

# Nitrofuran antibiotics: a review on the application, prohibition and residual analysis

M. VASS\*, K. HRUSKA, M. FRANEK

Veterinary Research Institute, Brno, Czech Republic

**ABSTRACT:** Nitrofurantoin antibiotics, employed for the treatment of bacterial diseases in livestock production, were banned from use in the European Union (EU) in 1995 due to concerns about the carcinogenicity of their residues in edible tissue. This review provides an overview of nitrofurantoin toxicity, metabolism, and also specific aspects of legislation surrounding their prohibition. Special attention is devoted to semicarbazide – a nitrofurantoin metabolite and food contaminant. Analytical procedures for nitrofurantoin analysis in various matrices and validation requirements for screening and confirmation methods with respect to EU regulations are also reviewed.

**Keywords:** nitrofurantoin; tissue bound metabolites; semicarbazide; bioavailability; mutagenicity; legislation; sample preparation; validation; detection methods

## List of abbreviations

**AHD** = 1-aminohydantoin; **AOZ** = 3-amino-2-oxazolidinone; **AMOZ** = 3-amino-5-morpholino-methyl-1,3-oxazolidinone; **CC<sub>α</sub>** = decision limit; **CC<sub>β</sub>** = detection capability; **EC** = European Commission; **EFSA** = European Food Safety Authority; **ELISA** = enzyme linked immuno-adsorbent assay; **ESI** = electro-spray ionisation; **EU** = European Union; **FTD** = furaltadone; **FZD** = furazolidone; **HPLC** = high performance liquid chromatography; **IC** = inhibition concentration; **LC** = liquid chromatography; **LOD** = limit of detection; **MS** = mass spectrometry; **NFT** = nitrofurantoin; **NFZ** = nitrofurazone; **NP** = nitrophenyl; **NPAHD** = 3-(2-nitrobenzylidenamino)-2,4-imidazolidinedione; **NPAMOZ** = 5-(morpholinomethyl)-3-(2-nitrobenzylidenamino)-2-oxazolidinone; **NPAOZ** = [3-(2-nitrobenzylidenamino)-2-oxazolidinone]; **NPSEM** = 3[(2-nitrophenyl)methylene]-hydrazinecarboxamide; **o-NBA** = *ortho*-nitrobenzaldehyde; **RASFF** = Rapid Alert System for Food and Feed; **SE** = solvent extraction; **SEM** = semicarbazide; **SPE** = solid phase extraction; **UV** = ultraviolet

## Contents

- |  |  |
|--|--|
| 1. Introduction  | 6. Legislation and analytical control                    |
| 2. Status and prohibition of nitrofurantoin use                  | 7. Sample preparation methods                            |
| 3. Metabolism and bioavailability of nitrofurantoin              | 8. Instrumental methods for nitrofurantoin determination |
| 4. Mutagenicity and toxicity of nitrofurantoin and semicarbazide | 9. ELISA methods for nitrofurantoin determination        |
| 5. Sources of nitrofurantoin and semicarbazide contamination     | 10. Concluding remarks                                   |
|  | 11. References   |

Supported by the Ministry of Agriculture of the Czech Republic (Grants No. NPV 1B53020 and MZE 0002716201)

\*Holder of the 2008 EuroResidue Award for Excellent Contributions in Residue Analysis

## 1. Introduction

Prior to the prohibition of nitrofurans, furazolidone was broadly used in European countries as an effective veterinary antibiotic, especially in pig husbandry. Residual control was based on the measurement of furazolidone concentration in blood and tissues. However, studies concerning the metabolism and toxicity of furazolidone and other nitrofurans revealed that the monitoring of residues based only on the detection of parent nitrofuran structures did not provide adequate data for the evaluation of real tissue contamination and their health risk (Vroomen et al., 1986, 1990). Due to fears of the carcinogenic effects on humans, nitrofurans were banned from use in livestock production in the European Union (Commission Regulation, 1995). In order to control the illegal use of nitrofuran antibiotics by measurement of residue levels in tissues, defined metabolic structures of the drugs were established as marker residues. Development of highly sensitive and specific analytical methods for the determination of nitrofuran residues has become increasingly challenging, with the implementation of new stringent regulation and validation requirements set by the EU (Commission Decision, 2002, 2003).

A key role in the development of sensitive methods for nitrofuran metabolites and monitoring strategies was played by the multi-national EU research project "FoodBRAND" (2000 to 2003), coordinated by the Department of Veterinary Science, Queen's University Belfast. This project provided the European Commission with analytical methods, analytical standards and training in the use of novel instrumental methods. In the framework of this project, the first immunoassays capable of nitrofuran metabolite detection were developed, providing simple alternative screening methods for convenient use, particularly in the food industry. FoodBRAND additionally examined data from an extensive survey of nitrofuran residues in pork meat in European countries, and was critical in exposing the global nitrofuran crisis in food production.

The aim of this review was to provide basic data on the potential effects of nitrofurans on human health and to summarise current methods for nitrofuran analysis with respect to regulatory requirements of the EU. Specific aspects of legislation are also discussed, including the EU's Rapid Alert System for Feed and Food which provides regulatory authorities with an effective tool for the

collection and exchange of information regarding contamination in food.

## 2. Status and prohibition of nitrofuran use

Nitrofurans, particularly furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT) and nitrofurazone (NFZ), belong to a class of synthetic broad spectrum antibiotics which all contain a characteristic 5-nitrofuran ring. Nitrofurans were commonly employed as feed additives for growth promotion, and mainly used for livestock (i.e. poultry, swine and cattle), aquaculture (i.e. fish and shrimp) and bee colonies in the prophylactic and therapeutic treatment of bacterial and protozoan infections such as gastrointestinal enteritis caused by *Escherichia coli* and *Salmonella* spp. (Draisci et al., 1997), fowl cholera and coccidiosis black heads (Mccalla, 1983; Draisci et al., 1997).

In 1995, the use of nitrofurans for livestock production was completely prohibited in the EU (Commission Regulation, 1995) due to concerns about the carcinogenicity of the drug residues and their potential harmful effects on human health (Mccalla, 1983; Vroomen et al., 1990; Van Koten-Vermeulen, 1993). Under EU regulation, countries with products intended for the EU are bound by the same regulations as locally produced food (Commission Decision, 2003), therefore food imported into the EU should be free of nitrofurans. The use of nitrofurans for livestock has also been prohibited in countries such as Australia, USA, Philippines, Thailand and Brazil (Khong et al., 2004).

Contrary to the complete ban of nitrofuran use in livestock production, the drugs are readily available for veterinary and human therapy: nitrofurazone is used for topical application on infected burns and skin infections (Vasheghani et al., 2008); furazolidone is available for the oral treatment of cholera (Roychowdhury et al., 2008), bacterial diarrhoea, and giardiasis (Petri, 2005); and nitrofurantoin is commonly used to treat infections of the urinary tract (Guay, 2008).

## 3. Metabolism and bioavailability of nitrofurans

Nitrofuran parent drugs, furazolidone, nitrofurazone, nitrofurantoin and furaltadone and their

related structures are depicted in Figure 1. These parent compounds metabolise rapidly after ingestion to form corresponding tissue bound metabolites (Nouws and Laurensen, 1990; McCracken et al., 1995). Due to this instability, effective monitoring of their illegal use has been difficult. The short *in vivo* half-life of the parent drugs (7 to 63 minutes) results in rapid depletion of nitrofurans in blood and tissue (Nouws and Laurensen, 1990). However, the formed metabolites (AOZ, AMOZ, AHD and SEM) bind to tissue proteins in the body for many weeks after treatment, making them more

practical for monitoring public compliance of the EU ban (Hoogenboom et al., 1991; Horne et al., 1996; McCracken and Kennedy, 1997a; Cooper et al., 2005a). Although the metabolism of nitrofurans is not well documented, a suggested mechanism is through cleavage of the nitrofurans ring, leaving the specific tail group covalently bound to tissue (Leitner et al., 2001). *In vivo*, these metabolites can be released by natural stomach acids (Hoogenboom et al., 1992); this fact is taken into consideration in the isolation of metabolites for residue analysis (see Chapter 7).

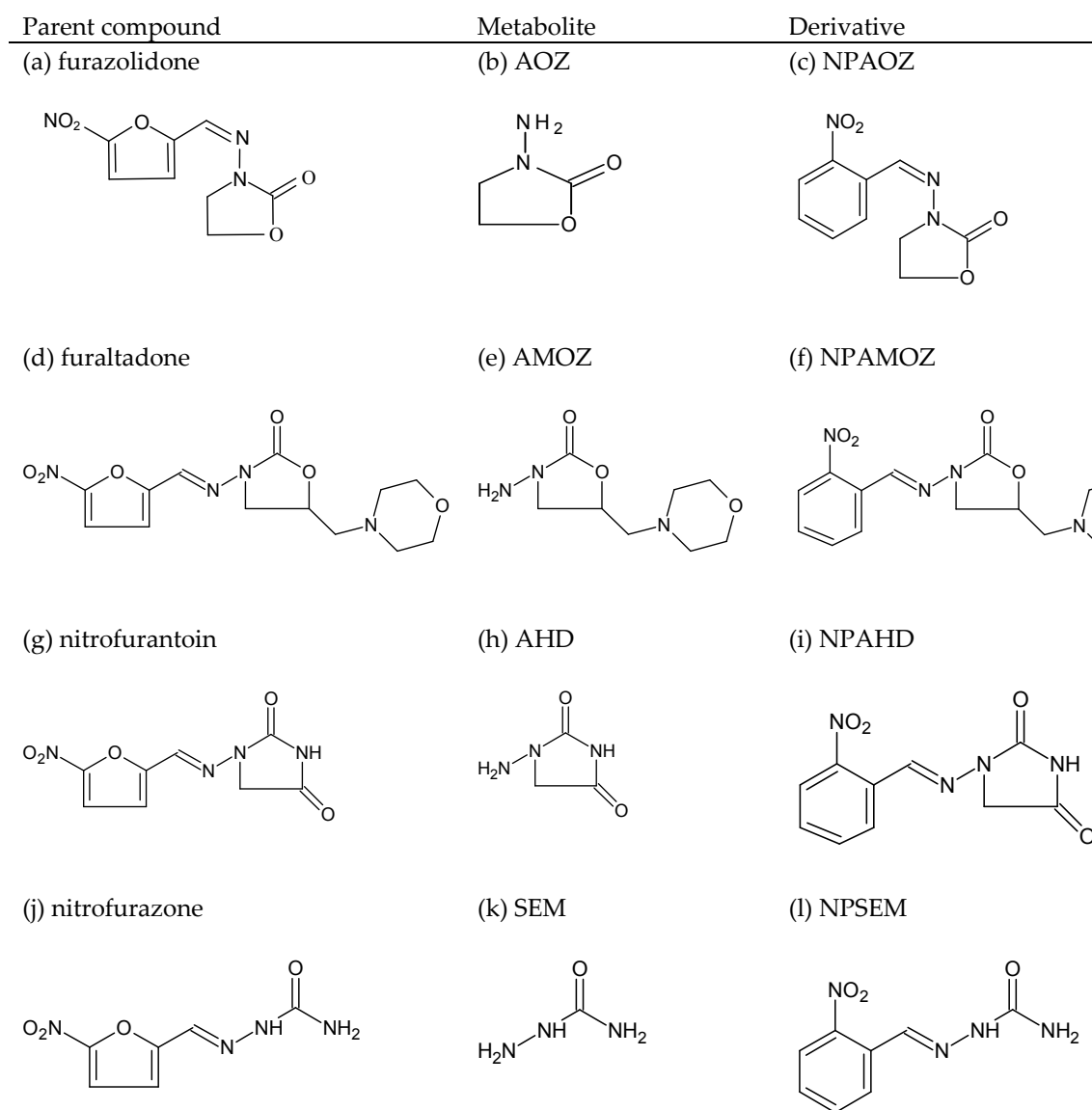


Figure 1. Structures of nitrofuran parent compounds, metabolites and nitrophenyl derivatives. AOZ (3-amino-2-oxazolidinone); NPAOZ (3-(2-nitrobenzylideneamino)-2-oxazolidinone); AMOZ (3-amino-5-morpholinomethyl-1,3-oxazolidinone), NPAMOZ (5-(morpholinomethyl)-3-(2-nitrobenzylideneamino)-2-oxazolidinone); AHD (1-aminohydantoin); NPAHD [3-(2-nitrobenzylideneamino)-2,4-imidazolidinedione]; SEM (semicarbazide); NPSEM 3[(2-nitrophenyl)methylene]-hydrazinecarboxamide

Studies examining the bioavailability of nitrofurans metabolites have demonstrated the possibility of residual transfer to secondary species. When rats were fed pig tissue containing radio-labelled ( $^{14}\text{C}$ ) furazolidone, 41% of the total amount consumed was made bioavailable to the rat (Vroomen et al., 1990). Bioavailability can occur through the ingestion of contaminated meat and animal products (such as eggs), even after cooking (Gottschall and Wang, 1995; McCracken and Kennedy, 1997b), as well as by transfer to the progeny of hens (McCracken et al., 2001, 2005a; Finzi et al., 2005) emphasising the health risk for consumers.

The stability of metabolites during the storage and cooking of meat was demonstrated recently (Cooper and Kennedy, 2007). Eight months storage did not have a significant effect on the residual concentration of nitrofurans in incurred muscle and liver pig samples. The authors determined that between 67% and 100% of the residues remained present in the tissue after cooking, frying, grilling, roasting and microwaving. Another study demonstrated that AOZ in egg was stable up to (at least) 12 months during storage at 4°C, and that 78% of AOZ occurs in the yolk as opposed to albumin (McCracken et al., 2001). Recently it was also found that 50% of total SEM residues in egg were found in the shell, which may be significant if an egg-shell product reaches the consumer (McCracken and Kennedy, 2007).

#### 4. Mutagenicity and toxicity of nitrofurans and semicarbazide

Mutagenicity and toxicity of nitrofurans are discussed not only in relation to their abuse in livestock production, but special attention has also been devoted to the toxicology of semicarbazide which has been found in food produced from raw materials not subjected to nitrofurazone administration (see Chapter 5).

Mutagenicity studies in the 1970's and 1980's revealed the potential effects of nitrofurans in bacterial and mammalian cells. It was suggested that endogenous nitro-reductase was responsible for the *in vitro* reduction of nitrofurans in *E. coli*, leading to the formation of cellular DNA lesions in the stationary phase of bacterial growth (Mccalla et al., 1971; Bryant and Mccalla, 1980). The formation of DNA adducts after bacterial replication causes the induction of error prone DNA repair processes,

indicating the mutagenic potency of the drug (Wentzell and Mccalla, 1980; Mccalla, 1983).

The toxicity and formation of mutagens in mammalian cells *in vitro* is less understood. Studies suggested that irreversible damage to the DNA of human epithelial cells (HEp-2) as well as hormone disturbances (reflecting endocrine dysfunction) occurred prevalently when cells were exposed to furazolidone (De Angelis et al., 1999; Ahmed et al., 2008). The majority of the information available describes *in vivo* studies which utilise mouse and rat models for examination of the effects of furazolidone and mainly nitrofurazone or its residue semicarbazide.

A major study conducted in 1988 examined groups of F344/N and B6C31 mice (of both sexes) fed nitrofurazone for a period of 14 days, 13 weeks or two years. Results showed clear evidence of carcinogenic activity as a direct consequence of nitrofurazone intake. This was demonstrated by an increased incidence of fibro-adenomas of the mammary gland in female mice, as well benign mixed tumours and granulosa cell tumours in the ovaries. Other common signs of toxicity in both species and genders of mice included convulsive seizures, osteoporosis, degenerative arthropathy and more commonly rough hair coats and lethargy, as well as a dose related decrease in feed consumption (Kari, 1988). In another study, no significant alterations in tested immunological or host resistance parameters were shown in B6C3F1 mice administered nitrofurazone for a consecutive 14 days at various low doses.

Nitrofurazone had significant reproductive effects on Swiss CD 1 mice fed 100, 350 and 750 ppm in feed over the course of 15 weeks. Overall, experimental mice showed fewer delivered litters, a large reduction in the average number of pups per litter and low birth weights were noted in high dose groups (750 ppm) in comparison to controls. Epididymal studies showed sperm concentrations of middle and high dose groups decreased by 20% and 98% respectively, and the percentage of sperm abnormalities tripled in comparison to control groups. The study concluded that adverse reproductive effects in male and female mice resulted from relatively low doses ( $\geq 100$  ppm) of nitrofurazone (George et al., 1996).

A clear majority of toxicity and carcinogenicity studies involve the administration of SEM into various species. Some of the side effects listed included lathyrism (a collagen cross linking disease) in rats

(Steffeck et al., 1972), death and foetus retardation in hamsters (Wiley and Joneja, 1978), tissue abnormalities such as haemorrhaging in the brain, liver and intestine, abnormalities in bone formation and underdeveloped testes in rats injected with low doses of SEM hydrochloride saline solution during various days of gestation (De la Fuente et al., 1983, 1986).

However, some conflicting results on carcinogenicity of the compound exist. Two studies showed a significant incidence of lung tumours in mice administered with low doses ( $\leq 0.1\%$ ) of SEM hydrochloride (Mori et al., 1960; Toth et al., 1975), whereas a separate controversial study reported that SEM hydrochloride produced negative results in regards to potential carcinogenicity, whilst stating a high death rate from animals fed large doses of nitrofurans (Ulland et al., 1973). More recent studies evaluating semicarbazide exposure effects on the endocrine pancreas determined small differences between control and experimental groups fed with low doses of hydrochloride semicarbazide (Cabrita et al., 2007).

The opinion of the European Food Safety Authority (EFSA) on the presence of the nitrofurazone metabolite semicarbazide in food has been published (European Food Safety Authority, 2005). On the basis of the difference in magnitude between experimental animals and humans (including infants), as well as the use of sensitive methodology (i.e. intraperitoneal administration of medicine resulting in direct exposure of the uterus to high concentrations of chemicals), the EFSA concluded that the issue of carcinogenicity is not a concern for human health at the concentrations of SEM encountered in food (European Food Safety Authority, 2005). Although, it should be noted that nitrofurazone, nitrofurantoin, furaldone and furazolidone are depicted on the State of California Proposition 65 Carcinogens List (US Environmental Protection Agency, 2008).

## 5. Sources of nitrofurans and semicarbazide contamination

The global nitrofurans crisis during 2002–2003 revealed frequent findings of tissue bound residues in poultry and aquaculture products imported to EU countries from Thailand, China, Taiwan, India, Vietnam, Ecuador and Brazil (Anon, 2008). Moreover, nitrofurans residues were also found

in poultry and pork muscle produced in European countries such as Portugal, Italy, Greece, Romania and Bulgaria (O'Keefe et al., 2004). Later inspection by EU authorities, revealed nitrofurans contamination in products originating from over nine countries in 2007, the highest incidences being from India (37%), China (37%), Bangladesh (10%) and Thailand (5%) in a variety of products including shrimp, honey and canned meat (European Commission, 2008). Despite strict legislation banning its use in food animal production in the EU, nitrofurans continue to be used due to their effectiveness and availability, as is evident from the European Commissions Rapid Alert System for Food and Feed (RASFF).

The RASFF, in place since 1979, provides regulatory authorities with an effective tool for the exchange of information regarding measures taken to ensure food safety in European Union countries (European Commission, 2008). Weekly overviews are available via the internet under three sections:

1. Alert notifications: sent when a food or feed product presents a serious risk on the market and when immediate action is required. Individual EU Member States have their own mechanisms to carry out the necessary measures.

2. Information notifications: concern a food or feed that was placed on the market and has been identified as a risk.

3. Border rejections (new category since 2008): concern food and feed consignments that have been tested and rejected at the external borders of the EU when a health risk was found. Such notifications are transmitted to all border posts in order to reinforce controls and to ensure that the rejected product does not re-enter the Community through another border post.

The notifications made regarding prohibited nitrofurans are published in the RASFF Weekly Overviews in 2007 and 2008 (until week 37) and listed in Tables 1–3. It is evident that aquaculture products from Asian countries are frequently contaminated by AOZ and SEM. The highest concentration of AOZ was 150  $\mu\text{g}/\text{kg}$  in frozen peeled black tiger shrimps from India. However, findings of nitrofurans at lower concentrations (10–63  $\mu\text{g}/\text{kg}$ ) were not rare.

The emerging issue of the presence of nitrofurazone metabolite SEM in edible tissue of non-animal origin has caused an increase in public awareness in recent years. From the total of nitrofurans metabolites notified by the RASFF, SEM was the highest with 48.9%, 60.9% and 71.0% of all nitrofurans notifications

Table 1. Nitrofurans in the Rapid Alert System for Food and Feed: Alert Notifications (week 1, 2007 to week 37, 2008)

Date	Notified by <b>Imported from</b>	Reference	Reason for notifying Notification basis and status
<b>2007</b>			
16/07	U.K. <b>GREECE</b>	2007.0478	nitrofurans (metabolite) – furaltadone ( <b>AMOZ</b> ) (1.8 µg/kg – ppb) in <b>sea bass</b> market control distribution on the market (possible)/withdrawal from sale
19/07	Netherlands <b>CHINA</b>	2007.0487	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) (1.2 µg/kg – ppb) in IQF PND <b>shrimps</b> ( <i>Trachypenaeus</i> spp.) border control – screening sample distribution on the market (possible)/destination of the product identified
09/08	Belgium <b>THAILAND</b>	2007.0538	nitrofurans (metabolite) nitrofurazone ( <b>SEM</b> ) (>1 µg/kg – ppb) in frozen freshwater <b>shrimps</b> ( <i>Macrobrachium rosenbergii</i> ) border control – screening sample distribution on the market (possible)/destination of the product identified
16/08	Belgium <b>INDIA</b>	2007.0559	nitrofurans (metabolite) nitrofurazone ( <b>SEM</b> ) (>1 µg/kg – ppb) in frozen <b>shrimps</b> ( <i>Macrobrachium rosenbergii</i> ) market control distribution on the market (possible)/product (to be) returned to dispatcher
23/08	Belgium <b>INDIA</b>	2007.0573	nitrofurans (metabolite) furazolidone ( <b>AOZ</b> ) (>1 µg/kg – ppb) in frozen peeled deveined raw black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) market control distribution on the market (possible)/product detained
11/12	Belgium <b>INDIA</b>	2007.0928	nitrofurans (metabolite) nitrofurazone ( <b>SEM</b> ) (>1 µg/kg – ppb) in raw frozen freshwater headless <b>scampi</b> ( <i>Macrobrachium rosenbergii</i> ) border control – screening sample distribution on the market (possible)/product (to be) returned to dispatcher
11/12	Belgium <b>INDIA</b>	2007.0931	nitrofurans (metabolite) nitrofurazone ( <b>SEM</b> ) (>1 µg/kg – ppb) in frozen headless <b>scampi</b> ( <i>Macrobrachium rosenbergii</i> ) border control – screening sample distribution on the market (possible) dispatched from the United Kingdom
<b>2008</b>			
07/02	Germany <b>CHINA</b> , via Belgium	2008.0149	nitrofurans (metabolite) furazolidone ( <b>AOZ</b> ) (15.2 µg/kg – ppb) in frozen cooked <b>pud shrimps</b> ( <i>Pandalus vannamei</i> ) official control on the market distribution on the market (possible)/product (to be) destroyed
20/02	Belgium <b>THAILAND</b> , via the U.K.	2008.0194	nitrofurans (metabolite) nitrofurazone ( <b>SEM</b> ) (11; 15 µg/kg – ppb) in frozen raw freshwater <b>shrimps</b> border control – screening sample distribution on the market (possible)
30/04	Latvia <b>HUNGARY</b>	2008.0495	nitrofurans (metabolite) nitrofurazone ( <b>SEM</b> ) (1.6 µg/kg – ppb) in <b>honey</b> dispatched from Hungary official control on the market distribution on the market (possible)/product (to be) withdrawn from sale
01/07	Belgium <b>INDIA</b>	2008.0785	nitrofurans (metabolite) nitrofurazone ( <b>SEM</b> ) (15 µg/kg – ppb) in frozen headless freshwater <b>shrimps</b> ( <i>Macrobrachium rosenbergii</i> ) border control – consignment released distribution on the market (possible)/reinforced checking
11/07	U.K. <b>INDIA</b>	2008.0832	nitrofurans (metabolite) furazolidone ( <b>AOZ</b> ) (150 µg/kg – ppb) in frozen raw peeled black tiger <b>shrimps</b> official control on the market distribution on the market (possible)/product (to be) destroyed

Table 2. Nitrofurans in the Rapid Alert System for Food and Feed: Information Notifications (week 1, 2007 to week 37, 2008)

Date	Notified by <b>Imported from</b>	Reference	Reason for notifying Notification basis and status (from April 2007)
<b>2007</b>			
05/01	U.K. <b>THAILAND</b>	2007.ABA	nitrofurans (metabolite) – nitrofurazone ( <b>SEM</b> ) in warm water <b>prawns</b>
09/01	Italy <b>CHINA</b>	2007.ABK	nitrofurans (metabolite) – nitrofurazone ( <b>SEM</b> ) in dried <b>hog casing</b>
12/01	Latvia <b>UKRAINE</b>	2007.ACI	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in natural bee <b>honey</b>
12/01	Netherlands <b>CHINA</b>	2007.ACU	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in frozen white <b>shrimp</b> ( <i>Penaeus vannamei</i> )
15/01	U.K. <b>MALAYSIA</b>	2007.ACY	nitrofurans (metabolite) – nitrofurazone ( <b>SEM</b> ) in frozen <b>prawns</b>
19/01	The Netherlands <b>INDIA</b>	2007.ADX	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in fresh frozen peeled tiger <b>prawns</b>
01/02	Belgium <b>INDIA</b>	2007.AHL	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in raw fresh frozen black tiger <b>prawns</b> ( <i>Penaeus monodon</i> )
13/02	U.K. <b>INDIA</b>	2007.AJZ	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in black tiger <b>prawns</b>
26/02	U.K. I <b>NDIA</b>	2007.AMP	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in black tiger <b>prawns</b> ( <i>Penaeus monodon</i> )
21/03	U.K. <b>INDIA</b>	2007.ASM	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in frozen black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> )
21/03	Spain <b>CHINA</b>	2007.ASP	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in tropical prawns ( <i>Penaeus vannamei</i> )
27/03	Belgium <b>INDIA</b>	2007.AUH	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) in frozen black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> )
13/04	U.K. <b>BANGLA- DESH</b>	2007.AXZ	nitrofurans (metabolite) – nitrofurazone ( <b>SEM</b> ) (9.1 µg/kg – ppb) in frozen raw brown head-on <b>shrimps</b> border control – screening sample information on distribution not (yet) available/product (to be) returned to dispatcher
13/04	U.K. <b>BANGLA- DESH</b>	2007.AYC	nitrofurans (metabolite) – nitrofurazone ( <b>SEM</b> ) (7.5 mg/kg – ppm) in frozen freshwater <b>shrimp</b> ( <i>Macrobrachium rosenbergii</i> ) market control no distribution / withdrawal from sale
13/04	U.K. <b>CHINA</b>	2007.AYD	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) (1.4 µg/kg – ppb) in frozen raw <b>tilapia</b> fillets market control distribution restricted to notifying country/recall from recipients
13/04	U.K. <b>BANGLA- DESH</b>	2007.AXZ	nitrofurans (metabolite) – nitrofurazone ( <b>SEM</b> ) (9.1 µg/kg – ppb) in frozen raw brown head-on <b>shrimps</b> border control – screening sample information on distribution not (yet) available / product (to be) returned to dispatcher
13/04	U.K. <b>CHINA</b>	2007.AYD	nitrofurans (metabolite) – furazolidone ( <b>AOZ</b> ) (1.4 µg/kg – ppb) in frozen raw <b>tilapia</b> fillets market control distribution restricted to notifying country/recall from recipients

Table 2 Part 2

Date	Notified by <b>Imported from</b>	Reference	Reason for notifying Notification basis and status (from April 2007)
19/04	Germany <b>INDIA</b>	2007.AZH	nitrofurantoin (metabolite) – furazolidone ( <b>AOZ</b> ) (3.6 µg/kg – ppb) in black tiger <b>prawns</b> ( <i>Penaeus monodon</i> ) border control – import rejected no distribution / product (to be) re-dispatched
27/04	Spain <b>CHINA</b>	2007.BBE	nitrofurantoin (metabolite) – furazolidone ( <b>AOZ</b> ) (2 µg/kg – ppb) and nitrofurantoin (metabolite) – nitrofurazone ( <b>SEM</b> ) (counter analysis: 1.8 µg/kg – ppb) in frozen <b>shrimps</b> ( <i>Penaeus vannamei</i> ) border control – import rejected no distribution/product (to be) re-dispatched
27/04	Spain <b>CHINA</b>	2007.BBF	nitrofurantoin (metabolite) – furazolidone ( <b>AOZ</b> ) (9.2; 2.1; 6.2 µg/kg – ppb) in raw <b>shrimps</b> ( <i>Penaeus vannamei</i> ) border control – import rejected no distribution/reinforced checking
18/06	Germany <b>INDIA</b>	2007.BMQ	nitrofurantoin (metabolite) – furazolidone ( <b>AOZ</b> ) (3.0 µg/kg – ppb) in black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) border control – import rejected no distribution/product (to be) re-dispatcher
26/06	Belgium <b>BANGLADESH</b>	2007.BOT	nitrofurantoin (metabolite) – furazolidone ( <b>AOZ</b> ) (4.6 µg/kg – ppb) and nitrofurantoin (metabolite) – furazolidone ( <b>AOZ</b> ) (>5 µg/kg – ppb) in black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) border control – import rejected no distribution/product (to be) destroyed
03/07	Belgium <b>INDONESIA</b>	2007.BQQ	nitrofurantoin (metabolite) – furazolidone ( <b>AOZ</b> ) (>5 µg/kg – ppb) in frozen cooked <b>shrimps</b> ( <i>Penaeus vannamei</i> ) company's own check distribution restricted to notifying country/product (to be) returned to dispatcher
19/07	Italy <b>BANGLADESH</b>	2007.BTO	nitrofurantoin (metabolite) – nitrofurazone ( <b>SEM</b> ) (1 µg/kg – ppb) in frozen fresh water headless shell on easy peel <b>shrimps</b> ( <i>Macrobrachium rosenbergii</i> ) border control – import rejected no distribution / product (to be) re-dispatched
27/07	Spain <b>CHINA</b>	2007.BUX	chloramphenicol (0.40 µg/kg – ppb) and nitrofurantoin (metabolite) nitrofurazone ( <b>SEM</b> ) (8.5 µg/kg – ppb) in dried <b>hog casing</b> border control – import rejected no distribution / product (to be) re-dispatched
02/08	Netherlands <b>INDIA</b> via the Netherlands	2007.0523	nitrofurantoin (metabolite) nitrofurazone ( <b>SEM</b> ) (1.2 µg/kg - ppb) in frozen fresh water headless <b>shrimps</b> ( <i>Macrobrachium rosenbergii</i> ) border control – screening sample distribution on the market (possible)/destination of the product identified
07/08	Netherlands <b>VIETNAM</b> via Germany	2007.0529	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (1.5 µg/kg - ppb) in frozen tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) border control – screening sample distribution on the market (possible)/destination of the product identified
13/08	Spain <b>CHINA</b>	2007.BXW	prohibited substances chloramphenicol (>8 µg/kg – ppb) and nitrofurantoin (metabolite) nitrofurazone ( <b>SEM</b> ) (8 µg/kg – ppb) in dried <b>hog casing</b> border control – import rejected no distribution / product (to be) re-dispatched



Table 2 Part 3

Date	Notified by <b>Imported from</b>	Reference	Reason for notifying Notification basis and status (from April 2007)
16/08	Belgium <b>INDIA</b>	2007.BYH	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (>1 µg/kg - ppb) in frozen peeled deveined raw black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) market control information on distribution not (yet) available/product detained
22/08	France <b>CHINA</b>	2007.BZO	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (1.7 µg/kg – ppb) in salted <b>pork casings</b> border control – screening sample no distribution / destination of the product identified
24/08	Netherlands <b>INDIA</b>	2007.CAJ	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (1.2 mg/kg – ppm) in frozen processed black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) border control – import rejected no distribution / product (to be) re-dispatched
27/08	Netherlands <b>INDIA</b>	2007.CAL	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (3.8 µg/kg – ppb) and nitrofurantoin (metabolite) nitrofurantoin (AHD) (0.3 µg/kg – ppb) in frozen black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) border control – import rejected no distribution
19/09	Netherlands <b>CHINA</b>	2007.CEF	nitrofurantoin (metabolite) nitrofurazone ( <b>SEM</b> ) (12.0 µg/kg – ppb) in frozen <b>shrimps</b> ( <i>Palaemon modestus heller</i> ) border control – import rejected no distribution/product (to be) re-dispatched
05/10	Germany <b>CHINA</b> , via Belgium	2007.0718	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (4.16 µg/kg – ppb) in frozen peeled tiger <b>shrimps</b> market control distribution on the market (possible)/withdrawal from recipient(s)
10/10	France <b>CHINA</b>	2007.CID	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (4.6 µg/kg – ppb) in frozen <b>rabbit meat</b> border control – screening sample no distribution/reinforced checking
24/10	Spain <b>CHINA</b>	2007.CKZ	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) in salted <b>lamb casings</b> border control – import rejected no distribution/product (to be) re-dispatched
21/11	Poland <b>CHINA</b>	2007.CQV	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) in salted <b>hog casings</b> border control – import rejected no distribution/product (to be) re-dispatched
7/12	U.K. <b>INDIA</b>	2007.CWG	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (11 µg/kg – ppb) in frozen black tiger shrimps border control – import rejected no distribution/product detained
11/12	Belgium <b>INDIA</b>	2007.0928	nitrofurantoin (metabolite) nitrofurazone ( <b>SEM</b> ) (> 1 µg/kg – ppb) in raw frozen freshwater headless <b>scampi</b> ( <i>Macrobrachium rosenbergii</i> ) border control – screening sample distribution on the market (possible) / product (to be) returned to dispatcher <b>2008</b>
<b>2008</b>			
03/01	Germany <b>CHINA</b>	2008.0004	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (1.13 µg/kg – ppb) in salted hog casings border control – screening sample distribution restricted to notifying country/product (to be) destroyed
28/02	Norway <b>INDIA</b>	2008.0229	nitrofurantoin (metabolite) furazolidone ( <b>AOZ</b> ) (3 µg/kg – ppb) in black tiger prawns ( <i>Penaeus monodon</i> ) border control – screening sample distribution restricted to notifying country / product (to be) detained

Table 2 Part 4

Date	Notified by <b>Imported from</b>	Reference	Reason for notifying Notification basis and status (from April 2007)
04/03	U.K. <b>INDIA</b>	2008.0257	nitrofuran (metabolite) furazolidone ( <b>AOZ</b> ) (5.5 µg/kg – ppb) and nitrofuran (metabolite) nitrofurazone ( <b>SEM</b> ) (1.3 µg/kg – ppb) in black tiger <b>prawns</b> ( <i>Penaeus monodon</i> ) official control on the market distribution restricted to notifying country/no stock left
25/04	U.K. <b>INDIA</b>	2008.0473	nitrofuran (metabolite) nitrofurazone ( <b>SEM</b> ) (1 µg/kg – ppb) in chilled head-on <b>scampi</b> ( <i>Macrobrachium rosenbergii</i> ) border control – screening sample no stock left / no action taken
05/06	U.K. <b>INDIA</b>	2008.0666	nitrofuran (metabolite) furazolidone ( <b>AOZ</b> ) (2.2 µg/kg – ppb) in frozen raw black tiger <b>prawns</b> official control on the market distribution restricted to notifying country / product (to be) withdrawn from sale
12/09	Germany <b>INDIA</b>	2008.1087	nitrofuran (metabolite) furazolidone ( <b>AOZ</b> ) (63.2 µg/kg – ppb) in raw head-on shell-on black tiger <b>shrimps</b> ( <i>Penaeus monodon</i> ) official control on the market distribution restricted to notifying country / product (to be) withdrawn from the market

in years 2004, 2005 and 2006, respectively, although a decline in 2007 (31%) was evident (Commission Regulation, 2002). Product notifications for semi-carbazide contamination have included not only food stuffs of animal origin such as aquaculture products (shrimp, prawn and crab), bovine and porcine tissue, poultry and chicken eggs but also in products such as baby food and flour (European Commission, 2008).

Findings of high volumes of SEM in baby food have caused great concern for infant health and resulted in the development of appropriate detection methods (De Souza et al., 2005; Ginn et al., 2006). It was found that azodicarbonamide, a foaming agent used in gasket production decomposes into gases (primarily nitrogen and carbon dioxide) during the heat treatment process and can leave trace amounts of residues such as biurea, urazole, cyanuric acid and cyamelide (European Commission, 2003). Moreover, studies have confirmed the presence of SEM as a by-product of azodicarbonamide treated gaskets in jarred foods (Stadler et al., 2004). Potentially susceptible products include jams, honey, fruit juices, pickles, sterilised products, mayonnaise, mustard, and ketchup. Currently azodicarbonamide is suspended from use in EU countries (Commission Directive, 2004).

The formation of SEM during the baking of bread (Becalski et al., 2004, 2006) and flour-coated poultry products (Hoenicke et al., 2004) was also confirmed when the use of azodicarbonamide as an additive in flour was examined. SEM formation has also been observed in samples such as carrageen (a seaweed extract used as a food additive), starch and egg white powder treated with hypochlorite solutions containing 12% active chlorine (Hoenicke et al., 2004). Hypochlorite is commonly used for carrageen bleaching or water disinfection and also as a disinfectant during egg breaking procedures (de la Calle and Anklam, 2005). SEM has also been found to occur naturally in particular crustaceans such as shrimp, prawn, and crab, generating queries over its suitability as a marker for detection purposes in these species (Pereira et al., 2004; Saari and Peltonen, 2004; Hoenicke and Gatermann, 2006).

Nitrofurazone has been found to accumulate over time in both avian eyes as well as the pig retina, and has been suggested as an alternative marker to SEM for the monitoring of NFZ abuse (Cooper and Kennedy, 2005; Cooper et al., 2005b). Samsonova et al. (2008) isolated proteins from rat liver and examined them for the presence of tissue bound metabolite, SEM. Albumin and glutathione S-transferase proteins contained high concentrations of bound

Table 3. Nitrofurans in the Rapid Alert System for Food and Feed: Border Rejections (week 1 to week 37, 2008)

Date	Notified by Imported from	Reference	Reason for notifying Action (to be) taken
<b>2008</b>			
22/01	Spain <b>CHINA</b>	2008.ADR	nitrofurans (metabolite) nitrofurazone (SEM) (1.9 µg/kg – ppb) in cooked shrimps product (to be) re-dispatched
22/02	Belgium <b>INDIA</b>	2008.AIQ	nitrofurans (metabolite) nitrofurazone (SEM) (>1; >1; 14.4; 21 µg/kg – ppb) in frozen raw headless shell-on freshwater shrimps product (to be) destroyed
13/03	Spain <b>CHINA</b>	2008.ALM	nitrofurans (metabolite) nitrofurazone (SEM) (10 µg/kg – ppb) in frozen peeled red shrimps ( <i>Solenocera melantho</i> ) product (to be) destroyed
21/03	Belgium <b>INDIA</b>	2008.AMZ	nitrofurans (metabolite) nitrofurazone (SEM) (6; 9; 13 µg/kg – ppb) in frozen raw headless fresh water shrimps ( <i>Macrobrachium rosenbergii</i> ) product (to be) re-dispatched or destroyed
19/03	Spain <b>VIETNAM</b>	2008.AMT	nitrofurans (metabolite) nitrofurazone (SEM) (2.3 µg/kg – ppb) in frozen Pangasius fillets product (to be) re-dispatched
04/04	Belgium <b>THAILAND</b>	2008.AOF	nitrofurans (metabolite) nitrofurazone (SEM) (4.8; 7.5; 11; 9.5 µg/kg – ppb) in frozen raw freshwater head-on shell-on shrimps ( <i>Macrobrachium rosenbergii</i> ) product (to be) re-dispatched or destroyed
25/04	Germany <b>INDIA</b>	2008.ARK	nitrofurans (metabolite) furazolidone (AOZ) (1.1 µg/kg – ppb) in shrimps ( <i>Penaeus monodon</i> ) product (to be) re-dispatched
28/05	U.K. <b>INDIA</b>	2008.AUP	nitrofurans (metabolite) furazolidone (AOZ) (18 µg/kg – ppb) in black tiger shrimps product (to be) destroyed
23/06	Italy <b>MALAYSIA</b>	2008.AZH	nitrofurans (metabolite) furazolidone (AOZ) (5.6 µg/kg – ppb) in raw frozen farmed black tiger prawns ( <i>Penaeus monodon</i> ) product (to be) re-dispatched or destroyed
25/07	Belgium <b>INDIA</b>	2008.BEF	nitrofurans (metabolite) nitrofurazone (SEM) (>1 µg/kg – ppb) in frozen raw freshwater king prawns product (to be) re-dispatched
06/08	Norway <b>INDIA</b>	2008.BFP	<i>Vibrio cholerae</i> NON O:1/NON O:139 and prohibited substances nitrofurans (metabolite) furazolidone (AOZ) (7.5 µg/kg – ppb) and nitrofurans (metabolite) nitrofurazone (SEM) (0.65 µg/kg – ppb) in frozen black tiger shrimps product (to be) re-dispatched or destroyed
11/08	Belgium <b>INDIA</b>	2008.BGM	nitrofurans (metabolite) nitrofurazone (SEM) (between 1.5 and 2.8 µg/kg – ppb) in frozen raw freshwater headless shell-on prawns shrimps product (to be) destroyed
04/09	Germany <b>CHINA</b>	2008.BJR	prohibited substance nitrofurans (metabolite) furazolidone (AOZ) (2.6 µg/kg – ppb) in single frozen tilapia fillet ( <i>Oreochromis niloticus</i> ) product (to be) re-dispatched
08/09	Italy <b>THAILAND</b>	2008.BKJ	prohibited substance nitrofurans (metabolite) nitrofurazone (SEM) (2.03 ≤ < 3.1 µg/kg – ppb) in frozen aquacultured breaded butterfly shrimps product (to be) re-dispatched or destroyed
09/09	Belgium <b>BANGLADESH</b>	2008.BKM	prohibited substance nitrofurans (metabolite) nitrofurazone (SEM) (>1 µg/kg – ppb) in fresh water headless shell-on shrimps ( <i>Macrobrachium rosenbergii</i> ) product (to be) re-dispatched or destroyed

SEM, suggesting their potential use as biomarkers for the detection of nitrofurazone exposure. Additionally, a novel method for the detection of biurea to discriminate between nitrofurazone and azodicarbonamide use in food products was described by Mulder et al. (2007).

## 6. Legislation and analytical control

Nitrofurans antibiotics have been included in Annex IV of Commission Regulation (EC) 1442/95 as compounds that are not permitted for use in the livestock industry (Commission Regulation, 1995).

The EU has established a minimum required performance limit (MRPL) of 1 µg/kg, for edible tissues of animal origin (Commission Decision, 2003). The illegal use of nitrofurans is controlled by official inspection and analytical services provided by laboratories following the recommendations specified by Council Directive 96/23/EC. According to this document, the EU Member States are required to set up monitoring plans and sampling procedures for given substances in live animals and their respective food products (Council Directive, 2008). Laboratories performing residual control usually deal with large numbers of samples, with a great variety of residues to be detected in a short period of time. The use of rapid screening methods can improve the effectiveness of residual control in both official and industrial laboratories, although contamination should be confirmed by a suitable instrumental method.

Although an MRPL of 1 µg/kg has been established, application and interpretation of this legislative to exports from 3<sup>rd</sup> countries into Europe has been rather complicated. Implementation of the new legislation has resulted in increasing trade restrictions to these countries and required large investments for newer and more sensitive instrumental equipment. Glenn Kennedy reported the situation regarding the so called “Zero tolerance to nitrofurans” and MRPL as follows: “*The EU has a policy of zero tolerance towards the use of nitrofurans in food-producing animals. According to the current legislation, any confirmed concentration of any of the metabolites is a non-compliance. Due to the export interests, 3<sup>rd</sup> countries were forced to accept the EU MRPL and as exporting countries reach the same performance limits as the EU laboratory with the lowest (i.e. most sensitive) threshold*” (Kennedy, 2004). Commission Decision (2003) describing the MRPL ruled out the use of unsatisfactory methods which are incapable of quantifying very low concentrations of nitrofurans metabolites, however, “*the MRPL concept did not include any provision for a maximum standard (for detection methods used)*” (Kennedy, 2004). In other words, the method is only required to be able to quantify concentration values up to 1 µg/kg, but the lowest concentration of analyte which should be quantifiable is not specified. This value is referred to as the decision limit,  $CC_{\alpha}$  (described below), and is determined by many laboratories using validation guidelines provided by the EU. However, the fluctuation of  $CC_{\alpha}$  between different instrumental methods and laboratories is a complicating fac-

tor in the application of this parameter to the zero tolerance requirements for nitrofurans residues in edible tissue.

In order to ensure quality and comparability of the analytical results generated by laboratories, a set of common performance criteria for residual analysis was incorporated into the European legislation. Commission Decision 2002/657/EC, sets guidelines for the validation of both screening and confirmatory analytical methods of analysis (Commission Decision, 2002). The commission decision implements the Council Directive 96/23/EC concerning the method performance and interpretation of results, for the fulfilment of key requirements set by the EU (Commission Decision, 2002).

According to this decision, performance characteristics such as detection capability ( $CC_{\beta}$ ), precision, selectivity and applicability/stability need to be assessed in order to classify a screening method as quantitative. For analytes which do not have a declared maximum residual limit (MRL), such as nitrofurans metabolites,  $CC_{\beta}$  is defined as the lowest concentration of a substance that can be measured by a method with an error probability  $\beta = 5\%$ . For quantitative screening, precision is acquired by the determination of variation coefficients. Inter-assay variation testing gives an indication of the precision of the assay over a longer period of time. Selectivity or specificity is the ability of a method to distinguish between the analyte being measured and other substances. Stability of the standard analyte in solution and in the matrix should be included in the validation process. For qualitative screening methods, only  $CC_{\beta}$ , selectivity and applicability need to be assessed. Any positive findings assessed using screening methods should always be re-analysed by a validated confirmatory method.

In order to classify the method as confirmatory, the decision limit ( $CC_{\alpha}$ ) and trueness/recovery must also be determined (Commission Decision, 2002).  $CC_{\alpha}$  is defined as the limit at which a substance can be concluded as positive with an error probability  $\alpha = 1\%$ . Information on the accuracy of a confirmatory method is determined by assessment of trueness. Trueness refers to the closeness of agreement between the averages recorded for a data set and is determined by the degree of deviation from the mean recovery. At least six aliquots of the matrix fortified with the target analyte at concentrations equal to or above the MRPL should be used to determine recovery yield (Commission Decision, 2002).

## 7. Sample preparation methods

As shown in Table 4, various sample preparation methods prior to analysis have been reported for a large variety of matrices such as in animal feeds (Barbosa et al., 2007), animal tissues (Verdon et al., 2007; Rodziewicz and Zawadzka, 2007; Chang et al., 2008; Cooper et al., 2008), chicken eggs (Szilagy and de la Calle, 2006; Cooper et al., 2007; Vass et al., 2008b), aquaculture products such as shrimp (Chu and Lopez, 2005) and prawn (Cooper et al., 2004b), water (Lui et al., 2007), and milk (Rodziewicz, 2008). Methods for the determination of semicarbazide presence or contamination not related to nitrofurans in matrices such as carrageenan and algae (Hoenicke et al., 2004), shrimp (Bock et al., 2007b), crayfish (Saari and Peltonen, 2004) flour (Becalski et al., 2004), salt (Pereira et al., 2004) and baby food (De Souza et al., 2005) have also been established.

Tissue bound nitrofurans metabolites are small molecular species which are usually derivatised using *ortho*-nitrobenzaldehyde (*o*-NBA) in order to increase molecular mass and improve the sensitivity of detection (Conneely et al., 2002). Prior to derivatisation, the release of bound metabolites from tissue is carried out under mildly acidic conditions (Figure 2b). Sample preparation based on this approach provides data on the total amount (free and bound) of nitrofurans metabolites in tissue. The resulting nitrophenyl (NP) derivatives of the respective metabolites (featured in Figure 1) are separated from a sample using various extraction methods.

Generally, sample preparation involves homogenisation, acid hydrolysis, derivatisation and extraction. Solvent extraction is a commonly used method for nitrofurans. Extraction of the nitrophenyl derivatives is carried out using a moderately polar organic solvent such as ethyl acetate and when necessary, a clean up step using a non polar solvent such as hexane is used to remove lipids from sample prior to detection. The use of solvent extraction in sample preparation for the detection of AOZ in eggs was first described by McCracken et al., (2001). Incurred egg samples were subjected to acid hydrolysis and derivatisation, followed by pH adjustment (using sodium hydroxide and dipotassium hydrogen *ortho*-phosphate) and double ethyl acetate extraction. The solvent was removed using nitrogen and the residue redissolved in diluted acetonitrile prior to detection by liquid chromatography-mass spectroscopy (LC-MS) (McCracken et al., 2001).

Solvent extraction methods used in porcine sample preparation vary depending on the nature of the sample and also the analyte. Several sample preparation methods employ sample pre-treatment prior to extraction. One approach, used to extract AOZ from porcine tissue, was to freeze and pulverise the muscle tissue into a fine powder using a food blender (McCracken and Kennedy, 1997a, b). Pre-treatment of fresh tissue samples (such as liver, kidney and muscle) with ice cold methanol and ethanol washes has also been utilised to isolate extractable metabolites, namely AOZ and AHD, from the sample (Horne et al., 1996; Cooper et al., 2005a). Other studies which did not employ sample pre-treatment, used multiple ethyl acetate extractions to ensure that

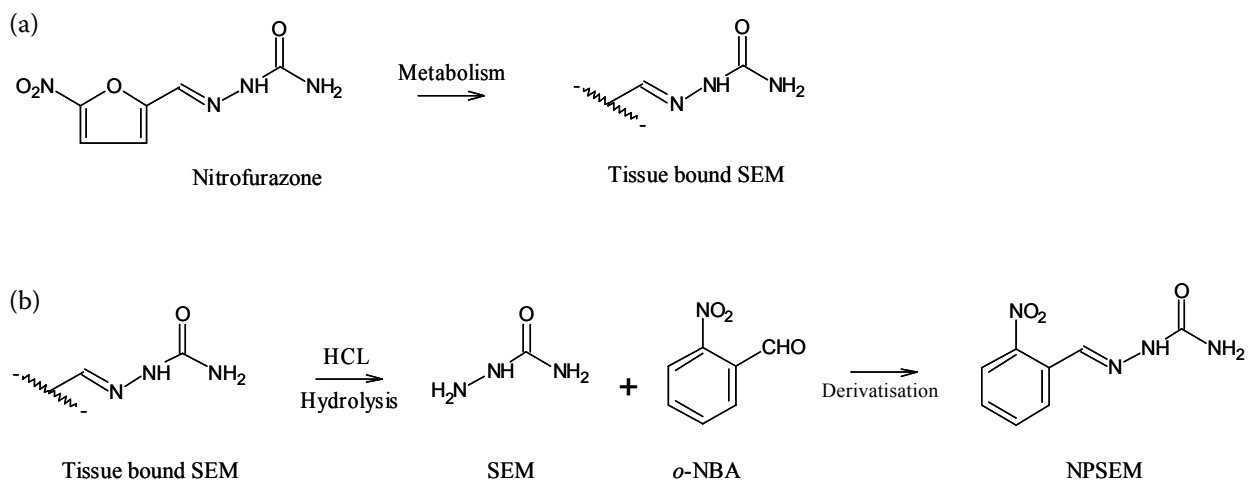


Figure 2. *In vivo* formation of tissue bound SEM (a). Release of bound SEM under mildly acidic conditions followed by SEM derivatisation to produce target analyte (b)

Table 4. Instrumental methods for the determination of nitrofurans

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Egg	AOZ	1 g of homogenised sample was derivatised (5 ml 0.1M HCl, 150 µl 50mM <i>o</i> -NBA in DMSO) and the pH adjusted (5 ml 0.1M K <sub>2</sub> HPO <sub>4</sub> , 0.4 ml 1M NaOH) followed by a 2 × 6 ml ethyl acetate extraction	HPLC separation used a C18 column (inj. vol. 25 µl, flow rate 0.2 ml/min) with MS/MS detection set in electrospray positive ionisation mode and multiple reaction monitoring mode	98–104%	Respective CC <sub>α</sub> and CC <sub>β</sub> AOZ: 0.14 and 0.44 AMOZ: 0.13 and 0.25 AHD: 0.21 and 0.60 SEM: 0.19 and 0.28	Cooper et al. (2008)
	AMOZ					
	AHD					
	SEM					
Egg	AOZ	1 g of sample homogenate underwent derivatisation (5 ml 0.2M HCl, 75 µl 100 mM <i>o</i> -NBA in MeOH) pH adjustment (pH 7, 0.5 ml), extraction and clean up using ethyl acetate (2 × 4 ml) and <i>n</i> -hexane (2 × 2 ml)	HPLC separation used a C18 column (flow rate 0.2 ml/min, inj. vol. 40 µl) with triple quadrupole MS/MS set in electrospray positive ionisation mode	95.2–102.1%	Respective CC <sub>α</sub> and CC <sub>β</sub> AOZ: 0.03 and 0.03 AMOZ: 0.05 and 0.06 AHD: 0.22 and 0.28 SEM: 0.20 and 0.25	Bock et al. (2007b)
	AMOZ					
	AHD					
	SEM					
Egg	AOZ	1 g of sample was homogenised with 4 ml H <sub>2</sub> O, after derivatisation (0.5 ml 1M HCl), 150 µl 50mM <i>o</i> -NBA in DMSO) the pH was adjusted (pH 7.4, 5 ml 0.1M K <sub>2</sub> HPO <sub>4</sub> , 0.4 ml 1M NaOH) and the residue was extracted (2 × 5 ml ethyl acetate)	HPLC separation using a C18 column (inj. vol. 5 µl) with MS/MS detection set in electrospray positive ionisation mode	70–115%	CC <sub>α</sub> ≤ 1 CC <sub>β</sub> ≤ 2	McCracken and Kennedy (2007)
	AMOZ					
	AHD					
	SEM					
Egg white powder	SEM	2 g of homogenised sample was derivatised (10 ml 0.2M HCl, 240 µl 10M <i>o</i> -NBA in MeOH) and the pH adjusted (pH 7: 10 ml 0.2M K <sub>2</sub> HPO <sub>4</sub> , 0.8 ml 2M NaOH). Extraction and clean up utilised SPE cartridges	HPLC used a reversed phase C18 separation column (flow rate 0.2 ml/min) with MS/MS detection using multiple reaction monitoring mode	85–187.5%	LOD = 0.15 and 0.4 for fresh egg and egg powder (respectively) LOQ = 0.2	Szilagyí and De la Calle (2006)
	SEM					
	SEM					
	SEM					
Egg white powder	SEM	Refer to Szilagyí and De la Calle (2006)	Refer to Szilagyí and De la Calle (2006)	105.3–121.3%	Various (interlaboratory validation)	De la Calle and Szilagyí (2006)
	SEM					
	SEM					
	SEM					
Egg	AOZ	Refer to McCracken and Kennedy (1997a)	Refer to McCracken and Kennedy (1997a)	NA	NA	McCracken et al. (2005a)
	AMOZ					
	AHD					
	SEM					

Table 4 Part 2

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Egg	FZD AOZ	For FZD refer to Yoshida and Kondo (1995) For AOZ refer to McCracken and Kennedy (1997a)	Refer to McCracken and Kennedy (1997a)	75–95%	LOD ~ 1	McCracken et al.(2001)
Egg Chicken liver muscle	NFZ FZD FAD	10 g of homogenised sample was extracted with 30 ml acetonitrile followed by blending then centrifugation. Purification was carried out by addition of 10 ml NaCl and 50 ml dichloro-methane, filtration with NaSO <sub>3</sub> and evaporation using a rotary evaporator. Clean up was conducted with 3 × 1 ml hexane	HPLC separation used a C18 column (flow rate 1 ml/min) and photodiode array detection, as well as a single quadrupole MS detector in atmospheric chemical ionisation mode	83.2–88.9%	LOD: NFZ = 3.2, FZD = 1.6 and FAD = 1.0	Draisci et al. (1997)
Egg Porcine serum	FZD	Samples (1 ml serum, 0.5 ml egg) were diluted (3 ml H <sub>2</sub> O) and extracted using Extrelut-3 SPE cartridges (eluted with 14 ml ethyl acetate). Following evaporation (with N), samples were redissolved with an acetonitrile-water mixture	LC separation used a C18 column (inj. vol. 10 µl, flow rate 0.2 ml/min) with MS detection and an atmospheric pressure chemical ionisation interface system	87.0–90.0%	LOD = 0.1	Yoshida and Kondo (1995)
Egg Chicken muscle	NFZ FDZ FAD	10 g of sample was homogenised and mixed with 40 ml dichloromethane, ethyl acetate or acetonitrile was added then centrifuged (twice). Extracts were evaporated. Muscle tissue was redissolved in dichloromethane and ether (5 ml). Egg was redissolved with acetonitrile and further extracted 3 times with petroleum ether and redissolved and in 5 ml dichloromethane and ether. Clean up was carried out using Sep-Pak SPE columns	LC separation used a C18 column (inj. vol. 40 µl, flow rate 1.1 ml/min) with UV detection at 362 nm	84–128%	LOD = 1.0 (NFZ) and 2.0 (FZD and FAD)	Kumar et al. (1994)
Egg	FZD	Egg homogenate (8 g) was acidified at pH4 and extracted with dichloromethane. After solvent evaporation the sample was treated with 10 ml acetone, then filtered and again evaporated. The extract was partitioned with hexane (3×), and dichloromethane. The organic layer was evaporated and resuspended with 200 µl water	HPLC was performed on a reversed phase C8 column (flow rate 1 ml/min) equipped with single beam UV/VIS spectrophotometer(365 nm)	93%	LOD = 1	Botsoglou (1988)

Table 4 Part 3

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Poultry muscle Shrimp	AOZ	1 g of homogenised sample was derivatised (5 ml 0.2M HCl, 75 µl 0.1M <i>o</i> -NBA in MeOH), extracted with ethyl acetate (2 × 4 ml), and redissolved in 250 µl of MeOH and ammonium format. Clean up was performed using 2 × 2 ml <i>n</i> -hexane	HPLC separation used a C18 column (flow rate 0.2 ml/min <sup>-1</sup> inj. vol. 50 µl) with triple quadrupole MS/MS set in electrospray positive ionisation mode	92.6–100.6%	Respective $CC_{\alpha}$ and $CC_{\beta}$ AOZ: 0.12 and 0.14 AMOZ: 0.13 and 0.15 AHD: 0.67 and 0.82 SEM: 0.70 and 0.88	Bock et al. (2007a)
	AMOZ					
	AHD					
	SEM					
Poultry muscle	AOZ	1 g of homogenised sample was derivatised 0.5 ml 1M HCl, 150 µl 50mM <i>o</i> -NBA in DMSO) and the pH adjusted (pH 7: 5 ml 0.1M $K_2HPO_4$ 0.3 ml 1M NaOH). The sample was extracted using ethyl acetate (5 ml and 3 ml)	LC separation used C8 and C18 columns (flow rate 0.2 ml/min) with triple quadrupole MS/MS detection set in electrospray positive ionisation mode	NA	$CC_{\alpha}$ and $CC_{\beta}$ ranged from 0.08–0.54 and 0.10–0.66 (respectively) for all analytes	Verdon et al. (2007)
	AMOZ					
	AHD					
	SEM					
	DNASH					
Poultry muscle egg	AOZ	1 g of homogenised sample was derivatised (5 ml 0.2M HCl, 50 µl 100 mM <i>o</i> -NBA in DMSO) and the pH adjusted (pH 7: 0.5 ml 0.3M $NaPO_3$ , 0.4 ml 2M NaOH). Extraction was performed using 4 ml ethyl acetate	HPLC separation used a C18 column (flow rate 0.2 ml/min) with triple quadrupole MS/MS set in electrospray positive ionisation mode	14.5–129.4%	LOQ was 0.5 for all analytes in egg and poultry	Finzi et al. (2005)
	AMOZ					
	AHD					
	SEM					
Poultry Porcine muscle	AOZ	5 g sample was homogenised with 250 ml 0.125M HCl and derivatised with 250 µl 50mM <i>o</i> -NBA in DMSO. The pH was adjusted and extraction used 15 ml ethyl acetate, a wash step with 1 ml hexane, and a clean up step using EN SPE cartridges	LC separation used a C18 column (inj. vol. 50 µl, 0.3 ml/min flow rate) with a triple quadrupole MS/MS detector set on electrospray positive ionisation and multiple reaction monitoring modes	85–123%	$CC_{\alpha}$ and $CC_{\beta}$ ranged from 0.11–0.21 and 0.19–0.36 (respectively)	Mottier et al. (2005)
	AMOZ					
	AHD					
	SEM					
Poultry liver muscle	AOZ	Refer to McCracken and Kennedy (1997a)	Refer to McCracken and Kennedy (1997a)	NA	$CC_{\alpha}$ range = 0.06–0.19	McCracken et al. (2005b)
	AMOZ					
	AHD					
	SEM					



Table 4 Part 4

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Porcine muscle liver	AOZ	1 g of sample was derivatised (9 ml 0.1M HCL, 150 µl 50mM <i>o</i> -NBA in DMSO), homogenised (with rinsing, 3 × 1 ml HCL), pH was adjusted (pH 7.2, 2.2 ml 0.3M NaPO <sub>3</sub> and 1M NaOH) and extraction performed using 2 × 8 ml ethyl acetate	Refer to Cooper et al. (2005a)	85.9–124.8%	CC <sub>α</sub> and CC <sub>β</sub> ≤ 1	Cooper and Kennedy (2007)
	AMOZ					
Porcine muscle liver kidney	AHD	1 g of sample, 1 ml water and 8 ml MeOH were homogenised. After centrifugation the pellet was washed with 3 × 4 ml MeOH and 2 × 4 ml ethyl ether. Both extracted fractions and supernatant were dried under N and resuspended with 4 ml water. Derivatisation (0.5 ml 1M HCl; 150 µl 50mM <i>o</i> -NBA in DMSO) was carried out and the pH adjusted (pH 7.4; 5 ml 0.1M K <sub>2</sub> HPO <sub>4</sub> ; 0.4 ml 0.8M NaOH). Extraction was carried out using 2 × 5 ml ethyl acetate	<p>LC-MS/MS conditions: C18 column with 50–100 µl inj. vol. and 0.4 ml/min flow rate, using an electrospray positive ionisation mode and multiple reaction monitoring mode.</p> <p>HPLC-UV conditions: C18 column with 20 µl inj. vol. and photodiode array detection</p>	NA	LC-MS/MS: LOD = 0.2–4 HPLC: LOD = 2–5	Cooper et al. (2005b)
	SEM					
	Parent drugs					
Porcine liver Prawns	AOZ	5 g of sample was washed with 1.5 ml water and 10 ml MeOH. After centrifugation the pellet was washed (3 × 10 ml MeOH and 2 × 10 ml ethyl ether), dried, and the pellet resuspended in 10 ml H <sub>2</sub> O. Derivatisation (1.5 ml 1M HCl and 100 µl 0.05 M <i>o</i> -NBA in DMSO) was followed by pH adjustment (pH 6.3) and ethyl acetate extraction (1 × 12 ml, 2 × 10 ml) SPE using Oasis MAX and Oasis HLB cartridges	<p>LC-MS/MS conditions: C18 column (inj. vol. 20 µl, 0.4 ml/min flow rate) with a triple quadrupole MS/MS detector set in electrospray positive ionisation mode.</p> <p>LC-UV conditions: C18 column (in. vol. 100 µl, 0.8 ml/min flow rate) with UV detection at 275 nm</p>	83.5–117% NA	NA	Conneely et al. (2003)

Table 4 Part 5

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Porcine liver	AOZ	3 ml protease enzyme digestion solution was added to 1 g sample after homogenisation. Samples were derivatised with 1 ml 1M HCl and 50 µl 0.5M <i>o</i> -NBA in DMSO, pH was adjusted (pH 6.3) and SPE was conducted using Oasis MAX and Oasis HLB cartridges	HPLC separation used a C18 column (inj. vol. 50 µl, flow rate 0.8 ml/min) in combination with UV/VIS detection	> 60%	NA	Conneely et al. (2002)
	AOZ AMOZ AHD SEM	10 g of sample homogenised with 90 ml 0.13M HCl. 10 ml homogenate was derivatised with 400 µl 50mM <i>o</i> -NBA in DMSO, the pH was adjusted (pH 7.4: 12.5 ml 0.1M $K_2HPO_4$ , 1.25 ml 0.8M NaOH) and after filtration, the sample was applied to EN SPE cartridges	HPLC separation used a C18 column (flow rate 0.5 ml/min) with a triple quadrupole MS/MS detector using an atmospheric pressure chemical ionisation interface system in multiple reaction monitoring mode	91.8–105.4%	Respective LOD and LOQ AOZ: 0.5 and 2.5 AMOZ: 0.5 and 2.5 AHD: 5.0 and 10.0 SEM: 3.0 and 10.0	Leitner et al. (2001)
Porcine	AOZ	Frozen tissue was pulverised to fine powder. 1 g sample, 1 ml water and 8 ml MeOH were homogenised. After centrifugation the pellet was washed with MeOH (3 × 4 ml) and ethyl ether (2 × 4 ml). Extract was dried under N and resuspended with 4 ml water. After derivatisation (0.5 ml 1M HCl, 140 µl 50mM <i>o</i> -NBA in DMSO) and pH adjustment (pH 7.4: 5 ml 0.1M $K_2HPO_4$ , 0.5 ml 0.8M NaOH), the sample was extracted (2 × 5 ml ethyl acetate)	HPLC separation using a reversed phase C18 column (inj. vol. 50 µl) with an MS detector set in electrospray positive ionisation mode	> 80%	LOD = 0.2–0.5	McCracken et al. (1997a)
	AOZ AMOZ	2 g of sample was mixed with 6 ml MeOH and water. After centrifugation the pellet was washed with MeOH (3 × 4 ml) and ethyl ether (2 × 4 ml). the extract was dried under N, resuspended with 6 ml water and derivatised (0.5 ml 1M HCl, 50 µl 50mM <i>o</i> -NBA in DMSO). The pH was adjusted (pH 7.4: 0.5 ml 0.1M $K_2HPO_4$ , 0.5 ml 1M NaOH) and the sample extracted with ethyl acetate (3 × 5 ml) and cleaned up using hexane (2 × 2 ml)	HPLC-UV conditions: C18 column (flow rate 0.8 ml/min) combined with UV detection and operated at 275 nm LC-MS conditions: C18 column (flow rate 0.5 ml/min) with single quadrupole MS detection and atmospheric pressure chemical ionisation operated in the positive ionisation mode	71–101%	HPLC-UV: LOD = 5 and 10 for AOZ and AMOZ (respectively) LC-MS: LOD = 10 (both analytes)	Horne et al. (1996)

Table 4 Part 6

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Porcine	AOZ	Refer to Hoogenboom et al. (1991)	Refer to Hoogenboom et al. (1991)	94.4–108.7%	NA	Gottschall and Wang (1995)
Porcine muscle liver	FZD	Frozen tissue was pulverised to fine powder. 2 g of sample was homogenised with 40 ml of buffer MeOH solution. After centrifugation the supernatant was evaporated and 25 ml dichloromethane was added. After mixing and centrifugation, the lower layer was again evaporated and the residue resuspended in 2 ml dichloromethane and 6 ml hexane. Extraction was carried out on Bond-Elut NH <sub>2</sub> cartridges	Thermospray LC-MS used a reversed phase C18 column (inj. vol. 50 µl, flow rate 1 ml/min and selected ion monitoring	62–76%	LOD = 1	McCracken et al. (1995)
Porcine liver kidney muscle	AOZ	Refer to McCracken and Kennedy (1997a)	Refer to McCracken and Kennedy (1997a)	NA	NA	McCracken et al. (1997b)
Porcine liver kidney muscle	AOZ	1 g of sample was washed with 6 ml MeOH and water(2 : 1). After centrifugation the pellet was washed with MeOH (3 × 4 ml), ethanol (2 × 4 ml and diethyl ether (2 × 4 ml). The pellet was dried under N, resuspended in H <sub>2</sub> O (4 ml) and derivatised (0.5 ml 1M HCl, 50 µl 50mM o-NBA in DMSO). After incubation the sample was extracted with ethyl acetate (3 × 4 ml) and the solvent evaporated and the process repeated (2 × 2 ml ethyl acetate). The final residue was redissolved in an acetonitrile/water mixture	HPLC separation used a C18-spher column (inj. vol. 100 µl) and was coupled to a single wavelength or diode array detector	80–100%	NA	Hoogenboom et al. (1992)

Table 4 Part 7

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results ( $\mu\text{g}/\text{kg}$ )	Reference
Porcine liver	FZD AOZ	1 g of frozen tissue was pulverised to a fine powder and washed with 6 ml of MeOH (3 $\times$ ), ethanol (2 $\times$ ) and diethyl ether. Following extraction, pellets were resuspended in H <sub>2</sub> O and mixed with <i>o</i> -NBA (50mM in DMSO) and HCl. After incubation, samples were extracted with ethyl acetate (2 $\times$ 2 ml) and evaporated. After repeating twice, the residue was redissolved in an acetonitrile/water mixture	HPLC separation used a C18-spher column (flow rate 1 ml/min) coupled to an online radioactive detector or diode array equipment	90–95%	NA	Hoogenboom et al. (1991)
Bovine muscle	NFZ NFT FZD FAD	10 g of sample was homogenised with 30 ml of acetonitrile and centrifuged. The supernatant was cleaned up with dichloromethane and ethyl acetate. The residue was redissolved in acetonitrile and hexane, which was then evaporated	LC separation used a reversed phase C18 column (inj. vol. 50 $\mu\text{l}$ ) with a UV/VIS diode array detector	60–110%	LOD = 1 LOQ = 2	Angelini et al. (1997)
Milk	AOZ AMOZ AHD SEM	1 g of raw milk was derivatised (9 ml 0.1M HCl, 100 $\mu\text{l}$ 100mM <i>o</i> -NBA), the pH was adjusted (pH 7.2, 1M NaOH) and the samples extracted with ethyl acetate (2 $\times$ 5 ml)	LC separation used a C18 column (inj. vol. 40 $\mu\text{l}$ , flow rate 0.2 ml/min) with an MS/MS detector set in electrospray positive ionisation mode and multiple reaction monitoring mode	91 - 107%	$CC_{\alpha}$ and $CC_{\beta}$ ranged from 0.12–0.29 and 0.15–0.37 (respectively) for all analytes	Rodziewicz (2008)
Milk	NFZ FZD FAD	Milk was deproteinised. 50 ml milk and 25 ml trichloric acid was filtered and the pH adjusted (pH 4.5–5) with NaOH, extraction was carried out on Sep-PakPlus C18 cartridges	HPLC used a C18 column (1 ml/min flow rate) with a coulometric detection system	85–98%	LOD = 0.5–0.8 ppb	Diaz et al. (1997)
Baby food	SEM	2 g of sample was derivatised (10 ml 0.2M HCl; 240 $\mu\text{l}$ 10M <i>o</i> -NBA in MeOH) and the pH adjusted (pH7: 10 ml 0.2M K <sub>2</sub> HPO <sub>4</sub> , 0.8 ml 2M NaOH). The extraction and clean up was performed using Strata SDB-L cartridges	HPLC used a reversed phase C18 separation column (flow rate 0.2 ml/min) with MS/MS detection using the multiple reaction monitoring mode	88.8–106.1%	NA	Ginn et al. (2006)

Table 4 Part 8

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Baby food	SEM	2 g of sample was hydrolysed and derivatised (10 ml 0.2M HCl, 240 µl 10M <i>o</i> -NBA in MeOH) and the pH adjusted (10 ml 0.2M K <sub>2</sub> HPO <sub>4</sub> , 0.8 ml 2M NaOH), extraction was carried out using Strata SDB/L cartridges	LC separation used a C18 reversed phase column (inj. vol. 10 µl, flow rate 0.2 ml/min) and MS/MS detector in multiple reaction monitoring mode	87.8–107.2%	LOD = 0.1 LOQ = 0.25	de Souza et al. (2005)
Blood	AOZ	1 ml of plasma serum was precipitated with MeOH and incubated with 1 ml 2M HCl and 25 µl 50mM <i>o</i> -NBA in DMSO, followed by extraction with ethyl acetate	LC separation used a C18 column (1 ml/min flow rate, inj. vol. 48 µl) with a MS detector containing a thermospray ionisation source and set in single reaction monitoring mode	NA	LOD = 0.3	Hoogenboom et al. (2002)
Crayfish	SEM	Crayfish samples were boiled prior to analysis. 1 g homogenised aliquots were treated with 5 ml 0.2M HCl and 50 µl 100mM <i>o</i> -NBA in MeOH. The pH was adjusted to 7 using 500 µl NaPO <sub>4</sub> and 300 µl 2M NaOH. Samples were extracted using 2 × 4 ml ethyl acetate	LC separation used a C18 column (inj. vol. 50 µl) with an ion trap mass spectrometer and an electrospray ionisation interface system set in a positive ionisation mode	NA	LOQ = 0.4	Saari and Peltonen (2004)
Shrimp	AOZ AMOZ AHD SEM	2 g of samples were homogenised with 5 ml MeOH (50%). After centrifugation, pellets were redispersed with 5 ml ethyl acetate and ethanol and the supernatant removed. After derivatisation (10 ml 0.125M HCl; 400 µl 50mM <i>o</i> -NBA in DMSO) and pH adjustment (pH 7.1; 1 ml 0.1M K <sub>2</sub> HPO <sub>4</sub> ), samples were extracted using SPE cartridges followed by hexane (10 ml) and ethyl acetate (3 × 4 ml) partitioning	Adapted from Leitner et al. (2001)	> 80%	NA	Chut and Lopez (2005)

Table 4 Part 9

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results ( $\mu\text{g}/\text{kg}$ )	Reference
Seaweed Prawn Shrimp Fish Egg Chicken Deer Milk powder Cheese, parmesan	SEM	Refer to Leitner et al. (2001)	Refer to Leitner et al. (2001)	NA	NA	Hoenicke et al. (2004)
Feed, animal	NFZ NFT FZD FAD	20 ml 74mM ammonium acetate was added to 5 g of homogenised sample. The pH was adjusted (pH 4.6) and the sample extracted with 30 ml ethyl acetate. After reconstitution in 2 ml acetone and MeOH, clean up was carried out using Sep-pack $\text{NH}_2$ cartridges	HPLC separation used a C18 column (flow rate 0.4 ml/min, inj. vol. 50 $\mu\text{l}$ ). Both tandem MS and photodiode array detection were used in electrospray positive and negative ionisation modes	NA	Respective $\text{CC}_\alpha$ and $\text{CC}_\beta$ LC-MS/MS = 7–21 and 20–50 LC-DAD = 47–76 and 150–300	Barbosa et al. (2007)
Feed, fish	FZD AOZ	FZD: 10 g of sample was blended with 25 ml methylene chloride and applied to an anhydrous $\text{NaSO}_4$ column twice; the eluate was evaporated and reconstituted in 1 ml acetonitrile and hexane ( $2 \times 1$ ml) was used for clean up AOZ: 1 g of sample was mixed with 6 ml MeOH/water, and then derivatised (4 ml water, 0.5 ml 1M HCl, 150 $\mu\text{l}$ 50mM <i>o</i> -NBA in DMSO) and pH was adjusted (pH 7, 5 ml 0.1M $\text{K}_2\text{HPO}_4$ , 0.3 ml 1M NaOH) followed by extraction using ethyl acetate ( $2 \times 4$ ml)	LC-MS/MS conditions: Separation on C18 column, with 50 $\mu\text{l}$ inj. vol., 0.4 ml/min flow rate; using electrospray positive ionisation and multiple reaction monitoring modes. HPLC-UV conditions: C18 column, with 20 $\mu\text{l}$ inj. vol. and photodiode array detection	87.7–102.8%	LOD = 0.4 and 0.05 for NFZ and AOZ	Hu et al. (2007)

Table 4 Part 10

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Eyes, avian	NFZ SEM	<p>Eyeballs were homogenised with water (2 × 3 ml) and derivatised using 4 ml 0.1M HCl 100 µl 100mM <i>o</i>-NBA in DMSO. Extraction was performed with 2 × 6 ml ethyl acetate. The sample was reconstituted in 4 ml acetonitrile and hexane and vortexed. The upper layer was removed and the acetonitrile evaporated again. Residues were redissolved in MeOH and water</p>	<p>HPLC separation used a C18 column (flow rate 0.2 ml/min) with MS/MS detection set in electrospray negative ionisation multiple reaction monitoring modes</p>	NA	NA	Cooper et al. (2005a)
Retina, porcine	AMOZ SEM AOZ AHD	<p>Retina (&lt; 100 mg) incubated overnight at 60°C with HCl and DSMO, , extraction with ethyl acetate, after evaporation residues were re-dissolved in metanol/water (50 : 50 v/v)</p>	LC-MS/MS	NA	<p>NA The use of retina is recommended for nitrofuran monitoring using only single MS or UV detection</p>	Cooper and Kennedy (2005)
Bread Flour	SEM	<p>1 g of sample was homogenised with 0.125N HCl (9 ml) and extracted using <i>n</i>-pentane (2 × 5 ml) and 200 µl <i>o</i>-NBA in MeOH. The pH was adjusted to 7 and extracted with ethyl acetate</p>	<p>HPLC separation used a C18 column (inj. vol. 10 µl, 0.15 ml/min flow rate) with MS/MS detector set using electrospray positive ionisation and multiple reaction monitoring modes</p>	NA	NA	Becalski et al. (2004)
Salt	AOZ AMOZ AHD SEM	<p>1 g of salt was mixed with hexane (2 × 3 ml). After centrifugation, the liquid phase was derivatised using 100 µl 0.2M HCl, 100 µl 0.1M <i>o</i>-NBA in MeOH. The residue was dried and redissolved in acetonitrile/water</p>	<p>HPLC separation used a C18 column (inj. vol. 40 µl, flow rate 0.2 ml/min) and a triple stage quadrupole MS detector set in electrospray positive ionisation and multiple reaction monitoring modes</p>	81.6–116.4%	Respective CC <sub>α</sub> and CC <sub>β</sub> 0.03–0.10 and 0.05–0.15	Pereira et al. (2004)
Food jar gaskets PVC	SEM	<p>50 mg pieces of lid gaskets were derivatised using 25 ml 0.125M HCl and 250 µl 50mM <i>o</i>-NBA in DMSO. After adjusting pH (7.1) with 1M NaOH and 1M K<sub>2</sub>PO<sub>4</sub> the sample was filtered and applied to LiChrolut EN SPE cartridges for extraction</p>	<p>HPLC separation used a C18 column (inj. vol. 50 µl, flow rate 0.3 ml/min) and triple stage quadrupole MS detector equipped with a TurboIonSpray ionisation source and set in multiple reaction monitoring mode</p>	NA	NA	Stadler et al. (2004)

NA = not available/not applicable; other abbreviations listed in the paper

the maximum amount of residue was extracted from the tissue (O’Keeffe et al., 2004). Although solvent extraction is effective, large amounts of high grade solvents can be costly.

Diblikova et al. (2005) compared the use of solvent extraction with a simplified sample preparation method for the analysis of animal tissue. The method eliminated the use of solvents and consisted of a simple protease digestion, homogenisation, derivatisation and a neutralisation step. When used in conjunction with matrix matched calibration standards, the method was very effective in detecting AOZ in tissue, although somewhat less sensitive when compared with a solvent extraction method. An excellent correlation between ELISA and LC-MS/MS was achieved (Diblikova et al., 2005).

Solid phase extraction (SPE) is used as an effective alternative to solvent extraction methods. SPE enables the analyte to be isolated and concentrated before its determination. A reduced amount of organic solvent is required during sample preparation, although it is time consuming and requires the pre-conditioning of cartridges. C18 cartridges containing octadecyl bonded encapped silica packaging are often used for reversed phase extraction of non-polar to moderately polar compounds, such as antibiotics. Szilagyi and de la Calle (2006) detected semicarbazide in egg tissue using solid phase extraction in conjunction with LC-MS/MS (Szilagyi and de la Calle, 2006). Conneely et al. (2002) on the other hand, implemented additional clean up measures for the detection of AOZ in pig liver. In this study, a protease digestion step was introduced to overcome matrix effects and allowed the simple application of filtered supernatants to SPE columns. In an attempt to remove the excess *o*-NBA from the sample, the use of two different SPE cartridges was implemented resulting in the removal of 99% of the excess *o*-NBA (Conneely et al., 2002).

## 8. Instrumental methods for nitrofurans determination

A summary of instrumental methods developed for the determination of nitrofurans and their residues in various matrices is presented in Table 4. These methods can be used for screening and confirmatory analysis as mentioned in Chapter 6. Earlier methods for nitrofurans determination utilised liquid chromatography with ultraviolet (UV) or UV photodiode array detection (Kumar et al.,

1994; Yoshida and Kondo, 1995; Angelini et al., 1997; Draisci et al., 1997). UV absorption detectors respond to substances that absorb light in the range 180 to 350 nm. Quantification relates the intensity of UV light to the concentration of solute at a fixed wavelength. However, due to the variety of complex matrices, the technique might not be specific enough to identify all analytes simultaneously (Draisci et al., 1997).

The coupling of high performance liquid chromatography and liquid chromatography electro-spray ionisation to tandem mass spectrometry (HPLC-MS/MS and LC-ESI MS/MS, respectively) has significantly advanced the capabilities of quantitative methods for the determination of nitrofurans in recent years (Balizs and Hewitt, 2003; Verdon et al., 2007; Rodziewicz, 2008). LC-MS/MS has been utilised in studies examining nitrofurans parent drugs and their metabolite contamination in eggs (Finzi et al., 2005; Szilagyi and de la Calle, 2006; Bock et al., 2007a), poultry (Finzi et al., 2005; Verdon et al., 2007), porcine tissue (McCracken et al., 1995; McCracken and Kennedy, 1997a; Leitner et al., 2001) as well as fish feed (Hu et al., 2007).

In general, LC-MS/MS uses reversed phase liquid chromatography with the incorporation of an atmospheric pressure ionisation system that enables mass spectrometry detection of high molecular, polar, non volatile and thermolabile analytes, with or without a derivatisation step (Balizs and Hewitt, 2003). This technique generally incorporates the use of an internal standard in the sample and a standard curve. The use of radioactive labelled internal standards enables the correction of errors resulting from matrix interference, chromatography and detection. Standard solutions of nitrofurans metabolites in methanol are stable for up to one year at 4°C (in the dark) and extracted samples can be stored up to three days prior to analysis (Bock et al., 2007a). LC-MS/MS is highly sensitive and the sample matrix can often interfere with the analyte signal. If the signal is distorted due to ion suppression, a control using matrix free blanks as well as biological control samples can be utilised to reduce error in determination (Balizs and Hewitt, 2003). A matrix matched calibration curve consisting of a series of blank samples fortified with SEM in increasing amounts was employed to minimise matrix interference in a study by Szilagyi and de la Calle (2006). Authors found that if sample clean up (using SPE cartridges) was sufficient, the matrix effect can be eliminated altogether.



LC-MS/MS was used as a confirmatory method for screening LC-UV (Conneely et al., 2002; Barbosa et al., 2007) and HPLC-UV (Horne et al., 1996; Cooper et al., 2005a), as well as antibody based methods (discussed in Chapter 9). Additionally, HPLC in combination with a porous graphite electrode has been used for relatively sensitive electrochemical detection of nitrofurantoin parent compounds (Diaz et al., 1997).

## 9. ELISA methods for nitrofurantoin determination

In an effort to provide a low cost, portable and high throughput screening method capable of sensitive nitrofurantoin metabolite determination, ELISA (Enzyme Linked Immunosorbent Assay) is becoming a favourable option. ELISA is based on the competition of the analyte or sample with an enzyme labelled component (tracer) for the binding site of an antibody in the wells of a microtitre plate. Highly sensitive and specific immunoassays allow qualitative as well as quantitative detection of derivatised nitrofurantoin metabolites, often without complicated clean up steps. A summary of developed ELISA methods for nitrofurantoin metabolites is given in Table 5.

The first polyclonal antibodies capable of AOZ detection were raised against a carboxy phenyl AOZ hapten and used in an assay specific for prawn tissue analysis (Cooper et al. 2004a,b). Samples of prawn were subjected to hydrolysis and derivatisation of the AOZ with *o*-NBA was followed by ethyl acetate extraction. A monoclonal antibody raised against the same hapten was used in a simplified ELISA procedure for the determination of AOZ in tissues (Diblikova et al. 2005). The ELISA used matrix matched calibration standards to reduce sample interference allowing the sensitive detection of AOZ in tissue homogenates without solvent extraction. A detection capability of  $0.3 \mu\text{g kg}^{-1}$  was achieved and a high correlation with LC-MS/MS was found (Diblikova et al., 2005; Franek et al., 2006). Recently, Chang et al. (2008) established an ELISA method for AOZ using derivatising agent *o*-NBA, solid phase extraction and a standard curve in PBS buffer. The resultant LOD was below  $0.3 \mu\text{g/kg}$  for fish, swine and chicken tissue.

Recent efforts in ELISA development have led to the production of other specific antibodies for nitrofurans. An ELISA for nitrofurantoin metabo-

lite AHD was also developed utilising a carboxy phenyl AHD hapten for antibody production. The resultant indirect ELISA demonstrated a relatively good level of sensitivity (LOD =  $0.2 \mu\text{g/kg}$ ) and was used for the detection of nitrofurans in drinking water (Lui et al., 2007). As yet, no method for the determination of nitrofurantoin derivative AMOZ has been described in literature, although commercial ELISA test kits for the detection of the metabolite are available.

Cooper et al. (2007) produced polyclonal antibodies against SEM. The semi-quantitative direct ELISA reached a detection capability of  $0.25 \mu\text{g/kg}$  for SEM in incurred chicken tissue. Vass et al. (2008a) also produced several polyclonal antibodies specific towards SEM, having comparable assay sensitivity and negligible interference with *o*-NBA. The ELISA was evaluated in porcine tissue and baby food as an effective screening assay (Vass et al., 2008a). In another study, the ELISA was validated for its applicability to screen egg samples (Vass et al., 2008b). The detection capability of the assay ( $0.30 \mu\text{g/kg}$ ) was comparable to LC-MS/MS methods and well below the MRPL of  $1 \mu\text{g/kg}$ . The assay enabled reliable monitoring of SEM in egg samples collected from incurred chickens during a 90-day period (Vass et al., 2008b). A similar approach was used for the development of a monoclonal based ELISA for SEM by Gao et al. (2007). Authors utilised 4-carboxybenzaldehyde as the derivatising agent to produce a 3-carboxy phenyl SEM hapten for antibody production. The resulting assay provided an LOD below  $0.2 \mu\text{g/l}$  in buffer, however, assay functionality was not demonstrated for use in sample analysis.

## 10. Concluding remarks

The presence of nitrofurantoin residues in meat, aquaculture and other products originating predominantly from non-European countries has been well documented in recent years by the European "Rapid Alert System for Food and Feed". From the data available, it appears that nitrofurantoin antibiotics are still used in some countries as growth promoters and prophylactic agents because they are cheap and effective. Therefore, sampling procedures and monitoring plans for regulatory laboratories are necessary to ensure consumer safety. Present European legislation does not permit any confirmed concentration of nitrofurantoin residues

Table 5. ELISA methods for the determination of nitrofurans

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results ( $\mu\text{g}/\text{kg}$ )	Reference
Egg	AOZ	1 g was homogenised and mixed with 3 ml $\text{H}_2\text{O}$ <u>Buffer extraction:</u> derivatisation: 150 $\mu\text{l}$ 5M HCl, 25 $\mu\text{l}$ 50mM <i>o</i> -NBA in DMSO, neutralisation: 0.3 ml of 2M NaOH, extraction and clean up: 4 ml ethyl acetate; 2 ml and 1 ml hexane <u>SE:</u> derivatisation: 0.5 ml 1M HCl, 25 $\mu\text{l}$ 50mM <i>o</i> -NBA in DMSO, neutralisation: 0.3 ml of 2M NaOH, extraction and clean up: 4 ml ethyl acetate; 2 ml and 1 ml hexane <u>SPE:</u> derivatisation: 0.5 ml 1M HCl, 25 $\mu\text{l}$ 50mM <i>o</i> -NBA in DMSO, neutralisation: 0.5 ml 0.3M $\text{Na}_3\text{PO}_4$ and 0.25 ml 2M NaOH, pH adjustment pH (7.1) and application to SDB/1 cartridges	Direct ELISA using monoclonal antibody specific for NPAOZ with a HRP enzyme detection system	62.8–90.2%	Respective LOD and $\text{CC}_{\beta}$ : buffer extraction = 0.162 and 0.6 SE = 0.089 and 0.3 SPE = 0.104 and 0.3	Franeek et al. (2006)
Egg	SEM	1 g of homogenised sample was derivatised (0.5 ml 1M HCl, 50 $\mu\text{l}$ 50mM <i>o</i> -NBA in DMSO) and neutralised (0.3 ml of 2M NaOH). Extraction and clean up was carried out using 5 ml ethyl acetate and 2 ml hexane	Direct ELISA using polyclonal antibody specific for NPSEM with a HRP enzyme detection system	79.4–110.0%	$\text{IC}_{50}$ = 0.18 $\mu\text{g}/\text{l}$ LOD and $\text{CC}_{\beta}$ were 0.13 and 0.3, respectively	Vass et al. (2008b)
Egg Chicken muscle liver	SEM	2 g of homogenised sample was derivatised (6 ml 0.1M HCl, 100 $\mu\text{l}$ 50mM <i>o</i> -NBA in DMSO) and digested using protease (1 ml, 10 mg/ml). Extraction was carried out using Strata XC SPE columns and the sample reconstituted in PBS buffer (0.6ml)	Direct ELISA using polyclonal antibodies specific for CPSEM were used with HRP enzyme detection	NA	$\text{CC}_{\beta}$ = 0.25	Cooper et al. (2007)
Porcine Chicken liver muscle Fish	AOZ	1 g of chopped sample was mixed with 5 ml $\text{H}_2\text{O}$ and derivatised (0.5 ml 1M HCl, 100 $\mu\text{l}$ 0.01M <i>o</i> -NBA in MeOH) and neutralised (5 ml 0.1M $\text{K}_2\text{HPO}_4$ , 0.2 ml $\text{HClO}_4$ ). The pH was adjusted pH (7.0) with NaOH and SPE Oasis MAX cartridges were used for extraction	Direct ELISA using polyclonal antibodies specific for <i>n</i> -phenylidene-AOZ were used with HRP enzyme detection	55.8–96.6%	$\text{IC}_{50}$ = 0.91 $\mu\text{g}/\text{l}$ <u>LOD values:</u> swine liver = 0.19, muscle = 0.17 chicken liver = 0.24, muscle = 0.15 and fish = 0.18	Chang et al. (2008)

Table 5 Part 2

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/kg)	Reference
Pork	SEM	0.5 g of homogenised sample was added to 2 ml H <sub>2</sub> O then derivatised (0.5 ml 1M HCl, 50 µl 50mM <i>o</i> -NBA in DMSO) and neutralised (2.5 ml 0.1M K <sub>2</sub> HPO <sub>4</sub> , 0.2 ml 1M NaOH). Extraction and clean up using 2.5 ml ethyl acetate and 20 µl MeOH and 0.5 ml hexane was carried out	Direct ELISA using polyclonal antibody specific for NPSEM with a HRP enzyme detection system	82.9–105.3%	IC <sub>50</sub> = 0.14 µg/l LOD and CC <sub>β</sub> for porcine tissue were 0.11 and 0.3, respectively	Vass et al. (2008a)
Shrimp Chicken Pork Beef	AOZ	1 g of sample was added to 2 ml protease solution and homogenised. Derivatisation (150 µl 5M HCl, 25 µl 50mM <i>o</i> -NBA in DMSO), neutralisation (0.36 ml of 2M NaOH) and pH adjustment (pH 7.1) were carried out prior to analysis	Direct ELISA using monoclonal antibody specific for NPAOZ with a HRP enzyme detection system	66–119%	CC <sub>β</sub> = 0.4	Diblikova et al. (2005)
Prawn	AOZ	1 g of homogenised sample was derivatised (5 ml 1M HCl, 100 µl 50mM <i>o</i> -NBA in DMSO) and neutralised (1ml 0.3M Na <sub>3</sub> PO <sub>4</sub> ). Extraction and clean up used 5 ml hexane followed by 2 × 8 ml ethyl acetate	Direct ELISA using polyclonal antibody specific for NPAOZ with a HRP enzyme detection system		IC <sub>50</sub> = 0.065 µg/l LOD and CC <sub>β</sub> = 0.05 and 0.4, respectively	Cooper et al. (2004b)
Water	AHD	NA	Indirect ELISA using polyclonal antibodies (specific for NFT) with HRP enzyme detection	88–103%	IC <sub>50</sub> = 3.2 µg/l LOD = 0.2	Lui et al. (2007)
NA	SEM	NA	Indirect ELISA using polyclonal antibodies (specific for CPSEM) with HRP enzyme detection	NA	IC <sub>50</sub> = 0.13 µg/l	Gao et al. (2007)
NA	AOZ	NA	Direct ELISA using monoclonal antibody specific for NPAOZ with a HRP enzyme detection system	NA	IC <sub>50</sub> range = 0.22–0.50 ng/ml	Vass et al. (2005)

in food commodities, although an MRPL of 1 µg/kg has been laid down by the European Commission for nitrofurans metabolites in edible tissues of animal origin. Detection of a parent nitrofurans or its metabolite below the concentration of 1 µg/kg requires enforcement action (product withdrawal, issue of alert notifications by the RASFF etc.) to be initiated. Regulatory authorities and producers are required to identify and eliminate the contamination source to ensure the chemical safety of foods available to the consumer.

## 11. REFERENCES

- Ahmed H.H., Ei-Aziem S.H.A., Abdel-Wahhab M.A. (2008): Potential role of cysteine and methionine in the protection against hormonal imbalance and mutagenicity induced by furazolidone in female rats. *Toxicology*, 243, 31–42.
- Angelini N.M., Rampini O.D., Mugica H. (1997): Liquid chromatographic determination of nitrofurans residues in bovine muscle tissues. *Journal of AOAC International*, 80, 481–485.
- Anon (2008): FoodBRAND and the nitrofurans crisis in global food production. Agri-Food and Biosciences Institute web site <http://www.afbini.gov.uk/index/services/diagnostic/services-diagnostic-analytical-services/foodbrand-introduction/foodbrand-the-nitrofurans-crisis.htm>
- Balizes G., Hewitt A. (2003): Determination of veterinary drug residues by liquid chromatography and tandem mass spectrometry. *Analytica Chimica Acta*, 492, 105–131.
- Barbosa J., Moura S., Barbosa R., Ramos F., da Silveira M.I.N. (2007): Determination of nitrofurans in animal feeds by liquid chromatography-UV photodiode array detection and liquid chromatography-ionspray tandem mass spectrometry. *Analytica Chimica Acta*, 586, 359–365.
- Becalski A., Lau B.P.Y., Lewis D., Seaman S.W. (2004): Semicarbazide formation in azodicarbonamide-treated flour: A model study. *Journal of Agricultural and Food Chemistry*, 52, 5730–5734.
- Becalski A., Lau B.P.Y., Lewis D., Seaman S. (2006): Semicarbazide in Canadian bakery products. *Food Additives and Contaminants*, 23, 107–109.
- Bock C., Gowik P., Stachel C. (2007a): Matrix-comprehensive in-house validation and robustness check of a confirmatory method for the determination of four nitrofurans metabolites in poultry muscle and shrimp by LC-MS/MS 1. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 856, 178–189.
- Bock C., Stachel C., Gowik P. (2007b): Validation of a confirmatory method for the determination of residues of four nitrofurans in egg by liquid chromatography-tandem mass spectrometry with the software InterVal. *Analytica Chimica Acta*, 586, 348–358.
- Botsoglou N.A. (1988): Determination of furazolidone in eggs by high-performance liquid-chromatography. *Journal of Agricultural and Food Chemistry*, 36, 1224–1227.
- Bryant D.W., Mccalla D.R. (1980): Nitrofurans induced mutagenesis and error prone repair in *Escherichia Coli*. *Chemico-Biological Interactions*, 31, 151–166.
- Cabrita A.M.S., Farinha R., Ramos A., Silva F.C.E., Patriocio J.A.B. (2007): Effects of semicarbazide exposure on endocrine pancreas morphology. *Toxicology Letters*, 172, 201.
- Chang C., Peng D.P., Wu J.E., Wang Y.L., Yuan Z.H. (2008): Development of an indirect competitive ELISA for the detection of furazolidone marker residue in animal edible tissues. *Journal of Agricultural and Food Chemistry*, 56, 1525–1531.
- Chu P.S., Lopez M.I. (2005): Liquid chromatography-tandem mass spectrometry for the determination of protein-bound residues in shrimp dosed with nitrofurans. *Journal of Agricultural and Food Chemistry*, 53, 8934–8939.
- Commission Decision (2002): Commission Decision 2002/657/EC of 12 August 2002 implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Communities*, L221, 8–36. <http://faolex.fao.org/docs/pdf/eur49615.pdf>
- Commission Decision (2003): Commission Decision 2003/181/EC of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Official Journal of the European Communities*, L71, 17–18. [http://eur-lex.europa.eu/pri/en/oj/dat/2003/l\\_071/l\\_07120030315en00170018.pdf](http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_071/l_07120030315en00170018.pdf)
- Commission Directive (2004): Commission Directive 2004/1/EC of 6 January 2004 amending Directive 2002/72/EC as regards the suspension of the use of azodicarbonamide as blowing agent. *Official Journal of the European Communities*, L7, 45–46. <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:007:0045:0046:EN:PDF>
- Commission Regulation (1995): Commission Regulation (EC) 1442/95 of 26 June 1995 amending Annexes I, II, III and IV of Regulation (ECC) No 2377/90 laying down a Community Procedure for the establishment

- of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal of the European Communities, L143, 26–30. [http://eur-lex.europa.eu/smartapi/cgi/sga\\_doc?smartapi!celexapi!prod!CELEXnumdoc&numdoc=31995R1442&model=guichett&lg=en](http://eur-lex.europa.eu/smartapi/cgi/sga_doc?smartapi!celexapi!prod!CELEXnumdoc&numdoc=31995R1442&model=guichett&lg=en)
- Commission Regulation (2002): Commission Regulation (EC) 178/2002 of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal of the European Communities, L31, 1–24. [http://eur-lex.europa.eu/pri/en/oj/dat/2002/l\\_031/l\\_03120020201en00010024.pdf](http://eur-lex.europa.eu/pri/en/oj/dat/2002/l_031/l_03120020201en00010024.pdf)
- Conneely A., Nugent A., O’Keeffe M. (2002): Use of solid phase extraction for the isolation and clean-up of a derivatised furazolidone metabolite from animal tissues. *Analyst*, 127, 705–709.
- Conneely A., Nugent A., O’Keeffe M., Mulder P.P.J., van Rhijn J.A., Kovacsics L., Fodor A., McCracken R.J., Kennedy D.G. (2003): Isolation of bound residues of nitrofurans from tissue by solid-phase extraction with determination by liquid chromatography with UV and tandem mass spectrometric detection. *Analytica Chimica Acta*, 483, 91–98.
- Cooper K.M., Kennedy D.G. (2005): Nitrofurans antibiotic metabolites detected at parts per million concentrations in retina of pigs – a new matrix for enhanced monitoring of nitrofurans abuse. *Analyst*, 130, 466–468.
- Cooper K.M., Kennedy D.G. (2007): Stability studies of the metabolites of nitrofurans antibiotics during storage and cooking. *Food Additives and Contaminants*, 24, 935–942.
- Cooper K.M., Caddell A., Elliott C.T., Kennedy D.G. (2004a): Production and characterisation of polyclonal antibodies to a derivative of 3-amino-2-oxazolidinone, a metabolite of the nitrofurans furazolidone. *Analytica Chimica Acta*, 520, 79–86.
- Cooper K.M., Elliott C.T., Kennedy D.G. (2004b): Detection of 3-amino-2-oxazolidinone (AOZ), a tissue-bound metabolite of the nitrofurans furazolidone, in prawn tissue by enzyme immunoassay. *Food Additives and Contaminants*, 21, 841–848.
- Cooper K.M., McCracken R.J., Kennedy D.G. (2005a): Nitrofurans accumulate in avian eyes – a replacement for semicarbazide as a marker of abuse. *Analyst*, 130, 824–827.
- Cooper K.M., Mulder P.P., van Rhijn J.A., Kovacsics L., McCracken R.J., Young P.B., Kennedy D.G. (2005b): Depletion of four nitrofurans antibiotics and their tissue-bound metabolites in porcine tissues and de-termination using LC-MS/MS and HPLC-UV. *Food Additives and Contaminants*, 406–414.
- Cooper K.M., Samsonova J.V., Plumpton L., Elliott C.T., Kennedy D.G. (2007): Enzyme immunoassay for semicarbazide – the nitrofurans metabolite and food contaminant. *Analytica Chimica Acta*, 592, 64–71.
- Cooper K.M., McCracken R.J., Buurman M., Kennedy D.G. (2008): Residues of nitrofurans antibiotic parent compounds and metabolites in eyes of broiler chickens. *Food Additives and Contaminants*, 25, 548–556.
- Council Directive (2008): Council Directive 96/23/EC of 29 April 1996, on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and Decisions 89/187/EEC and 91/664/EEC. Official Journal of the European Communities No. L 125, 10–32. Amended by (EC) No 806/2003 (Official Journal of the European Communities No. L 122, 16.05.2003, 1) amended by ACT of 2004 [http://www.wetgiw.gov.pl/old/UE/prawo/96\\_23/e9623rl.pdf](http://www.wetgiw.gov.pl/old/UE/prawo/96_23/e9623rl.pdf)
- De Angelis I., Rossi L., Pedersen J.Z., Vignoli A.L., Vincentini O., Hoogenboom L.A.P., Polman T.H.G., Stamatii A., Zucco F. (1999): Metabolism of furazolidone: alternative pathways and modes of toxicity in different cell lines. *Xenobiotica*, 29, 1157–1169.
- de la Calle M.B., Anklam E. (2005): Semicarbazide: occurrence in food products and state-of-the-art in analytical methods used for its determination. *Analytical and Bioanalytical Chemistry*, 382, 968–977.
- de la Calle M.B., Szilagyi S. (2006): Determination of semicarbazide in fresh egg and whole egg powder by liquid chromatography/tandem mass spectrometry: Interlaboratory validation study. *Veterinary Drug Residues*, 89, 1664–1671.
- De la Fuente M. (1986): Teratogenic effect of semicarbazide in Wistar rats. *Biology of the Neonate*, 49, 150–157.
- De la Fuente M., Hernanz A., Alia M. (1983): Effect of semicarbazide on the perinatal development of the rat: changes in DNA, RNA and protein content. *Experimental Clinical Pharmacology*, 5, 287–297.
- De Souza S.V.C., Junqueira R.G., Ginn R. (2005): Analysis of semicarbazide in baby food by liquid chromatography tandem mass spectrometry (LC-MS-MS) – In-house method validation. *Journal of Chromatography A*, 1077, 151–158.
- Diaz T.G., Cabanillas A.G., Valenzuela M.I.A., Correa C.A., Salinas F. (1997): Determination of nitrofurans, furazolidone and furaltadone in milk by high-performance liquid chromatography with electrochemical detection. *Journal of Chromatography A*, 764, 243–248.
- Diblikova I., Cooper K.M., Kennedy D.G., Franek M. (2005): Monoclonal antibody-based ELISA for the

- quantification of nitrofurantoin metabolite 3-amino-2-oxazolidinone in tissues using a simplified sample preparation. *Analytica Chimica Acta*, 540, 285–292.
- Draisci R., Giannetti L., Lucentini L., Palleschi L., Brambilla G., Serpe L., Gallo P. (1997): Determination of nitrofurantoin residues in avian eggs by liquid chromatography UV photodiode array detection and confirmation by liquid chromatography ionspray mass spectrometry. *Journal of Chromatography A*, 777, 201–211.
- European Commission (2003): Opinion of the Scientific Committee on Food on the 23<sup>rd</sup> additional list of monomers and additives for food contact materials. [http://ec.europa.eu/food/fs/sc/scf/out181\\_en.pdf](http://ec.europa.eu/food/fs/sc/scf/out181_en.pdf)
- European Commission (2008): Rapid Alert System for Food and Feed. [http://ec.europa.eu/food/food/rapidalert/index\\_en.htm](http://ec.europa.eu/food/food/rapidalert/index_en.htm)
- European Food Safety Authority (2005): Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to Semicarbazide in Food. *The EFSA Journal*, 219, 1–36. [http://www.efsa.eu.int/EFSA/Scientific\\_Opinion/afc\\_summ\\_ej219\\_semicarbazide\\_en1.pdf?ssbinary=true](http://www.efsa.eu.int/EFSA/Scientific_Opinion/afc_summ_ej219_semicarbazide_en1.pdf?ssbinary=true)
- Finzi J.K., Donato J.L., Sucupira M., De Nucci G., McCracken R.J., van Rhijn J.A., Kennedy D.G. (2005): Determination of nitrofurantoin metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry. *Journal Chromatography B Analytical Technology of Biomedical Life Sciences*, 824, 30–35.
- Franek M., Diblikova I., Vass M., Kotkova L., Stastny K., Frgalova K., Hruska K. (2006): Validation of a monoclonal antibody-based ELISA for the quantification of the furazolidone metabolite (AOZ) in eggs using various sample preparation. *Veterinarni Medicina*, 51, 248–257.
- Gao A., Chen Q., Cheng Y., Lei J., Zeng L. (2007): Preparation of monoclonal antibodies against a derivative of semicarbazide as a metabolic target of nitrofurantoin. *Analytica Chimica Acta*, 592, 58–63.
- George J.D., Fail P.A., Grizzle T.B., Heindel J.J. (1996): Nitrofurantoin: Reproductive assessment by continuous breeding in Swiss mice. *Fundamental and Applied Toxicology*, 34, 56–66.
- Ginn R., Wilson L., De Souza S.V.C., de la Calle M.B., Barbosa J., Berendsen B., Bockborn I., Brandtner M., Delahaut P., Doering T., Fuerst P., Griffin C., Gude T., Janosi A., Jaus A., Kennedy G., Mandix M., Hilla E.M., Plonevez S., Posyniak A., Saari L., van Bruijnsvoort M., Verdon E., Wohlfarth R. (2006): Determination of semicarbazide in baby food by liquid chromatography/tandem mass spectrometry: Interlaboratory validation study. *Journal of AOAC International*, 89, 728–734.
- Gottschall D.W., Wang R. (1995): Depletion and Bioavailability of [<sup>14</sup>C] Furazolidone Residues in Swine Tissues. *Journal of Agricultural and Food Chemistry*, 43, 2520–2525.
- Guay D.R. (2008): Contemporary management of uncomplicated urinary tract infections. *Drugs*, 68, 1169–1205.
- Hoenicke K., Gatermann R. (2006): How can zero tolerances be controlled? The case study of Nitrofurans. *Accreditation and Quality Assurance*, 11, 29–32.
- Hoenicke K., Gatermann R., Hartig L., Mandix M., Otte S. (2004): Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurantoin abuse? *Food Additives and Contaminants*, 21, 526–537.
- Hoogenboom L.A.P., Tomassini O., Oorsprong M.B., Kuiper H.A. (1991): Use of pig hepatocytes to study the inhibition of monoamine oxidase by furazolidone. *Food and Chemical Toxicology*, 29, 185–191.
- Hoogenboom L.A.P., Berghmans M.C.J., Polman T.H.G., Parker R., Shaw I.C. (1992): Depletion of protein-bound furazolidone metabolites containing the 3-amino-2-oxazolidinone side-chain from liver, kidney and muscle tissues from pigs. *Food Additives and Contaminants*, 9, 623–630.
- Hoogenboom L.A.P., van Bruchem G.D., Sonne K., Enninga I.C., van Rhijn J.A., Heskamp H., Huvneers-Oorsprong M.B.M., van der Hoeven J.C.M., Kuiper H.A. (2002): Absorption of a mutagenic metabolite released from protein-bound residues of furazolidone. *Environmental Toxicology and Pharmacology*, 11, 273–287.
- Horne E., Cadogan A., O'Keeffe M., Hoogenboom L.A.P. (1996): Analysis of protein-bound metabolites of furazolidone and furaltadone in pig liver by high-performance liquid chromatography and liquid chromatography mass spectrometry. *Analyst*, 121, 1463–1468.
- Hu X.Z., Xu Y., Yediler A. (2007): Determinations of residual furazolidone and its metabolite, 3-amino-2-oxazolidinone (AOZ), in fish feeds by HPLC-UV and LC-MS/MS, respectively. *Journal of Agricultural and Food Chemistry*, 55, 1144–1149.
- Kari R. (1988): NTP technical report on the toxicology and carcinogenesis studies of NFZ in F344/N rats and B6C3F1 mice (feed studies). U.S. Department of Health and Human Services, 1–186.
- Kennedy G. (2004): Analytical methods for nitrofurans: Lessons to be learned and new developments. In: Joint FAO/WHO Technical Workshop on Residues of Veterinary Drugs without ADI/MRL, Bangkok, Thailand, 87–91. <http://www.fao.org/docrep/008/y5723e/y5723e0n.htm#bm23>
- Khong S.P., Gremaud E., Richoz J., Delatour T., Guy P.A., Stadler R.H., Mottier P. (2004): Analysis of matrix-

- bound nitrofurans residues in worldwide-originated honeys by isotope dilution high-performance liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, 52, 5309–5315.
- Kumar L., Toothill J.R., Ho K.B. (1994): Determination of nitrofurans residues in poultry muscle tissues and eggs by liquid-chromatography. *Journal of AOAC International*, 77, 591–595.
- Leitner A., Zollner P., Lindner W. (2001): Determination of the metabolites of nitrofurans antibiotics in animal tissue by high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 939, 49–58.
- Lui W., Zhao C., Zhang Y., Lu S., Liu J., Xi R. (2007): Preparation of polyclonal antibodies to a derivative of 1-aminohydantoin (AHD) and development of an indirect competitive ELISA for the detection of nitrofurantoin residue in water. *Journal of Agriculture and Food Chemistry*, 55, 6829–6834.
- Mccalla D.R. (1983): Mutagenicity of nitrofurans derivatives – Review. *Environmental Mutagenesis*, 5, 745–765.
- Mccalla D.R., Kaiser C., Reuvers A. (1971): Breakage of bacterial DNA by nitrofurans derivatives. *Cancer Research*, 31, 2184–2188.
- McCracken R.J., Kennedy D.G. (1997a): Determination of the furazolidone metabolite, 3-amino-2-oxazolidinone, in porcine tissues using liquid chromatography-thermospray mass spectrometry and the occurrence of residues in pigs produced in Northern Ireland. *Journal of Chromatography B*, 691, 87–94.
- McCracken R.J., Kennedy D.G. (1997b): The bioavailability of residues of the furazolidone metabolite 3-amino-2-oxazolidinone in porcine tissues and the effect of cooking upon residue concentrations. *Food Additives and Contaminants*, 14, 507–513.
- McCracken R.J., Kennedy D.G. (2007): Detection, accumulation and distribution of nitrofurans residues in egg yolk, albumen and shell. *Food Additives and Contaminants*, 24, 26–33.
- McCracken R.J., Blanchflower W.J., Rowan C., Mccoy M.A., Kennedy D.G. (1995): Determination of furazolidone in porcine tissue using thermospray liquid chromatography-mass spectrometry and a study of the pharmacokinetics and stability of its residues. *Analyst*, 120, 2347–2351.
- McCracken R.J., Spence D.E., Floyd S.D., Kennedy D.G. (2001): Evaluation of the residues of furazolidone and its metabolite, 3-amino-2-oxazolidinone (AOZ), in eggs. *Food Additives and Contaminants*, 18, 954–959.
- McCracken R.J., van Rhijn J.A., Kennedy D.G. (2005a): Transfer of nitrofurans residues from parent broiler breeder chickens to broiler progeny. *British Poultry Science*, 46, 287–292.
- McCracken R.J., van Rhijn J.A., Kennedy D.G. (2005b): The occurrence of nitrofurans metabolites in the tissues of chickens exposed to very low dietary concentrations of the nitrofurans. *Food Additives and Contaminants*, 22, 567–572.
- Mori K., Yasuno A., Matsumoto K. (1960): Induction of pulmonary tumours in mice with isonicotinic acid hydrazid. *Gann*, 51, 83–89.
- Mottier P., Khong S.P., Gremaud E., Richoz J., Delatour T., Goldmann T., Guy P.A. (2005): Quantitative determination of four nitrofurans metabolites in meat by isotope dilution liquid chromatography-electrospray ionisation-tandem mass spectrometry. *Journal of Chromatography A*, 1067, 85–91.
- Mulder P.P.J., Beumer B., van Rhijn J.A. (2007): The determination of biurea: A novel method to discriminate between nitrofurazone and azodicarbonamide use in food products. *Analytica Chimica Acta*, 586, 366–373.
- Nouws J.F.M., Laurensen J. (1990): Postmortal degradation of furazolidone and furaltadone in edible tissues of calves. *Veterinary Quarterly*, 12, 56–59.
- O’Keeffe M., Conneely A., Cooper K.M., Kennedy D.G., Kovacsics L., Fodor A., Mulder P.P.J., van Rhijn J.A., Trigueros G. (2004): Nitrofurans antibiotic residues in pork the FoodBRAND retail survey. *Analytica Chimica Acta*, 520, 125–131.
- Pereira A.S., Donato J.L., De Nucci G. (2004): Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products. *Food Additives and Contaminants*, 21, 63–69.
- Petri W. (2005): Treatment of giardiasis. *Current Treatment Options in Gastroenterology*, 8, 13–17.
- Rodziewicz L. (2008): Determination of nitrofurans metabolites in milk by liquid chromatography-electrospray ionisation tandem mass spectrometry. *Journal of Chromatography B*, 867, 156–160.
- Rodziewicz L., Zawadzka I. (2007): Determination of nitrofurans metabolite residues in animal tissues by LC-MS/MS method. *Roczniki Panstwowego Zakladu Higieny*, 58, 625–632.
- Roychowdhury A., Pan A., Dutta D., Mukhopadhyay A.K., Ramamurthy T., Nandy R.K., Bhattacharya S.K., Bhattacharya M.K. (2008): Emergence of tetracycline-resistant *Vibrio cholerae* O1 serotype Inaba, in Kolkata, India. *Japanese Journal of Infectious Diseases*, 61, 128–129.
- Saari L., Peltonen K. (2004): Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS. *Food Additives and Contaminants*, 21, 825–832.

- Samsonova J.V., Douglas A.J., Cooper K.M., Kennedy D.G., Elliot C.T. (2008): The identification of potential alternative biomarkers of nitrofurazone abuse in animal derived food products. *Food and Chemical Toxicology*, 46, 1548–1554.
- Stadler R.H., Mottier P., Guy P., Gremaud E., Varga N., Lalljie S., Whitaker R., Kintscher J., Dudler V., Read W.A., Castle L. (2004): Semicarbazide is a minor thermal decomposition product of azodicarbonamide used in the gaskets of certain food jars. *Analyst*, 129, 276–281.
- Steffeck A.J., Verrusio C., Watkins C.A. (1972): Cleft plate in rodents after maternal treatment with various lathyrogenic agents. *Tetratology*, 5, 33–40.
- Szilagyi S., de la Calle B. (2006): Development and validation of an analytical method for the determination of semicarbazide in fresh egg and in egg powder based on the use of liquid chromatography tandem mass spectrometry. *Analytica Chimica Acta*, 572, 113–120.
- Toth B., Shimizu H., Erickson J. (1975): Carbamylhydrazine hydrochloride as a lung and blood-vessel tumor inducer in Swiss mice. *European Journal of Cancer*, 11, 17–22.
- Ulland B., Weisburg E.K., Weisburg J.H. (1973): Chronic toxicity and carcinogenicity of industrial chemicals and pesticides. *Toxicology and Applied Pharmacology*, 25, 446.
- US Environmental Protection Agency (2008): Chemicals known to the state that cause cancer or reproductive toxicity. Office of Environmental Health Hazard Assessment, USA, 1–18.
- Van Koten-Vermeulen J.E.M. (1993): Report of the 40<sup>th</sup> Meeting of the Joint FAO/WHO Expert Committee On Food Additives (JECFA), World Health Organisation, Geneva. 85.
- Vasheghani M.M., Bayat M., Rezaei F., Bayat A., Karimipour M. (2008): Effect of low-level laser therapy on mast cells in second-degree burns in rats. *Photomedicine and Laser Surgery*, 26, 1–5.
- Vass M., Kotkova L., Diblikova I., Nevorankova Z., Cooper K.M., Kennedy D.G., Franek M. (2005): Production and characterisation of monoclonal antibodies for the detection of AOZ, a tissue bound metabolite of furazolidone. *Veterinarni Medicina*, 50, 300–310.
- Vass M., Diblikova I., Franek M. (2008a): ELISA for semicarbazide and its application for screening in food contamination. *Analytica Chimica Acta*, 608, 86–94.
- Vass M., Diblikova I., Kok E., Stastny K., Frgalova K., Hruska K., Franek M. (2008b): In-house validation of an ELISA method for screening of semicarbazide in eggs. *Food Additives and Contaminants Part A – Chemistry Analysis Control Exposure & Risk Assessment*, 25, 930–936.
- Verdon E., Couedor P., Sanders P. (2007): Multi-residue monitoring for the simultaneous determination of five nitrofurans (furazolidone, furaltadone, nitrofurazone, nitrofurantoin, nifursol) in poultry muscle tissue through the detection of their five major metabolites (AOZ, AMOZ, SEM, AHD, DNSAH) by liquid chromatography coupled to electrospray tandem mass spectrometry – In-house validation in line with Commission Decision 657/2002/EC. *Analytica Chimica Acta*, 586, 336–347.
- Vroomen L.H.M., Berghmans M.C.J., Vanleeuwen P., Vanderstruijs T.D.B., Devries P.H.U., Kuiper H.A. (1986): Kinetics of C-14 furazolidone in piglets upon oral-administration during 10 days and its interaction with tissue macro-molecules. *Food Additives and Contaminants*, 3, 331–346.
- Vroomen L.H., van Bladeren P.J., Groten J.P., Wissink C.J., Kuiper H.A., Berghmans M.C. (1990): *In vivo* and *in vitro* metabolic studies of furazolidone: a risk evaluation. *Drug Metabolism Reviews*, 22, 663–676.
- Wentzell B., Mccalla D.R. (1980): Formation and excision of nitrofurans-dna adducts in *Escherichia-Coli*. *Chemico-Biological Interactions*, 31, 133–150.
- Wiley M.J., Joneja M.G. (1978): Neural tube lesions in offspring of hamsters given single oral doses of lathyrogens early in gestation. *Acta Anatomica*, 100, 347–353.
- Yoshida K., Kondo F. (1995): Liquid chromatographic determination of furazolidone in swine serum and avian egg. *Journal of AOAC International*, 78, 1126–1129.

Received: 2008–05–18

Accepted after corrections: 2008–09–29

## Corresponding Author:

Dr. Milan Franek, DrSc., Veterinary Research Institute, Hudcova 70, 621 00, Brno, Czech Republic  
Tel. +420 533 331 901, e-mail: franek@vri.cz, <http://www.vri.cz>