Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Road
Woodbridge, VA 22192-5755
703 590 7337 (Fax 703 580 8637)
smedley@cfr-services.com

consultants to the regulated industry

February 3, 2020

#910

Dr. Susan Carlson
Director, Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Dear Dr. Carlson:

SUBJECT: Transmittal of the NOMAD BIOSCIENCE GmbH –
GRAS Notice for THAUMATIN II
For use as Sweetener in Foods and Beverages

Enclosed you will find the GRAS notice for THAUMATIN II as produced by *Nicotiana benthamiana* as a food and beverage sweetener as submitted by NOMAD BIOSCIENCE GmbH.

I have provided a CD of the GRAS notice and all the cited references.

Should you have any questions on this filing, please contact me, at your convenience.

Sincerely,

[redacted]

Kristi O. Smedley, Ph.D.
Consultant to NOMAD BIOSCIENCE GmbH

Attachments

FDA Form 3667 (Hard Copy and CD-Copy)
THAUMATIN II GRN NARRATIVE of Notice (Hard Copy and CD-Copy)
Appendices (as appended to Narrative) (CD-copy)
Full Complement of References (CD-copy)

cc: Yuri Gleba, Nomad

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000910	Feb 4, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Yuri Gleba, Ph.D.	Chief Executive Officer

Company *(if applicable)*: Nomad Bioscience GmbH

Mailing Address *(number and street)*: Biozentrum Halle, Weinbergweg 22

City	State or Province	Zip Code/Postal Code	Country
Halle/Saale	Saxony-Anhalt	D-06120	Germany

Telephone Number	Fax Number	E-Mail Address
49 345 1314 2606	49 345 1314 2601	gleba@nomadbioscience.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position
Kristi O. Smedley, Ph.D.	Sponsor's US Regulatory Representative

Company *(if applicable)*: Center for Regulatory Services, Inc.

Mailing Address *(number and street)*: 5200 Wolf Run Shoals Rd.

City	State or Province	Zip Code/Postal Code	Country
Woodbridge	Virginia	22192	United States of America

Telephone Number	Fax Number	E-Mail Address
703-590-7337	703-580-8637	smedley@cfr-services.com

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

THAUMATIN II

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media with paper signature page

☐ Paper

If applicable give number and type of physical media

Submission consists of one (1) CD containing electronic files of GRAS Notice

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 738

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRN 775

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, see attached Designation of Confidential Information

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

THAUMATIN II is a plant-made non-caloric sweetening product comprised of the protein thaumatin II, intended for application to foods or beverages by itself or blended with other sweeteners to achieve the desired flavoring effect. THAUMATIN II is produced by a new plant-based process, but the component protein is (a) identical in amino acid sequence to commercially available thaumatin II (a.k.a. thaumatin 2) extracted from the fruit of the Katemfe bush, or made recombinantly by fermentation; (b) a simple polypeptide with no glycosylation or other non-natural post-translational modifications; and (c) produced in high purity. Extracted and recombinant thaumatins are allowed for use in multiple foods and beverages at application rates up to 150 ppm (mg/kg or mg/L) in the USA (FEMA GRASa 3732 and FEMA GRASa 3814), and up to 400 ppm in the EU (E 957) and in several other countries in Asia, Australasia, the Middle East and South America. As a sweetener, thaumatins (E 957) are allowed without limitation (Quantum satis) because the proteins are considered non-toxic. The same application rates are envisioned for THAUMATIN II as those for commercially available products. THAUMATIN II is not intended for use in infant formulas.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used (*CAS, EC*)	Registry No.[2]	Biological Source *(if applicable)*	
1	Thaumatin II CAS no. and UniProt entry no.	53850 34 3	P02884	Plant, recombinant	
2	Note: Extracted "thaumatin" consisting of single proteins or mixtures is CAS No. 53850-34-3. Recombinant THM-B is CAS No. 553850-34-3.				
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

Detailed information regarding the identity, manufacturing process, functionality, uses and safety of the notified substance is incorporated in the Notice documents provided.

3. Synonyms

Provide as available or relevant:

1	Thaumatin-2
2	
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that NOMAD BIOSCIENCE GMBH *(name of notifier)*

has concluded that the intended use*(s)* of THAUMATIN II *(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☐ _____ *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

_____ *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

_____ *(address of notifier or other location)*

_____ *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☒ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ---------- *(GRAS Affirmation Petition No.)*

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Kristi Smedley Digitally signed by Kristi Smedley DN: cn=Kristi Smedley, o=Center for Regulatory Services, Inc., ou, email=smedley@cfr-services.com, c=US Date: 2020.02.03 08:08:52 -05'00'	Kristi O. Smedley, Ph.D.	02/03/2020

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	GRN for THAUMATIN II as a non-caloric sweetener for use in foods and beverages (PDF of Notification)	Submission
	THAUMATIN II GRN References - For FDA Internal Review Only (PDFs of All Cited References - Not for Republication)	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany
Tel. 49 345 1314 2606
Fax. 49 345 1314 2601

3 February 2020

Antonia Mattia, Ph.D.
Director, Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice for THAUMATIN II Sweetener from *Nicotiana benthamiana*

Dear Dr. Mattia,

Nomad Bioscience GmbH ("Nomad"; "Notifier") is submitting this GRAS Notice for its **THAUMATIN II sweetener from *Nicotiana benthamiana*.** Nomad has concluded, under FDA's Final Rule pertaining to 21 CFR 170 (August 17, 2016), that the naturally occurring protein Thaumatin II when produced recombinantly in the plant species *Nicotiana benthamiana* is Generally Recognized as Safe (GRAS) for use as a food and beverage sweetener. The product could be used singly and in blends with sugar or other sweetening agents.

This Notice follows Notifier's **GRN 738** for its THAUMATIN product consisting of thaumatin I and/or thaumatin II proteins produced in edible plant species (e.g., spinach, leafy beet and lettuce), for which Nomad received from CFSAN a "No Questions" letter on 18 April 2018. GRN 738 established THAUMATIN as a GRAS sweetener in the USA, whereas before then the GRAS status pertained to thaumatins used only as flavor modifiers, as specifically described in FEMA GRASa No. 3732 (Oser 1984) for natural (extracted) thaumatins, and in FEMA GRASa 3814 (Smith 1996) for recombinant versions of these proteins.

Differences between this Notice and our prior GRN 738 include: (1) modification of the manufacturing process to expand the list of production host plants to include *Nicotiana benthamiana*; (2) selection of Thaumatin II as the preferred sweetening active ingredient; and (3) adjustment of product application rates and dietary intake estimates due to projected replacement of up to 50% (w/w) of sugar in food and beverages with Thaumatin II. Data and justifications for these changes are included in this submission.

In this Notice we describe production of Thaumatin II in the non-food plant species *Nicotiana benthamiana* (*N. benthamiana*). This host enables higher expression of recombinant proteins including thaumatins, is more amenable to controlled indoor cultivation, and yields better process economics. This host has been used to produce human biopharmaceuticals that have been shown safe in multiple clinical studies under FDA INDs. The same species has also been accepted by FDA for producing GRAS food antimicrobials such as Nomad's COLICIN (GRN 775; 26 October 2018), ENDOLYSIN (GRN 802; 10 May 2019) and SALMOCIN (21 October 2019). We describe here a process using the same host to produce Thaumatin II.

Regardless of production host plant, the amino acid sequence of Nomad's Thaumatin II is identical to the sequence of the same proteins extracted and purified from the fruit of the Katemfe tree (*Thaumatococcus daniellii*); the original source of thaumatins and the main source of commercial thaumatin-containing products. In the USA, EU, Japan and elsewhere, isolates of the Katemfe fruit have been marketed commercially since the mid-1970s under various trade names (e.g. Talin®; San Sweet T-100®), as low-calorie sweeteners and flavor modifiers. Results of extensive preclinical safety studies and human clinical exposure allowed adoption by the EU of a working maximum consumption level of 1.1 mg thaumatin/kg body mass per day as a highly safe level for all subpopulations of consumers (EFSA 2015). Based on that body of work, and the physicochemical equivalency of *Thaumatococcus*-derived and Notifier's recombinantly produced thaumatins, Nomad claimed the same maximum safe level of 1.1 mg/kg-day in GRN 738.

Since submission of GRN 738, Nomad has conducted blinded sensory evaluation studies in-house and at two independent sensory evaluation organizations. These studies confirmed that the threshold of sweetness detection of Nomad's Thaumatin II is equivalent to that of native proteins, and further, that Nomad's Thaumatin II can replace up to 50 wt% of the sucrose in test solutions while retaining the same sensation of sweetness. Therefore, the projected application rates and estimates for average moderate and average maximum levels of intake provided in this Notice take this new information into account.

In the current Notice, Nomad documents the identity, manufacturing process, product quality, safety, dietary exposure and potential risks from consumption of its THAUMATIN II product containing Thaumatin II as the high-intensity sweetener, when produced in the plant host *N. benthamiana*. While describing the manufacturing process and organoleptic properties, this Notice frequently references GRN 738 and GRN 775 for brevity and to rely on FDA's original review memoranda for our prior GRAS notices.

Our submission complies with the 7-part format prescribed by FDA in its Final Rule for the GRAS Notice process (August 17, 2016), and includes a CD containing PDFs of the following documents:

1. FDA Form 3667 Nomad Bioscience GRN for THAUMATIN II sweetener from *Nicotiana benthamiana*
2. GRN for THAUMATIN II sweetener from *Nicotiana benthamiana* (Parts 1-7), which includes:
   APPENDIX A: Nomad's Safety Data Sheet for THAUMATIN II from *Nicotiana benthamiana*
   APPENDIX B: THAUMATIN II *Nicotiana benthamiana* Manufacturing Process
   APPENDIX C: THAUMATIN II Characterization
3. Copies of references cited in the GRN

If the Agency has any questions or requires additional information to aid their review of Nomad's findings and GRAS conclusion, please contact us at the address listed above. For convenience, you may also contact our regulatory and product development representatives in the USA, Dr. Kristi Smedley at Center for Regulatory Services Inc., Woodbridge, VA (Tel 703-590-7337; Email smedley@cfr-services.com), and Dr. Daniel Tusé at DT/Consulting Group, Sacramento, CA (Tel 707-290-9528; Email daniel@dt-cg.com).

Sincerely,

Yuri Gleba, Ph.D.
Chief Executive Officer
Nomad Bioscience GmbH

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1. General Introduction and Claim of Exemption from Premarket Approval Requirements

Nomad Bioscience GmbH ("Nomad"; Notifier) THAUMATIN II product containing Thaumatin II protein sweetener is produced using a plant-based manufacturing process to match the amino acid sequence of naturally occurring thaumatin II protein. Thaumatins (both extracted and recombinant) have been extensively studied scientifically for many decades pre and post commercialization, and a large public database of information exists regarding manufacturing methods, functionality, applications and safety. This extensive public record enables comparison of Nomad's new product to the currently available thaumatin-containing products. Results of a literature search and analysis of publicly available information on which Nomad based its conclusions of the safety of its THAUMATIN product were included in GRN 738 and are updated in this Notice.

Native thaumatins found in the arils of the fruit of *Thaumatococcus danielli* have been used for centuries in West Africa as sweeteners and flavor modifiers. There is no ethnobotanical record of ill effects associated with consumption of impure thaumatins. Products containing industrially produced thaumatins have enjoyed an excellent safety record since their first use in human foods in the mid-1970s with expanded approvals in the 1990s. Nomad's plant-made thaumatins, including those produced in edible species as described in GRN 738 and those produced in *N. benthamiana* as described in the present submission, are identical in amino acid composition, sequence and structure to the native thaumatins.

This Notice provides exposure estimates from consumption of foods that would be treated with Nomad's *N. benthamiana*-produced THAUMATIN II sweetener and includes a corresponding risk assessment. Under any use scenario, we project that THAUMATIN II can be used safely at the levels needed for functionality.

Notifier therefore concludes that under the conditions of use described herein, *N. benthamiana*-produced THAUMATIN II is generally recognized as safe and therefore should be exempt from premarket approval procedures under 21 CFR 170.36(a)(I). THAUMATIN II is not intended for use in infant formulas.

1.1. Submission of Notice

This Notice is submitted in compliance with Subpart E of FDA's Final Rule of the GRAS Notification process (August 17, 2016) 21 CFR 170.203-170.285.

1.2. Name and Address of Notifier

NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale, Germany
Office: 49 345 1314 2606
Fax: 49 345 1314 2601

Notifier's US Representative
Kristi O. Smedley, Ph.D.
Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192
Office: 703-590-7337; Mobile: 703-786-7674
Fax: 703-580-8637
eMail: smedley@cfr-services.com

Notifier's US Representative
Daniel Tusé, Ph.D.
DT/Consulting Group
2695 13th Street
Sacramento, CA 95818
Telephone: 707-290-9528
Fax: 916-822-4124
Email: daniel@dt-cg.com

1.3. Common or Usual Name of the Notified Substance

THAUMATIN II

1.4. Conditions of Use

What is **claimed as GRAS in this Notice** is:

> A THAUMATIN II composition consisting of recombinant, *Nicotiana benthamiana*-produced Thaumatin II protein meeting the target Specification, for application to food and beverages as a sweetener singly and/or in combination with other caloric and/or non/low caloric sweeteners.

Native "thaumatin" is comprised of several related proteins, but the major sweetening properties reside in two very similar proteins, thaumatin I and thaumatin II. Thaumatins are naturally occurring proteins that can modify taste perception contextually, depending on their concentration and the properties of the food matrix into/onto which the thaumatins are applied. Nomad's product can be comprised of thaumatin I and/or thaumatin II, as described in GRN 738.

However, new manufacturing process and sensory information generated by Nomad since submission of GRN 738 suggests that thaumatin II can satisfy production and functionality requirements as a single active ingredient; therefore, this Notice focuses on a low-calorie, high-intensity sweetener comprised only of thaumatin II. Hence, Notifier's product "THAUMATIN II" is formulated to contain recombinant, *N. benthamiana* plant-produced, purified **Thaumatin II** (capitalized in this Notice to differentiate it from the same protein obtained from other sources). Thaumatin II can be applied to foods up to the safety limits allowed for thaumatins from native sources. The sources of information used to document and support Thaumatin II's properties, functionality, safety attributes and our GRAS conclusion are listed in Table 7-1.

This Notice pertains to Thaumatin II's use as a sweetener. In independent sensory evaluations, the threshold of detected sweetness of Notifier's Thaumatin II was in the same range as published values for highly pure thaumatins from other sources. Further, Thaumatin II was able to replace up to 50 wt% of the sugar in test solutions consisting of different Thaumatin II/sucrose ratios. Therefore, we anticipate consumption of Thaumatin II at levels considerably lower, but in any event not higher, than those specified for the currently available thaumatins (i.e., safe consumption up to 1.1 mg/kg-day body weight basis for all age groups, EFSA E957 October 28, 2015).

Additional intake and safety information for subpopulations potentially consuming THAUMATIN II, including children, adults and the elderly, is provided in Section 3 (Dietary Exposure) of this Notice.

1.5. Statutory Basis for Notifier's GRAS Conclusion

The statutory basis of the GRAS status in through scientific procedures in accordance with 21 CFR 170.30(b): GRAS Conclusion.

In accordance with the information provided in this Notice, it is Nomad Bioscience's conclusion that *Nicotiana benthamiana*-produced THAUMATIN II is generally recognized as safe when used as a sweetener on foods and beverages at application rates that would collectively amount to not more than 1.1 mg/kg-day (body weight basis) additive exposure in a typical human diet.

1.6. Not Subject to Preclearance

Notifier has concluded that THAUMATIN II as manufactured via its plant-based process using the host *N. benthamiana* is generally recognized as safe, and as such the substance is not subject to pre-market approval requirements of the Federal Food Drug and Cosmetic Act.

1.7. Availability of Information for FDA Review

All data and information that serve as a basis for the GRAS conclusions are included in this Notice.

1.8. Public Disclosure

The information provided in this Notice is publicly available and not subject to exception under 170.225(c)(8). All information contained in this Notice can be shared without restriction.

1.9. Certification

On behalf of Nomad Bioscience GmbH (Notifier), I certify that to the best of my knowledge, this GRAS Notice is complete, representative, and balanced with respect to the information provided, favorable or unfavorable, known to me and pertinent to the evaluation of the safety and GRAS status of our *Nicotiana benthamiana*-produced THAUMATIN II sweetener product.

Yuri Gleba, Ph.D.
Chief Executive Officer
NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany

2. Identity, Method of Manufacture, Specification, Technical Effect

2.1. Identity, Structural and Functional Information

Identity

In GRN 738, Notifier claimed substitutable use of thaumatin I or thaumatin II and/or their mixtures to achieve the desired sweetening effect. Recent studies summarized in this submission show that Thaumatin II can be produced more efficiently than Thaumatin I and also to possess a superior flavor profile relative to Thaumatin I, including fewer off-flavor notes. While development studies characterized both proteins, Notifier's commercial product will likely contain Thaumatin II as the preferred high-intensity sweetener.

The history of thaumatin development and commercialization was included in GRN 738 (Section 2.1, pg 9; Section 6.1, pp 15-16) and is only summarized here for brevity. The product Talin® was commercialized beginning in the 1970s by Tate & Lyle (UK) with a claimed sweetness of 1,600-2,700 times that of a 7-10% solution of sucrose (van der Wel 1972). A similar, mixture was sold in Japan under the brand San Sweet T-100®. Thaumatins are currently available from several vendors, including Naturex, Beneo Palatinit, Natex, KF Specialty Ingredients and several others. The chemistry and applications of thaumatins have been extensively described in several original and review articles (e.g. Lee 1987; Kim 1988; Hough 1993; Shallenberger 1993; Witty 1998; Zemanek 1995; Suami 1997; Greenly 2003). Table 2-1 lists the sweetness ranking of thaumatins relative to sucrose (from Greenly 2003).

Thaumatins I and II have similar properties, amino acid composition, sweetness, molecular weight (both are ~22 kDa) and highly similar amino acid sequences, differing by only 5 amino acid residues. Each protein is a single polypeptide chain of 207 amino acids with 8 intramolecular disulphide linkages (Suami 1997). X-ray crystallography of thaumatin I revealed the features of the protein's backbone (de Vos 1985; Kim 1988; Ogata 1992). Circular dichroism studies (van der Wel 1984) showed few α-helices, but many β-pleated sheet strands and bends. It is thought that the constrained structure of thaumatins is responsible for inducing taste sensation. Either heat denaturation or cleavage of the disulphide bridges results in loss of sweetness, suggesting that the tertiary structure of the protein triggers taste modulation through a highly stereoselective process (Kaneko 2001; Hough 1993). A molecular diagram of thaumatin II determined from crystallography at 0.99-Å resolution is shown in Figure 2-1 (from Masuda 2014).

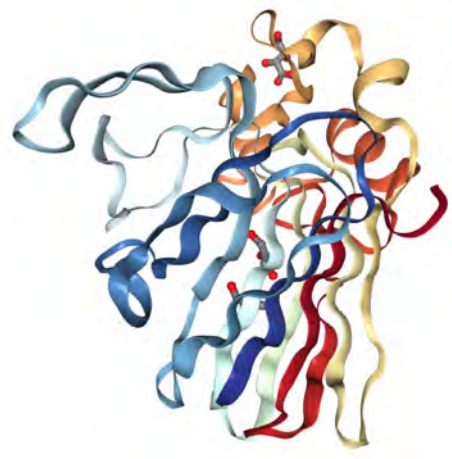

Figure 2-1 Structural model of thaumatin II

Table 2-1. Relative sweetness of natural and artificial sweeteners compared to sucrose

Sweetener	Sweetness/sucrose
Neotame	13,000
Thaumatin	3,000
Alitame	2,000
DHC	2,000
Sucralose	600
Saccharin	300
Stevia	300
Acesulfame-K	200
Aspartame	180
Glycyrrhizin	100
Cyclamate	30
HFCS	1.5
Honey	1.5
Sucrose	1.0
Tagatose	1.0
Xylitol	1.0
HSH	0.9
Maltitol	0.9
Erythritol	0.7
Isomalt	0.65
Mannitol	0.5
Trehalose	0.5
Sorbitol	0.6
Lactitol	0.4

Structural Information for Plant-Made Thaumatin II

The amino acid sequences of thaumatins I and II, including Notifier's plant-expressed thaumatins, were included in GRN 738 (Section 2.1, pg 10). The amino acid sequence of Thaumatin II is shown in Figure 2-2 (Faus 2008). Notifier's plant-expressed, mature Thaumatin II has an identical amino acid sequence to the corresponding protein from *Thaumatococcus* (APPENDIX C, Section C.2).

The sequence shown is for the mature protein; thaumatins including Thaumatin II are natively expressed as pre-proproteins containing N-terminal signal and C-terminal peptide additions. These precursors are processed *in planta* to yield the intermediate proprotein devoid of the N-terminal peptide but still containing the C-terminal addition. Subsequent processing *in planta* yields the nature-identical sequence of amino acids in the mature, isolated thaumatin. Thaumatin II does not contain glycosylation sites on its 207-aa backbone; hence, like the protein derived from *Thaumatococcus*, Notifier's Thaumatin II is a non-glycosylated polypeptide.

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ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTKGGKIWARTDCYFDD  60
SGRGICRTGDCGGLLQCKRFGRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMDFSPTTRG  120
CRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKRLCPDA  180
FSYVLDKPTTVTCPGSSNYRVTFCPTA                                    207
```

Figure 2-2. Amino acid sequence of mature Thaumatin II

A Safety Data Sheet (SDS) for THAUMATIN II (Thaumatin II produced in *N. benthamiana*) is found in APPENDIX A. The method applied to manufacture Notifier's Thaumatin II protein in the plant host *N. benthamiana* is described in APPENDIX B. Analytical methods used to define the physicochemical and functional properties of Notifier's recombinant Thaumatin II are summarized in APPENDIX C. Notifier's *Nicotiana*-expressed plant-made Thaumatin II conforms to its predicted composition and shares the amino acid sequence of the native *Thaumatococcus* protein.

Quantitative Composition

THAUMATIN II is prepared in bulk as a dry powder as described in APPENDIX B**.** The **Specification** for the bulk product is found in Table 2-2**.** Typically, THAUMATIN II is supplied as a solid of >95% purity. Thaumatin II is highly water soluble (>20% w/v). The bulk product is dissolved/diluted in water or other suitable food-compatible vehicle or directly mixed into foods or beverages to achieve the desired effect.

THAUMATIN can be used singly or in combination with other sweeteners and food additives to enhance the sweet flavor profile of foods and beverages. The amount of THAUMATIN II used is food- and application-specific and depends on the end result desired in each flavor formulation.

Mode of Action

The mode of action of thaumatins was reviewed in GRN 738 (Section 2.1, pg 11) and results of more recent studies are included in this submission. Thaumatins and other sweet-tasting proteins interact with taste receptors in the tongue to impart the neurophysiological sensation of sweetness. Sweet-tasting proteins as well as aspartame can be perceived by humans, apes, and Old World monkeys but not New World monkeys and rodents (Masuda 2013). Natural sweetness perception occurs when a sugar such as sucrose or other sweetener dissolves in saliva and binds to the heterodimeric T1R2–T1R3 receptors, which belong to the G-protein-coupled receptors (GPCRs) family (Li 2002; Margolskee 2001; Nelson 2001). These receptors have

multiple binding sites (Vigues 2008) that are activated upon interaction with compounds that elicit sweet taste. Each sweetener exhibits different binding properties on the same receptors (Fernstrom 2012), leading to the varying perceptions of sweetness for the different proteins.

In addition to their history of use by African, Western and Eastern cultures, thaumatins have been studied systematically for their flavor modifying properties *in vitro* and *ex vivo* as well as in animal studies *in vivo*. As defined in whole animal studies using various species and in isolated gustatory cells *in vitro*, thaumatins have a demonstrable effect on various groups of taste receptors and these interactions affect perception of taste (Bartoszewski 2003; Faus 2008; Glaser 2000; Greenly 2003; Hellekant 1996; Kaneko 2001; Kinghorn 1986; Lim 2012; Nagarajan 2017; Ohta 2011; Sardesai 1991; Schiffman 1995; Suami 1997; Tinti 2000; van der Wel 1972; Witty 1990). Thaumatins find multiple uses in food technology because these proteins can impart a sweet flavor, enhance desirable flavor notes, or mask or ameliorate unpleasant flavors in food components.

At the molecular level, the electrical charge distribution on the thaumatin molecules appears to mediate their interaction with the taste receptors. In addition to sweetness, thaumatins' effect in the presence of more complex flavor compounds suggests that flavor modification depends on the nature of the individual molecules present (Natex 2017). The strength of the interaction between the thaumatin molecules and the taste receptors may account for thaumatins' intensity and the duration of sweetness perception.

Natural thaumatin was originally found to consist of six closely related proteins (I, II, III, a, b and c), all with a MW of ~22 kDa (207 amino acids) (van der Wel 1972; Ledeboer 1984). Thaumatin I and II are robust because of their multiple disulfide bridges, but can lose their sweetness on heating at alkaline pH; the proteins are stable at neutral to acidic pH even at elevated temperatures (Lim 2012). The 3-D structure of thaumatin I (de Vos 1985; Ogata 1992) revealed that the protein consists of three domains: (a) an 11 strand, flattened β-sandwich (aa 1–53, 85–127 and 178–207, domain I); (b) a small disulfide-rich region (54–84, domain III); and (c) a large disulfide-rich region (128–177, domain II). Subsequently, the crystal structure of plant-extracted thaumatin II was determined at 1.27-Å resolution (Masuda 2011) and the structure of a recombinant version of the same molecule was determined at 0.99-Å resolution (Masuda 2014). These studies revealed very similar structures for thaumatin I and II and enabled subsequent in-depth structure-activity studies.

Modification of lysine residues in the structure of thaumatins was found to reduce their sweetness (Kaneko 2001). Phosphopyridoxylation of lysine residues Lys78, Lys97, Lys106, Lys137 and Lys187 markedly reduced sweetness. The intensity of sweetness was returned to that of native thaumatins by dephosphorylation of these phosphopyridoxylated lysine residues except Lys106. These lysine residues occurred in thaumatins, but not in non-sweet thaumatin-like proteins, suggesting that these lysine residues were required for sweetness.

The **sweetness threshold** of natural (plant extracted) thaumatins was reported by Masuda et. al. (2016; 2018) as **~50 nM** and sweeter versions have been generated by amino acid substitutions. Masuda (2016) found that a D21N mutation enhanced sweetness and reduced the sensory detection threshold from 50 nM to **31 nM**. Nomad determined a sweetness threshold of **37 nM** for its native-sequence, purified Thaumatin II.

2.2. Method of Manufacture

The THAUMATIN plant-based manufacturing process was described in APPENDIX B of GRN 738 and included expression and extraction of thaumatin I and/or II proteins in food species of plants. Plant hosts included *Spinacia oleracea* (spinach), *Beta vulgaris* (red beet) or *Lactuca sativa* (lettuce). These same hosts were found acceptable for the manufacture of antimicrobial proteins as food safety processing aids, as described in Notifier's GRN 593 and GRN 676. The safety features of these host species were detailed in GRN 593.

In the present submission, Notifier asserts that other plant hosts can be used to produce thaumatin proteins without impacting the safety or functionality of the final product. Such species include members of the genus *Nicotiana*, specifically the species ***N. benthamiana***. Reduction in the levels of *N. benthamiana*-associated impurities such as pyridine alkaloids that are not found in edible species hosts is achieved through the **process modification** described in APPENDIX B of this Notice. The modification enables inclusion of *N. benthamiana* to the list of candidate plant species for producing thaumatin proteins.

Nomad includes *N. benthamiana* as an additional host for its manufacturing process because the species is amenable to large-scale indoor cultivation and yields better process economics relative to its food-species counterparts. Nomad has described use of *N. benthamiana* in the manufacture of its food safety processing aids and has used scientific procedures to conclude GRAS status for the products COLICIN (GRN 775), ENDOLYSIN (GRN 802) and SALMOCIN (GRN 824). In the present Notice, Nomad asserts its GRAS conclusion for *N. benthamiana*-produced Thaumatin II.

Regardless of host used, the plant-derived biomass remaining after thaumatin protein extraction is treated and discarded (disposed) and is not used as a human food or animal feed product, additive or supplement.

2.3. Composition and Specification

Characteristic Properties

The characteristic properties of plant-made THAUMATIN II are summarized in the Specification. In recent studies Notifier has determined that the manufacture of Thaumatin II is more efficient than the manufacture of Thaumatin I. Additionally, Notifier has determined in sensory studies conducted in house and at independent sensory evaluation organizations, that Thaumatin II has superior flavor characteristics over Thaumatin I, including comparable sweetness with fewer/shorter-lasting off-flavor effects. Hence, a current target **Specification** for a product consisting of Thaumatin II as the preferred active ingredient is provided in Table 2-2.

As reported in GRN 738 (Section 6.2, pp 17 -21), Notifier studied at the molecular level the allergenic potential of plant-expressed thaumatins for use in food and determined, from published information, that Notifier's thaumatins should pose no higher allergenic risk than the *Thaumatococcus*-derived commercial thaumatins currently on the market (see current Notice's Section 6.1.1 Allergenic potential of THAUMATIN II).

Formulation

THAUMATIN II is provided in bulk as a dry powder, which the end-user can dissolve and/or dilute in water or other food-acceptable vehicle according to instructions and mixed in foods and beverages at levels sufficient to achieve the desired level of sweetness.

Content of Potential Human Toxicants in THAUMATIN II

Thaumatin proteins comprise an approved product and are non-toxic when used as intended. Process impurities derived from the manufacture of thaumatins in edible plants are also non-toxic as described in GRN 738. There are two potential human toxicants that may remain in low levels in the THAUMATIN II product when it is manufactured using the host plant *N. benthamiana*; namely, the alkaloids nicotine and anabasine. As discussed in GRN 775, the host plant *N. benthamiana* shares metabolic pathways with other members of the plant family Solanaceae, which includes tomato, pepper, potato, eggplant and others. All these plants contain low residual yet measurable levels of pyridine alkaloids.

The purpose of including a chromatographic purification step during downstream processing is to produce Thaumatin II with levels of residual alkaloids that are in the same range as the levels consumed in common vegetables in a typical diet.

In addition to residual alkaloids, some heavy metals may be present in low amounts in the final product. The residual levels of all impurities in the final product are low, and at those levels the impurities are not expected to pose a risk to any subpopulation of consumers when the product THAUMATIN II is used as specified.

Formulation excipients included in the final product are approved for food applications. There are no other known sources of potential human toxicants in THAUMATIN II.

Specification

The current Specification for THAUMATIN II is shown in Table 2-2. The process used to manufacture the product in *N. benthamiana* with this specification, including the current experimentally determined values used to justify this specification, are shown in APPENDIX B (THAUMATIN II Manufacturing Process).

Table 2-2. Specification of THAUMATIN II Product

THAUMATIN II (Thaumatin II) Bulk Product		
Parameter	**Specification limit**	**Method**
Appearance	Powder, yellowish to light tan	Visual
Minimum total thaumatin content (as percent of total protein)	≥ 95%	CGE (capillary gel electrophoresis)
Solubility (DI water)	≥ 200 mg/mL	Visual
pH of a 1% solution (excipient-dependent)	2.7 – 6.0	Potentiometric
Heavy metals (sum of Class 1 metals Pb, Cd, Hg, As)	≤ 5 ng/mg thaumatin	USP38<233>
Lead (Pb)	≤ 1 ng/mg thaumatin	USP38<233>
Host alkaloid: Nicotine	≤ 20 ng/mg thaumatin	HPLC/MS
Host alkaloid: Anabasine	≤ 5 ng/mg thaumatin	HPLC/MS
Bioburden	≤ 5,000 CFU total per g	USP32<61>
Agrobacterium (vector) per 10 g sample	0 (absent)	Selective plate-based assay
Undesirable microorganisms, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g sample	0 (absent)	USP32<1111>
Stability (dry powder; 0-20°C) as <3% loss of thaumatin protein	≥ 6 months	CGE; thaumatin peak in 0.1% (w/v) solution, T_m relative to T_0

2.4. Technical Effect and Suitability of Use

Notifier has determined that the intended use of THAUMATIN II does not raise safety concerns; as such, physical or technical data are not required. Nevertheless, technical effect information was developed by Notifier in its efforts to characterize its product, including residual impurities, and to assess Thaumatin II's sugar replacement potential. This information is therefore included in this Notice to enable additional estimates of application rates and dietary exposure (see Section 3 Dietary Exposure).

2.5. Overall Conclusion

The results of studies supporting this Notice show that Thaumatin II can be produced consistently using recombinant expression in the plant host *Nicotiana benthamiana*. The recombinant thaumatin can be readily purified from plant biomass and exhibits the characteristic properties of extracted thaumatin II. The molecular composition of Notifier's Thaumatin II is the same as that of their native counterpart. The gene sequence used in the expression vector encodes protein precursors that are correctly processed *in planta* to yield the same mature thaumatin protein that is isolated from *Thaumatococcus*, including identical amino acid sequence. Regardless of source, including the new host *N. benthamiana*, the mature Thaumatin II protein lacks glycosylation because the polypeptide backbone does not contain glycan addition sites.

The safety, physicochemical and taste-modifying properties of fermentation-derived recombinant thaumatins match those of native (extracted) thaumatins (Smith 1996) and both are available commercially. Ample evidence of safety of thaumatins exists in the published literature. Thaumatin's approval for use in food and beverages in the USA, EU countries, Japan, Australia, Israel and elsewhere underscore thaumatin's excellent record of safety obtained during more than 40-years of pre- and post-market surveillance.

In sum, the main difference between Notifier's *N. benthamiana*-produced Thaumatin II protein and commercially available extracted or fermentation-derived thaumatin II is the method of manufacture.

3. Dietary Exposure

3.1. Application rates of THAUMATIN II in various food categories

Average maximum application rates in all allowed uses

Thaumatin is listed in the Codex General Standard for Food Additives and permitted for general use in food (Codex Alimentarius 2016). In Europe, it is approved as a Sweetener or as a Flavour Enhancer (EFSA E 957) for use in a number of different categories. In the USA, natural (extracted) thaumatin has GRAS classification (FEMA GRASa No. 3732; Oser 1984) as a flavor modifier, as does a recombinant version of the proteins ("thaumatin B-recombinant"; FEMA GRASa 3814; Smith 1996).

Notifier's GRN 738 established plant-made thaumatin as a GRAS sweetener in the USA. In the EU and Japan, the acceptable daily intake (ADI) limit for thaumatin table-top sweeteners is "not specified" (*Quantum satis*) because the proteins are considered to be non-toxic. Based on their sweetening and flavor-modifying profile, thaumatins are applied to foods in the 1-400 ppm range, or 1-400 mg thaumatin/kg or /L food or beverage. When used at these maximum allowed safe levels and on the same foods, the use rates and dietary exposure of Notifier's product described in GRN 738 were expected to be the same as those for currently available thaumatin products. In the USA, FEMA's suggested application rates range from 1-150 ppm, because they exclude uses of thaumatin as a sweetener (Cohen 2016; Cohen 2018).

In GRN 738, Notifier used the more inclusive EU database for estimating the maximum safe dietary intake of thaumatins from THAUMATIN because it represented a higher potential intake scenario (1-400 ppm) by covering all food categories and use rates encompassed by EU statutes (EFSA 2015). Based on the use rates in those statutes, a safe maximum intake from a typical diet of thaumatin-treated products was defined as 1.1 mg/kg-day; for an adult person of 70 kg body mass, maximum safe intake would be 77 mg/person-day.

Average moderate application rate as a sweetener

Although the 1.1 mg/kg body mass/day upper limit of consumption of thaumatin in all uses provides a safety reference that is indicative of thaumatin's very low toxicity, it is unlikely that anyone would consume that amount, especially as a sweetener, as most uses of thaumatin are in flavor modification (Green 1999). Recently, Nomad conducted blinded sensory evaluation studies to determine the sweetening potency (intensity) of its Thaumatin II when mixed in various ratios with sucrose in highly pure water. A representative panel of results is shown in Figure 3-1. The perceived sweetness (intensity units relative to 10% sucrose w/v in water) is shown in the ordinate and the duration of the effect is shown in the abscissa (time in seconds).

Linear regression analyses of these data were then used to compare sweetness at various levels and to estimate the sugar replacement ratios. For example, it was calculated that 4.6 ppm Thaumatin II produced an equivalent sweetness sensation as 5% (w/v) sucrose, as it could replace 1/2 of the sugar in the positive control (10% sugar) with minimal sensory detection of the substitution by test subjects.

Stated another way, 4.6 mg Thaumatin II was found to be as sweet at 50 g of sucrose; meaning that at that concentration **Thaumatin II** is **>10,000-times** sweeter than sugar on a weight basis. Such results suggest that THAUMATIN II could be applied to some foods as a sweetener at very low rates, enabling potential replacement of up to one-half of the caloric sweetener typically added to food and beverages (Section 3.2).

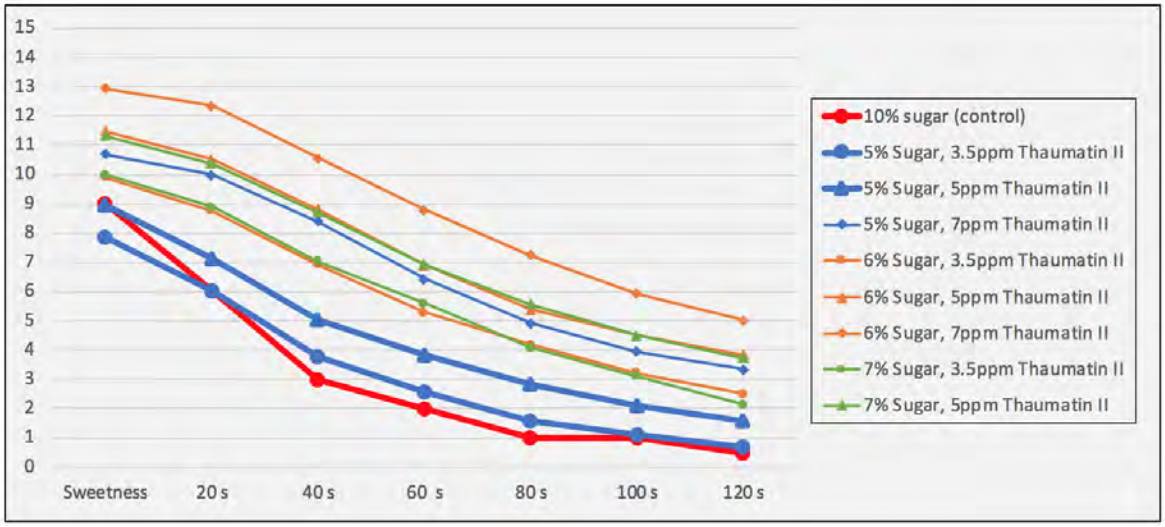

Figure 3-1. Thaumatin II sweetness and aftertaste

Sweetness intensity was evaluated by a panel of tasters in blinded fashion, after holding samples in their mouth for 5 seconds before expectorating. Sweet aftertaste was evaluated every 20 seconds for 2 minutes. 10% sucrose (w/v; sugar) was used as a control. Solutions with the same sugar content are grouped using the same color on the graph but using different patterns. Different levels of Thaumatin II are shown as different shape symbols on the graph markers.

Additional details of the methods used in these sensory evaluations and the results obtained are provided in APPENDIX C, Section C-7 Determination of Sweetness Intensity).

Application rates of THAUMATIN II sweetener to food and beverages

When used as a sweetener, the incorporation of *N. benthamiana*-produced THAUMATIN II into various foods is expected to include generally the same food categories and limits specified in the EU (EFSA 2015) and claimed in GRN 738, and as specifically defined in follow-up communications with the Agency, in particular **"Response to FDA's Dec 18, 2017 Request for Additional Information"** (January 11, 2018).

The original maximum safe application or use rates listed in GRN 738 are reaffirmed in this submission, and are summarized in Table 3-1.

Table 3-1. Estimated application rates of THAUMATIN II (Thaumatin II) sweetener

THAUMATIN II Use Category: Sweetener (*ppm thaumatin = mg/kg or mg/L food or beverage)			
Food Category	**Use level (ppm)* GRN 738**	**Use level (ppm)* This Notice**	**Comments**
Wine, beer and other fermented or distilled beverages	5	5	
Jams, jellies, marmalades, etc.	5	5	
Potato-based and similar snacks	10	10	
Breakfast cereals	10	10	
Chewing gum	10	10	If product contains other sweeteners
Chewing gum	50	50	If thaumatin is the only sweetener
Ice cream / other edible ices	50	50	
Cocoa and chocolate products	50	50	
Breath mints and similar	50	50	
Confectionary	50	50	
Decorations, coating and fillings	50	50	
Fine bakery items	30	30	
Food supplements (capsules, tablets, syrups and similar)	400	400	For use in food supplements only as a non-dietary ingredient
Table-top sweeteners	*Quantum satis* (10 ppm)	*Quantum satis* (10 ppm)	Includes liquid, powder and tablet formulations
Average usual level	**50 ppm**	**50 ppm**	Based on EFSA's safe use levels
Average maximum level	**400 ppm**	**400 ppm**	EFSA's *Quantum satis* is based on 10 mg/kg application rate.

3.2. Estimated dietary intake of THAUMATIN II in foods and beverages

Maximum estimated intake from all allowed uses

With respect to human dietary exposure, we expect no higher levels of consumption of Notifier's thaumatins per weight or volume of treated food as those allowed for extracted thaumatins. The European Food Safety Authority (EFSA) provides guidance on maximum permitted levels (MPL) of thaumatin on various foods and beverages according to Annex II to Regulation (EC) No 1333/2008 (OJEU 2011).

The EU's MPL for thaumatin food additive E 957 ranges from a low of 5 mg/kg in "flavored fermented milk products" (e.g. yogurt) to a high of 400 mg/kg in "food supplements supplied in a syrup-type or chewable form" (EFSA 2015). The same regulations allow the unlimited addition of thaumatin (*Quantum satis*[1]) when the protein is used as a table-top sweetener. It is envisioned that THAUMATIN II, regardless of production host, could be used at equivalent maximum safe application rates on various foods. Until GRN 738, thaumatins had not been considered a sweetener *per se* in the USA. As a flavor enhancer or modifier, FEMA cites use ranges from a low of 1 ppm for baked goods to a high of 150 ppm for chewing gum (Cohen 2016).

The application rates of thaumatins to various food products (maximum permissible level, MPL) were derived by assessing the consumption of each food type and estimating the additive intake to reach a maximum exposure of **1.1 mg thaumatin/kg body weight (bw) per day**, or **77 mg/day for a 70-kg person.** Consumption values were derived from typical European diets, taking into consideration country specific dietary preferences in Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden and the UK and various population age groups (**adult, infant, elderly**).

The original application rates allowed by EFSA (2008) were later amended (EFSA 2011) to include more food types and higher allowed thaumatin levels (OJEU 2011). Revision of statutes for expanded use was based on thaumatin's functionality and post-market safety record. Based on this safety record and on physicochemical equivalency, in GRN 738 Nomad claimed GRAS conclusion for its THAUMATIN product produced in edible plants when consumed at these same levels of intake. Additional detail is provided in Section 6.2.

Moderate estimated intake from use as a sweetener

In the USA, manufacturers are not required to provide information publicly regarding the quantity of low-calorie sweeteners added to food, with the exception of saccharin; hence, accurate estimations of the use and intake potential for THAUMATIN II are difficult. We therefore used an extreme model for estimating consumption of Thaumatin II in the **sweetener** category. We estimated current per capita intake of sugar (sucrose) as a reference and used our data on maximum sweetness replacement potential (Section C-7 Determination of Sweetness Intensity) to estimate intake if Thaumatin II were to **replace one-half of all the sugar**. As unlikely and extreme as this example might be (sugar and other caloric sweeteners have bulk and other rheological properties not replaceable by thaumatin or other high-intensity sweeteners), it helps illustrate an upper limit for THAUMATIN II's use and intake in the sweetener category.

Briefly, net consumption of sucrose per capita in the USA in 2018, adjusted for losses, was 18.3 kg (USDA ERS 2019b). Based on the **replacement potential** defined in Section 3.1, consumption of Thaumatin II would be **less than 1 g/person-year**, or **~2.5 mg/person-day**, which is <0.005% the amount of sucrose currently consumed. This estimated consumption rate is 1/30th (<3.25%) the safe daily maximum permitted level (MPL)

[1] With respect to food additives, the term "*Quantum satis*" indicates that no maximum level is specified. However, additives must be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided that they do not mislead the consumer. The EFSA *Quantum satis* designation was based on 10 mg/kg application rate.

allowed for all uses of thaumatin by an adult person (i.e. 77 mg/person-day). Although Thaumatin II's sweetening potency was not assessed in sensory evaluations against corn syrups (e.g., high-fructose corn syrup; HFCS), in 2018 the US per capita consumption of HFCS was ~10 kg, or about 1/2 of the refined crystalline sucrose consumed (USDA ERS 2019a). To complete the estimate, we added 50% to the projected daily sugar replacement value, for an estimated total intake of **3.75 mg/person-day** (2.5 mg replacing sucrose + 1.25 mg replacing corn syrup), which is <5% of the total safety based MPL for all uses of thaumatin. Although replacement of this level of caloric sweeteners is highly unlikely, even with an equally unlikely 100% market capture by Notifier's THAUMATIN II product, our "worst-case" model enables estimations of safety.

3.3. Additional exposure to thaumatins (intake not related to THAUMATIN product)

On the basis of the estimates presented in Section 3.2 for THAUMATIN II used as a sweetener, 3.75 mg thaumatin/person-day is expected to be the maximum intake contribution of Notifier's product in an extreme scenario where the product displaces one-half of caloric sweeteners but none of the existing thaumatins. Notifier does not have market segmentation information for thaumatin's various commercial uses, but no/low caloric high-intensity sweeteners (HIS) collectively represent a small fraction of all sweeteners, as reviewed by Sylvetsky et al. (2017). Assuming that 1/3 of thaumatin's current maximum safe use level is for food and beverage sweetening applications, the maximum safe daily per capita intake would be ~25 mg (1/3 of 77 mg). Hence, the additive consumer intake for thaumatin sweeteners (3.75 mg from THAUMATIN II plus 25 mg from existing sources) would be 28.75 mg.

A more likely scenario is one where Notifier's product displaces some of the current thaumatins and some of the current caloric sweeteners, although an accurate mix for market perturbation is difficult to predict. The commercial introduction of Notifier's THAUMATIN II product, irrespective of the manufacturing host used, is expected to displace, initially, thaumatin that is currently derived from extraction of the same proteins from *Thaumatococcus*. Should industrial thaumatin usage expand due to higher or more consistent availability of lower-cost THAUMATIN II, consumption of thaumatin would be expected to increase. Even if daily per capita intake were to double from 25 mg/day to 50 mg/day for sweetener uses, or from 77 mg/day to 150-200 mg/day for all uses, thaumatin's safety factor of 1,300 based on chronic toxicity studies suggest that any additional safety risk to consumers would be minimal (Hagiwara 2005; OJEU 2011; EFSA 2015). Additional perspective on the safety risks of such intake from Notifier's THAUMATIN II product and from all sources of thaumatin is provided in Section 6.2 (Safety in Relation to Dietary Intake of THAUMATIN II).

3.4. Dietary Exposure to Host- and Process-Derived Impurities

Although the safety of thaumatin proteins has been documented (Notifier's equivalency data and referenced studies in GRN 738, plus this Notice), the use of non-food plant species as manufacturing hosts introduces the potential for new risks not found in the original list of production plant varieties. A description of risk factors and their mitigation is found in Section 6 of this Notice.

On a protein basis, the THAUMATIN II product consisting of Thaumatin II produced in *N. benthamiana* has a purity of >95% (typically ~98%); hence the Specification was set at $\geq$95%. The residual host-derived constituents of chromatographically purified thaumatins are mainly proteinaceous, and there are no unusual proteins in the host that would introduce risk, especially at such low levels (Leffingwell 1999).

Assuming a worst-case scenario where the purity of the product is only 95% (i.e. the Specification minimum), at the thaumatin adult MPL of 77 mg/day, **proteinaceous host-derived impurities** would amount to <4 mg/day (i.e. 5% of 77 = 3.85). With a US food consumption average of 0.56 kg/day, the **maximum exposure to host-derived proteins would be ≤7 ppm/day** (3.85 mg protein impurity/0.56 kg-day food intake), which is

an insignificant level and not expected to impact either safety or nutritional content. Extending this calculation to the **moderate exposure** from Thaumatin II used in foods only as a sweetener (i.e., 3.75 mg/person-day), a 95%-pure product would expose consumers to **<0.335 ppm of host-derived proteins** (i.e. 0.187 mg protein impurity/0.56 kg/day food).

Some elemental impurities derived from plant growth media could be co-purified with thaumatin proteins (see Section 6.1.4 Process impurities in *N. benthamiana*-based THAUMATIN II manufacture). The Specification limit set for **total Class 1 heavy metal content** (sum of Pb, Cd, Hg, As) in *N. benthamiana*-purified Thaumatin II of 5 ng/mg would expose consumers to **<20 nanogram/person-day** of these metals in sweetening uses (3.75 mg Thaumatin II/day) and to **<400 ng/person-day** at the thaumatin MPL. Both levels are well below the **55 micrograms/person-day** combined oral permitted daily exposure **(PDE)** for Class 1 elements (ICH 2015).

The remaining inorganics include salts derived from the buffer (phosphate, NaCl) that are safe and allowed for food use. Hence, we assess that non-alkaloidal host- and process-derived impurities in *N. benthamiana*-produced THAUMATIN II would pose little to no risk to consumers.

The main organic impurities of concern are the alkaloids **nicotine** and **anabasine**, given their presence in *N. benthamiana* and their toxicity profiles. The other alkaloids potentially present, namely nornicotine and anatabine, have similar activities but are present at very low levels in the plant and pose no significant risk. Hence, our focus has been on assessing risk from the two main alkaloids present. The multi-batch average values for nicotine and anabasine (nanograms; ng) per milligram (mg) of Thaumatin II protein (i.e., ppm) were determined as described in APPENDIX C Section C-5. The results were used to support Specification limits of 20 ng/mg and 5 ng/mg for nicotine and anabasine, respectively, with a combined limit for total alkaloid established at 25 ng/mg protein. To estimate a **maximum exposure** level at the thaumatin adult MPL of 77 mg/person-day, the limit value (25 ng total alkaloid/mg protein) was multiplied by 77. Similarly, the **moderate exposure** from Thaumatin II sweetener was determined by multiplying the Specification limit value by 3.75 mg/person-day. The per capita daily exposure maxima at both intake levels are shown in Table 3-2.

Table 3-2. Estimated exposure to host alkaloids from Thaumatin II produced in *N. benthamiana*

Thaumatin consumption and estimated alkaloid exposure	Nicotine		Anabasine		Total Alkaloid
	Specification (ng/mg)	Daily exposure (ng/day)	Specification (ng/mg)	Daily exposure (ng/day)	Specification (25 ng/mg)
Thaumatin MPL (77 mg/d)	20	1,540	5	385	1,925
Sweetener exposure (3.75 mg/d)	20	75	5	18.75	93.75

Host alkaloid exposure potential was estimated from the maximum permitted level (MPL) from all uses of thaumatins (77 mg/adult person-day) as well as from consumption of Thaumatin II sweetener (3.75 mg/person-day). The Specification limits for nicotine (20 ng/mg) and anabasine (5 ng/mg) were multiplied by 77 mg (MPL) or by 3.75 mg Thaumatin II sweetener consumed per person-day, to arrive at the daily exposure estimates, in nanograms (ng) per person-day.

At the limit values the total alkaloid exposures are estimated to be <2 μg/person-day and <100 ng/person-day at the Thaumatin II MPL and sweetener use levels, respectively. These are comparable or lower exposures than from other sources of pyridine alkaloid-containing foods, consumer products and the environment. For comparison, several studies have reported the average per capita daily food-borne exposure to nicotine from consumptions of common vegetables as ~1,000 ng (900-1,300 ng)/person-day (Andersson 2003; Davis 1991; Domino 1993; Liu 2013; Moldoveanu 2016; Nielsen 2013; Siegmund 1999). Given Thaumatin II's maximum

alkaloid Specification limit of 25 ng/mg protein, and assuming full systemic distribution of alkaloids upon ingestion, a 70-kg person would experience <0.03 ppb level of alkaloids (Table 3-2, last column, ~2 μg total alkaloid/70 kg body mass). Moderate consumption of 3.75 mg/day of Thaumatin II as a sweetener by a 70 kg person would lead to 0.001 ppb concentration of total alkaloid.

For perspective, the amount of nicotine absorbed in second-hand (passive) smoke is 100 micrograms (μg)/day for those reporting exposure, and 20 μg/day for those not reporting exposure, due to the pervasive nature of environmental nicotine (Karačonji 2005). Even in a low-use state like California, 1/2 of consumers are exposed to second-hand cigarette smoke, and 1/3 are exposed to second-hand nicotine and related alkaloids emitted from vaping devices (Vuong 2019). Legally sold nicotine-containing products contain milligram (mg) levels of nicotine. A single cigarette may contain from 2 to >10 mg of nicotine, of which 85-90% is absorbed into the blood stream through inhalation (Digard 2013). Because exposure is very rapid, up to ~9 mg nicotine may be systemically absorbed within 5 minutes of smoking (Digard 2013). That level corresponds to up to 130 μg/kg nicotine exposure for a 70-kg person (130 ppb), which produces pharmacologic but not toxic effects. Nicotine-containing gum has ≥4 mg nicotine per dose (4.2 mg for Nicorette®) of which only 63% is absorbed via the oral route over a 45 min period (Digard 2013). This translates to 2.65 mg nicotine/70 kg person, or an exposure level of ~40 μg/kg (or ~38 ppb). This oral dose produces a mild, non-toxic pharmacologic effect. And these values are from only a single exposure to nicotine-containing products; multiple exposures per day are common.

Therefore, Thaumatin II alkaloid exposure is, at worst, thousands of times lower than from a single exposure to nicotine-containing consumer products. Although the nicotine analog anabasine may be similarly bioactive as nicotine, it is present at only a fraction of the level of nicotine in *N. benthamiana*-produced Thaumatin II, and at that level it too is not impactful on safety.

4. Information on Any Self-Limiting Levels of Use

THAUMATIN II has been reported to be 2,000 to 3,000-times (Table 2-1) sweeter than sucrose; therefore, a self-limiting level of use might be expected on the bases of (a) sensory saturation and (b) organoleptic tolerance. In addition, the cost of manufacturing Thaumatin II is higher than the cost of an equivalent sweetening level of sucrose or HFCS; hence, (c) economics may also self-limit use of this product.

5. Experience Based on Common Use in Food Before 1958

Notifier's GRAS Notice is based on scientific procedures. Thaumatins from *Thaumatococcus* have been used in indigenous human diets in West Africa for more than 200 years, as documented in British surgeon W.F. Daniell's report when he discovered the native use of *Thaumatococcus* pods in Nigeria in 1839 (Kinghorn 1986).

6. Basis for Conclusion of THAUMATIN II's GRAS Status

Notifier has used scientific procedures to conclude that its *N. benthamiana*-produced THAUMATIN II sweetener is GRAS under the conditions of intended use. Notifier has relied on detailed physicochemical analyses of its Thaumatin II protein and on extensive comparisons of its properties to a large volume of public information on thaumatins, including thaumatin's excellent safety record since these proteins were first commercially used as sweeteners and food flavor modifiers in multiple countries.

We conclude that the low residual levels of host alkaloids and elemental impurities in the *N. benthamiana*-produced THAUMATIN II product should not pose a risk to consumers, as those levels are lower than, or in

the same range as, the levels consumers are exposed to in their present diets or daily environment. We further assert that the additive content of alkaloids (THAUMATIN II-derived and dietary) should not expose consumers to undue risk because of the small contribution of the product to total alkaloid intake.

Section 6.1 through Section 6.5 of this Notice summarize the data and information on *N. benthamiana*-produced Thaumatin II that were used by Notifier to support its GRAS conclusion. In Section 7 (Supporting Data and Information), Table 7-1 presents a tabulated summary of this information from all sources.

6.1. THAUMATIN II Overall Safety

Prior to their broad approval for consumer use in the EU, Japan and other nations, thaumatin proteins were subjected to extensive safety assessments in animals and in humans, which have led to definition of application rates and daily exposure limits. Guidelines for thaumatin use and dietary exposure limits have been published in a number of regulatory documents issued by the European Food Safety Authority (EFSA 2015), based on reviews conducted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA 1983, 1986) and the EU Scientific Committee on Food (SCF 1985, 1989).

Thaumatin is considered safe for use in food and beverages. It is approved by EFSA in the EU (E 957) and in Japan, Australia, Israel and other countries as a food additive. In the USA thaumatin was granted GRAS status by FEMA (GRASa No. 3732, Oser 1984; and GRASa No. 3814, Smith 1996) as a flavor modifier, but not as a sweetener. FDA issued a "No Questions" letter to Nomad's GRAS Notice GRN 738 on 18 April 2018, for THAUMATIN (recombinant, plant-produced thaumatin I and/or II) as a food and beverage sweetener.

A discussion of thaumatin's safety from which a projection of Notifier's THAUMATIN safety can be drawn was presented in GRN 738 and is summarized here, with an expanded discussion on the safety of the new production host, the plant species *Nicotiana benthamiana*. Specifically, the safety profile of THAUMATIN II's **active ingredients** can be deduced from (1) their equivalence to commercially available thaumatins, and (2) published results of detailed studies on the safety of thaumatin conducted over the last 40 years.

The safety of the **gene expression system** and of residual **impurities from edible species of host plants** that Notifier uses to produce THAUMATIN were discussed in detail in GRN 593 (GRN 593 Section A.2.2; pp 25-28). The **impurities from *N. benthamiana*** were discussed in detail in GRN 775 (GRN 775 Section 6.1.1 – 6.1.6; pp 28-39) and are summarized herein for convenience. Regardless of host plant, the manufacturing processes defined in GRN 738 (THAUMATIN from edible species), GRN 593 (COLICIN from edible species) and GRN 775 (COLICIN from *N. benthamiana*) are analogous and differ mainly in the higher downstream purification applied for THAUMATIN II when using *N. benthamiana* as the host.

With respect to the thaumatin proteins themselves, studies by Higginbotham et al. (1983) and Hagiwara et al. (2005) have been pivotal to establishing safety and have helped craft regulations for the application rate and safe usage levels of these substances. Higginbotham et al. (1983) found that thaumatin, when used as a sweetener/flavor enhancer, was unlikely to be hazardous at the anticipated level of consumption. They studied the safety of thaumatin over a 7-year period when various purities and formulations of thaumatin were under development and the initial product was being commercially manufactured. A summary of their findings includes:

Thaumatin digestibility. Thaumatin was readily digested prior to absorption in rats. Groups of 20 animals were split into 2 x 10 animals each, and fed diets containing no (0%) or 5.73 wt% thaumatin, for 10 consecutive days with sampling at two time periods. The results of metabolic analyses showed that thaumatin was 89 to >90% digested when fed as a mixed additive to the diet. Digestibility was high even though thaumatins I and II have 8 intramolecular disulfide bridges.

Subacute toxicity in rats and dogs. Thaumatin-containing diets were fed to groups of 20 male and 20 female rats, and to groups of 4 male and 4 female dogs, at concentrations of 0%, 0.3%, 1.0% and 3.0% (w/w), daily for 13 weeks. Behavioral, food consumption, weight gain, clinical chemistry, cytology, ophthalmoscopy, urinalysis, and histopathology observations were conducted on all animals at every dose group. No adverse effects were found for any dose of thaumatin administered.

Teratogenicity. Thaumatin was not teratogenic when administered orally by gavage to groups of 20 pregnant rats at 0, 200, 600 and 2,000 mg/kg body weight/day from day 6 to 15 of gestation. No thaumatin- or dose-related adverse effects on the fetus were found.

Mutagenicity. Thaumatin was evaluated for mutagenic potential using two standard assays, the dominant-lethal test, and the Ames' *in vitro* mutagenicity assay. In the first study, groups of 15 male mice each of proven fertility were intubated daily for 5 consecutive days with thaumatin or negative or positive controls. Thaumatin administration had no effect on the incidence of dominant lethal mutations when administered at doses of 200 and 2,000 mg/kg-day. In the second study, the lack of mutagenic potential was confirmed in bacterial mutagenicity assays with *Salmonella typhimurium* (strains TA1535, TA1537, TA1538, TA98 and TA100) and *Escherichia coli* WP2, at levels of addition of 0.05–50 mg thaumatin/plate. All results were negative for mutagenicity.

No observed adverse effect level. The multi-species preclinical studies by Higginbotham et al. helped establish the **NOAEL** (no observed adverse effect level) for thaumatins at **1.4 g/kg** (dog) to **2.4-2.8 g/kg** (M and F rat). By conventional criteria, thaumatins are non-toxic. Allergenic risk potential was low.

Persistence of thaumatin proteins and genes. To complement Higginbotham (1983), Szwacka (2012) studied digestibility of thaumatins and the uptake of the thaumatin genes in animals fed transgenic fruit. The proteins were fully digested, and there was no gene transfer to the intestinal microflora.

6.1.1 Allergenic potential of THAUMATIN II

Amino acid sequences in Thaumatin II with similarity to known allergens

Because Notifier's Thaumatin II is identical in amino acid sequence and no less pure than commercial compositions containing thaumatin II, the allergenic risk for the recombinant proteins is deemed to be not higher than that of approved commercial products. Internal and external data were used to reach this conclusion. Baniulis et al. (2008) had conducted a computational analysis of thaumatin II allergenicity and assessed potentially antigenic elements in thaumatin-like family proteins. Of the various candidate antigenic domains in thaumatin II, only four amino acid residues (Thr12, Leu74, Gln133 and Thr161) were found to possess high surface exposure and antigenic propensity, in theory. Using a similar technique, Notifier conducted an updated search *in silico* for potentially antigenic domains in Thaumatin II.

The results of Notifier's sequence searches were included and discussed in detail in GRN 738 (Section 6.2, Allergenic potential of THAUMATIN, pp 17-21). The majority of identity hits were for thaumatin-like proteins ("TLP"), which are widely distributed across many genera. It is thought that TLP serve a protective function in the plants that synthesize them, including protection from fungal attack and/or stress; TLP may also be involved in fruit ripening (Barre 2000; Liu 2010). Table 6-1 summarizes the results of Notifier's amino acid sequence analyses performed *in silico*.

TLP are polypeptides of about 200 amino acid residues that share sequence similarity with thaumatins from *Thaumatococcus daniellii*. Due to their inducible expression by stresses like pathogen/pest attack, plant TLP are classified as the pathogenesis-related (PR) protein family 5 (PR5), which is one of 17 families of defense-

related PR proteins (Christensen 2002; van Loon 2006). The most closely matched amino acid sequences to those of *Thaumatococcus* thaumatin II protein are found in TLP in banana fruit (*Musa acuminata*). Of further interest is the finding that TLP, once thought to be present only in dicots, also appear in the fruits of monocots (e.g. banana) and may serve similar protective functions (Barre 2000; Liu 2010).

Table 6-1. Bioinformatic amino acid scan for allergenic sequences in Thaumatin II

Thaumatin	>35% allergen similarity at indicated search granularity			TLP Species Prevalence	Allergenicity Potential
	Full seq	80-mer	8-mer		
II	35 identified	33 similar seq	17 matches	Banana (17/17)	Low

The published literature show that TLP are consumed from multiple sources in our daily diet. Like any foreign protein, they have the potential for allergenicity. Yet, the lack of literature linking TLP to severe food allergies or anaphylactic reactions suggests that TLP, including thaumatins, present a low allergenic risk relative to other allergens in common foods, such as peanut, shellfish or gluten.

Allergenicity of *Thaumatococcus* thaumatins determined in preclinical and clinical studies

Results of prior allergenicity studies in humans and animals were included and discussed in detail in GRN 738 (Section 6.2, pp 19-21). Briefly, Higginbotham et al. (1983) performed detailed acute and sub-chronic toxicological evaluations of *Thaumatococcus*-extracted thaumatins, including determining the allergenic and anaphylactic potential of these proteins in rodents, NHP (non-human primates), and human volunteers. Their multi-species studies were comprehensive, and included evaluating the potential toxicity of purified thaumatin proteins and plant-derived impurities from *Thaumatococcus,* as well as formulation excipients. For example, they compared the safety of purified thaumatins I and II (95-98% protein) to that of less pure (78-85%) formulations.

These studies showed no evidence of thaumatin-related toxicity. Extensive preclinical and two human clinical studies verified the low allergenicity of thaumatins. Some commercial thaumatins from *Thaumatococcus* available even today may contain potentially allergenic and/or toxic impurities, including proteases, thaumatin variants and thaumatin-like proteins (some of which are known allergens), aluminum salts and other impurities and contaminants (Asherie 2008; Nikolic 2014).

In contrast, Notifier's THAUMATIN II is devoid of impurities and contaminants associated with *Thaumatococcus* extraction, and any residual host-derived proteins or other host components are found in low concentration (see APPENDIX C THAUMATIN II Characterization, for detail).

6.1.2 Host impurities

Edible plant species hosts

The acceptability and safety of food species of plants, such as **red or leafy beet, spinach** or **lettuce,** as manufacturing hosts for recombinant proteins was described in GRN 593 (Section A.2.2; pp 25-28), and the safe use of the same species in thaumatin production was described in GRN 738 (Section 6.1, pp 15-16). Edible food plants used in manufacturing recombinant thaumatins are in fact “food" and are therefore generally recognized as safe for ingestion at unrestricted levels.

Properties of *Nicotiana benthamiana* as a preferred manufacturing host

Although the original list of production hosts described in GRN 738 included only food (edible) species, Notifier has determined that additional plant species could be included in the manufacturing host list, and that these additional hosts also yield thaumatin proteins that are safe when produced using Notifier's manufacturing process, even though such species have not been traditionally consumed in human diets.

The concern in using non-food species derives from toxic components of the host that may remain in the final thaumatin product at levels to cause a safety risk. Therefore, 100% pure thaumatins produced in food species are expected to have equivalent safety features as 100% pure thaumatins produced in non-food species. Since thaumatins expressed in various food and non-food plant hosts have the same amino acid composition and do not undergo any differential postranslational modifications, an assessment of risk needs to be approached not from the standpoint of the safety of the thaumatin proteins themselves (established in GRN 738), but rather from potentially toxic levels of host and process impurities that may remain in the final product.

Genetic homogeneity. *Nicotiana benthamiana* is an ideal plant host for producing a wide range of biologics, including food additives. A thorough review of *N. benthamiana* was published by Goodin et al. (2008). Native to Western Australia, specimens of the species were reportedly first collected and brought to England aboard the *HMS Beagle* during the ship's third voyage (1843-1846). The specimens were eventually transferred to the Royal Botanic Garden, Kew, and the holotype first described as a new taxon in 1929 (Goodin 2008). Despite *N. benthamiana* having become a popular tool to study plant-pathogen interactions, relatively few accessions may have been the source of cultivars in use today.

The United States Department of Agriculture–Agricultural Research Service (USDA-ARS) National Plant Germplasm System has two accessions (plant introduction [PI] numbers 555478 and 555684), the Botanical Garden of Nijmegen (Netherlands) lists two accessions, the Institut fur Pflanzengenetik und Kulturpflanzen-forschung Gatersleben (IPK, Germany) has one, and the Australian Plant Genetic Resource Information Service (AusPGRIS) lists five accessions but only two are presently available for distribution. The Kentucky Tobacco Research and Development Center (KTRDC) at the University of Kentucky presently maintains six accessions of *N. benthamiana*, of which two are the USDA-ARS accessions and another two are almost certainly redundant (Goodin 2008).

Goodin et al. (2008) also used amplified fragment length polymorphism (AFLP)-based cluster analyses in an attempt to determine whether any genetic variation existed among 11 research accessions. They used DNA obtained from plants grown from seed submitted by researchers in five countries. When grown to maturity, no obvious differences in growth habit, foliage, or flowers were noted, with the exception of a single novel accession of *N. benthamiana* in the KTRDC collection, which was a much larger, coarser plant with larger flowers than the research accessions. Comparisons among the other 10 accessions averaged 0.924, indicating that they are very closely related and could possibly be derived from one source.

These published comparisons suggest that *N. benthamiana* accessions used in research and in the manufacture of plant-made pharmaceuticals are very similar in genotype and phenotype, including metabolic features. Therefore, importantly, with respect to the use of *N. benthamiana* for manufacturing food additives, it can be concluded that the exact cultivar used should not be impactful on the plant's compositional makeup or its inherent safety.

Virus and viral vector permissiveness. *Nicotiana benthamiana* is highly susceptible to infection by many plant viruses, at least in part due to a natural mutation in an RNA-dependent RNA polymerase gene (NbRdRP1m;

Yang 2004), making it a preferred species for studying plant-viral interactions. *Nicotiana benthamiana* EST (expressed sequence tags) are generally highly homologous to those of agriculturally relevant solanaceous crops, such as tomato, potato, pepper, and petunia, which collectively had a 2016-17 farm-gate commercial value of nearly US $7 billion in the United States alone (USDA ERS Cash Receipts by Commodity database, 2015-2018; https://data.ers.usda.gov/reports.aspx?ID=17845). As suggested by these USDA data, functional genomics projects concerned with host–pathogen interactions conducted in *N. benthamiana* will most likely reveal genes that play similar roles in agronomically important crops.

More recently, *N. benthamiana* has been the preferred host for heterologous protein expression in plants using plant virus-derived transient expression systems based on tobacco mosaic virus (TMV), potato virus X (PVX), and other vector backbones. A comprehensive review (Gleba 2014) catalogs efficient expression of heterologous proteins using *Agrobacterium*-mediated import of viral replicons, typically using *N. benthamiana* as the production host.

***Nicotiana benthamiana* as a biomanufacturing platform.** The homogeneity of the host, the ease of viral transduction for heterologous protein expression, and the high levels of accumulated protein product achievable after 7-10 days post infection, have made *N. benthamiana* a widely adopted "platform" species for plant-made pharmaceutical (PMP) R&D.

PMP is a rapidly developing field and its progress has been the subject of a number of reviews, many of which cover *N. benthamiana* as the preferred host (Cañizares 2005a; Cañizares 2005b; Chen 2011; Daniell 2009; Gleba 2004; Gleba 2005, 2007; Karg 2009; Klimyuk 2014; Komarova 2010; Lico 2008; McCormick 2008a; Mett 2008; Mortimer 2012; Pelosi 2012; Pogue 2010; Rybicki 2009; Sainsbury 2009; Saunders 2013; Smith 2009; Tusé 2011; Whaley 2011; Yusibov 2008) and others.

It is poignant that most of the currently announced or planned clinical trials of PMP are based on biopharmaceuticals produced using transient expression systems with *N. benthamiana* as the host. Those include recombinant biosimilars, antigens, and immunoglobulins produced using agroinfection (Icon Genetics/Nomad Bioscience, Germany; Mapp Biopharmaceutical, USA; Fraunhofer Institute for Molecular Biotechnology (IMB), USA; iBio, USA; University of Louisville/Intrucept Biomedicine, USA; PlantForm, Canada, etc.) or a non-viral transient system (Medicago, Canada).

Ease of scale-up and regulatory compliance. The popularity of transient systems reflects their speed and yields, as well as their scalability, and importantly, acceptance by the regulatory agencies. There are several plant-based cGMP-compliant or GMP-certified manufacturing facilities currently in operation, including those of Kentucky Bioprocessing (KBP), USA; iBio CMO, USA; Medicago, Canada; Icon Genetics, Germany; and Fraunhofer IMB, Germany. Manufacturing processes for biopharmaceuticals using *N. benthamiana* as the host are in compliance with quality and safety criteria specified in various FDA guidance documents and the Code of Federal Regulations (CFR), specifically CFRs treating GACP, cGMP and GLP.

Most of the PMP products that have or will enter clinical studies are manufactured in *N. benthamiana*; notably those manufactured by MAPP, KBP, Fraunhofer IMB, iBio CMO, University of Louisville/Intrucept Biomedicine, Icon Genetics and Nomad Bioscience. Universally, the products produced in *Nicotiana* have shown a high level of safety in clinical studies, even products whose release specifications for purity were set at only ≥90% (Tusé 2011). This means that *Nicotiana* host-derived impurities allowed in clinical products at up to 10% levels have failed to produce adverse events in clinical studies, even when the products were injectable vaccines co-administered with an adjuvant (McCormick 2008b; Tusé 2015).

Host impurities from *Nicotiana* species and their impact on safety

Humans and other animals consume a wide range of plants. Historical (evolutionary) trial and error, safety, nutritional value, ease of cultivation, and organoleptic qualities no doubt contributed to the selection of preferred species for dietary consumption. Plants of the genus *Nicotiana* share the main structural, physiological and biochemical constituents with all other land plants.

As extensively reviewed by (Leffingwell 1999), such constituents include (a) carbohydrates, including starch, sugars, sugar esters, cellulose and pectin; (b) nitrogenous constituents, including protein, soluble amino acids, nitrate, and certain alkaloids; (c) plastid pigments, including chlorophyll and carotenoids; (d) and isoprenoids and diterpenoids (both carotenoid-derived and non-carotenoid-derived), cembranoids and labdanoids; (e) phenolics, including polyphenols, lignin and various other phenolics; (f) sterols such as cholesterol and stigmasterol, and (g) various inorganics, including calcium, potassium, magnesium, sodium, chloride, and various other minerals that are absorbed from the soil (Leffingwell 1999).

These major structural, proteinaceous and biochemical components of *Nicotiana* species, including *N. benthamiana*, are shared with other plant species, including edible species, and are not considered inherently toxic. In fact, several studies have appeared in the peer-reviewed literature where members of the genus were assessed as a source of nutritional protein and other valuable biochemicals.

For example, in the proceedings of an Office of Technology Assessment conference on alternative uses of tobacco in agriculture, Wildman (1983) demonstrated from analytical studies that tobacco-derived protein fractions could be used as animal feed, with calculated nutritional value comparable or exceeding that of alfalfa. Wildman designed and described the agronomics for producing tobacco strictly for nutritional uses, including an extraction scheme for major plant components, each with directed end-uses. Even earlier, Kung et al. (1980) evaluated the nutritional content of tobacco leaf protein through analytical comparisons as well as animal feeding studies (rodent models). Kung fractionated total leaf protein from *N. tabacum* cv Maryland 609 into fraction I (FI), which contained ribulose 1,5-bis-phosphate carboxylase-oxygenase, and FII, comprised of soluble proteins from the cytoplasm and chloroplast. They compared the amino acid composition and nutritional value of FI and FII to milk-derived casein. Both *Nicotiana* protein fractions contained a full complement of essential amino acids, with only slight deficiencies in the sulfur-containing amino acids. Further, the protein efficiency ratio (PER; defined as animal weight gain per amount of protein consumed in feeding studies) of both FI and FII were comparable (within 10%) to the PER of casein.

In their discussion, Kung et al. (1980) cite similar nutritional studies with tobacco-derived FI and FII protein fractions, which showed that the *Nicotiana*-derived protein was similar in amino acid composition and nutritional value to soy protein (Kung 1978) and alfalfa leaf protein (Bickoff 1975), and better than other plant proteins, including rice, wheat and corn (Block 1951).

The above-cited studies did not report toxic effects as they were nutritional studies designed to assess animal weight gain. Conceivably, toxic metabolites from *Nicotiana* leaf preparations were removed in the extraction process or at least were reduced in concentration so as not to induce overt toxicity.

Notably, interest in *Nicotiana* as a source of nutritional protein occurred mainly in the 1970s and 1980s. The presence of toxicants and the availability of many other sources of vegetable protein may have discouraged additional research. This concept is supported by Siceloff (1981), who reviewed the impact of public policies on support for alternative uses of tobacco. However, Fu (2010) developed a modern tobacco purification process, as there may be renewed interest in low-cost plant proteins as meat analogs.

Of the minor constituents of *Nicotiana* species, it is the alkaloids that could have an impact on safety. Hence, potential toxicants need to be removed or diluted to ensure that the final product is safe. *Nicotiana* species synthesize a number of bioactive substances, some of which are toxic in sufficiently high doses. The major bioactive alkaloids in the genus include **nicotine**, **nornicotine**, **anabasine** and **anatabine**. Due in large part to tobacco variety improvement and tobacco safety research, the synthesis, accumulation and biological effects of these alkaloids have been extensively studied.

Wang et al. (2015) reported on genetic factors in *Nicotiana* associated with nicotine content. In commercial tobacco cultivars, nicotine represents 90–95% of the total alkaloid pool and is synthesized exclusively in tobacco roots, transported to leaves, and stored in leaf cell vacuoles by a multidrug and toxic compound extrusion (MATE) transporter. They reviewed various factors that control nicotine biosynthesis and accumulation, including plant genetics, environmental conditions, insect predation, mechanical injury and agronomical management measures, such as topping (decapitation of the apical meristem at the early stage of flowering) and suckering (removing the axillary buds of plants activated by topping).

Nicotine has two ring moieties, a pyrrolidine ring and a pyridine ring, derived from two branched pathways. The pyrrolidine ring is formed from the amino acids arginine or ornithine via putrescine and methylputrescine. The pyridine ring is derived from nicotinic acid, an intermediate of the pyridine nucleotide cycle. Lysine gives rise to piperidine, which with nicotinic acid gives rise to anabasine. The biosynthetic pathway of nicotine, nornicotine, anatabine and anabasine is summarized in Figure 6-1.

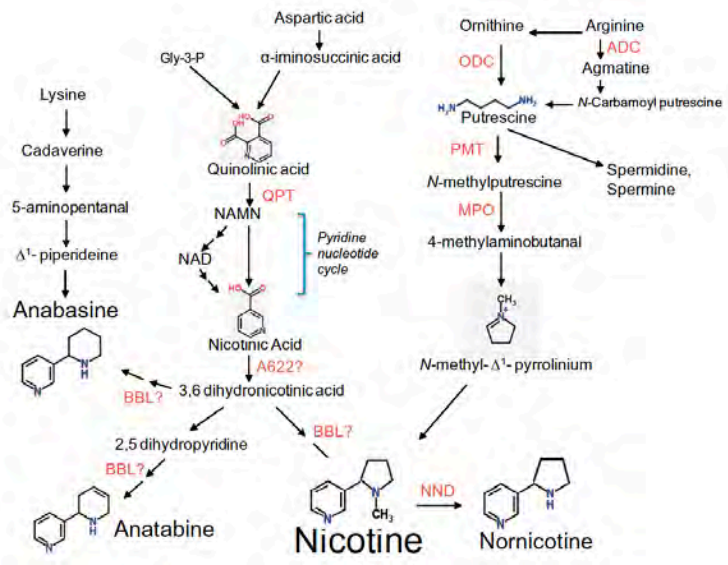

Figure 6-1. Biosynthetic pathway of major alkaloids in *Nicotiana* species

Schematic diagram of alkaloid biosynthesis in *Nicotiana* spp. Enzymes identified for key metabolic conversions are shown. A622: isoflavone reductase-like protein; ADC: arginine decarboxylase; BBL: berberine bridge enzyme-like; MPO: N-methylputrescine oxidase; NND: nicotine N-demethylase; ODC: ornithine decarboxylase; PMT: putrescine methyltransferase; QPT: quinolinate phosphoribosyltransferase (Dewey 2013).

Because the biosynthetic pathways for these alkaloids have been largely defined (Katoh 2005), it may be possible to knock out or suppress key genes in the pathway to yield varieties of *Nicotiana* having either very low (e.g. RNAi) or no nicotine and related alkaloids (e.g. gene knock-out) (Gavilano 2006; Hibi 1994; Hildreth 2011; Hung 2013; Kajikawa 2009; Shoji 2013; Takizawa 2007; Todd 2010; Wang 2008). Interspecific *Nicotiana* hybrids are also under development with the same goal (e.g. Ling 2012).

This opens the possibility of developing new varieties of *Nicotiana* that are devoid of potentially toxic constituents. These future hosts could be used to produce cruder (less purified) product formulations, akin to the purities currently acceptable when food species such as beet, spinach and lettuce are used to produce food additives and processing aids. Notifier is also working on controlling host alkaloids "upstream" by interfering with alkaloid biosynthetic pathways. These studies will continue with the goal of further reducing alkaloid content to below the level found in vegetables. In the meantime, Notifier has successfully reduced the levels of host alkaloids by "downstream" process controls (APPENDIX B). This Notice focuses on Thaumatin II purified from *N. benthamiana* using only downstream process controls.

The need for purification is borne from the pharmacological activity of residual host alkaloids. These alkaloids exert neuroactive effects and gathering evidence support their role in protecting the plant from insect predation (Steppuhn 2004; Todd 2010). Tobacco (*N. tabacum*) has a long history of human use for its neurostimulant effects (nicotinic acetylcholine receptor agonist) derived from these alkaloids, principally nicotine, which represents more than 90-95% of the total alkaloid content (Wang 2015). While several species and varieties of "tobacco" have been cultivated, there is no evidence that *N. benthamiana* was ever cultivated for any such purposes, probably because of the plant's morphology, its poor field performance, and the availability of hardier, larger species of tobacco with higher nicotine content.

The toxicities of *Nicotiana* alkaloids have been extensively studied, with variance due to multiple factors, including age (Okamoto 1994). For nicotine and its related analog nornicotine, the mouse and rat oral LD_{50} are in the range of 3-4 mg/kg and 50-60 mg/kg, respectively (see IPCS INCHEM 2012 http://www.inchem.org/documents/pims/chemical/nicotine.htm for comprehensive toxicity review). Human data from controlled toxicity studies are of course lacking, but the lower range of a human lethal dose is estimated to be 0.5 to 1.0 grams (~7-14 mg/kg), based on records of clinical overdoses (Mayer 2014). Table 6-2 from Baumung et al. (2016) summarizes some pharmacological threshold values for nicotine.

Table 6-2. Estimated pharmacological thresholds of nicotine

Species	Effect	Type of endpoint	Value [mg/kg bw]	Reference
Humans, i.v., acute	Heart rate acceleration	LOAEL	0.008[a]	EFSA[28] based on Lindgren *et al.*[26]
Humans, i.v., acute	Heart rate acceleration	BMDL for BMR = 1SD	0.013	Own modelling[c] based on data from Lindgren *et al.*[26]
Humans, chronic cigarette use	Addiction	Threshold	0.07[b]	Benowitz and Henningfield[29]
Humans (Children), dermal, acute	Various symptoms of intoxication	LOEL	0.01	EFSA[28] based on Woolf *et al.*[34]
Humans (Children), dermal, acute	Various symptoms of intoxication	BMDL10	0.004	Own modelling[c] based on data from Woolf *et al.*[34]
Various animal species, acute	Mortality (LD50 studies)	BMDL10[d]	3	Lachenmeier and Rehm[24]
Rats, 10-day study	Liver: fatty change	BMDL10	0.27	Own modelling[c] based on data from Yuen *et al.*[10]
Rats, 10-day study	Liver: focal necrosis	BMDL10	0.24	Own modelling[c] based on data from Yuen *et al.*[10]
Rats, 10-day study	Liver: dark cell change	BMDL10	0.21	Own modelling[c] based on data from Yuen *et al.*[10]
Rats, 10-day study	Pathological changes in liver	NOAEL	1.25	US EPA[61] based on data from Yuen *et al.*[10]

A key metric in Table 6-2 is the minimum dose of nicotine reported to induce a measurable pharmacologic effect in humans (increase in heart rate), considered to be the Lowest Observed Adverse Effect Level (**LOAEL**), which ranges from **8 to 13 μg/kg** Baumung 2016. Presumably, the No Observed Adverse Effect Level (**NOAEL**) in humans via the i.v. route would be **<8 μg/kg.** However, and importantly, these values are for intravenous bolus injection of nicotine, and hence due to ADME differences the NOAEL and LOAEL are both expected to be higher values via the oral route relative to the i.v. route.

Uptake of **nicotine** after oral administration, as would occur from food, cannot be directly compared with the uptake from pulmonary exposure. Oral exposure to nicotine leads to molecules reaching the gastrointestinal tract and being absorbed through the gut mucosa. However, in the acidic pH of the stomach, little nicotine is absorbed because the molecule is in its ionized state (Andersson 2003; Karačonji 2005). Intestinally absorbed nicotine molecules are transported via the hepatic portal vein to the liver, where extensive metabolism occurs (60-80% to cotinine; Benowitz 1999) before the alkaloid reaches the systemic circulation. Thus, nicotine will to some extent be metabolized by the bacteria and enzymes in the gastrointestinal tract, and to a large extent by the hepatic enzymes, before circulatory distribution. It has been calculated that 60-70 percent of orally supplied nicotine is metabolized by this type of first-pass metabolism before ever reaching the systemic circulation (Benowitz 1991).

Figure 6-2 is a pharmacokinetic profile showing nicotine bioavailability in human subjects through three different routes of administration, namely, inhalation via cigarette, intranasal spray (transmucosal), and orally from nicotine-containing chewing gum. The delay in absorption and the lower bioavailability of orally ingested nicotine is evident (Russell 1983).

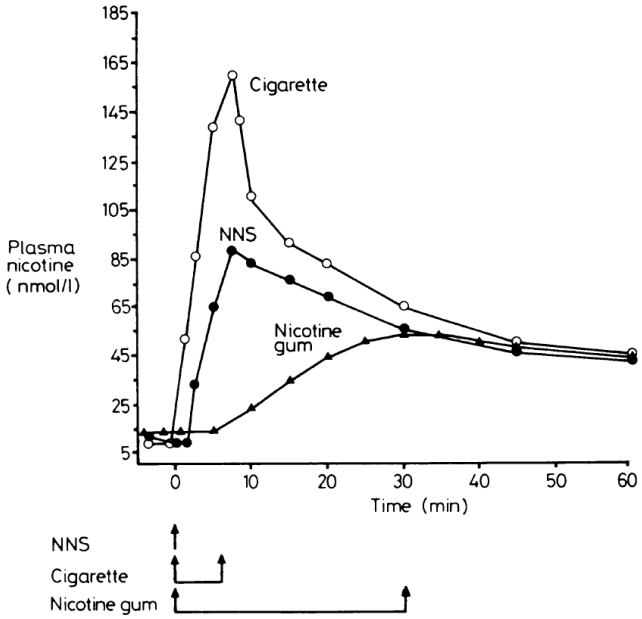

Figure 6-2. Bioavailability of nicotine in humans as a function of route of administration

Average plasma nicotine concentrations of three subjects after smoking a cigarette, taking nasal nicotine solution (NNS), and chewing nicotine gum. Doses of nicotine were 2 mg for nasal nicotine solution and nicotine gum and averaged 1.97 mg for the cigarette. Conversion: SI to traditional units-Nicotine: 1 nmol/ ~0.16 ng/ml (from Russell 1983).

There can be significant interindividual differences in the rates of nicotine metabolism in humans (Benowitz 1982). In studies with larger numbers of subjects, the half-life of systemically absorbed nicotine averaged 2-3 hours and that of cotinine, its major metabolite, about 17 hours (Benowitz 1996).

Because nicotine is rapidly metabolized and despite nicotine's physiological effects, it is unlikely that a person would overdose on nicotine through smoking, or nicotine replacement therapy (NRT) alone. The FDA stated in 2013 that *"There are no significant safety concerns associated with using more than one nicotine replacement therapy product (nicotine "patch" or nicotine gum) at the same time or using an NRT at the same time as another nicotine-containing product, including a cigarette"* (FDA 2013).

Anabasine (neonicotine) is a structural isomer (analog) of nicotine primarily found in the tree tobacco (*N. glauca*); it is present in *N. benthamiana* albeit at very low levels. It's acute oral LD_{50} is in the range of 200-300 mg/kg, rat (Toxnet: https://chem.nlm.nih.gov/chemidplus/rn/53912-89-3). Anabasine has been used medicinally to treat rectal prolapse in Russia, and as a urine biomarker for smoking cessation (Jacob 2002).

The concentration of nicotine and other alkaloids varies substantially among *Nicotiana* species and is impacted by genetics, cultivation practices, and biotic and abiotic stresses. The same is true for other species that biosynthesize these alkaloids. Several original articles and reviews have been published on the genetics and metabolism of alkaloid biosynthesis and accumulation. Saitoh et al. 1985 reported the alkaloid content of 60 *Nicotiana* species, including *N. benthamiana*. Similarly, Sisson and Severson (1990) reported on the alkaloid content across *Nicotiana* species.

Table 6-3 summarizes levels of nicotine and anabasine found in *N. benthamiana* by Notifier's own analyses compared to levels reported in the literature. Nicotine is the most abundant bioactive alkaloid in *N. benthamiana*, followed distantly by anabasine. Nicotine constitutes 80-90% of the total alkaloid content of *N. benthamiana* and anabasine 8-12% of the total; the typical ratio of nicotine to anabasine is ~10:1 (Sisson 1990). **Nornicotine** and **anatabine** may be present in trace amounts (<1% of total alkaloids each), and at the projected use levels of thaumatins these two alkaloids are not a risk. The genetic homogeneity of *N. benthamiana* cultivars suggests that alkaloid levels and ratios will remain consistent (Goodin 2008).

Table 6-3. Levels of nicotine and anabasine in *N. benthamiana*

A	**Notifier's Internal Data**		**Data From Literature**	
Sample	**Nicotine** content ng/g fresh weight	**Nicotine** content ng/g dry weight	**Nicotine** content ng/g fresh weight	**Nicotine** content ng/g dry weight
N. benthamiana Buffer 1	**61,830 ± 29,590**	**690,000 ± 340,000**	~500,000 (Todd 2010)	~14,000,000 (Sisson 1984) ~3,000,000 (Saitoh 1985)
N. benthamiana Buffer 2	**103,330 ± 15,610**	**1,140,000 ± 190,000**		
B	**Notifier's Internal Data**		**Data From Literature**	
Sample	**Anabasine** content ng/g fresh weight	**Anabasine** content ng/g dry weight	**Anabasine** content ng/g fresh weight	**Anabasine** content ng/g dry weight
N. benthamiana Buffer 1	**7,070 ± 1,290**	**80,000 ± 20,000**		~1,300,000 (Sisson 1984) (9.3% of nicotine)
N. benthamiana Buffer 2	**12,930 ± 400**	**140,000 ± 10,000**		

Panels A and B show the levels of nicotine and anabasine, respectively, found by Notifier in analyses of *N. benthamiana* using two different buffer extraction systems, compared to results reported in the literature.

6.1.3 Process modification to lower host alkaloid content in THAUMATIN II product

The downstream process used to reduce the level of nicotine and anabasine in the purified Thaumatin II protein expressed in *N. benthamiana* is an adaptation of the method described in GRN 738 for thaumatins produced in edible plants. The general approach was first described by Stephan et al. (2017) and is further detailed in APPENDIX B of this GRN, with adaptations for the properties of thaumatin proteins.

The process successfully reduces the level of host alkaloids in purified proteins relative to initial levels in the plant. Table 6-4 summarizes results from 3 batches showing average residual level of host alkaloids that are currently achievable. The process is subject to additional optimization that is expected to further reduce

alkaloid residues. Determination of alkaloid levels is discussed in APPENDIX C, Section C-5. The results were used to develop the upper alkaloid limits in the Specification, namely, 20 ng/mg protein for nicotine and 5 ng/mg protein for anabasine, for a combined limit of 25 ng alkaloid/mg Thaumatin II.

Table 6-4. Residual levels of *N. benthamiana* alkaloids in purified Thaumatin II

Protein	Residual alkaloid level in purified protein samples				
	Nicotine (ng/mg protein)		Anabasine (ng/mg protein)		No. of batches analyzed
	Single Batches	Mean ± SD	Single Batches	Mean ± SD	
Thaumatin II	14.09 [a]	14.41 ± 0.67	3.37 [a]	2.52 ± 1.55	3
	13.78 [a]		<3.84 [a]		
	15.37 [b]		0.34 [b]		

[a] Batches produced at bench scale at Notifier's facilities. [b] Batch produced at pilot-scale by Notifier's CRO.

For perspective, humans chronically ingest low levels of nicotine from their diet. Table 6-5 from Andersson (2003) summarizes the nicotine content of common vegetables as reported in multiple published studies.

Table 6-5. Average nicotine content in common vegetables

Sample	Data from literature		
	Nicotine content ng/g dry weight (Moldoveanu 2016)	Nicotine content ng/g dry weight	References
Cherry tomato, fruit	181.9 (tomato)	79-98 (a) 78-148 (b) 28-115 (c)	(a) Castro and Monji 1986 (d) Domino et al. 1993 (b) Davis et al. 1991 (e) Davis et al. 1986 (c) Liu et al. 2013 (f) Siegmund et al. 1999 (g) Andersson et al. 2003
Eggplant, fruit	174.3	1,000 (a) 52-150 (e)	
Potato (whole), tuber	42.6	71+/-59 (d) 76 (b) 45-123 (c)	
Cauliflower		3.8 (d)	
Sweet pepper		5.4 (g)	

Hence the natural dietary intake of nicotine can be calculated. Table 6-6 (Andersson 2003) summarizes the estimated food-borne nicotine exposure in Scandinavia; although daily consumption values for each vegetable sampled may differ from US consumption estimates, nicotine ingestion ranges from **900-1,300 ng/person-day** depending on dietary habits.

Table 6-6. Consumption, nicotine content and dietary nicotine exposure from common vegetables

Food Source	Average daily consumption (g/day)	Average nicotine content (µg/kg)	Average daily dietary nicotine exposure (ng/day)
TOMATO - Sweden - Denmark	21.6 62	4.4	95.0 273
POTATO - Sweden - Denmark	168.6 156	5.8	977.9 905
EGGPLANT - Sweden - Denmark	0.71¤ 2.7	34.4	24.4 93
SWEET PEPPER - Sweden - Denmark	0.27* 5.5**	5.4	1.5 30

¤ Swedish import of eggplant in 2000 (2 297 000 kg) divided with number of citizens in 2000 (8872294) and 365 days; * only green pepper; ** green, yellow or red pepper

In Section 3.4 of this Notice, Table 3-2, we presented the estimated maximum intake of alkaloids at the daily adult thaumatin MPL of 77 mg/person. At the Specification limit for total alkaloid (25 ng/mg protein), the estimated per capita daily exposure to all host alkaloids in that "worst case" scenario was <2 µg/person-day, which is in the same range of the level consumed daily in common vegetables.

Importantly, when THAUMATIN II is used only as a sweetener, with per capita consumption estimated at 3.75 mg per day, the total alkaloid intake would be <100 nanograms/day, or <10% of the level in vegetables.

6.1.4 Process impurities in *N. benthamiana*-based THAUMATIN II manufacture

Other than the low risk from natural alkaloids present in *N. benthamiana* discussed above, the only remaining potential risks would come from the **vector or induction process** used to express the thaumatin genes and/or any residual reagents added in the manufacturing process.

Agrobacterial vector and ethanol induction

Gene expression and thaumatin synthesis can be induced via infection of non-GM plants with agrobacteria or via chemical induction (e.g. ethanol) in transgenic hosts, as first defined in GRN 593 and subsequently described in APPENDIX B of GRN 738 (THAUMATIN from edible species). When agroinfection is used, the bacterial vectors carrying viral amplicons (TMV or PVX) with the thaumatin genes are recombinantly produced and become part of the manufacturing process. When ethanol induction is used, the thaumatin genes are a stable but silent trait in the host and are expressed when a dilute solution of ethanol is applied to the plant (Werner 2011). The ethanol is removed during thaumatin purification (data not shown).

Either agrobacterial induction process yields non-viable vector after the plant biomass is treated and the thaumatin protein is extracted. The question can be asked about potential safety risks from vector that may remain viable after bioprocessing. This issue can be addressed by noting that the *Agrobacterium* industrial strain used in the production of thaumatins is very similar to wild-type strains of *Agrobacterium* from which it was derived. Notifier employs a weakened strain of the vector that contains mutations that severely constrain its environmental viability and competitiveness, so it is environmentally safe.

Agrobacterium tumefaciens and related agrobacteria are natural soil inhabitants, ubiquitous plant colonizers, can effect lateral gene transfer in plants, and are present at levels as high as 30 million CFU/g of soil (Bouzar 1993; Krimi 2002; Mougel 2001; Penyalver 1999; Stockwell 1993; Vicedo 1993). *A. tumefaciens* builds

cellulosic bridges to plant structures, sticking to the plant during washing and consumption. Humans, therefore, unavoidably consume agrobacteria in their natural diet. No ill effects have been reported from consumption of agrobacteria present in produce (i.e. it is not an animal or human pathogen), even at levels far exceeding those that might result from the current manufacturing process.

Lastly, the agrobacterial vector carries genes that encode RNA amplicons, which replicate in the plant cells' cytoplasm and are translated by cellular machinery to yield thaumatin protein. The RNA replicons cannot integrate into the plant genome or be passed on via seed. They cannot integrate into the human genome or replicate in mammalian cells.

Humans are routinely exposed to significant amounts of TMV and PVX through consumption of uncooked vegetables (e.g. spinach, beets, collard greens, tomato, cucumber, etc.). A large percentage of smokers have serum antibodies against TMV (Liu 2013) with no ill effects.

Neither virus as used in these expression systems (Gleba 2014) has been implicated as a health risk. Therefore, even if viable vector became an impurity in the final product, the levels would be lower than what humans naturally consume from ingesting produce.

Abiotic process impurities

Purified Thaumatin II protein obtained from two bench-scale developmental batches and from one pilot-scale manufacturing batch have been analyzed for the **elemental impurities** of most concern in food (i.e. Class 1 heavy metals due to their high toxicities; see APPENDIX C, Section C.6 Analysis of Elemental Impurities). The results are summarized in Table 6-7.

These values were used to set Specification limits of 1 ng for Pb and 5 ng total Class 1 metals per mg Thaumatin II protein. Hence, hypothetical exposure to these most toxic metals (Pb, Cd, Hg, As) from consumption of Thaumatin II as a sweetener (3.75 mg/day) and at the maximum permitted level (MPL; 77 mg/day) are projected to be **<20 nanograms/person-day** and **<400 nanograms/person-day**, respectively.

Considering that the cumulative PDE for this combination of Class 1 elements is **55 micrograms/person-day** (ICH 2015), we conclude that the intake of heavy metal impurities from consumption of THAUMATIN II at either sweetener or MPL quantities will be inconsequential and will constitute a very low safety risk.

Table 6-7. Estimated exposure to elemental impurities in Thaumatin II

Heavy Metal Specification, Thaumatin II			Estimated Exposure		PDE
Element	**Class**	**Spec. Limit**	**Sweetener**	**MPL**	**(Oral/Daily)**
		(ng/mg protein)	**(ng/day)**	**(ng/day)**	**(μg/day)**
Pb	1	1	3.75	77	5
Sum of Pb, Cd, Hg, As	1	5	18.75	385	55

Specification limits for Pb and for all Class 1 heavy metals (Pb, Cd, Hg, As) were set at 1 ng/mg protein and 5 ng/mg protein, respectively, based on results of multi-batch analyses described in APPENDIX C, Section C-6. The Specification limits were multiplied by 77 or by 3.75, to estimate total potential exposure from consumption of Thaumatin II at the thaumatin MPL or at the level used as a sweetener; values are expressed as nanograms/person-day (ng/day). The estimated elemental intake values at each level of consumption are compared to the permitted daily exposure (PDE) for oral intake of Class 1 elements and are expressed in micrograms/person-day (μg/day; last column in the table; ICH 2015).

The conclusion that can be drawn with these new data is that sweetener consumption at 3.75 mg Thaumatin II/day would expose consumers to 3.75 x 1 = 3.75 ng/day of Pb and to 77 x 1 = 77 ng Pb/day from all uses. Likewise, exposure to all Class 1 heavy metals would lead to consumption of 3.75 x 5 = 18.75 ng heavy metals/day for sweetener uses and to 77 x 5 = 385 ng heavy metals/day from all uses.

Compared to the 5 micrograms/day (Pb) and 55 micrograms/day (sum of Class 1 elements) permitted daily exposures to these same heavy metals, respectively, exposure from Thaumatin II consumption would lead to less than 0.1% and ~1.5% Pb exposure from sweetener uses and from all uses, respectively, relative to the PDE. Similarly, consumption of Thaumatin II would expose consumers to <0.035% and ~0.7% of the Class 1 element PDE from consumption of the protein as a sweetener and from all uses, respectively.

For perspective, using the NHEXAS database, Ryan (2001) estimated the actual oral **daily intake** of heavy metals in Maryland residents as 28 **mg** for arsenic, 10 **mg** for cadmium, and 8 **mg** for lead. ASTSDR (1999) states that the mercury intake through food is approximately 3.5 **mg**/day. Compared to these reported levels of heavy metal intake and even the guidance limits (PDEs), the level of exposure to these elements from THAUMATIN II should be of little consequence with respect to health risks.

With respect to abiotic safety risks from **process reagents**, all processing aids, aqueous buffers/salts and solvents are selected from lists of approved food additives and food processing aids. Any residual levels of processing reagents would introduce no more risk than those derived from the consumption of other food products that also utilize them.

6.2. Safety in Relation to Dietary Intake of THAUMATIN II

We expect the same maximum levels of consumption of Notifier's Thaumatin II, whether produced in edible hosts or in *N. benthamiana*, per weight or volume of treated food as those allowed for all uses of extracted thaumatins. We also expect a much-reduced, moderate level of thaumatin consumption when Thaumatin II is used as a sweetener.

EFSA provides guidance on maximum permitted levels (MPL) of thaumatin on various foods and beverages according to Annex II to Regulation (EC) No 1333/2008 (OJEU 2011).

Table 6-8 lists the authorized **original and expanded-use levels for thaumatin (E 957)** in mg/kg food (w/w) and/or mg/L for beverages or non-solid foods (w/v) (EFSA 2015).

The same regulations allow the unlimited addition of thaumatin (*Quantum satis*) when the protein is used as a table-top sweetener. Although it excludes sweetener applications, FEMA's GRAS List (Cohen 2016; Cohen 2018) provides application rates for thaumatin in the USA.

Table 6-8. EFSA Currently authorized and extended use levels (2015) for thaumatin (E 957)

FCS category number	Food categories	Currently authorised MPL (mg/L or mg/kg as appropriate)	Currently authorised and change of use levels and proposed extension of use (mg/L or mg/kg as appropriate)	Restrictions/ exception
1.4	Flavoured fermented milk products including heat-treated products	5	5	Only as a flavour enhancer
3	Edible ices	50	50	Only energy reduced or with no added sugar
4.2.5	Jams, jellies and marmalades and similar products		5	Energy reduced or with no added sugar
5.1	Cocoa and chocolate products as covered by Directive 2000/36/EC	50	50	Only energy reduced or with no added sugar
5.2	Other confectionery including breath refreshening microsweets	50	50	Only cocoa or dried fruit based, energy reduced or with no added sugar
5.2	Other confectionery including breath refreshening microsweets	50	50	Only confectionery with no added sugar
5.3	Chewing gum	10	10	Only with added sugar or polyols, as flavour enhancer
5.3	Chewing gum	50	50	Only with no added sugar
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	50	50	Only confectionery with no added sugar
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	50	50	Only cocoa or dried fruit-based, energy-reduced or with no added sugar
6.3	Breakfast cereals		10	Energy reduced or with no added sugar
7.2	Fine bakery wares		30	
11.4.1	Table-top sweeteners in liquid form	*Quantum satis*[a]	*Quantum satis*[a]	
11.4.2	Table-top sweeteners in powder form	*Quantum satis*[a]	*Quantum satis*[a]	
11.4.3	Table-top sweeteners in tablets form	*Quantum satis*[a]	*Quantum satis*[a]	
12.1.2	Salt substitutes		15	
12.5	Soups and broths		15	
12.6	Sauces		15	
14.1.4	Flavoured drinks	0.5	5	Only water-based flavoured non-alcoholic drinks. As flavour enhancer only
14.2.1	Beer and malt beverages		5	
14.2.2	Wine and other products defined by Regulation (EEC) No 1234/2007 and alcohol-free counterparts		5	
14.2.4	Fruit wine and made wine		5	
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008		5	
14.2.7	Aromatised wine-based products as defined by Regulation (EEC) No 1601/91		5	
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % of alcohol		5	
15.1	Potato-, cereal-, flour- or starch-based snacks		10	
16	Desserts excluding products covered in category 1, 3 and 4	5	5	As flavour enhancer only
17.1	Food supplements supplied in a solid form, including capsules and tablets and similar forms excluding chewable forms		400	
17.2	Food supplements supplied in a liquid form		400	
17.3	Food supplements supplied in a syrup-type or chewable form	400	400	
18	Processed foods not covered by categories 1 to 17, excluding foods for infants and young children		10	Ready meals (chilled, frozen, dried) only

FCS: Food Categorisation System.
(a): Although permitted at *quantum satis* levels, a level of use of 10 mg/kg in table-top sweeteners (liquid, powder and tablet form) is considered an appropriate current level of use by the applicant and was used in the intake assessment.

Table 6-9 lists the **original and expanded-use levels of extracted and recombinantly produced thaumatins** for US food products. Notifier expects that THAUMATIN II can be used at equivalent application rates on various foods, as we claimed in GRN 738.

Table 6-9. FEMA Updated thaumatin use levels (2018)

	Thaumatin	Thaumatin B-Recombinant		Thaumatin	Thaumatin B-Recombinant
FEMA NO.	3732	3814	FEMA NO.	3732	3814
GRAS PUBLICATION	13	17	GRAS PUBLICATION	13	17
CATEGORY			CATEGORY		
Baked goods	7[a]/7[a]	1/1	Jams and jellies	7[a]/7[a]	2/5
Beverages, non-alcoholic	7[a]/7[a]	5/7[a]	Meat products	7[a]/7[a]	2/2
Beverages, alcoholic	7[a]/7[a]	5/7[a]	Milk products	7[a]/7[a]	3/6
Breakfast cereals	7[a]/7[a]	1/2	Nut products	7[a]/7[a]	5/7[a]
Cheeses	7[a]/7[a]	7[a]/7[a]	Other grains	7[a]/7[a]	
Chewing gum	150[a]/150[a]	150/150[a]	Poultry	7[a]/7[a]	2/5
Condiments and relishes	7[a]/7[a]	1/2	Processed fruits	7[a]/7[a]	2/5
Confections and frostings	7[a]/7[a]	2/5	Processed vegetables	7[a]/7[a]	2/5
Egg products	7[a]/7[a]	2/5	Reconstituted vegetables	7[a]/7[a]	2/5
Fats and oils	7[a]/7[a]		Seasonings and flavors	7[a]/7[a]	0.5/1
Fish products	7[a]/7[a]	5/7[a]	Snack foods	7[a]/7[a]	1/2
Frozen dairy	7[a]/7[a]	1/2	Soft candy	7[a]/7[a]	2/5
Fruit ices	7[a]/7[a]	2/5	Soups	7[a]/7[a]	2/5
Gelatins and puddings	7[a]/7[a]	1/2	Sugar substitutes		0[a]/0[a]
Granulated sugar			Sweet sauces	7[a]/7[a]	2/5
Gravies	7[a]/7[a]	2/5			
Hard candy	7[a]/7[a]	2/5			
Imitation dairy	7[a]/7[a]	7[a]/7[a]			
Instant coffee and tea	7[a]/7[a]	2/5			

Abstracted from FEMA GRAS™ 28 List (July 2018). Columns show allowed use levels for extracted and recombinant thaumatins in various food products. FEMA Updated average usual use levels (ppm)/average maximum use levels (ppm) for flavoring substances previously recognized as FEMA GRAS. Superscript "a" represents a new use level (Cohen 2016; Cohen 2018).

Consumption values used to develop current regulations in the EU for the safe daily intake of thaumatin were derived from typical European diets, taking into consideration country specific dietary preferences as well as various population age groups (**infant, adult, elderly**). In surveying the types of sweeteners applied to foods, and the consumption statistics for those foods in typical US diets (derived from USDA sources, including Haley 2011, and McConnell 2016), it was concluded that US and Western European diets were not sufficiently different to warrant separate calculations of consumption. Hence, the safety upper limits (MPL) found in extensive studies in the EU are applicable to domestic (US) consumption of thaumatin.

While near-term THAUMATIN II dietary intake levels are easier to project based on currently allowed application rates in many countries, future trends are more difficult to forecast and are the topic of several recent studies. Ng et al. (2012) tried to quantify the use of caloric and non-caloric sweeteners in US consumer packaged foods. They reported that of the 85,451 uniquely formulated foods purchased during 2005–2009, 75% contained sweeteners (68% with caloric sweeteners (CS) only, 1% with non-caloric sweeteners (NCS) only, 6% with both CS and NCS). CS were used in >95% of cakes/cookies/pies, granola/protein/energy bars, ready-to-eat cereals, sweet snacks, and sugar-sweetened beverages. NCS were in >33% of yogurts and sports/energy drinks, 42% of waters (plain or flavored), and most diet sweetened beverages.

Across unique products, corn syrup was found to be the most commonly listed sweetener, followed by sorghum, cane sugar, high fructose corn syrup and fruit juice concentrate (FJC). Ng et al. also reported that 77% of all calories purchased in the US in 2005–2009 contained CS and 3% contained NCS, while 73% of the volume of foods purchased contained CS and 15% contained NCS. Trends during this period suggest a shift towards the purchase of NCS-containing products.

More recently, a survey by Sylvetsky et al. (2017) that tracked US consumption of low-calorie sweeteners (LCS) showed that intake has increased significantly during the past several decades. The goal of the Sylvetsky et al. study was to describe LCS consumption in the United States and to characterize consumption by sociodemographic subgroups, source, frequency, eating occasion, and location. The sources of information were the National Health and Nutrition Examination Survey (**NHANES**) data from 2009-2010 and 2011-2012. Sylvetsky et al. found that total of 4,981 unique food and beverage items were consumed by participants in NHANES 2009-2010. Of these items, 126 contained LCS, including 57 beverages, 61 foods, and 8 packets. Similarly, 5,192 unique food and beverage items were reported in NHANES 2011-2012. Of these items, 147 contained LCS, including 74 beverages, 65 foods, and 8 packets.

Importantly, Sylvetsky et al. were not able to quantify the amount of LCS in LCS-containing products because manufacturers are not required to provide information regarding the quantity of LCS added, with the exception of saccharin. Due to the inability to quantify the amount of LCS in foods and beverages, intake (in grams) of LCS-containing products was estimated as a proportion of an individual's total intake of the specific product category (e.g., yogurts, desserts) reported in NHANES (Sylvetsky 2017).

The food categories in NHANES surveyed by Sylvetsky (2017) are very similar to the categories surveyed in the EU, with the possible exception that serving portions of some foods tend to be larger in the USA than they are in Europe; nevertheless, the EFSA application rates provide an accurate basis for estimating potential US intake of thaumatins based on the types of foods consumed.

Therefore, in GRN 738 Notifier assumed a "highest use" scenario for intake of thaumatin in the diet, and derived use levels from the broadest groups of foods that could be treated. Not coincidentally, the EFSA/EU database and use levels by food group presented the most comprehensive source of information, because thaumatin is already approved for such uses in the EU.

To summarize, an assessment of the potential dietary intake of thaumatins can best be derived from EU usage data, which show that, from all dietary sources, continuous thaumatin ingestion levels of up to 1.1 mg/kg (bw)-day in all age populations, or 77 mg/person-day for a 70-kg adult, are safe. The commercial introduction of Notifier's THAUMATIN II product, irrespective of the manufacturing host used, is expected to displace, initially, thaumatin that is currently derived from extraction of the same proteins from *Thaumatococcus*. Should industrial thaumatin usage expand due to availability of THAUMATIN II, consumption of thaumatin would be expected to increase. Even if daily per capita intake limit were to double to 150-200 mg from the current 77 mg MPL, thaumatin's safety factor of 1,300 based on chronic toxicity studies suggest that any additional safety risk to consumers would be minimal (Hagiwara 2005; OJEU 2011; EFSA 2015).

6.3. Occupational safety

With respect to occupational safety, Notifier's Thaumatin II protein is considered non-toxic. No higher-level precautions for personnel protection are suggested for handling, preparing or disposing of THAUMATIN II powder or liquid solutions. Only general precautions for handling powdered bulk substances and protein-containing waste solutions are suggested. A draft SDS for THAUMATIN II is appended to this Notice (APPENDIX A), where additional recommendations for storage, handling and disposal of this product are listed.

6.4. GRAS conclusion for *N. benthamiana*-produced THAUMATIN II sweetener

The results of clinical studies by Higginbotham et al. (1983) assessing the effects of occupational pulmonary exposure to thaumatin-containing substances, as well as the effects of oral ingestion of thaumatins, corroborate the results they obtained with multiple animal species that show that thaumatins are digestible, show no systemic toxicity when administered orally, and have low allergenic potential if delivered systemically but none if delivered orally.

The sub-acute (28-day with 14-day thaumatin plus 14-day lactose cross-over) ingestion study with human volunteers administered a total of 1.4 grams of thaumatin over a two-week period, with no ill effects. Similarly, the multi-year occupational exposure to low levels of thaumatin-containing dust in the workplace had no obvious toxic effects and only low measurable sensitivity, which was lower in prevalence than allergy to dust mites in the surveyed population.

Subsequently, Hagiwara et al. (2005) reported results of a 13-week (sub-chronic) feeding study of thaumatin in Sprague–Dawley rats. Thaumatin was administered at dietary levels of 0% (control), 0.3%, 1.0% and 3.0% to groups of 10 male and 10 female Crj:CD (SD) IGS rats. There were no treatment-related clinical signs or adverse effects on the survival rate, body weight, food consumption, water consumption and urinalysis, ophthalmology, hematology, or blood biochemistry data. No treatment-related alterations in gross pathology or organ weights were found in any group. On histopathological examination, sporadic spontaneous lesions known to occur in this strain of rats were the only findings, with no specific relation to the test substance.

Safety data available at the time helped support extensive reviews conducted by the EU Scientific Committee on Food (SCF 1985, 1989) and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 1983, 1986). Subsequently, Hagiwara et al. (2005) calculated the no-observed-adverse-effect-level (NOAEL) in rats to be a dietary intake of 2,502 mg/kg body weight-day for males and 2,889 mg/kg body weight-day for females. A study in dogs established a lower average NOAEL of 1,400 mg/kg body weight/day. Collectively, these studies have helped update the application rates of thaumatin. The levels of usage of thaumatin on various food groups, the consumption of each food type, and the anticipated total exposure to thaumatin as a food additive have since been aggregated and updated (EFSA 2015).

Based on the lowest NOAEL of 1,400 mg/kg bw-day for dogs calculated from results by Higginbotham et al. (1983) and Hagiwara et al. (2005) and others, EFSA applied a **safety factor of ~1,300 for E 957** (1,400 mg/kg bw-day in dogs and the then-current human maximum daily intake level of 1.03 mg/kg body wt-day) to arrive at an MPL for thaumatin by food product group, including solids, semi-solids and liquid beverages, for all age groups. The cumulative daily limit for chronic human consumption was set at 1.1 mg/kg-day, or 77 mg/day for a 70-kg adult person. At this intake level, thaumatin protein would represent <0.13% of the total adult daily protein intake (i.e. nutritionally insignificant). Even at 2x the current intake level, the safety factor would be nearly 700:1.

The safety of Thaumatin II protein comprising Notifier's THAUMATIN II product can be deduced by analogy to the thaumatin products evaluated in the studies cited herein. Higginbotham et al. (1983) evaluated thaumatin compositions of various degrees of purity, including commercial thaumatin (Thalin®) that contained 3% residual host impurities, including non-thaumatin proteins, carbohydrates, pectin, and glucuronoxylan from the arils of the source fruit. Ramsohoye (1989) found non-thaumatin protease and esterase impurities in commercial thaumatin products. In addition, because the original Tate & Lyle thaumatin extraction process involved the use of aluminum salts to assist with purification, the final product contained small amounts of the heavy metal. Importantly, in spite of these findings, the toxicity studies of Higginbotham et al. included evaluation of isolated host and process impurities, which proved to be innocuous with respect to safety. Since Notifier's submission of GRN 738 (THAUMATIN from edible plant species; 16 October 2017), no new literature was identified citing thaumatin safety concerns.

In the current Notice, Notifier has concluded using scientific procedures that its Thaumatin II protein produced in the host *Nicotiana benthamiana* is generally recognized as safe when used at up to 100% of the MPL allowed by current statutes for commercially available thaumatins. When used as a sweetener, Thaumatin II would be ingested at not more than 5% of the daily MPL (i.e., 3.75 mg/77 mg person-day). To ensure a high safety margin, host-derived alkaloids are monitored and controlled to minimize risk. In our target Specification for Thaumatin II, we have set 20 ng/mg and 5 ng/mg as the maximum nicotine and anabasine levels, respectively, and total alkaloids of ≤25 ng/mg protein in a final product. At the Specification limit, ingestion of the adult thaumatin MPL of 77 mg/day would lead to total alkaloids consumed of ~2 μg/person-day, or the equivalent of 1-2 extra servings of vegetables at the current average rate of US food consumption (0.56 kg food/day). At the same Specification limit, consumption of 3.75 mg Thaumatin II sweetener/person-day would result in exposure to <100 ng total alkaloid.

We present data from multiple public sources that these levels of pyridine alkaloids are below the threshold of pharmacologic or toxic activity even for intravenous administration. We reiterate that these low exposures from ingestion of residual alkaloids from Thaumatin II are exaggerated by the assumption of 100% adoption of Notifier's product in all food categories claimed and at the maximum permitted level (MPL) of the product.

Notifier describes a process modification introduced into its manufacturing method when using *N. benthamiana* as the expression host for thaumatins (APPENDIX B). The modification involves cool ambient temperature neutral extraction of biomass, heat treatment, and cation-exchange chromatography followed by diafiltration. These downstream steps effectively reduce the levels of host alkaloids and other impurities in the purified Thaumatin II protein.

We also present the content of Class 1 elemental impurities in purified Thaumatin II derived from analyses of multiple batches. Our process is undergoing optimization, yet current results enabled us to set a Specification limit of 5 ng total Class 1 metals (sum of Pb, Cd, Hg, As) per mg Thaumatin II protein. At that limit, total heavy metal exposure resulting from the consumption of Thaumatin II at the adult MPL (77 mg/day) would be <400 ng/person-day, whereas <20 ng/person-day elemental intake would be realized from Thaumatin II's use only

as a sweetener (3.75 mg/day). These levels pose no undue risk to consumers relative to the cumulative Class 1 metal PDE of 55 micrograms/day. Preliminary analyses of the less toxic Class 2 and Class 3 metals in a pilot-scale manufacturing batch of Thaumatin II have yielded very low impurity levels (sum of Ag, Bi, Cu, Mo, Sb and Sn = <1 ng/mg protein), and hence these additional elements are not included in the Specification.

There are no other identifiable toxic entities in *N. benthamiana* that we could find from extensive literature surveys or from our own analyses. The protein, lipid, carbohydrate and mineral components of the plant have been studied and are found in the public record, and reveal no unusual properties of the plant that are not also present in solanaceous plant species consumed as food.

Similarly, no allergenic or hypersensitivity inducing components have been reported for this species. In an average diet, exposure to residual *N. benthamiana* proteins would be ≤ 15 ppm/day (worst case); hence, host proteins are not a risk factor in the product.

Conclusion

The totality of evidence, from Notifier's internal studies and from the extensive public record cited herein and in GRN 738, suggests that consumer risk associated with ingestion of thaumatins themselves, or with ingestion of trace amounts of impurities derived from manufacturing Thaumatin II protein in *N. benthamiana*, is low. Consumers already ingest equivalent or even higher levels of the same impurities in their daily diet, and any contribution of additional alkaloids or elemental impurities from the use of THAUMATIN II as a sweetener is not expected to significantly raise dietary risk.

7. Supporting Data and Information

Multiple sources of information were used to support the conclusion that Notifier's THAUMATIN II product produced in the host *N. benthamiana* is GRAS. Table 7-1 lists the various data and other information discussed in this Notice and used in reaching this conclusion. Also listed in the table is whether the specific information cited was generated by Notifier and/or obtained from databases or references in the public domain.

The active ingredient Thaumatin II in Notifier's THAUMATIN II product is of the same molecular composition as the active ingredient in thaumatin II-containing products derived from extracts of *Thaumatococcus* or recombinant versions thereof, and has the same functional properties. Therefore, we draw analogies to those referenced products with respect to sweetening functionality, dietary use levels, and safety.

Table 7-1. Information supporting THAUMATIN II sweetener GRAS conclusion

Topic	Location in this GRN	Source of data or information	Availability
Thaumatin identity	Section 2.1	Suami 1997; van der Wel 1972; Lee 1987; Hough 1993; Zemanek 1995; Greenly 2003	Public
Thaumatin structure and function	Section 2.1	Suami 1997; de Vos 1985; Ogata 1992; van der Wel 1984; Hough 1993; Faus 2008	Public
Mode of action and mechanisms of taste perception	Section 2.1	Bartoszewski 2003; Faus 2008; Glaser 2000; Greenly 2003; Hellekant 1996; Kaneko 2001; Kinghorn 1986; Ledeboer 1984; Lim 2012; Masuda 2018; Nagarajan 2017; Ohta 2011; Sardesai 1991; Schiffman 1995; Suami 1997; Tinti 2000; van der Wel 1972; Witty 1990	Public

Topic	Location in this GRN	Source of data or information	Availability
Method of manufacture and QC	Section 2.2; APPENDIX B	Nomad Bioscience GmbH	This GRN
Host, vector and process impurities	Section 2.2; APPENDIX B; APPENDIX C	Nomad Bioscience GmbH	GRN 738 and This GRN
Composition and Specification	Section 2.3 Table 2-2	Nomad Bioscience GmbH	This GRN
Allowed maximum and moderate application rates; Estimated dietary intake; Additional exposure not related to Notifier's product	Sections 3.1, 3.2 and 3.3	OJEU 2011; EFSA 2008; EFSA 2011; EFSA 2015; Cohen 2016; Codex Alimentarius 2016; Nomad Bioscience GmbH	Public and This GRN
Dietary exposure to host- and process-derived impurities	Section 3.4	Nomad Bioscience GmbH	This GRN
Thaumatin overall safety; digestibility; potential for gene transfer to intestinal microflora	Section 6.1	JECFA 1983, 1986; EFSA 2015; SCF 1985, 1989; EFSA 2011; Higginbotham 1983; Hagiwara 2005; Szwacka 2012	Public
Estimation of thaumatin's NOAEL	Section 6.1	Higginbotham 1983; Hagiwara 2005	Public
Determination of thaumatin's allergenic potential	Section 6.1.1	a. Computational estimates / *in silico* modeling: Baniulis 2008; Nomad Bioscience GmbH b. Empirical studies, preclinical and clinical, Higginbotham 1983	a. Public and GRN 738 b. Public
Commonly consumed and environmentally contacted thaumatin-like proteins (TLP)	Section 6.1.1	Barre 2000; Liu 2010; Christensen 2002; van Loon 2006	Public and GRN 738
Allergenicity and occupational safety	Sections 6.1.1 and 6.4	Higginbotham 1983	Public
Safety in relation to allowed dietary intake of thaumatin	Sections 3.1, 3.2, 6.1 and 6.2	EFSA 2015; OJEU 2011; Codex Alimentarius 2016; Oser 1984; Smith 1996; Cohen 2016; Nomad Bioscience GmbH	Public and This GRN

Topic	**Location in this GRN**	**Source of data or information**	**Availability**
Potential additional exposure if consumption of low/no calorie sweeteners increases	Section 6.2	NHANES 2009-10, 2011-12 in Sylvetsky 2017; USDA ERS in Haley 2011, McConnell 2016; Ng 2012	Public
THAUMATIN II Safety Data Sheet (SDS)	APPENDIX A	a. Nomad Bioscience GmbH b. Sigma-Aldrich 2017; SFC 2006; SCBT 2015	a. This GRN b. Public
Thaumatin II analysis and characterization	APPENDIX C	Nomad Bioscience GmbH	This GRN

References

Adesina SK and JD Higginbotham. 1977. Studies on a novel polysaccharide gel from the fruit of Thaumatococcus daniellii (Benth). *Carbohydrate Research* 59(2):517-524.

Adesina SK and JB Harborne. 1978. Occurrence and identification of falvonoids in Thaumatococcus daniellii Benth. *Journal of Medicinal Plant Research* 34:323-327.

Andersson C, P Wennström and J Gry. 2003. Nicotine alkaloids in Solanaceous food plants. Nordic Working Group on Food Toxicology and Risk Evaluation. Nordic Council of Ministers. 92-893-0905-9. p 1-37.

Asherie N, C Ginsberg, A Greenbaum, S Blass and S Knafo. 2008. Effects of Protein Purity and Precipitant Stereochemistry on the Crystallization of Thaumatin†. *Crystal Growth & Design* 8(12):4200-4207.

ASTSDR. 1999. Public Health Statement: Mercury CAS#: 7439-97-6. CDC. https://www.atsdr.cdc.gov/PHS/PHS.asp?id=112&tid=24. Accessed Nov 21, 2018.

Baniulis D, V Stanys, G Vaitiekaitis, A Sasnauskas, D Gelvonauskienė and J Liobikas. 2008. Computational analysis of thaumatin-II allergenicity and prediction of antigenic elements of thaumatin-like family proteins. *Biologija* 54(3):202-207.

Barre A, WJ Peumans, L Menu-Bouaouiche, et al. 2000. Purification and structural analysis of an abundant thaumatin-like protein from ripe banana fruit. *Planta* 211(6):791-799.

Bartoszewski G, A Niedziela, M Szwacka and K Niemirowicz-Szczytt. 2003. Modification of tomato taste in transgenic plants carrying a thaumatin gene from Thaumatococcus daniellii Benth. *Plant Breeding* 122(4):347-351.

Baumung C, J Rehm, H Franke and DW Lachenmeier. 2016. Comparative risk assessment of tobacco smoke constituents using the margin of exposure approach: the neglected contribution of nicotine. *Sci Rep* 6:35577.

Benowitz NL, P Jacob, 3rd, RT Jones and J Rosenberg. 1982. Interindividual variability in the metabolism and cardiovascular effects of nicotine in man. *J Pharmacol Exp Ther* 221(2):368-372.

Benowitz NL, P Jacob, 3rd, C Denaro and R Jenkins. 1991. Stable isotope studies of nicotine kinetics and bioavailability. *Clin Pharmacol Ther* 49(3):270-277.

Benowitz NL. 1996. Cotinine as a biomarker of environmental tobacco smoke exposure. *Epidemiol Rev* 18(2):188-204.

Benowitz NL. 1999. Biomarkers of environmental tobacco smoke exposure. *Environ Health Perspect* 107 Suppl 2:349-355.

Bickoff EM, AN Booth, D de Fremery, et al. 1975. Nutritional evaluation of alfalfa leaf protein concentrate. In: *Protein Nutritional Quality of Foods and Feeds* Friedman M (ed). vol 2. Dekker, New York, p 319-340.

Block RJ and D Bolling. 1951. Amino Acid Composition of Proteins and Foods, Thomas CC (ed). 2nd edn, Springfield, Ill.

Bouzar H, D Ouadah, Z Krimi, et al. 1993. Correlative Association between Resident Plasmids and the Host Chromosome in a Diverse *Agrobacterium* Soil Population. *Applied and Environmental Microbiology* 59(5):1310-1317.

Cañizares MC, GP Lomonossoff and L Nicholson. 2005a. Development of cowpea mosaic virus-based vectors for the production of vaccines in plants. *Expert Rev Vaccines* 4(5):687-697.

Cañizares MC, L Nicholson and GP Lomonossoff. 2005b. Use of viral vectors for vaccine production in plants. *Immunol Cell Biol* 83(3):263-270.

Chen Q, J He, W Phoolcharoen and HS Mason. 2011. Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. *Hum Vaccin* 7(3):331-338.

Christensen AB, BH Cho, M Naesby, et al. 2002. The molecular characterization of two barley proteins establishes the novel PR-17 family of pathogenesis-related proteins. *Mol Plant Pathol* 3(3):135-144.

Codex Alimentarius. 2016. General Standard for Food Additives, CODEX STAN 192-1995 (Adopted in 1995. Revision 1997, 1999, 2001, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016). FAO/WHO.

Cohen SM, S Fukushima, NJ Gooderham, et al. 2016. FEMA GRAS Flavoring Substances 28 (Interim).

Cohen SM, G Eisenbrand, S Fukushima, et al. 2018. FEMA GRAS 28 Flavoring Substances.

Cusack M, AG Stephen, R Powls and RJ Beynon. 1991. Purification and characterization of thaumatopain, a cysteine protease from the arils of the plant Thaumatococcus daniellii. *Biochem J* 274 (Pt 1):231-236.

Daniell H, ND Singh, H Mason and SJ Streatfield. 2009. Plant-made vaccine antigens and biopharmaceuticals. *Trends Plant Sci* 14(12):669-679.

Davis RA, MF Stiles, JD deBethizy and JH Reynolds. 1991. Dietary nicotine: a source of urinary cotinine. *Food Chem Toxicol* 29(12):821-827.

de Vos AM, M Hatada, H van der Wel, H Krabbendam, AF Peerdeman and SH Kim. 1985. Three-dimensional structure of thaumatin I, an intensely sweet protein. *Proc Natl Acad Sci U S A* 82(5):1406-1409.

Dewey RE and J Xie. 2013. Molecular genetics of alkaloid biosynthesis in *Nicotiana tabacum*. *Phytochemistry* 94:10-27.

Digard H, C Proctor, A Kulasekaran, U Malmqvist and A Richter. 2013. Determination of nicotine absorption from multiple tobacco products and nicotine gum. *Nicotine Tob Res* 15(1):255-261.

Domino EF, E Hornbach and T Demana. 1993. The nicotine content of common vegetables. *N Engl J Med* 329(6):437.

EFSA. 2008. Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union*.

EFSA. 2011. Scientific Opinion on the Safety and Efficacy of thaumatin for all animal species. *EFSA Journal* 9(9):1-10.

EFSA. 2015. Scientific Opinion on the safety of the extension of use of thaumatin (E 957). *EFSA Journal* 13(11):4290.

Faus I and H Sisniega. 2008. Sweet-tasting proteins. *Biopolymers* 8:203-210.

FDA. 2013. Nicotine Replacement Therapy Labels May Change. FDA Consumer Health Information / U. S. Food and Drug Administration.

Fernstrom JD, SD Munger, A Sclafani, IE de Araujo, A Roberts and S Molinary. 2012. Mechanisms for sweetness. *J Nutr* 142(6):1134S-1141S.

Fu H, PA Machado, TS Hahm, RJ Kratochvil, CI Wei and YM Lo. 2010. Recovery of nicotine-free proteins from tobacco leaves using phosphate buffer system under controlled conditions. *Bioresour Technol* 101(6):2034-2042.

Gavilano LB, NP Coleman, LE Burnley, et al. 2006. Genetic engineering of *Nicotiana tabacum* for reduced nornicotine content. *J Agric Food Chem* 54(24):9071-9078.

Glaser D, M Wanner, JM Tinti and C Nofre. 2000. Gustatory responses of pigs to various natural and artificial compounds known to be sweet in man. *Food Chemistry* 68(4):375-385.

Gleba Y, S Marillonnet and V Klimyuk. 2004. Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Curr Opin Plant Biol* 7(2):182-188.

Gleba Y, V Klimyuk and S Marillonnet. 2005. Magnifection--a new platform for expressing recombinant vaccines in plants. *Vaccine* 23(17-18):2042-2048.

Gleba Y, V Klimyuk and S Marillonnet. 2007. Viral vectors for the expression of proteins in plants. *Curr Opin Biotechnol* 18(2):134-141.

Gleba YY, D Tuse and A Giritch. 2014. Plant viral vectors for delivery by *Agrobacterium*. *Curr Top Microbiol Immunol* 375:155-192.

Goodin MM, D Zaitlin, RA Naidu and SA Lommel. 2008. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. *Mol Plant Microbe Interact* 21(8):1015-1026.

Green C. 1999. Thaumatin: a natural flavour ingredient. *World Rev Nutr Diet* 85:129-132.

Greenly LW. 2003. A doctor's guide to sweeteners. *Journal of Chiropractic Medicine* 2(2):80-86.

Hagiwara A, H Yoshino, M Sano, et al. 2005. Thirteen-week feeding study of thaumatin (a natural proteinaceous sweetener), sterilized by electron beam irradiation, in Sprague-Dawley rats. *Food Chem Toxicol* 43(8):1297-1302.

Hahn S, A Giritch, D Bartels, L Bortesi and Y Gleba. 2015. A novel and fully scalable *Agrobacterium* spray-based process for manufacturing cellulases and other cost-sensitive proteins in plants. *Plant Biotechnol J* 13(5):708-716.

Haley S. 2011. Sugar and Sweeteners Outlook. USDA. Economic Research Service. vol SSS-M-270.

Hellekant G and V Danilova. 1996. Species differences toward sweeteners. *Food Chemistry* 56(3):323-328.

Hibi N, S Higashiguchi, T Hashimoto and Y Yamada. 1994. Gene expression in tobacco low-nicotine mutants. *Plant Cell* 6(5):723-735.

Higginbotham JD, DJ Snodin, KK Eaton and JW Daniel. 1983. Safety evaluation of thaumatin (Talin protein). *Food Chem Toxicol* 21(6):815-823.

Hildreth SB, EA Gehman, H Yang, et al. 2011. Tobacco nicotine uptake permease (NUP1) affects alkaloid metabolism. *Proc Natl Acad Sci U S A* 108(44):18179-18184.

Hough L. 1993. High-intensity, low-calorie sweeteners. In: *Low-calorie Foods and Food Ingredients* Khan R (ed). Blackie Academic & Professional, London.

Hung CY, L Fan, FS Kittur, et al. 2013. Alteration of the alkaloid profile in genetically modified tobacco reveals a role of methylenetetrahydrofolate reductase in nicotine *N*-demethylation. *Plant Physiol* 161(2):1049-1060.

ICH. 2015. Guidance for Industry Q3D Elemental Impurities.

Jacob P, 3rd, D Hatsukami, H Severson, S Hall, L Yu and NL Benowitz. 2002. Anabasine and anatabine as biomarkers for tobacco use during nicotine replacement therapy. *Cancer Epidemiol Biomarkers Prev* 11(12):1668-1673.

JECFA. 1983. Evaluation of certain food additives and contaninants. WHO. vol 696.

JECFA. 1986. Evaluation of certain food additives and contaminants. WHO. vol 733.

Kajikawa M, N Hirai and T Hashimoto. 2009. A PIP-family protein is required for biosynthesis of tobacco alkaloids. *Plant Mol Biol* 69(3):287-298.

Kaneko R and N Kitabatake. 2001. Sweetness of sweet protein thaumatin is more thermoresistant under acid conditions than under neutral or alkaline conditions. *Biosci Biotechnol Biochem* 65(2):409-413.

Karačonji IB. 2005. Facts about nicotine toxicity. *Arh Hig Rada Toksikol* 56(4):363-371.

Karg SR and PT Kallio. 2009. The production of biopharmaceuticals in plant systems. *Biotechnol Adv* 27(6):879-894.

Katoh A, H Ohki, K Inai and T Hashimoto. 2005. Molecular regulation of nicotine biosynthesis. *Plant Biotechnology* 22(5):389-392.

Kim S-H, A de Vos and C Ogata. 1988. Crystal structures of two intensely sweet proteins. *Trends Biochem Sci* 13(1):13-15.

Kinghorn AD, DD Soejarto and GE Inglett. 1986. Sweetening agents of plant origin*. *Critical Reviews in Plant Sciences* 4(2):79-120.

Klimyuk V, G Pogue, S Herz, J Butler and H Haydon. 2014. Production of Recombinant Antigens and Antibodies in *Nicotiana benthamiana* Using 'Magnifection' Technology: GMP-Compliant Facilities for Small- and Large-Scale Manufacturing. *Curr Top Microbiol Immunol* 375:127-154.

Komarova TV, S Baschieri, M Donini, C Marusic, E Benvenuto and YL Dorokhov. 2010. Transient expression systems for plant-derived biopharmaceuticals. *Expert Rev Vaccines* 9(8):859-876.

Krimi Z, A Petit, C Mougel, Y Dessaux and X Nesme. 2002. Seasonal fluctuations and long-term persistence of pathogenic populations of *Agrobacterium* spp. in soils. *Appl Environ Microbiol* 68(7):3358-3365.

Kung SD and TC Tso. 1978. Tobacco as a Potential Food Source and Smoke Material: Soluble Protein Content, Extraction, and Amino Acid Composition. *Journal of Food Science* 43(6):1844-1847.

Kung SD, JA Saunder, TC Tso, et al. 1980. Tobacco as a Potential Food Source and Smoke Material: Nutrional Evaluation of Tobacoo Leaf Protein. *Journal of Food Science* 45(2):320-322.

Ledeboer AM, CT Verrips and BM Dekker. 1984. Cloning of the natural gene for the sweet-tasting plant protein thaumatin. *Gene* 30(1-3):23-32.

Lee C-K. 1987. The Chemistry and Biochemistry of the Sweetness of Sugars. *Advances in Carbohydrate Chemistry and Biochemistry* 45:199-351.

Leffingwell JC. 1999. Basic Chemical Constituents of Tobacco Leaf and Differences among Tobacco Types. In: *Tobacco: Production, Chemistry, And Technology* Davis DL, Nielson MT (eds). Blackwell Science.

Li X, L Staszewski, H Xu, K Durick, M Zoller and E Adler. 2002. Human receptors for sweet and umami taste. *Proc Natl Acad Sci U S A* 99(7):4692-4696.

Lico C, Q Chen and L Santi. 2008. Viral vectors for production of recombinant proteins in plants. *J Cell Physiol* 216(2):366-377.

Lim TK. 2012. *Thaumatococcus daniellii*. In: *Edible Medicinal And Non Medicinal Plants: Volume 3, Fruits*. Springer Netherlands, Dordrecht, p 259-264.

Ling HY, AM Edwards, MP Gantier, et al. 2012. An interspecific *Nicotiana* hybrid as a useful and cost-effective platform for production of animal vaccines. *PLoS One* 7(4):e35688.

Liu JJ, R Sturrock and AK Ekramoddoullah. 2010. The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. *Plant Cell Rep* 29(5):419-436.

Liu R, RA Vaishnav, AM Roberts and RP Friedland. 2013. Humans have antibodies against a plant virus: evidence from tobacco mosaic virus. *PLoS One* 8(4):e60621.

Margolskee RF. 2001. Molecular mechanisms of bitter and sweet taste transduction. *J Biol Chem* 277(1):1-4.

Masuda T, K Ohta, F Tani, B Mikami and N Kitabatake. 2011. Crystal structure of the sweet-tasting protein thaumatin II at 1.27Å. *Biochem Biophys Res Commun* 410(3):457-460.

Masuda T, W Taguchi, A Sano, K Ohta, N Kitabatake and F Tani. 2013. Five amino acid residues in cysteine-rich domain of human T1R3 were involved in the response for sweet-tasting protein, thaumatin. *Biochimie* 95(7):1502-1505.

Masuda T, B Mikami and F Tani. 2014. Atomic structure of recombinant thaumatin II reveals flexible conformations in two residues critical for sweetness and three consecutive glycine residues. *Biochimie* 106:33-38.

Masuda T, K Ohta, N Ojiro, et al. 2016. A Hypersweet Protein: Removal of The Specific Negative Charge at Asp21 Enhances Thaumatin Sweetness. *Sci Rep* 6:20255.

Masuda T, S Kigo, M Mitsumoto, et al. 2018. Positive Charges on the Surface of Thaumatin Are Crucial for the Multi-Point Interaction with the Sweet Receptor. *Front Mol Biosci* 5:10.

Mayer B. 2014. How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century. *Arch Toxicol* 88(1):5-7.

McConnell M. 2016. Sugar and Sweeteners Outlook. USDA. Economic Research Service. vol SSS-M-329.

McCormick AA and KE Palmer. 2008a. Genetically engineered Tobacco mosaic virus as nanoparticle vaccines. *Expert Rev Vaccines* 7(1):33-41.

McCormick AA, S Reddy, SJ Reinl, et al. 2008b. Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. *Proc Natl Acad Sci U S A* 105(29):10131-10136.

Mett V, CE Farrance, BJ Green and V Yusibov. 2008. Plants as biofactories. *Biologicals* 36(6):354-358.

Moldoveanu SC, WA Scott and DM Lawson. 2016. Nicotine Analysis in Several Non-Tobacco Plant Materials. *Beiträge zur Tabakforschung International/Contributions to Tobacco Research* 27(2).

Mortimer E, JM Maclean, S Mbewana, et al. 2012. Setting up a platform for plant-based influenza virus vaccine production in South Africa. *BMC Biotechnol* 12:14.

Most BH, RJ Summerfield and M Boxall. 1978. Tropical Plants with Sweetening Properties Physiological and Agronomic Problems of Protected Cropping: 2. *Thaumatococcus daniellii*. *Economic Botany* 32(3):321-335.

Mougel C, B Cournoyer and X Nesme. 2001. Novel tellurite-amended media and specific chromosomal and Ti plasmid probes for direct analysis of soil populations of *Agrobacterium* biovars 1 and 2. *Appl Environ Microbiol* 67(1):65-74.

Nagarajan R. 2017. Amazing, super-sweet natural proteins: A look at three alternate natural sweeteners that could be safe for our health. O'Reilly Media. https://www.oreilly.com/ideas/amazing-super-sweet-natural-proteins. Accessed 7/1/2017 2017.

Natex. 2017. The Science Of Thaumatin. http://www.thaumatin.com/science-of-thaumatin. Accessed 8/16/2017 2017.

Nelson G, MA Hoon, J Chandrashekar, Y Zhang, NJ Ryba and CS Zuker. 2001. Mammalian sweet taste receptors. *Cell* 106(3):381-390.

Ng SW, MM Slining and BM Popkin. 2012. Use of caloric and noncaloric sweeteners in US consumer packaged foods, 2005-2009. *J Acad Nutr Diet* 112(11):1828-1834 e1821-1826.

Nielsen SS, GM Franklin, WT Longstreth, PD Swanson and H Checkoway. 2013. Nicotine from edible *Solanaceae* and risk of Parkinson disease. *Ann Neurol* 74(3):472-477.

Nikolic J, I Mrkic, M Grozdanovic, et al. 2014. Protocol for simultaneous isolation of three important banana allergens. *J Chromatogr B Analyt Technol Biomed Life Sci* 962:30-36.

Ogata CM, PF Gordon, AM de Vos and SH Kim. 1992. Crystal structure of a sweet tasting protein thaumatin I, at 1.65 Å resolution. *J Mol Biol* 228(3):893-908.

Ohta K, T Masuda, F Tani and N Kitabatake. 2011. The cysteine-rich domain of human T1R3 is necessary for the interaction between human T1R2-T1R3 sweet receptors and a sweet-tasting protein, thaumatin. *Biochem Biophys Res Commun* 406(3):435-438.

OJEU. 2011. Annex II Regulations: Amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union*.

Okamoto M, T Kita, H Okuda, T Tanaka and T Nakashima. 1994. Effects of aging on acute toxicity of nicotine in rats. *Pharmacol Toxicol* 75(1):1-6.

Oser BL, RA Ford and BK Bernard. 1984. Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment. 13. GRAS Substances. *Food Technology* 38(10):66-89.

Pelosi A, R Shepherd and AM Walmsley. 2012. Delivery of plant-made vaccines and therapeutics. *Biotechnol Adv* 30(2):440-448.

Penyalver R and MM Lopez. 1999. Cocolonization of the rhizosphere by pathogenic *Agrobacterium* strains and nonpathogenic strains K84 and K1026, used for crown gall biocontrol. *Appl Environ Microbiol* 65(5):1936-1940.

Pogue GP, F Vojdani, KE Palmer, et al. 2010. Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnol J* 8(5):638-654.

Ramsohoye PV and IA Kozlov. 1989. An investigation of enzyme activities found in thaumatin preparations. *International Journal of Food Science & Technology* 24(6):619-627.

Russell MA, MJ Jarvis, C Feyerabend and O Ferno. 1983. Nasal nicotine solution: a potential aid to giving up smoking? *Br Med J (Clin Res Ed)* 286(6366):683-684.

Ryan PB, KA Scanlon and DL MacIntosh. 2001. Analysis of dietary intake of selected metals in the NHEXAS-Maryland investigation. *Environ Health Perspect* 109(2):121-128.

Rybicki EP. 2009. Plant-produced vaccines: promise and reality. *Drug Discov Today* 14(1-2):16-24.

Sainsbury F, L Liu and GP Lomonossoff. 2009. Cowpea mosaic virus-based systems for the expression of antigens and antibodies in plants. *Methods Mol Biol* 483:25-39.

Saitoh F, M Noma and N Kawashima. 1985. The alkaloid contents of sixty *Nicotiana* species. *Phytochemistry* 24(3):477-480.

Sardesai VM and TH Waldshan. 1991. Natural and synthetic intense sweeteners. *The Journal of Nutritional Biochemistry* 2(5):236-244.

Saunders K and GP Lomonossoff. 2013. Exploiting plant virus-derived components to achieve *in planta* expression and for templates for synthetic biology applications. *New Phytol* 200(1):16-26.

SCBT. 2015. Thaumatin Safety Data Sheet. *Santa Cruz Biotechnology I*. SC-296528; CAS 53850-34-3.

SCF. 1985. Reports of the Scientific Committee for Food concerning sweeteners (opinion expressed by the SCF on 14 September 1984). In: *Food Science and Techniques Reports of the Scientific Committee for Food (16th series)*. Commission of the European Communities, Directorate-General for Health and Consumer Protection, Scientific Committee for Food, Brussels, Belgium.

SCF. 1989. Reports of the Scientific Committee for Food on Sweeteners (opinion expressed 11 December 1987 and 10 November 1988, and adopted 10 November 1988). In: *Food Science and Techniques Reports of the Scientific Committee for Food (21st series)*. Commission of the European Communities, Directorate-General for Health and Consumer Protection, Scientific Committee for Food, Brussels, Belgium.

Schiffman SS, BJ Booth, ML Losee, SD Pecore and ZS Warwick. 1995. Bitterness of sweeteners as a function of concentration. *Brain Research Bulletin* 36(5):505-513.

SFC. 2006. Thaumatin Safety Data Sheet. *Chemical SF*. CAS 53850-34-3.

Shallenberger RS. 1993. Amino Acids, Peptides, and Proteins. In: *Taste Chemistry*. Springer US, Boston, MA, p 250-252.

Shoji T and T Hashimoto. 2013. Smoking out the masters: transcriptional regulators for nicotine biosynthesis in tobacco. *Plant Biotechnology* 30(3):217-224.

Siceloff B. 1981. Tobacco for protein: a revolutionary upheaval? *NC Insight*:28-32.

Siegmund B, E Leitner and W Pfannhauser. 1999. Determination of the nicotine content of various edible nightshades (Solanaceae) and their products and estimation of the associated dietary nicotine intake. *J Agric Food Chem* 47(8):3113-3120.

Sigma-Aldrich. 2017. Thaumatin Safety Data Sheet. *Aldrich S*. T7638.

Sisson VA and RF Severson. 1990. Alkaloid Composition of the *Nicotiana* Species Beiträge zur Tabakforschung International/Contributions to Tobacco Research. vol 14, p 327.

Smith ML, WP Fitzmaurice, TH Turpen and KE Palmer. 2009. Display of peptides on the surface of tobacco mosaic virus particles. *Curr Top Microbiol Immunol* 332:13-31.

Smith RL, P Newberne, TB Adams, RA Ford, JB Hallagan and FEMA. 1996. GRAS Flavoring Substances 17. *Food Technology* 50(10):72-78, 80-81.

Stephan A, S Hahn-Lobmann, F Rosche, M Buchholz, A Giritch and Y Gleba. 2017. Simple Purification of *Nicotiana benthamiana*-Produced Recombinant Colicins: High-Yield Recovery of Purified Proteins with Minimum Alkaloid Content Supports the Suitability of the Host for Manufacturing Food Additives. *Int J Mol Sci* 19(1).

Stephen AG, R Powls and RJ Beynon. 1991. The relationship between thaumatin, a sweet protein and thaumatopain, a cysteine protease, from the arils of *Thaumatococcus daniellii*. *Biochem Soc Trans* 19:297S-297S.

Steppuhn A, K Gase, B Krock, R Halitschke and IT Baldwin. 2004. Nicotine's defensive function in nature. *PLoS Biol* 2(8):E217.

Stockwell VO, LW Moore and JE Loper. 1993. Fate of *Agrobacterium radiobacter* K84 in the environment. *Appl Environ Microbiol* 59(7):2112-2120.

Suami T, L Hough, T Machinami, N Watanabe and R Nakamura. 1997. Molecular mechanisms of sweet taste 7: The sweet protein, Thaumatin I. *Food Chemistry* 60(3):277-285.

Sylvetsky AC, Y Jin, EJ Clark, JA Welsh, KI Rother and SA Talegawkar. 2017. Consumption of Low-Calorie Sweeteners among Children and Adults in the United States. *J Acad Nutr Diet* 117(3):441-448 e442.

Szwacka M, W Burza, R Zawirska-Wojtasiak, et al. 2012. Genetically Modified Crops Expressing 35S-Thaumatin II Transgene: Sensory Properties and Food Safety Aspects. *Comprehensive Reviews in Food Science and Food Safety* 11(2):174-186.

Takizawa M, K Hori, K Inai, H Takase, T Hashimoto and Y Watanabe. 2007. A virus-induced gene silencing approach for the suppression of nicotine content in *Nicotiana benthamiana*. *Plant Biotechnology* 24(3):295-300.

Tinti JM, D Glaser, M Wanner and C Nofre. 2000. Comparison of Gustatory Responses to Amino Acids in Pigs and in Humans. *LWT - Food Science and Technology* 33(8):578-583.

Todd AT, E Liu, SL Polvi, RT Pammett and JE Page. 2010. A functional genomics screen identifies diverse transcription factors that regulate alkaloid biosynthesis in *Nicotiana benthamiana*. *Plant J* 62(4):589-600.

Tusé D. 2011. Safety of plant-made pharmaceuticals: Product development and regulatory considerations based on case studies of two autologous human cancer vaccines. *Human Vaccines* 7(3):322-330.

Tusé D, T Tu and KA McDonald. 2014. Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *Biomed Res Int* 2014:256135.

Tusé D, N Ku, M Bendandi, et al. 2015. Clinical Safety and Immunogenicity of Tumor-Targeted, Plant-Made Id-KLH Conjugate Vaccines for Follicular Lymphoma. *Biomed Res Int* 2015:648143.

USDA ERS. 2019a. Sugar and Sweeteners Yearbook, Table 52, updated 6/24/2019. https://www.ers.usda.gov/data-products/sugar-and-sweeteners-yearbook-tables/. Accessed October 2019.

USDA ERS. 2019b. Sugar and Sweeteners Yearbook, Table 51, updated 6/24/2019. https://www.ers.usda.gov/data-products/sugar-and-sweeteners-yearbook-tables/. Accessed October 2019.

van der Wel H and K Loeve. 1972. Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from *Thaumatococcus daniellii* Benth. *Eur J Biochem* 31(2):221-225.

van der Wel H, RB Iyengar, J van Brouwershaven, PD van Wassenaar, WJ Bel and FJ van der Ouderaa. 1984. Assignment of the disulphide bonds in the sweet-tasting protein thaumatin I. *Eur J Biochem* 144(1):41-45.

van Loon LC, M Rep and CM Pieterse. 2006. Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol* 44:135-162.

Vicedo B, R Penalver, MJ Asins and MM Lopez. 1993. Biological Control of *Agrobacterium tumefaciens*, Colonization, and pAgK84 Transfer with *Agrobacterium radiobacter* K84 and the Tra^{-} Mutant Strain K1026. *Appl Environ Microbiol* 59(1):309-315.

Vigues S, CD Dotson and SD Munger. 2008. The receptor basis of sweet taste in mammals. *Results Probl Cell Differ* 47:187-202.

Vuong T, X Zhang and A Roeseler. 2019. California Tobacco Facts and Figures 2019. Sacramento, CA: California Department of Public Health. May 2019.

Wang B, RS Lewis, J Shi, et al. 2015. Genetic Factors for Enhancement of Nicotine Levels in Cultivated Tobacco. *Sci Rep* 5:17360.

Wang ET, R Sandberg, S Luo, et al. 2008. Alternative isoform regulation in human tissue transcriptomes. *Nature* 456(7221):470-476.

Werner S, O Breus, Y Symonenko, S Marillonnet and Y Gleba. 2011. High-level recombinant protein expression in transgenic plants by using a double-inducible viral vector. *Proc Natl Acad Sci U S A* 108(34):14061-14066.

Whaley KJ, A Hiatt and L Zeitlin. 2011. Emerging antibody products and Nicotiana manufacturing. *Hum Vaccin* 7(3):349-356.

Wildman S. 1983. An Alternate Use for Tobacco Agriculture: Proteins for Food Plus a Safer Smoking Material. In: *Plants: The Potentials for Extracting Protein, Medicines, and Other Useful Chemicals - Workshop Proceedings*. U.S. Congress, Office of Techology Assessment, OTA-BP-F-23, Washington, D.C., p 63-77.

Witty M. 1990. Thaumatin II — a palatability protein. *Trends in Biotechnology* 8:113-116.

Witty M. 1998. New technologies for taste modifying proteins. *Trends in Food Science & Technology* 9(7):275-280.

Yang SJ, SA Carter, AB Cole, NH Cheng and RS Nelson. 2004. A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana*. *Proc Natl Acad Sci U S A* 101(16):6297-6302.

Yusibov V and S Rabindran. 2008. Recent progress in the development of plant derived vaccines. *Expert Rev Vaccines* 7(8):1173-1183.

Zemanek EC and BP Wasserman. 1995. Issues and advances in the use of transgenic organisms for the production of thaumatin, the intensely sweet protein from *Thaumatococcus danielli*. *Crit Rev Food Sci Nutr* 35(5):455-466.

APPENDIX A. THAUMATIN II Safety Data Sheet

NOMAD

DRAFT– THAUMATIN II SAFETY DATA SHEET
Version 2.0
Revision Date 10/30/2019
Print Date 10/30/2019

1. PRODUCT AND COMPANY IDENTIFICATION

1.1 Product identifiers

Product name	:	THAUMATIN II from plants (e.g. lettuce, red beet, spinach, *Nicotiana benthamiana*)
	:	CAS Number 53850-34-3 (extracted); CAS Number 553850-34-3 (recombinant)
	:	Consists of Thaumatin II of identical amino acid sequence to thaumatin II from *Thaumatococcus daniellii*

1.2 Functional uses

Identified uses	:	Sweetener for use in food and beverages

1.3 Manufacturer

Company	:	Nomad Bioscience GmbH Weinbergweg 22 Halle 02160, Germany
Telephone	:	+49 345 1314 2606
Fax	:	+49 345 1314 2601

1.4 Emergency telephone number

Emergency Phone Number	:	+49 345 1314 2424 in the EU (US emergency phone number to be provided)

2. HAZARDS IDENTIFICATION

2.1 Classification of the substance or mixture
Not a hazardous substance or mixture

2.2 GHS Label elements, including precautionary statements
Not a hazardous substance or mixture

2.3 Hazards not otherwise classified (HNOC) or not covered by GHS
None

2.4 HMIS Classification

Health hazard:	0
Flammability:	0
Physical hazards:	0

2.5 NFPA Rating

Health hazard:	0
Fire:	0
Reactivity hazard:	0

2.6 Potential Health Effects of Bulk Powder/Concentrate

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation
Skin	May be harmful if absorbed through skin. May cause skin irritation
Eyes	May cause eye irritation
Ingestion	May be harmful if swallowed

3. COMPOSITION/INFORMATION ON INGREDIENTS

3.1 Substances

CAS Registry Number	:	53850-34-3

No ingredients are hazardous according to OSHA criteria
No components need to be disclosed according to the applicable regulations

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4. FIRST AID MEASURES

4.1 Description of first aid measures

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration

In case of skin contact
Wash off with soap and plenty of water

In case of eye contact
Flush eyes with water as a precaution

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water

5. FIREFIGHTING MEASURES

5.1 Extinguishing media - Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide

5.2 Hazardous combustion products
Hazardous decomposition products formed under fire conditions – Carbon oxides

5.3 Advice for firefighters
Wear self-contained breathing apparatus for firefighting if necessary

5.4 Additional information
No additional information is available

6. ACCIDENTAL RELEASE MEASURES

6.1 Personal precautions, protective equipment and emergency procedures
As with any concentrated protein, avoid dust formation and inhalation of particulates or aerosols. For personal protection see Section 8

6.2 Environmental precautions
Product active ingredients are biodegradable. No special environmental precautions are necessary

6.3 Methods and materials for containment and cleaning up
Sweep up and shovel solid. Water-wash surfaces. Use closed containers for disposal of any unused product

6.4 Reference to other sections
For disposal see Section 13

7. HANDLING AND STORAGE

7.1 Precautions for safe handling
Provide appropriate exhaust ventilation during preparation and use. Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection

7.2 Conditions for safe storage, including any incompatibilities
Keep container tightly closed in a dry well-ventilated place. Recommended storage temperature 2-8 °C

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Control parameters

Components with workplace control parameters
Contains no substances with occupational exposure limit values

8.2 Exposure controls

Appropriate engineering controls
General industrial hygiene practice

Personal protective equipment

Eye/face protection

Use government tested and approved eye protection devices (e.g. NIOSH - US or EN 166 - EU)

Skin protection

Handle with gloves that are inspected prior to use. Use proper glove removal technique to avoid skin contact. Dispose of used gloves in accordance with applicable laws and good laboratory practices. Wash and dry hands

Body Protection

Wear lab coat or similar cover during preparation, application and disposal of product in keeping with specific practices in the work environment

Respiratory protection

Use type N95 (US) or type P1 (EN 143) dust masks, or respirators, depending on the product formulation and preparation and use environment. Use devices approved under appropriate government standards such as NIOSH (US) or CEN (EU)

Control of environmental exposure

Product components are biodegradable and will be diluted during use. No special procedures for controlling environmental exposure are recommended

9. PHYSICAL AND CHEMICAL PROPERTIES

9.1 Information on basic physical and chemical properties

a)	Appearance form:	Solid; yellowish-white to light tan
b)	Odor	No specific odor
c)	Odor threshold	No odor threshold identified
d)	pH	pH 2.7 – 6, depending on the formulation
e)	Melting point/freezing point	No data available
f)	Initial boiling point and boiling range	No data available
g)	Flash point	No data available
h)	Evaporation rate	No data available
i)	Flammability (solid, gas)	No data available
j)	Upper/lower flammability or explosive limits	No data available
k)	Vapor pressure	No data available
l)	Vapor density	No data available
m)	Relative density	No data available
n)	Water solubility	>25 g/L
o)	Partition coefficient: n-octanol/water	No data available
p)	Auto-ignition temperature	No data available
q)	Decomposition temperature	No data available
r)	Viscosity	No data available
s)	Explosive properties	No data available
t)	Oxidizing properties	No data available

9.2 Other safety information

No additional information available

10. STABILITY AND REACTIVITY

10.1 Reactivity

No data available

10.2 Chemical stability

Stable under recommended storage conditions

10.3 Possibility of hazardous reactions

No data available

10.4 Conditions to avoid
Strong oxidizing agents

10.5 Incompatible materials
No data available

10.6 Hazardous decomposition products
Other decomposition products – Carbon oxides. In the event of fire: See Section 5

11. TOXICOLOGICAL INFORMATION

11.1 Information on toxicological effects

Acute toxicity
No data available

Inhalation
No data available

Dermal
No data available

Skin corrosion/irritation
No data available

Serious eye damage/eye irritation
No data available

Respiratory or skin sensitization
No data available

Germ cell mutagenicity
No data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as a probable, possible or confirmed human carcinogen by IARC

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH

NTP: No component of this product present at levels greater than or equal to 0.1% is identified as a known or anticipated carcinogen by NTP

OSHA: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by OSHA

Reproductive toxicity
No data available

Specific target organ toxicity - single exposure
No data available

Specific target organ toxicity - repeated exposure
No data available

Aspiration hazard
No data available

Additional Information
RTECS: Not available. Product is not a hazardous substance or mixture

11.2 Potential health effects

Inhalation: May be harmful if inhaled. May cause respiratory tract irritation
Ingestion: May be harmful if swallowed
Skin: May be harmful if absorbed through skin. May cause skin irritation
Eyes: May cause eye irritation

12. ECOLOGICAL INFORMATION

12.1 Toxicity
No data available

12.2 Persistence and degradability
Active ingredients are destroyed by heat, acid, and by digestive and microbial enzymatic activity

12.3 Bioaccumulative potential
None anticipated

12.4 Mobility in soil
No data available

12.5 Results of PBT and vPvB assessment
PBT/vPvB assessment not available as chemical safety assessment not required/not conducted

12.6 Other adverse effects
No data available

13. DISPOSAL CONSIDERATIONS

13.1 Waste treatment methods

Product
Offer surplus and non-recyclable solutions to a licensed disposal company

Contaminated packaging
Dispose of any unused product

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

OSHA
No known OSHA hazards

SARA 302 Components
SARA 302: No chemicals in this material are subject to the reporting requirements of SARA Title III, Section 302

SARA 313 Components
SARA 313: This material does not contain any chemical components with known CAS numbers that exceed the threshold (De Minimis) reporting levels established by SARA Title III, Section 313

SARA 311/312 Hazards
No SARA Hazards

Massachusetts Right to Know Components
No components are subject to the Massachusetts Right to Know Act

Pennsylvania Right to Know Components
Thaumatins from plants. See Section 1.1

New Jersey Right to Know Components
Thaumatins from plants. See Section 1.1

California Prop. 65 Components
This product does not contain any chemicals known to State of California to cause cancer, birth defects, or any other reproductive harm

16. OTHER INFORMATION

Additional information

The information contained in this Safety Data Sheet is believed to be correct as of the time of its release. It should be used as a guide for safe handling, storage, preparation, and disposal of the product. Assessment of product safety under conditions of normal use is based on information available at the time. Because information in some categories is lacking, this SDS is not all-inclusive and is subject to periodic updates. Nomad Bioscience GmbH and its Affiliates shall not be held liable for any damage resulting from handling, use, disposal or from contact with the above product.

In-Process controls and quality assurance

Notifier applies rigorous in-process controls to manage the quality of process intermediates and final products throughout the manufacturing process. Materials not meeting pre-determined specifications are rejected. Product release is done after each batch passes identity and purity tests. A Quality Management system is in place to ensure conformance with industry standards and federal and local regulatory guidelines.

B.4. Manufacturing Facilities

Notifier can manufacture THAUMATIN II product at various locations in Europe and the United States. For commercial manufacture, semi-automated plant cultivation, inoculation, incubation and harvesting systems can be applied. Depending on the scale needed, Notifier can manufacture at its own facilities or use a contract manufacturing organization to produce and formulate thaumatin proteins meeting Notifier's specification. Features of an existing US facility's upstream and downstream processing capabilities include:

Upstream

- 80,000 sq ft of controlled growth space with 672 tables holding 30,240 plant trays in 3 levels. Each tray holds 104 plants; controlled conditions for the growth and harvest of transfected plants
- An automated plant transport system allowing movement, irrigation, lighting and environmental control (temperature and humidity) of trays for plant growth

Downstream

- 32,000 sq ft manufacturing area
- Linear scalability: 1 metric ton (mt)/shift pilot scale – 68 mt/shift commercial scale
- 75 L of Green Juice (post-grind/pre-clarification extract) per minute; continuous processing up to UF
- 35,000 L of tank storage capacity; heating and cooling control of in-process material
- Manufacturing clean rooms with controlled environments
- Computer-controlled processing and data collection
- Clarification options (UF/DF/Microfiltration/Nanofiltration/Reverse Osmosis)

Regardless of manufacturing venue, all substances, materials and reagents used in manufacturing THAUMATIN II by Notifier's process conform to food grade or higher standards. All processing equipment is high-grade stainless steel meeting food-industry criteria. All cleaning and sterilization procedures are validated in accordance with FDA guidelines for food-grade materials.

B.5. Waste Handling and Disposal

Waste streams containing plant-derived residuals are treated per local regulations and discarded. No by-products of the process are used in food or feed products, supplements, additives or processing aids.

B.6. Justification of Specification

A Specification for the plant-expressed THAUMATIN II product (final bulk) produced through the manufacturing process described herein and using *N. benthamiana* as the host plant is shown in Table 2-2. The current values for parameters used to establish and justify the target Specification for *N. benthamiana*-produced THAUMATIN II sweetener containing Thaumatin II as the active ingredient are shown in Table B-1.

The Specification limits for alkaloid impurities in the final product shown in Table B-1 were derived from analyses of two bench-scale and one pilot-scale Thaumatin II batches (discussed in APPENDIX C, Section C-5).

The Specification limits for lead (Pb) and the sum of Class 1 (Pb, Cd, Hg, As) elemental impurities were also established from results of analyses of two bench-scale developmental batches and one pilot-scale manufacturing batch (APPENDIX C, Section C-6). Limits for the less toxic Class 2 and Class 3 metals were not included in the Specification because analyses of the pilot-scale manufacturing batch yielded very low levels of these impurities (i.e. sum of Ag, Bi, Cu, Mo, Sb and Sn = < 1 ng total metal/mg Thaumatin II protein).

Table B-1. Justification of Specification Release Limits for THAUMATIN II Product

Summary of current THAUMATIN II Sweetener Conformance with Target Specification			
Parameter	**Method**	**Specification limit**	**Results of analyses [1]**
Appearance	Visual	Powder, yellowish-lt. tan	Conforms
Minimum total thaumatin content	CGE	≥ 95% of total protein	Ave: 97.7 ± 1.19% Range: 96.1% – 99.4%
Solubility (DI water)	Visual	≥ 200 mg/mL	Conforms
pH of a 1% solution in water	Potentiometric	2.7 – 6.0	Conforms
Heavy metals (sum of Class 1 metals Pb, Cd, Hg, As) [2]	USP38<233>	≤ 5 ng/mg thaumatin	Ave: 0.232 ± 0.048 ng/mg
Lead [2]	USP38<233	≤ 1 ng/mg thaumatin	Ave: 0.126 ± 0.22 ng/mg
Nicotine (per total thaumatin) [2]	HPLC/MS	≤ 20 ng/mg thaumatin	Ave: 14.41 ± 0.67 ng/mg
Anabasine (per total thaumatin) [2]	HPLC/MS	≤ 5 ng/mg thaumatin	Ave: 2.52 **±** 1.55 ng/mg
Bioburden	USP32<61>	≤ 5,000 CFU total per g	0 (absent)
Agrobacterium (CFU/10 g sample)	Selective plate based assay	0 (absent)	0 (absent)
Undesirable microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g final product	USP32<1111>	0 (absent)	0 (absent)
Stability of dry product (0-20°C) [3] as <3% loss of thaumatin protein	CGE; THM peak at T_m vs. T_0	≥ 6 months	> 11 months (at time of GRN submission)

[1] Results of analyses of dry, purified Thaumatin II protein were obtained from three non-consecutive batches. Methods of analysis and results to support the Specification are discussed in APPENDIX C.

[2] Elemental and alkaloid impurity Specification limits were derived from results of analyses of 3 non-consecutive developmental batches, including two bench-scale batches and one pilot-scale manufacturing batch.

[3] Stability results were interim at the time of submission and were based on samples in an on-going stability program. Stability is calculated from Thaumatin II protein peak determined by CGE for 1% (w/v) Thaumatin II solution in water at T_0 and at various durations of storage expressed in months (T_m) using samples from a minimum of 3 non-consecutive developmental batches. Product is considered stable if there is ≤3% loss of Thaumatin II protein at the time of sampling.

Thaumatin II was stable during storage under the conditions indicated. The percent purity values shown in Table C-1 are averages of two replicate analyses. The active ingredient in THAUMATIN II sweetener, Thaumatin II, was found stable for 12 months when stored at 4°C, with less than 3% loss of purity over that storage time. Some batches of Thaumatin II were also stable at room temperature (RT) for up to 11 months. Generally, cold storage (4°C-10°C) is expected to provide greater stability, hence enabling longer duration of product storage. Storage conditions will continue to be defined and optimized during product development.

Table C-1. Stability of Thaumatin II during storage

	Thaumatin II Purity During Storage							
	Storage at 4°C				**Storage at RT (~22°C)**			
Protein	**Time of storage (months)**				**Time of storage (months)**			
	0	9	11	12	0	9	11	12
Thaumatin II								
Batch 5	98.0			96.15				
Batch 17	96.2			95.35	96.2		94.85	
Batch 31	99.4	96.15			99.4	94.55		
Thaumatin protein purity (thaumatin protein as percent of total protein) was maintained when dry protein powders were stored over prolonged periods. None of the samples of Thaumatin II showed degradation fragments or aggregation upon storage at either 4°C or at room temperature (~22°C).								

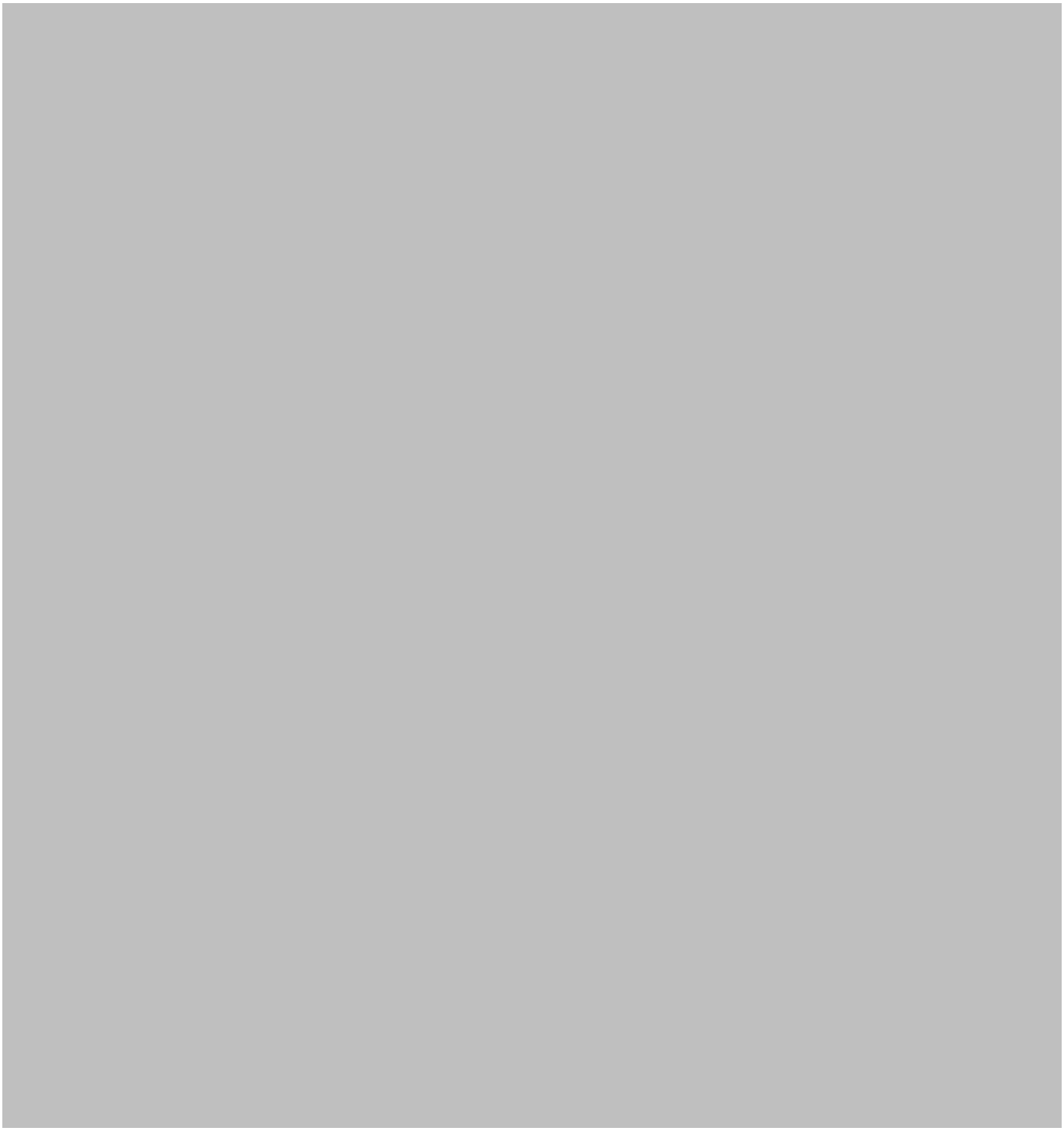
C.4. Manufacturing Yield and Consistency

The manufacturing yield, consistency, and product purity were determined from multiple non-sequential batches of Thaumatin II produced during product and process development at bench scale. Consistency is expected to increase as the process is upgraded and optimized at pilot-scale and eventually at industrial scale. Table C-2 summarizes multi-batch yield and purity of *N. nicotiana*-produced Thaumatin II.

Table C-2. Consistency of production yield and purity of Thaumatin II

Protein	**Yield of purified protein (mg/kg FW plant biomass)**		**Number non-consecutive batches analyzed**	**Thaumatin purity (% of total protein)**		**Number non-consecutive batches analyzed**
	Single batches	**Ave. ± SD**		**Single batches**	**Ave. ± SD**	
Thaumatin II (bench-scale)	710	550 ± 160	6	98.0	97.8 ± 1.55	4
	240			96.1		
	420			97.3		
	470			99.4		
	540					
	610					
Thaumatin II (pilot-scale)	117	-	1	98.2	-	1

Table C-2 lists results of analyses of individual production batches at developmental (bench) scale. The yield of purified protein per unit of biomass processed (fresh weight; FW) was determined for each batch, as well as the purify of the isolated protein obtained. Results from 6 independent production batches of Thaumatin II show the recoverable yield of protein and the resultant protein purity. In addition to data from bench-scale developmental studies, data from one pilot-scale run for Thaumatin II are included in Table C-2. Process scale-up focused on purity first, and results suggest that the purity release criterion of >95% can be achieved at larger scale.



C.9. Bioburden and Residual Vector

The bioburden level and level of residual *Agrobacterium* vector was determined for *N. nicotiana*-produced Thaumatin II from non-consecutive batches. Results shown in Table C-8 demonstrate that the isolates had no bioburden or residual *Agrobacterium* vector (inoculum), therefore meeting the Specification.

Table C-8. Multi-batch analyses of bioburden and residual *Agrobacterium* inoculum

Batch[1]	Bioburden (CFU/g Thaumatin II; average of 3 replicates per batch)[2]		
	Total aerobic microbial content	Total combined yeast and mold (TYMC)	Total residual *Agrobacterium* (inoculum) content
Batch A	0	0	0
Batch B	0	0	0
Batch C	0	0	0

[1]Batches analyzed were obtained from non-consecutive production runs

[2]Thaumatin II solution at 1 mg/ml stock, 100 µl aliquots plated in 3 replicates on different media. Total aerobic microbial count (TAMC) was determined in Soybean-Casein Digest Agar (Roth #X937.1) plates incubated at 33°C for 3 days. Total combined yeast and molds count (TYMC) was determined in Sabouraud-Glucose Agar (Roth #932.1) plates incubated at 20-25°C for 6 days. Residual *Agrobacterium* inoculum was determined in LB-Bacto Agar + 50 µg/ml rifampicin plates incubated at 28°C for 2 days. Agrobacteria containing binary plasmid were enumerated in plates of LB-Bacto Agar + 50 µg/ml rifampicin and 50 µg/ml kanamycin incubated at 28°C for 2 days.

C.10. Summary

APPENDIX C summarizes the various methods used to characterize Notifier's Thaumatin II protein expressed recombinantly in the plant host *Nicotiana benthamiana*. The sweet-tasting thaumatin protein that comprise Notifier's THAUMATIN II product is expressed with signal peptide to enhance its accumulation in plant tissue and facilitate isolation. The precursor protein is correctly processed *in planta* post-translationally to produce the final mature Thaumatin II protein.

All three methods described in GRN 738 (APPENDIX C, pp 47-51) for purifying thaumatins from edible plant biomass (heat/acid-induced precipitation, cool ambient temperature chromatography, or tartrate-induced crystallization) yielded highly pure product (95-98%). Cool neutral extraction followed by cation-exchange chromatography applied to *N. benthamiana*-produced process stream yielded similarly pure protein (~95-98%). The batch-to-batch yield in developmental studies has been generally reproducible and the process generates proteins of consistent quality. Improvements in overall yield and quality are expected as the process is scaled and optimized, as suggested by results of a pilot-scale manufacturing run with Thaumatin II.

The amino acid composition, amino acid sequence, and molecular mass of the resultant protein is identical to the corresponding thaumatins II found in *Thaumatococcus daniellii*, which forms the basis of currently marketed thaumatin products (GRN 738; this Notice).

The methods used to characterize thaumatins, summarized in GRN 738 and in this section of the Notice, are industry standard and are validated and conducted by licensed external analytical laboratories. Other release tests (e.g. pH with various excipients) are applied to each batch of Thaumatin II (not shown here) to ensure

that the product meets its specification. Purified Thaumatin II protein is stable for >6 months in storage as dry powders and are also stable (>3 months) when mixed and stored with sucrose. The stability program is in progress and longer stable-storage times should be supported by subsequent results.

Notifier's purification process is undergoing optimization during scale up. The residual host-derived alkaloid impurities in Thaumatin II are consistently low and preliminary results suggest that they can be further reduced during purification at larger (i.e. pilot) manufacturing scale, which employs more rigorous process controls. Even at the current level of total alkaloids, these impurities do not constitute a health risk due to their low concentration and the low level of ingestion of thaumatin proteins in the diet. Likewise, the levels of elemental impurities in Thaumatin II are not considered a health risk due to their low concentrations and the low per capita consumption of thaumatin.

It is important to note that thaumatin products approved as food additives in multiple countries are not 100% pure thaumatin, and contain a number of host- and process-derived impurities as well, including extraneous protein and heavy metals (Lim 2012; Cusack 1991; Stephen 1991; Adesina 1977; Adesina 1978). Regardless, the safety of the commercial products has been conclusively shown and, by comparison, the safety of Notifier's final product has also been affirmed (see Section 6.1 (overall safety), Section 6.1.1 (allergenic potential), and Section 6.2 (safety in relation to dietary intake).

The sweetness intensity, threshold of detection and other flavor characteristics, as well as the sugar-replacing potential of Notifier's Thaumatin II, have been defined under controlled conditions in three sensory evaluation studies conducted to date. These studies have determined that sucrose and potentially other caloric sweeteners of similar intensity (e.g., corn syrup) used in food and beverages could be replaced at up to 50 wt% with Notifier's Thaumatin II while retaining generally the same perceived level of sweetness. The functionality of Notifier's Thaumatin II as a high-intensity sweetener has therefore been verified. Importantly, consumption of these proteins at the projected rates of application to achieve sweetening is expected to be safe with respect to the intake of thaumatin itself and any residual host or process-derived impurities remaining in the final THAUMATIN II product.

END OF NOTICE.

Seventeen pages have been removed in accordance with the Privacy Act of 1974