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Supporting document 1

Safety assessment – Application A1323

Food derived from insect-protected soybean line COR23134

Executive summary

Background

Application A1323 seeks approval for the sale and use of food derived from soybean line COR23134 that has been genetically modified (GM) for protection from lepidopteran insect pests.

Insect protection is conferred by the expression of the *cry1B.34.*1, cry1B*.61.1* and *ipd083Cb* genes encoding three novel insecticidal proteins: Cry1B.34.1, Cry1B.61.1 and IPD083Cb, respectively. Cry1B.34.1 and Cry1B.61.1 are modified Cry family proteins from the soil bacterium *Bacillus thuringiensis*. IPD083Cb is an insecticidal protein from the giant maidenhair fern, *Adiantum trapeziforme* var. *braziliense*.

COR23134 also expresses the GM-HRA protein from soybean (*Glycine max*), which is used as a selectable marker.

Food Standards Australia New Zealand (FSANZ) has previously assessed the GM-HRA protein and the full length version of the Cry1B.34.1 protein, Cry1B.34. This is the first time FSANZ has assessed the Cry1B.61.1 and IPD083Cb proteins.

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

Soybean has a long history of safe use in the food supply. Soybean oil is widely used in cooking and as an ingredient in a wide range of manufactured products. Soybean seeds are also used to make soy milk, soy sauce, soy lecithin and meat substitutes such as tofu and tempeh.

Molecular characterisation

The genes encoding Cry1B.34.1 (*cry1B.34.1*), Cry1B.61.1 (*cry1B.61.1*), IPD083Cb (*ipd083Cb)* and GM-HRA (*gm-hra_1*) were introduced into soybean line COR23134 via *Agrobacterium*-mediated transformation. Detailed molecular analyses indicate a single copy of each of the four expression cassettes is present at a single insertion site in the COR23134 genome. There are no

extraneous plasmid sequences or antibiotic resistance 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

All four novel proteins (Cry1B.34.1, Cry1B.61.1, IPD083Cb and GM-HRA) are expressed throughout COR23134, with the highest levels expressed in leaf. Expression levels in seed were relatively low, compared to other tissues.

Characterisation studies confirmed that GM-HRA protein is highly similar to a protein previously assessed by FSANZ, while the truncated Cry1B.34.1 protein expressed in COR23134 contains an identical core region to the Cry1B.34 protein previously assessed by FSANZ. All proteins are rapidly degraded and heat inactivated, based on studies submitted with this application and/or conclusions from previous assessments. Bioinformatics studies for all four proteins confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens. Taken together, the evidence supports the conclusion that Cry1B.34.1, Cry1B.61.1, IPD083Cb and GM-HRA are not toxic or allergenic to humans.

Compositional analyses

Detailed compositional analyses were performed on COR23134. Statistically significant differences in mean values were found between seed from COR23134 and the non-GM control for 10 of the 70 analytes evaluated, however these differences were all within the range established for existing commercial non-GM soybean varieties. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in seed from COR23134 compared to non-GM soybean varieties available on the market.

Conclusion

No potential public health and safety concerns have been identified in the assessment of insect-protected soybean line COR23134. On the basis of the data provided in the present application and other available information, food derived from COR23134 is considered to be as safe for human consumption as food derived from conventional non-GM soybean varieties.

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List of Abbreviations

Abbreviation	Description
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
BSA	bovine serum albumin
COMPARE	COMprehensive Protein Allergen Resource
CTP	chloroplast transit peptide
DNA	deoxyribonucleic acid
dw	dry weight
ELISA	Enzyme Linked Immunosorbent Assay
FASTA	fast alignment search tool – all
FAO	Food and Agriculture Organisation of the United Nations
FSANZ	Food Standards Australia New Zealand
g	gram
GM	genetically modified
kDa	kilodalton
LLOQ	lower limit of quantitation
MT	million tons
NCBI	National Centre for Biotechnology Information
ng	nanogram(s)
nt	nucleotide
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PCR	polymerase chain reaction
RF	reading frame
RNA	ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-DNA	transfer DNA
µg	microgram(s)
USDA	United States Department of Agriculture
UTR	untranslated region

1 Introduction

Food Standards Australia New Zealand (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) soybean line COR23134, with the OECD Unique Identifier COR-23134-4. This soybean line has been modified for protection from lepidopteran insect pests.

Insect protection is conferred by the expression of the *cry1B.34.*1, cry1B*.61.1* and *ipd083Cb* genes encoding three novel insecticidal proteins: Cry1B.34.1, Cry1B.61.1 and IPD083Cb, respectively. Cry1B.34.1 and Cry1B.61.1 are derived from chimeric and modified gene sequences, respectively, from the soil bacterium *Bacillus thuringiensis*. IPD083Cb is an insecticidal protein from the giant maidenhair fern, *Adiantum trapeziforme* var. *braziliense*. COR23134 also expresses the GM-HRA protein from soybean, which is used as a selectable marker.

FSANZ has previously assessed the GM-HRA protein. Cry1B.34.1 is a truncated version of the Cry1B.34 protein, which has also been previously assessed by FSANZ. This is the first time FSANZ has assessed the Cry1B.61.1 and IPD083Cb proteins.

If approved, food derived from soybean line COR23134 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 soybean (*Glycine Max* (L.)), from the family Leguminosae. The non-GM soybean variety 93Y21 was used as the parental variety for the genetic modification described in this application and served as the conventional control for the purposes of comparative assessment with COR23134.

Soybean has a long history of safe human and animal consumption, and is the leading oilseed crop in the world, with total global production reaching 394.71 MT in 2023/2024 (USDA 2024). In many major soybean-producing countries, herbicide-tolerant GM soybean varieties are a major contributor to total production – for example, in 2024, 96% of all soybeans planted in the US were herbicide-tolerant GM soybeans[1].

Soybean production in Australia is comparatively minor, totalling 0.065 MT in 2023/2024 (USDA 2024), while New Zealand has no commercial soybean production. Australia and New Zealand are net importers of soybeans, with 2788.88 tonnes and 2352.68 tonnes imported respectively in 2022 (FAOSTAT 2024). No GM soybean lines are currently grown commercially in Australia.[2]

Whole soybeans are used to produce soy milk, tofu and soy sprouts, as well as fermented foods including miso, soy sauce, natto and tempeh. Soybeans may also be eaten with minimal processing, for example, as edamame, in which immature soybeans are boiled whole in their pods and served with salt. Soybean seeds are processed into two major products: oil and meal. Soybean oil is the second most consumed vegetable oil worldwide and accounts for 29% of global vegetable oil consumption (American Soybean Association 2023). It is used in a variety of manufactured foods, including cooking oil, shortening, margarine, salad dressings, frozen desserts and confectionery products. Soybean meal is a good source of protein and is primarily processed

[1] For more information please see USDA Economic Research Service: https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-united-states/recent-trends-in-ge-adoption

[2] Information on approved commercial releases of GM crops in Australia can be found on the website of the Office of the Gene Technology Regulator: https://www.ogtr.gov.au/

into livestock feed (pet and poultry food) and protein products such as soy flour, concentrates and isolates.

Soybeans are also a rich source of a number of bioactive phytochemicals, such as isoflavones and tocopherols (vitamin E) (Liu 2004), and are used as a source of these compounds for dietary supplements. Unprocessed (raw) soybean products are not suitable for food uses, due to the presence of anti-nutrients, such as phytic acid and lectins (OECD 2012). The heat applied during processing inactivates these anti-nutrients.

2.2 Donor organisms

2.2.1 Bacillus thuringiensis

The *cry1B.34.1 and cry1B.61.1* genes are derived from the Gram-positive bacterium *Bacillus thuringiensis*, a common soil bacterium which is ubiquitously distributed in the environment. *B. thuringiensis* expresses a number of insecticidal proteins, including the well-characterised Cry proteins. This bacterium has a long history of safe use in agriculture as a pesticide, as well as a history of safe use as a (US-EPA 2001; Nester et al. 2002; CERA 2011). Currently, there are approximately 44 biopesticide products based on *B. thuringiensis* registered in Australia (APVMA 2024) and 10 in New Zealand (ACVM 2024). While highly specific and toxic to their target insects, Cry proteins are innocuous to humans, vertebrates and plants (Bravo et al. 2007).

B. thuringiensis has been linked to human diarrheal illness due to its close relationship with the species *Bacillus cereus*. Some *B. cereus* strains contain enterotoxin genes and are pathogenic to humans (Ehling-Schulz et al. 2019; Biggel et al. 2022), However, it is unlikely *B. thuringiensis* subspecies or strains are causal agents of food-induced diarrhoea (Raymond and Federici 2017; Biggel et al. 2022).

2.2.2 Adiantum trapeziforme

The source of the *ipd083Cb* gene is the giant maidenhair fern, *Adiantum trapeziforme* var. *braziliense*. Members of the maidenhair fern family and other non-seed plants have been utilized for ethnomedicinal purposes from treating respiratory infections such as cough, colds and pneumonia. Many species of genus *Adiantum* L. are used in traditional medicine as infusions, decoctions, or pastes (Rastogi et al. 2018). The potential benefits of compounds from members of this genus is the subject of ongoing research (Rastogi et al. 2018). The applicant notes that there have been no reports of *A. trapeziforme* being poisonous to either humans or livestock.

2.2.3 Glycine max

The source of the *gm-hra_1* gene is soybean (*G. max*). As discussed in section 2.1 above, soybean has a long history of use in food.

2.2.4 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of COR23134. These genetic elements are non-coding sequences and are used to regulate the expression of the inserted genes.

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 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 transforming plasmid used in construction of COR23134, as well as its breeding history, were provided in the application as Confidential Commercial Information (CCI). While the full details of CCI cannot be provided in this public report, FSANZ had regard to this information in its assessment.

3.1 Transformation method

To create soybean line COR23134, conventional soybean variety 93Y21 was transformed with a plasmid containing the *cry1B.34.1*, *cry1B.61.1*, *ipd083Cb*, and *gm-hra_1* expression cassettes located within a transfer DNA (T-DNA) region.

Transformation of the 93Y21 line was achieved by co-culturing immature cotyledons[3] with *Agrobacterium tumefaciens* containing the transformation plasmid. Cotyledons were regenerated and cultivated over multiple steps on media containing an antibiotic to kill *Agrobacterium* and/or a herbicide for selection of plants containing the *gm-HRA_1* selectable marker gene. Once shoots and roots were established, healthy plants were selected, and PCR was used to confirm the presence of the T-DNA insert. Plants that were regenerated from transformation and tissue culture (designated T0 plants) were selected for further characterization.

Subsequent generations were further evaluated for insert integrity, trait efficacy, phenotypic characteristics and agronomic performance. Soybean line COR23134 was then selected.

3.2 Detailed description of inserted DNA

The T-DNA region from the transformation plasmid integrated into the soybean genome includes the *cry1B.34.1*, *cry1B.61.1*, *ipd083Cb*, and *gm-hra_1* expression cassettes.

3.3 Development of the soybean line from the original transformation

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of COR23134
- ensuring that the COR23134 event is incorporated into elite lines for commercialisation.

The generations analysed for the molecular characterisation and other analyses are listed in Table 1. The comparator used for all studies was soybean line 93Y21, which is isogenic to COR23134 except for the inserted T-DNA.

[3] Embryonic leaves that form within the seeds of seed plants and are the first to emerge during germination.

Table 1. COR23134 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Insertion copy number, insertion organization, and the absence of plasmid backbone sequences by SbS	Section 3.4	T1	93Y21
Insertion organisation and stability by Southern blot	Section 3.4.4	T1, T2, T3, T4, T5	93Y21
Mendelian inheritance by multi-generation segregation analysis	Section 3.4.4	T1, T2, T3, T4, T5, T6	93Y21
Sequence determination of Insert and its flanking genomic regions by Sanger sequencing	Section 3.4.3	T4	93Y21
Composition and expression analysis	Section 4.5 and Section 5	T5	93Y21

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

A range of analyses were undertaken to characterise the genetic modification in COR23134. These analyses focused on the nature and stability of the inserted DNA and whether any unintended re-arrangements or products may have occurred as a consequence of the transformation procedure.

To characterise the number of integration sites, organisation of the inserted DNA, and to confirm the absence of any unintended plasmid sequences, the applicant made use of Southern-by-Sequencing (SbS) technology (Zastrow-Hayes et al. 2015). SbS is a molecular characterization method that combines probe-based capture techniques with next-generation sequencing (NGS).

3.4.1 Number of integration site(s)

Genomic DNA was extracted from leaf tissue from:

- 4 plants containing the insertion from the T1 generation of COR23134,
- 6 null segregant plants from the T1 generation of COR23134,
- 1 plant from the non-GM 93Y21 line as a negative control, and
- 1 plant from the non-GM line 93Y21 spiked with the transformation plasmid as a positive control.

The DNA samples were used to construct sequencing libraries, hybridized with the capture probes (approximately 120 nucleotides long), then sequenced by Illumina MiSeq to a total read depth of at least 300x.

Bioinformatics programs were used to trim and ‘clean’ the sequencing data. The reads were aligned to the transformation plasmid sequence, including plasmid backbone. Bioinformatics programs were used to identify unique junctions attributable to inserted DNA. These junctions may be either between the inserted DNA and the genomic DNA (plasmid-genome junctions), or between two non-contiguous sequences derived from the transformation plasmid (plasmid-plasmid junctions).

SbS analysis of each of the 4 transgenic plants yielded sequencing reads that aligned to the intended insertion, and identified two unique plasmid-genome junctions, indicating that a single

copy of the intended insertion was integrated into the genome of COR23134.

Although some sequencing reads from the null segregant plants and the conventional control aligned to the transformation plasmid T-DNA and backbone sequences, these sequencing reads aligned only to the endogenous genetic elements present in the soybean genome. No junctions were detected in either the control or in the 6 null segregant plants.

The positive control sample resulted in sequence coverage across the entire length of the transformation plasmid with no detected junctions, as expected.

3.4.2 Absence of backbone and other sequences

The SbS analysis included a set of hybridisation probes to the backbone sequence of the transformation plasmid. Alignment of NGS reads from the controls or COR23134 to the plasmid sequences confirmed there was no integration of backbone sequences into COR23134, including the *spc* and *ntpIII* antibiotic resistance genes. Furthermore, there were no junctions identified between the plasmid backbone sequence and the soybean genome sequences, further demonstrating that no plasmid backbone sequences were incorporated into COR23134 soybean.

3.4.3 Insert integrity and site of integration

The SbS analysis indicated that COR23134 contains a single copy of the intended insertion. In addition to the two unique plasmid-genome junctions observed in the four transgenic plants, a single identical plasmid-plasmid junction was found, suggesting there is a deletion within the insertion. This was identified as a 21 bp deletion in a promotor region.

In addition to SbS, Sanger sequencing was used to sequence the insert and flanking soybean genomic regions. Genomic DNA was extracted from leaf tissue from 8 T4 COR23134 soybean plants and 1 plant from the non-GM 93Y21 line. PCR primers were designed to amplify seven overlapping PCR fragments spanning the insert and its 5' and 3' flanking genomic regions. The resulting PCR products were cloned into plasmids and sequenced by Sanger sequencing. The sequences of all seven overlapping fragments were combined to construct a consensus sequence for COR23134 soybean, which was compared to the sequence of the transformation plasmid T-DNA and a soybean reference genome.

The sequencing results confirmed the organisation of the insert in COR23134 is as expected, with the exception of the small 21 bp deletion identified by SbS. Furthermore, bioinformatic analysis of the flanking genomic sequences compared to the *G. max* genome confirmed the chromosomal location of the insert, and indicated that no endogenous genes were disrupted by the insertion.

3.4.4 Stability of the genetic changes in soybean line COR23134

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 event) 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 T-DNA insert in COR23134. Leaf-derived genomic DNA from five generations of COR23134 (T1, T2, T3, T4, T5) was extracted, digested with restriction enzyme *Bst1107 I* and hybridised with digoxigenin-labelled probes against *cry1B.34.1, cry1B.61.1, ipd083Cb,* and *gm-hra_1*. Non-GM soybean line 93Y21 was used as a negative control, while 93Y21 soybean spiked with the transformation plasmid served as a positive control.

Hybridisation of each probe to the digested genomic DNA from COR23134 showed an equivalent band fingerprint across all five generations. No unexpected bands were observed. The results of the

positive and negative controls were as expected. The consistency of these results across the analysed generations confirmed the inserted DNA is maintained stably in soybean line COR23134.

3.4.4.2 Phenotypic stability

Mendelian inheritance

Since the inserted T-DNA resides at a single locus within the COR23134 genome, it would be expected to be inherited according to Mendelian principles. Endpoint qualitative real-time polymerase chain reaction (PCR) was used on seed chip samples (from the T1, T2, T3, T4, T5 and T6 generations) to determine the presence or absence of the COR23134 insertion. The expected segregation ratios for each generation, based on Mendelian inheritance principles, were 3:1 for the T1, T2, and T3 generations, and homozygous positive for the T4, T5, and T6 generations.

A chi-square test was performed at the 0.05 significance level to compare the observed segregation ratios of T1, T2, and T3 generations of COR23134 soybean. A chi-square test was not performed for T4, T5 and T6 generations of COR23134 soybean as all plants were identified as positive as expected for a homozygous generation.

The results demonstrated the expected segregation ratio for each generation (Table 2), indicating the inserted DNA is present at a single locus in COR23134 and is inherited predictably according to Mendelian principles.

Table 2. Segregation results for six generations of COR23134

Generation	Expected segregation ratio (positive:negative)	Observed number of plants			Statistical analysis	
		Positive	Negative	Total	χ^2	p-value
T1	3:1	20	5	25	0.33	0.5637
T2	3:1	71	29	100	0.85	0.3556
T3	3:1	78	22	100	0.48	0.4884
T4	Homozygous positive	100	0	100	-	-
T5	Homozygous positive	100	0	100	-	-
T6	Homozygous positive	100	0	100	-	-

Expression of phenotype over several generations

Plants were also examined phenotypically using a herbicide tolerance evaluation. The herbicide diclosulam was applied to 100 plants from each of T4, T5, and T6 generations of COR23134 soybean at the V3 growth stage (Figure 1 in Section 4.5). The plants were inspected for visual herbicide injury to confirm the presence or absence of tolerance to herbicide treatment for each individual plant. The absence of injury corresponded to a herbicide-tolerant (positive) phenotype. These results were then compared to the endpoint PCR results, which confirmed the co-segregation of genotype and phenotype, and the intergenerational stability of the herbicide tolerance phenotype.

3.4.5 Reading frame analysis

A bioinformatic analysis of the COR23134 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel reading frames (RFs) had been created in COR23134 as a result of the DNA insertion, and whether any putative peptides encoded by the identified RFs have the potential for allergenicity or toxicity.

All sequences of ≥ eight amino acids (aa) in COR23134 soybean within the insertion or spanning the boundary between the insertion and its flanking genomic regions were translated in silico from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames.[4] A total of 1,695 RFs ≥ eight aa were identified.

It is highly unlikely that any of the identified RFs would be expressed *in planta*, therefore further analysis is not required.

3.5 Conclusion

Soybean line COR23134 contains a single copy of the intended DNA insertion, integrated at a single locus in the soybean genome. SbS and DNA sequencing analysis confirmed that the *cry1B.34.1, cry1B.61.1, ipd083Cb,* and *gm-hra_1* expression cassettes are present in the COR23134 genome with the expected sequence and organisation. No backbone sequences from the transformation plasmid are present. The inserted DNA is stably inherited and expressed across several breeding generations of COR23134. Bioinformatic analyses of the novel RFs created by the insertion did not raise any allergenicity or toxicity concerns.

4 Characterisation and safety assessment of novel substances

In considering the safety of novel proteins it is important to understand that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, because of anti-nutrient properties or triggering of 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, allergenic or anti-nutrient effects.

To effectively identify any potential hazards, knowledge of the characteristics, concentration and localisation of all newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects 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.

Four novel proteins are expressed in COR23134: (1) Cry1B.34.1, (2) Cry1B.61.1 and (3) IPD083Cb, which are insecticidal proteins and confer protection from lepidopteran insect pests, and (4) GM_HRA-1, which serves as a selectable marker.

4.1 Cry1B.34.1

The crystal (Cry) proteins are a family of pore-forming insecticidal proteins produced by the bacterium *B. thuringiensis.* Phylogenetic analyses has established that the diversity of the Cry family of proteins evolved by the independent evolution of three structural domains, and by swapping of domains between toxins (de Maagd et al 2003).

The Cry1B1.34.1 protein is a chimeric protein encoded by the *cry1B.34.1* gene, comprised of sequences from *B. thuringiensis*. The Cry1B.34.1 protein is the truncated form of the full length Cry1B.34 protein expressed in corn line DP910521 (assessed by FSANZ in application A1281). Both forms of the protein contain an identical three-domain toxin core, but the full length Cry1B.34 protein also contains a C-terminal crystal forming domain that is not present in the truncated Cry1B.34.1 protein. The *cry1B.34.1* gene prepared by the applicant encodes a protein of 665

[4] Evaluation of sequences stop-to-stop codon is a more conservative approach compared to the evaluation of start-to-stop codon sequences.

amino acids, with an apparent molecular weight of ~75 kDa.

Both the full length Cry1B.34 and truncated Cry1B.34.1 proteins, when expressed *in planta*, achieve protection from certain susceptible lepidopteran insect species by disrupting the midgut epithelium in these species. Upon ingestion of Cry1B.34.1 by the target pest, the protein binds to specific receptors in the brush border membrane of midgut epithelial cells, leading to the formation of ion-conducting pores in the apical membrane of these cells and subsequent cell death (Schnepf et al 1998).

4.1.1 Characterisation of Cry1B.34.1 expressed in COR23134 and equivalence to a microbially-derived form

The COR23134-derived Cry1B.34.1 protein was derived from whole plant tissue and purified using immunoaffinity chromatography. Cry1B.34.1 was also expressed in *E. coli* with a C-terminal hexahistidine tag and purified by immobilized metal affinity chromatography.

Data was provided to confirm the identity of the Cry1B.34.1 protein expressed in soybean event COR23134 (Table 3). The molecular weight, immunoreactivity and bioactivity of the microbially-derived Cry1B.34.1 protein were also analysed and were equivalent to those of the COR23134-derived protein.

Table 3. Cry1B.34.1 protein identity

Characteristic	Methodology	COR23134-derived	*E.coli*-derived	Notes
Molecular weight (kDa)	SDS-PAGE	~70	~70	
Immunoreactivity	Western blot	Yes	Yes	Cry1B.34.1 monoclonal mouse antibody
N-terminal amino acid sequence	Edman sequencing	APS	No	Matches expected sequence; N-terminal methionine absent as expected
Protein sequence	LC-MS	93.7% coverage	No	Matches expected sequence
Glycosylation	Glycoprotein staining	Not glycosylated	No	
Bioactivity	7-day bioassay using *Anticarsia gemmatalis* (velvetbean caterpillar)	LC_{50} of 0.0327 µg protein/cm^2 agar-based diet	LC_{50} of 0.0274 µg protein/cm^2 agar-based diet	Overlapping 95% confidence intervals

4.1.2 Safety of the introduced Cry1B.34.1

To support the safety of the Cry1B.34.1 protein, the applicant referred to studies performed using an *E. coli*-derived Cry1B.34 protein that were submitted as part of the DP910521 corn application (A1281; FSANZ 2024). The applicant provided the following justifications to support the safety data for the Cry1B.34 protein being applied to the Cry1B.34.1 protein:

1) The only difference between the truncated Cry1B.34.1 protein (665 amino acids, 75 kDa) expressed in COR23134 soybean and the full-length Cry1B.34 protein is that the crystal forming domain at the C-terminus of the full-length protein is not present in the truncated Cry1B.34.1 protein. Both proteins contain an identical three-domain toxin core, which is responsible for exerting the toxic effects of Cry proteins in lepidopteran insects.
2) The function of the crystal forming domain is to form crystalline structures that stabilize Cry proteins from environmental degradation until they are ingested by insects (Bravo et al

2007; Adalat et al 2017; Tetreau et al 2021). The crystal forming domain has no role in the toxic effects/mode of action of Cry proteins as it is cleaved within the insect midgut before the Cry protein is rendered active.

3) It can be assumed that Cry1B.34.1 and Cry1B.34 proteins perform similarly with respect to activity because they share identical toxin core sequences and the crystal forming domain which has been removed in Cry1B.34.1 has no role in the functional activity of the Cry protein. Further, as the crystal forming domain has no role in influencing protein characteristics such as digestibility or heat lability, it can be assumed that the Cry1B.34.1 protein would share similar digestibility and heat lability characteristics to the Cry1B.34 protein.
4) Cry proteins have a history of safe use in foods, a well-characterized mode of action and a spectrum of activity that does not target mammalian species (OECD 2010; Soberón et al 2016; Jurat-Fuentes and Crickmore 2017; Tetreau et al 2021). The binding of the active protein moiety is unique to corresponding receptors in the midgut of susceptible lepidopteran target pests, and mammals lack the receptors necessary to bind Cry proteins. Furthermore, Cry proteins are prone to digestion in the acidic environment of the mammalian digestive system, and thus are unlikely to remain intact in their active form following ingestion.

In considering the above, the applicant concluded that there is no scientific basis to suggest that the truncated Cry1B.34.1 protein would present a different toxicity or allergenicity profile from the full-length Cry1B.34 protein, and therefore, it is appropriate to bridge the safety data between the two proteins. The absence of the C-terminal crystal forming domain is unlikely to increase resistance of the protein to digestion or heat degradation. It has been suggested that truncation of *Bt* toxins can enhance their activity or affect a wider range of target insects, but there is no indication that truncation alters the host range of non-target insects (OECD 2010; Deist et al 2014).

The detailed safety assessment report for DP910521 is available on the FSANZ website.[5] In this assessment, studies on the potential allergenicity and toxicity of the full length Cry1B.34 protein (heat stability, digestibility, and acute oral toxicity) were submitted and assessed, and did not raise any safety concerns. Based on the rationale provided above, it is FSANZ's assessment that these conclusions can be applied to the truncated Cry1B.34.1 protein expressed in COR23134.

As part of the current application, the applicant also submitted bioinformatic studies for Cry1B.34.1 that looked for amino acid sequence similarity to known protein allergens and toxins.

Two separate searches for the Cry1B.34.1 sequence were performed using the 2023 COMprehensive Protein Allergen REsource (COMPARE)[6] database, from the Health and Environmental Science Institute. At the date of the search, there were 2,631 sequences in the database. The two searches were:

(a) An 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 aa to the database entries. Only matches of greater than 35% similarity over ≥ 80 aa were considered.
(b) An 8-mer exact match search – Emboss FUZZPRO was used to identify whether an 8 aa peptide match existed between the query sequences and sequences within the allergen database. Only matches of 100% similarity over 8 aa were considered.

The Cry1B.34.1 sequence was also compared *in silico* to (1) an internal toxin protein database

[5] https://www.foodstandards.gov.au/food-standards-code/applications/a1281-food-derived-herbicide-tolerant-and-insect-protected-corn
[6] COMPARE – http://comparedatabase.org/database/

consisting of a subset of sequences derived from the Swiss-Prot protein database[7], filtered to remove likely non-toxin proteins, and (2) the National Center for Biotechnology Information (NCBI) National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database. A BLASTP algorithm with a BLOSUM62 scoring matrix and an E-value threshold of 0.0001 was used.

No alignments were found between Cry1B.31.1 and any known allergens or toxins.

4.1.3 Conclusion

The Cry1B.34.1 protein expressed in COR23134 is a truncated version of the Cry1B.34 protein previously assessed by FSANZ, with an identical insecticidal toxin core region and mode of action. Based on this, the safety studies for Cry1B.34 previously considered by FSANZ can be applied to Cry1B.34.1. Bioinformatic analyses confirmed that Cry1B.34.1 has no amino acid sequence similarity to known toxins or allergens. Taken together this indicates that the Cry1B.34.1 protein is unlikely to be toxic or allergenic to humans.

4.2 Cry1B.61.1

Like Cry1B.34.1, the Cry1B.61.1 protein confers protection from certain susceptible lepidopteran insect pests by binding to specific midgut receptors and causing disruption of the midgut epithelium. The Cry1B.61.1 protein in COR23134 is encoded by the *cry1B.61.1* gene, which is a modified *cry1B*-class gene from *B. thuringiensis* (Horn et al. 2017). The *cry1B.61.1* gene prepared by the applicant encodes a protein of 656 amino acids, with an apparent molecular weight of ~74 kDa.

4.2.1 Characterisation of Cry1B.61.1 expressed in COR23134 and equivalence to a microbially-derived form

The COR23134-derived Cry1B.61.1 protein was derived from whole plant tissue and purified using immunoaffinity chromatography. To obtain sufficient quantities of Cry1B.61.1 for use in safety studies, Cry1B.61.1 was also expressed in *E. coli* with a C-terminal hexahistidine tag and purified by immobilized metal affinity chromatography.

Data was provided to demonstrate the equivalency of the COR23134- and *E. coli*-derived Cry1B.61.1 proteins, and is summarised in Table 4. The results demonstrated that *E.coli*-derived Cry1B.61.1 is structurally and biochemically equivalent to COR23134-derived Cry1B.61.1. It can be concluded that *E. coli*-derived Cry1B.61.1 is a suitable surrogate for use in the safety studies described in Section 4.2.2.

[7] UniProt – https://www.uniprot.org/

Table 4. Cry1B.61.1 protein identity and equivalence

Characteristic	Methodology	COR23134-derived	Recombinant protein	Notes
Molecular weight (kDa)	SDS-PAGE	~74 kDa	~74 kDa	
Immunoreactivity	Western blot	Yes	Yes	Cry1B.61.1 polyclonal rabbit antibody 12000
N-terminal amino acid sequence	LC-MS (COR23134). Edman sequencing (*E. coli*)	PSNRKNENEIINAL	PSNRKNENEI	Sequences as expected. N-terminal methionine absent as expected from both proteins.
Protein sequence	LC-MS	90.2% coverage	90.2% coverage	Sequences as expected
Glycosylation	Glycoprotein staining	Not glycosylated	Not glycosylated	
Bioactivity	7-day bioassay using *A. gemmatalis*	LC_{50} of 8.26 µg protein/cm^2 agar-based diet	LC_{50} of 2.95 and 3.19 µg protein/cm^2 agar-based diet (Lot # PCF-0062 and PCF-0067)	Overlapping 95% confidence intervals

4.2.2 Safety of the introduced Cry1B.61.1

Data were provided to assess the potential allergenicity and toxicity of Cry1B.61.1.

Bioinformatic analyses of Cry1B.61.1

In silico analyses were performed to compare the Cry1B.61.1 amino acid sequence to known protein allergens and toxins. The analyses were performed as described in section 4.2.1, and did not identify any biologically significant allergens or toxins with homology to Cry1B.61.1.

Susceptibility of Cry1B.61.1 to digestion with pepsin and pancreatin

The *in vitro* digestibility of *E. coli*-derived Cry1B.61.1 was determined in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), as well as in an additional sequential digestion in SGF followed by SIF. The conditions of each assay are briefly described below:

- SGF assay: The SGF reaction mixture containing 0.25 mg/mL of the test protein and pepsin (pepsin to protein ratio was 10 units:1 µg) at pH ~1.2 was incubated at 37°C for 0, 0.25 (or 0.5), 1, 2, 5, 10, 20 (or 30), or 60 minutes. Relevant controls including SGF only (no protein) and undigested protein only (no SGF) were also included. After incubation, the samples were analysed by SDS-PAGE and Western blot to detect protein bands.

- SIF assay: The SIF reaction mixture containing 0.25 mg/mL of the test protein and 0.5% (w/v) pancreatin at pH ~7.5 was incubated for 0, 0.25 (or 0.5), 1, 2, 5, 10, 20 (or 30), or 60 minutes. Relevant controls including SIF only (no protein) and undigested protein only (no SIF) were also included. After incubation, the samples were analysed by SDS-PAGE and Western blot to detect protein bands.

- Sequential SGF and SIF assay: The test protein was incubated in SGF with pepsin (pepsin to protein ratio was 8.6 units:1 µg or 10 units:1 µg) at pH ~1.2 for 5 minutes, then incubated in SIF containing 0.5% (w/v) pancreatin for 0, 0.25 (or 0.5), 1, 2, 5, 10, 20, or 30 minutes. After incubation in SGF/SIF, the samples were analysed by SDS-PAGE.

In the SGF assay, the 74 kDa Cry1B.61.1 protein was rapidly digested within 0.25 minutes. Low molecular weight bands (~2-10 kDa) remained detectable up to 60 minutes on SDS-PAGE. In the

SIF assay, the Cry1B.61.1 protein was digested into smaller fragments within 0.25 minutes. The smaller fragments remained detectable via Western blot for 60 minutes (the smaller fragments included a prominent band at a molecular weight slightly below that of undigested Cry1B.61.1 protein; this band was only slightly detectable after 30 minutes). In the sequential SGF and SIF assay, the low molecular weight bands (~2-10 kDa) observed in SGF digestion were digested within 1 minute of SIF digestion. The smaller fragments observed in SIF assay were also not visible in sequential SGF and SIF digestion.

Taken together, these results indicate that Cry1B.61.1 would be fully degraded by gastric and intestinal enzymes in the human digestive system.

Bioactivity of Cry1B.61.1 after exposure to heat

The thermal stability of Cry1B.61.1 was evaluated by assessing the functional activity of the heat-treated Cry1B.61.1 protein in a 7-day insect bioassay. *E. coli*-derived Cry1B.61.1 was heated at various temperatures (25, 50, 75, or 95˚C) for 30 minutes before incorporation into an artificial diet for *Chrysodeixis includens* larvae (soybean looper; Lepidoptera: Noctuidae), an insect sensitive to the Cry1B.61.1 protein. The insect larvae were exposed to the test diet containing the heat protein for 7 days (the larvae were refed on Day 4). A positive control diet containing the unheated protein and a control diet containing only the buffer were also included. The test diets contained a target concentration of 100 ng Cry1B.61.1 protein per mg diet wet weight. Each diet was provided orally to 20 individual *C. includens* larvae for a total of 7 days, with refeeding occurring on day 4. After 7 days, statistical analyses were conducted to evaluate insect mortality of the heat-treated test groups relative to the unheated test group.

Table 5. Bioactivity of heat-treated Cry1B.61.1 in a diet fed to insect larvae

Treatment description	Incubation condition	Number of observations	Total number of surviving organisms	Mortality (%)	p-value[a]	Weight of surviving organisms (mg)	
						Mean ± standard deviation	Range
Buffer control diet	-	20	20	0	-	15.6 ± 5.98	6.9 – 30.7
Unheated control diet	-	20	0	100	-	-	-
Test diet	25°C	20	0	100	1.0000	-	-
	50°C	20	0	100	1.0000	-	-
	75°C	20	16	20	<0.0001	14.0 ± 7.33	4.6 – 28.1
	95°C	20	17	15	<0.0001	14.5 ± 5.01	8.1 – 28.1

a. The p-value is derived from a Fisher's exact test comparing the test diets to the unheated control diet. A p-value of <0.05 indicates a statistically significant difference.

The results demonstrated that when heated to temperatures of ≥75°C for ~30 minutes, the bioactivity of Cry1B.61.1 was significantly reduced. Mortality of *C. includens* reduced to 20% and 15% relative to unheated control (P <0.0001) at Cry1B.61.1 protein temperatures of 75°C and 95°C, respectively (Table 5). No statistically significant decreases in protein activity were observed when Cry1B.61.1 protein was heated to 25°C or 50 °C (i.e., mortality remained at 100% relative to control). These data indicate that Cry1B.61.1 is heat labile at temperatures ≥ 75°C.

14-day acute oral toxicity study

Although the bioinformatic analyses, digestibility tests and heat susceptibility tests did not raise any safety concerns, a 14-day acute oral toxicity study in mice using *E.coli*-derived Cry1B.61.1 was submitted by the applicant as additional supporting information.

The vehicle control, BSA and Cry1B.61.1 protein formulations were administered on day 1 in three split doses, separated by 4 hours. The mice were fasted prior to and throughout dosing. A 2-week observation period followed dosing.

The study design and results are summarised in Table 6. All animals survived to study termination. There were no treatment related effects on body weight, clinical or gross observations. It was concluded that the oral LD_{50} of the Cry1B.61.1 protein was greater than 5000 mg/kg body weight.

Table 6. Acute oral toxicity study conducted with Cry1B.61.1

Species, sex, and number of animals	Test substance and control(s)	Route and dose	Parameters Evaluated	Summary of Results
Mice (approx. 7 weeks at start of dosing), 6 mice/sex/group	Test substance: Cry1B.61.1 protein expressed in *E. coli* (lot no. PCF-0062, purity: >95% on a total protein basis); Vehicle control: Deionized water Comparative control: BSA	Oral (gavage); 0 (vehicle control) or 5000 mg/kg bw of Cry1B.61.1 protein or BSA[a]	Body weight, clinical signs, gross pathology (external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera)	No mortality or any other treatment related effects reported. LD_{50}[b] = >5000 mg/kg bw

[a]BSA = bovine serum albumin; [b]LD_{50} = median lethal dose

4.2.3 Conclusion

A range of characterisation studies were performed on plant-derived Cry1B.61.1 confirming its identity, structure and biochemistry as well as equivalence of the corresponding protein derived from a bacterial expression system. The microbially-derived Cry1B.61.1 was also shown to be structurally, biochemically and functionally equivalent to the plant-derived Cry1B.61.1. Bioinformatic analyses showed Cry1B.61.1 did not share any meaningful homology with any known allergens or toxins. Cry1B.61.1 was heat labile at ≥ 75°C and susceptible to digestion by gastrointestinal enzymes. Additionally, an acute oral toxicity study in mice did not result in any treatment-related adverse effects. Taken together this indicates that the Cry1B.61.1 protein is unlikely to be toxic or allergenic to humans.

4.3 IPD083Cb

While current insect protection in GM crops relies heavily on proteins derived from *B. thuringiensis*, ferns have been revealed as an alternative source of proteins with insecticidal activity (Markham et al. 2006; Shukla et al. 2016; Simmons and Herman 2023; Wei et al. 2023).

IPD083Cb, isolated from the giant maidenhair fern (*Adiantum trapeziforme* var. *braziliense*), is an 853 amino acid, ~95 kDa protein encoded by the *ipd083Cb* gene. The IPD083Cb protein, when expressed *in planta*, provides protection from certain lepidopteran pests by disrupting their midgut epithelium, similar to Cry proteins from *B. thuringiensis*. However, competitive binding assays using brush border membrane vesicles from insect midguts have demonstrated that IPD083Cb likely binds to a distinct set of midgut receptor sites from the *Bt*-derived insecticidal proteins (including Cry2A.127, Vip3Aa, or a variant of Cry1Ab). This suggests that IPD083Cb is unlikely to share cross-resistance with insects resistant to Cry proteins (Liu et al 2019).

4.3.1 Characterisation of IPD083Cb expressed in COR23134 and equivalence to a tobacco-derived form

The COR23134-derived IPD083Cb protein was derived from whole plant tissue and purified using

immunoaffinity chromatography. As IPD083Cb is not able to be expressed as a soluble protein in *E. coli* (Liu et al. 2019), to obtain sufficient quantities of IPD083Cb for use in safety studies, IPD083Cb was also expressed in a tobacco-based protein expression system. Tobacco (*Nicotiana benthamiana*) is a common expression system for heterologous proteins due to the high yields it provides. IPD083Cb with a C-terminal hexahistidine tag was expressed in *N. benthamiana* and purified by immobilized metal affinity chromatography and anion exchange chromatography.

Data was provided to demonstrate the equivalency of the COR23134- and tobacco-derived IPD083Cb proteins, and is summarised in Table 7. The results demonstrated that tobacco-derived IPD083Cb is structurally and biochemically equivalent to COR23134-derived IPD083Cb. Bioactivity analysis also showed that the recombinant IPD083Cb protein had insecticidal activity against the target insect *A. gemmatalis*. It can be concluded that tobacco-derived IPD083Cb is a suitable surrogate for use in the safety studies described in Section 4.3.2.

Table 7: IPD083Cb protein identity and equivalence

Characteristic	Methodology	COR23134-derived	Recombinant protein	Notes
Molecular weight (kDa)	SDS-PAGE	~95 kDa	~95 kDa	
Immunoreactivity	Western blot	Yes	Yes	IPD083Cb polyclonal rabbit antibody R3373
N-terminal amino acid sequence	LC-MS	ADYSTLYR	ADYSTLYR	N-terminal methionine absent from both as expected; N-terminal alanine was acetylated for both proteins. Sequences as expected.
Protein sequence	LC-MS	95.7% coverage	87.5% coverage	Sequences as expected
Glycosylation	Glycoprotein staining	Not glycosylated	Not glycosylated	
Bioactivity	7-day bioassay using *A. gemmatalis*	LC_{50} could not be determined	LC_{50} of 2.49 µg IPD083Cb/cm^2 agar-based diet	

4.3.2 Safety of the introduced IPD083Cb

Data were provided to assess the potential allergenicity and toxicity of IPD083Cb.

Bioinformatic analyses of IPD083Cb

In silico analyses were performed to compare the IPD083Cb amino acid sequence to known protein allergens and toxins. The analyses were performed as described in section 3.4.5, and did not identify any biologically significant allergens or toxins with homology to IPD083Cb.

Susceptibility of IPD083Cb to digestion with pepsin and pancreatin

The *in vitro* digestibility of tobacco-derived IPD083Cb was determined in SGF and SIF, as well as in an additional sequential digestion in SGF followed by SIF. The conditions of each assay were as described for Cry1B.61.1 in section 4.2.2, except that the incubation period with SGF in the sequential assay was 10 minutes.

In the SGF assay, the 95 kDa IPD083Cb protein was rapidly digested within 0.25 minutes. A band migrating at ~60 kDa was detected by Western blot and was mostly digested within 5 minutes; the band was undetectable within 30 minutes. In SDS-PAGE analysis, low molecular weight bands

(~2-5 kDa) remained detectable for 60 minutes. In the SIF assay, the IPD083Cb protein was digested into smaller fragments (~20-60 kDa) within 0.25 minutes; these smaller fragments remained detectable *via* Western blot for 60 minutes. In the sequential SGF and SIF assay, the low molecular weight bands (~2-5 kDa) observed in SGF digestion were digested within 0.25 minute of sequential SIF digestion.

Bioactivity of IPD083Cb after exposure to heat

The thermal stability of IPD083Cb was evaluated by assessing the functional activity of the heat-treated tobacco-derived IPD083Cb protein in a 7-day insect bioassay. Tobacco-derived IPD083Cb was heated at various temperatures (25, 50, 75, or 95˚C) for 30 minutes before incorporation into an agar-based artificial diet for *A. gemmatalis* , an insect sensitive to the IPD083Cb protein. The test diets contained a target concentration of 50 µg IPD083Cb protein per cm^2 agar-based diet. A positive control diet containing the unheated protein and a control diet containing ultrapure water were also included. Each diet was provided orally to 24 individual *A. gemmatalis* larvae for a total of 7 days, with refeeding occurring on day 4. After 7 days, statistical analyses were conducted to evaluate insect mortality of the heat-treated test groups relative to the unheated test group.

Table 8. Bioactivity of heat-treated IPD083Cb in a diet fed to insect larvae

Treatment description	Incubation condition	Number of observations	Total number of surviving organisms	Mortality (%)	p-value[a]	Weight of surviving organisms (mg)	
						Mean ± standard deviation	Range
Buffer control diet	-	24	24	0	-	33.6 ± 7.71	18.8 – 50.7
Unheated control diet	-	24	0	100	-	-	-
Test diet	25°C	24	1	95.8	0.5000	0.00[b,c]	-
	50°C	24	1	95.7	0.4894	0.200[b]	-
	75°C	24	20	16.7	<0.0001	20.3 ± 9.56	5.0 – 40.5
	95°C	24	21	12.5	<0.0001	21.9 ± 9.90	3.5 – 40.7

a. The p-value is derived from a Fisher's exact test comparing the test diets to the unheated control diet. A p-value of <0.05 indicates a statistically significant difference.
b. The reported mean is the weight value of the one surviving organism.
c. A weight of 0 mg is possible for some live organisms due to limitations of the balance.

The results demonstrated that when heated to temperatures of ≥75°C for ~30 minutes, the bioactivity of IPD083Cb protein was significantly reduced (Table 8). Mortality of *A. gemmatali* was reduced to 16.7% and 12.5% relative to control (P <0.0001) at IPD083Cb protein temperatures of 75°C and 95°C, respectively. No statistically significant decreases in protein activity were observed when IPD083Cb protein was heated to 25°C or 50 °C (i.e., mortality was approximately 95% in both groups relative to the control). These data indicate that IPD083Cb is heat labile at temperatures ≥ 75°C.

14-day acute oral toxicity study

Although the bioinformatic analyses, digestibility tests and heat susceptibility tests did not raise any safety concerns, a 14-day acute oral toxicity study in mice using tobacco-derived IPD083Cb was submitted by the applicant as additional supporting information.

The vehicle control, BSA and IPD083Cb protein formulations were administered on day 1 in three split doses, separated by 4 hours. The mice were fasted prior to and throughout dosing. A 2-week observation period followed dosing.

The study design and results are summarised in Table 9. There were four unscheduled deaths in vehicle and BSA control groups attributed to dosing error. No other mortalities or treatment related effects were reported. There were no treatment related effects on body weight, clinical or gross observations. It was concluded that the oral LD_{50} of the IPD083Cb protein was greater than 5000 mg/kg body weight.

Table 9. Acute oral toxicity study conducted with IPD083Cb

Species, sex, and number of animals	Test substance and control(s)	Route and dose	Parameters Evaluated	Summary of Results
Mice (approx. 7 weeks at start of dosing), 6 mice/sex/group	Test substance: IPD083Cb protein expressed in tobacco (lot no. PCF-0061A, purity: >95% on a total protein basis); Vehicle control: Deionized water Comparative control: BSA[a]	Oral (gavage); 0 (vehicle control) or 5000 mg/kg bw of IPD083Cb protein or BSA[a]	Body weight, clinical signs, gross pathology (external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera)	Four unscheduled deaths in vehicle and BSA control groups attributed to dosing error. No other mortalities or treatment related effects were reported. LD_{50}[b] = >5000 mg/kg bw

[a]BSA = bovine serum albumin; [b]LD_{50} = median lethal dose

4.3.3 Conclusion

A range of characterisation studies were performed on plant-derived IPD083Cb confirming its identity, structure and biochemistry as well as equivalence of the corresponding protein derived from a heterologous plant expression system (tobacco). The tobacco-derived IPD083Cb was also shown to be functional. Bioinformatic analyses showed IPD083Cb did not share any meaningful homology with any known allergens or toxins. IPD083Cb was heat labile at ≥ 75°C and susceptible to digestion by gastrointestinal enzymes. Additionally, an acute oral toxicity study in mice did not result in any treatment-related adverse effects. Taken together this indicates that the IPD083Cb protein is unlikely to be toxic or allergenic to humans.

4.4 GM-HRA

COR23134 soybean expresses the GM-HRA_1 protein encoded by the *gm-hra_1* gene, a modified acetolactate synthase (*als*) gene from soybean. Soybean naturally contains a gene that encodes the enzyme acetolactate synthase (ALS). This enzyme is widely distributed in nature (Mazur et al. 1987) and catalyses the first common step in the biosynthesis of the essential branched-chain amino acids isoleucine, leucine and valine. ALS-inhibiting herbicides such as sulfonylureas and triazolopyrimidine block this synthesis, causing rapid cessation of plant cell division and growth (Brown 1990). However, changes to the amino acid sequence of ALS can result in tolerance to ALS-inhibiting herbicides.

The *gm-hra_1* gene in COR23134 soybean was derived from the soybean acetolactate synthase (*als*) gene by introducing two mutations in its amino acid sequence. These two amino acid changes render the GM-HRA_1 enzyme tolerant to ALS-inhibiting herbicides while retaining its ability to function as a key enzyme in the synthesis of branched chain-amino acids. In COR23134, the expressed GM-HRA_1 protein serves as a selectable marker during transformation which allows for the growth of tissue in the presence of a ALS-inhibiting herbicide.

The precursor GM-HRA_1 protein encoded by the *gm-hra_1* gene is 651 amino acids in length.

However, the coding region for the protein includes a 47 amino acid N-terminal chloroplast peptide protein sequence that is cleaved from the protein during processing, producing the mature GM-HRA_1 protein of 604 amino acids and ~65 kDa. The mature GM-HRA_1 protein in COR23134 soybean has two amino acid mutations compared to the native soybean ALS protein: alanine (A) at position 178 instead of proline (P); and leucine (L) at position 555 instead of tryptophan (W).

The applicant notes there are two versions of the modified *als* gene sequence for deployment in different soybean products: (1) the *gm-hra* gene which is expressed in soybean line DP305423 (A1018; FSANZ 2019); and (2) the *gm-hra_1* gene expressed in COR23134 soybean. The *gm-hra* gene encodes an extra five amino acids (MPHNT) at the N-terminus, however, both the *gm-hra* and the *gm-hra_1* genes produce the same mature protein *in planta* after cleavage of the chloroplast transit peptides (Appendix 1).

4.4.1 Safety of the introduced GM-HRA

As noted above, a GM-HRA protein has been previously assessed by FSANZ, in soybean line DP305423 (A1018; FSANZ 2009). The detailed safety assessment report for DP305423 is available on the FSANZ website.[8] This assessment, along with published literature (Mathesius et al. 2009) considered the potential allergenicity and toxicity of GM-HRA, and no safety concerns were raised. While the sequence of GM-HRA protein previously assessed by FSANZ contains an additional 5 amino acids at its C-terminus relative to the GM-HRA_1 protein, these 5 amino acids are cleaved after expression and do not form part of the mature protein. FSANZ therefore expects that the conclusions from the *in vitro* analyses in the previous assessment would also apply to the GM-HRA_1 protein expressed in COR23134.

The applicant has submitted further studies with this application which confirm that the GM-HRA protein is heat labile and susceptible to pepsin and pancreatin digestion. The heat susceptibility studies used *E. coli*-produced GM-HRA as a surrogate for the COR23134-produced protein. The equivalence of the bacterially expressed and plant expressed proteins was confirmed by a range of characterisation studies[9]. The applicant also submitted updated bioinformatic studies (January 2023) for GM-HRA that looked for amino acid sequence similarity to known protein allergens and toxins. The data submitted by the applicant does not alter conclusions reached in the previous assessment.

While the GM-HRA protein confers tolerance to certain herbicides, in COR23134 it was used as a selectable marker during transformation. As COR23134 is not intended to be used as a herbicide-tolerant line, an assessment of novel herbicide metabolites in herbicide-sprayed plants is not required.

4.4.2 Conclusion

The mature GM-HRA_1 protein expressed in COR23134 is highly similar to a GM-HRA protein previously assessed by FSANZ. Bioinformatic analyses confirmed that GM-HRA has no amino acid sequence similarity to known toxins or allergens. The protein was shown to be heat labile and susceptible to pepsin and pancreatin digestion. Taken together this indicates the GM-HRA protein is unlikely to be toxic or allergenic to humans.

4.5 Expression levels of novel proteins

For analysis of the expression levels of the Cry1B.34.1, Cry1B.61.1, IPD083Cb, and GM-HRA proteins in COR23134, tissues were collected from four replicate plots at each of six field-trial sites. These sites were in representative soybean-producing regions in North America during the

[8] https://www.foodstandards.gov.au/food-standards-code/applications/applicationa1018food4091
[9] apparent molecular weight, immunoreactivity, functional activity and glycosylation analysis

2022 growing season.[10] The following samples were collected: leaf (V5, R1 and R3 growth stages), flower (R1-R2 growth stages), root (R3 growth stage), forage (R3 growth stage) and seed (R8 growth stages). See Figure 1 for a summary of soybean growth stages.

Cry1B.34.1, Cry1B.61.1, IPD083Cb, and GM-HRA were extracted from tissues using standard methods and their expression levels were quantified in each tissue using a quantitative enzyme-linked immunosorbent assay (ELISA).

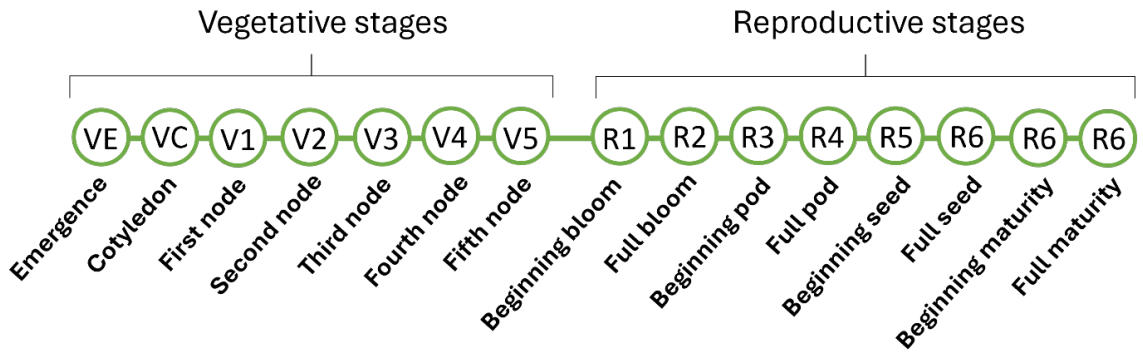

Figure 1. *Soybean growth stages.*

All four proteins were detected in soybean event COR23134. The mean level of each protein in each tissue type is shown on a dry weight (DW) basis in Figure 2. The key findings are:

- Cry1B.34.1 had the highest expression at the V5 stage in leaf tissue (190-960 ng/mg DW), which is the target tissue for lepidopteran pest consumption. Cry1B.34.1 was detected in the seed (140-210 ng/mg DW) at a lower level compared to leaf tissue. Root had the lowest level of Cry1B.34.1 expression (15-170 ng/mg DW).
- Cry1B.61.1 had the highest expression at the R1 stage in leaf tissue (250-1300 ng/mg DW). Cry1B.61.1 was detected in the seed (11-22 ng/mg DW) at a lower level compared to leaf tissue. Root had the lowest level of Cry1B.61.1 expression (<0.14 - 0.93 ng/mg DW).
- IPD083Cb had the highest expression at the R3 stage in leaf tissue (59-130 ng/mg DW). Seed had the lowest level of IPD083Cb expression (13-18 ng/mg DW).
- GM-HRA had the highest expression at the V5 stage in leaf tissue (<2.2-17 ng/mg DW). Seed had the lowest level of GM-HRA expression (<0.54-1.7 ng/mg DW).

For the full set of expression data, including ranges and standard deviations, refer to the Application dossier[11] (pages 155-156).

[10] Of the 6 field trial sites, 5 were located in the US: Iowa, Illinois, Indiana, Indiana, Nebraska, Pennsylvania, and one was located in Canada (Ontario).
[11] Application A1323 – https://www.foodstandards.gov.au/food-standards-code/applications/a1323-food-derived-insect-protected-soybean-line-cor23134

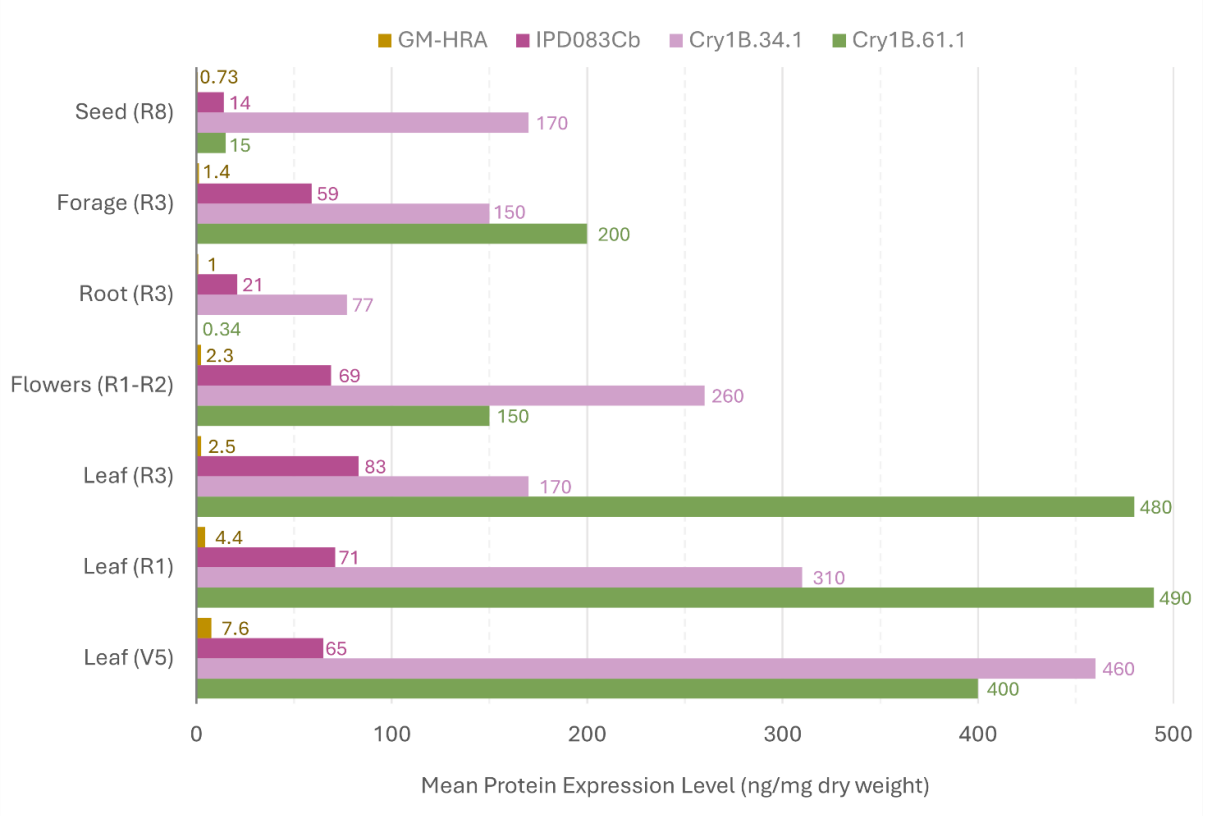

Figure 2. *Mean expression levels of the Cry1B.34.1, Cry1B.61.1, IPD083Cb and GM-HRA proteins in seven tissues from COR23134.*

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 antinutrients 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 GM and conventional soybean are outlined in the OECD revised Consensus Document on Compositional Considerations for New Varieties of soybean (OECD 2012). The analytes measured included moisture, crude protein, crude fat, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), carbohydrates (by calculation), ash, 19 amino acids, 24 fatty acids, 9 minerals, 8 vitamins, and 8 secondary metabolites and anti-nutrients (see Figure 3).

5.2 Study design

COR23134 soybean and a non-GM control of similar genetic background were grown and harvested from 8 field trial sites in North America during the 2022 growing season.[12] The sites were representative of soybean growing regions suitable for commercial production. Plants were grown under agronomic field conditions typical for each field site.

The field sites were established in a randomised complete block design with four blocks per site. Each block contained COR23134 soybean, non-GM control soybean, and four reference soybean lines selected from a total of 18 non-GM commercial soybean lines.[13] A herbicide treatment of quizalofop and fomesafen was applied to the COR23134 soybean, non-GM conventional control, and reference varieties. Given that there were eight sites each containing four replicates, a total of 32 samples for both COR23134 and the non-GM control were collected. For each of the reference varieties a total of 4, 8 or 12 samples were collected depending on the number of sites each reference variety was planted.

Seed from COR23134, the control and reference lines, were obtained at typical harvest maturity (R8 growth stage). Control and reference samples were collected prior to the collection of COR23134 samples to minimize the potential for contamination. Samples were chilled before being transferred to a freezer (≤-10ºC) or placed on dry ice within 3 hours of collection and shipped frozen to an analytical laboratory.

Compositional analyses were based on internationally recognised procedures included validated methods from the Association of Official Analytical Collaboration (AOAC) International, the American Association of Cereal Chemists (AACC), and the American Oil Chemists' Society (AOCS), or other published scientific methods.

A total of 74 analytes in seed were assessed (see Figure 3 for a complete list). In addition, moisture was also measured and used to convert the analyte values from fresh to dry weight, but was not analysed statistically. Of the 74 components measured, four gave results below the assay lower limit of quantification (LLOQ) (lauric acid, pentadecanoic acid, pentadecanoic acid and nonadecanoic acid; listed in grey in Figure 3) and were excluded from the statistical analyses. The undetectable nature of these 4 analytes is consistent with the OECD consensus document (OECD 2012) and with values in the AFSI crop composition database (AFSI 2024).

For the remaining 70 analytes, 'descriptive statistics' (mean, range and 95% confidence interval) were generated. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, North Carolina 2012). For 68 analytes, both COR23134 and the control soybean had <50% of samples below the LLOQ, and a linear mixed model analysis of variance was applied for combined data and locations, covering the eight replicated field trial sites. The across-site mixed model analysis included the fixed effect of the entry, and was adjusted by the random effects of the site, the replicate nested within the site, and the interaction between the site and the entry.

If both COR23134 and the control had at least two samples at a given site above the LLOQ, an individual-site 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, also provided, was used to determine if the differences were in line with the biological variation of soybeans.

For a further 2 analytes (isomer 1 of nonadecanoic acid and vitamin B1), >50% of either

[12] Of the 8 field trial sites, 7 were located in the US: Iowa (2 sites), Illinois, Indiana, Missouri, Nebraska, Pennsylvania, and one was located in Canada (Ontario).

[13] The 18 reference soybean lines: 92M35, 92B63, 92M72, BK291, P29T50, BK310, BK317, BK331N, P33T60, BK340, 93Y41, P34A50, P35A41, BK360, BK361, 93M62, BK370, and 93B82.

COR23134 or the control soybean samples were below the LLOQ. For these analytes, 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 soybean lines across sites for combined data and locations, covering the eight replicated field trial sites.

In assessing the statistical significance of any difference between COR23134 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. In cases where a given sample was below the LLOQ for an individual analyte, a value of half the LLOQ was assigned for statistical analysis.

Any statistically significant differences were evaluated further to assess whether they were likely to be biologically meaningful. Three ranges were used:

1. Compositional data from the non-GM reference varieties grown concurrently in the same trial as COR23134 and the control, were combined across all sites and used to calculate an instudy reference range for each analyte. This defines the variability in soybean varieties grown under the same agronomical conditions.
2. A literature range based on the natural variation of analytes from publicly available data was also considered (AFSI 2024; Kim et al. 2005; Lee et al. 2003; Morse et al. 1950; OECD 2012; Seguin et al. 2004; Taylor et al. 1999). This defines the variability in additional non-GM commercial soybean varieties grown in a wider range of agronomic conditions.
3. Any statistically significant differences between COR23134 and the control were compared to *tolerance intervals* derived from an in-house database containing compositional analyses from 81 non-GM commercial lines cultivated across 175 unique environments in North and South America, from 2005-2016. Tolerance intervals are expected (with 95% confidence) to contain at least 99% of the values for corresponding analytes of the conventional soybean population (Hong et al. 2014). This defines the variability in soybean varieties grown under a wide variety of agronomical conditions.

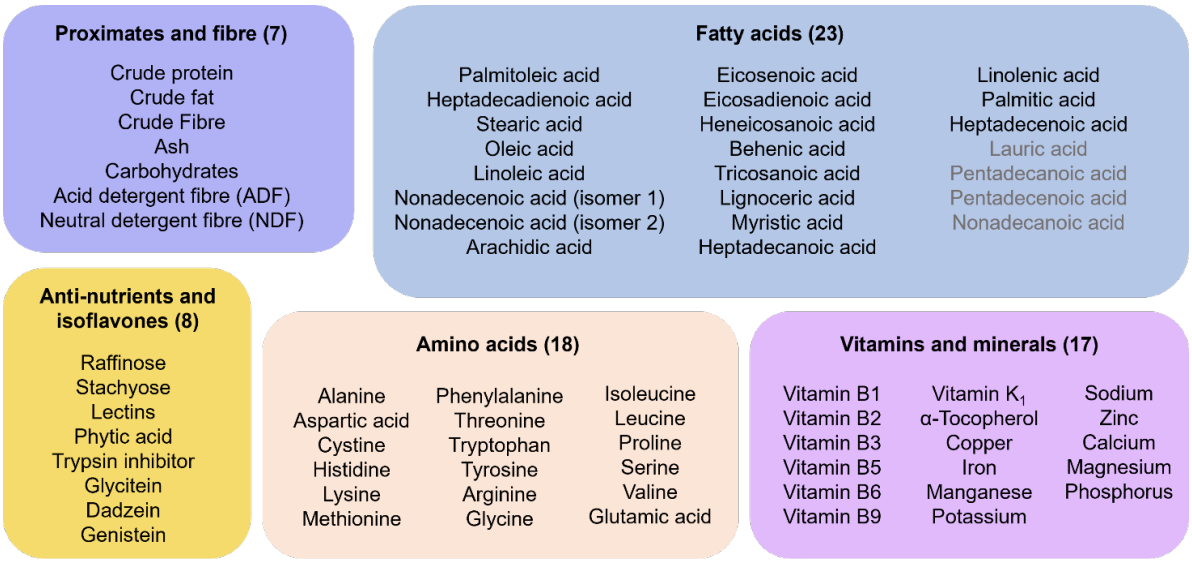

Figure 3. *Analytes measured in COR23134 seed. The analytes listed in grey text had >50% of samples below the LLOQ and were excluded from statistical analysis. The analytes listed in black text were analysed fully.*

5.3 Analyses of key components in seed

Of the 70 analytes for which mean values were provided, there were 23 for which there was a statistically significant difference ($p < 0.05$) between COR23134 and the non-GM control: crude protein, ash, carbohydrates, myristic acid, palmitic acid, heptadecanoic acid, heptadecenoic acid, linolenic acid, arginine, glutamic acid, glycine, isoleucine, leucine, proline, serine, valine, calcium, magnesium, phosphorus, vitamin B5, α-tocopherol, daidzein and genistein.

Of the 23 analytes with $p < 0.05$, 13 analytes (ash, carbohydrates, linolenic acid, arginine, glycine, proline, serine, valine, magnesium, phosphorus, vitamin B5, α- tocopherol, and daidzein), had FDR-adjusted P-values of >0.05, suggesting that the differences in these analytes were likely to be false positives. A summary of the remaining 10 analytes which had statistically significant FDR-adjusted P-values 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[14] (pages 159-177).

For the 10 analytes where a statistically significant difference was found, the deviation of the COR23134 mean from the control mean was less than 20% (Figure 4a). In addition, the COR23134 mean for all components was within the control range value, indicating that COR23134 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. In addition, the observed COR23134 means fall well within the natural variability seen in the range of values for conventional reference soybean varieties grown in the same growing season (dark grey lines; Figure 4 b-k), the publicly-available AFSI database (light grey bars, Figure 4 b-k), and the tolerance interval (pink shading, Figure 4 b-k). The differences reported here are therefore consistent with the normal biological variability that exists in soybean.

Overall, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in COR23134 when compared with conventional non-GM soybean varieties already available in agricultural markets. Seed from COR23134 can therefore be regarded as equivalent in composition to seed from conventional non-GM soybean.

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.

COR23134 is the result of a genetic modification to confer resistance to insect 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 of COR23134 compared with conventional non-GM soybean varieties. The introduction of food derived from COR23134 into the food supply is therefore expected to have negligible nutritional impact.

[14] The Application dossier can be found on the A1323 webpage – https://www.foodstandards.gov.au/food-standards-code/applications/a1323-food-derived-insect-protected-soybean-line-cor23134

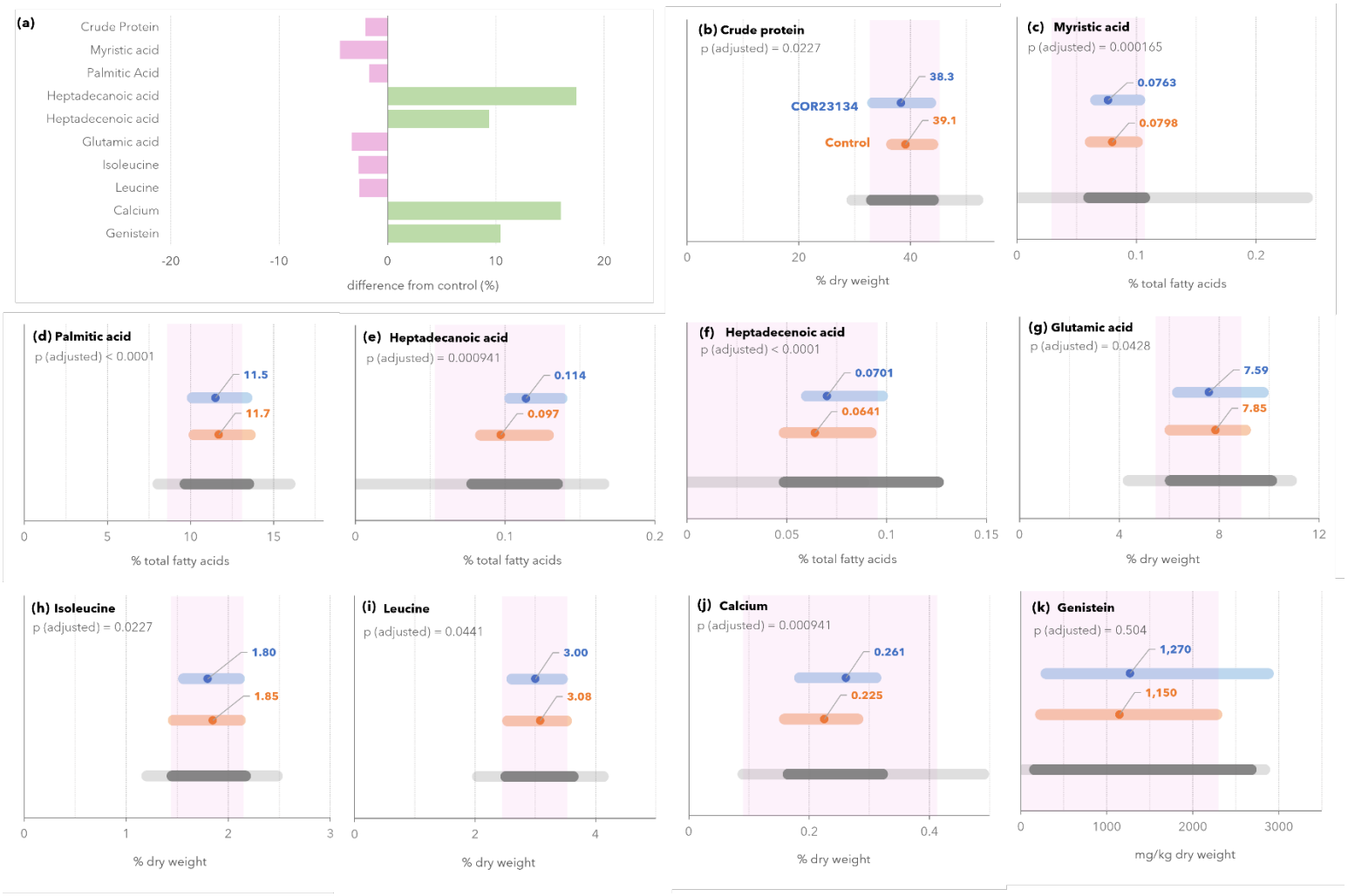

Figure 4. *Visual summary of statistically significant compositional differences between COR23134 and the conventional control soybean.* **(a)** *Deviation of the mean COR23134 value from the mean control value for each of the 10 analytes for which an FDR-adjusted statistically significant difference was found, expressed as a percentage of the mean control value.* **(b) – (k)** *Measured means (dots) and ranges (coloured bars) for COR23134 (blue) and the conventional control (orange) for the 10 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.*

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Appendix 1

GM-HRA and GM-HRA_1 protein sequence alignment

Sequence alignment of the deduced amino acid sequences of GM-HRA protein from the translation of the *gm-hra* gene in DP305423 soybean and the *gm-hra_1* gene in COR23134 soybean. The sequences are identical with the exception of five additional amino acids at the N-terminus of the protein encoded by the *gm-hra* gene. The chloroplast transit peptide sequence is underlined. Upon import in the plastid, the chloroplast transit peptide is removed from the precursor protein, forming the mature GM-HRA protein. As can be observed, the mature GM-HRA protein sequence is identical. The asterisks indicate the translational stop codons.

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gm-hra      MPHNTMAATA SRTTRFSSSS SHPTFPKRIT RSTLPLSHQT LTKPNHALKI   50
gm-hra_1    -----MAATA SRTTRFSSSS SHPTFPKRIT RSTLPLSHQT LTKPNHALKI   45

gm-hra      KCSISKPPTA APFTKEAPTT EPFVSRFASG EPRKGADILV EALERQGVTT  100
gm-hra_1    KCSISKPPTA APFTKEAPTT EPFVSRFASG EPRKGADILV EALERQGVTT   95

gm-hra      VFAYPGGASM EIHQALTRSA AIRNVLPRHE QGGVFAAEGY ARSSGLPGVC  150
gm-hra_1    VFAYPGGASM EIHQALTRSA AIRNVLPRHE QGGVFAAEGY ARSSGLPGVC  145

gm-hra      IATSGPGATN LVSGLADALM DSVPVVAITG QVARRMIGTD AFQETPIVEV  200
gm-hra_1    IATSGPGATN LVSGLADALM DSVPVVAITG QVARRMIGTD AFQETPIVEV  195

gm-hra      SRSITKHNYL ILDVDDIPRV VAEAFFVATS GRPGPVLIDI PKDVQQQLAV  250
gm-hra_1    SRSITKHNYL ILDVDDIPRV VAEAFFVATS GRPGPVLIDI PKDVQQQLAV  245

gm-hra      PNWDEPVNLP GYLARLPRPP AEAQLEHIVR LIMEAQKPVL YVGGGSLNSS  300
gm-hra_1    PNWDEPVNLP GYLARLPRPP AEAQLEHIVR LIMEAQKPVL YVGGGSLNSS  295

gm-hra      AELRRFVELT GIPVASTLMG LGTFPIGDEY SLQMLGMHGT VYANYAVDNS  350
gm-hra_1    AELRRFVELT GIPVASTLMG LGTFPIGDEY SLQMLGMHGT VYANYAVDNS  345

gm-hra      DLLLAFGVRF DDRVTGKLEA FASRAKIVHI DIDSAEIGKN KQAHVSVCAD  400
gm-hra_1    DLLLAFGVRF DDRVTGKLEA FASRAKIVHI DIDSAEIGKN KQAHVSVCAD  395

gm-hra      LKLALKGINM ILEEKGVEGK FDLGGWREEI NVQKHKFPLG YKTFQDAISP  450
gm-hra_1    LKLALKGINM ILEEKGVEGK FDLGGWREEI NVQKHKFPLG YKTFQDAISP  445

gm-hra      QHAIEVLDEL TNGDAIVSTG VGQHQMWAAQ FYKYKRPRQW LTSGGLGAMG  500
gm-hra_1    QHAIEVLDEL TNGDAIVSTG VGQHQMWAAQ FYKYKRPRQW LTSGGLGAMG  495

gm-hra      FGLPAAIGAA VANPGAVVVD IDGDGSFIMN VQELATIRVE NLPVKILLLN  550
gm-hra_1    FGLPAAIGAA VANPGAVVVD IDGDGSFIMN VQELATIRVE NLPVKILLLN  545

gm-hra      NQHLGMVVQL EDRFYKSNRA HTYLGDPSSE SEIFPNMLKF ADACGIPAAR  600
gm-hra_1    NQHLGMVVQL EDRFYKSNRA HTYLGDPSSE SEIFPNMLKF ADACGIPAAR  595

gm-hra      VTKKEELRAA IQRMLDTPGP YLLDVIVPHQ EHVLPMIPSN GSFKDVITEG  650
gm-hra_1    VTKKEELRAA IQRMLDTPGP YLLDVIVPHQ EHVLPMIPSN GSFKDVITEG  645

gm-hra      DGRTRY*                                                 656
gm-hra_1    DGRTRY*                                                 651
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