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Validation of a Commercial ELISA Kit for Screening 3-amino-2-oxazolidinone, A Furazolidone Antibiotic Residue in Shrimp

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AYKMMR and ASMS designed the work and analysed the data. Authors MMBP, DC, HI and MN conducted the experimental work. Author AYKMMR wrote the manuscript. All authors revised and approved the final manuscript.

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ABSTRACT

Furazolidone, a banned nitrofuran antibiotic, is still illegally used in aquaculture and livestock husbandry. A commercial Enzyme-linked Immunosorbent Assay (ELISA) from Europroxima, The Netherlands is described, and validated for the screening of 3-amino-2-oxazolidinone (AOZ), metabolite of furazolidone antibiotic in the muscle of shrimp. Research works was conducted at Veterinary Drug Residue Analysis Laboratory, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka-1349, between July 2018 and December 2019. Validation was done in accordance with the European Commission (EC) decision 2002/657/EC in terms of specificity, detection capability CCβ, selectivity, ruggedness, and stability. Homogenized sample was subjected to acid hydrolysis for releasing of tissue bound metabolite AOZ, and derivatized with o-nitrobenzaldehyde followed by ethyl acetate/n-hexane extraction and detection by ELISA reader. The detection capability CCβ of the method was lower or at 0.3 µg/kg accepting 0% false compliant result. Cut-off factor *Fm* and the positivity threshold value *T* was 0.716 and 0.982,

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respectively. Mean OD of blank sample *B* was1.225. The assay was valid because *T* was greater than *Fm* and smaller than *B*. The kit was stable even after ten months of storage at recommended temperature and showed good sensitivity. From the practical point of view, the kit studied has good reliability, and could be used in the routine application for the control of illegal use of the drug.

Keywords: AOZ; ELISA; residues; validation; furazolidone; shrimp.

1. INTRODUCTION

Furazolidone, furaltadone, nitrofurantoin, and nitrofurazone are veterinary drugs belong to the group nitrofuran. This group of synthetic antibiotics are frequently used in the poultry and fish production for their excellent antibacterial, pharmacokinetic and, growth-promoting properties [1,2]. Use of nitrofurans has been banned by the European Union (EU) and many other countries for food-producing animals because of their carcinogenic, teratogenic, and mutagenic effects on human health [2,3]. Bangladesh also banned the use of nitrofuran antibiotic in aquaculture in 2010 [4]. After ingestion, nitrofuran parent drugs furazolidone, nitrofurazone, furaltadone, and nitrofurantoin are rapidly metabolized to corresponding tissue bound metabolites-3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), and 1-aminohydantoïne (AHD). The half-life of these parent drugs is 7–63 min. However, the formed metabolites remain for many weeks after treatment [3,5,6]. According to European Commission Decision No. 2002/181/EC [7], there is no maximum residue limit (MRL) for nitrofuran antibiotics but the minimum required performance limit (MRPL). MRPL means minimum content of an analyte in a sample, which at least has to be detected and confirmed. The MRPL for AOZ in foods of animal origin is 1 µg/kg [7]. European Commission approved ELISA as a qualitative/screening method for screening nitrofuran metabolites in matrices. Commercial ELISA kits are developed to detect the metabolites of parent nitrofuran drugs. Usually, these kits are already validated by the manufacturer, but it is recommended that every laboratory should validate them to accommodate in their environment depending on the requirements of the regulatory authorities.

Shrimp/prawn is one of the major export items in Bangladesh. Targeting mainly for export, prawn and shrimp aquaculture has been expanded very fast over the last decades. Total shrimp and prawn production, including capture, have been increased from 1.60 lakh MT in 2002-03 to 2.46 lakh MT in 2016-17 [8]. In 2017, over 80% of produced shrimp was exported to the EU [9]. In the past, to maximize the production, nitrofuran drugs were used in prawn/shrimp aquaculture, both prophylactically and therapeutically [10]. Rapid Alert System for Food and Feed (RASFF) is the official network of national authorities from 27 EU member states that exchange information on the presence of potential health risk to consumers presented by a food product. Since 2005, the number of notifications of RASFF has been increased against exported seafood of many countries including Bangladesh, particularly for the *M. rosenbergii*. Bangladesh received around 120 notifications during the period 2002-2011for the presence of veterinary medicinal products of which 116 for SEM, 03 for AOZ and 01 for AMOZ. Data are retrieved from the web address- https://webgate.ec.europa.eu/rasff-window/portal. In the last few years, many Bangladeshi consignments of frozen prawn and shrimp had been rejected by the EU and USA due to the presence of nitrofuran drugs [11-14].

On January 22, 2018, Division of West Coast Imports, US Food and Drug Administration (FDA) refused the entry of four consignments of shrimp from a Bangladeshi company named “Sea Fresh” due to contamination with nitrofuran antibiotics [15]. Though the rejection is reduced significantly, and consciousness of using banned antibiotics among the farmers is increased, but there are chances to contaminate shrimp production chain through the food chain. Moreover, there are no authentic data about the local market. To meet the requirements and to identify the source of contamination and prevent the entrance of banned antibiotics in the shrimp value chain, Bangladesh Atomic Energy Commission (BAEC) in co-operation with International Atomic Energy Agency (IAEA) has established a new laboratory at Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), Savar, Dhaka.

This study was taken to validate the method of a commercial ELISA kit in accordance with the European Commission regulations 2002/657/EC

[16] for screening the presence of AOZ residues in shrimp before sending the samples to the confirmatory test by LC-MS/MS/MS.

2. MATERIALS AND METHODS

2.1 Chemicals and Standard Solutions

AOZ ELISA kit from Europroxima, The Netherlands, lot no. 6959 was used throughout the validation process. Test kit contained microplate having antibody- coated well, sample dilution buffer, dilution buffer, AOZ stock solution, NPAOZ standard solution, AOZ-conjugate, anti-AOZ-antibody, red chromogen stop solution, and washing buffer. Nitrophenyl derivatives of nitrofuran metabolites (NPAOZ, NPAMOZ, NPAHD, and NPSEM) were received from Fish Inspection and Quality Control (FIQC) laboratory, Savar, Dhaka, and other chemicals were bought from Sigma Aldrich (Germany).

2.2 Matrix

The muscle tissue of shrimp was used throughout the study. Several shrimp samples were collected from different areas of the country, and muscle /flesh was separated very carefully. Following all the procedure of ELISA described by manufacturer, optical density (OD) readings of these muscle extracts were recorded and compared to the OD of zero standards (only buffer). Sample having the closest OD reading to zero standards was selected as the negative control (blank sample).

2.3 Principle of AOZ ELIZA

The AOZ ELISA is a competitive enzyme immunoassay for the qualitative and semi-quantitative detection of AOZ. Well of the microtiter plate are pre-coated with antibody against AOZ. Horseradish peroxidase (HRP) labelled AOZ and standard solution or samples are added to the wells. Free AOZ from the samples or standards and AOZ-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation period of 30 minutes, the non-bound reagents are removed in a washing step. The amount of bound AOZ-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetra methyl benzidine, TMB). Bound AOZ-HRP conjugate transforms the colorless chromogen into a colored product. The substrate reaction is stopped by the addition of sulfuric acid. The color intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the AOZ concentration in the sample.

2.4 Standard Curve Preparation

To get an assumption about the sensitivity of the kit and selecting spiking concentration of blank samples, a standard dose response curve was prepared in buffer according to manufacturer's instruction. Stock NPAOZ standard solution supplied with the ELISA kit was used as calibration concentration. NPAOZ concentration at 0.01, 0.026, 0.064, 0.16, 0.40 and 1.00 µg/kg were used in duplicates. Percent responses (%B/Bo) were calculated, where B is the absorbance value of the bound calibrator and Bo is the absorbance value of the blank calibrator. The graph is in semi log format whose X-axis was in logarithmic scale, and created by plotting %B/Bo values in Y axis and AOZ equivalent concentration in X axis. Graphpad Prism software (V. 8.0.1) was used for making S-type graph. As expected, the concentration is inversely proportional to B/Bo. Lower the B/Bo, higher the AOZ concentration, because lower quantity of enzyme conjugate binds to the antibody sites on the microplate wells compared to the analyte. The half maximal inhibitory concentration (IC_{50}) and R squad value were determined. This IC_{50} value gives an index of sensitivity of the kit.

2.5 Spiking Blank Sample

According to European Commission guideline, the chosen concentrations of analyte for spiking blank samples should be lower or at the MRPL [17,18]. Since MRPL of AOZ for edible food is 1µg/kg, we fortified the blank samples with AOZ standard at 0.5 and 0.3 µg/kg to choose the target concentration for spiking blank samples.

2.6 Sample Extraction and Derivatization Procedure

Effective control of nitrofuran drug is based on the measuring of the metabolite of the drug after their release from tissues, and their derivatization [3]. Extraction and derivatization of tissue bound metabolite AOZ of parent furazolidone from spiked samples were carried out according to the kit manufacturer's instruction. Briefly, 1 gm of each homogenized samples was taken into 50 ml tube and 4 ml distilled water, 0.5 ml 1 M HCl and 150 µl derivative agent (10 mM 2-nitrobenzaldehyde in DMSO) were added. After short vortexing, they were mixed head over head

for 5 minutes. A 2.5 ml of 0.25M K_2HPO_4, 0.4 ml of 1 M NaOH and 5 ml ethyl acetate were added to the derivatized samples. After head to head carefully mixing for 1 minute, samples were centrifuged for 10 minutes at 2000 x g and 2.5 ml of ethyl acetate layer was transferred into a 4 ml glass tube. Extracted ethyl acetate layer was subjected to evaporation at 50ºC under nitrogen blow. Residues were dissolved in 1 ml hexane and 1 ml sample dilution buffer, and vortexed for 1 minute. The hexane layer was removed after centrifuge at 2000xg for 10 minutes. A 50 µl of the aqueous phase was used for ELISA.

2.7 Validation Protocol

To accommodate in our laboratory, assay of the ELISA kit was validated in accordance with the European Guideline for the validation of screening methods [17], which is based on the criteria of European Decision No. 2002/657/EC (16). The performance characteristics to be determined for validation were specificity, detection capability CCβ, selectivity, ruggedness, stability of kit, and analyte.

2.7.1 Specificity and detection capability (CCβ)

According to EC guideline CRL 2010 [17], 20 blank and 20 spiked samples were used to study specificity and detection capability. Spiking level (screening target concentration) will be the detection capability CCβ (smallest content of analyte that may be detected in a sample with an error probability of β*)* of the method, where less than or equal to 5% of false-compliant results may present. Since MRPL for AOZ in foods of animal origin is 1 µg/kg, we set the target concentration at half of the MRPL (0.5 µg/kg) and lower the MRPL (0.3 µg/kg). Twenty blank samples were spiked in each cases.

Detection capability and specificity can be determined by classical or statistical approach explained in the guideline of CRL/2010 [17]. In classical approach explained in Annex I, ranges of analytical responses for blank and spiked samples are evaluated. The cut-off level is the highest response for the spiked samples provided that it does not overlap with the lowest response for the blanks. If one response of spiked samples overlaps the lowest response of blank samples, then the false compliant rate will be 5% (β error).

The statistical approach explained in Annex II, took into account the β error of 5% in selecting detection capability [17,19]. The positivity threshold *T* and the Cut-Off value *Fm* were calculated accordingly. The positivity threshold *T* and the Cut-Off factor *Fm* are matrix specific.

$$T = B - 1.64 \times SDb, \quad (1)$$

where *B* is the mean, and SDb is the standard deviation of the optical densities (ODs) of the blank samples and,

$$Fm = M + 1.64 \times SDs, \quad (2)$$

where *M* is the mean, and SDs is the standard deviation of the ODs of the spiked samples.

The assay was considered valid only if *T* was greater than *Fm* and smaller than *B*.

2.7.2 Selectivity/Cross-reactivity

AOZ ELISA utilizes antibodies raised in mouse against protein conjugated AOZ. The reactivity pattern against four types of nitrofuran metabolites (AOZ, AHD, AMOZ, and SEM) were checked in the buffer by the manufacturer. We checked the cross-reactivity in the blank matrix against AHD, AMOZ, SEM, NPAHD, NPAMOZ, and NPSEM. Blank matrix was prepared following all the procedures of sample extraction avoiding the derivatization step. After complete extraction, dried residues was suspended in 500 µl of sample buffer, and spiked with AOZ and NPAOZ at 1 µg/kg (MRPL) concentration. Other nitrofuran metabolites and their nitrophenyl derivative were used at 10 µg/kg concentration for spiking the extracted blank matrix solution. Analytical responses were compared with blank and derivatized AOZ (i.e. NPAOZ).

2.7.3 Ruggedness

Ruggedness studies use the deliberate introduction of minor changes in the method and their consequences on the performance characteristics. We changed the amount of derivatization agent from 150 to 200 µl, incubation period from 1 hr to 2 hr and also changed the incubation period after adding conjugate solution from 30 minutes to 1 hr. Effect of these changes on analytical responses for blank and spiked samples was recorded.

2.7.4 Stability of analyte and kit

According to EC Guideline stability of the analyte should be tested both in matrix and in solution [16,17]. It is also mentioned that if the stability of

the analyte is known, there is no need to determine it again. We did not determine the stability of AOZ since its stability has already been determined by Cooper and Kennedy, 2007 [20] both in matrix and in stock solution. Stability of the kit was studied by keeping the kit at 2-8ºC for 10 months of first opening. Performance of the kit again checked in blank shrimp matrix kept at -20ºC before expiration date. Five blank and five fortified (spiked at 0.3 µg/kg) samples were analysed, and analytical responses were recorded.

3. RESULTS AND DISCUSSION

3.1 Standard Curve

An S–type graph was formed with IC50 value 0.0224-0.028 µg/kg and R-squared value 0.9996 (Fig. 1). If IC_{50} value is multiplied by method multiplication factor 2, then it will be around 0.05µg/kg. This sensitivity indicates the minimum detectable concentration of AOZ which is same to the manufacturer's declared limit of detection (LoD). Limit of detection was also calculated from 20 blank matrix spiked with AOZ at 0.3 µg/kg (Table 1). Highest response among the 20 spiked samples (0.692) was converted to %B/Bo and read from the standard curve. LoD was calculated multiplying the observed equivalent AOZ concentration (~0.025 µg/kg) with method multiplication factor 2 and found to be around 0.05 µg/kg.

3.2 Determination of Cut-off Value, Specificity and Detection Capability

3.2.1 Classical approach

For an effective screening with minimum false compliant rate, we set the target concentration at 0.5 (half MRPL) and 0.3 µg/kg. Analytical responses of blank and spiked samples at 0.3 µg/kg were summarized in Table 1. Among the samples spiked at 0.3 µg/kg, the highest response was 0.692 which did not overlap the lowest response of blank (1.001). Therefore, the Cut-Off value will be 0.692 and the detection capability CCβ of the method will be 0.3 µg/kg. Since a clear Cut-Off level is established at 0.3 µg/kg concentration. We did not consider the responses of spiked samples at 0.5 µg/kg for the rest of the study.

3.2.2 Statistical approach

The distribution of the ODs of blank samples and spiked samples is illustrated in Figs. 2 & 3. Difference of ODs between blank and spiked samples was so high that it allowed a good detection at 0.3 µg/kg. The mean OD for blank and spiked samples was presented in Table 2. From these data, mean response of blank samples *B*, mean response of spiked samples *M*, positivity threshold *T* and Cut-Off factor *Fm* were calculated. According to the guideline CRL/2010 [17], the detection capability CCβ is validated when the Cut-Off factor $Fm < B$. If $T < Fm < B$, the false-positive rate is greater than 5%. In the case of $Fm < T$, the false-positive rate is below 5%. The assay is valid only if $Fm < T$. The CCβ is equal to the concentration of the spiked samples at which less than or equal to 5% of false negative results remain. In this study, calculated Threshold value *T* and Cut-Off factor *Fm* were equal to 0.9823 and 0.716 OD respectably. Since $Fm<T$, the kit assay is valid for screening AOZ in muscle of shrimp with less than 5% false positive rate.

If *T* is chosen as the decision limit to determine the positivity of samples, the false positive rate is 0% (Fig. 2). If *Fm* is chosen as decision limit to determine negativity of samples, the false negative is also 0% (Fig. 2). Mean response of blank samples *B* and mean response of spiked samples *M* were 1.225±0.148 and 0.635±0.081.

Table 1. Optical density values (ODs) of blank and spiked samples, and determination of Cut-Off factor, specificity and detection capability according to classical approach (Annex I of CRL/2010)

SN	Blank samples	Spiked at 0.3µg/kg
1	1.035	0.515
2	1.167	0.549
3	1.079	0.584
4	1.06	0.590
5	1.162	0.692
6	1.001	0.650
7	1.128	0.648
8	1.071	0.638
9	1.149	0.685
10	1.196	0.566
11	1.440	0.644
12	1.306	0.663
13	1.369	0.670
14	1.335	0.667
15	1.269	0.645
16	1.235	0.644
17	1.256	0.678
18	1.316	0.678
19	1.357	0.625
20	1.575	0.678

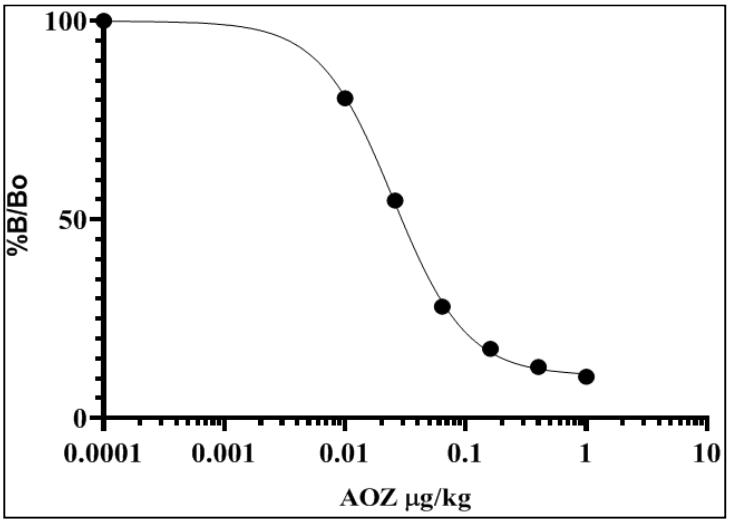

Fig. 1. Standard curve. X-axis is in logarithmic scale. Each point represents the mean of 02 replicates. R squared value 0.9996. IC_{50} is 0.0224-0.0279 µg/kg

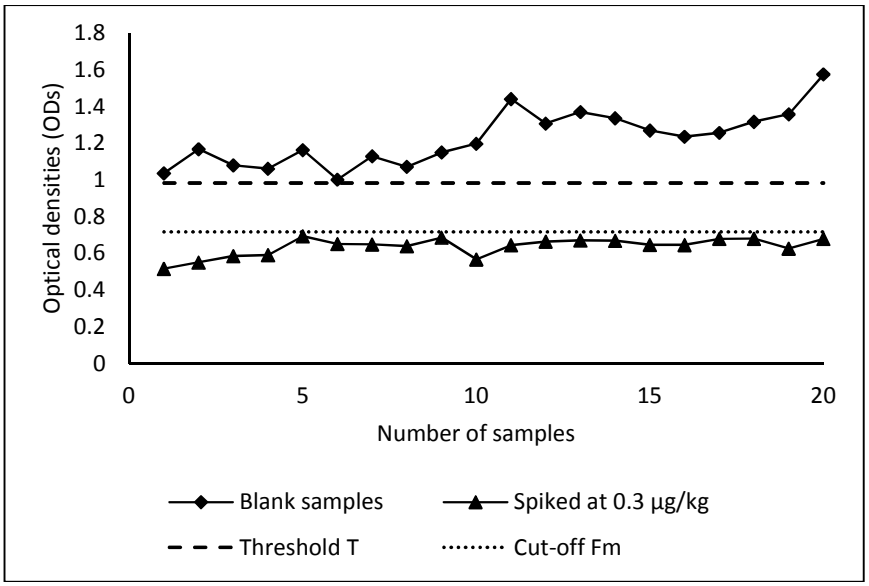

Fig. 2. Graphical illustration of the distribution of optical densities (ODs) of blank samples (shrimp) and blank samples spiked at 0.3 µg/kg with AOZ

OD respectably (Table 2). In accordance with the guideline, CCβ is validated because *Fm<B* where false compliant result is 0%. Therefore the detection capability CCβ of the ELISA kit for AOZ in muscle of shrimp was at or below 0.3 µg/kg.

Cut-off value or *Fm* value established from the classical and statistical approach was 0.692 and 0.716 respectively, which was close to each-other. However for better reliability *Fm* value 0.716, established from statistical method will be used for screening AOZ residues in shrimp. When the OD of one sample is greater than *Fm*, we can assume that the sample is screen negative. Critical interpretation of the result was not possible due to the lacking of validation report on the performance of ELISA kit from EUROPROXIMA in matrix, The Nederland. However, some researchers performed validation of AOZ ELISA kit from other manufacturers or made by themselves. First

ELISA for detection of AOZ residues in prawns was developed by Cooper, et al. [21] having CCβ below 0.4 µg/kg with 5% false positive rate in negative samples and a limit of detection 0.05 µg/kg. In house validation of AOZ ELISA from r-BIOPHARM was carried out by Eizabeta DS, et al. [22] and determined the CCβ 0.5 µg/kg with a limit of detection below 0.3µg/kg. On the contrary, the CCβ of the ELISA kit from EUROPROXIMA, which we used throughout this study, is at or lower 0.3 µg/kg (Table 2 and Fig. 2). We performed full validation for a single matrix shrimp, and the result suggests that AOZ kit from EUROPROXIMA showed better responses compared to other ELISA.

3.3 Selectivity/Cross Reactivity

Optical density (ODs) values of all the metabolite of nitrofuran and their derivatives except AOZ were within the range of optical density values showed by the blank matrix and did not overlap the cut-off value *Fm* (Tables 1, 2 and 3). Therefore, cross reaction of nitrofuran metabolites towards the antibody raised against the AOZ are noted as negligible. Response towards non-derivatized AOZ was also close to responses of the blanks. Response to derivatized AOZ was as usual as expected. This result also reflects the declaration of manufacturer about the selectivity of ELISA kit against different metabolites of nitrofuran where the cross reactivity pattern of the antibody used in AOZ ELISA were 100% for derivatized AOZ and <0.01% for derivatized AMOZ, SEM, and AHD.

3.4 Ruggedness Study

Effect of minor changes in method on the optical density values of blank and spiked samples are summarized in Table 4. Blank responses were greater than the Threshold value (*T*) 0.98 and responses of spiked samples were lower than cut off (*Fm*) value 0.716 (Table 2). Therefore, minor changes would not affect the performance characteristics of the method.

3.5 Stability of Analyte and Kit

Cooper and Kennedy [20] described that nitrofuran metabolite AOZ in muscle and liver of pig did not drop significantly during 8 months storage at –20ºC. Metabolite stock and working standard solutions in methanol were also stable for 10 months at 4ºC. In our study, after 10 months storage, we found mean OD of blank samples was 1.536 (n=5) while it was 0.744 for spiked samples at 0.3 µg/kg (n=5, data not shown). There is a clear difference of responses between blank and spiked samples. Early values for blanks were 1.001-1.575 (n=20), and for spiked were 0.515-0.692 (n=20). Responses of the kit at first stage and after ten months were similar. This result suggests that the kit is very stable if it is stored at the manufacturer's recommended temperature (2-8ºC). However, it needs to update the cut-off level (*Fm*) regularly using positive and negative control samples.

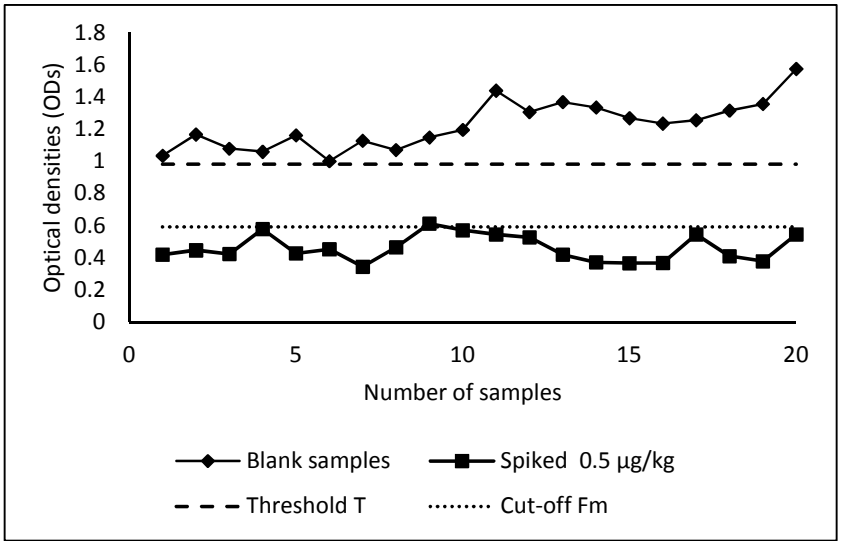

Fig. 3. Graphical illustration of the distribution of optical densities (ODs) of blank samples (shrimp) and blank samples spiked at 0.5 µg/kg with AOZ

Table 2. Determination of the threshold value T and cut off value Fm and detection capability CCβ for AOZ ELISA kit

Matrix	N_b	$B \pm SD_b$ (uOD)	$Ms \pm SD_s$ (uOD)	T(uOD)	N_s	Fm (uOD)	Nfp	CCβ (µg/kg)
Shrimp	20	1.225±0.148	0.635±0.081	0.9823	20	0.716	0/20	0.3

ELISA, enzyme linked immunosorbent assay; B, mean of blank samples; M_s, mean of spiked samples; SD_b, standard deviation of blank samples; SD_s, standard deviation of spiked samples; N_b, number of blank samples; N_s, number of spiked samples; Nfp, number of false positive; uOD, unit of optical density

Table 3. Cross reactivity pattern of AOZ ELISA to different nitrofuran metabolites and their derivatives. n=20 for blank and n=2 for spiked samples

Reactant/Analyte	Concentration (µg/kg)	Absorbance (OD)
Blank matrix (n=20)	0	1.001-1.575
AOZ	1	1.261
NP-AOZ	1	0.321
AHD	10	1.623
NP-AHD	10	1.493
AMOZ	10	1.593
NP-AMOZ	10	1.182
SEM	10	1.166
NP-SEM	10	1.57

Table 4. Factors that may affect the analytical response of ELISA in blank and spiked samples (Either stated, n = 2)

Factor	Variation	Mean OD	
		Blank	Spiked at 0.3 µg/kg
Derivatization agent	200µl instead of 150µl	1.198	0.638
Incubation period after derivatization	120 min instead of 60 min	1.192	0.593
Incubation period after adding conjugate solution	60 min instead of 30 min	1.274	0.640
Reading after completion of reaction	After 20 minutes	1.239	0.6121

4. CONCLUSION

Commercial ELISA kit from Europroxima, The Nederlands, has been validated in accordance with the requirements of European Regulations 2002/657EC [16]. Detection Capability CCβ has been determined using both classical and statistical methods described in the EU Guideline CRL2010 [17]. The kit passed the validation criteria successfully. Statistical method is more elaborated and acceptable method for determining cut-off value. It is therefore suggested that the laboratories should use the cut-off value (0.716) determined by statistical method for screening AOZ positive samples. Samples having optical density values higher than the cut-off value (towards blank) will be considered as screen negative. In practical, the kit is very suitable for monitoring AOZ residue, metabolite of furazolidone antibiotic, in muscle of shrimp. It does not require solid-phase extraction (SPE) procedure, hence reduces cost and time. Operator skill is very important for screening drug residues in matrices. For a skilled operator, the target concentration (detection capability) could be set at lower or at 0.3 µg/kg. During routine analyses, the recommendation is to calculate a daily threshold *T* and a daily cut-off factor *Fm*, which would be based on the analyses of negative and positive quality controls (QCs) on each day. The assay is valid only if *T* is greater than *Fm* daily.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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