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October 20, 2023

Via FedEx & CD-ROM

Dr. Susan Carlson
Director, Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740-3835

Re: GRAS Notice for Perfect Day's Brazzein from Fermentation by *Komagataella phaffii*

Dear Dr. Carlson:

We respectfully submit the attached GRAS Notice on behalf of our client, Perfect Day, Inc. (Perfect Day), for brazzein produced from fermentation by *Komagataella phaffii* (*K. phaffii*). Brazzein from fermentation by *K. phaffii* is intended for use as a general-purpose sweetening agent at levels consistent with good manufacturing practices (GMP). More detailed information regarding product identification, intended use levels, the manufacturing process, and safety of the ingredient is set forth in the attached GRAS Notice.

Perfect Day has determined that its brazzein produced from fermentation by *K. phaffii* is GRAS for its intended uses based on scientific procedures in accordance with 21 CFR § 170.30(b) and in conformance with the guidance issued by the Food and Drug Administration (FDA) under 21 CFR § 170.36, 81 Fed. Reg. 54960 (Aug. 17, 2016). Therefore, the use of brazzein produced by *K. phaffii* , as described in this GRAS Notice, is exempt from the requirement of premarket approval as set fortha in the Federal Food, Drug, and Cosmetic Act.a

The analytical data, published studies, and information that are the basis for this GRAS Notice are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001, or will be sent to the FDA upon request.

We look forward to the agency's review of this submission and would be happy to provide agency officials with any information they may need to complete their assessment. Thank you for your attention to this matter.

Cordially yours,

Evangelia C. Pelonis

GRAS Notice for Brazzein from Fermentation by *Komagataella phaffii*

Prepared for: Office of Food Additive Safety (FHS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Campus Dr.
College Park, Maryland 20740

Submitted by: Keller and Heckman LLP
1001 G St., NW
Suite 500W
Washington, DC 20001

On behalf of our client:

Perfect Day, Inc.
813 Heinz Ave.
Berkeley, California 94710

Date: October 20, 2023

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Part 1. Signed statements and certification

1. Applicability of 21 CFR part 170, subpart E

We submit this generally recognized as safe (GRAS) notice in accordance with 21 CFR 170, subpart E.

2. Name and address of the notifier

Company:	Perfect Day, Inc.
Name:	Srivats Rajagopal
Address:	M/s. Perfect Day India Private Limited No. 54, Bommasandra-Jigani Link Road, Industrial Area, Bangalore KA 562106
Phone:	974-182-1061
Email:	srivats.r@perfectday.com

All communications on this matter are to be sent to Counsel for Perfect Day, Inc.:

Evangelia C. Pelonis
Keller and Heckman LLP
1001 G Street, NW, Suite 500W
Washington, DC 20005
Tel: 202-434-4106
Fax: 202-434-4646
Email: pelonis@khlaw.com

3. Names of the notified substance

Brazzein

4. Applicable conditions of use of the notified substance

Perfect Day, Inc. intends to market brazzein produced from fermentation by *Komagataella phaffii* (*K. phaffii*) as a general-purpose sweetening agent in the United States for use at levels consistent with good manufacturing practices (GMPs). Perfect Day's brazzein from fermentation by *K. phaffii* is produced in accordance with current Good Manufacturing Practice ("cGMP") and hazard analysis and risk-based preventive control (HARPC) requirements at 21 CFR Part 117 Subpart C. It is not intended for use in infant formulas and meat and poultry products.

Most other high-intensity sweeteners have been approved by the FDA as general-purpose sweeteners without their uses being restricted to specific foods or use-levels. Hence, the foods to which high-intensity sweeteners are added and the use-levels are controlled by technological properties (e.g., relative sweetness when compared to sucrose or other sugars). Brazzein is a high intensity sweetener and is approximately 500 times as sweet as sucrose, therefore the intended

uses of brazzein produced from fermentation reflect those currently permitted for other high-intensity sweeteners (e.g. aspartame and steviol glycosides) in the U.S.

5. Basis for the GRAS determination

Keller and Heckman LLP, on behalf of Perfect Day, Inc., hereby notifies the Agency of its determination that its brazzein from fermentation by *Komagataella phaffii* is GRAS for its intended uses, consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (FD&C Act). This GRAS conclusion is based on scientific procedures in accordance with 21 CFR §170.30(a) and (b) and conforms to the guidance issued by the Food and Drug Administration (FDA) under 21 CFR §170.36, 81 Fed. Reg. 54,960 (Aug. 17, 2016). The statutory basis for our conclusion of GRAS status is through scientific procedures in accordance with proposed 21 CFR § 170.36. The GRAS status of brazzein from fermentation by *Komagataella phaffii* is based on data generally available in the public domain relating to the safety of brazzein produced from fermentation and the safety of the production strain, *K. phaffii* (also known as *Pichia pastoris*).

6. Exclusion from premarket approval

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

7. Availability of data and information

The information for this GRAS conclusion including analytical data, published studies, and information that are the basis for this GRAS determination are available to FDA upon request as required by 21 CFR § 170.225(c)(7)(ii)(A) or (B) by contacting Keller and Heckman LLP at the below address.

Keller and Heckman LLP
1001 G Street, NW, Suite 500W
Washington, DC 20005
Tel: 202-434-4106
Fax: 202-434-4646
Email: pelonis@khlaw.com

8. Applicability of FOIA exemptions

Perfect Day, Inc. is not claiming any information in Parts 2 through 7 of this document as trade secret, confidential or financial information that is privileged or confidential. Thus, all information and data in this submission are not exempt from the Freedom of Information Act (FOIA), 5 U.S.C. Section 552.

9. Certification

We certify on behalf of our client, Perfect Day, Inc., that this GRAS conclusion is based on representative data from Perfect Day, Inc. required for the safety and GRAS status for brazzein from fermentation by *Komagataella phaffii*. To the best of our knowledge, it is a

complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

10. Signature and name and title of the person signing this GRAS notice:

Date: October 20, 2023

Evangelia C. Pelonis
Partner
Keller and Heckman LLP

Part 2. Identity, method of manufacture, specifications, and physical or technical effect

1. Identity

Brazzein is a recently identified sweet protein derived from the plant *Pentadiplandra brazzeana* that has a sweetness approximately 500 times that of sucrose (Ming and Hellenkant, 1994). Naturally occurring brazzein is a 6.5-kDa, single-chain polypeptide consisting of 54 amino acids with four disulphide bridges which provide heat stability at temperatures as high as 80 °C for 4 hours (Ming *et al*., 1995). Brazzein isolated from *P. brazzeana* occurs primarily in two forms, a 54 amino acid protein in which the N-terminal glutamine has been converted to pyroglutamic acid, and a 53 amino acid protein in which the N-terminal glutamine has been removed entirely (Izawa et al., 1996). This notice describes a highly purified 53 amino acid (des-pyrE-brazzein) protein extract comprised of ≥ 90% brazzein via fermentation, using a yeast strain commonly used for production of protein used as an ingredient in a variety of foods, *Komagataella phaffii,* also known as *Pichia pastoris* (Heistinger *et al.*, 2020). The expression cassettes employed by Perfect Day encode only the 53 amino acid version of the protein so that the finished product described herein consists only of this version of the protein. The resulting product is a homogenous white to cream colored powder that can be incorporated into foods at usage levels matching other high intensity sweetener products.

Table 1: Sequence of Perfect Day's Brazzein from Fermentation with *K. phaffii*

DKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEY

2. Material specifications

(a) Host strain

The host microorganism used to construct the brazzein producing strain is the yeast *Komagataella phaffii* strain PD-B1. *K. phaffii* was initially known as *Pichia pastoris* and was reassigned as the sole member of genus *Komagataella* in 1995 (Yamada *et al*., 1995). It is notable for its ability to metabolize methanol (Ogata *et al*., 1969). The host strain is auxotrophic for histiadine production through deletion of the endogenous His4 gene, which is analogous to His4 in Saccharomyces cerevisiae. *K. phaffii* is classified as a Biosafety Level 1 (BSL-1) organism and is commonly utilized as a production strain to produce proteins utilized to achieve a variety of technical effects in foods worldwide.

(b) Production strain

To optimize expression of brazzein and obtain the purest product possible, Perfect Day employs several common and well-characterized genetic modification techniques: (1) the host strain was genetically modified with one or more expression cassettes to produce brazzein from *Pentadiplandra brazzeana*, a plant native to West Africa; (2) the genetic sequence for brazzein

has been codon-optimized for expression in the host yeast strain, but the amino acid sequence of the protein remains unchanged from the donor organism (other than removal of the N-terminal glutamine sequence); (3) a series of well understood and characterized endogenous or synthetic promoters that are used to promote the expression of the brazzein gene via methanol induction and (4) selective up-regulation or attenuation of endogenous transcription factors and/or inclusion of exogenous transcription factors from non-pathogenic and non-toxigenic source organisms. Each brazzein expression cassette contains: (1) DNA homologous to the desired integration site, (2) the codon-optimized 53 amino acid brazzein gene under the control of an endogenous or synthetic *K. phaffii* promoter, as well as signal and terminator sequences, and (3) the endogenous His4 (histidinol dehydrogenase) gene. The expression cassette is stably inserted into the host strain genome, as evidenced by multi-generational studies showing consistent levels of brazzein production. The expression cassette does not contain any antibiotic resistance genes or mobile genetic element.

3. Raw Materials and Processing Aids

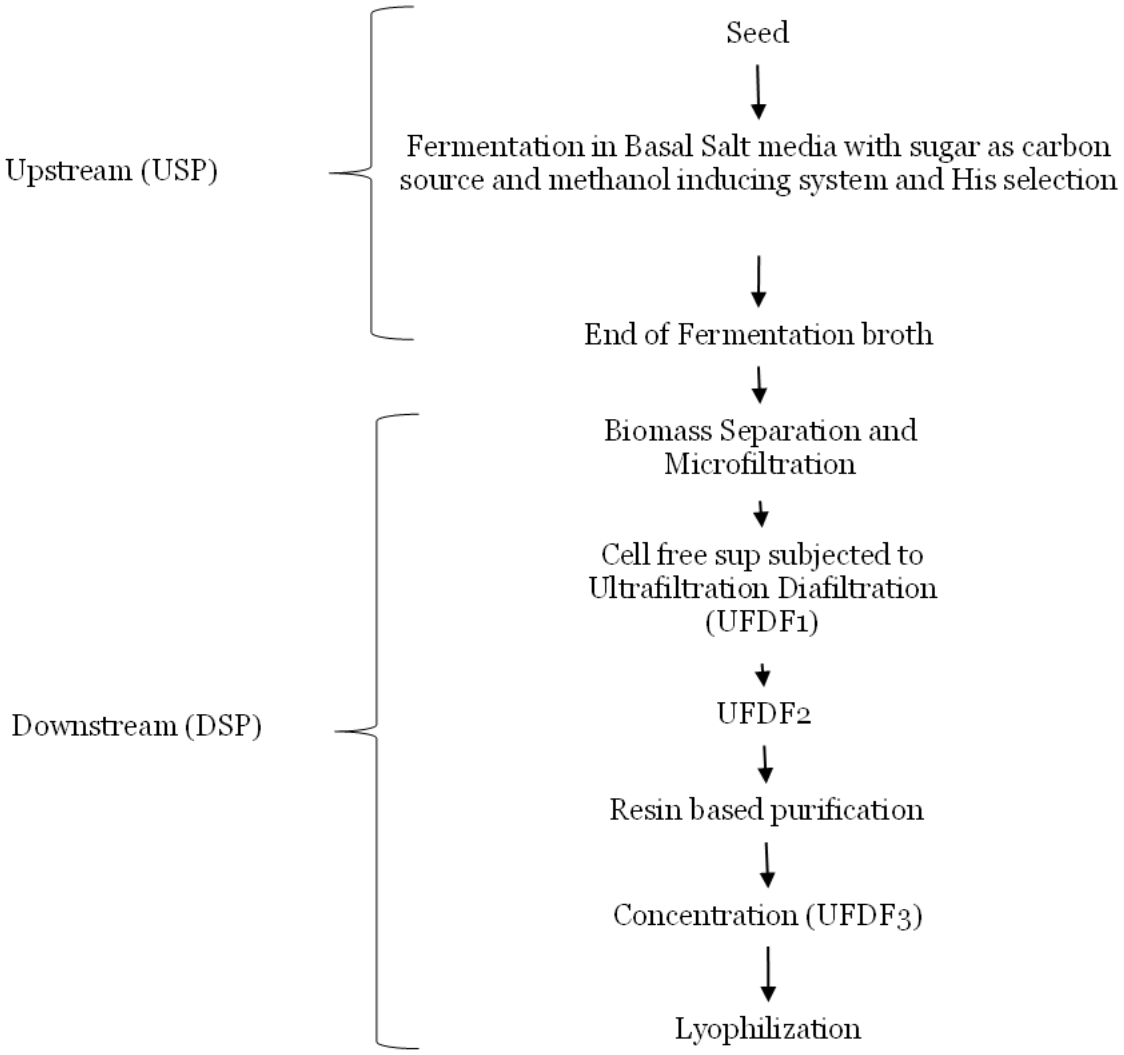
The raw materials, processing aids (*e.g.*, antifoam), filtration aids, and pH adjustors used in the fermentation and recovery processes are safe and suitable standard ingredients that meet predefined quality standards. The raw materials conform to either specifications set out in the Food Chemical Codex, 11th edition, 2018 or to other applicable regulatory standards.

(a) Description of the method of manufacture

Brazzein is manufactured under current good manufacturing practices (cGMP) for food (21 CFR Part 117, Subpart B), meets hazard analysis and risk-based preventive control (HARPC) requirements (21 CFR Part 117, Subpart C), and meets appropriate food grade specifications. Brazzein is manufactured by submerged fermentation of a pure culture of the yeast *K. phaffii* (previously *P. pastoris)* that has been genetically modified as described above. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by undesired microorganisms. During all steps of fermentation, physical and chemical control measures are taken, and microbiological analyses are conducted periodically to ensure the absence of foreign microorganisms and confirm the production strain's identity.

A new lyophilized stock culture vial of the modified *K. phaffii* production strain is used to initiate a seed fermentor at the beginning of production for each batch. Production then moves from a seed to production fermentor for the main fermentation phase. Brazzein is secreted from the yeast and remains solubilized in the fermentation media until the recovery process begins. Recovery begins immediately after the fermentation phase and is a multi-step process that begins with a primary solid/liquid centrifugation step to separate biomass from the fermentation media that contains soluble Brazzein. This step is followed by a micro filtration. The Brazzein containing solution is then subjected to ultrafiltration to remove high molecular weight impurities. The Brazzein is then concentrated via an ultrafiltration/diafiltration step and then lyophilized. The finished product is a white to off-white powder consisting of ≥ 90% Brazzein. The manufacturing process is summarized in **Figure 1**.

Figure 1. Manufacturing Process of Brazzein



4. Product Specifications and Batch Analyses

(a) Physical, Chemical, and Microbiological Specifications

The product specifications for brazzein produced from fermentation of *K. phaffii* are presented in **Table 2**.

Table 2. Physical and Microbiological Characteristics of Perfect Day's Brazzein Produced from Fermentation

Analyte	Value	Method
Total Protein	≥90%	AOAC 968.06, Dumas (N x 6.25)
Protein as Brazzein	≥90%	HPLC (Internal Method)
Moisture	≤10%	AOAC 925.09; AOAC 926.08
Ash	≤3%	AOAC 923.03
Fat	≤1.5%	AOAC 922.06
Total Carbohydrates	≤2 %	Calculation
pH	3.5-6.0	AOAC 981.12
Lead (Pb)	≤1.0 ppm	AOAC 2011.19; AOAC 993.14
Arsenic (As)	≤2.0 ppm	AOAC 2011.19; AOAC 993.14
Cadmium (Cd)	≤1.0 ppm	AOAC 2011.19; AOAC 993.14
Mercury (Hg)	≤1.0 ppm	AOAC 2011.19; AOAC 993.14
Ethanol	<10 ppm	Eurofins Internal Method (GC-FID)
Methanol	<10 ppm	Eurofins Internal Method (GC-FID)
Total Plate Count	<10,000 CFU/g	FDA BAM Ch. 3
Yeast	<50 CFU/g	FDA BAM Ch. 18
Mold	<50 CFU/g	FDA BAM Ch. 18
Coliform	<10 CFU/g	CMMEF: Ch. 8, 4th Ed.

(b) Batch Analyses

Data from the analysis of three non-consecutive lots of brazzein produced from fermentation, which demonstrate the consistency of manufacturing process and compliance with the physical and chemical specifications, are presented in **Table 3**.

Table 3. Physical, Chemical, and Microbiological Product Analysis for Three Non-Consecutive Lots of Perfect Day's Brazzein Produced from Fermentation

Analyte	Specification	Lot 1	Lot2	Lot 3
Total Protein	≥90%	95.5	98.1	97.4
Protein as Brazzein	≥90%	96.2	95.7	95.4
Moisture	≤10%	5.08	2.89	3.97
Ash	≤3%	<0.1	<0.1	<0.1
Fat	≤1.5%	<0.1	0.1	<0.1
Total Carbohydrates	≤2 %	<0.1	<0.1	<0.1
pH	3.5-6.0	3.94	4.10	4.23
Lead (Pb)	≤1.0 ppm	<0.005	<0.005	<0.005
Arsenic (As)	≤2.0 ppm	<0.01	<0.01	0.553
Cadmium (Cd)	≤1.0 ppm	<0.005	<0.005	<0.005
Mercury (Hg)	≤1.0 ppm	0.014	0.014	0.013
Ethanol	<10 ppm	<10	<10	<10
Methanol	<10 ppm	<10	<10	<10
Total Plate Count	<10,000 cfu/g	3,000	70	30
Yeast	<50 cfu/g	<10	<10	<10
Mold	<50 cfu/g	<10	<10	<10
Coliform	<10 cfu/g	<10	<10	<10

(b) Batch Analyses

Data from the analysis of three non-consecutive lots of brazzein produced from fermentation, which demonstrate the consistency of manufacturing process and compliance with the physical and chemical specifications, are presented in **Table 3**.

Table 3. Physical, Chemical, and Microbiological Product Analysis for Three Non-Consecutive Lots of Perfect Day's Brazzein Produced from Fermentation

Analyte	Specification	Lot 1	Lot2	Lot 3
Total Protein	≥90%	95.5	98.1	97.4
Protein as Brazzein	≥90%	96.2	95.7	95.4
Moisture	≤10%	5.08	2.89	3.97
Ash	≤3%	<0.1	<0.1	<0.1
Fat	≤1.5%	<0.1	0.1	<0.1
Total Carbohydrates	≤2 %	<0.1	<0.1	<0.1
pH	3.5-6.0	3.94	4.10	4.23
Lead (Pb)	≤1.0 ppm	<0.005	<0.005	<0.005
Arsenic (As)	≤2.0 ppm	<0.01	<0.01	0.553
Cadmium (Cd)	≤1.0 ppm	<0.005	<0.005	<0.005
Mercury (Hg)	≤1.0 ppm	0.014	0.014	0.013
Ethanol	<10 ppm	<10	<10	<10
Methanol	<10 ppm	<10	<10	<10
Total Plate Count	<10,000 cfu/g	3,000	70	30
Yeast	<50 cfu/g	<10	<10	<10
Mold	<50 cfu/g	<10	<10	<10
Coliform	<10 cfu/g	<10	<10	<10

Part 3. Dietary exposure

1. Estimate of Dietary Exposure

Perfect Day's Brazzein produced from fermentation is approximately 500 times sweeter than sucrose and is intended for use as a general-purpose sweetening agent, in accordance with cGMP. The majority of other high-intensity sweeteners (aspartame, steviol glycosides, etc.) have been approved by the FDA as general-purpose sweeteners without their uses being restricted to specific foods or use-levels. The foods to which high-intensity sweeteners are added and their use-levels are controlled by technological properties (e.g., sweetness potency). Brazzein has a sweetness intensity that is comparable to that of other high-intensity sweeteners (e.g., aspartame is approximately 200 times as sweet as sucrose, steviol glycosides are approximately 200-300 times sweeter than sucrose), and therefore the uses and use-levels of brazzein produced from fermentation are likely to reflect or be lower than those currently permitted for other high-intensity sweeteners in the U.S.

Numerous surveys have been conducted in various jurisdictions (U.S., Canada, Brazil, Australia, New Zealand, and countries in the European Union) to assess daily consumption estimates of other well-established high-intensity sweeteners in the marketplace (e.g., aspartame, cyclamate, saccharin, and sucralose). Renwick (2008) used the available post-market surveillance data for other high-intensity sweeteners as the basis for the assessment of dietary exposure for reb A, a commonly consumed steviol glycoside, by assuming full replacement of the currently approved intense sweeteners with the new sweetener. This intake assessment methodology yields intake estimates that, while conservative, are realistic in that they reflect actual post-market intakes of high-intensity sweeteners. Specifically, to estimate reb A intakes, Renwick calculated the post-market surveillance intake estimates for intense sweeteners presently used in the global marketplace as sucrose equivalents in various population groups (**Table 4**). The data used in these analyses were primarily derived from studies that used specifically designed food diaries combined with actual use-levels or approved levels in different foods and beverages. In order to predict dietary exposure to reb A, the intake estimates for the high-intensity sweeteners were adjusted for the sweetness intensity of reb A relative to sucrose (approximately 250). Herein, we adapt this approach to estimate exposure to brazzein from fermentation using the method described in Renwick, with the relative potency of brazzein assumed to be approximately 500x as described in the public literature (Ming and Hellenkant 1994).

Table 4. Estimated Consumption of Brazzein Using Renwick's (2008) Methodology of Intense Sweetener Intake

Population Group	Intakes of intense sweeteners (expressed as sucrose equivalents) (mg/kg bw/day)	Consumption estimates for Brazzein[a] (mg/kg bw/day)

	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic Adults	255	675	0.51	
Diabetic Adults	280	897	0.56	1.79
Non-diabetic Children	425	990	0.85	
Diabetic Children	672	908	1.34	1.82

[a] Approximately 500 times as sweet as sucrose

For non-diabetic adults, average and high-end intakes of brazzein of up to 0.51 and 1.35 mg/kg bw/day, respectively, were calculated. For diabetic adults, average and high-end intakes were slightly higher at up to 0.56 and 1.79 mg/kg bw/day. Average and high-end exposures to brazzein produced from fermentation in non-diabetic children were calculated to be up to 0.85 and 1.98 mg/kg bw/day, respectively. Average intakes were estimated to be slightly higher at 1.34 mg/kg bw/day in diabetic children compared to values for non-diabetic children (up to 0.85 mg/kg bw/day) while high-end values in diabetic children (1.82 mg/kg bw/day) were lower than high-end values in non-diabetic children (1.98 mg/kg bw/day).

As discussed in Part 6 below, Lynch *et al.* 2023 established a NOAEL in a 90-day rodent study of 978 mg/kg bw/day in male rats. This yields a margin of safety (MOS) of 493 when comparing the NOAEL from the Lynch study with the most conservative (e.g. highest exposure) EDI of 1.98 mg/kg bw/day calculated above. The test article description for Lynch *et al.* gives a minimum brazzein content of 30% (w/w test article), and assuming that brazzein is present at the minimum stated concentration a more conservative MOS of 148 (Lynch NOAEL of 978 * 0.30 brazzein content = 293 mg/kg bw/day brazzein; 293/1.98 mg/kg bw/day EDI = 148.2 MOS) can be calculated which clearly supports brazzein's status as GRAS for the intended uses described here.

Part 4 – Self-limiting levels of use

As with other high intensity sweeteners, brazzein produced from fermentation has self-limiting levels related to the desired sweetness intended for a particular food or beverage product. Therefore, the use of brazzein produced from fermentation as a general-purpose sweetener in foods is self-limiting based on its organoleptic properties.

Part 5. Experience based on common use in food before 1958

While the basis for this GRAS Notice is scientific procedures, rather than common use in food, we note that reports of locals consuming the fruit of *Pentadiplandra brazzeana* in its native habitat of West Africa have been documented (Hladik and Hladik 1988; Ming and Hellenkant 1994).

Part 6. Narrative

1. Overview of Safety of Brazzein Produced via Fermentation of *K. phaffii*

Considerations regarding safety of brazzein produced from fermentation of *K. phaffii* involve the safety of both the production organism (modified *K. phaffii*) and the safety of the end use product (brazzein). *K. phaffii* (previously *P. pastoris*) has a long history of safe use in industrial scale food protein production. The safety of this species as an industrial enzyme production organism has been reviewed multiple times; it is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under submerged fermentation conditions.

Brazzein safety has been established using scientific procedures including bioinformatics, and publicly available *in vitro* and *in vivo* studies. A search of publicly available, peer reviewed literature relating to brazzein and the production strain *K. phaffii* was conducted in October 2023. Databases searched included PubMed, ToxPlanet, Google Scolar, and Embase. Search terms were (and/or): brazzein; *Pentadiplandra brazzeana*; safety; toxicity; toxicology; consumption; genotoxicity; oral toxicity; acute toxicity; *Komagataella phaffii*; *Pichia pastoris*. These studies are summarized below and clearly establish that brazzein produced via fermentation is GRAS for its intended uses.

2. Safety of *Komagataella phaffii*

(a) Safety of the Parental Strain

K. phaffii is recognized as a non-toxin producing microorganism and is classified as a biosafety level 1 (BSL1) organism by the ATCC and has Qualified Presumption of Safety (QPS) status in the EU for use in enzyme production (EFSA, 2017), indicating that *K. phaffii* is a safe and suitable organism for production of food ingredients and is not capable of producing toxic metabolites when used for food protein production. Dried *P. pastoris* is also permitted for the addition to chicken feed as a source of protein under 21 CFR § 573.750. This information suggests that *K. phaffii* is non-pathogenic to humans and non-toxigenic and would therefore be a safe and suitable source organism for production of brazzein for its intended uses (Pariza and Johnson, 2001)

K. phaffii has a long history of safe use in industrial scale protein production for food use. FDA's GRAS Notice Inventory lists 4 notices involving production of exogenous proteins genetically modified *K. phaffii* or *P. pastoris* that have received "no questions" letters from FDA (**Table 5**).

Table 5. Summary of GRAS Notices Utilizing *K. phaffii* or *P. pastoris*

GRN No.	Enzyme	Use
204	Phospholipase C enzyme from *P. pastoris*	Degumming vegetable oils
737	Soy leghemoglobin from *P. pastoris*	Flavoring in meat analogs
967	Chicken egg white protein from *K. phaffii*	Substitute for egg-white protein and source of nutrition
1001	Bovine myoglobin from *P. pastoris*	Flavoring in meat analogs

Fraser *et al.* 2018 describes a series of toxicological studies on soy leghemoglobin produced using *P. pastoris* which is the subject of GRN 737. The study reports no adverse effects from administration of the test article to rodents in a 28 day repeat dose study, indicating that the production strain is from a safe lineage.

(b) Safety of Production Strain

The safety of the production strain used in the production of brazzein from fermentation using *K. phaffii* has been assessed using the principles for assessing the safety of microbially-derived enzymes for use in food production first set forth by Pariza and Johnson, 2001. This approach to the safety evaluation of food enzymes is widely accepted by the scientific community and regulatory agencies and includes an evaluation of the pathogenicity, toxigenicity, and the genetic modification techniques employed.

As described in Part 2, Section 2b, the host strain was modified with an expression cassette containing a codon optimized sequence for brazzein which has been stably inserted into the genome of *K. phaffii*. The cassette also contains appropriate, well-described regulatory sequences and a *His4* auxotrophic selection marker which can be recycled as needed. No antibiotics or antibiotic selection markers remain from the production strain construction process. Based on the long history of safety for the host strain and the nature of the genetic modifications made to the host and the long history of use of modified *K. phaffii* in industrial food enzyme production, it can be concluded that the production strain poses no risk to human health.

3. Safety of Brazzein

(a) Published Safety Studies

Two rodent studies were identified which exposed animals to brazzein. One of these studies was not conducted as Organisation for Economic Co-operation and Development (OECD) guideline (or similar) safety study and lacks the full battery of histopathological, blood chemistry, organ weight, etc. analyses typically undertaken for such a

guideline study. However, this report still represents relevant exposures to brazzein and is summarized here. The second study does describe an OECD 408, 90-day rodent study as well as in vitro genotoxicity studies and is described in detail below.

Kim *et al*. 2020 provided three (3) groups of mice with either brazzein dissolved in water, plain water or 10% sucrose in water *ad libitum* for 15 weeks. The authors report that brazzein did not cause adiposity hypertrophy when compared to mice who received sucrose, and that brazzein consumption did not disrupt glucose homeostasis, insulin resistance, or inflammation. No adverse effects of brazzein consumption were reported.

The toxicity of brazzein produced via fermentation with *K. phaffii* was assessed via a series of studies as reported by Lynch *et al.* 2023. Results are reported for several GLP studies including *in vitro* mutagenicity (OECD 471) and micronucleus (OECD 487) were reported as well as a 90 day repeat dose oral toxicity study in rats (OECD 408). Results were also reported for a 14-day range finding study and an *in silico* allergenicity assessment of brazzein protein. The test article was described as a preparation of brazzein produced via fermentation with *K. phaffii* containing 85% total protein (≥ 30% brazzein w/w %), 5% carbohydrates, 10% ash, and ≤ 1% fat.

To assess the allergenicity potential of brazzein, Lynch *et al.* 2023 also describes searches of the AllergenOnline database (www.allergenonline.org) maintained by the Food Allergy Research and Resource Program (FARRP) of the University of Nebraska-Lincoln and Allermatch (www.allermatch.org) database maintained by Wageningen University were conducted using the approaches described by Codex Alimentarius (Codex Alimentarius 2009) and FAO/WHO (FAO/WHO 2001). For both databases sliding 80-amino acid alignment, exact 8 amino acid, and full-length amino acid searches were conducted using default settings (percent identity >35%) and the FASTA36 algorithm. Brazzein did not share any significant sequence homology matches with any known allergens from the AllergenOnline Database or Allermatch Database. All matches shared less than 35% identity in all 80-amino acid sliding window searches, and no 8-amino acid exact matches were identified with any known allergens from the AllergenOnline Database or Allermatch Database.

An in vitro bacterial reverse mutation test was conducted to determine the mutagenicity potential of brazzein in 5 tester strains: *S. typhimurium* TA98, TA100, TA1535 and TA1537, and *E. coli* WP2 uvrA, in the presence or absence of S9 metabolic activation. A main test conducted using the plate incubation method and a confirmatory test conducted using the pre-incubation method both utilized 8 dose levels: 5,000, 1,580, 500, 158, 50, 15.8, 5 and 1.58 μg/plate) were conducted for all strains. Mean revertant colony counts for each treatment did not reach the level which would indicate that the treatment was mutagenic, which was defined for this study as a 2-fold or greater increase for strains TA98, TA 100, and WP2 uvrA and a 3-fold or greater increase for strains TA1535 and TA1537 when compared to the vehicle/negative control with or without S9 metabolic activation. As the test article did not induce signs of toxicity, the mean revertant colony counts for each strain tested with the vehicle/negative control were within the laboratory historical range, and the positive control substances caused expected substantial increases in revertant colony counts both with and without S9 metabolic activation

the test was considered valid, and the authors concluded that brazzein was not mutagenic under the conditions of the assay.

A micronucleus assay was undertaken to determine if brazzein induced structural or numerical chromosomal damage in primary human lymphocytes. An initial test for cytotoxicity indicated that the test article was not cytotoxic at the maximum suggested dose according to the OECD 487 guideline of 2,000 μg/mL. Therefore, this was set as the maximum dose for the main experiments which consisted of doses of 2,000, 1,000, 500, 250, 125, and 62.5 μg/mL including negative (cell culture media) and positive controls. Two separate experiments were undertaken, the first consisted of short term (4 hours) exposure of cells to the test article with and without S9 metabolic activation while the second consisted of long-term exposure (44 hours) without metabolic activation only. The highest 3 doses (2,000, 1,000, and 500 μg/mL) were chosen for microscopic analysis, and 1,000 cells were analyzed per dose per replicate. The test as a whole was considered to be valid as all conditions of validity were met: concurrent negative/vehicle controls were consistent with laboratory historical controls, concurrent positive controls induced a statistically significant increase in micronuclei, cell proliferation as measured by CBPI was ≥ 70% (≤ 30% cytostasis); and an adequate number of cells (≥ 500 cells per treatment) and concentrations (≥ 3) were analyzed. As the numbers of micronucleated cells exposed to brazzein were within or below the historical controls of the negative control and did not show a biologically relevant increase compared to the concurrent negative control and the $\chi 2$ test for trend did not show a concentration dependent statistically significant increase in micronuclei, brazzein was determined to not be clastogenic or aneugenic under the conditions of the test.

A 14-day range finding study was conducted using four groups of adult CRL: Sprague-Dawley rats (5/sex/group) which were fed diets prepared to contain 3000, 6000, and 12000 ppm of brazzein targeting daily intakes of 250, 500, and 1000 mg/kg/day respectively. The animals were observed at least once daily for viability, signs of gross toxicity, and behavioral changes, and weekly for a battery of detailed observations. Body weights were recorded twice during the acclimation period and on days 3, 7, 10, 14, and immediately prior to sacrifice. Individual food consumption was also recorded to coincide with body weights. Food efficiency and dietary intake were calculated. A gross necropsy was performed on all animals at study termination. The authors reported that there were no treatment related effects of brazzein at up to nominal doses of 1000 mg/kg body weight/day, including effects on mortality, clinical signs, body weight, body weight gain, or food consumption. No treatment related abnormalities were noted upon macroscopic examination at necropsy. Based on the results of the 14-day study, a dose of 1000 mg/kg body weight/day was chosen as the high dose for the 90-day dietary toxicity study.

For the 90-day study, Sprague-Dawley rats [Crl:CD(SD)] were administered brazzein in the diet at 0, 250, 500, and 1,000 mg/kg bw/day. To achieve the target doses, the dietary concentrations of brazzein were adjusted weekly on the basis of the previous weeks' food consumption and body weight data. Animals were observed for clinical signs three times daily, body weights were recorded twice in the first week of the study, once a week during the course of the study, and at necropsy. Food consumption was recorded twice during the initial week of and once a week for the remainder of the study. Ophthalmologic examinations were conducted prior to initiation of the study and at the beginning of Week 13. Functional observational battery (FOB) tests were conducted once prior to test substance administration, during week 1, and

monthly thereafter. Blood chemistry (including thyroid hormones), urinalysis, organ weights, and histopathology analyses were conducted per OECD 408 guidelines. No mortalities occurred during the study and there were no reports of clinical signs related to brazzein treatment. Hair loss (alopecia) was the chief finding, but the incidence of which was spread throughout the control and treatment groups of both male and female rats, indicating this was not a result of exposure to the test article. Brazzein treatment also had no adverse effect on FOB measurements, body weight, body weight gain, feed consumption, or feed efficiency. Actual exposure to brazzein calculated using recorded body weights and feed consumption were 0, 245, 490 and 978 mg/kg bw/day for males and 0, 245, 493, and 985 mg/kg bw/day for females. There were no indications of an adverse effect of treatment on the results of the hematological, clinical chemistry, or urinalysis parameters measured. No biologically significant effects of brazzein treatment were reported on either absolute or relative organ weights and histopathological examinations revealed no differences between treatment and control groups. Given the data reported, the authors concluded that a no observed adverse effect level (NOAEL) for the study could be considered to be the maximum dose tested, 978 mg/kg bw/day for males and 985 mg/kg bw/day for females.

(b) Unpublished Studies

In addition to the above described published studies, Perfect Day has undertaken a similar series of studies which, while unpublished, serve to support the GRAS status of brazzein produced via fermentation with *K. phaffii*. The test article in these unpublished studies is the ingredient described herein, and meets all identity, specification, and production processes as described in Part 2 above.

An OECD 471 Ames assay was conducted to determine the mutagenicity potential of brazzein in 5 tester strains: *S. typhimurium* TA98, TA100, TA1535 and TA1537, and tryptophan-dependent *E. coli* WP2, in the presence or absence of S9 metabolic activation (Anthem Study no. G23039). The main test conducted using the plate incubation and pre-incubation methods utilized 5 dose levels: 5,000, 1,580, 500, 158, and 50 μg/plate were conducted for all strains. Mean revertant colony counts for each treatment did not reach the level which would indicate that the treatment was mutagenic, which was defined for this study a 2-fold or greater increase for strains TA98, TA 100, and WP2 uvrA and a 3-fold or greater increase for strains TA1535 and TA1537 when compared to the vehicle/negative control with or without S9 metabolic activation. As the test article did not induce signs of cytotoxicity, the mean revertant colony counts for each strain tested with the vehicle/negative control were within the laboratory historical range, and the positive control substances caused expected substantial increases in revertant colony counts both with and without S9 metabolic activation the test was considered valid, and the study concluded that brazzein was not mutagenic under the conditions of the assay.

An OECD 473 *in vitro* chromosomal aberration assay in CHO-K1 cells was conducted (Anthem Study no. 23031). A dose finding study was conducted using 5 test concentrations between 5,000 and 156 μg/mL with (4 hours) and without (4 and 24 hours) S9 metabolic activation. There was no evidence of cytotoxicity at any tested concentration. For the main study, concentrations of 5,000, 2,500, and 1,250 μg/mL were used with (4 hours) or without (4 and 24 hours) S9 metabolic activation. 300 giemsa stained metaphases were scored for aberrations at

each concentration. There were no statistically significant (Fisher's exact test, $p<0.05$) increases in the number of observed CA, and brazzein was determined to be non-genotoxic (non-clastogenic) under the conditions of the assay.

An OECD 487 micronucleus assay was undertaken to determine if brazzein induced structural or numerical chromosomal damage in CHO-K1 cells (Anthem Study no. G23032). An initial range finding study indicated that the test article was not cytotoxic at any of the 6 concentrations tested (5,000-156 μg/mL). Doses for the main experiments were 5,000, 2,500, and 1,250 μg/mL including negative (cell culture media) and positive controls. Two separate experiments were undertaken, the first consisted of short term (4 hours) exposure of cells to the test article with and without S9 metabolic activation while the second consisted of long-term exposure (24 hours) without metabolic activation only. The test as a whole was considered to be valid as all conditions of validity were met: concurrent negative/vehicle controls were consistent with laboratory historical controls, concurrent positive controls induced a statistically significant increase in micronuclei, cell proliferation as measured by CBPI was ≥ 70% (≤ 30% cytostasis); and an adequate number of cells (≥ 500 cells per treatment) and concentrations (≥ 3) were analyzed. As the numbers of micronucleated cells exposed to brazzein were within or below the historical controls of the negative control and did not show a biologically relevant increase compared to the concurrent negative control and the $\chi 2$ test for trend did not show a concentration dependent statistically significant increase in micronuclei, brazzein was determined to not be clastogenic or aneugenic under the conditions of the test.

A series of *in vivo* single (OECD 423) and repeat dose (OECD 408) studies were conducted. For the single dose (acute) study, the maximum suggested dose under the guideline (2,000 mg/kg bw) was administered by gavage, followed by 14 days of observation (Anthem Study no. 21117) indicated that there were no signs of toxicity due to administration of the test article. Therefore, the LD_{50} of brazzein was determined to be >5,000 mg/kg bw.

A 14-day range finding study was conducted using four groups of adult Sprague-Dawley rats (5/sex/group) were administered 2000, 1000, 500, and 0 mg/kg bw/day brazzein (Anthem Study No. 21137). The animals were observed at least once daily for viability, signs of gross toxicity, and behavioral changes, and weekly for a battery of detailed observations. The study reported that there were no treatment related effects of brazzein at up to doses of 2,000 mg/kg body weight/day, including effects on mortality, clinical signs, body weight, body weight gain, clinical pathology, or food consumption. No treatment related abnormalities were noted upon macroscopic examination at necropsy. The NOAEL was determined to be the maximum dose of 2,000 mg/kg bw/day. Based on the results of the 14-day study, a dose of 1,000 mg/kg body weight/day was chosen as the high dose for the 90-day dietary toxicity study as this dose did not indicate any toxicity in the 90-day study and a dose of 1,000 mg/kg bw/day was expected to be sufficient to support the safety of brazzein given the expected human exposures detailed in Section 3 above.

For the 90-day study, Sprague-Dawley rats [CD(SD)IGS] were administered brazzein by gavage at 0, 250, 500, and 1,000 mg/kg bw/day (Anthem Study No. G22104). 28-day recovery groups were included for the control (0) and maximum dose (1,000) groups. All the animals were observed once a day for clinical signs, twice a day for mortality/morbidity and once a week

for detailed clinical examination. Cage rotation, body weight and feed consumption were performed at weekly intervals. Functional observation battery of tests/neurological examination was conducted during the acclimatization period (prior to first dosing), 4th, 8th & 13th week of the study for all main study groups and 17th week for recovery groups. Ophthalmological examination was performed before initiation of treatment for all the animals and vehicle control and high dose groups during 13th week of the study and 17th week of the study for recovery groups. Vaginal cytology was performed on day 90 (main) and day 118 (recovery). Urine samples were collected on day 89 from overnight fasted animals of main study groups and on day 114 (males), 113 (females) for recovery groups. At the end of experimental period (day 91 for main study groups and day 119 for recovery groups), blood, serum and harvested plasma specimens were analyzed for hematology, hormonal parameters, coagulation, and clinical chemistry parameters respectively. Subsequently, the animals were euthanized and subjected to gross pathological examination. Organs were collected and preserved in suitable fixative for histopathological evaluation. No mortalities occurred during the study and there were no reports of clinical signs related to brazzein treatment. Brazzein treatment also had no adverse effect on FOB measurements, body weight, body weight gain, feed consumption, or feed efficiency. There were no indications of an adverse effect of treatment on the results of the hematological, clinical chemistry, or urinalysis parameters measured. No biologically significant effects of brazzein treatment were reported on either absolute or relative organ weights and histopathological examinations revealed no differences between treatment and control groups. Given the data reported, the authors concluded that a no observed adverse effect level (NOAEL) for the study could be considered to be the maximum dose tested, 1,000 mg/kg bw/day.

(c) Interaction With Sweet Taste Receptors

Brazzein, along with traditional caloric and non-caloric sweeteners, acts by binding sweet taste receptor proteins. These tase receptors are typically described as G-protein coupled receptors (GCPRs) which consist of heterodimers of T1R2 and T1R3 (Lee and Owyang 2017). Brazzein in particular is thought to interact primarily with the T1R2 subunit (Walters and Hellekant 2008) though brazzein variants with amino acid mutations have been identified which interact preferentially with T1R3 (Kim *et al.* 2022).

While these receptors are primarily expressed on the tongue, they are known to also be expressed in the intestinal tract as well as in β-cells in the pancreas (Kojima and Nakagawa 2011). Recent evidence linking use of low-calorie alternative sweeteners have linked increases in insulinemia and insulin resistance to effects on these distal sweet taste receptors and changes in gut microbiome induced by artificial sweeteners (Kreuch *et al.* 2018; Lei *et al.* 2022). However, these effects are primarily reported to occur with small molecule artificial sweeteners such as saccharin and aspartame rather than naturally occurring low calorie sweeteners (steviol glycosides) and other sweet proteins such as thaumatin and monellin (Lee and Owyand 2017).

It is important to note, however, that these effects were not seen in the above described repeat dose studies. Lynch *et al.* 2023 did not report any effects on weight, weight gain, or blood chemistry parameters, and these results are further substantiated by the unpublished studies described above. Finally, Kim *et al.* 2020 describes a repeat dose study that was specially designed to monitor these parameters (food intake, insulin resistance, etc.) and indicates that

brazzein does not induce the same types of changes in sugar metabolism and insulin resistance as seen with other artificial sweeteners (Pepino *et al.* 2013; Greenwood *et al.* 2014; Mathur *et al.* 2020). Given the lack of evidence for increases in insulinemia and insulin resistance seen in multiple published and unpublished repeat dose oral toxicity studies, it is reasonable to conclude that brazzein is GRAS for its intended purpose as described herein.

4. Allergenicity

Perfect Day's brazzein from fermentation does not contain any protein from the 9 major food allergens as specified by the Federal Food, Drug and Cosmetic Act. Beyond the allergenicity report described in Lynch *et al.* 2023, Perfect Day commissioned a report from the Food Allergy Research and Resource Program (FARRP) which assessed the allergenic potential of brazzein as well as residual host cell proteins identified in the finished product. This report utilized well researched and accepted allergenicity databases and determined there was no concern with regard to allergenicity related to Perfect Day's brazzein.

Results of the bioinformatics search of the full-length 54 amino acid sequence of the protein in www.AllergenOnline.org by full-length FASTA version 35 identified only low identity matches of 30.4% to rice allergens with only a 46 amino acid alignment and 26% identity to a peanut defensin protein Ara h 12. Note that the product of Perfect Day has the first amino acid removed and is 53 amino acids, which does not impact this study. These results are not significant. A sliding 80 AA search did not identify any relevant match. Additional information from the product intended for food use includes a liquid chromatography-tandem mass spectrometry (LC-MS/MS) evaluation of trypsin digested peptides analyzed by the Protein Core Facility at the University of Nebraska. Individual peptides were identified by Dr. Phil Johnson using Peaks Software. The full-length sequences from UniProt of the host organism, *Komagataella pastoris* for the identified proteins were searched in the www.AllergenOnline.org database version 21 and results are described in this report. In addition, BLASTP searches of the full-length protein sequences for identified *K. pastoris* proteins were sequenced against the NCBI Protein database in March 2023 for relative identity matches and comparison for potential risks of allergy. Ten separate host cell proteins were identified, of which 3 host cell proteins returned a greater than 35% match using a sliding 80 amino acid search. One, a *K. pastoris* superoxide dismutase returned a match to olive tree pollen (55-60% match over 80 amino acids, 53-57% identity to full length protein). However, given the very low abundance of the host cell protein (~0.23% total protein) it is unlikely to represent a risk for olive pollen allergic individuals. Two other host cell proteins were identified which returned results for allergens typically associated with various fungi, however given that both proteins are low abundance (~0.1% of total protein each), these residual host cell proteins are unlikely to induce allergic reactions in consumers exposed to Perfect Day's brazzein. The conclusions of this report were that taking into consideration common identity matches to other species, and the abundance of the LC-MS-MS proteins in the final product there is no reason to suspect a risk of food allergy from the notified substance.

The results of both the FASTA comparisons to AllergenOnline and matches to the general Protein database by BLASTP are attached in the full report (**Appendix 1**).

5. Summary of Basis for GRAS Determination

Perfect Day, Inc. has determined that brazzein produced from fermentation by *K. phaffii* is GRAS for the intended use in food based on the following:

- The fact that brazzein is manufactured under cGMP for food (21 CFR Part 117, Subpart B), meets hazard analysis and risk-based preventive control (HARPC) requirements (21 CFR Part 117, Subpart C), and meets appropriate food grade specifications;
- That potential contaminants, such as heavy metals and pathogenic microbes, are either absent (not detected) or below toxicological and regulatory limits;
- The intended uses and the estimated consumption of brazzein;
- The long history of safe use of the production organism, *Komagataella phaffii* (formerly *Pichia pastoris*) in the industrial scale production of proteins used broadly in a number of foods, and data supporting the organism's non-pathogenic and non-toxigenic nature;
- Lack of allergenic proteins; and
- Specific toxicological studies undertaken using brazzein from fermentation.

Part 7. List of supporting data and information

1. Anthem Study no. G21140-Ames

2. Anthem Study no. G22002-CA

3. Anthem Study no. G22003-MN

4. Anthem Study no. 21117-Acute

5. Anthem Study no. 21137-14 day

6. Anthem Study no. 22104-90 day

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From: Pelonis, Evangelia C.
To: Kampmeyer, Christopher
Subject: FW: [EXTERNAL] FW: Questions for GRN 001167
Date: Friday, June 28, 2024 11:06:01 AM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png
2024-04-11 GRN 001167 Questions.pdf
Attachment 1 - HPLC Method Validation Report.pdf
Appendix 2-Insert Copy Number report.pdf
Attachment 3-Micro Analysis.pdf
Perfect Day Responses to GRN 1167 Questions (6-28-24).pdf

Dear Chris,

Please find attached our response to the additional questions from FDA regarding GRN 1167 along with related attachments.
Please let us know if FDA needs anything else or if FDA has any other questions.

Best,
Eve

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From: Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov>
Sent: Thursday, April 11, 2024 11:59 AM
To: Pelonis, Evangelia C. <pelonis@khlaw.com>
Subject: Questions for GRN 001167

Dear Ms. Pelonis,

We noted some questions during our review of GRAS Notice No. 001167—please see the attachment in this email.

We respectfully request a response within 10 business days. If you are unable to complete the response within that timeframe, please contact me to discuss further options. Thank you in advance for your attention to our comments.

Best regards,
Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
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June 28, 2024

Via Electronic Mail

Christopher Kampmeyer
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: Responses to the Agency's Questions Regarding Perfect Day's GRN 1167

Dear Dr. Kampmeyer:

On behalf of Perfect Day, this letter responds to questions posed in FDA's April 11, 2024 correspondence, regarding GRAS Notice (GRN) 001167 for brazzein produced by *Komagataella phaffii* expressing a gene encoding for brazzein from *Pentadiplandra brazzeana* (brazzein). For ease of reference, we reproduce each question below, followed by the relevant response.

Chemistry Questions

1. **The specifications provided for the notified substance (page 10) include a minimum of 90% of protein as brazzein and the results of three batch analyses (page 11) that demonstrate the brazzein content to be 95.4 to 96.2% of the total protein. Please discuss the identity of the remaining protein composition. For example, on page 23 the notifier discusses the allergenicity of *Komagataella phaffii* proteins and discusses several residual proteins that may each represent approximately 0.1 to 0.23% of the total protein.**

   Response: Peptides identified by LC-MS/MS from three production lots of Brazzein in the recombinant *K. phaffii* host were identified and arranged based on highest relative abundance. The amino acid sequences were gathered from the UniProt database and compare to the www.AllergenOnline.org database by a sliding 80mer window. Sequences with less than 0.1% relative match were not counted and are not included in the table. There were no matches from the 47 different 8 contiguous amino acid sequences. Three of the peptides had matches to AllergenOnline that were greater than 35% identity over 80 Amino acids. The most abundant protein was Brazzein, with no matches to allergens. The 97 kDa protein only had one peptide and therefore was not counted, but it also did not match anything in AOL. The 15 kDa protein did not match any known allergen. The 33 kDa protein was less abundant, had only one peptide identified as was discounted. The 10 kDa

protein had 11 peptides identified and was identified as a superoxide dismutase. There were 23 matches to the Ole e 5 protein of Olive pollen. With very low abundance it is 70 kDa and is unlikely to represent a risk for olive pollen allergic subjects.

The 120 kDa protein represented 0.18 percent in abundance. It did not have any matches to any AOL protein. The 4 kDa protein is a formate dehydrogenase at 0.11relative abundance. It did not match any AOL protein. The 5 kDa protein matched 13 proteins in AOL, primarily to fungal allergens of low potency and to mite, mosquito with high identities, but it represents 0.1% relative abundance and had very low identity matches to three fish collagens. The 3 kDa alcohol oxidase protein had one match over 35% identity to a dermal fungus, *Malassezio Mala* s 12, and represented only 0.1% relative abundance. Finally, the 8 kDa protein with 0.1% relative abundance did not match any known allergen.

All identified proteins are listed in Appendix 1-Allergenicity Report (pp. 9-13), which was included with Perfect Day's original GRAS Notice submission.

2. **The notifier states (page 8) that the raw materials used in the manufacture of the notified substance are safe, suitable, and conform to specifications in the Food Chemical Codex, 11t^{h} edition, 2018 or other applicable regulatory standards. Please provide a confirming statement that all raw materials and processing aids used in the manufacturing process are used in accordance with appropriate U.S. regulations, are GRAS for their intended use, or are the subject of an effective food contact notification.**

   Response: We confirm that all raw materials and processing aids used in the manufacturing process for Perfect Day's brazzein are used in accordance with appropriate U.S. regulation and are GRAS for their intended use.

3. **The use of antifoam agents and pH adjustors are noted on page 8; however, it is not clear where in the manufacturing process these are utilized. Please describe where in the manufacturing process the antifoam agents and pH adjustors are used.**

   Response: The antifoam agent is added to the starting medium and applied as needed during the process to maintain the functional effect. The pH adjustors are added during fed-batch fermentation.

4. **The manufacturing flow chart (Figure 1, page 9) includes a "resin-based purification" step that is not included in the description provided on page 8. Please confirm that this step is intended to be included in the manufacturing process description and describe the nature of this step (e.g., ion exchange or adsorption resin chromatography).**

   Response: Yes, this "resin-based purification" step is part of the manufacturing process description and should have been included in the narrative on page 8. A cation exchange resin is employed as a selective purification tool in the present purification process to produce high-quality protein. This process is essential to most protein purification techniques as it enables the isolation of a specific protein from a multitude of other

proteins present in the broth. Proteins are sorted using ion exchange chromatography based on their charge and isoelectric point.

5. **Please indicate whether specifications provided in Table 2 (page 10) that are presented as a percentage are on a dry weight/weight basis.**

   Response: All specifications in Table 2 are provided on a dry weight basis.

6. **Please confirm that all analytical methods used (e.g., chemical and microbiological), including the internal high performance liquid chromatography (HPLC) method for determination of protein as brazzein, listed in Table 2, page 10, are validated for their intended purpose.**

   Response: We confirm that all analytical methods used are validated for their intended purpose. With the exception of the internally developed HPLC method for determination of % brazzein as % protein, all methods are standard and validated methods for the analyte in the food matrix of interest here. The validation documentation for the HPLC method is provided in **Attachment 1** – HPLC Method Validation Report.

7. **We note that the specification limits for lead, arsenic, cadmium, and mercury are listed as ≤1.0 mg/kg, ≤2.0 mg/kg, ≤1.0 mg/kg, and ≤1.0 mg/kg, respectively. The results from the analyses of three batches of the notified substance demonstrated that lead and cadmium were consistently <0.005 mg/kg, mercury was detected in all batches at 0.013 to 0.014 mg/kg, and for arsenic two batches were determined to be <0.01 mg/kg and "lot 3" was determined to contain 0.553 mg/kg arsenic. Please discuss the potential reason for the elevated level of arsenic in "lot 3" and provide the current limits of quantitation for the method(s) used in the analyses for heavy metals. We note that specifications help to ensure that the ingredient is being manufactured in accordance with good manufacturing practices, and we would like to remind the notifier of FDA's recent "Closer to Zero" initiative that focuses on reducing dietary exposure to lead, arsenic, cadmium, and mercury from food. Further, we request that specifications for heavy metals be as low as possible and be reflective of the results obtained from the batch analyses. Please consider lowering the specifications for these heavy metals.**

   Response: Perfect Day has agreed to lower the heavy metal specifications as follows: lead from ≤1.0 mg/kg to ≤ 0.1 mg/kg, arsenic from ≤2.0 mg/kg to ≤0.6 mg/kg, cadmium from ≤1.0 mg/kg to ≤0.1 mg/kg, and mercury from ≤1.0 mg/kg to ≤0.1 mg/kg. It is likely that the "lot 3" outlier for arsenic is part of normal variation. Perfect Day is confident that they can meet the new lower specification for arsenic.

Microbiology Questions

1. **On page 7, the notifier states "*K. phaffii* was initially known as *Pichia pastoris* and was reassigned as the sole member of genus *Komagataella* in 1995 (Yamada *et al*., 1995)." The corresponding citation describing this reclassification of the species (listed below) was published in 2005. For the administrative record, please provide a statement that corrects the date listed in the notice.**

Kurtzman, C. (2005). Description of *Komagataella phaffii* sp. nov. and the transfer of *Pichia pseudopastoris* to the methylotrophic yeast genus *Komagataella*. International Journal of Systematic and Evolutionary Microbiology, 55, 973-976. doi: 10.1099/ijs.0.63491-0

Response: We note that Yamada *et al.,* 1995 was the first publication which proposed the establishment of a new genus (*Komagataella*) and was the first publication to suggest assigning *Pichia pastoris* into this new genus. Kurtzman 2005 notes that the Yamada paper did not review an adequate amount of sequence data relating to different methanol-reducing yeast strains, and therefore rejected the findings of Yamada. Both publications, despite potential drawbacks to the earlier analysis conducted in Yamada, serve to describe the reasons behind the reassignment of *Pichia pastoris*.

The type strain *Pichia pastoris*, now part of the genus *Komagataella* (Yamada *et al.,* 1995), was isolated in 1919 from a chestnut tree in France and described by A. Guillermond in 1920. The type strain was given the accession number NRRL Y-1603 for the US-based stock center and CBS704 for a European stock center. Later versions of *Pichia pastoris* were isolated by H. Phaff from trees in California (Phaff *et al.*, 1956). NRRL Y-1603 was used, along with other strains, by Phillips Petroleum to develop improved versions that were deposited back into the US stock center. One of these new strains, NRRLY-11430 (CBS7435), was the base strain for the development of *Komagataella phaffii* into a protein production platform (Cregg et al., 1985). Recent phylogenetic work, using molecular information such as 26S RNA sequence information (C. Kurtzman, 2005), established new species designations within the genus *Komagataella*. *K. phaffii* was shown to be descended from the strain isolated by Phaff in the US (C. Kurtzman, 2009). The NRRL Y-11430 strain was used by the company BioGrammatics (Carlsbad, CA, USA) to develop strain BG08 that was further modified to create BG10 through the loss of endogenous plasmids. This work by BioGrammatics is described, along with the genome sequence for BG10, in a recent publication (Sturmberger, *et al.,* 2016).

2. **Please provide the strain name for the *K. phaffii* production strain. Further, please state whether the production strain has been deposited in a recognized culture collection and provide the deposit designation. If it has not been deposited, please discuss how the production strain was taxonomically identified and verified.**

Response: The host strain NRRL Y-11430 was used by the company BioGrammatics (Carlsbad, CA, USA) to develop strain BG08 that was further modified to create BG10 through the loss of endogenous plasmids. This work by BioGrammatics is described, along with the genome sequence for BG10, in a recent publication (Sturmberger, *et al*. 2016). BG10 strain was further modified to BG12 by deleting the ORF of HIS4 gene making it a histidine auxotroph. The production strain has not been deposited in a recognized culture collection, but as the host strain was deposited and obtained from NRRL, there is no question as to the taxonomic identity of the strain.

3. **Please state whether the genome of the *K. phaffii* production strain has been sequenced. If the genome has been sequenced, please discuss whether the full genomic**

sequences are publicly available and provide the corresponding NCBI accession number.

Response: The host strain has been sequenced and can be found under accession number GCA_900235035.2. The modified production strain has not been sequenced fully.

4. **Please provide a detailed description of the *K. phaffii* production strain including genotypic (e.g., pathogenicity and toxigenicity) and phenotypic characteristics (e.g., production of antimicrobials, production of secondary metabolites), and whether this poses a safety concern. We note that the notifier provides broad statements regarding the species but does not characterize the production strain in any detail.**

Response: Extensive evaluations regarding the safety of the production strain have been undertaken and described in GRN 1167 and here in the response to questions from FDA. The European Food Safety Authority (EFSA) has determined that *K. phaffii* is appropriate for its Qualified Presumption of Safety (QPS) list (BIOHAZ 2018) due to its lack of pathogenicity and toxicity. There are no reports of mycotoxin or other secondary metabolite production in *K. phaffii*, and in fact is used as a production strain to produce an enzyme (fumonisin esterase) which is used to reduce the presence of harmful secondary metabolite in animal feed (FEEDAP 2018; FEEDAP 2020). The lack of reports of toxicity or pathogenicity, along with published whole genome sequence indicate that *K. phaffii* is not known to produce toxic secondary metabolites such as mycotoxins, is not known to be pathogenic, does not produce any antimicrobials of import, and is overall a safe and suitable production organism for food and feed ingredients.

5. **Please describe the construction of the *K. phaffii* production strain in more detail, including the following:**

   a. **Please clarify if the notifier has added any other genes on the cassette described on pages 7-8; if so, please briefly describe. For example, on page 7, the notifier states, "... the host strain was genetically modified with one or more expression cassettes"; however, on page 17, the notifier states "... the host strain was modified with an expression cassette";**

Response: The host was transformed with a single expression cassette which may have multiple copies integrated into the insertion site. This is the source of the statement "one or more expression cassettes". Subsequently, Perfect Day has confirmed that multiple copies of the expression cassette have been inserted into the genome in a single location. The copy number estimation report is provided in **Attachment 2** – Insert Copy Number Report and indicates a stable copy number of 11 for the insertion cassette.

We note that by using Droplet Digital PCR, the sample is partitioned into 20,000 nL sized droplets. This partitioning enables the measurement of thousands of independent amplification events within a single sample. Following PCR, each droplet is analysed or read to determine the fraction of PCR-positive droplets in the original sample. The data is then analysed using Poisson statistics to determine the target DNA template concentration in the original sample. As digital

droplet PCR is very sensitive and can even detect very low levels of target DNA, the NTC (which refers to no template control) showing 44 droplets positive for brazzein is a negligible count and cannot be considered as the preferred range for such estimation should be ≥ 1,500 counts. This is commonly observed in NTC sample and could be due to aerosol contamination of sample. Hence, this can be neglected as there is a huge error bar for no template control compared to other samples. Strain 34 which is the clone used for GRAS application is analysed in duplicates for which the data is represented in C04 & D04 which shows 16851 & 18311 positive droplets for brazzein.

b. Please state whether the donor gene(s) are *de novo* synthesized;

Response: Yes, the gene was *de novo* synthesized.

c. Please state the function of the His4 (histidinol dehydrogenase) gene that is used in the production strain cassette;

Response: The HIS4 gene catalyses the terminal step in the biosynthesis of histidine in the final strain. HIS4 auxotroph grows poorly in the minimal medium. Complementation of the HIS4 gene into the production strain helps in restoring the normal growth in the minimal media as well as helps in selection of transformants after integrating the expression cassette making it a GRAS strain.

d. Please state how the integration of the insert was confirmed. Further, on page 8, the notifier states, "The expression cassette is stably inserted into the host strain genome, as evidenced by multi-generational studies showing consistent levels of brazzein production," but does not describe this statement in greater detail. Please provide a narrative on the stability of the production strain (including stability of the production of brazzein);

Response: The stability of the insertion cassette is tested by gene copy number evaluation at the glycerol stock stage and the end of fermentation stage. Copy number (11 copies) of the strain were found to be stable until the end of fermentation. Apart from this, several batches were tested and the titer from this strain was consistent. **Attachment 2** contains data regarding copy number and the assay.

e. Please provide a description of how the notifier confirms the expression construct was transformed into the *K. phaffii* production strain.

Response: The host cell BG12 is an auxotrophic strain with a non-functional His4 gene. His- strains cannot be cultivated in minimal media as they are incapable of producing His amino acid for survival. Using this concept, after electroporation with the plasmid, transformants are plated onto YNB agar media (Yeast Nitrogen Base), which is a minimal media and the transformants in which the plasmid has been integrated will only grow in this media. These clones will be further confirmed by PCR and Brazzein gene copy number estimation.

6. **For the administrative record, please briefly specify how the purity of the *K. phaffii* production strain is ensured.**

   Response: The purity of the *K. phaffii* production strain is ensured by following internal SOPs involving regular culturing and monitoring of strains, periodic contamination checks of glycerol stocks prior to culture initiation, maintaining appropriate storage condition and following proper culture conditions such as temperature, pH, and nutrient requirements while growing. To maintain and assess purity, subculturing an aliquot of production strain in Potato Dextrose agar and Tryptic Soy Agar to detect any contamination by other organisms is carried out on a routine basis.

7. **In Table 2, the notifier lists specifications for microorganisms, including coliforms, but does not provide specifications for other common, notable foodborne pathogen analyses, such as *Salmonella* serovars (page 10). Further, on page 24, the notifier states, "... potential contaminants, such as heavy metals and pathogenic microbes, are either absent (not detected) or below toxicological and regulatory limits." We note that the notifier does not provide any specifications for pathogenic microorganisms. For the administrative record, please clarify if further analysis is performed to identify the genera or species of any presumptive positive result from the analysis of coliforms. If further analysis is not performed, please describe why analysis for coliforms is sufficient (and other methods employed during the manufacturing process to control for the presence of microorganisms).**

   Response: We are adding specifications for *Salmonella*, *Listeria*, and *E. coli*. Our specification and methods of analysis are provided below. We are also attaching additional microbiology testing on three batches in **Attachments 3** – Micro Analysis.

Analysis	Limit	Method of Analysis	Batch 1	Batch 2	Batch 3
E. coli	< 10 CFU/g	AOAC 991.14	<10 cfu/g	<10 cfu/g	<10 cfu/g
Salmonella spp.	Not detected in 10 g	USP <62>	Not detected in 10g	Not detected in 10g	Not detected in 10g
Listeria	Not detected in 10 g	FDA BAM Ch. 10.	Not detected in 10g	Not detected in 10g	Not detected in 10g

8. **For the administrative record, please discuss the differences in the results of the total plate count analyses in Lot 1 from Lots 2 and 3.**

   Response: Batch-to-batch variations in microbial load in finished products are a common occurrence in food products. However, microbial load should not exceed the set limit. Variations could be due to environmental factors including temperature, humidity, and

equipment's conditions and sampling tools that are used to collect the sample for testing and analysis.

The microbial limit for Lot 1 was observed to be 3,000 CFU/g, which is well within the plate count limit set and also observed that results of other microbial analysis which include, yeast mold, and coliform were well within the set limit.

9. **Please state whether any of the raw materials used in the fermentation are major allergens or derived from major allergens. If any of the raw materials used are major allergens or derived from major allergens, please discuss why these materials do not pose a safety concern.**

Response: None of the raw materials used in fermentation during production are themselves major allergens (tree nuts, peanuts, wheat, soy, milk, egg, shellfish, fish, and sesame), nor are they derived from a major food allergen.

10. **In Table 5, the notifier lists four GRAS notices, where the subject of the notice was a substance produced by a strain of *K. phaffii*, that have been submitted to FDA and have received "no questions" letters (page 17). We evaluated GRNs 001056 and 001104 and responded in letters respectively dated February 15, 2023, and October 17, 2023, stating that we had no questions at the time regarding the notifiers' GRAS conclusions. For the administrative record, please briefly discuss GRNs 001056 and 001104 in the context of the notifier's safety conclusion.**

Response: GRN 1167 contains a discussion of previous uses of the production organism (*K. phaffii* or *P. pastoris*) which have been previously reviewed by FDA and subsequently received no questions letters. The safety of the production organism in each case was reviewed, particularly with respect to Pariza and Johnson (Pariza and Johnson 2001), which is commonly used to assess the safety and appropriateness of microorganisms used in the production of food ingredients. Below, we assess Perfect Day's brazzein from fermentation using the Pariza "decision tree" method.

1. Is the production strain genetically modified?
   Yes. If yes, go to 2.

2. Is the production strain modified using rDNA techniques?
   Yes. If yes, go to 3.

3. Issues relating to the introduced DNA are addressed in 3a-3e.
   3a. Do the expressed product(s) which are encoded by the introduced DNA have a history of safe use in food?
   Yes. If yes, go to 3c.

   3c. Is the test article free of transferable antibiotic resistance gene DNA?
   Yes. If yes, go to 3e.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

Yes. If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?

Yes. If yes, go to 5.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?

Yes. If yes, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?

Yes.

If yes, the test article is ACCEPTED.

The results of the Pariza decision tree indicate that *K. phaffii* is a safe and suitable production organism for the production of brazzein for its intended uses. Additionally, the updated literature search (described in more detail in the response to Question #11) identified three additional safety evaluations of proteins produced in the same *K. phaffii* organism as Perfect Day's brazzein.

Freeman *et al.* (2024) describes a series of toxicological studies, including genotoxicity and 90 day repeat dose studies, of a different sweet protein (monellin from the Serendipity berry) produced using modified *K. phaffii* which, as with the published and unpublished repeat dose studies described in GRN 1167, indicate no adverse effects in rodents dosed up to the maximum dose in the study of 1,954 mg/kg bw/day. Peterson *et al.* 2024 describes a 14-day range finding study in rats using human lactoferrin produced using *K. phaffii*. This study again found that the protein produced using *K. phaffii* fermentation is safe up to the maximum dose tested of 2,000 mg/kg bw/day. Veselovsky *et al.* 2024 describes the administration of brazzein and monellin, each of which were produced using modified *P. pastoris*, to rodents with no adverse events reported at any dose, including effects on the gut microbiome of rats.

These new studies serve to further emphasize the safety of the production strain described in GRN 1167, and combined with the previous studies and the Pariza decision tree leave no doubt as to the suitability and safety of *K. phaffii* as a production organism.

11. Please provide an updated literature search including the date (month and year) the literature search was performed and discuss the safety of *K. phaffii*.

Response: An updated literature search was conducted in May 2024 to determine if additional relevant published safety information had become available subsequent to the submission of GRN 1167.

Databases searched: PubMed; Google Scholar: ToxNet; PubChem; SciFinder

Search terms used (alone or in combination): brazzein; *Pentadiplandra brazzeana*; sweet; protein; *Pichia pastoris*: *Komagataella phaffii*; toxicology; safety

Four relevant studies were identified from this search which have been published since the submission of GRN 1167. These studies are summarized below:

Veselovshy *et al.* 2024

Veselovshy 2024 describes a rodent study in which brazzein or monellin is administered to rats by gavage for 23 weeks at doses up to 21.4 mg/kg bw/day for brazzein. The purpose of the study was to monitor changes to the gut microbiome. Other than clinical observations, no additional toxicological parameters (blood chemistry, pathology, body weight, etc) were measured. No adverse effects were reported.

Hong *et al.* 2024

Hong 2024 describes the administration of brazzein to high fat diet induced female mice and the effects of brazzein on the mice and their offspring. 40 female C57BL/6 mice and divided into two groups: group 1 (30 animals) were started on a high fat diet, while group 2 (10 animals) received a control diet for 6 weeks. After 6 weeks, animals receiving the high fat diet were further divided into groups which received water containing 10% sucrose, brazzein (equivalent in sweetness to 10% sucrose), or control (pure water) and returned to a control diet. After an additional 6 weeks of receiving water *ad libitum* with sucrose or brazzein, animals were mated and followed through 21 days after birth of pups. Administration of the test solutions continued and through lactation and to the end of the study. Blood chemistry and liver histopathology indicated that there were no adverse events in any test group related to administration of brazzein.

Freeman *et al.* 2024

Freeman 2024 describes a series of toxicology studies in rats involving administration of a different sweet protein (monellin from Serendipity berry) produced in *K. phaffii*. While the test article is a different sweet protein, Freeman 2024 does describe a series of genotoxicity and oral repeat dose toxicology studies which indicate no adverse effects up to the maximum dose tested of 1,954 mg/kg bw/day in a 90 day study. While the test article consists primarily of a different sweet protein than is the subject of GRN 1167, it does serve to underscore the fact that *K. phaffii* is a safe and suitable production organism for brazzein and other food ingredients.

Peterson *et al.* 2024

Peterson 2024 describes a 14-day range finding study in rats which were administered human lactoferrin produced using *K. phaffii*. As with Freeman 2024, the test article is a different protein than the subject of GRN 1167, however as the production organism in Peterson 2024 is the same as utilized in GRN 1167, this is again a relevant safety publication. The test article was administered by gavage up to 2,000 mg/kg bw/day. Clinical observations, gross pathology, blood chemistry, and hematology parameters were evaluated, and the authors report no adverse effects at any dose.

The newly identified publications serve to further show that brazzein produced by *K. phaffii* is GRAS for its intended uses as described in GRN 1167.

Toxicology Questions

1. **Please clarify if the intended use of brazzein is substitutional for uses of brazzein preparation as specified in GRN 001142.**

   Response: Brazzein is an intensely sweet protein, with a potency several hundred (3-500x) that of sucrose. As such, any uses of brazzein which overlap between GRN 1142 and GRN 1167 would be substitutional and the exposure to brazzein in the diet would not increase from the uses described herein.

2. **The notice describes the details of an unpublished acute toxicity study conducted with the subject material up to a dose of 2,000 mg/kg body weight (bw). However, it notes that the LD50 of brazzein was determined to be >5,000 mg/kg bw. Please indicate how this is possible when the highest dose tested was 2,000 mg/kg bw.**

   Response: The correct LD_{50} for this study should be >2,000 mg/kg bw rather than >5,000. The confusion arose from language in the study report which was referring to the fact that the cutoff for Globally Harmonized System (GHS) for classification of acute hazards which includes a "non-categorized" Category 5 for compounds which show an acute LD_{50} between 2,000 and 5,000 mg/kg bw.

3. **Please describe if the sweetening effect of brazzein is transient, similar to polysaccharide-based sweeteners, or if the effects on taste receptors are sustained for a significant duration. Please note that such activity has been reported in sensory studies of other protein-based sweeteners, such as miraculin. Additionally, if the effect on sweet taste perception is sustained, please clarify whether any adverse effect on taste perception could result due to prolonged binding of brazzein with sweet taste receptors.**

   Response: There are no reports in the published literature of brazzein having similar long-lasting effects to that of miraculin. Miraculin is a glycoprotein while brazzein has no sugar moiety, and while both are known to bind the same sweet taste receptors (T1R2 and T1R3), miraculin is believed to be an antagonist for these receptors while brazzein binds as an

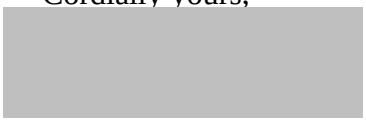
agonist. All indications are that brazzein's effects are transient, as are saccharide sweeteners and other low-calorie sweeteners such as steviol glycosides.

4. **The stability of brazzein raises some questions that need to be addressed. In the literature, there are reports that resistance of proteins to gastric digestion and thermal stability may be correlative indicators for potential allergenic risk (e.g., PMID: 9631091, 30134536, FAO/WHO (2001)). However, other recent publications conclude that protein stability is relevant for allergenicity of some proteins, but not for all (e.g., PMIDs: 31063834, 33473251, 21906650). Please provide a scientific narrative that addresses why the stability of brazzein (digestion and thermal) is not a cause for an allergenic safety concern when consumed orally.**

   Response: The resistance of proteins to digestion and the resulting potential effects on allergenicity have been debated in the scientific community and amongst scientific regulators. Both stable and unstable (i.e., digested) proteins may elicit allergenic reactions, and no validated methods exist for predicting *de novo* sensitization and/or production of specific IgE to the protein of interest. The allergenic potential of Perfect Day's brazzein produced via fermentation using modified *K. phaffii* was assessed (including residual production organism proteins) in the body of GRN 1167 and the full report from FARRP, which was attached to GRN 1167. This *in silico* approach has achieved consensus amongst the scientific community as the "best practice" for assessing risk of allergenicity of novel proteins and to predict potential cross-reactivity of novel proteins to known allergens. No matches to known allergens were identified in searches of publicly available databases when compared to the sequence of brazzein or of the identified residual host proteins. Therefore, based on the weight of evidence, while brazzein may be resistant to digestion, it is unlikely that this protein would pose any allergenic concern. Both published and unpublished 90 day repeat dose studies in rodents are described in GRN 1167 and these studies indicate brazzein is safe and have established NOAELs which were the maximum dose tested in each study. Based on the available information, the stability of brazzein would not be indicative of a safety concern for the intended uses.

We appreciate the Agency's continued review of this GRAS Notice. Please let us know if you have any other questions or if you need any additional information.

Cordially yours,



Evangelia C. Pelonis

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Attachment 1 Pages 1-29 were redacted in their entirety per FOIA exemption (b)(4)

Appendix 2 Pages 1-6 were redacted in their entirety per FOIA exemption (b)(4)

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Report Number: 4489874-0
Report Date: 11-May-2024
Report Status: Final

Certificate of Analysis

Perfect Day, Inc.

740 Heinz Ave
Berkley CA 94710 United States

Sample Name:	**SM22PDM04-002**	**Eurofins Sample:**	**14048253**
Project ID	PERFECT_DA-20240429-0008	**Receipt Date**	02-May-2024
PO Number	Product Development	**Receipt Condition**	Ambient temperature
Sample Serving Size	35 g	**Login Date**	29-Apr-2024
Description	white crystaline hygroscopic powder	**Date Started**	02-May-2024
		Sampled	Sample results apply as received
		Online Order	901-2024-E030210

Analysis	Result
ZME8J - Listeria monocytogenes [Abs Pres]/10g FDA BAM Ch. 10 (mod.)	
Listeria monocytogenes	Not Detected /10 g
Escherichia coli Count (Petrifilm)	
Escherichia Coli	<10 CFU/g
Salmonella USP	
Salmonella	Absent /10 g

Method References	Testing Location
Escherichia coli Count (Petrifilm) (ECPET)	**EML New Berlin** 2345 S 170th St New Berlin, WI 53151 USA
AOAC 991.14	
Salmonella USP (USPS2022)	**Eurofins Micro Lab - Madison** 6304 Ronald Reagan Ave Madison, WI 53704 USA
USP Current revision, Chapter 2022. To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix. **Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.	
ZME8J - Listeria monocytogenes [Abs Pres]/10g FDA BAM Ch. 10 (mod.) (ICO_EML_MA)	**Eurofins Micro Lab - Madison** 6304 Ronald Reagan Ave Madison, WI 53704 USA

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	**Edward Ladwig - President Eurofins Food Chemistr**

Eurofins Food Chemistry Testing Madison, Inc.
6304 Ronald Reagan Ave
Madison WI 53704
800-675-8375

Certificate of Analysis

Perfect Day, Inc.
740 Heinz Ave
Berkley CA 94710 United States

eurofins | Food Integrity & Innovation

Report Number:	**4489873-0**
Report Date:	**11-May-2024**
Report Status:	**Final**

Certificate of Analysis

Perfect Day, Inc.

740 Heinz Ave
Berkley CA 94710 United States

Sample Name:	**SM22PDM04-003**	**Eurofins Sample:**	**14048251**
Project ID	PERFECT_DA-20240429-0008	**Receipt Date**	02-May-2024
PO Number	Product Development	**Receipt Condition**	Ambient temperature
Sample Serving Size	35 g	**Login Date**	29-Apr-2024
Description	white crystaline hygroscopic powder	**Date Started**	02-May-2024
		Sampled	Sample results apply as received
		Online Order	901-2024-E030210

Analysis	**Result**
ZME8J - Listeria monocytogenes [Abs Pres]/10g FDA BAM Ch. 10 (mod.)	
Listeria monocytogenes	Not Detected /10 g
Escherichia coli Count (Petrifilm)	
Escherichia Coli	<10 CFU/g
Salmonella USP	
Salmonella	Absent /10 g

Method References	**Testing Location**
Escherichia coli Count (Petrifilm) (ECPET)	**EML New Berlin** 2345 S 170th St New Berlin, WI 53151 USA
AOAC 991.14	
Salmonella USP (USPS2022)	**Eurofins Micro Lab - Madison** 6304 Ronald Reagan Ave Madison, WI 53704 USA
USP Current revision, Chapter 2022. To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix. **Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.	
ZME8J - Listeria monocytogenes [Abs Pres]/10g FDA BAM Ch. 10 (mod.) (ICO_EML_MA)	**Eurofins Micro Lab - Madison** 6304 Ronald Reagan Ave Madison, WI 53704 USA

Certificate of Analysis

Perfect Day, Inc.

740 Heinz Ave
Berkley CA 94710 United States

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	**Edward Ladwig - President Eurofins Food Chemistr**

Eurofins Food Chemistry Testing Madison, Inc.
6304 Ronald Reagan Ave
Madison WI 53704
800-675-8375

eurofins | Food Integrity & Innovation

Report Number: 4495022-0
Report Date: 15-May-2024
Report Status: Final

Certificate of Analysis

Perfect Day, Inc.

740 Heinz Ave
Berkley CA 94710 United States

Sample Name:	**SM22PDM04-004**	**Eurofins Sample:**	**14048249**
Project ID	PERFECT_DA-20240429-0008	**Receipt Date**	02-May-2024
PO Number	Product Development	**Receipt Condition**	Ambient temperature
Sample Serving Size	40 g	**Login Date**	29-Apr-2024
Description	white crystaline hygroscopic powder	**Date Started**	02-May-2024
		Sampled	Sample results apply as received
		Online Order	901-2024-E030210

Analysis	Result
ZME8J - Listeria monocytogenes [Abs Pres]/10g FDA BAM Ch. 10 (mod.)	
Listeria monocytogenes	Not Detected /10 g
Escherichia coli Count (Petrifilm)	
Escherichia Coli	<10 CFU/g
Salmonella USP	
Salmonella	Absent /10 g

Method References	Testing Location
Escherichia coli Count (Petrifilm) (ECPET)	**EML New Berlin** 2345 S 170th St New Berlin, WI 53151 USA
AOAC 991.14	
Salmonella USP (USPS2022)	**Eurofins Micro Lab - Madison** 6304 Ronald Reagan Ave Madison, WI 53704 USA
USP Current revision, Chapter 2022. To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix. **Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.	
ZME8J - Listeria monocytogenes [Abs Pres]/10g FDA BAM Ch. 10 (mod.) (ICO_EML_MA)	**Eurofins Micro Lab - Madison** 6304 Ronald Reagan Ave Madison, WI 53704 USA

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	**Edward Ladwig - President Eurofins Food Chemistr**

Eurofins Food Chemistry Testing Madison, Inc.
6304 Ronald Reagan Ave
Madison WI 53704
800-675-8375

Certificate of Analysis

Perfect Day, Inc.
740 Heinz Ave
Berkley CA 94710 United States

From: Pelonis, Evangelia C. <pelonis@khlaw.com>
Sent: Thursday, August 22, 2024 6:33 PM
To: Kampmeyer, Christopher
Subject: FW: [EXTERNAL] FW: GRN 001167

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Chris,

The parent strain and production strain are both BG12.

Please let us know if you need anything else.

Best,
Eve

Evangelia C. Pelonis
Partner

direct 202.434.4106 pelonis@khlaw.com
Keller and Heckman LLP | 1001 G Street NW, Suite 500 West | Washington, DC 20001

khlaw.com
dailyintakeblog.com

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From: Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov>
Sent: Wednesday, August 21, 2024 1:00 PM
To: Pelonis, Evangelia C. <pelonis@khlaw.com>
Subject: RE: [EXTERNAL] FW: GRN 001167

Dear Eve,

I am writing with a follow up question to the below email amendment from July 19, 2024.

1. In the June 28, 2024, amendment, the notifier describes the construction of the **parent strain**, *K. phaffii* strain "BG12". In the July 19, 2024, amendment, the notifier confirms the identity of the **production strain** as *K. phaffii* strain "BG12". For the administrative record please confirm whether the strain names of the **parent strain** and **production strain** are both "BG12". If the strain names are different, please provide the strain name of the **production strain** used to produce brazzein. Our understanding is that *K. phaffii* strain "BG12" is the parent or host strain from which the production strain is derived.

Thank you,
Chris

Chris Kampmeyer, M.S.
Regulatory Review Scientist

Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
christopher.kampmeyer@fda.hhs.gov

From: Pelonis, Evangelia C. <pelonis@khlaw.com>
Sent: Friday, July 19, 2024 10:11 AM
To: Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov>
Subject: [EXTERNAL] FW: GRN 001167

Dear Chris,

Thanks for your email.
For number 1, we confirm that the production strain used to produce brazzein is BG12.
For number 2, Listeria refers to *Listeria monocytogenes.*

Please let us know if you need anything else.

Best,
Eve

Evangelia C. Pelonis
Partner

direct 202.434.4106 pelonis@khlaw.com
Keller and Heckman LLP | 1001 G Street NW, Suite 500 West | Washington, DC 20001

khlaw.com
dailyintakeblog.com

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From: Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov>
Sent: Thursday, July 18, 2024 3:38 PM

To: Pelonis, Evangelia C. <pelonis@khlaw.com>
Subject: GRN 001167

** EXTERNAL EMAIL **

Dear Eve,

After reviewing the June 28, 2024 amendment, we noted a couple follow-up questions. Could you please provide responses to the following?

1. In the June 28, 2024, amendment, when describing the production strain, the firm states, "The host strain NRRL Y-11430 was used by the company BioGrammatics (Carlsbad, CA, USA) to develop strain BG08 that was further modified to create BG10 through the loss of endogenous plasmids … BG10 strain was further modified to BG12 by deleting the ORF of HIS4 gene making it a histidine auxotroph"; however, based on the firm's response to microbiology question five, *K. phaffii* strain "BG12" is further modified to produce brazzein, as described in the GRAS notice and in the June 28, 2024, amendment. As such, our understanding is that *K. phaffii* strain "BG12" is the parent or host strain from which the production strain is derived. Therefore, please provide the strain name of the production strain used to produce brazzein.

2. Please specify whether *Listeria* refers to *Listeria* spp. or *Listeria monocytogenes*.

Best regards,
Chris

Chris Kampmeyer, M.S.
Regulatory Review Scientist

Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
christopher.kampmeyer@fda.hhs.gov