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**THE DETECTION, ACCUMULATION AND DISTRIBUTION OF
NITROFURAN RESIDUES IN EGG YOLK, ALBUMEN AND
SHELL.**

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4 **The detection, accumulation and distribution of**
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7 **nitrofurans residues in egg yolk, albumen and shell**
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36 Keywords: nitrofurans, eggs, LC-MSMS, eggshell, yolk, albumen.
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Abstract

Nitrofurans antibiotics have been banned for use in food producing animals in many countries including European Union, because of the threat they pose to human health. Research continues into the accumulation of these drugs in animal tissues and into the appropriate methods for their detection. In this study, an LC-MS/MS method is presented for the detection of the parent compounds, furazolidone, nitrofurantoin, furaltadone and nitrofurazone in eggs. The parent compounds are first extracted into ethyl acetate, fats are removed by partition between acetonitrile and hexane, and the concentrated sample is analysed by LC-MS/MS. Decision limits ($CC\alpha$) for the parents were $\leq 1 \mu\text{g kg}^{-1}$ for all four compounds. Within-day and between-day CVs are well within the limits stated in Commission Decision 2002/657/EC. The method provides an alternative to the testing of side-chain metabolites in eggs, which is particularly important in the case of nitrofurazone, where semicarbazide contamination of food has been attributed to sources other than nitrofurazone use. This method was used together with a method for the detection of the side-chain metabolite compounds, AOZ, AMOZ, AHD and SEM, to study the accumulation and distribution of the nitrofurans in eggs. Eggs were collected from 4 groups of hens that had been treated with one of the nitrofurans at a feed concentration of 300 mg kg^{-1} for 1 week. Parent compounds and metabolites were found in the yolk, albumen and shell. The albumen/yolk ratios for the parent compounds were 0.7, 0.82, 0.83 and 0.31 for furazolidone, furaltadone, nitrofurantoin and nitrofurazone, respectively. Ratios for the side-chain metabolites were 1.02, 1.06, 0.83 and 0.55 for AOZ, AMOZ, AHD and SEM, respectively. However, 50% of the total SEM residues were found in eggshell. This may be significant if eggshell products reach the consumer.

INTRODUCTION

Nitrofurans antibiotics were prohibited for use in food-producing animals in the European Union (EU) and other countries, in response to evidence that these compounds are both carcinogenic and mutagenic (van Koten-Vermeulen et al. 1993). In 2002 the EU had to impose restrictions on the import of poultry products from Thailand (Commission Decision 2002/251/EC) and Brazil (Commission Decision 2002/794/EC), countries whose food exports to the EU were found to contain nitrofurans residues. Since May 2003, more than 150 Rapid Alerts have been issued by the EU through its Rapid Alert System for Food and Feeds (http://europa.eu.int/comm/food/fs/sfp/ras_index_en.html) regarding the detection of nitrofurans in food from 29 countries. The presence of such residues in food, either through illegal use of the drugs or through inadvertent contamination, has made it essential for monitoring authorities to have appropriate detection methods in place to prevent such residues reaching the consumer.

An analytical method using LC-MS/MS was developed under the auspices of FoodBRAND (www.afsni.ac.uk/foodbrand), an EU funded project (Cooper et al. 2005a). This method and others, based on Solid-Phase Extraction (Leitner et al. 2001; Connely et al. 2003), are now used throughout the world to detect the marker residues of the nitrofurans drugs. Previous reports have shown that the tissue-bound side chain metabolites are the most suitable markers for nitrofurans detection in animal tissues, because they are more stable *in vivo* than the parent compounds and persist in tissues for many weeks (Cooper et al. 2005a). However, in the case of nitrofurazone (NFZ), whose side-chain metabolite is semicarbazide (SEM), this practice has proved unreliable, because SEM has been attributed to other sources of contamination in food

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3 (Kennedy et al., 2004; Pereira et al. 2004). Following a spate of positive SEM
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(Kennedy et al., 2004; Pereira et al. 2004). Following a spate of positive SEM detection in coated chicken products, the source of SEM contamination was attributed to the presence of azodicarbonamide, a flour treatment agent, used in the coating material. It did not therefore indicate the use of nitrofurazone in chicken production. Consequently, attention has focused upon finding alternatives to SEM as a marker for the detection of nitrofurazone abuse. Recently, it was reported that intact nitrofurazone accumulates in whole eyes of chickens treated with the drug (Cooper et al. 2005b). The detection of the parent drug in retina is therefore a realistic alternative to SEM to monitor for abuse in primary production. This, of course, is not possible for the analysis of processed meat products. Arising from the crisis in 2002 of nitrofurans contamination in imported poultry and shellfish, and the subsequent doubts over the use of SEM as a marker residue is the need for further information on the accumulation of nitrofurans in food products. This requirement goes hand-in-hand with the need for sensitive confirmatory methods for detecting appropriate residues, as required under Commission Decision 2002/657/EC.

As part of a continuing investigation into the most appropriate markers for nitrofurans detection and the development of confirmatory methods, an evaluation of the residues for the four main nitrofurans in chicken eggs was undertaken. It has been known for some time that furazolidone (FZD) accumulates as intact parent compound in chicken eggs (Botsoglou 1988, McCracken et al. 2001). Levels of the drug reached a plateau of approximately 300 $\mu\text{g kg}^{-1}$ after the fourth day of treatment, and parent compound could still be detected up to the ninth day after withdrawal from medication.

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3 Two analytical methods were required to monitor the accumulation of residues in
4 eggs. Firstly, the existing LC-MSMS method for nitrofurans metabolites in tissues
5 was applied to the analysis of eggs. Secondly, a new LC-MSMS method for the
6 detection of the intact parent compounds was developed. This new method is
7 presented for the analysis of whole egg, with validation data obtained using the
8 guidelines contained in Commission Decision 2002/657/EC. The accumulation of
9 parent compound and side-chain metabolites in yolk, albumen and shell is presented
10 in order to illustrate, that for poultry eggs, the analysis of intact parent compound
11 residues is feasible as part of a residue monitoring programme. The procedure is less
12 complicated than the detection of metabolites, and samples are processed rapidly, thus
13 providing a satisfactory alternative to metabolite testing. This is particularly
14 important in the case of nitrofurazone, because the parent compound can be
15 determined instead of semicarbazide, which has been shown to be insufficiently
16 specific as an indicator for nitrofurazone use.
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39 The study of nitrofurans accumulation in chicken eggs was extended to include the
40 analysis of the drugs and metabolites in eggshell. There are several varied
41 applications for the utilisation of eggshell waste from poultry production. In the US,
42 scientists have produced dietary phosphorous supplements for poultry, pigs and pets
43 (Miller 2001). Eggshell has been used to replace limestone as a source of calcium,
44 and has been combined with phosphoric acid to produce mono- and di-calcium
45 phosphate. The use of eggshell powder as a source of calcium has also been
46 evaluated for human nutrition (Schaafsma et al. 2000). It has been demonstrated that
47 eggshell matrix proteins enhance calcium transport in human intestinal epithelial cells
48 (Daengprok et al. 2002). Collagen, derived from eggshell membrane, is used in
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3 cosmetics, wound dressings, surgical implants and intrusive device coatings. Gelatine
4 is used in the food and confectionery industries, and sialic acid is a precursor for use
5 in anti-inflammatory drugs. The utilisation of eggshell waste is therefore another
6 route potentially for nitrofurantoin contamination to reach the consumer.
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15 The work presented therefore has three important implications. Firstly, that
16 confirmation of nitrofurantoin intact parent compounds in eggs is possible using
17 LC-MS/MS. Secondly, that intact parent compounds accumulate in eggs and are
18 appropriate markers for monitoring nitrofurantoin abuse in laying chickens. Thirdly, that
19 nitrofurans accumulate in eggshell and because of the utilisation of eggshell waste
20 there exists the potential for nitrofurans to reach the consumer by this route.
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34 **EXPERIMENTAL**

35 **Materials**

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37 Furazolidone (FZD), nitrofurazone (NFZ), furaltadone (FTD) and nitrofurantoin
38 (NFT) were obtained from Sigma-Aldrich (Poole, UK). The metabolite compounds:
39 3-amino-2-oxazolidinone (AOZ) and semicarbazide (SEM) hydrochloride were
40 obtained from Sigma-Aldrich; 1-amino-hydantoin (AHD) hydrochloride and 3-amino-
41 5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ) were obtained from Chemical
42 Synthesis Services (Craigavon, UK). D₄-Furazolidone, D₅-furaltadone,
43 ¹³C¹⁵N₂-nitrofurazone, D₄-AOZ, and D₅-AMOZ were also obtained from Chemical
44 Synthesis Services. ¹³C¹⁵N₂-SEM and ¹³C₃-AHD were obtained from Witega (Berlin,
45 Germany). All other reagents and solvents were analytical and HPLC grade,
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Determination of parent nitrofurans residues in eggs.

Samples of whole egg, yolk, albumen (5 g) or shell (0.5 g) were placed in 30 ml glass centrifuge tubes. Internal standard solution (50 μl of 1 $\mu\text{g ml}^{-1}$ solutions in methanol) containing D⁴-FZD, D⁵-FTD and ¹³C ¹⁵N₂ -NFZ was added to each tube. Ethyl acetate (12 ml) was added, and samples were homogenised using a Silverson high-speed emulsifier for 1 min. Following centrifugation at 2200 rpm for 15 min., supernatants were transferred to clean tubes and evaporated to dryness under nitrogen at 50 °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 evaporated under nitrogen at 50 °C. The dry residue was redissolved in 500 μl of methanol-water (1:1, v/v), transferred to Eppendorf vials and centrifuged at 10 000 rpm for 10 min. Aliquots (140 μl) were transferred to autosampler vials prior to analysis by LC-MS/MS.

LC-MS/MS analysis of parent nitrofurans.

An Agilent 1100 Series HPLC system (Agilent Technologies, USA) coupled to a Quattro Ultima[®] Platinum tandem mass detector (Micromass, UK) incorporating MassLynx[®] software were used for nitrofurans analysis. The mass spectrometer was operated in electrospray positive mode for the analysis of FZD and FTD, and in electrospray negative mode for NFT and NFZ. Data acquisition was performed in multiple reaction monitoring (MRM) mode. The time-sectored ion transitions, dwell times, cone voltages and collision energies are shown in Table 1. The source settings were as follows: source and desolvation temperatures were 120 and 350°C, respectively, cone and desolvation gas flows were 99 and 800 L hr⁻¹, respectively,

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3 capillary 3.2 kV, RF lens 5.0. Argon was used as collision gas and the multiplier was
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5 operated at 650 V.
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10 The HPLC analytical column was a Columbus C18 (2) 5 μ m, 2.0 x 150 mm
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12 (Phenomenex, UK). A binary gradient mobile phase was used at a flow rate of 0.2 ml
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14 min⁻¹. Solvent A consisted of 0.5 mM ammonium acetate and methanol (80:20, v/v),
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16 and solvent B was methanol. The gradient ran from 10 % solvent B initially to a
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18 maximum of 80% after 8 min, was held until 15 min, returning to 10 % after 15.5
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20 min. The sample injection volume was 5 μ l. Nitrofurans concentrations in samples
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22 were calculated by comparing the ratio of the nitrofurans base peak to the internal
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24 standard peak with the same ratio in the calibration curve standards.
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32 **Validation of the method for parent nitrofurans in eggs.**

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34 The method for the determination of parent nitrofurans compounds was validated for
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36 whole egg homogenates in accordance with the guidelines stated in Commission
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38 Decision 2002/657/EC. Whole egg homogenate, fortified with each of the nitrofurans
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40 (FZD, FTD, NFT and NFZ) at concentrations of 2, 3 and 4 μ g kg⁻¹ were analysed on
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42 three separate occasions (n=7). Samples were quantified against standard calibration
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44 curves in the range, 0, 1, 2, 5, 10 and 20 μ g kg⁻¹.
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51 **Determination of nitrofurans side-chain metabolites in eggs using LC-MS/MS.**

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53 The protein-bound, side-chain metabolites for each of the nitrofurans were determined
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55 using the extraction procedure reported previously for the determination of AOZ in
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57 eggs (McCracken et al. 2001). Identification and quantification was performed using
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59 liquid chromatography tandem mass spectrometry. The mass spectrometric
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3 conditions used were developed under the auspices of EU project, QLK1-CT-1999-
4 00142 known as “FoodBRAND” (Cooper et al. 2005a). Briefly, egg homogenate,
5 albumen, yolk (1 g) or shell (0.5 g) was mixed in a 50 ml centrifuge tube with internal
6 standard solution containing D⁴-AOZ, D⁵-AMAZ, ¹³C ¹⁵N₂-SEM and ¹³C₃-AHD (50 μl
7 of 100 ng ml⁻¹), 4 ml of water, 0.5 ml of 1 M hydrochloric acid and 150 μl of
8 2-nitrobenzaldehyde (50 mM in dimethylsulphoxide). The tube was stoppered and
9 the mixture incubated in a water bath at 37 °C for 16 hours. Nitrofurans side-chain
10 metabolites (AOZ, AMAZ, SEM and AHD) present in the egg sample were
11 derivatised by the 2-nitrobenzaldehyde to produce the corresponding nitrophenyl
12 derivative. Following the incubation, the pH of the mixture was adjusted to
13 approximately 7.4 by the addition of 5 ml of 0.1 M dipotassium hydrogen
14 orthophosphate and 0.4 ml of 1M sodium hydroxide. The nitrophenyl derivatives
15 were extracted by shaking twice with 5 ml of ethyl acetate. The solvent was removed
16 under nitrogen at 50°C and the residue redissolved in 0.5 ml of methanol:water (1:1,
17 v/v). The sample was transferred to a centrifuge tube and centrifuged at 10 000 rpm
18 for 10 min. An aliquot of the supernatant was transferred to an autosampler vial for
19 LC-MS/MS analysis.

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 **Treatment of laying hens with nitrofurans.**

48 Four groups of six laying hens were held in wire-floored cages. They were allowed
49 *ad libitum* access to fresh water at all times. Each group was fed a standard layers
50 diet for three days prior to commencement of the experiment. After this period one
51 group was fed a layer feed medicated at 300 mg kg⁻¹ with furazolidone. In the same
52 way the other groups were fed with furaltadone, nitrofurantoin or nitrofurazone. After
53 feeding for 1 week and with no withdrawal from treatment, eggs were collected for 2
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3 days from each cage. They were separated into yolk, albumen and shell, and analysed
4 for the presence of bound residues of AOZ, AMOZ, SEM and AHD, and for parent
5 compounds. The analysis for parent compounds was performed on the day of
6 sampling.
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12 13 14 15 **RESULTS AND DISCUSSION**

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17 Method validation results for the analysis of parent nitrofurans are shown in Tables 2
18 and 3. Calibration curves for standard solutions were linear in the range 1-20 $\mu\text{g kg}^{-1}$
19 (Table 2). Decision Limits ($\text{CC}\alpha$) for the parents were $<1 \mu\text{g kg}^{-1}$ for all four
20 compounds (Table 3). Detection Capabilities ($\text{CC}\beta$) were lower for furazolidone and
21 furaltadone by comparison with nitrofurazone and nitrofurantoin. Nevertheless,
22 confirmation of residues was possible at $2 \mu\text{g kg}^{-1}$ for all four compounds in all of the
23 replicates analysed. Intra and inter run CVs are well within the acceptable values
24 predicted by the Horwitz equation (Commission Decision 2002/657/EC).
25 Concentrations for furazolidone, furaltadone and nitrofurazone were calculated using
26 the appropriate internal standard. An internal standard was not available for
27 nitrofurantoin, however concentrations were calculated using the D⁴ furazolidone.
28 For this reason, recovery values for nitrofurantoin were lower than the other 3
29 nitrofurans.
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51 There are numerous methods reported for the determination of nitrofuran residues in
52 eggs. Kumar et al. (1994) reported an LC method with UV detection. The limits of
53 detection were $1 \mu\text{g kg}^{-1}$ for nitrofurazone and furazolidone; and $2 \mu\text{g kg}^{-1}$ for
54 furaltadone. This procedure, however, would not meet present EU criteria as a
55 confirmatory method. Draisci et al. (1997) confirmed nitrofuran residues in eggs
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3 using LC-ion spray mass spectrometry with an atmospheric pressure ionisation
4 interface. Limits of detection were 3.2, 1.6 and 1.0 $\mu\text{g kg}^{-1}$ for nitrofurazone,
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8 furazolidone and furaltadone, respectively. Only single protonated-molecular ions
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10 were monitored, and again this would not meet EU criteria as a confirmatory method.
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12 Since 2002, and the start of the crisis related to nitrofurantoin contamination in poultry
13 and shellfish, most residues monitoring programmes have focussed upon the detection
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15 of the side-chain metabolites. This was justified on the basis of the stability *in vivo* of
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17 metabolite residues compared to the parent compounds. However, this is not
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19 necessarily the case when considering other matrices such as eggs. Botsoglou (1988)
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21 and McCracken et al. (2001) demonstrated that intact furazolidone residues
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23 accumulate in eggs and may still be detected as such up to 9 days after withdrawal of
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25 medicated feed. A Minimum Required Performance Limit (MRPL) of 1.0 $\mu\text{g kg}^{-1}$ has
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27 been set for all four side-chain metabolites in poultry meat (Commission Decision
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29 2003/181/EC). Subsequently, Commission Decision 2005/34/EC extended the MRPL
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31 definition to cover all matrices. No MRPL has been established for the nitrofurantoin
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33 parent drugs in eggs, or in any other tissue. The developed method may find two
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35 potential applications. Firstly, it may be used as the primary test used to detect
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37 nitrofurantoin residues in eggs. Secondly, it is quicker and simpler than methods for the
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39 determination of the side chain metabolites. It may also be used to back up the results
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41 obtained using the more widely used method for the detection of the side chain
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43 metabolites. Specifically, it might be used to confirm the presence of nitrofurazone
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45 following the finding of semicarbazide in eggs. This would provide unambiguous
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47 evidence for the administration of nitrofurazone to the chickens and would remove
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49 any possible ambiguity associated with the use of semicarbazide as a marker. Stability
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51 experiments performed at this laboratory and reported elsewhere indicate that while
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3 SEM has a depletion half-life in eggs of 2.4 days, the half-life for parent nitrofurazone
4 is 1.1 days (Cooper et al., 2006). Parent nitrofurazone was detected in eggs up to 3
5 days post-withdrawal when chickens were fed at only 1% of the therapeutic dose (3
6 mg kg⁻¹). In eggs pasteurised for 4 min. at 64-66 °C, followed by spray drying at 180-
7 230 °C, parent nitrofurazone depleted by 65%. It is possible, however that albumen
8 powder containing over 5 µg kg⁻¹, SEM may contain detectable quantities of parent
9 nitrofurazone.
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22 The distributions of parent compound and side chain metabolite residues in yolk,
23 albumen and shell for each nitrofurazone are shown in Table 4. For all four drugs,
24 residues of parent compounds accumulated in yolk, albumen and shell. The chemical
25 basis for the detection of AOZ, AMOZ, AHD and SEM means that any parent
26 compound present in the sample will also be measured as its corresponding metabolite
27 together with any other moiety containing acid-releasable AOZ, AMOZ, AHD or
28 SEM. In this study, where there was no withdrawal from treatment before eggs were
29 collected, it was expected that most of the residues occurring in the yolk and albumen
30 might be present as intact parent compound. This was not the case. In terms of the
31 number of moles detected, the concentrations of side-chain metabolites greatly
32 exceeded that of the parent compound for all four nitrofurans. This suggests that over
33 the 6-day treatment period, and despite zero withdrawal, most of the drug was present
34 not as the parent – but as other moieties which contain the intact side-chain.
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36 McCracken et al. (2001) reported that the concentration of furazolidone in egg
37 homogenates stored at –20°C fell by 44% after 55 days. In the present study however,
38 eggs were analysed immediately after collection for parent compound residues. Any
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3 degradation therefore, occurred during the period of treatment, and much of it prior to
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5 deposition in the egg.
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8 For furazolidone the albumen/yolk ratio of parent residues was 0.7 compared to 1.02
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10 of AOZ. Similar values were found for furaltadone: 0.82 of parent and 1.06 for
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12 AMOZ; and for nitrofurantoin: 0.83 parent compared to 1.01 for AHD. Residue
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14 concentrations of nitrofurantoin were lower than the other nitrofurans, once again
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16 reflecting the poor absorption of this drug in chickens (McCracken et al. 2005). The
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18 concentration of residues and their distribution in yolk and albumen was quite
19
20 different in the case of nitrofurazone. Higher concentrations of parent compound
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22 were detected in yolk (73%) compared to albumen (23%), giving an albumen/yolk
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24 ratio of 0.31. The albumen/yolk ratio for SEM was 0.55. Reasons for the difference
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26 in distribution between compounds may be attributable to the physiochemical
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28 characteristics of the drugs and to the physiology of the hen and the formation of the
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30 egg. Kan and Petz (2000) compiled a database from the literature on drug residues in
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32 egg yolk and white. White/yolk ratio values for the four nitrofurans compare
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34 favourably with those reported here, but the authors concluded that there was no
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36 single factor apparent that determines the distribution of drug between yolk and
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38 albumen. The difference between the yolk/albumen ratio for nitrofurazone and the
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40 other nitrofurans therefore may not be significant. Contrary to this, Kan and Petz also
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42 discussed the striking difference in distribution between yolk and white of narasin and
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44 salinomycin (white/yolk ratios of 0.31 and <0.007, respectively). These large
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46 molecules (molecular weights: 765 and 751 for narasin and salinomycin, respectively)
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48 differ only by one methyl group. Similarly, the differences in distribution of the
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50 nitrofurans may in part be due to the molecular structure of the compounds. The most
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52 striking difference in residue distribution of the nitrofurans however, was that 50% of
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3 the total SEM residues found in the egg were detected in the shell. A mean
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5 concentration of 24.2 nmol g⁻¹ of SEM was found in shell compared to only 0.24 nmol
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7 g⁻¹ of parent nitrofurazone. Eggshells used as negative controls were free of SEM,
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9 which eliminates the possibility of SEM occurring by any means other than
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11 nitrofurazone treatment of the laying birds. This concentration is significantly higher
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13 than for the side-chain metabolites of the other nitrofurans and illustrates the potential
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15 for SEM contamination to occur in eggshell products. The concentration of residues
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17 in shell were slightly higher for furazolidone compared to furaltadone but did not
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19 amount to more than 10% of the total residues in the egg. The result reported for NFT
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21 in shell represents detection of the drug in only 1 of the six samples tested.
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29 The utilisation of eggshell waste is a growing industry. Eggshell is mainly composed
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31 of calcium carbonate (94%), but also contains small amounts of magnesium
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33 carbonate, calcium phosphate and organic matter including protein. AOZ, AMOZ,
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35 AHD and SEM have been considered as part of the side-chain metabolites of the
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37 nitrofurans that are bound to protein in the tissues of the animal. It is not known
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39 whether all of the metabolites detected in the eggshell are bound to the eggshell
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41 proteins, but it remains a possibility. However, it is clear that nitrofurans residues
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43 accumulate in eggshell and therefore pose a threat to health, where eggshell products
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45 are consumed.
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53 In conclusion, residues of nitrofurans are deposited throughout the stages of egg
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55 production: in the development of yolk, in the albumen and during the shelling
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57 process. These residues, at least with zero withdrawal from treatment, can be detected
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59 as intact parent compound. The method presented is suitable as a confirmatory
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3 procedure and can be used together with the established method for the metabolites to
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6 form an effective residue control programme.
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Table 1. Data acquisition parameters for the Multiple Reaction Monitoring (MRM) of parent nitrofurans.

Compound	Ion transition	Dwell time (sec)	Cone voltage (V)	Collision energy (eV)
Function: 1: Electrospray positive mode (0 – 20 min)				
Furazolidone	226.2 > 139.2	0.40	35	13
	226.2 > 209.3	0.60	35	13
D ₄ FZD	230.2 > 139.2	0.30	35	13
Function 2: Electrospray negative mode (0 – 20 min)				
Nitrofurazone	197.2 > 124.2	0.40	35	7
	197.2 > 150.2	0.70	35	7
¹³ C ¹⁵ N ₂ NFZ	200.2 > 153.2	0.70	35	7
Nitrofurantoin	237.1 > 152.2	0.40	35	12
	237.1 > 194.2	0.70	35	8
Function 3: Electrospray positive mode (5 – 20 min)				
Furaltadone	325.3 > 252.4	0.20	35	15
	325.3 > 281.3	0.20	35	9
D ₅ -FTD	330.3 > 286.3	0.20	35	10

Table 2. Typical values for calibration of parent nitrofurantoin standard solutions in the range 1-20 $\mu\text{g kg}^{-1}$

	Typical equation of curve	Correlation coefficient (r)
Nitrofurazone	$y = 0.7872x - 0.033$	0.9989
Furazolidone	$y = 0.8322x + 0.194$	0.9971
Furaltadone	$y = 0.9698x + 0.206$	0.9993
Nitrofurantoin	$y = 0.0781x + 0.011$	0.9977

Table 3. Summary of validation results for the determination of parent nitrofurans in whole egg homogenate.

	Fortification Level ($\mu\text{g kg}^{-1}$)	Overall Mean ($\mu\text{g kg}^{-1}$) n=21	Overall recovery (%)	Within Day CV	Between Day CV
Furazolidone	2.0	2.2	110	6.4	7.4
	3.0	3.3	110	7.0	6.8
	4.0	4.5	113	5.7	9.0
	CCα	0.7 $\mu\text{g kg}^{-1}$			
	CCβ	1.2 $\mu\text{g kg}^{-1}$			
Furaltadone	2.0	2.3	115	6.7	6.2
	3.0	3.3	110	4.8	4.9
	4.0	4.6	115	4.5	7.6
	CCα	0.5 $\mu\text{g kg}^{-1}$			
	CCβ	0.8 $\mu\text{g kg}^{-1}$			
Nitrofurazone	2.0	2.3	115	9.6	11.8
	3.0	3.3	110	10.1	12.9
	4.0	4.6	115	7.0	12.1
	CCα	1.0 $\mu\text{g kg}^{-1}$			
	CCβ	1.7 $\mu\text{g kg}^{-1}$			
Nitrofurantoin	2.0	1.5	75	10.0	23.5
	3.0	2.3	77	7.3	6.9
	4.0	2.8	70	7.3	8.4
	CCα	1.0 $\mu\text{g kg}^{-1}$			
	CCβ	1.7 $\mu\text{g kg}^{-1}$			

Table 4. The distribution of nitrofurans parent compound and side chain metabolite residues in yolk, albumen and shell.

	Parent compounds (Mean \pm SE, n=6)			Side-chain metabolites (Mean \pm SE, n=6)		
	Yolk	Albumen	Shell	Yolk	Albumen	Shell
Furazolidone						
nmol g ⁻¹	1.82 \pm 0.30	1.29 \pm 0.16	0.23 \pm 0.03	4.68 \pm 0.57	4.81 \pm 0.66	1.12 \pm 0.38
Furaltadone						
nmol g ⁻¹	1.83 \pm 0.08	1.50 \pm 0.10	0.11 \pm 0.03	2.93 \pm 0.23	3.10 \pm 0.14	0.28 \pm 0.07
Nitrofurantoin						
nmol g ⁻¹	0.28 \pm 0.02	0.23 \pm 0.03	0.15	1.47 \pm 0.12	1.49 \pm 0.11	0.17 \pm 0.03
Nitrofurazone						
nmol g ⁻¹	4.18 \pm 0.59	1.30 \pm 0.13	0.24 \pm 0.04	15.13 \pm 0.81	8.44 \pm 1.19	24.2 \pm 2.97

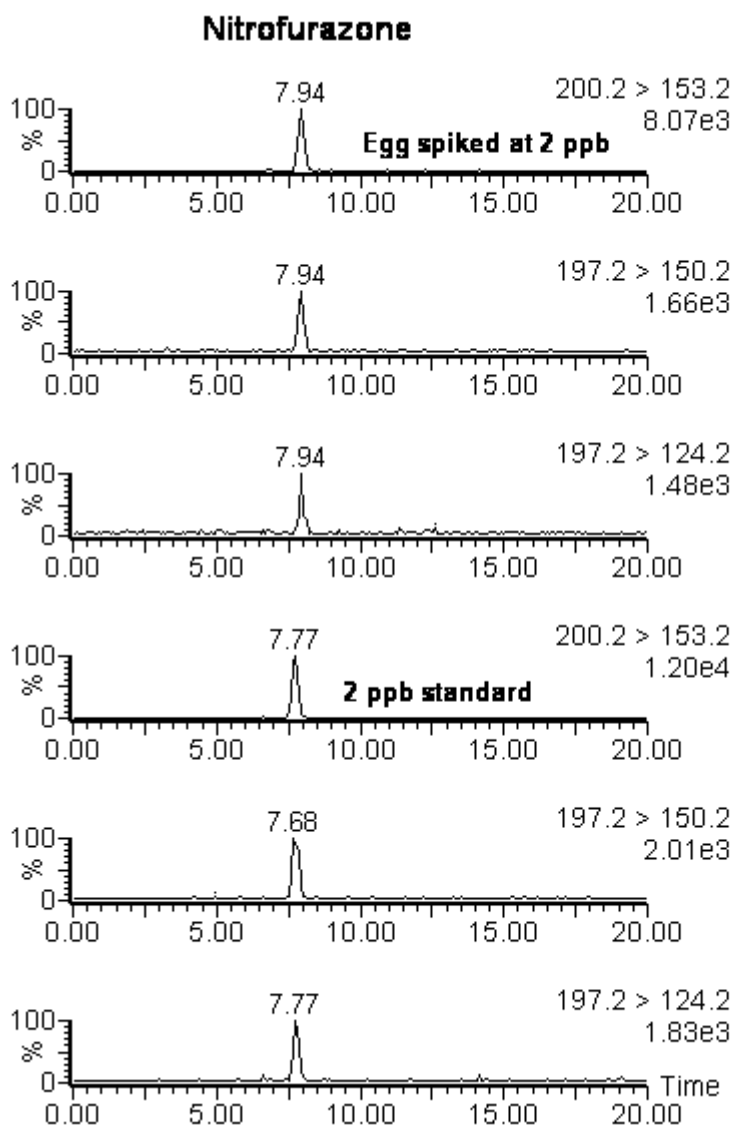


Figure 1a.

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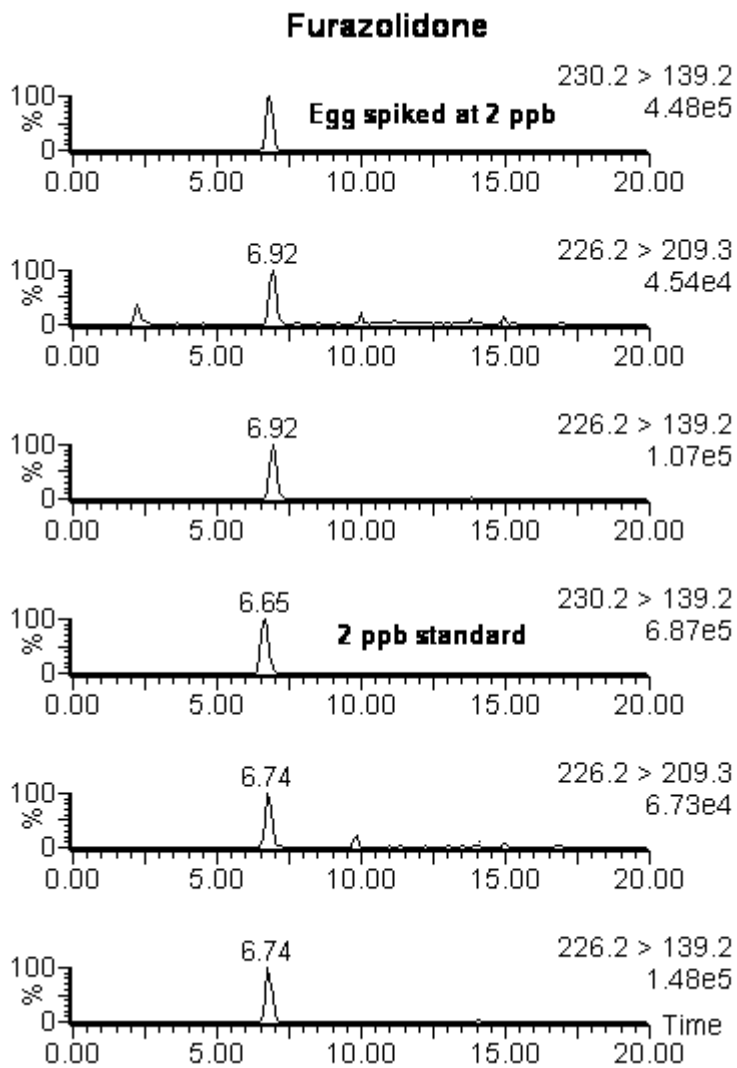


Figure 1b

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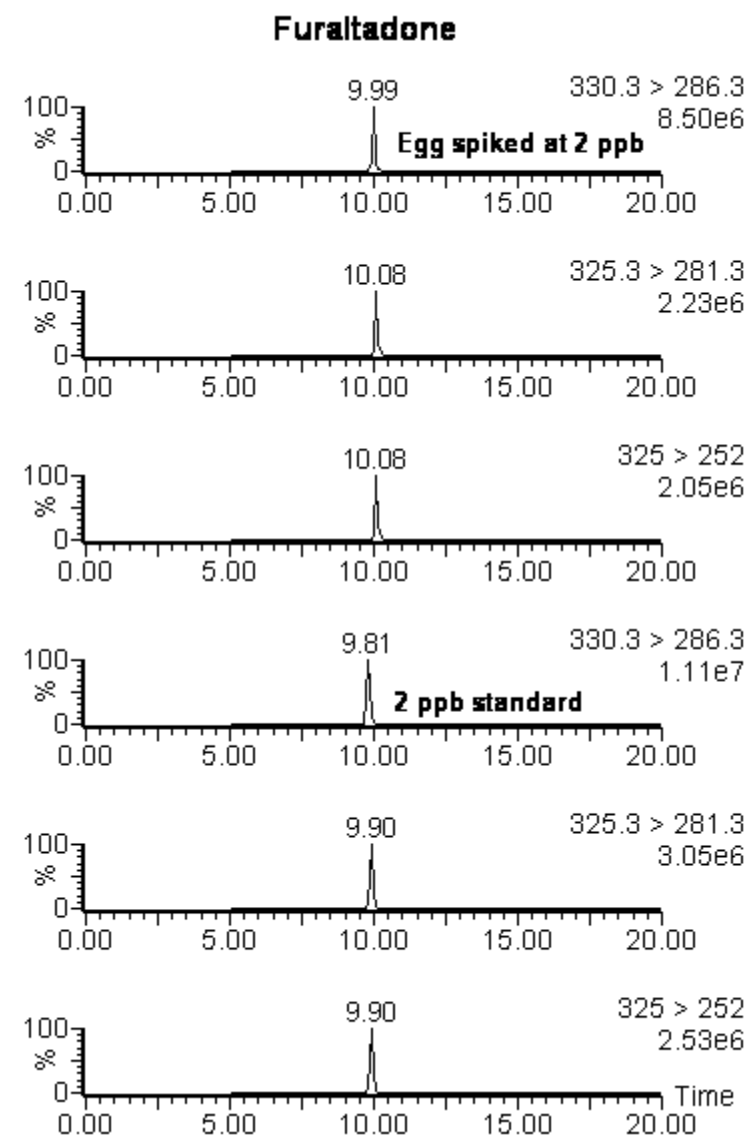


Figure 1c

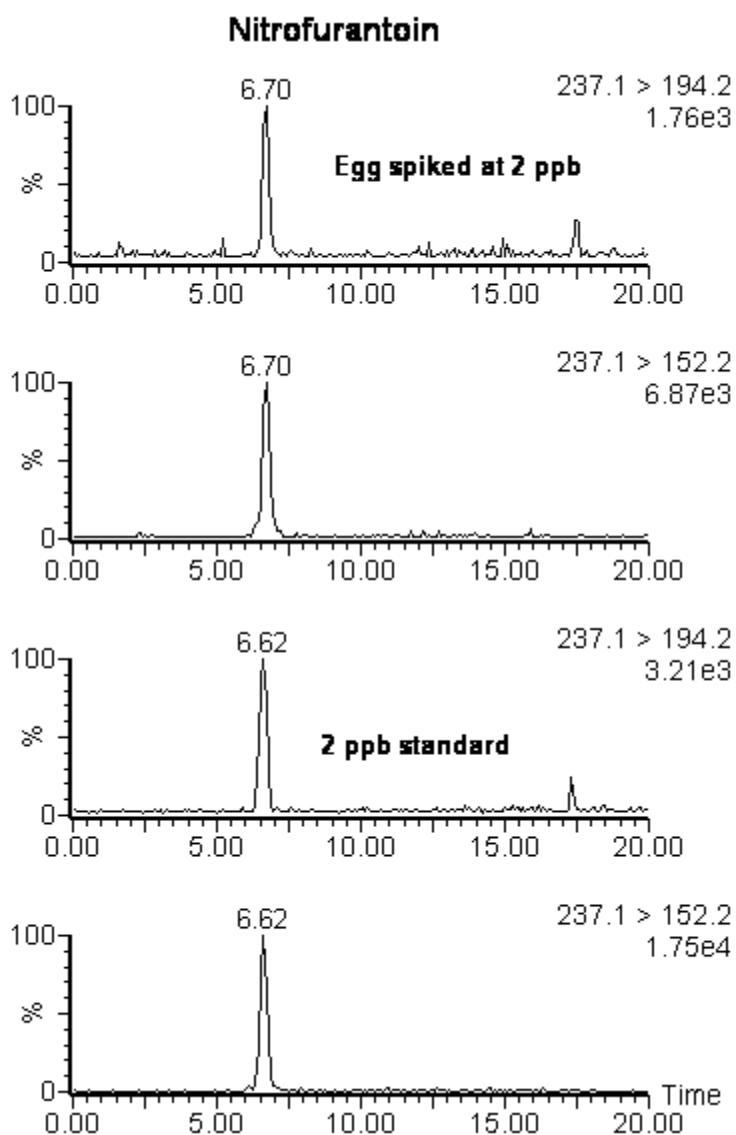


Figure 1d.

Legends to figures.

Figure 1a. Chromatograms (un-smoothed data) of MRM in electrospray negative mode of a blank egg extract spiked with nitrofurazone at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for nitrofurazone: $197.2>150.2$ and $197.2>124.2$; and $200.2>153.2$ corresponding to the internal standard $^{13}\text{C}^{15}\text{N}_2$ -nitrofurazone.

Figure 1b. Chromatograms (un-smoothed data) of MRM in electrospray positive mode of a blank egg extract spiked with furazolidone at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for furazolidone: $226.2>209.3$ and $226.2>139.2$; and $230.2>139.2$ corresponding to the internal standard D_4 -furazolidone.

Figure 1c. Chromatograms (un-smoothed data) of MRM in electrospray positive mode of a blank egg extract spiked with furaltadone at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for furaltadone: $325.3>281.3$ and $325>252$; and $330.3>286.3$ corresponding to the internal standard D_5 -furaltadone.

Figure 1d. Chromatograms (un-smoothed data) of MRM in electrospray negative mode of a blank egg extract spiked with nitrofurantoin at $2 \mu\text{g kg}^{-1}$, and similar chromatograms of a standard solution equivalent to $2 \mu\text{g kg}^{-1}$. Two transitions are shown for nitrofurantoin: $237.1>194.2$ and $237.1>152.2$.