FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Safety assessment – Application A1239

Food derived from EPA and DHA producing and herbicide-tolerant canola line LBFLFK

Executive summary

Background

Application A1239 seeks amendment to the Australia New Zealand Food Standards Code to permit the sale and use of food derived from genetically modified (GM) canola line LBFLFK. The canola line produces omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the seed and has tolerance to imidazolinone herbicides.

The production of EPA and DHA in LBFLFK is achieved by the introduction of ten genes encoding various desaturase and elongase enzymes involved in the fatty acid (FA) biosynthesis pathway. The genes were derived from microalgae, water moulds and moss.

Tolerance to imidazolinone herbicides is achieved through the expression of the acetohydroxy acid synthase (*AHAS*) gene from *Arabidopsis thaliana*. Imidazolinone tolerance was used for selection of putative transformants during the transformation stage as well as for selective post-emergence weed control during field production. The AHAS protein has been previously assessed by FSANZ.

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

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

Further assessment of n-3 LC-PUFAs in canola line LBFLFK was undertaken as part of a nutrition risk assessment, and is described in Supporting Document 2.

History of use

Canola is the second largest oilseed crop in the world behind soybean. It has a long history of safe use in the food supply. Canola oil is routinely used directly for cooking and as an ingredient in a variety of manufactured food products including salad and cooking oil, margarine, shortening and a range of prepared foods such as mayonnaise, sandwich spreads, creamers and coffee whiteners. Whole canola seeds are being used increasingly in

products such as breads. Canola meal provides a good source of protein in stock feed for a variety of animals, primarily pigs, poultry and dairy cattle.

Molecular characterisation

Canola line LBFLFK was generated through *Agrobacterium*-mediated transformation with a single T-DNA containing thirteen expression cassettes. Detailed molecular analyses indicate there are two insertion sites on different chromosomes and a single fully functional T-DNA exists at each insertion site. There are no extraneous plasmid sequences or antibiotic resistance genes present in this line.

The introduced genetic elements were shown by molecular techniques and phenotypic analyses to be stably inherited across multiple generations. The pattern of inheritance supports the conclusion that the introduced traits occur in two loci in the LBFLFK genome and are inherited according to Mendelian principles.

Characterisation and safety assessment of new substances

The LBFLFK expresses eleven new proteins – 10 desaturase and elongase proteins as well as the AHAS protein.

Eight of the ten proteins in the FA biosynthesis pathway were only detected in seeds of LBFLFK. This is expected since the genes encoding the proteins all have seed-specific promoters. The levels of all eight proteins were low. The remaining two proteins were below the levels of detection and quantification in LBFLFK seeds. The newly expressed AHAS protein, driven by a constitutive promoter, was quantifiable in every tissue except mature seed.

Bioinformatic studies confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens. Laboratory studies demonstrated the newly expressed proteins would be thoroughly degraded before being absorbed in the gastrointestinal tract. The proteins are also susceptible to heat denaturation and degradation at the high temperatures typically used in food processing.

Herbicide metabolites

The spraying of LBFLFK with imidazolinone herbicides produces the same metabolites that arise when non-GM canola is sprayed with the same herbicide.

Compositional analyses

Detailed compositional analyses were undertaken on seed, crude oil and refined, bleached and deodorized (RBD) oil from LBFLFK. Seed samples were harvested for seed composition analysis from 12 field trials across two growing seasons: winter 2014/2015 and spring 2015. Seeds from a further five field trials conducted in 2016 were used for oil compositional analysis. Seed and oil samples were analysed for proximates, FAs, amino acids, minerals, vitamins, phytosterols, and anti-nutrients. The levels of each analyte in canola line LBFLFK were compared to levels in: a) the non-GM parental line, Kumily; b) six non-GM commercial reference lines grown at the same locations; and c) levels published in the literature.

In seeds, a total of 28 analytes (including individual FAs) differed significantly between LBFLFK and Kumily. In oil, 24 (crude oil) and 22 (RBD oil) individual analytes (including FAs) differed significantly between LBFLFK and Kumily. The changes in FA profile in both seed and oil were consistent with those expected as a result of the introduction of the EPA and DHA producing trait.

Apart from the intended change to the FA profile and a slightly higher content of trans fatty acids (TFAs) in seed and crude oil, canola line LBFLFK is otherwise compositionally equivalent to conventional canola varieties.

Conclusion

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

Table of contents

Index of Figures

Index of Tables

List of Abbreviations

Abbreviation	Description
AHAS	acetohydroxy acid synthase
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
CI	confidence level
COMPARE	COMprehensive Protein Allergen REsource
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dw	dry weight
EPA	eicosapentaenoic acid
ELISA	enzyme-linked immunosorbent assay
FA	Fatty acid
fw	fresh weight
FASTA	fast alignment search tool – all
FSANZ	Food Standards Australia New Zealand
g	gram
GM	genetically modified
h	hours
ILSI	International Life Sciences Institute
kDa	kilodalton
LB	left border of T-DNA
LOD	limit of detection
LOQ	limit of quantitation
min	minutes
LC-PUFA	long-chain polyunsaturated fatty acid
MT	million tons
n-3	omega-3
NCBI	National Centre for Biotechnology Information
ng	nanogram
NGS	next generation sequencing
ns	not significant
OECD	Organization for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
PCR	polymerase chain reaction
PPP	plant produced protein
RB	right border of T-DNA
SE	standard error
T-DNA	transfer DNA
TFA	trans fatty acid
µg	microgram
UTR	untranslated region

1 Introduction

FSANZ has received an application from BASF Australia Ltd to amend the Australia New Zealand Food Standards Code. The amendment is to include food derived from the genetically modified (GM) canola line LBFLFK, with the OECD Unique Identifier BPS-BFLFK-2 (herein referred to as LBFLFK). This canola line has been genetically modified to introduce the pathway for production of the omega-3 (n-3) long-chain polyunsaturated fatty acid (LC-PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Coding sequence from ten genes in the fatty acid (FA) synthesis pathway has been introduced as follows:

- Δ12 desaturase [D12D(*Ps*)] from the *Phytophthora sojae*
- Δ6 desaturase [D6D(*Ot*)] from *Ostreococcus tauri*
- Δ6 elongase [D6E(*Tp*)] from *Thalassiosira pseudonana*
- Δ6 elongase [D6E(*Pp*)] from *Physcomitrella patens*
- Δ5 desaturase [D5D(*Tc*)] from *Thraustochytrium sp*.
- Ω3 desaturase [O3D(*Pir*)] from *Phytium irregular*
- Ω3 desaturase [O3D(*Pi*)] from *Phytophthora infestans*
- Δ5 elongase [D5E(*Ot*)] from *Ostreococcus tauri*
- Δ4 desaturase [D4D(*Tc*)] from *Thraustochytrium sp*.
- Δ4 desaturase [D4D(*Pl*)] from *Pavlova lutheri*

In addition, LBFLFK also contains the acetohydroxy acid synthase (*AHAS*) gene from *Arabidopsis thaliana* that confers tolerance to the broad spectrum imidazolinone herbicides (e.g.imazamox). The imidazolinone tolerance was used for selection of putative transformants during the transformation stage as well as for selective post-emergence weed control during field production. The AHAS protein has been previously assessed by FSANZ (A1064).

The applicant states the purpose of LBFLFK is to provide a sustainable and land-based source of EPA and DHA, to meet increased human consumption and demand on aquaculture.

Canola line LBFLFK will be cultivated in other countries such as Canada and the United States. The applicant has not stated any intent to cultivate LBFLFK in either Australia or New Zealand. It is therefore anticipated food products derived from LBFLFK will enter the Australian and New Zealand food supplies via imports from major canola-producing countries.

2 History of use

2.1 Host organism

The host organism is canola (*Brassica napus L.*) and the parental variety used for the genetic modification is Kumily; a spring variety. The Kumily host organism was used as the conventional control for the purposes of comparative assessment with LBFLFK.

Canola is a highly domesticated crop that has a history of safe consumption. Canola belongs to the Brassicaceae family, also commonly referred to as the mustard or cabbage family. A number of vegetable species including broccoli, cabbage, turnip and radish belong to this family.

Canola is the name used for rapeseed (*Brassica napus*, *Brassica rapa* or *Brassica juncea*) which has been conventionally bred to contain less than 2% erucic acid (a FA)[1] and less than 30 micromoles of glucosinolates per gram of seed solids (OECD 2001). It was first developed in Canada in the 1970s, in response to demand for food-grade rapeseed products and animal feed with improved palatability. Rapeseed-derived products that do not meet the compositional standard cannot use the trademarked term, 'canola'. In some countries, the term canola is not used and the low erucic acid crop is known generically as rapeseed.

Canola is one of the most important sources of the long-chain monounsaturated oleic acid (OA), omega-6 (n-6) LC-PUFA linoleic acid (LA), and n-3 LC-PUFA α-linolenic acid (ALA). It does not, however, produce other important LC-PUFAs such as EPA and DHA.

Canola is the second largest oilseed crop in the world behind soybean. In 2020, world production was 86.38 MT[2] and the major oilseed-rape producing countries were Canada (19.5 MT), the European Union (16.6 MT), China (14.0 MT), India (9.1 MT), Russian Federation (2.6 MT) and Ukraine (2.6 MT); and Australia is ranked 7th at 2.3 MT (FAOSTAT, 2020). In the same year, New Zealand canola production was minor at approximately 2204 tonnes. In 2020, Canada was the largest exporter of canola accounting for around 50% of world trade. While, Australia was the fourth largest exporter at about 8.2% of global trade. Australia's major export destinations in 2019/20 were Europe, China and Japan (FAOSTAT, 2020).

In Australia, canola is the third largest broad acre crop behind wheat and barley and the growing area extends from south-western Western Australia to south-eastern Australia and northern New South Wales. About 20% of Australia's canola crop is genetically modified (OGTR, 2021).

Canola seeds are processed into two major products, oil and meal. The oil is the major product for human consumption and has a long history of safe use. Canola oil is used in a variety of manufactured foods including salad and cooking oil, margarine, shortening and a range of prepared foods such as mayonnaise, sandwich spreads, creamers and coffee whiteners. Whole canola seeds are being used increasingly in products such as breads. The meal provides a good source of protein in stock feed for a variety of animals; primarily for pigs, poultry and dairy cattle. More recently, protein isolate from canola meal was approved as a new food ingredient in Australia and New Zealand under Application A1175.

2.2 Donor organisms

2.2.1 Microalgae

Microalgae are quite diverse in their lipid profiles and abundance, which in recent years has become a point of interest as it serves as a sustainable source of essential lipids for human nutritional products as well as aquaculture feed. Some microalgae are rich in n-3 LC-PUFAs which make them commercially important for various applications in nutraceuticals, pharmaceuticals and dietary supplements (Maltsev and Maltseva, 2021). In recent years, microalgae have been exploited as a source for FA synthesis genes that can be used in the genetic modification of other organisms, particularly plants (Zhou et al., 2007; Petrie and Singh, 2011; Napier et al., 2015; Ruiz-Lopez et al., 2015). Microalgae used as a source of FA synthesis genes in LBFLFK are discussed below.

[1] Codex Standard for Named Vegetable Oils (CODEX-STAN 210-1999) - https://www.fao.org/3/y2774e/y2774e04.htm#bm4.1
[2] million tons

Ostreococcus tauri

The DNA sequences encoding the delta-6 desaturase *D6D*(*Ot*) and delta-5 elongase *D5E*(*Ot*) genes are derived from *Ostreococcus tauri* (*O. tauri*), a unicellular marine green microalga. *O. tauri* is part of the marine phytoplankton community and is not directly consumed as food or feed. However, *O. tauri* is indirectly consumed by fish consumed by humans (Worden et al., 2004).

Pavlova lutheri

The DNA encoding the delta-4 desaturase *D4D*(*Pl*) gene was derived from *Pavlova lutheri* (*P. lutheri*), a marine microalga. *P. lutheri* is commonly used as marine feed in the aquaculture industries (Ponis et al., 2006). Although, *P. lutheri* is not consumed directly as food but may end up as human food indirectly via shellfish[3] consumption (Aranda-Burgos et al., 2014; Semura 1995; Gonzales-Araya et al., 2013; D'Souza et al., 2000; Kashenko 2007).

Thalassiosira pseudonana

The delta-6 elongase *D6E*(*Tp*) gene was sourced from *Thalassiosira pseudonana* (*T. pseudonana*), a marine diatom. Diatoms are photosynthetic eukaryotic microalgae (Wang and Seibert, 2017). *T. pseudonana* is not directly consumed as food but is often used as aqua feed for marine organisms such as prawns (D'Souza et al., 2000), pacific oysters (Thompson and Harrison, 1992; Thompson et al., 1996), clams (Li et al., 2002; Liu et al., 2016), copepods (Harris 1977), and basket cockle (Liu et al., 2009). Therefore, human consumption is would be indirect.

***Thraustochytrium* sp**

The DNA sequences encoding the delta-4 desaturase D4D(*Tc*) and delta-5 desaturase D5D(*Tc*) genes was derived from *Thraustochytrium* sp; a marine eukaryotic protist. They are heterotrophic in nature and accumulate large amounts of triacylglycerols with high proportions of LC-PUFAs, particularly DHA (Patel et al., 2020). *Thraustochytrium* sp are not directly consumed as food however, indirect human consumption may occur via consumption of mussels, clams and fish (Bergé and Barnathan, 2005).

2.2.2 Oomycetes

Oomycetes are a distinct class of fungus-like eukaryotic microorganisms, many of which are highly destructive plant pathogens (Fry and Grünwald, 2010). They are filamentous, saprophytic and reproduce via spores. They are also known as water moulds. Oomycetes used as donor organisms as a source for FA synthesis genes in LBFLFK are discussed below.

[3] Scallops, oysters, carpet shells, giant tiger prawns and sea urchins.

Phytophthora infestans

Phytophthora infestans (*P. infestans*), the source organism of the omega-3 desaturase O3D(*Pi*) gene is a plant pathogen that causes potato and tomato disease known as late blight (Fry and Grünwald, 2010). The asexual life cycle enables rapid population growth in susceptible host. *P. infestans* are not consumed as food or feed.

Phytophthora sojae

The source organism for delta-12 desaturase *D12D*(*Ps*) is *Phytohthora sojae* (*P. sojae*), a soil-borne pathogen that causes stem and root rot disease in soybean causing severe economic damages (Tyler 2007). *P. sojae* is not consumed as food or feed.

Phythium irregulare

The omega-3 desaturase *O3D*(*Pir*) was sourced from *Phythium irregulare* (*P. irregulare*); an oomycete that has been identified as a microorganism that can produce health beneficial, valuable PUFA rich oils. The biomass and oil derived from *P. irregulare* is used as an ingredient in food, feed and pharmaceuticals and is considered safe. However, it is not directly consumed as food or feed (Wu et al., 2013).

2.2.3 Physcomitrella patens

Physcomitrella patens (*P. patens*) or commonly known as moss was the source organism for delta-6 elongase *D6E*(*Pp*). *P. patens* is a model organism used for studies on plant evolution, development and physiology and has been increasingly used as a bio-factory for the production of biopharmaceutical products like glycosylated peptide hormone erythropoietin (EPO) (Simonsen et al., 2009). *P. patens* is not consumed as food or feed.

2.2.4 Arabidopsis thaliana

The donor organism for the acetohydroxy acid synthase *AHAS* gene is *A. thaliana* (common names: thale cress, mouse ear cress). *A. thaliana* is a small flowering plant belonging to the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish. Arabidopsis is widely used as a model organism in plant biology and genetics, and its genome has been fully sequenced.

Although a member of the mustard family, *A. thaliana* is not commonly cultivated or harvested for food due to its small size; it therefore does not have a history of significant human consumption. There are however no reports of *A. thaliana* being allergenic or a source of toxins. *A. thaliana* has been previously assessed by FSANZ as a donor organism in numerous other applications.

2.2.5 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of LBFLFK. These genetic elements are non-coding sequences that are used to regulate the expression of the ten genes for the FA synthesis pathway and the *AHAS* gene (refer to Appendix 1).

3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

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

3.1 Transformation method

To create LBFLFK, the canola variety Kumily was transformed with the LTM593-1qcz plasmid (Figure 1) using *Agrobacterium*-mediated transformation. LTM593 contains the 11 genes organised into 13 expression cassettes; two gene expression cassettes were introduced twice (O3D(*Pir*) and D5D(*Tc*)) with different seed-specific promotors, between the right border (RB) and left border (LB) region of the transfer DNA (T-DNA).

Following transformation, hypocotyl segments of Kumily were transferred to shoot regeneration medium containing imazethapyr (an imodazolinone herbicide) for selection of putative transformed shoots, which were then screened for the presence of the FA synthesis genes. Further evaluation of insert integrity, FA profile, gene expression, phenotypic characteristics and agronomic performance resulted in the selection of the canola line LBFLFK.

3.2 Detailed description of inserted DNA

The transformation process that generated the canola line LBFLFK makes use of the LTM593-1qcz plasmid. The complete plasmid is 60,074 bp comprising 16,064 bp vector backbone and 44,010 bp T-DNA (Figure 1). The T-DNA consists of thirteen expression cassettes located between a 328 bp RB and 136 bp LB. The expression cassettes contained within the T-DNA of LTM593 are further detailed in Table 1.

In order to facilitate a LC-PUFA biosynthesis pathway, a series of aerobic desaturations and elongations are required. To do this, seven desaturases and three elongases were introduced via genetic modification. Desaturases and elongases play critical roles in regulating the length and degree of unsaturation of FAs. Desaturases remove two hydrogen atoms from a FA, creating a carbon double bond, a process known as desaturation. While elongases facilitate the addition of two carbon atoms. Detailed steps involved in LC-PUFA biosynthesis are illustrated in Figure 2.

The transcription of all the FA synthesis enzyme genes is initiated by seed-specific promoters that confine the synthesis pathway to the seed. The coding sequences of all FA synthesis enzyme genes have been codon optimised for expression in *B. napus*. Information on the genetic elements in the T-DNA used for transformation is summarised in Table 1.

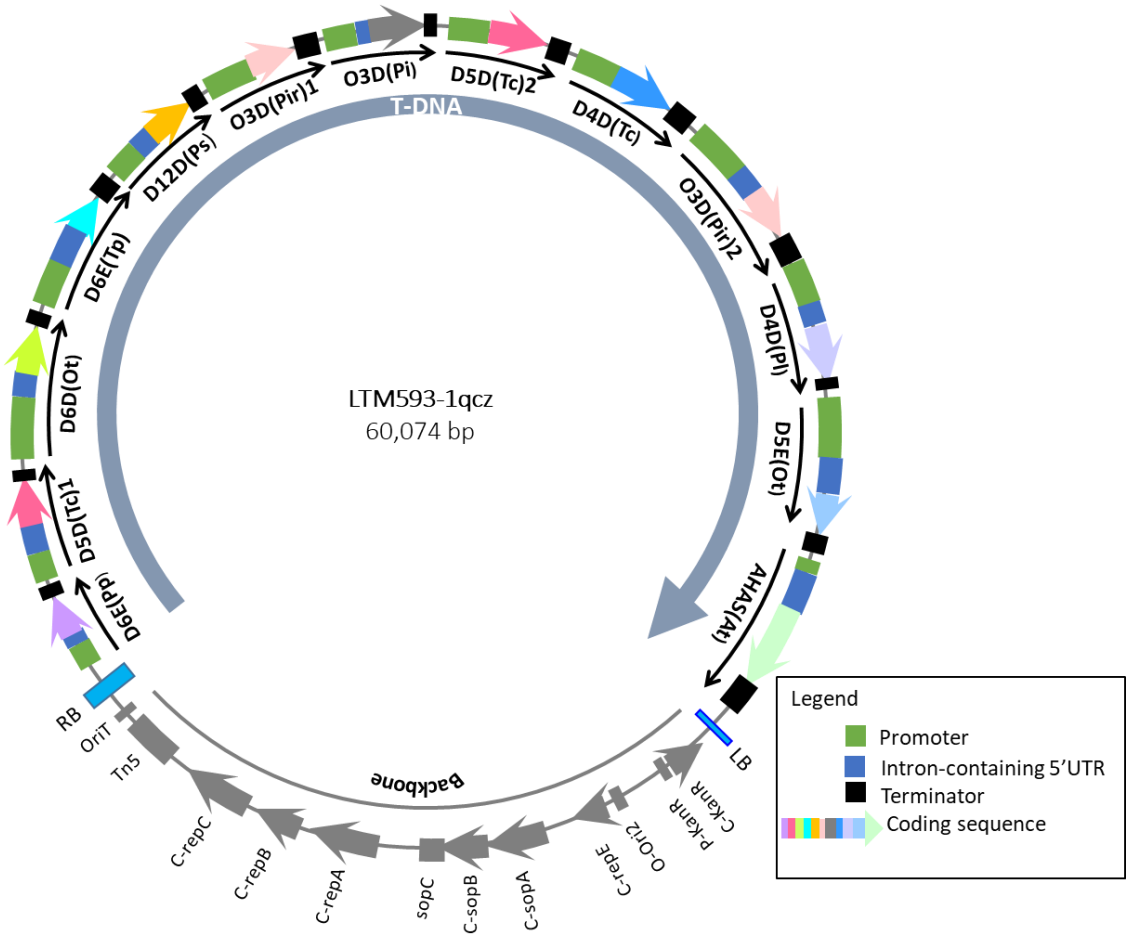

Figure 1: Plasmid map of LTM593-1qcz

The T-DNA contains thirteen expression cassettes (indicated by black arcs with arrowheads in the inner circle) named after the respective coding sequences. Where the same coding sequence is used twice [D5D(Tc) and O3D(Pir)], a suffix number is added to the cassette name in the inner circle. The vector backbone contains origin of replication, kanamycin-antibiotic resistance gene and several other elements that are required for preparing the plasmid, passaging through standard Escherichia coli (E.coli) and into the Agrobacterium. The vector backbone is not incorporated into the plant during transformation.

Table 1: Expression cassettes contained in the T-DNA of LTM593-1qcz
Expression cassettes are listed in the same order as presented in LTM593-1qcz (Figure 1).

Promoter (Drives transcription)	Intron-containing 5'UTR (Expression enhancer)	Coding sequence	Terminator (Polyadenylation and termination of transcription)
Promoter of unknown seed protein gene (*USP*) from *Vicia faba* (faba bean)	5'UTR from Arabidopsis locus At1g01170	Delta-6 elongase from *P. patens* *D6E*(*Pp*)	3'UTR derived from 35S Cauliflower Mosaic Virus (*CaMV35S*).
Promoter of sucrose-binding protein-related gene from faba bean	5'UTR of Arabidopsis locus At1g65090	Delta-6 desaturase from *O. tauri* *D6D*(*Ot*)	3'UTR of the cathepsin D inhibitor gene from *Solanum tuberosum* (potato)
Promoter of the conlinin gene from *Linum usitatissimum* (flax)	5'UTR from Arabidopsis locus At1g63190	Delta-5 desaturase 1 from *Thraustochytrium* sp. *D5D*(*Tc*)1	3'UTR of octopine synthase gene (*OCS*) from *Agrobacterium tumefaciens.*
Promoter of peroxiredoxin like protein gene (*PXR*) from flax	5'UTR of Arabidopsis locus At1g62290	Delta-6 elongase from *T. pseudonana* *D6E*(*Tp*)	3'UTR of *PXR*-like protein gene (*PER1*) from Arabidopsis
Promoter of napin gene from *B. napus*	5'UTR of Arabidopsis locus At5g63190	Delta-12 desaturase from *P. sojae* *D12D*(*Ps*)	3'UTR from the RuBisCO small subunit gene from *Pisum sativum*
Promoter of *SETL* gene from *B. napus*	None	Omega-3 desaturase 1 from *P. irregulare* *O3D*(*Pir*)1	3'UTR of *SETL* gene from *B. napus*
Promoter of *USP* gene from faba bean	5' UTR of Arabidopsis locus At1g01170	Omega-3 desaturase from *P. infestans* *O3D*(*Pi*)	3'UTR derived from *CaMV35S*
Promoter of *SETL* gene from *B. napus*	None	Delta-5 desaturase 2 from *Thraustochytrium* sp *D5D*(*Tc*)2	3'UTR of *SETL* gene from *B. napus*
Promoter of Arcelin-5 (*ARC5*) gene from *Phaseolus vulgaris* (Kidney bean)	None	Delta-4 desaturase from *Thraustochytrium* sp. *D4D*(*Tc*)	3'UTR of *ARC5* gene from kidney bean
Promoter of the *PXR* gene from flax	5'UTR of *AGO4* gene from Arabidopsis	Omega-3 desaturase 2 from *P. irregulare* *O3D*(*Pir*)2	3'UTR of *PER1* gene from Arabidopsis.
Promoter of the conlinin gene from flax	5'UTR of Arabidopsis locus At1g65090	Delta-4 desaturase from *P. lutheri* *D4D*(*Pl*)	3'UTR of *OCS* gene from *Agrobacterium tumefaciens.*
Promoter of the *FAE1* gene from *B. napus*	5'UTR from *Arabidopsis* locus At1g62290	Delta-5 elongase from *O. tauri* *D5E*(*Ot*)	3'UTR of *FAE1* gene from Arabidopsis
Promoter of ubiquitin gene (*Ubi4*) from *Petroselinum crispum* (Parsley)	5'UTR from *Ubi4* gene from parsley	*Arabidopsis thaliana* acetohydroxy acid synthase *AHAS*	3'UTR of *AHAS* gene from Arabidopsis

1, 2 = Where the same coding sequence is used twice [*D5D*(*Tc*) and *O3D*(*Pir*)], a suffix number is added to the expression cassette name to distinguish them.

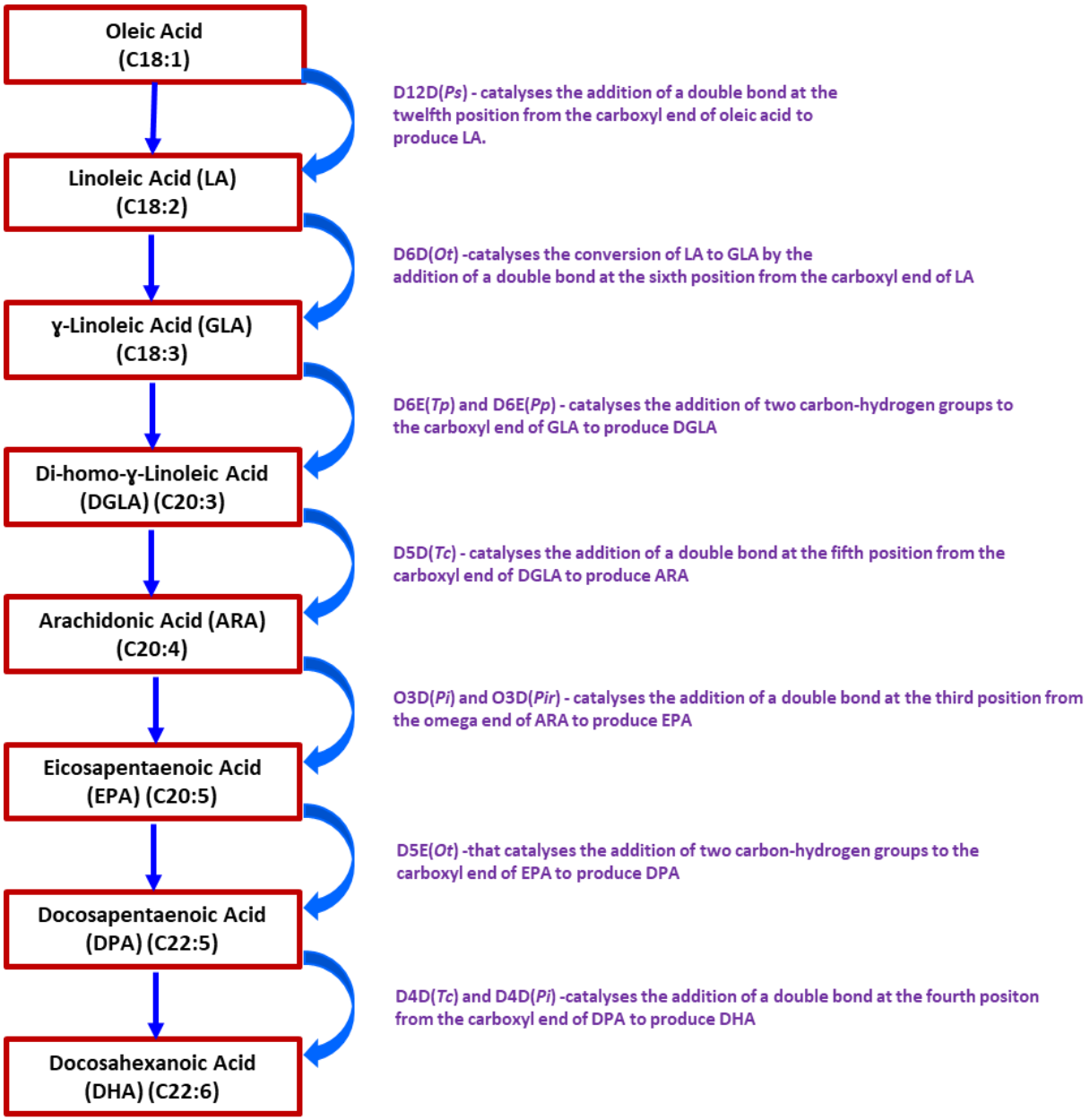

Figure 2: EPA and DHA biosynthesis pathway engineered into LBFLFK

3.3 Development of the canola line from the original transformation

A breeding program was undertaken for the purpose of:

- obtaining generations suitable for analysing the characteristics of LBFLFK; and
- ensuring that the LBFLFK event is stably inherited and expressed over several generations.

The breeding path from the original transformation (T0) and the specific generations of plants used in the characterisation of LBFLFK is shown in Figure 3. Table 2 indicates the analysis, generation and controls used in the characterisation of LBFLFK.

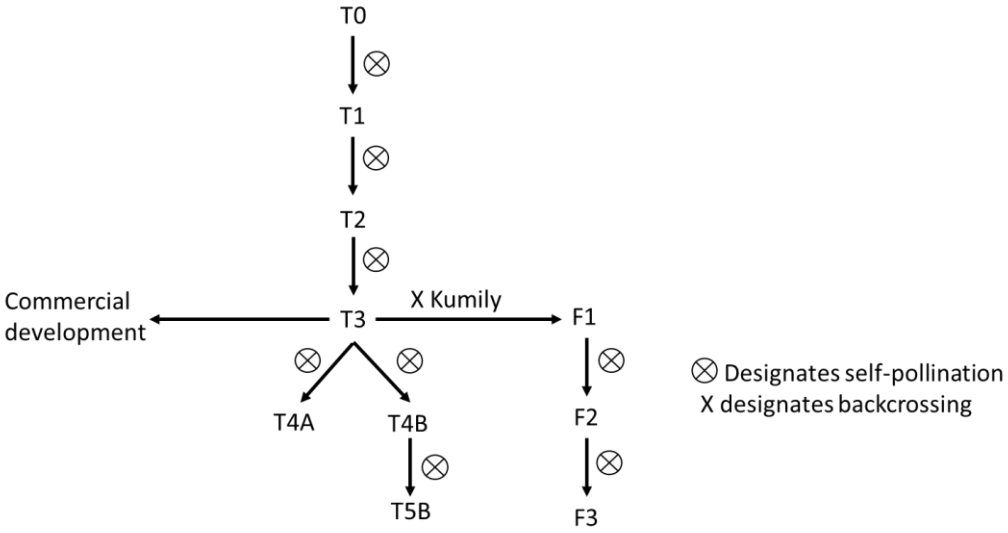

Figure 3: Breeding path used in the characterisation of the LBFLFK

Table 2: LBFLFK generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Number of integration sites	3.4.1	T3, T4, T5	*B. napus*-Kumily, LTM593
Absence of backbone and other sequences	3.4.2	T3	*B. napus*-Kumily, LTM593
Insert integrity and site of integration	3.4.3	T3	*B. napus*-Kumily,
Genetic stability	3.4.4	T3, T4, T5	*B. napus*-Kumily,
Mendelian inheritance	3.4.4.2	F2 & F3	*B. napus*-Kumily,
Expression of phenotype over several generations	3.4.4.2	T4	*B. napus*
Compositional analysis	5	T3, T4	*B. napus*-Kumily,

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

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

3.4.1 Identifying the number of integration site(s)

Next-generation sequencing (NGS) was performed on the seed-derived genomic DNA from LBFLFK and the parental line Kumily as control. Additionally, plasmid DNA served as a reference control for sequencing and a transformation plasmid spike was sequenced to assess the sensitivity of the NGS. Short sequence reads (~125 bp) were prepared and sequenced. Sufficient sequence fragments were obtained to cover the inserted T-DNA and selected *B. napus* endogenous genes, with a depth coverage of 160X and an adequate level of sensitivity[4].

Comparison of the sequence between LBFLFK and the Kumily control, detected four unique

[4] The NGS method was sufficiently sensitive to detect 100% of the spiked plasmid when present at 1/10th of a copy per genome equivalent or greater.

insert-flank junction sites, identifying two T-DNA integration sites in the genome of LBFLFK (Figure 4). No additional unique insert-flank junction sites were identified confirming that each T-DNA insertion site in LBFLFK consists of a single T-DNA copy from LTM593. As expected, no junctions were detected in the control, Kumily.

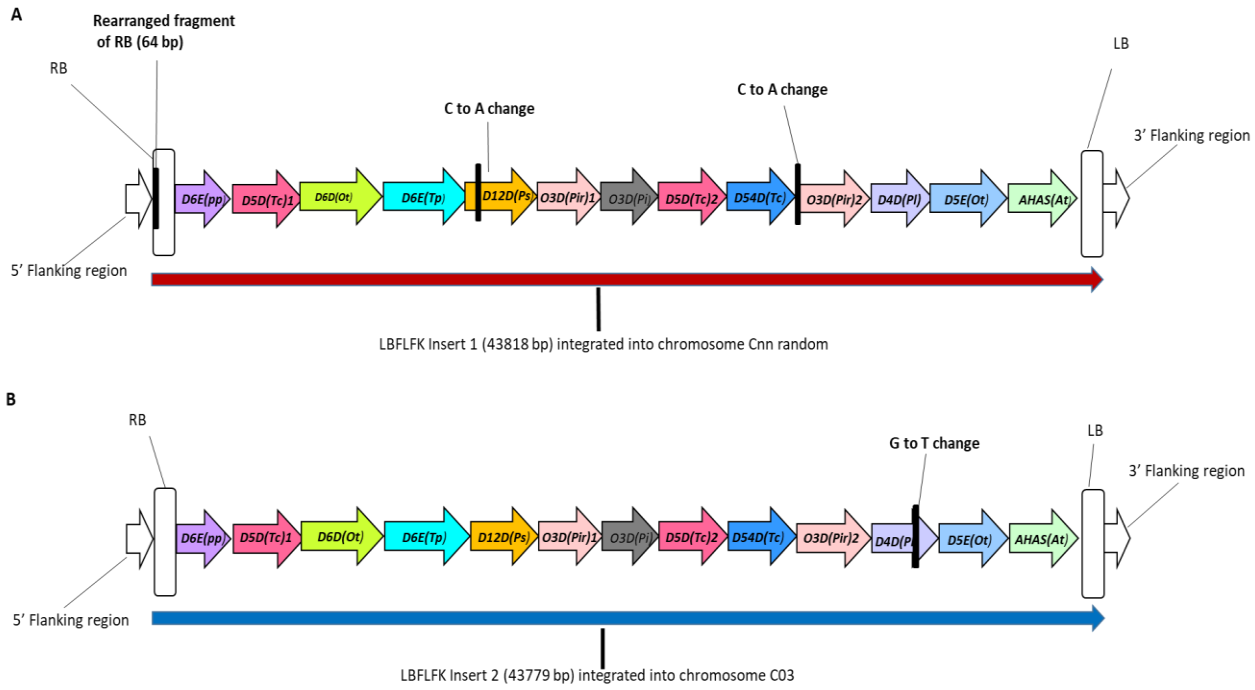

Figure 4: Insert and flanking sequences present in LBFLFK

3.4.2 Absence of backbone and other sequences

NGS reads from LBFLFK (T3) and the backbone sequence of LTM593 transformation plasmid were aligned. The results of this alignment confirmed there was no integration of LTM593 backbone sequences, including any antibiotic resistance genes, into the LBFLFK genome.

3.4.3 Insert integrity and site of integration

The integrity and adjacent canola genomic sequences of the two T-DNA inserts was examined by locus-specific PCR followed by Sanger sequencing. To analyse each locus, Bacterial Artificial Chromosome (BAC) clones containing LBFLFK Insert 1 and LBFLFK Insert 2 were generated from T3 leaf material.

A comparison of the sequences obtained from the 3' and 5' flanking regions of the two T-DNA inserts in LBFLFK with Kumily genome sequences identified Kumily sequences flanking the insertion sites. The identified sequences were further subjected to homology searches against the *B. napus* genome sequence from cultivar Darmor-*bzh* (Chalhoub et al., 2014). These searches located the two T-DNA inserts in two separate chromosomes; LBFLFK Insert 1 integrated into chromosome "Cnn random" and LBFLFK Insert 2 integrated into "C03" chromosome. In addition, an 8-bp deletion of the Kumily genome at the integration site of the LBFLFK Insert 1 (chromosome Cnn random) and a 31-bp deletion of the Kumily genome at the integration site of LBFLFK Insert 2 (chromosome C03) were identified.

The sequence analysis of the BAC clones confirmed that both T-DNA inserts contained all 13 gene expression cassettes without rearrangements. LBFLFK Insert 1 had a 184-bp deletion

of the 5’ end of the RB and a 72-bp deletion of the 3’ end of the LB. In addition, the first 64 bp in the RB of LBFLFK Insert 1 was determined to be a rearrangement of a short T-DNA RB-derived repeats. LBFLFK Insert 2 had a 184-bp deletion of the 5’ end of the RB and a 53-bp deletion of the 3’ end of the LB. Such rearrangements are an expected outcome of T-DNA integration (Gheysen et al., 1991; Mayerhofer et al., 1991).

The sequences of the T-DNA insert were determined to be identical to the vector reference sequence of the LTM593 except for two single nucleotide changes in LBFLFK Insert 1 and one nucleotide change in the LBFLFK Insert 2 (Figure 4):

- In LBFLFK Insert 1, one cytosine (C) to adenine (A) nucleotide change in the coding sequence of the *D12D*(*Ps*) gene which resulted in a phenylalanine to leucine amino acid substitution (F83L) in the D12D(*Ps*) protein. In addition, a cytosine to adenine nucleotide change was found in the promoter sequence p-*PXR*(*Lu*), which is a part of the *O3D*(*Pir*)2 expression cassette. This nucleotide change does not result in an amino acid substitution.
- In LBFLFK Insert 2, there was a guanine (G) to thymine (T) nucleotide change in the coding sequence of the *D4D*(*Pl*) gene. This change resulted in an alanine to serine amino acid substitution (A102S) in the D4D(*Pl*) protein.

These amino acid changes have no impact on the function or activity of the respective proteins.

3.4.4 Stability of the genetic changes in LBFLFK

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

3.4.4.1 Genetic stability

Genetic stability of the inserted DNA in LBFLFK was characterised through NGS combined with bioinformatics analysis. The junction sequences and sequencing read distributions over three LBFLFK generations (T3, T4, T5) were compared. Read depth distribution patterns across the entire T-DNA were uniform with similar peaks in read depth across the entire T-DNA sequence, and the same class of unique insert-flank junction sequences were observed in all three generations of LBFLFK tested. These results confirmed the genetic stability of the inserted DNA.

3.4.4.2 Phenotypic stability

Mendelian inheritance

The inserted DNA in LBFLFK would be expected to be inherited according to Mendelian principles (refer to Table 3). To confirm the segregation and stability of the inserted DNA, a Chi-square (X^2) analysis was undertaken over two generations starting with a LBFLFK T3 cross with Kumily (Figure 5). For the purpose of descriptive annotation, the alleles for the presence of the two T-DNA inserts were designated as 'A' and 'B' while those for the wild-type counterpart were designated as 'a' and 'b'.

Table 3: Expected genotype distribution in the F2 and F3 generations if inheritance follows Mendelian principles

Genotype	AABB	AABb	AAbb	AaBB	AaBb	Aabb	aaBB	aaBb	aabb
Expected segregation ratio	1/16	2/16	1/16	2/16	4/16	2/16	1/16	2/16	1/16

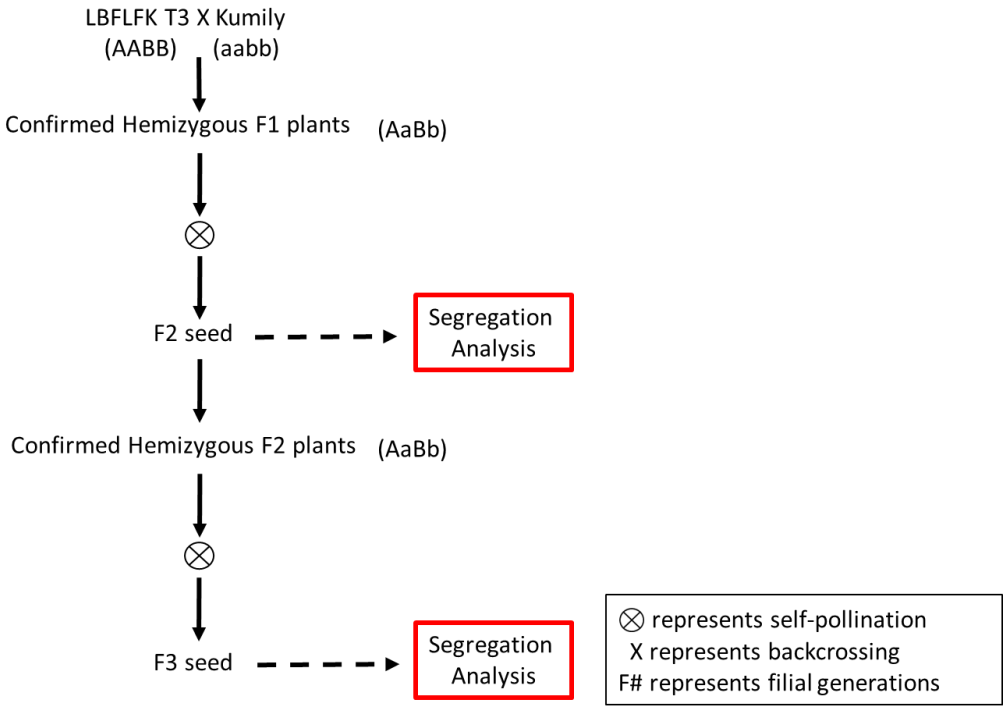

Figure 5: Breeding path used to assess inhertance and stability of LBFLFK

The observed ratio presented in Table 4A and 4B matches the expected results, indicating the inserted DNAs follow expected Mendelian inheritance rules. A p-value ≥ 0.05, at a 95% confidence level, shows the observed segregation matches the expected Mendelian inheritance pattern.

Table 4A: Results of segregation analysis of single F2 seeds.

Genotype	Locus LBFLFK Insert 1 (A)	Locus LBFLFK Insert 2 (B)	Expected segregation ratio	Observable Phenotype	Expected Phenotype	(Observed-Expected)^2/Expected
aabb	WT	WT	1/16	43	48	0.521
aaBB	WT	Hom	1/16	50	48	0.083
aaBb	WT	Het	2/16	105	96	0.844
Aabb	Het	WT	2/16	97	96	0.010
AAbb	Hom	WT	1/16	54	48	0.750
AaBb	Het	Het	4/16	200	192	0.333
AaBB	Het	Hom	2/16	98	96	0.042
AABb	Hom	Het	2/16	76	96	4.167
AABB	Hom	Hom	1/16	45	48	0.188

Chi squared Value X^2 = 6.938, p-value = 0.543

Table 4B: Results of segregation analysis of single F3 seeds.

Genotype	Locus LBFLFK Insert 1 (A)	Locus LBFLFK Insert 2 (B)	Expected segregation ratio	Observable Phenotype	Expected Phenotype	(Observed-Expected)^2/Expected
aabb	WT	WT	1/16	50	47.688	0.112
aaBB	WT	Hom	1/16	45	47.688	0.151
aaBb	WT	Het	2/16	100	95.375	0.224
Aabb	Het	WT	2/16	100	95.375	0.224
AAbb	Hom	WT	1/16	49	47.688	0.036
AaBb	Het	Het	4/16	189	190.75	0.016
AaBB	Het	Hom	2/16	84	95.375	1.357
AABb	Hom	Het	2/16	98	95.375	0.072
AABB	Hom	Hom	1/16	48	47.688	0.002

Chi squared Value X^2 = 2.195, p-value = 0.974

Expression of phenotype over several generations

The applicant provided NGS data combined with bioinformatics analyses showing the 11 inserted genes are expressed in multiple generations; T3, T4, and T5 (Section 3.4.4). LBFLFK expresses D12D(*Ps*), D6D(*Ot*), D5E(*Ot*), D5D(*Tc*), D6E(*Tp*), D4D(*Pl*), D4D(*Tc*) and O3D(*Pir*) proteins at multiple generations (T3, F1 and F2). D6E(*Pp*) and O3D(*Pi*) could not be detected due to expressions levels below the limit of quantification (LOQ) (Section 4.1.3). Additionally, LBFLFK displays imidazolinone tolerance or expresses the AHAS protein and displays the EPA and DHA producing trait across multiple growing season and regions (data provided in Section 5). Together this indicates the EPA and DHA producing trait and imidazolinone tolerance phenotype in LBFLFK are stable over several generations.

3.4.5 Open reading frame analysis

An *in silico* analysis of the flanking regions was undertaken to identify whether any novel open reading frame (ORF) had been created in LBFLFK as a result of the T-DNA insertions. Sequences spanning the 5' and 3' insert-flank junctions of the LBFLFK were translated from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames[5]. A total of 647 ORFs that corresponded to putative peptides of 30 amino acids or greater in length were investigated further to determine whether their amino acid sequence showed similarity with known allergen and toxin peptide sequences in established databases.

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

In addition to the junction regions, the entire LBFLFK T-DNA Insert 1 and T-DNA Insert 2 was translated *in silico* in all six reading frames. The resultant amino acid sequences cover any putative peptides present in the entire inserted DNA. These were used as query sequences in homology searches for known allergens and toxins in established databases.

These analyses are theoretical only as there is no reason to expect that any of the identified ORFs or putative peptides would, in fact, be expressed.

3.4.5.1 Bioinformatic analysis for potential allergenicity

The applicant has provided the results of *in silico* analyses using putative peptides present in the 5' and 3' insert-flank junctions sequences and the amino acid sequences encoded by all six reading frames present in the LBFLFK T-DNA inserts. These sequences were compared against known allergenic proteins listed in the COMprehensive Protein Allergen Resource (COMPARE[6]) database, from the Health and Environmental Science Institute. At the date of the search, there were 2,081 sequences in the allergen database (COMPARE 2019).

Three types of analyses were performed for this comparison:

(a) full length sequence search – a FASTA alignment using a BLOSUM50 scoring matrix. Only matches with E-scores of $\leq 1\times10^{-5}$ were considered;
(b) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids to the database entries. Only matches of greater than 35% similarity over ≥ 80 amino acids were considered;
(c) 8-mer exact match search - SeqMatchAll tool from the European Molecular Biology Open Software Suite (EMBOSS) (6) (version 6.6.0) was used to identify whether an 8 amino acid peptide match existed between the query sequences and sequences within the allergen database. Only matches of 100% similarity over 8 amino acids were considered.

The alignment of the 647 putative peptides present in the 5' and 3' insert-flank junctions with database sequences did not identify any matches. The results of this analysis support the conclusion that there were no matches of significance or concern.

3.4.5.2 Bioinformatic analysis for potential toxicity

The applicant performed two *in silico* comparative analyses using the NCBI non-redundant peptide sequence database (NCBI_NR_2020_906[7]) and an in-house toxin database to identify any potential amino acid sequence similarity to known toxins. At the date of the search, there were 195,624,353 sequences in the NCBI database and 187,356 sequences in the in-house toxin database. A FASTA algorithm was used with a BLOSUM50 scoring matrix and the E-value threshold conservatively set to 1×10^{-5}. No matches were found between the 647 putative peptides present in the 5' and 3' insert-flank junctions and any of the known protein toxins.

3.4.6 Conclusion

The data provided by the applicant showed that two DNA insertions occurred at two loci in the LBFLFK genome. DNA sequencing confirmed the presence of a single fully functional T-DNA at each insertion site in the LBFLFK genome. The introduced DNA is stably inherited from one generation to the next. No new ORFs were created by the DNA insertions that would raise potential allergenicity or toxicity concerns should they be expressed.

[6] http://comparedatabase.org/database/
[7] NCBI Resource Coordinators, 2016. Database resources of the National Center for Biotechnology Information. Nucleic Acids Research 44(Database issue), D7-D19.

4 Characterisation and safety assessment of new substances

The main purpose of the characterisation is to describe the nature of any new substances and their phenotypic and biochemical effects on the organism in which they are expressed, particularly in the parts of the organism consumed as food. Typically, the main focus of the characterisation is on newly expressed (or potentially expressed) proteins, but other (non-protein) substances may also be considered.

4.1 Newly expressed proteins

In considering the safety of newly expressed proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects.

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

To effectively identify any potential hazards, knowledge of the characteristics, detailed understanding of the biochemical function and phenotypic effects and concentration levels in the edible part of the plant is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

Two types of proteins were considered:

(a) the proteins expected to be directly produced as a result of the translation of the introduced genes. A number of different analyses were done to characterise these proteins and determine their *in planta* expression.
(b) those that may be potentially expressed as a result of the creation of ORFs during the transformation process (see Section 3.4.5).

4.1.1 AHAS protein

The AHAS protein encoded by *csr1-2* gene from plant *Arabidopsis thaliana* consist of 670 amino acids, where the first 64 amino acids represent the putative chloroplast transit peptide that is not part of the mature protein (606 amino acids). AHAS catalyses the first common step in the metabolic pathway for the biosynthesis of the branched chain amino acids valine, leucine and isoleucine (Stidham and Singh, 1991; Duggleby and Pang, 2000).The activity of AHAS is feedback-inhibited by the end product amino acids. The protein harbours two amino acid substitutions, A122T and S653N, known to interfere with the binding of imidazolinone herbicides (McCourt et al., 2006; Duggleby et al., 2008), resulting in imidazolinone tolerance, with no effect on either feedback inhibition by the branched-chain amino acids or normal catalytic function of the enzyme (Newhouse et al., 1992).

Several commercialised crops have herbicide tolerance conferred by alleles of the *AHAS* gene (e.g. Clearfield canola, Clearfield wheat, Clearfield sunflower, Clearfield lentils). The safety of AHAS enzymes expressed in crops has been extensively investigated (Mathesius et al., 2009; Chukwudebe et al., 2012). No reports of adverse effects due to exposure to AHAS enzymes have been reported.

Proteins with homologous amino acid sequence to AHAS are found in commonly consumed food such as *B. napus* (88.2% identity), chickpea (78.2% identity), apple (78.9% identity), and sunflower (77.6% identity). The AHAS protein has been previously assessed for safety by FSANZ[8].

4.1.2 Fatty acid desaturase and elongases

The desaturases and elongases expressed in LBFLFK are integral membrane proteins found in all organisms (i.e. plants, fungi, bacteria and animals). The desaturases catalyse the introduction of a double bond in the hydrocarbon chains of FAs to produce unsaturated FAs. The elongases catalyse the condensation step in the elongation pathway that result in the extension of the FA (Los and Murata, 1998; Hashimoto et al., 2008).

In silico analyses show the desaturases and elongases expressed in LBFLFK are homologous to proteins present in foods commonly consumed as part of the diet (Appendix 2).

4.1.3 Expression of new proteins in LBFLFK tissue

Tissues and seeds derived from LBFLFK (T4) together with the non-GM Kumily parent were sampled from 4 growing locations in the United States during the 2016 planting season. The tissues analysed were whole plants at different maturity stages, leaf tissue, root tissue, immature seed, mature seed, and pollen. There was a single replication of each plot (LBFLFK unsprayed, LBFLFK sprayed with imidazolinone herbicide, and the control variety Kumily) at each site. Field sites were representative of canola producing regions suitable for commercial production.

Protein was quantified using either an enzyme-linked immunosorbent assay (ELISA) or a Western blot that utilizes a polyclonal primary antibody. The choice of quantification method depended on its suitability based on the characteristics of each protein. ELISAs were used to determine the amounts of D12D(*Ps*), D6E(*Pp*), D5D(*Tc*), and D5E(*Ot*), while Western blot was used to determine the amounts of D6D(*Ot*), D6E(*Tp*), O3D(*Pir*), O3D(*Pi*), D4D(*Pl*), D4D(*Tc*), and AHAS [A122TS653N].

Canola seeds are the most likely tissue to enter the food supply, usually after processing into oil. Protein expression levels were measured in LBFLFK treated with imidazolinone as this is more representative of actual growing conditions for this GM canola. Table 5 shows the expression levels of the 11 newly expressed proteins in mature and immature seeds of LBFLFK. The applicant stated that none of the proteins involved in the EPA and DHA synthesis pathway were detected in other tissue types.

Eight of the ten proteins in the EPA and DHA biosynthesis pathway were detected in immature and/or mature seeds of LBFLFK. Expression of each protein is expected to be highest in the seed because expression is driven by seed-specific promoters. The levels of all eight proteins were low with D12D(*Ps*) having the lowest (4.24 µg/mg in immature seeds; 0.80 µg/mg in mature seeds) and D6E(*Tp*) with the highest expression (826.70 µg/mg in immature seeds; 682.75 µg/mg in mature seeds). D6E(*Pp*) and O3D(*Pi*) could not be detected in either immature or mature seeds. D5E(*Ot*) and D5D(*Tc*) could not be detected in immature seeds. D4D(*Pl*) and D4D(*Tc*) were detectable but not quantifiable for both immature and mature seeds. For the AHAS protein, expression was quantifiable in both immature and mature seeds.

[8] AHAS protein – A1064

Table 5: Mean protein expression levels in mature and immature seed of LBFLFK collected from 2016 field sites

Protein	Protein expression levels in LBFLFK (sprayed) immature seed (µg/g dry weight)		Protein expression levels in LBFLFK (sprayed) mature seed (µg/g dry weight)	
	Mean (SD) Range	LOQ LOD	Mean (SD) Range	LOQ LOD
D12D(*Ps*)	4.24 (2.77) <LOD - 9.89	2.97 0.70	0.80 (0.27) <LOQ - 1.50	0.55 0.13
D6D(*Ot*)	27.13 (23.53) <LOD - 77.15	15.77 5.26	26.83 (9.16) 15.94 - 44.35	11.77 3.92
D6E(*Pp*)	- <LOD	16.65 2.78	- <LOD	6.22 1.04
D6E(*Tp*)	826.70 (261.32) <LOQ - 1120.00	448.51 232.33	682.75 (100.10) 546.23 - 913.16	375.03 242.83
D5D(*Tc*)	- <LOD	28.03 11.21	<LOQ[1] <LOQ - 2.04	1.31 0.52
O3D(*Pi*)	- <LOD	19.03 7.26	- <LOD	71.02 27.08
O3D(*Pir*)	86.54 (31.79) <LOD - 169.37	87.51 33.37	172.56 (51.18) <LOQ - 288.98	117.60 37.37
D5E(*Ot*)	- <LOD - 16.24	13.03 2.84	7.58 (2.56) <LOQ - 12.68	4.87 1.06
D4D(*Pl*)	<LOQ[1] <LOD - 12.20	5.63 2.68	<LOQ[1] <LOD - 8.78	4.21 2.00
D4D(*Tc*)	- <LOD - 22.73	8.83 4.84	- <LOD - 10.30	9.23 3.80
AHAS [A122TS653N]	11.54 (3.53) <LOQ - 16.32	6.12 1.28	3.31 (0.45) <LOQ - 4.80	3.05 0.64

< LOQ = below the limit of quantification
< LOD = below the limit of detection
– = not applicable; datasets did not contain two or more quantifiable values.
[1] mean value fell below the limit of quantitation once <LOD and <LOQ results were substituted for their respective LOD and LOQ values.

4.1.4 Characterisation of proteins expressed in LBFLFK

Plant-produced proteins (PPP) isolated from immature seeds of LBFLFK were used for the characterisation studies. Kumily was used as the control substance for this study. Membrane fractions from yeast strains producing D6D or D5D encoded by the D6D(*Ot*) or D5D(*Tc*) genes, respectively, were used as positive controls for the *in vitro* enzyme activity assays.

The newly expressed proteins present in the PPP fraction of LBFLFK were characterised for their (i) apparent molecular weights and immunoreactivity by Western blot, (ii) identity by tryptic peptide mapping using mass spectrometry, (iii) glycosylation status by selective carbohydrate biotinylation and western blot, and (iv) enzyme activity by an *in vitro* assay.

Due to the low level of AHAS [A122TS653N] present in the LBFLFK PPP, immunopurified product of this protein isolated from the PPP was used for peptide mapping studies and glycosylation status analysis. Table 6 summarises the results from the characterisation studies.

Table 6: Characteristics of the newly expressed proteins in LBFLFK PPP

Protein	No. of amino acids	Predicted MW (kDa)	Apparent MW (kDa)	Immune-reactivity	Peptide mapping		Observed enzyme activity	Glycosylation status
					No. of Identified peptides	Sequence coverage (%)		
D12D(*Ps*)	398	45.6	41.8	Confirmed	11	51	Yes	Negative
D12D(*Ps*)[F83L]	398	45.5	41.8	Confirmed	10	46	Yes	Negative
D6D(*Ot*)	456	51.7	55.6	Confirmed	11	23	N/D	Negative
D6E(*Pp*)	290	33.4	N/D	N/D	N/D	N/D	Yes	N/D
D6E(*Tp*)	272	31.8	25	Confirmed	2	12	Yes	Negative
D5D(*Tc*)	439	49.8	46.8	Confirmed	7	31	N/D	Negative
O3D(*Pi*)	363	40.8	N/D	N/D	N/D	N/D	N/D	N/D
O3D(*Pir*)	361	40.4	38.1	Confirmed	8	37	N/D	Negative
D5E(*Ot*)	300	34.2	30.8	Confirmed	4	19	Yes	Negative
D4D(*Pl*)	445	49.1	50.7	Confirmed	3	9	Yes	Negative
D4D(*Pl*)[A102S]	445	49.1	50.7	Confirmed	3	9	Yes	Negative
D4D(*Tc*)	519	59.0	63.1	Confirmed	9	23	Yes	Negative
AHAS [A122TS653N]	606[2]	66.1	79.2	Confirmed	6[1]	15[1]	Yes	Negative[1]

Note: MW = Molecular Weight, N/D = Not Detected
[1] Results obtained from analysis of IP product.
[2] Size of the mature protein devoid the transit peptide sequence

Molecular weight. Western blot analysis with protein-specific antibodies determined the apparent molecular weights of all newly expressed proteins. The protein-specific antibodies used for D12D(*Ps*) and D4D(*Pl*) protein does not differentiate the variants present in LBFLFK: D12D(*Ps*) [F83L] and D4D(*Pl*) [A102S], respectively. The analysis showed that the apparent molecular weights of all newly expressed proteins were consistent with the predicted molecular weight except for the D6E(*Pp*) and the O3D(*Pi*) proteins, as these proteins were below the LOD and LOQ in the LBFLFK PPP. No signal was detected in the Kumily PPP control.

Immunoreactivity. Western blot analysis with protein-specific antibodies confirmed the identity of all newly expressed proteins except for D6E(*Pp*) and O3D(*Pi*) proteins. An immunoreactive band near the calculated mass of the D6E(*Pp*) and the O3D(*Pi*) proteins was not observed in the LBFLFK PPP. However, a strong signal was observed when using recombinant proteins consisting of a fusion of the amino- and carboxy termini of D6E(*Pp*) and O3D(*Pi*) as a positive control.

Peptide mapping. All newly expressed proteins were digested with trypsin and analysed via mass spectrometry (LC-MS/MS). The sequence coverage for all newly expressed proteins are detailed in Table 6. The peptides were identified with 95% confidence according to Mascot Score (>13). These results confirms the identity of all newly expressed proteins in LBFLFK except for D6E(*Pp*) and O3D(*Pi*).

Glycosylation analysis. None of the proteins present in the LBFLFK PPP fraction are glycosylated as analysed by selective carbohydrate biotinylation and Western blot.

Enzyme activity. Enzyme activity for the proteins present in the LBFLFK PPP fraction was demonstrated by *in vitro* assays for D12D(*Ps*), D12D(*Ps*) [F83L], D6E(*Pp*)[9], and D6E(*Tp*)[9], D5E(*Ot*), D4D(*Pl*), D4D(*Pl*) [A102S], D4D(*Tc*) and AHAS [A122TS653N]. Enzyme activity was not observed for the D6D(*Ot*), D5D(*Tc*), O3D(*Pir*)[9], and O3D(*Pi*)[9] proteins in LBFLFK PPP.

4.1.5 Safety of the newly expressed proteins

Data were provided to assess the potential toxicity and allergenicity of the newly expressed proteins.

Bioinformatic analyses

In silico analyses comparing the amino acid sequences from Insert 1 and Insert 2 of LBFLFK were performed by the applicant, using the same approach as outlined in Section 3.4.5.1. The search did not identify any known allergens with homology to sequences present in Insert 1 and Insert 2 of LBFLFK. No alignments had an E-score of ≤1×10^{-5} or met or exceeded the threshold of greater than 35% similarity over ≥ 80 amino acids and no eight amino acid peptide matches were shared between the compared sequences and proteins in the allergen database.

The applicant provided the results of *in silico* analyses comparing the amino acid sequences from Insert 1 and Insert 2 of LBFLFK to the NCBI_NR database and an in-house toxin database to identify any potential amino acid sequence similarity to known toxins, as described in Section 3.4.5.2. The full-length FASTA search with the NCBI_NR showed that most of the matches corresponded to the FA synthesis proteins from various organisms, which are not known toxins. When the amino acid sequences were compared to the toxin database, no alignments with an E-score of ≤1×10^{-5} were identified, indicating no significant homology with any known toxins.

Susceptibility of the newly expressed proteins to digestion with pepsin and pancreatin

The newly expressed proteins from LBFLFK were incubated with simulated gastric fluid (SGF) (10 U pepsin/µg protein at pH 1.2) for 0 - 60 min in a 38°C water bath (Thomas et al.,

[9] LBFLFK contains the newly expressed D6E from two different organisms; *Thalassiosira pseudonana* and *Physcomitrella patens*. The *in vitro* enzyme activity assay for D6E(*Tp*) and D6E(*Pp*) are identical and detects the presence of either protein. However, the relative contributions of the individual D6E proteins to the total activity cannot be determined. Similarly, the *in vitro* enzyme activity assay for O3D(*Pi*) and O3D(*Pir*) is unable to discriminate between the two O3D proteins.

2004). Controls included a no test protein control (pepsin only) and no pepsin control (test protein only) incubated for 0 and 60 min. The extent of digestion was determined by Western blot.

The newly expressed proteins were also incubated with simulated intestinal fluid (SIF) (~10 mg/ml pancreatin at pH 7.5) at 37.7°C for 0 - 60 min. Controls for this experiment included a no test protein control (pancreatin only) and no pancreatin control (test protein only) incubated for 0 and 60 min. The extent of digestion was determined by Western blot.

In the human digestive system, gastric digestion (pepsin) occurs before intestinal digestion (pancreatin). Therefore a sequential digestion was performed on proteins if incomplete digestions were observed in either the SGF or SIF assays. The results for the digestive fate analyses are summarised in Table 7. The data observed for D12D(*Ps*) and D4D(*Pl*) are representative of D12D(*Ps*) [F83L] and D4D(*Pl*) [A102S] respectively. This is because the desaturase assays and the antibodies used for Western blotting does not discriminate between the intended protein and the variant present in LBFLFK PPP.

Visual inspection of the pepsin and pancreatic digestion showed that D12D(*Ps*), D6D(*Ot*), D6E(*Tp*), D5D(*Tc*), O3D(*Pir*), D5E(*Ot*), D4D(*Pl*), D4D(*Tc*) and AHAS [A122TS653N] were rapidly degraded in SGF and/or SIF. Sequential digestions were performed for D6E(*Tp*), D5E(*Ot*), and AHAS [A122TS653N]. No data were reported for D6E(*Pp*) and O3D(*Pi*) proteins, as these proteins were below the LOD and LOQ in the LBFLFK PPP.

Stability to heat treatment

The effect of heat on the enzymatic activity and structural integrity of the newly expressed proteins present in the LBFLFK PPP was assessed. The LBFLFK PPP was heat treated for 5 and 20 min at temperatures ranging from 30 - 90ºC. Samples of LBFLFK PPP and Kumily PPP were kept on ice (~ 0ºC) for use as untreated controls.

The D4D(*Pl*), D4D(*Tc*), D12D(*Ps*), D5E(*Ot*), D6E(*Tp*) and AHAS [A122TS653N) were analysed by *in vitro* enzyme activity and Western blot. D6E(*Pp*) was only analysed for enzyme activity while D5D(*Tc*), O3D(*Pir*), D6D(*Ot*) were only analysed for structural integrity by Western blot. O3D(*Pi*) was not assessed since it was not observed previously in LBFLFK PPP (section 4.1.4). The result presented for D6E(*Pp*) is the same as shown for the D6E(*Tp*) protein as the *in vitro* enzyme assay is not able to discriminate the two D6E proteins.

The results for structural stability and bioactivity following heat treatment are summarised in Table 7. The data observed for D12D(*Ps*) and D4D(*Pl*) represents the data observed for D12D(*Ps*) [F83L] and D4D(*Pl*) [A102S] respectively. This is because the *in vitro* enzyme assays and the antibodies used for Western blotting does not discriminate between the two variants.

The data indicates that D12D(*Ps*), D6D(*Ot*), D6E(*Pp*), D6E(*Tp*), D5D(*Tc*), O3D(*Pir*), D5E(*Ot*), D4D(*Pl*), D4D(*Tc*) and AHAS(*At*) [A122TS653N] proteins are heat labile at temperatures greater than 50ºC.

Table 7: Summary of heat treatment sensitivity and digestive fate analysis of the newly expressed protein in LBFLFK

Protein	Susceptibility to digestion	Sensitivity to heat	
		Loss of enzyme activity	Loss of structural integrity
D12D(*Ps*)	Yes (SGF and SIF)	Yes (≥ 50ºC)	Yes (≥ 50ºC)
D6D(*Ot*)	Yes (SGF and SIF)	NA[1]	Yes (≥ 50ºC)
D6E(*Pp*)	NA[2]	Yes (≥ 50ºC)	NA[2]
D6E(*Tp*)	Yes (SIF; SGF followed by SIF)	Yes (≥ 50ºC)	Yes (≥ 70ºC)
D5D(*Tc*)	Yes (SGF and SIF)	NA[1]	Yes (≥ 50ºC)
O3D(*Pi*)	NA[2]	NA[1]	NA[2]
O3D(*Pir*)	Yes (SGF and SIF)	NA[1]	Yes (≥ 50ºC)
D5E(*Ot*)	Yes (SIF; SGF followed by SIF)	Yes (≥ 50ºC)	Yes (≥ 50ºC)
D4D(*Pl*)	Yes (SGF and SIF)	Yes (≥ 50ºC)	Yes (≥ 70ºC)
D4D(*Tc*)	Yes (SGF and SIF)	Yes (≥ 50ºC)	Yes (≥ 50ºC)
AHAS [A122TS653N]	Yes (leaf: SGF and SIF) and (PPP: SGF; SGF followed by SIF)	Yes (≥ 50ºC)	Yes (≥ 70ºC)

NA = not assessed
[1] Enzyme activity was not assessed because enzyme activity was not detectable in LBFLFK PPP (section 4.1.4).
[2] Loss of structural integrity to heat treatment or digestibility in SGF or SIF was not assessed because these proteins were not detected in LBFLFK PPP (section 4.1.4) and LBFLFK tissues (section 4.1.3).

4.1.6 Conclusion

LBFLFK expresses eleven new proteins. A range of characterisation studies were performed on LBFLFK confirming their identity, structure, biochemistry and function.

Eight of the ten proteins in the EPA and DHA biosynthesis pathway were only detected in immature and/or mature seeds of LBFLFK. This is expected since the genes encoding the proteins all have seed-specific promoters. The newly expressed AHAS [A122TS653N] protein, driven by a constitutive promoter, was quantifiable in both immature and mature seeds of LBFLFK. Two of the newly expressed proteins, D6E(*Pp*) and O3D(*Pi*) were reported to be below levels of detection and quantification in LBFLFK seeds.

Bioinformatic studies confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens and also confirmed the proteins share significant homology with proteins already consumed in food from other species.

The *in vitro* digestibility studies of the newly expressed protein suggest the proteins would be rapidly degraded in the gastrointestinal tract following ingestion. In addition, the heat denaturation studies suggest the proteins are not thermally stable and are functionally inactivated following heating. Taken together, the evidence indicates the newly expressed proteins are unlikely to be toxic or allergenic to humans should they be present in the diet.

The applicant has stated that LBFLFK will only be used for the production of highly refined canola oil for food use. Oil from canola line LBFLFK was shown, like other highly processed vegetable oils, to be devoid of all proteins, including the eleven newly introduced proteins assessed in this application (Section 5.4.2).

4.2 Herbicide metabolites

FSANZ has previously assessed the imidazolinone-tolerant form of AHAS (A1064) for its potential to generate novel metabolites in GM crops sprayed with imidazolinone herbicides. This assessment found the expression of the imidazolinone-tolerant form of AHAS is unlikely to lead to the production or accumulation of any novel herbicide metabolites in the plant. The spraying of LBFLFK with imidazolinone is therefore expected to produce the same metabolites that arise when non-GM canola is sprayed with the same herbicide.

5 Compositional analysis

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

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

5.1 Key components

Canola oil is the primary food product used for human consumption and is extracted from the seed by a number of processes of which solvent extraction is the most efficient and leaves about 2% - 4% residual oil in the meal (NSW DPI 2014). The components to be analysed for the comparison between GM and non-GM canola are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Canola (OECD, 2011b).

5.2 Study design

The applicant provided compositional data for:

- **Seeds**
  - LBFLFK seeds treated and non-treated with the imidazolinone herbicide for winter 2014/2015 and spring 2015 growing season.
- **Processed products**
  - defatted meal, pressed oil, crude oil (pressed + extracted oil) and refined, bleached and deodorised (RBD oil) from LBFLFK treated with the imidazolinone herbicide for 2016 growing season.

5.2.1 Seeds

LBFLFK was grown and harvested across two growing seasons; five winter field trials conducted in 2014/2015 and seven spring field trials in 2015. Each site was planted as a randomised complete block consisting of four replicates. Canola seeds were harvested at physiological maturity from the middle six rows of each plot. This included plots of LBFLFK

sprayed with Beyond herbicide (imidazolinone), non-sprayed LBFLFK, and Kumily. Additionally, six non-GM reference varieties[10], were also included in each site to generate reference ranges for each analyte. This aids in a determination of the normal variation found in canola for the various analytes measured. Planting and crop maintenance were done according to local agronomic practices at each site.

Harvested seeds from each trial were fumigated by a commercial fumigator prior to shipment to BASF Plant Science facility in Ames for post-harvest activity.

About 200 g of harvested seed were collected for each sample and were frozen at -80ºC for 12 h prior to grinding to a fine powder suitable for nutrient compositional analysis. An aliquot of approximately 30 g was kept separately for FA analysis and the remainder of the milled sample was used for all other laboratory analyses. Samples were maintained at

-20ºC until analysed. Methods of analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods.

The constituents of LBFLFK and Kumily were statistically analysed using a linear mixed model ANOVA method. Data were transformed into Statistical Analysis Software (SAS) data sets and analysed using SAS® software (SAS, version 9.0 of the SAS System for Linux, SAS Institute Inc.)[11]. For each analyte, 'descriptive statistics' (mean, standard error (SE), and range) were generated. Where a statistically significant difference (p-value < 0.05) was identified versus the parental variety, further context for interpreting the possible nutritional relevance of the difference was gathered through comparisons with the expected range for conventional canola as defined by means reported by the applicant for the reference varieties, the International Life Sciences Institute (ILSI)[12] Crop Composition Database, and peer-reviewed scientific literature.

Seed samples were analysed for proximates, FAs, amino acids, minerals, vitamins, phytosterols and anti-nutrients (glucosinolates, phytic acid, and phenolics). In total, 113 individual analytes were measured (Figure 6). Of these, 39 individual analytes had mean values below the assay LOQ and were excluded from the statistical analysis, leaving 74 individual analytes able to be quantified.

[10] Q2, 46A65, IMC105, IMC302, Wizzard, Orinoco
[11] SAS Institute Inc., 2017. SAS/STAT® Contextual Analysis 14.3: User's Guide. Cary, NC: SAS Institute Inc.
[12] ILSI, 2016, online. International Life Sciences Institute Crop Composition Database, Version 6. Accessed on May 16, 2017. available at https://www.cropcomposition.org/query/index.html

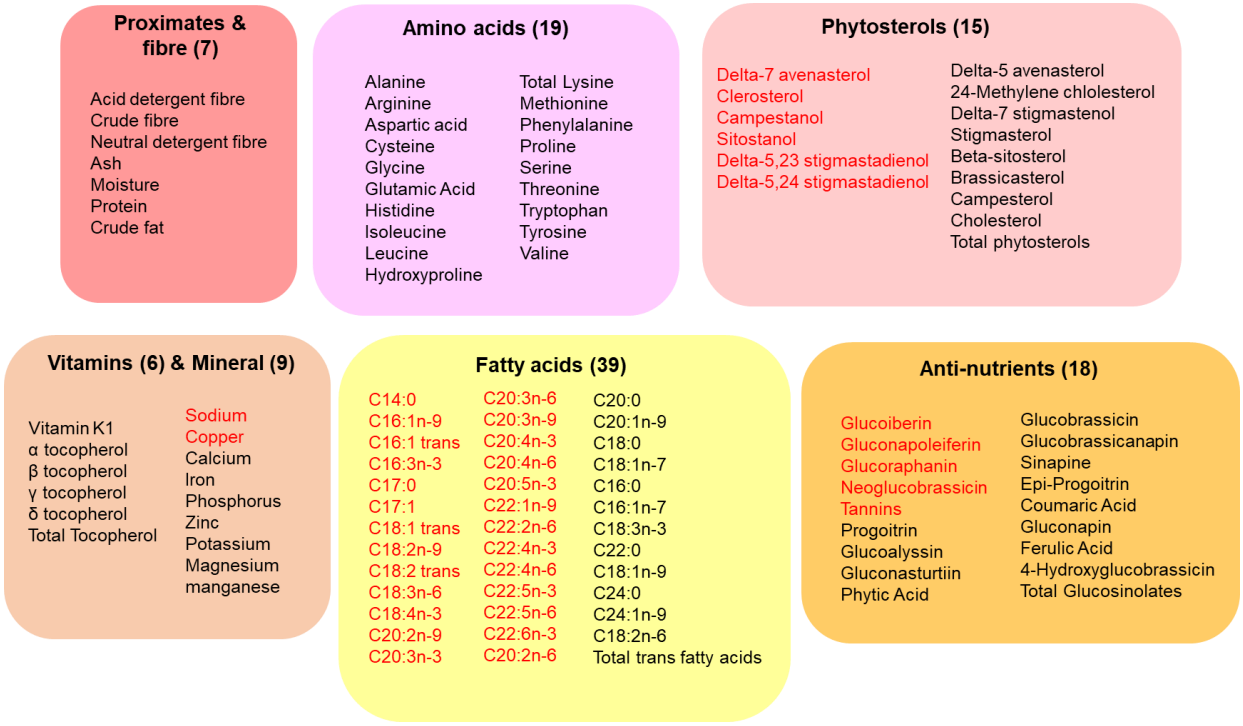

Figure 6: Analytes measured in seed samples. Analytes in red text were below the LOQ and excluded from statistical analysis

5.2.2 Processed products

Processed products analysed included defatted meal, crude oil, pressed oil and RBD oil. Since the primary purpose of canola oil is as cooking oil, FSANZ's assessment focused on the data for crude oil and RBD oil produced from LBFLFK seeds harvested from the 2016 growing season. Since crude oil is a blend of pressed and extracted oil comprising approximately 83% pressed oil, data for crude oil was taken as representative of pressed oil. As meal is still used primarily for animal feed, the data were noted by FSANZ but are not part of this report. The results from compositional analysis of the meal were unremarkable.

LBFLFK (sprayed), the parental control Kumily, and three non-GM reference varieties[13] were included in this study. Seeds for compositional analysis were harvested from five field trials during 2016. About 600g of the harvested seeds were processed into oil and meal for further analysis. The processing involved seed cleaning, seed pre-conditioning and flaking, seed cooking, pressing the flake to mechanically remove a portion of the oil, solvent extraction of the press-cake to remove the remainder of the oil.

The compositional data for oil derived from LBFLFK were statistically analysed using Student's paired t-Test with two-tailed distribution (Steel and Torrie,1976). For each analyte, 'descriptive statistics' (mean, standard deviation (SD), and range) were generated.
Oil samples were analysed for moisture, protein, amino acids, FAs, vitamins, minerals, vitamins, phytosterols. A total of 82 and 61 analytes including FAs were analysed for crude oil and RBD oil, respectively (Figure 7). Of these, 45 (crude oil) and 22 (RBD oil) individual analytes had mean values below the assay LOQ for LBFLFK and/or Kumily and were excluded from the statistical analysis, leaving 37 (crude oil) and 39 (RBD oil) individual analytes that were able to be quantified.

[13] IMC105, 46A65, Wizzard

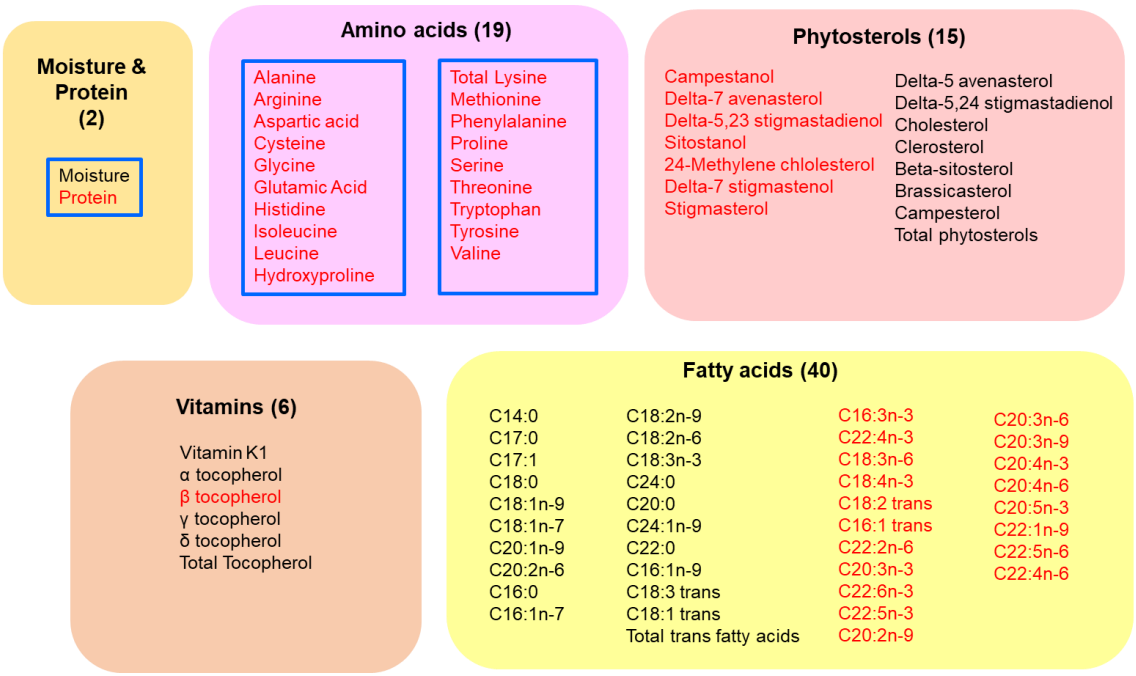

Figure 7: Analytes measured in crude oil and RBD oil [analytes presented in blue boxes were only tested in crude oil]. Analytes in red text were below the LOQ and excluded from statistical analysis

5.3 Key components in seeds

5.3.1 Fatty acids

There were no differences in FA composition between the two different growing seasons. Compositional data is therefore only presented from the spring 2015 growing season.

The levels of 39 individual FAs were measured. Of these, 26 individual FAs including EPA and DHA (refer to Table 8; shaded) were omitted from analysis because the results were consistently below the LOQ for both LBFLFK and/or Kumily.

The biosynthesis of EPA and DHA in LBFLFK is expected to produce FAs not normally present in non-GM canola. The biosynthesis of EPA and DHA also has impacts on the levels of the normal endogenous FA content of canola. The results from the FA analyses is therefore presented in three parts: endogenous FAs not impacted by the EPA and DHA producing trait; endogenous FAs impacted by the EPA and DHA producing trait; and FAs produced as a result of the new EPA and DHA producing trait (summarised in Table 8).

Table 8: Fatty acids analysed in LBFLFK and Kumily seeds

Canola endogenous FAs NOT impacted by the EPA and DHA producing trait	Canola endogenous FAs impacted by the EPA and DHA producing trait	FAs introduced by the EPA and DHA producing trait
Myristic acid (C14:0)	Palmitoleic acid (C16:1n-7)	C16:1 trans
Palmitic acid (C16:0)	Stearic acid (C18:0)	C18:2n-9
Cis-7 hexadecenoic acid (C16:1n-9)	Oleic acid/OA (C18:1n-9)	ɣ-linolenic acid/GLA (C18:3n-6)
Hexadecatrienoic acid (C16:3n-3)	Linoleic acid/LA (C18:2n-6)	Stearidonic acid/SDA (C18:4n-3)
Ginkgolic acid (C17:0)	Alpha-linolenic acid/ALA (C18:3n-3)	C20:2n-9
Margaroleic acid (C17:1)	Arachidic acid (C20:0)	Eicosatrienoic acid (C20:3n-3)
Eicosadienoic acid (C20:2n-6)	Gondoic acid (C20:1n-9)	Dihomo-gamma-linolenic acid/DGLA (C20:3n-6)
Erucic acid (C22:1n-9)	Behenic acid (C22:0)	Mead acid (C20:3n-9)
Docosadienoic acid (C22:2n-6)	Ligceric acid (C24:0)	Bishomostearidonic acid (C20:4n-3)
C18:2 trans	Nervonic acid (C24:1n-9)	Arachidonic acid/ARA (C20:4n-6)
	Total trans fatty acid/TFA	EPA (C20:5n-3)
	Cis-vaccenic acid (C18:1n-7)	C22:4n-3
	C18:1 trans	Adrenic acid (C22:4n-6)
		Clupanodonic acid (C22:5n-3)
		Osbond acid (C22:5n-6)
		DHA (C22:6n-3)

*Aqua shading represents individual FA not suitable for statistical analysis.

Endogenous fatty acids not impacted by the EPA and DHA producing trait

The mean values for C14:0, C16:1n-9, C16:3n-3, C17:0, C17:1, C18:2 trans, C20:2n-6, C22:1n-9[14] (erucic acid) and C22:2n-6 were consistently below the LOQ and were not included in the statistical analysis (Table 8). The mean value for C16:0 was above the LOQ but did not show any statistically significant differences.

Endogenous fatty acids impacted by the EPA and DHA producing trait

Table 9 summarises the individual FAs in LBFLFK that showed statistically significant differences compared to the parental control Kumily. Consistent differences were observed for the following FAs; C16:1n-7, C18:0, C18:1n-7, C18:1n-9, C18:2n-6, C18:3n-3, C20:0, C20:1n-9, C22:0, C24:0, C24:1n-9, and total trans fatty acids (TFAs). C18:1 trans was measured in LBFLFK but was consistently below LOQ in Kumily, therefore excluded from statistical analysis.

The mean values for C16:1n-7, C18:0, C18:1n-7, C18:3n-3, C20:0, C22:0, C24:0, C24:1n-9, in LBFLFK were well within the natural variability of the analytes in the reference varieties and publically available data. Therefore these statistically significant differences are not

[14] Canola varieties must have C22:1n-9 (erucic acid) below 2% (OECD, 2011b).

biologically meaningful.

The mean values for C18:1n-9 (oleic acid), C18:2n-6 (linoleic acid) and C20:1n-9 (gondoic acid) were consistently outside the ranges for the reference varieties and publically available data. This difference is expected and is consistent with the FA synthesis pathway being pushed towards the production of EPA and DHA.

Oleic acid is the starting substrate for the biosynthesis of EPA and DHA, hence the lower mean values in LBFLFK which are due to the conversion of oleic acid into more highly unsaturated FAs. The higher relative linoleic acid content is attributable to the newly expressed delta-12 desaturase from *Phytophthora sojae* [D12D*(Ps*)] that converts oleic acid to linoleic acid (Yilmaz et al., 2017).

In terms of TFAs, LBFLFK seed had a statistically significantly higher total level than Kumily and this was higher than the reference range. The increased total TFAs across seasons in LBFLFK represents only a marginal and low amount of the total FAs (0.3%) compared to the amount of TFAs produced as a result of conventional commercial processing of canola seeds to RBD oil[15]. FSANZ has assessed the increase in TFAs as a result of directing the biosynthesis pathway of canola toward n-3 LC-PUFA in a previous application[16]. A consideration of the consumption data of TFAs in the Australian and New Zealand diets indicated consumption of food derived from DHA canola does not pose a public health concern. Likewise, the difference in TFAs in LBFKFK compared to Kumily is not considered to be of concern.

Table 9: Statistically significantly different fatty acids (mean ±SE) between LBFLFK seed and control Kumily (Spring 2015)

Fatty acid	Kumily (% of total FAs)	LBFLFK(sprayed)[1] (% of total FAs)	*p*-value	Non GM reference varieties	Publically available data[3]
	Mean (SE) Range	Mean (SE) Range		Range	Range
Palmitoleic C16:1n-7	0.29 (0.011) 0.25–0.38	0.19 (0.011) 0.17–0.23	<0.001	0.18–0.28	0.16-0.40
Stearic C18:0	2.18 (0.061) 1.95–2.33	2.77 (0.061) 2.52–3.06	<0.001	1.73–2.23	1.5-2.77
Cis-vaccenic acid C18:1n-7	3.50 (0.1) 3.28–4.13	3.46 (0.1) 3.21–3.98	0.047	2.57–3.47	N/A
Oleic acid C18:1n-9	54.83 (0.74) 49.59–56.69	26.41 (0.74) 23.31–28.18	<0.001	55.21–76.44	53.19-69.45
Linoleic acid C18:2n-6	19.29 (0.47) 17.98–21.88	27.89 (0.47) 26.09–29.61	<0.001	5.81–23.23	14.13-25.68
Alpha-linolenic acid/ALA C18:3n-3	8.01 (0.21) 7.17–9.08	5.37 (0.21) 4.88–6.08	<0.001	1.97–8.52	1.79-11.97
Arachidic acid C20:0	0.7 (0.017) 0.64–0.78	0.66 (0.017) 0.60–0.73	<0.001	0.57–0.80	0.48-0.71
Gondoic acid C20:1n-9	1.03[2] 1.00–1.08	0.70[2] 0.68–0.73	<0.001	1.00–1.45	0.93-1.43
Behenic acid C22:0	0.34 (0.011) 0.29–0.41	0.26 (0.011) 0.22–0.30	<0.001	0.23–0.45	0.21-0.39
Lignoceric acid C24:0	0.19 (0.0078) 0.16–0.24	0.13 (0.0078) 0.11–0.15	<0.001	0.15–0.31	0.10-0.26
Nervoic acid C24:1n-9	0.13 (0.0079) 0.094–0.19	0.082 (0.0079) 0.066–0.11	<0.001	0.084–0.18	0.08-0.36
Total TFAs	0.062 (0.0065) 0.055–0.070	0.27 (0.0065) 0.25–0.32	<0.001	< LOQ–0.10	N/A

Mauve shading represents LBFLFK means that are significantly lower than the Kumily means, while the orange

[15] Further assessment on LBFLFK RBD oil is undertaken in Supporting Document 2.
[16] DHA canola – A1143

shading represents LBFLFK means that are significantly higher.
Red border represents FAs with mean values of LBFLFK consistently outside the ranges for reference varieties and publically available database.
N/A = Not available
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] Data were log-transformed. Means were back-transformed. Back-transformed SE is not provided.
[3] Represents combined data from ILSI and peer-reviewed scientific literature.

Fatty acids produced as a result of the new EPA and DHA producing trait

In addition to the FAs C20:5n-3 (EPA) and C22:6n-3 (DHA), a number of other FAs are associated with the EPA and DHA producing trait. These are: C16:1 trans, C18:2n-9, C18:3n-6, C18:4n-3, C20:2n-9, C20:3n-3, C20:3n-6, C20:3n-9, C20:4n-3, C20:4n-6, C22:4n-3, C22:4n-6, C22:5n-3 (docosapentaenoic acid; DPA)[17], and C22:5n-6. As expected, these FAs consistently had values below the assay LOQ in Kumily and other non-GM reference varieties. These FAs are present in other organisms and foods that are safely and routinely consumed. Their presence in LBFLFK canola therefore does not raise any safety concerns.

A summary of the mean values for the FAs produced as a result of the new EPA and DHA producing trait is provided in Table 10.

Table 10: Fatty acids (mean ±SD) produced in LBFLFK seeds as a result of the EPA and DHA producing trait (Spring 2015)

Fatty acid	LBFLFK (sprayed)[1] (% of total FAs)
	Mean (SE) Range
C16:1 trans	0.057 (0.0043) 0.050–0.060
C18:2n-9	1.12 (0.15) 0.96–1.38
C18:3n-6	1.60 (0.16) 1.44–1.82
C18:4n-3	0.26 (0.038) 0.21–0.33
C20:2n-9	0.33 (0.06) 0.27–0.43
C20:3n-3	0.067 (0.0093) 0.060–0.082
C20:3n-6	4.06 (0.38) 3.65–4.53
C20:3n-9	0.079 (0.016) 0.060–0.10
C20:4n-3	1.92 (0.27) 1.54–2.37
C20:4n-6	1.87 (0.25) 1.62–2.19
C20:5n-3 (EPA)	6.27 (0.46) 5.47–6.98
C22:4n-3	0.68 (0.12) 0.54–0.90
C22:4n-6	0.45 (0.042) 0.38–0.50
C22:5n-3 (DPA)	2.75 (0.15) 2.51–3.00
C22:5n-6	0.072 (0.017) 0.05–0.10
C22:6n-3 (DHA)	0.77 (0.12) 0.59–0.96

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond herbicide spray (35 g

[17] DPA is the metabolic intermediate between EPA and DHA.

a.i./ha) at the 3–4 leaf stage.

5.3.2 Other components

A summary of the statistically significant differences for the other components analysed in the seed is provided in Table 11 and are briefly described below.

Proximates

A total of seven proximates were measured. There were no statistically significant differences in the mean levels for proximates between LBFLFK and Kumily harvested during winter 2014/2015. Small but statistically significant differences were observed in means levels for acid detergent fibre, crude fibre and neutral detergent fibre from seeds harvested in spring 2015. However, the levels observed fall well within the natural variability of the analytes in the reference varieties and publically available data, therefore these small differences are not biologically meaningful.

Amino acids

A total of 19 amino acids were measured. There were no statistically significant differences in mean levels for amino acids between LBFLFK and Kumily for winter 2014/2015. For spring 2015, statistically significant differences were observed for aspartic acid and leucine. The differences were small in magnitude except for aspartic acid. However, the means observed for all amino acids were within the natural variability of the analytes in the reference varieties and publically available data, therefore these differences are not biologically meaningful.

Vitamins and minerals

Six vitamins were analysed. Fat-soluble vitamin K1 and tocopherols (including vitamin E) are found in processed edible oils and contribute to human health and nutrition. Statistically significant differences between LBFLFK and Kumily were observed for the delta-tocopherols and vitamin K1 in seeds harvested in winter 2014/2015. For spring 2015, a statistically significant difference was observed for vitamin K1 only. All measured mean values were within the natural variability of the analytes in the reference varieties and publically available data. No other statistically significant differences for vitamins were observed.

Nine minerals were analysed. The mean values for calcium and magnesium were statistically significantly lower in LBFLFK compared to Kumily across both growing season, with the differences small in magnitude. However means were just slightly outside the reference range, they were well within the range from publically available data. Therefore these statistically significant differences are not biologically meaningful. No other statistically significant differences in minerals were observed between LBFLFK and Kumily. All measured mean values were within the natural variability of the analytes in the reference varieties and publically available data.

Anti-nutrients

Canola seeds are much richer in phenolic compounds than other oilseeds (Naczk et al., 1998) but most of the phenolics remain in the meal after pressing, contributing to its dark colour, bitter taste and astringency. While glucosinolates are present in canola seed, these remain behind in the meal when the oil is extracted.

A total of eighteen anti-nutrients were measured. The mean values for epi-progoitrin, glucoiberin, gluconapoleiferin, coumaric acid, tannins, and glucoraphanin (2014/2015 and spring 2015) and neoglucobrassicin (spring 2015) were below the LOQ in LBFLFK and/or Kumiy, therefore were not subjected to statistical analysis.

The mean value of sinapine was statistically significantly lower while gluconapin and glucobrassicin were statistically significantly higher in LBFLFK compared to Kumily across both growing seasons. The means of glucoalyssin and glucobrassicanapin were statistically significantly lower in LBFLFK compared to Kumily in the winter 2104/2015 season only. The mean value for total glucosinolates was statistically significantly higher in LBFLFK compared to Kumily in spring 2015. The differences observed were small and all the mean values for LBFLFK were within the reference range. These differences are therefore not biologically meaningful. No other statistically significant differences for anti-nutrients were observed. All means values were within the natural variability of the analytes in the reference varieties and publically available data.

Phytosterols

Phytosterols are important micronutrients in human diets and have been shown to decrease cardiovascular morbidity by reducing cholesterol absorption (Kamal-Eldin and Moazzami, 2009). Canola has approximately twice the amount of phytosterol content of sunflower or soybean oils (Vlahakis and Hazebroek, 2000). The level of phytosterols is governed by the level of unsaturated FAs; an increase in unsaturation will result in the formation of higher levels of anti-oxidants to protect the oil (Unger, 2015). Therefore, an increase in phytosterols level is expected in LBFLFK. However, phytosterols are largely removed during the deodorisation stage of oil refining (NSW DPI 2014).

A total of fifteen phytosterols including total phytosterols were analysed. The brassicasterol, delta-5 avenasterol, delta-7 stigmastenol, stigmasterol, and the total phytosterols measurement showed statistically significant differences between LBFLFK and Kumily, in the winter 2014/15 growing season. While in spring 2015, beta-sitosterol, brassicasterol, campesterol, and total phytosterols showed statistically significant differences. Nonetheless, the mean values were within the reference range, therefore these differences are not biologically meaningful.

Interestingly, the mean values for some of the phytosterols were at least 2-3 fold higher than the values from the publically available range. However, the elevated levels were also observed in all reference varieties tested and can be attributed to differences in the method of analysis used. No other statistically significant differences for phytosterols were observed. All means values were within the natural variability of the analytes in the reference varieties and publically available data.

Table 11: Summary of statistically significant compositional differences (excluding fatty acids) between LBFLFK seeds and control Kumily

Analyte	Kumily	LBFLFK (sprayed)[1]	*p*-value	Non-GM reference varieties	Publically available data
	Mean (SE) Range	Mean (SE) Range		Range	Range
Proximates					
Acid detergent fibre (% dw)	11.73 (0.3) 10.95-13.1	11.32 (0.3)[b] 10.39-13.1	0.046	9.14–11.95	10.82-20.16
Crude fibre (% dw)	10.37 (0.19) 9.46–11.00	9.39 (0.19)[b] 8.90–10.24	<0.001	7.57-10.95	11.6-22.1
Neutral detergent fibre (% dw)	15.26 (0.26) 14.45–16.3	14.55 (0.26)[b] 13.65–15.40	0.031	12.45–15.7	14.28-24.32
Amino acid					
Aspartic acid (% dw)	2.06 (0.083) 1.83–2.33	2.14 (0.083)[b] 1.86–2.41	<0.001	1.55-2.5	1.51-2.55
Leucine	1.79 (0.042)	1.75 (0.042)[b]	0.007	1.53-2.18	1.47-2.35

Analyte	Kumily	LBFLFK (sprayed)[1]	*p*-value	Non-GM reference varieties	Publically available data
	Mean (SE) Range	Mean (SE) Range		Range	Range
(% dw)	1.66–1.96	1.64–1.88			
Vitamins and Minerals					
Delta-tocopherol (mg/100 g dw)	0.73 (0.045) 0.61–0.84	0.63 (0.045)[a] 0.52–0.73	0.017	0.41–1.07	0.143-0.5
Vitamin K1 (mg/100 g dw)	0.088 (0.01) 0.067–0.110	0.097 (0.01)[a] 0.075–0.130	0.027	0.038–0.110	N/A
	0.11[2] 0.091–0.21	0.12[2 b] 0.099–0.18	0.01	0.059-0.15	
Calcium (% dw)	0.33 (0.022) 0.25–0.40	0.30 (0.022)[a] 0.22–0.36	0.01	0.27–0.47	0.248-1.41
	0.32 (0.018) 0.25–0.42	0.29 (0.018)[b] 0.23–0.37	<0.001	0.30–0.52	
Magnesium (% dw)	0.33 (0.0081) 0.31–0.35	0.31 (0.0081)[a] 0.28–0.33	0.002	0.32–0.38	0.26-0.53
	0.34 (0.0075) 0.31–0.38	0.32 (0.0075)[b] 0.30–0.35	<0.001	0.32–0.40	
Anti-nutrients					
Glucoalyssin (µmol/g dw)	0.69 (0.076) 0.45–0.89	0.62 (0.076)[a] 0.45–0.81	0.027	0.077–0.71	0.05-9.8
Glucobrassicin (µmol/g dw)	0.26 (0.11) 0.11–0.37	0.59 (0.11)[a] 0.22–0.96	0.002	< LOQ–0.90	0.06-1.84
	0.314[2 b] 0.16–0.8	0.674[2 b] 0.39–1.52	<0.001	0.07–1.30	
Glucobrassicanapin (µmol/g dw)	0.24 (0.034) 0.13–0.33	0.21 (0.034)[a] 0.11–0.29	0.028	0.053–0.32	0.18-1.8
Gluconapin (µmol/g dw)	1.40 (0.14) 0.94–1.79	1.72 (0.14)[a] 1.53–1.92	0.006	0.96–3.51	0.10-6.84
	2.30 (0.22) 1.44–2.71	2.45 (0.22)[b] 1.61–3.57	0.002	0.88–5.87	
Sinapine (% dry weight)	1.00 (0.03) 0.93–1.11	0.89 (0.03)[a] 0.85–0.96	<0.001	0.76–1.08	0.26-1.12
	1.02 (0.031) 0.90–1.20	0.95 (0.031)[b] 0.87–1.10	<0.001	0.79–1.09	
Total glucosinolates (µmol/g dw)	11.62 (1.19) 7.16–15.48	12.78 (1.19)[b] 7.69–17.25	0.001	4.21–25.57	0.41-31.98
Phytosterols					
Beta-sitosterol (% dw)	0.47 (0.013) 0.42–0.53	0.44 (0.013)[b] 0.40–0.49	<0.001	0.40–0.57	0.10-0.21
Brassicasterol (% dw)	0.12 (0.0043) 0.11–0.13	0.078 (0.0043)[a] 0.067–0.087	<0.001	0.054–0.11	0.0210-0.0403
	0.12 (0.002) 0.10–0.13	0.072 (0.002)[b] 0.065–0.076	<0.001	0.052–0.110	
Campesterol (% dw)	0.25 (0.012) 0.22–0.31	0.24 (0.012)[b] 0.21–0.3	0.001	0.21–0.35	0.05-0.11
Delta-5 avenasterol (% dw)	0.01 (0.0015) 0.007–0.02	0.0065 (0.0015)[a] 0.0055–0.0090	0.022	0.0042–0.03	N/A
Delta-7 stigmastenol (% dw)	0.0025 (0.0008) 0.0018–0.0035	0.0068 (0.0008)[a] 0.0042–0.0097	<0.001	0.0018–0.01	N/A
Stigmasterol (% dw)	0.0051 (0.0005) 0.0027–0.0060	0.0041 (0.0005)[a] 0.0022–0.0055	0.003	0.0015–0.0050	0.0012-0.0056
Total phytosterols (% dw)	0.93 (0.039) 0.83–1.03	0.88 (0.039)[a] 0.77–1.01	0.01	0.78–1.18	N/A
	0.89 (0.027) 0.78–1.02	0.80 (0.027)[b] 0.71–0.91	<0.001	0.74–1.08	

Mauve shading represents LBFLFK means that are significantly lower than the Kumily means, while the orange shading represents LBFLFK means that are significantly higher.
dw = Dry weight; LOQ = Limit of quantification; N/A = Not available
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[2]Data were log-transformed. Means were back-transformed. Back-transformed SE is not provided
[a] Seeds collected from winter 2014/2015 growing seasons.
[b] Seeds collected from Spring 2015 growing season

5.4 Key components in crude oil and RBD oil

5.4.1 Fatty acids

A total of 40 individual FAs and total TFAs were analysed. Eighteen and nineteen individual FAs in crude oil and RBD oil (including DHA and EPA), respectively, were omitted from statistical analysis as they were below the LOQ in Kumily and other non-GM varieties. The statistically significant differences in FAs between oil from LBFLFK and Kumily are summarised in Table 12, and are briefly described below.

Endogenous fatty acids not impacted by the EPA and DHA producing trait

Statistically significant differences were observed for C14:0, C16:0, C16-1n-9, C17:0, C17:1, and C20:2n-6 in the oil fractions (except for C17:0 in crude oil). While the mean values of these FAs were just slightly outside the reference range, they were well within the range from publically available data. Therefore these statistically significant differences are not biologically meaningful.

Endogenous fatty acids impacted by the EPA and DHA producing trait

The FAs C16:1n-7 (palmitoleic acid), C18:0 (stearic acid), C18:1n-9 (oleic acid), C18:1 trans, C18:2n-6 (linoleic acid), C18:3n-3 (alpha-linolenic acid), C20:1n-9, C22:0, C24:0, and C24:1n-9 showed statistically significant differences in both crude and RBD oil fractions between LBFLFK and Kumily. While C20:0 and total TFAs showed statistically significance differences in crude oil fraction between LBFLFK and Kumily, it is important to note that only RBD oil is the food grade quality oil produced from LBFLFK grain (Andre et al., 2019). Further assessment on the nutrition risk of LBFLFK RBD oil is undertaken in Supporting Document 2.

The mean values for stearic acid, oleic acid, and linoleic acid were consistently outside the range for reference varieties and publically available data. This difference is expected and is consistent with the FA synthesis pathway being pushed towards the production of EPA and DHA. The mean values for C16:1n-7, C18:3n-3, C20:1n-9, C22:0, C24:0, and C24:1n-9 were still within the reference range and/or the range from publically available data.

Interestingly, the FAs C18:2n-9 and C20:2n-9 were not quantifiable in seeds of Kumily and the reference varieties (section 5.3.1) but were present in the processed oil fraction.

In terms of TFAs, LBFLFK RBD oil fraction had higher total levels relative to crude oil, however this increase was also observed in RBD oil from Kumily and other non-GM canola varieties. This increase is the result of isomerisation of FAs to *trans* fats that occurs spontaneously with heating at a faster rate (Wolff 1993; Chardigny 1996). As canola oils are diluted when used, the overall level of TFAs consumed would also be further reduced. Furthermore, TFAs are present in other refined non-GM vegetable oils, including soybean, sunflower and rice oils, and the TFAs content of LBFLFK RBD oil is not expected to vary significantly from these other retail vegetable oils. The increase in TFAs was considered for DHA canola in a previous application (A1143), including consumption data of TFAs in the Australian and NZ diets. The difference in TFAs is not considered to be of significance or concern.

Table 12: Statistically significantly different individual fatty acids (mean ±SD) between LBFLFK oil (crude oil and RBD oil) and control Kumily

Analytes	Control Kumily (% relative)	LBFLFK (sprayed) (% relative)	T-test[2]	Non-GM reference variety	Publically available data
	Mean[1] (SD)	Mean[1] (SD)	p-value[3]	Range[1]	Range
C14:0	0.056 (0.005)	0.061 (0.003)	0.0198	0.042–0.057	<LOQ–0.2
	0.056 (0.004)	0.063 (0.003)	0.0052	0.042–0.056	
C16:0	4.471 (0.140)	4.581 (0.045)	0.0211	3.592–4.090	2.5-7.0
	4.410 (0.140)	4.695 (0.046)	0.0001	3.56–4.041	
C16:1n-7	0.247 (0.015)	0.168 (0.007)	0.0000	0.190–0.211	0.16–0.4
	0.245 (0.012)	0.173 (0.011)	0.0000	0.191–0.211	
C16:1n-9	0.039 (0.004)	0.048 (0.004)	0.0001	0.040–0.046	<LOQ–0.6
	0.039 (0.004)	0.049 (0.004)	0.0001	0.039–0.045	
C17:0	0.048 (0.003)	0.049 (0.002)[NS]	0.3742	0.038–0.045	<LOQ-0.3
	0.047 (0.001)	0.052 (0.002)	0.0002	0.037–0.045	
C17:1	0.057 (0.003)	0.025 (0.006)	0.0000	0.052–0.058	<LOQ-0.3
	0.058 (0.002)	0.027 (0.003)	0.0000	0.051–0.058	
C18:0	2.290 (0.182)	2.994 (0.276)	0.0001	2.064–2.159	0.8-3.0
	2.280 (0.178)	3.100 (0.273)	0.0000	2.059–2.156	
C18:1n-9	58.417 (1.195)	27.880 (1.532)	0.0000	61.007–62.450	51.0-70.0
	59.048 (0.979)	29.554 (1.558)	0.0000	61.327–62.776	
C18:1 trans	0.021 (0.013)	0.042 (0.010)	0.0003	0.022–0.024	N/A
	0.029 (0.013)	0.047 (0.007)	0.0079	0.029–0.030	
C18:2n-6	20.003 (1.163)	28.732 (1.027)	0.0000	18.513–23.654	14.13-25.68
	19.603 (0.882)	29.560 (1.000)	0.0000	18.282–23.331	
C18:2n-9	0.026 (0.003)	1.321 (0.168)	0.0000	0.022–0.026	NA
	0.065 (0.005)	1.428 (0.165)	0.0000	0.055–0.068	
C18:3n-3	8.273 (0.470)	5.221 (0.551)	0.0000	2.760–7.482	5.0–14.0
	7.446 (0.452)	4.916 (0.504)	0.0000	2.538–6.886	
C18:3 trans	0.067 (0.004)	0.067 (0.003)[NS]	0.8524	0.041–0.067	N/A
	0.595 (0.038)	0.511 (0.068)	0.0079	0.207–0.532	
C20:0	0.723 (0.038)	0.676 (0.028)	0.0090	0.666–0.706	0.2–1.2
	0.207–0.532	0.700 (0.028)[NS]	0.1181	0.667–0.702	
C20:1n-9	1.106 (0.042)	0.748 (0.032)	0.0000	1.104–1.280	0.1–4.3
	1.117 (0.044)	0.795 (0.034)	0.0000	1.112–1.281	
C20:2n-6	0.070 (0.017)	0.113 (0.011)	0.0000	0.068–0.072	< LOQ–0.86
	0.058 (0.006)	0.114 (0.011)	0.0000	0.060–0.067	
C20:2n-9	<LOQ	0.338 (0.074)[NS]	NA	<LOQ	< LOQ–0.86
	0.035 (0.016)	0.373 (0.079)	0.0000	0.027–0.033	
C22:0	0.349 (0.013)	0.268 (0.012)	0.0000	0.314–0.372	<LOQ–0.6
	0.347 (0.013)	0.272 (0.012)	0.0000	0.313–0.370	
C24:0	0.192 (0.025)	0.125 (0.010)	0.0001	0.196–0.254	<LOQ–0.3

Analytes	Control Kumily (% relative)	LBFLFK (sprayed) (% relative)	T-test[2]	Non-GM reference variety	Publically available data
	Mean[1] (SD)	Mean[1] (SD)	p-value[3]	Range[1]	Range
	0.192 (0.021)	0.252 (0.017)	0.0000	0.192–0.252	
C24:1n-9	0.133 (0.013)	0.099 (0.013)	0.0004	0.133–0.148	< LOQ–0.4
	0.133 (0.014)	0.095 (0.013)	0.0001	0.132–0.148	
Total TFA	0.096 (0.012)	0.155 (0.013)	0.0000	0.066–0.094	NA
	0.626 (0.039)	0.594 (0.065)[NS]	0.2489	0.238–0.561	

Yellow shading represents mean values for RBD oil; Orange shading represents mean values for crude oil
Red border represents FAs with mean values of LBFLFK consistently outside the ranges for reference varieties and publically available data .
[1] LBFLFK, Kumily and reference varieties means were formed from ten replicate samples (n = 10).
[2] Student's paired t-Test (two-tailed distribution) was used to determine the probability of a significant difference between LBFLFK and Kumily means. [3] indicates p≤ 0.05
[NS] indicates statistically not significant; NA= Not available; LOQ = Limit of quantification

Fatty acids produced as a result of the EPA and DHA producing trait

In addition to the FAs C20:5n-3 (EPA) and C22:6n-3 (DHA), a number of other FAs are associated with the EPA and DHA producing trait. These are: C16:1 trans, C18:2n-9, C18:3n-6, C18:4n-3, C20:2n-9, C20:3n-3, C20:3n-6, C20:3n-9, C20:4n-3, C20:4n-6, C22:4n-3, C22:4n-6, C22:5n-3 (DPA), and C22:5n-6. As expected, these FAs consistently had values below the assay LOQ in Kumily and other non-GM reference varieties. These FAs are present in other organisms and foods that are safely and routinely consumed. Their presence in LBFLFK canola therefore does not raise any safety concerns.

A summary of the mean values for the FAs produced as a result of the new EPA and DHA producing trait is provided in Table 13.

Table 13: Fatty acids (mean ±SD) produced in LBFLFK oil as a result of the EPA and DHA producing trait

Fatty acid	LBFLFK sprayed Crude oil[1] (% of total FAs)	LBFLFK sprayed RBD oil[1] (% of total FAs)
	Mean (SD)	Mean (SD)
C16:1 trans	< LOQ	< LOQ
C18:3n-6	2.348 (0.361)	2.211 (0.348)
C18:4n-3	0.356 (0.041)	0.314 (0.037)
C20:3n-3	0.065 (0.015)	0.062 (0.013)
C20:3n-6	5.199 (0.438)	4.959 (0.450)
C20:3n-9	0.055 (0.015)	0.047 (0.014)
C20:4n-3	2.222 (0.314)	1.912 (0.283)
C20:4n-6	2.057 (0.283)	1.747 (0.251)
C20:5n-3 (EPA)	5.390 (0.429)	4.132 (0.321)
C22:4n-3	1.173 (0.176)	1.021 (0.156)
C22:4n-6	0.599 (0.061)	0.530 (0.058)
C22:5n-3 (DPA)	2.915 (0.328)	2.225 (0.240)
C22:5n-6	0.065 (0.009)	0.052 (0.008)
C22:6n-3 (DHA)	0.512 (0.069)	0.358 (0.039)

[1] LBFLFK and Kumily means were formed from ten replicate samples (n = 10); LOQ = Limit of quantification

5.4.2 Other components

The mean values for protein and amino acids in both crude oil and RBD oil were below the LOQ for all samples tested, therefore excluded from statistical analysis. A summary of the statistically significant differences for the other components analysed in the oil is provided in Table 14, and are briefly described below.

Vitamins

Six vitamins were analysed for both crude oil and RBD oil. A statistically significant difference in mean levels was observed for vitamin K1 in crude oil, while no difference was observed for RBD oil. The vitamin K1 values for both LBFLFK and Kumily in crude oil fractions were higher than the reference range but were well within the literature range for non-GM varieties. Therefore the differences observed are not biologically meaningful. All other vitamin mean values were within the natural variability of the analytes in the reference varieties and publically available data.

Phytosterols

A total of fifteen phytosterols including total phytosterols were analysed in both crude oil and RBD oil. The mean values for most of the individual phytosterols were low or below the LOQ (except campestenol and sitosterol). However, statistically significant differences were observed for brassicasterol, cholesterol, and delta-5 avenasterol for both crude oil and RBD oil, and delta-5,24 stigmastadienol and delta-7 stigmastenol in the crude oil fraction only (Table 14). The mean values for brassicasterol and delta-5 avenasterol in both LBFLFK and Kumily were slightly lower than the lower limit of the reference varieties but were within the ranges from publically available data. Therefore the differences observed are not biologically meaningful. All other phytosterol mean values were within the natural variability of the analytes in the reference varieties and publically available data.

Table 14: Summary of statistically significant compositional differences (excluding fatty acids) between LBFLFK oil and control kumily

Analytes	Control Kumily	LBFLFK (sprayed)	T-test[2]	Non-GM reference variety	Publically available data
	Mean[1] (SD)	Mean[1] (SD)	p-value[3]	Range[1]	Range
Crude oil					
Vitamin K1 (mg/100g fw)	0.10685 (0.02373)	0.13404 (0.03797)	0.0242	0.06309–0.10660	0.04-0.56
Brassicasterol (% fw)	0.1041 (0.0058)	0.0664 (0.0018)	0.0000	0.0769 – 0.0918	0.044–0.115
Cholesterol (% fw)	0.0035 (0.0007)	0.0029 (0.0004)	0.0198	0.0027 – 0.0039	<LOQ–0.012
delta-5 avenasterol (% fw)	0.0090 (0.0020)	0.0068 (0.0012)	0.0235	0.0088 – 0.0169	0.022–0.058
delta-5,24 stigmastadienol (% fw)	0.0034 (0.0003)	0.0037 (0.0003)	0.0183	0.0030 – 0.0044	N/A
delta-7 stigmastenol (% fw)	0.0014 (0.0008)	0.0051 (0.0019)	0.0004	0.0013 – 0.0027	<LOQ–0.012
RBD oil					
Brassicasterol (% fw)	0.0909 (0.0068)	0.0577 (0.003)	0.0000	0.0667 – 0.0813	0.044–0.115
Cholesterol (% fw)	0.0029 (0.0005)	0.0022 (0.0007)	0.0255	0.0023 – 0.0031	<LOQ–0.012

Analytes	Control Kumily	LBFLFK (sprayed)	T-test[2]	Non-GM reference variety	Publically available data
	Mean[1] (SD)	Mean[1] (SD)	p-value[3]	Range[1]	Range
delta-5 avenasterol (% fw)	0.0083 (0.0017)	0.0064 (0.0013)	0.0232	0.0076 – 0.0143	0.022–0.058

[1] LBFLFK, Kumily and reference varieties means were formed from ten replicate samples (n = 10).
[2] Student's paired t-Test (two-tailed distribution) was used to determine the probability of a significant difference between LBFLFK and Kumily means; [3] indicates p≤ 0.05.
Mauve shading represents LBFLFK means that are significantly lower than the Kumily means, while the orange shading represents LBFLFK means that are significantly higher.
N/A = Not available

5.5 Conclusion

Detailed compositional analyses were conducted on seed and oil (crude oil and RBD oil) from LBFLFK harvested across a total of three growing seasons. Harvested seeds and oil were analysed for proximates, FAs, amino acids, minerals, vitamins, phytosterols and anti-nutrients.

Seeds

A total 74 individual analytes with values above the LOQ were included in the analysis.

Of these, 28 individual analytes (including 12 FAs) showed statistically significant differences compared to the control Kumily. The changes in FA profile were consistent with those expected as a result of the new trait. Apart from the intended change to the FA profile and a slightly higher content of TFAs, seed from LBFLFK is otherwise compositionally equivalent to seed from conventional canola varieties.

Crude oil and RBD oil

A total of 38 (crude oil) and 33 (RBD oil) individual analytes with values above LOQ were included in the analysis. Of these, 24 (crude oil) and 22 (RBD oil) individual analytes (including FAs) showed statistically significant differences compared to the control Kumily. As with the seed analyses, these differences were consistent with those expected as a result of the new trait. The LBFLFK RBD oil fraction had higher total level of TFAs relative to crude oil, however this is from the refinement process. This increase was observed in RBD oil from both LBFLFK and Kumily and therefore is not considered biologically meaningful.

Apart from the intended change to FA profile, crude oil and RBD oil from LBFLFK are otherwise compositionally equivalent to oil from non-GM canola varieties.

6 Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

If the compositional analysis indicates biologically meaningful changes in the levels of certain nutrients in the GM food, additional nutritional assessment may assist to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply. Evidence indicates that feeding studies using target livestock species will add little to the safety assessment (see e.g. OECD 2003; Bartholomaeus et al., 2013; Herman and Ekmay, 2014).

In the case of canola line LBFLFK, there are significant changes in the FA profile of the seed and crude oil particularly in the production of n-3 LC-PUFAs. This is expected due to the nature of the genetic modification. Naturally preformed n-3 LC PUFAs are mainly available in the human diet through seafood and marine oils. The canola line LBFLFK will serve as an additional plant-based source of n-3 LC-PUFA for consumers.

FSANZ has assessed the increase in TFAs as a result of directing the biosynthesis pathway of canola toward n-3 LC-PUFA in a previous application (DHA canola, A1143). The previous assessment indicates that an increase in TFAs from canola line LBFLFK would not pose a public health concern. Further assessment is not required.

FSANZ has conducted a separate nutrition risk assessment of canola line LBFLFK to determine the potential risks associated with the intake of the n-3 LC-PUFAs by the general population and whether this may cause any adverse health effects (see Supporting Document 2).

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Appendix 1

Table 1.1: Description of the genetic elements contained in the T-DNA of LTM593-1qcz

Genetic elements	Relative position	Size (bp)	Source	Description & Function	References
RB	1-328	328	*Agrobacterium tumefaciens*	• RB sequence used to transfer the T-DNA region into the host genome • NCBI accession AF242881	(Barker et al., 1983)
IS	329-508	180	Synthetic	• Required for cloning of genetic elements	
D6E(Pp) expression cassette					
p-*USP*(*Vf*)	509-1192	684	*Vicia faba*	• Promoter region of an unknown seed protein gene (*USP*) • Directs transcription of the *D6E*(*Pp*) gene • NCBI accession HJ187156	(Bäumlein et al., 1991)
i-*At1g01170*	1193-1444	252	*Arabidopsis thaliana*	• Intron-containing 5'UTR sequence that enhances expression of D6E(*Pp*) protein • NCBI accession At1g01170	(Nakabayashi et al., 2005)
IS	1445-1446	2	Synthetic		
c-*D6E*(*Pp*)	1447-2319	873	*Physcomitrella patens*	• Coding sequence of the delta-6-elongase gene • NCBI accession AF428243	(Zank et al., 2000; Zank et al., 2002)
t-*CaMV35S*	2320-2535	216	*Cauliflower mosaic virus*	• 3'UTR region of the *CaMV*35S • Directs polyadenylation of the *D6E*(*Pp*) gene • NCBI accession AF234316	(Hajdukiewicz et al., 1994)
IS	2536-2627	92			
D5D(Tc)1 expression cassette					
p-*CNL*(*Lu*)	2628-3691	1064	*Linum usitatissimum*	• Promoter region of conlinin gene • Directs transcription of the *D5D*(*Tc*) gene • NCBI accession HJ187156	(Truksa et al., 2003)
i-*At5g63190*	3692-4068	377	*Arabidopsis thaliana*	• Intron-containing 5'UTR sequence that enhances the expression of D5D(*Tc*) protein • NCBI accession At5g63190	(Sharma et al., 2007; Wang et al., 2008)
IS	4069-4071	3			
c-*D5D*(*Tc*)1	4072-5391	1320	*Thraustochytrium sp*	• Coding sequence of the delta-5 desaturase gene • NCBI accession AF489588	(Qui et al.,, 2001)
t-*OCS*	5392-5583	192	*Agrobacterium tumefaciens*	• 3'UTR region of the octopine synthase gene from *Ti* plasmid pTi15955 • Directs polyadenylation of the *D5D*(*Tc*) gene • NCBI accession NC_002377	(MacDonald et al., 1991)
IS	5584-5718	135	Synthetic		
D6D(Ot) expression cassette					
p-*SBP*(*Vf*)	5719-7517	1799	*Vicia faba*	• Promoter region of a sucrose-binding protein-related gene. • Directs transcription of the *D6D*(*Ot*) gene • NCBI accession LQ576466	(Grimes et al.,, 1992; Heim et al., 2001)
i-*At1g65090*	7518-7972	455	*Arabidopsis thaliana*	Intron-containing 5'UTR sequence that enhances the expression of D6D(*Tc*) protein • NCBI accession At165090	(Braybrook et al., 2006)
IS	7973-7981	9			
c-*D6D*(*Ot*)	7982-9352	1371	*Ostreococcus tauri*	• Coding sequence of the delta-6 desaturase gene • NCBI accession AY746357	(Domergue et al., 2005)
IS	9353-9379	27			

Genetic elements	Relative position	Size (bp)	Source	Description & Function	References
t-*CATHD*(*St*)	9380-9614	235	*Solanum tuberosum*	• 3'UTR region of the cathepsin D inhibitor gene • Directs polyadenylation of the *D6D*(*Ot*) gene • NCBI accession HJ187168	(Hannapel 1993)
IS	9615-9692	78			
D6E(Tp) expression cassette					
p-*PXR*(*Lu*)	9693-11419	1727	*Linum usitatissimum*	• Promoter region of peroxiredoxin like-protein gene *PXR* • Directs transcription of the *D6E*(*Tp*) gene • NCBI accession HL700593	(Duwenig & Loyall, 2007)
i-*At1g62290*	11420-12265	846	*Arabidopsis thaliana*	• Intron-containing 5'UTR sequence that enhances the expression of D6E(*Tp*) protein • NCBI accession At1g62290	(Chen et al., 2002)
IS	12266-12278	13			
c-*D6E*(*Tp*)	12279-13097	819	*Thalassiosira pseudonana*	• Coding sequence of the delta-6 elongase gene • NCBI accession XM_002288445	(Ambrust et al., 2004)
IS	13098-13152	55			
t-*PXR*(*At*)	13153-13552	400	*Arabidopsis thaliana*	• 3'UTR region of the PXR-like protein gene *PER1* • Directs polyadenylation of the *D6E*(*Tp*) gene • NCBI accession HL700651	(Haslekås et al., 1998)
IS	13553-13721	169			
D12D(Ps) expression cassette					
p-*napA*(*Bn*)	13722-14385	664	*Brassica napus*	• Promoter region of a seed storage protein; napin A/B gene • Directs transcription of the *D12D*(*Ps*) gene • NCBI accession LQ576463	(Elerström et al., 1996; Rask et al., 1998)
i-*At5g63190*	14386-14762	377	*Arabidopsis thaliana*	• Intron-containing 5'UTR sequence that enhances the expression of D12D(*Ps*) protein in plant cells • NCBI accession At5g63190	(Sharma et al., 2007; Wang et al., 2008)
IS	14763-14768	6			
c-*D12D*(*Ps*)	14769-15965	1197	*Phytophthora sojae*	• Coding sequence of the delta-12 desaturase gene • NCBI accession GY508423	(Cirous & Bauer, 2006)
IS	15966-15983	18			
t-*rbcs*(*Ps*)	15984-16541	558	*Pisum sativum*	• 3'UTR region of the RuBisCO small subunit gene (*rbcS*) • Directs polyadenylation of the *D12D*(*Ps*) gene • NCBI accession AY572837	(Coruzzi et al., 1984; Smigocki 1991)
IS	16542-16633	92			
O3D(Pir)1 expression cassette					
p-*SETL*(*Bn*)	16634-17867	1234	*Brassica napus*	• Promoter region of *SETL*(*Bn*) gene • Directs transcription of the *O3D*(*Pir*)1 gene • NCBI accession HC307781	(Bauer & Senger, 2010)
IS	17868-17869	2			
c-*O3D*(*Pir*)1	17870-18961	1092	*Phythium irregular*	• Coding sequence of the omega-3 desaturase gene • NCBI accession FB753541	(Cheng et al., 2010)
IS	19962-18982	21			
t-*SETL*(*Bn*)	18983-19596	614	*Brassica napus*	• 3'UTR region of the *SETL*(*Bn*) gene • Directs polyadenylation of the *O3D*(*Pir*)1 gene • NCBI accession HC307782	(Bauer & Senger, 2010)
IS	19497-19674	78			

Genetic elements	Relative position	Size (bp)	Source	Description & Function	References
O3D(Pi) expression cassette					
p-*USP*(*Vf*)	19675-20358	684	*Vicia faba*	• Promoter region of an unknown seed protein gene *USP* • directs transcription of the *O3D*(*Pi)* gene • NCBI accession X56240	(Bäumlein et al., 1991)
i-*At1g01170*	20359-20610	252	*Arabidopsis thaliana*	• Intron-containing 5'UTR sequence that enhances expression of *O3D*(*Pi*) protein • NCBI accession At1g01170	(Nakabayashi et al., 2005)
IS	20611-20620	10			
c-*O3D*(*Pi*)	20621-21706	1086	*Phytophthora infestans*	• Coding sequence of the omega-3 desaturase gene • NCBI accession XM_002902553	(Wu et al., 2005)
IS	21707-21714	8			
t-*CaMV35S*	21715-21930	216	*Cauliflower mosaic virus*	• 3'UTR region of *CaMV35S* • Directs polyadenylation of the *O3D*(*Pi*) gene • NCBI accession AF234316	(Hajdukiewicz et al., 1994)
IS	21931-22065	135			
D5D(Tc)2 expression cassette					
p-*SETL*(*Bn*)	22066-23299	1234	*Brassica napus*	• Promoter region of *SETL*(*Bn*) gene • Directs transcription of the *D5D*(*Tc*)2 gene • NCBI accession HC307781	(Bauer & Senger, 2010)
IS	23300-23301	2			
c-*D5D*(*Tc*)2	23302-24621	1320	*Thraustochytrium sp*	• Coding sequence of the delta-5 desaturase gene • NCBI accession AF489588	(Qui et al., 2001)
IS	24622-24642	21			
t-*SETL*(*Bn*)	24643-25256	614	*Brassica napus*	• 3'UTR region of the *SETL*(*Bn*) gene • Directs polyadenylation of the *O3D*(*Pir*)1 gene • NCBI accession HC307782	(Bauer & Senger, 2010)
IS	25257-25402	146			
D4D(Tc) expression cassette					
p-*ARC5*(*Pv*)	25403-26553	1151	*Phaseolus vulgaris*	• Promoter region of Arcelin-5 (*ARC5*) gene • Directs transcription of the *D4D*(*Tc*) gene • NCBI accession JC056714	Goossens et al., 1994; Goossens et al., 1999)
IS	26554-26563	10			
c-*D4D*(*Tc)*	26564-28123	1560	*Thraustochytrium sp*	• Coding sequence of the delta-4 desaturase gene • NCBI accession GN042654	(Qui et al., 2001)
IS	28124-28736	13			
t-*ARC5*(*Pv*)	28137-28736	600	*Phaseolus vulgaris*	• 3'UTR region of Arcelin-5 (*ARC5*) gene • Directs polyadenylation of *D4D*(*Tc*) • NCBI accession Z50202	Goossens et al., 1994; Goossens et al., 1999)
IS	28737-28828	92			
O3D(Pir)2 expression cassette					
p-*PXR*(*Lu*)	28829-30555	1727	*Linum usitatissimum*	• Promoter region of peroxiredoxin like-protein gene *PXR* • Directs transcription of the *O3D*(*Pir*)2 gene • NCBI accession HL700593	(Duwenig & Loyall, 2007)
i-*AGO4*(*At*)	30556-31313	758	*Arabidopsis thalaiana*	• Intron-containing 5'UTR sequence that enhances the expression of O3D(*Pir*)2 protein	(Zilberman et al., 2003)

Genetic elements	Relative position	Size (bp)	Source	Description & Function	References
IS	31314-31328	15			
c-*O3D*(*Pir*)2	31329-32420	1092	*Phythium irregular*	• Coding sequence of the omega-3 desaturase gene • NCBI accession FB753541	(Cheng et al., 2010)
IS	32421-32476	56			
t-*PXR*(*At*)	32477-32878	400	*Arabidopsis thaliana*	• 3'UTR region of the PXR-like protein gene *PER1* • Directs polyadenylation of the *O3D*(*Pir*)2 gene • NCBI accession HL700651	(Haslekås et al., 1998)
IS	32877-33011	135			
D4D(Pi) expression cassette					
p-*CNL*(*Lu*)	33012-34075	1064	*Linum usitatissimum*	• Promoter region of conlinin gene • Directs transcription of *D4D*(*Pi*) gene • NCBI accession HJ187156	(Truksa et al., 2003)
i-*At1g65090*	34076-34530	455	*Arabidopsis thaliana*	• Intron-containing 5'UTR sequence that enhances the expression of the D4D(*Pi*) protein • NCBI accession At1g65090	(Braybrook et al., 2006)
IS	34531-34539	9			
c-D4D(*Pi*)	34540-35877	1338	*Pavlova lutheri*	• Coding sequence of the delta-4 desaturase gene • NCBI accession AY332747	(Tonon et al., 2003)
IS	35878-35898	21			
t-*OCS*	35899-36090	192	*Agrobacterium tumefaciens*	• 3'UTR region of the octopine synthase gene from *Ti* plasmid pTi15955 • Directs polyadenylation of the *D4D*(*Pi*) gene • NCBI accession NC_002377	(MacDonald et al., 1991)
IS	36091-36283	193			
D5E(Ot) expression cassette					
p-*FAE1*(*Bn*)	36284-37713	1430	*Brassica napus*	• Promoter region of fatty acid elongase (FAE1.1) gene • Directs transcription of the *D5E*(*Ot*) gene • NCBI accession HC474755	(Han et al., 2001)
i-*At1g62290*	37714-38560	847	*Arabidopsis thaliana*	• Intron-containing 5'UTR sequence that enhances the expression of D5E(*Ot*) protein. • NCBI accession At1g62290	(Chen et al., 2002)
IS	38561-38567	7			
c-*D5E*(*Ot*)	38568-39470	903	*Ostreococcus tauri*	• Coding sequence of the delta-5 elongase gene NCBI accession CS020159	(Crowe et al., 1994)
IS	39471-39486	16			
t-*FAE1*(*At*)	39487-39886	400	*Arabidopsis thaliana*	• 3'UTR region of the fatty *FAE1* gene • Directs polyadenylation of the *D5E*(*Ot*) gene NCBI accession HV571989	(Rossak et al., 2001)
IS	39887-40004	118			
AHAS(At) expression cassette					
p-*Ubi4*(*Pc*)	40005-40393	394	*Petroselinum crispum*	• Promoter region of ubiquitin (*ubi4-2*) gene • Directs transcription of *AHAS*(*At*) gene • NCBI accession X64345	(Kawalleck et al., 1993)
i-Ubi4(Pc)	40399-40986	588	*Petroselinum crispum*	• Intron-containing 5'UTR region of *ubi4* gene that enhances the expression of AHAS(*At*) protein • NCBI accession X64345	(Kawalleck et al., 1993)
IS	40987-40993	7		•	

Genetic elements	Relative position	Size (bp)	Source	Description & Function	References
c-*AHAS*(*At*)	40994-43006	2013	*Arabidopsis thaliana*	• Coding sequence of the acetohydroxy acid synthase (*AHAS*) gene • Modified to have substitutions S653N and A122T • NCBI accession NM_114714	(Mazur et al., 1987)
t-*AHAS*(*At*)	43007-43786	780	*Arabidopsis thaliana*	• 3'UTR region of the *AHAS*(*At*) gene • Directs polyadenylation of the *AHAS*(*At*) gene • NCBI accession NM_114714	(Mazur et al., 1987)
IS	43787-43786	88			
LB	43875-44010	136	*Agrobacterium tumefaciens*	• LB sequence used to transfer the T-DNA region into the host genome NCBI accession AF242881	(Barker et al., 1983)

IS=intervening sequence; p=promoter; i=intron-containing 5'UTR; c=coding sequence; t=transcription terminator

Appendix 2

Results of homology searches for each of the ten n-3 LCPUFA proteins introduced into LBFLFK

In silico analyses were conducted, where the sequence of each of the ten proteins in the n-3 LC-PUFA pathway was used to search for homologous sequences present in organisms used in food, food production or in animal feeds. The purpose of this search was to identify the similarity (sequence identity) of each protein to other proteins present in consumed foods or used in food production or animal feeds. The results are presented in Tables 2.1

Table 2.1: Amino acids similarity (sequence identity) between the newly expressed proteins in LBFLFK and other proteins found in food/feed sources

Protein	Accession	Scientific Name	Common Name	Identity %	Description
D12D(Ps)	KAE8821404.1	*Hordeum vulgare*	Barley	46.7	FA desaturase DES2
	XP_006647793.1	*Oryza sativa*	Japanese rice	46.4	FA desaturase 2
	ABF50053.1	*Zea mays*	Corn	47	FAD2
	AHA83525.1	*Mortierella alpina*	Fungi	45	Delta-12 FA desaturase
D6D(*Ot*)	XP_023931483.1	*Lingula anatina*	lamp shell	27.4	FA desaturase 2
	XP_029649716.1	*Octopus vulgaris*	Common octopus	27.3	Acyl-CoA 6-desaturase-like
	XP_027029033.1	*Tachysurus fulvidraco*	Yellow-head catfish	25	FA desaturase 2-like
	XP_015651259	*Oryza sativa*	Rice	25	Delta(8)-FA desaturase 2
	XP_003206387.2	*Meleagris gallopavo*	Turkey	25	Acyl-CoA (8-3)-desaturase-like
D6E(*Tp*)	XP_010779444	*Notothenia coriiceps*	Black rock-od	36	Elongation of very LC-FAFA protein 4-like
	XP_005470661.1	*Oreochromis niloticus*	Nile tilapia	33.1	Delta-6 fatty acyl desaturase isoform X1
	NP_001117039	*Salmo salar*	Atlantic salmon	28	Polyunsaturated FA elongase
D6E(*Pp*)	AB762292.1	*Thraustochytrium*	Marine microalgae	41.4	Delta-6 FA elongase
	AAF70417.1	*Mortierella alpina*	Fungi	42	LC-polyunsaturated FA elongation enzyme
	XM_014863863.2	*Equus asinus*	Donkey	33.8	Elongation of very LC - FAs protein 5 isoform X2
	XP_005479178.1	*Oreochromis niloticus*	Nile tilapia	33	Elongation of very long-chain FAs protein 4
	XP_014000130	*Salmo salar*	Atlantic salmon	35	Elongation of very long-chain FAs protein 4
D5D(*Tc*)	BAK08911.1	*Thraustochytrium aureum*	Marine microalgae	58.1	Delta-5 desaturase
	NP_001165752.1	*Salmo salar*	Atlantic salmon	26.2	Delta-6 fatty acyl desaturase
	XP_005470661.1	*Oreochromis niloticus*	Nile tilapia	26.2	FA desaturase-6
O3D(*Pir*)	XP_014790265.1	*Octopus bimaculoides*	California two-spot octopus	43	Omega-3 FA desaturase, ER like
	XP_008457364.1	*Cucumis melo*	Muskmelon	38	Acyl-lipid omega-3 desaturase (Cytochrome B5)
	XP_004490604.1	*Cicer arietinum*	Chickpea	33.8	Omega-3 FA desaturase, ER
	AEP37842.1	*Linum usitatissimum*	Flax seed	35	Omega-3 desaturase
O3D(*Pi*)	XP_014790265.1	*Octopus*	California two-	43.3	omega-3 FA desaturase, ER-

Protein	Accession	Scientific Name	Common Name	Identity %	Description
		bimaculoides	spot octopus		like
	MBD2277983.1	*Aphanizomenon flos-aquae*	Cynobacteria	36	FA desaturase
	NP_001233791.2	*Solanum lycopersicum*	Tomato	38.8	Omega-3 FA desaturase
	ABG22328.1	*Oryza sativa*	Rice	39.3	Omega-3 FA desaturase, chloroplast precursor
	NP_001302953.1	*Brassica napus*	Canola,	37.7	Omega-3 FA desaturase, ER
D5E(*Ot*)	KTG39645.1	*Cyprinus carpio*	Common carp	35.4	Hypothetical protein cypCar_00022757
	XP_014000130.1	*Salmo salar*	Atlantic salmon	34.8	Elongation of very long-chain FAs protein 4-like
	AF206662.1	*Mortierella alpina*	Fungi	30.8	LC-polyunsaturated FA elongation enzyme
	XP_029643977.1	*Octopus vulgaris*	Common octopus	37.7	Elongation of very long-chain FAs protein 4-like
D4D(*Tc*)	AAN75708.1	*Thraustochytrium aureum*	Marine microalgae	71.9	Delta 4-desaturase [
	AAC72755.1	*Mortierella alpina*	Fungi	30.4	Delta-5 FA desaturase
	XP_002699331.1	*Bos taurus*	Cow	24	FA desaturase 1 isoform X1
D4D(*Pl*)	ACB11556.1	*Pyropia yezoensis*	Japanese nori	28.3	Putative delta-5 FA desaturase, partial
	ACD10793.1	*Dicentrarchus labrax*	European sea bass	23.8	FA delta-6 desaturase