MYCOPHENOLIC ACID AS A FOOD AND FEED CONTAMINANT

M. Sc THESIS

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ABSTRACT

Mycophenolic acid (MPA) is a toxic metabolite produced by many Penicillium species among which are *P. brevicompactum* and *P. roqueforti*. *Byssochlamys nivea*, a yeast species, has also been reported to produce MPA.

MPA producing fungi are frequently isolated from maize and grass silage (animal feed) for example *P.roqueforti. Byssochlamys* species are also responsible for spoilage and degradation of fruits and silages.

P. brevicompactum has been isolated from a wide range of human foods. *P. roqueforti* is used in the production of blue-veined cheeses, thus MPA has been detected in most of this type of cheese.

Animals are exposed to MPA through the ingestion of contaminated feeds. MPA or majority of MPA producing fungi have been found in animal feed thus could be carried over to human foods of animal origin. MPA has not been detected in milk so far, but up to 0.23mg/kg has been found in sheep muscle tissue. The possibility for MPA occurrence in fruit is high but this has not been extensively studied yet. The major route of for human exposure is therefore dietary.

From the available data, exposure of humans and animals to MPA seems to be considerably high (>0.1mg/day for humans, 900mg/day for cattle and1.8mg/kg body weight for livestock in general). This could have health consequences especially considering that humans and animals are chronically exposed to this compound. The calculated Acceptable Daily Intake (ADI) for humans is 0.195mg and that for cattle is 1.8mg.

Due to the genotoxic nature of MPA (although results of genotoxicity assays are conflicting), the Margin of Exposure (MOE) of MPA for humans has been calculated which is 1000; thus it is a substance with a high risk for which urgent risk management measures are required.

The few clinical studies on animal subjects have also been strictly limited to the acute, rather than chronic, toxin exposure. Chronic exposure studies however will be more relevant to real life situation.

Human exposure to MPA has not been extensively studied, thus there are only a few data on its concentration in human food products. The consumption of blue-veined cheese is a major route of human exposure. However, the cumulative exposure from different food sources which should be of health concern has not been studied.

MPA has a number of therapeutic uses, the most important of which is, its use as an immunosuppressant drug in renal transplant, lupus nephritis, non-infectious ocular inflammation and in the management of difficult inflammatory bowel diseases.

1. INTRODUCTION

1.1 Introduction to Mycotoxins

Mycotoxins are secondary metabolites of microfungi/ filamentous fungi which are produced in the substrates on which they grow. Such substrates often include plants grown and stored for human or animal consumption as well as processed food. All mycotoxins are low-molecular-weight natural products (small molecules) and are capable of causing disease and death in humans and animals. Dietary, respiratory, dermal, and other exposures to mycotoxin produce the diseases collectively called mycotoxicoses. Some mycotoxins or their derivatives which have pharmacological activities are used as antibiotics, growth promotants, and other kinds of drugs and some others have been used as chemical warfare agents (1).

Mycotoxin-producing mould species are very common. Mycotoxins can enter the food chain in the field, during storage, or at later points. Mycotoxin problems are aggravated if shipping, handling, and storage practices are conducive to mould growth. Kuiper-Goodman (2) has graded mycotoxins as the most important chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives, or pesticide residues.

Mycotoxins can also be found in indoor air. It has been shown that spores in air-borne dust can cause ochratoxin exposure (3). Sterigmatocystin has been isolated from water-damaged wallpaper (4) and from damp carpeting (5). Trichothecenes have been found in aerosolized conidia (6) and others such as T-2, diacetoxyscirpenol, roridine A, and T-2 tetraol have been detected in the dust from office ventilation systems (7).

Although between 300 and 400 different mycotoxins have been identified, only a few are present in high concentrations or do have a significant health or economical impact (8, 9). The most common mycotoxins associated with human and veterinary diseases are aflatoxin, fumonisins, ochratoxin A, patulin, trichothecenes, and zearalenone. Aflatoxins are largely associated with commodities produced in the tropics and subtropics, such as cotton, peanuts, spices, pistachios and maize (10, 11). Ochratoxins are found in beverages such as beer and wine and patulin is found in mouldy fruits and vegetables, in particular rotting apples and figs (12, 13). Fusarium toxins (fumonisins, trichothecenes, zearalenone etc) are found in the grain of developing cereals such as wheat and maize (14, 15).

The susceptibility of animals (and humans) varies with species, age, nutrition, length of exposure and other factors as well (16, 17). Assessment of adverse health effects of mycotoxins is complicated by their co-existence in food and feed and their possible synergistic action (18). Mycotoxins may be carcinogenic (e.g. aflatoxin B_1, ochratoxin A, fumonisin B_1), oestrogenic (zearalenone and α and β zearalenols), neurotoxic (fumonisin B_1, ergot alkaloids), nephrotoxic (ochratoxins, citrinin, oosporeine), dermonecrotic (trichothecenes), immunosuppressive (aflatoxin B_1, ochratoxin A, T-2 toxin, patulin). Many mycotoxins show a non-specific action at the usual exposure levels, for example immunosuppression (17) but consequently increase susceptibility to other illnesses (19).

1.2 Introduction to Mycophenolic acid (MPA)

Mycophenolic acid (MPA) is a toxic metabolite produced by many Penicillium species such as *P. stoloniferum* (20), *P. viridicatum* (21), *P. brevicompactum* (22), *P. carneum* (23), *P. raciborskii*(24) and some strains of *P. roqueforti* (25). MPA and its precursors, 5-methylorsellinic acid and 5, 7-dihydroxy-4-methylphthalide, have also been identified as secondary metabolites of *Byssochlamys nivea* (a yeast species) (26). MPA (6-[4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl]-4-methyl-4-hexenoic acid, (Figure 1) is a polyketide compound and most likely the first antibiotic that was extracted from fungal culture and well described in scientific literature (27).

The mycotoxin, MPA is worth considering due to the fact that its main producer, *P. roqueforti*, is found in foods and feeds.

P. roqueforti is commonly present in silage, hence rations contain MPA. *Byssochlamys nivea* is often isolated from 3-4 months old silage as well (28). However, further work still need to be carried out to investigate its MPA production in naturally contaminated silage (26). Other mycotoxins such as patulin (29), roquefortine C (30, 31) are also known to contaminate maize and silage apart from MPA**.**

P. roqueforti is also the most important fungal strain used to produce blue-veined cheese as MPA production has been detected in Roquefort cheese and/or roqueforti isolated blue-veined cheeses (32, 33).

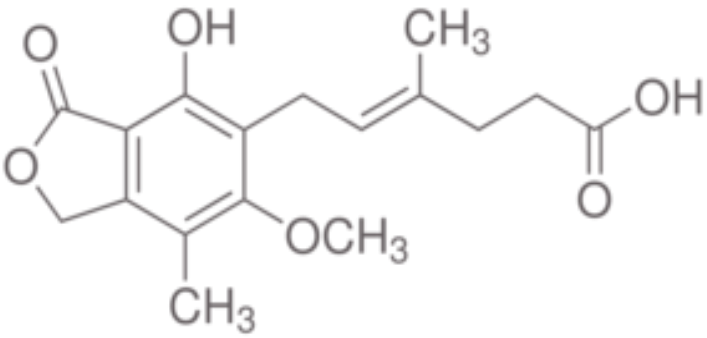

Figure 1: Mycophenolic acid

Systematic (IUPAC) name: (4*E*)-6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-1,3- dihydro-2-benzofuran-5-yl)-4-methylhex-4-enoic acid
Molecular weight: 320.33g/mol; Molecular formula: $C_{17}H_{20}O_6$

2. ANALYTICAL METHODS

One of the most important steps for the qualitative and quantitative determination of individual mycotoxins is the sample preparation and pre-concentration. Several different extraction procedures include solvent extraction (SE); solid-phase extraction (SPE); solid phase microextraction (SPME) and immunofiltration (34). Sampling is however difficult because of inhomogeneous distribution of individual mycotoxins in their substrates.

Due to differences in the types of feed and food as well as chemical diversity of mycotoxins and their simultaneous occurrence in sample, there is a need for rapid multi-analyte methods. Moreover, it is imperative that these methods are sensitive enough to detect mycotoxins below the legally imposed limits.

There are various screening methods such as immunoassay-based methods sensor and biosensor methods. The marker of the mycotoxin may be radioactive in radioimmunoassay (RIA) – rarely used now, or a chromogenic or fluorogenic compound reacting with enzyme in enzyme immunoassay (EIA, ELISA) or in fluorescence immunoassay (FIA), respectively. Another direct screening method involves the use of thin layer chromatography (TLC) (34).

The majority of chemical analytical methods used for accurate, selective and sensitive mycotoxin determination in various samples fall under the category of separation methods: chromatography, electrophoresis. High performance liquid chromatography (HPLC) with different detectors is one of them and it is frequently used both for routine analyses and as confirmatory method for novel or screening techniques (35, 36).

The mass spectrometer has become the detector of choice and preferably the tandem mass spectrometer (37**,** 18**,** 38). Fluorimetric detector for HPLC however is still very popular due to its sensitivity, selectivity, low price and ease of use. Other detectors for HPLC are also used, most especially UV-spectrometric.

Liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) has in the last ten years become the reference and main method in mycotoxin analysis. This is due to the development of efficient electrospray (ESI) and atmospheric pressure chemical ionization (APCI) interfaces for LC-MS coupling, the advancement in the field of mass analyzers. Other reasons are the ease of use and the reasonable costs of tandem mass spectrometers. Most newly developed LC-MS methods enable the analysis of concomitantly-occurring mycotoxins.

The strength of LC-MS methods lies in the possibility to perform multi-analyte analyses. An example is the heterogenous group of mycotoxins produced mainly by *Penicillium* species, among which are cyclopiazonic acid (CPA), mycophenolic acid (MPA) and roquefortin C. Although several methods exist for the determination of MPA (39), a full-scan and SRM LC-MS/MS method for the analysis of six *Penicillium* mycotoxins with low detection limits has been developed as well (39).

The LC-MS method has been employed already in a number of publications available on the detection of MPA in silage and meat products (40, 41) while enzyme immunoassay (EIA) was used to analyze the presence of MPA in milk and blue-veined cheese (42).

3. OCCURRENCE

3.1 Occurrence in cereals, grains and silage

Mycotoxins can appear in the food chain as a result of fungal infection of crops which are either eaten directly by humans or used as livestock feed. Mycotoxins greatly resist decomposition, being broken down in digestion or destroyed by temperature treatments, so they remain in the food chain, in meat and dairy products.

Occurrence of MPA in silage can affect both animal health and animal product safety which is of importance to human consumers. Maize and grass silage are frequently contaminated by fungal toxins, such as patulin (34), roquefortine C (30, 31), and mycophenolic acid (MPA) (43).

Silage is frequently contaminated with fungi of the genera *Monascus*, *Aspergillus*, and *Penicillium* (44). One of the most common moulds isolated from silage, *P. roqueforti*, can produce MPA. In a study by Schneweis et al (2000) MPA was found in 74 (32%) of 233 silage samples examined ranging from 0.02-35mg/kg with a mean of 1.4mg/kg and only 42% of the MPA-positive samples were contaminated by *P. roqueforti* (40), suggesting that one or more other fungal species could also synthesize this mycotoxin. *Monascus purpureus*, *Trichoderma viride*, *Geotrichum candidum*, *Paecilomyces variotii*, and *Byssochlamys nivea* are fungal species commonly recovered from ensiled maize (46, 47). *Byssochlamys* species are responsible for spoilage and degradation of fruits and silages and have been known to produce the mycotoxins patulin and byssochlamic acid (46). Puel et al (2005) however reported the production MPA and its precursors, 5-methylorsellinic acid and 5, 7-dihydroxy-4-methylphthalide in all of the *B. nivea* strains that were examined (26).

Mansfield et al (2007) examined the contamination of fresh and ensiled maize by multiple Penicillium mycotoxins namely patulin (PAT), cyclopionic acid (CPA), roquefortine C (ROC) and MPA. Silage was collected both at harvest and after ensiling. The frequency of contamination in the same silage samples was: ROC 60%, MPA 42%, CPA 37%, and PAT 23%. Of 120 samples tested, 15% contained no detectable levels of toxin, 25% contained only one toxin, 32% two, 18%, three, and 10% all the four. All the four were found in freshly harvested material, contrary to the belief that Penicillium toxins formation occurs only during storage (48).

P. brevicompactum was one of the four predominant Penicillium species found in samples of deteriorating barley, oat and wheat (49).

Grain dusts from farms and storage companies are generally used in animal feeding. These mycotoxin rich dusts can also accidentally contaminate stored grains. Fourteen grain dusts collected from farms and storage companies in Belgium were assayed and toxins co-occurred at uneven distributions with wide ranges of concentrations. Median concentrations exceeded 1 mg/kg for eight of fifteen mycotoxin screened MPA inclusive (50).

3.2 Occurrence of MPA in fermented foods

Fermented foods are traditionally produced in Europe and all over the world. Fermentation is a chemical process in which microorganisms including bacteria, yeast and mould (a class of fungi), convert carbohydrate like sugar into an acid or alcohol. Fermentation occurs naturally in different

foods including a variety of grains, fruits, juices, and other organic liquids. It is used to preserve food without losing nutrients (unlike other processing techniques).

Moulds are an important group of organisms that are responsible both as spoilers and preservers of foods. Moulds are frequently found in foods and can tolerate high concentrations of salt and sugar. Some of the fungi add flavors and colour to foods fo example *P. roqueforti* used in the production of blue-veined cheeses and others produce enzymes, such as amylase for bread making.

Dairy foods supply a wide range and a large amount of nutrients for the growth of both spoilage and pathogenic microorganism. Such growth can however be inhibited by organic acids produced by acidifying microorganism present in such foods as well as the water activity (aw) conditions.

Occurrence of fungal genera such as Aspergillus, Penicillum, Rhizopus, Fusarium and Mucor is a serious and frequent problem in the dairy industry because they grow satisfactorily at the yoghurt/air interface (51).

P. brevicompactum, an MPA producer has been isolated by Ndagijimana et al as a dominant species in contaminated industrial yoghurt. Their latter study confirmed the production of MPA by *P. brevicompactum* grown in yoghurt during storage at refrigeration temperature of 8^{o}C (52). Sources of microbial contamination during yoghurt production are contaminated starters, poorly cleaned filters, contaminated cups and lids, overall hygiene in the manufacturing process, contaminated flavouring materials, and air quality in packaging areas (53).

From the MPA analysis carried out by Usleber et al in raw bulk milk and pasteurized milk, no MPA was found (42).

Blue-veined cheese from the German market (n = 53) was also analyzed BY Usleber et al. 51 out of 53 analyzed contained MPA, although mostly (66%) at levels of <0.01mg/kg. MPA at 0.4-1.2mg/kg was found in Roquefort cheeses. Highest levels (4-11mg/kg) were found in a German soft cheese preparation. MPA levels in mycelium-rich parts of cheese were 3 times higher than in mycelium-free parts (42).

Engel et al in an experimental manufacture of blue cheese with *P. roqueforti* found a maximum MPA level 4 mg /kg in these cheeses. This was similar to a maximum level of 5mg/kg earlier detected in commercial cheese (29). In some other blue-veined cheeses however, MPA concentrations up to 15mg/kg have been detected (54).

In most cases however starter cultures are used in the production of these cheeses but traditional fermentationists might include potentially toxigenic strains.

Determination of mycotoxins in meat products is significantly more difficult than in cereal-based products because of the need to remove small proteins, interfering peptides, phospholipids, and other interferences from meat (45). Multi-mycotoxin detection methods have also not been developed for meat since selective purification methods inevitably remove some mycotoxin **(**41).

Mycotoxin in meat products may originate from two sources: carry-over from feed or spoilage, usually on dried meat products. The most common fungi contaminants of dried meat products are *Penicillum species, P. brevicompactum, P.chrysogenum, P.solitum, P. palitans,* P. nalgiovense *and P.*

nordicum being the most common (55,43,54-58). MPA was shown to be produced on meat inoculated with *P. brevicompactum* (45) but retail meat products have not yet been analysed for MPA. Levels found in these meat products (dry-cured ham and liver pâté) ranged from 0.19mg/kg in centre to 11mg/kg in surface of ham and from 0.15mg/kg in bottom to 14mg/kg in surface of pâté (45).

3.3 Occurrence in fruits

Byssochlamys species are responsible for spoilage and degradation of fruits (46). As already noted in the previous section *Byssochlamys nivea* can synthesize MPA, there is a high possibility of its occurrence in decaying or infested fruits.

Spoilage of pasteurized and canned fruit and fruit products caused by heat-resistant moulds has been reported repeatedly in recent years. Species most commonly implicated in fruit and fruit product disintegration *are Byssochlamys fulva, Byssochlamys nivea, Neosartorya fischeri, Talaromyces flavus, and Eupenicillium brefeldianum*. These organisms usually contaminate fruits on or near the ground. They can survive heat treatments used for fruit processing and can grow and spoil the products during storage at room temperature. Besides spoilage, the heat-resistant moulds produce a number of toxic secondary metabolites, such as byssotoxin A, byssochlamic acid, patulin, fumitremorgin A and C, verruculogen, fischerin (59)and MPA (26).

Twenty ginger (Zingiber officinale) rhizomes displaying visible mould growth (due to spoilage) were examined to identify the fungi and to evaluate the presence of fungal secondary metabolites. *P. brevicompactum* was the predominant species isolated from 85% of the samples. Mycophenolic acid was identified from corresponding plant tissue extracts. This is the first reported occurrence of mycophenolic acid in commercially sold ginger plant food products (60).

There is limited information on the occurrence of MPA in decaying fruits. Its presence however is much likely since *Byssochlamys nivea* is one of the fungi species responsible for fruit spoilage.

4. EXPOSURE

4.1 Animal exposure

As it has been established earlier, several mycotoxins produced by *Penicillium* species including cyclopiazonic acid (CPA), patulin (PAT), mycophenolic acid (MPA), and roquefortine C (ROC) are known to occur in maize based feeds including silage. Most of these toxins together with gliotoxin have also been investigated as possible contaminants of fodder for ruminants (61). The presence of MPA in maize and silage and fodder is an indication of animal exposure to this mycotoxin.

In a study conducted with silage, MPA was reported in 38 of 135 samples of maize silage and 36 out of 98 samples of grass silage with a mean concentration of 1.4mg/kg in the total number of samples examined (concentration ranged from 0.02-35mg/kg) (Table 9.1). The authors further stated that animal exposure of 1.8mg/kg body weight might result from eating about 25kg silage per day. This is equivalent to 10% of the dose given to patients undergoing immunosuppressive therapy (40).

In a recent study with sheep, Mohr et al. (2007) found that animals fed varying concentrations of MPA (10-300mg/kg/animal/day) for 44 days suffered no significant effects from exposure. This conclusion was drawn from the haematological and biochemical parameters of these animals that were measured. An oral application of up to 300 mg MPA/animal daily, which is equivalent to 5.4 mg/kg body weight, did not affect the sheep's general state of health and weight gain significantly There were also no indications for a ruminal reduction of MPA (62).

In a survey conducted in the Netherlands on the occurrence and the total dietary intakes of 20 different mycotoxins in feedstuff of dairy cows, MPA was found to be one of the mycotoxins with the highest incidence. Roquefortine C and MPA were only found in silage and ensiled by-product samples with the incidence between 7 and 19%. The average concentration of MPA in complete diets was 0.05mg/kg while the maximum concentration was 1.84mg/kg. Calculated average intake of MPA was 0.9mg/animal/day while the maximum daily intake was 32.3mg/animal (63) (Table 9.2).

Animals are also exposed to MPA from grain dusts used in animal feeding at a median concentration of >1mg/kg grain dust (50).

4.2 Human exposure

Human exposure to mycotoxins in general can occur by several ways, including ingestion, contact, and inhalation. Due to the fact that MPA or majority of MPA producing fungi have been found in animal feed, rotten fruits and some human food products such as blue-veined cheese, the major route of exposure for humans is ingestion of these foods or animal derived food products.

P. brevicompactum commonly encountered in indoor air, and an MPA producer has been isolated from a wide range of food such as cheese, ham, Italian fermented sausage, dried foods, bakery products, and cereal grains. It has also been detected in tap water (64).

P. roqueforti is commonly found in silage hence the production of MPA in animal feeds. MPA as high as 35mg/kg have been reported in silage samples (40). There is limited knowledge about the carry-over effects of MPA from silage to cow milk and tissue of food producing animals. It has however

been observed that after up to 300mg MPA/day was fed to sheep, up to 0.23mg/kg was found in muscle tissue (65). MPA and its glucuronide were also detected in the blood serum of these animals (62). Usleber et al found no detectable amounts of MPA in raw and pasteurized milk sampled (42). *P. roqueforti* however has been shown as one of the frequently isolated fungi from raw cow's milk having a fungi count of approximately 1000 colony forming units (66). This could be an implication of theoretical risk of post-secretory MPA production in milk. There may not be a major problem with MPA in drinking milk but with raw cheese production from raw milk (42).

Engel et al discovered MPA (up to 5mg/kg) in commercial blue-veined cheeses (33). MPA at 0.4-1.2mg/kg was found in Roquefort cheeses in German market and highest levels (4-11mg/kg) were found in a German soft cheese preparation by Usleber et al. This gives an estimate of 0.1mg daily intake assuming a daily consumption of 100g of blue-veined cheese per day. Lafont et al however discovered MPA concentrations up to 15mg/kg in some blue-veined cheeses (32).

Humans are exposed to MPA from stored grains which are contaminated with mycotoxin-rich grain dusts. These grain dusts have been found to contain a median of >1mg/kg MPA. They also give rise to airborne dust to workers in farms and storage companies where these grain dusts are produced. This represents an additional route of exposure which has not been so far completely investigated (50).

Apart from exposure to MPA through the sources already mentioned, humans are also exposed to MPA through the consumption of deteriorated, contaminated or spoiled fruit or vegetable whether these are canned or uncanned. The possibility also exists of carry-over of MPA consumed by other organisms to human where these serve as food thus increasing human exposure. There is however limited knowledge about these kinds of exposure.

5. MECHANISM OF ACTION OF MPA

Mycophenolate mofetil (MMF) is an immunosuppressive drug which is rapidly converted in the body to mycophenolic acid (MPA), hence the most extensive and actual literature regarding the mode of action originates from the medicinal use. The mechanism of action of MPA is based on interference with purine synthesis. MPA is a potent, reversible, noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) which is an enzyme that facilitates the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP), a precursor of guanine nucleotides. This blocks the de novo synthesis of guanosine nucleotides which are necessary substrates for DNA and RNA synthesis as demonstrated in Figure 2 below. Unlike other cell types which can use the salvage pathways, B and T lymphocytes are dependent upon the *de novo* pathway for the generation of guanosine (67, 68).The consequences of the reduction in guanine nucleotides in lymphocytes include the inhibition of DNA synthesis, and GTP-dependent metabolic events (69). These cytostatic effects lead to the inhibition of the proliferative responses of T- and B-lymphocytes to both mitogenic and allospecific stimulations as well as antibody formation by B- lymphocytes (70). This is the principal mechanism by which MPA exerts immunosuppressive effects.

In conclusion, three mechanisms may contribute to the efficacy of MPA in preventing allograft rejection and other applications.

First, MPA can induce apoptosis of activated T-lymphocytes, which may eliminate clones of cells responding to antigenic stimulation following organ transplantation (71).

Second, MPA also inhibits the glycosylation of lymphocytes (by inhibiting the synthesis of frucose- and mannose-containing saccharide components of membrane glycoproteins) thereby inhibiting the expression of adhesion molecules (such as selectins and integrins) and consequently the recruitment of lymphocytes and monocytes into sites of inflammation and graft rejection (71, 72). Although MPA may also inhibit the recruitment of leukocytes into sites of inflammation and graft rejection, it has no effect on the production or release of the cytokines (IL-1 and IL-2) associated with early T-cell signal transduction but rather blocks the coupling of these events to DNA synthesis and proliferation (70, 73). Hence, it is not effective in the treatment of ongoing acute rejection (73).

Third, by depleting guanosine nucleotides MPA also depletes tetrahydrobiopterin and decreases the production of nitric oxide (NO) by inducible NO synthase (iNOS) without affecting the activity of constitutive NO synthases. Through this pathway, MPA consequently suppresses tissue damage that is normally mediated by the production of peroxynitrite (activated macrophages produce NO and superoxide, which combine to generate tissue-damaging peroxynitrite) (71, 74).

By the last two mechanisms: inhibition of the recruitment of lymphocytes and monocytes into sites of inflammation and suppression of tissue damage, MPA exerts anti-inflammatory activity (74).

MPA does not only inhibit the proliferation of lymphocytes but also of fibroblasts, endothelial cells, and arterial smooth muscle cells (73). Clinically attainable concentrations of MPA suppress the proliferation of human arterial smooth muscle cells. These two properties of MPA may decrease the risk of lymphoma development and proliferative arteriopathy in long-term treatment (72).

In addition to preventing allograft rejection, MPA suppresses graft-versus-host reactions in lethal and nonlethal murine models (72). The efficacy of regimes including MMF in preventing allograft rejection, and in the treatment of rejection, is now firmly established. MMF is also efficacious in several experimental animal models of chronic rejection (71).

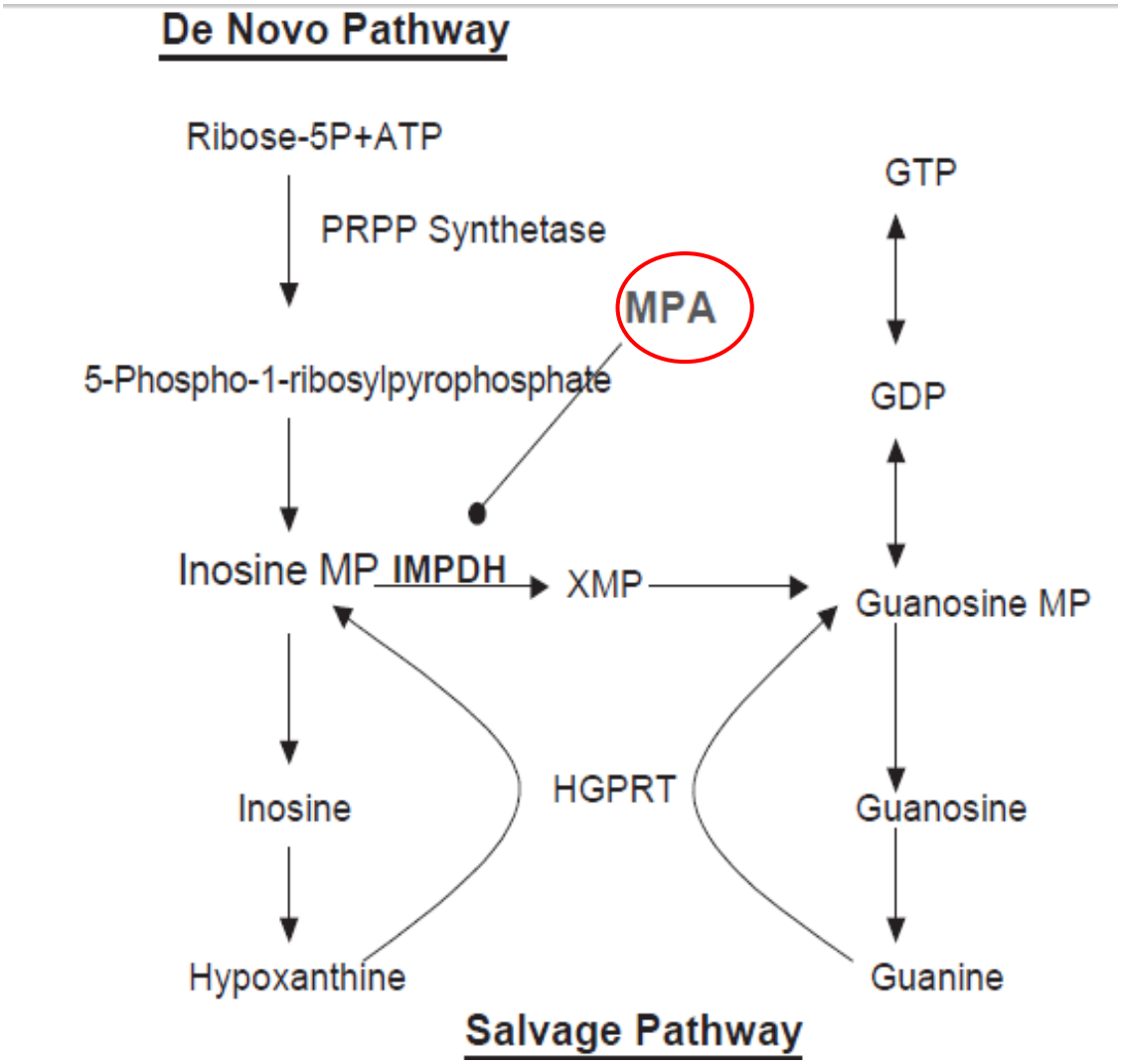

Figure 2: Schematic representation of the de novo and salvage pathways of purine biosynthesis Inosine monophosphate dehydrogenase (IMPDH) and the substrate inosine monophosphate take a central position in purine metabolism. This scheme focuses on the synthesis of guanine nucleotides from metabolites of the de novo and the salvage pathway (HGPRT=hypoxanthine guanosine phosphoribosyl transferase; XMP=xanthosine monophosphate; PRPP=phosphoribosyl pyrophosphate (75).

6. TOXICOLOGY OF MMF

6.1 Oral Toxicity, Reproductive Toxicity and Teratogenicity

Little information is available in the literature on the toxicology of MMF in animals.

The acute oral toxicity of MMF is low: 50% lethal doses (LD50) of MMF are 352mg/kg in rat, 1000mg/kg in mouse and> 6000mg/kg in rabbit (70). Acute oral exposure of monkeys at doses up to 1000 mg/kg bw also resulted in no deaths (76). Acute oral toxic doses however are not equivalent to chronic oral doses taken by transplant patients.

A study in female rats showed that 4.5 mg/kg bw/ day resulted in malformations of the head and eyes of the fetus. In addition, studies on rats and rabbits at a dose of 6 and 90 mg/kg bw/day respectively found fetal resorptions and malformations (76).

Rhesus monkeys developed hypoplastic anemia and severe intestinal disorders after being fed with daily doses of 150 mg/kg bw (77).

Large differences in the response of individual animals to MPA seem to be caused by the difference in the rate of conversion into the MPA-ß-glucuronide , which is easily excreted in the urine (78).

6.2. Mutagenic and Genotoxic Effects of MMF

Studies have been done to investigate the genotoxic potential of mycophenolic acid. Results from some of the assays done demonstrated the mutagenic activity of MPA while many others showed no genotoxicity with MMF.

In a standard battery of *in vitro* and *in vivo* mutagenicity tests performed, MMF was not genotoxic, with or without metabolic activation, in several assays. These assays included the bacterial mutation assay (Ames test), the yeast mitotic gene conversion assay, the mouse micronucleus aberration assay, or the Chinese hamster ovary cell (CHO) chromosomal aberration assay. In a single-plate Ames assay, MMF did not induce point mutations or primary DNA damage in the yeast mitotic gene conversion assay (with or without metabolic activation). In two assays for clastogenic effects, MMF was not mutagenic *in vivo* (mouse micronucleus assay) or *in vitro* with metabolic activation (Chinese hamster ovary [CHO] cell chromosomal aberration assay). The chromosomal aberrations that occurred *in vitro* without metabolic activation in the initial CHO cell chromosomal aberration assay were due to the markedly cytotoxic doses used. Therefore, this result was considered false (79).

The genotoxicity of mycophenolate sodium however has been reported in the mouse lymphoma/thymidine kinase assay, the micronucleus test in V79 Chinese hamster cells and the in vivo mouse micronucleus assay. Mycophenolate sodium was not genotoxic in the bacterial mutation assay (Ames test with *Salmonella typhimurium* TA 1535, 97a, 98, 100, & 102) or the chromosomal aberration assay in human lymphocytes. Mycophenolate mofetil generated similar genotoxic activity (80). Similar results were also found in the similar mutagenicity assays for MMF in other reports (81, 82).The genotoxic activity of MMF in the positive assays is probably due to the depletion of the nucleotide pool required for DNA synthesis as a result of the pharmacodynamic mode of action of MPA (inhibition of nucleotide synthesis)(80).

The most serious complication among patients undergoing immunosuppressive therapy is the risk of developing cancer. In order investigate whether the drugs used have mutagenic properties thus contributing to increased cancer risk, the mutagenic and cytotoxic effects of immunosuppressive drugs such as cyclosporine A, mycophenolate mofetil, tacrolimus, and the immunosuppressive agent sirolimus in human lymphocyte cultures were evaluated. Clinically relevant blood-drug concentrations were used in this study. The mutagenicity and cytotoxicity in the blood of kidney transplanted patients were also evaluated. Mutagenicity was tested by analyzing micronuclei using the cytokinesis-block micronucleus assay and cytotoxicity was evaluated by calculating the cytokinesis-block proliferation index. Results showed that mycophenolate mofetil and tacrolimus displayed more mutagenic effects in vitro than cyclosporine A or sirolimus. Transplanted patients also exhibited higher amounts of micronuclei (which denotes higher mutagenicity) and a considerable reduction in the cytokinesis-block proliferation index (which denotes higher cytotoxicity) compared with healthy persons (83).

In an experiment in which 17 mycotoxins produced by various Aspergillus and Penicillium species were screened for their mutagenic activity to *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537(Ames test), both with and without metabolic activation, no mutagenic activity was evident with MPA (84).

Mutagenicity of various mycotoxins and the efficiency of mutagenic mycotoxins in producing DNA single-strand breaks and chromosome aberrations were also examined in another study, using a mammalian cell line. MPA among some other mycotoxins has been found to induce 8-azaguanine-resistant mutations in a mammalian cell line although it had little effect on DNA single-strand at high concentrations (85).

7. MYCOPHENOLATE AS AN IMMUNOSUPPRESSANT IN HUMAN MEDICINE

The most important medical application of MPA is its use as an immunosuppressant drug although it has other therapeutic uses. MPA is therapeutically used as its 2-morpholinoethyl ester prodrug (86), known as myco-phenolate mofetil (MMF, RS-61443); this is to increase oral bioavalability. It is produced under the trade name CellCept by Roche. MMF is rapidly hydrolyzed after absorption to MPA by plasma and tissue esterase, and further metabolized to the MPA-ß-glucuronide (87). The primary indication of MMF is post-transplantational treatment, for the prevention of organ and tissue rejection, mostly renal transplantations (88). MPA, through its immunosuppressive action reversibly inhibits T- and B-lymphocyte proliferation (see chapter 5 for the mechanism of action). This makes them less effective at recognizing and attacking the transplanted organ, lowering the risk of the organ being rejected (89, 90, 66). In renal and hepatic allograft recipients, MMF is used on a lifetime basis at doses of 1-3 g per day (15-46mg/kg/day).

Multiple immunosuppressive drugs have been used to manage inflammatory eye disease when control cannot be achieved by corticosteroid alone. Ocular inflammation is a common cause of ocular morbidity and vision loss, with uveitis alone accounting for approximately 10% of new cases of blindness in the US (91). Immunosuppressive drugs are used to treat many potentially blinding cases of ocular inflammation, primarily in three settings: as corticosteroid-sparing therapy when the disease can be controlled with oral corticosteroids, but substantial toxicity is expected at the dose required; for inflammation that is resistant to corticosteroids; and for management of specific diseases expected to respond poorly with corticosteroids alone(92).

Mycophenolate mofetil, an immunosuppressive drug, is increasingly popular for management of various types of non-infectious ocular inflammation (92-94) by suppressing the immune system in the manner already enumerated above**.**

Lupus nephritis is an inflammation of the kidney caused by systemic lupus erythematosus (SLE), a disease of the immune system. SLE typically causes harm to the skin, joints, kidneys, and brain. Lupus nephritis, particularly the proliferative form, is among the most common and severe manifestations of systemic lupus erythematosus (SLE) leading to significant morbidity and mortality if left untreated (95). Therapy aims to prevent evolution to end-stage renal disease and reduce mortality by early induction of remission and long-term prevention of recurrence. Intermittent intravenous (IV) pulses of cyclophosphamide (CYC) in combination with IV oral steroids have been the standard of care for induction of remission, and long-term quarterly IV CYC pulses used as remission maintenance treatment (96, 97). However, the benefits of CYC have been limited by the significant drug-related toxicities including sustained amenorrhea and the possibility of no response or relapse in a substantial number of these patients (98)**.** In recent years, MMF has been considered an important alternative agent for lupus nephritis refractory to other treatments and has also been studied as an induction therapy agent with promising results and mild toxicity (99).

Both forms of the inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC) are characterized by a lifelong course of remissions and relapses. A number of these patients do not respond to steroid refractory or develop steroid dependence which requires the maintenance of immunosuppression. The most commonly used immunomodulatory medications are azathioprine

(AZA), or its metabolite 6-mercaptopurine (6MP). Approximately 10% of patients, however, will be intolerant of these drugs, resulting in their withdrawal and the need for an alternative immunomodulator (100). Up to 50% of CD and 20% of UC patients will also develop a severe acute episode of their disease requiring hospitalization(94) and almost half of these patients will require rescue therapy or surgery(101, 102) More recently, MPA has been employed in the management of difficult IBD cases (103, 104).

8. CLINICAL PRESCRIPTION

MMF(CellCept®) is available as capsules (250 mg), tablets (500 mg), a powder to be made up into an oral suspension (1 g/5 ml) and a powder to be made up into a solution for infusion (drip into a vein; 500 mg). It is used with cyclosporine and corticosteroids (other medicines used to prevent organ rejection). The medicine can only be obtained with a prescription (90).

The most serious risk associated with CellCept is the possible development of cancer, particularly lymphoma and skin cancer. The most common side effects with CellCept used in combination with ciclosporin and corticosteroids (seen in more than 1 patient in 10) are sepsis (blood infection), gastrointestinal candidiasis (a fungal infection of the stomach or gut), urinary tract infection (infection of the structures that carry urine), herpes simplex (a viral infection that causes cold sores), herpes zoster (a viral infection that causes chickenpox and shingles), leucopenia (low white blood cell counts), thrombocytopenia (low blood platelet counts), anaemia (low red blood cell counts), vomiting, abdominal (tummy) pain, diarrhoea and nausea (feeling sick). The full list of side effects reported with CellCept is in the Package Leaflet (90).

CellCept should not be used in people who may be hypersensitive (allergic) to mycophenolate mofetil or mycophenolic acid. It should not be used in women are breast-feeding. CellCept treatment is not recommended for use in pregnant women, and should only be started in women after a negative pregnancy test and if effective contraception is used before, during and for six weeks after CellCept treatment (90).

9. RISK ASSESSMENT OF MYCOPHENOLIC ACID

9.1 Risk Assessment of Mycophenolic acid in Feed

9.1.1 Hazard identification

Mycophenolic acid is a mycotoxin produced by many *Penicillium* species among which are *P. brevicompactum* and some strains of *P. Roqueforti*. Apart from fungal species, the yeast species *Byssochlamys nivea* is also produces MPA and it is one of the fungal species responsible for fruit spoilage. Mycophenolic acid has been detected in silage (animal feed*)* as *P. Roqueforti* is one of the most common moulds detected in silage. *P. Brevicompactum* has been found in samples of deteriorating barley, oat and wheat. These cereals can become an important source of MPA as they are used as part of animal feed. Grain dusts from farms and storage companies which are generally used in animal feeding have also been confirmed to contain MPA.

9.1.2 Hazard characterization: (Dose-response)

Dose-response data for MPA in animal feed is not available. In spite of this, MPA has been regarded as a hazardous substance in feed due to its immunosuppressive properties which may have measurable consequences on animal health. From the few toxicological studies done with animals it has been shown that at a low dose of 4.5mg/kg /day fed to female rats, MMF (MPA therapeutic form) causes malformation of the head and eyes in the fetus and at oral doses of 6 and 90 mg/kg/day fetal resorptions and malformations were noticed in female rats and rabbits respectively. Rhesus monkeys developed hypoplastic anemia and severe intestinal disorders at daily doses of 150 mg/kg which is three times the therapeutic doses (15-45mg/kg/day) used for renal transplant patients.

9.1.3 Exposure assessment

Table 9.1 gives the summary of data obtained in a study which involved the measurement of MPA concentration in maize and grass silage while Table 9.2 gives the overview of animal exposure to MPA.

Table 9.1: Occurrence of MPA in maize and grass silage (40). This table gives a mean MPA concentration present in 74 of 233 samples of silage examined to be in the range of 0.02 to 35mg/kg with a mean concentration of 1.4mg/kg.

Type of Silage	No of samples analyzed		Mean (range) concentration MPA (mg/kg)
	Total	Positive	
Maize	135	38	0.70 (0.02-23.0)
Grass	98	36	2.2 (0.02-35)
Total	233	74	1.4 (0.02-35)

Table 9.2: Animal Exposure Assessment from major animal feedstuff as found in articles

Animal/ Article Reference	Food product	Maximum MPA concentration (mg/kg)	Mean MPA Concentration (mg/kg)	Average daily intake (mg/kg body weight)	Maximum daily intake (mg/kg body weight)
Dairy Cows (63)	Complete Diets	1.84	0.05	1.5 E-03	0.054
Cattle (40)	Silage (Maize and Grass)	35	1.4	-	1.8
Animals (50)	Grain dust		>1.0	-	-

From table 9.2, the highest average daily intake is from silage which is 1.8mg/kg body weight. This is approximately 10% of the therapeutic dose used to effect immunosuppression in organ transplant patients. The assumption of the cattle's body weight in the calculations in this table is 500kg and that of the cow 600kg.

9.1.4 Risk Assessment

Cattles take 1.8mg/kg bw MPA which implies 1.8 x600mg/day. This is 900mg /day (0.9g/day). Since there is no dose-response data available for animal feed, risk assessment parameters will be calculated from human therapeutic doses.

No Observed Adverse Effect Level (NOAEL) =Low Observed Adverse Effect Level (LOAEL)/10 =15mg/kgbw/10 = 1.5mg/kgbw

Acceptable Daily Intake (ADI) for cattle = NOAEL/Safety Factors

Safety Factors=10(interspecies variability) x10 (intraspecies variability) x5 (teratogenic effects)

Thus ADI for MPA= NOAEL/500 = 1.5mg/kg body weight/500 = 3 E-3mg/kg/bw.

ADI for cattle = 3 E-3mg/kg/bw x 600kg (average body weight)

ADI =1.8mg/day

Cattles take 900mg/day instead of the ADI of 1.8mg/day (600 times the ADI).

9.2 Risk Assessment of Mycophenolic acid in Food

9.2.1 Hazard identification

Mycophenolic acid has been detected in fermented food products such as blue veined cheeses (produced with P. roqueforti) and contaminated yoghurt. *P. brevicompactum* commonly encountered in indoor air, and an MPA producer has been isolated from a wide range of food such as cheese, ham, Italian fermented sausage, dried foods, bakery products, and cereal grains. It has also been detected in tap water. *P. brevicompactum* has been found in samples of deteriorating barley, oat and wheat, hence may serve as a source of MPA production in these cereals which are also used as human foods. Grain dusts from farms and storage companies contain median concentrations of >1 mg/kg of MPA. These dusts contaminate stored grains which could serve as human food.

9.2.2 Hazard characterization: (Dose-response)

Dose-response data for MPA in food is not available.

9.2.3 Exposure assessment

The most available data on exposure assessment of MPA in food comes from the blue-veined cheese, hence the only data presented in table 9.3 below.

Table 9.3: Human Exposure Assessment. This table presents the amount of MPA found by different researchers in blue-veined cheese.

Article	Food Product	MPA concentration (mg/kg)
Engel et al (33)	Blue-veined cheese	≤ 5.0
Usleber et al (42)	Roqueforti cheese (blue veined cheese)	0.4 – 1.2
Usleber et al (42)	German soft cheese preparation (blue-veined cheese)	4.0 – 11.0
Lafont et al (25)	Some blue-veined cheese	Up to 15.0
Tangni et al (50)	Grain dust	>1.0

Using data from Usleber, and assuming human daily consumption of 100g blue-veined cheese, a daily intake of 0.1mg MPA could be estimated.

9.2.4 Risk Assessment

Based on the data available, risk assessment parameters can be defined.

NOAEL: Due to the fact that there is no dose-response data available, NOAEL can only be determined from the LOAEL of the human therapeutic dose which is 15mg/kg/day.

Human therapeutic dose is 15-45mg/kgbw/day.

NOAEL is one-tenth of the LOAEL (15mg/kg).

This is 1.5mg/kg body weight (bw).

Acceptable daily intake (ADI): This is NOAEL/Safety Factors

Safety Factors=10(interspecies variability) x10 (intraspecies variability) x5 (teratogenic effects)

Thus ADI for MPA= NOAEL/500 = 1.5mg/kg body weight/500 = 3 E-3mg/kg/bw.

ADI = 3 E-3mg/kg bw x 65kg (human average weight) = 0.195mg/day.

9.2.5 Margin of Exposure

Calculation of the margin of exposure (MOE) has been recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Food and Safety Authority (EFSA) for substances that are genotoxic and carcinogenic to support prioritization of risk management action (105, 106).

MOE is defined as the ratio of the NOAEL or benchmark dose lower confidence limit (BMDL) for the critical effect to the theoretical , predicted or estimated exposure dose or concentration (WHO, 2009).

A rule of an MOE of 10,000 or higher has been laid down by EFSA for the genotoxic or carcinogenic risk of a substance to be considered low and treated with low priority. However, the more the MOE falls below 10,000, the higher the risk and the more urgent the need for minimisation measures.

Calculation of MOE for MPA:

NOAEL (derived from the therapeutic dose) = 1.5mg/kgbw/day.

Estimated exposure dose is based on blue-veined cheese alone since that is the only available data for humans. This is 0.1mg day = 1.5 E-03mg/kgbw/day

MOE = NOAEL/ Exposure

MOE for MPA = 1.5mg/kgbwday/ 1.5 E-03mg/kgbw/day.

MOE for MPA = 1000

This is much below 10,000, hence MPA belongs to the group of substances with high risk add needs to be treated with urgency in the risk minimisation measures.

9. CONCLUSIONS

Mycophenolic acid occurs in silage. The consumption of silage is the major route through which livestock are exposed to this mycotoxin. Cattles have been shown to consume 1.8mg/kg MPA /day through silage.

MPA has been found in blue-veined cheeses due to the fact that P. roqueforti (a producer of MPA) is used in the production of this type of cheese. Up to 0.23mg/kg MPA has been detected in sheep muscle tissue fed with 300mg/kg MPA/day. This implies a high exposure of MPA to human consumers through mutton. Carry-over from feed to milk is also possible though this has not been detected yet.

However, there is limited data on the occurrence of MPA in human foods; studies have only been done on animal feedstuffs and some fermented food products meant for human consumption. Thus little is known about human exposure to this mycotoxin.

Although its acute toxicity appears to be low from a few animal studies already carried out, little is known about the effect of chronic exposure of animals and humans to MPA through food. Chronic exposure studies of animals and humans to MPA, however, may be more relevant to health issues than the acute toxicity.

Another issue that may be of considerable concern is the synergetic effects of MPA on other mycotoxins (or vice versa) due to the facts that different mycotoxin many times co-exist in the same food samples.

There is more data both on animal and human toxicology for myco-phenolate mofetil, the therapeutic form of MPA. MMF has been found teratogenic (causes deformations in animal foetus) in rats and rabbits at relatively lower doses.

There are conflicting results on the genotoxicity of MPA, but Ame's test consistently declares this compound non genotoxic. MOE of MPA is 1000; thus it is a substance with a high risk for humans. The risk of developing lymphomas or skin cancer may be probably due to the long-term use by patients, the possible synergetic action of other drugs on MMF since it is commonly used together with other drugs or as a result of its pharmacodynamic mode of action of MPA (inhibition of nucleotide synthesis).

From the calculations made in chapter nine, human and cattle exposure to MPA is high (> 0.1mg/day and 900mg/day respectively). MPA exposure to animals is so high because of its high concentration in silage which is the livestock major food.

10. RECOMMENDATIONS

More human food items should be screened for the occurrence and amount of MPA in them especially foods and fruits that could be naturally infested with MPA-producing fungi.

More studies should be done on the possibility of carry-over of MPA from animal feed to their milk or and muscle tissue.

More chronic animal exposure studies should be done with MPA for its hazard characterization in form of dose-response relationship..

More investigation should be done on the impact of chronic exposure on the teratogenic effect of MPA.

More studies should be done to investigate the synergetic effects of MPA on other mycotoxins (or vice versa) since they often co-exist.

EFSA should urgently set a limit for MPA in human and animal foods as it has been done for some other mycotoxins. Screening should be made mandatory for industries that produce these foods to ensure they comply with new EFSA limits for MPA.

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