JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Determination of Furaltadone and Nifursol Residues in Poultry Eggs by Liquid Chromatography–Electrospray Ionization Tandem Mass Spectrometry

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ABSTRACT: The use of nitrofurans as veterinary drugs has been banned from intensive animal production in the European Union (EU) since 1993. The objective of the present study was to evaluate the accumulation and depletion of furaltadone and nifursol and their side-chain metabolites 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ) and 3,5-dinitrosalicylic acid hydrazide (DNSAH) in eggs after administration of therapeutic and subtherapeutic doses of the drugs to laying hens during three consecutive weeks. LC-MS/MS, with positive and negative electrospray ionization methods, was used for the determination of parent compounds and metabolites in yolk and egg white and was validated according to criteria established by Commission Decision 2002/657/EC. The decision limit (CCα) and the detection capability (CCβ) of the analytical methodology for metabolites were 0.1 and 0.5 μg/kg for AMOZ and 0.3 and 0.9 μg/kg for DNSAH, respectively. For the parent compounds, CCα and CCβ were 0.9 and 2.0 μg/kg for furaltadone and 1.3 and 3.1 μg/kg for nifursol, respectively. The data obtained show that the parent compounds are much less persistent than their side-chain metabolites in either yolk or egg white. Between the studied metabolites, AMOZ is the most persistent and could be detected in either yolk or egg white three weeks following withdrawal from treatment.

KEYWORDS: *furaltadone, nifursol, poultry, eggs, LC-MS/MS*

INTRODUCTION

Nitrofurans are a large group of compounds that are characterized by a nitro group at position 5 of the nitrofuran ring. Furazolidone (FZD), furaltadone (FTD), nitrofurazone (NFZ), nifursol (NFS), and nitrofurantoin (NFT) are important members of this group of compounds that were widely used in veterinary medicine for intensive animal production, especially in pigs, poultry, and fish, because of their broad antimicrobial activity in the treatment of infections caused by *Escherichia coli* and *Salmonella*. Nifursol has been extensively used as a feed additive for the prevention of histomoniasis (black head disease) in turkeys.

Due to the toxicological hazard for human consumers (carcinogenicity and mutagenicity),[1] nitrofuran antibiotics were prohibited for use in intensive animal production in the European Union (EU) after 1993 for FTD, NFZ, and NFT[2] and in 1995 for FZD.[3] The use of NFS as a feed additive was forbidden in 2002.[4] To control its illegal use and to prevent its reaching the consumer, authorities have implemented specific measures that include the monitoring of food of animal origin.

In animal tissues, nitrofuran compounds are rapidly metabolized after administration, with a rapid depletion of the parent drug, but persistent metabolites are formed as residues bond to tissue proteins that are much more stable and can remain in tissues for months.[5−7]

The EU has established a Minimum Required Performance Level (MRPL) of 1 μg/kg for FZD, FTD, NFZ, and NFT metabolites.[8] Because NFS is no longer permitted, this MRPL level should also be adopted for the detection of the metabolite of this compound.[9]

For the detection of nitrofuran tissue-bound side-chain metabolites, LC-MS/MS methods are now used throughout the world in animal tissues and other matrices. These methods, which are based on those described by Leitner et al.[10] and Conneely et al.[11] and were developed under the auspices of the multinational EU research project FoodBRAND, can detect the nitrofuran metabolites as their nitrophenyl derivatives of 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-1,3-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), and semicarbazide (SEM), marker residues of furazolidone, furaltadone, nitrofurantoin, and nitrofurazone, respectively. For the NFS metabolite 3,5-dinitrosalicylic acid hydrazide (DNSAH), the same method can be used with fewer modifications.[9]

This method can detect all five metabolites, as demonstrated by Verdon and co-workers.[12] Nitrofurans can be used therapeutically for laying hens, generally via water or feed; in the case of feed, nitrofurans may reach the hens unintentionally as a result of cross-contamination during the manufacturing of premix, feed preparation in the mill, or transport.[13]

Received: December 16, 2011
Revised: April 3, 2012
Accepted: April 9, 2012
Published: April 9, 2012

After ingestion, these compounds reach the bloodstream and are distributed throughout the body. The amount of the compounds or their metabolites in each tissue depends on their characteristics, such as lipid solubility or pK_a. In the case of eggs, the deposition of drugs in the yolk and/or the white follows the same principles.

Nitrofuran parent drugs can be detected in eggs for some days after the cessation of administration.[14] In one study, laying hens were treated with FZD at 330 mg/kg for 14 days, and the parent compound residues could be detected 9 days after the withdrawal of administration.[15] In another study, FZD was administered for 11 days at 400 mg/kg. Four days after treatment had been suspended, the parent drug could no longer be detected. Nevertheless, AOZ, the FZD metabolite, persists for a much longer period.[14]

As part of a continuing investigation involving FTD after the nitrofuran crisis in Portugal,[16−18] a study about the accumulation and depletion of this compound in poultry eggs after the administration of therapeutic and subtherapeutic doses to laying hens was performed. To the best of our knowledge, there are no published studies of NFS accumulation in poultry eggs, and this study also included the evaluation of this compound. Thus, the present study uses LC-MS/MS methodologies to monitor the accumulation and distribution of FTD and NFS and their respective side-chain metabolites, AMOZ and DNSAH (Figure 1), in the yolk and white of poultry eggs following oral dosing to laying hens for 3 weeks. The analytical methods used for the parent compounds and metabolites were validated in accordance with Commission Decision 2002/657/EC.[19]

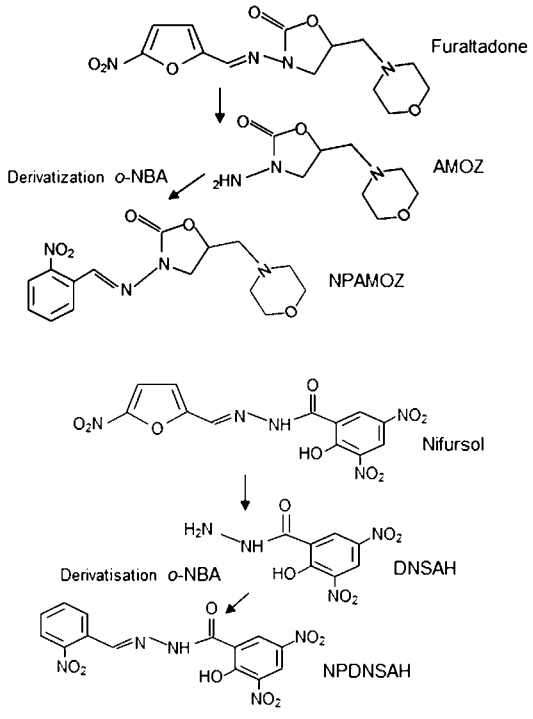

Figure 1. Structures of furaldone and nifursol, their metabolites, and corresponding nitrophenyl derivatives.

EXPERIMENTAL PROCEDURES

Reagents and Materials. All chemicals and solvents used were of analytical grade, except solvents used in mobile phases, which were of HPLC grade. Methanol, hydrochloric acid, trisodium phosphate dodecahydrate, sodium hydroxide, ammonium formate, ammonium acetate, ethyl acetate, acetonitrile, and hexane were supplied by Merck (Darmstadt, Germany). *o*-Nitrobenzaldehyde (*o*-NBA) was supplied by Sigma (Madrid, Spain). Water was demineralized using a Millipore purification system (Bedford, MA, USA).

Standards of AMOZ and 5-methylmorpholine-3-amino-2-oxazolidinone-fifth deuterated (AMOZ-d_5) were supplied by VSD of Belfast (Veterinary Sciences Division, Dard, U.K.). DNSAH was obtained from Mikromol LGC (Luckenwalde, Germany). Standards of furaltadone, nifursol, and nifuroxazide were purchased from Sigma.

A Moulinex mincer (Lisbon, Portugal), Mettler Toledo PC2000 and AE100 balances (Greifensee, Switzerland), a Memmert incubator (Buchenbach, Germany), a Heidolph Reax 2 overhead mixer (Schwabach, Germany), a Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), a Turbovap Zymark evaporator (Hopkinton, MA, USA), neutral alumina cartridges (Macherey-Nagel, Germany), and Whatman Mini-UniPrep PVDF filters (0.45 μm) (Clifton, NJ, USA) were used to perform the extraction and purification procedures.

An Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA), consisting of a binary pump, autosampler, and column oven, coupled to a Sciex API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA), both operating under Sciex Analyst software, version 1.4.2, was used for analyzing the yolk and white of poultry eggs.

An electrospray ion source in positive and negative mode was used for data acquisition in multiple reaction monitoring mode (MRM). The chromatographic separation was achieved with an Agilent Zorbax XDB C18, 3.5 μm, 150 × 2.1 mm column with an Agilent Zorbax XDB C8, 5 μm, 4 × 2.1 mm guard column.

Standard Solutions. Individual stock standard solutions of furaltadone and nifursol and respective metabolites (AMOZ and DNSAH), as well as the two internal standards (AMOZ-d_5 and nifuroxazide) were prepared in the appropriate volume of methanol to obtain solutions of 100 mg/L. Standard working solutions of the two internal standards were prepared at 100 μg/L. Standard working solutions of the nitrofurans, parent drugs, and metabolites were prepared at 100 and 10 μg/L. The stock and working standard solutions were stable for up to 6 months and 2 month, respectively, when stored in the dark at 4 °C.[16−18]

Furaltadone and Nifursol Administration to Laying Hens. *Medicated Feeds.* The four analyzed treatments consisted of three diets supplemented with furaltadone and nifursol and one unsupplemented basal diet. The basal diet was a corn- and soy-based diet, formulated according to the National Research Council specifications for laying hens.[20] In the control diet (diet C), nitrofurans were not incorporated. In the other three diets, compounds were incorporated at therapeutic concentrations of 150 mg/kg furaltadone (diet F) and 100 mg/kg nifursol (diet N) and at subtherapeutic concentrations of 15 mg/kg furaltadone plus 10 mg/kg nifursol (diet SubNF). The nitrofurans were mixed with the other ingredients at the final stage of diet preparation. The diets were in mash form. The laying hens had free access to feed and water.

Production of Incurred Eggs. A total of 20 19-week-old laying hens were individually housed in wire-floored cages. Each cage was provided with an individual feeder and two automatic pipet drinkers. The cages were located in a temperature-controlled room, and the photoperiod during the experiment was fixed at 16 h.

The laying hens were randomly assigned to one of the four experimental treatments (5 birds/treatment) for eight weeks between 19 and 27 weeks of age. From week 1 to week 5, the birds received the experimental diets and at least five eggs were collected per treatment on the last two days of weeks 3, 4, and 5. Thereafter, all of the birds received only the control diet (C), and at least five eggs were collected per treatment on the last two days of weeks 6, 7, and 8. After collection, the eggs were separated into the yolk and white, frozen at −20 °C, and transported to the laboratory within 4 days. Furaltadone and nifursol parent compounds were measured as soon as the samples arrived at the laboratory. For metabolites, the remaining samples were frozen at −20 °C and analyzed afterward. As nitrofurans

are particularly sensitive to light, measures were taken to prevent photodegration of the compounds in all phases of sample manipulation, transport, extraction, and injection procedures.

The experiments were carried out in accordance with the current ethical guidelines for the care of laboratory animals for investigation set by the Portuguese General Directorate of Veterinary.

Preparation of Eggs for the Determination of Furaltadone and Nifursol Parent Compounds. The extraction procedure followed that of McCracken et al.,[14] with no major modifications. A 5 g portion of egg yolk or white was fortified with nifuroxazide as internal standard (100 μL of standard working solution at 100 μg/L), and the sample was left to equilibrate for approximately 15 min. The extraction was performed with ethyl acetate (12 mL). The organic layer was transferred to clean tubes and evaporated to dryness under nitrogen. Acetonitrile (5 mL) was added to the dry residue, and hexane (3 mL) was added under vortexing. After centrifugation, the hexane layer was discarded, and the acetonitrile was evaporated under nitrogen at 45 °C. The dry residue was dissolved in 500 μL of methanol/water (1:1, v/v), vortexed briefly, transferred to Eppendorf vials, and centrifuged at 3500*g* for 5 min. Aliquots of the clear layer were transferred to LC vials prior to LC-MS/MS analysis. Highly concentrated samples were diluted as required for analysis.

Preparation of Eggs for the Determination of Furaltadone and Nifursol Metabolites. A 1 g portion of egg yolk or white was weighed into a 15 mL screw-top glass centrifuge tube and fortified with AMOZ-d_5 as internal standard (100 μL of standard working solution at 100 μg/L), and the sample was left to equilibrate for approximately 15 min. Then 5 mL of 0.2 M hydrochloric acid and 50 μL of 100 mM *o*-NBA in methanol were added, and the tube was closed and shaken manually. The tube was then placed (conditioned) in a Reax 2 agitator in an incubator for overnight hydrolysis at 37 °C. After cooling, 500 μL of 0.3 M trisodium phosphate dodecahydrate solution was added to the sample, and the pH was adjusted to 7 with 2 M sodium hydroxide. Then 4 mL of ethyl acetate was added to the sample, and the tube was shaken in a Reax 2 agitator for 20 min. The sample was centrifuged at 1200*g* for 10 min, and the organic layer was transferred to a 20 mL centrifuge tube. The previous step was repeated, and the two organic layers were mixed and evaporated to dryness at 45 °C under a nitrogen stream. The dry residue was dissolved in 500 μL of 10 mM ammonium formate (pH 4)/methanol (85:15, v/v), vortexed for 20 s, and transferred to a LC amber autosampler vial through a 0.45 μm PVDF filter. Highly concentrated samples were diluted as required for analysis.

Liquid Chromatography–Tandem Mass Spectrometry. For the determination of the FTD and NFS parent compounds, the samples were injected into the LC-MS/MS system with a flow rate of 350 μL/min. The mobile phase was composed of two solutions: [A] 10 mM ammonium formate (pH 4)/methanol (9:1, v/v) and [B] 10 mM ammonium formate (pH 4)/methanol (1:9, v/v). The following mobile phase gradient program was used: 0–1 min, 10% B; 1–16 min, from 10 to 45% B; 16–18 min, from 45 to 90% B; 18–20 min, from 90 to 10% B. The column oven was maintained at 40 °C, and the injection volume was 20 μL. The mass spectrometer was operated in positive electrospray ionization mode (ESI^+) for FTD and in negative mode (ESI^-) for nifursol. Nifuroxazide can be detected in both the negative and positive modes. Nitrogen was used for the curtain, collision, heater, and nebulizer gases at flow rates of 20, 3, 25, and 75 L/min, respectively. The ion source block temperature was 450 °C, and the electrospray capillary voltage was 4.5 kV. Because Commission Decision 2002/657/EC[19] requires four identification points for the identification of banned substances, the fulfillment criteria were obtained with one precursor ion and two product ions. Two transition reactions were monitored for each compound. For each internal standard, only one transition was controlled. These transitions are shown in Table 1. For the determination of AMOZ, DNSAH, and the respective internal standard AMOZ-d_5 as their nitrophenyl derivatives (NPAMOZ, NPDNSAH, and NPAMOZ-d_5), the same instrumental conditions that were described above for the parent compounds were used, except that only the positive ionization mode (ESI^+) was used. The controlled MRM transitions are shown in Table 1. A calibration curve with seven concentration levels was prepared by spiking blank egg yolk or white samples with standard working solutions. For the determination of nitrofuran metabolites (AMOZ and DNSAH), concentration points of 0 (blank sample), 0.5, 1, 2, 5, 10, and 20 μg/kg were used.

Table 1. Ion Transitions Used for Nitrofuran Parent Compounds and Metabolites Detection

		MRM diagnostic ions	
compound	ESI polarity	precursor ion (*m*/*z*)	product ions (*m*/*z*)
DNSAH[a]	pos	376	166; 211
AMOZ[a]	pos	335	291; 262
AMOZ-d_5[a]	pos	340	296
furaltadone	pos	325	280; 253
nifursol	neg	364	182; 226
nifuroxazide	pos	274	93
	neg	276	121

[a]As its nitrophenyl derivative.

For the determination of the FTD and NFS parent compounds, concentration points of 0 (blank sample), 2, 5, 10, 15, 20, and 25 μg/kg were utilized. These fortified calibration samples were prepared using the procedures described above for the extraction. To calculate the analyte concentrations, the ratio of the areas of the analyte and the internal standard in the sample were compared with the same ratio in the calibration curve.

Method Validation. For the method validation, the specificity, linearity, precision (repeatability and within-laboratory reproducibility), recovery, decision limit (CCα), and detection capability (CCβ) were determined according to Commission Decision 2002/657/EC.[19] The specificity was demonstrated by analyzing 20 blank whole egg homogenate samples before and after spiking with 5 μg/kg FTD or NFS for the parent compound method or 1 μg/kg NPAMOZ or NPDNSAH for the metabolite method to observe the eventual presence of interfering peaks of appropriate size at the retention time windows of the analytes for MRM monitoring of transitions of interest for each analyte.

For the linearity, accuracy, precision, and analytical limits, calibration curves were prepared with blank whole homogenized egg samples spiked in the concentration range of 0, 0.5, 1, 2, 5, 10, and 20 μg/kg for the metabolite method and in the range of 0, 2, 5, 10, 15, 20, and 25 μg/kg for the parent compound method. For each calibration level, six whole homogenized egg samples were prepared and analyzed every day for 3 days. For the determination of the metabolites AMOZ and DNSAH, concentrations of 0.5, 1, and 2 μg/kg were used. For the FTD and NFS parent compounds method, concentrations of 2, 5, and 10 μg/kg were utilized. Standard calibration curves (calibration curve without matrix) were also prepared in the same range as the fortified curves to be analyzed each day. The accuracy in terms of recovery for both methods was evaluated by comparing the data from the fortified curves with the corresponding standard calibration curves.

RESULTS AND DISCUSSION

Figures 2 and 3 show the typical chromatograms obtained after the application of the above-described analytical methods on eggs fortified with FTD and NFS and their metabolites.

The results obtained from the validation of the method for the parent compounds in eggs are summarized in Tables 2 and 3.

When specificity in the time window for controlled MRM transitions was evaluated, there was no observation of any important peaks capable of interfering with the detection and identification of analytes after comparison of the blank samples and the same samples fortified with FTD and NFS at the level of interest. The linearity of the chromatographic response for FTD and NFS was evaluated with the calibration curves

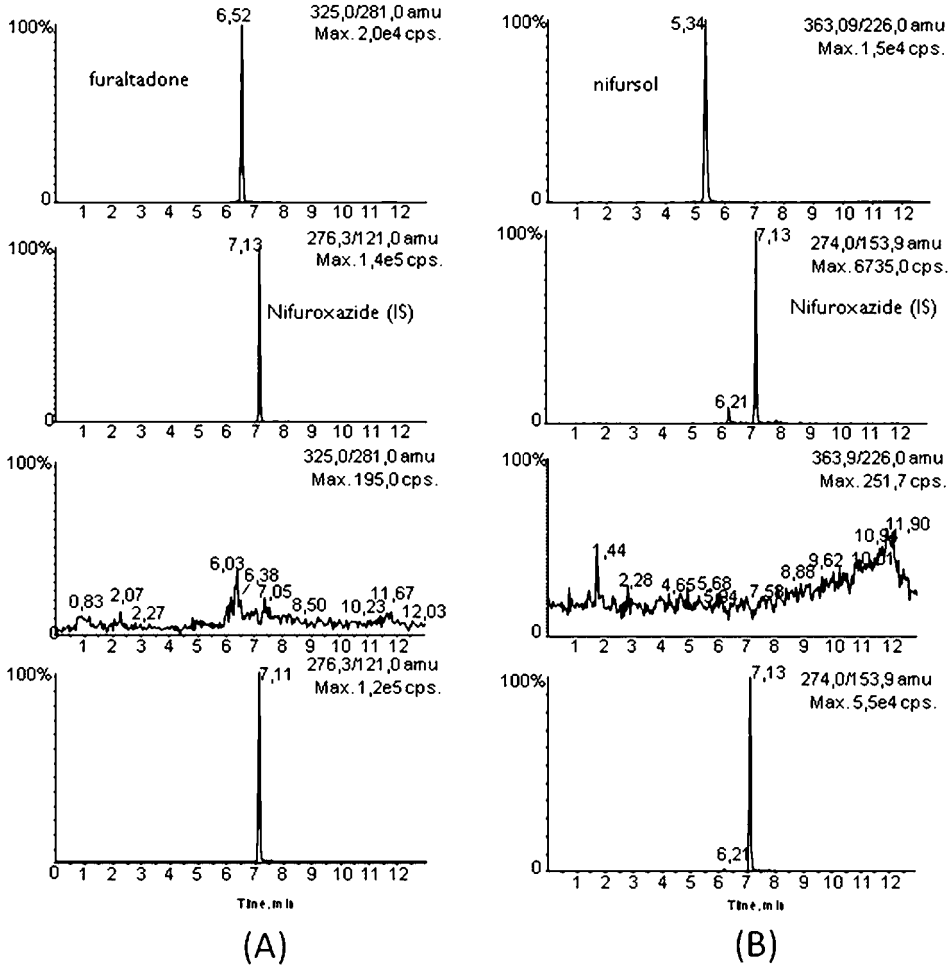

Figure 2. LC-(ESI$^+$)MS/MS chromatograms of incurred egg white with furaltadone compared with a blank sample (A), and LC-(ESI$^-$)MS/MS chromatograms of incurred egg yolk with nifursol also compared with a corresponding blank sample (B).

fortified with these compounds, and the determination coefficients (R^2) were ≥0.993. The precision in terms of repeatability and intralaboratory reproducibility was evaluated by the variation within a single day and between days by calculating the coefficient of variation (CV%) of the mean concentration results obtained from each analyte at the 2, 5, and 10 μg/kg concentration levels during the three days.

The values obtained are shown in Table 3 for the 2, 5, and 10 μg/kg level. According to Commission Decision 2002/657/EC,[19] the coefficient of variation for repeated analyses of spiked or incurred material should not exceed the level obtained after application of the Horwitz equation, but this equation for mass fractions lower than 100 μg/kg gave unacceptably high values. As low a value as possible should be used as a guideline. It was accepted that values lower than 23% are appropriate for this purpose. As can be seen in Table 3, all of the values for the repeatability and reproducibility are lower than 23%, except for the 2 and 5 μg/kg levels of NFS reproducibility. Nevertheless, the values obtained are significantly lower than those calculated with the direct application of the Horwitz equation, respectively 40.77 and 35.52% for 2 and 5 μg/kg levels.

The CCα and CCβ values obtained (Table 2) were appropriate to evaluate the analyte concentrations present in the samples.

For the method used to analyze the metabolites AMOZ and DNSAH in eggs, the validation data obtained are summarized in Tables 2 and 3. The decision limit, CCα, and the detection capability, CCβ, are below the EU MRPL of 1 μg/kg and are thus adequate for confirmatory analysis in eggs according to EU regulations. In terms of repeatability and intralaboratory reproducibility, the CV% was much better than the 23% considered to be the minimum adequate level. The accuracy in terms of the recovery of the method was measured at three concentration levels of the fortified calibration curve. The obtained values were within the criteria specified in Commission Decision 2002/657/EC.[19] In the evaluation of specificity, no interfering peaks of eligible size were observed, at the expected retention time windows, in the two MRM monitoring transitions of the nitrofuran side-chain metabolites tested.

The linearity for AMOZ and DNSAH was determined using the calibration curves from the validation process. The determination coefficients (R^2) were >0.996.

Previous studies of the accumulation of nitrofurans in eggs have highlighted the possibility of detecting the parent compounds to identify illegal use by producers, in contrast to other edible tissues in which a rapid depletion of the parent compounds occurs. The nitrofuran parent compounds are only sporadically detectable a few hours after the cessation of administration.[21] After administering 330 mg/kg of the compound for 14 days, Botsoglou[15] reported the detection of FZD in eggs 9 days after the withdrawal period. McCracken et al.[22] also

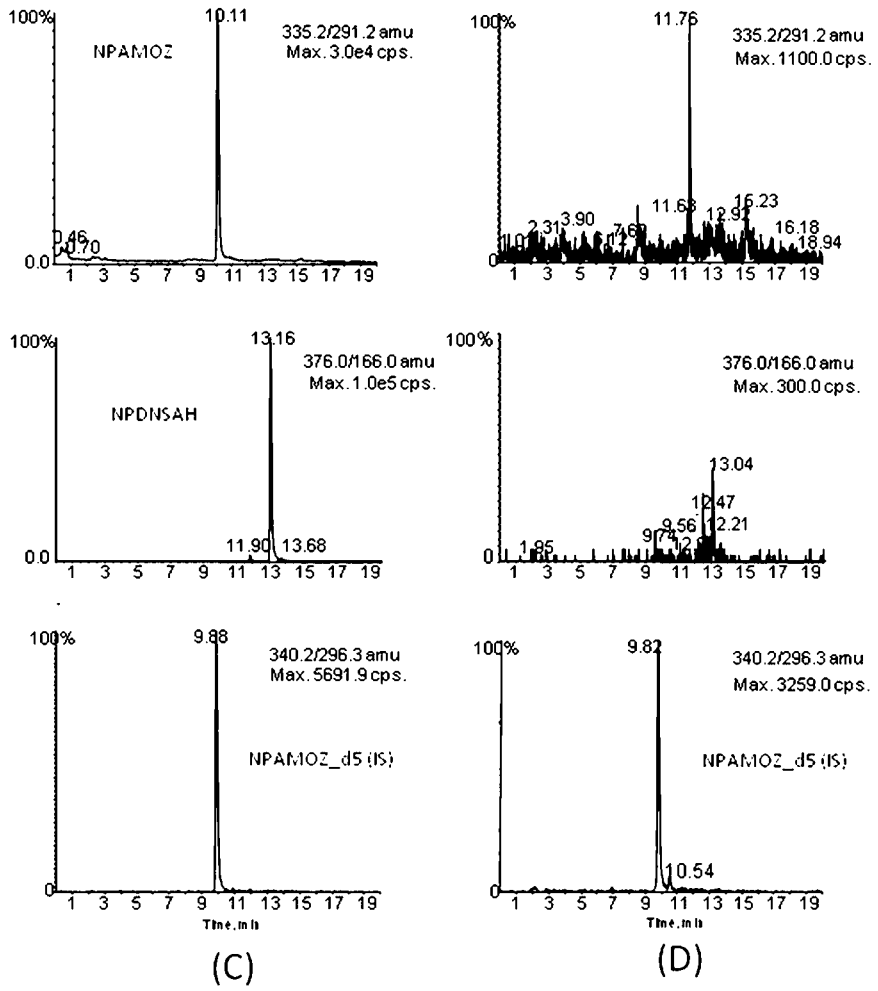

Figure 3. MRM chromatograms obtained after LC-ESI$^+$MS/MS for incurred egg white with AMOZ and DNSAH, biological markers of furaltadone and nifursol, respectively (C), and a blank sample (D).

Table 2. Decision Limit (CCα) and Detection Capability (CCβ) of the Analytes

analyte	CCα (μg/kg)	CCβ (μg/kg)
FTD	0.9	2.0
NFS	1.3	3.1
AMOZ[a]	0.1	0.5
DNSAH[a]	0.3	0.9

[a]As its nitrophenyl derivative.

Table 3. Coefficient of Variation (CV) of Repeatability, Within-Laboratory Reproducibility, and Recovery of FTD, NFS, and Their Side-Chain Metabolites

concentration level (μg/kg)	repeatability (CV%)	reproducibility (CV%)	recovery (%)
FTD			
2	14.5	18.4	73.1
5	9.0	11.5	76.6
10	5.8	8.3	79.2
NFS			
2	20.9	23.1	82.4
5	17.1	25.3	88.9
10	15.4	20.6	90.3
AMOZ			
0.5	11.4	11.7	93.7
1	9.8	9.6	94.3
2	8.2	6.8	95.1
DNSAH			
0.5	10.5	13.1	96.0
1	7.2	10.4	97.0
2	7.6	9.8	97.7

detected the presence of FZD in eggs 4 days after treatment with 400 mg/kg of the compound for 11 days.

Cooper et al.[21] detected parent compound residues in eggs for up to 6 days following withdrawal after administering 300 mg/kg of NFZ to laying hens for 16 days. Importantly, NFZ was detected in eggs 3 days after the cessation of treatment with subtherapeutic amounts (3 mg/kg) of the compound.

Nevertheless, these cited studies also demonstrated that the side-chain metabolites of the measured nitrofurans can be detected for a much longer time period than the parent compounds and that, for that reason, they are better markers for monitoring the abuse of these drugs in intensive animal production.

In the present accumulation and depletion study, no residues of FTD and NFS were detected in chickens that were fed the control diet. Table 4 shows the data concerning the accumulation and depletion of the side-chain metabolites AMOZ and DNSAH in the egg yolks and whites produced by laying

dx.doi.org/10.1021/jf205186y | J. Agric. Food Chem. 2012, 60, 4227–4234

Table 4. Mean Levels of Nitrofuran Side-Chain Metabolites AMOZ and DNSAH on Egg Yolk and White after Different Levels of FTD and NFS Administration

		AMOZ			DNSAH		
		mean value $n = 5$ (μg/kg)			mean value $n = 5$ (μg/kg)		
	weeks	yolk	white	ratio white/yolk	yolk	white	ratio white/yolk
AMOZ (FTD 150 mg/kg)	3	591	492	0.83			
	4	712	613	0.86			
	5	584	376	0.64			
	6	29	31	1.07			
	7	5.2	3.2	0.62			
	8	3.1	2.9	0.94			
DNSAH (NFS 100 mg/kg)	3				1602	178	0.11
	4				1530	223	0.15
	5				1654	259	0.16
	6				65	12	0.18
	7				2.1	ND	-
	8				ND	ND	-
AMOZ + DNSAH (FTD 15 mg/kg + NFS10 mg/kg)	3	76	65	0.86	95	18	0.19
	4	89	68	0.76	86	25	0.29
	5	73	60	0.82	98	24	0.24
	6	3.1	3.7	1.19	1.2	ND	
	7	1.1	ND		0.5	ND	
	8	0.2	ND		ND	ND	

Table 5. Mean Levels of Nitrofuran Parent Compounds in Yolk and White Egg after Different Levels of FTD and NFS Administration

		FTD			NFS		
		mean value $n = 5$ (μg/kg)			mean value $n = 5$ (μg/kg)		
	weeks	yolk	white	ratio white/yolk	yolk	white	ratio white/yolk
FTD (150 mg/kg)	3	372	245	0.7			
	4	445	253	0.6			
	5	334	228	0.7			
	6	ND	ND				
NFS (100 mg/kg)	3				213	73	0.3
	4				290	85	0.3
	5				312	92	0.3
	6				ND	ND	
FTD + NFS (15 + 10 mg/kg)	3	53	30	0.6	26	3,4	0.1
	4	64	45	0.7	43	6,9	0.2
	5	55	37	0.7	32	6,1	0.2
	6	ND	ND		ND	ND	

hens after the administration of 150 mg/kg FTD or 100 mg/kg NFS or the combined administration of 15 mg/kg FTD and 10 mg/kg NFS for 3 weeks. After 3 weeks, the treatment was withdrawn.

The data obtained for the parent compounds FTD and NFS are shown in Table 5. The parent compounds could be detected only in the accumulation phase and not in the depletion phase.

In previous papers,[15,21,22] the concentration of the nitrofuran parent compounds was shown to fall below the detection limit of the method at a maximum of 9 days after the withdrawal of treatment. As in this study, the eggs were collected only at the end of every week of the experiment, and thus the time period between the two sequential egg collections can explain this observation.

The egg white/yolk ratios obtained for FTD (Table 5) are in agreement with those described in the literature. Petz[23] found for this compound a value for the egg white/yolk ratio of approximately 0.5. For FZD, which has kinetics similar to FTD, Botsoglou et al.[15] determined a value of 0.7, whereas Furusawa[24] determined a value 0.6. For NFS, the ratios obtained from eggs from birds treated with 100 and 10 mg/kg are quite different. Much higher concentrations of the parent compound were found in the yolk compared to the white, yielding a ratio of approximately 0.3 when the laying hens were treated with 100 mg/kg NFS and approximately 0.05 when the administration was only 10 mg/kg.

As the deposition of drugs in the yolk, white, or both phases depends on the drug's characteristics, such as the rate of

dx.doi.org/10.1021/jf205186y | J. Agric. Food Chem. 2012, 60, 4227−4234

metabolism, lipid solubility, molecular weight, and pK_a of the compound and the binding to plasma proteins, the specific physicochemical characteristics of NFS relative to other compounds of this group may be responsible for the differences observed.

For the metabolites, the value obtained for the AMOZ white egg/yolk ratio was approximately 0.9 for both treatments (chickens treated with 150 or 15 mg/kg FTD) (Table 4). This is similar to the value of 1.06 reported by McCracken et al.[14] for the ratio after the administration of FTD at a feed concentration of 300 mg/kg for 1 week.

In the present study, a much higher concentration of DNSAH was found in the yolk than in the white, yielding a ratio of approximately 0.4. In this case, the egg yolk/white ratios from laying hens treated with 150 or 15 mg/kg NFS were identical. Zuidema et al.[25] reported that the disposition and depletion of the metabolites of NFS and FTD in muscle, kidney, liver, bile, and plasma were similar. This similarity was not observed in the eggs in this study.

In conclusion, laying hens treated with medicated feed containing FTD and NFS at therapeutic and subtherapeutic doses produced eggs with considerable amounts of the parent compounds and their side-chain metabolites, which could be detected by the LC-MS/MS analytical methods employed. The metabolites were more stable than the parent compounds and could be detected for 2−3 weeks after the cessation of treatment. In eggs, these observations are in agreement with the results of previous studies and indicate that the side-chain metabolite residues are better biological markers than the parent compounds for effectively monitoring illegal nitrofuran use.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Van Koten-Vermeulen, J. E. M.; Wouters, M. F. A.; van Leeuwen, F. X. R. *Report of the 40th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)*; WHOTRS 832; World Health Organization: Genève, Switzerland, 1993; pp 32−40.

(2) European Commission. Council Regulation No. 2901/93/EC of 18 October 1993, amending annexes to regulation (EEC) No 2377/90 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Union* **1993**, *L264*, 1−4.

(3) European Commission. Council Regulation No. 1442/95/EC of 27 Jun 1995, amending annexes to regulation (EEC) No 2377/90 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Communities* **1995**, *L143*, 26−31.

(4) European Commission. Council Regulation No. 1756/2002/EC of 23 September 2002, amending Directive 70/524/EEC concerning additives in feedingstuffs as regards withdrawal of the authorisation of an additive and amending Commission Regulation (EC) No 2430/1999, *Off. J. Eur. Union* **2002**, *L265*, 1−12.

(5) McCracken, R. J.; Blanchflower, W. J.; Rowan, C.; McCoy, M. A.; Kennedy, D. G. Determination of furazolidone in porcine tissue using thermospray liquid chromatography-mass spectrometry and a study of the pharmacokinetics and stability of its residues. *Analyst* **1995**, *120*, 2347−2351.

(6) Hoogenboom, L. A. P.; Berghmans, M. C. J.; Parker, R.; Shaw, I. C. Depletion of protein-bound furazolidone metabolites containing the 3-amino-2-oxazolidinone side-chain from liver, kidney and muscle tissues from pigs. *Food Addit. Contam.* **1992**, *9*, 623−630.

(7) Cooper, K. M.; Mulder, P. P.; Van Rhijn, J. A.; Kovacsics, L.; McCracken, R. J.; Young, P. B.; Kennedy, D. G. Depletion of four nitrofuran antibiotics and their tissue-bound metabolites in porcine tissues and determination using LC-MS/MS and HPLCUV. *Food Addit. Contam.* **2005**, *22*, 406−414.

(8) Commission Decision No. 2003/181/EC, of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Off. J. Eur. Communities* **2003**, *L71*, 17−18.

(9) Mulder, P. P. J.; Zuidema, T.; Keestra, N. G. M.; Kooij, P. J. F.; Elbers, I. J. W.; Van Rhijn, J. A. Determination of nifursol metabolites in poultry muscle and liver tissue. Development and validation of a confirmatory method. *Analyst* **2005**, *130*, 763−771.

(10) Leitner, A.; Zollner, P.; Lindner, W. Determination of the metabolites of nitrofuran antibiotics in animal tissue by high performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2001**, *939*, 49−58.

(11) Conneely, A.; Nugent, A.; O'Keeffe, M.; Mulder, P. P. J.; Van Rhijn, J. A.; Kovacsics, L.; Fodor, A.; McCracken, R. J.; Kennedy, D. G. Isolation of bound residues of nitrofuran drugs from tissue by solid-phase extraction with determination by liquid chromatography with UV and tandem mass spectrometric detection. *Anal. Chim. Acta* **2003**, *483*, 91−98.

(12) Verdon, E.; Couedor, P.; Sanders, P. Multi-residue monitoring for the simultaneous determination of five nitrofurans (furazolidone, furaltadone, nitrofurazone, nitrofurantoine, nifursol) in poultry muscle tissue through the detection of their five major metabolites (AOZ, AMOZ, SEM, AHD, DNSAH) by liquid chromatography coupled to electrospray tandem mass spectrometry—In-house validation in line with Commission Decision 657/2002/EC. *Anal. Chim. Acta* **2007**, *586*, 336−347.

(13) McCracken, R. J.; Van Rhijn, J. A.; Kennedy, D. G. The occurrence of nitrofuran metabolites in the tissues of chickens exposed to very low dietary concentrations of the nitrofurans. *Food Addit. Contam.* **2005**, *22*, 567−572.

(14) McCracken, R. J.; Kennedy, D. G. Detection, accumulation and distribution of nitrofuran residues in egg yolk, albumen and shell. *Food Addit. Contam.* **2007**, *24*, 26−33.

(15) Botsoglu, N. A. Determination of furazolidone in eggs by high performance liquid chromatography. *J. Agric. Food Chem.* **1988**, *36*, 1224−1227.

(16) Barbosa, J.; Ferreira, M. L.; Ramos, F.; Silveira, M. I. N. Determination of the furaltadone metabolite 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ) using liquid chromatography coupled to electrospray tandem mass spectrometry during the nitrofuran crisis in Portugal. *Accred. Qual. Assur.* **2007**, *12*, 543−551.

(17) Barbosa, J.; Moura, S.; Barbosa, R.; Ramos, F.; Noronha da Silveira, M. I. Determination of nitrofurans in animal feeds by liquid chromatography-UV photodiode array detection and liquid chromatography-ionspray tandem mass spectrometry. *Anal. Chim. Acta* **2007**, *586*, 359−365.

(18) Barbosa, J.; Freitas, A.; Moura, S.; Mourão, J. L.; Noronha da Silveira, M. I.; Ramos, F. Detection, accumulation, distribution and depletion of furaltadone and nifursol residues in poultry muscle, liver and gizzard. *J. Agric. Food Chem.* **2011**, *59*, 11927−11934.

(19) European Commission. Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Union* **2002**, *L221*, 8−36.

(20) National Research Council. *Nutrient Requirements of Poultry*, 9th rev. ed.; National Academy Press: Washington, DC, 1994.

(21) Cooper, K. M.; Kane, J.; Kennedy, D. G. Kinetics of semicarbazide and nitrofurazone in chicken eggs and egg powders. *Food Addit. Contam.* **2008**, *25*, 684−692.

(22) McCracken, R. J.; Spence, D. E.; Floyd, S. D.; Kennedy, D. G. Evaluation of the residues of furazolidone and its metabolite, 3-amino-2-oxazolidinone (AOZ), in eggs. *Food Addit. Contam.* **2001**, *18*, 954−959.

(23) Petz, M. Distribution of sulfaquinoxaline and three nitrofurans between yolk and egg white during medication and depletion. In *Residues of Veterinary Drugs in Food − Proceedings of EuroResidue II Conference*, Veldhoven; Haagsma, N., Ruiter, A., Czedik-Eysenberg, P. B., Eds.; NVVL (Netherlands Society for Nutrition and Food Technology): Veldhoven, The Netherlands, 1993; pp 528−532.
(24) Furusawa, N. Transference of dietary veterinary drugs into eggs. *Vet. Res. Commun.* **2001**, *25*, 651−662.
(25) Zuidema, T.; Mulder, P. P. J.; Van Rhijn, J. A.; Keestra, N. G. M.; Hoogenboom, L. A. P.; Schat, B.; Kennedy, D. G. Metabolism and depletion of nifursol in broilers. *Anal. Chim. Acta* **2005**, *529*, 339−346.