Biotechnology Notification File No. 000162
CVM Note to the File

Date: March 18, 2022

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To: Administrative Record, BNF No. 000162

Subject: Event NS-B5ØØ27-4 Canola

Keywords: Canola, *Brassica napus*, Canola meal, Docosahexaenoic acid, DHA, Omega-3 long-chain polyunsaturated fatty acids, Delta-12-desaturase, *Lachancea kluyveri*, Delta-6-desaturase, *Micromonas pusilla*, Delta-5-desaturase and Delta-4-desaturase, *Pavlova salina*, Omega-3-desaturase, *Pichia pastoris*, Delta-6-elongase and Delta-5-elongase, *Pyramimonas cordata*, Phosphinothricin N-acetyltransferase, *Streptomyces viridochromogenes*, Tolerance to glufosinate ammonium, OECD identifier NS-B5ØØ27-4, Nuseed Americas, Inc.

Purpose

This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000162. Nuseed Americas, Inc. (Nuseed) submitted a safety and nutritional assessment for a genetically engineered canola, transformation event NS-B5ØØ27-4, an amended assessment, and additional information afterwards. CVM evaluated the information in Nuseed's submissions to ensure that regulatory and safety issues regarding animal food derived from NS-B5ØØ27-4 canola meal have been resolved prior to commercial distribution. FDA's Center for Food Safety and Applied Nutrition summarizes its evaluation of uses of NS-B5ØØ27-4 canola in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by Nuseed as well as publicly available information and information in the agency's files. Here we discuss the outcome of the consultation for animal food derived from NS-B5ØØ27-4 canola meal, but do not intend to restate the information provided in the final consultation in its entirety.

Intended Effects

The first intended effect of the modification in NS-B5ØØ27-4 canola is to alter the fatty acid composition of the oil obtained from the new canola variety. To confer this trait, Nuseed introduced deoxyribonucleic acid (DNA) sequences for seven genes encoding a series of transmembrane proteins that convert oleic acid, which is normally present in

canola seed, to omega-3 long-chain polyunsaturated fatty acids (LCPUFA), primarily docosahexaenoic acid (DHA).[1] In addition, a codon-optimized gene from *Streptomyces viridochromogenes* that encodes the phosphinothricin N-acetyltransferase (PAT) protein confers tolerance to glufosinate ammonium herbicides.

Regulatory Considerations

The purposes of this evaluation are (1) to assess whether Nuseed has introduced into animal food derived from NS-B5ØØ27-4 canola meal a substance requiring premarket approval as a food additive and (2) to determine whether use of the meal from the new plant variety in animal food raises other regulatory issues with respect to the Federal Food, Drug, and Cosmetic Act (FD&C Act).

The Environmental Protection Agency (EPA) regulates herbicides under the FD&C Act and the Federal Insecticide, Fungicide, and Rodenticide Act. Under EPA regulations, the herbicide residues in NS-B5ØØ27-4 canola are considered pesticide residues.

Genetic Modification and Characterization

Introduced DNA and Transformation Method

Nuseed transformed cotyledonary petioles obtained from *Brassica napus* variety AV Jade with plasmid pJP3416_GA7-ModB using disarmed *Agrobacterium tumefaciens*-mediated transformation.[2] Nuseed states that the transfer-DNA (T-DNA) region within the plasmid contained eight expression cassettes and two spacer sequences between the left (LB) and right (RB) border sequences. These included:[3]

- Cassette 1: *Delta-6-desaturase* gene from *Micromonas pusilla* (D6D(Mp)) with regulatory elements, including *conlinin2* gene promoter from *Linum usitatissimum* and 5´ untranslated region from tobacco mosaic virus (TMV) 59 and *conlinin2* gene terminator from *L. usitatissimum*.
- Cassette 2: *Delta-5-elongase* gene from *Pyramimonas cordata* (D5E(Pc)) with regulatory elements, including *fatty acid elongase* gene promoter from *Arabidopsis thaliana* and 5´ untranslated region of TMV 59 and *lectin* gene terminator from *Glycine max*.

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[1] Crude oil obtained from NS-B5ØØ27-4 canola contained on average 21.04% alpha-linolenic acid, 2.53% stearidonic acid, 0.44% eicosapentaenoic acid, 1.05% docosapentaenoic acid, and 8.38% docosahexaenoic acid (as a percentage of the total fatty acids).

[2] Bhalla, P.L., and M.B. Singh. 2008. *Agrobacterium*-mediated transformation of *Brassica napus* and *Brassica oleracea*. Nature Protocols 3:181-189; Belide, S., X.R. Zhou, Y. Kennedy, G. Lester, P. Shrestha, J.R. Petrie, and S.P. Singh. 2013. Rapid expression and validation of seed-specific constructs in transgenic LEC2 induced somatic embryos of *Brassica napus*. Plant Cell Tissue Organ Culture 113:543-553.

[3] Seed-specific promoters were used to drive expression of the genes for the fatty acid desaturases and elongases, whereas a ubiquitous promoter was used to drive expression of the *pat* gene in all tissues. The coding sequences of the genes included in the T-DNA region of plasmid pJP3416_GA7-modB were optimized for codon usage in *B. napus* to enhance enzyme expression. The cassettes for the seven fatty acid biosynthesis genes were synthesized as a single unit, which was then cloned into vector pJP3416, which already contained the cassette for the *pat* gene, to generate GA7. The vector pJP3416_GA7-ModB was generated by switching the location of the two elongase genes and replacing the promoter region of the delta-6-desaturase gene as described in Petrie, J.R., X.-R. Zhou, A. Leonforte, J. McAllister, P. Shrestha, Y. Kennedy, S. Belide, G. Buzza, N. Gororo, W. Gao, G. Lester, M.P. Mansour, R.J. Mulder, Q. Liu, L. Tian, C. Silva, N.O.I. Cogan, P.D. Nichols, A.G. Green, R. de Feyter, M.D. Devine, and S.P. Singh. 2020. Development of a *Brassica napus* (Canola) crop containing fish oil-like levels of DHA in the seed oil. Front. Plant Sci. 11:article 727.

- Cassette 3: *Delta-5-desaturase* gene from *Pavlova salina* (D5D(Ps)) with regulatory elements, including truncated *napin* gene promoter from *B. napus* and 5´ untranslated region from TMV 59 and *nopaline synthase* gene terminator from *A. tumefaciens*.
- Spacer: Adenine/thymine (AT)-rich matrix attachment region from the root-specific *Rb7* gene from *Nicotiana tabacum*.
- Cassette 4: *Omega-3-desaturase* gene from *Pichia pastoris* (O3D(Pp)) with regulatory elements, including *conlinin1* gene promoter from *L. usitatissimum* and 5´ untranslated region of TMV 59 and *conlinin1* gene terminator from *L. usitatissimum*.
- Cassette 5: *Delta-4-desaturase* gene from *P. salina* (D4D(Ps)) with regulatory elements, including *conlinin2* gene promoter from *L. usitatissimum* and 5´ untranslated region of TMV 59 and *conlinin2* gene terminator from *L. usitatissimum*.
- Cassette 6: *Delta-12-desaturase* gene from *Lachancea kluyveri* (D12D(Lk)) with regulatory elements, including *conlinin1* gene promoter from *L. usitatissimum* and 5´ untranslated region of TMV 59 and *conlinin1* gene terminator from *L. usitatissimum*.
- Spacer: AT-rich matrix attachment region from the root-specific *Rb7* gene from *N. tabacum*.
- Cassette 7: *Delta-6-elongase* gene from *P. cordata* (D6E(Pc)) with regulatory elements, including *fatty acid elongase* gene promoter from *A. thaliana* and 5´ untranslated region of TMV 59 and *lectin* gene terminator from *G. max*.
- Cassette 8: *Phosphinothricin N-acetyltransferase* gene from *S. viridochromogenes* (*pat*) with regulatory elements, including 35S RNA gene promoter from cauliflower mosaic virus and *nopaline synthase* gene terminator from *A. tumefaciens*.

The vector sequence was confirmed by Sanger sequencing prior to being used for transformation of canola. Following transformation, petioles were grown in selection medium[4], plants were then regenerated and grown to maturity. The presence of the inserted T-DNA in leaf material was verified by polymerase chain reaction (PCR) amplification of the seven fatty acid synthesis genes and the *pat* gene, which was used as a selection marker. Additional breeding steps (up to seven generations) were conducted to generate plants used in the characterization of the genetic insertion, inheritance studies, and gene expression studies.

Nuseed characterized the number of T-DNA inserts, the organization of each insert that is present in NS-B5ØØ27-4 canola, and the absence of vector backbone sequences using vector-targeted sequencing, PCR-amplicon Sanger sequencing, and high-throughput sequencing. Nuseed concludes that NS-B5ØØ27-4 canola contains two T-DNA inserts, one in chromosome A02, while the other is present in chromosome A05. Nuseed notes that both inserts are needed for the "desired amount of DHA" to be present in the oil from NS-B5ØØ27-4 canola. Nuseed stated that genomic DNA sequences of both T-DNA inserts were verified and match the corresponding sequences in vector pJP3416_GA7-ModB and that no vector backbone is present. The T-DNA insert in the A02 chromosome contains the D6D(Mp), D5E(Pc), D5D(Ps), and O3D(Pp) expression cassettes, but not those for the other four genes. This T-DNA insert replaced 15 bp of *B. napus* genomic DNA from the 3´ untranslated region of a gene of unknown function. Nuseed states this deletion would not be expected to affect expression of this gene.

[4] The selection media contained timentin and cefotaxime to eliminate *A. tumefaciens* and phosphinothricin for selection of putative transformants.

Nuseed confirmed that two complete copies of the T-DNA region of the pJP3416_GA7-ModB plasmid were inserted into chromosome A05. The T-DNA sequences are in RB-LB:LB-RB orientation and are linked by 156 bp of palindromic LB sequence. The insert contains 40 bp of RB sequence upstream and 42 bp of RB sequence downstream. This insert replaced 20 bp of *B. napus* DNA in the second exon of the *Pto-Interacting* (*pti*) gene and according to Nuseed, disrupted expression of the *pti* gene. Nuseed states that this gene encodes a serine-threonine kinase involved in hypersensitive-response-mediated signaling and disease resistance.[5] Nuseed concludes based on data from its breeding program and field trial observations that disruption of this gene did not have any deleterious effects on NS-B5ØØ27-4 canola. Nuseed also demonstrated that NS-B5ØØ27-4 canola did not contain vector backbone or binary vector selection marker sequences.

The stabilities of the inserted T-DNA sequences in NS-B5ØØ27-4 canola across five self-pollinated generations (T3 through T7) were evaluated using junction-specific sequencing and Kompetitive Allele-Specific (KAS) PCR. In addition, Nuseed assessed inheritance in several outcrossed lines using phenotypic (percent LCPUFA (sum of EPA, DPA, and DHA) in the seed) and genotypic (junction-specific sequencing and KAS PCR). Nuseed concludes that the desired genotype segregated as two independent loci according to the expected Mendelian principles.

Nuseed performed bioinformatics analyses using the nucleotide sequences for the five junction sites (four canola genome-insert junctions and the junction between the two T-DNA inserts in chromosome A05) to determine whether insertion of the new DNA created any open reading frames (ORFs) that could encode for putative polypeptides. Nuseed evaluated the putative polypeptides against the National Center for Biotechnology Information (NCBI) protein database to determine the similarity of the putative polypeptides to known and putative toxins and anti-nutrients. Based on the results of the bioinformatics analyses, Nuseed concludes there are no new ORFs created as a consequence of the genetic changes that would have the potential to encode a protein with any significant similarity to a protein that would raise an adverse effect in consumers.

Protein Safety

Nuseed used a weight of evidence approach to demonstrate that the introduced proteins do not raise safety concerns. Nuseed performed bioinformatics analyses to ensure that the amino acid sequences of the proteins expressed in NS-B5ØØ27-4 canola matched the amino acid sequence of the proteins that are expressed in the donor organisms. Nuseed evaluated the safety of the donor organisms, including pathogenicity and production of toxins and anti-nutrients, and prevalence of the donor organisms in animal food. Nuseed concludes that the amino acid sequences of the expressed proteins are identical to the proteins produced by the donor organisms and that these organisms do not produce toxins or anti-nutrients that would raise safety concerns.

Nuseed also characterized the proteins that make up the inserted fatty acid biosynthetic pathway, including D12D(Lk) and O3D(Pp), initially identified in yeast species, and D6D(Mp), D5D(Ps), D4D(Ps), D6E(Pc), and D5E(Pc), found in microalgae. Several

[5] The deduced amino acid sequence for this gene shares high sequence identity (more than 80% identity) with at least eleven PTI-like sequences in *B. napus*.

methodologies were used to characterize the identity and safety of the expressed proteins.[6] The low expression level of the seven proteins in NS-B5ØØ27-4 canola led Nuseed to use indirect approaches to address the safety of these proteins. These included *in silico* translation of the known DNA sequences and comparison with protein databases; comparisons to similar enzymes present in food and their catalytic activities; the fatty acid biosynthetic pathway was functional in canola[7], Arabidopsis[8], and camelina[9]; and enzyme specificity as demonstrated in heterologous systems (yeast S288C, insect cells, *Arabidopsis thaliana*, or *Nicotiana benthamiana*).

Acyl-CoA Type Desaturases from Yeast

Nuseed indicates that the D12D(Lk) and O3D(Pp) proteins share high homology to their respective proteins from yeasts, fungi, and various crop plants, including canola, rice, soybean, flax, sunflower, and sesame. Additionally, Nuseed notes that yeast are commonly used in fermentation products that are used in foods and at least 69 species of yeasts and molds are listed in the inventory of microbial food cultures, including two species within the *Lachancea* genus and four within the *Pichia* genus.[10] Both of these desaturases contain conserved structural motifs, including transmembrane regions, histidine rich motifs that contain eight conserved histidine residues, and a conserved histidine following the C-terminal transmembrane region. Nuseed showed using *in vitro* assays, based on pepsin or pepsin followed by trypsin digestion, that both of these proteins were rapidly cleaved, at least 80% within 5 minutes. Additionally, Nuseed showed that the D12D(Lk) and O3D(Pp) proteins were heat inactivated within fifteen minutes at 95°C.[11] When the proteins were expressed in *P. pastoris*, the D12D(Lk) and O3D(Pp) proteins catalyzed the conversion of oleic acid to linoleic acid and linoleic acid to alpha-linolenic acid, respectively.

Algal Desaturases

The amino acid sequences for the D6D(Mp), D5D(Ps), and D4D(Ps) proteins contain conserved structural motifs that are present in front-end desaturases, including a cytochrome b5-like domain at the N-terminus of the protein, multiple transmembrane regions, and histidine rich motifs with seven conserved histidine residues and a glutamine residue. Nuseed showed using *in vitro* assays that 80% of these proteins were cleaved within ten minutes. Additionally, these three proteins were heat inactivated within fifteen minutes at 95°C. Overexpression of D6D(Mp), D5D(Ps), and D4D(Ps)

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[6] Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, quantification of pepsin-trypsin digested peptides by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), and *in vitro* and *in vivo* biochemical studies.

[7] Petrie, J.R., X.-R. Zhou, A. Leonforte, J. McAllister, P. Shrestha, Y. Kennedy, S. Belide, G. Buzza, N. Gororo, W. Gao, G. Lester, M.P. Mansour, R.J. Mulder, Q. Liu, L. Tian, C. Silva, N.O.I. Cogan, P.D. Nichols, A.G. Green, R. de Feyter, M.D. Devine, and S.P. Singh. 2020. Development of a *Brassica napus* (Canola) crop containing fish oil-like levels of DHA in the seed oil. Front. Plant Sci. 11: article 727.

[8] Petrie, J.R., P. Shrestha, X.-R. Zhou, M.P. Mansour, Q. Liu, S. Belide, P.D. Nichols, and S.P. Singh. 2012. Metabolic engineering plant seeds with fish oil-like levels of DHA. PLoS ONE 7(11):e49165.

[9] Petrie, J.R., P. Shrestha, S. Belide, Y. Kennedy, G. Lester, Q. Liu, U.K. Divi, R.J. Mulder, M.P. Mansour, P.D. Nichols, and S. Singh. 2014 Metabolic engineering *Camelina sativa* with fish oil-like levels of DHA. PLoS ONE 9(1):e85061.

[10] Bourdichon, F., S. Casaregola, C. Farrokh, J.C. Frisvad, M.L. Gerds, W.P. Hammes, J. Harnett, G. Huys, S. Laulund, A. Ouwehand, I.B. Powell, J.B. Prajapapti, Y. Seto, E. Ter Schure, A. Van Boven, V. Vankerckhoven, A. Zgoda, S. Tuijtelaars, and E.B. Hansen. 2012. Food fermentations: Microorganisms with technological beneficial use. International J Food Microbiol. 154: 87-97.

[11] In the typical crushing and oil extraction process for canola seeds, the temperatures in the cooker can reach 90°C for up to 40 minutes; distillation and stripper temperatures can reach 115°C; and the desolventizer-toaster temperatures can reach 110°C for up to 60 minutes.

proteins in yeast S288c cells resulted in the conversion of alpha-linolenic acid to stearidonic acid[12], eicosatetraenoic acid to eicosapentaenoic acid, and docosapentaenoic acid to docosahexaenoic acid, respectively.[13]

Algal Elongases

The amino acid sequences for the D6E(Pc) and D5E(Pc) proteins contain the conserved structural motifs of elongation-type ketoacyl synthases, which catalyze the addition of two carbon units to a fatty acid chain. Nuseed showed using *in vitro* assays that these proteins were rapidly cleaved, 95 and 75%, respectively, within five minutes. Additionally, the D5E(Pc) protein was heat inactivated within fifteen minutes at 95°C, while D6E(Pc) was inactivated after thirty minutes. Overexpression of the D6E(Pc) and D5E(Pc) genes in yeast S288c cells resulted in the conversion of stearidonic acid to eicosatetraenoic acid and eicosapentaenoic acid to docosapentaenoic acid, respectively.[14]

Phosphinothricin N-Acetyltransferase

Nuseed states that the safety of the PAT protein is well established and the biochemical characterization and toxicity of this protein has been addressed in previous safety assessments provided to regulatory authorities in different countries. Nuseed concludes, in part based on a review by Hérouet and coworkers, 2005[15], that the PAT protein is not toxic and it is digested during *in vitro* simulated gastric and intestinal fluid assays and, thus, does not raise a safety concern.

Expression Levels of Proteins in NS-B5ØØ27-4 Canola

Nuseed quantified the amounts of the proteins that were introduced into NS-B5ØØ27-4 canola.[16] Samples of whole plant (leaves or nodes), roots, flowers, and immature and mature seeds were obtained from NS-B5ØØ27-4 canola and AV Jade (control) plants that were planted at three field trial sites in 2016 in Victoria, Australia. Samples from five growth stages were analyzed by high-sensitivity liquid chromatography-multiple reaction monitoring-mass spectrometry. Nuseed reported that none of the target peptides (proxies for the seven fatty acid biosynthesis and PAT proteins) were present in extracts obtained from control. In addition, none of the target peptides for the fatty acid biosynthesis enzymes were detected in non-seed tissues of NS-B5ØØ27-4 canola. All

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[12] Petrie, J.R., P. Shrestha, M.P. Mansour, P.D. Nichols, Q. Liu, and S.P. Singh. 2010. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Δ6-desaturase with ω3-preference from the marine microalga *Micromonas pusilla*. Metabol. Engineering 12:233-240.

[13] Zhou, X.-R., S.S. Robert, J.R. Petrie, D.M.F. Frampton, M.P. Mansour, S.I. Blackburn, P.D. Nichols, A.G. Green, and S.P. Singh. 2007. Isolation and characterization of genes from the marine microalga *Pavlova salina* encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis. Phytochem. 68:785-796.

[14] Petrie, J.R., Q. Liu, A.M. Mackenzie, P. Shrestha, M.P. Mansour, S.S. Robert, D.F. Frampton, S.I. Blackburn, P.D. Nichols, and S.P. Singh. 2010. Isolation and characterisation of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. Marine Biotechnol. 12:430-438.

[15] Hérouet, C., D.J. Esdaile, B.A. Mallyon, E. Debruyne, A. Schulz, T. Currier, K. Hendricks, R.-J. van der Klis, and D. Rouan. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regul. Toxicol. Pharmacol. 41:134-149.

[16] Colgrave, M.L., K. Byrne, S.V. Pillai, B. Dong, A. Leonforte, J. Caine, L. Kowalczyk, J.A. Scoble, J.R. Petrie, S. Singh, and X.-R. Zhou. 2019. Quantitation of seven transmembrane proteins from the DHA biosynthesis pathway in genetically engineered canola by targeted mass spectrometry. Food Chem. Toxicol. 126:313-321.

eight of the peptides were detected in developing and/or mature seeds obtained from NS-B5ØØ27-4 canola. All of the expressed proteins, with the exception of D4D(Ps) were present in mature seeds at less than 1 microgram/milligram total protein. The D4D(Ps) protein was present from 1.34 to 1.55 micrograms/milligram total protein in mature seeds and ranged from 3.20 to 5.60 micrograms/milligram total protein in developing seed across the three field sites. The PAT protein was detected in all sampled tissues from NS-B5ØØ27-4 canola throughout the growing season. The PAT protein ranged from 0.023-0.033 micrograms/milligram total protein in mature seeds and from 0.325 to 0.605 micrograms/milligram total protein in developing seed across the three field sites.

Nuseed concludes that each of the newly expressed enzymes in NS-B5ØØ27-4 canola was fully characterized to confirm amino acid sequence, function, and specificity. Nuseed determined that these proteins are cleaved by pepsin or a combination of pepsin and trypsin and are denatured when exposed to 95°C. Additionally, Nuseed noted that each of the desaturases and elongases were expressed at low levels in mature NS-B5ØØ27-4 canola seed. Based on this information, Nuseed concludes that there is reasonable certainty of no harm resulting from use of NS-B5ØØ27-4 canola meal in animal food.

Animal Food Use

Canola (developed from *B. napus* and *B. rapa* varieties) refers to rapeseed varieties that contain low levels of erucic acid and glucosinolates. Canola is used primarily to produce oil for human food. Canola oil is low in saturated fatty acids and high in mono- and di-unsaturated fatty acids and is commonly used as cooking oil for frying, baking, and other food applications. Canola meal is a byproduct of oil crushing. The majority of canola meal is used in animal food, primarily for cattle and pigs, and, to a lesser extent, poultry, aquaculture, lamb, and other livestock. Industrial uses of canola are limited.

Nuseed states that use of oil derived from NS-B5ØØ27-4 canola in animal food will be addressed outside of the BNF process. Nuseed indicates that meal derived from the production of NS-B5ØØ27-4 canola oil will be used in animal food for cattle and pigs, and, to a lesser extent, poultry, aquaculture, and other livestock at the same inclusion rate as meal obtained from conventional canola varieties.

Composition

Scope of Analysis

Nuseed analyzed the nutrient composition of NS-B5ØØ27-4 canola, AV Jade (control), and other commercial canola varieties (conventional varieties) that were grown and harvested under similar conditions. Nuseed selected components for analysis from those recommended in the Organisation for Economic Cooperation and Development (OECD) canola composition consensus document[17], but also conducted additional analyses.

Study Design

Nuseed conducted field trials in 2015 at eight locations in canola growing regions of Australia. The canola varieties were planted using a randomized complete block design

[17] Organisation for Economic Cooperation and Development. 2011. Revised consensus document on compositional considerations for new varieties of low erucic acid rapeseed (canola): Key food and feed nutrients, anti-nutrients, and toxicants. Series on the safety of novel foods and feeds No. 24. ENV/JM/MONO(2011)55. OECD, Paris.

with five replicate plots at each field site. Seed samples of 350-400 grams were collected and pooled from the middle two rows of each plot.

Nuseed statistically compared each component for NS-B5ØØ27-4 canola with the control across locations using a linear mixed model with genotype as a fixed factor and site as a random factor. The firm notes that P-values were not reported for data when more than 30% of the values were less than the lower limit of quantitation (LLOQ) or missing data made the analyte results non-testable. Means, standard deviations, and analyte ranges were not reported if means were below the limits of detection, when more than 30% of values were <LLOQ, or where missing data made the effect non-testable. Single degree of freedom comparisons were used to test at $P \leq 0.05$ for differences between NS-B5ØØ27-4 canola and the control. Any observed differences between NS-B5ØØ27-4 canola and control were compared with the ranges obtained for the conventional varieties that were grown at the same locations, values for conventional canola varieties in the International Life Sciences Institute (ILSI) Crop Composition Database version 6[18] or in the scientific literature.

Nuseed also harvested NS-B5ØØ27-4 canola for compositional analysis of processed fractions from one location in Australia. The control was harvested from two locations near to the NS-B5ØØ27-4 canola site and the three samples were processed to obtain crude or solvent-extracted[19] meal. Each of the samples was divided into two batches prior to processing at Commonwealth Scientific and Industrial Research Organization Agriculture and Food facility. Compositional analyses were conducted on eight different samples: two crushes of crude meal from NS-B5ØØ27-4 canola, one crush of crude meal from control at each of the two locations, two crushes of solvent-extracted meal from NS-B5ØØ27-4 canola, and one crush of solvent-extracted meal from control at each of the two locations. Compositional analyses of meal samples included crude protein and 18 amino acids, fiber components (crude fiber, acid detergent fiber, and neutral detergent fiber), ash and minerals, crude fat and individual fatty acids, vitamin E, and key secondary metabolites and anti-nutrients. Means and individual values were reported for these components.

Results of Analyses – Seed

Nuseed reported that there were no statistically significant differences in the concentrations of crude protein and in arginine, cystine, glutamic acid, histidine, isoleucine, leucine, phenylalanine, serine, tryptophan, and valine between NS-B5ØØ27-4 canola and the control. Although statistically significant differences in the concentrations of alanine, aspartic acid, glycine, lysine, methionine, proline, threonine, and tyrosine were observed, Nuseed states the mean values were numerically similar and the means for these amino acids in NS-B5ØØ27-4 canola fell within the range of values reported for the conventional varieties grown under similar conditions and values reported in the ILSI database.

Nuseed reported a statistically significant increase in the levels of carbohydrates[20] in seed obtained from NS-B5ØØ27-4 canola when compared to the control. However, there were no statistically significant differences between NS-B5ØØ27-4 canola and the control for crude fiber, acid detergent fiber, and neutral detergent fiber. Nuseed

[18] The ILSI Crop Composition Database has become the Agriculture and Food Systems Institute Crop Composition Database and is available at www.cropcomposition.org.
[19] The solvent used for oil extraction was hexane.
[20] Carbohydrates were calculated as 100 – (moisture + crude protein + fat + ash).

highlights that the mean for carbohydrates in NS-B5ØØ27-4 canola falls within the range obtained from the conventional varieties grown under similar conditions and values reported in the ILSI database.

Nuseed states that slightly higher, but statistically significant, ash values were observed in seed obtained from NS-B5ØØ27-4 canola when compared to the control. Nuseed also reported statistically higher values for iron, potassium, and zinc and statistically lower values for calcium in seed from NS-B5ØØ27-4 canola when compared to the control. There were no significant differences in the concentrations of copper, magnesium, phosphorus, sodium, and sulfur. Nuseed highlighted that the means for ash and the minerals are numerically similar and fall largely within the range obtained from the conventional varieties grown under similar conditions. Nuseed concludes that it is unlikely these differences have any biological significance.

Nuseed reported values for eight glucosinolates and total glucosinolates that are present in canola seed. Five of the glucosinolates had mean values that were less than one micromole/gram dry weight. Progoitrin was at the highest concentration at 4.9 micromoles/gram dry weight and values were similar for NS-B5ØØ27-4 and control.[21] 4-hydroxyglucobrassicin was present at the second highest concentration in NS-B5ØØ27-4 canola and control (both were less than four micromoles/gram dry weight). Gluconapin levels were approximately two micromoles/gram dry weight and were slightly, but not significantly, higher in seed obtained from NS-B5ØØ27-4 canola when compared to the control. Although means for glucobrassicin were significantly higher for NS-B5ØØ27-4 canola, values for NS-B5ØØ27-4 canola and control were less than 0.3 micromoles/gram dry weight. Total glucosinolates were 11.88 and 12.07 micromoles/gram dry weight in seed obtained from NS-B5ØØ27-4 canola and the control, respectively. The mean values for total glucosinolates in samples obtained from NS-B5ØØ27-4 canola fell within the range of values obtained from the conventional varieties grown under similar conditions and fall within or below the values reported in the ILSI database.

Although there was a statistically significant difference in the mean values for sinapine, the mean value obtained for NS-B5ØØ27-4 canola fell within the range of values obtained for the control and the conventional varieties. No statistically significant differences were observed for ferulic acid and phytic acid in NS-B5ØØ27-4 canola when compared to the control and the mean values for these components in seed obtained from NS-B5ØØ27-4 canola were within the range of values obtained from the conventional varieties grown under similar conditions.[22] Nuseed reported statistically higher levels of total phytosterols in NS-B4ØØ27-4 canola when compared to the control (1.106 versus 1.025%). Five of the sterols had maximum values that were slightly higher than the upper limit that were reported in the OECD consensus document[23] and Nuseed attributes the increase in total phytosterols to small increases in these individual sterols. Nuseed attributes the variation in phytosterol values to environmental

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[21] These glucosinolates were epi-progoitrin, gluconasturtin, glucoalyssin, glucobrassicanapin, and glucobrassicin. For gluconapoleiferin and neoglucobrassicin more than 33% of the values were <LLOQ.

[22] Values for *p*-coumaric acid and soluble tannins were not reported because 69 and 98%, respectively, of the values were <LLOQ.

[23] Nuseed reports that 8 of 40 campesterol value, 20 of 40 delta-5 avenasterol value, 7 of 40 delta-7 avenasterol value, 5 of 40 sitosterol value, and 1 of 40 stigmasterol value in NS-B5ØØ27-4 canola exceeded the upper limit that is listed in the OECD consensus document.

conditions, natural variation, and differences in processing methods. Nuseed concludes that the differences are not biologically relevant.

Intended Effect – Modification of Fatty Acid Profile

Nuseed states that the five desaturases and two elongases were expressed in NS-B5ØØ27-4 canola to alter the fatty acid composition of the seed oil. Nuseed reported that crude fat and total fatty acids[24] levels were statistically lower in seed obtained from NS-B5ØØ27-4 canola when compared to the control. Nuseed concludes that the mean values for crude fat and total fatty acids in seed obtained from NS-B5ØØ27-4 canola fell within the ranges obtained from the conventional varieties grown under similar conditions.

Nuseed analyzed the fatty acid profile of the oil present in seeds obtained from NS-B5ØØ27-4 canola, control, and conventional varieties and reported results for 31 different fatty acid isomers.[25] Nuseed highlighted that statistical analysis was not performed on 25 of the fatty acid isomers because more than 33% of the values were less than LLOQ.[26] The values for (6Z,9Z)-octadecadienoic, gamma linolenic, stearidonic, eicosatrienoic, bishomostearidonic, EPA, (10Z,13Z,16Z,19Z)-docosa-10,13,16,19-tetraenoic, clupanodonic (DPA), osbond, and DHA acids were quantifiable in NS-B5ØØ27-4 canola, whereas most of the values for these fatty acid isomers, with the exception of DHA, were at or below the LLOQ in control and conventional varieties. Nuseed reported the proportions of oleic and linoleic acids decreased from 57.1 to 42.0% and 19.3 to 8.5% (as percent of total fatty acids), respectively, in seed obtained from NS-B5ØØ27-4 canola when compared to the control; whereas the proportion of alpha-linolenic acid and DHA increased from 11.2 to 21.0% and 0.15[27] to 8.4% (as percent of total fatty acids), respectively. Nuseed reported that omega-6 and omega-9 LCPUFAs were either negligible or minor components of the fatty acid profile of seed obtained from NS-B5ØØ27-4 canola and the control. Nuseed concludes that, with the exception of the intended changes in fatty acid composition, NS-B5ØØ27-4 canola seed is not materially different in composition from that from other canola varieties.

Results of Analyses – Processed Meal

Nuseed reported values for crude fat and total fatty acids levels in crude and solvent-extracted meals obtained from NS-B5ØØ27-4 canola and control. Nuseed noted that crude meal contained between 15.7 and 21.4% crude fat, whereas solvent-extracted meal contained less than 1% crude fat. As indicated above, Nuseed described the changes in the fatty acid profile of oil obtained from NS-B5ØØ27-4 canola and control. Nuseed states that use of oil derived from NS-B5ØØ27-4 canola in animal food in the United

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[24] Total fatty acids is the sum of 32 individual fatty acids that were analyzed by capillary column gas chromatograph.

[25] Thirty two fatty acids were included in the reference standards that were used in the capillary column gas chromatograph analysis. Nuseed provides results for fatty acids with chain lengths of at least 16 carbons plus C14:0.

[26] Fatty acid isomers with at least 33% of its values below LOQ (typically 0.01%): C16:1 *trans*, C16:3 n-3, C18:1 *trans*, C18:2 n-9, C18:2 *trans*, C18:3 n-6, C18:4 n-3, C18 total, C20:2 n-9, C20:3 n-3, C20:3 n-6, C20:3 n-9, C20:3 total, C20:4 n-3, C20:4 n-6, C20:4 total, C20:5 n-3, C22:1 n-9, C22:1 total, C22:2 n-6, C22:4 n-3, C22:4 n-6, C22:5 n-3, C22:5 n-6, and C22:5 total.

[27] Nuseed stated that conventional canola varieties do not produce DHA and suggests that the low levels of DHA in the control were due to cross-pollination within the experimental plots. Nuseed also noted that one of the conventional variety samples had a DHA value of 7.76% and attributed this to an error in either planting, sampling or sub-sampling for analysis. Data for this sample was removed from the dataset.

States will be addressed outside of the biotechnology consultation process. Thus, data for the individual fatty acids in processed meal from NS-B5ØØ27-4 canola are not summarized in this memo.

Although Nuseed reported results for both crude and solvent-extracted meal obtained from NS-B5ØØ27-4 canola and control, only values from solvent-extracted meal are discussed below.[28] Nuseed reported that values for crude protein in meals from NS-B5ØØ27-4 canola and control (53.0 and 54.6% of dry matter, respectively) were within 10% of each other and values for each of the 18 amino acids varied from each other by less than 8%. Values for carbohydrates were also within 10% with NS-B5ØØ27-4 canola meal being slightly higher. Levels of crude fiber, acid detergent fiber, and neutral detergent fiber were all lower in meal obtained from NS-B5ØØ27-4 canola when compared to the control, with the differences being less than 3.3 percent of dry matter. Nuseed reported that values for ash in both meals fell within 10% of each other, with NS-B5ØØ27-4 canola being slightly lower (5.90 versus 6.28 as percent of dry matter). Calcium levels were lower for NS-B5ØØ27-4 canola when compared to the control (0.52 and 0.65 percent of dry matter, respectively). Phosphorus levels were also lower for NS-B5ØØ27-4 canola when compared to the control (1.10 and 1.14 percent of dry matter, respectively). However, Nuseed indicates that there was considerable variation in the individual results for these minerals, thus suggesting that the differences were not biologically relevant.

Nuseed reported that seven of the ten glucosinolates that were analyzed were present at less than one micromole/gram in meals derived from NS-B5ØØ27-4 canola and the control. The concentrations of progoitrin were 9.91 and 10.01 micromole/gram, gluconapin were 3.85 and 4.94 micromole/gram, and 4-hydroxyglucobrassicin were 7.48 and 8.9 micromole/gram in meals obtained from NS-B5ØØ27-4 canola and the control, respectively. Nuseed highlighted that the levels of total glucosinolates did not exceed 30 micromoles/gram (dry weight basis). The concentrations of sinapine, ferulic acid, and phytic acid were lower in meal obtained from NS-B5ØØ27-4 canola when compared to the control. The concentrations of *p*-coumaric acid and soluble tannins were either below or slightly above the LOQ in meals obtained from NS-B5ØØ27-4 canola and the control.

Nuseed reported that levels of DHA were below the LLOQ in solvent-extracted meal from NS-B5ØØ27-4 canola. Nuseed states that meal containing about 2% fat is compositionally equivalent to conventional canola meal, except NS-B5ØØ27-4 canola meal contains a small (0.2%) amount of LCPUFA. Nuseed concluded that solvent-extracted meal derived from NS-B5ØØ27-4 canola can be used for animal food in the same manner as meal from conventional canola varieties.

Summary of Compositional Analyses

Nuseed states that, with the exception of the intended changes in fatty acid composition, NS-B5ØØ27-4 canola is not meaningfully different in composition and nutrient content from canola varieties now grown, marketed, and consumed in the United States. Nuseed concludes that meal derived from NS-B5ØØ27-4 canola is compositionally equivalent to conventional canola meal and is as safe for animal food as conventional canola meal.

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[28] Nuseed indicates that the oil from canola seed is the most valuable fraction of a canola crop and warrants crushing and solvent-extraction. Nuseed states solvent-extracted meals from NS-B5ØØ27-4 canola and conventional canola varieties are substantially similar to each other since there is only a very small amount of oil remaining in the meal.

Animal Food Labeling Considerations

It is a producer's or distributor's responsibility to ensure that labeling of the foods it markets meet applicable legal requirements, including disclosure of any material differences in the food. In evaluating the common or usual name appropriate for animal food ingredients derived from NS-B5ØØ27-4 canola meal, CVM considered that this new canola variety was genetically engineered to synthesize omega 3 long-chain polyunsaturated fatty acids, and that the intended use in animal food is as solvent-extracted meal. CVM recognizes that when used in animal food, the appropriate name for solvent-extract NS-B5ØØ27-4 canola meal is "canola meal".

Conclusion

CVM evaluated Nuseed's submissions to determine whether solvent-extracted meal derived from NS-B5ØØ27-4 canola raises any safety or regulatory issues with respect to its uses in animal food. Nuseed states that use of oil derived from NS-B5ØØ27-4 canola in animal food will be pursued outside of the biotechnology consultation process. Based on the information provided by Nuseed and other information available to the agency, CVM did not identify any safety or regulatory issues under the FD&C Act with respect to the use of solvent-extracted meal derived from NS-B5ØØ27-4 canola that would require further evaluation at this time. Should Nuseed change its intended uses to include other products derived from NS-B5ØØ27-4 canola in animal food in the United States, we recommend Nuseed contact CVM's Division of Animal Food Ingredients.

Nuseed concludes that meal derived from NS-B5ØØ27-4 canola and the animal foods derived from it are as safe as and, with the exception of the fatty acid profile, are not materially different in composition or relevant parameter from meal derived from conventional canola varieties. At this time, based on Nuseed's data and information, CVM considers Nuseed's consultation on solvent-extracted meal derived from NS-B5ØØ27-4 canola for use in animal food to be complete.

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