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Presence of nitrofurans and their metabolites in gelatine

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Abstract

Following the detection of semicarbazide (SEM) in gelatine by Italian Authorities, at levels exceeding by three times the reference point for action (RPA) of 1 μg/kg, set out by Commission Regulation (EU) 2019/1871 for nitrofurans and their metabolites, the European Commission mandated EFSA to investigate the available sources of nitrofurans and their metabolites in gelatine. European Commission also asked EFSA to provide approaches that would distinguish SEM occurring due to illegal treatment with nitrofurazone from SEM produced during food processing. The literature indicates that SEM, both free and bound to macromolecules, could occur also in food products such as gelatine, during food processing, arising from the use of disinfecting agents and/or from reactions of various food components and, therefore, SEM cannot be considered as an unequivocal marker of the abuse of nitrofurazone in animal production. It is recommended to investigate in more detail which processing conditions lead to the formation of SEM in gelatine during its production and what levels can be found. One potential approach to distinguishing between SEM from nitrofurazone and SEM from other sources in food products, such as gelatine, might be based on determining the ratio of bound:free SEM in a sample of gelatine. However, whether the ratio of bound:free SEM would unequivocally distinguish between SEM arising from nitrofurazone abuse or from other sources still needs to be demonstrated.

Keywords: semicarbazide, SEM, nitrofurazone, gelatine, nitrofurans, nitrofuran metabolites, analytical approach, illegal treatment

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Summary

Nitrofurans, that is, furazolidone, furaltadone, nitrofurantoin, nitrofurazone and nifursol, are synthetic broad-spectrum antimicrobial agents which are rapidly absorbed an extensively biotransformed, giving rise to protein-bound residues retaining the side chains 3-amino-2-oxazolidinone (AOZ) from furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) from furaltadone, 1-aminohydantoin (ADH) from nitrofurantoin, semicarbazide (SEM) form nitrofurazone and 3,5-dinitrosalicyclic acid hydrazide (DNSH) from nifursol.

These metabolites are routinely used as marker residues for control purposes of the prohibited treatment with nitrofurans of food-producing animals in the European Union (EU). However, in the case of gelatine, the industry has claimed, that SEM could be present by oxidation of amino acids in the gelatine as the consequence of processing.

Gelatine is widely used as a coating, binding, gelling and glazing agent in food, pharmaceuticals and cosmetic products. As detailed in the Regulation (EC) No 853/2004 laying down specific hygiene rules for food of animal origin, for the production of gelatine, intended for use in food, bones, hides and skins of farmed ruminant animals, pig skins, poultry skin, tendons and sinews, wild game hides and skins and fish skin and bones may be used. Gelatine is derived from the structural protein collagen found in these tissues and it is extracted, after acidic or basic treatment, with hot water.

No information has been retrieved on the nitrofurazone-mediated occurrence of SEM in tissues usually employed for gelatine manufacture. However, according to the findings on AOZ distribution and provided that SEM shows the same kinetic behaviour displayed by AOZ, it is expected that SEM could easily reach tissues via the systemic circulation. In addition, bones and cartilages are known target tissues of SEM, which in rats is associated with dose-related osteochondral lesions affecting several bones and the knee joint. These lesions are considered to reflect the SEM-mediated inhibition of lysyl oxidase. Based on the above considerations, it is plausible that the illegal treatment of target species with nitrofurazone will result in SEM distribution to and possibly its accumulation in tissues rich in collagenous fibres (e.g. cartilages, tendons, bones and skin) which are the main components of gelatine.

The determination of protein-bound residues of nitrofurans, based on release of the marker metabolites by acid hydrolysis of the azomethine bond, has been the basis for testing for abuse of nitrofuran drugs in food-producing animal tissues and in food products. More specifically for SEM in gelatine, confirmatory methods based on LC-ESI-MS/MS directed at animal and fish tissues are of most relevance.

No occurrence data on nitrofurans and their metabolites in gelatine have been reported to EFSA and no information from RASFF notifications, other than RASFF notification 2021.0223, were retrieved. This evaluation is based on data obtained from the available literature.

No specific studies have been retrieved concerning the occurrence of SEM or other nitrofuran markers in gelatine as a result of the treatment with nitrofurans of ruminants, pigs, poultry or fish, except from one report (Gong et al., 2020) investigating the presence of SEM, AOZ, AMOZ and AHD in 25 samples of commercial gelatine Chinese medicine. Two samples out of 25 were found to contain measurable levels of SEM and other nitrofuran metabolites (AMOZ or AOZ). Considering that the origin of the gelatine used as medicine could not be identified, no conclusions on the possible origin of SEM and the other nitrofuran metabolites were drawn.

Other than from a treatment of animals with nitrofurazone, SEM, both free and bound, could occur in food products, such as gelatine, during food processing, arising from the use of disinfecting agents and/or from reactions of various food components. Free SEM produced in food products during food processing may become bound to cellular constituents released by procedures such as chilling and freezing. Therefore, SEM, whether free or bound, determined in food products, including gelatine, cannot be considered as an unequivocal marker of the abuse of nitrofurazone in animal production.

The problem with SEM not being an unequivocal marker metabolite for nitrofurazone led to efforts to identify alternative approaches to establishing the abuse of nitrofurazone, either through sample preparation procedures to ensure that any measured SEM is from tissue-bound residues due to nitrofurazone use or through analytical procedures that would identify nitrofurazone use independently than by measurement of SEM.

While research has been undertaken on alternative markers to SEM for nitrofurazone in animal tissues and food products, no such alternative markers have been identified to date that would be appropriate to test for nitrofurazone and its metabolites in processed food products, such as gelatine.

One potential approach to distinguishing between SEM from nitrofurazone and SEM from other sources in food products, such as gelatine, might be based on the independent determination of total (bound and free) SEM and of bound SEM in a sample of gelatine. The basis for this approach is the observation that nitrofurazone treatment gives rise to a high proportion of protein-bound SEM whereas SEM from other sources gives rise to a high proportion of free SEM. Gelatine, being a highly processed food product, is potentially likely to contain SEM as an indirect contamination and, therefore, may contain a high proportion of non-protein-bound SEM (free SEM) when tested independently for both total and protein-bound SEM, providing the basis for discriminating as to the source of the SEM determined in the gelatine.

Applying this approach to the testing of samples of food products, such as gelatine, and the determination of whether measured SEM is from a nitrofurazone source or from a food processing source, it is recommended that, where SEM is determined in a sample, a further set of analyses might be undertaken to establish the ratio of bound:free SEM in the sample; where the proportion of bound SEM is higher, a nitrofurazone source might be suspected, whereas if the proportion of free SEM is higher, a non-nitrofurazone source might be suspected.

Research should be undertaken to establish whether this approach might be suitable for unequivocal identification of the source of SEM determined in gelatine. As an example, gelatine might be prepared, according to industrial processing standards, from animals both non-treated and treated with nitrofurazone. The processing of both types of gelatine might be carried out both with and without the use of hypochlorite and/or other potential sources of SEM. In addition, as a positive control, SEM might be added to the gelatine. Determination of total and bound SEM should be undertaken on samples of tissues used in the process and of gelatine at various stages of the processing procedure and used to establish whether the ratio of bound:free SEM would be a suitable method for distinguishing the source of SEM determined in gelatine.

More data are needed to establish the occurrence of SEM in gelatine and in tissues used for its production supported by information on the type of gelatine tested and the production process used. It is recommended to investigate in more detail which processing conditions lead to formation of SEM in gelatine during its production and what levels can be found.

In addition, since the toxicity of SEM is of concern, testing for SEM *per se* in food should be increased.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

RASFF notification 2021.0223 concerns the finding of semicarbazide (SEM, nitrofurazone – nitrofuran metabolite) in gelatine originated from Pakistan at a level of 3.72 μg/kg. Nitrofurans (including furazolidone) are listed in Table 2 (Prohibited substances) of Regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.[1] A reference point for action (RPA) is set out for nitrofurans and their metabolites (3-amino-2-oxazolidinone (AOZ) from furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) from furaltadone, 1-aminohydantoin- (AHD) from nitrofurantoin, semicarbazide (SEM) from nitrofurazone and 3,5-dinitrosalicyclic acid hydrazide (DNSH)) from nifursol at the level of 1 μg/kg for all.[2]

The presence of these substances in food of animal origin is mainly related to the prohibited treatment with nitrofurans. However, in the case of gelatine, the industry has claimed, that SEM could be present in gelatine by oxidation of amino acids in the gelatine as the consequence of processing.

In EFSA Scientific Opinion on nitrofurans and their metabolites in food,[3] the formation of SEM in foods treated with hypochlorite solution is mentioned in one study: SEM was formed in chicken, egg white powder, carrageenan, locust bean gum, gelatine and starch, after overnight treatment with a hypochlorite solution containing 1% active chlorine. The increase in SEM formation following hypochlorite treatment was in the range of the method variability for shrimps, milk, soybean flakes and red seaweed. Treatment with a hypochlorite solution containing 0.015% active chlorine resulted in only a little formation of SEM in carrageenan and starch.

1.1.2. Terms of Reference

In accordance with Article 31 of Regulation (EC) No 178/2002, the European Commission requests the European Food Safety Authority for a report on available sources of nitrofurans and their metabolites in gelatine. In case it is concluded that the presence of SEM in gelatine could be related to other sources than the illegal use of nitrofurans, to provide information on possible analytical approaches that could be applied in case of a finding of SEM in gelatine to distinguish the presence of SEM in gelatine related to an illegal treatment with nitrofurans and the presence of SEM due to processing.

1.2. Additional information

1.2.1. Nitrofurans and their metabolites in food with specific reference to SEM formation and its health effects

Nitrofurans are wide spectrum antibacterial agents sharing a nitrofuran ring which is coupled to a side chain via an azomethine bond. They were extensively employed in livestock species (cattle, pigs, poultry), aquaculture and bees. Despite the ban in the EU, USA, China and other countries, nitrofurans are still illegally used because of their documented efficacy, low cost and easy availability via the web.

A rapid kinetics has been reported in both mammalian and fish target species with *in vivo* half-life times of the parent compounds not exceeding a few hours; actually, intact nitrofurans become barely detectable in organs, tissues and excreta 12–24 h after treatment withdrawal. Nitrofurans are well absorbed and extensively metabolised in liver and other organs giving rise to several derivatives; unlike the parent molecules, part of them persist in edible tissues due to an extensive protein binding (Guichard et al., 2021). The main pathway (see Figure 1) involves the reduction of the nitro-group, giving rise to unstable reactive metabolites with an open nitrofuran ring, which may bind to DNA and tissue proteins. The latter, still retaining the side chain, are detectable for many weeks after treatment cessation.

[1] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1–72.
[2] Commission Regulation (EU) 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC. OJ L 289, 8.11.2019, p. 41–46.
[3] EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2015. Scientific Opinion on nitrofurans and their metabolites in food. EFSA Journal 2015;13(6):4140, 217 pp. https://doi.org/10.2903/j.efsa.2015.4140

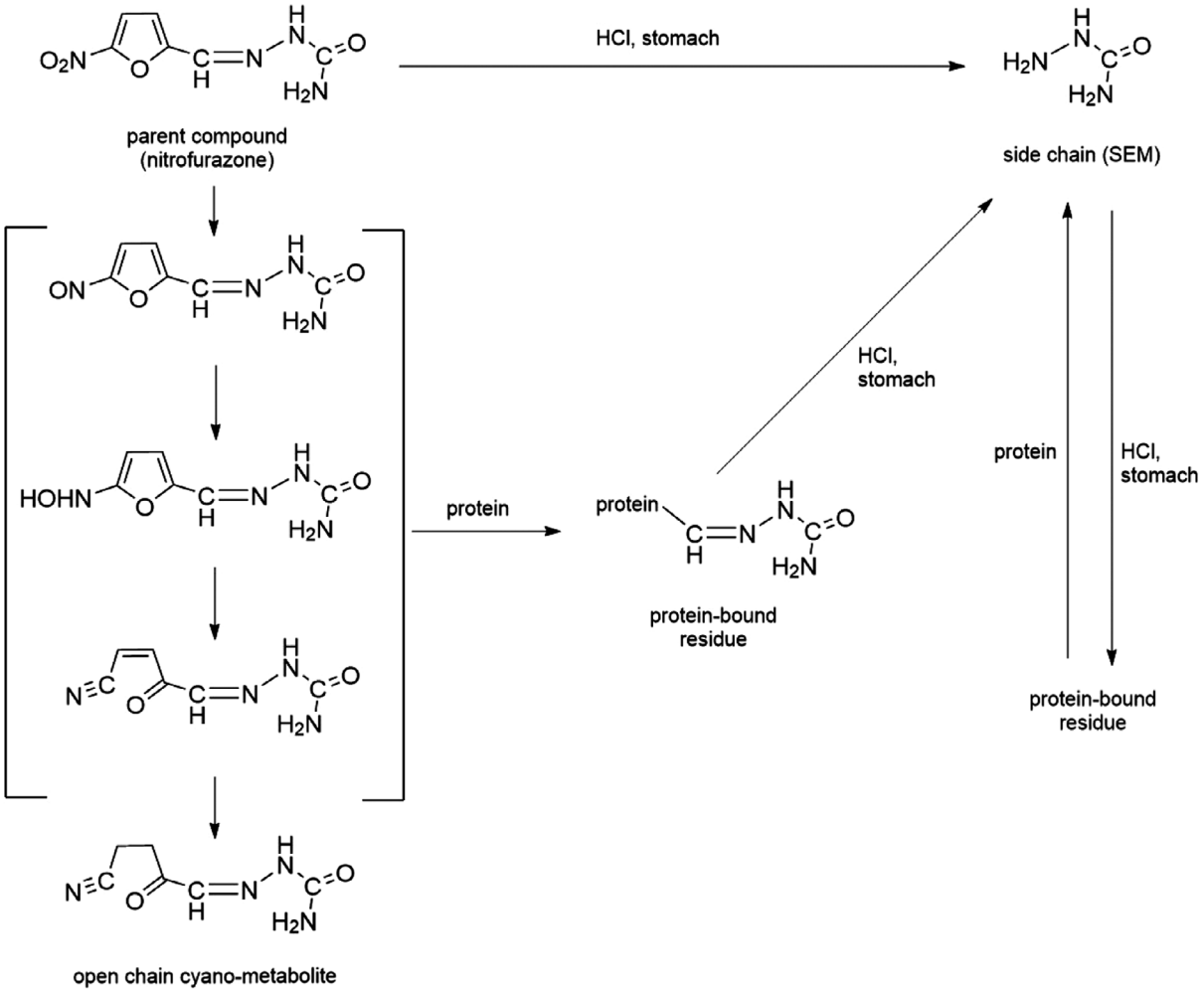

Figure 1: Putative formation of nitrofurazone-derived protein-bound residues from (i) reduction of the nitro-group and generation of an open chain cyano-metabolite (left) and (ii) release of the side chain (SEM) under acid hydrolysis (right). Unstable metabolites are between square brackets (modified from Hoogenboom et al., 2002)

Upon mild acid hydrolysis (even at gastric pH), the azomethine bond of the protein-bound residue is cleaved and the side chain released or, as an alternative, the side chain may be also released from the parent compound under acidic conditions (e.g. gastric pH); the side chain itself, may give rise to protein-bound residues after absorption and tissue distribution (Hoogenboom et al., 2002). The putative formation of protein-bound residues arising from nitrofurazone metabolism and/or SEM formation is depicted in Figure 1. After derivatisation, the side chains are used as marker residues for nitrofuran exposure for monitoring purposes (Vass et al., 2008). According to Liu et al. (2010), pigs fed with 400 mg furazolidone/kg diet for 7 days had plasma and urine concentrations of the marker residue 3-amino-2-oxazolidinone (AOZ) of the same order of magnitude (range 0.7–2 mg/kg) as those found in liver, muscle and kidney as early as 0.5 days after treatment withdrawal. This is consistent with a rapid absorption of the parent molecule followed by extensive biotransformation and transfer of metabolites containing the marker residue and the marker residue itself into the systemic circulation, with subsequent distribution in the body tissues and elimination via the kidney. Since measurable amounts of AOZ were detected in plasma and urine even 63 days after treatment cessation, both biological fluids may be suitable on-farm monitoring purposes. Kinetic studies in target species have been mainly focussed on furazolidone and scant information is available for the related compounds; nonetheless, it may be assumed that a similar fate may occur for all nitrofurans, including nitrofurazone (EFSA CONTAM Panel, 2015). In this respect, an Ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method has been developed and validated for the main nitrofuran marker metabolites AOZ, AMOZ, AHD and SEM in bovine,

ovine, porcine and equine plasma samples in view of the potential routine use of this matrix for control purposes; a CCα value for SEM was reported at 0.07 μg/kg (Radovnikovic et al., 2011).

In summary, nitrofurans are rapidly absorbed and extensively biotransformed, giving rise to protein-bound residues retaining the side chains (AOZ, AMOZ, SEM and AHD); the latter can be released under acidic conditions and after derivatisation are routinely used as marker residues for control purposes (see Section 1.2.4 below). Based on limited information, almost entirely related to studies on furazolidone and AOZ, it is assumed that free SEM or metabolites containing SEM may reach the plasma shortly after the exposure to nitrofurazone and distribute to all tissues.

The formation over time and the subsequent depletion of tissue-bound residues following nitrofurazone administration, measured as the marker residue SEM, have been reported in pigs (Cooper et al., 2005), chickens (McCracken et al., 2005) and fish (Chu et al., 2008) mainly in edible tissues (muscle, liver, kidney) but also in other tissues (e.g. pig retina) found to retain high amounts of SEM (mg/kg) for several weeks after treatment cessation. Of note, feeding of rats with swine muscle tissue containing non-extractable (protein-bound) radioactive residues of furazolidone led to the excretion of some of the radiolabel in urine and the recovery of radioactivity in rat tissues indicating the oral bioavailability of the bound residues (Vroomen et al., 1990; Gottschall and Wang, 1995); further studies revealed that the marker residue AOZ was found in organs of rats fed liver, kidney or muscle from furazolidone-treated pigs (McCracken and Kennedy, 2007). Finally, free AOZ was detected in the blood of rats fed non-extractable liver fractions from pigs treated with furazolidone, clearly indicating that AOZ can be released and absorbed from bound residues (Hoogenboom et al., 2002). Although no similar studies have been performed with other nitrofurans, and hence, there is no information on the bioavailability of their bound residues, the above studies underscore the importance of characterising the adverse effects of the marker residues (side chains) of such compounds.

The toxicity of SEM was recently reviewed in the opinion on nitrofurans and their metabolites in food (EFSA CONTAM Panel, 2015). In repeated dose toxicity studies, severe limbs malformation and osteochondral lesions were observed in rats already at the lowest tested dose (23 mg SEM/kg body weight (bw)/day). These lesions have been related to the SEM-mediated inhibition of lysyl oxidase (Dawson et al., 2002), an enzyme belonging to the group of amine oxidases involved in the proper formation of collagen by promoting covalent cross links between collagen molecules (Kuroyanagi et al., 2002). While the i.p. administration of SEM to pregnant rats resulted in embryotoxic and teratogenic effects at all tested dosages, an no observed adverse effect level (NOAEL) of 25 mg SEM/kg bw per day could be determined in the progeny of orally exposed rats for increased resorption rate and cleft palate (the only tested endpoints). Neurobehavioural effects were observed in young rats exposed to 40 mg SEM/kg bw for 10 days and no clear NOAEL could be identified for SEM effects on endocrine homoeostasis in peripubertal rats (circulating sex hormone levels, onset of puberty). The data on *in vitro* genotoxicity indicate that SEM is mutagenic in bacteria and mammalian cells, but the contrasting results on the *in vivo* clastogenic potential did not allow clear conclusions to be drawn on *in vivo* SEM genotoxicity. In line with a previous evaluation of the AFC Panel (EFSA, 2005), and recognising the limitations of the available studies, the CONTAM Panel (EFSA CONTAM Panel, 2015) concluded that there is limited evidence of SEM carcinogenicity only in mice (lung tumours). The International Agency for Research on Cancer (IARC) evaluated SEM in 1987 and concluded that it is carcinogenic in mice after oral administration and not classifiable as regards its carcinogenicity in humans (group 3), since no adequate data were available for humans and limited evidence was available for experimental animals (IARC, 1987).

A couple of studies on SEM genotoxicity have been published since the EFSA CONTAM Panel, 2015, evaluation. In one of them (Wang et al., 2016), it was demonstrated that SEM reacts rapidly with apurinic/apyrimidinic sites containing oligonucleotides to form covalently bonded DNA adducts in vitro and in *E. coli*. In a subsequent report (Wang et al., 2016), a dose-dependent DNA and RNA adduct formation was identified in organs from SEM-treated rats (0, 40 or 80 mg SEM/kg bw per day for 5 days). RNA adducts significantly prevailed over DNA adducts and, in both cases, the highest amounts were detected in the small intestine and the stomach, followed by large intestine, liver and kidney. The authors concluded that these results point to a direct genotoxicity of SEM, without the need for metabolic activation.

1.2.2. Gelatine: sources, chemical composition and processing

Gelatine is widely used as a coating, binding, gelling and glazing agent in food, pharmaceuticals and cosmetic products. As detailed in the Regulation (EC) No 853/2004 laying down specific hygiene rules for food of animal origin, for the production of gelatine intended for use in food, bones, hides

and skins of farmed ruminant animals, pig skins, poultry skin, tendons and sinews, wild game hides and skins and fish skin and bones may be used.[4] Pork skin is the most abundantly used raw material, accounting for the 45% of the world's and 80% of Europe's total gelatine production. In Europe, cattle skin follows at 15% and 5% is from pork and cattle bones, fish and chicken. Worldwide, 30% of gelatine is obtained from bovine hides, and 23% from bovine and porcine bones. Other sources include chicken and fish, but these account for only 1.5% of the world's annual gelatine production (Boran and Regenstein, 2010).

Gelatine is derived from collagen, which is a natural structural protein, predominantly found in the connective tissues of animals, constituting 20–25% of the total protein (Boran and Regenstein, 2010). It is a heterogeneous mixture of peptides derived from the parent collagen protein by the destruction of cross-linkages between the polypeptide chains along with some breakage of polypeptide bonds (Liu et al., 2015). Gelatine is rich in glycine, proline and hydroxyproline, and contains very low amount of threonine, histidine and methionine (Eastoe, 1955).

The main process used to produce gelatine is via mixing of the animal skin and bones with hot water (Eastoe, 1955). For industrial purposes, additional steps are performed that may vary considerably. The animal parts are first washed and degreased. They are then treated with an acidic or basic solution, depending on their origin from pig or bovine, respectively, to make the collagen easier to access and to separate it from other raw materials. Next, the processed animal parts are mixed in hot water to produce gelatine. Traces of fat and fine fibres and the finest particles are removed. In a last purification step, the gelatine is freed of calcium, sodium, residual acid and other salts. Finally, the water is evaporated from the liquid and the solid gelatine is ground into powder or other forms.[5] An example of gelatine extraction from pig skins is given in Figure 2 (modified from Chung et al., 1990). Regulation (EC) No 853/2004 Annex II, Section XIV details requirements for the production of gelatine.

[4] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. OJ L 139, 30.4.2004, p. 55–205.
[5] https://www.gelatine.org/en/gelatine/manufacturing.html

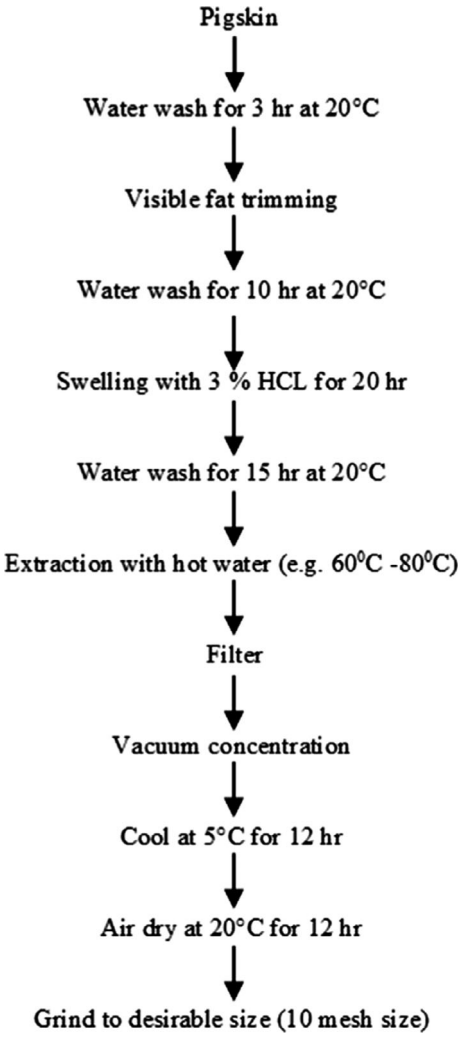

Figure 2: Flowchart of the process for pigskin gelatine extraction (modified from Chung et al., 1990)

Gelatine belongs to the food category 12.09 Protein hydrolysates in accordance with Annex II to Regulation (EC) No 1333/2008[6] on food additives and, therefore, all group I and group II (colours) food additives are permitted at *quantum satis* in gelatine, including carrageenan with some few exceptions. The mean use of carrageenan in foods of the category 12.09 is estimated at 2,152 mg/kg and the maximum use at 8,000 mg/kg (EFSA ANS Panel, 2018). European Pharmacopeia (2017) defines limits for impurities in gelatine for some heavy metals, microbial contamination, sulfur dioxides and peroxides.

1.2.3. Legislation

A comprehensive overview of the legislation relating to pharmacologically active substances in veterinary medicinal products (VMPs) covering 'allowed' and 'not allowed' and more specifically the group of 'prohibited substances' including *inter alia*, nitrofurans, is provided in the previous EFSA opinion (EFSA CONTAM Panel, 2015). More recently, Regulation (EU) No 2019/1871[7] established a Reference Point for Action (RPA) of 0.5 μg/kg for five nitrofuran metabolites AOZ, AMOZ, AHD, SEM and DNSH, that will be effective from November 2022 for testing for nitrofurans under the National Residue Control Plans of Member States, and repealing Decision 2005/34/EC. In line with Article 6 of this Regulation, 'where the results of official controls, including analytical tests, identify residues of

[6] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.
[7] Commission Regulation (EU) 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC (Text with EEA relevance) C/2019/7908 OJ L 289, 8.11.2019, p. 41–46.

prohibited or non-allowed substances at levels above, equal to or below the reference points for action, the competent authority shall carry out the investigations referred to in Articles 137(2) or (3) of Regulation (EU) 2017/625[8], and Articles 13, 16(2), 17 and 22 to 24 of Directive 96/23/EC to determine whether there has been illegal treatment with a prohibited or non-allowed pharmacologically active substance'.

In accordance with Regulation (EC) No 853/2004, 'Gelatine' means natural, soluble protein, gelling or non-gelling, obtained by the partial hydrolysis of collagen produced from bones, hides and skins, tendons and sinews of animals and 'collagen' means the protein-based product derived from animal bones, hides, skins and tendons manufactured in accordance with the relevant requirements of this Regulation. The production process for gelatine must ensure that the raw materials are treated in line with specifications provided in this regulation and/or come from establishments registered or approved pursuant to Regulation (EC) No 852/2004[9], including establishments from third countries.

The production process for gelatine must ensure that certain requirements are fulfilled including bone treatment with dilute hydrochloric acid (at a minimum concentration of 4% and pH < 1.5) over a period of at least 2 days followed by treatments including alkaline treatment with saturated lime solution (pH > 12.5) for a period of at least 20 days with a sterilisation step of 138–140°C during four-seconds or acid treatment (pH < 3.5) during 10 h minimum with a heat treatment step of 138°C minimum during at least 4 s, or heat-and-pressure process for at least 20 min with saturated steam of 133°C at more than 3 bars, or any approved equivalent process; other raw material can be treated with acid or alkali.

1.2.4. Methods of analysis

Many of the methods to test for residues of nitrofurazone and its metabolites in animals and animal products are methods that test for the class of nitrofurans and, in some cases, for residues of other drugs. These methods have been comprehensively reviewed in the EFSA scientific opinion on nitrofurans and their metabolites in food (EFSA CONTAM Panel, 2015); some of the material presented here is taken from that opinion, updated and modified to address specifically analytical techniques for nitrofurazone and its metabolites.

In summary, determination of protein-bound residues of nitrofurans, based on release of the marker metabolites by acid hydrolysis of the azomethine bond, has been the basis for testing for abuse of nitrofuran drugs in food-producing animal tissues and in food products. Most methods applied are multiresidue methods for the class of nitrofuran drugs, covering nitrofurazone as its marker metabolite SEM. Suitable methods are those that provide confirmatory testing for nitrofuran marker metabolites with sufficient sensitivity to satisfy regulatory requirements, currently at the minimum required performance limit (MRPL) value of 1.0 μg/kg for food of animal origin (Annex II of Commission Decision 2002/657/EC). It should be noted that, more recently, a Reference Point for Action (RPA) of 0.5 μg/kg has been established for five nitrofuran metabolites AOZ, AMOZ, AHD, SEM and DNSH, that will be effective from November 2022 for testing for nitrofurans under the National Residue Control Plans of Member States (see Section 1.2.2).

For nitrofuran marker metabolites, a combined acid hydrolysis with hydrochloric acid (HCl) and derivatisation with NBA is performed to release the protein-bound residues. This procedure may also detect parent compounds or non-bound metabolites containing the intact side chain that may be present in the sample. Where determination of only the protein-bound metabolites is required, a series of washing steps with organic solvents may be performed prior to the hydrolysis/derivatisation step (Hoogenboom et al., 1991). Following incubation at 37°C to complete the derivatisation, the sample is cooled and neutralised with phosphate buffer. The derivatised nitrophenyl marker metabolites are extracted with multiple aliquots of ethyl acetate (O'Keeffe et al., 2004). Some alternative approaches have been proposed for the release and derivatisation of marker metabolites, such as protease

[8] Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation) Text with EEA relevance. OJ L 95, 7.4.2017, p. 1–142.

[9] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. OJ L 139, 30.4.2004, p. 1–54.

digestion of samples and extraction of the derivatised marker metabolites using mixed-mode cation exchange SPE instead of ethyl acetate (Cooper et al., 2007; Stastny et al., 2009), accelerated solvent extraction with methanol/5% trichloroacetic acid (1/1, v/v) (Tao et al., 2012), incubation at 55°C instead of at 37°C (Verdon et al., 2007) or incubation in a microwave oven (Palaniyappan et al., 2013).

LC-MS/MS has become the most widely used methodology for confirmatory determination of nitrofuran marker metabolites in a broad range of sample types. Single quadrupole LC-MS has been shown not to be sufficiently sensitive (Cooper and Kennedy, 2005) or selective (Tribalat et al., 2006) for the determination of marker metabolites at the current MRPL of 1 μg/kg. Most of the published LC-MS/MS methods are multiresidue methods, covering AOZ, AMOZ, AHD and SEM, with some methods also including DNSH. Because the concern of this opinion is the determination of SEM in gelatine, confirmatory methods directed at animal and fish tissues are of most relevance. LC-ESI-MS/MS methods for the determination of AOZ, AMOZ, AHD and SEM in animal tissues have been applied to poultry and pork samples. The values of the decision limit (CCα),[10] CCβ,[11] limit of detection (LOD) and limit of quantification (LOQ) for SEM by these methods are 0.20–1.2, 0.34–1.4, 0.05–0.1 and 0.2–0.5 μg/kg, respectively (Finzi et al., 2005; Mottier et al., 2005; Xia et al., 2008; Ryad et al., 2013; Zhang et al., 2017). A method for five marker metabolites, including DNSH, in turkey muscle reported a CCα value of 0.20 μg/kg and a CCβ value of 0.25 μg/kg for SEM (Verdon et al., 2007). For fish, a number of methods are described with reported CCα values of 0.09–0.29 μg/kg and CCβ values of 0.36–0.48 μg/kg (Tsai et al., 2010; Tao et al., 2012) and LOD/LOQ values of 0.05/0.15 μg/kg (Zhang et al., 2015) for SEM. A number of methods that may be applied to testing for eight nitrofurans in fish and shellfish have been described, with CCβ of < 0.5 μg/kg (Chen et al., 2020) and LOQ of < 0.5 μg/kg (Yuan et al., 2020) for SEM.

Multi-analyte methods for nitrofuran metabolites and single-analyte methods for SEM, with determination by LC-ESI-MS/MS, have been developed for a range of other food matrices such as shrimp (Douny et al., 2013; Hossain et al., 2013), eggs and egg products (Bock et al., 2007; Śniegocki et al., 2008; Szilagyi and de la Calle, 2006; Stastny et al., 2009), honey (Khong et al., 2004; Tribalat et al., 2006; Lopez et al., 2007) and milk (Rodziewicz, 2008); for SEM, these methods report CCα values in the range of 0.2–0.9 μg/kg and CCβ values in the range of 0.25–1.0 μg/kg.

Recently, the European Union Reference Laboratory (EURL) for Residues of Antibacterial Substances and Dyes in Food (Anses, Fougères, France) published a method for AOZ, AMOZ, AHD, SEM and DNSH that satisfies the RPA of 0.5 μg/kg for these five nitrofuran metabolites. This method includes washing of the sample with methanol/water to remove the natural occurrence of compounds of interest, particularly SEM, as confirmatory methods should measure only the protein-bound metabolites; the CCα values for SEM ranged from 0.084 to 0.182 μg/kg in beef, pork and turkey muscle and in fish and shrimp tissue (Guichard et al., 2021).

Only one paper in the published scientific literature describes a method for the specific determination of nitrofuran metabolites in gelatine (Gong et al., 2020). This method describes a procedure for testing for AOZ, AMOZ, AHD and SEM and reports LOD/LOQ values of 0.2/0.6 μg/kg for SEM. The method follows the typical procedure described above for multi-nitrofuran methods, using hydrolysis with HCl and formation of nitrophenyl derivatives of the metabolites, and clean-up of sample extracts with a combination of dispersive SPE and reversed-phase SPE, prior to residue determination by LC-ESI-MS/MS. One addition to the typical procedure is a pretreatment of the gelatine sample with pepsin-HCl; this has the effect of increasing the recovery of the nitrofuran metabolites, in the case of SEM by approximately 30%.

2. Data and methodologies

2.1. Data

2.1.1. Occurrence data submitted to EFSA

No occurrence data on nitrofurans and their metabolites in gelatine have been reported to EFSA. No RASFF notifications other than RASFF notification 2021.0223 were retrieved on SEM in gelatine.

[10] CCα is the decision limit at and above which it can be concluded with an error probability of α that a sample is non-compliant.
[11] CCβ is the detection capability, meaning the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β.

2.1.2. Data provided to EFSA

Member States have been alerted by Italy through the RASFF notification, reference number 2021.0223 (Appendix A), on a case of contaminated gelatine with SEM imported from Pakistan. Details of the method of analysis followed have been provided to EFSA by the laboratory responsible for the analysis of the gelatine sample (see 'Documentation provided to EFSA').

Analytical results on nitrofuran metabolites in gelatine samples and the corresponding raw material were provided by gelatine manufacturers. However, due to limitations in detailed information of the raw materials used for the sample preparation, the processing of the raw material, the analytical methods used, etc., these data could not be used in the risk assessment.

2.1.3. Literature data

The previous evaluation of the CONTAM Panel was used for retrieval of relevant information and literature and a 'forward snowballing' approach was applied by all working group (WG) members in order to obtain any relevant information published more recently.

2.2. Methodologies

2.2.1. Occurrence data

The EFSA Data Warehouse was searched for the following residues: furazolidone, furaltadone, nitrofurantoin, nitrofurazone, nifursol, nitrofurans, AOZ, AMOZ, AHD, SEM and DNSH in gelatine and collagen.

2.2.2. Evaluation

The WG on nitrofurans and its metabolites in gelatine appraised narratively all the information available, in order to address the specific question of the mandate.

3. Assessment

3.1. Sources of nitrofurans and their metabolites including SEM in gelatine

As detailed in Section 1.2.1, according to EU Regulation 854/2003, gelatine is produced from bones, hides and skins, as well as tendons and sinews (as appropriate) of several animal species including farmed ruminants, pigs, poultry, wild game and fish. In general, there is scant information on the distribution of nitrofurans and/or their metabolites in non-edible tissues (EFSA CONTAM Panel, 2015) and most of the available studies were concerned with furazolidone and its marker metabolite AOZ (see Section 1.2.2). In particular, no information has been retrieved on the nitrofurazone-mediated occurrence of SEM in plasma or in tissues usually employed for gelatine manufacture. However, according to the findings on AOZ distribution mentioned above (Liu et al., 2010) and provided that SEM shows the same kinetic behaviour displayed by AOZ, it is expected that SEM could easily reach all such tissues via the systemic circulation. In addition, bones and cartilages are known target tissues of SEM, which in rats is associated with dose-related osteochondral lesions affecting several bones and the knee joint (Takahashi et al., 2010, 2014). These lesions are considered to reflect the SEM-mediated inhibition of lysyl oxidase (see Section 1.2.2).

Based on the above considerations, it is plausible that the illegal treatment of target species with nitrofurazone will result in SEM distribution to and possibly its accumulation in tissues rich in collagenous fibres (e.g. cartilages, tendons, bones and skin) which are the main components of gelatine.

No specific studies have been retrieved concerning the occurrence of SEM or other nitrofuran markers in gelatine as a result of the treatment with nitrofurans of ruminants, pigs, poultry or fish, except from one report (Gong et al., 2020) investigating the presence of SEM, AOZ, AMOZ and AHD in 25 samples of commercial gelatine Chinese medicine. Two samples out of 25 were found to contain measurable levels of nitrofuran metabolites, amounting in one case to 2.52 $\pm$ 0.24 μg SEM/kg and 6.27 $\pm$ 0.31 μg AOZ/kg, while in the other one 1.27 $\pm$ 0.17 μg SEM/kg and 9.53 $\pm$ 0.25 μg AMOZ/kg were detected. Considering that the origin of the gelatine used as medicine could not be identified, no conclusions on the possible origin of SEM and the other nitrofuran metabolites were drawn.

3.2. Possible sources of SEM other than as a metabolite of nitrofurazone

Unlike the metabolites AOZ, AMOZ, AHD and DNSH, which are highly specific marker metabolites for furazolidone, furaltadone, nitrofurantoin and nifursol, respectively, SEM has been found not to be an unequivocal marker metabolite for nitrofurazone. Almost at the same time as multi-nitrofuran metabolite methods based on LC-MS/MS, with sufficient sensitivity to test at MRPL of 1 μg/kg, were being developed in 2004/2005, the first reports on sources of SEM other than from nitrofurazone were published in the literature. It has been reported that SEM, or compounds from which SEM may be released, may occur in food from the following sources:

- as a reaction product formed from use of hypochlorite for disinfection or for bleaching of food products (Hoenicke et al., 2004; Xing et al., 2012);
- as a product from various chemical reactions in foods (Bendall, 2009; Abernethy, 2015; Yu et al., 2018);
- as a migration or breakdown product from azodicarbonamide previously permitted to be used as a blowing agent to foam the plastic sealing gaskets on metal lids of food jars (Stadler et al., 2004; Nestmann et al., 2005; Szilagyi and de la Calle, 2006);
- as a migration or breakdown product from azodicarbonamide previously permitted to be used as a flour treatment agent in bread production (Becalski et al., 2004, 2006; Noonan et al., 2005);
- as a reaction product formed during warm storage of food products such as dried egg powder and whey powder (Gatermann et al., 2004);
- as a product formed from the reaction of hydrazine with urea in milk, under acidic conditions, including those used in the analytical method (Abernethy and Higgs, 2013; Kuhn, 2014);
- released from a naturally occurring compound present in shrimps, prawns and crayfish (Saari and Peltonen, 2004; Van Poucke et al., 2011; McCracken et al., 2013; Li et al., 2015);
- released from precursors occurring in honey, such as the amino acid arginine (Khong et al., 2004; Crews, 2014).
- use as a chemical additive in gelatine intended for the preparation of drug formulations (e.g. capsules, tablets), in order to inhibit the cross-linking of collagen which would lead to a fall in drug dissolution and hence in the release of the active principles (Singh et al., 2002).

From this list of non-nitrofurazone sources of SEM, two potential sources, other than from a treatment of animals with nitrofurazone, are assumed to be the most relevant ones for food gelatine: from the use of hypochlorite for disinfecting or bleaching during production of gelatine or from chemical reactions occurring during the production of gelatine.

Hoenicke et al. (2004) carried out studies on a range of food types to establish the extent to which SEM could be formed in these food products by contact with hypochlorite. The foods, including a sample of gelatine, were supplemented with hypochlorite solution containing 0.015, 0.05, 1 or 12% active chlorine. The samples were allowed to incubate overnight before LC-APCI-MS/MS analysis of SEM after derivatisation with NBA. Treatment with hypochlorite solutions containing 0.015% (which is the concentration generally recommended for disinfection) or 0.05% (which is the concentration commonly used for bleaching) active chlorine did not result in a significant increase of SEM levels in the foods, including the sample of gelatine. Treatment with a hypochlorite solution containing 1% active chlorine resulted in the formation of SEM in the majority of foods, including in the sample of gelatine; the gelatine sample before treatment had a measured concentration of SEM of 1.6 μg/kg, but the explanation for this concentration of SEM in gelatine is not provided by the authors, although they refer elsewhere in the paper to an artefact of the analysis. After treatment of the gelatine sample with hypochlorite solution containing 1% active chlorine, the concentration of SEM in the sample was 5.1 μg/kg. To simulate a worst-case scenario, the samples were also treated with a hypochlorite solution containing 12% active chlorine; this treatment resulted in the formation of higher levels of SEM in all of the foods tested and, in the case of the sample of gelatine, a concentration of 450 μg/kg. The CONTAM Panel noted that the concentration of hypochlorite causing an increase in the level of SEM was at 20-fold higher than the level normally used in food processing.

The authors suggest that formation of SEM in the food samples following treatment with hypochlorite may be due to (a) degradation of nitrogen-containing substances having either an amidino or ureido group, such as arginine, histidine, citrulline, creatine and creatinine, or (b) reaction of urea with chloramine, which can originate from the reaction of hypochlorite with ammonia. They

tested this hypothesis that SEM might be formed from naturally occurring compounds in food during food processing, by treating arginine, histidine, citrulline, creatine, creatinine and urea with hypochlorite at 0.015% and 12% active chlorine. SEM was formed after treatment of arginine, creatine, creatinine and urea with 0.015% active chlorine and at much higher concentrations following treatment with 12% active chlorine.

The authors concluded that a possible formation of (tissue-bound) SEM was shown to occur in different kinds of nitrogen-containing foodstuffs after hypochlorite treatment and that it is possible to produce SEM in nearly all kinds of foodstuffs. Therefore, SEM could not be considered as an unequivocal marker for nitrofurazone abuse and, using available methods, it was not possible to distinguish between SEM related to nitrofurazone abuse and SEM formed during processing, since the latter was also detected by targeting the bound residues. The CONTAM Panel noted that the authors describe the independent determination of free and bound SEM for only one sample of food (carrageenan).

Kwon (2017) discusses the possibility of SEM that is determined in prawns/shrimp samples by triple quadrupole LC-MS/MS being an artefact of analysis, particularly as biurea may readily react with 2-nitrobenzaldehyde to form a derivative that may be incorrectly identified as NPSEM. He suggests that interference in the MS/MS analysis, when using a stable isotope as the internal standard, may be responsible for an incorrect assignment of SEM to natural occurrence in prawns/shrimp. However, using a largely similar analytical procedure, he determined four samples of freshwater prawns to contain SEM and considered this SEM to be a metabolite of nitrofurazone.

Abernethy (2015) described the formation of hydrazine in foods from the natural food components ammonia, hydrogen peroxide and acetone and the reaction of hydrazine, under hydrolysis in the presence of urea, to produce SEM. These studies suggested that, in foods containing urea compounds, SEM may be formed from hydrazine during the acid conditions that are an integral part of the methodology for determination of marker metabolites of nitrofurans.

Bendall (2009) described the formation of SEM in milk and whey when hypochlorite was used in conjunction with localised high pH conditions which can occur in dairy product processing. This author also described the formation of SEM in the presence of hydroxyurea.

Johnston et al. (2020) reported on a study to determine whether SEM was formed in chicken during processing, disinfection, chilling and freezing. The study arose because exported poultry was rejected at the point of entry by a U.S. trading partner due to the presence of SEM and the associated assumption that the SEM was indicative of nitrofurazone use. The study was designed to establish whether SEM residues in poultry might be a constituent or reaction product of processing agents and/or food ingredients and whether some portion of these non-nitrofurazone-associated SEM residues could become bound to tissue during processing.

For the study, poultry was collected at commercial slaughterhouses at three different processing points, post-evisceration, post-chilling and post-cut-up, and the poultry carcasses/parts were routinely washed with antimicrobial solutions, typically peroxyacetic acid and/or chlorine, at the three processing steps. Samples of poultry were analysed for total SEM and protein-bound SEM. Protein-bound SEM was determined by prewashing of the sample with alcohol prior to hydrolysis and derivatisation. SEM was not detected in any of the post-evisceration samples but was detected as total SEM in 92% of post-chilling samples and in 88% of post-cutup samples and as bound SEM in 29% of post-chilling samples and in 21% of post-cutup samples, indicating that the SEM resulted from chicken processing. The mean concentrations determined in the positive samples were 1.97 μg/kg total SEM and 0.44 μg/kg bound SEM. The duration of exposure to antimicrobial solutions during the chilling process was much longer (1.9–3.5 h) than exposure immediately following the evisceration (2–5 s) and after the cut-up (2–10 s) processing steps. This suggests that the duration of exposure to antimicrobial solutions during chilling played a critical role in the formation of detectable concentrations of SEM. During further frozen storage of the cut-up chicken parts, there was an increase, compared to the levels determined post-chilling, in the mean concentration of total SEM (2.31 μg/kg after 10 days, 2.70 μg/kg after 20 days and 1.67 μg/kg after 30 days of frozen storage) and of bound SEM (0.89 μg/kg after 10 days, 0.96 μg/kg after 20 days and 0.63 μg/kg after 30 days of frozen storage); the lower levels determined after 30 days of frozen storage were not significantly different to the levels determined after 10 and 20 days of frozen storage. These results suggest that when exported poultry products are analysed for SEM, the probability of detecting bound SEM may be increased as these products typically are frozen during transport.

In summary, SEM, both free and bound, can occur in food products, such as gelatine, during food processing, arising from the use of disinfecting agents and/or from reactions of various food components. Therefore, SEM, whether free or bound, determined in food products, including gelatine, cannot be considered as an unequivocal marker of the abuse of nitrofurazone in animal production.

3.3. Possible analytical approaches to distinguish between SEM in gelatine related to treatment with nitrofurazone and the presence of SEM due to food processing

The problem with SEM not being an unequivocal marker metabolite for nitrofurazone led to efforts to identify alternative approaches to establishing the abuse of nitrofurazone, principally through (a) sample preparation procedures pre-analysis for tissue-bound residues of SEM to ensure that any measured SEM corresponded unequivocally with nitrofurazone use and (b) analytical procedures that would identify nitrofurazone use independently than by measurement of SEM.

The first approach involved laboratories taking particular steps where positive screening results for SEM were found (Sanders, 2003; Points et al., 2015). Some examples of these steps include the following: (1) to address the issue of azodicarbonamide in flour, testing for the marker metabolite for nitrofurazone in breaded food products should be carried out on only the animal tissue part of the product, (2) to address the issue of natural occurrence of SEM in shellfish, only the inner core of products such as shrimps, prawns and crayfish should be tested for the marker metabolite for nitrofurazone, as the naturally occurring SEM occurs only in the outer part and (3) to address the issue of SEM being produced during food processing, the sample should be extensively washed with a range of organic solvents to remove any free SEM from the sample prior to the hydrolysis and derivatisation step for protein-bound SEM as the marker metabolite for nitrofurazone. The latter step is the one relevant to analysis of products, such as gelatine, for SEM as a marker metabolite of nitrofurazone; however, the finding that SEM generated during food processing may become bound to macromolecules in the food product (Hoenicke et al., 2004; Johnston et al., 2020) renders this step not useful in distinguishing the source of any measured SEM in such samples.

The second approach involved research to identify alternative analytes, matrices or analytical techniques to determining the presence of residues from nitrofurazone in samples. For example, determination of nitrofuran parent compounds, and marker metabolites, in pig and chicken eyes by LC-ESI-MS/MS has been described by Cooper and Kennedy (2005) and Cooper et al. (2008). This analytical approach was adopted to take advantage of the higher concentration of drug residues in eye tissues and, particularly, to establish the source of SEM as a nitrofuran marker metabolite through confirmation of the parent compound nitrofurazone in the eye. This approach of determining the nitrofuran parent compounds was used also by McCracken and Kennedy (2007) in a study on the accumulation of nitrofuran residues in egg yolk, albumen and shell, and by Pearson et al. (2016) to determine nitrofurazone specifically in dairy products, such as milk and milk powders. These methods for analysis of nitrofurazone parent compound were developed specifically to deal with the problem of SEM potentially arising in eggs/egg products and in dairy products from sources other than abuse of nitrofurazone in poultry and dairy animals. However, while this approach may be relevant to slaughter animals and testing of primary products, where the nitrofurazone parent compound may be detectable for a few days post-sampling, it is not suitable for testing for nitrofurazone-related residues in processed food products, such as gelatine, where persistence of nitrofurazone parent compound is highly unlikely.

Some research projects, partially funded by the EU, have been undertaken to attempt to identify alternative markers for nitrofurazone. One of these projects (Mulder et al., 2006) identified a number of metabolites in porcine muscle tissue during medication with nitrofurazone, primarily the cyano-metabolite which was detectable for up to 1 week after the withdrawal of nitrofurazone. Other metabolites, such as the aminofurfural hydrazone metabolite, were only qualitatively detected in muscle tissue at zero withdrawal time. The cyano-metabolite was proposed as an alternative marker metabolite for nitrofurazone, but it was recognised that its depletion in muscle tissue is relatively quick compared to the persistent tissue-bound adduct, SEM; in pigs treated with radiolabelled furazolidone, it was shown that the cyano-metabolite of furazolidone has a much shorter half-life than the protein-bound residues (Vroomen et al., 1987). Use of the cyano-metabolite as a marker for nitrofurazone was proposed also by Wang et al. (2010) for determination in muscle samples from nitrofurazone-treated channel catfish. The cyano-metabolite was measurable for up to 14 days after treatment with nitrofurazone, compared with only 4 days for the parent compound; the authors suggest that the cyano-metabolite could be used as an alternative confirmatory marker for monitoring the use of nitrofurazone in fish.

Another research project (Samsonova et al., 2008) studied the binding of nitrofurazone metabolites to proteins and considered that proteins with altered structure, due to the presence of the

metabolites, could be used as alternative biomarkers of nitrofurazone abuse. The aim of the research was to isolate and identify one or more potential biomarkers (proteins or peptides) implicated in the *in vivo* binding of nitrofurazone metabolites in liver tissue from treated rats. The authors identified albumin as having high concentrations of nitrofurazone metabolites (indicated by high SEM concentrations) and, because albumin has a relatively long elimination half-life, they considered that it might be sufficiently persistent *in vivo* to be a viable biomarker for the monitoring of nitrofurazone abuse. They proposed that the albumin fraction containing nitrofurazone metabolites might be used as an immunogen to produce antibodies that would bind only to albumin from animals that had been exposed to nitrofurazone; this work raised the possibility of a screening test for nitrofurazone that would not rely on the detection of SEM but on the whole adduct. However, further development of this approach towards a working analytical technique does not appear to have been published.

5-Nitro-2-furaldehyde may be released from nitrofurans by hydrolysis and, even though it is a generic analyte for nitrofurans, it was proposed (Bendall, 2009) that it might be used, in combination with a determination of SEM, to identify the source of SEM as being from nitrofurazone. A research group in China (Wang et al., 2014; Zhang et al., 2015, 2016, 2017) has proposed the determination of 5-nitro-2-furaldehyde as a marker metabolite for detecting illegal use of nitrofurazone in fish, crab and shrimp and have demonstrated that the method, based on LC-MS/MS, is sufficiently sensitive to meet the MRPL of 1 μg/kg. This method appears to be directed at nitrofurazone and, as such, is not appropriate for the determination of bound residues of nitrofurazone metabolites in processed food products, such as gelatine.

In summary, while research has been undertaken on alternative markers to SEM for nitrofurazone in animal tissues and food products, no such alternative markers have been identified to date that would be appropriate to test for nitrofurazone and its metabolites in processed food products, such as gelatine. The EURL has advised EFSA (EURL-Anses-Fougères, 2021, personal communication) that it intends to organise research on this topic that will attempt to identify biomarkers of nitrofurazone treatment using new approaches.

The EURL confirms its view that the presence of SEM in the animal-derived fraction of processed foods like gelatine may not be considered as a specific indicator for nitrofurazone abuse, as indirect contamination with or neo-formation of SEM are possible and have been observed in various food production processes.

One potential approach to distinguishing between SEM from nitrofurazone and SEM from other sources in food products, such as gelatine, might be based on the independent determination of total (bound and free) SEM and of bound SEM. This approach is based on the advice (see above) that the sample to be tested should be pretreated by solvent washing prior to hydrolysis/derivatisation to ensure that only protein-bound residues of SEM are measured; in the case of the gelatine sample subject to the RASFF report in January 2021, the analytical methodology supplied indicates that this step was not taken and that total SEM was determined in the sample.

The basis for this approach is the observation that nitrofurazone treatment gives rise to a high proportion of protein-bound SEM whereas SEM from other sources gives rise to a high proportion of free SEM. Gelatine, being a highly processed food product, is potentially likely to contain SEM as an indirect contamination and, therefore, may contain a high proportion of non-protein-bound SEM (free SEM) when tested independently for both total and protein-bound SEM, providing the basis for discriminating as to the source of the SEM determined in the gelatine (EURL-Anses-Fougeres, 2021, personal communication).

A couple of publications have identified these differences in the bound:free ratio of SEM from nitrofurazone treatment and from other sources. A study on Finnish crayfish (Saari and Peltonen, 2004) found traces of SEM in cooked samples, showing mean concentrations of total SEM of 4.2 ng/g ($n = 18$) and of bound SEM of 0.5 ng/g ($n = 12$). The authors proposed that the SEM determined was either due to it being a natural constituent of crayfish or of being formed during cooking; the results point to a non-nitrofurazone source of SEM having a much higher proportion of free SEM than of bound SEM. In studies related to the development of an ELISA for SEM, to be used both for analysis of SEM as a metabolite of nitrofurazone and as a food contaminant, Cooper et al. (2007) determined that three quarters of the SEM in poultry muscle arising from nitrofurazone ingestion was found in the form of tissue-bound residues while SEM arising from non-nitrofurazone sources, such as carrageenan or azodicarbonamide, was present predominantly as free SEM.

Applying this approach to the testing of samples of food products, such as gelatine, and the determination of whether measured SEM is from a nitrofurazone source or from a food processing source, it is recommended that, where SEM is determined in a sample, a further set of analyses might be undertaken to establish the ratio of bound:free SEM in the sample; where the proportion of bound

SEM is higher, a nitrofurazone source might be suspected, whereas if the proportion of free SEM is higher, a non-nitrofurazone source might be suspected.

Research should be undertaken to establish whether this approach might be suitable for unequivocal identification of the source of SEM determined in gelatine. For example, gelatine might be prepared, according to industrial processing standards, from animals both non-treated and treated with nitrofurazone. The processing of both types of gelatine might be carried out both with and without the use of hypochlorite and/or other potential sources of SEM from processing operations. In addition, as a positive control SEM might be added to the gelatine. Determination of total and bound SEM should be undertaken on samples of tissues used in the process and of gelatine at various stages of the processing procedure and used to establish whether the ratio of bound:free SEM would be a suitable method for distinguishing the source of SEM determined in gelatine.

3.4. Uncertainties

Overall, the CONTAM Panel considered that, at this stage, there is limited information to provide any advice with sufficient certainty.

4. Conclusions

- Scant information is available on the presence of nitrofuran markers in gelatine.
- It is plausible that SEM arising from the illegal treatment of target species with nitrofurazone would result in its distribution and possible accumulation in tissues rich in collagenous fibres (e.g. cartilages, tendons, bones and skin) which are the main sources of gelatine.
- SEM, both free and bound, could occur in food products including gelatine, as a result of food processing, arising from the use of disinfecting agents and/or from reactions with various food compounds. SEM produced in food products during food processing may become bound to cellular constituents released by procedures such as chilling and freezing. Therefore, SEM, whether free or bound, determined in food products, including gelatine, cannot be considered as an unequivocal marker of the abuse of nitrofurazone in animal production.
- One potential approach to distinguishing between SEM from nitrofurazone and SEM from other sources in food products, such as gelatine, might be based on determining the ratio of bound: free SEM in a sample of gelatine. However, whether the ratio of bound:free SEM would unequivocally distinguish between SEM arising from nitrofurazone abuse or from other sources still needs to be demonstrated.

5. Recommendations

- More data are needed to establish the occurrence of SEM in gelatine and in tissues used for its production, supported by information on the type of gelatine tested and the production process used.
- Research should be undertaken to
    - investigate in more detail which processing conditions lead to formation of SEM in gelatine during its production and what levels can be found.
    - establish whether the ratio of bound:free SEM would be a suitable method for distinguishing the source of SEM determined in gelatine.
    - develop analytical methods, other than the determination of SEM, to unequivocally identify the occurrence of residues in food samples related to the illegal use of nitrofurazone in animal production e.g. detecting the intact SEM adduct using antibodies or LC-MS/MS.
- In addition, since the toxicity of SEM is of concern, testing for SEM *per se* in food should be increased.

6. Documentation provided to EFSA

1) Response to EFSA request. June 2021. Submitted by Gelatine manufacturers of Europe (GME).
2) Response to EFSA request. May 2021. Submitted by Lab. Chimica Biotossicologica (UO Chimica) Istituto Zooprofilattico Sperimentale del Lazio e della Toscana M. Aleandri, Italy.
3) Response to EFSA request. June 2021. EU-RL for Antibiotic and Dye Residues Veterinary Medicinal Products at Anses - Laboratory of Fougeres, France.

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Abbreviations

AFC Panel	EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
AHD	1-aminohydantoin
AMOZ	3-amino-5-methylmorpholino-2-oxazolidinone
AOZ	3-amino-2-oxazolidinone
CAS	Chemical Abstracts Service
CCα	Decision limit
CCβ	Detection capability
DNSH	3,5-dinitrosalicyclic acid hydrazide
CONTAM Panel	EFSA Scientific Panel on Contaminants in the Food Chain
EURL	EU Reference Laboratory
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
i.p.	Intraperitoneal
LC-ESI-MS/MS	Liquid chromatography–electrospray ionisation-tandem mass spectrometry
LC-MS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
NBA	2-nitrobenzaldehyde
qs	*quantum satis*
RASFF	Rapid Alert System for Food and Feed
RPA	Reference point for action
SEM	Semicarbazide
SPE	Solid phase extraction
UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry
USA	United States of America