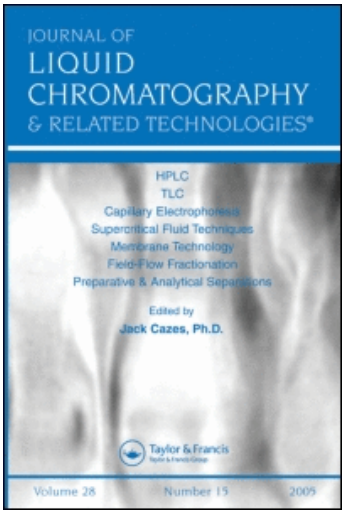

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J. R. Wang[a]; L. Y. Zhang[a]
[a] National Key Laboratory of Animal Nutrition, China Agricultural University, Beijing, P. R. China

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Simultaneous Determination and Identification of Furazolidone, Furaltadone, Nitrofurazone, and Nitrovin in Feeds by HPLC and LC-MS

J. R. Wang and L. Y. Zhang
National Key Laboratory of Animal Nutrition, China Agricultural University, Beijing, P. R. China

Abstract: An HPLC and LC-MS method for simultaneous determination of furazolidone, furaltadone, nitrofurazone, and nitrovin in feeds was developed. Samples were extracted with a mixture of acetonitrile and methanol with ammonia solution, and further clean-up was achieved with solid-phase extraction (SPE). Separation and quantitation of the analyte were developed with an acetonitrile-water gradient program and UV absorbance at 365 nm. LC-MS was in an electrospray positive ion mode. The limits of detection were 0.1 mg/kg for furazolidone and furaltadone, and 0.2 mg/kg for nitrofurazone and nitrovin; the limits of quantitation in feeds were 1 mg/kg for furazolidone and furaltadone, and 2 mg/kg for nitrofurazone and nitrovin.

Keywords: HPLC, LC-MS, Nitrofurans, Feeds

INTRODUCTION

Nitrofurans, such as furazolidone (FZD; *N*-[5-nitro-2-furfurylidene]-3-amino-2-oxazolidone), furaltadone (FTD; 5-morpholinometryl-3-[5-nitrofurfurylideneamino]-2-2 oxazolidinone), nitrofurazone (NFZ; 5-nitro-2-furaldehyde semicarbazone), nitrofurantoin (NFT; *N*-[5-nitro-2-furfurylidene]-1-aminohydantoin), and nitrovin (NTV; 2-[3-(5-nitro-2-furanyl)-1-[2-(5-nitro-2-furanyl) ethenyl]-2-propenylidene] hydrazine carboxamidine, are synthetic broad-spectrum antibiotic compounds. They have been widely used as feed

Address correspondence to Liying Zhang, National Key Laboratory of Animal Nutrition, China Agricultural University, No. 2. Yuanmingyuan West Road, Beijing 100094, P. R. China. E-mail: zhangly@mafic.ac.cn

additives for treatment of gastrointestinal infections (bacterial enteritis caused by *Escherichia coli* and *Salmonella*) in cattle, pigs, and poultry.[1] They are also used as growth promoters for food producing animals.[2] FZD, FTD, NFZ, and NTV can be used in feeds, while NFT is often used by drinking water or injection. NFZ and FZD have been used as therapy for pigs at levels between 800 and 1200 mg per kilogram of feed; NFT is used at levels of 400 mg/kg to 500 mg/kg for curing and 200 mg/kg to 300 mg/kg for defense purpose in broiler feeds;[2] and NTV has been used as a growth promoter in feeds for chickens, turkeys, pigs, and calves at levels varying between 10 and 25 mg of NTV per kilogram.[3] However, nitrofurans are toxic, mutagenic, and carcinogenic,[4]and many countries have taken steps to regulate these drugs in animal production. Despite bans imposed on nitrofurans in many industrialized countries from the mid-1990s onwards, extensive illegal use of these compounds in intensive animal production has persisted. Widespread use in livestock over three decades was based on relatively low cost, antibacterial efficacy, anti-protozoal activity.

The parent drugs are rapidly metabolized, both in vivo and in vitro, with half-lives in the range of a few hours.[5] Most of the published methods in the literature still rely on the analysis of drugs or their metabolites in tissues.[1,4,6–9] Feeds are regarded as an important source of nitrofuran contamination. Cases that have been investigated have disclosed indirect introduction of nitrofuran residues from animal protein. Therefore, it is imperative that a simple and accurate method to detect and confirm nitrofurans in feeds is available. The previously published methods for the analysis of nitrofurans in feeds were mostly with single drugs,[3,10,11] and a few experiments were conducted to simultaneously determine two[12,13] or three[14,15] kinds of nitrofurans in feeds by HPLC. However, there were no reports on the simultaneous determination of FZD, FTD, NFD, and NTV by HPLC; no LC-MS method for identification of these four nitrofurans in feeds has been reported so far. Therefore, the objective of this study was to develop a quick and simple analytical method which could simultaneously and unambiguously detect any of these compounds or their mixtures in feeds. At the same time, the cost of each determination was minimized.

EXPERIMENTAL

Materials and Reagents

Samples of drug free feeds for poultry and pigs were used in the spiked experiments, and all the feeds were made at the pilot workshop of the Ministry of Agricultural Feed Industry Centre (MAFIC) in Beijing, P.R. China.

All chemicals and solvents were analytical or HPLC grade, Milli-Q water, 18.2 MΩ cm. (Milli-Q reagent water system, Millipore Corp.). The standards of FZD, FTD, and NFZ were bought from Sigma Chemical Co.; NTV was a gift from China Institute of Veterinary Drug Control, Beijing.

The stock standard solutions of NFZ and FTD were prepared by dissolving in acetonitrile. The stock standard solution of FZD was prepared in 2% dimethyl formamide (DMF), and the stock standard solution of NTV was prepared by dissolving in acetonitrile containing a 1% ammonia solution. All the stock standard solutions were composed of 0.5 mg/mL of each pure standard product. All solutions were left in an ultrasonic bath for 5 min. Working standard solutions, comprised of 5 μg/mL of each drug, were made by mixing aliquots of these stock solutions and diluting them with acetonitrile. The stock standard solutions were stored at $+4^{\circ}$C in the absence of light, and working standard solutions were prepared fresh daily.

Extracted solution, consisting of a mixture of methanol and acetonitrile (1 + 1, v/v) containing 1% ammonia solution, was made by diluting 1 mL of ammonia solution in ca. 100 mL of equal amounts of methanol and acetonitrile. 5% and 15% methanol solutions were made by diluting 5 mL and 15 mL methanol in ca. 100 mL of water, respectively. 5% DMF methanol solution was made by diluting 5 mL of DMF in ca. 100 mL of methanol.

Solid phase extraction (SPE) columns Oasis® HLB 1 cc (30 mg), purchased from Waters (China), were used for clean-up.

Apparatus and Chromatographic Conditions

The HPLC and LC-MS systems used for method development consisted of Waters Associates: Alliance 2690 Separations Module, 2487 Dual λ Absorbance Detector, Micromass ZQ, and MassLynx™4.0 software (Waters company, USA). HPLC analytical column (stainless steel 4.6 × 150 mm), and guard column (4.6 × 20 mm) packed with 5 μm particle size (Waters Symmetry®, USA) were used. The mobile phase consisted of water and acetonitrile. Separation was optimized by changing the composition of the mobile phase in a gradient. Photometric detection was performed at 365 nm. The flow rate was at 1.2 mL/min. The LC-MS column (2.1 × 100 mm) which was used in the method development was packed with 3.5 μm particles of Symmetry MS C18 from Waters (China). The mobile phase for LC-MS was the same as that for HPLC, but the flow rate was at 0.24 mL/min. The MS was set to collect single-ion data in positive ion mode (ESI+) at m/z 226 for FZD, m/z 325 for FTD, m/z 199 for NFZ, and m/z 361 for NTV, respectively. The electrospray voltage was set at 3 KV; the entrance electrode voltage and the positions of the ion spray inlet were adjusted to provide optimum conditions. The clean-up system consisted of a vacuum manifold processing station of Agilent Technologies and a vacuum pump (Agilent Technologies, USA).

Sample Preparation and Extraction

Typical commercial feed formulations (premix, concentrated feed, and completed feed) for poultry and pigs were used in this study. These feeds were used to provide the presence of interfering peaks, carry out accurate

and precise experiments, and also were further used to examine the applicability of the method in medicated feeds. Nitrofurans are light sensitive and, during sample preparation and all subsequent stages of analysis, samples and standard solutions must be taken to avoid exposure to light as much as possible.

A volume of 1 mL of water or standard solution (mixing stock standard solutions) was added to 2 or 5 g of the samples, which were weighed into 50 mL screw-capped centrifuge tubes. The mixture was homogenized thoroughly and then evaporated under a stream of nitrogen gas to dryness at 60°C. A volume of 40 mL of extracted solution was added, and the tube was shaken at high speed until it was homogenized, then centrifuged for 10 min at 3,500 rpm after being shaken on a wrist action shaker for 30 min; the particulates were allowed to settle for a few minutes. Supernatant was filtered through a filter paper into 100 mL amber-coloured volumetric flasks, and the sediment was re-extracted with two additional 30 or 20 mL of extracted solutions. All the extracts were combined and diluted to volume with methanol. An aliquot of extract was concentrated, under a gentle stream of nitrogen gas, to near dryness in a 60°C water bath. An aliquot size was determined by concentration of sample (Table 1). A volume of 2 mL of water solution, containing of 1% DMF-2% methanol, was added to the concentrated extract, and the mixture was stirred and ultrasonically vibrated for 2 min; this solution was prepared for clean-up.

SPE Column Clean-Up

The column was conditioned with 1 mL of methanol, followed by 2 × 1 mL of water before the extract was applied. Care was taken not to let the SPE column run dry, for it will result in lower recoveries. The sample prepared by sample extraction was loaded, washed, and eluted. The flow rate through the SPE column was controlled at ca. 2 drops/s by applying positive pressure at the inlet, or conditioning and application of sample took place under gravity flow. When the eluting solvent passed through, the column was suctioned to dryness. The eluates were captured in a 10 mL tube and evaporated to

Table 1. Sample extract aliquot volume

Nitrofurans, g/kg	Extract aliquot, mL
10–<25	10
25–<100	5
100–<500	2.5
500–<1000	1
1000–2500	0.5

near dryness under a gentle stream of nitrogen gas at 60°C in a water bath; then, 1 mL of methanol was added to the tube and the solution was used for analysis.

Determination

The linearities of FZD, FTD, NFZ, and NTV were evaluated by samples spiked with a mixture of standard solutions to yield 0.2, 0.5, 1.0, 2.0, 5.0, 8.0, and 10.0 mg/kg. The standard calibration curves were generated by running working standard solutions, then plotting recorded peak areas versus the corresponding masses of analytes in the injected volumes. The precision was expressed with relative standard deviation (RSD) by means of injection of working standard solutions, as well as spiked feed samples, and the determination was repeated seven times. The recoveries were evaluated using five levels of concentration lying within the linearity range. These assays were also repeated seven times. The identification of FZD, FTD, NFZ, and NTV was analyzed by injecting 2 μL of the calibration standard solutions and sample extracts for LC-MS.

All samples exceeding the linear calibration range were diluted appropriately and reinjected on the same day of analysis.

RESULTS AND DISCUSSION

Liquid Chromatography and LC-MS

The analytical separation of FZD, FTD, NFZ, and NTV was performed with a reversed phase C_{18} column. Optimized chromatographic conditions were as follows: 365 nm was selected as detection wavelength according to previous work and literature data,[3,10–14] and the four nitrofurans can be detected and quantified, simultaneously. Acetonitrile had been initially tested as mobile phase to perform the separation; then, the ratio of acetonitrile to water was changed for isocratic separation. The four drugs cannot be baseline separated. With gradient programming, separation can be observed, i.e., FZD, FTD, and NFZ are easily eluted by the mobile phase (Table 2). NTV, however, was strongly retained and it was necessary to use a higher ratio of acetonitrile to shorten the retention time. The chromatogram of the four drugs is shown in Figure 1. It is also interesting to note that the retention-elution behavior of NTV is different between standard product and other commercial products. There is a single peak for standard NTV solution and there are two peaks for commercial products when they are dissolved in 0.1% ammonia-acetonitrile solution or 2% DMF-acetonitrile solution. Three products were selected from different markets and analyzed, and the same results were obtained. Although NTV is hard to dissolve in

Table 2. Mobile phase linear gradient program

Time (min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0	84	16
6	75	25
8	30	70
15	10	90
16	84	16

[a]Mobile phase A: water (Milli-Q water, 18.2 MΩ · cm).
[b]Mobile phase B: acetonitrile (HPLC grade).

water and ether, it can be dissolved in ethanol, dimethyl formamide, dimethysulfoxide, and other organic solvents.[2] However, it is more difficult for commercial products of NTV to be dissolved; and it was also hard to clean up the samples when dimethyl formamide or dimethysulfoxide were used as extraction solvents; moreover, the recoveries of FTD and FZD decreased. Adding some alkaline solvent improved NTV solubility, but alkaline solvent probably reacted with NTV products, and maintained an active balance. The ratio and distance of the two peaks change with pH of the mobile phase (Figure 2), but the total area of the two peaks changes little. The

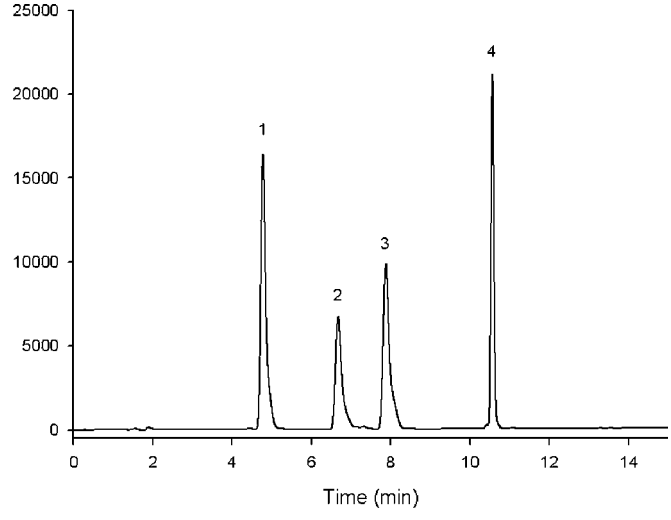

Figure 1. HPLC chromatogram of the nitrofurans. Elution order: 1, NFZ: nitrofurazone; 2, FZD: furazolidone; 3, FTD: furaltadone; 4, NTV: nitrovin. Wavelength: 365 nm; flow rate: 1.2 mL/min; mobile phase performed gradient programming given in Table 2.

NTV standard solution was stored at ambient temperature for more than two weeks, then determined by HPLC. There were also two peaks observed in the chromatogram (Figure 3). The structure of NTV has a cis-trans-isomerism with different chromatographic conditions; this will change in the alkaline solvent when stored for a long time at ambient temperature. These results were consistent with the report of the Analytical Methods Committee.[3]

LC-MS was performed for peak identification for each of the nitrofurans and the results are shown in Table 3. The ion chromatograms are shown in Figure 4. Electrospray in positive ion mode (ESI+) was chosen for the experiments. A source capillary voltage of 3.0 KV was employed. The source

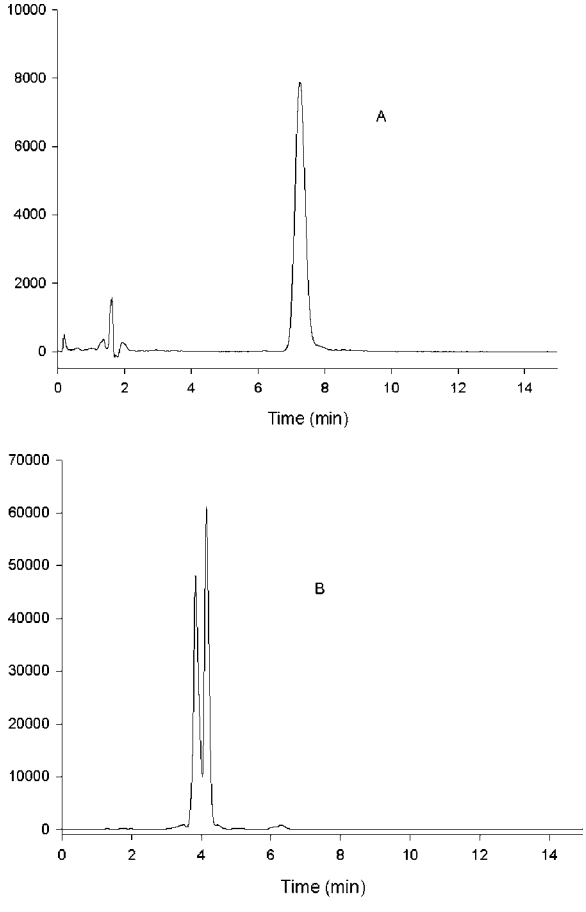

Figure 2. Chromatograms of the new fresh NTV standard solution; flow rate is 1.2 mL/min; A: the mobile phase ratio is 50 : 50 with 0.1% formic acid solution and acetonitrile; B: the mobile phase ratio is 50 : 50 with 0.2% formic acid solution and acetonitrile.

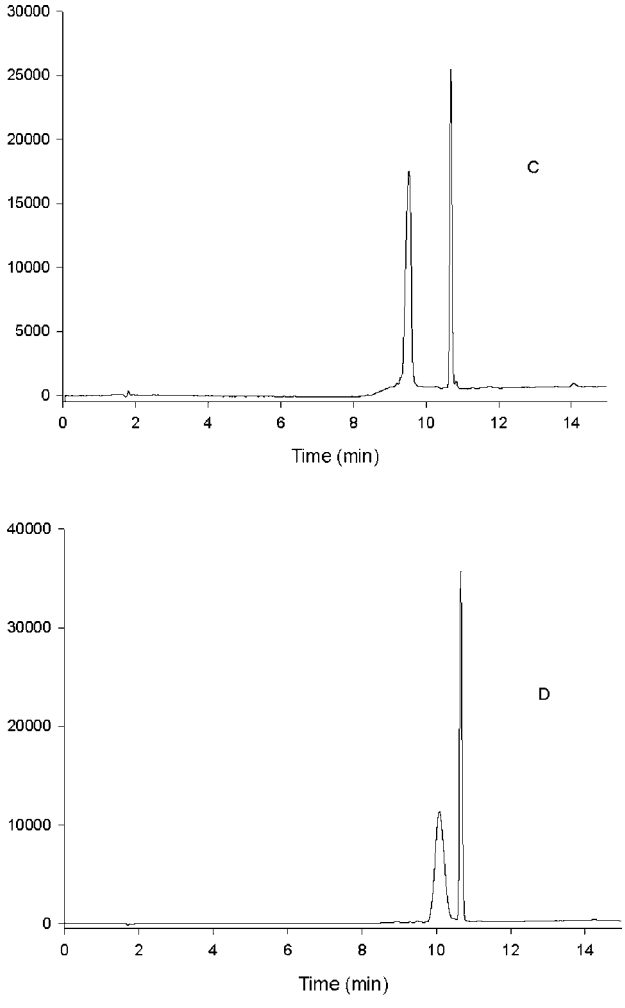

Figure 3. Chromatograms of the NTV standard solution which was storied for a long time; flow rate is 1.0 mL/min; the mobile phase ratio is 50 : 50 with water and acetonitrile. C: 2 weeks; D: 4 weeks

temperature of the heated transfer capillary was set at 100°C; the extractor voltage was 5 V; the desolvation temperature was set at 300°C; desolvation gas flow was 350 L/h and cone gas flow was 50 L/h. The mass spectrometer was scanned from $m/z = 50$ to 410 in positive ion mode. There was only one ion peak for either NTV standard or NTV commercial products, which is different from the results of the HPLC chromatogram. To some content, this explains the reason that NTV has two peaks but its specificity doesn't change when NTV products were dissolved in alkaline solvent, the mobile phase used at a different pH, or stored for a long time at ambient temperature.

Table 3. Fragment ion combinations used for multiple reaction monitoring

	Cone voltage (V)	Fragment ions (m/z)
Furazolidone	35	226,139,113,95
Furaltadone	25	325,281,127
	30	252,100
Nitrofurazone	30	199,182,156,102
Nitrovin	25	361
	30	149,58

Electric spray in positive ion mode (ESI+); source capitary voltage: 3.0 KV; source temperature:100; extractor voltage: 5 V; desolvation temperature: 300; desolvation gas flow: 350 L/h; cone gas flow: 50 L/h.

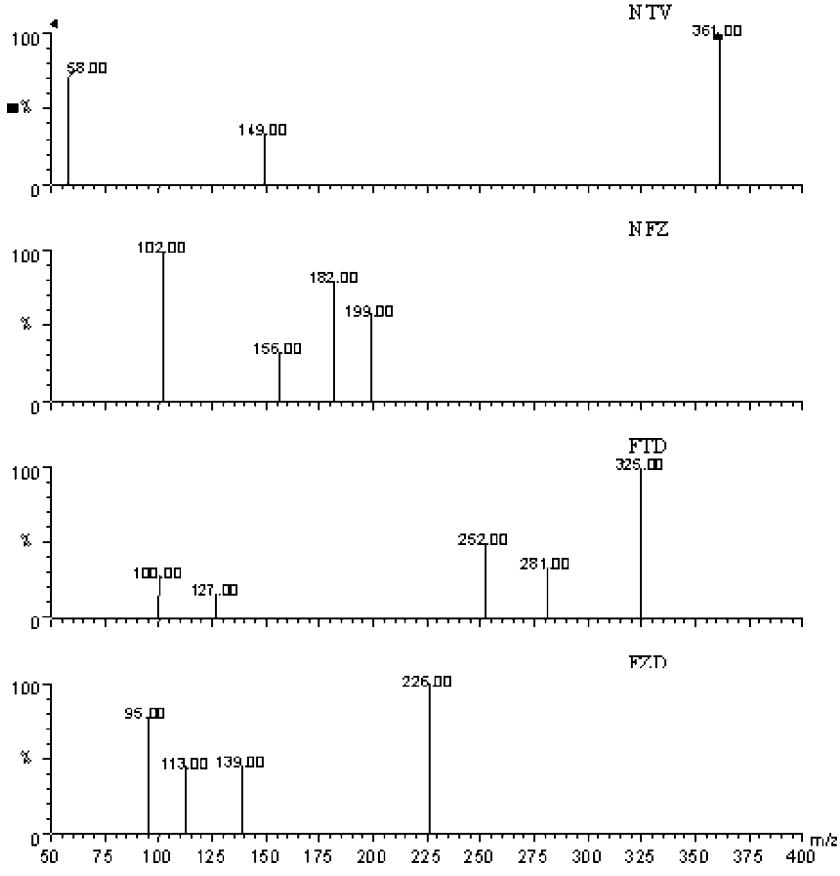

Figure 4. LC-MS spectrum of nitrofurans, LC-MS conditions were based on the conditions described in Table 3.

Choice of Extraction Solvent and Clean-Up

FZD, FTD, NFZ, and NTV are all nitrofurans and have a common 5-nitryl furan ring, but their solubilities in organic solvents are very different. NFZ and FTD are easy to dissolve in methanol and acetonitrile, while FZD and NTV are difficult to dissolve in organic solvents. The solubility of FZD and NTV could be improved when DMF or ammonia solution is added to an organic solvent. Different solvents had been used to extract nitrofurans in feeds. DMF was used often in previous work, but it is difficult to evaporate and will affect the analysis. Methanol and acetonitrile exhibit good solubility towards substances, whether polar or non-polar. Therefore, methanol and acetonitrile were chosen as the main extraction solvents. The efficiencies of extractions were compared among the four solutions, i.e., 1% DMF-methanol solution (1% DMF-MeOH), 5% DMF-methanol solution (5% DMF-MeOH), a mixture of methanol and acetonitrile (1 + 1, v/v) containing 5% DMF solution (5% DMF-MeOH/ACN) and a mixture of methanol and acetonitrile (1 + 1, v/v) containing 1% ammonia solution (1% ASN-MeOH/ACN). All methods were evaluated using solutions of all the drugs under evaluation at a concentration of 10 μg/mL. The results indicated that the mixture of methanol and acetonitrile (1 + 1, v/v) containing 1% ammonia solution was the best extraction solvent (Table 4).

The matrix of feed samples is very complicated, especially for premix and concentrated feeds. The sample extracts were difficult to analyze directly and the analytical column is also easily polluted because of the presence of other components. The traditional liquid-liquid extraction method needs more organic solvent, and the recovery and efficiency of clean-up are lower and/or not consitent; they are easily affected by the feed matrix. In recent years, solid phase extraction (SPE) is most frequently used for clean-up. The reverse phase C_{18} column (Oasis® HLB) has been used for clean-up. Compared to mixed-mode anion exchanger (MAX) and mixed-mode cation

Table 4. Sample extracted solution for nitrofurans

Extracted solution	Recovery[a] (%)	RSD[b] (%)
1% DMF-MeOH	25.2–60.9	5.6–18.9
5% DMF-MeOH	32.1–85.3	2.6–15.3
5% DMF-MeOH/ACN	45.7–89.4	3.6–9.4
1% ASN-MeOH/ACN	59.4–99.6	3.13–9.18

[a]The recovery included range of all the data of experiment.

[b]Repeated seven times.

The test samples including five kinds of feeds were used in the experiment.

DMF: dimethyl formamide; MeOH: methonal; CAN: acetonitrile; ASN: ammonia solution.

Table 5. The steps and purposes of solid phase extraction (SPE) in feed clean-up (triplicate analysis)

Steps	Operation	Purposes
Condition/ equilibration	1 mL methanol/ 1 mLwater	Prepares sorbent for use
Load	Dryness extraction and spiked 1 mL 1% DMF-2% methanol solution	Basic analytes are retained by reverse-phase mechanism
Wash 1	1 mL of 5% methanol	Removes salts
Wash 2	1 mL of 15% methanol	Removes other organic interference
Elute	1 mL of 5 DMF-methanol	Elutes analytes of nitrofurans

exchanger (MCX) columns, neither MAX column nor MCX column could achieve excellent recovery of nitrofurans, which exceeded 95% with the HLB column, for the standard solutions. The clean-up steps are shown in Table 5. Results show that the clean-up procedure is good for the pretreatment of feed samples, and there were no interference peaks observed near the target compounds.

Linearity and Accuracy

Regression analysis of the data, obtained by running 0.2–10 μg/mL working solutions and injecting 5 replicates, showed the detector response to be linear ($R^2 > 0.996$). The samples were spiked with different levels of nitrofurans to yield 2, 5, 10, 15, and 20 μg/g. The linearities were determined by comparing the analyses of spiked feed samples with those of standard solutions of FZD, NFZ, NFT, and NTV. Regression analysis of data for spiked samples also showed good linearity ($R^2 > 0.990$). The results suggested that the linearity of the method would be quantitative by an external standard method.

The accuracy of the method was evaluated by analyzing five sets of seven replicates of 2 to 5 g samples from each pig and poultry feed. Each set was previously spiked with four drugs at different levels, based on doses used in animal production (Table 6). Recoveries of these four drugs in feeds were satisfactory, except for NTV. The recoveries of FZD and FTD were higher than 90%. The recovery of NFZ was different among different types of feeds. In concentrate feeds for poultry and premixes for pig, the recoveries are higher than 90%, but, for the others, it is lower, especially in complete feed for pig (only 82.5%). This might be due to the higher content of protein and minerals in feeds, where the drugs are adhered strongly, so it is hard for them to be extracted completely. The recovery of NTV was relatively

Table 6. Recovery of nitrofurans from spiked feeds

Feeds	Drugs[a]	Spiked level (mg/kg)	Recovery (%, n = 7)			
			Maximum	Minimum	Mean	RSD (%)
Layer complete feed	FZD	50	101.2	89.7	93.3	5.26
	FTD	50	106.7	86.2	94.9	6.80
	NFZ	100	98.8	83.5	89.4	5.18
	NTV	15	79.2	63.4	70.4	6.37
Layer concentrated feed	FZD	100	99.2	87.1	92.5	4.99
	FTD	100	104.9	90.6	97.6	6.33
	NFZ	200	98.8	87.2	92.4	4.26
	NTV	30	65.8	54.7	59.6	5.46
Pig complete feed	FZD	100	101.4	86.8	92.1	5.96
	FTD	100	103.3	83.6	91.8	6.93
	NFZ	100	88.3	80.5	82.5	3.51
	NTV	20	67.2	55.9	62.4	7.81
Pig concentrated feed	FZD	300	101.6	87.7	92.5	4.36
	FTD	300	101.4	89.6	92.6	5.62
	NFZ	300	85.2	76.2	86.6	3.72
	NTV	60	54.6	65.5	61.9	4.27
Pig premix	FZD	1000	101.0	92.1	96.8	3.13
	FTD	1000	104.9	85.3	99.5	6.80
	NFZ	1000	101.3	85.3	95.5	5.97
	NTV	200	69.6	80.8	75.9	3.64

[a]FZD = furazolidone; FTD = furaltadone; NFZ = nitrofurazone; NTV = nitrovin.

low, which is the same as the result reported by Lin.[15] The solubility of NTV in the organic solvent is low, which is probably due to some form of binding between NTV and other components in the feeds, such as minerals and proteins; these maybe interact with ammonia solution and reduce its alkaline action, so NTV couldn't be extracted completely. If increasing the ratio of ammonia to improve the extracted efficiency of NTV, the other drugs will be destroyed and, even worse, the absorbance is lowered. The peak of NFZ will split into two or three peaks and the absorbance of FTD and FZD will decrease. Although the recovery of NTV was poor, the recoveries of the other three drugs were high. The extraction method for NTV needs further improvement.

Limit of Determination

The limits of detection of the four drugs were calculated as three times the peak-to-peak baseline noise (S/N) from the standard solutions. The limits

of detection were 0.1, 0.1, 0.2, and 0.2 mg/kg for FZD, FTD, NFZ, and NTV, respectively. The limits of quantitation in the feeds for the four drugs were calculated as S/N from drug free feed samples, spiked at different levels of drugs to determine the limit of determination. The results showed that the limits of quantitative determination by HPLC were 1.0, 1.0, 2.0, and 2.0 mg/kg for FZD, FTD, NFZ, and NTV in the feeds, respectively. The limits of identification by LC-MS were 0.1, 0.1, 0.2, and 0.2 mg/kg in feeds. In the past, the doses of the four drugs as growth promoters or therapeutic applications were all more than 10 mg/kg. Therefore, the limits described in this present study could be used to test and monitor nitrofurans in feeds efficiently.

Applications

In this study, the method of simultaneous determination of FZD, NFZ, FTD, and NTV in feeds was developed. Though feed is a complex matrix, it was found that SPE Oasis® HLB cartridges served as a simple and rapid technique to clean the feed samples for further analysis. The accuracy and linearity of the method were adequate for monitoring feed quality. This method is also could be used to identify and quantify the drugs in feeds for regulatory purposes in a rapid, simple, and economical way.

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