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Assessment of genetically modified maize MON 95379 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2020-170)

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Abstract

Genetically modified maize MON 95379 was developed to confer insect protection against certain lepidopteran species. These properties were achieved by introducing the *cry1B.868* and *cry1Da_7* expression cassettes. The molecular characterisation data and bioinformatic analyses do not identify issues requiring food/feed safety assessment. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize MON 95379 and its conventional counterpart needs further assessment. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Cry1B.868 and Cry1Da_7 proteins as expressed in maize MON 95379. The GMO Panel finds no evidence that the genetic modification impacts the overall safety of maize MON 95379. In the context of this application, the consumption of food and feed from maize MON 95379 does not represent a nutritional concern in humans and animals. Therefore, no post-market monitoring of food/feed is considered necessary. In the case of accidental release of viable maize MON 95379 grains into the environment, this would not raise environmental safety concerns. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of maize MON 95379. The GMO Panel concludes that maize MON 95379 is as safe as its conventional counterpart and the tested non-GM maize varieties with respect to potential effects on human and animal health and the environment.

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Summary

Following the submission of application EFSA-GMO-NL-2020-170 under Regulation (EC) No 1829/2003 from Bayer Agriculture BV (referred to hereafter as 'the applicant'), the Panel on Genetically Modified Organisms of the European Food Safety Authority (referred to hereafter as 'GMO Panel') was asked to deliver a Scientific Opinion on the safety of genetically modified (GM) insect protected maize (*Zea mays* L.) MON 95379 according to Regulation (EU) No 503/2013. The scope of application EFSA-GMO-NL-2020-170 is for import, processing and food and feed uses within the European Union (EU) of maize MON 95379 and does not include cultivation in the EU.

In this scientific opinion, the GMO Panel reports on the outcome of its risk assessment of maize MON 95379 according to the scope of the application EFSA-GMO-NL-2020-170. The GMO Panel conducted the assessment of maize MON 95379 in line with the principles described in Regulation (EU) No 503/2013 and its applicable guidelines for the risk assessment of GM plants. The molecular characterisation data establish that maize MON 95379 contains a single insert consisting of one copy of the *cry1B.868* and *cry1Da_7* expression cassettes. The quality of the sequencing methodology and data sets was assessed by the EFSA GMO Panel and is in compliance to the requirements listed in the EFSA Technical Note. Updated bioinformatics analyses of the sequences encoding the newly expressed proteins and open reading frames (ORFs) present within the insert or spanning the junctions between the insert and genomic DNA, do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the Cry1B.868 and Cry1Da_7 proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-derived Cry1B.868 and Cry1Da_7 proteins indicate that these proteins are equivalent, and the microbe-derived proteins can be used in the safety studies.

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize MON 95379 and its conventional counterpart needs further assessment. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Cry1B.868 and Cry1Da_7 proteins as expressed in maize MON 95379. The GMO Panel finds no evidence that the genetic modification impacts the overall safety of maize MON 95379. In the context of this application, the consumption of food and feed from maize MON 95379 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize MON 95379 is as safe as the conventional counterpart and non-GM maize varieties tested, and no post-market monitoring of food/feed is considered necessary.

Considering the introduced traits, the outcome of the agronomic and phenotypic analysis and the routes and levels of exposure, maize MON 95379 would not raise safety concerns in the case of accidental release of viable GM maize grains into the environment. The post-market environmental monitoring (PMEM) plan and reporting intervals are in line with the intended uses of maize MON 95379.

The GMO Panel considered the overall quality of the performed literature searches acceptable. The literature searches did not identify any relevant publications on maize MON 95379.

The GMO Panel concludes that maize MON 95379 is as safe as its conventional counterpart and the tested non-GM maize reference varieties with respect to potential effects on human and animal health and the environment.

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1. Introduction

The scope of the application EFSA-GMO-NL-2020-170 is for food and feed uses, import and processing of maize MON 95379 and does not include cultivation in the European Union (EU).

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

On 30 November 2020, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands application EFSA-GMO-NL-2020-170 for authorisation of maize MON 95379 (Unique Identifier MON-95379-3), submitted by Bayer Agriculture BV (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003¹. Following receipt of application EFSA-GMO-NL-2020-170, EFSA informed EU Member States and the European Commission, and made the application available to them. Simultaneously, EFSA published the summary of the application.²

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013³, with the EFSA guidance documents, and, when needed, asked the applicant to supplement the initial application. On 29 March 2021, EFSA declared the application valid.

From the validity date, EFSA and its Panel on Genetically Modified Organisms (hereafter referred to as 'the GMO Panel') endeavoured to respect a time limit of six months to issue a scientific opinion on application EFSA-GMO-NL-2020-170. Such time limit was extended whenever EFSA and/or its GMO Panel requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU Member States and to European Commission (for further details, see the section 'Documentation', below). In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC.⁴ The EU Member States had three months to make their opinion known on application EFSA-GMO-NL-2020-170 as of date of validity.

1.2. Terms of Reference as provided by the requestor

According to Articles 6 and 18 of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of maize MON 95379 in the context of its scope as defined in application EFSA-GMO-NL-2020-170.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation including the opinions of the nominated risk assessment bodies of the EU Member States.⁵ In addition to the present scientific opinion, EFSA was also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No 1829/2003, but not to give an opinion on them because they pertain to risk management.⁶

2. Data and Methodologies

2.1. Data

The GMO Panel based its scientific risk assessment of maize MON 95379 on the valid application EFSA-GMO-NL-2020-170, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by the Member States and relevant peer-reviewed scientific publications.

¹ Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, pp. 1–23.

² Available online: <https://open.efsa.europa.eu/questions/EFSA-Q-2020-00786>

³ Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, pp. 1–48.

⁴ Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, pp. 1–38.

⁵ Opinions of the nominated risk assessment bodies of EU Member States can be found at the Open EFSA Portal <https://open.efsa.europa.eu/questions>, querying the assigned Question Number.

⁶ These particulars are available online at: <https://open.efsa.europa.eu/study-inventory/EFSA-Q-2020-00786>

2.2. Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 503/2013, the applicable guidelines (i.e. EFSA GMO Panel, 2010a, 2011a,b, 2015, 2017; EFSA Scientific Committee, 2011) and explanatory notes and statements (i.e. EFSA, 2010, 2014, 2017, 2018, 2019a,b; EFSA GMO Panel, 2010b, 2018) for the risk assessment of GM plants.

For this application, in the context of the contracts OC/EFSA/GMO/2018/04, OC/EFSA/GMO/2018/02, OC/EFSA/GMO/2020/01 and EOI/EFSA/SCIENCE/2020/01–CT02GMO, the contractors performed preparatory work for the evaluation of the applicant's literature search, methods applied for the statistical analysis, completeness and quality of DNA sequencing information and statistical analysis of the 90-day toxicity study on maize MON 95379.

3. Assessment

3.1. Introduction

Maize MON 95379 expresses Cry1B.868, a chimeric protein containing domains from Cry1A, Cry1B and Cry1C naturally expressed in *Bacillus thuringiensis*, and Cry1Da_7, an optimised version of Cry1Da carrying four amino acids substitutions to increase its activity. The two Cry proteins expressed in maize MON 95379 provide protection against targeted lepidopteran pests including fall armyworm (*Spodoptera frugiperda*), sugarcane borer (*Diatraea saccharalis*) and corn earworm (*Helicoverpa zea*).

3.2. Systematic literature review as requested by Commission Regulation (EU) No 503/2013

The GMO Panel assessed the applicant's literature searches on maize MON 95379, which include a scoping review, according to the guidelines given in EFSA (2010, 2019b).

A systematic review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of application EFSA-GMO-NL-2020-170. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for maize MON 95379 at present. The GMO Panel considered the overall quality of the performed literature searches acceptable.

The literature searches did not identify any relevant peer-reviewed publications on maize MON 95379.

3.3. Molecular characterisation⁷

3.3.1. Transformation process and vector constructs

Maize MON 95379 was developed by a two-step process. In the first step, immature embryos of maize inbred line LH244 were co-cultured with a disarmed *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*) strain ABI containing the vector PV-ZMIR522223. In the second step, selected R2 lines were crossed with maize inbred LH244 line expressing Cre recombinase, which had been transformed with vector PV-ZMOO513642. In the resulting plants, the CP4 EPSPS cassette was excised by the Cre recombinase, and the Cre gene was subsequently segregated away, through conventional breeding, to obtain maize MON 95379. The plasmid PV-ZMIR522223 used for the transformation contains three expression cassettes between the right and left border of the T-DNA, containing the following genetic elements:

- The *cp4 epsps* expression cassette consisting of the promoter, 5' UTR and intron sequence of the tubulin (*TubA*) gene from *Oryza sativa*, the chloroplast transit peptide (TS-CTP2) of the *shkG* gene from *Arabidopsis thaliana*, the coding sequence of the *aroA* gene from *Agrobacterium* sp. encoding the CP4 EPSPS protein and the 3' untranslated sequence of the α -tubulin (*TubA*) gene from *Oryza sativa*. The cassette is flanked by *loxP* sites from bacteriophage P1.
- The *cry1B.868* expression cassette consisting of the promoter, 5' untranslated sequence and first intron sequences of the ubiquitin (*Ubq*) gene from *Zea mays*, the coding sequence of the Cry1B.868 gene from *B. thuringiensis* and the 3' untranslated sequence of a lipid transfer protein-like gene (LTP) from *Oryza sativa*.

⁷ Dossier: Part II–Section 1.2; additional information provided: 6 April 2021, 20/08/2021, 11 March 2021, 21/01/2022, 5 October 2022; spontaneous information: 6 April 2021.

- The *cry1Da_7* expression cassette consisting of the enhancer from the 35 S RNA of figwort mosaic virus (FMV), the promoter and 5' UTR sequences of the tonoplast membrane integral protein (*Tip*) gene from *Setaria italica*, the intron and flanking UTR sequence from the Actin 15 (*Act 15*) gene from *Oryza sativa*, the codon optimised coding sequence of the *cry1Da_7* gene from *B. thuringiensis*, and the 3' untranslated sequence of the *GOS2* gene from *Oryza sativa*.

The transformation vector PV-ZMOO513642, used to generate the line expressing the Cre recombinase, contains two expression cassettes between the right and left border of the T-DNA, containing the following genetic elements:

- The *cre* expression cassette consisting of the promoter, leader, intron and flanking 5' untranslated sequence of the *act1* gene from *Oryza sativa*, two partial regions of the *cre* recombinase gene from bacteriophage P1 interrupted by the second intron sequence from the light inducible (*LS1*) gene from *Solanum tuberosum* and the 3' untranslated sequence of the *Hsp17* gene from *Triticum aestivum*.
- The *nptII* cassette consisting of the 35 S promoter and leader sequence from cauliflower mosaic virus (CaMV), the coding sequence of the *nptII* gene from *Escherichia coli* and the 3' untranslated sequence of the *nos* gene from *Agrobacterium tumefaciens*.

The vector backbone contained elements necessary for the maintenance and selection of the plasmid in bacteria.

3.3.2. Transgene constructs in the GM plant

Molecular characterisation of maize MON 95379 was performed by next generation sequencing (NGS) and junction sequence analysis (JSA) in order to determine insert copy number and to confirm the absence of PV-ZMIR522223 plasmid backbone and the entire PV-ZMOO513642 plasmid, and NGS sequencing on PCR amplified fragments to determine size and organisation of the inserted sequences. Overall, the quality of the sequencing methodology and data sets was assessed by the EFSA GMO Panel and is in compliance with the requirements listed in the EFSA Technical Note (EFSA GMO Panel, 2018).

NGS/JSA of the whole genome indicated that maize MON 95379 contains a single insert, consisting of a single copy of the T-DNA in the same configuration as in the PV-ZMIR522223 transformation vector with the exception that *cp4 epsps* and one *loxP* site are absent. NGS/JSA also confirmed the absence of plasmid backbone sequences in the maize genome.

The nucleotide sequence of the entire insert of maize MON 95379 together with 1,000 bp of the 5' and 1,000 bp of the 3' flanking regions was determined. The insert of 13,322 bp is identical to the T-DNA of PV-ZMIR522223 vector, with the exceptions of the removed CP4 EPSPS cassette and the border regions: the entire right border region (330 bp) is absent in maize MON 95379, whereas the 5' end of the left border is deleted, with only 185 bp remaining identical to PV-ZMIR522223 plasmid.

A comparison with the pre-insertion locus indicated that 160 bp was deleted from the maize genomic DNA. The possible interruption of known endogenous maize genes by the insertion in maize MON 95379 was evaluated by bioinformatics analyses of the pre-insertion locus and of the genomic sequences flanking the insert. The results of these analyses do not indicate the interruption of any known endogenous gene in maize MON 95379.

The results of segregation (see Section 3.3.5) and bioinformatics analyses are compatible with a single insertion in the nuclear genome.

Updated bioinformatics analyses of the amino acid sequence of the newly expressed Cry1B.868 and Cry1Da₇ proteins reveal no significant similarities to toxins and allergens. In addition, updated bioinformatic analyses of the newly created open reading frames (ORFs) within the insert and spanning the junctions between the insert and genomic DNA indicated that two ORFs (frame 1_80 and frame 3_105) exceeded the allergenicity assessment threshold of 35% identity using an 80 amino acid sliding window approach. Both ORFs are found within the transcriptional unit of Cry1Da₇ coding sequence, but in a different reading frame and do not contain a start codon. In conclusion, these analyses indicated that the expression of any ORF showing significant similarities to toxins or allergens in maize MON 95379 is unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis for maize MON 95379 to microbial DNA. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.6.1.2.

3.3.3. Protein characterisation and equivalence

Maize MON 95379 expresses two new proteins, Cry1B.868 and Cry1Da_7, which confer protection against lepidopteran insects. Given the technical constraints in producing large enough quantities from plants, this protein was recombinantly produced in *B. thuringiensis*. A set of biochemical methods was employed to demonstrate the equivalence between the maize MON 95379 and *Bt*-derived Cry1B.868 and Cry1Da_7. Purified proteins from these two sources were characterised and compared in terms of their biochemical, structural and functional properties.

Cry1B.868 protein characterisation and equivalence

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that both plant and microbe-produced Cry1B.868 proteins had the expected molecular weight of ~126.8 kDa and were comparably immunoreactive to Cry1B.868 protein-specific antibodies. Glycosylation analysis demonstrated that none of the Cry1B.868 proteins were glycosylated. Amino acid sequence analysis of the intact plant-derived Cry1B.868 protein by mass spectrometry (MS) methods showed that the protein matched the deduced sequence as defined by the *cry1B.868* gene present in maize MON 95379. In addition, the MS data showed that the N-terminal methionine was truncated. Such modifications are common in eukaryotic proteins (e.g. Polevoda and Sherman, 2000). Functional equivalence was demonstrated by an insect feeding bioassay which showed that plant and microbe-derived Cry1B.868 protein samples had comparable insecticidal activity.

The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-derived Cry1B.868 proteins indicate that these proteins are equivalent. Therefore, the GMO Panel accepts the use of the Cry1B.868 protein produced in bacteria for the safety studies.

Cry1Da_7 protein characterisation and equivalence

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that both plant and microbe-produced Cry1Da_7 proteins had the expected molecular weight of ~132.1 kDa and were comparably immunoreactive to Cry1Da_7 protein-specific antibodies. Glycosylation analysis demonstrated that none of the two proteins were glycosylated. Amino acid sequence analysis of the intact plant-derived Cry1Da_7 protein by mass spectrometry (MS) methods showed that the protein matched the deduced sequence as defined by the *cry1Da_7* gene present in maize MON 95379. In addition, the MS data showed that the N-terminal methionine was truncated. Such modifications are common in eukaryotic proteins (e.g. Polevoda and Sherman, 2000). Functional equivalence was demonstrated by an insect feeding bioassay which showed that plant and microbe-derived Cry1Da_7 protein samples had comparable insecticidal activity.

The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-derived Cry1Da_7 proteins indicate that these proteins are equivalent. Therefore, the GMO Panel accepts the use of the Cry1Da_7 protein produced in bacteria for the safety studies.

3.3.4. Information on the expression of the insert

Protein levels of Cry1B.868 and Cry1Da_7 were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested in a field trial across five locations in the USA during the 2018 growing season. Samples analysed included forage (R5) and grain (R6).⁸ The mean values, standard errors and ranges of protein expression levels in grains (n = 20) and forage (n = 20) of the Cry1B.868 and Cry1Da_7 proteins used to estimate human and animal dietary exposure (see Section 3.5.5) are reported in Table 1.

Table 1: Mean values, standard errors and ranges of newly expressed proteins [$\mu\text{g/g}$ dry weight (dw) and $\mu\text{g/g}$ fresh weight (fw)] in grains and forage from maize MON 95379 (n = 20)

	Tissues	
	$\mu\text{g/g}$ dry weight (dw)	$\mu\text{g/g}$ fresh weight (fw)
Grain (R6)		
Cry1B.868	26 ^(a) \pm 3.5 ^(b) (7.8–77) ^(c)	23 \pm 3.1 (6.9–68)

⁸ BBCH scale describes phenological stages. BBCH 85–87 corresponds to approximately R5 stage of maize development and BBCH 87–99 corresponds to R6.

	Tissues	
	$\mu\text{g/g}$ dry weight (dw)	$\mu\text{g/g}$ fresh weight (fw)
Cry1Da_7	0.25 \pm 0.032 (0.13–0.64)	0.22 \pm 0.028 (0.11–0.56)
Forage (R5)		
Cry1B.868	110 \pm 8.2 (50–170)	32 \pm 2.5 (15–52)
Cry1Da_7	26 \pm 2.1 (13–50)	7.8 \pm 0.63 (3.8–15)

(a): Mean value.

(b): Standard error.

(c): Range.

3.3.5. Inheritance and stability of inserted DNA

Genetic stability of maize MON 95379 insert was assessed using NGS to sequence the insert and the flanking regions from five generations (F4, F5, F4F1, F5F1 and F6F1) while segregation analysis was performed by PCR-based analysis from three consecutive generations (F4F2, F4F3 and F4F4). The results indicate that all the plants tested retained the single copy of the insert and flanking regions, which were stably inherited in subsequent generations. The results support the presence of a single insertion, segregating in a Mendelian fashion.

3.3.6. Conclusion on molecular characterisation

The molecular characterisation data establish that maize MON 95379 contains a single insert consisting of one copy of the *cry1B.868* and *cry1Da_7* expression cassettes. Bioinformatics analyses of the sequences encoding the newly expressed proteins and other ORFs within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the Cry1B.868 and Cry1Da_7 proteins is considered adequate. The protein characterisation data comparing the structural, biochemical and functional properties of plant and microbe-derived Cry1B.868 and Cry1Da_7 proteins indicate that these proteins are equivalent and the microbe-derived proteins can be used in the safety studies.

3.4. Comparative analysis⁹

3.4.1. Overview of studies conducted for the comparative analysis

Application EFSA-GMO-NL-2020-170 presents data on agronomic and phenotypic characteristics, as well as on forage and grain/seed composition of maize MON 95379 (Table 2).¹⁰

Table 2: Main comparative analysis studies to characterise the maize MON 95379 provided in application EFSA-GMO-NL-2020-170

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic and phenotypic analysis	Field study, U.S., 2018, eight sites ^(a)	LH244 \times HCL617	17 ^(b)
Compositional analysis			

GM: Genetically modified.

(a): The field trials were located in Shelby, IA; Boone, IA; Jefferson IA; Clinton, IL; Champaign, IL; Clinton, IN; York, NE and Miami, OH.

⁹ Dossier: Part II – Section 1.3; additional information: 4 June 2021.

¹⁰ A study on pollen viability and morphology was also provided and considered by the GMO Panel as additional. (MSL0030195. Pollen Viability and Morphology Evaluation of Maize MON 95379 Grown in a 2018 U. S. Field Trial. Monsanto Company).

(b): Non-GM hybrid maize with their corresponding comparative relative maturity indicated in brackets were Agrigold A6267 (107), Agrigold A6472 (110), Agrigold A6574 (114), Dekalb DKC61-52 (111), Dekalb DKC62-06 (112), Dekalb DKC64-85 (114), Golden Harvest G09C43 (109), Golden Harvest G12J11-A (112), Kruger K-0708 (108), Lewis 1407 (107), Lewis 1613 (113), LG Seeds LG2549 (109), LG Seeds LG2636 (114), Mycogen Seeds 2H721 (112), Mycogen Seeds MY09V40 (109), Mycogen Seeds NH6769 (115) and Stone 5820 (108).

3.4.2. Experimental field trial design and statistical analysis

At each field trial site, the following materials were grown in a randomised complete block design with four replicates: maize MON 95379, the comparator LH244 × HCL617 and four non-GM reference varieties. The agronomic, phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010b, 2011a). This includes the application of a difference test (between the GM maize and the non-GM comparator) and an equivalence test (between the GM maize and the set of non-GM commercial reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).¹¹

3.4.3. Suitability of selected test materials

3.4.3.1. Selection of the test materials

As described in Section 3.3.1, inbred line LH244 was transformed to obtain MON 95379, which was then crossed with the inbred line HCL617 to produce the hybrid maize MON 95379 used in the comparative analysis.

The comparator used in the field trials is the non-GM maize hybrid LH244 × HCL617, which is isogenic to maize MON 95379 (as documented by the pedigree), and is therefore considered to be the conventional counterpart.

Maize MON 95379 and the conventional counterpart, both with a comparative relative maturity (CRM) of 111, which is considered appropriate for growing in environments across U.S., where the comparative field trials were conducted.

Commercial non-GM reference varieties with a CRM ranging from 107 to 115 were selected by the applicant and, at each selected site, four reference varieties were tested (see Table 2). On the basis of the provided information on relative maturity classes, the GMO Panel considers the selected non-GM reference varieties appropriate for the comparative assessment.

3.4.3.2. Seed production and quality

Seeds of maize MON 95379 and the conventional counterpart used in the 2018 field trials were produced from plants free of diseases, harvested and stored under similar conditions, before being sown in the field trial sites. The seed lots were verified for their identity via event-specific quantitative PCR analysis.

The grains were tested for their germination capacity at optimal and suboptimal temperature conditions.¹² Germination capacity of the GM maize MON 95379 was compared with the one of its comparators and the results¹³ of these studies indicate that the seed germination of maize MON 95379 was not different than that of its comparator.

3.4.3.3. Conclusion on suitability

The GMO Panel is of the opinion that the maize MON 95379, the conventional counterpart and the non-GM maize reference varieties were properly selected and are of adequate quality. Therefore, the test materials are considered suitable for the comparative analysis.

¹¹ In detail, the four outcomes are category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence).

¹² Optimal temperature condition corresponds to 25° and suboptimal 10°C for 7 days followed by 4 days at 25°C.

¹³ GM hybrid maize showed a mean germination of 100% for both temperature conditions while the non-GM comparator showed a mean of 99.5% and 99.3% under optimal and suboptimal temperature conditions, respectively.

3.4.4. Representativeness of the receiving environments

3.4.4.1. Selection of field trial sites

The selected field trial sites were located in commercial maize-growing regions of the United States of America. Climate and soil characteristics of the selected fields were diverse,¹⁴ corresponding to optimal, near optimal and suboptimal conditions for maize cultivation (Sys et al., 1993). Despite a limited variability on the soil texture, the GMO Panel considers that the selected sites, including the subset chosen for the compositional analysis, reflect commercial maize-growing regions in which the test materials are likely to be grown.

3.4.4.2. Meteorological conditions

Maximum and minimum mean temperatures and sum of precipitations were provided on a weekly basis. No exceptional weather conditions were reported at any of the selected sites; therefore, the GMO Panel considers that the meteorological data set falls within the historical range of climatic conditions normally occurring at these sites.

3.4.4.3. Management practices

The field trials included plots containing maize MON 95379, plots with the comparator and plots with non-GM maize reference varieties, mostly managed according to local agricultural practices. Despite not considered a normal agricultural practice, thinning was applied at two field trial sites¹⁵ to achieve a more homogeneous plant density across plots. The GMO Panel considers that the management practices including sowing, harvesting and application of plant protection product were acceptable for the selected receiving environments.

3.4.4.4. Conclusion on representativeness

The GMO Panel concludes that the geographical locations, soil and climatic characteristics, meteorological conditions and most of the management practices are typical for receiving environments where the tested materials could be grown.

3.4.5. Agronomic and phenotypic analysis

Data for 10 agronomic and phenotypic endpoints¹⁶ plus information on abiotic stressors, disease incidence and arthropod damage were collected from the field trial sites (see Table 2). The endpoint fruit count (ears/plant) was not analysed with formal statistical methods because of lack of variability in the data.

The statistical analysis (Section 3.4.2) was applied to nine endpoints, with the following results:

- The test of difference identified statistically significant differences between maize MON 95379 and the comparator for six endpoints (days to flowering, plant height, days to maturity, lodging, final stand count and seed weight). All these endpoints fell under equivalence category I.

3.4.6. Compositional analysis

Forage and grain harvested from the field trials (Table 2) were analysed for 78 constituents (nine in forage and 69 in grain), including those recommended by OECD (OECD, 2002). The statistical analysis as described in Section 3.4.2 was not applied to 15 grain constituents,¹⁷ because their concentration in more than half of the samples was below the limit of quantification.

¹⁴ Soil types of the field trials were silty clay loam, loam and silt loam (covering optimal and near-optimal conditions); soil organic carbon ranged from 1.0% to 2.7% (covering optimal, near-optimal and suboptimal conditions); soil pH ranged from 5.7 to 7.2 (covering optimal and near-optimal and conditions). Average temperatures and sum of precipitations during the usual crop growing season ranged, respectively, from 19.9°C to 24.5°C and from 368 mm to 858 mm.

¹⁵ Clinton, IN and York, NE.

¹⁶ Early stand count, days to flowering, plant height, days to maturity, lodging, fruit count (ears/plant), final stand count, moisture, yield and seed weight.

¹⁷ Caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (14:1), pentadecanoic acid (C15:0), pentadecenoic acid (15:1), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), γ -linolenic acid (18:3), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), sodium and furfural.

The statistical analysis was applied to a total of 63 constituents (nine in forage¹⁸ and 54 in grain¹⁹); a summary of the outcome of the test of difference and the test of equivalence is presented in Table 3.

Table 3: Outcome of the comparative compositional analysis of grains and forage from MON 95379 maize. The table shows the number of endpoints in each category

		Test of difference ^(a)	
		Not different	Significantly different
Test of equivalence ^(b)	Category I/II	31	32 ^(c)
	Category III/IV	–	–
	Not categorised	–	–
	Total endpoints	63	

(a): Comparison between MON 95379 maize and the non-GM comparator.

(b): Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.

(c): Endpoints with significant differences between MON 95379 maize and its non-GM comparator and falling in equivalence category I–II. For grains: carbohydrates, total fat, protein, acid detergent fibre (ADF), manganese, phosphorus, zinc, β -carotene, thiamine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, palmitic acid (C16:0), linoleic acid (C18:2), α -linolenic acid (C18:3), phytic acid and raffinose. For forage: carbohydrates, phosphorus.

For MON 95379 maize, statistically significant differences in the comparison with the non-GM comparator were found for 32 endpoints (two in forage and 30 in grains). All these endpoints fell under equivalence category I or II.

3.4.7. Conclusions of the comparative analysis

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis.

Taking into account the natural variability observed for the set of non-GM reference varieties, the GMO Panel concludes that:

- None of the differences identified in agronomic and phenotypic characteristics between maize MON 95379 and the conventional counterpart needs further assessment regarding potential environmental impact.
- None of the differences identified between maize MON 95379 and the conventional counterpart needs further assessment.

3.5. Food/Feed safety assessment²⁰

3.5.1. Effects of processing

Maize MON 95379 will undergo existing production processes used for conventional maize. No novel production process is envisaged. Based on the outcome of the comparative assessment, processing of the GM maize into food and feed products is not expected to result in products being different from those of conventional non-GM maize varieties.

¹⁸ Moisture, protein, total fat, ash, carbohydrates, acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium and phosphorus.

¹⁹ Proximates and fibre content (ash, carbohydrates, total fat, protein, moisture, acid detergent fibre (ADF), neutral detergent fibre (NDF) and total dietary fibre (TDF)), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, zinc), vitamins (β -carotene, thiamine, riboflavin, niacin, pyridoxine, folic acid, α -tocopherol), amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine), fatty acids (palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α -linolenic acid (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0)) and other compounds (ferulic acid, p-coumaric acid, phytic acid and raffinose).

²⁰ Dossier: Part II – Section 1.4, 1.5, 1.6, 2, 3, 4; additional information: 6 April 2021; 20/08/2021; 21/01/2022; 20/05/2022; 8 May 2022.

3.5.2. Stability of newly expressed proteins

Protein stability is one of several relevant parameters to consider in the weight-of-evidence approach in protein safety (EFSA GMO Panel, 2010c, 2011a, 2017, 2021). The term protein stability encompasses several properties such as thermal stability, pH-dependent stability, proteolytic stability and physical stability (e.g. tendency to aggregate), among others (Li et al., 2019). It has been shown, for example, that when characteristics of known food allergens are examined, a prominent trait attributed to food allergens is protein stability (Helm, 2001; Breiteneder and Mills, 2005; Foo and Mueller, 2021; Costa et al., 2022).

3.5.2.1. Effect of temperature and pH on newly expressed proteins

The applicant provided information on the effects of temperature on Cry1B.868 and Cry1Da₇ proteins. Independent samples of Cry1B.868 and CryDa₇ proteins were incubated for 15 or 30 min at 25°C, 37°C, 55°C, 75°C or 95°C followed by SDS-PAGE or by a bioassay measuring their activity. The studies showed that both Cry1B.868 and CryDa₇ proteins are unstable, evidenced by loss of functional activity and protein degradation, at temperatures $\geq 75^\circ\text{C}$. In relation to the effect of pH on the Cry1B.868 and Cry1Da₇ proteins, the molecular mass and immunoreactivity of the proteins was unchanged at pH 1.2, 7.5 and above 10.

3.5.2.2. *In vitro* protein degradation by proteolytic enzymes

The applicant provided information on *in vitro* protein degradation. Initially, a resistance to degradation by pepsin of the Cry1B.868 and Cry1Da₇ proteins was investigated in solutions at pH ~ 1.2 . The integrity of the test proteins in samples of the incubation mixture taken at various time points was analysed by SDS-PAGE followed by protein staining or by western blotting. The Cry1B.868 and Cry1Da₇ proteins were degraded by pepsin within 0.5 min of incubation. Transient peptide fragments at ~ 4 kDa were observed at different time points by SDS-PAGE. Secondly, the resistance to degradation by pancreatin of the Cry1B.868 and Cry1Da₇ proteins was also analysed in solutions at pH ~ 7.5 . The Cry1B.868 and Cry1Da₇ proteins were partially degraded after 5 min of incubation when analysed by western blotting. Finally, the applicant provided a voluntary study where the Cry1B.868 and Cry1Da₇ proteins were subjected to a sequential digestion, pepsin followed by pancreatin. The transient peptide fragments seen in the pepsin analysis were degraded within 0.5 min of exposure to pancreatin when analysed by SDS-PAGE. The sequential addition of digestive enzymes (i.e. gastric digestion conditions followed by an intestinal *in vitro* digestion) has been proposed as part of several alternative protocols to the classical pepsin resistance test to more closely simulate (within the inherent limitations of *in vitro* models) the physiological conditions of gastrointestinal digestion (EFSA GMO Panel, 2021). This is in line with Codex Alimentarius which indicated that alternative *in vitro* digestion protocols may be used where adequate justification is provided (Codex Alimentarius, 2009).

3.5.3. Toxicology

3.5.3.1. Testing of the newly expressed protein

Two proteins (Cry1B.868 and Cry1Da₇) are newly expressed in maize MON 95379 and were never assessed by the GMO Panel, in the context of its previous opinions, for the safety of humans and animals (i.e. farmed and companion animals).

The potential for a functional interaction among these two proteins has been assessed with regard to human and animal health. These insecticidal proteins act through cellular receptors found in target insect species. It is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high specific affinity to Cry proteins (Hammond et al., 2013; Koch et al., 2015; Jurat-Fuentes and Crickmore, 2017). On the basis of the known biological function of the individual newly expressed proteins, there is currently no expectation for their possible interactions relevant to the food and feed safety of maize MON 95379.

The GMO Panel assessed the safety profile of Cry1B.868 and Cry1Da₇ proteins, taking into account molecular characterisation and bioinformatic analyses (Section 3.3), the history of safe use for consumption of the newly expressed proteins, and *in vitro* (Section 3.5.2) and *in vivo* studies.

i) Molecular characterisation

The plant-produced Cry1B.868 and Cry1Da₇ proteins have been extensively characterised and their equivalence to the microbial-produced proteins was demonstrated (Section 3.3.3).

ii) Bioinformatic studies

No significant similarities of the Cry1B.868 and Cry1Da₇ proteins to toxins were identified (Section 3.3.2).

iii) History of safe use for consumption as food/feed of the newly expressed proteins

iii.a) Information on the source organism

The Cry1B.868 and Cry1Da₇ proteins' gene source organism is a ubiquitous soil bacterium (*B. thuringiensis*) and has been reported to protect plants by producing Bt toxins that inhibit insect and nematode growth; furthermore, Bt microbes are used as sprayed pesticide for pest control in agriculture.

iii.b) Information on structure, function and mode of action of the new proteins

Cry1B.868 and Cry1Da₇ are chimeric proteins combining domains from *B. thuringiensis* (Bt) Cry proteins (domain I and II from Cry 1Be; domain 3 from Cry1Ca and C-terminus from Cry1Ab). Cry1Da₇ differs by four amino acids from its parent Bt protein Cry1Da. Both Cry1B.868 and Cry1Da₇ proteins are intended for control of lepidopteran corn pests (including corn earworm, sugarcane borer and fall armyworm) via the classical 3D Cry proteins mode of action (activation by proteases) present in the target insect midgut and to binding to specific receptors on the brush border of the insect midgut (COGEM). It is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high-specific affinity to Cry proteins (Hammond et al., 2013; Koch et al., 2015; Jurat-Fuentes and Crickmore, 2017).

iii.c) Overall conclusion on the history of safe use for consumption

Based on the above information, the GMO Panel concludes that it is not possible to confirm a documented history of safe use for consumption of the Cry1B.868 and Cry1Da₇ proteins.

iv) *In vitro* studies

The outcome of *in vitro* studies to characterise the stability of newly expressed proteins has been described in Section 3.5.2.

v) *In vivo* studies

The outcome of an acute toxicity study and of a 28-day study for each Bt-produced Cry1B.868 and Cry1Da₇ protein is described below.

Acute studies

An acute toxicity study in CD-1 mice administered the *B. thuringiensis*-produced Cry1B.868 protein by gavage at the dose of 5,000 mg/kg bw showed no adverse effects.

An acute toxicity study in CD-1 mice administered the *B. thuringiensis*-produced Cry1Da₇ protein by gavage at the dose of 5,000 mg/kg bw showed no adverse effects.

28-day repeated dose toxicity studies

Upon EFSA request to further corroborate information on the history of safe use for consumption as food and/or feed of the Cry1B.868 and Cry1Da₇ proteins, the applicant provided a 28-day repeated dose toxicity study for each of the two proteins,²¹ which was assessed by the GMO Panel.

Cry1B.868 protein

The 28-day toxicity study in mice with the Cry1B.868 protein was conducted in accordance with OECD TG 407 (2008) and to the principles of good laboratory practice.

Groups of CrI:CD-1 mice (20/sex per group), 8- to 9-week old at the start of dosing were allocated to five groups. Groups were administered by oral gavage: the test substance (Cry1B.868 protein) at targeted nominal doses of 1,000, 100 or 10 mg/kg body weight (bw) per day (high, medium and low Cry1Da₇ protein groups); 1,000 mg/kg bw per day of bovine serum albumin (BSA) (BSA control group); and the vehicle.²² Mice were randomised to treatment groups (males and females separately)

²¹ Additional information 20/08/2021.

²² 4 mM sodium carbonate/bicarbonate, pH 10.0, 5 mM cysteine.

using a stratified randomisation scheme designed to achieve similar group mean body weights ($\pm 20\%$ of the mean for each sex). Due to behavioural characteristics, animals were singly housed.²³ The GMO Panel considers this justification acceptable.

The test substance used in this study was produced by a recombinant system and contained about 97% Cry1B.868 protein. The amino acid sequence analysis of the *B. thuringiensis* - produced Cry1B.868 used in this 28-day toxicity study by mass fingerprint analysis matched the deduced sequence as defined by the *cry1B.868* gene. This protein had the expected molecular weight and immunoreactivity to Cry1B.868 specific antibodies, was not glycosylated and showed functional activity.

The first 10 animals per group were subject to in-life procedures and observations and terminal procedures in accordance to OECD TG 407 (2008), except coagulation analysis; the remaining 10 animals per group were used to evaluate coagulation parameters, body weight, food consumption and clinical observation parameters only.

Deviations to the protocol reported in the study were considered minor deviations with no impact on the study results.

An appropriate range of statistical tests was performed on the results of the study and a detailed description of the methodology and of statistically significant findings identified in mice is reported in Appendix A.

One male mouse given 1,000 mg/kg per day was found dead on Day 3 of the study. Post-mortem investigations revealed a gavage error as the cause of death.

No Cry1B.868-related clinical observations or ophthalmology findings were seen.

A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation²⁴ for the parameter in mice of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or endpoints;
- exhibited no consistency with increasing dose levels.

No gross pathology findings related to the administration of the test item were observed at necropsy, and the microscopic examinations of a wide range of organs and tissues did not identify relevant differences in the incidence or severity of the histopathological findings related to the administration of the test item compared to the controls.

The GMO Panel concludes that no adverse effects were observed in this 28-day mouse toxicity study on the microbially produced Cry1B.868 protein, at doses up to 1,000 mg/kg bw per day.

Cry1Da_7 protein

The 28-day toxicity study in mice with the Cry1Da_7 protein was conducted in accordance with OECD TG 407 (2008) and to the principles of good laboratory practice.

Groups of CrI:CD-1 mice (20/sex per group), 8- to 9-week old at the start of dosing were allocated to five groups. Groups were administered by oral gavage: the test substance (Cry1Da_7 protein) at targeted nominal doses of 1,000, 100 or 10 mg/kg body weight (bw) per day (high, medium and low Cry1Da_7 protein groups); 1,000 mg/kg bw per day of bovine serum albumin (BSA) (BSA control group); and the vehicle²². Mice were randomised to treatment groups (males and females separately) using a stratified randomisation scheme designed to achieve similar group mean body weights ($\pm 20\%$ of the mean for each sex). Due to behavioural characteristics, animals were singly housed.²³ The GMO Panel considers this justification acceptable.

The test substance used in this study was produced by a recombinant system and contained about 97% Cry1Da_7 protein. The amino acid sequence analysis of the *B. thuringiensis*-produced Cry1Da_7

²³ From the Technical Dossier: Males were individually housed because male mice are often aggressive and not considered social; females were housed individually to avoid compromising data interpretation resulting from animals being treated differently.

²⁴ Although animals used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is 'adverse' account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardised effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (Historic Control Data).

used in this 28-day toxicity study by mass fingerprint analysis matched the deduced sequence as defined by the *Cry1Da_7* gene. This protein had the expected molecular weight and immunoreactivity to *Cry1Da_7* specific antibodies, was not glycosylated and showed functional activity.

The first 10 animals per group were subject to in-life procedures and observations and terminal procedures in accordance to OECD TG 407 (2008), except coagulation analysis; the remaining 10 animals per group were used to evaluate coagulation parameters, body weight, food consumption and clinical observation parameters only.

Deviations to the protocol reported in the study were considered minor deviations with no impact on the study results.

An appropriate range of statistical tests were performed on the results of the study and a detailed description of the methodology and of statistically significant findings identified in mice is reported in Appendix A.

Four intercurrent deaths occurred (2 female mice at 1000 mg/kg bw per day from the Cry group, 1 male from the BSA group, 1 male at 100 mg/kg bw per day from the Cry group). Post-mortem investigation showed two of the deaths (1,000 mg/kg bw per day Cry and 100 mg/kg bw per day Cry) to be related to the gavage dosing procedures. The cause of the remaining two deaths (1,000 mg/kg bw per day BSA or Cry) could not be determined.

No *Cry1Da_7*-related clinical observations or ophthalmology findings were seen.

A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation for the parameter in mice of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or endpoints;
- exhibited no consistency with increasing dose levels.

Decreased numbers of corpora lutea in the females and testicular tubular degeneration in the males were identified by the report authors as potential target lesions. These tissues were examined microscopically from all males and females in all groups. The examinations provided no evidence of an effect of treatment.

The GMO Panel concludes that no adverse effects were observed in mice in this 28-day toxicity study on *E. coli*-produced *Cry1Da_7*- protein, at doses up to 1,000 mg/kg bw per day.

Overall conclusion of the *Cry1B.868* and *Cry1Da_7* proteins safety:

Based on the above information, the GMO Panel did not identify indications that the *Cry1B.868* and *Cry1Da_7* proteins raise food and feed safety concerns in humans and animals.

3.5.3.2. Testing of new constituents other than proteins

Based on the outcome of the studies considered in the comparative analysis and molecular characterisation, no new constituents other than the newly expressed proteins have been identified in grains and forage from maize MON 95379. Therefore, no further food/feed safety assessment of components other than the newly expressed proteins is required.

3.5.3.3. Information on altered levels of food and feed constituents

Based on the outcome of the studies considered in the comparative analysis and molecular characterisation, none of the differences identified between maize MON 95379 and its non-GM comparator in grains and forage composition require further assessment.

3.5.3.4. Testing of the whole genetically modified food and feed

Based on the outcome of molecular characterisation and comparative analysis assessment, no compositional modifications, or indication of possible unintended effects relevant to food and feed safety have been identified for maize MON 95379. Therefore, animal feeding studies with food/feed derived from maize MON 95379 are not considered necessary by the GMO Panel (EFSA GMO Panel, 2011a). In accordance with Regulation (EU) No 503/2013, the applicant provided a 90-day feeding study in rats receiving diets containing maize MON 95379 grains.

In this study, pair-housed CrI:CD(SD) rats (16 per sex per group; 2 rats per cage) were allocated to three groups using a randomised complete block design with eight replications per sex. Groups were fed diets containing maize MON 95379 grains at 50% and 33% of inclusion level (the latter

supplemented with 17% of the non-GM comparator maize) and the non-GM comparator (inclusion level 50%).

The study was adapted from OECD test guideline 408 (2018), aligned with EFSA Scientific Committee guidance (EFSA Scientific Committee, 2011) and complied with the principles of good laboratory practice (GLP) with some minor deviations not impacting the study results and interpretation.

The stability of the test and control materials was not verified; however, in accordance to product expiration declared by the diet manufacturer, the constituents of the diets are considered stable for the duration of the treatment. The GMO Panel considered this justification acceptable. Diet preparation procedures and regular evaluations of the mixing methods guaranteed the homogeneity and the proper concentration of the test or control substances in them.

Event-specific PCR analysis confirmed the presence of the event in both the GM maize and diets and excluded the presence of the event in the respective controls. Both the GM maize and diets were analysed for nutrients, antinutrients and potential contaminants (e.g. selected heavy metals, mycotoxins and pesticides). Balanced diets were formulated based on the specifications for PMI Certified Rodent LabDiet[®] 5,002. Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance to OECD TG 408 (1998).

An appropriate range of statistical tests was performed on the results of the study. Detailed description of the methodology and of statistically significant findings identified in rats given a diet containing maize MON 95379 is reported in Appendix A.

There were no test diet-related incidents of mortality or clinical signs. No test diet-related adverse findings were identified in any of the investigated parameters. A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation²⁵ for the parameter in rats of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or end-points.
- exhibited no consistency with increasing dietary incorporation level.

No gross pathology findings related to the administration of the test diet were observed at necropsy, and the microscopic examinations of a wide range of organs and tissues did not identify relevant differences in the incidence or severity of the histopathological findings related to the administration of the test diet compared to the control group.

The GMO Panel concludes that no adverse effects were observed in rats in this 90-day toxicity study given diets containing maize MON 95379 up to 50% incorporation rate.

3.5.4. Allergenicity

The strategies to assess the potential risk of allergenicity focus: (i) on the source of the recombinant protein; (ii) on the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons; and (iii) on whether the transformation may have altered the allergenic properties of the modified plant. Furthermore, the assessment also takes into account potential adjuvant properties of the newly expressed proteins, which is defined as the ability to enhance an allergic reaction.

3.5.4.1. Assessment of allergenicity of the newly expressed proteins

A weight-of-evidence approach was followed, taking into account all the information obtained on the newly expressed protein, as no single piece of information or experimental method yield sufficient evidence to predict allergenicity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a, 2017; Regulation (EU) No 503/2013).

²⁵ Although animals used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is 'adverse' account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardised effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (Historic Control Data).

The *cry1B.868* and *cry1Da_7* genes encoding for the Cry1B.868 and Cry1Da_7 proteins originate from *B. thuringiensis* which is not considered a common allergenic source.

Updated bioinformatic analyses of the amino acid sequences of the *cry1B.868* and *cry1Da_7* proteins, using the criterion of 35% identity in a sliding window of 80 amino acids, revealed no relevant similarities to known allergens. The studies on protein stability of the Cry1B.868 and Cry1Da_7 proteins have been described in Section 3.5.2. In addition, the GMO Panel did not find an indication that the newly expressed proteins Cry1B.868 and Cry1Da_7 at the levels expressed in maize MON 95379 might be adjuvants.

Furthermore, the applicant provided information²⁶ on the safety of the Cry1B.868 and Cry1Da_7 proteins regarding their potential hazard to cause a celiac disease response. For such assessment, the applicant followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017). The assessment of the Cry1B.868 and Cry1Da_7 proteins identified no perfect or relevant partial matches with known celiac disease peptide sequences.

In the context of this application, the GMO Panel considers that there are no indications that the newly expressed Cry1B.868 and/or Cry1Da_7 proteins in maize MON 95379 may be allergenic.

3.5.4.2. Assessment of allergenicity of the whole GM plant or crop

The GMO Panel regularly reviews the available publications on food allergy to maize. However, maize is not considered a common allergenic food²⁷ (OECD, 2002). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM maize.

In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 3.3, 3.4 and 3.5), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from this GM maize MON 95379 with respect to that derived from the non-GM comparator and the non-GM reference varieties tested.

3.5.5. Dietary exposure assessment to new constituents

In line with Regulation (EU) No 503/2013, the applicant provided dietary exposure estimates to Cry1B.868 and Cry1Da_7 proteins newly expressed in MON 95379 maize. Dietary exposure was estimated based on protein expression levels reported in this application for MON 95379 maize, the current available consumption data and feed practices, the foods and feeds currently available in the market and the described processing conditions.

For the purpose of estimating dietary exposure, the levels of the two insecticidal proteins in MON 95379 maize grains and forage were derived from replicated field trials (four replicates from five locations, $n = 20$) in 2018 in the United States (see Section 3.3.4). Table 1 in Section 3.3.4 shows the protein expression levels used to estimate both human and animal dietary exposure.

3.5.5.1. Human dietary exposure

Chronic and acute dietary exposure to Cry1B.868 and Cry1Da_7 proteins newly expressed in MON 95379 maize were provided. The applicant followed the methodology described in the EFSA Statement 'Human dietary exposure assessment to newly expressed protein in GM foods' (EFSA, 2019a) to estimate human dietary exposure in average and high consumers making use of summary statistics of consumption.

Human dietary exposure was estimated across different European countries on different population groups: young population (infants, toddlers, 'other children'), adolescents, adult population (adults, elderly and very elderly) and special populations (pregnant and lactating women). Since no specific consumption data were available on commodities containing, consisting of or obtained from MON 95379 maize grains, a conservative scenario with 100% replacement of conventional maize by the GM maize was considered. Consumption figures for all relevant commodities (e.g. corn flakes, sweet corn,

²⁶ Additional information: 5 October 2022.

²⁷ Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

popcorn, etc.) were retrieved from the EFSA Comprehensive European Food Consumption Database (EFSA consumption database).²⁸ Corn oil was excluded from the assessment since no proteins are expected to be present in the oil.

Mean protein expression values on fresh weight basis are considered as the most adequate to estimate human dietary exposure (both acute and chronic) when working with raw primary commodities that are commonly consumed as processed blended commodities (EFSA, 2019a). Different recipes and factors were considered to estimate the amount of maize in the consumed commodities before assigning newly expressed protein levels to the relevant commodities.²⁹ No losses in the newly expressed proteins during processing were considered, except for certain commodities excluded from the exposure estimations (corn oil, corn starch, corn syrup).

The highest acute dietary exposure (high consumers) was estimated in the age class 'Other children' with exposure estimates of 350 µg/kg bw per day and 3.34 µg/kg bw per day for Cry1B.868 and Cry1Da_7 proteins, respectively. The main contributor to the exposure in the dietary survey with the highest estimates was corn grains.

The highest chronic dietary exposure (high consumers) was estimated in the age class 'Infants' with exposure estimates of 188 µg/kg bw per day and 1.80 µg/kg bw per day for Cry1B.868 and Cry1Da_7 proteins, respectively. The main contributor to the exposure in the dietary survey with the highest estimates was corn flakes.

Additional dietary exposure to the Cry1B.868 and Cry1Da_7 proteins might occur via the consumption of pollen supplements under the assumption that these supplements contain pollen from MON 95379 maize. Consumption data on pollen supplements are available for few consumers across eight different European countries.³⁰ However, since no data on the presence of newly expressed proteins in pollen were available, the potential dietary exposure to Cry1B.868 and Cry1Da_7 proteins from the consumption of pollen supplements could not be estimated.

3.5.5.2. Animal dietary exposure

Dietary exposure to Cry1Da_7 and Cry1B.868 proteins in maize MON 95379 was estimated across different animal species, as below described, assuming the consumption of maize products commonly entering the feed supply chain (i.e. maize grains, gluten feed, gluten meal and forage/silage). A conservative scenario with 100% replacement of conventional maize products by the maize MON 95379 products was considered. Mean levels (dry weight) of the newly expressed proteins in grains and forage from maize MON 95379 used for animal dietary exposure are listed in Table 1 (Section 3.3.4). Mean levels (dry weight) of the newly expressed proteins in maize gluten feed and gluten meal were calculated to be, respectively, 2.6 and 7.1-fold higher than in grain, based on adjusting factors that take into account the protein content in these feed materials relative to maize grain (OECD, 2002), and assuming that no protein is lost during their production/processing. The levels in forage were used as the silage values based on a conservative assumption that there is no protein loss. The applicant estimated dietary exposure to Cry1Da_7 and Cry1B.868 proteins via the consumption of maize grains, gluten feed and gluten meal in broiler and finishing pig and maize gluten feed, gluten meal and silage in lactating dairy cow, based on default values for animal body weight, daily feed intake and inclusion rates (percentage) of maize feedstuffs in diets/rations, as provided for the EU by OECD (2009). Estimated dietary exposure in the concerned animals is reported in Appendix B.

3.5.6. Nutritional assessment of GM food and feed/endogenous constituents

The intended trait of maize MON 95379 is insect protection against some lepidopteran pests, with no intention to alter nutritional parameters. Comparison of the composition of the maize MON 95379 with the non-GM comparator and non-GM reference varieties did not identify differences that would require further safety assessment. From these data, the GMO Panel concludes that maize MON 95379 is nutritionally equivalent to the non-GM comparator and the non-GM reference varieties used.

²⁸ <https://www.efsa.europa.eu/en/applications/gmo/tools>. Data accessed: February 2021.

²⁹ Example: 100 g of maize bread are made with approximately 74 g of maize flour, and a reverse yield factor of 1.22 from the conversion of maize grains into flour is used. This results in 20.9 µg of Cry1B.868 per gram of maize bread as compared to the 23 µg/g (fresh weight, see Section 3.3.4) reported as mean concentration in the maize grains.

³⁰ <https://www.efsa.europa.eu/en/food-consumption/comprehensive-database>. Data accessed: January 2022.

3.5.7. Post-market monitoring of GM food/feed

The GMO Panel concluded that maize MON 95379, as described in this application, does not raise any nutritional concern and is as safe as the non-GM comparator and the non-GM reference varieties tested. Therefore, the GMO Panel considers that post-market monitoring of food and feed from this GM maize, as described in this application, is not necessary.

3.5.8. Conclusion on the food and feed safety assessment

The proteins Cry1B.868 and Cry1Da_7 newly expressed in maize MON 95379 do not raise safety concerns for human and animal health. No interactions between the newly expressed proteins relevant for food and feed safety were identified. Similarly, the GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed proteins in maize MON 95379. The GMO Panel finds no evidence that the genetic modification impacts the overall safety of maize MON 95379. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of maize MON 95379 does not represent any nutritional concern, in the context of the scope of this application. The GMO Panel concludes that maize MON 95379, as described in this application, is as safe as the non-GM comparator and the non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

3.6. Environmental risk assessment and monitoring plan³¹

3.6.1. Environmental risk assessment

Considering the scope of application EFSA-GMO-NL-2020-170, which excludes cultivation, the environmental risk assessment (ERA) of maize MON 95379 mainly takes into account: (1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of viable maize MON 95379 grains during transportation and/or processing (EFSA GMO Panel, 2010a).

3.6.1.1. Persistence and invasiveness of the GM plant

Maize is highly domesticated, not winter hardy in colder regions of Europe, and generally unable to survive in the environment without appropriate management. Survival is limited mainly by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and cold climate conditions (OECD, 2003), even though occasional feral GM maize plants may occur outside cultivation areas in the EU (e.g. Pascher, 2016). Field observations indicate that maize grains may survive and overwinter in some EU regions, resulting in volunteers in subsequent crops (e.g. Gruber et al., 2008; Palaudelmàs et al., 2009; Pascher, 2016). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009). Thus, the establishment and survival of feral and volunteer maize in the EU is currently limited and transient.

It is unlikely that the intended trait of maize MON 95379 will provide a selective advantage to maize plants, except when they are infested by insect pests that are susceptible to the Cry1B.868 and/or Cry1Da_7 proteins. However, if this was to occur, this fitness advantage will not allow the GM plant to overcome other biological and abiotic factors (described above) limiting plant's persistence and invasiveness. Therefore, the presence of the intended trait will not affect the persistence and invasiveness of the GM plant.

In conclusion, the GMO Panel considers that maize MON 95379 will be equivalent to conventional maize hybrid varieties in their ability to survive until subsequent seasons, or to establish occasional feral plants under European environmental conditions in case of accidental release into the environment of viable maize MON 95379 grains.

³¹ Dossier: Part II – Sections 5 and 6; additional information: 6 April 2021.

3.6.1.2. Potential for gene transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer (HGT) of DNA or through vertical gene flow via cross-pollination from feral plants originating from spilled grains.

Plant-to-microorganism gene transfer

Genomic DNA can be a component of food and feed products derived from maize. It is well documented that such DNA becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However, bacteria in the digestive tract of humans and animals, and in other environments, may be exposed to fragments of DNA, including the recombinant fraction of such DNA.

Current scientific knowledge of recombination processes in bacteria suggests that horizontal transfer of non-mobile, chromosomally located DNA fragments between unrelated organisms (such as from plants to bacteria) is not likely to occur at detectable frequencies under natural conditions (for further details, see EFSA, 2009).

Homologous recombination is known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments to bacterial genomes. This requires the presence of at least two stretches of DNA sequences that are similar in the recombining DNA molecules. In the case of sequence identity with the transgene itself, recombination would result in gene replacement. In the case of identity with two or more regions flanking recombinant DNA, recombination could result in the insertion of additional DNA sequences in bacteria and thus confer the potential for new properties.

In addition to homology-based recombination processes, at a lower transformation rate, the non-homologous end joining and microhomology-mediated end joining are theoretically possible (Hülter and Wackernagel, 2008; EFSA, 2009). Independently of the transfer mechanism, the GMO Panel did not identify a selective advantage that a theoretical HGT would provide to bacterial recipients in the environment.

The bioinformatic analysis for event MON 95379 revealed no homology with known DNA sequences from bacteria which would facilitate homologous recombination.

In summary, there is no indication for an increased likelihood of horizontal transfer of DNA from maize MON 95379 to bacteria. Given the nature of the recombinant DNA, the GMO Panel identified no safety concern linked to an unlikely but theoretically possible HGT.

Plant-to-plant gene transfer

The potential for occasional feral maize MON 95379 plants originating from grain import spills to transfer recombinant DNA to sexually compatible plants and the environmental consequences of this transfer were considered.

For plant-to-plant gene transfer to occur, imported GM maize grains need to germinate and develop into plants in areas containing sympatric wild relatives and/or cultivated maize with synchronous flowering and environmental conditions favouring cross-pollination.

Maize is an annual predominantly cross-pollinating crop. Cross-fertilisation occurs mainly by wind (OECD, 2003). Vertical gene transfer from maize is limited to *Zea* species. Wild relatives of maize outside cultivation are not known/reported in Europe (Eastham and Sweet, 2002; OECD, 2003; EFSA, 2016, 2022; Trtikova et al., 2017). Therefore, potential vertical gene transfer is restricted to maize and weedy *Zea* species, such as teosintes, and/or maize-teosinte hybrids, occurring in cultivated areas (EFSA, 2016, 2022; Trtikova et al., 2017; Le Corre et al., 2020).

The potential of spilled maize grains to establish, grow and produce pollen is extremely low and transient (see Section 3.6.1.1). Therefore, the likelihood/frequency of cross-pollination between occasional feral GM maize plants resulting from grain spillage, and weedy or cultivated *Zea* plants is considered extremely low (EFSA, 2016, 2022). Even if cross-pollination would occur, the GMO Panel is of the opinion that environmental effects as a consequence of the spread of genes from occasional feral GM maize plants in Europe will not differ from that of conventional maize varieties for the reasons given in Section 3.6.1.1.

3.6.1.3. Interactions of the GM plant with target organisms

Taking the scope of application EFSA-GMO-NL-2020-170 into account (no cultivation), potential interactions of occasional feral maize MON 95379 plants arising from grain import spills with the target organisms are not considered a relevant issue.

3.6.1.4. Interactions of the GM plant with non-target organisms

Given that environmental exposure of non-target organisms to spilled GM grains or occasional feral GM maize plants arising from spilled maize MON 95379 grains is limited, and because ingested proteins are degraded before entering the environment through faecal material of animals fed GM maize, the GMO Panel considers that potential interactions of maize MON 95379 with non-target organisms do not raise any environmental safety concern. Interactions that may occur between the Cry proteins will not alter this conclusion.

3.6.1.5. Interactions with abiotic environment and biogeochemical cycles

Given that environmental exposure to spilled grains or occasional feral maize MON 95379 plants arising from grain import spills is limited, and because ingested proteins are degraded before entering the environment through faecal material of animals fed GM maize, the GMO Panel considers that potential interactions with the abiotic environment and biogeochemical cycles do not raise any environmental safety concern.

3.6.2. Post-market environmental monitoring

The objectives of a post-market environmental monitoring (PMEM) plan, according to Annex VII of Directive 2001/18/EC, are: (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the ERA are correct; and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment that were not anticipated in the ERA.

Monitoring is related to risk management, and thus, a final adoption of the PMEM plan falls outside the mandate of EFSA. However, the GMO Panel gives its opinion on the scientific rationale of the PMEM plan provided by the applicant (EFSA GMO Panel, 2011b).

As the ERA did not identify potential adverse environmental effects from maize MON 95379, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for maize MON 95379 includes: (1) the description of a monitoring approach involving operators (federations involved in import and processing), reporting to the applicant, via a centralised system, any observed adverse effect(s) of GMOs on human health and the environment; (2) a coordinating system established by CroLife Europe for the collection of information recorded by the various operators; and (3) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis for the duration of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of maize MON 95379. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan.

3.6.3. Conclusion of the environmental risk assessment and monitoring plan

The GMO Panel concludes that it is unlikely that maize MON 95379 would differ from conventional maize varieties in its ability to persist under European environmental conditions. Considering the scope of application EFSA-GMO-NL-2020-170, interactions of occasional feral maize MON 95379 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from maize MON 95379 to bacteria does not indicate a safety concern. Therefore, considering the introduced trait, the outcome of the agronomic and phenotypic analysis, and the routes and levels of exposure, the GMO Panel concludes that maize MON 95379 would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment.

The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of maize MON 95379.

4. Overall conclusions

The GMO Panel was asked to carry out a scientific assessment of maize MON 95379 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

The molecular characterisation data establish that maize MON 95379 contains a single insert consisting of one copy of the *cry1B.868* and *cry1Da_7* expression cassettes. The quality of the sequencing methodology and data sets was assessed by the EFSA GMO Panel and is in compliance to the requirements listed in the EFSA Technical Note. Updated bioinformatics analyses of the sequences

encoding the newly expressed proteins and other ORFs present within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of Cry1B.868 and Cry1Da_7 proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant- and microbe-derived Cry1B.868 and Cry1Da_7 proteins indicate that these proteins are equivalent and the microbe-derived proteins can be used in the safety studies.

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize MON 95379 and its conventional counterpart needed further assessment. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Cry1B.868 and Cry1Da_7 proteins as expressed in maize MON 95379. The GMO Panel finds no evidence that the genetic modification impacts the overall safety of maize MON 95379. In the context of this application, the consumption of food and feed from maize MON 95379 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize MON 95379 is as safe as the conventional counterpart and non-GM maize reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable grains from maize MON 95379 into the environment. The PMEM plan and reporting intervals are in line with the intended uses of maize MON 95379. Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the uses of maize MON 95379.

The GMO Panel concludes that maize MON 95379 is as safe as its conventional counterpart and the tested non-GM maize varieties with respect to potential effects on human and animal health and the environment.

5. Documentation as provided to EFSA

- Letter from the Competent Authority of the Netherlands received on 30th November 2020 concerning a request for authorization of the placing on the market of genetically modified maize MON 95379 submitted in accordance with Regulation (EC) No 1829/2003 by Bayer Agriculture BV (EFSA Ref. EFSA-GMO-NL-2020-170; EFSA-Q-2020-00786)
- The application was made valid on 29th March 2021
- Additional Information (Clock 1) was requested on 13th April 2021
- Additional Information (Clock 1) was received on 4th June 2021
- Additional Information (Clock 2) was requested on 21st June 2021
- Additional Information (Clock 2) was received on 20th August 2021
- Additional Information (Clock 3) was requested on 3rd September 2021
- Additional Information (Clock 3) was received on 3rd November 2021
- Additional Information (Clock 4) was requested on 25th November 2021
- Additional Information (Clock 4) was received on 21st January 2022 partial; 31st January 2022 complete
- Additional Information (Clock 5) was requested on 7th February 2022
- Additional Information (Clock 5) was received on 10th and 20th May 2022
- Additional Information (Clock 6) was requested on 1st July 2022
- Additional Information (Clock 6) was received on 5th August 2022

Supplementary information was provided on voluntary basis on 4th June 2021 and 10th May 2022.

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Abbreviations

ADF	acid detergent fibre
bp	base pair
bw	body weight
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
ERA	environmental risk assessment
FMV	figwort mosaic virus
fw	fresh weight
GLP	good laboratory practice
GMO	genetically modified organism
HGT	horizontal gene transfer
HR	homologous recombination
JSA	junction sequence analysis
LB	left border
MS	mass spectrometry
NDF	neutral detergent fibre
NGS	next generation sequencing
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring
RB	right border
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-DNA	transfer-deoxyribonucleic acid
TEV	tobacco etch virus
UTR	untranslated region

Appendix A – Statistical analysis and statistically significant findings in the 28-day toxicity study in mice on the microbially produced Cry1B.868 and Cry1Da_7 proteins and 90-day toxicity study in rats on maize MON 95379

A.1 Statistical analysis in the 28-day study on *B. thuringiensis*-produced Cry1B.868 protein in mice

The following endpoints were statistically analysed: body weights, body weight changes, food consumption, clinical pathology values (as applicable), absolute and relative organ weights, functional observational battery (FOB) data, locomotor activity and histopathological data. For all continuous endpoints, mean, standard deviation in terms of the standardised effect sizes (SES) of each dose group for each sex, variable and period or time interval were reported. The main statistical analysis compared each of the three test diet groups (high, medium and low Cry1B.868 protein groups) separately with the vehicle group.

The analysis was performed for male and female mice separately at 5% level of significance. Continuous endpoints were analysed with a linear mixed model (fixed effect: diet; random effect: block; for locomotor activity data, additional fixed effects were time and the interaction diet time); for endpoints measured on a discrete scale, the comparisons were performed with Fisher's exact test as appropriate. Ranges from historical control data were provided to aid the assessment of statistically significant differences between the test and control diet groups. Missing data were considered by the Panel and found not to have an impact on the results.

Table A.1: Statistically significant findings in 28-day study on *B. thuringiensis*-produced Cry1B.868 protein in mice

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
FOB and Motor activity Open Field Observations: Urination	Increased urinary marking in the male 100 mg/kg bw per day group.	No dose response – not observed at 1,000 mg/kg bw per day. Within normal variation. Not an adverse effect of treatment
Haematology, WBC and lymphocytes	Reduced absolute WBC and lymphocyte counts (30%) in top dose males	Within normal variation. Mean value in top dose males is above HCD mean. Not an adverse effect of treatment
Organ weight kidney	Decreased (10%) in top dose females.	Small magnitude. No associated changes in clinical chemistry or pathology. Not an adverse effect of treatment

A.2 Statistical analysis in the 28-day study on *B. thuringiensis*-produced Cry1Da_7 protein in mice

The following endpoints were statistically analysed: body weights, body weight changes, food consumption, clinical pathology values (as applicable), absolute and relative organ weights, functional observational battery (FOB) data, locomotor activity and histopathological data. For all continuous endpoints, mean, standard deviation in terms of the standardised effect sizes (SES) of each dose group for each sex, variable and period or time interval were reported. The main statistical analysis compared each of the three test diet groups (high, medium and low Cry1Da_7 protein groups) separately with the vehicle group.

The analysis was performed for male and female mice separately at 5% level of significance. Continuous endpoints were analysed with a linear mixed model (fixed effect: diet; random effect: block; for locomotor activity data, additional fixed effects were time and the interaction diet time); for endpoints measured on a discrete scale, the comparisons were performed with Fisher's exact test as appropriate. Ranges from historical control data were provided to aid the assessment of statistically significant differences between the test and control diet groups. Missing data were considered by the Panel and found not to have an impact on the results.

Table A.2: Statistically significant findings in 28-day study on *B. thuringiensis*-produced Cry1Da₇ protein in mice

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Body weight	Body weight gains were reduced in the mid- and top dose groups during some periods.	Final body weights were similar (within 3%). Within normal variation. Not an adverse effect of treatment.
Food consumption	Food consumption was reduced in the mid- and top dose male groups during some periods.	Similar to controls in the final week. No impact on final body weights. Not an adverse effect of treatment.
FOB and Motor activity Open Field Observations	Increased time to first step in top dose males (0.3 to 0.4 s)	Small magnitude. Within normal variation. Not an adverse effect of treatment.
	Increased animals showing grooming behaviour in mid-dose females.	Not evident at higher doses. Consistent with pretest results. Not an adverse effect of treatment.
Haematology – Mean Corpuscular Volume	Increased (5%) in low-dose males.	Small magnitude, not evident at higher doses. Not an adverse effect of treatment.
Haematology – Mean Corpuscular Haemoglobin (pg)	Decreased (5%) in low-dose females.	Small magnitude, not evident at higher doses. Total haemoglobin level same as controls. Not an adverse effect of treatment.
Clinical chemistry – Total protein, Globulin and Albumin	Increased (10%) in top-dose males	Small magnitude. Within normal variation. Not an adverse effect of treatment.

A.3 Statistical analysis of the 90-day study on maize MON95379 in rats

The following endpoints were statistically analysed: body weights, cumulative body weight changes, food consumption, clinical pathology values (as applicable), absolute and relative organ weights, microscopic findings, functional observational battery (FOB) data and motor activity data. For all continuous endpoints, the applicant reported mean, standard deviation in terms of the standardised effect sizes (SES) of each dose group for each sex, variable and period or time interval.

The main statistical analysis compared rats consuming high- and low-dose test diets with those consuming the control diet. The statistical analysis of continuous endpoints was performed using linear mixed models, applied separately for each parameter and period. For food consumption data (with cage-based observations), the model included treatment, sex and treatment-by-sex interaction as fixed effects; replicate-within-sex was the random effect.

Continuous data were investigated separately for each variable and period or time interval, according to a linear mixed model (LMM); then, pairwise comparisons, between each test and control group (separately for each sex, time interval if necessary), were performed using a t-test (at the 5% level of significance).

Treatment, sex and their interaction were defined as fixed effects.

Random effects included only the block (per sex) for food consumption, while for the other outcomes, the interaction block per treatment (per sex) was also considered in order to take into account the cage effect.

For locomotor activity data, a more complex model was used including the time effect and its interaction with the other fixed and random effects as additional factors.

For all the models, in case the sex-by-treatment interaction was significant (and in any case for sex-specific parameters), a sex-specific analysis was performed.

Finally, outcome proportions of incidence of functional observations were analysed with Fisher's exact test (at the 5% level of significance).

Historical control data were provided for organ weight and clinical pathology and used to assess statistical differences identified for such parameters in the study. Missing data were considered by the Panel and found not to have an impact on the results.

Table A.3: Statistically significant findings in 90-day study on maize MON 95379 in rats

Statistically significant parameter/ endpoint	Finding	GMO Panel interpretation
Haematology – Mean Corpuscular Haemoglobin (pg)	Increased (2%) in top-dose females.	Small magnitude. An increase is not adverse in isolation. Not an adverse effect of treatment.
Haematology – Reticulocyte count	Increased in low-dose (20%) and top-dose (10%) females	No clear dose response. Not adverse in isolation. No change in RBC count. Not an adverse effect of treatment.
Haematology – Eosinophil count.	Increased 30% in top-dose groups.	Not adverse in isolation. Within normal variation. Not an adverse effect of treatment.
Haematology – Haematocrit & total haemoglobin	Increased 3% in low-dose groups.	Not seen at top dose. Low magnitude. An increase is not adverse in isolation. Not an adverse effect of treatment.
Haematology – Prothrombin time	Increased 3% in low-dose groups.	Not seen at top dose. Low magnitude. Not an adverse effect of treatment.
Urinalysis	A range of changes in low-dose groups. Increased specific gravity (1%), osmolality (21%), creatinine (30%) and glucose (21%); decreased volume (30%)	Not seen at top dose. Within normal variation. No associated histopathology or clinical chemistry changes (BUN & creatinine within 10%). Not an adverse effect of treatment.
Ovary weight (absolute and relative to body weight)	Increased (15%) in top-dose females	Within normal variation (only 1/16 outside concurrent control range). No associated histopathology findings. Not an adverse effect of treatment.
Heart weight (absolute)	Decreased (6%) in low-dose groups.	Small magnitude. Not seen at top dose. No associated histopathology findings. Not an adverse effect of treatment.
Kidney weight (absolute and relative to body weight)	Decreased (5%) in low-dose groups.	Small magnitude. Not seen at top dose. No associated clinical chemistry or histopathology findings. Not an adverse effect of treatment.
Liver weight (relative to body weight)	Reduced (5%) in top dose groups.	Small magnitude. No associated clinical chemistry or histopathology findings. Not an adverse effect of treatment.

Appendix B – Animal dietary exposure

Table B.1: Animal dietary exposure to Cry1Da_7 and Cry1B.868 proteins ($\mu\text{g}/\text{kg}$ bw per day) based on the consumption of maize grains, gluten feed, gluten meal and silage

Animal species BW (kg)/total diet intake (kg dw)	Feed material	IR%	Cry1Da_7	Cry1B.868
Broiler 0.12/1.7	Grain	70	12.35	1,285
	Gluten feed	10	4.6	477.1
	Gluten meal	10	12.5	1,305
	Total	90	29	3,065
Finishing pig 100/3	Grain	70	5.25	546
	Gluten feed	20	3.9	405.6
	Gluten meal	10	5.3	555
	Total	100	14	1,505
Lactating dairy cow 650/25	Gluten feed	20 ^(a)	5	520
	Gluten meal	20	13.6	1,423
	Forage/Silage	60	600	2,538
	Total	100	619	4,478

(a): For lactating dairy cow, the allocation of ingredient was based on first using the ingredient with the higher protein expression until 100% of daily intake is achieved. Thus, in this scenario, 100% of the dairy cow diet was achieved without maize grain, and using gluten feed at 20% of inclusion rate, although OECD, 2009 indicate 30%, which would exceed the 100% of the total diet.