Determination of Nitrofuran Residues in *Tilapia* Tissue by Enzyme-Linked Immunosorbent Assay and Confirmation by Liquid Chromatography Tandem Mass Spectrometric Detection

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Furazolidone is a broad-spectrum antibiotic that is frequently used in aquaculture on account of its excellent antibacterial properties. In this study, both the enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were used to analyze the content of residual 3-amino-2-oxazolidinone (AOZ), a metabolite of furazolidone in *Tilapia* tissue. Homogenized fish samples were spiked with various amounts of AOZ, and following combined acid-hydrolysis and derivatization of the homogenized tissue with 2-NBA (2-nitrobenzaldehyde), sample clean-up was performed and the derived 2-nitrophenylmethylene-3-amino-2-oxazolidinone (NPAOZ) was analyzed. Using the LC-MS/MS method, a linear correlation between measured concentration Y and spiked concentration X was observed: $Y = 0.4518X - 0.0166$, $R^2 = 0.9972$. The linear equation for the ELISA method was $Y = 0.9322X + 0.5168$, $R^2 = 0.9066$. These results demonstrated that the ELISA method might overestimate the residual AOZ content at low concentrations. The detection limit and recovery of the known addition were 0.05 μg kg^{-1} and 108% for the LC-MS/MS method and 0.31 μg kg^{-1} and 305% for the ELISA method, respectively.

Keywords: Nitrofuran; Metabolite; ELISA; Furazolidone.

INTRODUCTION

Furazolidone [3-(5-nitrofurfurylideneamino)-2-oxazolidinone] is a nitrofuran antibacterial agent (antibiotic), and is frequently used as an antibacterial veterinary drug in the form of a food additive for the treatment of gastrointestinal infections, including bacterial enteritis caused by *Escherichia coli* and *Salmonella* in livestock, aquaculture and poultry. If nitrofuran remains in food, it causes mutagenesis, carcinogenicity and teratogenesis. Because of the toxicity of nitrofuran, the European Union (EU) prohibited the use of nitrofuran antibiotics in food-producing animals by listing them in Annex IV of Council Regulation 2377/90,[1,2] and furazolidone was added to Annex IV in June 1995. A provisional maximum residue limit (MRL) of 5 μg kg^{-1} was established by the EU for furazolidone,[3] and the total amount of nitrofuran residues in animals from foodstuffs should not exceed this legal limit. Drugs in Annex IV can no longer be registered for use in any farm animals in the EU.[4]

In previous studies, it has been reported that furazolidone overdose in mammals may not only cause muscle convulsions but also can lead to nerve breakage throughout the body.[5-7] Furazolidone can also inhibit sperm production in humans, mice and poultry.[8] The majority of a dose of the parent drug furazolidone taken by livestock is metabolized and destroyed by bacteria in the intestine and stomach; a smaller part is absorbed in the gut and transferred to the liver,[9] where the drug is broken down by enzymes, and an even smaller proportion is eliminated in its original form without being metabolized.[10]

Research into the half-life of furazolidone in pigs reported that half of the parent drug was depleted in 2.6 h;[11] the excretive pathway was 60-70% in the urine, 15% in the bile and 19% in the excrement.[12] Broilers were treated with medicated feed containing 50 mg kg^{-1} of nifursol over a 7-day period, and the results showed that nifursol could be

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detected only in the bile and the plasma 3 days after the cessation of treatment; it could not be detected in the liver, kidney or muscle samples of the broilers (limit of determination, LOD = 1-2 μg kg^{-1} for tissue).[13]

The absorption and elimination half-lives of furazolidone in the serum are 0.2 and 3.3 h, respectively,[14] and therefore the detection of the parent drug in the animal tissue is generally not very applicable or useful. Some residues are metabolites of furazolidone, one of which is a 3-amino-2-oxazolidinone (AOZ) side chain, which can be released from bound residues under acidic conditions and detected by reaction with 2-nitrobenzaldhyde to produce 2-nitrophenylmethylene-3-amino-2-oxazolidinone (NPAOZ)[15] (Fig. 1).

Many residue analysis methods have been described, including high-performance liquid chromatography-ultraviolet (HPLC-UV), liquid chromatography-mass spectrometry (LC-MS)[16] and liquid chromatography-tandem mass spectrometry (LC-MS/MS);[17] however LC-MS and LC-MS/MS are prohibitively expensive. Nowadays, a rapid and high-capacity screening method is required in order to process the large number of samples to be analyzed, and hence enzyme-linked immunosorbent assay (ELISA) is a common method more recently used in normal laboratories for this purpose. ELISA is cheaper to carry out than LC-MS/MS, and the ELISA method has a detection limit similar to that of LC-MS/MS.

Methods for the detection of NPAOZ in feeds and biological matrices have been detailed in the literature, and analysis has been performed by reversed-phase (RP) high-performance liquid chromatography-mass spectrometry. The ion source used for mass spectrometers is electrospray ionization (ESI), which is a soft ion source that produces signals for parent and daughter ions of the analyzed compound.

Another method commonly used to analyze AOZ residues is ELISA,[18] which involves the production of polyclonal antibodies capable of detecting AOZ following derivation with 2-nitrobenzaldehyde. However, a commonly-occurring problem of the ELISA method is one of false positive results.

In this study, we attempted to determine the presence of nitrofuran residues in *Tilapia* tissue by ELISA and confirm the results using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS).

EXPERIMENTAL

Chemicals and solvents

Several milligrams of AOZ were provided by Riedel-de Haën (Sigma-Aldrich Laborchemikalien Gmbh, D-30918 Seelze, Germany). An AOZ stock standard solution (10 mg L^{-1}) was prepared in methanol and stored at 4 °C in the dark. Standard solutions were then prepared from the stock solution at concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 μg L^{-1} AOZ in methanol. Methanol (HPLC gradient grade), ethyl acetate (HPLC grade) and dimethyl sulfoxide (DMSO, spectroscopy grade) were supplied by Merck (Darmstadt, Germany), and hydrochloric acid (pro analysis grade) by Riedel-de Haën (Seelze, Germany). Water was double-distilled prior to use. 2-nitrobenzaldehyde (2-NBA) and ammonium acetate were obtained from Fluka (Buchs, Switzerland), di-potassium hydrogen phosphate

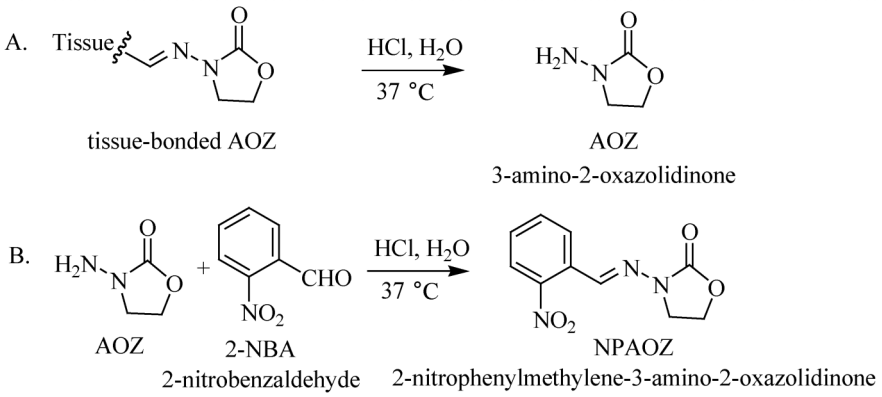

Fig. 1. The structures of tissue-bonded AOZ (3-amino-2-oxazolidinone), released type of AOZ, derivative reagent 2-NBA (2-nitrobenzaldehyde) and target derivative NPAOZ (2-nitrophenylmethylene-3-amino-2-oxazolidinone).

trihydrate from Merck, and sodium hydroxide from Riedel-de Haën.

HPLC analysis

Chromatographic analyses were performed using an high performance liquid chromatography system (HPLC, Agilent 1100 Series) and separation were achieved using an reversed-phase C_{18} column (4.6 × 150 mm, 5 μm, Agilent, ZORBAX SB-C_{18}). The auto-sampler was equilibrated at 20 °C. The analytes were separated with a mobile phase consisting of 0.5 mM ammonium acetate and 0.05% formic acid in water (eluent A) and methanol (eluent B) at a flow rate of 700 μL min^{-1}. The linear gradient program was: 0-0.5 min from 65 to 30% A; 0.5-6 min 30% A; 6-8 min from 30 to 65% A; 8-9 min 65% A (Table 1). The injection volume was 25 μL.

Electrospray ionization mass spectrometer (ESI-MS-MS)

Mass spectrometry analysis was carried out using a API 4000 tandem quadrupole mass spectrometer (Applied Biosystem, USA). The instrument was operated using an electrospray (ESI) source in positive mode. ESI parameters were: capiliary voltage 5500 V, source temperature 650 °C. Collision-induced dissociation was performed using argon as the collision gas at the pressure of 4×10^{-3} mbar in the collision cell. In all cases, $[M+H]^+$ ions were found to be the most abundant. These ions were selected as the precursor ions, the most abundant product ions were selected the most sensitive transition for quantification purposes and a second one for confirmation. It shows MS/MS transitions for quantification and confirmation for each of the selected compounds in Fig. 2. The parent/fragment ion combinations and the optimizing parameters of ion path entrance (DP), collisional focusing quadrupole (EP), offset on collision cell quads (CE) and Q3 entrance lens (CXP) are listed in Table 2. The dwell time for each fragmentation pathway was 150 ms. The electrospray voltage was set to 5500 V and the collision energy to 15 eV. Nitrogen was used as collision gas. Data acquisition was performed using Analyst 1.4.1 software for Applied Biosystem.

Enzyme immunoassay for the quantitative analysis of AOZ (reagents and equipment)

Each immunoassay kit contained sufficient material for 96 measurements. Each microtiter consisted of 96 wells, which were coated with capture antibodies against anti-AOZ-antibodies. There were six concentration levels (0, 50, 150, 450, 1350 and 4050 ng L^{-1} AOZ in aqueous solution) standards of AOZ. Each level were prepared, which included peroxidase-conjugated AOZ, anti-AOZ antibody, and substrate/chromogen (containing tetramethylbnezidine). A stop solution containing 1N sulfuric acid and a sample-washing buffer made with 10 mM phosphate buffer (pH = 7.4) were also prepared. The microtiter plate spectrophotometer was set at 450 nm.

Table 1. Optimized conditions for the analysis of AOZ in fish tissue by HPLC

HPLC gradient		
Time (min)	Eluent A (%) 0.5 mM ammonium acetate	Eluent B (%) 100% Methanol, HPLC grade
0	65	35
0.5	30	70
3.5	30	70
6.0	30	70
8.0	65	35
9.0	65	35
HPLC system		
Sample temperature	25 °C	
Injection volume	25 μL	
Column type	4.6 × 150 mm, 5 μm, Agilent, ZORBAX SB-C_{18}	
Column temperature	25 °C ± 2 °C	
Flow rate	700 μL min^{-1}	

Sample extraction (ELISA standard method)

4 mL H_2O, 0.5 mL 1 M HCl and 100 μL 10 mM 2-nitrobenzoic aldehyde (in DMSO) was added to a 1 ± 0.02 g sample of homogenized fish tissue. After thorough shaking, the tubes containing the samples were incubated at 37 °C overnight (for more than 16 h); 5 mL 0.1 M K_2PO_4, 0.4 mL 1 M NaOH and 10 mL ethyl acetate were then added and the tubes shaken vigorously for 30 sec then centrifuged 3000 g for 10 min at room temperature (20-25 °C). A 2.5 mL portion of the organic layer (ethyl acetate) was then transferred into a new vessel and reduced to dryness with N_2, after which the residue was dissolved in 1 mL n-hexane and mixed thoroughly with 1 mL sample buffer. The vessels were centrifuged again, then 50 μL of the aqueous layer was placed in each well.

Determination of antibody titer

All standards and samples (50 μL of each standard solution or prepared sample) were added to a sufficient num-

Table 2. Parent/fragment ion combinations used for multiple reaction monitoring

	Transition Reactions (*m/z*)	Conditions of MRM			
Analyte	NPAOZ	DP	EP	CE	CXP
Quantitation	236.2 → 134.0	58	10	18	11
Analyte confirmation	236.2 → 104.0	58	10	17	11

ber of wells in the microwell holder to ensure that each was run in duplicate, and the positions of the standards and samples were recorded. A new pipette tip was used for each standard or sample. 50 μL of the enzyme conjugate was placed in the bottom of each well, and 50 μL of the antibody was added to each well, followed by gentle mixing by rocking the plate manually, then incubation for 1 h at room temperature (20-25 °C) in the dark. 100 μL of the stop solution was then added to each well and the solutions gently mixed by manual rocking of the plate. The absorbance at 450 nm was then measured against an air blank and the results were read within 60 min of the addition of the stop solution.

RESULTS AND DISCUSSION

Validation of the method

The purpose of this study was to use liquid chromatography-tandem mass spectrometric detection to confirm the results obtained using the enzyme-linked immunosorbent assay method to extract AOZ residue from the tissue of *Tilapia* fish. The method used was as described in the illustration accompanying the ELISA kit. There were two groups of gradation standards: the first was the AOZ standard in each ELISA kit (0, 50, 150, 450, 1350, 4050 ng kg^{-1}) (Fig. 3), which was determined by two methods without direct extraction; the second was determined using spiked specific concentrations in the homogenized samples

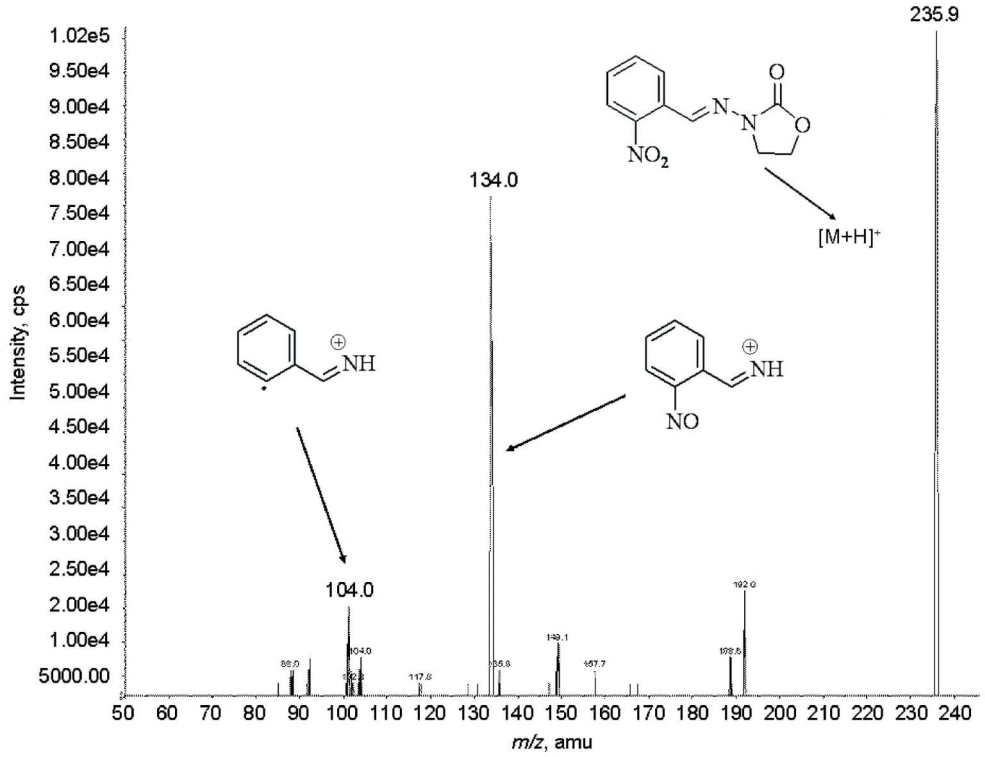

Fig. 2. Mass spectrum of NPAOZ and its typical fragmentation pattern.

of *Tilapia* tissue (0, 0.05, 0.1, 0.5, 1.0, 2.0, 4.0 μg kg^{-1}). After extraction using the ELISA standard method, the results were determined by antibody titer and confirmed by LC-ESI-MS/MS (Fig. 4).

Analysis of control fish tissue

Analysis of the control fish tissue by LC-MS/MS confirmed that no AOZ was present. The analysis result for the control fish tissue obtained by antibody titer was 0.14~0.48 μg kg^{-1}, with a mean of 0.3 μg kg^{-1} and a standard deviation (S.D.) of 0.11 μg kg^{-1} (Table 3). There must therefore have been some disturbance of the matrix in the ELISA method. Compared with the control group about the LC-MS/MS were all none determination.

Linearity (LC-ESI-MS-MS)

The AOZ standard in the ELISA kit was determined by LC-ESI-MS-MS, the result of which was perfect linearity ($y = 1.0003x + 0.0007$, $R^2 = 1$). The linearity of the determination of the gradient standard without fish tissue was $y = 0.8052x - 0.0241$, $R^2 = 0.9982$. External calibration curves obtained for the nitrofuran metabolites (n = 8) in the 0.05~4 μg kg^{-1} range were linear, with correlation coefficients $R^2 > 0.998$ for AOZ (Fig. 5).

Linearity (ELISA)

In the same way, the spiked specific concentration of

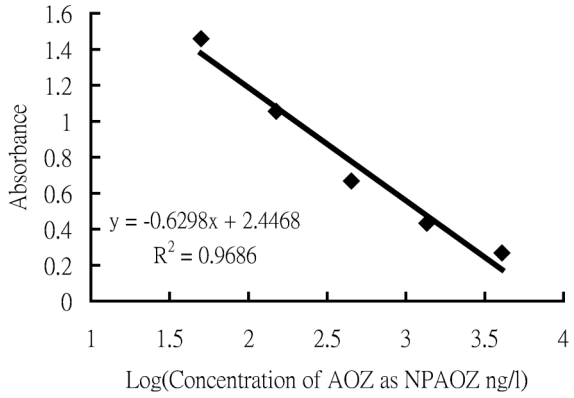

Fig. 3. ELISA standard curve for RIDASCREEN Nitrofuran (AOZ) (Art. No.: R3701) (Y = –0.6298X + 2.4468, $R^2 = 0.9686$).

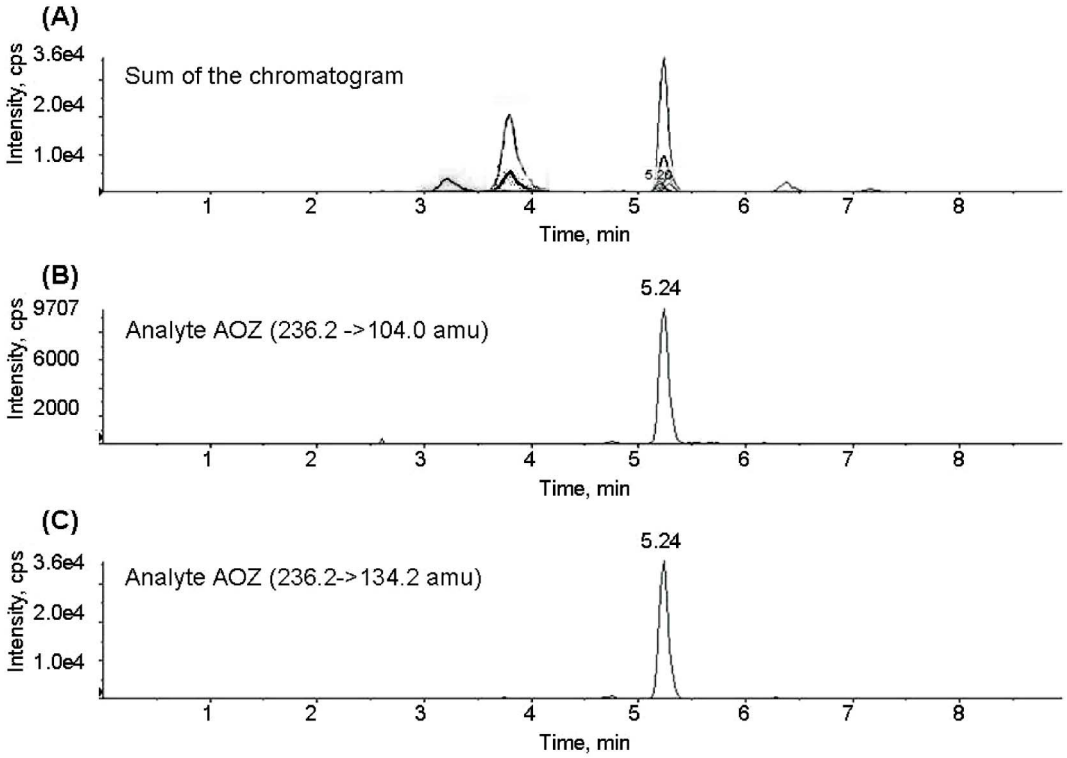

Fig. 4. LC-MS/MS chromatograms (MRM) of spiked 5.0 ng/g AOZ in *Tilapia* fish tissue. (A) sum of the chromatogram, (B) confirmation of analyte AOZ (236.2 → 104.0 amu), (C) quantification of analyte AOZ (236.2 → 134.0 amu).

Table 3. Precision of the ELISA and LC-MS/MS methods in control fish tissue

Control fish tissue group (n = 10)	ELISA (μg L^{-1})	LC-MS/MS (μg L^{-1})
1	0.25	ND
2	0.38	ND
3	0.34	ND
4	0.39	ND
5	0.48	ND
6	0.26	ND
7	0.20	ND
8	0.14	ND
Mean	0.30	0
S.D.	0.11	0

AOZ in the control fish tissue was determined as 0.05~4 μg kg^{-1}. Then, analysis of AOZ in the samples with and without tissue was performed by ELISA. The linearity of the proportion of AOZ in the tissue was $y = 0.9322x + 0.5168$ and $R^2 = 0.9066$, for which the percentage of extraction was 93% as compared with perfect extraction. The linearity was therefore worse and the intercept was too high (0.5168 μg kg^{-1}) (Fig. 6). In the spiked specific concentration analysis of samples without fish tissue, the percentage of extraction was found to be 170% and the intercept 0.3 μg kg^{-1}. Fish tissue samples must therefore cause a disturbance of the matrix in the ELISA method of about 0.2 μg kg^{-1}, and there must be some error in the ELISA method accounting for 0.3 μg kg^{-1}. Use of the ELISA method to determine the AOZ content in fish tissue therefore leads to overestimation of the true content (Fig. 7). Diblikova et al. published about the ELISA method that the detection capability was 0.4 μg kg^{-1} for shrimp, poultry, beef and pork muscle.[18] Furthermore, they showed the blank tissue which were overestimated 0.1-1.7 μg kg^{-1} and 0.1-1.4 μg kg^{-1} in shrimp and chicken, respectively.

CONCLUSION

This study involved the quarantine of aquaculture products for medicament determination, in which two commonly-applied methods were used to quantify AOZ in fish muscle tissue.

Electrospray ionization-tandem mass spectrometry was used for detection, and two fragment ions per analysis were used as additional identifiers, taking the EU regulations into account. The results of this study showed that the LC-MS/MS method resulted in a smaller distribution, but the equipment required for LC-MS/MS analysis is more expensive than that required for the ELISA method. In addition, sample extraction and pretreatment were more complicated in the ELISA method than in the LC-MS/MS method. For these reasons, LC-MS/MS equipment is commonly found in the laboratories of Taiwan, and the ELISA method is familiarly used for analysis of aquaculture products for medicament determination. If samples are found to be positive by the ELISA method, the results should then be reconfirmed by the LC-MS/MS method, as the AOZ content is overestimated when using the ELISA method.

Our future research will focus on reducing the error in

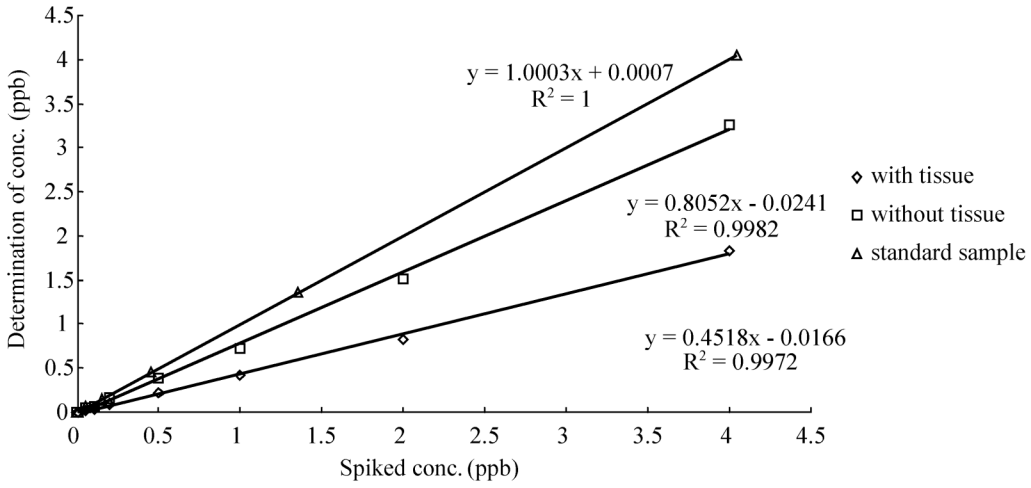

Fig. 5. Calibration curves for: (a) spiked specific concentrations in samples with fish tissue, (b) spiked specific concentrations in samples without fish tissue, (c) standard solutions in the ELISA kits.

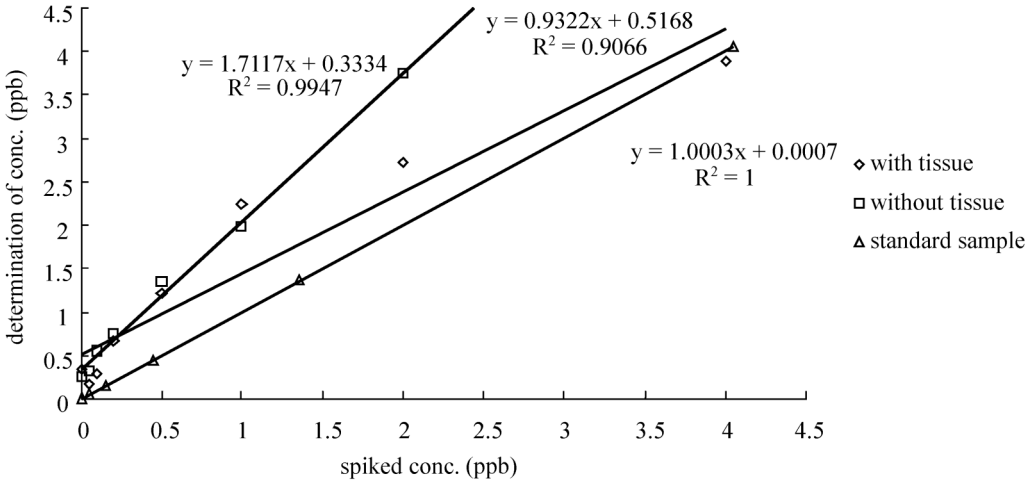

Fig. 6. Calibration curves for: (a) spiked specific concentrations in samples with fish tissue, (b) spiked specific concentrations in samples without fish tissue, (c) standard solutions in the ELISA kits.

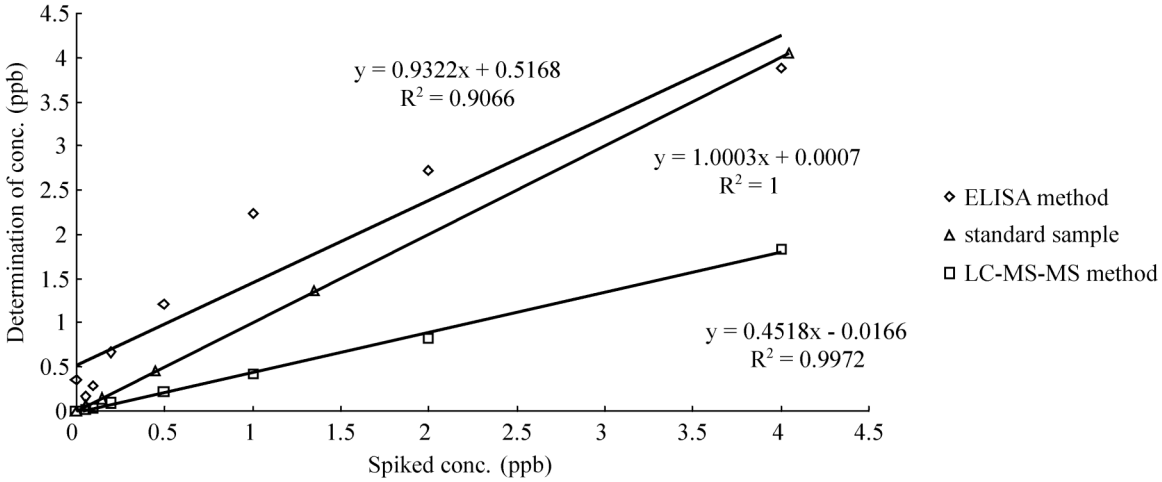

Fig. 7. Comparison of calibration curves for ELISA method, standard sample and LC-MS/MS method.

the results obtained by the ELISA method and modifying the method in order that the accuracy and speed matches that of the LC-MS/MS method.

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