FDA U.S. FOOD & DRUG ADMINISTRATION
CENTER FOR VETERINARY MEDICINE

# Biotechnology Notification File No. 000198
# CVM Note to the File

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**From:** Lei Dai, BVSc, Ph.D.

**To:** Administrative Record, BNF No. 000198

**Subject:** Event KWS20-1 Sugar Beet

**Keywords:** Sugar beet, *Beta vulgaris* L., *dmo*, *Stenotrophomonas maltophilia*, dicamba mono-oxygenase (DMO), Herbicide tolerance, dicamba, *pat*, *Streptomyces viridochromogenes*, Phosphinothricin N-acetyltransferase (PAT), Glufosinate, *cp4 epsps*, *Agrobacterium* sp. strain CP4, CP4 5-enolpyruvylshikimate-3-phosphate synthase, CP4 EPSPS, Glyphosate, OECD identifier: KB-KWS2Ø1-6, Bayer CropScience LP and KWS SAAT SE & Co. KGaA.

## Purpose

This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000198. Bayer CropScience LP and KWS SAAT SE & Co. KGaA (Bayer and KWS) submitted a safety and nutritional assessment for a genetically engineered (GE) sugar beet, transformation event KWS20-1 (hereafter referred to as KWS20-1 sugar beet), and additional information afterwards. CVM evaluated the information in Bayer and KWS's submissions to ensure that regulatory and safety issues regarding animal food derived from KWS20-1 sugar beet have been resolved prior to commercial distribution. FDA's Human Foods Program summarizes its evaluation of uses of KWS20-1 sugar beet in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by Bayer and KWS as well as publicly available information and information in the agency's files. Here we discuss the outcome of the consultation for animal food use, but do not intend to restate the information provided in the final consultation in its entirety.

## Intended Effects

The intended effects of the modifications in KWS20-1 sugar beet are tolerance to dicamba, glufosinate, and glyphosate herbicides. To confer tolerance to dicamba, Bayer and KWS introduced the *dmo* gene from *Stenotrophomonas maltophilia* that encodes dicamba mono-oxygenase (DMO). To confer tolerance to glufosinate, Bayer and KWS introduced the *pat* gene from *Streptomyces viridochromogenes* that encodes phosphinothricin N-acetyltransferase (PAT). To confer tolerance to glyphosate, Bayer

and KWS introduced the *aroA* gene from *Agrobacterium* sp. strain CP4 (*cp4-epsps* gene) that encodes CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS).

## Regulatory Considerations

The purposes of this evaluation are (1) to assess whether Bayer and KWS have introduced into animal food a substance requiring premarket approval as a food additive and (2) to determine whether use of the new plant variety in animal food raises other regulatory issues with respect to the Federal Food, Drug, and Cosmetic Act (FD&C Act).

## Genetic Modification and Characterization

### Introduced DNA and Transformation Method

Bayer and KWS transformed shoot segment tissues from a conventional sugar beet variety with plasmid PV-BVHT527462 using *Agrobacterium tumefaciens* mediated transformation. The transfer-DNA (T-DNA) region within plasmid PV-BVHT527462 contains the following three expression cassettes:

- Codon optimized *dmo* gene from *S. maltophilia*, which is preceded by: 1) enhancer sequence of a promoter region from Dalia Mosaic Virus; 2) promoter, leader, and intron sequences of a putative ubiquitin gene from *Cucumis melo*; and 3) targeting sequence and partial sequence of *rbcS* gene encoding the first 27 amino acids of the small subunit ribulose 1,5-bisphosphate carboxylase protein (RbcS) from *Pisum sativum*. The codon optimized *dmo* gene is followed by 3' untranslated region (UTR) sequence of an expressed gene of unknown function from *Medicago truncatula*.
- Codon optimized *pat* gene from *S. viridochromogenes*, which is preceded by promoter and leader sequences of the *CAB* gene from *Arabidopsis thaliana*, and followed by 3' UTR sequence of the *Hsp20* gene from *M. truncatula*.
- Codon optimized *cp4 epsps* gene from *Agrobacterium* sp. strain CP4, which is preceded by: 1) intron, 5′ UTR, and promoter sequences of the *SAM2* gene from *Cucumis melo*; and 2) targeting sequence of the *ShkG* gene from *A. thaliana*. The codon optimized *cp4 epsps* gene is followed by 3' UTR sequence of an expressed gene of unknown function from *M. truncatula*.

Following transformation, explants were grown in selection medium[1] and then grown in fresh medium that induced shoot development. The developed plants with normal phenotype were selected as the $T_0$ generation. These plants were self-pollinated and the further generations was screened for insert number, insert integrity, absence of vector backbone sequences before being transferred to soil for growth and further assessment. Bayer and KWS used a combination of techniques, including Southern blot analysis, directed sequencing, and polymerase chain reaction (PCR)[2] to characterize the insertion event in KWS20-1 sugar beet. Based on these analyses, Bayer and KWS conclude that a single copy of the inserted DNA containing the three intended gene expression cassettes

[1] The selection media contained DL-phosphinothricin for inhibition of the growth of untransformed plant cells and timentin for inhibition of *A. tumefaciens* growth.

[2] Bayer and KWS state that both the traditional PCR method and Kompetitive Allele Specific PCR (KASP), a homogenous, fluorescence-based genotyping variant of PCR, were used for characterization and segregation analysis of KWS20-1 sugar beet.

is present in the sugar beet genome, with a seven base pairs deletion of the sugar beet genomic DNA at the insertion site. Bayer and KWS state that this change is probably the result of double stranded break repair during the *Agrobacterium*-mediated transformation process. No plasmid backbone sequences were detected in the KWS20-1 sugar beet genome.

### Stability and Inheritance

Bayer and KWS confirmed stability of the insert in the KWS20-1 sugar beet genome by conducting Southern blot analysis using genomic DNA obtained from three generations of KWS20-1 sugar beet. Bayer and KWS also assessed segregation of the intended DNA using PCR. Chi-square statistical analysis was carried out to compare observed segregation ratios for the inserted DNA, as measured by KASP, to the expected segregation ratios for different generations. Bayer and KWS concluded that the inserted nucleotide sequence containing *dmo*, *pat* and *cp4 epsps* genes was stably integrated at a single locus and segregated according to Mendelian principles.

### Open Reading Frame Analysis

Bayer and KWS performed bioinformatics analyses using the nucleotide sequences obtained for the inserted DNA and their corresponding flanking genomic junction sequences to determine whether insertion of the introduced DNA created any potential open reading frames (ORFs) that could encode for putative polypeptides. Bayer and KWS reports that none of the putative polypeptides had alignments with proteins in its toxin database, TOX_2022[3], and that a search in its protein database, PRT_2022, did not identify any unintended polypeptides generated at the insertion site. Based on the results of bioinformatics analyses, Bayer and KWS conclude that the T-DNA insertion does not lead to the production of putative polypeptides that would raise animal food safety concerns.

## Protein Safety

Bayer and KWS highlight that the DMO protein has been expressed in several commodity crops and include by reference information on safety of DMO protein isoforms in these crops.[4] Bayer and KWS note that there were minor amino acid differences between the DMO proteins expressed in KWS20-1 sugar beet and in these crops, but these modifications are not anticipated to have an effect on the structure of the catalytic site, functional activity, immunoreactivity, or specificity of the protein. Bayer and KWS designated the isoform of the processed DMO protein produced by KWS20-1 sugar beet as DMO+27.1, which is identical to wild-type DMO in amino acid sequence, except for an additional leucine at position 2, and 27 additional amino acids on the N-terminus derived from RbcS. Bayer and KWS assessed the equivalence between KWS20-1 sugar beet-produced and *Escherichia coli*-produced DMO protein by comparing results from the analyses for amino acid sequence, molecular weight and

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[3] According to Bayer and KWS, TOX_2022 is a subset of sequences derived from the Swiss-Pro database that was selected using a keyword search to exclude non-toxin proteins.

[4] Some listed examples of completed consultations for DMO proteins that are highly similar (identical in structure of the catalytic site, function, immunoreactivity, and substrate specificity) to those produced in KWS20-1 sugar beet include BNF No. 000193 (corn), No. 000125 (soybean), No. 000135 (cotton). Detailed safety assessments on the DMO proteins were provided in BNF No. 000125 and No. 000135.

immunoreactivity, glycosylation, and specific enzymatic activity on dicamba. Bayer and KWS report that KWS20-1 sugar beet-produced and *E. coli*-produced DMO proteins are equivalent in terms of physicochemical and functional properties. Bayer and KWS conclude that previous safety assessments using *E. coli*-produced DMO protein are applicable to DMO protein that is expressed in KWS20-1 sugar beet. Additionally, Bayer and KWS carried out bioinformatics analysis and conclude that KWS20-1 sugar beet-produced DMO protein does not share structurally-relevant similarity with any sequences in the TOX_2022 database or proteins associated with adverse health effects in the GenBank protein database.

Bayer and KWS highlight that the PAT and CP4 EPSPS proteins expressed in KWS20-1 sugar beet are identical to the proteins present in BNFs that have completed FDA's Biotechnology Consultations on Food from GE Plant Varieties and includes by reference information on safety of these proteins in these crops.[5] Bayer and KWS note that the conclusions of the multi-step approach to the safety assessments described in previous consultations, including oral acute toxicology, digestibility, heat susceptibility, and sequence similarities to known toxins through bioinformatics analysis, are applicable to the PAT and CP4 EPSPS proteins expressed in KWS20-1 sugar beet.

### Protein Expression Level

Bayer and KWS quantified DMO, PAT, and CP4 EPSPS protein levels in various tissues of KWS20-1 sugar beet. The study included five field trial sites with four replicate plots at each site. Leaf (over-season leaf 1 (OSL1), over-season leaf 2 (OSL2), and tops) and root (over-season root 1 (OSR1), over-season root 2 (OSR2), and over-season root 3 (OSR3) representing final harvest) tissue samples were collected from each replicated plot at all field sites treated with dicamba, glufosinate, and glyphosate herbicides. Samples were prepared and analyzed using Enzyme-linked immunosorbent assay. Bayer and KWS report that mean DMO protein level in KWS20-1 sugar beet across all sites was highest in OSL1 at 140 micrograms/gram of tissue dry weight (DW) and lowest in OSR3 at 12 micrograms/gram DW. The mean PAT protein level in KWS20-1 sugar beet across all sites was highest in OSL1 at 25 micrograms/g DW and lowest in OSR3 at < limit of quantitation (LOQ) (0.125 micrograms/gram DW for root). The mean CP4 EPSPS protein level in KWS20-1 sugar beet across all sites was highest in OSL1 at 590 micrograms/gram DW and lowest in OSR3 at 100 micrograms/gram DW.

Based on the information provided in previous BNFs and in this notification Bayer and KWS state that the DMO, PAT, and CP4 EPSPS proteins were fully characterized in the multistep approach addressing the safety. It is also noted that these proteins were rapidly degraded in the presence of pepsin and pancreatin under physiological conditions, and rapidly lost activity upon heating. Additionally, no oral toxicity in mice was detected at the levels tested for the DMO, PAT, and CP4 EPSPS proteins. Taken

[5] Some listed examples of completed consultations for PAT protein, which has specificity for glufosinate, include BNF No. 000023 (canola), No. 000029 (corn), No. 000038 (sugar beet), No. 000046 (canola), No. 000055 (soybean), No. 000063 (rice), and No. 000086 (cotton); some listed examples of completed consultation for CP4 EPSPS protein, which has specificity for glyphosate, include BNF No. 000090 (sugar beet), No. 000104 (soybean), No. 000126 (corn), No. 000127 (canola), and No. 000173 (corn).

together, Bayer and KWS conclude that dietary exposure to DMO, PAT, and CP4 EPSPS proteins in KWS20-1 sugar beet poses no meaningful risk to animal health.

## Animal Food Use

Bayer and KWS state that KWS20-1 sugar beet is suitable for all animal food uses of conventional sugar beet. Bayer and KWS reference the Organisation for Economic Co-operation and Development (OECD) consensus document on compositional considerations of sugar beet[6] and state that sugar beet (*Beta vulgaris* L.) is primarily grown for the extraction of sucrose from its root tissue. The final product is granular sugar. The sugar refining process also yields sugar beet pulp and sugar beet molasses, which are used in animal food. The leafy sugar beet "tops" are usually left in the field, but they may occasionally be fed to ruminant animals.

## Composition

### Scope of Analysis

Bayer and KWS analyzed the nutrient composition of KWS20-1 sugar beet and a non-GE near isogenic control sugar beet that were grown and harvested under similar conditions. Compositional analyses on tops and root samples were reported for components listed in the OECD sugar beet composition consensus document.

### Study Design

Bayer and KWS conducted field trials in 2020 at five sites in the United States. A randomized complete block design with four replicate plots at each field site was used. The KWS20-1 sugar beet, the non-GE near isogenic control, and conventional commercial reference varieties were grown under normal agronomic field conditions for their respective regions. Bayer and KWS harvested tops and root from each replicate within each site for composition analysis. Tops and root were harvested at physiological maturity and shipped on dry ice from the field sites. A subsample for compositional analysis was obtained from tops and root samples from each replicate at each site, and then ground and stored prior to nutrient analyses.

For statistical analysis, Bayer and KWS combined composition data for each component from KWS20-1 sugar beet and the control across locations using a linear mixed model with site and replicate as random factors. T-test analyses were used to test at the level of $P \leq 0.05$ for differences between KWS20-1 sugar beet and control. Differences between KWS20-1 sugar beet and control were evaluated in context of variation within the conventional commercial reference varieties grown across multiple sites, and of natural variability defined by values for conventional corn varieties in the scientific literature or in the Agriculture & Food Systems Institute (AFSI) Crop Composition Database (CCDB). Results were all expressed on a dry matter basis prior to statistical analyses. Moisture of tops and root were not statistically analyzed.

### Results of Analyses

For tops, Bayer and KWS report values for proximates (crude protein, crude fat,

[6] Organisation for Economic Co-operation and Development. 2002. Consensus document on compositional considerations for new varieties of sugar beet: Key food and feed nutrients and antinutrients. OECD ENV/JM/MONO(2002)4. OECD, Paris, France.

carbohydrates by calculation, and ash), and crude fiber. No statistically significant differences were observed between KWS20-1 sugar beet and the non-GE near isogenic control for these components.
For root, Bayer and KWS chemically analyzed proximates, 18 amino acids, sucrose, fiber (crude fiber and pectin), three minerals (phosphorus, potassium, and sodium), and one secondary metabolite (oleanolic acid). Bayer and KWS noted that one component (sodium in root) was not statistically analyzed because more than 50% of the observations fell below the lower LOQ. Bayer and KWS report statistically significant differences between KWS20-1 sugar beet and the non-GE near isogenic control in the levels of eight components (lysine, proline, serine, threonine, total fat, ash, phosphorus, and potassium). For these components, the mean difference between KWS20-1 sugar beet and the control was less than the range of values for the control. In addition, KWS20-1 sugar beet mean component values were also within the range of values of the conventional commercial reference varieties grown across multiple sites and/or values in the AFSI CCDB. Bayer and KWS conclude that the differences in these components between KWS20-1 sugar beet and the control are not biologically meaningful from an animal food safety perspective.

### Summary of Compositional Analyses

Bayer and KWS conclude based on the results from the compositional analyses that tops and root obtained from KWS20-1 sugar beet are compositionally comparable to its non-GE near-isogeneic control and commercial reference varieties in the levels of key nutrients and secondary metabolites.

## Conclusion

CVM evaluated Bayer and KWS's submissions to determine whether KWS20-1 sugar beet raises any safety or regulatory issues with respect to its uses in animal food. Based on the information provided by Bayer and KWS and other information available to the agency, CVM did not identify any safety or regulatory issues under the FD&C Act that would require further evaluation at this time.

Bayer and KWS conclude that KWS20-1 sugar beet and the animal foods derived from it are as safe as and are not materially different in composition or any other relevant parameter from other sugar beet varieties now grown, marketed, and consumed. At this time, based on Bayer and KWS's data and information, CVM considers Bayer and KWS's consultation on KWS20-1 sugar beet for use in animal food to be complete.

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