Position papers

ACNFP advice on the safety of genetically modified GHB811 cotton for food and feed uses

Reference Number RP1232

Regulated Product Dossier Assessment

Assessment finalised: 5th of April 2024

Summary

Following the submission of application RP1232 to the Food Standards Agency (FSA) under assimilated Regulation (EC) No. 1829/2003 from BASF Agricultural Solutions Seed US LLC, FSA/FSS (Food Standards Scotland) were required to undertake a safety assessment on genetically modified GHB811 cotton. To support the safety assessment by FSA/FSS, the Advisory Committee on Novel Foods and Processes (ACNFP) provided advice to FSA/FSS on the data submitted for the authorisation of genetically modified GHB811 cotton, as outlined in this document.

GHB811 cotton is modified by the addition of the *2mepsps* and *hppdPfW336-1Pa* gene cassettes. The 2mEPSPS protein is modified from the wild-type maize (*Zea mays*) 3-enolpyruvylshikimate-3-phosphate synthase (*epsps*) by two mutations, T102I and P106S. EPSPS (and 2mEPSPS) are essential for the synthesis of some amino acids and aromatic compounds in plants and are targets for glyphosate herbicides. The mutations inserted into 2mEPSPS decrease glyphosate binding affinity, thereby conferring tolerance to glyphosate herbicides. The HPPD W336 protein is modified from the soil bacterium *Pseudomonas fluorescens* A32 4-hydroxyl-phenyl-pyruvate dioxygenase by the mutation G336W and confers improved tolerance to HPPD (4-hydroxyphenylpyruvate dioxygenase) inhibitors. HPPD is involved in tyrosine catabolism in aerobic organisms, and the formation of isoprenoids in anaerobic organisms.

Cotton is primarily used worldwide for its lint; however, raw, unprocessed cottonseed may be fed to ruminants as meal, or the seed can be processed into oil. Cottonseed oil has been in use since the 19th century and is considered to be a premium quality oil. The scope of the application is for the authorisation for import, processing, and food and feed use of herbicide tolerant GHB811 cotton. The application does not cover cultivation and therefore no GHB811 cotton will be grown in the UK.

In providing its scientific advice, the ACNFP considered data provided as part of application RP1232. The molecular characterisation determined that GHB811 cotton contained a complete T-DNA at a single locus, with no disruption of endogenous genes. Bioinformatics analyses of the insert and flanking regions (including the junctions between them) found no homology with known toxic or allergenic proteins, and found no sequences that could lead to horizontal gene transfer. Genetic stability of the transgenic locus, and phenotypic stability of transgenic protein expression were both confirmed. The field trials (including locations and management practices) for the production of test materials for the comparative analysis were considered appropriate, and no differences between the GHB811 cotton and the conventional counterpart or the non-GM reference varieties that would raise safety concerns were observed. Studies on both newly expressed proteins found no evidence of potential toxicology. Both proteins have well documented histories of safe use, and their source organisms are either commonly consumed by humans and animals, or are ubiquitous in the environment. The studies were performed using bacterially-produced proteins, and the ACNFP were satisfied that these proteins were equivalent to plant-produced proteins. No safety concerns were identified in the 90-day feeding study. Bioinformatics analysis of allergenicity potential found no relevant homology with known allergenic proteins. An independent, outside contractor assessed the outcomes and methodologies of all bioinformatic analyses and was satisfied that the methods and results were satisfactory.

The ACNFP concludes that considering the nature of the introduced traits, the lack of differences in the agronomic and compositional analyses, and the proposed levels of exposure, there is no evidence that the import, processing, and food and feed use of GHB811 cotton would raise any safety concerns. The ACNFP concludes that GHB811 cotton is as safe as its conventional counterpart.

1. Introduction

1.1 Background

On 26th August 2021, the Food Standards Agency (FSA) received application RP1232 (EFSA-GMO-ES-2018-154) for the authorisation of genetically modified herbicide tolerant GHB811 cotton (unique identifier: BCS-GH811-4), submitted by BASF Agricultural Solutions Seed US LLC (Florham Park, New Jersey) (hereafter referred to as “the applicant”) according to Regulation (EC) No. 1829/2003, as assimilated into UK law.

FSA/FSS checked the application for compliance with the relevant requirements of Regulation (EC) No. 1829/2003, and assimilated Regulation (EU) No. 503/2013, and on 6th September 2021, declared the application valid.

FSA and FSS would like to thank the following members of the Advisory Committee on Novel Foods and Processes (ACNFP) who participated in the assessment: Dr Camilla Alexander White, Dr Andy Greenfield, Dr Anton Alldrick, Alison Austin, Dr Mark Berry, Prof Dimitris Charalampopoulos, Prof Susan Fairweather-Tait, Prof Paul Fraser, Dr Hamid Ghouddusi, Prof Wendy Harwood, Prof Huw Jones, Dr Ray Kemp, Dr Elizabeth Lund, Emeritus Professor Harry McArdle, Rebecca McKenzie, Prof Clare Mills, Dr Lesley Stanley, Prof Hans Verhagen, Dr Maureen Wakefield, Prof Bruce Whitelaw, and Prof Pete Lund (co-opted member of ACNFP-PGT Subcommittee).

1.2 Terms of Reference

According to Articles 6 and 18 of assimilated Regulation (EC) No. 1829/2003, FSA/FSS were requested to carry out a scientific safety assessment of genetically modified GHB811 cotton for authorisation in the scope of the application, namely the import, processing, and food and feed use of GHB811 cotton.

FSA/FSS sought safety advice from the ACNFP on genetically modified GHB811 cotton, which will inform the FSA/FSS safety assessment. The FSA/FSS safety assessment is to be seen as the opinion requested under Articles 6(6) and 18(6) of that Regulation.

In addition to the present advice on the safety of genetically modified GHB811 cotton, the ACNFP were also asked to advise on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No. 1829/2003. These articles concern details that must be included in positive opinions/outcomes of assessment of GMO foods and feeds, including labelling details, any relevant conditions or restrictions, and monitoring plans.

2. Applicant details

Name: BASF Agricultural Solutions Seed US LLC

Address: 100 Park Avenue, 07932

Florham Park, New Jersey

USA

(represented by)

Name: BASF PLC

Address: 2 Stockport Exchange

Railway Road

Stockport

Cheshire

UK

SK1 3GG

3. Data and methodologies

3.1 Data

The data for application RP1232 submitted according to assimilated Regulation (EC) No. 1829/2003 and provided by the applicant at the time of submission are specified below. To inform the FSA/FSS safety assessment of genetically modified GHB811 cotton for food and feed uses in accordance with Articles 11 and 23 of assimilated Regulation (EC) No. 1829/2003, the ACNFP was asked to provide safety advice. It considered the requirements described in applicable guidance for the safety assessment of GM food and feed applications under assimilated Regulation (EC) No. 1829/2003, and based its scientific safety assessment on the data within application RP1232, additional information provided by the applicant, and any relevant peer-reviewed scientific publications.

3.2 Methodologies

The ACNFP conducted its assessment in accordance with the principles described in assimilated Regulation (EU) No. 503/2013, applicable guidance, explanatory notes, and statements (EFSA GMO Panel 2010; EFSA GMO Panel 2011; EFSA GMO

Panel, 2015a; EFSA GMO Panel, 2017). Independent contractors performed preparatory work and delivered reports on the methods applied by the applicant in performing sequencing and bioinformatics analyses.

4. Assessment

4.1 Molecular characterisation

The molecular characterisation section of the safety assessment considers the methods used to insert the transgenic material, the sequence and structure of the newly expressed protein(s), and the sequences at the insertion locus. Analyses performed by the applicant to determine insertion locus, copy number, and any deletions that occurred during the insertion of transgenic material are assessed. Bioinformatics analyses performed on the transgenic sequences are also assessed to ensure the newly expressed proteins do not raise any safety concerns. Additionally, the expression of the newly expressed proteins is assessed. Finally, bioinformatics analyses performed on the flanking regions either side of the inserted material (and the junctions between them) are assessed to ensure no sequences occur that could raise safety concerns.

4.1.1 Transformation process and vector constructs

Hypocotyl segments grown from Coker 312 cotton seeds were dissected and transformed with transformation vector pTSIH09 (and a non-oncogenic helper Ti-plasmid pEHA101) using *Agrobacterium tumefaciens* (strain C58C1Rif).

The T-DNA region of pTSIH09 contains two gene cassettes containing the *2mepsps* and *hppdPfW336-1Pa* genes. 2mEPSPS is modified from the wild-type maize (*Zea mays*) pyruvyl-shikimate synthase (*epsps*) by two mutations, T102I and P106S. These mutations decrease glyphosate binding affinity, allowing it to maintain sufficient enzymatic activity in the presence of the glyphosate (thereby conferring tolerance). HPPD W336 is modified from the *Pseudomonas fluorescens* A32 4-hydroxyl-phenyl-pyruvate dioxygenase by the mutation G336W (the coding sequence was also modified for cotton codon usage) and confers improved tolerance to HPPD inhibitors.

4.1.2 Molecular studies performed on GHB811 cotton

Southern blot analysis demonstrated that GHB811 cotton contains a single copy of the complete T-DNA at a single locus in chromosome A05. The absence of backbone vector sequences was also demonstrated by Southern blot analysis.

Some weak additional bands were observed when the gDNA was digested with *Sac*I or *Hind*III, but this is attributed to incomplete digestion.

The inserted sequence, and at least 1 kb of both flanking regions (5’ flanking region = 1138 bp, 3’ flanking region = 1241 bp), were sequenced. Bioinformatics analysis of the GHB811 insertion locus and the flanking sequences (BLASTn and BLASTx similarity searches against a *Gossypium hirsutum* genome database) indicated it is unlikely that endogenous genes are interrupted, or their transcriptional or translational activity altered. Additionally, bioinformatics analyses of the newly created open reading frames (ORFs) within the insert and spanning the junctions with the genomic DNA found no safety concerns. In the GHB811 insertion locus, 13 bp are observed which are not present in the GHB811 transgenic locus.

4.1.3 Transgenic protein expression

Expression levels of 2mEPSPS and HPPD W336 were determined by enzyme-linked immunosorbent assay (ELISA) on different plant matrices (leaf, root, pre-candle squares, pollen, immature bolls, and whole plant) during development, and in fuzzy seeds at harvest, from tissues harvested from GHB811 cotton plants grown in the USA (Texas, Mississippi, and North Carolina) in 2015.

Table 1. Mean values and ranges (n=12) of 2mEPSPS and HPPD W336 in GHB811 fuzzy cottonseed.

Treatment	**Fresh weight (µg/g)**	**Fresh weight (µg/g)**	**Dry weight (µg/g)**	**Dry weight (µg/g)**
2mEPSPS	**Average**	**Range**	**Average**	**Range**
CHM[a]	129.79 ± 38.41	65.07 – 205.88	145.11 ± 37.86	76.36 – 221.42
TIH[b]	132.94 ± 20.38	80.83 – 162.76	150.88 ± 27.87	86.67 – 198.93
HPPD W336	**Average**	**Range**	**Average**	**Range**

CHM[a]	26.45 ± 13.65	9.30 – 55.96	29.61 ± 14.96	10.91 – 62.33
TIH[b]	23.82 ± 8.46	10.27 – 39.46	27.01 ± 9.78	11.01 – 43.85

[a] CHM (conventional herbicide management) = no treatment with trait-specific herbicides.

[b] TIH (treated with intended herbicides) = isoxaflutole treatment before emergence (BBCH 00) and glyphosate treatment at 7-8 leaf growth stage (BBCH 17-18).

4.1.4 Genetic stability

Southern blot analysis confirmed the genetic stability of the insert over five generations (T1, T3, T4, BC1F2, and BC2F3). For each generation, the expected fragments were obtained demonstrating the structural stability of the insert.

Phenotypic stability of 2mEPSPS and HPPD W336 expression was determined for five generations using lateral flow strip analysis. 2mEPSPS and HPPD W336 expression was consistent across all generations tested. Event-specific PCR of the GHB811 insert was used to determine the genotype of five segregating generations of plants to calculate the segregation ratios. Chi-square analysis of the segregation data confirmed that the GHB811 insert is inherited in a predictable manner as expected for a single insert, consistent with Mendelian principles.

4.1.5 Conclusion on the molecular characterisation

The molecular characterisation data presented confirm that GHB811 cotton contains a single transgenic insert. Bioinformatics analyses of this insert, and the flanking sequences, raised no safety concerns. The genetic stability of the insert was confirmed over five generations. The expression levels of the transgenic proteins in fuzzy cottonseed were determined using suitable methodologies, and do not cause a safety concern.

4.2 Comparative analysis

The role of the comparative analysis is to compare the GM plant with its conventional counterpart, a non-GM plant with a similar genetic background, and several non-GM reference varieties with similar properties to the GM plant and

conventional counterpart. This comparison takes two forms; firstly, a comparison of the agronomic characteristics of the plant as it grows in the field which looks at the yields derived from the plants, as well as their observable characteristics such as height and colour, and a comparison of the composition of the plant after harvest which considers the nutritional value and safety of the genetically modified plant.

4.2.1 Experimental field trial design

GHB811 cotton, along with Coker 312 cotton (the non-GM conventional counterpart) and seven non-GM reference varieties (Acala Maxxa, DP399, FM958, FM966, FM989, ST457, and ST468) were grown at seven sites in 2014, and eight sites in 2015, all in the USA. Five field trials in 2014 and one in 2015 did not produce data and were removed from the study. The trial sites excluded in 2014 were due to unavoidable circumstances, equipment breakdowns, and adverse environmental conditions. The trial site excluded in 2015 was due to deficiencies in data collection and record keeping.

The field trials consisted of entries replicated four times in a randomised complete block design. The entries were;

- Non-GM conventional counterpart (Coker 312) with conventional herbicide treatment
- GHB811 cotton with conventional herbicide treatment
- GHB811 cotton with trait-specific herbicide treatment (one application of isoxaflutole at BBCH 00-13 and one application of glyphosate at BBCH 16-19)
- Three of the nine reference varieties with conventional herbicide treatment

The agronomic/phenotypic data and compositional data from these field trials were analysed as specified previously in guidance provided by EFSA (EFSA GMO Panel 2010; EFSA GMO Panel 2011; EFSA GMO Panel, 2015). This includes the application of a test of difference between GHB811 cotton and the conventional counterpart, and a test of equivalence between GHB811 cotton and the non-GM reference varieties.

4.2.2 Suitability of field trials and test materials

The field trial sites were representative of commercial cotton production in the USA, and represented diverse climates, soils, crop rotations, irrigation systems, and management practices. The trials were placed in prime, and marginal, cotton production areas.

Meteorological data were recorded weekly, and average monthly maximum and minimum temperatures and total monthly precipitation were recorded for each site. Weather conditions were generally normal however some exceptional weather events were recorded at some sites (mostly excessive rainfall)

GHB811 cotton and the conventional counterpart were produced following good agricultural practices and with quality assurance mechanisms in place to ensure genetic identity, purity, and health. All seed shipments were accompanied by a phytosanitary certificate issued by the Texas Department of Agriculture and prior to planting, seed health was ensured through acid delinting, and treatment with insecticidal and fungicidal coatings.

Analysis of 352 GHB811 cotton seeds by validated PCR methods determined seed purity as >96%. The absence of impurities from GHB811 cotton and the non-GM conventional counterpart was confirmed by analysing 3000 seeds (12 subsamples of 250 seeds) or 352 individual seeds. Potential impurities were at or below 0.99%. Germination rates observed across seed lots were appropriate to ensure the suitability of the seed material.

The ACNFP is satisfied that the field trials, and the materials used in the field trials are appropriate for the comparative assessment. The geographical locations, soil conditions, meteorological conditions, and the management practices used were all considered typical of the receiving environments where GHB811 cotton could be grown.

4.2.3 Compositional analysis (agronomic characteristics)

In the comparative analysis of agronomic characteristics, tests between GHB811 cotton not treated with the intended herbicide and the conventional counterpart found statistically significant differences in 15 of the 31 continuous endpoints measured (Table 2); however, for most of these, equivalence (or equivalence more likely than not) was demonstrated in the test for equivalence against the reference varieties. Only yield-lint, % lint, and lint length were found to have significant differences in the test of equivalence.

Table 2. Outcome of the comparative analysis of the continuous agronomic characteristics of GHB811 cotton.

Intended herbicide treatment[c]

	Test of difference[(a)]	**Test of difference[(a)]**

Test of equivalence[b]	Not different	Significantly different
Category I	23[d]	5
Category II	1[e]	1[g]
Category III	-	-
Category IV	-	1[h]
Total endpoints	24	7

Conventional herbicide treatment[c]

	Test of difference[a]	**Test of difference[a]**
Test of equivalence[b]	Not different	Significantly different
Category I	15	12
Category II	1[e]	1[f]
Category III	-	1[g]
Category IV	-	1[h]
Total endpoints	16	15

[a] Comparison between GHB811 cotton and the conventional counterpart (Coker 312)

[b] The test of equivalence with the reference varieties is categorised into four different outcomes; category I (equivalence with the reference varieties is

demonstrated), category II (equivalence is more likely than not), category III (equivalence is less likely than not), and category IV (non-equivalence is demonstrated)

[c] The intended herbicide treatment was one application of isoxaflutole at BBCH 00-13 and one application of glyphosate at BBCH 16-19

[d] See Appendix 1 for all parameters tested

[e] Days to 10% flower

[f] Yield-lint

[g] % lint

[h] Lint length

Tests between GHB811 cotton treated with the intended herbicide and the conventional counterpart found statistically significant differences in 7 of the 31 continuous endpoints tested (in the test of difference); stand count 2, days to first open bolls, heat units to first open bolls, bolls-% open, average boll weight, % lint, and lint length. Only % lint and lint length were found to be significantly different in the test for equivalence.

The Cochran-Mantel-Haenszel test showed no significant differences between GHB811 cotton and the conventional counterpart for all but one of the categorical agronomic and phenotypic endpoints. Statistically significant differences were seen only in disease stressor rating 3 (BBCH 61 to 69). The absolute differences were lower than the standard deviation within the conventional counterpart data and the appearance of an increase in disease stressor 3 can be attributed to one site and one type of stressor; verticillium wilt. Disease stressor ratings at other growth stages were not statistically different, so no biological relevance is attributed to this difference.

The absolute differences between GHB811 cotton (both treated with the intended herbicide and untreated) and the conventional counterpart for % lint, yield-lint, and lint length are all smaller than the standard deviation of the conventional counterpart for the same endpoint. All three were also within the range of the reference varieties. % lint is calculated from average boll weight, so differences found in average boll weight can impact the % lint statistics. Similarly, yield-lint is calculated from % lint and total cottonseed yield, so changes in % lint will impact yield-lint. Considering the magnitude of the differences, the natural variability, and the relevance of the endpoints to food and feed safety, the ACNFP considered

that the observed differences for % lint, yield-lint, and lint length did not change the safety of GHB811 cotton compared to the comparator.

4.2.4 Compositional analysis

In the comparative analysis of composition, GHB811 cotton fuzzy seeds harvested from the field trials were analysed for composition as recommended by the OECD (OECD, 2009). Tests between GHB811 not treated with the intended herbicide and the conventional counterpart found statistically significant differences for 11 composition analytes: carbohydrates, total dietary fibre, neutral detergent fibre, crude protein, C16:1 palmitoleic acid, C18:0 stearic acid, C20:0 arachidic acid, manganese, alpha-tocopherol, free gossypol, and total gossypol (Table 3). The test of equivalence between GHB811 not treated with the intended herbicide and the reference varieties found significant differences for neutral detergent fibre, C16:1 palmitoleic acid and dihydrosterculic acid.

Table 3. Outcome of the comparative analysis of GHB811 fuzzy cottonseed.

Intended herbicide treatment[c]

	Test of difference[(a)]	**Test of difference[(a)]**
Test of equivalence[(b)]	Not different	Significantly different
Category I	33[d]	14
Category II	1[e]	2[f]
Category III	-	-
Category IV	-	1[g]
Not categorised	3	-
Total endpoints	37[Appendix 1]	17[Appendix 1]

Conventional herbicide treatment[c]

	Test of difference[(a)]	**Test of difference[(a)]**
Test of equivalence[(b)]	Not different	Significantly different
Category I	39	9
Category II	-	2[f]
Category III	-	-
Category IV	1[g]	-
Not categorised	3	
Total endpoints	43 Appendix 1	11 Appendix 1

[a] Comparison between GHB811 cotton and the conventional counterpart (Coker 312)

[b] The test of equivalence with the reference varieties is categorised into four different outcomes; category I (equivalence with the reference varieties is demonstrated), category II (equivalence is more likely than not), category III (equivalence is less likely than not), and category IV (non-equivalence is demonstrated)

[c] The intended herbicide treatment was one application of isoxaflutole at BBCH 00-13 and one application of glyphosate at BBCH 16-19

[d] See Appendix 2 for all parameters tested

[e] Tyrosine

[f] Neutral detergent fibre and C16:1 palmitoleic acid

[g] Dihydrosterculic acid

Tests between GHB811 treated with the intended herbicide and the conventional counterpart found statistically significant differences for 17 composition analytes: moisture, carbohydrates, total dietary fibre, neutral detergent fibre, crude protein, cystine, methionine, C14:0 myristic acid, C16:1 palmitoleic acid, C18:0 stearic acid, C20:0 arachidic acid, calcium, manganese, alpha-tocopherol, free gossypol, total gossypol, and dihydrosterculic acid. The test of equivalence between GHB811 treated with the intended herbicide and the reference varieties found significant differences for neutral detergent fibre, C16:1 palmitoleic acid and dihydrosterculic acid.

The differences for neutral detergent fibre and C16:1 palmitoleic acid were smaller than the standard deviation of the reference varieties, so no biological relevance was given to these differences.

The absolute difference observed for dihydrosterculic acid (0.029% total fatty acid) was smaller than the standard deviation of the conventional counterpart (0.054%). No equivalence was demonstrated with the reference varieties; however, the mean values are within the range of the reference varieties and the ranges previously reported in the literature (OECD, 2009). Dihydrosterculic acid is an intermediate in the conversion of oleic acid to sterculic acid and malvalic acid, and the levels of these fatty acids in GHB811 cotton treated with the intended herbicide was not different to the conventional counterpart. Therefore, no biological relevance is attributed to the difference in dihydrosterculic acid.

The test of equivalence for sodium, C18:3 linolenic acid, and C24:0 lignoceric acid could not be performed as no variance was found within the reference varieties. However, GHB811 cotton mean values for sodium, C18:3 linolenic acid, and C24:0 lignoceric acid (both treated with the intended herbicide and untreated) were not different from the conventional counterpart, within the minimum and maximum ranges of the reference varieties, and within the ranges reported in the literature for the fatty acids (the values for sodium are lower than those published in the literature, but the same is true for the conventional counterpart and the reference varieties).

4.2.5 Conclusion on the comparative analysis

The ACNFP assessed the field trials used to generate material for the comparative analyses and considered the locations selected were representative of commercial cotton production, and that the meteorological conditions and management practices used during the field trails were appropriate.

The ACNFP also assessed the results from the comparative analysis, including all the significant differences between GHB811 cotton and its conventional counterpart, and found no safety concerns.

4.3 Food/feed safety assessment

The food/feed safety assessment covers the likelihood that the newly expressed protein(s), or the whole genetically modified food or feed, will cause safety concerns when consumed by humans and/or animals. This includes looking at the concentrations of newly expressed proteins in the final products that will be consumed, as well as the anticipated rates of consumption by humans and animals to understand the anticipated magnitude of exposure to any transgenic proteins. Any toxicological or allergenic effects that can be observed and any nutritional effects that consumption of the products may cause are also assessed.

4.3.1 Effects of processing

Cotton undergoes extensive processing to yield the final products intended for the food and feed market, and GHB811 cotton is expected to require the same processing steps as conventional cotton. Information on the processing steps was provided.

The concentrations of the newly expressed proteins, 2mEPSPS and HPPD W336, were determined in GHB811 cotton fuzzy seed and its processed fractions by ELISA. The protein concentrations in toasted meal, crude oil, and RBD (refined, bleached, and deodorised) oil were all ˂LLOQ. The concentration of 2mEPSPS ranged from 15.53 to 209.98 µg/g DW in fuzzy seed, linters, delinted seed, untoasted meal, and hull samples. The concentration of HPPD W336 ranged from 4.54 to 42.50 µg/g DW. Although the concentrations of both 2mEPSPS and HPPD W336 increase after treatment in untoasted meal, the concentrations are much lower than in fuzzy seed.

Table 4. Expression levels of 2mEPSPS and HPPD W336 in GHB811 cotton fuzzyseed and processed fractions.

Matrix	Treatment	2mEPSPS (µg/g DW)	HPPD W336 (µg/g DW)
Fuzzy seed	**Not treated**	150.66	42.50

	Treated	123.48	28.58
Linters	**Not treated**	15.69	8.38
	Treated	15.53	8.82
Delinted seed	**Not treated**	209.98	37.67
	Treated	209.87	31.10
Untoasted meal	**Not treated**	28.49	4.54
	Treated	58.88	11.62
Toasted meal	**Not treated**	˂LLOQ	˂LLOQ
	Treated	˂LLOQ	˂LLOQ
Hull	**Not treated**	67.01	16.62
	Treated	62.66	14.46
Crude oil	**Not treated**	N/A	N/A
	Treated	N/A	N/A
RBD oil	**Not treated**	N/A	N/A
RBD oil	**Treated**	N/A	N/A

4.3.2 Activity and stability of the newly expressed proteins

The studies on both newly expressed proteins were performed with bacterially-produced recombinant proteins rather than the proteins extracted directly from the plants, due to the low levels of protein that can be extracted from plant material. Structural and functional equivalence between bacterially-produced and plant-produced proteins was confirmed for both 2mEPSPS and HPPD W336 using mass spectrometry, immuno-reactivity experiments, peptide mapping and N-terminal sequencing, and quantitative activity assays.

The modified maize *2mepsps* gene encodes a 47 kDa protein consisting of 45 amino acids. The two modifications of 2mEPSPS described earlier have no effect on the enzymatic properties and it is expected to have the same safety profile as the wild-type protein, and a safety evaluation of 2mEPSPS has already been published by EFSA (EFSA, 2009; EFSA GMO Panel, 2014; EFSA GMO Panel, 2015b; EFSA GMO Panel, 2018). Additional studies were presented in the context of this application.

The enzymatic activities of wtEPSPS and 2mEPSPS were determined using the malachite green assay. The catalytic efficiency of 2mEPSPS is five times lower than wild type, but maintains a high affinity for phosphoenolpyruvate. The optimal pH for both wild type and 2mEPSPS was between 5.5 and 7.5. Enzymatic activities of 2mEPSPS increased linearly in increasing temperatures up to 60 °C, followed by a sharp decline and no activity at 75 °C. 2mEPSPS appeared less sensitive to heat inactivation, however it was still completely inactivated after 10 minutes at 60 °C. The effect of temperature on 2mEPSPS was also assessed using SDS-PAGE and western blot. The structure of 2mEPSPS was stable at 25 °C and remained in the soluble fraction. After treatment at 55 °C and above, the protein began to appear in the insoluble fraction and some minor degradation occurred. The 2mEPSPS protein is very rapidly (within 30 seconds) digested in SGF (pepsin at pH 1.2), and complete digestion in SIF (pancreatin at pH 7.5) is observed within a few seconds.

The wild-type *hppd* gene was isolated from *Pseudomonas fluorescens*, and was modified by a single mutation (G336W). The HPPD W336 protein has been previously assessed in the context of other applications (EFSA GMO Panel, 2015b) and no safety concerns were identified, however additional studies were undertaken in the context of this application.

The optimal pH for HPPD W336 was between 7.5 and 8.5. The activity of HPPD W336 was significantly reduced at 45 °C and 4 °C compared to 25 °C and 37 °C. The reduction in activity seen at 45 °C is likely due to increased instability. At higher temperatures, the protein is more unstable, and activity is abolished after

2.5 minutes incubation. SDS-PAGE showed no significant changes in HPPD W336 after heat treatment at 60 °C, 75 °C, or 90 °C for 10-60 minutes. The mutation at W336 at no effect on substrate specificity. The HPPD W336 protein is rapidly degrading upon incubation in SGF and SIF.

4.3.3 Toxicological testing of newly expressed proteins

Both 2mEPSPS and HPPD W336 have been subjects of previous assessments by EFSA (EFSA, 2009; EFSA GMO Panel, 2014; EFSA GMO Panel, 2015b; EFSA GMO Panel, 2018), and no safety concerns were raised. The ACNFP considered the toxicological safety of both newly expressed proteins during its safety assessment using the molecular characterisation data, bioinformatic analyses, and any *in vitro* or *in vivo* studies performed by the applicant.

Bioinformatic analyses of both newly expressed proteins (including their nucleotide and amino acid sequences) revealed no similarities with known toxins and raised no safety concerns.

Repeated dose toxicity studies using laboratory animals was not deemed necessary by the applicants for 2mEPSPS as it is highly homologous to wtEPSPS, which is naturally present in maize and therefore has a long history of safe use. A 28-day toxicity study in mice was performed using bacterially-produced HPPD W336. No treatment-related changes were observed.

4.3.4 Toxicological testing of new constituents other than the newly expressed proteins

No new constituents other than the newly expressed proteins, 2mEPSPS and HPPD W336, were identified in GHB811 cotton, therefore no assessment of any constituents other than the newly expressed proteins is required.

4.3.5 Toxicological testing of the whole genetically modified food or feed

In accordance with assimilated Regulation (EU) No. 503/2013, the applicant provided a 90-day feeding study of Sprague Dawley rats fed a diet consisting of 10% toasted GHB811 meal treated with the intended herbicide, the conventional counterpart (Coker 312), or a non-GM reference variety (FM966). The study was adapted from OECD TG 408 (OECD, 1998) with some small changes (the test substance and control substance were not tested for stability, however they were used within their expiration date). All procedures and observations were conducted in accordance with OECD TG 408.

No effects on survival, clinical observation, body weights, or any of the other parameters tested were observed during the study.

4.3.6 Assessment of allergenicity

In accordance with assimilated Regulation (EU) No. 503/2013, the applicant used a weight-of-evidence approach to assess the allergenicity potential of 2mEPSPS and HPPD W336 as no single method is sufficient to predict allergenicity (Codex Alimentarius, 2009).

Neither of the source organisms for the two newly expressed proteins (*Zea mays* – 2mEPSPS, and *Pseudomonas fluorescens* – HPPD W336) is considered allergenic.

Two *in silico* searches (an overall identity search and an 80-mer sliding window search) against the COMPARE database found no relevant identities between either newly expressed protein with known allergenic proteins. Two potential N-glycosylation sites were found in 2mEPSPS, however the presence of these sites is not necessarily predictive of potential glycosylation *in vivo*.

The amino acid sequences of the newly expressed proteins were divided into 9-mer blocks and then a search for a perfect sequence match to known CD peptide sequences, a search for the transglutaminase 2 (TG2) deamination motif (Q/E-X1-P-X2, X1 = L, Q, F, S, or E, X2 = Y, F, A, V, or Q), and a search allowing up to 3 sequence mismatches were performed. For the 2mEPSPS protein, only a partial match with a HMW-Glutenin epitope (with three mismatches) was found. For the HPPD W336 protein, no matches, complete or partial, were found.

The ACNFP considered the updated bioinformatics analyses and found no safety concerns for either newly expressed protein.

4.3.7 Anticipated intake/extent of use

In accordance with assimilated Regulation (EU) No. 503/2013, the applicant provided anticipated dietary intake of 2mEPSPS and HPPD W336 in GHB811 cotton by using protein expression levels in GHB811 treated with the intended herbicide (see Table 1) and international and national food consumption data.

The anticipated human intake of GHB811 cotton is considered to be negligible, based on data available on the consumption of cottonseed and derived products. The newly expressed proteins are not detectable in RBD oil derived from GHB811 cotton, therefore no dietary exposure is expected from consumption of RBD oil (or derived products such as dressings, bakery goods, and chocolate spreads).

Dietary exposure of goods other than cottonseed oil (such as cottonseed flour and linters) is also negligible as these are minor products in the UK market.

Anticipated animal dietary intakes were calculated using a worst-case scenario approach, assuming that all cotton commodities used for animal feed was solely from GHB811 cotton, the maximum percentages of cotton commodities were used to prepare animal feed, and the protein contents in the toasted cottonseed meal were not lower than the protein contents in the fuzzy cottonseeds. In data for the EU, the highest daily intakes were obtained for dairy cattle fed a diet that consisted of up to 10% cottonseed, 5% cottonseed meal, and less than 5% cottonseed hulls and by-products. This resulted in anticipated dietary protein intakes of 126 µg/kg bw/day of HPPD W336 and 694 µg/kg bw/day for 2mEPSPS.

4.3.8 Nutritional assessment

As the intended traits are for agronomic purposes only (tolerance to glyphosate herbicides and HPPD inhibitors), no change in the nutritional value of the product is of relevance. The only significant change observed in the comparative analysis was a reduction in the amount of dihydrosterculic acid in GHB811 cotton treated with the intended herbicide compared to the conventional counterpart, which does not therefore present a safety/nutritional concern.

4.3.9 Conclusion of the food/feed safety assessment

The ACNFP assessed the food/feed safety of the newly expressed proteins in terms of their toxicological potential, allergenic potential, and nutritional quality. It concluded that the newly expressed proteins shared no identity with known toxins and allergens, and the overall allergenicity of GHB811 cotton was not different to conventional cotton. The ACNFP concluded that based on the comparative analysis and the nutritional assessment, GHB811 cotton does not cause any nutritional concerns, and is as safe as conventional cotton varieties.

4.4 Environmental risk assessment and monitoring plan

4.4.1 Environmental risk assessment

The environmental risk assessment (ERA) of GHB811 cotton was considered by the Advisory Committee on Releases to the Environment (ACRE).

The scope of the application does not include cultivation and only covers the import, processing, and food and feed use of GHB811 cotton. No deliberate release of viable plant material or derived products is expected. Therefore, only

accidental release of viable GM seeds or propagating material during import, transportation, storage, handling, and processing will be considered.

ACRE considered the ability of GHB811 cotton to persist under GB environmental conditions, interaction of feral GHB811 cotton with the environment, and the potential for horizontal gene transfer (HGT) to the environment. ACRE concluded that GHB811 cotton would not raise safety concerns in the event of accidental release of viable seeds or propagating material into the environment.

ACRE's advice is available on the GOV.UK website.

4.4.2 Post-market environmental monitoring (PMEM) plan

The PMEM plan provided by the applicant proposes general surveillance to identify the occurrence of unanticipated adverse effects due to the unintended release of GHB811 cotton. Exposure (via accidental release) can be controlled by clean-up measures, and the application of current practices used for the control of any adventitious cotton plants, such as manual or mechanical removal, and the application of herbicides.

General surveillance will be predominantly based on collaboration with third parties, such as operators involved in the import, handling, and processing of GHB811 cotton. These third parties will report any potential unanticipated adverse effects to the authorisation holder, who will investigate.

The authorisation holder will submit an annual report including results of the general surveillance and any unanticipated adverse effects. If information that confirms an adverse effect becomes available, the authorisation holder will investigate, and based on a scientific evaluation, define and implement management measures to protect human and animal health, or the environment, as necessary.

ACRE considered the PMEM plan provided by the applicant, in conjunction with the ERA. As the ERA did not identify potential adverse effects to the environment, it was not considered necessary for case-specific monitoring to be implemented. The proposed PMEM plan and monitoring intervals are appropriate for the intended uses of GHB811 cotton.

5. Overall conclusions and recommendations

To support the safety assessment by FSA/FSS, the ACNFP was asked to provide advice on the data submitted for the authorisation for import, processing, and

food and feed use of genetically modified GHB811 cotton in accordance with assimilated Regulation (EU) No. 1829/2003. GHB811 cotton was modified by the addition of the *2mepsps* and *hppdPfW336-1Pa* gene cassettes. The 2mEPSPS protein is modified from the wild-type maize EPSPS protein which is a target for glyphosate herbicides. The modifications made in 2mEPSPS decrease glyphosate binding affinity, thereby conferring tolerance to glyphosate herbicides. The HPPD W336 protein is a modified HPPD protein and confers improved tolerance to HPPD (4-hydroxyphenylpyruvate dioxygenase) inhibitors.

The molecular characterisation data established that GHB811 cotton contains a single transgenic insert and bioinformatics analyses of this insert, and the flanking sequences, raised no safety concerns. The stability of the insert was confirmed over five generations. The expression levels of the transgenic proteins in fuzzy cottonseed were determined using suitable methodologies, and do not cause a safety concern.

The field trials used to generate material for the comparative analyses were deemed appropriate, and the locations selected were considered representative of commercial cotton production. The meteorological conditions and management practices used during the field trails were appropriate. The ACNFP also assessed the results from the comparative analysis, including all the significant differences between GHB811 cotton and its conventional counterpart, and found no safety concerns.

The food/feed safety of the newly expressed proteins was assessed, and no safety concerns were raised in terms of their toxicological potential, allergenic potential, and nutritional quality. Based on the comparative analysis and the nutritional assessment, GHB811 cotton does not cause any nutritional concerns.

Overall, the ACNFP concludes that GHB811 cotton is as safe as its conventional counterpart with respect to its potential effects on human and animal health.

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7. Appendices

7.1 Appendix 1

Results of the statistical analyses performed on all analytes tested in the comparative assessment of agronomic characteristics of GHB811 cotton.

Intended herbicide treatment

Category I (equivalence demonstrated)[a]

i. Not significantly different

Stand count 1, average % ground cover, heat units to 10% flower, final stand count, seed weight, average seeds per boll, total yield, yield-lint, average plant height, average 1st fruiting branch, average bolls per plant, average fruiting branch bolls per plant, average potential fruiting sites per plant, average

vegetative bolls per plant, average vegetative branches per plant, % fruit retention, % harvestable fruiting branch bolls, average node count, height to node ratio, lint micronaire, lint elongation, lint strength, and lint uniformity (23)

ii. Significantly different

Stand count 2, days to 1st open bolls, heat units to 1st open bolls, bolls - % open, and average boll weight (5)

Category II (equivalence more likely than not)

i. Not significantly different

Days to 10% flower (1)

ii. Significantly different

% lint (1)

Category III (equivalence less likely than not)

i. Not significantly different

N/A

ii. Significantly different

N/A

Category IV (non-equivalence demonstrated)

i. Significantly different

Lint length (1)

Conventional herbicide management

Category I (equivalence demonstrated)[a]

i. Not significantly different

Stand count 1, average % ground cover, heat units to 10% flower, days to first open bolls, heat units to 1st open bolls, seed weight, average seeds per boll, average 1st fruiting branch, average fruiting branch bolls per plant, average potential fruiting sites per plant, average vegetative branches per plant, % fruit retention, average node count, lint elongation, and lint strength (15)

ii. Significantly different

Stand count 2, bolls - % open, final stand count, average boll weight, total yield, average plant height, average bolls per plant, vegetative bolls per plant, % harvestable fruiting branch bolls, height to node ratio, lint micronaire, and lint uniformity (12)

Category II (equivalence more likely than not)

i. Not significantly different

Days to 10% flower (1)

ii. Significantly different

Yield-lint (1)

Category III (equivalence less likely than not)

i. Not significantly different

N/A

ii. Significantly different

% lint (1)

Category IV (non-equivalence demonstrated)

i. Significantly different

Lint length (1)

[a] The comparative analysis comprises a test of equivalence with the non-GM reference varieties and a test of difference with the conventional counterpart. The results of the test of equivalence are categorised into four groups; equivalence with the reference varieties is demonstrated, equivalence with the reference varieties is more likely than not, equivalence with the reference varieties is less likely than not, and non-equivalence with the reference varieties is demonstrated.

7.2 Appendix 2

Results of the statistical analyses performed on all analytes tested in the comparative assessment of GHB811 cotton.

Intended herbicide treatment

Category I (equivalence demonstrated)[a]

i. Not significantly different

Ash, crude fat, acid detergent fibre, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, valine, C16:0 palmitic acid, C17:0 heptadecanoic acid, C17:1 heptadecanoic acid, C18:1 oleic acid, C18:2 linoleic acid, C20:1 eicosenoic acid, C22:0 behenic acid, copper, iron, magnesium, phosphorus, potassium, zinc, malvalic acid, and sterculic acid (33)

ii. Significantly different

Moisture, carbohydrates, crude protein, total dietary fibre, cystine, methionine, C14:0 myristic acid, C18:0 stearic acid, C20:0 arachidic acid, calcium, manganese, α-tocopherol, free gossypol, and total gossypol (14)

Category II (equivalence more likely than not)

i. Not significantly different

Tyrosine (1)

ii. Significantly different

Neutral detergent fibre and C16:1 palmitoleic acid (2)

Category III (equivalence less likely than not)

i. Not significantly different

N/A

ii. Significantly different

N/A

Category IV (non-equivalence demonstrated)

i. Significantly different

Dihydrosterculic acid (1)

Not categorised

C18:3 linolenic acid, C24:0 lignoceric acid, and sodium (3)

Conventional herbicide management

Category I (equivalence demonstrated)[a]

i. Not significantly different

Moisture, ash, crude fat, acid detergent fibre, alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, C14:0 myristic acid, C16:0 palmitic acid, C17:0 heptadecanoic acid, C17:1 heptadecanoic acid, C18:1 oleic acid, C18:2 linoleic acid, C20:1 eicosenoic acid, C22:0 behenic acid, calcium, copper, iron, magnesium, phosphorus, potassium, zinc, malvalic acid, and sterculic acid (39)

ii. Significantly different

Carbohydrates, crude protein, total dietary fibre, C18:0 stearic acid, C20:0 arachidic acid, manganese, α-tocopherol, free gossypol, and total gossypol (9)

Category II (equivalence more likely than not)

i. Not significantly different

N/A

ii. Significantly different

Neutral detergent fibre and C16:1 palmitoleic acid (2)

Category III (equivalence less likely than not)

i. Not significantly different

N/A

ii. Significantly different

N/A

Category IV (non-equivalence demonstrated)

i. Not significantly different

Dihydrosterculic acid[b] (1)

ii. Significantly different

N/A

Not categorised

C18:3 linolenic acid, C24:0 lignoceric acid, and sodium (3)

[a] The comparative analysis comprises a test of equivalence with the non-GM reference varieties and a test of difference with the conventional counterpart. The results of the test of equivalence are categorised into four groups; equivalence with the reference varieties is demonstrated, equivalence with the reference varieties is more likely than not, equivalence with the reference varieties is less likely than not, and non-equivalence with the reference varieties is demonstrated.

[b] Dihyrosterculic acid in GHB811 cotton grown with conventional herbicide management is classified in category 4 (non-equivalence is demonstrated), however there is insufficient evidence of a difference with the conventional counterpart.

Abbreviations

Acronym	Definition
ACNFP	Advisory Committee on Novel Foods and Processes
ACRE	Advisory Committee on Releases to the Environment
ADF	Acid Detergent Fiber
BLAST	Basic Local Alignment Search Tool
bp	Base pair
bw	Body weight
CD	Coeliac disease
CHM	Conventional Herbicide Management

COMPARE	COMprehensive Protein Allergen REsource
DNA	Deoxyribonucleic acid
DW	Dry weight
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate
EU	European Union
FSA	Food Standards Agency
FSS	Food Standards Scotland
gDNA	Genomic DNA
GM	Genetically modified
GMO	Genetically modified organism
HPPD	Hydroxyphenylpyruvate dioxygenase
kDa	Kilodalton
LLOQ	Lower limit of quantification

NDF	Neutral Detergent Fibre
OECD	Organisation for Economic Co-operation and Development
ORFs	Open reading frames
PCR	Polymerase chain reaction
PMEM	Post-market environmental monitoring
PMM	Post-Market Monitoring
RBD	Refined, bleached, and deodorised
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
T-DNA	Transfer-deoxyribonucleic acid
TIH	Treatment with Intended Herbicide