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#### **Food Additives and Contaminants**



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# Residues of nitrofuran antibiotic parent compounds and metabolites in eyes of broiler chickens

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#### Abstract

Nitrofuran antibiotic residues in food continue to be of international concern. The finding of sources of semicarbazide (SEM), other than through the misuse of nitrofurazone, present a challenge to the use of SEM as a definitive marker residue for this drug. Detection of intact (parent) nitrofurazone would avoid confusion over the source of SEM residues. Broiler chickens were fed sub-therapeutic nitrofurancontaining diets and tissues analysed for parent compounds and metabolites by LC-MS/MS. Depletion half-lives in muscle were longer for tissue-bound metabolite residues, 3.4 days (AOZ, AMOZ) to 4.5 days (SEM), than total metabolite residues, 2.0 days (AOZ) to 3.2 days (SEM). Metabolite concentrations were higher in eyes than muscle. Metabolite half-lives in eyes ranged from 8.5 days (AHD) to 20.3 days (SEM). Nitrofuran parent compounds were also detected in eyes.. Furaltadone was detected in single eyes after 21 days withdrawal of a 6 mg kg<sup>-1</sup> furaltadone diet. When 50 eyes from broilers containing metabolites in muscle close to the 1  $\mu$ g kg<sup>-1</sup> MRPL were pooled into single samples, 1.2 ng furazolidone and 31.1 ng of furaltadone were detected, but nitrofurazone was not detected due to the long depletion half-life of SEM Further studies are required to improve LC-MS/MS nitrofurazone in muscle. sensitivity and refine the sample size necessary to use nitrofurazone detection in pooled eyes as a complement to SEM detection in muscle.

Keywords: nitrofurans, broiler chickens, eyes, metabolites, depletion, semicarbazide

#### Introduction

The monitoring of residues of nitrofuran metabolites in the food chain has been a major focus in the international control of veterinary drug residues for several years. Nitrofuran antibiotics were banned within the European Union (EU) (Commission Regulation 1995) due to concerns over their carcinogenicity and mutagenicity (Van Koten-Vermeulen et al. 1993) and should not be used in food-producing animals or be present in foods produced in, or imported into, the EU. However, improved liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmatory analytical methods developed under the EU funded research project FoodBRAND (http://www.afsni.ac.uk/foodbrand; Cooper et al. 2005b) were instrumental in uncovering the widespread abuse of nitrofurans in various world-wide food industries during 2001-02. These included primarily the poultry and aquaculture industries in S.E. Asia and S. America, in addition to isolated findings of nitrofurans in European pork (O'Keeffe et al. 2004) and poultry (Food Standards Agency 2003), and egg powders and honey from various countries (European Commission RASFF). These findings disrupted trade relations between the EU and some exporting countries. The measures taken included the adoption of Decisions that required the mandatory preimport testing of aquaculture products from Vietnam (Commission Decision 2002a), poultry and aquaculture products from Thailand (Commission Decision 2002b) and poultry products from Brazil (Commission Decision 2002c). In the wake of improved analytical techniques and subsequent changes to national legislation and monitoring programmes instigated by some exporting countries, the above decisions were revoked and the frequency of EU Rapid Alerts for nitrofurans gradually fell. The 18-month period from January 2005 produced 73 Rapid Alerts for nitrofuran metabolites (European Commission RASFF). Shrimps from S.E. Asia and India continue to be the most commonly violative foodstuff (58 Alerts), the remaining food sources being fish (5), honey (4), poultry meat (3), bovine (1), porcine (1) and baby food (1). The majority of cases concern findings of semicarbazide (SEM, 48 Alerts), the remaining being 3-amino-2-oxazolidinone (AOZ, 22) and 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ, 3).

The nitrofuran antibiotics are unstable in vivo, breaking down in edible tissues within hours (McCracken et al. 1995). However, they give rise to stable tissue-bound metabolites, which persist in muscle and liver for many weeks (Hoogenboom et al. 1991; Cooper et al. 2005b). These metabolites, for example SEM which is the metabolite of nitrofurazone (NFZ), are the marker residues used to indicate the use of, or contamination with, their parent compounds in animal tissues. However, the validity of SEM as a unique marker residue for NFZ has been called into question following recent evidence that SEM in food may arise from sources other than from this illegal antibiotic. In 2003 SEM was detected in baby food (EFSA, 2003). Subsequent investigations (Stadler et al., 2004) demonstrated that the SEM was a breakdown product of azodicarbonamide; the blowing agent used in the manufacture of plastic gaskets inside the jar lids. Azodicarbonamide is also a flour treatment agent used in the bread making process, which may account for SEM found in breaded chicken but not in the chicken meat (Kennedy et al., 2004; Pereira et al., 2004). Carrageenan, a widely used thickening agent derived from red seaweed, has also been implicated in the production of SEM under certain conditions (Hoenicke et al., 2004). Low levels of SEM, possibly of natural origin or a by-product of boiling, have also been observed in cooked crayfish samples (Saari and Peltonen 2004). Consequently, the presence of SEM can no longer be used as definitive proof of administration of NFZ to an animal

and some national authorities have become reluctant to take enforcement action on the basis of SEM detection.

One approach to overcoming this uncertainty surrounding SEM would be to find a depot of intact NFZ in tissues of NFZ-treated animals. This laboratory has demonstrated that the four major nitrofurans furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT) and NFZ all accumulate in chicken eggs and are not broken down rapidly as in muscle and liver (McCracken and Kennedy 2006). Our previous finding of nitrofuran metabolites, including SEM, at high concentrations (mg kg<sup>-1</sup>) in the retina/choroid layer in eyes of pigs suggested that at least some of these residues may be present in the form of their parent nitrofuran drugs (Cooper and Kennedy 2005). We subsequently demonstrated that NFZ does indeed accumulate in whole eyes of laying hens (Cooper et al. 2005a). In birds fed only 0.3 mg NFZ per kg of feed (approximately 0.1% of a therapeutic dose; National Office Of Animal Health Limited, 1992) the mean NFZ detectable by LC-MS/MS in a single eye was  $0.5 \text{ ng eye}^{-1}$ . The mean tissue-bound SEM concentration in muscle of these chickens was 3.3 µg kg<sup>-1</sup>. This suggested that, if multiple eyes could be pooled from chickens of a single flock, a test for parent NFZ could be developed with sensitivity comparable to that of tissuebound SEM in muscle. The current methodology for statutory monitoring of NFZ abuse in animal tissues is detection of tissue-bound SEM in muscle or liver and regulatory laboratories are required by EU legislation to set in place confirmatory methods which can quantify SEM at 1.0 µg kg<sup>-1</sup> or less (the Minimum Required Performance Limit, MRPL, set down in Commission Decision 2003/181).

The current study aimed to assess the suitability of testing for nitrofuran parent compounds in eyes of broiler chickens by addressing the following objectives:

- 1. To demonstrate the depletion profile of nitrofuran metabolite residues in broiler eyes, muscle and liver tissues following drug withdrawal.
- 2. To demonstrate the presence of nitrofuran parent compounds in eyes of nitrofuran-medicated broiler chickens.
- 3. To demonstrate the depletion profile of the nitrofuran parent compounds in broiler eyes following drug withdrawal.
- 4. To determine if monitoring nitrofuran parent compounds in pooled broiler eyes is comparable to monitoring their tissue-bound metabolites in muscle tissue.

#### Materials and methods

Materials and instrumentation

Internal standards D<sub>4</sub>-3-amino-2-oxazolidinone, D<sub>5</sub>-3-amino-5-morpholinomethyl-2oxazolidinone,  ${}^{13}C_3$ -1-aminohydantoin and  ${}^{13}C^{15}N_2$ -semicarbazide were supplied by Witega Laboratorien Berlin-Adlershof (Berlin, Germany). 3-Amino-2-oxazolidinone (AOZ), semicarbazide (SEM), furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT) and nitrofurazone (NFZ) were supplied by Sigma-Aldrich (Poole, UK). 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), and the nitrofuran parent compound internal standards D<sub>4</sub>-furazolidone, D<sub>5</sub>-furaltadone and <sup>13</sup>C<sup>15</sup>N<sub>2</sub>nitrofurazone were supplied by Chemical Synthesis Services (Belfast, UK). 1-Aminohydantoin (AHD) was a gift from Proctor and Gamble Pharmaceuticals USA.

Unless stated, all other chemicals and solvents were obtained from Sigma-Aldrich and were analytical and HPLC grade, respectively.

An Agilent 1100 Series HPLC system (Agilent Technologies, USA) coupled to a Quattro Ultima® Platinum tandem mass detector (Micromass/Waters, Manchester, UK), both operating under MassLynx® software, were used for tissue analyses. The mass spectrometer operated in electrospray positive and negative modes and data acquisition was in multiple reaction monitoring mode (MRM). The HPLC system was equipped with a Columbus C18(2) 3 µm, 2.0 x 150 mm column (Phenomenex, UK). A binary gradient mobile phase was used at a flow rate of 0.2 ml/min, solvent A being 0.5 mM ammonium acetate and methanol (80:20 v/v mix), solvent B being 100% methanol. Commercial crumbed feed for broiler chickens was supplied by McLarnons Ltd. (Randalstown, UK). Muscle and liver were minced in a Mini Prep Plus domestic food processor (Waring, Torrington, CT, USA) then were homogenised in a SL2 laboratory homogeniser (Silverson Machines Ltd, Chesham, England). Whole chicken eyeballs were homogenised in self-contained, disposable FOODcon7 homogenisation units using a FASTH21 high throughput, automated tissue homogenisation system (ConsulAR SA, Switzerland).

#### Production of incurred broiler tissues

Two-week old broiler chickens, purchased from a local supplier, were housed in deep litter pens in 4 groups of 43 birds. Birds were allowed *ad libitum* access to fresh water and feed at all times. Commercial broiler chick crumb feed was used to prepare four medicated diets containing NFT at 50 mg kg<sup>-1</sup> feed, FZD at 10 mg kg<sup>-1</sup> feed, FTD at 6 mg kg<sup>-1</sup> feed and NFZ at 1 mg kg<sup>-1</sup> feed. These concentrations were designed to produce tissue-bound nitrofuran metabolite residues in muscle close to the EU MRPL of 1  $\mu$ g kg<sup>-1</sup> after a drug withdrawal period of between 1 to 3 weeks. Approximate calculations were based on the limited literature data available for dietary nitrofuran levels and muscle residues in chickens (Zuidema et al. 2004; McCracken et al. 2005; Cooper et al. 2005a) and pigs (Cooper et al. 2005b). The four groups were fed a medicated diet for 7 days after which the medicated diets were withdrawn and replaced with conventional unmedicated broiler grower crumb feed for a further 21 days' withdrawal period. Birds (3 per group) were euthanised by cervical dislocation at intervals during the withdrawal period. Breast muscle and liver were sampled and stored at -20°C. Whole, intact eyeballs were removed and stored at -80°C. During the withdrawal period, muscle samples were analysed immediately for total nitrofuran metabolite concentration as described below. McCracken et al. (1997) demonstrated that approximately 60% of total AOZ residues in kidney of FZD treated pigs was present in the form of tissue-bound residues. Unpublished data from this laboratory demonstrates that approximately 80% of total SEM residues in muscle of NFZ treated chickens is present in the form of tissue-bound residues. Therefore, when total muscle metabolite concentrations had fallen to approximately 1 to 3  $\mu$ g kg<sup>-1</sup> in the present study, 25 birds from each group were euthanised and muscle, liver and eyes were sampled. These groups of 25 birds provided 50 eyes that could be pooled for analysis of nitrofuran parent compounds. The remaining birds continued on the withdrawal rations and were euthanised (3 per group) and tissues sampled every 3 to 4 days.

Tissue analysis by LC-MS/MS

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Tissue-bound nitrofuran metabolites were measured in 1 g samples of chicken muscle and liver essentially as described by Cooper et al. (2005b). Briefly, samples were homogenised and extractable residues were removed by repeated washing in methanol, ethanol and diethyl ether. Tissue-bound metabolites were hydrolysed in mild acid and derivatised with 2-nitrobenzaldehyde before extraction into ethyl acetate. Sample preparation and LC-MS/MS analysis were as described by Cooper et al. (2005b) with the exceptions that a Columbus C18 HPLC column and an additional <sup>13</sup>C<sub>3</sub>-AHD internal standard were used. Highly concentrated samples were diluted as required for analysis.

Total nitrofuran metabolites were measured in 1 g samples of chicken muscle as described by Cooper and Kennedy (2006). This method omits the sample solvent prewashing steps and employs minor differences in reagent volumes compared to the tissue-bound metabolite method.

Total nitrofuran metabolites were measured in single whole chicken eyes as described by Cooper et al. (2005a). Eyes were cut into four pieces and placed in FOODcon7 homogenisation units while still frozen in order to retain the vitreous humour. If allowed to defrost, the retina, which contains the vast majority of the ocular nitrofuran residues, partially disintegrates and is dispersed with the humour when the sclera is cut open.

Nitrofuran parent compounds were extracted from single whole chicken eyes essentially as described by Cooper et al. (2005a). Sliced eyes were homogenised in FOODcon7 units with 5 ml water fortified with 10 ng per eye mixed internal standard

and extracted into ethyl acetate, reconstituted in acetonitrile and washed with hexane. Dried extract was finally reconstituted in 100  $\mu$ l of methanol : water (50 : 50 v/v) and centrifuged at 13 000 rpm. LC-MS/MS conditions for the analysis of the four nitrofuran parent compounds were as described by McCracken and Kennedy (2006). Nitrofuran parent compounds were also analysed in pooled samples comprising 50 pooled eyes, homogenising 5 eyes in 6 ml water in each of 10 FOODcon7 units, fortifying with 1 ng per unit mixed internal standard. After transferring the contents to glass tubes and rinsing the units with 2 ml water, samples were extracted into ethyl acetate (9 + 7 ml) and reconstituted in acetonitrile (3 ml). The samples were washed with hexane (3 + 3 ml) before combining the ten acetonitrile extracts, evaporating to dryness and reconstituting the single combined extract (50 eyes) in 100  $\mu$ l of methanol : water (50 : 50 v/v) and centrifuging at 13 000 rpm.

#### **Results and discussion**

#### Nitrofuran metabolites in edible tissues

Table I presents the concentrations of total nitrofuran metabolites in broiler breast muscle during the first week of drug withdrawal. Depletion half-lives, calculated on the basis of linear regression of metabolite concentrations over this 7 day period, ranged from 2.0 days (AOZ) to 3.2 days (SEM). This is the first publication of total metabolite half-lives for all four of the nitrofuran drugs in broiler chickens. Zuidema et al. (2004) published total AOZ and AMOZ depletion data in broilers fed therapeutic doses. These graphs suggested AOZ and AMOZ half-lives were in the region of 4 days. It was on the basis of estimates made from the measurements of the total

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metabolite concentrations in the present study, that broilers were euthanised on days 8, 9 and 10 in order to produce muscle samples with tissue-bound nitrofuran metabolite concentrations close to the MRPL of 1  $\mu$ g kg<sup>-1</sup>. Groups of birds were euthanised on different days due to the varying concentrations of dietary nitrofurans employed and the variable metabolite half-lives observed.

#### [Insert TABLE I about here]

Whilst European legislation does not specify whether nitrofuran metabolites should be monitored in the form of tissue-bound or total fractions, analysis of tissue-bound metabolites became the norm in this laboratory, initially because of the cleaner extracts obtained following solvent washing. This approach was reinforced by AFSSA, the Community Reference Laboratory for nitrofuran drugs, which, following the discovery that SEM could arise from degradation of azodicarbonamide, used as a flour treatment agent, issued a statement in 2003 that "when testing composite food, only analyse the part of the product which is of animal origin, for example, only the meat part of breaded products...In the case of a non-compliant sample for total SEM, a sample must be reanalysed for the bound residues of SEM only" (Sanders 2003). Given that chicken muscle is the world's most popular meat, and that nitrofuran residues have been an issue of concern in the global poultry industry, there is a need for data on the depletion half-lives of the tissue-bound nitrofuran metabolites in edible broiler tissues. Figures 1 and 2 illustrate the depletion profiles and half-lives of the four major tissue-bound nitrofuran metabolites in muscle and liver respectively. (Note: the nitrofuran drugs were fed at different dietary concentrations, so absolute metabolite concentrations cannot be compared meaningfully across drug groups). Depletion half-lives for all metabolites were longer in muscle (ranging 3.4 to 4.5 days) than in liver (ranging 2.2 to 3.1 days), possibly reflecting the lower metabolic rate and slower cellular turnover of muscle tissue. This tissue difference was also evident in our previous studies of nitrofuran depletion in porcine tissues (Cooper et al. 2005). SEM exhibited the longest half-life in broiler muscle (4.5 days) and was detectable after 3 weeks withdrawal of a low contamination level diet (1 mg kg<sup>-1</sup>, normal therapeutic dose being 200-300 mg kg<sup>-1</sup>). Total nitrofuran metabolites in muscle (Table I) exhibited shorter half-lives than their corresponding tissue-bound metabolites (Figure 1), possibly due to the faster clearance rate of the "free" (solvent-extractable) residues portion compared with the tissue-bound residues.

#### [Insert FIGURE 1 and FIGURE 2 about here]

Nitrofuran metabolites in broiler eyes

The recent controversy over sources of SEM in foods, other than through abuse of NFZ, led this laboratory to investigate eyes as a potential depot of nitrofuran residues. We demonstrated that total nitrofuran metabolites were detectable at parts per million concentrations in retina of pigs fed therapeutic nitrofuran doses (Cooper and Kennedy 2005). We then proceeded to show that NFZ-treated laying hens had similarly high concentrations of total SEM in whole eyeballs (Cooper et al. 2005a) and that SEM was even detectable in a single eye when feeding a diet containing NFZ at only 0.03 mg kg<sup>-1</sup>. Figure 3 shows that AOZ, AMOZ, AHD and SEM residues can be detected by LC-MS/MS in single eyes of broiler chickens fed sub-therapeutic nitrofuran doses. The depletion half-lives of total metabolites in eyes are considerably longer (ranging 8.5 to 20.3 days) than the depletion half-lives for tissue-bound metabolites in muscle and liver (Figures 1 and 2). As is the case with some other xenobiotic residues such as the  $\beta$ -agonist clenbuterol (Malucelli et al. 1994), ocular tissue, specifically the retina, appears to be a metabolic "dead end" tissue where nitrofuran metabolites accumulate to

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high concentrations and where they are not subject to the higher cellular turn-over and clearance rates of other tissues such as muscle and liver. The pigment melanin has been identified as the site of binding of various drugs in pigmented ocular tissues (Ings 1984).

#### [Insert FIGURE 3 about here]

The concentrations of nitrofuran metabolites are also significantly greater in eyes (measured as ng per eye; each eye weighing approximately 1 g) than in muscle and liver (measured as ng per g tissue). The MRPL for the nitrofurans is  $1.0 \ \mu g \ kg^{-1}$  in poultry meat and aquaculture products (Commission Decision 2003). Commission Decision 2005/34/EC has recently harmonised the standards applied for monitoring banned substances, including the nitrofurans, in foods. The MRPL is now to be considered a "reference point(s) for action irrespective of the matrix tested." Furthermore, "where the results of analytical tests on products are below the MRPL... the products will not be prohibited from entering the food chain." Where recurrent sub-MRPL findings are observed, "the Commission shall bring the matter to the attention of the competent authority in the country of origin and shall make appropriate proposals" (Commission Decision 2005). Given the higher concentrations and longer half-lives of nitrofuran metabolites in eyes compared to muscle and liver, and the possibility of applying the MRPL as an reference point for action irrespective of the matrix tested, it is now possible, in principle at least, that a chicken may be condemned as unfit for human consumption on the basis of metabolites detected in its eyes long after the metabolite concentration in muscle or liver has fallen below the MRPL, further diminishing the temptation to misuse these drugs.

Nitrofuran parent compounds in broiler eyes

The major attraction of testing chicken eyes is their ability to retain the intact nitrofuran parent compounds in contrast to edible tissues in which the parent compounds are broken down inside a matter of hours (McCracken et al. 1995, Cooper et al. 2005b). We previously demonstrated that NFZ was detectable in eyes of laying hens (Cooper et al. 2005a). Table II now demonstrates that FZD, FTD and NFT are also detectable in single broiler chicken eves when no withdrawal period is observed. Whilst direct comparisons cannot be made between the absolute concentrations of the four nitrofuran parent compounds in eyes because of the differing dietary concentrations administered, we have shown that accumulation of nitrofuran parent compounds in broiler eyes is not restricted to NFZ. The failure to detect NFZ in single eyes in this study (Table II) may have been due to the fact that SEM has a long depletion half-life in muscle and therefore it was necessary to feed NFZ at a very low dietary level (1 mg kg<sup>-1</sup>) to achieve SEM muscle concentrations close to the MRPL within the confines of a short term study. NFZ was also the least sensitive of the nitrofuran parent compounds under LC-MS/MS ionisation. However, monitoring compliance with the prohibition on the use of NFZ in food-producing animals may still be feasible by measuring NFZ in the retina of larger food-producing animals (e.g. cattle and pigs).

#### [Insert TABLE II about here]

NFT was still detectable in a single eye after 3 d withdrawal of a 50 mg kg<sup>-1</sup> NFT diet.. FTD, by contrast, when fed at only 6 mg kg<sup>-1</sup> feed, was still detectable (0.08 ng eye<sup>-1</sup>) in a single eye after 21 days withdrawal (Table II; depletion half-life 3.4 days). The ability to detect FTD over such a period is due partly to FTD being the most sensitive of the nitrofuran parent compounds under the LC-MS/MS method described above.

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However, FTD does appear to be the most abundant nitrofuran parent compound in ocular tissues, given that in pigs fed 400 mg kg<sup>-1</sup> FTD, the parent compound was detected in isolated retina at mg kg<sup>-1</sup> concentrations, much higher than the  $\mu$ g kg<sup>-1</sup> concentrations found in FZD treated pigs (Cooper et al. 2005a).

Twenty-five broilers from each group were euthanised after 8, 9 or 10 days withdrawal when it was estimated (based on real-time analysis of total metabolites) that tissuebound metabolite concentrations in muscle would be close to the 1  $\mu$ g kg<sup>-1</sup> MRPL. Table III shows that two groups were below (AHD 0.8  $\mu$ g kg<sup>-1</sup>, SEM 0.4  $\mu$ g kg<sup>-1</sup>) and two groups above the MRPL (AOZ 1.4  $\mu$ g kg<sup>-1</sup>, AMOZ 1.6  $\mu$ g kg<sup>-1</sup>) on these dates. Processing and pooling 50 whole eyes into a single extract reconstituted in 100 µl of solvent proved to be easier than expected. Eyes were split across 10 groups of 5 eyes, each independently extracted and washed with hexane to remove the considerable orange oily fraction present in intact eyeballs, before combining to a single extract which could be dissolved in 100 µl of 50% methanol. Ultra-centrifugation further clarified the samples such that extracts of 50 eyes were of a similar clarity to extracts of a single eye and suitable for injection onto a HPLC column. However, greater ionisation suppression was evident during tandem MS analysis of extracts of 50 eyes compared to single eye samples (peak areas reduced by a factor of approximately 4). Comprehensive validation of the LC-MS/MS method is hindered by the enormous number of eyes required for the sample compositing process, however, the authors estimate that in a fortified sample of 50 broiler eyes, nitrofuran parent compounds can be detected down to approximately 4 ng NFZ, 2 ng NFT, 1 ng FZD and 0.2 ng FTD per pooled sample.

#### [Insert TABLE III about here]

FZD was detected at 1.2 ng in 50 pooled eyes (Table III) some 10 days after withdrawal of a diet containing 10 mg kg<sup>-1</sup> FZD (5% of a therapeutic dose). This equates to 0.02 ng eye<sup>-1</sup>, which would be undetectable in a single broiler eye using current LC-MS/MS techniques. In contrast, the stability of FTD was reinforced by the detection of 31.1 ng in 50 pooled eyes 10 days after withdrawal of a diet containing only 6 mg kg<sup>-1</sup> FTD. This equates to 0.62 ng eye<sup>-1</sup>, which is consistent with the detection of 0.6 ng FTD in single eyes at 7 days withdrawal (Table II). Pooled eyes were unavailable for testing for NFT. However, on the basis of the concentrations of NFT in single eyes (Table II), it can be estimated that NFT would have been detected in 50 pooled eyes at approximately 10 ng. Possibly the most significant result was the inability to detect NFZ in 50 pooled eyes after 8 days withdrawal of a 1 mg kg<sup>-1</sup> NFZ diet. This can again be attributed primarily to the long depletion half-life of SEM in muscle (4.5 days) that results in levels of SEM close to the MRPL being detected in muscle even following withdrawal of a low contamination level diet. This was compounded by the poor sensitivity of NFZ under the applied LC-MS/MS conditions. It may be possible that, if MS/MS sensitivity can be improved and samples analysed when SEM in muscle is closer to the 1  $\mu$ g kg<sup>-1</sup> MRPL rather than the 0.4  $\mu$ g kg<sup>-1</sup> in the current test samples, NFZ may be detectable in a 50 eyes pooled sample. However, a larger sample size may also be considered to improve test sensitivity. Given the scale of commercial broiler production facilities, obtaining 50 or 100 eyes from a chicken house (in which all birds receive the same feed and are therefore suitable for pooling into a single sample) would present little difficulty. However, it must be recognised that the testing of eyes for nitrofuran parent compounds is unlikely to replace the testing of edible tissues for nitrofuran metabolites as the routine method of nitrofuran residue monitoring. Imported poultry meat is rarely accompanied by the eyes from the

source bird. Nevertheless, the testing of nitrofuran parent compounds in pooled eyes from a poultry flock would be a valuable tool within a monitoring programme. It would remove any doubt over the source of nitrofuran metabolites and enable poultry producers to police their own supply chains and competent national authorities to carry out comprehensive investigative work within their domestic primary production facilities. Ultimately it would assist EU Member States to enforce fully the ban on the use of nitrofuran drugs in primary production. Furthermore, the higher concentrations of nitrofuran metabolites detected in eyes may permit laboratories lacking LC-MS/MS technologies to monitor nitrofuran abuse in their primary production using HPLC-UV detection as an alternative to monitoring tissue-bound metabolites in edible tissues, albeit at a level which would not meet EU confirmatory criteria.

This study has provided useful data on the depletion profiles and half-lives of the nitrofuran metabolites in broiler chicken muscle and liver. It has also demonstrated that higher concentrations of metabolites with longer half-lives are detectable through the analysis of whole eyes of broilers. This study, in conjunction with our previous work (Cooper et al., 2005a), has now shown that all four major nitrofuran parent antibiotics, as opposed to their metabolites, are detectable in broiler eyes, with FTD, in particular, being detectable even after 3 weeks withdrawal of a contamination level diet. This was the first attempt to measure nitrofuran parent compounds in pooled samples of eyes from broilers whose muscles contained nitrofuran metabolites close to the EU MRPL. Measuring FZD in 50 pooled eyes was shown to be an alternative to measuring tissue-bound AOZ in single muscle samples. FTD was detectable in single eyes of broilers with MRPL concentrations of AMOZ in their muscle. However, NFZ could not be detected in 50 pooled eyes of broilers with 0.4  $\mu$ g kg<sup>-1</sup> SEM in their muscle. Further

study is required to improve NFZ sensitivity under LC-MS/MS and to refine the sample size necessary to enable NFZ detection in pooled eyes to be applied as an alternative to SEM detection in muscle. Such a test would circumvent the problem of alternative sources of SEM in the food chain and would help protect both the consumer from eating meat of birds that have received a banned substance, and of poultry producers from being wrongly accused of administering nitrofurazone on the basis of semicarbazide detection in muscle.

<text>

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#### **TABLE AND FIGURE CAPTIONS:**

Figure 1. Depletion, during 3 weeks withdrawal, of tissue-bound nitrofuran metabolites, AOZ, AMOZ, AHD and SEM from breast muscle of broiler chickens fed, FZD (10 mg kg<sup>-1</sup>), FTD (6 mg kg<sup>-1</sup>), NFT (50 mg kg<sup>-1</sup> feed) and NFZ (1 mg kg<sup>-1</sup>) respectively for 7d. Mean  $\pm$  SE (n=3).

Figure 2. Depletion, during 3 weeks withdrawal, of tissue-bound nitrofuran metabolites AOZ, AMOZ, AHD and SEM from liver of broiler chickens fed FZD (10 mg kg<sup>-1</sup>), FTD (6 mg kg<sup>-1</sup>), NFT (50 mg kg<sup>-1</sup> feed) and NFZ (1 mg kg<sup>-1</sup>) respectively for 7d. Mean  $\pm$  SE (n=3).

Figure 3. Depletion , during 3 weeks withdrawal, of total nitrofuran metabolites AOZ, AMOZ, AHD and SEM from single whole eyes of broiler chickens fed FZD (10 mg kg<sup>-1</sup>), FTD (6 mg kg<sup>-1</sup>), NFT (50 mg kg<sup>-1</sup> feed) and NFZ (1 mg kg<sup>-1</sup>) respectively for 7d. Mean  $\pm$  SE (n=3).

Table I. Depletion, during first week of withdrawal, of total nitrofuran metabolites AOZ, AMOZ, AHD and SEM from breast muscle of broiler chickens fed FZD (10 mg kg<sup>-1</sup>), FTD (6 mg kg<sup>-1</sup>), NFT (50 mg kg<sup>-1</sup> feed) and NFZ (1 mg kg<sup>-1</sup>) respectively for 7d. Mean (n=3)  $\pm$  SE.

Table II. Detection of nitrofuran parent compounds in single whole eyes of broiler chickens fed FZD (10 mg kg<sup>-1</sup>), FTD (6 mg kg<sup>-1</sup>), NFT (50 mg kg<sup>-1</sup> feed) and NFZ (1 mg kg<sup>-1</sup>) for 7d. ND = not detected. Mean (n=3)  $\pm$  SE. Depletion half-life of FTD was 3.4 d.

Table III. Detection of nitrofuran parent compounds in 50 pooled eyes of medicated broiler chickens when tissue-bound nitrofuran metabolites in breast muscle (n=5) were close to the MRPL of  $1 \mu g kg^{-1}$ .









Withdrawal period (days)





Withdrawal period (days)

## Table I

	Total nitrofuran metabolites (μg kg <sup>-1</sup> muscle)						
Withdrawal (days)	AOZ	AMOZ	AHD	SEM			
0	31.9 ± 6.6	19.5 ± 4.3	18.0 ± 1.6	$4.8 \pm 0.3$			
3	7.5 ± 1.0	8.9 ± 1.0	$4.9 \pm 0.5$	1.9 ± 0.2			
7	2.8 ± 0.5	$3.0 \pm 0.2$	$2.6 \pm 0.5$	$1.0 \pm 0.2$			
Half-life (days)	2.0	2.6	2.5	3.2			

### **Table II**

	Nitrofuran parent compound (ng per eye)					
Withdrawal (days)	FZD	FTD	NFT	NFZ		
0	8.3 ± 7.4	10.4 ± 1.7	1.8 ± 0.7	ND		
3	ND	$2.0 \pm 0.2$	0.4 ± 0.1	ND		
7	ND	$0.6 \pm 0.2$	ND	ND		
14	ND	$0.2 \pm 0.1$	ND	ND		
17	ND	$0.3 \pm 0.2$	ND	ND		
21	ND	0.1 ± 0.0	ND	ND		

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0.2 \pm i \\
0.3 \pm 0.2 \\
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0.1 \pm 0.0
\end{array}$ 

# Table III

	Nitrofuran	mg kg <sup>-1</sup> feed	Withdrawal (days)	Tissue- bound metabolite	µg kg⁻¹ muscle ± SE	Nitrofuran parent	ng per 50 pooled eyes
	NFT	50	9	AHD	0.8 ± 0.1	NFT	Not tested
	FZD	10	10	AOZ	1.4 ± 0.1	FZD	1.2
	FTD	6	10	AMOZ	1.6 ± 0.1	FTD	31.1
_	NFZ	1	8	SEM	0.4 ± 0.1	NFZ	Not detected