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Determinations of Residual Furazolidone and Its Metabolite, 3-Amino-2-oxazolidinone (AOZ), in Fish Feeds by HPLC-UV and LC-MS/MS, Respectively

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The antibacterial drug furazolidone belonging to the group of nitrofuran antibacterial agents has been widely used as an antibacterial and antiprotozoal feed additive for poultry, cattle, and farmed fish in China. During application a large proportion of the administered drug may reach the environment directly or via feces. Although the use of furazolidone is prohibited in numerous countries, there are indications of its illegal use. It is known that furazolidone can be rapidly metabolized to 3-amino-2-oxazolidinone (AOZ) in the body of the target organism. In this study, a total of 21 fish feed samples, including 17 commercial fish feeds from local markets in China (representing 15 different formulations) and 4 fish feeds obtained from Germany and Turkey, respectively, are analyzed to determine whether the drug is still illegally used or commercially available feeds are contaminated by this drug. High-performance liquid chromatography (HPLC) and liquid chromatography−electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) methods have been implemented to determine furazolidone and its metabolite AOZ in fish feeds containing animal protein, respectively. An efficient and convenient cleanup method for the determination of furazolidone in fish feeds is developed, and a simple cleanup method for the determination of AOZ is used. Method recoveries for samples used were determined as 87.7−98.3% for furazolidone at two spike levels of 2.0 and 5.0 ng g^{-1} and as 95.6−102.8% for AOZ at spike levels of 0.4 and 0.8 ng g^{-1}. Limits of detections were 0.4 ng g^{-1} for furazolidone and 0.05 ng g^{-1} for AOZ. The established methods are therefore suitable for the determination of furazolidone and its metabolite AOZ in fish feeds at trace contamination levels. Using the established methods, all fish feed samples have been proved to be furazolidone negative; however, AOZ is tested in 16 of 17 fish feeds obtained from local markets in the Hubei province of China, with a positive rate as high as 94.1%.

KEYWORDS: Furazolidone; 3-amino-2-oxazolidinone (AOZ); fish feeds; high-performance liquid chromatography; liquid chromatography−mass spectrometry

INTRODUCTION

Nitrofurans are Shiff base derivatives of nitrofuraldehyde, known to be effective against a variety of pathogenic bacteria. Although the use of furazolidone is prohibited in numerous countries, there are indications of its illegal use. Furazolidone was one of the most commonly used nitrofurans in China, and it is known to be a genotoxic carcinogen. At present, available data concerning the identity and toxic potential of compounds released from bound furazolidone residues are still insufficient (*1*). The medicine has been widely applied as a feed additive in food-producing animals such as cattle, swine, poultry, and cultured fish and shrimps and for prophylactic and therapeutic treatment of diseases causally linked to bacteria or protozoa (*2*). Furazolidone is rapidly metabolized to 3-amino-2-oxazolidinone (AOZ) in vivo (*3*), leading to a significant decrease in plasma levels of the parent compound (*4*, *5*) and a concomitant accumulation of its metabolite in proteins, generating a stable adduct that can be detected in tissues over periods of up to 30 days (*6*). Contaminated animal feeds of furazolidone can cause deleterious health effects in the animals and, through "secondary exposure" of consumers to products deriving from these animals, may be harmful to human health. Animal feed produced in China contains mainly animal protein sources, primarily meal of discarded meat, bone, feather, blood, and especially fish and other seafood. The wastes from fish, shrimp, and crab are characterized by high ash and protein contents, with high biological value and balanced proportion of amino acids, whereas poultry and pig wastes present the highest crude fiber

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and lowest gross energy contents. The detection of AOZ would indicate that part of the fish feeds currently in use might originate from areas with a history of furazolidone application, resulting in AOZ residues in fish.

Following concerns about carcinogenicity and mutagenicity of furazolidone and its metabolite, AOZ, furazolidone has been placed in Annex of EC Regulation 2377/90/EEC (1995), which prohibits the use of certain chemicals in food-producing animals in the European Union (EU) and in products from third-world countries intended for EU markets (*7*). For the year 1996, an issued directive on residue testing within the EU (*8*) places considerable emphasis on the monitoring for banned substances on samples collected on-farm. The EU Member States are required to monitor food under their national supervisory schemes for the presence of potentially harmful furazolidone residues because no maximum residue limit (MRL) could be established for this drug due to its possible effects on human health (*9*).

Analysis for furazolidone in feedstuffs therefore may provide an effective method for monitoring their abuse on farms. Previously reported methods for the determination of furazolidone in feeds were based on either thin-layer chromatography (TLC) (*10*), colorimetric determination (*11*), or HPLC-UV measurements (*12*). These methods have limits of detection of around 50 μg g^{-1} and are apparently unsuitable for the determination of trace levels. Recently, HPLC-UV and LC-MS methods for the detection of furazolidone in animal feeds have been published (*13*). The HPLC-UV procedure is used to quantify furazolidone at medicated levels (200 mg kg^{-1}) and at contamination levels of 5 and 20 μg g^{-1}. The limit of detection is 1 mg kg^{-1}, and the recoveries range from 93.4 to 98.2%. The mass spectrometric procedure is used to analyze sample extracts at concentrations <5 μg g^{-1}, and the overall recovery from feed samples fortified at 1 μg g^{-1} is 93.8% (*13*). All of the above methods for the determination of furazolidone have limits of detection of ≥ 1 μg g^{-1} and are therefore unsuitable for the determination of trace levels. Moreover, the determination of AOZ, the metabolite of furazolidone in animal feeds, has not been reported so far. Therefore, there is an urgent need for rapid, high-capacity screening methods for the AOZ residue of furazolidone in animal feeds. Currently, only a few MS-based analytical methods for the detection of nitrofuran metabolites in biological tissues are known (*14*−*18*). Leitner et al. (*14*) report a liquid chromatography−electrospray ionization tandem mass spectrometry (LC-ESIMS/MS) method to analyze simultaneously the four nitrofuran antibacterial agents and their main metabolites, AOZ and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), in animal muscle tissues at detection limits of 0.5−5 ng g^{-1} and limits of quantitation (LOQ) ranging from 2.5 to 10 ng g^{-1}. McCracken and Kennedy (*13*) describe a method for the detection of furazolidone in animal feeds by HPLC-UV and by thermospray mass spectrometry. The authors give a limit of detection as 1 μg g^{-1} for feed samples. The same group presented a method for the detection of AOZ residues in porcine tissue, giving a limit of determination for liver and muscle as 10 ng g^{-1} (*15*). Recently, four nitrofuran metabolites including AOZ in meat have been quantitatively determined by isotope dilution LC-ESIMS/MS, and the procedure entails an acid-catalyzed release of protein-bound metabolites, followed by their in situ conversion into the 2-nitrobenzaldehyde (2-NBA) imine-type derivatives according to the method of Mottier et al., with detection limits of 0.11−0.21 ng g^{-1} (*16*). An application of a solid-phase extraction (SPE) procedure, developed for the isolation and clean-up of nitrophenyl-derivatized AOZ (NPAOZ), for the determination of AOZ by liquid chromatography with UV or tandem mass spectrometry in porcine liver tissue is reported by Conneely et al. (*17*).

In China, nitrofurans, especially furazolidone, were widely used in aquaculture. As a result of the decision concerning the hazardous effect of furazolidone residues in foodstuffs of animal origin, its use was explicitly prohibited in April 2002 by the Ministry of Agriculture of China as it is given in the list of Prohibited Drugs and Other Compounds for Food Animals (*19*). However, in July 2004, furazolidone and chloramphennicol, another drug prohibited in the above-mentioned list, were detected in fish feeds produced in Guangdong province, southern China, indicating that there is still illegal use of banned animal drugs in China.

The present work describes an effective and quantitative method for the determination of furazolidone in fish feeds by HPLC-UV and an improved method for the detection of AOZ in fish feeds by liquid chromatography−electrospray tandem mass spectrometry. To screen fish feed samples for illegal use of prohibited furazolidone and its metabolite, AOZ, 17 fish feed samples containing animal protein from the local markets in Hubei, China, and 4 fish feed samples from Germany and Turkey were collected and analyzed, respectively.

MATERIALS AND METHODS

Chemicals and Reagents. Methanol (HPLC gradient grade), *n*-hexane (HPLC grade), acetonitrile (HPLC grade), ethyl acetate (HPLC grade), methylene chloride (HPLC grade), and dimethyl sulfoxide (DMSO, spectroscopy grade) were supplied from Fisher (Fair Lawn, NJ). Acetic acid (pro analysis grade, p.a.), ammonium acetate (p.a.), formic acid (p.a.), phosphoric acid (p.a.), dipotassium hydrogen orthophosphate (p.a.), sodium hydroxide (p.a.), hydrochloric acid (p.a.), and sodium sulfate (p.a.) were from Hushi (Shanghai, China). Furazolidone (99% purity), AOZ (99% purity), and 2-NBA (98% purity) were from Sigma (Poole, Dorset, U.K.). Water was double-distilled prior to use.

Furazolidone was dissolved in acetonitrile/methanol (1:1, v/v) to give a stock solution of 250 μg mL^{-1}. Working standards of 0.01, 0.02, 0.05, and 0.1 μg mL^{-1} were prepared by serial dilutions of the stock with acetonitrile/methanol/water (17.5:17.5:65, v/v/v). AOZ stock solution was prepared in methanol, wrapped in aluminum foil, and stored at 4 °C for a maximum of 2 months.

Fish Feed Samples. Seventeen commercial fish feed samples collected from local markets in the Hubei province of China and representing 15 different formulations were used in the present study because the province has an old aquaculture production tradition. Four fish feed samples were obtained from Germany and Turkey. Samples were filtered through a U.S. 20 mesh screen to ensure sample uniformity.

Determination of Furazolidone in Fish Feed. The determination of furazolidone in fish feed was based on previous methods described by two different authors (*13*, *20*) with minor modifications. Ten grams of each sample was accurately weighed and placed in a 100 mL conical flask. Fortified samples at 1.0, 2.0, and 5.0 ng g^{-1} were prepared by adding an appropriate volume of furazolidone standard to the feeds. Twenty-five milliliters methylene chloride was added into each flask, and the flasks were shaken for 5 min on a whirl blender. An anhydrous sodium sulfate column was used for dehydration. The column was a syringe barrel column (10 mL) containing a frit (0.45 μm) and 4.0 g of anhydrous sodium sulfate. A pipet tip with 100 μL volume was attached to the end of the column. The sample extract was applied to the column, and the eluate was transferred into a 100 mL evaporating flask. The extraction step was repeated twice with additional 25 mL methylene chloride portions, and afterward the column was rinsed with a small amount (about 2 mL) of methylene chloride. The total eluate (75 mL) was concentrated almost to dryness at 50 °C in a water bath by a K−D vacuum rotary concentrator, and 1.0 mL of acetonitrile/

Table 1. Composition and Ramp of Mobile Phase for the Determination of NPAOZ

time (min)	flow rate (μL min^{-1})	A (%)	B (%)
0	250	80	20
4	250	50	50
10	250	20	80
14	250	80	20
20	250	80	20

Table 2. Optimization Parameters of MRM of the Analyte, NPAOZ

compound	transition reaction (*m/z*)	declustering potential (V)	collision energy (V)	dwell time (ms)
NPAOZ	236.3/104.1	80	31	100
	236.3/192.1	80	20	100
	236.3/134.1[a]	80	13	100

[a] The transition reactions were used for quantitative analysis.

water (8:2, v/v) followed by 1.0 mL of *n*-hexane was added to dissolve the residue. The resulting mixture was transferred to a 5 mL centrifuge tube and centrifuged at 800*g* for 10 min at room temperature (Avanti J-30I centrifuge, Beckman Coulter TM). The supernatant *n*-hexane was discarded with a pipet. Then, 1.0 mL of *n*-hexane was added into the tube again, shaken for 2 min, and centrifuged as described above. The lower clear phase was diluted to 1.0 mL with acetonitrile/methanol (1:1, v/v) for liquid chromatographic determination.

Determination of AOZ in Fish Feeds. The determination of AOZ in fish feeds followed the described methods by former authors (*17*, *21*) with some minor modifications. One gram of each sample was accurately weighed and placed in a 50 mL centrifuge tube. Fortified samples at 0.1, 0.4, and 0.8 ng g^{-1} were prepared by adding an appropriate volume of AOZ standard to the feeds, to which 6 mL of methanol/water (1:1, v/v) solution was added, and the mixture was shaken for 5 min in a mechanical shaker. Four milliliters of pure water and 0.5 mL of 1 mol/L hydrochloric acid were added, successively. Freshly prepared 2-NBA in dimethyl sulfoxide (50 mmol L^{-1}, 0.15 mL) was added to the samples followed by vortex mixing for 5 min. The mixture in the tube was placed in a water bath at 37 °C and incubated for 16 h. After cooling, the pH value of the mixture was adjusted to 7.0 with 5 mL of 0.1 mol L^{-1} dipotassium hydrogen orthophosphate and 0.3 mL of 1 mol L^{-1} sodium hydroxide. Then, 4 mL of ethyl acetate was added and followed by vortex mixing for 20 min and centrifugation at 1900*g* for 5 min at room temperature (Kubota KR/180FA centrifuge, Kubota Seisakusho Co. Ltd.). The ethyl acetate supernatant was collected in a 10 mL centrifuge tube. The samples were extracted for a second time with an additional 4 mL of ethyl acetate and centrifuged. The combined ethyl acetate extract was concentrated under a gentle nitrogen stream to near dryness at room temperature. The residue was dissolved in 0.1 mL of methanol/0.3 mL of 0.1% acetic acid mixture and centrifuged at 1900*g* for 20 min (Avanti J-30I centrifuge, Beckman Coulter TM). The supernatant was transferred to an injection vial for analysis.

HPLC-UV. The analysis and quantification of furazolidone in fish feeds were performed using liquid chromatography. The system consisted of a G1312B controller unit, a G1313A autosampler, a G1311A Quat pump, a G1316A column oven, and a G1315B diode array detector (DAD) (Agilent). All separations were performed using a C_{18} column (250 mm × 4.6 mm, 5 μm) (Agilent). The column temperature was maintained at 25 °C, and DAD detection was at 365 nm (*13*). The HPLC mobile phase used for furazolidone separation was 0.1% phosphoric acid in acetonitrile/water (40:60, v/v) and an isocratic HPLC gradient was carried out. The flow rate was set at 1.0 mL min^{-1}, and the sample injection volume was 20 μL. Furazolidone was eluted at ca. 5.51 min. Quantitative results were obtained by comparing peak areas of samples with those of standards.

LC-ESIMS/MS. A model API 3000 triple-quadrupole mass spectrometer (Applied Biosystems) with electrospray ionization (ESI) was used for the determination of AOZ. A Pinnacle II C18 column (150 mm × 2.1 mm, 5 μm) supplied by Restek was used with a gradient mobile phase of 0.25% formic acid in 0.5 mmol L^{-1} ammonium acetate (A) and acetonitrile/water (9:1, v/v) (B). The composition and ramp of the mobile phase are given in **Table 1**. The sample injection volume was 50 μL. NPAOZ was eluted at 8.57 min.

As the metabolite AOZ of furazolidone is a strong polar compound and is provided with atoms of high negativity, the sensitivity of ESI positive ion testing should be sufficient. During the test phase, qualitative and quantitative analyses were performed using multiple reaction monitoring (MRM) with the conditions of mass spectrum optimized. At the same time, the transition reactions with strong signal extended adequate sensitivity to the quantitative analysis, whereas other transition reactions served for qualitative analysis secondarily. After the optimization, the conditions of the mass spectrum were set as follows: ion spray voltage, 5300 V; curtain gas, 11 psi; probe temperature, 450 °C; nebulizer gas, 10 psi; auxiliary gas, 7 L/min. **Table 2**provides further information. The transition reaction 236.3/134.1 was used for quantitation, and the transition reactions 236.3/104.1 and 236.3/192.1 were used for confirmation.

Calibration Curves, Limits of Detection, and Recovery Rates. Samples for the generation of calibration curves were prepared by adding the furazolidone standard solution to the extract of blank fish feed sample. Calibration standards were run before each analytical series. Linear calibration curves were obtained for furazolidone at the spiking level of 1.0−10.0 ng g^{-1} with an r^2 correlation coefficient of >0.996. The linearity was checked by calculating the standard deviation of the average of response factors (peak area ratios divided by the corresponding analyte concentration ratios of all standards), which was <15% assuming a linear response. The method detection limit (MDL), defined as the concentration of analyte that yielded a peak-to-peak signal-to-noise ratio of at least 3:1, was determined by running a series of 10 negative feed extracts. A value of 0.4 ng g^{-1} was calculated and was comparable with reports using conventional extraction techniques (*13*). Recovery rates ranged from 87.7 to 98.3% at spike levels of 2.0 and 5.0 ng g^{-1} ($n = 10$). Correspondingly, samples for the generation of calibration curves were prepared by adding the extract of blank fish feed sample to NPAOZ standard solutions. Calibration standards were injected before the start of each analytical series. The linearity of the assay was established using NPAOZ standards, equivalent to an AOZ concentration of between 0.1 and 5.0 ng g^{-1} with an r^2 correlation coefficient of >0.997. The MDL was below 0.05 ng g^{-1} of sample for AOZ and was comparable to those of other studies using conventional extraction techniques (*14*−*16*, *21*). Recovery rates ranged from 95.6 to 102.8% at spike levels of 0.4 and 0.8 ng g^{-1} ($n = 10$).

RESULTS AND DISCUSSION

Method Development. The method to determine furazolidone in fish feed samples is principally described by former authors as indicated above, but several steps have been modified in this study. The main aspects regarding sample preparation and sample cleanup have been reconsidered during the development of the method used. In contrast to the described method by Robert et al. (*13*) to determine furazolidone in animal feed samples, the extraction in the present study is performed in multiple steps by liquid−solid extraction, replacing only one step by liquid−solid extraction; moreover, the cleanup in this study is performed in multiple steps by liquid−liquid extraction, replacing only one step by solid-phase extraction. Although these steps in the present study are time- and solvent-consuming, samples are thoroughly cleaned, the limit of detection is very low, and the recovery rate increases significantly.

The furazolidone metabolite, AOZ, has a low molecular mass of 102 g/mol. Due to the highly abundant mass spectrometric background noise in this mass, a low ionization efficiency of the analyte, and its nonspecific fragmentation behavior (predominatly loss of ammonia, water, or carbon dioxide), the MS detection sensitivity is relatively low. Consequently, derivatization of the free amino group of the target analyte must be

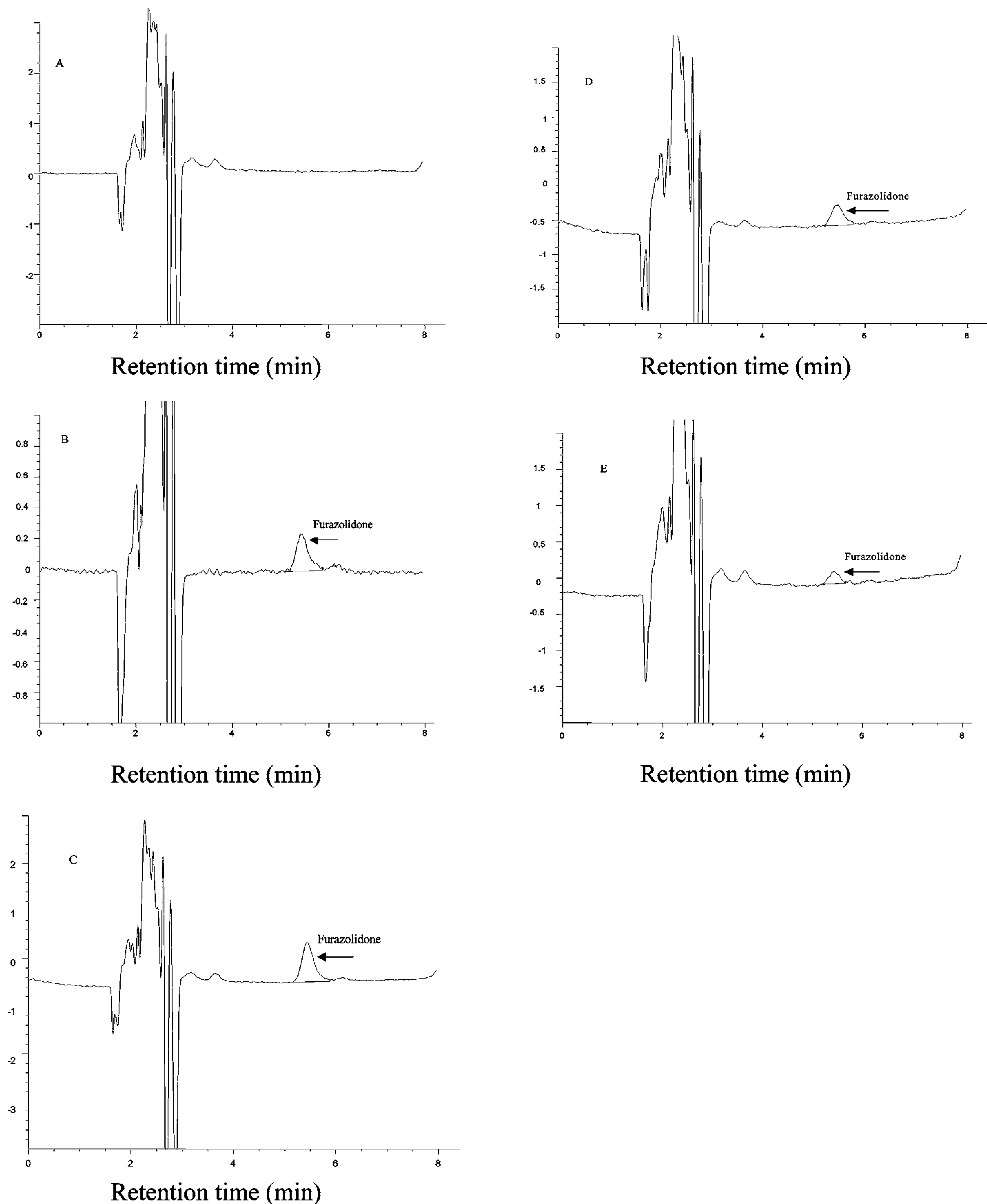

Figure 1. HPLC chromatograms of (**A**) blank fish feed extract, (**B**) 0.02 μg mL^{-1} furazolidone standard, (**C**) blank fish feed fortified with furazolidone at 5.0 ng g^{-1}, (**D**) blank fish feed fortified with furazolidone at 2.0 ng g^{-1}, and (**E**) blank fish feed fortified with furazolidone at 1.0 ng g^{-1}.

carried out. 2-NBA has been described by different authors (*14*, *17*) as a good derivative, and therefore 2-NBA was selected in this study as derivative. The present study describes a further modification of the methods stated by previous authors in that the sample preparation is performed without the use of solid-phase extraction, resulting in a good recovery rate of from 95.6 to 102.8% at spike levels of 0.4 and 0.8 ng g^{-1}. It can be assumed that fish feed as a matrix might be easier to handle than biological tissues.

Analysis of Fish Feeds. HPLC chromatograms of a blank fish feed extract, a furazolidone standard (0.02 μg mL^{-1}), and a blank fish feed extract fortified at 1.0, 2.0, and 5.0 ng g^{-1} are shown in **Figure 1**. LC-ESIMS/MS chromatograms of a blank fish feed extract, an AOZ standard (2.0 ng mL^{-1}), and a blank fish feed extract fortified at 0.4 and 0.1 ng g^{-1} are shown in **Figure 2**. All fish feed samples have been proved to be furazolidone negative in the test, proving that no illegal drug use exists among the 21 samples. However, AOZ was tested in

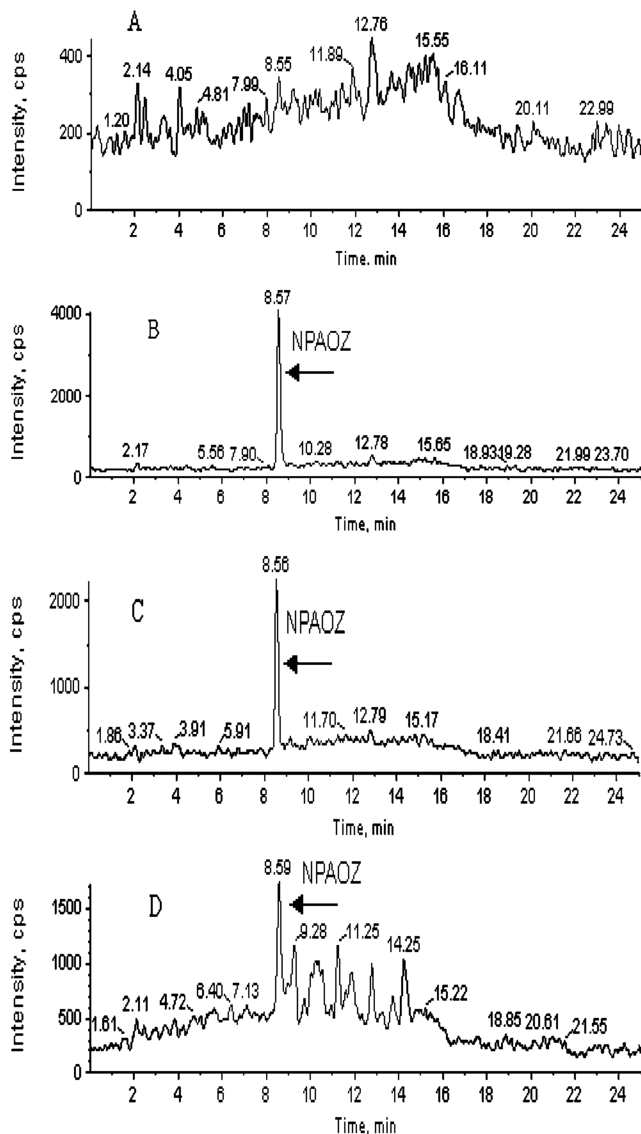

Figure 2. LC-ESIMS/MS chromatograms of (**A**) blank fish feed extract, (**B**) 2.0 ng mL^{-1} AOZ standard, (**C**) blank fish feed fortified with AOZ at 0.4 ng g^{-1}, and (**D**) blank fish feed fortified with AOZ at 0.1 ng g^{-1}.

Table 3. Survey Results of Furazolidone and Its Metabolite, AOZ

analyte	sample number	geometric mean (ng g^{-1})	median (ng g^{-1})	range (ng g^{-1})	positive rate (%)
furazolidone	21				0
AOZ	17 (local)	0.68	0.38	0.30–3.16	94.10
	4 (abroad)				0

16 of 17 fish feeds obtained from local markets of Hubei province, China, with a positive rate as high as 94.1% (**Tables 3** and **4**).

The results from **Tables 3** and **4** have shown that, although nitrofurans are banned in China, the residual AOZ in fish feeds may still cause new pollution sources and may have harmful effects on consumer health. The presence of AOZ in 17 local fish feed samples might be caused either by illegal use of the banned veterinary drug or by the use of polluted raw material. Therefore, illegal access to the banned medicines, misuse of feed additives, and hazardous additives have to be strictly controlled, which requires multidisciplinary inputs from the collaboration of the fields of human and veterinary medicine, agriculture, and academia and from national control agencies.

Table 4. Contents of AOZ in All Samples

sample no.	contents (ng g^{-1})	sample no.	contents (ng g^{-1})
1	0.332	12	0.300
2	0.326	13	0.300
3	0.371	14	0.314
4	0.323	15	0.382
5	1.290	16	0.336
6	1.411	17	ND[a]
7	1.230	18	ND
8	1.111	19	ND
9	3.160	20	ND
10	2.290	21	ND
11	2.510		

[a] ND, lower than the detection limit.

Given the direct links between feed safety and safety of foods of animal origin, it is essential that feed production and manufacture be considered as an integral part of the food production chain. In general, the forgoing outlines the responsibilities of both industry and national governments in ensuring the safety of feed and food. It is important to realize, however, that the large volume of international trade in foods of animal origin as well as feedstuffs adds an important international dimension to the control of animal feedstuffs. Furthermore, the World Trade Orgization's Agreement on the Application of Sanitary and Phytosanitary Measures advocates that national standards related to food safety be harmonized with international standards.

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