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A study of the kinetics of semicarbazide and nitrofurazone in chicken eggs and egg powders

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4 1 **A study of the kinetics of semicarbazide and nitrofurazone in**
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6 2 **chicken eggs and egg powders**
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Abstract

The accumulation, depletion and partitioning of semicarbazide (SEM) and its parent compound nitrofurazone (NFZ) in eggs were studied using hens fed NFZ at therapeutic and sub-therapeutic levels. Dietary NFZ correlated strongly with NFZ and total SEM in eggs, while 28% of observed SEM was present in the form of parent NFZ. Depletion half-life in eggs was 2.4 days for SEM and 1.1 days for NFZ. NFZ accumulated preferentially in the yolk (57-63%) as opposed to albumen, while 71-80% of SEM was found in yolk. In whole egg, 29% of SEM was present as tissue-bound residues compared with 80% in breast muscle. Whilst NFZ and SEM were partly degraded by pasteurisation and spray drying, sufficient NFZ remained to suggest it may be detectable in egg powders when SEM is observed at low $\mu\text{g kg}^{-1}$ concentrations. NFZ was detectable in whole eggs during ingestion of only 0.1% of the therapeutic NFZ dose, making detection of intact NFZ in eggs a feasible means to prove conclusively the administration of this banned compound.

Keywords: Nitrofurans, Nitrofurazone, Semicarbazide, Eggs, LC-MS/MS.

Introduction

The nitrofurans are a group of antibiotics that, during the 1990s, were prohibited within the European Union (EU) for use in food-producing animals (Commission Regulation (EC) 1442/95, 1995) because of their potentially carcinogenic and mutagenic effects (Van Koten-Vermeulen *et al.*, 1993). They were used previously in livestock for prevention and treatment of various gastrointestinal infections and as growth promoters. The nitrofurantoin parent compounds are metabolised rapidly by animals *in vivo* (McCracken *et al.*, 1995; Cooper *et al.*, 2005a), however, persistent tissue-bound metabolites are formed which can be released by mild acid hydrolysis and which can be used as marker residues for monitoring the abuse of these drugs (Hoogenboom *et al.*, 1991, Cooper *et al.*, 2005a, Bock *et al.*, 2007). For example, the stable tissue-bound metabolite 3-amino-2-oxazolidinone is monitored as a marker residue for its parent nitrofurantoin, furazolidone. The four major nitrofurantoin antibiotics are furazolidone, furaltadone, nitrofurantoin and nitrofurazone. Their marker residues, under current EU legislation, are 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD) and semicarbazide (SEM) respectively.

The widespread use of nitrofurantoin drugs in food production, particularly in the poultry and aquaculture industries, was uncovered in 2001 by laboratories involved in the EU-funded R&D project FoodBRAND. Despite mandatory testing of chicken and prawns from Thailand, Vietnam and Brazil, instigated by the European Commission in 2002, nitrofurantoin use was shown to be more widespread than initially thought and continues to be a problem for both the consumer and 3rd country producers wishing to export foods to the EU. For example, furaltadone contamination of the Portuguese poultry

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4 66 industry led to the destruction of 1.5 million birds in 2003. The European
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6 67 Commission has since issued notifications to Member States via its Rapid Alert
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8 68 System for Food and Feed concerning findings of nitrofurans in a wide variety of food
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10 69 products including fish, prawns, poultry, egg powders and honey (European
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12 70 Commission RASFF system).

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16 71 Approximately half of the nitrofurans-related EU Rapid Alerts issued during this recent
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18 72 “nitrofurans crisis” have concerned the detection of semicarbazide (SEM), the marker
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20 73 residue for nitrofurazone (NFZ). However, the validity of SEM as a definitive marker
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22 74 for NFZ has been questioned following the discovery that SEM in food may arise
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24 75 from sources other than this illegal veterinary antibiotic. In 2003 SEM was found in
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26 76 jars of processed baby foods. Subsequent investigations demonstrated that the SEM
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28 77 arose as a breakdown product of azodicarbonamide (ADA), a blowing agent used in
29
30 78 the manufacture of the plastic gaskets inside the lids of these, and other, glass jars
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32 79 (Stadler *et al.*, 2004). ADA is also used as a flour treatment agent in some countries
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34 80 to improve the yield and quality of dough during the bread making process. This may
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36 81 explain the presence of SEM in a wide range of breaded meat products (Kennedy *et al.*,
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38 82 2004; Pereira *et al.*, 2004). Carrageenan, a polysaccharide derived from red
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40 83 seaweed, is used widely as a stabilising and thickening agent in the food industry and
41
42 84 has also been implicated as a potential source of SEM under certain conditions of
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44 85 bleaching (Hoenicke *et al.*, 2004). Clearly, SEM can no longer be considered a
45
46 86 definitive marker residue proving administration of NFZ to an animal. The focus of
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48 87 nitrofurans residue research has, therefore, shifted recently to elucidating the kinetics
49
50 88 of both NFZ and SEM, and identifying potential alternative markers of NFZ abuse.
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52 89 One solution would be the identification of NFZ itself in the tissues of NFZ-treated
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54 90 animals. NFZ does not accumulate in edible tissues of NFZ-treated animals (Cooper
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3 91 *et al.*, 2005a) but we have recently demonstrated that it can be detected in the avian
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6 92 eye (Cooper *et al.*, 2005b).
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9 93 We have also shown previously that the parent nitrofurantoin furazolidone accumulates in
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11 94 chicken eggs and is not broken down rapidly as is the case in liver and muscle
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13 95 (McCracken *et al.*, 2001). Subsequent trials in this laboratory have shown that NFZ is
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15 96 also present in eggs of NFZ-treated hens (McCracken and Kennedy, 2007). Since
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17 97 May 2003, residues of SEM have been reported in dried powders derived from whole
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19 98 egg, egg yolk, and lysozyme derived from egg albumen, produced in France, Mexico,
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21 99 Israel, Brazil and India (European Commission RASFF system). It is not known if
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23 100 these residues arose from tissue-bound metabolites, parent nitrofurantoin compounds or,
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25 101 indeed, contamination of the product during production. Whilst there is a zero
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27 102 tolerance approach to nitrofurantoin residues within the EU, the stability of residues
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29 103 during industrial processing of foods will be a crucial issue in reassuring consumers
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31 104 that such residues will not reach their plates. The effects of the pasteurisation and
32
33 105 spray drying processes on nitrofurantoin residues in egg are not known. The current study
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35 106 begins to address these issues by measuring the concentration of NFZ and SEM in egg
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37 107 from NFZ treated chickens before and after pasteurisation and spray drying.
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45 108 Given the widespread use of eggs and egg powders throughout the global food
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47 109 industry, there is a need for further detailed information on the accumulation,
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49 110 depletion, stability and partitioning of NFZ and SEM in chicken eggs. Such
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51 111 information will inform both the discussion on the health risks posed by this drug and
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53 112 its metabolite, and the search for a definitive test for NFZ use.
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Materials and Methods

114 *Materials and instrumentation*

115 Internal standards D₄-3-amino-2-oxazolidinone, D₅-3-amino-5-morpholinomethyl-2-
116 oxazolidinone, ¹³C₃-1-aminohydantoin and ¹³C¹⁵N₂-semicarbazide were supplied by
117 Witega Laboratorien Berlin-Adlershof (Berlin, Germany). Standard 3-amino-2-
118 oxazolidinone (AOZ), semicarbazide (SEM) and nitrofurazone (NFZ) were supplied
119 by Sigma-Aldrich (Poole, UK) and 3-amino-5-morpholinomethyl-2-oxazolidinone
120 (AMOZ) and ¹³C¹⁵N₂-nitrofurazone by Almac Group (formerly Chemical Synthesis
121 Services; Craigavon, UK). 1-Aminohydantoin (AHD) was a gift from Proctor and
122 Gamble Pharmaceuticals, USA. All other reagents and solvents were analytical and
123 HPLC grade, respectively.

124 Feed for laying hens was supplied by McLarnons Ltd. (Randalstown, UK). Muscle
125 and liver were minced in a Mini Prep Plus domestic food processor (Waring,
126 Torrington, CT, USA). Tissues were homogenised in a SL2 laboratory homogeniser
127 (Silverson Machines Ltd, Chesham, England). A 915-1 Universal digital penetration
128 probe thermometer was obtained from Testo AG (Lenzkirch, Germany). Eggs were
129 dried in a pilot scale spray drier manufactured by Niro Atomizer (Copenhagen,
130 Denmark). An Agilent 1100 Series HPLC system (Agilent Technologies, USA)
131 coupled to a Quattro Ultima[®] Platinum tandem mass detector (Micromass/Waters,
132 Manchester, UK), both operating under MassLynx[®] software, were used for sample
133 analysis. The mass spectrometer operated in electrospray positive mode and data
134 acquisition was in multiple reaction monitoring mode (MRM). The HPLC system
135 was equipped with a Columbus C18 (2), 5 μm, 2.0 x 150 mm column (Phenomenex,
136 UK) for separation of both parent nitrofurans and their metabolites. For metabolite

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3 137 analysis, a binary gradient mobile phase, at a flow rate of 0.2 ml min⁻¹, used 0.5 mM
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5 138 ammonium acetate and methanol (80 : 20 v/v, solvent A) and methanol (solvent B),
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8 139 starting at 95% A/5% B and changing linearly to 25% A/75% B in 6 min. These
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10 140 conditions were held until 11 min. Initial conditions were restored by 11.5 min. The
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12 141 total run time was 20 min. The precursor/product ions monitored during analysis of
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14 142 nitrofurans metabolites are listed in Table 1. The source settings were as follows:
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16 143 capillary voltage 2.45 kV, RF lens 25.0 V, source temperature 120 °C, desolvation
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18 144 temperature 350 °C, cone nitrogen gas flow
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20 145 80 L hr⁻¹, desolvation gas flow 670 L hr⁻¹. Argon was used as the collision gas and
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22 146 the multiplier was operated at 650 V. The cone voltage was 35 V and the collision
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24 147 energy (7 to 16 eV) changed during analysis depending on the analyte. Sample
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26 148 injection volume was 25 µl. LC-MS/MS conditions for nitrofurazone analysis were
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28 149 as described previously (McCracken and Kennedy, 2007).
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35 [INSERT TABLE 1 ABOUT HERE]
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38 151 *Treatment of laying hens with NFZ*

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41 152 Broiler breeder hens (30) in mid-lay, purchased from a local supplier, were housed in
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43 153 wire-floored cages in five separate batteries of six birds. Hens were allowed *ad*
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45 154 *libitum* access to fresh water and feed at all times. Commercial broiler breeder layer
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47 155 crumb feed was used to prepare five medicated diets (one at therapeutic and four at
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49 156 sub-therapeutic levels) containing 300, 30, 3, 0.3 and 0.03 mg NFZ per kg of feed.
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51 157 NFZ powder was added to feed in a rotary mixer at 300 mg kg⁻¹, mixed for 30 min
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53 158 and subsequently diluted serially ten-fold in unmedicated feed. The five groups were
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55 159 fed medicated diets for 16 days when, with the exception of the 300 mg kg⁻¹ group,
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57 160 the experiment was terminated. On day 16, diet of the birds fed nitrofurazone at a
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4 161 concentration of 300 mg kg⁻¹ was withdrawn and replaced with conventional
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6 162 unmedicated feed, which was fed to these birds for a further 16 days to study the rate
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8 163 of depletion of both NFZ and SEM from eggs. Throughout the entire study, eggs
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10 164 were collected daily. Blank control eggs were collected from the same birds before
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12 165 introduction of NFZ-medicated feeds (day zero). Eggs were homogenised, or
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14 166 separated into yolk and albumen, on the day of collection and stored at -80°C
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18 167 Egg contents intended for spray drying were added to pools of whole egg, yolk or
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20 168 albumen stored at -80°C throughout the trial. When sufficient material was collected,
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22 169 the pooled samples were thawed to room temperature. Yolk and albumen pools were
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24 170 diluted with further yolk and albumen from locally reared hens that had not received
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26 171 NFZ. The whole egg pool was homogenised and divided into 1.0 kg aliquots and
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28 172 refrozen. The egg albumen pool could not be homogenised mechanically without
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30 173 dramatically changing its consistency, so it was stirred thoroughly for 2 h before
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32 174 being divided into 1.0 kg aliquots and refrozen. Upon thawing, the consistency of the
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34 175 egg yolk pool was too thick to allow homogenisation. Consequently, it was diluted
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36 176 50:50 (w/w) with distilled water before being homogenised, divided into 0.8 kg
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38 177 aliquots and refrozen. Sub-samples of each aliquot were also retained at -80°C for
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40 178 analysis by LC-MS/MS.
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51 *Pasteurisation and spray-drying of NFZ incurred eggs*

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54 181 Laboratory scale pasteurisation equipment capable of heating 1 L of liquid egg was
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56 182 not available so samples were heated in a water bath as an approximation of the
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58 183 conditions applied in the industrial pasteurisation process. Legislation governing
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60 184 pasteurisation typically requires egg to be maintained at 64-66°C for a minimum of 3

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3 185 min. Each aliquot of egg, yolk and albumen was thawed in a 37°C water bath.
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5 186 Samples were then transferred to a water bath held at 67°C. Sample temperature was
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8 187 monitored with a digital probe thermometer while gently stirring. Sample
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10 188 temperature was held at 64 to 66°C for a minimum of 4 min before re-freezing at
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12 189 -80°C. All samples remained fluid following thawing and pasteurisation.
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16 190 Egg spray drying was carried out in the Food Technology Centre at Loughry Campus,
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18 191 College of Agriculture, Food and Rural Enterprise, in a pilot scale spray drier.
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20 192 Pasteurised liquid egg samples were warmed to approximately 37°C over water
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22 193 before applying drop wise to the pre-heated spray drier. Drier inlet temperatures were
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24 194 in the range 180 to 230°C and outlet temperatures were 70 to 80°C during the drying
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26 195 process. Internal drier pressure was set at 6 kg cm⁻². Powdered egg was collected,
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28 196 cooled to room temperature and weighed to give approximate dry matter content.
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30 197 Spray dried samples were stored at -80°C. To enable assessment of the stability of
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32 198 NFZ and SEM as a result of spray drying, residue concentrations in liquid egg, on a
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34 199 molar basis, were converted to concentrations per unit dry weight based on the
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36 200 observed dry matter contents for each sample.
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44 45 46 202 *LC-MS/MS analysis of NFZ in eggs*

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50 203 NFZ concentration in eggs was determined using the method of McCracken and
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52 204 Kennedy (2007). Briefly, 5 g samples of whole egg contents, fortified with
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54 205 ¹³C¹⁵N₂-NFZ internal standard, were homogenised in ethyl acetate (12 ml). Following
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56 206 centrifugation, supernatants were evaporated to dryness under nitrogen. Acetonitrile
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58 207 (5 ml) and hexane (3 ml) were added while vortexing. The hexane layer was
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60 208 discarded and the acetonitrile evaporated to dryness. The residue was redissolved in

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4 209 0.5 ml of 50 % (v/v) methanol-water, before being centrifuged at 13 000 rpm for 10
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6 210 min. Aliquots of the clear, lower layer were transferred to HPLC microvials for
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8 211 LC-MS/MS analysis. Highly concentrated samples were diluted as required for
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10 212 analysis. Prior to LC-MS/MS analysis, microvials were centrifuged at 13 000 rpm for
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12 213 15 min to remove precipitated material. Egg yolk (liquid) was treated as described
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14 214 above with the exception that samples were vortexed for 2 min in ethyl acetate rather
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16 215 than homogenised. Following the hexane wash step, acetonitrile extracts of egg
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18 216 albumen (liquid) were transferred by Pasteur pipette to clean tubes to remove
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20 217 precipitated solids prior to evaporation. Egg powders were treated in the same
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22 218 manner as liquid samples, except for spray-dried albumen. Following
23
24 219 homogenisation and centrifugation of powdered albumen in ethyl acetate, supernatant
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26 220 was transferred to clean tubes by Pasteur pipette, centrifuged a second time and
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28 221 transferred again to clean tubes prior to evaporation. LC-MS/MS analysis was
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30 222 performed in electrospray negative mode using the following transitions: 197.2>124.2
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32 223 and 197.2>150.2 for NFZ, and 200.2>153.2 for $^{13}\text{C}^{15}\text{N}_2$ -NFZ. The Decision Limit
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34 224 (CC α) for NFZ in egg by this method was 1.0 $\mu\text{g kg}^{-1}$ and the detection capability
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36 225 (CC β) was 1.7 $\mu\text{g kg}^{-1}$ (McCracken and Kennedy 2007).
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227 *LC-MS/MS analysis of nitrofurans metabolites in eggs*

51 228 This analytical method is based on that of McCracken *et al.* (2001) for total AOZ
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53 229 residues (sum of solvent-extractable and tissue-bound metabolites) in egg. That
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55 230 protocol was expanded and validated to include AOZ, AMOZ, AHD and SEM in
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57 231 whole egg contents. Secondary validation (repeatability) was also performed on egg
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59 232 albumen and yolk separately. While the recommendation from the Community
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3 233 Reference Laboratory for nitrofurans is to monitor SEM in the form of tissue-bound
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5 234 residues, for the purposes of regulatory control, it was inappropriate to use that
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8 235 analytical approach with eggs in the current study. It was essential to study the total
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10 236 SEM concentration in eggs to enable a comparison to be made between total NFZ
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12 237 concentration and total SEM content of the egg. Were tissue-bound SEM to be
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15 238 measured, a poorer, and possibly erroneous, correlation may have been found between
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17 239 NFZ and SEM.

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20 240 Samples (1.00 ± 0.02 g) were weighed into 30 ml glass tubes. Mixed internal
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22 241 standard (50 μ l of a 100 ng ml⁻¹ solution of D₄-AOZ, D₅-AMOZ, ¹³C₃-AHD and
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24 242 ¹³C¹⁵N₂-SEM) was added to all samples, controls and standards. To all tubes were
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26 243 added hydrochloric acid (0.1 M, 5 ml) and 2-nitrobenzaldehyde (150 μ l, 50 mM in
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28 244 DMSO). Calibration standards were prepared by addition of mixed standard solutions
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30 245 to 30 ml glass tubes. Egg samples from nitrofuran-free chickens were fortified at
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32 246 1.0 μ g kg⁻¹ with mixed standard (100 μ l of a 10 ng ml⁻¹ solution) to act as recovery
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34 247 control samples. Blank egg samples were also included in every analytical run.
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36 248 Tubes were capped, vortex mixed for 20 sec and incubated overnight (approximately
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38 249 16 hr) in a water bath held at 37°C.

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41 250 All tubes were then adjusted to pH 7.2 ± 0.2 with 0.1 M di-potassium hydrogen
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43 251 orthophosphate (5 ml) and 1 M sodium hydroxide (0.4 ml). Liquid-liquid extraction
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45 252 was carried out using ethyl acetate (2 x 6 ml, repeatedly inverting the tubes for 1 min
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47 253 then centrifuging at 2000 rpm for 15 min) and the organic phase evaporated to
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49 254 dryness at 45°C under a stream of nitrogen. Residues were re-dissolved by vortex
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51 255 mixing for 1 min in methanol : water (50 : 50 v/v; 0.4 ml). Samples were transferred
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53 256 to 0.5 ml plastic centrifuge tubes and centrifuged at 13 000 rpm for 10 min. Aliquots

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4 257 (200 µl) of the clear, lower layer were transferred to HPLC microvials for LC-MS/MS
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6 258 analysis. Prior to LC-MS/MS analysis, microvials were centrifuged at 13 000 rpm for
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8 259 15 min to remove precipitated material. Highly concentrated samples were diluted as
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10 260 required for analysis. Egg powders were treated in the same manner as liquid
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12 261 samples, with the exception of spray-dried egg albumen. Powdered albumen required
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14 262 8 ml of hydrochloric acid to keep samples fluid during derivatisation and 8 ml of di-
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16 263 potassium hydrogen orthophosphate for neutralisation. During extraction into ethyl
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18 264 acetate, gently mixing only was necessary to avoid creation of a suspension. A third
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20 265 ethyl acetate extraction (6 ml) was necessary for recovery of sufficient supernatant
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22 266 from powdered albumen. LC-MS/MS analysis of nitrofurans metabolites was carried
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24 267 out using the conditions described above. Analyte concentrations in samples were
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26 268 calculated by comparing the ratio of an analyte base peak response to its appropriate
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28 269 internal standard response with the same ratio in calibration curve standards (0, 0.25,
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30 270 0.5, 1, 2 and 5 µg kg⁻¹).

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37 271 A limited number of eggs were also analysed for tissue-bound (non-extractable) SEM
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39 272 residues. Prior to the addition of hydrochloric acid and 2-nitrobenzaldehyde
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41 273 described above, samples (1 g) were repeatedly washed by vortex mixing for 10 sec in
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43 274 ice cold methanol (3 x 4 mL), ethanol (2 x 4 mL) and diethyl ether (2 x 4 mL), with
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45 275 centrifugation at 2000 rpm for 10 min between washes.

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53 277 *Validation of LC-MS/MS method for total nitrofurans metabolites in eggs*

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57 278 In accordance with the European Union's guidelines on analytical method validation
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59 279 for residue analysis (Commission Decision 2002/657/EC, 2002) that sets out the
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280 quality criteria for analytical methods for the analysis of veterinary drug residues in

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4 281 food of animal origin, the following validation was performed on the LC-MS/MS
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6 282 method for total nitrofurans metabolites in egg. Blank whole egg homogenate was
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8 283 fortified with each of the nitrofurans metabolites (AOZ, AMOZ, AHD and SEM) at
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10 284 concentrations of 0.5, 1.0 and 1.5 $\mu\text{g kg}^{-1}$, which correspond to half, one, and one and
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12 285 half times the EU Minimum Required Performance Limit (MRPL) of 1 $\mu\text{g kg}^{-1}$ for
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14 286 nitrofurans metabolites (Commission Decision 2003/181/EC, 2003). Eight replicates
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16 287 at each concentration were analysed on three separate occasions, each replicate being
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18 288 based on the fortification of a different nitrofurans-free egg sample. A total of 20
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20 289 different nitrofurans-free egg samples were also analysed in the course of this
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22 290 validation study. Secondary validation was also performed to measure the
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24 291 repeatability of the method when applied to egg albumen (white) and yolk. Albumen
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26 292 and yolk were fortified at 0.5 and 1.5 $\mu\text{g kg}^{-1}$ (n=7 at each concentration) and
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28 293 analysed on one occasion.
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Results and Discussion

Analytical method & performance characteristics

Summary method validation data for the analysis of nitrofurans metabolites in egg are shown in Table 2. Decision limits ($CC\alpha$) and detection capabilities ($CC\beta$) were $<0.6 \mu\text{g kg}^{-1}$ for AOZ, AMOZ, AHD and SEM. Within and between-run CVs were within the acceptable values predicted by the Horwitz equation (Commission Decision 2002/657/EC, 2002). All four nitrofurans metabolites can be quantified and confirmed below the EU Minimum Required Performance Limit of $1 \mu\text{g kg}^{-1}$ making this method suitable for confirmatory analysis in whole egg. Secondary validation of the method with egg yolk and albumen yielded recovery and within-run CVs comparable to those for whole egg (data not shown).

[INSERT TABLE 2 ABOUT HERE]

Accumulation of NFZ and SEM in eggs

The concentrations of both total SEM and NFZ rose rapidly in eggs, following the introduction of the medicated diets. Plateau concentrations of both compounds reached an approximate “steady state” after about 4 days feeding, irrespective of the dietary NFZ concentration (figures 1 and 2). These figures also illustrate that a significant proportion of the SEM detected in whole eggs was present in the form of parent NFZ. The chemical basis for the detection of SEM by LC-MS/MS ensures that any parent NFZ present in a sample will be measured as SEM as will any other moiety that is present, capable of releasing SEM following mild acid hydrolysis. One molecule of NFZ yields one molecule of SEM. If calculated on a molar basis,

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3 317 approximately 28% of the SEM present in eggs, during the period when residue
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5 318 concentrations were in a steady-state (days 4 to 16), was present as NFZ. NFZ
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7 319 concentrations in eggs from the 0.03 NFZ mg kg⁻¹ group were below the decision
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9 320 limit of the analytical method whilst total SEM was detectable only sporadically in
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11 321 this group, the mean concentration being 0.54 µg kg⁻¹. For this reason, these data are
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13 322 not presented on figures 1 and 2.
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19 323 [INSERT FIGURES 1 AND 2 ABOUT HERE]
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22 324 There was a strong linear correlation between the dietary NFZ concentration and the
23
24 325 plateau NFZ concentration (mean of all results between days 4 and 16) in whole eggs.
25
26 326 The dietary NFZ concentration (x, in mg kg⁻¹) was related to NFZ in eggs (y, in µg
27
28 327 kg⁻¹) as follows: $y = 2.3x + 1.4$ ($r^2 = 0.999$). A similar strong correlation was
29
30 328 observed between the dietary NFZ concentration (x in mg kg⁻¹) and the total SEM
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32 329 concentration in eggs (y in µg kg⁻¹): $y = 2.8x + 8.6$ ($r^2 = 0.999$), over the same time
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34 330 period.
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41 42 43 332 *Depletion of NFZ and SEM from eggs*

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46 333 The depletion of NFZ and total SEM from whole egg following withdrawal of 300 mg
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48 334 kg⁻¹ NFZ-medicated feed is shown in figure 3. The depletion half life of total SEM,
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50 335 calculated over the 16 day withdrawal period, was 2.4 days. Following this period,
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52 336 significant concentrations of SEM were still detectable in whole eggs (17.0 ± 4.9 µg
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54 337 kg⁻¹, mean \pm se). Standard errors (se) were calculated throughout as standard
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56 338 deviation divided by the square root of the number of samples tested. The depletion
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58 339 half-life of NFZ calculated over the first 6 days of withdrawal was 1.1 days, with the
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4 340 parent drug becoming undetectable following 8 days withdrawal. Given the more
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6 341 rapid clearance of NFZ from eggs, the proportion of SEM present in the form of
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8 342 parent NFZ (calculated on a molar basis) during this depletion phase dropped from
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10 343 25 % at zero withdrawal to 4 % following 6 days withdrawal.

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14 344 [INSERT FIGURE 3 ABOUT HERE]

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21 346 *Partitioning of NFZ and SEM between yolk and albumen*

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23
24 347 Table 3 shows the distribution of NFZ and SEM between the egg yolk and albumen
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26 348 compartments at three dietary NFZ levels when no withdrawal period was observed.
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28 349 Both parent and metabolite, but particularly SEM, were found preferentially in the
29
30 350 yolk. At the three dietary concentrations studied, approximately 75 % of SEM and
31
32 351 60 % of NFZ was located in the yolk. Table 3 also shows the percentage of SEM
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34 352 which was present as NFZ (calculated on a molar basis). Irrespective of the dietary
35
36 353 NFZ concentration, approximately 16 % of SEM in yolk was present as NFZ,
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38 354 compared with 36 % in albumen. This is consistent with the average value, reported
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40 355 above, that 28 % of SEM in whole egg is present as parent NFZ.

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46 356 [INSERT TABLE 3 ABOUT HERE]

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53 358 *Total versus tissue-bound SEM*

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56 359 This laboratory, which acted as the co-ordinating laboratory for the EU funded
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58 360 FoodBRAND project, strongly advocates the use of bound residue analysis for the
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60 361 regulatory control & monitoring of the nitrofurans drugs, particularly in the case of

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3 362 SEM. This study was exceptional, since we were interested in exploring the
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5 363 relationship between the accumulation of nitrofurazone in eggs and making
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7 364 comparisons with the accumulation of SEM. Accordingly, most of the analyses
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9 365 described here relate to total SEM – as it is presumed that tissue bound SEM is not
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11 366 nitrofurazone itself, but some metabolite formed from it, capable of forming covalent
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13 367 bonds with the protein matrix. Nonetheless, we were interested in determining the
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15 368 relative amount of SEM in egg that was present in the free- and tissue-bound forms.
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17 369 This is shown in Table 4. Tissue-bound SEM in eggs represents 28 and 31 % of the
18
19 370 total SEM in eggs derived from birds fed NFZ at 3 and 30 mg kg⁻¹, respectively. The
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21 371 concentration of total SEM (mean ± se) in the breast muscle of the 6 birds fed NFZ at
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23 372 0.3 mg kg⁻¹ was 4.2±0.3 µg kg⁻¹ and that of tissue-bound SEM was 3.3±0.3 µg kg⁻¹,
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25 373 indicating that approximately 80 % of the SEM was tissue-bound. This contrasts
26
27 374 markedly with the situation in eggs collected from the same birds. It is probable that
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29 375 the presence of NFZ in the eggs, which does not contribute to tissue-bound SEM
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31 376 accounts for the major part of the difference between these two figures.
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39 377 Despite finding that the greater proportion of SEM in eggs is not tissue-bound, it
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41 378 remains the case that regulatory laboratories should continue to test for bound
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43 379 nitrofurazone residues, especially in the case of SEM, to minimise the potential of
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45 380 inappropriate conclusions being drawn from the finding of SEM in eggs following an
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47 381 analysis for total residues - given the potential non-NFZ sources of SEM, as described
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49 382 in the introduction.
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55 383 [INSERT TABLE 4 ABOUT HERE]
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4 385 *Degradation of NFZ and SEM caused by spray drying*
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7 386 Retention of some egg powder within the spray drier mechanisms prevented accurate
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9 387 calculation of individual sample dry matter contents. However, the approximate
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11 388 values obtained (23 % dry weight for whole egg, 43 % for yolk, and 9 % for albumen)
12
13 389 compare favourably with standard values for egg solids (25 % for whole egg, 52 %
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15 390 for yolk, and 12 % for albumen). The quoted value of 52% dry weight for pure yolk
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17 391 is a conversion from the value of 45% solids for commercial yolk products which
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19 392 typically contain 17 % egg albumen.
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24 393 The effect of pasteurisation and spray drying on the concentrations of NFZ and total
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26 394 SEM in whole egg, yolk and albumen is shown in Table 5. Parent NFZ was
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28 395 considerably less stable than its metabolite SEM under the rigours of processing to
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30 396 dried powder form. Most of the NFZ in albumen was destroyed as was almost half
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32 397 the NFZ in yolk. This equated to a loss of 65 % of the NFZ in whole egg. It is,
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34 398 perhaps, surprising that major losses of SEM were also observed during processing.
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36 399 Some 72% of the SEM present in albumen was destroyed, leading to a loss of half the
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38 400 SEM in whole egg. This contrasts markedly with the finding that less than 15 % of
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40 401 SEM in pig muscle is destroyed by the cooking process reported by Cooper and
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42 402 Kennedy (2007). It may be postulated that the higher temperatures involved in spray
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44 403 drying caused SEM to react with other matrix components with the result that SEM
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46 404 could not be released by the mild acid hydrolysis applied during the analytical
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48 405 procedure. Residues of both NFZ and SEM were less susceptible to degradation
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50 406 when present in yolk as opposed to albumen. It should be noted that direct
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52 407 comparisons cannot be made in Table 5 between residue concentrations in whole egg,
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3 408 yolk and albumen because these pooled samples were derived from different
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6 409 populations of eggs.
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9 [INSERT TABLE 5 ABOUT HERE]
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12 411 Powdered egg albumen is the dried egg product most widely used in the food
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14 412 industry. Given the recently identified causes of non-NFZ-related SEM in food (e.g.
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16 413 azodicarbonamide and carrageenan), it would be beneficial to regulatory authorities
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18 414 and egg processors to be able to detect parent NFZ in egg powders which had tested
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20 415 positive for SEM. The LC-MS/MS method employed in this study, to detect NFZ in
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22 416 egg powders containing several hundred $\mu\text{g kg}^{-1}$, has not yet been adapted for use
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24 417 with egg powders containing low $\mu\text{g kg}^{-1}$ NFZ concentrations. However, the method
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26 418 used herein for NFZ analysis of liquid egg has Decision Limit ($CC\alpha$) of around
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28 419 $1 \mu\text{g kg}^{-1}$. If a similar sensitivity can be achieved in the analysis of powdered egg, the
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30 420 present data suggest that egg albumen powder containing in excess of $5 \mu\text{g kg}^{-1}$ SEM
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32 421 may also contain detectable amounts of parent NFZ. Detection of NFZ in whole egg
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34 422 and yolk powders is more promising. Following spray drying, the concentration of
35
36 423 NFZ fell to approximately one third of the remaining SEM concentration in yolk and
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38 424 one half in whole egg (Table 5). Consequently, yolk powder containing in excess of 3
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40 425 $\mu\text{g kg}^{-1}$ SEM and whole egg powder in excess of $2 \mu\text{g kg}^{-1}$ SEM may contain
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42 426 detectable amounts of NFZ.
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51 427 McCracken and Kennedy (2007) demonstrated the accumulation of intact NFZ in
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53 428 eggs when therapeutic doses of NFZ (300 mg kg^{-1} feed) were administered. We have
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55 429 now shown that NFZ is also detectable in eggs at sub-therapeutic (contamination)
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57 430 levels. When only 1% of the therapeutic dose is administered (3 mg kg^{-1} group), NFZ
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59 431 may be detectable in eggs for up to 3 days after withdrawal of contaminated feed
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3 432 (based on NFZ half-life of 1.1 days). Furthermore, NFZ is detectable in whole eggs
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5 433 even during ingestion of only 0.1% of the therapeutic NFZ dose (Fig. 1, 0.3 mg kg⁻¹
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8 434 group). This report may now enable proof of nitrofurazone use in egg layers and in
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10 435 processed products derived from the eggs.
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441 semicarbazide analysis" (2004/S39-033708). Grateful thanks are also due to Mark
442 Conlon (AFBI) for diligent chicken husbandry and sample processing.

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3 506 Table 1. LC-MS/MS precursor/product ion combinations (base peaks underlined)
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6 507 monitored in MRM ESI positive mode for the analysis of the nitrophenyl (2-NP)
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8 508 derivatives of four nitrofuran metabolites.
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10 509

| Compound | Precursor ion (m/z) | Product ions (m/z) |
|---|---------------------|----------------------|
| 2-NP-AOZ | 236.3 | 104.3, <u>134.3</u> |
| 2-NP-AMOZ | 335.3 | 262.3, <u>291.4</u> |
| 2-NP-AHD | 249.3 | 104.3, <u>134.2</u> |
| 2-NP-SEM | 209.2 | <u>166.2</u> , 192.2 |
| 2-NP-D4-AOZ (internal standard) | 240.3 | 134.3 |
| 2-NP-D5-AMOZ (internal standard) | 340.3 | 296.4 |
| 2-NP- ¹³ C ₃ -AHD (internal standard) | 252.0 | 134.0 |
| 2-NP- ¹³ C ¹⁵ N ₂ -SEM (internal standard) | 212.2 | 195.3 |

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4 510 Table 2. Summary of validation data for the determination of four nitrofuran
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6 511 metabolites in whole egg homogenate.
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| | Fortification Level $\mu\text{g kg}^{-1}$ | Overall Mean $\mu\text{g kg}^{-1}$ (n=24) | Overall recovery (%) | Within run CV | Between run CV | CCα ($\mu\text{g/kg}$) | CCβ ($\mu\text{g/kg}$) | |
|-------------|---|---|-------------------------------------|------------------------------|-------------------------------|--|---|------|
| AOZ | 0.5 | 0.51 | 102 | 7.4 | 1.7 | AOZ | 0.14 | 0.44 |
| | 1.0 | 1.03 | 103 | 4.5 | 1.6 | | | |
| | 1.5 | 1.54 | 103 | 4.2 | 1.4 | | | |
| AMOZ | 0.5 | 0.53 | 105 | 3.0 | 2.9 | AMOZ | 0.13 | 0.25 |
| | 1.0 | 1.07 | 107 | 3.1 | 3.9 | | | |
| | 1.5 | 1.60 | 107 | 3.5 | 1.5 | | | |
| AHD | 0.5 | 0.52 | 103 | 5.8 | 11.5 | AHD | 0.21 | 0.60 |
| | 1.0 | 1.02 | 102 | 6.3 | 11.6 | | | |
| | 1.5 | 1.57 | 104 | 7.1 | 4.5 | | | |
| SEM | 0.5 | 0.52 | 104 | 5.5 | 1.6 | SEM | 0.19 | 0.28 |
| | 1.0 | 1.01 | 101 | 5.2 | 1.5 | | | |
| | 1.5 | 1.47 | 98 | 3.9 | 1.9 | | | |

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4 513 Table 3. Partitioning of NFZ and SEM between egg yolk and albumen at different
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6 514 dietary NFZ levels with no withdrawal period (se = standard error).
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9 515

| | Mean NFZ $\mu\text{g kg}^{-1}$ | se (n=5) | NFZ Partitioning (%) | Mean total SEM $\mu\text{g kg}^{-1}$ | se (n=5) | Total SEM Partitioning (%) | Molar % of SEM present as NFZ |
|--|--------------------------------------|-------------|----------------------------|--|-------------|----------------------------------|-------------------------------------|
| 3 mg NFZ kg^{-1} feed | | | | | | | |
| Yolk | 10.8 | 0.7 | 63 | 27.8 | 2.3 | 79 | 15 |
| Albumen | 6.4 | 0.7 | 37 | 7.3 | 0.6 | 21 | 33 |
| 30 mg NFZ kg^{-1} feed | | | | | | | |
| Yolk | 81.6 | 16.2 | 57 | 224.0 | 29.2 | 80 | 14 |
| Albumen | 61.9 | 11.3 | 43 | 56.8 | 7.3 | 20 | 41 |
| 300 mg NFZ kg^{-1} feed | | | | | | | |
| Yolk | 712.9 | 112.3 | 58 | 1441.8 | 192.0 | 71 | 19 |
| Albumen | 515.2 | 58.7 | 42 | 575.1 | 77.8 | 29 | 34. |

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3 516 Table 4. Distribution of tissue-bound and total SEM in whole egg homogenates at
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6 517 two dietary NFZ levels (se = standard error).
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| NFZ in feed mg kg⁻¹ | Mean total SEM µg kg⁻¹ | se | Mean tissue-bound SEM µg kg⁻¹ | se | % Tissue- bound | n |
|---|--|-----------|---|-----------|----------------------------------|----------|
| 3 | 13.7 | 0.4 | 3.8 | 0.2 | 28 | 10 |
| 30 | 113.5 | 12.4 | 35.1 | 4.0 | 31 | 5 |

519 Table 5. Effect of pasteurisation and spray drying on NFZ and SEM concentrations in eggs (se = standard error). Whole egg, yolk and albumen
 520 pools were derived from different sample populations so concentrations cannot be compared across sample type.

521

| | NFZ $\mu\text{g kg}^{-1}$ dry weight | | | | | SEM $\mu\text{g kg}^{-1}$ dry weight | | | | | Molar % of SEM present as NFZ | | n |
|------------------|--------------------------------------|-------|--------------|------|---------------|--------------------------------------|-------|--------------|------|---------------|-------------------------------|--------------|---|
| | Before drying | se | After drying | se | % loss of NFZ | Before drying | se | After drying | se | % loss of SEM | Before drying | After drying | |
| Whole egg | 502.1 | 23.7 | 177.6 | 35.4 | 65 | 676.5 | 25.8 | 330.7 | 30.0 | 51 | 28 | 20 | 3 |
| Yolk | 267.6 | 15.1 | 151.9 | 3.6 | 43 | 608.7 | 17.7 | 507.6 | 3.7 | 17 | 17 | 11 | 4 |
| Albumen | 1085.6 | 203.7 | 44.5 | 6.8 | 96 | 954.3 | 148.1 | 264.5 | 1.8 | 72 | 43 | 6 | 4 |

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4 522 **FIGURE CAPTIONS**

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9 524 Figure. 1. Accumulation of nitrofurazone in whole egg homogenates of chickens fed
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11 525 four dietary nitrofurazone levels (0.3 to 300 mg kg⁻¹ NFZ in feed). Data are means ±
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13 526 standard error.

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18 528 Figure 2. Accumulation of total semicarbazide in whole egg homogenates of chickens
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20 529 fed four dietary nitrofurazone levels (0.3 to 300 mg kg⁻¹ NFZ in feed). Data are
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22 530 means ± standard error.

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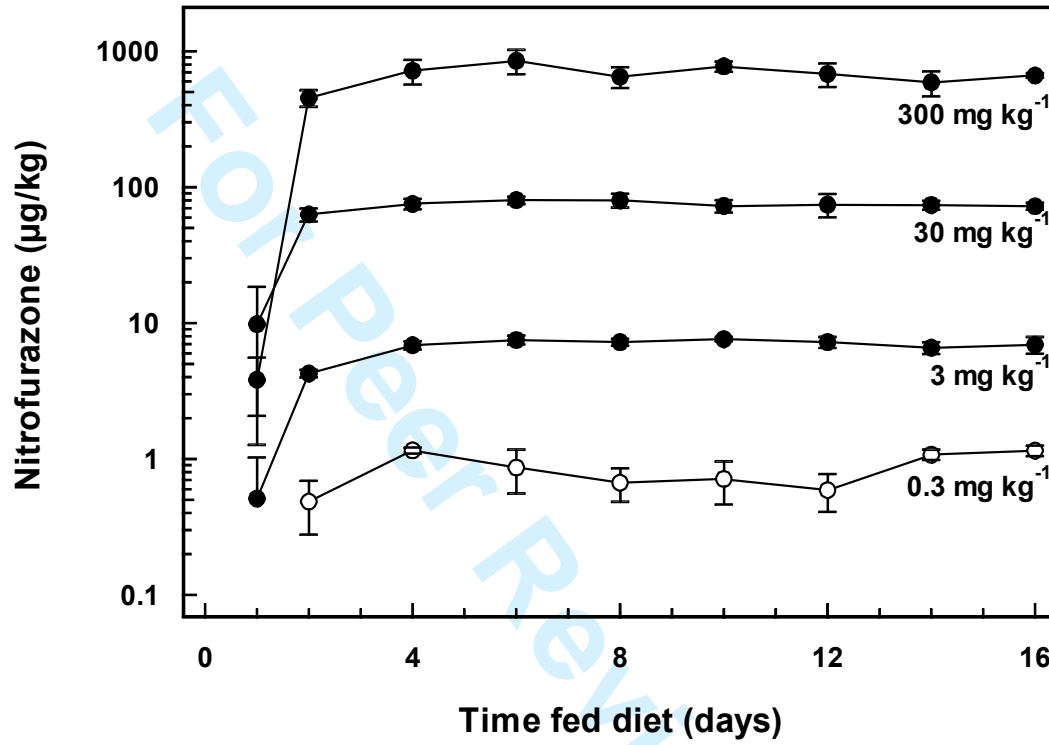
26
27 532 Figure 3. Depletion of nitrofurazone (NFZ) and total semicarbazide (SEM) from
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29 533 whole egg homogenates following withdrawal of 300 mg kg⁻¹ nitrofurazone-
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31 534 medicated feed. Data are means ± standard error.

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536 **FIGURE 1**

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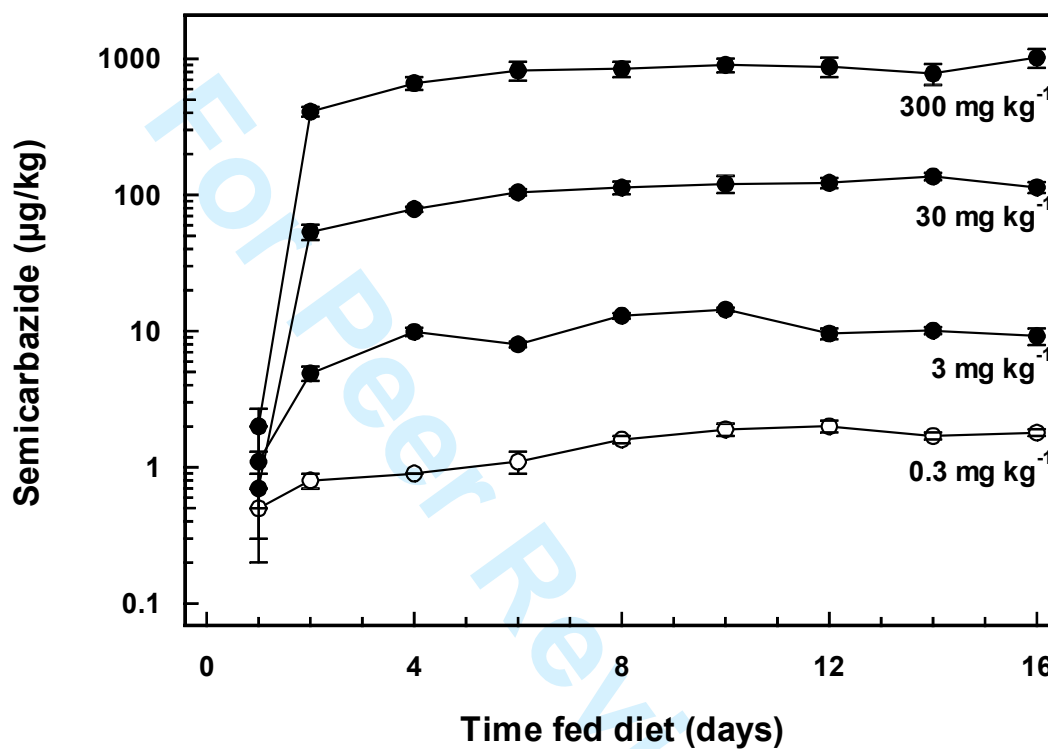


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540 **FIGURE 2**

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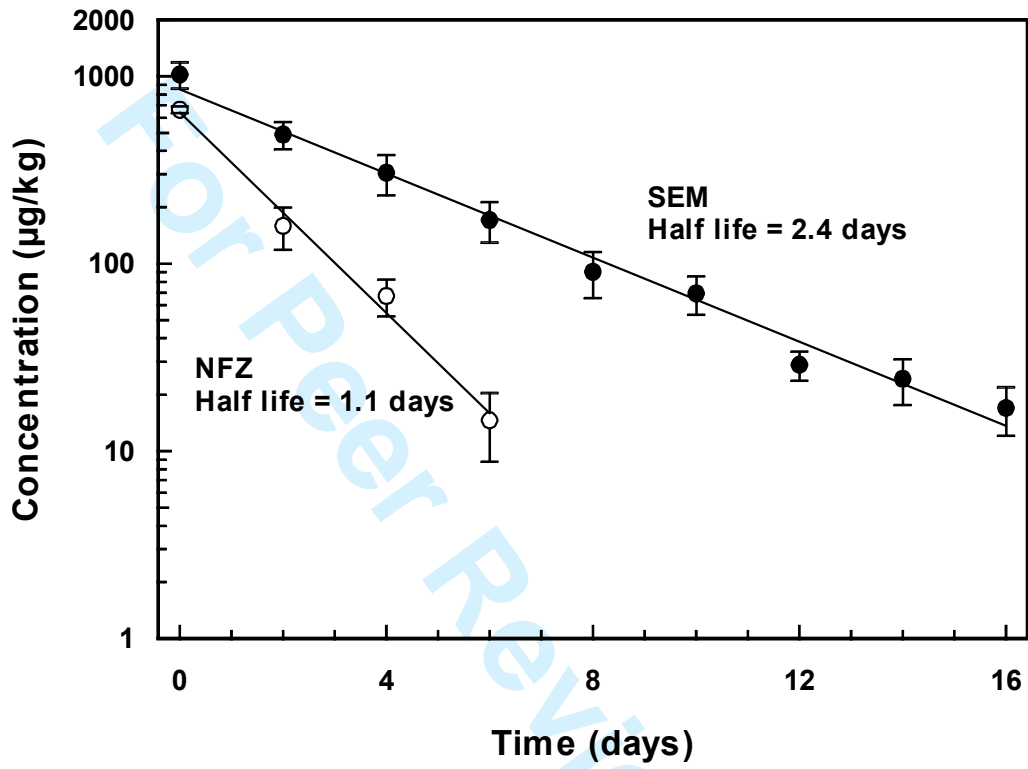


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FIGURE 3

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