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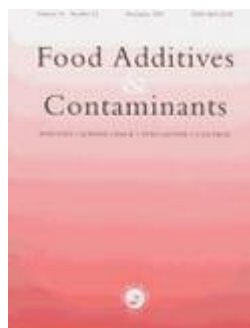
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Stability studies of the metabolites of nitrofuran antibiotics during storage and cooking

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Stability studies of the metabolites of nitrofurantoin antibiotics during storage and cooking.

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Abstract

Nitrofurans antibiotics cannot be used in food production within the EU because of their potential health risks to the consumer. The recent discovery of their widespread use in global food industries and the finding of semicarbazide in baby food as a result of packaging contamination have focussed attention on the toxicity and stability of these drugs and their metabolites. The stability of the nitrofurans marker residues 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD) and semicarbazide (SEM) were tested. Muscle and liver of nitrofurans treated pigs were cooked by frying, grilling, roasting and microwaving. Between 67 and 100% of the residues remained after cooking, demonstrating that these metabolites are largely resistant to conventional cooking techniques and will continue to pose a health risk. The concentration of metabolites in pig muscle and liver did not drop significantly during 8 months storage at -20°C . Metabolite stock and working standard solutions in methanol were also stable for 10 months at 4°C . Only a 10 ng ml^{-1} solution of SEM showed a small drop in concentration over this extended storage period.

Keywords: *nitrofurans, metabolites, stability, cooking, freezer storage, semicarbazide,*

FoodBRAND

Introduction

Food safety and the exposure of the consumer to chemical residues in their food are of growing concern and the subject of extensive worldwide research. The year 2001 saw the beginning of what became known as the “nitrofurans crisis” in world food production. The nitrofurans are a group of antibiotics prohibited within the European Union (EU) for use in food-producing animals (Commission Regulation 1995) because of their potentially carcinogenic and mutagenic effects on human health (Van Koten-Vermeulen et al. 1993). They have been used previously for prevention and treatment of various gastrointestinal infections and as growth promoters in livestock. Nitrofurans are metabolised rapidly by animals in vivo (McCracken et al. 1995) but persistent tissue-bound metabolites are formed which may be released by mild acid hydrolysis and used as marker residues (Hoogenboom et al. 1991). For example, the stable tissue-bound metabolite 3-amino-2-oxazolidinone is monitored as a marker residue for the parent nitrofurans furazolidone. The four main nitrofurans antibiotics are furazolidone, furaltadone, nitrofurantoin and nitrofurazone. Their marker residues are 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD) and semicarbazide (SEM) respectively.

The widespread use of nitrofurans drugs in food production, in particular the poultry and aquaculture industries, was uncovered by partner laboratories in the EU-funded R&D project FoodBRAND (www.afsni.ac.uk/foodbrand), primarily AFBI (formerly DARD) Belfast and RIKILT, Wageningen, The Netherlands. The first major nitrofurans food scare concerned prawns and chicken from Vietnam, Thailand and Brazil. This led the

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European Commission to instigate mandatory testing of consignments being imported to the EU from these countries (Commission Decision 2002a, b, c). These restrictions have now been lifted following proactive measures taken by the Governments concerned to reduce nitrofurans use in their food production industries and to implement effective residues monitoring programmes. However, nitrofurans use has been shown to be more widespread than initially thought. Furalfadone contamination of the Portuguese poultry industry led to the destruction of 1.5 million birds in 2002. Metabolites of furazolidone and furalfadone were discovered by the FoodBRAND project consortium (O’Keeffe et al. 2004) in pork meat purchased in Portugal, Greece and Italy. Furthermore, the European Commission issued notifications to Member States via its Rapid Alert System for Food and Feed concerning findings of nitrofurans in fish from Taiwan, crayfish and salted hog casings from China, prawns from Bangladesh, India and Indonesia, catfish from Thailand, egg powders from India, Brazil, Israel, France and Mexico, honey from Vietnam, Argentina, Turkey and various European countries, and poultry meat products from Argentina, Romania and Bulgaria. In light of these findings, the consumer will want to know what health risks are posed by nitrofurans residues in their food.

AOZ in tissues of pigs fed furazolidone has been shown to be bioavailable to the rat (McCracken and Kennedy 1997). It is likely that the acidic conditions in the human stomach would also liberate these potentially carcinogenic metabolite side chains and pose a threat to human health. Consequently, there is a “zero tolerance” towards the of nitrofurans in food-producing animals within the EU. The development of methods and legislation with regard to the monitoring of nitrofurans residues has recently been

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3 reviewed (Kennedy et al. 2003). Legislators and consumers may also wish to know if
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5 nitrofurans residues in food are destroyed when that food is cooked. Between 1995 and
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7
8 1999, Rose and co-workers in the Central Science Laboratory, Norwich demonstrated
9
10 that residues of a range of veterinary drugs have varying degrees of stability during
11
12 cooking and, therefore, that cooking influences the level of risk posed by such residues
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14 (Rose et al. 1999). However, with the exception of AOZ, nitrofurans stability during
15
16 cooking has not been assessed. McCracken and Kennedy (1997) demonstrated that AOZ
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18 residues were stable in meat, liver and kidney of furazolidone treated pigs following
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20 cooking by microwave, grilling and frying. However, no reports have been published
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22 concerning other nitrofurans residues.
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29 Furthermore, very little information is available concerning the stability of nitrofurans
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31 metabolite residues in food during long-term frozen storage. Residues inspection
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33 laboratories will wish to know if metabolite residues in incurred tissues degrade
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35 significantly during storage. In addition, the stability during refrigerated storage of
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37 standard solutions of the metabolites used for analytical monitoring has not been defined.
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39 The current study aims to determine the stability of the four major nitrofurans metabolites
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41 during cooking and storage.
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48 **Materials and methods**

49 *Materials and instrumentation*

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52 Internal standards D₄-3-amino-2-oxazolidinone, D₅-3-amino-5-morpholinomethyl-2-
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54 oxazolidinone, ¹³C₃-1-aminohydantoin and ¹³C¹⁵N₂-semicarbazide were supplied by
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3 Witega Laboratorien Berlin-Adlershof (Berlin, Germany). Standard 3-amino-2-
4 oxazolidinone (AOZ) and semicarbazide (SEM) were supplied by Sigma-Aldrich (Poole,
5 UK) and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) by Chemical Synthesis
6 Services (Belfast, UK). 1-Aminohydantoin (AHD) was a gift from Proctor and Gamble
7 Pharmaceuticals USA. Unless stated, all other chemicals were obtained from Sigma-
8 Aldrich.
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20 An Agilent 1100 Series HPLC system (Agilent Technologies, USA) coupled to a Quattro
21 Ultima[®] Platinum tandem mass detector (Micromass/Waters, Manchester, UK), both
22 operating under MassLynx[®] software, were used for sample analysis. The mass
23 spectrometer operated in electrospray positive mode and data acquisition was in multiple
24 reaction monitoring mode (MRM). The HPLC system was equipped with a Luna C18(2)
25 3 µm, 2.0 x 150 mm column (Phenomenex, UK). A binary gradient mobile phase was
26 used at a flow rate of 0.2 ml/min, solvent A being 0.5 mM ammonium acetate and
27 methanol (80 : 20 v/v mix), solvent B being 100% methanol. A Testo 915-1 Universal
28 Thermometer was obtained from, Testo AG, Lenzkirch, Germany. Tissues were minced
29 in a Mini Prep Plus domestic food processor (Waring, Torrington, CT, USA) before
30 homogenising in a SL2 laboratory homogeniser (Silverson Machines Ltd, Chesham,
31 England).
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51 *Production of incurred pig tissues*

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53 Weaned piglets, approximately 8 weeks of age, were divided into 4 groups and housed in
54 concrete floored pens. Four nitrofurantoin medicated feeds were prepared using
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3 furazolidone, furaltadone, nitrofurantoin and nitrofurazone at 400 mg per kg feed, the
4 recommended therapeutic dose for furazolidone preparations prior to it being banned in
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8 1995 (National Office of Animal Health Limited 1992). Medicated feed was provided *ad*
9
10 *libitum* for 10 days after which it was withdrawn and replaced with conventional
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12 unmedicated feed for a 6 weeks drug withdrawal period. At weekly intervals following
13
14 withdrawal of medicated feed, 3 pigs from each feed group were euthanized by captive
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16 bolt and samples of *Longissimus dorsi* muscle, liver and kidney removed and stored at
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18 -20°C.
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22 23 24 *Cooking stability study*

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27 Three muscle samples and 3 liver samples from one withdrawal time-point were selected
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29 from pigs fed each of the 4 medicated diets (6 weeks withdrawal for furazolidone,
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31 furaltadone and nitrofurazone, 3 weeks for nitrofurantoin). After thawing to room
32
33 temperature, muscle samples were cut into 4 portions (40 to 60 g). Three of these
34
35 portions were weighed immediately before and after cooking by grilling, microwaving or
36
37 roasting. Samples were then re-frozen at -20°C along with the fourth uncooked muscle
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39 sample. Liver samples were cut into 3 portions (30 to 50 g). Two of these portions were
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41 weighed immediately before and after cooking by frying or roasting. Samples were then
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43 re-frozen at -20°C along with the third uncooked liver sample. Cooking conditions were
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45 as follows.
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53 *Grilling.* Muscle was grilled for 8 min, turning once, under medium heat in a pre-heated
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55 domestic grill on a grill rack, which allowed meat juices to escape.
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Microwave. Muscle was microwaved for 2.5 min in a microwave-proof plastic cooking bag on a rotating plate in an 800 Watt domestic microwave oven at 50% setting, which was taken from a recipe for the cooking of meat..

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Roasting. Muscle and liver were roasted for 20 min at 170°C in a pre-heated fan-assisted domestic oven, in ovenproof plastic cooking bags on an uncovered tray on the middle shelf of the oven.

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Frying. Liver was fried for 2.5 to 3 min each side at medium heat setting on a domestic ceramic hob, in a minimal volume of sunflower oil to prevent burning.

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In all cases, excess juices and cooking oil were removed from raw and cooked tissues using absorbent tissue paper before weighing the samples. The maximum internal temperature of each sample was measured on completion of cooking by inserting a digital penetration probe thermometer into the centre of the sample. All samples, raw and cooked, were analysed by LC-MS/MS for total nitrofurans metabolites as described below.

Tissue storage stability study

The stability of total nitrofurans metabolites in incurred porcine muscle and liver was assessed during 8 months storage at -20°C. Five muscle samples and 5 liver samples were selected from pigs fed each of the 4 medicated diets. Tissues were homogenised in domestic food processor and divided into 4 separate aliquots to avoid repeated freeze-thawing. Tissues were stored at -20°C. At 0, 2, 4 and 8 month intervals an aliquot of each sample was thawed to room temperature and analysed for total nitrofurans

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3 metabolites as described below. For each of these 4 analytical runs, completely new sets
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5 of calibration standard solutions were prepared freshly from stock nitrofurans metabolite
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7 standard powders as described below. The use of fresh standards would ensure that any
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9 observed reduction in nitrofurans concentration in tissue would not be masked by a
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11 commensurate degradation of metabolites in stored standard solutions.
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15 16 17 18 *Standard storage stability study*

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20 The stability of solutions of each of the four nitrofurans metabolites, in methanol, was
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22 assessed at two concentrations (1.0 mg ml^{-1} and 10 ng ml^{-1}) during storage at 4°C for 0, 2,
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24 6, 8 and 10 months. At each time point, the concentration in the stored standards was
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26 measured against analytical standards, freshly prepared from powdered stocks. These
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28 powders were stored desiccated at room temperature throughout this period. Separate 1
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30 mg ml^{-1} stock solutions were prepared in methanol for each metabolite at each time-point.
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32 Mixed standard solutions ($10 \text{ }\mu\text{g ml}^{-1}$, $1 \text{ }\mu\text{g ml}^{-1}$, 100 ng ml^{-1} and 10 ng ml^{-1}) were
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34 prepared by volumetric serial dilution of the freshly prepared 1 mg ml^{-1} stock solutions at
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36 each time-point. Using the LC-MS/MS nitrophenyl derivatisation and extraction method
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38 described below, a calibration standard curve (consisting of duplicate analyses of 6
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40 standards in the range equivalent to 0 to 25 ng ml^{-1}) was prepared using the freshly
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42 prepared 10 ng ml^{-1} mixed standard solution. Each of the stored 10 ng ml^{-1} solutions
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44 were analysed and quantified, as five replicates, against this calibration curve. Each of
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46 the stored 1 mg ml^{-1} solutions were serially diluted to 10 ng ml^{-1} immediately prior to
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48 analysis (to bring them within the range of the standard curve), and were analysed and
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50 quantified, as five replicates, against the same calibration curve. Results obtained
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3 following the analysis of the dilutions made from the stored 1.0 mg ml^{-1} solutions are
4 presented after correction for the 100,000-fold dilution that was necessary to bring them
5 within the range of the standard curve.
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10 11 12 *Sample derivatisation and LC-MS/MS analysis*

13 This analytical method is based on the protocol for total nitrofurans metabolites (sum of
14 extractable and tissue-bound metabolites) developed by RIKILT Wageningen and AFBI
15 VSD Belfast as part of the FoodBRAND project and disseminated to the EU National and
16 Community Reference Laboratory networks and 3rd countries during 2001-2003.
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27 Tissues were minced in a domestic food processor and samples ($1.00 \pm 0.02 \text{ g}$) weighed
28 into 30 ml glass tubes. Mixed internal standard ($100 \mu\text{l}$ of a $1 \mu\text{g ml}^{-1}$ solution) was
29 added to all samples, controls and standards. To all tubes were added 0.1 M hydrochloric
30 acid (9 ml) and 2-nitrobenzaldehyde ($150 \mu\text{l}$, 100 mM in DMSO). Samples were
31 homogenised gently for 1 min in a laboratory homogeniser. The homogeniser was rinsed
32 with 3 x 1 ml 0.1 M hydrochloric acid, which was added to sample tubes. Calibration
33 standards were prepared by addition of mixed standard solutions to 30ml glass tubes.
34 Control muscle and liver tissues from nitrofurans-free pigs were fortified at $50 \mu\text{g kg}^{-1}$
35 with mixed standard ($50 \mu\text{l}$ of a $1 \mu\text{g ml}^{-1}$ solution) to act as recovery control samples.
36 Blank tissues were also included in every analytical run. Tubes were vortex mixed for 10
37 sec to disperse the froth produced during homogenisation, then were incubated overnight
38 (approximately 16 hr) in a water bath held at 37°C .
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3 All tubes were then adjusted to pH 7.2 ± 0.2 with 0.3 M tri-sodium orthophosphate (2.2
4 ml) and 1 M sodium hydroxide (approximately 6 drops). Liquid-liquid extraction was
5 carried out using ethyl acetate (2 x 8 ml, inversion for 1 min and centrifugation at 2000
6 rpm for 15 min at 4°C) and the organic phase evaporated to dryness at 50°C under
7 nitrogen. Residues were re-dissolved in methanol : water (50 : 50 v/v; 2 ml) and an
8 aliquot transferred to HPLC microvials (200 µl) and stored at 4°C. Prior to LC-MS/MS
9 analysis, microvials were centrifuged at 13 000 rpm for 15 min to remove precipitated
10 material. LC-MS/MS was carried out using the conditions described previously (Cooper
11 et al. 2005). Analyte concentrations in samples were calculated by comparing the ratio of
12 an analyte base peak response to its appropriate internal standard response with the same
13 ratio in calibration curve standards. Tissue sample chromatograms were clean, with no
14 interferences arising from cooking processes. This method has been subject to extensive
15 validation in a wide range of tissues (albeit uncooked). In all cases, $CC\alpha$ and $CC\beta$ were
16 less than the EU Minimum Required Performance Limit (MRPL, 1.0 µg/kg) for all of the
17 metabolites, indicating fitness-for-purpose and CVs obtained during validation, above
18 and below the MRPL, were less than 15%.
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Results and discussion

Cooking stability study

Data relating to the stability of the nitrofurans metabolites during cooking are shown in Table I. Each datum is a mean of three different tissue samples selected to contain similar concentrations of metabolites before cooking. All concentrations are based on the wet weight of tissue samples, following correction for loss of water during the cooking process, as judged from the figures contained in columns 4 and 5 of Table I. Internal cooking temperatures ranged from 68 to 98°C. Higher internal temperatures were achieved during roasting of liver (mean 92°C) than frying (84°C). Microwaving of muscle achieved the highest temperatures (mean 95°C) compared with roasting (86°C) and grilling (74°C).

[Insert TABLE I about here]

Concentrations of all four nitrofurans metabolites dropped during cooking of incurred liver, losses ranging from 6 to 33%. Losses were consistently greater following roasting of liver than frying, possibly due to the higher internal temperatures achieved during roasting. Concentrations of AOZ, AMOZ and SEM also dropped during cooking of incurred muscle (losses ranging from 7 to 15%) but no loss of AHD was observed. This was surprising, as anecdotal evidence previously suggested that AHD was the least stable of the nitrofurans metabolites. There was no clear correlation between the drop in metabolite concentrations in muscle observed during different cooking methods and their respective internal cooking temperatures. These data clearly demonstrate that all four major nitrofurans metabolites are sufficiently stable to survive conventional cooking procedures in sufficient quantities to continue to pose a health risk to the consumer. This

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3 finding agrees with McCracken and Kennedy (1997) who observed no significant
4 reduction in total AOZ in porcine liver or muscle following frying, grilling or
5 microwaving. For the consumer, the loss of (at best) one third of the chemical residues in
6 their food by cooking is of little comfort. Consumers want their meat to be residue-free
7 on the supermarket shelf, and on their plate.
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17 It could be argued that the tissue slices used for comparative cooking procedures did not
18 contain equal concentrations of residues prior to cooking. Various studies have
19 demonstrated that concentrations of some veterinary drug residues such as tilmicosin
20 (Beechinor and Bloomfield 2001), clenbuterol (Rose et al. 1995) and chloramphenicol
21 (Cooper et al. 1998) are not evenly distributed throughout a tissue sample. Non-
22 homogenous residue distribution potentially raises problems for residues surveillance, in
23 particular when intact A and B samples are sub-sampled from a tissue prior to tissue
24 homogenisation. In cases of prosecution of a food producer when a second quantitative,
25 confirmatory analysis is required, the second sample may give a different result to the
26 first sample. This may lead to confusion in the case of legal medicines where a
27 maximum residue limit (MRL) applies – sample A may be above the MRL while sample
28 B may be below. However, in the case of banned substances such as the nitrofurans,
29 such minor quantitative variations are not as problematic, at least in principle, since the
30 confirmation of any concentration of the substance is a non-compliance. . Furthermore,
31 inherent variation in tissue distribution can be minimised by sampling adjacent slices of a
32 tissue sample. This was the practice in the current study where adjacent tissue slices
33 were sub-sampled for each cooking procedure and the uncooked control sample.
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3 Furthermore, non-homogenous residue distribution within a tissue is not an issue in the
4 tissue storage stability study described below. In this case tissue samples were
5 thoroughly homogenised before dividing into aliquots for storage at -20°C .
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12 The presence of metabolites in cooked meat indicates that nitrofurans antibiotics have
13 been administered to the livestock or have contaminated the food product at a later stage.
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15 The metabolic breakdown of the nitrofurans drugs is complex and the routes leading to
16 toxicological effects in animals and humans are unclear. It is thought that the nitrofurans
17 bind covalently to macromolecules via the opening of the furan ring structure (Silva et al.
18 1993, Debnath et al. 1993). A variety of metabolite structures have been postulated,
19
20 some of which contain side chains including the marker residues measured in this study.
21
22 It is likely that the acidic conditions in the human stomach would liberate these residues,
23 making them available for absorption. When rats were fed tissues from furazolidone
24 treated pigs, AOZ was detected subsequently in the rat tissues (McCracken and Kennedy
25 1997). It is not clear to what extent the marker residues AOZ, AMOZ, AHD and SEM
26 are themselves toxic. For example, it has been suggested that the *in vivo* inhibition of the
27 enzyme monoamine oxidase (MAO) results from the formation of
28 β -hydroxyethylhydrazine from the ring cleavage of AOZ (Hoogenboom et al. 1991).
29
30 However, the MAO inhibitory effect of AMOZ is lower than that of AOZ (Hoogenboom
31 et al. 1994). The authors are unaware of any studies of the toxicity of AHD. Following a
32 major food scare in 2004 involving the discovery of SEM in baby food (SEM was a by-
33 product of the manufacture of the plastic gaskets used in the jar lids) the European Food
34 Safety Authority (www.efsa.eu/int) commissioned studies into the toxicity of SEM. The
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3 opinion of the EFSA Panel was that “*the issue of carcinogenicity is not of concern for*
4 *human health at the concentrations of SEM encountered in food*” (EFSA 2005).
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8 However, it must be emphasised that the marker metabolites measured in the present
9
10 study are merely fragments of many unidentified and potentially toxic metabolites that
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12 will be present in tissue of an animal treated with a nitrofurant antibiotic.
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15 16 17 *Tissue storage stability study*

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19 Data relating to the stability of the nitrofurant metabolites during long-term storage at –
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21 20°C are shown in Table II. At each time point (0, 2, 4 and 8 months storage) tissues
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23 were analysed using standards prepared freshly from stock powders. Each variable (for
24
25 example, AOZ concentration in muscle) was analysed statistically using the following
26
27 technique. A separate linear regression was fitted against storage time for each sample,
28
29 generating a regression coefficient (or slope). If the concentration within a variable falls
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31 significantly over time, then the slope of this regression should be negative.
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33 Consequently, the standard one tailed t-test was applied to the five regression coefficients
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35 (for example the five AOZ muscle samples) to test the null hypothesis that the nitrofurant
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37 concentration does not fall over time, against the alternative hypothesis that the
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39 concentration does fall (a one-tailed test).
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46 [Insert TABLE II about here]
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48 There was no significant reduction ($P > 0.05$) in the concentration of any of the four
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50 nitrofurant metabolites in either muscle or liver over the 8 months period of storage at
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52 -20°C. Residues inspection authorities, official control and research laboratories can be
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3 reassured that the total nitrofurantoin metabolite content of incurred tissue samples will not
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5 change significantly during extended storage at -20°C.
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11 *Standard storage stability study*

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13 Data relating to the stability of methanolic standard solutions of the nitrofurantoin
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15 metabolites during storage at 4°C are shown in Table III. Each variable (for example,
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17 AOZ concentration in 1 mg ml⁻¹ stock standard solution) was analysed statistically using
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19 the same linear regression and one-tailed t-test technique as applied above to the tissue
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21 storage data, the only difference being that the regression was applied within a replicate
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23 analysis as opposed to within a repeated analysis of a sample. No significant reduction
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25 (P>0.05) was seen in the concentration of any of the four nitrofurantoin metabolites in either
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27 1 mg ml⁻¹ or 10 ng ml⁻¹ methanolic solutions over the 10 month period of storage at 4°C
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29 with the exception of SEM in 10 ng ml⁻¹ solution (P = 0.015). The equation of the linear
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31 regression line for SEM in 10 ng ml⁻¹ solution was $y = -0.0127x + 1.0604$. The slope of
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33 this regression indicates that the concentration of a 10 ng ml⁻¹ SEM standard solution will
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35 fall by 5% in 3.9 months. The 1 mg ml⁻¹ stock SEM solution, however, was stable for at
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37 least 10 months. The authors therefore suggest that stock solutions of nitrofurantoin
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39 metabolites in methanol may be retained for 10 months, whilst working standard
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41 dilutions prepared from these stocks should be used for no longer than 4 months. Some
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43 laboratories currently place expiry dates of 1-2 months on nitrofurantoin working standards.
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51 This study demonstrates that such standards may be retained for at least 4 months.
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53 [Insert TABLE III about here]
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3 The various stability data presented here demonstrate that the metabolites AOZ, AMOZ,
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5 AHD and SEM, which are used as marker residues of their parent nitrofurans antibiotics,
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7 show remarkable chemical stability. They are resistant to conventional domestic cooking
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9 procedures and are not degraded in edible animal tissues during many months storage in
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11 the freezer or in pure standard solution in the refrigerator. Whilst this chemical stability
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13 is advantageous to the analyst monitoring the nitrofurans drugs in food production, it is to
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15 the detriment of the consumer of that food. This study highlights the need for continued
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17 vigilance in the global monitoring of nitrofurans abuse and possible routes of
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19 contamination.
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TABLE AND FIGURE CAPTIONS:

Table I. Effect of various cooking methods on the concentration ($\mu\text{g kg}^{-1}$) of total nitrofurans metabolites in pig muscle and liver. Data are means \pm standard errors of 3 different tissue samples from a single withdrawal date. Expected metabolite concentrations are corrected for weight loss during cooking.

Table II. Effect of long term storage at -20°C on the concentration ($\mu\text{g kg}^{-1}$) of total nitrofurans metabolites in pig muscle and liver. The figures in bold are the mean \pm SE of the normalised concentration (relative to the concentration at $t = 0$) measured in each tissue sample.

Table III. Stability of nitrofurans metabolites (1.0 mg ml^{-1} and 10 ng ml^{-1} in methanol) during storage at 4°C .

Table I

| Tissue | Nitrofuran metabolite | Treatment | Weight before (g) | Weight after (g) | Temp °C | $\mu\text{g kg}^{-1}$ before cooking | $\mu\text{g kg}^{-1}$ expected after cooking | $\mu\text{g kg}^{-1}$ found after cooking | Net change (%) during cooking |
|--------|-----------------------|-----------|-------------------|------------------|---------|--------------------------------------|--|---|-------------------------------|
| Liver | AOZ | Fry | 42.3 | 29.6 | 83.9 | | 75.3 ± 5.6 | 59.4 ± 6.6 | -21 |
| | | Roast | 46.9 | 27.3 | 94.1 | | 88.8 ± 1.9 | 59.6 ± 2.7 | -33 |
| | | Uncooked | | | | 51.3 ± 4.1 | | | |
| Muscle | AOZ | Grill | 54.2 | 37.2 | 75.3 | | 66.5 ± 5.3 | 59.7 ± 6.3 | -10 |
| | | Microwave | 41.3 | 24.8 | 85.7 | | 75.9 ± 5.6 | 63.9 ± 1.9 | -15 |
| | | Roast | 56.2 | 36.7 | 88.5 | | 69.7 ± 5.2 | 62.0 ± 4.6 | -11 |
| | | Uncooked | | | | 45.6 ± 2.8 | | | |
| Liver | AMOZ | Fry | 38.0 | 27.9 | 83.5 | | 44.6 ± 1.8 | 39.7 ± 2.2 | -11 |
| | | Roast | 37.8 | 23.8 | 89.5 | | 52.4 ± 4.1 | 38.9 ± 3.0 | -26 |
| | | Uncooked | | | | 32.8 ± 1.7 | | | |
| Muscle | AMOZ | Grill | 53.0 | 41.0 | 67.5 | | 93.6 ± 9.5 | 86.8 ± 7.3 | -7 |
| | | Microwave | 51.8 | 32.2 | 98.1 | | 116.2 ± 9.8 | 99.5 ± 2.1 | -14 |
| | | Roast | 50.4 | 32.5 | 85.1 | | 112.5 ± 10.9 | 94.4 ± 7.6 | -15 |
| | | Uncooked | | | | 72.3 ± 7.2 | | | |
| Liver | AHD | Fry | 44.4 | 32.5 | 82.2 | | 56.1 ± 2.1 | 46.5 ± 1.8 | -17 |
| | | Roast | 42.9 | 28.0 | 91.9 | | 62.6 ± 1.6 | 48.5 ± 0.6 | -22 |
| | | Uncooked | | | | 41.0 ± 1.2 | | | |
| Muscle | AHD | Grill | 45.7 | 32.8 | 76.8 | | 35.4 ± 1.7 | 36.6 ± 0.4 | +4 |
| | | Microwave | 40.8 | 25.6 | 96.7 | | 41.1 ± 2.2 | 41.4 ± 1.5 | +1 |
| | | Roast | 53.5 | 35.9 | 83.2 | | 38.0 ± 2.5 | 37.9 ± 1.7 | 0 |
| | | Uncooked | | | | 25.4 ± 1.2 | | | |
| Liver | SEM | Fry | 36.3 | 26.0 | 84.4 | | 57.8 ± 8.7 | 53.1 ± 5.4 | -6 |
| | | Roast | 39.6 | 23.6 | 93.4 | | 68.2 ± 8.2 | 51.8 ± 6.4 | -24 |
| | | Uncooked | | | | 40.2 ± 4.1 | | | |
| Muscle | SEM | Grill | 54.8 | 38.4 | 77.0 | | 343.3 ± 14.0 | 293.6 ± 10.4 | -14 |
| | | Microwave | 47.2 | 29.5 | 98.1 | | 387.5 ± 19.2 | 338.2 ± 12.3 | -13 |
| | | Roast | 55.6 | 37.0 | 87.3 | | 361.4 ± 15.8 | 320.3 ± 22.5 | -11 |
| | | Uncooked | | | | 240.9 ± 10.0 | | | |

Table II

| Nitrofuran metabolite | Muscle 0 months | Muscle 2 months | Muscle 4 months | Muscle 8 months | Liver 0 months | Liver 2 months | Liver 4 months | Liver 8 months |
|-----------------------------|-----------------|--------------------|--------------------|--------------------|----------------|--------------------|--------------------|--------------------|
| AOZ | 54.0 | 63.4 | 60.6 | 70.3 | 226.4 | 232.3 | * | 198.6 |
| | 65.3 | 72.7 | 76.1 | 86.3 | 219.8 | 208.4 | 253.4 | 210.6 |
| | 44.6 | 44.1 | 45.8 | 55.8 | 54.2 | 62.4 | 65.5 | 63.8 |
| | 35.6 | 36.7 | 40.6 | 43.6 | 39.7 | 40.5 | 47.0 | 44.4 |
| | 32.0 | 32.0 | 32.5 | 36.5 | 60.5 | 62.7 | 60.9 | 59.0 |
| <i>Normalised Mean ± SE</i> | 100 | 106.1 ± 3.2 | 109.4 ± 2.7 | 124.8 ± 2.9 | 100 | 103.6 ± 2.9 | 113.8 ± 3.9 | 102.1 ± 4.9 |
| AMOZ | 126.0 | 103.3 | 102.3 | 105.5 | 98.7 | 97.6 | 103.2 | 106.0 |
| | 86.6 | 79.8 | 86.2 | 81.5 | 93.7 | 80.7 | 90.0 | 104.1 |
| | 59.8 | 51.9 | 54.7 | 54.2 | 28.6 | 27.6 | 28.2 | 28.3 |
| | 53.3 | 45.6 | 51.7 | 52.3 | 27.5 | 24.4 | 23.6 | 25.2 |
| | 70.7 | 58.8 | 70.3 | 68.3 | 30.5 | 28.7 | 27.8 | 30.0 |
| <i>Normalised Mean ± SE</i> | 100 | 85.9 ± 1.6 | 96.9 ± 3.5 | 92.6 ± 2.3 | 100 | 92.9 ± 2.1 | 92.9 ± 3.2 | 101.5 ± 3.1 |
| AHD | 54.5 | 50.1 | 47.5 | 51.7 | 95.9 | 91.2 | 85.1 | 87.7 |
| | 50.7 | 55.5 | 53.5 | 53.8 | 116.7 | 101.3 | 113.7 | 109.6 |
| | 29.3 | 25.4 | 27.8 | 29.5 | 42.6 | 35.5 | 36.5 | 38.3 |
| | 27.9 | 27.8 | 28.5 | 27.8 | 41.9 | 41.8 | 38.2 | 41.0 |
| | 28.0 | 23.5 | 26.0 | 26.5 | 42.2 | 41.8 | 44.1 | 42.7 |
| <i>Normalised Mean ± SE</i> | 100 | 94.3 ± 4.1 | 98.9 ± 3.3 | 99.2 ± 1.9 | 100 | 92.0 ± 3.2 | 94.7 ± 3.4 | 94.9 ± 1.9 |
| SEM | 304.0 | 253.5 | 299.0 | 281.3 | 96.6 | 86.4 | 92.1 | 91.5 |
| | 260.6 | 274.7 | 251.4 | 252.9 | 74.9 | 72.4 | 72.8 | 79.1 |
| | 203.6 | 219.4 | 221.5 | 216.7 | 27.9 | 32.9 | 30.4 | 34.9 |
| | 210.5 | 202.9 | 188.0 | 185.4 | 39.7 | 41.2 | 46.2 | 47.1 |
| | 201.1 | 194.0 | 201.9 | 187.8 | 40.7 | 40.5 | 45.1 | 41.8 |
| <i>Normalised Mean ± SE</i> | 100 | 97.9 ± 3.8 | 98.7 ± 3.1 | 95.5 ± 2.8 | 100 | 101.5 ± 4.2 | 108.3 ± 4.1 | 109.4 ± 4.9 |

Table III

| Compound | Storage in solution at 4°C as | 0 months | 2 months | 6 months | 8 months | 10 months |
|----------|-------------------------------|-------------|-------------|-------------|-------------|-------------|
| AOZ | 1.0 mg ml ⁻¹ | 1.01 ± 0.01 | 0.97 ± 0.02 | 0.93 ± 0.01 | 0.94 ± 0.02 | 1.13 ± 0.01 |
| | 10.0 ng ml ⁻¹ | 10.1 ± 0.1 | 9.6 ± 0.2 | 9.2 ± 0.2 | 9.4 ± 0.3 | 11.0 ± 0.3 |
| AMOZ | 1.0 mg ml ⁻¹ | 0.99 ± 0.04 | 1.19 ± 0.03 | 1.25 ± 0.02 | 1.21 ± 0.03 | 1.11 ± 0.01 |
| | 10.0 ng ml ⁻¹ | 9.9 ± 0.4 | 10.9 ± 0.3 | 12.1 ± 0.1 | 11.6 ± 0.2 | 11.2 ± 0.2 |
| AHD | 1.0 mg ml ⁻¹ | 1.02 ± 0.03 | 1.02 ± 0.03 | 1.11 ± 0.04 | 1.15 ± 0.02 | 1.03 ± 0.03 |
| | 10.0 ng ml ⁻¹ | 10.2 ± 0.3 | 10.3 ± 0.1 | 10.3 ± 0.2 | 11.2 ± 0.2 | 9.6 ± 0.1 |
| SEM | 1.0 mg ml ⁻¹ | 1.05 ± 0.06 | 1.04 ± 0.04 | 1.00 ± 0.05 | 1.03 ± 0.03 | 1.03 ± 0.03 |
| | 10.0 ng ml ⁻¹ | 10.5 ± 0.6 | 10.5 ± 0.2 | 9.6 ± 0.3 | 9.9 ± 0.1 | 9.2 ± 0.3 |