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Development and Residue Screening of the Furazolidone Metabolite, 3-Amino-2-oxazolidinone (AOZ), in Cultured Fish by an Enzyme-Linked Immunosorbent Assay

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A sensitive and specific polyclonal enzyme-linked immunosorbent assay (ELISA) for the determination of tissue-bound metabolite 3-amino-2-oxazolidinone (AOZ) is described. The procedures allow for the detection of protein-bound AOZ in the form of a 2-nitrophenyl derivative (2-NP-AOZ) in the sample supernatant or extract after acid hydrolysis and derivatization with *o*-nitrobenzaldehyde. The polyclonal rabbit antibodies were produced with the immunogen hapten, 2-NP-HXA-AOZ, and the 50% inhibition values (IC_{50}) of 0.14 μg kg^{-1} of AOZ was achieved with the most sensitive antibody A0505. The mean lower detection limit of the ELISA method is about 0.025 μg kg^{-1}. According to the test preparation record, the detection limit is 0.1 μg kg^{-1}, which is well below the minimum required performance limits (MRPLs) for tissue-bound residues of AOZ at 1 μg kg^{-1} in the European Communities. In the present study, we investigated the use of homemade ELISA, a new immunoassay, to monitor the presence of the furazolidone marker residue in 370 samples of cultured fish. Adopting 0.3 μg kg^{-1} AOZ as a cutoff value, the ELISA has a sensitivity of 100% and a specificity of 98.5% versus high-performance liquid chromatography−mass spectrometry (HPLC−MS) at a cutoff of 0.3 μg kg^{-1} and gives no false-negative rate results. From the practical point of view, the homemade kit could be advantageously used for the screening of large groups of animal-edible tissue samples and the kit employed has good reliability even in routine application for the control of the illegal use of the drug.

KEYWORDS: Nitrofurans; furazolidone; 3-amino-2-oxazolidinone (AOZ); eel; enzyme-linked immunosorbent assay (ELISA); residues

INTRODUCTION

Furazolidone, as well as other nitrofuran antibacterial agents, were used for the treatment of gastrointestinal and dermatological infections, including salmonellosis in fish, shrimp, bees, swine, cattle, and poultry. The use of the nitrofuran drugs in food animal production has been banned within the European Union (*1*) because of potentially mutagenic, carcinogenic, and teratogenic effects on human health (*2*−*4*). The minimum required performance limits (MRPLs) for nitrofurans is set at 1 μg kg^{-1} in poultry meat and aquaculture products by Commission Decision 2003/181/EC (*5*) amending Decision 2002/657/EC (*6*).

It is recognized that nitrofurans are metabolized rapidly *in vivo* and stable tissue-bound metabolites are formed (*7*−*10*). Fragments of these metabolites may be released by mild acid hydrolysis and monitored as marker residues in edible tissues. Accordingly, 3-amino-2-oxazolidinone (AOZ) is monitored as a marker residue for the drug furazolidone (FZD); 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) is the marker residue for furaltadone (FTD); 1-aminohydantoin (AHD) is the marker residue for nitrofurantoin (NFT); and semicarbazide (SEM) is the marker residue for nitrofurazone (NFZ).

Residues of these nitrofurans could present a potential risk for public health safety. EU Member States are required to monitor compliance with the ban on nitrofurans through their annual national residues control plans. Trade restrictions arising from such findings prompted many food producers and regulatory authorities to instigate nitrofuran testing schemes. Effective surveillance for the illicit use of nitrofurans requires sensitive and satisfactory analytical techniques for their detection in muscle, kidney, or liver tissues from treated animals. Monitoring of illegal administration is by detection of the marker residue AOZ, which is the metabolite moiety derived from furazolidone (**Figure 1**). Various methods have been released for the determination of AOZ by high-performance liquid chromatography−ultraviolet (HPLC−UV) (*11*−*14*), liquid chromatography−mass spectrometry (LC−MS) (*9*, *15*), and liquid chromatography−tandem mass spectrometry (LC−MS/MS) (*12*, *14*, *16*−*18*).

However, widespread use of FZD in poultry and aquaculture products imported into Europe highlighted the urgent need for

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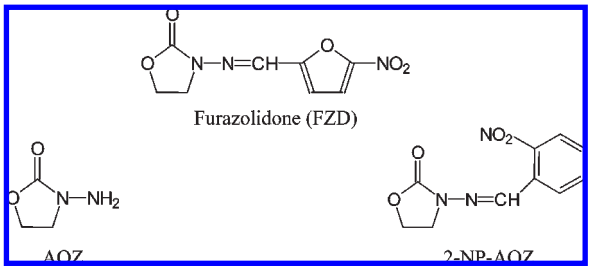

Figure 1. Structures of the parent furazolidone, the metabolite AOZ, and the target analyte 2-NP-AOZ.

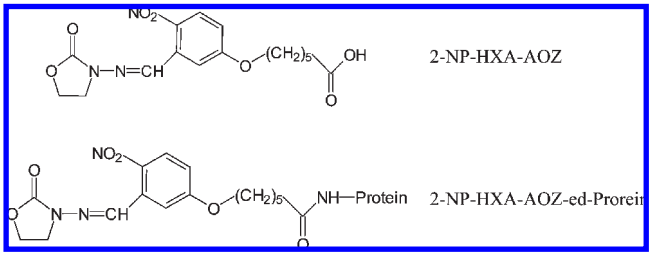

Figure 2. Immunogens used for antibody preparation.

rapid, high-capacity screening methods for tissue-bound residues of the nitrofurans. Methods such as enzyme-linked immunosorbent assay (ELISA) can provide an inexpensive, sensitive, and fast screening alternative for the detection of samples containing trace amounts of low-molecular-weight analytes, such as nitrofurans. Theoretically, specifically tailored antibodies are capable of detecting nitrofuran metabolites through the use of an enlarge target molecule, which can be formed by a simple derivatization step (*19*). The polyclonal antibodies developed by Cooper et al. using 3-([(3-carboxyphenyl)-methylene]-amino)-2-oxazolidinone) (CPAOZ) were incorporated into a competitive direct ELISA and involved AOZ being released from prawns, derivatized with *o*-nitrobenzaldehyde (*o*-NBA), extracted using ethyl acetate, and washed in hexane prior to detection. This polyclonal-based immunoassay provided a detection capacity for AOZ in prawns at 0.4 μg kg^{-1} (*20*). An ELISA test kit had been manufactured by R-Biopharm AG (Darmstadt, Germany) based on the polyclonal rabbit antibody mentioned above. Monoclonal antibodies raised against the same immunizing hapten, CPAOZ, by Diblikova et al. (*21*) exhibited comparable sensitivity. This method relied on the use of matrix-matched calibration standards to reduce sample interference and allowed for the sensitive determination of AOZ in shrimp, poultry, pork, and beef tissue homogenates without solvent extraction. Currently, an indirect ELISA format is based on polyclonal antibodies against 3-([(4-carboxyphenyl)-methylene]-amino)-2-oxazolidinone) (4-CPAOZ) by Chang et al. (*22*). The indirect ELISA test kit provided a new derivatizing agent, benzaldehyde, and using a phosphate-buffered saline (PBS) calibration standard, good precision and accuracy were observed. However, the Oasis MAX cartridges used to minimize matrix effects in the study were not fit for rapid screening and high throughput. As before, the detection capability was similar to that of Cooper et al. (*20*) and Diblikova et al. (*21*).

Objectives of the study are to develop a new assay for the determination of AOZ residues, a tissue-bound metabolite of the nitrofuran furazolidone, in cultured fish and to apply in field test screens. In this study, the polyclonal rabbit antibodies produced with the new immunogen hapten, 2-NP-HXA-AOZ (**Figure 2**), were highly sensitive to 2-NP-AOZ (**Figure 1**). Our work shows the advantages of good specificity, high sensitivity, and adequate reliability in high-throughput screening of AOZ residues.

MATERIALS AND METHODS

Reagents. AOZ, AHD, SEM, FZD, FTD, NFT, NFZ, *o*-NBA, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), bovine serum albumin (BSA), ovalbumin (OVA), porcine thyroglobulin (Tg), goat anti-rabbit IgG-horseradish peroxidase (HRP), sodium periodate, ethylene glycol, 1,6-diaminohexane, sodium borohydride, potassium phosphate dibasic, and Freund's complete/incomplete adjuvant were commercially available from Sigma-Aldrich (St. Louis, MO). 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 2-NP-AOZ, 2-NP-AMOZ, 2-NP-AHD, and 2-NP-SEM were obtained from Fluka (Tokyo, Japan). 6-[4-[Nitro-3-[[(2-oxo-3-oxazolidinyl)imino]methyl]]phenoxy]hexanoic acid (2-NP-HXA-AOZ, $C_{16}O_7N_3H_{19}$, FW 365) was provided by Professor Li Wen-Ren, the Department of Chemistry, National Central University (Taoyuan, Taiwan, Republic of China). Dimethyl sulphoxide (DMSO) and *n*-hexane of liquid chromatographic grades were purchased from TEDIA (Fairfield, OH). HRP was obtained from Roche (Switzerland). TMB ready-to-use substrate was obtained from Kem-En-Tec (Denmark). Deionized water was purified on a Milli-Q system (Millipore, MA). All other solvents and chemicals were of reagent-grade and were used without further purification.

Buffers and Solutions. The following buffers and solutions were used in the experiments: (1) 50 mM carbonate/bicarbonate buffer (pH 9.6) was used as a coating buffer; (2) 10 mM PBS containing 140 mM NaCl and 2.7 mM KCl (pH 7.4) was used in dialysis; (3) PBS with 0.05% Tween 20 (PBST) was used as a sample diluent and washing buffer; (4) 0.1 M potassium phosphate dibasic was used for sample extracts; (5) 10 mM *o*-NBA in DMSO was used for the derivatization of AOZ; and (6) 0.5 N HCl was used as a stopping reagent.

Instrumentation. A high speed refrigerated centrifuge and a tabletop centrifuge (Kubota 6900 and 5400, Tokyo, Japan) were used. The antibody was dispensed in microtiter plates using a μFill microplate dispenser (Bio-Tek, Winooski, VT). The microtiter plates were washed with the washing solution to remove unbounded antibodies using a 96PW microplate washer (Tecan, SLT, Salzburg, Austria). The absorbances of each well were measured with the EMax microplate reader (Molecular Devices, Sunnyvale, CA).

Preparation of AOZ Hapten Derivative−Protein Conjugates. The pale yellow powder, AOZ hapten derivative, 6-[4-[Nitro-3-[[(2-oxo-3-oxazolidinyl)imino]methyl]]phenoxy]hexanoic acid (2-NP-HXA-AOZ), was depicted in **Figure 2** and provided by Professor Li Wen-Ren, the Department of Chemistry, National Central University (Taoyuan, Taiwan, Republic of China). After dissolution of 10 mg of 2-NP-HXA-AOZ (27.4 μmol), 9.3 mg of *N*-hydroxysuccinimide (80.8 μmol), and 18.6 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (97 μmol), 1 mL of dry *N*,*N*-dimethylformamide (DMF) was added. The mixture (activated hapten derivative) was gently stirred at room temperature for 1 h. Different hapten−protein conjugates were synthesized as follows: (a) The activated hapten derivative (217 μL) was then added dropwise to BSA solution (20 mg in 4 mL of 0.1 N phosphate buffer at pH 8.0) by selecting a protein/hapten molar ratio equal to 1:20 (BSA/hapten). (b) The activated hapten derivative (338 μL) was added dropwise to OVA solution (20 mg in 4 mL of 0.1 N phosphate buffer at pH 8.0) by selecting a protein/hapten molar ratio equal to 1:20 (OVA/hapten). (c) The activated hapten derivative (109 μL) was added dropwise to Tg solution (20 mg in 4 mL of 0.1 N phosphate buffer at pH 8.0) by selecting a protein/hapten molar ratio equal to 1:100 (Tg/hapten). The resultant solutions were stirred for 1 h at room temperature, and the conjugates were then dialyzed against 3 L of 0.01 M phosphate buffer solution containing 0.14 M NaCl for 1 day with two changes of buffer. The dialyzed solution of immunogen was frozen at −20 °C until use.

Preparation of AOZ Hapten Derivative−HRP Tracer. A total of 2 mg of HRP was dissolved in 0.2 mL of a 5 mM sodium acetate buffer at pH 4.5, and 20 μL of a freshly prepared 46.8 mM solution of sodium periodiate in Milli-Q water was then added. The color rapidly changed from brown to green. This solution was incubated for 30 min at room temperature in the dark. A total of 2 μL of ethylene glycol was added to stop the reaction. After an additional incubation for 15 min, the reaction mixture, 100 μL of 1,6-diaminohexane solution (0.43 M, dissolved in 1 N

HCl, pH adjusted to 3.0), and 300 μL of Milli-Q water were added and mixed. Subsequently, this mixture was adjusted to pH 9.0 with sodium carbonate solution and was allowed to react for 1.5 h at room temperature in the dark. Finally, 32 μL of freshly prepared 0.26 M solution of sodium borohydride in Mili-Q water was added, mixed, and reacted for 1 h at room temperature in the dark. The resultant HRP solution was then dialyzed against 5 L of 0.01 M phosphate buffer solution containing 0.14 M NaCl with two changes of buffer.

2-NP-HXA-AOZ (2 mg), NHS (2 mg), and EDC (4 mg) were dissolved in dry DMF (1 mL). The mixture was gently stirred at room temperature for 3 h. The activated hapten was added while stirring to the dialyzed HRP in phosphate buffer to obtain a molar ratio of 10:1 (hapten/HRP). The conjugation mixture was stirred at room temperature for 1.5 h, and the formed tracer was concentrated using Centriprep (Amicon Ultra, 30 000 MWCO; Millipore). The tracer obtained was diluted with an equal volume of glycerol and stored until use (−20 °C).

Immunization of Rabbits. Two groups of New Zealand rabbits (in duplicate) were immunized by sc injection with 2-NP-HXA-AOZ-OVA and 2-NP-HXA-AOZ-Tg. Immunogen emulsions were prepared by repeatedly passing this mixture through a narrow-bored cylindrical Pyrex block, drilled at each end to accommodate two syringes. The first immunizations were composed of 500 μL of PBS containing 1 mg of immunogen emulsified in 500 μL of Freund's complete adjuvant. Subsequent immunizations, at 2−5 week intervals, were of the same volume, with complete adjuvant replaced by incomplete adjuvant. Rabbits were bled after each injection. The sera titer was detected by an indirect ELISA.

Determination of Antibody Titer. The 96-well microtiter plates (Costar, Cambridge, MA) were coated with 2-NP-HXA-AOZ-BSA overnight at 4 °C. The plates were washed 3 times with PBST and treated with 0.1% skim milk at 37 °C for 1 h. The plates were again washed with PBST and incubated with animal sera (100 μL/well) diluted in 0.1% BSA at 37 °C for 1 h. After incubation, plates were washed with PBST 3 times. Goat anti-rabbit IgG antibody conjugated with HRP was diluted (1/2000) in 0.1% BSA, and 100 μL of the resultant solution was added to each well. The plates were incubated at 37 °C for 1 h and washed again with PBST 3 times. The substrate solution TMB (100 μL) was added to each well and incubated at 37 °C for 10 min. The reaction was stopped by the addition of aqueous HCl (0.5 N). The optical density at a wavelength of 450 nm in each well was read using an ELISA reader.

Assessment of Antibody Sensitivity. The competitive ELISA format described was used to determine the sensitivity to free 2-NP-AOZ of test bleeds of the polyclonal antisera. Derivatization of tissues with *o*-nitrobenzaldehyde, yielding 2-NP-AOZ from a sample containing tissue-bound residues of furazolidone, is well-characterized and routinely used in mass spectrometric and HPLC determinations of AOZ. Therefore, the sensitivity and specificity data in this paper are calculated on the basis of derivatization from AOZ.

Optimum antiserum was determined previously by checkerboard titration. 2-NP-AOZ standard solutions having concentrations extending 9 orders of magnitude were prepared serially and run in triplicate. 2-NP-AOZ standards were prepared in the concentrations of 0.0256, 0.128, 0.64, 3.2, 16, 80, 400, 2000, and 10 000 μg kg^{-1}. The mean absorbance of each standard was normalized against the mean absorbance of the zero standard (B/B_0). The midpoint of each displacement standard curve was calculated to determine its IC_{50}, that is, the amount of substance that displaces 50% of the labeled antigen (enzyme conjugate) as compared to the IC_{50} of the antigen (2-NP-AOZ). The IC_{50} is thus the effective concentration of analyte that results in 50% enzyme conjugate inhibition.

The antibody most sensitive to 2-NP-AOZ was selected; a final bleed was taken from the appropriate rabbit; and sensitivity to 2-NP-AOZ was defined further by performing direct ELSIA analysis.

Antibody Preparation. The AOZ-specific antibodies were purified from high titer rabbit serum by affinity chromatography on a Protein A-Sepharose column (GE Healthcare, U.K.). The antibodies were concentrated using Centriprep (Amicon Ultra, 50 000 MWCO; Millipore). The antibody concentration was measured with a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Direct ELISA. A competitive enzyme immunoassay method for nitrofuran metabolite AOZ has been developed using an antiserum raised in rabbits by immunization against the 2-NP-HXA-AOZ derivative coupled to ovalbumin. The same derivative of the hapten was used, prepared by coupling to HRP to synthesize the enzymatic tracer. The 96-well microtiter plate was precoated with 4 μg/mL anti-AOZ polyclone antibody overnight at 4 °C in a coating buffer. The wells were blocked with 3% BSA in PBS for 2 h at room temperature the second day after washing them 3 times with washing buffer. The microtiter plates were dried at 20 °C and 25% RH for 4 h.

The quantitative nitrofuran metabolite AOZ ELISA kit contains the following components: a 96-well microtiter plate (spilt into 12 strips of 8 wells each), precoated with anti-AOZ antibody, AOZ−HRP enzyme conjugate, six 2-NP-AOZ standard solutions (0, 0.025, 0.05, 0.1, 0.2, and 0.5 μg kg^{-1}), derivative reagent, chromogen [3,3′,5,5′-tetramethylbenzidine (TMB)], extraction buffer, washing buffer, and stop solution.

Preparation of Fish Samples for ELISA. Fish samples were obtained from the local market, including *Oreochomis* sp. (Tilapia and Taiwan Tilapia), *Chanos chanos* (milkfish), and *Anguilla japonica* (eel). The fish was filleted; the skin and bones were removed; and the muscles were minced and frozen before being analyzed. Accurately weighted 1.0 g of homogenized fish muscle was put into a 15 mL disposable plastic tube (with screw caps), and samples were fortified with AOZ (when required). A total of 4 mL of deionized water, 0.5 mL of 1 N HCl, and 100 μL of 10 mM *o*-NBA in DMSO were added to each sample. Test tubes were vortexed for 60 s and incubated at 37 °C overnight (approximately 16 h). A total of 5 mL of 0.1 M K_2HPO_4, 0.4 mL of 1 N NaOH, and 5 mL of ethyl acetate were added to each sample, vigorously vortexed for 30 s, and centrifuged approximately at 3000 rpm for 10 min. The upper ethyl acetate layer (2 mL) was transferred into a 10 mL Quickfit glass tube, and the extract was evaporated under a gentle steam of nitrogen at 50 °C. The sample was reconstituted with 1 mL of *n*-hexane and mixed with 1.6 mL of PBST. After vortexing again (60 s), samples were centrifuged at 3000 rpm for 10 min. The upper hexane layer was discarded, and the remaining extract was used for ELISA determination. The measured data were corrected by a dilution factor of 4.

ELISA Procedure for Screening. A total of 50 μL of standard (0, 0.025, 0.05, 0.1, 0.2, and 0.5 μg kg^{-1} 2-NP-AOZ) or sample with 100 μL of AOZ−HRP enzyme conjugate was added to each well. Maximum binding was assessed by adding no inhibitor (zero standard) to the relevant wells. The plate was then incubated for 30 min at room temperature (19−25 °C) in the dark. After washing 3 times, the wells were emptied completely by inverting them onto absorbent paper; 100 μL each of chromagen (TMB) was added. The contents were mixed thoroughly and left to incubate for 20 min at room temperature in the dark. Afterward, 100 μL of stop solution (0.5 N HCl) was added to each well to stop the reaction. Absorbance values were measured at 450 nm, and the concentration of the analyte in a sample was calculated from the calibration curve, where the bound enzyme activity was expressed as the logit of the ratio (in present). Each concentration of AOZ (B) and the bound activity in the absence of unlabeled AOZ (B_0) were plotted versus the log of the AOZ concentration.

Assessment of Antibody Specificity. Specificity of the assay was estimated by measuring percent cross-reactivities (% CR), determined by measuring their IC_{50} values in the competitive direct ELISA described above using the midpoint of the 2-NP-AOZ standard curve. The CR values were calculated from the formula:

$$\%\ \mathrm{CR} = (\mathrm{IC_{50}\ 2\text{-}NP\text{-}AOZ}/\mathrm{IC_{50}\ structural\ analogue}) \times 100$$

ELISA Validation. Immunoassay validation was carried out using the limit of detection (LOD), the recovery (%) of the fortified AOZ, and coefficients of variation (CVs). The standard 2-NP-AOZ solution was diluted in PBST to obtain 5-point standard curves (0.025, 0.05, 0.1, 0.2, and 0.5 μg kg^{-1}) and expressed as underivatized AOZ. The determination of LOD was based on 20 blank samples accepting no false-positive rates (average + 3 SD). A total of 20 *A. japonica* (eel) samples were purchased in the local market, which have previously been proven to be free of AOZ and other nitrofuran metabolites using LC−MS determinations of nitrobenzaldehyde derivatives. No measurable AOZ residues were observed in additional blank samples by previous ELISA determinations. The recovery (%) was determined by the analysis of the above samples fortified with AOZ and calculated using the equation: measured concentration/fortified concentration × 100. According to the test preparation record,

Table 1. Comparison of Immunization Titer and IC_{50} Results of Polyclonal Antisera Produced with 2-NP-HXA-AOZ-OVA in Indirect ELISA

	B/B_0[a]			
	administration intervals			
2-NP-AOZ (μg kg^{-1})	W5[b]	B1W2[c]	B1W5[d]	B1W8[e]
0	100	100	100	100
0.05	93	93	88	82
0.15	90	75	74	70
0.45	87	57	58	57
1.35	81	49	47	47
4.05	75	42	41	40
titer	1600	4000	8000	8000
IC_{50}[f] for 2-NP-AOZ as AOZ (μg kg^{-1})		1.34	1.24	1.16
IC_{85}[g] for 2-NP-AOZ as AOZ (μg kg^{-1})	0.46	0.07	0.05	0.03

[a] Results are presented as the ratio B/B_0, where B is the amount of antibody bound in the present of a given concentration of a competitor and B_0 is the amount bound in the absence of a competitor. [b] A total of 5 weeks after the first vaccine. [c] A total of 2 weeks after the second booster. [d] A total of 5 weeks after the second booster. [e] A total of 8 weeks after the second booster. [f] IC_{50} = amount of unlabeled 2-NP-AOZ required to inhibit 50% of the 2-NP-AOZ−enzyme conjugate bound to the antiserum. [g] IC_{85} = 85% inhibitory concentration.

the detection limit is 0.1 μg kg^{-1}, which is well below the MRL for tissue-bound residues of AOZ at 1 μg kg^{-1} in the European Communities. With a request for strict measures, we adopted 0.3 μg kg^{-1} AOZ as a cutoff value < 3-fold compared to the MRL value and the spike recoveries were therefore determined by adding $^1/_3\times$, 1×, and 3× the MRL value, i.e., at 0.3, 1.0, and 3.0 μg kg^{-1}. Moreover, the precision of the ELISA was analyzed by repeated determination of the intra- and interassay CVs of the sample at the levels of 0.3, 1.0, and 3.0 μg kg^{-1}. Intra-assay variation was measured for 12 replicates of each concentration of AOZ-spiked eel. To measure interassay variation, each concentration of AOZ was determined on each of 3 different days.

Comparison of ELISA and Reference Methods. A total of 370 fish samples in field test screens were obtained from the local market, including *Oreochomis* sp. (Tilapia and Taiwan Tilapia), *C. chanos* (milkfish), and *A. japonica* (eel), and were processed to detect any nitrofuran metabolite AOZ residues. When 0.3 μg kg^{-1} AOZ was adopted as a cutoff value, the potentially positive samples would then require further confirmation analyzed by HPLC−MS.

RESULTS AND DISCUSSION

Characterization of AOZ Polyclonal Antibodies. Table 1 shows the comparison of immunization titer and IC_{50} results of AOZ polyclonal antisera produced with 2-NP-HXA-AOZ-OVA in indirect ELISA. The IC_{50} value was defined as the concentration of inhibitor required to inhibit color development by 50% compared to control wells containing no competitors, and IC_{85} was indicated as 85% inhibitory concentration. It is found in **Table 1** that increasing and lengthening the administration interval between booster immunizations could promote the titer of antibodies, simultaneously increasing the sensitivity of the antibodies to 2-NP-AOZ. For antisera B1W8, the IC_{50} was less than the other antisera and the IC_{85} decreased significantly more than 50% against antisera B1W2. Our efforts were focused on direct ELISA based on the most sensitive antibody A0505, which was selected for further characterization with regard to sensitive and specificity, and a final bleed was taken 8 weeks after the second booster and was purified from polyclonal antisera B1W8 using a Protein A-Sepharose column.

Analytical Performance of the ELISA Kit. A typical standard curve obtained using the direct ELISA is presented in **Figure 3**. The result of the AOZ−ELISA produced linear ranges from 0.025 to 0.5 μg kg^{-1}, and the mean lower detection limit of the ELISA method was about 0.025 μg kg^{-1}. According to the test preparation record, the sensitivity of the assay was evaluated by

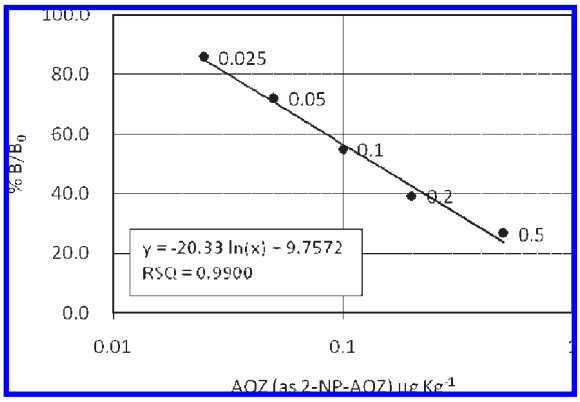

Figure 3. Calibration curve for AOZ ELISA. B = absorbance of each standard or sample. B_0 = absorbance of the standard = control.

Table 2. Cross-Reactivities Among 2-NP-AOZ and Related Compounds of Interest in Direct ELISA

competitor	cross-reactivity[a] (%)
2-NP-AOZ	100
2-NP-AMOZ, 2-NP-AHD, 2-NP-SEM	<0.1
AOZ, AMOZ, AHD, SEM	<0.1
o-NBA	<0.1
FAZ, FTD, NFT, NFZ	<0.1

[a] Cross-reactivity (%) = (IC_{50} of 2-NP-AOZ/IC_{50} of the competitor) × 100.

Table 3. Recovery Values Obtained in Fish Samples for the Determination of AOZ by Direct ELISA (n = 3)

forfified concentration (μg kg^{-1})	recovered concentration (μg kg^{-1})	recovery (%)
0.30	0.27 ± 0.05	90.2
1.00	1.18 ± 0.09	118.0
3.00	2.47 ± 0.19	82.5

examining fish tissue samples and the detection limit of this assay was 0.1 μg kg^{-1}, much lower than the regulatory limit by the European Commission (*5*). An IC_{50} of 0.14 μg kg^{-1} (AOZ in the form of 2-NP-AOZ) was achieved with excellent antibody A0505 in these direct ELISA conditions.

Specificity of the antibody A0505 in optimized assays was tested by the measurement of cross-reactivity using 2-NP-AOZ and related compounds as described. **Table 2** presents results as a percentage of cross-reactivity with respect to the target compound, 2-NP-AOZ. The data demonstrate that A0505 was highly specific for 2-NP-AOZ, with negligible cross-reactivity with other nitrofuran antibiotics, their metabolites, and corresponding nitrophenyl derivatives or the derivatizing agent *o*-NBA. The negligible assay response to *o*-NBA (CR < 0.1%) eliminates the need for separation of this reagent from the sample homogenate after derivatization.

Recovery was investigated by adding increasing amounts of AOZ (0.3, 1.0, and 3.0 μg kg^{-1}) to fish samples. The results indicated a recovery ranging from 82.5 to 118% (**Table 3**). Mean recovery thus indicated a reasonable parallelism and accuracy of the assay when applied to real samples.

Precision of the assay was assessed by replicate measurements of three known fish samples with a final AOZ concentration of 0.3, 1.0, and 3.0 μg kg^{-1}. The obtained mean values ± SD and CV (%) by replicate analyses (n = 12) in the same (intra-assay) and separate (interassay) runs are reported in **Table 4**. The CV (%) values were below 12%, demonstrating an acceptable level of precision.

Table 4. Intra- and Interassay Variations in Fish Sample AOZ Measurements with Direct ELISA

forfified concentration (μg kg^{-1})	mean[a] ± SD (μg kg^{-1})	CV[b] (%)
	Intra-assay (12 Replicates)	
0.30	0.30 ± 0.03	9.2
1.00	1.02 ± 0.09	8.9
3.00	3.06 ± 0.26	8.3
	Interassay (12 Replicates)	
0.30	0.31 ± 0.04	11.8
1.00	1.07 ± 0.12	11.0
3.00	2.91 ± 0.35	11.9

[a] AOZ concentration found. [b] Coefficient of variation.

Table 5. Comparison of AOZ Concentrations Determined by ELISA and HPLC−MS in Fish Tissue Samples (n = 370)

immunoassay screening cutoff concentration (0.3 μg kg^{-1})	confirmation cutoff concentration (0.3 μg kg^{-1})	number of samples
+[a]	+[a]	40
−[b]	+[a]	0
+[a]	−[b]	5
−[b]	−[b]	325
sensitivity[c]		100.0%
specificity[d]		98.5%

[a] Positive test result. [b] Negative test result. [c] Percent sensitivity = (true positives − false negatives)/(true positives) × 100%. [d] Percent specificity = (true negatives − false positives)/(true negatives) × 100%.

Application of the ELISA Kit in Field Trial Measurements. Field trials with 370 fish tissue samples from local meat markets were analyzed. The qualitative immunoassay reports each sample as either positive or negative based on predetermined cutoff concentrations. In the ideal diagnosis, results would be positive if the animal took the drug (true positive) and negative if the drug was not taken (true negative). However, false-positive or false-negative results can occur; therefore, it is imperative to interpret the results carefully. In context, the sensitivity is the proportion of true positives and the specificity is the proportion of true negatives (*23*, *24*). The sensitivity of a test is the ability to detect a class of drug, while the specificity is the ability to identify a particular drug. A high specific test gives few false-positive results and identifies individual drugs and/or their metabolites. High sensitivity is due to the ability of the test to detect the drug and/or its metabolite(s) and to reach the cutoff concentration for a positive report.

Table 5 demonstrated the performance of the ELISA in comparison to a confirmatory HPLC−MS method for the determination of AOZ in fish tissue. All 40 fish samples tested positive, demonstrating a consistent qualitative performance (no false negatives) in the analysis of these incurred samples that had confirmed the concentration ranging from 0.53 to 22.29 μg kg^{-1}. The AOZ ELISA had a sensitivity of 100% and a specificity of 98.5% versus HPLC−MS at a cutoff of 0.3 μg kg^{-1} for fish tissue detection.

Objectives of the study were to develop a new assay for the determination of AOZ residues, a tissue-bound metabolite of the nitrofuran furazolidone, in cultured fish and to apply in field test screens. The mean lower detection limit of the ELISA method was about 0.025 μg kg^{-1}. According to the test preparation record, the detection limit was 0.1 μg kg^{-1}, which was much lower than the MRPLs for tissue-bound residues of AOZ at 1 μg kg^{-1} in the European Communities (*5*). ELISA results showed a sensitivity of 100% and a specificity of 98.5%, demonstrating a consistent qualitative performance (no false negatives) in the analysis of incurred 370 samples. The presented ELISA method was similar to that of Cooper et al. (*20*), and the sensitivity of our ELISA method was superior to that described previously (*20*). In a previous study, the sample preparation procedure of our ELISA method would be simple and precise and the Oasis MX cartridges used were not fit for rapid screening and high throughput (*22*). In practice, several rapid assay kits were commercialized for nitrofuran metabolite AOZ residue detection in seafood, and the detection limits were compared in **Table 6**. Our results were presented and are as good as the competitors.

Table 6. Comparison of the Detection Limit with the Commercially Nitrofuran (AOZ) ELISA Test Kit

homemade	MaxSignal	RIDASCREEN	Charm
0.1 μg kg^{-1}	0.1 μg kg^{-1}	~0.1 μg kg^{-1}	0.3 μg kg^{-1}

In response to our concern, the ELISA test kit was evaluated for the screening of food animal production for AOZ residues in field samples and the tests were described as sufficiently sensitive and reproducible, simultaneously showing appreciable accuracy and precision. We presented findings on the developed assay with regard to excellent specificity of the AOZ measurements. From the practical point of view, the kit could be advantageously used to screen large groups of animal-edible tissue samples and the kit employed has good reliability even in routine application for the control of the illegal use of the drug. The antibody upon which this ELISA is based has now been incorporated into a commercially available ELISA test kit for AOZ manufactured by Taiwan Advance Bio-Pharm, Inc., Taiwan.

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