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Assessment of genetically modified oilseed rape 73496 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2012-109)

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Abstract

Oilseed rape 73496 was developed to confer tolerance to the herbicidal active substance glyphosate through the expression of the glyphosate acetyltransferase protein GAT4621. The molecular characterisation data and bioinformatic analyses identify no issues requiring food/feed safety assessment. None of the identified differences between oilseed rape 73496 and its conventional counterpart in the agronomic/phenotypic endpoints tested needs further assessment. Differences identified in seed composition of oilseed rape 73496 as compared to its conventional counterpart raise no safety and nutritional concerns in the context of the scope of this application. No safety concerns are identified regarding toxicity and allergenicity of the GAT4621 protein as expressed in oilseed rape 73496. No evidence is found that the genetic modification would change the overall allergenicity of oilseed rape 73496. Based on the outcome of the comparative and nutritional assessments, the consumption of oilseed rape 73496 does not represent any nutritional concern, in the context of the scope of this application. The implementation of a post-market monitoring plan is recommended to confirm the predicted consumption data and to verify that the conditions of use are those considered during the pre-market risk assessment. In the case of accidental release of viable oilseed rape 73496 seeds into the environment, oilseed rape 73496 would not raise environmental safety concerns. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of oilseed rape 73496. The GMO Panel concludes that oilseed rape 73496, as described in this application, is as safe as its conventional counterpart and the non-genetically modified oilseed rape reference varieties tested with respect to potential effects on human and animal health and the environment.

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Summary

The scope of application EFSA-GMO-NL-2012-109 is for food and feed uses, import and processing of the genetically modified (GM) herbicide tolerant oilseed rape 73496 within the European Union (EU).

In the present scientific opinion, the scientific Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA) (hereafter referred to as the 'GMO Panel') reports the outcome of its risk assessment of oilseed rape 73496 according to the scope as defined in application EFSA-GMO-NL-2012-109. The GMO Panel conducted the assessment of oilseed rape 73496 in line with the principles described in Regulation (EC) No 1829/2003 and its applicable guidelines for the risk assessment of food and feed from GM plants, including their environmental risk assessment.

The molecular characterisation data establish that oilseed rape 73496 contains a single insert consisting of one copy of the *gat4621* expression cassette, expressing the GAT4621 protein conferring tolerance to the herbicidal active substance glyphosate. Upon transformation, a region of chromosome C02 was potentially inverted and a putative *tpt* gene interrupted. The relevance of the gene interruption and potential chromosomal inversion for the risk assessment of oilseed rape 73496 is addressed. Bioinformatic analyses of the sequences encoding the newly expressed protein and open reading frames (ORFs) present within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and introduced trait is confirmed over several generations. The levels of the GAT4621 protein were obtained and reported adequately. The protein characterisation data of the plant- and microbe-produced GAT4621 protein indicate that both proteins are equivalent and thus that the microbial-derived protein (two batches) can be used in safety studies.

None of the identified differences between oilseed rape 73496 and its conventional counterpart in the agronomic/phenotypic endpoints tested needs further assessment. Among the differences identified in seed composition between oilseed rape 73496 and its conventional counterpart, the levels of N-acetylaspartate, N-acetylglutamate, N-acetylthreonine, free amino acid glycine, crude fibre, crude fat, acid detergent fibre, neutral detergent fibre, magnesium, pyridoxine, pantothenic acid and 4-hydroxyglucobrassicin were further assessed and found to raise no safety and nutritional concerns in the context of the scope of this application. No safety concerns are identified regarding the toxicity and allergenicity of the GAT4621 protein as expressed in oilseed rape 73496. No evidence is found that the genetic modification would change the overall allergenicity of oilseed rape 73496. Based on the outcome of the comparative and nutritional assessments, the consumption of oilseed rape 73496 does not represent any nutritional concern, in the context of the scope of this application.

The implementation of a post-market monitoring plan is recommended to confirm the predicted consumption data and to verify that the conditions of use are those considered during the pre-market risk assessment.

Considering the introduced trait, the outcome of the agronomic and phenotypic analysis and the routes and levels of exposure, oilseed rape 73496 would not raise safety concerns in the case of accidental release of viable GM oilseed rape seeds into the environment.

The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of oilseed rape 73496.

The GMO Panel concludes that oilseed rape 73496, as described in this application, is as safe as its conventional counterpart and the non-GM oilseed rape reference varieties tested with respect to potential effects on human and animal health and the environment.

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1. Introduction

The scope of application EFSA-GMO-NL-2012-109 is for food and feed uses, import and processing of the genetically modified (GM) herbicide tolerant oilseed rape 73496 within the European Union (EU).

1.1. Background

On 24 May 2012, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands the application EFSA-GMO-NL-2012-109 for authorisation of herbicide tolerant oilseed rape 73496 (Unique Identifier DP-Ø73496-4), submitted by Pioneer Hi-Bred International (hereafter referred to as 'the applicant') within the framework of Regulation (EC) No 1829/2003[1].

Following receipt of application EFSA-GMO-NL-2012-109, EFSA informed EU Member States and the European Commission, and made the application available to them. Simultaneously, EFSA published the summary of the application.[2]

EFSA checked the application for compliance with the relevant requirements of its guidance documents (see Section 2.2), and, when needed, asked the applicant to supplement the initial application. On 4 December 2012, EFSA declared the application valid.

From validity date, EFSA and its scientific Panel on Genetically Modified Organisms (hereafter referred to as 'the GMO Panel') endeavoured to respect a time limit of 6 months to issue a scientific opinion on application EFSA-GMO-NL-2012-109. Such time limit was extended whenever EFSA and/or its GMO Panel requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to EU Member States and the European Commission (for further details, see the section 'Documentation as provided to EFSA').

In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC[3]. The EU Member States had 3 months to make their opinion known on application EFSA-GMO-NL-2012-109 as of date of validity.

1.2. Terms of Reference as provided by the requestor

According to Articles 6 and 18 of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of oilseed rape 73496 in the context of its scope as defined in application EFSA-GMO-NL-2012-109.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation, and thus will be part of the EFSA overall opinion in accordance with Articles 6(5) and 18(5).

The relevant information is made available in OpenEFSA including the information required under Annex II to the Cartagena Protocol; a labelling proposal; a post-market environmental monitoring (PMEM) plan as provided by the applicant; and the method(s), validated by the Community reference laboratory, for detection, including sampling, identification of the transformation event in the food/feed and/or foods/feeds produced from it and the appropriate reference materials.[4]

2. Data and methodologies

2.1. Data

The GMO Panel based its scientific risk assessment of oilseed rape 73496 on the valid application EFSA-GMO-NL-2012-109, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by EU Member States and relevant peer-reviewed scientific publications.

[1] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.
[2] Available online: https://open.efsa.europa.eu/questions/EFSA-Q-2012-00617
[3] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, p. 1–38.
[4] https://open.efsa.europa.eu/questions/EFSA-Q-2012-00617

2.2. Methodologies

The GMO Panel carried out a scientific risk assessment of oilseed rape 73496 for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. The GMO Panel took into account the appropriate principles described in its applicable guidelines (i.e. EFSA GMO Panel, 2010a,b,c, 2011a,b) and explanatory notes (i.e. EFSA, 2014, 2017a,b) for the risk assessment of food and feed from GM plants, including their environmental risk assessment.

For the assessment of 90-day animal feeding study, the GMO Panel took into account the criteria reported in the EFSA Scientific Committee guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed (EFSA Scientific Committee, 2011) and the explanatory statement for its applicability (EFSA, 2014).

The GMO Panel also assessed the applicant's literature searches in accordance with the principles outlined in EFSA (2010, 2017a). In the frame of the contracts OC/EFSA/GMO/2013/01 and OC/EFSA/GMO/2014/01, contractors performed preparatory work and delivered reports on the methods applied by the applicant in performing bioinformatic and statistical analyses and toxicological studies, respectively.

3. Assessment

3.1. Molecular characterisation

3.1.1. Transformation process and vector constructs[5]

Oilseed rape 73496 was developed by biolistic transformation of microspores of oilseed rape (*Brassica napus* L.) line 1822B with a *Hind*III/*Not*I fragment named PHP28181A from plasmid PHP28181.

The PHP28181A fragment contains the *gat4621* (glyphosate acetyl transferase) expression cassette, containing the following genetic elements: the polyubiquitin (*UBQ10*) promoter of *Arabidopsis thaliana*; the *gat4621* gene; and the 3′ terminator sequence of a gene encoding the proteinase inhibitor II (*pin*II terminator) of *Solanum tuberosum*. The *gat4621* gene is a shuffled variant of three *gat* genes, isolated from *Bacillus licheniformis* strains 401, B6 and DS3 that has been codon-optimised for expression in plants.

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

3.1.2. Transgene constructs in the GM plant[6]

Molecular characterisation of oilseed rape 73496 was performed by Southern analysis, polymerase chain reaction (PCR) and DNA sequence analysis, in order to determine copy number, size and organisation of the inserted sequences, and to confirm the absence of plasmid backbone sequences. The approach used was acceptable both in terms of coverage and sensitivity.

Southern analyses indicated that oilseed rape 73496 contains a single insert, consisting of a single copy of the PHP28181A fragment used for transformation. The insert and copy number were confirmed by multiple restriction enzyme/probe combinations covering the insert and flanking regions. No signal was observed with probes corresponding to PHP28181 vector backbone sequences.

The nucleotide sequence of the entire insert of oilseed rape 73496, together with 2003 nucleotides of the 5′ and 2038 nucleotides of the 3′ flanking regions, was determined. The insert of 2109 bp is identical to the fragment of PHP28181A, except for the deletion of the first three base pairs of the 5′ end of the PHP28181A fragment. The possible interruption of known endogenous oilseed rape genes by the insertion in event 73496 was evaluated by bioinformatic analyses of the pre-insertion locus and of the genomic sequences flanking the insert. Sequence comparisons of the insert flanking sequences suggest an inversion of a region of chromosome C02. Indeed, the two flanking genomic border sequences were mapped to the reference genome sequence and located about 9.2 Mbp apart, and the 3′ flanking genomic border sequence in oilseed rape 73496 is on the opposite strand of chromosome C02 compared to the same sequence in the parental line. A SNP marker analysis showed no evidence

[5] Part II Scientific Information/Section A.2.1.

[6] Part II Scientific Information/Section A.2.2.2. Additional information 9/4/2013, 15/12/2015, 29/4/2016, 3/10/2016, 2/5/2017, 28/9/2018, 17/4/2019 and 9/7/2020.

of deletion of this region during the transformation process. However, the insertion resulted in the disruption of a putative gene, named *PGtpt* (*PredictedGenetpt*) showing similarity to a triose phosphate transporter (*tpt*). Southern blot analyses indicated that there are four copies of the *tpt* gene in oilseed rape and qRT-PCR analysis revealed a lower overall transcript level of the *tpt* gene family in leaf from oilseed rape 73496, compared to the control plant. These data suggest that the *PGtpt* gene has been interrupted in 73496 oilseed rape affecting most probably the level of transcripts for this gene in leaves. Further considerations on the relevance of the gene interruption and potential chromosomal inversion for the risk assessment of oilseed rape 73496 are provided in Sections 3.2.2 and 3.2.3.

The results of segregation (see Section 3.1.5) and bioinformatic analyses established that the insert is located in the nuclear genome.

Updated bioinformatic analyses of the amino acid sequence of the newly expressed GAT4621 protein revealed no significant similarities to toxins and allergens. In addition, updated bioinformatic analyses of the newly created Open Reading Frames (ORFs) within the insert and spanning the junctions between the insert and genomic DNA did not indicate significant similarities to toxins and allergens.

In order to assess the possibility for horizontal gene transfer by homologous recombination (HR), the applicant performed a sequence identity analysis of the inserted regions of bacterial origin in oilseed rape 73496. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.4.1.2.

3.1.3. Protein characterisation and equivalence[7]

Oilseed rape 73496 expresses one new protein, GAT4621, which is a glyphosate acetyl transferase conferring tolerance to the herbicidal active substance glyphosate.

Given the technical restrains in producing large enough quantities from plants, GAT4621 was recombinantly produced in *Escherichia coli*. A set of biochemical methods was employed to demonstrate the equivalence between oilseed rape and the two batches of *E. coli*-derived GAT4621 protein used in the different experiments presented in the dossier (see Section 3.3.3). The purified plant protein and *E. coli*-derived protein (two batches) were characterised and compared in terms of their physico-chemical, structural and functional properties.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that both plant- and microbe-produced GAT4621 proteins had the expected molecular weight of ~ 16.5 kDa and were comparably immunoreactive to GAT4621 protein-specific antibodies. Glycosylation detection analysis demonstrated that none of the GAT4621 proteins were glycosylated. Amino acid sequence analysis of the two GAT4621 proteins by mass spectrometry and N-terminal sequencing methods showed that they matched the deduced sequence as defined by the *gat4621* gene. These data also showed that the N-terminal methionine of both the plant- and microbial-produced GAT4621 proteins was truncated. Such modifications are common in eukaryotic proteins (e.g. Poledova and Sherman, 2000) and have been previously assessed by the GMO Panel for newly expressed proteins (EFSA GMO Panel, 2017). Due to the purified plant GAT4621 protein being inactive, the activity between the plant and *E. coli*-derived proteins could not be directly compared. The activity and substrate specificity of the two *E. coli*-produced GAT4621 was analysed by a biochemical *in vitro* activity assay.[8] The results from this assay confirmed the acetylation activity of the GAT4621 protein for the intended herbicide as well as for a number of amino acids. The activity of the plant-produced GAT4621 was indirectly demonstrated by the tolerance to the herbicidal active substance glyphosate and compositional analyses (see Section 3.2.3).

Based on these data, the GMO Panel accepts the use of the GAT4621 protein produced in bacteria for the safety studies.

[7] Dossier Part II Scientific Information/Section A.4.2. Additional information 18/12/2018.

[8] The substrate specificity of the *E. coli*-produced GAT4621 protein has been tested on a range of 21 different agrochemicals, 21 amino acids and 10 antibiotics under *in vitro* conditions (Annex 22_PHI-2006-184/017). GAT4621 protein has been shown to acetylate certain amino acids, such are aspartate, glutamate and threonine. Increased concentration of the N-acetylated forms of these three amino acids (N-acetylaspartate, N-acetylglutamate and N-acetylthreonine) in oilseed rape 73496 is indicative of an equivalent activity of the plant-produced GAT4621 compared to the *E. coli*-produced GAT4621 protein.

3.1.4. Information on the expression of the insert[9]

Levels of the GAT4621 protein were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested in a field trial across four locations in the USA and five locations in Canada during 2010 growing season. Samples analysed included whole plants (BBCH15, BBCH33 and BBCH65), roots (BBCH65) and seeds (BBCH90), from plants treated and not treated with the intended herbicide. The mean values, standard deviations and ranges of protein expression levels in seeds for plants treated (n = 32) and not treated with the intended herbicide (n = 36) are summarised in Table 1.

Table 1: Mean values, standard deviations and ranges of the GAT4621 protein in seeds [ng/mg dry weight (dw)] from oilseed rape 73496

Tissue	Intended herbicide treatment	
	Not treated	Treated
Seed (BBCH90)		
GAT4621	5.6[(a)] ± 1.1[(b)] (3.6–8.1)[(c)]	5.6 ± 1.1 (4.2–8.7)

(a): Mean.
(b): Standard deviation.
(c): Range.

3.1.5. Inheritance and stability of inserted DNA[10]

Genetic stability of the oilseed rape 73496 insert was assessed by Southern analysis of genomic DNA from five generations (T2, T3, T3F2, T3F3, F1) and segregation analysis of the glyphosate tolerance trait of oilseed rape 73496 from five generations (T3F2, BC1F1, BC2F1, BC3F1, F1). For the Southern analysis, the restriction enzyme/probe combinations used were sufficient to conclude that all the plants tested retained the single copy of the insert and flanking regions, which were stably inherited in subsequent generations. The results supported the presence of a single insertion, segregating in a Mendelian fashion.

3.1.6. Conclusion on molecular characterisation

The molecular characterisation data establish that oilseed rape 73496 contains a single insert consisting of one copy of the *gat4621* expression cassette. Upon transformation, a region of chromosome C02 was potentially inverted and a putative *tpt* gene interrupted. Further considerations on the relevance of the gene interruption and potential chromosomal inversion for the risk assessment of oilseed rape 73496 are provided in Sections 3.2.2 and 3.2.3. Bioinformatic analyses of the sequences encoding the newly expressed protein and other ORFs within the insert or spanning the junctions between the insert and genomic DNA indicate no significant similarities to toxins and allergens. The stability of the inserted DNA and of the introduced herbicide tolerance trait was confirmed over several generations. The levels of the GAT4621 protein were obtained and reported adequately. The protein characterisation data of the plant- and microbial-derived GAT4621 proteins indicate that these proteins are equivalent and thus that the microbial-produced protein (two batches) can be used in the safety studies.

3.2. Comparative analysis[11]

3.2.1. Choice of comparator and production of material for the comparative assessment

Application EFSA-GMO-NL-2012-109 presents data on agronomic/phenotypic characteristics, as well as seed composition, of oilseed rape 73496 derived from field trials performed in the USA and Canada during the 2010 growing season (Table 2). In addition, seed characteristics of oilseed rape 73496 were evaluated under laboratory (growth chamber) conditions.

Oilseed rape 73496 was obtained through the transformation of the double haploid male sterile maintainer line 1822B. The obtained GM oilseed rape, after restoration with line 1822R, was crossed

[9] Dossier Part II Scientific Information/Section A.2.2.3.
[10] Dossier: Part II Scientific information/Section A.2.2.4.
[11] Dossier: Part II Scientific information/Section A.3. Additional information: 17/9/2013.

with oilseed rape 5536F to produce the oilseed rape 73496 hybrid used in the agronomic/phenotypic field trials, in the seed germination tests and in the compositional studies (see Table 2). The comparator used in the studies was obtained by crossing the non-GM line 5536F with line 5676M, the latter sharing high similarity with line 1822B. The GMO Panel considers that the used comparator (5536F × 5676M) is a conventional counterpart suitable for the comparative analysis.

Table 2: Overview of comparative assessment studies with oilseed rape 73496 provided in application EFSA-GMO-NL-2012-109

Study focus	Study details	Comparator	Commercial non-GM oilseed rape reference varieties
Agronomic/phenotypic and compositional analysis	Field trials, 2010, North America	Conventional counterpart (5536F × 5676M)	6(a)
Agronomic and phenotypic analysis	Seed germination study	Conventional counterpart (5536F × 5676M)	2(b)

(a): The commercial non-GM oilseed rape reference varieties used in the 2010 field trials are 44A04, 44A89, 54H72 and the hybrids 45H73, 46H02 and 46A65.
(b): The commercial non-GM oilseed rape reference varieties used in the seed germination studies are 45H72 and 45H73.

The field trial sites were located in major oilseed rape growing areas in North America.[12] At each site, the following materials were grown in a randomised complete block design with four replicates: oilseed rape 73496, the conventional counterpart and three non-GM oilseed rape reference varieties, all treated with required maintenance pesticides (including conventional herbicides); and oilseed rape 73496 treated with the intended glyphosate herbicide and required maintenance pesticides (including conventional herbicides). In total, six commercial non-GM oilseed rape reference varieties were included in the field trials (Table 2).

3.2.1.1. Statistical analysis of field trials data

The agronomic/phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010a, 2011a). This includes, for each of the two treatments of oilseed rape 73496, the application of a difference test (between the GM oilseed rape and its conventional counterpart) and an equivalence test (between the GM oilseed rape and the set of commercial non-GM oilseed rape reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).

3.2.2. Agronomic/phenotypic analysis

3.2.2.1. Agronomic/phenotypic characteristics tested under field conditions

Twelve agronomic/phenotypic endpoints were collected from all field trial sites (see Table 2).[13]

The GMO Panel considered the adequacy of the selected endpoints for the identification of possible unintended effects related to the reorganisation at the insertion site and the *PGtpt* gene disruption (see Section 3.1.2). The information available from the published literature suggests that phenotypic changes may be associated with a deficiency of *tpt* gene activity in dicot plants (such as *Arabidopsis thaliana* and tobacco) indicating a possible impact on growth, biomass and yield (Häusler et al., 2000; Schneider et al., 2002; Bockwoldt et al., 2019). The agronomic/phenotypic data set included several endpoints that are directly related to those parameters (i.e. days to flowering, flowering duration, plant height, days to maturity and yield). Taking this into account, the GMO Panel did not identify the need to include additional specific endpoints in the agronomic/phenotypic analysis of oilseed rape 73496 and considers that the data set provided is adequate to conclude the agronomic and phenotypic analysis of oilseed rape 73496.

The endpoints were analysed as described in Section 3.2.1.1, with the following results:

[12] Four locations in the USA (Ephrata, Washington; Gardner, North Dakota; Northwood, North Dakota; and Velva, North Dakota); five locations in Canada (two in Rosthern, Saskatchewan; one each in Elm Creek, Manitoba; Dundurn, Saskatchewan; and Saskatoon, Saskatchewan).
[13] Early stand count, seedling vigour, days-to-flowering, flowering duration, plant height, lodging, days-to-maturity, pod shattering, yield, final stand count, disease incidence and arthropod damage.

- For oilseed rape 73496 (not treated with the intended herbicide), statistically significant differences with the conventional counterpart were identified for four endpoints (early and final population, flowering duration and disease incidence), which all fell under equivalence category I.
- For oilseed rape 73496 (treated with the intended herbicide), statistically significant differences with the conventional counterpart were identified for five endpoints (early and final population, flowering duration, plant height and lodging), which all fell under equivalence category I.

Regarding the indicators of possible growth retardation, statistically significant differences were found for flowering duration and plant height; both those endpoints, however, fell under equivalence category I.

3.2.2.2. Agronomic/phenotypic characteristics tested under controlled conditions

The applicant also reported data on seed characteristics of oilseed rape 73496. Seed germination tests with seeds harvested from oilseed rape 73496, its conventional counterpart and two commercial non-GM oilseed rape reference varieties, grown under field conditions, were performed to evaluate seed characteristics under growth chamber conditions.[14] The endpoint analysed was the number of germinated seed. The applicant found no statistically significant differences between oilseed rape 73496 and its conventional counterpart under cold and diurnal growing conditions. The germination rate of oilseed rape 73496 was significantly lower than that of its conventional counterpart under warm growing conditions, yet the range of values fell within that observed for the non-GM oilseed rape reference varieties.

3.2.3. Compositional analysis

Seeds from oilseed rape harvested from the field trials (Table 2) were analysed for 99 constituents, including those recommended by the OECD (OECD, 2001).

Since the GAT4621 protein was demonstrated to acetylate certain amino acids (see Section 3.1.3), the acetylated derivatives of several amino acids were also analysed in seeds. These were N-acetylaspartate, N-acetylglutamate, N-acetylthreonine, N-acetylglycine and N-acetylserine (hereafter referred as NAA, NAG, NAT, NAGly and NAS, respectively).

Additionally, 26 free amino acids were analysed, since free amino acids might be metabolically related to the levels of the acetylated amino acids.[15]

The GMO Panel assessed the adequacy of the compositional endpoint data set for the identification of potential unintended effects related to the reorganisation at the insertion site and the PGtpt gene disruption (see Section 3.1.2). Data in *Arabidopsis* and tobacco plants suggest that the potential deficiency of *tpt* gene would be compensated by both continuous accelerated starch turnover and export of neutral sugars from the stroma (Bockwoldt et al., 2019; Häusler et al., 2000; Schneider et al., 2002). The GMO Panel considers that the compositional endpoints evaluated (key nutrients, key toxicants and anti-nutrients) are comprehensive enough to capture potential modifications in metabolic pathways of the GM plant, induced by the above changes, that may be of relevance for food and feed assessment, and that no specific hypotheses requiring further compositional investigations are identified. The GMO Panel concludes that the provided compositional data set is adequate, and no additional endpoints are necessary to conclude on compositional analysis.

The statistical analysis was not applied to 28 compounds,[16] because more than 50% of the observations were below the limit of quantification.

[14] Three separate germination tests were conducted (warm: 25°C and 90% relative humidity for 10 days; cold: 10°C and 90% relative humidity for 12 days; and diurnal: cyclical setting of 10°C and 90% relative humidity for 16 h and then 25°C and 90% relative humidity for 8 h, repeated daily for 10 days).

[15] Alanine, arginine, asparagine, aspartic acid, alpha-aminobutyric acid, cystine, ethanolamine, gamma-aminobutyric acid, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, taurin, threonine, tryptophan, tyrosine, valine.

[16] Caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), heptadecadienoic acid (C17:2), (9,15) isomer of linoleic acid (C18:2), γ-linolenic acid (C18:3), nonadecanoic acid (C19:0), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), heneicosanoic acid (C21:0), erucic acid (C22:1), glucoiberin, epi-progoitrin, glucoraphanin, gluconapoleiferin, glucoalyssin, glucobrassicanapin, gluconasturtiin, 4-methoxyglucobrassicin, neoglucobrassicin), β-tocopherol and the free amino acids α-aminobutyric acid, cystine, hydroxyproline and taurine.

The statistical analysis was applied to the remaining 103 compounds,[17] with the following results (Table 3):

- For oilseed rape 73496 (not treated with the intended herbicide), statistically significant differences with the conventional counterpart were identified for 53 endpoints. Of those, two endpoints fell under equivalence category III/IV (pyridoxine and NAG), six endpoints were not categorised for equivalence (crude fibre, 4-hydroxyglucobrassicin, magnesium, NAA, NAT and the free amino acid glycine), while the other endpoints fell under category I/II. Brassicasterol fell under equivalence category III; however, no significant differences with the conventional counterpart were identified.
- For oilseed rape 73496 (treated with the intended herbicide), statistically significant differences with the conventional counterpart were identified for 56 endpoints. Of those, five endpoints fell under equivalence category III/IV (crude fat, ADF, NDF, pantothenic acid and NAG), six endpoints were not categorised for equivalence (crude fibre, 4-hydroxyglucobrassicin, magnesium, NAA, NAT and the free amino acid glycine), while the other endpoints fell under category I/II. NAGly, pyridoxine and brassicasterol fell under equivalence category III/IV; however, no significant differences with the conventional counterpart were identified.

Table 3: Outcome of the comparative compositional analysis in seeds of oilseed rape 73496. The table shows the number of endpoints in each category

		Test of difference[a]			
		Not treated[c]		Treated[c]	
		Not different	Significantly different	Not different	Significantly different
Test of equivalence[b]	Category I/II	45	45[d]	40	45[d]
	Category III/IV	1[e]	2[f]	3[e]	5[f]
	Not categorised	4[g]	6[h]	4[g]	6[h]
	Total endpoints	103		103	

(a): Comparison between oilseed rape 73496 and its conventional counterpart.
(b): Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.
(c): Not treated/treated with the intended herbicide glyphosate.
(d): Endpoints with significant differences between oilseed rape 73496 and its conventional counterpart and falling in equivalence category I-II. For both treated and not-treated: arachidic acid (C20:0), aspartic acid, cholesterol, crude protein, delta-tocopherol, eicosadienoic acid (C20:2), glucobrassicin, gluconapin, total glucosinolates, linoleic acid (C18:2), linolenic acid (C18:3), moisture, oleic acid (C18:1), palmitic acid (C16:0), palmitoleic acid (C16:1), phytic acid, progoitrin, stearic acid (C18:0), tannins-insoluble, tannins-soluble, tryptophan, niacin, phosphorus, zinc. Only non-treated: ADF, copper, eicosenoic acid (C20:1), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), NDF, nervonic acid (C24:1), potassium, free alanine, free asparagine, free gamma-aminobutyric acid, free glutamic acid, free glutamine, free histidine, free isoleucine, free leucine, free methionine, free phenylalanine, free proline, free serine and free valine. Only treated: alanine, glutamic acid, valine, calcium, manganese, carbohydrates, lignoceric acid (C24:0), folic acid and free lysine.
(e): Endpoints falling in equivalence category III-IV and with no significant differences between oilseed rape 73496 and its conventional counterpart. For both treated and not-treated: brassicasterol. Only treated: pyridoxine and NAGly.

[17] Proximates and fibre content (moisture, crude protein, crude fat, acid detergent fibre (ADF), crude fibre, neutral detergent fibre (NDF), ash, carbohydrates), fatty acids (myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), eicosadienoic acid (C20:2), eicosenoic acid (C20:1), behenic acid (C22:0), tricosanoic acid (C23:0), lignoceric acid (C24:0), nervonic acid (C24:1)), amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc), vitamins (vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B9 (folic acid), α-tocopherol, γ-tocopherol, δ-tocopherol and total tocopherols), glucosinolates (4-hydroxyglucobrassicin, glucobrassicin, gluconapin, progoitrin, total glucosinolates), acetylated amino acids (NAA, NAG, NAGly, NAS, and NAT), free amino acids (alanine, arginine, asparagine, aspartic acid, ethanolamine, gamma-aminobutyric acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) and other compounds (brassicasterol, campesterol, cholesterol, phytic acid, sinapine, stigmasterol, tannins-insoluble, tannins-soluble, total sterols, ß-sitosterol).

(f): Endpoints with significant differences between oilseed rape 73496 and its conventional counterpart and falling in equivalence category III-IV. Quantitative results for these endpoints are reported in Table 4.
(g): Endpoints not categorised for equivalence and with no significant differences between oilseed rape 73496 (both treated and not-treated) and its conventional counterpart: glycine, serine, NAS and sodium.
(h): Endpoints not categorised for equivalence and with significant differences between oilseed rape 73496 and its conventional counterpart. Quantitative results for these endpoints are reported in Table 4.

The GMO Panel assessed all significant differences between oilseed rape 73496 and its conventional counterpart, taking into account the potential impact on plant metabolism and the natural variability observed for the set of commercial non-GM oilseed rape reference varieties. Mean estimates for the endpoints showing significant differences between oilseed rape 73496 and its conventional counterpart and falling under category III/IV are shown in Table 4 together with endpoints with significant differences with the conventional counterpart where the equivalence test was not applied because of the lack of variation among the non-GM oilseed rape reference varieties.

Table 4: Quantitative results (estimated means and equivalence limits) for compositional endpoints in seeds that are further assessed based on the results of the statistical analysis

	Endpoint	Oilseed rape 73496		Conventional counterpart	Non-GM oilseed rape reference varieties	
		Not treated[(a)]	Treated[(a)]		Mean	Equivalence limits
Acetylated amino acids (µg/g DM)	NAA	1,860*	1,670*	3.4	2.3	–[(b)]
	NAG	27.3*	28.9*	0.83	1.10	0.59–2.05
	NAT	0.97*	0.84*	0.25	0.24	–[(b)]
Free amino acids (µg/g DM)	Glycine	0.039*	0.037*	0.048	0.046	–[(b)]
Other compounds	Crude fibre (% DM)	30.9*	32.2*	30.1	29.5	–[(b)]
	Crude fat (% DM)	43.9	45.2*	43.2	42.7	40.4–45.2
	ADF (% DM)	35.0*	36.0*	33.9	33.2	31.5–35.0
	NDF (% DM)	37.0*	37.7*	35.8	35.7	34.1–37.3
	Magnesium (% DM)	0.37*	0.38*	0.40	0.40	–[(b)]
	Pyridoxine (mg/kg DM)	4.9*	5.2	5.4	6.47	5.38–7.78
	Pantothenic acid (mg/kg DM)	6.4	5.7*	6.8	7.23	5.55–9.41
	4-Hydroxyglucobrassicin (µmol/g DM)	0.22*	0.29*	0.09	0.20	–[(b)]

For the GM oilseed rape, significantly different values are marked with an asterisk, while the outcomes of the test of equivalence are differentiated by greyscale backgrounds. Light and dark grey backgrounds correspond to equivalence category III and IV, respectively. A white background is used for outcomes other than III/IV: for equivalence category I/II (crude fat, ADF, NDF and pantothenic acid) and when the test of equivalence was not applied (in all other cases).
DM = dry matter; NAA = N-acetylaspartate; NAG = N-acetylglutamate; NAT = N-acetylthreonine.
(a): Not treated: treated only with conventional herbicides. Treated: treated with the intended herbicide glyphosate.
(b): Test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.

3.2.4. Conclusion on the comparative analysis

Taking into account the natural variability observed for the set of commercial non-GM oilseed rape reference varieties, the GMO Panel concludes that: 1) none of the differences identified in the agronomic/phenotypic endpoints tested between oilseed rape 73496 and its conventional counterpart needs further assessment; and 2) among the differences identified in seed composition between oilseed rape 73496 and its conventional counterpart, the levels of NAA, NAG and NAT, the free amino acid glycine, crude fibre, crude fat, ADF, NDF, magnesium, pyridoxine, pantothenic acid and 4-hydroxyglucobrassicin need further assessment regarding food and feed safety (see Sections 3.3.3 and 3.3.5).

3.3. Food/feed safety assessment

3.3.1. Effects of processing[18]

Oilseed rape 73496 will undergo existing production processes used for conventional oilseed rape. Several processed products from oilseed rape (refined bleached deodorised (RBD) oil, meal, protein isolates and whey fractions) were analysed to investigate the presence of different compounds, including N-acetyl amino acids.

Refined bleached deodorised (RBD) oil

Seeds from both oilseed rape 73496 and non-GM oilseed rape (comparator) were collected from eight different fields among those indicated in Section 3.2.1. Samples from each of the eight fields were combined into one bulk seed sample. RBD oil was produced from de-hulled and un-hulled seeds of oilseed rape 73496 treated and not treated with the intended herbicide.

Following OECD recommendations (OECD, 2001), the fatty acid profile of RBD oil (n = 2) was analysed together with the content in tocopherols.[19] As in seeds, the presence of acetylated derivatives of aspartic acid, glutamic acid, threonine, serine and glycine was also investigated in RBD oil; none of the five acetylated amino acids analysed was detected. Although small amounts of NAA were identified in crude oil (0.0419 μg/g), the absence of N-acetyl amino acids in RBD oil from oilseed rape 73496 was further confirmed in an additional study provided by the applicant.[20] The levels of GAT4621 protein in RBD oil were not measured based on the assumption that oil does not contain detectable amounts of protein.

Overall, the composition in fatty acids was similar in all samples of RBD oil analysed. Regarding the content in tocopherols, the RBD oil produced from oilseed rape 73496 (de-hulled and un-hulled, treated with the intended herbicide and not treated) contains higher content of total tocopherols (between 6% and 28% increase) than that produced from the non-GM comparator, in particular due to the increase of γ-tocopherol levels, the most abundant tocopherol in oilseed rape.

Meal

Following OECD recommendations (OECD, 2001), defatted toasted meal from oilseed rape 73496 and non-GM oilseed rape (comparator) (n = 2) was analysed for proximate and fibre composition, amino acids, secondary metabolites, anti-nutrients and glucosinolates.[21] The presence of GAT4621 protein was also investigated together with the presence of acetylated derivatives of aspartic acid, glutamic acid, threonine, serine and glycine.[20]

The concentration of GAT4621 protein was below the limit of quantification (0.22 ng/mg dry weight) in the samples of defatted toasted meal analysed. Higher levels of N-acetylated amino acids were quantified in the defatted toasted meal from oilseed rape 73496 seeds (de-hulled and un-hulled) as compared to the defatted toasted meal from a non-GM oilseed rape (Table 5). For the un-hulled seeds treated with the intended herbicide, the levels of quantified N-acetyl amino acids ranged between 0.4 μg/g for NAGly and 3,070 μg/g for NAA. As compared to the N-acetylated amino acid levels found in oilseed rape 73496 seeds, approximately two- to threefold higher concentration was detected in the toasted meal; this increase is probably due to the elimination of the crude fat (~ 40–45% dry weight) in the final product that also seems to indicate that processing has no effect on N-acetylated amino acid levels.

[18] Dossier Part II Scientific information/Section 3.5. Additional information: 29/8/2017, 20/6/2018.

[19] Annex 18_PHI-2011-088. Caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), heptadecadienoic acid (C17:2), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), (9,15), isomer of Linoleic acid (C18:2), linolenic acid (C18:3), γ-linolenic acid (C18:3), nonadecanoic acid (C19:0), arachidic acid (C20:0), eicosenoic acid (C20:1), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), heneicosanoic acid (C21:0), behenic acid (C22:0), erucic acid (C22:1), tricosanoic acid (C23:0), lignoceric acid (C24:0), nervonic acid (C24:1), α-tocopherol, γ-tocopherol, β-tocopherol, δ–tocopherol, total tocopherols.

[20] Additional information: 29/8/2017.

[21] Annex 18_ PHI-2011-088. Proximates and fibre content (crude protein, crude fat, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), ash, carbohydrates), amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine), glucosinolates (4-hydroxyglucobrassicin, glucobrassicin, gluconapin, progoitrin, total glucosinolates, glucoiberin, 4-Methoxyglucobrassicin, glucoraphanin, epiprogoitrin, glucoalyssin, gluconasturtiin, neoglucobrassicin, gluoconapoleiferin, glucobrassicinapin), phytic acid, sinapine, tannins-insoluble, tannins-soluble.

Table 5: Mean levels of N-acetylated amino acids (μg/g, fresh weight, n = 2) in defatted toasted meal from oilseed rape 73496 and non-GM oilseed rape

	Defatted toasted meal					
	Non-GM oilseed rape		Oilseed rape 73496			
	Un-hulled	De-hulled	Un-hulled		De-hulled De-hulled	
			Not treated[(a)]	Treated[(a)]	Not treated[(a)]	Treated[(a)]
NAA	14.5	16.8	3,190	3,070	3,690	3,480
NAG	2.37	2.57	50.8	53.7	61.9	56.8
NAT	0.759	0.571	3.23	2.90	1.25	1.41
NAGly	0.267	0.298	0.362	0.413	0.348	0.395
NAS	1.97	2.54	2.19	2.56	2.22	2.68

NAA: N-acetylaspartate; NAG: N-acetylglutamate; NAT: N-acetylthreonine; NAGly: N-acetylglycine; NAT: N-acetylserine.
(a): Not treated: treated only with conventional herbicides. Treated: treated with the intended herbicide glyphosate.

Protein isolate

Protein isolates from oilseed rape 73496 were produced simulating a new industrial method working under low temperature conditions ('cold press' processing technology) to combine high yield of oil and good meal protein quality.[20]

The presence of N-acetylated amino acids in protein isolates from oilseed rape 73496 and non-GM oilseed rape was investigated in two different studies using UPLC-MS/MS.[20,22] Table 6 reports the results of the study with the highest levels of five acetylated derivatives observed. In each study, only one protein isolate was produced and then analysed four times (technical replicates). In the protein isolates from non-GM oilseed rape, only NAA was quantified in one of the four technical replicates (mean = 0.008 μg/g).[20] In both studies, all N-acetylated amino acids were quantified in the protein isolates from oilseed rape 73496, except for NAGly, with the highest values reported for NAA (mean = 13.01 μg/g and 18.0 μg/g).

Additional information was provided to explain the relatively low levels of N-acetylated amino acids in the protein isolates as compared to those in seeds; this additional information showed that the N-acetylated amino acids are almost completely transferred to the supernatant (whey fractions) resulting from the precipitation and washing steps used in the production of the protein isolates from the pressed cake.[23] As an example, for the most abundant N-acetylated amino acid, NAA, the concentration decreased from 1,280 μg/g reported as mean concentration in the oilseed rape 73496 seeds to 18 μg/g in the protein isolate, i.e. less than 1.5% of the initial amount remained (see Table 6).

Table 6: Mean levels of N-acetylated amino acids (μg/g fresh weight, four technical replicates of one sample) in protein isolate produced from oilseed rape 73496 and a non-GM oilseed rape[(a)]

	Seeds		Protein isolate	
	Non-GM oilseed rape	Oilseed rape 73496	Non-GM oilseed rape	Oilseed rape 73496
NAA	0.169	1,280	0.008[(b)]	18.0
NAG	0.241	46.4	< LOQ[(c)]	0.611
NAT	0.251	1.54	< LOQ[(d)]	0.008[(b)]
NAGly	0.0614	0.157	< LOQ[(d)]	< LOQ[(d)]
NAS	0.405	2.19	< LOQ[(d)]	0.025

NAA: N-acetylaspartate; NAG: N-acetylglutamate; NAT: N-acetylthreonine; NAGly: N-acetylglycine; NAT: N-acetylserine.
(a): Mean levels from study PHI-2017-009. Another study (PHI-2018-024) provided additional levels of N-acetyl amino acids in different processed commodities including protein isolates; in the protein isolates, the reported mean levels (fresh weight, four technical replicates) were 13.01 μg/g for NAA, 0.4329 μg/g for NAG, 0.006753 μg/g for NAT, < LOQ (0.0125 μg/g) for NAGly and 0.007375 μg/g for NAS.

[22] Additional information: 20/6/2018.

(b): One sample above the LOQ (0.0125 μg/g fw) and three samples below the LOQ; for samples below LOQ half the value of the LOQ value was used to calculate the mean.
(c): LOQ = 0.0250 μg/g fw.
(d): LOQ = 0.0125 μg/g fw.

Whey fractions [23]

Whey fractions resulting from the production of protein isolates from conventional herbicide-treated oilseed rape 73496[22] were analysed by UPLC-MS/MS for the presence of NAA, NAG, NAGly, NAS and NAT. Three whey fractions are produced during the process, one from the protein precipitation step and the other two from washing the protein isolate pellet. N-acetylated amino acids are predominantly found in whey fraction 1 (see Table 7).

Table 7: Mean levels of N-acetylated amino acids (four technical replicates of one sample) in herbicide-treated oilseed rape 7496 seeds, in protein isolates and in the whey fractions obtained during the production of the protein isolates[23]

	Oilseed rape 73496 seeds	Protein isolate	Whey fraction 1	Whey fraction 2	Whey fraction 3
	(μg/g)		(μg/mL)		
NAA	1,323	13.01	140.8	20.40	2.919
NAG	49.57	0.4329	5.492	0.7435	0.1061
NAT	1.436	0.006753[(a)]	0.1301	0.01940	0.002391
NAGly	0.1533	< LOQ[(b)]	0.01407	0.001529	< LOQ[(c)]
NAS	1.961	0.007375[(a)]	0.1984	0.02584	0.002747

NAA: N-acetylaspartate; NAG: N-acetylglutamate; NAT: N-acetylthreonine; NAGly: N-acetylglycine; NAT: N-acetylserine.
(a): One sample above the LOQ (0.0125 μg/g fw) and three samples below the LOQ; for samples below LOQ half the value of the LOQ value was used to calculate the mean.
(b): LOQ = 0.0125 μg/g fw.
(c): LOQ = 0.001250 μg/g fw.

3.3.2. Stability of the newly expressed protein[23]

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

Effects of temperature and pH on the newly expressed protein

The effects of temperature and pH on the GAT4621 protein have been previously evaluated by the GMO Panel (EFSA GMO Panel, 2013). The GAT4621 lost most of its activity at temperatures greater than 53°C.

In vitro protein degradation by proteolytic enzymes

The resistance to degradation by pepsin of a microbial GAT4621 protein in solutions at pH ~ 1.2 has been previously assessed by the GMO Panel (EFSA GMO Panel, 2013). As described, the GAT4621 protein was degraded within the first 30 seconds of incubation, while less intensely staining bands corresponding to low-molecular weight fragments (≤ 3 kDa) were still visible throughout the incubation period.

[23] Dossier Part II Scientific information/Sections 4.2 and 5.1.

3.3.3. Toxicology

3.3.3.1. Testing of the newly expressed protein[24]

Oilseed rape 73496 expresses the new protein GAT4621, a glyphosate acetyltransferase conferring tolerance to the herbicidal active substance glyphosate. The GMO Panel has previously assessed this protein (EFSA GMO Panel, 2013), but was unable to conclude on its safety due to the lack of an adequate 28-day toxicity study. In the context of this application, the GMO Panel assessed the safety of the GAT4621 protein considering bioinformatic analyses (Section 3.1.2), protein characterisation (Section 3.1.3), in vitro studies (Section 3.3.2) and a new 28-day toxicity study spontaneously provided by the applicant.

Bioinformatics

Bioinformatic analysis of the amino acid sequence of the GAT4621 protein revealed no significant similarities to known toxins (Section 3.1.2).

28-day repeated dose toxicity study in the rat

The new 28-day repeated-dose toxicity study provided in the application was conducted in accordance with OECD TG 407 (2008) and the principles of Good Laboratory Practice (GLP).

Five groups of Crl:CD1(ICR) mice (10 per sex per group, individually housed, approximately 9-week old at study start dosing) were given 1) a standard diet (control group); 2) a diet containing the GAT4621 protein at the target dose of 100, 300 or 1,000 mg/kg body weight (bw) per day (low, intermediate and high dose test diet groups); and 3) a diet containing bovine serum albumin (BSA) protein at the target dose of 1,000 mg/kg bw per day (BSA control group). These groups are hereafter defined as main study groups. Ten additional animals/sex per group were dedicated for coagulation analysis and are mentioned hereafter as satellite study groups. The test and BSA diets were prepared by mixing to a standard rodent diet the test substance at 0.5, 1.5 or 5 g/kg diet, or the BSA at 5 g/kg diet. The GMO Panel noted that at the start of dosing, the age of the animals and the variation in body weights among animals were slightly outside the OECD TG 407 (2008) requirements. These were considered minor deviations with no impact on the study results.

The GAT4621 protein used in this study was produced by a recombinant system (*E. coli*, lot PCF-0041) equivalent to the protein newly expressed in oilseed rape 7496 (Section 3.1.3) since it was demonstrated to have the expected molecular weight and N-terminal sequence and a 98% coverage of the expected protein sequence at MALDI–MS.[25] The test substance used in this study contained 0.82 mg GAT4621/mg lyophilised powder. The test substance was stored frozen (–80°C) and considered stable for long storage. Levels of the GAT4621 or BSA protein were measured by ELISA in the diets at the time of mixing (Day 0) to assess their concentration and homogeneity; on Day 1, 6, 28 and 46 (high dose test diet only) to evaluate their stability. During the treatment period, all animals were given approximately 14 g/day (7 g twice a day/mouse) of control or test diets. Water was provided ad libitum.

In-life procedures and observations and terminal procedures were conducted in accordance with OECD TG 407 (2008). Ophthalmoscopy examinations, functional observational batteries (FOBs) and motor activities were recorded on main study groups only. Haematological and clinical chemistry analyses were performed on main study groups, while coagulation analysis was performed on satellite groups. Detailed necropsy examination, organ weight and histopathological examination (controls, BSA controls and high dose group) were conducted on main study groups only.

The results of the diet analyses revealed that the test diets met the expected GAT4621 concentrations at the time of diet formulation,[26] that these were homogenous and that all test diets were stable up to 28 days in terms of GAT4621 content.

In-life data endpoints, with the exception of total and ambulatory motor activity counts, were analysed by sex, using a two-sided analysis of variance (ANOVA) model; in case a statistically significant does effect was identified, pairwise comparisons (Dunnett's test) were done between the test groups and the control group and the BSA group, and between the BSA group and the control

[24] Dossier Part II Scientific information/Section A. 4.2. Spontaneous information 29/8/2017 and additional information 20/6/2018, 18/12/2018.
[25] 17 kDa by Western blot and 16.5 kDa by MALDI-MS.
[26] An anomalous value for concentration was noted on Day 28 in the high-dose diet but not confirmed on Day 46, therefore considered not relevant. Achieved concentrations for all the three diets were 84% of the nominal value.

group. Total and ambulatory motor activity counts were recorded prior to the initiation of the study and near the end of the feeding period for control, BSA and test diet groups and were analysed by sex and session, using a repeated measures ANOVA model (factors: treatment, time interval and time-by-treatment interaction), followed by pairwise group comparisons across the pooled time intervals if the main effect of treatment was significant; if the time-by-treatment interaction was significant, the pairwise comparisons were also conducted for each individual time interval. Categorical FOBs data were analysed using Fisher's test.

Based on feed consumption, the average GAT4621 consumption was 59.4, 183.6 and 595.5 mg/kg/bw per day in males and 75.1, 214.5 and 740.1 mg/kg/bw per day in females (low-, intermediate- and high-dose groups, respectively).[27] Animals from the BSA control group consumed 716.4 (males) and 832.7 (females) mg BSA/kg bw per day (based on nominal value).

There were no deaths. Isolated clinical findings observed in females from the BSA and low dose test group were considered incidental. The GMO Panel assessed the statistically significant findings observed in the treated groups and concluded that these are not adverse effects of the treatment with GAT4621 protein (see Appendix A, Table A.1). No gross pathological findings related to the treatment with GAT4621 protein were seen at necropsy. At microscopic examinations of selected organs and tissues, an increased incidence of mononuclear cell infiltrate was noted in the kidneys of males given the high dose test diet, as compared to controls. This finding was described as minimal; it is compatible with background microscopic findings in mice of this strain and age and considered not an adverse effect related to treatment with GAT4621 protein. No other relevant differences in the incidences and severity of the histopathological findings were noted between high dose test dose group and those given the control diets.

The GMO Panel concluded that no adverse effects related to the treatment were observed in mice exposed by diet to 595.5 (males) and 740.1 (females) mg GAT4621/kg bw per day for 28 days.

3.3.3.2. Assessment of altered levels of endogenous compounds – N-acetylated amino acids[28]

The GMO Panel assessed the altered levels of N-acetylated amino acids (NAA, NAG and NAT) observed in oilseed rape 73496 as compared to its conventional counterpart (Section 3.2.4) with regard to their relevance for food and feed safety taking into account available toxicological studies, other relevant information on the biological role and metabolism of N-acetylated amino acids and dietary exposure assessment.

Toxicological studies on N-acetyl amino acids

Rodent studies

The applicant provided toxicological studies on NAA, NAG and NAT, which were already assessed by the GMO Panel in the context of previous applications (EFSA GMO Panel, 2011c, 2013). A summary of these studies and the outcome of the GMO Panel assessment are presented in Table 8.

Although the applicant set the no observed adverse effect level (NOAEL) for NAA at 500 mg/kg bw per day (based on the 90-day repeated dose toxicity and in the two-generation reproductive toxicity dietary studies in rats), the GMO Panel had previously concluded that it is appropriate to use the intermediate dose level as the reference value for risk assessment considerations (see Table 8 and EFSA GMO Panel, 2011c, 2013). In particular, the most conservative NOAEL was chosen for risk characterisation (229.5 mg/kg per bw per day from 90-day repeated dose toxicity dietary study in male rats, see Table 8).

[27] Dietary exposures include a correction for the purity of the test item (84%).
[28] Dossier Part II Scientific information/Section A. 4.4. Additional information 28/4/2016, 29/4/2016, 28/8/2017 and 29/8/2017.

Table 8: Toxicological studies on N-acetylated amino acids provided by the applicant and outcome of the previous assessment by the GMO Panel

Compound	Study	Target dose	Outcome of previous GMO Panel assessments
NAA	Rat acute toxicity	2,000; 5,000[(a)]	No adverse effects at 2,000[(a)]; toxicity at 5,000[(a)]
	Rat 28-day repeated dose toxicity (dietary)	10/100, 100/500, 1,000[(b)]	NOAEL[(b)]: 852.3 (males); 890.1 (females)
	Rat 90-day repeated dose toxicity (dietary)	100, 250, 500[(b)]	NOAEL[(b),(c)]: 229.5 (males); 253.2 (females)
	Rat two-generation reproductive toxicity (dietary)	100, 250, 500[(b)]	NOAEL[(b),(c)]: 245.7(males F1), 269.1(females F1); 237.2 (males F2), 500 (females F2)
	Bacterial reverse mutation test (Ames test)	333, 667, 1,000, 3,333, 5,000[(d)]	Negative
	Mouse Bone Marrow Erythrocyte Micronucleus Test	500, 1,000, 2,000[(a)]	Negative
NAG	Rat acute toxicity	2,000[(a)]	No adverse effects at 2,000[(a)]
	Rat 28-day repeated dose toxicity dietary	100, 500, 1,000[(b)]	NOAEL[(b)]: 914.2 (males); 1,006.6 (females)
	Bacterial reverse mutation test (Ames test)	333, 667, 1,000, 3,333, 5,000[(d)]	Negative
	Mouse Bone Marrow Erythrocyte Micronucleus Test	333, 1,000, 2,000[(a)]	Negative
NAT	Rat acute toxicity	2,000[(a)]	No adverse effects at 2,000[(a)]
	Rat 28-day repeated dose toxicity dietary	100, 500, 1,000[(b)]	NOAEL[(b)]: 848.5 (males); 913.6 (females)
	Bacterial reverse mutation test (Ames test)	333, 667, 1,000, 3,333, 5,000[(d)]	Negative
	Mouse Bone Marrow Erythrocyte Micronucleus Test	500, 1,000, 2,000[(a)]	Negative

(a): mg/kg body weight.
(b): mg/kg body weight per day.
(c): The GMO Panel has previously concluded that it is appropriate to use the intermediate dose as the reference value for risk assessment consideration (EFSA GMO Panel, 2011a–c, 2013).
(d): µg/plate.

42-day feeding study in broiler on NAA[29]

To further support the safety evaluation of dietary oilseed rape 73496 in chickens, the applicant provided a 42-day feeding study on chicken for fattening with graded supplementation levels of pure NAA in the standard diets, to investigate growth performance and toxicological endpoints. Necropsy with major organ weights, haematology and routine clinical blood chemistry, histopathology of the salivary glands[30] and measurement of the content of NAA and aspartic acid in liver and muscle tissues were assessed. A total of 240 male chickens for fattening (day-old Ross 708) were randomly allocated to five dietary treatment groups with 48 chicks per treatment (four pens per treatment, 12 birds per pen) and fed standard diets[31] alone (carrier control group) or supplemented with NAA at three different levels (test groups), or with L-aspartic acid (Asp comparative control group) at 100 mg/kg bw per day (Asp comparative control group). Test diets were formulated, manufactured and characterised to provide target exposure to NAA of 25, 50 and 100 mg/kg bw per day, corresponding to an approximate supplemental 10%, 20% and 40% incorporation of oilseed rape 73496 meal in diets. Diets and water were offered ad libitum.

[29] Additional information 29/4/2016; 29/8/2017.
[30] Acinar cells hypertrophy of the salivary glands in both male and female rats orally exposed to 500 mg NAA/kg bw was reported in a 90-day rat study (see Table 8).
[31] Starter (0–21 days), grower (22–35 days) and finisher (36–42 days) diets formulated to meet the nutrient requirements of a commercial broiler in accordance with the National Research Council's Nutrient requirements for Poultry (NRC, 1994).

Statistical analysis on mortality, performance and toxicology endpoints was conducted. Differences were considered significant at a p-value of ≤ 0.05 and adjustment (FDR) was made across all endpoints within each pairwise comparison between diet groups. For mortality data, Fisher's exact test was conducted. For all continuous endpoints, if < 50% of non-missing data values were at a uniform value, a mixed model analysis was applied. For endpoints that were measured on a per pen basis, statistical modelling was conducted on a response variable at the pen level. For endpoints that were measured on an individual broiler basis, statistical modelling was conducted on a response variable at the individual broiler level. For mortality data, Fisher's exact test was conducted.

The target exposures to NAA were met, and even exceeded, during the entire phases of the study. The mean actual exposures were at least 28.4, 62.5 and 121.6 mg/kg bw per day, respectively, in the low, mean and high-dose test groups for the entire duration of the study, corresponding to an approximate supplemental 11%, 25%, 48% incorporation of 73496 oilseed rape meal in diets.

Overall mortality was low (4%) with no significant difference between the groups, and no adverse clinical signs were reported throughout the study. No statistically significant difference was seen in body weights and body weight gains, feed consumption and feed conversion, absolute and relative kidney and liver weights (pre-chilled), dressed carcass weights (post-chilled), breast, thigh, wing, leg and abdominal fat weights, when the NAA test groups and Asp-positive control group were compared to the negative control group. Moreover, there were no statistically significant differences in absolute and relative selected organ weights, or test substance-related effects on haematology, coagulation or clinical chemistry examined parameters when the NAA test groups, and Asp-positive control group were compared to the negative control group.

The microscopic examination of salivary glands showed the absence or attenuation of secretory units of one or more lobules in all groups, with a slightly higher incidence and severity in the lingual and sublingual glands of animals fed diets with NAA at the highest dose (test group). The lack of association of these findings with NAA consumption was confirmed by the outcome of an expert scientific opinion provided by the applicant,[32] based on the comparison of the histology of salivary glands of broilers from the present study with that of strain, age and gender-matched control broilers from other studies, fed either similar diets or standard commercial poultry diets (using a series of parasagittal step sections of the lingual salivary glands); all animals were sourced from the same breeder and housed under similar environmental conditions at the same test facility.

Variability with respect to the severity score of 'decreased glands' was observed among animals within the same treatment groups, and there were no differences in nuclear cytology or evidence of significant cellular pathology or other morphologic changes (e.g. inflammation, degeneration, necrosis) in any of the test animals that would suggest an adverse effect. Furthermore, the composition and cellularity of the salivary glands were highly dependent on the plane of section. Therefore, although subtle quantitative histological intergroup differences were observed in the salivary tissue of broiler chickens, they were considered a consequence of the normal variability of salivary gland histology and the variation in the plane of section of a dispersed gland, and no pathological alterations were observed to indicate a treatment-related effect of NAA on the salivary glands of broiler chickens under the conditions of this study.

There were no statistically significant differences in NAA or Asp concentrations in the breast or liver tissues of fasted or non-fasted broilers when the NAA test groups or the Asp comparative control group were compared to the carrier control group.

Based on the results of this study, the EFSA GMO Panel concludes that administration of diets containing NAA for 42 consecutive days at an average overall dose level of 121.6 mg/kg bw per day (highest dose evaluated) to broilers, did not cause adverse effects on mortality, growth performance or clinical and anatomic pathology variables and no effects on NAA and Asp tissue concentrations of male Ross 708 broiler chickens. The mean actual dose of 121.6 NAA mg/kg bw per day corresponds to an approximate supplemental 48% incorporation of 73496 oilseed rape meal in diets that exceed the standard incorporation of conventional oilseed rape into commercial animal's diets.

Therefore, the GMO Panel considers the use of oilseed rape 73496 as feed material safe for the broiler and that the incorporation of this GM oilseed rape into animal's diet has no limitations other than those of conventional oilseed rape.

[32] Additional information 29/8/2017.

Other information on N-acetylated amino acids.[33]

NAA and NAG are produced by the mammalian metabolism and are normal constituents of many foods and feedstuff (Hession et al., 2008), with NAA being detected in human bio-fluids as reported in the Human Metabolome (https://hmdb.ca/) and Chemical Entities of Biological Interest (https://www.ebi.ac.uk/chebi/) databases.

N-acetylation and de-acetylation of cellular proteins are widespread processes with a regulatory function in metabolism (Perrier et al., 2005; Smith and Denu, 2007; Hwang et al., 2010).

A number of both specific and unspecific N-acetyltransferases acetylate free amino acids, amines and drugs. The synthesis of NAA is known to occur in brain neurons and has important roles in the function of the central nervous system, following release into the extracellular fluid and uptake by glial cells, where it is hydrolysed to aspartic and acetic acid (Baslow, 2003; Baslow, 2010).

Hydrolase enzymes from the aminoacylase family catalyse the hydrolysis of acylated L-amino acids to their constituent L-amino acids and an acyl group. Aminoacylases genes (ACY1 and ASPA) are nearly ubiquitously present in organs and tissues, with sequences moderately to highly conserved across animal species (Yates et al., 2020). Acylase I and acylase II enzymes catalyse the stereospecific hydrolysis of N-acetylated amino acids, including NAA, and are identified in multiple tissues from various species including intestine, liver and kidneys, presumably mediating the catabolism of ingested N-acetylated amino acids under normal dietary conditions (Birnbaum et al., 1952; Birnbaum, 1955; Nadler and Cooper, 1972; D'Adamo et al., 1973; Endo, 1978; Endo, 1980; Daabees et al., 1984; Giardina et al., 1997; Giardina et al., 1999; Lindner et al., 2000; Arnaud et al., 2004; Hershfield et al., 2006; Surendran et al., 2006; Mersmann et al., 2011; Luna et al., 2013).

Deacetylation of NAA was shown to be rapid in studies in mice using radiolabelled NAA and L-aspartic acid; after intraperitoneal injection, both substances were metabolised at a similar rate (as determined by measurement of expired radioactive CO2) indicating a rapid hydrolysis of the N-acetyl group (Berlinguet and Laliberté, 1966). Studies in premature infants, rats, dogs and pigs with enterally or parenterally administered N-acetylated amino acids (cysteine, tryptophan, tyrosine, methionine, threonine, glutamine) have shown that the nutritional value of the N-acetylated amino acids was comparable to that of free amino acids, also suggesting an efficient de-acetylation (Boggs, 1978; Neuhäuser-Berthold et al., 1988; Gouttebel et al., 1992; van Goudoever et al., 1994; Arnaud et al., 2004; López-Pedrosa et al., 2007).

Dietary exposure assessment to N-acetyl amino acids

Human dietary exposure to N-acetyl amino acids

Humans are habitually exposed to N-acetyl amino acids since they are natural constituents of different foods; the presence of NAA and NAG has been described and quantified in a broad range of foods including meat, fish, eggs, brewed coffee, vegetables and fruits (Hession et al., 2008). Likewise, other N-acetyl amino acids, among them NAS and NAT, are described as frequent components of dietary proteins although concentration levels have not been reported (Persson et al., 1985; Van de Mortel et al., 2010a; Van de Mortel et al., 2010b). Table 9 shows a selection of foods with quantified levels of NAA and NAG; it can be seen that in several cases, the levels are similar or higher than those measured in the protein isolates from oilseed rape 73496.

Table 9: Selection of different foods with reported levels of NAA and NAG[(a),(b)] (complete food list in Hession et al., 2008), and levels of NAA and NAG as reported in seeds and protein isolates from oilseed rape 73496

	NAA (µg/g)	NAG (µg/g)
Soybean	0.3–0.7[(c)]	0.7–1.2
Stout beer	0.14	0.21
Brewed coffee	3.8	0.3
Brewed espresso coffee	15.4	1.8
Cocoa powder	26.8	62.2
Dark chocolate	4.5	10.2
Broccoli	0.09	0.8

[33] Dossier Part II Scientific information/Section A4.4. Additional information 10/9/2020 and 10/11/2020.

	NAA (μg/g)	NAG (μg/g)
Spinach	0.04	1.8
Whole egg	1.5	0.05
Ground chicken	4.7	0.07
Ground turkey	7.4	0.09
Canned sardines	10.2	0.2
Seeds from oilseed rape 73496(b)	1,670(c)	28.9
Protein isolate from oilseed rape 73496	13–18	0.43–0.61

NAA: N-acetyl aspartate; NAG: N-acetylglutamate.
(a): Levels of NAA and NAG are the result of two determinations.
(b): Seeds treated with the intended herbicide.
(c): Result expressed in dry weight.

Dietary intake of NAG and NAT

Even though the levels of NAG and NAT in oilseed rape 73496 were significantly higher than those present in the conventional counterpart, no dietary intake estimations were considered needed for these N-acetyl amino acids. This decision was based on 1) the relatively low levels of these N-acetyl amino acids as compared to those of NAA, 2) their very low levels or absence in oilseed processed commodities, e.g. protein isolates and RBD oil, 3) their presence in conventional foods, in particular NAG, at similar or higher levels than those present in the seeds, 4) toxicological information (see Table 8).

Dietary intake of NAA

Based on the levels of NAA described for the different conventional foods (see Table 9), the baseline intake of NAA was estimated across different ages classes in the European population using individual consumption data from the EFSA Comprehensive European Food Consumption database (EFSA consumption database).[34] In the young population (including adolescents) and in the adult population, the maximum dietary intake estimates (95th percentile) were 47.3 and 99.0 μg/kg bw per day, respectively (see Appendix B).

As today, oil is almost the only food commodity derived from oilseed rape regularly consumed by the European population,[35] although other food commodities derived from oilseed rape have been approved as novel food in recent years, e.g. protein isolates (EFSA NDA Panel, 2013) and rapeseed powder (EFSA NDA Panel, 2020). Different preparations of protein isolates from oilseed rape meal are found under commercial names (Puratein®, Supertein™, etc.). However, protein isolates from oilseed rape are literally absent in the European market as verified in Mintel's Global New Products Database.[36] This was further confirmed by the absence of consumption data for protein isolates/meat imitates from oilseed rape in the EFSA consumption database. The protein meal resulting from oil extraction is currently almost exclusively used as animal feed since the food industry is still often encountering diverse challenges, e.g. undesirable flavour/colour, functional properties of the proteins, etc., when using the protein fraction (Wanasundara et al., 2016; Chmielewska et al., 2020; Fetzer et al., 2020).

Different dietary intake scenarios for NAA were conducted taking into account the food commodities from oilseed rape available today in the market: RBD oil, protein isolates and oilseed rape powder:

1) N-acetyl amino acids are not present in RBD oil from oilseed rape 73496 (Section 3.3.1); therefore, consumption of RBD oil from the GM oilseed rape is not expected to contribute to the dietary intake of N-acetyl amino acids.
2) A conservative intake scenario was conducted assuming that protein isolates from oilseed rape 73496 could be used as protein supplements by the adult population. Using the highest concentration of NAA reported in protein isolates (18 μg/g, see Section 3.3.1.) and a daily consumption of 30 grams of protein isolate, this would result in an additional intake

[34] Accessed on January 2021. Consumption data from the UK were included in the EFSA Comprehensive European Food Consumption Database when the UK was a member of the European Union.
[35] From double low cultivars, i.e. varieties with a low content of erucic acid (< 2% expressed as percentage of total fatty acids) and reduced content of glucosinolates (< 25 mmol/kg at a moisture content of 9%) under Regulation (EC) No 2316/1999.
[36] The Mintel's GNPD is an online database which monitors new introductions of packaged goods in the market worldwide. It contains information of over 2.5 million food and beverage products of which more than 1,000,000 are or have been available on the European food market. Mintel started covering EU's food markets in 1996, currently having 25 out of its 28 member countries and Norway presented in the Mintel GNPD.

of ~ 8 μg/kg bw per day, considering a default body weight of 70 kg in adults (EFSA Scientific Committee, 2012). This additional intake based on the consumption of protein isolates from oilseed rape 73496 represents less than 10% of the maximum baseline dietary intake of NAA observed in adults.

3) No data were available on the presence of NAA in the recently approved novel food oilseed rape powder. In a worst-case scenario (overly conservative), it was assumed that oilseed rape powder might have a similar concentration of NAA to that in the seeds (~ 1,500 μg/g), i.e. no losses of NAA occur during the production of the oilseed rape powder. A dietary intake scenario was conducted considering the described proposed uses of oilseed rape powder in food products (EFSA NDA Panel, 2020), the concentrations of NAA analysed in the conventional foods (see Table 9) and the consumption of protein isolates as protein supplements. The EFSA consumption database was used as a source of individual consumption data. The maximum dietary intake estimates of NAA (95th percentile) were 992.9 and 444.8 μg/kg bw per day in the young population (including adolescents) and in the adult population, respectively (see Appendix C).

When using this dietary intake scenario in the risk assessment of NAA, one should take into account the overly conservative nature of the intake estimations. This mainly refers to the assumption that all NAA present in the seeds will also be present in the oilseed rape powder, with no losses during a production process that includes different washing and extraction steps (EFSA NDA Panel, 2020).

Animal dietary exposure to N-acetyl amino acids

Dietary exposure to N-Acetylaspartate (NAA), N-Acetylglutamate (NAG) and N-Acetylthreonine (NAT) in oilseed rape 73496 was estimated by the applicant across different animal species, as summarised below (for details, refer to Appendix D) following conservative approaches. Estimations of exposure to NAA, NAG and NAT are based on the assumption that the totality of oilseed rape products fed to animals is derived from oilseed rape 73496 (100% replacement scenario). Moreover, in the absence of a feed consumption database for animals (EFSA, 2019), estimations are based on default values for theoretical maximal inclusion rates of feed materials in diets, selected from the literature.

Dietary exposure to NAA, NAG and NAT in poultry, swine, cattle and sheep was estimated based on the consumption of oilseed rape 73496 meal.[37]

1) Background exposures to NAA in poultry, swine, cattle, sheep, salmon, dog and cat were estimated based on the consumption of simple diets (not nutritionally balanced) consisting of the combination of two conventional feed materials (i.e. maize grains and distillers grain with solubles, forage/silage from maize, alfalfa and grass, soybean, oilseed rape and fish meal) with known concentration of NAA; a comparison was also made with the exposures based on the consumption of simple diets containing oilseed rape meal 73496 as one of the combined feed materials in order to determine whether a safe comparative consumption could be established.[38] Among the different outcomes, the most conservative exposures were selected for further risk characterisation (see below), as reported in Table 10:

Table 10: Conservative Dietary Exposures to NAA

Animal species	**Simple diet: oilseed rape 73496 meal + conventional feed material**	
Daily feed intake (kg DM animal/kg body weight)	**Dietary exposure mg/kg bw**	**IR%**
Cattle		
Cattle for fattening (8/400)	14.5	Oilseed rape 73496 meal (20%) + grass silage (50%)
Swine		
Pig for fattening (2.20/60)	25.9	Oilseed rape 73496 meal (20%) + maize DDGS (75%)
Comp. animals		
Cat (0.06/3)	14.0	Oilseed rape 73496 meal (20%) + maize grain (25%)

[37] Dossier Part II Scientific information/Section B.
[38] Additional information: 6/12/2017.

2) Dietary exposure to NAA and NAG in calf was estimated based on the consumption of milk replacer, making the conservative assumption that 100% of the protein in milk replacer would be from oilseed rape protein isolates.[20]
3) Simulation of dietary exposure to NAA, NAG and NAT in ruminants (i.e. cattle for fattening, dairy cow and sheep/goat) was estimated based on the consumption of oilseed rape solubles (whey), alone or combined with oilseed rape meal. As oilseed rape protein isolate production is not a common industrial practice, soy protein isolate and the corresponding whey fraction productions were examined as a surrogate; theoretical inclusion rates for oilseed rape were derived from the literature, considering the reporting of adverse nutritional impact of soy solubles at experimental inclusion rates above 10% in diets.[22]

The GMO Panel notes that the incorporation in the diet of oilseed rape 73496 meal in substitution of conventional oilseed rape meal determines an increased exposure to NAA, NAG and NAT in all the animal species investigated and in all the proposed scenarios. Feed products other than meal for incorporation into the diet would include oilseed rape protein isolates and its by-product whey. However, these products, at the current status of knowledge, do not represent common ingredients currently used in animal rations and diets. To date, there is very little or no consumption of oilseed rape protein isolates as feed, due to relatively low protein yield and high costs of production compared to available alternatives (Campbell et al., 2016); however, their future use as feed cannot be excluded (e.g. as milk replacer for calves, but also in piglets). Whey from protein isolate production is highly diluted and most often considered a waste stream of the process; soluble products from whey could be produced and used as feed ingredient; however, this process requires substantial energy and therefore is not economical.[39] Therefore, the products were not considered for risk characterisation in the context of this application.

The GMO Panel notes that these human and animal intake estimates of N-acetyl amino acids only considers the food and feed commodities from oilseed rape that can currently be used in the European market, making use of consumption/feeding data of food and feed assumed to be replaced by food and feed from oilseed rape 73496. In the future, the intakes to N-acetyl amino acids might vary due to changes in consumption/feeding patterns and, above all, by the introduction in the market of new products from oilseed rape (e.g. seeds, whey fraction, etc.).

RISK CHARACTERISATION

Human risk characterisation

Human dietary exposure to NAA was estimated considering the presence of NAA in conventional foods and the processed foods from oilseed rape that can currently be in the European market (RBD oil, protein isolates and oilseed rape powder). The maximum dietary exposure to NAA combining the consumption of conventional food and considering the processed foods from oilseed rape 73496 was estimated in the age class 'Other children' (992.9 μg/kg bw day, 95th percentile dietary intake). In the adult population the maximum 95th percentile dietary intake estimate was 444.8 μg/kg bw day (age class 'Adults'). A NOAEL of 229.5 mg/kg bw day was derived for salivary glands hypertrophy in male rats in a 90-day repeated dose toxicity study with NAA (see Table 8, Section 3.3.3). The highest dietary exposure estimates in the age classes 'Other children' and 'Adults' provide Margin of Exposures (MoE) to the NOAEL of ca. 225 and ca. 500, respectively. These MoE are considered acceptable as they exceed the default 100 fold factor applied when extrapolating from animal data to humans and noting the extent of the information currently available on NAA (see Section 3.3.3.2). The GMO Panel concludes that the human dietary exposure to NAA as estimated from the combined consumption of conventional food and considering the processed food from oilseed rape 73496 is unlikely to present a risk to health in humans.

Animal risk characterisation

The levels of NAG and NAT were significantly higher in seeds from oilseed rape 73496 than in those the conventional counterpart (see Section 3.2.3) and noted to achieve even higher concentration in

[39] This waste stream is typically disposed of as municipal waste. In a limited number of cases where this is not possible due to both municipal restrictions and the inability to treat waste on site, a soy soluble product can be produced which could be used in animal diets (personal communication from Solae LLC, St Louis, Missouri, USA). The soy soluble product is produced by concentrating the soy whey via drying. Consumption of soy solubles produced from whey is not currently authorised as a feed ingredient in the EU, and no pending EU applications for either soy or oilseed rape solubles produced from whey fractions are known).

the defatted toasted meal from the GM oilseed rape, as compared to the non-GM one (Section 3.3.1). Based on the 28-day toxicity studies on NAG and NAT (Table 8) and the dietary intake estimates provided by the applicant (see *Animal dietary exposure to N-acetyl amino acids* above and Appendix D), the GMO Panel notes that the margin of exposure for NAG and NAT is at least 1,000 fold for all animal species. The GMO Panel concludes therefore that exposures to NAG and NAT via animal feed from oilseed rape 73496, as described in this application, pose no concerns to animal health.

The initial estimated exposures provided by the applicant indicates that NAA in animals via feed result in low MoEs (less than 100) to the most conservative NOAEL (229.5 in male rats from the 90-day toxicity study, see Table 8) for most species. The GMO Panel concluded that a potential concern on animal health could not be excluded and requested additional information from the applicant to address exposures to NAA from feed from oilseed rape 73496.

A 42-day study on NAA in the broiler to provide target exposure to NAA of 25, 50 and 100 mg/kg bw per day, corresponding to an approximate supplemental 10%, 20% and 40% incorporation of oilseed rape 73496 meal into commercial poultry's diets confirmed that there are no limitations in the use of oilseed rape 73496 as feed material at the currently used inclusion rate for poultry (around 20%). The GMO Panel did not consider it necessary to elaborate further the risk assessment on fish (no salivary glands are present in fish).

The applicant provided further exposure estimates in animal species other than poultry (see Appendix D), as well as generic information on the normal occurrence and metabolism of NAA in animals and on the function and physiology of salivary glands. Moreover, the applicant provided studies on the toxicokinetics of NAA in rats, goats and pigs, together with a proposal to base the assessment on a Compound Specific Assessment Factor (CSAF) approach.

Description of the CSAF based approach proposed, information on the toxicokinetics provided to support the exercise and the actual CSAF based assessment of NAA in feed from oilseed rape 73496 are summarised below. Additional details are given in Appendix E.

CSAF based approach (see also Appendix E)

The default assessment factor used when deriving an acceptable human exposure level from the no-observed adverse effect levels (NOAEL) in animal studies is 100. This factor accounts for differences in sensitivity between the experimental animal and the average human and for variations in sensitivity within the human population to protect sensitive sub-groups. This factor of 100 has also been utilised in the assessment of feed additives as an indicator of the expected margin between the NOAELs in laboratory animal studies and intakes in farm and domestic animals (MoE) (EFSA FEEDAP Panel, 2017a,b).

Where specific data are available, it is possible to derive Chemical Specific Adjustment Factors (also known as Chemical Specific Assessment Factors and Data Derived Evaluation Factors) to replace the default 100-fold assessment factor. The overall CSAF can be lower or higher than the default of 100. The concept was developed by comparing the findings seen in humans and experimental animals exposed to pharmaceuticals and was described in detail by the World Health Organisation (IPCS 2005). The CSAF approach splits the default factor of 100 into four separate factors addressing differences in toxicokinetics (how a compound is absorbed, metabolised, distributed and excreted) and toxicodynamics (how a specific level of exposure affects the target tissue). Each individual factor can be modified, if suitable data are available, and then combined to give the overall CSAF. CSAFs have been referenced by EFSA in the Scientific Opinions on Default values (EFSA Scientific Committee, 2012) and Uncertainty Analysis (EFSA Scientific Committee, 2018). A CSAF based approach has been used by EFSA in the re-evaluation of phosphates (EFSA FAF Panel, 2019).

Information on toxicokinetics submitted to EFSA (see also Appendix E)

To address the low margins between estimated exposures and the NOAEL of 229.4 mg/kg bw per day (see Section 3.3.3.2) and to support a CSAF approach, the applicant performed studies in goats (representative ruminant), pigs (representative monogastric animal) and rats (the species used in the toxicity studies) to investigate the toxicokinetics of NAA. The results have been used to develop a CSAF based assessment to determine if exposures to NAA from animal feed derived from oilseed rape 73496 present an acceptable risk. Details of the studies are presented in Appendix C.

The key results from the studies relevant to the CSAF approach are described below:

- NAA is a normal component of the blood plasma, present at similar levels in all three tested species;

- NAA is rapidly metabolised and there is no accumulation over 14 doses at 25 mg/kg bw per day in all species;
- in the goat, plasma levels of NAA are much lower than in the rat at the dose of 25 mg/kg bw per day;
- the plasma levels of NAA are similar in the pig and rat at a dose of 25 mg/kg bw per day;
- there were no notable differences between results in males and females.

CSAF based assessment of NAA in 73496 oilseed rape (see also Appendix E)

When performing a CSAF based assessment it is valuable if the mode of action underlying the adverse effect is well understood. The mode of action underlying the salivary gland hypertrophy seen in some rats exposed to NAA has not been investigated in detail by the applicant but generic information is available. This was used by the GMO Panel to develop on two scenarios (direct or systemic mode of action) detailed below.

Direct Mode of Action

Given the physiological mechanisms controlling the production of saliva and mechanisms producing hypertrophy the GMO Panel considered that salivary glands hypertrophy in rats exposed to NAA was likely due to a direct mode of action in the mouth. Data supporting this is presented in Appendix E.

On the basis that NAA acts on the salivary glands via a direct action in the mouth, the salivary glands findings would be essentially independent of absorption, distribution, metabolism and excretion. Therefore, both toxicokinetic factors can be removed leaving an overall **CSAF of 8** based on the revised toxicodynamic factors (see Table 11). This would apply when extrapolating from rats to any other species and life-stages.

When comparing exposures on a body weight basis the MoE between the highest predicted exposure and the chosen NOAEL (229.5 mg/kg bw per day see Table 8) is > 8 in all cases (Table 12) and therefore considered acceptable.

For some local effects, it is the concentration of the chemical in the feed or vehicle that is critical, rather than the dose in mg/kg bw per day. An additional assessment was therefore performed comparing the concentration of NAA in the diet in the 90-day rat study on NAA with the estimated NAA concentration in the feed; a MoE of 8 or more was identified, indicating an acceptable risk (Table 12).

The GMO Panel considers that based on the more likely mode of action of NAA on salivary glands, which involves direct action in the mouth, current and predicted exposures to NAA in animal feed do not pose a concern to animal health.

Systemic Mode of Action

As the mode of action of NAA on salivary glands has not been investigated in detail, the GMO Panel also performed a supplementary CSAF based exercise in the unlikely case of systemic mode of action, to determine if any significant risks might be missed by adopting the more likely direct mode of action approach. Using the data from the submitted toxicokinetic studies and noting that in a rat two-generation study on NAA there was no evidence of any sensitive life-stages (see Table 8) and that expression data on the main enzymes metabolising N-acetylated amino acids are widely conserved across species (see Other information on N-acetylated amino acids in Section 3.3.3.2), the CSAFs were adjusted by modifying the toxicokinetic factors, based on the available toxicokinetic data, but retaining the default toxicodynamic factors, as detailed in Table 11 below.

Table 11: Outline of the derivation of the overall CSAFs based on the toxicokinetic data and potential modes of action behind the effects of NAA on salivary glands

	Rat to standard species*		Standard species to different life stages or related species		Overall CSAF
	Toxicokinetic	**Toxicodynamic**	**Toxicokinetic**	**Toxicodynamic**	
Default	4	2.5	3.16	3.16	100
Direct action (all species and life-stages)	1	2.5	1	3.16	8

	Rat to standard species*		Standard species to different life stages or related species		Overall CSAF
	Toxicokinetic	Toxicodynamic	Toxicokinetic	Toxicodynamic	
Systemic action					
Ruminant	0.17	2.5	1	3.16	1.3
Swine	1	2.5	1	3.16	8
Other monogastric	1	2.5	3.16	3.16	25

*: Standard species are those used in the toxicokinetic studies i.e. goat for ruminants; pigs for swine and other monogastric animals.

The calculated MoEs and estimated exposures to NAA via feed are presented in Table 12. For ruminants, swine and horses, all scenarios based on feed incorporating defatted toasted meal from oilseed rape 73496 gave MoEs that are acceptable. For cats (the monogastric species with the highest estimated exposure) the only estimated exposures available were based on theoretical diets with a high (worst case) incorporation rate of 20% defatted toasted meal from oilseed rape (see Table 10); these gave a MoE of 16. The GMO Panel notes that this MoE is lower than the CSAF of 25 for other monogastrics (Table 11) by a factor of ca 1.5. Given the conservative nature of the exposure estimate (see section on Animal dietary exposure to N-acetyl amino acids above) the GMO Panel consider this to present an acceptable risk.

Table 12: Assessment of NAA based on predicted exposures from feed uses of oilseed rape using a CSAF approach

Type of effect	Species	CSAF (see Table 11)	Predicted high exposure mg/kg bw per day	MoE vs NOAEL (229.5 mg/kg bw per day)	Acceptable
Direct/local (more likely mode of action)	All, body weight basis (mg/kg bw per day)	8	25	9	Yes
	All concentration basis (ppm)	8	300–700 ppm	8–34$	Yes
Systemic	Ruminants	1.3	14.5	16	Yes
	Horse	8	11**	21	Yes
	Swine	8	25.9	9	Yes

$: NOAEL = 5,530 ppm; *LOAEL= 10,143 ppm* based on concentrations in the rat 90 day study on NAA.
**: Based on feed consumption values used by EFSA CONTAM Panel (2019).

Overall, the GMO Panel concludes that exposures to NAA by feed from oilseed rape 73496 in this application do not pose a concern to animal health, based on the acceptable outcomes from the likely direct mode of action of NAA in the mouth, supported by the similar outcomes in the case that the mode of action were systemic.

The GMO Panel notes that the intakes of NAA and, therefore, the outcome of the risk characterisation might vary in the future due to changes in consumption/feeding patterns and, above all, by the introduction in the market of new products from oilseed rape after the completion of this risk assessment (e.g. seeds, whey fraction, etc.).

3.3.3.3. Assessment of altered levels of compounds other than NAAs

Compositional analysis studies indicated that the levels of some endogenous compounds (other than NAAs) were altered in oilseed rape 73496 when compared to its conventional counterpart and showed a lack of equivalence with a set of non-GM oilseed rape reference varieties. These compounds were the free amino acid glycine, crude fibre, crude fat, ADF, NDF, magnesium, pyridoxine, pantothenic acid and 4-hydroxyglucobrassicin (see Section 3.2.4). The GMO Panel assessed the toxicological relevance of these findings taking into account the biological role of the compounds and the magnitude of the changes observed and concluded that they do not pose toxicological concern for

food and feed from oilseed rape 73496. Further information on the safety of these compounds is provided in Section 3.3.5.

3.3.3.4. Testing of the whole genetically modified food/feed[40]

Based on molecular characterisation studies and on the outcome of compositional analysis, the GMO Panel considered that animal studies on the whole food/feed are not necessary to conclude on the safety of this crop (EFSA GMO Panel, 2011a).

In particular, the assessment of the increased levels of N-acetyl amino acids noted at compositional analysis was based on specific NAA, NAG and NAT toxicological studies, dietary exposure assessment, information on the biological relevance and metabolism of these compounds and on a subsequent risk characterisation.

The applicant spontaneously provided a 90-day toxicity study in rats and a 42-day study in broilers receiving diets containing whole food/feed derived from oilseed rape 73496, which were assessed by the GMO Panel.

a) 90-day toxicity study in rats[41]

A total of 168 Crl:CD(SD) rats (84 per sex) were randomly allocated to seven treatment groups (one control group, two oilseed rape 73496 test groups – treated or untreated with the intended herbicide, respectively – and three reference variety test groups, n = 12/sex per group) according to a randomised complete block design. Animals were individually housed.

This study is adapted from OECD Test Guideline 408 (OECD, 1998) aligned with EFSA Scientific Committee guidance (2011) and complies with the principles of GLP, except for the lack of analytical determination of concentration, homogeneity and stability of the test item in the formulated diets. It is recognised that it may not always be technically possible to generate information on homogeneity and concentration for a test item administrated or formulated, and the lack of such data and its impact on the validity of a study should be justified (OECD, 2018). The GMO Panel acknowledges that there are no practical methods available to analytically determine these for complex test items such as oilseed rape meal and oil in formulated diets and considers adequate the application of proper diet preparation procedures and regular evaluations of the mixing methods. Based on the information received from the applicant, the GMO Panel considers that the diet preparation procedures in place in the facilities where the diets for this study were prepared guaranteed their homogeneity and the proper concentration of the respective test or control items. As regards the stability of the test, control and reference items (defatted toasted meals and oil) in the diet, the applicant considers that, in accordance with product expiration declared by the diet manufacturer, the constituents of the diets used in these studies are stable for the duration of the treatment. The GMO Panel considers this justification acceptable.

The diets contained around 19–24% (w/w) dehulled defatted toasted meal and around 1.5–2.1% oil from an appropriate conventional counterpart (control diet), from oilseed rape 73496 treated with the intended herbicide (IH test diet) or with conventional herbicides (CH test diet), or from three commercial oilseed rape varieties, respectively (reference diets). The seeds used to produce the test and control materials (i.e. defatted toasted meal) were sent to the processing facility in about one month from harvest, then maintained at room temperature for about one month and finally processed into defatted toasted meal. The oilseed fractions were introduced in substitution of other dietary ingredients and balanced diets were prepared according to the specifications for PMI Certified Rodent LabDiet#5002 within four months from processing. The identity of the GM materials (oilseed rape seeds and dehulled defatted toasted meal) and of the diets was confirmed by PCR, and ELISA was used to assess the presence of the GAT4621 protein in the diets. The test item, control and reference materials, as well as test, control and reference diets were analysed for proximates, amino acids, minerals, mycotoxins, pesticides and antinutrients. In addition, the concentrations of NAA, NAG, NAT, NAGly and NAS were measured in the diets and demonstrated to be higher in GM diets[42] when

[40] Dossier Part II Scientific information/Section A, 4.5.

[41] The study was also published (Delaney B, Appenzeller LM, Roper JM, Mukerji P, Hoban D and Sykes GP, 2014. Thirteen week rodent feeding study with processed fractions from herbicide tolerant (DP-Ø73496-4) canola, Food and Chemical Toxicology, 66, 173-184, ISSN 0278-6915, https://doi.org/10.1016/j.fct.2014.01.042

[42] Oilseed rape 73496 containing diets (CHT and IHT, respectively): NAA: 694 ± 5 and 609 ± 94 μg/g DM; NAG: 12.7 ± 1.3 and, 15.2±1. Control diet: NAA: 2.47 ± 0.66 μg/g DM; NAG: 1.55 ± 0.22 μg/g DM. Reference varieties diets: NAA up to 2.85 ± 0.61; NAG up to 1.63 ± 0.19 μg/g DM. Dossier: Part II, Section A, 4.5, Annex 41_PHI-2011-047.

compared to the control diet and reference varieties diets. In-life procedures and observations and terminal procedures were conducted in accordance to OECD Test Guideline 408.

Histopathology was carried out on control and GM diet fed rats; on gross lesions from all groups; and on the thymus from all females and one intercurrent death.

Three intercurrent deaths occurred during the treatment period; one male given the control diet and one male given a reference diet were killed due to urinary tract calculi; one male given the (CHT) GM diet was sacrificed due to osteoarthritis. These deaths were considered incidental.

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

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

Detailed description of statistically significant findings identified in rats given diets containing oilseed rape 73496 is reported in Appendix A, Table A.2.

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

The GMO Panel concludes that no treatment-related adverse effects were observed in rats after feeding diets including 19–24% dehulled defatted toasted meal and 1.5–2.1% oil from oilseed rape 73496 for 90 days. The GMO Panel notes that the applicant only tested one dose level. However, the dose tested was close to the highest possible without inducing nutritional imbalance according to the current knowledge, and in accordance with the limit test dose as described in OECD TG 408. Therefore, this is not considered to affect the above conclusions.

b) 42-day feeding study in broiler[43]

A 42-day feeding study with chickens for fattening (day-old Ross 708) was provided. Groups of animals given diets including meal from oilseed rape 73496, treated and untreated with the intended herbicide, were compared to animals given diets containing the conventional counterpart and to four non-GM commercial oilseed rape varieties (45H72, 45H73, 46A65, and 44A89). The chickens were fed starter (day 0–21), grower (day 22–35) and finisher diets (day 36–42) in mesh form containing 10%, 20% and 0% of rapeseed meal, respectively. Since all diets in the last experimental week were free of rapeseed meal, all statistical analyses of the zootechnical parameters were performed for the periods 0–35 days and 0–42 days. Since male and female birds were kept together in cages, the influence of gender on feed intake and feed:gain ratio could not be evaluated, and for all zootechnical parameters the potential interaction of treatment x sex could not be calculated. Thirty-five-day body weight, and feed:gain ratio did not show differences between the treated and untreated GM diets and the conventional counterpart, average body weight was 1,678 g, feed:gain ratio 1.85. These data were within the 95% confidence interval established by the four diets with non-GM commercial oilseed rape varieties (1,494–1,836 g bw, 1.59–1.88 feed:gain ratio), however, they were considerably below the reference values (2,005 g bw, 1.55 feed:gain ratio) published by the breeder company for the strain.[44] Consequently, the power of the study to detect adverse effects is reduced. The EFSA GMO Panel considered this study not sensitive enough to detect potential small adverse effects on performance, since the performance data (body weight, feed intake and feed to gain ratio) collected on day 35 (end of grower phase) were not considered sufficient to conclude on the safety of dietary rapeseed 73496 in chicken for fattening; moreover performance data reported were considerably below the reference values published by the breeder company Ross for the broiler strain 708 used in the study.

[43] The study was also published (McNaughton J, Roberts M, Rice D, Smith B, Hong B, Delaney B, Iiams C. Comparison of broiler performance and carcass yields when fed diets containing genetically modified canola meal from event DP-Ø73496-4, near-isogenic canola meal, or commercial canola meals. Poult Sci. 2014 Jul;93(7):1713–1723. https://doi.org/10.3382/ps.2013-03645).

[44] Aviagen, 2012. Ross 708 Broiler Performance Objectives. Available online: http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross708BroilerPerfObj2012R1.pdf

3.3.4. Allergenicity

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

3.3.4.1. Assessment of allergenicity of the newly expressed proteins[45]

A weight-of-evidence approach was followed, taking into account all the information obtained on the newly expressed protein, as no single piece of information or experimental method yield sufficient evidence to predict allergenicity (Codex Alimentarius, 2009, 2011a–c).

The *gat4621* gene originates from *B. licheniformis*, which is not considered to be an allergenic source.

Updated bioinformatic analyses[46] of the amino acid sequences of the GAT4621 protein, using the criterion of 35% identity in a sliding window of 80 amino acids, revealed no relevant similarities to known allergens. The studies on protein stability of the GAT4621 protein have been described in Section 3.3.2.

The EFSA GMO Panel has previously evaluated the safety of the GAT4621 protein and no concerns on allergenicity were identified in the context of the application assessed (EFSA GMO Panel, 2013). In addition, the GMO Panel did not find an indication that the newly expressed protein GAT4621 at the levels expressed in oilseed rape 73496 might be adjuvants.

In the context of this application, the EFSA GMO Panel considers that there are no indications that the newly expressed GAT4621 protein in oilseed rape 73496 may be allergenic.

3.3.4.2. Assessment of allergenicity of the whole GM plant[47]

The GMO Panel regularly reviews the available publications on food allergy to oilseed rape. However, to date, oilseed rape is not considered a common allergenic food[48] (OECD, 2011). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM oilseed rape.

In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed protein (see Sections 3.4.1, 3.4.2 and 3.4.3), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from oilseed rape 73496 with respect to that derived from its conventional counterpart.

3.3.5. Nutritional assessment

The intended trait of oilseed rape 73496 is herbicide tolerance, with no intention to alter the nutritional profile. However, levels of different compounds were significantly different from its conventional counterpart and showed a lack of equivalence with a set of non-GM reference varieties. This mainly refers to the levels of NAA, NAG, NAT, the free amino acid glycine, crude fibre, crude fat, ADF, NDF, magnesium, pyridoxine, pantothenic acid and 4-hydroxyglucobrassicin (see Section 3.2.3).

The safety assessment of the N-acetyl amino acids NAA, NAG and NAT is described in Section 3.3.3.2. For the remaining compounds, a nutritional assessment was conducted to assess their biological relevance, the role of oilseed rape as contributor to their total intake and the magnitude and direction of the observed changes.

3.3.5.1. Human nutrition

The main food commodity derived from oilseed rape regularly consumed by the European population is oil, which is typically devoid of fibre, proteins/amino acids, water-soluble vitamins (e.g.

[45] Dossier Part II Scientific information/Section A5.1.
[46] Additional information 9/7/2020.
[47] Dossier Part II Scientific information/Section A5.2. Additional information 2/12/14, 22/6/15.
[48] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

pyridoxine, pantothenic acid) and minerals. The presence in the European market of other oilseed rape derived food commodities such as protein isolates and oilseed rape powder can be currently considered as negligible; however, the nutritional assessment also considered the consumption of these commodities.

Overall, the relatively small increase in crude fat (~ 5%) is not considered relevant for human nutrition also based on the fact that no significant differences in the profile of fatty acids were observed as compared to its conventional counterpart. The decrease in free glycine (19–23%) which is not an essential amino acid does not represent nutritional concerns.

Slightly higher contents of fibre (crude fibre, ADF, NDF) were reported in seeds from oilseed rape 73496 as compared to that of its conventional counterpart (up to 7% for crude fibre). In the context of human nutrition, fibre is referred to as dietary fibre, which primarily includes non-starch polysaccharides (mainly cellulose, hemicelluloses, pectins and other hydrocolloids) and lignin (EFSA NDA Panel, 2010). While protein isolates typically contain very small amounts of fibre, this is one of the main components of oilseed rape powder (EFSA NDA Panel, 2020). The safety and tolerability of oilseed rape powder with special focus on its fibre content was confirmed during the risk assessment of this novel food (EFSA NDA Panel, 2020). The relatively small increase of fibre reported in seeds from oilseed rape 73496 is unlikely to represent any nutritional concern for humans.

Dietary reference values are set for both pyridoxine (vitamin B6)[49] and pantothenic acid (vitamin B5); deficiency of these vitamins is considered rare (EFSA, 2017c). Foods rich in vitamin B6 include grains, pulses, nuts, seeds, potatoes and meat and meat products. Together with cruciferous vegetables, foods rich in pantothenic acid include meat and meat products, eggs, nuts and avocados. Protein isolates and oilseed rape powder are not typical sources of these water-soluble vitamins. Therefore, the decrease in the levels of pyridoxine and pantothenic acid in seeds from oilseed rape 73496 does not raise any nutritional concern. Adequate intakes (AI) are also set for magnesium as it is involved in numerous physiological functions (EFSA NDA Panel, 2015). Foods rich in magnesium are nuts, whole grains and grain products, fish and seafood, several vegetables, legumes, berries, banana and some coffee and cocoa beverage preparations. Protein isolates and oilseed rape powder are not typical sources of this mineral. Therefore, the decrease (up to 7.5%) in the levels of magnesium in seeds from oilseed rape 73496 does not raise any nutritional concern.

Levels of the glucosinolate 4-hydroxyglucobrassicin in oilseed rape 73496 were around three times higher than in the conventional oilseed rape (up to 0.290 μmol/g DM). The maximum reported levels of total glucosinolates in oilseed rape 73496 were 2.59 μmol/g DM, values that were significantly higher than those in its conventional counterpart (1.81 μmol/g DM) but within the natural variability represented by non-GM oilseed rape reference varieties (see footnote to Table 3 in Section 3.2.3) and well below the maximum glucosinolate content of 25 μmol/g at a moisture content of 9% as set-out for double-zero oilseed rape varieties under Regulation (EC) No 2316/1999[50]. Considering the total glucosinolate levels in oilseed rape 73496 and that humans are typically exposed to them through the consumption of *Brassicaceae* vegetables (cauliflower, cabbages, broccoli, kale, Brussels sprouts, etc.), no safety concerns are identified related to the increase of 4-Hydroxyglucobrassicin.

Therefore, the changes in glycine, crude fibre, crude fat, ADF, NDF, magnesium, pyridoxine, pantothenic acid and 4-hydroxyglucobrassicin observed in seeds from oilseed rape 73496 are unlikely to represent any nutritional concern for humans.

3.3.5.2. Animal Nutrition

Oilseed rape is a valuable protein source of vegetable origin widely used in animal nutrition, mainly in ration formulations for farmed animal species (e.g. poultry, pigs, cattle and aquaculture). The main oilseed rape product entering the feed supply chain is the meal (mostly un-hulled but also de-hulled), left after the removal of the oil (e.g. solvent-extracted and expeller meal). Other oilseed rape products which may enter the feed chain are the oil and seeds, which may be used as part of the total rations. On the contrary, the production of oilseed rape protein isolates and concentrates is not a common industrial practice due to relatively low protein yield and high costs, compared to available alternatives (Campbell et al., 2016), although there is evidence that oilseed rape protein isolates have been a topic

[49] Pyridoxine is one of the derivatives considered as vitamin B6 together with pyridoxal, pyridoxamine and their respective phosphorylated forms, pyridoxine 5′-phosphate, pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate (PMP).

[50] Commission Regulation (EC) No 2316/1999 of 22 October 1999 laying down detailed rules for the application of Council Regulation (EC) No 1251/1999 establishing a support system for producers of certain arable crops.

of research also in feed.[51] Therefore, their future application cannot be excluded. One of the largest potential use of rape protein isolates for livestock could be reasonably as milk replacer, which is primarily used for calves and in piglets. The potential use of whey as a feed ingredient must be assessed together with the prevalence of protein isolate production. If oilseed rape protein isolate were to be produced, there is a possibility for oilseed rape solubles (whey) to be used as a feed ingredient. Although it is very unlikely that oilseed rape protein isolate and the corresponding whey fraction will be available as commercial feed products, this might be kept under monitoring in the next future.

4-Hydroxyglucobrassicin is a derivative of glucobrassicin, one of the several glucosinolates that can be found in Brassicaceae. The GMO Panel considers that the increased level reported in seeds of oilseed rape 73496 does not represent an issue for animal nutrition. The maximum reported levels of total glucosinolates in oilseed rape 73496, significantly higher than those in its conventional counterpart, fall within the natural variability represented by non-GM oilseed rape reference varieties, and are well below the maximum glucosinolate content set-out for double-zero rapeseed varieties under Regulation (EC) No 2316/1999 (see Section 3.3.5.1). Moreover, Mejicanos et al. (2016) reports 4-hydroxyglucobrassicin content of 1.2 and 0.3 μmol/g in oilseed rape meal from Brassica napus and Brassica juncea, showing a certain variability of these compounds across varieties which can be fed to animals.

Glycine is considered a non-essential amino acid, even though Wu, 2014 suggests that adequate provision of all amino acid is important to improve efficiency of animal production. The magnitude of the decrease observed in oilseed rape 73496 (treated and non-treated) as compared to the non-GM comparator does not constitute an issue for animal nutrition.

Dietary fibre (crude fibre, NDF, ADF) is considered essential for animal health due to its influence on gastrointestinal tract physiology in animals. The observed increase of crude fibre, ADF, NDF in oilseed rape 73496 (treated and not treated), as compared to the non-GM comparator does not constitute an issue for animal nutrition.

The observed increase of crude fat in treated oilseed rape 73496 as compared to the non-GM comparator does not constitute an issue for animal nutrition.

Magnesium is an essential mineral in animal nutrition, and the diet must supply the adequate amount to satisfy the requirement. Many feeds are a good source of magnesium, and several magnesium sources, among which magnesium oxide is the most used, are included in the diet when the content in feeds is not sufficient, or when antagonists to magnesium absorption are present, i.e. high level of potassium. The lower level of magnesium found in oilseed rape 73496 (treated and not treated with the intended herbicide) as compared to non-GM counterpart does not represent an issue for animal nutrition.

Pyridoxine, a form of vitamin B6, plays an essential role mainly in amino acid metabolism. Vitamin B6 is produced by microorganisms in intestinal tracts of animals, but whether significant quantities are absorbed and utilised is in doubt. Muscle, liver, vegetables, whole grain cereals and their by-products, are among the best sources of pyridoxine. The bioavailability of two common feed ingredients is 65% for soybean meal with corn varying from 45% to 56% (McDowell and Ward, 2008). The level of vitamin B6, as other vitamins, contained in all feeds is affected by processing, subsequent storage and presence of antagonist in some feeds, i.e. hydrazic acid in linseed meal. In ruminants, vitamin B6 is mainly obtained by microbial synthesis in the rumen. The decrease observed in the non-treated oilseed rape as compared to the non-GM comparator does not constitute an issue for animal nutrition, considering also that hydrosoluble vitamins can be added in the diet of animals.

Pantothenic acid is a component of enzymes involved in carbohydrate, fat and protein metabolism. This vitamin is found in several feeds, i.e. wheat and rice bran, yeast, but the quantity present is generally insufficient to satisfy nutrient requirements for most monogastric species, so as many other micronutrients is added to the diet of animals. Pantothenic acid, as many other hydrosoluble vitamins, is synthesised in the rumen. The decrease observed in the non-treated oilseed rape as compared to the non-GM comparator does not constitute an issue for animal nutrition.

3.3.6. Post-market monitoring of GM food/feed

In accordance with Article 6(5)(e) of Regulation (EC) No 1829/2003, based on the outcome of the risk assessment of oilseed rape 73496 and, in particular, on the safety assessment of NAA, EFSA

[51] http://www.canproingredients.ca/research_development.php

recommends to implement a PMM plan. This PMM plan should initially focus on the collection of import data to Europe of oilseed rape 73496 and/or its products, entering the food and feed supply chains. If imports are identified, consumption data should be collected for humans and animals (e.g. through dietary surveys) on oilseed rape 73496 and/or its food and feed products to confirm the predicted consumption data and to verify that the conditions of use are those considered during the pre-market risk assessment.

3.3.7. Conclusion on the food/feed safety assessment

The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the GAT4621 protein as expressed in oilseed rape 73496 and finds no evidence that genetic modification would change the overall allergenicity of oilseed rape 73496.

No safety concerns are identified with regards to the increased levels of the N-acetyl amino acids NAA, NAG and NAT in food and feed derived from oilseed rape 73496 as considered during this risk assessment. The GMO Panel recommends to implement a PMM plan to confirm the predicted consumption of oilseed rape 73496 and/or its food and feed products and the application of conditions of uses considered during the pre-market risk assessment.

Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of oilseed rape 73496 does not represent any nutritional concern, in the context of the scope of this application.

The GMO Panel concludes that oilseed rape 73496, as described in this application, is as safe as its conventional counterpart and the non-GM reference varieties tested.

3.4. Environmental risk assessment and monitoring plan[52]

3.4.1. Environmental risk assessment

Considering the scope of application EFSA-GMO-NL-2012-109, which excludes cultivation, the environmental risk assessment (ERA) of oilseed rape 73496 mainly takes into account: 1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and 2) the accidental release into the environment of viable oilseed rape 73496 seeds during transportation and/or processing (EFSA GMO Panel, 2010b).

3.4.1.1. Persistence and invasiveness of the GM plant

Oilseed rape (*Brassica napus* AACC) is an annual allotetraploid species (2n = 38, genome constitution AACC), which has probably evolved through hybridisation and polyploidisation between the two diploid species *Brassica rapa* (2n = 20, AA) and *Brassica oleracea* (2n = 18, CC). Oilseed rape seeds have the ability to survive in soils for more than 10 years (Hails et al., 1997; Begg et al., 2006; Lutman et al., 2004; Lutman et al., 2005; Lutman et al., 2008; Messéan et al., 2007; D'Hertefeldt et al., 2008; Gruber et al., 2008; Beckie and Warwick, 2010; Peltonen-Sainio et al., 2014; Belter, 2016) and demographic studies and surveys have shown the ability of oilseed rape (*B. napus*) seed to establish self-perpetuating populations outside agricultural areas, mainly in semi-natural and ruderal habitats in different countries (e.g. Crawley et al., 1993; Pascher et al., 2010, 2017; Devos et al., 2012; Bauer-Panskus et al., 2013; Hecht et al., 2014; Schulze et al., 2014; Katsuta et al., 2015; Bailleul et al., 2016; Busi and Powles, 2016; Franzaring et al., 2016; Nishizawa et al., 2016). Oilseed rape is generally regarded as an opportunistic species, which can take advantage of disturbed sites (e.g. mowed areas, semi-natural habitats) to germinate and capture resources rapidly. In undisturbed natural habitats, oilseed rape lacks the ability to establish stable populations over successive years, possibly due to the absence of competition-free germination sites (Crawley et al., 1993, 2001; Meffin et al., 2015) and exposure to biological and abiotic stressors likely limiting fitness (COGEM, 2013; Busi and Powles, 2016). Once established in competition-free germination sites, feral populations decline over a period of years (Crawley and Brown, 1995, 2004; Knispel et al., 2008; Squire et al., 2011; Banks, 2014; Busi and Powles, 2016). However, if habitats are disturbed on a regular basis, then feral populations can persist for longer periods (Pessel et al., 2001; Claessen et al., 2005a, 2005b; Garnier et al., 2006; Elling et al., 2009; Pascher et al., 2010; Banks, 2014) and can have the characteristics of a weed or ruderal (Banks, 2014). The persistence or recurrence of a population in one location is

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variously attributed to replenishment with fresh seed spills, to recruitment from seed emerging from the soil seedbank or shed by resident feral adult plants or to redistribution of feral seed from one location to another (Pivard et al., 2008a, 2008b; Banks, 2014; Bailleul et al., 2016). Banks (2014) showed that the substantial increase in small and large (100–1,000 plants) feral populations occurred throughout the studied area during study years in Scotland.

It is unlikely that the intended trait of oilseed rape 73496 will provide a selective advantage to oilseed rape plants, except when they are exposed to glyphosate-containing herbicides. Should these plants be exposed to such herbicides, their abundance may increase locally (Londo et al., 2010, 2011; Watrud et al., 2011), allowing the establishment of transient populations. However, the likelihood of such an event will be restricted to managed environments, which may occasionally be treated with such herbicides. Moreover, this fitness advantage will not allow oilseed rape 73496 to overcome other biological and abiotic factors (described above) limiting plant's persistence and invasiveness.

In conclusion, the GMO Panel considers it unlikely that oilseed rape 73496 will differ from conventional oilseed rape varieties in its ability to survive and establish feral populations under European environmental conditions in case of accidental release into the environment of viable oilseed rape 73496 seeds.

3.4.1.2. Potential for gene transfer

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

Plant-to-microorganism gene transfer

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

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

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

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

The updated bioinformatic analysis of the inserted DNA did not identify sufficient sequence identity with bacterial DNA (including the *gat4621* gene, which was originally derived from *B. licheniformis*, but which has been codon-optimised for expression in plants) that would facilitate homologous recombination-mediated gene transfer between plants and bacteria.

In summary, there is no indication for an increased likelihood of horizontal transfer of DNA from oilseed rape 73496 to bacteria. Given the nature of the recombinant DNA, the GMO Panel identified no safety concern linked to an unlikely but theoretically possible HGT.

Plant-to-plant gene transfer

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

Oilseed rape is an open pollinating crop plant capable of cross-pollinating with other *Brassica* crops (Eastham and Sweet, 2002). It can also spontaneously hybridise with sexually compatible feral and wild relatives. Several hybrids between oilseed rape and wild relatives have been reported in the scientific

literature. Evidence suggests that transgenes could readily introgress into *B. rapa*, *B. juncea* and *B. oleracea*, and is expected to be rare with *B. nigra*, *Hirschfeldia incana*, *Raphanus raphanistrum* and *Sinapis arvensis* (reviewed by Liu et al., 2013; Ellstrand et al., 1999, Ellstrand et al., 2013; FitzJohn et al., 2007; Devos et al., 2009; Tang et al., 2018). Under field conditions, transgene introgression has only been confirmed for *B. rapa* (Hansen et al., 2001, 2003; Jørgensen et al., 2004; Norris et al., 2004; Warwick et al., 2003, 2008; Jørgensen, 2007). For transgene introgression to occur, feral GM oilseed rape must require some overlap in flowering in time and space with compatible relatives. Subsequently, transgenes must be transmitted through successive backcross generations or selfing, so that they become stabilised into the genome of the recipient (de Jong and Rong, 2013; Garnier et al., 2014). Because of these barriers (Luijten et al., 2015), reported incidences of hybrids and backcrosses with *B. rapa* were found to be low in fields (Jørgensen et al., 2004; Norris et al., 2004; Warwick et al., 2008; Elling et al., 2009), or at ports, along roadsides and riverbanks (Saji et al., 2005; Aono et al., 2006, 2011; Yoshimura et al., 2006; Elling et al., 2009; Katsuta et al., 2015; Luijten et al., 2015).

The GMO Panel does not consider the occurrence of feral oilseed rape 73496 plants, pollen dispersal and consequent cross-pollination as environmental harm in itself, as there is no evidence that the intended trait will enhance the vertical gene flow potential, or fitness, persistence or invasiveness of feral oilseed rape 73496, or cross-compatible plants such as hybridising wild relatives. However, when exposed to glyphosate-containing herbicides, occasional cross-compatible plants that acquired the herbicide tolerance trait through vertical gene flow are likely to exhibit a selective advantage, which may lead to their increased abundance. The likelihood of such an event to happen will be restricted to managed environments, which may occasionally be treated with such herbicides, so that environmental impacts will be minimal. Therefore, the GMO Panel considers that the acquisition of the herbicide tolerance trait by cross-compatible plants would not create additional environmental impacts.

In conclusion, the GMO Panel considers that the likelihood of environmental effects because of the spread of genes from oilseed 73496 rape in Europe will not differ from that of conventional oilseed rape varieties.

3.4.1.3. Interactions of the GM plant with target organisms

Taking the scope of application EFSA-GMO-NL-2012-109 (no cultivation) and thus the absence of target organisms into account, potential interactions of feral oilseed rape 73496 plants arising from seed import spills with target organisms are not considered a relevant issue.

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

Given that environmental exposure of non-target organisms to spilled GM seeds or feral GM oilseed rape plants arising from spilled oilseed rape 73496 seeds is limited, and because ingested proteins are degraded to a great extent before entering the environment through faecal material of animals fed GM oilseed rape, potential interactions of oilseed rape 73496 with non-target organisms are not considered by the GMO Panel to raise any environmental safety concern.

3.4.1.5. Interactions with the abiotic environment and biogeochemical cycles

Given that environmental exposure to spilled seeds or feral oilseed rape 73496 plants arising from seed import spills is limited, and because most proteins are degraded before entering the environment through faecal material of animals fed GM oilseed rape, potential interactions with the abiotic environment and biogeochemical cycles are not considered by the GMO Panel to raise any environmental safety concern.

3.4.2. Post-market environmental monitoring

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

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

As the ERA does not identify potential adverse environmental effects from oilseed rape 73496, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for oilseed rape 73496 includes: 1) the description of an approach involving operators (federations involved in import and processing), reporting to the applicant, via a centralised system, any observed adverse effect(s) of GMOs on human health and the environment; 2) a coordinating system established by EuropaBio for the collection of information recorded by the various operators; and 3) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis and a final report at the end of the authorisation period.

The scope of the PMEM plan provided by the applicant is consistent with the intended uses of oilseed rape 73496. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan.

3.4.3. Conclusion on the environmental risk assessment and monitoring plan

It is unlikely that oilseed rape 73496 would differ from conventional oilseed rape varieties in its ability to persist under European environmental conditions. Considering the scope of the application EFSA-GMO-NL-2012-109, interactions of feral oilseed rape 73496 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from oilseed rape 73496 to bacteria does not indicate a safety concern. Therefore, considering the introduced trait, the outcome of the agronomic and phenotypic analysis, and the routes and levels of exposure, the GMO Panel concludes that oilseed rape 73496 would not raise safety concerns in the event of accidental release of viable GM oilseed rape seeds into the environment.

The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of oilseed rape 73496.

4. Conclusions

The GMO Panel was asked to carry out a scientific assessment of oilseed rape 73496 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

The molecular characterisation data establish that oilseed rape 73496 contains a single insert consisting of one copy of the *gat4621* expression cassette. Upon transformation, a region of chromosome C02 was potentially inverted and a putative *tpt* gene interrupted. The relevance of the gene interruption and potential chromosomal inversion for the risk assessment of oilseed rape 73496 is addressed. Bioinformatic analyses of the sequences encoding the newly expressed protein and other ORFs within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and introduced trait is confirmed over several generations. The levels of the GAT4621 protein were obtained and reported adequately. The protein characterisation data of the plant- and microbial-derived GAT4621 proteins indicate that both proteins are equivalent, and thus, that the microbial-produced protein (2 batches) can be used in the safety studies.

None of the identified differences in the agronomic/phenotypic endpoints between oilseed rape 73496 and its conventional counterpart needs further assessment. Among the differences identified in seed composition between oilseed rape 73496 and its conventional counterpart the levels of NAA, NAG and NAT, the free amino acid glycine, crude fibre, crude fat, ADF, NDF, magnesium, pyridoxine, pantothenic acid and 4-hydroxyglucobrassicin were further assessed and found not to raise nutritional and safety concerns.

No safety concerns are identified regarding toxicity and allergenicity of the GAT4621 protein as expressed in oilseed rape 73496. No evidence is found that the genetic modification would change the overall allergenicity of oilseed rape 73496. Based on the outcome of the comparative and nutritional assessments, the consumption of oilseed rape 73496 does not represent any nutritional concern, in the context of the scope of this application.

The implementation of a PMM plan is recommended to confirm the predicted consumption of oilseed rape 73496 and/or its food and feed products; and the application of conditions of uses considered during the pre-market risk assessment.

There is a low likelihood of environmental effects resulting from the accidental release of viable seeds from oilseed rape 73496 into the environment. The PMEM plan and reporting intervals are in line with the intended uses of oilseed rape 73496.

The GMO Panel concludes that oilseed rape 73496, as described in this application, is as safe as its conventional counterpart and the non-GM oilseed rape reference varieties tested with respect to potential effects on human and animal health and the environment.

5. Documentation as provided to EFSA

- Letter from the Competent Authority of The Netherlands received on 24 May 2012 concerning a request for authorization of the placing on the market of oilseed rape 73496 submitted in accordance with Regulation (EC) No 1829/2003 by Pioneer Overseas Corporation.
- Application EFSA-GMO-NL-2012-109 validated by EFSA, 4 December 2012.
- Request for supplementary information to the applicant, 20 February 2013.
- Request for supplementary information to the applicant on behalf of EURL-GMFF, 2 April 2013.
- Receipt of supplementary information from the applicant, 9 April 2013.
- Receipt of supplementary information, from the applicant to EURL-GMFF, 26 April 2013.
- Request for supplementary information, from EURL-GMFF to the applicant, 22 May 2013.
- Receipt of supplementary information, from the applicant to EURL-GMFF, 4 June 2013.
- Request for supplementary information to the applicant, 13 August 2013.
- Receipt of supplementary information from the applicant, 17 September 2013.
- Request for supplementary information to the applicant, 12 December 2013.
- Receipt of supplementary information from the applicant, 28 January 2014.
- Request for supplementary information to the applicant, 11 July 2014.
- Request for supplementary information to the applicant, 8 December 2014.
- Receipt of supplementary information from the applicant, 23 January 2015.
- Request for supplementary information to the applicant, 11 March 2015.
- Receipt of supplementary information from the applicant, 16 April 2015.
- Receipt of supplementary information from the applicant, 27 April 2015.
- Receipt of supplementary information from the applicant, 29 September 2015.
- Request for supplementary information to the applicant, 2 December 2015.
- Receipt of supplementary information from the applicant, 15 December 2015
- Request for supplementary information to the applicant, 29 February 2016.
- Receipt of supplementary information from the applicant, 29 April 2016.
- Request for supplementary information to the applicant, 19 May 2016.
- Receipt of supplementary information from the applicant, 3 October 2016.
- Receipt of supplementary information submitted spontaneously by the applicant, 22 November 2016.
- Request for supplementary information to the applicant, 2 December 2016.
- Request for supplementary information to the applicant, 16 December 2016.
- Receipt of supplementary information from the applicant, 2 May 2017.
- Receipt of supplementary and spontaneous information from the applicant, 29 August 2017.
- Request for supplementary information to the applicant, 6 October 2017.
- Receipt of supplementary information from the applicant, 6 December 2017.
- Request for supplementary information to the applicant, 19 December 2017.
- Receipt of supplementary information from the applicant, 20 June 2018.
- Request for supplementary information to the applicant, 3 July 2018.
- Receipt of supplementary information from the applicant, 28 September 2018.
- Request for supplementary information to the applicant, 29 October 2018.
- Request for supplementary information to the applicant, 13 November 2018.
- Receipt of supplementary information from the applicant, 18 December 2018.
- Receipt of supplementary information from the applicant, 10 January 2019.
- Receipt of supplementary information submitted spontaneously by the applicant, 16 April 2019.
- Request for supplementary information to the applicant, 27 May 2019.
- Request for supplementary information to the applicant, 23 April 2020.
- Request for supplementary information to the applicant, 18 May 2020.
- Receipt of supplementary information from the applicant, 9 July 2020.
- Receipt of supplementary information from the applicant, 10 September 2020.
- Request for supplementary information to the applicant, 7 October 2020.
- Receipt of supplementary information from the applicant, 8 October 2020.
- Request for supplementary information to the applicant, 14 October 2020.
- Receipt of supplementary information from the applicant, 10 November 2020.

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Abbreviations

ADF	acid detergent fibre
ATG	translational start codons
bp	base pair
bw	body weight
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
ERA	environmental risk assessment
FA	fatty acid
fw	fresh weight
GLP	good laboratory practice
GM	genetically modified
GMO	genetically modified organism
GMO Panel	EFSA Panel on Genetically Modified Organisms
HGT	horizontal gene transfer
HR	homologous recombination
IgE	immunoglobulin E
JSA	Junction Sequence Analysis
LOQ	limit of quantification
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NDF	neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-DNA	transfer-DeoxyriboNucleic Acid
UTR	untranslated region

Appendix A – Statistically significant findings in toxicological studies

Table A.1: Statistically significant findings in GAT4621 protein treated mice in the 28-day study

Statistically significant parameter/ endpoint	Finding	GMO Panel interpretation
Defecation score (count)	Decrease in males from the low and high dose groups, when compared to the basal control group only	Incidental, within normal variation, not an adverse effect of treatment.
Mean monocyte counts (%)	Decrease in the GAT4621 intermediate (–51%) and high (–44%) dose groups when compared to BSA control group. No statistically significantly differences in comparison to the basal control group.	No changes in the absolute mean monocyte count, no dose relationship. Expression of normal variability, not an adverse effect of treatment.
Mean eosinophil count (absolute)	Increase in GAT4621 high dose group males compared to both control groups (around 70%, 0.07 vs. 0.04)	All individual values within the concurrent control ranges. Expression of normal variability, not associated with significant changes in total White Blood Cell Count (WBC) not an adverse effect of treatment.
Mean alanine aminotransferase (U/L)	Increase in GAT4621 intermediate dose group females compared to both control groups (41 U/L vs 31 or 28 U/L in control and BSA control, respectively).	Minimal increase, no dose response, no consistent pattern of increased levels of other liver marker enzymes, no histopathology changes in liver. Not an adverse effect of treatment.
Mean thyroid/ parathyroid gland weights (g, % to final body weight, % brain weight)	Increase in the GAT4621 treated females compared to both controls (intermediate dose group: around +17% absolute and relative to brain weight, and +14% relative to final body weight; high-dose group: around +20% absolute and relative to final body weight)	Minimal increase, no microscopic correlates in the high dose group. Not an adverse effect of treatment.

Table A.2: Statistically significant findings in 90-day study on the whole food feed from oilseed rape 73496

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Body weight and related parameters (mean body weights, mean body weight gains, mean terminal body weights, feed intake, overall feed efficiency)	Increase in males (IHT and CHT diet groups)	Limited in magnitude (10 - 20% overall), within the normal variability. Not an adverse effect of treatment.
Body weight gain (g)	Increase in females (day 21–28, IHT and CHT diet groups)	Transient, not associated with differences in terminal body weight or cumulative body weight gains. Not an adverse effect of treatment.
Forelimb grip strength	Increased in males (IHT & CHT diet groups).	Small in magnitude (< 11%), within normal variation set by reference varieties groups. Not an adverse effect of treatment.
Serum calcium (mg/dL)	Increase in males (10.2 ± 0.5 CHT diet group vs. 9.9 ± 0.4 mg/dL control diet group)	Small in magnitude (3%), within normal variation. No changes in related parameters, including kidney histopathology. Not an adverse effect of treatment.

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Potassium (mEq/L)	Decrease in males (4.5 ± 0.2mEq/L IHT diet group vs. 4.8 ± 0.2 mEq/L control diet group).	Small in magnitude (6%), within normal variation set by reference varieties groups. No changes in related parameters, including kidney histopathology. Not an adverse effect of treatment.
Mean heart weight (g, % to body weight)	Decrease in females (8% IHT diet group as compared to control diet group)	Minimal, within normal variation, not associated with histopathological changes. Absolute and relative to brain weight values are unaffected. Not an adverse effect of treatment.

IHT diet: Intended Herbicide Test diet, i.e. containing ingredients from oilseed rape 73496 treated with the intended herbicide.
CHT diet: Conventional Herbicide Test diet, i.e. containing ingredients from oilseed rape 73496 treated with conventional herbicides.

Appendix B – Summary statistics of the baseline dietary intake of NAA (μg/kg bw per day) across European dietary surveys

	Dietary intake (μg/kg bw per day)						
	N	Mean dietary intake			95th percentile dietary intake		
		Min	Median	Max	Min	Median	Max
Infants	13	1.1	3.2	15.3	8.7	16.4	47.3
Toddlers	20	3.2	6.9	15.5	10.8	22.7	45.3
Other children	30	3.7	7.2	12.6	11.8	20.3	34.8
Adolescents	30	2.7	5.5	7.6	9.1	17.7	23.7
Adults	35	2.8	16.7	39.9	11.5	42.0	99.0
Elderly	25	2.8	15.7	46.0	9.4	35.6	92.9
Very elderly	17	4.3	14.9	43.3	12.2	36.4	52.7
Pregnant women	5	5.9	7.9	8.8	13.4	21.8	28.3
Lactating women	2	4.6	–	15.2	12.9	–	35.8

NAA: N-acetylaspartate.

Appendix C – Summary statistics of dietary intake of NAA (μg/kg bw per day) across European dietary surveys considering the presence of NAA in conventional foods, and the consumption of protein isolates and oilseed rape powder from oilseed rape 73496

	Dietary intake (μg/kg bw per day)						
	N	Mean dietary intake			95th percentile dietary intake		
		Min	Median	Max	Min	Median	Max
Infants	13	1.1	22.6	144.8	9.0	114.4	651.5
Toddlers	20	6.9	104.4	375.7	36.6	461.9	933.8
Other children	30	17.8	90.9	412.6	55.3	428.6	992.9
Adolescents	30	13.3	56.6	244.6	51.3	252.2	674.1
Adults	35	13.4	52.2	217.5	49.4	211.4	444.8
Elderly	25	11.2	47.9	210.2	32.9	175.3	418.6
Very elderly	17	14.8	44.7	201.3	54.2	193.5	305.1
Pregnant women	5	12.1	40.5	57.1	53.4	164.1	214.9
Lactating women	2	40.0	–	75.6	214.3	–	236.7

NAA: N-acetylaspartate.

Appendix D – Animal dietary exposure to N-acetyl amino acids via oilseed rape 73496 and derived feed

a) Technical Dossier: Part II, section B

Dietary exposure to N-Acetylaspartate (NAA), N-Acetylglutamate (NAG) and N-Acetylthreonine (NAT) in oilseed rape 73496 was estimated by the applicant across different animal species (i.e. poultry, swine, cattle and sheep), assuming the consumption of oilseed rape meal, the main rapeseed by-product entering the feed supply chain. A conservative scenario with 100% replacement of conventional oilseed rape meal by oilseed rape 73496 meal was considered. Mean levels (dry weight) of NAA, NAG and NAT in un-hulled toasted meal processed from oilseed rape 73496 seeds treated with the intended herbicide (i.e. glyphosate) were used as occurrence data (see Table D.1). Dietary exposure was based on estimates for animal body weight, daily feed intake and inclusion rates (percentage) of oilseed rape meal in diets (OECD, 2009). Estimated dietary exposures in livestock animals is reported in Table D.1.

Table D.1: Dietary exposure (DE) to NAA, NAG and NAT (mg/kg bw per day) in livestock animals based on the consumption of oilseed rape meal

Animal species		Body weight (kg)	Daily feed intake (kg DM/Animal)	Inclusion rate (%)	NAA[(a)]	NAG[(b)]	NAT[(c)]
					mg/kg bw per day	mg/kg bw per day	mg/kg bw per day
Poultry	Broiler	1.7	0.12	18	38.99	0.68	0.04
	Layer	1.9	0.13	10	21	0.37	0.02
	Turkey	7	0.50	20	43.90	0.77	0.04
Swine	Breeding	260	6	20	14.18	0.25	0.01
	Finishing	100	3	20	18.42	0.32	0.02
Cattle	Beef	500	12	–	–	–	–
	Dairy	650	25	10	13.43	0.23	0.01
Sheep	Ram/ewe	75	2.5	–	–	–	–
	Lamb	40	1.7	–	–	–	–

(a): NAA concentration in meal: as-is for poultry and swine (3070 mg/kg); dry weight basis for cattle and sheep (3,489 mg/kg).
(b): NAG concentration in meal: as-is for poultry and swine (53.7 mg/kg); dry weight basis for cattle and sheep (61 mg/kg).
(c): NAT concentration in meal: as-is for poultry and swine (2.90 mg/kg); dry weight basis for cattle and sheep (3.30 mg/kg).

b) Additional information: 6/12/2017

The applicant provided estimations of background exposures to NAA in poultry, swine, cattle, sheep, salmon, dog and cat, based on the theoretical consumption of simple diets (not nutritionally balanced) consisting of the combination of two selected conventional feed materials (i.e. maize grains and distillers grain with solubles, forage/silage from maize, alfalfa and grass, soybean, oilseed rape and fish meal) with known concentration of NAA; a comparison was made with the exposures based on the theoretical consumption of simple diets containing oilseed rape meal 73496 as one of the combined feed materials in order to determine whether a safe comparative consumption could be established. Concentration of NAA for selected feedstuffs and for dog and cat foods were used as occurrence (see Table D.2). Dietary exposure was based on estimates for body weight and daily feed intake obtained from EFSA's guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), combined with feedstuff inclusion rates obtained from OECD Guidance Document on Residues in Livestock (OECD, 2013), Canola Council of Canada (2015), Food and Agriculture Organization of the United Nations (FAO, 2017), Advisory Committee on Animal Feedingstuffs (ACAF Secretariat, 2001), Atti et al. (2007), Mandell et al. (1997), Windsor (2001) and Purina (personal communication). Estimated dietary exposures in farmed and companion animals is reported in Table D.2.

Table D.2: Dietary exposure (DE) to NAA (mg/kg bw per day) in food-producing and non-food-producing animals based on the consumption of simple diets consisting of the combination of two conventional feed materials

Simple diets	Animal daily feed intake (kg DM animal/kg body weight)	Inclusion rate (%)/NAA (mg/kg bw per day)								
		Maize grain	Maize forage/ silage	Alfalfa silage	Grass silage	Maize DDGS	Soybean meal	Fish meal	Oilseed rape meal	Oilseed rape 73496 meal
Maize Grain	Chicken for fattening 0.158/2	70% 0.058	NA	NA	NA	60% 0.585	40% 0.103	10% 0.274	18% 0.292	18% 49.7
	Laying hen 0.106/2	70% 0.039	10% 0.111	NA	NA	50% 0.333	25% 0.058	10% 0.184	10% 0.126	10% 18.5
	Turkey for fattening 0.176/3	50% 0.031	NA	NA	NA	50% 0.357	45% 0.068	10% 0.191	20% 0.224	20% 41.0
	Sow lactating 5.28/175	70% 0.022	20% 0.104	NA	20% 0.355	75% 0.274	30% 0.035	10% 0.105	20% 0.121	20% 21.1
	Pig for fattening 2.20/60	70% 0.027	NA	NA	NA	75% 0.333	30% 0.042	10% 0.127	20% 0.148	20% 25.6
	Cattle for fattening 8/400	80% 0.017	80% 0.235	25% 0.333	50% 0.569	30% 0.083	20% 0.022	5% 0.044	20% 0.083	20% 14.0
	Dog 0.25/15	45% 0.008	NA	NA	NA	NA	15% 0.011	NA	20% 0.063	20% 11.6
	Cat 0.06/3	25% 0.005	NA	NA	NA	NA	30% 0.014	NA	20% 0.071	20% 14.0
Maize DDGS	Chicken for fattening 0158/2	70% 0.585	NA	NA	NA	60% 0.527	40% 0.572	10% 0.743	18% 0.761	18% 50.1
	Laying hen 0.106/2	70% 0.333	10% 0.367	NA	NA	50% 0.295	25% 0.314	10% 0.440	10% 0.382	10% 18.8
	Turkey for fattening 0.176/3	50% 0.357	NA	NA	NA	50% 0.326	45% 0.364	10% 0.487	20% 0.520	20% 41.3
	Sow lactating 5.28/175	70% 0.274	20% 0.334	NA	20% 0.585	75% 0.252	30% 0.265	10% 0.334	20% 0.351	20% 21.3
	Pig for fattening 2.20/60	80% 0.333	NA	NA	NA	75% 0.306	30% 0.321	10% 0.406	20% 0.427	20% 25.9
Maize Forage/ Silage	Cattle for fattening 8/400	80% 0.235	80% 0.218	25% 0.535	50% 0.771	30% 0.285	20% 0.224	5% 0.245	20% 0.284	20% 14.2
	Dairy cow 20/650	30% 0.261	60% 0.252	40% 1.03	60% 1.27	30% 0.354	25% 0.262	5% 0.294	10% 0.302	10% 11.0
Grass Silage	Cattle for fattening 8/400	80% 0.569	80% 0.771	25% 0.869	50% 0.553	30% 0.619	20% 0.558	5% 0.580	20% 0.618	20% 14.5
	Dairy cow 20/650	30% 1.03	60% 1.27	40% 1.80	60% 1.02	30% 1.12	25% 1.03	5% 1.06	10% 1.07	10% 11.8
	Sheep/goat 1.2/60	30% 1.00	NA	40% 1.50	90% 0.995	30% 1.06	25% 1.00	10% 1.05	15% 1.04	15% 11.5
Fish Meal	Salmon 0.0021/012	NA	NA	NA	NA	NA	12% 0.156	32% 0.153	20% 0.211	20% 12.4

Note: NA: not applicable.
Concentrations (μg/g) of NAA for select feedstuffs: maize grain 1.04; maize forage/silage 13.6; alfalfa silage 63.3; grass silage 55.3; maize DDGS 11.1; soybean meal 1.42; fish meal 27.4; oilseed rape meal 16.5; oilseed rape 73496 meal 3489; dog food 2.81; cat food 9.50.

c) Additional information: 29/8/2017

The applicant provided estimations of exposure in calves based on the consumption of milk replacer, making the conservative assumption that 100% of the protein in milk replacer would be from oilseed rape protein isolate. Concentration of NAA and NAG in oilseed rape protein isolates were used as occurrence (see Table D.3). Estimated dietary exposures in calves are reported in Table D.3.

Table D.3: Dietary exposure to NAA and NAG (mg/kg bw per day) in calves based on the consumption of milk replacer (protein isolates)

Animal species	NAA[(a)]	NAG[(b)]
	mg/kg bw per day	mg/kg bw per day
Calf[(c)]	0.072	0.0024

(a): NAA concentration in oilseed rape protein isolates (18 mg/kg).
(b): NAG concentration in oilseed rape protein isolates (0.611 mg/kg).
(c): The total protein intake from milk replacer was estimated by multiplying the content of protein in milk replacer powder (EFSA GMO Panel, 2011c; BAMN, 2008) by the solids content in prepared liquid milk replacer (Krishnamoorthy and Moran, 2011) to determine the total protein content in the prepared liquid milk replacer. This was multiplied by consumption (high end value) of liquid milk replacer by a calf at 5 days of age (Krishnamoorthy and Moran, 2011) and divided by the average calf birth weight of a Jersey calf, one of the smaller breeds of cattle (Queensland Government, 2012) to determine the approximate total protein intake, 4 g/kg BW per day by a 5-day old calf.

d) Additional information: 20/6/2018

The applicant simulated estimations of exposure in ruminants (i.e. cattle for fattening, dairy cow and sheep/goat) based on the consumption of oilseed rape solubles (whey), alone or combined with oilseed rape meal. Dietary exposure was based on estimates for body weight and daily feed intake obtained from EFSA's guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), combined with oilseed rape inclusion rates obtained from OECD Guidance Document on Residues in Livestock (OECD, 2013). Since oilseed rape protein isolate production is not a common industrial practice, soy protein isolate and the corresponding whey fraction productions were examined as a surrogate; theoretical inclusion rates of 10% were indeed derived from the literature, considering the reporting of adverse nutritional impact of soy solubles (whey) at experimental inclusion rates higher than 10% in diets (Perry et al., 1976; van Eys, 2012). Concentration of NAA, NAG and NAT, in oilseed rape solubles (whey) were used as occurrence (see Table D.4).

Table D.4: Dietary exposure to NAA, NAG and NAT (mg/kg bw per day) in ruminants based on the 'theoretical' consumption of whey from oilseed rape protein isolate and oilseed rape meal, alone or combined

Animal daily feed intake (kg DM animal/ kg body weight)	Oilseed rape by-products (IR%)	NAA[(a)]	NAG[(b)]	NAT[(c)]
		mg/kg bw per day	mg/kg bw per day	mg/kg bw per day
Cattle for fattening 8/400	Oilseed rape meal (20%)	14.0	0.244	0.0132
	Oilseed rape whey (10%)	9.71	0.379	0.00897
	Oilseed rape meal + whey (20%) + 10%)	23.7	0.623	0.0222
Dairy cow 20/650	Oilseed rape meal (20%)	10.7	0.188	0.0101
	Oilseed rape whey (10%)	14.9	0.583	0.0138
	Oilseed rape meal + whey (20%) + 10%)	25.7	0.770	0.0239
Sheep/goat 1.2/60	Oilseed rape meal (20%)	10.5	0.183	0.00989
	Oilseed rape whey (10%)	9.71	0.379	0.00897
	Oilseed rape meal + whey (20%) + 10%)	20.2	0.562	0.0189

(a): NAA concentration in oilseed rape meal (3070 μg/g) and in Whey 1 Fraction of oilseed rape protein isolate production (140.8 μg/g).
(b): NAG concentration in oilseed rape meal (53.7 μg/g) and in Whey 1 Fraction of oilseed rape protein isolate production (5.49 μg/g).
(c): NAT concentration in oilseed rape meal (2.90 μg/g) and in Whey 1 Fraction of oilseed rape protein isolate production (0.13 μg/g).

Appendix E – NAA risk characterisation by the use of Chemical Specific Adjustment Factors (CSAF) with reference to exposures to N-acetylated amino acids found in oilseed rape 73496

Background

- The default assessment factor used when deriving an acceptable human exposure level from the no-observed adverse effect levels (NOAEL) in animal studies is 100. This factor accounts for differences in sensitivity between the experimental animal and the average human and for variations in sensitivity within the human population, to protect sensitive sub-groups. This factor of 100 has also been utilised an indicator of the expected margin between the NOAELs in laboratory animal studies and intakes in farm and domestic animals (EFSA FEEDAP Panel, 2017a,b).
- Where specific data are available, it is possible to derive Chemical Specific Adjustment Factors (also known as Chemical Specific Assessment Factors and Data Derived Evaluation Factors) to replace the default 100-fold assessment factor. The overall CSAF can be lower or higher than the default of 100. The concept was developed by comparing the findings seen in humans and experimental animals exposed to pharmaceuticals and was described in detail by the World Health Organisation (IPCS, 2005). The CSAF approach splits the default factor of 100 into four separate factors addressing differences in toxicokinetics (how a compound is absorbed, metabolised, distributed and excreted) and toxicodynamics (how a given exposure affects the target tissue) – see Figure E.1. Each individual factor can be modified, if suitable data are available, and then combined to give the overall CSAF.

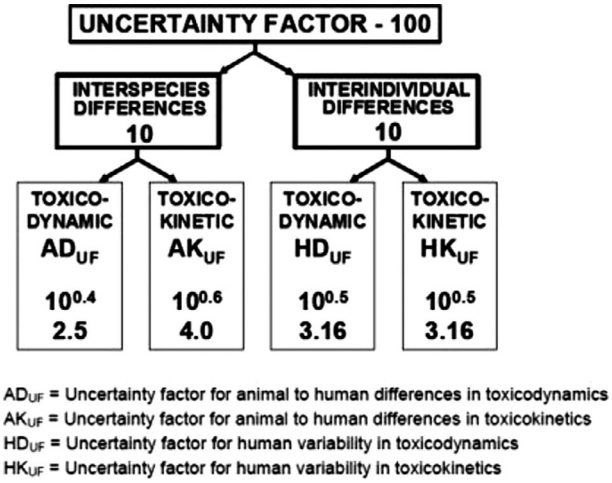

Figure E.1: Sub-division of the default 100-fold assessment factor (from IPCS, 2005)

- CSAFs have been referenced by EFSA in the Scientific Opinions on Default values (EFSA Scientific Committee, 2012) and on Uncertainty Analysis (EFSA Scientific Committee et al., 2018). A CSAF based approach has been used by EFSA in the re-evaluation of phosphates (EFSA FAF Panel et al., 2019). A CSAF based approach has been proposed by the applicant for the assessment of N-acetyl aspartate (NAA) present in feed derived from GM 73496 oilseed rape in the context of this application and this is described below.
- The CSAF approach as above described was developed for assessments of human safety extrapolating from experimental animal data. In the context of this dossier the extrapolation is conducted from experimental animals to a representative animal for an order or sub-order. In this evaluation the interspecies factors are considered to apply between rats and goats/pigs and the interindividual factors apply between pigs/goats and all respective other relevant species and life stages. The finding of concern (i.e. salivary gland hypertrophy) was seen in some but not all of the rats exposed at the effect dose in the relevant studies (see Table 8 in Section 3.3.3.2 of the scientific opinion) and can be considered as including some conservatism in affecting the more sensitive individuals. In addition, rats exposed during gestation, lactation and post-weaning and through maturity showed no greater sensitivity to the salivary gland

effects than those exposed as young adults, as noted in a rat two-generation reproductive toxicity study (see Table 8 in Section 3.3.3.2 of the scientific opinion).

Data submitted in support of a CSAF

- The applicant has performed comparison studies in goats (representative ruminants), pigs (representative monogastric) and rats (the test species) to investigate the toxicokinetics of NAA, and results are presented below.

  Goats: groups of Boer goats (3/sex per group) received NAA (99.9% pure) in gelatine capsules at a nominal dose of 25 mg/kg bw per day for 14 days. Blood samples were taken regularly pre-dosing and from 5 min to 12 h after dosing on days 1 and 14. Samples of blood plasma were analysed by UHPLC and LC/MS/MS for NAA and aspartic acid.[53]
  Pigs: groups of Landrace cross pigs (3/sex/group) received NAA (99.9% pure) in a small volume of feed at a nominal dose of 25 mg/kg bw per day for 14 days (actual dose 26 mg/kg bw per day). Blood samples were taken and analysed as described above for goats.[53]
  Rats: groups of Sprague Dawley rats (6/sex/group) received NAA (99.9% pure in water) by gavage at doses of 10, 25, 75, 250 or 500 mg/kg bw per day for 14 days. Blood samples were taken and analysed as described above for goats.[53]

- In addition to the toxicokinetic studies, relevant information was presented on:
  - the physiology and anatomy of salivary glands,
  - the background exposure to NAA in the diet,
  - the presence and function of NAA in animals,
  - the metabolism of NAA and on the expression levels of the main metabolising enzymes across species.

  The information confirmed that NAA was a natural component of the diet/feed but at levels much lower than those associated with oilseed rape 73496; that NAA was naturally present in the body of animals, with a function in the CNS; the initial step in the metabolism is a simple transamination to give aspartic acid and that the primary metabolising enzymes are present in a wide range of species (see also Section 3.3.3.2 of the Scientific Opinion).

- The key results from the toxicokinetic studies are presented in Table E.1 below and a summary comparison between rats, goats and pigs is presented in Table E.2 below. The were no notable differences between males and females of any species. The derivation of the relevant CSAF is outlined in Table E.3. Data are available only for the toxicokinetics of NAA across species in blood plasma with no data on levels in the salivary gland, therefore no adjustment of the toxicodynamic factors can be performed.

Potential modes of action

a) Direct mode of action in the mouth

When performing a CSAF based assessment it is valuable if the mode of action underlying the adverse effect is well understood. The mode of action underlying the salivary gland hypertrophy seen in some rats exposed to NAA (see Section 3.3.3.2) has not been investigated in detail, but generic information is available. The GMO Panel considered that given the physiological mechanisms controlling the production of saliva and mechanisms leading to hypertrophy it is likely that the findings in rats exposed to NAA were due to a direct mode of action of NAA in the mouth. Data supporting this include:

- Compensatory or adaptative hypertrophy is considered to represent a physiological response to a repeated/high stimulus (King, 2007; Mastorides & Maronpot, 2002).
- Factors resulting in stimulation of saliva excretion include taste and presence of food in the mouth. (https://www.britannica.com/science/human-digestive-system/Salivary-glands).
- Saliva is alkaline and buffers acid food, acid compounds stimulate secretion (vinegar/lemon juice). NAA is acidic – pKa ~ 3.5. (https://hmdb.ca/metabolites/HMDB0000812)
- Some studies indicated that substances may produce salivary gland hypertrophy when given in the diet but not by gavage. This effect was suggested to be an outcome of the exposure to the

[53] Additional information 10/9/2020.

test substance in the oral cavity rather than being a systemic effect by a limited number of studies (Wells and Voelkel, 1963; Burdock et al., 2000). In their studies, Wells and Voelkel and Burdock et al., administered the test substances in question (RP-1 and pancreatin), known to cause salivary gland enlargement when administered through the diet, both by diet and by gavage.

- Some chemicals can act systemically to increase saliva secretion (e.g. pesticides which result in increased levels of acetylcholine) but salivary gland hypertrophy is not a common finding in toxicity studies with these types of compounds. No reports of NAA having a similar activity likely to increase acetylcholine were identified.

On the basis that NAA acts on the salivary glands via a direct action in the mouth, the effects would be essentially independent of absorption, distribution, metabolism and excretion. Therefore, both toxicokinetic factors can be removed leaving a **residual CSAF of 8** based on the toxicodynamic factors. This would apply when extrapolating from rats to any other species and life-stage (see Table E.3 below).

b) Systemic MoA

As the mode of action of NAA on salivary glands has not been investigated in detail, a supplementary CSAF based approach assuming a systemic mode of action was performed to see if any significant risks might be missed by adopting a direct mode of action approach. Using the data from the submitted toxicokinetic studies and noting that in a rat two-generation study there was no evidence of any sensitive life-stages and that expression data on the main enzymes metabolising N-acetylated amino acids are widely distributed across species (Yates et al., 2020), adjustment of the CSAFs by modifying the toxicokinetic factors was evaluated for the various species but retaining the default toxicodynamic factors (see below and Table E.3).

Systemic CSAF for goats/ruminants

The toxicokinetic data on NAA (Table E.1 below) show that the peak plasma concentration in goats is over 20 times lower than in rats administered the same dose (25 mg/kg bw per day). The Area Under the Curve (AUC) is sixfold lower in goats than rats on day 1, and 33-fold lower on day 14. These data support a reduction in the interspecies toxicokinetic factor to 0.17 using the most conservative comparator of AUC on day 1 (see Table E.2 below).

The low systemic exposure to NAA in goats appears to be due to its degradation in the ruminant digestive tract as there is no initial peak and being a small molecule extensive absorption of NAA would be expected. The ruminant digestive tract is reported to be consistent across ruminant species and degradation in the digestive tract is independent of absorption and distribution. Therefore, there would be expected to be little variation across the ruminant species. Suckling ruminants might be outliers in terms of the toxicokinetics of NAA as they have a less developed ruminant digestive system, but as NAA is not lipophilic and is unlikely to concentrate in milk, exposures to NAA via milk are not considered to be significant compared to those from direct consumption of feed. An interindividual toxicokinetic factor of 1 would be supported.

In conclusion, an overall CSAF in ruminants would be 1.3 (0.17 × 2.5 × 1 × 3.16).

Table E.1: Results of the toxicokinetics of NAA in the plasma of goats, pigs and rats

Compound	Time point (h)	Species, route of exposure, dose (mg/kg bw per day)			
		Goat	Pig	Rat	
		Capsule	Feed (small amount)	Gavage	
		25	25	25	500
	Day 1				
NAA (ng/mL)	0*	45	47	71	74
	0.5	53	3,795	8,300	40,300
	1	58	4,930	4,525	77,400
	2	208	1,840	766	98,150
	12	51	42	65	296

Compound	Time point (h)	Species, route of exposure, dose (mg/kg bw per day)			
		Goat	Pig	Rat	
		Capsule	Feed (small amount)	Gavage	
		25	25	25	500
Cmax (ng/mL)	–	410	4,930	8,550	98,100
AUC 0–12 h (h.ng/mL)	–	1,700	8,545	10,160	314,335
Tmax (h)	–	4	1	0.25–0.5	2
Aspartic acid (ng/mL)$	0*	1,245	1,555	6,900	3,970
	0.5	993	1,415	4,530	4,680
	1	855	1,460	5,120	8,080
	2	1050	2,120	4,000	5,950
	12	970	1,650	4,300	3,830
	Day 14				
NAA (ng/mL)	0*	52	51	70	112
	0.5	53	4,700	9,915	52,300
	1	58	7,240	3,650	97,000
	2	130	2,570	4,400	98,500
	12	56	58	74	400
Cmax (ng/mL)	–	171	7,200	13,000	98,500
AUC 0–12 h (h.ng/mL)	–	625	12,430	10,300	318,350
Tmax (h)	–	2–4	1	0.25–0.5	2
Aspartic acid (ng/mL)	0*	1,415	1,550	4,730	1,260
	0.5	914	1,170	5,500	3,420
	1	952	2,520	6,060	5,430
	2	1,015	2,000	5,690	3,140
	12	1,150	2,115	5,620	1,720

*: Sample taken prior to dosing, representing the background concentration..
$: Aspartic acid is reported to be the primary metabolite of NAA and is itself rapidly metabolised.
AUC – Area Under the Curve – an integration of the concentration in plasma over time.
Cmax – The peak concentration in plasma – modelled to cover changes between sampling times.
Tmax – The time at which Cmax occurs.

Table E.2: Summary comparison of the mean toxicokinetic values of NAA on rats, goats and swine

Dose (mg/kg/bw per day)	Species			
	Goat	Pig	Rat	
	25	25	25	500
Day 1				
NAA Cmax (ng/mL)	410 (0.05)*	4,930 (0.6)	8,550	98,100
NAA AUC 0–12 h (h.ng/mL)	1,700 (0.17)	8,545 (0.84)	10,160	314,335
Day 14				
NAA Cmax (ng/mL)	171 (0.013)	7,200 (0.55)	13,000	98,500
NAA AUC 0–12 h (h.ng/mL)	625 (0.06)	12,430 (1.2)	10,300	318,350

*: Expressed as proportion of rat value at 25 mg/kg bw per day.

Systemic CSAF for pigs/swine

The toxicokinetic data on NAA (Table E.1) show that the peak concentration in pigs is lower, by a factor of < 2, on days 1 and 14 than that in rats administered the same dose (25 mg/kg bw per day). The AUC is lower in pigs than rats on day 1, but higher (1.2-fold) on day 14. These data indicate that the toxicokinetics of NAA in pigs is essentially the same in pigs and rats and support a reduction in the interspecies toxicokinetic factor to 1. The toxicokinetics in pigs (assuming a body weight 30 kg) and

rats (assuming a body weight 200 g) are similar and the key metabolic step(s) in the degradation of NAA is likely to be simple, as it is water soluble and would not require conjugation for excretion. NAA is a normal component of the blood of rats, goats and pigs and it is considered reasonable to assume that there will be no significant differences in the toxicokinetics of NAA between pigs and other swine. Suckling animals might be outliers in terms of the toxicokinetics of NAA, but as NAA is not lipophilic and is unlikely to concentrate in milk, exposures to NAA via milk are not considered to be significant compared to those from direct consumption of feed. A reduction in the interindividual toxicokinetic factor to 1 is proposed.

In conclusion, an overall CSAF for swine would be 8 ($1 \times 2.5 \times 1 \times 3.16$).

Other monogastric animals

As data are available only from two monogastric animals (rats and pigs), it is considered that any change from the default values for extrapolating from pigs to different species of monogastric animals is not appropriate, as it is not supported by any specific data.

Therefore, an overall CSAF for other monogastric animals would be 25 ($2.5 \times 3.16 \times 1 \times 3.16$).

Table E.3: Outline of the derivation of the overall CSAFs based on the toxicokinetic studies and potential modes of action behind the effects of NAA on salivary glands

	Rat to standard species*		Standard species to different life stages or related species		Overall CSAF
	Toxicokinetic	**Toxicodynamic**	**Toxicokinetic**	**Toxicodynamic**	
Default	4	2.5	3.16	3.16	100
Direct Action[$]	1	2.5	1	3.16	8
Systemic action					
Ruminant	0.17	2.5	1	3.16	1.3
Swine	1	2.5	1	3.16	8
Other monogastric	1	2.5	3.16	3.16	25

*: Standard species are those used in the toxicokinetic studies i.e. goat for ruminants; pigs for swine and other monogastric animals.
$: Considered to be the more likely mode of action for the salivary gland hypertrophy.