Rat feeding trials: A comprehensive assessment of contaminants in both genetically modified maize and resulting pellets

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ABSTRACT

We analyzed a comprehensive set of contaminants in MON810 and NK603 genetically modified (GM) maize, and their non-GM counterparts, used in a rat feeding study (the GMO90 + project). Both the maize grains and the manufactured pellets were characterized. Only minor differences in contaminant levels between GM and corresponding non-GM harvests were evidenced. Fumonisin and deoxynivalenol mycotoxins were the pollutants present in the highest amounts, with concentrations that were however largely below acceptance reference values. Our data reporting slightly lower levels of fumonisin in MON810 compared to its non-GM counterpart corroborate the lower susceptibility of insect resistant Bt maize to fumonisin-producing fungi. Traces of glyphosate (0.016 mg/kg) were evidenced in grains from NK603 treated crops. Regarding the pellets, analysis of more than 650 potentially toxic substances revealed low amounts of various mycotoxins, pesticides and heavy metals. Concentrations of contaminants quantified in the pellets were however far below the maximum level of residues values set by regulatory agencies, and no substantial differences in contaminants between GM and non-GM pellets were observed. Moreover, when comparing the contamination status of grains and pellets, we demonstrate yet again that characterizing the grains is actually not sufficient to foresee the quality of the produced pellets.

1. Introduction

Since the commercial release of genetically modified crops more than 20 years ago, the worldwide area devoted to them has steadily increased to reach 185.1 million hectares in 2016 where maize, soybean, cotton and rapeseed account for 99% of the worldwide GM acreage (ISAAA, 2016). To date, the two most prevalent GM crop traits are Bt-derived insect resistance and herbicide tolerance. The development of GM technology, which makes possible the transfer of genetic material across unrelated species, has raised numerous questions regarding the potential risks of GM crops for the environment and for human health (e.g. direct toxicity, allergenicity, gene transfer) (Domingo, 2016). Most of the developed countries have set up full and

detailed genetically modified organism (GMO) regulations that require the implementation of a risk assessment procedure before taking a decision on whether or not to approve the cultivation and use of a given genetically modified plant (GMP). The starting point of any safety assessment approach is the compositional analysis of GMPs with the aim to evaluate similarities and/or potential differences between a GM product and its conventional counterpart. As recommended by the consensus documents from the Organization for Economic Cooperation and Development (OECD), the compositional analysis usually encompasses the analysis of proximates, micronutrients, secondary metabolites, allergens and anti-nutrients. For maize crops, over 60 components have been listed in the OECD documents for analysis and statistical comparison (OECD, 2002). In addition to these components,

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several contaminants including mycotoxins, heavy metals, pesticide residues and persistent organic pollutants (POPs) also need to be investigated (Clarke et al., 2015). In this context, it should be mentioned that maize is considered to be one of the best substrates for the production of mycotoxins by toxigenic fungi (Chulze, 2010). The most frequently found mycotoxins in maize kernels are aflatoxins, ochratoxins, trichothecenes, zearalenone and fumonisins, for which maximal allowable concentrations in foodstuffs have been set by the European Commission (EC Regulation 1881/2007 and 1126/2007). In addition to these regulated mycotoxins, maize harvests can also be contaminated by "emerging mycotoxins", a group of chemically diverse mycotoxins for which to date no regulation exists and that includes enniatins, beauvericin and moniliformin. Climatic factors determine the balances that occur within toxigenic fungi populations and consequently their production of mycotoxins. Thus, deoxynivalenol and zearalenone are mainly encountered in maize grown in temperate areas, while hot and dry summers favor the contamination of kernels with fumonisins and aflatoxins (Rodrigues and Naehrer, 2012). Heavy metals of particular concern in relation to harmful effects on health are mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As). These trace elements are natural constituents of the earth's crust, but natural and industrial atmospheric deposition or agricultural inputs can lead to an increase in the amount of heavy metals in soils. Once present in agricultural soils, they are readily absorbed by crop roots and can be transported to the edible parts of the plants, where they accumulate. Although maize is known to accumulate low amounts of metals in its kernels when compared to other crops such as wheat (Wang et al., 2002), the potential presence of these contaminants should not be neglected in a safety assessment procedure. Occurrence of pesticide residues in maize can be related to three potential contamination sources: the use of pesticides to protect the crop during the cultivation, the contamination of the environment by pesticides previously applied for other purposes and the use of insecticides during storage and handling. Among these pesticides, glyphosate, the herbicide active compound present in weed killer mixtures such as Roundup®, is currently the subject of an increasing and controversial debate. However, in most maize crop management practices adopted in developing countries, pesticides (herbicides, fungicides and insecticides) are applied very early in the crop cycle, so that there is a large period between the last application and the harvest, which theoretically should not result in significant pesticide residues or metabolites in the harvested grains. Indeed, as evidenced by Clarke et al. (2015), the greatest concern regarding pesticide residues in maize is linked to insecticides applied during storage and handling, mainly organophosphorus compounds (chlopyriphos, pirimiphos-methyl) and/or pyrethroid derivatives (cypermethrin, deltamethrin) applied in combination with the piperonyl butoxide synergist. Lastly, POPs, with polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyl (PCBs) being the most frequent representatives, are also relevant when considering environmental contaminants in maize. Due to their resistance to degradation, these pollutants are transported by air and accumulate in different parts of the environment such as water and soil. POPs are highly lipophilic and accumulate in the fat tissue of all living organisms including maize plants (Kacalkova and Tlustos, 2011).

Significant precautions need to be taken when cultivating GM plants and near-isogenic control plants for toxicity studies to avoid the occurrence of different levels of contaminants in the two materials, including the cultivation in adjoining plots, identical agronomic practices (fertilizer, growth regulator and pesticide treatments) and the establishment of buffer zones. However, for some contaminants, these precautions are often not sufficient to ensure that harvests will be equally contaminated. Namely, this can be the case of contaminants that are transported by air (POPs) or that heterogeneously accumulate in soils (heavy metals and POPs). Moreover, in the case of mainly fumonisins and some mycotoxins, it is acknowledged that insect-resistant GM maize is less sensitive to contamination (Bakan et al., 2002; Abbas et al., 2013). Indeed, infection through insect damage is a major

infection pathway for *Fusarium verticillioides* and *Fusarium proliferatum*, the two main fungal species producing fumonisins (Picot et al., 2010). Reducing insect attacks is a key component of strategies implemented to control fumonisin contamination, and the use of GM maize such as Bt maize (which expresses Cry1ab genes of *Bacillus thuringiensis* that encode insecticidal proteins against lepidopteran pests) can be part of these strategies (Koch et al., 2015). Surprisingly, whereas differences in contaminant levels between diets can introduce significant biases in toxicological feeding studies, an accurate and comprehensive analysis of their occurrence is not always integrated into the analytical strategies published in scientific literature (Liu et al., 2012; Zhu et al., 2013; Cuhra, 2015; Chen et al., 2016; Fang et al., 2017). Detailed data are provided in few projects (Zeljenková et al., 2014; Delaney et al., 2013; Zeljenková et al., 2016), including the GRACE European project that gives open access to data sets *via* the CADIMA database (https://www.cadima.info/index.php/area/publicAnimalFeedingTrials). Other projects have restricted the contaminant analysis to the characterization of the GM and non-GM maize grains (Liu et al., 2012; Zhu et al., 2013; Fang et al., 2017), while the additional ingredients introduced during the pellet production can also be a source of contamination (Mesnage et al., 2015). Maize pellets are usually formulated with maize contents ranging between 12 and 33%, and up to 50% in exceptional cases. The remaining constituents include other cereals (such as wheat or barley) and/or legumes (such as soybean) that may have been sprayed with pesticides, may be sensitive to mycotoxin contamination, and/or may accumulate heavy metals.

The present study was realised in the framework of the GMO90⁺ (Genetically Modified Organisms 90-day rodent trial extended to 180-day) project. The GMO90⁺ project (http://recherche-riskogm.fr/fr/page/gmo90plus) was designed to evaluate potential health effects of two GMPs, namely MON810 (inclusion rates in pellets: 11% and 33%) resistant to insects by expression of a Cry protein encoded by the *Cry1Ab* gene of *Bacillus thuringiensis*, and NK603 (inclusion rates in pellets: 11% and 33%, with or without glyphosate treatment) tolerant to glyphosate by expression of a particular bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and their corresponding near-isogenic controls (non-GMPs). As the first step in the frame of this GMO90⁺ sub-chronic feeding study and complementary to a thorough compositional analysis of the maize grains and GM-based diets (Bernillon et al., 2018), we analyzed a comprehensive set of contaminants in the maize grains as well as in the pellets including the two previously mentioned maize GM varieties and their closest non-GM counterparts, each genotype pair (GM and non GM maize) being cultivated in the same environmental conditions. Furthermore, the associations between contaminant levels and GM traits as well as the associations between contaminant levels in grains and those in the pellets were investigated.

2. Materials and methods

2.1. Plant materials

For each of the considered GMPs and their closest non-GMP counterparts, crops were cultivated at two different spots to overcome production hazard and favor the availability of high quality harvests meeting the objectives of our project. Each crop was grown according to the rules of good agronomic practices. Grains of insect-resistant maize MON810 and its closest near-isogenic counterpart, hereafter named MON and ISOMON, were produced in Catalonia (Spain) at two spots during the growing season in 2014 (Fig. 1). Grains of glyphosate-tolerant maize NK603 and its closest near-isogenic counterpart, hereafter named NK and ISONK, were produced in Ontario (Canada) and in Minnesota (USA) during the growing season in 2014. NK was cultivated with (NK-G) or without glyphosate application (NK) during the crop cycle. The maize productions were jointly undertaken with the European G-TwYST project (https://www.g-twyst.eu/). In each growing

MAIZE PRODUCTION : Spain (Catalonia) 2 spots
Canada (Ontario) & USA (Minnesota)

SAMPLING
One mix of all large bags for each condition & for each site of production

ANALYSIS OF GRAINS (SGS, Hamburg, Germany)

- DNA analysis – GMO events
- Contaminants (POPs-Pesticides-Mycotoxins)

CHOICE OF BATCHES: Spain (Catalonia) spot2 & Canada (Ontario)

1 LARGE BAG OF EACH VARIETY CHOOSED RANDOMLY

PRODUCTION OF DIETS
(Safe Cie, Augy, France)

SAMPLING
4 random spots in each large bag

ANALYSIS OF DIET SAMPLES
(Eurofins, Nantes, France)

- DNA analysis – GMO events
- Contaminants (POPs-Pesticides-Herbicides-Mycotoxins)

ANALYSIS OF EMERGING MYCOTOXINS
(INRA MycSA, Villenave d'Ornon, France)
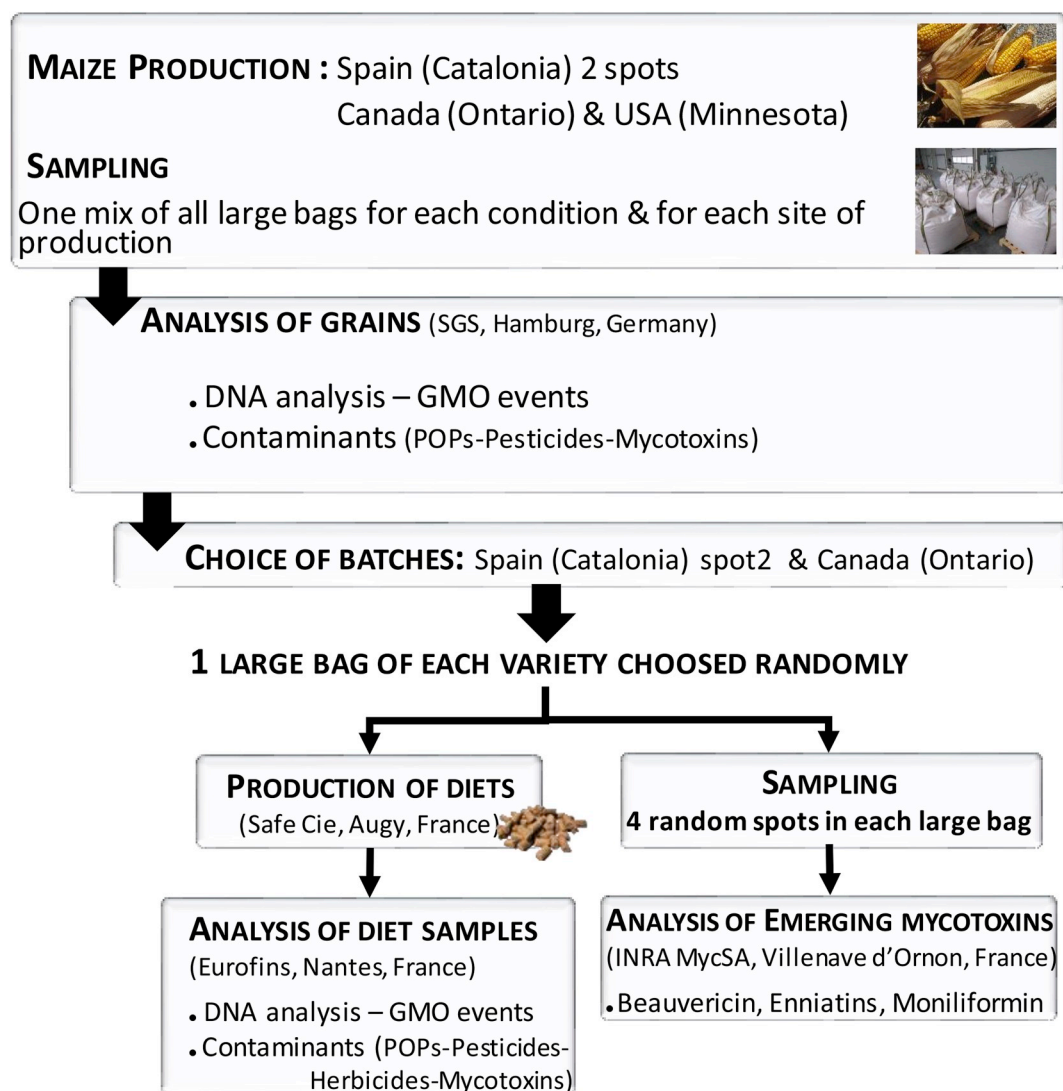
- Beauvericin, Enniatins, Moniliformin

Fig. 1. Sampling and analytical strategies for assessing the contamination status of maize grains and maize-based diet samples.

area, transgenic and near-isogenic control varieties were cultivated in adjoining plots under identical environmental conditions and with buffer zones to prevent pollen flow. As summarized in the supplementary information (Table S1), only herbicide treatments were applied at the pre- and post-emergence stages, and no insecticides or fungicides were used during the crop cycle, shipment or storage. The maize grains were sent to Germany (December 2014), where samples were taken for genetic as well as biochemical analyses (proximates and contaminants) to identify the best batches for the diet trials planned in the frame of the GMO90$^+$ and G-TwYST research projects. The production from Canada (8906R maize as MON crop and 8906 maize as closest near-isogenic counterpart (ISOMON), both from Pioneer) and from the Catalonia spot 2 (DKC6667YG maize as NK crop and DKC6666 maize as closest near-isogenic counterpart (ISONK), both from DE-KALB) was selected to produce the pellets used in the feeding trials.

2.2. Diet formulation

Harvested maize grains (moisture content < 14%) were stored at room temperature in large bags of 500 kg (Spain harvests) or 1000 kg (Canada harvests). Temperature, humidity and insect infestation were monitored to ensure a lack of biological deterioration of grains during storage (Magan & Aldred, 2007). Maize grains were milled (mesh size: 1 mm) to produce the diets. One large bag of maize grains of a given

harvest was used to produce the unique batch of the corresponding pellets. As summarized in Table 1, eight types of pellets were produced, encoded and vacuum packed in plastic bags (10 kg). The diets were isoproteic and isocaloric and adjusted to the dietary nutritional requirements of the specific rat strain used in the feeding study (Wistar Han RCC), while maintaining the same constant inclusion level of maize grain (33%) throughout all diets. In order to differentiate between the grains and the pellets, we added the prefix (d) to the denomination of the rat diets.

In addition to the milled maize, the formulation mainly consisted of

Table 1
Inclusion level of GM and non GM maize grains in the diets for rats.

DIET	MAIZE COMPOSITION
dISOMON	33% ISOMON
dMON11	22% ISOMON & 11% MON
dMON33	33% MON
dISONK	33% ISONK
dNK11	22% ISONK & 11% NK
dNK33	33% NKG
dNKG11	22% ISONK & 11% NKG
dNKG33	33% NKG

Table 2
Formulation of the diets for rats.

INGREDIENTS	%
Maize grain	33
Wheat flour	24.5
Wheat bran	15
Soybean meal	18
Soybean oil	3
CaCO$_3$	1.1
HPO$_4$Ca	0.65
Brewer's yeast	2
Mineral feed*	1
Vitamins*	1.4
L-lysine 78%	0.18
DL-methionine	0.15

*These values are calculated averages of product raw values and are an indication only.

other vegetable ingredients from organic sources, including wheat flower, wheat bran, soybean meal, and soybean oil, while it did not contain any animal-derived ingredients (Table 2).

Directly after production (Safe Cie, Augy, France), the eight types of pellets in their vacuum bags were sterilized with beta-irradiation at 29.2–35.8 kGy (Ionisos Cie, Dagneux, France).

2.3. Sampling and analytical strategy

The sampling and analytical strategies applied to grains and pellets are schematically shown in Fig. 1. As detailed in supplementary information (Table S2), 65 and more than 650 toxic substances were searched for in the maize grains and pellets, respectively. The list of the analyzed contaminants includes pesticide residues (an extensive list of active ingredients used in the production of fungicides, insecticides and herbicides including glyphosate and its main degradation product aminomethyl phosphonic acid/AMPA), POPs (PAHs and PCBs), heavy metals with safety concern (As, Hg, Cd and Pb) and mycotoxins (regulated mycotoxins produced by *Fusarium*, *Aspergillus* and *Penicillium* species and emerging mycotoxins). To ensure the accuracy and reliability of the results, most of the analyses were subcontracted to accredited laboratories, in the case of the grains to SGS (Hamburg, Germany) and in the case of the pellets to Eurofins (Nantes, France).

2.4. Confirmation of the GM trait and lack of unintended GM contamination in grains and pellets

The presence of expected and unexpected GMO events in grains and pellets was verified in three consecutive steps, as reported in Tables 3A and 3B. The analyses were performed by SGS GmbH (Hamburg, Germany). First, frequently used transgene elements (*35S* promoter, *Nos* terminator, *FMV*, *PMI*) were searched for. Second, event-specific detection was performed using real-time PCR for NK603, MON810 and maize varieties authorized for cultivation in Canada in 2014 (MON863, MON88017, MON89034, Bt176, GA21, Bt11, NK603, T25, TC1507, LY038, DAS40278-9, DAS59122, DP98140, SYN3272, MON87460 and CBH351). Third, the events with positive results in the second step were quantified. Results were reported as percentages relative to maize grain DNA.

2.5. Emerging mycotoxins

Emerging mycotoxins, including enniatins A, A$_1$, B, B$_1$, beauvericin and moniliformin, were analyzed using LC-MS/MS with procedures adapted from Sorensen et al. (2008) and Sulyok et al. (2006). Briefly, mycotoxins were extracted from 3 g of finely ground grains or pellets with 20 mL of an acetonitrile/water mixture (84/16, v/v). After 1 h of agitation, 5 mL were evaporated to dryness at 50 °C under a gentle

stream of nitrogen. Samples were reconstituted in 200 μL of acetonitrile/water (84/16, v/v) with 10 mM ammonium acetate before analysis. MS analysis and fragmentation experiments were performed using a QTrap 2000 system (Sciex, Villebond sur Yvette, France) equipped with an ESI source and an 1100 Series HPLC system (Agilent, Les Ulis, France). Two separate injections were performed. For the analysis of enniatins and beauvericin, a chromatographic separation was achieved on a Kinetex XB - C18 100 Å column (150 × 4.60 mm, 2.6 μm) (Phenomenex, Torrance, CA, USA) protected with a guard column of the same material and maintained at 45 °C. The mobile phase consisted of 10 mM ammonium acetate in H$_2$O (solvent A) and acetonitrile (solvent B). The gradient elution was: 70% B for 5 min, 70–95% B in 10 min, 95% B for 5 min, 95–70% B in 1 min, and 70% B for 5 min post-run equilibration. The injection volume was 5 μL. The flow rate was kept at 0.7 mL/min and a split was used, so that 350 μL/min was forwarded to the ESI source. Chromatographic separation of moniliformin was achieved on a ZIC-Hilic column (150 × 4.6 × 3.5 μm) (Merck, Darmstadt, Germany). The gradient elution was: 10% B for 1 min, 10–50% B in 10 min, 50% B for 5 min, 50-10% B in 1 min and 10% B for 5 min. The flow rate was 0.3 mL/min and the injection volume was 5 μL. Quantification was performed by external calibration with commercial standards (Sigma-Aldrich, City, France) and ranging from 1 to 100 ng/mL. The MS parameters used for the analysis of emerging mycotoxins are summarized in supplementary information (Table S3).

2.5.1. MRL values, calculation of the rat exposure to food contaminants and calculation of the hazard quotient

An MRL (Maximum Residue Level) value corresponds to a legally fixed maximum concentration for a particular active ingredient in a particular food or animal feed. In the case of pesticides, the MRL is the highest level of a pesticide residue that is legally tolerated in or on food or feed following a correct application (Good Agricultural Practice). Since these values may differ between agencies (European or other international bodies), we have considered the lowest value. The MRL acronym also takes into account the health risks related to the consumption of the food for humans or feed for animals leading to the point that MRL exceedance is not legal for trade but safe for human health.

If one knows the concentration of a chemical agent present in feed, the daily exposure dose can be calculated by using a conversion factor of 0.05 in the case of chronic studies in the frame of food safety experiments with rats (EFSA, 2012). For instance, 1 mg/kg of a particular contaminant in feed is equivalent to a dose of 0.05 mg/kg body weight per day (mg/kg bw/d) in rats. Almost all the toxic compounds exhibit a dose-response relationship with a threshold value of exposure. The so-called No Observed Adverse Effect Level (the NOAEL value) is the highest dose level of a substance that does not lead to toxicity in laboratory animals when these undergo a subchronic or chronic exposure to the compound. The Acceptable Daily Intake (ADI) value corresponds to a toxicological safety limit specifying the amount of a substance which can be ingested each day over the entire lifespan without any recognizable risks to the health of the consumer. The ADI value for compounds that exhibit a low toxicity effect, is generally calculated by dividing the NOAEL determined in animals (very often in rats) by an overall default uncertainty factor of 100. In the case of highly toxics compounds such as metals, a large number of reports are dealing with toxicity in humans with an expression as a Tolerable Weekly Intake (TWI), but for simplicity when available the TWI were normalized to daily intake for the rats by dividing by 7 the TWI value. When not available, the NOAEL in the rat was calculated from the ADI value for humans using the uncertainty factor of 100. Then, the ratio of the chronic daily intake of each contaminant to the corresponding NOAEL in the rat was calculated and designed as the Hazard Quotient (HQ) for a specific contaminant. A lack of negative health effects is expected when the HQ value is ≤ 1.

Table 3A

GMO identity of the harvested maize grains and search for unintended GMO contamination. Quantification results are expressed in percentages relative to maize DNA.

A-Maize harvests						
Origin		Canada	Canada	Canada (glyphosate)	Spain-2	Spain-2
Transgene		isogenic	NK603	NK603	isogenic	MON810
	Variety	Pio8906	Pio8906-R	Pio8906-R	DKC6666	DKC6667YG
Detection	NOS terminator	N	Positive	Positive	N	N
	35S promotor	Positive*	Positive	Positive	N	Positive
	FMV	N	N	N	N	N
	PMI	N	N	N	N	N
	MON863	N	N	N	N	N
	MON88017	N	N	N	N	N
	MON89034	N	N	N	N	N
	Bt176	N	N	N	N	N
	MON810	N	Positive*	N	N	Positive
	GA21	N	N	N	N	N
	Bt11	N	N	N	N	N
	NK603	N	Positive	Positive	N	N
	T25	N	N	N	N	N
	TC1507	N	Positive*	N	N	N
	LY038	N	N	N	N	N
	DAS40278-9	N	N	N	N	N
	DAS59122	Positive*	N	N	N	N
	DP98140	N	N	N	N	N
	SYN3272	N	N	N	N	N
	MON87460	N	N	N	N	N
	CBH351	N	N	N	N	N
Quantification (%)	NK603		90	100		
	MON810		< 0.1			90
	TC1507		< 0.1			
	DAS59122	0.6				

*: Unexpected result N: Negative.

3. Results

3.1. Check of the GMO identity of the harvested grains and produced pellets and lack of unintended GMO contamination

To ascertain the identity of the GM crops used in our study and the absence of unintended GM contamination in the non-GM harvests, we first investigated the occurrence of commonly used transgene elements and GM events authorized for cultivation in each cultivation area. In each case of positive occurrence, the amount of unexpected GM event was quantified and expressed as percentage relative to maize DNA. As evidenced by the analyses of transgene elements reported in Table 3A, the characterization of grains harvested in Spain (ISOMON and MON) did not reveal any unexpected results. However, regarding the Canada harvests, the *35S* promoter element was detected in the non-GM harvest ISONK. This unexpected result was associated with trace amounts (0.6%) of the DAS59122 event authorized for cultivation in Canada. In addition, NK may have contained trace amounts of two other GM events which were below the detection limit in the quantitative assay (< 0.1%). As expected, NK and NKG grains contained ≥90% of the NK603 event. Overall, these data allowed certifying the identity of the GM harvests and the lack of substantial contamination in non-GM grains.

A similar three-stage procedure was applied to the eight types of pellets (Table 3B). Search for commonly used transgene elements revealed three unexpected results: the occurrence of the *35S* promoter in dISONK and dISOMON and of the *Pat-Syn* transgene in dNK11. While confirming the GM origins of dMON11, dMON33, dNK11, dNK33, dNKG11 and dNKG33, the search and quantification of GM events authorized for cultivation in Spain and Canada allowed ascribing the unexpected results to a low contamination of dISOMON and dISONK with MON810 (0.2%) and of dNK11 with traces of TC1507 (0.15%) and

DAS59122 (0.1%). Although initially detected in dNK11, MON810 and RRsoy were below the detection limit of 0.1% in the quantitative analysis.

3.2. Contaminants quantified in maize grains

Among the 70 toxic analytes targeted in grains, only a few were detected in quantifiable amounts. The amounts of these contaminants are listed in Table 4. With regard to the 20 analyzed POPs, all molecules were in amounts below the limit of quantification (LOQ) for all harvests. Regarding pesticide residues, only glyphosate in the NKG harvest was present at a level above the detection limit. The concentration of 0.016 mg/kg was slightly higher than the LOQ (0.01 mg/kg), but largely below the maximum level of residue (MRL) reported as 1 mg/kg (EU pesticide database). Among the targeted heavy metals, only traces of Pb were detected in ISONK and NK grains, but the levels were below the MRL value. Regarding mycotoxins, deoxynivalenol was measured in all harvests with concentrations ranging from 0.26 to 0.77 mg/kg of dry weight. Zearalenone was detected in four of the harvests (NK, ISONK, MON and ISOMON), while fumonisins were mainly observed in maize cultivated in Spain (MON and ISOMON). Whatever the considered mycotoxin, the quantified values were in the same range for the varieties grown within the same site of production. Lastly, low amounts of the emerging mycotoxin beauvericin were quantified in the five batches of grains, while moniliformin was found in four of the five batches (no moniliformin was quantified in ISONK grains).

Overall, this comprehensive monitoring of possible contaminants in maize grains indicated that none of the harvests was contaminated with concentrations of toxic analytes that could raise safety concerns for rats if one compares the dose of exposure of a specific contaminant ingested with the NOAEL value in the rat reported by international agencies. Moreover, only minor differences in contaminant levels between GM

Table 3B

GMO identity of the maize-based diets and search for unintended GMO contamination. Quantification results are expressed in percentages relative to maize DNA.

Diets

		Unit	dISONK	dNK11	dNK33	dNK-G11	dNK-G33	dISOMON	dMON11	dMON33
Detection	plant_DNA_Cotton (species)		N	N	N	N	N	N	N	N
	plant_DNA_Rape (species)		N	N	N	N	N	N	N	N
	plant_DNA_Potato (species)		N	N	N	N	N	N	N	N
	plant_DNA_Rice (species)		N	N	N	N	N	N	N	N
	plant_DNA_Sugar beet (species)		N	N	N	N	N	N	N	N
	p35S		Positive*	Positive	Positive	Positive	Positive	Positive*	Positive	Positive
	tNos		N	Positive	Positive	Positive	Positive	N	N	N
	FMV promotor		N	N	N	N	N	N	N	N
	Bar		N	N	N	N	N	N	N	N
	Npt II		N	N	N	N	N	N	N	N
	Pat-syn		N	Positive*	N	N	N	N	N	N
	MON88017		N	N				N		
	MON89034		N	N				N		
	MON810		Positive*	Positive*	N	N	N	Positive*	Positive	Positive
	GA21		N	N				N		
	NK603		N	Positive	Positive	Positive	Positive	N	N	N
	T25		N							
	TC1507		N	Positive	N	N				
	DAS59122		N	Positive	N	N				
	SYN3272		N	N				N		
	SYN5307		N	N				N		
	MIR604		N	N				N		
	MIR162		N	N				N		
	RR soy qualitative		N	Positive*				N		
	MON87427		N	N				N		
	MON89788 soy		N	N				N		
	FG72 soy		N	N				N		
	MON87705 soy		N	N				N		
	A5547 soy			N						
	A2704-12 soy			N						
Quantification (%)	NK603	%		30	30	40	100			
	uncertainty NK603	%		10	30	13	30			
	MON810	%	0.2	< 0.1				0.2	30	85
	uncertainty MON810	%							10	25
	TC1507	%		0.15						
	DAS59122	%		0.1						
	RR soy	%		< 0.1						

*: Unexpected result N: negative.

and corresponding non-GM harvests were observed, even for crops grown in the presence of glyphosate. These differences related to mycotoxins, with slightly higher levels of fumonisins in ISOMON if compared to MON grains, and of deoxynivalenol and zearalenone in ISONK if compared to NK grains.

3.3. Contaminants quantified in pellets

More than 650 potentially toxic substances were monitored in the pellets (Table S2). Whereas the vast majority was below the detection limit, 14 substances could be quantified (Table 5). Five pesticides were detected in concentrations higher than the LOQ of the analytical methods (glyphosate, the two pyrethroids deltamethrin and cypermethrin and the two organophosphorus chlorpyriphos-methyl and pirimiphos-methyl insecticides) and a synergist commonly used in the formulation of insecticides (piperonyl butoxide). All diets were shown to contain a similar low level of glyphosate (close to 0.06 mg/kg), which, compared to the MRL value defined by the EU pesticide database (1 mg/kg), does not raise any safety concern. All diets were characterized by similar levels of pesticide residues below the corresponding MRL values. The dose of exposure of the rats was deduced from the concentration of each toxic compound in the feed (see

Table 4

Contaminants quantified in the maize grain harvests and associated toxicity reference values.

Compound	LOQ μg/kg	ISOMON	MON	ISONK	NK	NKG	MRL μg/kg	MRL source	NOAEL mg/kg bw/d (rat)	HQ
		μg/kg								
Glyphosate	10	< 10	< 10	< 10	< 10	16	1000	EU pesticide database	0.4 (a)	≤ 0.002
Lead	20	< 20	< 20	30	50	< 20	200	EC-Dir 1881/2006	0.0063 (b)	≤ 0.39
Σ Fumonisins	50	1474	1062	< 50	196	< 50	2000	EC-Dir 1881/2006	0.2 (a)	≤ 0.37
Deoxynivalenol	10	440	342	772	351	263	750	EC-Dir 1881/2006	0.1 (a)	≤ 0.38
Zearalenone	5	54	12	26	96	< 5	75	EC-Dir 1881/2006	0.1 (a)	≤ 0.048
Beauvericin	0.1	4	1.5	< 0.1	0.8	1	NE			
Moniliformin	0.1	18.6	6	< 0.1	0.2	4	NE			

a) calculated from human ADI value using a security factor of 100 fold between human and rat.
b) no effect on kidney in human is observed up to an exposure of 0.63 μg/kg bw/d; a correction factor of 10 for the rat takes into account the comparison of the similar lack of effect corresponding to a Pb-blood concentration (EFSA Journal 2010; 8(4):1570).

Table 5
Contaminants quantified in the maize-based pellets and associated toxicity reference values.

	Compound	Unit	LOQ	dISONK	dNK11	dNK33	dNKG11	dNKG33	dISOMON	dMON11	dMON33	MRL µg/kg	MRL source	NOAEL mg/kg bw/d (rat)	HQ
Pesticides	Piperonyl butoxide	µg/kg	–	260	280	470	420	460	430	400	370	NE	EU pesticide database	1 (a)	≤0.00055
	Chlorpyrifos-methyl	µg/kg	5	< 5	< 5	11	100	10	7	7	< 5	3000	EU pesticide database	1 (a)	≤0.0027
	Deltamethrin	µg/kg	–	45	55	26	35	23	23	20	48	2000	EU pesticide database	5 (a)	≤0.00033
	Cypermethrin	µg/kg	–	16	22	31	33	31	33	29	12	100	EU pesticide database	5 (a)	≤0.05
	Pirimiphos-methyl	µg/kg	–	210	210	400	360	350	400	370	220	500	EU pesticide database	0.4 (a)	≤0.0094
	Glyphosate	µg/kg	10	50	50	59	63	75	55	50	60	1000	gain report VM3070 /2011 USDA	0.4 (a)	≤0.0003
Heavy Metals	Arsenic	µg/kg	50	100	80	60	60	90	80	80	90	1000		0.21 (a)	
	Cadmium	µg/kg	5	76	73	72	75	74	72	75	76	200	EC-Dir 1881/2006	0.05 (a)	≤0.076
	Lead	µg/kg	20	53	67	57	53	49	119	110	130	200	EC-Dir 1881/2006	0.0063 (b)	≤1
Mycotoxins	Aflatoxin B1	µg/kg	0.1	0.2	0.2	0.2	0.2	< 0.1	0.2	0.2	0.2	2	EC-Dir 1881/2006	NE (c)	–
	Deoxynivalenol	µg/kg	50	187	149	154	174	150	227	194	163	750	EC-Dir 1881/2006	0.1 (a)	≤0.11
	Σ fumonisins	µg/kg	30	< 30	< 30	39	68	26	474	431	368	2000	EC-Dir 1881/2006	0.2 (a)	≤0.12
	Beauvericin	µg/kg	0.1	0.8	2.3	5.0	1.6	2.3	1.9	2.9	1.7	NE			
	Σ enniatins	µg/kg	0.5	5.4	6.9	6.6	7.0	7.2	4.2	5.6	5.5	NE			

a) calculated from human ADI value using a security factor of 100 fold between human and rat.
b) no effect on kidney in human is observed up to an exposure of 0.63 µg/kg bw/d; a correction factor of 10 for the rat takes into account the comparison of the similar lack of effect corresponding to a Pb-blood concentration (EFSA Journal 2010; 8(4):1570).
c) The European and international bodies have not set an ADI for aflatoxins since these substances have genotoxic carcinogenic effects, with no threshold.
d) LOAEL = 8 µg/kg bw/d for early markers of renal toxicity in pigs, the most sensitive animal species.

Materials and Methods). Each exposure value was compared to the NOAEL value and it can be noticed that the exposure was a factor 20- to 3000-fold below the threshold value that potentially could induce an adverse health effect.

Three heavy metals (Pb, Cd and As) were detected in all diets at amounts that were below the MRL values. The level of contamination corresponded to a safety factor of 13- and 330-fold for the Cd and As contamination, respectively. The highest HQ value was found for Pb contamination, but even in this case the value was equal to 1.

Various amounts of mycotoxins were measured in the diets. All diets were shown to contain traces of aflatoxin B_1 (between 0.1 and 0.18 µg/ kg) and deoxynivalenol (< 227 µg/kg). Fumonisins in concentrations lower than 500 µg/kg were detected in diets produced with grains from Spain. Regarding the emerging mycotoxins, enniatins and beauvericin (< 7.5 µg/kg) were observed in all diets, while no moniliformin was detected. Regardless of the type of diets and the mycotoxin, contamination levels were far below the MRL values reported in Table 5. The level of contamination corresponded to a safety factor of at the most 8-fold by comparison with the NOAEL values in the rat.

Altogether, our results indicate that the eight types of diets do not contain any toxic substances at levels that could raise safety concerns for rats. Only very slight differences were observed between GM and non-GM maize-based pellets of the same growing area or between NK and NKG maize-based ones.

4. Discussion

This study was undertaken to quantify a large set of environmental contaminants in maize grains and the resulting pellets formulated for rats from two GM maize varieties (containing the events MON810 or NK603) and their closest non-GM counterparts. Grains and pellets from maize NK603 cultivated with or without glyphosate were also compared regarding their levels of contaminants. Such a comprehensive study, applied on both grains and pellets, is rarely explicitly addressed in scientific studies about GM safety assessment, although several toxic substances (heavy metals, mycotoxins, pesticide residues and POPs) can contaminate maize grains and subsequently the produced pellets, and differences in these contaminant levels possibly influence the outcome of the feeding trials. In addition, chemical contaminants present in the ingredients used in the formulation can be introduced in the feed during the preparation of the pellets. Our data show that some contaminants occur in grains and pellets but, whatever the considered toxic substances, the determined amounts in pellets were lower than the MRL values set by the regulatory agencies. It is however important to note that MRL values were firstly set up to limit pesticide contamination in different plant or animal products and that the value for a pesticide may differ between maize, wheat, soybean and other ingredients. Moreover, the MRL value for contaminants that have not been intentionally added to feed, as for instance heavy metals, were calculated in a different way, i.e. by taking into account a dose-response relationship in animal experiments. Therefore, to estimate a potential health hazard, the rat exposure level was deduced from the level of the highest contaminated diet by using a conversion factor of 0.05 (EFSA, 2012), the obtained value being compared to that of NOAEL in the rat and leading to a ratio (HQ) lower than 1 in all cases.

With respect to the pollutants contaminating maize grains, fumonisins in Spanish harvests and deoxynivalenol in Canadian ones were present at the highest amounts, an observation that is in accordance with the acknowledged worldwide geographical distribution of mycotoxins in maize (Schatzmayr and Streit, 2013). Furthermore, our data describing slightly higher concentrations of fumonisins in ISOMON compared to MON corroborate the lower susceptibility of insect resistant Bt maize to *F. verticillioides* and *F. proliferatum,* which are the two main fungi responsible for fumonisin production (Ostry et al., 2010; Abbas et al., 2013). We also observed slightly higher levels of deoxynivalenol in grains with the ISONK genotype when compared to NK

and NKG and higher levels of zearalenone in NK grains when compared to ISONK and NKG. These data could be explained by differences in the composition of ISONK, NK and NKG grains, leading to a different suitability as a substrate source for the mycotoxin-producing *Fusarium* strains and more precisely for *Fusarium graminearum* and *Fusarium culmorum*, which are mainly responsible for deoxynivalenol and zearalenone production in maize. However, only limited differences were observed in grains in the frame of the metabolomics characterization performed on the same batches as those used in the present study (Bernillon et al., 2018). Another explanation could be fortuitous differences occurring during culture, harvest, transport or storage due to the random exposure of one or several cultures/harvests to pathogens. The contamination of NK but not NKG or ISONK with fumonisins may be taken as a supporting argument for this scenario. Data on glyphosate and AMPA residues in grains indicated that only NKG grains contained traces of this herbicide residue, which was not surprising, since glyphosate had been applied, although very early in the crop cycle. As highlighted by Cuhra (2015), published data on glyphosate residues in glyphosate-tolerant crops are very sparse and concern mainly soybeans. In contrast to the low levels of contamination of the maize grains with glyphosate, high levels of glyphosate and its metabolite AMPA have been reported to accumulate in glyphosate-tolerant soybean plant material (Bøh et al., 2014).

A close examination of the data obtained for the pellets shows that for environmental contaminants whose concentrations exceed the LOQ, levels were most often far below the respective acceptance references values. The levels were more than twice lower than the values reported by Mesnage et al. (2015) in their recent publication dealing with the potential occurrence of environmental toxic substances in rodent diets. However, there are two points that need to be taken into account. First, all diets of the present experiment contain quantifiable amounts of piperonyl butoxide (between 0.2 and 0.5 mg/kg), a compound used to enhance the efficiency of insecticides. While piperonyl butoxide does not yet have a harmonized classification in Europe, this pesticide synergist is considered an acute toxicant as category III by oral and dermal and category IV by inhalation exposure routes by the US Environmental Protection Agency. Furthermore, a recent publication (Vardavas et al., 2016) indicates that piperonyl butoxide, at relatively low doses, causes liver and kidney inflammation and induces genotoxicity in rabbits. Secondly, low amounts of several toxic substances, that considered individually do not raise any safety concern, were detected. The risks that may result from a mixture of toxic substances present at low levels in the diets remain unknown at the present time.

Surprisingly, when comparing the contamination status of maize grains and the corresponding pellets, several discrepancies can be evidenced. The only consistent positive correlation between the amount of toxic substances in grains and pellets is observed in the case of fumonisins. As shown in Fig. 2A, the predicted amounts of fumonisins in pellets calculated using the amounts quantified in grains show a strong correlation ($R^2 = 0.99$) with the amounts experimentally quantified in the pellets. For the other environmental contaminants quantified in grains, no clear relationship with the pellet data could be established. This lack of correlation is clearly illustrated in Fig. 2B for deoxynivalenol. The data for glyphosate were also surprising. Indeed, all diets were found to contain glyphosate, with slightly higher levels in NKG-based pellets, while glyphosate had only been detected in NKG grains. Similarly, all diets were characterized by the presence of Pb, while this heavy metal had only been quantified in ISONK and NK harvests. Lastly, some environmental contaminants that had not been detected in grains were shown to be present in diets. This was the case for insecticide residues, aflatoxins and enniatins as well as Cd and As. An explanation for this lack of consistency could be the sampling procedure of grains. The 500-g aliquot representing a grain sample contained randomly chosen grains from four different positions in each of the 0.5 t large bags, whereas the pellets corresponding to this sample were produced from the entire content of a single large bag. If

environmental contaminants, and in particular contaminants introduced during transport or storage, were not evenly distributed inside a given large bag and between large bags, contaminants found in the pellets may have been absent or below the detection limit in the grain aliquot. Such a sampling effect is expected to be more pronounced for weak contaminations close to the detection limit and less important for substantial, systematic contaminations. Therefore, it is not surprising that the association obtained for fumonisins, which showed the highest level of contamination of all substances, was the only consistent one. An additional explanation could be the introduction of contaminants in pellets through the other ingredients used in the formulation, in particular those derived from wheat and soybean. This may lead to the presence of novel contaminants, for example insecticides, or to additional amounts of the same contaminants, e.g. glyphosate, which is used on a large range of crops. Therefore, it is likely that the presence of glyphosate residues in all diets results from a weak contamination of the soybeans, that residues of storage insecticides were introduced by wheat and wheat products and that additional amounts of aflatoxins, leading to concentrations that in some diets exceeded the LOQ, were incorporated *via* soybeans.

Overall, based on the analytical data reported in this study and the lack of substantial differences in contaminants between GM and non-GM pellets as well as between NK and NKG pellets, we can anticipate that the traces of quantified toxic substances will not influence the comparisons foreseen in the GM90 + feeding trial. Nevertheless, the highly sensitive omics approaches that are planned to be implemented in the GM90$^+$ project with the aim of deciphering the complex (multivariate) physiological response pattern of rats when exposed to diets might reveal variations between rats fed with the different diets. It can not be excluded that specific compounds in the diets (formulated with 33% of maize) including contaminants below toxicological levels could interact with the rat physiology. Furthermore, very little is known regarding the potential toxicity that could result from the co-exposure to a mixture of contaminants, even though concentration of each individual contaminant is below its respective toxicological value (Abolaji et al., 2017).

In addition, our study gives yet again further evidence that it is not sufficient to analyze the different grain lots used for the production of otherwise identical pellets, but that it is essential to check the diets actually fed to rats for environmental contaminants. While a grain analysis focusing on a core set of parameters may help to save time and money by excluding grain lots prior to the pellet production process, this first step must be followed by a comprehensive characterization of the produced pellets.

Compliance with ethical standards

Conflict of interest

Conflicts of interest of the principal French investigators are declared on the public RiskOGM program website (http://recherche-riskogm.fr/en/page/partners-pdis).

Conflicts of interest of German investigators are declared on the public G-TwYST program website (https://www.g-twyst.eu/reports/dois).

Ethical approval

The GMO plant samples followed dedicated laboratory procedures concerning their identification and destruction. While this article is related to a project involving animals (Study approved by French Ethical Committee CETEA), it does not contain any study with animals performed by any of the authors.
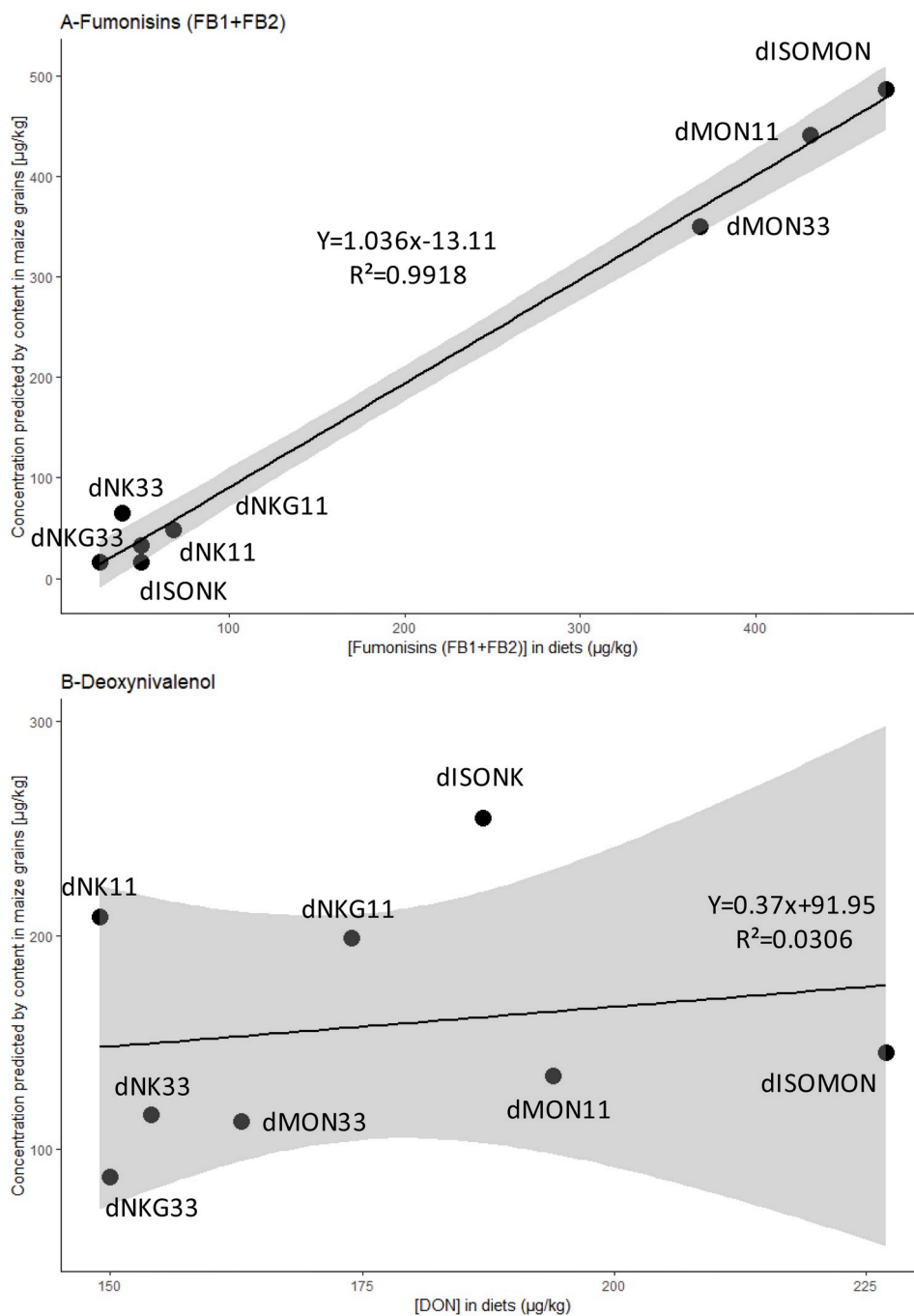
Fig. 2. Relationships between predicted concentrations of fumonisins (A) and deoxynivalenol (B) in maize-based pellets estimated with the values quantified in maize grains and actually quantified concentrations in pellets. Gray shadows represent confidence intervals for the regression.

Transparency document

Transparency document related to this article can be found online at

https://doi.org/10.1016/j.fct.2018.09.049.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.09.049.

List of Abbreviations

GM	Genitically modified
MRL	Maximum Residue Level
Bt	Bacillus thuringiensis
GMO	Genitically Modified Organism

GMP	Genetically Modified Plant
OECD	Organization for Economic Cooperation and Development
POPs	Persistent Organic Pollutants
Pb	Lead
Cadmium Cd	
Arsenic	As
Hg	Mercury
PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	PolyChlorinated Biphenyl
ESI	ElectroSpray Ionisation
HPLC	High Performance Liquid Chromatography
MS	Mass Spetrometry
PCR	Polymerase Chain Reaction
NOAEL	No Observed Adverse Effect Level
ADI	Acceptable Daily Intake
TWI	Tolerable Weekly Intake
HQ	Hazard Quotient
LOQ	Limit Of Quantification

References

Abbas, H., Zablotowicz, R., Weawer, M., Shier, T., Bruns, A., Bellaloui, N., Accinelli, C., Abel, C., 2013. Implications of Bt traits on Mycotoxin contamination in Maize: overview and recent experimental results in southern United States. J. Agric. Food Chem. 61, 11759–11770. https://doi.org/10.1021/jf400754g.

Abolaji, A.O., Awogbindin, I.O., Adedara, I.A., Farombi, E.O., 2017. Insecticide chlorpyrifos and fungicide carbendazim, common food contaminants mixture, induce hepatic, renal, and splenic oxidative damage in female rats. Hum. Exp. Toxicol. 36 (5), 483–493. https://doi.org/10.1177/0960327116652459.

Bakan, B., Melcion, D., Richard-Molard, D., Cahagnier, B., 2002 Feb 13. Fungal growth and fusarium mycotoxin content in isogenic traditional maize and genetically modified maize grown in France and Spain. J. Agric. Food Chem. 50 (4), 728–731. https://doi.org/10.1021/jf0108258.

Bernillon, S., Maucourt, M., Deborde, C., Chéreau, S., Jacob, D., Priymenko, N., Laporte, B., Coumoul, X., Salles, B., Rogowsky, P., Richard-Forget, F., Moing, A., 2018. Characterization of GMO or glyphosate effects on the composition of maize grain and maize-based diet for rat feeding. Metabolomics 14 (3), 36. https://doi.org/10.1007/s11306-018-1329-9.

Bøh, T., Cuhra, M., Traavik, T., Sanden, M., Fagan, J., Primiceri, R., 2014. Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans. Food Chem. 153, 207–215. https://doi.org/10.1016/j.foodchem.2013.12.054.

Chen, L., Sun, Z., Liu, Q., Zhong, R., Tan, S., Yang, X., Zhang, H., 2016. Long-term toxicity study on genetically modified maize with cry1Ac gene in a Wuzhishan miniature pig model. J. Sci. Food Agric. 96 (12), 4207–4214. https://doi.org/10.1002/jsfa.7624.

Chulze, S.N., 2010. Strategies to reduce mycotoxin levels in maize during storage: a review. Food Addit. Contam. 27 (5), 651–657. https://doi.org/10.1080/19440040903573032.

Clarke, R., Connolly, L., Frizzell, C., Elliott, C.T., 2015. Challenging conventional risk assessment with respect to human exposure to multiple food contaminants in food: a case study using maize. Toxicol. Lett. 238, 54–64. https://doi.org/10.1016/j.toxlet.2015.07.006.

Cuhra, M., 2015. Review of GMO safety assessment studies: glyphosate residues in Roundup ready crops is an ignored issue. Environ. Sci. Eur. 27, 20. https://doi.org/10.1186/s12302-015-0052-7.

Delaney, B., Karaman, S., Roper, J., Hoban, D., Sykes, G., Mukerji, P., Frame, S., 2013. Thirteen week rodent feeding study with grain from molecular stacked trait lepidopteran and coleopteran protected (DP-ØØ4114-3) maize. Food Chem. Toxicol. 54, 417–427. https://doi.org/10.1016/j.fct.2012.12.002.

Domingo, J.L., 2016. Safety assessment of GM plants: an updated review of the scientific literature. Food Chem. Toxicol. 95, 12–18. https://doi.org/10.1016/j.fct.2016.06.013.

EFSA, 2012. Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured Data. EFSA Journal 10 (3), 2579. https://doi.org/10.2903/j.efsa.2012.2579.

Fang, J., Feng, Y., Zhi, Y., Zhang, L., Zhou, Y., Jia, X., 2017. A 90-day toxicity study of GmTMT transgenic maize in Sprague-Dawley rats. Regul. Toxicol. Pharmacol. 85, 48–54. https://doi.org/10.1016/j.yrtph.2017.01.004.

ISAAA, 2016. Global Status of Commercialized Biotech/GM Crops: 2016. ISAAA Brief No. 52. Ithaca, NY. ISBN: 978-1-892456-66-4.

Kacalkova, L., Tlustos, P., 2011. The uptake of persistent organic pollutants by plants. Cent. Eur. J. Biol. 6 (2), 223–235. https://doi.org/10.2478/s11535-010-0116-z.

Koch, M.S., Ward, Jason M., Levine, Steven L., Baum, James A., Vicini, John L., Hammond, Bruce G., 2015. The food and environmental safety of Bt crops. Front. Plant Sci. 6, 283. https://doi.org/10.3389/fpls.2015.00283.

Liu, P., He, X., Chen, D., Luo, Y., Cao, S., Song, H., Liu, T., Huang, K., Xu, W., 2012. A 90-day subchronic feeding study of genetically modified maize expressing Cry1Ac-M protein in Sprague-Dawley rats. Food Chem. Toxicol. 50, 3215–3221. https://doi.org/10.1016/j.fct.2012.06.009.

Magan, N., Aldred, D., 2007. Post-harvest control strategies: minimizing mycotoxins in the food chain. Int. J. Food Microbiol. 119, 131–139. https://doi.org/10.1016/j.ijfoodmicro.2007.07.034.

Mesnage, R., Defarge, N., Rocque, L.M., Spiroux de Vendômois, J., Séralini, G.E., 2015. Laboratory rodent diets contain toxic levels of environmental contaminants: implications for regulatory tests. PLoS One 10 (7). https://doi.org/10.1371/journal.pone.0128429.

OECD, 2002. Consensus document on compositional considerations for new varieties of maize (Zea mays). Key Food and Feed Nutrients, Antinutrienst and Secondary Plant Metabolites. Organisation for Economic Cooperation and Development (OECD), Paris, France. www.oecd.org/dataoecd/15/63/46815196.pdf.

Ostry, V., Ovesna, J., Skarkova, J., Pouchova, V., Ruprich, J., 2010. A review on comparative data concerning Fusarium mycotoxins in Bt maize and non-Bt isogenic maize. Mycotoxin Res. 26, 141–145. https://doi.org/10.1007/s12550-010-0056-5.

Picot, A., Barreau, C., Pinson-Gadais, L., Caron, D., Lannou, C., Richard-Forget, F., 2010. Factors of the Fusarium verticillioides-maize environment modulating fumonisin production. Crit. Rev. Microbiol. 36, 221–231. https://doi.org/10.3109/10408411003720209.

Rodrigues, I., Naehrer, K., 2012. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. Toxins 4 (9), 663–675. https://doi.org/10.3390/toxins4090663.

Schatzmayr, G., Streit, E., 2013. Global occurrence of mycotoxins in the food and feed chain: facts and figures. World Mycotoxin J. 6 (3), 213–222. https://doi.org/10.3920/WMJ2013.1572.

Sørensen, J.L., Nielsen, K.F., Rasmussen, P.H., Thrane, U., 2008. Development of a LC-MS/MS method for the analysis of enniatins and beauvericin in whole fresh and ensiled maize. J. Agric. Food Chem. 56 (21), 10439–10443. https://doi.org/10.1021/jf802038b.

Sulyok, M., Berthiller, F., Krska, R., Schuhmacher, R., 2006. Development and validation of a liquid chromatography tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. RCM (Rapid Commun. Mass Spectrom.) 20, 2649–2659. https://doi.org/10.1002/rcm.2640.

Vardavas, A.;I., Stivaktakis, P.D., Tzatzarakis, M.N., Fragkiadaki, P., Vasilaki, F., Tzardi, M., Datseri, G., Tsiaoussis, J., Alegakis, A.K., Tsitsimpikou, C., Rakitskii, V.N., Carvalho, F., Tsatsakis, A.M., 2016. Long-term exposure to cypermethrin and piperonl butoxide cause liver and kidney inflammation and induce genotoxicity in New Zealand white male rabbits. Food Chem. Toxicol. 94, 250–259. https://doi.org/10.1016/j.fct.2016.06.016.

Wang, Q.R., Liu, X.M., Cui, Y.S., Dong, Y.T., Christie, P., 2002. Responses of legume and non-legume crop species to heavy metals in soils with multiple metal contamination. J. Environ. Sci. Health - Part A Toxic/Hazard. Subst. Environ. Eng. 37 (4), 611–621. https://doi.org/10.1081/ESE-120003241.

Zeljenková, D., Ambrušová, K., Bartušová, M., Kebis, A., Kovrižnych, J., Krivošíková, Z., Kuricová, M., Líšková, A., Rollerová, E., Spustová, V., Szabová, E., Tulinská, J., Wimmerová, S., Levkut, M., Révajová, V., Ševčíková, Z., Schmidt, K., Schmidtke, J., La Paz, J.L., Corujo, M., Pla, M., Kleter, G.A., Kok, E.J., Sharbati, J., Hanisch, C., Einspanier, R., Adel-Patient, K., Wal, J.M., Spök, A., Pöting, A., Kohl, C., Wilhelm, R., Schiemann, J., Steinberg, P., 2014. Ninety-day oral toxicity studies on two genetically modified maize Mon810 varieties in Wistar Han RCC rats (EU 7th Framework Programme project GRACE). Arch. Toxicol. 88, 2289–2314. https://doi.org/10.1007/s00204-014-1374-8.

Zeljenková, D., Aláčová, R., Ondrejková, J., Ambrušová, K., Bartušová, M., Kebis, A., Kovrižnych, J., Rollerová, E., Szabová, E., Wimmerová, S., Černák, M., Krivošíková, Z., Kuricová, M., Líšková, A., Spustová, V., Tulinská, J., Levkut, M., Révajová, V., Ševčíková, Z., Schmidt, K., Schmidtke, J., Schmidt, P., La Paz, J.L., Corujo, M., Pla, M., Kleter, G.A., Kok, E.J., Sharbati, J., Bohmer, M., Bohmer, N., Einspanier, R., Adel-Patient, K., Spök, A., Pöting, A., Kohl, C., Wilhelm, R., Schiemann, J., Steinberg, P., 2016. One-year oral toxicity study on a genetically modified maize Mon810 variety in Wistar Han RCC rats (EU 7th Framework Programme project GRACE). Arch. Toxicol. 90, 2531–2562. https://doi.org/10.1007/s00204-016-1798-4.

Zhu, Y., He, X., Luo, Y., Zou, X., Huang, K., Xu, W., 2013. A 90-day feeding study of glyphosate-tolerant maize with G2-aroA gene in Sprague-Dawley rats. Food Chem. Toxicol. 51, 280–287. https://doi.org/10.1016/j.fct.2012.09.008.