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CFSAN Note to the File

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To: Administrative Record, BNF No. 000165

Subject: Canola with transformation event LBFLFK (LBFLFK canola)

Keywords: Canola; *Brassica napus*; low erucic acid rapeseed; omega-3 long-chain polyunsaturated fatty acids (LCPUFAs); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); fatty acid desaturase; fatty acid elongase; imidazolinone herbicide tolerance; modified acetohydroxy acid synthase (AHAS); Unique identifier BPS-BFLFK-2; BASF Plant Science, L.P.

Summary

BASF Plant Science, L.P. (BASF) has completed a consultation with the Food and Drug Administration (FDA) on food derived from LBFLFK canola, with an altered oil composition and imidazolinone herbicide tolerance. The altered oil composition is conferred through seed-specific expression of genes encoding fatty acid desaturases and elongases from plants, oomycetes, and marine microorganisms. Together, the introduced enzymes enable biosynthesis of omega-3 long chain polyunsaturated fatty acids (LCPUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Imidazolinone herbicide tolerance is conferred through constitutive expression of the large subunit of a modified acetohydroxy acid synthase (AHAS) from *Arabidopsis thaliana*. This document summarizes BASF's conclusions and supporting data and information that FDA's Center for Food Safety and Applied Nutrition (CFSAN, we) evaluated pertaining to human food uses. FDA's Center for Veterinary Medicine summarizes its evaluation pertaining to animal food uses in a separate document.

BASF concludes:

- it has not introduced into human food a new protein or other substance that would require premarket approval as a food additive.
- oil from LBFLFK canola is as safe for human food use as other oils that contain LCPUFAs and are currently on the market, including menhaden oil; and, subject to the limitations in 21 CFR 184.1472, is generally recognized as safe (GRAS) for use in human food.[1]

[1] The Generally Recognized as Safe (GRAS) affirmation regulation for menhaden oil (21 CFR 184.1472) establishes limits on the use of menhaden oil to avoid total dietary exposures above 3.0 grams per person per day (g/p/d) of EPA and DHA combined. Paragraph (a)(3) limits maximum use levels of menhaden oil in specific food categories; paragraph (a)(4) restricts use of menhaden oil in combination with any other added oil that is a significant source of EPA or DHA.

- solvent-extracted meal from LBFLFK canola is comparable to and as safe for human food use as other solvent-extracted canola meals that are currently on the market.

CFSAN evaluated data and information supporting these conclusions and considered whether LBFLFK canola raises other regulatory issues involving use in human food under the Federal Food, Drug, and Cosmetic Act (FD&C Act). We have no further safety, nutritional, or regulatory compliance questions at this time about BASF's current intended uses of LBFLFK canola in human food.

Subject of the Consultation

Crop:	Canola (*Brassica napus*)
Designation:	LBFLFK
Intended trait:	Altered oil composition; biosynthesis of omega-3 LCPUFAs
Intended trait:	Tolerance to imidazolinone herbicides
Developer:	BASF Plant Sciences, L.P.
Submission received:	January 30, 2018
Amendments received:	June 4, 2019; August 12, 2019; December 15 and 18, 2019; April 14, 2020; June 10, 2020; October 28, 2020
Intended use:	Use of the oil subject to the limitations in 21 CFR 184.1472; general uses customary for solvent-extracted canola meal.
Transformation vector:	plasmid LTM593[2]
Expression cassette 1:	The D12D(*Ps*) cassette encodes a delta-12 desaturase from *Phytophthora sojae*. The D12D(*Ps*) enzyme catalyzes the conversion of 18:1 n-9 (oleic acid, OA) to 18:2 n-6 (linoleic acid, LA).
Expression cassette 2:	The D6D(*Ot*) cassette encodes a delta-6 desaturase from *Ostreococcus tauri*. The D6D (*Ot*) enzyme catalyzes the conversion of 18:2 n-6 (LA) to 18:3 n-6 (gamma-linolenic acid, GLA).
Expression cassette 3:	The D6E(*Tp*) cassette encodes a delta-6 elongase from *Thalassiosira pseudonana*, which differs from the published *T. pseudonana* sequence by a one amino acid substitution located outside of the domains responsible for protein functionality. The D6E(*Tp*) enzyme catalyzes the conversion of 18:3 n-6 (GLA) to 20:3 n-6 (dihomo-gamma-linolenic acid, DGLA).

[2] The gene sequences in the expression cassettes were based on sequences originally identified from plant, oomycetes, and marine microorganisms. Although the DNA sequences from oomycetes and marine microorganisms were modified for expression in canola, the predicted amino acid sequences of the encoded proteins are unchanged, with noted exceptions.

Expression cassette 4:	The D6E(*Pp*) cassette encodes a delta-6 elongase from *Physcomitrella patens*. The D6E(*Pp*) enzyme catalyzes the conversion of 18:3 n-6 (GLA) to 20:3 n-6 (DGLA).
Expression cassettes 5 and 6:	The D5D(*Tc*) cassettes encode a delta-5 desaturase from *Thraustochytrium* sp. The D5D(*Tc*) enzyme catalyzes the conversion of 20:3 n-6 (DGLA) to 20:4 n-6 (arachidonic acid, ARA).
Expression cassettes 7 and 8:	The O3D(*Pir*) cassettes encode an omega-3 desaturase from *Pythium irregulare*. The O3D(*Pir*) enzyme catalyzes the conversion of 20:4 n-6 (ARA) to 20:5 n-3 (EPA).
Expression cassette 9:	The O3D(*Pi*) cassette encodes an omega-3 desaturase from *Phytophthora infestans*. The O3D(*Pi*) enzyme catalyzes the conversion of 20:4 n-6 (ARA) to 20:5 n-3 (EPA).
Expression cassette 10:	The D5E(*Ot*) cassette encodes a delta-5 elongase from *Ostreococcus tauri*. The D5E(*Ot*) enzyme catalyzes the conversion of 20:5 n-3 (EPA) to 22:5 n-3 (docosapentaenoic acid, DPA).
Expression cassette 11:	The D4D(*Tc*) cassette encodes a delta-4 desaturase from *Thraustochytrium* sp. The D4D(*Tc*) enzyme catalyzes the conversion of 22:5 n-3 (DPA) to 22:6 n-3 (DHA).
Expression cassette 12:	The D4D(*Pl*) cassette encodes a delta-4 desaturase from *Pavlova lutheri*. The D4D(*Pl*) enzyme catalyzes the conversion of 22:5 n-3 (DPA) to 22:6 n-3 (DHA).
Expression cassette 13:	The AHAS(*At*) cassette encodes a modified version of the acetohydroxy acid synthase (AHAS) large subunit from *Arabidopsis thaliana* with two amino acid substitutions (S653N and A122T substitutions) compared to the published *A. thaliana* sequence. The modified AHAS enzyme confers imidazolinone herbicide tolerance.
Transformation method:	Agrobacterium-mediated transformation

Molecular Characterization

Confirmation of intended genetic change

BASF used a combination of analytical methods to determine the integrity and number of DNA insertions. Sequence reads from high-throughput sequencing of LBFLFK canola genomic DNA from three different generations were evaluated.[3] Sequence reads with similarity to the transformation plasmid underwent bioinformatic analysis to identify (1) the number of insertion sites, (2) the copy number of inserted T-DNA sequences, (3) the absence of vector backbone,

[3] According to BASF, high-throughput sequencing achieved 100% breadth of coverage, at least 50x estimated depth of coverage genome-wide, and at least 160x depth of coverage at the T-DNA insertion and at each of six single-copy endogenous reference genes. The sensitivity of the method was demonstrated by 100% coverage of the transformation plasmid in a control sample spiked with 0.1x equivalent copies of the plasmid DNA.

and (4) the stability of the genetic modification. The sequences, organization, and integrity of the T-DNA insertions, as well as the sequences of the flanking canola genome DNA, were determined by locus-specific PCR and Sanger sequencing.[4]

BASF identified four unique junctions between T-DNA and canola genomic sequences, consistent with the presence of two DNA insertions. Bioinformatic analysis showed the two T-DNA insertions (hereafter referred to as Insert 1 and Insert 2) are located on different chromosomes in the canola genome and identified short deletions of genomic DNA (8 bp and 31 bp, respectively) at both insertion sites. Both Insert 1 and Insert 2 contained all 13 intended gene expression cassettes. Sequence analysis revealed two single-nucleotide changes predicted to result in single amino acid substitutions in the expressed proteins: one in D12D(*Ps*) in Insert 1 and one in D4D(*Pl*) in Insert 2. BASF stated that the two amino acid substitutions have no impact on the function of the respective proteins. BASF concluded from its analyses that two largely intact copies of the transformation plasmid T-DNA were integrated into the LBFLFK canola genome.[5]

Absence of vector backbone DNA

BASF reported that high-throughput sequencing results confirmed the LBFLFK canola genome does not contain vector backbone DNA.

Open reading frame analysis

BASF analyzed the DNA sequencing results to identify potential open reading frames (ORFs) created as a result of the two DNA insertions. BASF reported that it identified 11 ORFs equal to or greater than 30 codons in length. Using the deduced amino acid sequences of these ORFs, BASF searched for similarities to allergens in the Food Allergen Research and Resource Program Allergen Protein Database (FARRP, January 2017).[6] BASF also searched for similarities to proteins in the National Center for Biotechnology Information GenBank non-redundant peptide sequence database (NCBI GenBank, May 2017).[7] The descriptions of proteins from the NCBI database search results were subsequently screened for toxin-related keywords and known toxins using the list published in 40 CFR 725.421 and for keywords related to enzymes and other proteins involved in the production of anti-nutrients in common food crops using the Organisation for Economic Co-Operation and Development (OECD) Consensus Documents for Compositional Considerations.[8] BASF stated that the results of its bioinformatic analyses support the conclusion that the deduced amino acid sequences of the identified ORFs

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[4] To facilitate Sanger sequencing, BASF generated a bacterial artificial chromosome library of the LBFLFK canola genome from T3 generation leaf tissue.

[5] BASF reported that both Insert 1 and Insert 2 contain short truncations of Left and Right Border (RB) sequences and that Insert 1 contains a rearranged fragment within its RB sequence. BASF concludes that these changes do not impact the integrity of the T-DNA expression cassettes.

[6] BASF defined significant homology to allergens as >35% identity over 80 amino acids, ≥ 8 contiguous, identical amino acids, or overall homology to a known allergen.

[7] To assess sequence similarity to proteins in the NCBI database, BASF used the Basic Local Alignment Search Tool for proteins (BLASTP) with an E-value cut-off set to ≤ 1.

[8] These included consensus documents for canola (2011), maize (2002), rice (2016), soybean (2012), sugar beet (2002), and sugarcane (2011).

do not share significant homology with known allergens, toxins, or proteins related to biosynthetic pathways or production of anti-nutrients.

Stability over multiple generations

BASF assessed the stability and inheritance of the two DNA insertions using the results from high-throughput sequencing across multiple generations (generations T3, T4, and T5) in combination with results from genotyping and chi-square statistical analysis of hemizygous, segregating populations (generations F2 and F3). BASF reported that the same four unique junction sites between T-DNA sequences and the LBFLFK canola genomic sequences were present in the tested generations, confirming stability of the two DNA insertions. BASF observed segregation ratios for Insert 1 and Insert 2 that were consistent with those expected for two independent loci inherited according to Mendelian laws of inheritance.

Introduced Proteins: fatty acid desaturases and elongases

Intended trait:	Altered oil composition; biosynthesis of omega-3 LCPUFAs
Source organisms:	• Oomycetes *(Phytophthora sojae, Phytophthora infestans, Pythium irregulare*) • Marine microorganisms (*Ostreococcus tauri, Pavlova lutheri, Thraustochytrium* sp., *Thalassiosira pseudonana*) • Moss (*Physcomitrella patens*)
Protein descriptions:	Fatty acid desaturases catalyze the formation of a double bond between two carbon atoms at specific positions of fatty acid carbon chains. The introduced desaturases are delta-12 desaturase, delta-6 desaturase, delta-5 desaturase, delta-4 desaturase, and omega-3 desaturase. Fatty acid elongases catalyze the addition of an ethyl group at a fixed position on the fatty acid carbon chain, thereby extending its length by two carbons. The introduced elongases are delta-6 elongase and delta-5 elongase.
Intended function:	In combination, the introduced desaturases and elongases progressively desaturate and elongate fatty acids to produce the desired omega-3 LCPUFAs.

Levels of introduced desaturases and elongases in LBFLFK canola seed

BASF measured the levels of the introduced fatty acid desaturases and elongases in LBFLFK canola seed collected from field trials. According to BASF, quantitative immunoassays were developed and validated for each of the introduced proteins.[9] In mature seed, levels of the introduced fatty acid desaturases and elongases ranged from below 1.0 microgram (µg)/gram

[9] An enzyme-linked immunosorbent assay was used to quantify the levels of D12D(*Ps*), D5D(*Tc*), D6E(*Pp*), and D5E(*Ot*); a Western blot analysis was used to quantify the levels of D6D(*Ot*), O3D(*Pir* and *Pi*), D4D(*Pl* and *Tc*), and D6E(*Tp*).

(g) dry weight (DW) for D12D(*Ps*) and D6E(*Pp*) up to 936 µg/g DW for D6E(*Tp*) and 561 µg/g DW for O3D(*Pir*). The remaining introduced desaturases and elongases were present at mean levels of less than 50 µg/g DW.[10]

Safety assessment of fatty acid desaturases and elongases in food

BASF relied on a weight of evidence approach to assess the safety of the introduced fatty acid desaturases and elongases in food. The evidence considered by BASF included enzyme substrate specificity, the presence of related fatty acid desaturases and elongases from other species in foods, their susceptibility to digestive enzymes and heat, the results of a systematic search of publicly available scientific literature about the enzymes and source organisms, and the results of bioinformatic analyses. BASF summarized the available evidence demonstrating that the introduced enzymes (and their source organisms) are not associated with human disease, toxicity, anti-nutritive effects, or allergenicity.

BASF characterized each of the ten introduced integral membrane enzymes using detergent-free membrane fraction samples prepared from crude extracts of immature LBFLFK canola seeds. Biochemical analyses included enzyme functional assays, Western blot analyses, tryptic peptide mapping, and glycosylation analyses. BASF provided the results of these biochemical analyses along with its conclusion that these results confirm the identity and activity of the introduced fatty acid desaturases and elongases in LBFLFK canola. BASF also discussed published evidence of the enzymatic activity of the individual fatty acid desaturases and elongases, including the results of an enzymatic conversion study showing the fatty acid substrate preferences of each when expressed in yeast.[11]

BASF summarized evidence showing that desaturases and elongases are found in a wide variety of plants, animals, and microorganisms, where they are involved in fatty acid biosynthesis. BASF conducted bioinformatic analyses to identify similar fatty acid desaturases and elongases in foods that are commonly consumed. Although the fatty acid desaturases and elongases introduced in LBFLFK canola are derived from source organisms that are not directly used in human food, BASF reported that the introduced enzymes share amino acid sequence and/or activity similarities with desaturases and elongases present in a variety of food crops and animals (e.g., fish), as well as in fungi used in production of edible oils (e.g., *Mortierella alpina*).

BASF also conducted bioinformatic analyses comparing the amino acid sequences of the introduced enzymes to allergens listed in the FARRP Allergen Online database (January 2017) [6] and to proteins in the NCBI GenBank non-redundant peptide sequence database (May 2017).[7] The descriptions of proteins from the NCBI database search results were subsequently screened for toxin-related keywords and known toxins using the list published in 40 CFR 725.421 and for keywords related to enzymes and other proteins involved in the production of anti-nutrients in common food crops using the OECD Consensus Documents for Compositional Considerations.[8] Based on the results of these analyses, BASF concluded that the introduced desaturases and

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[10] Protein expression levels for the introduced desaturases and elongases were similar in LBFLFK canola grown with or without imidazolinone herbicide treatment.

[11] Yilmaz, J.L. et al., (2017). Determination of Substrate Preferences for Desaturases and Elongases for Production of Docosahexaenoic Acid from Oleic Acid in Engineered Canola. *Lipids* **52**: 207-222.

elongases in LBFLFK canola do not bear significant homology to known or predicted allergens, toxins, or proteins that are themselves, or are associated with the biosynthesis or production of anti-nutrients. This conclusion is corroborated by the absence of evidence in the published literature associating the source organisms with human disease or the introduced proteins with toxicity, allergenicity or anti-nutritional properties in humans.[12]

BASF conducted *in vitro* digestive fate and heat sensitivity studies to further assess the safety of the introduced enzymes. Simulated gastric and intestinal fluid studies using membrane fractions prepared from crude extracts of immature seed showed that the desaturases and elongases are susceptible to digestion by pepsin, by pancreatin, or both pepsin and pancreatin. Heat sensitivity studies of the desaturases and elongases under conditions similar to commercial oil processing (at temperatures >50˚C) showed that the structural integrity[13] and/or enzymatic activity of the introduced proteins was disrupted. Protein sensitivity to degradation by gastric enzymes and to inactivation under commercial processing conditions (e.g., heat) supports a conclusion that the potential of the protein to be allergenic or toxic following consumption is low.[14]

Citing several publications, BASF further explained that the commercial food processing conditions (e.g., heat, pH change) used to produce seed oils and solvent-extracted meal disrupt the structure and activity of enzymes and that the levels of protein generally in food grade oil are below quantifiable concentrations. BASF concluded that dietary exposure to enzymatically active forms of the introduced desaturases and elongases from consumption of human food derived from LBFLFK canola seed oil and solvent-extracted meal will be negligible.

Based on the weight of this evidence, BASF concludes that the introduced desaturases and elongases do not raise any safety concerns with regard to human health.

Introduced Protein: modified AHAS

Intended trait:	Tolerance to imidazolinone herbicides
Source organism:	Plant (*Arabidopsis thaliana*)
Protein description:	The modified acetohydroxy acid synthase (AHAS) expressed in LBFLFK canola consists of the large subunit of AHAS, with two amino acid substitutions (A122T and S653N) compared to the *A. thaliana* AHAS
Intended function:	AHAS is an essential enzyme involved in the biosynthesis of branched-chain amino acids. The amino acid substitutions in the modified AHAS reduce its binding affinity to imidazolinone herbicides

[12] BASF acknowledged reports that the diatom *T. pseudonana* can produce a neurotoxin; however, the delta-6 elongase from *T. pseudonana* is not associated with toxicity.
[13] BASF notes that the appearance of protein aggregates (an indicator of structural integrity disruption) is typical of proteins subjected to heat, especially membrane proteins.
[14] Delaney, B. et al., (2008). Evaluation of protein safety in the context of agricultural biotechnology. *Food and Chemical Toxicology* **46**: 871-897.

Levels of modified AHAS in LBFLFK canola seed

BASF measured the levels of modified AHAS in seeds using a capillary-based quantitative Western blot method. BASF optimized and validated the assay parameters to ensure specific detection of the introduced, modified AHAS. BASF reported that while the level of modified AHAS is quantifiable in the immature seed, levels of modified AHAS ranged from below the limit of quantification (LOQ = 3.0 µg/g dry weight (DW)) to a maximum value of 3.5 µg/g DW in the mature seed. LBFLFK canola oil would not be expected to contain modified AHAS because of the processing steps in oil production. However, solvent-extracted meal produced from LBFLFK canola may contain residual modified AHAS. BASF concluded that dietary exposure to the enzymatically active form of modified AHAS from consumption of human food derived from LBFLFK canola seed oil and solvent-extracted meal will be negligible

Safety assessment of modified AHAS in human food

BASF relied on a weight of evidence approach to assess the safety of the modified AHAS in canola meal, considering its source, its structural and functional similarity to AHAS proteins present in crops that have a history of safe use in human food, and its lack of similarity to known toxins and allergens. The source of the modified AHAS gene is *Arabidopsis thaliana*, a member of the Brassicaceae family, that is not known to produce or contain toxins. The modified *A. thaliana* AHAS shares sequence and functional similarities with AHAS proteins present in camelina, radish, canola, clementine, chickpea, and apple. Moreover, BASF noted that while there are several naturally-occurring herbicide-insensitive AHAS alleles, there are no reports associating adverse effects from dietary exposure to these AHAS variants. Sequence comparisons by BASF did not identify similarities between the modified AHAS and known toxins or allergens.

BASF characterized the modified AHAS using both LBFLFK canola leaf protein extracts as well as extracts prepared from immature seed. Biochemical analysis of the modified AHAS included enzyme function and herbicide tolerance assays, Western blot analysis, tryptic peptide mapping, and glycosylation analysis. BASF provided the results of these biochemical analyses confirming the identity, activity, and reduced herbicide-binding affinity of the modified AHAS in LBFLFK canola seed. Digestive fate studies showed digestion of the protein within 0.5 minutes of incubation with pepsin; heat lability studies showed enzymatic inactivation within 5 minutes at temperatures ≥ 50˚C.

Based on the weight of evidence, BASF concludes that the modified AHAS does not raise any food safety concerns with regard to human health.

Intended Human Food Uses

Canola refers to varieties of rapeseed (of species *B. napus*, *B. rapa*, and *B. juncea*) that are low in erucic acid and glucosinolates.[15] Canola is used primarily as a source of edible seed oil. Canola seed also has minor uses as a source of protein isolates and lecithin.[16] LBFLFK canola (*B. napus*)

[15] See definition in 7 CFR 810.301 for canola seed and 21 CFR 184.1555(c) for canola oil.
[16] See GRAS Notice Nos. (GRNs) 327, 386, 533, 682, and 683.

has been genetically engineered to enable biosynthesis of LCPUFAs, including EPA and DHA, that would not otherwise be present in canola oil.

The omega-3 LCPUFAs EPA and DHA are commonly found in some fish oils, including menhaden oil. Menhaden oil is the subject of a GRAS Affirmation regulation (21 CFR 184.1472; menhaden regulation). Paragraphs (a)(3) and (a)(4) of the GRAS affirmation regulation for menhaden oil (21 CFR 184.1472) establish limits on the use of menhaden oil to avoid total dietary intakes above 3.0 grams/person/day of EPA and DHA. Paragraph (a)(3) limits maximum use levels of menhaden oil in specific food categories; these categories include but are not limited to baked goods; cereals and pastas; confections and candy; egg, fish, meat, and milk products; desserts and snack foods; dressings and spreads; gravies and sauces; and (non-alcoholic) beverages. The maximum levels of use in 21 CFR 184.1472(a)(3) are based on the composition of menhaden oil (13% EPA and 7% DHA) and the targeted dietary exposure limit of 3.0 g/p/d EPA and DHA. Paragraph (a)(4) restricts use of menhaden oil in combination with any other added oil that is a significant source of EPA or DHA.

Likewise, other fish,[17] yeast,[18] and algal[19] oils have been determined to be GRAS for uses in human food where the uses are substitutional for those listed in the menhaden regulation such that (1) the maximum levels of use are based on the EPA and DHA content of the oil and the targeted dietary exposure limit of 3.0 g/p/d or less of EPA and DHA and (2) the oil will not be used in combination with any other added oil that is a significant source of EPA or DHA. Accordingly, BASF explains that oil derived from LBFLFK canola is intended for use in human food subject to the limitations in the Generally Recognized as Safe (GRAS) affirmation regulation for menhaden oil (21 CFR 184.1472).

Human Food Nutritional Assessment

With the exception of altering the seed oil composition, the DNA insertions in LBFLFK canola are not expected to change levels of key nutrients, anti-nutrients, or toxicants. To assess the intended changes in seed oil composition as well as potential unintended changes in other key components relevant to safety or nutrition, BASF conducted field trials at multiple sites across three growing seasons (winter 2014/15, spring 2015, and 2016). Mature seed from LBFLFK canola, the parent variety Kumily (control), and six commercial reference varieties grown in winter 2014/15 and spring 2015 were collected and analyzed; levels of proximates and fiber, key vitamins and minerals, fatty acids, amino acids, phytosterols, anti-nutrients, and toxicants were determined.[20] Mature seed from LBFLFK canola, the control, and three reference varieties

[17] The GRAS status of certain fish oils when used in food in accordance with the menhaden oil regulation has been determined in GRAS Notices receiving "no questions" letters from FDA. These include salmon oil (GRN 146), fish oil-predominantly anchovy (GRNs 138, 193), and tuna oil (GRN 109).
[18] The GRAS status of oil from the EPA-rich *Y. lipolytica* oil when used in food in accordance with the menhaden oil regulation has been determine in GRN 355, which received a "no questions" letter from FDA.
[19] The GRAS status of oils from the *Schizochytrium* sp., when used in food in accordance with the menhaden oil regulation, has been determine in GRAS notices receiving "no questions" letters from FDA. These include GRNs 137 and 732.
[20] Components selected for analysis in seed were based primarily on the OECD consensus document on composition of low erucic acid rapeseed (canola) (OECD, 2011).

grown during the 2016 season were collected and processed into solvent-extracted meal, crude oil, and refined, bleached, and deodorized (RBD) oil using methods that BASF described as similar to conventional canola and representative of the expected commercial process for production of LCPUFA-containing canola products. Solvent-extracted meal and crude oil were analyzed for the same components as seed, except that the proximate analysis in the crude oil was limited to moisture and protein. RBD oil was analyzed for fatty acids, vitamins, and phytosterols. The analytical results from LBFLFK canola, control, and reference varieties were compared to each other and to published data and information.

Analysis of seed oil

Characterization of LBFLFK canola oil composition

BASF reported the levels of individual fatty acids in seed, crude oil, and RBD oil from LBFLFK canola, the control, and reference varieties on the basis of percentage of total fatty acids (TFA). The levels of individual fatty acids were similar for seed and crude oil. Slight differences were observed between crude and RBD oils associated with modest, predictable losses of LCPUFAs due to oil processing. For the purpose of this document, we summarize fatty acid composition results for RBD oil, the form consumed in human food.

BASF compared LBFLFK canola oil to edible oils ordinarily consumed in the human diet using data and information in Codex Standards for Named Vegetable Oils,[21] the ILSI Crop Composition Database,[22] FDA's GRAS Notice Inventory,[23] and published literature.[24] The composition of LBFLFK canola oil was first compared to oils from commercial canola varieties. Based on this comparison, the fatty acids in LBFLFK canola oil fell into three categories: (1) endogenous fatty acids *unaffected* by the altered metabolic pathway, (2) endogenous fatty acids *affected* by the altered metabolic pathway, and (3) polyunsaturated fatty acids *introduced* as a result of the altered metabolic pathway. BASF subsequently compared the composition of LBFLFK canola oil to oils with histories of safe use in human food, including fish, fungal, and algal oils.

The fatty acids in LBFLFK canola oil unaffected by the altered metabolic pathway include fatty acids with levels for which no differences were observed between LBFLFK canola seed and oil and the control, or, with levels consistent with the ranges reported for the reference varieties

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[21] Values for low erucic acid rapeseed (LEAR) oil in the Codex Standard for Named Vegetable Oils CODEX STAN 210-1999. Codex limits for mono-, di-, and poly-unsaturated fatty acids are not isomer-specific. Values represents the sum of isomers. Codex non-detect level is defined as ≤0.05%.

[22] International Life Science Institute (2016) Crop Composition Database Version 6.0. The ILSI database is now known as the Agriculture and Food Systems Institute (AFSI) Crop Composition Database; accessible at https://www.cropcomposition.org.

[23] Available at https://www.fda.gov/grasnoticeinventory.

[24] BASF cited the results of its analysis of ordinarily consumed foods, published in Andre et al., (2019). Safety assessment of EPA+DHA canola oil by fatty acid profile comparison to various edible oils and fat-containing foods and a 28-day repeated dose toxicity study in rats. *Food and Chemical Toxicology* **124**: 168-181.

and/or in published literature, if minor differences were observed.[25] These were palmitic, vaccenic, arachidic, 20:1 n-9, behenic, lignoceric, and alpha-linolenic (ALA; discussed below) acids, as shown in Table 1. Other fatty acids included in this category were those present at levels below the LOD[26] and those present at low levels (≤0.2%) in LBFLFK canola oil, [27] and consistent with levels observed for conventional canola. BASF concludes that endogenous fatty acids in LBFLFK canola oil unaffected by the altered metabolic pathway do not raise nutritional or safety concerns because their levels are comparable to levels in canola oil varieties with a history of safe use.

Table 1 - Endogenous canola fatty acids unaffected by the altered metabolic pathway (%TFA)

Fatty acid	LBFLFK canola RBD oil mean	Control variety mean	Reference varieties range of means	Codex canola oil range	Fish oil range of means*
16:0 (palmitic)	4.7	4.4	3.6-4.0	2.5-7.0	13.7-16.6
18:1 n-7 (vaccenic)	3.3	3.2	2.8-3.0	--	2.9-3.1
18:3 n-3 (ALA)	4.9	7.4	2.5-6.9	5.0-14.0	0.67-2.3
20:0 (arachidic)	0.70	0.72	0.67-0.70	0.2-1.2	0.21-0.30
20:1 n-9	0.80	1.1	1.1-1.3	0.1-4.3	1.0-2.3
22:0 (behenic)	0.27	0.35	0.31-0.37	ND-0.6	0.12-0.16
24:0 (lignoceric)	0.25	0.19	0.19-0.25	ND-0.3	0.07-0.12
Total *trans* fatty acids	0.59	0.63	0.24-0.56	--	1.3-1.7

Fatty acids present at ≤0.2% TFA (14:0, 16:1 n-7, 16:1 n-9, 17:0, 17:1, 20.2 n-6, 24:1 n-9) or below the LOD (22:1 n-9, 22:2 n-6) are not shown. *means for menhaden, salmon, and fish oil (not specified); -- value not available; Codex standard values for canola oil are sum of isomers; Codex non-detectable (ND) values defined as ≤0.05%.

[25] Initially, BASF identified 16:1 n-7 (palmitoleic), 18:3 n-3, 20:0 (arachidic), 24:1 n-9 (nervonic), 22:0 (behenic), 24:0 (lignoceric) acids, and total *trans* fat as affected based on observed statistical differences in seed or oil samples. Subsequently, because fatty acid values for LBFLFK canola oil were within ranges for canola oil reference varieties or in published literature, BASF concluded these fatty acids were not affected by the altered metabolic pathway.

[26] Including 22:1 n-9 (erucic acid) and 22:2 n-6.

[27] Including 14:0 (myristic acid), 16:1 n-9, 17:0 (margaric acid), 17:1 (margoleic acid), 20:2 n-6, and 24:1 n-9 (nervonic acid).

The fatty acids in LBFLFK canola oil affected by the altered metabolic pathway were those with mean values that consistently differed from the control and that were outside the range for the reference varieties and literature values. The introduced fatty acid desaturases and elongases enable production of LCPUFAs through the desaturation and elongation of their endogenous precursors, including oleic acid (OA) and linoleic acid (LA); accordingly, changes in the levels of these fatty acids would not be unexpected. As shown in Table 2, the level of oleic acid (mean 30%) in LBFLFK canola oil was lower than in the reference varieties, but it was still a predominant fatty acid and higher than fish oils, for which LBFLFK canola oil is intended to be a substitute. While the level of ALA in LBFLFK canola oil (mean 4.9%) was within the range of levels observed in reference varieties, the level of LA (mean 30%) was higher than in the reference varieties. Both essential fatty acids ALA and LA were present in LBFLFK canola oil at levels higher than fish oils but within the ranges reported for edible vegetable oils. For the remaining affected fatty acids (18:0, 18:2 n-9, and 20:2 n-9), differences between LBFLFK canola oil and control were small (<2%) and do not raise nutritional or safety concerns.

Table 2 - Endogenous canola fatty acids affected by the altered metabolic pathway (% TFA)

Fatty acid	LBFLFK canola RBD oil mean	Control variety mean	Reference varieties range of means	Codex canola oil range	Fish oil range of means*
18:0 (stearic)	3.1	2.30	2.1-2.2	0.8-3.0	2.9-3.4
18:1 n-9 (OA)	29.6	59.0	61.3-62.8	--	6.1-20.8
18:1 sum	--	--	--	51.0-70.0	--
18:2 n-6 (LA)	29.6	19.6	18.3-23.3	--	1.1-5.8
18:2 n-9	1.4	0.065	0.055-0.068	--	0.10-0.17
18:2 sum	--	--	--	15.0-30.0	--
20:2 n-9	0.37	0.035	0.027-0.033	--	0.064-0.13
20:2 sum	--	--	--	ND-0.1	--

*means for menhaden, salmon, and fish oil (not specified); -- value not available; Codex standard values for canola oil are sum of isomers; Codex non-detectable (ND) values defined as ≤0.05%.

Shown in Table 3, the fatty acids produced in LBFLFK canola as a result of the altered metabolic pathway are those that were consistently quantifiable in LBFLFK canola oil but that were not detected in control or reference varieties (LOQ = 0.02%). The introduced polyunsaturated fatty acids include the omega-6 fatty acids gamma-linolenic (GLA), dihomo-gamma linolenic (DGLA), and arachidonic (ARA); the omega-3 fatty acids eicosatetraenoic acid (ETA), docosapentaenoic (DPA), eicosapentaenoic (EPA), 22:4 n-3, and docosahexaenoic (DHA). Low levels (≤0.5%) of other polyunsaturated fatty acids are also present in LBFLFK canola oil, including stearidonic (SDA), eicosatrienoic (ETrA), mead, osbond, and adrenic. While not present in oil from traditional canola varieties, the fatty acids introduced in LBFLFK canola oil are present in LCPUFA-containing oils for which LBFLFK canola oil is intended to substitute, such as edible fish oil[17] and EPA-rich oil from *Y. lipolytica*.[18]

BASF compared levels of introduced omega-6 and omega-3 polyunsaturated fatty acids in LBFLFK canola oil with those in LCPUFA-containing oils that are GRAS under the conditions of their intended use in accordance with limitations in the menhaden oil GRAS Affirmation. As shown in Table 3, all but GLA and DGLA are present individually at levels consistent with (≤1% difference) or below those in fish oils.[28] The combined level of EPA plus DHA in LBFLFK canola oil (mean 4.5%) is lower than levels reported in fish oil and EPA-rich oil from *Y. lipolytica*; for example, the level of EPA plus DHA in LBFLFK canola oil is approximately one fifth that reported for fish oils (range of means 21% - 30%).

Table 3 - Fatty acids introduced as a result of the altered metabolic pathway (% TFA)

Fatty acid	LBFLFK canola RBD oil mean	Fish oil range of means*	*EPA-rich oil from Y. lipolytica***
18:3 n-6 (GLA)	2.2	0.17-0.32	0.14
18:4 n-3 (SDA)	0.31	1.6-3.0	0.15
20:3 n-3 (EtrA)	0.06	0.18-0.32	--
20:3 n-6 (DGLA)	5.0	0.16-0.24	1.36
20:3 n-9 (mead)	0.05	0.04-0.06	--
20:4 n-6 (ARA)	1.7	0.99-1.4	0.29
20:4 n-3 (ETA)	1.9	0.70-1.5	1.28
20:5 n-3 (EPA)	4.1	9.7-17.5	35.79
22:4 n-3	1.0	0.05-0.11	--
22:4 n-6 (adrenic)	0.53	0.10-0.22	--
22:5 n-3 (DPA)	2.2	1.5-2.5	1.52
22:5 n-6 (osbond)	0.05	0.45-0.53	--
22:6 n-3 (DHA)	0.36	11.4-13.2	0.10
EPA+DHA	4.5	21.1 – 30.7	35.9

*means for menhaden, salmon, and fish oil (not specified); **source of fatty acid profile is GRAS Notice No. GRN 355; -- value not available; LOQ = 0.02%.

In summary, consistent with the altered fatty acid pathway, LBFLFK canola oil contains a lower level of total monounsaturated fatty acids and a higher level of total polyunsaturated fatty acids compared to conventional canola oil. However, the mono- and poly-unsaturated fatty acid levels in LBFLFK canola oil are within the ranges observed in other vegetable oils commonly consumed as food. Levels of total saturated fatty acid in LBFLFK canola oil are increased slightly but remain below levels in fish oils. Compared to conventional canola oil, LBFLFK canola oil has both higher total omega-3 and omega-6 fatty acids, attributed to the introduced omega-3 and omega-6 LCPUFAs and to the increased levels of LA (18:2 n-6). The introduced LCPUFAs – which include DGLA, ARA, ETA EPA, DPA, and DHA – make LBFLFK canola oil qualitatively, but not quantitatively, similar to other omega-3 LCPUFA oils used in food. While the total level of omega-3 polyunsaturated fatty acids in LBFLFK canola oil, calculated as the sum of introduced omega-3 fatty acids plus ALA, is within the ranges for other omega-3 LCPUFA oils, the total level of EPA and DHA in LBFLFK canola oil is lower than levels in fish oils and EPA-

[28] GLA and DGLA are present at levels more comparable to, but slightly higher than those in *M. alpina*, an oil used in combination with DHA-containing oil in infant formula.

rich oil from *Y. lipolytica* (Table 3). The total level of DGLA is higher than levels in fish oils and EPA-rich *Y. lipolytica* oil.

Safety assessment of LBFLFK canola oil

BASF used a weight of evidence approach to assess the safety of the altered oil composition, considering data and information about the composition and use of LCPUFA-containing oils available in published literature, the 1997 Menhaden Oil Final Rule[29] and GRAS affirmation regulation,[1] and FDA's GRAS Notice Inventory.[23] In its submissions to FDA, BASF summarized published scientific literature documenting the presence of omega-6 and omega-3 LCPUFAs in foods with a history of safe use and reported the results of the compositional analyses for several edible oils and fat-containing foods, including fish and fish oils, dairy, eggs, and meat.[24] BASF concludes that the fatty acids in LBFLFK canola oil – including the introduced fatty acids – are currently consumed in human diets and present at comparable levels in other consumed oils.

The fatty acid profile of LBFLFK canola oil is qualitatively, but not quantitatively, similar to fatty acids in fish and other omega-3 LCPUFA oils. To assess the safety of these quantitative compositional differences, BASF estimated and compared the dietary exposure to individual LCPUFAs from LBFLFK canola oil, menhaden oil, and EPA-rich *Yarrowia lipolytica* oil. For its discussion of dietary exposure, BASF cited the estimated dietary exposure of 2.7 g/p/d to EPA and DHA from FDA's Menhaden Oil Final Rule (See 62 FR 30751 at 30754); this estimate is based on a combined level of approximately 20% EPA plus DHA in menhaden oil and a mean estimated dietary intake of menhaden oil (13 g/p/d) from its use at the maximum use levels in the food categories specified in the menhaden oil regulation. The dietary exposure to individual LCPUFAs from LBFLFK canola oil and menhaden oil were estimated using a 1:1 oil replacement scenario where the oil would be used at the maximum levels in the food categories specified in the menhaden oil regulation; BASF compared the dietary exposure to individual LCPUFAs from consumption of LBFLFK canola oil and menhaden oil at the estimated mean (13 g/p/d) and pseudo-90th percentile (26 g/p/d).[30] For the dietary exposure to individual LCPUFAs from EPA-rich *Y. lipolytica* oil, which contains almost twice the amount of total EPA and DHA (36% EPA and 0.1% DHA) as menhaden oil, BASF used an EPA plus DHA replacement scenario based on its use at levels corresponding to dietary exposures for EPA and DHA of approximately 3.0 g/p/d (estimated mean) and 6.0 g/p/d (estimated 90th percentile). BASF then discussed estimates of dietary exposure to both omega-3 and omega-6 LCPUFAs and other fatty acids present in these oils in the context of consumption of dietary fat (total) for the US population over 2 years of age.

The most abundant LCPUFA in LBFLFK canola is the omega-6 fatty acid, DGLA. BASF discussed the higher levels of DGLA (and its precursor GLA) in comparison with fish oils, noting

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[29] Final Rule Substances Affirmed as Generally Recognized as Safe: Menhaden Oil (62 FR 30751; June 5, 1997).

[30] Upper percentile intakes of substances in the diet are estimated to account for individuals who are considered "high level" consumers of specific foods that contain these substances. A crude approximation of intake of a substance at the 90th percentile can be obtained by doubling the calculated mean intake. An intake at the 90th percentile obtained in this manner is sometimes referred to as the "pseudo-90th percentile" intake, in order to distinguish it from an intake estimated from food intake distribution data.

that these fatty acids are present in fish- and animal-based foods (e.g., beef and chicken livers, cheese, and butter) at low levels and are normal components of the diet. BASF discussed the metabolism of DGLA, its presence in the human body, and the estimated dietary exposure to DGLA (<1 g/p/d at the mean) from consumption of LBFLFK canola oil at the maximum use levels in the food categories specified in the menhaden oil regulation. BASF also discussed published pre-clinical[31] and clinical[32] studies to support its conclusion that consumption of DGLA is safe at this level.

LBFLFK canola oil contains the omega-3 LCPUFAs EPA (4.1%) and DHA (0.4%), which are present in menhaden oil and known to have physiological effects on bleeding time, glycemic control, and LDL cholesterol levels. FDA affirmed the GRAS status of menhaden oil with limitations on its use to avoid dietary intakes above 3.0 g/p/d of EPA and DHA.[29] Use of LBFLFK canola oil (4.5% EPA and DHA) is estimated to provide approximately 0.6 g/p/d EPA and DHA (estimated mean) and approximately 1.2 g/p/d EPA and DHA (pseudo-90th percentile). Thus, the estimated mean and pseudo-90th percentile dietary exposures to EPA and DHA from consumption of LBFLFK canola oil when used at the maximum levels in the food categories specified in the menhaden oil regulation are below the targeted dietary exposure limit of 3.0 g/p/d EPA and DHA.

In addition to DGLA, EPA, and DHA, BASF estimated dietary exposure to other LCPUFAs that are present at levels above 1% TFA (e.g., ETA, ARA, DPA) in LBFLFK canola oil. As expected, given the observed compositional differences, estimates of dietary exposure to individual LCPUFAs from menhaden oil, EPA-rich *Y. lipolytica* oil, and LBFLFK canola oil showed some differences; however, in the context of total fat in the US diet, the contributions from individual fatty acids were small relative to baseline fatty acid intakes. The relative differences in dietary exposures to individual fatty acids were generally modest; differences were ≤ 2 g/p/d at the pseudo-90th percentile.

BASF also estimated dietary exposure to total EPA and DHA, saturated fat, monounsaturated fat, omega-6 polyunsaturated fat and omega-3 polyunsaturated fat from consumption of LBFLFK canola oil. BASF concludes that these estimated dietary exposures are minimal compared to the total fatty acid exposure from the diet and are comparable to one or more of the known omega-3 LCPUFA oils from marine and microbial sources.

BASF concludes that use of oil derived from canola event LBFLFK as an ingredient in consumer food items per the GRAS affirmed categories and inclusion levels for EPA and DHA described in 21 CFR 184.1472 may be considered generally recognized as safe based on scientific procedures with consideration of peer-reviewed publications and other publicly available information.

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[31] Kawashima et al., (2009). Subchronic (13-week) oral toxicity study of dihomo-gamma-linolenic acid (DGLA) oil in rats. *Food and Chemical Toxicology* **47**:1280-1286.

[32] Teraoka et al., (2009). Oral supplementation with dihomo-gamma-linolenic acid-enriched oil altered serum fatty acids in healthy men. *Bioscience, Biotechnology, and Biochemistry* **73**: 1453-1455; Tanaka et al., (2012). Oral supplementation with dihomo-gamma-linolenic acid (DGLA)-enriched oil increases serum DGLA content in healthy adults. *Lipids* **47**: 643-646.

Analysis of other key components

Aside from expected changes in fatty acid composition, BASF observed minor differences between LBFLFK canola and the control in the levels of key components in seed.[33] BASF notes that the mean levels of these components in LBFLFK canola were within ranges of natural variation, based on the levels observed in reference varieties grown concurrently in field trials and/or reported in publicly-available sources such as the ILSI Crop Composition Database[22] and peer-reviewed scientific literature. LBFLFK canola contains low levels of erucic acid and glucosinolates, consistent with quality standards for food-grade canola oil.[15] BASF concludes that the observed differences between LBFLFK canola and the control in the levels of other key components would not impact the safety of human food from LBFLFK canola.

Human Food Labeling Considerations

It is a producer's or distributor's responsibility to ensure that labeling of the foods it markets derived from LBFLFK canola meets applicable legal requirements, including disclosure of any material differences (for example, differences in function, composition, and nutritional or safety profiles) in the food as compared to its conventional counterpart. It is our understanding that LBFLFK canola may be used in various food applications. Depending on the particular food application, the altered fatty acid composition of the oil may be considered a material fact requiring disclosure under Sections 201(n) and 403(a)(1) of the FD&C Act. Companies marketing oil from LBFLFK canola or products containing oil from LBFLFK canola are advised to consult with CFSAN's Office of Nutrition and Food Labeling, Division of Food Labeling and Standards, to discuss any required or voluntary labeling including statements relating to attributes of this canola variety and products produced from it. Failure to do so may result in the misbranding of products produced from LBFLFK canola within the meaning of Sections 201(n) and 403(a)(1) of the FD&C Act.

Conclusion

Based on the information provided by BASF and other information available to CFSAN, we have no further safety, nutritional, or regulatory compliance questions at this time about BASF's current intended uses of LBFLFK canola in human food. We consider the consultation with BASF on LBFLFK canola to be complete.

Carrie H. Mcmahon -S
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Carrie McMahon, Ph.D.

[33] Based on comparisons between LBFLFK canola and the control using the Student's paired T-test with a significance level of $\alpha = 0.05$ (confidence level = 95%).