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CFSAN Note to the File

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To: Administrative Record, BNF No. 000193

Subject: Soybean with transformation event MON 94313 (MON 94313 soybean)

Keywords: Soybean, *Glycine max*, herbicide tolerance, dicamba, demethylase (*dmo*) gene, dicamba mono-oxygenase, DMO, *Stenotrophomonas maltophilia*, glufosinate, phosphinothricin-N-acetyltransferase *(pat)* gene, PAT, *Streptomyces viridochromogenes*, 2,4-dichlorophenoxyacetic acid (2,4-D), modified version of R-2,4-dichlorophenoxypropionate dioxygenase (*RdpA*) gene, FT_T.1, *Sphingobium herbicidovorans*, mesotrione, triketone dioxygenase (*TDO)* gene, TDO, *HPPD INHIBITOR SENSITIVE 1* (*HIS1*) gene, HIS1, *Oryza sativa*, Bayer CropScience LP, MON 94313, OECD unique identifier MON-94313-8

Summary

Bayer CropScience LP (Bayer) has completed a consultation with the Food and Drug Administration (FDA) on food derived from MON 94313 soybean with multiple herbicide tolerance traits.[1] MON 94313 soybean expresses dicamba mono-oxygenase (DMO) for tolerance to dicamba herbicide; phosphinothricin-N-acetyltransferase (PAT) for tolerance to glufosinate herbicide; a modified R-2,4-dichlorophenoxypropionate dioxygenase (RdpA) (referred to by Bayer as FT_T.1) for tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide; and triketone dioxygenase (TDO) for tolerance to beta-triketone herbicides such as mesotrione. This document summarizes Bayer'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.

Based on the safety and nutritional assessment Bayer has conducted, it is our understanding that Bayer concludes:

- it has not introduced into human food a new protein or other substance that would require premarket approval as a food additive, and

[1] The United States Environmental Protection Agency (EPA) registers pesticides (including herbicides) under the Federal Insecticide, Fungicide, and Rodenticide Act. Under the Federal Food, Drug, and Cosmetic Act, EPA establishes tolerances (maximum legally permissible levels) of residues of pesticides in food.

- human food from MON 94313 soybean is comparable to and as safe as human food from other soybean varieties.

CFSAN evaluated data and information supporting these conclusions and considered whether MON 94313 soybean raises other regulatory issues involving human food within FDA's authority under the Federal Food, Drug, and Cosmetic Act (FD&C Act). We have no further questions at this time about the safety, nutrition, and regulatory compliance of human food from MON 94313 soybean.

Subject of the Consultation

Crop	Soybean
Designation	MON 94313
Intended trait	Tolerance to dicamba herbicide
Intended trait	Tolerance to glufosinate herbicide
Intended trait	Tolerance to 2,4-D herbicide
Intended trait	Tolerance to mesotrione herbicide
Developer	Bayer CropScience LP
Submission received	September 7, 2022
Amendment(s) received	January 24 and September 29, 2023; January 11, February 14, and June 13, 2024
Intended use	General use in human food
Transformation plasmid	PV-GMHT529103[2]
Expression cassette 1	A demethylase (*dmo*) gene from *Stenotrophomonas maltophilia* encoding dicamba mono-oxygenase (DMO)
Expression cassette 2	A phosphinothricin-N-acetyltransferase (*pat*) gene from *Streptomyces viridochromogenes* encoding PAT
Expression cassette 3	A modified R-2,4-dichlorophenoxypropionate dioxygenase (*RdpA*) gene from *Sphingobium herbicidovorans* encoding FT_T.1
Expression cassette 4	A *Triketone dioxygenase (TDO)* gene is derived from *Oryza sativa* subsps. *japonica HPPD INHIBITOR SENSITIVE 1* (*HIS1*) gene, that is codon optimized for better expression in soybean
Method for conferring genetic change	*Agrobacterium*-mediated transformation

[2] The plasmid PV-GMHT529103 contains two T-DNAs. T-DNA I contains genes conferring herbicide tolerance traits and T-DNA II contains genes *splA* and *aadA* for selecting the transformants. After selecting transformants carrying both T-DNA I and T-DNA II, Bayer removed T-DNA II from the lead event, MON 94313 soybean, through segregation and selection for progeny that did not contain the selectable markers.

Molecular Characterization

Confirmation of intended genetic change

Following transformation and selection of event MON 94313, Bayer used next generation sequencing (NGS) and polymerase chain reaction (PCR) product sequencing approaches to assess the integrity and copy number of the inserted T-DNA I. Through comparison of MON 94313 soybean sequences to both conventional soybean genomic DNA and transformation plasmid sequences, Bayer identified two junction sequences and a single insertion of T-DNA I. Bayer separately confirmed the junction sequences by amplifying flanking genomic regions and directly sequencing the PCR products. Sequencing analysis confirmed that the insertion is a single and exact copy of the T-DNA I from the transformation plasmid. Bayer reported that the junction sequence analysis also revealed the deletion of 40 base pairs of genomic DNA at the insertion site in MON 94313 soybean.

Absence of vector backbone DNA

Bayer compared NGS reads from MON 94313 soybean to the transformation plasmid sequence. Bayer reported that the vector backbone sequences and the T-DNA II cassette containing the selectable marker genes are not present in MON 94313 soybean.

Inheritance and stability

Bayer analyzed the T-DNA I insert in MON 94313 soybean across multiple generations to determine its pattern of inheritance and generational stability. Bayer evaluated five generations of NGS data to assess the generational stability of MON 94313 soybean. Based on the NGS sequencing reads and PCR product sequencing comparison, Bayer found a single copy of the T-DNA I insert and an identical pair of junction sequences throughout the tested generations, confirming genomic stability of the inserted sequence. Bayer used PCR-based genotyping and chi-square analysis of three segregating generations to show that the T-DNA I insert is inherited according to Mendelian principles of inheritance for a single locus.

Open reading frame analysis

Bayer used bioinformatic analysis to evaluate whether unintended open reading frames (ORFs) were created because of the inserted DNA in MON 94313 soybean and, if so, whether putative expression products raised toxicity or allergenicity concerns in food. Bayer translated ORFs (from stop codon to stop codon, in all six reading frames) in the MON 94313 insert DNA and across flanking genomic DNA sequences. The resulting putative polypeptide sequences were compared to the sequences of known allergens[3], toxins[4], and to biologically active proteins[5] associated with adverse health effects for humans. Bayer found no relevant sequence similarities across the length of the putative polypeptides when compared to sequences in the allergen,

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[3] Bayer's allergen sequence dataset consists of allergen, gliadin, and glutenin sequences in "COMprehensive Protein Allergen REsource" (2021) database from the Health and Environmental Sciences Institute (HESI, https://comparedatabase.org).

[4] Toxin sequences were derived from the Swiss-Prot database (2021). Bayer described their dataset as "a subset of sequences selected using a keyword search and filtered to remove likely non-toxin proteins."

[5] Bayer derived protein sequences with biological activity from National Center for Biotechnology Information (2021) (NCBI, https://www.ncbi.nlm.nih.gov).

toxin, and protein databases.[6] The allergen sequence comparison also included identification of significant similarities with >35% identity across an 80 amino acid sliding window or identical matches of eight contiguous amino acid to sequences in the allergen database. Bayer thereby confirmed the absence of similarity of the ORFs to the sequences of allergens, toxins, or biologically active proteins that would raise food safety concern.

Introduced Protein: Dicamba mono-oxygenase (DMO)

Intended trait	Tolerance to dicamba herbicide
Source organism	*Stenotrophomonas maltophilia*
Protein description	DMO from *S. maltophilia* with an added chloroplast targeting sequence
Intended function	DMO catalyzes the demethylation of dicamba herbicide.

DMO safety assessment

Bayer used a multiplexed immunoassay to measure the concentration of DMO in MON 94313 soybean seed. The mean DMO protein expression level was 40 µg/g dry weight (dw) in seed, the part of the soybean plant consumed by humans as food.

FDA previously evaluated DMO proteins in consultations BNF 000125 (MON 87708 soybean), BNF 000135 (MON 88701 cotton), BNF 000148 (MON 87419 corn), and BNF 000173 (MON 87429 corn). The DMO in MON 94313 soybean is similar to DMOs in previous consultations, except for the differences in a few amino acids.[7] According to Bayer, these minor differences in amino acid sequences between the DMO protein expressed in MON 94313 soybean and the DMO proteins previously evaluated by FDA are not expected to affect catalytic site structure, function, immunoreactivity, or substrate specificity. Bayer therefore included data and information supporting the safety of DMO from these previous consultations by reference in its safety assessment of the DMO expressed in MON 94313 soybean. Bayer explained that the incorporated information demonstrates that DMO has a documented history of safe consumption; lacks similarity to known allergens, toxins, or other biologically active proteins known to have adverse effects on human health; is degraded by the digestive enzyme pepsin and pancreatin; loses activity after heat treatment; and is not acutely toxic. Bayer concluded that dietary exposure to DMO from MON 94313 soybean poses no meaningful risk to human health.

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[6] Relevant sequence similarity was determined by visual inspection of the sequence alignment, calculated percent identity, and the *E*-score of ≤$1e^{-5}$ to known allergen and toxin sequences.

[7] Minor differences among the DMO proteins include the presence of additional N-terminal residues remaining from partial processing of the chloroplast transit peptide (CTP), the presence of an additional alanine or leucine at position 2, and either a tryptophan or cysteine at position 112. Bayer used N-terminal sequence analysis to show that the mature MON 94313 DMO is present as a single isoform with the additional leucine but no additional N-terminal CTP residues.

Bayer has previously considered the potential for DMO to catalyze reactions with endogenous substances in plants, including soybean, and to produce unintended products that would raise food safety concerns. Bayer stated that DMO in MON 94313 soybean differs from wildtype DMO from *S. maltophilia* by one amino acid.[8] To ensure this single amino acid difference does not affect DMO substrate specificity, Bayer assessed the ability of a microbially-expressed MON 94313 DMO to demethylate dicamba herbicide and *o*-anisic acid, the soybean endogenous substance that is most structurally similar to dicamba. Bayer used an *in vitro* enzymatic assay and liquid chromatography-mass spectrometry to show that the microbially-expressed MON 94313 DMO is functionally active and converts dicamba to non-herbicidal 3,6-dichlorosalicylic acid but does not catabolize *o*-anisic acid, confirming that the single difference in the amino acid sequence in MON 94313 DMO does not affect its specificity for dicamba herbicide. These results are consistent with the results from previous analysis of a microbially-expressed MON 87708 DMO protein, which is identical to the wildtype DMO, except for an N-terminal histidine tag. Based on the results of the previous and current studies, Bayer concludes that MON 94313 DMO is specific to dicamba and unlikely to catalyze reactions with *o*-anisic acid-like endogenous soybean substances.

Introduced Protein: Phosphinothricin-N-acetyltransferase (PAT)

Intended trait	Tolerance to glufosinate herbicide
Source organism	*Streptomyces viridochromogenes*
Intended function	PAT catalyzes the acetylation of glufosinate herbicide.

PAT safety assessment

Bayer used a multiplexed immunoassay to measure the concentration of PAT in MON 94313 soybean seed. PAT was detected at a mean level of 3.8 µg/g dw in seed.

FDA has evaluated PAT in many previous consultations. Bayer incorporated data and information from BNF 000135 (MON 88701 cotton), BNF 000148 (MON 87419 corn) and BNF 000173 (MON 87429 corn) by reference, in support of its safety assessment of MON 94313 PAT.[9] Bayer explained that PAT proteins have a documented history of safe consumption; lack similarity to known allergens, toxins, or other biologically active proteins known to have adverse health effects for humans; are degraded by digestive enzymes such as pepsin and pancreatin; lose activity after heat treatment; and are not acutely toxic. Bayer concludes, based on the

[8] DMO in MON 94313 soybean has an additional leucine at position two in comparison to the wild-type DMO protein from *S. maltophilia*. This version of DMO, also present in MON 87429 corn, is referred to by Bayer as DMO+0.

[9] The PAT protein produced in MON 94313 soybean is identical to wildtype PAT from *S. viridochromogenes*, except for cleavage of the leading methionine.

weight of the evidence, that dietary exposure to PAT protein from MON 94313 soybean poses no meaningful risk to human health.[10]

Bayer referenced earlier consultations with FDA and published literature showing the mode-of-actions of PAT protein. Bayer noted that though PAT is capable of acetylating amino acids such as tryptophan and aminoadipate, PAT activity on these substances is very low compared to its activity on glufosinate. Bayer thereby concludes that PAT protein is highly specific to glufosinate.

Introduced Protein: Modified RdpA (FT_T.1)

Intended trait	Tolerance to 2,4-D herbicide
Source organism	*Sphingobium herbicidovorans*
Protein description	RdpA is described as an alpha-ketoglutarate-dependent non-heme iron dioxygenase. FT_T.1 is modified from FT_T, which is a modified RdpA. FT_T.1 differs from FT_T by three amino acids. The amino acid substitutions increase the enzymatic activity of FT_T.1 to 2,4-D herbicide.
Intended function	FT_T.1 catalyzes the double oxidation of 2,4-D herbicide.

Modified RdpA (FT_T.1) safety assessment

Bayer used a multiplexed immunoassay to measure the concentration of FT_T.1 in MON 94313 soybean seed. FT_T.1 was detected at a mean level of 6.1 µg/g dw in soybean seed.

FDA evaluated Bayer's safety assessment of FT_T in BNF 000173 (MON 87429 corn), from which FT_T.1 was derived. The FT_T.1 in MON 94313 soybean is similar to the FT_T protein in MON 87429 corn apart from differences in three amino acids. Bayer summarized a published study showing the structural similarity between FT_T.1 to FT_T, and noted that the iron binding site, an important structural property of Fe(II)/alpha-ketoglutarate-dependent dioxygenases, is conserved in both proteins.[11]

Given the similarities between FT_T.1 and FT_T, Bayer referenced BNF 000173 for the description of the source organism *S. cc* as well as results from *in vitro* digestibility, heat stability, and oral toxicity studies. Bayer noted that the source organism *S. herbicidovorans,* from which FT_T and FT_T.1 were derived, is ubiquitous in nature and its exposure to humans and animals is not known to cause allergenicity, pathogenicity or any other health concern.

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[10] Although PAT is not used as a plant-incorporated protectant (PIP) inert ingredient in MON 94313 soybean, Bayer notes its safety is nonetheless supported by the EPA exemption from the requirement of a tolerance for PAT in all food commodities when used as a PIP inert ingredient under 40 CFR 174.522.
[11] Larue, C. T., M. Goley, L. Shi, A. G. Evdokimov, O. C. Sparks, C. Ellis, A. M. Wollacott, T. J. Rydel, C. E. Halls, B. Van Scoyoc, X. Fu, J. R. Nageotte, A. M. Adio, M. Zheng, E. J. Sturman, G. S. Garvey and M. J. Varagona. 2019. Development of enzymes for robust aryloxyphenoxypropionate and synthetic auxin herbicide tolerance traits in maize and soybean crops. Pest Management Science 75(8):2086-2094.

Bayer stated that FT_T lacks similarity to known allergens, toxins, or other biologically active proteins known to have adverse health effects for humans; is degraded by the digestive enzymes pepsin and pancreatin; remains intact after moderate heat treatment of up to 37 ^{o}C but loses activity above 75 ^{o}C or higher temperature; and is not acutely toxic. Bayer further confirmed using bioinformatic data that the changes in amino acids in FT_T.1 exhibit no amino acid sequence similarities to know allergenic or toxic proteins. Based on the weight of the evidence, Bayer concluded that dietary exposure to FT_T.1 from MON 94313 soybean poses no meaningful risk to human health.

Because the amino acid modifications made to FT_T.1 are intended to alter its enzyme activity, Bayer characterized the enzyme activity parameters of FT_T.1. As expected, FT_T.1 has greater activity for 2,4-D herbicide than RdpA or FT_T. The experiment also assessed whether FT_T.1 can oxidize endogenous plant substances. Bayer initially selected endogenous plant substances from the NAPRALERT-2016 database[12] and from a literature search.[13] From these, Bayer identified potential FT_T.1 substrates based on their structural similarity to 2,4-D herbicide and their potential to fit in the FT_T.1 binding site. The spectrophotometry results of the *in vitro* enzyme activity assay showed that FT_T.1 did not oxidize the tested endogenous plant substances. Bayer then concluded that FT_T.1., like FT_T, is specific to herbicidal substrates and is unlikely to catalyze endogenous plant substances that would raise food safety concerns.

Introduced Protein: Triketone Dioxygenase (TDO)

Intended trait	Tolerance to beta-triketone herbicides such as mesotrione
Source organism	*Oryza sativa* subsp. *japonica*[14]
Protein description	TDO protein is derived from rice *HIS1* gene that encodes an Fe(II)/alpha-ketoglutarate-dependent non-heme iron dioxygenase.
Intended function	TDO protein oxidizes beta-triketone herbicides such as mesotrione to hydroxy-mesotrione and oxy-mesotrione. Oxidized reaction products are less inhibitory to enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD), an endogenous soybean enzyme required for biosynthesis of the amino acid tyrosine and downstream metabolism in plants.

[12] The NAtural PRoducts ALERT is a database derived from literature containing information on the chemistry of organisms, including plants.
[13] The same list of compounds was used by Bayer to assess the enzyme specificity of FT_T in BNF 000173, where Bayer previously reported that FT_T did not demonstrate activity toward the endogenous plant compounds identified as potential substrates.
[14] Maeda, H., K. Murata, N. Sakuma, S. Takei, A. Yamazaki, M. R. Karim, M. Kawata, S. Hirose, M. Kawagishi-Kobayashi, Y. Taniguchi, S. Suzuki, K. Sekino, M. Ohshima, H. Kato, H. Yoshida and Y. Tozawa. 2019. A rice gene that confers broad-spectrum resistance to beta-triketone herbicides. Science. 365(6451):393-396.

TDO safety assessment

Bayer used a multiplexed immunoassay to measure the concentration of TDO in MON 94313 soybean seed. TDO was detected at a mean level of 5.0 µg/g dw in seed.

Bayer relied on a weight of evidence approach to assess the safety of the TDO protein in MON 94313 soybean. Bayer compared the amino acid sequences of the soybean-expressed TDO and the HIS1 protein from japonica rice, explaining that these are nearly identical.[15] Moreover, the TDO catalytic site is comprised of structural motifs present in Fe(II)/alpha-ketoglutarate-dependent dioxygenases from across a broad range of organisms, including bacteria, fungi, plants, and vertebrates. RNA transcripts of HIS1 are present in rice leaves, which Bayer notes have a history of safe use in animal food.

Bayer conducted bioinformatics analysis to assess the toxicity and allergenicity of the TDO protein. Bayer compared the amino acid sequence of the TDO protein to the sequences of known allergens[3], toxins[4], and biologically active proteins[5] associated with adverse health effects for humans to identify significant sequence similarities (E score $\leq 1e^{-5}$) across the length of the proteins. The allergen sequence comparison also included identification of significant similarities with >35% identity across an 80 amino acid sliding window or identical matches of eight contiguous amino acid to sequences in the allergen database. Bayer concluded that the results from its bioinformatics comparison confirmed the absence of relevant amino acid sequence similarity between TDO and toxins, allergens, or biologically active proteins known to have adverse effects on human health.

Bayer conducted *in vitro* digestive fate and heat sensitivity studies to further assess the safety of the introduced TDO protein. Bayer treated microbially-expressed TDO with the digestive enzymes pepsin and pancreatin. Pepsin degraded at least 98% of intact TDO in 30 seconds and pancreatin degraded approximately 97% of the TDO in 5 minutes, confirming the degradation of TDO protein by these digestive enzymes. Heat sensitivity was determined by treating TDO protein with temperatures ranging from 25 to 95 ^{o}C and then assessing size and enzymatic activity. Size and enzymatic activity were measured by the mobility of TDO band on SDS page gel and the ability of TDO to convert mesotrione to oxidized mesotrione, respectively. Bayer reported that although TDO mobility did not change across the treated temperature range, the enzymatic activity of TDO was reduced to ≤1% relative to the unheated control when exposed to higher temperatures. Thus, while the size of the TDO enzyme did not change, its functionality decreased as the temperature increased. Bayer concluded that heat treatment during standard processing of soybean-containing foods will degrade the TDO protein functionality, and that TDO protein would consequently not be consumed as an active protein in human food products derived from MON 94313 soybean.

Bayer conducted both acute and 28-day repeat dose oral toxicity studies in mice to evaluate the potential toxicity of the TDO protein. Bayer reported the results of the acute oral toxicity study and stated that there was no evidence of adverse effects at intake levels of up to 2,000 mg TDO protein/kg body weight (bw), the highest dose tested. Similarly, Bayer reported that the repeat

[15] MON 94313 soybean-expressed TDO lacks an N-terminal methionine at position 1 compared to HIS1.

dose toxicity study had no direct test substance related adverse effects on survival and tolerance at dose levels up to 1000 mg TDO protein/kg bw/day. Bayer concluded that the No-Observed-Adverse-Effect-Level for TDO protein is greater than the anticipated human exposure level of TDO when consumed in food.

Bayer concluded that based on the weight of the evidence, dietary exposure to TDO from MON 94313 soybean poses no meaningful risk to human health.

Bayer investigated whether TDO protein in MON 94313 soybean could catalyze reactions with endogenous plant substances and produce unintended reaction products that would raise food safety concern. For this test, Bayer selected endogenous plant substances with structural similarity to mesotrione that potentially fit in the active site of TDO from NAPRALERT-2016 database[12] and scientific literature. Bayer then conducted an *in vitro* enzyme assay on the pre-selected commercially available substances as well as several beta-triketone herbicides. Bayer used spectrophotometry to assess enzymatic oxidation of the test substances. The results showed that TDO oxidized the beta-triketone herbicides mesotrione, tembotrione, and sulcotrione, but did not oxidize the tested endogenous plant substances. Bayer concluded that TDO protein is unlikely to catalyze the oxidation of endogenous soybean substances and thereby unlikely to produce unintended products that would raise food safety concerns.

Human Food Nutritional Assessment

Analysis of key nutrients, anti-nutrients, and toxicants

The intended herbicide tolerant traits in MON 94313 soybean are not expected to alter levels of key nutrients, anti-nutrients, or toxicants. To assess potential unintended changes in composition relevant to safety or nutrition, Bayer conducted field trials at multiple locations across United States in 2020. MON 94313 soybean was grown along with the conventional (non-genetically engineered) soybean variety A3555, which has a similar genetic background (control). Mature seeds from MON 94313 soybean and the control were collected and measured for proximates (crude protein, total fat ash, carbohydrates by calculation), acid detergent fiber, neutral detergent fiber, amino acids, fatty acids, minerals (calcium and phosphorus), vitamins (E and K1), secondary metabolites of isoflavone class (daidzein, genistein and glycitein) and anti-nutrients (phytic acid, raffinose, soybean lectin, stachyose and trypsin inhibitor). MON 94313 soybean values were compared to those of the control and to publicly available soybean composition data.

Bayer stated that fatty acids with values below the level of quantitation were excluded from the statistical analysis. Among the statistically analyzed components, the mean values of cystine, tryptophan, palmitic acid, linolenic acid, carbohydrates (by calculation), vitamin K1, and glycitein were statistically different between MON 94313 soybean and the control. The differences between mean values for MON 94313 soybean and the control, however, were less than the observed ranges in values from the control measured across the experimental sites. Moreover, the MON 94313 soybean mean values were within the range of natural variation

observed for soybeans varieties reported in the published scientific literature and in AFSI CCDB[16], and therefore likely not a food safety concern.

Bayer concludes that seed from MON 94313 soybean is compositionally equivalent to the control variety in levels of key nutrients and anti-nutrients and that its levels are within natural variation for soybean with respect to food safety.

Conclusion

Based on the information provided by Bayer and other information available to CFSAN, we have no further questions at this time about the safety, nutrition, and regulatory compliance of human food from MON 94313 soybean. We consider the consultation with Bayer on MON 94313 soybean to be complete.

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[16] Agriculture and Food Systems Institute (AFSI) Crop Composition Database; https://www.cropcomposition.org.