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## **Supporting document 1**

Safety assessment – Application A1270

Food derived from herbicide-tolerant and insect-protected corn line DP51291

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## **Executive summary**

### **Background**

Application A1270 seeks approval for the sale and use of food derived from corn line DP51291 that has been genetically modified (GM) for tolerance to the herbicide glufosinate and protection from coleopteran insect pests, primarily western corn rootworm (WCR).

Protection against corn rootworm is conferred by the expression of the *ipd072Aa* gene, from the bacterium *Pseudomonas chlororaphis*, encoding the IPD072Aa protein. This protein causes damage to the midgut epithelium of corn rootworm larvae, resulting in insect death. Tolerance to the herbicide glufosinate is achieved by the expression of the maize-optimised *mo-pat* gene, derived from the bacterium *Streptomyces viridochromogenes*, encoding the enzyme phosphinothricin acetyltransferase (PAT). DP51291 also expresses the phosphomannose isomerase (PMI) protein from *Escherichia coli* strain K-12 as a selectable marker. The IPD072Aa, PAT and PMI proteins have all been assessed previously by FSANZ.

This safety assessment addresses food safety and nutritional issues associated with the GM food. It therefore does not address:

- risks related to the environmental release of GM plants used in food production
- risks to animals that may consume feed derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

### **History of use**

Corn has a long history of safe use in the food supply. Examples of corn-derived food products include cornflour, starch products, breakfast cereals and high fructose corn syrup.

### **Molecular characterisation**

The genes encoding IPD072Aa (*ipd072Aa*), PAT (*mo-pat*) and PMI (*pmi*) were introduced into corn line DP51291 via a three-step transformation process. Molecular analyses indicate that a single copy of each of the linked *ipd072Aa*, *pat* and *pmi* cassettes is present at a single insertion site in the DP51291 genome. There are no extraneous plasmid sequences,

selectable marker cassettes or antibiotic resistance marker genes present in this line.

The introduced genetic elements were shown by molecular techniques and phenotypic analyses to be present within a single locus and stably inherited across multiple generations.

## **Characterisation and safety assessment of new substances**

### **Newly expressed proteins**

A range of characterisation studies were performed on the plant-derived IPD072Aa, PAT and PMI proteins to confirm their identity, structure, biochemistry and function, as well the equivalence of IPD072Aa to the corresponding protein produced in *E. coli*. All three proteins were expressed throughout the plant, including at a low level in grain. Updated bioinformatic analyses were consistent with previous analyses showing that none of the three proteins shared any meaningful homology with any known allergens or toxins. Taken together, the studies provided do not alter the conclusions reached in previous assessments that IPD072Aa, PAT and PMI proteins are neither toxic or allergenic in humans.

### **Herbicide metabolites**

For PAT, the metabolic profiles resulting from the novel protein/herbicide interaction have been established through a significant history of use. There are no concerns that the spraying of corn line DP51291 with glufosinate would result in the production of metabolites that are not also produced in non-GM crops sprayed with the same herbicide and already used in the food supply.

### **Compositional analyses**

Detailed compositional analyses were performed on DP51291. Statistically significant differences were found between grain from DP51291 and the control for 7 of the 68 analytes evaluated, however these differences were small and all within the range established for existing commercial non-GM corn cultivars. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in grain from DP51291 compared to non-GM corn cultivars available on the market.

### **Conclusion**

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant and insect-protected corn line DP51291. On the basis of the data provided in the present application and other available information, food derived from DP51291 is considered to be as safe for human consumption as food derived from non-GM corn cultivars.

# Table of contents

<b>EXECUTIVE SUMMARY</b> .....	<b>1</b>
<b>1 INTRODUCTION</b> .....	<b>5</b>
<b>2 HISTORY OF USE</b> .....	<b>5</b>
2.1 HOST ORGANISM .....	5
2.2 DONOR ORGANISMS .....	6
<b>3 MOLECULAR CHARACTERISATION</b> .....	<b>6</b>
3.1 TRANSFORMATION METHOD .....	7
3.2 DETAILED DESCRIPTION OF INSERTED DNA.....	7
3.3 DEVELOPMENT OF THE CORN LINE FROM THE ORIGINAL TRANSFORMATION .....	7
3.4 CHARACTERISATION OF THE INSERTED DNA AND SITE(S) OF INSERTION .....	7
3.5 CONCLUSION .....	11
<b>4 CHARACTERISATION AND SAFETY ASSESSMENT OF NOVEL SUBSTANCES</b> .....	<b>11</b>
4.1 NOVEL PROTEINS.....	11
4.2 HERBICIDE METABOLITES .....	17
<b>5 COMPOSITIONAL ANALYSIS</b> .....	<b>17</b>
5.1 KEY COMPONENTS .....	17
5.2 STUDY DESIGN.....	18
5.3 ANALYSES OF KEY COMPONENTS IN GRAIN .....	19
<b>6 NUTRITIONAL IMPACT</b> .....	<b>22</b>
<b>7 REFERENCES</b> .....	<b>23</b>

## Index of Figures

	<b>Title</b>	<b>Page</b>
Figure 1	Schematic figure of the insertion in DP51291	8
Figure 2	Stages of corn growth	13
Figure 3	Analytes measured in DP51291 grain samples	19
Figure 4	Summary of the 7 analytes for which the measured DP51291 mean was significantly different from the conventional control mean	21

## Index of Tables

	<b>Title</b>	<b>Page</b>
Table 1	Segregation results in five generations of DP51291	10
Table 2	IPD072Aa concentrations (ng/mg DW) in DP51291 tissues	13
Table 3	PAT concentrations (ng/mg DW) in DP51291 tissues	15
Table 4	PMI concentrations (ng/mg DW) in DP51291 tissues	16

## List of Abbreviations

Abbreviation	Description
ADF	acid detergent fibre
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUBstitution Matrix
bp	base pair
CI	confidence interval
COMPARE	COMprehensive Protein Allergen REsource
DNA	deoxyribonucleic acid
DW	dry weight
ELISA	enzyme-linked immunosorbent assay
FASTA	fast alignment search tool – all
FSANZ	Food Standards Australia New Zealand
g	gram
GM	genetically modified
HFCS	high fructose corn syrup
kDa	kilodalton
LLOQ	lower limit of quantitation
mg	milligram
MT	million tons
NCBI	National Centre for Biotechnology Information
NDF	neutral detergent fibre
ng	nanogram
NGS	next generation sequencing
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
PCR	polymerase chain reaction
SbS	Southern-by-sequencing
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TDF	total dietary fibre
µg	microgram
WCR	western corn rootworm

# 1 Introduction

FSANZ received an application from Corteva Agriscience Australia Proprietary Limited to vary Schedule 26 in the *Australia New Zealand Food Standards Code*. The variation is to include food from a new genetically modified (GM) corn line DP51291, with the OECD Unique Identifier DP-Ø51291-2. This corn line is tolerant to the herbicide glufosinate and protected against coleopteran insect pests, primarily western corn rootworm (WCR; *Diabrotica virgifera virgifera*).

Protection against corn rootworm is conferred by the expression of the *ipd072Aa* gene, from the bacterium *Pseudomonas chlororaphis*, encoding the IPD072Aa protein. Tolerance to the herbicide glufosinate is achieved by the expression of the maize-optimised *mo-pat* gene, derived from the bacterium *Streptomyces viridochromogenes*, encoding the enzyme phosphinothricin acetyltransferase (PAT). DP51291 also expresses the phosphomannose isomerase (PMI) protein from *Escherichia coli* strain K-12 as a selectable marker. The IPD072Aa, PAT and PMI proteins have all been assessed previously by FSANZ.

If approved, food derived from DP51291 corn line may enter the Australian and New Zealand food supply as imported food products.

## 2 History of use

### 2.1 Host organism

The host organism is corn (*Zea mays*) which is also referred to as maize. Corn was one of the first plants to be cultivated by humans (Ranum et al. 2014) and is now the world's dominant cereal crop, with global production of 1,151 MT<sup>1</sup> in 2022/23, ahead of wheat (788 MT) and rice (513 MT) (USDA 2023). Due to its economic importance, corn has been the subject of extensive study.<sup>2</sup>

The United States is the world's largest producer of corn, producing 349 MT in 2022/23 (USDA 2023). Canada produced 14.5 MT in 2022/23 (USDA 2023). Of the corn grown in the United States and Canada, an estimated 92% and ~90%, respectively, is GM.<sup>3,4,5</sup>

Corn is not a major crop in Australia or New Zealand – in 2021 these amounted to 0.306 and 0.209 MT respectively (FAOSTAT 2022). No GM corn is currently grown commercially in Australia or New Zealand.

To supplement their limited local production of corn, Australia and New Zealand import both corn grain and processed corn products. For example, in 2021 the imported quantities of corn flour into Australia and New Zealand were 11,626 and 1,284 tonnes respectively, while imports of corn oil totalled 1,106 and 122 tonnes respectively (FAOSTAT 2022).

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<sup>1</sup> million tons

<sup>2</sup> Refer to detailed reports published by the OECD (OECD 2002), the Grains Research and Development Corporation (GRDC 2017) and the Office of the Gene Technology Regulator (OGTR 2008).

<sup>3</sup> For more information please see USDA Economic Research Service: <http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx>

<sup>4</sup> USDA Grain Report, CA14062, 2014:

<https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agricultural%20Biotechnology%20Annual%20Ottawa%20Canada%207-14-2014>

<sup>5</sup> Statistics Canada, 2023: <https://www150.statcan.gc.ca/t1/tb1/en/tv.action?pid=3210004201>

Corn has a long history of safe consumption as food by humans<sup>6</sup>. Food products derived from processing of corn kernels include corn flour, meal, oil, starch and sweeteners such as high fructose corn syrup (HFCS). In Australia and New Zealand, corn starch is used in dessert mixes and canned foods, and HFCS is used in breakfast cereals, baking products, corn chips and extruded confectionary.

## 2.2 Donor organisms

### 2.2.1 *Pseudomonas chlororaphis*

The source of the *ipd072Aa* gene is the bacterial species *Pseudomonas chlororaphis* (Schellenberger et al. 2016). This Gram-negative, aerobic bacterium is ubiquitous in soil (Anderson et al. 2018). Due to its ability to suppress plant pathogens via the production of a variety of compounds (Arrebola et al 2019), *P. chlororaphis* has found widespread use as a seed-treatment and foliar-applied biopesticide (Kupferschmied et al. 2013, EFSA 2017, US-EPA 2017). As well as a history of safe use in agriculture, *P. chlororaphis* has a history of safe use as a gene donor for GM crops. GM tomatoes containing the *Accd* gene from *P. chlororaphis* (event 8338 tomato) were deregulated by the USDA in 1995 (USDA-APHIS 1995). Although other species from the *Pseudomonas* genus are plant and human pathogens (e.g. *P. aeruginosa*, *P. syringae*), *P. chlororaphis* is only distantly related to these species, and there is no evidence that it causes any toxic effects in humans or other mammals (Anderson et al. 2018).

### 2.2.2 *Streptomyces viridochromogenes*

The source of the *mo-pat* gene is the bacterial species *Streptomyces viridochromogenes*. This bacterium is Gram-positive, spore-forming, found in soil and water and is not pathogenic to humans or animals. *S. viridochromogenes* does not have a history of food use itself, but the *pat* gene has been used to confer resistance to glufosinate in multiple food-producing crops for almost three decades (CERA 2011).

### 2.2.3 *Escherichia coli*

The *pmi* gene is derived from the bacterial species *Escherichia coli*, a Gram-negative bacterium which is ubiquitous in the environment. *E. coli* strain K-12 is a non-pathogenic strain with a long history of use for laboratory and commercial applications including the production of pharmaceutical products (Baeshen et al. 2015) and food ingredients (e.g. Schedule 18 of the Code permits the use of chymosin derived from *E. coli* K-12 strain as a food processing aid). Despite the pathogenicity of certain *E. coli* strains, such as the enterohaemorrhagic *E. coli* group (e.g. 0157:H7) there are no toxicity or health concerns associated with strain K-12.

### 2.2.4 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of DP51291. These genetic elements are non-coding sequences and are used to regulate the expression of *ipd072Aa*, *mo-pat* and *pmi*.

## 3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety

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<sup>6</sup> A large proportion of corn produced is also used as animal feed.

assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA, including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Details of the specific genetic elements and plasmids used in construction of DP51291, as well as its breeding history and generations used for various analyses were provided in the application as Confidential Commercial Information (CCI). While the full details of CCI cannot be provided in this public report, FSANZ has given regard to this information in its assessment

### **3.1 Transformation method**

Three sequential transformation steps were used to construct corn line DP51291: an initial transformation using a single plasmid to insert a “landing pad” sequence into the corn genome; a second transformation using multiple plasmids to prepare the landing pad sequence for insertion of the intended trait genes, and a third transformation using a single plasmid to integrate the intended *ipd072Aa*, *mo-pat* and *pmi* expression cassettes into the prepared landing pad.

A corn line that contained the intended insertion, but without any unintended DNA insertions, was selected for subsequent development.

### **3.2 Detailed description of inserted DNA**

Corn line DP51291 contains inserted DNA that includes the *pmi*, *mo-pat*, and *ipd072Aa* expression cassettes.

### **3.3 Development of the corn line from the original transformation**

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of DP51291
- ensuring that the DP51291 event is incorporated into elite lines for commercialisation.

### **3.4 Characterisation of the inserted DNA and site(s) of insertion**

A range of analyses were undertaken to characterise the genetic modification in DP51291. These analyses focused on the nature and stability of the inserted DNA and whether any unintended re-arrangements or expression products may have occurred as a consequence of the transformation procedure.

The applicant made use of in-house methodology for some of the characterisation studies. The method combines hybridisation techniques with next generation sequencing (NGS) and has been termed Southern-by-Sequencing (SbS). Details of the methodology and proof of concept work is publically accessible in the following publications: Zastrow-Hayes et al. (2015) and Brink et al. (2019).

#### **3.4.1 Number of integration site(s)**

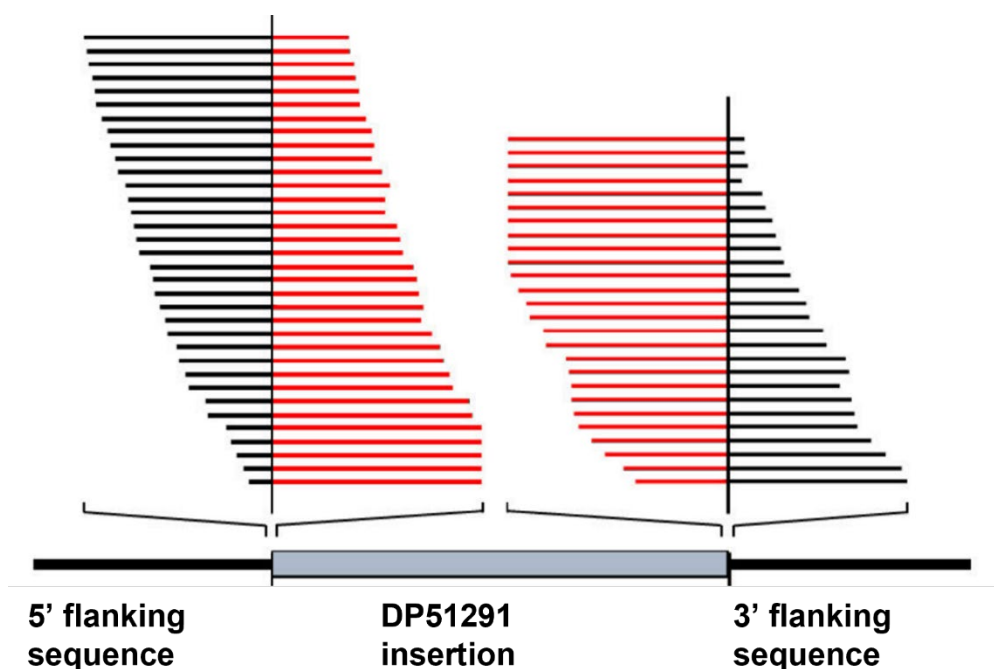
Leaf-derived genomic DNA from ten plants from the T1 generation of DP51291, along with DNA from a plant from the non-GM near-isoline PHR03 corn as a control, was analysed by

SbS. Positive control samples were generated using PHR03 DNA spiked with each of the plasmids used for development of DP51291.

NGS libraries were prepared on sheared genomic DNA that consisted of an average fragment size of 400 bp. The probe set was designed to collectively target all sequences within all plasmids. The DNA was enriched twice by hybridisation and was sequenced using an Illumina platform. Sufficient sequence fragments were obtained to cover the genomes being analysed, with a 100x depth of coverage.

The sequencing reads obtained by SbS were compared to the intended insertion sequence, the plasmid sequences, and to the endogenous corn genome to identify unique junctions attributable to inserted DNA. The ten DP51291 plants analysed by SbS consisted of 5 transgenic and 5 null segregant plants. SbS analysis of each of the 5 transgenic plants yielded sequencing reads that aligned to the intended insertion, and identified two unique genome-insertion junctions. This result indicated that a single copy of the intended insertion, with the intended organisation, was integrated into the genome of DP51291. No junctions were detected in either the control or in the 5 null segregant plants.

Figure 1 shows a schematic representation of the single insertion in DP51291 and the 5' and 3' flanking genomic sequences. The vertical black lines in this figure represent the two genome-insertion junctions. Representative junction reads, each containing a combination of insert and flanking sequence, are shown above each junction.



Figure

**Figure 1.** Schematic of the single copy insertion in DP51291. Red and black bars represent junction reads obtained from NGS sequencing.

### 3.4.2 Absence of backbone and other sequences

The SbS analysis used a set of hybridisation probes covering the backbone sequences for all of the plasmids used to create DP51291. Alignment of NGS reads from the controls or DP51291 to all plasmid sequences confirmed there was no integration of backbone sequences into DP51291, including any antibiotic resistance genes.



### 3.4.3 Insert integrity and site of integration

The SbS analysis indicated that DP51291 contains a single copy of the intended insertion, with the expected organisation, and no unintended sequences or rearrangements. PCR and sequencing analysis of the insert and flanking corn genomic regions confirmed that the organisation of the insert in DP51291 is as expected, with the exception of small deletions outside of the incorporated *pmi*, *mo-pat* and *ipd072Aa* gene cassettes. Given that these sequences are not part of the gene cassettes it is unlikely they would affect the function of the inserted genetic elements.

### 3.4.4 Stability of the genetic changes in corn line DP51291

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification (as produced in the initial transformation events) over successive generations. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

#### 3.4.4.1 Genetic stability

Southern blot analysis was used to show the genetic stability of the inserted *pmi*, *mo-pat* and *ipd072Aa* gene cassettes in DP51291. Leaf-derived genomic DNA from five generations of DP51291 (T1 – T5) was extracted, digested with a restriction enzyme that possesses a single recognition site within the DP51291 insertion, and hybridised with labelled probes specific for the *pmi*, *mo-pat* and *ipd072Aa* gene cassettes. Genomic DNA from the non-GM parental line PHR03 served as a negative control, and PHR03 DNA spiked with the plasmid used to transform DP51291 with *pmi*, *mo-pat* and *ipd072Aa* served as a positive control to confirm probe hybridisation.

Hybridisation of each probe to the digested genomic DNA from DP51291 showed equivalent bands of the expected sizes across all five generations. The consistency of these results confirmed the inserted DNA is stably maintained in corn line DP51291.

#### 3.4.4.2 Phenotypic stability

##### Expression of phenotype over several generations

The inheritance pattern was assessed in five generations of DP51291 (T1, T2, T3, T4, and T5), using 100 plants per generation. Plants from each generation were evaluated by both quantitative and qualitative polymerase chain reaction (PCR) assays, using primers targeting the *ipd072Aa*, *mo-pat*, and *pmi* genes, as well as other genetic elements associated with the insertion site.

Plants were also examined phenotypically by observing plant survival after exposure to glufosinate. Each plant was assessed visually for glufosinate-tolerance four to five days after application of glufosinate spray. The absence of injury corresponded to a herbicide-tolerant (positive) phenotype.

##### Mendelian inheritance

A Chi-square ( $\chi^2$ ) analysis was undertaken over several generations to confirm the segregation and stability of the insert in DP51291. Since the inserted DNA resides at a single locus within the DP51291 genome, the inserted DNA would be expected to be inherited according to Mendelian principles. The expected segregation ratios for each generation, based on Mendelian inheritance principles, were 1:1 for the T1 generation, 3:1 for the T2 and

T3 generations, and homozygous positive for the T4 and T5 generations. The results demonstrated the expected segregation ratio for each generation (Table 1). These results were compared to the results from the phenotypic analysis and the co-segregation of genotype and phenotype was confirmed.

These data support the conclusion that the inserted DNA is present at a single locus in DP51291 and is inherited predictably according to Mendelian principles in subsequent generations.

**Table 1: Segregation results in five generations of DP51291**

Generation	Expected segregation ratio (positive:negative)	Observed number of plants			Statistical analysis	
		Positive	Negative	Total	$\chi^2$	p-value
T1	1:1	49	51	100	0.04	0.845
T2	3:1	82	18	100	2.61	0.1060
T3	3:1	74	26	100	0.05	0.8174
T4	Homozygous positive	100	0	100	-	-
T5	Homozygous positive	100	0	100	-	-

### 3.4.5 Open reading frame analysis

A bioinformatic analysis of the DP51291 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel open reading frames (ORFs) had been created in DP51291 as a result of the DNA insertion, and whether any putative peptides or polypeptides present in the insert have the potential for allergenicity or toxicity.

All stop-codon bracketed reading frames of  $\geq 8$  amino acids (aa) in length spanning the 5' and 3' insert-flank junctions of DP51291, or contained within the insert itself, were translated *in silico* from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames<sup>7</sup>. A total of 771 theoretical ORFs  $\geq 8$  aa were identified and queried against allergen and toxin databases. These analyses are theoretical only, as it is highly unlikely that any of the identified ORFs or putative peptides would be expressed *in planta*.

#### 3.4.5.1 Bioinformatic analysis for potential allergenicity

The theoretical ORFs present in the 5' and 3' insert-flank junction sequences and the amino acid sequences encoded by all six reading frames in the DP51291 insert DNA were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource ([COMPARE](http://comparedatabase.org/database/)<sup>8</sup>) database, from the Health and Environmental Science Institute. At the date of the search (January 2022), there were 2,463 sequences in the allergen database.

A FASTA search algorithm (v35.4.4) (Pearson and Lipman, 1988) was used to identify alignments between the query sequences and the COMPARE database, using a BLOSUM50 scoring matrix and an E-value threshold of 100. Only matches with a linear identity of greater than 35% over 80 amino acids were considered. In addition, a search for  $\geq$  eight contiguous aa matches to the allergens from the COMPARE database was performed using EMBOSS

<sup>7</sup> Evaluation of sequences stop-to-stop codon is a more conservative approach compared to the evaluation of start-to-stop codon sequences.

<sup>8</sup> <http://comparedatabase.org/database/>

FUZZPRO.

Comparison of the 771 putative peptides against the COMPARE allergen database identified five ORFs that had  $\geq 35\%$  identity with nine allergens over an 80 aa sliding window. In addition, four ORFs produced 8 contiguous amino acid matches to allergens in the COMPARE database. These ORFs were identified using a highly conservative approach and, collectively, these alignments either lacked upstream promoters, lacked methionine residues (start codons), or possessed start codons in positions only a short distance from a stop codon, meaning that either transcription or translation of these ORFs would be highly unlikely. The risk of allergenic proteins with relevance to human safety being produced by these ORFs is negligible.

#### **3.4.5.2 Bioinformatic analysis for potential toxicity**

The putative peptides encoded by the junction and insert sequences were compared *in silico* to an in-house toxin database (updated in January 2022). This database is a subset of sequences derived from the UniProtKB/Swiss-Prot protein databases, filtered using keywords relating to potential toxicity or adverse health effects. The ORFs were also queried against the NCBI non-redundant (nr) protein database. For both the toxin and nr protein database searches, a BLASTP algorithm with a BLOSUM62 scoring matrix and an E-value threshold of 0.0001 was used.

No alignments were found between the 771 putative polypeptides and any known protein toxins. Twelve alignments were found between the ORFs and the NCBI nr protein database, but none of these proteins were related to any known toxic proteins. Therefore, these matches are of no significance or concern.

### **3.5 Conclusion**

Corn line DP51291 contains a single copy of the intended DNA insertion, integrated at a specific locus in the corn genome. SbS and sequencing results confirmed that the *pmi*, *mo-pat*, and *ipd072Aa* cassettes were inserted with the expected sequence and organisation. No backbone sequences from the plasmids used in the transformation are present, including any antibiotic resistance genes.

The inserted DNA is stably inherited and expressed across several breeding generations of DP51291. None of the new ORFs created by the modification raised any allergenicity or toxicity concerns.

## **4 Characterisation and safety assessment of novel substances**

### **4.1 Novel Proteins**

Only a small number of dietary proteins have the potential to impair health, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutrient or allergenic effects.

To effectively identify any potential hazards, knowledge of the characteristics, detailed understanding of the biochemical function and phenotypic effects and concentration levels in

the edible part of the plant is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

Three novel substances are expressed in DP51291: the IPD072Aa insecticidal protein, which provides protection against corn rootworm; the PAT protein, which affords tolerance to the herbicide glufosinate, and the PMI protein, which allows for growth on media containing mannose and acts as a selectable marker during the transformation process. In considering the safety of newly expressed substances it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects.

#### **4.1.1 IPD072Aa**

The IPD072Aa protein is an 86 amino acid, ~10 kDa protein isolated from *P. chlororaphis*, which possesses a potent inhibitory effect on the survival of western corn rootworm larvae (WCR; *D. virgifera*). Although IPD072Aa does not share structural similarity with any other known proteins, it produces a similar effect to the crystal (Cry) proteins from *Bacillus thuringiensis*, in disrupting the midgut cells of WCR larvae (Schellenberger et al. 2016, Jiménez-Juárez et al. 2023). However, the midgut receptors to which IPD072Aa binds are distinct from those used by Cry proteins and other related insecticidal proteins in commercial use (Jiménez-Juárez et al. 2023).

Given that the source organism for IPD072Aa, *P. chlororaphis*, has been widely used as a biopesticide for the past twenty years (Kupferschmied et al 2013, EFSA 2017, US-EPA 2017), it is likely that humans have been exposed to IPD072Aa during this period. The sequence of the IPD072Aa protein expressed in DP51291 is identical to that of IPD072Aa expressed in DP23211, which has been previously assessed by FSANZ (A1202; FSANZ 2020).

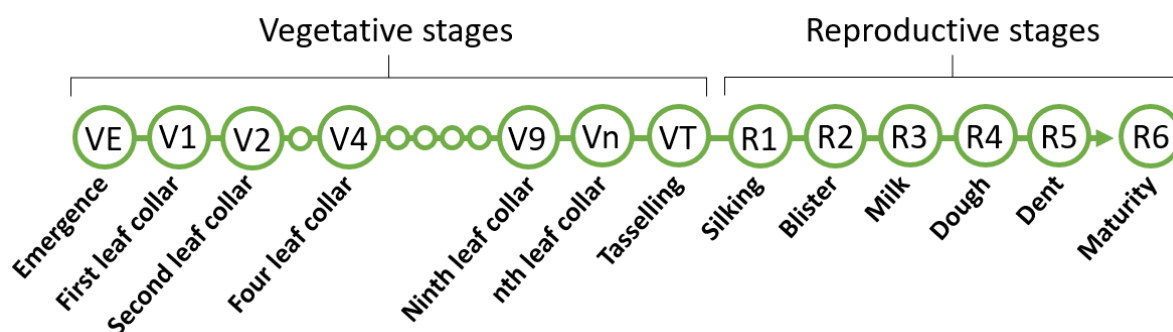
##### **4.1.1.1 Expression of IPD072Aa in DP51291 tissue**

For analysis of the expression levels of IPD072Aa protein in DP51291, tissues were collected from eight field-trial sites in representative corn-producing regions of the United States and Canada during the 2021 growing season<sup>9</sup>. Tissues were collected at varying stages of growth (see Figure 2 for a summary of corn growth stages). Samples from both glufosinate-treated or non-treated DP51291 were collected. Tissues were lyophilised, homogenised (except pollen samples) and stored frozen until analysis.

Expression levels of IPD072Aa were quantified in each tissue using a quantitative enzyme-linked immunosorbent assay (ELISA). Recombinant IPD072Aa was used as an analytical reference for plant-derived IPD072Aa. For each tissue analysed, four samples were processed from each of the eight field-trial sites.

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<sup>9</sup> Field sites for testing protein expression levels were in the following United States and Canadian states – Iowa, Illinois, Indiana, Missouri, Nebraska, Pennsylvania, Texas, and Ontario.



**Figure 2.** Stages of corn growth. Grain is harvested at maturity (R6).

Results from the ELISA showed that IPD072Aa was detected in glufosinate-treated DP51291, with the highest expression on a dry weight (dw) basis at the early reproductive stage in root tissue (200 ng/mg dw). This is the target tissue for corn rootworm larvae consumption (Table 2). IPD072Aa was detected in the grain (3.8 ng/mg dw) at a very low level compared to root tissue, and pollen had the lowest level of IPD072Aa expression (1.0 ng/mg dw). Similar levels of IPD072Aa were detected in DP51291 not treated with glufosinate (Table 2).

**Table 2: IPD072Aa concentrations (ng/mg dw<sup>1</sup>) in DP51291 tissues**

Tissue (Growth Stage <sup>2</sup> )	Mean	Range	Standard Deviation	LLOQ <sup>3</sup>
<b>DP51291</b>				
Root (V6)	76	17 - 140	31	0.11
Root (V9)	140	63 - 230	51	0.11
Root (R1)	180	66 - 330	85	0.11
Root (R4)	140	36 - 280	80	0.11
Leaf (V9)	69	23 - 140	33	0.054
Leaf (R1)	68	31 - 110	25	0.054
Leaf (R4)	53	18 - 120	28	0.054
Pollen (R1)	1.2	0.25 – 7.1	1.4	0.11
Forage (R4)	34	0.92 - 88	20	0.018
Grain (R6)	4.1	0.051 - 12	3.6	0.027
<b>Herbicide-Treated DP51291</b>				
Root (V6)	67	28 - 120	26	0.11
Root (V9)	130	33 - 260	61	0.11
Root (R1)	200	63 - 330	88	0.11
Root (R4)	140	39 - 300	82	0.11
Leaf (V9)	68	18 - 130	31	0.054
Leaf (R1)	68	22 - 120	28	0.054
Leaf (R4)	52	14 - 90	22	0.054
Pollen (R1)	1.0	0.35 – 2.5	0.62	0.11
Forage (R4)	33	9.8 - 62	17	0.018
Grain (R6)	3.8	0.24 - 11	3.2	0.027

1. dw - dry weight 2. Growth Stage abbreviations – see Figure 2 3. LLOQ – lower limit of quantitation

#### **4.1.1.2 Safety of the introduced IPD072Aa**

The IPD072Aa protein has been previously assessed by FSANZ in corn line DP23211 – Application A1202 (FSANZ 2020). In this application, studies on potential allergenicity and toxicity were submitted and assessed, and did not raise any safety concerns. Since the amino acid sequence of the IPD072Aa protein expressed in DP51291 is identical to the IPD072Aa sequence expressed in DP23211, no further safety evaluation is required other than the examination of updated bioinformatics searches.

Updated bioinformatic studies for IPD072Aa that looked for amino acid sequence similarity to known protein allergens and toxins were provided by the applicant (January 2022). The results do not alter the conclusions reached in the assessment of DP23211.

#### **4.1.2 PAT**

The *mo-pat* gene in DP51291 encodes the protein phosphinothricin N-acetyltransferase (PAT), which enzymatically inhibits phosphinothricin (PPT) (Strauch et al. 1988; Wohlleben et al. 1988). PPT is the active constituent of glufosinate ammonium herbicides and acts by irreversibly inhibiting the endogenous plant enzyme glutamine synthetase. This enzyme is involved in amino acid biosynthesis in plant cells and its inhibition causes accumulation of ammonia, leading to plant death. In glufosinate-tolerant GM plants, the introduced PAT enzyme chemically inactivates PPT by acetylation of the free ammonia group to produce N-acetyl glufosinate, allowing plants to continue amino acid biosynthesis in the presence of the herbicide (Hérouet et al. 2005).

The *mo-pat* gene in DP51291 has been codon optimised for expression in corn. The deduced amino acid sequence from translation of the *mo-pat* gene is identical to that produced from the *pat* gene in the source organism *S. viridochromogenes*. Both genes encode a 183 amino acid protein with a calculated molecular weight of ~21 kilodaltons (kDa).

The PAT enzyme has been used to confer glufosinate-tolerance in crops for approximately 25 years (CERA 2011). Since 2002, FSANZ has assessed and approved numerous events with *pat*-encoded glufosinate-tolerance. There have been no credible reports of adverse effects on human health since it was introduced into food.

##### **4.1.2.1 Expression of PAT in DP51291 tissue**

PAT expression was determined using an ELISA on the same processed tissue samples analysed for IPD072Aa (Section 4.1.1.1). Results from the ELISA (Table 3) show that PAT was detected in glufosinate-treated DP51291, with the highest expression in pollen in the early reproductive stage (R1 – 68 ng/mg dw). At maturity (R6), PAT was detected in the grain (5.8 ng/mg dw). Similar levels of PAT expression were detected in DP51291 not treated with glufosinate (Table 3).

**Table 3: PAT concentrations (ng/mg dw<sup>1</sup>) in DP51291 tissues**

Tissue (Growth Stage <sup>2</sup> )	Mean	Range	Standard Deviation	LLOQ <sup>3</sup>
<b>DP51291</b>				
Root (V6)	34	16 - 51	8.0	0.054
Root (V9)	26	14 - 36	6.2	0.054
Root (R1)	21	15 - 33	4.6	0.054
Root (R4)	10	6.6 - 17	2.3	0.054
Leaf (V9)	38	30 - 49	5.9	0.11
Leaf (R1)	40	32 - 50	4.5	0.11
Leaf (R4)	21	15 - 28	3.2	0.11
Pollen (R1)	67	58 - 83	7.5	0.22
Forage (R4)	15	11 - 22	2.7	0.036
Grain (R6)	5.7	2.3 – 9.0	1.8	0.054
<b>Herbicide-Treated DP51291</b>				
Root (V6)	33	23 - 42	5.9	0.054
Root (V9)	26	12 - 42	8.1	0.054
Root (R1)	20	9.9 - 28	4.4	0.054
Root (R4)	12	7.5 - 21	3.7	0.054
Leaf (V9)	37	23 - 47	6.5	0.11
Leaf (R1)	42	29 - 55	6.3	0.11
Leaf (R4)	22	16 - 28	2.9	0.11
Pollen (R1)	68	59 - 80	5.5	0.22
Forage (R4)	16	11 - 22	2.6	0.036
Grain (R6)	5.8	3.3 - 11	1.7	0.054

1. dw - dry weight 2. Growth Stage abbreviations – see Figure 2 3. LLOQ – lower limit of quantitation

#### 4.1.2.2 Safety of the introduced PAT

The PAT protein encoded by the *pat* or *mo-pat* gene has been considered in numerous previous FSANZ safety assessments, including 11 in corn. These assessments, together with the published literature, have established the safety of PAT and confirm it does not raise toxicity or food allergenicity concerns in humans (ILSI 2016; Hammond et al. 2011; Delaney et al. 2008; Hérouet et al. 2005).

Since the PAT protein expressed in DP23211 is identical in amino acid sequence to PAT protein expressed in previously assessed lines, no further safety evaluation is required other than the examination of updated bioinformatics searches.

The applicant has submitted updated bioinformatic studies for PAT that looked for amino acid sequence similarity to known protein allergens and toxins (January 2022). The results do not alter conclusions reached in previous assessments.

#### 4.1.3 PMI

The *pmi* gene in DP51291 encodes the enzyme phosphomannose isomerase (PMI), which

catalyses the interconversion of mannose 6-phosphate and fructose 6-phosphate. Expression of PMI allows plant cells to use mannose as a source of carbon, which assists with the identification of transformed cells (Negrotto et al. 2000).

The *pmi* gene encodes a 391 amino acid protein with a calculated molecular weight of ~43 kilodaltons (kDa). PMI has been assessed by FSANZ previously as a novel protein in five corn lines and one rice line.

#### 4.1.3.1 Expression of PMI in DP51291 tissue

PMI expression was determined using an ELISA on the same processed tissue samples analysed for IPD072Aa and PAT (Section 4.1.1.1). PMI was detected in glufosinate-treated DP51291, with the highest expression in leaf at the R4 growth stage (31 ng/mg dw). At maturity (R6), PMI was detected in the grain (3.7 ng/mg dw). Similar levels of PMI expression were detected in DP51291 not treated with glufosinate (Table 4).

**Table 4: PMI concentrations (ng/mg dw<sup>1</sup>) in DP51291 tissues**

Tissue (Growth Stage <sup>2</sup> )	Mean	Range	Standard Deviation	LLOQ <sup>3</sup>
<b>DP51291</b>				
Root (V6)	8.3	2.7 - 13	2.7	0.27
Root (V9)	6.9	2.9 - 12	2.3	0.27
Root (R1)	4.8	2.3 – 8.4	1.6	0.27
Root (R4)	3.7	2.4 – 6.3	1.0	0.27
Leaf (V9)	8.9	4.4 - 14	2.7	0.054
Leaf (R1)	13	7.2 - 26	4.6	0.054
Leaf (R4)	29	17 - 43	6.0	0.054
Pollen (R1)	29	19 - 37	4.4	1.1
Forage (R4)	9.2	6.8 - 12	1.3	1.8
Grain (R6)	4.1	1.7 – 9.3	1.7	0.27
<b>Herbicide-treated DP51291</b>				
Root (V6)	7.3	4.5 - 11	2.3	0.27
Root (V9)	6.9	2.8 - 11	2.0	0.27
Root (R1)	4.9	3.3 – 7.5	1.3	0.27
Root (R4)	3.9	2.3 – 6.0	1.1	0.27
Leaf (V9)	8.3	4.6 - 14	3.1	0.54
Leaf (R1)	15	7.8 - 28	5.2	0.54
Leaf (R4)	31	23 - 38	4.4	0.54
Pollen (R1)	30	22 - 37	4.3	1.1
Forage (R4)	9.2	6.2 - 13	1.4	1.8
Grain (R6)	3.7	2.0 – 5.7	0.94	0.27

1. dw - dry weight 2. Growth Stage abbreviations – see Figure 2 3. LLOQ – lower limit of quantitation

#### 4.1.3.2 Safety of the introduced PMI

The PMI protein has been previously assessed by FSANZ in five corn lines: 5307



(Application A1060; FSANZ 2012), MIR162 (Application A1001; FSANZ 2008a), 3272 (Application A580; FSANZ 2008b), MIR604 (Application A564; FSANZ 2006) and DP23211 (A1202; FSANZ 2020), as well as in rice line GR2E (Application A1038; FSANZ 2017). These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans. Since the PMI protein expressed in DP51291 is identical in amino acid sequence to the PMI protein expressed in previously assessed corn and rice lines, no further safety evaluation is required other than the examination of updated bioinformatics searches.

Updated bioinformatic studies for PMI that looked for amino acid sequence similarity to known protein allergens and toxins were provided by the applicant (January 2022). The results do not alter conclusions reached in previous assessments.

#### **4.1.4 Conclusion**

The data submitted by the applicant confirms that the IPD072Aa, PAT and PMI proteins expressed in DP51291 are identical to proteins previously assessed by FSANZ. All three proteins were expressed throughout the plant, including in grain. Updated bioinformatic analyses were consistent with previous analyses showing that none of the three proteins shared any meaningful homology with any known allergens or toxins. Taken together, the studies provided do not alter the conclusions reached in previous assessments for the three substances.

## **4.2 Herbicide metabolites**

FSANZ has assessed the novel herbicide metabolites for glufosinate in GM crops in multiple previous applications. These previous assessments indicate the spraying of DP51291 with glufosinate ammonium would result in the same metabolites that are produced in non-GM corn sprayed with the same herbicide. As no new glufosinate metabolites would be generated in corn event DP51291, further assessment is not required.

# **5 Compositional analysis**

The main purpose of compositional analyses is to determine if, as a result of the genetic modification, any unexpected change has occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analyses can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The classic approach to the compositional analyses of GM food is a targeted one. Rather than analysing every possible constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors such as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health.

## **5.1 Key components**

The key components to be analysed for the comparison of transgenic and conventional corn

are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Maize (OECD 2002), and include: proximates (protein, fat, fibre, ash, carbohydrates), amino acids, fatty acids, minerals, vitamins and the anti-nutrients phytic acid, raffinose, furfural and the phenolic acids ferulic acid and *p*-coumaric acid.

## 5.2 Study design

DP51291 (F1 generation), a non-GM control of similar genetic background (PHEJW/PHR03), and a total of 20 non-GM commercial reference lines were grown and harvested from eight field trial sites in the United States and Canada during the 2021 growing season<sup>10</sup>. The sites were representative of corn growing regions suitable for commercial production. The field sites were established in a randomised complete block design with four replicates per site. Each block contained DP51291 maize, control maize, and four reference lines selected from 5433, H3257, K-0204, P0574, 205-17, 207-27, G07F23, P0843, P1093, 5858, H3394, K-0608, 209-50, 6076, 6046, G10T63, G11A33, 6282, G12W66, and 6269. Plants were grown under agronomic field conditions typical for each growing region. A herbicide treatment of glufosinate was applied to the DP51291 maize.

At maturity, grain was harvested from all plots, with reference and control grain collected prior to glufosinate-treated DP51291 samples to minimise the potential for contamination. Following harvest, samples were chilled before being transferred to a freezer (<10°C) or dry ice and shipped frozen to an analytical laboratory with full identity labelling. Compositional analyses were performed based on internationally recognised procedures including official methods specified by the Association of Official Analytical Chemists (AOAC), the Analytical Oil Chemists' Society (AOCS), methods specified by the manufacturer of the equipment used for analysis, or other published scientific methods.

A total of 70 analytes in grain were assessed (see Figure 3 for a complete list, not including moisture). For 6 of these analytes (listed in grey in Figure 3) all samples of both DP51291 and control maize were below the assay lower limit of quantification (LLOQ) and were therefore not analysed statistically.

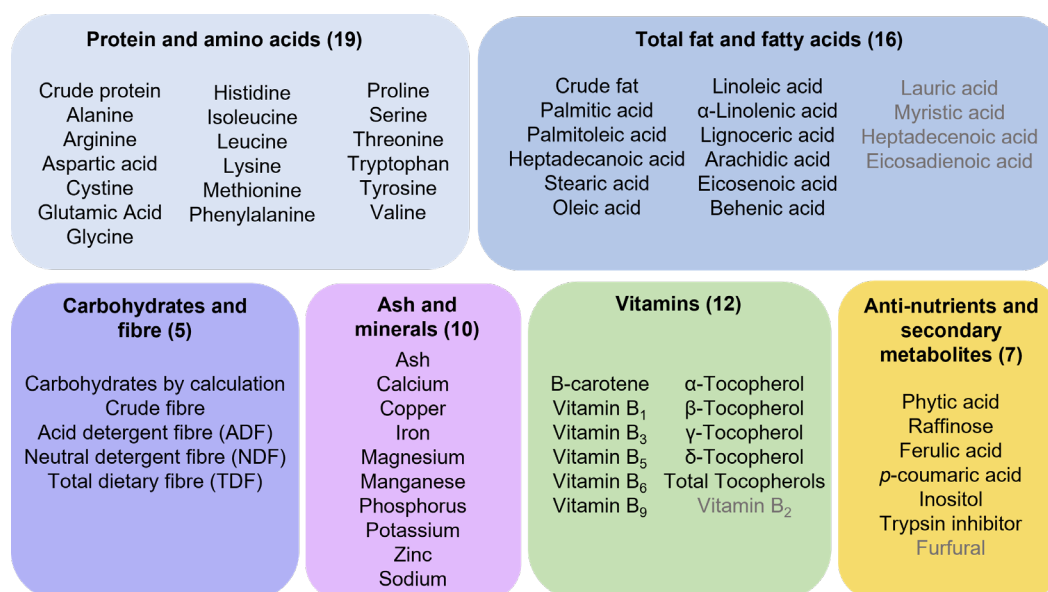
For the remaining 64 analytes, 'descriptive statistics' (mean, range and 95% confidence interval) were generated. For 59 of these analytes, where both DP51291 and the control maize had <50% of samples below the LLOQ, a linear mixed model analysis of variance was applied for combined data and locations, covering the eight replicated field trial sites. The mixed model analysis was also applied to the data from each site separately. Where statistically significant differences were observed in the combined data from all sites, analysis of the data from each site was used to determine if the differences were common to the majority of sites. For the remaining 5 analytes (heptadecanoic acid, behenic acid,  $\beta$ -tocopherol,  $\delta$ -tocopherol, and raffinose), >50% of either DP51291 or the control maize samples were below the LLOQ, Fisher's exact test was used to assess whether there was a significant difference in the proportion of samples below the LLOQ between the two maize lines across sites.

In assessing the statistical significance of any difference between DP51291 and the conventional control, a p-value of 0.05 was used. A further adjusted p-value was determined using the false discovery rate (FDR) method, as a consideration of the chance of false positives being observed with the testing due to the multiple analytes being analysed. In cases where the raw p-value was <0.05 but the FDR-adjusted p-value was >0.05, the difference was considered likely to be a false positive.

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<sup>10</sup> The location of the eight field trial sites: one site in each of Iowa, Illinois, Indiana, Missouri, Nebraska, Pennsylvania, Texas, and Ontario.

Any statistically significant differences between DP51291 and the control were compared to tolerance intervals derived from an in-house database containing compositional analyses from 196 non-GM commercial lines cultivated across 202 unique environments in North and South America, from 2003-2020. Tolerance intervals are expected (with 95% confidence) to contain at least 99% of the values for corresponding analytes of the conventional maize population (Hong et al 2014). In addition, compositional data from the non-GM reference varieties grown concurrently in the same trial as DP51291 and the control were combined across all sites and used to calculate an in-study reference range for each analyte. This reference range is useful to define the variability in corn varieties grown under the same agronomical conditions. Finally, the natural variation of analytes from publically available data was also considered (Watson 1982; OECD 2002; Codex 2019; Lundry et al 2013; Cong et al 2015; AFSI 2023). These data ranges assist with determining whether any statistically significant differences are likely to be biologically meaningful.



**Figure 3.** Analytes measured in DP51291 grain samples. Analytes listed in grey text had all samples below the LLOQ and were excluded from statistical analysis. The analytes listed in black text, as well as moisture, were analysed fully.

### 5.3 Analyses of key components in grain

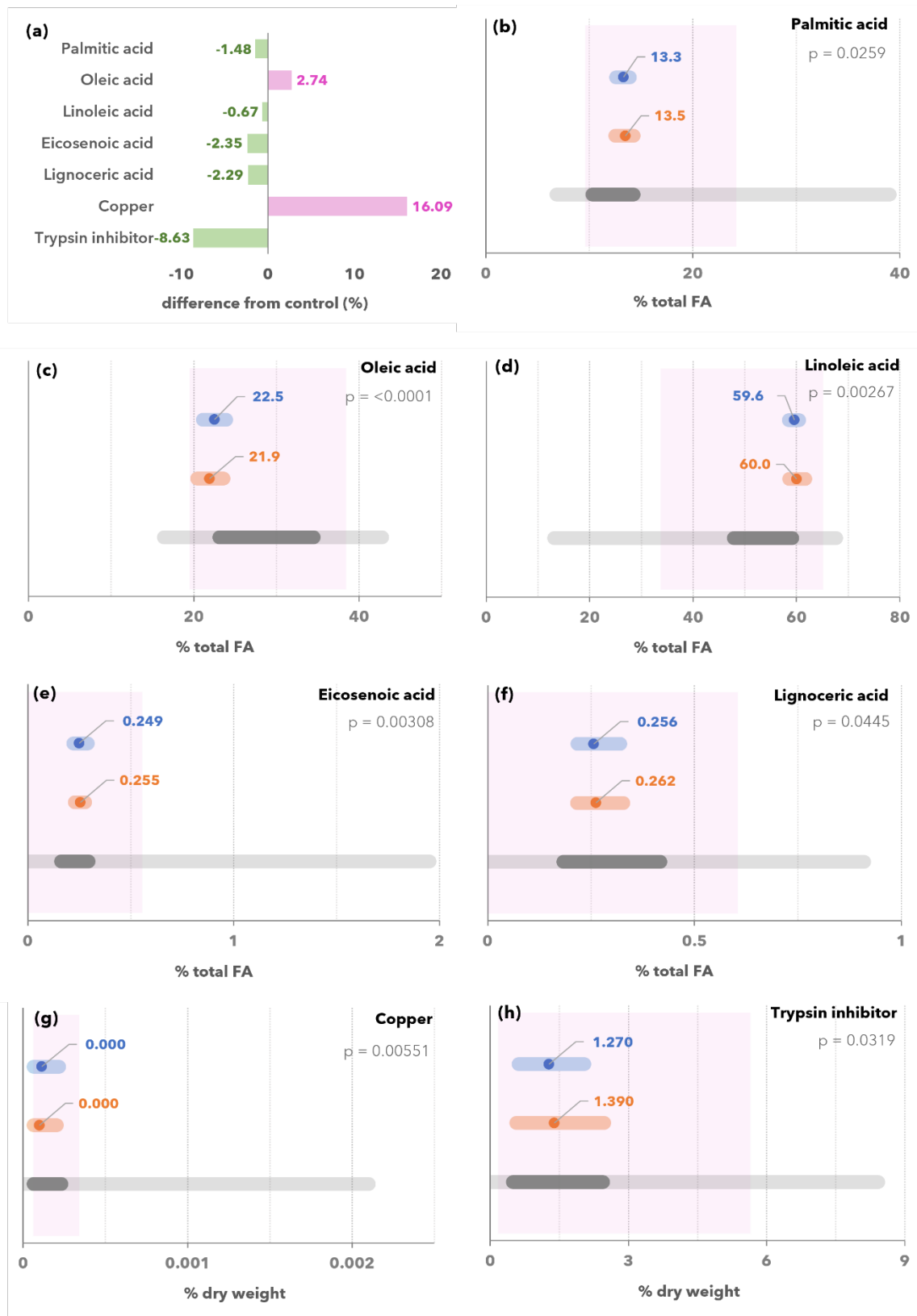
Of the 70 analytes measured in grain, mean values were provided for 64 analytes and of these, there were 7 for which there was a statistically significant difference ( $p < 0.05$ ) between herbicide-treated corn line DP51291 and the control: palmitic acid, oleic acid, linoleic acid, eicosenoic acid, lignoceric acid, copper, and trypsin inhibitor. A summary of these 7 analytes is provided in Figure 4. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the [Application dossier](#)<sup>11</sup> (pages 103 – 120).

Of these 7 analytes for which a statistically significant difference was found, all except oleic acid had FDR-adjusted  $p$ -values of  $>0.05$ , suggesting that the differences in these analytes were likely to be false positives. In addition, as can be observed in Figure 4 (panels b-h), the DP51291 mean for each of these 7 components was within the control range value, indicating that DP51291 has a smaller impact on the levels of these analytes than does

<sup>11</sup> <https://www.foodstandards.gov.au/code/applications/Pages/A1270---Food-derived-from-herbicide-tolerant-and-insect-protected-corn-line-DP51291.aspx>

natural variation within the conventional control. For all 7 analytes, including oleic acid, the observed DP51291 means fall well within the natural variability represented by the tolerance interval, in-study reference range and publicly available range (purple shaded area, dark grey and light grey bars, respectively, in Figure 4, b-h). The differences reported here are therefore consistent with the normal biological variability that exists in corn.

Overall, the compositional data support the conclusion that no biologically significant differences exist in the levels of key constituents in DP51291 when compared to conventional non-GM corn cultivars already available in agricultural markets. Grain from DP51291 can therefore be regarded as equivalent in composition to grain from conventional non-GM corn.



**Figure 4.** Visual summary of statistically significant compositional differences between DP51291 and the conventional control. **(a)** Percentage deviation of the mean DP51291 value from the mean control value for each of the 7 analytes for which a statistically significant difference was found. **(b) – (h)** Measured means (dots) and ranges (coloured bars) for DP51291 (blue) and the conventional control (orange) for the 7 analytes as labelled. The light and dark grey bars represent the publicly-available range of values and in-study reference range of values, respectively, for each analyte. The purple shaded range represents the tolerance interval for each analyte. Note that the x-axes vary in scale and unit for each component.

## 6 Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food, such as that presented in Section 5.

Where a GM food has been shown to be compositionally equivalent to conventional cultivars, the evidence to date indicates that feeding studies using target livestock or other animal species will add little to the safety assessment (OECD 2003; Bartholomaeus et al. 2013). If the compositional analysis indicates biologically significant changes, either intended or unintended, to the levels of certain nutrients in the GM food, additional nutritional studies should be undertaken to assess the potential impact of the changes on the whole diet.

DP51291 is the result of genetic modifications to confer tolerance to the herbicide glufosinate and protection against corn rootworm pests, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modification has not altered the nutrient composition DP51291 compared with conventional non-GM corn cultivars. The introduction of food derived from DP51291 into the food supply is therefore expected to have negligible nutritional impact.

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