batch corresponding to a different type of shrimps.

2.10. Evaluation of elimination of furazolidone in shrimps

2.10.1. Medicated-feed

Four grams of furazolidone (98% purity) powder was mixed with 1 kg commercial shrimp feed. Medicated-feed was coated with 3% water and then with 3% cod liver oil and kept frozen at −20 °C until use.

2.10.2. Conditions of shrimp contamination

An experiment was carried out at the College of Aquaculture and Fisheries, Can Tho University, Vietnam. The shrimp (average weight of 10 g) were obtained from extensive shrimp farms. Upon arrival, 256 shrimps *P. monodon* were stocked and acclimated to laboratory conditions in six composite tanks (capacity of 2 m^3) filled with 1.5 m^3 of 15 g/l natural aerated water. Shrimp were fed with commercial shrimp feed (35% crude protein), four times daily, but were starved one day before experimentation. After two weeks acclimation, three tanks were supplied with furazolidone medicated-feed and three with non-medicated-feed for 7 days. After 7 days medication, all shrimps were fed with non-medicated-feed for another 28 days (post-medication). During the acclimation and decontamination periods, water was cleaned and 50% of the water was exchanged by using prepared stock saline water. In contrast, during the contamination period, shrimps were kept in static water. Uneaten feed was removed before the next feeding. No mortality was observed during the experiment.

2.10.3. Sampling and muscle collection

Shrimps were sampled on day 0, after 96 h and 171 h medication (contamination) and after 24 h, 96 h, 7 days, 14 days and 28 days post-medication (decontamination). At each sampling time, the muscles from six shrimp per tank were collected on ice, pooled per tank and kept at −80 °C until assayed.

3. Results and discussion

3.1. Method development

The solid phase extraction step proposed by Leitner et al. (2001) was replaced by a double liquid–liquid extraction (LLE) with ethyl acetate, as previously proposed by McCracken and Kennedy (1997b). To compensate for the fact that the LLE extracts were less clean than the SPE extracts, a filtration step using a 5 μm Acrodisc® syringe filter was added at the end of the extraction protocol. The use of an extracted calibration curve corrected a possible loss of compounds that could be caused by poor resolubilisation of the extracts.

For the LC gradient, a mixture of acetonitrile/water + 0.1% acetic acid was used with the Symmetry C18 Waters LC column. The use of acetonitrile gave an enhanced sensitivity compared to methanol for that LC column. The separation of the target molecules and their respective internal standards was then achieved within a total runtime of 28 min.

3.2. Method validation

3.2.1. Limits of detection (LOD) and of quantification (LOQ)

As the MRPL of the four nitrofuran marker residues was 1 μg kg^{-1}, the values obtained for LOD and LOQ were satisfactory for AMOZ, AOZ and AHD (data not shown). However, a small peak of SEM was always

observed in "blank" samples. As a consequence, there was a systematic bias which considerably increased the y-intercept value, consequently increasing LOD and LOQ. The presence of SEM in shrimp samples may come from sources other than from the use of the parent drug nitrofurazone. Indeed, SEM has been found in foods after treatment with sodium hypochlorite, which is used in some food processing methods for disinfection or bleaching (Hoenicke et al., 2004). Furthermore, Pereira et al. (2004) demonstrated that some meats coated with flour were contaminated with SEM, the origin of which was due to azodicarbonamide (AZD) used in the industry as a dough conditioner. More recently, Van Poucke and co-workers demonstrated the natural occurrence of SEM in the shell of *Macrobrachium rosenbergii* prawns (Van Poucke et al., 2011). Considering these recent findings, SEM is not the most suitable marker for the control of nitrofurazone administration.

The Commission Decision 2002/657/EC promotes the use of Certified Reference Materials (CRM). Unfortunately, CRM of nitrofuran residues in shrimps or prawns are not yet available. For this reason, shrimp samples fortified with standard solutions were used to assess the accuracy of the method.

3.2.2. Calibration curves

For quantification, a linear regression was used and no "fit weighting" was applied. The correlation coefficients, R^2, associated with those curves were higher than 0.98 (Table 1), which is the minimum value we fixed for that parameter. We also established that only one point of the curve can deviate from the curve by more than 20% of the corresponding calculated value.

3.2.3. Selectivity and specificity

The absence of significant peaks was shown in the blanks and the presence of quantifiable peaks was seen in the QCs (blank shrimp samples spiked at the MRPL level of 1 μg kg^{-1}). When a peak was detected in the blanks, it was shown that the relative retention times and/or the transition ratios (ratio between the peak area corresponding to the first MRM transition and that of the second transition for an analyte) did not correspond to those of the four nitrofuran residues analysed here. For the QCs, it was also shown that the variations of relative retention times and of transition ratios corresponded to that of the calibration solution at a tolerance of ±2.5% for the RRTs, and ±20% for the transition ratios of ions with relative intensities higher than 50% of the base peak (Anonymous, 2002). The establishment of an acceptability criterion for concentration in the QCs was problematic. Indeed, those compounds being forbidden for use in the European Union are then classified in group A of Annex 1 of Directive 96/23/EC. For that reason, it was decided that if the signal/noise ratio (S/N) was equal or higher than 3 and if the relative retention time and ion ratio criteria are satisfactory, the analyte is considered present and the QC is accepted.

Table 1
Mass of precursor and daughter ions and collision energies followed in MS/MS for each compound analysed and their respective internal standard.

Native compound	m/z	Collision energies	Internal standards	m/z	Collision energies
NPAMOZ	335 > 291	10 eV	NPAMOZ-D5	340 > 296	10 eV
	335 > 128	15 eV			
NPAOZ	236 > 134	10 eV	NPAOZ-D4	240 > 134	12 eV
	236 > 104	18 eV			
NPSEM	209 > 166	10 eV	NPSEM-^{15}N, ^{13}C	212 > 168	10 eV
	209 > 192	10 eV			
NPAHD	249 > 134	12 eV	NPAHD-D2	251 > 134	12 eV
	249 > 104	15 eV			

3.2.4. Decision limit (CCα) and detection capability (CCβ)

The concentrations of the twenty blank samples extracted on day 1 were measured by integrating the area of the chromatographic peak at the retention time corresponding to the expected compound. Three times the concentration measured for the blanks can be used as decision limit (CCα) for each derivatised nitrofuran metabolite. The twenty samples spiked at a concentration equivalent to their respective decision limits were then analysed and the compounds were identified (on day 2). The detection capability (CCβ) is equal to the value of the decision limit measured in the spiked samples plus 1.64 times (β = 5%) the standard deviation for the measured content.

The decision limits and detection capabilities for each of the four nitrofuran residues presented in Table 2 were comparable to those already reported in the literature for meat (Mottier et al., 2005).

The detection capabilities met the legislation criteria since they were all below the MRPL. Concerning the CCα values, they were close to those of the CCβ, which implied quite a low standard deviation obtained with the developed method during the validation.

3.2.5. Repeatability

The Commission Decision 2002/657/EC specifies that, for mass fractions lower than 100 μg kg^{-1}, the application of the Horwitz equation gives unacceptably high values. Therefore, the CVs for concentrations lower than 100 μg kg^{-1} shall be as low as possible. In that case, Thomson's equation can be used (Thompson, 2000). According to that equation, the CVs must be below 22%. As shown in Table 3, this was the case for the four nitrofuran residues.

3.3. Elimination of furazolidone in shrimp muscles

Residues of furazolidone were quantified in the muscles of *P. monodon* from the contamination experiment (see Section 2.10). Residues of the furazolidone metabolite 3-amino-2-oxazolidone (AOZ) were measured as NP-AOZ from shrimp collected at day 0, after 96 h and 171 h of contamination, and after 24 h, 96 h, 7 days, 14 days and 28 days of decontamination. Results are presented in Fig. 1. Concentrations are expressed in μg residue/kg wet weight muscle.

It can be observed that antibiotic residue levels increased within one week of contamination. The maximum concentration measured for NP-AOZ was 874 ± 326 μg kg^{-1}. During the decontamination period, residue levels were decreasing. However, after 28 days decontamination, a concentration of 115 ± 37 μg kg^{-1} NP-AOZ was measured, corresponding to 13% of the maximum level observed for NP-AOZ.

These results demonstrate, as previously shown by Xu et al. (2006) in Nile tilapia (*O. niloticus*), that even if these drug levels in shrimp muscles are decreasing rapidly after one week contamination, residues can still be detected 28 days after the end of the treatment, which corresponds with the average time after which shrimps are sold. This confirms that furazolidone should not be used for shrimp treatment.

Table 2
Decision limits (CCα) and detection capabilities (CCβ) validation data.

(n = 20)	CCα (μg kg^{-1}) = decision limit	CCβ (μg kg^{-1}) = detection capability
NPAMOZ	0.20	0.29
NPAOZ	0.08	0.13
NPSEM	0.36	0.85
NPAHD	0.16	0.16

CCα and CCβ were calculated from data of twenty blank samples and twenty blank samples fortified with the nitrofuran compounds at a concentration equivalent to their respective decision limits (CCα), respectively.

Table 3
Repeatability validation data.

	Theoretical conc. (μg kg^{-1})	Number of determinations	Mean conc. (μg kg^{-1})	SD (μg kg^{-1})	CV[a] (%)
NPAMOZ	1	20	0.88 ± 0.18	0.06	6.8
NPAOZ	1	20	1.06 ± 0.20	0.07	6.4
NPSEM	1	20	1.24 ± 0.69	0.23	18.5
NPAHD	1	20	0.95 ± 0.31	0.10	11.0

Repeatability data were obtained by analysing twenty shrimp samples fortified at 1 μg kg^{-1} and analysed on the same day.
[a] CV obtained from the measurement of samples spiked at the MRPL value (1 μg kg^{-1}).

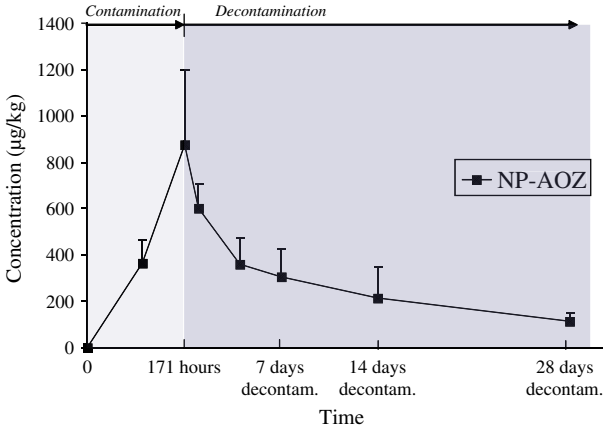

Fig. 1. NP-3-amino-2-oxazolidone (NP-AOZ) residues in the muscles of *Penaeus monodon* during a contamination period (up to 171 h) and a decontamination period (up to 28 days). Mean ± SD.

4. Conclusion

For the four nitrofurans analysed, the detection capability (CCβ) values obtained were lower than the fixed MRPL values, which are used as a limit of acceptability of imported products in EU, meaning that the developed method could reach suitable concentrations. The method is relatively sensitive for three of the four nitrofuran residues (AMOZ, AOZ and AHD) and could be improved for SEM. Indeed, traces of SEM can be detected in samples originating from animals untreated with nitrofurazone, meaning that another marker would be more suitable to monitor the use of this banned antibiotic.

In conclusion, a specific LC–MS/MS method for the analysis of four residues of nitrofurans in shrimps has been developed and validated according to the Commission Decision 2002/657/EC. In addition, the method has been applied to muscles of shrimps contaminated with furazolidone to evaluate the elimination of nitrofurans in real samples.

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