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Multidetermination of Four Nitrofurans in Animal Feeds by a Sensitive and Simple Enzyme-Linked Immunosorbent Assay

JUN LI, JU XIANG LIU, AND JIAN PING WANG*

College of Animal Science & Technology, Agricultural University of Hebei, Baoding Hebei, 071000 China

In this study, the polyclonal antibody against furazolidone was produced with furazolidone coupling to protein carriers by a diazotization method and glutaraldehyde reaction, respectively. The antibody obtained showed good specificity toward furazolidone and various cross-reactivity toward nitrofurantoin, nitrofurazone, and furaltadone. Then, an indirect competitive enzyme-linked immunosorbent assay (ELISA) based on the antibody was first developed for multidetermination of four nitrofurans in animal feeds. The limit of detection (LOD) of the method was 0.2−2.1 ng/g depending on the component. After simple extraction, the fortified swine and broiler chicken feed samples were detected with recovery ranges of 75.9−86.4%. Results obtained from ELISA were confirmed by high-performance liquid chromatography (HPLC) with ultraviolet detection. Analysis of the unknown feed samples indicates that ELISA can be a practical tool for screening of nitrofurans in animal feeds before confirmation by HPLC.

KEYWORDS: Furanzolidone; nitrofurans; ELISA; HPLC; feeds

INTRODUCTION

The nitrofurans, with a molecular structure of the characteristic 5-nitrofuran ring group, are a class of broad-spectrum antibacterial compounds that have been widely used as feed additives in poultry, swine, and aquaculture for the treatment of certain bacterial infectious for many years. They are still a group of important veterinary drugs in the Middle and Far East because they are cheap and effective (*1*). The most common nitrofurans (**Figure 1**) are furazolidone (**N1**), nitrofurantoin (**N2**), nitrofurazone (**N3**), and furaltadone (**N4**). These compounds and their metabolites as protein-bound residues (*2*−*4*) have been proven to show potential carcinogenic and mutagenic effects (*5*).

So, the European Union has prohibited the use of nitrofuran drugs in food-producing animals since 1993 and has established a minimum residue performance limit of 1 μg/kg for each nitrofuran metabolite in edible tissues. Subsequently, the use of nitrofurans for food animals has also been regarded as illegal in the United States, China, and most of other countries. Still, the nitrofurans can be illegally applied to food-producing animals in feeds. During 2002−2003, poultry and aquaculture products imported from Europe from some countries were determined to contain parent nitrofurans and their metabolite residues, and the residue of the **N1** metabolite was also detected in poultry meat from Portugal and pork meat from Italy and Greece (*6*).

Therefore, it is very important to monitor the illicit use of nitrofurans in animal feeds, and the development of sensitive and specific analytical methods is the aim of many researchers. Traditional analytical methods, enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), and liquid chromatography−mass spectrometry (LC-MS), have been used for the determination of residues of nitrofurans and/or their metabolites in a large variety of matrixes such as animal tissues (*7*−*14*), eggs (*15*, *16*), aquaculture products (*17*−*19*), milk (*20*, *21*), honey (*22*, *23*), and water (*24*). Because of the character of their metabolism, the nitrofuran metabolites have been regarded as marker residues in animal edible tissues or products, whereas animal feeds may be checked for the presence of their parent compounds. The residues of nitrofurans or their metabolites have been mostly due to the illicit use of high levels of nitrofurans as feed additives, feeding animals with nitrofuran-contaminated feeds at low levels of 30 μg/kg (*25*), or exposure to the nitrofurans-contaminated environment (*26*). Therefore, the analysis of animal feeds for the presence of nitrofurans is one of the most helpful means to inspect the abuse of them. Nevertheless, there have only been a few papers reported for

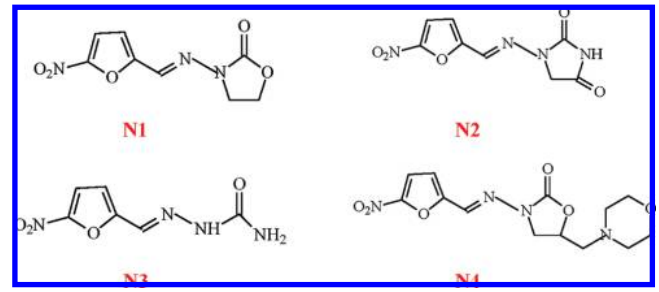

Figure 1. Molecular structures of furanzolidone (**N1**), nitrofurantoin (**N2**), nitrofurazone (**N3**), and furaltadone (**N4**).

* To whom correspondence should be addressed. Tel: 86312-7528477. Fax: 86312-7528369. E-mail: wjpcau@yahoo.com.cn.

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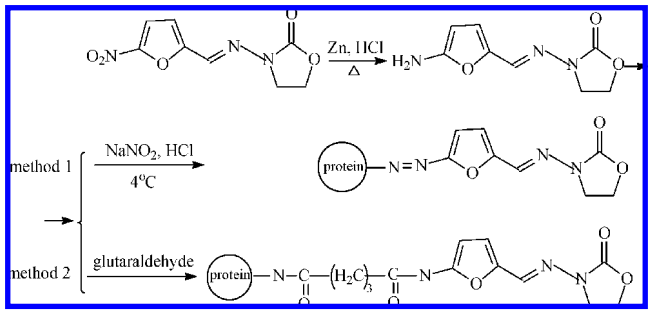

Figure 2. Process of **N1** conjugated with a carrier.

the determination of nitrofurans in animal feeds by HPLC (*27*−*30*). However, these methods are time-consuming, and sophisticated and expensive instruments are required as compared to ELISA. ELISA is a low-cost, portable, and sensitive method capable of screening large amount of samples; however, there has been no paper reported for the preparation of the antibody of parent nitrofurans or detection of nitrofurans in animal feeds by ELISA. Therefore, the objective of the paper was to develop a sensitive, simple ELISA method for simultaneously screening four nitrofurans in animal feeds.

MATERIALS AND METHODS

Reagents and Chemicals. The standards of **N1**, **N2**, **N3**, and **N4** and bovine serum albumin (BSA), OAlbumin (OA), and Freund's adjuvant were all from Sigma (St. Louis, MO). Other chemical reagents were all analytical grade or better and were from Beijing Chemical Co. (Beijing, China).

The extract solvent was a mixture of acetonitrile/methanol (1/1, v/v), and the standard stock solution (1 mg/mL) and working solution (1 μg/mL) of the four nitrofurans were prepared by extract solvent. HPLC mobile phase was a mixture of 200 mL of acetonitrile with 800 mL of 0.05 M sodium dihydrogen phosphate buffer (pH 4.0), filtered through a membrane of 0.2 μm and sparged before use. Phosphate-buffered saline (PBS) (pH 7.2) was prepared by dissolving 0.2 g of KH_2PO_4, 0.2 g of KCl, 1.15 g of Na_2HPO4, and 8.0 g of NaCl in 1000 mL of demineralized water.

Apparatus. The ultraviolet (UV)−vis spectrophotometer was model 751GW from Shanghai Analytical Instrument (Shanghai, China). The ELISA plate reader was model 550 from Bio-Rad (Hercules, CA). The HPLC system consisted of Waters 2695 liquid chromatography, UV detector, and a reversed phase Supelco C_{18} column (250 mm × 4.6 mm, 5 μm) (Waters Co., Milford, CT).

Synthesis of N1 Conjugates. N1 was coupled to carrier protein according to the diazotization reaction (method 1) and glutaraldehyde reaction (method 2) to compare the immunogens synthesized by different methods; the synthesis process is shown in **Figure 2**. Eighty milligrams of zinc powder and 10 mL of 1 M HCl were added into an acetonitrile/methanol solution containing 55 mg of **N1**, and the mixture was stirred at 80 °C until the solution turned dark red. The red solution was cooled down to 4 °C (solution A) for preparation of the **N1** conjugates.

Method 1. One milliliter of 0.5 M $NaNO_2$ was added into solution A dropwise, then the pH value of the mixture was adjusted to 1.0 with 1 M HCl, and the mixture was stirred for 2 h at 4 °C. Then, the excessive $NaNO_2$ was removed using ammonium sulfamate, and the pH value of the mixture was readjusted to 7.5 with 2 M NaOH. Finally, the mixture was added into 10 mL of PBS solution containing BSA (160 mg) or OA (80 mg) dropwise, and the mixture was allowed to react for 12 h at 4 °C to prepare the immunogen (**N1**-BSA) or coating antigen (**N1**-OA). The obtained conjugates were further purified by passing them through a homemade Sephadex G25 cartridge, and the eluate with a pink color was collected to be dialyzed against three changes of PBS for 3 days.

Method 2. After solution A was dropped slowly into 10 mL of PBS solution containing BSA (160 mg) or OA (80 mg), 100 μL of 25% glutaraldehyde was added, and the solution was mixed round for 4 h at room temperature to prepare **N1**-BSA or **N1**-OA. Then, the conjugates were treated as described above.

N1, BSA, and OA, and all of the conjugates were scanned, respectively, on a UV spectrophotometer to identify the conjugation.

Production of the Antibody against N1. The immunogens from both methods were all used to produce the polyclonal antibody. Briefly, six rabbits were immunized with **N1**-BSA (0.5 mg protein per kilogram of body weight) in Freund's complete adjuvant on the dorsal region subcutaneously and were boosted with **N1**-BSA in Freund's incomplete adjuvant at a 3 week interval. After five boosters, the rabbits were exsanguinated, and the serum was collected. Finally, the IgG was isolated using the saturated ammonium sulfate precipitation method for development of the indirect competitive ELISA.

Indirect Competitive ELISA Procedure. A checkerboard procedure was used to determine the optimal dilution of coating antigen and antibody. After that, each well of a microtiter plate was coated with 100 μL of **N1**-OA, incubated overnight at 4 °C, and then blocked with 1% fetal calf serum. The plate was washed three times with PBS, and then, 50 μL of the optimal antibody dilution and 50 μL of **N1** standard with series concentrations from working solution (or sample extractions) were added to the wells (in triplicate) for incubation for 1 h at 37 °C. The plate was washed as above. One hundred microliters of horseradish peroxidase-labeled goat antirabbit IgG was added before incubation for 30 min at 37 °C. After washes, 100 μL of the terramethylbenzidine (TMB) substrate system was added for 15 min of incubation at 37 °C. Finally, the reaction was stopped by addition of 50 μL of 2 M H_2SO_4, and the plate was read on an ELISA plate reader at 450 nm to develop the competitive inhibitory curve. In addition, the cross-reactivity toward **N2**, **N3**, and **N4** was determined.

Sample Preparation. An amount of 5 g of feed sample thoroughly minced by a grinder and 15 mL of water were added to a 100 mL glass jar and mixed on a shaker for 5 min. Then, 35 mL of extraction solvent was added, and the mixture was stirred violently on a variable speed reciprocal shaker for 30 min. After centrifugation for 10 min at 4000 rpm, the supernatant was allowed to pass through a homemade Alumina N cartridge (4 g) under gravity. The eluate was filtered through a 0.45 μm membrane and evaporated to dryness under a stream of nitrogen at 45 °C on a water bath. The dry residue was redissolved in 0.5 mL of mobile phase for HPLC analysis or reconstituted in 5 mL of extract solvent for analysis by ELISA before a 1:1000 dilution with PBS. The control blank swine and chicken feed samples were fortified for each analyte at levels of 20.0 and 40.0 mg/kg and assayed by ELISA and HPLC.

HPLC. A volume of 25 μL sample extract was injected into the chromatography for confirmation with HPLC operation conditions as follows: detection wavelength of 365 nm, flow rate of 1.2 mL/min with running time of 20 min. The quantification of the four nitrofurans was calculated according to the chromatogram peak areas of each analyte.

Unknown Samples. The swine feed samples ($n = 50$) and broiler chicken feed samples ($n = 50$) purchased from the markets of China were all analyzed by ELISA, and the positive samples were selected for further evaluation by HPLC because of its higher sensitivity.

RESULTS AND DISCUSSION

Conjugates Preparation and Antibody Performance. In this study, the first important work was the conjugation of **N1**-carrier. Because there is no chemical group available in the molecule of **N1**, which can be utilized to couple with a protein carrier, the nitryl was deoxidized to amidogen as the active chemical group first, and this step proved to be successful by a phenomenon that the reaction solution turned red from yellow. Then, the **N1** hapten was coupled to BSA and OA using the usual diazotization reaction and glutaraldehyde reaction. The resulting red color was always present throughout the process of coupling, purification, and dialysis. In addition, the curve diagram of the conjugate scanned by UV contained the characteristic peak of **N1** and BSA (**Figure 3**). All of these things suggested successful conjugation.

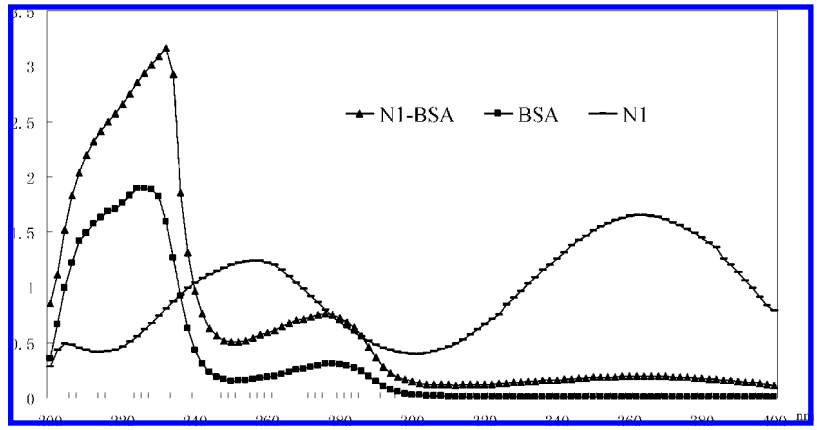

Figure 3. Scaning spectrum diagrams of **N1**, BSA, and **N1**-BSA (method 1) by UV.

Table 1. Cross-Reactivity of the Antibody from Two Methods toward Four Nitrofurans

	antibody from method 1			antibody from method 2	
competitors	IC_{50}[a] (ng/mL)	cross-reactivity[b] (%)	LOD (ng/g)	IC_{50} (ng/mL)	cross-reactivity (%)
N1	3.2	100	0.2	8.0	100
N2	3.4	94	0.3	10.0	80
N3	9.0	36	0.6	22.9	35
N4	28.4	12	2.1	200.0	4

[a] The IC_{50} was calculated from the competetive calibration curve in **Figure 4**. [b] The cross-reactivity of the anitbody was the ratio of the IC_{50} value of **N1** with that of each competetor.

The immunogens from two methods both produced the antibody toward **N1** with IC_{50} values of 3.2 (method 1) and 8.0 ng/mL (method 2), respectively, and the antibodies showed various cross-reactivity toward other nitrofurans (**Table 1**). These differences maybe were due to the specific conjugation method. In method 1, the hapten was coupled to carrier directly with only a diazo between them, which lead to higher sensitivity of the antibody to **N1** or its analogues. However, in method 2, the spacer arm of the five-carbon chain between hapten and carrier might stimulate the lymphocyte to produce the "bridge antibody". Consequently, the antibody obtained maybe showed partial specificity toward the spacer arm, which resulted in its lower sensitivity to **N1**. Although the sensitivity of the two antibodies was high enough to detect the nitrofurans in animal feeds, the antibody from method 1 was selected for the posterior experiment considering the detection of nitrofurans contaminated feeds at low levels. The competitive calibration curves by the antibody from method 1 for four analytes are shown in **Figure 4** with concentration ranges of 0.1−40 ng/mL.

Sample Extraction. A very important evaluation for any analytical procedure is the assessment of its sample preparation procedure. For the extraction of nitrofurans in feeds, cetonitrile (*28*), acetonitrile/methanol (*29*), and ethyl acetate (*30*) were once used as the extraction solvent followed nylon Millipore filter cleanup or solid phase extraction, which all gave good recoveries. In the present study, sample pretreatment involved an extraction with acetonitrile/methanol and a solid phase extraction by Alumina N cartridge. Results from the fortified blank samples suggested that the extraction solvent can extract the four analytes simultaneously with satisfactory results (**Table 2**) and that no impurity peaks in the chromatogram of blank feed extracts and no interfering peaks around the analyte peaks mean that the effect of sample pretreatment for nitrofurans in animal feeds was good and was appropriate for them but not for other impurities (data not shown).

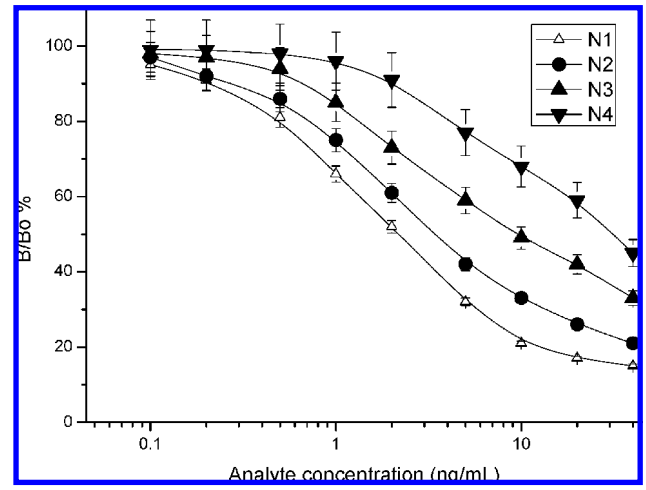

Figure 4. Competitive calibration curve for four analytes of the ELISA.

ELISA Analysis and HPLC Confirmation. By now, there have been only a few methods published for the determination of parent nitrofurans in animal feeds. McCracken and Kennedy (*27*) reported a HPLC-UV method only for **N1** with a limit of detection (LOD) of 1 mg/kg. Vinas et al. (*28*) developed a HPLC-DAD method for **N1**, **N2**, **N3**, and **N4** with a LOD of 2.1−2.7 μg/kg. Barbosa et al. (*30*) also determined the four analytes by HPLC-DAD with a LOD of 50−100 μg/kg. Wang and Zhang (*29*) developed a HPLC-UV method for the determination of **N1**, **N3**, **N4**, and Nitrovin with a LOD of 100−200 μg/kg.

This study first reported an ELISA method for simultaneous detection of four parent nitrofurans in animal feeds. Because of extensive cross-reactivity of the anti-**N1** antibody, the method can be used for multidetermination of four analytes. The LODs, defined as 10% of inhibition, for **N1**, **N2**, **N3**, and **N4** in feeds were 0.2−2.1 ng/g (or μg/kg), which was more sensitive than the published methods (**Table 1**). For determination of its accuracy and precision, the blank swine and chicken feed samples fortified for each analyte were assayed, and the results were satisfactory (**Table 2**). Furthermore, each analyte fortified at a low level of 30 μg/kg was also assayed with recoveries higher than 75% (data not shown). So, the anti- **N1** antibody-based ELISA can be utilized for screening animal feeds for the presence of one or more nitrofurans. However, if the sample contained more than one kind of nitrofuran, the method can only detect the total amount of nitrofurans and cannot verify the specific analyte. This demanded a confirmatory method for the ELISA.

Table 2. Recoveries of Four Nitrofurans Fortified in Blank Swine Feeds by ELISA and HPLC ($n = 6$)

			ELISA			HPLC		
analyte	sample	fortified (μg/g)	tested, μg/g	recovery, %	CV, %	tested, μg/g	recovery, %	CV, %
N1	swine feeds	20.0	15.7 ± 1.5	78.6	7.6	17.9 ± 0.8	89.5	4.2
	swine	40.0	32.9 ± 3.0	82.3	7.5	33.0 ± 1.4	82.6	3.5
	chicken	20.0	16.2 ± 1.6	81.2	8.2	17.4 ± 0.9	87.2	4.6
	feeds	40.0	33.8 ± 2.7	86.4	6.8	33.0 ± 1.2	82.5	3.1
N2	swine	20.0	16.2 ± 1.9	80.9	9.6	17.0 ± 0.5	84.9	2.6
	feeds	40.0	33.3 ± 3.1	83.2	8.1	32.6 ± 0.9	81.6	2.3
	chicken	20.0	16.5 ± 1.8	82.7	9.2	15.9 ± 0.6	79.4	2.8
	feeds	40.0	32.6 ± 3.2	81.5	7.9	32.1 ± 1.2	80.2	3.1
N3	swine	20.0	15.9 ± 2.0	79.5	10.0	18.3 ± 0.4	91.5	2.2
	feeds	40.0	32.2 ± 3.8	80.6	9.5	35.4 ± 1.1	88.6	2.8
	chicken	20.0	15.4 ± 2.0	77.1	9.8	18.1 ± 0.3	90.5	1.6
	feeds	40.0	31.4 ± 3.7	78.6	9.2	35.2 ± 0.8	87.9	1.9
N4	swine	20.0	15.3 ± 2.0	76.4	9.8	16.6 ± 0.7	82.8	3.4
	feeds	40.0	30.4 ± 4.6	75.9	11.5	33.6 ± 1.0	83.9	2.6
	chicken	20.0	15.7 ± 2.5	78.6	12.4	16.6 ± 0.6	83.2	3.2
	feeds	40.0	31.3 ± 4.2	78.2	10.6	32.2 ± 1.4	80.6	3.5

Table 3. Determination Results of the Unknown Feed Samples by ELISA and HPLC

	ELISA[a] (mg/kg)	HPLC (mg/kg)		
sample	total amount	**N1**	**N3**	**N4**
swine feeds	12	15		
	28	28	9	
	28		32	
chicken feeds	30	28		
	33			34
	37	44		
	>40	57		
	>40	102		

[a] The total amount of nitrofurans detected by ELISA was shown as the amount of **N1**.

In this paper, the HPLC-UV procedure was the confirmatory tool. The identification was by comparing the retention times of chromatographic peaks of the fortified samples or unknown samples with those of nitrofuran standards. Barbosa et al. (*30*) once proved that the pH of the mobile phase was fundamental to the separation effect of four nitrofurans, and they control pH with 14 mM ammonium acetate (pH 4.6) to obtain a good separation. In this study, 50 mM sodium dihydrogen phosphate buffer (pH 4.0) in the mobile phase also gave a satisfactory effect with analytes separated as sharp and symmetrical peaks. Retention times were 5.83, 8.97, 11.46, and 14.90 min for the standard of **N4**, **N3**, **N2**, and **N1**, respectively. The LODs, defined as signal/noise (S/N) ≥ 3, for **N1**, **N2**, **N3**, and **N4** were 2, 2, 3, and 5 ng/g, respectively. Blank feed samples fortified with each analyte were determined by HPLC with recovery results shown in **Table 2**. From the results, it could be said that both methods gave satisfactory recoveries and the HPLC method was more consistent and stable for the four analytes with a coefficient of variation lower than 5%. Therefore, the ELISA may be used as a simple routine screening method for a large number of feed samples and the samples with positive ELISA results were further verified by the qualitative and quantitative HPLC method.

Unknown Samples. For verifying the practicability of the ELISA, 100 unknown feed samples were detected with six chicken feeds and two swine feeds as positive. All of the ELISA positive samples were confirmed by HPLC with various levels of **N1**, **N3**, and **N4** except **N2** (**Table 3**), and **N1** illicit use was more extensive than the other two analytes. All of the negative ELISA samples were determined by HPLC with no samples containing analyte. From the results, it could be said that the illicit use of nitrofurans is still existent in animal husbandry in China especially in broiler chicken breeding.

From analysis of the blank and real feed samples, the ELISA procedure is a simple, sensitive, and accurate method for nitrofurans screening in animal feeds before confirmation and quantification by other instruments, such as HPLC.

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