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

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ABSTRACT: Nitrofuran 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 nitrofuran toxicity, metabolism, and also specific aspects of legislation surrounding their prohibition. Special attention is devoted to semicarbazide – a nitrofuran metabolite and food contaminant. Analytical procedures for nitrofuran analysis in various matrices and validation requirements for screening and confirmation methods with respect to EU regulations are also reviewed.

Keywords: nitrofurans; 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_α** = decision limit; **CC_β** = 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

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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 implimentation 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 instrumetal 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 nitrofuran 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).

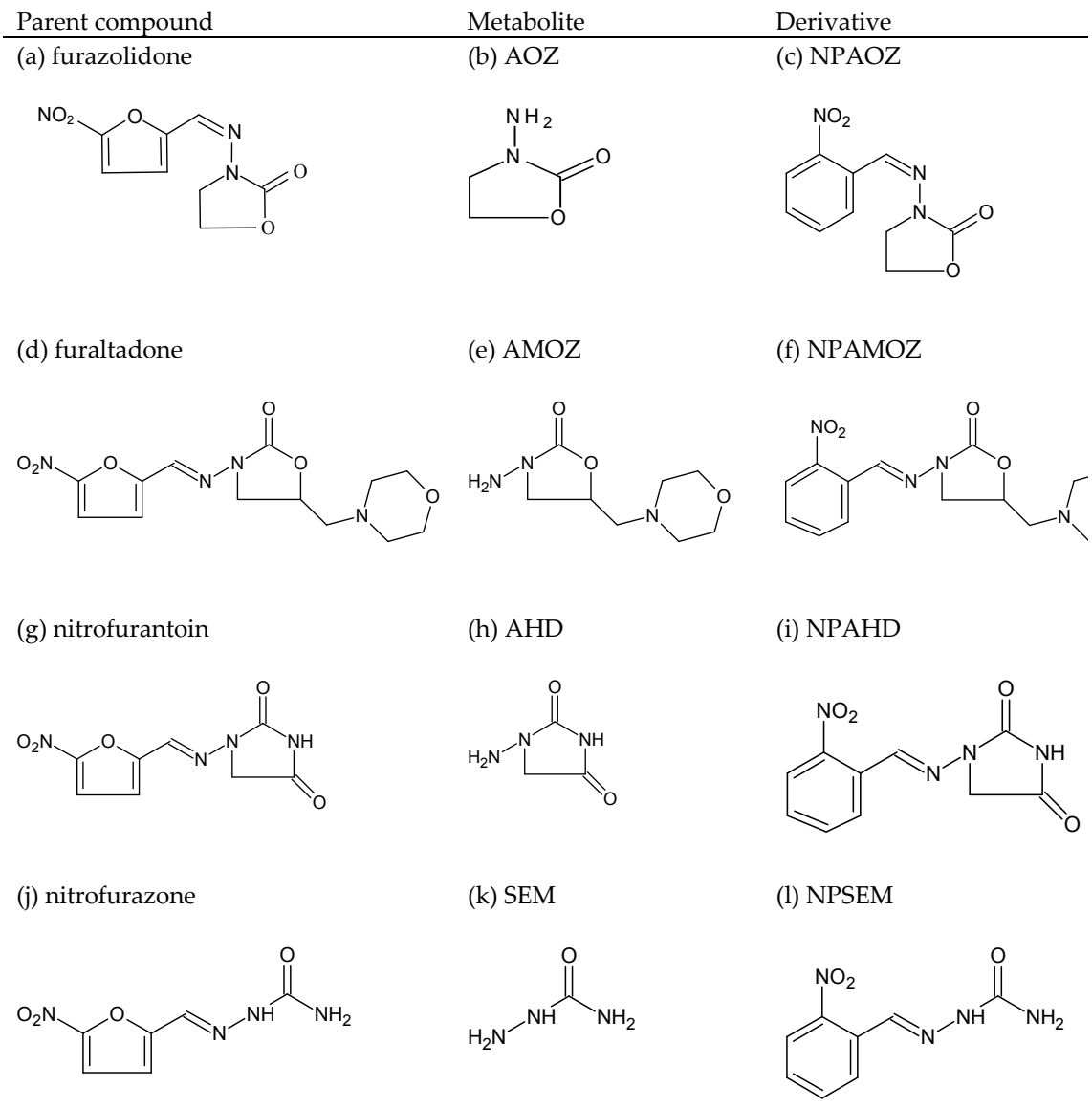

Figure 1. Structures of nitrofuran parent compounds, metabolites and nitrophenyl derivatives. AOZ (3-amino-2-oxazolidinone); NPAOZ (3-(2-nitrobenzylidenamino)-2-oxazolidinone); AMOZ (3-amino-5-morpholinomethyl-1,3-oxazolidinone), NPAMOZ (5-(morpholinomethyl)-3-(2-nitrobenzylidenamino)-2-oxazolidinone);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

Studies examining the bioavailability of nitrofuran metabolites have demonstrated the possibility of residual transfer to secondary species. When rats were fed pig tissue containing radio-labelled (^{14}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 eggshell 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 (≥ 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 (≤ 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, furaltadone and furazolidone are depicted on the State of California Proposition 65 Carcinogens List (US Environmental Protection Agency, 2008).

5. Sources of nitrofuran and semicarbazide contamination

The global nitrofuran 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, nitrofuran 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 nitrofuran 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 catergory 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 μg/kg in frozen peeled black tiger shrimps from India. However, findings of nitrofurans at lower concentrations (10–63 μg/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 nitrofuran metabolites notified by the RASFF, SEM was the highest with 48.9%, 60.9% and 71.0% of all nitrofuran 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 **Imported from**	Reference	Reason for notifying Notification basis and status
2007			
16/07	U.K. **GREECE**	2007.0478	nitrofuran (metabolite) – furaltadone (**AMOZ**) (1.8 µg/kg – ppb) in **sea bass** market control distribution on the market (possible)/withdrawal from sale
19/07	Netherlands **CHINA**	2007.0487	nitrofuran (metabolite) – furazolidone (**AOZ**) (1.2 µg/kg – ppb) in IQF PND **shrimps** (*Trachypenaeus* spp.) border control –screening sample distribution on the market (possible)/ destination of the product identified
09/08	Belgium **THAILAND**	2007.0538	nitrofuran (metabolite) nitrofurazone (**SEM**) (>1 µg/kg – ppb) in frozen freshwater **shrimps** (*Macrobrachium rosenbergii*) border control –screening sample distribution on the market (possible)/ destination of the product identified
16/08	Belgium **INDIA**	2007.0559	nitrofuran (metabolite) nitrofurazone (**SEM**) (>1 µg/kg – ppb) in frozen **shrimps** (*Macrobrachium rosenbergii*) market control distribution on the market (possible)/product (to be) returned to dispatcher
23/08	Belgium **INDIA**	2007.0573	nitrofuran (metabolite) furazolidone (**AOZ**) (>1 µg/kg – ppb) in frozen peeled deveined raw black tiger **shrimps** (*Penaeus monodon*) market control distribution on the market (possible)/product detained
11/12	Belgium **INDIA**	2007.0928	nitrofuran (metabolite) nitrofurazone (**SEM**) (>1 µg/kg – ppb) in raw frozen freshwater headless **scampi** (*Macrobrachium rosenbergii*) border control – screening sample distribution on the market (possible)/product (to be) returned to dispatcher
11/12	Belgium **INDIA**	2007.0931	nitrofuran (metabolite) nitrofurazone (**SEM**) (>1 µg/kg – ppb) in frozen headless **scampi** (*Macrobrachium rosenbergii*) border control – screening sample distribution on the market (possible) dispatched from the United Kingdom
2008			
07/02	Germany **CHINA**, via Belgium	2008.0149	nitrofuran (metabolite) furazolidone (**AOZ**) (15.2 µg/kg – ppb) in frozen cooked pud **shrimps** (*Pandalus vannamei*) official control on the market distribution on the market (possible)/product (to be) destroyed
20/02	Belgium **THAILAND**, via the U.K.	2008.0194	nitrofuran (metabolite) nitrofurazone (**SEM**) (11; 15 µg/kg – ppb) in frozen raw freshwater **shrimps** border control – screening sample distribution on the market (possible)
30/04	Latvia **HUNGARY**	2008.0495	nitrofuran (metabolite) nitrofurazone (**SEM**) (1.6 µg/kg – ppb) in **honey** dispatched from Hungary official control on the market distribution on the market (possible)/product (to be) withdrawn from sale
01/07	Belgium **INDIA**	2008.0785	nitrofuran (metabolite) nitrofurazone (**SEM**) (15 µg/kg – ppb) in frozen headless freshwater **shrimps** (*Macrobrachium rosenbergii*) border control – consignment released distribution on the market (possible)/ reinforced checking
11/07	U.K. **INDIA**	2008.0832	nitrofuran (metabolite) furazolidone (**AOZ**) (150 µg/kg – ppb) in frozen raw peeled black tiger **shrimps** 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 **Imported from**	Reference	Reason for notifying Notification basis and status (from April 2007)
2007			
05/01	U.K. **THAILAND**	2007.ABA	nitrofuran (metabolite) – nitrofurazone (**SEM**) in warm water **prawns**
09/01	Italy **CHINA**	2007 ABK	nitrofuran (metabolite) – nitrofurazone (**SEM**) in dried **hog casing**
12/01	Latvia **UKRAINE**	2007 ACI	nitrofuran (metabolite) – furazolidone (**AOZ**) in natural bee **honey**
12/01	Netherlands **CHINA**	2007.ACU	nitrofuran (metabolite) – furazolidone (**AOZ**) in frozen white **shrimp** (*Penaeus vannamei*)
15/01	U.K. **MALAYSIA**	2007.ACY	nitrofuran (metabolite) – nitrofurazone (**SEM**) in frozen **prawns**
19/01	The Netherlands **INDIA**	2007.ADX	nitrofuran (metabolite) – furazolidone (**AOZ**) in fresh frozen peeled tiger **prawns**
01/02	Belgium **INDIA**	2007.AHL	nitrofuran (metabolite) – furazolidone (**AOZ**) in raw fresh frozen black tiger **prawns** (*Penaeus mon*odon)
13/02	U.K. **INDIA**	2007.AJZ	nitrofuran (metabolite) – furazolidone (**AOZ**) in black tiger **prawns**
26/02	U.K. **I** **NDIA**	2007.AMP	nitrofuran (metabolite) – furazolidone (**AOZ**) in black tiger **prawns** (*Penaeus monodon*)
21/03	U.K. **INDIA**	2007.ASM	nitrofuran (metabolite) – furazolidone (**AOZ**) in frozen black tiger **shrimps** (*Penaeus monodon*)
21/03	Spain **CHINA**	2007.ASP	nitrofuran (metabolite) – furazolidone (**AOZ**) in tropical prawns (*Penaeus vannamei*)
27/03	Belgium **INDIA**	2007.AUH	nitrofuran (metabolite) – furazolidone (**AOZ**) in frozen black tiger **shrimps** (*Penaeus monodon*)
13/04	U.K. **BANGLADESH**	2007.AXZ	nitrofuran (metabolite) – nitrofurazone (**SEM**) (9.1 µg/kg – ppb) in frozen raw brown head-on **shrimps** border control – screening sample information on distribution not (yet) available/product (to be) returned to dispatcher
13/04	U.K. **BANGLADESH**	2007.AYC	nitrofuran (metabolite) – nitrofurazone (**SEM**) (7.5 mg/kg – ppm) in frozen freshwater **shrimp** (*Macrobrachium rosenbergii*) market control no distribution / withdrawal from sale
13/04	U.K. **CHINA**	2007.AYD	nitrofuran (metabolite) – furazolidone (**AOZ**) (1.4 µg/kg – ppb) in frozen raw **tilapia** fillets market control distribution restricted to notifying country/recall from recipients
13/04	U.K. **BANGLADESH**	2007.AXZ	nitrofuran (metabolite) – nitrofurazone (**SEM**) (9.1 µg/kg – ppb) in frozen raw brown head-on **shrimps** border control – screening sample information on distribution not (yet) available / product (to be) returned to dispatcher
13/04	U.K. **CHINA**	2007.AYD	nitrofuran (metabolite) – furazolidone (**AOZ**) (1.4 µg/kg – ppb) in frozen raw **tilapia** fillets market control distribution restricted to notifying country/recall from recipients

Table 2 Part 2

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

Table 2 Part 3

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

Table 2 Part 4

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

in years 2004, 2005 and 2006, respectively, although a decline in 2007 (31%) was evident (Commission Regulation, 2002). Product notifications for semicarbazide 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
2008			
22/01	Spain **CHINA**	2008.ADR	nitrofuran (metabolite) nitrofurazone (**SEM**) (1.9 µg/kg – ppb) in cooked shrimps product (to be) re-dispatched
22/02	Belgium **INDIA**	2008.AIQ	nitrofuran (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 **CHINA**	2008.ALM	nitrofuran (metabolite) nitrofurazone (**SEM**) (10 µg/kg – ppb) in frozen peeled red shrimps (*Solenocera melantho*) product (to be) destroyed
21/03	Belgium **INDIA**	2008.AMZ	nitrofuran (metabolite) nitrofurazone (**SEM**) (6; 9; 13 µg/kg – ppb) in frozen raw headless fresh water shrimps (*Macrobrachium rosenbergii*) product (to be) redispatched or destroyed
19/03	Spain **VIETNAM**	2008.AMT	nitrofuran (metabolite) nitrofurazone (**SEM**) (2.3 µg/kg – ppb) in frozen Pangasius fillets product (to be) re-dispatched
04/04	Belgium **THAILAND**	2008.AOF	nitrofuran (metabolite) nitrofurazone (**SEM**) (4.8; 7.5; 11; 9.5 µg/kg – ppb) in frozen raw freshwater head-on shell-on shrimps (*Macrobrachium rosenbergii*) product (to be) redispatched or destroyed
25/04	Germany **INDIA**	2008.ARK	nitrofuran (metabolite) furazolidone (**AOZ**) (1.1 µg/kg – ppb) in shrimps (*Penaeus monodon*) product (to be) re-dispatched
28/05	U.K. **INDIA**	2008.AUP	nitrofuran (metabolite) furazolidone (**AOZ**) (18 µg/kg – ppb) in black tiger shrimps product (to be) destroyed
23/06	Italy **MALAYSIA**	2008AZH	nitrofuran (metabolite) furazolidone (**AOZ**) (5.6 µg/kg – ppb) in raw frozen farmed black tiger prawns (*Penaeus monodon*) product (to be) redispatched or destroyed
25/07	Belgium **INDIA**	2008.BEF	nitrofuran (metabolite) nitrofurazone (**SEM**) (>1 µg/kg – ppb) in frozen raw freshwater king prawns product (to be) re-dispatched
06/08	Norway **INDIA**	2008.BFP	Vibrio cholerae NON O:1/NON O:139 and prohibited substances nitrofuran(metabolite) furazolidone (AOZ) (7.5 µg/kg – ppb) and nitrofuran (metabolite) nitrofurazone (**SEM**) (0.65 µg/kg – ppb) in frozen black tiger shrimps product (to be) redispatched or destroyed
11/08	Belgium **INDIA**	2008.BGM	nitrofuran (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 **CHINA**	2008.BJR	prohibited substance nitrofuran (metabolite) furazolidone (**AOZ**) (2.6 µg/kg – ppb) in single frozen tilapia fillet (*Oreochromis niloticus*) product (to be) re-dispatched
08/09	Italy **THAILAND**	2008.BKJ	prohibited substance nitrofuran (metabolite) nitrofurazone (**SEM**) (2.03<=>3.1 µg/kg – ppb) in frozen aquacultured breaded butterfly shrimps product (to be) redispatched or destroyed
09/09	Belgium **BANGLADESH**	2008.BKM	prohibited substance nitrofuran (metabolite) nitrofurazone (**SEM**) (>1 µg/kg – ppb) in fresh water headless shell-on shrimps (*Macrobrachium rosenbergii*) product (to be) redispatched 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

Nitrofuran 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 3rd 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, 3rd 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 nitrofuran 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_α (described below), and is determined by many laboratories using validation guidelines provided by the EU. However, the fluctuation of CC_α between different instrumental methods and laboratories is a complicating factor in the application of this parameter to the zero tolerance requirements for nitrofuran 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_β), 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 nitrofuran metabolites, CC_β 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_β, 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_α) and trueness/recovery must also be determined (Commission Decision, 2002). CC_α 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 (Szilagyi 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 nitrofuran abuse 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 nitrofuran 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 nitrofuran 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

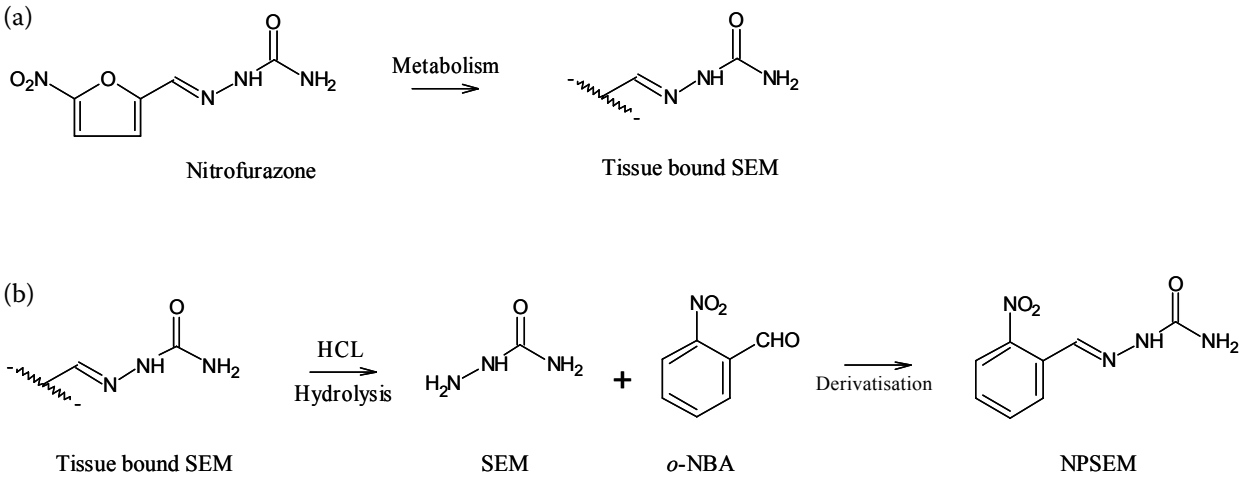

Figure 2. *In vivo* formation of tissue bound SEM (a). Release of bound SEM under mildly acidic conditions followed by SEM derivatisation to produce target analyte (b)

Table 4. Instrumental methods for the determination of nitrofurans

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (μg/kg)	Reference
Egg	AOZ AMOZ AHD SEM	1 g of homogenised sample was derivatised (5 ml 0.1M HCl, 150 μl 50mM *o*-NBA in DMSO) and the pH adjusted (5 ml 0.1M K_2HPO_4, 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_α and CC_β 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)
Egg	AOZ AMOZ AHD SEM	1 g of sample homogenate underwent derivatisation (5 ml 0.2M HCl, 75 μl 100 mM *o*-NBA in MeOH) pH adjustment (pH 7, 0.5 ml), extraction and clean up using ethyl acetate (2 × 4 ml) and *n*-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_α and CC_β 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)
Egg	AOZ AMOZ AHD SEM	1 g of sample was homogenised with 4 ml H_2O, after derivatisation (0.5 ml 1M HCl,150 μl 50mM *o*-NBA in DMSO) the pH was adjusted (pH 7.4, 5 ml 0.1M K_2HPO_4, 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%	$CC_\alpha \leq 1$ $CC_\beta \leq 2$	McCracken and Kennedy (2007)
Egg Egg white powder	SEM	2 g of homogenised sample was derivatised (10 ml 0.2M HCl, 240 μl 10M *o*-NBA in MeOH) and the pH adjusted (pH 7: 10 ml 0.2M K_2HPO_4, 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	Szilagyi and De la Calle (2006)
Egg Egg white powder	SEM	Refer to Szilagyi and De la Calle (2006)	Refer to Szilagyi and De la Calle (2006)	105.3–121.3%	Various (interlaboratory validation)	De la Calle and Szilagyi (2006)
Egg	AOZ AMOZ AHD SEM	Refer to McCracken and Kennedy (1997a)	Refer to McCracken and Kennedy (1997a)	NA	NA	McCracken et al. (2005a)

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 $NaSO_3$ 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_2O) 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 AMOZ AHD SEM	1 g of homogenised sample was derivatised (5 ml 0.2M HCl, 75 µl 0.1M *o*-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 *n*-hexane	HPLC separation used a C18 column (flow rate 0.2 ml/min^{-} inj. vol. 50 µl) with triple quadrupole MS/MS set in electrospray positive ionisation mode	92.6–100.6%	Respective CC_{α} and CC_{β} AOZ: 0.12 and 0.14 AMOZ: 013 and 0.15 AHD: 0.67 and 0.82 SEM: 0.70 and 0.88	Bock et al. (2007a)
Poultry muscle	AOZ AMOZ AHD SEM DNASH	1 g of homogenised sample was derivatised 0.5 ml 1M HCl,150 µl 50mM *o*-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_{α} and CC_{β} ranged from 0.08–0.54 and 0.10–0.66 (respectively) for all analytes	Verdon et al. (2007)
Poultry muscle egg	AOZ AMOZ AHD SEM	1 g of homogenised sample was derivatised (5 ml 0.2M HCl, 50 µl 100 mM *o*-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)
Poultry Porcine muscle	AOZ AMOZ AHD SEM	5 g sample was homogenised with 250 ml 0.125M HCl and derivatised with 250 µl 50mM *o*-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_{α} and CC_{β} ranged from 0.11–0.21 and 0.19–0.36 (respectively)	Mottier et al. (2005)
Poultry liver muscle	AOZ AMOZ AHD SEM	Refer to McCracken and Kennedy (1997a)	Refer to McCracken and Kennedy (1997a)	NA	CC_{α} range = 0.06–0.19	McCracken et al. (2005b)

Table 4 Part 4

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (μg/kg)	Reference
Porcine muscle liver	AOZ AMOZ AHD SEM	1 g of sample was derivatised (9 ml 0.1M HCL, 150 μl 50mM *o*-NBA in DMSO), homogenised (with rinsing, 3 × 1 ml HCl), pH was adjusted (pH 7.2, 2.2 ml 0.3M $NaPO_3$ and 1M NaOH) and extraction performed using 2 × 8 ml ethyl acetate	Refer to Cooper et al. (2005a)	85.9–124.8%	CC_α and $CC_\beta \leq 1$	Cooper and Kennedy (2007)
Porcine muscle liver kidney	AOZ AMOZ AHD SEM Parent drugs	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 *o*-NBA in DMSO) was carried out and the pH adjusted (pH 7.4: 5 ml 0.1M K_2HPO_4, 0.4 ml 0.8M NaOH). Extraction was carried out using 2 × 5 ml ethyl acetate	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. HPLC-UV conditions: C18 column with 20 μl inj. vol. and photodiode array detection	NA	LC-MS/MS: LOD = 0.2–4 HPLC: LOD = 2–5	Cooper et al. (2005b)
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_2O. Derivatisation (1.5 ml 1M HCl and 100 μl 0.05 M *o*-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	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. LC-UV conditions: C18 column (in. vol. 100 μl, 0.8 ml/min flow rate) with UV detection at 275 nm	83.5–117%	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 *o*-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 ul, flow rate 0.8 ml/min) in combination with UV/VIS detection	> 60%	NA	Conneely et al. (2002)
Porcine muscle	AOZ AMOZ AHD SEM	10 g of sample homogenised with 90 ml 0.13M HCL. 10 ml homogenate was derivatised with 400 μl 50mM *o*-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 *o*-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)
Porcine liver	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 *o*-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 (respetively) 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_2 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_2O (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 (μg/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×), ethanol (2×) and diethyl ether. Following extraction, pellets were resuspended in H_2O and mixed with *o*-NBA (50mM in DMSO) and HCl. After incubation, samples were extracted with ethyl acetate (2 × 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 μl) 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 μl 100mM *o*-NBA), the pH was adjusted (pH 7.2, 1M NaOH) and the samples extracted with ethyl acetate (2 × 5 ml)	LC separation used a C18 column (inj. vol. 40 μl, 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_α and CC_β 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 cartriges	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 μl 10M *o*-NBA in MeOH) and the pH adjusted (pH7: 10 ml 0.2M K_2HPO_4, 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 *o*-NBA in MeOH) and the pH adjusted (10 ml 0.2M K_2HPO_4, 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 *o*-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 *o*-NBA in MeOH. The pH was adjusted to 7 using 500 µl $NaPO_4$ 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 *o*-NBA in DMSO) and pH adjustment (pH 7.1; 1 ml 0.1M K_2HPO_4), 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	Chu and Lopez (2005)

Table 4 Part 9

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (µg/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 NH_2 cartridges	HPLC separation used a C18 column (flow rate 0.4 ml/min, inj. vol. 50 µl). Both tandem MS and photodiode array detection were used in electrospray positive and negative ionisation modes	NA	Respective CC_α and CC_β 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 $NaSO_4$ column twice; the eluate was evaporated and reconstitute in 1 ml acetonitrile and hexane (2 × 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 µl 50mM *o*-NBA in DMSO) and pH was adjusted (pH 7, 5 ml 0.1M K_2HPO_4, 0.3 ml 1M NaOH) followed by extraction using ethyl acetate (2 × 4 ml)	LC-MS/MS conditions: Separation on C18 column, with 50µl inj. vol., 0.4 ml/min flow rate; using electrospray positive ionisation and multiple reaction monitoring modes. HPLC-UV conditions: C18 column, with 20 µl 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	Eyeballs were homogenised with water (2 × 3 ml) and derivatised using 4 ml 0.1M HCl 100 µl 100mM *o*-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	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	NA	NA	Cooper et al. (2005a)
Retina, porcine	AMOZ SEM AOZ AHD	Retina (< 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)	LC-MS/MS	NA	NA The use of retina is recommended for nitrofuran monitoring using only single MS or UV detection	Cooper and Kennedy (2005)
Bread Flour	SEM	1 g of sample was homogenised with 0.125N HCl (9 ml) and extracted using *n*-pentane (2 × 5 ml) and 200 µl *o*-NBA in MeOH. The pH was adjusted to 7 and extracted with ethyl acetate	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	NA	NA	Becalski et al. (2004)
Salt	AOZ AMOZ AHD SEM	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 *o*-NBA in MeOH. The residue was dried and redissolved in acetonitrile/water	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	81.6–116.4%	Respective CC_α and CC_β 0.03–0.10 and 0.05–0.15	Pereira et al. (2004)
Food jar gaskets PVC	SEM	50 mg pieces of lid gaskets were derivatised using 25 ml 0.125M HCl and 250 µl 50mM *o*-NBA in DMSO. After adjusting pH (7.1) with 1M NaOH and 1M K_2PO_4 the sample was filtered and applied to LiChrolut EN SPE cartridges for extraction	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	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 nitrofuran 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 nitrofuran 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 nitrofuran 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 nitrofuran 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 nitrofuran parent compounds (Diaz et al., 1997).

9. ELISA methods for nitrofuran determination

In an effort to provide a low cost, portable and high throughput screening method capable of sensitive nitrofuran metabolite determination, ELISA (Enzyme Linked Immunosorbant 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 nitrofuran metabolites, often without complicated clean up steps. A summary of developed ELISA methods for nitrofuran 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 μg 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 μg/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 metabolite 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 μg/kg) and was used for the detection of nitrofurans in drinking water (Lui et al., 2007). As yet, no method for the determination of nitrofuran 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 μg/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 μg/kg) was comparable to LC-MS/MS methods and well below the MRPL of 1 μg/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 μg/l in buffer, however, assay functionality was not demonstrated for use in sample analysis.

10. Concluding remarks

The presence of nitrofuran 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 nitrofuran 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 nitrofuran residues

Table 5. ELISA methods for the determination of nitrofurans

Sample	Target analyte	Sample treatment	Determination technique	Recovery	Validation results (μg/kg)	Reference
Egg	AOZ	1 g was homogenised and mixed with 3 ml H_2O Buffer extraction: derivatisation: 150 μl 5M HCl, 25 μl 50mM *o*-NBA in DMSO, neutralisation: 0.3 ml of 2M NaOH (pH 7.1) SE: derivatisation: 0.5 ml 1M HCl, 25 μl 50mM *o*-NBA in DMSO, neutralisation: 0.3 ml of 2M NaOH, extraction and clean up: 4 ml ethyl acetate; 2 ml and 1 ml hexane SPE: derivatisation: 0.5 ml 1M HCl, 25 μl 50mM *o*-NBA in DMSO, neutralisation: 0.5 ml 0.3M Na_3PO_4 and 0.25 ml 2M NaOH, pH adjustment pH (7.1) and application to SDB/l cartridges	Direct ELISA using monoclonal antibody specific for NPAOZ with a HRP enzyme detection system	62.8–90.2%	Respective LOD and CC_β: buffer extraction = 0.162 and 0.6 SE = 0.089 and 0.3 SPE = 0.104 and 0.3	Franek et al. (2006)
Egg	SEM	1 g of homogenised sample was derivatised (0.5 ml 1M HCl, 50 μl 50mM *o*-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%	IC_{50} = 0.18 μg/l LOD and CC_β 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 μl 50mM *o*-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	CC_β = 0.25	Cooper et al. (2007)
Porcine Chicken liver muscle Fish	AOZ	1 g of chopped sample was mixed with 5 ml H_2O and derivatised (0.5 ml 1M HCl, 100 μl 0.01M *o*-NBA in MeOH) and neutralised (5 ml 0.1M K_2HPO_4, 0.2 ml $HClO_4$). 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 *n*-phenzlidene-AOZ were used with HRP enzyme detection	55.8–96.6%	IC_{50} = 0.91 μg/l LOD values: 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_2O then derivatised (0.5 ml 1M HCl, 50 µl 50mM *o*-NBA in DMSO) and neutralised (2.5 ml 0.1M K_2HPO_4, 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_{50} = 0.14 µg/l LOD and CC_β 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 *o*-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_β = 0.4	Diblikova et. al. (2005)
Prawn	AOZ	1 g of homogenised sample was derivatised (5 ml 1M HCl, 100 µl 50mM *o*-NBA in DMSO) and neutralised (1ml 0.3M Na_3PO). 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_{50} = 0.065 µg/l LOD and CC_β = 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_{50} = 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_{50} = 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_{50} 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 nitrofuran metabolites in edible tissues of animal origin. Detection of a parent nitrofuran 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.

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