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Assessment of an application on a detoxification process of groundnut press cake for aflatoxins by ammoniation

EFSA Panel on Contaminants in the Food Chain (CONTAM),
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Abstract

Following a request from the European Commission, the EFSA Panel on Contaminants in the Food Chain (CONTAM) provided a scientific opinion on an application for a detoxification process of groundnut press cake for aflatoxins by ammoniation. Specifically, it is required that the feed decontamination process is compliant with the acceptability criteria specified in the Commission Regulation (EU) 2015/786 of 19 May 2015. The CONTAM Panel assessed the data provided by the feed business operator with respect to the efficacy of the process to remove the contaminant from groundnut press cake batches and on information demonstrating that the process does not adversely affect the characteristics and the nature of the product. Although according to the literature the process may be able to reduce aflatoxin levels below the legal limits, the Panel concluded that the proposed decontamination process, on the basis of the experimental data submitted by the feed business operator, cannot be confirmed for compliance with the acceptability criteria provided for in Commission Regulation (EU) 2015/786 of 19 May 2015. The Panel recommended sufficient sample testing before and after the process, under the selected conditions, to ensure that the process is reproducible and reliable and to demonstrate that the detoxification is not reversible. In addition, genotoxicity testing of extracts of the treated feedingstuff and of the identified degradation products would be necessary. Finally, information on the transfer rate of AFB1 to AFM1 excretion in milk for animals fed the ammoniated product, in comparison to the starting material and on the ammoniation process changes of the nutritional values of the feed material should be provided.

Keywords: aflatoxin, ammoniation, groundnut press cake, decontamination process

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1. Introduction

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

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed[1] provides that the use of products intended for animal feed which contain levels of undesirable substances exceeding the maximum levels laid down in Annex I of that Directive is prohibited.

Directive 2002/32/EC provides also that Member States are to ensure that measures are taken to guarantee the correct application of any acceptable detoxification process on products intended for animal feed and the conformity of those detoxified products with the provisions of Annex I of that Directive.

In order to ensure a uniform assessment across the European Union of the acceptability of detoxification processes, acceptability criteria for detoxification processes have been established at Union level by Commission Regulation (EU) 2015/786 of 19 May 2015 defining acceptability criteria for detoxification processes applied to products intended for animal feed as provided for in Directive 2002/32/EC of the European Parliament and of the Council.

The acceptability criteria for detoxification processes established by the Regulation shall ensure that the detoxified feed does not endanger animal and public health and the environment and that the characteristics of the feed are not adversely altered by the detoxification process. The Regulation furthermore provides that the compliance of a detoxification process with those criteria shall be scientifically assessed by the European Food Safety Authority (EFSA) on a request from the Commission.

The Commission has received the application of a detoxification process of groundnut press cake for aflatoxins by ammoniation for assessment by EFSA of compliance with the acceptability criteria.

TERMS OF REFERENCE

In accordance with Art. 29(1) of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority for an assessment of an application of a detoxification process of groundnut press cake for aflatoxins by ammoniation, for compliance with the acceptability criteria provided for in Commission Regulation (EU) 2015/786 of 19 May 2015.

1.2. Interpretation of the Terms of Reference

EFSA received from the European Commission a request for a scientific opinion on the assessment of an application referring to feed detoxification processes to be compliant with acceptability criteria specified in the Commission Regulation (EU) 2015/786 of 19 May 2015.[2] In this context the term detoxification is interpreted as either decontamination by removing the contaminants or by chemical or biological processes able to reduce the toxicity of the contaminants present. This scientific opinion is dealing with a chemical decontamination process reported of groundnut press with the aim to reduce the level of aflatoxins below the legal limits.

The EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) concluded that the Terms of Reference provided by the European Commission were clear and that the opinion for the assessment of this chemical decontamination process should mainly focus on data:

- enabling the assessment of the efficacy of the process to remove the contaminants from the feed batches to ensure compliance with the requirements of Directive 2002/32/EC,
- demonstrating that the decontamination process does not adversely affect the characteristics and the nature of the feed, and
- showing that the process is irreversible and that no harmful reaction products of the contaminant are formed and transferred to food products.

[1] OJ L 140, 30.5.2002, p. 10.
[2] Commission Regulation (EU) 2015/786 of 19 May 2015 defining acceptability criteria for detoxification process applied to products intended for animal feed as provided for in Directive 2002/32/EC of the European Parliament and of the Council. OJ L 125, 21.5.2015, p. 10–14.

1.3. Additional information

The feed business operator has provided the European Commission with information on the proposed decontamination process and its effectiveness. This information should be provided as laid down in Directive 2002/32/EC.

1.4. Legislation

According to Directive 2002/32/EC, for feed materials a maximum content of 0.02 mg/kg applies. In compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals, a ML of 0.005 mg/kg applies. In compound feed for cattle (except dairy cattle and calves), sheep (except dairy sheep and lambs), goats (except dairy goats and kids), pigs (except piglets) and poultry (except young animals), a ML of 0.02 mg/kg applies. For all other compound feed, a ML of 0.01 mg/kg applies. According to Commission Regulation (EC) No 1881/2006, the maximum level for aflatoxin M1 (AFM1) in raw milk, heat-treated milk and milk for the manufacture of milk-based products is 0.050 μg/kg.

2. Data and methodologies

2.1. Data

The feed business operator has submitted information in support to its claim about the efficacy of the decontamination process consisting in filling the reactor with feed, adding ammonia and increasing the pressure and the temperature in order to achieve at the end of the process aflatoxin levels below the legally permitted levels. The CONTAM Panel based its assessment on the provided information (Documentation provided to EFSA No 1) to address the Terms of Reference.

Additional data were submitted by the feed business operator to EFSA (Documentation provided to EFSA No 2 and No 3) further to a request for clarification. In addition a technical meeting was held with the feed business operators.[3] No additional data were provided after the technical meeting.

Furthermore, relevant literature was considered.

2.2. Methodologies

The CONTAM Panel evaluated the acceptability of the proposed decontamination process as requested by the relevant regulations, specifically Directive 2002/32/EC and Commission Regulation (EU) 2015/786 with their Annexes. Any assessment is conducted in line with the principles described in the EFSA guidance on transparency in the scientific aspects of risk assessment (EFSA, 2009) and following the relevant existing guidance from the EFSA Scientific Committee.

3. Assessment

3.1. Identity of the contaminants

Aflatoxins are bisfuranocoumarin compounds produced primarily by toxigenic strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. *A. parasiticus* produces AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), whereas *A. flavus* mainly produces AFB1 and AFB2. *A. flavus* favours the aerial parts of the plants (e.g. leaves and flowers) while *A. parasiticus* is more adapted to a soil environment and is of more limited distribution than *A. flavus* (EFSA, 2007). Many other species closely related to *A. flavus* (*A. minisclerotigenes*, *A. korhogoensis*, *A. aflatoxiformans* and *A. texensis*) or to *A. parasiticus* (*A. novoparasiticus* and *A. arachidicola*) also produce aflatoxins B and G (Pildain et al., 2008; Adjovi et al., 2014; Carvajal-Campos et al., 2017; Frisvad et al., 2019; Singh and Cotty, 2019). In addition to the above-mentioned four aflatoxins, these fungi also form other substances such as aflatoxicol, versicolorin, sterigmatocystin, cyclopiazonic acid and kojic acid (Yu, 2012). AFB1 is known to be a genotoxic carcinogen and there are indications that similar effects apply to the AFB2, AFG1 and AFG2, albeit with different potencies (EFSA CONTAM Panel, 2020). In animals and humans, AFB1 is metabolised into AFM1 which in dairy cows is partly excreted into milk. AFM1 also showed genotoxic and carcinogenic effects, although with lower potency than AFB1. As a result,

[3] 11th meeting of the WG on feed detoxification processes https://www.efsa.europa.eu/sites/default/files/wgs/chemical-contaminants/contamwg-feeddetoxification.pdf

levels in feed should be low enough to avoid levels of AFM1 in milk that might be a risk for the consumer.

The applicant has not provided any analyses on the identification of other compounds, e.g. aflatoxin D1 (AFD1), or any information on the AFB1 to AFM1 conversion ratio in milk for cows or any other species fed the ammoniated products, in comparison to the starting material. Instead, the applicant referred to a review paper (Park, 1993).

3.2. Method of analysis

The feed business operator has submitted information on the analysis of aflatoxins (AFB1, AFB2, AFG1, AFG2 and sum of the four) performed by an accredited laboratory (Eurofins WEJ Contaminants GmbH (Hamburg): EN ISO/IEC 17025:2005 DAKKS D-PL-14602-01-00, for Eurofins Steins Laboratorium A/S, Ladelundvej 85, DK-6600 Vejen, DANMARK). The certificate of analysis indicated that the method used was 'EN 14123, mod./IAC-LC-FLD'.

EN 14123:2007 is a standard method for the determination of AFB1 and the sum of aflatoxins (AFB1, AFB2, AFG1 and AFG2) in hazelnuts, peanuts, pistachios, figs and paprika powder in foodstuffs. A test portion is extracted with methanol/water, sometimes with the addition of hexane or cyclohexane. The sample is filtered, diluted with phosphate-buffered saline and applied to an immunoaffinity column which contains antibodies to the four specific aflatoxins. The aflatoxins are retained by the column and are then eluted with methanol. Quantification is by reverse-phase high performance liquid chromatography with post-column derivatisation based on bromination using bromine that is generated electrochemically or by using pyridinium hydrobromide perbromide. Determination is performed using fluorescence detection.

The limit of quantification of the standard method is reported to be 0.8 ng/g for each aflatoxin or better (value derived from in-house and collaborative study), depending on the equipment used.

3.3. Decontamination process

3.3.1. Description of the process

A number of physical, chemical or biological treatments for reducing levels of aflatoxins in contaminated feed materials, principally cereal grains and oilseed meals, have been reported and reviewed (e.g., Park and Price, 2001; Karlovsky et al., 2016; Čolović et al., 2019). While some have achieved success in reducing aflatoxin levels in these feed materials, most are technically and/or economically impractical for commercial application (Kolton et al., 1979; Park and Liang, 1993). However, from the literature, it appears that the most commonly used process to date, and the method used by the applicant involves exposure of mycotoxin-contaminated feedingstuffs to ammonia under heat and pressure, followed by recovery of ammonia by evaporation (see Figure 1).

Reductions in the levels of aflatoxin of between 96% (Park et al., 1988) and 99% (Gardner et al., 1971; Norred, 1982) have been reported using this process, and as a result of these and other studies some countries permit ammoniation as a means of detoxifying contaminated feeds (Phillips et al., 1994). However, there is no generally accepted protocol for ammoniation; rather, differences in the levels of ammonia, temperature, pressure and water, together with the duration of exposure, the initial level of contamination and the substrate material have all been shown to influence the effectiveness of the method.

For example, Gomaa et al. (1997) observed significant reductions in aflatoxin content with increasing ammonia concentrations (0.25, 0.5 and 1% in aqueous concentrations) but no further reductions were observed when ammonia concentrations increased to 1.5% and 2%, for both total and individual aflatoxins (AFB1, AFB2, AFG1 and AFG2), suggesting a curvilinear response to the ammonia. The effect of temperature was illustrated in the study in which the reduction in the levels in AFB1 in roasted contaminated groundnuts was almost complete at a temperature of 150°C, while at temperatures of 90°C and 120°C the reduction in AFB1 was 19% and 58%, respectively.

In a series of studies, Gomaa et al. (1997) examined the effect of ammonia concentrations (0.25, 0.5, 1.0, 1.5 and 2.0% in aqueous solution), pressure (ambient or 2 bar) and temperatures (ambient or 121°C) on aflatoxin levels in cereal grains. Significant reductions in aflatoxin levels at low ammonia concentrations (0.25 and 0.5%) were observed, but the effects of increasing pressure were lost at higher ammonia concentrations. The study also showed that the reduction of aflatoxin was greater under the high pressure/high temperature treatment than the low pressure/low temperature treatment. Studies by other authors have confirmed that, under increased pressure, greater reductions

in aflatoxin levels may be achieved following exposure to ammonia compared to ambient pressure (Gardner et al., 1971; Park et al., 1984; Samarajeewa et al., 1990; Gomaa et al., 1997).

The moisture content of the material has also been identified as a factor influencing the reduction in aflatoxin content following ammoniation, with Jorgensen and Price (1981) concluding that the moisture level of the product and holding temperature were the main factors influencing the efficacy of aflatoxin decontamination.

In summary, Park and Price (2001) concluded that the application of ammonia (0.5–2.0%) to feed materials with a moisture content of 12–16%, under pressure (45–55 psi), and at temperatures of 80–100°C for 20–60 min, is the most reliable method of reducing aflatoxins in contaminated grains, oilseeds and the feedingstuffs derived from them.

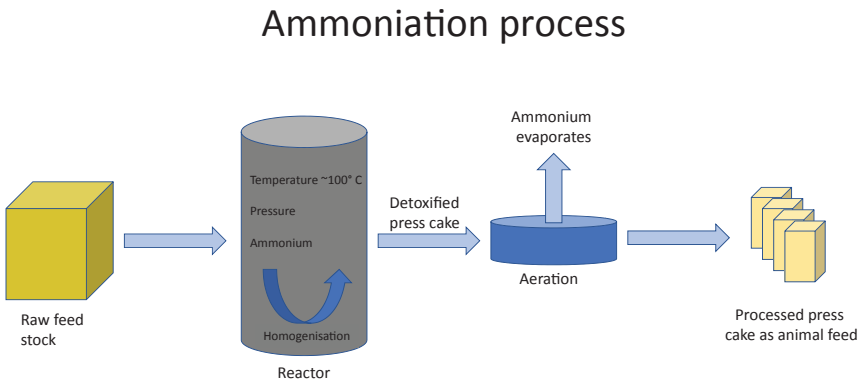


Figure 1: Summary of the detoxification process of groundnut press cake from aflatoxins by ammoniation

Two tests were presented by the applicant to study the dynamics of temperature, pressure, ammonia concentration and processing time on the reduction of levels of aflatoxin in groundnut cake.

Test 1: In the first test (carried out in 2017), peanut press cake from Senegal (initial total aflatoxin concentration of 147.9 μg/kg) was used. Initially, a 5-L reactor was employed, but due to difficulties in controlling the amount of ammonia gas applied a larger reactor (size not specified, but capable of handling 50 kg of the press cake) was used. For this study, 50 kg of the press cake, 0.6 L water and 2 kg 25% W/W NH_4OH were mixed for up to 180 min, at pressures of 100, 450 or 1,000 mbar. Heat was applied resulting in a product temperature of 103°C. The chamber was fitted with rotating knives to ensure homogenous mixing of the cake. The results of the test showed varying success in removing aflatoxins.

Table 1: Levels of aflatoxin in peanut press cake following exposure to ammonia under different pressures and maturation time

Test number	Pressure	Maturing time	Aflatoxin (μg/kg)				
	mbar	minutes	Total	B1	B2	G1	G2
1.1	1,000	20	11.3	9.6	0.9	0.8	< 0.1
1.2	450	40	20.7	18.6	1.6	0.5	< 0.1
1.3	100	120	27.9	25.0	2.2	0.7	< 0.1
1.4	100	180	8.3[(a)]	4.5	0.6	0.2	< 0.1

(a): The number does not correspond to the sum of the four aflatoxin.

In this test, short maturing time and high pressure seems equally effective as long maturing time and low pressure (see treatments 1.1 and 1.4 in Table 1). Both resulted in aflatoxin contents lower than the approved EU maximum in feed materials (20 μg/kg).

Test 2: For this test (carried out in 2018), similar equipment and processes were adopted, but the feed material was groundnut press cake from Malawi (initial aflatoxin concentration 286 μg/kg). During the test, samples of the feed were taken at 15-min intervals. The results of the study are given in Table 2.

Table 2: Levels of total aflatoxin in peanut press cake, over time, as a result of different levels of ammonia and pressure

Test number	Addition of Ammonia solution[(a)]	Pressure	Total aflatoxin content (μg/kg) after various reaction time				
			Time (min)				
			0	15	30	45	60
2.1	2	Ambient	286	141.8	80.3	110.4	89
2.2	3 + 1	Ambient	286	124	103	135	88.7
2.3	4	Ambient	286	155	76.4	58.9	86.9
2.4	2 + 2	2-bar	286	272.8	238	281	193.7
2.5	3 + 1	2-bar	286	103	43.9	43.9	75.7
2.6	4	2-bar	286	47.3	14.6	14.6	10.6

(a): Not further clarified by the applicant.

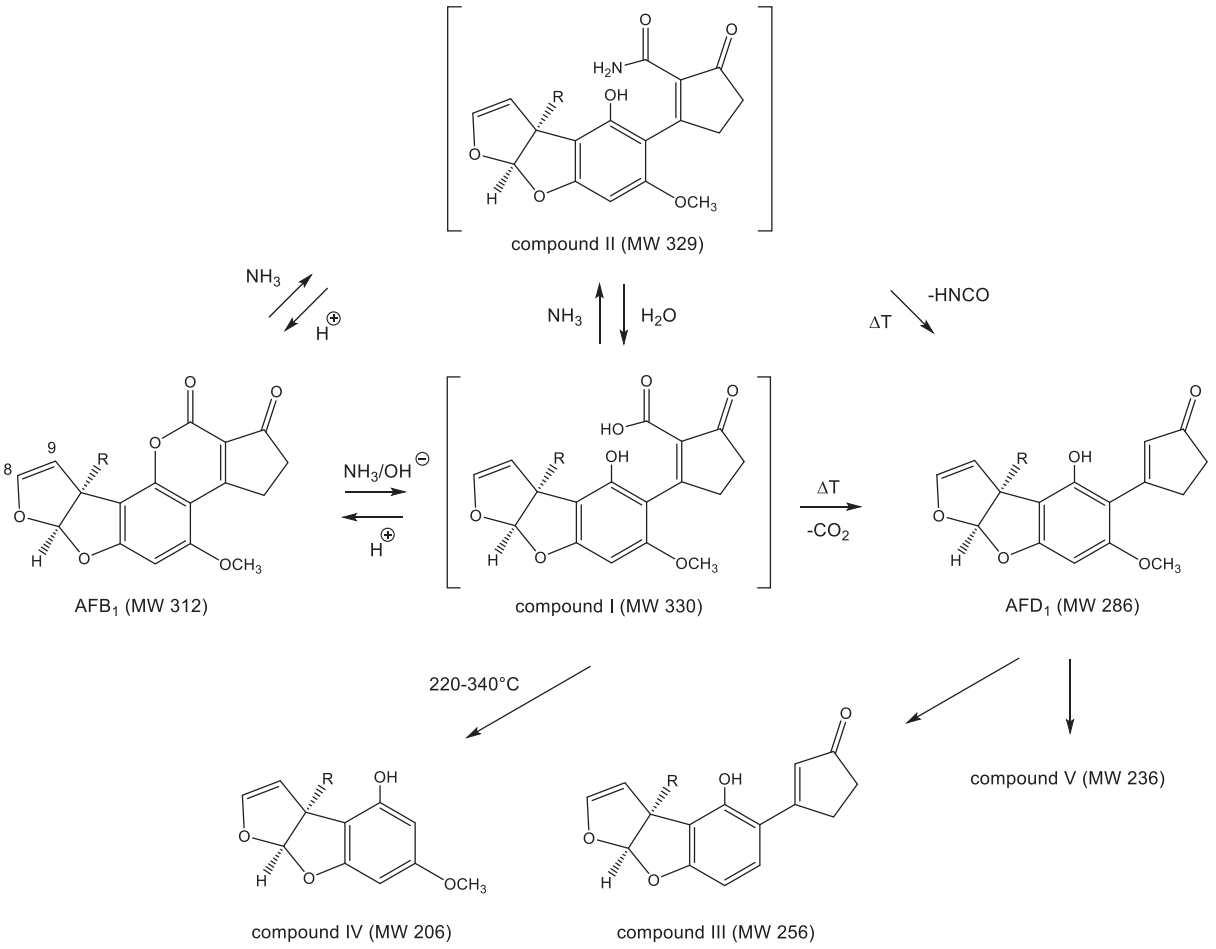
The authors of the report noted that in both tests there were some unexplained results. In particular, they concluded that the results of test 2.4 can only be explained by the fact that no NH_4OH had been added to reactor. In this study, only treatment 2.6 reduced aflatoxin content to less than 20 μg/kg.

3.3.2. Efficacy and irreversibility of the process

Under certain circumstances, and particularly where the degradation process is sub-optimal, the aflatoxin molecule may revert to its original state (see section 3.4). In an acid environment (e.g. the gastric system of animals) the amount of aflatoxin passing to the small intestine and absorbed may therefore be greater than would be indicated by analysis of the feed. No evidence was provided by the applicant that the proposed process is sufficient to ensure irreversibility.

3.4. Reaction products

The ammoniation of AFB1 gives rise to several degradation products, which were first disclosed in studies using pure AFB1, i.e. in the absence of any matrix from an agricultural commodity. The identified degradation products of AFB1 and their putative intermediates are depicted in Figure 2.



Compounds in square brackets are putative intermediates. MW: molecular weight.

Figure 2: Pathways of degradation of AFB1 during ammoniation

The ammoniation chemistry of AFB1 has been reviewed by Park et al. (1988) and Park (1993). Briefly, the first step of the chemical reaction with ammonia in the presence of water is assumed to be the opening of the lactone ring of AFB1, generating compound I and compound II, which exist in a chemical equilibrium. When conducted under mild conditions, e.g. at room temperature, this reaction is reversible upon acidification and leads back to AFB1 (Vesonder et al., 1975). However, under conditions of high temperature (e.g. 100°C) and pressure, compounds I and II release CO_2 and HNCO, respectively, leading to the decarboxylation product AFD1 (molecular weight 286) as first described by Lee et al. (1974). The trivial name AFD1 was chosen because this product arises from decarboxylation of AFB1. A structurally related product without the methoxy group and MW 256 (compound III) has also been reported, as has a further product with MW 206 (compound IV), which lacks both the lactone carbonyl and the cyclopentenone ring of AFB1 (Cucullu et al., 1976). No trivial names were assigned to these degradation products. When AFB1 is degraded in the absence of matrix, another product with MW 236 (compound V) has been detected by mass spectrometry, the chemical structure of which is yet unknown (Stanley et al., 1975). AFD1 and compounds III, IV and V are unable of reverting back to AFB1, and none of them exhibits chemical reactivity.

The formation of degradation products of AFB1 during ammoniation as described above was found to be markedly affected by the feed matrix. Again, early studies are reviewed in detail by Park et al. (1988). As with pure AFB1, the amount of unchanged AFB1 was, in general, very low after ammoniation of contaminated feeding materials. However, matrix components appeared to lower or eliminate the conversion of AFB1 to AFD1 and other products formed in model reactions with AFB1 alone. Instead, a high proportion of the original AFB1 was found to bind to matrix constituents in a non-extractable manner, as could be demonstrated by using radiolabelled AFB1 (Lee et al., 1979, 1984; Park et al., 1984). A possible explanation might be that the lactone ring of AFB1 reacts with amino and/or hydroxyl groups of the matrix and is thereby protected against decarboxylation.

The findings of these earlier studies were confirmed in a more recent investigation (Hoogenboom et al., 2001a). When peanut meal was spiked with radiolabelled AFB1 and subjected to high temperature/high pressure ammoniation, only 10% of the radiolabel could be extracted. The extract contained about 1% of the original amount of AFB1 but no degradation products. 90% of the radiolabel could not be extracted. When the ammoniated meal was fed to cows, the levels of radiolabelled material in milk and various tissues were much lower (milk 10-fold, liver 25-fold, kidney 3-fold) as compared with untreated meal. Urinary excretion of radioactivity was also strongly (10-fold) reduced with the ammoniated meal, whereas faecal excretion was increased. The authors conclude that these effects are caused by a reduced bioavailability of the degradation products. The AFB1 metabolite aflatoxin M1 was about 30-fold lower in the milk of cows receiving ammoniated feed as compared to feed without decontamination, probably due to the much lower level of AFB1 remaining after ammoniation. However, the ratio AFB1 in feed and AFM1 in milk increased in the case of the ammoniated meals, suggesting partial reversion of decontamination products.

In addition to demonstrating the degradation of AFB1 in the process of ammoniation, it is important to show that the treated materials are not toxic. Multiple studies have been performed to assess the toxicity of individual degradation products, as well as the toxicity of ammoniated feeding materials contaminated with AFB1. Both types of toxicity studies have been reviewed in detail by Park et al. (1988). In summary, selected isolated degradation products such as AFD1 and compound IV exhibit weak effects in some assays (Ames *Salmonella* mutagenicity test and *in vivo* covalent binding to rat liver DNA), but their toxic potency is several orders of magnitude below that of AFB1. Moreover, these products may be of low practical significance as they appear to be only formed in minute amounts in ammoniated feeds, where most of the AFB1-related material is bound to feed matrices in a non-extractable manner (see above). Hoogenboom et al. (2001b) showed a strong reduction in the mutagenic response of extracts from peanut meal ammoniated by commercial processes, supported by results for unscheduled DNA synthesis and Comet assay with rat hepatocytes. When ammoniated AFB1-contaminated feeding material was given to rats, mice, ducklings, swine, chickens, lambs, trout and dairy and beef cattle in acute and chronic trials, no toxic effects related to the ammoniation procedure were disclosed (Park et al., 1988). However, Neal et al. (2001) fed peanut meals treated by two commercial ammoniation processes to rats for 90 days and observed decreased growth and hepatic abnormalities for one of the meals. The authors suggested that this could also be due to differences in nutritional status due to effects on the proteins in the meal. It would be a useful supplement of the available whole animal feeding studies to investigate the fate of the non-extractable degradation products in *in vitro* model systems for digestion in ruminants and other animals receiving the decontaminated feed.

No analysis of the degradation products of AFB1 has been reported by the applicant for the two tests described in Section 3.3.1. The applicant has only referred to several studies on the formation and toxicology of degradation products cited in the Background section above.

3.5. Characteristics and nature of the processed groundnut press cake

Apart from the levels of aflatoxins in the treated press cake, the applicant has provided no further information on the characteristics of the peanut cake following ammoniation. However, Park (1993) reported that ammoniation resulted in increased levels of total, protein and non-protein nitrogen, ash, and soluble solids, and reduced levels of sulphur-containing amino acids, available lysine, and non-reducing sugars. As a result, the concentration of true protein is reduced (due to the additional non-protein nitrogen taken up by the feedingstuff). Park (1993) further concluded that production parameters, e.g. milk yield and egg quality, were not adversely affected by the treatment. Since ruminants are capable of utilising non-protein nitrogen, they tended to show benefits (improved weight gain and feed efficiency) while for non-ruminant species either no effect or decreased production values were observed. Lee and Cucullu (1978) reported that although ammonia is effective in degrading AFB1 in feedstuffs, it also forms a degradation product AFD1 which, although not completely non-toxic possesses lower toxicity than AFB1.

The characteristics and nature of the processed groundnut press cake were not described. Therefore, the comparison of the same batches before and after the decontamination by means of evaluation of the nutrients profile and other key characteristics was not feasible.

3.6. Uncertainty analysis

The CONTAM Panel considered that the lack of information does not allow uncertainty analysis.

4. Conclusions

The CONTAM Panel assessed the information made available in the documents submitted by the feed business operator and was of the view that insufficient information was available to conclude on the safety and efficacy of the proposed decontamination process for groundnut press cake for aflatoxins by ammoniation.

5. Recommendations

A substantial body of literature is available for aflatoxin decontamination of feeds by ammoniation. The safety and efficacy of the specific process need to be demonstrated by:

- sufficient sample testing before and after the process under the selected conditions to ensure that the process is reproducible and reliable.
- providing evidence that the method proposed is sufficient to ensure that the detoxification is not reversible.
- genotoxicity evaluation of the extracts of the treated products and of the identified degradation products.
- information on the transfer rate of AFB1 from detoxified feed to AFM1 excretion in milk for animals fed the ammoniated product, in comparison to the starting material.
- specifying if, and to what extent, the ammoniation process changes the nutritional values of the feed material.

6. Documentation as provided to EFSA

1) Reduction of aflatoxin in groundnut press cake. July 2020. Submitted by Global Green Developer.
2) Responses provided to EFSA questions of 04/08/2020. October 2020. Submitted by Global Green Developer.
3) Responses provided to EFSA questions 11/12/2020. February 2021. Submitted by Global Green Developer.

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Abbreviations

AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFD1	aflatoxin D1
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AFM1	aflatoxin M1
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