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Detection, Accumulation, Distribution, and Depletion of Furaltadone and Nifursol Residues in Poultry Muscle, Liver, and Gizzard

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ABSTRACT: Nitrofurans were broadly used as an extremely effective veterinary antibiotic especially in pig and poultry production farms. Because of fears of the carcinogenic effects on humans, the nitrofurans were banned from use in livestock production in many countries, including the European Union. The present study examines the accumulation, distribution, and depletion of furaltadone and nifursol and of their tissue-bound metabolites [3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) and 3,5-dinitrosalicylic acid hydrazine (DNSAH), respectively, in poultry edible tissues (muscle, liver, and gizzards) following administration to chickens of therapeutic and subtherapeutic concentrations of both compounds. Nitrofurans determination was performed by high-performance liquid chromatography−diode array detection and liquid chromatography−tandem mass spectrometry, respectively, for feeds and for poultry tissues. Furaltadone and nifursol, in very low concentrations, were found in samples of muscle, liver, and chicken's gizzard collected from slaughtered animals after 5 weeks of treatment and no withdrawal time period. When a withdrawal time period of 3 weeks was respected, no detectable nitrofuran parent compounds was observed in all of the studied matrices. For AMOZ, concentrations of 270 μg/kg in meat, 80 μg/kg in liver, and 331 μg/kg in gizzard were determined after administration of a medicated feed with furaltadone (132 mg/kg), 3 weeks after withdrawal of treatment. For DNSAH, the concentration values obtained are much lower than those observed for AMOZ. For meat, liver, and gizzard, DNSAH concentrations of 2.5, 6.4, and 10.3 μg/kg, respectively, were determined, after administration of a medicated feed with nifursol (98 mg/kg), 3 weeks after withdrawal of treatment. The gizzard could be considered a selected matrix for nitrofuran residues evaluation in poultry, due to its capacity of retaining either nitrofuran parent compounds or metabolites in higher concentrations, regardless of the administered dose or of the respected withdrawal time period.

KEYWORDS: furaltadone, nifursol, poultry, meat, liver, gizzard

INTRODUCTION

Food safety has become increasingly significant in the Western world, mostly due to the ever improving quality of life and the awareness of citizens for the right to obtain safe products for their health. One of the problems in human nutrition is the presence of drug residues in foods of animal origin. Intensive animal production has led to a significant increase in the use of antimicrobial agents for therapeutic, prophylactic, and growth promotion purposes in the veterinary field.[1,2] One area of concern has been that residues of veterinary drugs present in food may be bioavailable and may be absorbed by humans consuming meat containing incurred residues.[3]

Nitrofurans belong to a class of synthetic broad spectrum antibiotics, which all contain a characteristic 5-nitrofuran ring. They were commonly employed as feed additives for growth promotion and mainly used for livestock (i.e., poultry, swine, and cattle), aquaculture (i.e., fish and shrimp), and bee colonies in the prophylactic and therapeutic treatment of bacterial and protozoan infections.[4]

Because of fears of the carcinogenic effects on humans, in 1993, the nitrofurans furaltadone, nitrofurantoin, and nitrofurazone were banned from use in livestock production in the European Union by their inclusion in Annex IV of Council Regulation 2377/90/EEC.[5] Furazolidone was included in Annex IV in 1995. Nifursol has been prohibited as a feed additive by Council Regulation 2002/1756/EC.[6]

Recently, Commission Regulation no. 37/2010 of 22 December 2009[7] simplifies the existing information on the therapeutical classification of pharmacologically active substances, contained in the Annexes of Regulation (EEC) no. 2377/90[5] (all substances were now listed in one Annex by alphabetical order). For reasons of transparency, two separate tables were established: one for allowed substances listed in Annexes I, II, and III of former Regulation (EEC) no. 2377/90 and the other for prohibited substances listed on Annex IV.[5] Provisional maximum residue limits laid down in Annex III of Regulation (EEC) no. 2377/90,[5] for which the period of application has ended, were not incorporated into the new Regulation.[7]

The EU Member States are required to set up monitoring plans and sampling procedures for given substances in live animals and their respective food products. The illegal use of

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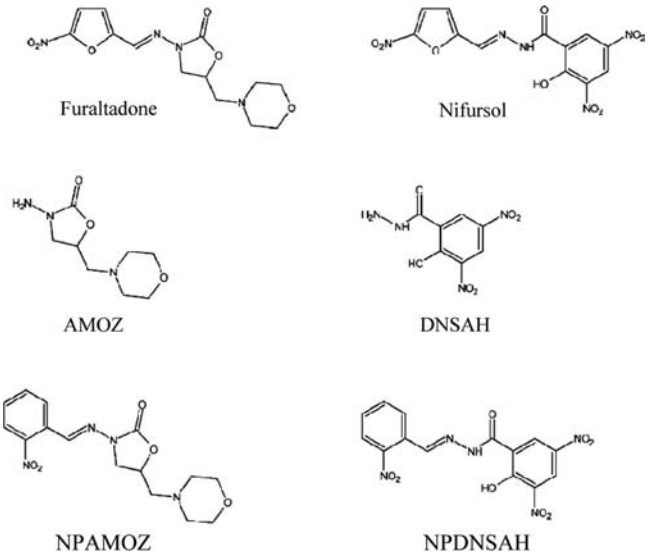

Figure 1. Chemical structures of furaltadone, nifursol, respective main metabolites, and *o*-NBA derivatives.

nitrofurans is controlled by official inspection and analytical services provided by laboratories following the recommendations specified by Council Directive 96/23/EC.[8]

Residues control was based on the detection of nitrofuran parent compounds concentration in tissues. However, studies concerning their metabolism revealed that they metabolize rapidly after administration with a short in vivo half-live (7−63 min).[9] After the FAO/WHO Bangkok Workshop on residues of banned substances held in 2004, testing for bound metabolites of nitrofurans was the standard approach in most countries.[10−12]

One of the ways to control the illegal use of nitrofuran antibiotics is by measurement of residue levels in tissues. The development of highly sensitive and specific analytical methods for the determination of nitrofuran metabolite residues has become increasingly challenging. A key role in the development of sensitive methods for nitrofuran metabolites and monitoring strategies was performed by the multinational EU research project "FoodBRAND".[4]

Metabolites of furazolidone and furaltadone were found by the FoodBRAND project consortium in pork meat purchased in Portugal, Greece, and Italy. Furthermore, the European Commission issued notifications to Member States via its Rapid Alert System for Food and Feed (RASFF) concerning findings of nitrofurans in fish from Taiwan, crayfish and salted hog casings from China, prawns from Bangladesh, India, and Indonesia, catfish from Thailand, egg powders from India, Brazil, Israel, France, and Mexico, honey from Vietnam, Argentina, Turkey, and various European countries, and poultry meat products from Argentina, Romania, and Bulgaria.[13]

In light of these findings, EU Member States are required to monitor compliance with the ban on nitrofurans through their annual national residues control plans. The introduction of the multiresidue detection of nitrofuran tissue-bound metabolites by LC-MS/MS for nitrofuran control in Portuguese Residues Monitoring Plan revealed the presence of 5-morpholinomethyl-3-amino-2-oxozolidinone (AMOZ), the bound residue of furaltadone, in a large number of samples, namely, in meat poultry samples.[14]

Furaltadone (Figure 1) belongs to the group of nitrofuran antibacterial agents, which have been widely used for the treatment of gastrointestinal infections (bacterial enteritis caused by *Escherichia coli* and *Salmonella*).[15] Furaltadone is rapidly metabolized in vivo creating a toxic metabolite, AMOZ (Figure 1), that has the property to be highly bound to proteins and thus stable for long periods of time (several weeks or even months).[11,16]

Nifursol (Figure 1) is used extensively as a feed additive for the prevention of histomoniasis.[17] As nifursol was rapidly metabolized to form the metabolic marker 3,5-dinitrosalicyclic acid hydrazide (DNSAH) (Figure 1), which can persist for a long time in vivo, detection of illegal use in animal production has focused on DNSAH detection methods[11,18,19] as well as for AMOZ.[11] These metabolites can be released from proteins under mildly acidic conditions and derivatized with *ortho*-nitrobenzaldehyde (*o*-NBA) to obtain the compounds 5-(morpholinomethyl)-3-(2-nitrobenzylidenamino)-2-oxazolidinone) (NPAMOZ) (Figure 1) and 2-hydroxy-3,5-dinitro-*N*′-(2-nitrobenzylidene)-benzohydrazide (NPDNSAH) (Figure 1) that can be used as markers of the illegal use of the referred nitrofuran compounds in food production animals.[20,21]

The improvement observed in the nitrofuran residues control has been pursuing, and the researchers have suggested the eye (retina) of the animals as a matrix of control,[22−24] according with what has already occurred with β-agonists.[25,26] Likewise, the fact that the nitrofuran concentrations in gizzard are absolutely unknown, as well as the use of gizzard in Portuguese diet (Portugal needs to import gizzards for domestic consumption because its production is not sufficient for the market demands), led to a study of the determination of nitrofurans in the referred matrix.

Thus, the present study, using a liquid chromatography−tandem mass spectrometry (LC-MS/MS) methodology, does the monitorization of the accumulation and distribution of furaltadone and nifursol and of their tissue-bound metabolites, respectively, AMOZ and DNSAH, on edible tissues (muscle, liver, and gizzard) of poultry following oral dosing. The procedures for parent compounds and metabolites were validated in accordance with Commission Decision 2002/657/EC.[27]

MATERIALS AND METHODS

Reagents and Materials. All chemicals and solvents used were of analytical grade except solvents used in the mobile phase that were high-performance liquid chromatography (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*-NBA was supplied by Sigma (Madrid, Spain). Water was demineralized using a Millipore purification system (Bedford, MA).

Standards of AMOZ and 5-methylmorpholino-3-amino-2-oxazolidinone-fifth deuterated (AMOZ-d5) were supplied by VSD of Belfast (Agri-Food and Biosciences Institute, United Kingdom). The 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), neutral alumina cartridges (Macherey-Nagel, Germany), and Whatman PVDF filters

(0.45 μm) Mini-Uniprep (Clifton, NJ) were used to perform extraction and purification procedures.

An Agilent 1100 Series LC system (Agilent Technologies, Palo Alto, CA) consisting of an binary pump, autosampler, and column oven, coupled to a triple quadrupole tandem mass spectrometer Sciex API 2000 (Applied Biosystems, Foster City, CA), both operating under Sciex Analyst software, version 1.4.2., were used for tissue analysis. The electrospray ion source in positive and negative mode was used with data acquisition in multiple reactions monitoring mode (MRM). Chromatographic separation was reached by using an Agilent Zorbax XDB C18 3.5 μm, 150 mm × 2.1 mm column with an Agilent Zorbax XDB C8 5 μm, 4 mm × 2.1 mm guard column.

For the feed analyses, an Agilent 1100 Series HPLC system with a diode array detector (DAD) (Agilent Techonologies, Waldbronn, Germany), a Lichrospher 60, RP-select B, 5 μm, 250 mm × 4 mm analytical column, and a Lichrospher 60, RP-select B, 5 μm, 4 mm × 4 mm pre-guard column (Merck) were used.

Standard Solutions. Individual stock standard solutions of the nitrofurans (furaltadone and nifursol) and nitrofuran metabolites (AMOZ and DNSAH), and the two internal standards (AMOZ-d5 and nifuroxazide) were prepared at 100.0 mg/L. All of these stock standards solutions were stored in the refrigerator and were considered stable for at least 6 months. Composite standard working solutions of the internal standards were prepared at 250, 50, and 1 μg/L. Composite standard working solutions of the nitrofurans were prepared at 50, 0.5, and 0.001 mg/L. Identical solutions but for the nitrofuran metabolites were prepared at the same concentrations as for the nitrofuran parent compounds. These composite standard working solutions were stored in the refrigerator and were shown to be stable for at least 1 month.

Samples. *Medicated Feeds.* The four treatments under analysis consisted of one basal diet not supplemented or supplemented with nitrofurans. The basal diet was a corn- and soy-based diet, formulated according to the National Research Council specifications for chickens.[28] Nitrofurans were not incorporated in the control diet (0 mg/kg), incorporated in therapeutic concentrations of 150 mg/kg furaltadone (diet F) and 100 mg/kg nifursol (diet N), and incorporated in subtherapeutic concentrations of 15 mg/kg furaltadone and 10 mg/kg nifursol (diet subNF). Nitrofurans were mixed with the other ingredients at the final stages of diet preparation. Diets were in mash form. The chickens always had free access to feed and water.

Production of Incurred Poultry Tissues. Although in Portugal the chicken meat for human consumption is mainly obtained from broilers slaughtered with 6−8 weeks of age, a small part of this meat is obtained from egg type chickens ending the laying period. The meat of these chickens is frequently used for industrial processing or as an ingredient in chicken soup. Egg type chickens are also a good animal model to use instead broiler chickens, since they consume less feed and have a similar digestive process and physiological characteristics. Thus, a total of 40 chickens (Isa Brown), 18 weeks of age, were housed in wire-floored cages, 1 hen for each cage. Each cage was provided with an individual feeder and two automatic pipette drinkers. The cages were located in a temperature-controlled room, and the photoperiod during the experiment was fixed at 16 h. Feed intake per kilogram of body weight was determined as the ratio between the mean feed intake by chicken during the experimental period and the body chicken weight at slaughter. The intake of drugs per kilogram of body weight was estimated using the concentration of nitrofurans in feeds and feed intake per kilogram of body weight and could be observed in Table 1.

Chickens were randomly assigned to the four experimental diets (10 birds/treatment) during 5 weeks (19−23 weeks of age). At 23 weeks of age, five birds per treatment were slaughtered by cervical dislocation and eviscerated. Samples (50−100 g) of breast muscles (pectoralis major and pectoralis minor), the liver, and gizzard were collected in dark plastic bags. The samples were transported in frozen or refrigerated conditions

Table 1. Intake of Feed (g Feed/kg Body Weight of Animal Per Day) and of Drug (mg Drug/kg Body Weight of Animal Per Day) per Chicken

treatment	feed (g/kg BW)	nifursol (mg/kg BW)	furaltadone (mg/kg BW)
control diet	64.9	0.0	0.0
F diet	69.8	9.2	0.0
N diet	66.2	0.0	6.5
subNF diet	66.0	0.9	0.9

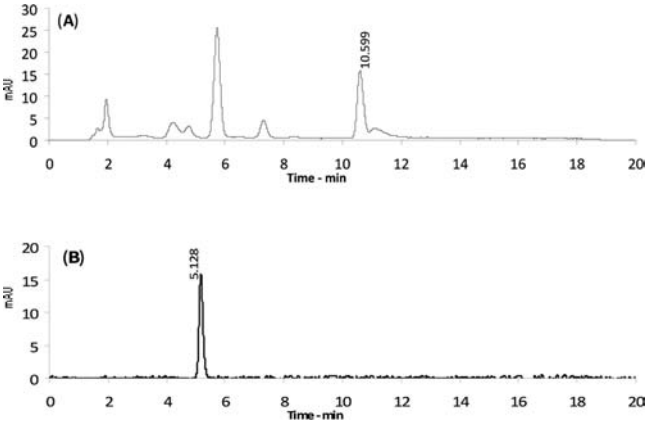

Figure 2. HPLC-DAD chromatograms of feed samples with nifursol (A; RT = 10.599) and furaltadone (B; RT = 5.128).

and, after reception in the laboratory, were kept frozen ($T = -20 \pm 2$°C) until analysis. The remaining five chickens of each treatment received, during the following 3 weeks, the control diet and at 26 weeks of age were slaughtered, and samples of breast muscles (pectoralis major and pectoralis minor), liver, and gizzard were collected as previously described. The experiments were carried out in accordance with current ethical guidelines for the care of laboratory animals for investigation set by the Portuguese General Directorate of Veterinary.

Determination of Parent Nitrofuran Compounds in Feed. For nifursol, the samples (5.0 ± 0.05 g) were spiked at 3 mg/kg with nifurozaxide and extracted for 30 min with 30 mL of a mixture of water: acetonitrile (50:50). A 5 mL aliquot of the previous extract was loaded in a neutral alumina cartridge for clean-up. The first 3 mL was discarded, and the following 2 mL was collected and transferred to an HPLC amber vial through a PVDF 0.45 μm filter. A volume of 50 μL was injected into the HPLC-DAD system, and a typical nifursol chromatogram sample is shown in Figure 2A.

For furaltadone determination, a portion of 1.0 ± 0.05 g of the samples was extracted with 50 mL of acetonitrile for 30 min. The resulting solution was filtered, and a 1 mL aliquot was collected and evaporated to dryness in an N_2 evaporator at 45 °C. The resulting extract was reconstituted in the same volume or more (as needed for dilution purposes) of mobile phase {14 mM ammonium acetate (pH 4.6) [A] with acetonitrile [B] 70:30 v/v} and transferred to an HPLC amber vial through a PVDF 0.45 μm filter. The mobile phase was composed of [A] and [B] at a flow rate of 1.2 mL/min. The gradient starts with 70% [A] for 1 min. It goes from 70 to 50% in 15 min, finishing at 70% [A] during 4 min with a total run time of 20 min. The DAD was set at 375 nm. A volume of 50 μL was injected into the HPLC-DAD system, and a typical furaltadone chromatogram sample is shown in Figure 2B.

Tissues Samples Preparation for Determination of Parent Nitrofuran Compounds. A portion of 5.0 ± 0.05 g of minced and mixed sample

Table 2. Ion Transitions Used for Nitrofuran Parent Compounds and Metabolites Detection

		MRM Diagnostic Ions	
compd	ESI polarity	precursor ion (m/z)	product ions (m/z)
NPDNSAH	pos	376	166; 211
NPAMOZ	pos	335	291; 262
NPAMOZ-d5	pos	340	296
furaltadone	pos	325	280; 253
nifursol	neg	364	182; 226
nifuroxazide	pos	276	93
	neg	274	121

was weighed into a 15 mL screw top glass centrifuge tube. Then, the sample was fortified with the composite standard working solution of internal standards at a concentration of 10, 100, or 500 μg/kg, depending on the expected concentration of the analytes, homogenized, and left to stand for about 15 min. Ethyl acetate (12 mL) was added, and samples were extracted for 20 min. Following centrifugation at 2000*g* for 15 min, the organic layer was transferred to clean tubes and evaporated to dryness under nitrogen at 45 °C. Acetonitrile (5 mL) was added to the dry residue, and hexane (3 mL) was added while vortexing. Following centrifugation, the hexane layer was discarded, and the acetonitrile was evaporated under nitrogen at 45 °C. The dry residue was redissolved in 500 μL of methanol/water (1:9, v/v), vortexed for 20 s, and transferred to an autosampler LC amber vial through a PVDF 0.45 μm filter.

Sample Preparation for Nitrofuran Metabolites. A portion of 1.0 ± 0.05 g of minced and mixed sample was weighed into a 15 mL screw top glass centrifuge tube. Then, it was fortified with the composite standard working solution of internal standards at a concentration of 10, 100, or 500 μg/kg, depending the expected concentrations of the analytes, homogenized, and left to stand for about 15 min. Afterwards, 5 mL of a 0.2 mol/L hydrochloric acid solution and 50 μL of a 100 mM *o*-NBA solution in methanol were added, and the tube was closed and shaken by hand. The tube was then put in the Reax 2 agitator in the incubator, and hydrolysis was done overnight at $T = 37 \pm 2$ °C. After it was cooled, 500 μL of a 0.3 M trisodium phosphate dodecahydrate solution was added to the sample, and the pH was adjusted to 7 ± 0.5 with a 2 M sodium hydroxide solution. Then, 4 mL of ethyl acetate were added to the sample, and the tube was shaken in the 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 $T =$ 45 °C under a nitrogen stream. The dry residue was redissolved in 500 μL of 10 mM ammonium formate (pH 4)/methanol (85:15, v/v), vortexed for 20 s, and transferred to an autosampler LC amber vial through a PVDF 0.45 μm filter.

LC-MS/MS. 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 mobile phase linear gradient program used was as follows: 0−1 min, 10% [B]; 1−16 min, from 10 to 45% [B]; 16−18 min, from 45 to 90% [B]; and 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 ionisation mode (ESI^+) for the analysis of furaltadone, NPAMOZ-d5, NPAMOZ, NPDNSAH, and nifuroxazide and in electrospray negative mode (ESI^-) for nifursol and nifuroxazide, using the TurboIonSpray source. Nitrogen was used as the curtain, collision, heater, and nebulizer gas at flow rates of 20, 5, 8, and 75 L/min, respectively. The ion source block temperature was set to $T = 450$ °C, and the electrospray capillary voltage

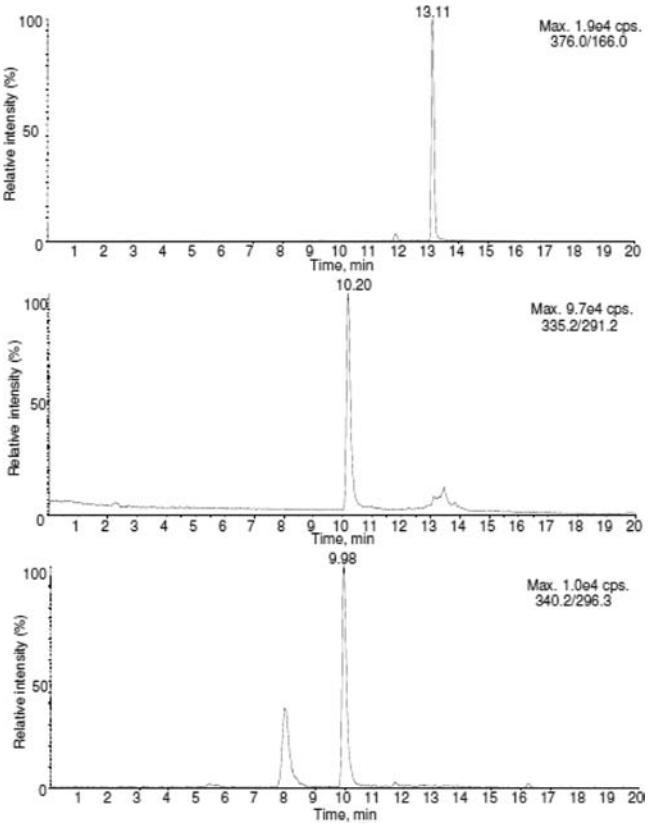

Figure 3. LC-MS/MS chromatograms of incurred gizzard samples with nifursol metabolite (DNSAH m/z 376 > 166, RT = 13.11), furaltadone metabolite (AMOZ m/z 335 > 291, RT = 10.20), and internal standard (AMOZ_d5 m/z 340 > 296, RT = 9.98).

was set to 4.5 kV. As the identification of banned substances requires four identification points, according to Commission Decision 2002/657/EC,[27] fulfillment criteria were obtained with one precursor ion and two product ions. Two transition reactions were monitored for each monitored compound. For each internal standard, only one transition was controlled. These transitions are shown in Table 2.

Given the fact that a large concentration range of analytes are expected to be observed, it was necessary to preanalyze one sample belonging to each group of animals at similar conditions of the experiment, to better anticipate the concentration of analytes present, add the most convenient quantity of internal standard for quantification, and, when necessary, dilute the final sample extract.

Calibration curves with 10 concentrations levels were prepared by spiking blank meat samples with a composite standard working solution. For the determination of nitrofuran metabolites (AMOZ and DNSAH), concentration points at 0 (blank sample), 5, 10, 20, 40, 80, 160, 320, 640, and 1280 μg/kg were used. For the determination of nitrofuran parent compounds (furaltadone and nifursol), concentration points at 0 (blank sample) 2, 3, 5, 10, 20, 40, 80, and 100 μg/kg were utilized. These fortified calibration samples were prepared using the above described procedures of extraction, the one for nitrofuran and the other for their metabolites. For the calculation of analytes concentration, the best five calibration points around the value of the analyzed sample were used.

The analyte identification was rechecked following the same criteria described above. For AMOZ identification and quantification, internal standard AMOZ-d5 was used, and for DNSAH, nifuroxazide was utilized. A typical chromatogram is shown in Figure 3.

The method used for the determination of nitrofuran metabolites (AMOZ and DNSAH) was previously described.[14] As it was already

Table 3. Summary of Validation Data

		μg/kg		CV %			
compd	matrix	CCα	CCβ	repeatability	reproducibility	recovery (%)	determination coefficient (r^2)
DNSAH	muscle	0.2	0.6	5.4	6.6	82.1	0.993
furaltadone	muscle	0.8	1.4	9.0	11.5	76.6	0.995
	liver	0.8	1.5	11.2	14.5	89.8	0.991
	gizzard	0.9	1.7	12.4	21.0	86.1	0.987
nifursol	muscle	0.9	1.3	17.1	25.3	88.9	0.993
	liver	1.1	1.7	15.3	28.2	85.5	0.990
	gizzard	1.2	1.9	19.9	27.9	80.6	0.981

Table 4. Nitrofuran Concentrations (mg/kg ± SD) of Medicated Feed (n = 3)

	nitrofuran concentrations	
diet	furaltadone	nifursol
control	0	0
F	132 ± 12	0
N	0	98 ± 5
subNF	14 ± 3	13 ± 3

validated for AMOZ, it was necessary to demonstrate its applicability for the determination of DNSAH. For this, blank poultry meat samples were fortified with DNSAH at concentrations of 5, 10, and 20 μg/kg and analyzed on three separate occasions, six samples each occasion. Samples were quantified against standard calibration curves in the range of 0, 5, 10, 20, 40, and 80 μg/kg. For specificity, 20 blank poultry meat samples of different origins were analyzed. The fully validated method is for poultry meat but has been extended to other different animal tissues including liver and gizzard, with satisfactory quantification results.

The evaluation of the applicability of the method for the determination of nitrofuran parent compounds residues in poultry meat, liver, and gizzard was done by testing specificity, linearity, precision accuracy, decision limit (CCα), and detection capability (CCβ). Specificity could be demonstrated by analyzing 30 blank samples (10 for each type of commodity) before and after spiking at a concentration level of 1.5 μg/kg. For linearity, accuracy, precision, and analytical limits, calibration curves were prepared using blank meat samples spiked at 1.5, 3, and 5 μg/kg and were analyzed on three separate occasions, six samples each occasion. Samples were quantified against standard calibration curves in the range of 0, 1.5, 3, 5, 10, and 20 μg/kg.

RESULTS AND DISCUSSION

Method validation results for DNSAH are presented in Table 3. Calibration curves were stable in the range of 5−80 μg/kg. Concerning specificity, no interfering peaks of eligible size were observed at the retention time window for the nitrofuran metabolite and for the two MRM monitoring transitions of interest.

Method validation results for the analysis of parent nitrofurans are shown in Table 3. The linearity of the individual calibration curves could be demonstrated by a linearity verification of the calibration curves. The determination coefficient (r^2) was better than 0.98 in all cases as shown in Table 3. Decision limits (CCα) for the parent compounds were equal or lower than 1.2 μg/kg for both compounds. Intra- and interday CVs are well within the acceptable values predicted by the Horwitz equation.[27] After the blank samples were analyzed for testing specificity, no interference from the matrix was observed that might disturb the detection of the MRM signals produced by the analytes.

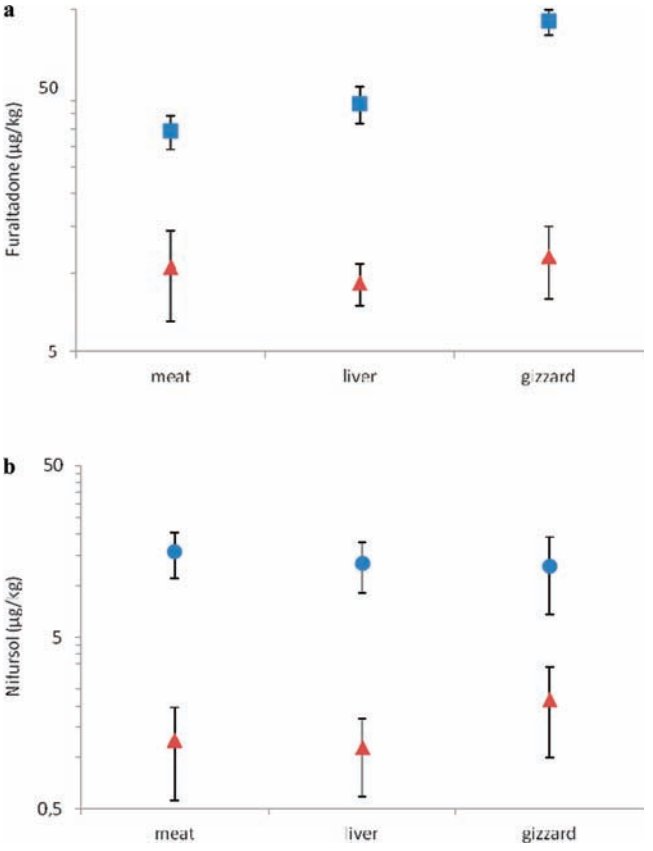

Figure 4. Tissues concentration of (a) furaltadone (means ± SDs) after the administration of diet F (■) and diet SubNF (▲) and (b) nifursol (means ± SDs) after the administration of diet N (●) and diet subNF (▲), with no withdrawal period.

Although the validation for the metabolites and parent compounds was made with calibration curves for lower quantifications, a test of homogeneity of the variances was performed in all of the range of concentrations used for quantification. With that, several working ranges were established to be used depending on the concentration needed to quantify.

The results obtained in feed samples are summarized in Table 4, and data from nitrofuran residues in chicken edible tissues are presented in Figures 4−6. Figures data are the average

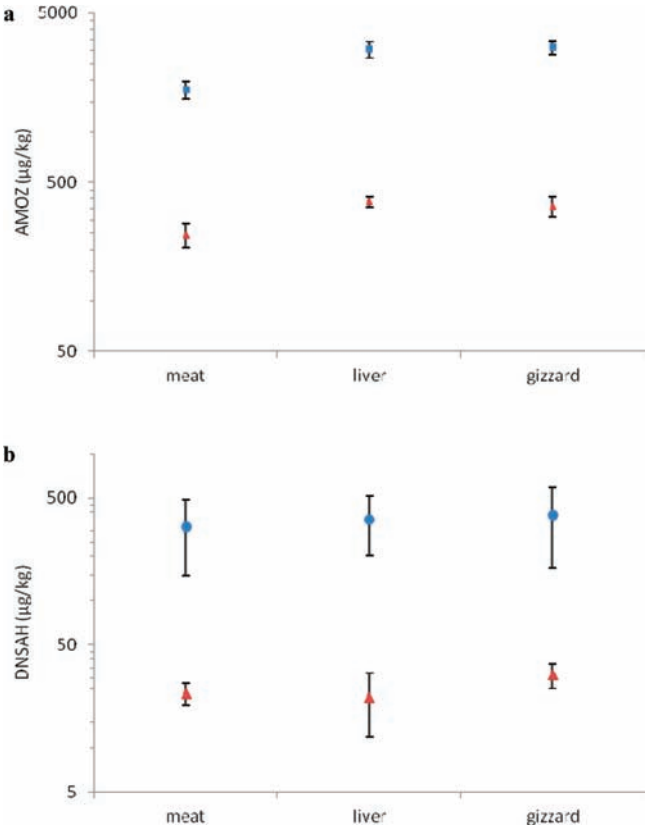

Figure 5. Tissues concentration of (a) AMOZ (means ± SDs) after the administration of diet F (■) and diet SubNF (▲) and (b) DNSAH (means ± SDs) after the administration of diet N (●) and diet subNF (▲), with no withdrawal period.

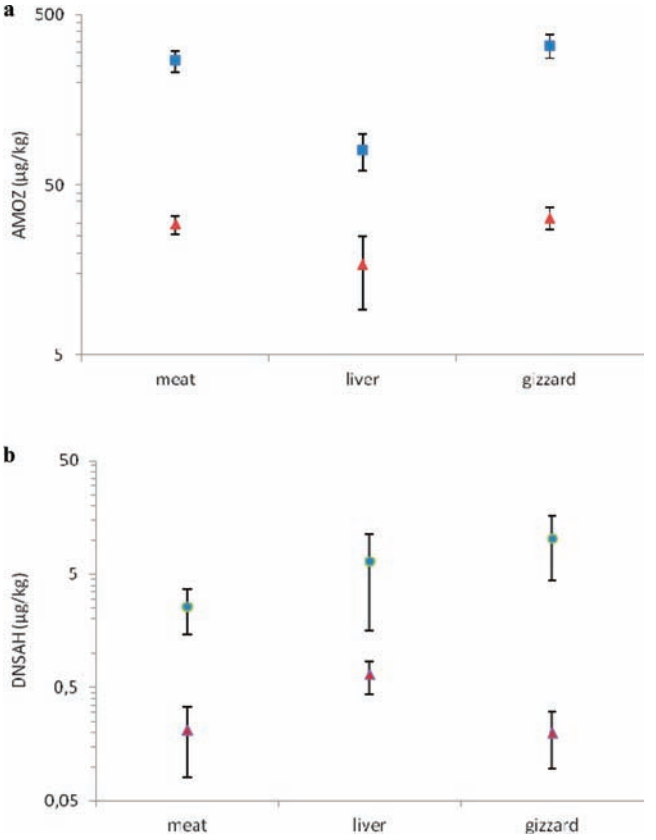

Figure 6. Tissues concentration of (a) AMOZ (means ± SDs) after the administration of diet F (■) and diet SubNF (▲) and (b) DNSAH (means ± SDs) after the administration of diet N (●) and diet subNF (▲), with 3 weeks of withdrawal period.

values of five samples, each determined in triplicate, and error bars are the standard deviations (SDs). All meat, liver, and gizzard nitrofuran parent compounds and metabolites residue data were statistically different with values of $p < 0.05$.

Feed nitrofuran concentrations for chickens confirm that the obtained values are in agreement with the initially proposed in medicated feed manufacturing. Regarding the data of samples of chicken meat, liver, and gizzard, it was verified that no detectable residues of nitrofurans were observed in chickens that were fed with the control diet. The presence of furaltadone and nifursol was only determined in edible tissue samples with no withdrawal time period (Figure 4).

When a 3 week withdrawal period was observed, it was not possible to detect any of the referred parent compounds, regardless of the ingested dose of diets F, N, and subNF. These previous results confirm that the nitrofurans have a very short half-life and are rapidly metabolized.[9] The major furaltadone and nifursol metabolites are, respectively, AMOZ and DNSAH.[11,14] As shown in Figures 5 and 6, AMOZ and DNSAH metabolites are much more persistent than related parent compounds. Both metabolites could be determined whether the withdrawal time period is 0 or 3 weeks, regardless of the nitrofuran concentrations in the diets eaten by the chickens. It could also be observed that the concentrations of metabolites in meat, liver, and gizzard are far higher than those of the respective parent compound. Taking into account the proportion of furatadone and nifursol concentrations eaten by the chickens, it is also possible to verify that AMOZ always shows higher concentrations than DNSAH, especially after a 3 week withdrawal time period (Figures 5 and 6).

For AMOZ, average concentrations of 270 μg/kg in meat ($n = 5$, SD = 37.0), 80 μg/kg in liver ($n = 5$, SD = 19.8), and 331 μg/kg in gizzard ($n = 5$, SD = 53.2) were determined ($p < 0.00$), when diet F was supplied. When chickens were submitted to diet SubNF, concentrations of 29 μg/kg in meat ($n = 5$, SD = 3.0), 17 μg/kg in liver ($n = 5$, SD = 7.2), and 32 μg/kg in gizzard ($n = 5$, SD = 3.1) were determined ($p < 0.03$). The referred data were obtained after 3 weeks of withdrawal time period for both diets.

For DNSAH, after a 3 week withdrawal time period, obtained data were much lower than those observed for AMOZ. For meat, liver, and gizzard, values of 2.5 ($n = 5$, SD = 1.4), 6.4 ($n = 5$, SD = 4.9), and 10.3 μg/kg ($n = 5$, SD = 6.1) and 0.2 ($n = 5$, SD = 0.12), 0.7 ($n = 5$, SD = 0.20), and 0.2 μg/kg ($n = 5$, SD = 0.21) were obtained, respectively, when F and subNF diets were administered ($p < 0.00$ in both cases). Data obtained are close to the EU nitrofuran MPRL (1 μg/kg). Thus, some labs may struggle to measure DNSAH, mainly after a 3 week withdrawal of a subtherapeutic diet.

Zuidema et al.[17] found a concentration exceeding 10 μg/kg for DNSAH in broilers liver after a 21 day withdrawal time period, following 7 days of treatment with 50 mg/kg nifursol, when for muscle the DNSAH residue concentrations were lower than 1 μg/kg. Taking into account the experimental differences, the values for DNSAH obtained by Zudeima et al.[17] and the data of the present study have proven to be in agreement.

McCracken et al.[29] found concentration values for AMOZ of 10 and 40 μg/kg approximately, in muscle and meat of broilers after 12 days of treatment with only 3 mg/kg furaltadone in feed. In the present study, it could be observed that very low levels of administered furaltadone and nifursol were still detectable after a 3 week withdrawal time period.

Regarding the analyzed edible matrices, AMOZ was found in the highest concentration in liver than in meat, which meets the data obtained in a previous study with pigs.[30] However, for the DNSAH metabolite, a similar distribution among liver and meat was observed with no relevance from other to another (Figures 5 and 6).

It seems possible to make the conclusion that AMOZ is more persistent than DNSAH. The fact that the administration of subtherapeutic concentrations of furaltadone and nifursol lead to detectable amounts of residues of these compounds in edible tissues is also a valid observation.

Nevertheless, it was noted that among the chicken-analyzed matrices, gizzard was the one in which either nitrofuran parent compounds or metabolites were determined in higher concentrations in almost every matrices, regardless of the administered diet or of the respected withdrawal period. The fat content of the gizzard seems to be the most relevant factor of this finding. In fact, the fat content of the gizzard ($\pm$21.8%) is about three times higher than in liver ($\pm$6.1%) and about 18 times higher than in muscle ($\pm$1.2%).[31]

In conclusion, the residues of nitrofurans are deposited in edible tissues, and detection of parent compounds is not always possible, especially in long-term detection. It is clear that the metabolites are much more persistent. After 3 weeks of treatment withdraw, a big contrast between residues levels for furaltadone and nifursol metabolites is evident. Last but not least, and because this is the first study that presents data of nitrofurans in gizzard, it seems that it might be considered a selected matrix for nitrofuran residues evaluation in poultry.

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