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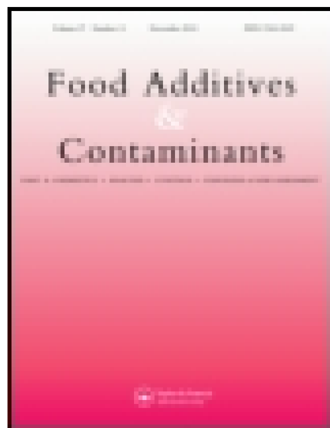

Food Additives & Contaminants: Part A
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tfac20

Mycotoxin contamination of animal feedingstuff: detoxification by gamma-irradiation and reduction of aflatoxins and ochratoxin A concentrations

Vita Di Stefano[a], Rosa Pitonzo[b], Nicola Cicero[c] & Maria Cristina D'Oca[a]

[a] Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), University of Palermo, Palermo, Italy

[b] Centro Grandi Apparecchiature (CGA), University of Palermo, Palermo, Italy

[c] Dipartimento di Scienze dell'Ambiente, della Sicurezza, del Territorio, degli Alimenti e della Salute (SASTAS), University of Messina, Messina, Italy

Accepted author version posted online: 25 Sep 2014.Published online: 31 Oct 2014.

To cite this article: Vita Di Stefano, Rosa Pitonzo, Nicola Cicero & Maria Cristina D'Oca (2014): Mycotoxin contamination of animal feedingstuff: detoxification by gamma-irradiation and reduction of aflatoxins and ochratoxin A concentrations, Food Additives & Contaminants: Part A, DOI: 10.1080/19440049.2014.968882

To link to this article: http://dx.doi.org/10.1080/19440049.2014.968882

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Food Additives & Contaminants: Part A, 2014
http://dx.doi.org/10.1080/19440049.2014.968882

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Vita Di Stefano[a]*, Rosa Pitonzo[b], Nicola Cicero[c] and Maria Cristina D'Oca[a]

[a]*Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), University of Palermo, Palermo, Italy;* [b]*Centro Grandi Apparecchiature (CGA), University of Palermo, Palermo, Italy;* [c]*Dipartimento di Scienze dell'Ambiente, della Sicurezza, del Territorio, degli Alimenti e della Salute (SASTAS), University of Messina, Messina, Italy*

(*Received 28 May 2014; accepted 17 September 2014*)

Mycotoxins are fungal secondary metabolites identified in many agricultural products screened for toxigenic moulds. They have been reported to be carcinogenic, teratogenic, tremorogenic, haemorrhagic and dermatitic to a wide range of organisms. With the increasing stringent regulations for mycotoxins imposed by importing countries such as those of the European Union, many cereals that are not safe for human consumption are used in formulations intended for animal feed. Gamma-rays are reported in the scientific literature to destroy ochratoxin A and aflatoxin in food crops and feed. The present study provides preliminary data for establishing the effect of dose of gamma-irradiation, ranging from 0 to 15 kGy, on aflatoxins and ochratoxin A reduction in commercial animal feed. The mycotoxin levels were determined by means of immunoaffinity clean-up (IAC) and HPLC with fluorescence detection (HPLC-FLD). The maximum reductions found at 15 kGy were 23.9%, 18.2%, 11.0%, 21.1% and 13.6% for ochratoxin A, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2, respectively. Results showed that the gamma-rays even at 15 kGy were not effective in the complete destruction of ochratoxin A and aflatoxins in the tested feed.

Keywords: mycotoxin; commercial animal feed; aflatoxins; ochratoxin A; gamma-irradiation

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Introduction

Feed supply is central to all animal production systems and any factor that affects the security of the feed chain is a significant constraint to production. Globally, mycotoxins have significant effects on human and animal health, with economic and international trade implications (Bryden 2007; Wild & Gong 2010; Zaki et al. 2012).

Poor harvesting practices, improper drying, handling, packaging, storage and transport conditions contribute to fungal growth and increase the risk of mycotoxin production. Mycotoxins are produced by some of the specific strains of filamentous fungi belonging to species of the genera *Aspergillus, Penicillium* and *Fusarium* that invade crops at the field level and grow on foods during storage under favourable conditions (temperature, moisture, water activity and relative humidity).

There is a database that reported more than 400 mycotoxins and fungal metabolites (Nielsen & Smedsgaard 2003); previously unknown metabolites have been discovered and they have assumed significance due to their deleterious effects on human beings, poultry and livestock. Human exposure results from consumption of plant-derived foods contaminated with toxins, and the carryover of mycotoxins and their metabolites into animal products such as milk, meat and eggs (Bryden 2007). The most important are aflatoxins (AFs) and ochratoxin A (OTA), sometimes detected in food products and animal feed produced in developing countries, whose climatic conditions favour mycotoxin production (Wild 2007).

AFs in particular pose a particular threat due to their widespread occurrence and toxicity (Williams et al. 2004). One of the most spectacular incidences of AFs toxicity (in animals) was first recognised in 1960 in UK when there was severe outbreak of so-called 'Turkey X Disease' in which over 100 000 turkey poults died. The cause of the disease was shown to be due to toxins in peanut meal imported from Brazil, subsequently found to contain AFB_1. The carcinogenicity of AFs was soon identified (Wogan & Newberne 1967) and the subsequent interest for this group of compounds increased exponentially, resulting in the publication of thousands of research papers (Bhat et al. 2010).

AFs are encountered in a wide range of important agricultural commodities, including wheat, rice, maize, sorghum, pearl millet, spices, oilseeds, tree nuts and milk (Bryden 2007). Of the nearly 18 different types of AFs identified to date, the IARC has classified four AFs (AFB_1, AFG_1, AFB_2, AFG_2) as Group 1 carcinogens (IARC 1993a, 1993b).

With respect to secondary exposure, i.e. exposure of consumers to mycotoxins and to their metabolites in primary animal products (meat, milk and eggs), the principal metabolite of AFB_1 is AFM_1, which is secreted in milk

*Corresponding author. Email: vita.distefano@unipa.it

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following consumption of AFB_1 by lactating cows (Rastogi et al. 2004; Britzi et al. 2013).

OTA is the second most important mycotoxin produced by the fungi *Aspergillus ochraceus* and *Penicillium verrucosum*. Wheat, rye, barley and grapes can be contaminated with OTA (Jørgensen 2005). It may also be present in some of the organs of animals that have been fed on contaminated feeds (Duarte et al. 2011). It has been estimated that pork and poultry products contribute to around 5% of the total OTA human exposure (Duarte et al. 2010).

OTA is deemed to be nephrotoxic, immunosuppressive, carcinogenic and teratogenic. The IARC has classified OTA as a compound possibly carcinogenic to humans (Group 2B) (EFSA 2006). OTA is also the causal agent for both endemic nephropathy and urothelial tumours.

Overall, there are a number of approaches that can be taken to minimise mycotoxin contamination in animal feeds and these involve prevention of fungal growth and, therefore, mycotoxin formation, and strategies to reduce or eliminate mycotoxins from contaminated commodities, especially feed additives.

With the increasingly stringent regulations for mycotoxins food content imposed by the importing countries, many cereals that are not safe for human consumption are used in formulations intended for animal feed.

Food irradiation has been recognised as a reliable and safe method for the preservation of food and feed, and for improving the hygienic quality and nutritional value of them (EPA 2013). A treatment at 3 and 10 kGy has been shown to be successful in reducing the microbial load and, thereby, toxin production (Farkas 1989; Tauxe 2001; D'Oca et al. 2009; Waje et al. 2009). However, there are conflicting opinions: some researchers found that gamma-ray treatments were effective in reducing mycotoxin concentration in different foods (Van Dyck et al. 1982; Refai et al. 1996; Herzallah et al. 2008; Jalili et al. 2012), however some were not effective (Frank & Grunewald 1970; Hooshmand & Klopfenstein 1995).

The aim of this study was to investigate the efficacy of gamma-radiation (^{60}Co) for detoxification of OTA and AFB_1, AFB_2, AFG_1 and AFG_2 in artificially contaminated feed samples. Mycotoxin levels were determined by means of immunoaffinity clean-up (IAC) and HPLC with fluorescence detection (HPLC-FLD).

Materials and methods

Chemicals

AFB_1, AFB_2, AFG_1, AFG_2 and OTA standards were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Syringe filters were Millex LCR, PTFE, 0.45 µm (Millipore, Milford, MA, USA); the C_{18} Luna HPLC column and security guard cartridge C_{18} were from Phenomenex (Torrance, CA, USA). The immunoaffinity columns used for OTA and AFs analysis were OCHRAPREP® and Aflatoxin EASI-EXTRACT®, respectively, from R-Biopharm Rhone (Glasgow, UK). All reagents used such us hexane, acetic acid and trifluoroacetic acid were of analytical grade; methanol, water and acetonitrile were of HPLC grade and purchased from Carlo Erba (Milan, Italy). Potassium chloride, potassium dihydrogen phosphate, anhydrous disodium hydrogen phosphate and sodium chloride were purchased from Fluka AG (St. Gallen, Switzerland).

Samples and sampling procedures

A commercial poultry feed sample composed of corn (40–50%), barley (15–25%), soybean meal (15–25%), pea (5–8%), dehydrated alfalfa meal (3%) and corn gluten meal (2–5%) was supplied by the Istituto Zooprofilattico Sperimentale (IZS) 'A. Mirri' of Palermo, Italy; 10 representative subsamples of the whole batch were used for analysis.

The absence of mycotoxins in the samples analysed was certified by IZS and verified by HPLC/FLD analysis before treatment with γ-radiations. A kitchen blender was used for the homogenisation of samples. All samples were kept in the dark under controlled moisture at –15°C.

Spiked sample preparation

A total of 10 feed subsamples were analysed; 10 g of each aliquots were spiked with AFB_1, AFB_2, AFG_1, AFG_2 and OTA at 25 ng g^{-1}, packaged in polyethylene bags and irradiated in the range between 0.5 and 15 kGy.

Gamma-ray treatment

Irradiation of the spiked feed samples (10 g each, packaged in polyethylene bags, 50 µm thickness) with gamma-rays was carried out at the Dipartimento di Energia, Ingegneria dell'Informazione e Modelli Matematici of Palermo in the 1014 Bq ^{60}Co, panoramic irradiator IGS-3, at a dose-rate of about 7.5 Gy min^{-1}; the irradiation chamber temperature during irradiation was 25°C. The bags containing the samples were enclosed in a plastic chamber with a wall thickness of 0.4 g cm^{-2}, which is suitable for establishing electronic equilibrium. The dose-rate of the Gammacell for the reference geometry was determined with the alanine reference transfer standard dosimeters from RISØ high-dose reference laboratory, with an expanded uncertainty of 2.8% at 95% confidence level. The applied doses were 0.5, 1.5, 3, 5, 10 and 15 kGy. Non-irradiated samples (0 kGy) were considered as control samples and kept separated. All samples were stored at –15°C until further HPLC analysis.

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Mycotoxin analysis

A total of 5 g of each feed sample was mixed with 20 ml methanol/water (80:20 v/v) and well shaken for 10 min. After filtration an aliquot of 5 ml was used for AF analysis and another 5 ml was used for OTA analysis. The same procedure was used both for irradiated and control samples.

Aflatoxin determination

AFB_1, AFB_2, AFG_1 and AFG_2 were determined by means of IAC and HPLC-FLD, according to AOAC Official Methods (AOAC 1995). A total of 5 ml from the filtrate was diluted with 40 ml PBS and mixed for 1 min. The diluted extract was passed through the AFLAPREP® IAC at a flow rate of about 2 ml min^{-1}, followed by 2 × 20 ml deionised water at the same flow rate. AFB_1, AFB_2, AFG_1 and AFG_2 were then eluted with 3 ml of acetonitrile and collected in a clean vial. The eluate was then evaporated to dryness under a gentle stream of nitrogen, and before HPLC/FLD analysis the sample must be derivatised in order to increase the natural fluorescence of AFG_1 and AFB_1.

AF derivatives were obtained by adding 400 µl hexane and 100 µl trifluoroacetic acid. Then, 2 ml of a solution of deionised water/acetonitrile (9:1, v/v) were added and vortexed for 30 s to allow the layers to separate. An aqueous layer containing AFs was filtered through a 0.45 µm syringe filter tip and analysed by HPLC/FLD.

Ochratoxin A determination

OTA was determined according to the methodology described by Villa and Markaki (2009). A total of 5 ml of filtrate was diluted with 40 ml PBS and mixed for 1 min. The diluted extract was passed through the OCHRAPREP® IAC at a flow rate of about 2 ml min^{-1}, followed by 2 × 20 ml deionised water at the same flow rate. OTA was then eluted with 3 ml of a solution of methanol/acetic acid (98:2, v/v) and collected in a clean vial. The eluate was then evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved immediately in 1 ml water/acetonitrile/acetic acid (60:40:2), filtered through a 0.45 µm syringe filter tip and analysed by HPLC/FLD.

HPLC analysis

Reversed-phase HPLC with fluorescence detection was used to determine AFB_1, AFB_2, AFG_1, AFG_2 and OTA. The instrumentation was an Agilent 1100 Series liquid chromatograph including a binary pump (Model G1312A; Agilent Technologies; Hewlett-Packard, Waldbronn, Germany), a fluorescence detector (Model G1312A; Agilent), a Rheodyne 7125 injection valve fitted with a 20 µl loop and a column temperature controller (Thermosphere TS-130; Phenomenex). Separation was achieved using a C_{18} 5 µm particle size column (Luna C_{18} (2) 100A, 150 × 4.6 mm), equipped with a security guard cartridge C_{18} (4 × 3.0 mm), which was maintained at 40°C. The mobile phase consisted of water/acetonitrile (70:30) for AFs and water/acetonitrile/acetic acid (60:40:2) for OTA. The mobile phase for AFs was filtered through Millipore filters (0.45 µm) before use, as well as the mobile phase for OTA. The detection of AFB_1, AFB_2, AFG_1 and AFG_2 was carried out at λ_{ex} 360 nm and λ_{em} 440 nm. The detection of OTA was carried out at λ_{ex} 335 nm and λ_{em} 465 nm. The flow rate was 1 ml min^{-1} and the retention times were 3.1 min for AFG_1, 3.6 min for AFB_1, 6.1 min for AFG_2, 7,7 min for AFB_2 and 18.2 min for OTA.

Preparation of standard solutions

Standard stock solutions of each mycotoxins were prepared in methanol at a concentration of 1000 ng ml^{-1}. These solutions were stored in dark glass bottles at –4°C. The working mixed standard solution was prepared daily from standard stock solutions using the mobile phase for dilution.

Preparation of phosphate-buffered saline (PBS)

PBS was prepared by dissolving 0.2 g potassium chloride, 0.2 g potassium dihydrogen phosphate, 2.92 anhydrous disodium hydrogen phosphate and 8.0 g of sodium chloride in 900 ml of distilled water. After adjusting the pH to 7.4 using 0.1 M HCl/0.1 M NaOH if necessary, the solution was made up to 1000 ml.

Calibration curve

To assess the matrix effect, six concentrations of matrix-matched calibrations (2, 4, 8, 15, 20 and 25 ng g^{-1}) were prepared by spiking feed sample with appropriate amounts of AFB_1, AFB_2, AFG_1, AFG_2 and OTA working solutions, and following the clean-up and derivatisation procedure.

The results show that the standard calibration graphs were linear over the same range as mentioned above; the linearity of the standard curves at three determinations of five concentration levels was reliable, between 0.996 for AFG_1 and 0.999 for OTA.

Calibration curves were obtained using the linear least squares regression procedure of the peak area versus the concentration. With regard to accuracy of the method applied, feed samples were spiked with three different levels of each mycotoxin at concentrations of 5, 10 and

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20 ng g^{-1} and then recovery and standard deviation (SD) were calculated (n = 4).

LOD values were in the range 0.012–0.022 ng g^{-1}; and LOQ values were in the range 0.040–0.074 ng g^{-1}.

Statistical analysis

Analysis of variance (ANOVA) was used to investigate the significant effects of gamma doses on the reduction of mycotoxins in animal feed samples. Comparison of the means was conducted using ANOVA with post-hoc Turkey's test at $p < 0.05$.

Results and discussion

The average recovery values of AFs ranged from 76.2% for AFG_2 to 89.4% for AFB_1, and between 92.1% and 94.0% for OTA (Table 1). The obtained recoveries for all mycotoxins were in line with the legislated levels described by the European Commission in 2006. The data obtained after irradiation at doses of 0.5, 1.5, 3, 5 and 10 kGy of 10 subsample of feed, artificially contaminated with a quantity of 25 ng g^{-1} of each mycotoxin, are reported in Table 2. The results show the weak relationship between radiation dose and the reduction of mycotoxins. Samples irradiated at 0.5, 1.5 and 3 kGy showed no significantly reduction. The effect of irradiation on mycotoxin reduction was significant by increasing the gamma-ray dose from 10 to 15 kGy. In the results, the greatest per cent reduction was obtained by irradiation at 15 kGy, which ranged from 11.0% for AFB_2 to 23.9% for OTA.

The minimal reduction of the content of AFs and OTA is probably due to the absence of water in the samples irradiated. The water content has an important role in the destruction of mycotoxins by gamma-rays.

Upon irradiation of foods, the primary reaction is the ionisation of water, which causes the water molecule to split into positively charged water radical and a negative free-solvated electron. The water radical then decomposes into a hydroxyl radical and a hydrogen ion. The reaction progresses until the end products of hydrated electrons, hydroxyl radicals, and hydrogen ion and hydrogen atoms are formed (Jalili et al. 2012). The addition of free radicals to double bonds, especially to those in aromatic or heterocyclic rings, is an energetically positive reaction that is expected to occur in AFB_1 and AFG_1. The solvated electron may also add to the aromatic and heterocyclic rings, or to the carbonyl group of the lactone ring in the structures of AFs and OTA. In fact, AFB_2 and AFG_2 in all the treatments showed lower reduction comparing with other mycotoxins which indicated that these two mycotoxins are more radio-resistant than the analogous AFB_1 and AFG_1 (Di Stefano et al. 2014).

There are conflicting literature data regarding the effect of gamma-ray on mycotoxin content in different foods. Refai et al. (1996) reported that radiation doses of 15 and 20 kGy are sufficient for the complete destruction of OTA from feed for yellow corn and soya bean samples, respectively. Aziz and Youssef (2002) showed that a dose of 20 kGy was sufficient for complete destruction of AFB_1 in peanut, yellow corn, wheat and cotton seed meal. In a report, corn grain samples contaminated with AFB_1 and AFB_2

Table 1. Recovery (%) results from the analysis of blank sample of animal feed spiked with OTA, AFB_1, AFB_2, AFG_1 and AFG_2 at three different levels.

	OTA		AFB_1		AFB_2		AFG_1		AFG_2	
Spike level (ng g^{-1})	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
5	92.1	3.1	89.4	7.3	80.1	6.2	82.9	4.9	76.2	3.9
10	93.4	3.7	78.3	5.9	81.5	7.9	79.9	6.1	77.4	5.8
20	94.0	2.9	80.1	2.7	82.0	5.9	82.7	3.1	78.4	3.9

Table 2. Reduction (%) (mean ± SD) of aflatoxins and ochratoxin A (ng g^{-1}) in feed samples spiked at 25 ng g^{-1} of each mycotoxin after irradiation treatment.

Radiation dose	Non-irradiated (0 kGy)	0.5 kGy	1.5 kGy	3 kGy	5 kGy	10 kGy	15 kGy
OTA	–	0.31 ± 0.09	2.65 ± 0.14	5.68 ± 0.71	10.68 ± 0.71	16.34 ± 0.22	23.90 ± 0.80
AFB_1	–	0.13 ± 0.09	1.10 ± 0.33	3.25 ± 0.04	7.25 ± 0.04	11.87 ± 0.66	18.25 ± 0.17
AFB_2	–	0.10 ± 0.07	0.88 ± 0.37	1.59 ± 0.11	2.69 ± 0.11	6.98 ± 0.34	10.99 ± 0.25
AFG_1	–	0.24 ± 0.43	2.12 ± 0.27	4.15 ± 0.69	8.15 ± 1.29	18.95 ± 1.13	21.10 ± 1.26
AFG_2	–	0.11 ± 0.21	1.22 ± 0.66	2.36 ± 0.31	4.36 ± 0.31	9.04 ± 0.94	13.62 ± 0.68

were totally decontaminated after exposure to 10 kGy of gamma-radiation (Aquino et al. 2005). Another study reported that in feed samples, reduction of AFs achieved were 40.1% and 42.7% after a dose of irradiation of 25 kGy (Herzallah et al. 2008).

In contrast with these studies, Frank and Grunewald (1970) found that the dosage required to eliminate AFB_1 totally from food and feed would be so high that it would cause a significant deterioration of quality and thus change the organoleptic properties of the irradiated product. Hooshmand and Klopfenstein (1995) have shown that gamma doses even up to 20 kGy did not significantly affect AFB_1 in wheat, corn or soybeans. Irradiation studies on almond samples have shown that 15 kGy dose was not sufficient to destroy AFs and OTA completely (Di Stefano et al. 2014). A related study reported that peanut meal exposed to gamma-rays at a dosage of 25 kGy showed no apparent difference from non-irradiated control meal when examined by a fluorescence test (Feuell 1966). These different results may be related to differences in sample matrices. Ghanem et al. (2008) shown that at a dose of 10 kGy, the per cent AFB_1 degradation reached the highest values at 58.6%, 68.8%, 84.6%, 81.1% and 87.8% for peanuts, peeled pistachios, unpeeled pistachios, corn and rice samples, respectively.

Conclusions

The effects of gamma-irradiation ranging from 0.5 and 15 kGy on the per cent reduction of AFB_1, AFB_2, AFG_1, AFG_2 and OTA in feed samples were evaluated. The results showed that reduction of mycotoxins at a dose of 15 kGy was less than 25%. These results could be related to the amount of AFs and OTA present in the samples before irradiation (Ghanem et al. 2008) and to the presence of water (Jalili et al. 2012). Therefore we found that gamma-ray treatment is not an effective method for complete degradation of OTA and AFs in animal feedingstuff. Unfortunately, it is not possible to increase the dose of ionising radiation since the FAO/IAEA/WHO Expert Committee on Food Irradiation already concluded in its report of 1981 that 'the irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological hazard, hence, toxicological testing of food so treated no longer required'. It is therefore concluded that the decontamination of mycotoxins by irradiation is necessary prior to their production from moulds (Di Stefano et al. 2014).

Acknowledgements

The authors thank MIUR and Dr. A. Parlato of the Dipartimento di Energia, Ingegneriadell'Informazione e ModelliMatematici of the University of Palermo for taking care of γ-ray irradiation in the IGS-3 irradiator.

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